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Hidden codes in mRNA: Control of gene expression by m^{6}A

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SUMMARY

Information in mRNA has largely been thought to be confined to its nucleotide sequence. However, the advent of mapping techniques to detect modified nucleotides has revealed that mRNA contains additional information in the form of chemical modifications. The most abundant modified nucleotide is *N*^{6}-methyladenosine (m^{6}A), a methyl modification of adenosine. Although early studies viewed m^{6}A as a dynamic and tissue-specific modification, it is now clear that the mRNAs that contain m^{6}A and the location of m^{6}A in those transcripts are largely universal, and are influenced by gene architecture, i.e., the size and location of exons and introns. m^{6}A can affect nuclear processes such as splicing and epigenetic regulation, but the major effect of m^{6}A on mRNAs is to promote degradation in the cytoplasm. m^{6}A marks a functionally related cohort of mRNAs linked to certain biological processes, including cell differentiation and cell fate determination. m^{6}A is also enriched in other cohorts of mRNAs and can therefore affect their respective cellular processes and pathways. Future work will focus on understanding how the m^{6}A pathway is regulated to achieve control of m^{6}A-containing mRNAs.

eTOC blurb

In this review, Murakami et. al. discuss the recently recognized concept of epitranscriptomic regulation with a focus on *N6*-methyladenosine (m^{6}A), the most abundant internal mRNA modification. They describe the early views of m^{6}A, how these views have changed, and highlight emerging concepts that fundamentally change our understanding of how m^{6}A functions.

INTRODUCTION

The search for regulatory elements in mRNA has primarily focused on sequence-specific RNA-binding proteins, which influence processes such as splicing, mRNA stability, and translation. However, some of the regulatory elements in mRNA are not evident simply by looking at the sequence. Instead, they are encoded by chemical modifications of

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DECLARATION OF INTERESTS

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nucleotides in mRNA. The most abundant chemically modified nucleotide in mRNA is *N*^{6}-methyladenosine (m^{6}A), which is also found in ribosomal RNA (rRNA) and small nuclear RNA (snRNA). m^{6}A was identified as a component of mRNA in the 1970s (Desrosiers et al., 1974; Perry and Kelley, 1974), but interest in the functional and physiological roles of m^{6}A, especially in mRNA, was stimulated by a study in *Arabidopsis*. In this study, the plant homolog of the mammalian m^{6}A-synthesizing enzyme METTL3 was depleted, resulting in marked developmental defects (Zhong et al., 2008). These pioneering studies revealed the first phenotypes of m^{6}A depletion in a multicellular organism, and demonstrated that m^{6}A has key roles in development.

Over the past 10 years, with the advent of transcriptome-wide mapping of m^{6}A and the identification of m^{6}A readers, a variety of functions and mechanisms of m^{6}A have been identified. Highly divergent models have been proposed to explain how mRNAs are selected for methylation and the effect of m^{6}A on mRNAs (Zaccara et al., 2019). Recently, the answers to these questions have become much clearer, resulting in a more coherent model for the role of m^{6}A in gene expression.

The major question is the function of m^{6}A on mRNAs that contain it. It is clear that m^{6}A has a major effects on splicing in a small number of transcripts (Wei et al., 2021). m^{6}A can also influence epigenetic silencing, e.g. in the case of endogenous retrotransposons (Liu et al., 2021; Xu et al., 2021). However, the major function of m^{6}A is to confer mRNA instability (Lasman et al., 2020; Zaccara and Jaffrey, 2020).

An early concept was that m^{6}A is a dynamic modification (Roundtree et al., 2017a). In this model, an mRNA could be methylated and demethylated during its lifecycle, much like phosphorylation and dephosphorylation of a protein. The transcriptome-wide distribution of m^{6}A was also described as tissue specific, thus potentially explaining tissue-specific patterns of gene expression (Zhang et al., 2021). However, as described below, it is likely that the transcriptome-wide distribution of m^{6}A is largely “hard-wired” by the gene architecture (i.e., length and distribution of exons and introns) of each gene. Although a subset of m^{6}A sites might be variable, the majority of m^{6}A sites are found in the same transcripts in the same location in every tissue (Garcia-Campos et al., 2019).

The major implication of the hard-wired distribution of m^{6}A is that m^{6}A marks a specific cohort of mRNAs whose stability can be jointly regulated. Many of the mRNAs that contain multiple annotated m^{6}A sites (e.g. four or more m^{6}A sites) are linked to pathways that control cell fate, differentiation, and morphogenesis (Ries et al., 2019). These results are consistent with earlier studies in yeast and *Arabidopsis* which showed m^{6}A deficiency leads to developmental defects (Agarwala et al., 2012; Zhong et al., 2008), as well as subsequent studies which showed that m^{6}A controls embryonic stem cell differentiation (Batista et al., 2014; Geula et al., 2015; Wang et al., 2014). Thus, the ability of m^{6}A to influence development is evolutionarily conserved.

m^{6}A can regulate biology by enhancing degradation of specific transcripts that play central roles in the cellular pathways. In many cases, m^{6}A-containing mRNAs are master regulators of specific signaling pathways. m^{6}A-mediated degradation of these mRNAs can alter

signaling or transcription, leading to indirect increases or decreases in gene expression. For example, m^{6}A residues are found on mRNAs encoding the tumor suppressor APC and transcription factors Nanog and Klf4. m^{6}A-mediated repression of APC attenuates cancer progression (Wang et al., 2021a), while the decreased expression of Nanog and Klf4 enables differentiation of naïve embryonic stem cells (Geula et al., 2015).

Here we describe how the current views of m^{6}A have emerged and we discuss the outstanding questions about m^{6}A pathway regulation (Figure 1).

m^{6}A is a low stoichiometry modification

m^{6}A mapping revealed the fundamental features of m^{6}A, including which transcripts contain m^{6}A and potential mechanisms by which certain sites are selected for methylation. The initial mapping methods (MeRIP-seq and m6A-seq) revealed ~13,000 thousand m^{6}A sites in ~6000 mRNAs (Dominissini et al., 2012; Meyer et al., 2012). These methods used m^{6}A antibodies to immunoprecipitate m^{6}A-containing fragments after shearing mRNA. These fragments were then sequenced by next-generation sequencing and mapped to the transcriptome. Subsequent mapping methods (miCLIP) crosslinked m^{6}A antibodies to RNA to achieve antibody-RNA adducts that created signature mutations adjacent to m^{6}A, providing the first single-nucleotide resolution maps of m^{6}A (Linder et al., 2015). These mapping methods have been used in many studies to show that m^{6}A is dynamic and markedly altered in different cellular contexts and disease states (Liu et al., 2020b). However, as will be described below, more recent studies have argued that m^{6}A dynamics may have been overestimated, or at least were performed with data that contained insufficient replicates to detect such differences (McIntyre et al., 2020).

m^{6}A mapping also revealed that identifying the methylation consensus sequence is not enough to reveal the sites of methylation in the transcriptome. Mapping identified the major m^{6}A consensus sequence, designated DRACH (D = A, G, U; R = A, G; H = A, C, U) (Linder et al., 2015). This consensus sequence is very similar to the sequence context predicted in the 1970s based on nuclease mapping experiments with metabolically labeled RNA (Wei et al., 1976). However, the DRACH sequence motifs are ubiquitous in mRNA, with the statistical frequency of one in every ~57 nucleotides. However, m^{6}A is much more rare (~1 in every thousand nucleotides) (Perry et al., 1975), indicating that most DRACH sites are not methylated. Thus, additional factors are needed beyond the consensus motif to target methylation to a DRACH site.

One mechanism that could account for the methylation of some DRACH sites versus others is local structure or other contextual features, as seen in yeast (Garcia-Campos et al., 2019; Schwartz et al., 2013). In the case of structure, methylation appears to be more likely to occur in less structured RNA (Sun et al., 2019), likely due to the greater accessibility to the m^{6}A writer complex. However, as described below, gene architecture (i.e. the size and location of exons and introns in a gene) appears to be particularly important for determining which DRACH sites will be methylated in mammals.

Designating adenosine residues or DRACH sites as either “methylated” or “not methylated” is an oversimplification. It is now clear that m^6A is a substoichiometric modification. In other words, at any given adenosine site in an mRNA, only a fraction of the copies of the mRNA actually contain m6A at that site. The low m^6A stoichiometry was first seen in early studies of Rous sarcoma virus using nuclease digestion and chromatography of Rous sarcoma virus RNA (Kane and Beemon, 1985). More recent methods, such as SCARLET, have allowed precise quantification of m^6A stoichiometry (Liu et al., 2013). In SCARLET, an mRNA of interest is site-specifically cleaved using a “SCARLET oligo.” This oligo hybridizes with the target mRNA to create a region of RNA-DNA hybridization that allows RNase H to cleave precisely at the 5’ side of the adenosine residue of interest. Next, this adenosine is selectively radiolabeled, and the RNA is then digested to single nucleotides and, analyzed by thin-layer chromatography. The radiolabeled A and m^6A derived from the exact position in the mRNA will appear as two separately resolved spots. The signal intensity from each spot can be used to calculate the m6A stoichiometry at this position. These experiments generally showed that most methylated sites exhibit low methylation stoichiometry with the highest stoichiometry of 20% at one m^6A residue in the *ß-actin* mRNA (Liu et al., 2013).

