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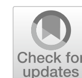
REVIEW



m6A RNA Methylation: Ramifications for Gene Expression and Human Health

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Abstract
Cellular transcriptomes are frequently adorned by a variety of chemical modification marks, which in turn have a profound influence on its functioning. Of these modifications, the one which has invited a lot of attention in the recent years is m6A RNA methylation, leading to the development of RNA epigenetics or epitranscriptomics as a frontier research area. m6A RNA methylation is one of the most abundant reversible internal modification seen in cellular RNAs. Studies in the last few years have not only shed light on the molecular machinery involved in m6A RNA methylation but also on the impact of this modification in regulating gene expression and hence biological processes. In this review, we will emphasize the biological impact of this modification in normal organismal development and diseases.

Keywords Transcriptome · Gene regulation · Reversible RNA modifications · m6A RNA methylation

Introduction

The flow of genetic information in biological systems from DNA to RNA to protein is explained by the central dogma of molecular biology, which forms the backbone of modern biology [1]. This flow of genetic information is with checks and balances and is often subjected to a wide variety of regulatory controls operational at multiple levels. Adding to this regulatory complexity is the reversible chemical modification of DNA and nucleosomal histones, which are collectively termed as epigenetic marks or signatures. One such well-studied epigenetic modification is DNA methylation, wherein the enzyme DNA methyl transferase adds the methyl group to cytosine in the CpG islands of DNA sequence, leading to transcriptional silencing [2]. The loss of DNA methylation, i.e., hypomethylation has been reported to promote tumorigenicity in various cancer types [3, 4]. Aside from DNA, the tails of nucleosomal histone proteins, which are essential for chromatin formation, are often subjected to numerous chemical modifications like acetylation, deacetylation, methylation, phosphorylation and ubiquitination [5]. These modifications are catalyzed by specific enzymes, for example, histone acetyl transferase and deacetylase, histone methyl transferase and demethylase facilitate acetylation, deacetylation, methylation and demethylation of histones respectively. Acetylation of histones 3 and 4 at specific amino acid residues, activate transcription by loosening up the chromatin, whereas methylation of histones 3 and 4 at specific positions can activate transcription in certain instances and repress in others [6].

Cellular RNAs, coding as well as non-coding, since their birth by transcription, have been known to be subjected to > 100 different chemical modifications, most irreversible or static in nature, such as 5′ cap, pseudouridine, 5-hydroxymethylcytosine, 5-methylcytosine to name a few, which often impacts their structure as well as function [7]. Intriguingly about two-thirds of these chemical modifications involve addition of methyl group and the structure and topology of the most common methylated nucleosides reported in eukaryotic messenger RNAs (mRNA) are illustrated in Fig. 1. The list includes N7-methylguanosine (m7G), which aids in translation, splicing, nuclear export and prevents degradation; 2′-O-methylation (Nm), which modulates translation efficiency; 5-methyl cytosine (m5C), which regulates translation and nuclear export; N1-methyladenosine (m1A), which impacts translation; N6-methyladenosine (m6A),

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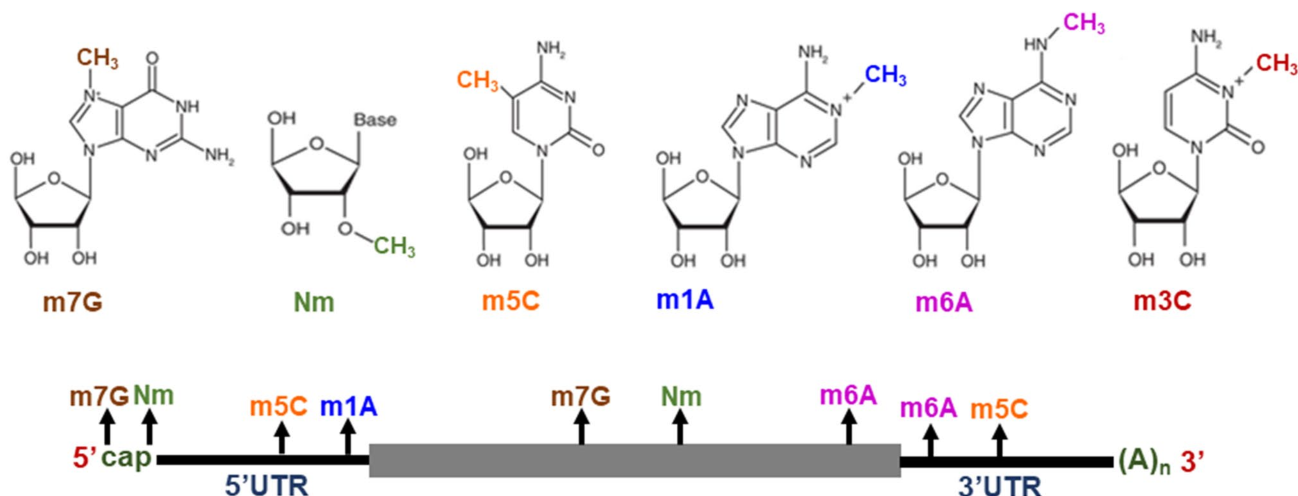

