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Epigenetics in Oncology

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RNA Epigenetics

1 RNA Modifications in Cancer Metabolism and Tumor Microenvironment

Ying Qing, Dong Wu, Xiaolan Deng, Jianjun Chen, and Rui Su

1.1 Introduction

Natural RNAs carry a plethora of chemical modifications which play central roles in regulating gene expression at the posttranscriptional level [1, 2]. The first identified RNA modification, pseudouridine (also known as the "fifth nucleotide"), was initially described by Davis et al. in 1957 [3]. To date, more than 170 types of chemical modifications have been identified in protein-coding RNAs and noncoding RNAs (ncRNAs) [1, 4–6]. However, only a few of these modifications have been validated over the past decades. Due to technical limitations as well as a lack of molecular biological evidence, RNA modifications were generally recognized as irreversible decorations. This research field did not move forward too much during the past several decades until 2011 when He and colleagues reported the discovery of fat mass and obesity-associated protein (FTO) as the first demethylase of mRNA m^6A modification, which revealed that RNA modifications are dynamic and reversible [7–10]. Such groundbreaking discovery substantially revived the

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RNA modification research field. It has been well documented that RNA modifications can be deposited by "writers", removed by "erasers", and recognized by specific "readers" that mediate diverse downstream biological effects [11]. The dynamic regulation of RNA modifications is emerging as a critical new layer of gene expression and is involved in many aspects of cancer metabolism and anti-tumor immune response [9, 12–14].

As the most prevalent internal modification on eukaryotic mRNAs, m^6A has attracted the most attention in the epitranscriptomics field and plays broad and critical roles in gene expression regulation [6]. In addition to m^6A, other types of modified nucleotides also exist in both mRNA and ncRNAs, including those generated by the addition of methyl group to the bases or ribose sugar, such as 7-methylguanosine (m^7G), N^1-methyladenosine (m^1A), and 5-methylcytosine (m^5C) (Fig. 1.1) [5, 15]. Other modifications such as pseudouridine (Ψ), 5-hydroxymethylcytosine (hm5C), and adenosine to inosine (A-to-I) editing have also been reported for their physiological and pathological roles in diverse biological processes [1, 6].

Via high-throughput technologies, the landscapes of m^6A modification in humans and mice were first delineated by methylated RNA immunoprecipitation sequencing (MeRIP-seq, also known as m^6A-seq) in 2012 [16, 17]. Thus far, it has become a reality to achieve high-resolution detection of m^6A decorations in various cell contexts with the development of several next-generation sequencing (NGS) methods, including DART-seq (deamination adjacent to RNA modification target sequencing), m^6A-REF-seq (m^6A-sensitive RNA-endoribonuclease facilitated Sequencing) [18, 19]. Most recently, Hu et al. developed m^6A-SAC-seq (m^6A-selective allyl chemical labeling and sequencing), which can measure m^6A modification at single-base resolution with very limited input RNA [20].

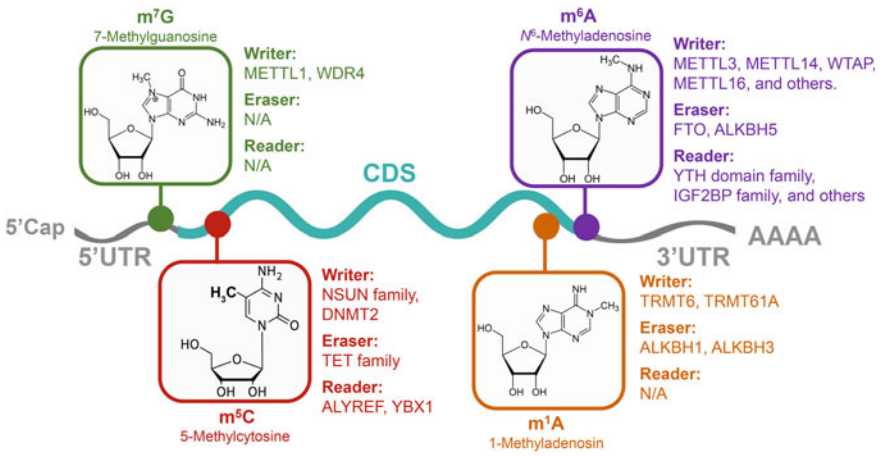

Fig. 1.1 Chemical structures of internal RNA modifications as well as the characterized writers, erasers, and readers

The mRNA m^6A methylation is mainly catalyzed by a multicomponent m^6A methyltransferase complex (MTC) consisting of 3 major subunits: methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms tumor suppressor 1-associated protein (WTAP) [6, 15, 21, 22]. The crystal structural studies demonstrate that METTL3 functions as the sole catalytic subunit, while METTL14 is required for recognizing substrate RNAs and deciding the methylation sites by forming a stable heterodimer with METTL3 [23–25]. WTAP and other accessory subunits, including vir like m^6A methyltransferase associated (VIRMA), RNA-binding motif protein 15 (RBM15), and zinc finger CCCH domain-containing protein 13 (ZC3H13), contribute to anchoring MTC in nuclear speckles and recognizing particular mRNA binding sites [26–32]. Although m^6A deposition in poly(A) RNA is catalyzed by the MTC under most circumstances, other m^6A writers have also been identified which could function either in a complex or alone to install m^6A marks. For example, phosphorylated CTD interacting factor 1(PCIF1) is a cap-specific m^6A writer that specifically catalyzes N^6-methylation of adenosine at the transcription start site in capped mRNAs to form N^6,2'-O-dimethyladenosine (m^6A_m) [33, 34]; while METTL16 was reported previously to install m^6A marks on U6 small nuclear RNA, *MALAT1* and *XIST* long-non-coding RNAs (lncRNAs), and MAT2A mRNA [35–38], our recent studies suggest that METTL16 can deposit m^6A into hundreds or even thousands of mRNA transcripts [39, 40]; METTL5 and Zinc finger CCHC-type containing 4 (ZCCHC4) are responsible for the deposition of m^6A on 18 s rRNA and 28 s rRNA, respectively [41, 42].

The methyl group can be selectively removed from m^6A-decorated RNAs by the demethylases FTO and alkB homolog 5 (ALKBH5) [8, 43], enabling reversible and dynamic regulation of m^6A. FTO could mediate the demethylation of multiple DNA and RNA methylation modifications, including m^6A, 3-methyluracil (m^3U), m^6Am, m^1A, and 3-methylthymine (3mT), [44, 45]. Amongst those decorations, the internal m^6A in mRNAs has been demonstrated as the major substrate of FTO [6, 10, 45–47]. In terms of ALKBH5, current studies indicate that it functions solely as an m^6A-specific demethylase [43, 48, 49].

While the dynamic balance between deposition and removal of m^6A is determined by writers and erasers, the biological consequences of mRNA m^6A modification are mediated by specific m^6A binding proteins (also known as "readers") [6, 10, 21, 47, 50–57]. Currently, two families of proteins, the YT521-B homology (YTH) domain family and the insulin-like growth factor 2 mRNA binding protein (IGF2BP) family, have been identified to primarily function as m^6A readers. The YTH family is comprised of 5 members, YTHDF1/2/3 and YTHDC1/2, and are among the earliest identified m^6A readers. They play versatile roles in almost every stage of mRNA metabolism, including mRNA splicing (YTHDC1), mRNA translation (YTHDF1/3 and YTHDC2), mRNA degradation (YTHDF2/3 and YTHDC2), and secondary structure modulation (YTHDC2) [50, 51, 53, 56, 58]. As opposed to the mRNA-decay-promoting function of the YTH family members (i.e., YTHDF2/3 and YTHDC2), IGF2BPs (including IGF2BP1/2/3) were recently identified as a new family of m^6A readers that could enhance the

stability and facilitate the translation of their target mRNAs [57]. Another class of m^6A readers, including heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1 (hnRNPA2B1), hnRNPC, and hnRNPG, were reported to regulate alternative splicing or processing of target transcripts indirectly by recognizing m^6A-induced RNA structural remodeling (also known as "the m^6A switch") [59–62].

RNA m^5C modification has been detected on rRNA, tRNA, mRNA, ncRNA, and enhancer RNA (eRNA) [63]. Different functions of m^5C modification have been described in different subclasses of RNA molecules [64, 65]. It has been illustrated that the NOL1/NOP2/SUN domain family members (NSUN) as well as DNA methyltransferase-like 2 (DNMT2) could function as RNA m^5C methyltransferases, and mRNA m^5C can then be recognized by the Aly/REF export factor (ALYREF) and Y-box binding protein 1 (YBX1) [63, 66, 67]. In addition, TET family proteins may oxidize m^5C into hm^5C in RNA [15, 68, 69]. Another methylation modification of RNA, m^1A, is documented to mainly occur on tRNA and rRNA, and impact tRNA functions and ribosome biogenesis [70, 71]. The methyl group of m^1A can be installed by tRNA methyltransferase 10A (TRMT10A) as well as the tRNA methyltransferase 6 non-catalytic subunit (TRMT6)– tRNA methyltransferase 61A (TRMT61A) complex and can be erased by ALKBH1 and ALKBH3 [72, 73]. Our knowledge of regulators of other modifications is still at infancy and the potential contribution of various RNA modifications to post-transcriptional gene regulation is largely underexplored.

Cells in a living organism constantly consume energy to build, maintain, and selectively destroy biological structures to adapt to the ever-changing environment. Thus, a stable and robust energy supply provided by homeostatic metabolism is necessary to maintain cell survival. Perturbation of metabolism leads to many human diseases, among which the most notable one is cancer. Cancer cells exhibit distinct metabolic characteristics that distinguish them from their normal counterparts. RNA modifications are pervasively involved in the maintenance of cellular metabolic homeostasis. More recently, technological advances have substantially expanded our understanding of the biological functions and underlying mechanisms of RNA modifications, especially m^6A, in the regulation of cancer metabolism [12, 74–76].

Cancer cell-associated metabolism reprogramming also plays an essential role in reshaping tumor microenvironment (TME). The TEM is a highly heterogenous ecosystem comprising not only the malignant cell compartment, but also immune cells, stromal cells, extracellular matrix, and blood vessels that have complex interactions with the tumor cells. Distinct immune cell populations, which can be broadly categorized into adaptive and innate immune cells, represent a critical component of TME by exerting both pro- and anti-tumorigenic functions, and are termed as tumor immune microenvironment (TIME). RNA post-transcriptional modifications dynamically shape TME/TIME via various mechanisms and impose profound influences on anti-tumor response and tumor progression [77, 78].

In this Chapter, we summarize the current knowledge on the biological functions and the molecular mechanism of RNA modifications (with a focus on m^6A)

in regulating tumor metabolism and microenvironment. Moreover, we also discuss the prospects of targeting epitranscriptomic regulators for cancer treatment.

1.2 m^{6}A Modification in Cancer Metabolism

Cellular metabolism is composed of myriad metabolic pathways that are orchestrated into a sophisticated metabolic network. Among all metabolic pathways, those involving the 3 major nutrients—carbohydrates, lipids, and amino acids—are located at the core of the network and are fundamental to bioenergetic homeostasis. Research on the metabolic functions of m^{6}A RNA modification has therefore focused on the metabolism of these key nutrients, including glucose metabolism, lipid metabolism, and amino acid metabolism.

1.2.1 Glucose Metabolism

Dietary carbohydrates are broken down into a few monosaccharides during digestion, including glucose, fructose, and galactose [79]. Glucose enters the tissue cells and serves as the primary fuel for ATP production and the major precursor for the synthesis of many biomolecules [80], whereas most fructose and galactose are transported to liver to be converted to glucose for further utilization [79]. Hence, glucose is central to carbohydrate metabolism. Glycolysis is the first process to utilize glucose to release energy, followed by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) to metabolize glucose to CO_2 and H_2O under the aerobic condition in normal cells. In contrast, tumor cells shift their metabolism to favor glycolysis over OXPHOS and excrete lactate as the end product instead of oxidizing glucose completely to CO_2 even in the presence of sufficient oxygen. This phenomenon was first described by Otto Warburg over 60 years ago (now called the Warburg effect or aerobic glycolysis), and represents the major reason why cancer is considered a metabolic disease [81, 82]. There is now accumulating evidence that RNA modifications are involved in the regulation of glucose metabolism at multiple steps (Fig. 1.2).

The m^{6}A writer METTL3 promotes glycolysis in various types of cancer via different mechanisms: METTL3 methylates solute carrier family 2 member 1 *(SLC2A1*, a glucose transporter) and hexokinase 2 (*HK2*, a glycolytic enzyme) to stabilize their mRNAs via the m^{6}A-IGF2BP2/3 pathway and promotes glycolysis in colorectal cancer (CRC) [83]; in gastric cancer (GC), a transcription coregulator, heparin binding growth factor (*HDGF*), can be methylated by METTL3 and recognized by IGF2BP3, leading to its elevated mRNA stability, and increased nuclear HDGF further activates the expression of *SLC2A4* (glucose transporter) and enolase 2 (*ENO2*, glycolytic enzyme) to enhance the glycolytic rate in GC cells [84]. METTL3 is overexpressed in esophageal squamous cell carcinoma (ESCC) and it installs m^{6}A onto a tumor suppressor adenomatous polyposis coli (APC) [85]. Then, YTHDF2 can recognize the m^{6}A-modified *APC* mRNA,

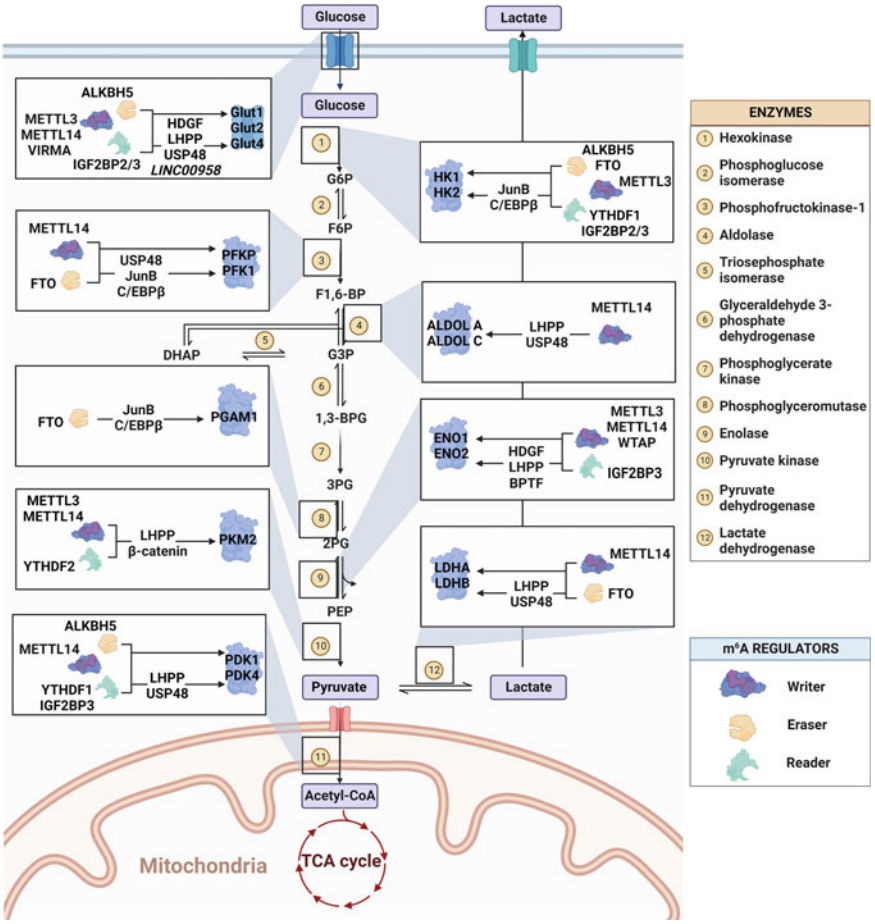

Fig. 1.2 The m^{6}A modification regulates cancer glucose metabolism via multiple mechanisms. The writers/erasers/readers of m^{6}A and their corresponding pathways involved in the modulation of aerobic glycolysis at various steps are shown. This image was created with BioRender (https://biorender.com/)

leading to the degradation of APC transcript. The METTL3/m^{6}A/YTHDF2 axis-mediated downregulation of APC further facilitates the expression of β-catenin and β-catenin-induced multiple glycolytic-related target genes, such as *MYC* and pyruvate kinase M2 (*PKM2*), and thereby promotes glucose consumption and lactate production [85]. In addition, METTL3 is also highly expressed in cervical cancer (CC) where it methylates *HK2* mRNA and recruits YTHDF1 to promote HK2 expression and thereby glycolysis [86]. There are also reports that METTL3 promotes the Warburg effect in non-small cell lung cancer (NSCLC) [87, 88] and hepatocellular carcinoma (HCC) [89].

METTL14, on the other hand, plays distinct roles in regulating cancer glucose metabolism in different types of cancer. METTL14 promotes aerobic glycolysis and growth of gastric cancer cells by methylating and downregulating the expression of the histidine phosphatase *LHPP* gene and impairing its glycolytic inhibitory and tumor suppressive function [90]. In contrast, METTL14 may play an opposite role and act as a tumor suppressor to attenuate glycolysis in metastatic renal cell carcinoma (RCC) and HCC [91, 92]. In RCC, METTL14 deposits m^6A modification on bromodomain PHD finger transcription factor (BPTF) and then mediates the decay of *BPTF* to silence aerobic glycolysis pathway to suppress RCC metastasis. In HCC, METTL14-induced m6A modification increases the stability of Ubiquitin-specific peptidase 48 (*USP48*), which subsequently stabilizes SIRT6 to suppress glycolysis and HCC tumorigenesis.

Other writers are also involved in the regulation of cancer aerobic glycolysis by modulating different targets. WTAP facilitates m^6A installation onto enolase 1 (*ENO1*, glycolytic enzyme) transcripts and promotes ENO1 expression and glycolysis in breast cancer cells [93]. VIRMA methylates lncRNA *LINC00958* which further interacts with *SLC2A1* mRNA and improves its stability to promote aerobic glycolysis in GC [94].

Removal of m^6A marks from RNAs involved in glucose metabolism by FTO and ALKBH5 represents another critical mechanism for epitranscriptomic regulation of cancer glucose metabolism. FTO has been documented to promote cancer aerobic glycolysis by two independent studies. We reported that, in leukemia cells, FTO directly demethylates two glycolytic genes *PFKP* and *LDHB* to facilitate aerobic glycolysis and leukemia cell growth [13]. A following study reports that FTO-mediated m^6A demethylation upregulates the expression of transcription factors (including *JunB* and *C/EBPβ*) to enhance chromatin accessibility of several glycolytic genes to indirectly elevate their expression and thereby promote glycolytic activity in murine tumor cells [14]. Yet results from another study support an inhibitory function of FTO on both glycolysis and tumorigenesis in lung adenocarcinoma via downregulating c-Myc expression [95]. Analogous to FTO, ALKBH5 is also reported to play different roles in regulating glycolysis in different types of cancer. Liu et al. revealed the glycolysis-promoting function of ALKBH5 in breast cancer whereas Li et al. demonstrated that ALKBH5 attenuates glycolytic activity and increases OXPHOS activity in cervical and liver cancer cells [96, 97]. Thus, whether m^6A erasers impose context-dependent impacts on cancer glucose metabolism and what the key context factors determining their functions require further investigation in future.

m^6A-dependent modulation of targets is executed by the readers, indicating that the functions of m^6A writers and erasers in glycolysis introduced above are ultimately realized by respective readers. For example, YTHDF1 and IGF2BP3 are both implicated to mediate the glycolysis-suppressive effects of ALKBH5 in cervical cancer by improving translation and increasing mRNA stability of the target transcript *PDK4* [97]. Additionally, IGF2BP2 is reported to promote aerobic

glycolysis in glioblastoma multiforme (GBM) and pancreatic ductal adenocarcinoma (PDAC) via enhancing the mRNA stability of CASC9/HK2 and GLUT1, respectively [98, 99].

1.2.2 Lipid Metabolism

Lipids comprise a diverse group of hydrophobic biomolecules, many of which are derived from fatty acids. Lipids not only form biological membranes but also function as energy storage molecules and precursors for signaling molecules. Therefore, lipids play an essential role in maintaining cell homeostasis [100]. Cancer cells need to double their lipids in each division cycle to generate two daughter cells. The lipids can either come from the circulation/microenvironment (lipids from dietary fat or produced by liver) or be generated inside cancer cells via de novo synthesis. Consequently, when lipid is in shortage in the tumor microenvironment, cancer cells often reactivate de novo lipid synthesis pathways to meet their metabolic demands [101, 102]. RNA m^6A modulators are involved in the regulation of de novo lipid synthesis to affect tumor metabolism and growth.

METTL3 positively regulates the mRNA stability of *LINC00958*, which (in addition to its function in glycolysis) upregulates critical enzymes in lipogenesis including SREBP1, FASN, SCD1, and ACC1, to promote lipogenesis and tumor progression in HCC [103]. METTL14 methylates another lncRNA, *lncDBET*, in bladder cancer to increase its expression and activates the PPAR pathway, which is presumed to subsequently augment lipid metabolism [104]. Whereas PPAR pathway is extensively related to lipid metabolism, this study did not test the direct relationship between the METTL14/*lncDBET*/PPAR pathway and lipid metabolism [104].

While serving as a major m^6A demethylase, *FTO* was mainly known as the first genetic locus unequivocally associated with obesity before the discovery of its m^6A demethylation activity, as reported by genome-wide association (GWAS) studies [105–108]. Given the close association of FTO with obesity and previous works that confirmed the regulation of adipogenesis by FTO/m^6A pathway, cancer researchers are trying to delineate the role of FTO in modulating cancer cell lipid metabolism [109, 110]. One recent publication suggests that FTO facilitates lipid droplet formation and exhibits an oncogenic effect in esophageal cancer (EC) by upregulating HSD17B11 (previously reported to induce lipid droplet aggregation and regulate lipid metabolism) via the m^6A/YTHDF1-dependent mechanism [111]. Whether this is true for other types of cancer and the proportion of FTO function in cancer that can be attributed to its regulation on lipid metabolism have yet to be elucidated. Another study in EC identified ALKBH5 as a tumor suppressor and hnRNPA2B1 as an oncogenic factor by suppressing or promoting the expression of de novo lipid synthesis enzymes *ACLY* and *ACC1* [112]. The recently recognized 18S rRNA m^6A writer, METTL5, has been shown to potentially regulate lipid biosynthesis pathways in cervical cancer cells, but its exact function needs to be further verified [113].

1.2.3 Amino Acid Metabolism

Glutamine has been increasingly appreciated for its roles in energy generation and intermediary metabolism of proliferating cancer cells [114, 115]. Even though glutamine is a nonessential amino acid (NEAA), many cancer cells display glutamine addiction and rely heavily on exogenous glutamine for various fundamental cell functions which are essential for cancer cell survival and growth [115–118]. For example, in VHL-deficient clear cell RCC (ccRCC), whereas cancer cells activate glycolysis, pyruvate generated during glycolysis is mostly converted to lactate. Therefore, VHL-deficient ccRCC cells require exogenous glutamine to fuel TCA cycle and biosynthesis of biomolecules such as nucleotides, amino acids, and fatty acids, and to produce glutathione and maintain redox balance [119, 120]. Genetic depletion of *FTO* in VHL-deficient ccRCC cells limits glutamine uptake and consumption via escalated methylation and decay of *SLC1A5*, a glutamine transporter, and impairs the viability of tumor cells [121]. In acute myeloid leukemia (AML), the m^6A reader IGF2BP2 regulates glutamine metabolism via stabilizing *MYC*, *GPT2*, and *SLC1A5* to promote leukemia initiation and progression [122].

In addition to the regulation of glutamine metabolism, our very recent study also uncovered the functions of m^6A writer METTL16 in reprogramming branched-chain amino acid (BCAA) metabolism in AML and promoting leukemogenesis [40]. METTL16 deposits m^6A onto *BCAT1* and *BCAT2*, two crucial transaminases for BCAA biosynthesis, to increase their expression, and knockout of *METTL16* diminishes BCAA metabolism, as demonstrated by metabolomic analysis with ^{13}C, ^{15}N-leucine labeling [40].

Serine is another NEAA whose metabolism has been recently shown to be critical for cancer cell proliferation. It provides benefits to cancer cells through facilitating protein synthesis and participating in the production of purine nucleotides, S-adenosyl methionine (SAM), sphingolipids, and phospholipids [123]. Expression of the first rate-limiting enzyme in serine biosynthesis, phosphoglycerate dehydrogenase (PHGDH), is controlled by different oncogenes and tumor suppressors. Wang et al. unveiled that deletion of *ALKBH5* in leukemia cells destabilizes *PHGDH* and *PSAT1* (another gene engaged in the serine biosynthesis pathway) mRNA in an m^6A/YTHDF2-dependent way, and forced expression of PHGDH restores the colony-forming capacity in *ALKBH5*-knockdown leukemia cells, implying that the regulation of serine metabolism accounts for at least part of the oncogenic function of ALKBH5 in leukemia [124].

1.3 Other RNA Modifications in Cancer Metabolism

Besides m^6A, additional layers of epitranscriptomic regulation by other RNA modifications also contribute to the metabolic reprogramming of cancer cells. However, the paucity of systematic studies on these modifications, especially when compared to those on m^6A, has made the metabolic functions of such modifications largely unrevealed. Thus, instead of delving into many modifications whose functions are

still unclear, we highlight a few modifications that have been more intensively studied for their functions in modulating cancer metabolism.

Methylation of cytosine at position 5 occurs on various types of RNA (e.g. mRNA and tRNA) and generates m^5C to guide cell metabolism in different aspects [125]. For instance, the rate-limiting glycolysis enzyme, PKM2, is decorated with m^5C in bladder cancer and such modification is recognized by the reader Aly/REF export factor (ALYREF). The binding of ALYREF stabilizes *PKM2* mRNA which in turn promotes aerobic glycolysis and tumor growth of bladder cancer [126]. When m^5C is deposited by its writer NSUN2 onto specific tRNA sites (the anticodon or variable loop), it helps to maintain the host cells like dermal fibroblasts at an anabolic state [127]. Exposure to external stress significantly suppresses the expression of NSUN2 and reduces m^5C levels in tRNA, resulting in global inhibition of protein synthesis and capturing the cells in a catabolic state by altering 3 vital metabolic pathways: amino acid synthesis, TCA, and methionine cycle [127]. A lately published work does elucidate the function of m^5C in mitochondrial $tRNA^{Met}$ for modulating cancer cell metabolism [128]. NSUN3, another m^5C writer, methylates mitochondrial $tRNA^{Met}$ to form m^5C and f^5C at position 34, which is required for the translation of mitochondrial-encoded components of the respiratory chain complex [128]. Therefore, a metabolic switch from OXPHOS to glycolysis is observed in mitochondrial $tRNA^{Met}$ m^5C-deficient oral cancer cells. Although m^5C-deficient cancer cells remain unaffected for their viability, they fail to metastasize effectively due to the loss of metabolic plasticity, indicating that mitochondrial $tRNA^{Met}$ m^5C is essential for the OXPHOS activity and metastasis of oral squamous cell carcinoma [128]. However, further systemic studies are warranted to determine whether m^5C also acts as a sensor in cancer cells to adapt cancer metabolism to environmental stress.

Similarly, another methylation mark of adenosine, m^1A, imposes different effects on cancer metabolism when present on distinct types of transcripts and in varying contexts. In cervical cancer, the ALKBH3-mediated removal of m^1A from the mRNA of ATP synthase F1 subunit delta (*ATP5F1D*, which encodes a crucial subunit of mitochondrial ATP synthase) is shown to improve glycolytic activity by promoting ATP5F1D translation in a YTHDF1/eRF3-dependent manner [129]. Furthermore, the mRNA stability of E2F transcription factor 1 (*E2F1*), a transcription factor that drives transcription of *ATP5F1D*, is compromised when modified with m^1A, augmenting the negative regulatory effect of glycolysis by the m^1A/ATP5F1D axis [129]. When installed into a subset of target tRNA by the TRMT6/TRMT61A complex in HCC, m^1A potentiates PPARδ translation to subsequently stimulate cholesterol synthesis and the downstream Hedgehog pathway, promoting the self-renewal of HCC stem cells and consequently facilitating tumorigenesis [130].

1.4 Regulation of Tumor Microenvironment by m^{6}A

TME is featured by its hostile hypoxic, nutrient-limiting, and immunosuppressive conditions. While having achieved extraordinary efficacy in some patient populations, current immunomodulatory strategies failed to bring benefits to most cancer patients as a result of unsuccessful reinvigoration of anti-tumor response, likely due to inadequate reprogramming of the immunosuppressive TME [131]. Metabolic reprogramming of cancer cells plays a dominant role in shaping the TME because cancer cells and tumor-infiltrating immune cells compete for the limited nutrients in the TME to maintain their functions and proliferation [132, 133]. Hence, the aforementioned functions of m^{6}A in modifying the metabolism of cancer cells also reshape their TME. For example, FTO deficiency in tumor cells diminishes cancer aerobic glycolysis and allows the function of CD8$^+$ T cells in the TME to be restored, leading to tumor growth inhibition [14]. Further, m^{6}A directly participates in the activation of immune cells and their infiltration into the TME and remodels the anti-tumor immune responses [77, 78]. Here we review our current knowledge of the m^{6}A-dependent pathways engaged in the dynamic evolution of TME to encourage further exploration of targeting such pathways as a therapeutic strategy itself for cancer treatment and/or as an adjuvant for cancer immunotherapy to improve clinical effects (Fig. 1.3).

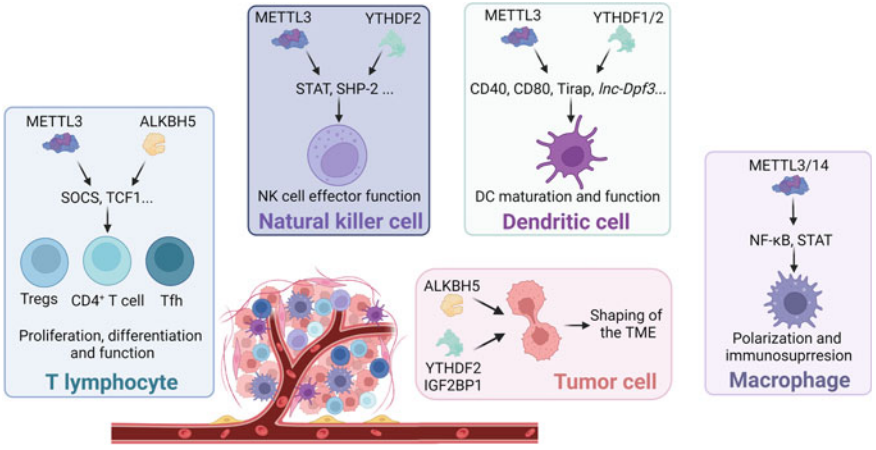

Fig. 1.3 The m^{6}A-dependent pathways are broadly implicated in the dynamic regulation of the tumor microenvironment. The key m^{6}A regulators can directly modulate the maturation, proliferation, and function of immune cells, including T cells, NK cells, dendritic cells, and macrophages, to reprogram TME. In addition, those m^{6}A regulators can also regulate the expression of immune checkpoint genes in tumor cells to alter immune cell infiltration and indirectly reshape TME. This image was created with BioRender (https://biorender.com/)

1.4.1 Roles of m^{6}A in T Cells

Cancer immunotherapies including adoptive cell therapy and immune checkpoint inhibitors (ICIs) primarily aim to enhance T cell activity as T cells are fundamental to anti-tumor immunity [134]. METTL3 is reported to be essential for physiological CD4$^+$ T cell development and function [135]. Genetic deletion of *Mettl3* in mouse CD4$^+$ T cells prevents methylation and efficient degradation of mRNAs of the SOCS family genes, which are responsible for restraining IL-7 mediated STAT5 activation. Thus, naive *Mettl3*-KO CD4$^+$ T cells displayed increased levels of SOCS family proteins, which impede STAT signaling and disrupt T cell proliferation and differentiation [135]. Regulatory T cells (Tregs) are a crucial immunosuppressive subset of T cells in the TME that block the anti-tumor activity of other effector T cells. Interestingly, METTL3 targets a similar group of SOCS proteins in Tregs to those reported in CD4$^+$ T cells [135] to promote IL-2/STAT5 signaling and maintain the immunosuppressive function of Tregs [136]. Furthermore, Mettl3 is required for the differentiation of follicular helper T cells (Tfh, specialized T cells that facilitate B cell function) in mouse [137]. Hence, the overall effects of METTL3 on T cells in the TME is a combined result of its functions on different T cell subpopulations. Future studies are required to comprehensively dissect those effects in the context of different cancer types. ALKBH5 is also implicated in preserving CD4$^+$ T cell-mediated immune response during autoimmunity, whereas its specific role in regulating tumor-infiltrating T cells remains to be further investigated [138].

1.4.2 Roles of m^{6}A in Natural Killer Cells

Natural killer (NK) cells represent a group of innate lymphoid cells that possess direct cytotoxic activity against various types of cancer cells [139]. Consequently, epitranscriptomic pathways that modulate NK cell functions may serve as potentially significant targets to improve anti-tumor immunity. Current studies have revealed the critical roles of METTL3 and YTHDF2 in mediating the biological functions of NK cells. Ythdf2 expression is upregulated in murine NK cells upon activation to potentiate NK cell effector functions [140]. Ythdf2 is required for homeostasis maintenance, terminal maturation, and IL-15-mediated survival and proliferation of NK cells, and loss of Ythdf2 significantly inhibits the immunosurveillance activity of NK cells [140]. Likewise, Mettl3 positively regulates NK cell effector functions in the TME, and the downregulation of Mettl3 in tumor-infiltrating NK cells impedes NK cell tumoricidal function and facilitates tumor progression [141].

1.4.3 Roles of m^{6}A in Dendritic Cells

Dendritic cells (DCs) are capable of processing and presenting tumor neoantigens to T cells and other anti-tumor immune cells in the TME to initiate a powerful anti-cancer immune response, and can thus be utilized to overcome tumor immune evasion [142]. DCs are necessary for T cell-mediated cancer immunity. YTHDF1 reduces the cross-presentation capacity of DCs through facilitating the translation of lysosomal cathepsins, thereby causing excessive degradation of tumor antigens [143]. Consistently, *Ythdf1*-depleted DCs show better presentation of tumor neoantigens and improved cross-priming of $CD8^+$ T cells, and *Ythdf1*-KO mice exhibit enhanced $CD8^+$ T cell-dependent anti-tumor immunity which synergizes with the ICI PD-L1 inhibitor, suggesting YTHDF1 to be a promising target to improve cancer immunotherapy [143]. Chemokine-driven migration is decisive for the amplitude and outcome of DC-mediated immune response, and YTHDF2 but not YTHDF1 is engaged in the regulation of C–C motif chemokine receptor 7 (CCR7)-induced DC migration [144]. In unstimulated mouse DCs, Ythdf2 recognizes and destabilizes m^{6}A-modified lncRNA *lnc-Dpf3*. Upon CCR7 stimulation, HIF-1α signaling is activated in DCs to shift energy metabolism toward glycolysis to allow migration, whereas *lnc-Dpf3* is concurrently upregulated to bind and inhibit HIF-1α activity as a negative feedback mechanism. The upregulation of *lnc-Dpf3* is fulfilled by CCR7-induced removal of m^{6}A from *lnc-Dpf3* and subsequently compromised binding of Ythdf2 to *lnc-Dpf3* [144]. Moreover, Wang and colleagues reported that Mettl3 promotes mouse DC physiological maturation and function in an m^{6}A/Ythdf1-dependent manner [145]. Mettl3 methylates *CD40*, *CD80*, and *Tirap* in DCs to increase their translation for efficacious priming of T cells and potent activation of DC-mediated T cell response. However, whether the same mechanism presents in the TME and can be exploited as a therapeutic target to improve immunotherapies warrant further investigation.

1.4.4 Roles of m^{6}A in Macrophages

Tumor-associated macrophages (TAMs) are highly heterogeneous and plastic. Despite often being considered to resemble more closely the anti-inflammatory (M2) subgroup, TAMs contain a continuum of functional status and can switch between anti-tumor M1 and pro-tumor M2 phenotypes under diverse TME contexts. Yin and colleagues found that *Mettl3* deficiency in TAMs leads to activation of NF-κB and STAT3 pathways and results in increased polarization to both M1 and M2 phenotypes, which in turn creates an immunosuppressive TME and promotes tumor progression and metastasis [146]. Correspondingly, the response to anti-PD-1 therapy is dampened by *Mettl3* depletion in mouse melanoma model, supporting the role of METTL3 in affecting clinical efficacy of immune checkpoint blockade (ICB) [146]. Conversely, a following study argued that *Mettl3* deficiency in myeloid cells limits the tumor progression and alleviates the immunosuppression of TME [147]. Mechanistically, METTL3 upregulation in TAMs and

other myeloid-derived suppressor cells (MDSCs) enhances the JAK-STAT3 signaling to facilitate the immunosuppressive functions of these cells [147]. Results from another work, on the other hand, favor that METTL3 promotes M1 polarization in macrophages and ablation of *Mettl3* in macrophages exacerbates the immunosuppression of TME and accelerates colorectal cancer growth in vivo [148]. Considering the high complexity and heterogeneity of TME, even a slightly different factor in the TME might significantly reverse the function of METTL3 in macrophage-related anti-tumor immunity. Thus, more comprehensive studies that consider additional TME factors, including impacts from other immune cells and the interaction with tumor cells, are required to fully clarify the discrepancy between previous reports. *METTL14* deletion in macrophages is shown to attenuate tumor infiltration and drive dysfunction of $CD8^+$ T cells, thereby impairing anti-tumor responses and promoting tumor growth [149].

1.4.5 Roles of m^6A in Immune Evasion of Tumor Cells

Besides its regulation of cellular metabolism, m^6A regulates tumor cells through additional mechanisms to contribute to cancer immune escape. Three independent studies jointly uncovered the multifaceted albeit consistent cancer-promoting roles of tumor intrinsic ALKBH5 in shaping TME: *Alkbh5* deficiency sensitizes murine melanoma and colon cancer cells to anti-PD-1 antibody by altering lactate levels and immune cell (including Tregs and MDSCs) recruitment in TME [150]; ALKBH5 directly demethylates PD-L1 in intrahepatic cholangiocarcinoma (ICC) cells to sustain PD-L1 expression and hence restrain T cell proliferation and anti-tumor cytotoxicity [151]; ALKBH5 is also required in GBM for hypoxia-induced TAM recruitment and immunosuppression [152]. Genetic knockdown or allosteric inhibition of IGF2BP1 in HCC cells causes apoptosis, decreases PD-L1 expression, and increases tumor infiltration of various types of immune cells including $CD4^+$/$CD8^+$ T cells, NK cells, and macrophages [153]. Another m^6A reader, YTHDF1, also exerts a tumor-promoting effect in GC by remodeling TME. Genetic depletion of YTHDF1 in GC tumors represses the infiltration of MDSCs, while potentiates the recruitment of mature DC cells, leading to the infiltrations of $CD4^+$/$CD8^+$ T cells [154].

1.5 Regulation of Tumor Microenvironment by Other Modifications

Adenosine deaminase acting on RNA 1 (ADAR1) is an RNA-editing enzyme that catalyzes the deamination of adenosine (A) to produce inosine (I). A recent study has proposed a new mechanism for how the loss of ADAR1 leads to a global reshaping of TIME and profoundly sensitizes tumors to immunotherapy

[155]. This finding identifies ADAR1 as a promising target to restore the sensitivity to ICB in cancer patients. The A-to-I RNA-editing of antizyme inhibitor 1 (AZIN1) was also reported to contribute to tumor progression in colorectal cancer by enhancing the invasive potential of cancer-associated fibroblasts and promoting tumor angiogenesis in the TME [156, 157]. In addition, the association between tRNA m^7G methyltransferase METTL1 and the prognosis of neck squamous cell carcinoma (HNSCC) has also been addressed. It was demonstrated that METTL1 was involved in the regulation of TME immune status through the PI3K/AKT/mTOR pathway [158]. Moreover, in PDAC patients, the m^5C regulator-related lncRNAs may be involved in the regulation of tumor-infiltrating lymph cells and such lncRNAs might be used to predict the overall survival of PDAC [159]. Nonetheless, such associations need to be experimentally verified. In this study, the authors neither characterized the exact function of m^5C in modulating TIME nor determined whether such m^5C regulator-related lncRNAs are indeed decorated with m^5C modification. At current stage, the relatively low abundance of these RNA modifications as well as the technical limitations still hamper the systematic analysis of their presence and functions in TME. Further validation is needed for a comprehensive understanding of how RNA modifications act in TME and thereby affect tumor progression.

1.6 Clinical Implications and Epitranscriptomic Drugs: A New Dawn for Epitranscriptomic Cancer Therapy?

Thanks to the substantial advancement in high-throughput sequencing methods, the epitranscriptomic landscapes of various modifications have been gradually revealed in cancers. Specifically, the discovery of multiple functions of key m^6A regulators in cancer initiation, progression, and maintenance, as well as in cancer stem cell self-renewal, immune evasion, and drug resistance, provides important insights and potential therapeutic targets for epitranscriptomic targeted cancer therapy. Indeed, multiple proof-of-principle studies from us and other colleagues have already developed several potent and specific small-molecule compounds targeting m^6A machinery, including FTO, METTL3, and IGF2BPs, for anti-tumor therapy [160–163]. More encouragingly, the small molecule drug targeting m^6A demethylase FTO currently under phase 1 and 2 clinical trials (NCT04989335; NCT05456269), is shown to be less cardiotoxic and synergize with hypomethylating agents, anthracycline drugs, and other types of chemotherapy [161]. Such novel epitranscriptomic strategies may bring new hope to patients who are resistant and/or less tolerant to current cancer therapies, yet many challenges remain to be overcome before epitranscriptomic cancer therapies can be applied in clinic. Expanding our knowledge of the reliance on differential epitranscriptomic pathways required for regulation of cancer metabolism and microenvironment will be instrumental to optimizing epitranscriptomic drugs to achieve more clinical benefits without causing severe side effects.

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RNA Modifications in Cancer Stem Cell Biology

2

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2.1 Introduction

Gene expression is a dynamically regulated process that involves multiple steps of control, from chromatin accessibility to protein degradation. Following transcription, RNA molecules are subject to various processing events, including RNA decoration by over 170 possible RNA modifications [1]. Although the existence of RNA modifications has been known for decades, only recently have the wide-ranging contributions of the RNA epitranscriptome to cellular biology been recognized [1, 2]. In particular, the discovery of N^6-methyladenosine (m^6A) reader and eraser proteins has revealed that m^6A is installed and removed dynamically and plays important roles in post-transcriptional control of gene expression [3–5]. Moreover, dysregulation of RNA modification machinery contributes to unique RNA epitranscriptomes of cancer tissues, promoting cancer-associated gene expression [6]. Of particular interest are changes to the RNA epitranscriptomes of cancer stem cells (CSCs) that are capable of self-renewal and are involved in metastasis, drug resistance, and cancer recurrence [7]. Here, we discuss the roles of m^6A, N^7-methyguanosine (m^7G), pseudouridine (Ψ), and inosine (I) RNA modifications in CSC biology and consider how RNA modification-mediated

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post-transcriptional control of gene expression supports the CSC state and promotes CSC adaptation to profound rewiring of the intracellular and extracellular environments of tumor cells.

2.2 Cancer Stem Cells

Tumor tissues are highly heterogenous and often resemble the hierarchical structure of corresponding healthy tissues [7–9]. At the top of this hierarchy are CSCs that are defined as tumor cells capable of self-renewal, asymmetric division, and reconstitution of the tumor bulk upon transplantation [7, 10]. The CSC state is often characterized by the expression of pluripotency factors that endow CSCs with stem-like properties and the required plasticity to sustain tumor progression. Stem-like gene expression is often accompanied by aberrant activation of signaling pathways that regulate the CSC state, including Hedgehog, Wnt, and Notch signaling pathways [10, 11]. Functionally, CSCs are characterized by unique properties that reinforce the stem-like state and preserve the pool of CSCs during cancer progression; for example, CSCs can acquire a quiescent state and limit the production of reactive oxygen species to avoid oxidative damage [11]. CSC plasticity also enables constant adaptation to the changing tumor environment, energy demands, and therapeutic challenge. Intratumoral hypoxia is a common feature of tumor tissues, and hypoxia-inducible factors (HIFs) are important regulators of CSC biology [9]. Expression of HIFs is elevated in CSCs, including glioblastoma stem cells (GSCs) [12]. HIF-mediated transcriptional programs modulate the expression of pluripotency factors, epithelial-to-mesenchymal transition (EMT), and resistance to therapy [9]. Although CSCs may share similar genetic alterations with their differentiated counterparts, CSCs often undergo extensive reprogramming of their DNA epigenomes that promotes the establishment and maintenance of the stem-like state [7]. For example, elevated expression of lysine-specific demethylase 1 (*LSD1*) is associated with remodeling of the histone methylation landscape and chromatin accessibility in leukemia stem cells (LSCs) [7]. It can be hypothesized that remodeling of the RNA epitranscriptome is another critical feature of transformation and maintenance of CSCs, which is the focus of this Chapter.

2.2.1 CSCs, EMT, and Drug Resistance

CSCs are also implicated in the defining events of cancer progression: EMT, invasion, metastasis, drug resistance, and recurrence [13]. EMT is characterized by the loss of cell-to-cell junctions, downregulation of cellular adhesion proteins—most notably, E-cadherin—and upregulation of mesenchymal markers, such as vimentin and N-cadherin [14]. Transforming growth factor β (TGF-β) is a potent inducer of EMT that activates a downstream signaling cascade culminating in the activation of SMAD transcription factors that orchestrate EMT gene expression [13, 15].

CSCs and the cells undergoing EMT share various similarities, including overlapping transcriptional profiles and acquisition of stem-like properties [10]. EMT gene expression programs contribute to cancer cell plasticity, invasive capabilities, and drug resistance—cellular features that also define the CSC state [10]. Moreover, metastatic disseminating cells often express stem cell markers and share transcriptomic similarities with CSCs [8, 16]. CSCs also play a major role in drug resistance due to their quiescent state, enhanced drug efflux, and increased capacity for DNA repair [8]. CSC plasticity enables rapid adaptation to the therapeutic challenge, leading to tumor recurrence with novel mechanisms of resistance across the tumor bulk. Given the links between EMT, metastasis, drug resistance and CSCs, we also discuss the roles of RNA modifications in these processes.

2.3 RNA Modifications in CSC Biology

Oncogenic transformation of normal cells involves profound changes to the cellular biology programs that establish the malignant state. As a prominent layer of post-transcriptional control of gene expression, the RNA epitranscriptome is rewired along the course of tumorigenesis to support the newly acquired cancer cell state, adaptation to the tumor microenvironment, and acquisition of stem-like properties in the case of CSCs [6, 17]. In this section, we discuss the roles of RNA modifications, including m^6A, m^7G, Ψ, and I, in the initiation and maintenance of the CSC state by context-dependent regulation of gene expression.

2.3.1 *N*^{6}-methyladenosine (m^6A)

M^6A is the most abundant internal modification in eukaryotic messenger RNAs (mRNAs) that is dynamically installed and removed by dedicated writer and eraser proteins, respectively [18]. M^6A is also by far the most studied RNA modification because of its far-reaching roles in cellular biology, development, and diseases as well as its high abundance and the existence of accurate detection methods [19, 20]. M^6A-decorated transcripts are recognized by m^6A reader proteins that orchestrate downstream processing of these transcripts by interacting with a plethora of other proteins, including the RNA stabilization, decay, and translation machineries [21]. In cancer, the altered balance of the expression and activity of m^6A writer, reader, and eraser proteins results in dysregulation of the m^6A epitranscriptome [22]. CSCs exploit the dynamic m^6A landscape to establish and maintain stem-like properties as well as to facilitate EMT and drug resistance [23]. While it is beyond the scope of this Chapter to cover all of the studies that have implicated m^6A in cancer progression (reviewed in refs. [22, 24, 25]), here, we focus on the roles of m^6A in the regulation of the CSC state and stem-like activity (Table 2.1).

Table 2.1 The roles of m^6A and its modification machinery in tumorigenesis and CSCs

Cancer type	Protein effector	Role in tumorigenesis	Target transcripts	Role in CSCs or stem-like activity	References
AML	METTL3	Oncogene	PTEN, MYC, BCL2	Controls the HSPC state and promotes leukemogenesis	[28]
AML	METTL3	Oncogene	SP1, SP2	Maintains myeloid differentiation block and leukemia	[32]
AML	METTL14	Oncogene	MYB, MYC	Promotes leukemogenesis and maintains LSCs	[33]
AML	YTHDF2	Oncogene	Tnfrsf1b	Promotes leukemogenesis and maintains LSCs	[34]
AML	YTHDC1	Oncogene	MYC	Suppresses myeloid differentiation and promotes leukemogenesis	[35]
AML	YTHDC1	Oncogene	MCM2-4, CHAF1A	Promotes leukemogenesis and maintains LSCs	[36]
AML	IGF2BP2	Oncogene	GPT2, SLC1A5, MYC	Promotes leukemogenesis; expression of IGF2BP2 is increased in $CD34^+$ LSCs	[37]
AML	ALKBH5	Oncogene	TACC3	Promotes leukemogenesis; expression of ALKBH5 is increased in $CD34^+$ LSCs	[38]
AML	FTO	Oncogene	ASB2, RARA	Inhibits AML cell differentiation and promotes leukemogenesis	[39]
AML	FTO	Oncogene	MYC, CEBPA	Promotes leukemogenesis	[40]

(continued)

Table 2.1 (continued)

Cancer type	Protein effector	Role in tumorigenesis	Target transcripts	Role in CSCs or stem-like activity	References
GBM	METTL3, METTL14	Tumor suppressor	ADAM19	Suppresses GSC tumorigenicity and stem-like gene expression	[48]
GBM	METTL3	Oncogene	SOX2	Promotes GSC tumorigenicity and expression of SSEA1	[49]
GBM	METTL3	Oncogene	OPTN	Promotes GSC tumorigenicity	[50]
GBM	YTHDF2	Oncogene	MYC, VEGFA	Promotes GSC tumorigenicity and expression of SOX2 and OLIG2	[47]
GBM	YTHDF2	Oncogene	LXRA, HIVEP2	Promotes GSC tumorigenicity	[51]
GBM	ALKBH5	Oncogene	FOXM1	Maintains GSC stem-like state and expression of SOX2, NANOG, and OCT4	[52]
CRC	METTL3, IGF2BP2	Oncogene	SOX2	Maintains CRC CSCs and expression of CSC markers	[53]
BC	METTL14	Tumor suppressor	NOTCH1	Suppresses TIC tumorigenicity; m^6A levels are decreased in $CD44^+$ TICs	[54]
HCC	YTHDF2	Oncogene	OCT4	Maintains HCC CSCs	[55]
ML	YTHDF3	Oncogene	CTNNB1	Maintains ML CSCs; expression of YTHDF3 is increased in $CD133^+$ CSCs	[56]
OC	FTO	Tumor suppressor	PDE4B, PDE1C	Expression of FTO is decreased in $ALDH^+/CD133^+$ CSCs; suppresses pluripotency marker expression	[57]

AML, acute myeloid leukemia; BC, bladder cancer; CRC, colorectal carcinoma; CSCs, cancer stem cells; GBM, glioblastoma; GSCs, glioblastoma stem cells; HCC, hepatocellular carcinoma, HSPCs, hematopoietic stem and progenitor cells; LSCs, leukemia stem cells; ML, melanoma; OC, ovarian cancer; TICs, tumor-initiating cells

2.3.1.1 The Roles of m^{6}A in Leukemia and LSCs

Hematopoiesis is one of the best understood processes depicting adult stem cell maintenance and differentiation into mature progeny, whereas dysregulation of hematopoiesis is a hallmark of leukemic transformation [26]. Dysfunctional hematopoietic progenitors that fail to differentiate into the hematopoietic lineage give rise to LSCs that drive leukemia progression [27]. Therefore, the hematopoietic system is a particularly suitable model for investigating the roles of m^{6}A in adult stem cell maintenance, tumorigenic transformation, and CSC biology. Experimental manipulation of the m^{6}A modification machinery has revealed the importance of m^{6}A in hematopoiesis; for example, overexpression of the m^{6}A writer *METTL3* promotes human CD34$^+$ hematopoietic stem and progenitor cell (HSPC) proliferation and suppresses myeloid differentiation in a catalytic activity-dependent manner (that is, requiring installation of m^{6}A) [28]. Accordingly, dysregulation of all classes of the m^{6}A modification machinery—writer, reader, and eraser proteins—has been described in acute myeloid leukemia (AML) (reviewed in refs. [29–31]). Gain- and loss-of-function studies of the m^{6}A modification machinery have, in turn, provided mechanistic evidence for m^{6}A-dependent regulation of leukemogenesis and the LSC state. Vu et al. found that silencing of *METTL3* suppressed MOLM-13 AML cell proliferation in vitro and leukemogenesis in vivo [28]. Mechanistically, METTL3 interacted with the mRNAs of important regulators of the stem-like state, including *PTEN*, *MYC*, and *BCL2*, promoting their translation efficiency in an m^{6}A-dependent manner [28]. Barbieri et al. also linked the regulation of *MYC* expression in AML cells to the methylation activity of METTL3 [32]. The authors found that METTL3 interacted with chromatin and installed m^{6}A to nascent transcripts of certain genes, including those encoding transcription factors SP1 and SP2 that are positive regulators of *MYC* expression. M^{6}A methylation increased the translation efficiency of the corresponding transcripts, promoting SP1 and SP2-mediated gene expression programs and leukemic cell proliferation [32]. METTL14, the protein that forms a heterodimer with METTL3 to install m^{6}A, is also involved in hematopoiesis and is downregulated during normal myelopoiesis [33]. Weng et al. used the MLL-AF9 (MA9) fusion-mediated AML mouse model, which represents spontaneous oncogene-induced leukemic transformation, and found that METTL14 promoted leukemic transformation, whereas depletion of METTL14 was associated with decreased frequency of LSCs [33]. Mechanistically, METTL14 stabilized *MYB* and *MYC* mRNAs and increased their translation efficiency in an m^{6}A-dependent manner [33]. Using a different mouse model of leukemogenesis driven by *Meis1* and *Hoxa9* oncogenes, Paris et al. found that the m^{6}A reader YTHDF2 maintained LSCs and promoted leukemic cell engraftment and initiation of AML in vivo [34]. Mechanistically, YTHDF2 interacted with *Tnfrsf1b* mRNA, encoding a tumor necrosis factor receptor 2 (TNFR2), and promoted its degradation [34]. Although the *Tnfrsf1b* mRNA was m^{6}A methylated, the direct role of m^{6}A in the interaction between *Tnfrsf1b* and YTHDF2 remains to be confirmed. Cheng et al. found that the m^{6}A reader YTHDC1 suppressed myeloid differentiation of various AML

cell lines in vitro and promoted leukemogenesis in patient-derived xenotransplantation (PDX) models in vivo [35]. Mechanistically, YTHDC1 formed liquid-like nuclear condensates that were required for leukemia growth. In particular, m^{6}A-dependent nuclear co-localization of *MYC* mRNA and YTHDC1 prevented *MYC* degradation by the nuclear exosome machinery [35]. Moreover, Sheng et al. found that YTHDC1 maintained the Lin$^-$cKit$^+$ and leukemic granulocyte-monocyte precursor (L-GMP) subpopulations of LSCs in MA9-mediated leukemia mice [36]. Mechanistically, YTHDC1 stabilized the mRNAs of key regulators of DNA replication, including *MCM4*, in an m^{6}A-dependent manner [36]. Weng et al. found that the m^{6}A reader IGF2BP2 was enriched in CD34$^+$ LSCs as compared to normal CD34$^+$ or leukemic CD34$^-$ cells [37]. IGF2BP2 promoted MA9-mediated colony formation in vitro and leukemogenesis in vivo in an m^{6}A-dependent manner. Mechanistically, IGF2BP2 stabilized the mRNAs of key effectors of glutamine metabolism, including *SLC1A5* and *GPT2*, as well as *MYC* and promoted their translation [37]. While the studies described above indicate that m^{6}A writer and reader activities and thus m^{6}A methylation are oncogenic in leukemia, other studies have also revealed oncogenic roles for m^{6}A erasers that reverse m^{6}A methylation. Shen et al. found that the m^{6}A eraser ALKBH5 promoted MA9-mediated cell immortalization in vitro and leukemogenesis in vivo [38]. Moreover, expression of *ALKBH5* was higher in CD34$^+$ LSCs as compared to CD34$^-$ AML blasts. Mechanistically, ALKBH5 interacted with *TACC3* mRNA, encoding an important regulator of tumorigenesis in various cancers, and stabilized *TACC3* in a catalytic activity-dependent manner [38]. Li et al. found that FTO promoted MONOMAC-6 and MV4-11 AML cell proliferation and prevented apoptosis, whereas overexpression of *Fto* accelerated MA9-mediated leukemogenesis in vivo [39]. Mechanistically, FTO interacted with *ASB2* and *RARA* mRNAs, encoding mediators of retinoic acid signaling, and promoted their degradation in a catalytic activity-dependent manner. Given that *ASB2* and *RARA* are also upregulated upon all-trans-retinoic acid-induced differentiation of leukemic cells, these data suggest that FTO activity in LSCs may prevent their differentiation and instead promote the CSC state [39]. Su et al. found that FTO also promoted the expression of *MYC* and *CEBPA* transcription factors in a catalytic activity-dependent manner, contributing to LSC self-renewal and AML progression [40, 41]. Together, these studies indicate that both m^{6}A methylation and demethylation can play oncogenic roles in AML progression, suggesting multimodal and context-dependent regulation of gene expression by the RNA epitranscriptome. The fate of an m^{6}A-decorated transcript may be starkly different depending on the m^{6}A reader that orchestrates the downstream processing of the given transcript, whereas the context of the tumor genetic background and microenvironment may influence the oncogenicity of protein effectors under the m^{6}A control. Nevertheless, AML dependency on the m^{6}A epitranscriptome indicates the potential for therapeutic targeting of the m^{6}A modification machinery, and the development of small-molecule compounds is ongoing. Targeting of METTL3 with a small-molecule inhibitor STM2457 reduces CD93$^+$ and L-GMP subpopulations of LSCs [42]. Pharmacological inhibition of IGF2BP2 with a small-molecule compound CWI1-2 suppresses MA9-mediated

colony formation ability in vitro as well delays leukemogenesis and prolongs survival in vivo [37]. Moreover, inhibition of FTO with FB23-2 promotes retinoic acid-induced myeloid differentiation of MONOMAC6 AML cells in vitro and suppresses leukemic progression in vivo [43]. Finally, FTO inhibitors CS1 and CS2 exhibit remarkable therapeutic efficacy in preclinical animal models of AML by substantially suppressing LSC self-renewal and AML progression [41].

2.3.1.2 The Roles of m^{6}A in Glioblastoma and GSCs

Glioblastoma (GBM) is the most aggressive type of brain cancer with a median survival of only 12 to 18 months [44]. The heterogeneous nature of GBM and the high rate of recurrence can be partially attributed to the existence of GSCs that are highly adaptive to the changing tumor microenvironment and drive therapy resistance [45, 46]. In GBM, the unique m^{6}A epitranscriptome distinguishes GSCs from normal neural stem cells (NSCs) [47]. Moreover, m^{6}A methylation critically regulates GSC self-renewal and tumorigenesis [48]. In particular, we found that silencing of *METTL3* or *METTL14* promoted GSC proliferation and sphere formation ability in vitro as well as tumorigenesis in vivo, whereas overexpression of *METTL3* or pharmacological inhibition of FTO had an opposite effect. Silencing of *METTL3* or *METTL14* was also associated with increased expression of a GSC marker *CD44* and oncogenes *EPHA3* and *KLF4* as well as decreased expression of GSC differentiation markers *GFAP* and *TUBB3* and a tumor suppressor *CDKN2A*. Furthermore, induction of GSC differentiation was associated with increased global m^{6}A levels. Our data thus indicate that m^{6}A methylation activity suppresses GBM tumorigenesis, whereas m^{6}A demethylation activity promotes it [48]. However, Visvanathan et al. found that expression of *METTL3* and the m^{6}A levels were higher in GSCs as compared to differentiated glioma cells (DGCs), suggesting that METTL3 and its methylation activity could promote the CSC state of glioma cells [49]. Silencing of *METTL3* in various GSC lines impaired sphere formation ability in vitro and tumorigenesis in vivo as well as suppressed the expression of a stemness-associated marker *SSEA1* [49]. These apparently conflicting results between our study and that of Visvanathan et al. indicate the complexity of m^{6}A-mediated regulation of the GSC state, which likely depends on the delicate balance of global m^{6}A methylation and is context specific. Indeed, different mechanisms of METTL3-mediated regulation of the GSC state have been described. We found that silencing of METTL3 was associated with reduced m^{6}A methylation but increased the expression of *ADAM19* mRNA, encoding an oncogenic metalloproteinase disintegrin [48]. Visvanathan et al. found that METTL3-mediated m^{6}A methylation of *SOX2* mRNA enabled human antigen R (HuR) binding to *SOX2*, leading to its stabilization and enhanced expression [49]. Lv et al. demonstrated that METTL3 inhibited mitophagy by promoting degradation of an autophagy adaptor *OPTN* mRNA in an m^{6}A-dependent manner, and enhanced GSC tumorigenicity [50]. In addition to METTL3, m^{6}A reader and eraser proteins have also been shown to regulate the GSC state. Dixit et al. found that YTHDF2 promoted GSC proliferation and sphere formation ability in vitro as well as tumorigenesis in vivo, whereas knockout of *YTHDF2* was associated with

decreased expression of GSC markers *SOX2* and *OLIG2* [47]. Mechanistically, YTHDF2 interacted with *MYC* and *VEGFA* mRNAs in an m^{6}A-dependent manner, leading to their stabilization and increased expression [47]. Moreover, Fang et al. found that YTHDF2 promoted the degradation of the transcription factor *LXRA* and *HIVEP2* mRNAs in an m^{6}A-dependent manner, disrupting cholesterol homeostasis and promoting GSC tumorigenicity [51]. Zhang et al. found that ALKBH5 promoted GSC proliferation and sphere formation ability in vitro, maintained the expression of pluripotency factors *SOX2*, *NANOG*, and *OCT4*, and suppressed GSC differentiation towards astrocytic and neuronal lineages upon serum exposure [52]. Mechanistically, ALKBH5 demethylated *FOXM1* mRNA, encoding a transcription factor involved in GBM tumorigenesis, and enabled HuR binding to *FOXM1*, leading to *FOXM1* stabilization, whereas m^{6}A methylation prevented such stabilization [52]. Interestingly, a long non-coding RNA (lncRNA) *LOC100507424* that shares complementarity with the *FOXM1* transcript enhanced ALKBH5-mediated m^{6}A demethylation of *FOXM1*, revealing a novel role for lncRNAs in modulating the m^{6}A epitranscriptome [52].

2.3.1.3 The Roles of m^{6}A in CSCs of Other Tumors

While LSCs and GSCs have served as well-characterized models of CSC biology, evidence for the importance of the m^{6}A epitranscriptome in shaping the CSC state in cancers other than leukemia and GBM is rapidly accumulating. Studying colorectal carcinoma (CRC), Li et al. found that METTL3 was required for the maintenance of the CSC pool as silencing of *METTL3* in SW620 and HCT116 CRC cell lines reduced stem cell frequency, sphere formation ability, and the expression of CSC markers, including epithelial cell adhesion molecule (*EpCAM*), *CD133*, and *CD44* [53]. Mechanistically, METTL3-mediated m^{6}A methylation of *SOX2* mRNA enabled *SOX2* interaction with IGF2BP2, which stabilized *SOX2* mRNA and thus promoted *SOX2* expression [53]. In bladder cancer, Gu et al. found that m^{6}A levels were lower in CD44$^+$ tumor-initiating cells (TICs) than in non-TIC bladder cancer cells [54]. Knockout of *METTL14* in primary CD44$^+$ bladder cancer TICs promoted their proliferation, sphere formation ability, and invasion in vitro as well as tumor initiation in vivo. Moreover, the loss of *METTL14* was associated with increased expression and stability of *NOTCH1* mRNA but the role of m^{6}A in such stabilization remains to be confirmed [54]. In hepatocellular carcinoma (HCC), Zhang et al. found that YTHDF2 promoted sphere formation ability of Hep3B and Huh7 HCC cells and maintained the CD133$^+$ liver CSCs [55]. Mechanistically, YTHDF2 promoted the translation of *OCT4* mRNA in an m^{6}A-dependent manner [55]. In melanoma, Xu et al. found that expression of the m^{6}A reader *YTHDF3* was increased in CD133$^+$ melanoma CSCs (isolated from CRMM1 and Mum2B cells lines) as compared to non-CSCs [56]. YTHDF3 was required for the maintenance of the ALDH$^+$ cell subpopulation of melanoma stem cells as well as promoted *SOX2* expression, sphere formation ability in vitro, and tumorigenesis in vivo. Mechanistically, YTHDF3 interacted with *CTNNB1* mRNA, encoding a transcription factor involved in melanoma tumorigenesis, and enhanced *CTNNB1* translation efficiency in an m^{6}A-dependent manner

[56]. In ovarian cancer, Huang et al. found that expression of *FTO* was decreased in $ALDH^+/CD133^+$ CSCs isolated from OVCAR5 and COV362 ovarian cancer cell lines [57]. FTO suppressed OVCAR5 cell sphere formation ability in vitro and tumor initiation in vivo. Moreover, *FTO* expression negatively correlated with the expression of pluripotency markers *NANOG*, *SOX2*, and *OCT4* as well as the CSC marker *ALDH1A1*. Mechanistically, FTO demethylated *PDE4B* and *PDE1C* mRNAs, encoding phosphodiesterases, and promoted their degradation, leading to increased cAMP levels and impaired cAMP signaling [57].

2.3.1.4 The Roles of m^6A in EMT

The dynamic m^6A epitranscriptome also influences cancer cell state transitions that are required for EMT, invasion, and metastasis. Although EMT may not necessarily require CSCs, cancer cells undergoing EMT often exhibit stem-like properties [9]. The rewiring of gene expression programs is an important aspect of EMT and is, in part, mediated by m^6A (Fig. 2.1). By treating HeLa and HepG2 cancer cell lines with TGFβ—an inducer of EMT—Lin et al. found that global mRNA m^6A levels increased in the cells undergoing EMT [58]. Deletion of *METTL3* was associated with decreased HeLa cell invasion vitro and increased expression of E-cadherin. Mechanistically, YTHDF1 interacted with m^6A-methylated *SNAI1* mRNA, encoding an EMT master regulator SNAIL, and enhanced *SNAI1* translation efficiency [58]. Yue et al. found that METTL3 suppressed E-cadherin expression and promoted mesenchymal marker expression as well as enhanced invasion of various gastric cancer cell lines in vitro and metastasis in vivo [59]. Mechanistically, m^6A methylation of the transcription factor *ZMYM1* mRNA enabled *ZMYM1* interaction with HuR, which stabilized *ZMYM1* and thus promoted its expression. In turn, ZMYM1 recruited the repressive transcriptional regulation complex CtBP/LSD1/CoREST to the promoter of E-cadherin and repressed its transcription [59]. Liu et al. found that *MIR100HG* lncRNA promoted EMT and metastasis in various CRC cell lines by interacting with an m^6A reader hnRNPA2B1 [60]. Specifically, *MIR100HG*/hnRNPA2B1 interacted with *TCF7L2* mRNA, encoding a transcriptional co-activator of the Wnt signaling pathway, and stabilized *TCF7L2* in an m^6A-dependent manner. Stabilization of *TCF7L2* was associated with decreased expression of E-cadherin and increased expression of mesenchymal markers in CRC cells [60].

2.3.1.5 The Roles of m^6A in Drug Resistance

The distinct features of CSCs, such as quiescence, plasticity, elevated expression of drug efflux pumps, and the ability to resist DNA damage, enhance the evasive capabilities of CSCs and mediate drug resistance. Moreover, CSCs exploit the RNA modification machinery and m^6A methylation to drive gene expression programs associated with drug resistance. Visvanathan et al. found that METTL3 conferred radioresistance to GSCs, as γ-irradiation of *METTL3*-silenced GSCs suppressed sphere formation more efficiently than did γ-irradiation of wild-type GSCs [49]. Mechanistically, GSC radioresistance resulted from m^6A-dependent stabilization of *SOX2* mRNA, leading to increased expression of *SOX2*, which in

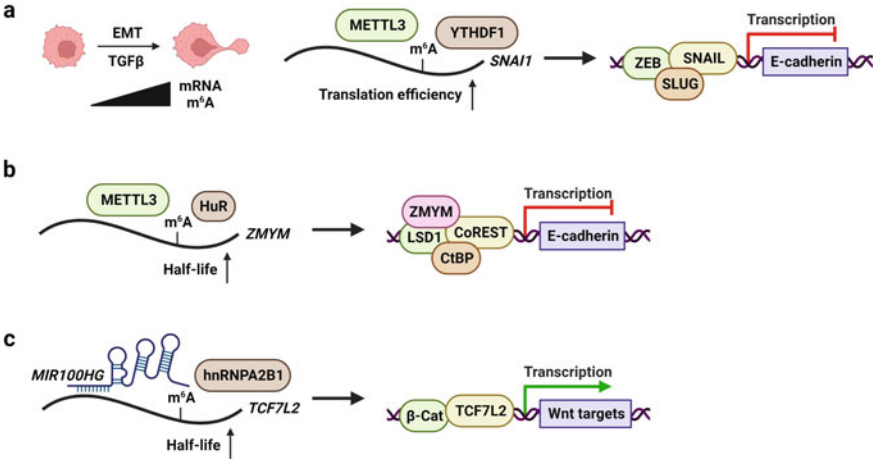

Fig. 2.1 The roles of m^{6}A in epithelial-to-mesenchymal transition (EMT). **a** Lin et al. found that HeLa and HepG2 cells undergoing TGFβ-induced EMT had higher mRNA m^{6}A levels as compared to untreated cells. Mechanistically, an m^{6}A reader YTHDF1 interacted with m^{6}A-methylated *SNAI1* mRNA, encoding an EMT master regulator SNAIL, and enhanced its translation efficiency in HeLa cells. In turn, SNAIL suppressed the expression of E-cadherin and promoted EMT. **b** Yue et al. found that an RNA-binding protein human antigen R (HuR) interacted with m^{6}A-methylated *ZMYM1* mRNA, encoding a transcriptional regulator, and stabilized *ZMYM1*, promoting its expression in gastric cancer cells. In turn, ZMYM1 interacted with a repressive transcriptional regulation complex CtBP/LSD1/CoREST and suppressed the expression of E-cadherin. **c** Liu et al. found that a long non-coding RNA *MIR100HG* and an m^{6}A reader hnRNPA2B1 interacted with m^{6}A-methylated *TCF7L2* mRNA, encoding a transcriptional co-activator of the Wnt signaling pathway, and stabilized *TCF7L2*, promoting its expression in colorectal carcinoma cells. In turn, TCF7L2 activated the Wnt signaling pathway and promoted EMT.

turn prevented the formation of γ-H2AX foci in GSCs [49]. Li et al. found that METTL3 was also required for CRC cell resistance to oxaliplatin [53]. Tang et al. found that expression of *ALKBH5* was decreased in the tissues of pancreatic ductal adenocarcinoma (PDAC) treated with gemcitabine as compared to untreated tumors, whereas overexpression of *ALKBH5* sensitized various PDAC cell lines to gemcitabine [61]. Subsequently, Zhang et al. showed that ALKBH5 negatively regulated the expression of *DDIT4-AS1* lncRNA in an m^{6}A-dependent manner, whereas depletion of *DDIT4-AS1* decreased the CD133$^+$ PDAC CSC population and sensitized PDAC cells to gemcitabine [62]. M^{6}A methylation has also been implicated in immune evasion and immunotherapy resistance. Paris et al. found that YTHDF2 mediated the resistance of preleukemic cells to tumor necrosis factor (TNF)-induced apoptosis [34]. Su et al. found that FTO mediated AML cell resistance to T cells by positively modulating the expression of the immune checkpoint regulator *LILRB4* [41]. Importantly, pharmacological inhibition of FTO with CS1 and CS2 sensitized human AML cells to T cell cytotoxicity [41]. Yan et al. found that FTO also mediated AML cell resistance to tyrosine kinase inhibitor (TKI) therapy by positively regulating the expression of pro-survival genes, including *BCL2*

and *MERKT*, whereas pharmacological inhibition of FTO with a small-molecule compound rhein sensitized AML cells to TKIs [63]. Moreover, Yang et al. found that FTO mediated the resistance of B16F10 melanoma cell line-derived tumors to anti-PD-1 antibody immunotherapy as well as to IFNγ-mediated cell killing in vivo [64].

2.3.2 N^7-methylguanosine (m^7G)

M^7G is a ubiquitous RNA modification that is best known for its role as the 5' cap of mRNA transcripts [65]. M^7G is also installed internally in several classes of RNA, such as mRNA and transfer RNA (tRNA), by an m^7G writer protein complex composed of METTL1 and WDR4 [66]. Although no reader or eraser proteins of m^7G have been identified thus far, its structural roles are evident. In tRNA, m^7G stabilizes the tertiary structure to maintain correct mRNA translation programs, whereas silencing of *METTL1* is associated with decreased expression of m^7G-modified tRNAs. Decreased expression of m^7G-modified tRNAs leads to suppressed global protein translation and especially translation of transcripts enriched for codons recognized by the affected tRNAs [66]. Notably, expression of *METTL1* is increased in various tumors as compared to normal controls (reviewed in refs. [66, 67]). Orellana et al. found that ectopic expression of METTL1 in embryonic murine fibroblasts carrying the SV40 T antigen promoted colony formation ability in vitro and tumorigenesis in vivo in an m^7G-dependent manner, indicating that METTL1 acts as an oncogene that can facilitate tumorigenic transformation [68]. Mechanistically, METTL1 enhanced the translation efficiency of genes enriched for AGA codons, with the corresponding tRNA *Arg-TCT* m^7G methylated and dysregulated in various tumors, indicating a potential oncogenic role for *Arg-TCT* under METTL1 control [68]. Chen et al. found that METTL1 promoted head and neck squamous cell carcinoma (HNSCC) cell proliferation, colony formation ability, invasion, and migration in vitro in an m^7G-dependent manner [69]. Interestingly, single-cell profiling of tumor transcriptomes revealed that *Mettl1* and *Wdr4* expression positively correlated with the CSC score, indicating that METTL1 and m^7G could promote the CSC state in HNSCC. Mechanistically, METTL1 positively regulated the PI3K/AKT/mTOR signaling pathway, promoting expression of its downstream effectors, including cyclin D1 and BCL2 [69]. Finally, Xia et al. found that WDR4 promoted HCC cell proliferation, invasion, and migration in vitro as well as tumor progression in vivo [70]. WDR4 also promoted expression of mesenchymal markers, suppressed expression of E-cadherin, and mediated HCC cell resistance to sorafenib [70].

2.3.3 Pseudouridine

Pseudouridine (Ψ) is another ubiquitously installed RNA modification that confers distinct structural features to the decorated transcripts in a context-dependent

manner [71, 72]. Although Ψ writer proteins, including dyskerin 1 (DKC1) and pseudouridine synthases (PUS), have been identified, it remains unknown whether any Ψ eraser proteins exist and whether Ψ is a reversible modification. Mutations in genes encoding Ψ writer proteins have been linked to developmental diseases, indicating the importance of Ψ to cellular programs governing differentiation [71]. Recently, dysregulation of the Ψ epitranscriptome has been also implicated in cancer progression and CSC biology. We found that PUS7 promoted GSC sphere formation ability in vitro and tumorigenesis in vivo in a Ψ-dependent manner [73]. Moreover, expression of *PUS7* was higher in GSCs as compared to NSCs or astrocytes. Mechanistically, PUS7 pseudouridylated certain tRNAs at position 50, leading to reduced translation efficiency of transcripts enriched for codons recognized by these tRNAs (Fig. 2.2a). Such codon-biased translation resulted in low expression of a tumor suppressor tyrosine kinase 2 (TYK2) in GSCs, promoting GSC tumorigenicity [73]. Guzzi et al. uncovered an alternative mechanism for Ψ-dependent regulation of protein translation involving tRNA fragments, termed mTOGs [74, 75]. The authors found that mTOGs—when pseudouridylated by PUS7 at position 8—suppressed aberrant protein translation by displacing the translation initiation factor eIF4A/G from the cap complex (Fig. 2.2b) [74]. Moreover, pseudouridylated mTOGs interacted with a poly(A)-binding protein PABPC1, hindering the recruitment of its partner PAIP1 and thus inhibiting translation [75]. Importantly, somatic loss of *PUS7* in myelodysplastic syndrome (MDS) patients with monosomy 7 (the *PUS7* gene is located on chromosome 7) was associated with decreased mTOG levels in HSPCs and increased protein translation in differentiated mononuclear cells [74]. Low levels of mTOGs increased the risk for leukemic transformation, whereas transfection of high-risk MDS HSPCs with pseudouridylated mTOGs reduced the malignant $CD34^+CD45RA^+CD123^+$ stem cell population and improved HSPC differentiation upon transplantation in vivo [75]. Given the importance of PUS7-mediated pseudouridylation in the maintenance of CSCs and tumorigenesis, we have identified a small-molecule inhibitor of PUS7, termed C17 (NSC107512), which impairs GSC cell proliferation in vitro and suppresses GBM tumorigenesis in vivo [73]. In addition to the RNA-independent mechanism of pseudouridylation by standalone PUS enzymes, RNA-dependent pseudouridylation catalyzed by DKC1 and associated guide RNAs also modulates tumorigenic transformation and cancer progression. Beneventi et al. found that expression of small Cajal body-specific RNA (scaRNA) *SCARNA15* was increased upon MYC-driven malignant transformation of human fibroblasts [76]. Depletion of *SCARNA15* in HEK293T cells abolished pseudouridylation of U2 small nuclear RNA (snRNA) at position 39, leading to dysregulated global splicing and suppressed anchorage-independent growth [76]. Moreover, McMahon et al. found that depletion of *SNORA24*—a small nucleolar RNA (snoRNA) required for pseudouridylation of 18S ribosomal RNA (rRNA) at positions 609 and 863—promoted RAS-driven HCC transformation in vivo [77]. Mechanistically, low expression of *SNORA24* was associated with reduced Ψ levels of 18S rRNA, affecting the biophysical properties of ribosomes and mRNA decoding [77]. These data suggest that decreased pseudouridylation of the RNA

component of ribosomes can promote HCC tumorigenesis, although the causal role of Ψ remains to be confirmed.

2.3.4 A-to-I Editing

Inosine is installed by adenosine deaminases acting on RNA (ADARs), especially ADAR1, that convert adenosines into inosines in a process termed A-to-I editing [78]. The installed inosine can affect the local structure of RNA and is subsequently read as guanine during translation, which may lead to incorporation of an alternative amino acid. Although the major role of A-to-I editing has been attributed to the modulation of the innate immune response, aberrant *ADAR1* expression and A-to-I editing activity can also promote cancer initiation and progression (reviewed in 79–81) [79–81]. Jiang et al. found that the expression of the p150 isoform of *ADAR1* was increased in the blast crisis phase of chronic myeloid leukemia (CML) as compared to normal cord blood, whereas silencing of p150 *ADAR1* in blast crisis CML progenitors suppressed their serial transplantation potential in vivo [82]. In their subsequent study, Jiang et al. demonstrated that ADAR1 suppressed maturation of *pri-miR-26a* into a tumor suppressor microRNA *miR-26a-5p* in an A-to-I editing-dependent manner [83]. Moreover, the authors found that A-to-I editing of the 3'-untranslated region (3'-UTR) of *MDM2* mRNA, encoding a negative regulator of p53, prevented *miR-155*-mediated silencing of *MDM2* expression [83]. Similarly, Zipeto et al. found that ADAR1 enhanced colony formation ability of chronic phase CML progenitors in vitro and engraftment of normal $CD34^+$ progenitors in vivo [84]. Mechanistically, ADAR1 negatively regulated the biogenesis of a tumor suppressor miRNA *let-7* in an A-to-I editing dependent manner [84]. These findings reveal a prominent role for ADAR1-mediated A-to-I editing in miRNA biogenesis and function during CML progression [82, 84]. In multiple myeloma, Lazzari et al. found that ADAR1-mediated A-to-I editing modulated the activity of the Hedgehog signaling pathway, which is often activated in CSCs [85]. In particular, A-to-I editing of *GLI1* mRNA, encoding a transcription factor that regulates the Hedgehog signaling pathway, affected the transcriptional activity of the GLI1 protein. In particular, the GLI1-R701G protein translated from edited *GLI1* mRNA activated a *GLI1*-promoter driven reporter more efficiently than did the GLI1 protein translated from unedited *GLI1* mRNA [85]. In GBM, Jiang et al. found that A-to-I editing events were more frequent in GSCs as compared to NSCs [86]. Likewise, the expression of *ADAR1* was increased in GSCs as compared to DGCs. ADAR1 promoted GSC proliferation and sphere formation ability in vitro as well as tumorigenesis in vivo. Mechanistically, A-to-I editing of *GM2A* mRNA, encoding a glycolipid transport protein, enhanced *GM2A* expression; GM2A activity in ganglioside catabolism was required to maintain the stem-like state of GSCs [86].

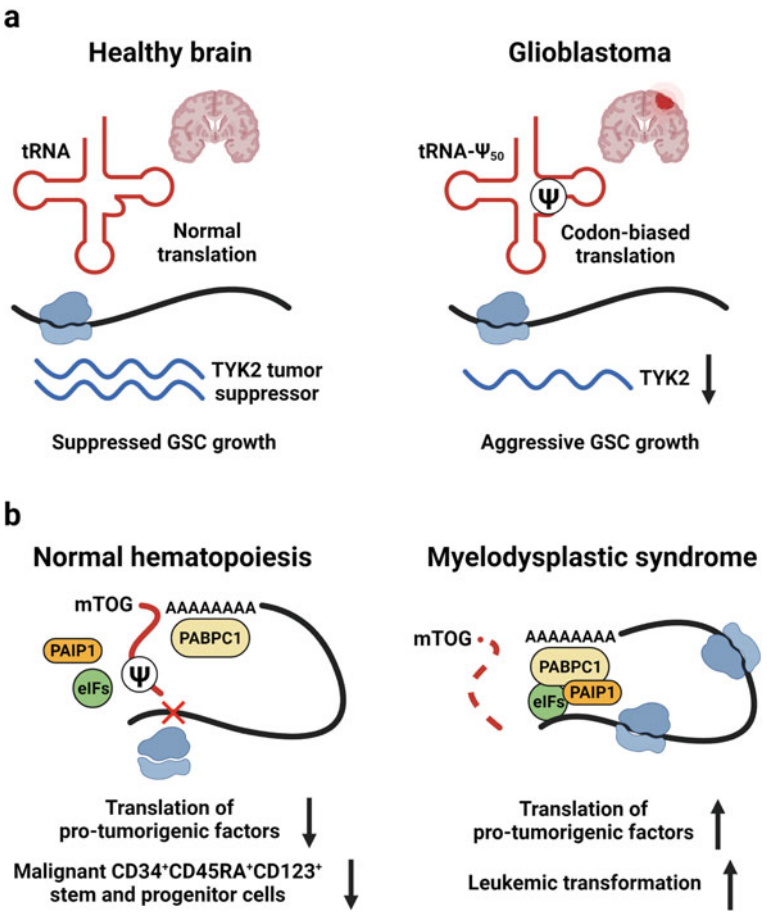

Fig. 2.2 The roles of Ψ in tumorigenesis and CSCs. **a** We found that expression of a Ψ writer *PUS7* was increased in glioblastoma stem cells (GSCs) as compared to normal neural stem cells (NSCs) and promoted glioblastoma tumorigenesis in a Ψ-dependent manner. Aberrant pseudouridylation of certain tRNAs at position 50 resulted in codon-biased translation, especially of transcripts that were enriched for codons decoded by the affected tRNAs. Consequently, expression of a tumor suppressor tyrosine kinase 2 (TYK2) was decreased in GSCs, promoting aggressive tumor growth. **b** Guzzi et al. found that tRNA fragments, termed mTOGs, critically regulated protein translation in hematopoietic stem and progenitor cells (HSPCs) in a Ψ-dependent manner. Specifically, mTOGs pseudouridylated at position 8 suppressed aberrant protein translation by preventing the interaction between a poly(A)-binding protein PABPC1 and its partner PAIP1. Moreover, pseudouridylated mTOGs displaced the translation initiation factor eIF4A/G from the cap complex. However, decreased expression of *PUS7* in patients with myelodysplastic syndrome (MDS) was associated with reduced mTOG levels and aberrant translation of pro-tumorigenic factors, promoting leukemic transformation. Transfection of high-risk MDS HSPCs with pseudouridylated mTOGs restored normal protein translation and suppressed the malignant $CD34^+CD45RA^+CD123^+$ stem cell population.

