

# **The role of m6A RNA modification in RNA regulation**

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## Zusammenfassung

N<sup>6</sup>-Methyladenosin (m<sup>6</sup>A) ist die häufigste interne Modifikation in eukaryotischer messenger-RNA (mRNA) und fungiert als dynamisches regulatorisches Signal, das zahlreiche Aspekte des mRNA-Stoffwechsels steuert, darunter das Spleißen, den nukleären Export, die Translation und den Abbau. Unter diesen Prozessen gilt insbesondere die Regulierung der mRNA-Stabilität als zentrale Funktion von m<sup>6</sup>A. In der Regel werden m<sup>6</sup>A-Stellen von Mitgliedern der YTH-Domänen-Protein-Familie erkannt, insbesondere von YTHDF1–3, die den Abbau der markierten Transkripte durch die Rekrutierung von Effektorkomplexen vermitteln. Trotz intensiver Forschung zu m<sup>6</sup>A-vermitteltem mRNA-Abbau bleiben zentrale Fragen ungeklärt: Welche spezifischen m<sup>6</sup>A-Stellen lösen den Abbau aus? Haben m<sup>6</sup>A-Modifikationen in verschiedenen Transkriptregionen dieselbe Wirkung? Und wie wird der jeweilige Abbauweg festgelegt? In dieser Arbeit beschreibe ich die Entdeckung und Charakterisierung eines neuartigen, translationsabhängigen mRNA-Abbauweges, der durch m<sup>6</sup>A-Modifikationen innerhalb der codierenden Sequenz (CDS) von Transkripten ausgelöst wird. Wir bezeichnen diesen Mechanismus als CDS–m<sup>6</sup>A-vermittelten Abbau (CMD). Im Gegensatz zu den bekannten m<sup>6</sup>A-abhängigen Abbauwegen, die auf der Erkennung durch Leserproteine basieren, wird CMD durch eine gestörte Decodierung des methylierten Codons durch das Ribosom ausgelöst. Dies stellt einen direkten mechanistischen Zusammenhang zwischen m<sup>6</sup>A und der Translation her, bei dem ribosomale Verlangsamung an modifizierten Codons den schnellen Abbau des Transkripts einleitet. Obwohl der Weg unabhängig von der initialen Erkennung des Lese-proteins funktioniert, zeigen unsere Daten, dass YTHDF2 nachgeschaltet in den Abbauprozess eingebunden ist, was auf eine mehrschichtige Regulation hindeutet.

Zudem beobachten wir, dass CMD-Zieltranskripte bevorzugt in Processing Bodies (P-Bodies) angereichert sind – zytoplasmatische Strukturen, die als Zentren für mRNA-Abbau und -Speicherung dienen. Diese subzelluläre Lokalisierung könnte eine zusätzliche regulatorische Ebene bei der Ausführung von CMD darstellen. Interessanterweise erfolgt die m<sup>6</sup>A-Methylierung in der CDS nicht zufällig: Neue Erkenntnisse zeigen, dass der Exon-Junction-Complex (EJC), der bei der Spleißung in der Nähe von Exon-Exon-Grenzen abgelagert wird, die m<sup>6</sup>A-Modifikation in seinem

Umfeld verhindert. Dies legt nahe, dass die Entscheidung zur Methylierung spezifischer Codons – und damit zur Einleitung von CMD – bereits co-transkriptionell im Zellkern getroffen werden kann, noch bevor die Translation im Zytoplasma stattfindet.

Die hier vorgestellte Arbeit liefert neue Einblicke in das Zusammenspiel von positionsspezifischer m6A-Deposition und Ribosomendynamik bei der Regulierung des mRNA-Schicksals. Sie zeigt, dass m6A-Modifikationen in der codierenden Sequenz nicht nur passive Marker sind, sondern aktiv Translationseffizienz und Transkriptstabilität koppeln. Dieser Mechanismus ist besonders relevant für Transkripte, die an zellulären Stressantworten, Entwicklungsvorgängen und Tumorentstehung beteiligt sind, und unterstreicht die physiologische Bedeutung von CMD bei der präzisen zeitlichen Steuerung genregulatorischer Programme.

## Abstract

N6-methyladenosine (m6A) is the most abundant internal modification found in eukaryotic messenger RNA (mRNA) and serves as a dynamic regulatory signal that controls multiple aspects of mRNA metabolism, including splicing, nuclear export, translation, and degradation. Among these, m6A's most prominent function is the regulation of mRNA stability. Typically, m6A sites are recognized by members of the YTH domain family of reader proteins, particularly YTHDF1–3, which facilitate transcript degradation by recruiting effector complexes. Despite extensive research into m6A-mediated decay, key questions remain unresolved: Which specific m6A sites trigger degradation? Do all m6A-modified regions contribute equally to mRNA destabilization? And how is the choice of decay pathway determined?

In this thesis, I describe the discovery and characterization of a novel, translation-dependent mRNA decay pathway driven by m6A modifications located specifically within the coding sequence (CDS) of transcripts. We term this mechanism CDS–m6A decay (CMD). In contrast to canonical pathways that require reader protein binding to initiate decay, CMD is triggered by impaired decoding of methylated codons by the ribosome. This establishes a direct mechanistic link between m6A and translation, where ribosomal slowdown or stalling at modified codons initiates rapid degradation of the transcript. This pathway operates independently of initial reader recognition, although we find that YTHDF2 acts downstream to facilitate transcript clearance, suggesting a multilayered control system. Our data also show that CMD targets are preferentially enriched in processing bodies (P-bodies), which serve as key cytoplasmic hubs for mRNA decay and storage. This spatial segregation may represent a protective or regulatory layer in the execution of CMD. Intriguingly, the deposition of m6A in the CDS is not random; recent findings reveal that the exon junction complex (EJC), deposited near exon–exon boundaries during splicing, restricts m6A methylation in its vicinity. This implies that the decision to methylate specific codons—and thus commit the transcript to CMD—can be made co-transcriptionally in the nucleus, long before translation occurs in the cytoplasm. The work presented here provides new insights into how site-specific m6A deposition and ribosome dynamics intersect to regulate mRNA fate. It reveals that CDS m6A modifications couple translation fidelity with transcript turnover. This pathway is especially relevant for transcripts involved in cellular stress responses, development, and

cancer, pointing to a broader physiological significance of CMD in controlling gene expression programs that require precise temporal regulation.

## Preface

The results presented in this thesis have been published previously or have been accepted for print.

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# 1 Introduction

## 1.1 The regulation of RNA and gene expression

The genetic information of an organism is stored in deoxyribonucleic acid (DNA). DNA consists of four chemical bases—adenine (A), thymine (T), guanine (G), and cytosine (C)—attached to a sugar-phosphate backbone. In eukaryotes, the double-stranded DNA is wrapped around specific proteins called histones to efficiently package and protect the genetic material. These histone-bound DNA segments are further organized into nucleosomes, which fold into chromatin and ultimately form chromosomes (reviewed in Felsenfeld and Groudine 2003).

Genetic information is converted from DNA into functional proteins through an ribonucleic acid (RNA) intermediate, in a process first described by Francis Crick in 1958 and known as the central dogma of molecular biology (Crick 1958, 1970). The central dogma consists of three main stages: replication, transcription, and translation. Each of these steps is tightly regulated to ensure that genes are expressed with the appropriate timing, location, and intensity. RNA is chemically similar to DNA but differs in two key aspects: it is typically single-stranded and contains uracil (U) instead of thymine. According to the central dogma, RNA serves merely as an intermediate between DNA and proteins. However, this is an oversimplified view. Not all RNAs are translated into proteins—non-coding RNAs have essential regulatory functions in gene expression themselves. Moreover, the flow of information from DNA to RNA to protein is not linear or proportional; many layers of regulation shape the final outcome. The term *gene expression* refers to the dynamic process by which information from a gene is used to synthesize a functional product, often a protein. This process is modulated at multiple levels, including through chemical modifications of DNA and RNA bases, or of amino acids in proteins.

### 1.1.1 Epitranscriptomics: regulatory functions of RNA modifications

The first RNA modification to be identified was pseudouridine initially identified as the fifth nucleotide, due to the high abundance in which it was found (Markham and Smith 1951; Yu and Allen 1959). Almost ten years had passed until it was finally identified as an isomer of uridine (Cohn 1960). Initial RNA modification studies focused on highly abundant RNA species such as ribosomal RNA (rRNA) and transfer RNA (tRNA), while

the first modifications in messenger RNA (mRNA) were reported in the 1970s. To date, over 170 types of RNA modifications have been identified (Machnicka et al. 2013). A major breakthrough in the field came with the development of high-throughput sequencing techniques, which enabled transcriptome-wide detection of mRNA modifications. This advancement gave rise to the field now known as *epitranscriptomics* (Saletore et al. 2012).

Among the many RNA modifications uncovered by the field of epitranscriptomics, *N*6-methyladenosine (m6A) has emerged as the most prevalent and functionally diverse modification in eukaryotic mRNAs. The following section traces the historical discovery and current understanding of m6A.

### **1.1.2 *N*6-methyladenosine**

m6A is a chemical derivative of adenosine and was first reported in bacterial DNA in 1955 (Dunn and Smith 1955). A few years later, in 1958, m6A was also detected in bacterial and yeast RNA (Adler, Weissmann, and Gutman 1958; Littlefield and Dunn 1958). It is now understood that these early observations corresponded to highly abundant rRNA species (Hayashi, Osawa, and Miura 1966; Starr and Fefferman 1964). Nearly two decades later, in 1974, the development of improved mRNA purification techniques enabled the first identification of m6A in mammalian mRNA (Desrosiers, Friderici, and Rottman 1974; Perry et al. 1975). To date, m6A is known to occur in nearly all RNA classes, including mRNA, tRNA, rRNA, circular RNA (circRNA), and microRNA (miRNA) (Liu and Pan 2016; Shi, Wei, and He 2019). With approximately 1 to 13 sites per transcript, m6A is the most prevalent internal modification in polyadenylated mRNAs and long non-coding RNAs in higher eukaryotes (Liu et al. 2023; Uzonyi et al. 2023),.

### **1.1.3 m6A detection**

To study the biological relevance and function of m6A, it is essential to identify its location across the transcriptome with high accuracy. Initially, only a few individual sites were known, all located within the conserved RRACH motif (where R = G or A; H = A, C, or U) (Horowitz et al. 1984; Kane and Beemon 1985; Harper et al. 1990). Nearly 40 years passed between the first detection of m6A in mRNA and the generation of the first transcriptome-wide maps. In 2012, two independent groups developed methods to globally map m6A using an m6A-specific antibody to enrich for methylated RNA fragments, followed by next-generation sequencing (NGS). These methods, known as

m6A-seq and MeRIP-seq (Figure 1.1), enabled the identification of thousands of methylation sites across the transcriptome (Dominissini et al. 2012; Meyer et al. 2012). While these early maps provided valuable insights into the distribution and general enrichment patterns of m6A, they were limited by relatively low resolution. The RNA fragments enriched for sequencing were typically 50–200 nucleotides long, making it impossible to determine the precise nucleotide harboring the m6A modification. Moreover, it remained unclear whether one or multiple m6A sites were present within any given enriched fragment.

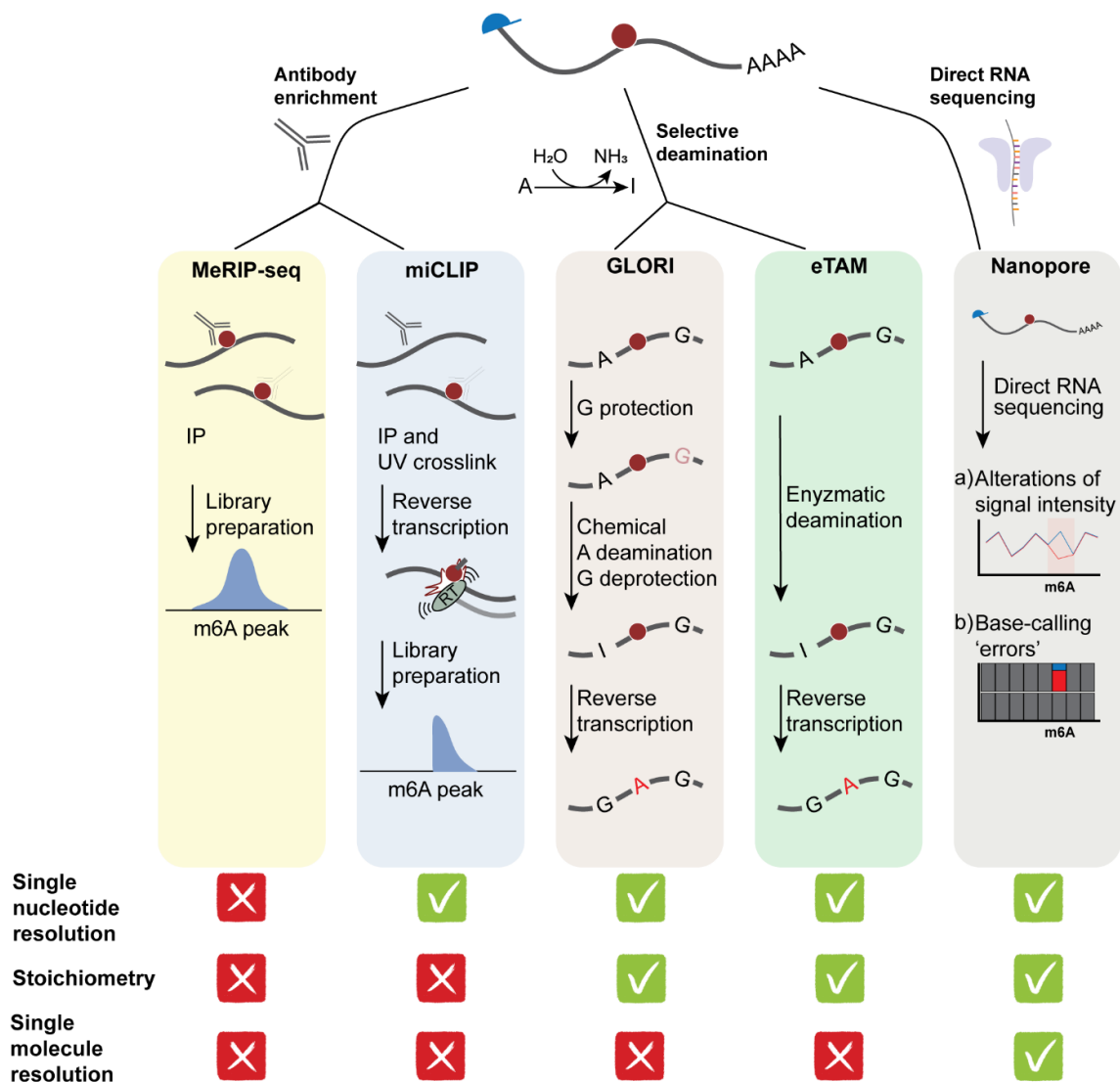


Figure 1.1 Overview of the most relevant m6A mapping techniques, highlighting their differences in resolution and quantification capabilities.

#### *1.1.3.1 m6A-CLIP and miCLIP*

A significant breakthrough occurred in 2015 with the development of two new methods: m6A-CLIP and miCLIP (Ke et al. 2015; Linder et al. 2015). These approaches are built upon the principle of ultraviolet (UV) crosslinking and immunoprecipitation (CLIP) (König et al. 2010). Instead of protein protein-specific antibody used in CLIP approaches, they utilize an m6A-specific antibody, thereby allowing for single-nucleotide resolution mapping of m6A sites. Despite this advancement, both methods faced several limitations. Due to the limited specificity of the m6A antibody, high background remained a persistent challenge. In addition, these protocols required large amounts of input RNA, and crucial information regarding the degree of methylation at each site—referred to as stoichiometry—could not be obtained.

In 2021, an improved protocol called miCLIP2 was introduced, incorporating both experimental improvements and a machine learning-based analysis tool called m6Aboost (Körtel et al. 2021). These innovations reduced the amount of input material required and enhanced the accuracy of m6A site prediction. However, similar to previous approaches, miCLIP2 still did not provide information on the stoichiometry of individual m6A sites.

#### *1.1.3.2 GLORI and eTAM-seq*

To overcome the limitations of antibody-based methods, particularly the lack of stoichiometric information and high background, a new class of techniques emerged based on adenosine deamination. These include both chemical and enzymatic approaches: glyoxal and nitrite-mediated deamination of unmethylated adenosines (GLORI) (Liu et al. 2023), and evolved TadA-assisted *N*6-methyladenosine sequencing (eTAM-seq) (Xiao et al. 2023). These methods are conceptually similar to bisulfite sequencing used for profiling 5-methylcytosine in DNA, where sodium bisulfite selectively deaminates unmethylated cytosines into uracils, allowing differentiation from methylated cytosines. In GLORI and eTAM-seq, only unmethylated adenosines undergo deamination, resulting in A-to-inosine (I) conversions, while m6A-modified adenosines remain unaffected. Upon next-generation sequencing, inosines are read as guanosines, enabling high-confidence identification of methylated versus unmethylated sites. Both methods demonstrate A-to-I conversion rates exceeding 99%, and crucially, they allow quantification of m6A stoichiometry at single-nucleotide resolution. Moreover, they require lower input material and detect a greater number of m6A sites compared to antibody-based techniques.

#### *1.1.3.3 Direct RNA sequencing for m6A mapping*

Nanopore direct RNA sequencing (DRS) has emerged as an even more powerful technology for mapping m6A and other RNA modifications. It enables the sequencing of native RNA molecules by translocating them through a nanopore with the help of a motor protein. As the RNA passes through, each nucleotide causes characteristic changes in the ionic current, which are used to identify the base (Garalde et al. 2018). Importantly, RNA modifications such as m6A also influence the current signal and dwell time, allowing their detection directly from the RNA, without the need for immunoprecipitation, chemical treatment, or cDNA synthesis (Joedicke et al. 2018). The first study demonstrating m6A detection using nanopore DRS was published in 2019 (Liu et al. 2019). Since then, numerous computational tools have been developed to improve modification calling accuracy from the raw signal data. These tools address the inherent complexity of signal interpretation, which remains a major challenge in the field (reviewed in Acera Mateos et al. 2023).

A major advantage of nanopore DRS lies in its capacity to detect RNA modifications directly on native RNA molecules, without requiring reverse transcription, amplification, chemical treatment, or antibody-based enrichment. This eliminates biases introduced by enrichment procedures and preserves the original modification landscape. In addition to providing isoform-resolved maps of m6A distribution, nanopore DRS allows for quantitative assessment of site-specific modification stoichiometry. Furthermore, by sequencing RNA molecules directly, the technology offers the unique potential to detect multiple types of RNA modifications simultaneously within a single experiment.

#### **1.1.4 m6A writer complex**

Understanding the biological significance of m6A requires not only accurate detection, but also a detailed knowledge of how the modification is installed. m6A is deposited on mRNA exclusively in the nucleus by a multicomponent methyltransferase complex, commonly referred to as the writer complex. This complex was first purified in the 1990s, leading to the identification of methyltransferase-like 3 (METTL3) as a key component (Bokar et al. 1994, 1997). Subsequent studies revealed that METTL3 forms a stable heterodimer with METTL14 in a 1:1 ratio (Liu et al. 2014; Ping et al. 2014; Śledź and Jinek 2016; Y. Wang et al. 2014; X. Wang et al. 2016; P. Wang, Doxtader, and Nam 2016). METTL3 serves as the catalytically active subunit; it contains a conserved methyltransferase domain with a DPPW (Asp-Pro-Pro-Trp) motif that transfers a methyl

group from S-adenosylmethionine (SAM) to target adenosines in RNA (Śledź and Jinek 2016). Although METTL14 is catalytically inactive, it stabilizes METTL3, enhances its enzymatic activity, and contributes to RNA substrate recognition and binding (Liu et al. 2014; Y. Wang et al. 2014; P. Wang et al. 2016).

Another core component of the writer complex is Wilms's tumor 1-associating protein (WTAP), which interacts with the METTL3/METTL14 heterodimer and localizes the complex to nuclear speckles (Ping et al. 2014). In addition to the core subunits, several regulatory proteins have been identified that modulate the specificity or localization of the complex. For example, RNA-binding motif protein 15/15B (RBM15/RBM15B), cbl proto-oncogene like 1 (HAKAI), and vir-like m6A methyltransferase-associated (VIRMA) contribute to site-specific deposition of m6A, while zinc finger CCCH-type containing protein 13 (ZC3H13) is essential for the nuclear retention of the writer complex (Deng and Ma 2024).

While the METTL3/METTL14 complex is responsible for depositing m6A modifications on mRNA, additional methyltransferases that install m6A on other RNA species have been identified. For example, METTL5, in complex with its cofactor tRNA methyltransferase activator subunit 11-2 (TRMT112), catalyzes m6A methylation at position A1832 of the 18S rRNA (van Tran et al. 2019). Zinc finger CCHC-type containing 4 (ZCCHC4) mediates m6A modification at position A4220 of the 28S rRNA (Ma et al. 2019; van Tran et al. 2019). Another m6A methyltransferase, METTL16, installs m6A on U6 small nuclear RNA (snRNA). Interestingly, METTL16 has also been shown to methylate a small subset of mRNAs, but unlike METTL3/METTL14, it targets a distinct sequence motif, different from the canonical motif recognized by the core writer complex (Pendleton et al. 2017; Shima et al. 2017; Warda et al. 2017).

### **1.1.5 Determinants of m6A site selection**

Deposition of m6A by the writer complex occurs in a sequence-dependent manner. Early studies identified the RRACH motif (R = A or G) as the core sequence for methylation, which was later refined to the DRACH motif (D = A, G, or U; R = A or G; H = A, C, or U) (Dominissini et al. 2012; Ke et al. 2015; Linder et al. 2015; Meyer et al. 2012). Some recent analyses further extended this to the DRACN motif (N = any nucleotide) (Körtel et al. 2021; Liu et al. 2023). However, not all DRACN sites are methylated equally. m6A deposition is enriched near stop codons and within long internal exons (Ke et al. 2015; Meyer et al. 2012). This spatial pattern of methylation can be partially attributed to

interactions between the m6A writer complex and epigenetic markers. For example, a study in 2019 showed that METTL14 can recognize the histone modification H3K36me3, a marker of actively transcribed gene bodies, which correlates with m6A deposition on RNA (Huang et al. 2019). METTL3 has also been shown to preferentially associate with broad H3K4me3 domains and super-enhancers, suggesting that chromatin context influences m6A placement (Li et al. 2023). Furthermore, both METTL3 and METTL14 can interact with other histone marks such as H3K27ac and H3K9me3 (Dou et al. 2023). Transcriptional kinetics also contribute to methylation patterning. A reduced elongation speed of RNA polymerase II (RNA Pol II) increases the accessibility of consensus motifs by minimizing RNA secondary structure formation, thus enhancing m6A deposition (Slobodin et al. 2017; Wang et al. 2024). In addition to co-transcriptional regulation, post-transcriptional events also influence m6A deposition. Recent studies have shown that the exon junction complex (EJC), which is deposited on mRNA during splicing near exon-exon junctions, can sterically hinder the binding of the writer complex. This leads to reduced m6A methylation near splice junctions and explains why m6A is preferentially enriched in long internal exons, where central regions remain accessible, unlike in short exons that are largely occluded by EJCs (He et al. 2023; Luo et al. 2023a; Uzonyi et al. 2023; Yang et al. 2022).

Once installed, m6A marks are interpreted by reader proteins that mediate downstream regulatory outcomes. These readers link m6A deposition to specific fates in RNA metabolism, such as altered stability, localization, or translation.

#### **1.1.6 m6A reader proteins: mediators of m6A function**

Specific RNA-binding proteins recognize m6A-modified transcripts and mediate their downstream effects; these proteins are collectively known as m6A readers. Several m6A reader proteins have been identified, each influencing RNA metabolism in distinct ways. m6A is recognized both in the nucleus and the cytoplasm, and it regulates RNA processing, localization, translation, and degradation (Figure 1.2) (reviewed in He and He 2021).

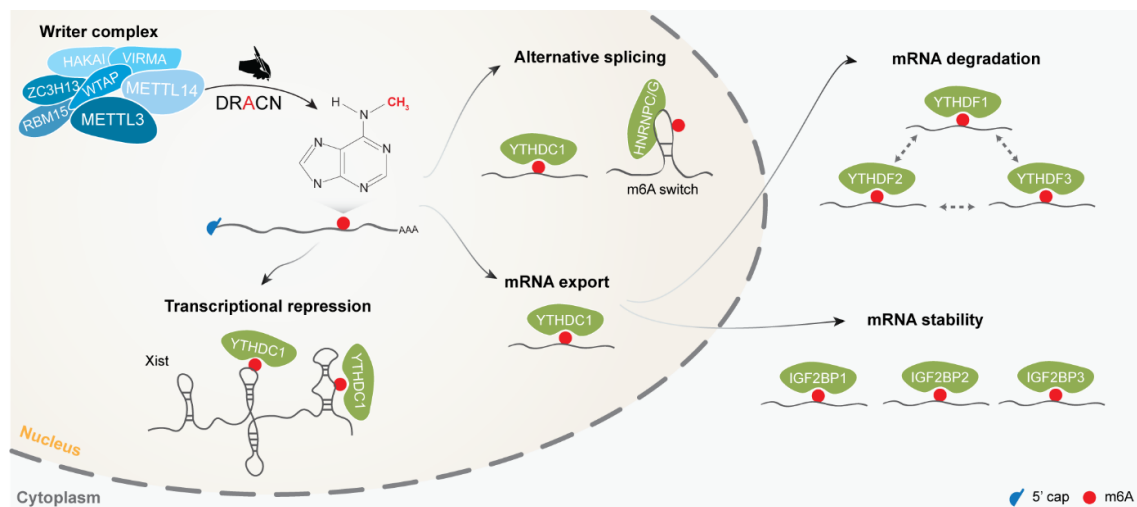


Figure 1.2 Overview of m6A functions in RNA metabolism. m6A is deposited in the nucleus by the writer complex in a sequence-dependent manner. It is recognized by reader proteins that regulate distinct steps of RNA metabolism, including alternative splicing, mRNA mRNA export, *Xist*-mediated transcriptional repression, mRNA stability, and degradation.

#### 1.1.6.1 YTH-domain containing reader proteins

The first m6A reader proteins were identified in 2012 using RNA pulldown assays with m6A-modified RNA probes. Two proteins—YTH N6-methyladenosine RNA binding protein F2 (YTHDF2) and YTHDF3—were shown to specifically bind methylated RNA (Dominissini et al. 2012). These proteins contain an RNA-binding module called the YT521-B homology (YTH) domain. Subsequent studies revealed that this highly conserved ~140 amino acid domain forms a specific binding pocket for m6A (Li et al. 2014; Zhu et al. 2014). Five proteins in mammals possess a YTH domain: the cytoplasmic YTHDF proteins (YTHDF1, YTHDF2, YTHDF3), and the more divergent nuclear proteins YTH N6-methyladenosine RNA binding protein C1 (YTHDC1) and YTHDC2. YTHDF proteins primarily function in the cytoplasm (Wang et al. 2015). They share high sequence homology, with a conserved C-terminal YTH domain and an N-terminal low-complexity region containing prion-like domains (Patil, Pickering, and Jaffrey 2018). These low-complexity domains enable liquid–liquid phase separation facilitating compartmentalized RNA regulation (Ries et al. 2019). Early studies proposed distinct roles for each paralog: YTHDF1 enhanced translation, YTHDF2 promoted mRNA degradation, and YTHDF3 functioned in both processes, supporting the other two

proteins (Li et al. 2017; Shi et al. 2017; Wang et al. 2015; X. Wang et al. 2014). However, recent reports suggest that all three YTHDF proteins may act redundantly to promote mRNA decay (Lasman et al. 2020; Zaccara and Jaffrey 2020).

YTHDC1 and YTHDC2 are less homologous outside of the YTH domain and are involved in a variety of biological functions. YTHDC1, localized in the nucleus, contains intrinsically disordered regions (IDRs) that may enable liquid–liquid phase separation. It plays key roles in mRNA export and alternative splicing (Kasowitz et al. 2018; Roundtree et al. 2017; Tsai et al. 2021; Xiao et al. 2016). Notably, YTHDC1 interacts with non-coding RNAs such as *Xist*, where it contributes to X-chromosome inactivation (Patil et al. 2016). In addition, YTHDC1 has been shown to interact with the histone methyltransferase SET domain bifurcated histone lysine methyltransferase 1 (SETDB1), a writer of the repressive H3K9me3 histone mark. Through this interaction, YTHDC1 contributes to the maintenance of genome stability by repressing retrotransposon activity (Liu et al. 2021; Xu et al. 2021). Beyond its physiological roles, YTHDC1 has emerging relevance in disease. It was recently identified as a potential therapeutic target in acute myeloid leukemia (AML) (Cheng et al. 2021), and may be implicated in other cancer types as well (Shi et al. 2021).

YTHDC2, expressed in both the nucleus and cytoplasm, is the most structurally distinct member of the YTH protein family. Its binding affinity for m6A is comparatively weaker than that of other YTH proteins (Saito et al. 2022; Xu et al. 2015), yet it has been implicated in several m6A-dependent processes. These include the regulation of spermatogenesis (Hsu et al. 2017), nuclear RNA stability (Hsu et al. 2017; Kretschmer et al. 2018; Li et al. 2022), and mRNA translation (Hsu et al. 2017; Mao et al. 2019).

#### *1.1.6.2 Reader proteins without YTH domain*

In addition to YTH domain-containing proteins, several other proteins have been reported as potential m6A readers. One well-characterized group is the insulin-like growth factor 2 mRNA-binding proteins 1–3 (IGF2BP1–3). These proteins recognize m6A-modified transcripts via their K homology (KH) RNA-binding domains. Unlike YTHDF proteins, whose binding closely follows the canonical m6A distribution, IGF2BP binding appears more broadly distributed and does not show strong positional enrichment. Functionally, IGF2BPs are reported to stabilize their m6A-modified RNA targets (Huang et al. 2018). More recently, IGF2BP3 has been shown to repress translation by redirecting m6A-containing transcripts from the translating pool into processing bodies (P-bodies) (Shan

et al. 2023). Additionally, IGF2BPs were found to bind and stabilize tumor suppressor transcripts—frequent targets of m6A-disrupting mutations—suggesting a role in oncogenic processes (Lan et al. 2025). Beyond canonical readers, fragile X messenger ribonucleoprotein 1 (FMRP) has been identified as an m6A reader that binds to RNA via its KH domains (Edupuganti et al. 2017). FMRP has been shown to mediate m6A-dependent regulation of translation, mRNA stability, and nuclear export (Edens et al. 2019; Edupuganti et al. 2017). However, subsequent studies suggest that FMRP's binding is influenced more by local RNA sequence and structure than by the presence of m6A itself (Zhang et al. 2018), indicating that its reader function may be indirect or conditional. In addition to direct readers, m6A can influence RNA–protein interactions indirectly by altering RNA secondary structure. This mechanism, termed the m6A-switch, refers to m6A-induced destabilization of local RNA structures that either expose or occlude protein-binding sites (Liu et al. 2015). For example, m6A modifications within hairpin loops can destabilize these structures, enabling access for heterogeneous nuclear ribonucleoprotein C (HNRNPC), thereby influencing mRNA abundance and alternative splicing (Liu et al. 2015). Similar mechanisms have been described for HNRNPG and HNRNPA2B1, which also rely on m6A-induced structural remodeling to regulate alternative splicing and primary microRNA (pri-miRNA) processing (Alarcón et al. 2015; Liu et al. 2017). In contrast, some proteins are repelled by m6A-induced structural changes—a concept referred to as anti-readers. Proteins such as Ras GTPase-activating protein-binding protein 1 (G3BP1), G3BP3, Ewing sarcoma breakpoint region 1 (EWSR1), and pumilio RNA binding family member 2 (PUM2) fall into this category, as m6A-modified regions hinder their binding, thereby influencing downstream regulatory outcomes (Liu et al. 2015; Vaidyanathan et al. 2017).

While m6A deposition and reader-mediated recognition are central to shaping transcript fate, their consequences are ultimately executed through downstream pathways such as mRNA decay. In the following section, I explore the mechanisms of cytoplasmic mRNA degradation, which serve as key effectors of m6A function.

## 1.2 Cytoplasmic mRNA decay

mRNA starts its life by being transcribed from DNA by RNA Pol II and subsequently undergoes several maturation steps, including 5' capping, splicing, 3' polyadenylation, chemical modifications, nuclear export, translation, and ultimately degradation. Each of

these steps is tightly regulated by numerous cellular factors and mechanisms. The lifespan of an mRNA varies widely and is often linked to its functional role. For example, housekeeping mRNAs are typically stable and long-lived, whereas regulatory or stress-responsive mRNAs are generally short-lived, enabling rapid changes in protein expression (Bae and Collier 2022). When an mRNA reaches the end of its functional life, it is degraded through tightly regulated decay pathways. mRNA decay plays a particularly important role during development, viral infections, and abrupt environmental changes, ensuring that transcripts are swiftly removed once they are no longer needed (Shehata et al. 2024; Vastenhouw, Cao, and Lipshitz 2019). Furthermore, aberrant or defective transcripts are selectively degraded to prevent the synthesis of potentially harmful proteins (Isken and Maquat 2007; Monaghan, Longman, and Cáceres 2023; Wolin and Maquat 2019).

In general, mRNA decay is initiated by deadenylation—the removal of the poly(A) tail, followed by decapping, which involves excision of the 5' 7-methylguanosine (m7G) cap. This is then followed by exonucleolytic degradation proceeding from the 5' end. In certain cases, decay is initiated by endoribonucleolytic cleavage, which generates uncapped 5' and 3' ends that are subsequently degraded by exonucleases (reviewed in Horvathova et al. 2017).

### **1.2.1 mRNA decay by deadenylation**

With the exception of metazoan replication-dependent histone mRNAs, all eukaryotic mRNAs possess poly(A) tails at their 3' ends. The poly(A) tail is bound by cytoplasmic poly(A)-binding protein (PABPC1), which promotes translation by enhancing ribosome recruitment (Passmore and Collier 2022). In addition to its role in translation, PABPC1 also interacts with components of the mRNA decay machinery and can facilitate deadenylation. Two major deadenylase complexes are responsible for shortening poly(A) tails in eukaryotes: the poly(A)-specific ribonuclease 2 (PAN2)–PAN3 complex and the carbon catabolite repression 4–negative on TATA-less complex (CCR4–NOT) complex (Tucker et al. 2001). The PAN2–PAN3 complex primarily acts on mRNAs with long poly(A) tails, trimming them in a regulated, non-degradative manner. In contrast, the CCR4–NOT complex plays a central role in initiating mRNA decay following deadenylation.

#### *1.2.1.1 Deadenylation by the PAN2–PAN3 complex*

The PAN2–PAN3 complex consists of a catalytically active subunit, PAN2, and a regulatory subunit, PAN3. PAN2 recognizes the poly(A) tail not through base-specific interactions but by sensing the stacked, helical conformation formed by the adenosines. PAN3, on the other hand, binds to PABPC, activating the deadenylase complex (Jonas et al. 2014; Wolf et al. 2014). Structural studies have shown that PAN2–PAN3 binding to PABPC requires PABPC dimerization (Schäfer et al. 2019). Since one PABPC molecule binds approximately 30 nucleotides, this requirement explains why PAN2–PAN3 only acts on long poly(A) tails and why deadenylation typically stops before the final PABPC molecule is displaced.

The physiological role of PAN2–PAN3-mediated trimming may include maintaining a stable cytoplasmic pool of PABPC, or priming aged mRNAs for degradation. Supporting this idea, long-lived mRNAs with shortened poly(A) tails have been observed in somatic cells, indicating that deadenylation can occur without immediate mRNA decay (Decker and Parker 1993).

#### *1.2.1.2 Deadenylation by CCR4–NOT complex*

The CCR4–NOT complex is a large multisubunit complex that orchestrates mRNA deadenylation and decay. CCR4–NOT transcription complex subunit 1 (CNOT1) serves as the central scaffold, anchoring various regulatory subunits (CNOT2, CNOT3, CNOT9, CNOT10, and CNOT11) as well as two distinct classes of deadenylases. These include:

- DEDD-type deadenylases (CNOT7 and CNOT8), which contain conserved Asp-Glu-Asp-Asp catalytic motifs and associate directly with CNOT1;
- EEP-type deadenylases (CNOT6 and CCR4–NOT transcription complex subunit 6 like (CNOT6L)), which contain endonuclease–exonuclease–phosphatase (EEP) domains and require interaction with CNOT7/8 for integration into the complex.

Each CCR4–NOT complex typically incorporates one deadenylase of each type, enabling cooperative and efficient poly(A) tail shortening (Shirai et al. 2014). Although both classes of exonucleases exhibit adenosine specificity, they differ in their interaction with PABPC. CNOT7/8 activity is inhibited by PABPC, whereas CNOT6/6L has been shown to displace PABPC from the poly(A) tail (Webster et al. 2018; Yi et al. 2018). While the full mechanism remains to be elucidated, the cooperative activity between the two deadenylase types likely facilitates poly(A) tail shortening beyond the final bound PABPC, thereby enabling the initiation of mRNA degradation—either through exosome-

mediated 3'–5' decay of the unprotected 3' end, or through subsequent decapping followed by 5'–3' degradation by 5'-3' exoribonuclease 1 (XRN1).

### **1.2.2 mRNA decay in 3' to 5' direction**

Following deadenylation or endoribonucleolytic cleavage, mRNAs can be degraded from the exposed 3' end by the cytoplasmic exosome complex (Anderson and Parker 1998). The exosome consists of a non-catalytic core composed of nine subunits—exosome component 1–9 (EXOSC1–9), which form a barrel-like structure with a central channel capable of accommodating single-stranded RNA (Liu, Greimann, and Lima 2006). This channel guides RNA toward the catalytic subunit—DIS3 homolog, exosome endoribonuclease, and 3'-5' exoribonuclease (DIS3), anchored at the base of the complex, which degrades RNA as it is threaded through. The exonucleolytic activity of the exosome is supported by the superkiller (SKI) complex, composed of superkiller viralicidic Activity 2-Like RNA helicase (SKIV2L), tetratricopeptide Repeat Domain 37 (TTC37), and two copies of WD repeat Domain 61 (WDR61). The SKI complex functions as a helicase, unwinding structured RNA and facilitating its delivery to the exosome. This interaction is bridged by HBS1-like protein 3 (HBS1L3), which links the SKI complex to the exosome and coordinates substrate handover (Kowalinski et al. 2016).

Recent studies have shown that the exosome primarily targets protein-coding RNAs (Tuck et al. 2020). Additionally, the SKI complex has been found to associate with ribosomes near the mRNA entry tunnel and is capable of extracting RNA from stalled ribosomes (Keidel et al. 2023; Kögel et al. 2022; Schmidt et al. 2016; Zinoviev et al. 2020). These findings suggest that the primary function of the cytoplasmic SKI–exosome complex is to degrade RNAs following endoribonucleolytic cleavage triggered by aberrant translation events.

### **1.2.3 Decapping and mRNA decay in 5' to 3' direction**

Similar to the poly(A) tail, nearly all eukaryotic mRNAs possess an m<sup>7</sup>G cap at their 5' end, which is linked to the first transcribed nucleotide via a unique 5'–5' triphosphate bond (Shatkin 1976). The removal of this cap, known as decapping, is a critical step in mRNA degradation. Decapping is mediated by the Nudix-domain-containing mRNA-decapping enzyme 2 (DCP2), whose catalytic activity requires binding to DCP1 (Wang et al. 2002).

The composition of the decapping complex is dynamic and context-dependent. Enhancer of mRNA decapping 4 (EDC4) functions as a scaffold protein, stabilizing the interaction between DCP1 and DCP2 (Chang et al. 2014). Additional proteins, such as proline-rich nuclear receptor coactivator 2 (PNRC2), EDC3, and like-Sm protein 14 (LSM14), act as decapping enhancers and can associate with the complex in a mutually exclusive manner. Furthermore, regulatory factors such as DEAD-box Helicase 6 (DDX6) and protein PAT1 homolog 1 (PATL1) interact with the decapping complex to fine-tune decapping activity, often linking it to translational repression or mRNA remodeling (Jonas and Izaurralde 2013).

In addition to their role in mRNA decay, many decapping-associated proteins are characterized by the presence of IDRs, which enable them to undergo liquid–liquid phase separation. This property contributes to the formation of P-bodies—cytoplasmic ribonucleoprotein (RNP) granules that concentrate translationally repressed mRNAs together with decapping and degradation factors (Luo, Na, and Slavoff 2018; Sheth and Parker 2003). P-bodies are conserved in eukaryotic cells and exhibit biophysical properties of biomolecular condensates. Their formation is tightly linked to the accumulation of untranslated, decay-competent mRNAs, and their size and number can vary depending on cellular conditions. Although P-bodies have long been associated with mRNA turnover, their precise function remains a topic of ongoing investigation. It is still unclear whether they serve as active sites of decay, storage depots for silenced transcripts, or both—roles that may depend on context and cell type (Aizer et al. 2014; Blake et al. 2024; Eulalio et al. 2007; Hubstenberger et al. 2017; Sheth and Parker 2003).

Decapping is closely coupled to deadenylation by the CCR4–NOT complex through at least two mechanisms. First, DDX6, a decapping enhancer, interacts directly with CNOT1 (the scaffold of CCR4–NOT) and forms a regulatory network with PATL1, EDC3, and LSM14A, creating a platform that coordinates deadenylation and decapping (Coller and Parker 2005; Mathys et al. 2014). Second, once deadenylation proceeds beyond the final PABPC molecule, it exposes a binding site for the LSM1–7–PATL1 complex, which promotes recruitment of the decapping machinery (Lobel, Tibble, and Gross 2019; Montemayor et al. 2020). Under normal conditions, the m<sup>7</sup>G cap is bound by the eukaryotic initiation Factor 4F (eIF4F) complex, which facilitates translation initiation (Schwartz and Parker 2000). How the decapping complex gains access to the cap remains incompletely understood. One possibility is that the removal of PABPC from the deadenylated RNA leads to destabilization of eIF4F via its eIF4G subunit, weakening cap

protection (Tarun and Sachs 1996). Alternatively, some decapping activators may interact with translation repressors to displace the eIF4F complex from the cap structure (Christie and Igreja 2023; Kinkelin et al. 2012). Decapping is followed by 5'–3' degradation of the mRNA by the XRN1 (Nagarajan et al. 2013). During the processes of deadenylation and decapping, translation initiation is blocked, but elongating ribosomes already engaged on the transcript can often complete translation (Hu et al. 2009). This may help explain why most cytoplasmic mRNA degradation proceeds in the 5'–3' direction.

