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CLASSICAL SIGNALING PATHWAYS (A CARDOSO & N CARLESSO, SECTION EDITORS)

The Role of RNA Epigenetic Modification in Normal and Malignant Hematopoiesis

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Abstract

Purpose of Review RNA epigenetic modifications have been identified as novel, dynamic regulators of gene expression, with important impacts on stem cell fate decisions. Here, we examine the functions of RNA modifications, with a focus on N^6-methyladenosine (m^6A), in hematopoietic stem cells under normal conditions and in malignancy.

Recent Findings The m^6A RNA modification is a critical regulator of hematopoiesis. Disruption of different elements of the m^6A machinery can skew the balance of self-renewal and differentiation in normal hematopoietic stem cells. The m^6A reader, writer, and eraser proteins are also overexpressed in myeloid leukemia, and disruption of their function impairs leukemogenesis. RNA m^6A modification governs important aspects of immune system function, including immune cell development, immune signaling, and recognition of RNA as foreign or self. In hematopoietic stem cells, endogenously derived double-stranded RNA can form in the absence of m^6A, inducing deleterious inflammatory pathways which compromise stem cell function.

Summary The RNA modification m^6A exerts a variety of functions in normal hematopoietic stem cells as well as leukemic cells. Pharmacologic modulation of different elements of the m^6A machinery provides a promising avenue for ex vivo expansion of hematopoietic stem cells in the transplant setting, as well as for leukemia therapy.

Keywords RNA modification · N^6-methyladenosine · m^6A · Hematopoiesis · dsRNA

Introduction

The past half century has witnessed burgeoning discoveries in the mechanisms regulating gene expression, thereby largely expanding the tenets of the central dogma. Post-transcriptional RNA modifications encompass more than 160 different chemical variations on the four canonical ribonucleotides, with diverse impacts on transcript function and regulation [1, 2].

Eukaryotic mRNA is well-known to be modified by a 5′-m^7G cap and 3′-polyadenylated tail, which facilitate sufficient output of translational products. In addition, mRNA can be exquisitely decorated by a collection of internal modifications [2]. The modified nucleotide N^6-methyladenosine (m^6A) was first identified as an abundant internal mRNA modification as early as the mid-1970s, and its principal methyltransferase, methyltransferase like 3 (METTL3), was discovered shortly thereafter [3–5]. We now know that mRNA can be modified by over 10 different chemical marks, including m^6A, N^6,2'-O-dimethyladenosine (m^6A_m), 5-methylcytosine (m^5C), and rarely N^1-methyladenosine (m^1A). Among these, m^6A is the most prevalent internal modification in mammalian cells, accounting for 1–3 adenosines per mRNA transcript on average [6].

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A contemporary renaissance in RNA epigenetics was unlocked by the publication of the first transcriptome-wide m^{6}A maps, which were derived by coupling RNA immunoprecipitation with an m^{6}A-specific antibody with next-generation sequencing (MeRIP-seq) [7, 8]. Across the transcriptome, m^{6}A is predominantly distributed throughout the coding region of mRNAs, with enrichment in the 3′-untranslated region (3′UTR) and near-stop codons with an RRACH (R = A/G, H = A/C/U) consensus motif.

Over the past decade, mechanistic studies facilitated by these techniques have implicated the m^{6}A modification in RNA processing, metabolism, and structure. Demonstrated functions include regulation of transcript stability, translational efficiency, micro RNA (miRNA) processing, long non-coding RNA (lncRNA) function, RNA/DNA hybridization, and RNA conformation [9–13]. While some studies have shown that m^{6}A guides RNA splicing by recruiting splicing factors, this remains a point of contention [14, 15].

Corresponding biologic phenotypes have garnered significant interest. Early studies demonstrated that perturbation of the m^{6}A modification exerts remarkable effects on stem cell populations. Constitutive deletion of *Mettl3* in mice results in early embryonic lethality with persistence of embryonic stem cells in "ground-state" or "naïve" pluripotency, implicating m^{6}A as a key regulator of self-renewal [16, 17]. This has prompted further investigation into the effects of m^{6}A on other stem cell populations in normal and diseased states.

As a result, emerging literature has begun to describe the role of the m^{6}A machinery in normal hematopoiesis and malignancy. The m^{6}A modification is an essential regulator of hematopoietic stem cell (HSC) self-renewal and differentiation, and various elements of the m^{6}A machinery have been implicated in myeloid malignancies including myelodysplasia (MDS) and acute myeloid leukemia (AML). In this review, we will describe the m^{6}A machinery and its functions within the hematopoietic system.

Overview of the m^{6}A Machinery

The establishment of transcriptome-wide methods for m^{6}A mapping has facilitated extensive characterization of the armament of m^{6}A reader, writer, and eraser proteins and their impacts on RNA metabolism.

Core components of the m^{6}A methyltransferase complex include METTL3, METTL14, WT1-associated protein (WTAP), and KIAA1429. m^{6}A is deposited co-transcriptionally, guided in part by binding of METTL14 to H3K36me3 [14, 18, 19]. The presence of m^{6}A can in turn regulate other histone modifications and mediate chromatin accessibility [20••]. RNA-binding motif protein 15 (RBM15) and RBM15B participate in the recruitment of the METTL3/14/WTAP complex to a subset of RNAs including the lncRNA *Xist*, which mediates X chromosome inactivation in an m^{6}A-dependent fashion [11]. METTL5, zinc finger CCHC-type containing 4 (ZCCHC4), and METTL16 are ancillary m^{6}A methyltransferases, which independently install m^{6}A on a few specific target transcripts including the 28S rRNA, spliceosomal U6 snRNA, and S-adenosyl methionine synthetase [21–24].

