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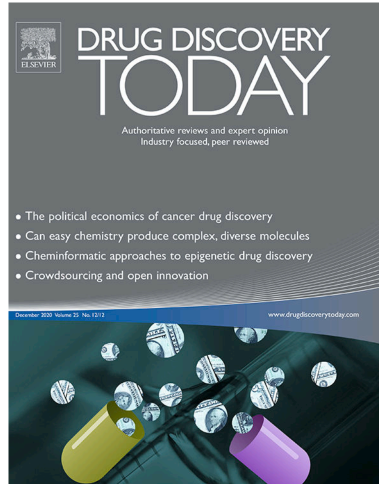

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m6A RNA methylation and beyond – the epigenetic machinery and potential treatment options

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m6A RNA methylation and beyond – the epigenetic machinery and potential treatment options

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Teaser: m6A is a newly discovered RNA signature that is involved in pathophysiology. Recent findings highlight the central role of this modification and open the way for the development of effective pharmacological approaches.

Highlights:

- We summarize recent findings on the roles of the m6A modification in pathophysiology.
- We discuss the role of m6a epitranscriptomics in stem cell maintenance, induction of the epithelial to mesenchymal transition (EMT) and tumor progression.
- We describe the state of the art in the design and validation of inhibitors of **{AuQ: Edit OK?}** m6A writers and erasers.

Author biographies

Dr Sabrina Garbo obtained her Master's Degree in Cell, Molecular Biology and Biomedical Sciences from the University of Rome Tor Vergata in 2017 and a PhD in Life Sciences from Sapienza University of Rome in 2021. She is currently a postdoctoral Researcher at Bambino Gesù Children's Hospital, Rome. Her scientific interest is focused on the role of long non-coding RNAs (lncRNAs) in epigenetic regulation and in RNA modifications that are involved in carcinogenesis.

Clemens Zwergel is currently a postdoc at the Sapienza University of Rome. He moved after his license to practise as a pharmacist

from his country of origin (Germany) to Exeter (UK), where he obtained a Diploma in Pharmaceutical Sciences. Before coming to Italy, he was then a Marie Curie fellow in France (Metz), where he obtained his EuroPhD within the RedCat network. Since 2010, his main research interest has involved the design and synthesis of small molecules that have potential applications in cancer or in neurodegenerative, metabolic, and infectious diseases.

Cecilia Battistelli obtained her Degree in Biological Sciences and a PhD in Human Biology and Genetics at the Sapienza University of Rome. She is currently a Researcher and Assistant Professor of Applied Biology in the Faculty of Pharmacy and Medicine at Sapienza University. In recent years, her research has focused on the involvement of epigenetics in the regulation of gene expression in both cellular differentiation and tumorigenesis, with the intent to develop new strategies to counteract tumor progression.

m6A is emerging as one of the most important RNA modifications because of its involvement in pathological and physiological events. Here, we provide an overview of this epitranscriptomic modification, beginning with a description of the molecular players involved and continuing with a focus on the role of m6A in the maintenance of stemness, induction of the epithelial to mesenchymal transition (EMT), and tumor progression. Finally, we discuss the state of the art regarding the design and validation of inhibitors of m6A writers or erasers to provide a background for future investigations and for the development of specific therapeutics.

Introduction

In recent decades, epigenetics has emerged as a fundamental level of gene expression regulation in development, differentiation, and pathophysiological states. The available knowledge on epigenetics is considerable. Nevertheless, the design and production of chemical drugs that can target specific epigenetic regulators, in order to re-establish the correct expression of genes that are dysregulated in disease, remains an open field of investigation [1–4].

In recent years, the scientific literature on the discovery and characterization of epigenetic regulatory processes that affect RNA molecules has grown exponentially. The increasing number of reports on RNA modification has given rise to a new era of epigenetics, referred to as 'RNA epigenetics' [5] or 'epitranscriptomics' [6].

Over the years, several types of modifications to RNA have been discovered: m6A (N6-methyladenosine), m1A (1-methyladenosine), m5C (5-methylcytidine), Ψ (pseudouridine), hm5C (5-hydroxymethylcytidine) [7]. In addition, several online databases that are related to RNA modifications have emerged: together, MODOMICS [8], RMBase [9], m6A-Atlas [10], and

RMDisease [11] provide information on chemical structures, biosynthetic pathways, modified residues on RNA sequences, novel nomenclature and relationships with diseases.

In this review, we focus on m6A and its role in physiology and disease, focusing on the need to design new molecules that are capable of selectively impairing the activity of the enzymes responsible for this RNA modification.

m6A players and functional role

m6A, the addition of a methyl group to carbon 6 of adenosine, is the most frequent modification on RNAs. New high-resolution methods, such as miCLIP-Seq, that allow the study of specific methylated sites on RNA have allowed the identification of m6A sites along RNA molecules [12, 13]. They are most frequently found in RRACH motifs (R = A/G, H = A/C/U) [14], where they were first detected at the 3' UTRs, 5' UTRs and in the coding sequences of mRNAs, or in DRACH motifs (D = A/G/U, R = A/G, H = A/C/U), or RAC motifs (R = A/G, H = A/C/U) [13].

As regards the proteins responsible for m6A modification (**Figure 1**), a pivotal role is driven by the RNA methyltransferase complex. This complex is mainly composed of m6A writers such as METTL3 (methyltransferase like 3), METTL14 (methyltransferase like 14) and WTAP (Wilm's tumor 1-associated protein). METTL3 was the first discovered member of the methyltransferase complex characterized by the presence of an S-adenosyl methionine (SAM)-binding domain); METTL14 is another methyltransferase that forms a heterodimer with METTL3; whereas WTAP is an auxiliary member of the core complex that lacks methyltransferase activity but which influences m6A deposition by METTL3–METTL14 [15].

In addition to these members, the methyltransferase-writing complex comprises adaptor proteins such as RBM15 (RNA-binding motif protein 15), which are essential for the initial recruitment of the writers onto pre-mRNAs. KIAA1429 (also called VIRMA for vir-like m6A methyltransferase associated) is another adaptor protein that is essential for bridging interactions with the other members of the complex [16]. ZC3H13 (Zinc finger CCCH domain-containing protein 13) was recently characterized and found to interfere positively with the binding between the adaptor protein RB15 and WTAP [17]. Among the RNA methyltransferases, METTL16 (Methyltransferase-like protein 16) is involved in the methylation of coding and non-coding RNAs (such as U6 snRNA) [18]. It is not part of the METTL3–METTL14 complex, and it does not recognize target RNAs via specific core motifs but through certain RNA secondary structures [19].

In addition to RNA methylation, RNA demethylation is also an open field of research and is mediated by m6A erasers. FTO (Fat mass and obesity-associated protein) was the first eraser discovered to be involved in the oxidative reversion of m6A to adenosine, even though it also has the ability to form reaction intermediates such as hm6A (N6-hydroxymethyadenosine) and f6A (N6-formyladenosine) [20]. FTO is localized both in the nucleus, where it affects RNA processing, and in the cytoplasm, suggesting an activity in cytosolic RNA metabolism [21]. The second m6A eraser to be discovered was ALKBH5 (Alkylated DNA repair protein alkB homolog 5), which is localized in the nucleus and plays a role in RNA metabolism and RNA export [22].

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m6A readers are RNA-binding proteins (RBPs) that are involved in the recognition of methylated RNAs and in the regulation of RNA processing, splicing, export, stability, translation, and storage through various mechanisms. The most-studied m6A readers are members of the YTH (YT homology) domain-containing family. These proteins have a high affinity for methylated RNA and are characterized by the presence of a hydrophobic pocket for binding to the modified m6A residue [23].