2.4 Mechanisms Underlying Dysregulation of RNA Modification Machinery

As described in the previous sections, remodeling of the RNA epitranscriptome in cancer primarily arises because of dysregulation of RNA modification machinery. It is thus important to define the upstream events that affect expression and activity of RNA modification machinery and to determine if these events could be targeted therapeutically. Among such events, metabolic reprogramming [64], hypoxia [87], chromatin remodeling [88], chromosome abnormalities and mutations [74, 75, 89, 90], inflammatory signaling [84], growth factor signaling [50, 51], and oncogene activity [70] have been shown to modulate expression and activity of RNA modification machinery. Yang et al. demonstrated that exposure of Mel624 melanoma cells to metabolic stress, such as starvation medium or low serum medium, was associated with increased expression of *FTO* and decreased global m^6A levels [64]. The authors found that autophagy mediators ATG5 and ATG7 as well as the NF-κB signaling pathway mediated starvation medium-induced upregulation of *FTO* [64]. Moreover, both m^6A erasers FTO and ALKBH5 require oxygen for their enzymatic reactions and contain catalytic iron centers; therefore, it can be hypothesized that oxygen availability under hypoxic tumor conditions as well as addiction of tumor cells, including GSCs, to iron metabolism may affect FTO and ALKBH5 catalytic activities [91–93]. Zhang et al. found that various breast cancer cell lines upregulated *ALKBH5* mRNA levels in an HIF-dependent manner when exposed to hypoxia [87]. Mechanistically, the hypoxic exposure of breast cancer cells was associated with decreased m^6A methylation of *NANOG* mRNA, promoting *NANOG* stabilization and expression. Silencing of *ALKBH5* suppressed hypoxia-mediated stabilization of *NANOG* as well as impaired ALDH1 activity and sphere formation ability of breast cancer cells [87]. Wang et al. found that chromatin remodeling could also affect expression of *ALKBH5* [88]. In particular, the genomic locus of *ALKBH5* was more accessible in $Lin^-CD34^+CD38^-$ LSCs as compared to normal human HSPCs; increased *ALKBH5* accessibility was in turn associated with higher *ALKBH5* mRNA levels in LSCs [88]. In addition to chromatin remodeling, chromosomal abnormalities may also affect expression of RNA modification machinery at DNA level. Guzzi et al. found that chromosome 7 monosomy frequently observed in MDS was associated with low *PUS7* expression in HSPCs because the *PUS7* gene is encoded by the chromosome 7 [74]. Likewise, mutations in genes encoding RNA modification machinery can disable their function and catalytic activity. Liu et al. identified a mutated METTL14-R298P variant, which was associated with decreased mRNA m^6A methylation levels in endometrial cancer tissues as compared to normal controls [90]. An in vitro enzymatic activity assay revealed impaired METTL14-R298P catalytic activity [90]. Extrinsic factors that activate intracellular signaling cascades can also influence expression of RNA modification machinery. Zipeto et al. found that inflammatory signaling via the JAK/STAT pathway enhanced *ADAR1* expression, with STAT transcription factors known to bind directly to the *ADAR1* promoter [84, 94]. Indeed, ectopic expression of *JAK2* in $CD34^+$ human HSPCs promoted *ADAR1*

expression and A-to-I editing activity, whereas pharmacological inhibition of JAK2 in K562 cells suppressed A-to-I editing activity [84]. Fang et al. found that epidermal growth factor receptor (EGFR) signaling induced the expression of *YTHDF2* in GSCs, whereas pharmacological inhibition of EGFR reduced protein but not mRNA levels of YTHDF2 [51]. Mechanistically, EGFR signaling-mediated stabilization of YTHDF2 was dependent on the activity of EGFR signaling effectors SRC and ERK1/2, whereas ERK1/2 phosphorylated YTHDF2 directly. Notably, the EGFR signaling pathway is often aberrantly activated in GBM, indicating tumor-specific dependencies that modulate activity of RNA modification machinery [95]. Lv et al. identified platelet-derived growth factor (PDGF) signaling as a positive regulator of *METTL3* expression in GSCs, whereas pharmacological inhibition of PDGF signaling was associated with decreased global m^6A methylation levels [50]. Mechanistically, the authors found that PDGF signaling activated *METTL3* expression via a transcription factor EGR1 that was shown to directly interact with a reporter harboring the promoter region of *METTL3* [50]. Finally, Xia et al. found that expression of *WDR4* in HCC was under the control of the MYC oncogene, which directly bound the *WDR4* promoter and activated *WDR4* transcription [70]. Given that expression of *MYC* is upregulated in various tumors, this finding indicates that oncogenes may play a direct role in the remodeling of the RNA epitranscriptome.

2.5 Considerations for Studying the Roles of RNA Modifications in CSC Biology

The rigor and reproducibility of RNA modification research in CSC biology is highly dependent on the quality of CSC preparation and handling. Whether cell lines or primary tumor tissues are used for CSC isolation, it is important to clearly define the CSC enrichment and characterization strategies, such as by specific marker expression and functional experiments. It is worth noting that different culture techniques can notably affect the status and purity of CSCs. For example, culturing mixed cancer cell populations in spheres tends to enrich for CSCs, whereas culturing in adherent monolayers promotes differentiation and expansion of non-CSCs [55, 87]. Such technical differences may result in distinct RNA epitranscriptomes and thus influence the downstream molecular analyses. Indeed, Huh7 and Hep3B HCC cells cultured in non-adherent spheres have higher expression of *YTHDF2* as compared to the same cells cultured in adherent monolayers [55]. Similarly, MDA-MB-231 and MCF-7 breast cancer cells have higher expression of *ALKBH5* when cultured in non-adherent spheres as compared to adherent monolayers [87]. Unique conditions associated with in vitro culture of CSCs, such as high oxygen and glucose levels as compared to tumor microenvironment, may also affect the functions of RNA modification machinery. As described earlier, oxygen levels have a major effect on the m^6A RNA epitranscriptome and may affect the m^6A modifiers that use oxygen in their enzymatic reactions, such as ALKBH5 and FTO [87, 91]. Finally, standardization of CSC characterization, both

in terms of their gene expression profiles and functional properties, would enhance reproducibility of CSC research as well as aid the design of clinical trials targeting CSCs. We anticipate that the rapidly advancing technology of high-throughput profiling of tumor tissues at single-cell level combined with in silico tools for characterization of tumor evolution will help better define the CSC state and its dependencies as well as refine the experimental approaches for studying CSCs.

2.6 Perspective

Our understanding of RNA modifications has greatly improved over the past decade. This can be partially attributed to the constantly improving high-throughput profiling methods that have enabled identification of RNA modification sites with high confidence [96, 97]. Indeed, a wealth of datasets is readily available as an invaluable resource for detailed mechanistic studies of molecular events driven by RNA modifications [98–101]. Moreover, development of experimental techniques that enable precise manipulation of individual RNA modifications will facilitate the elucidation of causal relationships between RNA modifications and downstream consequences. For example, catalytically inactive Cas13-based tools of epitranscriptomic editing for installation or removal of m^6A at desired RNA sites have been recently developed [102]. Another approach, termed single-cell CRISPR tiling, originally designed for interrogating gene regulatory elements and protein domains, enables mutational analysis in a high-throughput manner and could be applied for probing the functions of individual RNA modifications along the entire transcript [103, 104]. Finally, uncovering the roles of other RNA modifications, including N^1-methyladenosine and 5-methylcytosine, in CSC biology, as well as discovering additional effectors of the RNA modification machinery will further advance the knowledge of the regulatory roles of RNA epitranscriptomics and reveal novel targets for therapeutic development.

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3 Recent Advances of RNA m^{6}A Modifications in Cancer Immunoediting and Immunotherapy

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3.1 Introduction

Cancer has become a major public health problem worldwide and is expected to rank as the leading cause of death. According to the latest cancer statistics by the American Cancer Society, 1,958,310 new cancer cases and 609,820 cancer deaths are expected to occur in the United States by 2023 [1]. With rapid population growth and aging, as well as changes in the prevalence and distribution of the main risk factors for cancer, the incidence and mortality of cancer are progressively growing worldwide [2]. Cancer is a complex disease in which the outcome depends largely on the cross-talk between the tumor and its microenvironment, for example, the immune system. The first attempt of modulating the immune system to cure cancer can be traced back over a century ago [3]. It is now appreciated that the immune system plays a dual role in cancer; it not only conveys protective immunity but also facilitates malignant progression. This concept is now recognized as a fundamental theory of cancer immunology called "cancer immunoediting" [4, 5], which was developed from the cancer immunosurveillance theory formulated by Burnet and Thomas in 1957 [6–8]. Recently, a number of therapeutic approaches

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have been developed to unleash the immune system and control malignancy, such as immune checkpoint inhibitors (ICIs) and chimeric antigen receptor (CAR) T cell therapy, which have shown promising efficacy against certain cancers in the clinic [9, 10]. The recognition of the cancer immunotherapy field was convincingly sealed by the 2018 Nobel Prize awarded to James Allison and Tasuku Honjo for their ground-breaking work on ICIs as potential cancer therapeutic targets. However, despite the enormous promise of immunotherapy, only a small proportion of patients with limited subsets of cancer are responsive to immunotherapy [11–13]. In fact, our understanding of tumor immunity is only the tip of the iceberg, and many crucial questions and challenges remain and need to be addressed to achieve the great promise of cancer immunotherapy.

Epigenetic regulation, which includes DNA methylation, histone modification, and chromatin remodeling that alter DNA accessibility and chromatin structure, is largely involved in programming gene expression in diseases, including cancer [14]. Epigenetic therapy modulates both immune cells and tumor cells by preventing immune exhaustion and reverting cancer immunoediting [15–17]. The combination of ICIs with DNA methyltransferase inhibitors or histone deacetylases has been tested in clinical trials [18, 19]. Accordingly, epigenetic therapy has emerged as a promising combination partner for use with cancer immunotherapy. More recently, the discovery of mRNA modifications that powerfully impact transcript stability and translation adds another complex layer of gene regulation. So far, more than 170 types of chemical modifications have been identified in cellular RNAs. The most abundant and common internal mRNA modification is N^6-methyladenosine (m^6A) methylation [20]. Accumulating evidence indicates that RNA modifications play critical roles in almost every aspect of RNA metabolism as well as in various physiological and pathological processes, including immune system development and cancer pathogenesis [21–23]. Therefore, a deeper understanding of the mechanisms by which RNA modifications impact the cancer immunoediting process can provide insight into the therapeutic implications of targeting RNA modifications, making combined epigenetic therapy and immunotherapy an attractive approach to evade the limitations of immunotherapy alone.

In this chapter, we will provide a historical background of cancer immunoediting and immunotherapy and briefly introduce the regulatory factors of RNA modifications. We will also review and discuss the roles of RNA modifications in fine-tuning the innate and adaptive immune responses, as well as in regulating immune-associated molecules. Finally, we summarize the current state of the development of RNA modification-based immunotherapy for cancer.

3.2 Cancer Immunoediting and Immunotherapy

3.2.1 From Cancer Immunosurveillance to Cancer Immunoediting

Although cancer immunotherapy is a modern medical concept originating no later than a couple of decades ago, the first scientific attempts to modulate patients' immune systems to treat cancer can be traced back to 150 years ago, when two German physicians, Fehleisen and Busch, independently noticed significant tumor regression after erysipelas infection in cancer patients [24, 25]. A few decades later, in 1891, William Coley, who is known today as the Father of Immunotherapy, attempted to systematically study how to harness the immune system for treating cancer. He injected a mix of bacteria and bacterial products, which is known as Coley's toxins, into patients' tumors and yielded a remarkable result in more than 1,000 patients with several types of malignancies [26, 27]. However, Coley's toxins were gradually forgotten and even denied for several decades with the replacement of the more promising radiotherapeutic (1896) and chemotherapeutic (1942) approaches [28, 29]. Additionally, little was known about the composition and function of the immune system at that time. In 1909, even though Paul Ehrlich first conceived the concept that the immune system can recognize and eliminate nascent malignant cells, it was difficult to validate this idea at that time. It would take nearly five decades before the idea of immune control of cancer reemerged, with many advances in immunity and cancer research, such as the discovery of the histocompatibility antigens (1948) [30], the natural-selection theory of antibody formation (1955) [31], the acquired immune tolerance theory (1956) [32], the interferon (1957) [33, 34], the Bacille Calmette-Guérin (BCG) vaccine (1959) [35], tumor antigen (1965) [36], the existence of T cells and cellular immunity (1967) [37], dendritic cells (DCs, 1973) [38], and natural killer (NK) cells (1975) [39] (Fig. 3.1).

The milestone in cancer immunotherapy was the cancer immunosurveillance theory coined by Thomas and Burnet in 1957 [40]. The theory was mainly supported by the immune-mediated rejection of transplanted syngeneic tumors induced by chemical carcinogens or viruses [41, 42]. However, immunodeficient nude mice did not show an increased incidence of spontaneous or chemically induced tumors compared with wild-type mice [43–45], which significantly challenged this theory and led to the abandonment of the immunosurveillance hypothesis for two decades. It was not until the 1990s that the tumor immunosurveillance theory was revived (Fig. 3.1). Improved immunodeficient animal models, including mice lacking either interferon-γ (IFN-γ) or IFN-γ receptor, or signal transducer and activator of transcription (STAT)-1, the transcription factor required for IFN-γ receptor signaling, or perforin, or recombinase-activating gene 2 (RAG-2), showed that the immune system was involved in controlling primary tumor development, which supports the existence of cancer immunosurveillance [46–52]. In parallel to animal studies, observations in humans also support the

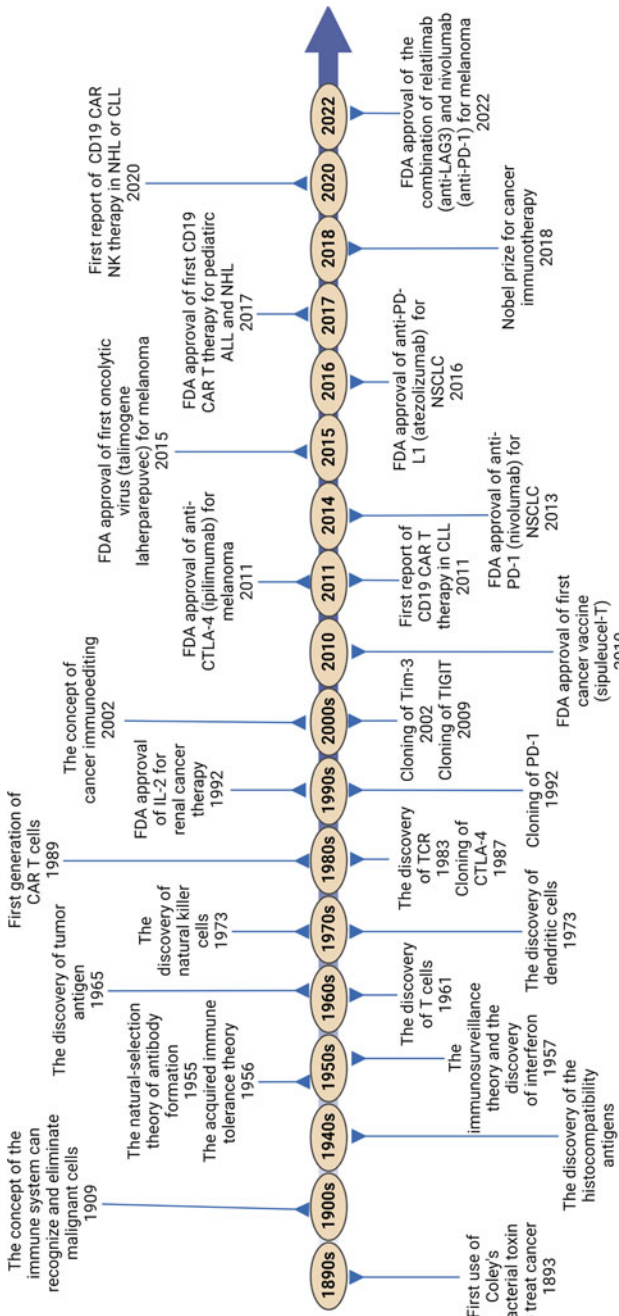

Fig. 3.1 **The history of cancer immunology and immunotherapy**. Abbreviations: CAR, chimeric antigen receptor; TCR, T-cell receptor; CTLA-4, cytotoxic T lymphocyte antigen-4; IL-2, interleukin-2; PD-1, programmed cell death-1; Tim-3, T cell immunoglobulin and mucin-domain containing-3; TIGIT, T cell immunoglobulin and ITIM domain; CLL, chronic lymphocytic leukemia; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand-1; ALL, acute lymphocytic leukemia; NHL, non-Hodgkin lymphoma; LAG-3, lymphocyte activation gene-3; NK cells, natural killer cells

presence of immunosurveillance in human cancers. Transplant patients or individuals with severe deficits of immunity have a higher risk of cancer development [53–55]. In addition, accumulating evidence demonstrates a positive correlation between immune cell infiltration and patient prognosis in many types of human cancers [56–58].

Although the immunosurveillance theory has been well-recognized since the 1990s, it could not completely explain why cancers still occur in immunocompetent individuals. Extensive studies showed that tumors derived from immunocompetent or immunodeficient mice grew avidly when transplanted into recipient immunodeficient mice [4, 59–61]. However, tumors derived from immunodeficient mice were nearly rejected when transplanted into immunocompetent mice, while tumors derived from immunocompetent mice grew progressively when transplanted into naive syngeneic immunocompetent hosts, indicating that tumors develop differently in the presence of the immune system [4, 59–61]. These findings also suggest that the immune system not only protects the host against tumor formation but also shapes tumor immunogenicity. In 2002, Robert Schreiber and colleagues coined the term "cancer immunoediting" to more accurately describe the dual role of the immune system [5] (Fig. 3.1). This dynamic process is composed of three phases: elimination, equilibrium, and escape [5, 62, 63]. The elimination phase is an updated version of immunosurveillance, in which both the innate and adaptive immune systems work together to recognize and eliminate tumor cells before they become clinically visible. Transformed cells express stress-induced molecules such as tumor antigens and natural killer group 2D (NKG2D) ligands, which can be recognized by $CD8^+$ T cells through antigen presentation by DCs and NK cells, respectively [59, 64]. These activated effector cells kill the tumor cells through a range of effector molecules, including IFN-α/β/γ [4, 47, 65, 66], perforin [48, 49], Fas/FasL [67], and TNF-related apoptosis-inducing ligand (TRAIL) [68]. However, the mechanisms by which the immune system controls the developing tumor are not fully understood. Occasional tumor cells that have evaded immunosurveillance will enter the equilibrium phase, in which the immune system prevents tumors from outgrowing and also sculpts tumor immunogenicity. In equilibrium, the immune system maintains tumor cells in a dormant state and applies selective pressure on the residual tumor cells, which will occur for a long time, perhaps throughout the lifetime of the host [69]. The molecular mechanisms that trigger immune-mediated tumor dormancy and immunogenicity remain poorly understood.

As a result of immunoediting, these tumor cells have acquired the ability to escape immune recognition and/or destruction and emerge as progressively growing even in the presence of an immune system. Tumor cell escape can occur through many different mechanisms, such as loss of antigens and major histocompatibility complex (MHC)-I proteins which make them become "invisible" to the immune system [70]. Tumors can also upregulate the expression of many resistant genes to defend against the cytotoxic effects of immunity, including anti-apoptotic molecule BCL-2 and pro-oncogenic transcription factor STAT3 [71, 72].

On the other hand, tumors can create an immunosuppressive "tumor microenvironment" by recruiting regulatory immune cells, such as regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), through various cytokines and chemokines, such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), interleukin 6 (IL-6), macrophage colony-stimulating factor (M-CSF), prostaglandin E2 (PGE2), C–C motif chemokine ligand 2 (CCL2), CCL17, CCL22, chemokine (C-X-C motif) ligand 2 (CXCL2), and CXCL12, to help protect the tumor from attack by the immune system [73, 74]. Evasion of tumor cells from immune destruction has been proposed as the eighth hallmark of cancer [75].

3.2.2 Immunotherapy: A New Road for Cancer Treatment

Recognizing the concept of cancer immunoediting laid the foundation for the development of many types of immune-based cancer therapies (Fig. 3.2), including monoclonal antibodies and ICIs, adoptive cell therapies (ACTs), oncolytic virus therapy, cancer vaccines, and non-specific immunotherapies such as interleukins and interferons [76]. Targeting ICIs, such as cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), and programmed cell death-1 ligand-1 (PD-L1), has demonstrated promising clinical efficacy and durable responses in a broad spectrum of cancers [77]. As of September 2022, the United States Food and Drug Administration (FDA) has approved eight ICIs that target CTLA-4, PD-1/PD-L1, or LAG-3, including ipilimumab (Yervoy), pembrolizumab (Keytruda), nivolumab (Opdivo), cemiplimab (Libtayo), atezolizumab (Tecentriq), avelumab (Bavencio), durvalumab (Imfinzi), and relatlimab (Opdualag) for the clinical treatment of 17 different types of malignancies, including advanced-stage melanoma, non-small cell lung carcinoma (NSCLC), and bladder cancer [78]. Apart from CTLA-4 and the PD-1/PD-L1, novel immune checkpoints, such as lymphocyte activation gene-3 (LAG-3) [79], T cell immunoglobulin and mucin-domain containing-3 (TIM-3) [80], B and T cell lymphocyte attenuator (BTLA) [81], T cell immunoglobulin and ITIM domain (TIGIT) [82], V-domain Ig suppressor of T cell activation (VISTA) [83], and leukocyte immunoglobulin-like receptor B4 (LILRB4) [84] have been gradually discovered, followed by strategies to target using specific monoclonal antibodies, some of which are being tested in the clinic.

Adoptive cell-therapy (ACT), also known as cellular immunotherapy, is a method of treatment that uses one's own immune cells to eliminate cancer, including tumor-infiltrating lymphocyte (TIL) therapy, engineered T cell receptor (TCR) therapy, CAR T or CAR NK cell therapy. The initial successful clinical applications of ACT are based on the use of autologous TILs in patients with metastatic melanoma [85] and allogeneic donor lymphocyte transfusions for patients with relapsed chronic myelogenous leukemia [86] during the 1980s. Today, ACTs, particularly CAR T and CAR NK cell therapy, are improving and providing new treatment options to cancer patients. The first CAR T cell therapy, Kymriah (tisagenlecleucel, CTL019; Novartis) received FDA approval for the treatment of

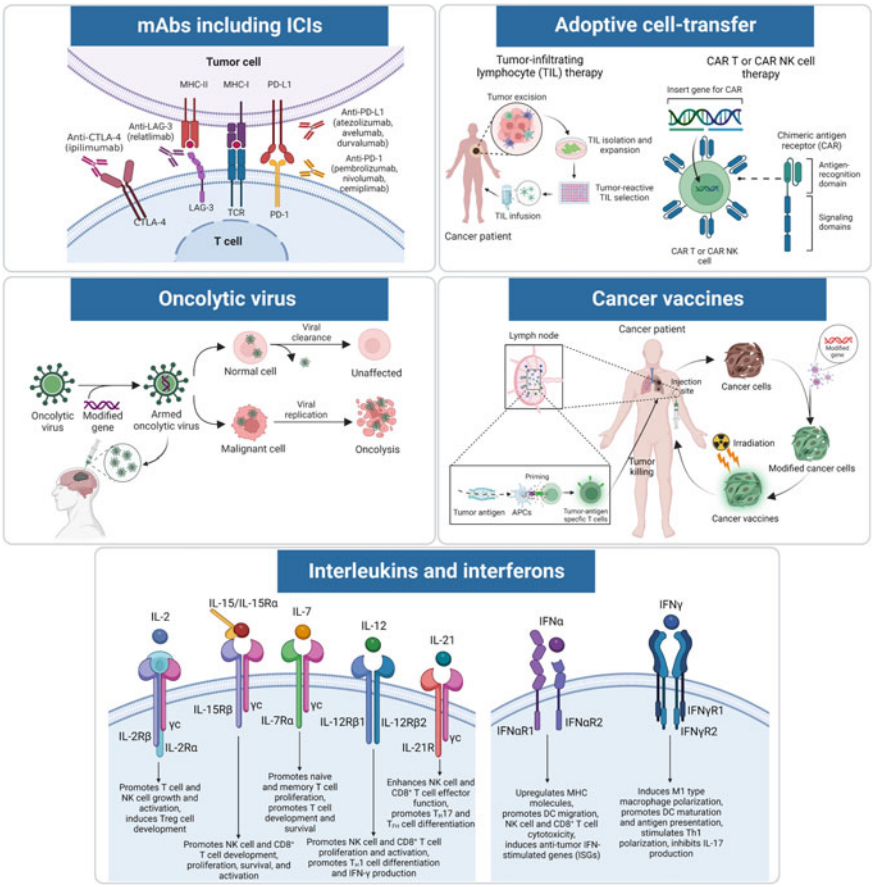

Fig. 3.2 Approaches for cancer immunotherapy. Cancer immunotherapies include the use of monoclonal antibodies (mAbs) and immune checkpoint inhibitors (ICIs), adoptive cell therapies (ACTs), oncolytic virus therapy, cancer vaccines, and non-specific immunotherapies such as interleukins and interferons. The United States Food and Drug Administration (FDA) has approved eight ICIs that target CTLA-4, PD-1/PD-L1, or LAG-3. Adoptive cell-transfer–based therapy is a method of treatment that uses one's own immune cells to eliminate cancer, including tumor-infiltrating lymphocyte (TIL) therapy and chimeric antigen receptor (CAR) T or CAR NK cell therapy. Oncolytic viruses are a form of immunotherapy that uses a genetically engineered or naturally existing virus that can selectively replicate in cancer cells and then kill them without affecting the normal cells. Cancer vaccines are made with cells from the patient's own tumor, which are genetically modified, irradiated in the lab, and then infused into the same patient to stimulate anti-tumor immunity with tumor antigens. Interleukins, including IL-2, IL-15, IL-7, IL-12, and IL-21 can promote NK cell and T cell development, differentiation, proliferation, survival, and activation, which enhance antitumor immunity. Interferons, such as IFN-α and IFN-γ, can upregulate MHC molecules, promote DC maturation and antigen presentation, and enhance NK cell and $CD8^+$ T cell cytotoxicity, which exert anti-tumor activity by boosting both innate and adaptive immune responses. Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen-4; LAG-3, lymphocyte activation gene-3; MHC, major histocompatibility complex; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; TCR, T-cell receptor; CAR, chimeric antigen receptor; NK, natural killer; APCs, antigen-presenting cells; MHC, major histocompatibility complex

patients with B-cell precursor acute lymphoblastic leukemia (B-ALL) in 2017. CD19-targeted CAR T cell therapy has induced complete remissions of disease in up to 90% of patients with relapsed or refractory B-ALL. Since 2017, the FDA has approved the use of six CAR T cell immunotherapies in the United States, including Kymriah (tisagenlecleucel, 2017), Yescarta (axicabtagene ciloleucel, 2017), Tecartus (brexucabtagene autoleucel, 2020), Breyanzi (lisocabtagene maraleucel, 2021), Abecma (idecabtagene vicleucel, 2021), and Carvykti (ciltacabtagene autoleucel, 2022) for the treatment of patients with ALL, diffuse large B-cell lymphoma, transformed follicular lymphoma, primary mediastinal or high-grade B-cell lymphoma, or multiple myeloma.

In the past five years, innovative CAR engineering strategies have led to a breakthrough in CAR T cell persistence and cancer cell clearance [87]. However, antigen escape, on-target off-tumor effects, and CAR T cell-associated toxicities, such as cytokine release syndrome (CRS) and neurologic toxic effects, still limit the therapeutic efficacy of CAR T cell therapy [87]. In addition, CAR T cell therapy is a rather expensive and personalized treatment option and usually time-consuming to manufacture. Therefore, it is of great interest to generate cost-effective, "universal", and "off-the-shelf" products. As such, CAR NK cells are an ideal candidate for this purpose [88]. Compared to CAR T cells, CAR NK cells offer several significant advantages in that they have low on-target/off-tumor toxicity, reduced risk for CRS, and less neurotoxicity [89]. CAR NK cells use multiple mechanisms to kill target tumor cells, such as in a CAR-dependent manner, CD16-mediated antibody-dependent cell-mediated cytotoxicity (ADCC), and natural cytotoxic activity against tumor cells. CAR NK cells have a reduced risk for graft-versus-host disease (GVHD) and thus are suitable to produce "off-the-shelf" products [90, 91]. Moreover, CAR NK cells can be generated from multiple sources, including the NK92 cell line, peripheral blood mononuclear cells (PBMCs), umbilical cord blood mononuclear cells (UCBMCs), $CD34^+$ hematopoietic progenitor cells (HPCs), and induced pluripotent stem cells (iPSCs) [90]. So far, CAR NK cells have shown promising effects for the treatment of certain types of cancer, including hematopoietic malignancies and solid tumors [89, 92–97].

Cancer immunotherapy has revolutionized cancer treatment and improved the survival of patients in the clinic. However, only approximately 30% of patients benefit from immune checkpoint therapies, and some patients are resistant to CAR T cell therapy [13, 98]. As a new regulatory layer of gene expression in post-transcriptional levels, m^6A RNA methylation has been shown to affect multiple aspects of mRNA metabolism. Therefore, elucidation of the epigenetic mechanisms of m^6A modifications in tumor cells and immune cells may help to better understand resistance mechanisms of immunotherapy and provide us with great opportunities for developing combinational therapies.

3.3 Regulatory Factors of RNA Modifications

To date, more than 170 chemical modifications have been shown in protein-coding and non-coding RNAs [99]. However, the functions and regulatory mechanisms of the majority of RNA modifications are less studied. It was not until 2012 when next-generation sequencing (NGS) methods were developed for the transcriptome-wide detection of m^{6}A modifications [100, 101].Several other common RNA modifications were also discovered and mapped, including N^6, 2-O-dimethyladenosine (m^{6}Am, 2015) [102], 5-methylcytosine (m^{5}C, 2012) [103], 5-hydroxymethylcytosine (hm^{5}C, 2016) [104], N^1-methyladenosine (m^{1}A, 2016) [105, 106], and N^4-acetylcytidine (ac4C, 2018) [107], 7-methylguanosine (m^{7}G, 2019) [108], adenosine-to-inosine editing (A-to-I, 2015) [109], and pseudouridine (ψ, 2014) [110, 111]. These RNA modifications can impact the sequence, structure, splicing, export, translation, and stability of target RNAs.

The biological functions of RNA modifications are mediated by a group of proteins, called RNA-modifying proteins, which comprises three groups: "writers", the enzymes that install RNA chemical marks; "erasers", the enzymes that remove them; and "readers", the proteins that selectively recognize and bind to specific RNA chemical modifications [22]. For example, m^{6}A is installed by RNA methyltransferases methyltransferase-like (METTL) 3, METTL14, and Wilms' tumor 1-associated protein (WTAP) (writers) [20], removed by the demethylases fat mass and obesity-associated protein (FTO) and alkB homolog 5 and RNA demethylase (ALKBH5) (erasers) [112, 113], and decoded by m^{6}A-binding proteins, such as YT521-B homology (YTH) domain-containing proteins and the insulin-like growth factor-2 mRNA-binding proteins (IGF2BPs) [114–116]. The proteins involved in RNA modifications and the dynamic control of RNA modifications in regulating gene expression have been extensively reviewed elsewhere as well as in this book [20, 117].

3.4 RNA Modifications in Fine-Tuning the Innate and Adaptive Immune Responses

The immunoediting theory demonstrates that both innate and adaptive immunity work together to recognize and eliminate tumor cells. Innate immune cells, including DCs and macrophages, can sense and recognize transformed cells by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectin receptors [118]. The release of endogenous molecules by dying tumor cells serves as danger-associated molecular patterns (DAMPs), which trigger DCs and other antigen-presenting cells (APCs) to secrete proinflammatory cytokines, and uptake and present tumor antigens for the induction of adaptive immune responses [118]. The adaptive immunity, comprised of CD4$^+$ and CD8$^+$ T cells, is crucial to the elimination of tumor cells in the late stage of host defense responses and generates more specific anti-tumor immunity, as well as induces long-term immune memory. Innate immune cells, including NK cells, NKT cells, γδ T

cells, and innate lymphoid cells (ILCs) also play critical roles in immune surveillance against tumors in the early stages [119–121]. Recently, numerous studies revealed that RNA modifications, mainly the m^6A modification, are significantly involved in regulating innate and adaptive immune responses, including immune cell development, differentiation, and effector functions (Fig. 3.3).

3.4.1 Dendritic Cells

DCs recognize antigens through DAMPs via PRRs. DCs exposed to modified RNA, such as nucleosides modified with m^5C, m^6A, m^5U, s^2U, or pseudouridine, express significantly fewer cytokines and activation markers than those treated with unmodified RNA [122], indicating that nucleoside modifications suppress the potential of RNA to activate DCs. Tumor cells are usually characterized by higher levels of RNA methylation [123]. This provides a logical rationale to use specific inhibitors that discharge the RNA methylation in the free RNA released by dying tumor cells to enhance the function of DCs. When immature DCs uptake antigens, they migrate to secondary lymphoid organs, such as tumor-draining lymph nodes (DLNs), and present antigens to helper T cells or effector T cells to trigger tumor-specific cytotoxic T lymphocyte (CTL) responses. Once mature, DCs upregulate the expression of various co-stimulatory molecules, such as CD40, CD80, and CD86, and increase the production of proinflammatory cytokines and chemokines [124]. The abundance of m^6A modifications increases during DC maturation due to the upregulation of m^6A methyltransferases METTL3, METTL14, and WTAP in mature DCs [125]. Although METTL3 does not affect DC development in vitro and in vivo, as evidenced by the similar frequencies of CD11c-positive cells in the splenocytes and bone marrow-derived DCs (BMDCs) from *Mettl3* WT (*Mettl3*$^{fl/fl}$) mice and *Mettl3* knockout (KO) (*Mettl3*$^{fl/fl}$CD11c^{Cre}) mice, depleting or silencing of *Mettl3* in DCs impaired the maturation and activation of DCs, via downregulating the translation of key transcripts, including CD40, CD80, CD86, MHC-II, and inflammatory cytokines IFN-γ and IL-12 [125]. METTL3 in DCs was also required for DC function in promoting T-cell proliferation in vitro and in vivo [125]. Mechanistically, METTL3-mediated m^6A modifications promoted the mRNA translation of *Tirap*, *Cd80*, and *Cd40* through YTHDF1 [125]. Another study reported consistent results that METTL3 was highly expressed in myeloid DCs. Knockdown of METTL3 using small hairpin RNAs (shRNA) in DCs reduced the expression of MHC-II and co-stimulatory molecules such as CD80 and CD86 [126]. In addition, transferred METTL3 shRNA-infected DCs into recipient mice that underwent heart transplantation significantly prolonged allograft survival in the cardiac transplantation model [126], suggesting the therapeutic potential of METTL3 in immune regulation.

DC trafficking towards DLNs was reported to be governed by chemokine and chemokine receptor interactions, such as CCL19/21–CC-chemokine receptor 7 (CCR7) [127]. Liu et al. identified a long non-coding RNA (lncRNA) lnc-Dpf3 in DCs that suppressed CCR7-mediated DC migration [128]. DC-specific deletion of

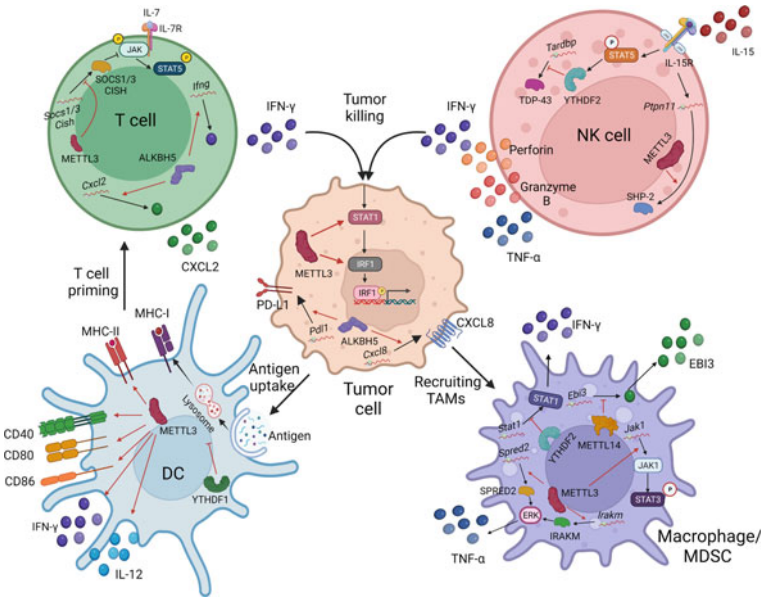

Fig. 3.3 RNA modifications in fine-tuning the innate and adaptive immune responses. In T cells, METTL3 inhibits the expression of the SOCS family proteins (SOCS1, SOCS3, and CISH), which enhances the activation of IL-7-mediated JAK/STAT5 signaling and ultimately promotes the homeostasis and differentiation of CD4+ T cells. In addition, ALKBH5 decreases the m^{6}A modification on *Cxcl2* and *Ifng* mRNA, thereby increasing their stability and enhancing CD4+ T cell effector functions. In NK cells, YTHDF2 controls the IL-15-mediated survival of NK cells by inhibiting the stability of *Tardbp*, a negative regulator of cell division. METTL3 positively regulates the production of IFN-γ, TNF-α, and granzyme B of NK cells by targeting *Ptpn11* (encoding SHP-2), a critical mediator for IL-15-induced ERK activation. In DCs, the costimulatory molecules, such as CD40, CD80, and CD86, proinflammatory cytokines, such as IFN-γ and IL-12, and MHC-II are positively regulated by METTL3. YTHDF1 promotes the translation of mRNAs that encode enzymes related to phagosome and lysosome pathways, which destruct antigens and limit antigen cross-presentation. In macrophages and MDSCs, METTL3 regulates the activation and M1-type polarization by targeting IRAKM and SPRED2, respectively. METTL3-mediated m6A modification on *Jak1* mRNA promotes its mRNA translation and enhances the downstream STAT3 signaling. METTL14 inhibits the expression of Ebi3, an inhibitory cytokine in macrophages. YTHDF2 decreases the stability of Stat1 mRNA and downregulates IFN-γ–STAT1 signaling. In tumor cells, ALKBH5 removes the m6A modification in the 3′UTR of Pdl1 mRNA and reduces its degradation, thus inhibiting the cytotoxicity of T cells. ALKBH5 also facilitates CXCL8 expression in GBM cells, which recruits TAMs to generate an immunosuppressive tumor microenvironment. METTL3 in tumor cells stabilizes the transcripts involved in IFN-γ-STAT1-IRF1 signaling and enhances their sensitization to IFN-γ. Abbreviations: METTL3, methyltransferase-like 3; SOCS, suppressor of cytokine signaling 1; CISH, cytokine-inducible SH2 containing protein; ERK extracellular signal regulated kinase; Tardbp, TAR DNA binding protein. Ptpn11, tyrosine-protein phosphatase non-receptor type 11; JAK, Janus kinase; STAT5, signal transducer and activator of transcription 5; ALKBH5, AlkB homolog 5, RNA demethylase; CXCL2, C-X-C motif chemokine ligand 2; NK cells, natural killer cells; YTHDF2, YTH N6-methyladenosine RNA binding protein 2; DCs, dendritic cells; MHC-II, major histocompatibility complex-II; IRAKM, interleukin-1 receptor-associated kinase M; SPRED2, sprouty related EVH1 domain containing 2; Ebi3, Epstein-Barr Virus-induced 3; TAMs, tumor-associated macrophages; MDSCs, myeloid-derived suppressor cells

lnc-Dpf3 (*lnc-Dpf3*$^{fl/fl}$*Itgax*^{Cre}) promoted CCR7-triggered DC migration. Interestingly, CCR7 stimulation reduced the m^{6}A modification levels in lnc-Dpf3, which prevented its degradation by m^{6}A reader YTHDF2 [128]. Cross-presentation is the process in which DCs present extracellular antigens via MHC-I molecules to CD8$^+$ T cells [129]. It has been shown that loss of YTHDF1 in DCs enhanced the cross-presentation of neoantigens and the cross-priming of CD8$^+$ T cells in vivo [130]. YTHDF1 depletion attenuated the translation of mRNAs that encode enzymes related to the phagosome and lysosome pathways [130]. These enzymes degraded proteins in the phagosome, which destructed antigens and limited antigen cross-presentation in DCs [129]. As a result, YTHDF1$^{-/-}$ or DC-specific deletion of YTHDF1 inhibited tumor growth, suggesting that YTHDF1 could be a therapeutic target for cancer immunotherapy.

3.4.2 Macrophages

Macrophages play a major role in the recognition and clearance of transformed cells via phagocytosis [131]. However, macrophages can infiltrate into solid tumors and modulate T-cell function to either favor or inhibit tumor growth. These so-called tumor-associated macrophages (TAMs), which comprise both anti-tumorigenic (M1 type) and pro-tumoral (M2 type) cells, are recruited to the sites where the tumor develops under the influence of tumor-derived chemokines [131]. The m^{6}A writer METTL3 has been reported to regulate macrophage polarization and tumor growth and metastasis [132–135]. *Mettl3* depletion in macrophages promotes B16 melanoma tumor growth and lung metastasis by creating an immunosuppressive microenvironment with an abundance of M2 type TAMs and regulatory T (Treg) cells in tumors [132]. Mechanistically, METTL3 depletion impaired the YTHDF1-mediated translation of SPRED2, a negative regulator of ERK signaling, thereby increasing the activation of the ERK, NF-κB, and STAT3 signaling pathways [132]. Tong et al. performed pooled clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) screens in macrophages and identified the m^{6}A writer METTL3 as a positive regulator of macrophage activation in terms of TNF-α production upon lipopolysaccharide (LPS) stimulation [133]. Loss of METTL3 in macrophages promoted tumor growth by facilitating the polarization of TAMs toward the M2 type of macrophages [133]. METTL3 deficiency resulted in reduced m^{6}A methylation of *Irakm* mRNA, a negative regulator of the TLR4 signaling, inhibited its degradation, and therefore suppressed the TLR4 signaling pathway [133]. However, a very recent study reported inconsistent results that *Mettl3*-deficient in TAMs inhibited tumor progression [135]. Loss of METTL3 inhibited the infiltration of CD206$^+$ M2 type TAMs but promoted the infiltration of CD103$^+$ conventional type 1 DCs (cDC1), accompanied by an increased number of tumor-infiltrating transcription factor 1 (TCF1)$^+$ stem-like CD8$^+$ T cells and IFN-γ-expressing effector CD8$^+$ cytotoxic T cells [135]. METTL3-mediated m^{6}A modification on *Jak1* mRNA promoted its mRNA translation mediated by YTHDF1 and enhanced the

downstream STAT3 signaling [135]. These controversial results suggest that the m^{6}A modification by "writer" METTL3 plays a complicated role in regulating TAMs in the tumor microenvironment (TME), which needs to be further clarified. A recent study developed a highly potent small molecule inhibitor against METTL3 [136]. This METTL3 inhibitor suppressed the growth of acute myeloid leukemia (AML) in vitro and in vivo [136]. Whether this inhibitor could regulate TAMs reprogramming and inhibit solid tumor progression in mice still needs further investigation.

C1q$^+$ TAMs, which were identified by single-cell RNA sequencing (scRNA-seq), are a new cluster of M2 type TAMs that express a set of immunomodulatory factors, including *Cxcl10*, *Nectin1*, and *Ebi3* [137]. This C1q$^+$ TAM population showed upregulated RNA m^{6}A methylation-associated transcripts, including *Mettl3* and *Mettl14* [137]. Ablation of METTL14 in macrophages elevated EBI3 expression in C1q$^+$ TAMs, inhibited IFN-γ production in CD8$^+$ T cells, and promoted tumor growth in several tumor models, including B16-OVA melanoma, MC38 colon cancer, and LLC lung carcinoma models [137]. Consistent with these results, knockdown of *METTL14* in human monocytic cell line THP-1 cells promoted M2 type polarization of macrophages [138]. These studies indicate that orchestrating macrophage immunosuppressive capacity through epitranscriptional regulation is a promising approach for cancer immunotherapy.

Besides m^{6}A writers, the m^{6}A eraser FTO also contributes to the regulation of polarization and function of macrophages [139]. Unlike the scenario where it was observed that METTL3 was upregulated in M1 macrophages but had no change in M2 macrophages [134], FTO expression decreased in both M1 and M2 macrophages. FTO knockdown using small interfering RNA (siRNA) in the RAW264.7 macrophage cell line and bone marrow-derived macrophages (BMDMs) suppressed the polarization of both M1 type and M2 type macrophages [139]. Mechanistic studies demonstrated that FTO knockdown suppressed M1 type polarization by downregulating STAT1, and inhibited M2 type polarization by inhibiting STAT6 and peroxisome proliferator-activated receptor-γ (PPARγ) [139].

There are several studies about the m^{6}A readers in macrophages. An early study showed that YTHDF2 expression was increased in RAW264.7 cells upon LPS stimulation [140]. Knocking down YTHDF2 promoted the expression of LPS-induced inflammatory cytokines, such as IL-6, TNF-α, IL-1β, and IL-12 [140], suggesting that YTHDF2 is an inhibitor of M1 type macrophage polarization. Our group recently reported that YTHDF2 plays a critical role in shaping the TME through reprogramming TAMs. Ablation of YTHDF2 in macrophages reprogrammed TAMs towards the tumor-suppressive M1 type and enhanced their cross-presentation ability to CD8$^+$ T cells, thereby suppressing tumor growth via CD8$^+$ T cell-mediated anti-tumor immunity [141]. YTHDF2 expression was regulated in TAMs through the IL-10 − STAT3 signaling pathway. In addition, we found that YTHDF2 deficiency increased the stability of STAT1 mRNA and upregulated the IFN-γ–STAT1 signaling pathway, which helps to reprogram macrophages into the M1 type [141]. IGF2BPs are another class of m^{6}A readers [116]. A recent study showed that IGF2BP2$^{-/-}$ BMDMs had increased expression

of inflammatory cytokines, such as *Il1b*, *Il6*, *Il12*, *Ifng*, and *Tnf* upon LPS stimulation [142], indicating that deletion of IGF2BP2 promotes M1 type macrophage polarization. In addition, when BMDMs were treated with IL-4, IGF2BP2 deficiency impaired M2 type polarization, as evidenced by decreased expression of *Arg1*, *Cd206*, *Ym1*, *Fizz1*, and *Tgfb1* [142]. Mechanistic studies showed that IGF2BP2 regulated macrophage polarization by stabilizing tuberous sclerosis 1 (TSC1) and PPARγ [142].

3.4.3 NK, NKT, and γδ T Cells

NK cells, NKT cells, and γδ T cells bridge the innate and adaptive systems. NK cells are the major innate lymphoid cells that mediate anti-viral and anti-tumor immunity. NK cells mediate cytotoxicity against MHC class I–deficient tumor cells by ADCC or perforin/granzyme-mediated cytotoxicity [143]. They can also produce a variety of cytokines and chemokines, including IFN-γ, TNF-α, MIP-1α and -β, which further elicit adaptive immune responses [144]. Our group reported that YTHDF2-mediated m^6A methylation plays multifaceted roles in NK cell immunity [145]. YTHDF2 was highly expressed in NK cells compared with other immune cells, such as T cells, B cells, and myeloid cells [145]. YTHDF2 expression was upregulated in NK cells upon stimulation with IL-15, murine CMV (MCMV) infection, and tumor challenge. Deletion of *Ythdf2* in NK cells promoted tumor growth and MCMV infection, and inhibited NK cell homeostasis and terminal maturation, indicating an extensive role of YTHDF2 in NK cells [145]. In addition, YTHDF2 was positively regulated by IL-15–STAT5 signaling in NK cells and contributed to IL-15–mediated NK cell survival, proliferation, and effector functions [145]. Further mechanistic studies identified *Tardbp* (TAR DNA-binding protein 43), a negative regulator of cell division, as the functional target of YTHDF2 in NK cells [145]. Similarly, Song et al. reported that METTL3 also showed a positive regulatory role in NK cells [146]. METTL3 was downregulated in tumor-infiltrating NK cells in patients with hepatocellular carcinoma and ovarian cancer [146]. METTL3 expression positively correlated with NKG2D, T-bet, and IFN-γ, but negatively correlated with T cell immunoreceptor with Ig and ITIM domains (TIGIT) in NK cells, suggesting that METTL3 contributes to the effector function of NK cells [146]. *Mettl3* deletion in NK cells promoted tumor growth and metastases and disturbed NK cell homeostasis at a steady state, similar to YTHDF2 [145, 146]. METTL3 also contributed to the responsiveness of NK cells to IL-15, as evidenced by decreased cell proliferation and survival of METTL3-deficient NK cells upon IL-15 stimulation [146]. The target of METTL3 in NK cells was SHP-2 (encoded by *Ptpn11*), a tyrosine phosphatase that is involved in the signaling pathways of a variety of growth factors and cytokines, including IL-15 [147]. NK cell proliferation and secretion of IFN-γ, TNF-α, and Granzyme B were inhibited when treated with SHP-2 inhibitor SHP099 [146]. However, the mechanism by which METTL3 regulates SHP-2 expression still needs further investigation.

NKT cells are defined as specialized populations of αβ T cells that co-express some receptors of the NK cells, which makes them feature characteristics of both conventional T cells and NK cells. Based on the TCR repertoire, NKT cells are divided into two subsets: Type I and Type II. Type I NKT cells, also known as invariant natural killer T (iNKT) cells or classical NKT cells, express a restricted TCR α-chain (Vα24-Jα18 in humans, Vα14-Jα18 in mice) and are coupled with diverse TCR β-chains, including Vβ11 in humans and Vβ2, Vβ7, and Vβ8 in mice [148]. iNKT cells recognize glycosphingolipid α-galactosylceramide (α-GalCer) presented by non-polymorphic MHC class I-like molecule, CD1d on APCs [149, 150]. Type II NKT cells are also CD1d-restricted but express a more diverse TCR repertoire and recognize non-α-GalCer molecules presented by CD1d molecules [148]. NKT cells are critical mediators of tumor immunosurveillance [151]. Activated NKT cells rapidly produce a variety of cytokines, including IFN-γ, TNF-α, IL-4, IL-10, and IL-17 that regulate anti-tumor immune responses [152]. A very recent study reported the essential role of METTL14 in iNKT cell development and function [153]. They used CD4 T cell-specific deletion of *Mettl14* conditional knockout (cKO) mice. Loss of METTL14 results in almost the absence of iNKT cells in the thymus, spleen, and liver [153]. METTL14 deficiency also impaired the function of mature iNKT cells, such as the IFN-γ and IL-4 production [153]. Mechanistic studies demonstrated that METTL14 deficiency induced more cell apoptosis of double-positive (DP) thymocytes by enhancing the p53-mediated apoptotic pathway and impairing the responses to IL-2/IL-15 and TCR stimulation [153]. However, the mechanisms by which other m^{6}A modification regulations affect NKT cell immunity remain for further investigation.

γδ T cells express the γδ T-cell receptor (γδTCR) but not αβTCR. They are an "unconventional" T-cell subset as they recognize a broad range of antigens without the presence of MHC molecules [154]. γδ T cells play dual roles in cancer development. They can attack tumor cells directly by perforin/granzyme-mediated cytotoxicity or indirectly by their production of IFN-γ [155]. On the contrary, γδ T cells produce IL-17 to promote tumor development through recruiting immunosuppressive neutrophils, macrophages, or MDSCs [156–158]. A recent study showed that RNA m^{6}A eraser ALKBH5 regulated the development of γδ T cells [159]. They generated *Alkbh5*$^{f/f}$ *Lck*-Cre cKO mice and found that ALKBH5 deficiency resulted in more expansion of γδ T cells in both the thymus and peripheral tissues [159]. However, the ablation of ALKBH5 did not affect the production of IFN-γ and IL-17 by γδ T cells, as well as the proliferation and apoptosis of γδ T cells [159]. METTL14 deficiency mainly promoted the proliferation of γδ T cell precursors by enhancing m^{6}A RNA modification on the Notch signaling components *Jagged1* and *Notch2* mRNAs to decrease their stability and expression[159]. Although ALKBH5 plays a critical role in the cell-fate decision of γδ T cells, whether it regulates the anti-tumor functions of γδ T cells remains to be determined.

3.4.4 Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are newly identified members of the lymphoid lineage that are involved in the maintenance of tissue homeostasis [160]. Three groups of ILCs have been characterized based on the differences in their phenotypes and functional properties [160]. Group 1 ILCs, which consist of NK cells and ILC1 cells, produce type 1 cytokines such as IFN-γ and TNF-α; group 2 ILCs (ILC2s) produce type 2 cytokines such as IL-4, IL-5, IL-9, and IL-13; and group 3 ILCs (ILC3s), which includes ILC3s and lymphoid tissue-inducer cells (LTi cells), produce IL-17, IL-22, and GM-CSF [160, 161]. The anti-tumor role of ILCs has not yet been fully understood, mainly due to their scarcity in the TME [119, 162]. Patients with severe combined immunodeficiency (SCID) lacked all ILC subsets in peripheral blood following non-myeloablative allogeneic hematopoietic stem cell transplantation [163]. However, no particular susceptibility to disease was observed in these patients, suggesting a possible redundancy in the protective immune function of human ILCs [163]. A distinct class of anti-tumor immune response has been demonstrated through the engagement of unconventional ILC1-like cells (ILC1ls) and type 1 innate-like T cells (ILTC1s), which expressed high levels of granzyme B and exhibit potent cytotoxicity against tumor cells [164]. We recently reported that ILC1s control leukemia stem cell (LSC) differentiation and limit the development of acute myeloid leukemia through IFN-γ [165]. Using mouse models that are deficient either in liver ILC1s or NK cells has revealed that ILC1s are essential to control LSC-derived tumor metastasis, whereas NK cells are less effective in this model [166], suggesting that complementary but different roles may exist within group 1 ILCs in local tumor immunosurveillance. Different from group 1 ILCs, recent advances in mouse models revealed the paradoxical role of ILC2s and ILC3s in tumor development, which has been reviewed elsewhere [119, 162, 167]. Overall, there is much more to learn about the involvement of ILCs in tumor immunosurveillance. Currently, studies on the role of RNA modifications in ILCs are very limited. A recent study reported that ALKBH5-dependent m^6A demethylation of *Nr4a1* mRNA contributes to the maintenance of ILC3 homeostasis [168]. Therefore, a more comprehensive understanding of how RNA modification and its modulators regulate the development, phenotype, and function of ILCs will expand our knowledge of the biological roles of ILCs in regulating tissue homeostasis and tumor immunosurveillance.

3.4.5 T Cells

T cells, generally containing $CD4^+$ T and $CD8^+$ T cells, play a pivotal role in anti-tumor immune responses. Naïve $CD4^+$ T cells differentiate into Th1, Th2, Th17, and T follicular helper (Tfh) effector cells, as well as immunosuppressive Treg cells upon exposure to various signals [169]. The balance between the effector T cells and Treg cells orchestrates immune homeostasis, which contributes to the progression of cancer [170]. Naïve $CD8^+$ T cells differentiate into cytotoxic

effector cells and long-lived memory cells [171]. An early study reported that *Mettl3*-deficient naïve CD4$^+$ T cells exhibited a defect in T cell proliferation and reduced differentiation of Th1 and Th17 cells, an increase in Th2 cells, but no changes in Treg cells [172]. The ablation of METTL3 in T cells also disrupted T cell homeostasis in vivo, as evidenced by naive METTL3-deficient T cells failing to undergo homeostatic expansion and remaining in the naive state [172]. T cell homeostasis is primarily maintained by IL-7/STAT5 signaling and TCR-mediated ERK/AKT signaling [173], which is negatively regulated by the suppressor of the cytokine signaling (SOCS) family genes, including *Socs1*, *Socs3*, and *Cish* [174–176]. Loss of METTL3 resulted in decreased JAK1 and STAT5 phosphorylation upon IL-7 stimulation but increased *Socs1*, *Socs3*, and *Cish* mRNA levels and half-life [172], suggesting that METTL3 controls T cell homeostasis through blocking IL-7 mediated STAT5 activation. METTL3 does not affect Treg cell development [172]. However, *Mettl3* deficient Treg cells lost their ability to suppress effector T cells [177]. Mechanistically, the METTL3-mediated m^{6}A RNA modification specifically targeted the IL-2/STAT5/SOCS pathway and sustained the suppressive functions of Treg cells [177]. Similar to METTL3, deletion of METTL14 (*Mettl14*$^{fl/fl}$; Cd4-Cre mice) also leads to a global loss of m^{6}A mRNA levels in T cells [178]. METTL14 cKO mice developed spontaneous colitis by 6 weeks of age, which was associated with a higher frequency of proinflammatory Th1/Th17 cells and defective Treg cells [178].

T cell development, activation, and survival are also controlled by TCR signaling [173, 179]. A very recent study reported that WTAP-mediated m^{6}A RNA modification controls TCR signaling and the survival of T cells [180]. Similar to METTL14 cKO mice [178], WTAP cKO mice (*Wtap*$^{fl/fl}$; Cd4-Cre mice) also developed colitis at a young age [180]. WTAP cKO mice had increased IFN-γ^+IL-17A$^+$ pathogenic T cells but reduced RORγt$^+$Helios$^-$ induced Treg (iTreg) cells [180], indicating that loss of WTAP impairs Treg cell function and causes inflammation of the gut. In addition, WTAP cKO mice had impaired thymocyte development and reduced abundance of CD4$^+$ and CD8$^+$ T cells [180]. Further studies showed that Wtap-depleted CD4$^+$ T cells underwent more apoptosis in the presence of TCR stimulation [180]. Transcriptome analyses revealed that the encoding Ca2$^+$ channel *Orai1* and the programmed cell death-inducing kinase *Ripk1* are the two targets of WTAP in T cells [180]. Collectively, these studies highlight the critical role of m^{6}A "writers" in governing T cell development, survival, differentiation, and functions.

Tfh cells are critical for germinal center formation, B cell development, and antibody generation [181]. They also favor anti-tumor immunity by promoting CTL activity [182]. The role of m^{6}A modifications in Tfh cell development and function is not consistent. One study showed that knockdown of METTL3 or METTL14 in CD4$^+$ T cells with shRNA could promote Tfh differentiation upon lymphocytic choriomeningitis virus (LCMV) infection [183]. However, another study using METTL3 cKO mice found that loss of METTL3 in CD4$^+$ T cells inhibited Tfh cell differentiation, germinal center responses, and proliferation after

LCMV challenge [184]. In Tfh cells, METTL3-mediated m^{6}A modification regulated the stability of *Tcf7* mRNA, eventually maintaining TCF-1 expression and securing the differentiation of Tfh cells [184].

In addition to m^{6}A writers, the m^{6}A "eraser" enzyme ALKBH5 is also involved in regulating T cell development and function [185]. The expression of ALKBH5 but not FTO was upregulated in Th1, Th2, Th17, and Treg cells compared with naïve CD4$^+$ T cells. However, the ablation of ALKBH5 in CD4$^+$ T cells (*Alkbh5*$^{fl/fl}$; Cd4-Cre mice) did not disrupt T cell development in the thymus and peripheral lymphoid tissues. Loss of ALKBH5 also did not affect CD4$^+$ T cell activation, apoptosis, proliferation, and cytokine secretion, indicating that the absence of ALKBH5 does not disrupt T cell development at a steady state. However, deletion of ALKBH5 in T cells inhibited the homeostatic expansion of naïve T cells and IFN-γ production, thereby alleviating T cell-mediated autoimmune colitis and experimental autoimmune encephalomyelitis [185]. Further mechanistic studies indicated that ALKBH5 ablation increased m^{6}A modification on *Cxcl2* and *Ifng* mRNA, decreasing their stability in CD4$^+$ T cells, thereby suppressing CD4$^+$ T cell function. Current studies have highlighted the importance of m^{6}A writers and erasers in regulating T cell immunity. Therefore, it will be interesting to characterize whether and how m^{6}A readers control T cell homeostasis or functionality.

3.4.6 B Cells

Despite T cells being the major adaptive immune cells for controlling tumor growth, B cells, which mediate humoral immunity and are responsible for the production of antibodies, are also involved in tumor progression. As APsC, B cells can present tumor antigens to induce tumor-specific T cells and drive T cell expansion [186, 187]. B cells can also provide co-stimulation signals to activate T cells, such as CD40, CD80, and CD86 [188]. Furthermore, B cells produce antibodies that promote antibody- and complement-mediated killing of tumor cells, Fc-mediated phagocytosis by macrophages, and ADCC by NK cells [189]. Finally, B cells produce cytokines to enhance the activity of NK cells and cytotoxic T-cells [189]. However, B cells also exert pro-tumor effects. For example, there is a distinct subset of B cells, named B regulatory (Breg) cells, which exert immune-modulatory functions through the production of immunosuppressive cytokines TGF-β, IL-10, and IL-35 [190, 191]. Thus, B cells are a heterogeneous population with diverse functions, contributing to both pro- and anti-tumor immunity. Understanding the diverse roles of B cells in cancer will yield novel avenues for cancer immunotherapy.

Early B cell development has been reported to be regulated by RNA m^{6}A methylation [192]. METTL14 deficiency (*Mettl14*$^{fl/fl}$; *Mb1*-Cre mice) had undetectable mature B cells in the spleen and peritoneal cavity, and only 25% of B cells in the bone marrow, indicating that loss of METTL14 impairs B cell development [192]. Further studies indicated that METTL14 blocked B cell development

at the late large pre-B cell stage. METTL14 deficiency blocked IL-7-induced pro-B cell proliferation and transition to the large pre-b stage [192]. In the same study, they also generated mice with YTHDF2 deficiency in B cells (*Ythdf2*$^{fl/fl}$; *Mb1*-Cre mice). Loss of YTHDF2 only caused a mild reduction (20%) of peripheral B cells. Although YTHDF2 has a mild effect on B cell development, a recent study revealed that YTHDF2 plays a critical role in the early transition of pre-germinal center (GC) state GC cells [193]. YTHDF2 was also required for effective antibody-mediated immune response and GC formation [193]. Interestingly, loss of *Mettl3* in hematopoietic stem cells did not affect B cell development [194, 195]. Since METTL3 is the core catalytic component of the methyltransferase complex responsible for m^{6}A modification, it is odd to know that METTL3 was not essential for the development of B cells. Further studies using B cell-specific cKO mice for METTL3 are needed to clarify the role of METTL3 in B cell development.

Whether and how m^{6}A modifications modulate B cell-mediated tumor immunity has not been described. However, m^{6}A modification is involved in the development of diffuse large B-cell lymphoma (DLBCL), an aggressive type of non-Hodgkin lymphoma that develops from B cells [196]. DLBCL tissues have higher levels of m^{6}A RNA methylation as well as METTL3 expression compared with inflammatory lymph glands. Silencing METTL3 using shRNA inhibited the proliferation and promoted cell apoptosis of DLBCL cells in vitro and suppressed tumor growth in vivo. Mechanistic studies showed that knockdown of METTL3 inhibited the expression of pigment epithelium-derived factor (PEDF), a component upstream of the Wnt pathway, by decreasing m^{6}A methylation in PEDF mRNAs. PEDF overexpression abolished the inhibitory effects of METTL3 knockdown on DLBCL cell proliferation and xenograft growth.

3.5 Regulation of Immune-Associated Molecules by RNA Modifications

The immune system exerts an immunoediting function through multiple layers. At the cellular level, an intact immune response includes many subsets of leukocytes as described above. Myeloid cells recognize the transformed cells or cancer cells and orchestrate the initiation of inflammation and protective anti-tumor adaptive immune responses. At the molecular level, numerous key immune-associated effector molecules, such as cytokines, are important mediators of immune responses and are involved in shaping the TME. Indeed, many of these immune-associated molecules are regulated by RNA modifications.

3.5.1 Type I Interferons

Type I interferons (IFNs), including IFN-α and β, are known for their crucial role in antiviral immunity [197]. Mounting evidence indicates that type I IFNs produced by malignant cells or immune cells in the TME are involved in cancer

immunosurveillance [198]. Many types of cells in the TME, including tumor cells [199, 200], tumor-infiltrating DCs [201–203], TAMs [204, 205], cancer-associated fibroblasts (CAFs) [206], and tumor endothelial cells [207], can produce type I IFNs in response to viruses, endogenous nucleic acids, and synthetic oligoribonucleotides/oligodeoxyribonucleotides. Type I IFNs can induce tumor cell apoptosis and inhibit tumor cell proliferation and metastasis [208]. They can also upregulate MHC-I and tumor-associated antigen (TAA) in tumor cells, resulting in increased recognition and uptake of TAA by APCs thereby enhancing $CD8^+$ T cell-mediated tumor killing [208]. In addition, type I IFNs stimulate anti-tumor immune responses by promoting DC differentiation, maturation, and migration into lymph nodes [66, 209], increasing NK cell maturation, activation, and cytotoxicity [210, 211], and inhibiting Treg cell differentiation and suppressive function [212, 213]. Recombinant IFN-α2 has long been approved by the United States FDA to treat cancer since 1986. Currently, type I IFNs have been widely used alone or in combination with other immunotherapeutic agents for the treatment of solid and hematologic malignancies [214].

It has been shown that both *IFNA* and *IFNB* mRNA are modified by m^6A [215]. Deletion of METTL3 or YTHDF2 reduced the m^6A modifications on *IFNA* and *IFNB* and increased their stability [215]. Another study reported similar results that depleting METTL14 also induced more IFN-β expression [216]. In contrast, the deletion of ALKBH5 increased the m^6A modifications and reduced IFN-β expression [216]. These studies indicate that m^6A acts as a negative regulator of type I IFNs. Of note, in head and neck squamous cell carcinoma, ALKBH5 overexpression inhibited retinoic acid-inducible gene I (RIG-I)-mediated IFNα secretion and promoted tumor progression, indicating a novel mechanism of immune microenvironment regulation mediated by m^6A modification through the ALKBH5/RIG-I/IFNα axis [217].

3.5.2 IFN-γ

IFN-γ is the sole member of the type II interferon that is produced by numerous cells of innate and adaptive immunity, including NK cells, ILC1s, iNKT cells, γδ T cells, and effector $CD4^+$ and $CD8^+$ T cells [218]. IFN-γ exerts both pro-tumor and anti-tumor activities. Early studies demonstrated that neutralizing IFN-γ or mice lacking IFN-γ receptor (IFNGR) and STAT1 inhibited the development of transplanted tumors and carcinogen-induced sarcomas [46–48]. IFN-γ could also induce apoptosis of tumor cells and LSCs [165, 219]. However, IFN-γ was later found to induce the expression of inhibitory molecules, such as PD-L1, PD-L2, and indoleamine 2,3-dioxygenase 1 (IDO1) in tumors and other immune cells [220–222], which limit anti-tumor immunity. IFN-γ expression is tightly regulated by epigenetic, transcriptional, post-transcriptional, and post-translational modifications, which have been reviewed elsewhere [223, 224]. For example, *IFNG* is silenced in naive T cells via methylation and hypoacetylation [225, 226]. At the

post-transcriptional level, *IFNG* mRNA is also negatively regulated by the microRNAs, such as miR-29, miR-146a, and miR-142-3P [227–229]. However, the effects of m^{6}A modifications on the *IFNG* mRNA lifecycle remain largely unknown. A recent study showed that *Ifng* mRNA had several m^{6}A modification sites in mouse CD4$^+$ T cells, which were removed by ALKBH5. Ablation of ALKBH5 decreased stability and expression of *Ifng* [185]. However, the detailed mechanism by which the m^{6}A regulators control *Ifng* expression remains unclear. m^{6}A modification has been shown to regulate the downstream signaling of IFN-γ. For instance, knockout of METTL3 or METTL14 in tumor cells enhanced their sensitization to IFN-γ by stabilizing the transcripts involved in the IFN-γ-STAT1-IRF1 signaling pathway [230]. Depletion of FTO also increased the sensitivity of melanoma cells to IFN-γ [231]. A recent study evaluated the association of IFN-γ with m^{6}A RNA methylation in human skin cutaneous melanoma (SKCM) in the TCGA database [232]. They found that the expression of IFN-γ had a positive association with *WTAP*, *YTHDC2*, *RBM15*, and *FMR1* expression levels, whereas a negative correlation with *METTL3*, *METTL16*, *YTHDF1*, *YTHDF2*, *YTHDF3*, and *FTO* [232], indicating that IFN-γ has potent interactions with m^{6}A regulators.

3.5.3 TGF-*β*

TGF-β plays an important role in the regulation of numerous cellular functions, including development and progression of cancer. TGF-β transduces signals through a heteromeric complex of type I and type II TGF-β receptors and subsequently induces the phosphorylation and activation of SMAD proteins, which translocate to the nucleus and induce transcription of their target genes [233, 234]. In addition to the canonical Smad-mediated signaling, TGF-β also activates several other signaling pathways such as TAK1 (TGF-β-associated kinase 1), Erk (extracellular signal regulated kinase), p38/MAPK (mitogen-activated protein kinase), and Akt [235]. TGF-β plays a dual role in tumorigenesis. At the early stage, TGF-β inhibits tumor cell cycle arrest and induces tumor cell apoptosis. However, at the late stage, TGF-β promotes tumor migration, invasion, and epithelial-mesenchymal transition (EMT) [236]. TGF-β also plays an essential role in regulating anti-tumor immune responses and remodeling the TME [236, 237]. For example, TGF-β induces the differentiation of the immune-suppressive Treg cells, which limits the anti-tumor response [238]. TGF-β promotes the polarization from M1 type towards M2 type TAMs [239]. TGF-β also inhibits NK cell proliferation, activation, and functions via SMAD proteins [240–243], while Smad4 can also positively regulate NK cell homeostasis and maturation and antitumor immunity in a TGF-β-independent manner [240]. Tumor cells escape from immune surveillance by producing TGF-β, resulting in reduced expression levels of several cytolytic cytokines, including granzyme A, granzyme B, perforin, and IFN-γ in cytotoxic T cells (CTLs), which inhibit CTL-mediated tumor cytotoxicity [244].

It has been reported that the 5′UTR of TGF-β1 mRNA was m^{6}A hypermethylated in LPS-activated Kupffer cells, which was mediated by METTL3/

METTL14 [245]. m^{6}A-modified TGF-β1 mRNA did not affect its degradation and YTHDF1/YTHDF3-mediated translation but promoted m^{6}A-mediated cap-independent translation [245]. This study suggests a novel mechanism by which m^{6}A modification regulates gene expression. TGF-β is a major regulator of EMT, which drives cancer metastasis [246]. A recent study found that the expression of METTL3 and m^{6}A RNA modification were increased during TGF-β-induced EMT in lung cancer cells [247]. Knockdown of METTL3 inhibited TGF-β-induced EMT phenotypes, such as the morphological conversion of the cells and enhanced cell migration [247]. Mechanistic studies revealed that METTL3 knockdown reduced the mRNA stability of JUNB, one of the critical transcriptional regulators of EMT [247]. This study suggests that m^{6}A methyltransferase METTL3 is essential for TGF-β-induced EMT of lung cancer cells. In contrast, RNA demethylase ALKBH5 inhibited TGF-β-induced EMT in NSCLC [248]. Mechanistically, ALKBH5 decreased TGFβR2 and SMAD3 expression and mRNA stability by erasing their m^{6}A modification [248]. Similarly, YTHDF2 knockdown promoted EMT in pancreatic cancer cells through the Hippo-YAP/TAZ pathway [249]. YTHDF2 knockdown up-regulated total YAP and p-YAP protein levels [249].

In addition to cytokines, chemokines, which are produced by tumor cells, immune cells, and stromal cells, contribute to regulating the infiltration of different immune cells into the TME and affect tumor immunity [250]. C-X-C motif chemokine ligand 1 (CXCL1) is a chemokine that recruits MDSCs into the TME via binding to its receptor C-X-C motif chemokine receptor 2 (CXCR2) [251]. A recent study reported that METTL3 in colorectal cancer recruited accumulation in tumors via the CXCL1/CXCR2 axis [252]. Mechanistically, METTL3 promoted m^{6}A-BHLHE41 to drive CXCL1 transcription in colorectal cancer cells [252].

3.6 Targeting RNA Modifications for Cancer Immunotherapy

Most of the m^{6}A regulatory factors have been reported to be expressed at abnormally high levels in tumors, which leads to an immunosuppressive TME, and serve as oncogenes to promote cancer progression. Therefore, attempts have been made to target m^{6}A regulatory factors for cancer therapy. Here we discuss current strategies in targeting m^{6}A modifications for cancer immunotherapy (Fig. 3.4).

3.6.1 Developing Small Molecular Inhibitors Targeting m^{6}A Regulators

3.6.1.1 FTO Inhibitors

Once the methyltransferases "writers" and demethylases "erasers" of the m^{6}A modification were discovered, investigators started to screen small-molecule inhibitors targeting those m^{6}A regulators. The first reported was the FTO inhibitor in 2012 [253]. The group started with virtual screening using the crystal structure

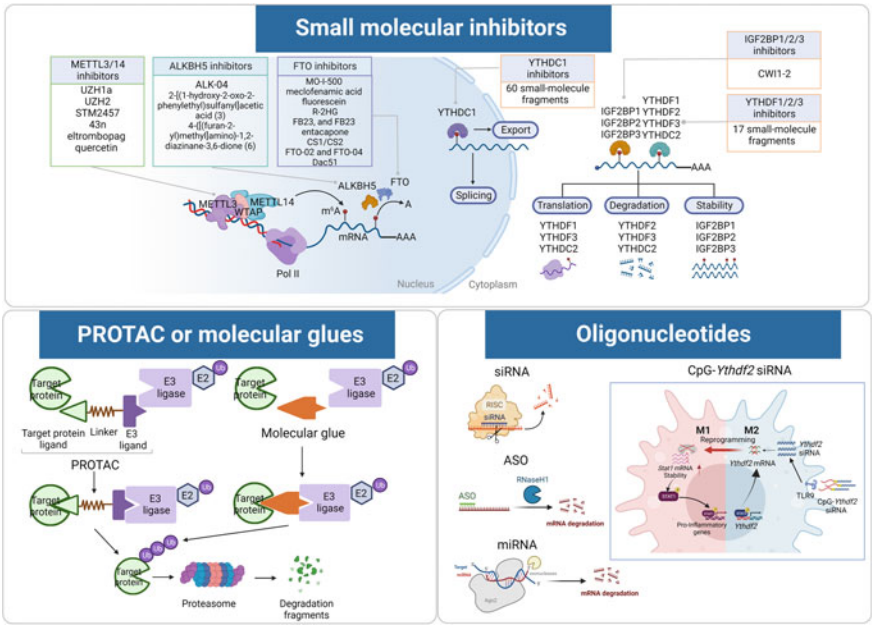

Fig. 3.4 Strategies in targeting the m^{6}A modification for cancer immunotherapy. A series of small-molecule inhibitors targeting m^{6}A regulators such as METTL3/14, FTO, and ALKBH5 have been developed. Currently, there are no inhibitors that target the YTH (YT521-B homology) family readers. However, several fragment ligands targeting YTHDC1 and YTHDF2 have been reported. One inhibitor that targets IGF2BP2 has been reported recently. PROTAC is a heterobifunctional molecule that consists of a protein comprising a ligand for the target protein, a ligand for an E3 ubiquitin ligase, and a connection linker. PROTAC recruits the E3 ligase to the protein of interest and induces its ubiquitination and degradation. Molecular glues are a class of small molecules that act as adhesives by allowing the target protein to bind to an E3 ubiquitin ligase and recruit target proteins for their ubiquitination and subsequent degradation by the proteasome. Oligonucleotide-based therapeutics, using antisense oligonucleotides (ASOs), siRNA, and microRNAs (miRNAs), are emerging as a new class of targeted anti-cancer drugs that induce gene silencing. A CpG-*Ythdf2* siRNA was developed for specific delivery into TLR9-positive cells, such as TAMs, which induces the gene silencing of *Ythdf2* and reprograms M2 TAMs to M1 TAMs. Abbreviations: METTL3/14, methyltransferase-like 3/14; ALKBH5, AlkB homolog 5, RNA demethylase; FTO, fat mass and obesity-associated protein; YTHDF, YTH N6-methyladenosine RNA binding protein; IGF2BP, insulin-like growth factor 2 mRNA binding protein; PROTAC, proteolysis targeting chimera; siRNA, small interfering RNA; ASO, antisense oligonucleotides; miRNA, microRNA; CpG, cytosine-phosphate-guanine; TLR-9, toll-like receptor 9; RISC, RNA-induced silencing complex; TAMs, tumor-associated macrophages

of FTO in complex with m^{3}T as a docking target, and found one potent compound, the natural product rhein, exhibited good inhibitory activity on m^{6}A demethylation by FTO [253]. Later, several FTO inhibitors, such as MO-I-500 [254], meclofenamic acid (MA) [255], fluorescein [256], 2-hydroxylglutarate (R-2HG) [257], FB23 and FB23 [258], entacapone [259], CS1/CS2 [260], FTO-02 and FTO-04 [261], and Dac51 [262] were established.

Functional studies revealed that some of those aforementioned FTO inhibitors not only suppress cancer cell proliferation and cancer stem cell self-renewal but also improve anti-tumor immunity. For example, FTO was highly expressed in subtypes of AML cells and served as an oncogene in promoting leukemogenesis by targeting several tumor suppressors, such as ankyrin repeat and SOCS box containing 2 (ASB2) and retinoic acid receptor alpha (RARA) via reducing m^6A levels in these mRNA transcripts [263]. Subsequently, Su et al. found that R-2HG exhibited broad and intrinsic anti-tumor activity in leukemia by inhibiting FTO demethylase activity, thereby suppressing MYC and CCAAT/enhancer-binding protein alpha (CEBPA)-associated pathways [257]. Su et al. further identified two effective small-molecule compounds (CS1 and CS2) that specifically target FTO with high efficacy [260]. CS1 and CS2 exerted potent anti-leukemic effects in vitro and in vivo by suppressing FTO activity, leading to the activation of apoptosis signaling and inhibition of the MYC pathways [260]. Moreover, this study also found that CS1 and CS2 reprogrammed the immune response by reducing the expression of immune checkpoint genes, especially leukocyte immunoglobulin-like receptor (*LILRB4*) in AML cells, which sensitized human AML cells to T cell cytotoxicity [260].

A recent study further emphasized the immune-inhibitory role of FTO in tumor immunity. FTO expression was negatively associated with several activated T cell-related genes, including *CD3D*, *GZMA*, *GZMB*, and *TNFRSF18* in melanoma samples from TCGA. Knockdown of FTO in B16 melanoma cells and lung cancer cell line LLC inhibited tumor growth, followed by elevated $CD8^+$ T cell infiltration and increased $CD8^+$ T cell-mediated cytotoxicity, indicating that tumor-intrinsic FTO functions as a suppressive molecule that restricts T cell activation and effector states. Treatment with FTO inhibitor, Dac51, inhibited tumor growth, increased T cell infiltration, and synergized with anti-PD-L1 blockade [262].

3.6.1.2 ALKBH5 Inhibitors

Unlike FTO that demethylates not only internal m^6A on mRNAs, but also m^6Am on mRNAs, m^1Am on tRNAs, m^3T on single-stranded DNAs (ssDNAs), and 3-methyluracil (m^3U) on single-stranded RNAs (ssRNAs) [112, 264–266], ALKBH5 was found to be responsible for only the m^6A demethylation on mRNAs [113], which makes it an ideal specific target for m^6A modification. ALKBH5 is dysregulated in multiple malignancies and plays a dual role in tumor development based on the type of cancer [267]. For example, ALKBH5 decreased m^6A methylation of NANOG mRNA, increasing its mRNA stability, and thus promoting breast cancer development [268]. ALKBH5 was overexpressed in glioblastoma (GBM) stem-cell-like cells (GSCs) and its overexpression demethylated *FOXM1*, resulting in increased FOXM1 expression, which is critical for GSC growth and self-renewal [269]. ALKBH5 also selectively promoted tumorigenesis and cancer stem cell self-renewal in AML but not for normal hematopoiesis [270, 271]. Several studies also revealed the tumor suppressor role of ALKBH5. ALKBH5 inhibited cell adhesion

by repressing ITGA6 expression in bladder cancer [272]. ALKBH5 prevented pancreatic cancer tumorigenesis and progression by targeting the Wnt pathway and PER1, respectively [273, 274].

ALKBH5 is also involved in regulating tumor immunosurveillance and in remodeling the TME. Tumor-intrinsic ALKBH5 removed m^{6}A modification in 3′UTR of *PDL1* mRNA and reduced its degradation, thus inhibiting cytotoxicity of T cells and mediating immune escape of intrahepatic cholangiocarcinoma (ICC) cells [275]. Tumor-intrinsic ALKBH5 upregulated extracellular lactate content and inhibited the accumulation of Tregs and MDSCs in the TME, which enhanced the efficacy of anti-PD-1 therapy and suppressed tumor growth in melanoma and colon cancer mouse models [276]. Tumor-intrinsic ALKBH5 facilitated hypoxia-induced paraspeckle assembly and CXCL8 expression in GBM cells, which recruited TAMs to generate an immunosuppressive TME, thereby promoting tumor evasion [277].

Although ALKBH5 displays controversial and context-dependent roles in cancer progression, pharmacological inhibition of ALKBH5 has shown antitumor effects in certain types of cancer. A specific small-molecule inhibitor of ALKBH5, named ALK-04, was identified by in silico screening. Mice treated with ALK-04 significantly reduced tumor growth when combined with PD-1 immunotherapy in a B16 melanoma mouse model [277]. However, detailed information regarding this inhibitor, such as chemical structure and biochemical inhibition, has not yet been disclosed. Two compounds, 2-[(1-hydroxy-2-oxo-2-phenylethyl)sulfanyl]acetic acid (3) and 4-as-1,2-diazinane-3,6-dione (6), were identified as ALKBH5 inhibitors through high-throughput virtual screening of the library of 144,000 preselected compounds [278]. These two inhibitors suppressed cell proliferation of three leukemia cell lines, including HL-60, CCRF-CEM, and K562 [278].

3.6.1.3 METTL3/METTL14 Inhibitors

m^{6}A is installed mainly by a multicomponent methyltransferase complex, which contains the catalytic subunit METTL3 and other auxiliary subunits such as METTL14 and WTAP [279, 280]. As the key catalytic subunit of the methyltransferase complex, METTL3 and METTL14 are crucial for both normal physiological and pathophysiological events, including cancer. Accumulating evidence in recent years demonstrated that METTL3 acts as an oncogene in cancer. For example, METTL3 and METTL14 are both essential for the development and maintenance of AML and the self-renewal of LSCs [281, 282]. In liver cancer, METTL3 promoted liver cancer progression through YTHDF2-dependent post-transcriptional silencing of SOCS2 [283]. The disclosure of the METTL3-METTL14 cocrystals provided structural information for designing inhibitors that target this methyltransferase [279]. To date, several METTL3 inhibitors have been reported, including UZH1a [284], UZH2 [285], STM2457 [136], 43n [286], eltrombopag [287], and quercetin [288].

UZH1a possesses inhibitory activity against METTL3 with an IC_{50} of 280 nM. The molecular weight of UZH1a is relatively low (558 g/mol) and ideal for the

uptake of cells [284]. Treatment of the AML cell line MOLM-13 with UZH1a induced a dose-dependent decrease of m^6A methylation [284]. Subsequently, the same group optimized UZH1a and obtained UZH2, a UZH1a analogue, with IC^{50} of 5 nM for METTL3 [285]. Further functional studies are required to explore its role in cancer. Recently, STM2457 was discovered as the first-in-class, selectively, and highly potent inhibitor of METTL3 with an IC_{50} of about 16.9 nM [136]. STM2457 displayed excellent anti-leukemic effects in vitro and in *vivo*. Treatment with STM2457 reduced tumor growth and increased cell apoptosis in human and mouse AML models but not in normal non-leukemic hemopoietic cells [136]. STM2457 also significantly prolonged the survival of the mice without significant toxicity [136]. This is the first bioavailable METTL3 inhibitor that shows in vivo activity and therapeutic efficacy against tumors, indicating that targeting RNA methyltransferases is a promising strategy for AML therapy. Later, three other inhibitors targeting METTL3, including two allosteric inhibitors of the METTL3-14, 43n (IC_{50} of 2.81 μM) and eltrombopag (IC_{50} of 7.04 μM) [286, 287], and one natural product inhibitor, quercetin (IC_{50} of 2.73 μM) were reported [288]. However, whether these RNA methyltransferase inhibitors have potent anti-tumor effects in vivo needs further investigation.

3.6.1.4 m^6A Reader Inhibitors

YTH family proteins, whose YTH domain recognizes and binds m^6A-containing RNA, are the main "readers" of the m^6A modification. In mammals, there are five members in the YTH family, namely, YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3. YTHDF1 promotes the translation of m^6A-containing mRNAs [115], YTHDF2 regulates mRNA stability [114], and YTHDF3 promotes the translation and degradation of mRNA [289, 290]. YTHDC1 mainly regulates splicing and mediates the export of m^6A-containing mRNAs [291, 292]. YTHDC2 facilitates the mRNA translation or degradation depending on its targets [293, 294]. Currently, there are no inhibitors that target the YTH family readers due to the similarity of their binding sites. A recent study performed a complete comparison of the binding sites of the three YTHDF family members based on their crystal structure in complex with GG(m^6A)CU-RNA [295]. Indeed, a high degree of similarity among the YTHDF binding sites and the very similar conformation adopted by their recognition loops as well as the nearly identical position of the RNA backbone were observed [295]. Therefore, the robust conservation of the YTHDF binding site and its RNA binding mode indicates the impossible task of designing selective binders for just one of the three YTHDF proteins based solely on the binding pocket residues. Notably, in contrast to the principal model of diversity function, a recent study proposed a unified model that the YTHDF proteins are believed to mediate mRNA degradation [296]. Therefore, a pan-YTHDF inhibitor may be more advantageous than a selective one; however, the specific function of the inhibitor and the detailed mechanism need to be well-defined.

