Review

Refining the role of N⁶-methyladenosine in cancer
Jonas Koch and Frank Lyko

N⁶-methyladenosine (m⁶A) is the most abundant internal modification of eukaryotic mRNAs. m⁶A affects the fate of its targets in all aspects of the mRNA life cycle and has important roles in various physiological and pathophysiological processes. Aberrant m⁶A patterns have been observed in numerous cancers and appear closely linked to oncogenic phenotypes. However, most studies relied on antibody-dependent modification detection, which is known to suffer from important limitations. Novel, antibody-independent, quantitative approaches will be critical to investigate changes in the m⁶A landscape of cancers. Furthermore, pharmaceutical targeting of the m⁶A writer Methyltransferase-like 3 (METTL3) has demonstrated the potential to modulate cancer cell phenotypes. However, the enzyme also appears to be essential for the viability of healthy cells. Further refinement of therapeutic strategies is therefore needed to fully realize the potential of m⁶A-related cancer therapies.

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N⁶-methyladenosine (m⁶A) is the most investigated internal modification of eukaryotic mRNAs. It is catalyzed by a multiprotein complex, with Methyltransferase-like 3 (METTL3) being the catalytically active writer component. The modification has important roles in physiological and pathophysiological processes, including cellular differentiation and cancer development. Cancer-related changes of m⁶A and their potential role(s) in tumorigenesis have been described elsewhere [1–4]. In this mini-review, we focus on recent developments in the characterization of molecular mechanisms of m⁶A-dependent transcript regulation, particularly the m⁶A-dependent compartmentalization of mRNAs into phase-separated condensates. We also highlight the technical challenges of measuring cancer-associated changes in m⁶A patterns and discuss novel developments that might enable the quantitative mapping of m⁶A transcriptomes at single-base resolution. Finally, we provide an updated assessment of the role of METTL3 as an oncogene or tumor suppressor in different cancer entities. We discuss novel developments in the use of METTL3 inhibitors for cancer therapy, with a particular focus on the therapeutic potential of m⁶A-dependent condensates and METTL3 inhibition as an emerging strategy in cancer immunotherapy.

Molecular mechanisms of N⁶-methyladenosine-dependent transcript regulation

m⁶A has been shown to affect the fate of its target transcripts in multiple ways, including alternative splicing, nuclear transport, stability, and translation (Figure 1). These m⁶A-dependent functions are usually translated by m⁶A reader proteins that directly or indirectly bind to m⁶A sites [5]. Reader proteins of the YTH family essentially comprise the nuclear YTHDC1 reader and the cytoplasmic YTHDF1–3 readers that directly bind to m⁶A sites via their YTH domain. While earlier studies reported distinct roles for distinct YTHDF proteins, a more recent model described all cytoplasmic readers to have redundant binding sites and to function essentially in mRNA decay by networking with components that are linked to the Carbon catabolite repression 4-negative on TATA-less (CCR4-NOT) degradation complex [5–7]. In contrast, the nuclear YTHDC1 reader was shown to affect essential steps in pre-mRNA processing, including alternative splicing and nuclear export of transcripts [5,7]. Interestingly, insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs) were also shown to bind m⁶A-modified transcripts, most likely via their KH domain [8]. Together with the co-factors HuR and MATR3, IGF2BPs can promote the stabilization of their target transcripts, thereby facilitating their translation [8,9]. Since m⁶A also affects the secondary structures and the accessibility of its transcripts, other nondirect m⁶A reader proteins were described to mediate m⁶A-related functions [6]. For example, heterogeneous nuclear ribonucleoproteins (hnRNPs) hnRNPC and hnRNPG belong to this class of indirect reader proteins.

