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It's complicated… m^{6}A-Dependent Regulation of Gene Expression in Cancer

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Abstract

Cellular function relies on multiple pathways that are coordinated to ensure the proper execution of gene expression networks. Failure to coordinate the multiple programs active in the cell can have catastrophic consequences and lead to diseases such as cancer. At the post-transcriptional level, RNA modifications play important roles in the regulation of gene expression. N6-methyladenosine (m^{6}A) is the most abundant internal messenger RNA (mRNA) modification and has gained increasing interest in the last few years as a dynamic regulator of RNA metabolism. Modifications regulate all stages of the RNA life cycle, from transcription to decay. Recent studies have pointed to the role of RNA methylation in cancer initiation and progression, and aberrant modification has served as a biomarker of early-stage diagnosis in several cancers. Here, we review the regulation of m^{6}A, disruptions to methylation-dependent pathways that influence carcinogenesis, and potential avenues for m^{6}A-related therapeutic strategies.

Keywords

m^{6}A; epitranscriptome; gene regulation; cancer

Introduction:

Organismal development and homeostasis are fully dependent on changes in gene expression programs in response to both internal and external cues. After transcription, mRNA is subjected to multiple processing steps necessary for generating an RNA that can be translated. At each processing step, multiple pathways act to regulate how much product is generated from each mRNA, and ultimately, how a cell behaves. One of the regulatory mechanisms employed in the cell is the addition of post-transcriptional modifications to mRNAs. RNA modifications can directly influence RNA structure or modulate interactions with other molecules, contributing to complex regulatory networks. To date, over 150 modifications have been identified in RNA molecules (1) and methylation represents over 66% of known modifications (2). N6-methyladenosine (m^{6}A), one of several methyl

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modifications identified in mRNAs (3–10), is the most abundant modification found internally on mRNAs and long non-coding RNAs (lncRNA). This review will focus on the mRNA modification m^6A, and how disruption of m^6A-dependent regulatory pathways impacts cancer.

Section 1: m^6A methylation

1.1 The N6-methyladenosine modification:

Conserved throughout eukaryotic species, m^6A-dependent pathways play critical roles in development (reviewed in (11)). Although m^6A was first detected in mRNAs in the early 1970s (12–19), it was thought to be static and the role of this modification remained poorly understood. Recent discovery of fat mass and obesity-associated protein (FTO) as a m^6A demethylase (20), and the development of methods to map sites of modification (21,22) renewed the interest in mRNA methylation. In mRNAs, the majority of m^6A sites are found in a well-defined sequence context (RRACH; R can be A or G, H can be A, C or U) (23). m^6A sites are enriched near the last transcribed exon (a position that often correlates with the STOP codon), in large exons, and near the 5′ end (21,22,24,25), suggesting that addition of the m^6A modification is a regulated process (Figure 1A). In each cell type, while a large fraction of expressed genes can be modified, the majority of potential m^6A sites (RRACH motifs) are unmethylated, and a number of m^6A sites are only partially modified (26). This stoichiometry suggests a rheostat-like mechanism for fine-tuning gene regulation that can rapidly respond to cellular changes (26). The m^6A modification tends to be enriched in transcript isoforms with shorter 3′ untranslated regions (UTRs), and addition of m^6A modifications have been observed to impact tandem alternative polyadenylation (APA) site usage for some transcripts (24,27). Deposition of m^6A is co-transcriptional (Figure 1B), with the majority of m^6A sites determined by the time the mRNA is released into the nucleoplasm. A sub-optimal rate of transcription results in greater m^6A deposition on mRNAs, which impacts the rate of translation (24,28,29).

In addition to m^6A, methylation at position 6 of adenosine also occurs in the modification N6,2′-O-dimethyladenosine (m^6A_m). Unlike m^6A, which is an internal modification, m^6A_m is found at the first nucleotide adjacent to the 7-methylguanosine cap (5′ m^7GpppAm) (Figure 1A) (9). The m^6A_m modification is also found in viral RNAs, and it is thought that this addition is catalyzed by a host enzyme (13). Formation of m^6A_m in mRNA follows the synthesis of the 5′ m^7GpppAm and m^6A_m has not yet been observed in rRNA or tRNA (30). A recent report demonstrated that mRNAs modified with m^6A_m are more resistant to the mRNA-decapping enzyme 2 (DCP2) than those with the A_m modification alone (9) (Figure 1C).

Both m^6A and m^6A_m are regulated by the opposing activities of enzymes that introduce or remove these modifications, known as “writers” (methyltransferases) and “erasers” (demethylases), respectively. Furthermore, the presence or absence of these modifications can create or destroy binding sites for “readers” (RNA Binding Proteins). Decoding of m^6A by these reader proteins leads to downstream effects in all stages of the RNA lifecycle (Figure 1B and 1C).

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1.2 Writing the m^{6}A modification - the m^{6}A methylases:

m^{6}A modification of mRNA is catalyzed by a nuclear RNA methyltransferase complex. Initial characterization of m^{6}A methylation activity in HeLa cells identified two protein components required for modification: one component of approximately 200 kDa containing the enzyme methyltransferase-like 3 (METTL3), and a second unit of approximately 875 kDa (31). METTL3, the catalytic unit, forms a stable heterodimer with methyltransferase-like 14 (METTL14). This stable heterodimer interacts with an adaptor complex that includes zinc finger CCCH domain-containing protein 13 (Zc3h13); Wilms Tumor 1-associated protein (WTAP); Virilizer, Hakai and RNA binding motif protein 15 (RBM15) (32,33) (Figure 2A). The adaptor complex proteins play crucial roles in proper cellular localization of the methylase complex (34) and interactions of the METTL3/METTL14 heterodimer with mRNAs (27,32,33). Loss of function of components of the adaptor complex in multiple organisms suggests proteins in this complex may serve additional, m ^{6}A independent roles (33).

The core components of the methylation complex have been observed to re-localize under stress conditions (28,35). Upon ultraviolet (UV) radiation damage the heterodimer METTL3/METTL14, but not WTAP, is recruited to sites of UV damage (Figure 2B). Recruitment of METTL3/METTL14, which requires ADP-ribose polymerase (PARP), results in rapid, transient accumulation of m^{6}A at sites of damage. Accumulation of m^{6}A is required for early recruitment of DNA polymerase kappa (Pol κ), an enzyme implicated in both nucleotide excision repair and trans-lesion synthesis. Loss of METTL3 results in delayed repair of ultraviolet-induced cyclo-butane pyrimidine adducts and elevated sensitivity to ultraviolet light (35). In addition to UV damage, the methylation complex has also been observed to re-localize in response to thermal stress. Upon heat-shock, METTL3 and DiGeorge Syndrome Chromosomal Region 8 (DGCR8) form foci that co-localize with Heat Shock Factor 1 (HSF1) (Figure 2C). Under these conditions, binding of METTL3 is enriched at heat shock protein genes, resulting in the co-transcriptional marking of 70 kilodalton heat shock protein (Hsp70) mRNA for subsequent mRNA degradation (28). The activity of the methylation complex is controlled by transcription factor zinc finger protein 217 (ZFP217). This transcription factor binds to the promoter of m^{6}A targets and sequesters METTL3, disrupting the formation of the methylation core complex and resulting in lower activity of the methylation complex (36) (Figure 2A).

The enzyme methyltransferase-like 16 (METTL16), which occupies a central role in the regulation of both splicing machinery and of the levels of *S*-adenosyl-L-methionine (SAM), is also an m^{6}A writer. In addition to modifying the U6 snoRNA (37,38) METTL16 is also responsible for the modification and regulation of the Methionine Adenosyltransferase 2A (*MAT2A*) mRNA (37,39). *MAT2A* RNA levels are regulated in response to the availability of SAM, a common co-substrate involved in methyl group transfers. Under normal conditions, METTL16 binds to, and modifies, conserved hairpin structures in the 3′ UTR of *MAT2A*. When SAM is limiting, prolonged occupancy of METTL16 of a regulatory hairpin stimulates splicing of a retained intron and subsequent export and translation of the mRNA (37). An alternative model of *MAT2A* mRNA regulation proposes that METTL16-dependent m^{6}A modification of the 3′-UTR of *MAT2A* is read by the protein YTH Domain

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Containing Protein 1 (YTHDC1) to control the stability of the *MAT2A* mRNA. Differences observed in certain cell lines in response to cycloleucine (cLEU), an inhibitor of SAM synthesis, suggests that *MAT2A* RNA stability is controlled through multiple mechanisms, depending on cell type (39). In addition to *MAT2A*, other targets of METTL16 include ncRNAs (7SK, y-RNAs, vtRNA), lncRNAs (metastasis associated lung adenocarcinoma transcript (*MALAT*) and X-inactive specific transcript (*Xist*)) and pre-mRNAs (38). Interestingly, interaction between METTL16 and *MALAT* occurs at the triple helix at the 3′ UTR of noncoding RNA, suggesting METTL16 can interact with triple-stranded RNAs (40). METTL16 interaction sites are enriched in introns, at sites where m^6A levels are METTL16 dependent (37,38). Analysis of METTL16 homologs suggests that targeting of the *MAT2A* mRNA by METTL16 to regulate the levels of SAM occurred later in the evolutionary development of vertebrates (37).

