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Repeat-Associated Non-ATG Translation: Molecular Mechanisms and Contribution to Neurological Disease

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Abstract

Microsatellite mutations involving the expansion of tri-, tetra-, penta-, or hexanucleotide repeats cause more than 40 different neurological disorders. Although, traditionally, the position of the repeat within or outside of an open reading frame has been used to focus research on disease mechanisms involving protein loss of function, protein gain of function, or RNA gain of function, the discoveries of bidirectional transcription and repeat-associated non-ATG (RAN) have blurred these distinctions. Here we review what is known about RAN proteins in disease, the mechanisms by which they are produced, and the novel therapeutic opportunities they provide.

Contents

1. EXPANDING MECHANISMS IN MICROSATELLITE EXPANSION DISORDERS

Microsatellite expansion mutations cause more than 40 neurological and neuromuscular diseases, including myotonic dystrophy (DM), *C9ORF72* amyotrophic lateral sclerosis/frontotemporal dementia (C9 ALS/FTD), Huntington's disease (HD), and multiple types of spinocerebellar ataxia (SCA). These tri-, tetra-, penta-, or hexanucleotide repeats can expand when passed from generation to generation and also during an individual's life span through somatic instability (Cleary et al. 2006, Lopez Castel et al. 2010). These mutations can be located in protein-coding regions, within introns, or in the 5′ or 3′ untranslated regions (UTRs) of their respective genes (Gijselinck et al. 2016, Harley et al. 1992, Trottier et al. 1994). In general, repeats located in noncoding gene regions can be 10–100 times longer than those found in open reading frames (ORFs). In most expansion diseases, longer repeat lengths correlate with decreased ages of onset and increased disease severity (Cleary & Ranum 2014, Cleary et al. 2018, Gijselinck et al. 2016, Harley et al. 1992, Trottier et al. 1994).

1.1. RNA Gain of Function, Protein Gain of Function, and Protein Loss of Function

Traditionally, repeat expansion mutations have been thought to cause disease through the toxic gain-of-function (GOF) properties of mutant proteins or RNAs or through protein loss-of-function (LOF) mechanisms. For dominantly inherited disorders, microsatellite expansion mutations located in noncoding regions have typically been considered to involve RNA GOF mechanisms. In this mechanism, GC-rich expansion transcripts form secondary structures that can sequester disease-specific RNA-binding proteins (RBPs) and disrupt their normal functions. The best example of this RNA GOF mechanism is in myotonic dystrophy types 1 and 2 (DM1

and DM2). Hairpin-forming CUG and CCUG expansion RNAs transcribed from expansion mutations located in the *DMPK* 3′ UTR or *CNBP* intron 1 sequester muscleblind-like (MBNL) proteins in nuclear RNA foci, resulting in the loss of MBNL function and downstream RNA processing abnormalities (Kanadia et al. 2006, Lander et al. 2001, Miller et al. 2000, Mohan et al. 2014, Nelson et al. 2013, Scotti & Swanson 2016, Tóth et al. 2000, Wheeler & Thornton 2007). In contrast, expansions located in protein-coding regions can take on new functions and also disrupt normal protein function, for example, in HD and SCA1 (Labbadia & Morimoto 2013, Orr & Zoghbi 2007). Recessively inherited diseases involving protein LOF mechanisms include the intronic GAA and 5′ UTR CGG repeats, which decrease expression of the *FXN* and *FMR1* genes involved in Friedreich ataxia and fragile X syndrome (FXS), respectively (Bidichandani et al. 1998, Campuzano et al. 1996, Hagerman 2013, Hagerman & Hagerman 2013). The discoveries that many of these expansion mutations are bidirectionally expressed and that both coding and noncoding expansion mutations can express proteins in multiple reading frames by repeat-associated non-ATG (RAN) translation have changed our fundamental understanding of the molecular mechanisms of repeat expansion disorders.

1.2. Bidirectional Transcription

Bidirectional transcription at repeat expansion loci was first described for DM1 (Cho et al. 2005) and SCA8 (Moseley et al. 2006). In DM1, small RNAs expressed in the antisense direction were detected (Cho et al. 2005), and in SCA8, antisense transcripts containing CAG expansion were shown to express a polyGln expansion protein that accumulates in human and mouse brains (Moseley et al. 2006). Although most research and therapeutic strategies are focused on expansion RNAs or proteins expressed in the sense direction, antisense transcripts have also been reported in a growing number of diseases, including fragile X–associated tremor/ataxia syndrome (FXTAS), HD, Huntington's disease-like 2, SCA7, DM2, and C9 ALS/FTD (Cleary et al. 2018).

1.3. RAN Translation

The discovery of RAN translation added further complexity to possible mechanisms involved in microsatellite expansion disorders. RAN translation was first discovered while studying the CAG•CTG expansion mutations in SCA8 and DM1. Zu et al. (2011) showed in transfected cells that both CAG and CUG expansion transcripts can produce unexpected homopolymeric proteins in all three reading frames in the absence of an AUG start codon. Furthermore, they observed that polyGln, polyAla, and polySer are expressed from CAG expansions, and polyLeu, polyAla, and polyCys proteins are expressed across CUG expansion transcripts. Additionally, these authors provided evidence for the accumulation of novel polyAla and polyGln RAN proteins in SCA8 and DM1, respectively (Zu et al. 2011). RAN proteins are now known to accumulate in eight distinct neurodegenerative and neuromuscular diseases and, in addition to SCA8 and DM1, have been reported in FXTAS (Todd et al. 2013), HD (Bañez-Coronel et al. 2015), and C9 ALS/FTD (Ash et al. 2013, Mori et al. 2013, Zu et al. 2013) and recently in DM2 (Zu et al. 2017), Fuchs endothelial corneal dystrophy (Soragni et al. 2018), and SCA31 (Ishiguro et al. 2017) (**Table 1**).