Fig. 1 Spectrum of methylated nucleosides in eukaryotic messenger RNA. Top panel depicts the chemical structure of the various methylated nucleosides. Bottom panel highlights the distribution of the methylated nucleosides to the mRNA cap structure, 5′ or 3′ untranslated region (UTR) or the coding region of mRNA. m7G (7-methylguanosine); Nm (2′-O-methylation); m5C (5-methylcytosine); m1A (N1-methyladenosine); m6A (N6-methyladenosine); m3C (3-methylcytidine)

which is critical for various aspects of mRNA metabolism; and 3-methylcytidine (m3C), whose function is yet to be uncovered [8]. Of these methylation events, the one that has attracted immense attention and is the focus of this review is the chemical modification termed as 'N6-methyladenosine' or 'm6A', as it adds a methyl group to the nitrogenous base at the sixth position of the adenine residue of the RNA. m6A RNA methylation has emerged as one of the most abundant internal modification of RNAs and was first discovered in eukaryotic messenger RNAs in Novikoff hepatoma cells [9] and mouse L cells [10]. However, the precise function of m6A mark remained elusive and it was just regarded as any other post transcriptional modification, presumed to have a role in RNA processing. The dynamic and reversible nature of this RNA modification came to the fore, when a demethylating enzyme, FTO (fat mass and obesity-associated protein) which removes the methyl group from RNA was reported [11]. The reversibility of m6A mark, unlike other previously known modifications, which were irreversible in nature, gives it an added flexibility desired for regulating gene expression. Not only in messenger RNAs, m6A has been reported to be present in all classes of cellular RNAs, be it ribosomal RNAs, transfer RNAs and various non-coding RNAs like microRNAs, long non-coding RNAs, circular RNAs [12–15]. m6A RNA methylation has emerged as a key regulator of various post transcriptional gene regulatory processes like RNA splicing, stability, export, degradation and also as a signal for translational initiation machinery to act upon it to start protein synthesis [16]. Various reports also have shown the importance of this reversible RNA methylation in development and as well diseases like cancer, diabetes [17–20]. m6A modification is evolutionarily conserved, but only adenosine residues present in the consensus motif, RRACH (where R = G/A, H = A/C/U, underlined letter stands for adenosine being modified to m6A) are methylated [21]. Topologically, this modification is predominantly clustered in regions near 3′-UTR, stop codons [22, 23], long internal exons and 5′-UTR [24]. It was also observed in introns [25] and are added to nascent pre-mRNA associated with chromatin for their cytoplasmic turnover rate [26].

The in-depth study of m6A pathway was revolutionized in 2012 by two research groups in parallel, wherein they developed technique for mapping and detecting m6A methylome, referred to as m6A-seq or MeRIP-seq, i.e., immunoprecipitation of RNAs with m6A mark using an anti-m6A antibody, followed by next-generation sequencing [22, 27, 28]. This was further facilitated by development of methods such as PA-m6A-SEQ (photo-crosslinking-assisted m6A-sequencing) [29], SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography) [30] and LC–MS/MS (liquid chromatography linked to tandem mass spectrometry) [31]. In addition, bioinformatics tools such as SRAMP [32], TargetM6A [33], RNA-methylPred [34], iRNA-methyl [35] and pRNAm-PC [36] have been helpful in predicting m6A sites. Taken together these technological advancements for detection and prediction of m6A sites have made a huge impact on the nascent field of RNA epigenetics or epitranscriptomics [37–39].

A multi-subunit methyl transferase complex, i.e., m6A 'writers', adds the methyl group to adenosine; demethylases, i.e., m6A 'erasers' aids in the removal of methyl group and specific RNA binding proteins, i.e., m6A 'readers' recognize the m6A deposits and bind to the m6A methylated RNA and

modulate specific downstream functions. In this review, we attempt to summarize the key players involved in m6A RNA methylation pathway and their implications in human health.

m6A 'Writers'

m6A 'writers' are complex of methyl transferases, which install the m6A mark onto specific sites on RNA (Fig. 2). Using a combination of labeling and chromatographic techniques, the presence of internal methylated adenosines in eukaryotic messenger RNAs was first demonstrated [9, 24]. Following this, employing in vitro methylation together with mutational studies, the consensus motif associated with m6A deposition was identified to be 'RRACH' (where R = G/A, H = A/C/U, underlined letter stands for adenosine being modified to m6A) motif and the frequency of deposition was ~ 1 to 3 methyl group per mRNA molecule, primarily in the 3′ end of mRNA [21, 40–42]. The first indication for the existence of a RNA methyl transferase in nucleus was provided by in vitro methylation of prolactin mRNA upon incubation with HeLa nuclear extract in the presence of S-adenosyl methionine (SAM) [41]. However, the signals which regulate the deposition of m6A were not fully understood till 2018, when Bertero et al., for the first time showed that TGFβ can modulate m6A dynamics in human stem cells [43]. In another work published in 2017, R2 hydroxyglutarate (R2HG), an oncometabolite having anti-tumor activity in certain leukemia and gliomas, inhibits the demethylase, FTO, leading to increased m6A levels and decrease in stability of CEBPA and MYC transcripts [44]. Taken together, these reports, shed some light on the external and internal cues regulating the dynamics and specificity of m6A methylation.