A recent m^6A mapping method, called MAZTER-seq, maps m^6A and provides estimates of m^6A stoichiometry on a transcriptome-wide scale (Garcia-Campos et al., 2019). MAZTER-seq takes advantage of a unique ribonuclease called MazF that cleaves mRNA at ACA sequence motifs only if the first adenosine is not N6-methylated. The MAZTER-seq method thus examines global patterns of ACA site resistance to MazF cleavage. This analysis is complicated because ACA motifs are relatively widespread, and therefore MazF treatment leads to high fragmentation of RNA, which prevents unambiguous mapping of sequence data to the reference genome. However, careful analysis can reveal the percent of cleaved and uncleaved ACA sites at ~16% of all previously known m^6A In a MAZTER-seq analysis of mouse mRNA, a set of previously mapped m^6A sites showed a predicted median stoichiometry of ~26% (Garcia-Campos et al., 2019).

However, the median stoichiometry of m^6A may be even lower. As the sensitivity of m^6A mapping increases, more m^6A sites can be identified, typically reflecting m^6A sites of low stoichiometry. For example, DART-seq maps considerably more m^6A sites than other methods (Meyer, 2019). This method involves expressing a fusion protein comprising an m^6A-binding YTH domain fused to the APOBEC RNA-modifying enzyme. This fusion protein causes C→U conversion at the C next to m^6A in proportion to m^6A stoichiometry. Unlike MAZTER-seq, DART-seq allows stoichiometric measurement of m^6A in any sequence context. DART-seq analysis revealed that as many as ~100,000 sites contain m^6A, but most have very low stoichiometry (Figure 2A).

Thus, it is likely that every DRACH site could be methylated to some degree, and DRACH sites should not be considered methylated or unmethylated, but instead should be considered along a wide spectrum of methylation. At any given site, the stoichiometry is likely to be relatively low, but there may be a small subset of DRACH sites that have methylation as high as ~20%, and possibly even higher.

The low stoichiometry of m^6A has important implications for how m^6A could affect mRNA. Since current thinking is that the major function of m^6A in cytosolic mRNA is to induce mRNA degradation (Zaccara and Jaffrey, 2020), the expression of a gene with a single mapped m^6A site would likely not be meaningfully reduced by m^6A unless the stoichiometry is very high, i.e, most of the encoded transcripts indeed contain m^6A at that site. Instead, m^6A is likely to be important primarily for genes that have multiple mapped m^6A sites, since multiple mapped sites would make it more likely that most transcripts have at least one m^6A site for m^6A-mediated mRNA degradation.

Methylation patterns are linked to gene architecture

Mapping studies have also shown that gene architecture (i.e., length and distribution of exons and introns within a gene) is directly linked to global patterns of mRNA methylation. In one of the initial m^6A mapping studies, the authors noted that methylation was disproportionately enriched in the regions of transcripts that corresponded to long internal exons (Figure 2B) (Dominissini et al., 2012). Internal exons are on average ~120 nucleotides, but in some cases can be several hundred or even several thousand nucleotides (Movassat et al., 2019). These long internal exons have a disproportionate likelihood of containing m^6A and thus a higher density of m^6A when corrected for their longer length (Dominissini et al., 2012). Thus, long internal exons may create signals that recruit the methyltransferase complex. Notably, some m^6A sites are located in normal sized exons. These m^6A sites may exhibit lower stoichiometry than m^6A in long internal exons, but this has not been specifically demonstrated.

In addition to long internal exons, m^6A is enriched near the stop codon (Meyer et al., 2012) (Dominissini et al., 2012). The stop codon itself is unlikely to trigger m^6A formation in the nucleus since stop codons are presumably only recognized by translating ribosomes in the cytosol. Although every spliced mRNA has a terminal exon-exon junction, not all mRNAs have mapped m^6A sites near their terminal exon-exon junction. This suggests that additional features lead cause some terminal exon-exon junctions to be more likely to be methylated than others. This feature might be the length of the terminal exon, since the length of the terminal exon is correlated to the levels of m^6A near the stop codon (Ke et al., 2015). Thus, the process of m^6A formation near the terminal exon-exon junction is likely to be caused by events related to the splicing of the last exon or transcription termination.

Overall, methylation of DRACH sites in mRNA is driven, at least in part, by gene architecture. Currently the major question is the precise mechanism by which gene architecture is linked to methylation. This process may involve RNA polymerase II, whose C-terminal domain undergoes phosphorylation in a manner that is related to its location in the gene body, including its location in exons (Nojima et al., 2015). The C-terminal domain has been shown to bind numerous proteins involved in mRNA processing in a phosphorylation-dependent manner (Harlen and Churchman, 2017). Conceivably, RNA polymerase II could bind the m^6A writer complex as well. Alternatively, the m^6A writer complex may bind to genomic regions corresponding to long internal exons. Long internal exons are enriched in the H3K36me3 histone modification (Huang et al., 2019). H3K36me3 was shown to bind METTL14 and proposed to account for selective methylation of the

encoded transcripts (Huang *et al.*, 2019). However, H3K36me3 binding to METTL14 was not observed in a separate study (Vermeulen et al., 2010). Ultimately resolving how gene architecture is related to recruitment of the m^{6}A writer complex will provide insights into the pathways that regulate m^{6}A levels, and will reveal why only some DRACH sites are preferentially methylated.

Is m^{6}A dynamic?

m^{6}A is widely described as a dynamic modification, but the evidence for its dynamics is highly limited and has been challenged. A potential caveat is that the term dynamic may have different meanings. For example, dynamic may refer to the ability of m^{6}A to appear on a transcript, be removed, reappear, then removed in multiple cycles during the lifetime of the single mRNA molecule. This seems unlikely. Methylation occurs only once, i.e. co-transcriptionally (Ke et al., 2017c; Salditt-Georgieff et al., 1976), and demethylation, if it can occur, would be limited to the nucleus as the putative erasers, such as ALKBH5, are predominantly in the nucleus (Zaccara et al., 2019). Thus, rather than being dynamic in this respect, m^{6}A acquires a specific pattern in the nucleus, and retains that pattern throughout its subsequent cytosolic life. In this respect, m^{6}A is not dynamic.

Another definition for dynamic can refer to an increase in m^{6}A levels in mRNA after a stimulus. Importantly, this type of dynamics would not be due to methylation of existing cytosolic transcripts. Since m^{6}A formation is co-transcriptional and nuclear, a stimulus would only change the methylation of any newly made transcripts and would therefore alter the fate of only the newly methylated mRNAs, rather than the pre-existing mRNAs. A variety of different conditions have been suggested to lead to an increase in methylation, including heat shock and DNA damage (Xiang et al., 2017; Dominissini et al., 2012; Zhang et al., 2020).

However, assessing these dynamics by simply measuring total m^{6}A levels in mRNA is not trivial. m^{6}A can be quantified by mass spectrometry, but this requires extensive purification of mRNA from ribosomal RNA and small nuclear RNA, which contain m^{6}A and comprise >90% of m^{6}A in the cell (Legrand et al., 2017). Evidence is usually not presented to document mRNA purity. Thus, differences in m^{6}A between two samples may reflect different levels of contaminating ribosomal RNA. Dot blotting and ELISA-based methods are also widely used due to their simplicity but these methods also suffer from rRNA contamination. Surprisingly, many studies measure m^{6}A in total RNA, with researchers not realizing that they are measuring predominantly m^{6}A from rRNA. These problems make many of the claims of m^{6}A dynamics unsubstantiated.

Another way to detect m^{6}A dynamics is to use m^{6}A mapping datasets that compare different cellular conditions. m^{6}A mapping involves calling m^{6}A peaks based on thresholds that indicate whether m^{6}A-immunoprecipitated reads are above background levels (Figure 2C). In principle an m^{6}A site that shows a larger peak in one condition compared to another would indicate an m^{6}A site that has changed and is thus may be dynamic. However, traditional m^{6}A mapping method produce m^{6}A peaks with variable heights even between replicates (Dierks et al., 2021; McIntyre et al., 2020). In some cases, and m^{6}A site might not

be called because the peak height is just below the threshold for calling these m^{6}A sites in one dataset, but is just above the threshold and called in another dataset. This may give the false appearance of a complete gain or loss of an m^{6}A site. However, based on a statistical analysis of m^{6}A peak heights in previously published studies, Mason and colleagues argued that the vast majority of those reported alterations in m^{6}A were simply due to the noise in peak height measurements rather than true differences in m^{6}A (McIntyre et al., 2020).

