Fragile X-associated tremor/ataxia syndrome (FXTAS): Pathology and mechanisms

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Abstract

Since its discovery in 2001, our understanding of fragile X-associated tremor/ataxia syndrome (FXTAS) has undergone a remarkable transformation. Initially characterized rather narrowly as an adult-onset movement disorder, the definition of FXTAS is broadening; moreover, the disorder is now recognized as only one facet of a much broader clinical pleiotropy among children and adults who carry premutation alleles of the *FMR1* gene. Furthermore, the intranuclear inclusions of FXTAS, once thought to be a CNS-specific marker of the disorder, are now known to be widely distributed in multiple non-CNS tissues; this observation fundamentally changes our concept of the disease, and may provide the basis for understanding the diverse medical problems associated with the premutation. Recent work on the pathogenic mechanisms underlying FXTAS indicates that the origins of the late-onset neurodegenerative disorder actually lie in early development, raising the likelihood that all forms of clinical involvement among premutation carriers have a common underlying mechanistic basis. There has also been great progress in our understanding of the triggering event(s) in FXTAS pathogenesis, which is now thought to involve sequestration of one or more nuclear proteins involved with microRNA biogenesis. Moreover, there is mounting evidence that mitochondrial dysregulation contributes to the decreased cell function and loss of viability, evident in mice even during the neonatal period. Taken together, these recent findings offer hope for early interventions for FXTAS, well before the onset of overt disease, and for the treatment of other forms of clinical involvement among premutation carriers.

Keywords

fragile X; premutation; RNA toxicity; microRNA; DGCR8; neurodegeneration

The fragile X gene

The fragile X mental retardation 1 (*FMR1*) gene, located near the end of the long arm of the X chromosome (Xq27.3), is one of a growing number of genes with dynamic mutations that involve expansion of a trinucleotide repeat element [128,160]. For the *FMR1* gene, the CGG trinucleotide repeat lies in the 5′ untranslated region (5′UTR) of the gene (Fig. 1). Within the general population, the trinucleotide repeat element ranges from ~5 to 40 CGG repeats, with a mode of 29–30 repeats. Interestingly, this same mode and distribution is observed for rhesus monkeys [4]; however, repeat sizes are generally much smaller in non-primate mammals (e.g., 9–11 CGG repeats for the mouse *Fmr1* gene). One possible explanation for

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the increase in repeat size may be a mediating role of FMRP in the improvement of cognition during primate evolution.

The significance of the *FMR1* gene lies in the fact that expansions of the repeat element to more than 200 CGG repeats (full mutation range) give rise to fragile X syndrome, the leading heritable form of intellectual disability, through a process of hypermethylation of the promoter and repeat region, and consequent gene silencing. The resulting absence of the *FMR1* protein (FMRP), important for synaptic development and plasticity [103], is the proximal cause of the disorder.

Smaller expansions, from 55 to 200 CGG repeats (premutation range), paradoxically result in much higher levels of gene activity, with mRNA levels in lymphocytes that can exceed the levels found for normal alleles by 5–10-fold [152–154]. Prior to the discovery of the elevated mRNA levels, the importance of the premutation allele was generally thought to be its propensity for expansion to the full mutation range during matrilineal transmission. One remarkable feature of this expansion is its dependence on the size of the maternal allele, and on the number of AGG (interruptions) within the CGG-repeat element [43,45,46,105,124,169]. Indeed, for maternal alleles in the 70–80 CGG-repeat range, two AGG interruptions, spaced approximately 10 repeats apart, are associated with a nearly eight-fold reduction in transmission of a full mutation allele relative to the same size allele with no interruptions (~10%, 2 AGGs versus 80%, no AGGs)[168]. The basis of this dramatic effect is not understood, though it is likely due to altered structural features of the AGG-containing DNA that reduce the propensity of the CGG region to slip during replication and/or repair [122,126,163]. However, the influence of AGG interruptions on the risk of repeat expansion is of critical importance for genetic counseling [168], and demonstrates an intriguing complexity of the allelic instability mechanism.

The puzzle of clinical involvement in premutation carriers

Following the discovery of the *FMR1* gene in 1991, the basis for genetic anticipation for fragile X syndrome—termed the "Sherman paradox"—was resolved: expansion of the unstable CGG-repeat element leads to more cases of the syndrome with succeeding generations (Fig. 2). From this perspective, the significance of the "premutation" is that it defines a range of repeat sizes that are increasingly unstable (favoring further expansion) during transmission. For many years, there was no *a priori* expectation for clinical involvement in the premutation range, since the gene is generally not methylated. However, it is now clear that there are multiple, distinct clinical phenotypes among carriers of premutation alleles (Fig. 2); at least two, fragile X-associated primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS), are specific to the premutation range. Because these clinical phenotypes are largely limited to the premutation range (i.e., not present in those who lack FMRP production), they must have a distinct pathogenic mechanism from that of fragile X syndrome. This singular observation, coupled with the elevated expression of the CGG-repeat–containing *FMR1* mRNA [101,152,153], led to the proposal that the premutation disorders are the direct result of a gain-of-function toxicity of the *FMR1* mRNA [94,96, recent reviews: 66,111,132,151].

Clinical presentations among premutation carriers

There are at least four domains of clinical involvement seen in some, but not all carriers of premutation alleles (Fig. 2): (*i*) neurodevelopmental problems, including developmental delay, social and behavioral deficits (e.g., ADHD; autism spectrum disorder; intellectual disability (ID)), and childhood seizures; (*ii*) FXPOI; (*iii*) increased psychiatric problems among adults; (*iv*) FXTAS, and associated neurological and medical problems, including

hypertension, autonomic dysfunction, migraine headaches, sleep apnea, and immune-mediated disorders [166,6,20,69,70]. Since all of these forms of involvement have been extensively reviewed, their features will only be briefly summarized in the following paragraphs.

Neurodevelopmental phenotypes

There is mounting evidence that some children who are carriers of premutation alleles experience a range of developmental problems; including ID, attention problems or ADHD, autism and autism spectrum disorder (ASD), and increased frequency of seizures in childhood [11,21,27,28,49,57,66,114,131]. In a study of autism and ASD in premutation boys, Farzin et al. [49] found a higher rate of ASD in boys with the premutation presenting as probands compared with sibling (non-premutation) controls (79% versus 0%; p < 0.001); probands also had more ADHD symptoms than controls (93% versus 13%, p < 0.0001). Non-proband premutation carriers, identified through cascade testing, also had elevated levels of ASD and ADHD relative to controls.

As noted, childhood seizures constitute a significant co-morbidity of the premutation, and more broadly, a co-morbidity of ASD. In a survey of 57 premutation males and 199 premutation females, Bailey et al. [11] found that 8% of males (> 6 yr) had been treated for seizures. In their survey, premutation males were also more likely to have presented with developmental delay, and attention and behavioral problems than age-matched controls. More recently, Chonchaiya et al. [27] found that seizures occurred more frequently among premutation carrier probands (28%) than for non-proband carriers (0%), controls (0%), or population estimates (1%).