Figure 1
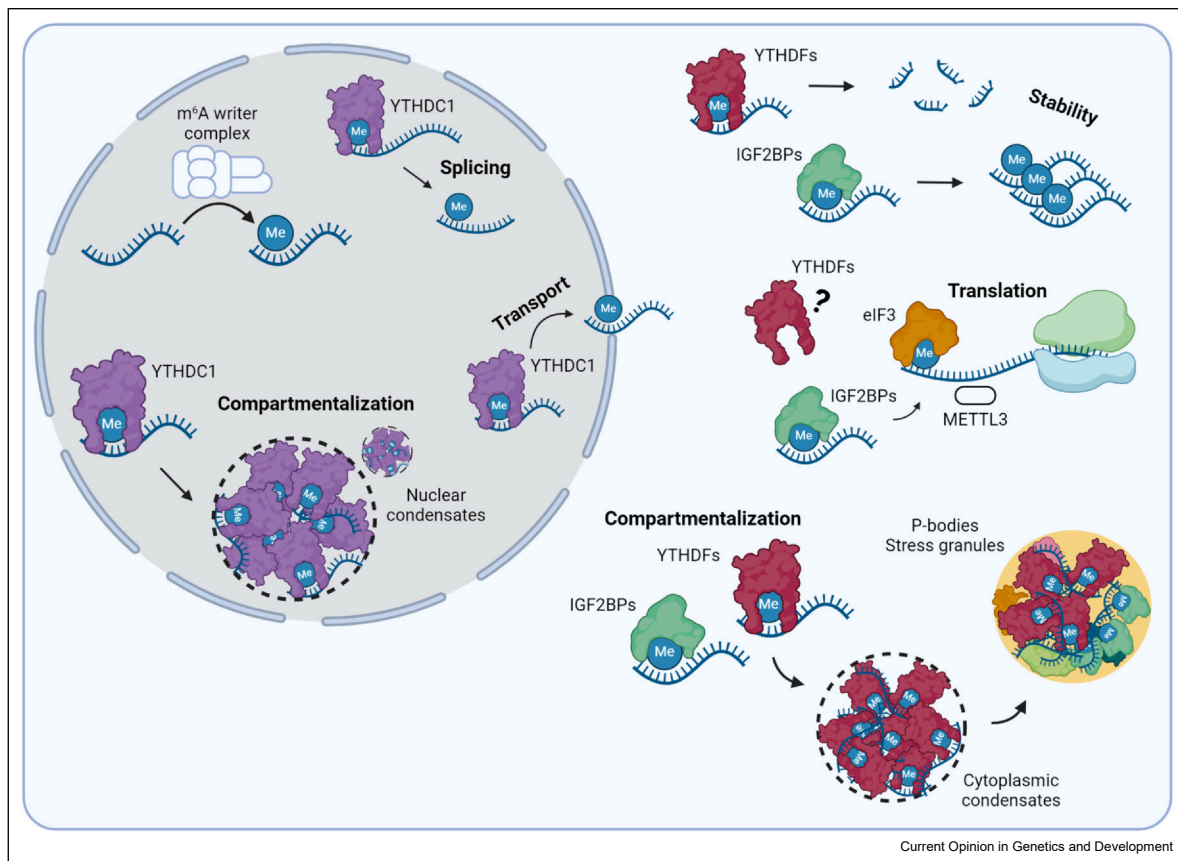
Molecular mechanisms of m^{6}A-mediated mRNA regulation. The fate of m^{6}A-modified mRNAs is regulated by m^{6}A readers essentially comprising the nuclear YTHDC1 protein and the cytosolic YTHDF proteins. YTHDC1 was shown to control splicing and nuclear export of mRNAs. Furthermore, the reader was found to promote the recruitment of mRNAs into nuclear YTHDC1-m^{6}A condensates, thereby affecting the stability of the transcripts. The most current model for cytoplasmic YTHDF proteins proposes an essential role in mRNA decay, while their role in translation needs further validation. Also, YTHDF proteins were shown to undergo phase separation and to further fuse with cytoplasmic condensates, including stress granules and P-bodies. Thereby, they promote the phase separation potential of bound transcripts and affect their localization and regulation. Mechanisms by which m^{6}A-modified mRNAs are regulated by IGF2BPs comprise stabilization, translation, as well as compartmentalization. Created with BioRender.com.