Fragment-based drug discovery (FBDD) is a powerful approach to developing potent small-molecule compounds starting from chemical fragments, which have a low molecular weight (less than 300 Da) [297]. Compared to high-throughput

screening (HTS), FBDD exhibits several attractive advantages such as saving experimental costs, offering diverse hits, and exhibiting multiple ways to develop novel compounds [297]. Several fragment ligands targeting m^{6}A readers, such as YTHDC1 and YTHDF2 have been reported. Bedi et al. first confirmed that m^{6}A-dependent protein–RNA interaction sites can be druggable using FBDD [298, 299]. They presorted about 60 small-molecule fragments with the micromolar affinity that bind to the m^{6}A reader domain of YTHDC1. They also reported 17 small molecule ligands that compete with m^{6}A for binding to the m^{6}A-reader domain of YTHDF2 [295]. Based on the knowledge obtained from these fragments, further hit optimization studies are needed to develop a potent inhibitor for YTHDF2.

The IGF2BP protein family, including IGF2BP1/2/3, which stabilizes m^{6}A-containing mRNAs and promotes their translation through their K homology domains, was recently identified as a new class of m^{6}A reader proteins [116]. IGF2BPs play oncogenic roles as m^{6}A readers in solid cancers, such as cervical cancer and liver cancer [116]. Recently, Weng et al. reported the oncogenic role and the therapeutic targeting of IGF2BP2 in AML [300]. IGF2BP2 promoted AML development and self-renewal of leukemia stem/initiation cells by regulating expression of critical targets, such as MYC, GPT2, and SLC1A5, in the glutamine metabolism pathways in an m^{6}A-dependent manner [300]. In this study, they developed an effective small-molecule inhibitor (namely CWI1-2) through structure-based virtual screening that preferentially binds to IGF2BP2 and inhibits its interaction with m^{6}A-modified target transcripts [300]. CWI1-2 showed promising anti-leukemia efficacy in vitro and in vivo with no toxicity, suggesting that CWI1-2 is an effective and safe compound targeting IGF2BP2 for AML treatment [300]. To our knowledge, this is the first reported functional inhibitor that targets an m^{6}A reader protein. However, since IGF2BP family proteins share the same m^{6}A binding domain [116], whether this CWI1-2 inhibitor specifically binds to IGF2BP2 without affecting other IGF2BP family members needs further evaluation.

3.6.2 PROTAC (Proteolysis Targeting Chimera)-Based or Molecular Glue Degrader-Based Inhibitors

Besides small-molecular inhibitors, targeted protein degradation provides additional approaches for targeting those undruggable proteins. The most well-known approaches are proteolysis targeting chimeras (PROTACs) and molecular glues. PROTAC is a heterobifunctional molecule that consists of a protein comprising a ligand for the target protein, a ligand for an E3 ubiquitin ligase, and a connection linker [301]. PROTAC recruits the E3 ligase to the protein of interest and induces its ubiquitination and degradation [301]. The main advantages of PROTACs over small-molecular inhibitors include their high selectivity, stronger sustained efficiency, and potential to degrade the “undruggable” targets. PROTACs have been successfully used in both solid tumors—via targeting the androgen receptor (AR), estrogen receptor (ER), focal adhesion kinase (FAK), and P38

[302–305], and hematological tumors—via targeting bromodomain and extraterminal (BET), Bruton's tyrosine kinase (BTK), BCR-ABL, and cyclin-dependent kinases (CDKs) [306–309]. Recently, ribonuclease targeting chimera (RIBOTAC) has become a promising strategy for RNA degradation, which is similar to the structure of the PROTAC molecule [310]. C5-RIBOTAC, which binds with the model of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) attenuator hairpin, selectively induced SARS-CoV-2 RNA degradation and attenuated viral activity by recruiting ribonuclease [311]. However, the approach by which a PROTAC degrades m^6A regulators merits further investigation.

Molecular glues are a class of small molecules that act as adhesives by allowing the target protein to bind to an E3 ubiquitin ligase and recruit target proteins for their ubiquitination and subsequent degradation by the proteasome [312]. In contrast to the PROTACs, in which two ligands are connected by a flexible linker, molecular glues directly enhance complex formation between an E3 ligase and a target protein by squeezing between protein–protein interfaces. In addition, molecular glues are much smaller than PROTACs and, therefore, are expected to have higher membrane permeability and improved cellular uptake [312]. Several molecular glues have been reported. Krönke et al. reported the first molecular glue lenalidomide that induces the degradation of IKZF1/3 through the E3 ligase CUL4 [313]. Arylsulfonamides, such as E7820, indisulam, and tasisulam, bind to the cullin-RING ligase substrate receptor DCAF15 and induce the degradation of splicing factor RBM39 [314]. Manumycin polyketides act as molecular glues between RING E3 ligase UBR7 and neosubstrate tumor-suppressor P53 [315]. Several inhibitors, such as CR8, HQ461, and dCeMM have been identified as molecular glues that promote CDK12-DDB1 interaction to trigger cyclin K degradation [316–318]. Therefore, molecular glue provides an attractive method for developing inhibitors targeting m^6A regulators.

3.6.3 Gene Silencing Using Oligonucleotide-Based Therapeutics

Oligonucleotide-based therapeutics (ONTs), using antisense oligonucleotides (ASOs), siRNA, and microRNAs (miRNAs), are emerging as a new class of targeted anti-cancer drugs. Over one hundred clinical trials are ongoing using oligonucleotides in oncology [319]. ONTs have shown the effective ability to specifically target those undruggable proteins, such as the MYC gene family (c-MYC, MYCN, and MYCL) [320, 321], RAS gene family (HRAS, KRAS, and NRAS) [322], STAT3 [323–325], and BCL-2 [326]. Therefore, gene silencing using ONTs is a promising strategy for targeting m^6A regulators in tumor cells and immune cells. Although gene silencing of those m^6A regulators has shown promising effects in tumor cell lines and multiple preclinical tumor models, whether they have anti-tumor effects in the clinic needs further investigation.

Our group has previously identified YTHDF2 as a novel negative regulator of M1 type macrophage polarization, which can reshape the TME to be more inflammatory for tumor eradication [141]. Based on our findings, we developed

a strategy for targeting YTHDF2 specifically in TAMs by CpG-siRNA. CpG oligodeoxynucleotides (ODNs) are TLR9 agonists that can selectively be delivered into TLR9-positive cells, such as TAMs [141]. Our study showed that macrophages could efficiently take up CpG-*Ythdf2* siRNA and reprogrammed M2 type TAMs to M1 type TAMs. Intra-tumoral delivery of CpG-*Ythdf2* siRNA showed promising anti-tumor effects in B16-OVA melanoma and MC38 colon cancer models [141]. In addition, CpG-*Ythdf2* siRNA can synergize with anti-PD-L1 blockade therapy. Our proof-of-concept study supports the clinical translation of the CpG-*Ythdf2* siRNA strategy alone for in situ immunotherapy against cancer or its combination with anti-PD-L1 therapy [141].

3.7 Conclusions and Perspectives

In this chapter, we briefly introduced the history of cancer immunoediting and cancer immunotherapy and summarized the current progress in understanding the roles and mechanisms of m^{6}A regulators in immune cells and immune-associated molecules and their effects on anti-immune responses. We also discussed the current strategies targeting RNA modifications for cancer immunotherapy, including small molecular inhibitors, PROTAC-based or molecular glue degrader-based inhibitors, and ONTs. As discussed above, m^{6}A modifications not only regulate tumor growth but also modulate the TME, especially immune evasion. Therefore, a more comprehensive understanding of how m^{6}A modulation regulates the interplay between immune cells and tumor cells will expand our knowledge of the biological roles of m^{6}A in cancer immunoediting and help to develop m^{6}A-based drugs for immunotherapy.

Although m^{6}A modifications can regulate both innate and adaptive immunity, such as the activation and antigen-presentation activity of DCs, macrophage polarization and cross-presentation, NK cell maturation, activation, and effector function, the development of γδ T cells, T cell development, homeostasis, and effector function, as well as B cell development, the contributions of this fine-tuned regulation on tumor evasion and immunoediting remain largely unknown. Currently, the majority of phenotypes that are regulated by m^{6}A modifications were studied using tumor or immune cells in which one of the essential components of the m^{6}A regulator was deleted. Identifying which of the m^{6}A regulators is the dominant factor that drives the anti-tumor immunity in cancer remains to be explored. Future studies using CRISPR/Cas9-based screens that target RNA modification regulators in tumor cells or specific immune cells in the context of the TME will help to identify key m^{6}A regulators that are associated with tumor evasion and immunoediting.

The discovery of m^{6}A modifications and their roles in RNA metabolism as well as in various physiological and pathological processes has expanded our knowledge of epigenetics and emerged as a new mechanistic layer of gene expression regulation. However, our knowledge regarding m^{6}A modifications is still in its infancy. The development of modulators targeting m^{6}A modifications has rapidly

grown in recent years. Studies have proven that certain small molecule inhibitors to modulate m^6A regulators have shown favorable functional outcomes in preclinical mouse models. In addition, only a few m^6A regulators have been confirmed to be druggable and could serve as therapeutic targets for cancer treatment. Efforts are needed to move these promising inhibitors forward into the clinic. Nowadays, artificial intelligence (AI)-assisted techniques have been widely used for drug discovery [327]. Therefore, it would be more efficient to develop novel and effective therapeutic agents that inhibit m^6A regulators with AI-based drug discovery approaches.

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4 Recent Advances in RNA m^{6}A Modification in Solid Tumors and Tumor Immunity

Michelle Verghese, Emma Wilkinson, and Yu-Ying He

4.1 Introduction

Targeted molecular therapies have revolutionized cancer treatments and represent the culmination of decades worth of work aimed at identifying molecular changes that are both unique to cancer cells and spare normal healthy cells [1]. Key therapies resulting from these efforts include the development of vemurafenib for BRAF-mutant melanoma, imatinib for BCR-ABL in chronic myelogenous leukemia, and erlotinib for EGFR-mutant lung cancer [2–4]. While these advances have expanded treatment options, patients can develop resistance to these therapies, signifying the need for new therapeutic avenues. One such example is the development of epigenetic therapeutics.

Epigenetic dysregulation is a noted hallmark of cancer and can lead to malignant changes in gene expression [5]. In past years, studies on epigenetic changes on DNA have encompassed the majority of epigenetics research. However, in more recent years, analogous, reversible, epigenetic modifications on RNA have

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been characterized, and current work suggests that they too play an important role in promoting cancer progression. Though modifications on RNA were discovered in the 1960s, recent advances in sequencing technologies have expanded our understanding of these modifications [6, 7].

RNA modifications can occur on several types of RNAs, including mRNA, rRNA, tRNA, and non-coding RNAs, and over 100 modifications have been identified thus far [8, 9]. The deposition and recognition of chemical modifications on RNA species is catalyzed by a set of regulatory proteins, which include writers, erasers, and readers. The proteins involved in this regulatory machinery can serve either tumor suppressive or oncogenic roles in various types of cancer, suggesting that they may represent a new category of therapeutic targets [10, 11] Thus, a more thorough understanding of RNA modifications and their distinct role in cancer development and progression is critical to the development of these new therapeutics.

In this chapter, we will focus on the most abundant mRNA modification, N^6-methyladenosine (m^6A), and its role in solid tumors. We will begin by surveying the various roles of m^6A and its regulatory enzymes in different cancer types. Then, we will discuss the emerging role of m^6A in anti-tumor immunity. Finally, we will summarize the effects of m^6A RNA methylation in response to cancer therapies.

4.2 m^6A mRNA Modification

m^6A RNA methylation was discovered in the 1970s [12]. This reversible modification consists of the addition of a methyl group onto the 6th nitrogen of the adenosine nitrogenous base ring structure [13]. m^6A RNA methylation is the most prevalent chemical modification in eukaryotic mRNA, and is enriched specifically on long internal exons, near stop codons, and on 3' untranslated regions (UTRs) [14].

The regulation of m^6A mRNA methylation is mediated by three classes of proteins: writers, erasers, and readers (Fig. 4.1). Writer enzymes catalyze the addition of the methyl group onto the RNA molecule; erasers catalyze the removal of the methyl group; and reader proteins recognize the modification. m^6A writers include methyltransferase-like family members 3 and 14 (METTL3, METTL14), Wilms-tumor 1-associated protein (WTAP), RBM15, KIAA1429, and ZC3H13, which together can form a complex [15]. m^6A erasers, or demethylases, include the fat mass and obesity associated (FTO) and Alkb homologue 5 (ALKBH5) [16, 17]. m^6A readers include YTH binding proteins 1, 2 and 3 (YTHDF1, YTHDF2, YTHDF3), heterogeneous nuclear ribonucleoprotein A2/B1 and C (HNRNPA2B1, HNRNPC), YTH domain-containing 1 and 2 (YTHDC1, YTHDC2), and IGF2BPs [18, 19].

Together, these proteins work in concert to regulate the deposition of m^6A and dictate the downstream fate of the mRNA transcripts. In doing so, they serve

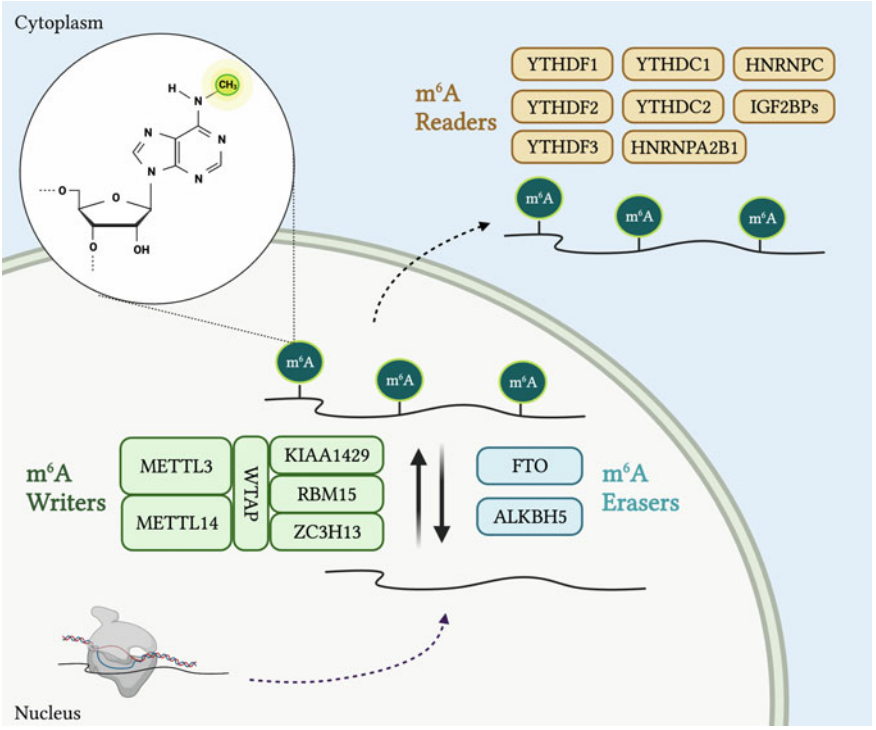

Fig. 4.1 m^{6}A regulatory proteins. After transcription of mRNA in the nucleus, m^{6}A is deposited by a multicomponent writer complex and removed by eraser proteins. Downstream, m^{6}A readers detect the presence of m^{6}A on mRNA transcripts to modulate mRNA metabolism

important roles in mRNA metabolism by driving processes including mRNA translation, nuclear processing, and decay [20, 21]. m^{6}A regulatory proteins have been shown to have a role in many biological processes, including embryogenesis, aging, and cancer [22, 23]. In cancer, these proteins have been found to play context-dependent roles in either promoting or inhibiting tumorigenesis [6].

4.3 m^{6}A in Solid Tumor Tumorigenesis

Here, we will highlight the roles of m^{6}A in several solid tumors and highlight the potential tumor suppressive or oncogenic function of m^{6}A regulatory proteins (Fig. 4.2).

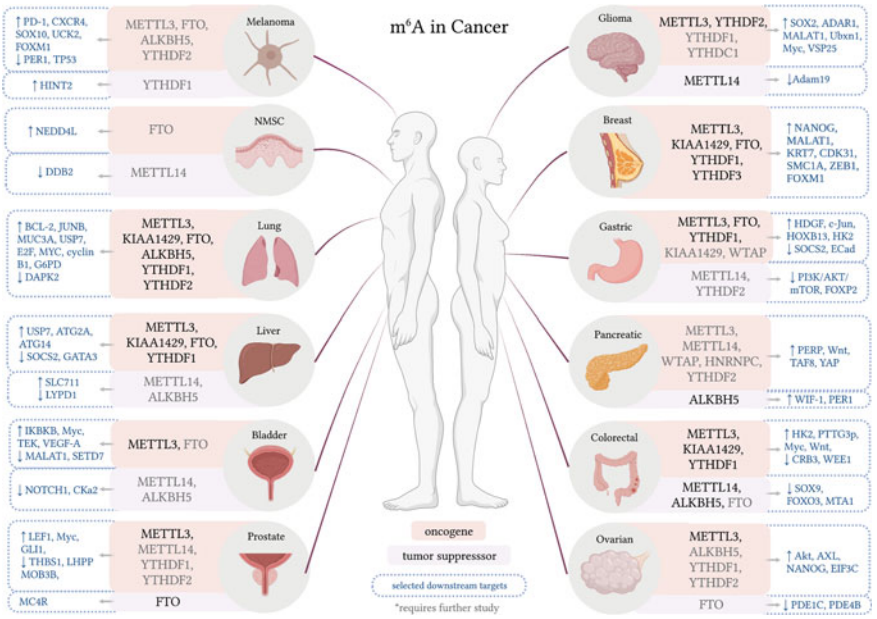

Fig. 4.2 The role of m^{6}A modifications in cancer. m^{6}A regulator proteins depicted in red windows represent those with oncogenic roles in cancer, while those in purple windows are tumor suppressive. Regulators depicted in grey text represent those needing additional studies to validate their role in cancer. Genes depicted in blue texts represent established downstream targets of the oncogenic or tumor suppressive m^{6}A regulators within each cancer type.

4.3.1 Breast Cancer

Research on the role of m^{6}A in breast cancer has shown that METTL3, KIAA1429, FTO, YTHDF1 and YTHDF3 can act as oncogenes. While preliminary studies suggest that METTL14, YTHDC1 and YTHDF2 may also be oncogenic, additional studies are needed to further assess this role. Finally, the roles of the m^{6}A eraser ALKBH5 and other m^{6}A reader proteins IGF2BPs have yet to be studied in the context of breast cancer.

i. *Writers*

Several studies have found that the m^{6}A writer METTL3 is oncogenic in breast cancer. One study found that METTL3 promotes m^{6}A methylation on the transcript of the apoptosis regulator *Bcl-2,* resulting in BCL-2 upregulation, reduced apoptosis, and breast cancer proliferation [24]. METTL3 was also found to promote m^{6}A deposition on the *Sox2* transcript and promote the expression of SOX2, a transcription factor which has been shown to be involved in maintaining tumor cell stemness [25].

Additionally, several other studies have shown that METTL3 can promote breast cancer through the regulation of the epithelial-mesenchymal transition (EMT), a pro-metastatic process. *Xu* et al. found that in breast cancer cells, METTL3 was bound by ZNF217, which led to decreased m^{6}A methylation on the pro-metastatic *Nanog* mRNA transcript, leading to increased NANOG protein expression, which then promoted EMT initiation, migration, and invasion [26]. Similarly, *Zhao* et al. found that METTL3 was overexpressed in breast cancer tissues and could promote the expression of long non-coding RNA metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) in an m^{6}A-dependent manner, which promoted EMT and tumor cell invasion [27]. Furthermore, METTL3 has been shown to promote metastasis in breast cancer cells by increasing the expression of keratin 7 (KRT7), a key protein in breast cancer lung metastasis [28]. In contrast, *Shi* et al. found that METTL3 could act as a tumor suppressor in breast cancer by upregulating collagen type III alpha 1 chain (COL3A1) via m^{6}A methylation, which inhibits metastasis [29]. These studies highlight the potential context-specific role of METTL3 in breast cancer metastasis.

With regards to other m^{6}A writer complex proteins, KIAA1429 has been found to be upregulated in breast cancer and to have an oncogenic role in breast cancer through regulation of the cell cycle regulator CDK1, and recent work has similarly implicated KIAA1429 in promoting breast cancer EMT and metastasis [30, 31]. Other studies have shown that KIAA1429 can stabilize *Smc1a* mRNA in an m^{6}A-dependent manner, leading to increased SMC1A expression and downstream upregulation of SNAIL to promote breast cancer EMT, migration and invasion [32]. Conversely, METTL14 was shown to be downregulated in the peripheral blood of breast cancer patients [33]. *Peng* et al. showed that the oncogenic kinase AURKA protects METTL14 from ubiquitin–proteasome-mediated degradation and strengthens IGF2BP2 binding to m^{6}A, leading to increased m^{6}A on *Drosha*, a catalytic subunit of the microprocessor complex with a role in tumorigenesis [34]. Increased m^{6}A on *Drosha* promoted *Drosha* mRNA stability and increased DROSHA expression, which enhanced a stemness phenotype and was associated with a poor prognosis for breast cancer [34]. Additional studies are needed to further assess the roles of KIAA1429 and METTL14 in breast cancer, and more research is needed to evaluate how other m^{6}A writers function in breast cancer.

ii. *Erasers*

The m^{6}A eraser FTO has been shown to be upregulated in breast cancer and may serve a potentially oncogenic role. Specifically, FTO has been shown to demethylate the pro-apoptotic tumor suppressor gene *Bnip3* to induce its degradation and promote breast cancer progression [35]. Other work has shown that FTO is upregulated in HER2-positive breast cancer and can accelerate cell invasion and migration by upregulating expression of ARL5B through inhibition of microRNA miR-181b-3p [36]. Additional research is needed to understand the role of other m^{6}A erasers, namely ALKBH5, in breast cancer.

iii. *Readers*

Most of the research on m^{6}A reader proteins in breast cancer has focused on YTHDF1 and YTHDF3. YTHDF1 has been found to contribute to breast cancer progression. *Sun* et al. showed that YTHDF1 is overexpressed and oncogenic in breast cancer and can promote S-phase entry, DNA replication, and DNA repair, in concert with METTL14 and E2F8 [37]. Another study found that YTHDF1 binds to m^{6}A-modified *Foxm1*, an EMT-promoting gene, and increases its translation, ultimately promoting breast cancer progression and metastasis [38]. YTHDF3 has also been shown to be upregulated in breast cancer tissues and to contribute to breast cancer progression [39]. YTHDF3 has been shown to promote migration, invasion, and EMT in triple-negative breast cancer by enhancing the mRNA stability of the oncogenic zinc finger transcription factor *Zeb1* in an m^{6}A-dependent manner, leading to increased ZEB1 expression [40]. Accordingly, upregulated YTHDF3 and ZEB1 were associated with poorer survival [40]. YTHDF3 can also regulate the expression of genes involved in brain metastasis in breast cancer, including ST6GALNAC5, GJA1, EGFR, and VEGFA, in an m^{6}A-dependent manner [41].

In addition, other m^{6}A readers may also play important roles in breast cancer tumorigenesis. *Zhou* et al. showed that the m^{6}A reader protein YTHDC1 is negatively regulated by tumor suppressor EMP3, and YTHDC1 was hypothesized to promote DNA replication and DNA damage repair, thereby promoting breast cancer [42]. However, the mechanism by which YTHDC1 promotes oncogenesis was not elucidated [42]. It has additionally been shown that YTHDF2 can interact with the MAPK pathway to induce EMT in breast cancer [43]. More research is needed to further elucidate the roles of YTHDC1 and YTHDF2 in breast cancer, as well establish the role of IGF2BPs in breast cancer tumorigenesis, which have yet to be considered.

4.3.2 Gastric Cancer (GC)

In gastric cancer, several m^{6}A regulators including METTL3, FTO, and YTHDF1 have been shown to be oncogenic. While some studies suggest that METTL14 may be a tumor suppressor in gastric cancer, additional work is needed to further explore these findings. Additionally, there are conflicting reports regarding whether ALKBH5 serves an oncogenic or tumor suppressive role in gastric cancer, and therefore future research is required to clarify this. Furthermore, few studies have looked at the role of KIAA1429, METTL16, WTAP, YTHDF2 or IGF2BPs in gastric cancer, which represents an area where more research is needed.

i. *Writers*

Studies on m^{6}A writers in gastric cancer (GC) have generally focused on METTL3 and have identified that METTL3 plays an oncogenic role in GC progression.

One study found that METTL3 could promote *Sphk2* mRNA translation in an m^{6}A-dependent manner, which inhibited the expression of the downstream zinc finger transcription factor KLF2 and promoted GC cell proliferation and migration [44]. METTL3 also led to the promotion of gastric cancer by reducing the mRNA stability of the cytokine signaling suppressor *Socs2* in an m^{6}A-dependent manner, resulting in reduced SOCS2 expression, which then promoted gastric cancer cell proliferation [45].

METTL3 has also been found to promote GC metastasis. *Yue* et al. found that METTL3 could deposit m^{6}A on *Zmym1* mRNA transcripts, which resulted in increased *Zmym1* mRNA stability and translation [46]. Mechanistically, ZMYM1 repressed the *E-cadherin* promoter to facilitate EMT and metastasis, as loss of E-cadherin can lead to EMT [46, 47]. *Wang* et al. also found that METTL3 upregulation promoted m^{6}A on *Hdgf* mRNA, which increased HDGF expression and promoted tumor angiogenesis [48]. Furthermore, *Liu* et al. showed that knockdown of METTL3 could suppress xenograft tumor growth and lung and lymph node metastasis [49]. Mechanistic studies revealed that METTL3 stabilized *Pbx1* transcripts to induce the expression of the GTP cyclohydrolase GCH1, which increased the biosynthesis of tetrahydrobiopterin BH4, a metabolite which has been shown to lead to malignant progression [49, 50].

Fewer studies have looked at other m^{6}A writer proteins in GC, and therefore more research is needed in this area. *Miao* et al. found that the m^{6}A writer KIAA1429 serves an oncogenic role in GC by promoting the mRNA stability of *c-Jun*, an oncogenic transcription factor, in an m^{6}A-dependent manner [51]. METTL16 has also been shown to promote proliferation of GC by increasing the mRNA stability and promoting the expression of the cell-cycle gene *cyclin D1* in an m^{6}A-dependent manner [52]. WTAP has been found to be upregulated in GC and contributes to GC progression by promoting glycolytic capacity and Warburg metabolism [53]. Mechanistically, WTAP promoted m^{6}A RNA methylation on hexokinase *Hk2* transcripts, increasing its stability and expression [53]. In contrast, METTL14 has been found to be downregulated in GC, while overexpression of METTL14 inhibits the PI3K/AKT/mTOR pathway to suppress GC progression, suggesting that METTL14 serves a tumor suppressive role in GC [54].

ii. *Erasers*

Both the m^{6}A erasers, FTO and ALKBH5, have been studied in the context of GC. In particular, high mRNA expression of *Fto* and *Alkbh5* has been associated with poor survival in GC [55]. FTO can lead to GC progression by decreasing m^{6}A on *Myc* mRNA, leading to MYC stabilization [56]. *Guo* et al. reported increased FTO expression in GC, and found that FTO may increase the expression of the androgen receptor repressor HOXB13 by reducing m^{6}A levels, which can lead to GC proliferation and invasion through activation of the PI3K/AKT/mTOR pathway; however the mechanism was not fully elucidated in this study [57]. It has been shown that FTO can promote GC metastasis by decreasing m^{6}A on the cell surface regulator *Integrin-B1* and enhancing its expression; this m^{6}A-dependent

regulation of *Integrin-B1* by FTO in GC is believed to promote migration and invasion [58]. FTO has also been shown to promote proliferation and metastasis of GC by demethylating the mRNA of membrane protein *Caveolin-1* to promote its degradation, which served to modulate mitochondrial metabolism and promote tumor growth [59].

Zhang et al. showed that ALKBH5 could demethylate transcripts of the lncRNA *Neat1*, which led to increased expression of EZH2, a subunit of the polycomb repression complex, to promote GC invasion and metastasis [60]. In contrast, another study found that ALKBH5 served a tumor suppressive role in GC, wherein ALKBH5 decreased the expression of PKMYT1, a serine/threonine kinase that serves to promote invasion and migration of GC [61]. Further studies will be needed to determine the role ALKBH5 plays in GC.

iii. *Readers*

In GC, high expression of YTHDF1 has been found to be associated with high-risk GC subtypes [62, 63]. Mechanistically, YTHDF1 has been shown to promote the translation of the WNT receptor FZD7 in an m^6A-dependent manner, leading to activation of the WNT/β-catenin oncogenic pathway [63]. *Chen* et al. also showed that YTHDF1 expression was associated with a poor prognosis in GC, and found that YTHDF1 can promote the translation of the ubiquitin-specific protein USP14 in an m^6A-dependent manner, promoting GC development [64]. Similarly, IGF2BP2 has also been shown to be elevated in GC; elevated IGF2BP2 increased IGF1R expression in an m^6A-dependent manner, which then activated the RhoA-ROCK signaling pathway and promoted GC progression [62]. In contrast, the expression of YTHDF2 has been shown to be lower in GC, and YTHDF2 has been found to inhibit GC cancer growth by reducing the expression of the transcription factor FOXP2 [65]. Future studies should aim to evaluate the roles of other m^6A reader proteins in GC.

4.3.3 Lung Cancer

In lung cancer, METTL3, KIAA1429, FTO, ALKBH5, YTHDF1 and YTHDF2 have been found to act as oncogenes. The role of METTL14 in lung cancer tumorigenesis is uncertain. Moreover, few studies have looked at YTHDC1, YTHDC2, or other m^6A readers, which therefore represents an additional area of future study in lung cancer.

i. *Writers*

Previous studies have found that the m^6A writers METTL3, METTL14, and KIAA1429 have roles in lung cancer tumorigenesis. METTL3 has been found to be overexpressed in lung adenocarcinoma (LUAD) and can promote tumor growth

and survival [66]. *Zhang* et al. also showed that METTL3 expression is upregulated in non-small cell lung cancer (NSCLC) and promotes NSCLC tumorigenesis by increasing the translation of oncogene BCL-2 in an m^{6}A-dependent manner [67]. Furthermore, METTL3 was found to be upregulated during TGF- β-induced EMT in lung cancer, and METTL3 increased the expression of the EMT transcriptional regulator JUNB in an m^{6}A-dependent manner promoting EMT [68]. *Xu* et al. further revealed that METTL3 is upregulated in LUAD and promotes cancer cell proliferation by inhibiting ferroptosis, an iron-dependent form of programmed cell death [69]. In contrast, *Wu* et al. found that METTL3 was downregulated in LUAD, where it played a tumor-suppressive role by enhancing the expression of the tumor suppressor FBXW7 in an m^{6}A-dependent manner [70].

Similarly, while several studies have delved into the role of METTL14 in NSCLC, the contrast in findings suggests that the role of METTL14 in NSCLC remains uncertain. *Shen* et al. identified a mechanism in which METTL14 bound to *Notch1* mRNA, resulting in increased *NOTCH1* expression and NSCLC progression [71]. Another study also found that METTL14 played a role in promoting EMT in NSCLC by decreasing expression levels of E-cadherin [72]. In contrast, *Mao* et al. showed that METTL14 may play a tumor suppressive role in LUAD by depositing m^{6}A on the tumor suppressive lncRNA Human leukocyte antigen complex group 11 (*Hcg11*) transcript and increasing *Hcg11* stability [73].

Furthermore, KIAA1429 was shown to be highly expressed in NSCLC and promoted tumor growth by suppressing the expression of *Dapk3* in an m^{6}A-dependent manner [74]. *Zhao* et al. showed that KIAA1429 was also highly expressed in LUAD, which increased the expression of the mucin MUC3A in an m^{6}A-dependent manner to promote tumor proliferation in LUAD [75]. These findings suggest that KIAA1429 may be oncogenic in lung cancer, but additional studies are required.

ii. *Erasers*

Studies have suggested that ALKBH5 is upregulated in NSCLC and plays a role in promoting NSCLC progression. *Guo* et al. showed that upregulated ALKBH5 in NSCLC can demethylate transcripts of the oncogene *Ube2c,* which promotes UBE2C translation and led to UBE2C-mediated repression of autophagy [76]. *Zhu* et al. found that ALKBH5 is upregulated in NSCLC and promoted cancer progression by demethylating transcripts and reducing expression of tumor suppressive *Timp3* [77]. Similarly, *Tsuchiya* et al. found that upregulated ALKBH5 in NSCLC can reduce TIMP3 expression via an IGF2BP-dependent pathway, leading to the inhibition of apoptosis, increased tumorigenicity, and poor prognosis [78]. ALKBH5 has also been found to be increased in lung cancers that are driven by mutant KRAS and a loss of LKB1, and promoted disease progression by stabilizing the expression of the oncogenes SOX2, SMAD7, and MYC in an m^{6}A-dependent manner [79].

FTO has been found to be oncogenic in NSCLC and increased the expression of the oncogenic ubiquitin-specific protease USP7 via m^{6}A demethylation of *Usp7*

transcripts [80]. *Wang* et al. also found a tumorigenic role for FTO in NSCLC by upregulating the expression of the E2F transcription factor-1 (E2F1), which promoted NSCLC growth and metastasis [81]. In LUAD, *Ding* et al. showed that FTO overexpression enhanced LUAD cell progression and activated cell migration [82]. In contrast, *Yang* et al. showed that WNT/β-catenin was bound to the FTO promoter in LUAD, resulting in decreased FTO expression [83]. FTO downregulation, in this study, served to promote tumor growth by increasing m^6A on the *Myc* transcript, which increased MYC translation in a YTHDF1-dependent manner [83]. Overall, while the literature suggests that FTO is oncogenic in lung cancer, additional research will be needed to evaluate how FTO expression is modulated during these processes.

iii. Readers

Both YTHDF1 and YTHDF2 have been suggested to play oncogenic roles in LUAD. In KRAS/TP53-mutant LUAD, YTHDF1 has been shown to promote the translation of the cell-cycle gene *Cyclin B1* in an m^6A dependent manner, promoting tumor proliferation [84]. *Sheng* et al. showed that YTHDF2 promoted lung cancer cell growth by promoting translation of *G6pd* mRNA and enhancing the pentose-phosphate pathway, while *Li* et al. found that YTHDF2 promoted LUAD progression by promoting the decay of *Axin1* transcripts and activating WNT/β-catenin signaling [85, 86]. Furthermore, *Zhao* et al. also showed that YTHDF2 was upregulated in LUAD, but found that YTHDF2 inhibited the invasion and metastasis of LUAD by binding m^6A on *Fam83d* mRNA and degrading these transcripts, which contributed to inhibition of the oncogenic FAM83D-TGFβ1-pSMAD2/3 pathway [87].

YTHDC1 has been previously found to be recruited to m^6A residues on lncRNA *Malat1*, which promoted its recruitment to nuclear speckles, which in turn promoted the transcription of oncogenes including *Twist2*, *Jun*, *JunB*, and *Pim1* [88]. *Ma* et al. further revealed that YTHDC2 expression is decreased in LUAD and plays an antitumor role in LUAD by targeting a subunit of the cysteine/glutamate antiporter system Xc^-, solute carrier 7A11 (SLC7A11), leading to impaired uptake of cysteine, which is a key amino acid that cancer cells rely on for metabolism [89, 90] The authors also showed that YTHDC2 can induce ferroptosis in LUAD cells via m^6A-mediated destabilization of subunits of the cystine/glutamate antiporter system Xc^- [89, 91]. Accordingly, further studies are needed to evaluate the roles of YTHDC1 and YTHDC2 in lung cancer.

4.3.4 Colorectal Cancer (CRC)

Studies have found that METTL3, KIAA1429, and YTHDF1 may act as oncogenes in CRC, while METTL14 and ALKBH5 may serve as tumor suppressors. The roles of FTO, IGF2BP2, and IGF2BP3 have not been studied in-depth, so further research is needed in these areas. Additionally, the roles of other m^{6}A readers, such as YTHDC1, also requires further study.

i. *Writers*

METTL3 has been found to function as an oncogene in colorectal cancer. Several studies have suggested that METTL3's oncogenic role in CRC is through modulation of metabolic activity. METTL3 has been shown to drive glycolytic metabolism in CRC by stabilizing *Hk2* and *Slc2a1* transcripts in an m^{6}A-dependent manner, leading to enhanced tumor progression [92]. Similarly, METTL3 can also promote the translation of the glucose transporter GLUT1 in an m^{6}A-dependent manner, which enhances glucose metabolism and contributes to CRC tumorigenesis [93]. *Zheng* et al. showed that METTL3 increased the expression of PTTG3P in an m^{6}A-dependent manner, which resulted in increased glycolysis and proliferation [94]. Other studies have found that METTL3 can promote CRC progression by directly regulating oncogenic pathways. Accordingly, *Li* et al. showed that METTL3 stabilizes transcripts of the stem cell marker *Sox2* via m^{6}A [95]. *Pan* et al. showed that METTL3 regulated CRC progression by suppressing the translation of *Crb3* transcripts via m^{6}A, which functioned to inhibit the Hippo pathway [96]. METTL3 has also been shown to promote CRC by enhancing the expression of the oncogene MYC through m^{6}A [97].

In contrast, some studies have indicated a tumor suppressive role for METTL14 in CRC. *Yang* et al. showed that METTL14 plays a tumor suppressive role in colorectal cancer by targeting lncRNA *XIST* in an m^{6}A-dependent manner, leading to its downregulation [98]. Another study showed that METTL14 mediated the degradation of the transcription factor *Sox9* in an m^{6}A-dependent manner, which inhibited EMT and metastasis of colorectal cancer [99]. *Wang* et al. also showed that METTL14 functions as a metastasis suppressor by downregulating the oncogene ARRDC4 in an m^{6}A-dependent manner [100].

Furthermore, KIAA1429 has been shown to be oncogenic in CRC. *Ma* et al. showed that KIAA1429 promoted CRC proliferation by negatively regulating the expression of the cell checkpoint regulator WEE1 via an m^{6}A-dependent mechanism [101]. *Zhou* et al. also found that KIAA1429 increased the expression of the sirtuin protein SIRT1 to promote the growth and motility of CRC [102]. Lastly, RBM15 has been found to act as an oncogene in CRC by modifying transcripts of the immune cell adaptor protein *myD88* [103].

ii. *Erasers*

Several studies have suggested that ALKBH5 may play a tumor-suppressive role in CRC. *Wu* et al. showed that decreased ALKBH5 expression is associated with a poor prognosis, and that ALKBH5 reduces m^6A on the transcript of the tumor suppressor *Foxo3*, resulting in increased *Foxo3* stability and expression [104]. *Yang* et al. further showed that the overexpression of ALKBH5 inhibited CRC invasion in vitro and metastasis in vivo, but a mechanism was not elucidated [105]. Furthermore, FTO has been shown to be tumor-suppressive in CRC by inhibiting the expression of metastasis-associated protein 1 (MTA1) [106]. It has also been suggested that FTO may play a role in the regulation of PD-L1 in CRC, but it is unclear whether this regulation was oncogenic or tumor suppressive [107]. More research is therefore needed to further explore the role of m^6A erasers in CRC tumorigenesis.

iii. *Readers*

In terms of reader proteins, YTHDF1 has been found to be highly expressed and oncogenic in CRC. *Bai* et al. showed that YTHDF1 may activate the WNT/β-catenin pathway [108]. *Wang* et al. showed that YTHDF1 increases the translation of RhoA activator ARHGGEF2, resulting in CRC cell growth and metastasis [109]. Lastly, *Nishizawa* et al. found that YTHDF1 is overexpressed in CRC and suggested that YTHDF1 expression is driven transcriptionally by the oncogene c-MYC [110].

Furthermore, IGF2BP2 can promote the expression of YAP in an m^6A-dependent manner, which leads to downstream activation of ERRB2, repression of apoptosis, and CRC progression [111]. It has been further shown that IGF2BP3 may play a role in promoting CRC cell cycle and angiogenesis by reading m^6A on *Cyclin D1* and *Vegf* transcripts, promoting their stability and expression [112].

4.3.5 Liver Cancer

In liver cancer, METTL3, KIAA1429, FTO, and YTHDF1 have been found to serve as oncogenes, while METTL14 and ALKBH5 may act as tumor suppressors. Additionally, while some studies have researched the role of other m^6A writer proteins in liver cancer, such as METTL16, RBM15, and WTAP, more studies are needed to fully establish their roles. Lastly, future research must also assess the roles of other m^6A reader proteins, such as YTHDC1 or YTHDC2, in liver cancer tumorigenesis.

i. *Writers*

Studies have shown that METTL3 can play an oncogenic role in hepatocellular carcinoma (HCC). METTL3 has been shown to repress the expression of HCC

tumor suppressor SOCS2 through an m^{6}A-YTHDF2-dependent mechanism, contributing to HCC progression [113]. It has been also shown that the SUMOylation of METTL3 could contribute to the progression and metastasis of liver cancer by regulating *Snail* mRNA expression in an m^{6}A-dependent manner [114]. *Wang* et al. additionally suggested that METTL3 may promote HCC proliferation, migration, and invasion by regulating the expression of USP7 via m^{6}A [114]. High expression of METTL3 in HCC was also shown to promote characteristics of vascular mimicry through regulation of the Hippo pathway in an m^{6}A-dependent manner [115].

Liu et al. highlighted the opposing roles that METTL3 and METTL14 may play in HCC, wherein METTL3 was found to be oncogenic, while METTL14 was found to be tumor suppressive [116]. METTL14 can inhibit the migration, invasion, and EMT of HCC by modulating the EGFT/PI3K/AKT signaling pathway via m^{6}A [117]. Similarly, *Fan* et al. showed that METTL14 plays a tumor suppressive role in HCC by targeting SLC711 in an m^{6}A-dependent manner, which stimulates ferroptosis in HCC [118].

Other m^{6}A writer proteins have also been studied in the context of HCC tumorigenesis. *Su* et al. found that METTL16 has a tumor-promoting role in liver cancer potentially via interactions with translation machinery eIF3a/3b, but a mechanism was not fully elucidated here [119] KIAA1429 was shown to promote HCC migration and invasion by upregulating m^{6}A on the mRNA of the DNA binding inhibitor *Id2*; this upregulation inhibited ID2 expression [120]. *Lan* et al. additionally showed that KIAA1429 promotes liver cancer development by upregulating m^{6}A on the pre-mRNA of the tumor suppressor *Gata3*, leading to *Gata3* degradation [121]. RBM15 has also been shown to promote HCC proliferation by mediating m^{6}A deposition on the HCC oncogene *Yes1*, which ultimately increased YES1 expression and activated the MAPK pathway [122].

In addition, other writer proteins have been investigated for their roles in liver cancer. WTAP has been shown to facilitate HCC progression by reducing the expression of the HCC tumor suppressor ETS1 in an m^{6}A-dependent manner [123]. *Li* et al. further showed that WTAP decreased LKB1 expression in an m^{6}A-dependent manner, resulting in decreased phosphorylation of AMPK and inhibition of autophagy [124]. ZC3H13 can act as a tumor suppressor in HCC, as ZC3H13-mediated m^{6}A modification of *Pkm2* transcripts leads to reduced *Pkm2* stability and PKM2 expression, resulting in reduced PKM-2-dependent glycolytic signaling in HCC [125]. Finally, a more recently discovered m^{6}A methyltransferase, ZCCHC4, has been shown to play an oncogenic role in HCC, but a mechanism was not elucidated [126].

ii. *Erasers*

FTO is suggested to play an oncogenic role in HCC. *Wang* et al. revealed that FTO can cooperate with RALYL to remove m^{6}A on the mRNA of *Tgfβ2*, thus stabilizing *Tgfβ2* transcripts, leading to the activation of the PI3K/AKT and STAT3 pathways and enhancement of HCC stemness [127]. Similarly, *Bian* et al. revealed

that high AMD1 expression in HCC served to increased FTO expression; increased FTO further upregulated NANOG, SOX2, and KLF4 through m^6A-demethylation and promoted HCC stemness [128]. FTO can also demethylate *Pkm2*transcripts, leading to increased PKM2 expression and downstream increases in aerobic glycolysis and cancer progression [125]. Lastly, ALKBH5 has been shown to play a tumor suppressive role in HCC by m^6A-mediated inhibition of the oncogene LY6/PLAUR Domain Containing 1 (LYPD1) [129]. However, more research is needed to elucidate the role of ALKBH5 in liver cancer.

iii. *Readers*

YTHDF1 has been shown to be upregulated in HCC and correlated with a poor prognosis [130]. *Li* et al. revealed that YTHDF1 can bind to m^6A-modified transcripts of the autophagy-related genes *Atg2a* and *Atg14* and enhance their translation, leading to increased autophagy and HCC malignancy [131]. Similarly, *Luo* et al. found that YTHDF1 could promote the growth of HCC via the PI3K/AKT/mTOR pathway and could play a role in inducing EMT; however, a mechanism was not elucidated [132]. YTHDF1 has also been shown to facilitate EMT in HCC, by activating AKT/glycogen synthase kinase (GSK)-3β/β-catenin signaling [133].

In addition, YTHDF2 has also been found to be upregulated in HCC, and high YTHDF2 expression has been shown to be associated with cancer recurrence in HCC [134]. *Hou* et al. showed that YTHDF2 promoted the mRNA decay of *Il11* transcripts in an m^6A-dependent manner, which promoted inflammation, the disruption of vasculature normalization, and malignancy of HCC [135]. In contrast, *Zhong* et al. found that YTHDF2 can act as a tumor suppressor in HCC by promoting the decay of *Egfr* mRNA, resulting in decreased cell proliferation [136]. To date, research has primarily focused on YTHDF1 and YTHDF2; future studies should therefore investigate the roles of other m^6A reader proteins in HCC.

4.3.6 Glioma

Glioma is a deadly form of brain cancer with limited treatment options. Interestingly, much research has been done regarding the role of m^6A in glioma tumorigenesis and m^6A represents a promising therapeutic target for this deadly cancer. A critical process for glioblastoma tumorigenesis is the development and maintenance of glioblastoma stem cells (GSCs), and several studies have found that the dysregulation of m^6A levels in GSCs can modulate glioblastoma.

i. *Writers*

Several studies have demonstrated that m^6A writer proteins play important roles in glioma pathogenesis. However, there are conflicting reports regarding the tumor suppressive or oncogenic role of METTL3 in GSC formation and glioblastoma

tumorigenesis. For example, *Cui* et al. found that METTL3/14 serves a tumor suppressive role in glioblastoma stem cell development, as knockdown of METTL3/14 resulted in increased GSC formation [137]. Mechanistically, these authors also found that knockdown of METTL14 resulted in decreased m^{6}A on the GSC-promoting gene *Adam19*, resulting in increased *Adam19* expression and GSC formation [137]. While these studies have found that METTL3/14 decreases GSC formation, other studies have discovered that METTL3 may serve an oncogenic function. *Visvanathan* et al. led a contrasting study wherein they found that METTL3 is increased in GSCs and leads to m^{6}A-dependent increases in *Sox2* mRNA stability, which promotes pluripotency and GSC maintenance [138]. *Lv* et al. also found that platelet-derived growth factor (PGDF) forms an axis with METTL3 to increase m^{6}A mRNA levels and decrease the expression of the mitophagy-promoting and tumor-suppressive gene optineurin (Optn) [139]. *Tassinari* et al. found that METTL3 was upregulated in glioblastoma and led to m^{6}A/YTHDF2-dependent increases in the pro-tumorigenic deaminase ADAR1 [140]. *Chang* et al. discovered that METTL3 was increased in IDH-wildtype gliomas and promoted tumorigenesis through m^{6}A-dependent stabilization of MALAT1 and downstream activation of NF-κB signaling [141]. Furthermore, another study proposed a novel mechanism by which METTL3 promotes glioblastoma tumorigenesis by promoting m^{6}A-mediated nonsense-mediated decay and alternative splicing [142]. Interestingly, *Zepecki* et al. found that m^{6}A is decreased in GSCs by miR-145, which lead to a novel miRNA-mediated increase in translation; however, the functional role of the decrease in m^{6}A enrichment in GSC function was not discerned [143]. The disparity in the function of METTL3 in glioblastoma tumorigenesis suggests that its role may indeed be context-dependent and requires further study.

ii. *Erasers*

For m^{6}A erasers, two studies found that pharmacologic inhibition of FTO resulted in decreased GSC formation, suggesting that m^{6}A mRNA levels are critical for GSC development and maintenance and highlighting the potential efficacy of FTO inhibitors in glioblastoma treatment [137, 144]. However, the oncogenic role of FTO in GSCs may be different in glioblastoma cells, as *Zhang* et al. found that decreased FTO expression was associated with worse glioblastoma grade and poor survival and that re-expression of FTO led to decreases in glioblastoma cell growth [145]. Mechanistically, these authors found that FTO serves its tumor suppressive function by promoting the maturation of miR-10a in an m^{6}A/HNRNPA2B1-dependent manner, leading to downstream inhibition of the pro-survival PI3K/AKT/mTOR pathway [145].

iii. *Readers*

The role of YTHDF1 in regulating glioma tumorigenesis is not well-studied. Accordingly, *Xu* et al. found that YTHDF1 expression was increased in advanced-stage glioma patients, and that knockdown of YTHDF1 led to decreased glioma cell proliferation [146]. However, the targets of m^6A-dependent YTHDF1 were not evaluated in this study [146]. *Yarmishyn* et al. also replicated these findings and found that YTHDF1 was increased in glioblastoma, but also did not determine whether this function was m^6A-dependent [147]. Compared to YTHDF1, more studies have found roles for YTHDF2 in glioma tumorigenesis and GSC maintenance and formation. *Fang* et al. found that YTHDF2 expression was associated with worse overall survival for glioblastoma patients, and that YTHDF2 is stabilized through EGFR/SRC/ERK [148]. Furthermore, these authors found that YTHDF2 promotes glioblastoma tumorigenesis by promoting the m^6A-dependent mRNA decay of *Lxrα* and *Hivep2*, which leads to increases in glioblastoma cell survival and cholesterol dysregulation [148]. YTHDF2 was also found to promote glioma progression through the m^6A-mediated mRNA decay of *Ubxn1*, which is a negative regulator of NF-κB signaling; therefore, the YTHDF2-mediated loss of *Ubnx1* expression resulted in the activation of NF-κB signaling and glioma progression [149]. In terms of GSC maintenance, *Dixit* et al. found that YTHDF2 was increased in GSCs and assumes a novel role in promoting the mRNA stability of *Myc* and *Vegfa* in an m^6A-dependent manner to promote GSC maintenance [150].

There is little research done on other m^6A regulators, which therefore represents a critical area of research. One study found that WTAP, HNRNPA2B1 and HNRN2PC were highly increased in glioblastoma samples compared to healthy controls [151]. Furthermore, YTHDC1 was found to promote the protein expression of VSP25, which further upregulates JAK-STAT signaling, in an m^6A-dependent manner, to promote glioma tumorigenesis [152].

4.3.7 Other Cancers

Here, we will highlight several cancers that have fewer reported roles for m^6A in their development than the cancers summarized above, including bladder cancer, ovarian cancer, prostate cancer, pancreatic cancer, melanoma, and non-melanoma skin cancers. These cancers represent areas of research that require further study.

4.3.8 Bladder Cancer

Research on m^6A modification in bladder cancer is primarily focused on METTL3 and FTO. Several studies have indicated that METTL3 is an oncogene in bladder cancer. *Han* et al. showed that METTL3 may play an oncogenic role in bladder cancer; its interaction with the microprocessor protein DGCR8 contributed to maturation of pri-miR221/222, leading to reduced levels of the tumor suppressor

PTEN and leading to bladder cancer progression [153]. METTL3 was also found to be oncogenic in bladder cancer as METTL3 installed m^{6}A on regulators in the NF-κB pathway (IKBKB and RELA), the Myc pathway (MYC), and transcription elongation (AFF4), leading to their increased expression [154]. *Wang* et al. showed that METTL3 promotes m^{6}A methylation on tyrosine kinase endothelial (TEK) and vascular endothelial growth factor A (VEGF-A) to increase their expression, which contributes to bladder cancer progression and angiogenesis [155]. Lastly, *Xie* et al. also showed that METTL3 was oncogenic in that it promoted the mRNA degradation of tumor suppressors *Setd7* and *Klf4* [156].

Several studies have also investigated the role of FTO in bladder cancer; however, the results are constrasting. For example, FTO has been shown to promote bladder cancer tumorigenesis via regulation of metastasis-associated lung adenocarcinoma transcript 1 *Malat1* mRNA in an m^{6}A-dependent manner, leading to decreased MALAT1expression [157]. Similarly, *Song* et al. showed that FTO decreased m^{6}A on *Pycr1* transcripts and stabilized them to promote bladder cancer progression [158]. However, *Yi* et al. found that FTO may be a tumor suppressor in bladder cancer, and that the activity of FTO was modulated by the RNA-binding protein SFPQ [159].

Far fewer studies have considered the roles of other m^{6}A proteins in bladder cancer progression. *Gu* et al. showed that METTL14 played a tumor suppressive role in bladder cancer by decreasing the mRNA stability of the bladder cancer oncogene *Notch1* in an m^{6}A-dependent manner, decreasing its expression [160]. Another study instead showed that METTL14 played an oncogenic role in bladder cancer, but a mechanism was not identified here [161]. *Yu* et al. showed that ALKBH5 may play a tumor suppressive role in bladder cancer by reducing the stability of *Ck2a* mRNA in an m^{6}A-dependent manner, which led to decreased bladder cell proliferation via reduced glycolysis [162]. However, more studies are needed to elucidate the roles of METTL14 and ALKBH5 in bladder cancer. Additionally, research is needed to investigate the potential roles of reader proteins in bladder cancer progression.

4.3.9 Ovarian Cancer

Most of the research on the role of m^{6}A in ovarian cancer has focused on METTL3. Taken together, these studies suggest that METTL3 is an oncogene in ovarian cancer. *Bi* et al. showed that METTL3 promoted the maturation of miRNA miR-126-5p, which can activate the PI3K/AKT/mTOR pathway and promote ovarian cancer oncogenesis [163]. Similarly, *Liang* et al. showed that METTL3 can promote ovarian cancer progression through regulation of the AKT pathway, and additionally showed that high METTL3 expression was associated with a high pathological grade [164]. One study found that METTL3, independently of METTL14 and WTAP, was correlated with poor outcomes, and that METTL3 regulated the expression of genes associated with ovarian cancer via m^{6}A, including *Eif3c*, *Axl, Csf-1*, FZD10 in TOV-112D, and *Crl-11731d* [165]. Lastly, METTL3

has been shown to stimulate the translation of the receptor tyrosine kinase AXL, which promoted EMT of ovarian cancer [166]. In terms of METTL14, more studies are needed to further understand its role in ovarian cancer as well as the roles of other m^{6}A writer proteins. One study found that METTL14 expression was reduced in ovarian cancer and that METTL14 suppresses ovarian cancer progression by promoting m^{6}A on troponin associated protein (*Troap*) mRNA, reducing its stability and inhibiting *Troap* expression [167].

Several studies have looked at the role of m^{6}A erasers and readers in ovarian cancer. *Jiang* et al. found that ALKBH5 was highly expressed in ovarian cancer tissue and ALKBH5 was found to demethylate m^{6}A on *Nanog,* a gene that promotes pluripotentcy and stemness, and increased its expression, contributing to ovarian cancer progression [168]. In contrast, FTO has been shown to be decreased in ovarian tumors. FTO was found to suppress ovarian cancer stem cell stemness and tumorigenesis via inhibition of cAMP signaling, which was mediated by reduced m^{6}A and decreased stability of phosphodiesterase *Pde1c* and *Pde4b* transcripts [169]. In terms of readers, *Liu* et al. found that YTHDF1 could promote ovarian cancer progression by binding to m^{6}A on translation initiation factor *Eif3c* transcripts, which increased translational output and promoted ovarian cancer tumorigenesis [170]. YTHDF2 has additionally been shown to play a role in ovarian cancer. *Xu* et al. found that YTHDF2 was degraded by the tumor suppressor FBW7, and that YTHDF2 itself acts as an oncogene in ovarian cancer, although a mechanism for the oncogenic role of YTHDF2 was not elucidated here [171]. Similarly, *Li* et al. showed that YTHDF2 was highly expressed in ovarian cancer tissues and could promote ovarian cancer progression by regulating the expression of the microRNA miR-145; however, the mechanism was not further explored here [172].

4.3.10 Prostate Cancer

Much of the research on m^{6}A in prostate cancer has focused on METTL3, with fewer studies considering other writer proteins, erasers, or readers. Namely, METTL3 has been shown to play an oncogenic role in prostate cancer. *Ma* et al. showed that METTL3 was associated with a poor prognosis and deposited m^{6}A on *Lef1* transcripts, which led to increased LEF1 expression, activated WNT signaling, and prostate cancer progression [173]. METTL3 has also been shown to deposit m^{6}A on *Myc* transcripts, leading to increased MYC expression and progression of prostate cancer [174]. *Cai* et al. showed that METTL3 played an oncogenic role in prostate cancer by increasing m^{6}A and the expression of a protein component of the hedgehog pathway, GLI1, thus inhibiting cell apoptosis and promoting prostate cancer growth [175].

Some studies that have evaluated the role of METTL3 in prostate cancer have specifically focused on the role of METTL3 in bone metastases. *Li* et al. showed that METTL3 could promote bone metastasis of prostate cancer by regulating the expression of *Integrin β1* through m^{6}A, which increased ITGB1 expression,

METTL3-mediated binding to collagen-I, and prostate cancer cell tumor motility [176]. *Lang* et al. showed that METTL3 increased the expression of lncRNA *Pcat6* in an m^{6}A-dependent manner, and PCAT6 subsequently upregulated the expression of *Igf1r* and promoted bone metastasis [177]. Few studies have considered other m^{6}A writer proteins in prostate cancer. In one study, METTL14 was shown to promote prostate cancer tumorigenesis by inhibiting expression of thrombospondin 1 (*Thbs1*) mRNA in an m^{6}A-dependent manner [178].

Studies suggest that FTO may act as a tumor suppressor in prostate cancer. *Zhu* et al. showed that FTO was downregulated in prostate cancer tissues and may inhibit prostate cancer cell invasion, though a mechanism was not elucidated [179]. *Li* et al. also showed that FTO was downregulated in prostate cancer, and similarly suggested that FTO acts as a tumor suppressor in prostate cancer via interactions with the melanocortin 4 receptor (MC4R) [180].

Finally, few studies have investigated reader proteins in prostate cancer. *Du* et al. showed that YTHDF2 played a role in binding m^{6}A on Mps one binder kinase activator (*Mob3b*) transcripts to induce their degradation, which promoted prostate cancer tumor growth [181]. YTHDF2 has also been shown to mediate the degradation of tumor suppressors *Llpp* and *Nkx3*-1 transcripts through binding to m^{6}A, leading to prostate cancer progression [182]. Additional studies are needed to investigate the roles of other m^{6}A reader proteins in prostate cancer.

4.3.11 Pancreatic Cancer

Few studies have looked at the roles of m^{6}A writer proteins and readers in pancreatic cancer. One study found that METTL3 expression was increased in pancreatic tumor samples and that METTL3 knockdown decreased cancer cell proliferation and migration, but a mechanism was not elucidated [183]. METTL14 has been found to promote pancreatic cancer progression by targeting the mRNA of the p53 effector *Perp* in an m^{6}A-dependent manner, thus reducing its expression [184]. WTAP was found to be upregulated in pancreatic cancer by the activity of WTAPP1, which then led to activated Wnt signaling, though a mechanism was not identified [185]. HNRNPC was also shown to mediate increased expression of a pro-metastatic splice isoform, TAF8, via m^{6}A, which promoted pancreatic cancer metastasis [186].

Many studies on the role of m^{6}A modifications in pancreatic cancer have focused on the m^{6}A eraser ALKBH5, which, taken together, suggest that ALKBH5 is an important tumor suppressor in pancreatic cancer. *Tang* et al. showed that ALKBH5 inhibits pancreatic cancer by targeting the Wnt inhibitory factor 1 WIF-1 and increasing its expression via m^{6}A demethylation [187]. ALKBH5 has also been shown to inhibit pancreatic cancer progression by increasing the expression of *Per1* transcripts through m^{6}A demethylation, which led to the activation of the tumor suppressive signaling pathway ATM-CHK2-p53/CDC25C to inhibit pancreatic cancer cell growth [188]. Another study found that ALKBH5 could enhance stability and expression of a lncRNA, *Kcnk15-As1*, which could exert

tumor suppressive effects in pancreatic cancer by regulating KCNK15 and PTEN [189]. Lastly, *Huang* et al. found that ALKBH5 regulated the mRNA decay of the ubiquitin ligase *Fbxl5* and mitochondrial iron importer *Slc25a28*, which promoted iron metabolism and protected against pancreatic cancer [190]. Fewer studies have looked at the role of FTO in pancreatic cancer. *Tang* et al. found that FTO promoted prostate cancer by enhancing stability and increasing the expression of MYC proto-oncogene *bHLH* transcripts via m^6A demethylation [191]. Further studies will be needed to clarify the role of FTO in pancreatic cancer.

Lastly, the m^6A reader YTHDF2 has been shown to promote pancreatic cell EMT via regulation of YAP signaling, but a mechanism was not determined in this study [192]. Additional studies will be needed to further investigate the roles of m^6A writer proteins and readers in pancreatic cancer.

4.3.12 Melanoma

Few studies have investigated the role of m^6A modifications in melanoma. *Yang* et al. showed that FTO is oncogenic in melanoma and mediates the m^6A demethylation of melanoma-promoting genes including *Pdcd-1*, *Cxcr4*, and *Sox10* transcripts, leading to their stabilization [193]. *Hao* et al. showed that ALKBH5 promoted uveal melanoma and metastasis via m^6A demethylation of oncogene *Foxm1* mRNA, increasing its stability and expression [194]. With regards to m^6A writer proteins, METTL3 has been shown to promote the transcript stability of the uridine-cysteine kinase *Uck2* via increased m^6A, which led to increased UCK2 expression and promoted the metastasis of melanoma cells through the WNT/β-catenin signaling pathway [195]. With regards to m^6A readers, *Jia* et al. showed that YTHDF1 acted as a tumor suppressor in ocular melanoma by recognizing m^6A RNA methylation on the mRNA of the tumor suppressor *Hint2* and promoting its translation [196]. *Yu* et al. showed that YTHDF2, in contrast, acted as an oncogene in ocular melanoma by recognizing m^6A RNA methylation on the mRNA of the tumor suppressors *Per1* and *Tp53* and promoting their degradation [197]. In general, studies suggest that m^6A RNA methylation plays a role in regulating melanoma development and progression, but more work will be needed to establish the explicit roles of m^6A regulators in melanoma.

4.3.13 Non-melanoma Skin Cancers

Non-melanoma skin cancers (NMSCs) comprise all cancers of the skin excluding melanoma. While the incidence of NMSCs is high, there remains a paucity of research regarding the role of m^6A in NMSC development and progression. Two recent studies have begun to address this gap in knowledge. *Yang* et al. found that METTL14 is down-regulated in human and mouse skin tumors and in response to UVB exposure through selective autophagy, and loss of METTL14 results in decreased m^6A on the global genome repair gene *Ddb2*, resulting in decreased

YTHDF1-mediated DDB2 translation, decreased repair of UVB-induced DNA damage, and increased skin tumorigenesis [198]. Furthermore, *Cui* et al. found that the m^{6}A demethylase FTO is up-regulated in arsenic-induced skin lesions and promotes arsenic-induced skin tumorigenesis [199]. The authors also found that *Nedd4l*, a ubiquitin ligase that can regulate Wnt signaling, is a downstream target of FTO-mediated m^{6}A demethylation, resulting in decreased *Nedd4l* stability and tumor progression [199].

4.4 Anti-tumor Immunity

The tumor microenvironment (TME) is the cellular environment surrounding a tumor, and is composed of stromal cells, infiltrating immune cells, blood vessels, and extracellular matrix [200]. The immune cells within the TME can be either pro- or anti-tumorigenic, depending on the context. Adaptive immune cells within the TME are B cells and T cells; innate immune cells can include macrophages, dendritic cells, neutrophils, and NK cells [200]. In this chapter, we will focus on the role of m^{6}A mRNA modification in the immune cells of the TME and cover studies in which m^{6}A in tumor cells modulates the activity of immune cells in the TME to promote tumor progression or suppression. The role of m^{6}A in immune cells in general, as well as the role of m^{6}A in nonimmune stromal cells in the TME, is reviewed in detail elsewhere [201].

4.4.1 Overview of m^{6}A in Immunity

m^{6}A has been shown to play important roles in various stages of immune system function, including innate immunity, adaptive immunity, and development, and some key studies will be highlighted here as the area has been previously reviewed in depth [202]. Much of the research with regards to m^{6}A in innate immunity has evaluated the role of m^{6}A during the cellular response to viral or foreign RNA (Fig. 4.3). In particular, it has been shown that type I interferon-B (IFN-B) production triggered by double-stranded DNA (dsDNA) or human cytomegalovirus is regulated by METTL14 and ALKBH5 [203]. Specifically, it was shown that METTL14 played a role in reducing interferon-B (*Ifnb1*) mRNA stability via m^{6}A, while ALKBH5-mediated m^{6}A demethylation had the opposite effect [203]. This suggests that m^{6}A regulators play a role in shaping immunity to both viral and nonmicrobial dsDNA. In support of this finding, another study also showed that following viral stimulation, METTL3 and YTHDF2 both played a role in restricting the Type I interferon response via m^{6}A-modification of *Ifnb* mRNA, which reduced its stability [204].

Viral RNAs can also be m^{6}A-modified, and it is suggested that this may play a role in allowing them to avoid immune detection. *Lu* et al. showed that human metapneumovirus itself is m^{6}A methylated, and that depletion of m^{6}A induced a higher expression of type I interferons and of cytoplasmic RNA sensor RIG-I

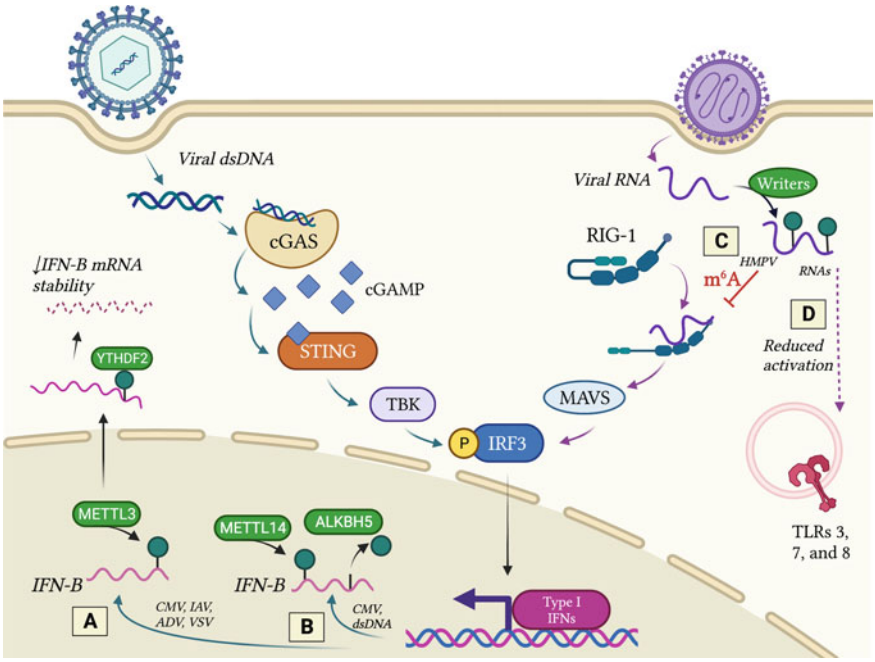

Fig. 4.3 **m^{6}A modifications in innate immunology. A** METTL3 and YTHDF2 could promote *Ifnb1* transcript degradation via m^{6}A, thus restricting the Type I IFN response after infection with cytomegalovirus (CMV), Influenza A virus (IAV), adenovirus (ADV), or vesicular stomatitis virus (VSV). **B** Infection with DNA virus cytomegalovirus (CMV) or nonviral dsDNA could lead to modulation of m^{6}A on type I IFN-B by METTL14 and ALKBH5. **C** Inhibition of m^{6}A on viral RNA from human metapneumovirus (HPMV) could increase type I interferon response via RNA sensor RIG-I. **D** m^{6}A could reduce the ability of RNAs to activate toll-like receptors (TLRs) 3, 7 and 8

[205]. Similarly, it has also been shown that m^{6}A-deposition on RNAs reduces the immunogenicity of the RNA, specifically in terms of its ability to activate toll-like receptors (TLRs) in dendritic cells [206].

Lastly, m^{6}A regulators also play a role in the regulation of adaptive immune responses, with specific studies focusing on the T cell response. A key study from *Li* et al. showed that METTL3 mediates T cell homeostasis by depositing m^{6}A on *Socs* family transcripts, increasing their stability and expression [207]. Increased SOCS-family proteins led to the inhibition of STAT5 activation and helped promote T cell homeostasis [207]. In this study, m^{6}A was additionally found to play a role in the degradation of *Socs* mRNA as part of T cell reprogramming mechanisms [207]. Finally, m^{6}A RNA methylation also plays important roles in immune cell development. *Zheng* et al. showed that METTL14 played a key role in promoting B cell development, especially in response to IL-7, via dysregulating gene expression and the large-pre-B to small-pre-B cell transition [208]. In summary,

m^{6}A RNA methylation has been shown to play a role in several facets of immune system regulation and function.

4.4.2 Immune Cell Intrinsic Mechanisms

a. *Macrophages*

Macrophages are important phagocytes in the immune system; when found in the TME, they are often referred to as tumor-associated macrophages (TAMs) (Fig. 4.4). TAMs can take the form of M1 macrophages, which function primarily to phagocytose antigens, or M2 macrophages, which are immune-suppressive and participate in healing [200]. While M1 macrophages have anti-tumor effects, M2 macrophages are oncogenic and can promote tumor growth through the secretion of cytokines, such as IL-4 [200]. *Dong* et al. established that C1q$^+$ TAMs can cross-talk with intratumoral T cells [209]. Furthermore, the authors showed that METTL14 and YTHDF2 are enriched in C1q$^+$ TAMs and serve to maintain the equilibrium of cytotoxic and dysfunctional CD8$^+$ T cell subpopulations to maintain anti-tumor functions [209]. Accordingly, knockout of METTL14 in C1q$^+$ TAMs promoted tumor progression by inhibiting CD8$^+$ T cell infiltration, and caused dysfunctional development and activation of the CD8$^+$ T cells [209].

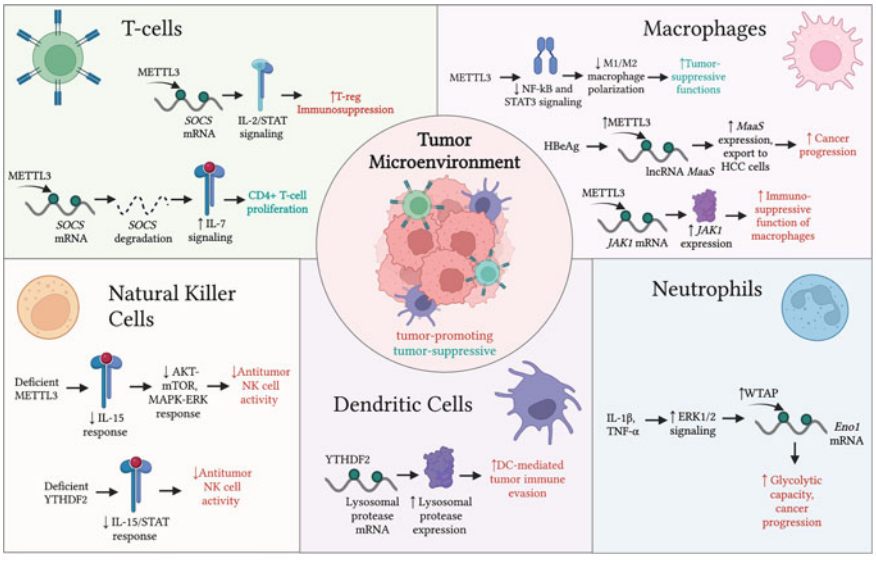

Fig. 4.4 m^{6}A modifications in tumor immunity. Highlighted are the roles of m^{6}A modifications in immune cells within the tumor microenvironment: T-cells, Macrophages, Natural Killer cells, Dendritic cells, and Neutrophils. Processes in red depict those which are tumor-promoting, while processes in green are tumor-suppressive

Some studies have indicated that METTL3 may play a role in promoting immunosuppressive functions in macrophages. In a study of Hepatitis B virus (HBV)-related HCC cancerous tissues, *Tao* et al. showed that an oncogenic lncRNA MAAS was upregulated in M2 macrophages and contributed to HCC progression [210]. Mechanistically, the HBV-associated antigen HBeAg stabilized and enhanced the expression of MAAS in M2 macrophages through METTL3-mediated m^6A modification of *MaaS* [210]. MAAS was then transported to HCC tumor cells from the M2 macrophages via exosomes, promoting HCC proliferation [210]. Similarly, METTL3 was upregulated via lactylation in tumor-infiltrating myeloid cells (TIMs), a population that includes TAMs as well as tumor-associated neutrophils, myeloid-derived suppressor cells, and regulatory dendritic cells [212]. Mechanistically, METTL3 increased the expression of JAK1 in an m^6A-dependent manner, leading to upregulated JAK1 and increased STAT3-mediated transcription of pro-tumoral genes, ultimately promoting the immunosuppressive functions of the TIMs [212]. However, in contrast, another study revealed that depletion of METTL3 in macrophages enhanced TAM infiltration into tumors, creating an immunosuppressive microenvironment [211]. Moreover, depletion of METTL3 enhanced the activation of NF-κB and STAT3, which led to M1- and M2-like macrophage polarization and contributed to increased tumor growth [211].

b. *Natural Killer cells*

Natural killer (NK) cells are innate lymphocytes that play an important role in mounting a defense against virus-infected cells and tumor cells, primarily by secreting cytolytic perforin granules which induce apoptotic cell death in the target cell [213]. Tumor-infiltrating NK cells have been shown to display decreased expression of METTL3 [214]. In most NK cells, METTL3 is required for the NK response to the cytokine IL-15, which engages the AKT-mTOR and MAPK-ERK pathways downstream of the IL-15 receptor [214]. Therefore, the downregulation of METTL3 in tumor-infiltrating NK cells impaired their effector functions and maturation ability [214]. This result indicates the importance of METTL3 in maintaining homeostasis and anti-tumor immunity in NK cells [214].

Ma et al. additionally showed that deficiency of YTDHF2 in NK cells impaired their antitumor immune functions [215]. In particular, YTHDF2 was found to be upregulated in response to IL-15 stimulation and was shown to be required for NK cell maturation, survival, and effector functions [215]. Mechanistically, YTHDF2 expression was positively regulated by STAT5 downstream of the IL-15 receptor, and through a positive feedback loop, YTHDF2 was required for optimum IL-15/STAT5 signaling in activated NK cells [215]. More research will be needed to assess the roles of other m^6A regulators apart from METTL3 and YTHDF2 in NK cells during the anti-cancer response.

c. *T cells*

Various subpopulations of T cells can be found within the TME. Cytotoxic CD8$^+$ T cells are responsible for targeting tumor cells for destruction through recognition of a specific tumor antigen. Additionally, CD4$^+$ helper T cells aid in CD8$^+$ T cell activation and response via IL-2 and IFNγ secretion [200]. Finally, regulatory T cells (Tregs) are also found in the TME, but typically serve to inhibit antitumor immune responses and thus can contribute to cancer progression [200].

Research on m^{6}A in T cell function within cancer has mainly focused on Tregs. In the tumor microenvironment, Tregs can play a cancer-promoting role by suppressing CD8$^+$ mediated-tumor-cell killing. *Tong* et al. revealed that METTL3 added m^{6}A on *Socs* mRNA, regulating the IL-2-STAT5 signaling pathway, which is essential to the suppressive function of Tregs [216]. This result suggests that it is possible that depletion of m^{6}A in tumor-infiltrating Tregs may be promising therapeutically [216]. Future work should continue to evaluate how m^{6}A functions in T cells during immune responses to cancer, with a special focus on CD8$^+$ T cells, given that they play a key role in cancer cell killing and have not yet been studied significantly in this area.

d. *Dendritic Cells*

Dendritic cells are professional antigen-presenting cells that play a critical role in presenting antigens to T cells and B cells, which leads to the generation of the adaptive immune response. Within the TME, dendritic cells also function to present tumor antigens to T cells in order to induce anti-tumor T cell immunity [217]. *Han* et al. showed that YTHDF1 in dendritic cells recognizes m^{6}A-modified transcripts of lysosomal proteases and promotes their translation [218]. These lysosomal proteases in dendritic cells serve to suppress cross-presentation of tumor neoantigens and promote tumor immune evasion [218]. Additional studies are needed to further understand the functions of YTHDF1 in dendritic cells in tumor immunity and to evaluate the roles of other m^{6}A regulators.

e. *Neutrophils*

Neutrophils are leukocytes mainly involved in the innate immune response. Similar to macrophages, neutrophils can differentiate and serve in pro- or anti-tumor roles [219]. *Ou* et al. identified a mechanism by which a neutrophil subpopulation, C5aR1$^+$ neutrophils, promoted glycolytic capacity and tumor progression of breast cancer [220]. Specifically, C5aR1$^+$ neutrophils were found to secrete IL1B and TNFα, which activate ERK1/2 signaling [220]. Activation of ERK signaling increased WTAP expression and subsequently increased m^{6}A deposition on *Eno1* and increased glycolytic capacity [220]. Increased glycolytic capacity can contribute to cancer progression as cancer cells much more readily rely on Warburg

metabolism [221]. As in the case of dendritic cells, additional studies are needed to evaluate the roles of other m^{6}A regulators in neutrophils in tumor immunity.

4.4.3 Tumor Intrinsic Mechanisms

Changes in m^{6}A modifications and their writers, erasers, and readers within tumor cells themselves can also indirectly modulate the anti-tumor immune response. For example, tumor cells can express programmed death ligand 1 (PD-L1), which interacts with PD-1 on immune cells and contributes to immune evasion. *Liu* et al. showed that METTL3-mediated m^{6}A methylation elevated the expression of circIGF2BP3 in NSCLC cells, which led to subsequent upregulation of PD-L1 expression on the surface of the NSCLC cells and contributed to NSCLC immune escape by inhibiting anti-tumor CD8$^+$ T cell infiltration [222]. Similarly, *Wan* et al. showed that METTL3, in conjunction with IGF2BP3, increased PD-L1 expression in breast cancer cells in an m^{6}A-dependent manner, which led to the inhibition of anti-tumor T cell activation and promoted tumor immune evasion [223].

Additionally, m^{6}A in tumor cells can affect immune cell development and infiltration. It has been previously shown that CRC tumors deficient in METTL3 or METTL14 had increased infiltration of anti-tumor CD8$^+$ T cells and increased production of cytokines IFNγ and CXCL-10, suggesting that METTL3 and METTL14 play a role in altering the tumor microenvironment [224]. Other studies have also revealed a similar function for METTL3 in tumor cells acting in promoting immune escape. *Shen* et al. showed that tumors with low METTL3 expression displayed increased infiltration of dendritic cells, as well as increased expression of MHC and co-stimulatory molecules [225]. Another study revealed that METTL3 could promote the mRNA degradation of *Socs2* in an m^{6}A-dependent manner, which inhibited the polarization of M1 macrophages, the macrophage subset needed to inhibit glioma cell proliferation [226]. However, in glioma, the histone demethylase JMJD1C was found to promote the expression of the microRNA miR-302a, which inhibits METTL3, and therefore promoted M1 macrophage polarization [226]. Similarly to METTL3, *Liu* et al. revealed that FTO also plays a role in promoting tumor immune escape by restricting CD8$^+$ T cell activation and effector functions [227]. Mechanistically, FTO upregulated the expression of the transcription factors *c-Jun*, *JunB,* and *C/ebpβ* through m^{6}A demethylation, and this enhancement promoted glycolysis in tumors and dampened T cell effector functions [227].

Regarding other immune cell types, *Bai* et al. showed that YTHDF1 is upregulated in gastric cancer cells and can inhibit the recruitment of dendritic cells to suppress the anti-tumor response [228]. Mechanistically, YTHDF1 has been found to decrease the expression of interferon gamma receptor-1 (IFNGR1) and decrease tumor cell responsiveness to IFNγ stimulation [228]. Furthermore, *Li* et al. found that YTHDC2 appeared to be tumor suppressive in HNSCC, and YTHDC2 expression was positively correlated with immune infiltration of B cells,

$CD8^+$ T cells, $CD4^+$ cells, neutrophils, and dendritic cells in HNSCC [229]. Furthermore, Another study showed that low expression of WTAP in gastric cancer cells was correlated with a high T cell immune response to GC [230]. However, the role of WTAP in T cell immune responses is not well-studied. Much of the research in this area focuses on METTL3 and METTL14, so future work should continue to study this while also investigating the potential importance of other m^{6}A regulators in cancer cells in the context of the immune response.

4.5 Therapeutic Responses

Here, we will highlight the role of m^{6}A in therapeutic responses and survey the role of m^{6}A and m^{6}A regulatory proteins in predicting response or sensitivity to various cancer therapies.

4.5.1 Targeted Therapy

Targeted therapies describe therapies that target genes or proteins in cancer cells that contribute to cancer progression (Fig. 4.5). One category of targeted therapies is kinase inhibitors, which are drugs that target and inhibit oncogenic kinases, enzymes which catalyze the phosphorylation of a target substrate. Since the development of the first kinase inhibitor, imatinib, for the treatment of BCR-ABL CML, kinase inhibitors have shown great promise in cancer therapeutics [231]. Other kinase inhibitors and their targets include EGFR inhibitors for lung cancer (gefitinib, erlotinib), HER2/EGFR inhibitors for breast cancer (lapatinib), BRAF inhibitors for melanoma (vemurafenib, dabrafenib), and Btk inhibitors for hematologic malignancies (ibrutinib), among many others [232]. Inhibitors targeting tyrosine kinases, such as EGFR, are referred to as tyrosine kinase inhibitors (TKIs). Studies on the role of m^{6}A in the therapeutic response to kinase inhibitors are limited; further research should expand on both the regulatory protein studies and the types of kinase inhibitors and cancers considered.

a. *Gefitinib*

Gefitinib is a selective EGFR TKI used to treat advanced or metastatic NSCLC [233]. *Xiao* et al. showed that FTO was upregulated in serum exosomes of gefitinib-resistant (GR) patients, compared with those of gefitinib-sensitive patients [234]. Mechanistically, exosomal FTO promoted the expression of the high-affinity gefitinib transporter ABCC10 in an m^{6}A-dependent manner, contributing to gefitinib resistance [234]. Furthermore, the authors also showed that the administration of GR exosomes could decrease gefitinib sensitivity of recipient cells in an FTO-dependent manner [234].

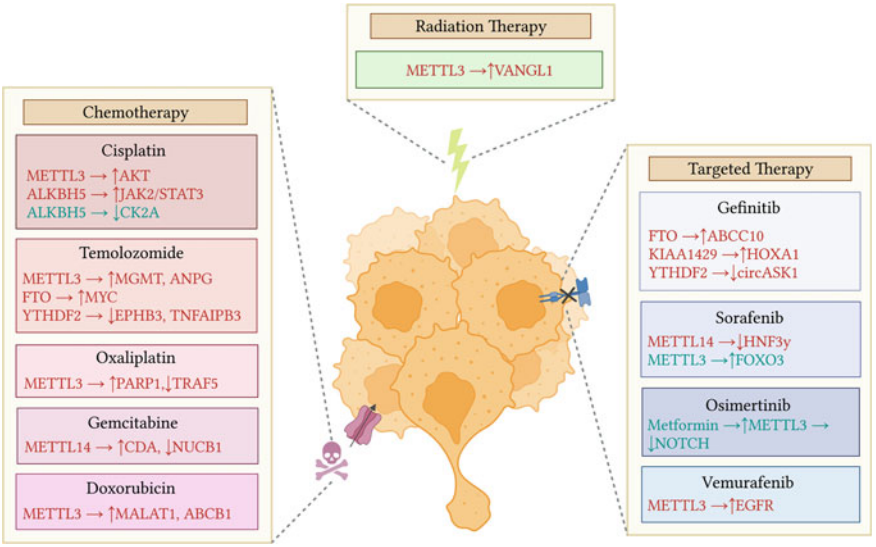

Fig. 4.5 m^{6}A modifications in responses to chemotherapy, targeted therapy, and radiation. Depicted are the roles of m^{6}A modifications and their regulators in response to chemotherapy, targeted therapy, and radiation therapy. Processes depicted in red text represent those promoting resistance to therapy, while processes in green text represent those promoting sensitivity to therapy

Tang et al. showed that KIAA1429 was upregulated in gefitinib-resistant NSCLC cells and promoted gefitinib resistance of NSCLC cells by enhancing the stability of *Hoxa1* transcripts through m^{6}A modification on the 3' UTR [235]. Another study by *Wang* et al. found that circular RNA, *circAsk1*, which encodes for a protein isoform of ASK1 that can decrease gefitinib resistance, was downregulated in gefitinib-resistant cells via YTHDF2-mediated cleavage of m^{6}A-modified circASK1 [236].

b. Osimertinib

Osimertinib is a EGFR TKI used for the treatment of advanced or resistant NSCLC [237]. *Li* et al. showed that treatment with metformin, an antidiabetic agent, promoted METTL3-mediated activation of *Let7b* maturation, which resulted in increased NSCLC sensitivity to osimertinib through suppression of NOTCH signaling [238].

c. Sorafenib

Sorafenib is a TKI used to treat advanced renal cell, liver, and thyroid cancer [239]. *Zhou* et al. showed that HNF3γ is downregulated in HCC via METTL14-mediated m^{6}A modification of *Hnf3γ* transcripts [240]. Decreased HNF3γ expression reduced the transactivation of two sorafenib influx transporters, OATP1B1 and

OATP1B3, which enhanced sorafenib resistance in HCC [240]. Another study showed that in sorafenib-sensitive HCC cells, METTL3 promoted the expression of the autophagy regulator FOXO3 in an m^{6}A/YTHDF1-dependent manner [241]. METTL3 was decreased in sorafenib-resistant HCC, leading to the downregulation of FOXO3, increased autophagy, and HCC survival [241]. Lastly, it was reported that a circRNA, circSORE, was upregulated in sorafenib-resistant HCC cells in an m^{6}A-dependent manner; this upregulation functioned to induce sorafenib resistance by activating the Wnt/β-catenin pathway [242].

d. Vemurafenib

PLX4032, or vemurafenib, is a BRAF inhibitor used in the treatment of melanoma [2]. *Bhattarai* et al. identified an increase in METTL3 expression in PLX4032-resistant melanoma cells, which contributed to PLX4032 resistance by increasing EGFR expression in an m^{6}A-dependent manner [243].