The fate of a methylated RNA depends not only on the cellular context and on the direct recruitment of m6A readers, but also on other RBPs that can act as indirect m6A readers. These RBPs play multiple roles in the regulation of RNA metabolism, such as splicing, processing, RNA export, stability and translation. YTHDF1 (YTH domain-containing family protein 1) positively regulates m6A-edited mRNA translation [24], working in collaboration with YTHDF3 (YTH domain-containing family protein 3) through interaction with the 40S and 60S ribosomal subunits [25]. YTHDF2 (YTH domain-containing family protein 2) promotes mRNA decay [26] through a de-adenylation process at the 3' end of m6A-edited mRNAs, preventing aggregation into polysomes. YTHDC1 (YTH domain-containing protein 1) acts as a recruiter of splicing factors such as SRSF3 (Serine/arginine-rich splicing factor 3) and SRSF10 (Serine/arginine-rich splicing factor 10) [27]. HNRNPC/G (Heterogeneous nuclear ribonucleoproteins C1/C2/G) ribonucleoproteins are able to bind to m6A-pre-mRNA and to modulate alternative splicing [28, 29]. In some contexts, m6A-modified mRNAs can undergo compartmentalization into processing bodies (p-bodies) [26] or stress granules [30]. IGF2BPs (Insulin Growth Factor 2 Binding Proteins) are involved as m6A readers in translational regulation or mRNA decay within p-bodies, or in RNA storage into stress granules [31].

m6A modification is also involved in altering the secondary structure of RNA, an aspect known as the m6A switch. When m6A is deposited at a stem-loop structure, destabilization of the base pairing between m6A and U occurs, facilitating the interaction between RNAs and hnRNPs (heterogeneous nuclear ribonucleoproteins). Coherently, it has also been reported that m6A-modified RNAs are less structured than non-methylated RNAs [32].

Another exciting aspect is that the epitranscriptome might influence the epigenome. A recent paper shows that m6A modification on carRNAs (chromosome-associated regulatory RNAs) disrupts the chromatin conformational state and downstream transcription [33]. Altogether, these findings indicate that complex epigenetic mechanisms that are associated with RNAs might influence RNA stability and expression, indicating that epitranscriptomics is a newly discovered layer of gene regulation.

m6A and non-coding RNAs

Although most of the published studies on m6A modification focus on mRNAs, m6A can affect the expression and function of non-coding RNAs. microRNAs (miRNAs) are small non-coding RNA molecules that have an essential function in modulating post-transcriptional gene expression. The m6A modification is involved in the biogenesis of miRNAs, starting from their processing and export but also influencing their function on target mRNAs. In mammalian cells, miRNA processing is affected by m6A modification, as reported in a study indicating that

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methylation of NSUN2 (NOP2/Sun RNA Methyltransferase 2) has a negative effect on different steps of miR-125b biogenesis [34]. In line with this, Berulava and colleagues [35] showed that m6A modification affects the stability and/or processing of some miRNAs. m6A is essential for the recognition of primary microRNAs (pri-miRNAs) by their processing machinery [36]. Methylated pri-miRNAs are recognized by the reader hnRNPA2B1 (Heterogeneous nuclear ribonucleoprotein A2/B1), which recruits DGCR8 (DiGeorge syndrome critical region 8) [37] and thus regulates miRNA biogenesis (more positively than negatively). In addition, some miRNAs are also involved in the regulation of genes related to m6A modification (writers, erasers, and readers).

There is initial evidence that long non-coding RNAs (lncRNAs) may also be subjected to m6A modification. lncRNAs are non-coding RNAs that are involved in the regulation of gene expression at the transcriptional, post-transcriptional and translational levels. KCNK15-AS1 (KCNK15 antisense RNA 1) is an lncRNA that is significantly downregulated and highly m6A-modified in pancreatic cancer tissues. It seems that, in this context, m6A decreases the stability of this tumor suppressor lncRNA and thus modifies the expression of epithelial to mesenchymal transition (EMT) markers [38]. In another study, the lncRNA RP11 was found to be highly represented in colorectal carcinoma (CRC) cells and tissues, and associated with the stability of the EMT transcriptional factor Zeb1 (Zinc finger E-box-binding homeobox 1). In CRC cells, the m6A-modified lncRNA RP11 is involved in a complex composed of m6A-RP11, the reader hnRNPA2B1, and Siah1 and FBXO45 mRNAs, which encode Zeb1 negative regulators and whose stability is downregulated when complexed in this way [39]. X-inactive specific transcript (XIST) is a master lncRNA that is involved in X chromosome inactivation through epigenetic silencing mechanisms. It is now known that XIST is also subject to m6A modification and that this is essential for proper XIST-mediated repression of target genes [40].

m6A and stemness

The m6A RNA modification is also deeply involved in stem cell biology and cell differentiation. Embryonic stem cells (ESCs) are pluripotent stem cells in which m6A modification is essential for cell commitment. Indeed, METTL3-depleted cells are unable to exit pluripotency, mainly because m6A modification destabilizes mRNAs that encode pluripotent promoters such as IGFBPs, SOX2 and NANOG [26, 41, 42]. Conversely, m6A deposition also destabilizes mRNAs encoding developmental modulators that contribute to the maintenance of stemness and self-renewal ability [43]. m6A modification seems to inhibit the binding of Hu antigen R (HuR) to mRNAs. HuR stabilizes mRNAs because it is able to prevent the interaction between the miRNA–RISC complex and the mRNA 3'UTR [26]. This is one of the reasons why these m6A-edited RNAs are less stable. Members of the YTH domain family are also involved in this destabilization process. YTHDF1-3 readers bind to m6A RNA to transport it into p-bodies for degradation [26].

Embryonic neural stem cells (NSCs) play a pivotal role during nervous system development, and m6A RNA modification exerts a central function in neuronal differentiation.

m6A can contribute positively to both the proliferation and the differentiation of NSCs by affecting the expression level of histone modifiers and by influencing the number of repressed or activated genes that are involved in proliferation and differentiation [42, 44]. Nevertheless, m6A can negatively influence neurogenesis through the m6A-dependent degradation of brain-derived neurotrophic factor (BDNF) mRNA [45]. METTL3 expression is also negatively correlated with differentiation and reduction of proliferation in hematopoietic stem progenitor cells (HSPCs) and leukemia cells [46]. This is due to the m6A-dependent translational regulation of specific m6A-edited mRNAs that are involved in tumorigenesis and myeloid differentiation, such as BCL2, c- MYC, and PTEN. m6A also controls the identity of hematopoietic stem cells (HSC) by modulating symmetric commitment through regulation of c-MYC expression [47].

A paper by Xu and coworkers [48] addressed a novel aspect of epitranscriptomics in mouse ESCs. These authors showed that binding of METTL3 is associated with the two heterochromatin marks H3K9me3 (mediated by SETDB1 and its regulator TRIM28) and H4K20me3 (mediated by SUV420H1 and SUV420H2). Remarkably, in mammals, constitutive heterochromatin forms over repetitive elements, including endogenous retroviruses (ERVs) such as the intracisternal A particle (IAP)-type family of endogenous retroviruses. METTL3 regulates heterochromatin formation on IAPs via its catalytic activity and it is specific for METTL3-targeted IAPs, as demonstrated by knock-out and rescue experiments. However, the METTL3-dependent chromatin modifications observed in this paper are due to the direct interaction between METTL3 and SETDB1/TRIM28, an interaction that is independent of METTL3 catalytic activity. This work provides the first evidence of chromatin-associated METTL3-induced histone modifications that are involved in the modulation of gene expression, at least in mouse ESCs.

m6A in the epithelial to mesenchymal transition (EMT)

The EMT is a cellular process in which epithelial cells undergo a profound reprogramming of gene expression and significant phenotypic changes in order to become mesenchymal cells [49]. This biological process is physiologically essential during embryogenesis, development, differentiation, wound healing and tissue regeneration [50], but is also involved in pathological events such as fibrosis and cancer metastasis. In general, m6A modifications appear to perform a pro-EMT function by inducing the expression of fundamental EMT genes (such as transcription factors).

The Snail family of EMT-inducing transcription factors includes SNAI1 (Snail), SNAI2 (Slug), and SNAI3 [51]. Snail (SNAI1) is a master transcription factor of EMT because it is sufficient to promote this cellular process, to modify cell morphology, to stimulate the expression of mesenchymal genes (such as matrix metalloproteinases MMP2 and MMP9, Vimentin and Fibronectin) and to repress epithelial genes (such as E-cadherin and HNF4a in liver) by directly binding to their promoters [52]. There is some evidence that expression of the master transcription factor Snail is also under the control of m6A modification during EMT. Snail

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mRNA is subjected to a high m6A modification rate during EMT in cancer cells, resulting in an increase in its translation [53].

