FEBS Letters

REVIEW ARTICLE

Adenosine methylation as a molecular imprint defining the fate of RNA

Philip Knuckles[1] and Marc Bühler[1,2]

1 Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
2 University of Basel, Switzerland

Correspondence
M. Bühler, Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland
Fax: +41 61 69 60438
Tel: +41 61 69 73976
E-mail: marc.buehler@fmi.ch

(Received 20 April 2018, revised 14 May 2018, accepted 17 May 2018, available online 31 May 2018)

doi:10.1002/1873-3468.13107

Edited by Wilhelm Just

Multiple lines of evidence suggest the RNA modification N^6-methyladonsine (m^6A), which is installed in the nucleus cotranscriptionally and, thereafter, serves as a reversible chemical imprint that influences several steps of mRNA metabolism. This includes but is not limited to RNA folding, splicing, stability, transport and translation. In this Review we focus on the current view of the nuclear installation of m^6A as well as the molecular players involved, the so called m^6A writers. We also explore the effector proteins, or m^6A readers, that decode the imprint in different cellular contexts and compartments, and ultimately, the way the modification influences the lifecycle of an RNA molecule. The wide evolutionary conservation of m^6A and its critical role in physiology and disease warrants further studies into this burgeoning and exciting field.

Keywords: cotranscriptional regulation; m^6A; RNA modifications

Eukaryotic gene expression is regulated at the transcriptional and post-transcriptional levels. Nascent transcripts are subject to additional levels of regulation resulting in their processing and/or decay. RNA maturation events often involve chemical modifications that expand the basic A, U, C and G nucleosides. As of 2017, 163 distinct RNA post-transcriptional modifications have been reported and this number continues to grow [1]. Among the best studied modifications in eukaryotes are the 5′ 7-methylguanylate cap and the 3′ poly(A) tail of mRNA which have important regulatory functions in RNA export, translation and stability.

Our understanding of internal RNA modifications and their functions is unfortunately not as advanced. In eukaryotes, m^6A is the most abundant internal modification in mRNA and has been studied for well over four decades. In mammalian cells 0.1–0.4% of adenosines are m^6A modified [2,3]. A current estimation of modified sites per transcript is 1–3 m^6As in mammalian cells [4,5] and 1.4-2.0 m^6As in *Arabidopsis* [6]. These approximations likely underrepresent the actual amount of m^6A modified sites as these tend to cluster together and are therefore not detectable by meRIP-seq. m^6A is highly conserved and has important physiological functions in different organisms including yeast, plants, flies and mammals. Interestingly *Schizosaccharomyces pombe* and *Caenorhabditis elegans* have evolved without this molecular pathway, as many of the core components are absent in these organisms [7,8].

Technological breakthroughs have recently allowed the genome-wide mapping of m^6A [4,5,9,10] and revealed a degenerate consensus motif, DRACH (where D = A, G or U; R = A or G; H = A, C or U), which is consistent with earlier studies [11,12]. Yet, low complexity of this motif implies the involvement of other sequence or structural motifs in guiding the methylation machinery to target RNAs. Another key recent finding was the discovery that the m^6A modification is a reversible imprint (not to be confused with

Abbreviations
AML, acute myeloid leukaemia; CPT, camptothecin; DMS, dimethylsulphate; mESC, mouse embryonic stem cell; MIRES, m^6A-induced ribosome engagement site.

genomic imprinting where a gene is expressed in a parent of origin-specific manner), with the characterization of the demethylase fat mass and obesity-associated (FTO) [13,14] and a second m^{6}A demethylase alkB homologue 5 (ALKBH5) [15]. A reversible modification on RNA opened the exciting possibility of dynamic regulation and simultaneously sparked a lively debate over the extent of this dynamism [16–18].

In this review we will focus on consolidating the recent advances made towards understanding the molecular players involved in m^{6}A deposition. The list of m^{6}A functions continues to expand within different cellular contexts, cell types and organisms. In fact, m^{6}A has been linked to essentially every single process within the RNA life cycle. Here we attempt to summarize the best-described functions. We briefly discuss the structural effects of m^{6}A as well as the specific protein binders and the way they can influence the fate of a particular RNA species.

m^{6}A writers

Core enzymatic components

The proteins that install m^{6}A can be thought of as RNA writers, just as enzymes that chemically modify N-terminal tails of core histones are referred to as chromatin writers. Identified in humans, the methyltransferase (MTase) that catalyses m^{6}A is methyltransferase like 3 (METTL3, also called MT-A70) [19]. A second, highly related protein, methyltransferase METTL14 was later characterized [20–22]. It forms a stable heterodimer with METTL3 and is essential for m^{6}A deposition *in vivo*. Intriguingly, this work found that physical association between METTL3 and METTL14 has a synergistic effect, resulting in higher activity of METTL3 alone. Recent structural studies have further examined the interactions and molecular activities of METTL3 and METTL14 [23–25], revealing that only METTL3 contains catalytic activity, whereas METTL14 has a degenerate active site that is unable to accommodate donor and acceptor substrates in the context of a heterodimer, and is probably inactive [25]. Instead METTL14 serves as an RNA-binding platform, which enhances METTL3 enzymatic activity by binding substrate RNA and by positioning the methyl group for transfer to adenosine, explaining the synergistic effect observed *in vitro*.

Expanding list of *in vivo* regulators of the core MTase complex

While there is detailed structural data of the subunits that compose a minimal catalytic core of the complex, studies in several organisms have shown that additional factors are essential for *in vivo* methylation of mRNA [22,26–30]. Prominent examples are Wilms tumour 1-associated protein (WTAP) and Vir-like m^{6}A methyltransferase associated (VIRMA), as their ablation has drastic effects on global mRNA m^{6}A levels. WTAP is proposed to ensure the stability and localization of the MTase heterodimer to nuclear speckles [22,28]. VIRMA (Vir) was first characterized as having a role in sex determination in *Drosophila* [31] but its molecular function remains to be determined in the context of m^{6}A. In *Drosophila,* the RNA binding protein (RBP) Nito promotes m^{6}A incorporation and also has a role in the sex determination and dosage compensation pathways [28]. Mammals have a Nito homologue, RNA Binding Motif 15 (RBM15) and a paralogue RBM15B, which are both suggested to regulate m^{6}A [27]. Mapping their binding sites using individual-nucleotide resolution UV cross-linking and immunoprecipitation (iCLIP) showed similar binding patterns [27]. It is thought that RBM15 and RBM15B recruit the MTase complex to its target transcripts *via* direct binding to U-rich sequences on mRNA. In humans this function is important to control X chromosome inactivation through the m^{6}A modification of *XIST* RNA, which is essential for its transcriptional silencing activity. The E3 ubiquitin-ligase that binds to E-cadherin, Hakai, was recently found to interact with other subunits of the m^{6}A methyltransferase complex in plants [30]. Down regulation of Hakai in plants led to a reduced level of m^{6}A, but its direct role within the MTase complex remains to be elucidated. The latest described factor involved in m^{6}A deposition is Zc3h13 [32–34]. Zc3h13 is essential for the localization of the MTase complex in mammalian cells and sex determination in flies. In mouse embryonic stem cell (mESC), Zc3h13 is necessary for self-renewal. Importantly, Zc3h13 bridges the interaction of Wtap and Rbm15 in both organisms. Consistent with an earlier study examining the WTAP interactome in the context of splicing and cell cycle regulation [35], the above mentioned proteins (WTAP, VIRMA, RBM15, HAKAI and ZC3H13) form a stable complex now referred to as MACOM (methylation associated complex, Fig. 1) [34]. MACOM weakly interacts with the Mettll3/14 dimer [20,34] but is nevertheless indispensible for the correct targeting of m^{6}A to mRNA.

