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m^6A RNA methylation in eukaryotic cells

m^6A metylace RNA v eukaryotických buňkách

Bachelor´s thesis

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Poděkování

Především děkuji svému školiteli Davidu Staňkovi za podnětné konzultace, rady a návrhy během tvorby mé bakalářské práce. Taktéž děkuji Davidovi a všem kolegům z Oddělení biologie RNA za vlídné přijetí mne do kolektivu a za každodenní pomoc, kterou mi poskytují při mém začínajícím vědeckém snažení. V neposlední řadě děkuji také své rodině za podporu při mých studiích a svým přátelům za společně strávené chvíle při studiu.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 9. 5. 2019

Abstrakt

N6-metylace adenosinu (m^6A) je nejčastější modifikací mRNA u eukaryot. Na RNA je vytvářena metyltransferázami během transkripce, navíc může být odstraněna konkrétními demetylázami. Přítomnost těchto demetyláz umožňuje vratnost modifikace a její potenciální dynamičnost, proto by se N6-metyladenosin mohl účastnit regulace genové exprese. Od objevu první m^6A-specifické demetylázy FTO, m^6A modifikace začala být oblíbeným tématem ve výzkumu biologie RNA. N6-metyladenosin je přítomen v mRNA ale také v různých nekódujících RNA. Analýza distribuce m^6A na mRNA odhalila obohacení kolem 3' nepřekládané oblasti a pravděpodobně kolem sestřihových míst. Zatím byly objeveny dvě m^6A metyltransferázy, METTL3/METTL14 metyltransferáza je hlavním metyltransferázovým komplexem a metyltransferáza METTL16 metyluje jen malou skupinu RNA. Také jsou známy dvě demetylázy – FTO a ALKBH5. Navíc byly identifikovány proteiny vázající m^6A, které spolu sdílí strukturní doménu YTH. m^6A slouží jako buněčný signál ovlivňující různé kroky metabolismu RNA, například sestřih RNA, jaderný export, translaci nebo odbourávání RNA. Některé tyto vlivy jsou zprostředkovány m^6A-vázajícími proteiny, ale mohou se účastnit i jiné mechanismy. Přítomnost m^6A modifikace na RNA může také ovlivňovat sekundární strukturu dané RNA a tím i přístup některých RNA-vazebných proteinů, tento regulační mechanismus se nazývá „m^6A switch".

Klíčová slova

N6-metyladenosin, m^6A, RNA modifikace, RNA metylace, epitranskriptom

Abstract

The N6-methylation of adenosine (m^6A) is the most abundant modification in eukaryotic mRNA. This modification is deposited on RNA co-transcriptionally by the methyltransferase complexes and can also be "erased" by specific demethylases. The existence of m^6A demethylases makes the modification reversible and potentially dynamic, therefore, m^6A could have a function in gene expression regulation. Since the discovery of the first m^6A demethylase FTO, the m^6A has become a hot-topic in RNA-biology research. m^6A is found in mRNAs but also in various non-coding RNAs. Analysis of m^6A distribution on mRNAs revealed the enrichment of m^6A in proximity of a stop codon, in 3' UTRs and possibly around 5' and 3' splice-sites. So far two m^6A methyltransferases have been discovered in vertebrates, METTL3/METTL14 complex is the major methyltransferase and METTL16 deposits m^6A just on a specific subset of RNAs. Additionally, two m^6A demethylases are known – FTO and ALKBH5. Finally, members of protein family with a so-called YTH RNA binding domain were identified as m^6A binding proteins. m^6A serves as a signal affecting various steps of RNA metabolism such as mRNA splicing, nuclear export, translation or RNA degradation. Some of the effects are clearly mediated by the m^6A binding proteins, but also other mechanisms can be involved. m^6A presence on RNA can also modify the RNA secondary structure, changing the accessibility of the RNA to various RNA-binding proteins, this regulatory mechanism is called m^6A switch.

Keywords:

N6-methyladenosine, m^6A, RNA methylation, RNA modifications, epitranscriptome

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Glossary

3’ UTR	3’ untranslated region
5’ UTR	5’ untranslated region
CDS	coding sequence
hnRNP	heterogeneous nuclear ribonucleoprotein
KO	knock-out
lncRNA	long non-coding RNA
m^6A	N6-methyladenosine
m^6Am	N6 ,2'-O-dimethyladenosine
MAT2A-RI	MAT2A-retained intron isoform
MeRIP-Seq	m^6A-specific methylated RNA immunoprecipitation, sequencing
miCLIP	m^6A individual nucleoside resolution cross-linking and immunoprecipitation
miRNA	microRNA
ncRNA	non-coding RNA
P-body	processing body
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
snRNA	small nuclear RNA
ssRNA	single-stranded RNA

Introduction

Multicellular organisms are composed of many various cell types which differ dramatically in their biological characteristics including morphology and metabolism. Besides, cells can change markedly during development or even in response to a changing outer environment and conditions. All this cellular flexibility is a product of a complex regulation of gene expression. There are many steps during gene expression which can be dynamically controlled, starting with transcription that is modulated by transcription factors and by the epigenetic marks including DNA methylation or various histone modifications (reviewed in Branco et al., 2012; Jones, 2012; Rothbart and Strahl, 2014; Strahl and Allis, 2000; Suzuki and Bird, 2008). Moreover, there are many post-transcriptional steps that can be finely tuned, such as RNA splicing, polyadenylation, export, translation or degradation.

For several decades, epigenetic modifications of DNA and histones have been studied extensively as signals affecting the gene expression. The existence of different chromatin-modifying enzymes which counteract each other enables the cells to dynamically change the patterns of chromatin marks and therefore change the transcription output (reviewed in Klose et al., 2006; Kohli and Zhang, 2013; Rothbart and Strahl, 2014; Shi, 2007). In contrast to DNA modifications, covalent modifications of RNA have been for many years considered stable and generally independent on the broader cellular context, therefore not important for the gene expression regulation (reviewed in Fu et al., 2014). The view on the RNA modification dynamics has changed rapidly in 2011 after the discovery of a protein called Fat-mass and obesity-associated factor (FTO), the RNA demethylase with the affinity toward N6-methylated adenosine (m^6A) in single-stranded RNA (ssRNA) regions (Jia et al., 2011). m^6A is probably the most abundant internal modification of mRNA in eukaryotes (Wei et al., 1975b). The discovery of the m^6A demethylase raised the question whether this very abundant modification could be dynamically regulated and thus also involved in regulation of gene expression. Recently, the research of m^6A methylation of various RNAs has become a very competitive and rapidly evolving field of RNA biology. Possible dynamics of m^6A and other other post-transcriptional modifications, which have been identified within mRNAs such as N1-methyladenosine (m^1A), 5-methylcytosine (m^5C), N4-acetylcytidine (ac^4C), N6 ,2'-O-dimethyladenosine (m^6Am) and pseudouridine (Ψ) (Arango et al., 2018; Carlile et al., 2014; Desrosiers et al., 1974; Dominissini et al., 2016; Squires et al., 2012; Wei et al., 1975a) gave rise to the idea of a new regulatory information layer in a cell called epitranscriptome.

It is estimated, that 0.1-0.4 % of total adenosines in mammalian transcriptome are N6-methylated (Wei et al., 1975b). To a smaller extent, m^6A modification is also present in various non-coding RNAs (ncRNAs) (Dominissini et al., 2012; Meyer et al., 2012). The distribution of m^6A residues within the transcripts is not random and it follows specific enrichment patterns (Dominissini et al., 2012; Meyer et al., 2012). This differential density of m^6A across the transcript is likely connected to the signaling function of the modification, therefore the precise determination of the m^6A density patterns could help us understand the biological relevance and function of m^6A modification. Moreover, the m^6A modification can be found throughout the transcriptomes of eukaryotic organisms from yeasts (Schwartz et al., 2013) to plants (Nichols, 1979), mammals (Dominissini et al., 2012; Meyer et al., 2012) or insects (Lence et al., 2016)., or even in some bacterial (Deng et al., 2015) and viral RNAs (Beemon and Keith, 1977; Canaani et al., 1979). Not just the m^6A connected protein machineries but also the m^6A distribution features on RNAs are often conserved throughout the species, suggesting the evolutionary old and important role of m^6A (Dominissini et al., 2012; Schwartz et al., 2013; Stoilov et al., 2002; Zhang et al., 2010).

