

DISEASES OF UNSTABLE REPEAT EXPANSION: MECHANISMS AND COMMON PRINCIPLES

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Abstract | The list of developmental and degenerative diseases that are caused by expansion of unstable repeats continues to grow, and is now approaching 20 disorders. The pathogenic mechanisms that underlie these disorders involve either loss of protein function or gain of function at the protein or RNA level. Common themes have emerged within and between these different classes of disease; for example, among disorders that are caused by gain-of-function mechanisms, altered protein conformations are central to pathogenesis, leading to changes in protein activity or abundance. In all these diseases, the context of the expanded repeat and the abundance, subcellular localization and interactions of the proteins and RNAs that are affected have key roles in disease-specific phenotypes.

Pathogenic mutations that affect nucleotide repeats were first described in 1991 when the causative mutations in **fragile X syndrome** (FRAXA; also known as fragile site mental retardation 1, FMR1)¹ and **spinal and bulbar muscular atrophy** (SBMA)² were identified as trinucleotide repeat expansions. The list of neurological disorders that are caused by unstable repeats has increased to include not only trinucleotide repeats, but also tetranucleotide³ and pentanucleotide repeats⁴, leading to designation of this class of diseases as disorders of unstable repeat expansion. Here we explore the pathogenic mechanisms in diverse classes of such diseases: diseases that are caused by expansions of non-coding repeats, which result in loss of protein function; those that are caused by expansions of coding repeats, which result in altered protein function; and a recently described class of diseases that are caused by expansions of non-coding repeats, which result in altered RNA function.

Within the group of diseases that are caused by a loss-of-function mechanism, the normal function of the disease-associated gene product provides insights into pathogenesis. Decreased or abolished expression of molecules that are involved in translational control

(FRAXA), signalling (FRAXE), and mitochondrial function (**Friedreich ataxia** (FRDA)) have widespread pathogenic effects, as might be expected. In diseases that are caused by glutamine-encoding repeats — polyglutamine diseases — the same toxic moiety is shared between nine different disease proteins, but these diseases are clinically distinct. Differences are determined not only by the repeat length, but also by the intrinsic function of the disease-causing protein. Finally, in diseases caused by expanded non-coding repeats that are mediated at the RNA level, there is emerging evidence that pathogenic RNA species induce aberrant RNA–protein interactions, leading to neuronal and systemic dysfunction. Our critical examination of the pathogenesis of this diverse group of disorders discusses these similarities within classes, and some themes that are shared between classes. We discuss molecular mechanisms in the context of these common principles, with the hope of providing insights into areas of opportunity for therapeutic intervention.

Diseases caused by loss of protein function

Fragile X syndrome. FRAXA is one of the most common forms of inherited mental retardation^{5,6} (TABLE 1).

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Table 1 | **Inheritance patterns and clinical features of unstable repeat expansion disorders**

Disease	Inheritance	OMIM number	Main clinical features
FRDA	AR	229300	Sensory ataxia, cardiomyopathy, diabetes
FRAXA	XL	309550	Mental retardation, macroorchidism, connective tissue dysplasia, attentional and behavioural abnormalities
FRAXE	XL	309548	Mild mental retardation or learning impairment
SCA1	AD	164400	Ataxia, slurred speech, spasticity, cognitive impairment
SCA2	AD	183090	Ataxia, slow saccades, decreased reflexes, polyneuropathy, motor neuropathy, infantile variant
SCA3	AD	109150	Ataxia, parkinsonism, severe spasticity
SCA6	AD	183086	Ataxia, dysarthria, nystagmus, tremor
SCA7	AD	164500	Ataxia, retinal degeneration, cardiac involvement in infantile variant
SCA17	AD	607136	Ataxia, behavioural changes or psychosis, intellectual deterioration, seizures
DRPLA	AD	125370	Ataxia, epilepsy, choreoathetosis, dementia
SBMA	XL	313200	Motor weakness, swallowing difficulty, gynecomastia, hypogonadism
HD	AD	143100	Severe movement abnormalities, chorea, dystonia, cognitive decline, psychiatric features
DM1	AD	160900	Myotonia, weakness, wasting, cardiac conduction abnormalities, testicular atrophy, insulin resistance, cataracts, congenital form, potentially severe CNS involvement with mental retardation
DM2	AD	602668	Similar to DM1*, no congenital form
FXTAS	XL	309550	Tremor/ataxia, parkinsonism, cognitive deficits
SCA8	AD	608768	Ataxia, slurred speech, nystagmus
SCA12	AD	604326	Tremor, ataxia, dementia
SCA10	AD	603516	Ataxia and seizures
HDL2	AD	606438	Similar to HD