The factors essential for the deposition of m^{6}A continue to be characterized, emphasizing that our understanding of this intricate pathway is incomplete. Additional players involved in m^{6}A deposition could yet be identified. Supporting this idea, global m^{6}A levels vary in different tissues, developmental

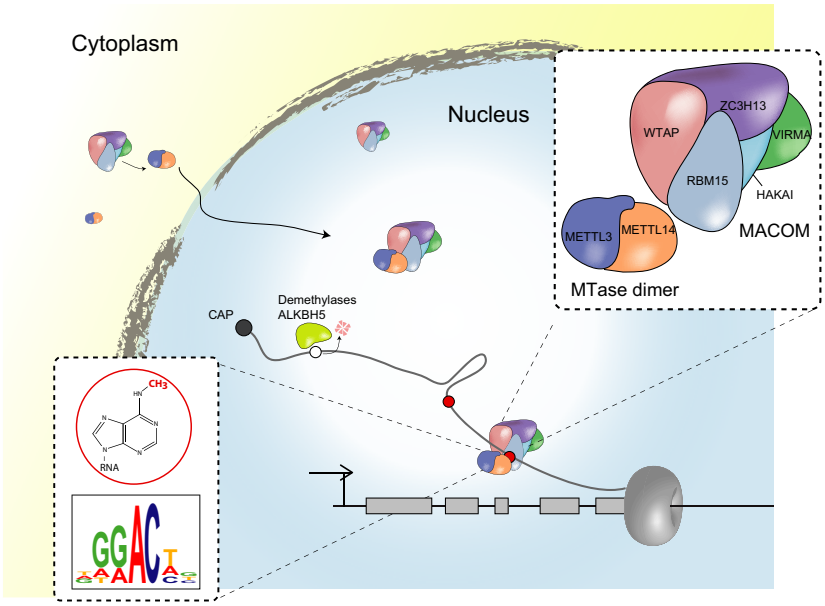

Fig. 1. Nuclear installation of m^{6}A. In the most updated model, m^{6}A installation is coupled to transcription and is catalysed by an MTase dimer (METTL3 and METTL14) where METTL3 is the active catalytic subunit. The consensus motif methylated by METTL3 is DRACH (where D = A, G or U; R = A or G; H = A, C or U). A complex termed MACOM composed of WTAP, VIRILIZER, HAKAI, RBM15 and ZC3H13 is essential for *in vivo* deposition of m^{6}A. MACOM is most likely essential in localizing the MTase to the nucleus and to its RNA targets. Nuclear proteins which have demethylase activity have been characterized including ALKBH5 and FTO.

stages and cell states [4,5], as do the levels of writers and erasers [36]. It is possible that cell-type-specific modulators could regulate the Mettl3/14 complex, altering its activity, specificity or localization. Alternative MTase's with different specificities could also install m^{6}A in a cell-type-specific manner. For example several studies have described Mettl16 as having m^{6}A activity modifying coding and noncoding RNAs [37–39].

Spatiotemporal installation of m^{6}A

Evidence for m^{6}A on pre-mRNA

Early exploratory studies of m^{6}A supported a model where installation occurs early in the life of RNA. Pioneering reports examining adenovirus type 2 RNA methylation suggested early RNA processing and m^{6}A installation are coupled, with methylation occurring soon after transcription [40]. Detection of m^{6}A in pre-mRNA of an endogenous mammalian gene supported a similar model for mammalian RNAs, where methylation occurs either cotranscriptionally, or very soon after transcription [41]. Furthermore, Bokar and colleagues demonstrated that the methylation activity resides in the nuclear fraction of HeLa cell extracts [19].

A breakthrough in our general understanding of the m^{6}A landscape came with the advent of genome-wide m^{6}A profiling technology using antibody-based approaches coupled to massive parallel sequencing [4,5]. These comprehensive works revealed the widespread distribution of the modification. In mouse and human cells approximately 10 thousand peaks could be identified within coding and noncoding RNAs. In addition, the distribution and global abundance of m^{6}A were shown to vary depending on cell type, developmental stage and environmental stimulus. These studies revealed evolutionary conservation of prominent features of m^{6}A distribution, including methylation at a degenerate consensus motif, enrichment in 3′UTRs and a sharp peak close to stop codons. Subsequent methodological refinements including ultraviolet light cross-linking and site-specific antibody induced mutagenesis improved the resolution to the nucleotide level, and revealed m^{6}A on small nucleolar RNAs (snoRNAs) [9,10].

An important additional observation from the genome-wide m^{6}A profiling studies was the identification of enrichment peaks within introns, despite the starting RNA material not being enriched for unspliced pre-mRNAs. This finding is again supportive of RNA methylation happening at early stages of RNA

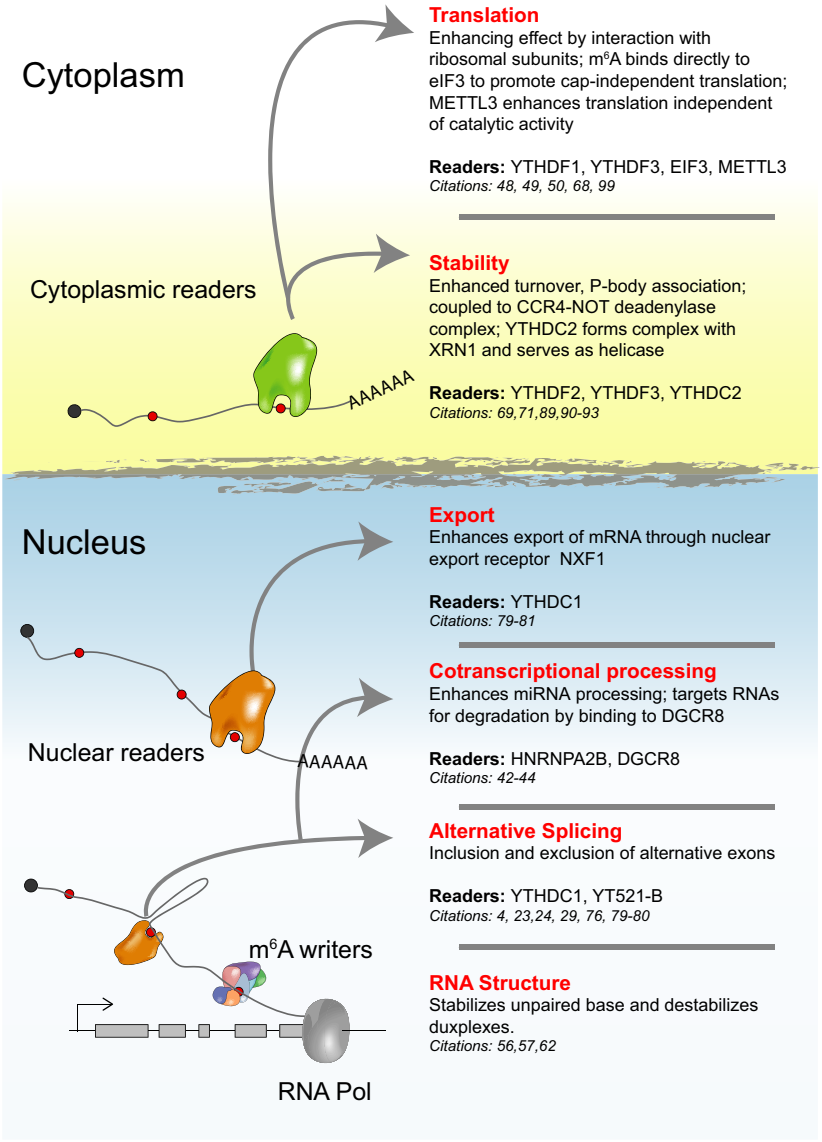

Fig. 2. Functional implications of m^{6}A modified RNA. Since transcription, m^{6}A modified RNAs are subject to effects altering their structure, splicing, processing, nuclear export, stability and translation at distinct cellular compartments. Some of these effects are mediated by reader proteins including YTH family proteins that contain a dedicated motif to recognize the methyl moiety as well as others such as HNRNPA2B and EIF3.

processing. To directly quantify m^{6}A within growing nascent pre-mRNA chains, one study used a method to enrich for chromatin-associated pre-mRNA (CA-RNA) using stringent purification conditions [42]. This study then compared m^{6}A using a cross-linking and immunoprecipitation approach of chromatin-associated, nuclear, and cytoplasmic RNA fractions. Surprisingly, pre-mRNA enrichment did not show a massive increase in the number of m^{6}A modified introns in CA-RNA relative to nuclear or cytoplasmic fractions, suggesting the methylation machinery has a preference for exonic sequences (the sequence of events regarding methylation and splicing and interplay between these processes is further discussed below). Additionally, the content of m^{6}A in cytoplasmic mRNA was largely indistinguishable from that in the newly synthesized CA-RNA or nucleoplasmic mRNA. This suggests m^{6}A is mostly deposited when RNA is still associated with chromatin. The authors further interpreted these observations as indicative of m^{6}A

levels remaining unchanged in the three cellular compartments studied and by extension proposed that quantitatively little methylation or demethylation occurs on cytoplasmic mRNA. This view is at odds with the idea of m^{6}A being a dynamic modification and is currently a matter of debate [16–18].

Using an alternative approach to isolate nascent RNA transcripts through BrU labelling, a recent report profiling m^{6}A found the mark to reside primarily in intronic sequences [43]. Perhaps the discrepancy between abundance of m^{6}A in introns compared to previous work is due to differing efficiencies in capturing unspliced pre-mRNA. In the same study, a comparison between CA-RNA and 15 min BrU-labelled RNA shows the latter to be more substantially unspliced. Supportive of intronic deposition is our observation that Mettl3 ChIP-seq data sets very often localize the protein within intronic regions albeit, high confidence peaks are often broad and span several features, likely due to the cross-linking strategy used [44]. Additionally, METTL3/14 and WTAP PAR-CLIP profiling experiments often colocalized the writers within introns (~ 29–34%) [20]. All together, these findings are consistent with a model of early cotranscriptional deposition of the m^{6}A mark as RNA is newly transcribed.

