Chapter 4
RNA N^6-Methyladenosine Modification in Normal and Malignant Hematopoiesis

Hengyou Weng, Huilin Huang, and Jianjun Chen

Contents

Abstract As the most abundant internal modification in eukaryotic messenger RNAs (mRNAs), N^6-methyladenosine (m^{6}A) modification has been shown recently to posttranscriptionally regulate expression of thousands of messenger RNA (mRNA) transcripts in each mammalian cell type in a dynamic and reversible manner. This epigenetic mark is deposited by the m^{6}A methyltransferase complex (i.e., the METTL3/METTL14/WTAP complex and other cofactor proteins) and erased by m^{6}A demethylases such as FTO and ALKBH5. Specific recognition of these m^{6}A-modified mRNAs by m^{6}A-binding proteins (i.e., m^{6}A readers) determines the fate of target mRNAs through affecting splicing, nuclear export, RNA stability, and/or translation. During the past few years, m^{6}A modification has been demonstrated to play a critical role in many major normal bioprocesses including self-renewal and differentiation of embryonic stem cells and hematopoietic stem cells, tissue development, circadian rhythm, heat shock or DNA damage response, and sex determination. Thus, it is not surprising that dysregulation of the m^{6}A machinery is also

H. Weng · H. Huang · J. Chen (✉)
Department of Systems Biology, Beckman Research Institute of City of Hope, Monrovia, CA, USA

The Gehr Family Center for Leukemia Research, Beckman Research Institute of City of Hope, Monrovia, CA, USA
e-mail: jianchen@coh.org

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closely associated with pathogenesis and drug response of both solid tumors and hematologic malignancies. In this chapter, we summarize and discuss recent findings regarding the biological functions and underlying mechanisms of m^{6}A modification and the associated machinery in normal hematopoiesis and the initiation, progression, and drug response of acute myeloid leukemia (AML), a major subtype of leukemia usually associated with unfavorable prognosis.

Keywords m^{6}A demethylases · m^{6}A modification · Normal hematopoiesis · AML

Abbreviations

AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
BMT	Bone marrow transplantation
CLIP	Cross-linking immunoprecipitation
CMP	Common myeloid progenitor
EHT	Endothelial-to-hematopoietic transition
GMP	Granulocyte/macrophage progenitor
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
lncRNA	Long noncoding RNA
LSCs	Leukemia stem cells
LSCs/LICs	Leukemia stem/initiating cells
m^{6}A	*N*^{6}-methyladenosine
miCLIP	m^{6}A individual-nucleotide-resolution cross-linking and immunoprecipitation
mRNA	Messenger RNA
MTC	Methyltransferase complex
pri-miRNA	Primary microRNA
rRNA	Ribosomal RNA
snoRNA	Small nucleolar RNA
SNPs	Single-nucleotide polymorphisms
snRNA	Small nuclear RNA
TF	Transcription factor
tRNA	Transfer RNA
TSS	Transcriptional start site

4.1 Introduction

Since the 1960s, over 150 modified RNA nucleotide variants have been identified in both protein-coding and noncoding RNAs, such as N^6-methyladenosine (m^6A) in messenger RNA (mRNA) and primary microRNA (pri-miRNA) [1–4], N^1-methyladenosine (m^1A) in mRNA and transfer RNA (tRNA) [5–7], 5-methylcytosine (m^5C) and 5-hydroxymethylcytosine (hm^5C) in mRNA and long noncoding RNA (lncRNA) [8–11], and pseudouridine (ψ) in tRNA, ribosomal RNA (rRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) [12–16] (Figs. 4.1 and 4.2). Of them, m^6A is the most prevalent and abundant internal