*Dystrophia myotonica 1 (DM1) differs from DM2 in that it also has a congenital form, and potentially severe CNS involvement with mental retardation. AD, autosomal dominant; AR, autosomal recessive; DRPLA, dentatorubral-pallidoluysian atrophy; FRDA, Friedreich ataxia; FXTAS, Fragile X tremor/ataxia syndrome; HD, Huntington disease; HDL2, Huntington disease-like 2; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; XL, X-linked.

POLYRIBOSOME

A string of 80S ribosomes that are bound to an mRNA molecule.

DENDRITIC SPINES

Mushroom-shaped structures on neuronal dendrites that receive synaptic input and have postsynaptic densities. Changes in spine shape are thought to be important for modulating synaptic strength.

LONG-TERM DEPRESSION

A long-lasting decrease in synaptic strength to below baseline levels. It is generally induced in the hippocampus by repetitive low-frequency stimulation. It is thought to result from changes in postsynaptic receptor density, although changes in presynaptic release might also have a role.

GROUP 1 mGluRs

Postsynaptic G-protein coupled receptors for the excitatory neurotransmitter glutamate. These receptors have many roles in synaptic plasticity.

SYNAPTIC PRUNING

The selective elimination of weak synapses during brain development.

SYNAPTIC PLASTICITY

The capacity for alterations in synaptic connections between neurons, including changes in the nature, strength or number of interneuronal connections, which subserves learning and memory.

Most cases are caused by expansion of a CGG repeat in the 5' UTR of the fragile X mental retardation 1 gene (*FMR1*), resulting in transcriptional silencing and loss of the protein product of *FMR1*, FMRP⁷ (FIG. 1a; TABLE 2). Tracts of more than 200 CGG repeats (full mutation) give rise to FRAXA, whereas intermediate alleles of 60–200 repeats (premutation), initially believed to be benign, have recently been recognized to cause a clinically distinct syndrome, fragile X tremor/ataxia syndrome (FXTAS)⁸ (see below).

FMRP is generally cytoplasmic, although it can shuttle between the nucleus and cytoplasm. It is enriched in neurons and contains three characteristic RNA-binding domains: two KH domains (KH1 and KH2) and an RGG box. FMRP associates with POLYRIBOSOMES in an RNA-dependent manner as part of a large messenger ribonucleoprotein (mRNP) particle⁹, which indicates a role in translational regulation of its RNA targets. Indeed, FMRP suppresses translation both *in vitro* and *in vivo*^{10,11}, and a point mutation in the KH2 domain that perturbs its association with polyribosomes causes a severe FRAXA phenotype^{12,13}.

FMRP and its mRNA also localize to DENDRITIC SPINES, where FMRP associates with polyribosomes and is upregulated in response to stimulation by metabotropic glutamate receptors (mGluRs)¹⁴. FMRP could therefore have an activity-dependent role in the localization and translation of specific RNA targets at synapses. *Fmr1*^{-/-} mice show subtle behavioural abnormalities and selective enhancement of mGluR-dependent LONG-TERM DEPRESSION (LTD)¹⁵. The function of FMRP in translational repression is thought to balance the stimulatory effects of GROUP 1 mGluRs. The absence of FMRP results in over-amplification of the mGluR response and increased LTD¹⁴. These alterations in dendritic function are coupled to morphological abnormalities: FRAXA patients have abnormally long, immature dendritic spines¹⁶, which are present at an increased density, indicating a potential role for FMRP in synaptic maturation and PRUNING (FIG. 1b). Together, such alterations in synaptic development and activity-dependent SYNAPTIC PLASTICITY could underlie many of the features of FRAXA. This is supported by recent studies in a *Drosophila* model of the disease, which showed rescue of neuronal and behavioural phenotypes on administration of mGluR antagonists¹⁷.