The developmental presentations raise a very important question regarding mechanism: is the developmental involvement due to an earlier manifestation of the same RNA-toxicity mechanism that leads to FXTAS, and likely to FXPOI; or is it caused by incipient FMRP deficiency (fragile X-spectrum disorder); or a combination of the two mechanisms? In a small cohort (n=10) of boys with either premutation or intermediate CGG-triplet expansions, Aziz et al. [8] observed a striking resemblance in clinical presentation to boys with fragile X syndrome. Using a semi-qualitative measure of protein (hair root analysis), the authors observed that FMRP expression was essentially normal in all participants where it was assessed. By contrast, Goodlin-Jones et al. [57] and Tassone et al. [155] found lowered FMRP levels in premutation carriers with developmental delays. More recent measurements with animal models of the premutation have provided evidence for lowered FMRP with higher CGG-repeat number in the premutation range [22, review: 14], raising the possibility of FMRP insufficiency as an added contribution to developmental involvement in some premutation carriers.

Fragile X-associated premature ovarian insufficiency (FXPOI)

Just prior to the discovery of the *FMR1* gene, Cronister et al. [35] had observed that there is an approximately 20-fold increase in the incidence of premature ovarian failure (infertility prior to age 40) in heterozygous carriers (~20% versus 1% in the general population). Now known as FXPOI [reviewed in: 148], the reproductive condition is recognized as the leading heritable form of early ovarian insufficiency/infertility. The association of FXPOI with premutation CGG-repeat expansions raises the prospect that this condition is also (along with FXTAS) due to an RNA gain-of-function toxicity [32,82,167]. Studying the mechanism of FXPOI has proven to be more difficult than for FXTAS, due to the relative paucity of suitable human samples or appropriate animal models. However, Hoffman et al. [82] have utilized a knock-in (KI; premutation CGG repeat) mouse model that displays features of FXTAS, suggesting that it should be useful, from a mechanistic standpoint, for

studying FXPOI as well. Using the KI model, the authors investigated the basis for altered hormone levels that are suggestive of a reduced residual follicle pool in the premutation carriers. Hoffman et al. [82] demonstrated that the development and establishment of the primordial follicle pool is grossly normal, but that there is an accelerated loss of follicles of all follicle classes, suggesting that the problem is intrinsic to the ovary. In addition, there are abnormalities of the follicles, including reduced size and number of granulosa cells. These observations provide an important foundation for further investigation of the pathogenic mechanism of FXPOI, and the determination of the extent to which there is mechanistic overlap with FXTAS.

Adult-onset psychiatric problems

Psychiatric involvement in adult premutation carriers, particularly depression and anxiety [144,53,52], was initially thought to be related to the stress of raising an affected (FXS) child. This involvement is now understood to be an intrinsic feature associated with premutation carriers (~40%)[133,19,20,79,80,134,142], although such problems can be exacerbated by the difficulty of raising an affected child or dealing with a parent with FXTAS [141,10]. Psychiatric problems generally intensify for those individuals who develop features of FXTAS [9]. Younger adult male carriers also manifest lowered activity of the amygdala on functional MRI (fMRI) compared to controls [78]; this lowered activity has also been shown to correlate with mild deficits in FMRP [80].

Fragile X-associated tremor/ataxia syndrome (FXTAS)

The neurodegenerative phenotype in older adult carriers of premutation alleles comprises core features of intention tremor and gait ataxia [67,94]; as well as associated white matter changes and global brain atrophy (Fig. 3); intranuclear neuronal and astrocytic inclusions; parkinsonism; peripheral neuropathy; and cognitive decline [1,23,30,59,60,67,97,116,146,2,63,114, reviews: 15,3,25,109,110].

Neuropathology

Based on studies of post-mortem CNS tissue from nineteen cases (18 cases with FXTAS, 1 premutation carrier without FXTAS), three general features of the neuropathology of FXTAS have emerged [59,60,150]: (*i*) significant cerebellar and cerebral white matter disease, consistent with MRI findings (Fig. 3); (*ii*) associated astrocyte pathology, particularly evident in sub-cortical cerebral white matter; (*iii*) the presence of solitary, generally spherical intranuclear inclusions, detected with either H&E or ubiquitin immunohistochemistry (IHC), present in both neurons and astrocytes (but not oligodendroglia), and in broad distribution throughout the brain and brainstem. Further details of the neuropathology are summarized in Table 1; however, several observations are noteworthy [59,60,150]. First, although both males and females with FXTAS possess inclusions, there tend to be far fewer inclusions in females, presumably due to the effects of the normal X chromosome; we also note that one female carrier without clinical evidence of FXTAS nevertheless possesses small numbers of inclusions in various brain regions. Second, the inclusions are exclusively located in the nuclei of cells; that is, no cytoplasmic inclusions have ever been observed in any brain tissue from a human case of FXTAS; this property of FXTAS inclusions is in stark contrast to the substantial numbers of cytoplasmic inclusions in the fly model of the premutation [96]. Third, although white matter changes in the MCP are observed in only ~60% of cases, there is histopathological evidence of disease of the MCP in nearly all cases (7/8) where there is available tissue. Fourth, there is a highly significant association between the number of inclusions in both neurons and astrocytes, and the number of CGG repeats within the premutation range. Finally, although MCP disease and Purkinje cell (PC) dropout are prominent features of the neuropathology of FXTAS,

there is a paucity of inclusions in pontine nuclei; however, there is substantial pontine volume loss and disease, and despite substantial PC dropout, inclusions are only rarely observed in PCs. These last observations suggest that the presence of inclusions is not necessary for cellular dysfunction.

Contemporaneous with the discovery of the clinical disorder was the observation of elevated, CGG-repeat-containing *FMR1* mRNA [101,152,153], indicating that FXTAS is due to a very different molecular mechanism than the protein-deficiency mechanism responsible for FXS. The remainder of this review will address this mechanism and recent findings regarding FXTAS pathology that have revised our thinking on a disorder thought to be limited to the CNS.

Current understanding of the pathogenesis of FXTAS

The discovery of FXTAS [67], and the mounting evidence supporting a role for the CGG-repeat-containing mRNA in triggering the disorder, has raised a number of questions related more broadly to the family of premutation-associated disorders. First, the role of the RNA may well be restricted to the initial triggering events in FXTAS pathogenesis, thus leaving open the question of what happens next; that is, what downstream processes are actually involved with cellular dysregulation and loss of neuronal-cell viability? Second, although FXTAS has been the principal focus of research on pathogenic mechanisms in the premutation range; it is likely that most, and perhaps all of the additional forms of clinical involvement in the premutation range will involve the same RNA-toxicity mechanism, at least initially. In particular, FXPOI displays the same apparent restriction to the premutation range, suggesting that FMRP insufficiency is not a primary driver of the loss of ovarian function. Third, although little is known regarding the basis for partial penetrance of FXTAS in the aging premutation population, genetic background effects are doubtless influencing the appearance and severity of the disorder. In this regard, it is interesting that risk factors for related neurodegenerative disorders may also influence risk for FXTAS (e.g., APOE4 allelotype, [143]) or related adult clinical involvement (e.g., corticotrophin releasing hormone receptor 1 polymorphisms, [91]). Fourth, one puzzling feature of clinical involvement in FXTAS is the greater frequency of associated medical findings, such as hypothyroidism and fibromyalgia [134,29], in women with FXTAS relative to males of similar age and stage of neurological involvement.

