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m6A RNA modification as a new player in R-loop regulation

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RNA:DNA hybrids that form across genomes control a wide range of biological processes. A new study shows that *N*6-methyladenosine (m6A) modification on the RNA moiety of these hybrids regulates their formation and genome integrity. This opens up a new avenue of research where the RNA modifications (the “epitranscriptome”) can help control genome maintenance.

R-loops are tripartite structures that form co-transcriptionally, with the nascent RNA hybridizing back to the DNA template (RNA:DNA hybrid), leaving a displaced non-hybridized single-stranded DNA (ssDNA) (Fig. 1). Besides their prevalence in highly transcribed genes, R-loops also accumulate in repeats such as centromeres, telomeres and retrotransposons. R-loops are beneficial for various biological processes such as transcription initiation and termination, replication, telomere maintenance and immunoglobulin class switch recombination¹. Yet, they are also potent inducers of DNA damage² and roadblocks for repair³, thereby making R-loops a source of genome instability. Tight regulation of R-loop levels is therefore critical. This is achieved by both preventing their co-transcriptional formation and favoring their removal via specific nucleases (e.g. RNase H) and helicases (e.g. Senataxin, FANCM)^{1,2}. In this issue, Abakir et al. propose an unexpected new regulatory pathway where *N*6-methyladenosine deposition on the RNA moiety of the RNA:DNA hybrid contributes to R-loop removal⁴.

*N*6-methyladenosine (or m6A) is thought to be the most prevalent internal mRNA modification in eukaryotes. This reversible mark plays key roles in the post-transcriptional control of gene expression by affecting nearly every steps of RNA metabolism such as splicing, export, translation, and degradation (Fig. 1). m6A is added co-transcriptionally by “writers” (e.g. METTL3), recognized by specific proteins called “readers” (e.g.: YTHDF1-3, YTHDC1-2) and removed by “erasers” (e.g. FTO)⁵. Importantly, the diverse regulatory functions of m6A are

mainly determined by the identity of the m6A-bound reader. The list of physiological processes affected by m6A is extending and includes response to DNA damage⁶, virus infection, cell differentiation, brain development as well as cell growth and tumor progression^{5,7,8}.

m6A regulates R-loop stability

In their study, Abakir *et al.* adapted the antibody-based DRIP technique (that immunoprecipitates RNA:DNA hybrids) to purify m6A-modified RNA:DNA hybrids with the available m6A antibody (m6A-DNA immunoprecipitation, or m6A-DIP) in human pluripotent stem cells⁴. m6A-DIP-seq and DRIP-seq datasets largely overlapped and displayed preferential accumulation on the same genomic features (transcribed regions, repeated sequences), suggesting that most RNA:DNA hybrids are m6A-modified. Importantly, DRIP followed by m6A-DIP-qPCR, also retrieved m6A-modified RNA:DNA hybrids, albeit less efficiently, further attesting that hybrids can be m6A-modified. Furthermore, a recent study by Yang *et al.* also corroborated the physical presence of m6A-modified RNA within R-loops even though their results rather indicate that only a subset of R-loops are m6A-modified⁹. The definitive answer regarding the prevalence of m6A RNA modification in RNA:DNA hybrids will therefore require the development of alternative reagents and strategies, since efficiency of the m6A-DIP-seq procedure relies greatly on the specificity of the antibody and includes a denaturation step which could in theory selectively affect the integrity of a subset of R-loops.

In this study, Abakir *et al.* also identified YTH-domain family member 2 (YTHDF2) to be the main reader of m6A-modified R-loops. One of their key observation is that YTHDF2 functions by preventing the accumulation of m6A-modified R-loops. It remains to be determined whether YTHDF2 destabilizes R-loop via the recruitment of known RNA:DNA hybrids nuclease/helicase or by specifically targeting the endoribonuclease RNaseP/MRP, which has

been shown to promote the cytoplasmic decay of specific m6A-containing mRNAs¹⁰. This hypothesis is appealing since RNAseP depletion triggers replication stress and genome instability in flies¹¹, two processes also involving R-loops. By contrast, Yang *et al.* recently proposed that instead of downregulating R-loop stability, m6A rather promotes R-loop formation at transcription termination sites⁹. The discrepancy between these studies calls for additional work to characterize in which instance m6A modifications destabilize or promote R-loop formation.

Consequences on genome stability, and beyond

One of the most remarkable finding of this study is that depletion of YTHDF2 and METTL3 (the writer that deposits m6A) triggered increased γ H2AX level, a marker of DNA double-strand breaks, suggesting that pathological R-loops accumulation in the absence of the m6A RNA methylation pathway challenges genomic integrity. This is in line with a large number of studies that clearly points towards the potential of R-loops to induce DNA double-strand breaks². Moreover, dysregulation of R-loops is emerging as a critical factor driving genome instability in a large variety of pathological contexts, including following oncogenic stress¹², in Kaposi's sarcoma-associated herpesvirus infected cells, in neurological disorders associated with trinucleotide repeat expansion (such as Huntington's disease and Fragile X syndrome) and in multiples other inherited ataxias (e.g. ataxia with oculomotor apraxia 2)². The use of existing drugs targeting the m6A pathway (e.g.: FTO inhibitors) could therefore be envisaged as a new therapeutic approach to treat R-loops-related diseases¹³.

Beyond genome stability, the finding that m6A methylation controls R-loop levels across the genome considerably expands the biological functions of the m6A modification pathway to potentially all R-loop-related functions. These include DNA topology, as R-loops were recently

proposed to relieve superhelical stress, immunoglobulin class switch recombination (and therefore the immune response), replication initiation and transcription^{1,2,14}. Additionally m6A also accumulates at sites of UV-induced DNA damage⁶, raising the interesting possibility that this modification also regulates R-loops during DNA repair and thus the frequency of chromosomal translocations¹⁵.

In summary, the results presented by Abakir *et al.* unveil an unexpected interplay between RNA modifications (the epitranscriptome) and the maintenance of genome integrity. Re-analyzing the pathological contexts implicating dysregulation of the m6A RNA pathway through the prism of genome instability will therefore warrant further investigation (Fig. 1).

Figure 1: Expanding the functions of the m6A RNA pathway.

m6A is the most abundant internal modification of eukaryotic mRNA. This co-transcriptionally added epitranscriptomic mark plays multiple roles in mRNA metabolism and expectedly, m6A has been associated with a wide variety of pathologies. Notably, nascent RNA can also hybridize back to the DNA template to form the so-called “R-loop”. Abakir *et al.* propose that R-loops are m6A-modified on their RNA moiety. The m6A-RNA reader YTHDF2 is recruited to destabilize RNA:DNA hybrids through a mechanism that remains to be determined (action of nucleases, helicases, RNaseP). The tight regulation of R-loops level is critical to ensure genome stability, and accordingly, stabilization of m6A-modified R-loops (upon YTHDF2 depletion) leads to increased DNA double-strand break formation. This broadens the previously acknowledged functions of m6A pathway in RNA metabolism to the maintenance of genome integrity and opens up new therapeutic strategies for R-loops related diseases.

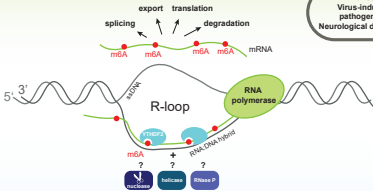
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EPITRANSCRIPTOME

m6A-associated pathologies

Cancer
Virus-induced
pathogenesis
Neurological disease (?)



R-loop diseases

Cancer
Neurological disorders
Autoimmune diseases

GENOME STABILITY