Abstract
The N6-methyladenosine (m6A) modification of mRNA has a crucial function in regulating pluripotency in murine stem cells: it facilitates resolution of naïve pluripotency towards differentiation.

An old modification rediscovered: m6A in messenger RNA
Epigenetic modifications of DNA and histones have been well investigated as crucial factors in the regulation of gene expression. However, the study of similar chemical modifications of RNA is still in its infancy. Among them, N6-methyladenosine (m6A) is the most abundant internal modification of the messenger RNA of almost all eukaryotes and of viruses that replicate in nuclei. Despite its existence being reported 40 years ago [1], the biological function and significance of m6A have only recently entered the research spotlight. The m6A modification was found to be dynamic, with the discovery of the alpha-ketoglutarate-dependent dioxygenase FTO and RNA demethylase ALKBH5, which are two demethylases that remove the methyl group from m6A within RNA, as well as the characterization of N6-adenosine-methyltransferase subunits METTL3 and METTL14, two methyltransferases that methylate adenosine residues to form m6A in RNA molecules [2-4] (Figure 1a). From these reports, methylation giving rise to m6A was demonstrated as the first confirmed reversible RNA modification, which sparked the re-emergence of research interest in this modification. Transcriptome-wide profiling of m6A further revealed its distribution pattern in mammalian cells and tissues [5,6]. It has a predominant sequence consensus and is strongly enriched around stop codons, and within long internal exons and at transcription start sites. These features suggest that m6A performs functional roles in regulation.

As the most abundant internal modification in mammalian mRNA, m6A is involved in multiple aspects of RNA metabolism, including RNA stability, translation, splicing, transport and localization (Figure 1a). One of the best-defined functions of m6A came alongside the discovery and characterization of the first m6A-specific binding protein, the YTH domain-containing family protein 2 (YTHDF2) [5,7]. YTHDF2 alters the overall stability of m6A-containing mRNAs by causing their relocalization to specialized mRNA-decay machineries, where they are committed to degradation. Other data also suggest roles for m6A in mRNA export and translation.

The modification m6A is essential in mammals and has been suggested to impact cell differentiation and development. Researchers have been probing the involvement of m6A in mouse and human embryonic stem cells (ESCs), but have produced somewhat conflicting results. Wang and colleagues [8] had reported in 2014 that knockdown of Mettl3 and Mettl14 in murine ESCs (mESCs) resulted in reduced m6A abundance and defective cell regeneration. Yet, in a more recent study, Batista and colleagues [9] found that complete knockout of Mettl3 in mESCs led to improved self-renewal and resulted in blocked differentiation. In the latest paper on this topic, published in Science, Geula and colleagues [10] confirm the crucial roles of m6A methylation in the differentiation of mESCs and present a detailed demonstration of how depletion of m6A drives mESCs at naïve or primed pluripotency states to divergent fates.

