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REVIEW

Molecular Pathophysiology of Fragile X-Associated Tremor/Ataxia Syndrome and Perspectives for Drug Development

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Abstract Fragile X-associated tremor/ataxia syndrome (FXTAS) is an inherited neurodegenerative disorder manifesting in carriers of 55 to 200 CGG repeats in the 5′ untranslated region (UTR) of the fragile X mental retardation gene (*FMR1*). FXTAS is characterized by enhanced *FMR1* transcription and the accumulation of CGG repeat-containing FMR1 messenger RNA in nuclear foci, while the FMRP protein expression levels remain normal or moderately low. The neuropathological hallmark in FXTAS is the presence of intranuclear, ubiquitin-positive inclusions that also contain FMR1 transcript. Yet, the complete protein complement of FXTAS inclusions and the molecular events that trigger neuronal death in FXTAS remain unclear. In this review, we present the two most accepted toxicity mechanisms described so far, namely RNA gain-of-function and protein gain-of-function by means of repeat-associated non-AUG translation, and discuss current experimental and computational strategies to better understand FXTAS pathogenesis. Finally, we review the current perspectives for drug development with disease-modifying potential for FXTAS.

Keywords FXTAS · Premutation · RNA toxicity · RNA-binding protein · RAN translation · FMRpolyG

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Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an inherited and age-dependent neurodegenerative disorder characterized by late-onset, progressive intention tremor and gait ataxia [1]. In addition to these core clinical features, FXTAS is commonly associated with parkinsonism, autonomic dysfunction, cognitive decline, dementia, emotional liability, and peripheral neuropathy, as well as white matter abnormalities and global brain atrophy that can be observed on magnetic resonance imaging [1, 2]. FXTAS is linked to a dominant mutation on the X chromosome, leading to milder clinical presentation in female patients and higher disease penetrance at about 45 % in males compared to 16 % only in females [3].

FXTAS patients are carriers of a trinucleotide CGG repeat expansion in the 5′ untranslated region (UTR) of the fragile X mental retardation gene (*FMR1*). The CGG repeat locus typically contains a variable number of repeats between 3 and 45 in the general population and expands to a pathogenic range named premutation from 55 to 200 repeats causing FXTAS [4]. In contrast, the full mutation above 200 repeats results in DNA methylation and gene silencing leading to fragile X syndrome (FXS), the most frequent genetic cause for neurodevelopmental disorder [5]. The *FMR1* premutation is also associated in women to a different clinical phenotype characterized by premature ovarian insufficiency, as well as high frequency of thyroid disease, hypertension, seizures, peripheral neuropathy, and fibromyalgia [3]. Due to large differences in disease manifestation, FXTAS is often misdiagnosed and many patients are identified only after a grandchild is diagnosed with FXS [6]. Patient treatment is currently limited to symptom management, with limited effects on disease progression [7, 8]. On this basis, understanding the molecular mechanisms of FMR1 premutation toxicity is essential for the development of disease-modifying therapies and improving disease prognosis.

At the molecular level, FXTAS is characterized by elevated levels of *FMR1* transcription and accumulation of the CGG repeat-containing FMR1 messenger RNA (mRNA) in nuclear foci, while the expression levels of the encoded protein FMRP remain normal or moderately low due to a decrease in translational efficiency [9–11]. As indicated by detailed analysis of transgenic mouse models and patient samples, the increase in FMR1 transcription level is strongly correlated with the numbers of CGG repeats, whereas FMRP expression level shows a weak, inverse correlation with repeat number [12, 13]. Both may contribute to the clinical profile of FXTAS patients, and there may be in fact a continuum of overlapping clinical and molecular phenotypes between FXTAS and FXS, leading to the proposal of a "fragile X spectrum disorder" [14].

The neuropathological hallmark in FXTAS is the presence of well-delineated intranuclear, eosinophilic, ubiquitin- and αB-crystallin-positive inclusions, which contain the FMR1 transcript. These inclusions are present not only in neurons and astrocytes with a broad distribution throughout the central nervous system but also in various peripheral tissues. Specifically, inclusions have been observed in the cerebral cortex, basal ganglia, hippocampus, thalamus, substantia nigra, and ependymal and choroid plexus nuclei; in the spinal cord and autonomic ganglia; in the heart and kidney; and in the thyroid, pineal, pituitary, and testicular glands [15–18]. The presence of intranuclear inclusions in various structures of the central and peripheral nervous systems is consistent with the broad range of neurological symptoms going from dementia to dysautonomia. A second prominent neuropathological feature consists in white matter disease, characterized by spongiosis in subcortical and deep white matter with corresponding axonal loss and glial cell loss [16]. Patients also present a moderate to severe loss of Purkinje cells, yet the remaining Purkinje cells only rarely present intranuclear inclusions—or only in older patients [19].

The frequency of cells presenting intranuclear inclusions is increasing with CGG expansion size, but it is still under debate whether these inclusions are toxic per se or if they are simply a side product of the neutralization of toxic FMR1 transcripts [16]. Attempts to characterize the protein complement of intranuclear inclusions present in FXTAS indicates that ubiquitinated proteins account only for a minor fraction, suggesting that the inclusions do not result from a failure of the nuclear proteosomal system [20]. Detailed histological studies found that neurons with intranuclear inclusions are not structurally altered, whereas in astrocytes, the presence of inclusions is associated with increased size of the nucleus [21]. In fact, the presence of inclusions is not associated with the typical morphological signature indicative of progressive neuronal death.