The best characterized m^{6}A readers are the YTH domain-containing family of proteins. Among these, YTH *N*^{6}-methyladenosine RNA-binding protein 1 (YTHDF1) and YTH domain-containig 2 (YTHDC2) predominantly promote translational efficiency, while YTHDF2 accelerates mRNA decay by trafficking target mRNAs to P bodies for degradation [9, 10, 25]. YTHDF3 facilitates the functions of both YTHDF1 and 2 and also enables translation of circular RNAs (circRNAs) [26–29]. YTHDC1 participates in RNA splicing, circRNA synthesis, *Xist*-mediated X chromosome inactivation, and global regulation of chromatin accessibility [11, 20, 29, 30]. Additional readers include eukaryotic initiation factor 3 (eIF3), insulin-like growth factor 2-binding protein (IGF2BP) family proteins, and heterogeneous nuclear ribonucleoprotein (hnRNP) family proteins hnRNPA2B1, hnRNPC, and hnRNPG [12, 31–35]. For a description of their functions, we recommend a detailed review by Meyer and Jaffrey [36].

While alkB homolog 5, RNA demethylase (ALKBH5) has been firmly established as an m^{6}A eraser, the second proposed m^{6}A demethylase, fat mass and obesity associated (FTO), has a higher affinity for cap-associated m^{6}Am, and its true substrate remains unclear [37–40].

Role of the m6A Methyltransferases in Hematopoiesis

METTL3 and METTL14 in Hematopoietic Stem Cells

An important role for m^{6}A modification in homeostatic hematopoiesis was first demonstrated in zebrafish models, as *mettl3*-deficient embryos die early during development due to profound hematopoietic failure secondary to an arrested endothelial-to-hematopoietic transition (EHT). In zebrafish, this critical cell fate decision marks the emergence of the first hematopoietic stem and progenitor cells from the hemogenic endothelium in the aorta-gonad-mesonephros (AGM). *Mettl3*-deficient zebrafish fail to navigate this transition, as endothelial identity is maintained in the AGM at the expense of primitive hematopoiesis. This effect is mediated by the stabilization of *notch1a* transcripts, which are normally targeted for degradation by m^{6}A during the EHT [41••].

In a subsequent study, tissue-specific Cre-Lox models of *Mettl3* deletion in endothelial and hematopoietic tissues driven by the *Vec* and *Vav* promoters, respectively, was

performed. Analysis of mice at E10.5 recapitulated the hematopoietic phenotype in *Vec*-Cre but not *Vav*-Cre mice [42]. This led to the suggestion that the observed hematopoietic phenotype in *Mettl3* deficient mice is exclusively attributable to defects within the Vec^+ endothelial compartment, and not due to effects in hematopoietic tissues. However, this interpretation is limited as analysis was performed prior to the onset of complete *Vav* expression in hematopoietic tissues at approximately E11.5.

Indeed, in our own analysis, we found that *Vav*-Cre^+-$Mettl3^{fl/fl}$ mice die during embryogenesis due to profound hematopoietic failure, with rare surviving pups exhibiting profound cytopenias and bone marrow aplasia [43••]. Our findings are in keeping with multiple preceding models of *Mettl3* deletion in adult mice. Three independent groups characterized *Mx1*-Cre^+-$Mettl3^{fl/fl}$ mice, in which *Mettl3* deletion is induced in the hematopoietic tissues of adult mice in response to polyinosinic:polycytidylic acid (pI:pC) treatment. Consistently across these studies, loss of METTL3 results in profound hematopoietic failure, with resultant peripheral blood cytopenias, reduced marrow cellularity, spleen hypertrophy, and extramedullary hematopoiesis [44–46].

Despite defective hematopoiesis, both E14.5 *Vav*-Cre^+-$Mettl3^{fl/fl}$ and adult *Mx1*-Cre^+-$Mettl3^{fl/fl}$ mice have significantly expanded bulk HSPC populations, identifiable by the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) surface markers. This finding suggests an arrest in hematopoietic differentiation in m^6A-deficient mice. Within the LSK compartment, there is a further enrichment of LSK subpopulations with immunophenotypes normally characteristic of self-renewing HSCs with long-term reconstitution potential. Despite the increased proportion of phenotypic HSCs in both models, these cells were deficient in functional assays including in vitro colony formation and hematopoietic reconstitution in competitive transplantation assays [43–46]. Taken together, these findings indicate that loss of METTL3 in the hematopoietic system results in accumulation of phenotypic HSCs that have profoundly reduced hematopoietic potential.