Taken together, bidirectional transcription and RAN translation blur the previous mechanistic lines drawn between protein-coding and noncoding classifications of microsatellite expansion mutations. Mutations previously considered to be noncoding and suggested to involve an RNA GOF mechanism (O'Rourke & Swanson 2009, Ranum & Cooper 2006, Todd & Paulson 2010) can express up to six mutant proteins, e.g., as in C9 ALS/FTD (Cleary & Ranum 2013, 2014; Cleary et al. 2018). Similarly, expansion mutations located in ORFs can express up to five

Table 1 Repeat expansion diseases and RAN proteins

Diseases	Gene	Expansion	RAN	Cells	Patient tissues
C9orf72 ALS/FTD	*C9orf72*	G_4C_2	$polyGA_S$	×	Hippocampus, frontotemporal neocortex, cerebellum
			$polyGP_S$	×	Hippocampus, frontotemporal neocortex, cerebellum, spinal cord
			$polyGR_S$	×	Hippocampus, frontotemporal neocortex, cerebellum
	C9orf72$_{AS}$	G_2C_4	$polyPR_{AS}$	×	Hippocampus, frontotemporal neocortex, cerebellum, spinal cord
			$polyPA_{AS}$	×	Hippocampus, frontotemporal neocortex, cerebellum
			$polyGP_{AS}$	×	Hippocampus, frontotemporal neocortex, cerebellum, spinal cord
DM1	*DMPK*	CTG	$polyL_S$	×	ND
			$polyC_S$	×	ND
			$polyA_S$	×	ND
	DM1$_{AS}$	CAG	$polyQ_{AS}$	×	Myoblast, skeletal muscle, leukocytes
			$polyS_{AS}$	×	ND
			$polyA_{AS}$	×	ND
DM2	*CNBP*	CCTG	polyLPAC	×	Neurons, activated microglia in gray matter
	DM2$_{AS}$	CAGG	polyQAGR	×	Oligodendrocytes in white matter
HD	*HTT1*	CAG	$polyQ_S$	×	Caudate, gray matter of FCX
			$polyS_S$	×	WMB caudate and putamen, WM of FCX and CB
			$polyA_S$	×	WMB caudate and putamen, WM of FCX and CB
	HD$_{AS}$	CTG	$polyL_{AS}$	×	WMB caudate and putamen, WM of FCX and CB
			$polyC_{AS}$	×	WMB caudate and putamen, WM of FCX and CB
			$polyA_{AS}$	×	WMB caudate and putamen, WM of FCX and CB
FECD	*TCF4*	CTG	$polyL_S$	ND	ND
			$polyC_S$	×	Corneal endothelium
			$polyA_S$	ND	ND
	TCF4$_{AS}$	CAG	$polyQ_{AS}$	ND	ND
			$polyS_{AS}$	ND	ND
			$polyA_{AS}$	ND	ND
FXTAS	*FMR1*	CGG	$polyG_S$	×	Hippocampus, frontal cortex, cerebellum
			$polyR_S$	ND	ND
			$polyA_S$	×	ND
	FMR1$_{AS}$	CCG	$polyA_{AS}$	×	Hippocampus, frontal cortex, cerebellum, midbrain
			$polyP_{AS}$	×	Hippocampus, frontal cortex, cerebellum, midbrain
			$polyR_{AS}$	×	ND

(*Continued*)

Table 1 (*Continued*)

Diseases	Gene	Expansion	RAN	Cells	Patient tissues
SCA8	*ATXN8*	CAG	$polyQ_S$	×	Purkinje cells
			$polyS_S$	×	Cerebellar white matter regions, brain stem, frontal cortex
			$polyA_S$	×	Purkinje cells
	ATXN8OS	CTG	$polyL_{AS}$	×	ND
			$polyC_{AS}$	×	ND
			$polyA_{AS}$	×	ND
SCA31	*BEAN*	TGGAA	$polyWNGME_S$		Purkinje cells
	$SCA31_{AS}$	TTCCA	$polyFHSIP_{AS}$		ND

The × indicates RAN proteins have been found in cell culture experiments. Abbreviations: ALS, amyotrophic lateral sclerosis; CB, cerebellum; DM, myotonic dystrophy; FCX, frontal cortex; FECD, Fuchs endothelial corneal dystrophy; FTD, frontotemporal dementia; FXTAS, fragile X–associated tremor/ataxia syndrome; HD, Huntington's disease; ND, not determined; RAN, repeat-associated non-ATG; SCA, spinocerebellar ataxia; WM, white matter; WMB, white matter bundle.

additional sense and antisense RAN proteins (Bañez-Coronel et al. 2015). Additionally, many of the polyGln expansion disorders are known to express both sense CAG and antisense CUG expansion transcripts, which could trigger RNA GOF features similar to those previously reported in DM1 and SCA8. Now that we know many repeat expansion mutations can undergo this type of promiscuous expression, it is critical to determine which expansion disorders express RAN proteins and when and where they accumulate in order to understand their contribution to disease.

2. WHEN AND WHERE RAN PROTEINS ACCUMULATE: CLUES FROM THE SCENE OF THE CRIME

Comparisons across the growing number of microsatellite expansion disorders now known to express RAN proteins have revealed common themes. First, RAN proteins have been primarily reported to accumulate in the central nervous system (CNS) (Cleary & Ranum 2014, Cleary et al. 2018). Within the brain, selected RAN proteins have distinct patterns of accumulation. For example, in DM2, the LPAC and QAGR RAN proteins are expressed in gray and white matter, respectively (Zu et al. 2017). Similarly, in HD, the ATG-initiated polyGln protein and four different HD RAN proteins are found in the caudate and putamen, but the HD RAN proteins show additional accumulation in the white matter bundle regions of the caudate and putamen (Bañez-Coronel et al. 2015). Additionally, Ayhan et al. (2018) recently described how SCA8 polySer RAN protein preferentially accumulates in cerebellar and cortical white matter regions. Curiously, polySer accumulates primarily in oligodendrocyte-like cells in the white matter regions, while the SCA8 polyGln and polyAla are found in Purkinje cells and other neurons. These patterns of accumulation are not explained by differences in transcript expression, as each of these proteins is expressed from the same CAG expansion transcript. These data suggest that the expression of RAN proteins may be differentially regulated and that cell-specific factors and/or frame-specific differences in flanking sequence may modulate RAN translation. In SCA8, eIF3F is elevated in white matter regions that show high levels of SCA8 RAN polySer accumulation, and knocking down eIF3F expression decreases polySer accumulation. In contrast, eIF3F knockdown did not decrease the translation of proteins with an upstream ATG or ATG-like sequence. This or similar differential effects that depend on sequence context may explain why some RAN proteins preferentially accumulate in white matter regions.