Why do CGG repeats become toxic upon expansion? How do they trigger neuronal death? Even though these questions are not fully elucidated yet, significant progress has been achieved with the identification of two different mechanisms involving RNA- and protein-mediated toxicity, presented hereafter. The identification of these two toxic processes is opening new perspectives for the development of innovative therapies, with the capacity to halt and possibly reverse disease progression.

RNA-Mediated Toxicity

Multiple microsatellite expansion diseases such as myotonic dystrophy type 1 (DM1) and type 2 (DM2), spinocerebellar ataxia types 8, 10 and 31, Huntington's disease, and FXTAS have in common the presence of intranuclear RNA inclusions [22]. It was first established in DM1, where a CTG repeat is present in the 3′UTR of the DMPK gene, that repeat expansion in a non-protein-coding region can lead to RNA toxicity by a mechanism which was later found to occur in a relatively similar manner in other repeat expansion diseases. In essence, this toxicity mechanism consists in the sequestration of various proteins at the repeat site on the transcripts, leading to depletion of these proteins from the common pool and to their accumulation in the form of nuclear inclusions. Such a mechanism is taking place in FXTAS, and the identification of the proteins sequestered in nuclear inclusions is an essential step for understanding how toxicity does occur.

A biochemical analysis performed on aggregate-containing nuclei extracted from FXTAS patient brain material identified more than 20 different proteins including the RNA-binding proteins hnRNP A2/B1 and MBNL1 and the chaperones HSP70, HSP27, and αB-crystallin [20]. Nuclear inclusions also contained structural proteins such as lamin A/C, GFAP, and golli-MBP, as well as proteins like MBP which presence in the nucleus had never been reported before. The presence of the splicing factor MBNL1 is particularly interesting as it is the one protein sequestered in DM1 and DM2, which depletion from the intracellular pool leads to splicing alterations and clinical symptoms [23]. However, a role for MBNL1 in FXTAS has been excluded because no alterations in splicing events regulated by MBNL1 were observed in human brain material from FXTAS patients [24]. Another interesting case is hnRNP A2/B1, which is a DNA- and RNA-binding protein involved in a large number of RNA processing mechanisms including transcription, mRNA splicing, and microRNA biogenesis. HnRNP A2/B1 plays several key roles in neuronal functioning and reduction in its expression level contributes to the severity of symptoms in several neurodegenerative diseases including Alzheimer's disease [25]. On this basis, depletion of hnRNP A2/B1 from the cytoplasmic pool upon binding to RNA containing expanded CGG repeats was thought to be an important factor driving toxicity in FXTAS. This hypothesis was confirmed using transgenic *Drosophila* expressing expanded CGG repeats, where the overexpression

of hnRNP A2/B1 was sufficient to rescue the neurodegenerative phenotype [26]. Furthermore, it was observed in primary neurons expressing an expanded CGG repeat that depletion of hnRNP A2/B1 from the cytoplasmic pool is leading to alteration in the RNA dendritic transport pathway [27].

Other proteins were identified which follow the same model of toxicity, where protein binding to RNA containing expanded CGG repeats leads to depletion from the active pool and alterations in the RNA processing machinery. Among them, the protein Sam68, officially named KHDRBS1, is a nucleo-cytoplasmic protein involved in multiple cellular processes including the regulation of alternative splicing. Sam68 was found to bind to expanded CGG repeats and thereby to lose its splicing-regulatory function, which in turn leads to splicing alterations in FXTAS patients [24]. Interestingly in neurons, Sam68 is a key regulator of activity-dependent splicing of neurexin 1, a cell adhesion molecule expressed at synapses with essential function in the assembly of synapses and synaptic circuits [28]. Sam68 is also involved in synaptodendritic post-transcriptional regulation of beta-actin and its acute knockdown results in fewer excitatory synapses in the hippocampal formation [29].

Another level of dysregulation resulting from protein sequestration by CGG repeat-containing mRNAs has to do with microRNAs (miRNAs), which are essential for RNA silencing and post-transcriptional regulation of gene expression [30]. Along the miRNA processing pathway, the enzymatic complex DROSHA-DGCR8 has a key function for the processing of pri-microRNAs into pre-miRNAs. DROSHA-DGCR8 was found to associate specifically with CGG repeats of pathogenic size, and its sequestration within nuclear inclusions results in reduced levels of miRNAs not only in cultured cells but also in the brain of FXTAS patients [31]. Alterations in miRNA expression levels have also been observed in lymphocytes collected from FXTAS patients and in the brain of a *Drosophila* model for CGG expansion [32, 33]. Interestingly, overexpression of DGCR8 was sufficient to rescue the cellular toxicity induced by expression of expanded CGG repeats in mouse primary neurons, indicating that DROSHA-DGCR8 sequestration is a major liability for the cells [31]. Moreover, DGCR8 is required for proper regulation of gene expression in neurons, and a modest decrease in DGCR8 expression levels obtained by deletion of one Dgcr8 allele is sufficient to induce alterations in the density, morphology, and electrophysiological properties of cortical neurons in mice [34]. DGCR8 was also found recently to participate in the regulation of neuronal morphogenesis [35]. Altogether, these results suggest that DROSHA-DGCR8 sequestration and the resulting alterations in miRNA expression may not only contribute to the late-onset neurodegenerative phenotype in FXTAS but also to the cognitive and psychiatric symptoms, which manifest in patients sometimes long before the onset of neurodegenerative symptoms [36, 37].

