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# The m$^6$A RNA modification in AML

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## Abstract

**Purpose of review—**In recent years, the N$^6$-methyladenosine (m$^6$A) modification of RNA has been shown to play an important role in the development of acute myeloid leukemia (AML) and the maintenance of leukemic stem cells (LSCs). In this review we summarise the recent findings in the field of epitranscriptomics related to m$^6$A and its relevance in AML.

**Recent findings—**Recent studies have focused on the role of m$^6$A regulators in the development of AML and their potential as translational targets. The writer METTL3 and its binding partner METTL14, as well as the reader YTHDF2, were shown to be vital for LSC survival, and their loss has detrimental effects on AML cells. Similar observations were made with the demethylases FTO and ALKBH5. Of importance, loss of any of these genes has little to no effect on normal haemopoietic stem cells, suggesting therapeutic potential.

**Summary—**The field of epitranscriptomics is still in its infancy and the importance of m$^6$A and other RNA-modifications in AML will only come into sharper focus. The development of therapeutics targeting RNA-modifying enzymes may open up new avenues for treatment of such malignancies.

### Keywords

m$^6$A; AML; RNA modifications; epitranscriptomics

## 1 Introduction

RNA is amenable to nucleotide modifications that influence aspects of its metabolism such as stability, exportability, localisation and translation[1]. Over 170 chemically distinct

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epimarks have been identified on RNA species, representing a dynamic mechanism of gene regulation at the epitranscriptomic level[2]. The selective methylation of adenosine residues at position N(6) (referred to as $N^6$-methyladenosine or $m^6A$) constitutes the most abundant and conserved internal modification in eukaryotic mRNAs. $m^6A$ deposition on mRNA is catalysed either post- or co-transcriptionally by methyltransferases and associated complexes known as “writers”, is recognised by RNA-binding proteins termed “readers”, and is removed by demethylating enzymes or “erasers”. Following the advancement of transcriptome-wide $m^6A$-mapping technologies, its presence has been identified on approximately a quarter of all mRNAs, with the majority decorated with only a single $m^6A$ despite numerous sites fulfilling the conserved motif DRACH (D=G/A/U, R=G/A, H=A/U/C), whereas a few may contain 20 or more $m^6A$ sites with a transcriptome-wide pronounced density near stop codons or the 3’ untranslated region (UTR)[3–5].

The main $m^6A$ installer is the enzyme Methyltransferase Like 3 (METTL3), which deposits $m^6A$ on mRNA via its S-adenosylmethionine (SAM)-binding domain and primarily in association with Methyltransferase Like 14 (METTL14), forming the heterodimeric $m^6A$-METTL Complex (MAC)[6, 7]. This enzymatic core interacts with a more extensive multicomponent regulatory complex, termed the $m^6A$-METTL-associated complex (MACOM), comprising several proteins including Wilms Tumor 1 Associated Protein (WTAP), Vir Like $m^6A$ Methyltransferase Associated (VIRMA or KIAA1429), Cbl Proto-Oncogene Like 1 (CBLL1 or Hakai), RNA Binding Motif Protein 15 (RBM15), and Zinc Finger CCCH-Type Containing 13 (ZC3H13)[8]. The fate of $m^6A$ modified transcripts is mediated by readers, including the YT512-B Homology (YTH) domain-containing protein family (YTHDC1/2, YTHDF1/2/3,) and Insulin Like Growth Factor 2 mRNA Binding Protein family (IGF2BP1/2/3)[9, 10], the Fragile X Mental Retardation Protein (FMRP), the Eukaryotic Translation Initiation Factor 3 subunit h (eIF3h) and members of the Heterogeneous Nuclear Ribonuclease (HNRNP) family (HNRNPA2B1, HNRNPC, HNRNPG)[11]. The reversibility of $m^6A$ modifications is attributed to two demethylases proposed as erasers of $m^6A$, the Fat Mass and Obesity Associated protein (FTO) and AlkB Homologue 5 RNA Demethylase (ALKBH5).

In acute myeloid leukemia (AML), genetic aberrations and molecular imbalances accrued in myeloid progenitors or blasts lead to their uncontrolled clonal expansion and accumulation in the bone marrow. The cancer is maintained by leukemic stem cells (LSCs), a portion of which also serves as the persisting reservoir in resistance to current treatment or disease relapse frequently observed in patients. The role of $m^6A$ in driving or maintaining the leukemic state has recently started to be unravelled[11]. Importantly, most of the MACOM subunits represent genetic vulnerabilities of AML cells as indicated by CRISPR loss-of-function screens[12, 13]. Multiple studies have shown that METTL3 depletion in mammalian cells leads to an acute deficit of $m^6A$ on polyadenylated RNA in line with the enzyme being the primary mRNA $m^6A$ installer, and the paramount importance of specific and balanced dosage of this modification on RNA in normal development and homeostasis has been confirmed for several physiological processes including haematopoiesis[14–16].