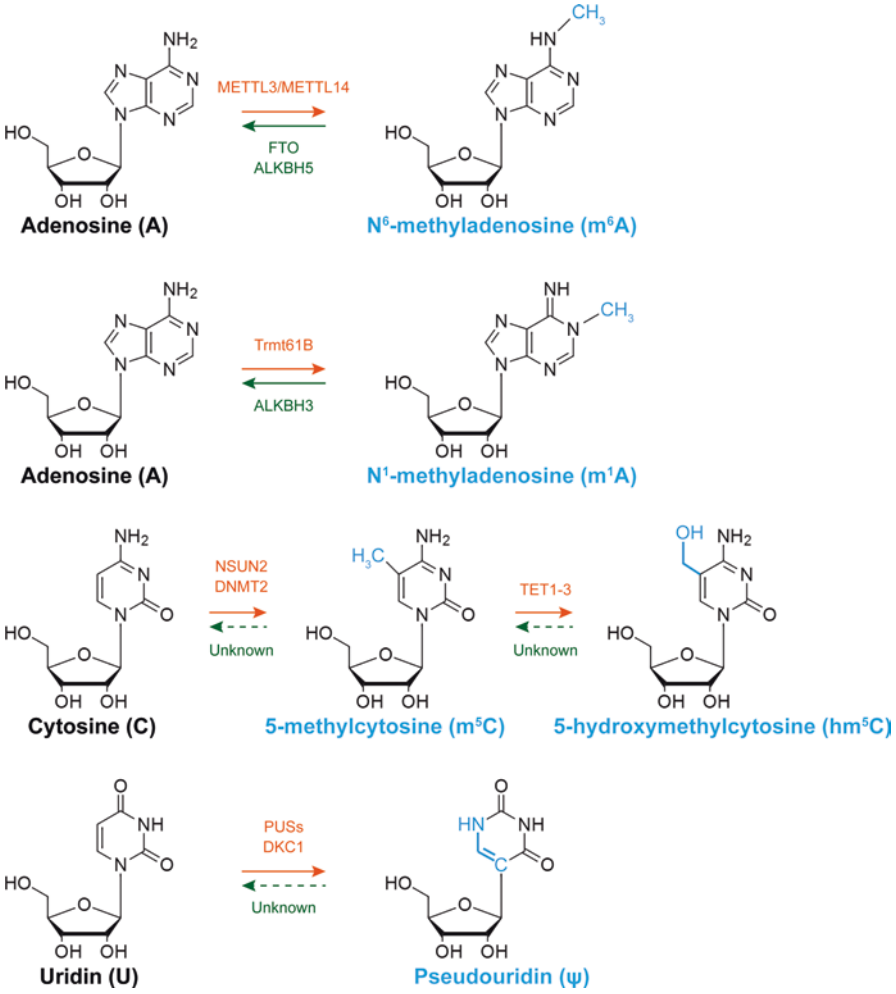

Fig. 4.1 Chemical structures of representative modified RNA nucleotide variants. Modifications are shown in blue. The known writer (in orange) and eraser (in green) proteins are also indicated

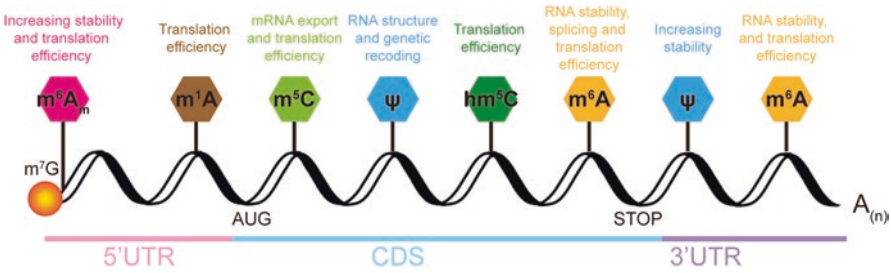

Fig. 4.2 Chemical modifications in eukaryotic mRNA. A schematic representation of common chemical modifications across eukaryotic mRNA transcript including 5′ untranslated region (5′UTR), coding region (CDS), and 3′UTR. Reported roles of these modifications are summarized on top of the corresponding modifications. Note that the same modification in different mRNA regions may have different functions in regulating mRNA fate

modification on eukaryotic mRNAs. m^6A RNA modification was first identified in the 1970s [1–3]. However, due to the lack of knowledge on its dynamic regulation and no high-throughput technology available to map m^6A modification to the RNA transcriptome, little attention had been paid to this RNA mark until 2011, when the fat mass and obesity-associated protein (FTO) was identified as a genuine demethylase of m^6A modification [17], which implies that m^6A modification is a reversible and dynamic process analogous to the well-studied modifications on DNA and histone [18]. Subsequent development of high-throughput m^6A sequencing technologies further facilitates the understanding of m^6A modification in a transcriptome-wide view, revealing that m^6A modification may affect more than 7000 mRNAs in individual transcriptomes of mammalian cells, with a special enrichment in the 3′ untranslated regions (UTRs) near the stop codons of mRNAs and with a consensus sequence of RRACH (R = G or A; H = A, C, or U) [19, 20]. Such findings strongly suggest that m^6A modification may have important biological functions. Indeed, emerging data demonstrate that m^6A modifications in mRNAs or noncoding RNAs influence RNA fate and functions and are critical for many normal and pathological bioprocesses including self-renewal and differentiation of embryonic stem cells, tissue development, circadian rhythm, heat shock or DNA damage response, sex determination, and tumorigenesis, as reviewed elsewhere [4, 16, 21–37]. Evidence is emerging that m^6A modification and the associated machinery also play essential roles in tumorigenesis and drug response (see reviews [16, 38, 39]). Here we summarize recent advances on our understanding of the functions and underlying molecular mechanisms of the m^6A machinery in normal hematopoiesis and AML pathogenesis and drug response.

4.2 Regulators of m^{6}A Modifications

Similar to other epigenetic modifications, m^{6}A modification is regulated by its formation and removal catalyzed by the methyltransferases and demethylases, known as "writers" and "erasers," respectively [16, 40, 41]. m^{6}A marks are installed by a multicomponent methyltransferase complex (MTC) consisting of the core methyltransferase-like 3 and 14 (METTL3 and METTL14) heterodimer and their cofactors including Wilms' tumor 1-associating protein (WTAP), vir like m^{6}A methyltransferase associated (VIRMA, also known as KIAA1429), RNA-binding motif protein 15 (RBM15), and zinc finger CCCH domain-containing protein 13 (ZC3H13) [22, 42–49] (Fig. 4.3). Structural studies demonstrated that METTL3 is the sole catalytic subunit, while METTL14 offers an RNA-binding scaffold to allosterically activate and enhance the catalytic activity of METTL3 [50–52]. WTAP, VIRMA, RBM15, and ZC3H13 are the regulatory subunits of the MTC to facilitate m^{6}A installation *in cellulo*. In addition, the METTL3 homolog METTL16 (methyltransferase-like 16) was recently shown to control cellular S-adenosyl methionine (SAM) level and install m^{6}A marks onto the U6 small nuclear RNA [53, 54]. FTO, previously known to function as a demethylase for N^3-methylthymidine in single-stranded DNA and N^3-methyluridine in single-stranded RNA in vitro [55, 56], was identified as the first m^{6}A demethylase that could demethylate m^{6}A in both DNA and RNA in vivo [17]. A recent study reported that FTO also demethylates m^{6}A$_m$, a modification exclusively found at the first encoded nucleotide after the 7-methylguanosine cap structure of mRNAs [57]. AlkB homolog 5 (ALKBH5) is the second identified m^{6}A demethylase that was found to be highly expressed in the testes [23]. Both FTO and ALKBH5 belong to the AlkB subfamily of the Fe(II)/2-oxoglutarate (2OG) dioxygenase superfamily that requires 2OG and molecular oxygen as co-substrates and ferrous iron Fe(II) as a cofactor to catalyze the oxidation of a substrate [56, 58].