While most decapping pathways in mammals are triggered by deadenylation, certain transcripts can undergo specialized, deadenylation-independent decapping (Chen and Shyu 2011). One well-characterized example is nonsense-mediated mRNA decay (NMD). In this pathway, recognition of a premature termination codon (PTC) by the translation machinery triggers rapid recruitment of decay factors, including the decapping complex, leading to 5' cap removal without the need for prior deadenylation (Eberle et al. 2009). This allows for the swift degradation of faulty transcripts before they can produce truncated, potentially harmful proteins.

#### **1.2.4 Endoribonucleolytic decay**

In addition to deadenylation and decapping, some mRNAs are degraded through endoribonucleolytic cleavage, a process in which endonucleases introduce internal cuts within the transcript (Garneau, Wilusz, and Wilusz 2007). This cleavage generates exposed 5' and 3' ends, which are subsequently degraded by XRN1 and the exosome, respectively. Endoribonucleolytic decay is considered one of the most efficient mRNA degradation pathways, enabling the rapid removal of unwanted or defective transcripts (Wilusz and Wilusz 2004).

In mammalian cells, this process is mediated by a set of specialized nucleases. While the identity of the responsible endonucleases varies depending on context—and remains incompletely defined in many cases—their activity represents an important alternative to the canonical deadenylation-first decay pathways. For example, nonsense-mediated mRNA decay factor SMG6 mediates endoribonucleolytic cleavage during NMD, ZC3H12A (also known as MCPIP1) and RNase L are active in immune-related RNA decay, argonaute RISC catalytic component 2 (AGO2) carries out microRNA-guided cleavage when near-perfect base pairing is present, and inositol-requiring enzyme 1 alpha (IRE1 $\alpha$ ) functions during the unfolded protein response by targeting ER-associated

mRNAs (Burke et al. 2022; Huntzinger et al. 2008; Jung et al. 2017; Le Thomas et al. 2021; Lichawska-Cieslar et al. 2023).

### **1.2.5 mRNA decay regulators and cis-acting determinants**

During cellular and developmental transitions, the demand for specific mRNAs fluctuates, and their stability is tightly regulated. In some cases, bursts of transcription increase mRNA and protein production, while in others, rapid degradation of no longer needed transcripts is essential. mRNA decay activators play a key role in this process by recruiting components of the decay machinery, often in a sequence-specific manner, to target transcripts for degradation (Schoenberg and Maquat 2012).

One well-studied example of a sequence-specific mRNA decay activator is the microRNA-induced silencing complex (miRISC), (Shang et al. 2023). This complex includes an AGO protein bound to a miRNA that is partially complementary to sequences in the target mRNA. Binding of miRISC to the target transcript leads to translational repression and recruitment of the mRNA decay machinery (Nakanishi 2022). This process is mediated by glycine-tryptophan repeat-containing protein of 182 kDa (GW182/TNRC6) adapter proteins, which interact directly with the CCR4–NOT complex via CNOT1 and CNOT9 (Wilczynska and Bushell 2015). In addition, decapping factors such as PATL1, DCP1, and DDX6 are also recruited, although it remains unclear whether this occurs through direct interaction with miRISC or indirectly via CCR4–NOT (Nishihara et al. 2013).

Another class of decay activators targets transcripts with AU-rich elements (AREs) in their 3' untranslated regions (3'UTRs). AREs are short sequences enriched in adenine and uracil, often containing multiple copies of the AUUUA motif (Chen and Shyu 1995). These elements are recognized by specific RNA-binding proteins (RBPs) such as tristetraprolin (TTP) and KH-type splicing regulatory protein (KHSRP), which then recruit deadenylases, decapping enzymes, and the exosome to promote decay (Lai et al. 2019; Sandler and Stoecklin 2008; Stoecklin, Mayo, and Anderson 2006). Interestingly, the fate of ARE-containing transcripts can vary depending on the associated RBP. For example, human antigen R (HuR) binds to AREs and stabilizes the transcript, delaying degradation even after deadenylation (Peng et al. 1998).

Further, several additional cis-acting elements and structural features contribute to mRNA decay regulation in mammalian cells. Iron-responsive elements in the UTRs of iron metabolism genes bind iron regulatory proteins to either stabilize or destabilize

transcripts, depending on iron availability (Rouault 2006; Sanchez et al. 2011). Upstream open reading frames, found in over half of human mRNAs, regulate translation and can also trigger decay by stalling scanning ribosomes (Akulich et al. 2019; Zhong et al. 2024). Other features, such as stem-loop structures or Staufen-binding sites, influence stability by recruiting decay-promoting factors, including RNA-binding proteins or the helicase up-frameshift protein 1 (UPF1) in the case of Staufen-mediated decay (Gowravaram et al. 2019; Oberstrass et al. 2024).

### 1.2.6 m6A-dependent mRNA decay

Another important layer of mRNA decay regulation involves chemical modifications of RNA, particularly *N*6-methyladenosine. As previously described, YTHDF proteins, and especially YTHDF2, act as cytoplasmic m6A readers that recruit decay machinery to methylated transcripts. Three distinct pathways have been identified through which YTHDF proteins promote mRNA degradation (Figure 1.3).

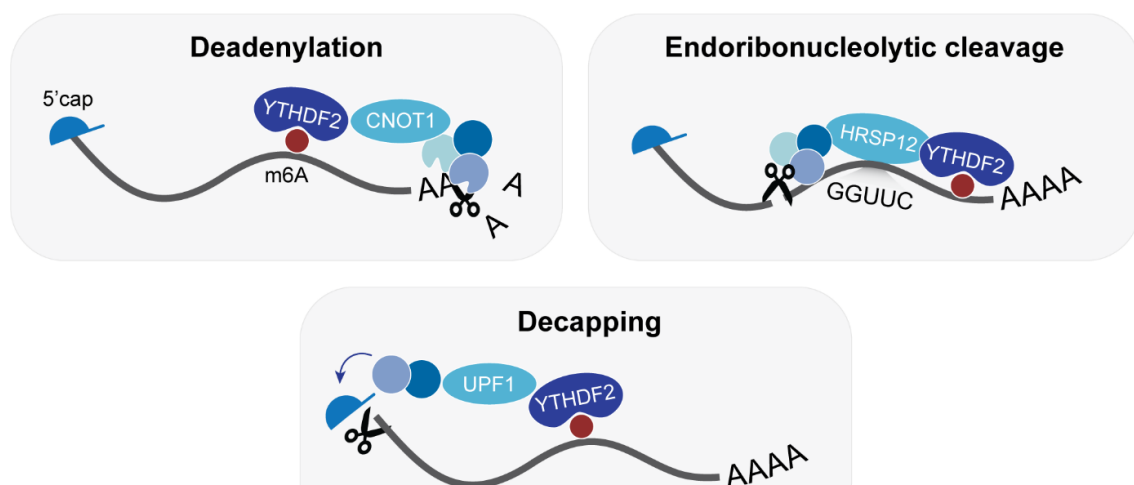


Figure 1.3 Overview of three distinct mechanisms by which YTHDF2 promotes m6A-mRNA degradation: deadenylation via the CCR4–NOT complex, endoribonucleolytic cleavage through HRSP12 and RNase P/MRP, and decapping mediated by the UPF1 and DCP1/2 complex.

The first pathway was described in 2016, when it was reported that YTHDF2 directly interacts with CNOT1, thereby recruiting the CCR4–NOT deadenylation complex to initiate degradation of m6A-modified mRNAs (Du et al. 2016). This interaction occurs between the N-terminal region of YTHDF2 and the SH domain of CNOT1. A few years later, an alternative route for YTHDF2-mediated decay was identified, involving the endoribonuclease ribonuclease P/MRP (RNase P/MRP) (Park et al. 2019). In this

pathway, YTHDF2 promotes binding of the heat-responsive protein 12 (HRSP12) adaptor protein to the target mRNA, which contains a conserved GGUUC motif required for recognition. HRSP12 then bridges YTHDF2 to the RNase complex, leading to cleavage of the transcript. More recently, YTHDF2 was also shown to interact with UPF1, a key factor in NMD. The N-terminal half of YTHDF2 interacts with UPF1, leading to rapid degradation of m6A-marked transcripts through a pathway that is dependent on PNRC2, a factor known to bridge UPF1 and DCP1A to promote decapping. This mechanism is functionally distinct from canonical UPF1–SMG5/7-mediated NMD (Boo et al. 2022). Cytoplasmic mRNAs are constantly navigating a balance between decay and translation, with both processes tightly interconnected. To fully understand how modifications such as m6A regulate transcript fate, we must also examine the translational state in which these decay pathways operate.

### **1.3 mRNA translation**

Once an mRNA transcript is processed and exported to the cytoplasm, it faces a tightly regulated balance between two competing fates: translation or degradation. While Chapter 1.2 focused on the mechanisms of mRNA decay, this chapter will shift to the other major outcome—translation, the process by which the genetic information encoded in mRNA is used to synthesize proteins. Translation not only defines the functional output of gene expression, but also interfaces with decay pathways, particularly during cellular stress, development, and in response to mRNA modifications such as m6A.

The fundamental principles of translation were established in the mid-20th century, laying the foundation for modern molecular biology. In 1954, George Gamow proposed the idea of a triplet code to explain how nucleotide sequences could specify amino acids (Gamow 1954). A few years later, Francis Crick introduced the central dogma and speculated about the existence of adaptor molecules—later discovered to be tRNAs—that decode mRNA (Crick 1958). Experimental confirmation of translation came in 1961, when Nirenberg and Matthaei demonstrated that poly-uracil RNA (poly-U) directs the synthesis of a poly-phenylalanine peptide *in vitro* (Nirenberg and Matthaei 1961). By 1965, the full genetic code—mapping 64 codons to 20 amino acids—had been essentially deciphered. Today, translation is understood as a dynamic, multi-step process involving ribosomes, tRNAs, and various regulatory factors. It is typically divided into three stages: initiation, elongation, and termination (Dever and Green 2012).

Initiation refers to the assembly of the translation machinery on the mRNA. The small subunit of the ribosome first binds near the 5' end of the mRNA and scans in the 3' direction to locate the start codon, typically AUG in eukaryotes. Upon start codon recognition, the initiator tRNA, carrying methionine, binds to the start site, followed by recruitment of the large ribosomal subunit to form the complete 80S ribosome. This process is tightly regulated by a set of eukaryotic initiation factors (Sonenberg and Hinnebusch 2009). During elongation, the nascent polypeptide chain is extended by the sequential addition of amino acids. Specificity is achieved through aminoacyl-tRNAs, which deliver the appropriate amino acid corresponding to each codon. The ribosome contains three key tRNA-binding sites: the A site (aminoacyl), P site (peptidyl), and E site (exit). Each aminoacyl-tRNA binds to the A site, guided by codon–anticodon pairing, and the ribosome catalyzes the formation of a peptide bond between the incoming amino acid and the growing chain. The ribosome then translocates to the next codon, moving the peptidyl-tRNA to the P site and the deacylated tRNA to the E site for release (Noller 2005). Termination occurs when a stop codon (UAA, UAG, or UGA) is recognized in the A site. This triggers the recruitment of release factors, which catalyze the hydrolysis of the bond between the polypeptide chain and the tRNA, releasing the newly synthesized protein. Subsequently, the ribosomal subunits, mRNA, and release factors dissociate (Sonenberg and Hinnebusch 2009).

Beyond its canonical role in protein synthesis, translation is increasingly recognized as a regulatory hub that influences mRNA fate, including stability, localization, and degradation (Monaghan et al. 2023; Shoemaker and Green 2012).

### **1.3.1 Interplay between translation and mRNA stability**

In eukaryotes, translation and mRNA decay are tightly coupled processes. Recent developments in live-cell imaging and single-molecule resolution techniques have enabled simultaneous observation of translation and degradation on individual mRNA molecules, providing new insights into their dynamic relationship. Both experimental data and mathematical modeling suggest that translation can reduce mRNA stability and that this effect is closely tied to ribosome flux (Dave et al. 2023). In parallel, various decay pathways monitor the translational state of ribosomes, targeting transcripts for degradation under specific conditions (Figure 1.4).

#### *1.3.1.1 Codon optimality-mediated decay (COMD)*

Over the past decade, initially in budding yeast and later in mammals, it has been shown that translation elongation can be influenced by codon identity, with certain codons—referred to as optimal codons—promoting more efficient translation and increased mRNA stability, while non-optimal codons tend to slow translation and promote mRNA decay (Caponigro, Muhlrads, and Parker 1993; Hoekema et al. 1987; Wu and Bazzini 2023). Codon optimality is determined by how efficiently a given codon recruits aminoacyl-tRNA to the ribosomal A site, which can vary between species, tissues, and environmental conditions (Presnyak et al. 2015). Mechanistically, recent structural studies revealed that CNOT3, a subunit of the CCR4–NOT complex, can directly interact with the ribosome in both yeast and humans. Specifically, CNOT3 binds to the empty E site of ribosomes where both the A and E sites are unoccupied. This supports the model in which mRNA stability is governed by competition between aminoacyl-tRNA and CNOT3 for ribosome access (Absmeier et al. 2023; Buschauer et al. 2020). Once recruited, the CCR4–NOT complex promotes not only deadenylation but also decapping and 5'–3' exoribonucleolytic decay, potentially through DDX6 (Barrington et al. 2023; Radhakrishnan et al. 2016; Weber and Chang 2024).

#### *1.3.1.2 mRNA decay caused by elongation stalling*

Beyond codon optimality, ribosome stalling during elongation also activates quality control pathways that degrade both the mRNA and associated nascent polypeptides. Such stalling can arise from strong RNA secondary structures or chemical damage to the transcript—a process known as no-go decay (NGD)—or from transcripts lacking stop codons, referred to as non-stop decay (NSD) (Doma and Parker 2006; Frischmeyer et al. 2002; van Hoof et al. 2002; Suryo Rahmanto et al. 2023). Both pathways are tightly integrated with the ribosome-associated quality control (RQC) system, which also ensures ribosome recycling and polypeptide clearance (Bengtson and Joazeiro 2010; Ito-Harashima et al. 2007).

Stalled ribosomes are initially recognized by the E3 ubiquitin ligase zinc finger protein 598 (ZNF598; Hel2 in yeast), which ubiquitinates proteins on the 40S subunit. In yeast, this ubiquitination recruits the endonuclease Cue2, which cleaves the mRNA at the A site. While the corresponding nuclease is not well defined in humans, NEDD4-binding protein 2 (N4BP2) has been proposed as a potential functional analog. NGD typically relies on XRN1 for 5'–3' decay, whereas NSD engages the SKI complex and the exosome for 3'–

5' degradation, especially when the 3' end is uncapped or abnormally structured (reviewed in Monaghan et al. 2023).

Ribosome rescue in both NGD and NSD is facilitated by the HBS1-like translational GTPase (HBS1)–Pelota homolog (PELO) complex, aided by the ATP-binding cassette sub-family E member 1 (ABCE1) ATPase, which dissociates ribosomes from stalled mRNA. While NGD primarily focuses on ribosome rescue, NSD more directly targets the aberrant mRNA for decay (reviewed in Monaghan et al. 2023). In addition to decay, stalled ribosomes can trigger translational repression. The factor endothelial differentiation-related factor 1 (EDF1) senses collided ribosomes and recruits the GRB10-interacting GYF protein 2–eukaryotic translation initiation factor 4E homologous protein complex (GIGYF2–4EHP) complex, which inhibits further rounds of translation on the problematic mRNA (Hickey et al. 2020; Sinha et al. 2020).

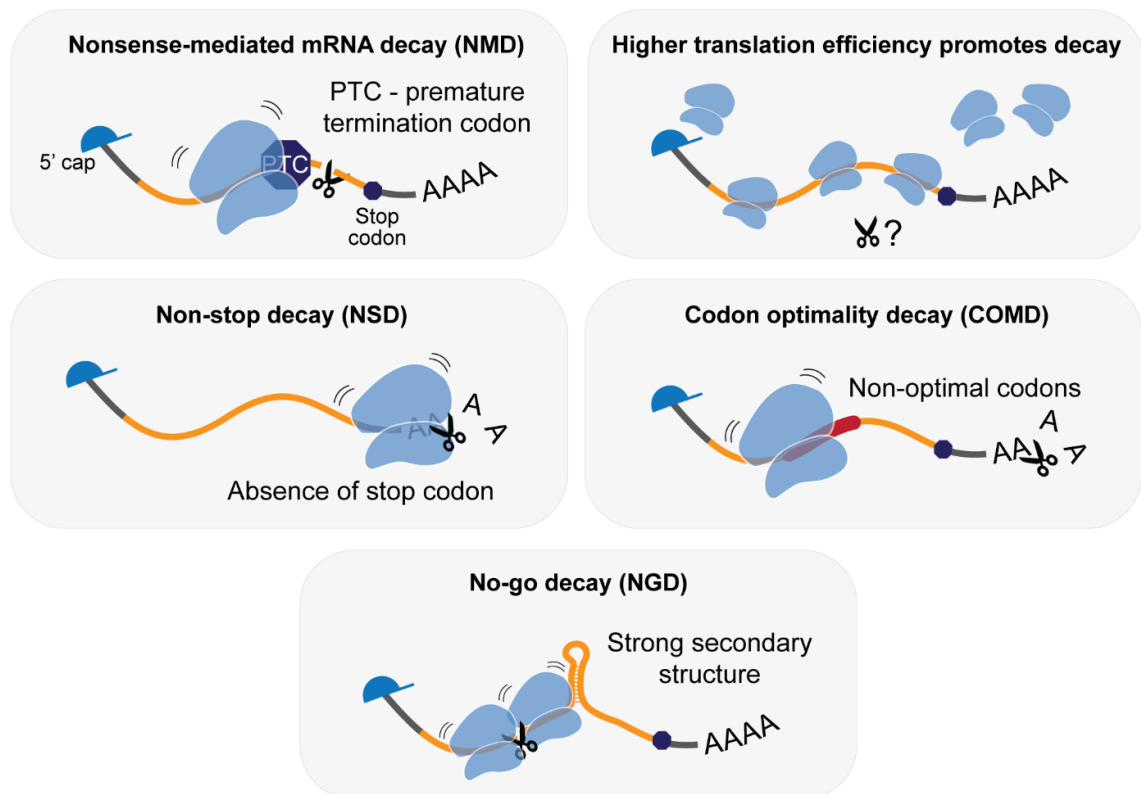


Figure 1.4 Overview of translation-dependent mRNA decay pathways. These include nonsense-mediated mRNA decay (NMD), triggered by premature termination codons (PTCs); non-stop decay (NSD), due to the absence of a stop codon; no-go decay (NGD), caused by strong secondary structures; and codon optimality-mediated decay (COMD), initiated by ribosome stalling at non-optimal codons. Additionally, higher translation efficiency has also been linked to accelerated mRNA decay in certain contexts.

### *1.3.1.3 Nonsense-mediated mRNA decay (NMD)*

The final stage of translation—termination—is also surveilled by a specialized quality control machinery. NMD is triggered when translation terminates prematurely at a premature termination codon (PTC), provided that a downstream EJC is located more than ~55 nucleotides away, although the applicability of this rule can vary depending on context. The central factor in this pathway is UPF1, which can associate with the terminating ribosome near the E site. Its activity is stimulated by UPF2 and UPF3, which enhance UPF1's RNA-binding and ATPase activity. Once a PTC-containing transcript is identified, UPF1 interacts with members of the SMG protein family, which mediate recruitment of the CCR4–NOT complex and decapping enzymes, leading to degradation of the transcript. Similar to NGD and NSD, NMD can also trigger degradation of the associated nascent polypeptide through the RQC pathway (reviewed in Monaghan et al. 2023).

Together, these examples highlight the close connection between translation and mRNA fate, providing a foundation for understanding how m6A modifications can further influence translation efficiency and selectivity.

### **1.3.2 Interplay between translation and m6A**

Our understanding of m6A modification has advanced rapidly over the past decade, with improvements in mapping and functional assays allowing for increasingly precise insights. Alongside this progress, however, a number of controversial questions have emerged—one of the most debated being whether m6A has a direct impact on translation. Early studies suggested that the reader proteins YTHDF1 and YTHDF3 enhance translation. YTHDF1 was proposed to promote translation through its interaction with the initiation factor eIF3, while YTHDF3 was believed to function cooperatively with YTHDF1 (Li et al. 2017; Shi et al. 2017; Wang et al. 2015; X. Wang et al. 2014). However, more recent work has challenged this model. Two studies demonstrated that all three YTHDF proteins (YTHDF1–3) appear to play redundant roles in mRNA decay, rather than having distinct effects on translation. According to this newer model, any observed enhancement of translation by YTHDF proteins is likely an indirect consequence of changes in mRNA stability (Lasman et al. 2020; Zaccara and Jaffrey 2020). Another m6A reader, YTHDC2, has been reported to increase translation efficiency while also promoting mRNA decay, although its specificity for m6A and mechanism of action remain less well defined (Hsu et al. 2017). In parallel, it was proposed that eIF3 could

directly bind m6A in the 5' untranslated region (5'UTR) of certain transcripts, enabling cap-independent translation under stress or other conditions (Meyer et al. 2015). Yet, more recent biochemical, structural, and single-molecule studies found that a single m6A in the 5'UTR does not significantly alter translation dynamics. While an interaction between m6A and eIF2 $\alpha$  was observed, it contributed only marginally to translation initiation (Guca et al. 2024). Finally, recent work revealed an indirect mechanism by which m6A can regulate translation efficiency. The reader protein IGF2BP3 binds to m6A-modified transcripts and relocates them from the actively translating pool into P-bodies, thereby reducing translation without promoting decay (Shan et al. 2023).

Taken together, the regulatory landscape of m6A-modified mRNAs is shaped by their interactions with translation and decay machineries. However, many mechanistic questions remain unresolved, particularly regarding how the position of m6A sites dictates their function. This thesis seeks to address these gaps through different experimental approaches.

## **1.4 Aims of the study**

m6A is the most abundant internal modification in eukaryotic mRNA and plays a critical role in regulating transcript stability. Several m6A-mediated decay pathways have been identified, including deadenylation via interaction with the CCR4–NOT complex, endoribonucleolytic cleavage through HRSP12, and decapping mediated by UPF1. However, it remains unclear how specific m6A sites are routed into distinct decay pathways and what determines the functional consequences of m6A deposition at different transcript positions. Notably, m6A is highly enriched near stop codons but largely excluded from CDS. Recent studies suggest that exon–exon junctions and the EJC, deposited during splicing, limit m6A deposition in CDS regions. Yet, the biological rationale behind this spatial restriction remains unresolved.

My thesis aims to dissect how the location of m6A sites influences their function and to uncover how specific features guide m6A-modified transcripts toward distinct regulatory fates. To this end, I combine transcriptome-wide m6A maps with mRNA half-life measurements to determine whether m6A sites in different transcript regions differ in their capacity to destabilize transcripts. I further develop a targeted reporter system to test the local effect of m6A in the vicinity of stop codons, allowing to manipulate site placement and measure consequences on stability. Finally, to investigate whether m6A

within coding regions influences translation efficiency or ribosome behavior, I apply ribosome profiling.

Together, these approaches aim to define the functional principles governing m6A-mediated decay and clarify how site positioning contributes to post-transcriptional gene regulation.

## 2 Publications

### 2.1 Publication 1: m6A sites in the coding region trigger translation-dependent mRNA decay

#### 2.1.1 Summary

Chemical modifications of mRNA, particularly *N*6-methyladenosine (m6A), have emerged as critical regulators of RNA metabolism. In this work, we uncovered a previously uncharacterized mechanism by which m6A marks within the coding region of transcripts accelerate mRNA turnover. This decay process, which we term CDS–m6A-mediated decay (CMD), is distinct in both kinetics and dependency: it is initiated during translation and requires ribosomal stalling at m6A-modified codons. This translational slowdown coincides with increased enrichment of target transcripts in processing bodies (P-bodies) and involves the m6A reader protein YTHDF2. CMD preferentially affects mRNAs encoding developmental regulators and retrogene products, indicating a context-specific role for CDS m6A in fine-tuning gene expression programs.

#### 2.1.2 Zusammenfassung

Chemische Modifikationen von mRNA, insbesondere *N*6-Methyladenosin (m6A), haben sich als entscheidende Regulatoren des RNA-Stoffwechsels herausgestellt. In dieser Arbeit haben wir einen bislang nicht beschriebenen Mechanismus identifiziert, durch den m6A-Markierungen innerhalb der codierenden Region von Transkripten den Umsatz von mRNA beschleunigen. Dieser Abbauprozess, den wir als CDS–m6A-vermittelte Degradation (CMD) bezeichnen, unterscheidet sich sowohl in seiner Kinetik als auch in seiner Abhängigkeit: Er wird während der Translation eingeleitet und erfordert ein Anhalten der Ribosomen an m6A-modifizierten Codons. Diese Verlangsamung der Translation fällt mit einer erhöhten Anreicherung der Zieltranskripte in sogenannten Processing Bodies (P-Bodies) zusammen und beinhaltet das m6A-erkennende Protein YTHDF2. CMD betrifft bevorzugt mRNAs, die für Entwicklungsregulatoren und Retrogenprodukte kodieren, was auf eine kontextspezifische Rolle von m6A in der codierenden Region bei der Feinabstimmung von Genexpressionsprogrammen hinweist.

### **2.1.3 Statement of contribution**

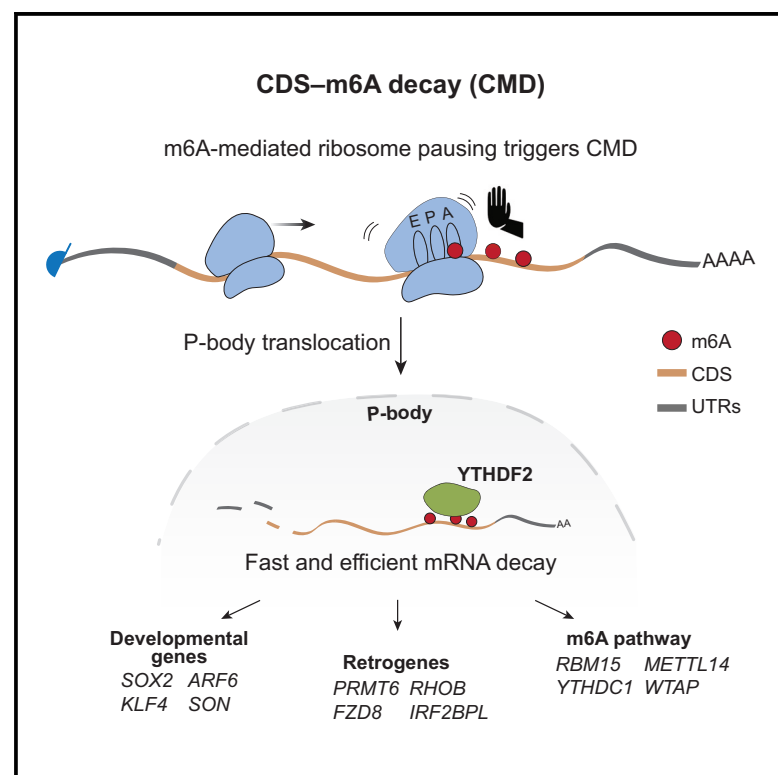
As one of the shared first authors of this publication, I was involved in the conceptual development and design of the study. My primary contributions consisted of performing experiments, including RNA-seq experiments upon m6A depletion and various protein knockdowns, SLAM-seq experiments to measure mRNA half-lives, the design and optimization of the reporter system, and the ribosome profiling experiments. In addition, I contributed to data interpretation, figure preparation, and was actively involved in writing and revising the manuscript.

Supervisor confirmation

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# m6A sites in the coding region trigger translation-dependent mRNA decay

## Graphical abstract



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## In brief

Zhou et al. discovered a specific role of adenosine modifications in the coding region of mRNAs. These chemical alterations slow down the movement of the ribosome during translation and trigger degradation of the corresponding mRNAs.

## Highlights

- m6A in the coding sequence triggers mRNA degradation via CDS-m6A decay (CMD)
- Decay relies on ribosome pausing at codons with m6A in the ribosomal A site
- The CMD targets are selectively enriched in processing bodies (P-bodies)
- CMD controls the expression of developmental regulators and retrogenes



Article

# m6A sites in the coding region trigger translation-dependent mRNA decay

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## SUMMARY

**N<sup>6</sup>-Methyladenosine (m6A) is the predominant internal RNA modification in eukaryotic messenger RNAs (mRNAs) and plays a crucial role in mRNA stability. Here, using human cells, we reveal that m6A sites in the coding sequence (CDS) trigger CDS-m6A decay (CMD), a pathway that is distinct from previously reported m6A-dependent degradation mechanisms. Importantly, CDS m6A sites act considerably faster and more efficiently than those in the 3' untranslated region, which to date have been considered the main effectors. Mechanistically, CMD depends on translation, whereby m6A deposition in the CDS triggers ribosome pausing and transcript destabilization. The subsequent decay involves the translocation of the CMD target transcripts to processing bodies (P-bodies) and recruitment of the m6A reader protein YT521-B homology domain family protein 2 (YTHDF2). Our findings highlight CMD as a previously unknown pathway, which is particularly important for controlling the expression of developmental regulators and retrogenes.**

## INTRODUCTION

The epitranscriptome comprises RNA modifications that confer specific properties on the modified RNA.<sup>1</sup> In mammals, N<sup>6</sup>-methyladenosine (m6A) is the most prevalent internal messenger RNA (mRNA) modification with crucial roles in RNA metabolism.<sup>2–8</sup> m6A controls important physiological functions but is also associated with several diseases.

Methylation at the N<sup>6</sup> position of adenine is catalyzed in the nucleus by the m6A writer complex with the proteins METTL3, METTL14, WTAP, and RBM15.<sup>9–11</sup> The methyltransferase METTL3 catalyzes the m6A modification, while METTL14 is catalytically inactive but facilitates the binding of METTL3 to the target RNA.<sup>12,13</sup> m6A preferentially occurs in the RNA sequence motif DRACH (where D refers to G, A, or U; R refers to G or A; and H refers to U, A, or C).<sup>4,14–17</sup> However, this motif reflects the true distribution of m6A only to a limited extent, as not all DRACH motifs are always modified. Moreover, m6A is also found at non-

DRACH motifs, especially GGACG, expanding the consensus to DRACN (where N refers to any nucleotide).<sup>14,18</sup>

m6A modifications are present throughout transcripts and enriched around stop codons and after the last splice site.<sup>16,19</sup> This is partly explained by recent reports that the exon junction complex (EJC) blocks m6A methylation around splice sites that are mostly found in the coding sequence (CDS).<sup>20–23</sup> To date, no distinct functions have been assigned to m6A sites in individual transcript regions.

m6A controls many steps in the RNA life cycle. The most prominent function is m6A-mediated RNA decay, which controls RNA stability in the cytoplasm.<sup>8,24,25</sup> Three main pathways are known based on either deadenylation, decapping, or endoribonucleolytic cleavage. All three pathways involve the m6A reader protein YT521-B homology domain family protein 2 (YTHDF2) that specifically binds m6A in RNAs. Although many studies have focused on YTHDF2, its close paralogs YTHDF1 and YTHDF3 fulfill similar functions.<sup>26</sup> In the deadenylation pathway,



YTHDF2 interacts with the carbon catabolite repression 4-negative on TATA-less (CCR4-NOT) complex to trigger deadenylation and degradation of m6A-containing transcripts.<sup>8</sup> In the decapping pathway, YTHDF2 interacts with UP frameshift 1 (UPF1), which in turn recruits the decapping machinery to initiate RNA decay.<sup>27</sup> Although UPF1's function in nonsense-mediated mRNA decay (NMD) is connected to translation, its role in m6A-mediated decay is independent of translation. In the internal cleavage pathway, YTHDF2 interacts with heat-responsive protein 12 (HRSP12, encoded by the gene *RIDA*) at m6A sites with nearby HRSP12-binding sites containing GGUUC motifs, which induces endoribonucleolytic cleavage by the ribonuclease P/mitochondrial RNA processing (RNase P/MRP) complex.<sup>28</sup> Despite this knowledge, it remains unclear how a pathway is selected for a specific m6A site.

Here, we investigate how the location of m6A sites affects RNA degradation. Unexpectedly, we find that CDS m6A sites are far more potent in destabilizing RNAs than m6A sites in other transcript regions. We termed this previously uncharacterized pathway CDS-m6A decay (CMD). The target transcripts for CMD are recognized during translation, recruited to processing bodies (P-bodies), and bound by YTHDF2. CMD thereby controls the expression of developmental regulators and safeguards against retrogenes.

## RESULTS

### The METTL3-METTL14 writer complex shows an inherent preference for GGACN motifs *in vivo* and *in vitro*

To study m6A function, we first examined DRACN methylation *in vivo*. Using m6A individual-nucleotide resolution UV crosslinking and immunoprecipitation (miCLIP2) data from HEK293T cells,<sup>14</sup> we found that, consistent with previous reports,<sup>9</sup> GGACN 5-mers are highly modified whereas more A/U-rich 5-mers are rarely methylated (Figures S1A and S1B). This was further supported by nanopore direct RNA sequencing (RNA-seq) analyses with m6Anet<sup>29</sup> as well as m6A data from glyoxal and nitrite-mediated deamination of unmethylated adenosines (GLORI)<sup>18</sup> (Figures S1C and S1D).

To investigate the specificity of the METTL3-METTL14 writer complex, we established a high-throughput *in vitro* methylation assay (Figure 1A). In brief, we generated 11,664 *in vitro* transcripts (IVTs, 183 nucleotides [nt] each), representing 3,488 human loci, that were chosen around DRACN motifs ("selected DRACN") with or without m6A from different transcript regions (Table S1). Furthermore, being natural transcript sequences, the IVTs contain additional DRACN motifs downstream of the selected sites (Figure S1E). We then added a recombinantly reconstituted METTL3-METTL14 writer complex<sup>30</sup> and analyzed the resulting methylation profile using miCLIP2<sup>14</sup> (Table S2). We observed precise peaks at the DRACN motifs that specifically appeared after *in vitro* methylation (Figures 1B, 1C, S1F, and S1G). By contrast, adenosines in a non-DRACN context remained unchanged (Figures S1F and S1H). Surprisingly, we saw *in vitro* methylation also for some DRACN sites that had been unmodified *in vivo* (Figure S1F), indicating that methylation is prevented *in vivo* (see below).

Among the different DRACN 5-mers, the strongest *in vitro* methylation occurred on GGACN—similar to *in vivo*—suggesting that this preference is intrinsic to the METTL3-METTL14 complex (Figures 1B, S1H, and S1I). This agrees with previous reports that GGACN motifs are the most methylated *in vivo*<sup>9,31</sup> and *in vitro*.<sup>30</sup> Exchanging the three most methylated DRACN motifs to weakly methylated DRACN 5-mers, and vice versa, consistently reduced or enhanced methylation, respectively (Figures S1J and S1K). Moreover, a strong correlation across DRACN motifs suggested that a major part of the *in vivo* methylation pattern is driven by the intrinsic specificity of the writer complex (Figures 1D and S1L). We note that some 5-mer motifs ending on uridine (U) achieved higher miCLIP2 signal *in vivo*, even though both datasets equally rely on UV crosslinking. A possible candidate for tuning the *in vivo* methylation specificity is WT1-associated protein (WTAP), a writer complex subunit that is not present in our reconstituted complexes and preferentially binds to GACU motifs.<sup>9</sup> Overall, we conclude that the *in vivo* preference for GGACN motifs is inherent to the METTL3-METTL14 writer complex.

### m6A sites from all transcript regions are equally methylated *in vitro*

As previously described, we found that *in vivo* m6A sites are predominantly enriched more than 200 nt away from splice sites on long exons, as well as around stop codons and in 3' untranslated regions (3' UTRs) (Figures 1E and 1F).<sup>16,20–23,32</sup> In the *in vitro* miCLIP2 data, however, we found no differences between DRACN motifs originating from different regions (Figures 1E, 1F, and S1M). Consistent with certain factors influencing methylation *in vivo*, recent reports have shown that the EJC prevents m6A deposition close to splice sites,<sup>20–23</sup> explaining the *in vivo* m6A enrichment in long exons and 3' UTRs since splice sites mostly occur in the CDS.<sup>33</sup>

### The impact of m6A sites on RNA decay is independent of the DRACN motif

To study the impact of m6A sites, we performed RNA-seq to quantify transcript expression in HEK293T cells upon depletion of m6A using the METTL3 inhibitor STM2457 for 9 h (Table S2).<sup>34</sup> Consistent with m6A destabilizing transcripts,<sup>6,26,35</sup> the transcripts harboring most DRACN motifs exhibited the strongest increase in abundance (Figure S1N).

Among the DRACN 5-mers, transcripts harboring GGACN motifs were the most increased (Figure S1O), matching their strongest methylation *in vitro* and *in vivo*. To control for varying methylation levels of DRACN motifs, we used partial correlations. These showed that only the total number of m6A sites but not the specific DRACN harboring those sites was strongly correlated with the changes in RNA abundance (Figure 1G). We conclude that the likelihood of adenosine being methylated depends on the DRACN 5-mer and its distance to the nearest splice site; however, once the modification is made, the RNA is primed for destabilization regardless of that sequence.

### CDS m6A sites have the strongest effect on transcript stability and abundance

Since m6A varies along transcripts, we decided to investigate how m6A sites in different regions affect RNA stability.

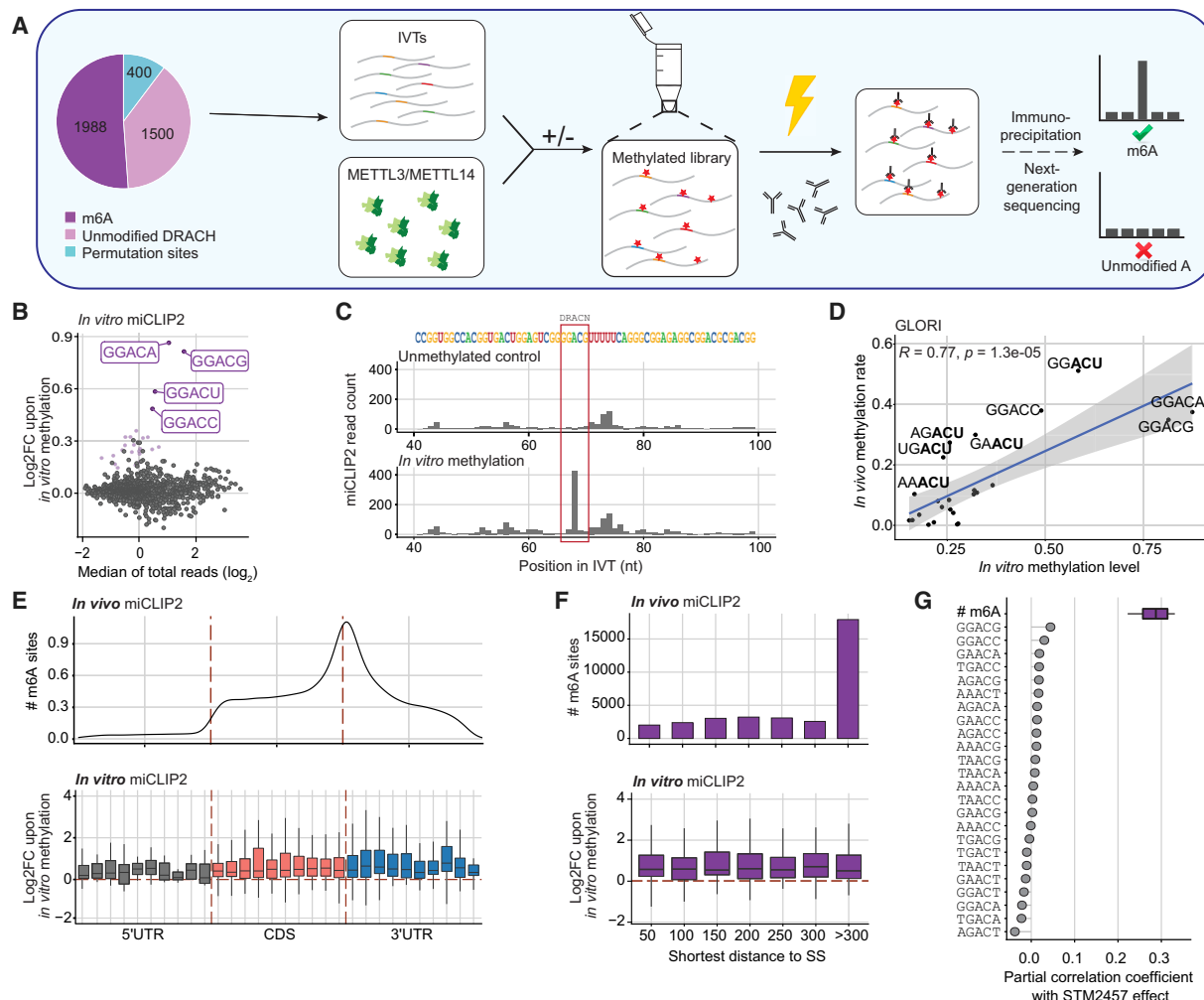


**Figure 1. High-throughput *in vitro* methylation reveals the intrinsic preferences of the METTL3-METTL14 writer complex**

(A) 11,664 *in vitro* transcripts (IVTs) representing transcript regions around m6A ( $n = 1,988$ ), unmodified DRACH (from *in vivo* miCLIP2;  $n = 1,500$ ), and permuted sites (exchange of stronger for weaker DRACH motifs and vice versa,  $n = 400$ ) were methylated using reconstituted METTL3-METTL14 writer complex and analyzed using miCLIP2.<sup>14</sup>

(B) Differential methylation (median  $\log_2$ -transformed fold change [ $\log_2$ FC] of METTL3-METTL14-treated vs. untreated samples) for all 5-mers against median miCLIP2 read counts ( $\log_2$ ). DRACH motifs in purple.