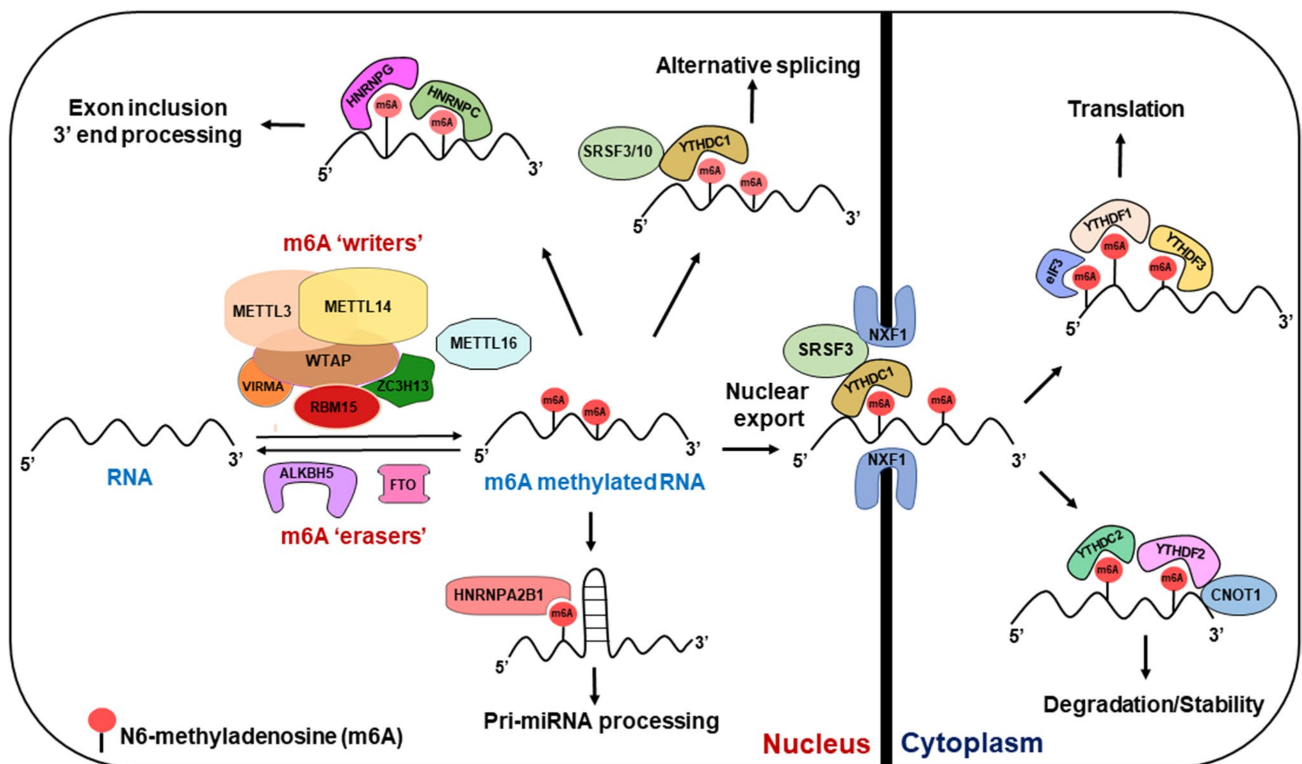

Fig. 2 Schematic representation of reversible m6A RNA methylation and m6A-mediated regulation of gene expression. m6A 'writer' complex or the methyl transferase complex is composed of METTL3/METTL14/WTAP along with other auxiliary proteins such as VIRMA, ZC3H13 and RBM15; facilitates the deposition of m6A methyl mark on cellular RNAs in the nucleus; the writer METTL16 is a specific methyl transferase for U6 small nuclear RNA. m6A 'readers' or m6A binding proteins consist primarily of proteins such as YTHDF1-3, YTHDC2 and eIF3, which are cytoplasmic readers, and HNRNPA2B1, HNRNPC, HNRNPG and YTHDC1, which are nuclear readers. m6A readers binds to the target RNAs in a m6A-dependent manner and determine the downstream fate of the transcripts by regulating various steps of RNA metabolism such as splicing, 3′-end processing, primary microRNA (pri-miRNA) processing, nuclear export, degradation, stability and translation. m6A readers act in conjunction with specific proteins to elicit their function, for example SRSF3 facilitates the nuclear export by serving as an adaptor between the export protein NXF1 and reader YTHDC1; SRFSF3/10 facilitate m6A-dependent splicing functions of the reader YTHDC1. m6A 'erasers' or demethylase consist of FTO and ALKBH5, both of which oxidatively remove the m6A signatures from the transcripts in the nucleus, thus making the process reversible

Methyl Transferase like 3 (METTL3)

Bokar et al. uncovered different components of the SAM-dependent methyl transferase complex (MTase complex), using classical biochemical techniques and named them as MT-A1 (30 kDa), MT-A2 (200 kDa) and MT-B (875 kDa) [42]. The 200 kDa fragment of the complex, now termed as methyl transferase like 3 (METTL3) has a SAM-binding site and is essential for m6A methylation activity [42, 45]. METTL3 functions as heterodimer with methyl transferase like 14 (METT14), and exists in a multi-subunit complex with other auxiliary factors like Wilm's tumor associated protein (WTAP). METTL3 via its impact on m6A deposition and hence RNA homeostasis, has been reported to influence normal organismal development and several human diseases like cancer (Table 1).

m6A deposition often serves as molecular switches, referred to as 'm6A switch', by facilitating the binding of specific RNA binding proteins and such interactions are important for cellular RNA transactions [46, 47]. During development, METTL3 and METTL14 were implicated in regulating cell fate transition in embryonic stem cells [48, 49] and their knockdown led to embryonic lethality in mice [50]. METTL3 also played role in regulation of osteogenic differentiation [51–53] and may be a potential therapeutic target in reversing osteoporosis condition [54]. Interestingly, it was also reported to enhance long term memory in mice [55] and mediate T-cell homeostasis [56]. METTL3 was also shown to have role in autophagy of cardiomyocytes, wherein the enzymes with opposing activities such as methyl transferase, METTL3 and demethylase, ALKBH5 were demonstrated to regulate autophagy via TFEB, which in turn regulated the expression of both METTL3 and ALKBH5 [57]. Inhibition of m6A methylation either by knockdown of METTL3 or depletion of consensus sequences had an impact on the expression level of circadian clock genes [58, 59]. Furthermore, METTL3 also promotes processing of primary microRNA in a m6A-dependent manner and its depletion shows global reduction in mature microRNAs [60]. METTL3 also facilitates translation of mRNA in a m6A-dependent manner and promotes a variety of cancer types [61–66]. Not only linear RNAs, METTL3-dependent m6A marks were found extensively in a subset of circular RNAs and were reported to promote translation initiation of the circular RNAs [14, 15].