Evidence thus far indicates that loss of METTL3 destabilizes HSC identity rather than reinforcing it. *Mx1*-Cre^+-$Mettl3^{fl/fl}$ phenotypic HSCs showed enhanced metabolic and proliferative activity, exiting quiescence and entering the cell cycle [44, 45]. This is accompanied by diminished expression of hallmark HSC self-renewal genes [44]. Single-cell RNA sequencing of *Mx1*-Cre^+-$Mettl3^{fl/fl}$ HSPCs demonstrated the emergence of two novel "HSC-like" populations, one of which exhibited diminished expression of core HSC self-renewal genes. Comparison with wildtype HSPC subtypes showed that $Mettl3^{-/-}$ HSC populations most closely resembled wildtype multipotent progenitors. Functional analyses via transplant of sorted populations demonstrated that phenotypic $Mettl3^{-/-}$ HSCs engrafted preferentially in the MPP compartment and eventually disappeared in long-term analyses, suggesting limited self-renewal capacity [45••]. Mechanistic studies of this phenotype have centered predominantly on expression of *Myc*, which is downregulated in $Mettl3^{-/-}$ HSCs. Global MYC downregulation and altered segregation of MYC at the level of individual cell divisions impair the capacity for lineage commitment of HSCs, instead favoring self-renewal via symmetric replication [45, 46].

In comparison with *Mx1*-Cre^+-$Mettl3^{fl/fl}$ mice, *Mx1*-Cre^+-$Mettl14^{fl/fl}$ mice exhibit an attenuated hematopoietic phenotype. HSC frequency is unaltered in primary *Mx1*-Cre^+-$Mettl14^{fl/fl}$ compared with controls. Engraftment potential is diminished in competitive reconstitution assays but to a lesser degree than that of $Mettl3^{-/-}$ cells [47]. METTL3 and METTL14 form a heterodimer, and METTL3 is typically degraded upon loss of METTL14. Independent roles of METTL3 or METTL14 or residual METTL3 function following *Mettl14* deletion may account for this difference.

Overall, these findings highlight the functional importance of the m^6A methyltransferase complex in the normal hematopoietic system. Both METTL3 and METTL14 are necessary for normal hematopoiesis, and dysregulation of either produces dramatic impairment of HSC function. The above described model systems and their corresponding phenotypes are summarized in Table 1.

METTL3 and METTL14 in Myelopoiesis and Myeloid Malignancies

The m^6A methyltransferase subunits METTL3 and METTL14 have been described as important regulators of normal and malignant myelopoiesis. While this has been of interest for the development of therapeutics, observations have varied across experimental systems, and it remains to be seen whether proof of concept experiments will translate to effective clinical therapies.

Initial in vitro experiments in human $CD34^+$ cord blood progenitors, which are enriched for HSPCs, showed that loss of either METTL3 or METTL14 promotes spontaneous myeloid differentiation [48]. Furthermore, METTL3 and METTL14 are overexpressed in multiple subtypes of acute myeloid leukemia and are recurrently identified as essential genes in genome-wide CRISPR-Cas9 dropout screens in experimental models of AML [54••]. Indeed, loss of METTL3 or METTL14 in both murine AML models and human AML cell lines attenuates proliferation, diminishes engraftment, and delays mortality following transplantation into recipient mice. Furthermore, loss of either methyltransferase component results in the acquisition of mature myeloid cell surface markers and mature myeloid morphology [47, 48, 54].

A variety of mechanisms accounting for METTL3-mediated inhibition of myeloid differentiation have been identified. Vu et al. found that METTL3 was essential for expression of the oncogenes MYC and BCL2, as well as suppression

Table 1 Summary of the phenotypes in loss-of-function studies of m^{6}A regulators in normal hematopoiesis