Another example of early nuclear RNA processing events coupled to m^{6}A deposition is the microRNA (miRNA) biogenesis pathway. Reports linking miRNA maturation to m^{6}A identified an enrichment for the modification on primary miRNA sequences in nuclear RNA [45,46]. Recently, our own work has shown that Mettl3 and Dgcr8, the RNA binding cofactor of Drosha necessary for pri-miRNA cleavage, often colocalize throughout the genome at miRNA encoding loci, as well as other RNA species including coding, long noncoding RNAs (lncRNAs) and snoRNAs [44]. Early deposition of m^{6}A is intriguing because it allows the machinery that recognizes and acts upon the modification to influence the processing and fate of RNAs subsequently, as will be discussed further.

Nuclear localization of the m^{6}A installation machinery

Identification of the proteins required for m^{6}A installation enabled a subcellular interrogation of this process. Using microscopy, the METTL3/METTL14 dimer and associated factor WTAP were localized to nuclear speckles [20,22,47]. Interestingly, WTAP knockdown in human cells resulted in loss of endogenous METTL3/METTL14 nuclear localization and not *vice versa*. This experiment suggested WTAP has a critical role in guiding the catalytic dimer to its RNA targets (Fig. 1). Other subunits of MACOM, the WTAP containing complex [34], have also been shown to have a role in localizing the Mettl3/14 dimer. Loss of function of Zc3h13 (discussed below), for example results in Mettl3 and Mett14 re-localizing to the cytoplasm, as revealed by imaging and cell fractionation experiments in mESCs [48]. Given that the disruption of Zc3h13 and other MACOM components severely reduces m^{6}A levels on mRNA, it is tempting to conclude that localizing the MTase dimer to the correct nuclear compartment is indispensable to install the mark.

The use of chromatin immunoprecipitation (ChIP) experiments to localize m^{6}A writers revealed important insights into the localization of the machinery with respect to the genome. ChIP-sequencing of METTL proteins in mammalian cells demonstrate their proximity to chromatin [44,49]. The profiles of METTL3 binding are variable in these reports, perhaps due to differences in organisms, cell states (mESC vs. human acute myeloid leukaemia [AML] cells) and cross-linking strategies. Whereas Mettl3 binding in mESCs showed a strong bias towards the 3′ end of target genes, in accordance with m^{6}A enrichment at the 3′UTRs and stop codons, in human AML cells METTL3 bound predominantly to transcription start sites (TSS) and to far fewer targets. Interestingly, METTL3 binding often did not correlate with METTL14 targets in AML cells, raising the provocative idea that they might have independent roles on chromatin in human AML cells. Notably, the role of m^{6}A deposition at the TSS in the malignant cells was suggested to promote translation of target mRNAs essential to maintain AML growth. A similar model had been suggested for 5′UTR methylation of heat shock genes promoting cap-independent translation upon heat stress (discussed below) [50,51]. Supporting the notion of an environmentally responsive m^{6}A writing machinery, Mettl3 binding radically changes upon heat shock in mESCs, selectively re-localizing to heat shock response genes [44].

Although the predominant view is that m^{6}A is installed in the nuclear compartment, METTL3 itself has been described to associate with ribosomes and promote translation in human cancer cells [52]. This study suggested the machinery could localize outside of the nucleus and associate with specific mRNAs. It was also proposed that METTL3 influences translation, independent of its catalytic activity and of its association with METTL14 and WTAP. These observations would be consistent with the idea that stimuli such as cellular stress or oncogenic transformation can alter the localization of the m^{6}A writers, prompting

them to modify novel mRNA targets. These can fall within noncanonical genic locations, such as the 5′UTR of mRNAs, thereby altering translation dynamics. In addition, it is possible that the methylation machinery can even shuttle to the cytoplasm to *de novo* methylate mRNAs.

Transcriptional mode influences m^{6}A deposition

Given that METTL3/METTL14 deposition of m^{6}A is an early RNA processing event, it is tempting to speculate that it might be directly linked to the act of transcription. Protein–protein interaction studies do not point towards direct associations of m^{6}A writers with the core transcriptional machinery under normal physiological conditions [26,34,35]. Recently, however, it was found that downstream members of the TGFβ pathway, the SMAD2/3 transcriptional effectors, directly interact with METTL3/METTL14 and WTAP in human pluripotent stem cells [53]. In this study, SMAD2/3 proteins were shown to promote the recruitment of m^{6}A writers to TGFβ target mRNAs that are essential for pluripotency, thereby destabilizing them and allowing the rapid transition out of pluripotency upon differentiation. Importantly, this study implies that transcriptional modulators can influence the target specificity of m^{6}A writers and warrants additional interrogation of yet to be identified regulatory binding partners of the METTL3/METTL14 and MACOM complexes.

A study investigating the relationship between transcription and translation efficiency proposes these two processes are coupled and that the former regulates the latter [54]. In addition, the authors propose that communication between these compartmentalized processes is mediated by m^{6}A on mRNAs. Mainly through pharmacological manipulation (low level camptothecin (CPT) treatment, which slows down RNA polymerase II [RNAPII] [55]), the authors show that slowly transcribed genes are more likely to be m^{6}A modified. Furthermore, METTL3 coprecipitated with RNAPII upon CPT treatment. These results suggest augmented cotranscriptional m^{6}A modification if RNAPII elongation is impeded. Although this is an attractive idea, more evidence is needed to support such a model. Future studies should address how the m^{6}A machinery is coupled to transcription. For example METTL3 has been shown to be post-translationally modified, phosphorylated and SUMOylated at several sites [33,47]. Such modification could impact catalytic activity, as has been shown when METTL3 is SUMOylated, as well as binding to interaction partners.

Intrinsic effects of m^{6}A

m^{6}A alters RNA secondary structure

The most direct effects of the m^{6}A modification on structure and base pairing of RNA are subtle. Perhaps this partially explains why it has been challenging to assign functions to the modification *in vivo*. The methyl group at the *N*6 position of adenosine does not alter Watson–Crick A•U base pairing [56], but nevertheless affects RNA structure by stabilizing unpaired bases and destabilizing duplexes. In solution, the 6-methyl group on an adenine base is known to exist in two conformations *anti* and *syn*, with the latter being energetically favoured [57]. NMR studies revealed that m^{6}A•U base pairing requires flipping and trapping of the methylamino group into an energetically unfavourable spring-loaded *anti* orientation [58]. In this model m^{6}A acts as a compressed spring that is locked into place by its paired context [58] resulting in a net destabilization of the duplex. In unpaired positions, m^{6}A stacks better than an unmodified base, thereby stabilizing stretches of single-stranded RNA.

Global *in vivo* secondary structure profiling to examine the impact of m^{6}A

Recent methodological advances permit the examination of RNA secondary structure at the genome-wide level and to single-nucleotide resolution *in vivo* (comprehensively reviewed in Ref. [59]). One strategy employs an alkylating reagent [dimethylsulphate (DMS)] that reacts with unpaired adenosines and cytidines and blocks reverse transcription. Coupled to massive parallel sequencing, prematurely terminated cDNA can be used as a proxy for DMS modification and unpaired RNA [60,61]. Another chemical approach termed SHAPE (selective 2′-hydroxyl acylation analysed by primer extension) uses chemicals that attack the sugar-phosphate backbone and also block reverse transcription [62]. Using an adaptation of SHAPE (icSHAPE), which allows *in vivo* RNA structure determination for all four bases, the influence of m^{6}A on RNA structure was examined [63]. In mESCs comparison of icSHAPE signals at m^{6}A-modified vs. unmodified sites revealed stronger icSHAPE reactivity (suggesting unpaired RNA) at positions surrounding the modified A. Moreover, in *Mettl3* KO mESCs canonical motif sites that lost m^{6}A also widely lost icSHAPE signal, suggesting a gain in pairing and secondary structure [63]. These findings are consistent with structural studies showing that m^{6}A has a destabilizing effect on paired bases. Future work including

FEBS Letters **592** (2018) 2845–2859 © 2018 The Authors. *FEBS Letters* published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.

other genome-wide secondary structure probing strategies such as DMS-sequencing will be important to further characterizing the role of m^{6}A in regulating RNA secondary structure *in vivo*.