1.3 Erasing m^6A - The m^6A demethylases:

While the m^6A modification had been known for some time, the recent identification of m^6A demethylases raised the possibility that this modification is dynamic and reversible. To date, two m^6A RNA demethylases have been identified: FTO and ALKB homolog 5 (ALKBH5), both members of the α-ketoglutarate (αKG)-dependent dioxygenase enzyme family. The FTO gene was first identified in genome-wide association studies and was shown to be linked to both obesity and Body Mass Index (BMI) (41–43). Glucose and amino-acid deprivation have been shown to regulate FTO expression (44). In 2011, it was demonstrated that FTO is able to demethylate m^6A both *in vitro* an *in vivo* (20). Crystallographic and biochemical analyses revealed the preference of FTO for single-stranded RNA (ssRNA) (45,46). Subsequent studies revealed that FTO oxidizes m^6A in RNA to N6-hydroxymethyl adenosine (hm^6A) and N6-formyl adenosine (f^6A) in a step-wise manner (47). Recently, it was demonstrated that FTO preferentially demethylates m^6A_m over m^6A (9). Altogether these studies suggest that demethylation of m^6Am may lead to an increase in diversity near the 5′ cap (9). ALKBH5 was the second identified m^6A demethylase (48). Like FTO, it was found to localize to nuclear speckles (20,48). Loss of ALKBH5 was shown to impact a number of RNA processing steps, including mRNA export and assembly of mRNA processing factors in nuclear speckles. ALKBH5-deficient male mice have increased m^6A in mRNA and show aberrant spermatogenesis and impaired fertility (48). Finally, several crystal structures of ALKBH5 demonstrate the preference of the enzyme for a ssRNA binding model and provide evidence for why hm^6A and f^6A have not been observed during demethylation with ALKBH5 (49–51). Although the m^6A writers show a preference for the m^6A consensus sequence RRACH, this sequence is not required for substrate selectivity by FTO and ALKBH5 (52). Additionally, while FTO is expressed highly in the brain (53) ALKBH5 is primarily expressed in the testes, with lower levels in other tissues (48). It remains to be understood how these two enzymes work together.

1.4 Interpreting the presence of m^6A - m^6A Readers:

The functional outcome of an RNA transcript is determined by interactions between the transcript and trans-acting factors, such as RNA binding proteins and small RNAs. RNA modification by m^6A can modulate regulatory interactions through three mechanisms (Figure 1B): introduction of a binding site for proteins that preferentially interact with

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methylated RNA (m^6A Readers), removal of a binding site of proteins that bind unmodified RNAs (Anti-m^6A Readers), and alteration of RNA secondary structure, impacting how single-stranded or double-stranded RNA binding proteins interact with RNA (m^6A-switch Readers). Reader proteins interpret changes in modification status, and affect a number of downstream cellular processes, including transcriptional efficiency, RNA processing, and RNA decay (Figure 2D).

The first m^6A readers identified were proteins belonging to the YTH domain-containing family (22), a highly conserved ssRNA binding domain in eukaryotes (54). In humans, the YTH domain family is composed of the YTH domain-containing family (YTHDF) proteins YTHDF1, YTHDF2, YTHDF3, and the YTH domain-containing (YTHDC) proteins YTHDC1 and YTHDC2 (55). These proteins interact with RNA in the presence of m^6A in cell-type specific interactions. YTHDF1 plays a role in increasing translation efficiency (56) and in axon regeneration has been shown to contribute to peripheral nerve-induced global protein translation (57). Conversely, m^6A recognition by YTHDF2 promotes mRNA degradation through interactions with the CCR4-NOT complex (58,59). YTHDF3 facilitates both translation and decay pathways through its interactions with YTHDF1 and YTHDF2, respectively (60). Interestingly, while YTHDF1, YTHDF2, and YTHDF3 each have their own unique target mRNAs, they also share a set of common mRNAs (56,60). The nuclear m^6A reader YTHDC1 has been shown to regulate mRNA splicing through recruitment of pre-mRNA splicing factors (61) and independent of its role in splicing, can also affect the transport of mRNAs from the nucleus (62). YTHDC1 is also essential for the function of *Xist*, a long non-coding RNA involved in X-chromosome inactivation (63). The protein YTHDC2 localizes in the cytoplasm of germ cells, and facilitates the switch from mitosis to meiosis in both male and female gametogenesis (64–67). Several structural studies have shed light on recognition of m^6A by the YTH family proteins. Analogous to the PHD and Tudor domain-containing enzymes which recognize methyl-lysine and methyl-arginine on histones tails, the YTH proteins contain a hydrophobic pocket and an “aromatic cage” to bind m^6A-containing RNAs (68–71).

The insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1/2/3) recognize m^6A modified RNAs and promote stability and storage of target RNAs (72). In addition to the presence of m^6A, other features such as the location of the modified base can also determine interactions. The eukaryotic initiation factor 3 (eIF3) interacts with m^6A-modified 5’UTR through a multi-subunit interface. This interaction recruits the 40S preinitiation complex to stimulate translation initiation, and allows for cap-independent translation of mRNA’s with m^6A modified 5′ UTR (21). Conversely, the presence of m^6A can also interfere with reader binding. The proteins RasGAP SH3 Domain Binding Protein 1 and 2 (G3BP1 and G3BP2) are strongly repelled by the presence of m^6A and binding of G3BP1 to mRNA enhances stability (73). Interestingly, the effect of m^6A on G3BP1 binding is context dependent. While m^6A in the context of its consensus sequence GGACU strongly repelled G3BP1 binding, the presence of m^6A in a GAACU sequence does not have a noticeable effect (73). Binding of direct m^6A readers, such as Fragile X Mental Retardation 1 (FMR1), can also be context dependent (73). While the m^6A modification does not affect Watson-Crick base pairing, the presence of the methyl group influences RNA secondary structure (74) and *in vivo* presence of m^6A favors a ssRNA conformation (75). These structural alterations

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facilitate binding of several proteins: heterogeneous nuclear ribonucleoprotein C (HNRNPC) and heterogeneous nuclear ribonuclearprotein G (HNRNPG), proteins responsible for pre-mRNA processing and mRNA maturation; as well as HNRNPA2B1, which interacts with the microprocessor protein DGCR8 to facilitate maturation of miRNAs (24,76,77).

Several of the readers mentioned above, as well as the writer and eraser proteins mentioned earlier, have been implicated in the development of disease. Below, we discuss the expanding role m^{6}A-dependent post-transcriptional regulation has in the development and progression of cancer.

Section 2: m^{6}A in Cancer

Although cancer is a highly diverse disease, cancer cells share a number of traits. These "hallmarks" include factors such as dysregulated metabolism, limitless replication, and chronic inflammation (78,79). A number of m^{6}A writers, readers, and erasers have been shown to play key roles in development and stem cell differentiation. Alterations in these pathways or dysregulation of epitranscriptomic regulators can drive tumorigenesis. While m^{6}A modifications have been implicated in a number of cancers, it is interesting to note that while some enzymes play similar oncogenic or tumor-suppressor roles across tissue types, others function in a more tissue-specific manner (Table 1). Here, we expand upon the disruptions to methylation-dependent pathways observed in cancer, as well as the effects altered m^{6}A-regulation have on driving cancer phenotypes.