Protein	Genetic alteration (method)	System	Phenotypes	References
METTL3	RNA interference (Morpholino)	Zebrafish embryo	• Endothelial-to-hematopoietic transition ↓ • HSPC emergence ↓	[41••]
	Endothelial KO (*Vec-Cre*)	Murine embryo (E10.5 AGM)	• HSPC and HEC frequency ↓ • In vitro CFU-forming ability and in vivo repopulating ability ↓	[42]
	Hematopoietic KO (*Vav-Cre*)	Murine embryo (E14.5 fetal liver)	• BM failure and perinatal lethality in homozygous knockout embryos • Cellularity, erythroid maturation, terminal myeloid differentiation ↓ • LSK and LT-HSC absolute number ↑ • In vitro CFU-forming and serial replating ability ↓ • In vivo repopulating ability ↓	[43••]
	pIpC-inducible KO (*Mx1-Cre*)	Adult mouse	• Pancytopenia, splenomegaly, extramedullary hematopoiesis • BM cellularity, terminal differentiation of megakaryocyte and erythroid ↓ • BM phenotypic HSC frequency and absolute number ↑ • In vitro CFU-forming ability of HSCs ↓ • In vivo repopulating ability of purified HSCs ↓	[44–46]
	Myeloid KO (*Lysm-Cre*)	Adult mouse	• BM and SP cellularity, complete blood count, no$^{\triangle}$ • Homeostatic and lipopolysaccharide-induced myelopoiesis, no$^{\triangle}$	[46••]
	RNA interference (shRNA)	Human CD34$^+$ HSPC (cultured ex vivo)	• CFU-forming ability and cell growth ↓ • Apoptosis, no$^{\triangle}$ • Myeloid differentiation ↑	[48]
METTL14	Tamoxifen-inducible KO (Cre^{ERT})	Adult mouse	• Complete blood count, no$^{\triangle}$ • In vivo repopulating ability of BM cells ↓	[47]
	pIpC-inducible KO (*Mx1-Cre*)	Adult mouse	• BM cellularity and BM LSK frequency, no$^{\triangle}$ • In vivo long-term repopulating ability of BM cells ↓	[44]
	RNA interference (shRNA)	Human CD34$^+$ HSPC (induced toward myelopoiesis ex vivo)	• CFU-forming ability ↓ • Cell growth, apoptosis, no$^{\triangle}$ • Monocytic differentiation ↑	[47]
YTHDF2	pIpC-inducible KO (*Mx1-Cre*)	Adult mouse	• BM LSK frequency and absolute number ↑ • Complete blood count, no$^{\triangle}$ • In vivo long-term repopulating ability of BM cells ↑	[49–51]
	Hematopoietic KO (*Vav-Cre*)	Adult mouse	• BM LSK absolute number ↑ • In vitro CFU-forming ability of BM cells, no$^{\triangle}$ • Equivalent total bone marrow chimerism in competitive transplantation • Preferential engraftment of HSPC and mature myeloid compartments	[50, 51]
	Tamoxifen-inducible KO (Cre^{ERT})	Adult mouse	• BM LT-HSC absolute number ↑ • In vivo long-term repopulating ability of purified LT-HSC ↑	[51]
	RNA interference (shRNA)	Human CD34$^+$ HSPC (cultured ex vivo)	• HSC frequency and absolute number and CFU-forming ability↑ • Apoptosis ↓ • In vivo repopulating ability ↑ • In vivo multilineage hematopoiesis upon xenotransplantation, no$^{\triangle}$	[49]
ALKBH5	pIpC-inducible KO (*Mx1-Cre*)	Adult mouse	• BM cellularity, complete blood count, no$^{\triangle}$ • Frequency, absolute number and distribution of HSPCs, no$^{\triangle}$ • In vitro CFU-forming ability and differentiation potential of HSPCs, no$^{\triangle}$ • In vivo repopulating ability of BM cells, no$^{\triangle}$ • In vivo long-term repopulating ability of purified LSK cells, no$^{\triangle}$	[52]
	Constitutive KO (CRISPR-Cas9)	Adult mouse	• Female-to-male ratio in homozygous knockout pups ↑ • BM cellularity, complete blood count, no$^{\triangle}$ • Frequency, absolute number and distribution of HSPCs, no$^{\triangle}$	[53]

Table 1 (continued)

Protein	Genetic alteration (method)	System	Phenotypes	References
	RNA interference (shRNA)	Human CD34$^+$ HSPC (cultured ex vivo)	• In vivo repopulating ability of BM cells minimally ↑ • In vivo HSC differentiation upon transplantation minimally ↑ • CFU-forming ability, no$^\triangle$ • Myeloid differentiation, no$^\triangle$	[52]

↑, enhanced phenotype; ↓, attenuated phenotype; no$^\triangle$, no difference compared with controls. *HSPC* hematopoietic stem and progenitor cell, *KO* knockout, *AGM* aorta-gonad-mesonephros, *HEC* hemogenic endothelial cell (CD31$^+$ c-Kit$^+$), *CFU* colony-forming unit, *BM* bone marrow, *LSK* Lin$^-$ Sca-1$^+$ c-Kit$^+$, *LT-HSC* long-term hematopoietic stem cell (CD34$^-$ Flk-2$^-$ Lin$^-$ Sca-1$^+$ c-Kit$^+$), *pIpC* polyinosinic:polycytidylic acid, *HSC* hematopoietic stem cell, *SP* spleen

of the PI3K/Akt signaling pathway, which normally promotes hematopoietic differentiation [48]. Barbieri et al. found that METTL3 is recruited to transcription start sites by the transcription factor CEBPZ. Promoter-bound METTL3 methylates emerging transcripts, enhancing the translation of key oncogenes such as SP1 by alleviating ribosome stalling [54••]. Weng et al. showed that METTL14 is downregulated by the myeloid transcription factor SPI1 (PU.1), which facilitates differentiation via downregulation of the m^{6}A target genes *Myb* and *Myc* [47]. The experimental systems used in these studies and the observed mechanisms of leukemogenesis are summarized in Table 2.

While these data support a role for METTL3 as a regulator of myelopoiesis, results have varied across different models. As described above, loss of METTL3 in mice results in deficient myeloid reconstitution and depletion of myeloid progenitors, which contrasts with the pro-myeloid differentiation phenotype seen in vitro in human CD34$^+$ cells [43–45]. Lee et al. further parsed the role of METTL3 in myeloid cells in vivo by performing myeloid-specific deletion of *Mettl3* driven by the *LysM* promoter. These mice showed no quantitative deficits in peripheral blood, bone marrow, or spleen counts, and myeloid lineage cells retained normal phenotype and function [46••]. As such, it will be important to reconcile the results of experiments across different model systems to fully resolve the role of METTL3 in normal and malignant myelopoiesis.

m^{6}A Readers in Normal Hematopoiesis and Malignancy

YTHDF2 Expands Functional Hematopoietic Stem Cells

While perturbation of the m^{6}A writer proteins has allowed for interrogation of the global effects of RNA methylation on hematopoiesis, studying individual reader proteins allows for further mechanistic dissection of these phenotypes. *Mx1*-Cre$^+$-*Ythdf2*$^{fl/fl}$ (*Ythdf2*$^{-/-}$) mice have a striking hematopoietic phenotype that differs from m^{6}A methyltransferase-deficient mice in important ways.