Finally, whereas the initial focus of FXTAS has been in the aging adult population, recent animal studies indicate that many features of the neuronal and astrocytic cellular pathology are occurring in the neonatal period [24,26,36]. These findings suggest that FXTAS may simply be one end-stage outcome of a cellular dysregulation that may be life-long, and that the developmental problems experienced by some children who are premutation carriers may be manifestations of this early, non-degenerative process. All of these considerations will be important for developing treatment approaches for the premutation-associated disorders, including FXTAS, and raise the possibility that the sub-clinical features of cellular dysregulation may be remediable long before any neurodegenerative features develop.

Initial events in the pathogenesis of FXTAS and other premutation-associated disorders

The neurodegenerative disorder, FXTAS, principally affects carriers of premutation alleles of the *FMR1* gene; the pathogenic mechanism, gain-of-function "toxicity" of the elevated levels of CGG-repeat-containing mRNA, is completely distinct from the gene-silencing mechanism (FMRP insufficiency) leading to fragile X syndrome [recent reviews:

151,132,111,66]. As currently envisioned, toxicity is thought to arise through partial sequestration of one or more RNA-binding proteins by the expanded CGG repeat, thus leading to a functional insufficiency of the sequestered proteins (Fig. 4). The paradigm for such a process is myotonic dystrophy, wherein the mRNA produced by the myotonic dystrophy protein kinase (*DMPK*) gene harbors a CUG repeat in its 3′UTR region, which, when substantially expanded, gives rise to many of the features of myotonic dystrophy (DM) [44,120,158]. Notably, no mutations causing DM have been reported within the DMPK coding region, arguing against a direct role of the protein in the pathogenesis of DM; nor have coding mutations in the *FMR1* gene been linked to FXTAS, though such mutations do lead to fragile X syndrome [31,38,64,119]. Furthermore, an intronic CCTG-repeat expansion in an unrelated gene, *ZNF9*, leads to a highly similar form of myotonic dystrophy (DM2) [112,130].

For the CUG-repeat element in the DMPK gene, expression in an unrelated reporter gene was capable of recapitulating a number of key phenotypic domains of DM, including defects in a muscle chloride channel and the formation of CUG-RNA–containing nuclear "foci." Thus, the expanded CUG-containing mRNA itself appeared to be responsible for the phenotype; the presence of the foci suggested that the CUG-repeat element was interacting with one or more proteins, such that the abnormal interaction would produce both clinical and histopathological features of DM.

The preponderance of evidence suggests that the triggering event in FXTAS pathogenesis involves the sequestration of one or more proteins by the expanded CGG repeat in the *FMR1* mRNA 5′UTR [recent reviews: 132,111,65]. Candidates for the sequestered proteins include hnRNP A2/B1 [93,145], one of the most abundant members of the family of heterogeneous nuclear ribonucleoproteins, and thought to play a role in binding to and translocating the myelin basic protein mRNA [98]; Purα [95,7], a transcriptional activator also thought to be involved in the control DNA replication; Sam68 [140], an RNA-binding protein that belongs to the "signal transduction and activation of RNA" (STAR) family [16]; and DGCR8, part of the "microprocessor" complex that processes micro (mi)RNA precursors in the nucleus [42,139].

For all of the above-mentioned proteins, there is evidence for *in vitro* association with CGG-repeat-containing RNA. Although both hnRNP A2/B1 and Purα appear to bind preferentially, at least *in vitro,* to shorter (~20 CGG) repeat RNAs [139], both proteins do appear to have mediating effects on *Drosophila* models of CGG-repeat–induced neurodegeneration [95,145]. Notwithstanding their role(s) in mitigating the phenotypes in animal models, roles for Purα and hnRNP A2/B1 have yet to be demonstrated in human disease or in murine models of FXTAS. For Sam68, there is clear evidence of insufficiency in animals and humans; with altered splicing noted in FXTAS patients [140]. However, a critical test of these candidate proteins will be whether they have any direct primary or secondary role in FXTAS pathogenesis in humans.

DGCR8 sequestration and dysregulation of microRNA biogenesis

As an extension of their initial study [140] of CGG-binding proteins identified through *in vitro* association with CGG-repeat RNA, Sellier and co-workers identified DGCR8 (DiGeorge syndrome critical region 8; [48,127,147]) as another protein that binds with relatively high specificity to CGG-repeat RNA [139]. Among the more than 30 RNA-binding proteins derived from mouse brain nuclei that were identified using their binding protocol, including the aforementioned hnRNP A2/B1, Purα, and Sam68 [7,93,95,140,145], 10 proteins were found to bind preferentially to premutation CGG repeats (60 and 100 CGG repeats). Of these 10, only three colocalized within the nuclear RNA aggregates that were

associated with premutation alleles, and only one, DGCR8, was specific for such aggregates. Interestingly, both hnRNP A2/B1 and Purα were preferentially recruited to the short (20 CGG-repeat) RNAs, and neither protein demonstrated any significant colocalization with the CGG RNA aggregates. For Purα, absence of colocalization in the RNA aggregates is consistent with its absence from the nuclear inclusions in mouse models of FXTAS [54].

The potential significance of DGCR8 lies in its central role in micro(mi)RNA biogenesis [40,51,62,71,107,108,161]. DGCR8 acts as an anchor for the type III RNase, DROSHA, which cleaves primary miRNAs (pri-miRNAs) in the nucleus to produce precursor miRNAs (pre-miRNAs)[40,51,62,71,107,108,161]; the pre-miRNAs are subsequently exported from the nucleus to the cytoplasm, where they are converted into mature miRNAs by DICER. The DGCR8-DROSHA complex is referred to as the "microprocessor," and is a key player in the production of the vast array of miRNAs in the cell. Insufficiency of the microprocessor function, as would be expected if DGCR8 were partially sequestered, would be expected to dampen production of mature miRNAs, and in the current context would lead to neuronal dysfunction and cell loss [37,39,50,72,77,83,136,138,147].

In their study of the DGCR8-CGG-repeat interaction, Sellier et al. [139] made several key observations that support a central role of sequestration of DGCR8 in the pathogenesis of FXTAS; specifically, they demonstrated the following:

i. DGCR8 preferentially binds to the expanded CGG-repeat RNAs, consistent with a CGG-repeat threshold for RNA toxicity, and for clinical involvement, at the lower end of the premutation range [81,149].

ii. miRNA levels are globally depressed both in cultured cells expressing the expanded-repeat RNA and in human brain tissue from FXTAS patients. Moreover, consistent with reduced function of the DGCR8-DROSHA microprocessor, levels of the corresponding pri-miRNAs were elevated in the human brain samples.

iii. Overexpression of DGCR8 reversed both the decreased dendritic complexity and the lowered neuronal cell viability [e.g., 26] resulting from expression of expanded CGG repeats.

iv. Expression of DROSHA does not reverse the RNA toxicity, suggesting that DGCR8, but not DROSHA, is limiting microprocessor function in the presence of the expanded CGG-repeat RNA.

