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New developments in RAN translation: insights from multiple diseases

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Since the discovery of repeat-associated non-ATG (RAN) translation, and more recently its association with amyotrophic lateral sclerosis/frontotemporal dementia, there has been an intense focus to understand how this process works and the downstream effects of these novel proteins. RAN translation across several different types of repeat expansions mutations (CAG, CTG, CCG, GGGGCC, GGCCCC) results in the production of proteins in all three reading frames without an ATG initiation codon. The combination of bidirectional transcription and RAN translation has been shown to result in the accumulation of up to six mutant expansion proteins in a growing number of diseases. This process is complex mechanistically and also complex from the perspective of the downstream consequences in disease. Here we review recent developments in RAN translation and their implications on our basic understanding of neurodegenerative disease and gene expression.

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An expanding field: updates on RAN-associated disorders

The discovery of repeat associated non-ATG (RAN) translation in 2011 [1] has changed our understanding of the mechanisms of microsatellite expansion diseases and of protein translation. We previously reviewed the discovery of RAN translation and early research findings [2]. These previous studies demonstrated that RAN translation, coupled with bidirectional transcription, from expanded repeats is capable of producing a startling array of toxic expansion proteins. Here we focus on recent developments in RAN translation, its mechanistic underpinnings and efforts to understand the impact of RAN proteins in neurologic disease.

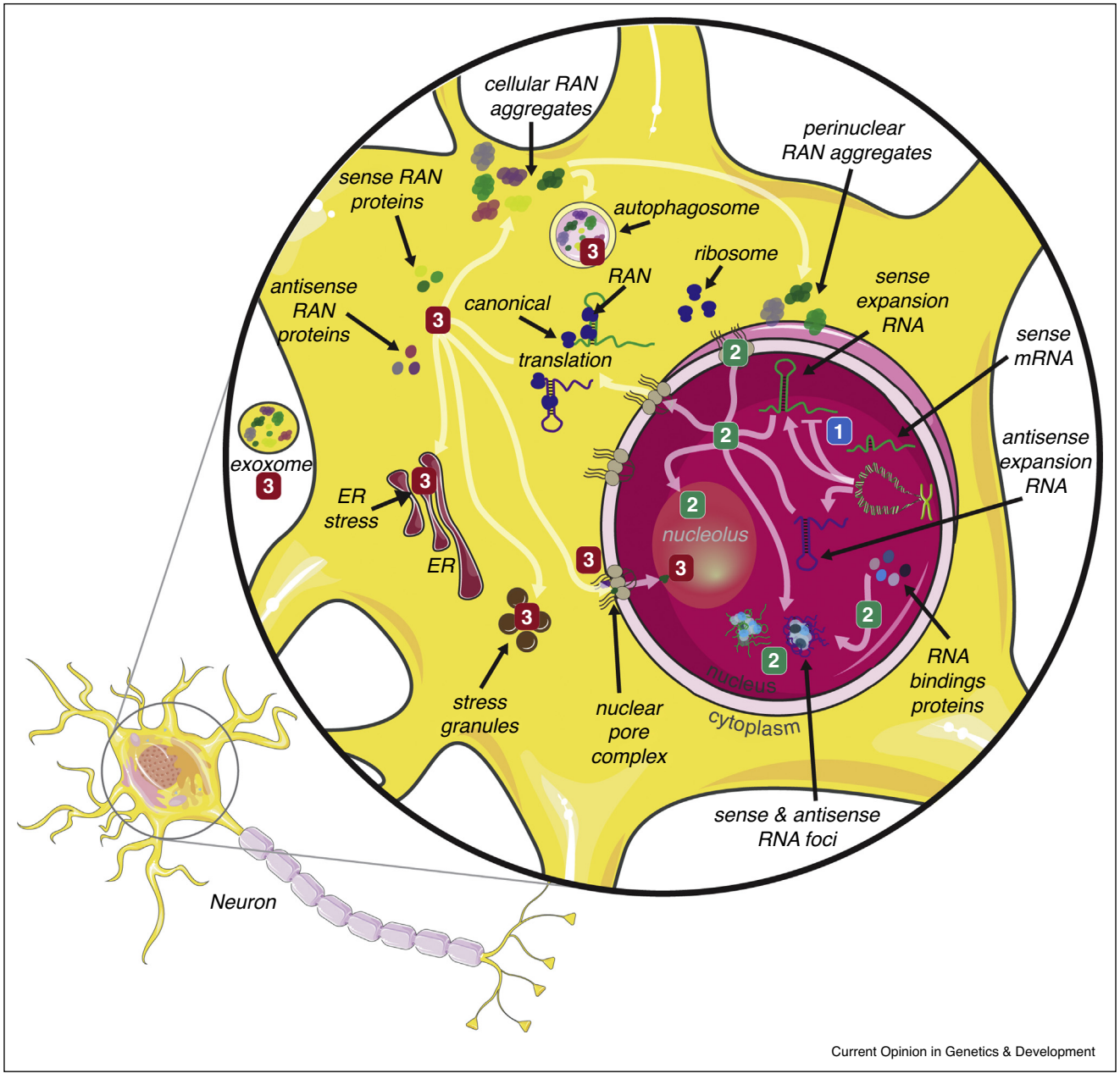
RAN translation and the resulting toxic homopolymeric proteins were first described in spinocerebellar ataxia type 8 and myotonic dystrophy type 1 [1]. This discovery was followed by descriptions of homopolymeric RAN proteins in fragile X tremor ataxia syndrome (FXTAS) [3] and dipeptide RAN proteins in C9of72 amyotrophic lateral sclerosis (ALS)/frontotemporal dementia (FTD) [4–8]. While research on the effects of RAN dipeptide repeat (DPR) proteins in C9-ALS/FTD has produced the largest amount of data and interest to date, RAN translation has been observed in other diseases and interest in its impact and mechanisms is growing (Figure 1).