While the prevalence and distribution of m^{6}A are determined by writers and erasers, the m^{6}A-dependent functions are mediated by m^{6}A-binding proteins, the so-called readers, which through specific recognition and binding to m^{6}A-modified mRNAs determine the fate of these transcripts [16, 21, 59] (Fig. 4.3). The YT521-B homology (YTH) domain family of proteins, including YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2, are among the first identified m^{6}A readers that possess a conserved m^{6}A-binding pocket [19, 60–66]. Of them, YTHDC1 was found to be located in the nucleus, playing a role in splicing regulation, XIST-mediated X-chromosome silencing, and nuclear export of m^{6}A-modified mRNAs [46, 63, 67]. The other YTH family proteins are all cytoplasmic m^{6}A readers regulating mRNA fate through different mechanisms: YTHDF2 promotes degradation of target mRNAs [60, 68], and YTHDF1 promotes translation of target mRNAs [61], while YTHDF3 and YTHDC2 can both mediate mRNA decay and enhance translation [66, 69–73]. We recently identified insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; including IGF2BP1/2/3) as a new family of m^{6}A readers that could promote stability and translation of their target mRNAs [32], distinct

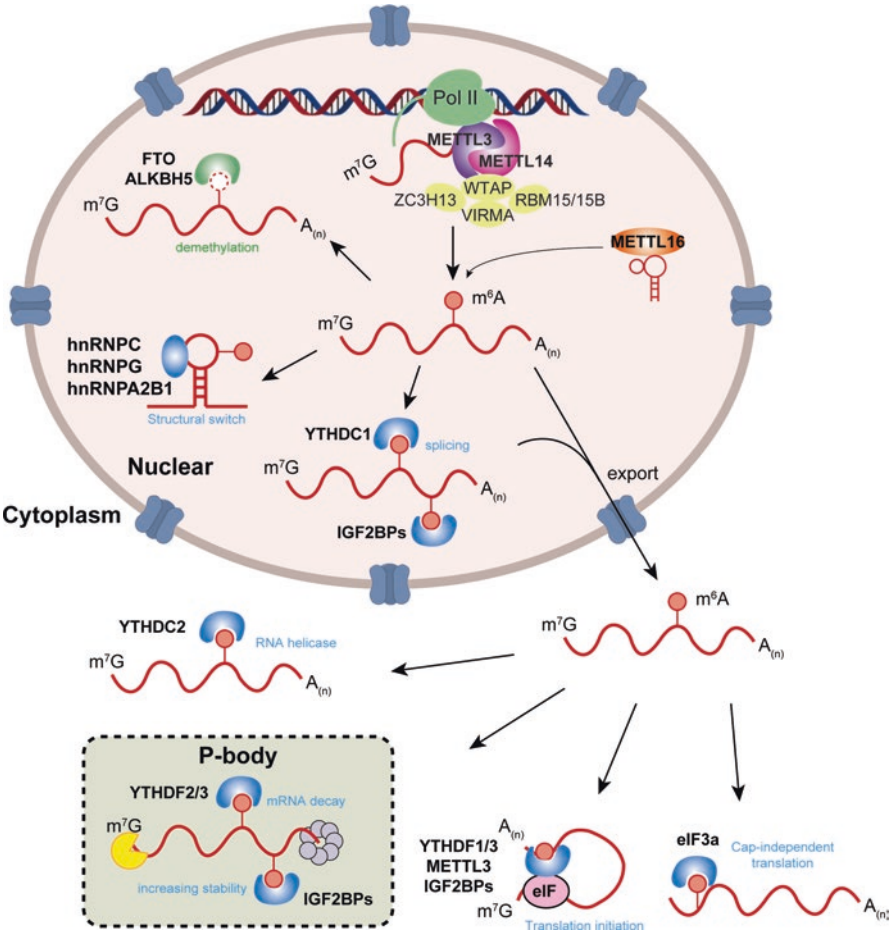

Fig. 4.3 Roles of m^{6}A RNA modification in determining mRNA fate. In nuclear, the methyltransferase complex composed of METTL3, METTL14, WTAP, VIRMA, RBM15, and ZC3H13 deposits m^{6}A marks co-transcriptionally onto newly transcribed RNAs, while METTL16 is responsible for m^{6}A deposition on the U6 snRNA. FTO and ALKBH5 function as m^{6}A demethylases to remove m^{6}A marks in selected sites of RNA. M^{6}A-mediated structural switch of mRNA recruits hnRNP family proteins including hnRNPC, hnRNPG, and hnRNPA2B1. Nuclear reader protein YTHDC1 recognizes m^{6}A to mediate alternative splicing. IGF2BP reader proteins bind to m^{6}A mRNAs to stabilize these nascent transcripts. After exporting to cytoplasm, m^{6}A-modified mRNAs could be subjected to degradation by YTHDF2 or YTHDC2 or protected by IGF2BP proteins and loaded to translation machinery. YTHDF1, YTHDF3, as well as METTL3 and eIF3a could also promote translation of m^{6}A mRNAs

from the functional manners of the YTH family proteins. The K homology (KH) domains of IGF2BPs are required for their recognition of m^{6}A and are critical for their oncogenic functions. Interestingly, ELAV-like RNA-binding protein 1 (ELAVL1, also known as HuR), an mRNA stabilizer that was previously reported

