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Relax, Don't RAN Translate It

Katherine M. Wilson,[1,2] Bhavana Muralidharan,[1,2,3] and Adrian M. Isaacs[1,2,*]
[1]UK Dementia Research Institute at UCL, Gower Street, London WC1E 6BT, UK
[2]Department of Neurodegenerative Disease, Queen Square UCL Institute of Neurology, London WC1N 3BG, UK
[3]Brain Development and Disease Mechanisms, Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, India, 560065
*Correspondence: a.isaacs@ucl.ac.uk
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The (GGGGCC)n repeat expansion in *C9orf72*, which is the most common cause of frontotemporal dementia and amyotrophic lateral sclerosis, is translated through repeat-associated non-AUG (RAN) translation. In this issue of *Neuron*, Cheng et al. (2019) report that the helicase DDX3X, which unwinds (or relaxes) RNA, suppresses RAN translation and toxicity.

The frontotemporal dementia (FTD)- and amyotrophic lateral sclerosis (ALS)-causative $(GGGGCC)_n$ repeat expansion in intron 1 of *C9orf72* is proposed to drive toxicity through several modes of action (Balendra and Isaacs, 2018). One potential mechanism is production of dipeptide repeat proteins (DPRs) by repeat-associated non-AUG (RAN) translation of the expanded repeats. This non-canonical translation occurs in all three reading frames, in both the sense and antisense directions, leading to the production of five different DPRs: poly-GA, poly-GP, and poly-GR from sense transcripts and poly-GP, poly-PR, and poly-AP from antisense transcripts. One possible therapeutic intervention could be to reduce RAN translation. Yet, the mechanism by which RAN translation occurs is still not fully understood. Targeted genetic screens have started to identify genes that regulate RAN translation (Goodman et al., 2019; Linsalata et al., 2019; Yamada et al., 2019). In this issue of *Neuron*, Cheng et al. (2019) now report the first genome-wide screen for modulators of *C9orf72* RAN translation. They first created a reporter cell line expressing the *C9orf72* intronic sequence directly upstream of the repeats, followed by $(GGGGCC)_{70}$ and an eGFP tag in the poly-GA frame. Using this reporter cell line, a genome-wide CRISPR-Cas9 knockout screen was carried out using fluorescence-activated cell sorting to monitor changes in poly-GA-eGFP levels. This identified 76 enhancer and 145 suppressor genes. Gene Ontology analysis identified an enrichment in genes involved in pathways relevant to protein translation and degradation: translation initiation, RNA transport, the proteasome complex, RNA binding, and helicases. To validate hits from the initial screen, a dual luciferase cell line was used in which cells express NanoLuciferase (NanoLuc) via RAN translation as well as firefly luciferase (FLuc) via canonical (AUG) translation. 48 genes were re-tested in this secondary screen, and knockdown of the RNA helicase DDX3X caused the greatest change in NanoLuc levels, increasing RAN translation by more than 2-fold while slightly reducing AUG-FLuc translation. DDX3X is a conserved DEAD-box RNA helicase shown to be involved in RNA transcription, splicing, export, and translation, as well as having roles in cell cycle, tumorigenesis, and stress granule formation (Linder and Jankowsky, 2011). Additionally, loss-of-function mutations in DDX3X have been identified as a cause of X-linked recessive intellectual disability (Snijders Blok et al., 2015). Knockdown of DDX3X caused an increase in RAN-translated NanoLuc signal in all three sense frames; however, no change in antisense RAN translation was observed, indicating specificity for sense GGGGCC repeats. The increase in DPR levels was also observed when *in vitro* transcribed GGGGCC repeat-NanoLuc RNA was transfected into DDX3X knockdown cells, indicating an effect on RAN translation and not transcription. Polysome fractionation revealed a striking change in distribution of repeat RNA, from monosomes in controls to translating polysomes in DDX3X siRNA-treated cells. No change in the polysome distribution of the AUG-FLuc RNA was observed, and no major changes in global translation were identified using puromycin incorporation. These results point toward a specific role of DDX3X in repeat RNA translation. The RNA helicase activity of DDX3X is dependent on ATPase activity. To ascertain whether ATPase activity was required for the effect of DDX3X on DPR levels, wild-type DDX3X and ATPase-defective mutant forms of DDX3X were overexpressed in cells. Only the wild-type DDX3X could lower DPR levels, suggesting a dependence on ATPase activity for modulation of RAN translation. Recombinant DDX3X was then used to show direct binding of repeat RNA to DDX3X and that GGGGCC repeat RNA could stimulate DDX3X ATPase activity.