2.1 Disruption of m^{6}A-dependent pathways in cancer:

m ^{6}A is a critical regulator of both self-renewal and differentiation in cells (25, 32, 36), and many of these same proteins that drive development can also become oncogenic when dysregulated. Genetic mutations, epigenetic reprogramming, and metabolic reprograming can all lead to changes in gene expression that allow cancer cells to overcome pathways that ensure tissue homeostasis. Several mechanisms are discussed below that have been shown to lead to either a loss- or gain-of-function in m^{6}A pathways in cancer.

2.1.1 Impact of Mutations and SNP variants on m^{6}A-dependent pathways:

Analysis of patient data from the Cancer Genome Atlas (TCGA) found that mutations and/or copy number variations (CNVs) of m^{6}A writer, reader, and eraser genes were strongly associated with the presence of TP53 mutations in acute myeloid leukemia (AML) patients. Furthermore, these mutations were associated with poorer patient outcomes. Because TP53 is such a strong driver of cancer, additional studies may help elucidate the link between m^{6}A writers/readers/erasers and TP53 in the progression of AML (80). A study on CNVs of patients with pancreatic adenocarcinoma identified loss of one copy of YTHDC2 as a potential candidate for pancreatic cancer susceptibility (81). While no complete loss of function has been described for METTL3 and METTL14, a highly conserved arginine of METTL14 is frequently mutated to proline in endometrial cancer. This mutation disrupts substrate recognition and reduces methylation activity (82) (Figure 3A). Genomic variations involved in the regulation of components of the m^{6}A machinery have also been implicated in cancer. Single nucleotide polymorphisms (SNPs) in the first intron of FTO (rs7206790, rs8047395, rs9939609 and rs1477196) have been reported to be

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significantly associated with an increased risk of breast cancer (83) and individuals with the AT or AA genotype at rs9939609 are more likely to develop malignant pleural mesothelioma (MPM) when compared to the TT genotype (84).

2.1.2 Transcriptional regulation of m^{6}A pathway components: Transcription factors are critical regulators of gene expression, and dysregulation of transcription factors is widespread in cancer (85). In Mixed Lineage Leukemia (MLL)-rearranged AML, when compared to non-MLL-rearranged AML samples or normal controls, FTO expression is upregulated through binding of MLL and particularly MLL-fusion proteins to CpG sites in the FTO locus (Figure 3B) (86). During myeloid differentiation, the transcription factor SPI1 is upregulated, repressing expression of METTL14, and indirectly of METTL14 targets that require m^{6}A for mRNA stability. In AML cells, SPI1 expression is suppressed, resulting in the upregulation of METTL14 and enhancement of self-renewal and proliferation (87) (Figure 3C).

2.1.3 Post-transcriptional regulation: Expression of m^{6}A-dependent pathway components is also disrupted at the RNA level by post-transcriptional pathways. MicroRNAs (miRNAs) are single-stranded non-coding RNAs 19–25 nt in length that regulate gene expression by base-pairing to complementary sites in the 3′ UTR of mRNAs (88). In clinical samples of non-small cell lung cancer (NSCLC), levels of METTL3 are higher in tumors when compared with corresponding peri-tumor tissues (89). Interestingly, the levels of METTL3 are negatively correlated with the levels of miR-33a, a miRNA that targets the 3′ of METTL3 and is expressed at low levels in NSCLC (Figure 3D) (89). Expression of miR-33a was found to inhibit the proliferation of A549 and NCI-H460 cells, and downregulate the expression of the oncogenes EGFR, TAZ, MAPKAPK2, and DNMT3A at the protein level (89). In colon cancer, YTHDC2 is upregulated and promotes translation of HIF-1α through unwinding of the 5′ UTR and contributes to colon tumor metastasis (90).

2.1.4 Environment changes: The tumor microenvironment influences tumor survival and progression, and m^{6}A-dependent regulation of gene expression has been shown to respond to multiple extracellular cues. One important factor of the tumor microenvironment is oxygen availability. Under conditions of hypoxia, expression of ALKBH5 and ZNF217, two proteins that lead to reduction of m^{6}A levels, is upregulated in breast cancer cells in a HIF-1α-dependent manner (91–93). Additionally, hypoxia has been shown to affect the splicing of YTHDC1 in gynecological tumor cell lines. Under hypoxia, changes in the splicing of YTHDC1 generate isoforms targeted by nonsense-mediated decay (NMD) which results in lower expression levels of the YTHDC1 protein. Signaling pathways also have an impact on m^{6}A-dependent gene regulation. In human embryonic stem cells, Mothers Against Decapentaplegic Homolog 2 and Homolog 3 (SMAD2 and SMAD3), intracellular effectors of the Transforming growth factor beta (TGFβ) signaling pathway, promote binding of the methylation complex to a subset of transcripts involved in early cell fate decisions. m^{6}A modification of these transcripts primes them for fast turn-over upon differentiation (94). While a direct link between TGFβ and m^{6}A has not yet been described in cancer, this signaling pathway is essential for cancer progression (95). The cellular

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metabolic environment can also lead to changes in m^{6}A-dependent regulation of gene expression. A powerful example is the accumulation of 2-hydroxyglutarate (2HG) in cells, a metabolite generated by mutations to isocitrate dehydrogenase (IDH). Mutations to IDH1 or IDH2 are present in approximately 80% of grade II-III gliomas (96–98), 50% of sporadic central and periosteal chondrosarcomas (99), 20–40% of angioimmunoblastic T cell lymphomas (AITL) (100), 10% to 20% of intrahepatic cholangiocarcinomas (101), and 12–16% of AML patients (102). Mutations in IDH have also been detected in hepatocellular carcinoma (HCC) (103) and prostate cancer (104). While wild type IDH enzymes catalyze the oxidative decarboxylation of isocitrate to generate α-ketoglutarate (αKG) using $NADP^+$ as a cofactor, mutations to IDH1 (Arg^{132}) or IDH2 (Arg^{140} or Arg^{172}) confer a neomorphic, gain-of-function activity to the enzymes, leading to the production of the onco-metabolite 2HG (105). The oncometabolite 2HG, is structurally similar to αKG and functions as a competitive inhibitor of enzymes in the αKG-dependent dioxygenases family, a group that includes DNA and histone demethylases as well as the RNA demethylases FTO and ALKBH5 (Figure 3E). Interestingly, there may be cell and cancer-specific sensitivity or resistance to 2HG. In AML cells overexpressing FTO, 2HG displays anti-tumor effects. Similar sensitive and resistant phenotypes were also observed in glioblastoma cells, and taken together, may explain the responses of certain AML and glioblastoma patient populations to standard chemotherapy regimens (106). Finally, m^{6}A-methylases have also been implicated in the process of arsenite-induced cell malignant transformation. In cells exposed to sodium arsenite, high levels of METTL3/METTL14 were shown to be correlated with the down-regulation of several miRNAs known to be involved in both small cell and non-small cell lung cancer. This suggests that external pollutants can also influence m^{6}A-dependent pathways and play a critical role in driving carcinogenesis (107).

2.2 Dysregulation of m^{6}A-dependent pathways contributes to the cancer phenotype

Dysregulation of m^{6}A-dependent regulatory pathways leads to changes in expression in multiple pathways, including regulators of gene expression. Defects in such processes may contribute to cancer development and tumorigenesis. Below we describe some of the targets dysregulated as a consequence of a loss or gain of m^{6}A regulation and their contributions to development of the cancer phenotype.

2.2.1 Transcription factors controlled through m^{6}A-dependent pathways:

c-MYC, which lies at the crossroads of many growth-promoting signaling pathways, is under tight transcriptional control (108). MYC expression is correlated with self-renewal activity, and is considered a marker for stem cells (109). Additionally, c-MYC and N-MYC have been shown to participate in reprogramming fibroblasts into induced pluripotent stem cells (110). Because MYC drives promoter escape and the transcription of numerous genes, even small changes in c-MYC levels, including changes in mRNA half-life dependent upon regulation by m^{6}A, may have a global impact on cellular phenotype. In fact, m^{6}A-dependent c-MYC dysregulation has now been described in multiple cancers.