Multiple groups have examined *Mx1*-Cre$^+$-*Ythdf2*$^{fl/fl}$ mice, with concordant results. Phenotypic HSCs are dramatically expanded in *Ythdf2*$^{-/-}$ marrow, strongly resembling the phenotype seen in *Mx1*-Cre-*Mettl3*$^{fl/fl}$ mice. However, whereas *Mettl3*-deficient mice experience profound cytopenias and hematopoietic failure, *Ythdf2*$^{-/-}$ HSCs maintain normal trilineage hematopoiesis with only minor changes in peripheral blood counts. Colony-forming capacity is preserved, and functional repopulating HSCs are fourfold enriched in *Ythdf2*$^{-/-}$ marrow by limiting dilution assays [49, 50]. *Ythdf2*$^{-/-}$ HSCs also expand more readily in response to stressors such as myeloablative 5-fluorouracil treatment and radiation [49]. While *Ythdf2*$^{-/-}$ marrow engrafts in equal proportions with wildtype marrow in competitive transplantation, *Ythdf2*$^{-/-}$ cells exhibit advantages in repopulation of the HSC, myeloid, and erythroid compartments with a relative deficit in T cell engraftment (Table 1) [50••].

The enhanced HSC proliferation with preserved repopulating capacity seen in *Mx1*-Cre$^+$-*Ythdf2*$^{fl/fl}$ mice is compelling as it points to a viable avenue for the ex vivo expansion of hematopoietic stem cells for transplant applications. Indeed, in preliminary studies, shRNA-mediated knockdown of *YTHDF2* in human CD34$^+$ cord blood results in up to 15-fold expansion of HSCs, providing a promising proof of concept for therapeutic applications [49].

YTHDF2 was identified as a putative oncogene based on its overexpression in bulk AML cells, with enriched expression in leukemia-initiating cell (LIC) subpopulations. Interestingly, while loss of YTHDF2 enhances HSC activity in normal cells, *Ythdf2* deletion impairs leukemogenesis in murine AML models and human AML cell lines and reduces LIC frequency. The loss of leukemogenic activity following *Ythdf2* deletion in AML is attributable to upregulation of TNF receptor 2, which sensitizes leukemic cells to TNF-induced apoptosis (Table 2) [50••]. The preferential disruption of leukemogenesis with preserved normal hematopoiesis seen in *Mx1*-Cre$^+$-*Ythdf2*$^{fl/fl}$ mice identifies another promising opportunity for therapeutic intervention.

Table 2 Summary of the pro-leukemogenic mechanisms of m^{6}A regulators

Protein	AML models	Mechanism	References
METTL3	Murine *MLL-AF9/Flt3-ITD* leukemia	• Maintains survival, blocks differentiation, promotes replating in vitro	[54••]
	MOLM-13 (*MLL-AF9*, *FLT3-ITD*)	• Localizes to transcription start sites of active genes via CAATT-box binding factor CEBPZ, induces co-transcriptional m^{6}A modification mainly in coding region • Enhances translation of transcription factor SP1 which regulates *c-MYC* expression	[54••]
	MOLM-13 (*MLL-AF9*, *FLT3-ITD*)	• Enhances translation of *c-MYC*, *BCL2*, and *PTEN* mRNAs • Maintains survival and proliferation, blocks differentiation, promotes leukemogenesis in vivo	[48]
METTL14	Murine *MLL-AF9* leukemia	• Maintains self-renewal/proliferation of leukemia stem/initiating cells, promotes AML propagation in vivo	[47]
	MONO-MAC-6 (*MLL-AF9*, *TP53*)	• Increased METTL14 expression via reduced expression of its suppressor protein SPI1 (PU.1) in myeloid leukemia cells	[47]
	MV4-11(*MLL-AFF1*, *FLT3-ITD*) NB4 (*PML-RARA*, *KRAS*, *TP53)*	• Enhances mRNA stability and translation of *MYB* and *MYC* independently of YTHDF proteins • Maintains survival and proliferation, blocks differentiation	
YTHDF2	Murine *Meis1/Hoxa9* leukemia	• Destabilizes targeted transcripts, reduces expression of genes associated with the loss of leukemogenic potential • Downregulates TNFR2, partly accounting for the resistance of preleukemic cell to TNF-induced apoptosis • Promotes AML initiation and leukemic stem cell (LSC) propagation in vivo	[50••]
	THP-1 (*MLL-AF9*)	• Maintains survival, promotes leukemogenesis in vivo	[50••]
FTO	MONO-MAC-6 (*MLL-AF9*, *TP53)* NB4 *(PML-RARA*, *KRAS*, *TP53)*	• Reduces mRNA stability of *ASB2* and *RARA* • Inhibits ATRA-induced differentiation of acute promyelocytic cells	[55]
	NOMO-1 (*MLL-AF9*, *KRAS*, *TP53*)	• Enhances mRNA stability of *MYC* and *CEBPA* • Inhibited by R-2-hydroxyglutarate (R-2HG) in sensitive cell lines • Maintains proliferation of R-2HG-sensitive leukemic cells	[39]
ALKBH5	Murine *MLL-AF9* leukemia	• Maintains LSC self-renewal, promotes AML development and LSC maintenance in vivo	[52, 53]
	MOLM-13 (*MLL-AF9*, *FLT3-ITD*)	• Enhanced *ALKBH5* expression via increased chromatin accessibility and recruitment of active Pol II and transcription factor MYB, mediated by histone demethylase KDM4C action on repressive histone mark H3K9me3 in the promoter region • Enhances stability of receptor tyrosine kinase *AXL* transcripts, mediated by YTHDF2 • Maintains survival and proliferation, blocks differentiation, promotes leukemogenesis in vivo	[52]
	MOLM-13 (*MLL-AF9*, *FLT3-ITD*) NOMO-1 (*MLL-AF9*, *KRAS*, *TP53*) MONO-MAC-6 (*MLL-AF9*, *TP53)*	• Reduces mRNA stability of *TACC3* whose translational product regulates downstream p21 and MYC pathways	[53]