(C) Exemplary IVT (*IER5*, chr1:181,089,254–181,089,314). Selected DRACH motif in red box.

(D) Correlation between methylation level *in vivo* (GLORI) (Figure S1B) and differential methylation *in vitro*. Linear regression (blue line), 95% confidence level interval (gray shading), Pearson correlation coefficient ( $R$ ), and associated  $p$  value are shown.

(E) Scaled density of *in vivo* m6A sites (miCLIP2) in 5' UTR, CDS, and 3' UTR and corresponding *in vitro* methylation levels ( $\log_2$ FC as in B).

(F) Number of m6A sites *in vivo* relative to shortest distance to splice site (SS) and corresponding *in vitro* methylation levels of top four miCLIP2 motifs (Figure S1B).

(G) Partial correlation for overall m6A sites and different DRACH motifs with transcript abundance after m6A depletion (9 h).

Surprisingly, mRNA half-life data from HEK293T cells<sup>36</sup> showed that the half-lives were the shortest for transcripts with m6A sites in the CDS, with more m6A sites progressively increasing the effect (Figure 2A). The same was visible in published transcript half-lives from mouse embryonic stem cells (mESCs; Figure S2A).<sup>37</sup> Importantly, if m6A was depleted using *Mettl3* knockout (KO),<sup>37</sup> transcripts with CDS m6A sites were more stabilized than those with 3' UTR m6A sites (Figure S2B). The half-life changes upon *Mettl3* KO<sup>37</sup> positively correlate with transcript abundance changes upon m6A depletion<sup>38</sup> (Figure S2C), supporting the use of RNA-seq data as a read-out for investi-

gating mRNA stability. Transcripts with CDS m6A sites were most elevated upon m6A removal, confirming the crucial role of m6A sites in this region (Figure S2D).

To study the dynamics of RNA degradation, we investigated transcriptome changes in HEK293T cells treated for 6, 9, or 24 h with the METTL3 inhibitor STM2457 (Table S2). To our surprise, we found that after 6 h of m6A depletion, only transcripts harboring CDS m6A were increased in abundance and hence stabilized (Figure 2B). By contrast, transcripts with m6A sites in the 5' and 3' UTRs increased only after 9 and 24 h (Figures 2C and 2D). Nonetheless, CDS m6A sites had the strongest effect

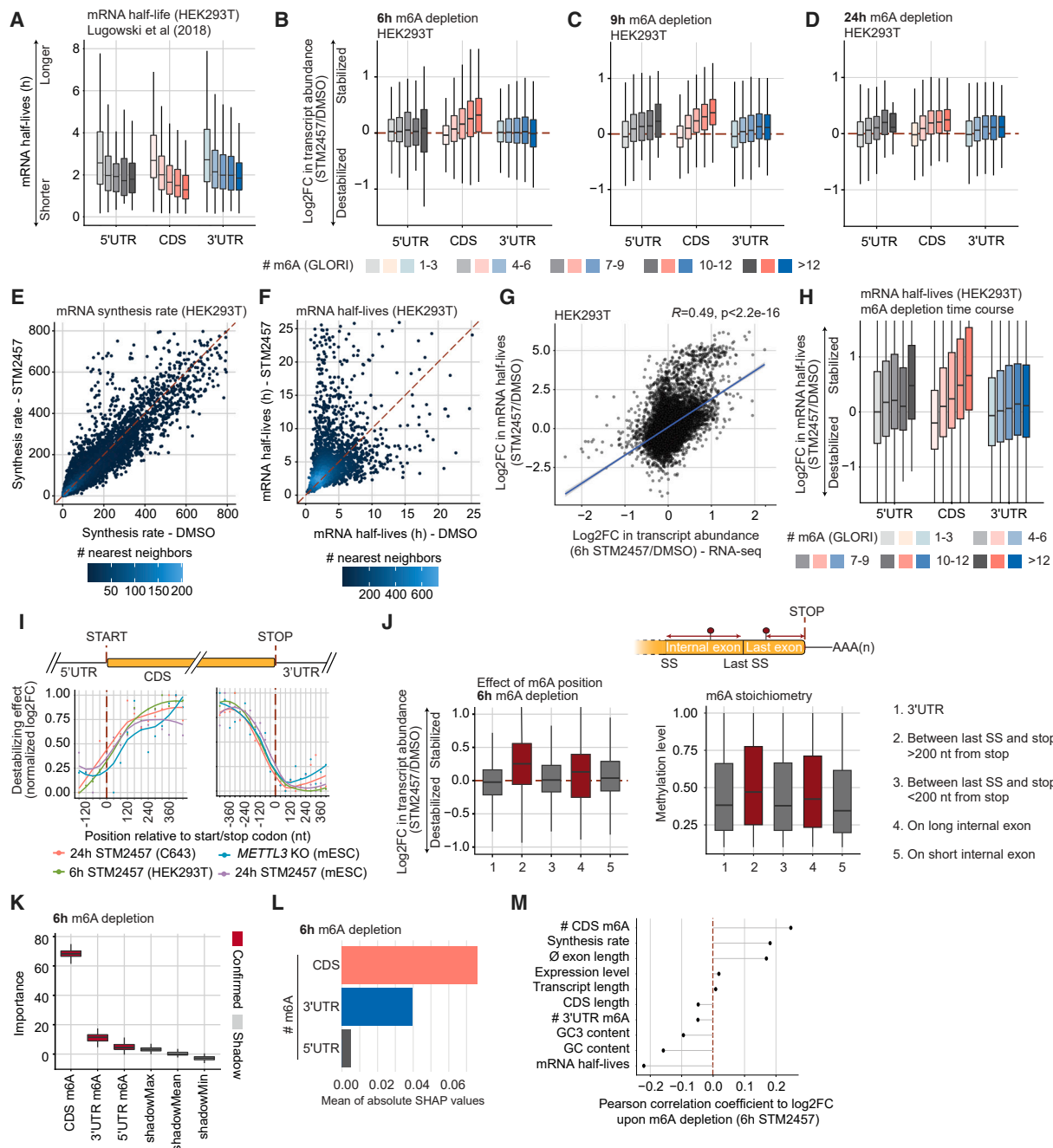


**Figure 2. CDS m6A sites strongly affect RNA stability and abundance**

(A) mRNA half-lives in HEK293T cells<sup>36</sup> grouped by m6A in regions (GLORI).<sup>18</sup>  
(B–D) Log<sub>2</sub>FC in transcript abundance upon m6A depletion for 6 h (B), 9 h (C), or 24 h (D), grouped as in (A).  
(E and F) Correlation of synthesis rates (E), and mRNA half-lives (F), from SLAM-seq.  
(G) Correlation between changes in RNA stability (SLAM-seq) and transcript abundance after m6A depletion (6 h) in HEK293T cells. Linear regression (blue line), Pearson correlation coefficient (R), and associated p value are shown.  
(H) Log<sub>2</sub>FC in mRNA half-lives in m6A-depleted HEK293T cells grouped as in (A).  
(I) Normalized log<sub>2</sub>FC in abundance of transcripts with m6A sites relative to CDS start (START) and end (STOP) in multiple datasets after m6A depletion.  
(J) Log<sub>2</sub>FC in abundance after m6A depletion (6 h, left) and m6A stoichiometry (right) for transcripts with m6A sites relative to last splice site and stop codon. Long and short exons refer to >3,000 nt and ≤3,000 nt, respectively.

(legend continued on next page)

also at these later time points. The CDS-specific effect was corroborated when explicitly controlling for potentially confounding m6A sites in the 3' UTR (Figures S2E–S2J). It was also observed in a time course experiment with mESCs after m6A depletion,<sup>14</sup> in which CDS m6A sites showed the strongest response, particularly at the earlier time points (Figures S2K–S2M). Thus, transcripts with CDS m6A sites not only receive greater stabilization but also react faster to m6A depletion.

To validate the effect on RNA stability, we measured mRNA synthesis rates and half-lives upon m6A depletion (3–6 h) in HEK293T cells using thiol (SH)-linked alkylation for metabolic sequencing (SLAM-seq).<sup>39</sup> Whereas mRNA synthesis rates appeared unaffected, we observed a global increase in mRNA half-lives upon m6A depletion (Figures 2E and 2F). Furthermore, the changes in mRNA half-lives correlated well with the changes in total RNA abundance (Figure 2G), supporting that altered mRNA stability underlies the observed transcript abundance changes. Importantly, transcripts with CDS m6A sites were more stabilized than transcripts with m6A sites in the UTRs (Figures 2H, S2N, and S2O), mirroring our observations on total transcript abundance.

A possible explanation for the stronger effects of CDS m6A sites are differences in stoichiometry. To address this, we used the stoichiometric information from GLORI data for HEK293T cells.<sup>18</sup> Generally, CDS m6A sites were slightly more abundant and more frequently methylated (Figure S2P). Nonetheless, when summing up the stoichiometry into an overall methylation level, we found that even the transcripts with the most m6A methylation in the 5' and 3' UTRs were not increased after m6A depletion for 6 h (Figure S2Q). By contrast, transcripts harboring the same overall methylation level in the CDS were strongly increased (Figure S2Q). Thus, differences in the m6A stoichiometry cannot explain the fast and efficient decay triggered by CDS m6A sites.

We observed that the destabilizing effect of m6A sites gradually increases from the beginning of the CDS and decreases toward its end, reaching its maximum more than 200 nt away from start and stop codons (Figures 2I, S2R, and S2S). This may partially reflect the distribution of splice sites, as transcripts with m6A sites further away from splice sites showed a stronger increase in abundance after m6A depletion (Figure S3A), matching the higher stoichiometry at these sites (Figure S3B). Indeed, transcripts with m6A sites within long exons or after the last splice site in the CDS exhibited the greatest effects (Figure 2J). For example, the *CGRRF1* transcript contains a cluster of m6A sites 144 nt upstream of the stop codon and is rapidly stabilized after m6A depletion for 6 h (Figures S3C and S3D). Overall, these results support the destabilizing effect of CDS m6A sites.

To confirm the relevance of CDS-m6A sites, we used two independent machine-learning-based feature selection strategies (Boruta and Shapley additive explanations for tree-based machine learning models, TreeSHAP).<sup>40,41</sup> Both approaches consistently ranked the number of CDS m6A sites as the most

important feature for predicting changes in transcript abundance after 6 h of m6A depletion (Figures 2K and 2L). To identify potential covariates, we correlated the transcript features to the changes upon m6A depletion. The number of CDS m6A showed the highest correlation, followed by moderate associations with synthesis rate, average exon length, GC content, and mRNA half-life (Figure 2M). However, irrespective of the covariates, transcripts with CDS m6A sites were strongly upregulated upon m6A depletion (Figures S3E–S3M). Using an alternative approach based on conditional quantile normalization,<sup>42</sup> the effect of CDS m6A sites also remained (Figures S3N–S3X). Overall, our data reveal a so far unknown pathway for m6A-mediated mRNA degradation that acts rapidly and specifically via CDS m6A sites. We termed this pathway CDS-m6A decay (CMD).

### CMD relies on translation

The link to the CDS raises the question whether translation is involved in CMD. To answer this, we first investigated transcriptome profiles after translation inhibition using emetine.<sup>43</sup> Of note, the effect of translation inhibition scaled with the m6A sites in the CDS but not in the 5' and 3' UTRs (Figure 3A). To further explore this, we combined 6 h m6A depletion with translation inhibition for the last 2 h using emetine and selected eight transcripts that were rapidly upregulated in our time course data and hosted between eight and 27 CDS m6A sites (Table S3). Quantitative real-time PCR (real-time qPCR) confirmed the immediate increase of all eight transcripts upon m6A depletion for 6 h (STM2457; Figure S4A). All eight transcripts also increased, albeit less, when translation was inhibited for 2 h (emetine, Figures S4A and S4B). Importantly, the effect was abolished if transcripts were first depleted of m6A (STM2457 + emetine vs. STM2457, Figure 3B). To generalize these findings, we performed RNA-seq experiments on the same samples (Table S2; Figure S4C). Similar to the published data, transcripts with CDS m6A sites were upregulated upon translation inhibition (Figure 3C). Importantly, the effect was lost in the m6A-depleted cells, supporting that CMD and translation are functionally connected (Figure 3D).

Next, we constructed a plasmid reporter expressing green fluorescent protein (GFP) fused in-frame to a 565-nt fragment of *CGRRF1* with last two exons up to the stop codon (Figure 3E; Table S4). The last exon contains a cluster of five m6A sites upstream of the stop codon (Figure S3D). The reporter also expresses mCherry from a bidirectional promoter to control for transfection efficiency (Figure 3E). Flow cytometry confirmed that the wild-type (WT) reporter emitted increased GFP signal after m6A depletion for 6 h (Figures 3F and S4D), consistent with stabilization of the reporter transcript. This was abolished when mutating the CDS m6A sites (Figure 3F). Importantly, moving the stop codon upstream of the m6A sites was also sufficient to abrogate the response (Figure 3F), strongly supporting that CMD relies on translation. We strengthened this observation

(K) Importance score for predicting changes after m6A depletion (6 h) based on m6A sites in transcript regions. Red, significant difference to shadow features (light gray).

(L) Mean absolute SHAP values for number of m6A sites (GLORI) in transcript regions.

(M) Pearson correlation coefficients for number of CDS m6A sites and covariates with transcript abundance upon m6A depletion (6 h).

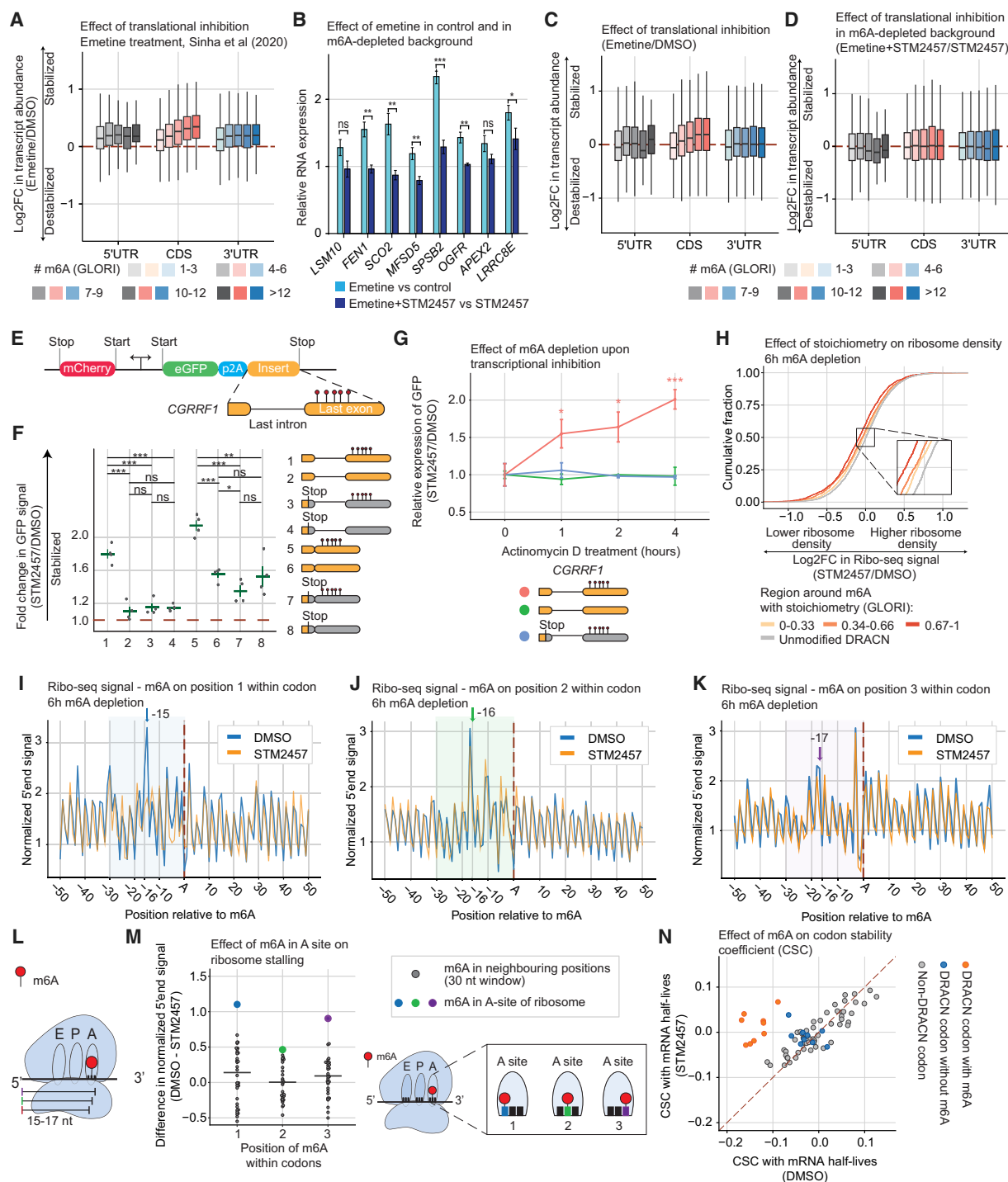


**Figure 3. Translation plays a crucial role in CMD**

(A) Log<sub>2</sub>FC upon translation inhibition (1.8  $\mu$ M emetine, 2 h)<sup>43</sup> for transcripts grouped by m6A in regions (GLORI). (B) Real-time qPCR for relative expression changes upon translation inhibition (emetine) in m6A-depleted (STM2457, 6 h) and control (DMSO) HEK293T cells. Fold changes of normalized Ct values ( $\Delta$ Ct, normalized to *ACTB*) are displayed as mean  $\pm$  standard deviation of mean (SD). \**p* value < 0.05, \*\**p* value < 0.01, \*\*\**p* value < 0.001, ns, non-significant; two-sided Student's *t* test on log<sub>2</sub>-transformed data; *n* = 3 biological replicates. (C and D) Log<sub>2</sub>FC upon translation inhibition (1.8  $\mu$ M emetine, 2 h) in control (C) and m6A-depleted condition (D) for transcripts as in (A). (E) *CGRFR1* plasmid reporter. GFP is fused in-frame to *CGRFR1* (565 nt) fragment containing five m6A sites in the last exon. Expression of mCherry and GFP from bidirectional promoter was measured using flow cytometry.

(legend continued on next page)

with two additional reporter constructs in which we moved the stop codon upstream of the m6A sites (Figure S4E; Table S4).

To investigate the role of splicing, we removed the intron from the *CGRRF1* reporter. This resulted in a stronger response to m6A depletion compared with the WT reporter (Figure 3F), indicating that—consistent with the impact of the EJC<sup>20–23</sup>—the reporter might be more methylated. Nonetheless, removing the m6A sites or moving the stop codon equally counteracted the increase in GFP signal upon m6A depletion (Figure 3F).

To confirm the link to RNA stability, we measured *CGRRF1* reporter RNA decay after transcription inhibition with actinomycin D. Indeed, the WT reporter RNA was less stable than the variants with mutated m6A sites or moved stop codon (Figures S4F–S4I). Importantly, the WT reporter was stabilized upon m6A depletion, while both mutant reporters were unaffected (Figure 3G). Overall, these results consistently support the primary role of translation in CMD.

### Codons with m6A in the ribosomal A site trigger ribosome pausing

To investigate local changes in translation, we performed ribosome profiling (Ribo-seq)<sup>44</sup> and matched RNA-seq in HEK293T cells upon m6A depletion (Table S2). First, we analyzed translational efficiency (TE, i.e., the ratio of Ribo-seq to RNA-seq signals) and found that transcripts with higher TE increased more after 6 h of m6A depletion only if m6A sites were present in the CDS but not in the 3' UTR (Figures S4J and S4K). Next, we used the ribosome footprints to assess local ribosome densities. m6A depletion resulted in a decrease in ribosome densities at m6A sites—scaling with m6A stoichiometry—but not at unmethylated DRACN motifs (Figure 3H), suggesting that ribosomes are slowed in the vicinity of CDS m6A sites. This effect was strongest for m6A located on the third position within codons and weakest on the second position (see below, Figure S4L). Interestingly, the reduction was strongest on transcripts that were upregulated after 6 h of m6A depletion (Figure S4M). Analysis of published Ribo-seq data for *Mettl3* KO mESCs<sup>45</sup> yielded similar results (Figure S4N).

Taking a closer look, we observed a peak in ribosome footprints around position –16 relative to the CDS m6A sites (corresponding to the DRACN adenine in the A site) that was reduced upon m6A depletion (Figures 3I–3L). This scaled with m6A stoichiometry (Figures S5A–S5F)—while no changes were visible upstream of unmodified DRACN motifs (Figures S5G–

S5J). Of note, local ribosome pausing was most prominent when m6A was located on the first and third position of codons (Figures 3I–3M). Consistently, transcripts harboring m6A sites on the first and third codon position were slightly more upregulated upon m6A depletion (Figures S5K and S5L). To experimentally test this, we generated *CGRRF1* reporter variants with all m6A sites either at the first, second, or third codon position (Table S3). Indeed, m6A on the first and third codon position led to a bigger increase in GFP signal upon m6A depletion than at the second codon position (Figure S5M).

When looking at specific codons, we observed an increase in transcript abundance for all the codons that overlap with DRACN (Figure S5N). To delineate m6A from other effects (e.g., codon optimality), we calculated codon stability coefficients (CSCs),<sup>46</sup> i.e., the correlation between codon frequency and transcript stability, for all codons. The CSCs of unmethylated codons (including codons with DRACN overlap but without modification in GLORI) were similar for transcript half-lives in the presence and upon m6A depletion (Figure 3N), arguing against a general impact of codon optimality on the m6A-mediated changes. By contrast, m6A-methylated codons showed a negative correlation to transcript half-lives in the presence of m6A, while their CSCs came close to 0 upon m6A depletion (Figure 3N). Overall, our data suggest that ribosomes pause when m6A-containing codons are in the ribosomal A site and that this is functionally connected to CMD.

### The m6A reader YTHDF2 is involved in CMD

The protein YTHDF2 acts in several m6A-mediated decay pathways.<sup>6,8,26–28,47</sup> We therefore mapped YTHDF2 RNA binding using iCLIP2<sup>48,49</sup> in HEK293T cells with and without 12 h m6A depletion (Table S2) and identified 364,280 YTHDF2 binding peaks.<sup>50</sup> Stronger YTHDF2 peaks frequently resided within DRACN motifs (Figure S6A). Similarly, significantly reduced peaks after m6A depletion were mostly located in DRACN (Figures 4A, S6B, and S6C). Moreover, DRACU motifs that are more methylated *in vivo*, m6A sites with higher m6A stoichiometry, and clustered m6A sites exhibited the strongest YTHDF2 binding (Figures 4B, S6D, and S6E). Within transcripts, most m6A-dependent YTHDF2 peaks resided within the CDS, followed by the 3' UTR and 5' UTR (Figure 4C). Overall, these results suggest that YTHDF2 directly binds m6A in all transcript regions.

(F) Fold changes in GFP levels (normalized to mCherry) after m6A depletion (16 h). *p* value is calculated on normalized fold changes of GFP levels and visualized as in (B). *n* = 4 biological replicates.

(G) Real-time qPCR of *GFP* mRNA upon m6A depletion (16 h) after transcriptional inhibition (actinomycin D, 5  $\mu$ g/mL, 0–4 h) for WT reporter (salmon), m6A mutant (green), and moved stop mutant (blue). *p* value is calculated on fold changes of normalized Ct values ( $\Delta$ Ct, normalized to *mCherry* mRNA). Data are visualized as in (B). *n* = 3 biological replicates.

(H) Log<sub>2</sub>FC in ribosome densities after m6A depletion (6 h) around m6A sites (GLORI, binned by stoichiometry) compared with unmodified DRACN (light gray). (I–K) Normalized ribosome footprints (5' ends of Ribo-seq reads) in control and m6A-depleted cells at m6A sites with stoichiometry > 0.3 on positions 1 (I), 2 (J), and 3 (K) of codons.

(L) Positioning of m6A site in ribosomal A site corresponds to 5' ends of ribosome footprints at positions –15, –16, or –17 relative to m6A site for m6A at position 1, 2, or 3 of codon.

(M) Difference in normalized 5' end signal between control and m6A-depleted (6 h) condition in 30-nt window (highlighted in I–K) for transcripts with m6A in codon position 1, 2, and 3. m6A in ribosomal A site is colored.

(N) Correlation of codon stability coefficients (CSCs) for codons within and outside the DRACN (gray) between control and m6A-depleted condition. DRACN codons are divided into methylated (orange, GLORI) and unmethylated (blue).

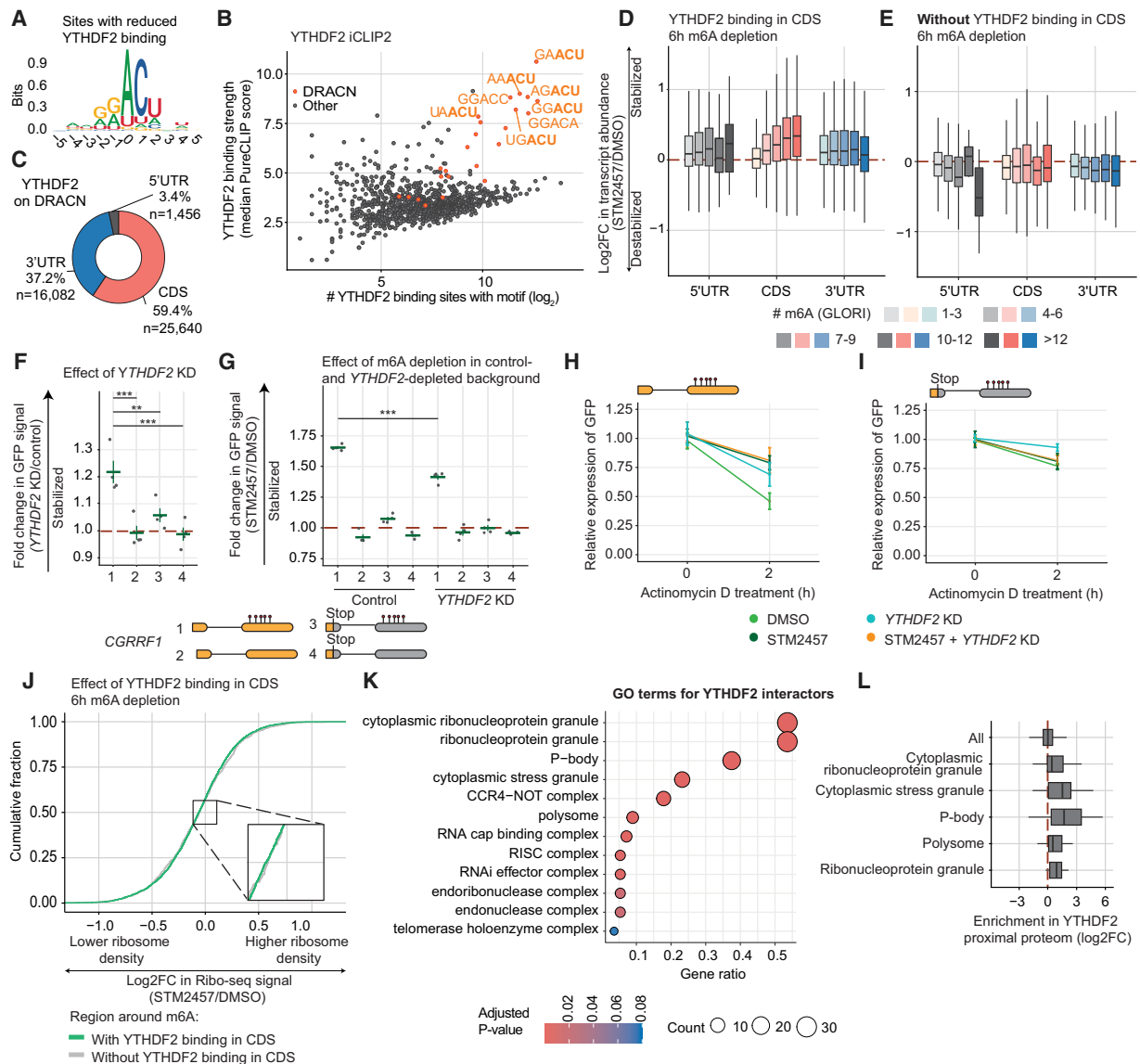


**Figure 4. YTHDF2 contributes to all m6A-mediated RNA decay pathways**

(A) Sequence logo for significantly reduced YTHDF2 peaks after m6A depletion (12 h).  
 (B) YTHDF2 binding strength (median PureCLIP score) for given motif. DRACN (orange) and DRACU (bold) are indicated.  
 (C) YTHDF2 peaks on DRACN across transcript regions.  
 (D and E) Log<sub>2</sub>FC after m6A depletion (6 h) for transcripts with (D) or without (E) YTHDF2 binding in CDS, grouped by m6A in regions (GLORI).  
 (F and G) Fold changes in GFP levels (normalized to mCherry) after YTHDF2 KD (48 h) (F) and after m6A depletion (16 h) (G) in control (left) and YTHDF2-depleted conditions (right). \*p value < 0.05, \*\*p value < 0.01, \*\*\*p value < 0.001, ns, non-significant; two-sided Student's t test on fold changes; n = 4 biological replicates.  
 (H and I) Real-time qPCR of GFP mRNA upon transcriptional inhibition (actinomycin D, 5 µg/mL, 0–4 h) in control (light green), m6A-depleted (dark green), YTHDF2 KD (blue), and m6A-depleted and YTHDF2 KD (yellow) conditions for WT reporter (H), and moved stop variant (I). Fold changes of normalized Ct values (ΔCt, normalized to mCherry mRNA) are displayed as mean ± SD.  
 (J) Log<sub>2</sub>FC in ribosome densities after m6A depletion around m6A sites with (green) and without (gray) YTHDF2 binding in CDS.  
 (K) Top 12 GO terms of YTHDF2 interaction partners.  
 (L) Enrichment of cellular compartments for YTHDF2 proximity proteomics, measured as a log<sub>2</sub>FC.

To learn about the role of YTHDF2 in CMD, we combined 6 h m6A depletion and YTHDF2 knockdown (KD) in HEK293T cells. Real-time-qPCR showed that the eight selected CMD targets increased upon 6 h m6A depletion as well as upon YTHDF2 KD (Figure S6F; Table S3), indicating that YTHDF2 is involved in their

degradation. Accordingly, the effect of m6A depletion was significantly reduced upon YTHDF2 KD (Figure S6G). To generalize these findings, we performed RNA-seq on the same samples (Table S2). Consistent with YTHDF2 acting in multiple pathways, the transcript increase upon YTHDF2 KD was connected to m6A

sites in all regions and almost completely abolished upon m6A depletion (Figures S6H and S6I). Interestingly, the stabilization of transcripts with CDS m6A sites after m6A depletion for 6 h was reduced in *YTHDF2*-depleted cells (Figures S6J and S6K). Moreover, CDS m6A sites only showed an effect when bound by YTHDF2, whereas transcripts with CDS m6A sites but without YTHDF2 binding were mildly decreased (Figures 4D, 4E, S6L, and S6M). This effect was not due to differences in m6A stoichiometry between YTHDF2-bound and unbound transcripts (Figure S6N).

Next, we analyzed effects on the *CGRRF1* GFP reporter construct. We found that the WT reporter showed an increase in GFP signal upon *YTHDF2* KD, while the constructs with mutated m6A sites or moved stop codon remained unaffected (Figure 4F). Moreover, the increase upon m6A depletion was alleviated in *YTHDF2* KD cells (Figure 4G). Real-time qPCR measurements after actinomycin D treatment confirmed that the WT reporter RNA was stabilized after *YTHDF2* KD cells (Figures 4H and 4I) and that the effect of m6A depletion was diminished in *YTHDF2* KD conditions (Figures S6O and S6P), supporting that YTHDF2 contributes to CMD.

### CMD targets localize to P-bodies

To address how YTHDF2 acts in CMD, we first analyzed our Ribo-seq data but did not observe a difference in ribosome pausing between YTHDF2-bound and unbound CDS-m6A sites (Figures 4J and S6Q). To learn about downstream events, we identified YTHDF2 interaction partners with proximity proteomics<sup>51</sup> in HEK293T cells (Figure S6R; Table S5). Gene Ontology (GO) analyses revealed multiple proteins from P-bodies (Figures 4K and 4L; Table S5) in line with previous observations.<sup>8,26</sup> P-bodies are cytoplasmic granules that contain translationally repressed mRNAs as well as RNA-regulatory proteins involved in RNA decay.<sup>52</sup>

To investigate the connection to P-bodies, we analyzed transcriptome-wide data on P-body transcripts in HEK293T cells.<sup>53</sup> Strikingly, transcripts more enriched in P-bodies harbored more CDS m6A sites but not UTR m6A sites (Figure 5A). We next defined the CMD targets based on increased abundance solely upon m6A inhibition for 6 h (termed 6 h targets), as well as the 9 h and 24 h targets that likely gathered the collected targets of other m6A-mediated decay pathways (Table S6). Indeed, the 6 h targets were preferentially localized in P-bodies (Figure 5B). The same was observed for the transcripts increased upon translation inhibition (emetine targets; Figure 5B). By contrast, the 9 h and 24 h targets were not enriched in P-bodies (Figure 5B), suggesting that P-bodies play a specific role in CMD. Whereas the overall YTHDF2 targets (i.e., increased upon *YTHDF2* KD) were not specifically associated with P-bodies (Figure 5B), transcripts with YTHDF2 binding in the CDS were clearly enriched in P-bodies in an m6A-dependent manner (Figures 5C, S6S, and S6T).

Next, we visualized P-bodies using immunofluorescence staining against DCP1a in HEK293T cells (Figure S7A). Notably, we found that depletion of m6A (6 and 24 h) reduced P-body number and signal intensity (Figures 5D, 5E, S7B, and S7C), consistent with CMD targets no longer being translocated to P-bodies. Depletion of *YTHDF2* showed only a slight reduction

(Figures 5F, 5G, S7D, and S7F), suggesting that YTHDF2 may act after P-body translocation.

Overall, we propose that CMD is specifically triggered by CDS m6A sites that cause ribosome pausing and initiate translocation of CMD targets to P-bodies for subsequent decay.

### CMD is distinct from other known pathways and involves RNA decapping

Three main pathways are known to initiate RNA decay by deadenylation, internal cleavage, or decapping, and these involve the factors CCR4-NOT transcription complex subunit 1 (CNOT1), HRSP12, or mRNA-decapping enzyme 1/2 (DCP1/2), respectively.<sup>8,27,28</sup> To address their contribution to CMD, we analyzed transcriptome changes upon *CNOT1* and *HRPS12* KD as well as *DCP2* KO in HEK293T or HeLa cells.<sup>28,54,55</sup> To differentiate the pathways, we assessed the impact on m6A-containing transcripts and compared specific target sets, based on increased transcript abundance solely upon m6A inhibition (6 h, 9 h, and 24 h targets), translational inhibition (emetine targets), and RNA-binding protein depletion (YTHDF2, DCP2, CNOT1, and HRSP12 targets, Table S6).

In the *CNOT1* KD, we observed mild effects on m6A sites in all transcript regions, most visible in the 3' UTR (Figure 6A), indicating that the deadenylation pathway can be triggered by m6A in all regions. Consistently, the CNOT1 targets harbored equal numbers of m6A sites in the CDS and 3' UTR (Figure S7G). By contrast, only transcripts with 3' UTR m6A sites increased in the *HRSP12* KD (Figures 6B and S7H), particularly those also harboring the HRSP12-binding motif GGUUC<sup>28</sup> (Figure S7H). Furthermore, the HRSP12 targets harbored more m6A sites in the 3' UTR than in the CDS (Figure S7G). The impact was even more pronounced for transcripts with YTHDF2 binding (Figure S7I), confirming that HRSP12 and YTHDF2 act together in this pathway.

In the *DCP2* KO, exclusively transcripts with CDS m6A sites were increased in abundance (Figure 6C), resembling the effect of m6A depletion for 6 h. A functional connection was also evidenced by the increased number of CDS m6A sites in the *DCP2* targets that reached levels similar to the emetine targets (Figure S7G), suggesting conjoined roles of decapping and translation in the CMD pathway.

The decapping of m6A-containing transcripts has been connected to the NMD factor UPF1.<sup>27</sup> To test a role in CMD, we analyzed mRNA half-lives upon *UPF1* KD.<sup>56</sup> The upregulation of known NMD targets<sup>57</sup> confirmed efficient UPF1 depletion in this dataset (Figure S7J). We found an increase in half-lives for transcripts with m6A sites in all transcript regions (Figure 6D). This was also reflected in the equal enrichment of m6A sites in the CDS and 3' UTR (Figure S7G). The effects were thus clearly distinct from the CDS-specific effects observed upon *DCP2* KO, suggesting that DCP2 but not UPF1 is involved in CMD. Together with the previous observation that m6A-mediated decay via UPF1 does not require translation,<sup>27</sup> we conclude that CMD involves decapping via DCP2 but is independent of UPF1.

As a complementary approach, we analyzed how the CMD (6 h) targets as well as the 9 h and 24 h targets behaved in the different KD/KO datasets. Indeed, the 6 h targets were increased upon *DCP2* KO, *YTHDF2* KD, and inhibition of translation (Figure 6E). By contrast, *CNOT1* KD, *UPF1* KD, and *HRPS12*

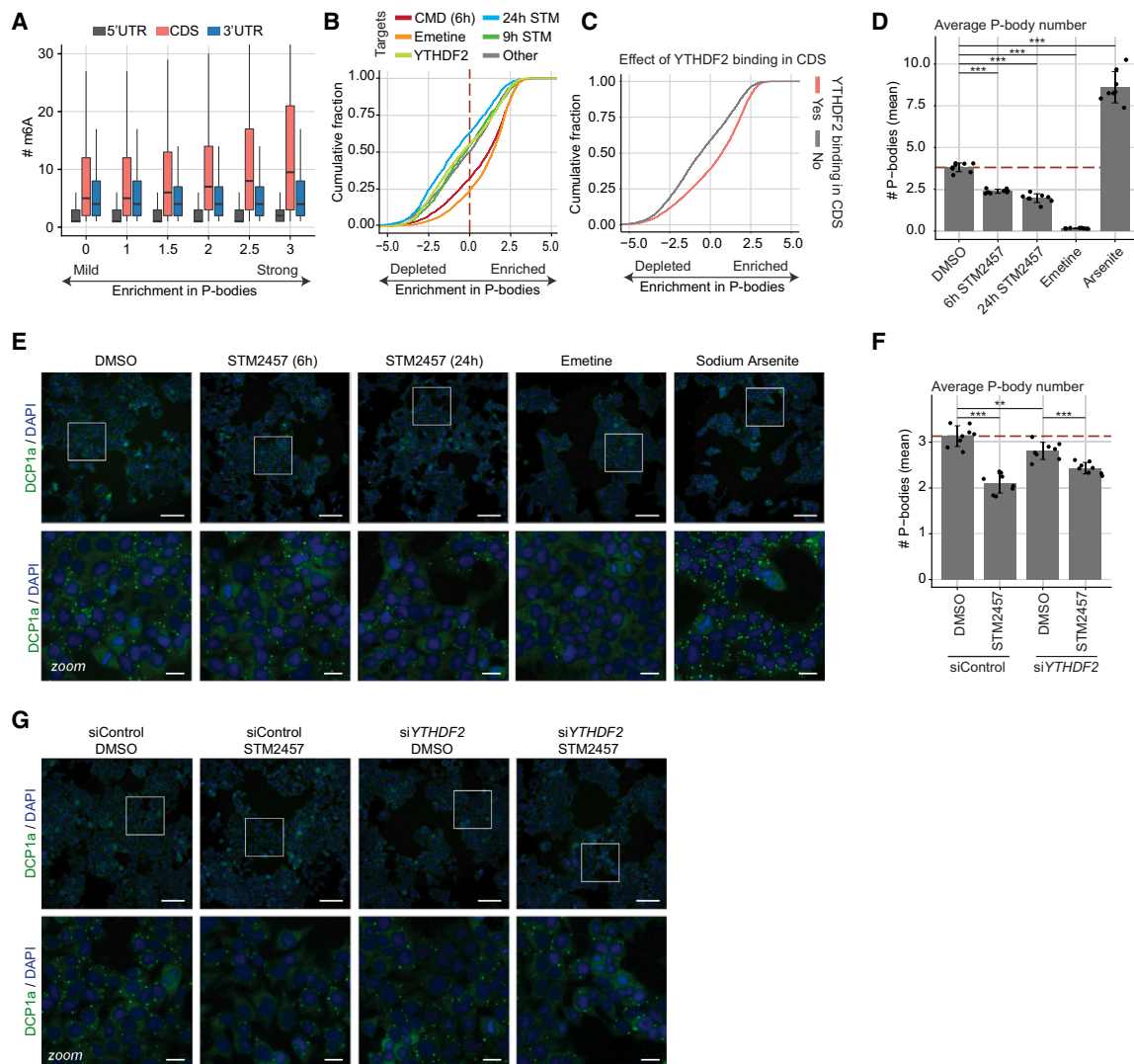


**Figure 5. CMD targets localize to P-bodies**

(A) m6A in regions (GLORI) for transcripts binned by P-body enrichment ( $\log_2FC$ ).<sup>53</sup> x axis refers to lower boundary of bin.

(B) P-body enrichment for 6, 9, 24 h, emetine, and YTHDF2 targets.