Following on from this cell line and *in vitro* data, Cheng et al. (2019) next investigated whether DDX3X modulation could affect RAN-translation-induced toxicity *in vivo*. Here, they utilized a *Drosophila* model expressing $(GGGGCC)_{58}$ in the fly eye, which causes mild eye degeneration. Expression of either mutant *belle* (the *Drosophila* DDX3X homolog) or *belle* RNAi knockdown exacerbated the eye degeneration phenotype and led to increased poly-GP levels. These results suggest that DDX3X may regulate RAN translation and associated toxicity *in vivo*. Importantly, in addition to these overexpression studies, Cheng et al. (2019) next investigated the effect of DDX3X on endogenous RAN translation in *C9orf72* patient cells. In both *C9orf72* patient lymphoblasts and induced pluripotent stem cells (iPSCs), levels of poly-GP were greatly increased by knockdown of DDX3X. All these data in various systems, including patient cells, point to a role for DDX3X in regulating RAN translation of *C9orf72* GGGGCC

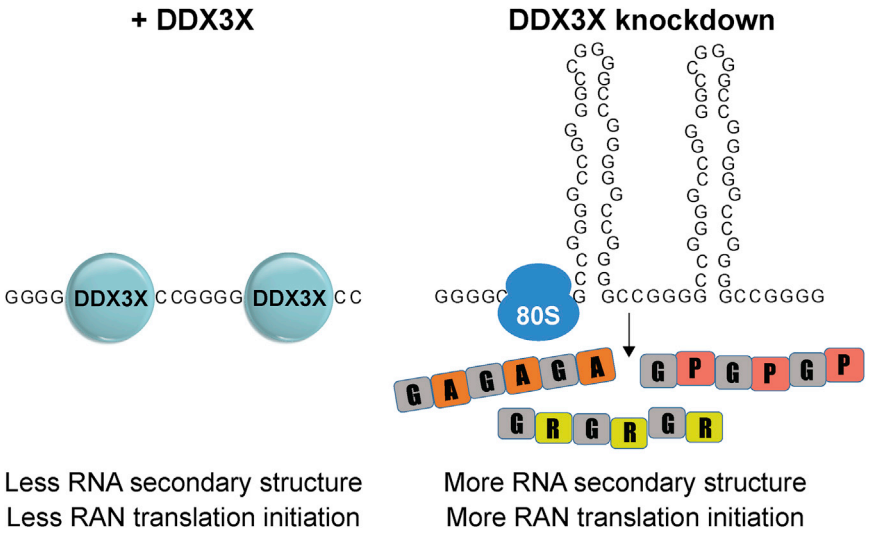

Figure 1. Loss of DDX3X RNA Helicase Activity Increases *C9orf72* Repeat RAN Translation
The (GGGGCC)n repeat expansion in C9orf72 can be translated via repeat-associated non-AUG (RAN) translation in each reading frame to produce poly-GA, poly-GP, and poly-GR dipeptide repeat proteins (DPRs). The RNA helicase activity of DDX3X reduces (GGGGCC)n RAN translation. Conversely, upon loss of DDX3X, the repeat RNA forms secondary structures that act as IRES elements and/or stall scanning ribosome complexes, leading to increased RAN translation and DPR levels.

repeats, as knockdown of DDX3X consistently increases DPR levels. However, for there to be therapeutic relevance, the opposite also needs to be demonstrated—that overexpression of DDX3X can reduce DPR levels. Cheng et al. (2019) address this question in two systems. First, they report that overexpression of DDX3X decreases poly-GP in their dual luciferase assay. Second, they show that DDX3X overexpression can reduce poly-GP in *C9orf72* patient iPSC-derived motor neurons. Impressively, this reduction was sufficient to reduce both the increased sensitivity to glutamate-induced cell death and the disrupted nucleocytoplasmic transport observed in *C9orf72* patient iPSC neurons.