Methyl Transferase like 14 (METTL14)

Results on structure-based computational studies from two different groups revealed the existence of methyl transferase like 14 (METTL14), an additional m6A methyl transferase bearing significant sequence and structural homology with METTL3; however, the functions of METTL14 remained elusive [67, 68]. Study on m6A RNA methylation employing mouse embryonic stem cells demonstrated that m6A writers function as a multi-subunit complex with METTL14 and METTL3 acting in tandem as heterodimers to deposit m6A on target genes and knocking down both, led to derailment of m6A methylation in mRNA transcripts and loss of self-renewal ability [49]. The interaction between METTL3 and METTL14 was further reinforced by crystal structure-based analysis, whereby METTL14 was shown to be required for binding of METTL3 to its RNA substrate [69, 70]. Further to this, Schöller et al., in 2018, provided insight into the overall architecture of this heterodimer complex and their interactions and requirement of splicing regulator WTAP (Wilm's tumor-1 associated protein) for their nuclear localization. This work also shed some light on function of METTL14, particularly the role of carboxyl terminus RGG domain [71]. In higher eukaryotes, the existence of homologs of METTL14 and other proteins involved in mammalian m6A RNA methylation showcased their evolutionary and functional significance [72].

METTL14 impacts developmental processes and a plethora of human diseases (Table 1). The deletion of METTL14 in mouse highlighted its role in early embryonic development [73] and spermatogenesis [74]. Neuron-specific deletion of METTL14 in adult mice brain had a profound effect on its learning ability, which gave further validation to the importance of the m6A mRNA modification during embryonic and postnatal development [75]. METTL14 deletion in neuron stem cell (NSC) in a mouse model displayed severe proliferation and differentiation changes affecting normal neuronal development [76]. Aberrant regulation of METTL14 was implicated in tumor metastasis in hepatocellular carcinoma [77] and malignant hematopoiesis [78]. Strikingly, a cross-talk between histone modification and m6A RNA methylation was revealed in 2019 by Huang et al., where it was reported that histone H3 trimethylation at lysine 36, a mark for transcriptional elongation, directly binds to METTL14 and guides the methyl transferase complex to nascent transcripts for m6A deposition [79].

Table 1 Role of m6A writers, readers and erasers in human diseases

S no.	m6A player	Name of gene	Disease	Expression pattern	Function in disease	References
1	Writer	METTL3	Gastric cancer	–	Inhibits proliferation, invasion and migration of gastric cancer cells and activates apoptosis pathway	[162]
2	Writer	METTL3	Liver cancer	Upregulated	Downregulates expression of SOCS2 (Suppressor of Cytokine Signaling 2) via reader YTHDF2 and marks it for degradation	[65]
3	Writer	METTL3	Bladder cancer	Upregulated	Upregulates the expression of genes MYC, AFF4, RELA, IKBKB and promotes proliferation and invasion	[163]
4	Writer	METTL3	Bladder cancer	Upregulated	Enhances the maturation of pre-mir-221/222 which in turn facilitates the reduction of PTEN (Phosphatase and tensin homolog), thus promoting proliferation	[164]
5	Writer	METTL3	Melanoma	Upregulated	Upregulates the expression of MMP2 (matrix metallopeptidase2) and promotes cell invasion	[165]
6	Writer	METTL3	Glioma	Upregulated	Upregulates the expression of genes involved in maintenance of Glioma Stem Cells and tumorigenesis	[166, 167]
7	Writer	METTL3	Acute myeloid leukemia	Upregulated	Essential for AML growth and promotes translation of target transcripts such as c-Myc, BCL2 (B Cell lymphoma -2) and PTEN (Phosphatase and tensin homolog) mRNA	[61, 64]
8	Writer	METTL3	Lung cancer	Upregulated	Promotes translation of oncogenic mRNAs such as EGFR (epidermal growth factor receptor) and the Hippo pathway effector TAZ	[62, 63]
9	Writer	METTL3	Ovarian cancer	Upregulated	Promotes Epithelial- mesenchymal transition (EMT) and translation of receptor tyrosine kinase AXL	[168]
10	Writer	METTL3	Endometrial cancer	Downregulated	Promotes cancer growth by activating Akt signaling pathway	[169]
11	Writer	METTL3	Pancreatic cancer	–	Promotes resistance against chemo/radio therapy in pancreatic cancer by altering splicing regulator and ubiquitination-related protein expression	[170]
12	Writer	METTL3	Renal cell carcinoma	Downregulated	Regulates cell proliferation and cell invasion	[171]
13	Writer	METTL3	Osteoporosis	–	Regulates the expression of PTH (parathyroid hormone) and Pth1r (parathyroid hormone receptor-1)	[66]
14	Writer	METTL3	Gastric cancer	Upregulated	Promotes metastasis	[172]
15	Writer	METTL3	Hepatoblastoma	Upregulated	Promotes disease progression via CTNNB1 (Catenin Beta 1) expression	[173]
16	Writer	METTL3	Prostate cancer	Upregulated	Promotes growth and invasion by regulating hedgehog pathway	[174]
17	Writer	METTL3	Rheumatoid arthritis	Upregulated	Biomarker in Rheumatoid arthritis patients	[175]

Table 1 (continued)