RAN proteins in Huntington disease and other polyglutamine disorders

Huntington disease (HD), a progressive neurodegenerative disorder characterized by severe movement, cognitive and behavioral alterations, is caused by a CAG·CTG expansion in the *HTT* gene [9]. The mutant huntingtin mHTT protein, which contains a polyglutamine expansion tract, is expressed from the sense-strand as part of a large ATG-initiated open reading frame (ORF). While it is clear that the HD CAG·CTG expansion mutation causes disease [10], HD research has almost exclusively been focused on the mutant huntingtin (mHTT) polyglutamine expansion protein because it was thought to be the only mutant protein that could be expressed across the expansion mutation. The first examples of RAN translation were found to occur across expansion mutations located in non-coding gene regions (SCA8, DM1, *C9orf72* ALS/FTD, and FXTAS). Because the HD expansion is much smaller and is located within a large canonical ORF that could inhibit RAN translation, it was unclear if RAN translation could also occur across the HD mutation.

In 2015, Bañez-Coronel *et al.* [11••] answered this question and reported that four additional novel homopolymeric RAN expansion proteins (polyAla, polySer, polyLeu and polyCys) expressed from both sense and antisense transcripts accumulate in HD human autopsy brain samples. These HD-RAN proteins were prominently found in brain regions showing neuronal loss, cell