A second common feature of RAN protein diseases is that the patterns of accumulation are highly variable and often involve focal sites of accumulation and pathology. The patchy accumulation is evident in C9 ALS/FTD, DM2, HD, and SCA8 (Ash et al. 2013; Ayhan et al. 2018; Bañez-Coronel et al. 2015; Mori et al. 2013; Moseley et al. 2006; Zu et al. 2013, 2017). For example, in HD, RAN proteins can be difficult to detect in some cerebellar regions and highly abundant in other regions (Bañez-Coronel et al. 2015). This variation is seen between patients as well as when comparing different sections of fixed tissue from the same brain regions in the same patient. In DM2 and SCA8, sites of DM2 QAGR and SCA8 polySer RAN protein accumulation occur in regions with neurodegeneration and white matter abnormalities, respectively (Ayhan et al. 2018, Zu et al. 2017). Although it is not yet clear what triggers the focal accumulation of RAN proteins, it is possible that focal regions of stress (Cheng et al. 2018, Green et al. 2017), a reported modulator of RAN translation, may lead to focal increases of RAN protein expression.

In C9 ALS/FTD, six different RAN proteins are expressed and accumulate in C9 human autopsy brain samples (Ash et al. 2013, Mori et al. 2013, Zu et al. 2013). Although histological studies frequently report GA dipeptide protein aggregates as the most abundant dipeptide repeat (DPR), differences in antibodies, antibody affinities, and antigen retrieval methods required for individual C9 RAN proteins make it difficult to quantitatively compare abundance. More quantitative and objective methods will be required to definitively measure the expression levels of individual C9 RAN proteins and assess the impact of therapeutic interventions on their levels. A particular challenge for the field has been to detect antisense C9 RAN proteins, especially in mice. In addition to the brain, GP proteins are also found in cerebrospinal fluid (CSF) and peripheral blood mononuclear cells. In human patients, GP levels in the CSF are relatively stable (Gendron et al. 2017, Lehmer et al. 2017), and in mice they have been shown to correlate with aggregates in the brain and to be reduced when treated with antisense oligonucleotides (ASOs) (Gendron et al. 2017), making GP proteins an important pharmacodynamic biomarker that can be used to assess therapeutic efficacy (Gendron et al. 2017, Westergard et al. 2016). More work is needed to form a complete picture of when and where C9 RAN proteins accumulate.

Another important question involves when RAN proteins are first expressed and how long it takes before they accumulate and contribute to disease. For many diseases, increases in repeat length result in increased severity and decreased ages of onset. Although RAN proteins can be expressed from relatively short (e.g., HD, 40–100 repeats) and much longer (e.g., DM2, 75–11,000 repeats) expansion mutations, in general, RAN translation increases with repeat length (Ash et al. 2013; Mori et al. 2013; Sellier et al. 2017; Todd et al. 2013; Zu et al. 2011, 2013). These data suggest that patients with longer repeat length may express RAN proteins earlier and at higher levels. Consistent with this hypothesis, cases of HD with longer repeat lengths (~100) that are associated with juvenile onset show much higher levels of RAN proteins in human autopsy samples than the adult-onset cases with shorter repeats (Bañez-Coronel et al. 2015). Additionally, in both SCA8 and C9 ALS/FTD mouse models, RAN protein aggregates are detected prior to overt onset of symptoms, and their accumulation increases with age and disease severity (Ayhan et al. 2018, Liu et al. 2016). Furthermore, RAN proteins, but not TDP-43 pathology, are observed prior to the onset of symptoms in several C9 ALS/FTD patients (Vatsavayai et al. 2016), supporting the presymptomatic expression of RAN proteins. Although these data are consistent with a role for RAN proteins as a driver of disease, the interplay between expansion RNAs and RAN translation is likely to also influence when and where RAN proteins are first expressed. This relationship was recently explored in DM2, where RAN translation of CCUG expansions was shown to be modulated by MBNL1 (Zu et al. 2017). The authors proposed a two-stage model of disease, the nuclear sequestration failure model, which posits that the initial stage of nuclear retention of expansion RNAs and RBP depletion is followed by a later disease stage, where expansion RNAs are exported

into the cytoplasm and undergo RAN translation, worsening the disease. Understanding when RAN proteins first appear in microsatellite diseases and if or at what stage their accumulation contributes to individual diseases will be important for the development of effective therapies and the timing of their application in patients.

3. RAN PROTEIN TOXICITY

Since their initial discovery there has been an enormous effort to understand the role of RAN proteins in disease. Studies in which expanded repeats are overexpressed in human cell lines (e.g., HEK293T, T98) have shown that the expression of several types of repeats is toxic to cells, but they do not distinguish the contribution of RNA versus protein toxicity (Bañez-Coronel et al. 2015; Soragni et al. 2018; Stopford et al. 2017; Zu et al. 2011, 2017). Driving higher levels of expression of specific RAN proteins in particular reading frames with an ATG-initiation codon has also been shown to increase cell death and decrease cell viability in comparison to non-ATG or vector controls (Todd et al. 2013; Zu et al. 2011, 2013). Experiments showing that the overexpression of individual RAN proteins in cells is toxic independent of the expansion RNA have been confirmed using nonhairpin-forming alternative codon sequences (May et al. 2014; Wen et al. 2014; Zu et al. 2013, 2017). This approach has demonstrated that the RAN proteins from multiple repeat expansions (CAG, CUG, CCUG, CAGG, G4C2, G2C4) are toxic independent of the toxic effects of the expansion RNAs (Bañez-Coronel et al. 2015; May et al. 2014; Zu et al. 2011, 2013, 2017) when they are overexpressed in cells. Although RAN proteins are toxic in these simple toxicity and viability assays using cell-based models, both RNA transcript and protein expression levels are often artificially high, making it critical to study toxicity in complex systems under physiological conditions. An examination of toxicity studies for FXTAS, HD, and C9 ALS/FTD provides examples of the complexity of RAN protein toxicity.