(C) P-body enrichment for transcripts with (red) and without (gray) YTHDF2 binding in the CDS.

(D) P-bodies per cell after treatment with DMSO, STM2457 (6 or 24 h, 20  $\mu$ M), emetine (1 h, 1.8  $\mu$ M), or sodium arsenite (1 h, 0.1 mM) are displayed as mean  $\pm$  SD. \*p value < 0.05, \*\*p value < 0.01, \*\*\*p value < 0.001, ns, non-significant; two-sided Student's t test; n = 8 biological replicates.

(E) Representative immunofluorescence images for DCP1a (green) and DAPI (blue) for each condition from (D) (scale bar, 100  $\mu$ m). Enlargement of boxed area shown below (scale bar, 20  $\mu$ m).

(F) P-bodies per cell after m6A depletion for 6 h after YTHDF2 KD. Data represented as in (D).

(G) Representative immunofluorescence images for each condition from (F). Data represented as in (E).

KD did not affect the 6 h targets but stabilized the 9 h and 24 h targets (Figures 6E, S7K, and S7L). The delineation of CMD from the other pathways was further strengthened when overlaid with the P-body transcriptome.<sup>53</sup> Indeed, the DCP2 targets were enriched in P-bodies, while CNOT1, UPF1, and HRSP12 targets were not (Figure 6F). The enrichment in P-bodies directly correlated with an increased abundance after m6A depletion for 6 h, emetine treatment, and DCP2 KO (Figures 6G and S7M–S7R).

Overall, these results support that DCP2, YTHDF2, and translation contribute to CMD, which is distinct from the other known m6A-mediated decay pathways.

### The targets of CMD are important for developmental processes

Interestingly, the CMD targets include the transcripts of many regulatory factors such as Hox proteins (*HOXA9*, *HOXA11*, *HOXD13*), developmental signaling factors (*KLF4*, *SOX2*,

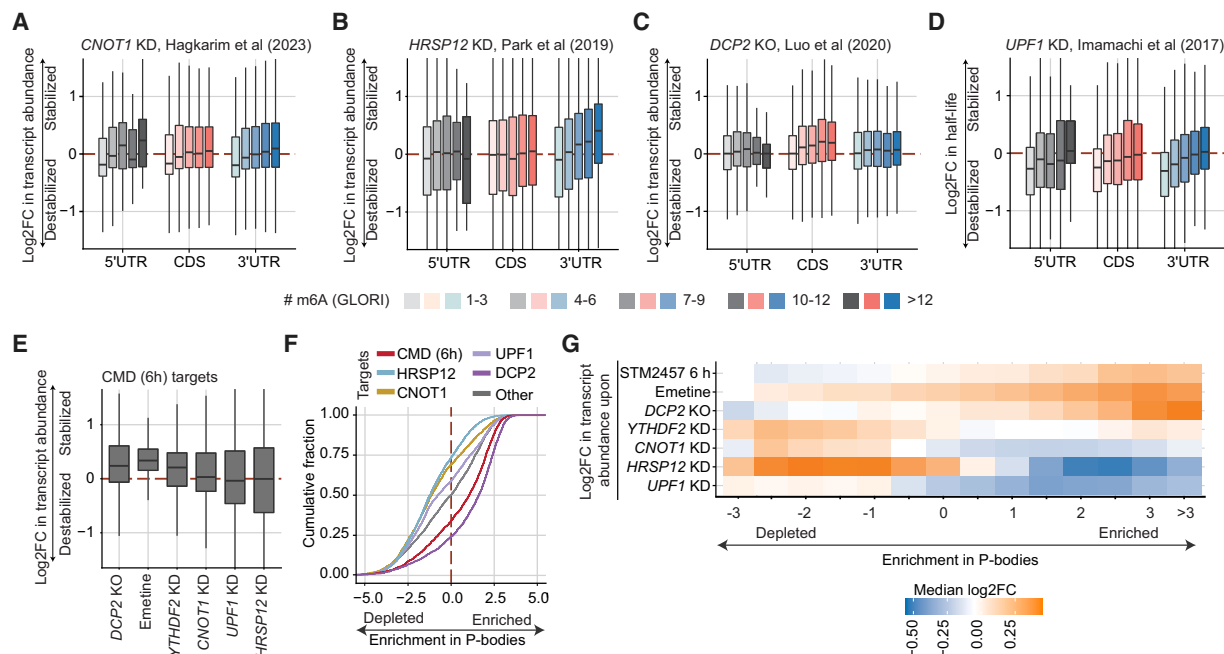


**Figure 6. CDS-m6A decay is distinct from other known pathways and involves RNA decapping**

(A–C) Log<sub>2</sub>FC in abundance upon *CNOT1* KD (A),<sup>54</sup> *HRSP12* KD (B),<sup>28</sup> and *DCP2* KO (C)<sup>55</sup> for transcripts grouped by m6A in transcript regions (GLORI).

(D) Log<sub>2</sub>FC in half-lives upon *UPF1* KD<sup>56</sup> for transcripts as in (A)–(C).

(E) Log<sub>2</sub>FC in transcript abundance upon protein KDs and translation inhibition (emetine) for CMD (6 h) targets.

(F) P-body enrichment for 6 h targets compared with UPF1, HRSP12, DCP2, and CNOT1 targets.

(G) P-body enrichment for transcripts increased upon m6A depletion (6 h), translational inhibition, and depletion of RNA-binding proteins. Color key represents median log<sub>2</sub>FC upon treatment.

*NOTCH1/2*, and *BMP4*), and also proteins from m6A deposition itself (*RBM15*, *WTAP*, and *VIRMA*; Figure 7A; Table S6). Consistently, the 6 h targets in HEK293T cells are associated with GO terms related to development and morphogenesis (Figure 7B; Table S6). This is conserved for transcripts with increased abundance in mESCs under m6A depletion for 3 h<sup>38</sup> (Table S6), including the pluripotency factors *Nanog*, *Esrrb*, and *Sox2* (Figures 7C and 7D; Table S6).

To estimate the full scope of CMD, we identified 6,324 annotated human and 8,139 mouse transcripts with more than 10 CDS GGACN motifs that reside at least 200 nt away from splice sites, and hence, should be efficiently methylated. Consistent with the experimentally identified CMD targets, the transcripts with excess CDS GGACN motifs are enriched in GO terms related to development and differentiation (Figures 7E and 7F). Together, this suggests that CMD accounts for a major part of m6A's reported function in controlling developmental genes.<sup>58</sup>

### Splicing constrains CDS m6A sites to safeguard the transcriptome against retrogenes

Since m6A within the CDS is constrained by the EJC at splice sites,<sup>20–23</sup> we speculated that CMD protects against inefficiently spliced or un-spliced transcripts. In support of this hypothesis, we found that intron-less transcripts host more CDS m6A sites than intron-containing transcripts (Figure 7G). Moreover, they show a globally increased abundance after 6 h of

m6A depletion (Figure 7H). This supports that splicing prevents m6A deposition in the CDS and thereby protects transcripts against CMD.

Among the intron-less genes are many retrogenes that have originated from spliced parental transcripts.<sup>59</sup> Interestingly, these are selectively increased after 6 h of m6A depletion, whereas their parental counterparts are unchanged (Figure 7I). Consistent with that, the regene transcripts harbor more CDS m6A sites (Figure 7J), even though they are directly derived from the parental genes. It is therefore conceivable that CMD safeguards the transcriptome against retrogenes that receive high levels of CDS m6A.

## DISCUSSION

In this study, we found that CDS m6A sites trigger an immediate and efficient translation-dependent RNA decay by a unique pathway that we termed CMD. We suggest that ribosomes pause when m6A-modified adenosines are positioned in the ribosomal A site. This is sensed by a yet-to-be-identified mechanism leading to the translocation of CMD targets to P-bodies, which primes the transcripts for decay. Our data suggest that YTHDF2 does not contribute to ribosome pausing but is related to the subsequent decay. Furthermore, CMD is independent of the key players of other known pathways, namely UPF1, CNOT1, and HRSP12.<sup>8,27,28</sup> Although CMD partially resembles the pathway involving RNA decapping and YTHDF2 binding,<sup>27</sup>

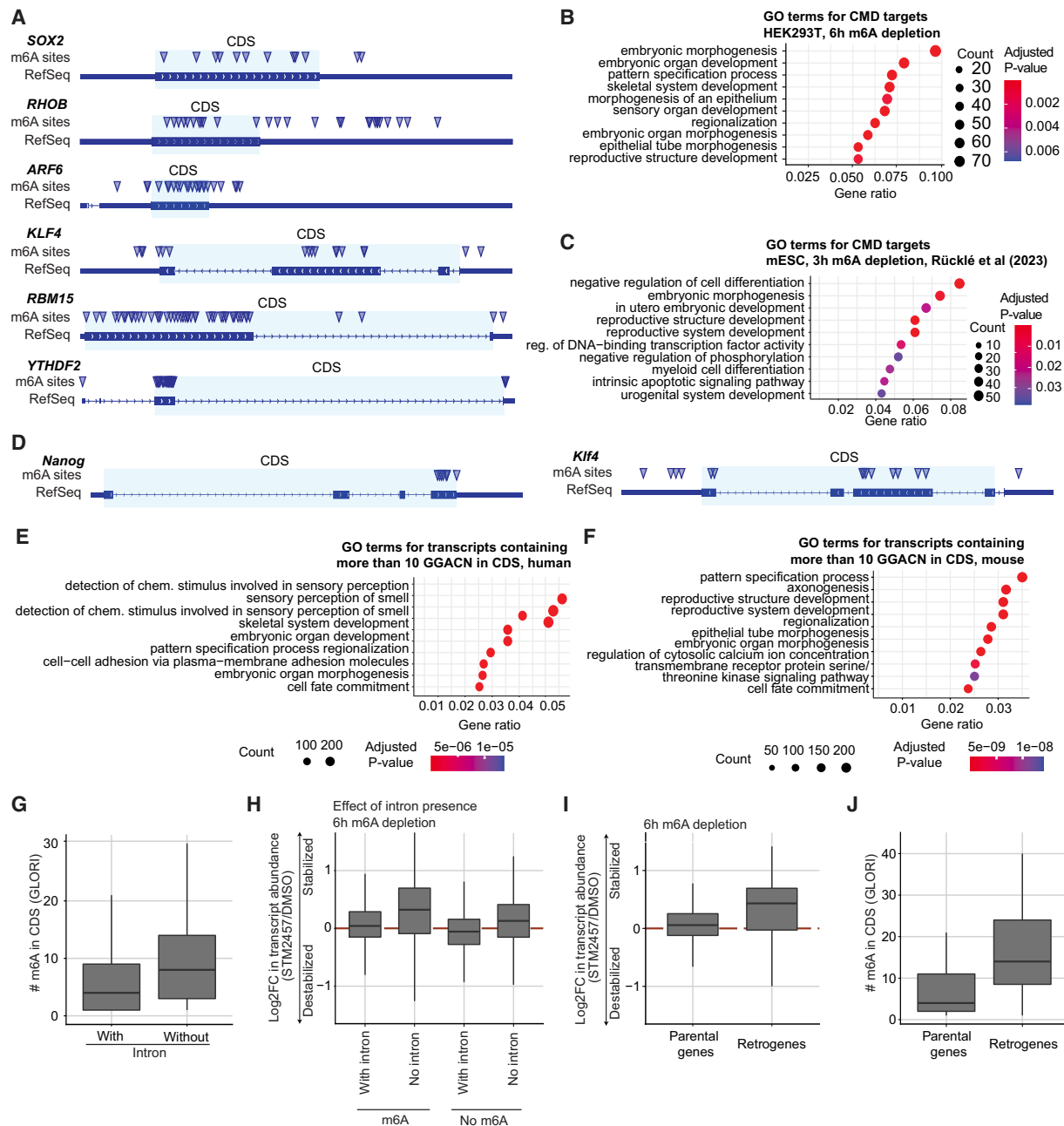


**Figure 7. The targets of CDS-m6A decay are important for developmental processes**

(A) Exemplary human CMD targets. m6A sites (dark blue triangles; miCLIP2 + GLORI) and CDS (light blue) are highlighted. (B and C) Top 10 GO terms of CMD targets identified upon m6A depletion for 6 h in HEK293T (B) and 3 h in mESCs (C).<sup>38</sup> (D) Exemplary murine CMD targets. m6A sites (miCLIP2) and CDS are highlighted as in (A). (E and F) Top 10 GO terms of human (E) and murine (F) transcripts with more than 10 GGACN motifs in CDS. (G and H) CDS m6A sites (GLORI; G) and log<sub>2</sub>FC in transcript abundance for transcripts with or without introns and m6A sites (GLORI; H). (I and J) log<sub>2</sub>FC in transcript abundance (I) and CDS m6A sites (GLORI; J) for retrogenes and parental genes.

it clearly differs in its dependence on translation and its delimitation from UPF1.

In support of our model, it has been shown that the translocation speed of bacterial and eukaryotic ribosomes can be modulated by m6A affecting codon-anti-codon pairing.<sup>25,60,61</sup> The

pausing could be recognized by pathways related to codon optimality-mediated RNA decay (COMD)<sup>62</sup> or No-go-decay.<sup>63</sup> In COMD, the RNA helicase DEAD-box helicase 6 (DDX6) promotes RNA decapping upon recognition of slow ribosomes,<sup>64,65</sup> in line with the role of DCP2 in CMD. Furthermore, DDX6 has

been identified as an interaction partner of YTHDF2.<sup>26,66</sup> Other possible players include zinc finger protein 598 (ZNF598), which recognizes collided ribosomes<sup>67,68</sup> during ribosome-associated quality control—including the degradation of associated mRNA transcripts—as well as the ribosomal protein receptor for activated C kinase 1 (RACK1), which interacts with YTHDF2.<sup>69,70</sup>

Several players of CMD were previously linked to P-bodies. For instance, DCP2 (and also DDX6) are used as markers of P-bodies,<sup>71,72</sup> and DCP2 has been proposed to regulate the stability of m6A-rich transcripts that are concentrated in P-bodies.<sup>6,73–75</sup> Interestingly, YTHDF proteins have also been found in P-bodies<sup>6,8,76</sup> and a link between P-bodies, DCP2, decapping, and m6A-mediated RNA decay has been suggested.<sup>6,8,77</sup> Our findings tie to these observations and suggest that specifically transcripts with CDS m6A sites are recruited to P-bodies. YTHDF2 is not primarily required for the formation of P-bodies and rather contributes to CMD by other means.

Our findings have broad consequences for gene expression and transcriptome maintenance. As the EJC represses m6A deposition,<sup>20–23</sup> CDS m6A sites are restricted to transcripts that harbor particularly long exons or generally lack introns. A special class of intron-less genes are retrogenes. Although retrogenes are considered important drivers of genome evolution, cells need to control their expression.<sup>78</sup> In comparison to the intron-containing parental genes, the transcripts of retrogenes harbor more CDS m6A sites and are strongly targeted by CMD, establishing an efficient safeguarding mechanism. Furthermore, many retrogenes are located on the mammalian X chromosome.<sup>79</sup> Interestingly, m6A contributes to X chromosome inactivation<sup>80</sup> and the stabilization of X-chromosomal transcripts,<sup>38</sup> highlighting how m6A coordinates X-chromosomal gene expression at multiple levels.

Besides retrogenes, the CMD targets encode many epigenetic regulators and key transcription factors from development and differentiation. This is consistent with a recent study that linked m6A-mediated RNA stability to cell-identity pathways<sup>58</sup> and the observation that m6A deposition is critical for early mammalian development.<sup>81,82</sup> Moreover, CMD may play a primary role during the maternal-to-zygote transition. Intriguingly, the rapid decay of maternal transcripts in this sensitive phase of early embryonic development depends on DCP2, METTL3, and YTHDF2.<sup>83–86</sup> Moreover, it is accompanied by the dynamic remodeling of P-bodies and related granules in the developing oocyte.<sup>87</sup> The direct coupling of translation and RNA decay via CMD offers a tight control for the expression of regulatory proteins by a feed-forward loop<sup>88</sup> whereby more efficiently translated mRNAs are degraded quickly, whereas any delay in translation is counterbalanced by an instant stabilization of transcripts. Additionally, reducing transcript half-life quickens the response to a change in transcription rate.<sup>58</sup> Hence, CMD provides flexible yet robust control of CDS m6A-containing transcripts.

### Limitations of the study

Our study provides multiple lines of evidence that CDS m6A sites trigger a specific mRNA decay pathway. However, it remains unclear how ribosome pausing at m6A is sensed and how this results in translocation to P-bodies. It will be important to explore

whether the sensing is connected to general quality control, codon optimality, or other yet unknown mechanisms. Furthermore, future work will be required to delineate the contribution of YTHDF2 and other RNA decay factors. Such analyses are complicated by the partial redundancy of the different pathways and multiple YTHDF paralogs.

### RESOURCE AVAILABILITY

#### Lead contact

For further inquiries or to request resources and reagents, please contact the lead contact, Julian König ([julian.koenig@uni-wuerzburg.de](mailto:julian.koenig@uni-wuerzburg.de)).

#### Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#).

#### Data and code availability

- Nanopore sequencing data were deposited in the SRA database under the BioProject accession number SRA: PRJNA1043631. All other high-throughput data were added to GEO with the SuperSeries accession number GEO: GSE248575. Western blot images are available at Mendeley Data: <https://doi.org/10.17632/246j5kmf66.1>, and imaging data are available at the public OMERO web server: <https://omero.imb.uni-mainz.de/pub/zhou2024>. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (identifier PRIDE: PXD054286).
- Source code has been deposited at [https://github.com/ZarnackGroup/Publications/tree/main/Zhou\\_et\\_al\\_2024](https://github.com/ZarnackGroup/Publications/tree/main/Zhou_et_al_2024) and is also available on Zenodo at <https://doi.org/10.5281/zenodo.13968916>.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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### AUTHOR CONTRIBUTIONS

Y.Z. performed most bioinformatic analyses. M.Č. and P.H.-K. performed most experiments. N.M. and M.H. performed *in vitro* methylation assays. M.M. established and helped with Ribo-seq experiments. N.K. and H.B. performed YTHDF2 iCLIP2 experiments. I.S.N.-d.V. and C.D. generated and analyzed nanopore direct RNA-seq data. A.B. established and performed next-generation sequencing data processing. K.K., A.O., and Š.V. performed

proximity proteomics experiments and data analysis. Y.Z., M.C., P.H.-K., K.Z., and J.K. designed the study and wrote the manuscript. All authors read and commented on the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                                                   | SOURCE                       | IDENTIFIER                           |
|---------------------------------------------------------------------------------------|------------------------------|--------------------------------------|
| <b>Antibodies</b>                                                                     |                              |                                      |
| Rabbit polyclonal anti-m6A                                                            | Synaptic Systems             | Cat# 202003; RRID: AB_2279214        |
| Rabbit polyclonal anti-YTHDF2                                                         | AVIVA SYSTEMS BIOLOGY        | Cat# ARP67917_P050; RRID: AB_2861185 |
| Rabbit monoclonal anti-DCP1a [EPR13822]                                               | Abcam                        | Cat# ab183709; RRID: N/A             |
| Mouse monoclonal anti-Alpha-Tubulin                                                   | Sigma Aldrich                | Cat# T5168; RRID: AB_477579          |
| IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody                                   | LI-COR Biosciences           | Cat# 926-68070; RRID: AB_10956588    |
| IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody                                  | LI-COR Biosciences           | Cat# 926-32211; RRID: AB_621843      |
| Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 | Thermo Fisher Scientific     | Cat# A-11034; RRID: AB_2576217       |
| Goat anti-YTHDF2 (S-14)                                                               | Santa Cruz Biotechnology     | Cat# sc-162427; RRID: AB_2217480     |
| Goat anti-HA-probe (Y-11)                                                             | Santa Cruz Biotechnology     | Cat# sc-805; RRID: AB_631618         |
| <b>Bacterial and virus strains</b>                                                    |                              |                                      |
| DH5alpha                                                                              | Invitrogen                   | Cat# 18265017                        |
| <b>Chemicals, peptides, and recombinant proteins</b>                                  |                              |                                      |
| Human METTL3/METL14 recombinant protein                                               | Meiser et al. <sup>30</sup>  | N/A                                  |
| STM2457 (METTL3 inhibitor)                                                            | Biomol GmbH                  | Cat# Cay34280                        |
| Emetine dihydrochloride                                                               | Sigma-Aldrich                | Cat# E2375                           |
| Sodium arsenite                                                                       | Sigma-Aldrich                | Cat# S7400                           |
| Phusion® High-Fidelity DNA Polymerase                                                 | New England Biolabs          | Cat# M0530                           |
| Turbo DNase                                                                           | Invitrogen                   | Cat# AM2238                          |
| HRP-Conjugated Streptavidin                                                           | Thermo Scientific™           | Cat# N100                            |
| Biotin                                                                                | Sigma-Aldrich                | Cat# B4501                           |
| Doxycycline hyclate BioChemica                                                        | Applchem                     | Cat# A2951                           |
| TurboFect                                                                             | Invitrogen                   | Cat# R0531                           |
| Dynabeads™ MyOne™ Streptavidin C1                                                     | Invitrogen                   | Cat# 65001                           |
| Doxycycline hyclate BioChemica                                                        | Applchem                     | Cat# A2951                           |
| Phosphatase Inhibitor Cocktail I                                                      | Sigma Aldrich                | Cat# P2850                           |
| Pepstatin A                                                                           | Glenthams                    | Cat# GE3524                          |
| Leupeptin, hemisulfate                                                                | Applchem                     | Cat# 2183                            |
| PMSF                                                                                  | Applchem                     | Cat# A0999                           |
| Urea                                                                                  | Applchem                     | Cat# A1360                           |
| <b>Critical commercial assays</b>                                                     |                              |                                      |
| RNeasy PLUS Mini Kit                                                                  | Qiagen                       | Cat# 74136                           |
| RevertAid First Strand cDNA Synthesis Kit                                             | Thermo Fisher                | Cat# 10161310                        |
| High Sensitivity D1000 ScreenTape                                                     | Agilent                      | Cat# 5067- 5582                      |
| High Sensitivity RNA ScreenTape                                                       | Agilent                      | Cat# 5067-5579                       |
| Qubit RNA HS Assay                                                                    | Invitrogen                   | Cat# Q32852                          |
| Luminaris HiGreen qPCR Master Mix, low ROX                                            | Thermo Fisher Scientific     | Cat# K0971                           |
| RNA Clean and Concentrator Kit                                                        | Zymo Research                | Cat# R1016                           |
| Stranded mRNA Prep Ligation Kit                                                       | Illumina                     | Cat# 20040534                        |
| Direct RNA sequencing kit                                                             | Oxford Nanopore Technologies | Cat# SQK-RNA002                      |
| MinION R9.4.1 flow cells                                                              | Oxford Nanopore Technologies | Cat# OXNFTLO-MIN106D                 |

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### Continued

| REAGENT or RESOURCE                                   | SOURCE              | IDENTIFIER    |
|-------------------------------------------------------|---------------------|---------------|
| FuGENE HD transfection reagent                        | Promega             | Cat# E2312    |
| RNAiMAX Transfection Reagent                          | Invitrogen          | Cat# 13778150 |
| Q5 Site-Directed Mutagenesis Kit                      | New England Biolabs | Cat# E0552S   |
| Direct-zol RNA Miniprep Kit                           | Zymo Research       | Cat# D2050    |
| SLAMseq Kinetics Kit – Anabolic Kinetics Module       | Lexogen             | Cat# 061.24   |
| QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD with UDI | Lexogen             | Cat# 191.24   |

### Deposited data

|                                                                                                                          |                                          |                                                                                                                       |
|--------------------------------------------------------------------------------------------------------------------------|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|
| Nanopore sequencing data                                                                                                 | This study                               | GEO: PRJNA1043631                                                                                                     |
| RNA-seq, Ribo-seq and matched RNA-seq, YTHDF2 iCLIP2, SLAM-seq and <i>in vitro</i> miCLIP2 data                          | This study                               | GEO: GSE248575                                                                                                        |
| RAW imaging data of the Opera Phenix Imaging System                                                                      | This study, OMERO database               | <a href="https://omero.imb.uni-mainz.de/pub/zhou2024">https://omero.imb.uni-mainz.de/pub/zhou2024</a>                 |
| Original Western Blot data and raw microscopy images                                                                     | This study, Mendeley Data                | <a href="https://doi.org/10.17632/246j5kmf66.1">https://doi.org/10.17632/246j5kmf66.1</a>                             |
| RNA-seq data for the 12 h STM2457 treatment                                                                              | Körtel et al. <sup>14</sup>              | GEO: GSE163500                                                                                                        |
| RNA-seq data for <i>CNOT1</i> KD                                                                                         | Hagkarim et al. <sup>54</sup>            | GEO: GSE141496                                                                                                        |
| RNA-seq data for <i>DCP2</i> KO                                                                                          | Luo et al. <sup>55</sup>                 | GEO: GSE143662                                                                                                        |
| RNA-seq data for emetine                                                                                                 | Sinha et al. <sup>43</sup>               | GEO: GSE149565                                                                                                        |
| RNA-seq data for <i>HRSP12</i> KD                                                                                        | Park et al. <sup>28</sup>                | PRJNA482875                                                                                                           |
| Half-live measurements for <i>UPF1</i> KO                                                                                | Imamachi et al. <sup>56</sup>            | DRA: DRA003675                                                                                                        |
| Half-live measurements in HEK293T                                                                                        | Lugowski et al. <sup>36</sup>            | GEO: GSE99517                                                                                                         |
| Half-live measurements in mESC                                                                                           | Ke et al. <sup>37</sup>                  | GEO: GSE86336                                                                                                         |
| P-body purification data                                                                                                 | Hubstenberger et al. <sup>53</sup>       | ArrayExpress: E-MTAB-5477                                                                                             |
| Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository | This study                               | PXD054286                                                                                                             |
| common Repository of Adventitious Proteins, cRAP                                                                         | The Global Proteome Machine Organization | <a href="http://www.thegpm.org/crap">http://www.thegpm.org/crap</a>                                                   |
| Human genome (hg38)                                                                                                      | Genome reference                         | <a href="https://www.gencodegenes.org/human/release_31.html">https://www.gencodegenes.org/human/release_31.html</a>   |
| Mouse genome (mm10)                                                                                                      | Genome reference                         | <a href="https://www.gencodegenes.org/mouse/release_M23.html">https://www.gencodegenes.org/mouse/release_M23.html</a> |
| Code for all the analysis in this study                                                                                  | This study                               | <a href="https://doi.org/10.5281/zenodo.13968917">https://doi.org/10.5281/zenodo.13968917</a>                         |

### Experimental models: Cell lines

|                                                 |            |        |
|-------------------------------------------------|------------|--------|
| Human: HEK293T                                  | DSMZ       | ACC305 |
| Human: HEK Flp-In™ T-REx™                       | Invitrogen | R78007 |
| Human: HEK Flp-In™ T-REx™ 3xHA-miniTurbo-YTHDF2 | This study | N/A    |
| Human: HEK Flp-In™ T-REx™ 3xHA-miniTurbo        | This study | N/A    |

### Oligonucleotides

|              |            |     |
|--------------|------------|-----|
| See Table S7 | This study | N/A |
|--------------|------------|-----|

### Recombinant DNA

|              |            |     |
|--------------|------------|-----|
| See Table S4 | This study | N/A |
|--------------|------------|-----|

### Software and algorithms

|                            |                                  |                                                                                                                                   |
|----------------------------|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| Guppy v6.4.6               | Nanopore basecalling tools       | <a href="https://timkahlke.github.io/LongRead_tutorials/BS_G.html">https://timkahlke.github.io/LongRead_tutorials/BS_G.html</a>   |
| FastQC v0.11.8 and v0.11.9 | Quality control tool of NGS data | <a href="https://www.bioinformatics.babraham.ac.uk/projects/fastqc">https://www.bioinformatics.babraham.ac.uk/projects/fastqc</a> |

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**Continued**

| REAGENT or RESOURCE                                    | SOURCE                                                                        | IDENTIFIER                                                                                                                                                                                                                                                        |
|--------------------------------------------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| FASTX-Toolkit v0.0.14                                  | command line tools for Short-Reads FASTA/FASTQ files preprocessing            | <a href="http://hannonlab.cshl.edu/fastx_toolkit/">http://hannonlab.cshl.edu/fastx_toolkit/</a>                                                                                                                                                                   |
| Seqtk v1.3                                             | tool for processing sequences in the FASTA or FASTQ format                    | <a href="https://github.com/lh3/seqtk">https://github.com/lh3/seqtk</a>                                                                                                                                                                                           |
| Flexbar v3.4.0 and v3.5.0                              | Roehr et al. <sup>89</sup>                                                    | <a href="https://github.com/seqan/flexbar">https://github.com/seqan/flexbar</a>                                                                                                                                                                                   |
| STAR v2.7.3a & v2.7.8a                                 | Dobin et al. <sup>90</sup>                                                    | <a href="https://github.com/alexdobin/STAR">https://github.com/alexdobin/STAR</a>                                                                                                                                                                                 |
| BEDTools v2.27.1 and v2.30.0                           | Quinlan and Hall <sup>91</sup>                                                | <a href="https://bedtools.readthedocs.io/en/latest/">https://bedtools.readthedocs.io/en/latest/</a>                                                                                                                                                               |
| bedGraphToBigWig                                       | Kent et al. <sup>92</sup>                                                     | <a href="https://genome.ucsc.edu/goldenPath/help/bigWig.html">https://genome.ucsc.edu/goldenPath/help/bigWig.html</a>                                                                                                                                             |
| rtracklayer v1.52.0                                    | Lawrence et al. <sup>93</sup>                                                 | <a href="https://www.bioconductor.org/packages/release/bioc/html/rtracklayer.html">https://www.bioconductor.org/packages/release/bioc/html/rtracklayer.html</a>                                                                                                   |
| DESeq2 v1.26.0, v1.32.0 and v1.34.0                    | Love et al. <sup>94</sup>                                                     | <a href="https://bioconductor.org/packages/release/bioc/html/DESeq2.html">https://bioconductor.org/packages/release/bioc/html/DESeq2.html</a>                                                                                                                     |
| cliProfiler v1.6.0                                     | Zhou and Zarnack <sup>95</sup>                                                | <a href="https://www.bioconductor.org/packages/release/bioc/html/cliProfiler.html">https://www.bioconductor.org/packages/release/bioc/html/cliProfiler.html</a>                                                                                                   |
| ppcor v1.1                                             | Kim <sup>96</sup>                                                             | <a href="https://cran.r-project.org/web/packages/ppcor/index.html">https://cran.r-project.org/web/packages/ppcor/index.html</a>                                                                                                                                   |
| minimap2 v2.22-r1101                                   | Li <sup>97</sup>                                                              | <a href="https://github.com/lh3/minimap2">https://github.com/lh3/minimap2</a>                                                                                                                                                                                     |
| nanopolish v0.13.3                                     | Software package for signal-level analysis of Oxford Nanopore sequencing data | <a href="https://github.com/jts/nanopolish">https://github.com/jts/nanopolish</a>                                                                                                                                                                                 |
| m6Anet v2.0.1                                          | Hendra et al. <sup>29</sup>                                                   | <a href="https://m6anet.readthedocs.io/">https://m6anet.readthedocs.io/</a>                                                                                                                                                                                       |
| Samtools v1.9 and v1.10                                | Danecek et al. <sup>98</sup>                                                  | <a href="https://github.com/samtools/samtools">https://github.com/samtools/samtools</a>                                                                                                                                                                           |
| Subread tool suite v2.0.0 and v2.0.3                   | Liao et al. <sup>99</sup>                                                     | <a href="https://subread.sourceforge.net/">https://subread.sourceforge.net/</a>                                                                                                                                                                                   |
| Trimmomatic v0.39                                      | Bolger et al. <sup>100</sup>                                                  | <a href="http://www.usadellab.org/cms/?page=trimmomatic">http://www.usadellab.org/cms/?page=trimmomatic</a>                                                                                                                                                       |
| Parallel Fastq Dump v0.6.7                             | parallel fastq-dump wrapper                                                   | <a href="https://github.com/rvalieris/parallel-fastq-dump">https://github.com/rvalieris/parallel-fastq-dump</a>                                                                                                                                                   |
| Boruta v7.0.0                                          | Kursa and Rudnicki <sup>40</sup>                                              | <a href="https://cran.r-project.org/web/packages/Boruta/index.html">https://cran.r-project.org/web/packages/Boruta/index.html</a>                                                                                                                                 |
| treeshap v0.1.1                                        | Lundberg et al. <sup>41</sup>                                                 | <a href="https://cran.r-project.org/web/packages/treeshap/index.html">https://cran.r-project.org/web/packages/treeshap/index.html</a>                                                                                                                             |
| clusterProfiler v4.0.0                                 | Wu et al. <sup>101</sup>                                                      | <a href="https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html">https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html</a>                                                                                                   |
| GRAND-SLAM v2.0.5f                                     | Jürges et al. <sup>102</sup>                                                  | <a href="https://erhard-lab.de/software">https://erhard-lab.de/software</a>                                                                                                                                                                                       |
| grandR v0.2.5                                          | Rummel et al. <sup>103</sup>                                                  | <a href="https://grandr.erhard-lab.de/">https://grandr.erhard-lab.de/</a>                                                                                                                                                                                         |
| PureCLIP v1.3.1                                        | Krakau et al. <sup>50</sup>                                                   | <a href="https://github.com/skrakau/PureCLIP">https://github.com/skrakau/PureCLIP</a>                                                                                                                                                                             |
| QuantStudio Design and Analysis Desktop Software 2.8.0 | Applied Biosystems                                                            | <a href="https://www.thermofisher.com/de/de/home/technical-resources/software-downloads/quantstudio-3-5-real-time-pcr-systems.html">https://www.thermofisher.com/de/de/home/technical-resources/software-downloads/quantstudio-3-5-real-time-pcr-systems.html</a> |
| FlowJo v10.8.1                                         | BD Life Sciences                                                              | <a href="https://www.flowjo.com/">https://www.flowjo.com/</a>                                                                                                                                                                                                     |
| DIA-NN version 1.8                                     | Demichev et al. <sup>104</sup>                                                | <a href="https://github.com/vdemichev/DiaNN">https://github.com/vdemichev/DiaNN</a>                                                                                                                                                                               |
| <b>Other</b>                                           |                                                                               |                                                                                                                                                                                                                                                                   |
| Acclaim™ PepMap™ 100 C18 HPLC Columns                  | Thermo Fisher Scientific                                                      | Cat# 164946                                                                                                                                                                                                                                                       |
| Aurora Ultimate 25×75 C18 UHPLC column                 | Ionoptics                                                                     | N/A                                                                                                                                                                                                                                                               |
| The Butterfly Heater: PST-BPH                          | Phoenix S&T                                                                   | PST-BPH                                                                                                                                                                                                                                                           |

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

HEK293T (ATCC, CRL-3216) cells were grown and cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, #21969035), supplemented with 10% fetal bovine serum (FBS, PAN-Biotech, #P40-47500), 1% L-glutamine (Thermo Fisher Scientific, #25030081), and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific, #10378016). Cells were incubated at 37°C with 5% CO<sub>2</sub> and routinely monitored for mycoplasma contamination.

## METHOD DETAILS

### High-throughput *in vitro* methylation assay

#### Design of oligonucleotide library

The oligonucleotide library was obtained from Twist Biosciences and included 11,999 synthesized single-stranded (ss) DNA oligonucleotides (200 nt) with a defined sequence (Table S1). Each oligonucleotide includes a T7 promotor (18 nt, used for *in vitro* transcription), a transcript region of interest (146 nt), a unique molecular identifier (UMI, 15 nt), and the L3 linker sequence (21 nt). The resulting *in vitro* transcripts were 183 nt long, including 1 nt from the T7 promoter. Transcript regions were selected based on miCLIP2 data from HEK293T cells<sup>14</sup> to contain miCLIP2-detected m6A sites or unmodified adenosines in a DRACN context between positions 62–72 from the oligonucleotide start. In total, we selected 1,988 m6A sites in a DRACN motif ("m6A") and 1,500 unmodified adenosines in a DRACH motif ("unmodified DRACH"). Additionally, we selected 50 m6A sites in weak DRACH motifs that were exchanged by 5 different strong DRACH motifs ( $n=250$ ), as well as 50 m6A sites in strong DRACH motifs that were exchanged by 3 different weak DRACH motifs ( $n=150$ ), generating a total of 400 permuted sequences ("Permutation"). Each transcript sequence was represented by three different oligonucleotides with distinct UMIs (technical replicates). 335 additional oligonucleotides ("Other usage") were excluded from data analysis.

#### Preparation of *in vitro* transcribed RNA library

To first generate double-stranded (ds) DNA templates, 10 ng of ssDNA oligonucleotide library was amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, #M0530) with primers annealing to the universal T7 and L3 sequences (Table S7). TapeStation 2200 in combination with D1000 Screen tapes (Agilent, #5067-5582) was used to verify amplification and dsDNA library was purified using MyONE Silane beads (Thermo Fisher Scientific, #37002D). The dsDNA library was used as input for *in vitro* transcription using the HiScribe T7 High Yield RNA Synthesis 26 Kit (New England Biolabs, #E2040S) with the reaction setup for short transcripts (<0.3 kb). After DNA digestion of the DNA template with 0.04 U/μl Turbo DNase (Invitrogen, #AM2238) for 15 min at 37 °C and cleanup with the RNeasy Mini Kit (Qiagen, #74104), RNA concentration was estimated by Qubit RNA HS Assay (Invitrogen, #Q32852). Purified *in vitro* transcripts (IVTs, 183 nt) were used as input in the *in vitro* methylation assay. Note that positional numbering in the IVTs is given relative to the start of the designed DNA oligonucleotide.

#### *In vitro* methylation assay

Human METTL3 and METTL14 were recombinantly expressed in Sf9 cells and purified as described.<sup>30</sup> 89 nM of IVT were incubated with 38 nM protein complex for 2 h at 37 °C with shaking at 300 rpm in reaction buffer containing 50 mM Tris, 1% glycerol, 20 mM KCl, 10 μM n-Dodecyl-β-D-maltoside, and 3.5 μM SAM. The mixture was heat-inactivated by incubation at 85 °C for 10 min. Phenol/chloroform extraction was performed twice to remove proteins from the mixture. The RNA concentration was increased by repeated extraction with 3 vol of 1-butanol. Residual butanol was evaporated, and the RNA precipitated in 0.5 M ammonium acetate by addition of 2.5 eq absolute ethanol and incubation at –20 °C for 1 h. The RNA was pelleted by centrifugation for 45 min at 21,000 g and 0 °C. The supernatant was removed, and the dried RNA was resuspended in ddH<sub>2</sub>O. Two independent replicates of the *in vitro* methylation were performed.

#### *In vitro* miCLIP2 library generation and sequencing

The miCLIP2 protocol was performed as described before<sup>14</sup> and was adjusted to the use of the *in vitro* transcribed RNA. Briefly, 500 ng purified RNA from the *in vitro* methylation assay was used as input for the miCLIP2 protocol. Since fragment size was <200 nt and the L3 linker sequence was already included in the designed oligonucleotides, the initial RNA fragmentation and L3 linker ligation steps of the protocol were omitted. RNA crosslinking and immunoprecipitation with an m6A-specific antibody (Synaptic Systems, #202003), protein-RNA complex purification, proteinase K digestion, RNA isolation, reverse transcription, and preparation of the sequencing library were performed as described before.<sup>14</sup> The *in vitro* methylation miCLIP2 libraries were pooled equimolarly and sequenced on an Illumina NextSeq 500 sequencing machine as 141-nt + 25-nt paired-end reads including a 6-nt sample barcode and 5+4-nt UMIs at the beginning of the first read (R1) as well as a 15-nt oligonucleotide UMI at the beginning of the second read (R2). Sequencing yielded 12–16 million reads per sample (Table S2).

#### Drug treatments and siRNA transfection

Depletion of m6A in HEK293T cells was performed using the METTL3 inhibitor STM2457 (obtained from STORM Therapeutics or BioMol, #Cay34280).<sup>34</sup> Treatment was performed by adding medium supplemented with 20 μM STM2457 in DMSO 0.2% (v/v) or with DMSO 0.2% (v/v) alone as control for 6–24 h.

Inhibition of translational elongation in HEK293T cells was performed using 1.8 μM emetine dihydrochloride (Sigma-Aldrich, E2375) in DMSO for 1–2 h. For the combination of m6A depletion and translational inhibition, 1.8 μM emetine was added for the last 1–2 h of a 6 h incubation period with 20 μM STM2457 or DMSO.

Small interfering RNAs (siRNAs) targeting *YTHDF2* GTAGCACAGAAGTTGCAAGCAA[dT][dT] or non-targeting control siRNAs (negative control, UGGUUUACAUGUCGACUAA[dT][dT]) were transfected into the cells 24 h after seeding using RNAiMAX (Invitrogen, #13778150) following the manufacturer's instructions at a final concentration of 20 nM. Alternatively, the RNAiMAX reverse transfection protocol was used, where the cell suspension was added to the siRNA/RNAiMAX mixture before seeding. Cells were harvested 48 h after transfection for further analysis. To combine the siRNA knockdown with m6A depletion, 42 h after transfection, cells were treated with 20 μM STM2457 or DMSO for additional 6 h.

## SLAM-seq

### Cell viability optimization

HEK293T cells were seeded to a 96-well plate. At 80% confluency, cells were treated with STM2457. After 3 h cells were labeled by varying concentrations of 4-thio-uridine ( $s^4U$ ). Cell viability was assessed 6 h later using CellTiter-Glo Luminescent Cell Viability Kit (Promega, G7570), according to the manufacturer's protocol. Luminescence was measured using Tecan Infinite P200 Pro plate reader.