Here we employ an outline of m$^6$A writers, readers and erasers to summarize the role this modification has only recently been shown to play in the pathogenesis and maintenance of AML.

## 2 m$^6$A Writers

### a METTL3

In experiments involving human cord blood-derived CD34+ hematopoietic stem/progenitor cells (CB-HSPCs), m$^6$A loss by METTL3 knock-down (KD) was shown to promote terminal myeloid differentiation *in vitro* while decreasing cell proliferation. In contrast, overexpression of wild-type METTL3 inhibits myeloid differentiation and promotes cell growth, an effect which is not phenocopied by a catalytically inactive form of the enzyme[15]. Importantly, *METTL3* mRNA and protein levels are significantly more abundant in human patient-derived AML cells than in primary CB-HSPCs. METTL3 depletion in AML cell lines induces differentiation and apoptosis, with *in vivo* experiments showing delayed AML progression in recipient mice[15]. These effects are traced to increased m$^6$A which alters the translation of oncogenic *c-MYC, BCL2* and *PTEN* mRNAs in AML models[15]. Cell differentiation upon METTL3 depletion appears to be orchestrated by the phosphatidylinositol 3-kinase (PI3K)/AKT pathway[15]. In an independent study, we demonstrated that, in AML, METTL3 installs m$^6$A co-transcriptionally in a METTL14-independent fashion. The gene essentiality of METTL3 was validated by demonstrating that its disruption by either knock-out (KO) or KD reverses the myeloid differentiation block in AML cells[16]. Ectopic expression of METTL3 rescued the leukemic self-renewing phenotype, while a catalytically inactive mutant failed to recapitulate this result[16]. Engraftment of human leukemic cells into immunocompromised mice was also markedly impaired following targeting of METTL3, leading to significantly longer survival[16].

We identified METTL3 and METTL14 associating with chromatin fractions in the AML cell line MOLM13, and we observed them binding to transcriptional start sites (TSSs) of different coding genes, with the CCAAT-box specifically enriched under METTL3 peaks[16]. We further identified CCAAT-binding factor CEBPZ as the recruiter of METTL3 to these TSSs[16]. Contrary to other m$^6$A-containing mRNAs which generally exhibited enhanced transcription upon METTL3 depletion, the transcripts from METTL3-bound genes were translated less efficiently. Mapping of ribosomal pausing sites on the pertinent mRNAs indicated stalling at GAN codons in METTL3-depleted cells[16]. One oncogenic driver requiring METTL3-mediated m$^6$A for enhanced translation is *SP1* which itself regulates *c-MYC* expression and which is also shown to be a dependency of AML[16]. Although METTL3 depletion has been reported to compromise normal haematopoiesis[15], the enzyme's essentiality in AML maintenance makes it a promising potential therapeutic target especially because inhibition by small molecule is not expected to achieve global reduction of METTL3 levels similar to gene deletion; rather, the response to a small molecule can be both dosage-dependent and reversible, with minimum or no effects on normal haematopoietic cells.

### b METTL14

While previously believed to possess weak methyltransferase activity, METTL14 is now thought to mainly act as an RNA-binding scaffold and allosteric activator to METTL3[17]. METTL14 is highly expressed in CB-HSPCs and is downregulated upon myeloid differentiation[17]. Importantly, when compared to healthy donor cells, METTL14 levels are higher in primary AML samples and in AML cell lines, while also being higher in AML than in non-haematological cancers[17]. These results are indicative of METTL14's role in maintaining the self-renewing capacity of LSCs. METTL14 is indeed essential for the preservation of HSPCs and AML's proliferative capacity as its silencing triggers myeloid differentiation, with its expression seemingly maintained by MLL-rearranged chimeras and diminished by differentiation-inducing agents (e.g. all-trans retinoic acid, ATRA)[17].

More specifically, *Mettl14* KO/KD disrupts MLL/AF9-induced cell immortalization *in vitro*, whereas ectopic expression of wild-type, but not mutant, protein increases transcriptomewide $m^6A$ thereby promoting MLL/AF9-mediated immortalization[17]. The LSC frequency is also shown to be lower in *Mettl14* KO murine cells vs controls, leading to delayed leukemia onset and prolonged survival *in vivo*[17]. Mechanistically, SPI1 binds the *METTL14* TSS and is negatively correlated with METTL14 expression; its silencing leads to an increase in METTL14 mRNA and protein levels in both HSPCs and human AML cells whereas its overexpression yields the reverse[17]. METTL14 expression positively modulates expression of oncogenic MYB and MYC by mediating $m^6A$ deposition; the latter is lost following METTL14 disruption which also produces a concomitant downregulation of the oncogenes[17]. Conversely, overexpression of METTL14 increases expression of both MYB and MYC, implicating this axis in leukemogenesis[17].

