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Spotlight

Mo' m^1A, mo' problems

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CAG-repeat expansions underlie fatal neurodegenerative disorders. In a lodestar study published in a recent issue of *Nature*, Sun et al.[1] identify a writer and eraser of N^1-methyladenosine (m^1A) modifications of CAG-repeat RNA. They establish that m^1A modifications in CAG-repeat expanded RNA promote neurodegeneration and aberrant phase transitions of TDP-43. These findings suggest therapeutic strategies for CAG-repeat expansion disorders.

Nucleotide-repeat expansion disorders are a group of diseases with a single, defined genetic cause: an expansion of a nucleotide-repeat region in the genome.[2] The repeat sequence can be located in coding or non-coding regions, and the length of the repeat correlates with age of onset and severity of disease.[2] Although the genetic basis of repeat-expansion disorders is clear, how disease results from the repetitive sequences is complex and multimodal, and so understanding the mechanisms of disease pathogenesis is an area of active research.[2]

Huntington's disease (HD), several spinocerebellar ataxias, and spinal-bulbar muscular atrophy are some of the diseases caused by CAG-repeat expansions. These are often referred to as the "PolyQ" diseases as CAG is the codon for glutamine, and polyglutamine inclusions have been characterized in disease models and patient tissue.[2] Additionally, intermediate-length repeats in CAG-repeat-containing genes have been connected to amyotrophic lateral sclerosis (ALS),[3,4] which typically presents with TDP-43 proteinopathy.[5] TDP-43 is an RNA-binding protein with a prion-like domain that is important for RNA splicing and processing, but in the degenerating neurons of ALS patients, TDP-43 undergoes an aberrant phase transition and accumulates in cytoplasmic inclusions.[5] Intriguingly, a recent preprint finds evidence that altered TDP-43 function and pathology may also be an aspect of HD,[6] thereby providing another link between CAG-repeat expansion disorders and TDP-43. In a recent paper, Sun et al.[1] explore the post-transcriptional methylation at N^1 of adenosine (m^1A) within CAG-repeat RNAs and illuminate a surprising connection between this modification, neurodegeneration, and aberrant phase transitions of TDP-43.

Dynamic post-transcriptional modifications of RNA can regulate RNA stability and function.[1] However, how CAG-repeat RNA might be post-transcriptionally modified has remained incompletely understood. Sun et al. begin their studies by searching for post-transcriptional modifications of CAG-repeat RNAs of various lengths in human (HEK293T) cells.[1] They found that the levels of m^1A modification increased with repeat length. Increasing levels of m^1A with repeat length were also observed *in vivo* utilizing *Drosophila* expressing $(CAG)_{27}$ or $(CAG)_{78}$, and *C. elegans* expressing $(CAG)_{19}$, $(CAG)_{40}$, or $(CAG)_{67}$. Further, expression of CAG repeats in mice revealed a specific correlation of m^1A levels with regions of the brain vulnerable to neurodegeneration. The m^1A levels on $(CAG)_{140}$ RNA were much higher than $(CAG)_7$ RNA in the striatum, a region severely affected in HD, but this difference was not observed in the cortex. These findings indicated that m^1A modification of CAG-repeat RNA could be problematic in HD and other CAG-repeat expansion disorders.

m^1A modifications are installed by "writer" enzymes and removed by "eraser" enzymes. However, it was not clear which m^1A writer and eraser enzymes modify CAG-repeat RNA. There were two candidate m^1A erasers (ALKBH1 and ALKBH3) and three candidate m^1A writers (TRMT6-TRMT61A, TRMT61B, and TRMT10C). Ectopic expression of ALKBH3 in human (HEK293T) cells led to an almost 10-fold decrease in the levels of m^1A in $(CAG)_{38}$ RNA. Short hairpin RNA (shRNA)-mediated knockdown of TRM61A reduced m^1A levels in $(CAG)_{38}$ RNA by about half. This writer function was confirmed *in vivo* by knockdown of the W02A11.1 gene, the nematode ortholog of TRMT61A, which reduced m^1A levels in $(CAG)_{67}$ repeat RNA in *C. elegans*. Thus, TRMT61A is a writer and ALKBH3 is an eraser for m^1A modifications of CAG-repeat RNA (Figure 1).