MYC expression is dysregulated in the context of tumors with IDH mutations and 2HG accumulation. In leukemia and glioma cells overexpressing FTO, 2HG displays anti-tumor effects. Mechanistically, inhibition of FTO by 2HG leads to an accumulation of m^{6}A on

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MYC transcripts, destabilizing MYC mRNA and leading to downregulation of MYC signaling pathways (Figure 4A). This feedback mechanism could serve to further downregulate FTO. As 2HG is also able to inhibit the Ten-eleven translocation (TET) demethylases, this may explain why IDH and TET2 mutations are not observed together in AML (106).

Conversely, stabilization of MYC can drive cellular proliferation and tumorigenesis. In AML, MYB and MYC, are stabilized through suppression of SP1 and over-expression of METTL14 (87). High levels of METTL14 expression correlate with increased deposition of m^6A on MYB and MYC transcripts, suggesting that the increase in transcript half-life and stability is mediated through the actions of an m^6A reader protein, whose identity remains unknown (Figure 4B).

An increase in MYC stability can also be driven by overexpression of ‘readers’ with a stabilizing role. The IGF2BPs are frequently amplified or highly expressed in various human cancers. Reduction in individual IGF2BP levels *in vivo* repressed expression of MYC, and led to phenotypes that mimic MYC silencing, such as inhibited cancer cell proliferation, colony formation ability and cell migration/invasion (72) (Figure 4C).

METTL3/METTL14 and FTO have also been shown to be co-expressed with members of the Ccaat-enhancer-binding protein (C/EBP) family. C/EBP proteins are widely expressed CCAAT box binding transcription factors that regulate a number of cellular processes, including energy metabolism, inflammation, hematopoiesis, cellular proliferation, and cellular differentiation in a context-specific manner (110). Binding sites for C/EBPs are present in the promoter regions of numerous genes that are expressed in myeloid cells (111) and are thought to be important negative regulators of cell proliferation (112). In mouse primary leukemia driven by *MLL-AF9* fusions, METTL3 was found to be closely co-expressed and co-localized with CEBPZ, which is required for the recruitment of METTL3 to transcriptional start sites on chromatin (113). Additionally, expression of the transcription factor CEBPA was correlated with FTO expression. Inhibition of FTO by 2HG lead to an accumulation of m^6A on CEBPA transcripts, which is recognized by the reader YTHDF2 and targeted for degradation. This feedback mechanism could serve to further downregulate FTO in AML (106).

The transcription factor Forkhead Box Protein M1 (FOXM1), an important component in cell-cycle regulation, is also an m^6A target implicated in the development of cancer (114). In glioblastoma, levels of FOXM1 mRNA and protein are regulated by ALKBH5. This demethylase is highly expressed in cell lines or patient-derived primary glioblastoma cultures. High expression of ALKBH5 correlates with poor prognosis in glioblastoma patients. While knockdown of ALKBH5 had no effect on growth of non-glioma cells, loss of ALKBH5 activity inhibited growth and self-renewal of glioblastoma stem cells. Upregulation of ALKBH5 results in loss of m^6A in the *FOXM1* mRNA, enabling interaction with HuR, and leading to an increase in FOXM1 levels (Figure 4D). Interestingly, the interaction between *FOXM1* and ALKBH5 is mediated by the lncRNA FOXM1-AS (114).

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Another class of transcription factors that are frequently dysregulated in cancer are the Hypoxiainducible factor (HIF) transcription factors, which serve as regulators of cellular response to hypoxic conditions. HIF-1α activates the transcription of genes that play critical roles in angiogenesis and adaptive responses (115). Translation of HIF-1α is promoted by the m^{6}A reader YTHDC2 (90) (Figure 4E). Finally, transcription factors that contribute to a stem cell state are also co-opted in cancer cells through dysregulation of m^{6}A-dependent pathways. In breast cancer, hypoxia dependent over-expression of ALKBH5 leads to reduced m^{6}A methylation of mRNAs coding for core pluripotency factors such as NANOG. Decreased m^{6}A levels in NANOG mRNA result in increasing the stability of the mRNA, and consequent accumulation of the protein, linking the ALKBH5, and m^{6}A-dependent pathways to the establishment of breast cancer stem cells (92).

2.2.2 Disruption of m^{6}A-pathways impacts post-transcriptional regulation:

Up-regulation or down-regulation of miRNAs can have tumor-suppressor or oncogenic effects that are context- or tissue-specific (116). Patients with portal vein tumor thrombus (PVTT) (indicative of late-stage HCC) showed significantly reduced METTL14 levels compared to patients with non-metastatic tumors, or metastatic tumors without PVTT. Knockdown of METTL14 in HCC cells resulted in a decrease in migration and invasiveness. The unprocessed pri-miR126, a metastasis-associated miRNA, accumulated in METTL14 knockdown cells, while it was more rapidly processed in cells overexpressing METTL14 (Figure 5A). When m^{6}A was immunoprecipitated directly, it was found that METTL14 overexpression significantly increased the amount of pri-miR-126 modified by m^{6}A, indicating that this miRNA is a direct target of the methyltransferase complex (117).

In HCC, the expression of YTHDF2 is negatively correlated with expression of miR-145, a microRNA that is expressed at lower levels when compared to adjacent peritumorous tissues. In a HepG2 cellular model, it was found that overexpression of miR-145 suppressed YTHDF2 at the mRNA and protein levels, thereby increasing the levels of m^{6}A in cells, and decreasing cellular proliferation (Figure 5B). miR-145 has multiple cellular targets, and it remains to be determined if it can affect the RNA targets of other m^{6}A writers, readers, and erasers (118).

2.2.3 Signaling pathways affected by m^{6}A-dependent pathway dysregulation:

Cancer is driven by alterations that allow cells to escape mechanisms that normally control their survival and proliferation. Many of these alterations affect signaling pathways that control cell growth and division. In T cell homeostasis, the balance between the IL-7-mediated JAK–STAT and TCR-mediated ERK/AKT signaling pathways is controlled by m^{6}A-dependent pathways (119). In multiple cancers, changes in gene expression of components of this signaling pathway have been linked to changes in m^{6}A activity. METTL3 mRNA and protein expression was found to be elevated in multiple AML cell lines relative to normal hematopoietic cells (120). Upregulation of METTL3 results in higher levels of m^{6}A in the PTEN mRNA, leading to an increase in PTEN protein. Increased PTEN activity reduces PI3K/AKT pathway signaling cascades and differentiation (Figure 6A). When cells were transplanted into immunodeficient recipient mice after knockdown of METTL3, animals showed a delay in leukemia development as compared to a control group.

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In renal cell carcinoma (RCC), downregulation of METTL3 significantly promotes cell growth and colony formation. In RCC cells, expression of p-PI3K, p-AKT, p-mTOR, and p-P70 is significantly higher. Additionally, it was observed that vimentin, beta-catenin, and N-cadherin levels were significantly higher in METTL3 knockdown cells, suggesting that the epithelial-mesenchymal transition (EMT) pathway may also be altered by METTL3 knockdown (121).

In pancreatic cancers, YTHDF2 expression level is associated with poor patient prognosis, and expression increases with progression from stage I to stage IV. In several model pancreatic cancer (PC) cell lines, YTHDF2 is upregulated at both the mRNA and the protein level. Upon YTHDF2 knockdown, PC cells show reduced colony formation, lower colony density and reduced growth curves. In these cells, the protein levels for GSK3b and CyclinD1 were downregulated, leading to the hypothesis that YTHDF2 acts through the Akt/GSK3b/CyclinD1 pathway. Interestingly, YTHDF2 knockdown cells showed increased migration, invasion, and adhesion abilities in PC. This EMT upregulation occurs through the yes-associated protein (YAP) gene, although the exact link between YTHDF2 and YAP remains to be determined (122).

In the HCC-derived cell lines Huh-7 and HepG2, knockdown of METTL3, decreased cell proliferation and colony forming capacity while overexpression led to accelerated cell migration in a transwell assay. Gene expression studies in METTL3 KD cells identified 15 genes that were consistently upregulated in both cell types. Suppressor of Cytokine Signaling 2 (SOCS2), a member of the JAK-STAT signaling pathway, is modified by m^6A and is a direct downstream target of METTL3. In HCC, SOCS2 down-regulation is significantly correlated to advanced TNM staging. SOCS proteins affect cellular response to cytokines and growth factors by regulating the JAK/STAT signaling pathway. Dysregulation of METTL3 may result in altered SOCS2 expression, leading to carcinogenesis (123) (Figure 6B). The EGFR signaling pathway is also regulated through proteins in the m^6A pathway.