Notably, in TGFβ-induced EMT, Snail expression is impaired in METTL3-expressing cancer cells [54]. The TGFβ pathway, which is able to induce EMT, appears to be involved in the regulation of gene expression through epitranscriptomics, leading to rapid and reversible gene modulation. In this context, SMAD2/3, the main effectors of this pathway at the transcriptional level, are able to interact with the m6A reader in order to regulate target genes such as Nanog [55].

In lung cancer with TGFβ-induced EMT, an increase in the expression level of METTL3, as well as an accumulation of m6A on RNA molecules such as JUNB, can be observed [56]. In EMT of gastric carcinoma, METTL3 is overexpressed to an extent that is related to the patient's prognosis in terms of survival and recurrence **{AuQ: Edit OK?}**. Indeed, ZMYM1 mRNA, one of the METTL3 targets, has been described as being stabilized by m6A modification. This causes a higher expression of this factor, which forms a complex with CtBP/LSD1/CoRest and in turn epigenetically represses epithelial genes, thus inducing EMT [57]. In non-small cell lung cancer (NSCLC), the m6A eraser ALKBH5 appears to have an anti-EMT property associated with a downregulation of YAP mRNA [58].

Even though the majority of scientific works suggests an oncogenic role for METTL3 and an accumulation of m6A modifications in tumoral EMT, some data show that METTL has a tumor-suppressive function. In tumoral EMT of renal carcinomas, METTL3 has been shown to have a negative effect the EMT process, cell proliferation and cell cycle progression [59].

m6A in cancer

Different studies focusing on distinct cancer contexts (**Figure 2**) have highlighted the impact of m6A modification on cancer growth, self-renewal of cancer stem cells, and tumor development [60].

Glioblastoma

In glioblastoma, the reduction of METTL3 and m6A levels enhances stem-like cell growth, self-renewal, and the ability to form brain tumors. Molecularly, this modification is related to the upregulation of ADAM19, EPHA3, and KLF4 and the repression of CDKN2A, BRCA2, and TP53I11. Coherently, METTL3 overexpression in these cells or pharmacological inhibition of the demethylase FTO counteract glioblastoma progression [61], suggesting that RNA demethylases represent a potential therapeutic approach for these tumors [62]. A recent study indicates, however, that METTL3 has an oncogenic effect in glioblastoma cells by modulating alternative splicing [63], leading to an increase in the production of BCL-XS and NCOR2a isoforms and to the inhibition of cancer stem cell (CSC) growth. Moreover, METTL3 has been observed to play an oncogenic role by methylating the 3'-UTR of SOX2 mRNA, thereby increasing the stability of this mRNA and promoting cell-stem-like properties and poor patient prognosis [64]. METTL3 silencing reduces SOX2 expression, enhances tumor cell sensitivity to γ-irradiation *in vitro*, inhibits glioblastoma tumor growth and prolongs survival

in mice [65]. Concerning erasers, ALKBH5 mRNA **{AuQ: Edit OK?}** is overexpressed in glioblastoma CSCs and is associated with a poor prognosis. Its knockdown increases m6A levels on FOXM1 mRNA and decreases its binding to HuR, thus stabilizing FOXM1 mRNA **{AuQ: Edit OK?}** [66]. Moreover, ALKBH5 silencing inhibits the proliferation of CSCs and MV1035, inhibits ALKBH **{AuQ: Edit OK?}**, and reduces the invasiveness of glioblastoma [67].

Hepatocellular carcinoma

In hepatocellular carcinoma (HCC), many m6A regulators are significantly overexpressed (for example, through gene amplification) [63] and correlate with poor prognosis and tumor recurrence. Coherently, METTL3 dysregulation seems to be linked to copy number variation and low rates of methylation at its gene promoter, leading to increased expression **{AuQ: Edit OK?}** [63].

By contrast, some research studies have indicated that low expression of METTL3 and YTHDF2, or downregulation of METTL14 **{AuQ: Edit OK?}**, confers metastatic capabilities [65] through regulation of the metastasis-associated miR126 [68] and through the m6A-dependent regulation of cysteine sulfinic acid decarboxylase (CSAD), glutamicoxaloacetic transaminase 2 (GOT2) and suppressor of cytokine signaling 2 (SOCS2) [67]. Expression levels of METTL3 and YTHDF1 are upregulated in HCC and are directly related to poor prognosis, tumor volume, distant metastasis, high histological grade and neoplasm stage, and to m6A in SNAIL coding sequence which promotes EMT in HCC cells [53]. Accordingly, it has been reported that YTHDF1 may be implicated in PI3K/AKT and Wnt/β-catenin signaling and in regulation of the p53 pathway, and that its depletion significantly decreases the expression of N-cadherin, pAKT(S308), pAKT(S473), pGSK-3β, β-catenin, c- MYC, TCF-1, cyclin D1, CD44 and vimentin and upregulates E-cadherin [69].

Functionally, METTL3 depletion impairs cell proliferation, anchorage-independent growth, cell migration, and metastasis formation *in vivo* [70]. Specifically, METTL3 inhibits expression of the tumor suppressor gene SOCS2 through a mechanism involving m6A and the reader YTHDF2 [71]. Moreover, the expression of both VIRMA and WTAP are elevated in HCC **{AuQ: Edit OK?}**, allowing an increase in proliferative capacity of HCC cells that correlates with silencing of the mRNAs ETS1 and ID2 [72]. WTAP is particularly highly expressed in HCC and its expression is inversely correlated to that of the oncosuppressor gene ETS1. Under normal conditions, ETS1 mRNA is recognized by the RNA-binding protein HuR, which stabilizes the mRNA, leading to translation of the protein, which is involved in the proper induction of p21 and p27. Instead, overexpression of WTAP in HCC causes a high rate of m6A modification on ETS1 mRNA, causing instability and activation of degradation machineries [47]. In another study, high expression of METTL3 and high rate of m6A modification on the lncRNA LINC00958 stabilize this transcript and enhance its sponge activity on the miR 3619-5p; this upregulates the expression of hepatoma-derived growth factor (HDGF) and triggers HCC lipogenesis and carcinogenesis [43]. Moreover, the m6A modification in the 3'UTR of EGFR that is recognized by YTHDF2 impairs the stability of EGFR **{AuQ: Edit OK?}** and MEK and ERK pathway.

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Concerning the role of ALKBH5, it has been reported that is protein is downregulated in HCC **{AuQ: Edit OK?}** and is related to a decrease in cell proliferation, invasion abilities, and metastasis formation *in vivo*. Moreover, in HCC, ALKBH5 contributes to the direct post-transcriptional inactivation of LYPD1, an oncoprotein expressed in high-grade cancer [73]. FTO is frequently overexpressed in HCC; however, it has also been reported that the sirt1 protein can induce the deacetylation and stabilization of RANBP2, which in turn can sumoylate and target FTO for degradation. This effect decreases the stability of HCC oncosuppressor gene mRNAs [33].

Gastric cancer

In gastric cancer, upregulation of METTL3 and reduced expression of ALKBH5 and FTO **{AuQ: Edit OK?}** are associated with poor patient prognosis and with advanced tumor stage and grade [74]. Oncogenic upregulation of METTL3 stimulates EMT and metastasis formation, and this modifier acts not only as a methylation writer but also as a reader [75]. In different gastric cancer cell lines, METTL3 knockdown impacts cell proliferation, colony formation, migration, and invasion abilities and induces the apoptotic pathways through BAX and active caspase-3 while decreasing BCL-2 expression [76]. Moreover, METTL3 downregulation reduces AKT phosphorylation and, in turn, p70S6K and cyclinD1 expression. METTL3-induced m6A on zinc finger MYM-type containing 1 (ZMYM1) mRNA by enhances the stability of this transcription factor **{AuQ: Edit OK?}** and promotes the EMT program, cell migration and E-cadherin inhibition [67]. As regards the expression, regulation and stability of non-coding RNA, the lncRNA NEAT1 undergoes m6A modification in gastric cancer [77]. However, ALKBH5 reduces m6A levels by promoting NEAT1 upregulation and the development of a malignant gastric cancer phenotype. The role and regulation of the tumor suppressor BATF2, which acts on ERK phosphorylation, has also been explored; m6A modification on BATF2 mRNA influences its stability and translation. IGF2BP3 directly binds to the m6A site on HDGF mRNA, thereby enhancing its stability and promoting tumor angiogenesis and liver metastasis [67]. Moreover, the preprotein translocator factor SEC62 and the HDGF can be m6A-modified and stabilized, thereby promoting cell proliferation and impairing apoptosis or favoring angiogenesis and metastasis, respectively [74].