m^{6}A decoders

YTH proteins as m^{6}A readers

Following the conceptual framework of m^{6}A writers selectively decorating RNAs, 'reader' proteins can recognize, decode and influence RNA fate (Fig. 2). The best-described m^{6}A readers are the YTH domain family of proteins, initially identified by RNA affinity chromatography coupled to mass spectrometry using m^{6}A modified RNA as bait [4]. The YTH domain has been identified by sequence comparison in 174 different proteins expressed in eukaryotes [64]. Vertebrate YTH proteins can be classified into three categories: YTHDF (YTH domain-containing family proteins) family, YTHDC1 (YTH domain-containing protein 1, also called DC1) and YTHDC2 (YTH domain-containing protein2, also called DC2). Humans have three YTHDF family members, and a single copy of YTHDC1 and YTHDC2 (for an excellent review refer to [65]). Structural studies have shown that the methyl moiety of m^{6}A is selectively recognized by an aromatic cage that is formed by two tryptophans and a leucine [66–68] or three tryptophans [69]. The selective binding of YTH proteins to methylated RNA was further confirmed by gel shift assays where the YTH domain preferentially binds methylated RNA oligonucleotides and *in vitro* pull-down assays confirming enhanced binding to methylated vs. nonmethylated RNA [4,21,67,70]. Transcriptome-wide binding studies of endogenous YTH proteins using CLIP methods demonstrated that most YTH proteins bind to the m^{6}A mammalian motif (DRACH) in RNA with a nearly complete overlap for the YTHDF members [27,70,71]. YTHDC1 is localized to a specific nuclear compartment, the YT body, where it was first proposed to regulate splicing [72]. YTHDC1 binds to nuclear RNAs including *XIST*, which is highly m^{6}A modified. As discussed above, m^{6}A is required for X chromosome gene silencing [27] and ablating METTL3 or RBM15 impairs this process. Strikingly, artificial tethering of YTHDC1 to *XIST* rescues *XIST*-mediated silencing upon loss of m^{6}A. YTHDF1–3 are cytoplasmic proteins that have been proposed to have independent roles in regulating mRNA stability and translation (discussed below) [70,71,73], despite the fact that YTHDF proteins are highly structurally related and share high amino acid identity. Whether these proteins are functionally fully redundant or indeed have specialized functions remains to be further investigated.

Expanding the m^{6}A reader list

In addition to the YTH domain proteins, other m^{6}A readers have been described. The initial m^{6}A RNA pull-down assays also identified the RRM domain protein ELAVL1 [4]. A more recent systematic mass-spectrometry-based report screened for m^{6}A interactors in various cell types and sequence contexts [74]. Prominent proteins described in this work, as expected, included YTH family proteins and the ALKBH5 demethylase. Novel factors included FMR1 and its paralogues FXR1 and FXR2 as sequence-context-dependent m^{6}A readers. Interestingly, some proteins were repelled specifically by the presence of the methyl moiety on RNA including G3BP1 in various cell types and mRNA sequence contexts.

m^{6}A-dependent regulation of splicing

A role for m^{6}A in the process of pre-mRNA splicing has been apparent since the beginning of the 1980s when this modification was found at position 43 of the U6 small nuclear RNA (U6 snRNA, an abundant RNA critical for splicing) [75]. Until very recently the MTase that catalyses m^{6}A on U6 snRNA was unknown, although it was clear that the enzyme was distinct from the MTase that methylated mRNA in HeLa nuclear extracts (later identified as Mettl3) [76]. The elusive MTase, METTL16, that methylates U6 snRNA was eventually identified [38]. Interestingly, METTL16 has evolved additional roles in vertebrates including the crucial homeostasis maintenance of methyl donor S-adenosylmethionine (SAM). In humans the SAM synthetase MAT2A generates SAM from methionine and ATP. There are two MAT2A RNA isoforms, a cytoplasmic mRNA and a nuclear retained-intron isoform (MAT2A-RI), which is subject to decay [77]. In conditions of low cellular SAM, METTL16 m^{6}A methylates MAT2A pre-mRNA on a conserved hairpin and promotes splicing of the MAT2A retained intron. The balance in isoforms then shifts to the cytoplasmic transcripts, which promotes SAM synthesis. Of note, a recent report suggests that m^{6}A regulation of MAT2A may also be mediated through mRNA degradation by recognition of the reader protein YTHDC1 [39].

The role of Mettl3-mediated m^{6}A in splicing has been best characterized in *Drosophila* [28,29,78]. In particular, m^{6}A is necessary for correct splicing and to achieve maximal Sex lethal (*Sxl*) expression, the

master regulator of sex determination. Sxl then suppresses dosage compensation in females by inhibiting the translation of Male-specific lethal-2 (Msl-2), which is required for the upregulation of transcription from the single male X chromosome [8]. The functional role of m^{6}A in splicing of *Sxl* was addressed in three studies and all coincided on sex determination phenotypes with varying penetrance depending on the m^{6}A component ablated. Furthermore, the *Drosophila* YTH family member m^{6}A reader, YT521-B recently renamed Ythdc1, is necessary for correct exon exclusion and to achieve maximum *Sxl* expression in females [28,29]. Finally, a splicing defect in *Sxl* and a sex determination phenotype is also apparent in flies with impaired expression of the MACOM component Zc3h13/*Flacc* [34]. In addition to m^{6}A installation, MACOM components have additional important roles. In flies it was found through mRNA profiling experiments that MACOM components showed a much stronger perturbation upon knock down when compared to the MTAses [34]. In human cells, a study has shown that WTAP expression is cell cycle regulated, and that it controls the stability of cyclin A2 mRNA [79]. Therefore, we propose that future studies should identify mutations disrupting the interaction between MACOM and the MTAse duplex to cleanly dissect m^{6}A independent functions.

In humans the nuclear reader protein YTHDC1 has a role in the inclusion of alternative exons [80]. YTHDC1 interacts with pre-mRNA splicing factors, including splicing enhancer-binding SR proteins (Fig. 2). Mechanistically, m^{6}A present in alternative exons induces their inclusion by recruiting YTHDC1, which in turn recruits the splicing factor Serine and arginine-rich splicing factor 3 (SRSF3) [80]. YTHDC1 also inhibits pre-mRNA binding of the exon-skipping factor SRSF10 by competitively binding to SRSF3 and occupying SRSF10 RNA landing sites. In conclusion, although m^{6}A has been clearly linked to pre-mRNA splicing, more work will be required for a full mechanistic understanding.

Nuclear export of methylated mRNA

Another key aspect that has been linked to m^{6}A modification is nuclear export. For example RNA viruses exploit the endogenous methylation machinery to modify their mRNAs. Zika and HIV-1 are extensively m^{6}A methylated and exploit the modification to enhance nuclear export and other processing steps during replication [81,82]. In noninfected human cells, the m^{6}A-binding protein YTHDC1 mediates export of methylated mRNA from the nucleus to the cytoplasm [83]. As described above, YTHDC1 interacts with SRSF3, which can also interact with the nuclear export receptor NXF1 (Fig. 2). Inhibition of YTHDC1 does not affect global m^{6}A levels in mRNA, but instead results in accumulation of the mark in nuclear mRNA and in depletion from the cytoplasmic pool. This finding was supported by tethering YTHDC1 to a synthetic mRNA, which facilitated its export and concomitantly increased cytoplasmic abundance and translation. Expanding on the reader-tethering assay, future studies on the role of m^{6}A in RNA transport should include single-molecule imaging techniques. These types of experiments will be crucial to yield quantitative information on the rate at which transport is enhanced.

Turnover of methylated RNA

One of the best-described functions for m^{6}A so far is in controlling RNA stability. In this respect, the modification is remarkably versatile in employing distinct mechanisms and molecular partners for turnover, depending on target RNA and cellular compartment (Fig. 2). Some have proposed that modulating RNA turnover is the main function of m^{6}A, at least in some cellular contexts such as human cancer cells and mESCs, [16,17] and that its role in pre-mRNA splicing is minor.

The physiological importance of m^{6}A-mediated regulation of RNA stability has been nicely exemplified in the context of mESCs, where Mettl3 is necessary to terminate naïve pluripotency [84]. *Mettl3* KO mESCs fail to terminate their naïve state and instead undergo aberrant and restricted lineage priming at the post-implantation stage, eventually resulting in embryonic lethality. Thus, by directly reducing mRNA stability of key naïve pluripotency transcripts, m^{6}A controls the transition from naïve to primed pluripotency.

Mechanisms of m^{6}A-mediated RNA decay

From nucleus to cytoplasm, the earliest example of m^{6}A-mediated RNA turnover occurs cotranscriptionally [44]. As previously mentioned, the miRNA biogenesis machinery can bind and degrade non-miRNA targets in the nucleus in an m^{6}A-dependent manner [85–91]. In this context, m^{6}A presumably confers specificity and facilitates the recognition of RNA structures by DGCR8. The molecular mechanism by which m^{6}A aids DGCR8/DROSHA to recognize and cleave its targets has been addressed and is in part mediated by the reader protein HNRNPA2B [46]. This cotranscriptional regulation mechanism is responsive to the

FEBS Letters **592** (2018) 2845–2859 © 2018 The Authors. *FEBS Letters* published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.

environment as the players involved can rapidly relocalize to essential genes. This is the case for *Hsp70* during heat shock where there is massive accumulation of Mettl3 and Dgcr8 [44]. Future studies should examine the response of these players to other environmental stimuli or conditions that globally alter the transcriptome.