Additional insight about the molecular function of YTHDF proteins came from a combination of structural and cell biological analyses. YTHDF1–3 are paralogs that share high sequence similarity. Besides the C-terminal YTH domain, they also possess low complexity and intrinsically disordered regions [10]. It was shown that upon interaction of multiple readers with poly-m^{6}A-modified transcripts, the readers were scaffolded and stabilized via their low complexity regions, which promoted liquid–liquid phase separation into condensates. These dynamic m^{6}A-mRNA-reader condensates could further separate into P-bodies or stress granules, thereby adding a novel layer of m^{6}A-dependent transcript regulation [11] (Figure 1). Additional findings showed that the depletion of YTHDF1 and YTHDF3 inhibits stress granule formation [12] and that m^{6}A mediates the length-dependent enrichment of transcripts in stress granules [13]. Furthermore, the IGF2BP readers were found to affect transcript regulation via their translocation into P-bodies and stress granules, thereby promoting their stabilization [9] (Figure 1). Most recently, the IGF2BP3 reader was found to regulate the partitioning of m^{6}A-marked transcripts into actively translating and nontranslating pools. It was shown that polysome-associated transcripts are generally low in m^{6}A sites, while P-body-associated transcripts are hyper-m^{6}A-methylated. Global reduction of m^{6}A in HeLa cells caused a translocation of mRNAs from P-bodies to polysomes [14].

Similar to YTHDF proteins, YTHDC1 also possesses an internal YTH domain and intrinsically disordered regions that were shown to promote the formation of nuclear YTHDC1-m^{6}A condensates via phase separation (Figure 1). In acute myeloid leukemia (AML) cells, YTHDC1 was found to recruit oncogenic MYC transcripts into

condensates, thereby protecting them from degradation via the PAXT-exosome complex and promoting AML cell survival and differentiation suppression [15]. It was also shown that the recognition of m6A-modified MALAT1 transcripts affects the composition of nuclear speckles, thereby regulating gene expression in esophageal cancer cells [16]. Taken together, these studies illustrate how phase separation and regulation via compartmentalization into nuclear and cytoplasmic condensates affect the fate of poly-m6A-marked transcripts.

Finally, while most m6A-related effects on transcript regulation appear to be mediated by m6A reader proteins, it was also shown that m6A can directly promote cap-independent translation of mRNAs via different mechanisms (Figure 1). eIF3 facilitates cap-independent translation by directly binding to m6A in the 5′UTR of transcripts, while METTL3 promotes mRNA circularization to initiate translation [6,17].

Challenges in the detection of cancer-associated N6-methyladenosine changes

To date, little is known about cancer-associated changes in m6A levels and distribution. A major problem for the robust detection of these differences is the quantification of m6A in the transcriptome. Many studies have used dot blot and Enzyme-linked immunosorbent assays (ELISA) for the global quantification of m6A levels in cell lines and patient samples. However, these techniques rely on m6A antibodies that are known to be cross-reactive [18,19]. Furthermore, purification of mRNA from total RNA samples is challenging as contaminating rRNA species, that also contain m6A and make up more than 90% of the cellular RNA, can be difficult to deplete [20].

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) allows for a more rigorous quantification of global m6A levels. By the use of external calibrations, spike-in measurements, and radio-labeled internal standards, LC-MS/MS achieves high sensitivity and measures RNA modifications within the femtomole range [21]. The parallel detection of rRNA-specific modifications (e.g. m_2^6A) allows for an estimation of contaminating rRNA amounts between different mRNA sample preparations. Thus, measured differences in m6A levels between mRNA samples are reliable, and most likely not the result of different levels of contaminating rRNA. Using this approach, we have recently shown a pronounced global reduction of m6A methylation levels in bladder cancer tissues compared with para-tumoral tissues (n = 20) [22]. On average, m6A levels were decreased by nearly 50% in tumor samples. Expression analyses of the m6A writer complex revealed that METTL3 was upregulated, while several subunits were downregulated in the tumor samples, suggesting that a stochiometric imbalance within the writer complex might cause the observed global m6A decrease [22].