Figure 1



Cellular consequences of expanded microsatellite repeats and repeat-associated non-ATG (RAN) translation. Although microsatellite repeats are expressed in a wide variety of cells, the majority of RAN proteins have been identified in the central nervous system (see Box 1). While this generalized illustration utilizes a neuron, RAN proteins are found in a variety of CNS cell types (neurons, astrocytes, microglia, motorneurons, *etc.*). Bidirectional transcription has been observed in a growing number of microsatellite repeat expansion diseases [96–99] resulting in the expression of both sense and antisense expansion transcripts. Three non-exclusive disease mechanisms have been proposed for the majority of microsatellite expansion diseases. (1) **Loss-of-function** (blue)—reduction in mRNA transcript resulting in reduced C9orf72 protein levels. The presence of expanded repeats in the DNA may result in epigenetic silencing and/or transcriptional inhibition of the expansion transcript that reduces the available levels of the gene's canonical protein product. Protein loss-of-function has been proposed for the C9orf72 hexanucleotide repeats [35,39,100–102]. (2) **RNA gain-of-function** (green)—sequestration of RNA-binding proteins into nuclear foci by the expanded RNA repeats lowers the available RBP concentration and reduces its normal cellular function. In DM1, CUG expansion RNAs sequester MBNL proteins from their normal splicing targets and MBNL loss-of-function leads to alternative splicing dysregulation and pathogenesis [37,52,59••,62•,63••,103]. The expanded repeat transcript may also interact with, and alter the function of other cellular components, such as the interaction between G_4C_2 expansion RNA and proteins of the nuclear pore complex [59••]. (3) **Protein gain-of-function** (red)—the production of up to six toxic RAN proteins from the sense and antisense expansion transcripts results in defects in cellular processes, including protein homeostasis. In C9-ALS/FTD, sense and antisense dipeptide RAN proteins have been shown to lead to nucleolar dysfunction, ER stress, stress granules, altered autophagy, transcellular transmission, disruption of nucleocytoplasmic defects and nuclear envelope defects [4,7,104,105••].

Box 1 RAN proteins and human disease.

Spinocerebellar ataxia type 8 (SCA8: CAG·CTG)—a dominantly inherited slowly progressive ataxia with cerebellar degeneration that is characterized by gait and limb ataxia, nystagmus, and dysarthria. SCA8 is caused by expansion of a CAG·CTG repeat in the overlapping *ATXN8OS* and *ATNX8* genes on chromosome 13. The RAN protein, **SCA8-poly-Alanine**, expressed from the antisense CAG expansion transcript, was detected in the cerebellar Purkinje cells of post-mortem SCA8 human brains [1].

Myotonic dystrophy type 1 (DM1: CAG·CTG)—a dominantly inherited multisystemic neuromuscular disease that is characterized by myotonia, muscle wasting, cardiac defects, cataracts and central nervous system alterations. DM1 is caused by expansion of CAG·CTG repeat with the 3′ UTR of the *DMPK* gene on chromosome 19. The RAN protein, **DM1-poly-Glutamine** expressed from the antisense CAG expansion transcript was detected in myoblasts, skeletal muscle and blood of DM1 patients [1].

C9of72 amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD: GGGGCC·GGCCCC)—a dominantly inherited motor neuron (ALS) and/or temporal lobar degenerative (FTD) disease that is characterized by upper or lower motor neuron degeneration (ALS) and/or progressive changes in behavior, executive dysfunction and language impairment (FTD). C9-ALS/FTD is caused by an expansion of a GGGGCC·GGCCCC repeat within the 1st intron of the *C9orf72* gene on chromosome 9. The RAN proteins, **C9-poly-GlycineProline**, **C9-poly-GlycineArginine** and **C9-poly-GlycineAlanine** expressed from the sense GGGGCC expansion transcript and **C9-poly-AlanineProline, C9-poly-ProlineArginine and C9-poly-GlycineProline** expressed from the antisense GGCCCC expansion transcript were detected in the brains, spinal cord and motor cortex of postmortem C9-ALS/FTD patients [4,5,7,8,49,104]. The **C9-poly-GylcineProline** RAN protein has also been detected in the CSF from C9-ALS/FTD patients [106].

Fragile X tremor ataxia syndrome (FXTAS: CGG·CCG)—a dominantly inherited late-onset disease primarily affecting males characterized by tremor, ataxia, parkinsonism and cognitive decline. FXTAS is caused by premutation (55–200 repeats) expansion of a CGG·CCG repeat in the 5′ UTR of the *FMR1* gene on the X chromosome. The RAN protein, **FXTAS-poly-Glycine** expressed from the sense CGG expansion transcript were detected in frontal cortex and hippocampal neurons in FXTAS postmortem brain tissues [3] and **FXTAS-poly-Proline** and **FXTAS-poly-Alanine** expressed from the antisense CCG expansion transcript [27] were detected in hippocampus, cortex and cerebellum of FXTAS postmortem tissue.