Taken together, the results of Sellier et al. [139] strongly implicate DGCR8 sequestration as a central, if not sole mode of transduction of CGG-repeat "toxicity" (Fig. 4). However, several things should be considered regarding mechanism. First, whereas DGCR8 undoubtedly plays an important role in mediating RNA toxicity, additional proteins, including the above-mentioned hnRNP A2/B1, Purα, and Sam68, may still play ancillary roles in the initiation of FXTAS pathogenesis. Although, as noted above, Sam68 sequestration does lead to splicing alterations in humans—and hence may play some role in the development and/or the domains of clinical involvement in FXTAS—its overexpression is not capable of reversing the abnormal cell phenotype [139]; thus, Sam68 sequestration is not sufficient for neuronal pathogenesis.

Possible alternative mechanisms for the initiation of FXTAS pathogenesis

The foregoing section has described a model for the initial events in FXTAS pathogenesis whereby the toxicity of the expanded CGG-repeat RNA is due to its partial sequestration of one or more RNA-binding proteins. In the case of DGCR8 sequestration, the consequent depression of miRNA production likely has multiple, as-yet-unspecified downstream effects leading to cellular dysregulation, though the haploinsufficiency of DGCR8 in 22q deletion

syndrome [147] may provide a useful foundation for understanding the consequences of depressed miRNA production. However, despite the preponderance of evidence supporting such a sequestration model, several additional processes have been proposed as candidates for an initial event in the disease process.

RNA-mediated protein aggregation

Renoux and Todd [132] have suggested that the CGG-repeat–containing RNA could initiate a conformational transition in one or more proteins that possess prion-like domains [104,132], triggering a cascade of aggregation reminiscent of amyloid plaque formation in Alzheimer's disease [104,132]. Indeed, King et al. [104] observed that hnRNP A2/B1, which is found in the intranuclear inclusions of FXTAS [60,59,93,145], ranks among RNA-recognition–motif proteins as highly "prionogenic." Such a model, if demonstrated to participate in FXTAS pathogenesis, would address one potential problem with a sequestration model involving hnRNP A2/B1; namely, its very high abundance in cells, which would seem to rule out its participation in a sequestration process, but would actually favor a prion-like nucleation process. One lingering concern with the prion-domain model is that nearly all of the proteins with prion domains listed by King et al. [104] tend to form cytoplasmic inclusions; whereas the inclusions in FXTAS are exclusively nuclear in humans [60,59] and the premutation mouse [14,159,164]. A second concern with the aggregation model, at least with respect to the participation of hnRNP A2/B1, is the observation of Sofola et al. [145] that overexpression of hnRNP A2/B1 actually reduces the neurodegenerative phenotype.

Antisense-RNA effects on expression

One interesting, albeit unresolved issue regarding FXTAS pathogenesis is what role, if any, is played by the antisense transcripts generated at the *FMR1* locus [106,102,116]. Although the initial reports by Ladd et al. [106] and Khalil et al. [102] differed in some details regarding the exact nature of the antisense transcripts, the two studies were remarkably consistent in their general observations regarding the transcripts. The RNA species are produced by RNA pol II, are spliced and polyadenylated, are exported from the nucleus, and may code for one or more small peptides. Moreover, levels of the antisense transcripts generally track with relative production of the sense *FMR1* transcript—elevated in the premutation range and absent in hypermethylated, full mutation alleles. Thus, the CGG-repeat element appears to regulate the expression of both sense and antisense transcripts, a remarkable finding in light of the fact that the more 5′ of the two antisense promoters (upstream in the antisense direction) lies more than 10 kb upstream (in the second intron of the *FMR1* RNA) of the CGG-repeat element.

Two observations raise the possibility of a functional role for the antisense RNA in FXTAS pathogenesis. First, Ladd et al. [106] observed that a specific splice isoform of the antisense transcript is only found with transcripts from premutation alleles. Determination of what role, if any, this isoform plays in FXTAS pathogenesis will necessarily require additional study. However, the altered isoform distribution could reflect a dysregulation of splicing either by Sam68 [140], or by the splice modulator, MBNL1, which is sequestered in DM; both Sam68 and MBNL1 are found in the intranuclear inclusions of FXTAS [93,140]. Second, Khalil et al. [102] observed that the presence of the antisense transcript markedly affected cell proliferation in culture; siRNA-mediated knockdown of the transcript altered both cell-cycle progression and increased apoptosis, whereas overexpression of the antisense RNA resulted in cell proliferation. Taken together, these intriguing results point to some functional importance of the antisense transcript; however, its precise role remains to be determined.

Role of reduced FMRP levels

Although the moderately reduced FMRP levels observed for large premutation alleles (> 120–150 CGG repeats) in both humans and in animal models cannot be playing a primary role in FXTAS pathogenesis, a functional FMRP insufficiency in some carriers may contribute to aspects of the premutation phenotypes, particularly with respect to domains of reduced cognition and abnormal behavior observed in both adults and children with premutation alleles [155,57,80]. Moreover, there could well be focal deficits of FMRP in the CNS that would not be properly gauged by the study of peripheral tissues (e.g., [47,129]). More recently, Iliff et al. [92] demonstrated impaired activity-dependent FMRP translation and enhanced mGluR-dependent long term depression in premutation mice, suggesting the possibility of a mixed phenotype, particularly in the upper portion of the premutation range. In this regard, in an elegant study in which an expanded CGG repeat was expressed ectopically in mouse cerebellar Purkinje cells, Hashem et al. [73] demonstrated that expression of the CGG-repeat RNA is both necessary and sufficient to produce the intranuclear inclusions of FXTAS and the associated neuronal cell death, where FMRP levels remain at wildtype levels.

RAN-mediated expression of alternative peptides

Todd, Oh et al. [157] have recently proposed an alternative model for FXTAS pathogenesis in which "toxic" peptides are produced through a process of repeat-associated, non-AUG–initiated (RAN) translation [125,170]. The authors provide evidence that translation initiation upstream of the CGG-repeat element results in the production of truncated peptides that possess a polyglycine (polyGly) stretch (GGC-codon repeat in the +1 frame), and that such peptides are toxic to the cell. The polyGly peptides are shown to accumulate in the intranuclear inclusions in both fly models and in human FXTAS cases. RAN translation has also been observed in association with the G_4C_2 hexanucleotide repeat expansion (C9orf72 locus) that causes amyotrophic lateral sclerosis and frontotemporal dementia [5]. The importance of this intriguing mechanism to FXTAS neurodegeneration remains to be determined. One puzzling aspect of the RAN translation model is that the "NIH" mouse [47] (Table 2), which does not support RAN translation [157], nevertheless displays substantial Purkinje cell dropout, similar to FXTAS in humans; whereas the "Dutch" mouse model, which does support RAN translation, does not exhibit Purkinje cell loss. Clearly, additional studies are warranted, and it may turn out that both RAN translation and RNA toxicity play roles in specific features of the FXTAS phenotype.

Cellular dysregulation

Although there is now compelling support for an RNA-toxicity/sequestration model for the initial triggering step in FXTAS pathogenesis, beyond the general notion that miRNA dysregulation (e.g., DGCR8 sequestration) would disrupt numerous additional cell functions, the RNA-toxicity model does not provide a specific explanation for why neuronal cells sicken and ultimately die. For such an explanation, one would need either to identify specific miRNAs that, when up-regulated, reverse the loss of neuronal viability; or to directly identify the more critical modes of dysregulation. Although the loss of the normal nuclear lamin architecture has been described in cell culture [4], the consequences of this altered architecture have not been further identified. Two additional, more promising modes of cellular dysregulation have now been identified, namely, altered mitochondrial function [123,135,99], and altered neuronal morphology and electrical activity [24].