S no.	m6A player	Name of gene	Disease	Expression pattern	Function in disease	References
18	Writer	METTL3	Type 2 diabetes	Upregulated	Regulates the expression of Fasn (Fatty acid synthase) mRNA and promotes fatty acid metabolism	[176]
19	Writer	METTL3	Osteosarcoma	Upregulated	Promotes cell progression through Wnt-pathway by regulating LEF1 (lymphoid enhancer-binding factor 1) expression	[177]
20	Writer	METTL3	Non-small-cell lung carcinoma	Upregulated	Promotes metastasis and drug resistance through YAP (yes-associated protein 1) translation via regulating MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) activity	[178]
21	Writer	METTL3	Oral cell carcinoma	Upregulated	Promotes tumorigenesis by accelerating c-MYC stability	[179]
22	Writer	METTL3	Bladder cancer	Upregulated	Promotes proliferation and migration via binding of m6A reader YTHDF2, degrades tumor-suppressor genes such as SETD7 (SET domain-Containing 7), and KLF4 (Kruppel-Like Factor 4)	[180]
23	Writer	METTL3	Colorectal cancer	Downregulated	METTL3 knockdown promotes growth and migration; overexpression suppressed growth via p38/MAPK pathway	[181]
24	Writer	METTL3	Colorectal cancer	Upregulated	METTL3 promotes cancer progression by stabilizing CCNE1 (Cyclin E1) expression	[182]
25	Writer	METTL3	Colorectal carcinoma	Upregulated	Regulates SOX2 (SRY-Box Transcription Factor 2) expression via m6A reader protein IGF2BP2 and prevents degradation and promotes self-renewal, progression	[183]
26	Writer	METTL3	Hand-foot-and-mouth disease	Upregulated	Interacts with RNA-dependent RNA *polymerase* (RdRp3D) and stabilizes its expression and facilitate enterovirus type 71 viral replication	[184]
27	Writer	METTL3	Lung cancer	–	Promotes migration and invasion of liver cancer cells	[185]
28	Writer	METTL3	Infectious mononucleosis	–	Have a role in expression of genes involved during lytic cycle of Kaposi's sarcoma-associated herpesvirus virus	[186]
29	Writer	METTL3	Acquired Immuno-deficiency syndrome	–	Suppresses viral replication in T-cell and regulates the interaction between rev protein and RRE (Rev response element) RNA	[187]
30	Writer	METTL3	Lung cancer	–	Suppresses Influenza A virus replication	[188]
31	Writer	METTL3	Hepatoblastoma	–	Modulates hepatitis B virus (HBV) gene expression via RNA intermediate pregenomic RNA (pgRNA)	[189]
32	Writer	METTL3	Zika fever	–	Negatively regulates Zika viral infection	[190]
33	Writer	METTL14	Zika fever	–	Negatively regulates Zika viral infection	[190]

Table 1 (continued)

S no.	m6A player	Name of gene	Disease	Expression pattern	Function in disease	References
34	Writer	METTL14	Hepatoblastoma	–	Modulates hepatitis B virus gene expression via RNA intermediate pregenomic RNA	[189]
35	Writer	METTL14	Acquired Immuno-deficiency syndrome	–	Suppresses HIV-1 replication in T-cell and regulates the interaction between rev protein and Rev response element (RRE) RNA	[187]
36	Writer	METTL14	Endometrial cancer	Mutated	Promotes cancer growth by activating Akt signaling pathway	[169]
37	Writer	METTL14	Acute myeloid leukemia	Upregulated	Promotes cell survival and maintenance by positively regulating expression of MYB and MYC	[78]
38	Writer	METTL14	Colorectal cancer	Downregulated	METTL14 regulates growth and migration via miR-375/Yes-associated protein 1 (YAP1) pathway and miR-375/SP1 pathway	[191]
39	Writer	METTL14	Hand-foot-and-mouth disease	Upregulated	Interacts with RNA-dependent RNA *polymerase* (RdRp3D) and stabilizes its expression and facilitate enterovirus type 71 viral replication	[184]
40	Writer	WTAP	Cholangio-carcinoma cells	Upregulated	Involved in metastasis	[192]
41	Writer	WTAP	Glioma	Upregulated	Related with poor prognosis in patients	[193]
42	Reader	IGF2BP	Cervical cancer Liver cancer	Upregulated	Stabilizes the expression of oncogenic gene MYC and promotes cell proliferation and invasion	[194]
43	Reader	IGF2BP1	Ovarian cancer Liver cancer	Upregulated	Involved in the stability of SRF and promotes cancer	[195]
44	Reader	IGF2BP2	Colorectal carcinoma	Upregulated	Regulates SOX2 expression via m6A reader protein IGF2BP2; prevents its degradation and promotes self-renewal	[183]
45	Reader	YTHDF1	Hepatocellular carcinoma	Upregulated	Involved in progression of cancer	[196]
46	Reader	YTHDF1	Colorectal cancer	Upregulated	Promotes proliferation of cancer cells	[197]
47	Reader	YTHDF2	Prostate cancer	Upregulated	Promotes proliferation and migration of cancer cells	[108]
48	Reader	YTHDF2	Infectious mononucleosis	Upregulated	Have a role in expression of genes involved during lytic cycle of Epstein-Barr virus	[186]
49	Reader	YTHDF2	Bladder cancer	Upregulated	Promotes proliferation and migration via binding of m6A reader YTHDF2, degrades tumor-suppressor genes such as SETD7 (SET Domain-Containing 7) and KLF4 (Kruppel-Like Factor 4)	[180]
50	Reader	YTHDF1–3 YTHDC1	Hand-foot-and-mouth disease	Upregulated	Promotes Enterovirus-71 replication	[184]
51	Reader	YTHCD2	Colon cancer	Upregulated	Promotes the translation of HIF1α (Hypoxia-inducible factor *1-alpha*) and is involved in metastasis	[118]

Table 1 (continued)