Lung cancer

METTL3 is expressed at high levels and promotes cell growth, survival, and invasion by inducing the translation of several mRNAs such as EGFR, YAP and TAZ [75]. In non-small cell lung carcinoma, the sumoylated form of METTL3 is unable to catalyze the deposition of m6A, resulting in the enhancement of carcinogenesis [78]. METTL3 also enhances BRD4 translation by forming an mRNA loop with the translation factor EIF3, suggesting that METTL3 **{AuQ: Edit OK?}** acts as a writer and as a reader [79]. METTL3 depletion impairs tumor development and TGFβ-induced EMT by enhancing the mRNA stability of JUNB, whereas high expression of FTO improves the mRNA stability of ubiquitin-specific peptidase 7 (USP7) and enhances the expression of myeloid zinc finger 1 (MZF1). ALKBH5 affects cell proliferation and invasion by

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reducing m6A of FOXM1 mRNA and promoting its expression [67]. Moreover, YTHDF2, which is upregulated in lung cancer, directly binds the 3' of the 6-phosphogluconate dehydrogenase (6PGD) and SOCS2 transcripts to facilitate m6A modification, mRNA translation and lung cancer development [65]. Depletion of METTL3 inhibits the survival and proliferation of lung cancer cells by increasing BAX and decreasing BCL-2 levels. Moreover, METTL3 silencing decreases the phosphorylation of AKT, thereby affecting cell growth and apoptosis. FTO knockdown inhibits cell proliferation and invasion, and promotes apoptosis, in lung squamous cell carcinoma as its expression promotes the stability of myeloid zinc finger 1 (MZF1) [74].

Breast cancer

Elevated expression of METTL3 correlates with tumor size and TNM stage, and stimulates the expression of HBXIP, conferring aggressiveness upon breast tumor cells [80]. Furthermore, aberrant regulation of HBXIP is able to stimulate METTL3 expression through the negative regulation of let-7g, forming a positive regulatory loop between the two genes [80]. Recently, metformin was shown to decrease m6A levels through downregulation of METTL3 expression mediated by miR-483-3p. METTL3 repression allows the rescue of p21 expression, which is involved in the inhibition of breast cancer cell proliferation through this molecular axis [81]. Moreover, under hypoxic conditions, the expression of ALKBH5 increases, inducing loss of m6A in the mRNA of NANOG and increasing the stability of this mRNA **{AuQ: Edit OK?}** in breast CSCs [82]; by contrast, ALKBH5 knockdown significantly reduces the number of breast CSCs and tumor initiation capacity [65]. Furthermore, ZNF217, a factor capable of sequestering METTL3, inhibits m6A on NANOG, KLF4, and SOX2 mRNAs in breast cancer cells, enhancing the development of CSCs [83]. More recently, high METTL3 levels in breast cancer samples and cell lines have been associated with tumor development, whereas METTL3 silencing decreases cell proliferation rate, accelerates cell apoptosis, and impairs tumor growth *in vivo*. These effects are in part due to a loss of methylation on BCL-2 after METTL3 silencing, which in turn leads to a decrease in BCL-2 expression. FTO is also upregulated in breast cancer tissues, where it mediates m6A demethylation in the 3'UTR of BNIP3 mRNA and induces its degradation.

Pancreatic cancer

The oncogenic microRNA 25-3p is positively regulated during biogenesis by the deposition of m6A on its precursor (pre-miR); the mature form of microRNA 25-3p inhibits PHLPP2, thus promoting AKT-p70S6K signaling [84]. Moreover, the YTHDF2 levels are higher in pancreatic cancer tissues than in normal tissues [85]; YTHDF2 interacts with the AKT–GSK3b–CyclinD1 pathway, and thus stimulates cell proliferation, whereas its knockdown regulates YAP expression, EMT, cell migration, and cell proliferation. Accordingly, WTAP promotes cell migration, invasion, and chemoresistance by stabilizing focal adhesion kinase mRNA and activating the Fak–PI3K–AKT and Fak-Src-GRB2-Erk1/2 pathways [86]. The m6A reader IGF2BP2 is associated with poor prognosis and can stabilize the lncRNA DANCR, resulting in the promotion of cancer cell stemness [67]. Depletion of METTL3 or overexpression of

ALKBH5 in pancreatic cancer cells enhances cell sensitivity to common chemotherapeutic agents such as gemcitabine, 5-fluorouracil, and cisplatin, as well as to radiotherapy approaches [87]. Moreover, after UV exposure, poly (ADP-ribose) polymerase (PARP) recruits METTL3 and METTL14 to DNA damage sites for nucleotide excision repair, DNA damage repair, and cell survival [65].

Colorectal carcinoma

Methylation of SOX2 transcript in CRC cells allows the recognition of IGF2BP2 by m6A readers, preventing the degradation of SOX2 mRNA. METTL3 downregulation activates p-p38 and p-ERK, while METTL3 inhibits the proliferation, migration and invasion of CRC cells via the p38/ERK pathway. METTL3, which is upregulated in metastatic tumors [88], prolongs the half-life of chromobox 8 (CBX8) mRNA and promotes the expression of leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), causing chemoresistance. METTL3 **{AuQ: Edit OK?}** can methylate pri-miR-1246, promoting its maturation, while downregulating the tumor suppressor gene SPRED2 (sprouty related EVH1 domain containing 2). METTL14 is downregulated in CRC and inhibits tumor growth, cell migration and invasion via the miR-375–YAP1 and miR-375–SP1 pathways [67]. Moreover, in this tumor, the expression of the reader YTHDF1 is induced by c-MYC, whereas YTHDF1 silencing impairs the Wnt/β-catenin signaling pathway and tumor growth [39, 88, 89]. METTL3 stimulates lncRNA-RP11, which promotes cell proliferation by upregulating the expression of zinc finger E-Box binding homeobox (ZEB). This lncRNA also associates with ribosomes and promotes the translation of oncogenic mRNAs, such as EGFR and TAZ, promoting cancer growth and invasiveness. YTHDC2 upregulates the expression of metastasis-related proteins such as hypoxia-inducible factor 1a by promoting mRNA translation and thereby metastasis [65].

Urogenital cancers

In ovarian cancer, METTL3 activity is associated with EMT as it stimulates the translation of AXL **{AuQ: Edit OK?}**, a member of the Tyro3-Axl-Mer (TAM) receptor tyrosine kinase subfamily, thereby enhancing cell migration and invasion [90]. Moreover, YTHDF1 increases the m6A-dependent translation of eIF3C and promotes the onset of metastasis. In endometrial tumors, m6A methylation is decreased due to METTL14 mutation or hampered METTL3 expression, resulting in increased cell proliferation and tumorigenicity through activation of AKT pathway (via negative regulation of AKT antagonist PHLPP2 and increased levels of the activator mTORC2) [67].