The transcriptional activator protein Pur-alpha is another protein that is sequestered in the nucleus: Pur-alpha was shown to bind strongly to CGG repeat RNA in vitro, to be present in intranuclear inclusions in patient brain tissue, and its overexpression in *Drosophila* was sufficient to abolish the neurodegenerative phenotype caused by CGG repeats [38, 39]. Here again, dysregulation of Pur-alpha activity upon sequestration is highly susceptible to deteriorate neuronal activity, as indicated by the severe neurodevelopmental phenotype observed in human carriers of mutations in Pur-alpha, including mental retardation, encephalopathy, and seizures [40, 41].

Yet, CGG repeat expansion in the premutation range is a mutation with limited penetrance. What are the genetic and environmental factors that determine individual susceptibility to CGG expansion? Even though this question may be too vast to answer, especially if one would consider the interactions between genes and environment, it may be of interest to mention a study which identified the suppressor effect of TDP-43 on premutation toxicity in *Drosophila*. This effect is related to TDP-43 capacity to interact with hnRNP A2/B1 and prevent the alterations in RNA splicing that are triggered by hnRNP A2/B1 sequestration [42]. Extrapolating from that, one could speculate that individuals with high TDP-43 expression levels would have a better resilience to CGG expansion. In contrast to that, specific environmental factors such as exposure to neurotoxins, chemotherapy, or general anesthesia are suspected to precipitate the onset of FXTAS disease [43, 44].

The few proteins described above help to understand the RNA toxicity model, where the sequestration of specific proteins at the CGG repeat RNA alter various intracellular processes which may individually or synergistically lead to symptoms. Yet, this model is far from being complete, especially with regards to possible differences between cell types and tissues, and a more granular characterization of proteins sequestered at the CGG repeat RNA could improve our understanding of the disease, especially at early stages, and identify targets for pharmacological intervention.

Many techniques have been recently developed with the aim of discovering novel protein-RNA interactions, starting from either a known protein or a known RNA. The so-called RNA-centric approaches, where RNA is the bait, are used to identify proteins which associate with a specific RNA. Two different strategies have been developed: RBP screening within protein libraries, e.g., protein arrays [45], and RNA affinity capture coupled with mass spectrometry for protein identification, with various protocols such as CHART [46], MS2-BioTRAP [47], and ChIRP [48]. Complementary experimental approaches such as RNA FISH, widely used for detecting expanded repeats in diseases with RNA foci, can also be applied to study FXTAS RNA-protein interactions and inclusion formation [49].

To complement the experimental approaches which are typically work intensive, computational methods have been

developed to support the discovery of proteins involved in protein-RNA complexes. Especially, the algorithm catRAPID is designed to predict which proteins bind to a specific RNA sequence; it differentiates spurious interactions from those with high affinity and can be used to characterize associations with repeat expansion [50, 51]. catRAPID was initially trained on a set of about 1000 protein-RNA complexes available in the Protein Data Bank to discriminate interacting and non-interacting molecules using the information contained in primary structures. Predictions of secondary structure, hydrogen bonding, and van der Waals' are combined together to calculate the interaction propensities. The algorithm was tested against the non-nucleic-acid-binding dataset NNBP (area under ROC curve of 0.92) [52], the non-coding RNA database NPInter (area under the ROC curve of 0.88) [53], and a number of interactions validated by RNA immunoprecipitation (RIP) and cross-linking immunoprecipitation (CLIP) approaches [54–57].

Applied to the CGG repeat RNA, catRAPID predicted the sequestration of several proteins including MBNL1 and the heterogeneous nuclear ribonucleoproteins (hnRNP) A1, A2/B1, C, D, E, and G, whereas proteins such as FMRP, CUGBP1 and Sam68 were predicted not to show any specific interaction with the CGG repeat RNA (Fig. 1a) [55]. This prediction is in very good agreement with prior experimental data showing that the proteins MBNL1 and hnRNP A1, A2/B1, C, D, E, and G colocalize with intranuclear RNA aggregates, whereas the proteins FMRP and CUGBP1 do not [24]. In silico modeling was also employed to investigate protein binding to CGG repeats in the wild-type (21 repeats) and pre-mutation range (79 repeats). The interaction propensity was found to increase for hnRNP A2/B1 upon repeat expansion; in contrast, the ribonuclease H2 subunit A (RNH2A) was found to have a low interaction propensity for both wild-type and expanded CGG regions (Fig. 1b). The results are also in agreement with experimental evidence showing that hnRNP A2/B1 interacts with long CGG repeats and has protective role in FXTAS [26, 58] while RNH2A only binds upon re-annealing of nascent *FMR1* transcript to template DNA strand [59]