In lung adenocarcinoma (LUAD), up-regulated METTL3 directly promotes the translation of several known oncogenes, including EGFR, TAZ, MK2 and DNMT3a. METTL3 enhances the translation of its targets by interacting with eukaryotic initiation factor 3 (eIF3) in an RNA-independent manner. Most interestingly, this effect occurred independently of the binding partner METTL14, or the catalytic domain of METTL3 (124).

Section 3: Clinical Treatments and Drugability

3.1 Treatment sensitization or resistance:

Acquisition of resistance to therapeutic drugs by cancer cells represents one of the major obstacles in cancer treatment. Changes in gene expression mediated through alteration in m^6A-dependent pathways can lead to changes in sensitivity to chemo and radio therapy and thus impact treatment regimens. In pancreatic cancer, knockdown of METTL3 resulted in higher sensitivity to gemcitabine, 5-fluorouracil, cisplatin, and irradiation (125). Microarray data and GO-term analysis identified a number of targets of METTL3, including genes involved in the MAPK signaling cascades, RNA splicing, and cell-cycle regulation. In

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glioblastoma cells, knockdown of METTL3 decreased proliferation, increased apoptosis, and increased sensitivity to temozolomide and irradiation (126). Finally, in colorectal cancer, knockdown of YTHDF1 resulted in inhibition of cell proliferation and increased sensitivity to both 5-flurouracil and oxaliplatin. Further analysis revealed that c-Myc was associated with the 5′ transcription start site of YTHDF1. Upon c-Myc knockdown, YTHDF1 was inhibited in a dose-dependent manner, but other YTH family proteins were unaffected (127). Given the important role that MYC plays in a number of cancers, further exploration of this relationship could increase our understanding of how m^{6}A regulation plays a role in cancer. m^{6}A-dependent pathways might also have a direct role in sensitivity to therapies that target DNA, due to its role in DNA repair (28).

3.2 Direct Drugability:

In addition to modulating the efficacy of traditional cancer treatments, a number of studies have investigated the direct drugability of the m^{6}A pathway. The natural product Rhein acts as a competitive inhibitor of FTO and can prevent recognition of m^{6}A sites by FTO in cells (128). However, one drawback of Rhein is its poor selectivity. Subsequent research identified the FTO inhibitor meclofenamic acid (MA) (129). The selectivity of MA towards FTO relies in part on the interaction of MA with the FTO nucleotide recognition lid, a structural feature that is absent in other ALKBH family members, including ALKBH5. This loop provides a hydrophobic interaction surface for the inhibitor. Treatment of cells with the ethyl ester form of MA (MA2), results in increased m^{6}A methylation of mRNA, indicating this compound is able to achieve good levels of cellular penetration. Upon FTO knockdown, m^{6}A levels were not further affected, indicating this compound does not act through the ALKBH5 demethylation pathway. In glioblastoma cells, treatment with MA2 inhibited tumor progression (130). A third inhibitor of FTO, characterized by Toh *et al.*, exhibits 30 to 130-fold selectivity over other members of the αKG-dependent dioxygenase family, including ALKBH5, PHD2, and KDM4A. Crystallography revealed that hydrogen-bonding interactions with the backbone of Glu234 (2.9 Å) in FTO play an important role in selectivity, as the inhibitor is unable to have similar backbone interactions with other AlkB family members (average distance ~ 4.6 Å) (131). Finally, the compound IOX3 was found to decrease protein expression of FTO in C2Cl2 cells. However, reduced expression of FTO did not appear to have a significant effect on cellular m ^{6}A levels. In this case, it may be difficult to discern the effects of FTO inhibition from inhibition of other αKG-dependent dioxygenases, including the PHD family, which are targets of IOX3 (132). The ability to target m^{6}A-dependent pathways might allow for the development of combination therapies. In AML cells, knockdown of FTO substantially enhanced ATRA-induced cell differentiation, suggesting that inhibiting FTO in combination with ATRA treatment represents a promising therapeutic strategy to treat leukemia (86).

Section 4. Preliminary Clinical Observations:

While our understanding of the role of m^{6}A writers, readers, and erasers in some cancers has dramatically improved, in several clinical studies, m^{6}A writers and erasers have been implicated in tumorigenesis, although the affected underlying pathways are still unknown. In patients with gastric cancer, it was observed that high FTO levels significantly correlated

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with lymph node metastasis, high TNM stage, and poor prognosis. In cell culture models, FTO overexpression led to increased cellular proliferation and migration phenotypes (133). In patients with HCC, the reader protein YTHDF1 was found to be overexpressed and was positively correlated with late-stage cancer and poor patient survival rates (134). Similarly, YTHDF2 was found to be upregulated in gastric cancer tissues and shRNA knockdown of YTHDF2 in MGC-803 cells was found to inhibit proliferation and promote apoptosis (135). In cervical cancer, it was observed that cancer tissue samples had decreased m^6A levels as compared to paired normal tissue, and that these decreased levels showed strong correlation with cancer progression and patient outcomes.

In cellular models, knockdown of METTL3/METTL14 or overexpression of FTO and ALKBH5 promoted cell proliferation. Conversely, knockdown of FTO and ALKBH5 or overexpression of METTL3 and METTL14 suppressed cancer development (136). Indeed, FTO expression varies with the sub-type of breast cancer, with tumors from patients with hormone receptor (HR)-negative and (human epidermal growth factor receptor 2 (HER2) overexpression breast cancer displaying higher levels of FTO expression when compared to HR positive and HER2 negative breast cancer (137). Additionally, the levels of m^6A writers, readers, and erasers, as well as the levels of m^6A-modified RNA, may serve as a potential prognostic biomarkers to assist in the selection of treatment options and assess patient's outcomes (138,139). Taken together, these clinical observations provide a number of interesting, unanswered questions. Further studies are needed to explore the underlying mechanisms.

Conclusions:

RNA post-transcriptional modifications can influence RNA structure and attract or repel RNA binding proteins. As a consequence, RNA post-transcriptional modifications are involved in all stages of the RNA life cycle, and have significant impact on the output of gene expression programs. Throughout development, and in maintaining homeostasis, several pathways are regulated in part by pathways dependent on RNA modifications. These pathways are often co-opted by the cancer cells, and disruption of RNA post-transcriptional regulation contributes to both establishment and progression of cancer. In this review, we focused on m^6A, and the emerging principles of how m^6A methylation contributes to cancer progression and metastasis. During development, the RNA modification m^6A has a significant impact on cell identity, and in mouse embryonic stem cells, loss of m^6A modification can result in different phenotypes depending on cellular identity (25,140,141). This early observation suggested that cellular identity is a key determinant of the phenotypic consequences of disrupting m^6A-dependent gene regulation, a phenomenon also observed in cancer studies. Considering the heterogeneity observed in tumors, it becomes critical to understand how m^6A-dependent pathways interact with both intracellular factors, such as metabolite levels or RNA binding proteins present, and extracellular factors such as oxygen availability or signaling pathways in order to effectively target these pathways in a therapeutic purpose. Future work aimed at understanding epitranscriptomic dysregulation and cancer tumorigenesis may increase our understanding of malignant transformations and aid in the design of novel therapeutics to treat cancer. (See Table 1.)