An important mechanism of m^{6}A-mediated RNA decay functioning in the cytoplasm employs the reader protein YTHDF2 [70]. In this study RNA half-life profiling revealed a pronounced increase in stability in YTHDF2 knockdown cells compared to control samples. The effect correlated well with YTHDF2 binding, where RNAs with more binding displayed increased stabilization. Importantly, YTHDF2 colocalizes with three markers (DCP1a, GW182 and DDX6) of processing bodies (P bodies) in the cytoplasm [70]. The highly related m^{6}A reader protein YTHDF3 has been reported to synergize with YTHDF1 and YTHDF2 by potentiating their binding to target RNAs thereby promoting their effects on translation and decay, respectively [73]. A separate study found that m^{6}A-modified RNAs exhibit accelerated deadenylation mediated by the CCR4–NOT deadenylase complex [92]. The deadenylation is mediated through the direct recruitment of the CCR4–NOT complex by YTHDF2. Another reader protein that was recently shown to serve as an intermediary between m^{6}A and the RNA degradation machinery is YTHDC2. This cytoplasmic reader and RNA helicase is essential for meiosis in mice [93–96]. Data suggesting that Ythdc2 mediate m^{6}A-dependent RNA degradation includes the observation that m^{6}A decorated transcripts are upregulated in *Ythdc2* knockout testes compared to wild-type controls [93,94]. Biochemical characterization of Ythdc2 binding partners identified the exoribonuclease Xrn1 as the top associated factor in mouse testis protein extracts in addition to Meioc, a highly conserved meiosis-specific protein. In sum, the usage of m^{6}A reader proteins to target a particular subset of transcripts for expedited degradation is a conserved strategy employed in diverse cell types and executed by varying molecular partners.

Another way in which m^{6}A regulates RNA stability, independent of direct reader proteins, is through its effect on secondary structure (discussed above). One example is the RBP HuR which binds to U-rich regions in the 3′-UTR of transcripts [97] and can block miRNA guided Argonaute complexes, thus preventing degradation [98]. mESC transcripts encoding developmental regulators are highly m^{6}A decorated [21,84]. One study proposed that HuR binding is impaired in m^{6}A modified transcripts allowing miRNA-mediated repression [21]. Accordingly, Mettl3 knockdown results in decreased Ago2 binding to a known methylated transcript and its concomitant stabilization.

m^{6}A effect on translation

A growing body of work supports the hypothesis that m^{6}A also serves as means to communicate nuclear transcriptional information to the downstream translation machinery [54,71] (Fig. 2). In yeast and human cells, when examining the distribution of m^{6}A modified mRNAs there was a noticeable enrichment in ribosome associated fractions. Conversely, nonribosome-associated fractions were depleted for m^{6}A [71,99]. A study using single-molecule methods to probe the effect of m^{6}A on mRNA showed changes in translational dynamics [100]. Although m^{6}A base pairs with uridine during decoding, methylated codons have slower translation-elongation dynamics, leading the authors to propose that the presence of an m^{6}A within a codon slows down cognate-tRNA decoding by acting as a barrier to tRNA accommodation [100].

On the other hand, a comprehensive examination of the functional role of the m^{6}A reader YTHDF1 showed that it enhances the translation efficiency of m^{6}A modified RNAs [71], through direct interactions with initiation factors and ribosomal subunits. High-throughput sequencing experiments uncovered a positive correlation between the ribosome association of YTHDF1 bound mRNAs and the number of YTHDF1-binding sites on the target mRNAs. Furthermore, tethering experiments revealed 72% increased translation efficiency for YTHDF1-tethered transcripts [71]. Whether such effects on translation are restricted to the subset of transcripts bound by YTHDF1 remains an open question. Notably, analysis of translation efficiency in WT vs. *Mettl3* KO ESCs by ribosome profiling revealed only a minor yet significant increase in translation efficiency in KO cells, on both methylated and unmethylated transcripts [84].

m^{6}A as a mediator of cap-independent translation during stress

Although the majority of m^{6}A shows a 3′ bias and is close to the stop codon, substantial methylation is detectable in 5′ UTRs. Antibody-based m^{6}A profiling experiments have been shown to also detect a related RNA modification, N^6,2′-O-dimethladenosine (m^{6}Am). This modification has the unique property that it is present only when the first cap-adjacent nucleotide is an adenosine and is thought to enhance stability of mRNAs [101]. m^{6}A is not detected at the first position of mRNA and can therefore be readily distinguished

from m^{6}Am by position in single-nucleotide resolution approaches [102]. It has been proposed that mRNAs containing m^{6}A in their 5′ UTR can be translated in a cap-independent manner [50,51]. In cap-independent translation, mRNAs do not require eIF4E and are translated under normal resting conditions, as well as under environmental stress, viral infection, or other disease conditions [103]. m^{6}A in the 5′ UTR can bind eukaryotic initiation factor 3 (eIF3) and thus act as an m^{6}A-induced ribosome engagement site (MIRES), which promotes translation of mRNA independent of the presence of a cap structure. Interestingly, cells subjected to heat stress exhibit increased m^{6}A methylation (and not m^{6}Am) within the 5′UTR of newly transcribed mRNAs, selectively on stress-inducible mRNAs. The 5′ accumulation was observed also when cells were exposed to UV radiation, supporting a generalized mechanism for other forms of stress [50]. The accumulation of 5′UTR methylation was proposed to be mediated through nuclear translocation of YTHDF2, where it protects methylated adenosines from eraser FTO [51]. Recently, however, a report suggested that *in vivo*, FTO demethylates m^{6}Am as its preferred substrate. It remains to be tested if the nuclear translocation and shielding effect of YTHDF2 prevents demethylation by ALKBH5.

Conclusions and Perspectives

The field of RNA modifications has recently garnered a great deal of attention. Among the most prominent modifications, m^{6}A stands out as multi-purpose signal on RNA. Deposited early during transcription by multiprotein writer complexes that are responsive to cell context and environmental cues, m^{6}A functions in key steps of mRNA metabolism. Intrinsic properties of RNA are affected by the modification, including base-pairing stability and secondary structure. The repertoire of proteins that can interact with a given RNA is also different once it is modified. Reader proteins affect early RNA processing events including splicing, poly-A usage and cotranscriptional cleavage [10]. In the cytoplasm, m^{6}A drastically influences mRNA turnover. Cytoplasmic YTH proteins mediate association to the decay machinery. It is also evident that m^{6}A can affect translation dynamics, in some cases potentiated through direct recruitment of translation initiation factors.

Despite significant progress in recent years, important basic questions regarding m^{6}A biology remain elusive. For example a better understanding of the rules that define the methylation machinery's specificity is urgently needed. It remains unclear which determinants make an mRNA a good substrate for methylation. Although the catalytic core of the methylation machinery and its obligate cofactors have been characterized with some detail, it is likely that m^{6}A biogenesis is a carefully orchestrated process that requires multiple additional factors and cell-specific regulators. We therefore anticipate that additional players involved in m^{6}A deposition will be discovered and characterized in the future.

Besides the advancement of our mechanistic understanding of m^{6}A biology, its physiological roles are also becoming increasingly evident in multiple organisms and cell types (description of which unfortunately is beyond the scope of this review). It has also become abundantly clear that, as with many fundamental cell biological processes, m^{6}A can be hijacked in several forms of cancer. A growing body of work suggests that m^{6}A is implicated in glioblastoma [104–106]. There is also a clear link of m^{6}A to leukaemia. For example METTL3 inhibition in human myeloid leukaemia cell lines induces differentiation, apoptosis and delays leukaemia in transplantation experiments [49,107]. Some forms of acute megakaryoblastic leukaemias are mediated by a chromosomal translocation of *RBM15* with the *MAL* gene, where the fusion protein drives the development of haematological malignancy [108]. It is thus tempting to speculate that a RBM15 fusion protein could re-direct the m^{6}A machinery to new targets, thereby tilting the transcriptome towards malignancy. Hence, manipulation of the m^{6}A pathway as a novel approach towards cancer therapy might be not too far-fetched.

Acknowledgements

We are grateful to Alex Tuck and Sarah Carl for comments on the manuscript. Work performed in MB's laboratory is supported by the Novartis Research Foundation and the Swiss National Science Foundation NCCR RNA & Disease (grant no. 141735). We apologize to colleagues whose work we were not able to cite due to space constraints.

References

1 Boccaletto P, MacHnicka MA, Purta E, Pitkowski P, Baginski B, Wirecki TK, De Crécy-Lagard V, Ross R, Limbach PA, Kotter A *et al.* (2018) MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic Acids Res* **46**, D303–D307.

2 Wei CM, Gershowitz A and Moss B (1975) Methylated nucleotides block 5′ terminus of HeLa cell messenger RNA. *Cell* **4**, 379–386.