This high level of agreement between in silico predictions and prior experimental data supports further use of catRAPID in the context of pathogenic repeat sequences. On this basis, specific discrepancies between predictions and data can shed new light on experimental results and suggest novel and targeted experiments. For example, the protein Sam68 is colocalized with intranuclear aggregates in vitro [24], but its sequence analysis does not predict any interaction with CGG repeat RNA. This suggested that Sam68 is a secondary binder to the CGG repeat RNA/protein complex, being included in the complex via protein-protein interaction rather than direct binding to the RNA sequence. This hypothesis was pursued in silico: Sam68 protein partners were retrieved using the protein-protein interaction database MINT and were evaluated for their binding propensity to CGG repeat RNA. Interestingly, the cold-inducible RNA-binding protein (CIRBP) and polypyrimidine tract-binding protein 2 (PTBP2) exhibit high binding propensities which are further increasing with CGG repeat numbers, suggesting that CIRBP and PTBP2 may be involved in Sam68 sequestration at the early nucleation stages [55]. In such a case, in silico modeling

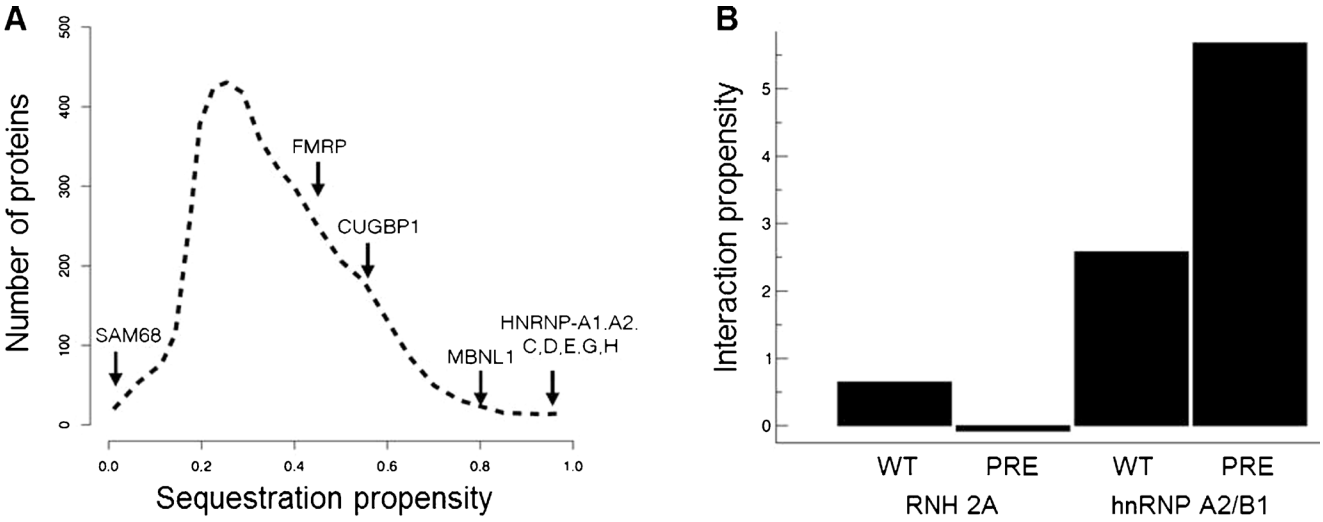

Fig. 1 **a**. Distribution across the whole human proteome of the sequestration propensity on CGG repeat RNA. catRAPID analysis of every human protein sequence for their capacity to bind CGG repeat RNA was able to identify, among others, the proteins MBNL1 and hnRNP A1, A2/B1, C, D, E, G and H, which were previously shown to colocalize with intranuclear inclusions. The sequestration propensity score takes into account the interaction strength and the average protein abundance in the cell. **b**. CatRAPID analysis applied to the WT (21 repeats) and premutation (PRE, 79 repeats) CGG expansion predicts, respectively, strong binding of hnRNPA2/B1 and no binding of RNH 2A to the expanded CGG allele. In the calculations, amino acids 32-242 of RNH2A (Uniprot entry O75792) and 21-104 of HNRNPA2/B1 (RRM1 of P22626) have been used

helps to formulate a specific hypothesis which is readily testable with experimental methods.

RAN Protein Toxicity

An additional perspective on the pathophysiological mechanisms of microsatellite repeat disorders appeared in the literature in 2011, with the discovery of non-canonical translation initiation at microsatellite repeat sites. Investigating the CTG repeat present in the Ataxin 8 gene (ATXN8), which expansion leads to spinocerebellar ataxia type 8, Laura Ranum and her team observed that translation could occur at the repeat site in the absence of any upstream translation initiation codon [60]. Repeat-associated non-AUG (RAN) translation was initiated on both sense and antisense transcripts, for each in the three possible reading frames, leading to expression of potentially six different peptides. Since the discovery of the biological mechanism, RAN translation has been found to occur in the context of the CGG trinucleotide repeat present in the FMR1 gene [61], the GGGGCC (G4C2) hexanucleotide repeat present in the C9ORF72 gene [62–64], and the CAG repeat present in the huntingtin (HTT) gene [65].