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Supplementary Material

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Highlights:

- N6-methyladenosine (m^6A) plays an important role in regulating gene expression
- m^6A-dependent pathways may have either a tumor-suppressive or oncogenic role
- Targeting m^6A-dependent pathways represents a promising new therapeutic route

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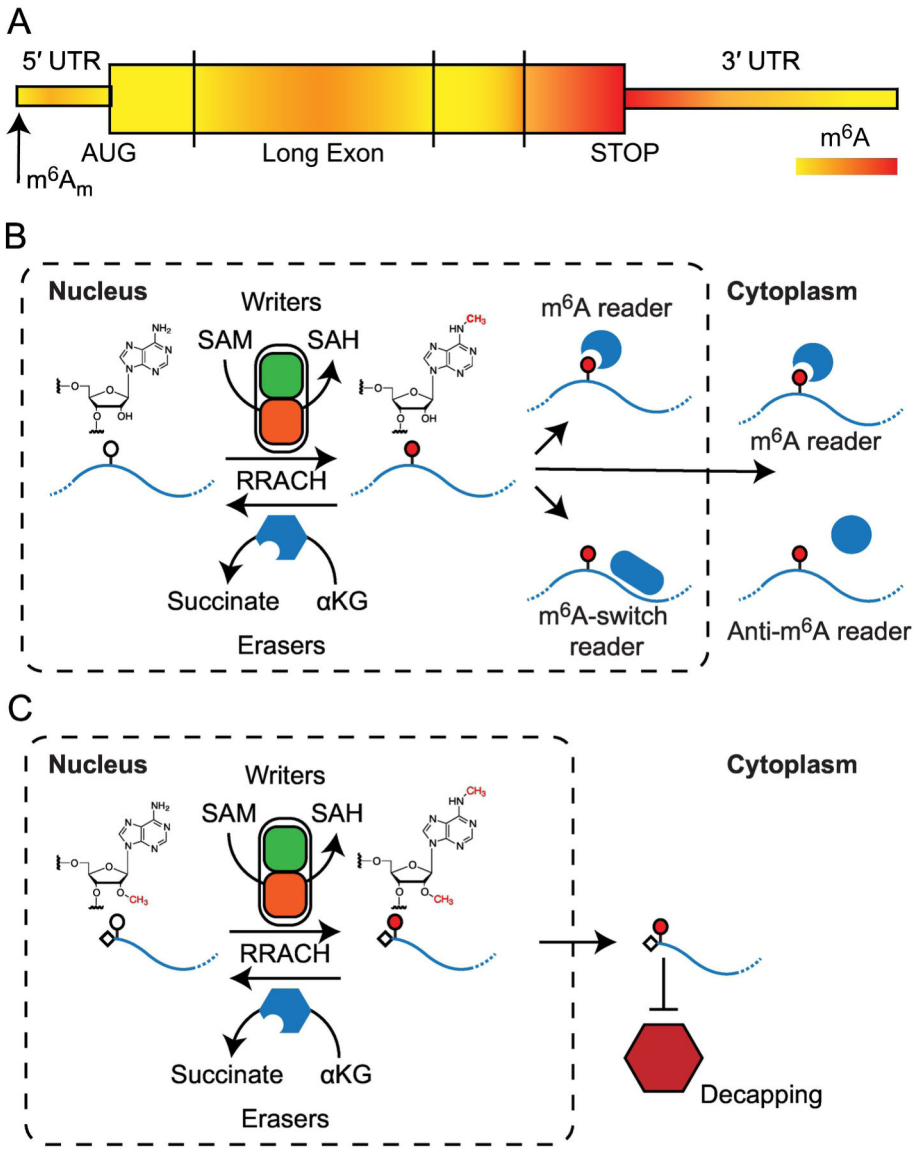

Figure 1: Cycle of methylation and demethylation.

(A) The modified bases N6-methyladenosine (m^6A) and N6,2′-O-dimethyladenosine (m^6A_m) are present in distinct regions of mature mRNA. **(B)** Addition of m^6A to RNA requires the co-factor SAM and is catalyzed by the methylase complex (writers). The modification is removed through the actions of the αKG-dependent demethylases (erasers). The presence or absence of m^6A affects the activity of numerous RNA binding proteins (readers) that modulate the downstream processing of the RNA. **(C)** Formation of m^6A_m occurs in the nucleus. Similar to m^6A, levels of $m\ ^6A_m$ are determined by a balance between writer and eraser activity. The presence of m^6A_m confers resistance to decapping activity in the cytoplasm.

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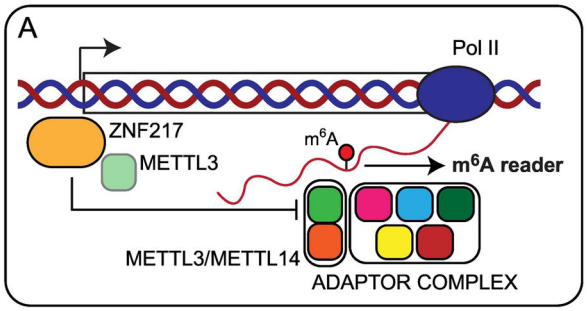

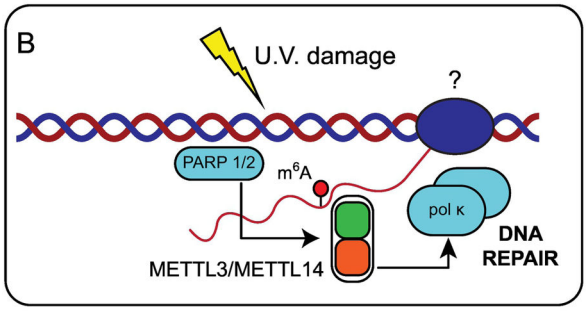

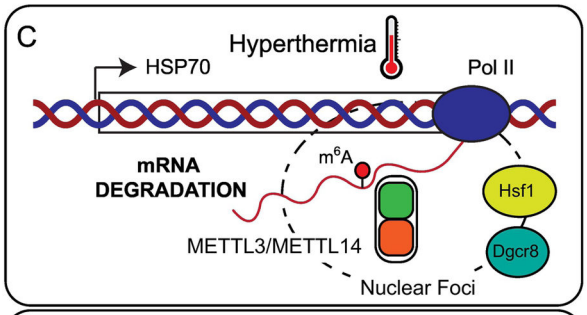

D

	Protein	Loc.	Function
m^{6}A reader	YTHDC1	(N)	Splicing/Transport
	YTHDF1	(C)	Translation
	YTHDF2	(C)	Degradation
	YTHDF3	(C)	Translation/Degradation
	YTHDC2	(C)	Translation/Degradation
	IGFBP1-3	(C)	Stability
	eIF3	(C)	Translation
Anti-m^{6}A reader	G3BP1	(C)	Stability
m^{6}A-switch reader	HNRNPG	(N)	mRNA maturation
	HNRNPC	(N)	mRNA maturation
	HNRNPA2B1	(N)	miRNA maturation

Figure 2. N6-methyladenosine regulatory complexes.

The METTL3/METTL14 proteins have been observed to interact with other cellular proteins. **(A)** The adaptor complex, which includes zinc finger CCCH domain-containing protein 13 (Zc3h13) Wilms Tumor 1-associated protein (WTAP) Virilizer, Hakai, and RNA binding motif protein 15 (RBM15), facilitates programed deposition of m^{6}A during development. Additionally, the transcription factor zinc finger protein 217 can disrupt the formation of the core complex by sequestering METTL3. **(B)** Accumulation of m^{6}A also occurs in response to UV damage and promotes recruitment of DNA damage repair factors.

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(C) Under hyperthermia, recruitment of METLL3/METTL14 to heat shock foci marks transcripts for rapid turnover. **(D)** Three types of RNA binding proteins that interact with RNA in a m^6A-dependent manner have been described: 1) m^6A readers, proteins that interact with the modified m^6A base; 2) proteins repelled by the presence of m^6A; and 3) proteins that interact with RNA after m^6A-induced rearrangement of RNA structure. These proteins are present in both the cytoplasm and the nucleus, and determine RNA metabolism.

