RNA MODIFICATIONS

Site-specific m^{6}A editing

The N^6-methyladenosine modification on RNA affects almost all steps of RNA metabolism. A new approach, using the CRISPR-based technology to modulate m^{6}A level in mRNA, enables direct functional interrogation of site-specific m^{6}A.

Jiangbo Wei and Chuan He

Reversible RNA methylation has recently emerged as a post-transcriptional regulator of RNA metabolism and function. Among more than 150 known chemical marks on cellular RNA, N^6-methyladenosine (m^{6}A) is a modification that exists in multiple RNA species, including messenger RNAs (mRNA), ribosomal RNAs (rRNA), and spliceosomal small nuclear RNAs (snRNA), and is the most abundant internal mRNA modification in most eukaryotic cells. The majority of m^{6}A on mRNA is installed co-transcriptionally in the consensus motif RRACH (R = A or G; H = A, C, or U) by the 'writer' complex consisting of METTL3, METTL14 and WTAP. The methyl group of m^{6}A on mRNA can be removed by the 'eraser's FTO and ALKBH5, and thereby the methylation-dependent processes can be reversed and controlled. There are also 'reader' proteins that recognize and preferentially bind to the m^{6}A-containing mRNAs, recruiting other RNA-binding proteins to affect the fate of the methylated mRNAs. Despite recent functional characterizations of mRNA m^{6}A, the field lacks tools to dissect contributions of specific m^{6}A sites on the modified RNAs. In this issue, Liu et al.[1] report an 'm^{6}A editing' approach that allows installation or removal of the methyl group at specific m^{6}A sites without altering the primary sequencing.

Early transcriptome-wide m^{6}A mapping revealed that m^{6}A is widely distributed in approximately 7,000 mRNAs and is enriched at stop codons and at 3′ untranslated regions (UTR)[2]. m^{6}A has also been shown to occur at the 5′ UTR and start codons[3]. Previous work has taken advantage of the type VI CRISPR-associated RNA-guided ribonuclease (RNase) Cas13 and tethered the m^{6}A reader proteins fused to catalytically inactive Cas13b (dCas13b) to specific RNA sites[4]. Translation and transcript stability could be tuned using this approach. However, modulation of m^{6}A levels on specific methylated RNA to dissect functional roles of the methylation has not been achieved.

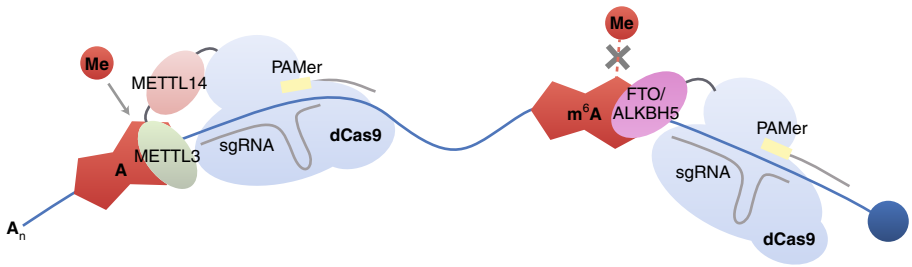

Fig. 1 | Depiction of Site-specific m^{6}A editing. Engineered 'writers' and 'erasers' are constructed by fusing METTL3–METTL14 fusion protein and demethylases (FTO or ALKBH5) to dCas9, respectively. With the presence of a protospacer adjacent motif (PAM) supplied by an antisense oligonucleotide (PAMer), these engineered 'writers' and 'erasers' can be directed by sgRNA to specific sites of targeting transcript to modulate m^{6}A levels. (A, adenosine; A$_n$, poly-A tail.)

To study context-dependent m^{6}A regulation[5], Liu et al.[1] developed a method to achieve programmable editing of epitranscriptome by coupling m^{6}A regulator enzymes with RNA-targeting dCas9 (Fig. 1). They linked two methyltransferase domains from METTL3 and METTL14 and constructed the engineered m^{6}A 'writer' by fusing these two fusion proteins to dCas9. This engineered 'writer' can be directed to the desired region on the transcript of interest by a single guide RNA (sgRNA) to achieve single-site methylation. Liu et al.[1] used this construct to induce a site-specific methylation targeting 5′ UTR of *Hsp70* mRNA and demonstrated that the 5′ UTR m^{6}A promotes cap-independent translation under stress conditions. Moreover, the authors also found that site-specific m^{6}A installation onto the 3′ UTR, but not the 5′ UTR, of endogenous *Actb* transcripts leads to the degradation of the targeting mRNA, supporting the concept that a single m^{6}A modification at different regions could exhibit distinct effects on mRNA. Following the successful engineering of m^{6}A 'writer', Liu et al.[1] fused the full-length ALKBH5 or FTO to dCas9 to achieve the targeted removal of m^{6}A. They showed that removal of the internal m^{6}A at A2577 of the long noncoding RNA *Malat1* results in the destabilization of its stem structure.