m6A as a fate determiner in murine embryonic stem cells
Geula and colleagues started by screening for factors essential for maintenance of pluripotency and identified several candidates. Among them, METTL3 emerged as a crucial component for regulation of stem cell pluripotency. The pluripotent stage can be further divided into two states: a ground, naïve state, and a differentiation-
prepared, primed state. The authors then separately investigated the pluripotent stem cells at naïve (ESC) and primed (epiblast stem cell, EpiSC) states along with the respective impacts of m^6^A. To dissect the roles of METTL3 and m^6^A in the naïve pluripotent state, heterozygous Mettl3^+/−_ mice were generated, and homozygous Mettl3^−/−_ mESCs were obtained from embryos. In contrast to wild-type cells, Mettl3^−/−_ cells generated partially differentiated embryoid bodies (EBs), failed to produce mature neurons and were unable to proceed into a primed state upon corresponding inductions in vitro. Similarly, when these cells were injected into immunodeficient mice in vivo, the teratomas generated were poorly differentiated. These data indicate that the depletion of METTL3, and hence m^6^A, blocks differentiation in mESCs and keeps them in a so-called hyper-naïve state. By contrast, the depletion of METTL3 and m^6^A in the primed EpiSCs during a primed pluripotent state produced the opposite spectrum of effects - this depletion led to minimal self-renewal and fast differentiation, ultimately disrupting the stability of the primed state and resulting in cell death. Thus, the roles of depletion of METTL3 in this state are to reduce stem cell self-renewal and push them towards differentiation (Figure 1b).
This seemingly contradictory effect was explained by subsequent high-throughput sequencing analysis. Transcriptome-wide profiling of m\(^6\)A (m\(^6\)A-seq) was performed in ESCs, EBs and mature mouse cells. The results revealed that 80% of transcripts of naïve pluripotency genes and multiple lineage-commitment genes are methylated. Thus, m\(^6\)A affects the genes governing both the naïve and primed states. Subsequent measurements of the global level and life-time of transcripts revealed that methylation shortens the half-life of modified mRNAs and reduces their abundance. Therefore, depletion of METTL3 increases the abundance and dominance of already-expressed genes, which leads to the observed phenotypes. For instance, in the naïve state, pluripotency genes dominate. Without m\(^6\)A methylation, cells become stuck at the so-called hyper-pluripotent state, with high levels of expression of pluripotent transcripts. By contrast, at the primed state, lineage-commitment genes prevail, and removing m\(^6\)A further tips the balance towards differentiation (Figure 1b).

Taking their findings further, the authors carefully measured the expression of both pluripotency and differentiation genes in Mettl3\(^−/−\) embryos before they died. It was found that the widespread expression of the pluripotency marker Nanog (encoding homeobox protein Nanog) was prolonged; and, although the expression of the differentiation marker Pou5f1 (encoding transcription factor Oct4) was maintained, other lineage-commitment genes were not upregulated in the way that they are in wild-type embryos. Thus, knockout cells were in naïve-like state, with a certain level of priming, exhibiting resistance to progression of differentiation. These in vivo findings are in good accord with the observed mESC phenotypes.

**Unifying mechanisms under a plethora of phenotypes**

All the studies performed to date demonstrate that the presence of m\(^6\)A reduces the stability of methylated mRNA transcripts in mESCs. Phenotypes are determined by the dominating type of transcripts, and m\(^6\)A depletion works to intensify the trend. Although the authors did not explain how m\(^6\)A affects mRNA stability, previous studies indicate that YTHDF2 could be partially responsible [6]. However, regulating mRNA stability is but one confirmed function of m\(^6\)A. In this work, Geula and colleagues additionally suggest that m\(^6\)A might increase the splicing efficiency of unfavored splicing events. Other aspects of RNA processing could also be affected by methylation and contribute to the observed phenotypes. In principle, with each discovered m\(^6\)A-specific binding protein (or m\(^6\)A ‘reader’), there will be a corresponding function associated with m\(^6\)A. Therefore, further research to identify and characterize m\(^6\)A-specific binding proteins is important for uncovering the functions of mRNA m\(^6\)A methylation. Studies of these reader proteins and their roles could provide underlying mechanisms for cell differentiation and development phenotypes associated with m\(^6\)A.

**Concluding remarks**

In summary, the comprehensive work presented by Geula et al. reveals m\(^6\)A as a timely maintainer of the balance between pluripotency and lineage priming factors, thus ensuring orderly differentiation of mESCs. The authors have shown that m\(^6\)A in mRNA might work as a ‘plug-in’ to other pre-existing pathways by altering downstream gene expression. In this manner, RNA modifications can promote a fast response to external cues during times of cellular transformation or differentiation. We fully anticipate additional future discoveries that connect modifications of mRNA with the regulation of gene expression in cell differentiation and development in this fast-growing field.

**Abbreviations**

EB: Embryoid body; EpiSC: Epiblast stem cell; mESC: Murine embryonic stem cell; m\(^6\)A: N\(^6\)-methyladenosine.

**Competing interests**

The authors declare that they have no competing interest.

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