4.5.2 Chemotherapy

Chemotherapy is a type of cancer treatment that utilizes drugs to target cancer cells. Chemotherapeutic drugs are classified by their mode of action; categories can include alkylating agents, platinating agents, antimetabolites, and antimicrotubular agents. Mechanistically, these agents can interfere with DNA, RNA or protein synthesis or function, leading to cell death. Notably, these effects can be toxic towards normal cells as well [244]. Chemotherapy is typically part of the first-line therapy given to cancer patients, often in conjunction with surgery and/or radiation. While the role of m^{6}A in chemotherapy has been more researched than its role in kinase inhibitor therapy, the effects of many m^{6}A regulators with chemotherapeutic drugs have yet to be investigated.

a. *Cisplatin*

Cisplatin is a widely used chemotherapeutic alkylating agent [245]. *Shi* et al. showed that METTL3 expression was upregulated in cisplatin-resistant NSCLC tissues and that METTL3 promoted NSCLC chemoresistance by upregulating AKT1 expression via m^{6}A modification of *Akt1* mRNA [246]. Another study found that ZC3H13 expression was downregulated in HCC, which promoted cisplatin sensitivity by reducing *Pkm2* mRNA stability in an m^{6}A-dependent manner [125].

ALKBH5 has also been implicated in the therapeutic response to cisplatin. *Nie* et al. demonstrated that ALKBH5 was upregulated in cisplatin-resistant epithelial ovarian cancer, and promoted cisplatin resistance by facilitating activation of the JAK2/STAT3 signaling pathway via a positive feedback loop with HOXA10 [247]. In contrast, *Yu* et al. found that ALKBH5 was downregulated in bladder cancer tissues and sensitized bladder cancer cells to cisplatin [162]. In particular, ALKBH5

functioned as a tumor suppressor by reducing *Ck2a* mRNA stability in an m^6A-dependent manner, which ultimately inhibited bladder cancer cell proliferation [162].

Some studies have found that YTHDF1 may play a role in promoting cisplatin resistance. *Chen* et al. showed that YTHDF1 was upregulated in CRC tissues and contributed to cisplatin resistance by promoting glutaminase 1 (GLS1) translation in an m^6A dependent manner [248]. Subsequently, increased GLS1 stimulated glutamine uptake and metabolism and promoted cisplatin resistance [248]. Further, another study showed that m^6A/YTHDF1-mediated upregulation of TRIM29 promoted cancer stem cell-like features in cisplatin-resistant ovarian cancer cells [249]. In another study, *Shi* et al. showed that YTHDF1 depletion in lung tumors rendered the tumors resistant to cisplatin treatment [250].

b. Temozolomide

Temozolomide is an alkylating agent used primarily for treatment of astrocytoma and glioblastoma multiforme [258]. In glioma, *Shi* et al. found that METTL3 promoted resistance to temozolomide, a DNA damaging agent, by increasing the expression of the DNA damage repair genes MGMT and ANPG in an m^6A-dependent manner [259]. In contrast, however, *Xiao* et al. found that FTO regulated MYC to promote temozolomide resistance in glioma, and that FTO inhibition, rather, led to increased temozolomide sensitivity [260]. Whether FTO-mediated regulation of MYC was m^6A-dependent was not evaluated, however [260]. Furthermore, YTHDF2 has been found to promote temozolomide resistance in glioma by increasing the mRNA decay of *Ephb3* and *Tnfaipb3* in an m^6A-dependent manner, leading to the activation of both NF-κB and PI3K/AKT signaling, contributing to temozolomide resistance [261].

c. Oxaliplatin

Oxaliplatin is an alkylating agent and platinum analog used for the treatment of colorectal and gastric cancer [251, 252]. *Lan* et al. found that METTL3 was upregulated in CRC cells by the activity of M2-tumor associated macrophages (TAMs), which serve to promote oxaliplatin resistance by inhibiting TRAF5-mediated necroptosis [253]. In gastric cancer, *Li* et al. found that $CD133^+$ cancer stem cells (CSCs) could promote oxaliplatin resistance by upregulating METTL3, which led to increased stability of *Parp1* transcripts and improved base excision repair pathways [254].

d. Gemcitabine

Gemcitabine is an antimetabolite cytidine analog drug used primarily in the treatment of pancreatic cancer [255]. One study found that the treatment of pancreatic cancer cells with gemcitabine resulted in increased expression of METTL14, and

METTL14 promoted gemcitabine resistance by increasing the expression of cytidine deaminase (CDA) [256]. Upregulation of METTL14 in gemcitabine-resistant pancreatic cancer cells was facilitated by p65 binding to the METTL14 promoter [256]. Further, *Hua* et al. described a mechanism by which METTL3 promoted degradation of the calcium binding protein *NucB1* transcript in an m^{6}A/YTHDF2-dependent manner, which led to reduced expression of NUCB1 and promoted chemoresistance in pancreatic ductal adenocarcinoma [257].

e. Doxorubicin

Doxorubicin is an anthracycline used for multiple cancers including breast cancer [262]. METTL3 has been previously found to be highly expressed in doxorubicin-resistant breast cancer [263]. In terms of mechanism, METTL3 was found to promote doxorubicin resistance by depositing m^{6}A on *Malat1*, which recruited factor E2F1 and activated the expression of AGR2, a member of the protein disulfide isomerase family [263]. Furthermore, *Yang* et al. found that IGF2BP3 promoted the mRNA stability of *Abcb1* in an m^{6}A-dependent manner, leading to increased ABCB1 expression and CRC chemoresistance to doxorubicin [264].

f. Other chemotherapies

Additional studies have considered other types of chemotherapy. One study showed that METTL3 increased the expression of *Sec62* in an m^{6}A-dependent manner, and SEC62 upregulation was associated with the promotion of CRC stemness and chemoresistance to fluorouracil and oxaliplatin via activation of the Wnt/β-catenin signaling [265]. *Zhang* et al. showed that METTL3 increased the mRNA stability of *Cbx8* transcripts in an m^{6}A/IGFBP1-dependent manner, promoting cancer stemness and decreased chemosensitivity to oxaliplatin and irinotecan in colon cancer [266]. Other studies have shown that YTHDF1 can coordinate with METTL14 to enhance *E2f8* mRNA stability, which led to increased DNA replication, DNA damage repair, and chemoresistance of breast cancer to doxorubicin, cisplatin, and Olaparib, a PARP inhibitor [37].

4.5.3 Radiation

Radiation therapy is used for many types of solid tumors and involves the administration of high energy waves to target cancer cells. *Hao* et al. showed that upon irradiation, METTL3 upregulated the expression of VANGL1 in an m^{6}A-dependent manner in lung adenocarcinoma [267]. Subsequently, VANGL1 mitigated the effects of radiotherapy by activating the BRAF/TP53BP1/RAD51 pathways, thereby protecting DNA from damage [267]. Furthermore, in glioma, *Visvanathan* et al. found that METTL3 was increased in glioma stem cells (GSCs) and promoted resistance to radiotherapy, and knockdown of METTL3 in GSCs

resulted in increased response to γ-irradiation, as evidenced by decreased DNA repair [138].

4.5.4 Immunotherapy

Immunotherapy describes a new form of cancer therapy that utilizes the immune system to destroy cancer cells. In particular, immune checkpoint inhibitors are a category of immunotherapy drugs which target checkpoints, or regulations that keep the immune system controlled and verify when to correctly induce a response [268]. Cancer cells can inappropriately activate these immune checkpoints, such that the immune system is unable to mount an appropriate response. Thus, checkpoint inhibitors are a promising immunotherapeutic target [268]. Two types of checkpoint inhibitors are anti-PD1 therapies (Pembrolizumab, nivolumab) and anti-CTLA4 therapies (ipilimumab).

a. Anti-PD1 therapy

Anti-PD1 therapy is a type of immunotherapy that targets the programmed cell death receptor (PD-1) to destroy cancer cells (Fig. 4.6). *Wang* et al. showed that knockdown of METTL3 and METTL14 could enhance the response to anti-PD1 treatment in colorectal cancer and melanoma by stabilizing the mRNA transcripts of *Stat1* and *Irf1* and elevating their expression [224]. Notably, STAT1 and IRF1 are chemokines with roles in promoting intratumor $CD8^+$ TILs and IFNγ expression and are important for the tumor killing function of anti-PD1 therapy [224].

It has also been reported that knockdown of ALKBH5 can enhance the efficacy of anti-PD1 therapy in melanoma and colon cancer. ALKBH5 has been found to recruit MDSCs and Tregs through changes in the lactate content of the tumor microenvironment, which can play a role in altering responses to immunotherapy [269]. Further, *Yang* et al. showed that knockdown of FTO sensitized melanoma cells to anti-PD1 treatment [193]. Mechanistically, FTO inhibition led to the suppression of melanoma-intrinsic *Pdcd1*, *Cxcr4*, and *Sox10* genes, which contributed to the response to anti-PD1 immunotherapy [193].

b. Anti-CTLA4 therapy

Fewer studies have looked at the potential role of m^6A and m^6A machinery in response to anti-CTLA4 therapies. A study by *Huang* et al. used a principal component analysis of m^6A modification patterns in HCC samples to determine that an m^6A-scoring signature could be associated with a response to anti-PD1 or anti-CTLA4 immunotherapies [270]. This preliminary finding indicates that more research is needed to assess the potential role of m^6A in the response to anti-CTLA4 therapies.

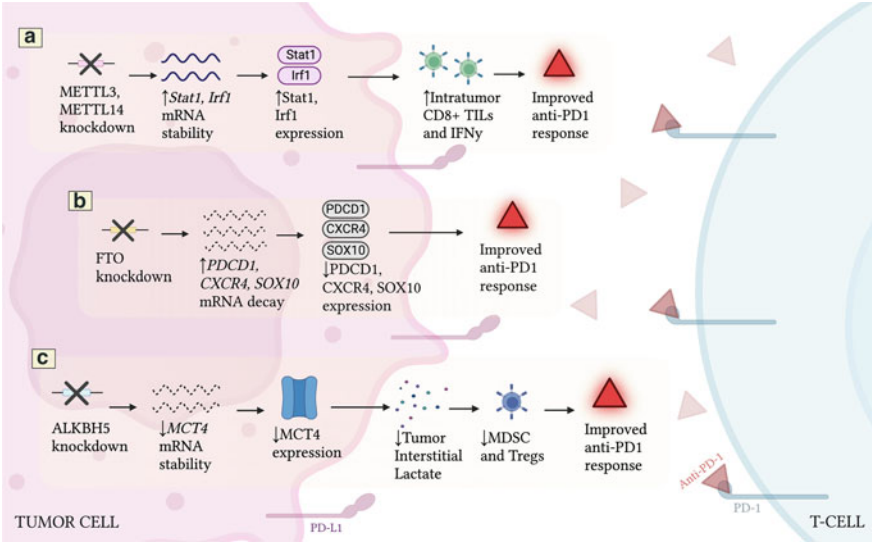

Fig. 4.6 **m^{6}A modifications in anti-PD1 immunotherapy.** Highlighted are the roles of m^{6}A modifications in responses to anti-PD1 immunotherapy. **a** Knockdown of METTL3 and METTL14 led to increased mRNA stability of the chemokines *Stat1* and *Irf1* via m^{6}A modifications, which increased their expression and led to recruitment of tumor-killing CD8 + tumor-infiltrating lymphocytes (TILs) and expression of IFNγ to improve the anti-PD1 response. **b** Knockdown of FTO led to mRNA degradation of the melanoma oncogenes PDCD1, CXCR4, and SOX10 via m^{6}A, which reduced their expression and improved the response to anti-PD1. **c** Knockdown of ALKBH5 led to reduced mRNA stability of the lactate transporter MCT4 via m^{6}A, reducing its expression and leading to reduced interstitial tumor lactate, which contributed to reduced myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) to improve the anti-PD1 response

4.5.5 Hormone Therapy

Very few studies have looked at m^{6}A modifications in hormone therapy and is an important area of future study. A few studies have looked at tamoxifen, a selective estrogen receptor modulator. *Liu* et al. showed that METTL3 expression was increased in tamoxifen-resistant breast cancer cells, and functioned to promote resistance. Mechanistically, METTL3 deposited m^{6}A on adenylate kinase 4 *Ak4* transcripts, which upregulated AK4 expression and led to increased ROS, p38 activation, and tamoxifen resistance [271].

Petri et al. found that HNRNPA2B1 was upregulated in breast cancer tissue, and the overexpression of HNRPNPA2B1 in these tissues promoted resistance to tamoxifen [272]. Another study found that YTHDF2 was downregulated in tamoxifen-resistant breast cancer cells, which led to the stabilization and increased translation of *Atf3* mRNA; this increased translation then increased the expression of efflux pump ABCB1, thus contributing to tamoxifen resistance [271].

4.6 Conclusions and Future Directions

In this chapter, we summarize the roles of m^6A and m^6A machinery in the development and progression of various types of cancer, anti-tumor immunity, and therapeutic responses. Taken together, the literature on m^6A in cancer formation and progression indicates that across different cancer types, m^6A writers, erasers, and readers play context-dependent roles, modulating various oncogenic or tumor suppressive pathways. For example, in some cases, an m^6A regulator can act as an oncogene in one cancer type, while it can serve as a tumor suppressor in another. Even within the same cancer type, some m^6A regulators have been shown across different studies to have both pro-tumorigenic and anti-tumorigenic functions. Despite these context-dependent functions, a few themes emerged when considering the literature overall. For example, METTL3 and FTO most often appear to be pro-tumorigenic in cancer. In contrast, we see that METTL14 is often tumor-suppressive across different solid tumor types. Additionally, m^6A readers seem to be frequently less studied in tumor biology than writers and erasers, even though the present research suggests that they do play important roles. Overall, as the cancer epitranscriptomics field progresses, more studies are needed to clarify the roles of m^6A enzymes in various cancer types, as well as identifying whether any one function is dominant and of greater phenotypic importance than another.

While m^6A and its regulators have been considerably studied in tumor biology, they have been less studied in tumor immunology. We emphasize that m^6A plays both cancer cell-intrinsic and immune cell-intrinsic roles in anti-tumor immunity. m^6A can affect immune cell development and infiltration, and frequently acts in favor of immune escape. Some themes from these studies include a role for METTL3 in macrophages in modulating tumor immunity, and YTHDF1 as a promoter of tumor immune evasion in dendritic cells. However, there is still much more work to be done in this area to identify how these various enzymes and immune cells act in concert in the dynamic tumor microenvironment. Determining the relative importance of these activities is another area of study to determine if one enzyme may be an appropriate therapeutic target for immunotherapy.

Finally, emerging evidence implies that m^6A has an important function within the therapeutic response to various types of cancer therapies, including targeted therapy, chemotherapy, radiation, and immunotherapy. While the roles and functions of the m^6A regulators are diverse, in many cases, these regulators seem to play a role in promoting resistance to therapies, specifically chemotherapies and targeted therapies. Thus, inhibiting m^6A enzymes may be promising in reducing resistance, but more research is needed to assess this strategy, including identifying any potential off-target effects. Additionally, few studies have looked at the role of m^6A in immunotherapy, especially for therapies other than anti-PD1 therapy. More research is needed here as immunotherapy continues to gain prominence as a cancer treatment. Additionally, non-T cell-focused immunotherapies, such as dendritic cell- and macrophage-based immunotherapies, have also begun to show promise. Future studies are needed to understand how m^6A is regulated in these cells as therapies are developed in these immune cell types.

In summary, compelling research suggests that m^{6}A RNA modification plays an important role in cancer development progression, anti-tumor immunity, and therapeutic responses. We now have a better understanding of the vast number of biological pathways that are modulated via m^{6}A-dependent RNA metabolism and how this dysregulation can promote cancer. However, part of the task going forward will be to understand which of these functions are phenotypically dominant or significant and thus may represent a feasible therapeutic target.

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Data Availability Data from this study are available from the corresponding author upon request.

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5 Recent Advances in Adenosine-to-Inosine RNA Editing in Cancer

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5.1 Introduction

In recent years, we have seen an explosion of interest in the field of enzymatic RNA modifications, also called *epitranscriptome*. There are more than 170 known RNA modifications across all types of RNAs in different domains of life [14], and the number of chemically distinct modifications is still growing. RNA editing is one of the most prevalent and abundant forms of post-translational RNA modification observed in normal physiological processes and diseases including cancer. RNA editing can be achieved through the insertion or deletion of nucleotides or deamination of nucleobases, generating either standard nucleotides such as uridine (U) or the rare nucleotide inosine (I). The most widespread type of RNA editing in mammals, with millions of editing sites detected in humans, is hydrolytic deamination of adenosine-to-inosine (A-to-I). Previously, an unknown enzymatic activity was found to be responsible for the unwinding of double-stranded RNA (dsRNA) in oocytes and embryos of *Xenopus laevis* [7]. Subsequent studies revealed that this unwinding activity is carried out by adenosine deaminase acting on RNA (ADAR), which eventually established the field of A-to-I RNA editing [8, 96, 143].

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A-to-I editing is one of the most abundant RNA modifications that is carried out by the ADAR family of proteins in humans [10]. The ADAR family comprises ADAR1, ADAR2 and ADAR3 for which ADAR1 and ADAR2 are responsible for all known A-to-I editing sites, whereas the catalytic activity of ADAR3 has yet to be established [20, 70, 89, 90, 123]. ADAR1 is ubiquitously expressed in many tissue types whereas ADAR2 is enriched in the brain [70, 90]. Interestingly, transcription of the *ADAR1* gene occurs at alternative promoters, one of which is interferon (IFN)-inducible that would translate to a full-length ADAR1p150 isoform that is primarily localized to the cytoplasm owing to an additional N-terminal nuclear export signal (NES), and the other promoter being constitutively expressed which translates to a shorter-length ADAR1 p110 isoform that is primarily localized to the nucleus [40, 101]. Both isoforms contain the C-terminal catalytic adenosine deaminase domain, three dsRNA-binding domains (dsRBD) and Zβ domains, whereas p150 contains an additional N-terminal Z-RNA binding domain—Zα which overlaps the NES. In contrast, ADAR2 only has two dsRBD domains and the catalytic deaminase domain. Importantly, homodimerization of ADAR1 and ADAR2 monomers is essential for A-to-I editing [26, 109, 139]. The third dsRBD of ADAR1 and the first dsRBD of ADAR2 are required for their respective homodimerization [99, 109]. On the other hand, ADAR3 is not able to homodimerize, which may explain its lack of catalytic activity [26]. A-to-I editing sites can be found in coding and non-coding regions of pre-mRNA transcripts as well as non-coding RNAs such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), among others. However, the vast majority of A-to-I editing sites are in introns and untranslated regions (UTRs) harbouring long and perfect dsRNA structures formed by inverted *Alu* repetitive elements [92]. In contrast, the editing of coding sequences is highly selective, possibly due to the formation of imperfect dsRNA structure by the edited exon and upstream/downstream complementary sequence, termed the editing site complementary sequence (ECS). Dysregulated A-to-I editing impacts human diseases, such as neurological diseases and cancer [18, 21, 37, 47, 86, 141]. In general, ADAR1 is upregulated in multiple cancer types and largely functions as an oncogene; whereas empirically, ADAR2 acts as a tumour suppressor [18, 19, 21, 24, 42, 47]. In this review, we will discuss how A-to-I RNA editing is regulated and the mechanisms underlying RNA editing dysregulation in cancer. We will also discuss how ADAR enzymes and A-to-I RNA editing contribute to cancer initiation and development by affecting functions of cancer-associated genes and cancer immunity.

5.2 ADAR Enzymes and A-to-I RNA Editing: Altering Functions of Cancer-Associated Genes

5.2.1 Dysregulated A-to-I RNA Editing in Cancer

For decades, scientists have invested tremendous effort into the identification of genomic changes such as somatic mutations, gene amplifications and fusion genes that drive tumour initiation and progression using whole-genome sequencing (WGS) or exome sequencing (exome-seq). A-to-I RNA editing, which cannot be detected by either WGS or exome-seq, introduces changes in the RNA sequences encoded by the genome, refuting the Central Dogma that DNA dictates each nucleotide in RNA. It is thought that the frequency of RNA editing alterations in cancer is considerably larger than anticipated, comparable or even higher than the number of somatic mutations recorded. Until recently, most studies on A-to-I RNA editing have been conducted in the central nervous system. For example, A-to-I editing was found to occur at precise locations within exons of several ion channels and receptors, such as glutamate-gated channels, the voltage-gated Kv1.1 potassium channel, and the serotonin 5-HT2C receptor, leading to protein recoding and changes in protein function [68]. However, the recent advent of next-generation sequencing-based RNA sequencing (RNA-seq) to demarcate entire transcriptomes has revealed a more global role for RNA editing in both normal development and diseases. Accumulating evidence now suggests that RNA editing is dysregulated in a wide variety of cancers and increasingly supports a role for ADAR-mediated A-to-I RNA editing in cancer development and progression [18, 46, 102, 103]. Interestingly, whether a particular cancer type is associated with an over-editing (a global increase in A-to-I editing) or under-editing (a global reduction in A-to-I editing) pattern or whether alterations in RNA editing are oncogenic or tumour suppressive, appears to be influenced by multiple variables. As a consequence of downregulation of all 3 ADAR RNA editing enzymes (ADAR1, 2, 3), an under-editing pattern was observed in brain tumours and contributed to tumour development [103]. In contrast, several transcriptomic analyses reported that thyroid carcinoma, head and neck squamous cell carcinoma, lung adenocarcinoma, and breast invasive carcinoma, demonstrated over-editing patterns in tumours [46, 102]. A-to-I RNA editing level can also vary among different subtypes of a particular cancer. For example, kidney chromophobe (KICH) and kidney renal papillary cell carcinoma (KIRP) subtypes demonstrate an under-editing pattern, whereas kidney renal clear cell carcinoma (KIRC) is associated with an over-editing phenotype. Other variables such as patient age, gender and ethnicity, sample size or preparation, sequencing platform, approach or coverage might result in conflicting conclusions. For example, Han et al. analyzed the RNA-seq datasets from TCGA (The Cancer Genome Atlas) and reported that among a total of 26,389 informative A-to-I editing sites in 285 stomach adenocarcinoma and 33 normal samples, there was only a small subset (<100) of editing sites demonstrating differential editing levels between matched tumours and normal samples [46]. However, by sequencing 14 matched pairs of gastric cancer and adjacent non-tumour samples

with a higher level of sequencing coverage, we found 1,203 A-to-I editing sites distributed across 923 genes were recurrently and significantly under- or over-edited in tumours [19]. In addition, the commonly used bulk RNA-seq analysis of patient samples reflects the averaged RNA editing frequency across thousands of cells that are mainly from tumour tissue or tumour-associated stroma. Therefore, tumour and/or tumour microenvironment heterogeneity may also cause difficulties in defining a particular cancer type over-edited or under-edited.

Although it remains unclear whether monitoring the global activity of RNA editing may be useful for early cancer detection or tracking cancer progression, we and others have reported an appreciable number of A-to-I RNA editing sites with potential clinical relevance. These editing sites may contribute to personalized medicine by better classifying and diagnosing cancers and potentially revealing new targets for therapeutics. In the following section, we will discuss these functionally important RNA editing sites/events in more detail.

5.2.2 Protein Recoding Editing Events

The vast majority of A-to-I editing sites in humans and primates are in introns and UTRs of pre-mRNA transcripts. Therefore, nonsynonymous sites, resulting in protein recoding, only make up <1% of total editing sites in humans [114]. Here, we will discuss several exemplary ADAR-mediated recoding events which have known functional relevance to cancer. One of the best-studied protein recoding editing targets is *AZIN1* which encodes antizyme inhibitor 1. In the cell, ornithine decarboxylase (ODC) catalyzes the rate-limiting conversion of ornithine to putrescine in polyamine biosynthesis. Antizyme negatively regulates polyamine biosynthesis by binding to and degrading ODC [36]. Interestingly, AZIN1 is an ODC homolog that binds to antizyme with greater affinity than ODC [36], thereby stabilizing ODC by inhibiting antizyme-mediated ODC degradation. In our study, we found that editing of *AZIN1* resulted in a serine-to-glycine substitution at residue 367 located in β-strand 15 (β15) of the AZIN1 protein which likely induces a conformational change that may explain the increased cytoplasmic-to-nuclear translocation and gain-of-function phenotypes of edited AZIN1 protein in hepatocellular carcinoma (HCC) cells [21]. Compared with wild-type (unedited) AZIN1 protein, the edited form has a stronger affinity to antizyme, and the resultant higher AZIN1 protein stability promotes cell proliferation through the neutralization of antizyme-mediated degradation of ODC and cyclin D1 (CCND1) which are oncoproteins and crucial positive regulators of G1/S transition [91, 110]. These findings suggest that protein recoding RNA editing of *AZIN1* is a potential driver in HCC. Later on, we and others found that the increased editing of *AZIN1* is also implicated in other cancer types such as esophagus squamous cell carcinoma (ESCC), colorectal cancer (CRC), and non-small-cell lung cancer (NSCLC) [60, 111, 129]. Notably, we recently developed an antisense oligonucleotide (ASO) ASO3.2 which specifically targets the *AZIN1* ECS which is located in exon 12 of *AZIN1* transcript. ASO3.2 was found to effectively inhibit ADAR1-mediated *AZIN1* editing

with no adverse effect on splicing or translation of *AZIN1* [136]. More importantly, treatment of ESCC and NSCLC cell lines with ASO3.2 significantly reduced cell viability in vitro and tumour incidence and growth in vivo [136]. Hence, targeting *AZIN1* editing or other functionally important editing targets using RNA therapeutics such as ASO may hold great promise for the treatment of cancer.

Unlike *AZIN1*, mRNA encoding α3 subunit of the gamma-aminobutyric acid type A receptor ($GABA_A$) (*GABRA3*) is a substrate of both ADAR1 and ADAR2 [42, 98]. Editing of *GABRA3* results in an isoleucine-to-methionine substitution at position 342 (I/M site) located within transmembrane (TM) domain 3 which may alter the environment of the channel. Interestingly, Gumireddy et al. reported that edited GABRA3 was only found in non-invasive breast cancer cell lines MCF7 and SKBR3 but not in invasive cell lines such as MBA-MD-231, MBA-MD-436 and MDA-MB-453. Infringingly, the introduction of edited GABRA3 into invasive breast cancer cells markedly reduced migration and invasion capabilities. Furthermore, overexpression of edited GABRA3 suppresses the phosphorylation of protein kinase B (AKT), whereas unedited GABRA3 promotes AKT activation which in turn promotes invasive potential of breast cancer cells.

In another study, we found that gastric cancer (GC) pathogenesis is closely associated with ADAR-mediated RNA editing with antagonising effects between two primary editing enzymes ADAR1 and ADAR2 (i.e. ADAR1 functions as an oncoprotein whereas ADAR2 functions as a tumour suppressor through their catalytic domains) [19]. Furthermore, we demonstrated that *PODXL* (podocalyxin-like protein 1) undergoes a histidine to arginine (His-to-Arg) substitution at codon 241 as a result of ADAR2 editing, conferring a loss-of-function phenotype which counteracts the oncogenic potential of unedited PODXL in vitro as well as in vivo. As such, being able to inhibit ADAR1 enzyme activity or restore ADAR2 activity could be an attractive therapeutic opportunity for the treatment of GC. We also found that HCC displays an aberrant A-to-I editing profile that is positively correlated to differential expression of ADAR1 and ADAR2 in HCC compared to normal liver tissues [18]. ADAR1-high and ADAR2-low patients tend to have an increased risk of liver cirrhosis and post-operative cancer recurrence with poor prognoses. Further, in vitro and in vivo tumourigenicity assays also demonstrated oncogenic and tumour suppressive potentials of ADAR1 and ADAR2, respectively, in HCC.

Chen and colleagues reported *IGFBP7* (insulin-like growth factor binding protein 7) transcript as a specific ADAR2 substrate [24]. They found ADAR2 was downregulated in ESCC and forced overexpression of ADAR2 in ESCC cell lines could induce apoptosis as well as inhibit tumour growth both in vitro and in vivo, and catalytically inactive ADAR2 could not rescue the pro-apoptotic phenotype in ESCC cells. The authors proposed that editing by ADAR2 may stabilize the IGFBP7 protein through altering the protease recognition site of matriptase. In addition, ADAR2 overexpression resulted in IGFBP7-dependent inhibition of Akt signalling [24].

Comparing WGS and RNA-seq data of CRC, Han et al. found a tumour-associated editing event in *RHOQ* (Ras Homolog Family Member Q) transcript

which results in a conversion of asparagine to seine at codon 136 of RhoQ protein [47]. CRC cells expressing edited RhoQ demonstrated increased invasive potential in vitro that was exacerbated with additional *KRAS* G12D mutation. Furthermore, CRC patients with a higher frequency of *RHOQ* editing in their tumours and KRAS mutations demonstrated a recurrence tendency.

More recently, we reported that ADAR2 binds to a dsRNA structure formed between edited exon 6 and an ECS located within intron 6 of *COPA* (coatomer subunit α) transcript [132]. The resultant editing by ADAR2 leads to an isoleucine-to-valine substitution at position 164 which confers a functional switch from a tumour-promoting wild-type (unedited) COPA to a tumour suppressive (edited) $COPA^{I164V}$ that harbours a dominant negative effect. $COPA^{I164V}$ appears to be less stable than $COPA^{WT}$ probably owing to a conformation change and $COPA^{I164V}$ likely deactivates the PI3K/AKT/mTOR pathway through downregulation of caveolin-1 (CAV1)—an activator of the PI3K/AKT/mTOR signalling cascade, without changes to the *CAV1* transcripts abundance [132]. However, an earlier study found $COPA^{I164V}$ to be tumour-promoting instead that significantly increases cell viability, wound healing and invasive potential compared to $COPA^{WT}$ in normal breast and breast cancer cell lines (MCF10A and MDA-MB-231) as well as SLR25 renal cancer cells [104]. Hence, further investigations are required to resolve these different observations across different cell types.

Altogether, protein recoding editing occurring in a cancer-associated gene may (1) promote its oncogenic ability, (2) suppress its tumour-suppressive ability, or (3) turn it from tumour-promoting to tumour suppressive or vice versa.

5.2.3 Editing Occurring in Non-coding Regions of mRNAs

Recent advancements of sequencing technologies and bioinformatic pipelines contributed to the identification of significantly increased number of A-to-I editing sites. Intriguingly, a vast majority of A-to-I sites reside within non-coding regions of transcripts such as introns and 3'UTRs. Of note, these non-coding editing sites are enriched in regions containing *Alu* elements, which belong to a class of repetitive DNA elements known as short interspersed nuclear elements (SINEs) [4, 10, 46, 69], (Levanon et al., 2004b), [105, 108, 115, 116, 120]. This discovery led to a focus and paradigm shift in A-to-I editing research from protein recoding to a myriad of other biological and molecular processes which involve base pairing, including RNA splicing, miRNA metabolism and miRNA targeting specificity. In this regard, we will discuss some known implications of non-coding RNA editing in the context of cancer.

5.2.3.1 Editing of Intronic Sequences Affects RNA Splicing and Backsplicing

In cells, inosine bases are recognized as guanosine residues by cellular machineries such as splicing machinery. As such, it is conceivable that editing of *cis* splicing elements within introns would affect splicing decisions. For example, an AU to

IU (recognized as GU) change would generate a de novo donor site (5' splice site), whereas editing of AA to AI (AG) could be recognized as an acceptor site (3' splice site). Moreover, editing of the adenosine branch point could influence splicing decisions. For example, Beghini et al. found that editing of a putative adenosine branch point of *PTPN6* (SH2 domain-containing tyrosine phosphatase) pre-mRNA led to intron 3 retention, resulting in the translation of non-functional PTPN6 protein. This editing mediated *PTPN6* intron 3 retention was found to be associated with acute myeloid leukaemia (AML) [11].

Recently, we found that ADAR1 and ADAR2 could regulate a hundred high confidence splicing events through differential mechanisms in the context of their target transcripts [135]. In this study, we found that ADAR1 could edit an intronic splicing silencer (ISS) of the *CCDC15* transcript to repress exon 9 inclusion by facilitating the recruitment of serine-arginine rich splicing factor 7 (SRSF7). Intriguingly, full-length CCDC15 harbours oncogenic functions. Although ADAR1 is established to be an oncoprotein, in the context of CCDC15, ADAR1 may function as a tumour suppressor by repressing full-length CCDC15 expression while increasing the expression of exon 9-skipped CCDC15 (CCDC15Δex9). In contrast, ADAR2 may employ differential mechanisms of exon-cassette regulation of its target transcripts. For example, secondary structure prediction of the *RELL2* transcript showed a perfect base-paired region formed between a GA-rich region of exon 3 and the polypyrimidine tract (Py) at intron 2. ADAR2 binds to this dsRNA structure to prevent the association of the U2AF65 splicing factor to the polypyrimidine tract (Py-tract), resulting in the exclusion of *RELL2* exon 3. As such, exon 3-skipped *RELL2* (*RELL2*Δ*ex3*) transcript generates a pre-termination codon (PTC) within exon 5 which leads to nonsense-mediated decay (NMD). Since RELL2 is known for its anti-metastatic and pro-apoptotic tumour suppressive roles that are shown in breast cancer and HEK293T cells respectively, suppressing RELL2 expression by ADAR2 would be pro-oncogenic. Together, these observations demonstrate the complex nature of ADAR1 and ADAR2 in the context of splicing and tumourigenesis even though they are known to be an oncoprotein and tumour suppressor, respectively. Although not studied in the context of cancer, another noteworthy example of ADAR-mediated splicing alteration is the autoregulatory capability of ADAR2 [33, 119]. ADAR2 can edit its own pre-mRNA at intron 4 which generates a de novo proximal 3' splice site. As such, an additional 47 nucleotides will be incorporated into the mature *ADAR2* transcript, leading to a pre-termination codon (PTC) that translates to a non-functional truncated form of ADAR2.

Apart from the regulation of canonical splicing by ADARs, we and others have also investigated whether and how ADAR and A-to-I RNA editing regulate backsplicing (circular RNA biogenesis) which could also contribute to tumourigenesis [127]. CircRNAs differ from canonical linear RNA in that they have a covalently closed loop structure and are generated by splicing between a downstream 5' donor site to an upstream 3' acceptor site [3, 29]. This process, termed 'backsplicing' requires spatial proximity of splice sites that may not necessarily be sequential and is usually facilitated by RNA-binding proteins (RBPs) that associate with

flanking introns and/or base-pairing formed within flanking introns termed reverse complementary matches (RCMs) [63, 155]. Several established functions of circRNAs include competition with canonical linear RNA splicing, serving as miRNA sponges, interacting with RBPs and even translating to small peptides [71]. Importantly, aberrant expression of circRNAs have shown to be implicated in various diseases including cancer [73]. It has been suggested that ADARs could suppress the generation of circRNAs by editing and "melting" the dsRNA [63, 128]. However, another study claimed that ADARs alone had no major effect on circRNA biogenesis [1]. In our study, we identified a total of 650 and 868 circRNAs that are regulated by ADAR1 and ADAR2, respectively. Intriguingly, both ADAR1 and ADAR2 could regulate circRNAs in both directions. As aforementioned, circRNAs are generated by backsplicing that is facilitated by the formation of RCMs in flanking introns. As such, ADARs either stabilize or destabilize these RCM secondary structures through correcting A:C mismatches to I(G)-C pairs or by creating I(G).U wobble pairs, respectively. In addition, we also showed that editing can facilitate the binding of RNA-binding proteins such as PTBP1 to regulate backsplicing. Importantly, ADARs-mediated bidirectional regulation of circRNAs likely exists in multiple cancer subtypes. Furthermore, depletion of *circCHEK2*, *circGALK2* and *circSLC39A8* in EC109 (esophageal carcinoma) and SNU398 (hepatocellular carcinoma) cells significantly inhibit tumourigenesis, demonstrating the functional relevance of ADAR-regulated circRNAs to cancer.

5.2.3.2 Editing Occurring Within 3′ UTRs Affects miRNA Targeting

Editing can also affect the targeting specificity of miRNAs, especially multiple editing sites are found within 3'UTR of transcripts. For example, the 3'UTR of *ARHGAP276* (Rho GTPase Activating Protein 26) proto-oncogene undergoes extensive ADAR1-mediated editing which would prevent the binding of miR-30b-3p and miR-573 [145]. Also, Zhang et al. overexpressed miR-200b in editing-low or editing-high human breast cancer cell lines, followed by observation of MDM2 downregulation [153]. In editing-low cells, there was approximately 40% reduction in MDM2 expression upon overexpression of miR-200b when compared to miR-control. Interestingly, this was not the case for editing-high cells as overexpression of miR-200b showed negligible knockdown of MDM2 compared to miR-control. In another study, Nakano et al. reported that dihydrofolate reductase (DHFR) was positively correlated with ADAR1 expression in MCF-7 breast cancer cell line owing to editing of 3'UTR of *DHFR* transcript which blocks the binding of miR-25-3p and miR-125a-3p [93]. Importantly, ADAR1 knockdown significantly increased the sensitivity of MCF-7 cells to methotrexate—a competitive inhibitor of DHFR. In a separate study, Nakano et al. found that ADAR1-mediated editing could also generate de novo miRNA targeting sites. The group found that 3'UTR editing of *AhR* (aryl hydrocarbon receptor) transcript created a binding motif for miR-378 which could explain the negative correlation between AhR and ADAR1 expression observed in HCC patient samples and cell lines [94]. Together, these examples showcase the important role of ADAR-mediated editing on miRNA metabolism and targeting specificity in the context of tumourigenesis.

5.2.4 Editing of miRNAs

MiRNAs are small non-coding RNAs that are ~ 22nt in length that serve as guide molecules for RNA silencing. Regulation of miRNA occurs at multiple levels with two RNAse III proteins—Drosha in the nucleus and Dicer in the cytoplasm. In brief, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II for which the subsequent secondary hairpin loop structure is cleaved by the micro-processor complex comprising Drosha and DGCR8 to precursor-miRNA (pre-miRNA) in the nucleus [43]. The pre-miRNA is then exported to the cytoplasm and is further processed by the cytoplasmic Dicer to generate mature miRNA molecules to be loaded to Argonaute to form the RNA-induced silencing complex (RISC) for subsequent miRNA-targeted translation repression or mRNA decay. A-to-I editing by ADARs was shown to suppress miRNA biogenesis, as well as to alter the target specificity of miRNA [97]. In the context of cancer, ADAR-mediated miRNAs were reported to be tumour-suppressive or promoting. We will discuss some reported studies on the crosstalk between RNA editing and miRNAs in cancer.

One such example is miR-376a-5p [27]. Particularly, the miR-376 family which comprises pri-mir-376a-1, pri-mir-376a-2, pri-mir-376b and pri-mir-376c undergoes extensive ADAR1-mediated editing which redirects the targeting of mature miRNA molecules to other mRNA substrates [67]. In this study, unedited miR-376a-5p uniquely targets Ras-related protein Rap-2A (RAP2A) whereas edited miR-376a-5p targets the Autocrine Motility Factor Receptor (AMFR). Furthermore, only the unedited miR-376a-5p could repress RAP2A in glioma cell lines. In addition, edited miR-376-5p is negatively correlated with the tumour volume in glioma patients. Further investigations revealed that overexpression of unedited miR-376a-5p significantly increased invasive potential of glioma cells in vitro and in vivo compared to edited miR-376a-5p, a similar phenotype to either overexpressing AMFR or knocking down RAP2A in vitro and in vivo [27]. Another example is miR-455-5p [130]. Previously, it was reported that loss of ADAR1 contributed to more aggressive melanoma in an editing-independent mechanism [95]. Subsequently, a miRNA-editing mediated pathway was uncovered to promote the aggressive melanoma phenotype. The group found that ADAR1 editing of pri-miR-455 confers poorer affinity for Drosha and Dicer explains the reduction in mature miR-455-5p levels as a result of the perturbation of miR-455-5p biogenesis. Further in vivo studies by the group found that mice treated with edited miR-455-5p indeed showed significantly less aggressive melanoma phenotypes (e.g. lung metastases) compared to mice treated with unedited miR-455-5p [130]. Apart from ADAR1, ADAR2 was also shown to be involved in miRNA editing. ADAR2 was found to influence miRNA biogenesis, affecting expression levels of ~ 90 miRNAs in glioma cells [137]. ADAR2-specific editing of the oncogenic pri-miR-222/221/21 inhibited maturation both in vitro and in vivo, contributing to the inhibition of cell proliferation and migration [137]. Together, these examples demonstrate tumour suppressive roles of ADAR1 and ADAR2 in the context of miRNA maturation.

Apart from the tumour-suppressive role of ADARs on miRNAs, there are also reports of oncogenic roles of ADARs on certain miRNAs. One example is the let-7 family of miRNAs. Zipeto et al. investigated the mechanism driving leukaemia stem cell self-renewal. In brief, the upregulation of ADAR1, owing to increased JAK2 signalling, was found to promote the transformation of chronic phase (CP) chronic myeloid leukaemia (CML) to a more aggressive, therapeutic-resistant blast crisis phase (BC) CML. Co-transfection of JAK2 with BCR-ABL1, which is a known driver of BC CML transformation, impaired let-7 miRNA biogenesis along with a dramatic increase in self-renewal capacity and decreased survival times in vivo. Subsequent profiling using RNA-seq data revealed that ADAR1 overexpression significantly upregulated genes associated with stem-cell pluripotency and these genes are targets of the let-7 miRNA family. Further transduction studies with wild-type ADAR1 and its editing-deficient mutant ($ADAR1^{E912A}$) revealed editing of the + 3 position of let-7 precursors affected its biogenesis to mature let-7 miRNAs, probably through preventing cleavage by the DROSHA/DGCR8 micro-processor. As such, increased JAK2 signalling in CML aberrantly upregulated ADAR1 expression which resulted in over-editing of let-7 family precursors that impaired their maturation to functional miRNAs. Since these miRNAs are required to downregulate genes associated with self-renewal and pluripotency such as *LIN28B*, over-editing of let-7 potentially facilitated the transition to BP CML. In another example, miR-381 editing was found to be positively correlated with ADAR1 expression in NSCLC [2]. Further studies found that edited miR-381 contributed significantly to tumourigenesis and migration compared to unedited miR-381. Liu et al. found that miR-214 undergoes ADAR2-mediated editing in HCC patients and cell lines [82]. Further in vitro studies indicated that edited pri-miR-214 suppressed the biogenesis of mature miR-214-3p, leading to upregulation of the RAB15 oncogene. Finally, Wang et al. identified an editing event of an established tumour suppressor miR-200b-3p across cancer subtypes which showed an adverse correlation between the editing level of miR-200b-3p and survival times in patients [148]. Further investigations showed that edited miR-200-3p redirected its target mRNA to a tumour suppressor known as *LIFR* (Leukaemia Inhibitory Factor Receptor), and functional studies showed that either *LIFR* knockdown or overexpression of edited miR-200b-3p promoted tumour migration and invasion [148].

5.3 ADAR1 and A-to-I RNA Editing: Key Player in Suppressing Cancer Immunity

RNA editing of duplex RNAs, particularly dsRNA structures formed by *Alu* elements such as those present in 3' UTRs of pre-mRNA is an important regulator of immunity. For instance, the p150 isoform of ADAR1 is induced by interferon during the antiviral immune response, and itself plays a major role in regulating antiviral immunity [40, 149]. In contrast, the role of ADAR2 in immune regulation has been much less studied. ADAR2 was recently found to edit the genome

of Borna disease virus (BoDV) in the nucleus, marking it as self and reducing immune activation of BoDV infection, and enhancing infection efficiency [151]. As such, in the following sections, we will highlight the importance of ADAR1 as a negative regulator of the innate immune system and enforcer of immune homeostasis via establishing tolerance to self-derived nucleic acids.

5.3.1 ADAR1 is Required for Self-tolerance to Endogenously Derived dsRNA

5.3.1.1 Immune Tolerance and dsRNA Sensing Pathways

The innate immune system serves as a first line of defence, where intracellular sensors known as Pattern Recognition Receptors (PRRs) are strategically positioned in various subcellular compartments to sense and react to immune challenges by recognizing signals such as damage associated molecular patterns (DAMPs), or pathogen associated molecular patterns (PAMPs) to elicit protective responses. In the case of antiviral immunity, PRRs such as toll-like receptors (TLRs) preferentially localize to endolysosomal compartments, and RIG-I-like receptors (RLRs) family (including RIG-I, MDA5, and LGP2) sense cytoplasmic nucleic acids. Herein, we will focus on the innate immune response mounted against immunogenic nucleic acids that form dsRNA structures, such as cell-intrinsic sources like *Alu* stem loops formed in 3'UTRs of mRNAs or endogenous retroelements [15, 16, 150].

The dsRNA sensing pathway is a critical innate immunity defence against RNA virus infection. Extracellular dsRNA is sensed by TLRs, while intracellular dsRNA in the cytoplasm is sensed by RLRs, OAS1 and PKR. Sensing of dsRNA by MDA5 & RIG-I signals the recruitment of adaptor molecule MAVS, which results in nuclear localization of phosphorylated IRF3 (IFN regulatory factor 3), activator protein 1 (AP-1) and NFκB, inducing the upregulation of Type 1 IFN response genes (most importantly, IFNβ) and other inflammatory cytokines. Secreted IFNβ can bind its receptors IFNAR1/2 in *cis* or *trans* to activate the JAK/STAT pathway and induce transcription of interferon-stimulated genes (ISGs) for antiviral activity, perpetuating the type I IFN response feedback loop. PKR is an important effector of the antiviral response. Upon sensing dsRNA, PKR dimerizes, auto-phosphorylates itself and catalyzes the phosphorylation of eIF2a to cause translational arrest, preventing further viral protein production in viral-infected cells [107, 138]. Activation of canonical antiviral dsRNA sensors MDA5 and PKR is not restricted to viral infection but also identified as major sensors during loss of self-tolerance, responding to self-derived nucleic acids in interferonopathies and cancers [62, 80, 106]. Overall, there exists a network of dsRNA sensors ready to enforce various responses against intracellular dsRNA that cumulate to antiviral ISG expression, inflammation, translational inhibition, and apoptosis (Fig. 5.1).

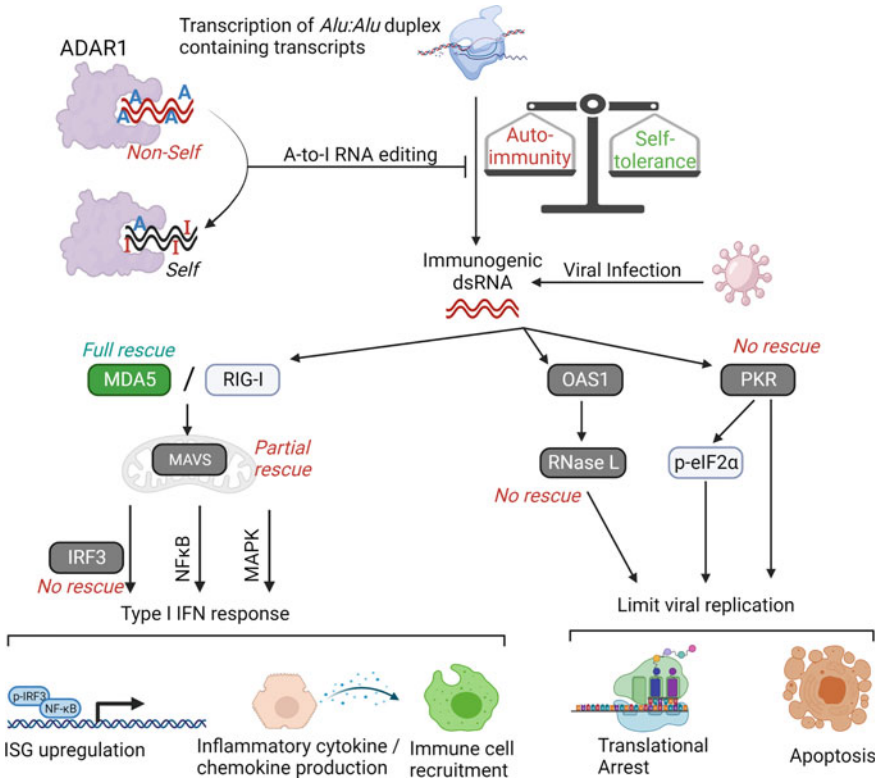

Fig. 5.1 The dsRNA sensing pathway is triggered by immunogenic dsRNA arising from viral infections or endogenous transcription of Alu elements that form dsRNA structures. Immunogenic dsRNA is sensed by cytoplasmic sensors MDA5, RIG-I, OAS1 and PKR for pathway activation, culminating in activation of the type I IFN response or limitation of viral replication. Self-derived dsRNA is marked as "self" by A-to-I RNA editing catalyzed by ADAR1, establishing self-tolerance, and preventing aberrant activation of the dsRNA sensing pathway. Results of studies involving concurrent deletion of *Adar1* and various dsRNA sensors/pathway mediators and the capacity of each factor to rescue embryonic lethality in *Adar1* knockout (KO) mice are shown

5.3.1.2 ADAR1 Suppresses dsRNA Sensing for Self-tolerance

The embryonic lethality observed in multiple ADAR1-null mouse models establishes the role of ADAR1 in innate immune regulation. At the cellular level, one of the immune tolerance mechanisms enforcing immune homeostasis is that ADAR1-mediated A-to-I editing marks endogenous dsRNA structures as 'self' by altering Watson–Crick A–U base pairs to the weaker I.U wobble base pairs, which destabilizes dsRNA and prevents its sensing primarily by the MDA5–MAVS pathway, which triggers IFN responses [28, 79]. In this section, we will discuss the current scientific consensus on ADAR1-mediated immune regulation and its underlying mechanisms, and how it contributes to immune suppression in cancer.

In humans, ADAR1 deficiency has been reported in Aicardi-Goutières syndrome (AGS), a lethal childhood interferonopathy involving harmful IFN production resulting in neurological defects [117]. Several earlier studies showed that deletion of *Adar* (encoding ADAR1) in mice led to embryonic lethality around E12.5 accompanied with liver disintegration, widespread apoptosis, and aberrant IFN production [49, 147]. However, concurrent deletion or deactivation of PKR and OAS1-RNase L pathways failed to rescue embryonic lethality derived from ADAR1 deficiency [147] (Fig. 5.1). Later, other groups employed similar strategies to identify the main sensor which can recognize unedited dsRNA as 'non-self' to trigger IFN responses. In two comprehensive studies, concurrent deletion of ADAR1 (*Adar*) and various dsRNA sensors including MDA5 (*Ifih1),* RIG-*I* (*Ddx58),* PKR *(Pkr),* or STING (*Tmem173*) or pathway signalling factors including MAVS (*Mavs)*, IRF3 (*Irf3)*, IFNAR1 (*Ifnar1),* or STAT1 (*Stat1*) was conducted to monitor the potential rescue of the embryonic lethal phenotype [85, 106] (Fig. 5.1). As a result, MDA5 was identified as the primary dsRNA sensor responsible for the aberrant activation of the antiviral immune response caused by ADAR1 deficiency [79, 106]. It is also reported that ADAR1-mediated editing of dsRNA suppresses PKR activation [39]. This finding was further confirmed by Chung and colleagues who conducted a mechanistic investigation and showed that ADAR1 could inhibit PKR activation due to endogenous transcripts during IFN stimulation, which is dependent on both ADAR1's dsRNA binding and catalytic activities.

Apart from the abovementioned findings that A-RNA forming right-handed double helices can be sensed by canonical RLRs such as MDA5 and PKR, Z-RNA forming left-handed double helices was recently found to be involved in IFN activation following ADAR1 deletion [31, 54, 61, 65]. ADAR1 and ZBP1 (Z-RNA binding protein) are the only two IFN-inducible proteins known to bind to Z-RNA likely via their respective Zα domains, and *Zbp1* was upregulated following loss of ADAR1 [61]. Mutations in the Zα domain of ADAR1, along with loss of ADAR1 p150 expression, were found in AGS patients, suggesting that ADAR1 may suppress IFN response triggered by Z-RNA [55]. Indeed, mice modelling this condition (*Adar1*$^{mZ\alpha/-}$) were found to exhibit early postnatal lethality, haematological abnormalities, pathology of the liver, kidney and spleen, upregulated expression of ISGs, and increased apoptosis in small intestine and colon [61, 87]. However, double knockout (KO) of *Zbp1* and *Mavs*, but not the single KO of either *Zbp1* or *Mavs* in *Adar1*$^{mZ\alpha/-}$ mice could completely ameliorate IFN activation, suggesting that ZBP1 and MDA5-MAVS pathway may work in concert to govern Z-RNA-induced IFN response arising from loss of ADAR1. In *Adar1* KO mice, concurrent deletion of *Zbp1* failed to rescue the embryonic lethality, and only delayed it by one day [31, 65]. Jiao and colleagues reported that ZBP1 responds to Z-RNA accumulation resulting from ADAR1 deficiency, which then acts through the ZBP1-RIPK3 pathway to induce necroptosis in *Adar1-/-* (*Adar*$^{tm1a(EUCOMM)Wtsi}$) mice [65]. Accordingly, the Zα domain of ADAR1 p150 isoform facilitates the A-to-I RNA editing of *Alu* duplex RNAs, preventing ZBP1-mediated cell death. Unexpectedly, concurrent deletion of ZBP1

downstream necroptosis and apoptosis pathway mediators *Mlkl* and *Casp8* in *Adar1*$^{mZ\alpha/-}$ mice did not rescue lethality, as such mice did not survive past P2 and exhibited cell death in the colon, raising the possibility of ZBP1-mediated activation of other cell death mechanisms [31].

In sum, ADAR1 deletion in mice results in embryonic lethality with excessive IFN signalling, apoptosis and liver disintegration [49, 146, 147]. Due to the lack of RNA editing, immunogenic dsRNAs, such as those arising from the expression of endogenous retroviral elements (EREs), trigger IFN response and embryonic lethality by activating A-RNA sensors such as MDA-5 and PKR [5, 53, 79, 106, 144]. More recently, ADAR1 was also shown to prevent the accumulation of endogenous Z-RNAs that are enriched in the 3'UTRs of ISG transcripts. Loss of ADAR1 results in Z-RNA accumulation and activation of the Z-RNA sensor ZBP1, which led to RIPK3-mediated necroptosis and possibly other ZBP1-associated cell death pathways. Altogether, ADAR1 is an essential enforcer of immune tolerance to endogenous dsRNA by acting as a negative regulator of innate immunity through suppressing various dsRNA sensing mechanisms involving sensor proteins MDA5, RIG-I, PKR and/or ZBP1.

5.3.2 Cellular Origins and Structures of Immunogenic dsRNA Recognized and Edited by ADAR1

The immune regulatory roles of ADAR1 have been extensively studied. However, the identity of the immunogenic, self-derived dsRNAs that accumulate in response to the loss of ADAR1 remains an ongoing research topic. This presents a significant knowledge gap, as cancer cells often exhibit accumulation of self-dsRNA, which may occur due to the loss of epigenetic silencing mechanisms [72]. As ADAR1 has been shown to be a suppressor of innate immunity arising from self-dsRNA sensing, this presents a potential promising vulnerability in cancers that may be exploited for the development of cancer therapeutics. In this section, we will review possible sources of immunogenic dsRNA, the existing studies on identifying such immunogenic RNA species, and their relevance to human health and disease, particularly cancer.

5.3.2.1 Duplex RNAs Derived from Repetitive Elements

The dsRNA sensing pathway not only protects cells from viral infection, but also plays an important role in protecting the integrity of the genome; failure of which contributes to genome instability, a hallmark of cancer. Repetitive elements (REs) comprise a large proportion of the non-coding compartment of the human genome, previously thought to be "junk" DNA, that were incorporated ancestrally and retain their ability to replicate and re-insert themselves into the genome [84]. As re-insertion is detrimental to genomic integrity, REs are normally silenced in adult mammals to prevent RE expansion. While there is still debate surrounding the true function of REs, various cellular strategies are employed to enforce the silencing of REs in the genome. These include epigenetic silencing via methylation

of CpG sites of REs and subsequent heterochromatin occlusion of transcription machinery [76]. However, de-repression of REs is a feature of cancer and contributes to tumourigenesis (Fig. 5.2). Loss of RE silencing leads to overexpression and accumulation of various REs such as LINEs (long interspersed nuclear elements), SINEs and hERVs (human endogenous retroviruses) in various cancers [48]. Expression of REs can produce transcripts capable of forming secondary structures, which can be recognized by intracellular PRRs. Failure in RE silencing results in RE activity, genomic instability, and RE-derived dsRNA accumulation with subsequent immune activation, dubbed the "transcription of repeats activate interferons" (TRAIN) phenomenon [74]. Upon DNA hypomethylation induced by DNA methyltransferase inhibitors (DNMTis), hERV-derived dsRNAs activate the MDA5-MAVS pathway for inflammatory antiviral response and such process has been described in various cancers [13, 25, 118]. In a recent study, Mehdipour and colleagues found that ADAR1 restricts the viral mimicry response to epigenetic therapy-induced inverted-repeats *Alu* elements, and depletion of ADAR1 in patient-derived cancer cells augments the efficacy of epigenetic therapy, inhibiting tumour initiation and growth [88].

In sum, transcripts derived from deregulated, overexpressed REs in various cancers can form immunostimulatory secondary structures that are recognized by innate immune sensors, including MDA5. The functional consequence of endogenous dsRNA accumulation is the activation of antiviral immune responses resulting in inflammation. Thus, existing evidence demonstrates that REs in the genome

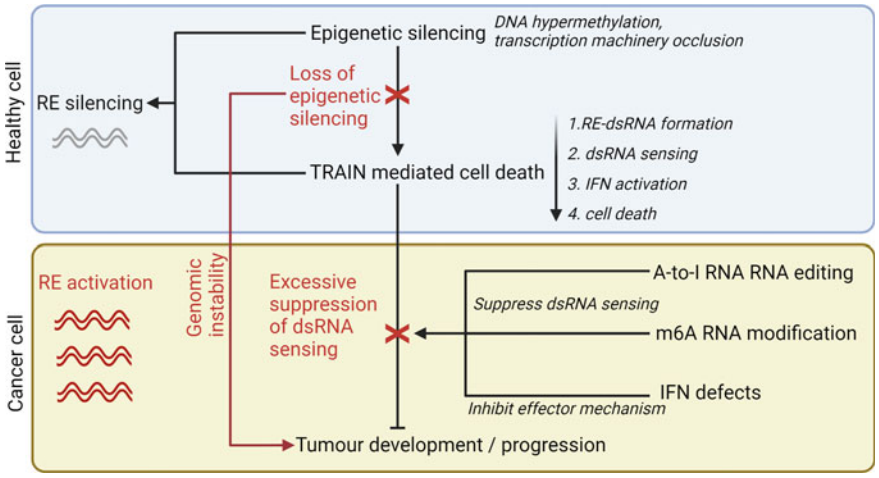

Fig. 5.2 Under physiological conditions, epigenetic silencing is the first layer of defence against activation of repetitive elements (REs) in the genome. Upon de-repression of REs, the transcription of repeats activates interferons (TRAIN) response mediated by dsRNA sensing pathways resulting in the activation of type I IFN response for cell death of cells with re-activated REs. In cancer, TRAIN mediated cell death is overcome by excessive dsRNA tolerance mediated by various mechanisms including overactive ADAR1-mediated A-to-I RNA editing

present a highly likely source of immunogenic, self-derived dsRNA, which has implications in cancer immunology. These nucleic acid species are highly dysregulated in cancer and various strategies exist to silence their transcription, to prevent their aberrant accumulation, sensing by MDA5 and other dsRNA sensors, and resultant detrimental IFN activation, or TRAIN. We suggest that in healthy cells, TRAIN results in activation of IFN response and cell death in response to RE activation. However, TRAIN is dependent on sensing of dsRNA formed from RE activation, and along the multistep process of tumourigenesis, dysregulation of immunoregulatory mechanisms, such as A-to-I RNA editing by ADAR1, hampers the TRAIN response and allows cancer cells to tolerate increased dsRNA content without IFN activation (Fig. 5.2).

5.3.2.2 Endogenously Transcribed Transcripts Forming dsRNA Structures

While the first line of defence against endogenous dsRNA is mainly enforced by epigenetic silencing, de-repression of REs can occur under certain disease conditions causing failure in epigenetic silencing. Furthermore, epigenetic silencing does not exhaustively eliminate all endogenously produced dsRNA structures, as even physiological transcription can produce nucleic acids that form dsRNA structures. As such, ADAR1 serves as a secondary defence mechanism against aberrant IFN activation mounted against self-dsRNA. However, the mechanism in which A-to-I RNA editing by ADAR1 suppresses the sensing of dsRNA by cytosolic sensors is an ongoing research topic. *Alu* elements are RNA species of roughly 300 nucleotides that can exist autonomously or as part of mRNAs, particularly in the 3'UTRs of mRNA transcripts [9, 32, 50]. *Alu* elements are known to form secondary structures, such as when two *Alu* elements in opposite orientations interact in *trans* to base-pair to form dsRNA, a common occurrence in mammalian systems [22]. This can occur when *Alu* elements embedded within 3'UTRs of mRNA transcripts interact with complementary *Alu* elements in lncRNAs [41]. Given the propensity of ADAR1 for *Alu* element editing and their potential dsRNA forming capacities, paired inverted *Alu* elements are a proposed source of immunogenic dsRNA. Interestingly, Bowling and colleagues showed that in MYC-driven triple-negative breast cancer (TNBC), spliceosome-targeted therapies cause widespread cytoplasmic accumulation of mis-spliced, intron-retained mRNAs, many of which form dsRNA structures. This process further triggers antiviral signalling and extrinsic apoptosis in TNBCs via dsRNA sensors [15]. A more recent study from Li et al. reported an additional source of immunogenic dsRNA that is formed by overlapping genes transcribed in opposite directions, also known as *cis*-natural antisense transcripts (*cis*-NATs) [77].

Apart from the abovementioned A-RNA, Z-RNA is another potential RE-derived dsRNA species edited by ADAR1 that contributes to endogenous RNA-induced immunity through ZBP1 activation [30, 64]. Interestingly, these Z-RNA species were found to be inverted SINEs that are highly enriched in the 3'UTR of ISG transcripts following ADAR1 depletion. The ADAR1 p150 isoform was shown to bind to dsRNA structures of Z-RNA for suppression of immunogenicity

through its Z-RNA binding domain (resulting in sequestration of Z-RNA) with partial dependence on RNA editing activity [154].

5.3.2.3 Current Understanding of How ADAR1 and RNA Editing Suppress Innate Immunity

Currently, the scientific consensus on how ADAR1 acts as an immune protector is that A-to-I RNA editing of dsRNA and introduction of inosine into RNA molecules lead to the formation of I.U wobble base pairs which are less stable than conventional A-U base pairing. Over-editing of dsRNA could drastically increase the I.U content of potentially immunostimulatory dsRNA by altering A-U, resulting in destabilization and reduced availability for dsRNA sensors. Multiple studies have shown that I.U containing dsRNA (IU-dsRNA) might undergo specific cleavage [122] and they could inhibit MDA5 and RIG-I activity and reduce downstream IRF3 activation resulting in suppressed ISG expression and apoptosis induced by synthetic dsRNA analogue Poly(I:C) [57, 142]. The importance of inosine in suppressing IFN activation to dsRNA suggests that the RNA editing functions of ADAR1 as the crux of ADAR1's role in IFN suppression, compared to the editing-independent functions of ADAR1 [28, 39, 152]. This might be due to direct binding and occupancy of MDA5 and RIG-I dsRNA sensors by IU-dsRNA, precluding their binding and sensing of immunostimulatory dsRNA. Physiologically, RNA editing might additionally reduce the total immunostimulatory dsRNA content in cells via introduction of inosine, reducing their stability, and inducing their specific cleavage. Further, the generic alterations introduced in IU-dsRNA were sufficient to suppress Poly(I:C) induced IFN activation and cell death. This also implies a "quantity-over-quality" model of A-to-I RNA editing mediated immune suppression, where the extent of generic RNA editing of a broad range of dsRNA structures is more important to the suppression of dsRNA sensing, rather than RNA editing of specific immunostimulatory dsRNA species. However, other studies suggest that A-to-I RNA editing in paired inverted *Alu* elements is infrequent; instead, purifying selection of long dsRNA species results in the removal or transcriptional inactivation of such sequences in the genome [6, 10]. Nonetheless, some long, perfect dsRNA species escape purifying selection, and are potentially strong activators of MDA5 owing to its structure. However, such endogenous long perfect dsRNA does not trigger autoimmune responses due to heavy RNA editing by ADAR1 [6]. This suggests a "quality-over-quantity" model where most potential immunogenic dsRNAs are not suppressed by ADAR1 given their low editing frequency; but certain rare long, perfect dsRNA species (the perfect substrate for MDA5) are key immunostimulatory dsRNA suppressed by ADAR1 RNA editing. In addition, Liddicoat et al. proposed that edited dsRNA that strongly binds to RNA sensors may outcompete other (unedited and yet unidentified) transcripts [79]. Moreover, recent animal studies suggested that ADAR1 may also suppress RNA sensing and IFN signalling via unknown, editing-independent functions in vivo [reviewed in [112]. This remains a topic which is worth further investigation.

5.3.3 ADAR1 and A-to-I RNA Editing: Leading to Cancer Immunosuppression

5.3.3.1 Cancer Cells Accumulate Cellular dsRNA

Recent developments highlight the internal immune milieu of cancer cells as an important factor for their survival, a previously unexplored strategy for cancer therapy. Reactivation of REs in the genome occurs commonly in cancer cells, to the detriment of genomeicintegrity. For example, hypomethylation is a common occurrence in human cancers that results in the activation of *Alu* elements [74]. While innate immunity-dependent, inflammatory defence mechanisms against double-stranded RNA arising from RE transcripts exist, cancer cells employ various strategies to circumvent these obstacles, such as dysregulation of RNA editing or interference with lethal type 1 interferon (IFN) response mechanisms (Fig. 5.2). We previously reviewed the process of RE de-repression in cancer resulting in RE activation and hence dsRNA accumulation. Here, we highlight the importance of RNA biology in contributing to cancer cell-autonomous inflammation and how cancer cells subvert endogenous self-RNA tolerance mechanisms to remain viable despite repetitive element expression.

The immunology of cancer has been a subject of extensive research. It is now evident that many cancer cells employ various strategies for immune evasion and resistance to immune checkpoint blockade (ICB). Furthermore, the interaction between immune cells and cancer cells in the tumour microenvironment facilitates both of those processes and can even promote carcinogenesis. However, many of these studies focus largely on the effects of extrinsic factors (immune cells and signals) on tumour cells. One largely underappreciated facet of cancer immunity is the study of the internal immune milieu within cancer cells. Recent work has revealed that the innate immune status of cancer cells is paramount to their survival, which highlights the importance of RNA biology (and its dysregulation) to carcinogenesis. Various groups have recently uncovered that certain cancer cells exhibit unique sub-inflammatory immune states kept in check by immune suppressive mechanisms such as ADAR1-mediated editing that once removed, predisposes them to lethal inflammatory responses [38, 62, 80]. It was recently discovered that certain tumour cells themselves can produce high levels of endogenous IFN and exhibit enhanced production of unedited immunostimulatory self-dsRNA intrinsically [80]. Interestingly, these characteristics arise in a cell-autonomous manner and cumulate to create an internal immune environment poised towards excessive inflammation and subsequent cell death dependent on the type 1 IFN response [80]. The working consensus is that immunostimulatory self-dsRNA arises from silenced endogenous repetitive elements hidden in the human genome, which are derepressed during tumourigenesis for overexpression and can form immunostimulatory secondary structures. This repetitive element-derived dsRNA can trigger anti-inflammatory antiviral responses which contribute to cancer endogenous IFN production, cumulating in lethal cancer cell-autonomous inflammation [38, 62, 80]. Non-cancerous cells do not tolerate RE expression, the resultant dsRNA accumulation nor endogenous IFN production, and are hence eliminated. However, cancer

cells remain viable and even thrive under these conditions [80]. Given the evidence that different immune states within cancer cells can cumulate to predispose them to dependency on immune suppressive mechanisms, the removal of such tolerance mechanisms is a viable strategy for cancer therapy and a promising potential vulnerability to be exploited [38, 62, 80]

Despite comprehensive strategies in place to limit the activity of REs, thereby preserving genomic integrity, cancer cells persistent in the face of these defences. Indeed, viable tumours expressing high levels of RE transcripts, RE-derived immunostimulatory dsRNA, and resultant IFN response, evidence the development of resistance to TRAIN and IFN-mediated elimination [74]. Several possible mechanisms in which cancer cells remain viable despite accumulation of dsRNA exist. This includes impairment of IFN signalling mechanisms, or upstream excessive modifications to accumulated dsRNA, such as N^6-methyladenosine (m^6A) modification or A-to-I RNA editing by ADAR1 (Fig. 5.2). IFN signalling is a key component of antitumour activity and a major mediator of TRAIN induced cell death. However, defects in IFN signalling can occur in cancers that protect them from cell death, including genetic insults like homozygous deletion of 9p21 in various cancers such as melanoma, ALL, NSCLC and HNSCC [34, 51, 52, 56, 74, 83, 100, 121]. Besides aberrations to IFN signalling mechanisms, activating ligands can also be modified to reduce initial dsRNA sensing and pathway activation. RNA m^6A modification plays important roles in regulating mRNA behaviour and has recently been shown to participate in regulating innate immunity to RNA by reducing secondary structure forming capacity and increasing RNA binding protein accessibility, which has been shown to suppress TLR3 and RIG-I dsRNA sensor activation in vitro [44, 66, 81, 131, 133]. Nonetheless, while other mechanisms of IFN resistance are also important, this review will focus on the immunosuppressive role of ADAR1 in limiting IFN activation in cancer.

5.3.3.2 Cancer Cells Depend on ADAR1 to Suppress Immune Activation for Survival

The function of ADAR1 to suppress innate immune activation and IFN production was recently discovered to play a prominent role in cancer immunity [38, 62, 80]. While ADAR1 is necessary under physiological conditions for establishing tolerance to self-derived dsRNA, and suppression of the IFN response after infection to prevent its overactivation, these immunosuppressive functions were found to be hijacked by cancer cells to survive despite the accumulation of cytoplasmic dsRNA. Thus, A-to-I RNA editing by ADAR1 mediates excessive self-tolerance in cancer cells, preventing IFN activation and cell death. This dependency on ADAR1 presents a unique vulnerability that can be exploited to sensitize cells to immunotherapeutic interventions.

Analysis of genome-scale shRNA screening datasets of lung cancer cell lines identified three cell lines dependent on ADAR1 for survival (HCC366, NCI-H1650 and NCI-H196) [38]. Deletion of ADAR1 in these cells resulted in stark reduction in cell viability. Surprisingly, the impairment of the MDA5/MAVS pathway did not rescue cell lethality, suggesting that in contrast to earlier studies in transgenic mice

models, ADAR1 dependency was independent of MDA5-mediated dsRNA sensing in these cell lines. Additionally, inhibition of the cGAS/STING pathway did not result in the rescue of cell lethality either. Instead, it was shown that another cytoplasmic dsRNA sensor, PKR, was highly activated following ADAR1 depletion, and concurrent deletion of PKR in ADAR1-null cells rescued the lethal phenotype in ADAR-dependent cell lines [38]. The introduction of both editing competent ADAR1 p150 and editing defective E912A ADAR1 p150 isoforms could rescue cancer cell lethality in ADAR1-null cell lines, demonstrating that ADAR1 suppresses PKR activation in lung cancer cells through both its catalytic functions and editing-independent functions, such as binding to PKR [38, 39]. In A549 human lung cancer cells, in vitro inhibition of the OAS-RNaseL pathway (despite retention of the MDA5-MAVS pathway) in ADAR1 KO cells instead rescued the cell lethality [78].

Expounding on this discovery, other studies demonstrated that patient tumours exhibit cell-intrinsic ISG-positive signatures due to endogenously produced IFN [80]. This phenomenon poises cells to accumulate dsRNA that can trigger sensing mechanisms, which when immunosuppressive ADAR1 is removed, leaves cells vulnerable to lethality. By analysing primary tumours in the TCGA database with low IFN producing immune cell infiltration, it was discovered that some tumours with ISG-positive signatures can themselves produce IFN endogenously. Analysis of Patient-Derived Xenograft (PDX) tumours demonstrated cancer-cell endogenous ISG and IFN upregulation of human origin, confirming that cancer cells with ISG-positive signatures demonstrate chronic endogenous IFN production. The establishment of cancer cell-derived IFN was found to be dependent on constitutive STING signalling, which also upregulates ISGs including dsRNA sensors such as MDA5, RIG-I, and PKR [80]. Thus, this creates a curious circumstance where cancer cells are theoretically highly sensitive to dsRNA-induced IFN response and resultant cell death but seem to resist such acute activation of IFN to avoid cell lethality. A screen of vulnerabilities in these cells revealed an exquisite dependence on ADAR1 for maintaining cell viability. Thus, ADAR1 deletion in these ISG-positive signature cells resulted in decreased cell viability. A complex signalling program involving STING and IFNAR1 was found to be required for establishing the ISG-positive signature in these cells, and MAVS and PKR were required for exaggerated IFN production following ADAR1 depletion [80] (Fig. 5.3).

Overall, the immunosuppressive function of ADAR1 was discovered to be hijacked by cancer cells, particularly those exhibiting endogenous IFN production and ISG-positive signatures to counteract TRAIN mediated cell death. Interestingly, various studies have identified different dsRNA sensors as mediators of ADAR1 deletion-mediated cancer cell lethality, including MDA5-MAVS, PKR, STING, and OAS-RNaseL pathways in different contexts, suggesting that ADAR1 dependency in different cancers operate in diverse ways.

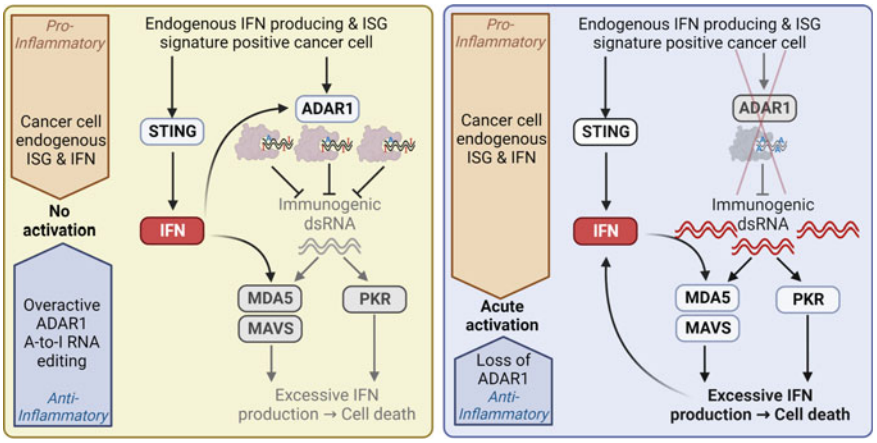

Fig. 5.3 Endogenous IFN production in cancer cells is tolerated due to ADAR1. (Left) Activation of the STING pathway is required for establishing chronic endogenous IFN production resulting in ISG-positive signature in cancer cells. Such cell-intrinsic IFN production results in increased expression of dsRNA sensors MDA5 and PKR. Under physiological conditions, this cellular state would result in excessive IFN production and cell death but is tolerated in cancer cells due to excessive A-to-I RNA editing and immunosuppression by ADAR1. Thus, cancer cells with ISG-positive signatures are dependent on ADAR1 for maintaining cell viability. (Right) When ADAR1 is ablated, lack of tolerance mechanisms for accumulated endogenous dsRNA is sensed by elevated levels of dsRNA sensors resulting in excessive IFN production and cancer cell death. *Adapted from [62, 80]

5.3.3.3 Ablation of ADAR1 Can Sensitize Cells to Cancer Therapeutics

Cancer cells were found to hijack the immunosuppressive functions of ADAR1 and establish excessive self-tolerance, to survive accumulation of immunogenic dsRNA, chronic endogenous IFN production, ISG positivity and upregulated dsRNA sensor expression, and finally the resultant acute activation of the IFN response mediated cell death. While such a cellular state of chronic, low-grade ISG positivity enhances the action of immunosuppressive mechanisms and immune evasion to render them resistant to immune checkpoint blockade (ICB) [12]; this also creates a poised state of high susceptibility to acute IFN activation and cell death which is held at bay by ADAR1. Resistance to epigenetic and ICB cancer therapeutics is an emerging problem despite vigorous research. Currently, efforts to circumvent such acquired resistance have been hampered by failure of IFN sensing or antigen presenting pathways. The dependency of some cancer cells on ADAR1 presents an attractive target for tipping the scales towards acute IFN activation and cancer cell killing. Inhibition of ADAR1-mediated A-to-I editing of endogenous cytosolic dsRNA alleviates the suppression of the type 1 IFN response, and the resultant elevated levels of non-edited dsRNA are sensed by MDA5-MAVS and PKR, triggering the dsRNA sensing pathway that cumulates in type 1 IFN and inflammatory cytokine production, cell cycle arrest and cell death [62].

Epigenetic therapeutics leverage on activating the IFN response to mediate cancer cell killing, by depressing REs in the genome that form dsRNA structures and serve as ligands for dsRNA sensor activation such as MDA5. However, various mechanisms described can hamper this response and confer resistance to epigenetic therapies. DNMTi treatment of CRC cells demonstrated that activated inverted *Alu* repeats forming dsRNA structures are the main source of immunogenic dsRNA that are bound by MDA5 for IFN activation [88]. As an ideal ligand for ADAR1-mediated A-to-I RNA editing, ADAR1 was indeed found to contribute to DNMTi resistance in CRC by editing and destabilizing these inverted *Alu* repeat stem-loop structures, suppressing MDA5 activation [75, 88]. The cytoplasmic p150 isoform of ADAR1 is upregulated upon IFN activation. Under physiological conditions, this negative feedback mechanism suppresses immune responses after infection to restore immune homeostasis. However, DNMTi treatment also results in prolonged upregulation of ADAR1 p150, which counterproductively counteracts the desired IFN response and cancer cell killing in a negative feedback loop. Consequently, ablation of ADAR1 enhanced MAVS activation and ISG upregulation in DNMTi treated cells, reduced the effective treatment dose in vitro, and exhibited strong inhibition of cancer initiation and progression in in vivo xenograft models [88].

In a recent screen for cell vulnerabilities for sensitization to immunotherapeutics, ADAR1 was identified in an in vivo CRISPR screen of transplantable B16 tumours in immunocompetent mice [62]. Deletion of *Adar1* alone in B16 cells demonstrated limited growth inhibition in vitro and in vivo but exhibited profound growth inhibition in xenograft models when treated with anti-PD-1 antibody treatment. As such, *Adar1* depletion was found to sensitize cells to ICB. Loss of *Adar1* was further associated with IFN activation in xenografted tumours including reduction in immunosuppressive cell types (myeloid-derived suppressor cells, tumour-associated neutrophils, and M2 macrophages), and increased recruitment and activation of $CD8^+$ T cells and NK Cells, as well as IFNβ and IFNγ content. In addition to sensitizing cells to ICB, *Adar1*-null cells also demonstrated increased sensitivity to IFN, which inhibited cell viability and promoted apoptosis accompanied by increased production of cytokines and chemokines. In fact, IFN signalling was required for sensitization of *Adar1* -null cells to ICB [62]. In concordance with the requirement of MDA5-MAVS pathway for increased IFN production in ADAR1 KO CRC cells [80], MDA5-MAVS signalling was required for IFNβ production in *Adar1* KO B16 cells [62]. However, PKR was the major enforcer of growth inhibition of *Adar1* KO B16 cells after IFN exposure. When treated with ICB, *Adar1* KO cells required only MDA5 for enhanced ISG upregulation and immune cell infiltration, but both MDA5 and PKR mediate the sensitization of B16 cells to ICB during loss of *Adar1*. Finally, *Adar1* deletion was found to overcome multiple mechanisms of acquired ICB resistance such as loss of *B2m, H2-K1, Nlrc5 and Jak2* [62].

Major challenges to the therapeutic translation of these findings are the obligate requirement for ADAR1 in physiological processes and the current lack of specific drugs limiting its viability as a target for inhibition in patients. However,

it was recently shown that only a small portion of A-to-I RNA editing events were required for maintaining homeostasis in mice models [17]. Nonetheless, one strategy to circumvent the unavailability of ADAR1 as a druggable target was the induction of Z-DNA to activate ZBP1 for activation necroptosis. In a screen for Z-DNA inducers, CBL0137 was identified as an effective DNA intercalator capable of inducing Z-DNA formation, a ligand for ZBP1 activation to drive necroptosis in cancer cells [154]. CBL0137 treatment demonstrated the ability to overcome ICB resistance, albeit through TME activation than within resistant cancer cells intrinsically [154].

In conclusion, ADAR1 is a potent immunosuppressive mechanism imperative for establishing tolerance to self-nucleic acids and maintaining immune homeostasis. While the specific immunogenic dsRNA species edited and suppressed by ADAR1 have yet to be identified (if A-to-I RNA editing mediated immunosuppression is dependent on a specific subset of dsRNAs), existing evidence suggests that the major immunogenic dsRNAs arise from intramolecular *Alu* elements forming stem-loop double-stranded structures in *cis* that can bind to and activate dsRNA sensors like MDA5 and PKR [23]. Further, the mechanism in which A-to-I RNA editing limits the immunogenicity of dsRNAs remains unclear, although inosine content of transcripts has been associated with their degradation, possibly reducing the total immunogenic dsRNA content in the cell. Finally, ADAR1 is often upregulated in various cancers, in conjunction with loss of epigenetic control and genomic instability, and is associated with maintaining cancer cell viability and conferring resistance to ICB through its immunosuppressive functions. This creates a dependency of certain cancers (particularly ISG signature positive, endogenous IFN producing cancer cells) on ADAR1 that can be exploited to sensitize cells to ICB induced cell death. However, there are currently no therapeutic strategies to specifically inhibit ADAR1, which is a major challenge to translating the current findings into clinical applications. We suggest that instead of targeting ADAR1 itself, targeting A-to-I RNA editing events via ADAR1 regulators presents a more accessible strategy for circumventing this issue.

5.4 Factors and Mechanisms Regulating A-to-I RNA Editing and Their Implications in Cancer

Although it has been demonstrated that dysregulated A-to-I editing contributes to cancer, the precise regulatory mechanisms governing this critical cellular process have yet to be fully elucidated. However, previous studies revealed that regulation of A-to-I editing is multifaceted, weaving an intricate network of auto- and trans regulations. It is apparent that tipping of any regulatory components will have profound effects on A-to-I editing, which in turn contributes to both normal and aberrant physiological conditions. A complete understanding of this intricate regulatory network may ultimately be translated into new therapeutic strategies against diseases driven by perturbed RNA editing events. Previously described transcriptional (e.g. IFN-induced transcription of *ADAR1 p150*), post-transcriptional

(miRNA-mediated silencing of *ADAR1* mRNA, alternative splicing of *ADAR1* and *ADAR2*) and post-translational (e.g. post-transcriptional modification such as sumoylation, ubiquitination, and phosphorylation) regulatory mechanisms governing ADAR expression and activity have been reviewed previously [59]. In this section, we will focus on recent findings on how other non-ADAR regulators, mostly RNA-binding proteins (RBPs), regulate A-to-I editing and discuss their functional relevance to cancer.

5.4.1 Types of RBPs Involved in A-to-I RNA Editing Regulation

RBPs are extensively involved in all aspects of RNA metabolism. It is not surprising that many RBPs may potentially act as regulators of A-to-I RNA editing, albeit to different extents. Indeed, differential editing analysis conducted using RNA-seq datasets generated as part of the ENCODE project [134], consists of individual depletion of more than 200 RBPs in both HepG2 and K562 cancer cell lines revealed varying degrees of editing level changes. Of note, RBPs that induce a small degree of changes in editing are likely indirect regulators and are manifestations of its involvement in other aspects of RNA processing. On the other hand, there are also RBPs, when depleted, that induce extensive changes in editing frequency. These RBPs tend to possess the ability to directly influence ADAR's catalytic activity and can be classified into numerous categories, ranging from splicing factors (e.g. SRSF9) [126], microRNA processing factors or ribonucleases (e.g. DROSHA) [113], mitochondrial proteins (e.g. DAP3) [45] and RNA helicases (e.g. DHX9) [58]. Importantly, each RBP possesses a unique pattern of editing regulation, either as predominant enhancers or repressors of editing [45, 126] or bidirectional regulators which regulate editing in both directions [58]. Deciphering its mechanisms of regulation is key for the understanding of how cancer cells may potentially hijack the editing regulatory functions of RBPs for their survival.