In cervical cancer, FTO is highly expressed and enhances chemotherapy and irradiation resistance by regulating the expression β-catenin through the reduction of m6A on its transcript, thus increasing the expression of excision repair cross-complementation group 1 (ERCC) [91]. Moreover, FTO interacts with E2F transcription factor 1 and MYC transcripts, enhancing their translation and promoting cell proliferation and migration [67]. In bladder cancer, METTL3 acts as an oncogene and promotes the expression of its direct positive regulator, AFF4, as well as of CDCP1 and MYC **{AuQ: Edit OK?}**. For these reasons, the

inhibition of METTL3 activity could be a valuable strategy to counteract tumor growth [92]. Moreover, METTL3 accelerates the biogenesis of pri-miR-221/222, which antagonizes the expression of PTEN [93], and also induces the translation of ITGA6, favoring cell adhesion, migration, and invasion [74]. In prostate cancer, METTL3 is highly expressed and promotes m6A modification and GLI family zinc finger 1 (GLI1) expression, thus favoring cancer development [67]. In addition, YTHDF2 is upregulated in prostate cancer, whereas its silencing impairs cell proliferation and migration in cells that have high methylation levels. Furthermore, a molecular axis involving miR-493-3p has been identified as a factor acting upstream of YTHDF2 that is crucial cell proliferation and migration inhibition in prostate cancer [94].

Melanoma

Upregulation of METTL3 promotes the invasion and migration of human melanoma cells by enhancing the expression of matrix metallopeptidase 2 (MMP2) [67]. In melanoma cells, FTO allows the upregulation of PD-1, CXCR4, and SOX10 through the suppression of YTHDF2 degradation, and impairs the efficacy of the immune-therapeutic approach [95].

Osteosarcoma

Abnormal deposition of m6A has been associated with cell proliferation, inhibition of differentiation, invasion, and metastasis formation [75]. Upregulation of lncRNA PVT1 is associated with advanced **{AuQ: Edit OK?}** tumor stage and size, and promotes cancer onset and development. PVT1 acts as a competing endogenous RNA (ceRNA), activates STAT signaling, and interacts with MYC [75]. ALKBH5-mediated m6A demethylation has been reported to stabilize, thereby promoting osteosarcoma growth, and relates with poor prognosis, whereas the transcription of PVT1 is not affected in cancer **{AuQ: Edit OK?}** [75]. METTL3 silencing reduces the mRNA level of lymphoid enhancer-binding factor 1 (LEF1) and then inhibits the activity of the WNT/β-catenin signaling pathway [67].

Leukemia

In acute myeloid leukemia (AML), WTAP supports tumor growth and the expression of METTL3 and RBM15 is extremely high [61]. Conversely, METTL3 depletion is related to the induction of apoptosis and cell differentiation while delaying leukemia progression [61]. In apparent contrast to these findings, the demethylase FTO is highly expressed in some AML types and stimulates cell growth, proliferation and viability [96], as well as regulating cell differentiation and apoptosis [97]. At the molecular level, FTO inhibits the expression of ASB2 and RARA genes, both associated with leukemia cell differentiation induced by trans-retinoic acid. Despite the global decrease in m6A levels in cells expressing FTO at high levels, context-dependent enrichment of m6A was observed at distinct genomic loci. METTL3 has also been reported to induce translation of c-MYC, BCL-2, PTEN and SP1 [98]. In the same context, METTL14 induces the translation of MYB and MYC, so enhancing the self-renewal of leukemia stem cells [99].

m6A modification can also negatively regulate the expression of oncogenes, and in some cases, acts as an epigenetic tumor-suppressor mechanism. In endometrial cancer, m6A inhibits the AKT pathway by degrading mTORC2, thereby affecting cell proliferation, anchorage-independent growth, migration, and invasion [100], and by translating PHLPP2, a negative AKT regulator. In breast cancer, m6A positively regulates the expression of NANOG and CSC proliferation [82], whereas high levels of FTO expression induces cell proliferation, migration, invasion and metastasis by inducing BNIP3 degradation. In glioblastoma, high levels of ALKBH5 facilitate expression of the FOXM1 oncogene, which enhances CSC proliferation and tumor development, and in HCC, m6A promotes EGFR degradation through YTHDF2 [101]. Coherently, the overexpression of METTL14 can inhibit cell proliferation and metastasis *in vivo*, as well as migratory and invasive capabilities *in vitro*, through the regulation of microRNA-126 [68]. In accordance with this anti-cancer effect of m6A, in HCC, miR-145 upregulates m6A levels by targeting YTHDF2 and, in turn, suppressing cell proliferation [102]. Also, in CRC, METTL3 may play an anti-oncogenic role by targeting p38/ERK signaling pathways [103], and overexpression of YTHDF1 in CRC cells is associated with a malignant phenotype and with poor prognosis, tumor depth and size [89]. In pancreatic cells, the FTO demethylase is overexpressed *in vivo*, and its knockdown impairs cell proliferation and promotes apoptosis [104]. In gastric cancer, high levels of FTO demethylase are associated with a low grade of cell differentiation, onset of metastasis, and poor patient prognosis [105].

These apparently controversial aspects of m6A activity are likely to be due to a sophisticated scenario. They depend on the specific modified target gene, on the different m6A readers and on the variable expression of these readers **{AuQ: Edit OK?}**, which can act as both negative and positive regulators, enhancing or repressing gene expression, mRNA translation and stabilization. In addition, it is worth mentioning that the complexity of the tumor microenvironment and its heterogeneity can influence tumor cell behavior.

m6A inhibition: a new frontier for cancer treatment

The described role of m6A suggests that epigenetic regulators are crucial for the reversion of misregulated processes. An RNA methylation-targeted system using a Cas13-directed methyltransferase has potential and has been used with encouraging results **{AuQ: Edit OK?}**, but targeting m6A with small molecules is also necessary [106].

When compared with the 'traditional', well-established DNA- or histone-related epigenetic modulators, relatively little is currently known about RNA modifiers, particularly with respect to pharmacological approaches that could be used to inhibit their activity specifically.

From a medicinal chemistry perspective, numerous scientific papers have been published on the erasers FTO and ALKBH5. These two enzymes belong to the 2-oxoglutarate (2OG) and iron-dependent oxygenases (2OGX) [107–109]. Thus, unspecific inhibitors, such as the 2OG competitors N-oxalyl-glycine **1** and fumarate **2**, were the first molecules tested, but are of limited interest because of their apparent lack of selectivity [109, 110].

Rhein **3** is the first FTO inhibitor that, despite possessing two acidic phenolic hydroxyl groups, is neither a 2OG mimic nor a metal chelator but is able to alter m6A methylation levels [111]. A crystal structure is available and compound **3** appears to be selective over 2OG-dependent hydroxylases such as prolyl 4-hydroxylase, HDAC3 histone deacetylase and APOBEC3 DNA deaminases. In a first rough screen, this compound was not cytotoxic and possessed an IC_{50} of approximately 30 µM against FTO [111]; however, successive more accurate measurements resulted in an IC_{50} of 9.0 µM [112]. Nevertheless, it was later revealed that Rhein **3** can also act as an inhibitor of AlkB isoforms, probably by occupying 2OG sites within these enzymes [113].

Next, Aik *et al*. [112] used a differential scanning fluorometry and chromatography-based approach to identify 2OG analogs from a small library of about 150 compounds. They identified and crystallized two compounds, **4** and **5**, that were capable of binding to the active site of FTO and exhibited IC_{50} values of 3.3 and 2.8 µM, respectively [112]. Interestingly, all of the 2OG analogs identified by Aik *et al*. [112] **{AuQ: Edit OK?}** possess a metal chelating group and are confirmed as 2OG competitors. Although not highly selective, these compounds are valuable tools in the development of selective inhibitors that will allow better investigations of FTO-mediated demethylation mechanisms. Indeed, compound **5**, also known as IOX3 in clinical trials as an inhibitor of hypoxia-inducible factor prolyl-hydroxylases (PHDs) (IC_{50} 1.4 µM for PHD2), was identified as an FTO inhibitor [112] and has been studied in more in detail [114]. McMurray *et al*. [114] **{AuQ: Edit OK?}** not only confirmed the IC_{50} value of 2.8 µM but also observed a decrease in FTO expression, as well as that of other 2OG oxygenases **{AuQ: Edit OK?}**, in C2C12 mouse myoblast cells; this effect could not be confirmed in mice. However, the effects of IOX3 treatment on m6A methylation remain vague because the phenotypic effects cannot be clearly attributed to inhibition of FTO and may also be due to inhibition of other 2OG oxygenases, including the PHDs.