To overcome this noise, as well as variability in sample-to sample preparation, Schwartz and colleagues developed m^{6}A-Seq2, which uses barcoded samples that are processed in a single reaction (Dierks et al., 2021). This markedly improves detection of true differences in m^{6}A between samples. Notably, neither m^{6}A-Seq2 nor MAZTER-Seq showed clear differences in m^{6}A distribution on commonly expressed genes between different tissues or in differentiating embryonic stem cells. Approximately 10% of m^{6}A sites showed potential tissue specificity, but these differences may have reflected low expression levels of the mRNAs harboring these m^{6}A sites. Thus, these highly quantitative methods suggest that most m^{6}A sites are conserved, at least in the tested tissues and experimental conditions.

The overall similarity of the m^{6}A maps between cell types and samples supports the idea that m^{6}A is largely “hard-wired,” as described above. An important implication is that m^{6}A mapping may not therefore be needed for most studies. A high-resolution m^{6}A map from one tissue may be used to reasonably predict the likely location of m^{6}A in any other tissue.

Because of the data suggesting that m^{6}A is largely similar between tissues, any proposed differentially regulated m^{6}A sites need to be rigorously documented. In particular, the exact m^{6}A stoichiometry in each condition needs to be measured using methods such as SCARLET. This type of data is generally lacking in the field but is needed to convincingly identify specific dynamic m^{6}A sites and the magnitude of the difference in their methylation status. Identification of these sites will provide a readout that can be used to test molecular pathways that lead to induction of m^{6}A.

m^{6}A reversibility

m^{6}A dynamics could occur through m^{6}A demethylases in the nucleus before being transported to the cytosol. Although this model is appealing, the relevance of demethylases to m^{6}A biology is unclear and inconsistent, except in testes which show marked spermatogenesis defects in *Alkbh5* knockout mice (Zheng et al., 2013). *Alkbh5* knockout mice otherwise appear normal, suggesting that ALKBH5-mediated m^{6}A demethylation is not needed for normal cellular functions or development outside germ cells. Indeed, quantitative m^{6}A mapping assays have suggested that only ~2% of m^{6}A sites might change stoichiometry in somatic cells upon depletion of ALKBH5 (Koh et al., 2019). Conversely, overexpression of ALKBH5 only showed slight decreases of m^{6}A in a small number of transcripts (Garcia-Campos et al., 2019). However, even for these slightly affected sites, it is not clear why these sites and not other m^{6}A sites are affected by ALKBH5. Additionally, it is not clear if the small change in m^{6}A stoichiometry is meaningful for any transcript. Precise measurements of m^{6}A stoichiometry in control and ALKBH5-depleted conditions will help address the importance of ALKBH5-mediated demethylation on specific mRNAs.

The connection between another demethylase, FTO, and m^{6}A is also unclear. Although FTO was first shown to demethylate m^{6}A (Jia et al., 2011), this activity is exceptionally low. It is now known that FTO exhibits markedly more efficient demethylation of m^{6}Am (Mauer et al., 2017), a modified nucleotide that can be found at the first transcribed nucleotide position in mRNA and snRNA. The major target of FTO is the m^{6}Am residues in snRNA, where FTO maintains snRNAs in a demethylated state (Mauer et al., 2019). In FTO-deficient cells, snRNAs have highly m^{6}Am-methylated snRNAs, which can affect snRNA-dependent process, such as splicing.

Since FTO can demethylate both m^{6}A and m^{6}Am, several groups have tried to detect which of these are regulated by FTO in cells. To address this, quantitative m^{6}A mapping methods have been used. These studies have clearly shown that m^{6}A sites are not affected in FTO-depleted cells (Garcia-Campos et al., 2019; Koh et al., 2019). In contrast, transcriptome-wide analysis show clear increases in the methylation of snRNAs, which reflect increased m^{6}Am, after FTO depletion. (Koh et al., 2019; Mauer et al., 2019). Thus, these transcriptome-wide quantitative m^{6}A measurements fail to show that FTO appreciably demethylates m^{6}A.

However to definitively determine if the effects of FTO depletion are due to increases in m^{6}A or m^{6}Am (or both), genetic approaches could be used. *FTO* knockout mice exhibit diverse phenotypes including partial neonatal lethality (Hess et al., 2013; Kim et al., 2021) and FTO-deficient cells show altered gene expression and 3'UTR lengths (Bartosovic et al., 2017) (Mauer *et al.*, 2017). To determine if the effects of FTO are mediated by increasing m^{6}A or m^{6}Am, parallel experiments should be performed with FTO depletion in combination with either METTL3 or PCIF1 depletion. If the effects of FTO are mediated by increasing m^{6}A, then FTO depletion should have no effect in *METTL3* deficient cells. Similarly, if the effects of FTO depletion are due to increased m^{6}Am, then FTO depletion effects would be lost in PCIF1-depleted cells. These controls are important to determine if the biological effects are mediated by m^{6}A or m^{6}Am.

A multiprotein m^{6}A writer complex

m^{6}A dynamics could be mediated by regulation of the ~1 MDa m^{6}A writer complex, which contains METTL3 as its catalytic component (Bokar et al., 1997). METTL3 and its adapter component WTAP were first shown to assemble into a multiprotein writer complex in yeast (Agarwala et al., 2012) and plants (Zhong et al., 2008), and subsequently confirmed in mammalian cells (Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014; Wang et al., 2014). Several additional components were identified, including VIRMA, ZC3H13, RBM15, HAKAI, and METTL14 (Guo et al., 2018; Patil et al., 2016; Ruzicka et al., 2017; Schwartz et al., 2014). Except for METTL14, little is known about these proteins. METTL14 was initially proposed to be a separate methyltransferase that catalyzes m^{6}A alongside METTL3 (Liu et al., 2014). However, it is now clear that METTL3 and METTL14 form a single functional heterodimeric enzyme (Wang *et al.*, 2014); (Sledz and Jinek, 2016; Wang et al., 2016a; Wang et al., 2016b). Structural studies showed that METTL3 comprises the catalytic SAM-binding component, while METTL14 appears to position the RNA for

methylation and allosterically activates METTL3. Thus, m^6A catalysis is mediated by a single heterodimeric methyltransferase which comprises METTL3 and METTL14.

The mechanism by which the other writer complex components control m^6A levels is not clear. Depletion of any of these proteins results in delocalization of METTL3 from nuclear speckles and loss of most, if not all, m^6A in mRNA (Bawankar et al., 2021; Knuckles et al., 2018; Ping et al., 2014; Yue et al., 2018). Thus, these components may have a role in placing METTL3 near nascent RNA for co-transcriptional methylation.

It will be important to determine if the writer complex components can be regulated by signaling pathways to activate or inactivate the writer complex to achieve different levels of methylation throughout the transcriptome, or possibly even on unique subsets of mRNAs.

Cytoplasmic readers: YTHDF1, 2, and 3 bind all m^6A sites

m^6A in mRNA exerts its effects by binding to m^6A “reader” proteins. The most well-established m^6A readers are YTHDC1 (“DC1”), which is found in the nucleus, and YTHDF1, 2, and 3 (DF1, DF2, DF3), which are cytosolic. Besides these four proteins, YTHDC2 is the only other mammalian protein to contain the YTH domain, which is thought to bind m^6A. However, YTHDC2 is primarily found in germ cells (Wojtas et al., 2017) and may not bind or regulate m^6A sites in vivo (Saito et al., 2022) (Li et al., 2022). In contrast, DC1 is poised to mediate nuclear processing events due to its nuclear localization, while DFs can regulate cytoplasmic mRNA processing.

DF1, DF2, and DF3 are paralogs that have high amino acid identity across their entire length. These proteins are primarily composed of low complexity domain sequence, comprising several P/Q/N-rich patches and other low-complexity regions (Patil et al., 2017). The C-terminal region contains the YTH domain (Figure 3A). The high proportion of low-complexity domain sequence is consistent with these proteins functioning in intracellular condensates. Recent studies have demonstrated that the three DF proteins are enriched in P-bodies, stress granules, and other cytosolic condensates, and that the DF proteins undergo phase separation when they interact with polymethylated RNAs (Ries et al., 2019; Zaccara and Jaffrey, 2020). Thus, DF protein function and regulation is likely related to condensate biology.

Recently, the binding properties and function of DF proteins have undergone a major revision, which has reshaped our understanding of both the DF proteins and m^6A more generally. The prevailing model was that each of these proteins binds a subset of m^6A sites throughout the transcriptome (Shi et al., 2017; Wang et al., 2015). That is, some of these m^6A sites are uniquely bound by DF1, some by DF2, some by DF3, and some sites are bound by combinations of two or three of the YTHDF proteins. In this model, there was no clear pattern or mechanistic basis for the distinct binding events. Thus, the actions of any single m^6A site are unpredictable until the DF reader(s) that bind to it are identified.