5.4.2 Mechanisms of A-to-I RNA Editing Regulation by RBPs

5.4.2.1 RNA Binding as Prerequisite for A-to-I RNA Editing Regulation

RNA binding by non-ADAR proteins appears to be an important prerequisite for A-to-I RNA editing regulation for some RBPs. RBPs may bind exactly at or near editing sites, which in turn may either act as competitive binders or recruiters of ADAR proteins, repressing or enhancing A-to-I RNA editing levels, respectively. Mapping transcriptome-wide binding patterns of RBPs is a critical step to unravel the mechanism of how an RBP regulates A-to-I RNA editing levels. The enhanced crosslinking and immunoprecipitation coupled with high-throughput sequencing (eCLIP-seq) is one such technique for the robust identification of authentic RNA

binding sites [140]. Investigators can utilize eCLIP-seq datasets to establish relationships between location of RBP binding with its respective location of regulated editing sites. Analysis of ILF3, TROVE2, XRCC6, AUH and PUS1 eCLIP-seq datasets revealed that the binding of the respective RBPs is significantly closer to its differentially edited sites induced upon their respective depletion than expected by chance, suggesting that binding of these RBPs at or around the editing sites, in general, is a prerequisite for editing regulation [113]. Consistent with this observation, overexpression of an ILF3 dsRNA-binding impaired mutant failed to significantly induce RNA editing changes in HEK293T, relative to wild-type ILF3 [35]. Similarly, Freund et al. also plotted the RNA binding locations of 19 ADAR-interacting proteins as identified by BioID around its respective regulated RNA editing sites, using the publicly available eCLIP-seq and RNA-seq datasets. The analysis revealed that 11 of these proteins are significantly enriched at or near differentially edited sites induced upon their depletion [35]. These studies suggest that the RNA binding ability of some RBPs facilitates A-to-I RNA editing regulation.

A-to-I RNA editing sites are mostly localized within dsRNA structures formed by inverted *Alu* repeats [76]. Given that some RBPs are found at or around editing sites that it regulates, it is expected that its RNA binding to *Alu* elements should strongly correlate with the extent of editing level change induced upon its depletion. However, analysis results indicate a poor correlation between the extent of RBP binding to *Alu* elements and the change in editing levels, upon its respective depletion [113]. This therefore suggests that binding within *Alu* elements, which is mostly at or near the regulated editing sites, does not fully account for its editing regulation, suggesting that either the RBP of interest has alternate RNA binding-independent mechanisms of regulation or that RNA binding further away from editing sites and outside of *Alu* elements can also influence editing levels. The next two sections will focus on RBP binding-independent mechanisms of regulation.

5.4.2.2 RBP-Mediated Regulation of ADAR Expression Levels

RBPs can regulate ADAR expression levels to influence A-to-I RNA editing levels. A couple of studies have demonstrated a few examples of RBPs that regulate editing through this mechanism. For instance, *fmr1* KO zebrafish models exhibited increased *Adar2* mRNA expression and A-to-I RNA editing levels, suggesting that its gene product fragile X mental retardation protein (Fmrp), is a repressor of Adar2-catalyzed RNA editing in zebrafish [125]. Another example is the TDP-43 protein which is encoded by the *TARDBP* gene. Silencing of *TARDBP* gene significantly reduced *ADAR1* mRNA and protein expression levels in HepG2 but not in K562 cells. Mechanistically, Quinones-Valdez et al. demonstrated using chromatin immunoprecipitation coupled with sequencing (ChIP-seq) that TDP-43 interacts with two chromatin regions that coincide with the first exons of both ADAR1p110 splicing isoforms [113]. Luciferase assays further revealed that these bound elements may serve as promoter or enhancer regions, suggesting that TDP-43 may facilitate *ADAR1* transcription [113]. Despite these examples, influencing *ADAR* transcription by RBPs as a mechanism of A-to-I RNA editing regulation

seems uncommon, at least in the case of cancer cell lines. Individual depletion of more than 200 RBPs in HepG2 and K562 followed by analysis of *ADAR1/2/3* mRNA expression levels revealed that almost all RBPs do not significantly regulate *ADAR* mRNA expression levels [113]. Furthermore, regulation of *ADAR* expression by RBPs at the post-transcriptional and protein level seems to be also uncommon, since depletion of only a few RBPs (31 and 15 RBPs in K562 and HepG2, respectively) induced A-to-I RNA editing level changes in more than 10% of testable editing sites [113]. In other words, depletion of most RBPs did not at least mimic the number of differentially edited sites as observed in *TARDBP*-depleted HepG2 cells. The lack of extensive or global changes in editing levels upon depletion of RBPs is indicative that most RBPs likely regulate A-to-I RNA editing largely through an ADAR expression change-independent mechanism.

5.4.2.3 Competitive Binding with ADAR Monomers

Homodimerization of ADAR1 and ADAR2 is crucial for its A-to-I RNA editing activity [26]. Homodimerization of ADAR1 and ADAR2 appears to be RNA-independent and is mediated through protein–protein interactions of ADAR monomers [139]. ADAR-interacting RBPs, especially proteins that bind interacting interfaces of ADAR monomers, can potentially inhibit the formation of ADAR homodimers (Fig. 5.4). The RBP Dicer, which is involved in the formation of miRNAs for RNA-induced silencing complex (RISC), interacts with monomeric ADAR1 [99]. The Dicer within the Dicer/ADAR1 heterodimer exhibited increased catalytic efficiency of pre-miRNA cleavage, possibly by releasing its auto-inhibitory effects upon ADAR1 interaction [99]. At the same time, this interaction also regulates ADAR1 catalytic activity, since ADAR1 monomers are catalytically inactive [99]. Apart from Dicer, DAP3 [45] and SRSF9 [126] were also found to inhibit the homodimerization of ADAR1 and ADAR2, respectively (Fig. 5.4). Identification of ADAR-interacting proteins is a key step to identify RBPs that may regulate editing through inhibition of homodimerization, or possibly facilitating formation of homodimers. This can be done through ADAR1 and ADAR2 co-immunoprecipitation coupled with mass spectrometry (Co-IP/MS), an effective strategy to uncover novel ADAR-interacting partners [58].

One RBP is not just restricted to one mechanism of A-to-I RNA editing regulation. Investigators may infer this likelihood from its pattern of editing regulation. RBPs that are bidirectional regulators are likely to possess different mechanisms of regulation. The DEAH box helicase 9 (DHX9) exerts a unique pattern of editing regulation, in which it enhances ADAR1-specific while represses ADAR2-specific RNA editing sites [58], suggesting that DHX9 likely regulates ADAR1 and ADAR2 through distinct mechanisms (Fig. 5.4). In fact, many RBPs depleted as part of the ENCODE project [134] are bidirectional regulators of A-to-I RNA editing [113], again suggesting that many RBPs are likely to possess more than one mechanism of editing regulation. However, RBPs with a largely predominant direction of editing regulation can have different mechanisms of regulation as well. For example, DAP3 was found to inhibit the homodimerization of ADAR1 but not

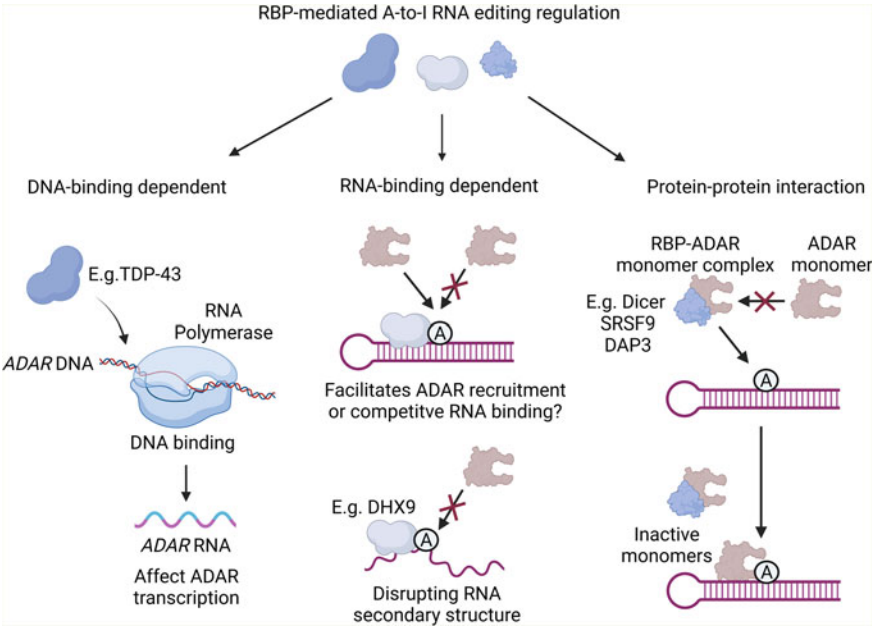

Fig. 5.4 RBPs regulate A-to-I RNA editing primarily through three different mechanisms: DNA-binding, RNA-binding dependent mechanisms, or through protein–protein interaction. A small group of RBPs can regulate ADAR expression at the transcriptional level. Some RBPs bind close to editing sites to perhaps facilitate ADAR recruitment or act as competitors of ADAR binding while RNA helicases may disrupt RNA secondary structure to inhibit its recruitment. Other RBPs repress editing by inhibiting ADAR homodimerization

ADAR2, while inhibiting the binding of ADAR2 to RNA substrates in an RNA binding-independent manner [45].

5.4.3 Implications of RBP-Mediated A-to-I RNA Editing Dysregulation in Cancers

Dysregulation of A-to-I RNA editing is one of the key drivers of tumourigenesis and is not just a manifestation of cancer development. Dysregulation of RBP expression levels has been shown to be one of the ways cancer cells gain survival advantage. Han and colleagues found that DAP3, which is over-expressed in many cancer types, functions as an oncogene partially through A-to-I RNA editing repression at an ADAR2 editing site within the *PDZD7* coding region [45]. A-to-I RNA editing at this editing site recodes the original stop codon to tryptophan, leading to an 18 amino acid extension at the c-terminus of PDZD7. DAP3 over-expression in ESCC tumours results in accumulation of the more tumourigenic shorter PDZD7 protein isoform [45]. Another example is SRSF9 which functions predominantly as an ADAR2 editing repressor. SRSF9 promotes cell survival by

repressing editing on RNA transcripts involved in cell metabolism, cell cycle and DNA repair [126].

The studies mentioned demonstrated how RBPs contribute to cancer cell's growth and survival through regulation of A-to-I RNA editing. However, editing regulation by some RBPs seemed to be cancer-type specific, as highlighted previously [113]. Notably, cancer-type specific expression levels of RNA transcripts partially account for the difference in editing profiles [113]. This means that an RBP that confers a growth advantage in one cancer cell type may not be the case for the other, as the expression levels of RNA transcripts containing a potent editing site may be much lower for the latter. Furthermore, as tumours are highly heterogenous, each tumour may contain clusters of cancer cells that depend on different RBPs for survival advantage due to different RNA expression levels between the clusters. This highlights the complexity of RBP-involved spatiotemporal regulation of A-to-I RNA editing in the cancer context.

5.5 Conclusions

A-to-I RNA editing catalyzed by ADARs is an important facet of cancer biology. RNA editing is often dysregulated in various cancers, and specific RNA editing events can have both oncogenic and tumour-suppressive effects in different contexts. They can lead to protein recoding, altered miRNA targeting, changes in miRNA biogenesis, canonical RNA splicing as well as backsplicing. However, the vast majority of knowledge on the functional relevance of specific RNA editing events to cancer was acquired using in vitro cell culture models. Evidence from in vivo models would be required for a better understanding of the physiological or disease impact of A-to-I RNA editing in the context of the organism. Further, a greater understanding of cell and tissue specificity of ADAR behaviour is needed, as we have observed variation in the effects of A-to-I RNA editing in different contexts. Nonetheless, the advent of single-cell RNA-seq (scRNA-seq) provides unprecedented opportunities for exploring RNA editing changes in different cell types in tumour or tumour-associated stroma at single-cell resolution.

On top of reviewing the effects of specific RNA editing events on cancer, we also highlight the importance of more generic A-to-I RNA editing activity by ADAR1 on cancer immunity. Overall, ADAR1-mediated A-to-I RNA editing suppresses the immunogenicity of dsRNAs. These dsRNAs likely originate from REs, specifically *Alu* elements that form dsRNA by intramolecular base pairing of *Alu* repeats [88]. The formation of immunogenic dsRNAs is likely in *cis*, through transcription of inverted repeat *Alu* sequences within the same transcript to form double-stranded stem-loop structures; rather than in *trans* [23]. The RNA editing function of ADAR1 is likely important for immunosuppression because editing deficient ADAR1 mice present similar phenotypic changes to ADAR1-null mice, and inosine content in dsRNA is critical in suppression of MDA5 and downstream IRF3 activation [79, 142]. Edited, inosine containing transcripts are thought to be less stable and targeted for specific degradation, reducing the total

dsRNA cytosolic content [75, 122, 124]. However, it is also possible that edited IU-dsRNA occupies and occludes dsRNA sensors such as MDA5 in competition with unedited immunogenic transcripts [142]. Besides its catalytic ability, the editing-independent functions of ADAR1 were also shown to suppress dsRNA mediated IFN production [154]. Whether editing of a small specific subset of dsRNA species, or generic and promiscuous editing of a wide range of dsRNA species results in the suppression of IFN responses is a topic of debate [6, 142]. While we now have a greater appreciation of the immunosuppressive action of ADAR1 in self-tolerance and its implications in cancer, there remain many details that have yet to be elucidated. First, the precise mechanism in which ADAR1 suppresses the immunogenicity of self-derived dsRNAs has yet to be determined. Next, it is unknown if either (or both) "quantity-over-quality" or "quality-over-quantity" models of self-derived dsRNA species edited by ADAR1 are accurate. Due to this, it remains a challenge to identify specific RNA species or commonalities among such immunogenic dsRNA that could serve as potential therapeutic targets.

Finally, A-to-I RNA editing is regulated by diverse mechanisms. We highlighted the role of RBPs in fine-tuning the A-to-I RNA editing level. RBPs can enhance, repress or regulate A-to-I RNA editing in both directions. Such regulations can occur via binding in proximity to regulated RNA editing sites—via competitive binding for editing repression or ADAR recruitment for editing enhancement, or without binding to RNA—by modulating ADAR expression levels or binding to ADAR monomers. However, due to the fact that RNA structures of editing substrates and the crystal structures of the full-length ADAR1 and ADAR2 proteins remain unclear, a complete understanding of the role of RBPs in this intricate regulatory network to shape the RNA editome is an ongoing research topic.

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RNA Modifications in Hematologic Malignancies

6

Yashu Li, Wen Tian, and Haojian Zhang

6.1 Introduction

It is well known that chemical modification plays an important role in almost all biological processes by regulating macromolecules such as DNA, RNA and proteins. In the central dogma of genetics, RNA serves as a ubiquitous mediator for information transfer from DNA to protein. Along with the development of better techniques for characterizing RNA, the complexity of RNA was appreciated by scientists since the 1960s, and the concept that RNA could be modified post-transcriptionally was introduced [1]. Indeed, the research about RNA modification started in the 1950s when Davis and Allen reported the presence of pseudouridine (Ψ) in the fractions of the ribonucleic acid from yeast [2]. Since then, over 150 different types of chemical modifications in RNA was identified [3]. However, given the features of RNA such as rapid turnover and instability, the functional importance of RNA modification was ignored by scientists in the past decades, and the study related to RNA modification was also limited due to the lack of high-throughput techniques for RNA modification. The revival of this field began with the discovery of fat mass and obesity-associated protein (FTO) as the first RNA demethylase, which uncovers that RNA modification is reversible and controlled by dedicated and typically highly conserved enzymatic machineries [4]. In 2012, two groups developed the high-throughput sequencing technology for profiling RNA N^6-methyladenosine (m^6A) at the transcriptome-wide level [5, 6],

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which further ignited this field. Since then, increasing evidences indicates that RNA modifications exert critical roles in various physiological and pathological conditions.

Hematopoietic system is maintained by hematopoietic stem cells (HSCs) that possess sufficient self-renewal capacity and the potential of differentiation into all lineages of blood cells. The homeostasis of this system is tightly controlled at multilayers such as the transcriptional and post-transcriptional levels [7–9], and its imbalance frequently causes hematologic diseases such as leukemia and bone marrow failure. For instance, acute myeloid leukemia (AML) is an aggressive and fatal type of hematologic malignancy that is characterized by uncontrolled expansion of poorly differentiated myeloid cells [10]. Accumulating evidences including studies from our laboratory indicates that RNA modification is involved in hematologic malignancies [11, 12]. Herein, we summarize recent advances in different RNA modifications, the development of detection methods, roles and mechanisms of RNA modifications in hematologic malignancies.

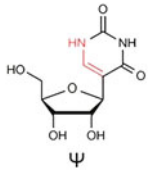
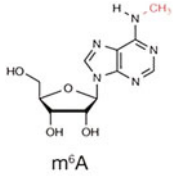
6.2 RNA Modifications

Many chemical modifications have been described in different RNAs, including small nuclear RNA (snRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), and messenger RNA (mRNA). These modifications confer distinct properties to the RNA by impacting RNA structure, RNA-RNA and RNA–protein interactions, function, and gene expression. Here, we focus on the more recent discoveries on four of these modifications: pseudouridine (Ψ), N^6-methyladenosine (m^6A), 5-Methylcytosine (m^5C), and N^1-methyladenosine (m^1A) (Table 6.1), especially on eukaryotic mRNA.

6.2.1 Pseudouridine

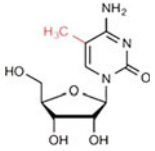
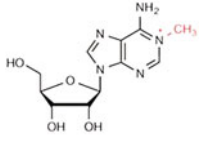
Pseudouridine (Ψ) is the first RNA modification identified by Davis and Allen in 1950s [2], and is considered as the fifth RNA nucleotide. Compared with uridine, the bases of Ψ and ribose are connected by C–C bond rather than N–C bond such that Ψ has an extra hydrogen bond donor on the non-Watson–Crick edge and contributes to its RNA stability. Pseudouridine is one of the most abundant post-transcriptional modifications. Quantitative liquid chromatography followed by mass spectrometry (LC–MS) analysis shows that pseudouridine reaches levels of ~0.3–0.4% of all uridines, and is likely very prevalent (0.2–0.6%) in mammalian mRNA [13]. Pseudouridine is widely present in most RNA species. Uridine is transformed into pseudouridine by a class of enzymes known as pseudouridine synthase [14, 15]. So far, fourteen pseudouridylases have been identified in eukaryotic cells, including 13 pseudouridine synthases (PUSes) and dyskerin pseudouridine synthase 1 (DKC1). These enzymes exhibit specific cellular localization, which confer them the ability to catalyze specific RNA targets. For instance, PUS1

Table 6.1 Features of four representative RNA modifications

Types	Writer	Eraser	Reader	Distribution	Biological function
 Ψ	DKC1, PUS	Unknown	Unknown	mRNA (3'UTR, CDS, 5'UTR), rRNA, snRNA, miRNA	RNA biogenesis, structure, stability and gene expression
 m^{6}A	METTL3-METTL14-WTAP-ZC3H13-RBM15/15B-KIAA1429	ALKBH5, FTO	YTHDF1/2 YTHDC1/2 IGF2BP1-3	mRNA (CDS, 3'UTR), tRNA, rRNA, snRNA	RNA stability, localization, transport, shearing, translation and gene expression

(continued)

Table 6.1 (continued)

Types	Writer	Eraser	Reader	Distribution	Biological function
 m^{5}C	NSUN	TETs, ALKBH1	ALYREF, YBX1	miRNA, tRNA, rRNA, mRNA (CG-rich regions, regions immediately downstream of translation initiation sites)	tRNA structure and stability, rRNA structural conformation and translational fidelity, mRNA metabolism
 m^{1}A	TRMT6-TRMT61A, TRMT61B, TRMT10C	ALKBH3 ALKBH1	YTHDF2	mRNA (around the start codon upstream of the first splice site)	The structure and function of tRNA and rRNA

predominantly localizes in nuclear and mainly modifies tRNA, mRNA, snRNA and ncRNAs [16]; DKC1 is part of ribonucleoprotein complex and requires RNA guide for its catalytic activity, and the main targets of DKC1 are rRNA, snRNA, snoRNA and TERC [17, 18]. Notably, erasers and readers for pseudouridine remain unknown.

Pseudouridine plays important roles in RNA biogenesis, structure, stability and gene expression. Pseudouridine in rRNA is required for binding to the internal ribosome entry site and for translational fidelity [19]. Pseudouridylation on tRNA contributes to maintaining its structure and stability [20]. Pseudouridine can affect mRNA splicing by fine-tuning branch site interactions [21]. However, the effect of pseudouridine in mRNA remains largely unclear. Currently, the physiological and pathological roles of pseudouridine are gradually appreciated. Modified nucleotides with pseudouridine ablates the potential of RNA to activate dendritic cells [22], which launches a new era for RNA modification application in RNA therapy and RNA vaccine.

6.2.2 N^6-Methyladenosine

The methylation of adenosine at position N^6 (m^6A) was first identified in the 1970s. Analysis of the purified poly(A)$^+$ mRNA using enzyme digestion, acid hydrolysis and chromatographic techniques has shown that the methylated nucleotide (53%) is due to m^6A [23]. Currently, m^6A is the best characterized RNA modification, and is also the most abundant one in mammalian mRNA and long noncoding RNA. Its level reaches 0.1–0.4% of total adenine (m^6A/A), and it is estimated that about 15,000 human genes have methylated mRNA, and each transcript contains around 3 to 5 or more m^6A modification sites [24]. Recent developed high-throughput techniques revealed that m^6A modification sites have a typical consensus motif DRACH and are mainly enriched in the coding sequence and 3'untranslated region [5, 6].

RNA m^6A modification is reversible, which was revealed by a recent study showing that FTO has efficient oxidative demethylation activity targeting the abundant m^6A residue in RNA in vitro [4]. Since then, accumulating evidences has uncovered that this reversible modification is highly dynamic, and is regulated by a set of m^6A modifiers including writers, erasers, and readers. The m^6A methyltransferase complex, also called writer, is a multicomponent nuclear complex composed of two methyltransferase-like proteins (METTL3 and METTL14) and several regulatory proteins (WTAP, RBM15/RBM15B, VIRMA, CBLL1, and ZC3H13) [25–29]. FTO and ALKBH5 are the main demethylases that selectively remove m^6A marks [4, 30]. m^6A readers including YTH domain-containing protein 1–2 (YTHDC1/2), YTH domain-containing family member 1–3 (YTHDF1/2/3), and insulin-like growth factor-2 mRNA-binding protein (IGF2BP) family IGF2BP1/2/3, are responsible for recognizing this modification and determining

RNA fates [31–34]. Notably, it becomes clear that m^6A readers and their associated protein machineries are very important for precisely exerting their biological functions under different conditions [35].

RNA m^6A Writer

RNA m^6A writer METTL3-METTL14 complex catalyzes the transfer of methyl group from S-adenosylmethionine (SAM) to the sixth N atom of RNA adenosine. In this complex, METTL3 acts as the sole catalytical protein, and METTL14 maintains the structural stability of METTL3/METTL14 heterodimer [36]. METTL3 has the leading helix structure (LH) domain, nuclear localization signal (NLS) domain, and CCH-type zinc finger domain (ZFD). LH and NLS mediate the interaction of METTL3 with METTL14 [25, 36, 37]. ZFD serves as the target recognition domain and fulfills the methyltransferase activity of the METTL3-METTL14 complex [38]. The subunits of this complex play important roles in its activity and specificity. For instance, WTAP promotes METTL3-METTL14 heterodimer to enter the nuclear plaque and stabilize it [26]; RBM15 facilitates the recruitment of m^6A writer complex to specific RNA [39]; VIRMA plays a significant role in m^6A modification concentrated near 3'UTR and stop codon [40].

RNA m^6A Erasers

Both FTO and ALKBH5 function as RNA m^6A demethylases and belong to the ALKB family [4, 30]. Their demethylation activity requires both α-KG and Fe^2. FTO was previously studied as an important protein in metabolic disorders such as diabetes and obesity, until a recent study from Dr. He's group reported that FTO has an efficient oxidative demethylation activity [4]. FTO can also catalyze the demethylation of m^6Am on mRNA and snRNAs, and m^1A on tRNA, which may be determined by FTO's access to different RNA substrates due to its cellular distribution. In the nuclear, FTO has a higher affinity for m^6A, but a higher affinity for m^6Am in the cytoplasm [41]. ALKBH5 is another demethylated enzyme that specifically recognizes RNA m^6A [30]. Interestingly, several works from our and other groups show that ALKBH5 deficiency in mice does not substantially affect the health status except for the defect of spermatogenesis in mice, which makes ALKBH5 a suitable therapeutic target [12, 30, 42].

RNA m^6A Readers

RNA m^6A readers recognize m^6A sites and exert a key function in regulating distinct mRNA fates. The m^6A readers including YTHDF1/2/3 and YTHDC1/2 contain the same YTH domain that recognizes m^6A, and are classified into one group. YTHDF1 and YTHDF3 mainly regulate the translation of their target transcripts [43], whereas YTHDF2 facilitates its m^6A target degradation [44]. Similarly, YTHDC1/2 executes a different function in fine-tuning m^6A-tagged mRNA. YTHDC1 mainly participates in the export of m^6A-tagged mRNA from the nucleus to the cytoplasm [31, 45–47]. It can also mediate mRNA splicing through recruiting splicing factors serine/arginine-rich splicing factor 3 (SRSF3)

and SRSF10 [31]. YTHDC2 regulates the translation and stability of its m^6A targets [48–50].

IGF2BP1-3 are another group of m^6A readers. These readers contain 4 repetitive KH protein domains (KH1-4), and KH3/4 domains are required for m^6A recognition [34]. It is known that IGF2BPs can stabilize their m^6A target mRNAs and also enhance their translation [34]. Our recent work reveals that YBX1 is an important component of IGF2BP regulatory protein machinery. We found that YBX1 cooperates with IGF2BPs to promote the stability of m^6A-tagged transcripts [51]. Several heterogeneous nuclear ribonucleoproteins (HNRNPs) including HNRNPC, HNRNPG, and HNRNPA2/B1 may also function as m^6A readers. For instance, HNRNPA2B1 functions as an m^6A nuclear modification reader that can regulate primary microRNA processing and alternative splicing [52]. Overall, m^6A readers are key in determining mRNA fates. In the future, identifying the cofactors of m^6A modifiers would be also very critical.

6.2.3 RNA 5-Methylcytosine

RNA m^5C methylation is another known methylated ribonucleoside that occurs at position 5 of the cytidine residues of coding and noncoding RNA. It was first observed in the RNA of E. Coli [53]. Compared to m^6A, m^5C is a much rarer RNA modification, and based on bulk analysis, its level ranges from 0.03–0.1% of cytosines (Wiener D, 2021). m^5C marks on tRNA, mRNA, rRNA, miRNA and enhancer RNA (eRNA), and plays distinct functions in regulating the fates of these different RNA types. For instance, m^5C regulates tRNA structure and stability; methylation of rRNA affects its structural conformation and translational fidelity, subsequently modulating translation efficiency [54]. The role of m^5C on mRNA metabolism is gradually revealed. A recent study indicates that m^5C modification is enriched in regions immediately downstream of translation initiation sites and in CG-rich regions, and has conserved, tissue-specific and dynamic features across mammalian transcriptomes. Moreover, m^5C marks regulate mRNA export that is specifically recognized and mediated by the mRNA export adaptor ALYREF. This work also reveals that ALYREF functions as a m^5C reader [55].

Currently, several m^5C writers have been identified. The enzymes that are responsible for RNA m^5C modification belong to the NOL1/NOP2/SUN domain (NSUN) family, including NSUN1-7 [56]. DNA methyltransferase-like 2 (DNMT2) also catalyzes m^5C formation on RNA [57]. These m^5C writers have their specific RNA targets. For example, NSUN1 and NSUN5 catalyze 28S rRNA, while NSUN2, NSUN6 and DNMT2 modify cytoplasmic tRNA. NSUN3 and NSUN4 target mitochondrial tRNA and rRNA, respectively, while NSUN7 is responsible for m^5C modification of eRNA [57]. Moreover, m^5C formation in mRNAs is mainly catalyzed by NSUN2, which modulates ALYREF's nuclear-cytoplasmic shuttling, RNA-binding affinity and associated mRNA export [55]. Recent studies found that NSUN6 can also act as a methyltransferase for mRNA in humans and animals [58, 59]. NSUN6 primarily targets 3'UTR at the consensus

sequence motif UCCA and catalyzes m^5C sites. Ribosome profiling showed that NSUN6-specific methylation correlates with translation termination [59].

Recent studies also reveal the erasers and readers of RNA m^5C. Enzymes of ten-eleven translocation (TET) family not only oxidize 5mC in DNA, but also possess the activity of oxidizing RNA m^5C to 5-hydroxymethylcytidine (hm^5C). TET-mediated oxidation in mRNA promotes global protein synthesis or decreases stability [60, 61]. For instance, in infection-induced myelopoiesis, Tet2 mediates oxidation of mRNA m^5C for Adar1 targeting and represses Socs3 expression, a key negative regulator of the JAK-STAT pathway that is critical for cytokine - induced myelopoiesis; Tet2 deficiency leads to the transcriptome-wide appearance of methylated cytosines, including ones in Socs3 3'-UTR [61]. TET-mediated oxidation is also observed on m^5C in tRNAs. In Tet2-deficient mouse embryonic stem cells, the level of hm^5C in tRNA was significantly decreased when compared with wild-type cells [62]. Conversely, induced expression of the catalytic domain of Tet2 caused an increase of hm^5C and a decrease of m^5C in tRNAs. Similar to ALKBH5, ALKBH1 is also a 2-oxoglutarate- and $Fe2^+$-dependent dioxygenase. ALKBH1 can function as a m^5C demethylase and covert m^5C to hm^5C at position 34 of tRNA, which subsequently affects mitochondrial translation; ALKBH1-knockout cells exhibit a strong reduction in mitochondrial translation and reduced respiratory complex activities [63]. In terms of m^5C readers, ALYREF is the first one identified as a mRNA m^5C reader. In a recent study, RNA affinity chromatography and mass spectrometry were performed to search for specific mRNA m^5C-binding proteins [55]. ALYREF, the mammalian mRNA export adaptor, was observed to be enriched by m^5C-containing oligonucleotide. This study confirmed that ALYREF functions as a specific mRNA m^5C-binding protein regulating mRNA export [55]. Interestingly, another RNA-binding protein, YBX1, was also identified as a m^5C 'reader' recognizing m^5C-modified mRNAs through the indole ring of W65 in its cold-shock domain. Further, YBX1 maintains the stability of its target mRNA by recruiting ELAVL1. NSUN2 and YBX1 have been demonstrated to drive the pathogenesis of human urothelial carcinoma of the bladder by targeting the m^5C methylation site in the HDGF 3'-UTR [64]. During zebrafish maternal-to-zygotic transition, YBX1 plays a key role in stabilizing m^5C-modified maternal mRNAs [65]. Overall, the regulatory machinery of RNA m^5C is still being explored.

6.2.4 N^1-Methyladenosine

Methylation of adenosine at position 1, that is, N^1-methyladenosine (m^1A), was initially observed in RNA from mammalian and plant sources in 1960s [66, 67]. It was later identified in tRNA, rRNA and mRNA [68, 69]. RNA m^1A can be converted to m^6A under alkaline conditions, which brings a big challenge for m^1A detection and quantification in mRNA [70]. In a recent study, using LC–MS/MS to quantify m^1A in pure mRNA preparations under controlled conditions, m^1A/A molar ratios ranging from about 0.015–0.054% were observed in several

human and mouse cell lines [71]. Based on other bulk analyses, m^1A level is about tenfold lower than m^6A [72, 73]. However, Safra and colleagues reported that m^1A is present in a low number of mRNA within the cytosol [74]. Thus, the level of m^1A is still controversial.

The current known methyltransferases catalyzing m^1A include tRNA methyltransferase 6 non-catalytic subunit (TRMT6)-RNA methyltransferase catalytic 61A (TRMT61A) complex for mRNA and mitochondrial tRNA. TRMT61B is dominantly localized to the mitochondria, and methylates mitochondrial rRNA and tRNA [75, 76]. TRMT10B and TRMT10C catalyze methylation at position 9 for mitochondrial tRNAs [77]. RNA m^1A shares some regulators with m^6A. For instance, m^6A reader YTHDF2 can also bind to m^1A with a relative low affinity [78], suggesting that the YTH domain-containing protein family might be the reader of m^1A, which needs to be further validated.

Both ALKBH1 and ALKBH3 function as m^1A erasers, and ALKBH3 is the only known eraser for m^1A on mRNA. ALKBH3 is known as a DNA/RNA demethylase [79, 80], and a recent study confirmed that m^1A on mRNA can be erased by ALKBH3. In an in vitro demethylation assay, recombinant ALKBH3 protein exhibits robust demethylation activity against a synthetic RNA substrate containing a site specifically incorporated m^1A modification [73]. Transcriptome profiling assay showed that ALKBH3 has minimal sequence preference and acts globally in the transcriptome, and manipulation of ALKBH3 protein levels could change the mRNA m^1A/A ratio in different cell lines [73]. ALKBH1 not only acts as a m^5C demethylase, but also mediates the demethylation of m^1A in tRNAs [81]. He and colleagues found that A tRNA-binding motif exists in ALKBH1, and it preferentially demethylates m^1A in a stem-loop structure of tRNA and possesses effective tRNA m^1A demethylation activity in vitro and inside cells. Using CLIP sequencing assay, they showed that ALKBH1 binds m^1A58-containing tRNAs. The ALKBH1-mediated tRNA demethylation controls the utility of the target tRNAs in translation, thereby directly influencing protein synthesis [81].

m^1A mainly affects the structure and function of tRNA and rRNA, due to the methyl adduct and a positive charge. Human rRNA and tRNA contain many different m^1A modification sites. For instance, m^1A at position 58 of tRNA affects its structure, stability and function in translation [81]. m^1A is associated with translation initiation sites in the human transcriptome [71]. m^1A preferentially enriches around the start codon upstream of the first splice site, and affects translation efficiency in response to physiological conditions. Nevertheless, the role of m^1A remains largely unknown, which needs to be explored in the future.

6.3 Methods for Mapping RNA Modifications

Detection of RNA modifications is always a key issue in this field. LC–MS is a powerful technique for detecting and quantifying the modified RNA; however, it is limited to measure specific sites. In addition, it is difficult for LC–MS to detect modifications that are relatively lower in mRNA. For instance, other RNA

modifications (non-m^6A) are rarer in mRNA and more pervasive in both tRNA and rRNA. This feature brings severe constraints on the ability of LC–MS to detect the abundance and dynamics of these rare modifications. In the past 10 years, the revival of RNA modification field largely attributes to the development of high-throughput technologies for detecting these modifications at the transcriptome level [5, 6]. In the following, we briefly summarize the methods for profiling RNA modifications at the transcriptome level (Table 6.2).

Table 6.2 A summary of mapping methods for RNA modifications

Detection methods	Advantages	Limitations	Refs.
MeRIP-seq/ m^6A-seq	First method for global view of m^6A	Large amounts of starting RNA Low resolution	[5]
miCLIP-seq	Single-nucleotide resolution	Large amounts of input RNA Non-specific binding of antibody	[82]
m^6A-LAIC-seq	Quantify m^6A stoichiometry	Low resolution	[83]
m^6A-seq2	Allows quantification across genes and samples	Low resolution	[84]
SLIM-seq	Low input material High sensitivity	Low resolution	[85]
MAZTER-seq/ m^6A-REF-seq	Single-nucleotide resolution Quantitative tracking of m^6A	Only detect 16%–25% of m^6A sites	[86]
DART-seq	Allow detection of m^6A accumulation low amounts of input RNA	Low sensitivity Only identify partial m^6A signal	[87]
m^6A-label-seq	Single-nucleotide resolution	Low efficiency Lack stoichiometric information	[88]
m^6A-SEAL-seq	Less false-positive signals	Lack stoichiometric information Unstable efficiency	[89]
m^6A-SAC-seq	Single-nucleotide resolution Quantitative tracking of m^6A	Low specificity and efficiency	[90]
m^1A-ID-seq	Single-nucleotide resolution	Large amounts of input RNA Unstable efficiency caused by enzyme	[73]
m^1A-seq	Single-nucleotide resolution	Large amounts of input RNA	[71]
m^1A-MAP	Single-nucleotide resolution High sensitivity and accuracy	Inaccuracy in low abundant RNA	[72]

(continued)

Table 6.2 (continued)

Detection methods	Advantages	Limitations	Refs.
m^1A-Seq-TGIRT	Single-nucleotide resolution	Limited sensitivity Large amounts of input RNA	[74]
m^1A-IP-seq	Single-nucleotide resolution	Large amounts of input RNA	[91]
m^1A-quant-seq	Single-nucleotide resolution Quantitative tracking of m^1A	Large amounts of input RNA	[91]
RBS-seq	Single-base resolution Potentially quantitative	Insensitive in low abundant RNA Signal loss caused by chemical modifications	[92]
Aza-IP	Single-base resolution High accuracy	Low chemical labeling efficiency	[93
m^5C miCLIP	Single-base resolution High sensitivity	False-positive detection by highly overexpressed enzymes	94]
Pseudo-seq/Ψ-seq	Single-nucleotide resolution Quantitative tracking in transcriptome-wide	Low chemical labeling efficiency Dropout in low abundant RNA	[95, 96]
CeU-Seq	Allow evaluation of the dynamics of Ψ	Non-specific chemical conversions	[13]

6.3.1 M^6A Mapping

The first high-throughput strategy for sequencing RNA m^6A, called MeRIP or m^6A-seq, was developed by two groups in 2012 [5, 6]. Briefly, mRNA is fragmented into 100–200 nt in size, and the m^6A-modified fragments for next generation sequencing are immunoprecipitated by anti-m^6A antibody due to its high sensitivity and selectivity. These studies, for the first time, identified over 7,000 mRNA transcripts labeling with m^6A modification in mammalian cells, and showed that m^6A positions are enriched near the stop codon and 3' UTR in the conserved DRACH motif [5, 6]. However, there are some limitations for this strategy. For instance, it cannot precisely identify m^6A residues at high or single-nucleotide resolution, and it is also limited with low input samples. Since then, researchers have attempted to address these issues. The miCLIP method enables to detect m^6A sites at single-nucleotide resolution based on UV cross-linking and immunoprecipitation [82]. This method uses the property that cross-linked proteins to RNA in living cells could cause mutations or truncations in the cDNA during reverse transcription. Similar to MeRIP, miCLIP also requires a large amount of starting RNA samples. Moreover, commercial antibodies from various manufacturers may result in various mutations or truncations, making it difficult to identify the true m^6A location.

Recently, two techniques, m^6A-seq2 and SLIM-seq, were established to map m^6A profiling using small amounts of RNA samples. m^6A-seq2 uses different tags

to fragmented RNA from several samples before combining them for immunoprecipitation. As a result, m^{6}A-seq2 decreases the amount of RNA needed for each sample as well as technical variability across samples due to manipulation [84]. Our group developed a highly sensitive and efficient super-low-input m^{6}A sequence (SLIM-seq) to study the m^{6}A landscape of rare cell populations [85]. This method combines the benefits of m^{6}A-LAIC-seq [83] and Smart-seq, showing high sensitivity and efficiency to low input RNA materials.

All the methods above depend on m^{6}A antibody. However, they have some limitations including cross-reactivity to other RNA modifications, and limited utility for quantification of m^{6}A stoichiometry. Recently, several mapping strategies have been developed to overcome these limitations. These m^{6}A antibody-independent approaches include MAZTER-seq [86], m^{6}A-REF-seq [97], DART-seq [87], m^{6}A-label-seq [88], m^{6}A-SEAL [89], SAC-seq [90]. MAZTER-seq and m^{6}A-REF-seq rely on the ability of RNase, MazF, to achieve recognition of m^{6}A at single-base resolution. However, the detected m^{6}A sites are limited by the cleaving capacity of MazF, and only 16%–25% of the m^{6}A sites can be detected. DART-seq is another m^{6}A mapping method that relies on enzyme activity [87]. This method uses APOBEC1, a cytosine deaminase, to edit m^{6}A-adjacent cytidine to uracil guided by YTH domain, and then identify the m^{6}A signal through high-throughput sequencing and analysis. The method can detect 79% of the edited signal in mRNA as low as 10 ng. However, the method only detects partial m^{6}A signal recognized by the YTH domain.

The inert chemical property of RNA m^{6}A poses a big challenge to detect m^{6}A site; however, a lot of efforts try to alter m^{6}A chemical feature in developing antibody-free m^{6}A mapping approach. m^{6}A-SEAL converts m^{6}A into dm^{6}A, which subsequently is labeled with biotin and pulled down by streptavidin [89]. This approach avoids cross-reactivity to other RNA modifications caused by m^{6}A antibody. However, m^{6}A-SEAL can only detect part of m^{6}A sites due to the conversion efficiency of m^{6}A to dm^{6}A. m^{6}A-label-seq is a metabolic labeling method that converts m^{6}A signal into N^1, N^6-cyclized adenosine (cyc-A) through multichemical reactions. The cyc-A can cause mismatches during reverse transcription and then be identified by next generation sequencing [88]. Also, m^{6}A-label-seq can only be applied to cells, not directly to RNA. Recently, m^{6}A-SAC-seq is a method developed by He and colleagues to detect and quantify m^{6}A levels at single-nucleotide-resolution in transcriptome-wide level, using the demethylation activity of Dim1/KsgA family proteins to convert m^{6}A to m$^6{}_2$A. The method can quantitatively detect m^{6}A modification using input RNA as low as 30 ng [90]. Although it seems that m^{6}A-SAC-seq could overcome the current technological bottleneck for m^{6}A mapping, the specificity and efficiency of Dim1 remain an important issue.

6.3.2 M^1A Mapping

Recently, several groups have independently developed transcriptome-wide approaches to map m^1A modification, including m^1A-ID-seq [73], m^1A-seq [71], m^1A-MAP [98], m^1A-Seq-TGIRT [74], m^1A-IP-seq and m^1A-quant-seq [91].

Unlike m^6A modification, m^1A modification can cause mismatch or extension termination during reverse transcription, therefore, m^1A sites can be identified at single-base resolution by commercial antibody and sequencing. The principles for the current m^1A mapping methods are similar. Firstly, RNA is fragmented and subjected to immunoprecipitation using m^1A antibody, then RNA fragments are converted to cDNA for sequencing, or treated to induce partial m^1A-to-m^6A or demethylated A before cDNA synthesis. m^1A-ID-seq successfully achieves high-resolution m^1A detection at the whole transcriptome-wide level in human cells, and identifies a total of 901 m^1A modification sites present on mRNA and non-coding RNA, and found that m^1A modifications were mainly present in the 5' UTR [73]. m^1A-MAP can detect 473 m^1A sites in the 293 T transcriptome, and finds that a major group of m^1A sites are enriched in 5' UTR, a small subset of GUUCRA tRNA-like m^1A sites relatively even distributed in the transcriptome [98]. The study also shows, m^1A sites in the 5' UTR, particularly those located at the first and second nucleotide of mRNA transcripts are associated with increased translation efficiency; in contrast, m^1A in the CDS of mitochondrial mRNA inhibits translation [98].

Similar to the above two methods, m^1A-seq also needs to fragment RNA and then use antibodies to enrich the fragments containing m^1A sites. The difference is that this method further converts m^1A to m^6A through Dimroth rearrangement instead of AlkB-assisted demethylation of m^1A. Transcriptome-wide mapping of m^1A revealed that m^1A is present in highly structured regions around the start codon and positively correlates with translation efficiency and protein level. Moreover, the distribution pattern of m^1A peaks is highly conserved in all mouse and human cell types examined, which suggests a positive and dynamic role of m^1A in translation initiation in mammalian cells [71, 99]. m^1A-Seq-TGIRT uses a similar principle as m^1A-seq does. However, due to read duplication and significant RNA degradation, m^1A-Seq-TGIRT only detect m^1A site in a low number of mRNAs, which provides conflicting results [74]. In addition, both m^1A-IP-seq and m^1A-quant-seq utilize evolved reverse transcriptase, which gives rise to higher mutation rates at the m^1A sites. These two strategies confirm many of the previously reported sites, and also identify hundreds of new m^1A sites in human mRNA [91]. Besides, in order to estimate m^1A stoichiometry, spike-in synthetic m^1A oligonucleotides are used with various m^1A fractions in m^1A-quant-seq, which allows to quantify m^1A stoichiometries at individual sites in the transcriptome [91]. Taken together, these findings indicate that m^1A may present at relatively high stoichiometry on a relatively large subset of mRNAs.

6.3.3 M^5C Mapping

The first m^5C detection method was developed in 2012, named RNA-Bisulfite-sequence(RBS-seq) [92]. m^5C can be detected by a modified bisulfite treatment, confirming the presence of m^5C modifications on mRNA. Currently, this method is the most widely used in m^5C detection at single-base resolution. However, it also has some limitations. For instance, chemical modification may damage mRNA stability and integrity, and affect the detection of m^5C signals. Therefore, bisulfite-independent m^5C detection methods have also been developed, including Aza-IP [93], miCLIP of m^5C [94] and m^5C-RIP [100]. Aza-IP exploits the covalent bond formed between an RNA methyltransferase and the cytidine analog 5-azacytidine to recover RNA targets by immunoprecipitation. The m^5C modification sites detected by this method are highly coincident with the data from RBS-seq, which illustrates the accuracy of this method. m^5C miCLIP uses a mutated version of NSUN2 protein to induce the formation of an irreversible covalent cross-link between NSUN2 and the methylated cytosine in its target RNA. miCLIP of m^5C identifies about 300 m^5C sites in HEK293 cells. Thus, different techniques to map m^5C in different studies give very different conclusions about m^5C abundance. Developing a new strategy for m^5C mapping is still necessary in the future.

6.3.4 Pseudouridine Mapping

Recently, methods for transcriptome-wide mapping of pseudouridine modification are developed. Three groups developed conceptually similar techniques (named as Pseudo-seq or Ψ-seq) for profiling pseudouridine on the mammalian transcriptome, as pseudouridine can be selectively modified with N-cyclohexyl-N'-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulphonate (CMC) to generate a block to reverse transcriptase one nucleotide 3' to the pseudouridylated site [95, 96]. Briefly, mRNA is treated with CMC that covalently binds to U, G, and pseudouridine residues, and the CMC-treated RNA is incubated at alkaline pH, leading to hydrolysis of the less stable U-CMC and G-CMC adducts. RNA is fragmented to 80–150 nt, and ligate 3' adaptor, followed by reverse transcription with expecting premature termination at Ψ-CMC sites. Both treated sample and input RNA that is not treated with CMC are used for next generation sequencing. These mapping approaches identify known modification sites as well as many novel sites in noncoding RNAs, and reveal hundreds of pseudouridylated sites in mRNAs in yeast. Interestingly, the majority of pseudouridines in mRNA are regulated in response to environmental signals, such as nutrient deprivation in yeast and serum starvation in human cells [95]. More recently, Li and colleagues developed a new approach, N_3-CMC–enriched pseudouridine sequencing (CeU-Seq), which combines the CMCT chemical labeling with a biotin pulldown method [13]. They identified 2,084 Ψ sites within 1,929 human transcripts. Applying CeU-Seq to the mouse transcriptome reveals conserved and tissue-specific pseudouridylation [13].

So far, hundreds to thousands of Ψ sites have been detected using each approach, however, the overall overlap between sites detected in different studies is very low. This is most likely because of higher experimental noise caused by non-specific chemical conversions.

6.4 RNA Modification in Hematologic Malignancies

The imbalance of hematopoietic homeostasis can lead to the development of various blood diseases such as leukemia, bone marrow failure and myelodysplastic syndrome. Genetic or epigenetic alterations occur in hematopoietic stem/progenitor cells (HSPCs) and transforms them into leukemia stem cells (LSCs), which subsequently initiate the development of hematologic malignancies, such as myeloid leukemia. Accumulating evidence showed that RNA modifications play key roles in regulating hematologic malignancies (Fig. 6.1).

6.4.1 RNA m^{6}A Dysregulation is Critical for the Development of Myeloid Leukemia

It is known that RNA m^{6}A modification controls the fate determination of HSCs in normal hematopoiesis. Recent studies indicate that HSPCs differentiation towards erythroid, myeloid, and lymphoid cells is substantially impaired upon METTL3 deletion [101–103]. Deletion of *Mettl3* in *Vav-Cre;Mettl3$^{fl/fl}$*

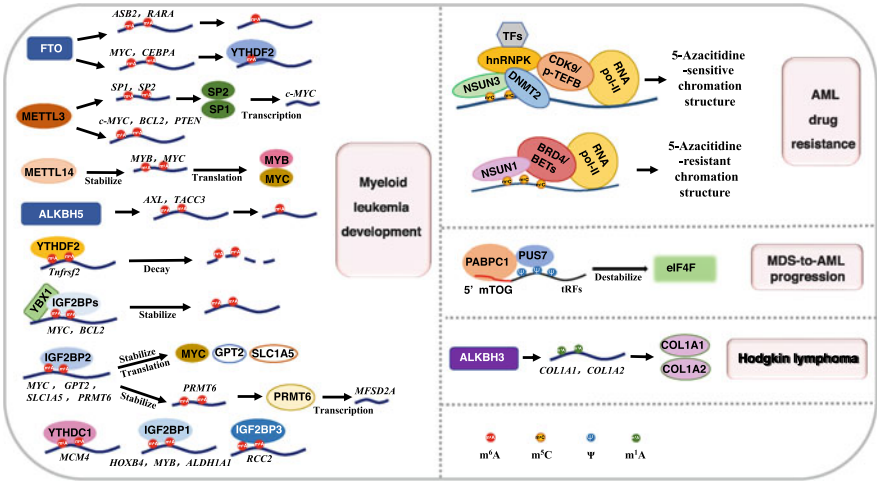


Fig. 6.1 The roles of RNA modifications in hematologic malignancies. m^{6}A modification dysregulation can enhance myeloid leukemia development; m^{5}C modification regulates drug sensitivity of leukemia cells; Ψ is clinically associated with leukemic transformation; Gain of m^{1}A in the transcriptome of Hodgkin lymphoma cells is associated with poor clinical outcome

mice caused hematopoietic failure with the expansion of functionally defective $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) HSPCs in the fetal liver. Similarly, m^6A is also essential for maintaining HSC function in adult hematopoiesis. Induced deletion of *Mettl3* in adult hematopoietic system in *Mx1-Cre;Mettl3*$^{fl/fl}$ mice blocks the normal differentiation and causes accumulation of phenotypical HSCs with long-term hematopoietic disorder and impaired hematopoietic reconstitution potential [104–106]. Knockdown of *METTL3* or *METTL14* also significantly inhibit the proliferation and promotes the differentiation of human umbilical cord blood derived $CD34^{+}$ HSPCs [107, 108]. Overall, these studies revealed a developmental stage-specific requirement for m^6A in hematopoiesis.

In general, myeloid leukemia is initiated by LSCs that are transformed from HSPCs due to dysregulated molecular networks. Altered RNA m^6A modification has been observed in myeloid leukemia. Compared with normal HSPCs or other types of tumor cells, the expression levels of m^6A modifiers, such as METTL3, METTL14, FTO, ALKBH5 and YTHDF2, are obviously increased in AML [108, 109]. FTO is the first reported m^6A modifier to play an oncogenic role in AML [110]. It highly expressed in different subtypes of AML. Knockout or inhibition of FTO can significantly inhibit the self-renewal of leukemia stem cells. Mechanistically, FTO regulates the stability of downstream mRNA targets, including *MYC*, *CEBPA*, *ASB2* and *RARA* in an m^6A-dependent manner [110, 111]. Since then, more and more efforts were done to uncover the role of other m^6A modifiers in leukemia. Overexpression of METTL3 inhibits leukemia cell differentiation and increases cell growth; conversely, deletion of METTL3 in human myeloid leukemia cells can induce cell differentiation and promote apoptosis, and delay in vivo leukemia development [108, 109, 112]. METTL3-mediated m^6A modification promotes the translation of downstream targets including *BCL2, c-MYC*, and *PTEN* [108]. In another study, an alternative mechanism was revealed by performing ChIP-seq assay. METTL3, independent of METTL14, binds to chromatin and localizes to promoter regions of some active genes, and subsequently recruits CEBPZ and regulates the translation of downstream oncogenic drivers SP1 and SP2 [109]. SP1 in turn regulates the expression of c-MYC and ultimately promotes the occurrence and development of leukemia [109]. Weng et al. reported that METTL14 is also overexpressed in AML patients and plays a critical oncogenic role in AML development/maintenance and LSC self-renewal, through promoting the RNA stability and translation of its critical targets such as *MYC* and *MYB* in an m^6A-dependent manner [113].

ALKBH5 is another major demethylase, besides FTO, for mRNA m^6A. Recently, we and others reported the key and selective role of ALKBH5 in leukemia stem cell maintenance, but not required for normal hematopoiesis [12, 42]. Through analyzing chromatin accessibility during leukemogenesis, we find that ALKBH5 is regulated by chromatin state alteration in leukemogenesis. Histone demethylase KDM4C could reduce H3K9me3 levels to increase chromatin accessibility, and facilitate the recruitment of MYB and Pol II to *ALKBH5* promoter region [12]. We also confirmed that Alkbh5 loss significantly inhibits AML development and progression. Moreover, ALKBH5 affects *AXL* mRNA stability in

an m^{6}A-dependent way and activates downstream PI3K/AKT pathway in AML. AXL belongs to the TAM (TYRO3, AXL, MER) receptor kinase family, and has been reported that AXL can phosphorylate FLT3 in AML to promote the pathological progress of AML [114]. In the same time, Shen et al. found that ALKBH5 knockdown accelerates the degradation of *TACC3* mRNA, an oncogenic factor described to be critical in the growth of various cancer cells [42, 115]. Interestingly, we found that AlKBH5 is dispensable for adult hematopoiesis and HSC function. Serial transplantation assay indicates that Alkbh5 loss does not affect HSCs' self-renewal, differentiation and long-term hematopoietic function [12, 42]. Therefore, the two complimentary studies uncover the important and selective role of ALKBH5 in the pathogenesis of AML and LSCs maintenance.

The roles of other m^{6}A readers or other factors in leukemogenesis are also being recognized recently. YTHDC1 knockout severely inhibits the development and maintenance of AML by regulating MCM4, which is a critical regulator of DNA replication [116]. YTHDF2 deletion extends the half-life of m^{6}A-modified transcripts including Tnfrsf2 to selectively compromise AML initiation and propagation without harming normal hematopoiesis [117]. Interestingly, recent works further add the complexity about the role of YTHDF2 in hematopoiesis. Two early studies found that YTHDF2 depletion significantly expands hematopoietic stem cells in mouse and human umbilical cord blood without skewing lineage differentiation preference or leading to hematopoietic malignancy [118, 119]. However, Mapperley and colleagues recently found that *Ythdf2*-deficient HSCs display chronic activation of inflammatory pathways, resulting in a progressive myeloid bias, loss of lymphoid potential and HSC expansion with functional defect of long-term reconstitution [117]. The role of IGF2BPs in leukemia is also being uncovered recently. IGF2BP1 deficiency promotes myeloid differentiation and decreases tumorigenic potential of AML cells by affecting HOXB4, MYB and ALDH1A1 [120]. IGF2BP3 loss significantly induces AML cell apoptosis, inhibits proliferation, and attenuates the ability of AML cells to develop leukemia in vitro and in vivo [121, 122]. Recently, we found that YBX1 interacts with IGF2BPs to stabilize m^{6}A targets, MYC and BCL2. Upon YBX1 loss, *MYC* and *BCL2* undergo accelerated decay, thus compromising AML cells [35]. Weng et al. reported very recently that IGF2BP2 is also overexpressed in AML and its increased expression is associated with poor prognosis in AML patients; through enhancing expression of critical targets (e.g., *MYC*, *GPT2*, and *SLC1A5*) in the glutamine metabolism pathways as an m^{6}A reader, IGF2BP2 promotes AML development and self-renewal of LSCs [123]. Recently, we also reported the role of IGF2BP2 in maintaining the function of human and murine LSCs. IGF2BP2 stabilizes *PRMT6* mRNA via m^{6}A-mediated manner, then PRMT6 suppresses lipid transporter MFSD2A expression by catalyzing H3R2me2a modification and regulates AML stem cells by modulating DHA level [124]. Taken together, these studies uncover the similar functional roles of different m^{6}A modifiers (writers, erasers, and readers) in leukemia.

These studies above provide a rationale for targeting some m^{6}A modifiers as a potential therapeutic strategy in clinic applications. A small molecule inhibitor

STM2457 of METTL3 was identified by a high-throughput screening. STM2457 is able to bind to the SAM binding pocket of METTL3, which blocks SAM binding and shows significant anti-leukemic effects in preclinical AML models [35, 125]. Dr. Chen and colleagues also developed a set of FTO inhibitors. FB23 and FB23-2 were found to directly bind to FTO, and selectively inhibit FTO's m^6A demethylase activity [126]. Another two molecules, CS1 and CS2, have been screened out, and can suppress m^6A demethylase activity of FTO by occupying the catalytic pocket [127]. CWI1-2, a selective IGF2BP2 inhibitor, was developed very recently, which also showed a promising therapeutic efficacy in treating AML in preclinical animal model studies [123]. Overall, these findings provide great promising potential for clinical application of these inhibitors.

6.4.2 Other RNA Modifications in Hematologic Malignancies

Compared with m^6A, the levels of other RNA modifications are relatively lower, and currently, their biological function in normal and malignant hematopoiesis remains elusive. A recent study focusing on myelodysplastic syndrome (MDS) and AML found that RNA m^5C methyltransferases interact with different partners to form distinct complexes and active chromatin structures at nascent RNA and associate with drug sensitivity of leukemia cells [128]. NSUN3 and DNMT2 directly bind hnRNPK that interacts with CDK9/P-TEFb and the lineage-determining transcription factors including GATA1 and SPI1/PU.1 to recruit RNA-polymerase II at nascent RNA, which results in the formation of 5-Azacitidine (AZA)-sensitive chromatin structure. However, NSUN1 binds BRD4 and RNA-polymerase II to form an active chromatin structure that is insensitive to 5-AZA, but hypersensitive to the BRD4 inhibitor JQ1 and to the downregulation of NSUN1 by siRNAs [128]. This study reveals a complex role of RNA m^5C in hematologic malignancies.

The role of pseudouridine in cancer is gradually appreciated. Transfer RNA-derived fragments (tRFs) are emerging small noncoding RNAs that are commonly altered in cancer. A recent work found that in patients with MDS, pseudouridylation of mini tRFs containing a 5' terminal oligoguanine (mTOG) selectively inhibits aberrant protein synthesis and promotes engraftment and differentiation of HSPCs [129]. The Ψ writher PUS7-driven Ψ enables mTOG binding to polyadenylate-binding protein cytoplasmic 1 (PABPC1) and destabilization of the translation initiation complex (eIF4F). More interestingly, mTOG dysregulation causes aberrantly increased translation of mRNA containing pyrimidine-enriched sequences at 5'UTR in malignant MDS-HSPCs, and is clinically associated with leukemic transformation and reduced patient survival [129].

ALKBH3 is the RNA m1A demethylase and plays an important role in DNA repair and cancer cell proliferation. In a recent study, He and colleagues found that deletion of Alkbh3 does not impair the reconstitution capacity of HSCs in both primary and secondary transplantation. Although increased expression of ALKBH3 was observed in aged HSPCs, and skews HSC differentiation without affecting the reconstitution capacity of aged HSCs [130]. Interestingly,

epigenetic loss of ALKBH3 in Hodgkin lymphoma targets collagen, conferring poor clinical outcome [131]. Through investigating cancer-specific DNA methylation changes at the promoter regions of m^6A modifiers, m^1A writers TRMT6, TRMT61A, TRMT10C, TRMT61B, and eraser ALKBH3, a recent study found that the ALKBH3 promoter CpG island is commonly methylated in Hodgkin lymphoma cell lines among different cancer cell line types, and is associated with its mRNA downregulation. ALKBH3 hypermethylation is associated with shorter overall survival of Hodgkin lymphoma patients [131].

6.5 Conclusions

The field of RNA modification continues to exponentially grow, and it also becomes clear that RNA modifications play critical roles in the pathogenesis of hematologic malignancies. It is definitely necessary to further explore and clarify the distinct roles of different modifications under different contexts. Interestingly, many scientific questions appear. For example, how does RNA modification sense and change in response to various environments? It also remains unknown how the transcripts are selected by different modifiers. Our recent work implies that the related cofactors or RBPs in the protein machineries are key in determining their target specificity. Overall, it is very promising that targeting RNA modification may be an attractive therapeutic approach for hematologic malignancies.

In addition, developing better methods to comprehensively and quantitatively map the landscape of different RNA modifications, especially in a single cell level, will be necessary in the future. All of the current mapping strategies have their advantages and limitations. In addition, it is necessary to establish standard criteria for bioinformatic analysis in this field. Some controversial findings might be attributed, at least some certain, to differences in the computational strategies used by different studies. To improve the reliability in characterizing RNA modification, in vitro, transcribed RNA product is introduced as a negative control to reduce the false positive resulting from sequencing bias or RNA structure [132]. Taken together, we believe that, in the future, the biological roles of these different RNA modifications will be characterized and understood more clearly.

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Part II
Histone Epigenetics

The SWI/SNF Complex: A Frequently Mutated Chromatin Remodeling Complex in Cancer

7

Vinh The Nguyen, Mathewos Tessema, and Bernard Ellis Weissman

7.1 Introduction

7.1.1 The SWI/SNF Complex and Chromatin Remodeling

Within eukaryotic cells, DNA does not exist as a free-floating macromolecule. Instead, it exists within tightly packed structures called nucleosomes, where ~147 bp of DNA is wrapped around a core of histone proteins. Without this structure, the large amount of DNA in a cell would stretch it by a significant amount. To be economical and better protect the genome, cells pack their DNA within a small nuclear space by packing it around nucleosomes. Because of this arrangement, nucleosomes control global gene expression as their presence generally prevents the binding of transcription factors, the proteins responsible for activating or inactivating the expression of specific genes. Consequently, elaborate cellular machinery works in concert with transcription factors to mobilize nucleosomes to control gene expression, a process termed chromatin remodeling. The mammalian SWI/SNF family of chromatin-remodeling complexes is made

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from proteins encoded by 29 different genes and assembled into 11–15 subunit complexes that serve as key regulators of nucleosome positioning. SWI/SNF complexes use an ATPase domain of either SMARCA4 (BRG1) or SMARCA2 (BRM) to generate energy through the hydrolysis of ATP to slide or eject nucleosomes. Since different SWI/SNF complexes are made from distinct combinations of protein subunits, they are complicated molecular assemblies with diverse structures and functions.

7.1.2 The Three Types of SWI/SNF Complexes

Mammalian SWI/SNF complexes are classified into three broad types or subfamilies, based upon the distinct subunits forming the complex: the canonical BRG1/BRM-associated factor (BAF, also called cBAF); the polybromo-associated BAF (PBAF); and the BICRA or BICRAL-containing and BRD9-containing (GBAF) complex also known as non-canonical BAF (ncBAF) (Fig. 7.1) [1]. All three complexes contain the core subunits, including SMARCC1, SMARCC2, SMARCD1, and either of the ATPases SMARCA4 (BRG1) or SMARCA2 (BRM). Critically, they also contain numerous variable subunits that provide each of the complexes with a distinct identity [2, 3]. Heterogeneity occurs within each subfamily owing to the differential use of related subunits, which are often encoded by multi-gene families, such that hundreds or even thousands of subtly different SWI/SNF complexes might exist. Although the recognition of the increasing complexity of composition has complicated research in the field, discovering the functions and mechanisms of SWI/SNF complexes and the unique roles they play within broader cancer cell signaling has energized the research community.

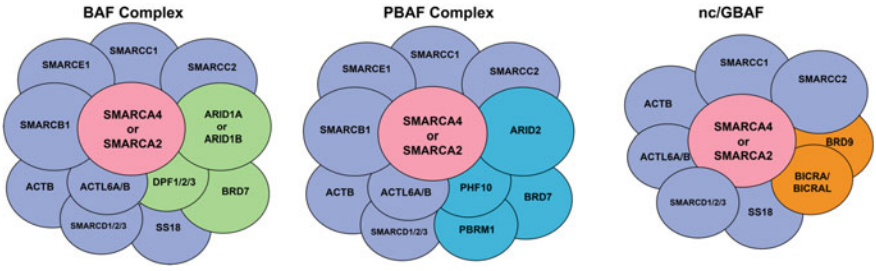

Fig. 7.1 The 3 major subtypes of SWI/SNF chromatin remodeling complexes. Each complex consists of ATPase subunit (pink), commonly shared subunits (violet) and subunits that define each subtype- BAF (green), PBAF (blue) and nc/GBAF (orange)

7.1.3 Critical Knowledge Gaps Exist in the Translational Understanding of Mutant-SWI/SNF Complexes in Human Cancer

Cancer genome sequencing studies have discovered mutations in members of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodeling complex in 20% of human cancers, including a wide range of pediatric cancers (Fig. 7.2) [4]. Although defined by three major subtypes (see Sect. 7.1.2), at least nine distinct SWI/SNF complexes have been reported, each imparting specific effects on cell growth and differentiation [5]. Many SWI/SNF subunits are tumor suppressors for which loss of function drives oncogenic phenotypes in diverse cancers [4]. Yet how the loss of one or more subunits impacts the composition/function of the nine SWI/SNF complexes and subsequently fuels tumor development remains unresolved. For example, a seminal study in 1998 demonstrated mutations in SMARCB1 (also known as SNF5/INI1/BAF47), a core subunit of the SWI/SNF complex, in 95% of pediatric rhabdoid tumors (RT), poorly differentiated and clinically aggressive tumors that arise in the kidney and the central nervous system [5, 6]. SMARCB1 mutations also drive development of two other aggressive tumors of children and young adults- epithelioid sarcomas and renal medullary carcinomas [4]. Beyond the common *SMARCB1* mutation, these tumors appear genetically simple and uniform, bearing no other recurrent driver mutations with primarily diploid genomes. In addition, the cells of origin for these tumors remain unknown. Thus, while RT provides a powerful, simple model for understanding SWI/SNF-driven tumorigenesis, the absence of an established cell of origin and the lack of complex genotypes limits our understanding of critical stages in SWI/SNF-driven tumorigenesis and selective therapeutic vulnerabilities.

7.2 The Components of the SWI/SNF Complex

7.2.1 Overview

Several key features distinguish the 3 distinct types of SWI/SNF chromatin remodeling complexes. Each complex has unique subunits that define it such as ARID1A/B for BAF, ARID2/PBRM1 for PBAF, and BIRCA/BRD9 for GBAF [1, 3]. A significant amount of research in the field has focused on the role of the catalytic subunits in the normal functions of the complex. BRG1, encoded by the *SMARCA4* gene, is a large protein (1647 amino acids) composed of multiple domains. Primary sequence analyses have identified multiple domains including an evolutionarily conserved catalytic ATPase domain, as well as a conserved C-terminal bromodomain and AT-hook motif [7, 8]. BRM, encoded by its ortholog *SMARCA2* gene, is a similarly large protein (1590 amino acids), that shares a large amount of similarity in protein sequence to BRG1 along with the same key domains [7, 8].

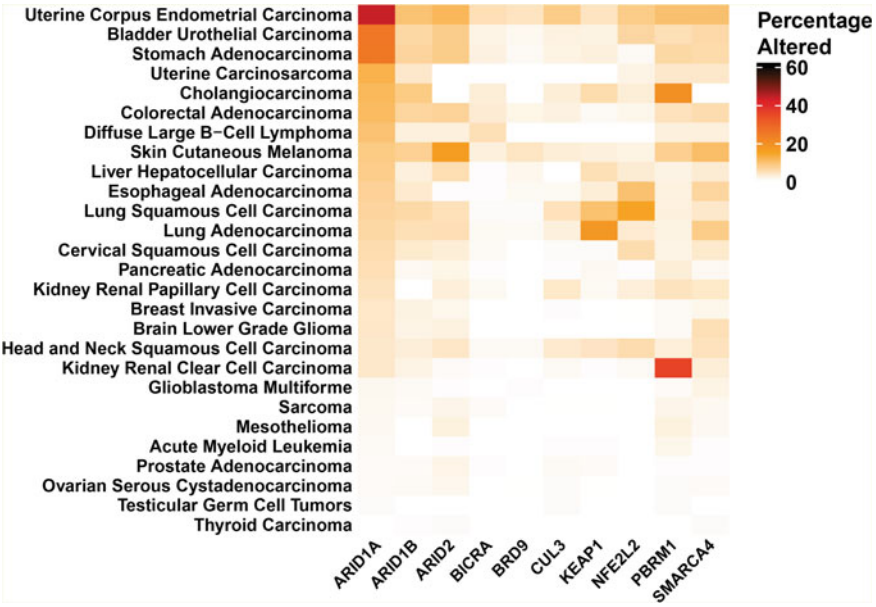

Fig. 7.2 SWI/SNF subunit mutation frequencies in cancer. Data from the TCGA Pan cancer project

7.2.2 ATPase Subunits

The bromodomains of the BRG1/BRM proteins are likely involved in the recognition of acetylated lysines within histone H3 and H4 tails [9, 10]. Modifications within target promoters may serve as an interaction surface for the assembly and/or recruitment of bromodomain complexes including SWI/SNF [10]. Because the BRG1/BRM proteins are found within the context of many multi-protein complexes, multiple regions of these proteins are dedicated to their interaction with other SWI/SNF subunits and nucleosomes [11, 12]. BRG1/BRM interact with a diverse group of nuclear proteins involved in a wide range of processes such as nuclear receptors, members of the transcriptional machinery, chromatin-modifying enzymes, tumor suppressors, and proteins critical for genomic stability and maintenance [3].

Because they serve as the core subunits of the SWI/SNF complexes, BRG1/BRM have large roles to play in the global transcriptional regulation of the cell and thus many cellular functions processes (Fig. 7.3) [13]. BRG1/BRM contains many DNA binding motifs which assist in their function as a chromatin remodeler. Multiple interactions likely mediate recruitment and stabilization of the SWI/SNF remodeling complex to gene-specific promoters through direct or indirect interactions involving one or more BAF proteins or transcription factors [14–18]. Many studies have suggested there is preferential recruitment of BRG1/BRM and BAF subunits to certain DNA motifs associated with distinct functions [11, 13,

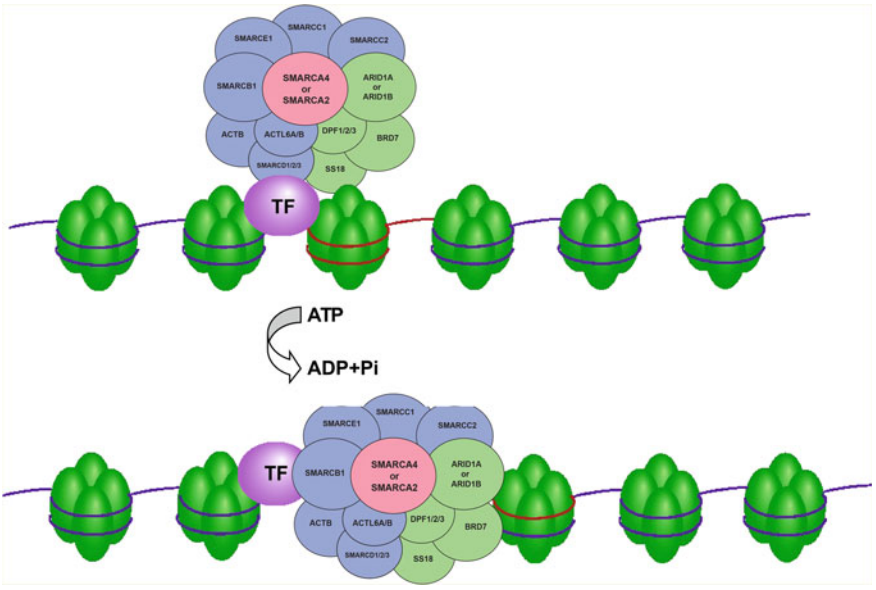

Fig. 7.3 Chromatin remodeling by the SWI/SNF complex. The complex uses the energy generated by ATP hydrolysis to physically slide nucleosomes apart on chromatin to open space for RNA transcription machinery

19]. Recruitment of BRG1/BRM has been shown to control numerous cellular processes such as differentiation, proliferation, signaling, and apoptosis [1, 3, 5, 20].

7.2.2.1 SMARCA4

BRG1, the main ATPase subunit of all 3 types of SWI/SNF complexes, is one of the most commonly mutated chromatin remodeling ATPases in cancer [2, 21]. Generally, BRG1 loss is not due to hypermethylation or gene silencing but by mutations or deletions in malignancies such as non-small cell lung cancers, stomach adenocarcinomas, and bladder cancers. Paradoxically, multiple reports have found BRG1 overexpressed in breast and colon cancers [8, 10, 22, 23]. Tumor subtypes with the highest percentage of BRG1 loss include small cell ovarian cancer, hypercalcemic type (SCCOHT) and SMARCA4 deficient thoracic sarcomas (SMARCA4-DTS) where greater than 95% of the tumors have loss of BRG1 protein expression [24–27]. An important feature of SCCOHTs is the loss of SMARCA4 in the absence of mutations in other classic driver genes such as KRAS and TP53 [28, 29]. Thus, SCCOHT serves as an important model for identifying mechanisms driving tumorigenesis following loss of SMARCA4 and its associated chromatin remodeling activity. One study by Orlando et al. used a multi-omics approach in an SCCOHT cell model to find that SMARCA4 re-expression induced a gene and protein signature similar to a reversal of an epithelial-to-mesenchymal

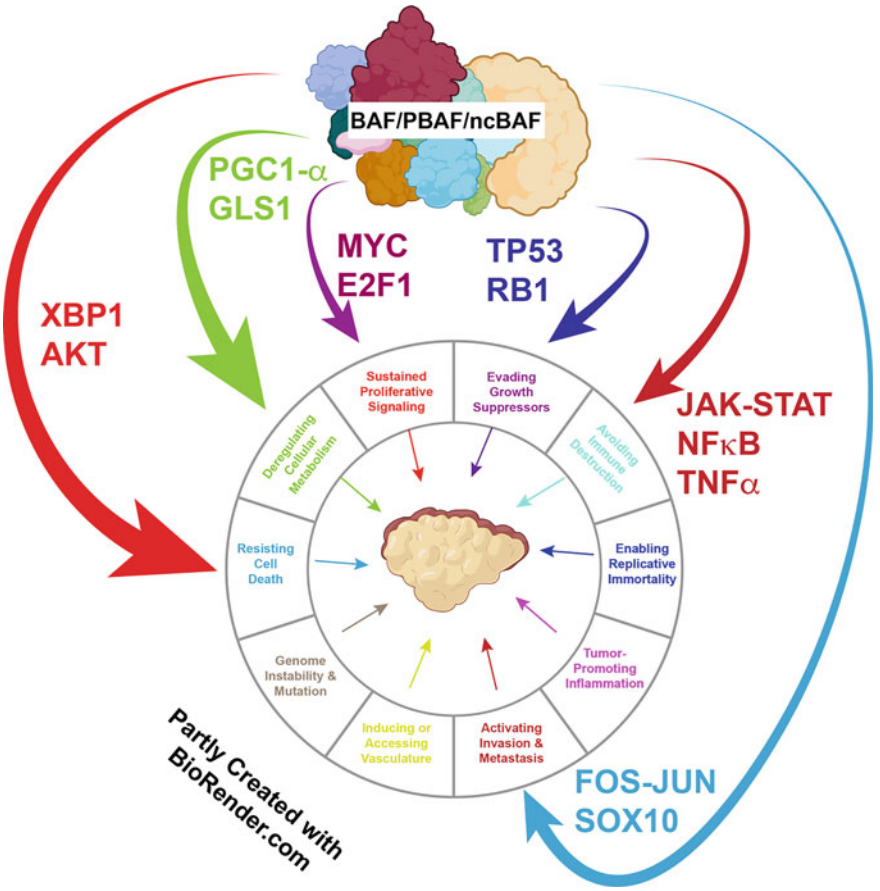

Fig. 7.4 Hallmarks of cancer impacted by mutations in subunits of the SWI/SNF complex. Studies have shown that at least 6 of the 10 hallmarks of cancer are activated by mutations in SWI/SNF subunits

transition (EMT) [14]. Of interest, chromatin sites that gained chromatin accessibility after SMARCA4 re-expression correlated with open chromatin sites in other epithelial-originating TCGA tumors [14]. They also reported that the expression of epithelial targets induced by SMARCA4 depended upon the activity of the AP-1 transcription factor. A recent study reported similar results and expanded the effects of SMARCA4 loss to alterations in the 3-dimensional chromatin architecture [16]. Together, these findings support a model where SMARCA4 loss alters transcription factor binding and the associated 3-D enhancer landscapes to promote disease development in cancer and other diseases. Whether this paradigm will prove a common one for tumorigenesis driven by mutations or loss in all SWI/SNF components remains an important question.

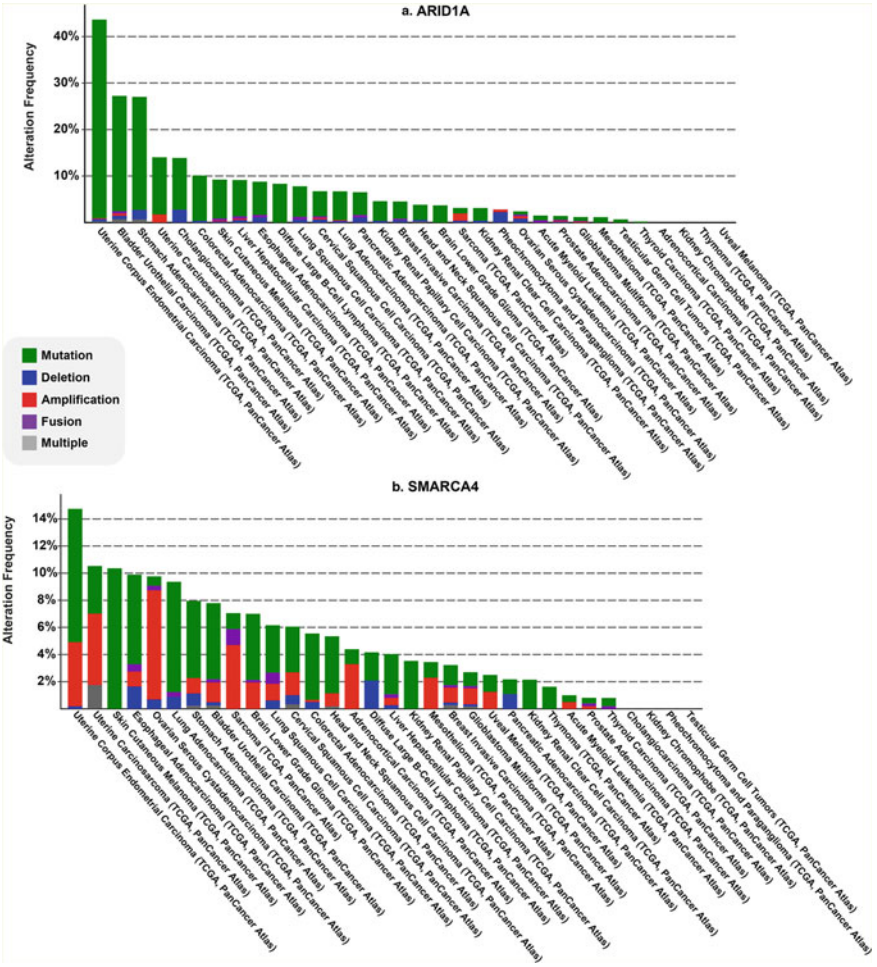

Fig. 7.5 The SWI/SNF subunit, ARID1A, is frequently mutated in human cancers. Mutation frequencies are derived from the TCGA Pan-cancer project [69] and visualized using cBioPortal [88, 90]

7.2.2.2 SMARCA2

BRM, along with BRG1, serves as one of the two mutually exclusive ATPase subunits for two (BAF and ncBAF) of the three SWI/SNF complex subfamilies [30]. While SMARCA4 usually behaves like a tumor suppressor, the role of SMARCA2 loss in tumor development remains less clear. A range of studies implicate SMARCA2 as a tumor suppressor; other reports suggest that SMARCA2 function may differ depending on the cancer type and the disease stage [31]. The existence of alternative splicing forms of the *SMARCA2* gene, leading to the appearance of truncated functional, loss of function, or gain-of-function forms of

the BRM protein suggests a far more complicated mode of action [31, 32]. A limited number of reports have described *SMARCA2* mutations or deletions in cancers, including adenoid cystic carcinoma [33–35], non-melanoma skin cancer [36, 37], hepatocellular carcinoma [38] and head and neck squamous cell carcinoma [39]. In addition, 3 case studies have shown *SMARCA2* fused to other classic fusion oncogenes such as *CREM* and *NR4A3*, with one report showing the fusion associated with poorer patient outcomes [40–42]. However, loss of SMARCA2 expression most frequently occurs via epigenetic silencing [31, 43], through multiple mechanisms such as promoter methylation [44], promoter polymorphisms [45–47] or HDAC/EZH2-driven mechanisms [48–51]. Of import, the development of clinically useful epigenetic inhibitors has led to reports of potential targeted therapies for cancer by reversing the silencing of *SMARCA2* [18, 40, 50, 52, 53].

Many tumors with frequent loss of SMARCA4 expression due to mutation or deletion also display epigenetic silencing of SMARCA2 such as SCCOHT [49, 54], SMARCA4-DTS [25], some cases of dedifferentiated [55] and undifferentiated [56] endometrium carcinomas, undifferentiated renal cell carcinoma [57] and non-small cell lung cancer [58]. In contrast, some SMARCA4-deficient cancers and cell lines retain SMARCA2 expression. Multiple reports screening for synthetic lethality in these cell lines identified SMARCA2, leading to a paradigm that SMARCA2 serves as a potential therapeutic vulnerability for the cancers [59, 60]. Several groups have now reported successful growth inhibition of SMARCA4-deficient cell lines in culture and *in vivo* models with drugs or PROTACs directed against SMARCA2 [61–64].

7.2.3 Subtype-Defining SWI/SNF Components

7.2.3.1 ARID1A

The most frequently mutated SWI/SNF subunit is ARID1A [1, 65]. Both ARID1A and its paralog ARID1B are subunits with relatively unknown functions/mechanisms and are being actively researched. ARID1A and ARID1B both contain an ARID DNA-binding domain. There is a BAF domain with a yet undescribed function that has been speculated to have ubiquitin ligase activity [66]. The earliest reports of ARID1A's tumor-suppressor role were the discovery that ~50% of ovarian clear cell carcinomas and endometriosis-associated ovarian carcinomas contain inactivating *ARID1A* mutations [67, 68]. Mutations of *ARID1A* have since been observed at high frequency in several studies, including uterine endometrial carcinoma (34%), colorectal cancers (10%), as well as cancers of the bladder (29%), stomach (34%) (14), cholangiocarcinomas (27%), neuroblastomas (11%), and pancreas (~5%) [69]. Many of these mutations are loss of function suggesting ARID1A is a tumor suppressor, but one study found that ARID1A-deficient cancer cells are vulnerable to inhibition of the antioxidant glutathione (GSH) and the glutamate-cysteine ligase synthetase catalytic subunit (GCLC) in an ovarian cancer model. Inhibition of GCLC decreased GSH in ARID1A-deficient cancer cells, leading to apoptosis [70]. In breast cancer, one group found deletion of ARID1A

resulted in loss of Histone Deacetylase 1 (HDAC1) binding, and resulting BRD4-driven transcription and growth which they suggested contributed to resistance to Estrogen Receptor-alpha (ER) antagonists [71]. In liver cancer, it was found that deletion of ARID1A conferred resistance to tumor initiation, but accelerated tumor progression in established tumors [72].

Mutations of *ARID1A* are most frequently truncating mutations (frameshifts and nonsense mutations) and no missense mutations expected to be of high functional impact have currently been reported in the ARID domain. The few missense mutations present are generally predicted to have a low or medium functional impact and are distributed uniformly over the gene [73, 74]. However, hypermethylation of the ARID1A promoter has been observed in many breast cancers, suggesting epigenetic silencing mechanisms are also common [75]. In ovarian cancer, mutation of *ARID1A* frequently co-occurs with activating mutations of *PI3K*.

7.2.3.2 ARID1B

ARID1B is mutated in several cancers, again mostly truncating mutations like ARID1A. However, *ARID1B* mutations are not as frequent as those of *ARID1A*. This discrepancy between mutation rates may reflect important functional differences between these two genes and/or ARID1B expression in fewer cell types. In general, the roles of ARID1A and ARID1B in tumorigenesis remain unclear. ARID1B and ARID2, a PBAF-exclusive ARID subunit (see below), participate in widespread cooperation to repress hundreds of genes. Additionally, there is competition between ARID1A and the other ARID family members; gene expression changes following the loss of one ARID component depend on the function of an alternative ARID component. Overall, these reports suggest that ARID1A has distinct regulatory roles in comparison to ARID1B and ARID2 [76].