### c WTAP

WTAP acts as a binding partner for MAC and MACOM protein elements and helps direct the complex at nuclear speckles[18]. Similarly to METTL3 and METTL14, WTAP is overexpressed in AML samples and AML cell lines compared to normal mononuclear cells. WTAP is strikingly upregulated in both METTL3 KD and overexpression scenarios, indicating a precise dosagedependent regulatory mechanism of WTAP homeostasis[19]. Overexpression of METTL3 does not increase WTAP mRNA levels, however, it mediates WTAP downregulation whether catalytically active or not[19]. METTL3 KD on the other hand leads to increased levels of both WTAP mRNA and protein[19]. In the absence of METTL3, WTAP upregulation alone does not suffice in driving proliferative growth in AML cells, further narrowing its functional role to its involvement in the $m^6A$ methylation complex[19].

## 3 $m^6A$ Readers

### a YTH domain-containing proteins

The effects of $m^6A$ on RNA molecules are dependent on reader proteins the modified transcripts interact with. YTHDC1 is the only nuclear member of the protein family and localises at nuclear speckles. In the cytoplasm, YTHDF1 and YTHDF3 enhance mRNA translation, while $m^6A$ recognition by YTHDF2 leads to mRNA degradation[20–

22]. Interestingly, YTHDC2 can both enhance mRNA translation and mediate mRNA degradation. In the context of AML, YTHDF2 is upregulated in patient-derived AML samples compared to healthy controls[23*]. The protein is essential for LSC self-renewal and maintenance of AML and whereas its depletion has no detrimental effects on HSPCs and instead causes their expansion, it compromises the ability of LSCs to expand and thus propagate AML[23*, 24]. Furthermore, *YTHDF2* KO cells show upregulation of the m$^6$A-modified *TNFRSF1B* gene (encoding TNF Receptor Superfamily Member 1B); when conditional YTHDF2 KO pre-leukemic cells were treated with TNF, they showed significant increase in apoptosis compared to controls, suggesting YTHDF2 as a potential therapeutic target[23*]. YTHDF1 and YTHDF2 have a large number of overlapping target mRNAs, suggesting a complex regulatory mechanism controlling their two distinct functions mentioned above[25]. A recent study showed that members of the YTHDF family preferentially bind m$^6$A modified mRNAs, while YTHDC1 has a higher affinity for lncRNAs, suggesting a potentially distinct role in their regulation[26].

### b IGF2BP1/2/3

In contrast to YTHDF2, the members of the IGF2BP family increase the stability of modified mRNAs. However, there are conflicting reports about whether IGF2BP1/2/3 bind – and hence are direct readers of – m$^6$A. Some studies suggest that by directly binding mRNA at the GG(m$^6$A)C consensus sequence via their K Homology (KH) domain, IGF2BPs promote oncogenesis[9]. In contrast, other work using pull-down experiments has shown that IGF2BPs interact directly with YTHDF proteins, thus binding to the modification indirectly, via an m$^6$A-binding intermediary[27]. Alternatively, m$^6$A binding by IGF2BPs has also been suggested to be a result of conformation changes in mRNA caused by the modification[28].

Similarly to IGF2BPs, FMRP was also thought to interact with m$^6$A[29], however, it has recently been shown to directly bind to YTHDF2[30]; thus, its role and status as an m$^6$A reader remain elusive.

## 4 m$^6$A Erasers

Conflicting reports exist about FTO's involvement in the demethylation of m$^6$A. The initial studies demonstrated that FTO was highly expressed in a subset of AMLs (e.g. those with chromosomal rearrangements or NPM1 mutations)[31]. The study then showed that through its eraser activity, FTO can regulate the expression and decrease the stability of ABS2 and RARA mRNA[31]. RARA is involved in leukemogenesis and ABS2 inhibits the growth of AML and promotes differentiation; as a result, increased levels of FTO lead to a decrease in differentiation and an increase in pro-survival signalling in AML cells. Other studies have suggested that FTO positively regulates the expression of AML oncogenes such as *MYB* and *CEBPA*[32]. FTO KD led to human AML being sensitised to treatment with ATRA, which induced LSC differentiation[31]. The potential of drug inhibition of FTO in AML has also been investigated using two different compounds. Su *et al*[32] reported that when treating AML cells with R-2-Hydroxyglutarate (R-2HG) the results were similar to FTO KD, and sensitized the cells to commonly used chemotherapy agents both *in vitro* and *in*