Intriguingly, expression of CAG-repeat RNA selectively reduced expression of the ALKBH3 eraser. Expression of CAG-repeat RNA in human (HEK293T) cells or mice did not change TRMT61A protein levels. By contrast, ALKBH3 mRNA and protein were reduced in CAG-repeat-expressing human (HEK293T) cells. In mice expressing $(CAG)_{140}$, ALKBH3 expression was selectively reduced in the striatum compared to $(CAG)_7$ mice, whereas expression of ALKBH3 was similar in the cortex of $(CAG)_{140}$ and $(CAG)_7$ mice. Thus, the CAG-repeat expansion reduces expression of the ALKBH3 eraser in brain regions that are selectively vulnerable in HD.

It is key to confirm that the observed correlation between m^1A levels and repeat length is linked to levels of neurodegeneration. The identification of the ALKBH3 eraser and TRMT61A writer provided tools to do so *in vivo*. In *C. elegans*, expression of $(CAG)_{67}$ led to degeneration of the neuron network. This neurodegeneration was reduced when worms expressed the ALKBH3 eraser or were depleted of the W02A11.1 writer. Likewise, in *Drosophila*, $(CAG)_{78}$-expressing animals had extended lifespan when ALKBH3 was upregulated in neurons,

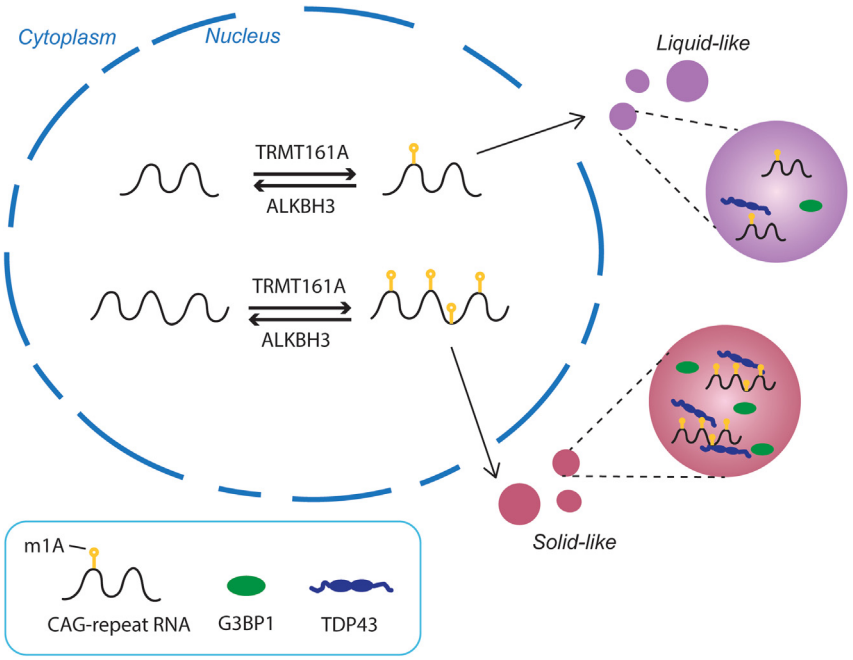

Figure 1. m^1A modification of CAG-repeat RNA alters RNA-protein interactions and phase separation

CAG-repeat RNAs are modified via methylation at the N^1 position of adenosine (m^1A) by TRMT61A methylase. ALKBH3 is a demethylase that can remove this post-transcriptional modification. Longer CAG-repeats are m^1A-modified to a greater extent than shorter repeats. Longer, more-m^1A-modified repeat RNAs interact to a greater extent with TDP-43 and G3BP1 and create more solid, aggregate-like structures in the cytoplasm.

whereas a demethylase-dead mutant of ALKBH3 conferred no protection.