The biological relevance of the m^6A modification on RNAs is supported by the abnormal phenotypes of the cells or whole organisms after the m^6A deposition or erasure is impaired. Knock-out (KO) of the methyltransferase protein METTL3 or METTL16 is embryonic-lethal (Batista et al., 2014; Mendel et al., 2018). Knock-outs of demethylases have milder but still distinct effect, FTO KO in mice leads to postnatal growth retardation and overall dysregulation of energy homeostasis (Fischer et al., 2009), while ALKBH5 KO causes infertility (Tang et al., 2018; Zheng et al., 2013). m^6A modification was as well connected to the various biological processes such as gametogenesis (Hongay and Orr-Weaver, 2011; Lin et al., 2017; Tang et al., 2018; Xu et al., 2017; Zheng et al., 2013), stem-cell differentiation (Batista et al., 2014; Bertero et al., 2018; Chen et al., 2015; Geula et al., 2015; Wang et al., 2014b), zygotic development (Mendel et al., 2018; Zhao et al., 2017) , tumorigenesis (Cui et al., 2017; Li et al., 2017b; Zhang et al., 2016, 2017) or even meiosis and sporulation in yeast (Schwartz et al., 2013). But how are the m^6A-deposition sites determined? What are the molecular mechanisms behind the various effects of this modification and is m^6A methylation pattern really changed in response to cellular stimuli?

Here I present so far known information about distribution of m^6A modification on mRNAs, the enzymes depositing, removing or recognizing m^6A modification and about the effects of m^6A modification on the metabolism of modified transcripts. This text is focusing mainly on the

molecular mechanisms underlying the various effects of m^{6}A modification on RNA and on the importance of m^{6}A modification in RNA metabolism in the context of mammalian, mostly human and murine cells. However, at least some of the mechanism seem to be conserved across the eukaryotes.

m^{6}A distribution patterns on different RNAs

The m^{6}A marks were shown to affect different aspects of RNA metabolism, such as splicing (Louloupi et al., 2018; Tang et al., 2018; Xiao et al., 2016; Xu et al., 2017; Zheng et al., 2013), nuclear export (Roundtree et al., 2017), RNA stability (Du et al., 2016; Ke et al., 2017; Tang et al., 2018; Wang et al., 2014a, 2014b) and translation (Barbieri et al., 2017; Li et al., 2017a; Lin et al., 2017; Meyer et al., 2015; Wang et al., 2015). Determining the specific position of m^{6}A within the transcripts and quantifying the methylated transcripts could lead to a better understanding of m^{6}A functional role and the underlying mechanisms by which it affects the fate of various RNAs. Our knowledge about localization and quantity of m^{6}A residues in RNAs is dependent on the limitations of the methods used for the identification of m^{6}A methylated sites. Because the N6-methyl group of m^{6}A does not interfere with Watson-Crick base pairing of adenosine (Roost et al., 2015) (Figure 1) and therefore it generally does not lead to an introduction of a mutation during reverse-transcription, more sophisticated methods than RNA sequencing had to be developed for transcriptome-wide m^{6}A mapping (Aschenbrenner et al., 2018). The first used methods such as m^{6}A-seq or MeRIP-Seq (m^{6}A-specific methylated RNA immunoprecipitation, sequencing) and currently widely used miCLIP (m^{6}A individual nucleoside resolution cross-linking and immunoprecipitation) are all based on RNA fragmentation, anti-m^{6}A antibody recognition of m^{6}A, enrichment of the recognized fragments by immunoprecipitation (IP) and finally next-generation sequencing of the fragments (Dominissini et al., 2012; Linder et al., 2015; Meyer et al., 2012). miCLIP can have a single-nucleotide resolution and is independent of the bioinformatic consensus-based prediction of m^{6}A sites, therefore this method can identify m^{6}A residues outside the common consensus motif (Linder et al., 2015). However, the usage of an antibody still introduces a bias in the method – often used antibodies cannot discriminate between different ribose modifications at the 2'-position, therefore between m^{6}A and m^{6}Am. Moreover, nucleotides adjacent to an m^{6}A site or secondary structures can affect the antibody affinity to the site. Finally, due to the enrichment steps, above-mentioned methods cannot provide the precise information about quantity of methylated vs unmethylated transcripts (reviewed in Hartstock and Rentmeister, 2019).

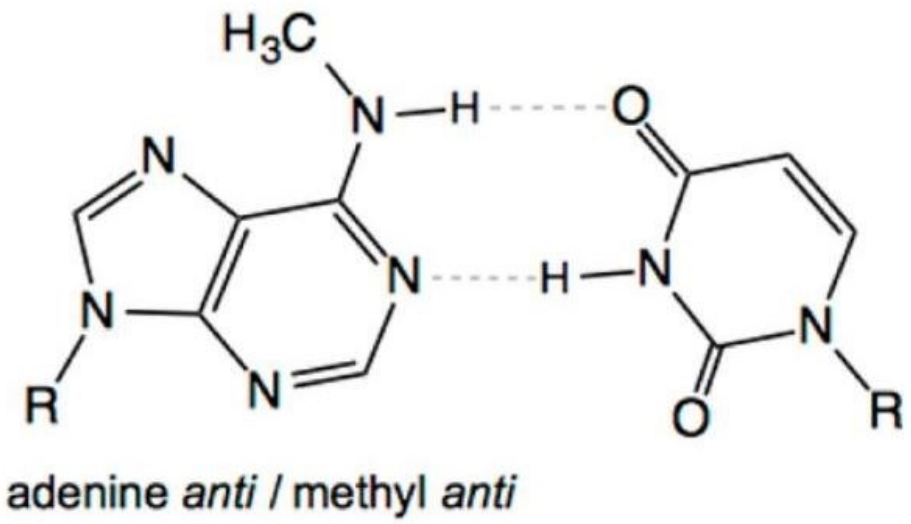


Figure 1 Structure of N6-methylated adenine base-paired with uracil within RNA duplex. N6-methyladenine (left) base and methylamino group are both in anti conformation adopting a Watson-Crick base pairing with uracil (right). Adopted from (Roost et al., 2015).

First transcriptome-wide m^{6}A mapping experiments were performed in 2012, using the newly established m^{6}A-seq and MeRIP-Seq methods and identified the m^{6}A consensus sequence RRACH (R stands for A or G; H for A, C or U), where the central adenine is methylated (Dominissini et al., 2012; Meyer et al., 2012). However, only some of the sequences consistent with the m^{6}A consensus are methylated within the transcripts, implying that other signals are also needed to determine the m^{6}A methylation site (Dominissini et al., 2012). Later, the major methyltransferase complex METTL3/METTL14 was identified and was shown to specifically recognize the m^{6}A consensus sequence (Liu et al., 2014).

m^{6}A residues were found in various groups of RNAs – messenger RNAs (Dominissini et al., 2012; Meyer et al., 2012), ribosomal RNAs (rRNAs) (Liu et al., 2013), long non-coding RNAs (lncRNAs) (Dominissini et al., 2012; Meyer et al., 2012), small nucleolar RNAs (snoRNAs) (Linder et al., 2015), microRNAs (miRNAs) (Alarcón et al., 2015a) or some small nuclear RNAs (snRNAs) (Karijolich and Yu, 2010; Pendleton et al., 2017; Warda et al., 2017). Most of those RNAs have the majority of m^{6}A residues within the m^{6}A consensus sequence but specific m^{6}A sites which do not localize in the consensus sequence are also found (Pendleton et al., 2017; Warda et al., 2017). snRNAs and rRNAs harbor several m^{6}A residues and none of them localizes in the consensus sequence (Karijolich and Yu, 2010; Liu et al., 2013; Pendleton et al., 2017; Warda et al., 2017). Considering, that the METTL3/METTL14 methyltransferase complex was shown to be consensus-specific, the m^{6}A residues outside of the consensus are probably deposited by a different methyltransferase complex (Liu et al., 2014).

Analysis of m^{6}A distribution within mRNA transcripts revealed a significant enrichment of m^{6}A marks in the proximity of a stop codon (Dominissini et al., 2012; Linder et al., 2015; Meyer et al., 2012; Schwartz et al., 2014) and at 3' untranslated region (3' UTR) (Ke et al., 2015; Meyer et al., 2012). First used methods also showed m^{6}A peaks close to a transcription start site (Dominissini et al., 2012) however, those results were later shown to be due to the limited specificity of the used antibody and the recognition of m^{6}Am methylation mark, which in some mRNAs follows directly the guanosine 5' cap structure (Linder et al., 2015; Schwartz et al., 2014). Coding sequence (CDS) of mRNAs is relatively depleted of m^{6}A, with the highest levels within the long internal exons, suggesting a possible role of m^{6}A modification in exon definition during splicing (Dominissini et al., 2012; Ke et al., 2017; Schwartz et al., 2014). Moreover, differentially spliced exons and introns showed an enrichment of m^{6}A methylation (Dominissini et al., 2012). Some of the m^{6}A sites are organized into clusters of 150 – 500 bp, which contain up to 15 m^{6}A residues (Linder et al., 2015).