The m^{6}A Erasers in Hematopoietic Malignancies

Several observations have implicated either gain or loss of function of the m^{6}A erasers in myeloid malignancies. Both FTO and ALKBH5 were found to be overexpressed in various subtypes of AML, launching investigation into their possible role as oncogenes [52, 53, 55]. In contrast, in an analysis of copy number variation in acute myeloid leukemia, loss of *ALKBH5* was proposed to correlate with poorer prognosis [56].

FTO and ALKBH5 could also hypothetically be downregulated in the context of isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations in myeloid malignancies, which occur in approximately 20–30% of patients with MDS or AML [57, 58]. IDH1/2 normally catalyzes the conversion of isocitrate to α-ketoglutarate (α-KG), a necessary substrate of the α-KG-dependent dioxygenase family of enzymes, which includes both FTO and ALKBH5. Substitutions in key catalytic site residues endow mutant IDH1/2 with neomorphic activity, resulting in aberrant conversion of α-KG to 2-hydroxyglutarate (2-HG), which competitively inhibits the α-KG-dependent dioxygenases [59–62]. It therefore stands to reason that inhibition of FTO or ALKBH5 might be important in the pathophysiology of IDH1/2-mutant leukemias. In keeping with this hypothesis, preliminary experiments

showed that overexpression of mutant IDH1/2 in cell lines resulted in a 2-HG-dependent increase in the overall amount of m^{6}A modification.

These preliminary observations provided the basis for further investigation of FTO and ALKBH5 in myeloid malignancies.

FTO in Myeloid Malignancies

In patient samples, *FTO* is overexpressed in AML subtypes bearing particular translocations or mutations, such as the *PML-RARA* fusion oncogene. Enforced *Fto* expression correspondingly enhances leukemogenesis in murine AML models bearing these translocations, while *Fto* knockdown prolongs survival. This effect is mediated by FTO-mediated downregulation of *ASB2* and retinoic acid receptor alpha (*RARA*), two m^{6}A target genes that are typically upregulated during both normal hematopoiesis and ATRA-mediated differentiation therapy in *PML-RARA* acute myeloid leukemia [55]. This presents a simple model whereby *FTO* overexpression regulates the expression of key leukemia genes via particular m^{6}A sites.

The proposed role of FTO in *IDH1/2*-mutant malignancies is more complex. Su et al. broadly assessed the mechanism of *IDH1/2* mutations in human leukemias by directly treating an array of human cell lines with 2-HG. Surprisingly, they found that 2-HG largely inhibits cell growth, viability, and leukemic activity in transplant assays in a subset of human leukemia cell lines, while other cell lines are resistant to this effect. At baseline, 2-HG-sensitive cell lines are distinguishable from resistant lines by increased expression of *FTO*, globally decreased m^{6}A levels, and downstream activation of *MYC* with suppression of *ASB* and *RARA*, in keeping with previous findings. By contrast, 2-HG-resistant cell lines are characterized by decreased levels of 5-hmC, implicating inhibition of the DNA demethylase ten-eleven translocation 2 (TET2) rather than either of the RNA demethylases [39]. To explain the counterintuitive anti-tumor activity of an apparent oncometabolite, they assessed gene expression in TCGA human AML samples bearing IDH1/2 mutations. In doing so, they found that *MYC* is overexpressed independently of *FTO* levels in both IDH1/2-mutant patient samples and 2-HG-resistant cell lines, but not in 2-HG-sensitive cells. Pharmacologic inhibition of MYC restores sensitivity to 2-HG in this context, suggesting that MYC overexpression is necessary to protect IDH1/2-mutant cells and 2-HG-resistant cells from the inherent anti-tumor activity of 2-HG (Table 2) [39].

It will be important to determine to what extent this dichotomy between 2-HG-resistant and 2-HG-sensitive leukemias is consistently recreated across a spectrum of patient samples. In this regard, patient-derived xenograft models may be of interest. Furthermore, there are several established murine models of *IDH1/2* mutations which may allow for further mechanistic validation in vivo.