Mitochondrial dysfunction

Premutation-associated mitochondrial dysfunction has now been demonstrated in both human (fibroblast) and mouse (hippocampal neurons) cultured cells, and in the post-mortem

CNS tissues from individuals who have died with FXTAS [99,123,135]. In addition to a lowered oxidative phosphorylation capacity, there is evidence for a deficit in mitochondrial import of nuclear-encoded mitochondrial proteins, and this latter deficit appears to be due to reduced loading of zinc into the metalloproteases that process proteins for import [123]. General mitochondrial dysfunction could explain the higher level of oxidative stress observed in CNS tissue [135]. Taken together, these observations would provide one explanation for the reduced neuronal viability, since neuronal cells are exquisitely sensitive to mitochondrial dysfunction due to their high metabolic demands. Using hippocampal neurons obtained from neonatal premutation KI mice, Kaplan et al. [99] were able to demonstrate that the premutation neurons possessed fewer mitochondria and that the mobility of these mitochondria was reduced. This form of dysfunction would be expected to have a strong negative impact on the growth of dendritic arbors as well as of synaptic function, consistent with the earlier observations of Chen et al. [26] of reduced dendritic growth for premutation (mouse) neurons.

Dysregulation of neuronal activity

Another form of dysregulation observed in the neonatal mice (cultured hippocampal neurons) is altered neuronal electrical activity, characterized by burst-type network firing behavior and altered Ca^{2+} regulation [26,24]; the latter is also observed in iPSC fibroblast-derived neurons from human premutation carriers [113]. The burst-type patterns of firing across networks of cultured mouse premutation neurons, likely related to the increased seizure activity in children who are premutation carriers, is completely reversed with either mGluR5 inhibitors, or the GABA$_A$ receptor positive modulator, allopregnanolone [24]. These observations are encouraging from the perspective of potential therapeutic intervention, since many of the drugs now in trials for fragile X syndrome may also prove efficacious for the premutation disorders.

Mouse models reveal early developmental phenotypes for premutation *Fmr1* alleles

One of the major unresolved questions regarding the diverse phenotypes among premutation carriers is whether there is a common underlying molecular mechanism. Specifically, does the RNA toxicity that appears to trigger the late-adult-onset neurodegenerative disorder, FXTAS, also initiate the early developmental, decidedly non-degenerative, domains of involvement (e.g., autism, seizures) and the ovarian dysfunction in carrier females? The most revealing observations thus far have come from mouse models in which the native CGG-repeat element (~9–11 repeats) has been replaced by a much larger repeat in the premutation range [17,165](Table 2). Two recent studies of premutation mice suggest that primary ovarian dysfunction could, indeed, be the result of an RNA-toxicity mechanism [82,118]; results that would be consistent with the infertility phenotype in women with expanded CGG-repeat alleles being restricted to the premutation range.

With regard to the neurodevelopmental phenotypes, the mouse models have been transformative, having provided substantial evidence that expression of the premutation CGG-repeat RNA leads to early molecular and behavioral phenotypes. Much of the work at the molecular level has involved studies of cultured neuronal and astrocytic cells derived from neonatal mice, where defects in both neuronal morphology (reduced dendritic growth; altered mitochondrial number and distribution) and electrical activity have been noted [24,26,99,129]. Moreover, Cunningham et al. [36] have demonstrated *in vivo* in mouse embryos that premutation alleles lead to impaired neocortical development, as reflected by both delayed neuronal maturation and altered neuronal migration. Thus, from the molecular perspective, the premutation influences CNS development and function at a very early stage,

and could therefore be causal in the developmental involvement, including the propensity for seizures, experienced by children who carry a premutation allele.

Young premutation (KI) mice have also demonstrated problems both in motor control/ coordination and in various cognitive functions. The mice display early (mild) deficits in both a ladder rung task designed to detect gait/coordination problems [89], and in a forelimb reaching task designed to probe forelimb coordination [41]. Perhaps the most exciting developments in the mouse studies have come in the study of specific domains of cognition that were designed to mirror deficits observed in humans. In a series of elegant studies, the premutation mice (mainly female heterozygotes) were shown to have deficits in spatiotemporal processing in which distinct domains became manifest at different times, and in a manner that also depended on the size of the CGG repeat [18,41,85,90,88]. These results are particularly significant in light of parallel studies in young women with the premutation who demonstrated both attention problems and deficits in magnitude estimation tasks [58]. Boys and young-adult men with the premutation had earlier been shown to have deficits in executive function, working memory, and attention tasks [34,33], which is similar to the findings of Moore et al. [121] in older adult carriers without FXTAS.

Developments in the pathology of FXTAS

Beyond the CNS

FXTAS had until recently been considered fundamentally to be a CNS disorder; with principal neuropathologic findings of intranuclear neuronal and astrocytic inclusions [18,41,59,60,85,90], as well as white matter disease and loss of brain volume [1,23,30,60,94,100,74–76,162] that point to subtle alterations in brain structure and white matter that may precede overt symptoms of the disorder [12,115]. However, an early indication that the pathology of FXTAS may extend beyond the CNS were the observations that intranuclear inclusions were present in both the anterior and posterior pituitary [61,117], and in testicular Leydig and myoid cells in two men who had died with FXTAS [61]. Gokden et al. [56] later reported the presence of intranuclear inclusions in broad distribution in the peripheral nervous system, including ganglion cells of the adrenal medulla, myenteric ganglia of the stomach, and subepicardial autonomic ganglion of the heart.

More recently, in a comprehensive analysis of the distribution of non-CNS inclusions, Hunsaker et al. [86] reported inclusions in many peripheral locations, including in numerous neuroendocrine and somatic organs, in premutation carriers who had died with FXTAS (Fig. 5). Importantly, they demonstrated a very similar distribution in the premutation KI mouse, indicating that the animal model may prove useful for the study of non-CNS aspects of the premutation disorders. Recognition of this broad, non-CNS distribution of inclusions is of importance for at least two reasons. First, the formation of morphologically similar inclusions, in an exclusively nuclear distribution and in a wide range of cell types, argues that the molecular mechanisms for inclusion formation are present in many types of non-neural cells. This is useful both for molecular studies in non-CNS tissues such as fibroblasts [4,55,123,135], and for the use of more convenient cell types to study the effects of various candidate therapeutic agents. Second, the distribution of inclusions may reflect non-CNS co-morbid features associated with, or preceding the onset of, FXTAS. Thus, the hypothyroidism in many women with FXTAS [29] could reflect a direct disease process within the thyroid. Similar arguments could also be made for peripheral neuropathy [114]; cardiac arrhythmias [67,94]; hypertension [29,69]; and various forms of autonomic dysfunction, including erectile dysfunction [61], sleep apnea [70], migraine headaches [6], and immune mediated disorders [166].