Importantly, the finding that the DF proteins can bind different m^6A sites is perplexing since the YTH domains in the three DF proteins are nearly identical. The crystal structures of YTH domains of three DF proteins show identical interactions with m^6A. A biophysical

study of all three YTH domains shows similar intrinsic molecular dynamics and affinity to m^{6}A (Li et al., 2020a). Thus, the reported differences in binding to m^{6}A was contradictory to the similar sequence and binding properties of the DF proteins. No mechanism was proposed for these putative differences in binding preference.

After extensive reanalysis of the CLIP data from the previous studies, we recently showed that the DF paralogs bind all m^{6}A sites in an essentially equivalent manner (Zaccara and Jaffrey, 2020). Our study concluded that there were no sites that were uniquely bound by one DF protein versus another DF protein. Several problems were identified in the earlier studies in which m^{6}A sites were designated as “DF1 unique” or “DF2 unique” (Wang et al., 2015). Each of these unique sites were shown to have considerable reads for the other DF proteins based on the same PAR-CLIP datasets used to identify sites as unique (Zaccara and Jaffrey, 2020). Direct comparison of CLIP datasets showed that peaks derived from DF1, DF2, DF3, and m^{6}A CLIP datasets show peaks that are essentially indistinguishable on essentially all genes.

The main problem with the earlier analyses was that they relied on calling binding sites based on thresholds. In this approach, many peaks are just above or below the threshold, and thus these borderline peaks are essentially arbitrarily called or eliminated as binding sites (Figure 2C). In the more recent studies, the CLIP datasets were reanalyzed without using thresholds. Instead, the analysis was based on the binding of each DF paralog at each m^{6}A site in the transcriptome. To measure binding, the CLIP reads that overlapped each m^{6}A site was used (Figure 3B). When analyzed this way, it was clear that all m^{6}A sites show essentially equivalent binding to DF1, DF2, and DF3. Another independent study in mouse embryonic stem cells also reached the same conclusion based on strong correlations of the binding properties of all three DF paralogs (Lasman et al., 2020). Thus, different m^{6}A sites are not linked to specific readers—each m^{6}A shows equivalent ability to bind any DF protein.

Although the m^{6}A directly binds each DF paralog (Patil et al., 2018), m^{6}A can indirectly affect the RNA-binding of other proteins. m^{6}A has a tendency to reduce RNA structure due to the low stability of m^{6}A•U basepairs (Liu et al., 2015). The subsequent unfolding of RNA near m^{6}A sites has been proposed to explain the m^{6}A-dependent binding activity of several RNA-binding proteins, such as hnRNPC, hnRNPA2B1, and IGF2BP3 (Sun et al., 2019) (Wu et al., 2018) (Liu et al., 2015). Conceivably, any RNA-binding protein that binds to a single-stranded motif will show enhanced binding if m^{6}A is near the binding site. It is currently unclear if any of these m^{6}A-dependent “structure switches” have an impact on physiology.

Redundant functions for YTHDF1, 2, and 3

Another fundamental change in the m^{6}A field was the recognition that the DF proteins do not have distinct functions, but instead serve the same overall function of promoting mRNA degradation. The prevailing idea was that DF1 and DF3 enhance translation, while DF2, typically the most abundant of these paralogs, promotes mRNA degradation (Shi et al., 2017; Wang et al., 2015). Thus, the model suggested that m^{6}A can either cause mRNA

degradation or enhanced translation depending on the bound DF protein. The conclusion that DF1 promotes translation was based on ribosome profiling data in DF1-depleted cells, which showed reduced translation of DF1-binding mRNAs. Additionally, DF1 was shown to bind translation initiation factor eIF3 (Wang et al., 2015). eIF3 binds in the 5' UTR to position the ribosome near start codons for translation. The overall mechanism was problematic since eIF3 recruitment to mRNA stop codons and 3'UTRs, which is where m^6A is generally located, is not consistent with the mechanism by which eIF3 promotes translation (Lee et al., 2015).

The function of the DF proteins, and thus the view of the function of m^6A, was fundamentally revised in recent studies (Kontur et al., 2020; Lasman et al., 2020; Zaccara and Jaffrey, 2020). Technical problems were identified in the original ribosome profiling studies of DF1 (Wang et al., 2015). The reanalysis showed that the individual replicates, when analyzed separately, were divergent in their conclusions. (Zaccara and Jaffrey, 2020). Therefore, the ribosome profiling studies were replicated, which showed that there was no effect on m^6A mRNA translation upon depletion of any or all DF proteins (Lasman et al., 2020; Zaccara and Jaffrey, 2020). In a recent study, He and colleagues re-examined the effect of DF1 depletion on the translation of m^6A-contaiing mRNAs, which were defined using a new method for mapping m^6A sites. In this study they showed no statistically significant change in translation of methylated vs. non-methylated mRNAs (Hu et al., 2022). Overall, these studies suggest that DF1 does not promote translation of m^6A RNAs.

To determine the function of the DF proteins, protein interactome analysis was used. This approach showed that all three DF paralogs have highly similar binding partners, most notably proteins associated with the CCR4-NOT RNA degradation complex (Figure 3C) (Zaccara and Jaffrey, 2020). Interactions with eIF3 were not detected as significant interactors. The finding that the DF proteins have the same interactors suggested that they may have the same function. When the DF proteins were depleted individually, m^6A mRNA stabilization was generally weak. However, when all three paralogs were depleted, there was considerable stabilization of m^6A mRNAs (Lasman et al., 2020; Zaccara and Jaffrey, 2020). Notably, this effect was in proportion to the number of annotated m^6A sites. These studies supported the new model that DF proteins function together in a redundant manner to promote mRNA degradation.

Studies using mouse genetics came to the same overall conclusion, showing that the DF proteins function together to mediate the essential role of m^6A in embryogenesis (Lasman et al., 2020). Lasman and colleagues expressed DF1, DF2, or DF3 separately in DF triple knockout mouse embryonic stem cells. Re-expression of any DF rescued the differentiation defect of the triple knockout cells. This result showed that DF proteins interchangeably mediate the effects of m^6A in promoting embryonic stem cell differentiation. Furthermore, they concluded that the tissue-specific phenotypes seen upon knockout of different DF paralogs during mouse development can be explained by the expression pattern of each DF protein in these different tissues (Lasman et al., 2020). In another study, DF protein redundancy was also shown to promote zebrafish embryonic development (Kontur et al., 2020). Overall, these studies support the physiologic relevance of DF protein redundancy in mediating the effects of m^6A.

Although the overall function of the DFs is redundant, this does not mean they are identical or completely interchangeable. These are slight differences in the amino acid sequences in the low-complexity domains of these paralogs. These differences could result in differences in phase separation behavior, interaction with specific proteins, or regulation by post-translational modification. If any DF-specific function is observed, it will be important to determine the precise mechanism for these differences given the high similarity of these paralogs.

Does this mean that m^6A does not affect mRNA translation rates at all? Indirect mechanisms following m^6A-mediated mRNA degradation could lead to translational changes. For example, the expression of ribosomal genes were elevated in single or triple DF knockout embryonic stem cells (Lasman et al., 2020). This could lead to increases in translation. In neurons, DF1 depletion does not affect translation under normal conditions, but affects translation induced by potassium depolarization (Shi et al., 2018). These effects can readily be explained by m^6A-mediated degradation of specific mRNAs that encode regulators of these translation pathways. In the case of AML (acute myeloid leukemia), reduced translation was seen upon depletion of m^6A (Vu et al., 2017). However, depletion of m^6A causes AML cells to undergo differentiation, which itself leads to changes in the expression levels and translation of diverse transcripts (Spevak et al., 2020). Thus, in the absence of a plausible molecular mechanism that links DF proteins to translation, effects of m^6A on translation are more likely to be indirect effects of m^6A. It should be noted that m^6A in the 5'UTR has also been shown to promote translation by directly interacting with eIF3, a major translation initiation factor (Meyer et al., 2015). However, the number of mRNAs with 5'UTR is low (Boulias et al., 2019), making it likely that this mechanism is only relevant for a few mRNAs.

Together, these newer studies have revealed a unified model in which the primary function of m^6A is to bind to DF proteins and to promote the degradation of m^6A-modified mRNAs (Figure 3D). Although the function of m^6A is now clearly linked to controlling mRNA stability, questions remain regarding whether all m^6A sites are equally able to induce mRNA degradation, and how DF-mediated degradation could be activated.