7.2.3.3 PBRM1

PBRM1 is an exclusive (signature) subunit of the polybromo-associated BAF (PBAF) subfamily of SWI/SNF complexes. Its name comes from the six bromodomains within the protein, a defining feature of the PBAF complex [74]. Bromodomains are protein domains that recognize acetylated lysines such as those found on the N-terminal tales of histones. In renal clear cell carcinoma (RCC), mutation or loss of PBRM1 occurs in ~41% of cases. Furthermore, hypermethylation of the PBRM1 promoter is generally absent in RCC, indicating that inactivation occurs primarily through mutation or deletion. Some tumors do contain missense mutations, although their functional impacts remain uncertain [77].

7.2.3.4 ARID2

ARID2 encodes another PBAF signature subunit. ARID2 is not a homolog of ARID1A/B, but is instead mutually exclusive with ARID1A/B, although they both share an ARID domain that is used for binding to DNA. ARID2 is frequently mutated in other malignancies but is not targeted in renal clear cell carcinoma

as frequently as PBRM1, suggesting that PBRM1 has an important and distinct functional role as a member of the PBAF complex in kidney cells. ARID2 has been reported to contribute to gene repression and is frequently mutated in melanoma, non-small-cell lung cancer, as well as in ~18% of hepatitis-associated hepatocellular carcinomas [78, 79].

7.2.3.5 SMARCB1

The one subunit in which an SWI/SNF subunit clearly serves as a tumor suppressor is SMARCB1 where its biallelic inactivation occurs in nearly all cases of malignant rhabdoid tumors (MRT), a rare but highly lethal childhood cancer [1, 3, 80]. The classic loss of heterozygosity observed for SMARCB1 leads to aberrant activation of many signaling pathways such as Wnt/β-catenin, and importantly, impairs the ability of BAF/PBAF complexes to regulate the placement and function of Polycomb repressive complexes [5, 13, 19]. As a result, the repressive mark H3K27me3 accumulates at the tumor suppressor p16/INK4A (CDKN2A) locus [81, 82]. These tumors have among the lowest mutational loads of any human tumor suggesting that SMARCB1 loss is the primary oncogenic event in this cancer [83]. Re-expression of SMARCB1 reverses Polycomb silencing at the tumor suppressor $p16^{INK4A}$ locus, leading to cellular senescence [13, 19]. In addition to MRTs, SMARCB1 loss appears to play a role in several cancers including prostate cancer, epithelioid sarcomas, familial schwannomatosis, poorly differentiated chordomas, and renal medullary carcinomas [80]. Of interest, our recent study implicated a different contribution to tumorigenesis of SMARCB1 loss in cancers arising from other initiation events [84]. Because chordomas lose $p16^{INK4A}$ expression prior to SMARCB1 inactivation, re-expression of SMARCB1 in poorly differentiated chordomas does not cause growth arrest. Rather, the cells re-express differentiation programs characteristic of SMARCB1-positive chordomas [84]. Thus, as discussed below, the cellular context in which loss of SWI/SNF component often determines the phenotypic effects.

7.2.3.6 BRD9 and BICRA/BICRAL

The ncBAF or GBAF complex is defined by the presence of the BRD9 subunit, as opposed to BRD7 in the other SWI/SNF complexes, and BIRCA or BIRCAL, previously known as GLTSCR1/1L [85–87]. Unlike the defining components of the BAF and PBAF complexes, overexpression of BRD9, BICRA, and BICRAL occurs in many human cancers, e.g., bladder, head, and neck and lung squamous, with only a limited number, such as melanoma and glioblastoma, showing loss of expression through deletion or truncating mutations [88, 89]. A second significant feature of the ncBAF complex is the absence of the SMARCB1 subunit [85–87]. Thus, ncBAF provides the only SWI/SNF remodeling activity in SMARCB1-deficient tumors, such as MRT and familial schwannomas. This dependence has led to the emergence of treatments targeting BRD9 in SMARCB1-deficient cancers along with those dependent upon BRD9 overexpression [1, 89]. Although many cancers in the Pan-cancer studies also show BICRA overexpression/loss (1.5%) or

BICRAL amplification (3%), their roles in tumorigenesis remain uncharacterized [69].

7.2.4 Other SWI/SNF Subunits

Surprisingly, next-generation sequencing studies, such as the TCGA and the MSK-IMPACT, have found limited mutations in other SWI/SNF subunits [69, 90, 91]. The best-studied example of another SWI/SNF mutation driving a rare cancer comes from synovial sarcoma, a rare, aggressive cancer found in the extremities of children and young adults [92]. However, one of the most intriguing mutations involving SWI/SNF subunits is the fusion oncogenes possessing the SS18 subunit found in synovial sarcomas [93]. Multiple studies have established that an in-frame fusion of the SS18 to the C-terminal end of SSX1 or SSX2 is the pathogenomic driver of this cancer [94, 95]. The mechanism by which this fusion protein caused synovial sarcoma remained ill-defined until a remarkable study by Kadoch and Crabtree in 2018 [93]. They showed that the SS18–SSX protein competes with the normal SS18 protein present in SWI/SNF complexes resulting in the loss of SMARCB1 from the BAF and PBAF complexes [93]. This finding implicated the loss of SMARCB1 as the underlying mechanism driving synovial sarcoma development, similar to the tumorigenesis of MRT. However, in 2018, McBride et al. reported that SS18-SSX-containing SWI/SNF complexes acquired oncogenic properties by changing the location of BAF and PBAF complexes from enhancers to polycomb domains [96]. The resulting loss of PRC2-mediated repression led to the activation of gene expression programs driving cellular transformation [96]. Subsequently, another report from this group demonstrated that a second oncogenic fusion protein, EWS-FLI1, can also alter the function of SWI/SNF complexes to drive the development of Ewing's Sarcoma, another pediatric cancer [97]. Reports have found a significant level of mutations of several other SWI/SNF complex components in human tumors such as, *SMARCE1* in clear cell meningiomas [98], *BCL7B/C* in gastric and renal cancers [99, 100] and *SMARCC2* in gastric cancers and gliomas [101–103]. As the number and depth of human tumor sequencing studies increase, we anticipate that mutations in other SWI/SNF subunits will appear, especially in rare cancers with limited numbers of samples. In addition, functional and biochemical analyses may identify other oncogenic fusion genes in human tumors that drive tumorigenesis through interactions with the SWI/SNF complex.

To summarize, most of the research on the SWI/SNF complex has been focused on the discovery of frequently mutated subunits in specific cancers. Some are strongly associated with mutations in SWI/SNF components like MRTs with *SMARCB1* mutations and loss and SCCOHTs with *SMARCA4* mutations and loss where their inactivation is the only mutation driving carcinogenesis. Other cancers, such as, NSCLC have multiple mutations in SWI/SNF subunits, predominantly *SMARCA4, ARID2*, and *ARID1A/B* [104]. Unfortunately, due to the mutational

complexity of many tumors, mechanistic studies have been difficult to tease out with some exceptions such as MRT and SCCOHT.

7.3 Mechanisms by Which Altered SWI/SNF Complex Activities Drive Cancer Development

Despite the increasing number of studies characterizing the impact of mutations in SWI/SNF subunits on tumor initiation, progression, and metastasis, studies have not fully identified and dissected the mechanisms by which aberrant SWI/SNF complex activities drive these oncogenic changes. The absence of a single subunit from the complex clearly alters nucleosome positioning by disrupting normal patterns of nucleosome placement [105–107]. However, how these changes in nucleosome localization lead to the initiation and progression of human cancer remains unclear. The current literature has identified multiple mechanisms driving the development of SWI/SNF-mutant tumors such as alterations in enhancer and super-enhancer sites, changes in the interactions between the complex and histones, aberrant activities of key oncogenic pathways such as growth regulation by the RB and TP53 tumor suppressors or the E2F1 and CMYC oncogenes or interactions with non-coding RNAs [1–3]. Chromatin remodeling plays a central role in regulating many cellular functions such as gene transcription, DNA damage repair, energy production, and metabolism. Thus, aberrations of normal SWI/SNF activities in cancer cells can impact multiple Hallmarks of Cancer, as shown in Fig. 7.4. Many of these hallmarks are affected by altered activities of transcription factors and their associated signaling pathways (Fig. 7.4) [14, 15, 65, 73, 102, 108–122]. We explore several of the better-characterized mechanisms below.

7.3.1 Altered SWI/SNF Complex Activity Leads to Alterations in the Histone Modification Landscape

Disruption to SWI/SNF complex functions by mutations in subunits is associated with the loss of distal and proximal gene enhancers and genome-wide alterations to H3K27me3 occupancy. H3K36 and H3K27 methylation states are antagonistic; in embryonic stem cells, H3K36me2 antagonizes PRC2-mediated H3K27me2 and H3K27me3 domains genome-wide [123]. Similarly, in prenatal, male germ cells, loss of H3K36me2 is accompanied by increased H3K27me3 and transcriptional downregulation suggesting a dynamic relationship that can play a role in cellular differentiation [124]. A gain-of-function H3K36M mutation has been described in chondroblastomas, head and neck cancers, and Sotos Syndrome where it associates with altered H3K27me3 distribution [125–128]. Aberrant H3K36me2/3 deposition has also been linked to the loss of H3K27me3 at *HOX* genes in AML [129]. Recognition of H3K36me by the PRC2 subunit PHF19 is required for efficient enzymatic activity of the PRC2 complex [130]. However, a recent report also showed a repression of EZH2 when it senses H3K36me3 [131]. Despite

well-established mechanistic linkages between H3K36 methylation and H3K27 methylation and well-known alterations to H3K27me3 occupancy associated with SWI/SNF loss, the extent of H3K36 methylation involvement in establishing a pathogenic epigenetic state in response to SWI/SNF loss remains unknown.

Two recent reports showed a novel and exciting link between SWI/SNF complex activity and H3K27 mutations that drive the development of diffuse midline gliomas [117, 132]. About 80% of these cancers possess an H3K27M mutation in one allele in the histone H3 variant, *H3.3* or the canonical *H3.1* gene [133–135]. These studies demonstrate that epigenetic change induced by the expression of H3.3K27M in DMG relies on the activity of SMARCA4-containing SWI/SNF complexes. Specifically, recruitment of the SOX10 transcription factor induces the expression of genes driving tumorigenesis including cellular proliferation and remodeling of the extra cellular matrix. Of particular interest, the dependence upon SWI/SNF chromatin remodeling activity did not extend to SMARCA2-containing complexes, another example of functional differences between the 2 ATPase paralogs. Although both reports found a dependence upon PBAF complexes for H3.3K27M-driven tumorigenesis along with a lack of reliance on ncBAF complexes, they differed upon a requirement for BAF complexes [117, 132]. These studies expand the gamut of potential interactions between mutations in SWI/SNF complex subunits and those involving histone modifications in the development of human cancer.

7.3.2 SWI/SNF Complex Loss Alters Key Transcription Factor Occupancy

While the chromatin remodeling activity of SWI/SNF predicted these findings, the non-random nature of the nucleosome positioning changes was unexpected. Recent studies from several laboratories have established that aberrant SWI/SNF complexes preferentially affect enhancers and/or super-enhancers [13, 19, 136, 137]. Some of these studies have further implicated these changes as drivers of altered differentiation in SWI/SNF mutant cancers [13, 107, 136, 138]. The mechanisms that determine SWI/SNF interactions with enhancers/super-enhancers and whether mutations in different subunits affect the same and/or different sites remain unknown.

With the alterations in histone positioning stemming from the loss of SWI/SNF, DNA accessibility to transcription factor (TF) binding sites changes. Furthermore, SWI/SNF has been implicated in the function of TFs, including the prominently-described interaction between AP-1 and SWI/SNF. AP-1 (activator protein-1), a dimeric TF involved in many cellular processes, does not display cell type specificity [139–141]. AP-1 exists as either a homodimer or heterodimer TF made up of a variety of family members: FOS (4 genes), JUN (3 genes), ATF (11 genes), and MAF (6 genes) [139, 141]. All AP-1 family members share a characteristic DNA-binding basic leucine-zipper domain (bZIP) that directs them to their common DNA sequence motif [139, 141]. The first study connecting AP-1 and SWI/

SNF described SMARCA4's role in transcriptional repression of the *c-FOS* gene [142]. Subsequent studies have shown SWI/SNF subunits, such as ARID1A/B [76, 143, 144], SMARCB1 [13], and SMARCA4 [138, 145, 146], and AP-1 function at common enhancer sites. A recent report also showed that the re-expression of SMARCB1 in MRT cell lines also affected AP-1 localization and activity accompanied by changes in the 3-D structure of enhancers [16]. Despite many noted interactions between AP-1 and SMARCA4, their cooperative biology in cancer cells remains unclear.

TEAD, a highly conserved TF family with a pivotal role in development [147], is also linked to SWI/SNF. In mammals, four TEAD members (TEAD1-4) share a conserved binding motif, show distinct expression patterns in both tissue and developmental stage, and may regulate numerous developmental processes including cardiogenesis, myogenesis, and neural crest, notochord, and trophectoderm differentiation [148–152]. Numerous cancer types over-express TEAD and, in partnership with YAP/TAZ, may induce the expression of proliferation-promoting and anti-apoptotic genes [147]. In primary human mammary epithelial cells, TAZ/TEAD complexes interact with SMARCA2 to drive basal cell differentiation [153]. In squamous cell carcinoma, SWI/SNF directly recruits YAP/TEAD to chromatin to drive tumor initiation and maintenance [154]. In contrast, SWI/SNF inhibits the interaction between TAZ and TEAD in MCF10A and HEK293T cell lines grown under low mechanical stress conditions [145]. Therefore, depending on the context, SWI/SNF functions as either a positive or negative regulator of the YAP/TAZ-TEAD pathway, requiring further investigation. Of note, cooperative binding of AP-1 and TEAD to enhancers modulates differentiation of mouse embryonic stem cells and oncogenesis [155, 156]. Whether they function together in concert with SWI/SNF subunit mutations to drive SCCOHT and RT oncogenesis remains to be addressed.

7.4 Targeting SWI/SNF Complex Abnormalities for Clinical Use

With the prevalence of mutations in SWI/SNF subunits in multiple human cancers, the efforts to identify targeted therapies for SWI/SNF-mutant cancers have exploded. Drugs inhibiting SWI/SNF dependencies such as inhibitors of the EZH1 and/or EZH2 histone methyltransferases have moved into the clinic for some SWI/SNF mutant tumors, a synthetic lethality in a subset of cancers with loss of expression of specific SWI/SNF subunits [10, 157, 158]. Other studies have exploited putative synthetic lethality with these tumors such as tyrosine kinase inhibitors and immune checkpoint inhibitors [120, 159]. In addition, drugs targeting SMARCA2 and/or SMARCA4 in cancers where they constitute a dependence such as in DMG (see Sect. 7.3.1) have also been developed [87]. We cover some of the major efforts to generate effective therapies for SWI/SNF-mutant cancers as well as the value of SWI/SNF subunits as prognostic and therapeutic markers below.

7.4.1 SWI/SNF Abnormalities as Diagnostic and Prognostic Biomarkers

Mutations in SWI/SNF complex subunits occur in over 20% of human cancers making the complex the second most frequent target of genetic alteration next to TP53. Thus, it has become an attractive target for improving the clinical management of cancer [160]. In addition, SWI/SNF-mutant tumors are often poorly differentiated, aggressive, and have very limited options for effective treatment [161, 162]. Therefore, the development of strategies targeting these molecular abnormalities to improve the diagnosis, prognosis, treatment, and/or prevention strategies is urgently needed and could benefit many cancer patients. The following sections discuss the emerging and promising ways we can exploit SWI/SNF abnormalities for clinical use.

7.4.1.1 SWI/SNF Germline Mutations

Germline mutations of the genes encoding for SWI/SNF subunits have been reported in patients with pediatric MRT, SCCOHT, and clear cell renal carcinoma, while somatic mutations occur in various malignancies [6, 24, 26, 67, 78, 79, 98, 163]. Because some of these germline mutations may lead to hereditary predispositions, clearly defining the underlying genetic basis and optimizing screening approaches have become important for the development of genetic counseling strategies for the early identification of high-risk individuals. For example, a subset of *SMARCB1* and *SMARCA4* inactivation mutations found in nearly all MRT and SCCOHT cases, respectively, are germline and constitute promising candidate biomarkers to screen for hereditary predisposition [98, 164, 165]. *SMARCB1* mutations have also been identified in other hereditary syndromes predisposing to the development of familial schwannomatosis or SWI/SNF-related meningiomas [166, 167]. Germline alterations of SMARCB1 occur in up to 35% of patients with rhabdoid tumors and confer a predisposition to MRTs with almost 100% penetrance [98]. In this hereditary setting, designated rhabdoid tumor predisposition syndrome type 1, MRTs present at a younger age, with widespread disease, and more rapid disease progression [168]. Thus, successful use of SMARCB1 mutation-based screening to identify high-risk individuals for MRTs will help to design strategies for closer monitoring, early detection, and early management of the disease to ultimately improve the treatment efficacy and survival of these patients. Similarly, germline *SMARCA4* mutations are also under consideration for the surveillance of high-risk individuals at risk for hereditary SCCOHT [165].

7.4.1.2 SWI/SNF Somatic Mutations

Somatic mutations of genes encoding SWI/SNF complex subunits occur in many cancers types including genital tract carcinomas, intrahepatic cholangiocarcinomas, hepatocellular carcinoma, pancreatic ductal carcinoma, melanoma, and many other tumors described in Sect. 7.2 [67, 78, 79, 169, 170]. Among female genital tract tumors, inactivating mutations of the *SMARCA4*, *SMARCB1*, *ARID1A*, and *ARID1B* genes with loss of expression of the corresponding proteins commonly

appear in endometrioid endometrial cancers, clear cell carcinomas in the uterine corpus and ovary, and other endometriosis-associated ovarian neoplasms such as seromucinous tumors [67, 68]. Overall, deficiencies of these four SWI/SNF subunits occur in up to 70% of endometrial carcinoma cases regardless of differentiation though loss of expression of these proteins are generally restricted to the undifferentiated component. This finding, along with the impact of SWI/SNF inactivation in cellular maturation and epithelial-mesenchymal transition (EMT) processes, suggests that its loss may drive the poor differentiation and the common "rhabdoid" morphology of many other SWI/SNF deficient tumors [14, 55, 105]. Whether they are the cause or consequence of the poor differentiation, the loss of SMARCA4, SMARCB1, or combined ARID1A/ARID1B expression will provide important diagnostic biomarkers as these changes rarely occur in other high-grade uterine malignancies [49]. Reliable immunohistochemistry (IHC) assays are widely used clinically to screen for loss of SMARCB1 (INI-1) or SMARCA4 (BRG1) proteins to support or confirm the often-difficult differential diagnosis of these poorly differentiated tumors [98, 165, 171]. Interestingly, the loss of ARID1A expression found in these tumors can also occur in the non-malignant (hyperplastic) adjacent tissues indicating the mutation as an early event in the development of these tumors [171]. This feature makes loss of ARID1A expression in these cancers an attractive target for preventive and/or therapeutic strategies, a topic discussed in the following section.

7.4.2 Discovery and Targeting of Therapeutic Vulnerabilities Created by Mutant-SWI/SNF Subunits

SWI/SNF mutations often inactivate the targeted gene leading to loss of function and/or complete loss of protein expression. As a result, directly targeting them for therapy requires activation of the specific protein in the affected cell type, a technically challenging approach compared to successful inhibition of activated oncogenes such as EGFR and EML4-ALK [172, 173]. However, the role of SWI/SNF as the major chromatin remodeling complex regulating epigenetic modification and expression of a broad range of genes makes downstream pathways attractive indirect targets for cancer therapy. First, the SWI/SNF complex primarily functions as a major chromatin regulator that promotes the accessibility of enhancer and promoter regions allowing gene expression. The complex also protects against abnormal repression by antagonizing the recruitment of polycomb repressive complexes (PRC1 and 2) [82, 174, 175]. Thus, its loss of function promotes cancer development through abnormal repression of the chromatin, making SWI/SNF-mutant tumors more sensitive to inhibitors of epigenetic regulators. Supporting this premise, we and others have used in vitro and in vivo studies to demonstrate increased sensitivity to epigenetic drugs including inhibitors of histone deacetylases (HDACs), bromodomains (BRDs), EZH2, and other epigenetic regulators in SWI/SNF deficient SCCOHT, MRT, lung, and other cancers [48, 50, 176].

Second, the impact of SWI/SNF inactivation on the expression of thousands of genes creates unique therapeutic vulnerabilities. Genes or pathways that become critical for the survival of the SWI/SNF-mutant cells can result in novel synthetic lethalities, providing new therapeutic opportunities. The genes and pathways shown to become synthetic lethal in SMARCA4 deficient cancers include SMARCA2, CDK4/6, Aurora kinase, and oxidative phosphorylation [60, 146, 177, 178]. Thus, identifying the key survival markers in SWI/SNF deficient tumors using shRNA, CRISPR, and drug or compound libraries should uncover candidate inhibitors for targeted therapy. These approaches prove more beneficial to SWI/SNF abnormalities that drive or contribute to the development of the specific cancer type such as the ARID1A mutation in genital tract cancers described in Sect. 7.2.3.1. Demonstrating the utility of these approaches, two recent studies uncovered that SMARCA4 loss or inhibition creates a synthetic lethality in diffuse midline glioma, a highly aggressive uniformly fatal pediatric cancer with a critical cancer driver H3K27M mutation [117, 132]. The H3K27M mutation prevents H3K27 methylation normally mediated by PRC2, creating genome-wide epigenetic abnormalities and reliance on SMARCA4 for survival. The creation of a synthetic lethal vulnerability by SMARCA4 further accentuates the antagonistic roles of PRC2 and SWI/SNF complexes as an important mechanism in the development and therapeutic targeting of this deadly cancer.

Despite the tremendous potential of exploiting SWI/SNF complex loss for the treatment of cancer patients, the discovery and targeting of therapeutic vulnerabilities of these tumors is an emerging field. Most of the promising discoveries have happened in the last few years; their clinical use and tolerability as durable therapeutic options remain untested. An important caution or consideration in the discovery of the therapeutic vulnerabilities of mutant SWI/SNF subunits arises from the likely context dependence of these targets, i.e., they may only work against a specific SWI/SNF subunit and cancer type (see Sect. 7.5.1). Therefore, careful evaluation and validation of discoveries are needed prior to clinical application.

7.4.3 SWI/SNF Abnormalities in Non-oncologic Diseases

The clinical use of SWI/SNF abnormalities is not limited to cancer. Studies have shown that SWI/SNF abnormalities also contribute to some developmental disorders such as Coffin–Siris and Nicolaides–Baraitser syndromes, neurodevelopmental and Autism Spectrum-like disorders, Type 2 diabetes, and some infectious and metabolic diseases including HIV, hepatitis B virus (HBV), inflammatory bowel diseases, and the hematological disorder neutrophil-specific granule deficiency [179–181]. Thus, the discovery and development of clinical benefits for cancers with SWI/SNF defects have the potential to be easily adopted or modified for use in non-cancer diseases with these abnormalities.

7.5 Unanswered Questions

7.5.1 How Does Cellular Context Dictate Preferential Loss of SWI/SNF Subunits in Human Cancers?

Multiple lines of evidence implicate the epigenomic state of a cell strongly as a key determinant of the functional outputs of mutant-SWI/SNF complexes. While mutations of SWI/SNF subunits may occur in approximately 20% of all human tumors, their distribution does not fall evenly among cancers [3, 69]. For example, *ARID1A* mutations appear frequently in ovarian, endometrial, and ovarian cancers while PBRM1 mutations occur frequently in renal cell carcinomas [182, 183]. In addition, SWI/SNF subunit mutations may also drive tumor development by themselves as in SCCOHT and MRT or drive progression as in LUAD and thoracic sarcomas [10]. As discussed in Sects. 7.3 and 7.4.3, while germline inactivating mutations in SWI/SNF subunits like *SMARCA4* and *SMARCB1* lead to familial cancer syndromes, germline missense mutations in the same subunits generally lead to neurological and developmental disorders [10, 184]. Finally, germline inactivating mutations of *SMARCA4* generally lead to SCCOHT but can also cause MRT in ~5% of patients [10, 27, 185, 186]. Taken together, these studies point to a more complicated mechanism for how SWI/SNF subunit mutations contribute to human tumor development and emphasize the need for more studies on the influence of cellular context.

7.5.2 How Do Post-Translational Modifications of Subunits Affect SWI/SNF Complex Activities?

While the paradigm for regulation of histone functions lies in their post-translation modifications, i.e., the histone code, surprisingly few studies have assessed the potential effects of post-translational modifications on the activities of SWI/SNF complex. As early as 2003, Bourachot et al. demonstrated that the ability of SMARCA2-containing complexes to inhibit cell growth relied upon its acetylation [187]. In 2014, Wang et al. reported that CARM1, a type I protein arginine methyltransferase, methylates SMARCC1 resulting in increased metastatic potential in human breast cancers [188]. A later report showed that this modification of SMARCC1 also occurs in ovarian cancer, providing a new mechanism to sensitize these tumors to PARP inhibition [189]. A 2021 report demonstrates that SMARCC1 methylation leads to changes in super-enhancers in triple-negative breast cancer to promote metastasis in concert with inhibition of anti-tumor immunity [190]. Guo et al. have shown that LSD-regulated lysine methylation in SMARCC1 stabilizes SWI/SNF complexes, providing another key post-translational modification [191]. Other reports have demonstrated p38 mitogen-activated protein kinase (MAPK)-mediated regulation of the SMARCD3 subunit [192] and regulation of SMARCA4 activities by its phosphorylation by

CK2, calcineurin, and PKCβ [193–195]. Considering the large numbers of missense mutations in SWI/SNF subunits across human cancers that can eliminate or initiate sites of post-translational modification, a comprehensive evaluation of the functional impacts of these sites seems critical (Fig. 7.5) [23, 69, 90, 196].

7.5.3 SWI/SNF Subunits-Tumor Suppressors and/or Oncogenes?

Another evolving area of research comes from recent reports suggesting that some SWI/SNF components may also act as oncogenes. As discussed in 2.3.6, several reports have implicated increased expression of BIRCA/L, BRD9 and BCL7B as drivers of tumorigenesis, consistent with their increased mRNA levels in many human cancers [16, 88, 90, 100]. In addition, overexpression of SWI/SNF complex subunits, such as ARID1A and SMARCA4, has been found in liver cancer and breast cancer, respectively [72, 197]. Indeed, if one examines the changes in SMARCA4 expression across human cancers in the Pan-Cancer project, many human tumors show its overexpression rather than mutations/deletions (Fig. 7.5b), [69, 88, 90]. Other studies have shown functional consequences of SMARCA4 overexpression such as activation of SOX4 in basal-like breast cancers and altered expression of lineage-specific transcription factors in neuroendocrine prostate cancer [198, 199]. It still remains unclear how overexpression of SWI/SNF subunits alters the complex's activities. Few studies have assessed the stoichiometry of SWI/SNF complexes, the average number of complexes per cell, and subunit levels that might limit complex formation. More importantly, as studies implicate targeting of SWI/SNF subunits for the treatment of human cancers, the significant growth inhibition of normal cells and tissues caused by the loss of these proteins brings up concerns about serious side-effects [31, 115, 200–204].

7.6 Summary

The frequent mutations of subunits of the SWI/SNF chromatin remodeling complex in ~20% of human tumors uncovered by the TCGA have opened an exciting new field of cancer epigenetics. However, the mechanisms that account for how mutant SWI/SNF subunits can drive the initiation of cancer, in some cases as the only mutation, as well as promote tumor progression and metastasis remain complicated and elusive. The broad roles that the SWI/SNF complex plays in basic cellular functions further impede progress in identifying the key downstream pathways driving tumorigenesis. These limited mechanistic insights have also hampered the increased efforts to discover drugs and other therapies targeting the changes in the epigenome induced by anomalous SWI/SNF complex activities. Because functional studies have implicated many SWI/SNF subunits as tumor suppressors consistent with their inactivation by mutations and deletions in tumors, their use as targets for therapy encounters the same problems seen for

other tumor suppressor genes such as *TP53*, *VHL*, *RB1*, or *KEAP1*. This knowledge has translated into limited novel treatment options. Thus, progress in finding drugs that specifically target cancers with relevant SWI/SNF subunit mutations has come from the inhibition of the key downstream signaling pathways or targeting targets of synthetic lethality. As the oncogenic mechanisms employed by mutant SWI/SNF complexes emerge, they will lead to insights into the roles of chromatin remodeling in normal and abnormal cells and reveal new therapeutic targets for precision medicine for the large number of patients with SWI/SNF-mutant cancers. The generation of new cell lines from these cancers and genetically engineered mouse models for in vivo studies will also accelerate the discovery of efficacious drugs by optimizing high-throughput screening efforts and validation by preclinical studies.

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8 Histone Readers and Their Roles in Cancer

Hong Wen and Xiaobing Shi

In eukaryotic cells, DNA is wrapped around histone proteins to form nucleosomes, which are further packaged into higher-order chromatin structures in the nucleus [1]. Each nucleosome contains an octamer of four types of histones (H2A, H2B, H3, and H4) and approximately 147 bp of DNA [2]. The histone proteins are subjected to a wide variety of post-translational modifications (PTMs), including methylation, acetylation, phosphorylation, ubiquitination, and many others [3]. Most of these PTMs occur in the unstructured amino-termini of histone proteins, with less found in the core or carboxyl-terminal regions. The N-terminal regions of histones, often termed as 'histone tails', are largely unstructured and protrude out from the nucleosomal cores, thus presenting PTMs to enzymes and reader proteins [4]. Histone readers are defined protein domains with well-organized three-dimensional structures that can "read" histone PTMs in a sequence-dependent manner [5]. Most reader domain-containing proteins are either components of chromatin-associated protein complexes, or are themselves histone-modifying enzymes or chromatin remodelers. Therefore, recognition of histone PTMs by reader proteins plays an important role in all chromatin-templated processes such as transcription, replication, DNA damage response and repair, and regulation of chromatin dynamics. Not surprisingly, dysregulation and misreading of histone PTMs are frequently found in various human diseases including cancer. In this Chapter, we will outline major classes of histone readers, delineate

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their biochemical and structural features in histone recognition, and describe how dysregulation of histone readout leads to human cancer.

8.1 Readers of Histone Methylation

Methylation can occur on both lysine and arginine residues. Lysine is subjected to mono- (Kme1), di- (Kme2), and trimethylation (Kme3), while arginine is subjected to monomethylation (Rme1) and symmetric (Rme2s) or asymmetric dimethylation (Rme2a). On histones, most methylation occurs on six lysine residues on histones H3 and H4, and correlations between these methylation events and gene transcription have been well established [6]. Methylation, especially di- and trimethylation, on histone H3 lysine 4 (H3K4), H3K36, and H3K79 is often linked to transcriptional activation whereas methylation on H3K9, H3K27, and H4K20 is associated with gene repression. Adding methyl groups onto lysine or arginine residues does not change their positive charge, thus methylation does not affect the charge interaction between histones and DNA. Therefore, the function of this modification is believed to be executed mainly through interaction with reader proteins [4].

Since the discovery of the chromodomain (CD) as the first histone methylation reader [7, 8], numerous methylation readers have since been identified. CD belongs to the larger "Royal family" of protein modules that also include Tudor, PWWP, and MBT domains [9], and members of all these domain classes have later been shown to also recognize methylated histone lysine, with some Tudor domains also binding to methylated arginine. Among the known histone readers, the plant homeodomain (PHD) zinc fingers probably comprise the largest reader family, with more than 100 distinct PHD fingers exhibiting histone-binding activity [10]. Other histone methylation readers include bromo-adjacent homology (BAH), CW-type zinc finger (zf-CW), ADD (ATRX-DNMT3-DNMT3L), and a few ankyrin and WD40 repeats [11].

One common feature of all histone methylation readers is a hydrophobic pocket for substrate recognition [5]. This pocket is typically formed by two to four aromatic residues, which are often positioned perpendicular to each other and surround the methyl group. Recognition of different methylation states (me1, me2, or me3) is primarily determined by the cavity size of the hydrophobic pocket. Readers with a larger pocket are mostly selective for trimethylation, and readers with a smaller pocket can only accommodate the state of lower methylation. In addition, the composition of the amino acids forming the walls of the cage also contributes to the specificity of the methylation state. Replacing the aromatic residues in the cage with negatively charged ones (such as aspartate or glutamate) alters the substrate preference from trimethylation to lower methylation or, sometimes, the unmethylated state [5]. Another common feature shared by methylation readers, with a few exceptions, is site specificity. Compared with acetylation readers, methylation readers are generally more specific to the sites of methylation. Unlike the cage recognition of methyllysine, the mechanism for

site specificity varies among different readers, largely determined by extensive interactions between readers and flanking sequences to the methylated ligand [5].

8.1.1 The Royal Family Protein Domains

The "Royal family" refers to protein modules that are structurally related to the Tudor domain [9, 12]. The Royal family members include CD, Tudor, MBT, chromo barrel, and PWWP domains [9], all of which are known to function as histone methylation readers. Below we briefly introduce the biochemical and structural features of these domains in methylation recognition.

8.1.1.1 Chromodomain (CD) and Chromo Barrel Domain (CBD)

In 2001, the Jenuwein and the Kouzarides labs independently reported that the HP1 CD bound to histone H3 trimethylated at lysine 9 (H3K9me3) [7, 8], and soon after several structures were solved revealing the mechanism of the CD-H3K9me3 interaction [13–15]. CD is a small domain of 40–50 amino acids consisting of three β strands packed against a helix [16]. The histone peptide adopts a β-strand conformation and inserts into the CD, completing the beta-sandwich architecture. The methyl group is caged by three conserved aromatic residues, and the side chain of H3K9 is almost fully extended and surrounded by residues that are also conserved in many other CDs, suggesting that other CDs may also bind methylated lysine [14, 15]. Indeed, the CDs of Chromebox (CBX) proteins were later found to bind methylated H3K9 and/or H3K27 [17]. These canonical CDs preferentially bind to Kme3 over Kme2, as trimethylation provides additional polar and van der Waals interactions. Another subclass of CD resides in the CHD (chromo-ATPase/helicase-DNA-binding) proteins, with two CDs in tandem. In contrast to canonical CDs that bind to the repressive Kme3 (i.e., H3K9me3 and H3K27me3), the tandem CDs of CHD1 bind to H3K4me3, a mark for gene activation [18–20]. The two CDs of CHD1 each contain a large sequence insertion and cooperate to interact with methylation specifically on H3K4 [18]. The chromo barrel domain (CBD) is structurally similar to the CD [16]. The best-studied CBD protein is MRG15, which has been shown to interact with H3K36me3 [21]. Different from CD, CBD does not have the C-terminal α-helix and instead has two additional β-strands. Nevertheless, recognition of methyl groups by CBD is still achieved through the three aromatic residues conserved in the canonical CD [21]. The MSL3 CBD interacts with H4K20me2 and possibly also H3K36me3 in a similar manner, whereas the MOF CBD does not bind to histones, as it lacks the conserved aromatic residues [22–26].

8.1.1.2 Tudor Domain

The Tudor domain is approximately 60 amino acids in length, slightly longer than CD [9]. Tudor domains can be categorized into functional units consisting of either a single domain or tandem Tudor domain (TTD). Most Tudor domains fold into a four- to five-stranded antiparallel β-barrel, with a hydrophobic pocket

formed by 4–5 aromatic residues for methylation recognition [27]. Despite the similar overall protein folding of individual Tudor, the substrate specificity varies significantly among different Tudor domain proteins [28]. For example, the single Tudor domains of PHF1 and PHF19 recognize H3K36me3, the UHRF1 TTD binds to H3K9me3, the SGF29 TTD binds to H3K4me3, the 53BP1 TTD binds to H4K20me2, and the JMJD2A TTD recognizes H3K4me3 and H4K20me3 [28–37]. Interestingly, in most TTDs, the aromatic residues for methyllysine recognition reside in only one of the two Tudor domains [27]. Besides methyllysine, some Tudor domains are also reported to bind methylarginine. For example, the TDRD3 Tudor recognizes H3R17me2a and H4R3me2a for gene activation [38], and the extended Tudor domains of germline-specific proteins SND1 and TDRD1 recognize methylated arginine on PIWI proteins, contributing to piRNA biogenesis [39, 40].

8.1.1.3 PWWP Domain

PWWP is named for a Pro-Trp-Trp-Pro motif within the domain [41]. The PWWP domain consists of ~130 amino acids, much longer than CD and Tudor domains. The N-terminal half of the domain resembles a barrel-like structure with five antiparallel β strands and the C-terminal half forms a helical bundle [42]. The PWWP domain was initially characterized as a DNA binding motif [43–46], and was later found to recognize histone methylation as well [47]. PWWP is best known as a reader for H3K36 methylation [48], although some PWWP domains are also known to bind H4K20me3 and H3K79me2. The PWWP domains of BRPF1, DNMT3A, DNMT3B, PSIP, MSH6, and HDGF2 have all been shown to bind H3K36me3 [48–54]. Interestingly, we found that the PWWP domain of ZMYND11 recognizes H3K36me3 specifically on the histone variant H3.3, and such specificity is achieved through the cooperation of PWWP with its adjacent BD and PHD domains [55, 56]. As H3K36 is in proximity to DNA in the nucleosomal context, both histone and DNA bindings contribute to the association of PWWP proteins with chromatin. Indeed, many PWWP domains bind to methylated nucleosomes with much higher affinity than histone or DNA alone, indicating a synergy between these two interactions [48]. The histone-binding pocket and the DNA-binding patch are separated into different regions of the domain, suggesting that the PWWP domain adopts distinct mechanisms and interfaces to engage the histone and DNA bindings synergistically [52, 57, 58].

8.1.1.4 MBT (Malignant Brain Tumor) Domain

The MBT domain consists of ~100 amino acids, and forms a four-stranded β-barrel followed by an extended "arm" of helices and a short strand [28, 59]. All known MBT domains exist in repeats, ranging from 2 to 4 times, with one domain packed against another. Notably, although each MBT repeat contains an aromatic cage, only the second repeat binds methyllysine [60–62]. Different from all other Royal family members, MBT appears to preferentially bind to monomethylated lysine, although some MBT members can recognize Kme2 modifications. This is because the aromatic cage in MBT is not intact, with an acidic residue replacing

an aromatic residue [60–62]. Another unique feature of the MBT domain is that it shows little sequence specificity for substrate recognition. Most MBT domains can bind monomethylated lysine on almost all major methylation sites on histones [63]. Structural studies revealed that MBT domains interact with the methyl group via a "cavity insertion recognition mode", but make few contacts with histone residues beyond the methylated lysine [60–62].

8.1.2 PHD Finger

The plant homeodomain (PHD) finger is a cysteine-rich module characterized by a C4HC3 motif that coordinates two zinc ions [64]. It is approximately 50–80 amino acids in length, and composed of a small two-stranded β-sheet flanked by one or two α-helices [65]. In 2006, the Gozani and Allis laboratories independently found that the PHD fingers of the ING2 and BPTF proteins are histone methylation readers specific for H3K4me3 [66, 67]. Structural studies from the Kutateladze and Patel labs revealed that the histone H3 tail is bound in an extended conformation and makes extensive contact with the PHD fingers, providing a high degree of site specificity [68, 69]. Besides H3K4me3 inserted into a hydrophobic cage, the R2 residue of H3 occupies an adjacent groove and the A1 residue is also bound in a small hydrophobic cavity [68, 69]. Such a coordination of all residues in the A-R-T-K^{me3} motif of the H3 tail is conserved among many PHD fingers, as well as some other H3K4me3 readers such as the CW-type zinc finger. Typically, the H3R2 binding pocket contains acidic amino acids, which restrain the guanidinium group of R2 through ionic and hydrogen-bonding interactions. Therefore, many H3K4me3-binding PHD fingers, such as ING2, are sensitive to methylation on H3R2. For the RAG2 PHD finger, however, the acidic residues are replaced with a tyrosine, which can interact with the symmetric methylation on R2, thus further enhancing its H3K4me3 binding [70, 71]. In addition to the H3K4me3-binding PHD fingers, there is also a subfamily of PHD fingers that recognize unmodified H3K4 (H3K4me0), which will be discussed in Sect. 8.3.2.

8.1.3 BAH Domain

The Bromo Adjacent Homology (BAH) domain is a conserved motif of 85–140 amino acids [72]. Initial indications that the BAH domain may interact with nucleosomes came from genetic screens and mutation studies of the yeast Sir3, and this was further proved by the crystal structure of Sir3 BAH in complex with nucleosome [73–76]. The Sir3 BAH domain forms contacts with a large area of the histone octamer (including all four histones), and its binding to the nucleosome is sensitive to covalent modifications on H4K16 and H3K79 [73, 76]. A few months later, the Gozani lab reported that the BAH domain of mammalian ORC1 is a reader of H4K20me2 [77]. The specificity for H4K20me2 is mediated by an aromatic dimethyl-lysine-binding cage and multiple intermolecular contacts involving

the bound peptide. The BAH domain prefers dimethylation over trimethylation with one of the aromatic residues replaced with a Glu, which forms a hydrogen bond with the dimethylammonium [77]. Of note, H4K20 in *S. cerevisiae* is not methylated, and correspondingly, the H4K20me2 binding activity is not conserved in the budding yeast Orc1. Instead, the yeast Orc1 BAH domain shares a similar structure and some of the nucleosome-binding properties with Sir3 [74, 75, 78]. Nevertheless, the histone methylation reader activity is conserved among several other BAH-containing proteins, from humans to plants. Studies from the Wang and Song labs showed that DNMT1 BAH1 binds to H4K20me3, and the BAH domains of human BAHCC1 and BAHD1 recognize H3K27me3 [79–81]. In addition, the BAH domains of the plant-specific histone reader SHL and the DNA methyltransferase ZMET2 were also shown to recognize H3K27me3 and H3K9me2, respectively [82, 83].

8.1.4 Other Methylation Readers

Besides the reader domains described above, there are a few other protein domains that also show binding activity to methylated histones. However, this activity may not be conserved among all the family members. For example, The CW-type zinc finger (zf-CW) of ZCWPW1 is a reader of H3K4me3, with its structure partially resembling the PHD finger bound to the histone H3 tail [84]. The ATRX-DNMT3A-DNMT3L (ADD) domain of ATRX recognizes the N-terminal sequence of H3 with unmodified K4 (H3K4me0) and H3K9me3, and the readout of both marks is important for its localization to pericentromeric heterochromatin [85–88]. In addition, the ankyrin repeats of G9a and GLP recognize H3K9me2/1, and this recognition stimulates the H3K9 methyltransferase activity of G9a/GLP facilitating the spreading of this repressive mark [89, 90]. Finally, the WD40 repeats of EED bind H3K27me3, H3K9me3, and a few other sites [91, 92]. EED is a core component of the PRC2 complex that methylates H3K27. The binding of EED to H3K27me3 stimulates the enzymatic activity of EZH2 [93].

8.2 Readers of Histone Acetylation

Lysine acetylation is probably the most frequent histone PTM. Many lysine residues of all four core histones can be acetylated, on both the flexible tails and the globular domain regions [94, 95]. The most studied acetylation sites on histones include K9, K14, K18, K23, K27, K36, and K56 of H3 and K5, K8, K12, and K16 of H4. Acetylation brings two changes to the lysine residue: neutralizing the positive charge of lysine, and adding a bulky group to the ε-amino group of the side chain. In general, acetylation is associated with active transcription, because neutralizing the positive charge of lysine weakens the interaction between histones and the negatively charged DNA, leading to a more open chromatin structure [94]. Like methylation, acetylation also functions as a docking site for reader proteins.

Relative to the diverse families of histone methylation readers, however, only a few families of histone acetylation readers have been discovered to date. The first family is the bromodomain (BD) identified by the Zhou group in 1999 [96], and the second is the YEATS domain family that we identified in 2014 [97]. In addition, some double PHD fingers (DPF) have also been shown to bind histone H3 in an acetylation-sensitive manner [98, 99].

8.2.1 Bromodomain (BD)

In 1999, the Zhou laboratory first reported that the BD of the histone acetyltransferase PCAF binds acetylated histones [96]. Soon after, studies from many labs further demonstrated that BD is an acetyllysine-binding domain [100]. BD is evolutionarily conserved from yeast to human, and is approximately 110 amino acids in length [101]. In humans, there are 46 BD-containing proteins, most of which are associated with histone-modifying enzymes, chromatin-remodeling complexes, and other chromatin-associated protein complexes. Despite sequence variations, all BD modules share a conserved fold of four-helix bundle with two interconnecting loops [102]. Two conserved tyrosine residues from the two loops form an aromatic acetyllysine binding pocket. Almost all BD modules contain an invariable asparagine that forms a hydrogen bond with the acetyllysine carbonyl oxygen [102]. In general, an isolated BD binds to acetyllysine with weak affinity and low site specificity. However, some BDs, such as the first BD of the testis-specific BRDT and possibly also other BET family bromodomain proteins, have a wider aromatic pocket that can accommodate two acetyllysines simultaneously [103]. This binding mode not only increases binding affinity but also enhances selectivity toward the H4 tail, as the H4 tail is frequently multi-acetylated. A subfamily of the BDs exists in tandem. For example, the TFIID subunit TAF1 contains double bromodomains (DBD) with each BD binding to an acetylated lysine, thus promoting TAF1 binding selectively to the heavily acetylated histone H4 with high affinity [104]. In addition, BDs are often found adjacent to other reader domains, with the PHD finger as the most common partner. Such arrangements allow multivalent readout of histone PTMs by the linked binding modules.

8.2.2 YEATS Domain

The YEATS domain, named for its five founding members (Yaf9, ENL, AF9, Taf14, and Sas5), is evolutionarily conserved from yeast to human [105]. In 2014, we first reported that the YEATS domain of AF9 binds to acetylated histone H3, with a strong preference for H3K9ac [97]. The co-crystal structure revealed that AF9 YEATS adopts an eight-stranded immunoglobulin fold and utilizes a serine-lined aromatic "sandwiching" cage for the specific readout, a novel acetyl recognition mechanism that is distinct from those of BDs [97]. Follow-up studies from many labs (including ours) demonstrated that acetylation binding is a

common feature of all YEATS domains, all of which adopt a similar fold and the same serine-lined "sandwiching" mechanism for acetylation binding [106–110]. Most YEATS domains display a binding preference towards acetylation on K9, K18, and K27 of H3, suggesting that the conserved "RK" motif is critical for substrate specificity. The only exception is Gas41, which can also recognize acetyllysine in the "GK" motif, which can be seen on H3K14 and multiple sites on H4 [107, 111]. Interestingly, the YEATS domain also exhibits binding activity to other types of acylation on histones, including propionylation, butyrylation, and crotonylation [112–114]. Notably, compared to acetylation on the same sites, the YEATS domains bind to acylations with ~ 2–eightfold higher affinity. Structurally, the YEATS domain can bind to the acylation with longer side chains as the acyl-binding pocket is end-open [114, 115]. In contrast, the pocket in BD is end-blocked, which can hardly tolerate side chains longer than acetylation [116, 117].

8.2.3 Double PHD Finger (DPF)

Besides the two well-characterized acetylation reader families discussed above, double PHD fingers (DPF) have also been shown to bind to histones in an acetylation-sensitive manner. The DPF modules of DPF3b and DPF2 bind to the H3 tail, and the interactions are promoted by acetylation on H3K14 [98, 99]. Structurally, the two adjacent PHD fingers fold as one functional cooperative unit, with the H3 peptide lying across a surface shared by the two domains. The second PHD finger engages the first four amino acids of histone H3, whereas the first PHD module anchors H3K14ac in a hydrophobic pocket with an aspartic acid forming a hydrogen bond with the acetylamide [99]. Of note, such a recognition mode is distinct from the mechanisms employed by BD or YEATS domains in acetylation binding or other PHD fingers in methyl-lysine recognition. Nevertheless, such a binding activity is conserved in DPF-containing proteins like MOF and MORF, and extends to the recognition of other types of acylation, such as crotonylation [118, 119].

8.3 Other Histone Readers

8.3.1 Histone Phosphorylation Readers

Phosphorylation of serine and threonine is another PTM commonly seen on histones. However, in contrast to the extensively characterized phosphorylation of cytosolic proteins in various signaling pathways, much less is known about the roles and regulators of histone phosphorylation. Currently, only a few proteins are known to bind phosphorylated histones. One example is the 14–3-3ζ protein that binds to H3S10ph, where S10ph is restrained through multiple hydrogen bonding and ionic contacts with two arginine residues and a tyrosine [120]. Another is the

BIR domain of Survivin that recognizes H3T3ph, with T3ph placed in a small, positively charged patch composed of a lysine and a histidine [121–123]. The BRCT domain is also known as a phospho-protein binding domain [124], and the BRCT domain of MDC1 has been shown to bind H2AX phosphorylation, a mark for DNA damage [125, 126]. Notably, histone phosphorylation often occurs near methylated or acetylated lysine residues (for example, H3K4, H3K9, and H3K27). Because of the size and chemical feature of the phosphor group, it often repels the readers that otherwise bind to the methylated or acetylated lysines [127, 128].

8.3.2 Readers of Unmodified Histones

Some protein domains have been shown to bind unmodified histone tails, especially the H3 tail. These include a subfamily of the PHD finger, the ZZ domain, the ADD domain of DNMT3A/3L, and the WD40 domain of WDR5. Like their modified counterparts, the unmodified H3 tail also represents a chromatin state and thus is biologically important.

8.3.2.1 PHD Finger

Besides a large number of PHD fingers that recognize H3K4me3, a subfamily of PHD fingers bind to the unmodified H3K4 (H3K4me0). Both types of PHD fingers share similar overall structure and conserved mechanisms for interacting with the N-terminal amino acids of H3. The major difference arises from the distinct coordination of H3K4. While the H3K4me3 is accommodated in a hydrophobic pocket formed by several aromatic residues, the unmethylated K4 is bound by a set of hydrogen bonds and salt bridges formed with the acidic residues clustered on the surface of the protein [5]. The K4me0-binding cavity is relatively narrow and cannot accommodate methyl groups, thus methylation on H3K4 disrupts the interaction [129]. Proteins containing this type of PHD finger include LSD1, UHRF1, TRIM24, TRIM33, the speckled protein family, and the first PHD finger within the KDM5 family proteins [129–136]. In addition, the second PHD finger in the DPF module of the DPF proteins and MOZ/MORF proteins, as well as the first PHD finger in the PHD-zinc-knuckle-PHD (PZP) module of AF10/17, the BRPF proteins, and the JADE family proteins also bind to H3K4me0 in a similar manner [98, 99, 118, 130, 137–139].

8.3.2.2 ZZ Domain

The ZZ-type zinc finger (ZZ) is comprised of ~50 amino acids and topologically belongs to a large family of RING fingers, which also includes other zinc fingers, such as the PHD finger [140]. Proteins containing the ZZ domain are implicated in various cellular processes, although the function of the ZZ domain remained unclear. Recently, we found that the ZZ domains of p300 and ZZZ3, a subunit of the ATAC histone acetyltransferase complex, function as a reader of the unmodified histone H3 tail [141, 142]. Structural studies revealed a unique H3-binding mechanism that differs from the binding mechanisms of other known H3K4me0

readers. Particularly, H3A1 occupies a highly negatively charged binding site, whereas H3K4 is sandwiched between the aromatic ring of phenylalanine and the negatively charged carboxylic group of aspartate, which forms a salt bridge with the ammonium group of H3K4 [141, 142]. Such an arrangement most likely accounts for the indifference of the ZZ domain toward PTMs on H3K4. This binding mode is conserved in some other nuclear ZZ proteins such as HERC2 and ZZEF1 [143, 144], but not cytoplasmic ZZ proteins involved in protein degradation signaling [145].

8.3.2.3 Other H3K4me0-Binding Domains

The ADD domains of DNMT3L and DNMT3A have been shown to specifically interact with the amino terminus of histone H3 [146, 147]. Like the H3K4me0-binidng PHD finger, this interaction is strongly inhibited by methylation at H3K4. Structural studies revealed that the ADD domain binds to the unmodified H3 peptide using a mechanism typical of PHD fingers in H3K4me0 binding [146, 147]. Importantly, the ADD domain of DNMT3A interacts with and inhibits enzymatic activity of the catalytic domain, and H3 binding induces activation of de novo DNA methylation [85, 148]. The WD40 repeats in WDR5, a component of the MLL H3K4 methyltransferase complex, bind to H3 tails with unmodified H3K4 and mono- or di-methylated H3K4 with comparable affinities [149–152]. Unlike PHD finger and the ZZ domain, symmetric methylation on H3R2 further enhances the binding of WDR5 to the H3 tail [153], suggesting that WDR5 may mediate the crosstalk between H3K4 and H3R2 methylation. Interestingly, the WD40 repeats of RbAP46/48 bind to unmodified H4 but not the H3 tail [154], suggesting that the WD40 repeats have evolved diverse functions.

8.4 Histone Readout in Cancer

Proper interpretation of epigenetic marks by reader proteins is critical for maintaining genome stability and proper control of essentially all chromatin-templated processes. Therefore, dysregulations of histone readout are frequently observed in various human diseases including cancer [155]. In this section, we will describe the common mechanisms that cause histone readout dysregulation leading to cancer initiation and progression. These include genetic mutations and dysregulations of histone readers, fusion proteins of histone readers caused by chromosomal translocations, and misreading of histone marks caused by oncogenic histone mutations.

8.4.1 Mutations and Dysregulations of Histone Readers

The de novo DNA methyltransferases DNMT3A and DNMT3B are frequently mutated in various types of human cancer, with the R882 mutations in the methyltransferase domain of DNMT3A being the most frequent alteration [156–159].

The PWWP reader module of DNMT3A is also frequently mutated in patients with leukemia and paraganglioma [160, 161]. The PWWP domain recognizes H3K36me2/3 and is important for DNMT3A and DNMT3B to localize to the chromatin regions enriched with these marks [44, 49, 162, 163]. Recurrent mutations in the PWWP domain suggest that misinterpretation of H3K36me2/3 underlies dysregulation of DNA methylation in tumorigenesis. Proper recognition of H3K36me3 by PWWP domain-containing DNA damage repair proteins, such as MSH6, provides a mechanism to safeguard genome stability [51]. Both MSH6 and H3K36me3 are essential for chromatin recruitment of the mismatch recognition protein MutSα [51]. Mutations of MSH6 are frequently observed in colorectal and uterine cancer, and cells lacking SETD2 and H3K36me3 display microsatellite instability and an elevated spontaneous mutation frequency [51].

Previously, we identified the PHD-Bromo-PWWP tandem reader cassette of ZMYND11 as a histone variant H3.3-specific reader of H3K36me3 [56]. ZMYND11 brings transcription coregulators to a gene body to suppress the transcriptional elongation of some target genes, and promote intron retention of others [55, 56]. It is a putative tumor suppressor and is frequently deleted in cancers [164]. ZMYND11 copy-number loss has been reported in several hematological malignancies, including ALL (acute lymphoblastic leukemia), CML (chronic myeloid leukemia), CLL (chronic lymphoid leukemia), MM (multiple myeloma), and MDS (myelodysplastic syndrome) [165]. In addition, reduced expression of ZMYND11 is correlated with poor outcomes in breast cancer patients [56]. A ZMYND11-related protein, ZMYND8, is also dysregulated in various human cancers. ZMYND8 is mutated with high frequency in mismatch repair-deficient colorectal cancers, and also downregulated in breast, prostate, and nasopharyngeal cancers [166, 167]. Mechanistically, the PHD-Bromo-PWWP cassette of ZMYND8 does not bind H3K36me3, but instead recognizes the dual histone marks of H3K4me1 and H3K14ac [168]. ZMYND8 binds to gene enhancers with the dual marks and suppresses the expression of oncogenes through recruiting the histone H3K4 demethylases KDM5C in breast cancer and KDM5D in prostate cancer cells, respectively [169, 170]. Loss of ZMYND8 results in overactivation of enhancers with elevated level of H3K4me3 and increased expression of enhancer RNAs, leading to overactivation of target genes involved in tumor initiation and metastasis.

Another family of proteins frequently dysregulated in cancer is the YEATS domain family. In humans, there are four YEATS domain-containing proteins. Besides the AF9 and ENL paralogue proteins being common fusion partners of MLL in acute leukemia (discussed in the next section), recurrent somatic mutations of the ENL YEATS domain have been reported in Wilms' tumor and pediatric AML [171–173]. These hotspot ENL mutations are unique, small, in-frame insertion or deletion mutations in the C-terminus of the YEATS domain, which are located distant from its Kac-binding pocket. We've shown that these are gain-of-function mutations promoting aberrant gene expression through self-reinforced chromatin recruitment, a process highly dependent on the reader function of the YEATS domain [174]. The markedly enhanced chromatin binding of ENL

YEATS mutants leads to increased recruitment and targeting of the super elongation complex (SEC) and RNA polymerase II onto target genes, resulting in aberrant activation of ENL targets, such as key developmental regulator HOX family genes, and impaired cell-fate decision. The other two YEATS-containing proteins, YEATS2 and GAS41, are amplified or overexpressed in a variety of cancers, including non-small cell lung cancer (NSCLC). GAS41 is also amplified in brain tumors, including 23% of glioblastoma and 80% of astrocytoma, sarcoma, colorectal, and gastric cancers [175–177]. Both YEATS2 and GAS41 are subunits of a HAT complex, the ATAC and Tip60 complex, respectively. High levels of these reader proteins lead to the overactivation of the associated HAT, and upregulation of target genes that promote oncogenesis [106–108]. GAS41 is also associated with the SRCAP complex, which regulates gene expression through genome-wide deposition of the histone variant H2A.Z [107].

8.4.2 Fusion Proteins of Readers Resulting from Chromosomal Translocations

Chromosomal translocations are catastrophic genomic alterations that cause tumorigenesis, especially in hematological malignancies and sarcomas [178]. The juxtaposition of a proto-oncogene to the promoter or enhancer of another gene often leads to sustained oncogene activation. In other cases, recurrent chromosomal translocations juxtapose the coding regions of two separate genes in the frame, thus producing fusion proteins associated with new functions and oncogenic transformation. Fusion proteins involving histone reader modules are commonly seen in cancers, suggesting that the recruitment of fusion proteins to the wrong places in chromatin is a significant contributor to pathogenesis.

Mixed lineage leukemias caused by recurrent chromosomal rearrangements involving the *MLL* gene at chromosome 11q23 have been extensively studied [179–181]. MLL translocations are found in ~10% of acute leukemia patients of all ages but are most prevalent (~80%) in infant ALL [179, 182]. In MLL-rearranged leukemia, the N-terminus of MLL is fused to the C-terminus of one of more than 80 partners, including the two YEATS domain proteins AF9 and ENL [183, 184]. AF9 and ENL are stoichiometric subunits of SEC and the DOT1L complex [185–190]. The chimeric oncoproteins of MLL-AF9 and MLL-ENL thus hijack the transcription elongation machinery to promote proto-oncogenic gene expression [181, 189, 191]. Interestingly, we found that the remaining wild-type allele of ENL, but not AF9, is required for tumor maintenance and that the histone acetylation binding activity of the YEATS domain is indispensable for its function [110, 192].

AF10 is another subunit of the DOT1L complex that is also involved in chromosomal translocations in acute leukemias. AF10 is commonly fused with CALM (clathrin assembly lymphoid myeloid leukemia) and MLL. In these cases, the N-terminal PZP reader domain of AF10 is often deleted, whereas the C-terminal octapeptide-motif leucine zipper (OM-LZ) domain of AF10 is retained in the

fusion proteins. The OM-LZ domain interacts with and recruits DOT1L, leading to aberrant *HOXA* gene activation and leukemic transformation [138, 193–195].

A recurrent chromosomal translocation t(10;17)(p15;q21) was recently reported in a subset of AML patients [196–198] that produces an abnormal chimeric gene. The chimeric gene encodes an in-frame fusion of the N-terminal fragment of ZMYND11 to the full-length MBTD1, a subunit of the Tip60 complex. The resultant ZMYND11-MBTD1 fusion still contains the PHD-Bromo-PWWP reader cassette of ZMYND11, thus retaining the H3.3K36me3 reader activity. As the fusion protein is stably incorporated into the Tip60 complex, the ZMYND11 reader cassette leads to mistargeting of the associated HAT activity, thus driving high-level expression of proto-oncogenes that are normally suppressed by ZMYND11 in leukemia stem cells. Therefore, both the H3.3K36me3-reading and the Tip60-interacting activities of the ZMYND11-MBTD1 fusion protein are required for its transformation activity [199, 200].

The t(15;19)(q14;p13.1) chromosomal translocation drives a highly aggressive squamous cancer called NUT midline carcinoma (NMC). This translocation results in the chimeric fusion protein BRD4-NUT (nuclear protein in testis) [201, 202]. The fusion protein is anchored to chromatin with pre-existing histone acetylation by the BRD4 BDs, and the NUT moiety recruits the histone acetyltransferases p300 and CBP to acetylate the nearby histones. The hyperacetylated chromatin in turn leads to the spreading of BRD4-NUT and its associated transcription factors to stimulate gene expression in the hyperacetylated chromatin domains [203, 204]. The broad BRD4-NUT binding domains can be larger than 1 Mb in size in the chromosomal regions containing the *MYC* and *TP63* genes, the expression of which is required for maintaining the highly proliferative and undifferentiated state of NMC cells. BET bromodomain inhibitors can displace the BRD4-NUT fusion protein from chromatin, and ultimately lead to squamous differentiation and arrested proliferation of the NMC tumors [205–207].

8.4.3 Misreading of Histone Marks Caused by Oncohistones

Somatic missense mutations of histone genes have recently been identified across several cancer types, including glioma, chondroblastoma, giant-cell tumor of bone, sarcoma, and lymphoma [208–212]. The histone proteins encoded by these somatic mutations exhibit oncogenic activity, and are thus called “oncohistones” [209, 213]. The most well-studied oncohistones are H3K27M, H3K36M, and H3.3G34V/R/W/L mutants; these oncohistones are tissue-type specific and mostly found in pediatric cancers [213]. For example, in aggressive pediatric high-grade gliomas (pHGGs), H3K27M mutations occur preferentially within the hindbrain and H3G34R/V mutations in the forebrain. Although affecting only one out of 32 alleles of H3-encoding genes, the H3 K-to-M mutations have a dominant effect, inhibiting the corresponding histone methyltransferases *in trans*, with H3K27M and H3K36M mutations resulting in a global reduction in H3K27me3

and H3K36me3, respectively [214, 215]. In contrast, H3G34 mutations only affect H3K36 methylation *in cis*, without a global effect [216, 217].

Oncohistones cause chromatin deregulation partially through perturbation of histone readout. Due to the global reduction in histone methylation, H3 K-to-M mutations presumably abrogate the binding of methylation readers to the corresponding lysine, such as the binding of ZMYND11 to H3.3K36me3 in the H3K36M mutant cancers. Although H3G34R does not cause global H3K36me3 reduction or widespread transcriptional changes, this mutation reduces the binding of ZMYND11 to the H3.3K36me3 in vitro, and impairs chromatin recruitment of ZMYND11 in G34R mutant tumor cells [56, 218]. Interestingly, the same oncohistone mutation in pediatric glioblastoma creates a docking site for ZMYND8, which binds to the mutant histones and suppresses genes related to the major histocompatibility complex (MHC) class II immune pathway [219]. ZMYND8 knockout or correcting the H3G34R mutation in the mutant glioblastoma cells can derepress MHC class II response genes. How the oncohistones alter histone readout and promote tumorigenesis in different cell types is an active area of research.

8.5 Concluding Remarks

Since the discovery of BD in 1999, we have gathered a wealth of information regarding the molecular basis of histone readers in substrate recognition. Currently, three types of acetylation readers and almost ten families of histone methylation readers have been discovered, with structures of almost all known readers solved. We suspect that most histone readers have already been discovered. However, studies to understand the biological consequences of histone readout have just begun. So far, most of our knowledge is obtained from studies of readers in complex with histone peptides, whereas histone readout in cells relies on combinatorial readout of multiple PTMs in a nucleosomal environment. In addition, DNA in the nucleosome and RNA produced from transcription also contribute significantly to many reader-histone interactions. Future studies to investigate histone readout in the context of nucleosome and chromatin fiber and in different biological and disease settings will provide more insights into these epigenetic events, and may help to identify more biomarkers and novel drug targets for disease treatment. For example, various small molecule inhibitors targeting the BET family bromodomain inhibitors have advanced to pre-clinical and clinical development. In addition, recent studies from our labs and others have shown that the YEATS domain is also pharmacologically tractable, and small molecule inhibitors and PROTAC degraders targeting the YEATS domains of AF9, ENL, and GAS41 have shown good anticancer activity [220–225]. We anticipate that more inhibitors targeting histone acetylation and methylation readers will be developed in the next decade for treating cancer and possibly also developmental diseases.

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Polycomb Repressive Complex 2 in Oncology

9

Yiran Guo, Yao Yu, and Gang Greg Wang

9.1 Introduction to PRC2, EZH2, and H3K27me3

Chromatin-based genome organization and modulation play a crucial role in a wide range of biological processes, which at least include organismal development, cell pluripotency and differentiation, tissue homeostasis and damage repair after injury, cell–cell and cell-microenvironment interaction, immunity, and tumorigenesis [1–16]. A group of so-called polycomb proteins, together with their regulatory factors and downstream effectors, represent one of the most important cellular machineries for achieving dynamic gene expression modulation and for determining the epigenetic states.

Initially, polycomb group (PcG) genes were identified in *Drosophila* as a set of genes that are critically involved in the control of body patterning and segmentation during embryogenesis [6, 10, 12]. Later on, extensive biochemical characterization of PcG-encoded proteins led to the determination of at least two functionally distinct multi-subunit complexes, termed Polycomb Repressive Complex 2 (PRC2) [1, 7, 17–19] and Polycomb Repressive Complex 1 (PRC1) [1, 5,

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20]. PCR2 core complex is composed of Enhancer of Zeste Homolog 2 (EZH2) or related Enhancer of Zeste Homolog 1 (EZH1), Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 Homolog (SUZ12), and Retinoblastoma Binding Protein 4 or 7 (RBBP4 or RBBP7) (Table 9.1). PRC2 core complex is highly conserved throughout metazoan evolution. In addition to the kingdom of animals, unicellular eukaryotes such as Tetrahymena (a ciliate and early-branching eukaryote) and plants such as fungi and Arabidopsis thaliana all have core components of PRC2 [21–23], indicating early emergency of PRC2 before diversification of eukaryotes. Extensive loss-of-function studies demonstrated that PRC2 plays multifaceted roles in a range of DNA-templated processes, such as transcription, X chromosome inactivation and gene imprinting, DNA replication, and DNA damage repair [1, 7, 11, 12, 19, 24–29], and RNA-based cell function regulation [30–33]. Biochemically, PRC2 serves as a sole enzymatic complex for catalyzing or 'writing' di- and tri-methylation of histone H3 lysine 27 (H3K27me2/3), which is executed by a Su(var)3–9, Enhancer of Zeste, and Trithorax (SET) domain located at the C-terminus of EZH2 or EZH1. Recent structural characterization, especially that based on cryogenic electron microscopy (cryo-EM), showed that PRC2

Table 9.1 Summary of PRC2 core subunits and associated factors

	Protein	Function	References
PRC2 core component	Enhancer of Zeste Homolog 2 (EZH2) or Enhancer of Zeste Homolog 1 (EZH1)	(i) Canonical role: Sole lysine methyltransferase class serves as the enzymatic subunit of PRC2 for catalyzing H3K27me3, with the catalytic activity residing in a SET domain; (ii) can methylate nonhistone proteins; (iii) EZH2 has additional noncanonical roles as transcriptional coactivator	Anwar et al. [39], Blackledge and Klose [1], Glancy et al. [40], Guo et al. [26], Kim and Kingston [5], Margueron and Reinberg [7], Pasini and di Croce [9], Piunti and Shilatifard [11], Wang and Wang [29], Yu et al. [19]
	Suppressor of Zeste 12 homolog (SUZ12)	(i) Using a VEFS domain at C-terminus, SUZ12 mediates the assembly of PRC2; (ii) using an N-terminal region, it enhances the binding of PRC2 core to DNA/RNA	Blackledge and Klose [1], Cao and Zhang [17], Glancy et al. [40], Guo et al. [26], Hojfeldt et al. [41], Kim and Kingston [5], Piunti and Shilatifard [11], Youmans et al. [42]

(continued)

Table 9.1 (continued)

	Protein	Function	References
	Embryonic ectoderm development (EED)	EED has a H3K27me3 'reader' module, WD40 repeats. EED is essential for allosteric activation of PRC2 upon binding to H3K27me3, a catalytic product of PRC2, thereby mediating Polycomb domain spreading via 'reading' and 'writing' H3K27me3	Blackledge and Klose [1], Chammas et al. [43], Glancy et al. [40], Guo et al. [26], Han et al. [44], Kim and Kingston [5], Margueron et al. [45], Piunti and Shilatifard [11], van Mierlo et al. [46]
	Retinoblastoma binding protein 4 and 7 (RBBP4, also known as RbAp48, and RBBP7 or RbAp46)	They can directly bind to nucleosome and guide PRC2 to maintain its position at specific target loci	Blackledge and Klose [1], Chammas et al. [43], Glancy et al. [40], Guo et al. [26], Kim and Kingston [5], Piunti and Shilatifard [11], Xu and Min [47], Yu et al. [19]
Accessory factor specific to PRC2.1	Polycomb-like (PCL), including PHF1, MTF2 and PHF19 (also known as PCL1, PCL2 and PCL3, respectively)	(i) Using a C-terminal chromodomain, PCL binds to SUZ12 and forms the PRC2.1 subcomplex; (ii) the N-terminal domains of PCLs are chromatin-associating modules and can mediate or enhance PRC2.1 engagement to chromatin regions, such as those containing CpG-rich DNA elements and H3K36me3/2	Blackledge and Klose [1], Cai et al. [48], Chammas et al. [43], Chen et al. [49], Glancy et al. [40], Guo et al. [26], Kim and Kingston [5], Li et al. [50], Piunti and Shilatifard [11]
	Elongin BC and Polycomb repressive complex 2 associated protein (EPOP)	(i) A C-terminal region of EPOP and the ZnB-Zn domain of SUZ12 mediate formation of PRC2.1 subcomplex; (ii) it links PRC2.1 with Elongin BC together to repress transcription	Beringer et al. [51], Blackledge and Klose [1], Chammas et al. [43], Glancy et al. [40], Guo et al. [26], Kim and Kingston [5], Liefke et al. [52], Shilatifard [11], van Mierlo et al. [46]

(continued)

Table 9.1 (continued)

	Protein	Function	References
	PRC2-associated LCOR isoform 1 (PALI1) and PALI2	(i) It promotes PRC2 activity; (ii) PALI1 and EPOP are mutually exclusive in binding of PRC2	Blackledge and Klose [1], Chammas et al. [43], Conway et al. [53], Glancy et al. [40], Guo et al. [26], Hauri et al. [54], Kim and Kingston [5], Piunti and Shilatifard [11], Zhang et al. [55]
Accessory factor specific to PRC2.2	AE binding protein 2 (AEBP2)	(i) It stabilizes the binding of PRC2 at the targets; (iii) it binds to DNA directly via the embedded Zinc fingers	Blackledge and Klose [1], Chammas et al. [43], Glancy et al. [40], Guo et al. [26], Kasinath et al. [56, 57], Kim and Kingston [5], Piunti and Shilatifard [11]
	Jumonji and AT-rich interaction domain containing 2 (JARID2)	(i) It has intrinsic activities for binding DNA, RNA, nucleosome and H2AK119ub, a catalytic product of PRC1; (ii) it can be methylated by PRC2 to induce JARID2-K116me3 which can be bound by EED for allosteric stimulation of PRC2.2	Blackledge and Klose [1], Chammas et al. [43], Glancy et al. [40], Guo et al. [26], Kasinath et al. [56, 57], Kim and Kingston [5], Piunti and Shilatifard [11], Zhang et al. [58]

exhibits an exquisite molecular architecture for conducting H3K27 methylation (Fig. 9.1), a process subjected to delicate regulation such as allosteric stimulation [34–38].

First, EZH2 or related EZH1 (Table 9.1), which belongs to the SET domain family of lysine methyltransferases (KMTs), acts as enzymatic subunit of PRC2 for depositing the H3K27me2/3 marks to maintain a gene-repressive state [1, 7, 17–19]. EZH2 contains several distinct domains, with its C-terminal SET domain harboring intrinsic KMT activity. EZH2's CXC domain, characterized by three C-X(6)-C-X(3)-C-X-C motifs, precedes the catalytic domain and is required for

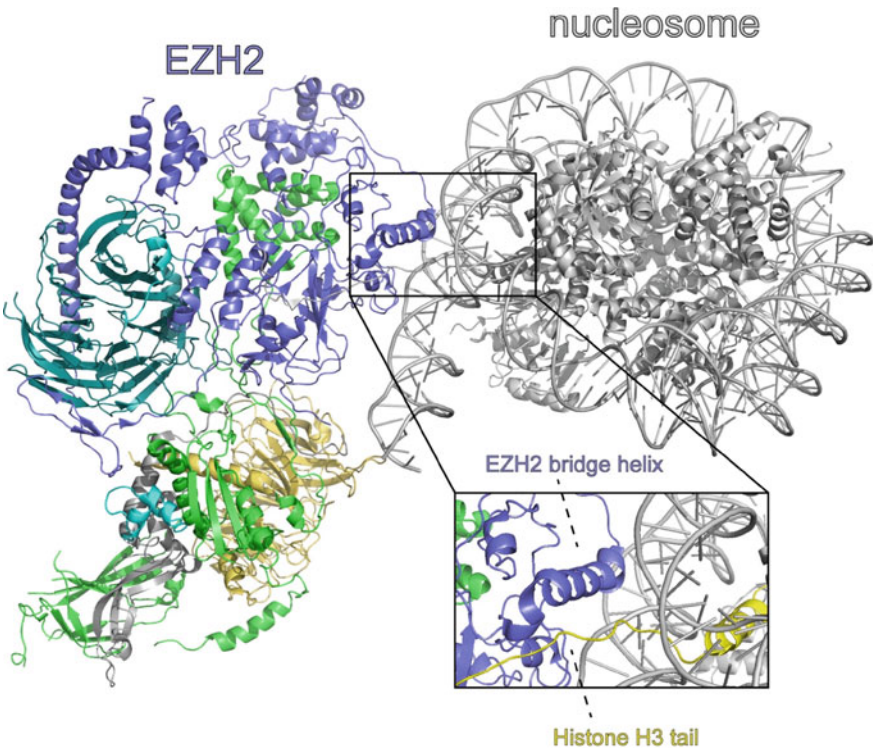

Fig. 9.1 The structure of PRC2-AEBP2-JARID2 complex bound to H2AK119ub1-modified nucleosome (PDB code: 6wkr). EZH2 is shown in blue and the nucleosome in gray. The EZH2 bridge helix recognizing histone H3 tail is highlighted in the lower right where histone H3 tail is shown in yellow

positioning the substrate (H3K27-containing histone tail) towards EZH2's catalytic center formed by SET. Moreover, two SANT domains of EZH2 allow it to bind DNA [59] while the ncRBD (non-coding RNA binding domain) and a DNA-binding domain can mediate EZH2 interaction with non-coding RNAs (ncRNAs) [60], all of which can provide the molecular force for PRC2 engagement and/or stabilization on chromatin. Interestingly, PRC2 can auto-methylate EZH2 at its lysines 510, 514, and 515, which are located within a disordered loop between EZH2's SANT2L and CXC domains, and such EZH2 auto-methylation activates PRC2 and enhances the methyltransferase activity [19, 61].

On the other hand, both EED and SUZ12 are required for EZH2 to operate as a KMT and, in their absence, EZH2 is autoinhibited [44–46]. In particular, EED is essential for allosteric activation of PRC2 upon EED binding to H3K27me3, the enzymatic product of PRC2, which immediately suggests a feedforward loop for spreading H3K27me3 along the chromatin [35, 37, 38, 45]. PRC2 stimulation by EED is achieved via its WD40 repeat domain, a 'reader' module that can directly

bind H3K27me3, leading to a conformational change of EZH2, which then disrupts the auto-inhibition of EZH2 and enhances the catalytic activity. The mammalian SUZ12, which contains two functional modules (namely, the N-terminal extension domain and the C-terminal VEFS domain), not only mediates the assembly of PRC2 core complex but importantly enhances binding of PRC2 core onto DNA or RNA [26, 41]. While SUZ12's VEFS domain is sufficient to assemble a stable minimally-active PRC2 core with EZH2 and EED, SUZ12's N-terminal extension module provides an additional protein–protein interaction platform to recruit accessory cofactors for regulating PRC2 (see below sections) [17, 42]. Some ancestor SUZ12 protein such as its orthologue in Tetrahymena lacks the N-terminal domain and is primarily made up by just VEFS [62]. Lastly, RBBP4 or RBBP7 directly binds to nucleosome and can recruit and/or stabilize PRC2 binding onto target chromatin, which is also critical for full KMT activity of PRC2. Also, additional roles for RBBP4/7 come from its histone modification 'sensing' activity because RBBP4/7 binding to nucleosome can be suppressed in the presence of histone H3 lysine 4 methylation (H3K4me), a gene activation-associated histone mark [19, 26, 47].

Various interfaces within PRC2 can bind to histone, DNA, RNA and accessory cofactor protein [1, 5, 11, 26, 40]. Based on accessory cofactors (Table 9.1), PRC2 can be classified into distinct subcomplexes, namely PRC2.1 and PRC2.2, which contain unique cofactor components. Besides PRC2 core, a PRC2.1 subcomplex contains its specific cofactors, including one of three Polycomb Like (PCL) family proteins such as PHD Finger Protein 1 (PHF1), Metal Response Element Binding Transcription Factor 2 (MTF2) and PHD Finger Protein 19 (PHF19), as well as EPOP (stand for Elongin BC and Polycomb Repressive Complex 2 Associated Protein) or PALI1/2 (stand for PRC2-Associated Ligand-dependent Co-repressor Isoform 1 or 2). A PRC2.2 sub-complex contains specific cofactors JARID2 (stand for Jumonji- and AT-rich Interaction Domain Containing Protein 2) and AEBP2 (stand for AE Binding Protein 2). Accessory cofactors of PRC2 are important for PRC2's targeting to the genome and/or its activity regulation. For example, JARID2 has intrinsic activities to mediate binding the mono-ubiquitination of histone H2A lysine 119 (H2AK119ub1, which is bound by JARID2's ubiquitin-interacting (UI) motif) [63], DNA and/or RNA. Likewise, the Extended Homologous (EH) region of PCL proteins can fold into a winged-helix structure and bind unmethylated DNA within CpG island (CGI) or DNA non-specifically [50, 64, 65] while their Tudor domain binds to histone H3 lysine 36 di- and tri-methylation (H3K36me2/3) [48, 66, 67], all of which are involved in the regulation of chromatin targeting and/or enzymatic activity of PRC2. There exists a canonical axis involving PRC2.1 → H3K27me3 → canonical PRC1 (cPRC1, which contains CBX2/4/6/7/8, a 'reader' of H3K27me3) → H2AK119ub1, as well as a separate axis involving noncanonical PRC1 (ncPRC1) → H2AK119ub1 → PRC2.2 (which contains JARID2, a 'reader' of H2AK119ub1) → H3K27me3. PRC2.1, PRC2.2, ncPRC1, cPRC1, and their coregulators and downstream readers (those specific to H3K27me3 and H2AK119ub1) act in concert to establish so-called polycomb domains in the genome [65, 68–73].

Recent works using histone site mutation in Drosophila [74, 75] and mouse embryonic stem cells (ESCs) [76] pointed to an essential role for H3K27 methylation in gene repression. First, H3K27me3 can serve as a docking site to directly recruit 'readers' for gene silencing and chromatin compaction; additionally, H3K27me3 can cross-talk with other chromatin modifications, thereby influencing transcription and epigenetic states. For example, H3K27me3 and H3K27 acetylation (H3K27ac) cannot coexist, thus providing a mechanism for repressing the gene-activation-related H3K27ac. In support, loss of PRC2 and H3K27me3 often results in global increase of H3K27ac [77–79]. Similar antagonism exists between PRC2 and H3K36me2/3 [80–82] or trithorax group (TxG) proteins, which mediates H3K4 mono-, di- and/or tri-methylation (H3K4me1/2/3) [1, 11]. H3K36me2/3 also provides a chromatin targeting mechanism for de novo DNA methyltransferases DNMT3A and DNMT3B [83]. Thus, rather complex interplays exist among H3K27me2/3, H3K36me2/3, H3K4me1/2/3, histone acetylation and DNA methylation, as well as the related chromatin-modulatory machineries. Readers shall also refer to comprehensive review articles published elsewhere [1, 3, 5, 7, 8, 11–13, 26, 81, 83–85].

In the following sections, we focus on how PRC2 and associated factors contribute to the initiation, development and malignant progression of cancers. We also discuss about recent advances in targeting EZH2 and PRC2 as therapeutics.

9.2 EZH2/1 Alterations in Cancer

EZH2, a catalytic subunit of PRC2, plays a crucial role in cancer development. Although this review focuses mainly on PRC2-dependent functions of EZH2, multiple types of action modes exist by which EZH2 exerts its multifaceted effect on transcriptomic and epigenomic regulation [8, 29, 86]. First and in a PRC2-dependent manner, EZH2 serves as a PRC2 catalytic submit to mediate H3K27 methylation for gene repression. Second and also in a PRC2-dependent fashion, EZH2:PRC2 complex can methylate non-histone proteins such as EZH2 automethylation [19, 61] and methylation of JARID2 [87] and GATA4 [88]. Third, EZH2 can modulate gene expression in a PRC2-independent fashion, either by direct methylation of non-histone protein or via interaction with coactivators and transcription factors (TFs) to activate target genes in a methylation-independent manner [29, 39, 86, 89]. Moreover, EZH2 forms interaction with RNAs and can regulate RNA stability and modification, mediating transcriptomic regulation [32, 33]. While EZH2:PRC2-mediated H3K27 methylation and related gene-repressive roles are widely known as canonical function, EZH2's PRC2-independent and methyltransferase-independent functions in transcriptomic and (epi)genomic regulation start to be appreciated, which are often referred to as EZH2's non-canonical functions [29, 39, 86, 89].

Depending on different contexts, EZH2 is recognized as an oncogene or tumor suppressor gene (TSG). In many tumor types, overexpression or gain-of-function (GOF) mutation of EZH2 frequently occurs, leading to enhancement

in probably both canonical and non-canonical activities of EZH2. EZH2:PRC2-mediated H3K27me2/3 is linked to repressed expression of transcripts related to anti-proliferation, differentiation, and tumor immunity. Meanwhile, EZH2's TSG function was also well established, partly due to the identification of EZH2 loss-of-function (LOF) mutation in human cancers and various EZH2 LOF studies in animal models. For instance, EZH2 missense mutations and/or truncation were centered on the EZH2 SET domain in hematological malignancies, which impairs EZH2:PRC2-mediated H3K27me2/3 deposition [90–93].

In the below sections, we discuss about cancer-related EZH2 deregulation and dependency seen in various cancers, especially those advanced ones that typically show poor prognosis in the clinic.

9.2.1 EZH2 Overexpression in Human Cancers

Dysregulation of the EZH2 gene, located at chromosome 7q36.1, is frequent in a wide range of human cancers, which can be caused by copy number variation (CNV), somatic mutation, transcriptional change, and alteration in regulatory microRNA [94–98]. For example, SOX4 and miR-101 regulate EZH2 expression at transcriptional and post-transcriptional levels, respectively [97, 99]. Also, EZH2 activity is highly intertwined with proliferative signals of cells. For example, E2F tightly regulates EZH2 expression [94]; likewise, cellular activities of c-Myc and EZH2, both of which are known to be associated with the cell cycle status, are regulated in a coordinated fashion, partly via PTEN-AKT signaling, a node often abnormally activated in cancer [100].

Initially, Varambally and coworkers reported CNV (amplification) of the EZH2 locus and genomic loss of EZH2-targeting miR-101 in hormone-refractory metastatic prostate cancer (PCa), which lead to elevated EZH2 at both mRNA and protein levels [97, 101]. They further showed that ectopic expression of EZH2 in PCa cells induced repression of target genes, a process that relied on the SET domain [101]. Later, a number of reports further showed that EZH2 overexpression is frequent in many other cancers such as B-cell lymphomas [98, 102, 103] and solid tumors [95], which will not be discussed in this Chapter. Please also note that cancer may exhibit exquisite EZH2 dependency even if it is not overexpressed. Indeed, EZH2 was found to be essential for tumorigenesis of MLL-rearranged acute leukemia [104–109] or cancers carrying LOF mutation of SWI/SNF remodelers [108, 110, 111]. Overall, enhanced EZH2:PRC2 functionality is linked to optimal silencing of TSGs and poor prognosis of cancer patients [60, 95, 108, 112]. A TSG locus often repressed by EZH2:PRC2 is INK4A/ARF [109, 113, 114], which produces the p16(INK4a) and p19(ARF) proteins via alternative splicing to, respectively, inhibit cyclin-dependent kinase 4 and 6 (CDK4/6) and activate p53 by interfering with the p53 antagonist MDM2 [115]. In addition to TSGs, EZH2:PRC2 also represses transcripts related to terminal cell differentiation [96, 107, 116, 117]. More recently, EZH2:PRC2 has been reported to silence the immunity-related transcripts, such as those involved in the innate immune system

and major histocompatibility complex class I or II (MHC-I or MHC- II) antigen processing and presentation [118–120], as well as endogenous retroviruses (ERVs) [121, 122]. ERV is a potent inducer of anti-viral response. Thus, it has become increasingly clear that EZH2:PRC2 and H3K27me3 function to repress a suite of genes related to anti-proliferation, cell differentiation and immune response, thus keeping cancer cells in a highly proliferative, less differentiated state while helping produce an immunologically silenced tumor microenvironment (TME) at the same time.