as an indirect m^{6}A-binding protein [22], was found to be a cofactor of IGF2BPs and may mediate the mRNA stabilizing effect of IGF2BPs [32]. The heterogeneous nuclear ribonucleoprotein (HNRNP) HNRNPA2B1 was previously reported to regulate alternative splicing and primary microRNA processing as an m^{6}A reader [74]; however, recent structural study suggested an "m^{6}A switch" mechanism rather than direct m^{6}A binding for the protein [75]. Two other members of the HNRNP family proteins, namely HNRNPC and HNRNPG, were shown to recognize m^{6}A-induced changes in mRNA secondary structures [76]. Other proteins were also recently reported to be m^{6}A interactors, including FMR1 and LRPPRC [77, 78], although the exact mode of binding still needs to be clarified.

4.3 Roles of m^{6}A in Normal Hematopoiesis and HSC Self-Renewal

Hematopoiesis is a tightly regulated dynamic process where mature blood cells are generated from a small pool of multipotent hematopoietic stem cells (HSCs) [79, 80]. During the past few decades, it has been well acknowledged that transcriptional regulation by a variety of hematopoietic transcription factors (TFs) plays a big role in regulating the multistep normal hematopoiesis [81–83]. In particular, during myelopoiesis where HSCs are differentiated into myeloid progenitors and eventually mature myeloid cells, the sequential actions of master TFs are required to specify and re-enforce each cell fate decision. For instance, PU.1 (also known as SPI1, the product of the oncogene SPI1) is a transcriptional master regulator of myeloid cells which plays an essential role in generating early myeloid progenitors (i.e., common myeloid progenitors, CMPs), while the basic region leucine zipper transcription factor C/EBPα is required for the production of granulocyte/macrophage progenitors (GMPs) from CMPs [80, 84].

In recent years, emerging studies reveal m^{6}A modification at the RNA level as an additional layer of the posttranscriptional regulation in governing HSC activity and normal hematopoiesis (see Fig. 4.4). During the endothelial-to-hematopoietic transition (EHT) of zebrafish embryogenesis, a key developmental event leading to the formation of the earliest hematopoietic stem and progenitor cells (HSPCs), m^{6}A modification was reported to play a role [30]. Deficiency of mettl3 in zebrafish embryos leads to decreased levels of m^{6}A and blockage of HSPC emergence, likely due to the reduced m^{6}A modification on the arterial endothelial genes *notch1a* and *rhoca* that delayed YTHDF2-mediated mRNA decay of these transcripts [30]. Vu and colleagues used short hairpin RNAs (shRNAs) to knock down *METTL3* expression in human HSPCs and observed cell growth inhibition and increase of myeloid differentiation [34]. Conversely, overexpression of wild-type, but not catalytically dead mutant of METTL3, promotes proliferation and colony formation and inhibits myeloid differentiation. We recently showed that METTL14 is highly expressed in murine HSCs and Lin$^-$ Sca-1$^+$c-kit$^+$ (LSK) cells and was downregulated during

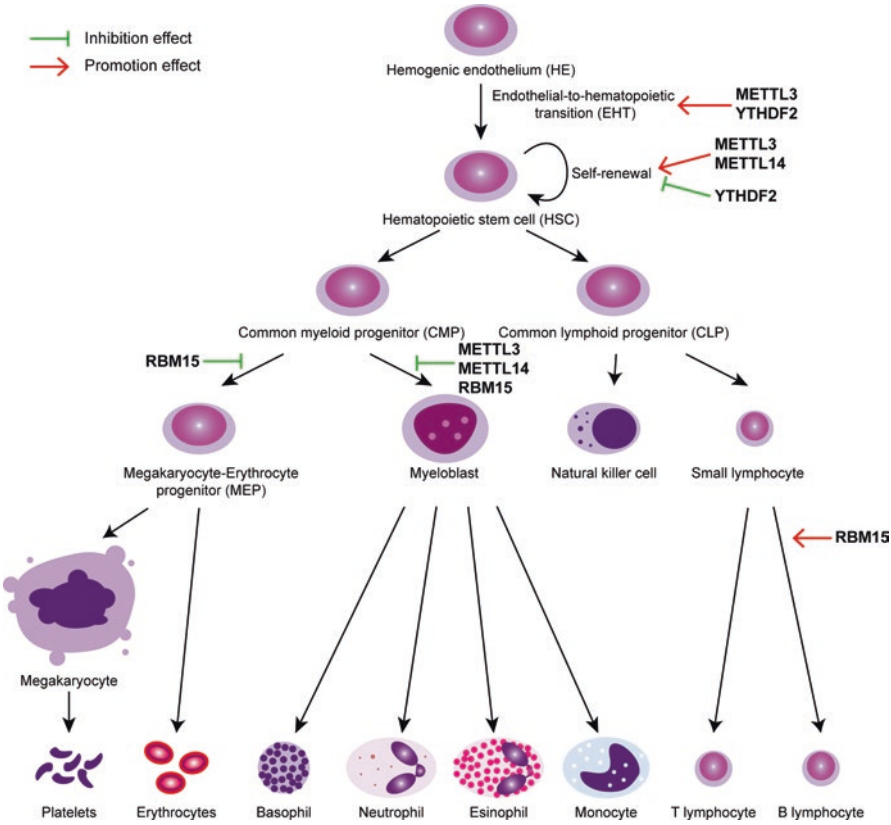

Fig. 4.4 Modifiers of m^{6}A RNA modification in normal hematopoiesis. METTL3 and METTL14 promote self-renewal of HSC and inhibit myeloid differentiation. In contrast, YTHDF2 inhibits HSC self-renewal. RBM15 inhibits myeloid and megakaryocytic differentiation while promotes B cell expansion, although it is unclear whether these effects are m^{6}A-related