Huntington's disease (HD: CAG·CTG)—a dominantly inherited progressive neurodegenerative disorder characterized by movement abnormalities, cognitive decline and psychiatric problems. HD is caused by CAG·CTG expansions in the 1st exon of the *HTT* gene on chromosome 4. The RAN proteins, **HD-poly-Alanine** and **HD-poly-Serine** expressed from the sense CAG expansion transcript and **HD-poly-Leucine** and **HD-poly-Cysteine** expressed from the antisense CTG expansion transcript were detected in striatum, frontal cortex and cerebellum of postmortem HD patients [11••].

death and microglial activation, including the striatum. While HD-RAN proteins accumulate in brain regions that overlap sites of polyGln accumulation, including the caudate and putamen, HD-RAN protein staining is also found in several white matter regions in the absence of detectable polyGln accumulation [11••] suggesting HD-RAN proteins play a role in previously reported HD white matter abnormalities [12–16]. Bañez-Coronel *et al.* [11••] also observed robust RAN, but almost no detectable polyGln, accumulation within the severely affected cerebellar layers of juvenile onset HD cases. Cell culture studies using codon replacement constructs demonstrated that polySer, polyLeu and polyCys expansion proteins are toxic to neural cells independent of CUG and CAG RNA gain-of-function effects. Additional studies showed repeat length thresholds for polySer and polyAla protein accumulation at 35 and >52 repeats. Because 35 repeats is close to the disease threshold for HD, it is possible that polySer expression is a trigger of disease and that the longer repeats associated with juvenile onset HD result from the expression of a toxic cocktail containing multiple RAN and polyGln expansion proteins. It is also possible that CUG and CAG RNA gain-of-function effects contribute to HD [11••].

Taken together these data suggest that RAN proteins contribute to HD and may be uniquely responsible for specific changes including white matter abnormalities and neurodegenerative changes in severely affected juvenile onset cases. Approaches to reduce the production or increase the turnover of the mHTT polyGln expansion protein have also been the focus of many studies of HD [17–21]. One recently described approach, which ameliorated behavioral phenotypes in HD mice, was to target the accumulation of mHTT and other SUMOylated proteins by decreasing levels of PIAS1, an E3 SUMO ligase that targets mHTT [22••]. It will be interesting to determine if posttranslational modifications such as SUMOylation and ubiquitination [22••,23,24] occur on the HD-RAN proteins and if these or other pathways also affect HD-RAN protein turnover.

The discovery that RAN translation occurs in HD raises the possibility that RAN proteins also contribute to eight other CAG polyglutamine expansion disorders. Consistent with this possibility, Scoles *et al.* [25] showed RAN proteins are produced using minigenes containing sequence flanking the CAG·CTG expansion mutation causing spinocerebellar ataxia type 2 (SCA2). These authors showed that SCA2 RAN protein accumulation is influenced by the sequence context 3′ of the repeat. Similarly, minigenes containing sequence upstream of the CAG repeat in SCA3, HD, DM1 or HDL2 also express RAN proteins [1]. Further research will be required to test if, similar to HD, RAN proteins are also found in patient tissues with these diseases. There are also additional regulatory connections between the polyglutamine disorders that affect protein translation. For example, the MID1-translation regulatory protein complex binds and regulates the translation of CAG expansions in the canonical ATG-initiated frame in a length dependent manner in HD and several SCAs [26]. It will be interesting to determine if this regulatory mechanism also influences RAN translation.

Fragile X tremor ataxia syndrome

In 2013, Todd *et al.* demonstrated that the CGG repeat expansion in FXTAS can, in cell culture, produce a polyglycine (FMR-polyGly) and a poly-alanine (FMR-polyAla) protein with FMR-polyGly accumulating in fly, mouse and patient samples [3]. More recently this group demonstrated that homopolymeric proteins are also expressed in the antisense direction generating polyPro, polyArg, and polyAla in cell culture with at least two of these proteins accumulating in human autopsy tissue [27]. Similar to previous results, cell culture studies show steady state levels of these proteins increase with repeat length.

