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Reading RNA methylation codes through methyl-specific binding proteins

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N^6-methyladenosine (m^6A) is a prevalent modification of eukaryotic mRNAs. It regulates yeast cell fate and is essential to the development and fertility of metazoans. Although its presence in mRNA has been known since the early 1970s, the function of m^6A remained a mystery until the spate of discoveries in the past three years. Here, we focus on the discovery of m^6A "readers" (proteins that specifically recognize m^6A), and their functions in tuning mRNA stability, as well as the broader significance of such m^6A-dependent regulation of gene expression.

Eukaryotic mRNAs (mRNAs) not only encode precise protein sequence, but also convey information for their processing, transportation, translation, and decay, thereby collectively creating a complex layer of gene regulation at the post-transcriptional stage. Known mechanisms involving RNA structure, microRNA, and translation regulation all contribute to post-transcriptional regulation of gene expression. Established regulatory modes of mRNA involve short RNA sequences in untranslated regions (UTRs) as exemplified by AU-rich element (stability), iron-responsive element (translation), zipcode element (localization), micro RNA seeding sequences (translation and stability), and the mRNA cap and polyadenylated tail. In this paper, we discuss a new mechanism of regulation: reversible internal RNA methylation.

Essential Description of m^6A

N^6-methyladenosine (m^6A) is a major internal (non-cap) modification of eukaryotic mRNA (mRNA).[1] On average, every mRNA has three m^6A residues within a context of G(m^6A)C (70%) or A(m^6A)C (30%).[2] Recent advances in m^6A-sequencing technology have also revealed m^6A enrichment in long exons and around the stop codon.[3-5] Despite the widespread distribution of m^6A sites over 7000 human transcripts, the m^6A content of individual mRNAs is non-uniform; each m^6A site can be non-stoichiometric while some mRNAs are free of m^6A.[1] m^6A is post-transcriptionally installed by an m^6A methyltransferase complex[1,6] in coordination with other mRNA processing events, namely 3' polyadenylation, 5' capping, and splicing. In addition to previously identified subunit METTL3, we have recently revealed two other components of mammalian m^6A methltransferase complex: METTL14 forms a stable heterodimer with METTL3 as the enzymatic core; WTAP interacts with the heterodimer to affect their methyltransferase activity inside cells.[7] The discovery of two functionally significant m^6A demethylases (FTO[8] and AlkBH5[9]) has defined mRNA methylation as a reversible process. METTL3 is essential for proper meiotic entry of budding yeast,[10,11] the viability of plants,[12] fruit flies,[13] and human HeLa cells,[6] while defects in FTO and

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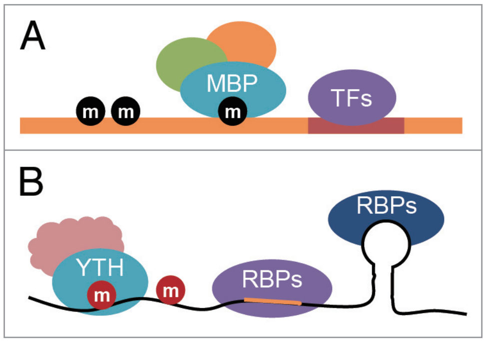

Figure 1. Specific binding proteins recognize DNA and RNA methylations. (**A**) The methyl-binding proteins (MBP) recognize mammalian DNA methylation (black ball). The figure also illustrates the methylation state with binding of MBP and proteins (green and orange) that interact with MBP as well as the various transcriptional factors (purple) that control transcription. (**B**) The YTH domain family proteins (blue) selectively recognize internal RNA methylation (red ball). The fate of mRNA is controlled by the interplay of methyl-specific binding proteins, protein factors (pink) that interact with YTH domain family proteins, and other RNA-binding proteins (RBPs, dark blue and purple) that recognize RNA sequence and/or structure.

AlkBH5 affect body weight and fertility respectively, thus demonstrating the physiological importance of m^6A.

Considering m^6A as a new layer of information on top of the primary sequence, methylation, and demethylation resemble writing and erasing. Yet a mechanism to read out the methylation information must exist. m^6A can either repel RNA-binding proteins that interact with A or be recognized by methyl-specific binding proteins or "readers." Potential readers have been suggested in early pull-down experiments using methylated RNA probes.[3] We have characterized the YTH domain family proteins as m^6A readers and provided the first functional role of m^6A through this reading process.[14] The analogy between "writer/eraser/reader" and methyltransferase/demethylase/selective binding partners, though not scientifically accurate, is advantageous in order to abstract the working pattern of all chemical modifications of DNA, mRNA, and proteins. Comparable to studies of DNA methyl-CpG binding proteins (MeCPs),[15] we believe that "readers" provide clues to uncover the mechanism and cellular function associated with m^6A.

YTH Domain Family as m^6A-Specific Binding Proteins

The YTH domain is a newly discovered domain that binds to short, degenerated, and single-stranded RNA sequences.[16] The YTH domain family has 174 members in various eukaryotic species.[17] In humans, the YTH domain family (YTHDF) contains three members, YTHDF1, YTHDF2, and YTHDF3. YTHDF2 and YTHDF3 were selectively identified using synthetic RNA bait containing m^6A.[3] We also discovered YTHDF1 with a slightly different bait sequence. All three proteins have significantly higher affinity for methylated probes as measured by gel shift assay, and thus are m^6A-specific binders.