S no.	m6A player	Name of gene	Disease	Expression pattern	Function in disease	References
52	Eraser	FTO	Acute promyelocytic leukemia	Upregulated	Promotes leukemogenesis by downregulating the expression of ASB2 (Ankyrin Repeat and SOCS Box Containing 2) and RARA (Retinoic Acid Receptor Alpha)	[198]
53	Eraser	FTO	Lung squamous cell carcinoma	Upregulated	Promotes the expression of transcription factor MZF1 (Myeloid Zinc Finger 1); promotes proliferation and metastasis	[199]
54	Eraser	FTO	Cervical squamous cell carcinoma	Upregulated	Confers chemo and radio therapy resistance by regulating the expression of β-catenin gene	[200]
55	Eraser	FTO	Pancreatic cancer	Upregulated	Regulates the expression of c-MYC and is necessary for the proliferation of the cancer cells	[201]
56	Eraser	FTO	Renal cell carcinoma	Downregulated	Have anti-tumorigenic role by stabilizing the expression of PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and suppressing tumor growth	[202]
57	Eraser	FTO	Heart failure	Downregulated	Leads to degradation of contractile proteins and disrupts contractile function of heart	[150]
58	Eraser	FTO	Breast cancer	Upregulated	Reduces the expression of BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) pro-apoptosis gene; involved in cancer cell growth	[203]
59	Eraser	FTO	Premature ovarian insufficiency syndrome	Downregulated	Role in infertility and can serve as a potential biomarker for Premature ovarian insufficiency syndrome	[18]
60	Eraser	FTO	Type 2 diabetes	Upregulated	Potential biomarker for type 2 diabetes	[17]
61	Eraser	ALKBH5	Breast cancer	Upregulated	Promotes the expression of NANOG (*Nanog* Homeobox), KLF4 (Kruppel-Like Factor 4) and maintains the pluripotency of cancer stem cells	[159, 160]
62	Eraser	ALKBH5	Glioma	Upregulated	Regulates the expression of transcription factor FOXM1 (Forkhead Box M1) and essential for proliferation and tumorigenesis	[204]

Wilm's Tumor-1 Associated Protein (WTAP) and Other Writers

Wilm's tumor-1 associated protein (WTAP) first came into picture as an interaction partner of Wilm's tumor-1 (WT1) protein [80]. WTAP was first identified as a regulatory subunit of m6A methyl transferase complex and its knockdown inhibited methyl transferase activity of METTL3-METTL14 complex demonstrating the importance of WTAP in m6A installation [81].

Extensive work done to probe the protein–protein interactions, led to the discovery of other protein partners involved in this dynamic modification, like KIAA1429 or VIRMA (Vir like m6A methyl transferase associated), which is the human homolog of *Drosophila's* Virilizer protein) [82, 83], RBM15 (RNA binding protein 15) [84] which interact with methyl transferase complex via WTAP. Furthermore, RBM15, interacts with BAF155 subunit of chromatin remodeling complex BAF and recruits m6A methyl transferase complex to BAF155 to modulate its stability and regulate cortical development [85]. RBM15 is also critical for deposition of m6A by METTL3 on X-inactive specific transcript (XIST), which promotes XIST-mediated transcriptional repression [84]. Another

component of the complex KIAA0853 or ZC3H13, is a zinc finger protein, which was identified in mouse embryonic stem cell and functions as a recruiter protein facilitating localization of methyl transferase complex to nucleus and its ablation led to loss of self-renewing ability of the mouse embryonic stem cells due to failure of the methyl transferase complex to reach the nucleus [86]. WTAP and other m6A writers, too, have a role in various human pathologies (Table 1).

Methyl Transferase like 16 (METTL16)

Aside from METTL3-METTL14-WTAP m6A writer complex, mammalian cell bear additional methyl transferase—METTL16, which is involved in m6A deposition on a limited subset of RNAs and U6 small nuclear RNA [87]. Interestingly, METTL16, modulates SAM homeostasis by depositing m6A in 3′-UTR of SAM synthetase (MAT2A) transcript and regulates its expression through altered splicing and stability [88]. Using crosslinking studies, m6A methylation targets of METTL16 like long noncoding RNA, U6 snRNA and mRNAs were deciphered [89]. In contrast to METTL3-METTL14 heterodimer complex, METTL16 works as a homodimer [90] and mouse embryo lacking METTL16 showed severe deregulation in transcriptomes and embryonic lethality [91].

m6A 'Readers'

Gene regulatory impact of m6A deposition are mediated by specific proteins termed as m6A 'readers', which read the m6A signature in the RNA transcript and elicit the downstream response and fate of the transcript either in nucleus or cytoplasm (Fig. 1). Dominissini et al. presented the first evidence for the existence of RNA binding proteins that recognize and bind to the m6A mark in RNAs [27]. A majority of the mammalian m6A 'readers' bear the YTH (YT521-B homology) domain, which has an aromatic cage for specifically accommodating the methyl mark. Aside from this, numerous other proteins, which lack the YTH domain, can also function as m6A 'readers' like IGF2BP, HNRNP, eIF3 to name a few. Facilitated by structural studies, YTH family of proteins were suggested to bind to m6A signature [92] with a preferential binding to the consensus sequence, GG(m^6A)C [93]. Like m6A writers, most of the reader proteins show an aberrant expression pattern in a variety of human diseases and have a role in disease progression and facilitation (Table 1).

YTH Domain-Containing Family Protein 1 (YTHDF1)

The cytoplasmic m6A 'reader' protein, YTHDF1, binds to m6A marks on transcripts and promotes protein synthesis through its interaction with the translation initiation machinery, particularly the translation initiation factor, eIF3 [94]. YTHDF1 was reported to regulate translation of several m6A marked neuronal transcripts like ROBO3.1, which helps in axon guidance during neuronal development [95]. Furthermore, it also enabled the translation of certain target transcripts in response to neuronal excitation in mouse brain's hippocampal region and thus influencing learning and memory processes [96]. Notably, pluripotency of induced pluripotent stem cells (iPSCs) was regulated in a m6A-dependent manner by YTHDF1, via binding and promoting translation of JAK2 transcript, to maintain the stemness [97].