Because the first m^{6}A distribution analysis used total cellular RNA, where the nascent, unspliced mRNAs are under-represented, there was not much data about methylation of intronic regions, hence they were believed to be rarely methylated (Meyer et al., 2012). Later, techniques for specific isolation of the nascent RNA were used to assess the methylation patterns of the nascent transcripts. Nevertheless, data from various research groups are not consistent. For example, analysis of the partially spliced RNA fraction bound to chromatin by Ke et al. (Ke et al., 2017) revealed that although the nascent mRNAs already contain the m^{6}A residues in the exonic sequences, introns are very rarely methylated. However, using bromouridine (BrU) metabolic labelling and isolation of labelled nascent transcripts, Louloupi et al. (Louloupi et al., 2018) showed that more than 50 % of m^{6}A marks is located in intronic regions. They also showed the enrichment of m^{6}A residues in exonic regions near splice junctions in nascent mRNAs, but those sites are not enriched anymore in the steady-state mRNAs. Both groups (Ke et al., 2017; Louloupi et al., 2018) detected m^{6}A peaks in the nascent mRNA molecules, which are then not present in mature transcripts, suggesting that m^{6}A in this regions acts as a transient mark potentially with a function during mRNA maturation (Louloupi et al., 2018).

It was shown that the general m^{6}A distribution patterns on mRNAs are highly conserved between human and mice and often the m^{6}A residues are localized at the orthologous positions in the transcripts (Dominissini et al., 2012; Meyer et al., 2012). Moreover m^{6}A sites are often located in the evolutionarily conserved sequences (Meyer et al., 2012). Those observations suggest an

important functional role of this modification (Dominissini et al., 2012). Several research groups also tried to compare the m^{6}A mapping results for various samples, showing that m^{6}A distribution patterns are mostly similar across diverse conditions and different cell types (Dominissini et al., 2012; Schwartz et al., 2014) but the analysis also revealed the subsets of transcripts that exhibit a distinct cell-type or treatment-dependent methylation patterns (Anders et al., 2018; Chen et al., 2015; Dominissini et al., 2012). On the other hand, the m^{6}A methylation was shown to be non-stoichiometric, meaning that not every molecule of a specific transcript is methylated (considering a specific position) and instead there is a ratio of N6-methylated vs unmethylated (m^{6}A/A) transcripts (Horowitz et al., 1984; Liu et al., 2013). Therefore, it is also reasonable to estimate and compare the methylation ratios in various cell types and conditions. According to MeRIP-Seq data by Meyer et al., in contrast to the high conservation of m^{6}A distribution ,the overall methylation levels differ widely between various cell types and their differentiation stages, with highest methylation levels in brain, liver and kidney and during neuronal maturation (Meyer et al., 2012). However, Schwartz et al. suggest that those differences are simply due to the changes in expression levels of m^{6}A-methylated transcripts (Schwartz et al., 2014). The comparison restricted to the transcripts with relatively stable expression levels across the tested conditions shows no significant changes in m^{6}A levels (detected by m^{6}A-seq) (Schwartz et al., 2014). Nevertheless, the methods for more precise quantification of m^{6}A containing transcripts must be used to confirm the estimations based on MeRIP-Seq and m^{6}A-seq data (Hartstock and Rentmeister, 2019).

The number of methylation sites differ between the transcripts, from highly methylated ones to the transcripts which have just one or no m^{6}A site (Meyer et al., 2012). The genes encoding m^{6}A containing RNAs are not clustered in a specific functional category, instead they are involved in many various cellular functions, for example signaling cascades, transcriptional regulation or RNA metabolism (Meyer et al., 2012). Interestingly, in coherence with the high levels of m^{6}A in brain tissue, many of the highly m^{6}A methylated transcripts belong to the genes connected with neurodevelopmental and neurological disorders (Meyer et al., 2012). Between the genes completely lacking m^{6}A methylation, there is an enrichment of housekeeping genes, for example genes important for translation such as ribosomal proteins, chromatin regulation, splicing and mitochondrial metabolism (Ke et al., 2017; Schwartz et al., 2014). The trend of lacking methylation is conserved between yeasts and mammals (human, mouse and yeast transcriptomes were

compared) for some Gene Ontology (GO) categories such as genes for ribosomal or splicing proteins and GTPases (Schwartz et al., 2014).

m^{6}A methyltransferases (m^{6}A writers)

METTL3/METTL14 complex

In 1997, METTL3 (originally named MT-A70) was recognized as a core protein of methyltransferase responsible for m^{6}A methylation of mammalian RNAs (Bokar et al., 1997). Later, another protein METTL14 was shown to form a complex with METTL3 (Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). Together, METTL3-METTL14 heterodimer functions as a cellular m^{6}A methyltransferase (Liu et al., 2014; Wang et al., 2014b) and is essential for the methylation of most of the m^{6}A sites on mRNA (Ke et al., 2017; Knuckles et al., 2017) and some non-coding RNAs (Alarcón et al., 2015a).

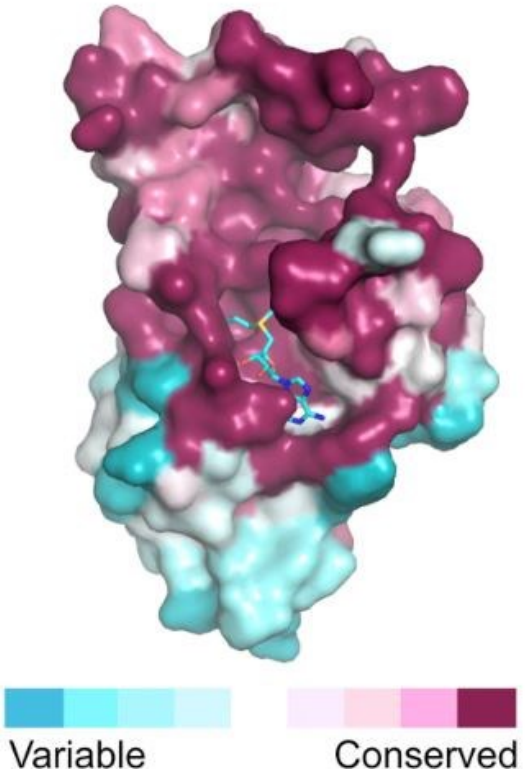


Figure 2 Degree of sequence conservation projected on the surface of methyltransferase domain of METTL3 (MTD3). MTD3 is highly conserved around the SAM (blue sticks) binding site. Adopted from (Wang et al., 2016).

METTL3 is the only component of the methyltransferase complex able to bind the methylation substrate S-adenosylmethionine (SAM), with its highly conserved binding pocket (Figure 2) and therefore execute the methylation reaction (Wang et al., 2016). It also contains a zinc-finger domain responsible for the recognition of the target sequence on RNA – the m^{6}A consensus sequence RRACH, where the central adenine is methylated (Huang et al., 2018b). Other component of the methyltransferase core, METTL14, facilitates the contact with RNA and is essential for structural stability and proper function of METTL3 (Wang et al., 2016) (Figure 3). In a recent study Huang et. al (Huang et al., 2019) also suggest a role of METTL14 in recognition and binding of chromatin modifications, however no direct structural data are yet available to support this hypothesis.