Zu *et al.* [1] previously showed, even small changes in flanking sequence can influence RAN translation across CAG repeat expansions. The role of flanking sequence is further highlighted in studies of the Fragile X tremor ataxia syndrome (FXTAS) [27,28••,29•]. Kearse *et al.* [28••] demonstrated that translation of the FMR-polyGly initiates preferentially at close-cognate start codons upstream of the repeat, in a repeat-length independent manner. In contrast, translation in the polyAla reading frame is repeat-length dependent and initiates within the expanded repeat with no close-cognate initiation codon requirement. Additionally, translation in both reading frames was shown to be cap-, eIF4E- and eIF4A-dependent. The authors suggest these findings supports canonical pre-initiation complex loading and ribosome scanning and that ribosome stalling caused by secondary CGG RNA structure facilitates translation initiation in both the polyAla and polyGly reading frames [28••]. In contrast, RAN translation in the polyArg frame was not detected yet interestingly translation in this frame could be detected in the absence of the CGG repeats. Taken together, these data highlight the role of frame specific effects of flanking sequence on protein translation across FXTAS CGG repeats.

In 2011, Zu *et al.* [1] showed by mass spectrometry that the N-terminal amino acid for the polyAla RAN protein expressed across expanded CAG repeats began with the amino acid alanine at various sites within the repeat tract. In contrast, mass spectrometry performed by Sellier *et al.* [29•] on the FMR-polyGly protein showed that initiation occurs at an upstream ACG close cognate start codon and that the N-terminal amino acid is a methionine in mammalian cells. These data suggest a canonical protein initiation mechanism in the polyGly reading frame but with the use of a close cognate initiation codon. Ribosome profiling studies have led to the identification of thousands of additional putative upstream open reading frames (uORFs) that use similar close cognate codons [30,31]. It will be interesting to determine if translation of the FMR1 uORF in the +1 reading frame inhibits translation of FMRP in the major ORF, as has been shown for other uORFs [32,33]. Additionally, Sellier *et al.* [29•] showed with conditional mouse models that neuronal expression of the FMRpolyGly protein, but not the CGG_{EXP} RNA alone, decreases survival in mice and that both the polyGly repeat tract and the C-terminal sequence contribute to toxicity in cell culture. They also show that the C-terminal region of the FMR-polyGly protein interacts with and disrupts the nuclear lamina protein, LAP2β [29•], which may underlie previous observations of disorganized nuclear lamina structure in FXTAS [34]. Taken together, these findings highlight the different roles that flanking sequences can play in both the expression and toxicity of repeat expansion proteins.

C9orf72 ALS/FTD

Since the discovery of the $G_4C_2{\cdot}G_2C_4$ expansion in the *C9orf72* gene as the most common known genetic cause of both ALS and FTD, research into disease mechanisms have focused on three main areas: (1) haploinsufficiency of the *C9orf72* gene caused by decreased expression of the expansion allele [35–40]; (2) RNA gain-of-function effects leading to sequestration of key RNA-binding proteins [36,40–45,46•]; and (3) protein gain-of-function effects caused by the RAN dipeptide repeat (DPR) proteins [4–8,36,37,46•,47–49]. Comprehensive reviews on ALS and *C9orf72* ALS/FTD have been previously written [50–54]. Here we will focus on recent developments on the effects of the RAN dipeptide proteins and outstanding questions and new tools that will allow a better understanding of their impact on disease.

A first basic set of questions is where and how does translation of the DPRs initiate and are there frame specific differences that may result in the use of both canonical and novel RAN translation mechanisms? Examination of the flanking sequences around the hexanucleotide repeat shows ATG initiation codons upstream of the antisense G_2C_4 repeats in both the ProlineArginine (PR) and GlycineProline (GP) reading frames (see Fig. S3 in Ref. [6]). This finding raises the question of whether or not these upstream ATG initiation codons are used in these reading frames and, if so, how these N-terminal flanking sequences would affect the function and toxicity of these proteins. While it is not clear if a mixture of canonical translation and RAN translation occurs in the antisense direction, a mixture of canonical and RAN mechanisms in C9-ALS/FTD would have parallels to both HD [11••] and FXTAS [28••].