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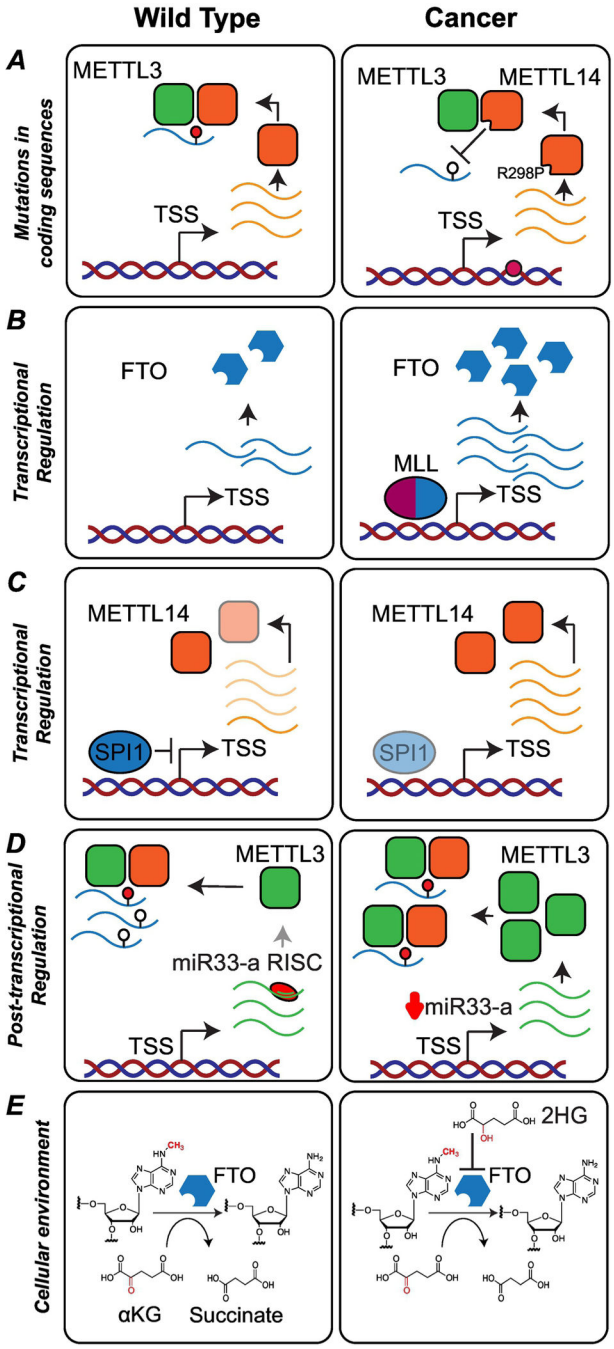

Figure 3. Cancer-induced disruptions of m^{6}A-dependent pathways.

(A) Mutation of a highly conserved arginine in METTL14 (R298P) is frequently observed in endometrial cancer. This mutation diminishes methylation activity by disrupting substrate recognition. **(B)** In Mixed Lineage Leukemia (MLL)-rearranged acute myeloid leukemia (AML), FTO expression is up-regulated through binding of MLL-fusion proteins to CpG sites in the FTO locus. **(C)** In individuals with AML, the transcription factor SPI1, a repressor of METTL14, is suppressed, resulting in the upregulation of METTL14 and enhancement of self-renewal and proliferation. **(D)** Production of METTL3 can be repressed

post-transcriptionally by microRNA-mediated gene silencing. In non-small cell lung cancer, miR-33a, a regulator of *METTL3*, is expressed at low levels, resulting in higher levels of METTL3 protein. **(E)** Mutations in IDH enzymes lead to the production of the onco-metabolite 2-hydroxyglutarate (2HG). 2HG is structurally similar to αKG, and acts as a competitive inhibitor of the m^6A demethylases FTO and ALKBH5.

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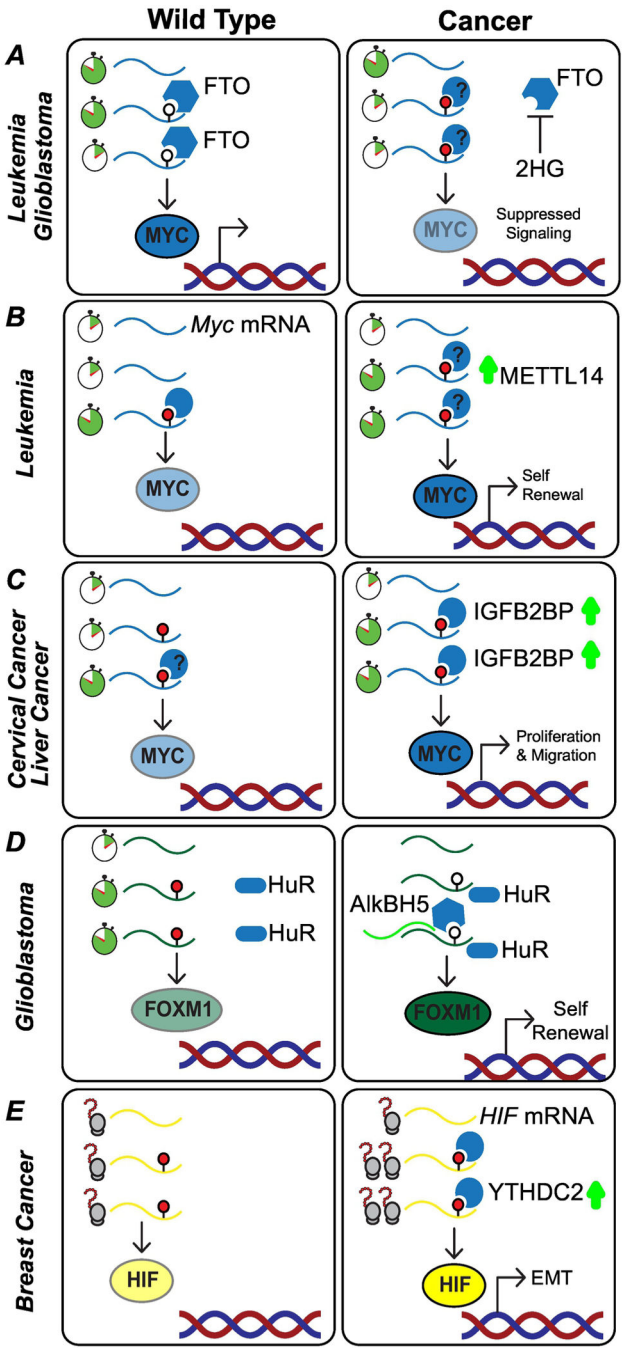

Figure 4: m^{6}A-dependent pathways regulate control of cellular transcription factors.

(A) Mutations in IDH lead to the production of the onco-metabolite 2-hydroxyglutarate (2HG). Accumulation of 2HG inhibits the activity of the m^{6}A demethylase FTO, leading to downregulation of MYC and suppressed MYC signaling. **(B)** Upregulation of METTL14 in individuals with acute myeloid leukemia (AML) leads to increased deposition of m^{6}A. Stabilization of *MYC* mRNA, through interaction with an unknown reader protein, drives a self-renewal phenotype in the cancer cells; **(C)** In cervical cancer and hepatocellular carcinoma (HCC) cells, the m^{6}A reader IGFB2BP stabilizes *MYC mRNA*, leading to

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increased cellular proliferation; **(D)** In glioblastoma cells, the lncRNA FOXM1-AS mediates the interaction between HuR and the demethylase ALKBH5, increasing expression of the transcription factor FOXM1 and driving a self-renewal phenotype. **(E)** Translation of the transcription factor HIF-1α is promoted by the m^6A reader YTHDC2. HIF activates the transcription of genes that play a role in angiogenesis and adaptive response.

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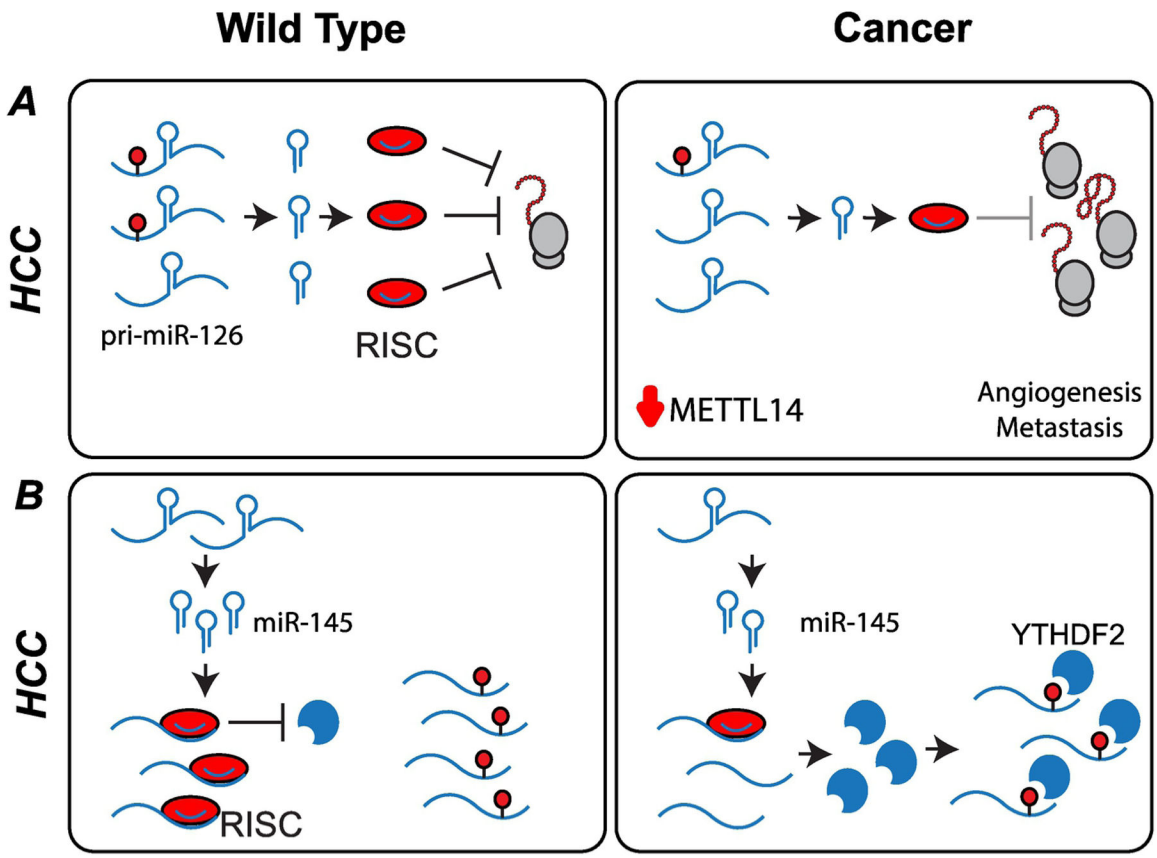