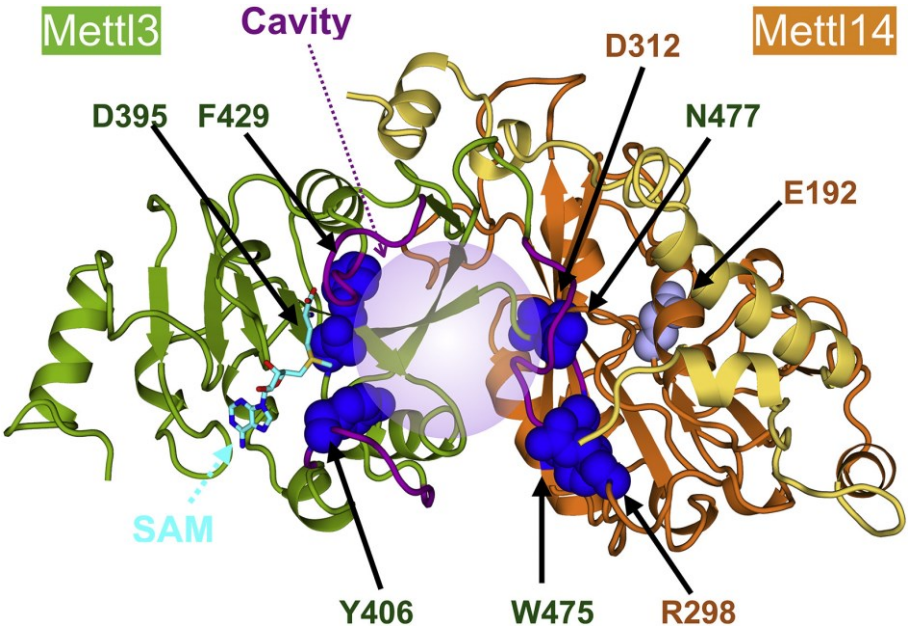


Figure 3 Annotated structural model of MTD3-MTD14 complex. MTD3 (green) and MTD14 (orange and yellow) are methyltransferase domains of METTL3 and METTL14. MTD3 forms the SAM (cyan sticks) binding site. The interface of MTD3 and MTD14 (purple sphere) surrounded by loops (purple) might accommodate the RNA substrate. Residues labeled by blue spheres were shown to be essential for enzymatic activity of the complex. Grey sphere shows the MTD14 residue, which does not impair the enzymatic activity of the complex. Adopted from (Wang et al., 2016).

METTL3-METTL14 dimer was shown to interact transiently with several other proteins, together forming the complete METTL3/METTL14 methyltransferase complex *in vivo*. Five METTL3-METTL14-interacting proteins are known so far: WTAP, VIRMA, RBM15 or RBM15B, ZC3H13 and HAKAI (Patil et al., 2016; Ping et al., 2014; Schwartz et al., 2014; Yue et al., 2018). While those proteins are not essential for the methylation activity of METTL3/METTL14 complex *in vitro* (Liu et al., 2014), they can modulate its activity, site specificity and localization in living cells. (Ping et al., 2014). WTAP is a METTL3/METTL14 complex regulatory subunit, known to be essential for the localization of the complex in nuclear speckles and even the methylation activity *in vivo* (Ping et al., 2014). The function and importance of other mentioned proteins is less known. ZC3H13 was reported to stabilize the interaction of several METTL3/METTL14 complex components (Knuckles et al., 2018) and its knock-down also resulted in the cytoplasmic localization of the METTL3/METTL14 complex components (Wen et al., 2018). The interaction of the methyltransferase components with VIRMA and RBM15/RBM15B might be important for the recruitment of the complex to a specific sites of methylation (Patil et al., 2016; Yue et al., 2018). HAKAI was shown to interact with the METTL3/METTL14 complex (Yue et al., 2018), but its function is not yet fully understood.

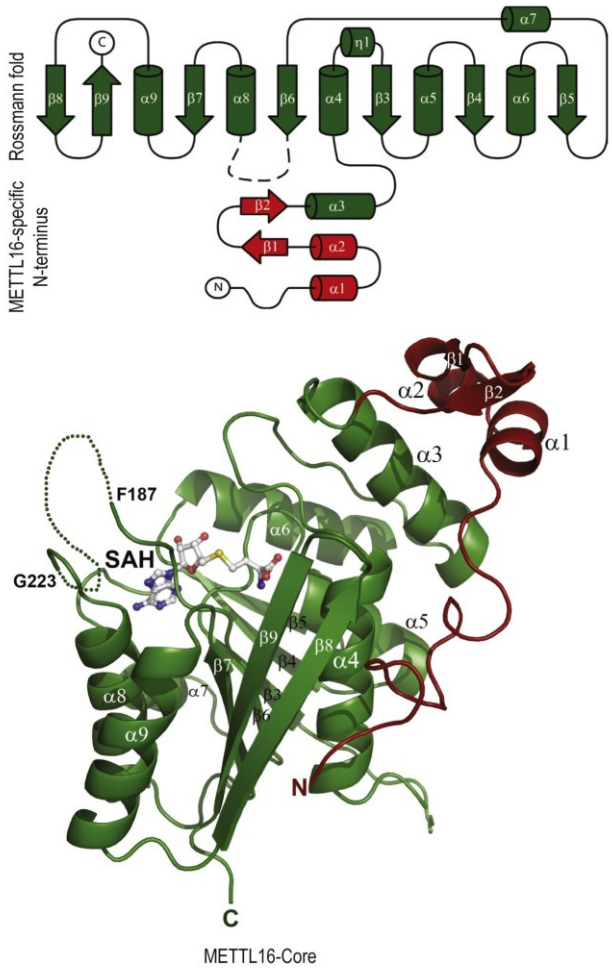
As mentioned previously, METTL3/METTL14 complex is localized into nuclear speckles (Liu et al., 2014; Ping et al., 2014). Nuclear or also so-called splicing speckles are nuclear membrane-less bodies enriched for proteins involved in transcription regulation or RNA processing, such as transcription factors, splicing factors or chromatin remodeling factors, and are suggested to integrate different steps of gene expression regulation (reviewed in Galganski et al., 2017). Interestingly, METTL3/METTL14 localization into speckles is RNA-dependent and is disrupted after the RNase treatment (Ping et al., 2014).

Since just a minority of sequences consistent with the m^{6}A consensus sequence is methylated, some other mechanism for recognition of those specific target sites by methyltransferase complex needs to be present (Dominissini et al., 2012). So far it was shown by several research groups, that the deposition of m^{6}A by METTL3/METTL14 complex happens co-transcriptionally (Barbieri et al., 2017; Bartosovic et al., 2017; Bertero et al., 2018; Huang et al., 2019; Ke et al., 2017; Knuckles et al., 2017). This is also in agreement with the findings of m^{6}A residues in intronic regions of nascent mRNAs (Louloupi et al., 2018) and the mostly co-transcriptional timing of splicing (reviewed in Herzel et al., 2017). However, the exact mechanism of the m^{6}A target site recognition by the METTL3/METTL14 is not yet fully understood. It has been shown by Liu et al. that the methyltransferase complex does not have a distinct structural preference (Liu et al., 2014). The research of Bertero et al. and Barbieri et al. suggest, that the complex is recruited to the methylation sites by specific transcription factors (Barbieri et al., 2017; Bertero et al., 2018). It could also be guided to the target sequence by specific miRNAs (Chen et al., 2015), or, as suggested recently, the methyltransferase complex binds directly to the chromatin at a site of transcription where METTL14 recognizes H3K36me3 histone marks. Then the contact of the METTL3/METTL14 complex with a nascent RNA is facilitated by its interaction with the elongating form of polymerase II (Huang et al., 2019). After the methyltransferase complex is recruited to the specific target site, methylation reaction occurs within the m^{6}A consensus sequence RRACH (Liu et al., 2014).

METTL16

In 2017 another m^{6}A methyltransferase, METTL16, was discovered (Pendleton et al., 2017; Warda et al., 2017). METTL16 is not a part of the METTL3/METTL14 complex and methylates just a small subset of m^{6}A sites on RNAs, independently of the m^{6}A consensus sequence (Pendleton et al., 2017; Warda et al., 2017). It is localized in the nucleus (Brown et al., 2016). The METTL16 protein itself has a SAM-binding site at the N-terminus (Mendel et al., 2018; Ruszkowska et al.,

2018) (Figure 4) and displays a methyltransferase activity toward RNA in vitro (Warda et al., 2017). The crystal structure revealed also a positively charged groove of METTL16 N-terminal domain which could be responsible for RNA binding (Mendel et al., 2018; Ruszkowska et al., 2018) (Figure 5) and disordered loop essential for catalytic activity of METTL16 (Mendel et al., 2018) (Figure 4). *In vivo*, METTL16 probably acts as a homodimer (Ruszkowska et al., 2018). So far, other proteins, which would interact and form a methyltransferase complex together with METTL16 were not discovered.



A search for METTL16 RNA substrates revealed several interesting METTL16 binding sites and potential role of METTL16 in post-transcriptional regulation: METTL16 associates with U6 small nuclear RNA (U6 snRNA), an important component of the spliceosome. It is responsible for a specific N6-methylation of A43 of U6 snRNA during its early maturation stages (Warda et al., 2017). m^6A43 is localized in a highly conserved region of U6, which participates in binding 5' splice site of pre-mRNA during splicing, therefore it is possible that this specific m^6A modification is important for proper splice site recognition and might even play a regulatory role in splicing (Warda et al., 2017).