In addition to the above-mentioned canonical functions (i.e., EZH2:PRC2 and resultant H3K27me3), increasing amount of evidence also points to EZH2's non-canonical functions unrelated to methyltransferase domain or gene repression (for details, please see Sect. 9.2.5). For example, EZH2 overexpression does not correlate well with expected increase of H3K27me3 in triple-negative breast cancer (TNBC) [123], indicative of a PRC2-independent role. EZH2 harbors several cryptic transactivation domains (TADs), which can directly bind TFs and (co)activators for mediating gene activation [124–127]. EZH2's TADs are located within the amino acids 135–250 and carry typical, partially disordered Φ-Φ-x-x-Φ motifs, in which Φ represents a hydrophobic residue and x can be any residue. TADs of EZH2 were reported to directly interact with (co)activators, such as the histone acetyltransferase (HAT) p300, and oncogenic TFs, such as c-Myc in hematological cancer [127] and androgen receptor (AR) in PCa [126]. Post-translational modifications (PTMs) of EZH2 (please also see Sect. 9.2.4), such as serine and threonine phosphorylation, play vital roles in regulating EZH2 activity and potentially regulating EZH2:PRC2 assembly/disassembly, which may serve as a regulatory mechanism for switching EZH2 between canonical and noncanonical complexes, the detail of which remains to be fully understood.

9.2.2 Gain-Of-Function (GOF) Mutation of EZH2 in B-cell Lymphomas and Melanomas

Besides EZH2 overexpression, its GOF mutation is also associated with enhanced PRC2 activity. GOF mutation of EZH2 was detected in approximately 10–20% of germinal center (GC)-derived B-cell lymphomas, such as follicular lymphoma and diffuse large B-cell lymphoma (DLBCL), and roughly 3% of melanoma patients [108, 128–131]. GOF mutation of EZH2 is heterozygous and primarily targets the tyrosine 641 (Y641) located in the SET domain of human EZH2 [128–131]. Y641X-mutated EZH2 (X represents F, C, H, S, or N) does not affect PRC2 assembly and instead, shows an altered activity in H3K27 methylation, that is, enhanced capability for depositing H3K27me2/3 and a reduced activity in generating H3K27me1 [108, 128, 129, 131] (Fig. 9.2a). Such preference of Y641X-mutated EZH2 contrasts with that of wildtype (WT) EZH2, which exhibits the highest activity in inducing H3K27me1 from unmethylated H3K27 and yet, a much more reduced capability for depositing H3K27me2/3. Thus, EZH2-Y641X can act in concert with WT-EZH2 to progressively methylate H3K27 towards a

highly methylated state leading to globally increased H3K27me3 in cancer, which explains why EZH2-Y641X mutation is primarily heterozygous in patients [108, 128–131]. Two relatively rare GOF mutations of EZH2, A677G and A687V, were found in about 1–2% of lymphomas, which show similar PRC2 enhancement effect as EZH2-Y641X [108, 132–134].

Using murine models with Ezh2-Y641F, researchers substantiated the roles of EZH2 GOF mutation, either alone or in combination with other cooperative mutations, in promoting tumorigenesis, which was concurrent with alterations of epigenetic and gene expression profiles [130, 135–140], immunity and TME [141, 142]. In particular, EZH2-Y641X-associated global increase of H3K27me3 often resulted in a stably repressive, more compacted state at target chromatin [138, 139]. Counterintuitively, EZH2-Y641X can also lead to upregulation of certain target genes, possibly due to an effect on diluting H3K27me3 'reader' or effector proteins such as PRC1 [130, 142].

9.2.3 Loss-Of-Function (LOF) Mutation of EZH2, Centered at Its Catalytic Domain, in Hematological Neoplasms

Direct sequencing of patient samples also found LOF mutation or deletion of EZH2 (Fig. 9.2b) among certain subtypes of hematological cancers and disorders, which include myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), acute myeloid leukemia (AML), and T-cell acute lymphoblastic lymphoma (T-ALL) [90–93, 143]. Many of these reported EZH2 mutations were either deletion, nonsense, or frameshift-generating aberrant EZH2 transcripts, indicative of a LOF nature. In agreement, EZH2 missense mutations occur mainly at those amino acids critical for enzymatic activity or allosteric activation of PRC2 (namely, SET and adjacent CXC domains) [16, 43, 90–92]. Deletion of EZH2 was found to be correlated with a poor prognosis in patients with MDS [92] and associated with chemotherapy resistance of AML and T-ALL cells [91, 144, 145]. Studies using murine models carrying LOF or knockout of Ezh2, either alone or in combination with other leukemia-associated mutation (such as $NRAS^{G12D}$), have demonstrated that EZH2 inactivation changed the epigenetic landscape of cancer-initiating cells, contributed to leukemic transformation, and predisposed animals to develop heterogeneous malignancies (such as murine MDS, MDS/MPN and T-ALL) [93, 146–148]. In particular, EZH2 loss leads to reduced H3K27me2/3 and increased H3K27ac, and the latter histone mark is 'read' by Bromodomain and Extra-Terminal (BET) domain containing proteins such as BRD4, which then act to substantiate active transcription of oncogenic pathways, such as JAK/STAT or NOTCH1, to promote oncogenesis [93, 147, 149, 150]. Some of EZH2 inactivation-associated phenotypes can be recapitulated by inactivation of another PRC2 core component, such as Suz12 loss in murine T-ALL models [151], pointing to a tumor-suppressive role of EZH2:PRC2 in these diseases. Interestingly, when EZH2 is absent or non-functional, the remainder of H3K27me2/3 could be maintained by EZH1, the only other available enzymatic subunit of PRC2 [147,

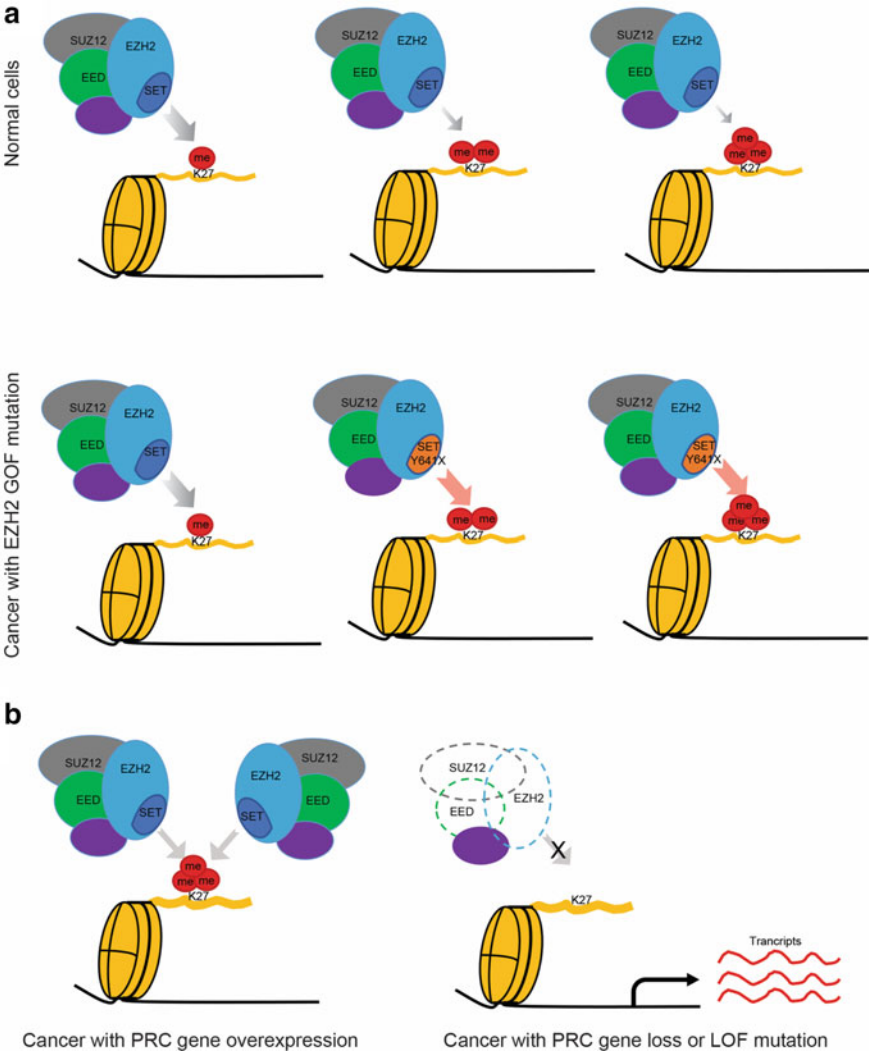

Fig. 9.2 Deregulation of EZH2:PRC2-mediated H3K27me3 deposition in cancers. **a** Top: in normal cells, PRC2 has the highest activity in depositing H3K27me1 and relatively lower activities in depositing H3K27me2 and H3K27me3. Bottom: cancer cells with EZH2-Y641X GOF mutations show the globally increased H3K27me3 level. WT EZH2:PRC2 and that assembled by EZH2 Y641X mutant act in concert to enhance progressive methylation reactions towards H3K27me2/3. **b** Overexpression (left) or depletion and LOF mutations (right) of PRC2 core subunits in various human cancers

152, 153]. In animals with Ezh2 insufficiency, Ezh1 activity was found to be essential for the maintenance of diseases [147]. What was interesting here is that deletion of Ezh1 alone did not cause tumorigenic transformation but combinational loss of Ezh1 and Ezh2 abolished repopulating capacity of leukemia-causing stem cells [147]. A similar EZH1 requirement and co-essentiality was reported in other tumors carrying PRC2:EZH2 inactivation [146, 150], indicating a potential treatment strategy. These studies also suggest that a redistributed pattern of H3K27me3/2 due to the remainder EZH1:PRC2 complex, together with global H3K27me3 loss and H3K27ac gain caused by EZH2:PRC2 inactivation, leads to oncogenic transformation. Gu and coworkers further reported that, in both murine and human leukemias, EZH2-deficient leukemia-initiating cells exhibited epigenetic alterations and a rewired metabolic program, as exemplified by aberrant activation of Branched Chain Aminotransferase 1 (BCAT1), an EZH2:PRC2 target and critical enzyme that catalyzes the reversible transamination of branched-chain amino acids (BCAA, which are essential amino acids such as valine, isoleucine and leucine) [146]. These works provide a critical glimpse to how EZH2:PRC2 loss can alter epigenetic, transcriptomic and metabolic profiles of cancer cells.

9.2.4 Post-Translational Modification (PTM) of EZH2 Regulates Its Activity and Stability

Many amino acids within EZH2 are amenable to PTMs such as phosphorylation, acetylation, methylation, *O*-GlcNAcylation, and ubiquitination. PTMs play a critical role in regulating EZH2 activity and stability [154].

Previously, Cha et al. reported phosphorylation of EZH2 at serine 21 (EZH2-pS21) by PI3K/AKT signaling in breast cancer [155]. EZH2-pS21 was shown to inhibit EZH2:PRC2 methyltransferase activity by attenuating EZH2 binding to histone H3, resulting in de-repression of PRC2 target genes. Similarly, Li and colleagues demonstrated that AMPK phosphorylates EZH2 at threonine 311 (EZH2-pT311) to inhibit EZH2 binding with SUZ12, which resulted in de-repression of oncogenes in ovarian and breast cancers [156]. On the other hand, phosphorylation of human EZH2 threonine 345 (EZH2-pT345) by CDK1/2 was found to be required for EZH2-mediated H3K27me3 in PCa [157]. EZH2 phosphorylation may also affect its activity on nonhistone targets. In glioblastoma multiforme stem-like cells, AKT-induced EZH2-pS21 was reported to promote the methylation of STAT3 by EZH2 [158]. During tumor progression, methylated STAT3 has the enhanced activity by increasing STAT3 tyrosine phosphorylation [29]. Despite these advances, additional studies are merited to dissect the mechanisms underlying the altered KMT activity of EZH2. Moreover, EZH2 phosphorylation is likely to be involved in regulating the PRC2-independent function of EZH2 (for details, see Sect. 9.2.5). In advanced PCa with activated PI3K/AKT signaling, EZH2-pS21 appeared to turn EZH2 into a transcriptional (co)activator, which acts independently of PRC2:EZH2 [89]. The transcriptional (co)activator role of EZH2 was also described in natural killer/T-cell lymphoma where EZH2

Y244 is phosphorylated by JAK3 [159]. EZH2-pY244 was reported to be absent from in its repressor form, EZH2:PRC2 [124].

Acetylation and methylation are additional PTMs recently detected in EZH2 that can regulate its activity/stability in cancer. K348 of EZH2 can be acetylated by P300/CBP Associated Factor (PCAF), a lysine acetyltransferase [160]. EZH2-K348ac, which enhances EZH2 stability, potentiated EZH2:PRC2 activity in target gene repression and strengthened lung cancer metastasis [160]. Later, Zeng et al. found that di-methylation of EZH2-K307 by SMYD2 improved its stability in breast cancer, as a result, stabilized EZH2 promoted the proliferation and invasion of breast cancer cells by TSG repression [161]. On the contrary, monomethylation of EZH2-K735 mediated by SETD2 was reported to promote EZH2 degradation, which then suppressed PCa metastasis [162]. Another reversible PTM in EZH2 is *O*-GlcNAcylation, deposited by *O*-linked *N*-acetylglucosamine transferase (OGT), on the hydroxyl group of serine or threonine side chain. This type of modification was reported to affect EZH2 stability and activity [154, 163, 164]. In addition, ubiquitination is a well-known PTM event that covalently adds ubiquitin, a 76-amino-acid-long polypeptide, onto protein substrate for promoting proteosome-mediated degradation or modulating target protein functions. In cancers, mechanisms exist to either promote or inhibit EZH2 stability by antagonizing or inducing its ubiquitination [154].

9.2.5 Non-canonical, PRC2-Independent Roles of EZH2 in Transcriptional Activation

Aside of being a catalytic subunit of EZH2:PRC2 (see Sects. 9.2.1 and 9.2.2), EZH2 also acts independently of PRC2 and can exert non-canonical gene-activation effect by interacting with TFs and transcriptional (co)activator in various diseased settings, notably cancer [165, 166]. In the AR-positive PCa, EZH2 interacts with AR and functions as a coactivator of AR [89, 167, 168]. Using a castration-resistant PCa (CRPC) cell line (an androgen-independent LNCaP cell derivative) as a model, Xu and colleagues initially demonstrated that a subset of EZH2-bound targets were devoid of H3K27me3, indicating a lack of active PRC2 at these sites [89], instead, these EZH2- 'solo' sites are co-bound by AR and H3K27ac [89]. Knockdown (KD) of EZH2 or AR, but not the PRC2 cofactor (SUZ12), caused decreased occupancies of AR, EZH2 and gene-active histone marks at their shared EZH2- 'solo' sites leading to decreased expression [89]. These results supported a direct role of EZH2 in stimulating transcription of a subset of AR target genes in PCa. Here, PRC2-independent methyltransferase activity was also proposed to be necessary since the enzymatically inactive mutant of EZH2 failed to rescue the EZH2 loss-associated defect in target gene activation [89]. Different from the report of Xu et al. [89], a separate study showed that a non-catalytic function of EZH2 in PCa exists independently of both PRC2 and its methyltransferase activity because many of EZH2-activated genes were not down-regulated by the EZH2 enzymatic inhibitor [168]. In the latter study, authors

also demonstrated that EZH2 binds directly to the AR promoter activating AR expression [168]. Furthermore, both studies found that the phosphorylation level at EZH2 S21 was elevated in PCa cell lines. A more recent work used 22Rv1 cells, a classic model of CRPC, and reported that EZH2- 'solo' sites account for a large majority of mapped EZH2 targets, part of which were co-bound by AR and AR splice variant 7 (AR-V7), a constitutively active form of AR [126]. Interestingly, a proportion of relapsed PCa tumors after AR-pathway inhibitor (ARPI) treatment exhibited loss of luminal identity with activation of stem cell and neuronal programs [169]. In the ARPI-treated cells, AR bindings are reprogrammed to regulate stem cell and neuronal gene networks [169]. Here, EZH2-T345 phosphorylation by CDK1 enables EZH2 to co-occupy those sites with re-targeted AR, forming a non-canonical AR:EZH2 complex, together with SUZ12 but not EED, to promote gene activation [167]. Notably, those de novo AR targets found in ARPI-resistant cells are not accessible in the original CRPC cells [167].

Although the gene activation function of EZH2 has been proposed, several key questions remain unaddressed. First, what is the molecular determinant as to EZH2's dual activities and switching between the PRC2-related repression function and the gene-activation-related one? Second, how does EZH2 interact with AR in PCa, and how is EZH2 recruited or retargeted to those 'solo' sites? Does EZH2 stimulate transcription in other cancers? To decipher the molecular mechanisms, researchers have further found that EZH2 harbors multiple cryptic TADs capable of interacting directly with components of active transcription machinery [124, 127]. These TADs (within amino acids 135–250) cover the SRM and SANT1 domains of EZH2, which are also involved in PRC2 assembly and H3K27 methylation. A crystal structure of EZH2-EED binary complex indicates that EZH2's TADs are entirely sequestered [124]. TADs of EZH2 have typical, partially disordered Φ-Φ-x-x-Φ motifs (Φ: hydrophobic residues, x: any residues), mimicking the TADs of archetypal acidic activators such as p53 and VP16 [124, 127]. Unlike the TADs of p53 and VP16, the EZH2-TAD in the native state is structurally 'locked' in an α-helix bundle structure by the SANT1-Binding Domain (SBD) of EZH2, making EZH2-TADs unable to interact with TFs or coactivators [124]. EZH2-TADs can potentially be unlocked by cancer-specific phosphorylation such as EZH2-pS21 and EZH2-pY244, which weakens the SBD−SANT1 complex and makes the EZH2-TAD disordered, thereby exposing TAD core fragment for cofactor binding [124]. It has been speculated that EZH2-TAD can mediate protein oligomerization with co-activators such as P300 to promote transcription activation [124]. In consistent, Wang et al. reported that EZH2-TADs are functionally important in MLL-rearranged leukemia [127] and CRPC [126]. In MLL-rearranged leukemia, EZH2 interacts with c-Myc, p300, SWI/SNF and RNA polymerase II (Pol II) through its TAD at the mapped EZH2- 'solo' sites [127]. EZH2- 'solo' sites exhibit features of transcriptional action instead of repression in MLL-r leukemia [127] and CRPC [89, 126]. EZH2 depletion decreased both EZH2 and c-Myc recruitment to their co-binding sites [127]. Importantly, the EZH2-TAD-defective mutants failed to rescue EZH2 loss-caused defects in cancer cell growth [126,

127]. Taking advantage of the proteolysis targeting chimeras (PROTAC) technology, an EZH2 PROTAC or degrader termed MS177 was identified, which effectively depletes EZH2 and associated factors, which include PRC2 and TFs namely, c-Myc in MLL-r leukemias [127] and AR/AR-V7 in CRPC cells [126]. Accordingly, MS177 showed much better therapeutic effect than the matched EZH2 inhibitor in both in vitro and in vivo settings [126, 127].

EZH2 interaction with MYC (c-Myc or N-Myc) and its PRC2-independent targeting also exist in other tumors, such as Peripheral T cell lymphoma (PTCL), neuroblastoma, and small cell carcinoma [127, 170, 171]. Interaction with EZH2 boosts the transcription activity of MYC. Depletion of EZH2, but not inhibition of its methyltransferase activity, impaired transcription regulated by MYC [170, 171]. Also, EZH2 can protect N-Myc from FBW7a-mediated degradation in neuroblastoma [171], therefore stabilizing N-Myc protein and promoting tumorigenesis. In PTCL, overexpressed N-Myc appears to hijack EZH2 to form the EZH2:N-Myc complexes for enhancing tumor progression [170]. This interaction might be regulated by EZH2-pT487, a PTM induced by CDK1; and CDK1 is a direct target of EZH2 and N-Myc, indicating a feedforward loop [170]. Consistent to this notion, the gene activation functions of EZH2 were also reported in natural killer/T-cell lymphoma where EZH2 Y244 is phosphorylated by JAK3 [159], also, EZH2 associated with RelA/RelB to activate NF-kB signaling in estrogen receptor-negative breast cancers [172–174]. While both EZH2 and TRIM28 are well known as corepressors, they actually also form a non-conventional complex with SWI/SNF subunits in breast cancer cell lines, and in this case, they stimulated transcription of genes related to mammary cancer stem cell biology and mammosphere formation [175]. The TRIM28 interaction region within EZH2 was mapped to the amino acids 385–618, which encompass the pre-SET domain and not TAD [175]. Thus, noncanonical gene activation-related functions of EZH2 appear complex and context-dependent, and many disease-associated mutations of EZH2 or cofactor probably need to be re-visited with consideration of EZH2's non-canonical functions, which awaits further investigation.

9.2.6 EZH1 Mutations in Thyroid Tumors

Besides EZH2, EZH1 also can serve as the enzymatic subunit of PRC2. A study indicated that the Ezh1 gene arose from an event of Ezh2 gene duplication [176]. EZH1:PRC2 and EZH2:PRC2 often compensate one another [147, 152, 153]. Recent structural characterization of EZH1:PRC2 revealed a role in chromatin compaction [177], in addition to H3K27me3 deposition [147, 152, 153]. Thyroid tumors with follicular growth pattern often harbor RAS mutations and PPARG rearrangement, and in those clinically benign and RAS-negative follicular-patterned thyroid tumors, two hotspots of EZH1 mutations were identified [178, 179]. Functions of these EZH1 mutations are largely unknown and were speculated to be LOF ones [178, 179], which merits further investigation.

9.3 Alteration of Core Subunit (SUZ12 and EED) or Axillary Cofactor of PRC2 Occurs Frequently in Cancers, Enhancing Oncogenesis

9.3.1 Cancer-Associated Upregulation of EED and SUZ12

Alteration and mutation of SUZ12 are frequent in human cancers. Previously, it has been shown that SUZ12 expression is controlled by proliferation-related TFs, such as E2F1 and TCF/beta-catenin [180, 181]. Increased binding by TCF4 at the SUZ12 promoter was found to be correlated with SUZ12 overexpression in tumors compared to normal tissues [180]. In non-muscle-invasive bladder cancer (NMIBC), E2F1 directly binds the proximal EZH2 and SUZ12 promoters, upregulating transcription of both [181]. Akin to EZH2 overexpression in cancer, upregulation of SUZ12 has been reported in a range of tumors, including breast cancer, epithelial ovarian (EOC), colorectal carcinoma (CRC), head-neck squamous cell carcinoma (HNSCC), gastric cancer, non-small cell lung cancer (NSCLC), and bladder cancer [180–187]. Ectopic expression of SUZ12 is generally positively correlated with tumor cell proliferation, migration, invasion and aggressive clinical features, indicating the oncogenic function of SUZ12 overexpression. Genetic depletion of SUZ12 in a number of cancer models led to suppressed tumor growth and induced apoptosis [182, 183, 185, 186]. Mechanistically, SUZ12 suppresses the expression of critical TSGs. For example, disruption of SUZ12 in gastric cancer cells decreased H3K27me3 at KLF2 and E-cadherin, two TSGs of gastric cancer, leading to elevated TSG expression and impaired tumor growth [187]. Likewise, EED was also found overexpressed in CRC and breast cancer [185, 188]. Compared to primary breast cancers, samples of breast cancer lymph node metastasis displayed overexpression of EED, which downregulates TSGs such as E-cadherin to promote epithelial-mesenchymal transition (EMT) in lymph node metastatic process [188]. Thus, elevated SUZ12 and EED in cancer can enhance PRC2 activity, in agreement with what was observed for EZH2 overexpression.

9.3.2 Inactivation Mutation of EED or SUZ12 in Hematological Cancers and Malignant Peripheral Nerve Sheath Tumors (MPNST)

SUZ12 or EED can also act as a bonafide TSG under different contexts. In particular, LOF mutation of SUZ12 and EED, such as deletion and frameshift mutation, occurs frequently in human T-ALL; these lesions caused PRC2 disruption and global H3K27me3 loss, which then led to aberrant activation of JAK3 and/or NOTCH1 signaling [93, 151, 189]. In support, Broux et al. used murine models and showed that Suz12 loss cooperates with JAK3 activating mutation to promote development of T-ALL [151]. EZH2 LOF mutation was also detected in T-ALL [93, 189]. Unlike a relatively higher rate of EZH2 mutations found in myeloid

malignancies and disorders [90–93], EED or SUZ12 mutation was reported to be markedly lower or rare in these cancers [147, 190], but EED's H3K27me3-binding and EZH2-binding interfaces were preferentially targeted by mutations in myeloid cancers [43, 191]. These observations highlight an involvement of PRC2:EZH2 inactivation during genesis of hematological malignancy and disorder such MDS, MNP, and T-ALL.

LOF mutation of SUZ12 and EED is also common in patients with MPNSTs, a rare aggressive sarcoma showing poor prognosis [78, 192]. SUZ12 and EED LOF mutations in MPNSTs are biallelic. As expected, MPNSTs harboring such SUZ12 or EED inactivation mutation showed H3K27me3/2 reduction (almost a complete loss), increases of H3K27ac and H3K36me2, and aberrant activation of PRC2 targets including multiple homeobox-containing TFs and master regulators [78, 192, 193]. Re-introduction of EED or SUZ12 into MPNSTs restored H3K27me3 and decreased tumor cell growth. Somatic alterations of the TGSs, CDKN2A, and NF1, co-occur frequently with PRC2 inactivation, suggesting their cooperation in causing MPNST [78]. Please note that PRC2 inactivation often leads to upregulation of CDKN2A, indicative of a selection pressure during MPNST evolution. Using animal models, De Raedt et al. reported that Suz12 inactivation acts in concert with as mutation of Nf1, a Ras GTPase-activating protein, to induce a wider spectrum of murine tumors that resembled MPNSTs, gliomas and/or melanomas [194]. Unlike mutational spectrum of PRC2 (i.e. somatic mutation of EZH2, EED or SUZ12) in myeloid malignancies, LOF mutation of EZH2 or EZH1 was never reported in MPNSTs [78, 192]. This points to distinctive dependency on PRC2 subunits under various contexts. A recent investigation showed that additional depletion of EZH1/2 did not significantly alter transcriptomic profiles of MPNST cells harboring SUZ12/EED inactivation [150]. In the ipNF05.5 plexiform neurofibroma cell line (a model mimicking cell origin of MPNSTs), transcriptomic changes caused by EZH1/2 loss closely resembled those by SUZ12/EED loss [150]. These observations thus argued against a PRC2-independent function of EZH2 and supported that EZH2 and EZH1 are redundant and only act as a PRC2 factor in the context of MPNSTs.

SUZ12 was also found downregulated in hepatocellular carcinoma (HCC) where SUZ12 silencing promoted the migration and invasion of HCC cells [195]. Likewise, EED downregulation was reported in squamous cell carcinoma (SCC) [196]. Lung adenocarcinoma (ADC) and SCC are regarded as two segregated entities of NSCLC with unique genetic profiles. Conversion of ADC to SCC has been associated with targeted therapy for ADC [197]. Relative to ADC, SCC showed higher EZH2 but a lack of EED at the protein, but not transcriptional, level, indicating a mechanism interfering with EED translation or protein stability [196]. Additionally, gain of H3K27ac and H3K4me3 at squamous lineage genes, such as Sox2, ΔNp63, and Ngfr, was reported in SCC lacking EED [196]. Although EED appeared a TSG in SCC, EZH2 was regarded as an oncogene in both ADC and SCC [196]. Thus, SUZ12 or EED loss might lead to loss of EZH2:PRC2 complex

and yet promote PRC2-independent functions of EZH2 to support SCC progression, as discussed in Sect. 2.5. Further investigation is warranted to dissect the underlying oncogenic mechanism.

Lastly, other PRC2 core factors such as RBBP4 and RBBP7 were also found mutated and altered in tumors, for which readers shall refer to a recent review [43].

9.3.3 Mutations Affecting Auxiliary Cofactors of PRC2 in Cancer

PRC2 can be divided into PRC2.1 and PRC2.2 subcomplexes, based on their specific accessory factor (Table 9.1). As mentioned above, PRC2 auxiliary cofactors can profoundly influence the PRC2 targeting to the genome [70, 198]. Their deregulations are also very common in cancers.

In endometrial stromal sarcomas (ESS) and ossifying fibromyxoid tumor (OFMT), PHF1, which encodes a PRC2.1-specific auxiliary cofactor, undergoes aberrant gene rearrangement, which generates a chimeric gene between PHF1 and one of its fusion partners [199–201]. Here, PHF1's fusion partners include JAZF Zinc Finger 1 (JAZF1), Enhancer of Polycomb homolog 1 (EPC1), EP400, MYST/Esa1 Associated Factor 6 (MEAF6), and Transcription Factor Binding To IGHM Enhancer 3 (TFE3) [199–202]. Interestingly, most of these PHF1 fusion partners (EP400, EPC1, JAZF1, and MEAF6) are members of an evolutionarily conserved NuA4/TIP60 acetyltransferase complex. NuA4/TIP60 mediates histone acetylation and/or H2A.Z deposition during gene activation [203–205]. In ESSs, chromosomal rearrangements also exist to generate in-frame fusion between another PRC2 cofactor (SUZ12 or EZHIP) and the NuA4/TIP60 complex subunit, (JAZF1 or Malignant Brain Tumor Domain-containing 1 (MBTD1)) [206–210]. Further investigations of these fusions point to a common pathway leading to PRC2 misregulation. First, Hofvander et al. found that the genes most upregulated after overexpression of aberrant PHF1 fusions in fibroblast cells largely overlapped with those in OFMT tumors versus normal samples, suggesting a causal role for PHF1 fusion in this disease [200]. Further, the EPC1-PHF1 and JAZF1-SUZ12 chimeric proteins are able to assemble a mega-complex that contains both NuA4/TIP60 and PRC2,and this caused a decrease of PRC2-generated H3K27me3, increase of histone acetylation, and elevated expression of PRC2 target genes such as HOX gene clusters [211, 212]. Thus, EPC1-PHF1 and JAZF1-SUZ12 context-dependently act as potent transcription activator by recruiting NuA4/TIP60 acetyltransferase complex, which in turn antagonizes PRC2. This mechanism is likely to be applicable to other ESS/OFMT-associated fusions such as JAZF1-PHF1, MEAF6-PHF1, and MBTD1-EZHIP.

MBTD1 was also found aberrantly fused to ZMYND11, a reader of H3.3-specific H3K36me3 [213, 214], in a subset of human AMLs [215]. Two groups independently demonstrated that ZMYND11-MBTD1 induces leukemogenesis through hijacking NuA4/TIP60 to block PRC2-mediated H3K27me3 deposition, as a result, PRC2- silenced proto-oncogenes such as Hox genes and Meis1 were

reactivated [216, 217]. In this scenario, a PWWP domain retained in ZMYND11-MBTD1 fusion directly binds H3.3K36me3, and because H3.3K36me3 is associated with active transcription, ZMYND11-MBTD1 effectively links the oncogene transcription together with histone acetylation, thereby establishing a feed-forward loop for activation [217]. Deregulated crosstalk between PRC2 and histone acetyltransferase machinery is a common theme during oncogenesis.

In refractory AMLs, loss of MTF2/PCL2 de-repressed MDM2, an antagonist and E3 ubiquitin ligase of p53, which then conferred chemoresistance due to defect in cell cycle regulation and apoptosis in the MTF2-deficient $CD34^{+}CD38^{-}$ cells [218]. Conversely, MTF2 expression or MDM2 inhibition re-sensitized refractory leukemic cells to chemotherapeutics and prevented relapse in AML patient-derived xenograft (PDX) models, supporting a TSG role of MTF2 [218]. However, MTF2 function in cancers is context-dependent. For example, MTF2 was also found to be highly expressed in gliomas where its overexpression promotes proliferation and enhances colony formation [219]. In glioma, MTF2 overexpression led to upregulation of EZH2 and EED thus altering the chromatin state [219].

PHF19 is often overexpressed in cancers. In multiple myeloma (MM), PHF19 overexpression/amplification was found associated with malignant progression and poor prognosis [80, 220]. Mechanistically, PHF19 is critical for maintaining proper H3K27me3 landscape because PHF19 depletion led to loss of broad H3K27me3 domains as well as de-repression of PRC2 targets involved in cell cycle inhibition and tumor suppression [80]. In MM, oncogenic effect of PHF19 relies on its various modules required for PRC2 interaction and chromatin binding, thus pointing to a role for PHF19 in the chromatin targeting of PRC2 in this disease [80]. In glioblastoma (GBM), PHF19 can sustain β-catenin levels by directly repressing Seven In Absentia Homolog 1 (SIAH1), an E3 ubiquitin ligase of β-catenin [221]. In PCa and HCC, PHF19 also promotes tumor cell growth, metastasis, and invasion [222, 223].

AEBP2 and JARID2 are subunits specific to PRC2.2 (Table 9.1). Compared to PRC2.1, the PRC2.2 subcomplex has higher chromatin-binding affinity and higher enzymatic activity; thus, PRC2.2 dysregulation can profoundly affect chromatin and transcriptional states. For example, JARID2 was reported as an oncogene and overexpressed in different cancers. In rhabdomyosarcomas (RMS), the hallmark fusion protein, PAX3-FOXO1, directly binds the JARID2 gene, leading to its upregulation [224]. And a high level of JARID2 was found associated with metastasis whereas JARID2 depletion resulted in reduced cell proliferation and myogenic differentiation, coupled with increased expression of Myogenin (MYOG) and Myosin Light Chain (MYL1) in RMS cell lines [224]. Mechanistically, JARID2-PRC2.2 complex deposits H3K27me3 at the MYOG and MYL1 promoters, repressing their expression [224]. In ovarian cancer and HCC models, JARID2 was also found overexpressed compared to normal controls and can potentiate tumor cell proliferation, migration, invasion and metastasis [225, 226], here, JARID2 reduces expression of the TSGs such as Phosphatase and Tensin homolog (PTEN) while inducing expression of the oncogenes such as PI3K and AKT [225, 226]. In leukemia, colon cancer and uterine adenocarcinoma, a

missense mutation of SUZ12 (R103P/Q) disrupted binding to JARID2 and yet enhanced binding to MTF2, leading to a PRC2.2-to-PRC2.1 switch and altered PRC2 occupancy [198]. Quite distinct from what was seen in cancers with JARID2 over-expression, JARID2 also acts a TSG in chronic myeloid disorders—its loss by genomic deletion is common among non-malignant MPN and MDS [227]. In a murine MPN model, JARID2 loss caused disease progression to secondary AML with the reduced survival of mice [227]. Mechanistically, JARID2 recruits PRC2.2 to repress self-renewal-related pathways in hematopoietic stem/progenitor cells, and its loss de-represses self-renewal genes such as Runx1t1 and Mycn, thus promoting malignant transformation and AML progression [227]. AEBP2 was also reported to enhance cancer progression in ovarian cancers [228]. Knockout of AEBP2 inhibited tumor cell proliferation and increased cell sensitivity to cisplatin, a chemotherapy medication used to treat cancers [228].

9.4 PRC2 Malfunction Caused by Onco-Histone and Inhibitory Factor

9.4.1 H3K27M Onco-Histone

Often, PRC2 malfunction in cancer is not directly caused by a mutation of its subunit per se, but rather that on its substrate. Lysine-to-methionine mutation at lysine 27 of histone H3, either H3.1 or H3.3 variant (termed H3K27M onco-histone) is a driver of high-grade pediatric glioma [229–231]. H3K27M was detected in about 80–85% of diffuse intrinsic pontine glioma (DIPG) cases [229–231]. H3K27M acts as a dominant negative inhibitor of PRC2. Although this H3 mutant accounts for only 3–10% of total H3 in cells, it significantly impairs genome-wide deposition of H3K27me2/3 by inhibiting or restricting PRC2 activity, meanwhile, focal gain of H3K27me3 at certain PRC2 sites was also observed [232–235]. Please note that EZH2 is automethylated at K510 and K514 and such auto-methylated EZH2 exhibits a higher level of methyltransferase activity by inducing PRC2 allosteric activation, which is required for spreading of H3K27me2/3 [61]. EZH2 auto-methylation is impeded by H3K27M in glioma [61]. Nevertheless, residual H3K27me2/3 does exist in cells expressing H3K27M, suggesting an incomplete impairment of PRC2 functionality [236]. Moreover, remaining H3K27me2/3 in H3K27M-expressing glioma cells was actually shown to be critical for tumorgenicity because blockade of residual PRC2 activity by EZH2 enzymatic inhibitors impaired tumor cell proliferation, making PRC2 an attractive therapeutic target in H3K27M-bearing DIPGs [236, 237]. Indeed, in the presence of H3K27M, PRC2 and the remaining H3K27me3 marks were found to be rather restricted and significantly enriched at PRC2 nucleation sites, which often contain CpG islands (CGI) [61, 233, 235, 237]. The retained PRC2 and H3K27me3 at nucleation sites then induced persistent repression of CGI-containing TSGs such as INK4A/p16 [232, 233, 236, 238] (Fig. 9.3). In parallel, H3K27M-caused global loss of distal H3K27me3 resulted in de-repression of PRC2 targets and repetitive elements,

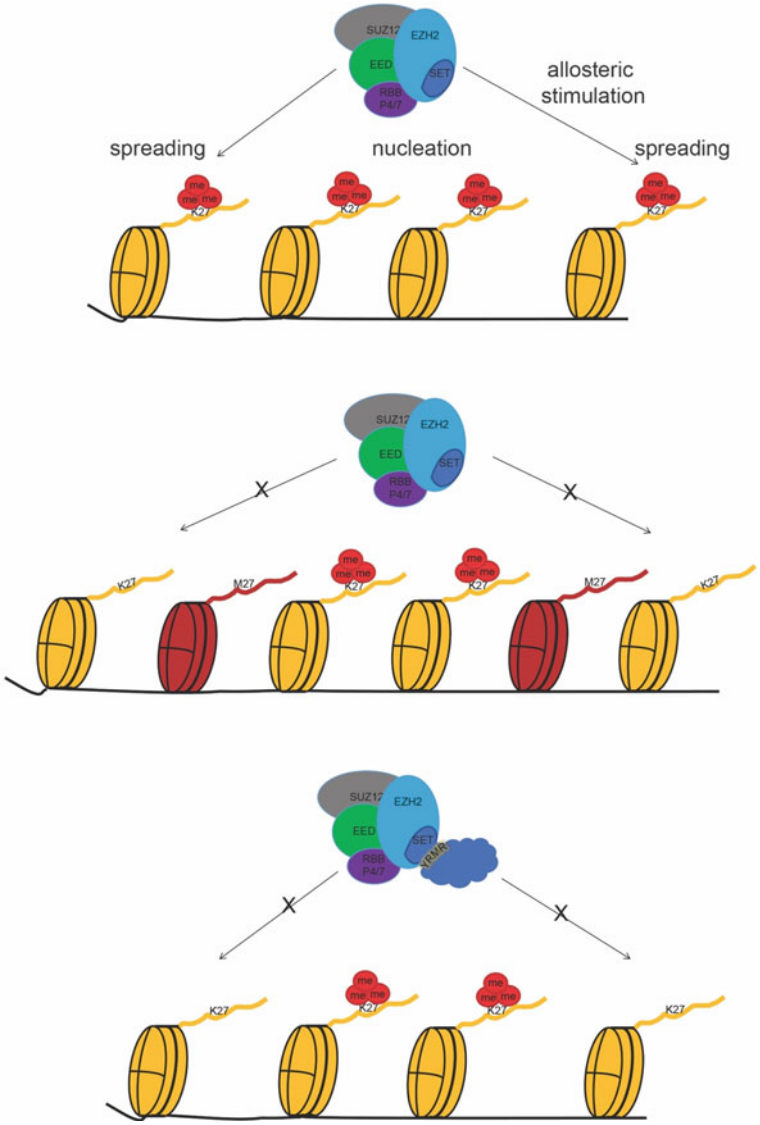

Fig. 9.3 PRC2 malfunction caused by inhibitory onco-histone and cofactor in cancer. Top: in normal cells, PRC2 deposits H3K27me3 at nucleation sites, followed by PRC2/H3K27me3 spreading via an allosteric stimulation mechanism. Middle: in tumor cells, the H3K27M onco-histone inhibits H3K27me3 spreading, without affecting the initial targeting of PRC2 or nucleation of H3K27me3. Bottom: like H3K27M, EZHIP is abnormally expressed in certain tumor, which acts to inhibit H3K27me3 spreading

which also affected cell differentiation and proliferation or oncogenesis [233, 236, 239–241].

The molecular mechanism by which H3K27M inhibits PRC2 in the context of chromatin is a topic of hot debate. It has been proposed that H3K27M exhibits enhanced binding affinity to EZH2, thereby limiting PRC2 mobility on chromatin [36, 242]. A structural study revealed that the methionine residue of H3K27M is located in the pocket of EZH2 SET domain that normally accommodates the target H3K27 residue [36]. Although the charge property and configuration of the methionine side chain are similar to a SET-bound lysine, a 16-fold increase in PRC2 binding to H3K27M peptide (with Kd measured at ~3.3 μM) was observed when compared with WT H3 peptide (with Kd measured at ~52 μM) [36]. However, other studies argued that H3K27M-EZH2 interaction is rather transient and that H3K27M-containing nucleosomes exhibit only slightly increased affinity to PRC2 [235, 243]. Moreover, the findings with EZHIP, a PRC2-inhibitory protein that uses a H3K27M mimic peptide to make contact with EZH2 in a fashion similar to H3K27M, also disagreed with the above model of limiting PRC2 mobility on chromatin, since EZHIP does not incorporate into chromatin but still robustly impedes PRC2 enzymatic activity in cells [244–246]. A different model was then hypothesized that H3K27M causes a defect in PRC2 allosteric activation [61, 235, 237]. Deficiency in PRC2 allosteric activation can explain the impaired PRC2 catalysis without prolonged association with H3K27M, possibly due to a failure in releasing S-Adenosyl-L-homocysteine (SAH), the enzymatic product generated from the methyl group donor, S-Adenosylmethionine (SAM) [61, 235, 237].

Effects of H3K27M on epigenetic and transcriptomic profiles are complex. Besides global loss of H3K27me3, H3K27M can additionally induce changes of H3K27me1, H3K27ac, and DNA methylation [233, 238, 239, 247], all of which may alter gene-expression programs. Gain of H3K9me3 in the absence of H3K27me2/3 suggests a possible compensation, transitioning from facultative heterochromatin to constitutive heterochromatin in order to maintain the chromatin integrity [233]. Additionally, the altered landscape of H3K27me3 can affect the pattern of binding by H3K27me3-specific readers, thereby eliciting subsequent effects on gene expression and epigenome changes [233]. Further investigation is needed to unravel the mechanisms by which H3K27M exerts its oncogenic effects.

By introducing a K27M mutation into endogenous H3F3A locus (which encodes one of the only two H3.3 variants in human cells), investigators further uncovered how H3K27M influences neural development and neural stem cell (NSC) biology [247–249]. Using human embryonic stem cells (hESCs) without any other DIPG-associated mutation, Kfoury-Beaumont et al. illustrated that H3K27M leads to a global loss of H3K27me3 and concordant decrease of DNA methylation in hESCs [249]. Phenotypically, H3M27M mutant cells were vulnerable to tumorigenesis because they showed enhanced proliferation and enhanced self-renewal, along with anomalous differentiation by interfering with mesodermal and ectodermal gene-expression profiles and blocking neuroectoderm differentiation [249]. Using induced pluripotent stem cells (iPSCs), Haag and colleagues

differentiated human iPSCs to various disease-relevant neural cell types, including NSCs and oligodendrocyte progenitor cells (OPCs) [248]. Here, H3K27M increased proliferation and apoptosis primarily in NSCs, and only NSCs, but not OPCs, gave rise to tumors upon induction of H3K27M and TP53 inactivation, recapitulating human DIPGs [248]. Mechanistically, H3K27M is enriched at bivalent domain promoters and poised enhancers leading to activation of developmental genes in iPSCs, which may explain the diverse outcomes observed in different cell types [248]. Consistent with this notion, single-molecule imaging studies with pediatric glioma-derived nucleosomes harboring H3K27M showed direct interaction between H3K27M and MLL1, the H3K4me3 'writer', which leads to genome-wide redistribution of H3K4me3 and imbalanced bivalent chromatin status [250]. In NSCs, H3K27M maintains the expression of genes involved in pluripotency and proliferation, leading to a premature activation of OPC programs that together may cause tumor initiation [248]. Single-cell RNA-seq-based studies also found that glioma cells bearing H3K27M primarily resemble OPC-like population and that more differentiated malignant cells are the minority [251]. But in hindbrain NSCs, H3K27M at enhancers caused a focal H3K27ac loss and the reduced expression of neurodevelopmental genes, despite the global gain of H3K27ac [247]. Altogether, these studies revealed that H3K27M profoundly alters the chromatin modification landscape and transcriptomic programs, which likely acts in a cell type-dependent manner to drive tumorigenesis by arresting cell differentiation at certain developmental stages.

9.4.2 Alteration of EZHIP (Also Known as CXorf67 and CATACOMB), a PRC2 Inhibitor

Another type of tumor in the hindbrain, termed Posterior Fossa Group A Ependymomas (PFA-EPN), exhibits ectopic expression of EZHIP in more than 90% cases, which also display globally decreased H3K27me3 in tumor. EZHIP directly binds PRC2 and inhibits PRC2 function in a fashion similar to H3K27M [244–246, 252]. Specifically, EZHIP contains a conserved peptide that mirrors the H3K27M tail [244, 252] (Fig. 9.3). Unlike H3K27M, EZHIP is not incorporated into chromatin and still causes a H3K27M-like effect, suggesting that PRC2 inhibition is most likely due to defect in PRC2 allosteric activation, rather than directly restricting PRC2 within specific chromatin locations [36, 246, 253]. Ectopic expression of EZHIP in PFA de-repressed PRC2 target genes, especially those involved in neurodevelopment [244]. Normally, EZHIP is expressed predominantly in the gonads during placental development [254]. While depletion of Ezhip in mice led to a global increase of H3K27me2/3 deposition in germ cells (both sperm and oocyte), only females showed fertility, indicating its physiological function during oocyte maturation [254]. Regulation of EZHIP expression was linked to DNA (de)methylation [252], however, the mechanism responsible for EZHIP upregulation in PFA awaits investigation. In a subset of ESS, recurrent chromosomal rearrangement involving reciprocal t(X;17)(p11.2;q21.33) translocation produces

an in-frame fusion between MBTD1 and EZHIP genes [207]. This represents another possible mechanism for ectopic EZHIP upregulation in disease.

9.5 H3K27me3 Readers

In addition to the above-mentioned WD40 repeats of EED, a core subunit of PRC2 [45] (see Sect. 9.1 and Table 9.1), the chromodomain (CD) harbored within chromobox (CBX) subunit of canonical PRC1 (cPRC1) [255] is a classic reader of H3K27me3. Due to the limited space, readers shall refer to other articles as to how H3K27me3 recruits cPRC1 for mediating transcriptional repression and/or chromatin compaction [1, 5, 8, 11, 40].

Recently, another class of H3K27me3-specific readers, namely, bromo-adjacent homology (BAH) domain, was identified in animal cells [256–258], plant [259–262] and fungi (Neurospora crassa) [263], lending strong support for existence of another conserved mechanism for 'sensing' PRC2-generated H3K27me3 marks [21, 26]. One of such H3K27me3 readers, BAH and coiled-coil containing 1 (BAHCC1), was found overexpressed in AML, T-ALL, and B-ALL where it recognizes H3K27me3 for mediating and/or stabilizing the repression of Polycomb target genes [257]. BAHCC1 depletion, or H3K27me3-engagement-defective mutation at the BAHCC1 BAH module, significantly suppressed leukemia cell proliferation whereas BAHCC1 LOF did not affect the growth of K562 chronic myeloid leukemia cells or colony-forming capabilities of normal hematopoietic stem/progenitor cells, pointing to a cancer-specific dependency [257]. BAHCC1 and H3K27me3 were found colocalized at TSGs such as CDKN1A and CDKN1C, as well as differentiation-related master regulators and TFs including HOX, GATA, KLF, and SMAD [257]. H3K27me3 binding by BAHCC1 was found to be required for target gene repression. Mechanistically, BAHCC1 acts as a scaffold, recruiting histone deacetylase (HDAC) and HDAC-associated corepressors [257, 264]. The recruited HDAC complexes then act to induce or enforce repression by erasing histone acetylation [257].

Bromo Adjacent Homology Domain Containing 1 (BAHD1), another BAH-based H3K27me3 reader in mammalian cells, also 'senses' H3K27me3 via a C-terminal BAH protein module [256, 258], assembles a multi-subunit complex with HDAC1/2 and MIER1/2/3 [265], and mediates heterochromatin formation via its multifunctional scaffold role [266]. A recent work further showed that MIER1/2/3, a core cofactor of the BAHD1 complex, directly binds the histone H2A:H2B dimer in the context of a histone octamer [267], furthermore, gel filtration-based analysis of the MIER1:HDAC1:BAHD1 complex co-purified C1QBP (also known as ASF/SF2-associated protein p32, a multi-functional protein with a recently reported function as a chaperone of histones H3 and H4), as well as histones H2A, H2B, H3, and H4 [268]. These results suggested that the BAHD1 complex may play a role as a histone chaperone. In agreement with the H3K27me3-reading role of the BAHD1 BAH domain and HDACs as a BAHD1 cofactor, a vast majority of histone H3 co-purified with the MIER1:HDAC1:BAHD1 complex carried H3K27me2/3

and also showed a lack of acetylated histone lysines [267]. Consistent with a role for PRC2 and H3K27me3 in TSG repression, BAHD1 was recently shown to enforce the cell cycle arrest in G1 phase of breast cancer cells thus potentiating tumor cell proliferation and invasion [269].

Altogether, the engagement and functional readout of H3K27me3 by mammalian BAH module-based readers (BAHCC1 and BAHD1) lead to induction, maintenance, and/or enforcement of a histone deacetylated state at H3K27me3-targeted regions [26]. BAH-containing reader complexes and CD-based H3K27me3 reader complexes (PRC1) use distinct molecular mechanisms for repressing genes, i.e., histone deacetylation by BAHCC1/BAHD1-HDAC complexes and H2A ubiquitination and chromatin compaction by cPRC1,therefore, these H3K27me3 downstream readers may cooperate and even synergize in inducing gene silencing and heterochromatin formation. Moreover, it is worthy of mentioning that PRC1, when bound to H3K27me3, can additionally regulate 3D chromatin structure and mediate the formation of compacted chromatin, termed as PRC1-associated domains [270–275]. Thus, there exists a complex interplay among PRC2, PRC1, readers/effectors, 3D chromatin structure, and gene and epigenome regulation.

9.6 Inhibition of EZH2 and PRC2 as Cancer Therapeutic

Established roles of EZH2 and PRC2 during oncogenesis have greatly motivated the effort in developing the EZH2 and PRC2 inhibitors. In this section, we briefly discuss about recent progress in blockade agents and inhibitors specific to EZH2, EED, and PRC2, some of which are currently in pre-clinical and clinical evaluations. Proteolysis-targeting chimeras (PROTAC)-based compounds for inducing EZH2 or PRC2 degradation also emerged and showed early promises in cancer treatment.

9.6.1 Catalytic Inhibitors and PROTACs/Degraders of EZH2

Multiple small-molecule inhibitors were developed to block the catalytic activity of EZH2 in a S-adenosyl-methionine (SAM) competitive manner [276, 277]. Tazemetostat (formerly known as EPZ-6438) was recently approved by FDA for the treatment of locally advanced or metastatic epithelioid sarcoma in adults and adolescents (aged $\geq$ 16 years), which represents the first targeted therapy approved for treating epithelioid sarcoma in USA [111, 278–280]. Moreover, GSK126, CPI-1205, CPI-0209, SHR2554, DS-3201, and PF-0682149712 have been or are currently being evaluated in clinical trials for the treatment of various tumors [277, 281, 282]. GSK126 effectively suppressed the proliferation of DLBCL cells with EZH2 GOF mutation both in vitro and in xenografted animal models [283]. CPI-1205 had robust antitumor effect in a Karpas-422 xenografted model of DLBCL [284] and is currently being evaluated in phase I/II clinical trials for solid tumors

[86]. CPI-0209 is a second-generation inhibitor of EZH2 currently under evaluation in phase I/II clinical trials for DLBCL, T-cell lymphoma, mesothelioma, and CRPC [285]. PF-06821497 inhibited the growth of tumor in a Karpas-422 xenografted mouse DLBCL model [286] and is under the phase I clinical trials for small lung cancer, CRPC and follicular lymphoma [287]. SHR2554 is being investigated as a single-agent therapy in mature lymphoid neoplasms and in phase I/II clinical trials for the treatment of lymphoid neoplasm, solid tumors, and lymphoma [287–289]. DS-3201 is now put into several clinical trials for patients with leukemia, T-cell lymphoma, PCa and small-cell lung cancer [290]. Moreover, preclinical investigation showed that UNC1999, a dual EZH2 and EZH1 inhibitor, selectively killed DLBCL cells harboring the EZH2 GOF mutation [291] and delayed the growth of MLL-r leukemia in mice [109]. C24 was a close analog of UNC1999 and acts as an EZH2-selective inhibitor [292].

Hydrophobic tagging (HyT) has been established as a means for degrading protein-of-interest (POI) [282]. By appending a hydrophobic tag that activates the unfolded protein response, the degradation of the POI is induced in the HyT approach [293]. By connecting C24 to hydrophobic groups via a linker, Ma et al. discovered that MS1943 can serve as an EZH2-selective degrader to decrease EZH2 protein levels and to suppress the growth of TNBC cells [282].

Apart from HyT-based degrader, PROTACs recently emerge as a feasible approach to target POI for polyubiquitination and degradation via the proteasome [293]. PROTAC has been an attractive therapeutic agent that mediates the formation of a ternary complex, which consists of a heterobifunctional small molecule (i.e., PROTAC), a POI, and a hijacked E3 ubiquitin ligase. A series of EZH2-targeted PROTACs were developed to induce the degradation of EZH2 together with other PRC2 components, including SUZ12, and EED, in cells. Based on C24, MS177 [127] and U3i [294] have been uncovered as potential therapeutic PROTACs to suppress EZH2-dependent tumorigenesis. Wang et al. recently found that MS177 robustly degrades both canonical EZH2:PRC2 and noncanonical EZH2:c-Myc complexes in MLL-r acute leukemia cells, thus simultaneously abolishing these two prominent oncogenic circuitries [127], likewise, effect of MS177 on EZH2 and N-Myc was seen in a neuroblastoma cell line carrying N-Myc amplification [127]. Later on, a similar tumor-killing effect by MS177 was reported in CRPC where MS177 effectively degrades EZH2:PRC2 and non-conventional partners of EZH2 in CRPC, i.e., AR and AR-V7 [126]. Meanwhile, U3i was shown to have a high affinity for EZH2:PRC2 and killed TNBC cells efficiently by inducing cell apoptosis [294]. Based on EPZ-6438, several more EZH2 PROTACs were developed—consistent with what was observed with MS177 in acute leukemia, AR-positive PCa and neuroblastoma [126, 127], E7 strongly inhibited both the canonical and noncanonical functions of EZH2 in lymphoma and lung cancer cells, which included PRC2-dependent repression function and PRC2-independent transactivation function [295]. YM281 significantly induced cell death in DLBCL and other subtypes of lymphoma [296]. MS8815 caused robust EZH2 degradation in a concentration- and time-dependent manner in multiple TNBC cell lines

and patient-derived TNBC cells, which can be a promising means of cancer treatment [297]. As discussed above, PRC2-independent functions of EZH2 have been associated with transcriptional activation rather than the PRC2-related methyltransferase activity for gene repression. Partly due to failure to target noncatalytic functions of EZH2, EZH2 catalytic inhibitors often show limited and/or slow-acting effects [109, 126, 127, 296]. In almost all cases, EZH2 PROTACs generally display unique, fast-acting, more potent and more consistent effects on killing tumor cells, than the matched enzymatic inhibitors or EED degraders (see below section) [126, 127, 296], thus offering an attractive therapeutic avenue.

9.6.2 Inhibitor and PROTACs Specific to EED

Besides developing the EZH2 inhibitors and degraders, those for targeting EED have also emerged as potential strategies for PRC2 inhibition. EED protein consists of seven copies of the WD40 repeat and contains a top and bottom pocket at each end [298]. EED inhibitors can be designed on either the top or bottom pocket. A series of inhibitors, including EED-226 [299], A395 [300], BR-001 [301], EEDi-5285 [302], EEDi-1056 [303], were developed for targeting the top pocket of EED. Most of these EED inhibitors had tumor-killing effect in DLBCLs. Moreover, inhibitors targeting the bottom pocket of the EED protein were also developed, including SAH-EZH2 [304], Astemizole [305], Wedelolactone [306] and DC-PRC2in-01 [307], which were efficacious to suppress growth of the tested cancer models. EED degraders, based on EED inhibitor and PROTAC, were also developed in recent years, including UNC6852 [308] and EED PROTAC #1 and #2 [309]. EED PROTACs can mediate the degradation of EED and EED-associated PRC2 components including EZH2 and SUZ12, resulting in H3K27me3 loss and anti-proliferative effects in various tumor models.

9.6.3 PRC2 Gene Mutation as a Predictive Factor of Immune Therapy

A recent study discovered the DNMT1 synthetic lethality with PRC2 inactivation in MPNSTs, which carry the PRC2 inactivation mutation such as SUZ12 or EED loss [122]. In this scenario, PRC2 inactivation and DNMT1 loss or inhibition (achieved by DNMT1 inhibitor) amplify de-repression of endogenous retrotransposons (ERVs), subsequently inducing viral mimicry response, IFN-γ signaling activation, and cell death partly through a PKR-dependent double stranded-RNA (dsRNA) sensor mechanism [122]. Cooperation of PRC2 and DNMTs was previously reported in cancers [310]. It was posited that DNA methylation acts as a safeguard against ERV reactivation in PRC2-loss cancers to promote oncogenesis, which then can be therapeutically exploited by DNMT1-targeted therapy [122]. Therapeutic intervention via DNMT inhibitors can potentially be applied to other PRC2-inactivated cancers. In support, a preclinical study showed that PRC2

mutation (especially that of EZH2) is correlated with survival benefit of advanced-staged patients treated with immune checkpoint inhibitors [311]. These results illustrated that PRC2 mutations may serve as surrogate markers to predict immune therapy effect, which awaits additional studies.

In summary, mechanistic studies and understanding of EZH2- and PRC2-mediated gene and chromatin (de)regulation have uncovered one of the most central pathways used by cancer to gain growth advantages during the course of tumorigenic initiation, evolution, and malignant progression. Recent development of more potent and more specific pharmacological agents shall aid in development of novel treatment of aggressive cancers. We look forward to seeing new and exciting advances along these lines in years to come.

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Epigenetic (De)regulation in Prostate Cancer

10

Chenxi Xu, Shuai Zhao, and Ling Cai

10.1 Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer in the men and one of the leading causes of cancer-related death [1]. According to Globocan estimates, approximately 1.4 million of new PCa cases were diagnosed worldwide in 2020 [1]. Androgen receptor (AR), a nuclear hormone receptor and transcription factor that mediates signaling of testosterone and 5-α-dihydrotestosterone (DHT), not only is essential for normal prostate development but also remains a main driver in PCa [2]. Accordingly, androgen deprivation therapy (ADT) has been a backbone of treatment for PCa patients [3]. However, most PCa patients eventually become refractory to ADT and, almost inevitably, the disease develops into castration-resistant prostate cancer (CRPC) [2]. Patients with CRPC often develop metastases (termed metastatic CRPC or mCRPC), mainly in the skeleton, and become incurable due to a lack of effective therapy [4]. CRPC exhibits a diverse disease presentation with variable outcomes, including neuroendocrine PCa (NEPC) [5]. NEPC is relatively rare at initial diagnosis of PCa but its incidence increases dramatically following ADT (thus termed therapy induced-NEPC or t-NEPC) and ranges from ~17 to 30% based on different clinical studies [6]. The available therapy for CRPC is very limited, therefore, it remains an urgent task to dissect precise mechanisms responsible for CRPC progression and develop new methods to overcome therapy resistance.

Genetic and epigenetic alterations, as well as changes in tumor microenvironment (TME), collectively contribute to malignant transformation of cancer

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[7]. Whole genome sequencing (WGS) studies in patient samples have identified numerous genomic alterations in primary and advanced PCa, which include the loss of tumor suppressor genes such as PTEN, RB, and TP53, amplification of oncogenes such as AR and MYC, formation of structural variants such as TMPRSS2-ERG, and mutations of genes important for various signaling pathways [8–14]. On the other hand, epigenetics, here by a relatively loose definition, refers to biological processes that regulate gene expression and function without altering DNA sequences [15]. Genetic information or DNA of mammalian cells is organized in the form of chromatin and the building unit of chromatin is termed as nucleosomal core particle, which consists of a histone octamer (two copies of each of the four core histones H2A, H2B, H3, and H4) surrounded by double-stranded DNA [16]. Epigenetic regulations include DNA methylation, histone modification, chromatin remodeling, amongst other mechanisms. DNA methylation mainly occurs in CpG dinucleotides in which a methyl group is added to the 5'-carbon position of cytosine by a family of DNA methyltransferases (DNMTs) [17]. Histone modification refers to covalent post-translational modification (PTM) of histone proteins. The cellular machineries that mediate the epigenetic regulation can be grouped as writers, erasers, and readers [18]. The writer refers to an enzyme that deposits a specific histone modification or DNA methylation. The common writer enzymes include histone acetyltransferases (HATs), histone methyltransferases (HMTs), and DNA methyltransferases (DNMTs). Histone modifications and DNA methylation are reversible and can be removed by erasers. Common erasers include histone deacetylases (HDACs), histone demethylases (HDMs), and TET family proteins. Readers refer to a set of specialized proteins that recognize and bind specific histone modification or DNA methylation and convey epigenetic information to downstream effectors [18]. The change in chromatin accessibility is mediated by chromatin remodeling complexes such as the SWI/SNF complex [19]. DNA methylation, histone modifications, and chromatin remodeling are important epigenetic mechanisms to mediate gene expression, and their deregulations act independently and/or cooperatively in the process of PCa initiation and progression and during the development of drug resistance [20–23]. In this book chapter, we will use prostate cancer as an example to highlight the recent discoveries on how deregulation in DNA methylation and histone modification modifiers and chromatin remodelers contributes to the cancer initiation and progression, as well as potential targeting strategies, as summarized in Fig. 10.1.

10.2 DNA Methylation in Prostate Cancer

Patterns of cytosine methylation within CpG dinucleotide sequences are important for regulating gene expression, DNA damage repair, DNA recombination, and DNA replication [24]. Cytosine methylation is de novo 'written' by DNMT3A and DNMT3B and maintained by DNMT1, which transfer a methyl group from S-adenosylmethionine, a methyl donor, to the 5'-carbon of the cytosine ring to

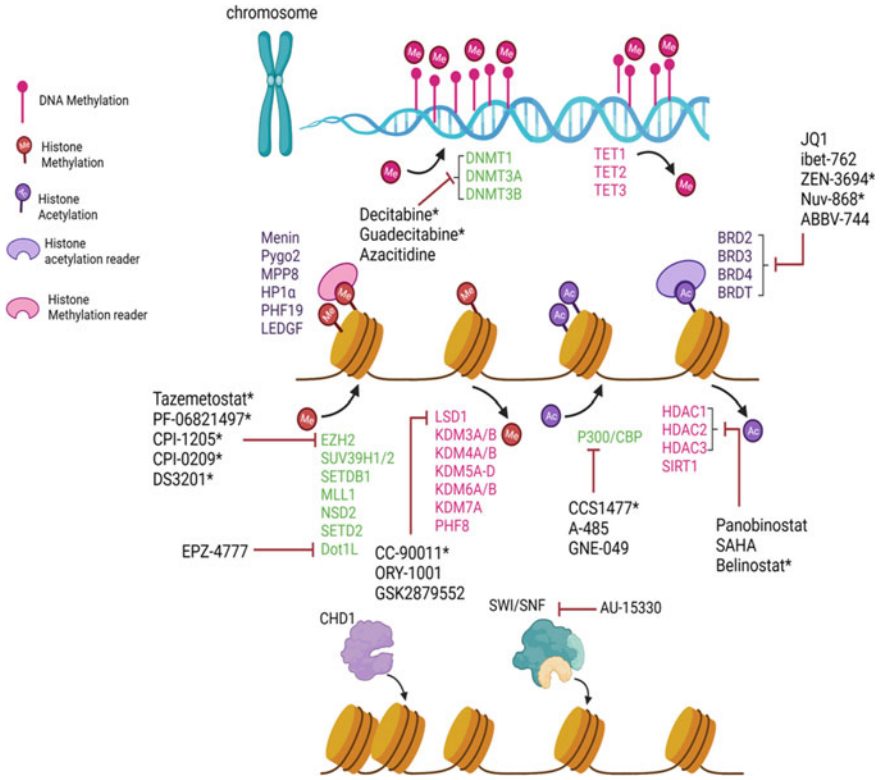

Fig. 10.1 Overview of key epigenetic regulators and targeting drugs in PCa. This schematic illustration only covers a set of selectively focused epigenetic modifications including DNA methylation (top), histone methylation and acetylation (middle), and chromatin remodeling (bottom) involved in PCa. Certain key writers, erasers and readers, as well as the respective targeting compounds, are listed. *, the compound used in the ongoing clinical trials in PCa as shown in Table 10.1. Many other histone modifications, epigenetic modifiers, and targeting drugs, which might have equally important functions in PCa, are not listed. The figure is created with BioRender.com

form 5-methylcytosine (5mC) [17]. 5mC can be 'erased' through sequential oxidation by Ten-eleven translocation (TET) family proteins, with the assistance of base excision repair (BER) pathway, first to 5-hydroxymethyl cytosine (5hmC), then 5-formyl cytosine (5fC) and finally 5-carboxyl cytosine (5caC) [25]. CpG islands refer to the CpG-rich regions of ~200 bp to several kilobases in length, usually located near the promoters of highly expressed genes [26]. CpG islands are often subject to hyper-methylation in human tumors including PCa, compared to normal tissues [27]. Hypermethylated CpG islands are often associated with target gene silencing [17].

Table 10.1 Selective ongoing clinical trials of epigenetic drugs for the treatment of PCa

Trial ID	Drug name	Phase	Conditions	Status
DNMT inhibitors				
NCT05037500	Decitabine/Cedazuridine	I/II	mCRPC (+Enza)	Recruiting
NCT02998567	Guadecitabine (SGI-110)	I	CRPC (+Pembrolizumab)	Active
LSD1/KMD1A inhibitors				
NCT04628988	CC-90011	I	mCRPC (+Abi/Pred)	Recruiting
EZH2 inhibitors				
NCT03460977	PF-06821497	I	mCRPC (alone or with SOC)	Recruiting
NCT04179864	Tazemetostat	I/II	mCRPC (+Enza or Abi/Pred)	Recruiting
NCT04846478	Tazemetostat	I	mCRPC (+Talazoparib)	Recruiting
NCT04104776	CPI-0209	II	mCPRC	Recruiting
NCT03480646	CPI-1205	I/II	mCRPC (+Enza or Abi/Pred)	Active
NCT04388852	DS3201 (Valemetostat)	I	CRPC (+Ipilimumab)	Recruiting
p300/CBP inhibitor				
NCT03568656	CCS1477	I/II	mCRPC (+Enza or Abi or Daro)	Recruiting
HDAC inhibitors				
NCT04703920	Belinostat	I	mCRPC (+Talazoparib)	Recruiting
BET protein inhibitors				
NCT05252390	NUV-868	I/II	mCRPC (alone or +Enza or Olaparib)	Recruiting
NCT04471974	ZEN-3694	II	mCRPC (+Enza and Pembrolizumab	Recruiting
NCT04986423	ZEN-3694	II	CRPC (+Enza)	Recruiting

Source https://clinicaltrials.gov/

Abbreviation in the table: mCRPC, Metastatic castration-resistant prostate cancer; Enza, Enzalutamide; Abi, Abiraterone acetate; SOC, Standard of Care; Pred, Prednisone/Prednisolone; Daro, Darolutamide

Mechanistically, 5mC can regulate gene expression by recruiting readers such as methyl-CpG binding proteins (MBP1, MBP2, MBP3), Kaiso, and methyl CpG-binding protein-2 (MeCP2) [28–31]. Once bound to promoter-associated 5mC, methyl-CpG-binding domain (MBD) family proteins can further recruit corepressors such as HDACs, thereby mediating repression of target gene transcription [32]. Meanwhile, DNA methylation can profoundly modulate binding of various DNA-binding proteins such as transcription factors (TFs) and thus directly influences gene activity [33]. In this case, 5mC may either inhibit or promote TF binding [33, 34].

In 1987, Bedford and van Helden reported that global DNA methylation was significantly lower in metastatic PCa compared to benign prostatic hyperplasia (BPH) [35]. This is the first study to demonstrate a correlation between global hypomethylation and metastatic potential of PCa. In general, promoter hypomethylation and gene body hypermethylation are positively correlated with gene expression [36]. In PCa, DNA hypomethylation has been linked to higher expression of genes and usually occurs later in PCa progression and contributes to tumor metastasis [37]. Over the last decade, several studies have compared genome-wide DNA methylation patterns in benign prostate tissues and various stages of PCa [11, 38–41]. Very recently, Zhao et al. used whole genome bisulfite sequencing (WGBS) and further confirmed that advanced PCa is overall less methylated than primary PCa, which is predominantly less methylated than benign prostate [42]. Numerous oncogenic factors involved in various signaling pathways are reported to be hypomethylated in PCa including Urokinase plasminogen activator (uPA) [43], Heparanase (HSPE) [44], Cytochrome P450 1B1 (CYP1B1) [45], and wingless-related MMTV integration site 5A (WNT5A) [46]. In the aforementioned study, Zhao et al. demonstrated promoter hypomethylation at AR and key androgen-responsive genes including KLK3 and NKX3-1 in advanced PCa compared to benign prostate samples [42]. Global DNA hypomethylation has also been proposed to be correlated with genetic instability by promoting formation of more open chromatin [47]. Therefore, DNA hypomethylation potentially contributes to PCa development and progression through altering transcriptomics and/or genome stability.

Conversely, DNA hypermethylation is one of the most commonly observed phenomena and best characterized epigenetic alterations in PCa. A plethora of genome-wide DNA methylation analyses have been performed, which suggest that differential methylation among distal and genic regions is one of the key drivers of PCa tumorigenesis and progression [48]. For example, Zhao et al. reported that about 22% of CRPC tumors exhibit a hypermethylation subtype termed a CpG methylator phenotype (CMP), which is enriched with somatic mutations of DNMT3B, TET2, BRAF, and IDH1 [42]. In contrast to promoter hypomethylation, hypermethylation of gene promoters often coincides with transcriptional repression of target genes, most of which are tumor suppressor genes (TSGs) such as adenomatosis polyposis coli (APC) [49], retinoic acid receptor β (RARβ) [50] and RAS-associated domain family 1 (RASSF1) [51, 52], DNA damage repair-related genes such as Glutathione S-Transferase Pi 1 (GSTP1) [53, 54] and O-6-methylguanine-DNA methyltransferase (MGMT) [55], hormonal response-related genes such as AR [56], ER-α [57] and ER-β [58], as well as genes involved in cell cycle control, apoptosis and cell adhesion such as cyclin dependent kinase inhibitor 2A (CDKN2A) [59], Target of Methylation-induced Silencing 1 (TMS1) [60] and E-cadherin (CDH1) [61, 62].

Measurement of global or targeted DNA methylation can be used as biomarkers to help the risk stratification of PCa patients. For instance, ConfirmMDx, an epigenetic test that evaluates the methylation status of three genes, GSTP1, APC, and

RASSF1, can distinguish true negative prostate biopsies from occult PCa, therefore avoiding unnecessary repeat prostate biopsies of patients [63]. Likewise, the methylation panel of GSTP1, together with those of GAS6 and HAPLN3, was identified by Patel et al. to separate PCa and benign prostate tissues in a sensitive and specific way [64].

While 5mC is commonly associated with transcriptional repression, further conversion to 5hmC is related to transcriptional activation [65]. The laboratory of Dr. Felix Feng has recently profiled 5hmC among 145 PCa samples representing different disease stages (including 52 localized PCa and 93 mCRPC) and integrated datasets of WGS, WGBS, and RNA-seq from the same samples [66]. In this study, 5hmC in mCRPC was shown to mark the activation of major cancer driver genes (such as AR and EZH2) at both gene-regulatory sites and their downstream binding sites [66], consistent with the transcriptional changes during disease progression captured by RNA-seq [67]. Since metastatic PCa is notoriously heterogeneous and hard to biopsy, circulating tumor DNA (ctDNA) obtained from liquid biopsy provides an alternative approach for understanding the underlying tumor biology [68, 69]. Indeed, 5hmC level in ctDNA represents the individual advanced tumors, making it a potential liquid biomarker for mCRPC [66, 67]. A very exciting work done by Chen et al. performed plasma DNA methylome analysis from patients with localized (60 samples) and metastatic PCa (175 samples) using the cell-free methylated DNA immunoprecipitation sequencing (cfMeDIP-seq) technology [70, 71]. They observed global hypermethylation in metastatic samples, together with hypomethylation in the pericentromeric regions [70]. This study indicated that the cell-free DNA methylome may accurately distinguish different disease stages, highlighting a potential use in PCa diagnosis and prognosis [70].

Deregulation of DNMT occurs commonly in cancer and is associated with tumorigenesis [72]. DNMT1 is often overexpressed in localized and metastatic PCa when compared to BPH [73, 74]. Increased expression of DNMT3A and DNMT3B led to hypermethylation at a substantial subset of CpG sites in PCa [75]. TET2 binds AR and TET2 loss is correlated with PCa progression [76]. To target aberrant DNA hypermethylation, DNMT inhibitors such as Azacytidine and Decitabine have been developed and tested in various types of cancers including PCa [77]. Currently, there are two ongoing clinical trials using Decitabine/Cedazuridine together with Enzalutamide (NCT05037500), or Guadecitabine (SGI-110) together with Perbrolizumab (NCT02998567), for the treatment of CRPC patients (see Table 10.1).

10.3 Histone Methylation in Prostate Cancer

Histones are a group of highly conserved, highly basic proteins that are responsible for chromatin organization and compaction by folding DNA into the nucleus [78]. There are four core histones, namely, H2A, H2B, H3, and H4. Amino acid residues of histones, especially those in the unstructured N-terminal tails, are known to be

potential sites for various types of post-translational modifications (PTMs), such as methylation, acetylation, phosphorylation, and ubiquitination [79]. Histone methylation and acetylation have been intensively studied in cancer and hence, we will focus the next sections on the recent findings showing the involvement of these modifications in PCa.

Histone methylation and acetylation are established, respectively, by histone lysine methyltransferases (KMTs) and histone acetyltransferases (HATs), and can be removed, respectively, by histone lysine demethylases (KDMs) and histone deacetylases (HDACs). Histone methylation is critically involved in a wide variety of biological processes ranging from transcriptional regulation to heterochromatin formation [80]. Lysine (Lys or K) and arginine (Arg or R) residues serve as the most common sites of methylation. Here we mainly discuss about lysine methylation. The histone H3 lysine 4, 9, 27, 36, and 79 (H3K4, H3K9, H3K27, H3K36, and H3K79) and histone H4 lysine 20 (H4K20) are among the most extensively studied sites of histone lysine methylation. Lysine can be mono-, di-, or trimethylated (Kme1, Kme2, or Kme3) on its ε-amine group [80]. The degree of histone lysine methylation is controlled by the relative activities of site-specific methyltransferases and demethylases.

10.3.1 H3K4 Methylation

Highly methylated H3K4, such as H3K4me3 and H3K4me2, is localized to the promoter-proximal regions of actively transcribed genes, with H3K4me3 exhibiting a more punctuate pattern than H3K4me2 [81, 82]. H3K4me3 and H3K4me2 are associated with active transcription. H3K4me1, on the other hand, is a marker of gene enhancer [83, 84]. While the poised enhancers are marked by the presence of both H3K4me1 and H3K27me3 and a lack of H3K27 acetylation (H3K27ac), those active ones carry both H3K4me1 and H3K27ac and a lack of H3K27me3 [83, 84]. In mammalian cells, methylation of H3K4 is catalyzed by the KMT2/MLL family methyltransferases: KMT2A/MLL1, KMT2D/MLL2, KMT2C/MLL3, KMT2B/MLL4, KMT2F/SETD1A, and KMT2G/SETD1B. Another SMYD family of H3K4 methyltransferases includes SMYD1, SMYD2, and SMYD3. On the contrary, H3K4 demethylation is primarily catalyzed by KDM1A/LSD1, KDM1B/LSD2, KDM5A/JARID1A, KDM5B/JARID1B, KDM5C/JARID1C, and KDM5D/JARID1D [18].

The H3K4 methyltransferase KMT2A/MLL1 can function as a co-activator for AR in CRPC [12, 85]. Here, AR directly interacts with the MLL complex via the menin subunit, and the inhibition of menin-MLL complex suppresses AR signaling [85]. Notably, a phase I clinical trial with revumenib (SNDX-5613), a potent and selective oral inhibitor of the menin–MLL interaction, demonstrated strong efficacy in MLL-rearranged or NPM1-mutated acute leukemia [86]. It would be worthwhile to test this inhibitor in advanced PCa. KMT2D/MLL2 is mutated in 8.6% of PCa cases [12]. KMT2D/MLL2 promotes prostate tumor growth through epigenetically activating LIFR and KLF4, which are involved in

PI3K/AKT and epithelial-mesenchymal transition (EMT) pathways [87]. KMT2F/SETD1A promotes tumor cell proliferation in metastatic CRPC by regulating FOXM1 transcription [88]. Upregulation of SMYD3 expression occurs frequently in multiple cancer types including PCa [89]. SMYD3 was shown to promote prostate tumorigenesis through upregulation of AR expression [90].

LSD1 (also known as KDM1A) is the first identified histone demethylase [91] and also represents one of the most widely studied KDMs in cancers including PCa [92]. LSD1 is overexpressed in patients with advanced PCa compared to normal tissues [93]. LSD1 was reported to interact with AR and activate AR signaling through demethylating histone H3K9 methylation [94–96]. Cai et al. demonstrated that LSD1 can play dual roles in PCa [97]. In the presence of high concentration of androgen, LSD1 acts as a co-repressor of AR expression by inducing demethylation of H3K4me1 and H3K4me2 at AR intronic region, and additionally, AR recruits LSD1 for mediating silencing of AR-repressed gene targets [98]. However, in CRPC cells where androgen level is generally low, the expression of AR and AR-repressed genes is increased due to an impaired recruitment of LSD1, which in turn promotes CRPC cell proliferation [98]. LSD1-mediated epigenetic reprogramming also drives PCa progression through regulating CENPE expression [99]. However, it remains to be elucidated as of the molecular determinants responsible for LSD1 specificity towards H3K4 versus H3K9 demethylation under different biological contexts. In addition to its role in demethylating histone substrates, LSD1 also demethylates a number of non-histone substrates such as p53 and FOXA1. LSD1-mediated repression of p53 functionality was reported to be relevant during oncogenesis [100]. More recently, LSD1 was shown to demethylate FOXA1, an oncogenic TF and functional partner of AR in PCa, at its residue K270, which positively regulates FOXA1's binding to chromatin targets and then enhances AR's transcriptional activity [101]. LSD1 blockade, alone or in synergy with AR antagonist treatment, dramatically decreased PCa growth in vivo [101]. LSD1 also has demethylase-independent functions to promote survival of the AR-independent prostate cancer [102].

A number of LSD1 inhibitors, which include CC-90011 [103], SP-2577 [104], ORY-1001 [105], TCP [106] and GSK-2879552 [107], have been reported and are under clinical evaluation for the treatment of cancers, either alone or in combination with other drugs [77, 108]. An ongoing clinical trial (NCT04628988) is testing the LSD1 inhibitor CC-90011 together with ADT in mCRPC (Table 10.1).

The KDM5 family of Jumonji domain-containing KDMs is H3K4-specific demethylase and also frequently altered in PCa [109]. For instance, KDM5A is upregulated in PCa [109]. KDM5B was reported to be frequently upregulated in PCa tissues and associated with AR to regulate its transcriptional activity [110]. KDM5B is also a key regulator of PI3K/AKT signaling in PCa through directly binding the *PIK3CA* promoter [111]. Loss of KDM5B results in a significant reduction of P110α and PIP3 levels and inhibition of the proliferation of PCa cells [111]. KDM5C overexpression in PCa was found to be associated with a reduced biochemical recurrence-free survival in patients after prostatectomy [112]. KDM5C is highly expressed in metastatic PCa and promotes tumor growth

and metastasis [113]. In contrast, KMD5D, a male-specific protein, was shown to suppress the invasion of PCa cells via demethylating H3K4me3 on the promoters of invasion-associated genes or recruiting co-repressor ZMYND8 [114]. Furthermore, KDM5D is associated with altered docetaxel sensitivity in PCa through modulating the AR signaling [115]. Loss of KDM5D expression results in aggressive PCa and confers a poorer prognosis in PCa patients [116]. Taken together, these findings underscore the roles of KDMs in PCa initiation, progression and metastasis, indicating that KDMs might represent attractive therapeutic targets.

The reader proteins for H3K4 methylation also play a role in PCa progression. For example, an in vivo open reading frame (ORF) screening identified PYGO2, a H3K4me2 and H3K4me3 reader, as a driver for metastatic PCa [117]. While overexpression of PYGO2 promotes PCa tumor growth and invasion, depletion of PYGO2 has the opposite effects in vitro and in vivo [117]. Upregulation of PYGO2 is associated with higher Gleason score and metastasis in PCa patients, further indicating PYGO2 as a potential therapeutic target in advanced PCa [117]. A recent follow-up study showed that targeting PYGO2 through genetic or pharmacological inhibition enhanced cytotoxic T cell responses and sensitized PCa to immunotherapy [118].

10.3.2 H3K9 Methylation

H3K9 methylation is deposited by the KMT1 writer enzymes (including KMT1A/SUV39H1, KMT1B/SUV39H2, KMT1C/G9a/EHMT2, KMT1D/GLP/EHMT1, KMT1E/SETDB1, and KMT1F/SETDB2), recognized by readers such as HP1α and MPP8, and eliminated by erasers such as the KDM3 (KDM3A-C/JHDM2A-C) and KDM4 (KDM4A-E/JMJD2A-E) family enzymes [119]. H3K9me2 or H3K9me3 is a gene silencing-related histone PTM and often found in high levels at constitutive heterochromatin regions [120]. H3K9me3 mediates transcriptional silencing of various transposable elements (TEs) and regular genes as well. Malfunction of H3K9 methylation-associated regulators and modifier enzymes can profoundly affect either the level or the genomic distribution of H3K9 methylation, thus resulting in de-regulation of gene expression and/or genome instability during the course of pathogenesis [119]. H3K9 methylation writers, readers, and erasers were reported to influence PCa development and progression by a variety of different mechanisms.

KMT1B/SUV39H2 is the writer responsible for H3K9me1/2 deposition and its overexpression was reported in PCa [109]. Through yeast two hybrid screening, SUV39H2 was identified as a coactivator of AR [121]. Similarly, the H3K9me3 writer KMT1E/SETDB1 was also upregulated in PCa [122, 123]. Knockdown of SETDB1 induced the G0/G1 cell cycle arrest and inhibited PCa cell proliferation and migration [123]. Dutta et al. discovered a NKX3.1-G9a-UTY transcriptional axis, which is essential for prostate differentiation [124]. More studies, however, are required to decipher the role of this axis in PCa. Notably, H3K9 methylation was found to be essential to drive antiandrogen resistance in advanced

PCa [125]. ADT induced de-repression of retroelements (REs) leading to a phenomenon termed viral mimicry, which activates the immune-related signaling pathways and thus inhibits tumor growth [125]. Overexpression of H3K9me3 writers (for example, GLP or G9a) conferred drug resistance through mediating repression of antiandrogen-induced activation of REs, whereas inhibition of these writers and readers (such as CBX5, also known as HP1α) restored RE expression and abolished antiandrogen resistance [125]. Therefore, a combined treatment with antiandrogen and inhibitors targeting H3K9me3 regulators serves as a promising therapeutic strategy for treating CRPC, based on the linkage between H3K9me3-mediated RE silencing and tumor microenvironment.

The H3K9me3 reader HP1α showed elevated expression in NEPCs, which was found to be associated with poor prognosis [126]. HPα promoted neuroendocrine trans-differentiation and silencing of HP1α inhibited NEPC cell proliferation [126]. Another H3K9me3 reader MPP8 was reported to repress E-cadherin through binding to H3K9 methylation and promote EMT phenotypes in PC3 cells [127].

H3K9 methylation is removed by the KDM3 and KDM4 family of erasers, which are often highly expressed in PCa [128]. KDM3A (also known as JMJD1A) regulates the activities of AR and c-Myc and promotes prostate cancer progression [129]. Acetylation of KDM3A by P300 blocks its degradation and enhances AR activity in CRPC [130]. In addition, KDM3A promotes alternative splicing of AR to generate the constitutively active AR spliced variant 7 (AR-V7), the formation of which represents one of the main ADT resistance mechanisms [131]. Collectively, multifaceted roles of KDM3A in regulating the AR activation, c-Myc activity, and AR-V7 alternative splicing suggested it to be a promising therapeutic target for PCa. KDM3B (also known as JMJD1B) was identified as a key regulator of cell proliferation in CRPC cells by a focused shRNA screening [132]. KDM3C has been shown to have a synthetic lethal relationship with AR and the AR-negative PCa cells are sensitive to KDM3C inhibition [133]. Knockdown of PHF8, another H3K9 demethylase, showed that it plays a role in PCa cell proliferation, migration, and invasion [128].