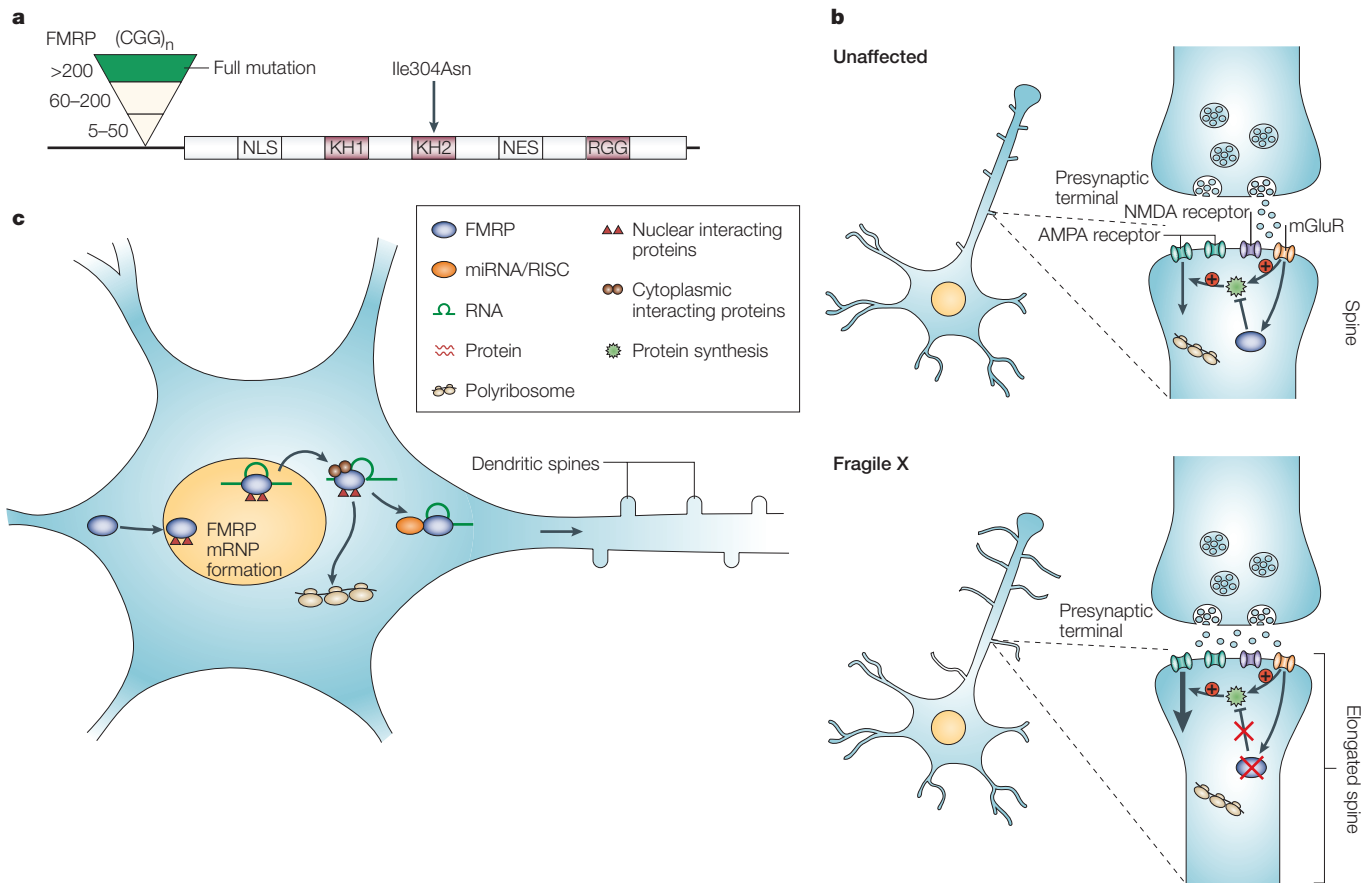


Figure 1 | Mechanisms of pathogenesis in fragile X syndrome. Expansion of CCG repeats in the fragile X mental retardation 1 (*FMR1*) gene that encodes FMRP underlies fragile X syndrome (FRAXA). Repeats that contain >200 copies (full mutation) lead to loss of FMRP expression. FMRP contains two domains that bind RNA: the KH2 domain and the RGG box. The Ile304Asn mutation in the KH2 domain, which prevents FMRP from binding targets that contain the kissing complex motif, gives rise to a severe mental retardation phenotype. **a** | Abnormal dendritic spine morphology in patients with FRAXA. An increased density of long, immature dendritic spines indicates that FMRP has a role in synaptic maturation and pruning, possibly through its regulation of gene products that are involved in synaptic development. FMRP might also have a regulatory role in activity-dependent translation at the synapse. Stimulation of postsynaptic metabotropic glutamate receptors (mGluRs) results in increased protein synthesis and subsequent internalization of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, which is important in the expression of long-term depression. FMRP, which is also upregulated by mGluRs, serves to dampen this process. The absence of FMRP in FRAXA results in over-amplification of this response. **b** | FMRP modulates the translation of its targets, probably through its association with the RNA-induced silencing complex (RISC). FMRP is transported to dendritic spines, together with its associated RNAs and proteins. mRNP, messenger ribonucleoprotein particle; NES, nuclear export signal; NMDA, *N*-methyl-D-aspartate; NLS, nuclear localization signal.