Beyond the premutation

One of the long-standing predictions of the RNA-toxicity model is that, strictly speaking, this pathogenic mechanism should not be limited to the premutation, but only to expanded alleles where there is significant, continued *FMR1* mRNA production. This prediction raises the possibility that those individuals who are mosaic for unmethylated, full mutation alleles [152–154] may retain an increased risk of developing features of FXTAS. Recently, intranuclear inclusions have been identified *post-mortem* in three full mutation males [87]. Although the inclusions were present at very low levels, as were mRNA levels, and none of the three cases displayed overt features of FXTAS; these cases demonstrate the possibility that some individuals may present with FXTAS even though they have alleles that lie within the full mutation range. Moreover, Loesch et al. [116] have described a male with an unmethylated, full-mutation allele, who presented with neurological (significant gait ataxia, intention tremor and parkinsonism), cognitive, and radiological (periventricular and middle cerebellar peduncle white matter disease) features supporting a diagnosis of definite FXTAS (diagnostic criteria: [94]). Several cases of FXTAS have also been reported for individuals with gray-zone alleles (45 to 54 CGG repeats), particularly in association with elevated *FMR1* mRNA levels [68,113]. Therefore, the range of CGG-repeat lengths giving rise to FXTAS must extend beyond the premutation range, albeit still in accord with the RNA-toxicity mechanism.

Conclusions

Our view of FXTAS has evolved rapidly over the past several years, as the data from animal models, large clinical cohorts, and more sophisticated imaging methods help to form a more detailed picture of the nature of the disorder. Although it is becoming increasingly evident that the initial triggering event for FXTAS involves the expanded CGG-repeat mRNA, what has been more surprising, from the animal models, is how early those events lead to dysregulation during development. This emerging picture of a unifying pathogenic mechanism—for neurodevelopment, for loss of fertility, and for the eventual neurodegenerative process leading to FXTAS—will radically change our thinking as to when, and by what means, to treat all of the premutation-associated disorders. Such a unifying concept is quite encouraging, since it suggests that therapeutic intervention for FXTAS could occur long before any symptoms emerge.

Despite the encouraging picture revealed through mechanistic unification, there is a lingering difficulty with our understanding of FXTAS and the other premutation-associated disorders; that is, we cannot predict who will develop any of the particular phenotypes. In the mouse models, nearly every animal displays abnormal mitochondrial function, and altered morphological and electrical properties of neurons; however, only a minority of children will develop behavioral or cognitive impairment, only ~20% of women will suffer FXPOI, and only about half of older males will experience the neurodegenerative phenotype. What we really need to understand is why some individuals develop these premutation disorders, whereas others with identical CGG repeats do not. It is likely, although not established, that part of the answer is to be found in genetic background effects, and with differences in intrinsic susceptibility. However, there are likely to be additional determinants, such as environmental effects (smoking, exposures to environmental toxins, etc.) that will help to determine who will or will not experience these disorders. Therefore, part of the task in understanding the pathogenesis of FXTAS is to identify those factors that determine penetrance; such knowledge will be critical for ultimate success in treatment.

