Relax, don't RAN translate it

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The (GGGGCC)n repeat expansion in *C9orf72*, which is the most common cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), 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, supresses RAN translation and toxicity.

The FTD- and 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 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). 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 FACS 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 over two-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 towards a specific role of DDX3X in repeat RNA translation. The RNA helicase activity of DDX3X is dependent on ATPase activity. To ascertain if ATPase activity was required for the effect of DDX3X on DPR levels, wildtype DDX3X and ATPase-defective mutant forms of DDX3X were overexpressed in cells. Only the wildtype 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, the authors next investigated whether DDX3X modulation could affect RAN translation-induced toxicity in vivo. Here, they utilised a *Drosophila* model expressing $(GGGGCC)_{58}$ in the fly eye, which causes mild eye degeneration. Expression of either mutant *belle* (the *Drosophila* DDX3X homologue) 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 over-expression studies, the authors next investigated the effect of DDX3X on endogenous RAN translation in *C9orf72* patient cells. In both *C9orf72* patient lymphoblasts and 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 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. The authors address this question in two systems. Firstly, they report that overexpression of DDX3X deceases poly-GP in their dual luciferase assay. Secondly, 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 supressed 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 supresses 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 utilisation 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 complex 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. Indeed, Linsalata and colleagues state that this was their original expectation. 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, 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.

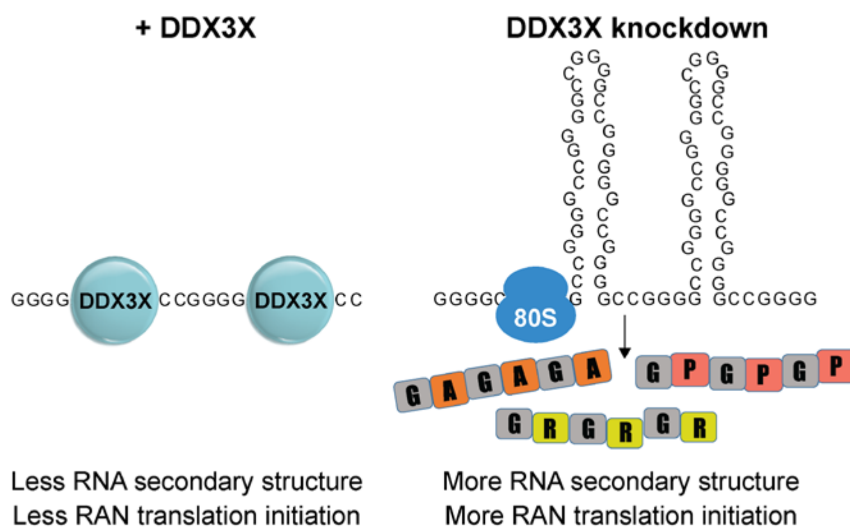

Figure 1. Loss of DDX3X RNA helicase activity increases *C9orf72* repeat RAN translation
The (GGGGCC)n repeat expansion in C9orf72 can be translated via repeat-associated non-AUG (RAN) translation, in each reading frame, to produce poly-GA, poly-GP and poly-GR dipeptide repeat proteins (DPRs). The RNA 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.

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