On the molecular level, a global reduction of m6A likely affects transcript-specific m6A signatures that impact the fate of a cell (Figure 2). For investigating the differences in the transcriptome-wide distribution of m6A sites, quantitative m6A mapping approaches need to be pursued. Again, most studies rely on antibody-dependent mapping techniques for the detection of m6A sites. The immunoprecipitation of RNA using m6A-recognizing antibodies allows for an enrichment of modified mRNAs that are sequenced, before modification peaks are called. However, these methods also suffer from limited antibody specificity that include cross-reactivity with other RNA modifications, and from poor resolution, since m6A modification sites can only be localized to regions spanning 200–300 nucleotides [18,19]. To counteract these disadvantages, antibody-independent mapping techniques are being established. Nanopore sequencing enables direct RNA sequencing by pulling RNA molecules through a membrane-embedded protein pore. Changes in the constantly measured current intensity result in specific profiles that allow for the computational identification of the respective nucleotide, while the molecule passes the pore. Modified RNA nucleobases such as m6A have characteristic current intensity profiles, which allows their direct detection [23,24]. Another emerging method is GLORI, which allows unbiased, antibody-independent, base-resolution, and transcriptome-wide m6A mapping on standard next-generation sequencing platforms [25]. The method uses glyoxal and sodium nitrite for the deamination of unmodified adenosines into inosines under conditions where m6A remains resistant to deamination. With this approach, more than 170 000 m6A sites with a median methylation level of approximately 40% were identified in the transcriptome of the human HEK293T cell line. One-third of the detected m6A sites appeared in clusters that were characterized by higher methylation levels and important for gene regulatory functions [25]. Thus, GLORI has the potential to become the gold standard for quantitative profiling of m6A in the transcriptome. Comparative modification mapping of mRNAs from tumor and matched control samples should be used to establish the epitranscriptomic landscape of human cancers and to define m6A-dependent, cancer-associated pathways.

N6-methyladenosine/METTL3 as a therapeutic target

In recent years, numerous studies have investigated the role of METTL3 in various cancer entities (Table 1). For most tumors, METTL3 was found to have tumor-promoting functions. However, the protein was also shown to have tumor-suppressive roles in endometrial and kidney cancers. In bladder, breast, colorectal, and thyroid cancers, both tumor-suppressive and oncogenic functions of METTL3 were reported.

Figure 2
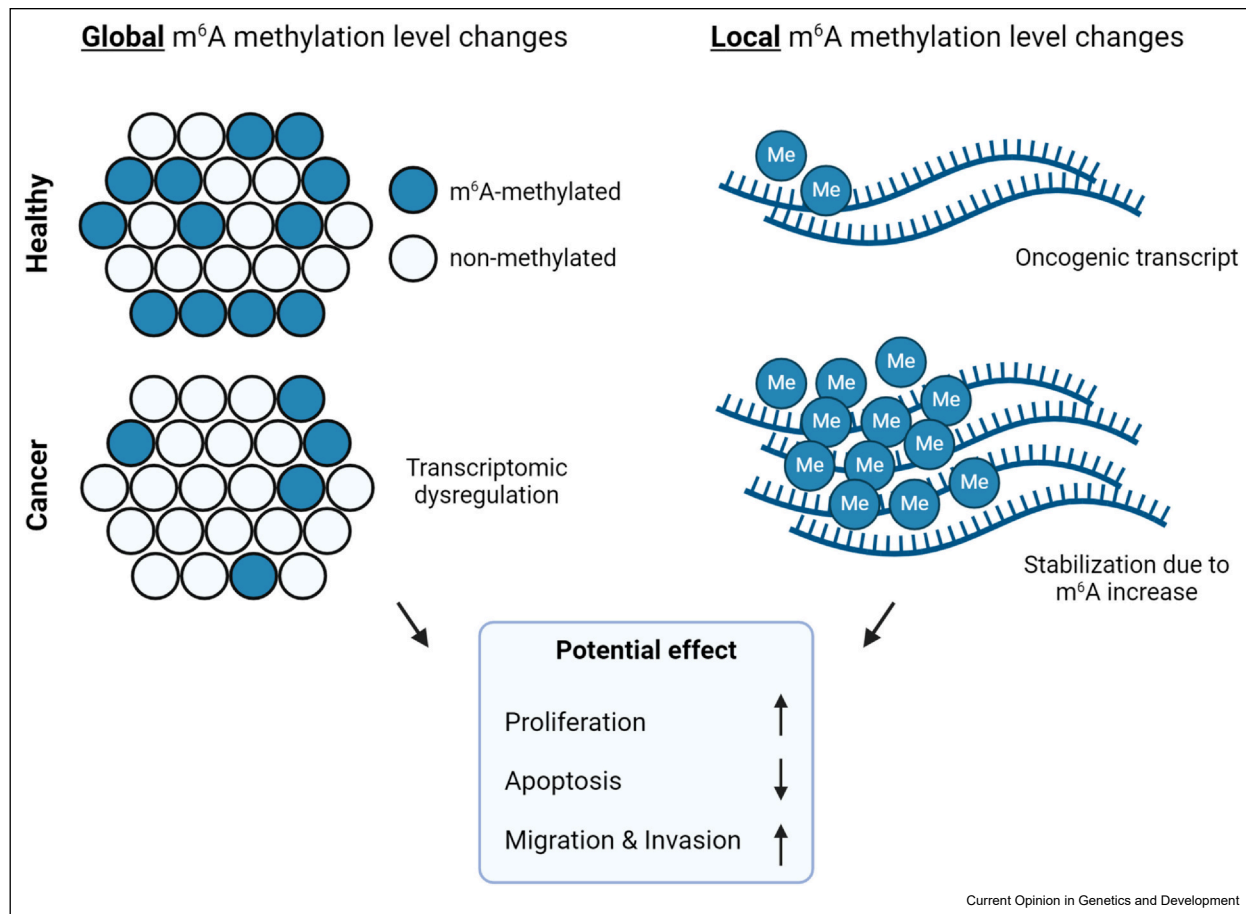
Cancer-associated changes of m⁶A. Numerous studies have reported global changes of m^{6}A methylation levels and patterns in different tumor entities, potentially causing a transcriptomic dysregulation that promotes oncogenic phenotypes. Prominent examples include the global reduction of m^{6}A in bladder cancer [22] (left panel) and the increased m^{6}A modification levels of the ZMYM1 mRNA that enhanced its stability, thereby promoting gastric cancer metastasis [64] (right panel). Created with BioRender.com.