FMR1 RAN translation products

The CGG repeat in the FMR1 gene is located in the 5′UTR of the transcript and is therefore not a target for the standard translation machinery. Using transgenic flies and human cells, Todd and co-workers described that translation can be initiated by the CGG repeat itself in an AUG independent manner, in at least two of the three possible reading frames of the sense transcript [61].

FMRpolyG is the main RAN translation product, which has been detected in flies, transgenic mouse models, human cultured cells, and FXTAS patient tissues. FMRpolyG consists of a homopeptidic glycine domain, the translation product of the expansion in the glycine-encoding frame (GGC), together with the in-frame translation product of the 5′UTR of FMR1 [61]. Remarkably, FMRpolyG expression was observed in vitro not only with repeat expansions of pathological size (over 55 repeats) but also with expansions of 30 repeats only, a length which is common in the general population and regarded as non-pathological. Using different antibodies directed against the C-terminal 5′UTR translation product, FMRpolyG was detected specifically in brain tissue from FXTAS patients but not from control, demented, and non-demented autopsy cases [61, 66]. FMRpolyG was detected in multiple areas of the brain and peripheral tissues as intranuclear aggregates which are often, but not always, colocalized with ubiquitin-positive inclusions. FMRpolyG was also reported in tissues from patients with fragile X-associated primary ovarian insufficiency [67].

Another RAN translation product was identified in vitro, in the alanine-encoding frame (GCG). In this case, the protein was detected as a GFP fusion protein after removal of a stop codon in the 5′UTR shortly after the CGG repeat [61]. Under native conditions, FMRpolyA protein would consist of the polyalanine domain and a short C-terminal segment of 16 amino acids only, corresponding to the in-frame translation of the 5′UTR up until the stop codon. It is not known whether FMRpolyA expression occurs in patients, a question which could be answered using antibodies generated specifically against this 16 amino acid peptide.

The last potential RAN translation product on the FMR1 sense transcript is in the arginine-encoding frame (GGC). There is no stop codon present in that frame in the 5′UTR of FMR1, such that the theoretical FMRpolyR protein would be de facto a high molecular weight (HMW) variant of FMRP, with an extra N-terminal part including the poly-arginine domain and translation of the 5′UTR. RAN translation in the arginine-encoding frame was detected neither in vitro using GFP fusion protein as detection system nor in transgenic *Drosophila* using mass spectrometry for protein identification [61], and there is no report known to us of a HMW FMRP variant.

RAN translation was found to occur also from antisense transcripts including the CTG repeats in the ATXN8 gene, the G4C2 repeat in C9ORF72, and the CAG repeat in the HTT gene [60, 65, 68]. Antisense transcription at the FMR1 locus leads to the expression of ASFMR1, a RNA molecule which is spliced, polyadenylated, and exported to the cytoplasm, which covers the repeat site and is therefore a candidate for RAN translation [69]. Similar to FMR1 mRNA, the expression level of ASFMR1 changes with repeat expansion, with elevated expression level in cells bearing premutation alleles and expression silencing in cells with full mutation alleles. The presence of RAN translation at the CCG repeat on ASFMR1 transcripts has not been reported so far but would lead to the expression of ASFMRpolyP (CCG), polyA (GCC), and polyR (CGC) proteins. Knowing whether these RAN proteins are produced in FXTAS patients will be very important for a complete understanding of the disease pathophysiology.

RAN Protein Toxicity in FXTAS

The mechanisms of repeat expansion toxicity have been intensively investigated in the context of the polyglutamine and polyalanine-associated diseases, for which expansions occur in the middle of a protein coding region and lead to toxic gain-of-functions. A generic mechanism emerged from these studies, where polyQ and polyA expansions lead to the expression of misfolded proteins that are poorly degraded, accumulate, form aggregates, and interfere with specific intracellular processes, leading ultimately to cell toxicity and age-dependent

neurodegeneration. A similar mechanism could be driven by FMRpolyG and contribute to FXTAS disease.

In vitro experiments on human cells indicated that RAN translation drives the expression of FMRpolyG at similar levels for constructs with pathological repeat expansion sizes (>55 repeats) and non-pathological expansion size (30 repeats) [61]. In contrast, FMRpolyG-induced toxicity was observed for long FMRpolyG proteins expressed from constructs with long CGG expansions and not for short FMRpolyG expressed from constructs with 30 repeats only. The intracellular expression profile also differed with repeat numbers: long FMRpolyG formed cytoplasmic and intranuclear inclusions, whereas short FMRpolyG expression was diffuse throughout the cytoplasm. FMRpolyG inclusions stained positive for ubiquitin and chaperone HSP70, indicating the protein quality control system recognized long FMRpolyG as misfolded protein and targeted it for degradation. Finally, mutations which reduced proteasome activity worsened FMRpolyG toxicity, whereas overexpression of chaperone proteins such as HSP70 reduced it [70].

These observations suggest that the length of the polyglycine domain is an essential determinant of aggregate formation and toxicity and is possibly the element linking repeat expansion size with disease severity. Indeed, glycine is a weak hydrophobic amino acid, and glycine homopeptides are characterized by poor solubility in water and rapid formation of insoluble aggregates for pure peptides as short as nine successive glycines [71]. When homopeptides are expressed in combination with a soluble protein such as YFP, a much longer hydrophobic stretch is required to trigger aggregation. For example, the expression of the fusion protein Ala70-YFP resulted in small and dispersed cytoplasmic aggregates which were not observed upon expression of Ala30-YFP protein [72]. PolyQ-YFP proteins exhibited a similar length-dependent aggregation phenotype, indicating that the size of the hydrophobic homopeptide domain is a major determinant for protein aggregation and cellular toxicity [73, 74].