### SLAM-seq experiment

For determining mRNA half-lives in HEK293T cells, the SLAM-seq Kinetics Kit - Anabolic Kinetics Module (Lexogen, #061.24) was used according to the manufacturer's recommendations. Briefly, HEK293T cells were seeded in a 24-well plate. At 80% confluency, cells were treated with STM2457 inhibitor or DMSO. After 3 h, the metabolic labeling was performed by addition of 100  $\mu M$   $s^4U$ . After 0–3 h incubation, cells were directly lysed in TRIzol reagent (Thermo Fisher Scientific, #15596026) and RNA isolation was performed using phenol-chloroform extraction. RNA was resuspended in the elution buffer provided in the Lexogen anabolic kit. Exactly 5  $\mu g$  of RNA was subjected to iodoacetamide treatment. Next-generation sequencing library preparation was performed with QuantSeq 3'mRNA-Seq V2 Library Prep Kit FWD (Lexogen) with Unique Dual Indices (12 nt) following Lexogen's standard protocol (0191UG444V0111).

Libraries were prepared with a starting amount of 200 ng and amplified in 15 PCR cycles. Libraries were profiled in a High Sensitivity DNA on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit 1x dsDNA HS Assay Kit, in a Qubit 4.0 Fluorometer (Life Technologies). All 24 samples were pooled in equimolar ratio and sequenced on a NextSeq 2000 P3 (100 cycles) FC, SR for 1x 114 cycles plus 2x 12 cycles for the dual index read.

### RNA isolation

Following indicated treatments, cells were washed twice with 1x PBS on ice and pelleted at 1,500 g for 5 min at 4 °C. Total RNA was isolated from cell pellets using RNeasy Plus Mini Kit (Qiagen, #74136) according to the manufacturer's instructions.

RNA for preparation of the RNA-seq matching to Ribo-seq library was isolated from crude cell lysate using Direct-zol RNA Miniprep kit (Zymo Research, D2050). Possible residual DNA contamination was removed by TURBO DNase (Ambion, AM2238) digestion. RNA was further purified using RNA Clean and Concentrator kit (Zymo Research, R1016) according to the manufacturer's instructions.

### RNA quality control and sequencing (RNA-seq)

RNA quality was determined by analyzing the RNA Integrity Number (RIN) through the utilization of a 4200 TapeStation instrument (Agilent Technologies, #G2991BA) equipped with RNA ScreenTapes (Agilent Technologies, #5067-5576). Next generation sequencing (NGS) library preparation was performed with Illumina's Stranded mRNA Prep Ligation Kit following Stranded mRNA Prep Ligation ReferenceGuide (June 2020) (Document # 1000000124518 v00). Libraries were prepared with a starting amount of 500 ng (6 h m6A depletion and *YTHDF2* KD) or 1000 ng (9 h and control RNA-seq matched to Ribo-seq, emetine experiment). Libraries were amplified with 9 PCR cycles (9 h m6A depletion, emetine, and control RNA-seq matched to Ribo-seq) or 10 PCR cycles (6 h m6A depletion and *YTHDF2* KD). Except for 9 h m6A depletion, ERCC spike-in mix (Thermo Fischer Scientific, #4456740) was added to the starting material for quality control. Two post-PCR purification steps were performed to exclude residual primer and adapter dimers. Libraries were profiled in a DNA 1000 chip on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit 2.0 Fluorometer (Life Technologies). Samples were pooled in equimolar ratio and sequenced on an Illumina NextSeq 500 or NextSeq 2000 sequencing machines with one or two dark cycles upfront as 79-nt or 116-nt single-end reads (Table S2).

### Nanopore direct RNA sequencing

PolyA+ RNA was isolated from total RNA of DMSO-treated HEK293T cells as described previously.<sup>105</sup> 200 to 400 ng polyA+ RNA was used for library preparation employing the Direct RNA sequencing kit (SQK-RNA002, Oxford Nanopore Technologies). Libraries were sequenced on MinION R9.4.1 flow cells for 72 h with high-accuracy basecalling (Guppy 6.4.6) enabled.

### cDNA synthesis and quantitative PCR (real-time qPCR)

RevertAid Reverse Transcriptase (Thermo Fisher Scientific, #10161310) was used to convert 500 ng of total RNA into complementary DNA (cDNA) using oligo(dT)<sub>18</sub> primers (Thermo Fisher Scientific, SO131) following manufacturer's instructions. Real-time quantitative PCR (qPCR) was carried out on a QuantStudio™ 5 Real-Time PCR device (Applied Biosystems) using the Luminaris HiGreen qPCR Master Mix, low ROX (Thermo Fisher Scientific, K0971) following manufacturer's instructions. Target-specific forward and reverse primers (0.3  $\mu M$  each; Table S7) were used with 2  $\mu l$  of 1:10 diluted cDNA as template in a 20  $\mu l$  reaction volume. Raw cycle threshold (Ct) values were obtained using the QuantStudio™ Design and Analysis Desktop Software (Applied Biosystems). The relative quantification of transcript abundance was calculated using the  $2^{-\Delta\Delta Ct}$  method, using *ACTB* as housekeeping control.

### Generation of the CMD fluorescent reporter plasmids

The pM-eGFP-p2A-K0-RFP vector was generously provided by Ramanujan S. Hegde (MRC Laboratory of Molecular Biology, Cambridge, UK).<sup>106</sup> Initially, the K0 and RFP components were excised using Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's recommendations, resulting in a plasmid that retained only the GFP-p2A segment. Inserts encompassing the last exon, last intron, and part of the penultimate exon of the *CGRRF1*, *THAP7* and *PELO* genes were amplified by PCR with specific primers (Table S7) from total DNA isolated from HEK293T cells. The wildtype fragments were inserted in-frame downstream of the GFP open reading frame and p2A site,<sup>107</sup> using a homemade Gibson Assembly master mix (IMB Protein Production Core Facility).<sup>108</sup> The dual promoter plasmid (pTRE3GBi\_EF1A\_Tet3G\_IRES\_Puro-hGRADEmCherry)<sup>109</sup> was generously provided by Michaela Müller-McNicoll (Institute for Molecular Biosciences, Frankfurt, Germany). Fragments with GFP fused to all mutant insert clones were inserted to dual promoter plasmid in the opposite orientation to mCherry using Gibson assembly. The P2A motif serves to separate the GFP and CGRRF1 (THAP7, PELO) protein products.

Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used to generate a set of mutant plasmids according to the manufacturer's recommendations. In order to analyze m6A-dependent effects, five A>G point mutations were introduced to disrupt all the m6A sites (CGRRF1\_m6Amut). To investigate the influence of translation on CMD, a stop codon was introduced into the penultimate exon to precede the m6A sites, shifting them from CDS to 3' UTR. Additionally, to examine the role of splicing, the intron was deleted, also in combination with previous mutants. All reporters are listed in Table S4, and all oligonucleotides used for cloning are listed in Table S7.

### CMD fluorescent reporter assay using flow cytometry

HEK293T cells were transfected with 150 ng of the indicated reporter plasmids in a 96-well plate setup using FuGENE HD transfection reagent (Promega, E2312) according to the manufacturer's recommendations, and treated with 20  $\mu$ M STM2457 or DMSO for 15 h post-transfection. For knockdown of *YTHDF2*, one day prior transfection of the reporter plasmids, siRNA transfection was performed.

Cells were washed once with DPBS, trypsinized and resuspended in flow cytometry buffer (DPBS, 2 mM EDTA, 1% BSA). GFP and mCherry fluorescence were measured on a LSRFortessa SORP (BD Life Sciences) using 488 nm excitation and the 530/30 BP filter for GFP and 561 nm excitation and 610/20 BP filter for mCherry detection. Dead cells were excluded by DAPI staining (excitation 405 nm, 31 450/50 BP). 20,000 cells have been recorded per sample. Data analysis was done using FlowJo (v10.8.1) (BD Life Sciences).

Median levels of GFP signal were normalized to median levels of mCherry within each sample. The difference in signal after treatment for all mutants was calculated by dividing the median level ratio upon STM2457 treatment by the median level ratio upon DMSO. To facilitate inter-experiment comparisons, the results of all mutants were further normalized to the levels of a control plasmid containing only GFP and mCherry. For statistical testing, unpaired two-tailed Student's *t* tests were performed on *n*=4 replicates.

### CMD reporter RNA stability measurement upon actinomycin D treatment

HEK293T cells were seeded in a 24-well plate. Upon reaching 80% confluency, they were transfected with 500 ng of the indicated reporter plasmids using FuGENE HD transfection reagent (Promega, E2312) according to the manufacturer's recommendations, and treated with 20  $\mu$ M STM2457 or DMSO and incubated overnight. After 15 h, cells were treated with transcriptional inhibitor actinomycin D (5  $\mu$ g/ml) for the time indicated. Following treatments, cells were harvested, RNA was isolated, and cDNA was generated as previously described. Changes in levels of GFP mRNA were measured by real-time qPCR and were normalized to mCherry mRNA levels produced from the same promoter as an internal control (primers in Table S7). Data was normalized to T0.

### Ribosome profiling (Ribo-seq)

Ribosome profiling was performed in HEK293T cells following a modified protocol based on McGlincy and Ingolia<sup>110</sup> and Buchbender et al.<sup>49</sup> HEK293T cells were plated in 10-cm cell culture dishes at  $24 \times 10^3$  cells/cm<sup>2</sup>. At 70% confluency, cells were treated with 20  $\mu$ M STM2457 or DMSO for an additional 6 h. At 1 h prior to cell lysis, 10% extra FBS was added to the cell culture medium to boost translation. Cells were treated with cycloheximide (CHX) at 100  $\mu$ g/ml for 3 min at 37°C directly before lysis. Cells were washed with ice-cold PBS/CHX once and lysed in 400  $\mu$ l of polysome lysis buffer (20 mM Tris-HCl pH7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 1 mM DTT, 100  $\mu$ g/ml cycloheximide, 2 U/ml TURBO DNase (Thermo Fisher Scientific, AM2238). In addition, 100  $\mu$ l of crude cell lysate was taken for preparation of the matched RNA-seq library. Next, the lysate was treated with RNase I (Thermo Fisher Scientific, AM2295) for 45 min at room temperature (24°C) with rotation at 500 rpm. Nuclease digestion was stopped by adding SUPERase In RNase inhibitor (Invitrogen, AM2696). Ribosomes were isolated by sucrose cushion and centrifugation with a TLA100.3 rotor at 100,000 rpm, 4°C for 1 h. RNA was purified from the resuspended ribosomal pellet using the Direct-zol kit (Zymo Research) according to the manufacturer's instructions for purifying total RNA including small RNAs. The eluted RNA was precipitated with ethanol. For the 32 purification of ribosome-protected fragments, RNA samples were run on a 15% polyacrylamide TBE-urea gel for 65 min at 200 V next to synthesized marker oligonucleotides of defined length (26 nt and 34 nt). The gel regions between 26–34 nt were excised for each sample and RNA was eluted for 1 h at room temperature in the extraction buffer (300 mM sodium acetate pH 5.5, 1 mM EDTA, 0.25% (v/v) SDS) followed by phenol-chloroform extraction and RNA precipitation with isopropanol. T4 polynucleotide kinase was used to remove 2'-3' cyclic phosphate from ribosomal footprints in the following

reaction mixture for 1 h at 37°C: 3.5  $\mu$ l purified RNA, 0.5  $\mu$ l T4 PNK buffer (10x), 0.5  $\mu$ l T4 PNK (10 U/ $\mu$ l) and 0.5  $\mu$ l SUPERase In RNase Inhibitor (20 U/ $\mu$ l). 3'-linker ligation was performed by adding the following components directly to the dephosphorylation reaction: 3.5  $\mu$ l 50% w/v PEG-8000, 0.5  $\mu$ l T4 RNA ligase buffer (10x), 0.5  $\mu$ l pre-adenylated L3-App linker (/rApp/AGATCGGAAGAGCGGTT CAG/ddC/, 20  $\mu$ M), 0.5  $\mu$ l T4 Rnl2 truncated K227Q (New England Biolabs, M0351S, 200 U/ $\mu$ l), and incubating for 3 h at 22°C. Unligated 3'-linker was removed from the reaction by the addition of 0.5  $\mu$ l yeast 5'-deadenylase (New England Biolabs, M0331S, 10 U/ $\mu$ l) and 0.5  $\mu$ l RecJ exonuclease (New England Biolabs, M0264S, 10 U/ $\mu$ l). The reaction was incubated for 45 min at 30°C. The RNA was purified with Oligo Clean and Concentrator kit (Zymo Research) according to the manufacturer's instructions. For reverse transcription, positive and negative controls were included. The negative control was performed as the reaction where the RNA sample was substituted with 8  $\mu$ l of 100 nM L3-App linker ("no-input"). As a positive control, dephosphorylated and L3-App linker-ligated 26 nt and 34 nt control RNA oligonucleotides were used. The mixture of 8  $\mu$ l L3-App-ligated RNA, 1  $\mu$ l RT oligo primer (2.5  $\mu$ M) and 1  $\mu$ l dNTP mix (10 mM each) was incubated for 5 min at 70°C and then held at 25°C until the addition of the following reagents: 4  $\mu$ l RNase-free water, 4  $\mu$ l RT buffer (5x), 1  $\mu$ l DTT (0.1 M), 0.5  $\mu$ l RNase inhibitor and 0.5  $\mu$ l SuperScript III (Life Technologies, 18080085). Reverse transcription was performed at the following conditions: 25°C for 5 min, 55°C for 60 min, 80°C for 5 min, and held at 4°C. To remove RNA, alkaline hydrolysis was performed. The second adapter ligation to the 3' end of the cDNA was performed as described before.<sup>49</sup> Briefly, after reverse transcription, cDNA clean-up was performed using MyONE Silane beads. Next, a second linker ligation was performed using different L#clip2.0 oligonucleotides for each sample. 5'-linker-ligated cDNA was cleaned up using MyONE Silane beads. Different from the published protocol,<sup>49</sup> we omitted PCR preamplification, followed by the first ProNex size selection before preparative PCR amplification, and instead performed just one PCR amplification step with an optimized number of cycles. After PCR, different samples were multiplexed in equal molarities. The library was then purified and size selected on ProNex magnetic beads. The libraries were sequenced on an Illumina NextSeq 500 as 92-nt single-end reads including a 6-nt sample barcode as well as 5+4-nt UMIs generating more than 40 million reads (Table S2).

### YTHDF2 iCLIP2

*In vivo* iCLIP2 for YTHDF2 was performed as described before.<sup>49</sup> Briefly, HEK293T cells treated with DMSO or STM2457 for 12 h were irradiated with 150 mJ/cm<sup>2</sup> in a UV-C crosslinker (CL-1000 Ultraviolet Crosslinker, UVP) at 254 nm. Crosslinked cells were lysed and RNA was digested to create 50–200 nt long fragments. YTHDF2 protein immunoprecipitation was performed with an YTHDF2-specific antibody (AVIVA SYSTEMS BIOLOGY, ARP67917\_P050) conjugated to protein A beads. Protein–RNA complexes were visualized using 5' end radioactive labeling of the precipitated RNA, followed by SDS-PAGE and transfer to a nitrocellulose membrane. Protein–RNA complexes were recovered from the membrane and treated by proteinase K to get protein-free RNA. cDNA was generated by reverse transcription, with termination occurring at the crosslinked site, thereby generating truncated cDNAs. MyONE beads were used to clean cDNA twice and the library was subsequently subjected to PCR amplification and ProNex size selection for amplification and purification, respectively. iCLIP2 libraries were sequenced on an Illumina NextSeq 500 sequencing device as 92-nt single-end reads including a 6-nt sample barcode as well as 5+4-nt UMIs (Table S2).

### Immunofluorescence and Opera Phenix High Content Imaging

HEK293T cells were grown in Poly-L-Lysine (PLL)-coated Cell Carrier Ultra 96-well plates (PerkinElmer, #6055302) for 48 h. For the YTHDF2 siRNA knockdown, cell suspension was added to the RNAiMAX/siRNA reverse transfection mixture before seeding into the 96-well plate. As indicated, cells were treated with STM2457 (BioMol, #Cay34280, 20  $\mu$ M) for 6 h and 24 h, emetine dihydrochloride (Sigma, #E2375, 1.8  $\mu$ M) for 1 h, sodium arsenite (Sigma, #S7400, 0.1 mM) for 1 h, or DMSO as a control for the respective times before the end of the 48 h growth period. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at RT. After each of the following incubation steps, cells were washed three times with PBS. Permeabilization was achieved by incubation with 0.1% Triton X100 in PBS for 5 min. After incubation for 45 min in blocking solution (PBS + 10% horse serum), the primary antibody (rabbit-anti-DCP1a, #ab183709, Abcam, 1:500) was incubated over night at 4°C in blocking solution. The secondary antibody (Goat-anti-Rabbit-Alexa488, #A11034, Thermo Fisher Scientific, 1:500) was applied for 1 h at room temperature (RT) in the dark. Optionally, DRAQ5 (Invitrogen, #65-0880-92, 1:1000) was added to the secondary antibody mixture, but was not included in the analysis. After washing, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min and cells were stored in PBS at 4°C until image acquisition. Imaging was realized using the Opera Phenix spinning disc high-content screening microscope (Perkin Elmer) equipped with two 16-bit sCMOS cameras and a 20x 0.4 NA air objective. Signals were detected in widefield mode in two z-planes of 1  $\mu$ m distance to account for different focus positions across the well plate at 405/435–480, 488/500–550 or 561/570–630 nm ex/em, respectively.

Maximum projections of the individual images were analyzed with the Harmony Software (version 5.2) using the Spot Analysis pipeline (Figure S7A). Briefly, cell nuclei were identified via DAPI staining and diffuse cytoplasmic staining of DCP1a was used for segmentation of the cellular borders. DCP1a-positive cytoplasmic loci (P-bodies) were detected using the spot analysis algorithm (Type C) and quantified for each segmented cell individually. The parameters of the image analysis sequences are available upon request. For each condition, 8 replicates were analyzed, counting five images per well, resulting in up to 4000 individual cells for analysis in each replicate. Results are shown as the mean of 8 replicates with standard derivation of the mean.

### SDS-PAGE and Western blot

Western blot was used to validate the knockdown of *YTHDF2* in HEK293T cells after reverse siRNA transfection for 48 h in 12-well plates in parallel to the P-body quantification experiments. Cells were washed twice with PBS on ice and lysed in modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS [all Sigma], 1x cOmplete Protease Inhibitor Mix [Roche] and 1% TURBO DNase [Thermo Fisher Scientific]) for 2 min at 37 °C and 20 min on ice. Cell debris were precipitated by centrifugation at 16,000 ×g for 10 min at 4 °C and concentration of the cleared protein lysate was measured with the BCA Protein Assay Kit (Thermo Fisher Scientific). 6 µg of lysate were separated with a NuPAGE 4–12% Bis-Tris Mini Protein Gel (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane (VWR International) using the NuPAGE Novex Gel System in combination with the XCell II Blot-Modul (Invitrogen). After transfer, the membrane was dried for 15 min at 37 °C and processed with the Revert 520 Total Protein Stain (LI-COR Biosciences) according to the manufacturer's instructions. After detection of the Revert 520 signal with the Odyssey M imaging system (LI-COR Biosciences), the membrane was blocked in blocking solution (5% [w/v] milk powder [Roth], 0.1% Tween20 in PBS [PBS-T]). Primary antibodies (anti-YTHDF2, AVIVA systems, #ARP67917\_P050, 1:5000; anti-alpha-Tubulin, Sigma, #T5168, 1:10000) were incubated overnight at 4 °C in blocking solution, and fluorophore- and species-matching secondary antibodies (Goat anti-Mouse IRDye 680RD, #926-68070; Goat anti-Rabbit IRDye 800CW, #926-32211; both 1:20000, LI-COR Biosciences) were incubated for 60 min at room temperature in the dark. After primary and secondary antibody incubation steps, the membrane was washed three times with PBS-T, proceeding with imaging at the respective fluorescent channels on the Odyssey M imaging system (LI-COR Biosciences). Signals were analyzed with the Emperia Studio Software v3.2 (LI-COR Biosciences).

### YTHDF2 proximity proteomics

#### Preparation of stable cell lines with inducible expression of miniTurbo-YTHDF2 fusion protein

The sequence encoding 3xHA-miniTurbo was PCR amplified from the miniTurbo-NLS\_pCDNA3 plasmid (Addgene: #107172)<sup>51</sup> using the forward 5'tataagcttGCCACCATGTACCCGTAT'3 and reverse 5'tagatcc CTTTTCGGCAGACCG'3 primers. The PCR product was inserted into pcDNA<sup>TM</sup>5 /FRT/TO (Invitrogen, #V652020) by standard cloning procedures using BamHI and HindIII restriction sites. For the N-terminal 3xHA-miniTurbo-YTHDF2 constructs, coding sequence (ORF) of *YTHDF2* was PCR-amplified from cDNA prepared from RNA isolated from HEK293T using forward (5'-actagatccATGTCGGC-CAGCAGCCTC-3') and reverse (5'-aacctcgaGTCATTTCCCACGACCTTG-3') primers and subcloned via BamHI and XhoI restriction sites to the 3xHA-miniTurbo-pcDNA5 construct by standard cloning procedure.

To prepare stable cell lines with inducible expression of the fusion proteins, HEK Flp-In<sup>TM</sup> T-REx<sup>TM</sup> 293 (Invitrogen, #R78007) cells were grown to 70% confluency in 6-well plates and co-transfected with 400 ng of the corresponding expression vector (pcDNA5/FRT/TO<sup>TM</sup> encoding 3xHA-miniTurbo-YTHDF2 and 3xHA-miniTurbo, respectively) and 3.6 µg of pOG44 Flp-Recombinase expression vector (Invitrogen, #V600520) (ratio 1:9) using 5 µl of TurboFect reagent (Invitrogen, #R0531) following the manufacturer's protocol. One day after transfection, cells were transferred to a 150 mm dish and selected with 40 µg/ml of hygromycin B in DMEM with 10% FBS until individual clones were formed. Doxycycline (dox)-inducible expression of the tagged proteins were confirmed by Western blot with anti-YTHDF2 (S-14, Santa Cruz #sc-162427) or anti-HA tag (HA-probe [Y-11], Santa Cruz #sc-805) antibodies.

#### Biotin proximity labelling and streptavidin pull-down assay

For each replicate experiment, two 100-mm cell culture dishes were grown to 70% confluency, protein expression was induced with 100 ng/ml of doxycycline (Applichem, #A2951) and after 24 h incubation, 50 µM biotin (Sigma, #B4501-5G) was added for 20 min to the medium to allow *in vivo* biotinylation. Cells from the two dishes were harvested and combined as one biological replicate, flash-frozen and short-term stored at -80 °C until affinity purification was performed. At least three independent biological replicates were prepared per cell line. Lysis was carried out in 750 µl of modified RIPA buffer (20 mM Tris-HCl pH 7.5; 130 mM NaCl; 2 mM EDTA; 20 mM NaF; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with protease inhibitors incl. 0.1% (v/v) - Pepstatin A (Glenthams, #GE3524-25MG), Leupeptin, hemisulfate (Applichem, #2183), 1% [v/v] PMSF (Applichem, #A0999,025) and 1% (v/v) of Phosphatase Inhibitor Cocktail I (Sigma Aldrich, #P2850-5ML). Lysates were clarified by centrifugation at 15,000 g for 8 min at 4 °C. Labeling efficiency was tested via Western blot. 25 µl of Dynabeads<sup>TM</sup> MyOne<sup>TM</sup> Streptavidin C1 (Invitrogen, #65001) were used per replicate to efficiently pulldown biotin-labelled proteins. Beads were equilibrated by washing three times with 1 ml RIPA buffer. Lysates (0.5 mg/ml) were mixed with the equilibrated beads and incubated for 1 h at RT on a rotary shaker. The beads were washed at RT two times for 8 min with 1.5 ml RIPA buffer. Subsequently beads were briefly washed with 500 µl Tris-urea buffer (20 mM Tris-HCl pH 7.5; 2 M urea) and finally washed for 8 min with 20 mM Tris-HCl buffer (pH 7.5). Samples were processed for MS/MS analysis.

#### Protein mass spectrometry sample preparation and data acquisition

Bead-bound protein complexes were mixed with 2% SDS solution and heated to 50 °C for 20 min after which the proteins were reduced using 500 mM DTT at 95 °C for 15 min. Resulting protein solutions were subjected to filter-aided sample preparation (FASP) as described elsewhere<sup>111</sup> using 0.75 µg of trypsin (sequencing grade; Promega). Peptides were then cleaned by liquid-liquid extraction (3 iterations) using water-saturated ethyl acetate.<sup>112</sup> Cleaned FASP eluate was evaporated completely in SpeedVac concentrator (Thermo Fisher Scientific). Resulting peptides were analyzed by LC-MS/MS. LC-MS/MS analyses of all peptides were done using UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) connected to timsTOF Pro spectrometer (Bruker). Prior to LC separation, tryptic digests were online concentrated and desalted using trapping column (Acclaim<sup>TM</sup> PepMap<sup>TM</sup> 100 C18, dimensions 300 µm ID, 5 mm long, 5 µm particles, Thermo Fisher Scientific). After washing of the trapping column with 0.1% formic

acid (FA), the peptides were eluted (flow rate - 300 nL/min) from the trapping column onto an analytical column (Aurora C18, 75  $\mu$ m ID, 250 mm long, 1.6  $\mu$ m particles, heated to 50°C, Ion Opticks) by 60 min linear gradient program (3–42% of mobile phase B; mobile phase A: 0.1% FA in water; mobile phase B: 0.1% FA in 80% ACN). Equilibration of the trapping column and the analytical column was done prior to sample injection to sample loop. The analytical column was placed inside the Butterfly Heater (Phoenix s&t) and its emitter side was installed inside the CaptiveSpray ion source (Bruker) according to the manufacturer instructions with the column temperature set to 50°C. MSn data were acquired in data independent acquisition (DIA) mode with base method m/z range of 100–1700 and 1/k0 range of 0.6–1.6 V  $\times$  s  $\times$  cm<sup>-2</sup>. Precursor range was defined to m/z 40–1000 with equal windows sizes of 26 Th (including 1 Th overlap) using two steps each PASEF scan and cycle time of 100 ms locked to 100% duty cycle.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Data processing for high-throughput *in vitro* methylation assays

#### Raw data processing

Basic quality controls were done with FastQC (v0.11.8) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and read pairs were filtered based on sequencing qualities (Phred score) in the barcode and UMI regions in R1 using the FASTX-Toolkit (v0.0.14) ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) and seqtk (v1.3) (<https://github.com/lh3/seqtk/>). All read pairs with a Phred score <10 in the considered regions were removed from further analysis. Read pairs were de-multiplexed based on the sample barcode, which is found on positions 6–11 of R1 using Flexbar (v3.4.0).<sup>89</sup> Barcode and UMI regions as well as adapter sequences were trimmed from read ends using Flexbar requiring a minimal overlap of 1 nt of read and adapter and adding UMIs to the read names. Read pairs with R1 or R2 shorter than 15 nt were removed from further analysis.

For each sample, R1 was de-multiplexed further based on the oligonucleotide UMI, which is found on positions 1–15 of R2 using basic Bash commands and seqtk (v1.3) (<https://github.com/lh3/seqtk/>). This resulted in one FASTQ file of R1 per oligonucleotide per sample. The reads of each oligonucleotide were mapped to the respective oligonucleotide sequence using STAR (v2.7.3a)<sup>90</sup> and allowing up to 4% of the bases to be mismatched (`--outFilterMismatchNoverReadLmax 0.04 --outFilterMismatchNmax 999`), discarding reads with more than one possible location (`--outFilterMultimapNmax 1`) and prohibiting soft-clipping at the 5' end (`--alignEndsType Extend5pOfRead1`). Subsequently, the position upstream of every mapped read (i.e., the crosslink site) was extracted using BEDTools (v2.27.1)<sup>91</sup> `bamtoBed`, `shift` (with parameters `-m 1 -p -1`) and `genomecov` (with parameters `-bg -strand + -5`). Resulting `bedGraph` files were converted to `bigWig` files using `bedGraphToBigWig` of the UCSC tool suite (v385).<sup>92</sup>

#### Differential methylation analysis for *in vitro* miCLIP2 data

The 47,996 `bigwig` files with the miCLIP2 crosslink signal were imported with `rtracklayer` (v1.52.0).<sup>93</sup> For each oligonucleotide, all positions from 50 nt upstream to 90 nt downstream of the selected site were considered for the differential methylation analysis. To this end, we compared miCLIP2 crosslink counts at each position on each oligonucleotide between METTL3–METTL14-treated and unmodified control samples with DESeq2 (v1.32.0).<sup>94</sup> All technical and biological replicates with more than 60 reads for a transcript region were considered as replicates. Differential methylation level *in vitro* is presented as log<sub>2</sub>-transformed fold change (log<sub>2</sub>FC) between METTL3–METTL14-treated samples versus unmodified controls.

### Analysis of *in vivo* m6A sites

#### Calculation of *in vivo* methylation ratio

m6A sites were taken from GLORI (170,239 m6A sites in HEK293T [Supplementary Data 1] from Liu et al.),<sup>18</sup> miCLIP2 (25,456 m6A sites in HEK293T [GSE163500]) datasets as indicated.<sup>14,18,113</sup>

For each dataset, m6A sites were assigned to different genes and transcript regions by the function `metaGeneProfile()` from the R/Bioconductor package `cliProfiler` (v1.6.0)<sup>95</sup> with human annotation from GENCODE release 31. m6A sites that were assigned to any transcript region were kept for the following analysis. Transcripts with at least one assigned m6A site were selected. Subsequently, the DRACN sites on the selected transcript located more than 200 nt away from a splice site were used for calculating the methylation rate (m6A-to-A ratio). The m6A-to-A ratio was calculated as:

$$\text{m6A-to-A ratio} = \frac{\text{m6A sites on given 5mer}}{\text{number of given 5mer}}$$

#### Partial correlation between m6A DRACN 5-mers and overall m6A numbers

The partial correlation was performed with the R package `ppcor` (v1.1).<sup>96</sup> The number of m6A on each DRACN 5-mers together with the overall m6A number on transcripts were used as input to calculate their partial correlation to the log<sub>2</sub>-transformed fold change in transcript abundance upon 9 h m6A depletion. The *p* value for the partial correlation was corrected with the Benjamini-Hochberg method.

#### Metagene profiles

Metagene profiles for the distribution of m6A sites within transcripts and m6A assignment to transcript regions were done with the R/Bioconductor package `cliProfiler` (v1.6.0).<sup>95</sup>

### Nanopore direct RNA sequencing data analysis

All base-called Nanopore direct RNA sequencing reads were aligned to the Ensembl reference transcriptome (release 102) using minimap2 (v2.22-r1101).<sup>97</sup> The respective alignment files served as input to segment the nanopore signal and to anchor them using nanopore-polish (v0.13.3). All subsequent steps were performed as outlined in the m6Anet tutorial using version 2.0.1 (<https://m6anet.readthedocs.io>).<sup>29</sup> For downstream analyses, we used all m6A sites with a mean m6Anet probability >0.6 for the two WT replicates and a distance >200 nt to the next splice site.

### Data processing for RNA-seq data

#### Processing of raw data

Basic quality controls were done using FastQC (v0.11.8 or v0.11.9) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Prior to mapping, possibly remaining adapter sequences were trimmed using Cutadapt (v1.18 or v4.0)<sup>114</sup> in 9 h and 24 h m6A depletion, DMSO control, as well as emetine-treated samples in both m6A-depleted and DMSO control conditions. A minimal overlap of 3 nt or 5 nt between read and adapter was required and only reads with a length of at least 50 nt or 30 nt after trimming were kept for further analysis. A NextSeq-specify quality trimming was applied to the 3' end of reads in emetine-treated samples and their controls. Since 24 h m6A depletion samples were sequenced with only one dark cycle, an additional 1 nt was trimmed at the 5' end of their reads (--cut 1). Emetine-treated samples and their controls were also sequenced with only one dark cycle upfront, but did not require trimming of an extra base at the 5' end of their reads.

Reads were mapped using STAR (v2.7.3a)<sup>90</sup> allowing up to 4% of the bases to be mismatched and with a splice junction overhang of 1 nt less than the sequenced read. Genome assembly and annotation of GENCODE<sup>115</sup> release 31 were used during mapping. In the case ERCC spike-ins were added during library preparation, their sequences and annotation (<http://tools.thermofisher.com/content/sfs/manuals/ERCC92.zip>) were used in combination with those from GENCODE. Secondary hits were removed using Samtools (v1.9 or v1.10).<sup>98</sup> Exonic reads per gene were counted using featureCounts from the Subread tool suite (v2.0.0)<sup>99</sup> with non-default parameters --donotsort -s2 -T4.

Genes differentially expressed between conditions were detected using the DESeq2 R/Bioconductor package (v1.26.0 or v1.34.0)<sup>94</sup> in an R environment (v3.6.0 or v4.1.2).<sup>116</sup>

All RNA-seq datasets generated in this study are listed in Table S2.

#### Re-analyses of public RNA-seq data

In this study, we reanalyzed data for *CNOT1* KD, *DCP2* KO, emetine treatment and *HRSP12* KD (GSE141496, GSE149565, PRJNA482875, and GSE143662).<sup>28,43,54,55</sup>

Raw data were downloaded from the NCBI Gene Expression Omnibus (GEO) using Parallel Fastq Dump (v0.6.7). The adapter sequences were removed with Trimmomatic (v0.39).<sup>100</sup> Individual samples were then aligned to the respective genome (assembly version GRCh38.p12 for all human samples, GRCm38.p6 for all mouse samples) and its annotation (GENCODE release 31 for all human samples, GENCODE release M23 for all mouse samples) using STAR (v2.7.8a).<sup>90</sup> When running STAR, up to 4% mismatches were allowed per read and only the unique mapping reads are used for the downstream analysis. Aligned reads were counted into genes using featureCounts (v2.0.1)<sup>99</sup> with default settings.

#### Boruta and SHAP analysis

The Boruta and SHAP analysis was done in R (v4.1.0) with the packages Boruta (v7.0.0) and treeshap (v0.1.1).<sup>41</sup> Only transcripts with at least one m6A site in any transcript region were used. To conduct the Boruta analysis, the numbers of m6A sites in different regions for each transcript were used to predict the log<sub>2</sub>-transformed fold change in transcript abundance or RNA half-lives upon the corresponding treatment. To conduct the SHAP analysis, the numbers of m6A sites in different regions for each transcript were used to predict the log<sub>2</sub>-transformed fold change in transcript abundance or RNA half-lives upon the corresponding treatment using a random forest regression algorithm (R package ranger v0.12.1).

### Processing of Ribo-seq data

#### Processing of public Ribo-seq data

The adapter sequences were removed with Trimmomatic (v0.39).<sup>100</sup> Individual samples were then aligned to the respective genome (assembly version GRCh38.p12 for all human samples, GRCm38.p6 for all mouse samples) and its annotation (GENCODE release 31 for all human samples, GENCODE release M23 for all mouse samples) using STAR (v2.7.8a).<sup>90</sup> When running STAR, up to 8% mismatches were allowed per read, soft-clipping was prohibited on the 5' end of reads, and multi-mapping reads were allowed to be aligned to a maximum of 5 positions. Following genomic alignment, the coverage of 5' ends of the reads was calculated with BEDTools (v2.30.0)<sup>91</sup> and converted to bigWig file format with bedGraphToBigWig (v4).<sup>92</sup>

#### Processing of Ribo-seq data generated in this study

Basic quality controls were done with FastQC (v0.11.9) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and reads were filtered based on sequencing qualities (Phred score) in the barcode and UMI regions using the FASTX-Toolkit (v0.0.14) ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) and seqtk (v1.3) (<https://github.com/lh3/seqtk>). All reads with a Phred score <10 in the considered regions were removed from further analysis. Reads were de-multiplexed based on the sample barcode, which is found on positions 6–11 using Flexbar (v3.5.0).<sup>89</sup> Barcode and UMI regions as well as adapter sequences were trimmed from read ends using Flexbar requiring a minimal overlap of 1 nt of read and adapter and adding UMIs to the read names. Reads shorter than

15 nt were removed from further analysis. Mapping and removal of duplicate reads were done as described for iCLIP data in Chapters 3.4 and 4.1 of Busch et al.<sup>117</sup> Genome assembly and annotation of GENCODE<sup>115</sup> release 31 were used during mapping with STAR (v2.7.3a).<sup>90</sup> Read start positions were extracted using BEDTools (v2.29.2)<sup>91</sup> bamtobed and genomecov with parameters -bg -strand + -5 for reads on the forward strand and -bg -strand - -5 for reads mapped to the reverse strand. Resulting bedGraph files were converted to bigWig files using bedGraphToBigWig of the UCSC tool suite (v385).<sup>92</sup>

#### Calculation of translation efficiency (TE)

First, we counted the reads for both Ribo-seq and RNA-seq data only on CDS regions with featureCounts from the Subread tool suite (v2.0.3). Then, the count tables from both Ribo-seq and RNA-seq data were merged, and DESeq2 sizeFactor calculation was used to normalize the read counts for each sample. Subsequently, translation efficiency was calculated as:

$$TE = \frac{\text{normalized read counts in Ribo-seq (mean over replicates)}}{\text{normalized read counts in RNA-seq (mean over replicates)}}$$

#### Calculation of ribosome density

First, we summed the Ribo-seq read 5' ends within a 101-nt window around the selected sites. This aggregated value was then divided by the number of Ribo-seq read 5' ends within the CDS on the corresponding genes, resulting in the ribosome density around the selected sites.

#### YTHDF2 iCLIP2 data analysis

Basic quality controls were done with FastQC (v0.11.8) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and reads were filtered based on sequencing qualities (Phred score) in the barcode and UMI regions using the FASTX-Toolkit (v0.0.14) ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) and seqtk (v1.3) (<https://github.com/lh3/seqtk/>). All reads with a Phred score below 10 in the considered regions were removed from further analysis. Reads were de-multiplexed based on the sample barcode, which is found on positions 6 to 11 using Flexbar (v3.4.0).<sup>89</sup> Barcode and UMI regions as well as adapter sequences were trimmed from read ends using Flexbar requiring a minimal overlap of 1 nt of read and adapter and adding UMIs to the read names. Reads shorter than 15 nt were removed from further analysis. The downstream analysis was done as described in Chapters 3.4, 4.1 and 4.2 of Busch et al.<sup>117</sup> Genome assembly and annotation of GENCODE release 31<sup>115</sup> were used during mapping with STAR (v2.7.3a).

Peak calling was done on merged samples from STM2457 treatment and control using PureCLIP (v1.3.1)<sup>50</sup> with default parameters. To identify YTHDF2 binding sites that significantly change upon STM2457 treatment, we used the bin-based method as described<sup>14</sup> to correct for transcript-level changes. In short, transcripts were stratified into 22 bins according to their expression change upon STM2457 treatment. All YTHDF2 binding sites in the transcripts of the same bin, i.e., with a similar change in transcript abundance, were then tested collectively using DESeq2 (v1.32.0).<sup>94</sup> Since the majority of YTHDF2 binding sites on A and R of DRACN received a downregulation upon STM2457 treatment, we moved the binding sites on R of DRACN by 1 nt. Finally, all YTHDF2 binding sites on DRACN were considered as the m6A-dependent YTHDF2 binding sites for the downstream analysis. Since the majority of YTHDF2 binding sites on A and R of DRACN received a downregulation upon STM2457 treatment, we moved the binding sites on R of DRACN by 1 nt. Finally, all YTHDF2 binding sites on DRACN were considered as the m6A-dependent YTHDF2 binding sites for the downstream analysis.

#### Definition of target transcript sets

First, transcripts with at least one m6A in GLORI data for HEK293T cells were extracted. Subsequently, we identified significantly upregulated targets in the different treatments, requiring a log2FC >0.1 and an adjusted *p* value <0.05 (Benjamini-Hochberg correction) in the respective comparison, including *CNOT1* KD, *DCP2* KO, emetine treatment, *YTHDF2* KD and *HRSP12* KD. To define the CMD targets (6 h targets), genes were required to be significantly upregulated upon 6 h m6A depletion with log2FC >0.1 and adjusted *p* value <0.05 and to maintain a log2FC >0 at 9 h and 24 h m6A depletion. For 9 h targets, genes had to be significantly upregulated upon 9 h m6A depletion but not already after 6 h m6A depletion, whereas 24 h target genes had to be upregulated significantly exclusively after 24 h m6A depletion. These additional rules were implemented to identify genes that exhibited a response solely at the later time points. Mouse CMD targets were defined as genes being significantly upregulated upon m6A depletion for 3 h in mESC.<sup>38</sup>

#### GO enrichment analysis

GO enrichment analysis was performed with the R package clusterProfiler (v4.0.0).<sup>101</sup> Human and mouse CMD targets, as defined above, were used as input. In addition, transcripts with more than 10 CDS GGACN motifs that reside more than 200 nt away from splice sites were selected from the mouse and human transcriptome and were used as input. A union of expressed genes (mean normalized reads [baseMean] ≥ 10) from all used RNA-seq experiments were used as the background ("universe") to calculate the GO enrichment. Significantly enriched GO terms with adjusted *p* value <0.1 (Benjamini-Hochberg correction) are listed in Table S6.

### Analysis of intron-less genes and retrogenes

Intron-less genes were identified based on GENCODE annotation (release 31) as genes in which all annotated protein-coding transcripts have only one annotated CDS exon as intron-less genes. The retrogene list was downloaded from Table S1 in Simpson et al.<sup>118</sup> The retrogenes and their parental genes in human were used for the corresponding analysis.

### Processing and analysis of SLAM-seq data

Basic quality controls were done with FastQC (v0.11.9) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed with Trimmomatic (v0.39).<sup>100</sup> Individual samples were then aligned to the human genome (assembly version GRCh38.p12) and its annotation (GENCODE release 31 for all human samples) using STAR (v2.7.8a).<sup>90</sup> When running STAR, outSAMattributes was set to All, alignEndsType was set to EndToEnd and only the uniquely mapping reads were used for the downstream analysis. The resulting BAM files were subsequently processed with GRAND-SLAM (v2.0.5f)<sup>102</sup> and grandR (v0.2.5)<sup>103</sup> for estimating transcript half-lives.