Zheng *et al*. [115] developed a series of dihydroxyfuran sulfonamides that are able to inhibit FTO. Their best compounds, **6a** and **6b**, inhibited this enzyme in the µM range (IC_{50} 4.9 and 8.7 µM, respectively), whereas they do not seem to inhibit the related PHDs. Zheng *et al*. [115] also assessed the performance of compound **6b** towards m6A alteration at the cellular level, observing a 9.3% increase at 25 µM in HeLa cells [115]. For the first time, they disclosed the possibility that FTO modulation might be beneficial in an anticonvulsant mouse model, providing the first lines of evidence that their compound **6b** might be beneficial in the treatment of epilepsy. Strong evidence of miRNA involvement in epilepsy has been found [116] and a small molecule that can cross the blood–brain barrier may offer an advantage over the use of siRNAs [117]. These promising effects of **6b** observed in a 6 Hz epilepsy mouse model cannot yet be linked to FTO modulation, because appropriate relative functional assays have not yet been conducted, but they do open the way for additional investigations.

Chlororesorcinol analogs are used as safe hair dyes and have also been found to inhibit FTO. N-CDPCB **7** was discovered in a virtual screening of o-catechol derivatives. This compound **7** was able to increase cellular m6A methylation in 3T3-L1 cells, exhibiting an IC_{50} of 4.95 µM against FTO [118]. Although other chlororesorcinol analogs have been described

after this pivotal study [119], their selectivity over other demethylases has not yet been disclosed.

Meclofenamic acid (MA) **8a** is a known approved non-steroidal anti-inflammatory COX1/2 inhibitor [120]. Interestingly, after being identified in a high-throughput fluorescence polarization screening of older known drugs, this compound was shown to inhibit FTO demethylation selectively (IC_{50} of approximately 8 μM) in a dose-dependent manner in HeLa cells [120]. Crystallographic studies revealed that the presence of a β-hairpin motif as part of the FTO nucleotide recognition lid (NRL) is crucial for the interaction between FTO and **8a**, which occurs via two crucial hydrogen bonds with an amino group in Ser229 and a water molecule bridge with Lys216 [120]. The selectivity of FTO might be explained by the fact that other demethylases such as ALKBH5 lack this motif. Although **8b**, an ester of **8a**, had no observable FTO inhibition **{AuQ: Edit OK?}** activity *in vitro*, the results *in vivo* were much better than those for **8a**, suggesting that the ester is able to penetrate better into the cells, where hydrolysis releases the active MA that is capable of inhibiting FTO [120]. In a subsequent study, **8b** was evaluated in glioblastoma stem cell (GSC)-grafted animals, in which suppression of glioblastoma progression and extension of lifespan were observed, opening a new avenue for the potential treatment of glioblastoma by inhibiting mRNA m6A modification [61].

A subsequent rational design approach guided by crystal structure led to the more potent MA **8a** analogs, FB23 **8c** (IC_{50} 0.06 μM, 140-fold more potent than MA) and its PK-optimized derivative, the hydroxamate derivative FB23-2 **8d** (IC_{50} 2.6 μM), which were shown to inhibit AML cell proliferation and to enhance differentiation in cellular and mouse models [97]. In more detail, **8c** is a highly potent FTO inhibitor that leads to demethylation of m6A RNA *in vitro*. With the aid of crystallography studies, the previously identified compound **8a** with known hydrogen bonds at Ser229 was extended at a dichloro-substituted benzene ring with a bulky five-membered heterocyclic ring. This substitution allowed the compound to occupy a deep pocket within the FTO molecule fully **{AuQ: Edit OK?}**, forming hydrogen bonds with Glu234FTO, a residue that had been **{AuQ: Edit OK?}** previously identified as important for the substrate affinity and specificity for FTO over ALKBH5 [121].

The subsequent transformation of the acid of FB23 **8c** into a hydroxamate resulted in FB23-2 **8d**, which possesses better physiochemical properties. This compound **8d** inhibited cell proliferation not only in a panel of AML cell lines but also in a patient-derived xenograft mouse model at a single-digit micromolar level [121]. Han *et al*. [121] further demonstrated that the effects of their inhibitors were associated with specific downstream targets, such as MYC, CEBPA, RARA and ASB2 RNA transcripts. To sum up, these authors provided proof-of-concept that small molecules might be beneficial for the treatment of AML by regulating the expression of critical genes and signaling pathways through modulation of the m6A methylation levels of mRNA transcripts.

Wang *et al.* [122] discovered a fluorescein derivative, FL6 **9**, as a selective inhibitor of FTO (IC_{50} 6.60 μM). Compound **9** occupies the substrate-binding site and the hydrophobic cavity of FTO in a similar manner to **8a**, and is thus selective for FTO over ALKBH5. As this compound

possesses fluorescent properties, it is a valuable tool in chemical biology, for example, in photolabeling studies [122].

Toh *et al*. [123] used an approach based on crystal structure modeling to identify compound **10** as a potent and highly selective FTO inhibitor that has strong interaction with Glu234FTO in the nucleotide-binding site, an interaction that was confirmed by thermal shift and crystallographic studies [123]. These authors identified a hydrazone similar to compound **10 {AuQ: Edit OK?}** as a '2OG binding component' that is predicted to chelate iron to the active site in FTO, with an appropriate side chain for extension into the nucleotide binding site [123]. Variously substituted pyridyl side chains within the compound **10** series interact strongly with Glu234FTO, allowing specific compounds to occupy the substrate and cofactor binding site **{AuQ: Edit OK?}**. The best compound in the series, **10a**, has an IC_{50} of 0.81 µM against FTO and is 30- to 130-fold selective for FTO **{AuQ: Edit OK?}** over other AlkB subfamilies [123]. Furthermore, it is also inactive against other human 2OG oxygenases, such as PHD2 and JMJD2A [123]. Cell-based assays in HeLa cells indicated that **10b**, the more cell-permeable ethyl ester derivative of **10a**, increased the level of m6A methylation by 19% at 10 µM or by 36% at 50 µM via FTO inhibition [123]. However, further in-depth studies have not yet been conducted.

Another selective FTO inhibitor was discovered via a multiprotein dynamic combinatorial chemistry approach. Interestingly, this compound also possesses a pyridine core. The computational approach led Das *et al*. [124] to the selective inhibitor **11**, which has an IC_{50} of 2.6 µM against FTO. **11** is up to 80-fold selective for FTO over the ALKB family and other structurally related human 2OG oxygenases, such as JMJD2A [124].

Entacapone **12** is a known inhibitor of catechol-O-methyltransferase (COMT), and like MA, provides another example of the repurposing an old approved drug. In this case, entacapone is known for its use in Parkinson's disease along with levodopa. A virtual screening approach on an FDA-approved drug database led to the identification of **12** as an FTO inhibitor with an IC_{50} of 3.5 µM [125]. Compound **12** reduced body weight and lowered fasting blood glucose concentrations in a diet-induced obese mouse model. Peng *et al.* [125] demonstrated that the forkhead box protein O1 (FOXO1) mRNA transcription factor is a direct substrate of FTO. In addition, they showed that entacapone modulates gluconeogenesis and thermogenesis in mice by acting on the FTO–FOXO1 regulatory axis. In the mouse model mentioned above, relatively high doses of **12** were necessary, probably because the compound has a very short half-life, and thus this compound warrants further studies after pharmacokinetic and pharmacodynamic optimization. Besides metabolic issues, the selectivity of this compound for FTO over other demethylases was not disclosed.

To summarize, numerous crystal structures of FTO inhibitors are known, and several interactions that are crucial for the recognition of FTO inhibitors have been described, such as those with Ser229 and Glu234. Building on this rather detailed knowledge, we can expect the development of more potent and selective inhibitors in due course.