Additional sequence analysis of the *C9orf72* repeat region predicts unique C-terminal flanking regions for five of the six RAN proteins [6]. Immunodetection of these C-terminal regions confirms that they are expressed and accumulate in C9 patient autopsy tissue [6]. While these unique C-terminal regions increase the complexity of the C9-RAN proteins beyond the DPR repeat motifs, C9-RAN protein toxicity studies have typically focused

on the DPR repeat motifs alone or as a fusion protein with a C-terminal reporter. It will be important to broaden studies of C9-RAN protein toxicity to include both the DPR repeat motifs and the corresponding endogenous flanking sequences.

Numerous cellular targets and interacting partners of RAN DPR proteins have been identified including RNA-binding proteins, nucleoporin proteins, low-complexity domain proteins, ribosomal proteins, translation initiation and elongation factors [55–58]. Of particular interest to the field has been the direct interaction of *C9orf72*-RNA with nuclear pore complex (NPC) proteins [59••] and the disruption of nucleocytoplasmic transport by C9-RAN protein [60,61,62•,63••] (see Haeusler *et al.* [50] and Boeynaems *et al.* [64] for reviews). Disruptions to the nucleocytoplasmic machinery are thought to play a role in the adult-onset neurodegeneration observed in ALS/FTD as the NPC has many long-lived structural components that can deteriorate during aging [65] and post-mitotic cells, such as neurons, cannot rely upon mitotic nuclear envelope disassembly for nuclear waste clearance. However, it is not yet clear if the NPC and nucleocytoplasmic transport disruptions in C9-ALS/FTD contribute directly to cellular pathogenesis, enhance age-related defects and/or exacerbate existing nuclear RNA loss-of-function and cytoplasmic protein gain-of-function mechanisms. Nevertheless, the connection between the NPC and nucleocytoplasmic transport defects extends beyond C9-ALS/FTD to amyloid-like protein aggregates in other diseases [66] as well as aggregates from other microsatellite diseases including DM1 [67], HD [66,68–70], DRPLA [71,72] and several polyglutamine expansion disorders [73,74].

To better understand the impact and biology of RNA gain-of-function and RAN protein effects in C9-ALS/FTD, four independent groups developed *C9orf72* BAC transgenic mouse models. While mice from each of these models showed the expected molecular phenotypes of sense and antisense RNA foci and RAN protein accumulation, surprisingly these models had very different phenotypic outcomes. Models developed by O'Rourke *et al.* [75•] and Peters *et al.* [76•] did not develop phenotypic or neurodegenerative features of the disease. A third study by Jiang *et al.* [77•] showed mice with 110 or 450 repeats did not develop behavioral, physiological or neuropathological evidence of motor neuron disease out to 18 months of age. However, deficits in spatial learning, anxiety and mild neuronal loss in the hippocampus without gliosis or TDP43 mislocalization were detected in lines with 450 repeats [77•]. In contrast, BAC transgenic mice developed by Liu *et al.* [78••] develop both the key phenotypic and molecular features of the disease. These C9-BAC mice show, in a repeat-length and expression dependent manner, features of both ALS and FTD including decreased survival, paralysis, muscle denervation, motor-neuron loss, anxiety-like behavior and cortical and hippocampal neurodegeneration. These mice express *C9orf72* sense and antisense transcripts and RNA foci are found throughout the CNS. Additionally, RAN protein accumulation increases with age and disease severity and TDP-43 inclusions are found in degenerating brain regions in end-stage animals.

While the striking phenotypic differences between these models are puzzling, in the end they may provide fundamental insights into the onset and triggers of both ALS and FTD. Possible reasons for the phenotypic differences between the BAC mouse models include: (1) the absence of the full length gene and 3′ UTR regulatory regions in two models [76•,77•]; (2) possible cis-modifier differences on individual BAC transgenes; (3) differences in mouse genetic background—Liu *et al.* [78••] used FVB whereas B6 backgrounds were used in the other studies [75•,76•,77•]; (4) differences in spatial or temporal expression of both the sense and antisense transcripts. Regarding the last point, in the Liu *et al.* model, sense foci were found throughout the CNS whereas antisense foci were found in regions that later showed neurodegeneration [78••]. These data suggest that antisense transcripts or antisense RAN proteins may be especially pathogenic. These mice can be used tease apart the most important molecular mechanisms of this complex disease and test the efficacy of therapeutic strategies, not just by reversing one or more molecular markers of disease, but by reversing the disease itself.