Figure 4 Scheme and structure of the methyltransferase domain and N-terminus of METTL16 crystalized with S-adenosyl homocysteine (SAH). The catalytical domain of METTL16 (green) consists of conserved Rossman fold with disordered loop (dotted line) containing the catalytical residues. N-terminal module (red) is METTL16-specific and essential for its enzymatic activity. SAH is depicted in grey sticks. Adopted from (Mendel et al., 2018)

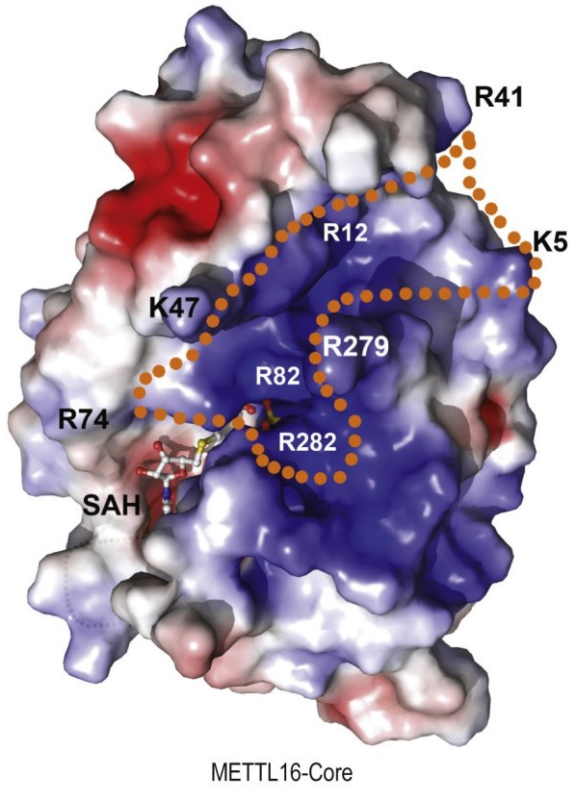


Figure 5 Electrostatic charge-representation of surface of METTL16 methyltransferase domain. Positively charged groove (blue) is extended in catalytic pocket and probably binds RNA. SAH is shown in grey lines. Adopted from (Mendel et al., 2018)

Moreover, in vertebrates METTL16 was shown to play a major role in SAM homeostasis (Mendel et al., 2018; Pendleton et al., 2017; Shima et al., 2017). In the cell SAM is generated from methionine and ATP by a SAM synthetase called MAT2A (Murray et al., 2016). METTL16 binds MAT2A pre-mRNA (Pendleton et al., 2017; Shima et al., 2017; Warda et al., 2017) and regulates the MAT2A protein levels and therefore SAM abundance in the cytoplasm (Pendleton et al., 2017; Shima et al., 2017). There are two research groups addressing the molecular mechanism of METTL16-dependent regulation of intracellular SAM levels. According to their findings, METTL16 recognizes a methylation target site within the MAT2A pre-mRNA and this site is m^6A methylated by METTL16 in normal conditions, however when the intracellular SAM levels are low, METTL16 reacts to the low levels of the methyl donor and does not proceed with the methylation reaction (Pendleton et al., 2017; Shima et al., 2017). It is known that there are two transcript isoforms of MAT2A gene, one is translated into a functional protein while the other contains a retained intron (MAT2A-RI). MAT2A-RI does not get exported from the nucleus and is subjected to a nuclear decay pathway (Bresson et al., 2015). According to the findings of Pendleton et al. low levels of SAM promote splicing of the retained-intron, which leads to the production of translated isoform of MAT2A and thus higher levels of MAT2A synthetase in the cell (Pendleton et al., 2017). On the other hand, the results of Shima et al. suggest, that there are no SAM-dependent changes in MAT2A mRNA splicing. They also show that the m^6A modification deposited by METTL16 on MAT2A mRNA is recognized by the m^6A binding protein YTHDC1. Based on those findings, Shima et al. proposed a model, where YTHDC1 recognizes m^6A methylated MAT2A mRNA and subjects it for the degradation. When SAM levels are low, MAT2A mRNA is not methylated and therefore not recognized and not degraded (Shima et al., 2017). However, YTHDC1 was shown

to play a role mostly in splicing regulation (Xiao et al., 2016) which would be more consistent with the hypothesis by Pendleton et al. Aditionally, results by Mendel et al. also show change in splicing of MAT2A mRNA in METTL16 knock-out mice embryos (Mendel et al., 2018).

METTL16 was also shown to bind other various non-coding RNAs (ncRNAs) (Warda et al., 2017) and mainly intronic sequences of pre-mRNAs (Pendleton et al., 2017; Warda et al., 2017), for example cancer-associated lncRNA MALAT1 or XIST lncRNA important for X chromosome inactivation (Warda et al., 2017).

The recognition mechanism of the METTL16 target sites is probably based on a combination of structural and sequence preference. Mendel et al. showed that methylation happens preferentially on the consensus nonamer UACm^{6}AGAGAA (Mendel et al., 2018), which was detected as a methylated sequence in both, U6 snRNA and MAT2A pre-mRNA (Pendleton et al., 2017). Additionally, the targeted adenosine must be unpaired, but in otherwise structured region for example in a bulge within a stem or between two stems (Mendel et al., 2018). The suggested structural preference of METTL16 is also supported by the fact that binding sites of METTL16 in MALAT1 and MAT2A were detected among a secondary-structured regions and also other METTL16 substrates are highly structured (Warda et al., 2017). On the other hand, bioinformatic analysis of other METTL16 binding sites did not show any enrichment of any sequence motif (Pendleton et al., 2017).

It was shown that after the METTL16 knock-down, m^{6}A signal on many transcripts is reduced, however since specific m^{6}A methylation of MAT2A by METTL16 affects cellular SAM levels, the change in m^{6}A levels on some transcripts might be due to the comprised effectivity of methylation itself and might be independent on direct METTL16 binding (Warda et al., 2017).

m^{6}A demethylases (m^{6}A erasers)

So far two mammalian enzymes are known to exhibit efficient m^{6}A demethylase activity. The first m^{6}A demethylase, fat mass and obesity-associated protein (FTO), was identified in 2011(Jia et al., 2011) and then in 2013 another demethylase, α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) was discovered (Zheng et al., 2013). Both enzymes belong to the AlkB protein family (Gerken et al., 2007; Kurowski et al., 2003; Zheng et al., 2013). Members of this family contain the non-heme Fe(II) and the oxidative demethylation process is alpha-ketoglutarate (α-KG)-dependent (Aravind and Koonin, 2001). Several other proteins of AlkB family were

previously described to oxidatively demethylate N-methylated DNA, lot of them acting in DNA repair pathway (Duncan et al., 2002; Falnes et al., 2002; Trewick et al., 2002). Both demethylases, FTO and ALKBH5, are localized in the nuclear speckles and in the nucleoplasm (Berulava et al., 2013; Jia et al., 2011; Zheng et al., 2013).

Both, FTO and ALKBH5 show differential expression levels in various tissues. FTO is highly expressed in brain (Fischer et al., 2009), whereas the highest levels of ALKBH5 are expressed in testes (Zheng et al., 2013), where ALKBH5 is most abundant in spermatocytes (Tang et al., 2018). Both demethylases probably act on a specific subset of m^6A residues or methylated transcripts (Bartosovic et al., 2017; Tang et al., 2018) but how those specific sites are recognized is not known.

The recent knowledge about FTO and ALKBH5 role in expression regulation is based mostly on the experiments using depletion, knock-out or over-expression of the m^6A demethylase. Knock-down of FTO or ALKBH5 results in the significant increase of the overall levels of m^6A modified adenosines in mRNA (Jia et al., 2011; Zheng et al., 2013) and in impaired expression of various genes (Bartosovic et al., 2017; Zheng et al., 2013). ALKBH5 knock-out mice display spermatogenesis defects and massive cell death by apoptosis in testes (Tang et al., 2018; Zheng et al., 2013), no other significant defects neither during knock-out mice development nor in adults were observed, suggesting a specific role of ALKBH5 in spermatogenesis (Tang et al., 2018). FTO knock-out in mice leads to a postnatal growth retardation (Fischer et al., 2009).