YTH Domain-Containing Family Protein 2 (YTHDF2)

Chuan He's lab first shed light on the functional relevance of the cytoplasmic reader protein, YTHDF2, highlighting its role as a regulator of mRNA stability by promoting degradation of m6A marked transcripts and reported its colocalization with decay proteins like DCP1A, GW182 and DDX6 in P-bodies [98]. Furthermore, YTHDF2 interacts with CNOT1 leading to the recruitment of CCR4-NOT complex and subsequent deadenylation and degradation of m6A marked transcripts [99]. Structural studies have further illuminated the binding of this reader protein to methylated adenosine on its target RNAs [100, 101]. YTHDF2 has functions in development and disease and this has been emphasized by multiple studies. Interestingly, in response to heat shock stress, YTHDF2 moves to the nucleus and protects the m6A marks in 5′-UTR of stress-induced transcripts from demethylation, which subsequently leads to their enhanced translation [102]. YTHDF2 functions in adult stem cell maintenance and in ex vivo expansion of hematopoietic stem cells (HSC), by regulating the stability of many mRNAs crucial for HSC self-renewal [103]. Its deletion causes embryonic lethality in mice and in heterozygous condition exhibited severe neural deregulation affecting embryonic brain development [104]. In zebrafish and mice model, YTHDF2 deficiency has been reported to cause dysregulations in maternal to zygotic transition process [105] and female specific infertility respectively [106]. Like other m6A players, YTHDF2 has also been reported to have aberrant expression pattern in a plethora

of pathological conditions such as hepatocellular carcinoma [107], prostate cancer [108] and in liver cancer [65] (Table 1).

YTH Domain-Containing Family Protein 3 (YTHDF3)

The cytoplasmic m6A reader, YTHDF3, works in close co-operation with the other cytoplasmic readers, YTHDF1 and YTHDF2, in deciding the fate of m6A bearing transcripts and often there is an overlap between targets of YTHDF3 with targets of YTHDF1 and YTHDF2 [109, 110]. It acts in co-operation with YTHDF1 and promotes translation efficiency of the targets, and by mediating decay of target transcripts, its synergy with YTHDF2 is displayed [109].

YTH Domain-Containing Protein 1 (YTHDC1)

YTHDC1 is a nuclear m6A reader, unlike other members of this protein family, which are cytoplasmic readers. It modulates alternative splicing of target transcripts via exon inclusion in a m6A-dependent manner, by binding and recruiting the splicing factor, SRSF3 to pre-mRNAs and in turn blocking SRSF10 from binding [111]. Apart from pre-mRNA splicing regulation, YTHDC1 also functions in nuclear mRNA export of methylated target mRNAs, by facilitating their binding to nuclear export factor 1 (NXF1) in a SRSF3-dependent manner [112]. Interestingly, YTHDC1 plays a pivotal role in maintaining intracellular levels of SAM, via degradation of m6A marked methionine adenosyltransferase 2A (MAT2A) transcripts, wherein the m6A deposition is catalyzed by the writer, METLL16, in a SAM-dependent manner [113]. Furthermore, this reader protein is essential for mice embryo development and fertility, particularly oocyte growth and maturation, via its impact on alternative splicing and polyadenylation, in a m6A-dependent manner [114]. In addition to this, YTHDC1 mediates XIST-mediated transcriptional silencing by binding to methylated XIST long non-coding RNA [84].

YTH Domain-Containing Protein 2 (YTHDC2)

The cytoplasmic reader protein, YTHDC2, is characterized by the presence of 3′–5′ RNA helicase activity, in addition to the YTH domain and plays an essential role in male and female fertility in mice via regulation of methylated transcripts to modulate meiosis in germ cells [115, 116]. YTHDC2 exerts its regulatory impact by enhancing translation efficiency of its targets by interacting with the ribosomal small subunit as well as by destabilization and degradation of the target transcripts via interacting with and recruiting 5′–3′ exoribonuclease, XRN1 to the transcripts [117]. The expression of YTHDC2 is elevated in several cancer cell lines and its role in colon tumor metastasis was reported [118] (Table 1).

Insulin-like Growth Factor 2 Binding Protein (IGF2BP)

As opposed to the YTH domain-containing m6A readers, the insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; IGF2BP1/2/3) represent a distinct family of m6A readers which target a large number of transcripts, by selectively binding to the m6A mark via their K homology domain [119]. Mechanistically, unlike the mRNA destabilization function of the reader, YTHDF2, IGF2BPs promotes mRNA stability and translation of its targets [119]. IGF2BPs play critical oncogenic roles in cancer cells, by stabilizing m6A methylated mRNAs of oncogenic targets like MYC [119].

Heterogeneous Nuclear Ribonucleoproteins (hnRNPs)

HnRNPs are a large family of RNA binding proteins having roles in multiple facets of RNA metabolism including readers of m6A methylation [120]. HNRNPA2B1 acts in concert with METTL3 and influences alternative splicing outcome of a subset of transcripts, as well as promotes primary microRNA processing of a subset of primary microRNA transcripts [121]. However, the precise binding specificity of hnRNPA2B1 to m6A methylation is a matter of debate and a combination of structural, biochemical and bioinformatics studies suggest that instead of direct binding to m^6A mark, the mark possibly facilitates accessibility of hnRNPA2B1 to certain binding sites on the RNA in the vicinity of the m6A mark, the so called concept of ‘m6A switches’ [122]. Further support to this concept was provided by studies on the reader protein, hnRNPG, wherein m6A mark increases the accessibility of the surrounding RNA sequence to bind hnRNPG, instead of a direct binding to the m6A signature [123].

Eukaryotic Initiation Factor 3 (eIF3)

eIF3 serves as a m6A reader by binding specifically to m6A signatures in 5′-UTR of target transcripts and promotes their cap-independent translation [124]. Such a regulatory mechanism is frequently used during cellular stresses whereby 5′-UTR m6A increases leading to increase in cap-independent translation of mRNAs like HSP70 mRNA [124].