To understand how the m^1A modification contributes to CAG-repeat toxicity, a candidate m^1A "reader" protein, TDP-43, was examined. A hallmark of diseases with TDP-43 proteinopathy is TDP-43 inclusions in the cytoplasm.[5] Expression of $(CAG)_{38}$ RNA or $(CAG)_{22}$ RNA in human (U2OS) cells redistributed TDP-43 to cytoplasmic foci. Moreover, there were greater levels of TDP-43 and $(CAG)_{38}$ RNA co-localization in cytoplasmic foci than with the shorter $(CAG)_{22}$ RNA. The levels of co-localization between TDP-43 and both RNAs in cytoplasmic foci were reduced by the expression of ALKBH3 or the depletion of TRMT61A, indicating that m^1A modification is important for the interaction between TDP-43 and CAG-repeat RNA in cells. Further, a short repeat, $(CAG)_{16}$, that normally does not colocalize with TDP-43 and is nuclear localized when unmodified, migrated to the cytoplasm and co-localized with TDP-43 when modified with three m^1A bases. This finding agreed with *in vitro* data, where TDP-43 directly bound to m^1A-modified CAG-repeat RNA more strongly than unmodified CAG-repeat RNA. Additionally, the TDP-43/CAG-repeat foci in the cytoplasm also co-localized with G3BP1, a key stress granule component. This colocalization increased with CAG-repeat length and with the level of m^1A modification within CAG repeats (Figure 1). These findings indicate that m^1A-modified CAG-repeat RNA might drive an aberrant phase transition of TDP-43.

To assess this possibility, Sun et al.[1] assessed the properties of TDP-43/CAG-repeat foci in cells. TDP-43/CAG repeat foci were more resistant to treatment with 1,6-hexanediol (a chemical that disrupts liquid-like condensates) and recovered more slowly after photobleaching when CAG-repeat lengths increased. The lower mobility of TDP-43 co-localized with m^1A-modified CAG repeats was supported by *in vitro* experiments. TDP-43 droplets containing $(CAG)_7$-3m^1A fused more slowly and recovered from photobleaching more slowly than TDP-43 droplets containing $(CAG)_7$ with zero or one m^1A modifications. Thus, m^1A-modified CAG-repeat RNA drives an aberrant phase transition of TDP-43, which is likely connected to disease pathogenesis (Figure 1).[5]

In conclusion, Sun et al.[1] provide a wealth of data implicating m^1A modification of CAG repeats in the pathology of CAG-repeat disorders. The authors identified that ALKBH3, an m^1A eraser, was reduced in the striatal tissues of HD mouse models. It will be important to determine whether ALKBH3 expression is reduced in the striatum of HD patients and to further disentangle the relationship between ALKBH3 expression levels and the various modalities of CAG-repeat toxicity. It is also necessary to understand where repeat-expansion products are localized in cells. Repeat-expanded RNAs were originally characterized as nuclear residents, which form foci that co-localize with nuclear RNA-binding proteins including MBNL1.[7] However, Sun et al.[1] now add to a growing body of work indicating that CAG-repeat expanded RNAs are residents of cytoplasmic aggregates.[8,9] For example, CAG-repeat expanded RNAs can initially localize to the nucleus but shift into the cytoplasm over time, where they are translated and then accumulate in aggregates with their repeat polypeptide products and TDP-43, but these structures do not contain G3BP1.[9] Another study found that CAG-repeat expanded RNA forms cytoplasmic foci, which appear to disrupt protein synthesis and are rapidly degraded by lysosomes.[8] Further work is needed to fully understand how CAG-repeat RNA directly contributes to pathology in CAG-repeat expansion disorders, but the emerging evidence suggests an important role for cytoplasmic aggregates containing CAG-repeat expanded RNA.[1,8,9]

The study by Sun et al.[1] illuminates possible therapeutic strategies for CAG-repeat expansion disorders. For example, decreasing the TRMT161A writer or increasing the ALKBH3 eraser to reduce m^1A levels in CAG-repeat RNA mitigates neurodegeneration in *C. elegans* and *Drosophila* models of CAG-repeat expansion disorders. Thus, genetic or pharmacological approaches to reduce TRMT161A activity or increase ALKBH3 activity could be therapeutic. With growing links between TDP-43 dysregulation and CAG-repeat RNA expansions,[1,3,4,6] methods to reverse aberrant TDP-43 phase separation and restore functional TDP-43 to

the nucleus could also serve as promising therapeutic strategies in CAG-repeat expansion disorders and beyond.[5,10]

DECLARATION OF INTERESTS

J.S. is a consultant for Dewpoint Therapeutics, ADRx, and Neumora. J.S. is a shareholder and advisor at Confluence Therapeutics.

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