Next-generation sequencing and advances in RNA–protein complex isolation technique have greatly empowered transcriptome-wide studies of RNA-binding proteins. By an integration of photoactivatable–ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP),[18] ribosome profiling,[19] and mRNA lifetime profiling results, current conclusions about YTHDF2 are: (1) YTHDF2 indeed binds m^6A inside the cell since YTHDF2-binding sites agree well with m^6A sites; (2) there is no direct interaction between YTHDF2 and polysomes; (3) the major function of YTHDF2 is to accelerate the decay of its targets (by roughly 30% as determined by YTHDF2 knockdown); (4) the YTH domain at the C terminus of YTHDF2 is sufficient to recognize m^6A while the N-terminal domain involves localizing the RNA to processing bodies where RNA decay can occur.[20] Results using a reporter system show that binding of YTHDF2 to its RNA target takes place in parallel with or at a late stage of deadenylation, which is a prerequisite step of eukaryotic mRNA decay.[21] Taken together, these results have led to a mechanistic model in which YTHDF2 transduces m^6A code into an RNA turnover signal by its modular structure and then conveys its bound RNA to decay machinery.

Recent studies of YTHDF2 homolog in yeasts may support a conserved role of the YTH domain protein in controlling mRNA turnover.[5,22,23] Mmi1, the YTHDF2 homolog in fission yeast (*Schizosaccharomyces pombe*), selectively degrades meiotic mRNA transcripts by RNA surveillance machinery during vegetative growth.[22] In budding yeast (*Saccharomyces cerevisiae*), m^6A conditionally accumulates during sporulation induced by nutrition starvation.[24] Ydr374c (Pho92 or MRB1), the corresponding YTH domain protein in budding yeast, has also been shown as a m^6A reader protein.[5] It regulates the stability of Pho4 mRNA, an important transcription factor in the phosphate signal transduction (PHO) pathway, by binding to its 3'UTR in a phosphate-dependent manner.[23] Mechanistically, Pho92 interacts with the Pop2-Ccr4-Not deadenylation complex, which coincides with our observation that YTHDF2 co-localizes with Pop2 (CNOT7) in human HeLa cells. Hence, these studies in yeast shed light on a possible role of m^6A in nutrition metabolism via regulation of mRNA stability.

m^6A-Dependent Control of mRNA Stability

To better understand the effect of m^6A, it is worth discussing how m^6A-dependent control of mRNA stability compares to other means of gene expression regulation. Based on transcriptome-wide measurements of RNA levels from cell populations, temporal mRNA level changes in response to external stimuli was suggested to be primarily determined by the change of transcription rate.[25] However, the change of mRNA degradation rate is important in order to define sharp responses. We have also observed that transcripts bearing m6A have inherently shorter lifetimes than non-targets.[26] Genes can be roughly classified into housekeeping genes whose protein production is always in demand, or regulatory genes whose protein production is conditional or strictly controlled at low abundance. Given that some mRNAs encoding housekeeping genes are free of m6A (globin, histone)[1] and that YTHDF2 RNA targets are enriched with regulatory genes such as transcription factors,[14] it is possible that m6A represents one mechanism that imposes precise control over the expression of those regulatory genes.

At the transcriptional level, transcriptional factors (TFs) recognize the genomic sequence. At the same time, DNA methylations or other forms of chemical modifications exist in high eukaryotes that specific reader proteins can recognize in order to exert additional regulation of gene expression (**Fig. 1**). Nature seems to use the same "trick" to gain additional control of gene expression at the mRNA level. Various RNA-binding proteins, parallel with TFs on DNA, exist to affect transport, storage, splicing, and stability of mRNAs. Now, we show that reader proteins also exist to recognize internal mRNA methylation, which provide additional complexity as well as the means to affect eventual protein production (**Fig. 1**). Chemical methylations can be added or removed on mRNA.[6-9] Therefore, these m6A codes are dynamic, reversible, and dependent on cell type and state. Such a feature of m6A might be extremely important during dynamic cell differentiation process, such as embryonic[13] and neuronal[27] development. It is highly possible that m6A codes can function in concert with all other RNA sequence elements as well as RNA-binding proteins, again analogous to the interplays between TFs and 5mC reader proteins on DNA.[28]

Future Directions

YTHDF1 and YTHDF3 have already been identified as m6A-specific binding proteins;[14] therefore, functional characterizations of these two YTHDF proteins are ongoing. Given the high-sequence similarities between YTHDFs, it is possible that these proteins are degenerate to some extent. We have shown that YTHDF2 mediates the translocation of m6A-containing RNA from the translatable pool to non-ribosomal mRNA–protein particles; however, we do not know if m6A-containing RNAs are targeted by YTHDF2 before any translation or after they exit translation. In addition, various RNA-binding proteins coat mRNAs; it is therefore tempting to envision that m6A readers work in concert with other sequence-specific RNA-binding proteins to collectively decide the fate of mRNA. It will be valuable to characterize the protein interactome of YTHDF2 as well as the other two YTHDF proteins.

Finally, knockdown of YTHDF2 causes reduced viability of HeLa cells. However, without knockout model organisms such as knockout mouse the exact physiological function of YTHDF2 remains unknown. Future investigations in this area are necessary. The discovery and characterization of m6A reader proteins represent critical steps in order to understand reversible m6A-dependent regulation at the RNA level,[26] but there are still vast knowledge gaps between molecular details and the biological necessity of m6A that require further research and connection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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