Acknowledgments

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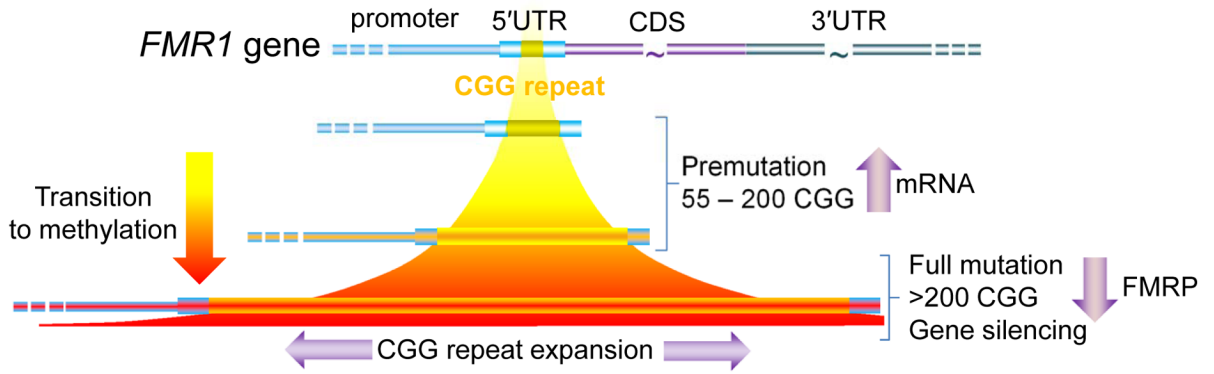
Fig. 1.
Schematic of the CGG-repeat element within the 5′ untranslated region (5′UTR) of the
FMR1 gene. The modal number of CGG repeats in the general population is approximately
30; 45–54 CGG repeats are generally referred to as "gray zone" alleles. Premutation alleles
are generally unmethylated (yellow); however, partially methylated alleles are observed near
the upper end of the premutation range (indicated by red shading). Full mutation alleles are
generally, although not always methylated. CDS, protein coding sequence.
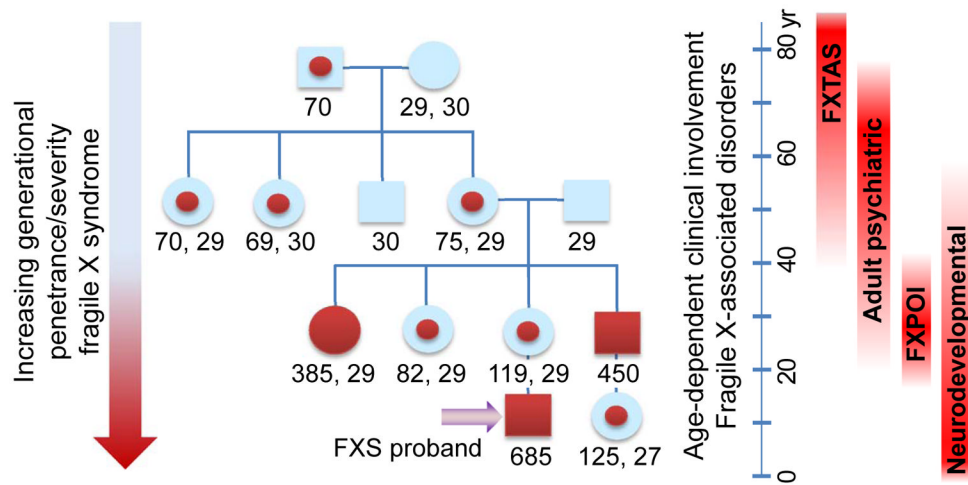
Fig. 2.
Representative fragile X pedigree displaying intergenerational shift from premutation to full mutation alleles, and consequent increase in disease penetrance and severity (genetic anticipation), due to CGG-repeat instability during transmission. Numbers below family members indicate CGG-repeat size (two alleles for females); red dots, premutation; solid red symbols, full mutation/fragile X syndrome. Premutation alleles are associated with at least four phenotypic domains, with typical age ranges as indicated to the right side of the figure.
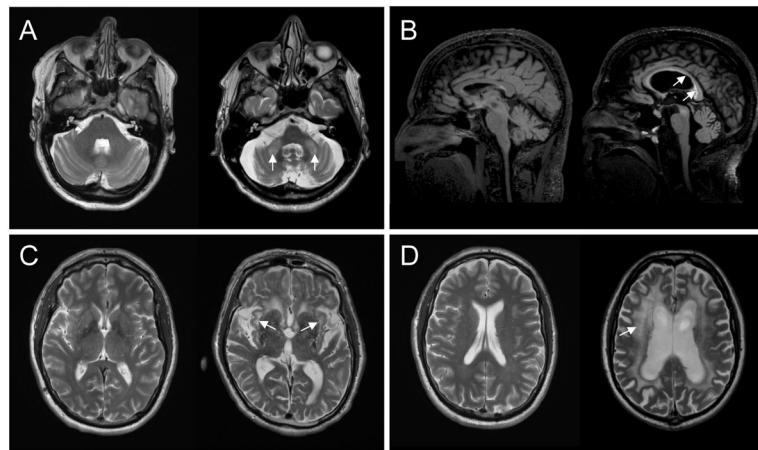
Fig. 3.
MRI images of white matter and structural abnormalities associated with FXTAS [1,3,23,30,162]. For each pair of images, a normal control (left) and FXTAS case (right) are represented. The white arrows indicate increased signal intensity on T2 turbo spin-echo sequences in the middle cerebellar peduncle (MCP) (**a**), sub-insular white matter (**c**), and cerebral white matter (**d**). Thinning and increased signal of the trunk and splenium of the corpus callosum (**b**; arrows) are from the T2 FLAIR sequence. Cases depicted in this figure are males, as follows: (**a**) 70 yr, FXTAS stage 3, 96 CGG repeats; (**b**) same as **a**; (**c**) 65 yr, stage 3, 91 CGG repeats; (**d**) 78 yr, stage 4, 116 CGG repeats. The control used for all images and comparisons was a 68-year-old man with 32 CGG repeats. Images kindly provided by Patrick Adams.
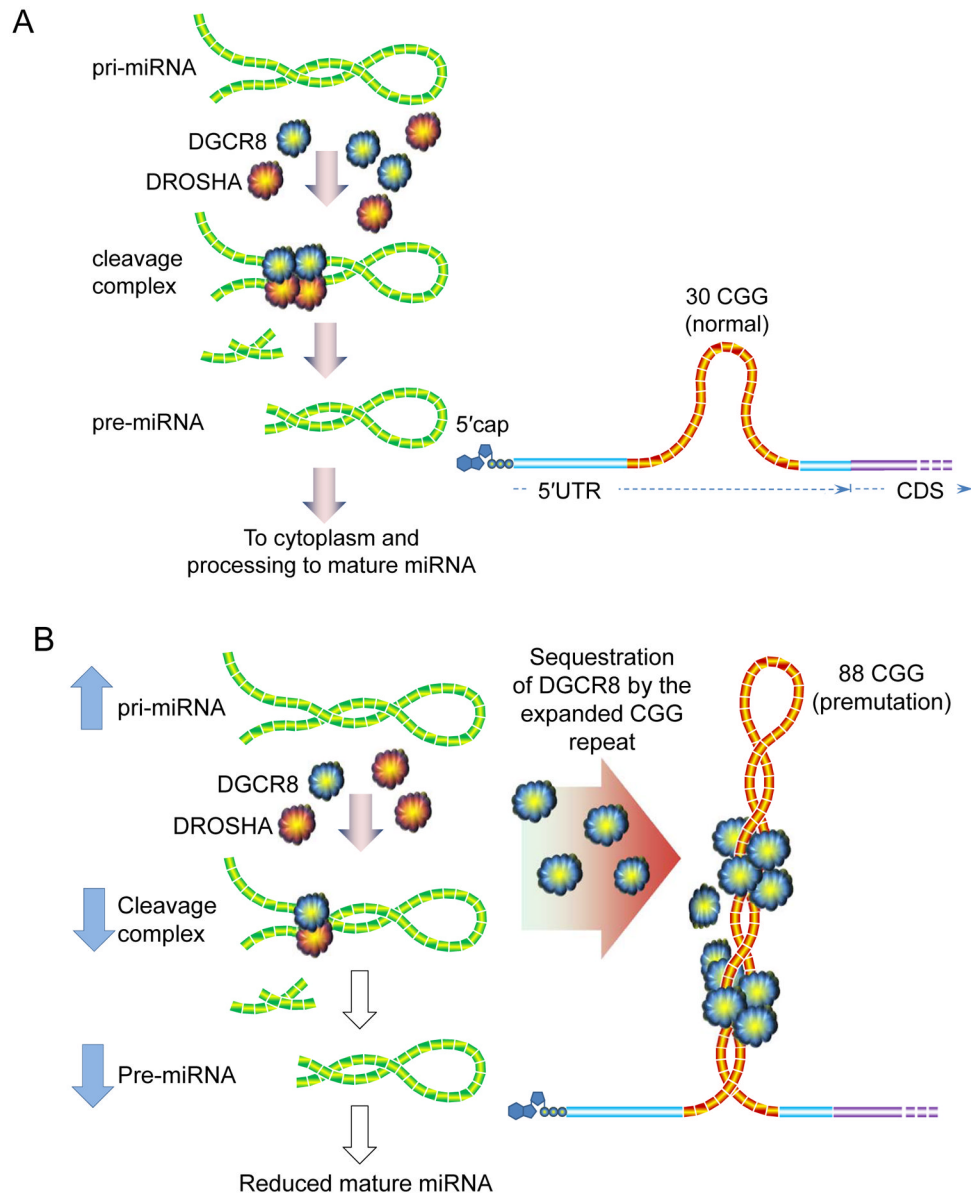
Fig. 4.
Schematic of the role of DGCR8 in the pathogenesis of FXTAS [139]. (**a**) DGCR8 binds to pri-miRNA in the nucleus, recruiting DROSHA (and other proteins) to form the "microprocessor" complex; binding of DGCR8 is cooperative and targets a specific (imperfect) helix region of the pri-miRNAs. Cleavage yields precursor miRNAs (pre-miRNAs), which exit the nucleus for further processing into mature miRNAs. Normal *FMR1* alleles do not interact appreciably with DGCR8. (**b**) Expanded CGG-repeat RNA forms stable helical stems, which recruit (sequester) DGCR8, thus preventing normal levels of pri-miRNA processing. Consequently, nuclear pri-miRNA levels are increased and mature, cytoplasmic miRNAs are decreased. The 88 CGG-repeat allele depicted in the figure is near the modal value for individuals with FXTAS.
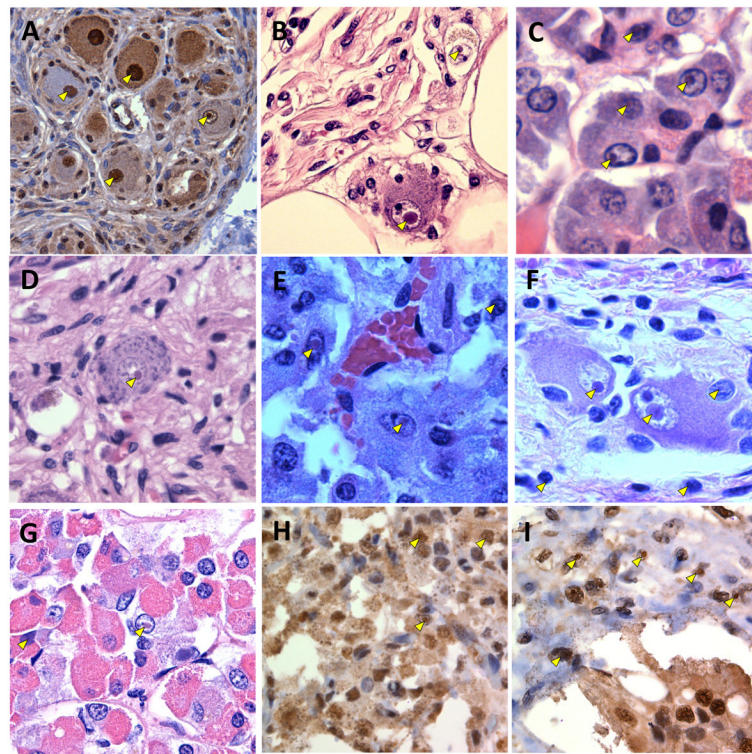
Fig. 5.
Representative examples of intranuclear inclusions in *post-mortem,* non-CNS tissues/organs
from FXTAS patients; for more details of the range of non-CNS tissues bearing inclusions
in both humans and premutation KI mice, see text and Table 2 of Hunsaker et al. [86]. For
each image, arrowheads point to inclusions. (**a**) Cardiomyocytes (ubiquitin; 400x); (**b**)
cardiac autonomic ganglion cell (H&E, 600x); (**c**) pancreas (ubiquitin, 1000x); (**d**) intestinal
wall (H&E, 400x); (**e**) adrenal medulla (H&E, 600x); (**f**) myenteric plexus, stomach (H&E,
400x); (**g**) anterior pituitary (H&E, 400x); (**h**) testicular Leydig cells (ubiquitin, 400x); (**i**)
renal distal tubule (ubiquitin, 400x). Images kindly provided by Claudia Greco.