3 Rottman F, Shatkin AJ and Perry RP (1974) Sequences containing methylated nucleotides at the 5′ termini of messenger RNAs: possible implications for processing. *Cell* **3**, 197–199.
4 Dominissini D, Moshitch-moshkovitz S, Schwartz S, Ungar L, Osenberg S, Cesarkas K, Jacob-hirsch J, Amariglio N, Kupiec M, Sorek R *et al.* (2012) Topology of the human and mouse m^{6}A RNA methylomes revealed by m^{6}A-seq. *Nature* **485**, 201–206.
5 Meyer KD, Saletore Y, Zumbo P, Elemento O, Mason CE and Jaffrey SR (2012) Resource comprehensive analysis of mRNA methylation reveals enrichment in 3 0 UTRs and near stop codons. *Cell* **149**, 1635–1646.
6 Wan Y, Tang K, Zhang D, Xie S, Zhu X, Wang Z and Lang Z (2015) Transcriptome-wide high-throughput deep m^{6}A-seq reveals unique differential m^{6}A methylation patterns between three organs in *Arabidopsis thaliana*. *Genome Biol* **16**, 272.
7 Wang C, Zhu Y, Bao H, Jiang Y, Xu C, Wu J and Shi Y (2016) A novel RNA-binding mode of the YTH domain reveals the mechanism for recognition of determinant of selective removal by Mmi1. *Nucleic Acids Res* **44**, 969–982.
8 Roignant JY and Soller M (2017) m^{6}A in mRNA: an ancient mechanism for fine-tuning gene expression. *Trends Genet* **33**, 380–390.
9 Linder B, Grozhik AV, Olarerin-George AO, Meydan C, Mason CE and Jaffrey SR (2015) Single-nucleotide-resolution mapping of m^{6}A and m^{6}Am throughout the transcriptome. *Nat Methods* **12**, 767–772.
10 Ke S, Alemu EA, Mertens C, Gantman EC, Fak JJ, Mele A, Haripal B, Zucker-Scharff I, Moore MJ, Park CY *et al.* (2015) A majority of m^{6}A residues are in the last exons, allowing the potential for 3′ UTR regulation. *Genes Dev* **29**, 2037–2053.
11 Dimock K and Stoltzfus CM (1977) Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. *Biochemistry* **16**, 471–478.
12 Schibler U, Kelley DE and Perry RP (1977) Comparison of methylated sequences in messenger RNA and heterogeneous nuclear RNA from mouse L cells. *J Mol Biol* **115**, 695–714.
13 Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang YG *et al.* (2011) N^6-Methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol* **7**, 885–887.
14 Bartosovic M, Molares HC, Gregorova P, Hrossova D, Kudla G and Vanacova S (2017) N^6-methyladenosine demethylase FTO targets pre-mRNAs and regulates alternative splicing and 3′-end processing. *Nucleic Acids Res* **45**, 11356–11370.
15 Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, Vågbø CB, Shi Y, Wang WL, Song SH *et al.* (2013) ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell* **49**, 18–29.
16 Darnell RB, Ke S and Darnell JE (2017) Pre-mRNA processing includes N^6 methylation of adenosine residues that are retained in mRNA exons and the fallacy of "RNA epigenetics". *RNA* **24**(3), 262–267.
17 Rosa-Mercado NA, Withers JB and Steitz JA (2017) Settling the m^{6}A debate: methylation of mature mRNA is not dynamic but accelerates turnover. *Genes Dev* **31**, 957–958.
18 Zhao BS, Nachtergaele S, Roundtree IA and He C (2017) Our views of dynamic. *RNA J* **24**, 268–272.
19 Bokar JA, Shambaugh ME, Polayes D, Matera AG and Rottman FM (1997) Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N^6-adenosine)-methyltransferase. *RNA* **3**, 1233–1247.
20 Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L and Jia G (2014) A METTL3 – METTL14 complex mediates mammalian nuclear RNA N^6-adenosine methylation. *Nat Chem Biol* **10**, 93–95.
21 Wang Y, Li Y, Toth JI, Petroski MD, Zhang Z and Zhao JC (2014) N^6-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. *Nat Cell Biol* **16**, 191–198.
22 Ping XL, Sun BF, Wang L, Xiao W, Yang X, Wang WJ, Adhikari S, Shi Y, Lv Y, Chen YS *et al.* (2014) Mamalian WTAP is a regulatory subunit of the RNA N^6-methyladenosine methyltransferasme. *Cell Res* **24**, 177–189.
23 Wang P, Doxtader KA and Nam Y (2016) Structural basis for cooperative function of Mettl3 and Mettl14 methyltransferases. *Mol Cell* **63**, 306–317.
24 Wang X, Feng J, Xue Y, Guan Z, Zhang D, Liu Z, Gong Z, Wang Q, Huang J, Tang C *et al.* (2016) Structural basis of N^6-adenosine methylation by the METTL3-METTL14 complex. *Nature* **534**, 575–578.
25 Śledź P and Jinek M (2016) Structural insights into the molecular mechanism of the m^{6}A writer complex. *eLife* **5**, e18434.
26 Schwartz S, Mumbach MR, Jovanovic M, Wang T, Maciag K, Bushkin GG, Mertins P, Ter-Ovanesyan D, Habib N, Cacchiarelli D *et al.* (2014) Perturbation of m^{6}A writers reveals two distinct classes of mRNA methylation at internal and 5′ sites. *Cell Rep* **8**, 284–296.
27 Patil DP, Chen C-K, Pickering BF, Chow A, Jackson C, Guttman M and Jaffrey SR (2016) m^{6}A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* **537**, 369–373.
28 Lence T, Akhtar J, Bayer M, Schmid K, Spindler L, Ho CH, Kreim N, Andrade-Navarro MA, Poeck B, Helm M *et al.* (2016) m^{6}A modulates neuronal functions and sex determination in Drosophila. *Nature* **540**, 242–247.