Regulation of nuclear mRNA processing with a nuclear m⁶A reader

A major question is whether the effects of m^6A are primarily mediated by the nuclear m^6A reader, DC1, or the DF cytosolic readers. Insights into this question can be seen in the using datasets such as the Broad Institute's Dependency Map (DepMap) (Tsherniak et al., 2017). DepMap is a large scale loss-of-function database in which the degree of dependency of over 10,000 genes was assessed in over 1000 cell lines using CRISPR-mediated knockout. Every gene is assigned a dependency score for each cell line which relates to the degree that the gene knockout reduces cell proliferation or survival. Using DepMap datasets, genes whose effects on growth are strongly correlated with a gene of interest across 1000+ cell lines can be readily identified. This co-dependency analysis can identify genes with similar functions or which operate in similar pathways (Shimada et al., 2021). In the case of *METTL3*, the genes that show the most similar profile of gene dependency across the thousand cell lines are the other components of the m^6A writer complex, i.e., *METTL14*, *VIRMA*, *ZC3H13*,

RBM15 and *WTAP*. However, the next most correlated gene is *YTHDF2*, the most abundant of the three DF paralogs in the majority of cell lines (Figure 3E). Importantly, DC1 is not identified among the top 100 genes that show co-dependency with METTL3. These data suggest that DF2, likely in concert with the other redundant DF paralogs, mediates the effects of METTL3 depletion, especially with respect to cell growth and proliferation assayed in the DepMap. Importantly, this does not rule out roles for DC1 in mediating other effects of m^6A which are not linked to cell proliferation or survival.

Although DF proteins appear to mediate major aspects of m^6A biology, DC1 also regulates m^6A-dependent functions in the nucleus. Prior to its connection to m^6A, DC1 was identified in a yeast two-hybrid screen as an interactor of TRA-2β, a component of the spliceosomal complex (Hartmann et al., 1999; Imai et al., 1998). Nearly all nuclear mRNA processing events have been linked in some way to m^6A and DC1, including splicing, nuclear export, polyadenylation site selection, nuclear RNA degradation, and epigenetic regulation (Figure 4) (Mirza and Jaffrey, 2020; Roundtree et al., 2017b; Xiao et al., 2016). Like the DF proteins, DC1 is primarily composed of low-complexity sequence and forms biomolecular condensates upon binding m^6A RNA (Cheng et al., 2021). This property likely accounts for the localization of DC1 in nuclear condensates. The DC1 condensates were originally referred to as YT bodies (Nayler et al., 2000), and partially overlap with nuclear speckles (marked by SC35) and super enhancer condensates (marked by BRD4), and some structures that overlap with neither (Cheng et al., 2021).

A major target of DC1 are nuclear noncoding RNAs, such as *MALAT1* and *XIST*, based on CLIP studies (Patil et al., 2016). This preferential enrichment of DC1 on nuclear ncRNA likely reflects the long residence time of ncRNA in the nucleus compared to the short time that mRNAs are found in the nucleus. Nevertheless, DC1 could still have access to mRNA during or shortly after transcription, which can thus provide the opportunity for DC1 to regulate nuclear mRNA processing.

The function of DC1 has been studied by depleting DC1 in cells. Although mRNA processing has been shown to be affected by DC1 depletion, some of these effects could be indirect. Since DC1 is bound to noncoding RNAs that contribute to nuclear structures like nuclear speckles (Patil et al., 2018), depletion of DC1 could impact nuclear architecture and nuclear processing bodies. In this way, depletion of DC1 would appear to affect different aspects of mRNA metabolism.

Consistent with the idea that DC1 depletion can have indirect effects are studies of DC1 interactions with MALAT1 (Patil et al., 2016; Wang et al., 2021b). In this study, DC1 was found to localize to nuclear speckles in part by its ability to bind *MALAT1*. When the m^6A-containing region of *MALAT1* was deleted, DC1 was no longer efficiently localized to nuclear speckles. Importantly, DC1 depletion also caused the nuclear speckles to show altered protein composition, indicating that DC1 is needed for the proper assembly of nuclear speckles. These studies highlight the potentially broad impact of depleting DC1 in the nucleus.

One potential way to determine if an effect of DC1 on an mRNA is direct, is to determine if the effect of DC1 is proportional to the number of m^6A sites in the mRNA. This is known to be the case with the DF proteins which promote m^6A-mediated degradation in proportion to the number of m^6A sites in the transcript. In contrast, the proposed direct effect of m^6A and DC1 in mediating nuclear export or nuclear degradation (Widagdo et al., 2021) has not been shown to correlate with the number of m^6A sites. The idea that m^6A is needed for nuclear export is problematic since non-methylated mRNAs are also clearly exported from the nucleus. It should be noted that there is data supporting the idea these effects of DC1 are direct, including data showing that DC1 binds components of the mRNA splicing and nuclear degradation complex (Imai *et al.*, 1998; Roundtree *et al.*, 2017b).

An additional function for DC1 is to control splicing. In Drosophila, m^6A promotes the splicing of *sex-lethal* (*Sxl*) by binding Drosophila DC1 (Haussmann et al., 2016; Kan et al., 2017; Lence et al., 2016). In mammals, DC1 was originally found to promote exon skipping of *Srsf3* splicing reporter gene (Hartmann *et al.*, 1999). More recent study found DC1 depletion influences >2,000 alternative splicing events in a manner that was dependent on its YTH domain (Xiao et al., 2016). These effects may reflect DC1 recruiting SRSF3 to m^6A sites to promote exon inclusion. Binding of DC1 to m^6A simultaneously leads to displacement of SRSF10, a factor that promotes exon skipping (Xiao et al., 2016).

However, other studies have suggested that m^6A has a very limited role in splicing regulation. In *Mettl3*$^{-/-}$ mouse embryonic stem cells, approximately 3% of all exons exhibit alternative splicing (Ke et al., 2017a), and of these less than 100 contain an m^6A site (Geula *et al.*, 2015; Ke et al., 2017b). These data suggest a minimal role for m^6A as a direct regulator of splicing.

To distinguish direct from indirect effects of m^6A a recent study developed a chemogenetically destabilized METTL3 that allows METTL3 protein to rapidly degraded after addition of a small molecule (Wei *et al.*, 2021). This acute METTL3 depletion blocks co-transcriptional m^6A formation but does not give time for indirect effects to occur, such as changes in gene expression or alteration in nuclear architecture. Acute METTL3 depletion caused pronounced effects on the splicing of a small number of transcripts, including several related to the m^6A pathway. In particular, DC1 and WTAP both contain an m^6A-containing exon that becomes alternatively spliced upon m^6A depletion, allowing production of full-length functional protein in a DC1-dependent manner (Ke *et al.*, 2017c; Wei *et al.*, 2021). This study emphasizes how acute depletion of METTL3, using either chemogenetic approaches or newly described METTL3 inhibitors (Yankova et al., 2021), can help identify direct effects of m^6A on nuclear mRNA processing events.

Connecting m^6A to epigenetic regulation and transcription

DC1 may also have important roles in regulating epigenetic silencing (Figure 4). DC1 is bound to m^6A sites on *XIST*, a noncoding RNA that mediates X chromosome inactivation in female mammalian cells (Engreitz et al., 2013). DC1 promotes XIST-mediated gene silencing (Patil et al., 2016), although notably the effects of m^6A and DC1 are not as pronounced as other XIST regulators such as SPEN (Nesterova et al., 2019). Notably, *XIST*

forms liquid-like condensates associated with low-complexity domain proteins (Cerase et al., 2019). DC1 may thus contribute to the formation of these *XIST* biomolecular condensates.

DC1 has also been shown to contribute to epigenetic silencing in other contexts, such as silencing of highly m^{6}A-methylated retrotransposons (Liu et al., 2021) and endogenous retroviruses (Xu et al., 2021). In both cases, DC1 facilitates the deposition of the repressive H3K9me3 mark by facilitating recruitment of the histone 3 lysine 9 (H3K9) tri-methyltransferase SETDB1 and its cofactor TRIM28 (Xu et al., 2021) to nascent mRNA. It should be noted that another study found that the same retrovirus family is also degraded by DF proteins in cytoplasm (Chelmicki et al., 2021). These results suggest that m^{6}A suppresses retroviruses both through epigenetic suppression and mRNA degradation.

m^{6}A and DC1 have also been shown to have the opposite effect and promote gene activation. YTHDC1 recruits KDM3B to nascent m^{6}A mRNA to promote H3K9me2 demethylation and enhance gene expression of approximately 30% of m^{6}A-containing transcripts (Li et al., 2020b). However, non-m^{6}A genes were also regulated by this pathway, and the relationship between m^{6}A number and gene activation was not addressed. It remains unclear how DC1 can promote H3K9 methylation in one context and suppress H3K9 methylation in another.

m^{6}A can also regulate gene expression through methylation of enhancer RNAs (eRNAs) and other chromatin-associated regulatory RNAs (carRNAs). Upon binding DC1, these RNAs can regulate gene expression from nearby promoters. However, the effects of DC1 binding to these RNAs were opposite in different studies (Lee et al., 2021; Liu et al., 2020a) . In one study, DC1 binding to m^{6}A on carRNAs, which normally upregulate transcription from the nearby genes. As a result, DC1 attenuated transcription from the nearby promoter (Liu et al., 2020a). In another study, DC1 interacted with eRNA to enhance transcription by forming enhancer-associated condensates that recruited the transcriptional coactivator BRD4 (Lee et al., 2021). As with the studies of DC1 and H3K9 methylation, it is not clear why the effects of m^{6}A appear to be different in these different contexts.