myelopoiesis, showing reduced expression in CMP and GMP progenitors and especially in mature myeloid cells [33]. Consistent with the expression pattern, depletion of *METTL14* expression in human HSPCs by shRNAs promotes myeloid differentiation in vitro. Moreover, by utilizing a *Mettl14* conditional knockout mouse model, we demonstrated that induced deletion of *Mettl14* impairs HSC self-renewal ability in vivo. Such effects were mediated by decreased *MYB* and *MYC* expression owing to the reduction of m^{6}A modification on these transcripts upon *METTL14* knockdown/knockout. Considering the role of MYB [85, 86] and MYC [87, 88] transcription factors in regulating HSC self-renewal and differentiation, METTL14-mediated m^{6}A regulation on the mRNA transcripts of these TFs adds a new layer of complexity to the regulatory networks in normal hematopoiesis.

A very recent study took advantage of *Mettl3* and *Mettl14* conditional knockout mice to investigate the roles of these m^{6}A writer proteins on regulation of HSC self-renewal in adult mouse bone marrow [89]. They found that deletion of *Mettl3*

alone or together with *Mettl14* in the hematopoietic system substantially increases HSC frequency in the bone marrow; in contrast, deletion of *Mettl14* alone has little effect [89]. Conditional deletion of *Mettl14* and especially that of *Mettl3* suppresses HSC self-renewal activity in recipient mice. Notably, although deletion of either *Mettl3* or *Mettl14* deletion leads to significant reduction of donor-derived myeloid cells in the peripheral blood, only deletion of *Mettl3* results in a significant reduction of B- and T-cell lineage [89].

RBM15, recently identified as a component of the m^{6}A methyltransferase complex, was also reported to play a role in normal hematopoiesis [90–92]. Conditional knockout of *Rbm15* in adult mice blocks B cell differentiation and results in an increase of the Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) HSPCs and an expansion of myeloid and megakaryocytic cells in spleen and bone marrow, demonstrating a role of RBM15 in hematopoietic development [90]. Rbm15 is expressed at highest levels in HSCs and inhibits myeloid differentiation and megakaryocytic expansion through stimulation of the Notch signaling and regulation of *MYC* expression, respectively [91, 92].

YTHDF2 is the first well-characterized m^{6}A reader that promotes mRNA decay of target transcript with m^{6}A modification [60]. Li and colleagues studied the role of YTHDF2 in adult stem cell maintenance and reported an increase of HSCs in *Ythdf2* conditional knockout mice as well as in human umbilical cord blood upon *YTHDF2* knockdown [93]. Such effects are partially mediated by the stabilization of mRNA transcripts encoding TFs critical for stem cell self-renewal. Overall, the studies of m^{6}A in normal hematopoiesis are just in the beginning. It is of great interest to explore the functions and underlying mechanisms of other m^{6}A modulators, including other writers, erasers, and readers, in stem cell biology and normal hematopoiesis.

4.4 Dysregulation of m^{6}A Regulators in Malignant Hematopoiesis

Dysregulation of the regulatory networks of normal hematopoiesis, such as incorrect activity of the hematopoietic TFs attributed to either aberrant expression or mutation, breaks the balance between HSC self-renewal and differentiation and places the progenitor cells at a higher risk of developing leukemia [80, 94]. Acute myeloid leukemia (AML) is a clonal hematopoietic disorder where a stem cell-like self-renewal capacity is gained and the differentiation capacity is blocked [95, 96]. According to the Cancer Statistics 2017, the 5-year survival of AML between 2007 and 2013 is 26.9%, much lower than other types of leukemia and many other common cancer types [97]. For instance, during the same period, the 5-year survival rate is 89.7% for breast cancer and 66.9% for chronic myeloid leukemia (CML). Therefore, there is an urgent need to better understand the mechanisms underlying AML leukemogenesis and, based on the gained knowledge, to develop effective targeted therapies.

The first evidence of a role of m^{6}A modification on leukemia, specifically on AML, came from the study of FTO, a major m^{6}A demethylase [31]. *FTO* was previously known as a gene associated with fat mass, adipogenesis, and body weight [98–100], and single-nucleotide polymorphisms (SNPs) in *FTO* were linked to higher risk of developing cancers including leukemia and lymphoma by large-scale epidemiology studies [101–103]. We found that *FTO* is highly expressed in certain subtypes of AML, including those carrying t(11q23)/*MLL* rearrangements, t(15;17)/*PML-RARA*, *FLT3*-ITD, and/or *NPM1* mutations. Modulation of *FTO* expression by depletion of *FTO* or forced expression of wild-type FTO (but not catalytically inactive mutant) could significantly influence AML cell survival and leukemogenesis and affect the response of AML cells to all-trans retinoic acid (ATRA) [31]. Importantly, the oncogenic function of FTO in AML relies on its m^{6}A demethylase activity. By reducing m^{6}A abundance on the transcripts of *ASB2* and *RARA*, two genes with reported roles in cell proliferation and drug response of leukemia cells, FTO posttranscriptionally regulates expression of *ASB2* and *RARA* through reducing m^{6}A abundance, thereby decreasing mRNA stability of these transcripts. These data provide compelling evidence on the role of FTO in leukemogenesis and establish a first link between m^{6}A modification and leukemia pathogenesis (Fig. 4.5) [31].