The link between surface hydrophobicity, formation of oligomers, and toxicity has been thoroughly investigated because protein misfolding and aggregate formation is the hallmark of many diseases including numerous neurodegenerative conditions [75, 76]. Toxicity results from a combination of surface hydrophobicity and size, with the most toxic aggregates having high hydrophobicity and small size [77]. Small aggregates exhibit a higher surface hydrophobicity than large ones and have therefore a higher capacity to interfere with various signaling processes [78]. Small hydrophobic aggregates can, for example, penetrate membrane and cause ionic leakage such as calcium influx or interfere with intracellular signaling pathways. To support the idea that macromolecular properties such as surface hydrophobicity are the main drivers for toxicity, a genome-wide analysis was performed comparing disease-associated mutations with neutral sequence variations. This analysis indicated that mutations increasing the aggregation potential of proteins are more often associated with human diseases than neutral sequence variations [79].

Aggregation-prone proteins are recognized by the protein folding quality control system and targeted for degradation by the ubiquitin-proteasome system (UPS); in good agreement, FMRpolyG was detected in the intranuclear inclusions which are positive for ubiquitin and chaperone HSP70, suggesting that FMRpolyG itself is ubiquitinated [61, 70]. Yet, FMRpolyG expression alters the activity of the UPS itself, as revealed by the abnormal accumulation of a G76V-ubiquitin-GFP protein used as a reporter system for the monitoring of UPS activity [70]. Inhibition of the UPS by aggregation-prone protein is not a novel observation but was already reported for alpha-synuclein [80], mutant huntingtin [81, 82], Abeta oligomers [83], GFAP oligomers [84], and the toxic conformation of prion protein (Prpsc) [85, 86]. Thus, there are common toxicity mechanisms shared by aggregation-prone proteins, and the same intracellular toxicity mechanisms may be at play for all the RAN proteins that form aggregates independent of the gene where the repeat expansion occurs.

Within each cell, the loss of control over the degradation of aggregation-prone proteins is possibly the starting point of a long series of toxic insults impacting various molecular processes. Toxicity is related to intrinsic factors such as surface hydrophobicity and structural flexibility, as well as extrinsic factors such as intracellular localization. For example, the accumulation of aggregation-prone peptides in the cytoplasm, but not in the nucleus, was found to have a strong inhibitory effect on the nucleo-cytoplasmic transport of both proteins and RNA molecules, leading to severe alterations in cytoplasmic mRNA content and protein localization [87]. Mitochondrial dysfunction, altered calcium regulation, and altered zinc transport were also described in FXTAS disease [88–90]. Such dysfunctions could happen as a direct consequence of the expression of hydrophobic and aggregation-prone peptides, which could form pores and disrupt membrane integrity, or as a downstream effect of nucleo-cytoplasmic transport inhibition and its impact on protein expression. Mitochondrial dysfunction in FXTAS has been established using primary fibroblasts obtained from FXTAS patients and non-symptomatic premutation carriers. Interestingly, the specific deficits in the oxidative phosphorylation pathway observed in FXTAS patients were also observed in aged and young premutation carriers, indicating that mitochondrial dysfunction may precede the onset of symptoms [91, 90]. Furthermore, specific measurements such as ATP-linked oxygen uptake, coupling, citrate synthase activity, and mitochondrial network organization were correlated with the number of CGG repeats, suggesting that the measurement of specific mitochondrial parameters could serve as a biomarker with a potential prognosis value [91].

Mechanism of RAN Translation in FXTAS

The exact molecular mechanism of RAN translation is far from being fully elucidated. Especially, it is unknown whether there is a unique mechanism valid for all repeat expansion sites or if it is the same mechanism that initiates translation in different reading frames at one expansion site. In the context of the CGG expansion in the FMR1 gene, RAN translation in the glycine-coding frame was shown to occur in mammalian cells for expansions as short as 30 repeats, the modal size in the human population. RAN translation level was not increased, rather decreased, with longer expansions of 50 and 88 repeats [61], suggesting that the CGG repeat is not inducing translation on its own but in conjunction with other elements in close proximity. Indeed, RAN translation at this site in the glycine frame is depending on the presence of pseudo AUG codons which are located −11, −22, and −38 bp 5′ to the expansion. One pseudo AUG codon was sufficient for RAN translation initiation, but the translation level was stronger when at least two were present [61]. The experimental observations were different for RAN translation in the alanine frame, which was detected from constructs with 88 repeats but not 30 and did not require translation initiation upstream of the repeat. It suggests that two different mechanisms support RAN translation in the glycine and alanine frames.

How could translation initiate at the CGG repeat in the 5′ UTR of FMR1 gene? There are at least three possible hypotheses and so far limited experimental data to support one or the other.