A major goal moving forward is to understand the mechanism for the divergent findings of m^{6}A in different assays. Since the observed effects of DC1 depletion could be indirect consequences of alteration of nuclear structure, the use of acute inhibition approaches will be useful to distinguish direct from indirect effects of m^{6}A. These approaches may help to create a more coherent model of how m^{6}A effects transcription.

Future directions and perspectives

Our understanding of m^{6}A has advanced considerably in the past few years. m^{6}A was previously viewed as a dynamic modification that showed tissue-specificity, thus allowing m^{6}A to encode unique patterns of gene expression. Although there is clear evidence for regulation, the overall m^{6}A landscape appears highly similar across tissues. This conservation of m^{6}A sites is likely because gene architecture, which is conserved in all tissues, is a major driver of m^{6}A formation. m^{6}A-marked mRNAs define a cohort of

functionally related transcripts that are coordinately regulated, via control of m^{6}A writing or reading, as part of various developmental and other processes. If m^{6}A induction exists, it will be important to determine if these pathways affect all m^{6}A sites, e.g. by globally increasing the efficiency of methylation, or if m^{6}A induction can be targeted to specific sites. In either case, clear documentation of the exact change in stoichiometry of specific adenosine residues will needed. As the field adopts quantitative mapping of m^{6}A, coupled with site-specific validation, we expect that questions of whether m^{6}A is dynamic, whether it can be demethylated, as well as the circumstances, can be answered. Another major question is whether the m^{6}A writer complex is regulated. The writer complex comprises many proteins of unknown function. It seems likely that these proteins could be regulated in ways to influence m^{6}A writing. It will be important to determine if the writer complex is regulated at the level of its assembly, or its recruitment to the transcription machinery, or through other mechanisms.

It remains possible that m^{6}A writing is relatively constant, but the regulation occurs primarily at the level of m^{6}A reading. In this way, the degradation of m^{6}A-marked mRNAs is increased or decreased depending on specific cellular pathways. In this case, the overall m^{6}A level can change depending on the efficiency of m^{6}A-mediated degradation. A major goal is to identify the signaling pathways that can regulate the degradation activity of DF proteins.

Part of understanding the m^{6}A pathway and how m^{6}A reading is regulated is to examine the potential function of protein interactors of DF proteins. DF proteins have a large number of interactors based on proximity labeling experiments, including IGF2BP proteins, PRRC2A, FMRP and others (Wu et al., 2019; Zaccara and Jaffrey, 2020; Zhang et al., 2018; Youn et al., 2018). Thus, DF proteins may exist as a “reader complex” and could be regulated via these interaction partners. Notably, these same interactors have also been shown to be m^{6}A readers in some studies based on their ability to be pulled down with m^{6}A-containing RNA probes (Edupuganti et al., 2017; Huang et al., 2018; Wu et al., 2019). Thus, it is not clear if their binding to m^{6}A mRNA was due to their ability to bind to DF proteins, which are known m^{6}A interactors. Further understanding of these DF-binding proteins will likely reveal insights into the regulation of m^{6}A reading.

Another major question is to understand the molecular basis for the unusual topology of m^{6}A, most notably its enrichment near stop codons and in long internal exons. It will be important to understand the mechanism for the specificity of m^{6}A deposition and if gene structure is guiding this effect. It will be important to determine if the location of m^{6}A in an mRNA influences the functional effects of m^{6}A, or if all m^{6}A re functionally equivalent in mediating m^{6}A-mediated degradation.

Although it is clear that the major effect of m^{6}A is to mediate degradation of a cohort of m^{6}A-marked mRNAs, an important future direction is to resolve the nuclear effects of m^{6}A, especially in light of the numerous contradictory studies regarding m^{6}A and epigenetic regulation (Figure 4). The indirect effect of m^{6}A depletion on nuclear architecture could be profound and thus the use of newer methods for acute m^{6}A depletion using chemogenetic

approaches (Wei et al., 2021) or new specific METTL3 inhibitors (Yankova et al., 2021), should help to resolve direct from indirect effects.

In addition to the basic molecular mechanisms of m^6A reading and writing, an important future direction is to understand how m^6A affects cellular physiology. Since there are so many different m^6A-containing mRNAs, deregulation of diverse m^6A-containing mRNAs have been used to explain the phenotypes of m^6A depletion in cells and tissues. However, it is possible that the effects of m^6A can be rationalized based on its ability of regulate the expression of a handful of master regulatory proteins that control major signaling pathways. As mentioned above, m^6A is found on transcripts that encode the tumor suppressor APC and the pluripotency transcription factor Nanog and Klf4, which regulate tumor progression and differentiation, respectively. Control of these pathways could contribute to the proliferative and differentiation effects associated with m^6A depletion. m^6A is also found on transcripts encoding interferon-ß (Rubio et al., 2018; Winkler et al., 2019) as well as *Stat1* and *Irf1* (Wang et al., 2020), all of which play central roles in the type I interferon pathway. Thus, by controlling these master regulators, m^6A suppresses an entire interferon-regulated gene expression network. It will be important to determine the major pathways controlled by m^6A and how these act together to mediate the physiological effects of m^6A.

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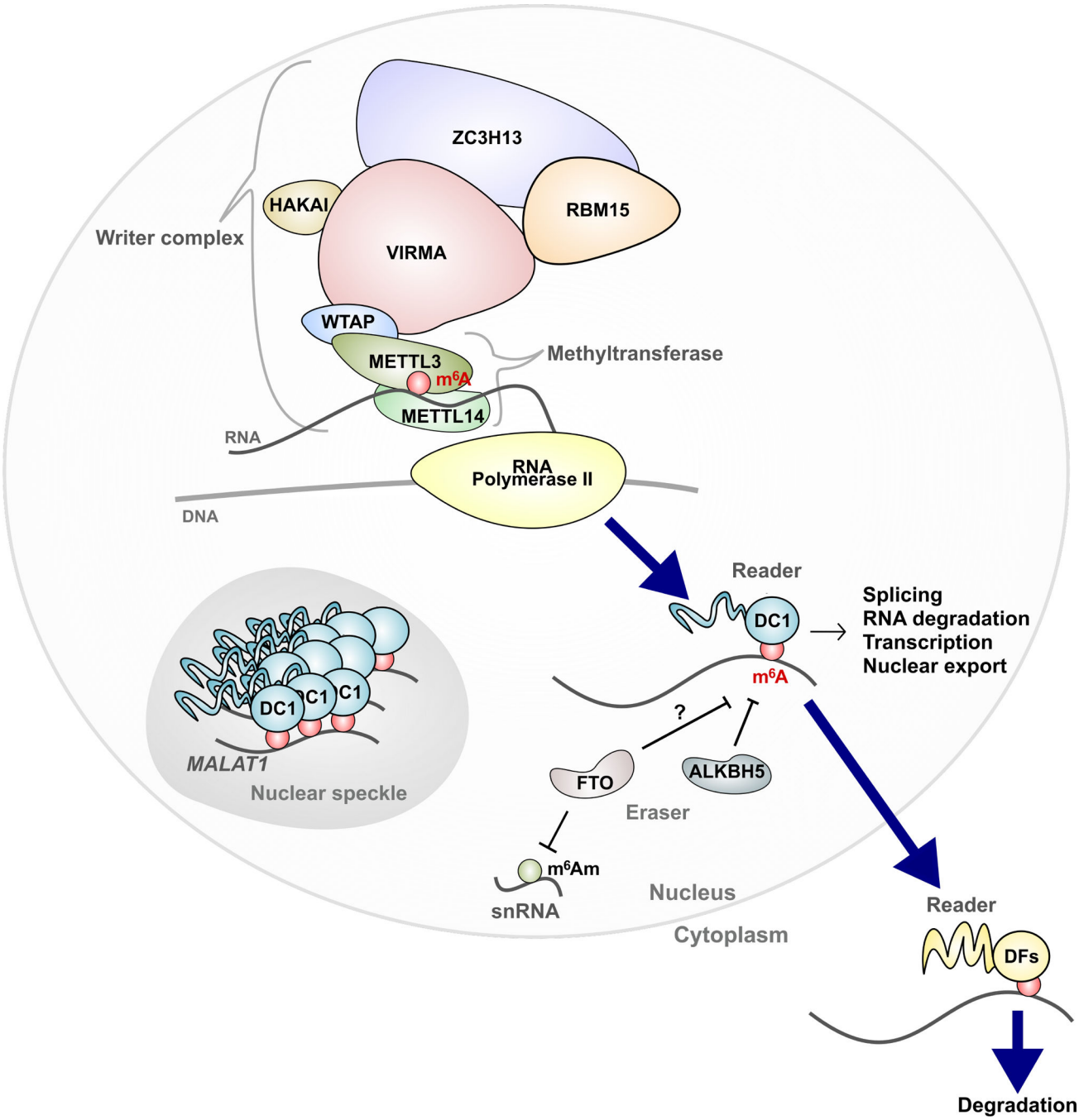