3.1. Toxicity of Fragile-X Tremor/Ataxia Syndrome RAN Proteins

The toxicity of the FMR-polyglycine (polyGly) protein in FXTAS has been explored across several model systems where phenotypes are strongly linked with neurodegeneration, such as rough eye phenotypes for *Drosophila* models and gait abnormalities, anxiety, cage behavior, and neurodegenerative changes for mouse models. To demonstrate the toxicity of FMR-polyGly, Todd and coworkers (2013) generated *Drosophila* models overexpressing repeats in different sequence contexts capable of producing CGG expansion RNAs and/or FMR-polyGly protein. Toxicity, as assessed by the rough eye phenotype, increased with increased production of FMR-polyGly and was suppressed when protein production was prevented. More recently, Sellier and coworkers (2017) generated two mouse lines: one expressing both expanded CGG transcripts and FMR-polyGly and another line containing a mutant 5′ UTR that only expresses CGG transcripts. The mice expressing both RNA and FMR-polyGly, but not the ones expressing just RNA, developed locomotor deficits, including decreased mobility and obesity, and they died at 10 months of age. Using a doxycycline-inducible mouse model, Castro and coworkers (2017) showed that expression of 90 CGG repeats outside of the context of the *FMR1* gene was sufficient to recapitulate FXTAS-like behavioral phenotypes (e.g., seizures, anxiety, and motor deficits). Anxiety-like phenotypes but not motor defects in mice were reversed immediately after stopping the expression of this transgene. These changes were accompanied by a reduction of ubiquitinated FMR-polyGly inclusions in hippocampal regions but not in the cerebellum. These results suggest a contribution of FMR-polyGly to neurodegeneration in this overexpression model; however, the contributions of other RAN protein species and the expansion RNAs were not excluded in this model. The toxicity of

FMR-polyGly, and to a lesser extent FMR-polyAla, has been linked with ubiquitin-proteasome system (UPS) impairment (Oh et al. 2015). FMR-polyGly was also found to recruit and alter the cellular distribution of Lap2β, which plays a role in organizing the nuclear lamina architecture, in FTXAS patients and induced pluripotent stem cell (iPSC)-derived neurons (Sellier et al. 2017). The antisense FMR-polyPro and FMR-polyAla were also shown to accumulate in FXTAS brain tissue (Krans et al. 2016) and likely contribute to protein toxicity in FXTAS. Given the number of FXTAS RAN proteins identified in patient brain tissue, understanding the toxicity and contribution of individual RAN proteins will be important for understanding the etiology of the disease and also for the development of effective therapeutic treatment strategies.

3.2. Huntington's Disease and Polyglutamine Toxicity

The toxicity of the polyglutamine (polyGln) expansion protein, mutant huntingtin, has been a major focus in HD and other polyGln disorders and has been reviewed extensively elsewhere (Bates et al. 2015, Figiel et al. 2012, Switonski et al. 2012, Underwood & Rubinsztein 2008, Walsh et al. 2005). PolyGln adopts amyloid-like fibril structures in vitro and in mammalian cells, which is necessary for its toxicity (Bevivino & Loll 2001, Poirier et al. 2005, Schaffar et al. 2004, Scherzinger et al. 1999). Recent findings on the structure of polyGln in expanded Huntingtin exon 1 in intact neurons showed that amyloid-like polyGln fibrils interact with the endomembrane (Bäuerlein et al. 2017). Interactions of polyGln fibrils with the endoplasmic reticulum (ER) alter ER organization and dynamics, which may play a role in the increased ER stress previously described in cellular and mouse models of HD (Duennwald & Lindquist 2008, Y. Jiang et al. 2016, Kouroku et al. 2002, Leitman et al. 2013). Although the mutant huntingtin protein is clearly toxic, four additional HD RAN proteins were also recently shown to accumulate in human autopsy samples. These HD RAN proteins are toxic to cells and found in brain regions with neurodegenerative changes, suggesting that they may also contribute to disease (Bañez-Coronel et al. 2015). A more complete understanding of the expression, distribution, and contribution of sense and antisense RAN proteins and RNAs to HD will be required to understand the pathophysiology of the disease. Additionally, the possibility that RAN proteins may also be expressed in the other eight polyGln disorders needs to be investigated.

3.3. Toxicity of *C9orf72* Amyotrophic Lateral Sclerosis/Frontotemporal Dementia RAN Proteins

A tremendous amount of work has been done to study the toxicity and contribution of individual DPR proteins, produced from the *C9orf72* G4C2 repeats, to ALS/FTD phenotypes. Toxicity studies have shown marked differences between the five DPR repeat motifs. GR and PR proteins have been described as highly toxic species due to their arginine-rich and shared biophysical properties. These properties favor binding to low-complexity domains (LCDs), including LCDs found in hnRNPA1/2 and directly interacting with TIA-1, a stress granule marker (Kwon et al. 2014, Lee et al. 2016, Lin et al. 2016). Exposure of human cell lines to synthetic PR_{20} and GR_{20} resulted in significant decreases in cell viability (Kwon et al. 2014). The toxicity of synthetic PR_{20} and GR_{20} was explained by the cellular uptake of these peptides followed by their migration to nucleoli and disruption in precursor messenger RNA (mRNA) splicing and RNA biogenesis, possibly through binding to the LCD of hnRNPA2 (Kwon et al. 2014, Lin et al. 2016). In addition, overexpression of $(PR)_{50}$ but not $(GA)_{50}$ caused substantial increase in death of primary cortical motor neuron cultures and iPSC-derived neurons (Wen et al. 2014). Overexpression of $(PR)_{50}$ was also shown to lead to its accumulation in nucleoli and correlate with stress granule formation (Wen et al.

2014). Understanding the toxicity of and interactions between the individual RAN proteins will be important for understanding the mechanisms of disease.