More recently, we showed that, by targeting FTO directly, R-2-hydroxyglutarate (R-2HG), a previously reported oncometabolite produced by mutant isocitrate dehydrogenase 1/2 (IDH1/2) enzymes [104–106], exhibits a broad and intrinsic antitumor activity in leukemia. By inhibiting the m^{6}A demethylase activity of FTO, R-2HG results in an increase of m^{6}A abundance on FTO target genes, such as *MYC* and *CEBPA*, leading to decay of these transcripts in R-2HG sensitive cells (see Fig. 4.5). Our data indicate that FTO/MYC homeostasis controls the sensitivity of leukemic cells to 2HG: high abundance of FTO confers R-2HG sensitivity, whereas hyper-activation of MYC signaling renders leukemic cells resistant to R-2HG [37]. Consistent with this notion, pharmaceutical or genetic inhibition of MYC signaling by JQ1 or *MYC* shRNAs can resensitize R-2HG-resistant leukemic cells to R-2HG. In addition, we showed that R-2HG treatment or FTO inhibition also sensitized AML cells to first-line chemotherapy drugs such as ATRA, Azacitidine (AZA), Decitabine, and Daunorubicin ([37] and unpublished data). Collectively, our studies demonstrate the critical role of FTO in leukemia pathogenesis and drug response and also highlight the therapeutic potential of targeting FTO signaling for AML treatment.

Subsequently, the dysregulation of the m^{6}A installing machinery was also reported to be involved in AML pathogenesis [107] (see Fig. 4.6). METTL3 and METTL14 were both expressed at a higher level in AML than in the vast majority of other cancer types according to The Cancer Genome Atlas (TCGA) genome-wide gene expression datasets [33–35]. We found that METTL14 is aberrantly overexpressed in certain subtypes of AMLs, such as those carrying t(11q23)/*MLL* rearrangements, t(15;17)/*PML-RARA*, and t(8;21)/*AML1-ETO* [33]. We next conducted in vitro and in vivo gain- and loss-of-function studies and demonstrated that METTL14 plays a critical oncogenic role in AML pathogenesis [33]. Depletion of

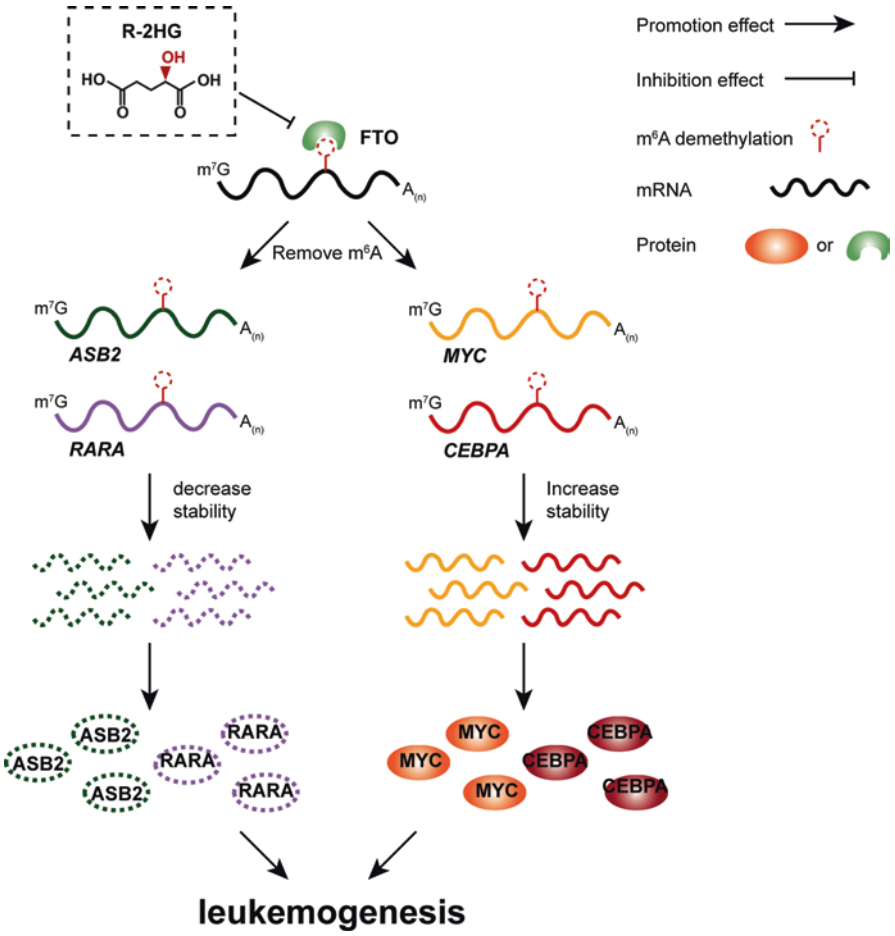

Fig. 4.5 Role of FTO in leukemogenesis. FTO removes m^6A modification on its target mRNAs (e.g., *ASB2*, *RARA*, *MYC*, and *CEBPA*), resulting in the decreased or increased stability of these transcripts, and promotes leukemogenesis. R-2HG inhibits FTO and exhibits antileukemia effects

METTL14 inhibited survival and proliferation of AML cells, promoted myeloid differentiation, and suppressed leukemic oncofusion protein (e.g., MLL-AF9, MLL-AF10, and AML1-ETO9a)-mediated immortalization of normal HSPCs. The opposite is true when wild type, but not mutant METTL14 (i.e., R298P), was forced expressed. Moreover, knockdown of *METTL14* significantly inhibited progression of human AML cells in xenotransplantation recipient mice, while inducible knockout of *Mettl14* greatly inhibited AML development and maintenance in bone marrow transplantation (BMT) recipient mice [33]. High-throughput RNA-seq and transcriptome-wide m^6A-seq, coupled with gene-specific m^6A-qPCR assays, cross-linking and immunoprecipitation (CLIP) assays, luciferase reporter and mutagenesis assays, mRNA stability assays, and polysome profiling assays, demonstrated