Recent articles also showed that FTO can sufficiently demethylate m^6Am residues, which are found adjacent to the 5' cap of mRNAs and snRNAs (Mauer et al., 2017, 2019). Whether this modification is a major modification regulated by FTO is a subject of discussion.

m^{6}A binding proteins and effect of the m^{6}A modification on RNA metabolism

The effect of m^{6}A modification on fate of methylated RNA is extensively studied. It was shown that m^{6}A modification might affect RNA splicing (Louloupi et al., 2018; Tang et al., 2018; Xu et al., 2017; Zheng et al., 2013), nuclear export (Zheng et al., 2013), translation (Barbieri et al., 2017; Li et al., 2017a; Lin et al., 2017; Meyer et al., 2015) or stability (Ke et al., 2017; Wang et al., 2014a, 2014b). Those effects on RNA metabolism are determined by differential recruitment or binding blockage of regulatory RNA binding proteins. The RNA binding proteins can directly recognize m^{6}A residue (so-called m^{6}A readers) (Xu et al., 2015) or, more interestingly, they can bind to specific m^{6}A-dependent secondary structures on RNAs (Liu et al., 2015, 2017). The mechanism, when m^{6}A controls binding of the proteins by structural change of the m^{6}A modified region is called m^{6}A switch (Liu et al., 2015). The recognition of m^{6}A modification by specific regulatory proteins is probably region-dependent, meaning that different proteins bind preferentially to different parts of RNA such as 5' UTRs or CDS (Anders et al., 2018; Wang et al., 2014a; Xiao et al., 2016; Zhou et al., 2015). This region preference of m^{6}A binding proteins can explain why differently localized m^{6}A marks can have different effects on the RNA metabolism. Moreover, the expression, localization and binding properties of each m^{6}A reader can be dependent on the presence and binding of other readers and on the cell type and it can be modulated by various signaling pathways. This makes the m^{6}A-dependent regulation network very complex and hard to study.

m^{6}A switch

It was shown that the m^{6}A residues within the RNA double-stranded structures such as stems can alter the thermal stability of those structures (Kierzek and Kierzek, 2003; Roost et al., 2015). Since many RNA binding proteins have a structural preference for either single-stranded or structured regions (Ray et al., 2013), the presence of the m^{6}A modification can affect their binding to surrounding regions. This m^{6}A switch effect was shown to regulate the binding of heterogeneous nuclear ribonucleoprotein C and G (HNRNPC, HNRNPG), which are important for various RNA maturation steps (Hofmann and Wirth, 2002; König et al., 2010; McCloskey et al., 2012; Wang et al., 2011; Zhao et al., 2008). m^{6}A destabilizes the stem secondary structure and therefore reveals

the ssRNA binding motif of those proteins (Liu et al., 2015, 2017). Additionally, RNA binding of another hnRNP, HNRNPA2/B1, was recently suggested to be modulated by m^{6}A switch mechanism (Wu et al., 2018). HNRNPA2/B1 was showed to affect RNA splicing and miRNA processing of m^{6}A methylated transcripts (Alarcón et al., 2015b).

m^{6}A binding proteins (readers)

The only so far known m^{6}A binding structural module is the YTH (YT521-B homology) domain (Xu et al., 2015). Proteins with YTH domain are found exclusively in eukaryotes and the amino acid sequence of the YTH domain is highly conserved across the species (Stoilov et al., 2002). Those proteins are classified into a so-called YTH protein family (Stoilov et al., 2002). So far, there are 5 proteins in human known to posses the YTH RNA-binding domain: YTHDC1 (also known as YT521-B), YTHDC2, YTHDF1, YTHDF2, YTHDF3 (Xu et al., 2014), and for example 13 proteins in *Arabidopsis thaliana* (Bhat et al., 2018; Stoilov et al., 2002).

YTH domain binds m^{6}A within the single stranded RNA regions independently on the presence of m^{6}A consensus motif (Xu et al., 2015), except for YTHDC1 protein, which shows a slight preference for m^{6}A consensus (Theler et al., 2014; Xu et al., 2014, 2015; Zhang et al., 2010). NMR structures of YTHDC1 protein revealed, that the conserved hydrophobic residues form a cavity in YTH domain and a so-called aromatic cage (Theler et al., 2014; Xu et al., 2014) (Figure 6). The aromatic cage composed of two tryptophan (W377, W428) and the leucine (L439) sidechains is responsible for m^{6}A recognition and binding (Xu et al., 2014). Both tryptophan residues are strictly conserved in YTH proteins, the leucine residue is replaced by another tryptophan at the corresponding position in YTHDF protein family, however it does not affect the function of the aromatic cage (Li et al., 2014; Xu et al., 2015). The tryptophan W428 side chain interacts with the m^{6}A methyl group, which provides the YTH domain specificity for the N6-methylated adenosine versus unmodified nucleotide (Xu et al., 2014). Also, the widely conserved positively charged amino acid residues surrounding the aromatic cage contribute to the RNA binding by the interaction with phosphate groups of RNA (Theler et al., 2014; Zhang et al., 2010). Outside the YTH domain, no sequence similarity is found between the YTHDC1, YTHDC2 and YTHDF family (Stoilov et al., 2002). Other domains of the YTH proteins probably determine their various localization and their interacting partners, affecting the function of those proteins in m^{6}A modified RNA metabolism (Zhang et al., 2010).

Some other proteins such as a group of insulin-like growth factor 2 mRNA-binding proteins IGF2BPs were suggested to directly bind m^{6}A modified RNAs, but the structural data are needed to further validate those findings (Huang et al., 2018a).

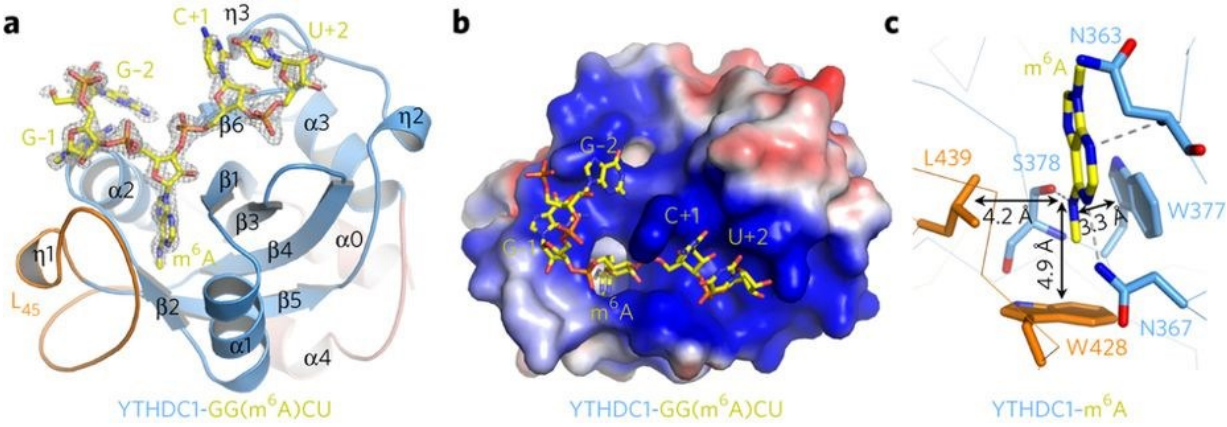


Figure 6 Structure of the YTHDC1 YTH domain in complex with m^{6}A-containing RNA pentamer (GG(m^{6}A)CU). m^{6}A residue is accommodated in cavity in so-called aromatic cage, visible in (c), which is formed by the sidechains of W377, W428 and L439. The RNA strand resides in positively charged groove of YTH domain. Color code: (a) The protein structure is shown in blue with loop L45 in orange and two helices (a0, a4) in pink, RNA pentamer is shown in yellow sticks. (b) Positive electrostatic potential on the YTH domain surface shown in blue, negative potential in red. (c) Protein residues in m^{6}A binding pocket are depicted in orange and blue sticks, m^{6}A residue in yellow sticks, hydrogen bonds are shown in grey dashes. Distances between N6 of m^{6}A and residues forming aromatic cage are shown in black. Adopted from (Xu et al., 2014).