Figure 5: Post-transcriptional regulation of gene expression by miRNA.

(A) Downregulation of METTL14 in hepatocellular carcinoma (HCC) cells leads to an increase in unprocessed pri-miR-126. Mature miR-126 is associated with metastasis. **(B)** In HCC cells, lower expression of miR-145 allows for expression of YTHDF2 protein, decreasing the levels of m^6A in cells, and increasing cellular proliferation.

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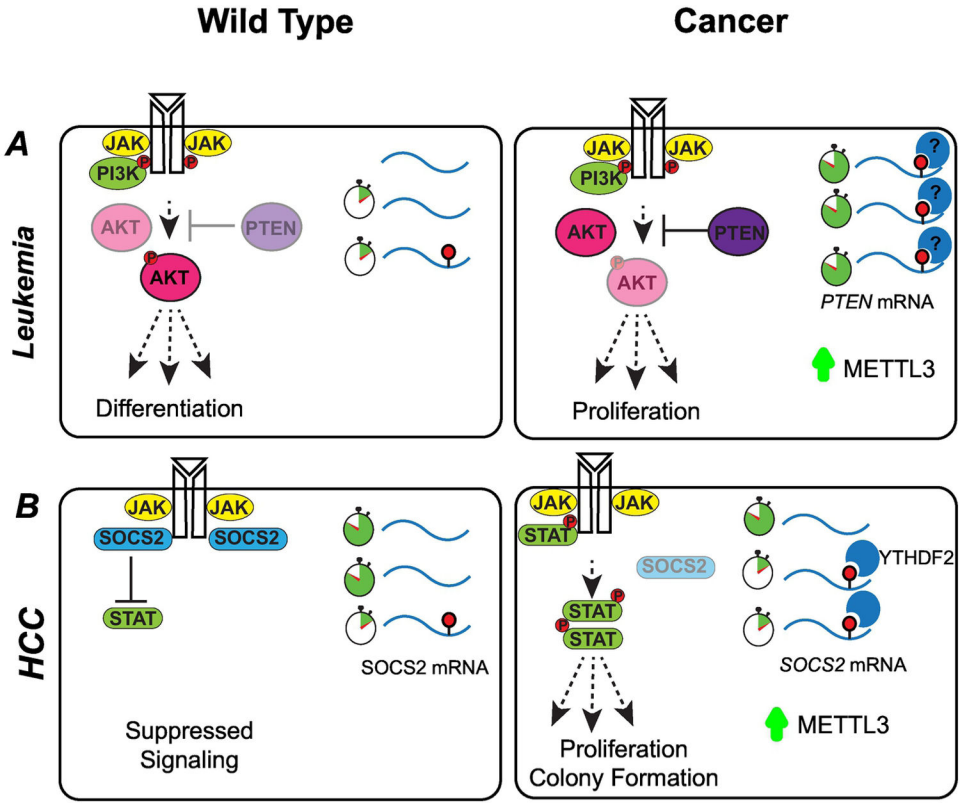

Figure 6: m^{6}A-dependent pathways influence signaling cascades.

(A) METTL3 is expressed more abundantly in acute myeloid leukemia (AML) cells than healthy hematopoietic stem cells. Upregulation of METTL3 results in increased mRNA methylation and higher expression levels of factors critical for the regulation of apoptosis and differentiation. For example, increased PTEN activity reduces PI3K/AKT pathway signaling, leading to proliferation and maintenance of the hematopoietic stem cell program. **(B)** In hepatocellular carcinoma (HCC) cells, increased METTL3 results in increased expression of SOCS2, a member of the JAK-STAT signaling pathway. Increased levels of SOCS2 lead to cellular proliferation and carcinogenesis.

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Table 1:

m^6A regulators associated with cancer.

Cancer Type	Enzy me	Oncogene/Tumor Suppresor	Pathway(s) Affected	Hallmark of Cancer	Reference
Acute Myeloid Leukemia	METTL3	oncogene	c-Myc, Blc2, PTEN	Limitless replicative potential	[120]
	METTL3	oncogene	CEBPZ, c-Myc, SP1, SP2	Limitless replicative potential	[113]
	METTL3	oncogene	MYB/MYC	Increased cell proliferation	[87]
	METTL14	oncogene	MYB/MYC	Increased cell proliferation	[87]
	FTO	oncogene	ASB2 and RARA	Increased cell proliferation	[86]
	FTO	oncogene	MYC/CEBPA	Increased cell proliferation	[106]
	IGF2BP	oncogene	MYC	Increased cell proliferation	[72]
Breast Cancer	FTO	unknown	unknown	Unknown	[137]
	AlkBH 5	oncogene	HIF	Chronic inflammation	[92]
Cervical Cancer	FTO	oncogene	unknown	Increased cell proliferation	[136]
Colorectal Cancer	YTHDF1	oncogene	c-Myc	Increased cell proliferation	[127]
	YTHDC2	oncogene	HIF-1α	Metastasis	[90]
Gastric Cancer	FTO	oncogene	unknown	Metastasis	[133]
	YTHDF2	oncogene	unknown	Increased cell proliferation	[135]
Glioblastoma	METTL3	tumor-suppressor	ADAM19	Cell growth and invasiveness	[130]
	METTL14	oncogene	ADAM19	Cell growth and invasiveness	[130]
	AlkBH5	oncogene	FOXM1	Cell-cycle regulation	[114]
Hepatocellular Carcinoma	METTL3	tumor-suppressor	SOCS2	Increased cell proliferation	[123]
	METTL14	tumor-suppressor	miR-126	Angiogenesis	[117]
	YTHDF1	unknown	unknown	Unknown	[134]
	YTHDF2	oncogene	miR-145	Increased cell proliferation	[118]
Lung adenocarcinoma	METTL3	oncogene	EGFR, TAZ	Increased cell proliferation	[124]
Malignant Pleural Mesothelioma	FTO	unknown	unknown	Unknown	[84]
Non-Small Cell Lung Cancer	METTL3	oncogene	miR-33-a	Increased cell proliferation	[89]

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Cancer Type	Enzy me	Oncogene/Tumor Suppresor	Pathway(s) Affected	Hallmark of Cancer	Reference
Pancreatic Cancer	METTL3	oncogene	mitogen-activated protein kinase cascades	Evading apoptosis Increased cell proliferation	[125]
	YTHDC2	unknown	unknown	Unknown	[81]
	YTHDF2	oncogene tumor suppressor	EMT/YAP p-AKT/GSK3b/C yclinD1	Tissue invasion / metastasis Cell proliferation	[122]
Renal Cell Carcinoma	METTL3	tumor-suppressor	EMT PI3K-Akt-mTOR	Increased cell proliferation, tissue invasion, and metastasis	[121]