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Loud and Clear Epitranscriptomic m^{1}A Signals: Now in Single-Base Resolution

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In this issue of *Molecular Cell*, Li et al. (2017) map N^1-methyladenosine (m^{1}A) with base precision, parsing the methylome into subsets differing in location, sequence-structure features, and catalyzing enzymes, thereby aiding functional investigation.

Conceptual and technological breakthroughs since 2011 have introduced the novel notion that internal chemical modifications of mRNA and non-coding RNA are abundant, dynamic, and reversible events, which constitute essential regulatory elements in a growing number of RNA processing steps such as splicing, transport, translation, and decay.

The epitranscriptome, as this ensemble is now known, comprises a growing number of chemical adducts—N^6-methyladenosine (m^{6}A), 5-methylcytidine (5mC), 5-hydroxymethylcytidine (5hmC), pseudouridine (Ψ), 2′-*O*-methylation (Nm), and m^{1}A—that elaborate the RNA alphabet to embed transcripts with additional information (Roundtree et al., 2017). Based on mass spectrometry, m^{6}A and Ψ are the most abundant modifications, present at ~0.4%–0.6% of their respective unmodified nucleotides.

Dedicated cellular machineries that install ("writers"), remove ("erasers"), and recognize ("readers") the various RNA modifications are being discovered, revealing essential roles for mRNA modification in many cellular, developmental, and disease processes. Modifications exert their effect by altering charge, base-pairing potential, secondary structure, and protein-RNA interactions that, in turn, shape the outcome of gene expression by modulating RNA processing. While a large body of knowledge regarding the roles of m^{6}A and its mechanisms of action has accumulated in the last five years, research into other newly recognized modifications is still at its early phases.

The bottleneck in the field lies in development of robust methods for transcriptome-wide mapping of modifications with single-base precision, preferably based on orthogonal principles for validation. The goal of high-throughput stoichiometry has not been met yet. It is noteworthy that m^{6}A mapping, even at high resolution, still solely relies on an antibody.

Last year, two studies used mass spectrometry and a methodology based on immunocapturing and massively parallel sequencing to identify and map m^{1}A, a new epitranscriptome mark that occurs on human and mouse transcripts and is enriched at the 5′ UTR (Dominissini et al., 2016; Li et al., 2016). Now, in a paper in this issue of *Molecular Cell*, Li et al. (2017) provide base-resolution maps of m^{1}A that validate and refine these earlier studies, reporting distinct classes of m^{1}A in nuclear- and mitochondrial-encoded transcripts, aiding their functional investigation.

Yi and colleagues developed an improved method for m^{1}A mapping with base precision that relies on m^{1}A-induced misincorporation during reverse transcription with TGIRT (thermostable group II intron reverse transcriptase), performed on RNA fragments pre-enriched with anti-m^{1}A antibody. Importantly, the advantage in using TGIRT is its higher processivity and mutation frequency at m^{1}A sites. *In vitro* demethylation by AlkB of pre-enriched RNA fragments results in decreased misincorporation rates. High-confidence m^{1}A sites were those demonstrating a reproducible AlkB-dependent reduction in mutation rates.

The improved method identified hundreds of m^{1}A sites, most of which are in the 5′ UTR of mRNA transcripts, including, for the first time, a minor subset that occurred in the first and second transcribed nucleotides (5′ cap). Only those sites in the 5′ UTR and cap, but not those in the CDS nor in the 3′ UTR, correlated with higher translation efficiency, based on ribosome profiling. The study found that sites fall into three subsets defined by their location, identity of the respective writer enzyme, and sequence-structure features: TRMT6/61A-independent and -dependent m^{1}A sites in nuclear-encoded mRNA and TRMT61B-dependent m^{1}A sites in mitochondrial-encoded mRNA. TRMT6/61A-independent m^{1}A sites constitute the largest subset and are strongly enriched in the 5′ UTR. TRMT6/61A-dependent m^{1}A sites conform to a GUUCRA tRNA-like motif and T-loop-like structures, are evenly distributed along transcript segments, and constitute roughly 10% of all identified sites. Lastly, TRMT61B-dependent m^{1}A sites are primarily located within the coding region of mitochondrial mRNA, where they were found to inhibit translation. Importantly, the identity of the methyltransferase responsible for installing m^{1}A in 5′ UTRs remains unknown. See Table 1 for summary of results.

A study by another group published in *Nature* at the same time took a similar approach, but surprisingly reached a very different conclusion (Safra et al., 2017). Starting with antibody-based enrichment, Safra et al. used TGIRT to generate m^{1}A-induced misincorporations and Dimroth m^{1}A-to-m^{6}A rearrangement to eliminate them and were only able to identify a handful of m^{1}A sites in mRNA,

Table 1. Summary of Results: Subsets of the m^{1}A Methylome

RNA Type	Segment	Writer	Motif	Function
Nuclear-encoded mRNA	5′ cap (+1, +2)	Unknown	N/A	Correlated with increased translation
	Enriched in 5′ UTR	Unknown	None	
	All transcript segments	TRMT6/61A	tRNA T-loop like	Unknown
Mitochondrial-encoded mRNA	CDS	TRMT61B	None	Decreased translation

leading them to conclude that this mark is largely absent from mRNA and possibly avoided by cells due to its inhibitory effect on translation. Why then did Safra et al. fail to identify m^{1}A in mRNA and especially the largest subset in 5′ UTRs? At present, we can only speculate that subtle—but impactful—differences in the experimental protocol and analytical pipeline are at fault. Whereas Li et al. used competitive elution, a tailored TGIRT buffer, AlkB for demethylation, and adaptors with unique molecular identifiers (UMIs), Safra et al. used bulk extraction, the commercial buffer, Dimroth rearrangement, and adaptors devoid of UMIs. These differences affect m^{1}A enrichment, the enzyme's propensity to generate mutations, demethylation efficiency, and RNA integrity, and in turn data analysis. Differences in the analytical criteria themselves, such as coverage thresholds, misincorporation types, and read collapse, could also have a major effect on the outcome, especially in such datasets of relatively low coverage. Lack of mention of UMIs in the Safra study is especially troubling, and indeed a large percent age of their reads is collapsed during analysis, potentially affecting the calculated mutation rate considerably. While both studies agree on the existence of tRNA-like sites, the Yi study identified many more of those and with overall higher mutation frequencies, suggesting its methodology is more sensitive. Furthermore, the results by the Schwartz group are not in line with mass spectrometry measurements of m^{1}A in mRNA, which cannot be accounted for by only 10–15 methylated sites.

Study of RNA modifications requires a thorough understanding of their chemical properties and of the tools used for their detection and mapping. TGIRT-generated misincorporation is by no means a gold standard, and the full spectrum of its properties has not been characterized. The evolution of pseudouridine mapping provides a lesson that may also apply in this case: while initial attempts uncovered only a limited number of sites that did not overlap and were incommensurate with mass spectrometry measurements (that were not even conducted in these studies) (Schwartz et al., 2014), only later was a more robust chemical labeling approach, which also addressed the requirement for pre-enrichment, developed (Li et al., 2015). While single-nucleotide resolution maps are poorer in terms of number of sites, they are richer in terms of information required for functional investigation. The high-quality m^{1}A maps generated by Yi and colleagues have opened the door for mechanistic studies of the function of m^{1}A in RNA metabolism.

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