YTHDC1 affects RNA splicing and nuclear export

Many studies have suggested a connection between m^{6}A modification and alternative splicing. First, m^{6}A is enriched in long internal exons (Dominissini et al., 2012; Ke et al., 2017), close to the 3' and 5' splice sites (Louloupi et al., 2018; Zhao et al., 2014), it is also more abundant in differentially spliced exons and introns (Dominissini et al., 2012). m^{6}A methyltransferase proteins and demethylases – METTL3/METTL14 complex, ALKBH5, FTO – localize in nuclear speckles (Jia et al., 2011; Liu et al., 2014; Ping et al., 2014; Zheng et al., 2013), the main reservoir of splicing factors (Galganski et al., 2017). Next, depletion of the methyltransferase protein METTL3 or demethylases FTO and ALKBH5 affect the alternative splicing patterns of many genes (Bartosovic et al., 2017; Dominissini et al., 2012; Louloupi et al., 2018; Ping et al., 2014; Tang et al., 2018; Xu et al., 2017; Zhao et al., 2014; Zheng et al., 2013). It was shown that m^{6}A modification can affect structure-dependent accessibility to the hnRNP family proteins HNRNPC, HNRNPG and HNRNPA2/B1 (Liu et al., 2015, 2017; Wu et al., 2018) All the mentioned hnRNP proteins are

involved in splicing regulation (Alarcón et al., 2015b; Hofmann and Wirth, 2002; König et al., 2010; Wang et al., 2011). Finally, recent studies revealed a mechanism of a direct effect of m^{6}A reader YTHDC1 on alternative splicing by influencing the splice site selection (Xiao et al., 2016).

YTHDC1 is the only human YTH protein predominantly localized in the nucleus, where it forms specific dot-like foci called YT bodies. YT bodies are often adjacent to the nuclear speckles (Nayler et al., 2000). In lower concentration YTHDC1 is also dispersed in the nucleoplasm (Hartmann et al., 1999; Nayler et al., 2000). It interacts with several splicing factors such as TRA2B, KHDRBS1 (alternative name SAM68), SRSF1/3/7/9/10 (Hartmann et al., 1999; Imai et al., 1998; Xiao et al., 2016). YTHDC1 preferentially binds m^{6}A in exonic regions near the splice sites (Xiao et al., 2016). Overexpression or depletion of YTHDC1 causes the changes in alternative exon exclusion/inclusion ratio of some mRNAs in concentration-dependent manner (Hartmann et al., 1999; Kasowitz et al., 2018; Rafalska et al., 2004; Xiao et al., 2016; Zhang et al., 2010). The binding of YTHDC1 to its recognition motif within the alternative exon results in the exon inclusion (Xiao et al., 2016; Zhang et al., 2010). Mechanistically, after YTHDC1 is bound, it recruits the splicing factor SRSF3 to the target region of pre-mRNA and SRSF3 then promotes inclusion of alternative exon. Moreover, SRSF10 binding to RNA, which would promote exon skipping, is repressed by YTHDC1 (Xiao et al., 2016). SRSF3 recruited by YTHDC1 to mRNA can further interact with the nuclear RNA export factor 1 (NXF1) and therefore promote the m^{6}A-dependent RNA export to the cytoplasm (Roundtree et al., 2017). As mentioned earlier YTHDC1 was also suggested to be involved in m^{6}A-dependent regulation of intracellular levels of SAM (Shima et al., 2017). Additionally, it was shown that YTHDC1 plays a role in XIST-mediated gene silencing. XIST RNA is heavily m^{6}A methylated, those m^{6}A residues are preferentially recognized by YTHDC1 and its binding is necessary for the silencing function of XIST (Patil et al., 2016). However, the exact mechanism of YTHDC1 effect on XIST is so far not known.

Cytoplasmatic YTH proteins regulate RNA translation and degradation

m^{6}A methylation is found more often in the transcripts with shorter half-lives (Tang et al., 2018; Zhao et al., 2014) and the m^{6}A density is negatively correlated with mRNA half-life (Schwartz et al., 2014). Additionally, the half-lives of the N6-methylated transcripts increase after the knock-down of various METTL3/METTL14 methyltransferase components (Bertero et al., 2018; Liu et al., 2014). The changes in m^{6}A levels affect the translation efficiency of specific m^{6}A target mRNAs, the translation efficiency can be changed in both directions – increased or decreased, since the

effect seems to be transcript-specific (Lin et al., 2017; Wang et al., 2015, 2014b). Although those effects can be partially a product of an overall dysregulation of a complex regulatory network, specific mechanisms of m^{6}A-dependent regulation of RNA stability and translation through m^{6}A reader proteins have been proposed.

YTHDC2 is an m^{6}A reader with cytoplasmic localization (Abby et al., 2016; Soh et al., 2017) with the possible enrichment in perinuclear region (Kretschmer et al., 2018). It contains several structural domains, for example the helicase domain with ATPase-dependent 3'→5' RNA helicase activity (Wojtas et al., 2017). YTHDC2 was shown to associate in an RNA-independent manner with several proteins, among them the XRN1 exonuclease (Kretschmer et al., 2018; Wojtas et al., 2017) and meiosis-specific protein MEIOC (Abby et al., 2016; Soh et al., 2017; Wojtas et al., 2017). MEIOC was shown to affect mRNA stability during meiosis (Abby et al., 2016; Soh et al., 2017). YTHDC2 also binds to the small ribosomal subunit even when the whole ribosome is assembled, in the vicinity of RNA entrance and exit site (Kretschmer et al., 2018). Some research groups speculate that those YTHDC2 interactors could affect the metabolism of YTHDC2-bound RNAs (Abby et al., 2016; Kretschmer et al., 2018; Soh et al., 2017; Wojtas et al., 2017). For example, YTHDC2 targets show altered stability after MEIOC knock-out (Soh et al., 2017) similarly to YTHDC2 knock-out (Wojtas et al., 2017). Nevertheless, the general effects of YTHDC2 binding or mechanisms beyond them are not fully understood.

Together, YTHDF family proteins are also suggested to control translation efficiency and stability of mRNAs, but the underlying mechanism are likely to be different from YTHDC2-dependent regulation (Du et al., 2016; Li et al., 2017a; Wang et al., 2014a, 2015; Zhou et al., 2015). YTHDF2 is localized in cytoplasm in processing bodies (P-bodies) (Wang et al., 2014a), where the RNA degradation process takes place (Sheth and Parker, 2003). It is composed of two domains: C-terminal RNA binding domain and N-terminal P/Q/N-rich domain, both domains are required for YTHDF2 proper function (Wang et al., 2014a). C-terminal domain selectively recognizes m^{6}A modified RNAs and the N-terminal domain targets the YTHDF2 in P-body (Wang et al., 2014a). N-terminal domain of YTHDF2 was also shown to interact with CNOT1 protein, subunit of the CCR4-NOT mRNA deadenylase complex (Du et al., 2016). Via this interaction CCR4-NOT deadenylase is recruited to the m^{6}A methylated transcripts recognized by YTHDF2 and those transcripts are deadenylated and subjected to degradation (Du et al., 2016). Additionally, it was shown that the YTHDF2-targeted transcripts get accumulated specifically in the translatable

polysome fraction after depletion of YTHDF2, which implies a role of YTHDF2 in translational repression (Wang et al., 2014a).

Not much is known about the YTHDF1 function. It binds m^6A in 3' UTR region and might be responsible for translation efficiency enhancement of a specific subset of mRNAs (Wang et al., 2015). This effect is probably connected to the association of YTHDF1 with small ribosomal subunit and the translation initiation factors such as eIF3 complex (Wang et al., 2015). Interestingly, YTHDF1 and YTHDF2 have some common RNA targets, Wang et al. showed that YTHDF1 is bound earlier than YTHDF2, this mutual regulation could be used in the cell for a fast response of protein production – the protein would be translated rapidly and at the same time, the short half-life of mRNA could help achieve the expression steady-state faster (Wang et al., 2015).

YTHDF3 was shown to interact with ribosomal proteins and to enhance the translation efficiency. Interestingly, the translation is enhanced cooperatively by YTHDF1 and YTHDF3, there is no enhancement of translation for YTHDF3-specific targets (Li et al., 2017a). Considering that YTHDF3 was also shown to interact with both YTHDF2 and YTHDF1 and increase their binding specificity, YTHDF3 could have a main role in modulating the function of the other two YTHDF proteins (Li et al., 2017a).

YTHDF2 and YTHDF3 also play specific regulation roles in the stress conditions. YTHDF2 localization is changed during the heat shock response. It is translocated into the nucleus where it is suggested to bind N6-methylated adenosines within the 5' UTR of stress-induced transcripts and therefore restrict it from demethylation by FTO (Zhou et al., 2015). The m^6A residue at 5' UTR promotes cap-independent translation (Meyer et al., 2015; Zhou et al., 2015). Under oxidative stress YTHDF3 selectively recognizes a subset of 5' UTR methylated transcripts and localizes them into the stress granules (Anders et al., 2018). YTHDF family proteins were also shown to bind m^6A modified viral RNAs and affect the production of viral particles probably by various mechanisms (Gokhale et al., 2016; Kennedy et al., 2016; Tirumuru et al., 2016).