Proline Rich Coiled-Coil 2A (PRRC2A)

PRRC2A is a novel m6A reader, bearing proline rich coiled-coil domain, and was reported to modulate oligodendrocyte specification and myelination, primarily by binding and stabilizing its target OLIG2 mRNA in a m6A-dependent manner [125].

m6A 'Erasers'

Erasers are RNA demethylases which remove the m6A mark installed by the writer complex and thus makes the m6A methylation reversible and dynamic in nature (Fig. 2). Even though the m6A writer, METTL3 was discovered decades ago, but the first m6A eraser, fat mass and obesity-associated protein (FTO) was reported only in 2011, and was shown to exhibit m6A demethylase activity [11]. Soon after this, the second m6A eraser, AlkB homology 5 (ALKBH5) was reported [126]. Interestingly, the substrate specificity of the m6A erasers is defined by a m6A-mediated substrate discrimination mechanism [127].

Fat Body Mass and Obesity-Associated Protein (FTO)

Pioneering work from Chuan He's lab was instrumental in uncovering the m6A erasers involved in removing the m6A mark from methylated transcripts. In 2008, employing in vitro experiments, recombinant FTO was demonstrated to catalyze oxidative demethylation of 3-methylthymine in single-stranded DNA and 3-methyluracil in single-stranded RNA substrates, with a greater preference being for the RNA [128]. FTO is an iron(II) and α-ketoglutarate dependent dioxygenase, homologous to the DNA repair protein, AlkB, and has been reported to be associated with obesity and increased body mass in humans [129, 130], as well as other developmental processes like cilia formation [131]. In 2011, Jia et al., showed the demethylase activity of FTO in vivo and its depletion led to increase in m6A levels of polyadenylated RNAs [11]. Furthermore, FTO demethylates its substrate through an oxidative process via formation of two intermediate products, N6-hydroxylmethyladenosine and N6-formyladenosine, whose function is a matter of further examination [132]. FTO shuttles between nucleus and cytoplasm mediated by interaction with exportin 2 (XPO2) and is suggestive of its different substrates in different cellular compartments [133]. Strikingly, a report suggested that FTO can also demethylate $m6A_m$, present at the first encoded nucleotide after the 7-methylguanosine cap in a subset of mRNAs and destabilizes them [134].

Multiple research studies have established the role of FTO in several facets of RNA metabolism such as pre-mRNA splicing and degradation [135–139] and its impact on sculpting the transcriptome [140, 141]. FTO modulates adipogenesis in a m6A-dependent manner, by regulating proliferation and differentiation of preadipocytes and impacts triglyceride metabolism [142–145]. Aside from adipogenesis, FTO impacts other developmental processes in a m6A-dependent manner, like bone development [146], postnatal brain development in mice where it is essential for adult neural stem cells proliferation [147, 148] and local mRNA translation in axons [149]. Increased expression of FTO was reported to reverse ischemic induced heart failure in mouse models [150] and skeletal muscle specific FTO deletion both under in vitro and in vivo scenarios, demonstrated its requirement for mitochondrial biogenesis [151] and for modulating lipid accumulation in skeletal muscle [152]. Interestingly, glycogen synthase kinase 3 (GSK3) was shown to regulate pluripotency in mouse embryonic stem cells through modulating m6A level by phosphorylating FTO, leading to its polyubiquitination and degradation [153]. FTO was suggested to contribute to pancreas islet β cells impairment and inhibition of its activity might be a potential target for the treatment of diabetes [154]. In accordance with and supporting this finding, elevated expression of FTO was often observed in diabetic patients [17]. Circadian rhythm is closely linked to obesity and FTO was shown to regulate the circadian rhythms by forming complexes with cryptochromes [155, 156]. Interestingly, last year a rather surprising function of FTO was uncovered. In a m6A-dependent manner, it was shown to modulate biogenesis of small nuclear RNAs and may influence RNA splicing since small nuclear RNAs are integral part of spliceosomes, which mediate pre-mRNA splicing [134]. FTO is aberrantly expressed in multiple cancer types and has been shown to influence various aspects of cancer development, progression and therapeutic resistance in a m6A-dependent manner (Table 1).

AlkB Homology 5 (ALKBH5)

ALKBH5, the second m6A eraser, identified by Chuan He's group in 2013, like FTO, belongs to the AlkB family of proteins and oxidatively reverses the m6A modification [126]. Initial eraser function of ALKBH5 was probed by in vitro experiments as in case of FTO; however, eventual in vivo studies using ALKBH5 knockout mice revealed its role in spermatogenesis and fertility in males [126, 157]. Interestingly, expression of ALKBH5 is stimulated under oxygen limiting hypoxic conditions, in a hypoxia-inducible factor (HIF)-dependent fashion and this mediates enrichment of

breast cancer stem cells in hypoxic tumor microenvironment [158–160]. Furthermore, a role for ALKBH5 in recurrent miscarriages was reported, wherein it controls trophoblast invasion at the maternal–fetal interface by modulating the stability of CRY61 transcripts, in a m6A-dependent manner [161]. Like, FTO, ALKBH5 has diverse functional and regulatory role in human diseases like cancer (Table 1).

Conclusions and Future Perspectives

The discovery of reversible m6A RNA methylation a few years back and subsequent functional characterization of the writers, readers and erasers of the m6A mark, has taken the field of gene expression by storm and has emerged as one of the key regulators of gene expression via its impact on virtually every aspect of RNA processing. Not only does it influence normal developmental processes, it also impinges upon a plethora of human diseases. Despite this, several pertinent questions are yet to be addressed to get a holistic view of this regulatory process. Some of the key questions are: What are the regulators of m6A methylation? What are the extracellular and intracellular signaling cues which regulate the m6A writers, readers and erasers? How is the substrate specificity of m6A writing, reading and erasing regulated? What is the cross-talk between m6A methylation and other reversible modifications of RNA? What are the fine and interwoven regulatory networks operating between m6A methylation and other RNA processing events? Deciphering the answers to these questions will provide a deeper insight into the role of m6A RNA methylation and in particular propel designing of novel therapies targeting this regulatory pathway.

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