G QUARTET
A secondary structure observed in G-rich RNA in which four consecutive guanosine residues bind to each other to form an intramolecular stem-loop structure.

RNA-INDUCED SILENCING COMPLEX
A multi-protein complex that functions in RNAi pathways to mediate the cleavage of target RNAs.

ARGONAUTE PROTEINS
A family of proteins that are essential for diverse RNA-silencing pathways.

DICER PROTEINS
A highly conserved family of RNaseIII enzymes that mediate dsRNA cleavage. This produces the small RNAs that direct target silencing in RNAi pathways.

MICRORNA
A form of ssRNA typically 20–25 nucleotides long that is thought to regulate the expression of other genes, either through inhibiting protein translation or degrading a target mRNA transcript through a process that is similar to RNA interference.

Much effort has been put into identifying the mRNA targets of FMRP^{18–20}. Candidate targets are involved in cytoskeletal structure, synaptic transmission and neuronal maturation, including the mRNA that encodes microtubule-associated protein 1B (**MAP1B**) — a protein that has an important role in microtubule stability. Targets of *Drosophila melanogaster* FMR1 include *futsch*, a MAP1B homologue²¹, and the *rac1* and *pickpocket* mRNAs, which are involved in synaptic function and development^{22,23}. There has also been interest in determining how FMRP interacts with its mRNA targets and whether a common RNA motif is involved. Darnell *et al.* found that FMRP binds through its RGG box to a class of RNAs that contain G QUARTETS¹⁹. However, a more recent study provided evidence that the KH2 domain of FMRP mediates its association with polyribosomes in neurons through an

RNA target motif termed the FMRP kissing complex, which indicates the involvement of more than one class of RNA target motif²⁴.

Work in flies and mammals indicates that the RNAi pathway has a key role in the regulation of translation by FMRP^{25–27}. FMR1 associates with Argonaute 2 (**AGO2**) and the RNA-INDUCED SILENCING COMPLEX (RISC) — two integral components of the RNAi machinery^{25,26}. Furthermore, FMRP interacts with the mammalian ARGONAUTE PROTEIN EIF2C2 and associates with DICER PROTEIN activity²⁷. One favoured model is that FMRP binds specific RNA ligands and subsequent recruitment of RISC and MICRORNAs (miRNAs) facilitates selective translational control of these targets²⁸ (FIG. 1c). The future identification of other FMRP targets at the synapse is needed to improve our understanding of the molecular pathogenesis of FRAXA.