Summary and discussion

In the last few years, m^{6}A methylation and potential epitranscriptomic regulations have become a hot-topic in RNA biology. m^{6}A modification can be found throughout various species and is abundant in eukaryotic RNAs (Beemon and Keith, 1977; Canaani et al., 1979; Deng et al., 2015; Dominissini et al., 2012; Lence et al., 2016; Meyer et al., 2012; Nichols, 1979; Schwartz et al., 2013; Sommer et al., 1976). In mRNAs, it is enriched within the specific regions such as 3' UTR (Ke et al., 2015; Meyer et al., 2012), differentially spliced and long exons (Dominissini et al., 2012; Ke et al., 2017; Schwartz et al., 2014) or the proximity of splice junctions (Louloupi et al., 2018). It is worth noticing, that the regions enriched for m^{6}A are often bound by RNA-processing factors. It was shown that m^{6}A presence can modulate the binding of RNA-processing factors and therefore change the metabolism of m^{6}A modified transcripts including alternative splicing (Alarcón et al., 2015b; Liu et al., 2015, 2017; Xiao et al., 2016), nuclear export (Roundtree et al., 2017), translation (Wang et al., 2015) efficiency or RNA degradation (Du et al., 2016). This modulation of RNA metabolism is achieved by at least two different mechanisms – directly by the structural change of RNA region promoted by m^{6}A (so-called m^{6}A switch mechanism)(Liu et al., 2015, 2017; Wu et al., 2018) or it is mediated by specific m^{6}A binding proteins (Du et al., 2016; Roundtree et al., 2017; Wang et al., 2015; Xiao et al., 2016). m^{6}A is also present on various non-coding RNAs (Alarcón et al., 2015a; Dominissini et al., 2012; Karijolich and Yu, 2010; Linder et al., 2015; Meyer et al., 2012; Warda et al., 2017) but its function on those RNAs is so far not well known. Interestingly, it was shown that m^{6}A modification of miRNAs could be essential for their biogenesis (Alarcón et al., 2015a) and specific m^{6}A site in U6 snRNA might be functionally important for 5' splice-site recognition (Warda et al., 2017).

Several enzymes are known to be involved in m^{6}A modification of RNA. METTL3/METTL14 is a major methyltransferase complex, which methylates adenosine within the m^{6}A consensus sequence RRACH (Liu et al., 2014). METTL16 is responsible for some m^{6}A sites outside the established m^{6}A consensus, so far it was shown to deposit the m^{6}A in U6 snRNA splice-site recognition region (Warda et al., 2017) and in SAM synthetase MAT2A mRNA, where the m^{6}A is important for regulation of intracellular SAM levels (Pendleton et al., 2017; Shima et al., 2017). So far known m^{6}A demethylases ALKBH5 and FTO are suggested to demethylate just specific m^{6}A sites and specific subsets of RNAs (Bartosovic et al., 2017; Tang et al., 2018). Finally, proteins with the YTH domain such as human YTHDC1-2 and YTHDF1-3 directly bind m^{6}A (Xu et al., 2014, 2015) and each of the proteins has its specific effect on metabolism of the target RNAs (Du et al.,

2016; Roundtree et al., 2017; Wang et al., 2015; Xiao et al., 2016). Some other RNA binding proteins outside the YTH protein family were suggested to bind m^{6}A (Huang et al., 2018a), however more data about their binding properties and effects on RNA metabolism are needed. Recently, FTO was suggested to preferentially demethylate the m^{6}Am modification which is adjacent to the 5' cap of some RNAs (Mauer et al., 2017, 2019). Since some m^{6}A readers were suggested to cooperate with FTO (Zhou et al., 2015), it would be interesting to test, whether they are truly m^{6}A specific or they can also bind m^{6}Am.

It is known that the specific localization of m^{6}A site determines the effect m^{6}A has on the RNA. However, it is not clear how the sites for m^{6}A deposition are determined. METTL3/METTL14 methyltransferase binding was shown to be sequence-specific (Liu et al., 2014), but the recognized consensus is much more abundant in the RNA, than actual m^{6}A modification, therefore not every consensus sequence is recognized and modified by the methyltransferase (Dominissini et al., 2012). The specificity of METTL16 recognition is a little higher since METTL16 binding requires specific sequence features but also a specific secondary structure of the target region (Mendel et al., 2018). It is a subject of discussion whether the m^{6}A site is determined just based on the sequence-related signals or whether the other signals are involved, potentially connecting the m^{6}A-related regulation to for example regulation of transcription or epigenetics. Indeed, it was suggested that METTL3/METTL14 recruitment to its target site could be mediated co-transcriptionally by the transcription factors (Barbieri et al., 2017; Bertero et al., 2018) or it could even be dependent on the histone modifications surrounding the transcribed region (Huang et al., 2019). On the other hand, there are groups which claim that the majority of m^{6}A sites can be successfully predicted using just the sequence information (Schwartz et al., 2013).

Another so far unclear feature of m^{6}A modification is its dynamics. The existence of demethylases could provide cells with a potential of changing the m^{6}A patterns under certain conditions independently on the primary signals which define the m^{6}A target sites. Studies of m^{6}A demethylases showed their differential expression in various tissues and tissue specific effects of the demethylase knock-out, suggesting that reversibility of m^{6}A modification is important for some tissue-specific processes (Fischer et al., 2009; Tang et al., 2018). Data from m^{6}A mapping experiments suggest that overall distribution of m^{6}A modification is generally stable throughout the cells in various conditions or differentiation stages (Batista et al., 2014; Dominissini et al., 2012; Schwartz et al., 2014) but it can probably change distinctly under stress conditions such as heat

shock or ultraviolet-induced damage as observed by (Meyer et al., 2015; Xiang et al., 2017; Zhou et al., 2015). Moreover, the m^{6}A modification is non-stoichiometric (Horowitz et al., 1984; Liu et al., 2013) and it is possible, that the ratio of methylated and demethylated transcripts is changed more dynamically. The methods widely used for transcriptome-wide m^{6}A mapping such as miCLIP are not sensitive enough to quantitative changes in m^{6}A levels and other methods must be established to precisely quantify the m^{6}A/A ratios in various conditions (Hartstock and Rentmeister, 2019).

Not just the m^{6}A modification itself, but also the m^{6}A readers can be regulated in response to certain cellular processes or outer stimuli, resulting in different m^{6}A-dependent regulation of specific transcripts. It was shown that the m^{6}A reader YTHDC1 is a kinase target and its phosphorylation alters its localization and activity (Rafalska et al., 2004). Similarly, m^{6}A binding protein YTHDF2 is translocated from cytoplasm to the nucleus during the heat shock response, where it probably gains totally different function (Zhou et al., 2015).

m^{6}A was shown to be important for many biological processes such as pluripotency exit (Batista et al., 2014; Bertero et al., 2018; Chen et al., 2015; Geula et al., 2015; Wang et al., 2014b), spermatogenesis (Lin et al., 2017; Tang et al., 2018; Xu et al., 2017; Zheng et al., 2013), maternal-to-zygotic transition in zebrafish (Zhao et al., 2017), stress responses (response to heat shock and UV damage), (Meyer et al., 2015; Xiang et al., 2017; Zhou et al., 2015), viral infection (Gokhale et al., 2016; Kennedy et al., 2016; Tirumuru et al., 2016), tumorigenesis (Cui et al., 2017; Li et al., 2017b; Zhang et al., 2016, 2017) or even for meiosis and sporulation in yeast (Schwartz et al., 2013). All those processes require fast and distinct changes of gene expression. Considering for example the possible functional interplay of YTHDF m^{6}A readers, which can together provide a quick change in protein production by increasing the translation efficiency and decreasing the half-life of the transcript (Li et al., 2017a), we can speculate that m^{6}A-dependent regulation of RNA metabolism is partially responsible for the fast changes in gene expression during the mentioned processes.

Taken together, m^{6}A modification of RNA is a unique RNA modification with dual function. It directly modulates the RNA structure and its accessibility for RNA binding proteins. Furthermore, similarly to epigenetic modifications, m^{6}A serves as a signal on RNA, possibly further increasing the coding capacity.

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