It is an attractive model in which RAN translation is regulated by specific factors and mechanisms. This would allow for specific targeting of RAN translation in multiple repeat expansion diseases. Interestingly, the role of DDX3X in another repeat expansion disease has already been investigated. However, in a confusing turn, Todd and colleagues reported the opposite role of DDX3X on RAN translation of the $(CCG)_n$ repeat in *FMR1* (Linsalata et al., 2019). *Drosophila* expressing $(CGG)_{90}$-EGFP had eye toxicity rescued by *belle* knockdown. DDX3X knockdown suppressed RAN translation *in vitro* using a $(CGG)_{100}$ NanoLuc assay and improved survival of primary rat neurons expressing $(CGG)_{100}$. Thus, they conclude that DDX3X knockdown suppresses RAN translation and associated toxicity. How can DDX3X have opposing effects on RAN translation of *C9orf72* and *FMR1* expanded repeats? In the first pioneering description of RAN translation it was shown that the secondary structure of the repeat RNA plays an important role. Two non-mutually exclusive mechanisms have been suggested to initiate RAN translation: cap-dependent scanning of the pre-initiation complex (PIC) and cap-independent utilization of an internal ribosome entry site (IRES) (Cheng et al., 2018; Green et al., 2017; Tabet et al., 2018). RNA secondary structure is relevant for both mechanisms, as it is known to be important for IRES-mediated translation, and repeat RNA secondary structures have also been suggested to slow the scanning PIC for long enough to recruit the 60S subunit and initiate RAN translation. In either scenario, an RNA helicase that unwinds the repeat RNA structure would be expected to reduce RAN translation (Figure 1), as reported by Cheng et al (2019). Indeed, Linsalata and colleagues state that this was their original expectation (Linsalata et al., 2019). Their explanation for the opposite result is that the region immediately upstream of the *FMR1* CGG repeats is also GC rich and that DDX3X activity is required to unwind this sequence in order to allow the PIC access to the repeats. Therefore, the requirement to unwind the upstream sequence trumps the need to maintain the secondary structure of the repeats themselves. Some unwinding of the repeats is presumably needed to allow translation to proceed after initiation has occurred. Consequently, a complex balance of maintaining and resolving repeat structure may ultimately be required for effective RAN translation. What is clear is that the effects of DDX3X are both sequence and context specific. It may also be challenging to directly target DDX3X therapeutically given its diverse roles in the cell, which suggests a relatively broad range of targets, but further work will determine whether specificity can be achieved. In summary, the innovative genome-wide CRISPR/Cas9 screen described here has identified new modifiers of GGGGCC repeat RAN translation, which help shed new light on this mysterious form of unconventional translation.

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Signal Amplification in *Drosophila* Olfactory Receptor Neurons

Byoung Soo Kim[1] and Greg S.B. Suh[1,2,*]
[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea
[2]Skirball Institute of Biomolecular Medicine, Department of Cell Biology, Neuroscience Institute, New York University School of Medicine, New York, NY, USA
*Correspondence: seongbaesuh@kaist.ac.kr
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Olfactory receptor neurons (ORNs) transform scant chemical inputs into significant neural signals. This transformation requires signal amplification. In this issue of *Neuron*, Ng et al. (2019) identified a mechanism by which the signals evoked by pheromones are amplified in the ORNs that selectively promote courtship behavior in *Drosophila*.

In non-primate males, sensing pheromones by chemosensory organs is the essential first step in eliciting sexual arousal and attraction. The sensitivity of chemosensory organs to pheromones is markedly enhanced in older *Drosophila* males, matching periods of increased reproductive maturity (Lin et al., 2016). This sensory enhancement would be also important because a scant quantity of pheromones is released from females. Pheromones emitted by *Drosophila* females are primarily detected by selected populations of olfactory receptor neurons (ORNs) in males, which transmit the converted chemical information to the higher brain centers. It is unclear, however, where and how the enhancement of the sensory signal occurs and how the age of male flies contributes to the signal amplification.

In vertebrates, odorant receptors expressed in chemosensory olfactory neurons are G-protein-coupled receptors (GPCRs) (Buck, 1996). Upon odorant binding, the GPCRs activate the G-protein-coupled transduction cascade and cyclic AMP synthesis that promote the opening of cyclic nucleotide-gated (CNG) channels, thereby stimulating the activity of ORNs. Through the signal transduction cascade, the response to ligands is amplified, and the influx of cations, including calcium, into the cytoplasm of ORNs is elevated, thus generating more substantive neurotransmission. On the other hand, olfactory receptors in *Drosophila* comprise three families: the 7-transmembrane odorant receptors (ORs), gustatory receptors (GRs) (Vosshall and Stocker, 2007), and the P-loop-containing ionotropic glutamate receptor (IRs) (Benton et al., 2009).

While the vertebrate chemosensory receptor system amplifies the signal through the GPCR transduction cascade, it is unclear whether the chemosensory input is amplified in *Drosophila* sensory neurons. The *Drosophila* 7-transmembrane ORs were reported as a ligand-gated ion channel that is activated directly by environmental odorants (Sato et al., 2008; Wicher et al., 2008) but may also promote signal amplification through the G-protein signaling cascade and CNG channels (Wicher et al., 2008). Another study suggested, however, that ORs function strictly as an ion channel and that the synthesis of second messengers from G-protein signaling may not be involved (Sato et al., 2008). The structurally related GRs have been shown to form a ligand-gated ion channel in another insect species (Sato et al., 2011). The P-loop-containing IRs also form a ligand-gated ion channel that confers depolarization in ORNs upon its direct binding to odorants (Benton et al., 2009). The insect chemosensory receptors that are ligand-gated ion channels and may not be coupled to the G-protein cascade have a limited role in augmenting the negligible ligand-evoked signal into substantive neurotransmission. How the sensory signal initiated by the ion channel is amplified in the insect sensory neurons has been a mystery. In this issue of