The first hypothesis involves standard cap-dependent recruitment of translation initiation factors and ribosomal subunits for the formation of the pre-initiation complex. CGG repeats are known to form hairpins and under specific conditions, G-quadruplex structures, which are very stable two- and three-dimensional structures, respectively, that are difficult to unfold [92, 93]. They form a physical obstacle for the pre-initiation complex forcing the scanning ribosome to pause [94], allowing time for non-canonical translation initiation at nearby pseudo AUG codons [95]. Whereas this model seems plausible for RAN translation initiation in the glycine frame, which is depending on the presence of pseudo AUG codons just before the repeats, it would not explain initiation in the alanine-coding frame.

A second hypothesis involves an internal ribosome entry site (IRES) located in the 5′UTR of FMR1. IRES are not conserved sequences but rather dense RNA tertiary structures that have the capacity to interact with ribosomal proteins and initiate translation [96]. There are multiple families of IRES which share limited homology, and it has not been possible yet to find universal rules to describe IRES structure and mechanism [97]. The presence of an IRES in the 5′UTR of FMR1 was characterized using a standard mapping approach, where various segments of the 5′UTR were evaluated for their capacity to induce translation of a reporter gene in a bicistronic construct. This approach identified a 21 nt pyrimidine-rich region that is essential for cap-independent translation initiation and is located ~100 nt before the CGG expansion (−232 to −212 before the ATG) [98]. The result was confirmed in another study which also identified that the CGG repeats contribute to the IRES (repeat ablation reduced IRES activity by 50 %), that the CGG repeats and flanking sequences are themselves IRES elements (able to induce cap-independent translation in the absence of the pyrimidine-rich region), and that IRES-dependent FMRP expression is responsive to various types of cell stimulation [99]. There are multiple families of IRES, which differ among other things by their capacity to initiate translation in the absence of AUG codon. The IRES structure including the CGG repeats, the flanking sequences, and the pyrimidine-rich region could therefore have the capacity to trigger expression of both FMRpolyG and polyA peptides.

Finally, a third hypothesis involves the action of a cryptic promoter located within the 5′UTR of FMR1. The presence of a cryptic promoter was identified in the context of experiments performed to characterize the IRES element in FMR1 leader. Dobson and his colleagues observed that, in the presence of the 5′UTR of FMR1, about 15 % of the expression of a reporter gene persisted after excision of the promoter from the vector, whereas expression was absent in the presence of unrelated 5′UTRs [99]. FMR1 cryptic promoter could be responsible for the expression of a yet unidentified transcripts leading to RAN translation.

Therapeutic Perspectives

Genetic diseases such as FXTAS were perceived until recently as untreatable medical conditions, and still now, pharmacological care is limited to symptom management with limited effects on disease progression [7, 8]. This perspective is changing with the emergence of new therapeutic modalities progressing at a rapid space from bench to bedside, including gene silencing and gene editing technologies, a new class of small molecules to prevent RNA-mediated toxicity, and proteasome activation approaches.

The use of gene silencing for drug development has long lived on promises, but this technology has finally progressed to the point where the first drugs have reached the market and many others are in clinical development. Gene silencing was initially based on DNA or RNA oligonucleotides, which suffered from rapid degradation and limited cell penetration in vivo, but these limitations have been circumvented with the development of modified nucleotide analogues and advanced vectorization methods. This technology is now being developed not only for peripheral diseases but also for neurodegenerative disorders, with a clinical trial for Huntington's

disease (HD) initiated in 2015 leading the trail in this field (trial identifier: NCT02519036). This clinical trial builds upon multiple independent studies showing that a reduction in the expression level of mutant huntingtin protein (HTT) in the brain helped the elimination of intracellular protein aggregates and led to significant cognitive and locomotor improvements [100]. In January 2016, the FDA has granted orphan drug designation for the HTTRx drug, indicating a strong belief in its capacity to show efficacy in patients.

In the context of FXTAS, targeted degradation of FMR1 mRNA would have the capacity to prevent both the RNA-mediated and the RAN protein-mediated toxicity, but the absence of FMRP is known to cause FXS so this approach may not be beneficial for the patients. Notwithstanding this, a narrow therapeutic window may exist for precise gene expression control in FXTAS: a modest and intermittent reduction in FMR1 mRNA levels would help the cell to eliminate toxic RNA and RAN protein aggregates while preserving a level of FMRP sufficient for normal neuron activity. In support of this idea, it was observed in the context of Huntington's disease that a short, 2-week treatment with antisense oligonucleotides triggered long-lasting therapeutic benefits, with improvement of the locomotor function and the absence of polyQ aggregates persisting for at least 9 months after the end of treatment [101]. As concerns FMRP levels, a detailed analysis of methylation mosaicism in FXS patients indicated that residual FMRP expression was associated with less severe forms of the disease [102], suggesting that a moderate and transient reduction in FMRP level may be well tolerated.

A more direct but still futuristic approach consists in genome editing and removal of the CGG repeat expansion. The fundamentals of such an approach were established in vitro using embryonic stem cells (ESCs) and induced pluripotent cells (iPSCs) derived from FXS patients, in combination with the genome editing tool clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9). In brief, the introduction of a targeted DNA double-strand cleavage a few nucleotides upstream the CGG repeat site in *FMR1* triggered deletions of variable sizes leading to reduction or complete ablation of the CGG repeats, and reactivation of the expression of *FMR1* gene [103].