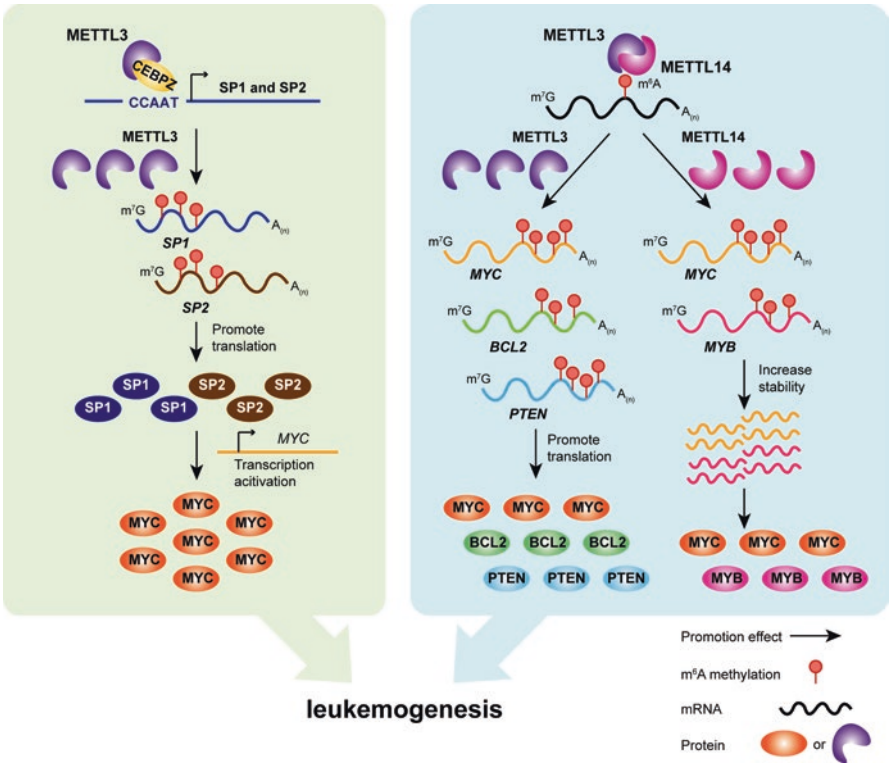

Fig. 4.6 Roles of m^{6}A writer genes in leukemogenesis. METTL3 and METTL14 function as oncogenes in AML through depositing m^{6}A modification on their target transcripts (e.g., *MYC*, *BCL2*, and *PTEN* for METTL3 and *MYB* and *MYC* for METTL14) to enhance translation and/or increase mRNA stability of these transcripts. METTL3 can also be recruited to promoter of its target gene by CEBPZ and deposit m^{6}A modification on the coding region of the target transcripts (e.g., *SP1* and *SP2*) to promote their translation and eventually leads to *MYC* activation

that METTL14 posttranscriptionally regulates the expression of its critical target mRNA transcripts, such as *MYB* and *MYC*, two well-known TF genes involved in leukemogenesis, in an m^{6}A-dependent manner [33]. Silencing of *METTL14* reduces m^{6}A abundance of *MYB* and *MYC* transcripts, especially near the 3′ end of the mRNAs, resulting in decreased mRNA stability and translation of these transcripts. Such effects were not mediated by the YTH family proteins, and therefore other readers (such as IGF2BPs [32]) may mediate the effect of METTL14 on *MYB* and *MYC*. Furthermore, we also identified SPI1 (also called PU.1), a transcriptional master regulator of myelopoiesis, as a negative regulator of *METTL14* expression in AML [33]. Taken together, our work reveals a previously unappreciated SPI1-METTL14-MYB/MYC signaling axis in leukemogenesis and highlights the critical roles of METTL14 and m^{6}A modification in malignant hematopoiesis [33].

Meanwhile, two other groups have demonstrated that METTL3 also plays an essential oncogenic role in AML, by showing that depletion of *METTL3* expression

results in cell growth inhibition, cell cycle arrest, and induction of differentiation and apoptosis, whereas overexpression of wild-type *METTL3*, but not a catalytically inactive mutant, promotes proliferation in AML cells and primary blasts [34, 35]. Similar to METTL14, METTL3 is also required for AML development as demonstrated by the xenotransplantation assay data that shRNA-mediated knockdown or CRISPR/Cas9-mediated knockout of *METTL3* substantially inhibited AML progression and prolonged survival in recipient mice [34, 35]. Such findings support the oncogenic role of METTL3 as an m^{6}A-catalyzing enzyme. Despite of the similar functions of METTL3 reported by the two groups, distinct underlying mechanisms of METTL3 in AML were reported [34, 35]. Vu et al. performed m^{6}A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP), RNA-seq, and Ribo-Seq, and identified *MYC*, *BCL2* and *PTEN* as direct RNA targets of METTL3, whose m^{6}A abundances were substantially reduced when *METTL3* was knocked down [34]. However, upon *METTL3* depletion, while expression of *MYC*, *BCL2*, and *PTEN* transcripts showed a great increase, the corresponding protein levels decreased on day 3 after shRNA transduction and recovered 1 day later [34]. Although the authors proposed that an alternative internal ribosome entry site (IRES)-mediated translational mechanism may be involved to reactivate translation of *MYC* and *BCL2* under METTL3 depletion-induced cell apoptosis, it remains unclear why the expression changes of these target genes at the mRNA level are inconsistent with that at the protein levels. A systematic understanding of the changes and functions of m^{6}A readers under this circumstance may help to address this question. Different from Vu's study [34], Barbieri et al. found that METTL3 and METTL14 could bind chromatin, mainly localizing to the transcriptional start sites (TSSs) of coding genes characterized by bimodal H3K4me3 peaks [35]. However, METTL3 and METTL14 did not bind the same TSSs, suggesting independent regulation on target genes. CEBPZ, a transcription factor critical for hematopoietic differentiation, recruits METTL3, but not METTL14, to chromatin [35]. Promoter-bound METTL3 induces m^{6}A modification within the coding region of the associated mRNA transcript, such as *SP1* and *SP2*, and enhances their translation by relieving ribosome stalling [35].