KDM4A and KDM4D form complexes with ligand-bound AR and function as AR coactivators [134]. KDM4A (also known as JMJD2A) is a coactivator of E2F1 and regulates PCa metabolism through transcriptional regulation of pyruvate dehydrogenase kinase (PDK) [135]. Overexpression of KDM4A is positively correlated with Gleason score and metastasis in human PCa [136]. Kim et al. further identified a KDM4A-ETV1-YAP1 axis that operates to promote PCa initiation [136]. KDM4B interacts with AR and regulates its stability and activity [137]. In addition, KDM4B promotes alternative splicing to generate AR-V7, thus contributing to the development of CRPC [138]. Furthermore, KDM4B promotes CRPC cell proliferation through physically interacting with c-Myc to activate a set of metabolic genes such as LDHA [139]. Overexpression of KDM4B may act to promote the recruitment of AR to the enhancer of c-Myc gene and induces its expression, which causes anti-androgen resistance [140]. Together, targeting

KDM4B may theoretically repress the nodes of AR-FL, AR-V7, and c-Myc, making it an attractive therapeutic strategy in PCa. In fact, small-molecule inhibitors targeting KDM4 were shown to suppress PCa cell proliferation [141, 142].

In summary, H3K9 methylation and its various regulators profoundly affect PCa cell proliferation and survival, as well as PCa-TME interaction, through different molecular mechanisms. Targeting the H3K9 methylation-related modifiers and regulators provides promising clinical strategies to fight against PCa, which awaits additional studies.

10.3.3 H3K27 Methylation

Trimethylation of histone H3 lysine 27 (H3K27me3) is another histone PTM closely associated with transcriptional repression [143]. H3K27me3 deposition is carried out by Polycomb Repressive Complex 2 (PRC2), a multi-subunit methyltransferase complex that contains Enhancer of Zeste Homolog 2 (EZH2) as the catalytic subunit. PRC2 core complex is composed of EZH2 or related EZH1, EED, SUZ12, and RbAP46/48 [144]. To gain a comprehensive view of PRC2 function in development and cancer, please refer to recent comprehensive reviews [145–147].

EZH2 mRNA and protein levels were found to be progressively increased in metastatic PCa samples compared with benign prostate tissues [148]. In a clinical PCa cohort, higher expression of EZH2 was found to be correlated with a worse prognosis [148]. EZH2 overexpression promotes the proliferative and invasive capacity of PCa cells [148, 149]. Loss of EZH2-targeting microRNAs such as micro-RNA-101, which negatively regulates EZH2 at a posttranscriptional level, contributes to overexpression of EZH2 during PCa progression [149].

As an oncogenic driver of PCa, overexpression of EZH2 inhibits the expression of TSGs through deposition of H3K27me3, which then promotes tumorigenesis [150]. In addition to this canonical role of EZH2 in H3K27me3 deposition and gene silencing, EZH2 also carries non-canonical oncogenic functions independent of PRC2 and H3K27me3 [151]. For instance, EZH2 can methylate STAT3 to promote its activity in glioblastoma stem-like cells [152]. EZH2 methylates elongin A to regulate target gene transcription in embryonic stem cells [153]. Besides its canonical function to repress TSGs in PCa, EZH2 was reported to be involved in transcriptional activation of AR target genes, and PI3K/AKT phosphorylation of EZH2 at serine 21 was proposed to promote such a gene-activation effect by EZH2 [154]. EZH2 was further demonstrated to directly bind the AR gene promoter to activate its transcription, thereby potentiating AR signaling in PCa [155]. A more recent study from the same group showed that EZH2 can methylate FOXA1 at the K295 residue to enhance FOXA1's stability, thereby regulating the cell cycle-related genes in PCa cells [156]. Furthermore, Yi et al. demonstrated that EZH2 increases rRNA 2'-O methylation and regulates translation through its direct interaction with fibrillarin in PCa [157]. EZH2 also plays a significant role in regulating lineage plasticity, drug resistance, and antitumor immunity [158–168].

Because of the aforementioned important roles of EZH2, many EZH2 inhibitors, such as DZNeP, GSK126, UNC1999, EPZ-6438, PF-06821497, CPI-1205, CPI-0209, and DS3201, have been developed and tested in preclinical and clinical settings [169–171]. Tazemetostat (EPZ-6438) is the first FDA-approved EZH2 inhibitor for the treatment of epithelioid sarcoma and follicular lymphoma [172]. In the domain of mCRPC, there are a couple of ongoing clinical trials, either with EZH2 inhibitors alone or together with antiandrogen treatment, PARP inhibitor or immune checkpoint blockade (ICB) therapy (NCT03460977, NCT04846478, NCT04179864, NCT03480646, NCT04104776, NCT04388852; Table 10.1). Despite much effort, there seems a lack of efficacy of EZH2 inhibitors in solid tumors, which could be explained, at least partially, by the enzymatically independent functions of EZH2.

Interestingly, a gene-activation-related function EZH2 has been linked to an intrinsic transactivation activity harbored within a cryptic transactivation domain (TAD) of EZH2 [173, 174]. Here, EZH2 TAD was found to directly interact with p300 and c-Myc, which then act to mediate gene activation [173, 174]. To more thoroughly inhibit multifaceted activities of EZH2 in cancers, a Proteolysis-Targeting Chimera (PROTAC)-based degrader specific to EZH2 was recently developed for blocking both H3K27me3-dependent and H3K27me3-independent tumor-promoting functions of EZH2 [174, 175]. Wang et al. further reported that, in advanced PCa, EZH2's TAD associates with both AR and AR-V7 to recruit the transactivation-related machineries at target sites that lack binding of PRC2 and H3K27me3 [175]. These non-canonical target sites of EZH2 and AR/AR-V7 were found to be enriched for the clinically relevant oncogenes, notably CDK2 and MYBL2 (also known as B-Myb) [175]. EZH2 TAD facilitates EZH2's recruitment to these non-canonical target oncogenes, stimulating their activation to enhance CRPC growth in vitro and in vivo [175]. And when compared to the matched EZH2 catalytic inhibitor, the EZH2 degrader MS177 showed superior antitumor efficacy, presumably through on-target depletion of both EZH2's canonical (EZH2:PRC2) and non-canonical (EZH2TAD:AR/AR-V7:co-activators) complexes in PCa [175]. EZH2-targeting PROTACs emerge as a promising therapeutic method for treating the aggressive cancers [174, 175].

H3K27me demethylases include KDM6A (or UTX), KDM6B (or JMJD3), and KDM7A. KDM6A interacts with AR in advanced PCa [12]. Although KDM6A was found mutated in metastatic CRPC, its exact function in PCa progression or AR signaling remains elusive. A role for KDM6B was also implicated in PCa [176, 177]. KDM7A is upregulated in PCa, which is correlated with Gleason score [178]. KDM7A directly interacts with AR and regulates expression of its downstream target genes [178]. Knockdown of KDM7A inhibits PCa cell proliferation in vitro and in the tumor xenografted animal model, indicating that targeting KDM7A might be a possible strategy to treat advanced PCa [178].

10.3.4 H3K36 Methylation

H3K36 methylation is involved in a range of biological processes including transcriptional regulation, mRNA splicing, DNA replication, and DNA repair, and it also cross-talks with other epigenetic modifications, such as DNA methylation and H3K27me3 [179, 180]. H3K36me2 is enriched at the 5' regions of actively transcribed genes and intergenic area, while H3K36me3 is mainly localized to the gene body of actively transcribed genes [179–181]. There exist several H3K36me2-specific writers in the human cells, which include ASH1L and the nuclear receptor binding SET domain (NSD) family proteins, NSD1, NSD2 (also known as WHSC1 and MMSET) and NSD3. In contrast, SETD2 is the sole H3K36me3 writer [179]. H3K36 methylation is removed by the KDM2 (KDM2A and KDM2B) and KDM4 (KDM4A-KDM4D) subfamilies of erasers [80]. H3K36 methylation can be recognized by readers that contain the PWWP, Chromodomain, or Tudor domain [180].

Deregulation of NSD family writer is known to be involved in development of human cancers including PCa [182]. For example, NSD1 and NSD2 were both shown to interact with AR, enhancing transactivation of AR signaling in PCa [183, 184]. The role of NSD2 in PCa has been extensively studied. NSD2 mediates constitutive activation of NF-κB signaling by proinflammatory cytokines in CRPC through direct interaction with NF-κB, which involves NSD2's enzymatic activity [185]. NSD2 is overexpressed in PCa and required for PCa tumor growth [185]. In addition, NSD2 has been found to be overexpressed in metastatic PCa compared to primary tumors and associated with disease stage and biochemical recurrence [186]. NSD2, together with PTEN loss, promotes PCa metastasis [186]. Aytes et al. further showed NSD2 to be a conserved driver of metastatic PCa through analysis of genetically engineered mouse models (GEMM) of PCa and correlative study of human PCa patient datasets [187]. Interestingly, H3K36me2 has recently been associated with epithelial plasticity and metastasis in pancreatic ductal adenocarcinoma (PDAC) through the H3K36 to H3M36 mutation (H3K36M), which directly and globally inhibits this mark [188]. via CRISPR-Cas9 screening, the authors discovered both NSD2 and KDM2A (the writer and eraser of H3K36me2) to be involved in regulating EMT and tumor metastasis of PDAC [188]. NSD2 also cross-talks with other epigenetic players. For instance, EZH2 regulates the expression of NSD2 through repressing the NSD2-targeting microRNAs [189]. On the other hand, the oncogenic function of EZH2 was mediated by NSD2, which affects PCa tumor formation and metastasis [189]. NSD2 has also been shown to be involved in regulating immune infiltration in PCa [190]. NSD3 is amplified in lung squamous cell carcinoma (LUSC) and functions as a key regulator of tumorigenesis [191]. In breast cancer, NSD3-mediated H3K36 methylation is crucial for tumor initiation and metastasis [192]. However, the function of NSD3 in PCa remains to be identified.

SETD2 serves as the sole methyltransferase for writing H3K36me3 [193] and is frequently mutated in a variety of human cancers [180]. Via H3K36me3 deposition, SETD2 has been shown to be involved in regulation of DNA damage repair

and mRNA splicing [194]. Additionally, SETD2 also methylates non-histone substrates to regulate oncogenesis. For example, Yuan et al. reported that loss of SETD2 cooperates with PTEN deletion to promote PCa metastasis in an EZH2-dependent manner [195]. Mechanistically, SETD2 methylates EZH2 at its K735 residue, an event that promotes degradation of EZH2, an oncoprotein in PCa, and thus inhibits progression of PCa [195]. The K735R mutant of EZH2 (a SETD2 methylation deficient mutant) induces metastatic PCa in mice [195]. These results, thus, provide an explanation for the known antagonism between SETD2-mediated H3K36me3 and EZH2-catalyzed H3K27me3 [195–197].

PHF19, a component of PRC2 complex, promotes the binding and spreading of PRC2 complex into active chromatin region through its Tudor domain-mediated recognition of H3K36me3, thereby promoting gene silencing during development [198–201]. PHF19 also plays a role in many cancers including PCa [202, 203]. In vitro studies with the AR-negative PCa indicated that PHF19 regulates cell proliferation, invasiveness and angiogenesis [203]. Another H3K36 methylation reader, LEDGF (or PSIP1), is overexpressed in PCa and knocking down LEDGF sensitizes chemo-resistant PCa cells to taxanes [204]. The exact functions of these H3K36 methylation readers in PCa tumorigenesis and progression warrant more detailed study in future.

The H3K36 methylation erasers include the family members of KDM2 and KDM4. KDM2B has recently been shown to play a role in PCa cell motility [205]. The four KDM4 family members (namely, KDM4A, KDM4B, KDM4C, and KDM4D) can act as the erasers of both H3K36me2/3 and H3K9me2/3 methylation [206, 207], as mentioned in the previous H3K9 methylation section. KDM4 proteins can form complex with AR and promote its activity in an enzymatic-dependent fashion [96, 134, 137, 141].

10.3.5 H3K79 Methylation

H3K79 methylation, located inside the globular domain of histone H3, is associated with the actively transcribed genes [18]. Disrupter of telomeric silencing 1L (DOT1L) is the only identified writer for H3K79 methylation [208]. So far, eraser of H3K79 methylation has not been reported. Very recently, Menin is identified as the first H3K79me2 reader protein [209]. Menin is a well-known component of MLL complex, the H3K4 methylation writer, and also a critical regulator of AR signaling [85]. The expression of Menin is higher in CRPC when compared to primary PCa and benign prostate tissues, which also correlates with poor overall survival of PCa patients [85]. How exactly such a newly identified function of Menin as a H3K79me2 reader is involved in PCa development warrants further investigation. H3K79me2 and H3K79me3 marks participate in the regulation of gene transcription, cell cycle progression and DNA damage response [210]. Besides its function in the development and maintenance of MLL-rearranged (MLL-r) leukemia [211], DOT1L has also become a promising therapeutic target for treating the solid tumors [212]. DOT1L was identified as a novel cofactor

for ERα and regulates transcription of ERα target genes in breast cancer [213]. DOT1L inhibition impairs the growth of antiestrogen-resistant breast cancer cells [213]. Vatapalli et al. found that DOT1L expression was significantly increased in PCa relative to normal prostate and correlated with Gleason score and poor clinical outcome in multiple datasets of PCa patients [214]. Interestingly, DOT1L blockade specifically reduces the viability of AR-positive PCa cells, including enzalutamide-resistant ones [214]. Mechanistically, DOT1L and AR co-bind a distal enhancer of MYC, thus promoting the MYC expression in AR-positive PCa cells; conversely, treatment with the DOT1L inhibitor promotes AR and MYC degradation via upregulation of the E3 ubiquitin ligases HECTD4 and MYCBP2, thus impairs the PCa tumor growth [214]. Taken together, these studies provide a foundation for rationalizing the targeting of DOT1L in endocrine therapy-resistant AR-positive or ERα-positive cancers.

10.4 Histone Acetylation in Prostate Cancer

Histone acetylation is closely associated with transcriptional activation and this histone PTM is achieved through the transfer of an acetyl group to the histone lysine by HATs [215]. Conversely, HDACs conduct deacetylation reaction by the removal of an acetyl group off lysine [215]. There are over 40 different histone lysine residues that have been reported to be modified by acetylation [216]. Histone acetylation influences numerous physiological and pathogenic processes [217]. For instance, HATs and HDACs act as AR's coactivator and corepressor, respectively, thus profoundly affecting the AR-mediated gene transcription in PCa [218]. H3K27ac is a marker for active enhancers and promoters. Patterns of global AR binding and H3K27ac are reprogrammed in mCRPC compared to localized PCa [219], suggesting the importance of chromatin states in governing tumor progression. In addition, H3K27ac profiling before and after AR-targeted therapy revealed that a subset of H3K27ac peaks are associated with treatment resistance [220].

Super-enhancers are defined as a cluster of enhancers formed by exceedingly high levels of binding of master transcription factors and mediator complex, also marked with strong histone acetylation such as H3K27ac [221], which play a crucial role as oncogenic drivers in various tumor types including PCa. Activation of AR-associated enhancers is well correlated with histone acetylation in PCa [222].

10.4.1 Histone Acetyltransferases (HATs)

Classic HATs include p300, CREB-binding protein (CBP), TIP60, GCN5, and PCAF. These HATs are involved in AR signaling and PCa tumorigenesis, which are nicely summarized in a recent review [223]. Here, we focus on p300 and CBP, the two highly homologous HAT proteins, each containing two ZZ-type zinc finger (ZZ) domains and three cysteine/histidine-rich (CH) domains (CH1, CH2, and CH3), a bromodomain (BD) that recognizes acetylated residues, and a

catalytic histone acetyltransferase (HAT) domain that catalyzes lysine acetylation [224]. p300 and CBP are well-known AR coactivators through directly acetylating AR to enhance AR's transcriptional activity [225]. They are overexpressed and involved in the progression of PCa [226, 227]. A number of CBP/p300 inhibitors have recently been developed. For example, GNE-049, a CBP/p300 bromodomain inhibitor, inhibits the growth of CRPC in vitro and in vivo [228]. A-485, a highly selective catalytic inhibitor of p300/CBP, potently downregulated the AR transcriptional output in both androgen-sensitive PCa and CRPC cells [229]. Furthermore, A-485 inhibited the growth of LUCaP-77 xenograft, a patient-derived AR-positive CRPC model [229]. In addition, A-485 treatment greatly increases the efficacy of PD-L1 blockade in a syngeneic PCa mouse model through inhibiting the secretion of exosomal PD-L1 [230]. More recently, CCS1477, another CBP/p300 inhibitor targeting the bromodomain, exerts anti-tumor activity through regulating AR, AR-V7 and c-MYC nodes and it is currently in a phase I/II clinical trial NCT03568656 (Table 10.1) [231]. In summary, these findings underscore CBP/p300 as a compelling therapeutic target, either alone or in combination with other drugs, for the treatment of advanced PCa.

10.4.2 Histone Deacetylases (HDACs)

HDACs remove acetylation of histones and non-histone substrates [217]. So far, 18 different HDACs have been identified, which comprise four major classes, namely, HDAC Class I, II, III, and IV [232]. Overexpression of HDACs is observed in different types of cancers, including PCa [233].

Class I HDACs include HDAC1, 2, 3, and 8, which are highly expressed in PCa compared to normal prostate tissue [234]. HDAC inhibitors block AR-mediated activation of target genes in both hormone-sensitive and refractory PCa [235]. Similarly, HDAC3 inhibitors were shown to block the activity of AR-V7, inhibiting the growth of 22Rv1 xenografted tumors [236]. A very recent study further demonstrated that selective elimination of senescent neutrophils through HDAC inhibitors delays PCa progression in vivo [237]. Class III HDACs, namely sirtuins (SIRT1–7), are nicotinamide adenine dinucleotide (NAD+)-dependent and different from other zinc-dependent HDAC classes [238]. The function of sirtuins in PCa is complex and context-dependent. For example, loss of SIRT1 results PIN formation [239], and SIRT1 inhibits PCa cell proliferation through AR deacetylation [218, 240]. In contrast, Huang et al. showed that lowering SIRT1 leads to inhibition of PCa growth [241].

HDAC inhibitors, which cover five different classes of compounds (namely, hydroxamic acids, aliphatic acids, cyclic peptides, benzamides, and sirtuin inhibitor), have been developed and tested in clinical trials [242]. Suberoylanilide hydroxamic acid (SAHA) was the first FDA-approved HDAC inhibitor for the treatment of cancer in the US [243]. SAHA treatment has been found to inhibit the proliferation of PCa cell lines in vitro and the progression of PCa tumors

in vivo [244–246]. Although HDAC inhibitors showed efficacy for some hematological malignancy subtypes, a majority of the clinical trials involving HDAC inhibitors in PCa failed due to high toxicity and a lack of specificity [247]. There are still ongoing clinical trials with HDAC inhibitor in PCa [248]. For example, the FDA-approved HDAC inhibitor Belinostat is currently tested together with PARP inhibitor Talazoparib in mCRPC (NCT04703920, Table 10.1). Nevertheless, different HDACs can exert either tumor-promoting or anti-tumorigenic functions [238], therefore demanding more mechanistic studies to provide guidance on how to employ class-specific HDAC inhibitors, either as a single-agent therapy or in combination with other therapies, to treat advanced PCa.

10.4.3 Histone Acetylation Readers

The bromodomain-containing family proteins recognize acetylated lysine such as histone acetylation to regulate gene expression [249]. The bromodomain and extra terminal domain (BET) subfamily of bromodomain-containing proteins include BRD2, BRD3, BRD4, and BRDT, all of which contain two tandem bromodomains (BD1 and BD2) [250, 251]. BD1 and BD2 play an important role in regulating transcription via recognizing acetylated lysine residues of histones and non-histone proteins [252]. BRD4, the best-characterized BET family protein, recruits the elongation factor p-TEFb to stimulate RNA polymerase II-dependent transcription [253]. BET proteins are frequently overexpressed in various types of human cancer including PCa [249, 251, 254, 255]. They play a critical role in tumorigenesis and represent a class of attractive therapeutic targets for cancer treatment [250].

Pan-BET inhibitors, such as JQ1, I-BET151, I-BET762, ABBV-075, OTX-015, and ZEN-3694, were developed to block interaction between BD domain and the acetylated residue, which were shown to affect a large variety of cellular processes and have antitumor effects in numerous preclinical and clinical models [250]. In PCa, BRD4 physically interacts with the N-terminal domain of AR and promotes AR-related gene-expression programs in CRPC [256]. JQ1, an inhibitor of BET family member proteins, inhibited the binding of BRD4 to AR enhancers globally, thereby repressing the AR-mediated gene transcription [257]. Welti et al. reported that the nuclear BRD4 level increases significantly over disease progression and its higher expression at diagnosis is associated with a worse clinical outcome [258]. Furthermore, they found that BET inhibitors reduce AR and AR-V7 signaling in patient-derived xenograft model of CRPC [258]. Furthermore, BET inhibitors also overcome antiandrogen resistance in advanced PCa [257, 259]. In addition, BRD4 seems to have AR-independent functions [260, 261]. BRD4 also recognizes the acetylated non-histone proteins such as NF-κB and TMPRSS2-ERG [262, 263]. Treatment of the BET inhibitor OTX-015 increases the efficacy of radiation therapy (RT) and overcomes radio resistance through blocking DNA repair [264]. In summary, these aforementioned studies lay a strong foundation for the future clinical exploration of BET inhibitors.

The mechanisms for resistance to BET inhibitors have been reported, some of which involve PCa-associated somatic mutation of SPOP, an E3 ligase substrate binding protein [265–267]. Due to its frequent mutation in PCa, mutant SPOP failed to induce the ubiquitination and proteasomal degradation of BET domain proteins [265–267]. This resistance mechanism then led to activation of AKT-mTORC1 signaling, which can be overcome by combination treatment with AKT inhibitors [267].

PROTAC-based small-molecule degrader for specifically targeting BET domain proteins have also emerged [268, 269]. For Instance, ARV-771, a potent pan-BET degrader, more potently suppressed the AR signaling and tumor progression in the CRPC-xenografted mouse models, compared to the original inhibitor [268]. ZBC-260, another BET degrader, preferentially affects AR-positive PCa cells over AR-negative ones, and suppresses PCa growth in vitro and in vivo [270].

Interestingly, a study based on CRISPR-Cas9-directed protein domain scanning has previously uncovered that the BD1 and BD2 domains of BRD4 have distinct functions [271]. Accordingly, selective inhibitors targeting either BD1 or BD2 have recently been developed [272, 273]. While the BD1-specific inhibitor iBET-BD1 phenocopies the pan-BET inhibitors in a majority of the tested cancer cell lines, the BD2-specific inhibitor iBET-BD2 is predominantly effective in models of inflammatory and autoimmune disease [273]. The activity of another BD2-specific inhibitor, ABBV-744, is predominantly restricted to AML and AR-positive PCa [272]. ABBV-744 displaces BRD4 from AR-containing super-enhancers, inhibits the AR-dependent transcription, and reduces tumor growth in PCa xenografts [272]. There are a plethora of ongoing clinical trials with inhibitors or degraders targeting BET domain proteins [274]. In mCRPC, a number of trials with the pan-BET inhibitor ZEN-3694 are currently underway (Table 10.1). A completed trial (NCT02711956) showed that ZEN-3694 plus Enzalutamide demonstrated acceptable tolerability and potential efficacy in mCRPC patients, and further prospective study is required [275]. Interestingly, a phase I/II clinical trial with NUV-868, a BD2-selective oral small-molecule BET inhibitor, is ongoing in mCRPC patients, either alone or in combination with Enzalutamide or Olaparib (NCT05252390, Table 10.1).

10.5 Chromatin Remodeling in Prostate Cancer

Chromatin accessibility is regulated by chromatin-remodeling complexes, which use the energy from ATP hydrolysis to slide or eject nucleosomes [19]. There are four subfamilies of chromatin-remodeling complexes, namely, Chromodomain Helicase DNA-binding (CHD), Inositol Requiring 80 (INO80), Imitation Switch (ISWI), and Switch/Sucrose Non-Fermentable (SWI/SNF, also known as BRG1/BRM associated factor (BAF) complex) [19]. In this section, we mainly highlight the recent discoveries on SWI/SNF and CHD remodeling complexes in PCa.

Alteration of the SWI/SNF complex subunit occurs in about 20–25% of all cancers including PCa [276, 277]. The catalytic ATPase components, SMARCA4

(BRG1) and SMARCA2 (BRM), mediate ATP hydrolysis to reposition nucleosomes at non-coding regulatory elements, thereby enabling the transcription factors to bind DNA and promote gene expression [278]. The mutant SWI/SNF complex usually enhances oncogenic transcriptional programs, making it a promising therapeutic target [279]. BRG1 has been reported to be overexpressed in PCa and associated with tumor progression [280–282]. Sandoval et al. identified a strong interaction between TMPRSS2-ERG and BAF complex in VCaP cells, a PCa cell line containing endogenous TMPRSS2-ERG fusion [283]. They further found that this interaction drives retargeting of BAF complex globally to ETS target sites,meanwhile, the ATP-dependent catalytic activity of BAF complex is required for ERG's chromatin association, ERG's downstream target gene expression and ERG's oncogenic function in PCa [283]. The interdependency between ERG and BAF complex indicates that TMPRSS2-ERG-positive PCa could be sensitive to BAF complex inhibitors. Indeed, Xiao et al. reported that the treatment of AU-15330, a PROTAC degrader of BRG1 and BRM, dismissed the main transcription factors such as AR, FOXA1, ERG and MYC from chromatin, disrupted their super-enhancer and promoter looping, inhibited the down-stream oncogenic pathways, and induced strong inhibition of CRPC growth, either alone or in combination with enzalutamide [284]. BRD9, a component of non-canonical SWI/SNF complex (ncBAF), has recently been shown to interact with AR, regulate its activity, and promote tumor progression [285]. In addition, the expression of BAF57 is positively correlated with Gleason score and functions as a critical regulator of AR [286, 287].

Chromatin-remodeling complex often intertwines with other signaling pathways. For example, a synthetic lethal relationship was characterized between PTEN and BRG1 by the Qin laboratory [288]. In this study, they found that PTEN loss stabilized BRG1 via inhibiting the AKT/GSK3b/FBXW7 pathway, which drove a tumor-promoting transcriptome through chromatin remodeling [288]. Additionally, PTEN-deficient PCa is sensitive to the treatment with BRG1 antagonist, indicating a therapeutic strategy [288].

Another chromatin remodeler CHD1 is altered in 7–10% of PCa cases [12, 289]. CHD1 has been reported to play significant roles in PCa in a context-dependent manner [290, 291]. CHD1 depletion leads to the defects of DNA double-strand break (DSB) repair via homologous recombination (HR) and thus sensitizes PCa cells to PARP inhibitors [292]. Coordinate loss of MAP3K7 and CHD1 occurs in 10–20% of localized PCa, correlating with poor disease-free survival [293]. Likewise, combined loss of MAP3K7 and CHD1 increased tumor growth and decreased survival in LNCaP xenografted animal models [293]. In addition, CHD1 loss alters AR binding and regulates different sets of target genes to promote PCa [294]. Zhang et al. identified that depletion of CHD1 leads to lineage plasticity and antiandrogen resistance through an in vivo shRNA screening [295]. In a mCRPC patient cohort, low CHD1 expression is correlated with a worse response to the second-generation antiandrogen treatment [295].

Interestingly, both SWI/SNF complex and CHD1 have been shown to play an important role in cancer immunotherapy including PCa [296, 297]. Since PCa is

known to be immune-cold [298], it would be worthwhile to test whether targeting chromatin remodeling pathway makes PCa sensitive to immunotherapy.

10.6 Conclusions and Future Perspectives

This chapter provides an overview of epigenetic alterations, focusing on DNA methylation, histone methylation and acetylation, and chromatin remodeling during the development and progression of PCa. AR-mediated signaling is one of the most critical drivers for PCa initiation and progression. Various histone-modifying enzymes regulate ligand-dependent or ligand-independent transcription of AR target genes through interacting with AR. Additionally, epigenetic regulators are also critically involved in the activation of other oncogenic signaling pathways, independent of AR signaling and usually in a more advanced setting of PCa such as mCRPC. Due to the reversible nature of epigenetic modifications, targeting epigenetic regulators has become a promising therapeutic intervention. Table 10.1 lists some of the ongoing clinical trials for testing drug candidates targeting epigenetic factors in patients with mCRPC. Development of epigenetically centered therapy alone generally showed less efficacy in PCa, probably because of the heterogeneity nature of this disease. However, we remain optimistic about potential combination therapies with other drugs, such as antiandrogen, PARP inhibitor, and immune therapy, for the treatment of patients with advanced PCa. We look forward to new exciting development along these lines in the years to come.

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Part III
DNA Epigenetics

Ten-Eleven-Translocation Genes in Cancer

11

Yadong Wang, Xujun Wang, and Jun Lu

11.1 Introduction

The TET family genes were initially discovered in 2003 as a form of rare chromosomal translocation in acute myeloid leukemia (AML) between chromosome 10 and 11, creating a fusion gene between TET1 and Mixed Lineage Leukemia (MLL) gene [1]. It is now recognized that the TET family of proteins includes three members, TET1, TET2, and TET3. While the translocation involving TET1 suggests a role for this family of proteins in cancer, the biochemical activity of these proteins as dioxygenase to oxidize 5'-methylcytosine (5mC) in DNA was revealed in 2009 [2, 3]. As DNA 5mC plays important roles in epigenetic regulation in many organisms, including mammalian species, the discovery of TET's biochemical activity leads to a breakthrough in understanding DNA demethylation through both a passive dilution mechanism and an active demethylation pathway involving mismatch repair pathway genes. As methylation is often associated with silenced gene expression, demethylation by TET proteins can lead to upregulation of target gene expression. Beyond catalyzing serial oxidation of 5mC, all three TET proteins contain large domains in addition to their catalytic domain, therefore

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mediating protein–protein interactions to regulate gene expression and other cellular functions. One of such interactions, involving TET and histone deacetylase (HDAC), paradoxically leads reduction of target gene expression [4]. With both positive and negative impacts on gene expression, TET proteins can have complex influences on downstream genes, which could lead to differential outcomes based on cellular context, cellular metabolic states, and micro-environmental factors. The biochemical functions of TET proteins have been covered extensively in excellent prior reviews such as [5–7]. There are also multiple excellent reviews of TET family genes in cancer, such as [7–10]. To complement the existing literature, this review will focus on roles of TET proteins in cancer with an emphasis on three aspects of biology with significant unresolved questions, namely the functions of TET mutations, the role of TET2 in hematopoietic malignancies, and the role of TET proteins in regulating anti-tumor immunity in solid cancers.

11.2 The Genetics of TET Mutations in Cancer

Assisted by the revolution in deep sequencing technologies, prevalent mutations in TET2 were discovered in 2009 in hematopoietic malignancies [11, 12]. In AML and myelodysplastic syndromes (MDS), TET2 mutations can range from ~15% to ~30% in patient cohorts. In a subgroup of myeloproliferative neoplasms, chronic myelomonocytic leukemia (CMML), TET2 mutation frequency could be as high as ~50%. In lymphoid malignancies, TET2 mutations tend to be less frequent but nevertheless can be found in both lymphomas and leukemias. Surveying the COSMIC database, the distribution of TET2 mutations in all cancer types as well as in hematopoietic malignancies has an obvious skew toward nonsense substitutions (generating early stop codons) and frameshift mutations, which together account for more than 50% of total mutations (Fig. 1A). This feature is characteristic of genetic inactivation of tumor suppressors. The majority of mutations in TET2 occurs in a monoallelic form, although biallelic mutations are also present, leading to early recognition of TET2 as a haploinsufficient tumor suppressor within the hematopoietic system [11, 12]. Considering that the catalytic domain of TET proteins is at the C-terminus, the frameshifts and early stop codons can clearly lead to a truncated protein with loss of catalytic activity by TET2. Missense substitutions in TET2 are also observed in abundance (~40% of mutations), a fraction of which is located in the catalytic domain, presumably impacting the catalytic function. Missense substitutions beyond the catalytic domain are also present in large numbers, but the molecular and biological consequences of such mutations are mostly unknown. It can be speculated that some of these mutations may lead to changes in interaction between TET2 and other partner proteins, alter TET2 protein or RNA stability, or lead to other structural changes in the protein. Which and how many of such mutations bear functional consequences? Although a small number of TET2 missense mutations have been functionally characterized in terms of biochemical activity [13], future studies could further elucidate the functions of such mutations in cancer. Additionally, a small percent of synonymous mutations is also

present in TET2 (<3%). Although synonymous mutations may simply reflect the background mutation rates and are often not considered as functionally important, they do affect codon usage, which in turn may affect translational efficiency and drive oncogenesis [14]. Recent studies also found that codon usage not only affects translation but also mRNA stability [15]. Considering the dose-sensitive functions of TET2 as a haploinsufficient tumor suppressor, it is feasible that a fraction of synonymous mutations in TET2 can play a functional role in determining TET2 gene expression levels. This possibility awaits further investigation.

TET1 and TET3 are also mutated with high frequencies in cancers (Fig. 1D). However, in contrast to TET2's clear loss of function mutation signature, nonsense substitutions, and frameshift mutations are at much lower levels (Fig. 1B, C). For TET1, these two types of mutations account for ~9% of mutated cases, and for TET3, ~7%. Missense substitutions are the dominant forms of observed mutations, accounting for ~52% for TET1 and 39% for TET3. In the case of TET3, missense substitution rates are only slightly more than twofold higher than the synonymous mutation rate (~18%), suggesting that the majority of TET3 mutations in cancer represents background mutation rates and is likely not selected for. However, it is possible that a subset of TET3 mutations may play functional roles in cancer. It is also possible that in certain cancer types, there is functional selection for TET3 mutations. For example, in hematopoietic malignancies, although the overall rate of TET3 mutation is low (<2% of cases), the ratio of missense mutation to synonymous mutation is substantially higher than 2 (Fig. 1C). Whether this is simply due to a low number of cases impacting the reliability of the statistics or reflects an enrichment of functional TET3 mutations in hematopoietic malignancies could be further studied. For TET1, missense substitution rates are substantially higher than twofold of synonymous mutation rate in all cancers (~12%) and in hematopoietic malignancies (Fig. 1B), suggesting a functional selection for missense alterations in TET1. Whether these mutations represent gain or loss of function or introduce neomorphic functions could be interesting areas of investigation.

11.3 TET2 in Hematopoietic Malignancies

TET2 is one of the most frequently mutated tumor suppressors in hematopoietic malignancies. What are the precise roles of TET2 in the initiation of transformation? The most well-recognized role of TET2 mutations is to initiate a pre-malignant expansion of mutant hematopoietic stem cells (HSCs) that paves way for additional mutation accumulation towards full transformation. This model is supported by the discovery of widespread clonal hematopoiesis in healthy human individuals, in which TET2 is the second most frequently mutated gene alongside DMNT3A [16–20]. The frequency of TET2 mutations is likely underestimated in the initial studies of clonal hematopoiesis, with technical limitations of the exon capture platforms to miss some of TET2's coding sequences. Clones with TET2 mutation expand and accumulate with increasing age [16–20]. Given that

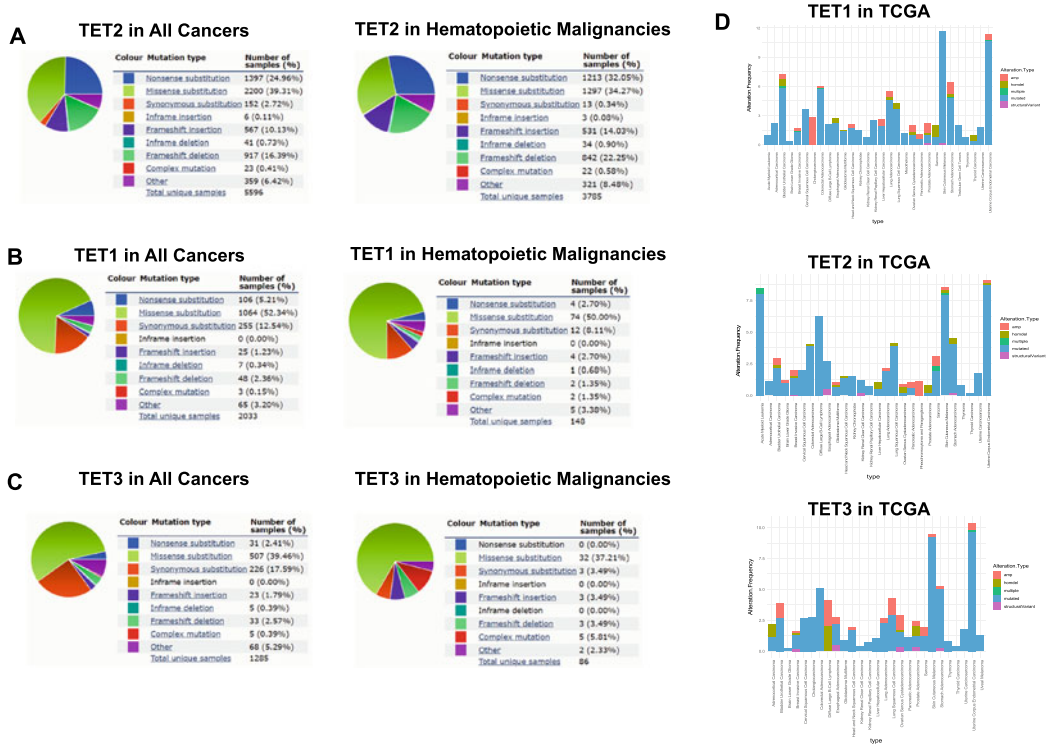

Fig. 11.1 Mutation patterns of TET genes in cancer. **A**, **B**, **C** The mutation patterns of TET2, TET1, and TET3 from the COSMIC database were plotted to show their mutation types and frequencies, for all cancer types (left) and hematopoietic malignancies (right). Mutation frequency represents the frequency of the indicated mutation type among all mutations detected. **D** The mutation rates of TET1, TET2, and TET3 in the indicated cancer types were plotted. Data were obtained from the TCGA database

the vast majority of individuals over the age of 50 have detectable clonal mutations in their hematopoietic cells [21], and the reported frequencies of TET2 in clonal hematopoiesis, the frequency of TET2 mutant clonal hematopoiesis likely exceeds a quarter of the general aged population. This role of TET2 mutation leading to pre-malignant expansion of mutant HSCs is also supported by multiple mouse knockout (KO) studies, in which loss of Tet2 leads to an expanded pool of functional HSCs, with full transformation to leukemia being rare and with long latency [22–25]. Alongside the expansion of functional HSCs, Tet2 KO mice show the expansion of the myeloid lineage particularly monocytic cells in the form of myeloid proliferative neoplasms. This phenotype is consistent with the high frequency of TET2 mutations in CMML. These data and phenotypes raise two questions that are not yet fully resolved in the field, which are discussed in detail below.

First, how does TET2 gatekeep HSC expansion in a dosage-dependent manner? The TET2 mutation patterns in human strongly implicate that the catalytic function of TET2 is important both in clonal hematopoiesis and in hematopoietic malignancies. Several murine studies have also supported the idea that the catalytic function of Tet2 is critical for safeguarding HSC expansion and malignant transformation. Zhao et al. demonstrate that catalytic activity dead Tet2 cannot rescue either aberrant serial colony replating activity of Lin-Kit + hematopoietic stem and progenitor cells (HSPCs) or CMML-like phenotypes in a Tet2 KO background, whereas wild-type Tet2 can [26]. Cimmino et al. show that the treatment of vitamin C, which can enhance the catalytic function of Tet2 as well as some other demethylases such as histone demethylases [27–29], can phenocopy the genetic restoration of Tet2 by suppressing the serial replating activity of Lin-Kit + HSPCs in both Tet2 heterozygous and homozygous KO background [30]. This activity of vitamin C is likely acting on both Tet2 (with one allele in the heterozygous KO) and Tet3 [30], although the involvement of other vitamin C targets cannot be fully excluded. However, what is downstream of Tet2's catalytic activity in safeguarding HSCs is not fully elucidated. While Tet2 loss clearly alters the 5hmC and 5mC landscape in the genomic DNA of HSPCs [31], Tet2 is also recognized for its ability to interact with RNA and catalyze RNA modification [32–34]. Whether DNA or RNA is the main functional target of Tet2's catalytic activity in HSCs is still debatable. Indeed, during evolution, not all species have DNA 5mC mechanisms. For example, Drosophila does not have abundant 5mC in the genomic DNA and the mammalian homologs of DNA methyltransferases Dmnt1 and Dmnt3 are absent in the Drosophila genome. Yet, a Tet homolog capable of 5hmC modification of RNA was found and is required for Drosophila development [35]. It is also worth noting that Drosophila has functional hematopoietic cells, albeit with a rudimental hematopoietic system compared to those of mammals. It is therefore possible that Tet enzymes' function on RNA rather than DNA could be the evolutionary root, and may even underlie the function of Tet2 in HSCs. In addition, evidence implicating a catalytic-independent function of Tet2 in suppressing HSC expansion is also present. Cai et al. demonstrated that reducing the genetic dose of Morrbid, a long non-coding RNA downstream of IL-6, can nearly completely rescue HSC

expansion in Tet2 heterozygous knockout mice [36]. IL-6 has been previously established as being suppressed by Tet2 via Tet2-Hdac2 interaction without an obvious involvement of Tet2's catalytic activity on DNA [4]. Therefore, it is still not fully clear whether Tet2's catalytic activity is required for HSC gatekeeping. It is also possible that the RNA-based 5mC oxidation activity of Tet2 is involved in the regulation of factors such as IL-6, therefore providing a united framework for the existing literature. Lastly, mechanistically, how downstream targets of Tet2 can be sensitive to the dosage of this gene is still largely unclear.

Second, how does pre-malignant expansion of TET2-mutant HSCs pave the way for full transformation? Although it is clearly possible that TET2 mutation may also occur after other oncogenic mutations, with one example being JAK2 mutation preceding TET2 mutation in some myeloproliferative neoplasm cases [37], the human genetic data of clonal hematopoiesis argue that TET2 loss is a first hit event in probably the vast majority of hematopoietic malignancies involving TET2 mutations. The general model is that TET2-mutation-driven pre-malignant HSPC expansion leads to higher chances of acquiring additional co-operating oncogenic events, eventually leading to full transformation. Consistent with this model, it was found that Tet2 KO HSPCs accumulate more mutations [38]. This can be simply due to more chances of mutation through increased cell doublings during HSPC expansion, and/or could be due to active maintenance of genomic integrity by Tet2. The findings that there is a ~30% increase of mutation frequency in genomic regions with Tet2-depedent 5hmC signals [38] suggest that there is a contribution by the latter possibility, which is also consistent with the fact that Tet2 mutant zebrafish HSPCs are more sensitive to the PARP1 inhibitor [39]. However, this data may also be interpreted as 5hmC serving as a marker for DNA damage, which has been shown in HeLa cells [40]. What is even less clear is how frequently such additional mutations cooperate with TET2 loss in driving transformation (a cooperative mutation model) instead of those mutations relying on TET2 loss for acquisition but driving oncogenesis without further dependence on TET2 status (a serial mutation model). This question unfortunately cannot be easily sorted out by examining human genetic data in terms of mutation co-occurrence between TET2 and other oncogenic mutations, because both cooperative model and serial model can display oncogene co-occurrence. Therefore, direct experimental testing is required for understanding the functional co-operation, which is far from being thoroughly tested. One example of cooperation is between Tet2 loss and Flt3-ITD, a mutant receptor tyrosine kinase, that led to accelerated AML formation [41]. The mechanism for this cooperation was found to be cooperative epigenetic regulation of Gata2 that depends on both genetic lesions [41]. This Tet2-Flt3 cooperation bears some similarity to an earlier two-hit model for AML in which one mutation (Flt3) alters the growth factor signaling pathway [42], but differs in a way that the second mutation (Tet2) does not lead to inhibition of differentiation but rather impacts the epigenome. Another example is N-Ras mutation that frequently co-occur with TET2 mutations in CMML [13]. Although an earlier study found that shRNA-based knockdown of Tet2 in $Nras^{G12D/+}$ mice did not result in detectable

functional cooperation in CMML onset [13], a more recent study showed cooperation in $Nras^{G12D/+}Tet2^{+/-}$ mice, with increased HSPC expansion and accelerated CMML lethality [43]. The molecular mechanism underlying this NRas-Tet2 cooperation is unclear, but this relationship also features a genetic lesion in the growth factor signaling pathway. However, cooperation with a growth signaling mutation is not a must. TET2 KO has been shown to cooperate with AML1-ETO especially the splicing variant AML1-ETO9a [44] in leukemogenesis, although the cooperative mechanism is unclear. In the case of genetic cooperation between MLL-AF9 and miR-125b [45], miR-125b overexpression accelerates AML leukemogenesis by MLL-AF9 and can downregulate Tet2 via targeting its 3'UTR [45, 46]. The cooperative mechanism was found to be due to a substantial VEGFA upregulation in a Tet2-reduction-dependent way. Although it is unclear whether Tet2 loss can cooperative with MLL-AF9 directly, these data suggest an alternative functional downstream effector of Tet2 loss. Through these examples, it seems that the cooperative oncogenic events with Tet2 loss are quite diverse without a simple rule. It also seems that there could be multiple downstream events of Tet2 that leads to genetic cooperativity. Considering Tet2's molecular function in regulating epigenetic and epitranscriptomic landscapes, maybe it should not come as a surprise that there could be multiple downstream effectors of Tet2 in transformation that depend on its partner genetic lesions, the initiating cell for the transformation, and possibly environment factors such as inflammatory states. Whether this model is true will await further experimental investigations.

11.4 TET Genes in Regulating Anti-Solid-Tumor Immune Responses

The frequencies of TET gene mutations in solid cancers (Fig. 1D), coupled with early findings that reduced 5hmC levels are a hallmark of melanoma [47], suggest that TET genes play widespread functional roles in solid tumors in addition to their roles in hematopoiesis. However, the exact roles of TET proteins in solid cancers are overall relatively understudied. A key question is whether there are unifying principles that can explain the diverse TET protein functions in regulating tumors.

One important aspect that TET genes regulate solid tumors is via regulating the immune responses. This can occur with clonal hematopoiesis where immune cells could inherit mutant TET2 from HSCs, thereby eliciting mutant immune programs. Several studies have shown that TET2 loss in hematopoietic cells can promote anti-tumor immune response. In murine melanomas, loss of Tet2 in myeloid cells leads to enhanced T cell responses against the tumor cells [48], via dysregulated Arg1 expression that is likely controlled by Tet2's catalytic activity on DNA 5hmC levels. Loss of Tet2 in T cells can also promote anti-cancer immunity. This was first shown in anti-CD19 CAR-T cells, in which TET2 mutation or experimental loss in the engineered T cells promotes the efficacy of CAR-T responses [49]. A later study using murine models of melanoma reached similar conclusion of improved anti-tumor responses from Tet2-null T cells [50]. These experimental

roles of Tet2 initially seem to at odds with human genetic studies of solid cancers, in which increased levels of clonal hematopoiesis are associated with solid cancer and poorer survival [51]. This difference may be due to several factors. First, therapies for solid cancer patients could be the cause of TET2 mutations, as there are association between therapy use and the increased levels of clonal hematopoiesis [51]. Second, the functional studies mentioned above are tested in immune cells with complete or near complete Tet2 loss, and with either all perturbed immune cells or a large number of them harboring Tet2 loss. These conditions represent the extremes of clonal hematopoiesis, with both high mutant allele frequency and biallelic TET2 mutations. Whether functional responses can be seen with heterozygous Tet2 KO or with lower frequencies of mutant cells needs to be addressed. Third, it is possible that the response of different cancer types to TET2 mutant immune cells may differ between cancer types. Further studies would be needed to examine these possibilities.

The role of cancer-cell-intrinsic TET2 in shaping the immune environment could be potentially opposite to the effects of TET2 mutations in the hematopoietic system. Loss of Tet2 in colon and melanoma models leads to reduced anti-tumor responses, via Tet2's control of the interferon response pathway [52]. This function may help to explain the loss-of-function Tet2 mutation patterns in solid tumors (Fig. 1A), in which the loss of anti-tumor response via loss of TET2 may be one of the reasons TET2 mutations are selected for during tumorigenesis. An interesting question arises as to what happens when TET2 is lost in both solid cancer cells and in HSCs, which could be further examined. However, it is also important to emphasize that the experiments above were performed using TET2 KO cancer cells. The genetic mutation of TET2 in solid cancers may not be biallelic or uniform across a single tumor, two effects that have not been systematically studied. If anti-tumor effect is the major reason for TET2 loss in solid cancer, one would expect that there will be differential selection for TET2 loss based on mutational burden, which is often associated with the availability of neoantigens. These questions would require future investigations.

11.5 Conclusions

14 years after the discoveries of TET protein's biochemical activity and TET mutations in human cancers, much progress has been made toward understanding TET functions in hematopoietic malignancies and solid cancers. Mutations in TET genes are frequent in human cancer specimens with mutational patterns suggesting functional importance for TET2 and TET1 mutations in both hematopoietic and solid cancers, and for TET3 in certain cancer subtypes. Yet, many important questions remain even in the most well-studied case: TET2 in hematopoietic malignancies. In solid cancers, much awaits exploration to better understand TET genes' functions and form new principles. Inspirations may also come from evolutionary cues or cancer-resistant species to further guide the thinking on the origin of TET genes and their relationship with cancer.

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12 Inferring the Cancer Cellular Epigenome Heterogeneity via DNA Methylation Patterns

Sheng Li

12.1 Introduction

Tumor cells evolve through space and time, generating genetically and phenotypically diverse cancer cell populations that are continually subjected to the selection pressures of their microenvironment and cancer treatment [1, 2]. This intra-tumor heterogeneity remains a major challenge in cancer molecular subtype classification and selection of appropriate treatment [3, 4]. Several studies on cancer cell states suggest that intra-tumor heterogeneity is organized into signatures [5–8]. Comprehensive profiling of intra-tumor heterogeneity can uncover key oncogenic drivers in specific tumor cell clones and enable therapy-resistant clones to be matched with more effective treatment regimens, such as sequential or concurrent combination therapy. Thus, *understanding intra-tumor heterogeneity is essential for improving the efficacy of cancer treatment*. Gene regulation of the mammalian genome occurs through complex interactions involving non-coding cis-regulatory elements, trans-regulatory factors, and epigenetic modifications. These epigenetic modifications constitute the epigenome, i.e., DNA methylation, histone modification, and chromatin remodeling, are essential for cellular functions and cell state transition. Notably, cell-to-cell variability in the epigenome can increase the sampling space for cancer cells to survive therapy and has been associated with clinical outcomes in several cancer types [2, 9–11]. This chapter will also review the cancer cellular epigenome heterogeneity based on DNA methylation patterns. The first part will discuss epigenome-level heterogeneity's biological significance and clinical relevance. The second part will cover epigenome technologies and analytical

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strategies to infer cellular epigenome heterogeneity and evolution, including different matrics and the clinical metadata associated with it. The intrinsic and extrinsic sources of cellular epigenome heterogeneity were also discussed. More profound knowledge of intra-tumor epigenome heterogeneity is expected to facilitate prognostic biomarker development, identify novel therapeutic targets, and support the development of epigenetic therapy.

12.2 Why Study Cancer Heterogeneity and Evolution?

Cancer evolution is a process by which cancerous cells develop and change over time [1, 2]. This process is driven by the increased genetic and epigenetic heterogeneity of the malignant cells and the selection of cells with increased fitness [3]. The heterogeneity characterizes the existence of multiple cellular states within a tumor that differ in their genetic and epigenetic makeup [4, 5]. During somatic cell evolution, selection occurs when cancer cells with beneficial mutations or epimutations survive and proliferate more than cancer cells without those [6]. This can lead to the accumulation of advantageous mutations and epimutation over time, allowing the population of cancer cells to become fitter, i.e., cellular adaptation [7]. Those mutations and epimutations contribute to a competitive advantage of cancer cells to the ever-changing microenvironment due to aging, therapy, and lifestyle [8–10]. A set of evolutionary models beyond Darwin's principles of cancer evolution [11] underpin the significance of understanding the cellular heterogeneity to anticipate and address cancer initiation and progression. Cancer heterogeneity and evolution provide insight into how cancer develops and progresses for improving cancer diagnosis, developing targeted treatments, and improving patient outcomes. The mechanisms by which cancer cells evolve and become more heterogeneous nominate strategies for more effective treatment [12]. Furthermore, studying cancer evolution trajectories identifies which cancer cells are more likely to be resistant to treatment and may help to predict the course of the disease.

12.3 Why Study Epigenetic Heterogeneity in Cancer Evolution?

Epigenetic mechanisms—including DNA cytosine methylation (5mC), histone modification, and chromatin remodeling—are essential for gene expression, genome organization, and cellular function. Cell-to-cell variability in the epigenome of the same individual, i.e., epigenetic intra-tumor heterogeneity such as DNA methylation epigenetic allele heterogeneity, can increase the sampling space for cancer cells to survive therapy and has been associated with clinical outcomes in several cancer types [13–16]. The past two decades have witnessed the rapid development of computational tools for functional genomic data analysis to extract the identity of the key 5mC dynamics and transcriptional programs that underpin changes in cell state or behavior [17–24]. Such changes could result

from differentiation programs in development [25], response to signaling ligands or metabolite/chemical exposure in differentiated cells [26, 27], immune response [28, 29], de-differentiation in response to experimental cellular reprogramming [30], or pathogenesis such as cancer [13, 31, 32], all which are expected to be the manifestation of changes in gene expression programs. While such cell state changes are reflected in analyses of RNA-sequencing (RNA-seq) datasets generated in cells before and after the change, a list of differentially expressed genes (DEGs) does not reveal which gene was the initial driver of the change. That is, *the key genes are shrouded by the great number of DEGs that are downstream effectors of the transcriptional regulatory programs.* Thus, more information is needed to understand changes in cell state. At the same time, rapid growth in the number of ever-more-sophisticated genome-wide molecular assays that spawn a dizzying array of big datasets derived from a plethora of cell types in innumerable states and conditions exposes a growing need for this information to be translated into useful knowledge for the research community, to improve fundamental understanding of how cells work and to harness this knowledge in ways that will lead to clinical impact. Here, we will review the recent effort in cancer DNA methylation data mining, with the goal of dissecting the functional role of 5mC in chromatin organization and gene regulation for cancer development.

12.3.1 Gene Regulation and Epigenetic Allelic Heterogeneity

The regulation of gene expression across the mammalian genome occurs through multi-tiered complex interactions between non-coding *cis*-regulatory elements (e.g., enhancers, promoters, and insulators), *trans*-regulatory factors [e.g., transcription factors (TFs) and miRNAs], and epigenetic modifications of DNA and histones that constitute the epigenome [33, 34]. DNA cytosine methylation affects gene expression and cell differentiation. Studies in hematopoietic stem/progenitor cells (HSPCs) and embryonic stem cells (ESCs), T-cells, and induced pluripotent cells showed that 5hmC, which is enriched in gene bodies and enhancers, can modulate enhancer activity and transcriptome reprogramming [35–40]. Development of advanced epigenome technology and data mining software uncovered the essential role of 5mC in normal cellular function and its contribution to cancer when disrupted [13, 20–22, 41].

5mC-based epigenetic allelic heterogeneity—where each combination of methylated CpGs detected at a given locus represents a specific epigenetic allele or epiallele—is associated with transcriptome heterogeneity and disease progression in cancer patients [13, 41]. *The concept of 'epigenetic allelic heterogeneity' goes far beyond simply designating genomic regions as highly or poorly methylated.* A cell population may consist of epigenetically distinct subpopulations where each epiallele could correlate with different behavioral potential, e.g., epialleles could prime for a particular differentiation program or pathogenic change such as neoplastic transformation (Fig. 12.1). Recent advances in elucidating chromatin interactions

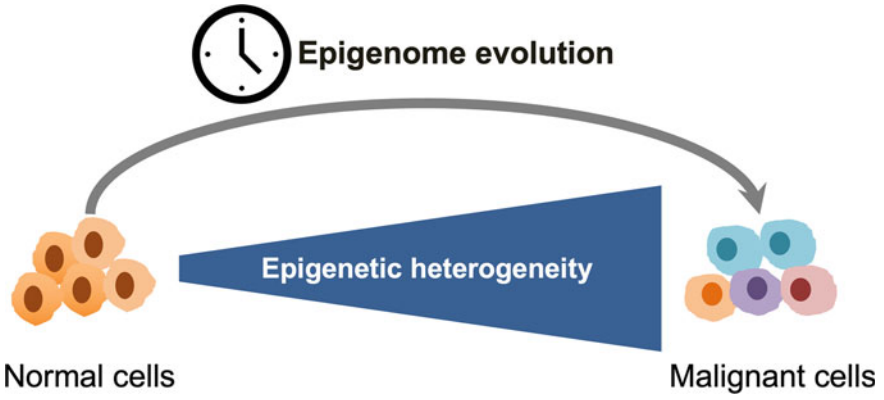

Fig. 12.1 Wide-spread somatic evolution and epigenetic heterogeneity in hematopoietic and solid tumors

through next-generation sequencing (NGS) methods have illuminated the hierarchical organization of the genome within the nucleus and shown that the DNA methylation patterns can promote or prevent the access of TFs to specific DNA sequences [42], move genes between transcriptionally active or inactive [34, 43] nuclear compartments, and build or remove the insulation between neighboring genomic regions [44]. Changes in the epigenome and chromatin organization can impact precise transcriptional regulatory programs to change cell differentiation status or lead normal cells into the neoplastic transformation [45]. Efforts to understand the molecular basis of these failures have focused on developing machine learning and statistical approaches to identify the TFs, miRNAs, and epigenomic changes that contribute to cellular differentiation and disease states [20, 21, 46, 47].

12.3.2 Clinical Relevance of Epigenetic Heterogeneity

Emerging studies suggest that epigenetic heterogeneity in cancer evolution is at a high level of clinical relevance (Fig. 12.2). First, epigenetic changes are thought to be involved in cancer development and progression. The disruption of epigenetic regulations contributes to the maintenance and survival of cancer stem cells [48, 49]. These mechanisms include enhanced plasticity, loss of cell identity, disruption of self-renewal and differentiation balance, and the acquisition of de novo self-renewal from differentiated cells [48]. Second, epigenetic heterogeneity has been linked to cancer treatment resistance [50–52]. Third, there is growing evidence that epigenetic heterogeneity plays a role in cancer evolution. Higher epigenetic heterogeneity is associated with poor clinical outcomes in hematopoietic and solid tumors, such as acute myeloid leukemia [13, 41], lymphocytic leukemia [15, 53], lymphomas [14], gliomas [54]. Thus, epigenetic heterogeneity can impact the

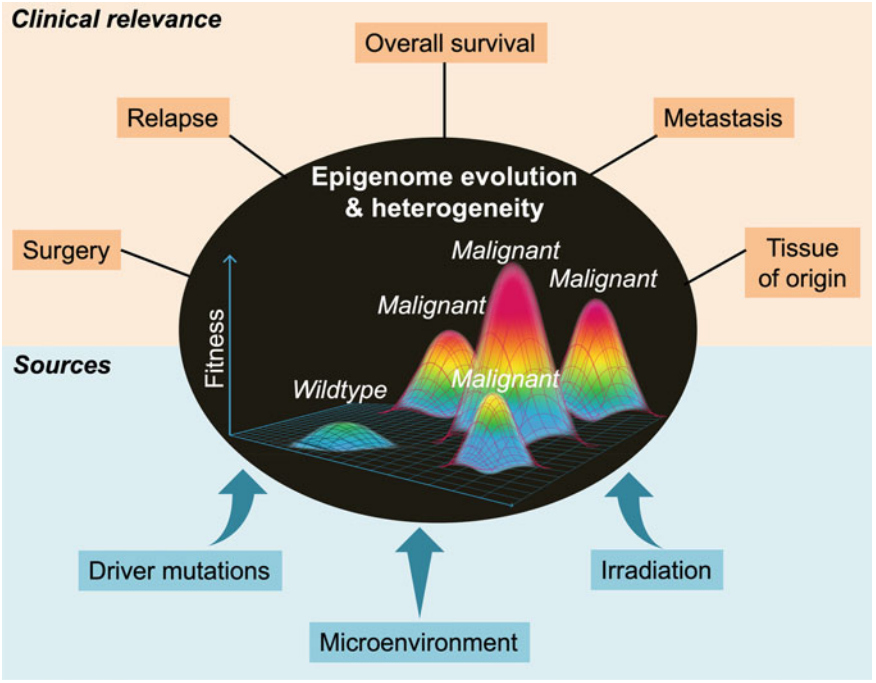

Fig. 12.2 Clinical relevance and sources of cancer somatic evolution and epigenetic heterogeneity

prognosis of cancer. If specific epigenetic changes are associated with more aggressive disease, then patients with those changes may be more likely to experience poorer outcomes. Indeed, epigenetic heterogeneity based on DNA methylation patterns of 26 loci has been developed as biomarkers for predicting leukemia patient event-free survival [41].

Epigenetic heterogeneity may help to improve our understanding of how cancer evolves and to develop new treatments. Epigenetic alterations are reversible and increasing endeavor to drug development targeting the epigenome leads to at least nine FDA-approved drugs for epigenetic therapy. FDA-approved 5′-azacytidine (AzaC, a DNA hypomethylation agent) and Enasidenib [55] have been shown to reverse epigenetic heterogeneity in *Tet2*- and *Idh2*-mutant AML mouse models. Specifically, either a global hypomethylating agent (AzaC) or targeted therapy (Enasidenib) for specific mutation-induced cancer can reduce epigenetic heterogeneity in vivo [41]. This suggests that clonal reduction achieved by decreasing epigenetic heterogeneity might reduce the selective pool size for malignant cells to adapt to the harsh conditions induced by other therapy. Thus, epigenetic heterogeneity challenges the concept of a one-size-fit-all strategy for cancer therapy. Reversing the epigenetic heterogeneity can enable clonal reduction and may serve as a clonal reduction strategy to sensitize the cancer cells to other treatment.

Clonal reduction by reversing heterogeneity using epigenetic therapy suggests that combination therapy including epigenetic drugs and standard therapy may provide an important clinical tool to benefit patients. Optimal treatment of AML arising in elderly patients remains a challenge. On a related note, in 2018, the FDA approved Venetoclax (BCL2 inhibitor) in combination with AzaC for treating elderly patients with AML who are aged ≥75 years or unfit for intensive chemotherapy. While single-agent Venetoclax has had modest activity in AML [56, 57], AzaC plus Venetoclax improved median overall survival by 5 months in previously untreated patients with AML who were ineligible for intensive induction therapy compared to AzaC plus placebo [58]. Furthermore, combination therapy of Ivosidenib (IDH1 mutation inhibitor) and AzaC improves median overall survival by 16 months in *IDH1*-mutated AML [59]. Thus, in 2022, FDA approved the combination for treating newly diagnosed AML. Collectively, these exciting results suggest that understanding epigenetic heterogeneity can provide novel strategies to address cancer evolution.

12.3.3 The Biological Significance of Epigenetic Heterogeneity

DNA methylation is one of the most well-studied epigenetic mechanisms, and it plays an essential role in regulating gene expression [60]. DNA methylation and demethylation modulate gene activities by corroboratively impacting transcriptional factors binding [42], enhancer-promoter interaction [31, 61], chromatin domain boundary [44, 62], and drives cell fate decisions including self-renewal [63] and differentiation [64]. DNA methylation is a process that can be heritable and reversible [65]. The heritability is largely enabled by DNA methylase—the writers of DNA methylation. DNMT1s for maintenance for heritability during mitosis [66] and DNMT3s for de novo methylation in response to the environment [67]. DNA methylation erasers, including the TET family proteins, oxidase the methylated cytosines and eventual DNA active demethylation [31, 32, 35]. Epigenome plasticity owns much to the epigenetic erasers. Epigenetic therapy can reverse aberrant DNA methylation [68, 69].

Studying epigenetic heterogeneity in cancer evolution is critical. First, epigenetic dysregulation is a hallmark of cancer [70]. The selection of disrupted epigenetic regulation was observed in human cancers [13, 41, 71]. Thus, understanding the role of epigenetic changes in cancer evolution provides insight into the mechanisms of disease initiation and progression. Second, epigenetic heterogeneity can impact the response of cancer cells to treatment. If specific epigenetic changes confer resistance to treatment, cancer cells with those changes may be more likely to survive and proliferate. The proposed mechanisms include epigenetic plasticity or phenotypic inertia.

Epigenetic plasticity in clonal evolution has been thoroughly reviewed previously [72]. For example, chromatin organization at the level of 'topologically associated domains' (TADs) plays a critical role in gene regulation for proper enhancer-promoter interaction [73]. TAD boundaries are enriched for genomic

insulator CCCTC-binding factor (CTCF) and CTCF binds to the DNA sequence in a DNA methylation-dependent manner. Thus, IDH-mutant gliomas that are hypermethylated exhibit insulator dysfunction and lead to potent enhancer to abnormally activate oncogene PDGFRA to drive aberrant proliferation [44]. Furthermore, abnormal DNA methylation is also exhibited in AML patients with 3D genome alterations, i.e., TAD boundary disruption due to loss of CTCF binding associated with DNA hypermethylation, and the hypomethylating agent AzaC partially dissociates AML-specific chromatin interactions [62]. Thus, epigenetic plasticity that leads to oncogene activation provides malignant cells alternative paths to escape immune surveillance and therapy.

Besides epigenetic plasticity, phenotypic inertia was proposed as an alternative mechanism to confer fitness mediated by epigenetic dysregulation. Specifically, phenotypic inertia happens when the cells do not efficiently respond to microenvironmental stress, e.g., nutrition deprivation or an acidic environment, by inhibiting proliferation or activating apoptosis. For example, epigenetically disrupted cells of melanoma patient-derived xenograft models showed limited phenotype changes related to stress and higher proliferation than control cells without epigenetic disruption [74]. This suggests that epigenetic heterogeneity can provide malignant or premalignant cells with a competitive advantage by deficiently sensing or responding to harsh conditions and thus enhancing their tolerance to environmental stress. This mechanism applies to an early stage of environmental stress where the tolerant cells will not halt proliferation and delay apoptosis but instead continue to expand the pool size for long-term adaptation.

Taken together, multiple mechanisms that been suggested to convey fitness of the premalignant and malignant cell via increased epigenetic heterogeneity, either by epigenetic plasticity or phenotypic inertia. These mechanisms are likely to be time-sensitive, and either or both strategies are likely to assist the somatic evolution during the life history of tumors.

12.4 What is Epigenetic Heterogeneity Based on DNA Methylation Patterns?

Epigenetic heterogeneity exists when a population of cells exhibits different phenotypes despite having the same genotype. This is due to the fact that the cells can express different genes depending on the epigenetic state of the cells. Epigenetic allele generally refers to a variant of a DNA fragment that is caused by epigenetic modifications, rather than changes in the DNA sequence. The distinct DNA methylation patterns that define epigenetic alleles can serve as the basic unit to quantify epigenetic heterogeneity. DNA methylation patterns can be phased to determine which allele is methylated and in what combination at each locus [75]. This can be useful for determining the inherited pattern of methylation marks, as well as for identifying the mechanism of epigenomic reprogramming in areas of the genome that are differentially methylated between individuals. Epigenetic alleles were initially defined in the plant evolution [76, 77] and later adapted for

the mammalian epigenome [78]. Briefly, for any adjacent *n* CpG sites, the total number of distinct DNA methylation patterns equals to the 2 states (methylated or unmethylated states) to the power of *n*. So given four adjacent CpG sites, there will be up to 16 distinct DNA methylation patterns. For a given locus with multiple CpG sites spanning by the same sequencing reads, the frequency of each distinct DNA methylation pattern could be derived from the corresponding sequencing read count divided by the total read count of all the patterns. Thus, epigenetic heterogeneity is the term used to describe the variations in epigenetic marks between cells. The epigenetic allele frequency can be used as a probability to measure and infer cellular epigenome heterogeneity.

12.4.1 Epigenome Technology for Detecting DNA Methylation Patterns

Epigenome technology advancement witnesses the increasingly profound understanding of epigenetic heterogeneity**.** *Bulk-cell single-base* DNA methylation microarray profiling of clinical specimens from large cohorts of cancer patients revealed high inter-tumor heterogeneity of the same type of cancer, which was widely used to stratify patients beyond genetic lesions [79, 80]. The inter-tumor epigenetic heterogeneity was revealed by bulk DNA methylation profiling across multiple cancers. By examining thyroid, lung, breast, kidney (Wilm's tumor), and colon cancers, respectively, multiple samples of the same type of cancer exhibit significantly increased inter-tumor variations than its matched tissue normal specimens [81]. The results suggested a model of hypervariability of DNA methylation that contributes to tumor heterogeneity due to loss of epigenetic stability. Such polymorphic DNA methylation underlies a putative mechanism for cellular-level cancer somatic evolution beyond cell differentiation. The *multi-region bulk-cell epigenome profiling* of the same patient depicts the intra-tumor heterogeneity and somatic evolution during treatment [82]. Bulk-cell profiling for intra-tumor heterogeneity assessment relies on multi-regional sampling and spatial variation, which is not always feasible in genomic studies due to biopsy challenges.

Bulk-cell bisulfite sequencing enables *single-base, single-molecule* resolution DNA methylation analysis and intra-tumor heterogeneity inference. Bisulfite converts unmethylated cytosine into uracil, thus, in the post-sequencing output, the cytosines changing to thymine will be considered unmethylated cytosines [83]. Given the low DNA input requirement and effectiveness in methylation detection, bisulfite sequencing has been widely used for DNA methylation pattern-based intra-tumor heterogeneity assessment. Though the cell-to-cell variation is inferred instead of directly measured by bisulfite sequencing, especially through reduced representation bisulfite sequencing (RRBS), where genomic regions with high or median CpG density are enriched for deep sequencing and DNA fragments sampling (Fig. 12.3). Bulk-cell sequencing can only infer the single-cell heterogeneity, so it is unable to directly distinguish the heterogeneity contributed by allele-specific methylation vs. cell-to-cell variations.

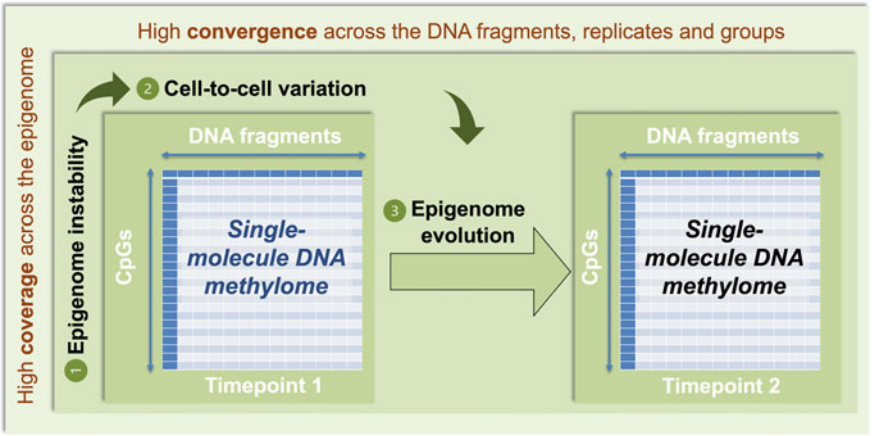

Fig. 12.3 *Single-molecule* DNA methylation characterizes epigenetic heterogeneity and epigenome evolution

The emerging *single-cell, single-base* bisulfite sequencing enables direct assessment of epigenetic heterogeneity, though the epigenetic heterogeneity analysis is limited by the data sparsity and comprising strategy. Currently, at least 18 published protocols aim to provide single-cell level DNA methylation sequencing [84]. The single-cell sequencing has great potential in dissecting intra-tumor heterogeneity at unprecedented resolution, while at the current stage, the limitations in extreme data sparsity and lack of convergence among cells prevail. Thus, the commonly attempted strategies to address the challenges of data science for single-cell methylation sequencing include (1) data aggregation by genomic bins or specific genomic regions for data sparsity; (2) pair-wise comparison of single-cell similarity to address the low common CpGs across single cells; (3) data imputation (Fig. 12.4).

Oxford nanopore direct DNA sequencing is a new and exciting technology that allows for the direct sequencing of DNA without the need for expensive and time-consuming PCR amplification. This technology uses an electric field to force DNA molecules through a tiny pore, and as the DNA passes through the pore, it is sequenced. The long-range *phased* DNAme patterns will inform whether multiple promoter regions that are far apart are co-methylated under the same condition, reflecting the transcription factor binding and regulatory activities [85]. Such long-range DNA methylation patterns identify co-modulated genes. NS is a powerful tool for cancer molecular subtype classification to simultaneously sequence the genome and DNA methylome [86]. Combining NS and scRRBS is a less costly alternative for validating *phased* DNAme patterns genome-wide. Here, we will primarily focus on the epiallele heterogeneity measurement using bulk-cell bisulfite sequencing and with the extension to single-cell epigenomics.

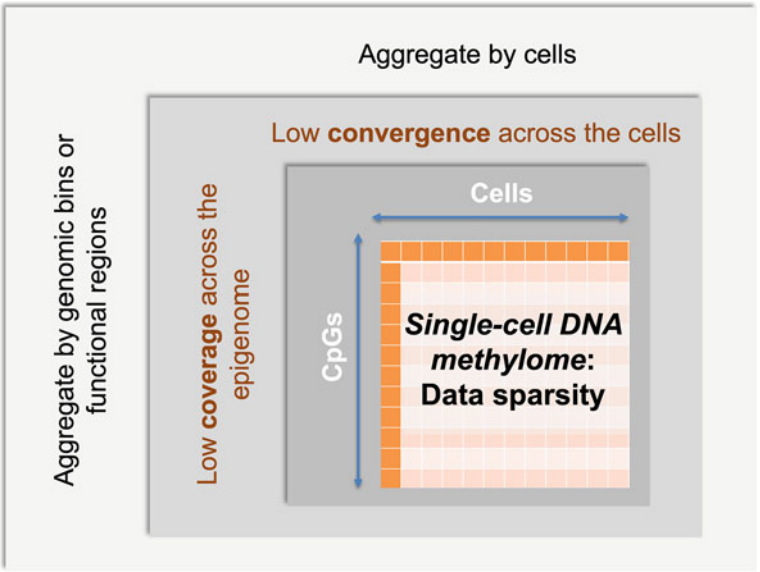

Fig. 12.4 Challenges in and solutions for single-cell DNA methylation sequencing data sparsity

12.4.2 Intra-tumor Heterogeneity Based on DNA Methylation Patterns

The first description of intra-tumor heterogeneity based on DNA methylation patterns focuses on the *MLH1* promoter [87] via bisulfite sequencing. Growing interests over the last decade focus on intra-tumor epigenetic heterogeneity and its clinical relevance in various solid and hematopoietic tumors. Such endeavors stimulate three directions of research advances utilizing DNA methylation patterns: (1) cell-to-cell epigenome variations; (2) epigenome instability focusing on the disordered methylation patterns; and (ss3) cellular somatic evolution inference (Fig. 12.3). These methods differ from the conventional DNA methylation percentage measurement [78], and they address aspects of epigenetic heterogeneity that are distinct from the CpG island methylator phenotype (CIMP) [88]. Levels of epigenetic heterogeneity in different tumors can be low or high even when the average DNA methylation level in a genomic region is the same [78]. These epiallele-based analyses characterize distinct but related aspects of epigenetic heterogeneity using DNA methylation patterns, providing a complimentary understanding of somatic evolution during cancer progression.

12.4.2.1 Cell-to-Cell Epigenome Variations

The cell-to-cell epigenome variations quantify the randomness of the distinct DNA methylation patterns within a cell population. The systemic inference of cell-to-cell

variability of DNA methylation patterns across the genome within a cell population, termed methylation entropy [89], was initially proposed using single-base, single-resolution bisulfite sequencing data in 2011. The frequency of each epiallele exhibiting a distinct DNA methylation pattern can be used as a probability for Shannon entropy to measure epigenetic heterogeneity. In 2012, epigenetic polymorphism or epipolymorphism was defined as the presence of two or more distinct DNA methylation patterns within a single tumor. Specifically, it calculates the probability of detecting distinct DNA methylation patterns when sampling two DNA fragments from a population of cancer cells [78]. In general, the epigenetic heterogeneity measured by epipolymorphism or methylation entropy is the highest with semi-methylation, i.e., the regional average methylation rate is around 50%. Such methylation level provides the highest chance of observing more distinct DNA methylation patterns. Epigenetic heterogeneity is the lowest with the 0 or 100% methylation, as there is only one possible DNA methylation pattern (fully unmethylated or methylated patterns). Thus, epipolymorphism was refined based on its relationship with regional average methylation level by median epipolymorphism across methylation levels [14]. These approaches quantify the heterogeneity based on the patterns of adjacent CpG sites and rely on the single-molecule information provided by bisulfite sequencing and can enhance the sensitivity of measurement in highly heterogeneous tumors. These metrics measure the cell-to-cell variations and treat each DNA methylation pattern equally. Other metrics computed the proportion of sites with semi-methylation with [90] or without [16] estimated allele-specific methylation. These metrics considered each CpG site independently. Thus, such metrics can be computed from both bisulfite sequencing data and DNA methylation microarray data and serves as surrogate variables to infer the cell-to-cell variations.

Ependymoma, AML, diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), and Ewing sarcoma exhibit substantial epigenetic heterogeneity inferred by cell-to-cell variations. The level of epigenetic heterogeneity is associated with the patient outcome of the treatment and disease progression. The methylation entropy of ependymoma is higher in aggressive tumors than in non-aggressive tumors and the matched normal tissues [89]. Furthermore, such heterogeneity changes during the cancer evolution, as the relapsed AMLs and DLBCLs exhibited lower heterogeneity than their counterparts at diagnosis, measured by epipolymorphism, suggesting that cancer therapy can pose selection on the epialleles, and the relapsed malignant cells with higher fitness expand at the relapse stage. The inferred cell-to-cell variation based on semi-methylation rate also significantly elevated in the metastatic tumor at diagnosis in ES. Furthermore, the CLL patients with higher pre-treatment epigenetic heterogeneity are associated with a more aggressive progression, i.e., a shorter time to receive their first treatment from their initiation specimen collection [90]. Thus, higher cell-to-cell variations likely provide a growth advantage in a subset of malignant cells to escape cancer therapy.

12.4.2.2 Epigenome Instability

The epigenome instability featured by local disordered DNA methylation patterns measures a related but distinct aspect of epigenetic heterogeneity compared to metrics for cell-to-cell variation. For example, epimutation rate, previously named the proportion of disordered reads (PDR), specifically considers the DNA fragments containing adjacent CpGs exhibiting both methylation and unmethylation [15, 53]. The metric assumes that a stable epigenetic location should show high fidelity in the process of DNA methylation and demethylation; thus, the methylation status of CpGs in close proximity should be consistent. Most cancers comprise compromised epigenetic regulation due to somatic mutations, and the dysfunction of epigenetic regulators can decrease the somatic cells' epigenome fidelity when writing and erasing the epigenetic marks. Thus, epimutation serves as an effective way to measure epigenome instability.

Epigenome instability has been shown to accelerate genetic evolution by facilitating adaptive potential and leading to worse clinical outcomes. For example, CLL patients with higher epigenome instability experienced adverse failure-free survival after treatment [15]. Epigenome instability is also associated with the tissue of origin in ES patients at the spine. The application of single-cell bisulfite sequencing in hematopoietic and solid tumor further revealed the significance of epigenome instability in cancer progression. For instance, the heightened epigenome instability is widespread across cell populations in CLL. In contrast, the level of epigenome instability is low and inconsistent in normal B cells despite low convergent CpG coverage [53]. Furthermore, higher epigenome instability is also observed in more aggressive glioma [54].

12.4.2.3 Epigenome Evolution

Epigenome evolution inference answers a different question compared to the other two metrics mentioned above: to what extent do the somatic cells evolve at the epigenome scale? DNA methylation can serve as a molecular clock during cancer evolution [91]. Understanding the dynamics of epiallele change over time during cancer progression portrays the selection of the various epigenome within a heterogeneous tumor cell population over time, given the intrinsic changes (i.e., genetic mutations) and microenvironmental changes (i.e., therapy, lifestyle, and aging). For example, the epiallele repertoire shift measures the epialleles that are present in a population of cells and change over time, which can be calculated through combinatorial entropy [13]. This method quantifies changes in the epialleles present within a population of cells over time. Therefore, the epiallele repertoire shift provides valuable information regarding the evolving epigenomic landscape. Such epiallele repertoire shifts during different time points in the course of cancer progression, e.g., from diagnosis to relapse, can inform how cancer cells adapt to the changing environment and become resistant to treatment at the epigenome level.

Epigenome evolution is linked to cancer relapse and aggressiveness. The higher level of pre-treatment epiallele repertoire shift is associated with adverse AML patients' outcomes, i.e., shorter duration from diagnosis to relapse and worse

event-free survival, suggesting the selection of cells carrying epiallele may convey fitness to escape leukemia therapy [13, 41]. Beyond the epigenome evolution in hematopoietic malignancy, later-stage solid tumors, i.e., lung adenocarcinomas (ADC), also exhibit more abundant epiallele shift than precancer for lung ADC (i.e., atypical adenomatous hyperplasia), preinvasive lung cancer (i.e., ADC in situ), and minimally invasive ADC [71]. Furthermore, the enhanced epipolymorphism of the genomic loci exhibits epiallele selection in later-stage ADC also indicates that the stochastic epigenome contributes to the evolution of the epigenome [71]. Collectively, the epigenome evolution in hematopoietic and solid tumors captured by epigenetic allele repertoire shift suggests the selection of more malignant phenotype in the ever-changing tumor microenvironment, perhaps benefiting a subset of the cell populations to expand through an evolutionary bottleneck.

12.4.2.4 Sources of Epigenetic Heterogeneity

Somatic driver mutations' status is widely associated with the level of epigenetic heterogeneity. Somatic mutations in genes encoding DNA methylation eraser *TET2* and its regulator *IDH2* in AML, while the loss of *Tet2* and *Idh2* mutation drives up epigenetic heterogeneity before overt malignant transformation in genetically engineered mouse models [41]. These results suggest a causal role of somatic mutations in enhancing epigenetic heterogeneity and expanding the sampling space for the premalignant cells to explore and adapt to the microenvironment better. Furthermore, microenvironmental and therapeutic stress also can drive epigenome instability. For instance, the epigenome instability enhances as the specimen collecting sites approach the tumor center of glioma, where hypoxia frequently occurs [54]. Notably, in vitro models show that hypoxic and irradiation stress enhance the epigenome instability of the glioma cells. The corresponding enhancement of epigenome instability also enriched for cell identity and stress pathways, which may increase the adaptive potential of responding to environmental stress. Collectively, these findings embarked on the assessment of intrinsic and extrinsic factors that modulate cancer epigenetic heterogeneity.

12.5 Conclusion and Future Directions

DNA methylation patterns provide the basis to infer cellular epigenome heterogeneity. The studies of various hematopoietic and solid tumor evolution underpin the biological and clinical significance of cellular epigenome heterogeneity beyond genetic heterogeneity. The advancement of epigenome technology, including bulk-cell, single-cell, and long-read sequencing, enables the detection of DNA methylation patterns to infer epigenome heterogeneity. Major metrics for characterizing and inferring cellular epigenome heterogeneity (i.e., cell-to-cell epigenome variations, epigenome instability) and epigenome evolution were discussed here

in detail. The clinical relevance of and driving forces for epigenetic heterogeneity highlights the significance of assessing DNA methylation patterns in cancer research.

However, the understanding of cellular epigenome heterogeneity and evolution remains incomplete. Multiple questions important for addressing cancer initiation and progression deserve future investigations. Biologically, what is the functional impact of epigenetic heterogeneity? And what are the other intrinsic and extrinsic sources of epigenetic heterogeneity that contribute to cancer evolution? What is the epigenetic heterogeneity and evolution in the microenvironment? And to what extent such heterogeneity drives cancer evolution? How does age-related inflammation promote or eliminate cancer cell heterogeneity? Furthermore, recognition of DNA hydroxymethylation in mammalian DNA in 2009 spurred studies of its potential roles in genome function, revealing 5hmC to be a key intermediate in the DNA oxidative demethylation pathway that also functions as an independent and stable epigenetic modification linked to transcriptional activation [92, 93]. Studies in hematopoietic stem/progenitor cells (HSPCs) and embryonic stem cells (ESCs), T-cells, and induced pluripotent cells showed that 5hmC, which is enriched in gene bodies and enhancers, can modulate enhancer activity and transcriptome reprogramming [35–38, 40, 94]. How does 5hmC contribute to epigenetic heterogeneity, and how does 5hmC epigenetic heterogeneity contribute to transcriptome heterogeneity? Technically, how to accurately measure the cancer epigenome atlas and effectively address the sparsity of genomic coverage and genome heterogeneity? Clinically, how shall epigenetic heterogeneity and epigenome evolution reshape personalized medicine, especially for customized dosage and timing of the epigenetic therapy, maximize the benefit for cancer patients and minimize treatment side effects? This is in light of the recent success of genetic-evolution-guided cancer therapy in brain tumors, whose treatment outcome using radiology is comparable to the higher-dosage group. Addressing these questions will help reveal the mechanisms that provide an additional layer of fitness beyond genetic heterogeneity during cancer evolution. Such knowledge is expected to contribute to ‘evolution-blocking’ strategies to mitigate disease progression.

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