ALKBH5 in Normal Hematopoiesis and Myeloid Malignancies

While a single study showed that loss of ALKBH5 copy number in AML is associated with inferior prognosis, reanalysis of the same patient data by two independent groups ultimately showed a relatively low rate of *ALKBH5* deletions. Furthermore, reanalysis showed that *ALKBH5* is in fact overexpressed in these AML patient samples, with increased expression correlating to diminished survival [52, 53, 56].

Additional experiments demonstrated that *ALKBH5* is preferentially overexpressed within the leukemia-initiating cell compartment and in post-relapse patient samples. *ALKBH5* was shown to be necessary for AML cell proliferation and survival in vitro, as well as for leukemogenic activity in vivo. By contrast, *ALKBH5* appeared dispensable for normal hematopoiesis, as *Alkbh5*-deficient mice maintain normal proportions of HSPCs with preserved peripheral blood counts and repopulating potential in competitive transplant assays (Table 1) [52, 53]. This again exposes a leukemia-specific dependence on a component of the m^{6}A machinery which may represent a therapeutic vulnerability.

Preferential expression of *ALKBH5* in leukemic cells is facilitated by widespread alterations in chromatin accessibility. The *ALKBH5* locus is preferentially depleted of the repressive histone mark H3K9me3 in leukemic cells, with a corresponding enrichment of the H3K9 demethylase KDM4C. Loss of KDM4C concordantly impairs AML cell proliferation and clonogenic capacity, closely resembling ALKBH5 depletion phenotypes [52]. At the level of downstream targets, depletion of ALKBH5 results in downregulation of the pro-proliferative receptor tyrosine kinase AXL, whose transcripts display increased m^{6}A levels after the deletion of *ALKBH5* [52]. *TACC3* is another m^{6}A-modified transcript whose stability is dependent on *ALKBH5* expression. Loss of TACC3 function also resembles loss of ALKBH5, resulting in diminished leukemic activity in vitro and in vivo (Table 2) [53]. These findings highlight an integrated effect of epigenetic chromatin and RNA modifications in myeloid malignancy.

RNA Modification and Immune Function

Recently, m^{6}A RNA modification has been found to play a significant role in immune regulation, broadening our understanding of the function of m^{6}A in the hematopoietic system.

While m^{6}A modification of endogenous RNAs impacts immune cell function and development in several contexts, many studies have also investigated how m^{6}A modification of exogenous viral RNAs modulates the host immune response during infection [63]. The roles of m^{6}A in the immune response have been summarized in depth in a recent review by Shulman and Stern-Ginossar [64]. Here, we will highlight a

few relevant studies which offer a general overview of m^{6}A function in immune regulation and how this might globally impact hematopoiesis.

m^{6}A in "Self" Versus "Non-self" Recognition

In certain contexts, the m^{6}A modification has been hypothesized to function in distinguishing RNAs as "self" versus "non-self." Viral single-stranded or double-stranded RNAs (dsRNAs) are well-established pathogen-associated molecular patterns (PAMPs), which are typically identified as non-self during the innate immune response by pattern recognition receptors (PRRs) such as the DExD/H-box helicase 58 (DDX58, also known as RIG-I) or interferon induced with helicase C domain 1 (IFIH1, also known as MDA5). PRRs in turn activate immune signaling pathways including interferon signaling to coordinate the antiviral response.

Using human metapneumovirus (HMPV) as a model, Lu et al. show that depletion of m^{6}A from viral RNA facilitates their detection by PRRs. Loss of m^{6}A consequently enhances HMPV immunogenicity and attenuates infectivity [65]. In theory, manipulation of m^{6}A could therefore facilitate adjuvant or vaccine development. Chen et al. similarly showed that m^{6}A mediates recognition of circRNAs as "self" in mammalian cells. While m^{6}A-modified circRNA is non-immunogenic, unmodified circRNA activates RIG-I, mitochondrial antiviral signaling (MAVS) protein filamentation, and interferon signaling to generate antigen-specific T and B cell responses [66••].

In our recent paper, we found that loss of m^{6}A in *Vav*-Cre$^+$-*Mettl3*$^{fl/fl}$ mice results in the formation of endogenously derived double-stranded RNAs. These dsRNA species are likely detected as "non-self" by PRRs, activating dsRNA response pathways including MDA5-RIG-I, PKR-eIF2a, and 2′-5′-oligoadenylate synthetase-ribonuclease L (OAS-RNaseL). This cell-intrinsic innate immune response contributes to the hematopoietic failure phenotype observed in these mice and can be partially rescued by knockdown or deletion of downstream immune mediators [43••]. Of note, these findings were found in *Vav*-Cre mice but not described in *Mx1*-Cre-mediated models of *Mettl3* deletion; the latter depend on induction of Cre expression with the dsRNA mimetic pI:pC, which would mask any dsRNA response phenotype in comparisons between control and experimental mice. In keeping with our findings, previous studies have shown that the incorporation of m^{6}A or other modified nucleotides m^{5}C, m^{5}U, s^{2}U, or Ψ in RNA suppresses their detection by Toll-like receptors or RIG-I [67, 68].