METTL3 has been proposed as a potential drug target due to its described oncogenic roles in most investigated tumors (Table 1). In AML, small-molecule-based inhibition of METTL3 via STM2457 led to a reduced oncogenic phenotype of AML cells. Mechanistically, the m^{6}A methylation levels of leukemogenic transcripts were reduced upon inhibitor treatment, thereby interfering with their expression and translation. *In vivo* METTL3 inhibition caused impaired engraftment and prolonged survival in a mouse leukemia model without significant side effects [26]. STM2457-based inhibition of METTL3 also reduced the oncogenic phenotype of small cell lung cancer cells and could reverse chemoresistance both *in vitro* and *in vivo* at a concentration that did not affect the proliferation of healthy lung epithelial cells [27]. Additional studies indicated therapeutic effects in a variety of solid tumor entities [28–31]. The molecular mode of action of METTL3 inhibition remains to be fully understood.

Considering the basic cellular functions of m^{6}A and the role of METTL3 as the predominant, if not only, m^{6}A mRNA methyltransferase in mammalian cells, it will be important to resolve whether METTL3 is a tumor-specific oncogene or whether it is more broadly required for cellular viability. METTL3 knockout mice completely lack m^{6}A methylation and fail to terminate naïve pluripotency, resulting in early embryonic lethality [32]. The recent identification of catalytically active METTL3 isoforms in CRISPR/Cas9 knockout cells further suggests that METTL3 is essential for the viability of cells [33]. This observation is also consistent with dependency analyses that characterize METTL3 as a pan-essential gene [22,33]. Pan-essential genes are often misidentified as tumor-selective genes due to limited preclinical modeling, and their drugs are often characterized by a low therapeutic index, where efficacy is limited by off-target toxicities as well as difficulties in patient stratification [34]. Strategies for increasing the therapeutic index for

Table 1

Functional role of METTL3 in selected tumors. Representative studies were cited.