29 Haussmann IU, Bodi Z, Sanchez-Moran E, Mongan NP, Archer N, Fray RG and Soller M (2016) m^{6}A potentiates Sxl alternative pre-mRNA splicing for robust Drosophila sex determination. *Nature* **540**, 301–304.
30 Růžička K, Zhang M, Campilho A, Bodi Z, Kashif M, Saleh M, Eeckhout D, El-Showk S, Li H, Zhong S *et al*. (2017) Identification of factors required for m^{6}A mRNA methylation in Arabidopsis reveals a role for the conserved E3 ubiquitin ligase HAKAI. *New Phytol* **215**, 157–172.
31 Niessen M, Schneiter R and Nothiger R (2001) Molecular identification of virilizer, a gene required for the expression of the sex-determining gene sex-lethal in *Drosophila melanogaster*. *Genetics* **157**, 679–688.
32 Guo J, Tang H-W, Li J, Perrimon N and Yan D (2018) Xio is a component of the *Drosophila* sex determination pathway and RNA *N*^{6}-methyladenosine methyltransferase complex. *Proc Natl Acad Sci U S A* **115**, 3674–3679.
33 Du Y, Hou G, Zhang H, Dou J, He J, Guo Y, Li L, Chen R, Wang Y, Deng R *et al*. (2018) SUMOylation of the m^{6}A-RNA methyltransferase METTL3 modulates its function. *Nucleic Acids Res* **04**, 1–14.
34 Knuckles P, Lence T, Haussmann IU, Jacob D, Kreim N, Carl SH, Masiello I, Hares T, Villaseñor R, Hess D *et al*. (2018) Zc3h13/Flacc is required for adenosine methylation by bridging the mRNA-binding factor Rbm15/Spenito to the m^{6}A machinery component Wtap/Fl(2)d. *Genes Dev* **32**, 415–429.
35 Horiuchi K, Kawamura T, Iwanari H, Ohashi R, Naito M, Kodama T and Hamakubo T (2013) Identification of Wilms' tumor 1-associating protein complex and its role in alternative splicing and the cell cycle. *J Biol Chem* **288**, 33292–33302.
36 Chang M, Lv H, Zhang W, Ma C, He X, Zhao S, Zhang Z-W, Zeng Y-X, Song S, Niu Y *et al*. (2017) Region-specific RNA m^{6}A methylation represents a new layer of control in the gene regulatory network in the mouse brain. *Open Biol* **7**, 170166.
37 Warda AS, Kretschmer J, Hackert P, Lenz C, Urlaub H, Höbartner C, Sloan KE and Bohnsack MT (2017) Human METTL 16 is a N^6-methyladenosine (m^{6}A) methyltransferase that targets pre-mRNAs and various non-coding RNAs. *EMBO Rep* **18**, 2004–2014.
38 Pendleton KE, Chen B, Liu K, Hunter OV, Xie Y, Tu BP and Conrad NK (2017) The U6 snRNA m^{6}A methyltransferase METTL16 regulates SAM synthetase intron retention. *Cell* **169**, 824–835.e14.
39 Shima H, Matsumoto M, Ishigami Y, Ebina M, Muto A, Sato Y, Kumagai S, Ochiai K, Suzuki T and Igarashi K (2017) S-Adenosylmethionine synthesis is regulated by selective N^6-adenosine methylation and mRNA degradation involving METTL16 and YTHDC1. *Cell Rep* **21**, 3354–3363.
40 Chen-Kiang S, Nevins JR and Darnell JE (1979) N-6-methyl-adenosine in adenovirus type 2 nuclear RNA is conserved in the formation of messenger RNA. *J Mol Biol* **135**, 733–752.
41 Carroll SM, Narayan P and Rottman FM (1990) N^6-methyladenosine residues in an intron-specific region of prolactin pre-mRNA. *Mol Cell Biol* **10**, 4456–4465.
42 Ke S, Pandya-jones A, Saito Y, Fak JJ, Vågbø CB, Geula S, Hanna JH, Black DL, Darnell JE Jr and Darnell RB (2017) m^{6}A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes Dev* **31**, 990–1006.
43 Louloupi A, Ntini E, Conrad T and Orom UA (2018) Transient N-6-methyladensosine transcriptome sequencing reveals a regulatory role of m^{6}A in splicing efficiency. *bioRxiv* 242966 [PREPRINT].
44 Knuckles P, Carl SH, Musheev M, Niehrs C, Wenger A and Bühler M (2017) RNA fate determination through cotranscriptional adenosine methylation and microprocessor binding. *Nat Struct Mol Biol* **24**, 561–569.
45 Alarcón CR, Lee H, Goodarzi H, Halberg N and Tavazoie SF (2015) N^6-methyladenosine marks primary microRNAs for processing. *Nature* **519**, 482–485.
46 Alarcón CR, Goodarzi H, Lee H, Liu X, Tavazoie S and Tavazoie SF (2015) HNRNPA2B1 is a mediator of m^{6}A-dependent nuclear RNA processing events. *Cell* **162**, 1299–1308.
47 Schöller E, Weichmann F, Treiber T, Ringle S, Flatley A, Feederle R, Bruckmann A and Meister G (2018) Interactions, localization and phosphorylation of the m^{6}A generating METTL3- METTL14-WTAP complex. *RNA* **24**, 499–512.
48 Wen J, Lv R, Ma H, Shen H, He C, Wang J, Jiao F, Liu H, Yang P, Tan L *et al*. (2018) Zc3h13 regulates nuclear RNA m^{6}A methylation and mouse embryonic stem cell self-renewal. *Mol Cell* **69**, 1028–1038.e6.
49 Barbieri I, Tzelepis K, Pandolfini L, Shi J, Millán-Zambrano G, Robson SC, Aspris D, Migliori V, Bannister AJ, Han N *et al*. (2017) Promoter-bound METTL3 maintains myeloid leukaemia by m^{6}A-dependent translation control. *Nature* **552**, 126–131.
50 Meyer KD, Patil DP, Zhou J, Zinoviev A, Skabkin MA, Elemento O, Pestova TV, Qian SB and Jaffrey SR (2015) 5′ UTR m^{6}A promotes cap-independent translation. *Cell* **163**, 999–1010.
51 Zhou J, Wan J, Gao X, Zhang X, Jaffrey SR and Qian SB (2015) Dynamic m^{6}A mRNA methylation directs translational control of heat shock response. *Nature* **526**, 591–594.
52 Lin S, Choe J, Du P, Triboulet R, Gregory Correspondence RI and Gregory RI (2016) The m^{6}A

methyltransferase METTL3 promotes translation in human cancer cells a methyltransferase METTL3 promotes translation in human cancer cells. *Mol Cell* **62**, 335–345.

53 Bertero A, Brown S, Madrigal P, Osnato A, Ortmann D, Yiangou L, Kadiwala J, Hubner NC, de Los Mozos IR, Sadee C *et al.* (2018) The SMAD2/3 interactome reveals that TGFβ controls m^{6}A mRNA methylation in pluripotency. *Nature* **555**, 256–259.

54 Slobodin B, Han R, Calderone V, Vrielink JAFO, Loayza-puch F, Elkon R, Agami R and Methylation N (2017) Transcription impacts the efficiency of mRNA translation via co-transcriptional N^6-adenosine article transcription impacts the efficiency of mRNA translation via co-transcriptional. *Cell* **169**, 326–337.e12.

55 Dujardin G, Lafaille C, de la Mata M, Marasco LE, Muñoz MJ, Le Jossic-Corcos C, Corcos L and Kornblihtt AR (2014) How slow RNA polymerase II elongation favors alternative exon skipping. *Mol Cell* **54**, 683–690.

56 Kierzek E and Kierzek R (2003) The thermodynamic stability of RNA duplexes and hairpins containing N^6-alkyladenosines and 2-methylthio-N6-alkyladenosines. *Nucleic Acids Res* **31**, 4472–4480.

57 Engel JD and von Hippel PH (1974) Effects of methylation on the stability of nucleic acid conformations: studies at the monomer level. *Biochemistry* **13**, 4143–4158.

58 Roost C, Lynch SR, Batista PJ, Qu K, Chang HY and Kool ET (2015) Structure and thermodynamics of N^6-methyladenosine in RNA: a spring-loaded base modification. *J Am Chem Soc* **137**, 2107–2115.

59 Bevilacqua PC, Ritchey LE, Su Z and Assmann SM (2016) Genome-wide analysis of RNA secondary structure. *Annu Rev Genet* **50**, 235–266.

60 Ding Y, Tang Y, Kwok CK, Zhang Y, Bevilacqua PC and Assmann SM (2014) In vivo genome-wide profiling of RNA secondary structure reveals novel regulatory features. *Nature* **505**, 696–700.

61 Rouskin S, Zubradt M, Washietl S, Kellis M and Weissman JS (2014) Genome-wide probing of RNA structure reveals active unfolding of mRNA structures in vivo. *Nature* **505**, 701–705.

62 Watts JM, Dang KK, Gorelick RJ, Leonard CW, Bess JW, Swanstrom R, Burch CL and Weeks KM (2009) Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature* **460**, 711–716.

63 Spitale RC, Flynn RA, Zhang QC, Crisalli P, Lee B, Jung J, Kuchelmeister HY, Batista PJ, Torre EA, Kool ET *et al.* (2015) Structural imprints in vivo decode RNA regulatory mechanisms. *Nature* **519**, 486–490.

64 Zhang Z, Theler D, Kaminska KH, Hiller M, Grange PDe, Pudimat R, Rafalska I, Heinrich B, Bujnicki JM and Stamm S (2010) The YTH domain is a novel RNA binding domain. *J Biol Chem* **285**, 14701–14710.

65 Patil DP, Pickering BF and Jaffrey SR (2017) Reading m^{6}A in the transcriptome: m^{6}A-binding proteins. *Trends Cell Biol* **28**, 1–15.

66 Xu C, Wang X, Liu K, Roundtree IA, Tempel W, Li Y, Lu Z, He C and Min J (2014) Structural basis for selective binding of m^{6}A RNA by the YTHDC1 YTH domain. *Nat Chem Biol* **10**, 927–929.

67 Xu C, Liu K, Ahmed H, Loppnau P, Schapira M and Min J (2015) Structural basis for the discriminative recognition of N^6-Methyladenosine RNA by the human YT521-B homology domain family of proteins. *J Biol Chem* **290**, 24902–24913.

68 Theler D, Dominguez C, Blatter M, Boudet J and Allain FHT (2014) Solution structure of the YTH domain in complex with N^6-methyladenosine RNA: a reader of methylated RNA. *Nucleic Acids Res* **42**, 13911–13919.

69 Li F, Zhao D, Wu J and Shi Y (2014) Structure of the YTH domain of human YTHDF2 in complex with an m^{6}A mononucleotide reveals an aromatic cage for m^{6}A recognition. *Cell Res* **24**, 1490–1492.

70 Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G *et al.* (2013) N^6-methyladenosine-dependent regulation of messenger RNA stability. *Nature* **505**, 117–120.

71 Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, Weng X, Chen K, Shi H and He C (2015) N^6-methyladenosine modulates messenger RNA translation efficiency. *Cell* **161**, 1388–1399.

72 Rafalska I, Zhang Z, Benderska N, Wolff H, Hartmann AM, Brack-Werner R and Stamm S (2004) The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation. *Hum Mol Genet* **13**, 1535–1549.

73 Shi H, Wang X, Lu Z, Zhao BS, Ma H, Hsu PJ and Liu C (2017) YTHDF3 facilitates translation and decay of N^6-methyladenosine-modified RNA. *Cell Res* **27**, 315–328.

74 Edupuganti RR, Geiger S, Lindeboom RGH, Shi H, Hsu PJ, Lu Z, Wang SY, Baltissen MPA, Jansen PWTC, Rossa M *et al.* (2017) N^6-methyladenosine (m^{6}A) recruits and repels proteins to regulate mRNA homeostasis. *Nat Struct Mol Biol* **24**, 870–878.