The proposed effect of m^{6}A modification on dsRNA formation and recognition resonates with the adenosine-to-inosine (A-to-I) editing function of the adenosine deaminase acting on RNA (ADAR). Interestingly, ADAR1 editing activity prevents the formation of endogenously derived dsRNA and consequent MDA5 activation, and ADAR1 activity is negatively correlated with m^{6}A deposition transcriptome wide [69, 70]. Notably, *Adar1*-deficient embryos die of hematopoietic failure and exhibit an expansion of functionally defective phenotypic HSCs that closely mimics the phenotype of *Mettl3*$^{-/-}$ hematopoiesis [71]. It is therefore reasonable to hypothesize that both m^{6}A modification and A-to-I editing prevent dsRNA formation and thereby mediate a common phenotype in hematopoietic stem cells—this possibility will need to be investigated further.

The dsRNA response can play an important role in malignancy, as Ishizuka et al. found that loss of ADAR1 in tumor cells results in a sensitization of cells to immunotherapy, overcoming resistance to checkpoint blockade. This effect is mediated in part by the formation of endogenous dsRNAs which triggers a deleterious immune response [72]. In this way, suppression of endogenous dsRNA formation can be considered a mechanism of immune evasion in cancers. It will be of interest to determine whether perturbation of m^{6}A could similarly promote dsRNA formation and anti-tumor effects in solid or hematologic malignancies.

m^{6}A in Immune Cell Signaling and Development

The m^{6}A modification has also been shown to modulate the development and function of immune cells. Li et al. showed that m^{6}A modification on mRNAs controls T cell homeostasis by regulation of *Socs* mRNA stability in *CD4*-Cre-*Mettl3*$^{fl/fl}$ mice [73]. The m^{6}A modification level is increased during dendritic cell (DC) maturation and is required for DC maturation during an immune response to lipopolysaccharide (LPS) in a YTHDF1-dependent manner [74]. However, as discussed above, Lee et al. have shown that m^{6}A RNA modification is not required for the maintenance or function of mature myeloid cells in *Lyzm*-Cre-*Mettl3*$^{fl/fl}$ mice [46••]. These findings demonstrate the importance of m^{6}A for immune cell development and function and further illustrate the context dependence of m^{6}A function in hematopoiesis and the immune response.

Other RNA Modifications in Hematopoiesis

In this review, we have focused on the role of m^{6}A in hematopoiesis as it is the most common mRNA modification and its function has been the most extensively characterized. However, additional RNA modifications have also been found to influence hematopoiesis. While the tumor suppressor TET2 is known to suppress leukemogenesis by hydroxylating DNA 5-mC to 5hmC, TET2 was also recently found to promote myelopoiesis in the context of infection by demethylating RNA 5-mC [75]. Several tRNA modifications impact hematopoiesis as well. Pseudouridylation of tRNA

governs HSC commitment and influences disease pathophysiology in MDS, and the m^1A modification modulates erythropoiesis [76, 77]. DNMT2 mediates methylation of cytosine on $tRNA^{Asp}$ and is essential for hematopoiesis [78]. In general, tRNA metabolism can influence HSPC quiescence and other aspects of hematopoiesis, and it is possible that these functions are dependent on specific modifications [79, 80].

Future Directions

The many studies presented here demonstrate the essential function of m^6A RNA modification in the hematopoietic system. Nevertheless, many questions remain regarding the relationship between m6A and malignant hematopoiesis.

First, it is notable that all components of the m^6A machinery—writers, readers, and erasers—have now been found to be overexpressed in various subtypes of AML. For instance, METTL3, METTL14, YTHDF2, ALKBH5, and FTO have all been reported to be upregulated in MLL-rearranged leukemias, and perturbation of any of these proteins disrupts leukemogenicity. It therefore appears that a model whereby leukemia is promoted via simple gain or loss of total m^6A content is unlikely. Instead, global upregulation of the m^6A machinery in leukemia might be indicative of a generalized state of increased RNA metabolism and turnover. Under these conditions, compromising any element of the m^6A machinery might be deleterious. Alternatively, each individual component of the m^6A machinery may govern distinct leukemogenic mechanisms. It will be important to determine which of these regulators ultimately represent the best therapeutic target.

Other elements of the m^6A machinery are likely to be mechanistically relevant in malignancy. The methyltransferase adapter RBM15 is a component of the fusion oncogene RBM15-MKL in acute megakaryoblastic leukemia [81]. Interestingly, *Mettl3* deletion results in the accumulation of megakaryocytic progenitors in mice, directly implicating m^6A in the regulation of this lineage [46••]. It remains to be seen whether RBM15-MKL promotes leukemogenesis via an m^6A-mediated mechanism. WTAP has been described as an oncogene in AML, but this function has not yet been tied directly to m^6A. Interestingly, the m^6A-dependent lncRNA *Xist* is essential for normal hematopoiesis and functions as a tumor suppressor in hematologic malignancies [82]. It is possible that the role of m^6A as a regulator of HSC cell fate decisions is partially mediated by effects on *Xist* or its reader YTHDC1.

Conclusions

The RNA modification m^6A affects RNA function through a variety of mechanisms. Beyond direct effects on transcript stability and translational efficiency, the m^6A modification can influence transcript conformation and recognition by immune regulators and interact with other epigenetic modifiers to regulate global chromatin structure. Further investigation will be necessary to determine the extent to which these different effects contribute to the regulation of hematopoiesis and leukemogenesis. It is clear that RNA modifications offers an avenue for the development of therapeutics for the ex vivo expansion of hematopoietic stem cells as well for the treatment of myeloid malignancies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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