To dissect RNA GOF and RAN toxicity in C9 ALS/FTD, Mizielinska and coworkers (2014) developed several *Drosophila* lines that expressed pure G_4C_2 repeats or stop codon–interrupted sequences that expressed RNA but not RAN proteins. Although the stop codon interruptions did not alter the G-quadruplex structures of RNA transcripts and foci formation, eye degeneration and lethality were observed only for lines expressing the pure repeats. In addition, the work from several research groups demonstrated that *Drosophila* lines expressing transcript-containing alternative codons for arginine-containing PR and GR proteins, but not GP, GA, or PA RAN proteins, showed neurodegenerative eye phenotypes and a significant increase in lethality (Mizielinska et al. 2014, Wen et al. 2014). More recently, Zhang and coworkers (2018) showed that GFP-GR_{100} mice develop age-dependent neurodegeneration, brain atrophy, and motor and memory deficits. In this AAV mouse model, GFP-GR_{100} protein was found to colocalize with ribosomal subunit and initiation factor eIF3η, suggesting that polyGR could impair ribosome function and protein translation. The mechanism of polyGR toxicity may be related to its ability to induce stress granule formation and impair stress granule disassembly (Kwon et al. 2014, Lee et al. 2016, Zhang et al. 2018). Its toxicity may also be related to posttranslation modifications of arginine-containing C9 RAN proteins, as polyGR colocalizes with asymmetrical dimethylarginine immunoreactivity in regions of neurodegeneration (Sakae et al. 2018). Dimethylarginine modifications are associated with the elevated levels of protein degradation found in neurodegenerative disorders. C9 ALS/FTD patients also accumulate symmetric arginine dimethylated proteins, which colocalize with p62, suggesting possible defects in stress granule degradation in these patients (Chitiprolu et al. 2018). Additionally, $(GR)_{80}$ has been shown to dysregulate mitochondrial function, possibly by binding to mitochondrial ribosomal proteins, which causes increased oxidative stress and DNA damage in iPSC-derived spinal motor neurons (Lopez-Gonzalez et al. 2016). Although GR and PR may be highly toxic, it is important not to overlook the contribution of other C9 RAN proteins.

3.3.1. PolyGA toxicity. The C9 polyGA DPR protein has been shown to have only moderate toxicity in various cell culture, zebrafish, and mouse models compared to polyGR and polyPR (Kwon et al. 2014; Lee et al. 2016; Lin et al. 2016; May et al. 2014; Ohki et al. 2017; Zhang et al. 2014, 2016). Nevertheless, AAV delivery and overexpression of GFP-$(GA)_{50}$ in the CNS in mice produce several neurological phenotypes, including brain atrophy, neuronal loss, astrogliosis, and behavioral deficits (including hyperactivity, anxiety-like behavior, and motor and cognitive defects) (Zhang et al. 2016). Unlike other C9 DPRs, polyGA forms cytoplasmic filamentous aggregates in C9 ALS/FTD patient tissue (Zhang et al. 2014) that have recently been shown to be composed of densely packed twisted ribbons (Guo et al. 2018). In vitro, short GA peptides of 6 or 15 repeats adopt amyloid-like structures that are toxic to neurons (Chang et al. 2016, Flores et al. 2016). Disruption of these amyloid structures in cell culture by inclusion of a proline residue after every five GA repeats in a GFP-$(GA)_{50}$ construct resulted in a more diffuse signal and decreased its toxicity. Toxicity of the polyGA protein may be related to the interaction and recruitment of other proteins and structures within cells, including proteasome subunits (e.g., PSMB6 and PSMC4), UPS-related proteins HR23A/B, the trafficking protein Unc119, and other nucleocytoplasmic transport proteins (Guo et al. 2018; Khosravi et al. 2017; May et al. 2014; Zhang et al. 2014, 2016). In cultured mouse neurons, polyGA expression results in caspase-3 activation, decreased proteasome activity, and increased levels of binding immunoglobulin protein (BIP), phosphorylated protein kinase RNA–like ER kinase (PERK), and transcription factor C/EBP homologous protein (CHOP) (Zhang et al. 2014). The activation of ER stress by polyGA is further supported by the fact that ER stress inhibitors (e.g., salubrinal and TUDCA) provide protection

against polyGA-induced toxicity. In addition, C9 polyGA is believed to act as a seeding template for other C9 RAN protein aggregations. For example, $(GR)_{80}$ was recruited to cytoplasmic $(GA)_{80}$ aggregates in HeLa cells, iPSC-derived neurons, and flies, which partially suppressed the Notch pathway–associated toxicity of $(GR)_{80}$ in flies (Yang et al. 2015). In addition, coexpression of polyGA induces the aggregation of polyGP and polyPA, which normally show diffuse signals. Additionally, polyGP and polyGA colocalize as aggregates in human autopsy brain tissue (Lee et al. 2017), further supporting a role for polyGA in the recruitment of other proteins in human disease.

3.3.2. Toxicity of other dipeptide repeat proteins. Toxicity studies using cellular and animal models indicate that C9 polyGP and polyPA are likely the least toxic C9 DPR species. These two proteins are uncharged, have a diffuse cellular localization in cell culture, and have been shown to have a compact flexible coil structure (Lee et al. 2016). The GP DPR does localize to discrete cytoplasmic and nuclear foci in some *Drosophila* models (Freibaum et al. 2015, Lee et al. 2016, Mizielinska et al. 2014, Wen et al. 2014), suggesting limited aggregation activity. This DPR also appears to affect the functionality of the UPS and increases cell death in the presence of the proteasome inhibitor MG-132 (Yamakawa et al. 2015). The limited toxicity of the GP DPR may explain why its levels do not correlate with disease onset and clinical and disease outcome measures (Gendron et al. 2017), although GP has been proposed as a pharmacodynamic biomarker for *C9orf72* expression and mutation carrier status. Despite limited toxicity data, polyPA was reported to modulate the GA-dependent toxicity in cells and chick spinal cords (Lee et al. 2017), suggesting a complicated relationship between C9 DRPs, protein aggregation, and toxicity. Given that overwhelming the cellular protein degradation system has been proposed as a disease mechanism for C9 ALS/FTD, it is possible that all C9 DPRs may contribute to disease, apart from their individual inherent toxicity or biophysical properties, simply through their unintended expression and accumulation. Additionally, it will be important to clarify if the toxicity of specific DPRs or RAN proteins is specific to particular cell types (e.g., neurons versus glia), in order to understand if the neurodegeneration seen in the disease is cell autonomous or involves neighboring glial cells.