Figure 1. Writing and reading m^{6}A

m^{6}A is deposited co-transcriptionally by a writer complex. The catalytic subunit METTL3 forms a heterodimer with METTL14 to form the minimal methyltransferase core complex. METTL3/14 is a component of a larger writer complex, and each protein is required for m^{6}A deposition in the cell. YTHDC1 (DC1), a nuclear m^{6}A-binding protein, is assembled into nuclear condensates via m^{6}A-containing RNA, including the nuclear noncoding RNA MALAT1. These condensates, such as nuclear speckles, are important for various processing steps that most mRNAs undergo in the nucleus. DC1 also binds and regulates specific m^{6}A

RNA to regulate splicing in some transcripts, or to influence epigenetic marks, transcription, RNA stability, or nuclear export. In the nucleus, m^6A may be erased, particularly in testes where the nuclear m^6A eraser ALKBH5 is enriched. FTO is an efficient demethylase for m^6Am in snRNA, and may also demethylate m^6A. Once exported to the cytoplasm, m^6A is bound by DF proteins, which comprise YTHDF1, YTHDF2, and YTHDF3, which all promote mRNA degradation (Bawankar et al., 2021; Schöller et al., 2018; Knuckles et al., 2018).

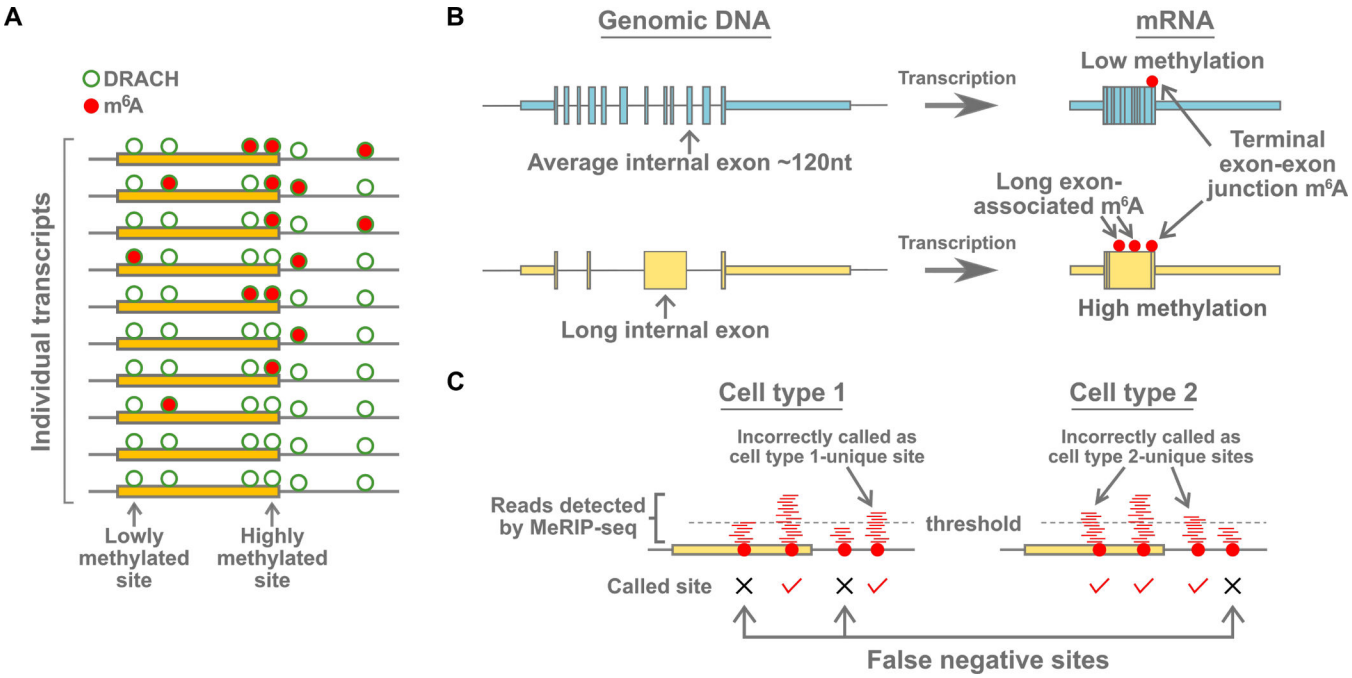

Figure 2. Deposition and detection of m^{6}A

(A) m^{6}A is a low stoichiometry modification. At any given m^{6}A site in a specific mRNA, only a small number (usually less than 20%) of the transcript copies in the cell will have m^{6}A at that site. Most of the DRACH m^{6}A consensus sites are not methylated (green circle). Additionally, some DRACH sites are more methylated than others, and all DRACH sites are likely methylated to some degree, although the stoichiometry is likely very low. The molecular mechanism that causes some DRACH sites to be more methylated than others, even in the same transcript, are not fully understood. Although m^{6}A mapping may reveal multiple m^{6}A sites for a given mRNA, the individual transcripts that comprise the m^{6}A annotation are typically methylated only at a subset of sites. Newer single-transcript analysis methods have revealed the stochastic nature of methylation on individual transcripts.

(B) Gene architecture influences m^{6}A deposition. Shown (left) are examples of two genes, one containing a long internal exon. After transcription (right), m^{6}A is preferentially formed on the regions of transcripts corresponding to long exons. m^{6}A is also enriched near the terminal exon-exon junction, particularly in mRNAs with long 3'UTRs. The correlation of m^{6}A with these genomic features suggests that the writer complex is regulated by other events that are responsive to gene architecture.

(C) Putative differences in m^{6}A are sometimes artifacts of the methods used to call m^{6}A sites from mapping data. Many studies have used mapping methods such as MeRIP-seq to map m^{6}A and to compare transcriptome-wide distributions of m^{6}A between two different cell conditions. These methods map reads (red lines) immunoprecipitated by an m^{6}A-binding antibody. m^{6}A sites are often called based on whether there are a sufficient number of reads above a threshold. However, the number of reads that map to any m^{6}A sites can be very variable, even between replicates (McIntyre et al., 2020). Thus, the same m^{6}A site in one sample might be called in one sample, but not the other, if the number of reads

just passes or misses the threshold. For this reason, the differences in the transcriptome-wide distribution of m^6A in many experiments may have been highly overestimated.