In addition to METTL3 and METTL14, other m^{6}A methyltransferases or cofactors of the m^{6}A methyltransferase complex have also been implicated to function in AML. A genome-wide CRISPR-Cas9 screening identified METTL16, as well as METTL3 and METTL14, as critical genes for AML survival [35], while detailed study is yet to be done. Before it was identified as a component of the m^{6}A methyltransferase complex [43], WTAP was shown to be upregulated in AML and is a novel client protein of HSP90 [108]. Knockdown of WTAP in AML cells led to reduced proliferation and promotion of phorbol 12-myristate 13-acetate (PMA)-induced myeloid differentiation and inhibited growth of human leukemia cells in xenograft mice [108]. RBM15 was found to be a fusion partner of the MKL1 gene in t(1,22)(p13;q13) acute megakaryoblastic leukemia (AMKL), a subtype of pediatric AML [109]. It is therefore reasonable to speculate that RBM15 may play a role in leukemogenesis, possibly related to m^{6}A modification, for which further studies are warranted.

4.5 m^{6}A and Leukemia Stem Cells

Malignant stem cells are considered as the potential origin of and a key therapeutic target for AML, similar to what is believed for other cancer types [110–115]. Leukemia stem cells (LSCs) are defined as cells with two important properties: (1) capable of engrafting and initiating the disease when transplanted into immunodeficient animals and can self-renew by giving rise to leukemia in serial transplantations and (2) produce non-LSC bulk blasts that resemble the original disease but are unable to self-renew [116]. During the last few decades, investigators have been dedicated to the characterization of LSCs using different combinations of cell surface markers. It was found that LSCs immunophenotypically resemble certain normal hematopoietic progenitor populations and usually reside in the CD34$^+$CD38$^-$ fraction, although approximately 25% of AML cases lack CD34 expression [116]. It is believed that therapeutic targeting and eliminating of LSC is the key to eradicate leukemia and achieve long-term remissions.

Retroviral transduction of the *MLL-AF9* oncofusion gene into mouse HSPCs followed by transplantation into recipient mice represents one of the best mouse models for AML LSC studies [116]. By using this model, we have shown that depletion of either *Fto* or *Mettl14* in mouse HSPCs could inhibit leukemia initiation in primary BMT recipients and maintenance in secondary transplantations [31, 33], suggesting a role of these m^{6}A modifying proteins on AML LSC function. Indeed, we conducted limiting dilution assays using bone marrow cells harvested from MLL-AF9 primary leukemia mice to directly evaluate the effect of *Mettl14* depletion on the frequency of leukemia stem/initiating cells (LSCs/LICs) and found that the estimated LSC/LIC frequency was significantly reduced when *Mettl14* was knocked out [33]. While our data provide the first link between m^{6}A modification and LSC self-renewal [33], further systematic studies are warranted to better understand the role of m^{6}A modification on LSC biology.

4.6 Conclusions and Perspectives

Evidence is emerging that m^{6}A modification and the associated machinery play essential roles in both normal and malignant hematopoiesis, including the self-renewal and differentiation of normal and malignant HSCs. Systematical studies of the functions and underlying molecule mechanisms of individual m^{6}A writer, eraser, and reader genes will further advance our understanding of the complex networks and molecular mechanisms underlying normal and malignant hematopoiesis. In addition, it is also important to understand how expression of individual m^{6}A regulators is regulated during normal and malignant hematopoiesis.

The impact of m^{6}A modification on the fate of its embedded RNA mediates the functions of such modification. Therefore, it is of great importance to identify key functionally important RNA targets (including mRNA, lncRNA, etc.) that when

manipulated could phenocopy or reverse the effects of knockdown or overexpression of individual m^{6}A regulators. For instance, recent studies show the oncogenic *MYC* transcript as an important target of many m^{6}A regulators in cancer including leukemia [32–34, 37], highlighting the importance of precise regulation of *MYC* expression at the posttranscription level in normal development and the big impact of its dysregulation in tumorigenesis. Further systematic identification and functional studies of all critical target genes of m^{6}A modification by combined use of high-throughput sequencing such as CLIP-seq, m^{6}A-seq, and RNA-seq, followed by the validation assays and in vitro and in vivo functional studies, are warranted. Such knowledge will be not only important for the understanding of the effects of manipulation of individual m^{6}A regulator but also may lead to the discovery of novel therapeutic targets for AML and other types of leukemia.

As demonstrated by the published data thus far, m^{6}A regulators represent promising targets for treatment of cancer, including leukemia. Development of effective and selective inhibitors targeting oncogenic m^{6}A regulators (e.g., FTO and METTL14) that play more essential roles in leukemogenesis, especially in self-renewal of LSCs/LICs, than in normal hematopoiesis may hold great therapeutic potential in treating leukemia, especially when in combination with other therapeutic agents.

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