Therapeutics

The demonstration of bidirectional transcription and RAN translation in a growing number of diseases highlights the potential pathogenic effects of both sense and antisense RNAs and RAN proteins. A challenge for the field is to understand the consequences of individual RAN proteins versus RNA gain-of-function mechanisms and the contributions of sense versus antisense genes to clarify the most important therapeutic targets. To date, research for most expansion diseases has focused on blocking the expression of the initially discovered 'sense' gene [17,19,79–83]. Because most microsatellite expansions are late onset, therapeutic benefit may be achieved by targeting sense transcripts alone, as this approach would lower the overall burden of expansion RNAs and RAN proteins. For example, antisense oligonucleotide (ASOs) treatment against the intronic region of the sense *C9orf72* transcript in C9 BAC mice developed by Jiang *et al.* were shown to decrease both sense RNA foci and levels of poly-GA and poly-GP RAN proteins and the mild behavioral deficits in these mice [77•]. While studies for *C9orf72* suggest that targeting the sense transcript does not alter levels of antisense foci in patient cells or mouse models [77•,84,85••], decreases in sense expansion transcripts resulted in increased antisense transcript expression in another microsatellite expansion disorder,

spinocerebellar ataxia type 7 (SCA7) [86]. Additional studies will be required to understand if targeting just one strand could potentially upregulate and exacerbate pathogenic effects from the other strand.

Alternative therapeutic approaches have focused on targeting pathways downstream of the expansion RNA and RAN proteins, such as nucleocytoplasmic transport defects [59••,61,62•] or protein quality control processes that affect mutant protein aggregation and turnover [21,22••,23,24,87]. These strategies may be effective but also may have unintended consequences. For example, small molecule and peptide inhibitors aimed at disrupting MBNL1 or other RNA binding proteins to CUG expansion RNAs in DM1 [88–93], may alleviate MBNL1 loss-of-function effects but may also increase the available pool of expansion transcripts for RAN translation.

Given the complex downstream consequences of microsatellite expansion mutations and their sense and antisense RNA and RAN protein products (Figure 1), preventing expression of the repeats is also an attractive strategy as it could block transcription and RAN protein production in both directions. Targeting of a *C9orf72* transcript transcription factor, *SUPT5H*, in patient-derived cells reduced both sense and antisense RNA foci as well as poly-GP protein with little apparent effect of other transcripts [85••]. Earlier experiments showed SPT5 expression in yeast is required for transcription through expanded CAG repeats [94]. Additional studies showed knockdown of *Supt4h* expression in Huntington's mice results in decreased expression of mutant *Htt* mRNA, reduced Htt protein aggregates and phenotypic improvements [95]. It will be important to understand how blocking sense transcription affects antisense transcripts and the more recently discovered HD-RAN proteins. Finally, the development of effective therapeutic strategies will require the use of models that accurately mimic the temporal/spatial expression patterns, the physiological levels of expansion RNAs and RAN proteins and the phenotypic changes found in patients.

Conclusions

RAN proteins have now been reported in five microsatellite expansion diseases (see Box 1) [1,3–8,11••], a number likely to grow with improved detection techniques. Animal and cell culture models are being used to determine the roles that RAN proteins play in disease and the mechanisms that regulate their translation. Understanding how RAN translation works and being able to modulate RAN protein expression will allow us to test the contribution of these proteins to disease and provides additional therapeutic targets. Because repetitive sequences make up approximately 50% of the human genome it is possible that RAN proteins may be widely expressed and have normal roles in cellular function.

Conflict of interest statement

Dr. Cleary has nothing to disclose. Dr. Ranum has three pending patents related to RAN translation ("Use and treatment of di-amino acid repeat-containing proteins associated with ALS", "Nucleotide repeat expansions-associated polypeptides and uses thereof" & "Methods for diagnosing Huntington's disease").

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