Together, Liu et al.[1] demonstrate that the m^{6}A enzyme-dCas9 can serve as a versatile toolbox to explore the regional effects of m^{6}A methylation. It would be attractive to utilize this system to achieve site-specific methylation and demethylation of key transcripts of development- or disease-related pathways in the future. Although various CRISPR-based systems have been investigated in recent years to target RNA, a CRISPR–Cas-inspired RNA targeting system (CIRTS) has recently been developed as a platform for engineering and delivering diverse programmable RNA effectors to target transcripts[6]. CIRTS is composed of a single-stranded RNA-binding protein, an RNA hairpin-binding protein, an effector protein, and a guide RNA. Components of CIRTS are built entirely from the human genome, and the protein part is much smaller than that of natural CRISPR–Cas systems. Engineered m^{6}A 'reader' proteins have been successfully constructed and delivered via CIRTS to modulate the translation or stability of target transcripts. Site-specific installation or removal of m^{6}A by an engineered 'writer' or 'eraser' with CIRTS could offer a humanized system to manipulate the human RNA epitranscriptome.

These new tools offer a way to more precisely understand the exact roles of

methylation at different regions. For instance, FTO has been shown to mediate demethylation of internal mRNA m^{6}A as well as N^6, 2′-O-dimethyladenosine (m^{6}A$_m$), a modification found on the second base adjacent to the 5′ cap (cap-m^{6}A$_m$) in a portion of mRNAs and snRNAs[7–9]. The cap-m^{6}A$_m$ and internal m^{6}A share identical chemical structures in the base moiety. The biological function of cap-m^{6}A$_m$ in mRNA remains unclear, as both transcript-stabilizing and translation-promoting roles have been previously proposed[7] but have yet to be confirmed[9,10]. The function of cap-m^{6}A$_m$ could be context or transcript specific. The new technique reported by Liu et al.[1] may allow functional characterization of cap-m^{6}A$_m$ on specific transcripts or in specific biological processes. ❐

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Competing interests
C.H. is a scientific founder of Accent Therapeutics and a member of its scientific advisory board.

MICROBIOLOGY

T(oo)bAd

An unusual terpene nucleoside, 1-TbAd, made by pathogenic mycobacteria acts as an antacid to block mycobacterial degradation in host cell vacuoles. The antacid activity acts to reduce acidity by neutralizing the pH of these degradative cell organelles.

Priscille Brodin and Eik Hoffmann

To fight infectious bacteria, a molecular understanding of the complex defense strategies that those pathogens deploy to disarm host defense mechanisms is needed. Since the pioneering studies of Élie Metchnikoff on phagocytes[1], there has been a particular focus on the specific mechanisms allowing pathogenic bacteria to fight the harsh acidic conditions in organelles called phagosomes, which are dedicated to microbe degradation in macrophages. In the case of *Mycobacterium tuberculosis* (*Mtb*), responsible for tuberculosis in humans, the ability to survive under these phagosomal conditions has been attributed to direct inhibition of the vacuolar proton pump (H^+ v-ATPase) at the phagosomal membrane, which usually generates a hostile acidic internal environment[2]. The direct neutralization on the H^+ v-ATPase was already shown to be mediated through the action of the bacterial protein phosphatase A or by hijacking host cellular pathways enabling its proteasomal degradation upon CISH-mediated ubiquitination[3,4]. More broadly, many mycobacterial products and virulence factors have been shown to interfere with and to arrest phagosome acidification[5]. Buter et al. now reveal that to fight the host-mediated acid defense, *Mtb* has 'another trick up its sleeve'. In fact, it appears that *Mtb* has the ability to release a unique terpene nucleoside called 1-tuberculosinyl adenosine (1-TbAd), which acts in neutralizing the acidic pH of host vacuoles[6].

1-TbAd is synthesized by the adenosine transferase encoded by the *Rv3378c* gene, which had been identified previously in a transposon screen for the identification of bacterial factors involved in control of phagosomal acidification[7]. Indeed, the *Rv3378c* knockout mutant was completely unable to halt the acidification of the phagosome[7]. Consistent with this, the authors show that expression of the Rv3377c and Rv3378c loci in a heterologous mycobacterium (*M. kansasii*), which initially lacks 1-TbAd, led to a growth advantage in acidic conditions, specifically arguing for a role of 1-TbAd in pH control.

How does 1-TbAd really impact on phagosomal pH? To decipher this, the authors generated a series of chemical analogs of 1-TbAd with different lipid moieties and modifications on the adenosine. They went on to study the effects of beads coated with these different 1-TbAd analogs on phagosomal pH in macrophages and demonstrated that only synthetic 1-TbAd was able to prevent acidification of bead-containing phagosomes.

Upon investigation of its spatial localization within *Mtb*-infected macrophages, 1-TbAd was found to be anchored by its lipid moiety on the bacterial surface (around 90%) but also shed in the spatially constrained phagosomal lumen (by around 10%) (Fig. 1). Next, using a model membrane system, the authors showed that 1-TbAd was able to cross lipid membranes and become specifically trapped at low pH (pH = 5).

Surprisingly, when investigating phagosomal morphology by electron microscopy after extemporaneous addition of large amounts of synthetic 1-TbAd, the authors observed massive swelling of those vacuoles, similar to the lysosomotropic effect of the antimalarial agent chloroquine. Corroborating this, a *Mtb* mutant deleted for Rv3378c localized in a more compact vacuole, whereas 1-TbAd-expressing mycobacteria were in larger phagosomes at 4 d post-infection in macrophages. It is now widely accepted that subsequent to the blockage of phagosome acidification, the *Mtb*-containing vacuole ruptures, enabling growth and replication of the bacilli[8,9]. Whether the observed impact by 1-TbAd on