3.4. Modifiers of C9 Dipeptide Repeat Toxicity

Identifying modifiers of C9 DPR toxicity is likely to provide insight into underlying disease mechanisms and aid in the development of therapeutic strategies. One of the first C9 RAN protein toxicity modifier screens was performed in yeast, a system that recapitulates the toxicity of polyPR and polyGR (Jovicic et al. 2015). Two unbiased screens for modifiers of $(PR)_{50}$ toxicity identified dozens of suppressors and enhancers, including multiple genes encoding proteins involved in nucleocytoplasmic transport (e.g., MTR10, an import receptor, and NDC1, a key component of the nuclear pore complex). Similar screens for polyGR toxicity modifiers revealed 133 gene suppressor candidates, also including genes involved in nucleocytoplasmic transport (Chai & Gitler 2018). However, the majority of polyGR regulators were genes involved in rRNA processing and ribosome synthesis, supporting distinct toxicity mechanisms for polyPR and polyGR RAN proteins. Similar modifiers were generated by a $(PR)_{20}$ clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated protein 9 (CRISPR-Cas9) screen in human cells and primary mouse neurons, which included genes related to the ER, proteasome, RNA-processing pathway nucleocytoplasmic transport, and chromatin modification (Kramer et al. 2018). The same group further investigated two potent modifiers: *RAB7A*, an endolysosomal trafficking gene, and Tmx2, an ER-resident transmembrane thioredoxin protein. Although the knockdown of *RAB7A* in HeLa cells altered PR_{20} subcellular localization, *TMX2* knockdown resulted in the upregulation of

prosurvival unfolded-protein-response-pathway genes, suggesting that Tmx2 mitigates toxicity by modulating the ER stress response. Interestingly, the C9ORF72 protein may itself be a modifier of polyGR and polyPR toxicity. Studies on *C9ORF72*$^{+/-}$ and *C9ORF72*$^{-/-}$ induced pluripotent stem cell–derived motor neurons (iMNs) showed that they degenerated faster than controls and overexpression of the A or B isoforms of C9ORF72 protein induced the clearance of $(PR)_{50}$ aggregates in iMNs (Shi et al. 2018). The interplay between C9 RAN proteins, modifiers, and other cellular components has been proposed to form a feedforward loop that may exacerbate disease. Based on work in *Drosophila*, the expression of C9 RAN proteins but not expansion RNAs was shown to cause the cytoplasmic accumulation of TDP-43, which in turn triggers karyopherin-α2/cytoplasmic mislocalization and nucleocytoplasmic defects that further increase RAN protein levels (Solomon et al. 2018). Overall, protein transport and clearance pathway genes have been identified from modifier screens for other microsatellite expansion diseases (Todd et al. 2010, Vossfeldt et al. 2012), suggesting that targeting these pathways may generate therapeutics that work across multiple diseases.

4. MECHANISM OF RAN TRANSLATION

Although there has been tremendous interest in the role of RAN proteins in disease, there is also growing interest in understanding how RAN translation occurs. Canonical translation initiation is a complex, highly regulated, step-wise process that typically includes the following: recognition of a 5′ methyl-7-guanosine (m^7G) cap structure, binding of a number of eukaryotic initiation factors (eIFs), scanning for an AUG or a near-cognate AUG codon by preinitiation complex (PIC) (43S) containing small (40S) ribosomal subunit and initiator methionine-transfer ribonucleic acid (Met-tRNAi), irreversible hydrolysis of eIF2-GTP to release eIF2-GDP and other eIFs, and recruitment of the large (60S) subunit to form the 80S ribosome where elongation begins (Harding et al. 2000, Sonenberg & Hinnebusch 2009). Alternative initiation pathways have also been described such as internal ribosome entry sites (IRESs), in which structured RNAs directly recruit PIC to the start codon to circumvent the scanning process, especially under conditions where eIFs are inhibited (Komar & Hatzoglou 2011). Near-cognate start codons with one nucleotide that is different from the AUG are also used in mammalian cells for translation initiation (Hinnebusch 2014). The fact that many microsatellite expansion mutations are GC-rich sequences that form secondary structures similar to IRESs suggests that RAN translation may behave in an IRES-like manner. Additionally, as RAN products have been detected in patient tissues across multiple reading frames in multiple diseases, it is possible that RAN translation uses both canonical scanning and initiation and alternative initiation pathways, depending on the reading frame.

4.1. Current Mechanistic Understanding of RAN Translation

The current understanding of the mechanisms of RAN translation initiation has been garnered primarily from work on SCA8 CAG, FXTAS CGG, and *C9orf72* G_4C_2 expansions. In the original paper, Zu et al. (2011) showed that RAN translation was repeat length dependent and dependent on structured RNAs. Additionally, the authors ruled out RNA editing and frame-shifting mechanisms that could explain the expression of these proteins using canonical rules. Mass spectrometry of polyAla showed that RAN proteins expressed in cell culture can initiate in that reading frame throughout the repeat track, whereas RAN proteins expressed from the polySer or polyGln reading frame began close to or at the beginning of the repeat tract. Furthermore, Zu et al. showed that sequence context affected the expression of RAN proteins in individual reading frames and that stop codons placed immediately in front of the CAG expansion blocked the expression of

the RAN polyGln but not RAN polyAla or RAN polySer proteins. Using in vitro rabbit reticulocyte lysates, Zu et al. (2011) showed that RAN translation only occurred across transcripts and in reading frames with close cognate initiation codons, and in these cases a Met-tRNA$_i^{Met}$ was used. Translation in this in vitro system was much more limited than the promiscuous translation observed in a variety of sequence contexts in mammalian cells, raising the possibility that modeling RAN translation in cell-free in vitro systems may not mimic what is occurring in disease.

Using a nanoluciferase reporter to monitor RAN translation, Kearse and coworkers (2016) showed that RAN translation of FXTAS CGG repeats is also repeat length dependent. Additionally, they showed translation initiation in the polyGly and polyAla reading frames and utilized cap-dependent scanning mechanism to initiate near-cognate start codons upstream (e.g., ACG or GUG) or within the repeat. The cap-dependent mechanism of polyGly translation initiation across CGG repeats was supported by multiple lines of evidence: significant reductions in polyGly translation products when using a nonfunctional A-cap in the *FMR1* mRNA; reduced translation efficiency with m^{7}G-cap analog titration, indicating the involvement of the eIF4E protein; and the inhibition of translation in two frames of the CGG repeat by an eIF4A inhibitor, showing the requirement of eIF4A helicase. Interestingly, the insertion of three stop codons upstream of CGG repeats significantly reduced polyGly but not polyAla expression in transfected cells. Furthermore, FMR-polyGly expression occurs independently of the repeat itself, in contrast to sense FMR-polyAla and antisense protein (polyPro, polyArg, and polyAla) expression. Taken together, these data provide additional evidence for reading frame–specific differences in FXTAS.