### Processing of YTHDF2 proximity proteomics data

DiaPASEF data were processed in DIA-NN (version 1.8)<sup>104</sup> in library free mode against modified cRAP database (based on <http://www.thegpm.org/crap>; 112 sequences in total) and UniProtKB protein database for *Homo sapiens* (downloaded on 03.05.2023, number of protein sequences: 20,593). No variable, carbamidomethylation as fixed modification and trypsin/P enzyme with one allowed missed cleavages were set during the library preparation. False discovery rate (FDR) control was set to 1% FDR. MS1 and MS2 accuracies as well as scan window parameters were set based on the initial test searches (median value from all samples ascertained parameter values). MBR was switched on. Reported protein intensities were further processed using the software container environment (<https://github.com/OmicsWorkflows>). Processing workflow is available upon request. Briefly, it covered: a) removal of decoy hits and contaminant protein groups, b)  $\log_2$  transformation of protein group intensities, c) loessF normalization of intensities, d) differential expression using limma<sup>119</sup> statistical test (v3.52.2).

### Statistics

All statistical analyses were performed using R. All boxplots in this study are defined as follows: boxes represent quartiles, center lines denote medians, and whiskers extend to most extreme values within 1.5× interquartile range. All statistical tests performed in this study were two-tailed. All indicated replicate numbers refer to independent biological replicates. No data was excluded from the analysis, unless stated otherwise. All adjusted *p* values were calculated using the Benjamini-Hochberg procedure.

**Supplemental information**

**m6A sites in the coding region trigger**

**translation-dependent mRNA decay**

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# SUPPLEMENTAL INFORMATION

## m6A sites in the coding region trigger translation-dependent mRNA decay

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## Supplementary Figures

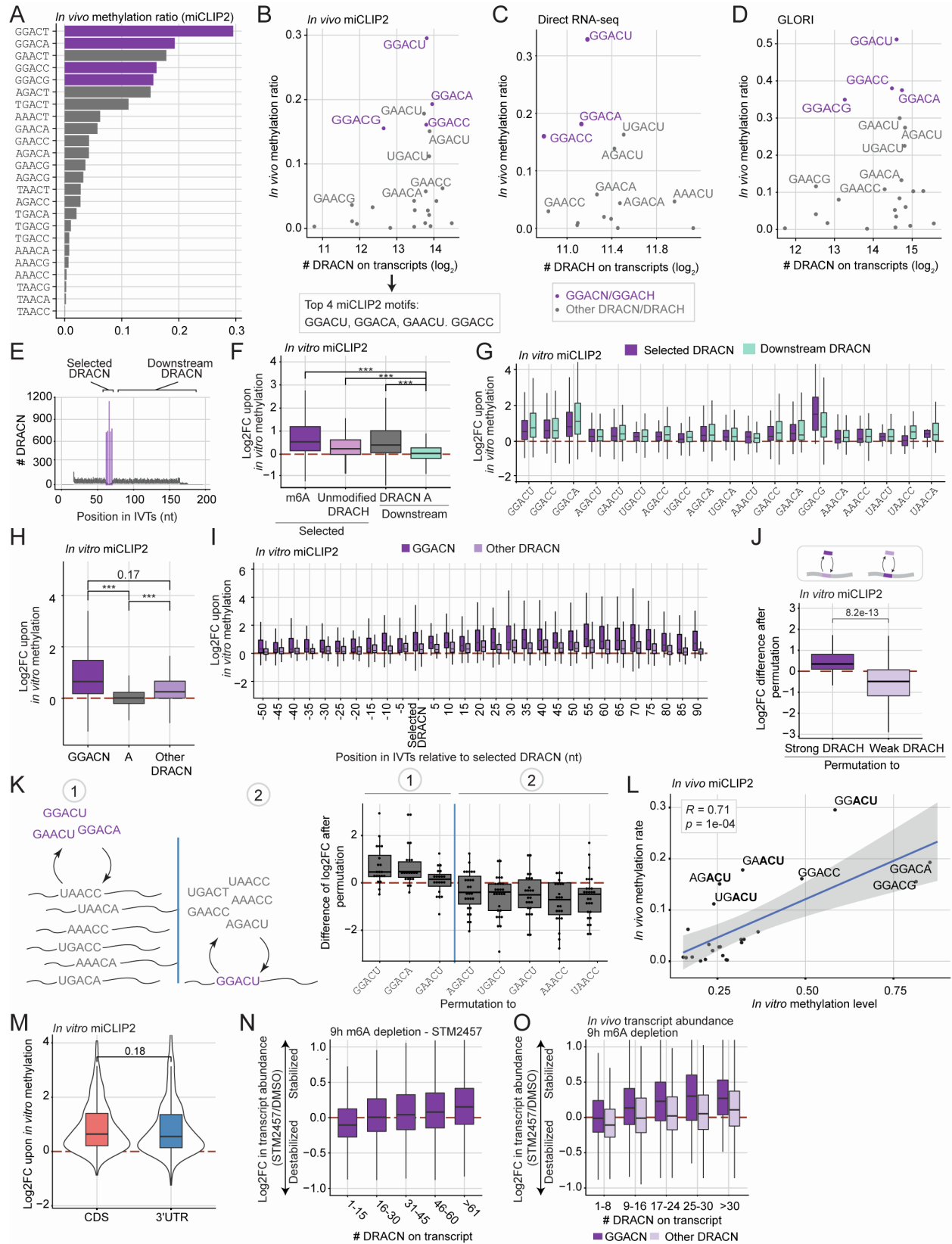


**Figure S1. High-throughput *in vitro* methylation reveals the intrinsic preferences of the METTL3–METTL14 writer complex (Related to Figure 1).**

**(A–D)** Ratio of methylated A compared to all A in DRACN motifs calculated from miCLIP2 (A, B) and GLORI (D),<sup>1</sup> and in DRACH motifs from nanopore direct RNA sequencing (C). DRACN/DRACH and GGACN/GGACH motifs are highlighted in gray and purple, respectively. The top four miCLIP2 motifs are shown.

**(E)** Selected DRACN motifs ( $n=3,888$ , including 3,488 from human transcript loci and 400 permuted sequences) are located in a 10-nt window around position 66 (relative to the start of the DNA oligonucleotide). Additionally occurring downstream DRACN motifs were used as controls (downstream DRACN sites,  $n=7,881$ ).

**(F)** Log<sub>2</sub>-transformed fold changes (log<sub>2</sub>FC) in methylation levels of the *in vitro* methylated library compared to unmethylated control at selected and downstream DRACN or control adenosines (A). \*\*\*  $p < 0.001$ ; Wilcoxon rank-sum test. Box plot elements hereafter, unless otherwise specified, include center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range.

**(G)** Log<sub>2</sub>FC in *in vitro* methylation levels for selected and downstream DRACN motifs.

**(H)** Log<sub>2</sub>FC in *in vitro* methylation levels for GGACN, unmethylated A (first A downstream of selected DRACN site), and other DRACN motifs. \*  $p$  value  $< 0.05$ , \*\*  $< 0.01$ , \*\*\*  $< 0.001$ , ns, non-significant; two-sided Student's  $t$ -test on log<sub>2</sub>-transformed fold changes.

**(I)** Comparison of log<sub>2</sub>FC in *in vitro* methylation levels between GGACN and other DRACH motifs, according to the position (higher boundary of previous 5-mer bins) in the IVT relative to selected DRACN motif. Selected and downstream sites show similar methylation patterns with a preference for GGACN motifs.

**(J)** Log<sub>2</sub>FC in *in vitro* methylation levels upon exchange of a weak DRACH motif to different strong DRACH variants (left) and vice versa (right) in a fixed sequence context.

**(K)** Log<sub>2</sub>FC in *in vitro* methylation levels upon exchange of weak DRACH motifs (UAACC, UAACA, AAACC, UGACC, AAACA, UGACA) by strong DRACH motifs (GGACU, GAACU, GGACA) on the left, and strong (GGACU) by weak (UAACC, UGACT, AAACC, GAACC, AGACU) on the right.

**(L)** Scatter plot shows correlation between methylation levels *in vivo* (miCLIP2) and differential methylation *in vitro*. GGACN are shown in black, with DRACU highlighted in bold. The blue line and grey shadow indicate linear regression and derivation, respectively. The Pearson correlation coefficient ( $R$ ) and associated  $p$  value are shown.

**(M)** Log<sub>2</sub>FC in *in vitro* methylation level for top four miCLIP2 motifs originating from CDS or 3' UTR.

**(N)** Log<sub>2</sub>FC in abundance after m6A depletion (20  $\mu$ M STM2457, 9 h) for transcripts grouped by number of DRACN motifs.

**(O)** Log<sub>2</sub>FC in transcript abundance upon m6A depletion (9 h) categorized by the number of DRACN motifs and motif strength (color shading).

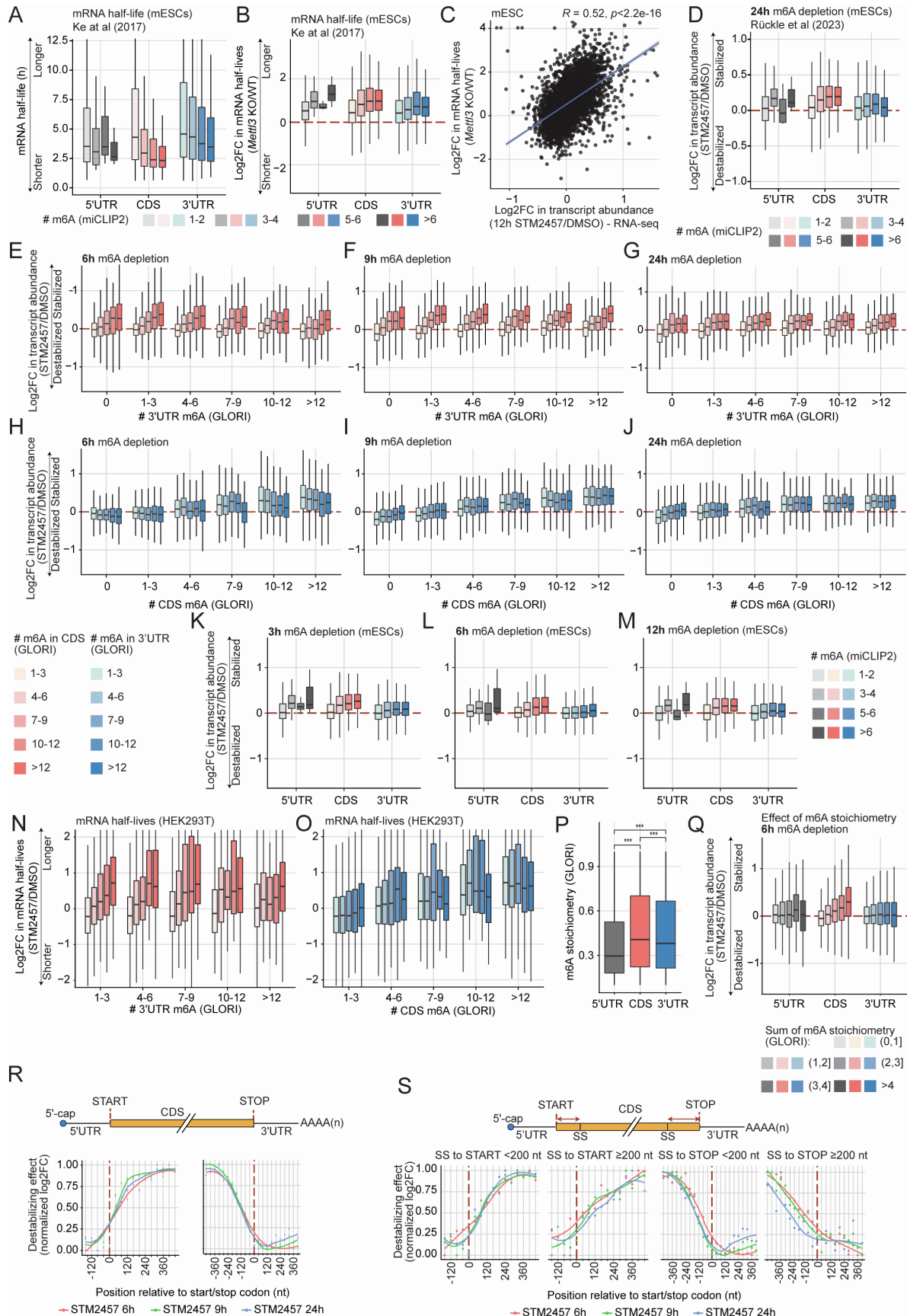


**Figure S2. CDS m6A sites have a dominant effect on transcript stability and abundance (Related to Figure 2).**

**(A)** Half-lives of transcripts grouped by number of m6A sites in each transcript region in mESCs<sup>2</sup> (m6A sites from miCLIP2).<sup>3</sup>

**(B)** Log2FC in half-lives between *Mettl3* KO and WT in mESCs for transcripts grouped by number of m6A sites (miCLIP2) in each transcript region.

**(C)** Correlation between changes in RNA stability<sup>2</sup> and transcript abundance after m6A depletion (12 h) in mESCs.<sup>4</sup> Pearson correlation coefficient (*R*) and associated *p* value are shown. Blue line indicates linear regression.

**(D)** Log2FC in half-lives after m6A depletion (24 h) in mESCs<sup>4</sup> for transcripts grouped by number of m6A sites (miCLIP2) in each transcript region.

**(E–J)** Given that a transcript may contain m6A sites within both its CDS and 3' UTR, we aimed to address this issue when investigating their individual effects. To accomplish this, we constructed plots based on number of m6A sites in 3' UTR (E–G) and CDS (H–J). Within these categories, we further explored how variations in the number of m6A sites within the CDS and 3' UTR, respectively, might impact our observations. Our plotted data illustrates that, irrespective of the number of m6A sites present in 3' UTR of a transcript, they do not significantly impact the overall outcome. (E–G) Log2FC in transcript abundance upon m6A depletion for 6 h (E), 9 h (F), 24 h (G) grouped by number of m6A sites (GLORI) in 3' UTR (x-axes) and CDS (shading). (H–J) Log2FC in transcript abundance upon m6A depletion for 6 h (H), 9 h (I), 24 h (J) grouped by number of m6A sites (GLORI) in CDS (x-axes) and 3' UTR (shading).

**(K–M)** Log2FC in transcript abundance upon m6A depletion for 3 h (K), 6 h (L), 12 h (M) grouped by number of m6A sites (miCLIP2) in each transcript region in mESCs.

**(N)** Log2FC in mRNA half-lives upon m6A depletion grouped by number of m6A sites (GLORI) in 3' UTR (x-axes) and CDS (shading). Data represented as in E–J.

**(O)** Log2FC in mRNA half-lives upon m6A depletion grouped by number of m6A sites (GLORI) in CDS (x-axes) and 3' UTR (shading). Data represented as in E–J.

**(P)** Although the number of m6A sites in the CDS (*n* = 60,489) was higher than in the 3' UTR (*n* = 56,198), the plot shows that the stoichiometry of CDS sites is higher. \* *p* value <0.05, \*\* <0.01, \*\*\* <0.001, ns, non-significant; two-sided Student's *t*-test on m6A number.

**(Q)** Log2FC in abundance after m6A depletion (6 h) for transcripts grouped by the sum of stoichiometries (GLORI) of m6A sites in the 5' UTR, CDS or 3' UTR.

**(R)** Scatter plot showing normalized log2FC in abundance of transcripts with m6A sites in specified positions relative to the CDS start (START) and end (STOP) after 6 h, 9 h, and 24 h of m6A depletion.

**(S)** Scatter plot showing normalized log2FC in abundance of transcripts with m6A sites in specified positions relative to the CDS start (START) and end (STOP) taking into account distance of the closest splice site (SS) after 6 h, 9 h, and 24 h of m6A depletion.

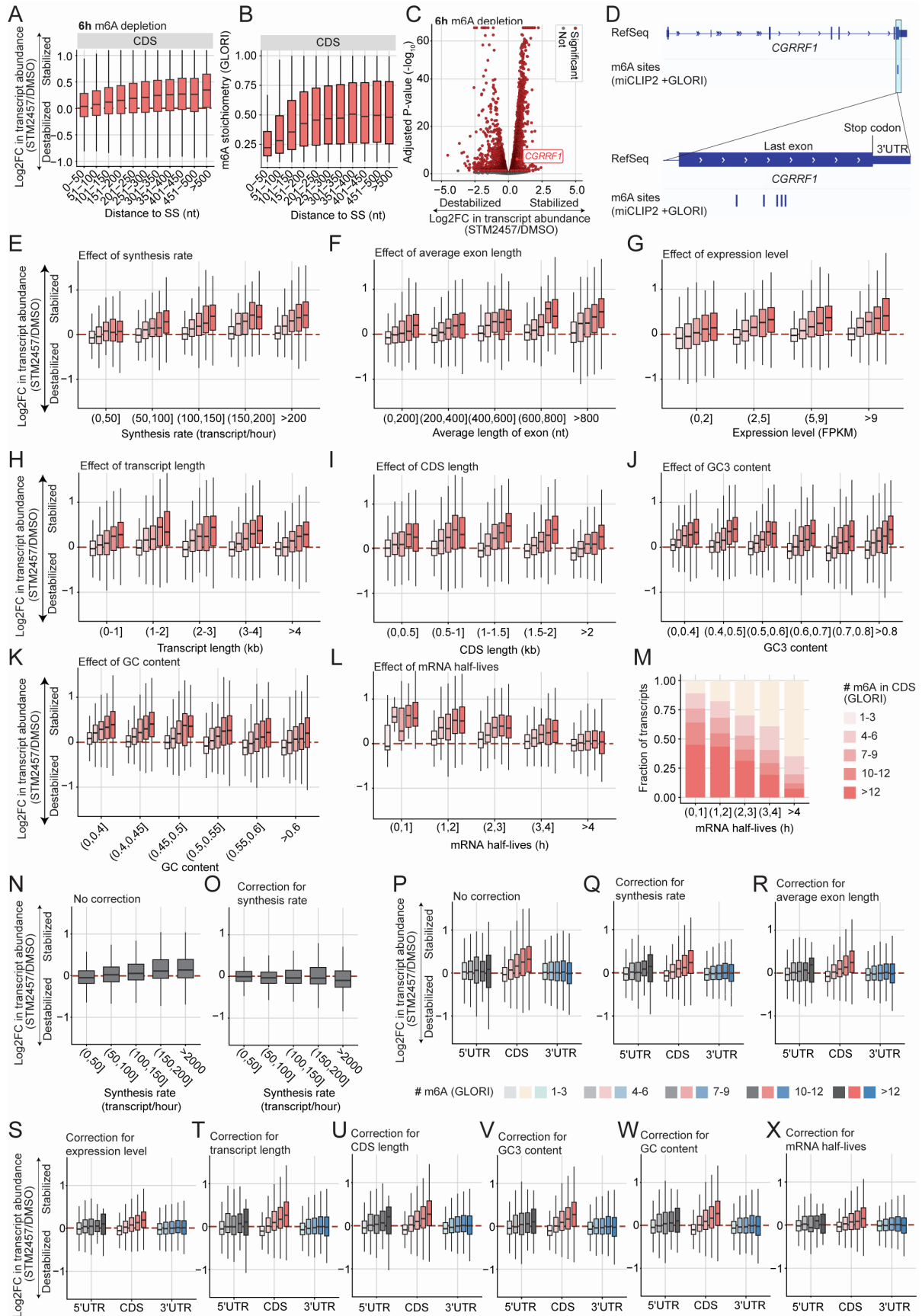


**Figure S3. CDS m6A sites are the primary driver of transcript stabilization (Related to Figure 2M).**

**(A)** Log2FC in transcript abundance after m6A depletion (6 h) for transcripts with m6A sites at increasing distance to the closest splice site (SS).

**(B)** Stoichiometry of CDS m6A sites (GLORI) at increasing distance to the closest splice site.

**(C)** Volcano plot showing log2FC in transcript abundance after 6 h of m6A depletion against adjusted  $p$  values (Benjamini–Hochberg correction). Transcripts with  $p$  values  $<0.05$  are colored. *CGRRF1* is highlighted as an example.

**(D)** Genome browser view of the *CGRRF1* gene. CDS m6A sites (miCLIP2 and GLORI) in the last exon of the CDS are highlighted.

**(A)** Summary plot of mean absolute SHAP values (feature importance score) for number of m6A sites (GLORI) in each transcript region, predicting the transcript abundance changes after m6A depletion.

**(E–L)** To control for the potential confounding factors, we binned all genes based on the covariates and then explored the effect of CDS m6A sites separately within each bin. Box plots show log2FC in transcript abundance upon m6A depletion (6 h) grouped by their synthesis rate (E), average exon length (F), expression level (G), transcript length (H), CDS length (I), GC3 content, i.e., the GC content in the third codon position (J), GC content (K), and half-lives (L, x-axis), and number of CDS m6A sites (color shading). Irrespective of the covariates, transcripts with CDS m6A sites were strongly upregulated upon m6A depletion.

**(M)** Stacked bar plot showing the distribution of transcripts divided by their half-lives (x-axis) according to the number of CDS m6A sites (color shading).

**(N–X)** As an alternative approach for addressing potential confounding effects, we corrected for the above-mentioned covariates using conditional quantile normalization. Shown is the effect of CDS m6A sites on transcript abundance with and without the correction.

**(N, O)** Log2FC in transcript abundance upon m6A depletion (6 h, y-axis) without correction (N), and with correction for synthesis rate (O) for transcripts grouped by synthesis rate (x-axis). The apparent association with synthesis rate is lost after conditional quantile normalization.

**(P–X)** Log2FC in transcript abundance upon m6A depletion for 6 h grouped by the number of m6A sites (GLORI) in each transcript region without correction (P), and with correction for synthesis rate (Q), average exon length (R), expression level (S), transcript length (T), CDS length (U), GC3 content (V), GC content (W), and mRNA half-lives (X). The effect of CDS m6A sites remains after the correction for these covariates.

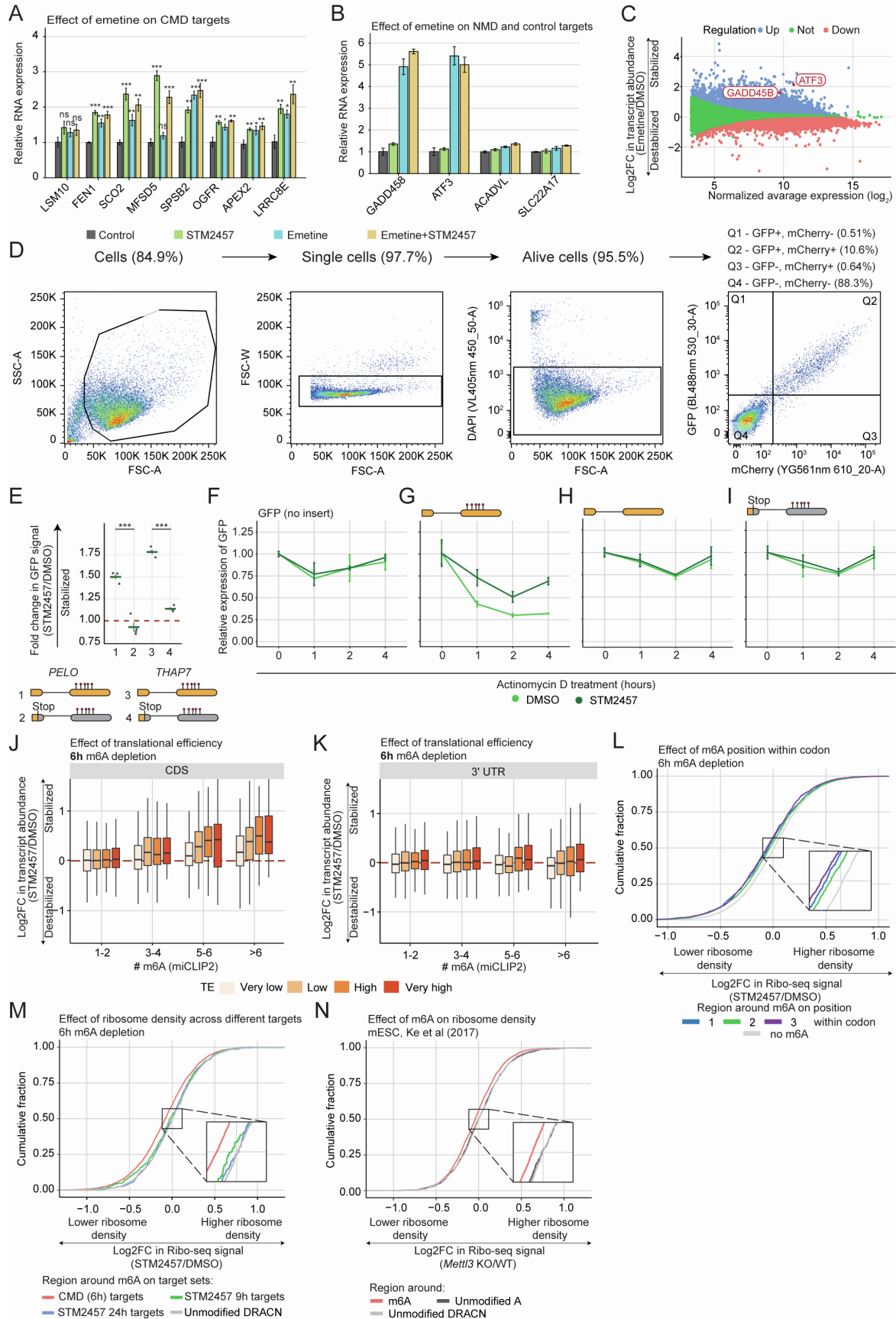


**Figure S4. Translation plays a crucial role in CDS-m6A decay (CMD) (Related to Figure 3A–H).**

**(A)** Quantitative real-time PCR (RT-qPCR) to quantify the relative changes in expression of eight CMD targets after m6A depletion (6 h), inhibition of translation (1.8  $\mu$ M emetine, 2 h), or a combination thereof compared to control (DMSO) in HEK293T cells. Normalized Ct values ( $\Delta$ Ct, normalized to *ACTB* expression) are displayed as mean  $\pm$  standard deviation of mean (s.d.m.). \*  $p$  value  $<0.05$ , \*\*  $<0.01$ , \*\*\*  $<0.001$ , ns, non-significant; two-sided Student's  $t$ -test on normalized Ct values;  $n=3$  biological replicates.

**(B)** As confirmation of emetine activity, RT-qPCR shows the relative changes in expression of two NMD and two control targets after m6A depletion (6 h), inhibition of translation (1.8  $\mu$ M emetine, 2 h), or a combination thereof compared to control (DMSO) in HEK293T cells. Data represented as in A.

**(C)** Scatter plot showing log2FC in transcript abundance upon inhibition of translation (1.8  $\mu$ M emetine, 2 h). Two known targets of nonsense-mediated mRNA decay (NMD) are highlighted.

**(D)** Gating strategy of exemplary sample (m6A mutant without intron in DMSO, replicate 2) for flow cytometry. Briefly, cell population was always gated out from the debris first (SSC-A vs. FSC-A). From the selected cell population, doublets and aggregates were removed (FSC-W vs. FSC-A). Next, a subset of live cells was selected based on DAPI signal (DAPI vs. FSC-A). Gating scheme for separating GFP and mCherry-positive cells is indicated by reference lines, divided cell populations based on the fluorescent signal: Q1, GFP+, mCherry-; Q2, GFP+, mCherry+; Q3, GFP-, mCherry+; Q4, GFP-, mCherry-. The gating was set to include 99.5% of the cells of the negative controls.

**(E)** Fold changes in GFP levels (normalized to mCherry) between variants of *PELO* and *THAP7* reporters after m6A depletion (16 h). \*  $p$  value  $<0.05$ , \*\*  $<0.01$ , \*\*\*  $<0.001$ , ns, non-significant; two-sided Student's  $t$ -test on fold changes;  $n=4$  biological replicates.

**(F–I)** RT-qPCR to quantify the relative changes in expression of *GFP* mRNA upon transcriptional inhibition (actinomycin D, 5  $\mu$ g/ml, 0–4 h) in control (light green) and m6A-depleted conditions (dark green) for reporter mutant without insert (empty GFP, F), WT reporter (G), m6A site mutant (H), and mutant with stop codon moved upstream of m6A sites (I). Normalized Ct values ( $\Delta$ Ct, normalized to *mCherry* expression) are displayed as mean  $\pm$  s.d.m.;  $n=3$  biological replicates.

**(J, K)** Log2FC in transcript abundance after m6A depletion (6 h). Transcripts are grouped by number of m6A sites (miCLIP2) in CDS (J), and 3' UTR (K) and further stratified based on translational efficiency (TE, color shading) calculated from Ribo-seq and matched RNA-seq data.

**(L)** Log2FC in ribosome densities after m6A depletion in a 101-nt window around m6A for sets of targets identified based on m6A located in position 1 (blue), 2 (green), and 3 (purple) of the codons compared to unmodified DRACN (gray).

**(M)** Log2FC in ribosome densities after m6A depletion in a 101-nt window around m6A for sets of targets identified based on the stabilization after m6A depletion for 6 h (red), 9 h (green), and 24 h (blue) compared to unmodified DRACN (gray).

**(N)** Log2FC in ribosome densities between *Mettl3* KO and WT in mESCs<sup>2</sup> in a 101-nt window around m6A sites (eTAM-seq, red)<sup>4</sup> compared to unmodified A (dark gray) and unmodified DRACN (light gray).



**Figure S5. m6A in ribosomal A-site leads to destabilization of transcripts (Related to Figure 3I–N)**

**(A–C)** Metaprofile of normalized 5' ends of ribosome footprint reads in control (DMSO) cells relative to m6A sites with different stoichiometries (color coded) in positions 1 (A), 2 (B), and 3 (C) of the codon (schematic shown in J).

**(D–F)** Metaprofile of normalized 5' ends of ribosome footprint reads in m6A-depleted cells (6 h) relative to m6A sites with different stoichiometries (color-coded) in positions 1 (D), 2 (E), and 3 (F) of the codons.

**(G–I)** Metaprofile of normalized 5' ends of ribosome footprint reads (Ribo-seq) in control and m6A-depleted cells relative to the unmodified A in DRACN in position 1 (G), 2 (H), and 3 (I) of the codons.

**(J)** Positioning of the m6A site within A-site of the ribosome results in varying positions of the 5' ends of ribosome footprints: –15, –16, or –17 relative to the m6A site, depending on whether it occupies positions 1, 2, or 3 of the codons.

**(K)** Log2FC in abundance after m6A depletion (6 h) for transcripts with m6A sites at positions 1 (blue), 2 (green), and 3 (purple) of the codons.

**(L)** Log2FC in abundance after m6A depletion (6 h) for transcripts divided by number of CDS m6A sites (x-axis) at positions 1 (blue), 2 (green), or 3 (purple) of the codons.

**(M)** Log2FC in GFP signal upon m6A depletion (16 h) of *CGRRF1* variants with all five m6A sites at position 1 (blue), 2 (green), or 3 (purple) of the codons.

**(N)** CDS m6A sites in all codon positions and codon types similarly affect RNA stability, except stop codons. Log2FC in abundance upon m6A depletion for 6 h for transcripts with m6A at positions 1 (blue), 2 (green), and 3 (purple) of the codons, and in stop codons (gray). Consistent with the reduced effect of the second codon position, transcripts with m6A in the codon GAC were least increased.

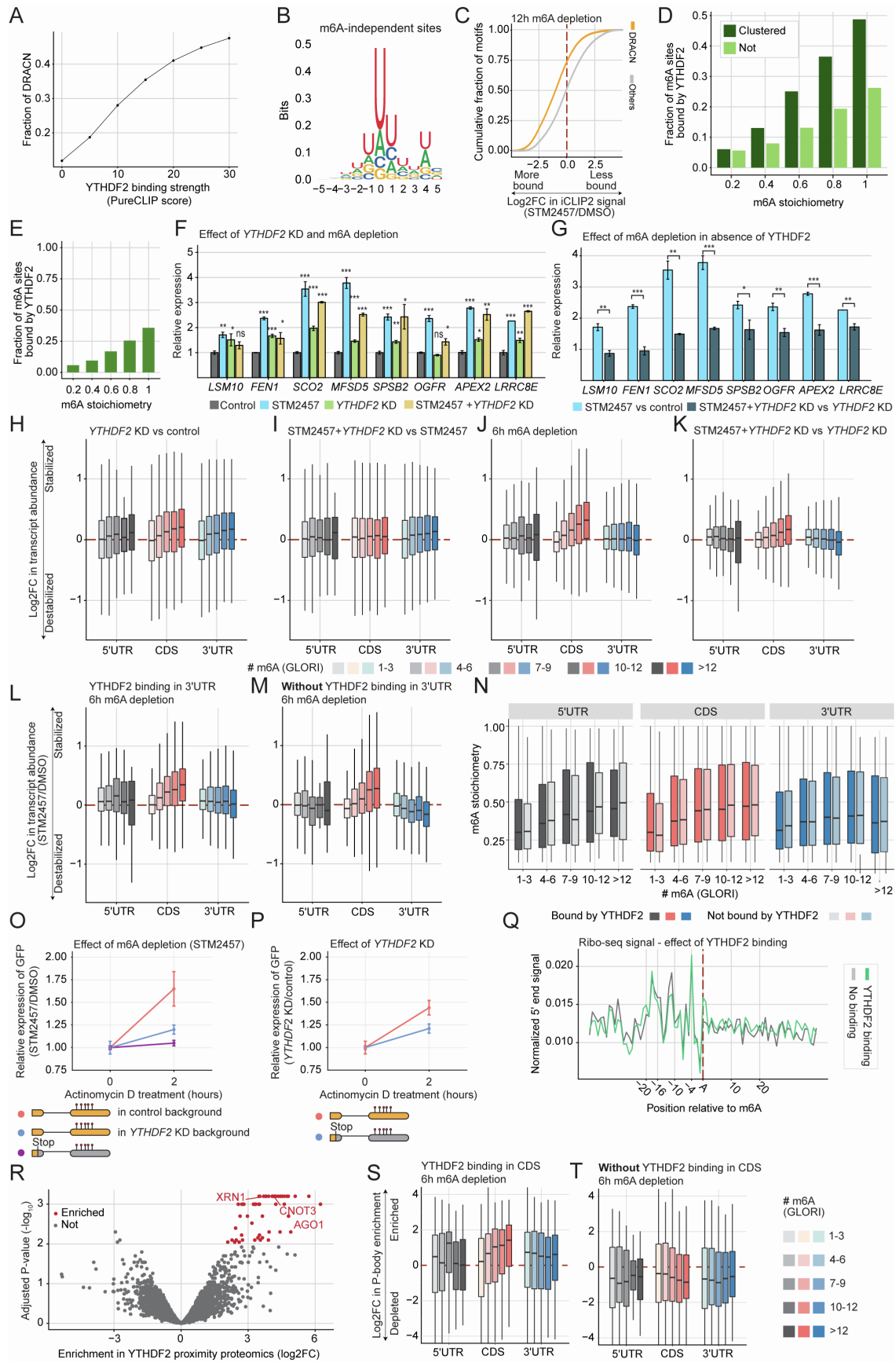


**Figure S6. YTHDF2 contributes to all m6A-mediated RNA decay pathways including CMD (Related to Figure 4).**

**(A)** Scatter plot shows YTHDF2 binding strength relative to fraction of DRACN motif.

**(B)** Sequence logo for m6A-independent YTHDF2 binding sites. Unchanged YTHDF2 peaks after m6A depletion are not located at DRACN.

**(C)** Log2FC of iCLIP2 signal after m6A depletion (12 h) for binding sites at DRACN motifs (orange) and others (gray). YTHDF2 peaks on DRACN showed a global decrease after m6A depletion.

**(D)** YTHDF2 binding to clustered (darker) compared to non-clustered (lighter) m6A sites binned according to stoichiometry. An m6A cluster entails the presence of at least three m6A sites within a 100-nt window, as defined in <sup>1</sup>.

**(E)** YTHDF2 binding scales with m6A stoichiometry (GLORI).

**(F)** RT-qPCR to quantify relative changes in expression of eight CMD targets after m6A depletion (6 h), *YTHDF2* knockdown (KD, 48 h), or a combination thereof compared to control HEK293T cells. Normalized Ct values ( $\Delta$ Ct, normalized to *ACTB* expression) relative to control are displayed as mean  $\pm$  s.d.m.. \*  $p$  value <0.05, \*\* <0.01, \*\*\* <0.001, ns, non-significant; two-sided Student's  $t$ -test on  $\Delta$ normalized Ct value;  $n=3$  biological replicates.

**(G)** RT-qPCR to quantify relative changes in expression upon m6A depletion (STM2457, 6 h) in *YTHDF2*-depleted and control cells. Data represented as in F.

**(H–K)** Log2FC in transcript abundance upon *YTHDF2* KD (H), *YTHDF2* KD in m6A-depleted cells (6 h; I), m6A depletion (6 h; J), and m6A depletion (6 h) in *YTHDF2*-depleted cells (K). Transcripts are grouped by number of m6A sites (GLORI) in each transcript region.

**(L, M)** Log2FC in transcript abundance after m6A depletion (6 h) for transcripts with (L) or without (M) YTHDF2 binding in 3' UTR, grouped by number of m6A sites (GLORI) in each transcript region.

**(N)** Stoichiometry of YTHDF2-bound (darker) and unbound (lighter) m6A sites (GLORI) in different transcript regions.

**(O)** RT-qPCR to quantify the relative changes in expression of *GFP* mRNA upon m6A depletion (16 h) after transcriptional inhibition (actinomycin D, 5  $\mu$ g/ml, 0–2 h) for WT reporter (salmon), WT reporter in *YTHDF2* KD conditions (purple), and moved stop mutant (blue). Fold changes of normalized Ct values ( $\Delta$ Ct, normalized to *mCherry* mRNA) are displayed as mean  $\pm$  s.d.m.;  $n=3$  biological replicates.

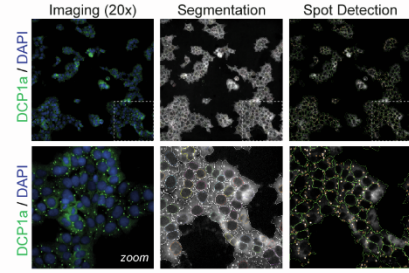
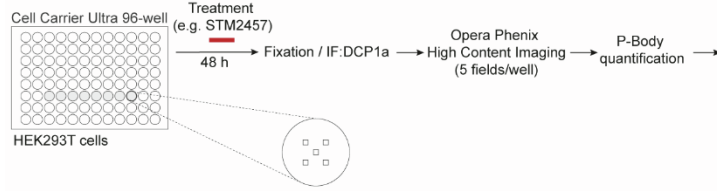
**(P)** RT-qPCR to quantify the relative changes in expression of *GFP* upon *YTHDF2* KD (48 h) after transcriptional inhibition (actinomycin D, 5  $\mu$ g/ml, 0–2 h) for WT reporter (salmon) and moved stop mutant (blue). Data represented as in O.

**(Q)** Metaprofile of 5' ends of ribosome footprint reads in control cells relative to m6A site with (green) and without (gray) YTHDF2 binding.

**(R)** Volcano plot showing log2FC in protein enrichment in YTHDF2 proximity proteome against adjusted  $p$  values (Benjamini–Hochberg correction). Proteins with  $p$  values <0.05 are colored. XRN1, CNOT3 and AGO1 are highlighted as exemplary P-body proteins.

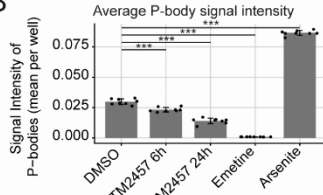
**(S, T)** Log2FC in P-body enrichment after m6A depletion (6 h) for transcripts with (T) or without (U) YTHDF2 binding in CDS, grouped by number of m6A sites (GLORI) in each transcript region.