Table 1

Summary of CNS pathology in FXTAS[a]

Inclusions	CNS pathology
Physical characteristics Exclusively intranuclear; identifiable with H&E or ubiquitin IHC; 2–5 μ, solitary, generally spherical; distinct from nucleolus	**Gray and white matter pathology** Cortical atrophy, patchy softening and loss of deep white matter, brainstem and pontine atrophy. Coexistence of Parkinson disease as evidenced by pale substantia nigra with concomitant Lewy bodies microscopically. White matter disease is distinct from that associated with vascular disease; broadly distributed in cerebrum, brainstem, and cerebellum. Variable degrees of spongiosis in subcortical and deep white matter; middle cerebellar peduncles, deep cerebellar white matter.
Location within cell types/CNS Identified in neurons and astrocytes within all regions of cerebral cortex; and within amygdaloid-hippocampal complex including dentate gyrus, ependymal cells, brain stem nuclei, cerebellum, and choroid plexus. Rare in microglial cells, not seen in oligodendroglial cells. Infrequent in neurons of pontine nuclei; more common in pontine astrocytes. Not observed within Betz cells of motor cortex; only rarely (single case [b], rare inclusions) within cerebellar Purkinje cells, occasionally in internal granular cells; not within motor neurons of spinal cord or oligodendrocytes. Strong correlation between inclusion counts and CGG-repeat size within the premutation range.	**Cellular pathology** Reactive astrocytes bearing enlarged, watery-appearing nuclei with inclusions in gray and white matter; axonal loss on Bielschowsky or neurofilament staining and myelin pallor on LFB-PAS staining. Glial cell loss of varying degree of severity and axonal swellings/spheroids. Mild to severe Purkinje cell loss, Bergmann gliosis.
Composition[c] More than 30 proteins identified by mass spectroscopy and IHC, revealing no dominant protein species; ubiquitinated proteins represent minor component and appear to be mono-ubiquitinated. Identified proteins include (among others): αB-crystallin, lamin A/C, various neurofilament proteins, myelin associated proteins, α-tubulin, GFAP, γH2AX, SAM68, DGCR8, DROSHA, MBNL1, hnRNP A2/B1, and poly-glycine-containing peptides. The following proteins of significance to neurodegenerative disorders have NOT been detected in the inclusions: α-synuclein, tau isoforms, polyglutamine peptides, FMRP, and CUGBP1. TDP 43 has not been reported to be in inclusions, and PURα is questionable (see: [139]). The inclusions also contain the *FMR1* mRNA (elements of 5′UTR, coding sequence and 3′UTR).	

[a]Case series for 11 males [59,60] and 8 females [150]

[b]Case 9, [59]

[c][59,60,93,96,139,140,156]

Table 2

Comparison of the neuropathology for two principal premutation knock-in mouse lines used to model FXTAS

Feature	NIH mouse[a]	Dutch mouse[a]
Mouse strain(s)	C57/BL6	C57/BL6J
Constructs[b]	Expanded CGG-repeat elements inserted into mouse 5′UTR; NIH construct retains more of the mouse sequence proximal to the CGG repeat **Dutch:** acggcN$_{13}$GGGGGCGTGCGGCAGCG_**(CGG)$_n$**CTGGGC*CTCGAG*cgcct **NIH:** taacgGCCCAAACGGCC **(CGG)$_n$** CGGCCCGGGCGGCCgacggc	
Inclusion formation	Inclusions present; morphologically and immunochemically similar to those in the Dutch mouse, although in much lower numbers	Inclusion numbers comparable to or exceeding those found in humans with FXTAS; broad distribution
***Fmr1* mRNA and FMRP**	Both mouse lines have comparable mRNA elevations; however, FMRP levels appear to be lower in the NIH mouse – as low as 15% of WT in some brain regions	
Cerebellar pathology	Evidence of Purkinje cell pathology: abnormal calbindin staining, swollen axons and torpedoes, Purkinje cell dropout	No evidence of Purkinje cell dropout; no Bergmann gliosis, though inclusions are in Bergmann glia
Prenatal neocortical development	Not examined	Embryonic premutation mice display migration defects in the neocortex and altered expression of neuronal lineage markers
Dendrite and spine morphology	Reduced complexity of dendritic branching patterns and increased spine density	Reduced complexity of dendritic branching patterns but no changes in spine density
Anxiety	Lower anxiety (Open field)	Increased anxiety (Plus maze)

[a] Designations follow Todd et al. [157]. (NIH mouse: [47,82,129]; Dutch mouse: [7,13,17,22,36,137,164,165]; general review: [84])

[b] Human reference, GenBank: L19476.1; NIH and Dutch constructs following summary in Todd et al. [157]. For the Dutch mouse, upper case letters indicates identity with the human sequence (30 nt 5′, and 12 nt 3′ of the CGG repeat); lower case, mouse sequence. Note that the corresponding sequence in Todd et al. [157] contains an extra C adjacent to the CGG repeat (removed: underscore, gray highlight). Red italics indicate a *Xho* I site. For the NIH mouse, lower case represents mouse sequence; additional, inserted non-mouse sequence is underlined; double underline indicates *Sfi*I sites. Again, an extra C adjacent to the CGG repeat (underscore, gray highlight) in Todd et al. (2013) has been removed. The gray-highlighted "GG" dinucleotide in the NIH sequence is subject to some uncertainty (GG vs CC).

[c] The TAA stop codon (red) prevents upstream translation in the +1 codon frame