75 Epstein P, Reddy R, Henning D and Busch H (1980) The nucleotide sequence of nuclear U6 (4.7 S) RNA. *J Biol Chem* **255**, 8901–8906.

76 Shimba S, Bokar JA, Rottman F and Reddy R (1995) Accurate and efficient N-6-adenosine methylation in spliceosomal U6 small nucelar RNA by HeLa cell extract in vitro. *Nucleic Acids Res* **23**, 2421–2426.

77 Bresson SM, Hunter OV, Hunter AC and Conrad NK (2015) Canonical Poly(A) polymerase activity

promotes the decay of a wide variety of mammalian nuclear RNAs. *PLoS Genet* **11**, e1005610.
78 Kan L, Grozhik A V, Vedanayagam J, Patil DP, Pang N, Lim KS, Huang YC, Joseph B, Lin CJ, Despic V *et al*. (2017) The m^{6}A pathway facilitates sex determination in Drosophila. *Nat Commun* **8**, 15737.
79 Horiuchi K, Umetani M, Minami T, Okayama H, Takada S, Yamamoto M, Aburatani H, Reid PC, Housman DE, Hamakubo T *et al*. (2006) Wilms' tumor 1-associating protein regulates G2/M transition through stabilization of cyclin A2 mRNA. *Proc Natl Acad Sci U S A* **103**, 17278–17283.
80 Xiao W, Adhikari S, Dahal U, Chen Y, Hao Y, Sun B-F, Sun H, Li A, Ping X-L, Lai W-Y *et al*. (2016) Nuclear m^{6}A reader YTHDC1 regulates mRNA splicing. *Mol Cell* **61**, 507–519.
81 Lichinchi G, Zhao BS, Wu Y, Lu Z, Qin Y, He C and Rana TM (2016) Dynamics of human and viral RNA methylation during Zika virus infection. *Cell Host Microbe* **20**, 666–673.
82 Lichinchi G, Gao S, Saletore Y, Gonzalez GM, Bansal V, Wang Y, Mason CE and Rana TM (2016) Dynamics of the human and viral m(6)A RNA methylomes during HIV-1 infection of T cells. *Nat Microbiol* **1**, 16011.
83 Roundtree IA, Luo G, Zhang Z, Wang X, Zhou T, Cui Y, Sha J, Huang X, Guerrero L, Xie P *et al*. (2017) YTHDC1 mediates nuclear export of N^6-methyladenosine methylated mRNAs. *eLife* **6**, 1–28.
84 Geula S, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, Hershkovitz V, Peer E, Mor N, Manor YS *et al*. (2015) m^{6}A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science* **347**, 1002–1006.
85 Han J, Pedersen JS, Kwon SC, Belair CD, Kim YK, Yeom KH, Yang WY, Haussler D, Blelloch R and Kim VN (2009) Posttranscriptional crossregulation between Drosha and DGCR8. *Cell* **136**, 75–84.
86 Macias S, Plass M, Stajuda A, Michlewski G, Eyras E and Cáceres JF (2012) DGCR8 HITS-CLIP reveals novel functions for the microprocessor. *Nat Struct Mol Biol* **19**, 760–766.
87 Heras SR, Macias S, Plass M, Fernandez N, Cano D, Eyras E, Garcia-Perez JL and Cáceres JF (2013) The Microprocessor controls the activity of mammalian retrotransposons. *Nat Struct Mol Biol* **20**, 1173–1181.
88 Knuckles P, Vogt MA, Lugert S, Milo M, Chong MMW, Hautbergue GM, Wilson SA, Littman DR and Taylor V (2012) Drosha regulates neurogenesis by controlling Neurogenin 2 expression independent of microRNAs. *Nat Neurosci* **15**, 962–969.
89 Johanson TM, Keown AA, Cmero M, Yeo JHC, Kumar A, Lew AM, Zhan Y and Chong MMW (2015) Drosha controls dendritic cell development by cleaving messenger RNAs encoding inhibitors of myelopoiesis. *Nat Immunol* **16**, 1134–1141.
90 Rolando C, Erni A, Grison A, Beattie R, Engler A, Gokhale PJ, Milo M, Wegleiter T, Jessberger S and Taylor V (2016) Multipotency of adult hippocampal NSCs in vivo is restricted by Drosha/NFIB. *Cell Stem Cell* **19**, 653–662.
91 Macias S, Cordiner RA, Gautier P, Plass M and Cáceres JF (2015) DGCR8 acts as an adaptor for the exosome complex to degrade double-stranded structured RNAs. *Mol Cell* **60**, 873–885.
92 Du H, Zhao Y, He J, Zhang Y, Xi H, Liu M, Ma J and Wu L (2016) YTHDF2 destabilizes m6A-containing RNA through direct recruitment of the CCR4-NOT deadenylase complex. *Nat Commun* **7**, 1–11.
93 Hsu PJ, Zhu Y, Ma H, Guo Y, Shi X, Liu Y, Qi M, Lu Z, Shi H, Wang J *et al*. (2017) Ythdc2 is an N^6-methyladenosine binding protein that regulates mammalian spermatogenesis. *Cell Res* **27**, 1115–1127.
94 Wojtas MN, Pandey RR, Mendel M, Homolka D, Sachidanandam R and Pillai RS (2017) Regulation of m^{6}A transcripts by the 3′→5′ RNA helicase YTHDC2 is essential for a successful meiotic program in the mammalian germline. *Mol Cell* **68**, 374–387.e12.
95 Bailey AS, Batista PJ, Gold RS, Grace Chen Y, de Rooij DG, Chang HY and Fuller MT (2017) The conserved RNA helicase YTHDC2 regulates the transition from proliferation to differentiation in the germline. *eLife* **6**, e26116.
96 Jain D, Puno MR, Meydan C, Lailler N, Mason CE, Lima CD, Anderson KV and Keeney S (2018) Ketu mutant mice uncover an essential meiotic function for the ancient RNA helicase YTHDC2. *eLife* **7**, e30919.
97 Lebedeva S, Jens M, Theil K, Schwanhäusser B, Selbach M, Landthaler M and Rajewsky N (2011) Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. *Mol Cell* **43**, 340–352.
98 Kundu P, Fabian MR, Sonenberg N, Bhattacharyya SN and Filipowicz W (2012) HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. *Nucleic Acids Res* **40**, 5088–5100.
99 Bodi Z, Bottley A, Archer N, May ST and Fray RG (2015) Yeast m^{6}A methylated mRNAs are enriched on translating ribosomes during meiosis, and under rapamycin treatment. *PLoS One* **10**, 1–13.
100 Choi J, Ieong KW, Demirci H, Chen J, Petrov A, Prabhakar A, O'Leary SE, Dominissini D, Rechavi G, Soltis SM *et al*. (2016) N^6-methyladenosine in mRNA disrupts tRNA selection and translation-elongation dynamics. *Nat Struct Mol Biol* **23**, 110–115.

101 Mauer J, Luo X, Blanjoie A, Jiao X, Grozhik AV, Patil DP, Linder B, Pickering BF, Vasseur J-J, Chen Q *et al*. (2016) Reversible methylation of m6Am in the 5′ cap controls mRNA stability. *Nature* **541**, 371–375.

102 Schibler U and Perry RP (1977) The 5′-termini of heterogeneous nuclear RNA: a comparison among molecules of different sizes and ages. *Nucleic Acids Res* **4**, 4133–4150.

103 Stoneley M and Willis AE (2004) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene* **23**, 3200–3207.

104 Cui Q, Shi H, Ye P, Li L, Qu Q, Sun G, Sun G, Lu Z, Huang Y, Yang CG *et al*. (2017) m^{6}A RNA methylation regulates the self-renewal and tumorigenesis of glioblastoma stem cells. *Cell Rep* **18**, 2622–2634.

105 Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, Chen Y, Sulman EP, Xie K, Bögler O *et al*. (2017) m^{6}A demethylase ALKBH5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. *Cancer Cell* **31**, 591–606.e6.

106 Visvanathan A, Patil V, Arora A, Hegde AS, Arivazhagan A, Santosh V and Somasundaram K (2017) Essential role of METTL3-mediated m^{6}A modification in glioma stem-like cells maintenance and radioresistance. *Oncogene* **37**, 522–533.

107 Vu LP, Pickering BF, Cheng Y, Zaccara S, Nguyen D, Minuesa G, Chou T, Chow A, Saletore Y, MacKay M *et al*. (2017) The N^6-methyladenosine (m^{6}A)-forming enzyme METTL3 controls myeloid differentiation of normal hematopoietic and leukemia cells. *Nat Med* **23**, 1369–1376.

108 Jaffrey SR and Kharas MG (2017) Emerging links between m^{6}A and misregulated mRNA methylation in cancer. *Genome Med* **9**, 2.