A different line of research is targeting specifically the deleterious effects of nuclear protein sequestration at CGG expansion sites. A new class of small molecules is being developed, which are characterized by their high affinity for CGG hairpins and their capacity to act as chaperones and reduce protein sequestration. These compounds were selected on the basis of their capacity to inhibit DGCR8 binding to CGG hairpins in vitro [104]. Tested in a cellular assay, they triggered a dose-dependent reduction in CGG-positive intranuclear inclusions and prevented the mRNA splicing defects associated with Sam68 sequestration at CGG hairpins [104, 105]. The development of this new class of compounds is at an early stage with no information available yet regarding in vivo activity; nevertheless, it would be very interesting to know if these molecules are able to prevent RAN translation and FMRpolyG-mediated toxicity.

Similar to many other toxic aggregation-prone proteins, FMRpolyG is targeted for degradation by the UPS and is simultaneously an inhibitor of the UPS. This leads to intracellular accumulation of FMRpolyG and toxicity and suggests that increased proteasome activity could prevent this toxic process. Protein homeostasis is maintained by the coordinated activity of stress response pathways which are highly conserved in the evolution and regulate protein synthesis, folding, trafficking, and degradation. Especially, the heat shock response (HSR) is rapidly induced by environmental and physiological stress conditions and is under control of the master regulator heat shock transcription factor 1 (HSF1). HSF1 is itself maintained in a monomeric inactive state by chaperone proteins such as HSP90 and HSP70.

The antibiotic geldanamycin and its derivative 17-AAG (also called tanespimycin) are HSP90 inhibitors that have been tested in various cellular, insect, and mammalian models of protein aggregation diseases. By promoting the release of HSF1 from its complex with HSP90, geldanamycin and 17-AAG trigger expression of chaperone molecules able to bind misfolded proteins, neutralize their toxicity, and promote their degradation. This was observed in a mouse model of the polyQ expansion disease spino bulbar muscular atrophy (SBMA), which upon treatment with 17-AAG or its oral form 17-DMAG presented reduced motor neuron degeneration and significant improvements in locomotor function [106, 107]. Similarly, treatment with 17-AAG in a mouse model of the polyA expansion disease oculopharyngeal muscular dystrophy (OPMD) promoted degradation of the toxic protein by the UPS, reduced aggregate formation, and increased in vitro and in vivo cell survival [108]. Similar results have also been obtained in the field of amyloid diseases, supporting the idea that pharmacological UPR activation has a strong therapeutic potential for aggregating protein diseases [109] and could be on this basis very useful against FMRpolyG toxicity. There are, however, some difficulties related to long-term toxicity of UPR activation and cellular adaptation leading to treatment resistance [110]. Targeting a specific process in the protein degradation pathway such as selective inhibition of the deubiquitinating enzyme USP14 may prove more translatable to patients [86].

Conclusion

At least two different pathogenic mechanisms have been identified for the toxicity of neurodegenerative diseases related to microsatellite expansions, including protein gain-of-function as in the case of Huntington's disease and RNA gain-of-

function as initially described for myotonic dystrophy or FXTAS. The RNA gain-of-function hypothesis consists in the sequestration of RNA-binding proteins by the mutant mRNA, leading to depletion of these proteins from the pool required for normal gene expression and mRNA processing. RNA-mediated sequestration of specific nuclear factors was shown to be intrinsically toxic, in that overexpression of these factors could compensate sequestration and prevent toxicity. Protein gain-of-function was not considered for repeat expansion located in 5′ and 3′UTRs such as FXTAS, until the discovery of the non-conventional RAN translation mechanism which leads to the expression of aggregation-prone polypeptides with demonstrated toxicity. These two mechanisms are not mutually exclusive and can certainly occur at the same time within a cell. On this basis, FXTAS pathophysiology would be best described with a two-hit model, where protein sequestration by mutant transcripts creates a liability for RAN protein toxicity.

Acting upon one of these two mechanisms may be sufficient to reduce the amount of molecular dysfunction to a level that is sustainable for the cells and prevent neurotoxicity. This being said, there is no easy drug target identified so far for the treatment of FXTAS. By targeting the root cause of the disease, genome editing would prevent both RNA-mediated and RAN protein-mediated toxicity, but in spite of tremendous progress in this field in the recent years, there is no technology available yet for repeat removal in vivo in non-dividing cells like neurons. In contrast to that, gene silencing technology has already reached patients for the treatment of specific conditions and is in clinical development for Huntington's disease. This technology could be easily adapted for use in FXTAS patients; however, there is a fundamental antagonism between promoting FMR1 transcript degradation for the treatment of FXTAS and preserving FMRP expression for cognitive function. Finally, the use of drugs developed for their anti-tumoral activity such as 17-AAG, which has already been tested in a phase 2 clinical trial against breast cancer [111] may be the most pragmatic way forward, targeting specifically RAN protein toxicity.

In this context, more fundamental research is still needed to increase our understanding of the molecular pathophysiology of FXTAS and other neurodegenerative conditions. The emergence of in silico prediction tools such as catRAPID is paving the way toward a new form of research in biology, where computer simulation is expanding research capacity and throughput and helping deciphering the complexity of biological processes.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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