Entity	Oncogene	Tumor suppressor	Reference
Acute myeloid leukemia	X		[44]
Cervical cancer	X		[45]
Gastric cancer	X		[46]
Head and neck cancer	X		[47]
Liver cancer	X		[48]
Lung cancer	X		[49]
Melanoma	X		[50]
Pancreatic cancer	X		[51]
Prostate cancer	X		[52]
Ovarian cancer	X		[53]
Bladder cancer	X	X	[54] (Oncogene), [55] (Suppressor)
Breast cancer	X	X	[56] (Oncogene), [57] (Suppressor)
Colorectal cancer	X	X	[58] (Oncogene), [59] (Suppressor)
Thyroid cancer	X	X	[60] (Oncogene), [61] (Suppressor)
Endometrial cancer		X	[62]
Kidney cancer		X	[63]

METTL3 inhibitors potentially include efforts that reduce their systemic exposure, such as tumor-selective drug delivery systems, local drug administration, or pro-drug approaches. Challenges and opportunities related to the targeting of pan-essential genes or pathways have been reviewed elsewhere [34,35].

An alternative, potentially more tumor-selective strategy might be the targeting of m^{6}A-dependent, pathophysiological condensates. For example, the protection of m^{6}A-modified MYC transcripts via YTHDC1-mediated compartmentalization into nuclear condensates was shown to promote AML progression [15]. Thus, targeting of these condensates might have significant therapeutical potential. Small molecules or peptides, that inhibit the interaction between m^{6}A readers and their methylated targets, could prevent the initiation of oncogenic condensates or promote their dissociation. While it remains to be resolved to what extent phase-separated compartments are druggable, it has been shown that chemotherapeutics can selectively cluster in condensates [36]. Furthermore, screening platforms for the identification of so-called condensate modifying drugs (c-mods) have been established in order to restore physiological condensate states in diseases [37,38].

METTL3 inhibition in cancer immunotherapy

Most recently, the role of METTL3 in the tumor microenvironment (TME), immune escape mechanisms, and antitumor immunity has become a topic of high interest. When the novel METTL3 inhibitor STM3006 was used to decrease global m^{6}A methylation levels in a human ovarian and a mouse breast cancer cell line, it induced the formation of double-stranded RNA (dsRNA). dsRNA sensing and Retinoic acid-inducible gene 1 (RIG-I)-like receptor signaling triggered a cell-intrinsic interferon response that increased antitumor immunity [39]. In *in vitro* killing assays, METTL3 inhibition enhanced the potency of CD8+ T cells and led to increased tumor cell killing. Furthermore, METTL3 inhibition was found to be as efficacious as PD-1 checkpoint blockade, and the combinatory use of both treatments even outperformed the single agents in a range of immunocompetent mouse models comprising hematologic and solid cancers [39]. Thus, METTL3 inhibitors were suggested to augment antitumor immunity in cancer therapies.

In bladder cancer, RIG-I was identified as a downstream target of YTHDF2 [40]. YTHDF2 mediated the degradation of RIG-I transcripts, leading to immune evasion of bladder cancer cells. *In vivo*, YTHDF2-deficient bladder cancer cells were more susceptible to a stronger immune response and characterized by increased CD8+ T-cell infiltration, thereby also boosting the efficacy of Bacillus Calmette-Guerin immunotherapy [40]. These findings describe an oncogenic METTL3/YTHDF2/RIG-I axis that has a potential role in antitumor immunity.

Finally, METTL3 was also shown to promote non-alcoholic fatty liver disease–derived hepatocellular carcinoma by reducing tumor infiltration of CD8+ T-cells [41]. STM2457 and anti-PD-1 combination therapy restored CD8+ T-cell infiltration, thereby promoting tumor regression [41]. In colorectal and non–small-cell lung cancer, METTL3 mediated the formation of an immunosuppressive TME, characterized by an accumulation of myeloid-derived suppressor cells and decreased potency of CD4+ and CD8+ T-cells. METTL3 inhibition led to the reprogramming of the TME favoring the infiltration of potent immune cells and potentiated the effects of anti-PD-1 therapy [42,43]. Taken

together, these findings reinforce a role for METTL3 in anticancer immunity and refine the therapeutic potential of METTL3 inhibitors.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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