*vivo*; additionally, the treatment with R-2HG negatively affected the ability of AML cells to proliferate and increased their differentiation. Another small molecule FTO-inhibitor, FB23-2, was later developed and exhibited results similar to genetic silencing of FTO and treatment with R-2HG – decrease of proliferation and increase of LSC differentiation, thereby stalling AML maintenance[33**]. However, a recent study has shown that FTO has a significantly higher catalytic activity when demethylating $m^6A_m$, compared to $m^6A$, with the rate of demethylation of the former by FTO being 100-fold higher[34]. Moreover, while the data suggesting FTO is only acting on $m^6A_m$ is compelling, an increase of ~20% in $m^6A$ is observed when FTO is depleted[31]. Despite the strong data supporting the role of FTO in AML and its potential as a therapeutic target, further studies are needed to clarify its molecular function and specificity as an $m^6A$ eraser.

The second $m^6A$ eraser, ALKBH5, is localized in the nucleus, suggesting that its demethylase activity is focused on newly formed mRNA prior to its export to the cytoplasm[35]. Unlike FTO, ALKBH5 has no activity towards $m^6A_m$[34]. ALKBH5 is overexpressed in LSCs compared to normal blasts and is correlated with poor prognosis in patients. Wang and colleagues[36] observed that the *ALKBH5* chromatin locus is more accessible in AML compared to healthy controls. They further investigated proteins which bind the *ALKBH5* promoter region and observed enrichment of histone modifiers, including the H3K9me3 demethylase Lysine Demethylase 4C (KDM4C). KDM4C can increase chromatin accessibility and transcription factor binding to the promoter site, thus increasing the expression of ALKBH5[36]. *In vitro* KDM4C depletion phenocopied the depletion of ALKBH5[36].

Another study investigating the specific role of ALKBH5 in AML observed that ALKBH5 is often overexpressed in patients and this correlates with poor prognosis[37*]. Depletion of ALKBH5 in both AML cell lines and primary $Lin^-CD34^+$ cells was shown to decrease the survival of the cancer and increase the apoptosis of cancer cells[37*]. Overexpression of *ALKBH5* showed the reverse effect by promoting the growth of cancer cells; this phenotype was not recapitulated with catalytically inactive ALKBH5[37*]. *In vivo* studies using *Alkbh5* KO mice showed that the lack of ALKBH5 leads to delayed onset of AML and decrease in the AML load[36, 37*]. Secondary transplantation of *Alkbh5* KO cells did not lead to AML, suggesting *Alkbh5* plays an important role for the maintenance of AML by controlling the self-renewal of leukemic stem cells[37*]. Further support for the role of *ALKBH5* in LSC self-renewal is that, in its absence, human and mouse leukemic cells both exhibit reduced propagation after xenograft transplantation as well as diminished colony-forming potential. These studies also demonstrate that ALKBH5 is not essential for normal haematopoiesis and thus highlight its potential as a therapeutic target.

Two different explanations have been put forth to account for the action of *ALKBH5*. One suggested mechanism is via *TACC3*, an oncogene and upstream regulator of MYC[37*] and an alternative mechanism of action is through the *ALKBH5*-$m^6A$-*AXL* kinase signalling pathway[36]. Both studies suggest that ALKBH5 plays a role in the control of AML cell growth by impacting mRNA stability[36, 37*].

There is, however, some conflicting evidence relating to the role of *ALKBH5* in AML progression. Based on genome-wide CRISPR screens, ALKBH5 was proposed as either a tumour suppressor gene or as non-essential for AML[15, 16]. A possible explanation for the discrepancies could be the difference in AML models used.

## 5 Conclusions

The complex network of $m^6A$ writers, readers and erasers plays a clear and crucial role in the development and maintenance of AML. Since targeting most of the relevant enzymes seems to specifically inhibit the survival of LSCs, it makes them potential targets for AML treatment as demonstrated recently with the FTO inhibitors. A number of groups and biotechnology companies are now racing to develop small molecule inhibitors against these. The first in the RNA-methyltransferase inhibitor class is a METTL3-specific bioavailable small molecule whose strong potential as a treatment for AML has been exhibited through work from our own group[38**]. The success of such an inhibitor molecule would open a new field in anti-cancer therapy. Of note, all current studies focusing on $m^6A$ and other RNA-modifications are heavily relying on antibody-specificity and there is need for the development of technologies complimenting or substituting antibody-based methods. Some examples here include pipelines for detection of RNA methylation on native RNA using Oxford Nanopore sequencing[39–41] or our recently published meCLICK-seq[42*].

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**Key points**

- $m^6A$ is important for myeloid leukemogenesis
- changes in $m^6A$ levels affect specifically LCSs but not normal HSCs
- $m^6A$ regulators are promising therapeutic targets for AML