A

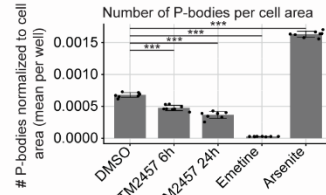


Harmony Software (v5.2) - Spot Analysis Pipeline

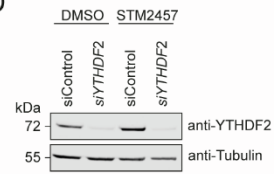
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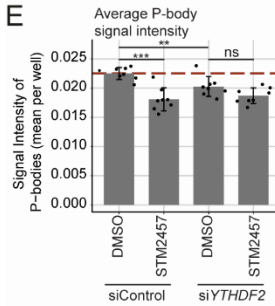
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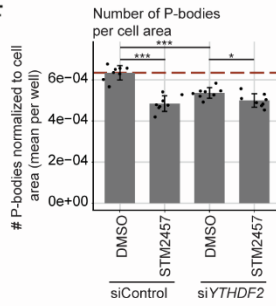
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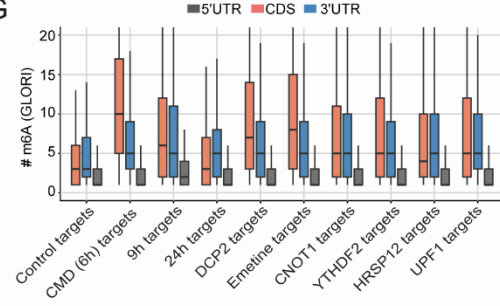
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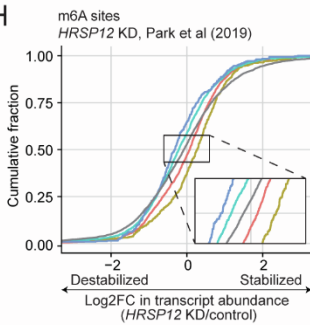
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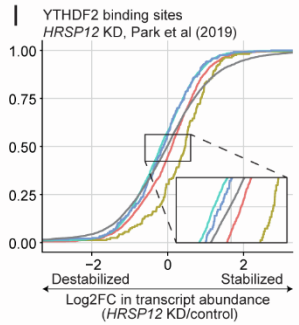
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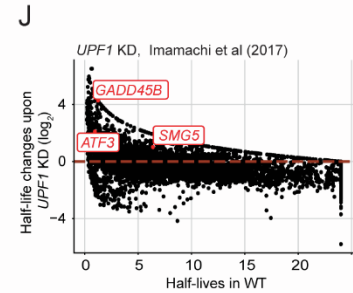
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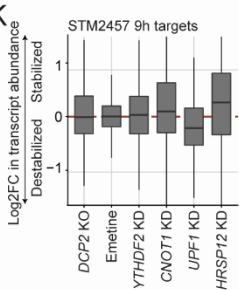
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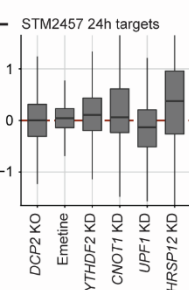
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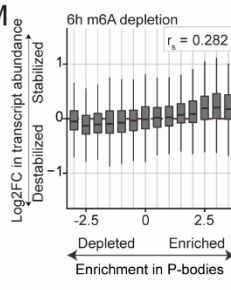
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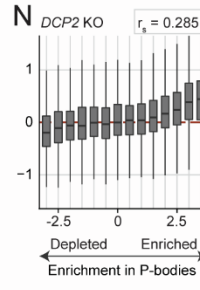
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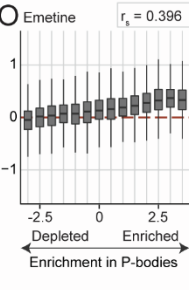
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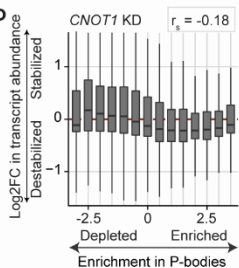
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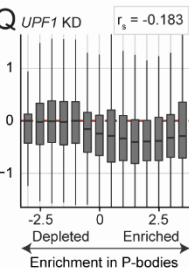
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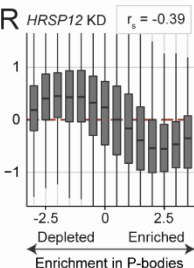


**Figure S7. CMD targets localize to P-bodies and are degraded through a pathway distinct from any previously known mechanisms (Related to Figures 5 and 6).**

Immunofluorescent staining of DCP1a was performed to visualize P-bodies, and the Opera Phenix High Content Imaging system was used to analyze P-body numbers and signal intensities.

**(A)** Experimental setup and exemplary representation of the image analysis pipeline, including segmentation and spot detection, using the DCP1a signals.

**(B, C)** Average signal intensities for detected P-bodies (B) and average numbers of P-bodies normalized to the area of each cell (C) are displayed as mean  $\pm$  s.d.m.. \*  $p$  value  $<0.05$ , \*\*  $<0.01$ , \*\*\*  $<0.001$ , ns, non-significant; two-sided Student's  $t$ -test;  $n=8$  biological replicates.

**(D)** Western blot for YTHDF2 and tubulin, validating the YTHDF2 knockdown on the protein level.

**(E, F)** Average signal intensities for detected P-bodies (E) and average numbers of P-bodies normalized to the area of each cell (F). Data represented as in B and C.

**(G)** Number of m6A sites (GLORI) in transcript regions for sets of targets identified based on the stabilization upon the corresponding treatment.

**(H, I)** Log2FC in transcript abundance upon *HRSP12* KD<sup>5</sup> for transcripts with m6A sites (H) and with m6A sites bound by YTHDF2 (I) in CDS and 3' UTR, with or without GGUUC motif in the vicinity.

**(J)** Scatter plot showing log2FC in half-lives upon *UPF1* KD against half-lives under control conditions (WT).<sup>6</sup> Three known targets of UPF1-mediated nonsense-mediated mRNA decay (NMD) are highlighted.

**(K, L)** Log2FC in transcript abundance upon RNA-binding protein KDs and inhibition of translation (emetine) for 9 h targets (K), and 24 h targets (L).

**(M–R)** Distribution of log2FC in transcript abundance upon m6A depletion (6 h, M), *DCP2* KO (N)<sup>7</sup>, translational inhibition (O),<sup>8</sup> *CNOT1* KD (P),<sup>9</sup> *UPF1* KD (Q),<sup>6</sup> and *HRSP12* KD (R),<sup>5</sup> for transcripts binned by their log2FC in P-body enrichment. Values on the x-axis refer to the lower boundary. Spearman's rank correlation coefficients ( $r_s$ ) are shown.

## Supplementary Tables

**Table S3. Selected CMD targets for real-time qPCR validation (Related to Figures 3B, S4A, B, and S7F, G).**

For each selected CMD target, the gene name and gene ID are given next to the log<sub>2</sub>-transformed fold change upon 6 h of m6A depletion (STM2457), the number of m6A sites in the CDS and the number of m6A sites in the 3' UTR (GLORI).<sup>1</sup> Real-time qPCR was used to evaluate the effect of m6A depletion, translational inhibition (emetine) or *YTHDF2* KD.

| Ensembl gene ID | Gene name     | Log2FC upon 6 h STM2457 | # CDS m6A | # 3' UTR m6A |
|-----------------|---------------|-------------------------|-----------|--------------|
| ENSG00000181817 | <i>LSM10</i>  | 0,50                    | 8         | 1            |
| ENSG00000168496 | <i>FEN1</i>   | 0,91                    | 13        | 9            |
| ENSG00000284194 | <i>SCO2</i>   | 1,43                    | 13        | 0            |
| ENSG00000182544 | <i>MFSD5</i>  | 1,34                    | 23        | 9            |
| ENSG00000111671 | <i>SPSB2</i>  | 1,08                    | 8         | 1            |
| ENSG00000060491 | <i>OGFR</i>   | 0,85                    | 14        | 2            |
| ENSG00000169188 | <i>APEX2</i>  | 0,90                    | 10        | 6            |
| ENSG00000171017 | <i>LRRC8E</i> | 1,18                    | 27        | 3            |

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## **2.2 Publication 2: m6A in the coding sequence: linking deposition, translation, and decay**

### **2.2.1 Summary**

*N*6-methyladenosine (m6A) within coding sequences (CDS) has emerged as a distinct regulatory mark that couples translation to mRNA decay. In this review, we outline how CDS–m6A is deposited in a site-specific manner and how exon junction complexes restrict its positioning near exon-exon boundaries. We discuss evidence showing that ribosomes slow down at m6A-modified codons, which promotes the recruitment of reader proteins such as YTHDF2 and triggers transcript degradation. This decay mechanism, observed across multiple systems, operates during active translation and leads to P-body enrichment of target RNAs. CDS–m6A predominantly affects transcripts involved in development, stress response, and gene regulation, suggesting a role in fine-tuning expression under dynamic cellular conditions.

### **2.2.2 Zusammenfassung**

*N*6-Methyladenosin (m6A) in codierenden Sequenzen (CDS) hat sich als eine eigenständige regulatorische Markierung etabliert, die Translation mit mRNA-Abbau koppelt. In diesem Übersichtsartikel beschreiben wir, wie CDS–m6A ortsspezifisch eingebaut wird und wie Exon-Junction-Komplexe dessen Positionierung in der Nähe von Exon-Exonat

-Grenzen begrenzen. Wir diskutieren Hinweise darauf, dass Ribosomen an m6A-modifizierten Codons langsamer elongieren, was die Rekrutierung von Leserproteinen wie YTHDF2 fördert und den Abbau der Transkripte auslöst. Dieser Abbauprozess, der in verschiedenen Systemen beobachtet wurde, findet während der aktiven Translation statt und führt zur Anreicherung der Zieltranskripte in P-Bodies. Betroffen sind vor allem Transkripte, die an Entwicklung, Stressantwort und Genregulation beteiligt sind, was auf eine Rolle von CDS–m6A bei der Feinabstimmung der Genexpression unter dynamischen zellulären Bedingungen hinweist.

### **2.2.3 Statement of contribution**

As the first author, I led the conception, organization, and writing of the review. I conducted the literature search, drafted the manuscript, and prepared the figures. The co-

authors contributed through critical discussions, suggestions for content, and revisions of the text. I coordinated the integration of feedback and finalization of the manuscript.

Supervisor confirmation

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## Review

m<sup>6</sup>A in the coding sequence: linking deposition, translation, and decayMiona Ćorović <sup>1</sup>, Peter Hoch-Kraft<sup>1</sup>, You Zhou<sup>2</sup>, Sarah Hallstein<sup>1</sup>, Julian König <sup>1,2</sup>, and Kathi Zarnack <sup>2,\*</sup>

**N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) is the most abundant internal modification in mRNA and plays a crucial role in regulating mRNA turnover. This review explores the characteristics of m<sup>6</sup>A sites in the coding sequence (CDS) and their influence on mRNA decay, with a focus on the newly discovered CDS–m<sup>6</sup>A decay (CMD) pathway – a translation-dependent mechanism that promotes rapid and efficient degradation. Further, we examine how splicing-associated factors influence m<sup>6</sup>A deposition and discuss the enrichment of CMD targets in processing bodies (P-bodies). We emphasize the interplay between m<sup>6</sup>A modification and decay, and propose that targeting the CMD pathway could offer novel therapeutic strategies for diseases such as cancer and metabolic disorders.**

m<sup>6</sup>A influences RNA turnover

RNA begins its life with transcription and ends with degradation, and undergoes multiple processing steps during its maturation. This dynamic progression, collectively known as RNA turnover, controls the lifespan of RNA molecules and ensures that transcripts are available when needed and degraded when they are no longer required. By modulating RNA stability and degradation, RNA turnover enables cells to adapt to environmental and developmental cues.

A key player in this regulatory process are **RNA modifications** (see [Glossary](#)), of which m<sup>6</sup>A is the most abundant internal modification in mRNA. By influencing crucial RNA processing events – including splicing, nuclear export, and most notably mRNA stability – m<sup>6</sup>A ultimately shapes the lifespan and functional availability of mRNAs [1]. Although m<sup>6</sup>A sites are distributed throughout mRNAs, emerging evidence suggests that their impact is highly context-dependent. In particular, m<sup>6</sup>A modifications within the **coding sequence (CDS)** have been identified as potent signals for rapid mRNA degradation [2]. In this review we explore the timing of m<sup>6</sup>A deposition and the unique characteristics of CDS m<sup>6</sup>A sites, and highlight their role in driving mRNA decay.

m<sup>6</sup>A deposition: a multi-layered process tied to transcription and splicing

m<sup>6</sup>A is a sequence-specific modification that primarily occurs within the DRACN consensus motif (D = A, G, or U; R = A or G; N = any nucleotide) [3,4]. Its deposition is catalyzed by the m<sup>6</sup>A writer complex where METTL3 acts as the active methyltransferase. METTL14, although catalytically inactive, stabilizes METTL3 and facilitates RNA binding [5,6]. Additional components, including WTAP, VIRMA, HAKAI, ZC3H13, and RBM15/RBM15B, regulate the stability, localization, substrate specificity, and catalytic efficiency of the complex [7]. Once placed, the m<sup>6</sup>A site in the RNA is recognized by reader proteins, including YTH domain-containing proteins such as YTHDF1–3 and YTHDC1, which mediate diverse regulatory outcomes.

The exon-junction complex restricts m<sup>6</sup>A deposition in the CDS

Accurate mapping of m<sup>6</sup>A is essential for understanding its biological functions. Early methods such as methylated RNA immunoprecipitation sequencing (MeRIP-seq) and m<sup>6</sup>A individual

## Highlights

The exon-junction complex (EJC) restricts METTL3 activity in the coding sequence (CDS), and thereby shapes the N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) landscape.

CDS m<sup>6</sup>A modifications trigger rapid, translation-dependent mRNA degradation via CDS–m<sup>6</sup>A decay (CMD).

Target transcripts for CMD are enriched in processing bodies (P-bodies).

The intricate link between m<sup>6</sup>A deposition, translation, and decay enables efficient gene expression regulation.

CMD modulates the expression of physiologically relevant genes in healthy conditions and in disease, particularly in cancer.

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nucleotide resolution crosslinking and immunoprecipitation (miCLIP) employed antibody-based enrichment to profile m<sup>6</sup>A sites transcriptome-wide [3,8,9]. More recently, advanced techniques – including glyoxal and nitrite-mediated deamination of unmethylated adenosines (GLORI), evolved TadA-assisted m<sup>6</sup>A sequencing (eTAM-seq), and nanopore direct RNA sequencing – have provided enhanced resolution and enabled the quantification of **m<sup>6</sup>A stoichiometry** [10–15].

Intriguingly, transcriptome-wide mapping showed that m<sup>6</sup>A is not randomly distributed across mRNAs and is preferentially enriched in long internal exons and near the stop codon [8,16–19]. This pattern cannot be attributed solely to the frequency of DRACN motifs nor to the specificity of the m<sup>6</sup>A writer complex because *in vitro* methylation works equally well for m<sup>6</sup>A sites from all transcript regions [2]. This implies that additional factors shape the m<sup>6</sup>A patterns observed *in vivo*. Indeed, three independent studies reported that the exclusion of m<sup>6</sup>A from regions near to splice sites is mediated by the **exon-junction complex (EJC)** which protects proximal DRACN motifs from methylation by the m<sup>6</sup>A writer complex (Figure 1) [16–18]. They propose that m<sup>6</sup>A deposition follows an exclusion-based model in which METTL3 methylates DRACN motifs by default unless inhibited [20]. Supporting this, Luo *et al.* utilized a deep learning model for m<sup>6</sup>A site prediction and *in silico* mutational modeling to show that the exon–intron boundary specifically inhibits m<sup>6</sup>A deposition on adjacent exons, a phenomenon that is partially explained by presence of EJCs [19].

Although compelling evidence argues that EJCs influence m<sup>6</sup>A deposition, other findings suggest that m<sup>6</sup>A can be added before splicing and may even play a role in regulating **alternative splicing** (see **Outstanding questions**). For instance, Ke *et al.* [21] reported that nearly all m<sup>6</sup>A deposition occurs on pre-mRNA during transcription and mRNA processing, often before splicing takes place. They argue that m<sup>6</sup>A does not directly regulate splicing. By contrast, Louloui *et al.* [22] provided evidence that m<sup>6</sup>A plays a role in splicing regulation and showed that it is enriched in introns associated with alternative splicing. Consistently, the m<sup>6</sup>A reader protein YTHDC1 has emerged as a central regulator of splicing [23–25]. In addition, the m<sup>6</sup>A reader HNRNPG has been reported to modulate alternative splicing through interactions with RNA polymerase II (RNAP II) [26]. Beyond these mechanisms, m<sup>6</sup>A may represent an ancient mode of splicing regulation. Mendel *et al.* [27] demonstrated that m<sup>6</sup>A deposition at the 3' splice site – catalyzed by the alternative methyltransferase METTL16 – prevents U2AF1 binding in *Caenorhabditis elegans*, thereby inhibiting splicing.

The interplay between m<sup>6</sup>A deposition and splicing presents an apparent paradox: although m<sup>6</sup>A has been implicated in splicing regulation, its deposition can also be influenced by the EJC which is recruited only after splicing has been completed. This raises important questions about the timing and sequence of events in mRNA processing. Specifically, does m<sup>6</sup>A mark transcripts co- or post-transcriptionally, and is it predominantly added before or after splicing?

#### m<sup>6</sup>A deposition occurs both co- and post-transcriptionally

Emerging evidence indicates that m<sup>6</sup>A is deposited cotranscriptionally. Notably, one study investigating the influence of the EJC on m<sup>6</sup>A deposition addressed the apparent discrepancy in deposition timing. By analyzing m<sup>6</sup>A levels in chromatin-associated RNA and polyadenylated mRNA, the authors suggest that m<sup>6</sup>A is initially deposited cotranscriptionally at low stoichiometry and relatively evenly across the transcript. This is followed by locally restricted METTL3/14 activity, guided by the EJC, which shapes the characteristic m<sup>6</sup>A pattern observed in mature transcripts [16]. To enhance the resolution, Tang *et al.* [28] developed two methods – 4sU-m<sup>6</sup>A-LAIC-seq [4-thiouridine (4sU)-coupled m<sup>6</sup>A-level and isoform-characterization sequencing] and

#### Glossary

**Alternative splicing:** a regulatory mechanism during RNA processing in which different combinations of exons are joined to produce multiple transcript variants from a single gene to increase proteomic diversity.

**Coding sequence (CDS):** the region of an mRNA that is translated into protein; the CDS is flanked by untranslated regions (UTRs) at both the 5' and 3' ends.

**Exon-junction complex (EJC):** a multiprotein complex that is deposited upstream of exon–exon junctions during splicing and which influences mRNA export, translation, and surveillance.

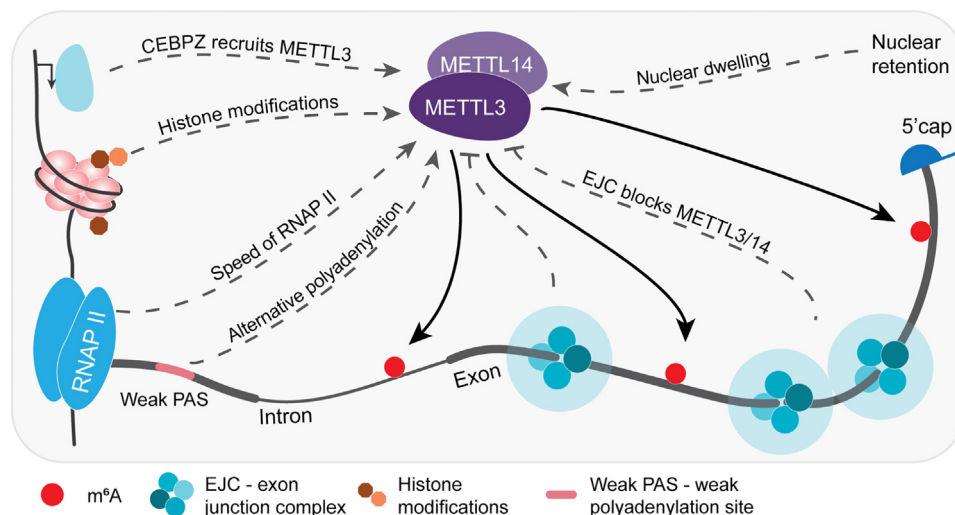
**m<sup>6</sup>A stoichiometry:** defined either as the fraction of transcripts that are methylated at a specific N6-methyladenosine (m<sup>6</sup>A) site (site-specific) or as the average level of m<sup>6</sup>A modification across all sites within a transcript (transcript-wide).

**Oncogene:** a gene that has the potential to cause cancer; when activated by mutation or overexpression it can drive uncontrolled cell proliferation and tumorigenesis.

**Phase separation:** a biophysical process in which biomolecules demix into distinct compartments that lack membrane boundaries, and organize cellular components such as RNA and proteins into dynamic condensates (e.g., stress granules and processing bodies, P-bodies).

**Ribosomal aminoacyl (A)-site:** one of three key sites in the ribosome during translation. Incoming aminoacyl-tRNAs are positioned at the A-site to decode mRNA codons.

**RNA modifications:** covalent chemical changes to RNA molecules that do not alter the underlying sequence but can modulate RNA fate and function. These modifications, including m<sup>6</sup>A, carry epitranscriptomic information that is analogous to epigenetic marks on DNA.



Trends in Genetics

**Figure 1. Regulatory layers controlling N6-methyladenosine (m<sup>6</sup>A) deposition.** Schematic overview of factors that regulate m<sup>6</sup>A deposition: CEBPZ (a transcription start site-binding protein), histone modifications, sequential polyadenylation, nuclear retention, and reduced RNA polymerase II (RNAP II) elongation speed promote METTL3/14 recruitment to specific transcript regions [31,32,34–37]. By contrast, the exon-junction complex (EJC) blocks METTL3/14 access to mRNA, thereby restricting m<sup>6</sup>A deposition [16–19].

4sU-GLORI – that enable the quantification of m<sup>6</sup>A levels in both nascent and steady-state RNAs. Their findings indicate that m<sup>6</sup>A deposition can occur both co- and post-transcriptionally, although a significant portion of the methylation takes place post-transcriptionally. Interestingly, they also observed that nuclear retention enhances m<sup>6</sup>A modification, once again supporting the notion that m<sup>6</sup>A deposition occurs provided that mRNA remains accessible to the writer machinery in the nucleus [29].

Recently, Sethi *et al.* developed dFORCE (direct fractionated observation of RNA coupled to elongation), a single-molecule direct pre-mRNA sequencing technique that can simultaneously detect m<sup>6</sup>A modifications and pre-mRNA processing status [30]. By analyzing cotranscriptional, post-transcriptional, and catalytic intermediate RNA fractions, they show that m<sup>6</sup>A deposition begins during transcription, continues post-transcriptionally, and completes before splicing. Interestingly, depletion of m<sup>6</sup>A around exon–exon junctions is already evident before splicing, suggesting that factors other than the EJC restrict methylation at these sites.

Several studies have also reported that m<sup>6</sup>A deposition is influenced by RNAP II itself. For instance, it was shown that transcription elongation kinetics directly influence the efficiency of m<sup>6</sup>A deposition: slowed RNAP II movement creates an opportunity for METTL3/METTL14 binding to nascent RNA, thereby enhancing m<sup>6</sup>A levels [31,32]. Wang *et al.* [31] further demonstrated that RNAP II pausing reduces secondary structure formation in nascent transcripts, and thus exposes consensus motifs for methylation. Beyond regulation by transcription speed, the m<sup>6</sup>A writer complex can modulate RNAP II activity. In *Drosophila*, Mettl3 promotes RNAP II pause release at promoter-proximal regions, establishing a feedforward loop that amplifies transcriptional output [33]. This bidirectional crosstalk positions m<sup>6</sup>A as both a sensor and effector of transcriptional states.

The link between the m<sup>6</sup>A machinery and transcription also extends to chromatin-associated factors. METTL3 is recruited to the transcription start sites of **oncogene** loci in acute myeloid leukemia (AML) through interactions with the DNA-binding protein CEBPZ. Notably, the majority of m<sup>6</sup>A modifications resulting from this interaction occur within the CDS of the associated transcripts [34]. In addition, METTL14 recognizes histone H3 lysine 36 trimethylation (H3K36me3), a marker of transcriptionally active chromatin, to guide m<sup>6</sup>A deposition near to stop codons [35]. This process is complemented by the preference of METTL3 for superenhancers and broad H3K4me3 domains, which further links transcriptional activity to elevated m<sup>6</sup>A levels and reduced transcript stability [36]. In addition, METTL3 and METTL14 interact with other histone marks, such as histone H3 lysine 27 acetylation (H3K27ac) and H3K9me3, highlighting their distinct chromatin-binding preferences and roles in regulating gene expression [37].

Taken together, recent methodological advances have significantly deepened our understanding of m<sup>6</sup>A deposition, revealing a complex and dynamic landscape shaped by multiple layers of regulation. These studies collectively demonstrate that m<sup>6</sup>A is not confined to a single point in RNA processing, and can occur both co- and post-transcriptionally. However, key questions remain. What governs the precise timing of deposition? Is it consistent across different m<sup>6</sup>A sites or is it shaped by context-specific features? Continued exploration will be essential to resolve these open questions.

### m<sup>6</sup>A sites in the CDS trigger efficient translation-dependent decay

Although the exact timing of m<sup>6</sup>A deposition remains debated, a key emerging concept is that m<sup>6</sup>A modifications in different regions of an mRNA influence its stability in distinct ways. In the cytoplasm, m<sup>6</sup>A is primarily recognized by the YTHDF family of reader proteins. Although YTHDF2 has been the most extensively studied and is known to initiate various decay pathways (Figure 2) [38], recent studies revealed that YTHDF1 and YTHDF3 also bind to m<sup>6</sup>A-marked transcripts in the cytoplasm and function redundantly in promoting mRNA degradation [39–40]. Previously characterized m<sup>6</sup>A-mediated degradation mechanisms include deadenylation (via interaction with the CCR4–NOT complex), decapping (through UPF1), and endoribonucleolytic cleavage (mediated by HRSP12 encoded by *RIDA*) [41–43]. Our recent study identified an additional faster and more potent degradation pathway that specifically targets m<sup>6</sup>A sites within the CDS [2]. We demonstrated that m<sup>6</sup>A sites across all regions promote decay, but CDS m<sup>6</sup>A sites do so with higher efficiency. Consequently, only transcripts with CDS m<sup>6</sup>A sites are immediately stabilized upon acute m<sup>6</sup>A depletion, whereas those with m<sup>6</sup>A elsewhere respond only upon prolonged m<sup>6</sup>A depletion. Notably, the magnitude of transcript stabilization upon acute depletion correlates with the transcript-wide m<sup>6</sup>A stoichiometry in the CDS. By contrast, even transcripts with the highest levels of methylation in the 5′ or 3′ untranslated regions (UTRs) remain unaffected in the acute setting. Nonetheless, the potential modulatory role of UTR sites on CMD remains to be explored.

The newly identified pathway – termed CDS-m<sup>6</sup>A decay (CMD) – relies on active translation, whereby transcripts with higher translation efficiency undergo more degradation. Consequently, inhibition of translation stabilizes CDS m<sup>6</sup>A-containing transcripts in a similar manner to m<sup>6</sup>A depletion. Mechanistically, CDS m<sup>6</sup>A sites influence ribosome dynamics – we demonstrated that translation pauses when an m<sup>6</sup>A-modified codon is positioned in the **ribosomal aminoacyl (A)-site**, indicating impaired decoding [2].

Structural studies have provided important insight into how the m<sup>6</sup>A modification affects translation. X-ray crystallography of *Thermus thermophilus* 30S ribosomal subunits showed that m<sup>6</sup>A forms Watson–Crick base pairs with U in the A-site, although the 6-methyl group was unresolved,

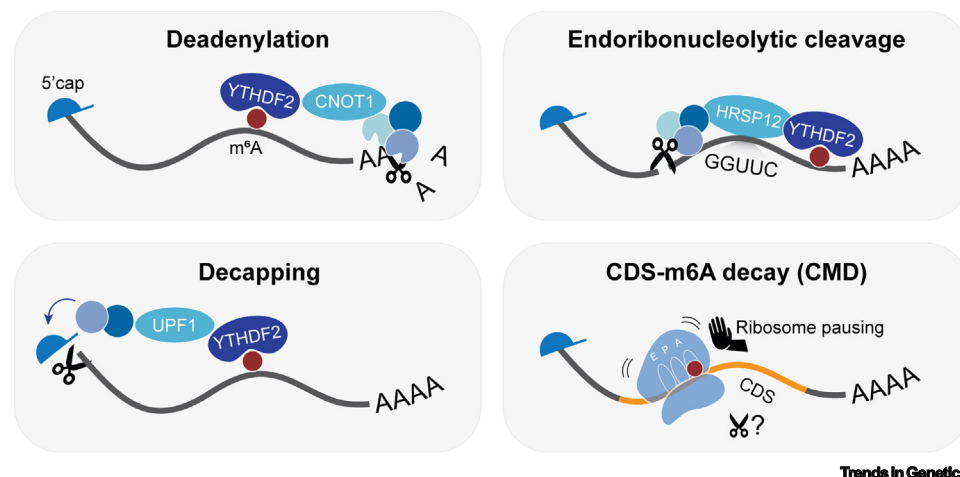


**Figure 2.** Overview of *N*<sup>6</sup>-methyladenosine (*m*<sup>6</sup>A) decay pathways. *m*<sup>6</sup>A acts as a destabilizing mark on mRNA. Upon recognition by the *m*<sup>6</sup>A reader YTHDF2, different decay pathways are initiated depending on its interacting partners [38]. Interaction with CNOT1 recruits the CCR4–NOT complex to promote deadenylation [41]. Association with UPF1 triggers decapping [42], whereas interaction with HRSP12 leads to endoribonucleolytic cleavage which requires the RNA sequence motif GGUUC for HRSP12 binding [43]. In a recently described pathway, termed CDS–*m*<sup>6</sup>A decay (CMD), ribosomes pausing at *m*<sup>6</sup>A sites within the coding sequence (CDS) initiate decay. However, the precise downstream degradation mechanisms involved in CMD remain to be elucidated [2]. Abbreviations: A, P, and E, the aminoacyl, peptidyl, and exit tRNA-binding sites of the ribosome, respectively.

probably due to rotational flexibility [44]. In *Escherichia coli*, *m*<sup>6</sup>A has been found to promote dynamic conformations that are rejected by the ribosome and increases tRNA drop-off [45], and this provides a possible mechanistic explanation for CMD-related ribosome pausing.

Two recently published studies by Murakami *et al.* and Linder *et al.* have further advanced our understanding of CDS *m*<sup>6</sup>A-dependent mRNA decay [46,47]. Murakami *et al.* applied ribosome profiling together with nascent RNA sequencing to dissect the role of translation in *m*<sup>6</sup>A-mediated turnover. They demonstrated that *m*<sup>6</sup>A within A-site codons induces ribosomal stalling and provokes ribosome collisions characterized by a unique conformational signature. This in turn recruits YTHDF proteins and triggers transcript decay. In parallel, Linder *et al.* uncovered a pan-epitranscriptomic interaction between CDS *m*<sup>6</sup>A and the 5-methoxycarbonylmethyl-2-thiouridine (mcm<sup>5</sup>s<sup>2</sup>U) modification on the corresponding tRNA. They found that ribosome pausing is especially pronounced at specific codons, and that mcm<sup>5</sup>s<sup>2</sup>U enhances the decoding of *m*<sup>6</sup>A-modified codons. In its absence – such as in some cancers – ribosome collisions and associated decay are markedly increased. Taken together, these findings support a model in which *m*<sup>6</sup>A deposition within the CDS acts as a regulatory switch that couples translation dynamics to selective mRNA decay, where ribosome stalling, codon context, and tRNA modification status jointly determine the susceptibility of transcripts to CMD (Figure 3).

#### Multiple RNA decay pathways recognize unprocessive ribosomes

Changes in translation dynamics, including ribosome pausing or acceleration, are recognized by specific surveillance systems (Figure 4). Three major pathways that monitor aberrant translation are no-go mRNA decay (NGD), non-stop mRNA decay (NSD), and nonsense-mediated mRNA decay (NMD) [48]. NGD is triggered by ribosome stalling due to structural barriers, GC-rich sequences, or damaged RNA bases [49–53], whereas NSD targets mRNAs lacking a stop

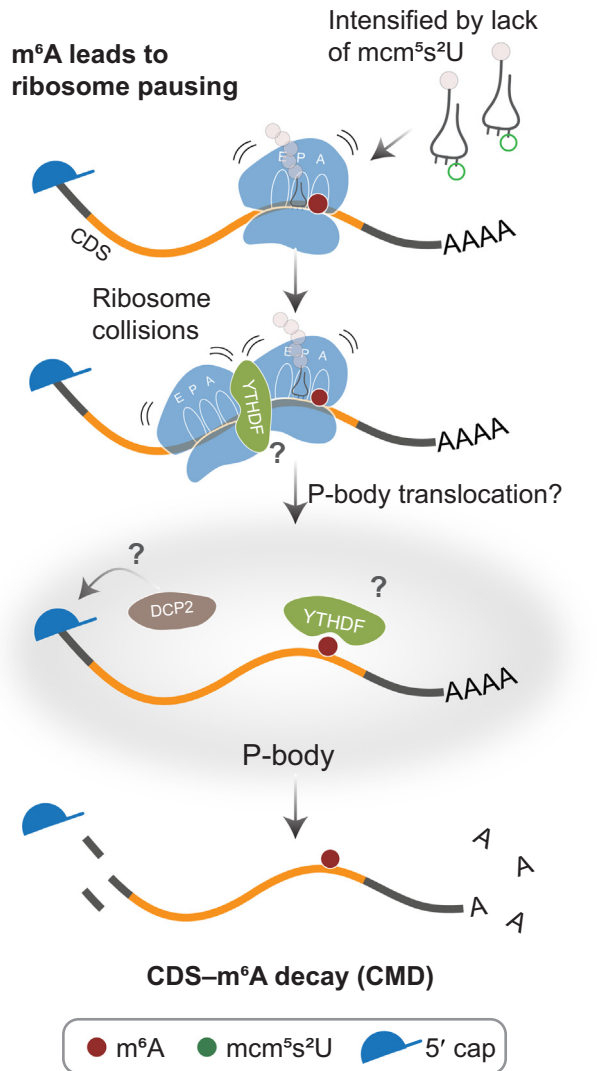
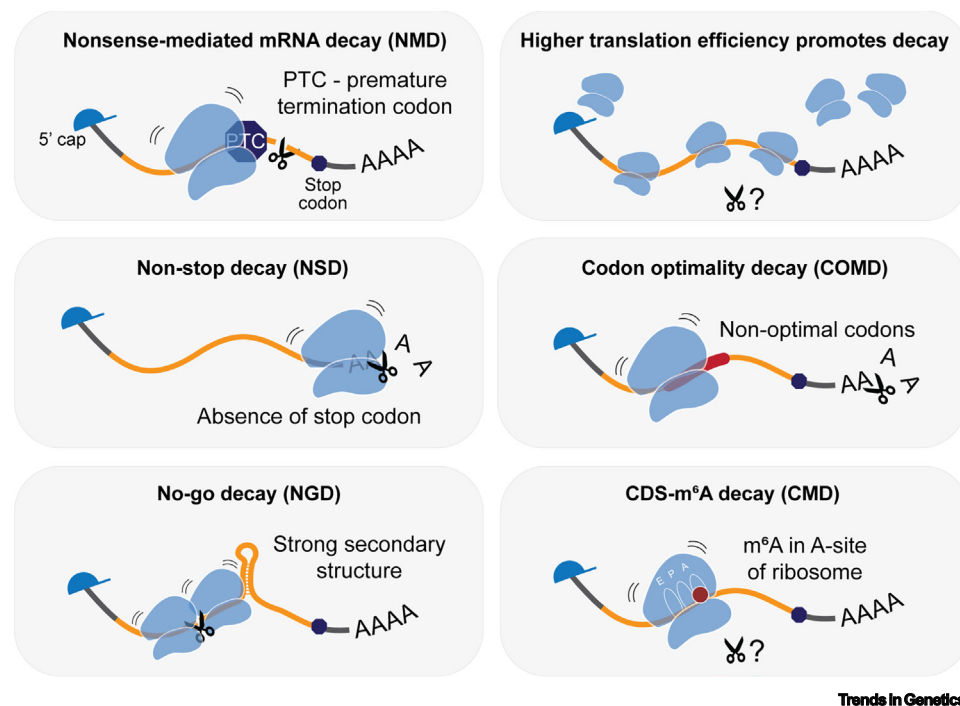


**Figure 3. Proposed mechanisms and open questions in coding sequence (CDS)-N6-methyladenosine (m<sup>6</sup>A)-dependent mRNA decay (CMD).** m<sup>6</sup>A modification within ribosomal aminoacyl (A)-site codons slows down ribosome decoding, which may result in ribosome pausing or stalling. This impaired translation can trigger downstream decay via multiple mechanisms that remain unresolved. YTHDF proteins may be recruited to stalled ribosomes or bind downstream, and either promote decay directly or via translocation to processing bodies (P-bodies). Ribosome collisions – potentially intensified by loss of tRNA modifications such as 5-methoxycarbonylmethyl-2-thiouridine (mcm<sup>5</sup>s<sup>2</sup>U) – amplify decay signals. It remains unclear whether degradation occurs at the site of pausing, within P-bodies, or both, and whether decapping enzymes such as DCP2 play a role in this process [2,46,47]. Abbreviations: A, P, and E, the aminoacyl, peptidyl, and exit tRNA-binding sites of the ribosome, respectively.

codon, leading to ribosome stalling at poly(A) sequences. The third pathway, NMD, recognizes premature termination codons and degrades faulty mRNAs [48]. Distinct mechanisms ensure mRNA decay in all pathways. In yeast NGD, the Dom34-Hbs1 complex recognizes stalled ribosomes and promotes subunit dissociation, thereby allowing the endonuclease Cue2 to cleave mRNA at the A-site, after which the exosome and Xrn1 degrade the resulting fragments. The mechanism is less well defined in humans, but ZNF598 and N4BP2 have been proposed to take over analogous functions [49,54,55]. NSD relies on the protein HBS1 and the SKI complex (including SKIV2L) to recruit the RNA exosome for 3'-to-5' degradation [56,57]. NMD involves phosphorylation of the RNA-binding protein UPF1 that triggers SMG6-mediated cleavage, and the fragments are degraded by exonuclease XRN1 and the exosome [58].

Beyond these pathways, translation-related factors also contribute to mRNA degradation. One such mechanism is codon optimality-mediated decay (COMD) in which stretches of non-



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**Figure 4. Translation-coupled mRNA decay mechanisms.** Schematic overview of mRNA decay pathways that are linked to translation (reviewed in [48]). Nonsense-mediated mRNA decay (NMD) is triggered by premature termination codons (PTCs) located upstream of exon-junction complexes (EJCs) in the coding sequence (CDS), leading to the degradation of faulty transcripts. Non-stop decay (NSD) targets transcripts lacking a stop codon, resulting in ribosome stalling at the poly(A) tail and recruitment of decay factors. No-go decay (NGD) is initiated when ribosomes stall due to strong secondary RNA structures or other obstacles, leading to endonucleolytic cleavage. Codon optimality-mediated decay (COMD) is driven by the presence of non-optimal codons which reduce translation speed and promote mRNA degradation [59]. CDS-N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) decay (CMD) occurs when m<sup>6</sup>A is located in the ribosomal aminoacyl (A)-site within the CDS, causing ribosome pausing and initiating decay [2]. High translation efficiency has also been linked to increased mRNA turnover, although the mechanistic basis remains unclear [60]. Abbreviations: A, P, and E, the aminoacyl, peptidyl, and exit tRNA-binding sites of the ribosome, respectively.

optimal codons slow down decoding. This slowdown triggers mRNA decay via DDX6-mediated decapping, even in the absence of strong ribosome stalling or collisions characteristic of NGD [59]. In addition, a recent study using single-molecule imaging demonstrated that increased ribosome flux correlates with reduced mRNA stability, reinforcing the close connection between translation and decay [60].

Despite similarities between NGD, NSD, NMD, and COMD, each pathway follows distinct molecular routes to ensure mRNA degradation via different regulatory frameworks. Although CDS-m<sup>6</sup>A decay does not seem to directly overlap with these pathways, it is possible that shared molecular features or upstream signals contribute to its function. Exploring these connections will be important to clarify how ribosome pausing translates into selective mRNA decay in CMD.

### CMD targets are enriched in P-bodies

P-bodies are cytoplasmic granules formed via **phase separation** that serve as hubs for translational repression, mRNA storage, and degradation, thereby playing a crucial role in post-transcriptional gene regulation [61]. Although P-bodies have been extensively studied, their exact function in RNA metabolism remains a subject of debate. Early work proposed that these structures function primarily as decay hubs because they are enriched in RNA decay proteins

[62]. However, subsequent studies suggest that visible P-bodies are not essential for RNA decay [63] and that P-bodies serve a dual role in both RNA storage and degradation [64]. More recent evidence has been conflicting, and suggests that P-bodies either function predominantly as storage sites without active decay [65] or are solely dedicated to mRNA decay without allowing mRNA exit [66]. This ongoing debate underscores the need for higher-resolution methodologies to resolve whether P-bodies serve exclusively as storage or decay centers, or both.

Notably, the m<sup>6</sup>A reader YTHDF2 is enriched in P-bodies [39,67,68], and m<sup>6</sup>A has been shown to enhance the ability of YTHDF2 to partition into intracellular phase-separated compartments, including P-bodies [68]. Moreover, mRNAs enriched in P-bodies exhibit higher levels of m<sup>6</sup>A compared to actively translating mRNAs. This process is mediated by the m<sup>6</sup>A reader IGF2BP3, which directs m<sup>6</sup>A-rich transcripts to these granules [69].

Our recent study provides new insights into this relationship. We report that an increasing number of m<sup>6</sup>A sites in the CDS correlates with greater mRNA enrichment in P-bodies, whereas m<sup>6</sup>A sites in the UTRs do not have the same effect. Notably, CMD target transcripts also accumulate in P-bodies, whereas the target transcripts of the other m<sup>6</sup>A-mediated decay pathways do not specifically locate there. Consistently, transcripts with YTHDF2 binding in their CDS are selectively enriched in these granules. Our findings also connect CDS–m<sup>6</sup>A decay to DCP2, a key decapping enzyme enriched in P-bodies, suggesting that the CMD target transcripts may undergo decapping. Moreover, m<sup>6</sup>A depletion leads to a reduction in both the number and intensity of P-bodies, further suggesting a functional link between CDS–m<sup>6</sup>A decay and these granules [2].

Despite these associations, the precise role of P-bodies in CMD remains unclear [2]. Although we observed that transcripts targeted by CMD are enriched in P-bodies, it is unclear whether this localization represents a functional step in decay, a downstream consequence of transcript processing, or transient storage or sequestration. The timing and functional relevance of P-body localization in this context remain to be elucidated. Adding further complexity, Safieddine *et al.* [70] demonstrated that the mRNA content of P-bodies dynamically changes across the cell cycle, often independently from cytoplasmic expression levels, indicative of active recruitment mechanisms. Moreover, RNA profiles in P-bodies shift during human embryonic stem cell differentiation [71]. This plasticity raises the question whether m<sup>6</sup>A plays a role in shaping these differences by influencing which transcripts are retained, stored, or degraded within P-bodies. Future studies should dissect exactly when and how CDS m<sup>6</sup>A sites direct mRNAs to P-bodies and clarify their precise contributions to mRNA metabolism.

### Physiological relevance of CMD and its targets

Because m<sup>6</sup>A deposition within the CDS is highly restricted – partly due to the EJC – only specific transcripts are selectively targeted by CMD [2]. A compelling example is provided by retrogenes – genes derived from the retrotransposition of spliced parental mRNAs [72]. Unlike their intron-containing counterparts, retrogenes lack the structural barriers that limit m<sup>6</sup>A deposition, resulting in elevated m<sup>6</sup>A levels. Consequently, retrogene transcripts are more susceptible to rapid degradation, whereas their parental mRNAs remain stable, highlighting an elegant mechanism of mRNA quality control [2].