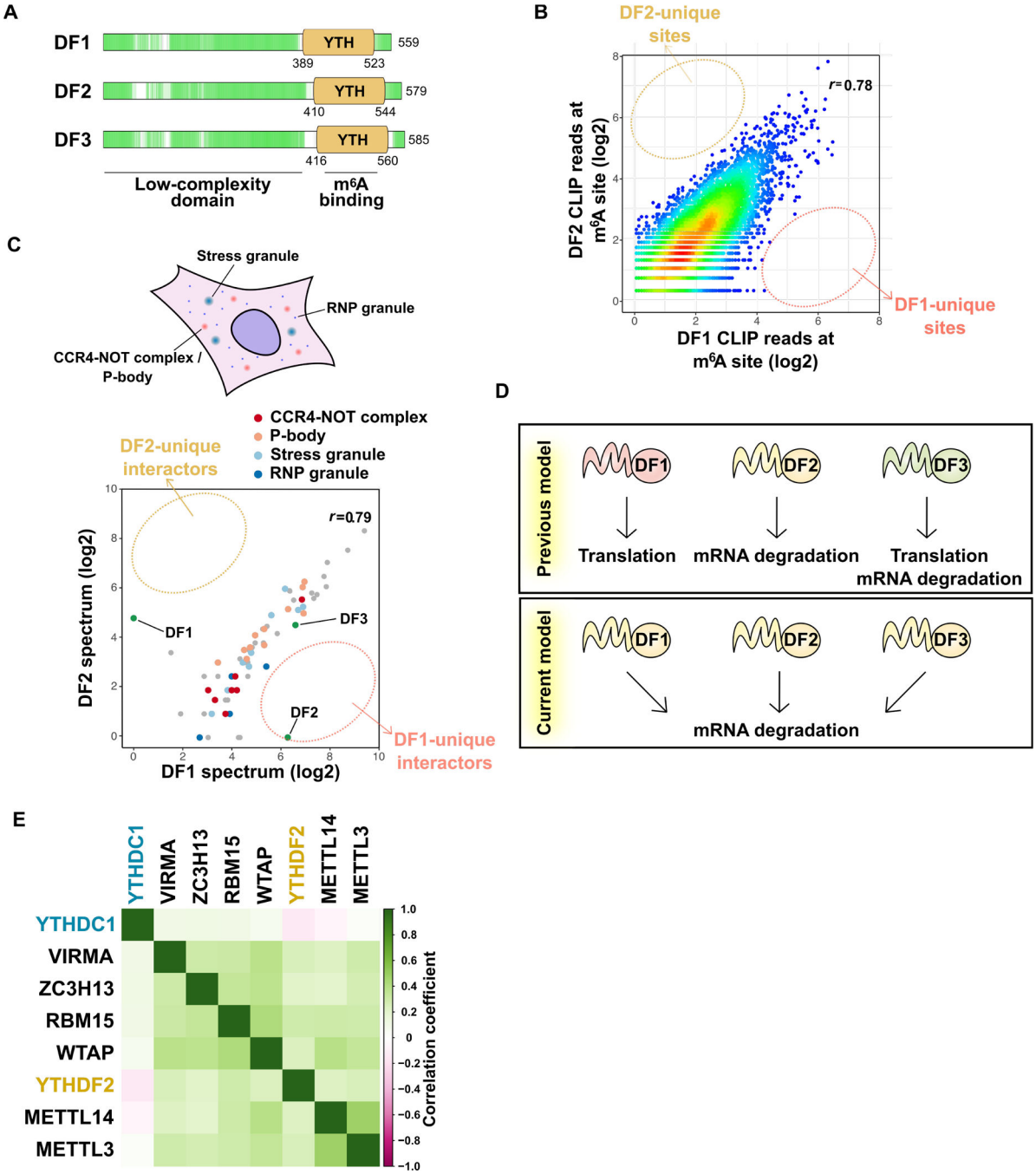

Figure 3. YTHDF proteins function together to promote degradation of m^{6}A-marked mRNAs

(A) DF proteins are low-complexity domain-containing “m6A reader” proteins. All three paralogs are highly similar and contain a proline-, glutamine-, and asparagine-rich low-complexity domain (green) followed by m^{6}A-binding YTH domain. The low-complexity domain allows these three DF proteins to localize to granule structures such as RNP granules, P-body, and stress granules.

(B) All DF paralogs bind to all m^{6}A sites, with no DF paralog showing preferential binding to any m^{6}A site. Shown is a pairwise comparison of DF1 and DF2 iCLIP coverage at each

single nucleotide-resolved m^{6}A site in HEK293T cells. The color indicates the density. *r*, Pearson correlation coefficient. Similar results are seen when comparing DF3 to either DF1 or DF2 (Zaccara and Jaffrey, 2020).

(C) All three DF paralogs likely serve the same function since they interact with same sets of proteins. Pairwise comparison of the probabilities of interaction for DF1- and DF2-interacting proteins determined by proximal labeling and proteomics (Youn et al., 2018). Proximal proteins were detected by DF1 or DF2 C-terminally tagged with promiscuous biotin ligase (BirA). The average probability of interaction for either DF1 or DF2 or both higher than 0.9 are shown. *r*, Pearson correlation coefficient.

(D) Revised model of the YTHDF protein function. DF1 and DF3 were previously shown to promote translation, while DF2 and DF3 enhance mRNA degradation. More recent studies show all three DF proteins redundantly promote mRNA degradation.

(E) DF2 is the major protein mediating the effects of m^{6}A on cell survival and proliferation. DepMap analysis reveals genes that show similar patterns of gene dependency as METTL3 across over 1000 different cell types. Members of the writer complex show the highest similarity to METTL3 in the different cell lines. The DF2 also shows a similar type of gene dependency as METTL3. In contrast, the pattern of cell dependency on DC1 shows poor correlation with METTL3. Dependency scores for m^{6}A writers and readers for each cell line were analyzed by pairwise comparison. Pearson correlation coefficients were visualized in the matrix heatmap upon hierarchical clustering.

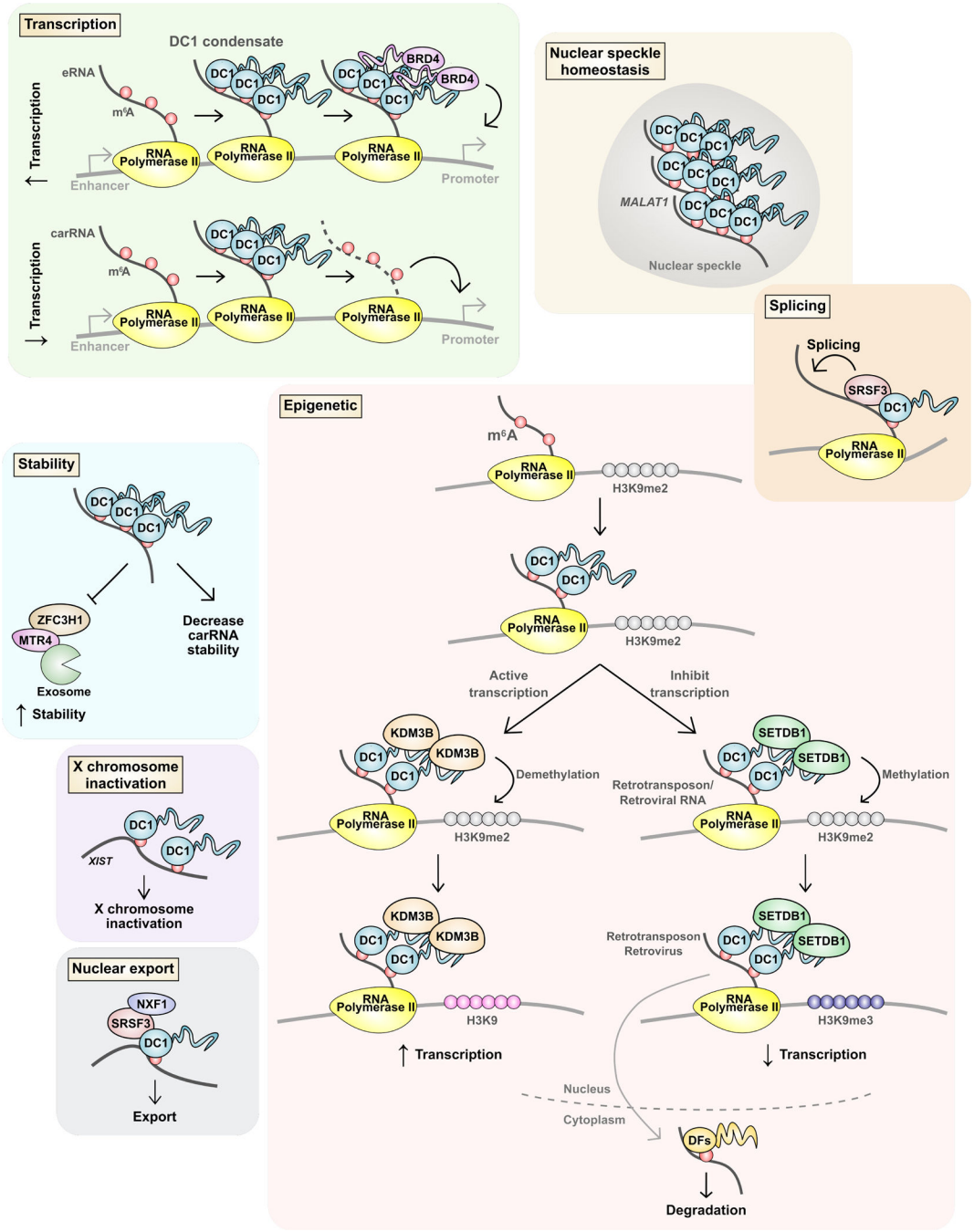

Figure 4. DC1 regulates diverse nuclear processing events through m^{6}A

DC1 has been reported to exert a wide range of effects on mRNA. Some studies suggest transcription regulation by DC1. DC1 can enhance transcription by binding m^{6}A-containing enhancer RNAs (eRNAs). DC1 forms a condensate with eRNAs that recruits BRD4. DC1 has also been shown to bind “chromatin-associated regulatory RNAs” (carRNAs), which broadly refers to many types of nuclear RNAs including eRNAs. DC1 was shown to mediate the degradation of carRNAs and subsequently reduce nearby gene expression. DC1 also has been shown to increase and decrease methylation of H3K9. In some studies, DC1 binds m^{6}A

mRNA and recruits KDM3B to demethylate H3K9me2 and enhance transcription. Other studies show that DC1 binds retrotransposon RNA and recruits SETDB1 to form H3K9me3 to reduce transcription. DC1 has also been shown to control splicing, RNA stability, nuclear export, and to promote X chromosome inactivation. DC1 also binds to the non-coding RNA *MALAT1* which allows DC1 to have an important role in maintaining the protein composition of nuclear speckles.