Several research groups have recently investigated translational mechanisms for the *C9orf72* expansion RNAs. Several groups showed that RAN translation across both G4C2 and G2C4 repeats is repeat length dependent (Mori et al. 2013, Zu et al. 2013). Recently, two groups have reported that the translation of *C9orf72* sense G4C2 expansion RNAs is 5′ cap and eIF4E dependent and uses an upstream near-cognate CUG codon (Green et al. 2017, Tabet et al. 2018). In addition, Tabet and coworkers (2018) showed that the presence of a uORF on misspliced transcripts influences the expression of RAN proteins in all three reading frames in transfected cells, possibly through preventing its interaction with PIC. In this model, initiation occurs at a close cognate CUG, and frameshifting is proposed to lead to expression in the alternative reading frames. In contrast, Cheng et al. (2018) used a bicistronic reporter system with multiple stop codons inserted just upstream of the second ORF $(G4C2)_{70}$-nLuc and detected significant expression, supporting a cap-independent mechanism. Translational efficiency also varies between the C9 reading frames, with the GA frame translated more efficiently than GP and GR frames in a cell-free system. C9 RAN protein expression levels also appear to be affected by repeat length, although these findings are still somewhat controversial. Similar to the frame-specific differences in FXTAS, C9 polyGP DPR production was remarkably influenced by repeat length, while no striking difference in expression levels was observed for polyGA and polyGR between 30 and 66 repeats (Tabet et al. 2018). In contrast, Green and coworkers (2017) reported that the expression of polyGA increased with repeat length in their system. Thus, whether the *C9ORF72* G4C2 repeat mRNA utilizes a cap-dependent and/or IRES-like mechanism in vivo, and which one is dominant if both occur, remains to be investigated.

Two recent publications have provided clues as to possible translation initiation factors involved in RAN translation. First, a novel SCA8 polySer protein was recently shown to accumulate and form aggregates in the white matter regions of SCA8 mouse and human autopsy brain tissue (Ayhan et al. 2018). White matter accumulation of polySer and QAGR has also been reported for HD and DM2 (Bañez-Coronel et al. 2015, Zu et al. 2017), suggesting that a specific factor in white matter regions may favor RAN translation. Because eIF3F was reported to be elevated in white matter regions (Mills et al. 2013), Ayhan and coworkers (2018) tested this factor in

RAN translation and showed that knockdown of eIF3F influenced SCA8 RAN translation. The mammalian eIF3 complex, of which eIF3F is a noncore subunit, is essential for most forms of cap-dependent and cap-independent translation. Interestingly, eIF3F knockdown reduced steady state levels of polySer and polyAla expressed from constructs lacking ATG start codons but not from constructs with ATG start or ATG-like codons. Similarly, a decrease of polyGP levels produced from G4C2 repeats was found in eIF3F knockdown cells, suggesting eIF3F may act as a RAN transactivating factor of RAN translation in C9 ALS/FTD and SCA8 (Ayhan et al. 2018). Second, Sonobe and coworkers (2018) recently showed that eIF2A is at least partially used for translation of the *C9orf72* polyGA DPRs. Although knockdown of eIF2A decreased translation of nanoLuciferase polyGA in chick embryo neural cells, its knockout did not completely abolish polyGA translation, suggesting other translation initiation factors are also involved. When canonical cap-dependent translation is limited by eIF2α phosphorylation, eIF2A becomes increasingly involved in translation, especially for mRNAs with a uORF. Understanding additional factors regulating RAN translation may help explain the regional distribution of specific RAN proteins and their contribution to neurodegeneration and disease.

4.2. Stress and RAN Translation

The integrated stress response (ISR) is activated by cells in response to various diverse stress stimuli and leads to decreased global protein synthesis and the expression of select genes to promote cellular recovery (Kroemer et al. 2010, Pakos-Zebrucka et al. 2016). The critical step in this pathway is the phosphorylation of eIF2α (Clemens 2001, Harding et al. 2000, Holcik & Sonenberg 2005), which leads to the attenuation of cap-dependent translation and activation of noncanonical translation initiation, such as those driven by IRESs. Given the uncertain mechanistic underpinnings of RAN translation, there has recently been considerable focus on effects of the ISR on RAN translation.

Introduction of ER stress via thapsigargin (an ER calcium pump inhibitor), tunicamycin (a global translation inhibitor), or oxidative stress (sodium arsenite) significantly increases expression of polyGly and polyAla from *FMR1* CGG repeats (Green et al. 2017). The expression of C9 RAN proteins was also unregulated in primary rat cortical neurons under ER stress induced by thapsigargin (Green et al. 2017). Cap-independent RAN translation of $(G4C2)_{70}$ and intronic repeat mRNA was also recently shown to be upregulated by various stress stimuli, including oxidative stress (via sodium arsenite) and unfolded protein stress (MG132 induced) (Cheng et al. 2018). Additionally, recent results show that C9 polyGA translation utilizes eIF2A, a noncanonical translation initiation factor, and is associated with ISR induction, which can lead to the attenuation of conventional cap-dependent translation (Sonobe et al. 2018). Taken together, these data suggest a feedforward mechanism in which ISR induction in neural cells triggers eIF2α phosphorylation and eIF2A use, which in turn leads to increased DPR synthesis. As C9 RAN proteins are aggregation prone and their expression induces ISR, this pathway may feed back on itself and lead to further RAN protein production and the acceleration of disease progression. Understanding the precipitating event in this cycle may provide a valuable future target for therapeutics.

5. THERAPEUTIC APPROACHES AND RAN TRANSLATION

For repeat expansion diseases, there are many potential therapeutic targets, but the complex nature of these diseases has hampered the development of effective therapies. Potential targets range from repeats in the genomic DNA to sense and antisense expansion RNAs, a plethora of RAN proteins, and affected downstream cellular pathways.