Additional targets provide further insights into the biological significance of CDS–m<sup>6</sup>A decay. The pathway regulates the transcripts of key developmental genes such as *SOX2*, *SON*, and *KLF4*, as well as components of the m<sup>6</sup>A machinery itself, including *RBM15*, *METTL14*, and *YTHDC1* mRNAs, suggesting an autoregulatory mechanism.

### Potential role of CMD in cancer biology

Importantly, CMD targets cancer-related transcripts such as *EP300*, *ARID1A*, and *NOTCH1/2* [2], potentially also linking disturbances of the pathway to tumorigenesis. Moreover, the m<sup>6</sup>A writer METTL3 has itself been implicated as an oncogene in various tumors [73]. A small-molecule inhibitor targeting METTL3, now widely used in m<sup>6</sup>A research, is currently being developed as a therapeutic for AML. Inhibition of METTL3 has shown efficacy in eradicating leukemia cells *in vitro* and in delaying AML progression in preclinical models [74]. The compound is now undergoing Phase 1 clinical trials (NCT05584111). Although specific CMD-related effects on carcinogenesis remain to be elucidated in future studies, it is conceivable that CMD may contribute to cancer biology, given the physiological relevance of its targets.

Building on this, Linder *et al.* demonstrated that the tRNA modification mcm<sup>5</sup>s<sup>2</sup>U, that is implicated in tumorigenesis, metastasis, and therapy resistance in various cancers, accelerates decoding of m<sup>6</sup>A-modified codons and prevents ribosome stalling, whereas its absence leads to ribosome collisions. This points to a potential role for the mcm<sup>5</sup>s<sup>2</sup>U/m<sup>6</sup>A axis, and more broadly for CDS m<sup>6</sup>A-mediated decay, in cancer-related translation control [46].

In addition, it is important to note that enrichment of CMD target transcripts in phase-separated compartments, such as P-bodies, may influence RNA accessibility for targeted interventions. Although further research will be necessary to determine whether this property can be leveraged therapeutically, it is intriguing that some chemotherapeutics selectively partition into condensates, potentially affecting their efficacy [75].

Finally, a recent study has suggested that m<sup>6</sup>A may play a role not only in RNA degradation but also as a stabilizing mark in the context of tumorigenesis. Interestingly, synonymous mutations in cancer that disrupt m<sup>6</sup>A sites – somatic mutations that alter the adenosine residues without changing the encoded amino acid – are significantly enriched in tumor-suppressor genes such as *BRCA2* and *CDKN2A*. These mutations result in destabilization of the corresponding transcripts due to the loss of IGF2BP binding [76]. Consistently, several studies reported that m<sup>6</sup>A readers from the IGF2BP family stabilize m<sup>6</sup>A-modified transcripts [77–79].

### Concluding remarks

In summary, m<sup>6</sup>A modification plays a pivotal role in regulating RNA turnover. Notably, the impact of m<sup>6</sup>A on mRNA fate is highly context-dependent, and varies with its position along the transcript. m<sup>6</sup>A sites located within the CDS trigger a rapid and potent decay pathway that is intricately coupled to translation. This coupling provides a mechanism for the precise fine-tuning of gene expression during dynamic processes such as development. Moreover, m<sup>6</sup>A deposition is constrained by exon–intron boundaries, primarily through the protective role of the EJC, resulting in preferential enrichment of m<sup>6</sup>A in long exons and intronless genes. This distribution may serve as a safeguard mechanism, as exemplified by the differential m<sup>6</sup>A levels observed between retrogenes and their parental counterparts. Ultimately, elucidating the interplay between m<sup>6</sup>A deposition and its downstream effects in the cytoplasm will be crucial for deciphering the broader roles of m<sup>6</sup>A in gene regulation and may pave the way for innovative therapeutic strategies.

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### Outstanding questions

To what extent is m<sup>6</sup>A deposited before splicing is complete, and how can this be reconciled with evidence that the EJC (that is assembled post-splicing) restricts m<sup>6</sup>A deposition in splice-adjacent regions?

What determines the timing of m<sup>6</sup>A deposition on individual sites and transcripts? Does this timing influence transcript fate?

How does m<sup>6</sup>A within A-site codons impair decoding efficiency, and does this lead to a transient pausing or stable ribosome stalling? How are such changes in ribosome dynamics sensed, and what roles do YTHDF proteins play in this process?

Do P-bodies serve as active decay sites or transient storage hubs, and is the observed P-body enrichment of transcripts a downstream consequence of CMD?

How and where are CMD targets ultimately degraded? Is DCP2-mediated decapping involved?

How is the nuclear history of transcripts connected to their effect in the cytoplasm?

Can targeting CMD be a therapeutic strategy for diseases such as cancer?

Is CMD regulated in a tissue- or cell type-specific manner, and how do context-dependent factors such as translation rates, P-body dynamics, and the differential functions of m<sup>6</sup>A reader proteins modulate its activity?

## Declaration of interests

The authors declare no competing interests.

## Resources

<https://clinicaltrials.gov/ct2/show/NCT05584111>

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### 3 Discussion

In this study, we investigated the impact of m6A RNA modification on mRNA stability, with a particular focus on how the position and timing of individual m6A sites influence the choice of the decay pathway. Our findings reveal a novel role for m6A within the CDS, where it promotes rapid and efficient mRNA decay. Unlike previously characterized pathways, this decay is not initiated by a canonical m6A reader protein, but instead appears to be translation-dependent, with m6A positioned in the ribosomal A site leading to translational slowdown and subsequent mRNA degradation. While the YTHDF2 reader protein is not required to trigger this pathway, our data suggest it may function downstream, potentially in a P-body-associated role. Understanding this mode of m6A-mediated decay expands our view of how translation and RNA modifications intersect to regulate gene expression, with potential implications for processes such as cellular stress response, development, and disease.

In the following sections, I will discuss the proposed mechanism in more detail and outline future directions that could help resolve the outstanding questions surrounding this newly identified decay pathway.

#### 3.1 m6A in the CDS leads to ribosome slowdown

To place our observations into a broader molecular context, I will first discuss how various mRNA modifications influence translation dynamics and ribosome behavior. I will then focus on m6A in bacteria as an example that may reveal conserved principles of translation-coupled decay, even across evolutionary distances.

##### 3.1.1 RNA modifications modulate translation dynamics and mRNA fate

The impact of RNA modifications on translation efficiency is increasingly recognized as a major determinant of mRNA stability. Among these, pseudouridine ( $\Psi$ ), *N*-methylpseudouridine (*m*1 $\Psi$ ), and 8-oxoguanosine (8-oxoG) have been shown to influence elongation dynamics, decoding fidelity, and ribosome behavior—linking the modification landscape to post-transcriptional control.  $\Psi$ , for instance, slows translation by disrupting tRNA positioning in the ribosome. Structural and biochemical studies have shown that  $\Psi$  substitution within UUU codons impairs Tu translation elongation factor (EF-Tu) GTPase activation and causes disorder in the tRNA's 3' CCA end (Eyler et al. 2019). In contrast, *m*1 $\Psi$  maintains elongation rates but affects codon recognition

accuracy, resulting in altered amino acid incorporation depending on the codon context (Monroe et al. 2024). 8-oxoG, a marker of oxidative damage, stalls the ribosome by interfering with essential conformational transitions in the small ribosomal subunit, independent of its codon position (Simms et al. 2014). Similarly, m5C shows an inverse correlation with ribosome association, suggesting a repressive role in translation (Schumann et al. 2020). Additional modifications, such as *N*1-Methyladenosine (m1A) and *N*4-acetylcytidine (ac4C), have been proposed to enhance translation, though their prevalence in human mRNA remains debated. m1A near start codons correlates with higher protein output (Dominissini et al. 2016), while ac4C at the wobble position boosts translation efficiency in both bacterial and human systems (Arango et al. 2018).

Together, these findings emphasize a common principle: mRNA modifications exert codon-specific and context-dependent effects on translation, which in turn influence mRNA stability. This interplay forms the basis of coupled translation-decay mechanisms such as the one we describe for CDS–m6A decay, in which decoding inefficiency triggers targeted degradation.

### **3.1.2 m6A can impact translational decoding in bacteria**

Building on the broader understanding of mRNA modifications in translation efficiency, studies have also highlighted the specific structural contributions of m6A. In bacterial systems, m6A has been shown to influence codon-anticodon interactions and RNA structure, offering insights that may also extend to eukaryotic translation regulation. X-ray crystallographic analyses of *Thermus thermophilus* 30S ribosomal subunits soaked with m6A-modified short RNAs revealed that m6A can form canonical Watson-Crick base pairs with U in the ribosomal A site. This observation supports the idea that m6A, like other canonical nucleotides, can engage in base pairing during translation, suggesting its functional role in the translational process. However, at the available resolution (3.35–3.45 Å), the 6-methyl group of m6A remained unresolved, likely due to the rotational flexibility around the C-N bond of the methyl group. This lack of resolution hints that the structural contribution of m6A may be influenced by the local conformational dynamics of the RNA and ribosome, implying that m6A's effects on translation could be highly context-dependent and influenced by the flexibility of the RNA structure in the ribosomal context (Choi et al. 2016).

A recent study in *Escherichia coli* further supports the idea that m6A modifies RNA structure in a way that impacts translation efficiency (Jain et al. 2023). This study revealed

that m6A destabilizes codon-anticodon interactions, leading to an increased rate of tRNA drop-off. Specifically, m6A was shown to reduce the stability of the codon-anticodon complex, promoting dynamic conformations that are rejected by the ribosome, even though the standard codon-anticodon geometry is maintained. This destabilization results in less efficient tRNA binding and elongation, offering a potential mechanistic explanation for how m6A could slow translation and promote decay. The observation that m6A promotes dynamic, non-standard RNA conformations adds a layer of complexity to its role, suggesting that the modification might induce subtle but functionally significant changes in RNA structure that influence ribosomal function and, by extension, translation efficiency.

Taken together, these structural insights in bacteria provide a mechanistic framework for understanding how m6A modifies RNA structure and function might work in eukaryotes as well. They suggest that m6A's role in translation is not solely dependent on its ability to form base pairs with U, but also on its capacity to induce conformational flexibility and destabilize critical RNA-RNA interactions, which may ultimately regulate both translation and mRNA stability.

### **3.2 m6A in the CDS leads to efficient mRNA decay**

The role of m6A modifications in regulating cytoplasmic mRNA stability is well established. Canonical decay pathways are triggered upon recognition of m6A marks by YTHDF reader proteins, which promote transcript degradation through either CCR4–NOT-mediated deadenylation, HRSP12-dependent endonucleolytic cleavage, or UPF1-mediated decapping (Boo et al. 2022; Du et al. 2016; Park et al. 2019). In this study, we identified a novel mechanism—CDS–m6A decay (CMD)—in which m6A sites within coding regions promote rapid and efficient mRNA degradation through impaired ribosome decoding, independent of canonical reader-mediated initiation. Notably, CMD-targeted transcripts are enriched in P-bodies, suggesting compartmentalization as a potential regulatory feature.

Following publication of our study, two subsequent studies provided independent evidence for a translation-dependent decay pathway driven by CDS-localized m6A (Linder et al. 2025; Murakami et al. 2025). While each work emphasizes different mechanistic aspects, together they reveal a coherent picture of m6A-induced transcript destabilization linked to ribosomal dynamics. All three studies—ours, Murakami et al.

(Murakami et al. 2025), and Linder et al. (Linder et al. 2025)—demonstrate that m6A-containing codons impair ribosome progression, as shown by high-resolution ribosome profiling. Murakami et al. further employed the more sensitive OTTR ribosome profiling technique, allowing them to detect ribosome collisions more robustly. In contrast, our profiling data revealed ribosome pausing at m6A sites but did not strongly indicate collisions, possibly due to methodological sensitivity differences. Each study also supports the positional specificity of m6A function. CDS m6A sites exert stronger destabilizing effects than 3' UTR sites, a conclusion consistently observed across all three studies. The effect is codon specific, with the most pronounced destabilization occurring when m6A is positioned at the third nucleotide of a codon corresponding to the ribosomal A site. Linder et al. further refined this observation by focusing on three specific codons and demonstrating that decoding efficiency—and hence degradation—is modulated by the presence of the tRNA modification 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U). In contrast, both our study and that of Murakami et al. support a broader model in which CDS-m6A sites contribute to transcript decay transcriptome-wide and across all methylated codons.

In the following chapters, I will explore possible molecular mechanisms underlying CMD, evaluate how our findings intersect with those of Murakami et al. and Linder et al., and propose future experimental directions to dissect the interplay between m6A, ribosome dynamics, and decay.

### **3.2.1 CMD is independent of previously known m6A-dependent degradation pathways**

Over the past 15 years, m6A modification has been extensively studied, particularly for its role in promoting mRNA degradation in the cytoplasm. Three main degradation pathways have been described, all initiated by the recognition of m6A by YTHDF reader proteins (Boo et al. 2022; Du et al. 2016; Park et al. 2019). However, it remains unclear how these pathways are distinguished from one another, or what determines which pathway is triggered in a given context. This uncertainty prompted us to investigate whether the position of m6A sites might play a role in specifying the pathway.

Our results revealed a strikingly rapid and robust degradation response specifically associated with m6A sites located in the coding sequence, suggesting the presence of a mechanistically distinct decay pathway. This form of decay exhibits key features that set it apart from previously described YTHDF-dependent mechanisms. Most notably, it

requires active translation to be triggered—an essential prerequisite that is not shared by canonical m6A-driven decay processes. In addition, transcripts undergoing this form of decay are consistently enriched in P-bodies, whereas targets of other m6A-associated pathways do not show this localization pattern. These observations point to a unique set of molecular requirements. Supporting this idea, depletion of key effectors involved in the three major YTHDF2-mediated m6A decay pathways—CNOT1, HRSP12, and UPF1—has minimal impact on these targets. These effectors are central to distinct, previously characterized mechanisms of m6A-driven decay: CNOT1 anchors the CCR4-NOT deadenylation complex recruited by YTHDF proteins to initiate transcript destabilization (Du et al. 2016); HRSP12 mediates endonucleolytic cleavage through the RNase P/MRP complex (Park et al. 2019); and UPF1, best known for its role in NMD, has also been implicated in translation independent YTHDF2-associated mRNA turnover (Boo et al. 2022). The insensitivity of our target transcripts to the depletion of these effectors highlights a clear mechanistic divergence from canonical YTHDF2 pathways. Taken together, the combination of translation dependency, P-body localization, and independence from canonical YTHDF2 effectors strongly suggests the involvement of a distinct molecular machinery—potentially engaging alternative RNA-binding proteins or decay effectors. Our findings thus support the existence of a specialized degradation pathway triggered by m6A sites in the coding sequence, functionally and mechanistically divergent from established m6A-driven decay mechanisms.

Interestingly, Murakami et al. (Murakami et al. 2025) do not explicitly make this distinction in their study. While our approach relies on short-term m6A depletion to isolate and observe the immediate effects of CMD, their study predominantly uses long-term depletion or knockout models. Despite this, they also report that a substantial portion of m6A-dependent decay is translation-dependent and propose that translation-dependent decay may, in fact, account for the majority of previously described m6A-triggered degradation. However, there remains a clear subset of m6A-mediated decay events—particularly those involving 3'UTR sites—that do not require translation.

Thus, our findings suggest that CMD represents an additional, highly potent and rapid pathway within the broader landscape of m6A-dependent RNA decay. Given its fast kinetics and specificity, CMD may play a key role in fine-tuning gene expression during acute stress or developmental transitions. Whether CMD employs known m6A readers in a novel context or involves as-yet-uncharacterized factors remains an open question.

Future studies with higher resolution and temporal precision will be essential to further disentangle these distinct pathways and clarify their respective roles in gene regulation.

### **3.2.2 Position of m6A within the codon can impact the degradation**

Taking into account that m6A in eukaryotes is deposited in a specific sequence – DRACN – ten possible internal codons can be methylated. These include four codons with m6A at the first position (ACA, ACT, ACG, and ACC), two at the second position (AAC and GAC), and four at the third position (GAA, GGA, AAA, AGA). In our study, we found that m6A in any of the three codon positions can induce ribosome pausing and subsequent mRNA decay, albeit with varying strengths. Specifically, m6A at the second position had a slightly weaker effect on mRNA stability, whereas m6A at the third position had the strongest impact. This pattern was consistently observed across three independent approaches: RNA-seq, Ribo-seq, and reporter assays. Moreover, our findings align with structural studies in *E. coli* (Jain et al. 2023), which also reported the weakest effect for second-position m6A, and results from Murakami et al (Murakami et al. 2025).

In contrast, Linder et al. (Linder et al. 2025) observed more codon-specific variability in m6A's impact on translation. Whereas we observed consistent effects across all codons—with some variation in magnitude—they reported significantly stronger translation slowdown for AGA, AAA, and GAA codons under specific conditions. These codons are decoded by tRNAs bearing mcm5s2U at position U34, a modification that enhances wobble pairing. In cells lacking the enzymes that install mcm5s2U, Linder et al. showed that decoding of m6A-containing codons is dramatically impaired, leading to ribosome collisions. While this modification clearly intensifies the translation defect, it may act as an additional factor on top of the general slowdown caused by m6A that we observe.

Together, these findings underscore a potential interplay between mRNA methylation and tRNA modification. Whether the tRNA modification machinery directly intersects with the m6A-mediated decay pathway we describe remains uncertain. Nonetheless, this highlights how multiple layers of regulation—m6A modification, tRNA modifications, and codon context—may converge to modulate decoding speed, thereby influencing the stability and fate of m6A-modified transcripts.

### **3.2.3 m6A sites induce ribosome collisions**

All three studies agree that impaired ribosome decoding is the trigger for initiating m6A-dependent mRNA decay. However, the nature of this impairment—how it is recognized

and how it triggers subsequent decay—remains unclear. In our data, the ribosome pausing effect appeared modest, suggesting that ribosomes may only be slowed at m6A sites, similar to what has been described for codon optimality-mediated decay (COMD). In contrast, the studies by both Murakami et al. and Linder et al. (Linder et al. 2025; Murakami et al. 2025) report evidence of more pronounced ribosome stalling and, in some cases, ribosome collisions, suggesting that our ribosome profiling method may have lacked the sensitivity to detect these events. Murakami et al. further supported their findings using disome profiling, where they observed an increased number of disome footprints around m6A sites. Interestingly, these disome footprints differed from classical collision signatures, indicating a conformational shift in the leading ribosome. This altered ribosome state may reflect a distinct type of slowdown or stalling, unique to the m6A context, and may serve as a key molecular signal for triggering downstream decay. What remains unresolved is how this conformational change is sensed and translated into a degradation response. In our study, we systematically knocked down a panel of known ribosome-associated quality control (RQC) factors and analyzed relevant public datasets, yet we found no strong evidence linking known RQC pathways to m6A-mediated decay in the coding sequence. This could be due to incomplete knockdowns, or it may point to the involvement of a distinct collision-sensing mechanism. The observed ribosome conformation shift in CMD may itself be a critical mechanistic feature. A similar ribosome state has been described in COMD, where it enables the binding of CNOT3—a subunit of the CCR4-NOT complex—to the ribosomal E site and initiates mRNA decay (Zhu et al. 2024). Specifically, when both the A and E sites are unoccupied, this conformation allows CNOT3 to engage the ribosome and recruit decay factors (Bae and Collier 2022; Müller et al. 2025; Zhu et al. 2024). Although COMD is generally considered independent of ribosome collisions, this overlap in ribosomal features raises the possibility that CMD and COMD might share core mechanistic elements. DDX6, another CCR4-NOT-associated factor implicated in COMD, may also play a role. Whether these proteins are involved in sensing or executing CMD remains an open question, but their involvement would provide a potential link between ribosome conformation and targeted decay. Notably, Murakami et al. (Murakami et al. 2025) propose a different model in which the altered ribosome conformation facilitates the recruitment of YTHDF reader proteins. They further hypothesize that YTHDF proteins may communicate with the ribosome via interaction with the RQC factor RACK1.

However, the evidence for this interaction is limited, and even the authors note that the precise role of YTHDF proteins in this context remains unclear.

### **3.2.4 What is the role of YTHDF proteins in CDS-m6A decay?**

Three well-established m6A-mediated decay pathways rely on YTHDF reader proteins to recognize methylated transcripts and initiate degradation (Lee et al. 2020). However, the pathway described in this study—translation-dependent CDS-m6A decay—appears to differ fundamentally, as it is triggered by impaired ribosome decoding rather than direct YTHDF binding. This raises the question: Is YTHDF2 still involved, and if so, what is its role?

Our data indicate that YTHDF2 is not essential for initiating this pathway but may act downstream of the ribosomal slowdown. We find that CMD targets, particularly those bound by YTHDF2, are enriched in P-bodies—suggesting a possible link between YTHDF2 function and subcellular localization. This is consistent with prior work implicating YTHDF2 in P-body recruitment and dynamics (Ries et al. 2019; X. Wang et al. 2014; Zaccara and Jaffrey 2020). Moreover, our proximity labeling data identify multiple P-body-associated proteins as YTHDF2 interactors, supporting a model in which YTHDF2 contributes to mRNA fate after translation has been impaired.

Recent studies provide additional mechanistic context for how YTHDF2 could be linked to ribosomal events. One report identified an interaction between YTHDF2 and the ribosomal protein RACK1 (Moch et al. 2023), suggesting that YTHDF2 may be recruited to ribosomes that have stalled due to codon usage or modification-induced pausing. Murakami et al. extended this idea, proposing that YTHDF2 operates at ribosomes stalled by m6A-containing codons, potentially recognizing paused complexes via RACK1 (Murakami et al. 2025). However, these studies do not resolve the timing or structural requirements of YTHDF2 recruitment—whether it binds during translation or post-release into granules remains incompletely understood.

A key challenge in interpreting these findings lies in distinguishing CDS-m6A decay from other YTHDF2-associated pathways. The observed necessity for YTHDF2 may reflect overlapping decay mechanisms rather than a specific role in the pathway we describe. Indeed, it remains possible that YTHDF2 functions as a general effector of m6A-induced mRNA clearance, regardless of the initiating signal (reader recognition vs. ribosome stalling).

Taken together, these findings suggest that YTHDF2 contributes to the downstream processing of CDS-m6A-modified transcripts, possibly by facilitating their retention or degradation in P-bodies. However, its precise mechanistic role remains to be fully defined. Future structural and functional studies—particularly of ribosomes paused at m6A-modified sites—will be critical for clarifying how and when YTHDF2 engages these transcripts during their decay. Whether YTHDF2 is passively recruited to these stalled complexes or actively directs their localization and decay remains an open question, which may intersect with the role of P-bodies.

### **3.2.5 Can the IGF2BP protein family have a role in CMD?**

In our study, we primarily focused on the role of YTHDF proteins, particularly YTHDF2, as cytoplasmic m6A readers that mediate mRNA degradation. However, the cytoplasm contains additional m6A reader proteins, including the IGF2BP family (IGF2BP1–3), which are generally associated with transcript stabilization (Huang et al. 2018). Intriguingly, recent work has shown that IGF2BP3 can bind m6A-modified mRNAs and relocate them from the translating pool to P-bodies, thereby repressing translation and possibly delaying degradation (Shan et al. 2023). In our dataset, we observed that transcripts undergoing CMD are enriched in P-bodies, but we found no direct evidence of degradation occurring within these granules. This raises the possibility that P-bodies serve as transient holding sites rather than decay centers. One speculative model is that CMD-targeted transcripts are sequestered in P-bodies shortly after export to the cytoplasm. In this case, they could be bound by IGF2BP proteins, which interact with core P-body components and are known to suppress translation. Such a mechanism could act as a regulatory buffer: the transcripts are protected from translation and decay while in P-bodies, and only upon specific cellular signals are they released into the translating pool. Once ribosomes engage with these transcripts, the presence of m6A in the coding region could trigger their rapid degradation through the canonical CMD pathway. In this context, IGF2BPs might not counteract decay, but instead temporally regulate it by controlling access to translation.

This model is currently speculative and unsupported by direct evidence. However, it is consistent with the observed P-body enrichment of CMD targets and the demonstrated ability of IGF2BP3 to traffic m6A-mRNAs to P-bodies. Future studies examining the interplay between IGF2BP proteins, translation entry, and CMD would be valuable to test this hypothesis.

### 3.2.6 What is the role of P-bodies in CDS-m6A decay?

P-bodies are dynamic, membraneless RNA granules that form through liquid–liquid phase separation, driven by interactions between RNA molecules and proteins containing intrinsically disordered or low-complexity regions. They are enriched in components of the mRNA decay and translational repression machinery, including decapping enzymes, exonucleases, and various RNA-binding proteins. Importantly, P-body assembly is tightly linked to changes in cellular translation activity, with their formation increasing when global translation is suppressed. As such, P-bodies are thought to serve as hubs for selective mRNA storage, silencing, or degradation in response to cellular signals and stress (reviewed in Luo et al. 2018).

Upon translational stress, mRNAs are frequently sequestered into P-bodies, where they are thought to be either degraded or temporarily stored. Emerging evidence suggests that m6A modifications can modulate this localization, particularly via interactions with YTHDF reader proteins (Ries et al. 2019). Transcripts bearing multiple m6A sites are more likely to accumulate in P-bodies, while unmethylated or sparsely methylated transcripts typically remain engaged with polysomes (Shan et al. 2023). Our findings build on this by demonstrating that CDS–m6A–marked transcripts—specifically those subject to the translation-dependent CMD pathway—are selectively enriched in P-bodies. In contrast, transcripts targeted by other m6A decay routes do not exhibit the same degree of enrichment, suggesting that P-body localization may be a distinctive feature of CMD. This observation supports the idea that P-bodies are not merely general decay compartments but may serve as specialized processing sites for translation-stalled transcripts marked by CDS m6A.

While historically viewed as decay centers, P-bodies are increasingly recognized for their nuanced roles in mRNA storage, protection, and post-transcriptional regulation. Their RNA composition changes dynamically across cell types and states, including cell cycle transitions and differentiation (Hubstenberger et al. 2017). Notably, recent work has shown that pluripotent and differentiated cells contain P-bodies with distinct mRNA profiles and functional roles (Jiang et al. 2023). Interestingly, many CMD targets in our study include developmental regulators. This raises the possibility that during certain stages of differentiation, these transcripts may be selectively routed to P-bodies—either for temporary protection from translation-dependent decay or for targeted degradation, depending on context.

At present, the precise mechanistic role of m6A in promoting P-body localization remains unresolved. It is unclear whether m6A actively recruits decay machinery to P-bodies, alters the binding affinity of specific RNA-binding proteins, or simply marks transcripts that are translationally stalled and consequently rerouted. Deciphering how P-bodies integrate signals from translational slowdown and m6A modification will be crucial for understanding both CMD and broader principles of post-transcriptional regulation.

### **3.2.7 Overview of the proposed mechanism**

Our findings, together with recent studies by Murakami et al. and Linder et al., converge on a mechanistic model in which m6A modifications within the coding sequence impair ribosomal decoding, ultimately triggering selective mRNA decay. This process—termed CDS–m6A decay—is mechanistically distinct from classical m6A-mediated decay pathways that are initiated through direct recognition of methylated sites by YTHDF reader proteins. Instead, CDS–m6A decay (CMD) appears to be initiated by altered ribosome dynamics—specifically, a slowdown or stalling event that arises during translation elongation when m6A-modified codons enter the ribosomal A site. This effect is most pronounced when m6A occupies the third nucleotide position of a codon, potentially disrupting correct tRNA accommodation or decoding kinetics.

While ribosome stalling appears to serve as the initiating trigger, how this signal is sensed and transduced into a degradation response remains unclear. While our data do not support a major role for known RQC pathways, we identify candidate factors that may mediate this response. In particular, the CCR4-NOT component CNOT3 emerges as a plausible effector, potentially acting at the ribosomal E site in response to conformational shifts associated with translational pausing. Additionally, although YTHDF2 is not required to initiate CMD, it may act at a later stage to direct stalled or marked transcripts toward decay or storage pathways, especially given its consistent association with P-bodies and its proximity to P-body-associated proteins in our interactome data. A notable feature of CMD is the selective enrichment of its target transcripts within P-bodies. This suggests that subcellular compartmentalization is not merely a consequence but may be an integral component of the pathway, enabling spatial separation of stalled transcripts from active translation zones and facilitating regulated degradation or storage. Whether entry into P-bodies reflects commitment to decay, a transient holding pattern during stress, or a decision point between reinitiation and decay remains an important open question. Moreover, the timing and structural prerequisites for YTHDF2 recruitment—

whether co-translationally at the ribosome or post-release into granules—are not yet fully defined.

Together, these findings support a translation-dependent m6A decay mechanism that integrates RNA modification, ribosome behavior, and spatial regulation. This layered mechanism expands the known repertoire of m6A-driven transcript fate decisions and highlights the coding sequence as a functionally active site for post-transcriptional control. In the next chapters, I will explore the physiological relevance of this pathway, how it contributes to cellular adaptation during stress and differentiation, and how its dysregulation could be implicated in disease.

### **3.3 Physiological relevance of CMD**

Efficient regulation of mRNA stability is essential for maintaining cellular homeostasis, and m6A methylation plays a pivotal role in this process. m6A has been implicated in nearly every stage of mRNA metabolism—from co-transcriptional deposition in the nucleus, where it can influence alternative splicing and nuclear export, to cytoplasmic functions such as translation and degradation (Du et al. 2016; Jiang et al. 2021; Lence et al. 2016; Li et al. 2017; Lin and Gregory 2014; X. Wang et al. 2014; Zheng et al. 2013). Traditionally, many of these effects were thought to stem from active regulatory mechanisms involving the recruitment of methyltransferases or demethylases. However, a recent study from the Schwartz lab by Dierks et al. (Dierks et al. 2025) presents a compelling alternative: that many observed changes in m6A levels and their consequences on mRNA distribution may instead be attributed to passive processes shaped by RNA metabolism itself (Hallstein and König 2025). In this work, the authors develop a minimal yet powerful model—m6ADyn—based on two core assumptions: that m6A promotes mRNA decay, and that this decay occurs primarily in the cytoplasm. By incorporating production, export, and degradation rates for both methylated and unmethylated transcripts, their model recapitulates numerous experimental observations previously attributed to active m6A dynamics. Notably, they show that differences in m6A levels between nuclear and cytoplasmic compartments can be explained by m6A-dependent degradation in the cytoplasm, without invoking differences in export efficiency. For instance, genes with higher nuclear localization exhibit higher m6A levels simply because their transcripts are less exposed to cytoplasmic decay. This framework reinforces the idea that CMD is not just a molecular mechanism but a physiologically

embedded process: transcripts that are efficiently exported and translated are also the ones most susceptible to m6A-triggered degradation. Such a link between translation and decay allows CMD to act as a dynamic buffer for gene expression, fine-tuning mRNA abundance in response to cellular conditions. The m6ADyn model thus highlights how CMD could serve as a passive yet robust means of shaping transcriptomes during processes like differentiation, stress, or oncogenic signaling, by coupling metabolic constraints to m6A-driven decay.

### **3.3.1 Spatial control of CMD through m6A deposition patterns**

m6A is not evenly distributed across mRNAs but instead follows a specific and regulated pattern: it is preferentially enriched near the stop codon and within the last exon, as well as in long internal exons (He et al. 2023; Luo et al. 2023b; Meyer et al. 2012; Uzonyi et al. 2023; Yang et al. 2022). This distribution suggests that the deposition of m6A is not random but instead influenced by specific features of the mRNA transcript architecture. Recent studies have demonstrated that m6A is notably depleted near exon-exon junctions, a pattern that is likely due to steric hindrance or competition from the EJC, which assembles at these sites following splicing (He et al. 2023; Uzonyi et al. 2023; Yang et al. 2022). Supporting this model, a recent study employing a deep learning framework for m6A site prediction combined with *in silico* mutational analysis showed that exon-intron boundaries act as inhibitory signals for m6A deposition. This inhibition appears to be at least partially mediated by the presence of the EJC, further reinforcing the idea that the splicing machinery plays a critical role in shaping the m6A epitranscriptomic landscape (Luo et al. 2023b). Together, these findings underscore the importance of transcript structure and processing in guiding site-specific m6A deposition. The question arises: why does this pattern of m6A deposition exist in the first place? Given that m6A sites within CDS can initiate efficient, translation-coupled mRNA decay, it is likely that certain transcripts must be shielded from this degradation pathway to ensure proper gene expression. This is particularly relevant for transcripts involved in developmental processes, which often require tight temporal control and rapid, dynamic shifts in expression levels. Indeed, our study has shown that many of the transcripts affected by CDS-m6A-mediated decay encode key developmental regulators, suggesting that this mechanism serves as a fine-tuning tool during critical cell state transitions.

### 3.3.2 Coupling between translation efficiency and CMD

In our analysis, we observed that the strength of decay triggered by m6A modifications within the CDS appears to correlate with translation efficiency: transcripts that are more actively translated tend to be more robustly degraded through this pathway. This observation suggests a potential coupling between translation and decay, consistent with the idea that increased ribosome occupancy and impaired decoding trigger m6A-mediated degradation. At the same time, many developmentally regulated transcripts targeted by this form of decay do not exhibit high translation efficiency under steady-state conditions. This suggests that the pathway is not constitutively active but may be selectively engaged during specific cellular states. For example, during differentiation, stress responses, or signaling events, transcription and translation of certain transcripts may transiently increase, triggering rapid decay through this mechanism. As the stimulus resolves and translation slows, the decay signal may diminish, stabilizing transcript levels. Such a mechanism could serve as a self-limiting system: high translation leads to enhanced decay, preventing excessive accumulation of transiently induced transcripts, while low translation preserves them. This dynamic regulation may provide temporal precision to gene expression programs and contribute to cellular homeostasis during changing physiological conditions.

### 3.3.3 Translational stress and cancer: Functional consequences of CDS-m6A decay

We observed that several cancer-related genes, such as *EP300*, *ARID1A*, *KLD4*, and *NOTCH1/2*, are targeted by the CDS-m6A-mediated decay pathway, indicating a potential link between this mechanism and certain cancers. While our study did not directly investigate this connection, it raises intriguing possibilities for future research into the role of m6A in cancer progression. Supporting this, Linder et al. demonstrated that decoding m6A leads to ribosome slowdown, with the effect being particularly strong when m6A is located at the last position of the codon (Linder et al. 2025). Additionally, they showed that the mcm5s2U modification on tRNA, present in the anticodon position opposite m6A in the codon, can modulate decoding speed. When mcm5s2U is present, decoding speeds up, while its absence significantly slows down translation, causing ribosome collisions. This tRNA modification has been reported to be associated with certain cancer types, particularly when it is absent or reduced, and the authors suggest a potential link between the mcm5s2U/m6A system and cancer. Although this connection

is currently based on correlation and requires further investigation, it is an intriguing association that warrants closer examination. On the other hand, Murakami et al., in their study of CDS-m6A-mediated decay, propose a connection between this pathway and stress responses (Murakami et al. 2025). Previous studies have suggested that stress activates alternative pathways that stabilize m6A-containing transcripts (Anders et al. 2018; Li et al. 2024; Luo et al. 2023b). However, Murakami et al. argue that this connection is more indirect and occurs through changes in translation levels. Specifically, during stress, global translation repression takes place, which reduces the likelihood of ribosome stalling during decoding, thereby stabilizing m6A-modified transcripts.

Although current data primarily link CMD to stress and cancer contexts, its core mechanistic features—translation-coupled decay, position-specific m6A deposition, and subcellular compartmentalization—are also central to many disease-relevant processes, including differentiation and RNA granule dynamics (Advani and Ivanov 2020; Bosco 2018; Hanson and Collier 2018; Hubstenberger et al. 2017). It is therefore plausible that CMD contributes more broadly to disease pathology, particularly in conditions where m6A deposition, translation efficiency, or tRNA modification profiles are disrupted. Future studies will be required to determine whether CMD represents a generalizable mechanism of post-transcriptional control under physiological and pathological conditions.

Together, these findings point toward a complex and context-dependent relationship between m6A, translation dynamics, and RNA decay. In particular, they highlight how modulation of tRNA modifications or translational activity may influence the engagement of CMD, with implications for stress responses and cancer progression.

### **3.3.4 CMD safeguards the transcriptome from retrotransposons**

One intriguing aspect of CMD is its apparent role in transcriptome surveillance, particularly in targeting retrogenes—copies of protein-coding genes generated via reverse transcription and reintegration into the genome (Zou, Guo, and He 2011). Unlike their parental genes, retrogenes lack introns and thus exon-exon junctions, making them more susceptible to CDS m6A deposition due to the absence of EJC, which normally suppresses methylation near splice sites. Consistent with this, we observe that retrogenes harbor significantly higher levels of m6A in their coding regions and are more strongly destabilized by the CMD compared to their intron-containing counterparts. This mechanism may have broader evolutionary implications. By selectively degrading

intronless, retrotransposed RNAs, m6A-mediated decay could act as a post-transcriptional filter, preventing aberrant gene expression and maintaining genomic integrity. It is plausible that such a system evolved as a defense against uncontrolled activation of retroelements, reinforcing a layer of quality control at the level of translation. Supporting this idea, a recent study in human embryonic stem cells showed that loss of METTL3—the methyltransferase responsible for m6A installation—results in derepression of endogenous retrotransposons such as SVA\_D and LTR5\_Hs (Zhang et al. 2025). These elements act as enhancers of naïve pluripotency genes and were upregulated through epigenetic changes, including reduced H3K9me3 and increased chromatin accessibility. While that study focused on transcriptional regulation, our findings raise the possibility that stabilization of these transcripts due to impaired CDS-m6A decay may further contribute to their functional activation.

In sum, our data suggest that CMD plays a dual role: regulating short-lived, developmentally responsive transcripts under normal conditions and acting as a defense mechanism against the expression of retrotransposed or intronless elements that might otherwise escape post-transcriptional control.

### **3.4 Conclusion and outlook**

In this work, we presented a novel pathway in which m6A leads to degradation of the mRNA, relying on translation, P-bodies, YTHDF2 reader protein, and possibly decapping via DCP2. Shortly after us, two additional groups published the same observation: that CDS-m6A sites lead to rapid and strong degradation of mRNAs. By presenting this pathway, we have demonstrated an additional mechanism by which m6A contributes to the regulation of gene expression and the maintenance of transcriptome homeostasis. We showed that it helps eliminate unwanted transcripts—such as retrotransposons—and primarily affects short-lived transcripts involved in developmental changes.

However, none of the three studies, including ours, can yet answer all questions regarding the exact mechanism of this pathway. Open questions include how exactly the m6A modification is recognized by the ribosome, how it impairs decoding, and to what extent. Is the resulting ribosome stalling strong enough to induce collisions, or do collisions occur as a side effect of multiple m6A sites positioned in close proximity or due to the absence of specific tRNA modifications? Furthermore, it remains unclear how the paused or stalled ribosome is detected, how this leads to decay, what the connection to P-bodies is,

and what the precise role of the YTHDF2 reader protein entails. Future work will be crucial to further elucidate this pathway and to understand its mechanism in greater detail. It will also be important to investigate how it interfaces with other m6A-mediated decay pathways, and whether UTR-localized m6A sites play a modulatory role in this context. It will also be valuable to explore whether CMD is evolutionarily conserved. The principles underlying CMD—such as translation-coupled decay, position-sensitive m6A deposition, and ribosome stalling—are mechanistically plausible across species. In bacteria, m6A can impair codon-anticodon pairing, while in eukaryotes, it triggers decay via ribosomal slowdown, suggesting a shared vulnerability to methylation-induced decoding defects. Given that translation-coupled quality control exists in yeast, plants, and metazoans, it is plausible that CMD-like pathways are more broadly conserved but contextually regulated. Comparative studies across species could reveal whether CMD represents a deeply rooted feature of RNA metabolism or a more specialized adaptation in higher organisms.

Taken together, this thesis highlights the complexity and versatility of m6A as a regulatory mark, particularly within the CDS, and its critical role in shaping gene expression outcomes. While many mechanistic details remain to be uncovered, the insights gained here lay the groundwork for future discoveries at the intersection of RNA modification, translation, and decay. As our understanding of m6A biology continues to grow, so too does its potential to inform therapeutic strategies targeting RNA regulation in health and disease. These insights may guide therapeutic strategies aiming to modulate transcript stability—for instance, by targeting m6A writers or erasers, modifying codon usage, or manipulating tRNA modification status in disease-relevant contexts.



## 4 List of Abbreviations

**3' UTR** – 3' Untranslated Region

**5' UTR** – 5' Untranslated Region

**A/P/E site** – Aminoacyl/Peptidyl/Exit sites of the ribosome

**ARE** – AU-rich element

**CDS** – Coding sequence

**CLIP** – Crosslinking and immunoprecipitation

**CMD** – CDS–m6A-mediated decay

**COMD** – Codon optimality–mediated decay

**DRS** – direct RNA sequencing

**EJC** – Exon Junction Complex

**eTAM-seq** – evolved TadA-assisted N6-methyladenosine sequencing

**GLORI** – glyoxal and nitrite-mediated deamination of unmethylated adenosines

**IDR** – Intrinsically disordered region

**KD** – Knockdown

**m6A** – *N*6-methyladenosine

**mcm5s2U** – 5-methoxycarbonylmethyl-2-thiouridine

**mRNA** – Messenger RNA

**NGS** – Next-generation sequencing

**NGD** – No-go decay

**NMD** – Nonsense-mediated decay

**NSD** – Non-stop decay

**P-body** – Processing body

**PTC** – Premature translation termination codon

**RBP** – RNA-binding protein

**RNA Pol II** – RNA Polymerase II

**RQC** – Ribosome quality control

**tRNA** – transfer RNA



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## **6 Curriculum vitae**



## **7 Acknowledgements**