To date, little is known about specific ALKBH5 inhibitors. Very recently, however, the imidazobenzoxazin-5-thione MV1035 **13** was identified via a virtual screening approach [126]

using the SPILLO-PBSS screening method on a proteome-wide scale, which allows the identification of off-target interactions [127]. Despite being described as a sodium channel blocker, this compound exhibited (independently from its previous target) promising effects in U87 glioblastoma cells. It was able to act against migration and invasiveness by inhibiting ALKBH5, an RNA demethylase that can be considered an interesting target in fighting glioblastoma aggressiveness. The treatment of U87 glioblastoma cells with MV1035 **13** led to reduced CD73 protein expression. This is an indirect proof of ALKH5 inhibition as CD73 is a downstream target that is known to be overexpressed in several tumors, including glioblastoma [128]. Even though MV1035 **13** was evaluated successfully in several glioblastoma cancer cell lines, with an increased level of N6-methyladenosine (m6A) RNA being observed, no IC_{50} value for ALKBH5 and no data for selectivity for ALKBH5 over other similar enzymes such as FTO were given. Furthermore, the selectivity of MV1035 **13** is questionable because Azambuja *et al*. [129] state that, when their screening method was used, compound **13** probably inhibits the DNA repair protein AlkB homolog 2 (ALKBH2), which was abundantly expressed in GBM cell lines and demonstrated to be responsible for temozolomide resistance [129] **{AuQ: Edit OK?}**. Consequently, further studies on potency and selectivity are necessary for MV1035 **13**.

Just very recently, an ALKBH5-selective inhibitor was shown to modulate lactate and suppressive immune cell accumulation in the tumor microenvironment, being effective even in a mouse model. Unfortunately, the chemical structure and biochemical inhibition data for this inhibitor have not yet been disclosed [130].

As outlined above, METTL3 inhibition could be beneficial in cancer and other diseases. To date, very little has been published in the literature regarding small molecule inhibitors, but there seem to be interesting compounds developed by a university spin-off Storm Therapeutics in the pipeline and close to a phase 1 clinical trial. Apart from a press release (https://www.stormtherapeutics.com/news-events/news/storm-therapeutics-selects-first-in-class-clinical-candidate-targeting-mettl3/), however, no data have been disclosed yet.

Bedi *et al*. [131] described a virtual screening approach on almost 4000 adenosine derivatives as potential METTL3 inhibitors. Their best compound **14**, a SAM mimic, was disclosed as the first small molecule inhibitor of METTL3. The adenosine derivative **14** had an IC_{50} of 8.7 µM in the homogeneous time resolved fluorescence (HTRF) assay and good ligand efficiency (0.24). Although the crystal structure of the **14**–METTL3 complex was also disclosed, the therapeutic potential of this compound remains somewhat limited because of target promiscuity and cell penetration issues. In a pre-print, the same research team revealed a selective and cell-permeable nanomolar inhibitor of METTL3, named UZH1a **15a**, as a first optimization step that provides promising biochemical and cellular data against AML [132]. They used a protein-structure-based optimization technique to develop, synthesize and test UZH1a **15a** (IC_{50} 0.28 µM) and its 100-fold less potent enantiomer UZH1b **15b**. Crystallographic data showed that the inhibitor **15a** fills the pocket of the adenosine moiety of SAM but not the pocket of the SAM methionine with a conformational rearrangement involving Lys513. Pharmacological inhibition of METTL3 with **15a** resulted in the induction of

apoptosis in AML MOLM-13 cells (IC_{50} 11 μM), whereas the survival capacity of osteosarcoma U2OS (IC_{50} 67 μM) and embryonic kidney HEK293T cells (IC_{50} 87 μM) seemed to be less influenced by altered m6A levels. Screening revealed that compound **15a** has no inhibitory effect on several kinases **{AuQ: Edit OK?}**, but that this compound does inhibit other methyltransferases, such as DOT1L, G9a, PRDM9, PRMT1 SETD2, and SMYD3, at a level similar to that of METTL3 inhibition **{AuQ: Edit OK?}**. So, the specificity of UZH1a **15a** still needs to be improved; nevertheless, **15a** can be considered as a good starting point for the development of more specific and potent METTL3 inhibitors.

To the best of our knowledge, small-molecule modulators of m6A readers have not yet been described, but based on the fundamental role of these molecules in pathophysiological cellular events, urgent efforts in this field of investigation appear to be necessary.

Conclusions and perspectives

Interestingly, despite being known since the mid-1970s, m6A epitranscriptomics has received much less attention than the well-known and extensively studied DNA and histone epigenetic modifications. Histone modifications in particular have seen a considerable boost in the development of clinical drug targets, small molecule modulators, and even approved drugs. By contrast, RNA-modifying proteins have received much less attention, and relatively little is known about them yet. Recently, however, interest in studying the most abundant modification on eukaryotic mRNA, the m6A modification, has grown rapidly. Abnormal levels of m6A methylation have often been associated with tumor pathogenesis and progression [133, 134]. However, the precise mechanisms and modes of action through which this methylation mark occurs remain elusive. Our understanding of the complex, dynamic and reversible interplay among m6A readers, writers, and erasers is still in its infancy. Nevertheless, in just a few years, RNA methylation has increased from a small niche topic to a hot area of rapidly evolving active research known as 'RNA epigenetics' or 'epitranscriptomics'.

In the present review, we shed light on the various key players that are involved in m6A methylation. As is the case for the classical players in DNA-related epigenetics, key players in RNA-related epigenetics are classified into readers, writers, and erasers. A heterodimer of METTL3 and METTL14, together with additional subunits, is part of the writing complex. METTL16 is also a writer, methylating stem-loop structured RNAs preferentially. With regard to the erasers, we have focused on FTO and ALKBH5, which demethylate m6A RNAs through an oxidative mechanism. Last but not least, readers such as YTHDF1/2/3, YTHDC1/2, and IGF2BP1−3 recognize m6A-containing RNAs and have been studied extensively. All the enzymes mentioned above are capable of mediating downstream processes, and, as reported in a broad spectrum of recent literature, the misregulation of any of these players and of their downstream regulatory networks may affect RNA metabolism directly or indirectly, resulting in aberrant cellular processes. Many biological questions regarding the mechanism and function of m6A methylation remain unanswered, and a multidisciplinary effort will be necessary to solve these queries. Although pharmacological tools that can specifically inhibit

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METTL3 activity are interesting targets from a medicinal chemist's point of view, such compounds have not been fully developed and optimized. Besides the identification of potential drug leads **{AuQ: Edit OK?}**, the development of selective chemical probes would certainly boost the level of understanding of the complex biology behind RNA methylation.

To date, mainly random compounds with other known activities or even approved drugs have been tested as m6A modifiers. In the main, no straightforward design approach has yet been applied towards m6A modulators. An approach based on the screening of a small fragment library and subsequent medicinal chemistry optimization with the aid of crystallography could help to speed up the drug design process for potent and selective m6A modulators. These m6A modulators are needed because numerous pieces of evidence in recent biological studies have underlined their potential as novel, innovative therapeutic agents. We are still at the beginning of the journey, and in the years to come, we can expect interest in the field of epitranscriptomics, from both biological and therapeutic points of view, to continue to grow rapidly.

Conflict of Interest

The authors declare that the research involved in writing this review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

All of the authors contributed to the composition and revision of this review article.