Expansion RNAs, currently the primary focus of much research, have been targeted by various techniques, including ASOs, small molecules, drugs, and alterations of upstream processes (Cleary et al. 2018, Connelly et al. 2016, Gao & Cooper 2013, Taylor et al. 2016). ASO strategies are being tested and/or developed for numerous expansion disorders, including DM1, HD, SCA2, SCA3, and C9 ALS/FTD (Gao & Cooper 2013, J. Jiang et al. 2016, Moore et al. 2017, Sah & Aronin 2011, Scoles et al. 2017, Wheeler et al. 2012). In C9 ALS/FTD, a single dose of an ASO targeting the sense transcript to a C9 BAC mouse model decreased expanded *C9ORF72* transcript levels, sense foci, and RAN protein levels (polyGA and polyGP) (J. Jiang et al. 2016). Even 6 months following ASO injection, these mice still demonstrated beneficial effects, such as lowered polyGP and polyGA levels, despite the expansion RNA returning to preinjection levels. An important consideration for any of the ASO-based approaches is the bidirectional nature of expression from the expanded repeat, especially given that most ASOs target either sense or antisense transcripts but not both.

One interesting approach is targeting transcription of the repeat rather than expansion RNAs by either directly deactivated Cas9 (Pinto et al. 2017) or upstream cellular processes (Cheng et al. 2015, Kramer et al. 2016). The use of deactivated Cas9 and guide RNAs against the repeat itself was recently shown to impede the transcription of expanded microsatellite repeats and rescue molecular and cellular phenotypes in patient-derived cells as well as muscle phenotypes in a mouse model of DM (Pinto et al. 2017). Targeting transcription was also attempted using knockdown of the SUPT4H1/SUPT5H transcriptional elongation factor complex, which reduces the transcription of genes with long stretches of expanded repeats (Cheng et al. 2015, Jiang & Cleveland 2016, Kramer et al. 2016). Genetic deletion or ASO knockdown reduction of SUPT4H1 levels in 2Q175 or R6/2 mice decreased mutant expanded HTT mRNA and Htt aggregates and resulted in phenotypic recovery (Cheng et al. 2015). A similar approach in C9 ALS fibroblasts and C9 iPSC-derived cortical neurons reduced both sense and antisense RNA foci as well as polyGP protein (Kramer et al. 2016). The applicability of this particular approach was recently brought into question, as a detailed examination of RNA abundance following SUPT4H1 depletion by RNAi demonstrated a global reduction in RNA (Naguib et al. 2019). Small-molecule approaches to targeting transcription have also been applied to expansion diseases, including the use of actinomycin D and furamidine to reduce the transcription of expansion RNA in DM model systems (Jenquin et al. 2018, Siboni et al. 2015). Although these drugs may primarily reduce transcription of the repeat, they may also act on other events such as the expression of RBPs or disruption of RBP-expansion RNA interaction (Jenquin et al. 2018). An alternative approach is to inhibit the nuclear export of C9 expansion RNAs by targeting SRSF1, which prevents the interaction of expansion RNAs with nuclear export receptors and has been shown to prevent neurodegeneration and locomotor deficits in flies (Hautbergue et al. 2017). It is important to note that, due to the multiple pathogenic mechanisms involved in repeat expansion diseases, targeting one process may have unintended consequences on others. For example, inhibiting sequestration of expansion RNAs by RBPs may allow the transcript to be exported to the cytoplasm, where transcripts can undergo RAN translation and exacerbate disease. Ultimately, understanding the nature, extent, and cellular consequences of the on- and off-target effects of each of these approaches will be critical for the development of effective clinical treatments for expansion diseases.

Surprisingly, given the wealth of knowledge on RAN proteins, especially in C9 ALS/FTD, there are limited therapeutic strategies aimed directly at the proteins. Treatment with antiGA antibodies has been shown to inhibit intracellular polyGA aggregation in cell culture and block seeding activity in brain extracts (Zhou et al. 2017). However, there are more strategies aimed at the downstream consequences of RAN proteins. For example, targeting expansion-associated

nucleocytoplasmic transport defects is neuroprotective in both C9 ALS/FTD (Hautbergue et al. 2017, Zhang et al. 2015) and HD (Grima et al. 2017) mouse models. Overexpression of the small heat shock protein B8 (HSPB8), which modulates the autophagy-mediated disposal of misfolded aggregation-prone proteins, was also recently shown to decrease the accumulation of most C9 RAN proteins (Cristofani et al. 2018). Salubrinal, a UPS inhibitor, and other compounds that reduce ER stress enhance cell survival in neurons expressing polyGA (Zhang et al. 2014). Given the increasing wealth of knowledge regarding RAN proteins and their downstream consequences, many potential therapeutic targets are likely to come to light and advance to preclinical testing in short order. Clearly, a better understanding of the mechanisms of RAN translation and the role of individual RAN proteins in disease will increase the pace and breadth of future therapeutic opportunities.

SUMMARY POINTS

1. Bidirectional transcription and RAN translation have expanded the traditional coding versus noncoding disease mechanism classification of microsatellite repeat diseases.
2. RAN proteins with a variety of repeat motifs accumulate in brain regions in multiple microsatellite repeat diseases.
3. Individual RAN proteins can show patterns of accumulation that are highly variable and often involve focal sites of accumulation and pathology.
4. The expression of RAN proteins can occur prior to the overt onset of symptoms and may represent long-term stress on the cellular system.
5. Toxicity varies greatly between individual RAN proteins expressed from the same repeat expansion, and RAN proteins may cause toxicity directly or through interactions with other proteins.
6. Nucleocytoplasmic transport, the ER, and protein clearance pathways are affected by the expression of individual RAN proteins.
7. RAN translation initiation can be variable and share characteristics of both canonical and noncanonical translation pathways depending upon the repeat.
8. ER stress and eIF2α phosphorylation lead to increases in RAN protein levels.

FUTURE ISSUES

1. The best tools must be identified to quantitatively and reliably examine RAN proteins in both model systems and patients.
2. The individual RAN proteins that are the most reliable biomarkers of disease and the best candidates for therapeutic interventions must be identified.
3. It must be determined when and where RAN proteins are first expressed and begin to accumulate and how they ultimately contribute to disease in humans.
4. The number of repeat expansion diseases and the common molecular mechanisms between them must be determined.

5. We must address the questions of how frequently RAN translation occurs in humans, including healthy individuals, and whether RAN translation plays a role in translation regulation.

DISCLOSURE STATEMENT

L.P.W.R. and L.N. are inventors on pending patents related to RAN translation and/or the detection of RAN proteins.

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