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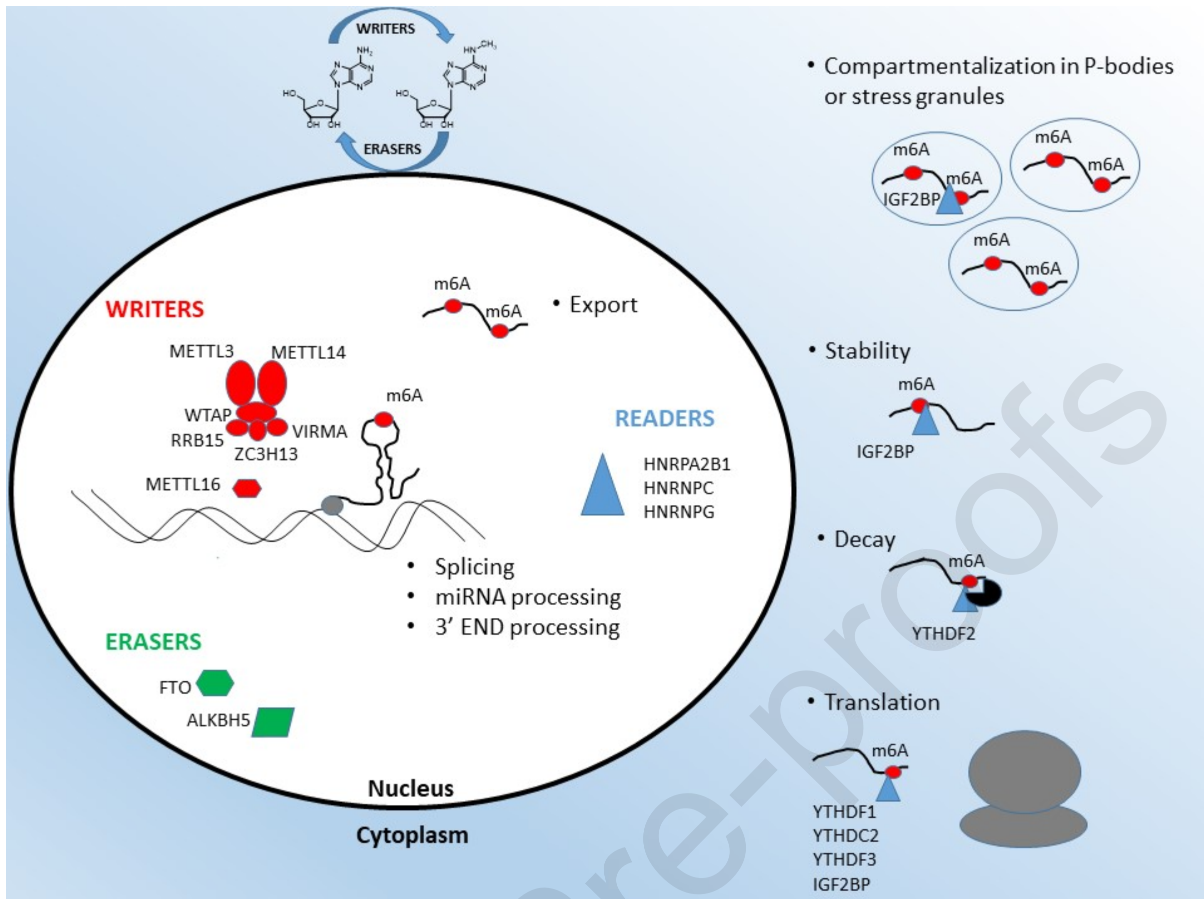

Figure 1. Model of the m6A modification scenario within the cell, showing the molecular players and the involvement of this epigenetic modification in RNA compartmentalization, stability, and translation. m6A writers are shown as red ovals and a red polygon, readers as blue triangles, and erasers as green polygons. m6A modifications are represented as red circles, P-bodies as grey ovals, and the ribosome as a grey complex.

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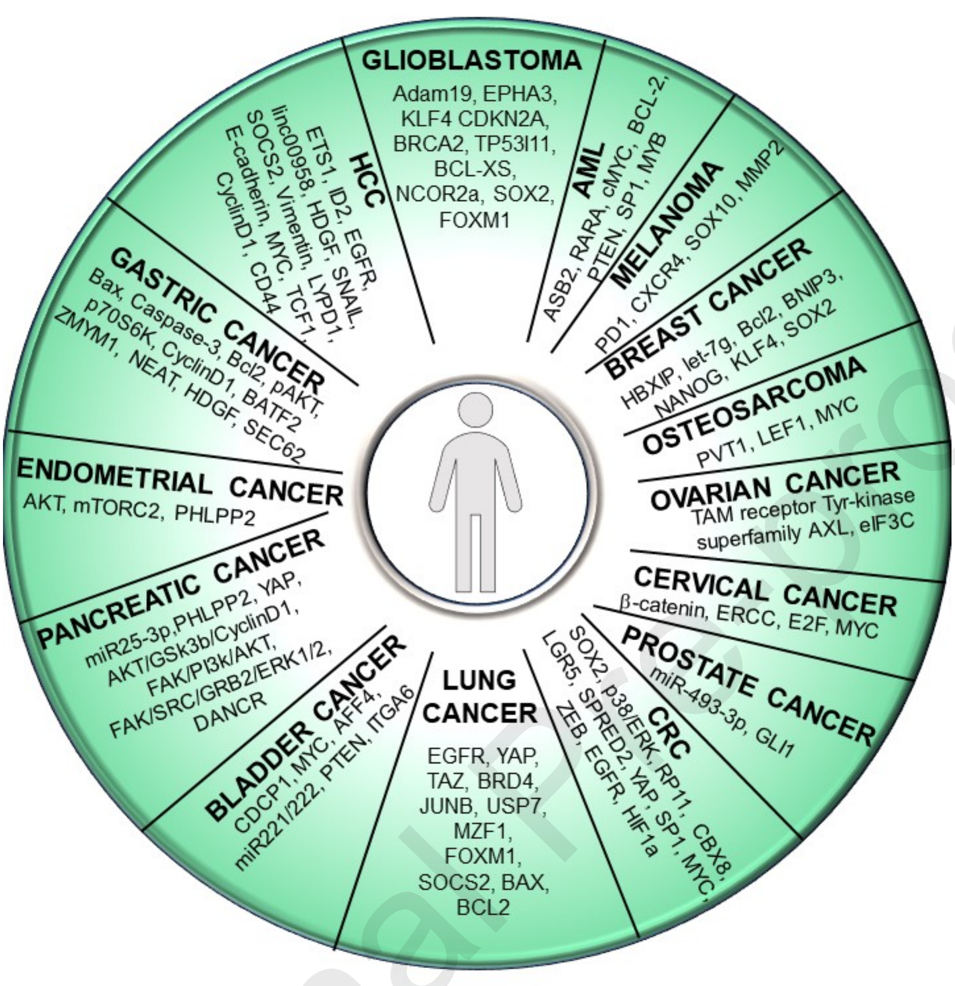

Figure 2. Genes regulated by the addition or removal of the m6A modification in different cancer types. The genes that are modulated in each cancer type are listed.

Table 1. Overview of the m6A modulators described in the text.

Molecular structure	Targets	Reference(s)
HO–C(=O)–C(=O)–NH–CH$_2$–C(=O)–OH **1**	Unspecific (FTO, ALKH5)	[109, 110]

2	Unspecific (FTO, ALKH5)	[109, 110]
3	FTO (IC_{50} = 9.0 μM), other ALK enzymes	[111, 112]
4	FTO (IC_{50} = 3.3 μM)	[112]
5	FTO (IC_{50} = 2.8 μM)	[112]
6a R^1= $-CH_3$ **6b** R^1= $-CH_2CH_3$	FTO (**6a** IC_{50} = 4.9 μM, **6b** IC_{50} = 8.7 μM)	[115]
7	FTO (IC_{50} = 4.95 μM)	[118]
8a R^1= -H, R^2= -H **8b** R^1= $-CH_2CH_3$, R^2= -H **8c** R^1= -H, R^2= **8d** R^1= -NHOH, R^2=	FTO (**8a** IC_{50} = 8 μM, **8b** inactive, **8c** IC_{50} = 0.06 μM, **8d** IC_{50} = 2.6 μM)	[120]

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9	FTO (IC$_{50}$ = 6.60 µM)	[122]
10a R^{1}= -CH_3 **10b** R^{1}= -CH_2CH_3	FTO (**10a** IC$_{50}$ = 0.81 µM, **10b** IC$_{50}$ not given)	[123]
11	FTO (IC$_{50}$ = 2.6 µM)	[124]
12	FTO (IC$_{50}$ = 3.5 µM)	[125]
13	ALKH5 (IC$_{50}$ not given)	[126–128]
14	METTL3 (IC$_{50}$ = 8.7 µM)	[131]
15a (R), **15b** (S)	METTL3 (**15a** IC$_{50}$ = 0.28 µM, **15b** IC$_{50}$ = 28 µM)	[132]

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