Table 2 | **Molecular features of unstable repeat expansion disorders**

Disease	Mutation/ repeat unit	Gene name (protein product)	Putative function	Normal repeat length	Pathogenic repeat length
Diseases that are caused by loss of protein function					
FRDA	(GAA) _n	<i>FRDA</i> (frataxin)	Mitochondrial iron metabolism	6–32	200–1,700
FRAXA	(CGG) _n	<i>FMR1</i> (FMRP)	Translational regulation	6–60	>200 (full mutation)
FRAXE	(CCG) _n	<i>FMR2</i> (FMR2)	Transcription?	4–39	200–900
Diseases that are caused by altered protein function					
SCA1	(CAG) _n	<i>SCA1</i> (ataxin 1)	Transcription	6–39	40–82
SCA2	(CAG) _n	<i>SCA2</i> (ataxin 2)	RNA metabolism	15–24	32–200
SCA3 (MJD)	(CAG) _n	<i>SCA3</i> (ataxin 3)	De-ubiquitylating activity	13–36	61–84
SCA6	(CAG) _n	<i>CACNA1A</i> (<i>CACNA1_A</i>)	P/Q-type α 1A calcium channel subunit	4–20	20–29
SCA7	(CAG) _n	<i>SCA7</i> (ataxin 7)	Transcription	4–35	37–306
SCA17	(CAG) _n	<i>SCA17</i> (TBP)	Transcription	25–42	47–63
DRPLA	(CAG) _n	<i>DRPLA</i> (atrophin 1)	Transcription	7–34	49–88
SBMA	(CAG) _n	<i>AR</i> (androgen receptor)	Steroid-hormone receptor	9–36	38–62
HD	(CAG) _n	<i>HD</i> (huntingtin)	Signalling, transport, transcription	11–34	40–121
Diseases that are caused by altered RNA function					
DM1	(CTG) _n	<i>DMPK</i> (DMPK)	RNA-mediated	5–37	50–1,000
DM2	(CCTG) _n	<i>ZNF9</i> (ZNF9)	RNA-mediated	10–26	75–11,000
FXTAS	(CGG) _n	<i>FMR1</i> (FMRP)	RNA-mediated	6–60	60–200 (premutation)
Diseases of unknown pathogenic mechanism(s)					
SCA8	(CTG) _n	<i>SCA8</i> (transcribed/untranslated)	Unknown	16–34	>74
SCA10	(ATTCT) _n	Unknown	Unknown	10–20	500–4,500
SCA12	(CAG) _n	<i>PPP2R2B</i> (<i>PPP2R2B</i>)	Phosphatase regulation	7–45	55–78
HDL2	(CTG) _n	<i>JPH3</i> (junctophilin 3)	PM/ER junction protein	7–28	66–78

CACNA1A, calcium channel, voltage-dependent, P/Q type, α 1A subunit; *DMPK*, dystrophin myotonia protein kinase; *DRPLA*, dentatorubral-pallidolusian atrophy; *DM*, dystrophin myotonia; *ER*, endoplasmic reticulum; *FRDA*, Friedreich ataxia; *FMRP*, fragile X mental retardation protein; *FMR1*, fragile X mental retardation 1; *FMR2*, fragile X mental retardation 2; *FXTAS*, fragile X tremor/ataxia syndrome; *HD*, Huntington disease; *HDL2*, Huntington disease-like 2; *MJD*, Machado–Joseph disease; *PM*, plasma membrane; *PPP2R2B*, protein phosphatase 2 (formerly 2A) regulatory subunit B; *SBMA*, spinal and bulbar muscular atrophy; *SCA*, spinocerebellar ataxia; *TBP*, TATA box binding protein; *ZNF9*, zinc-finger protein 9.

CpG ISLANDS
Sequences of at least 200 bp that contain both a GC content that is greater than 50% and a high CpG frequency.

FERRITIN
An iron-storage protein.

ATAXIA
The inability to coordinate movement.

ACONITASE
An enzyme that catalyses the reversible hydration of *cis*-aconitase to yield citrate or isocitrate. Aconitases are involved in the citric acid cycle.

IRON-SULPHUR (FE-S) CLUSTERS
Co-factor-like species that are common in nature and are involved in many cellular processes, such as electron transfer, catalysis, gene regulation and the sensing of iron and oxygen.

Fragile X syndrome E (FRAXE). FRAXE patients have variable, subtle phenotypes that include mild mental retardation and learning difficulties²⁹ (TABLE 1). Expansion of a CCG repeat in the 5' UTR of the fragile X mental retardation 2 (*FMR2*) gene, which leads to hypermethylation of the CpG ISLAND and transcriptional silencing^{30–32}, underlies FRAXE (TABLE 2). *FMR2* is a member of a family of serine-rich and proline-rich proteins that are known or potential transcription factors^{33–35}. Characterization of *Fmr2*^{-/-} mice provided evidence for roles of *FMR2* in regulating nervous system development and differentiation, sensory perception, synaptic plasticity, and memory³⁶. A *D. melanogaster* *FMR2* orthologue,

lilliputian, is essential for *D. melanogaster* embryogenesis and is involved in the transforming growth factor β 1 (TGFB), mitogen-activated protein kinase (MAPK) and phosphoinositide 3 kinase (PI3K) signalling pathways^{37,38}. Defects in the MAPK pathway are associated with X-linked mental retardation syndromes, indicating that *FMR2* might also function in these pathways in mammals.

Friedreich ataxia. *FRDA*, the most common inherited ATAXIA, is caused by expansion of a GAA repeat in the first intron of *FRDA* (also known as *FXN*) (TABLES 1,2). This gene encodes frataxin³⁹, a highly conserved protein that associates with the inner mitochondrial

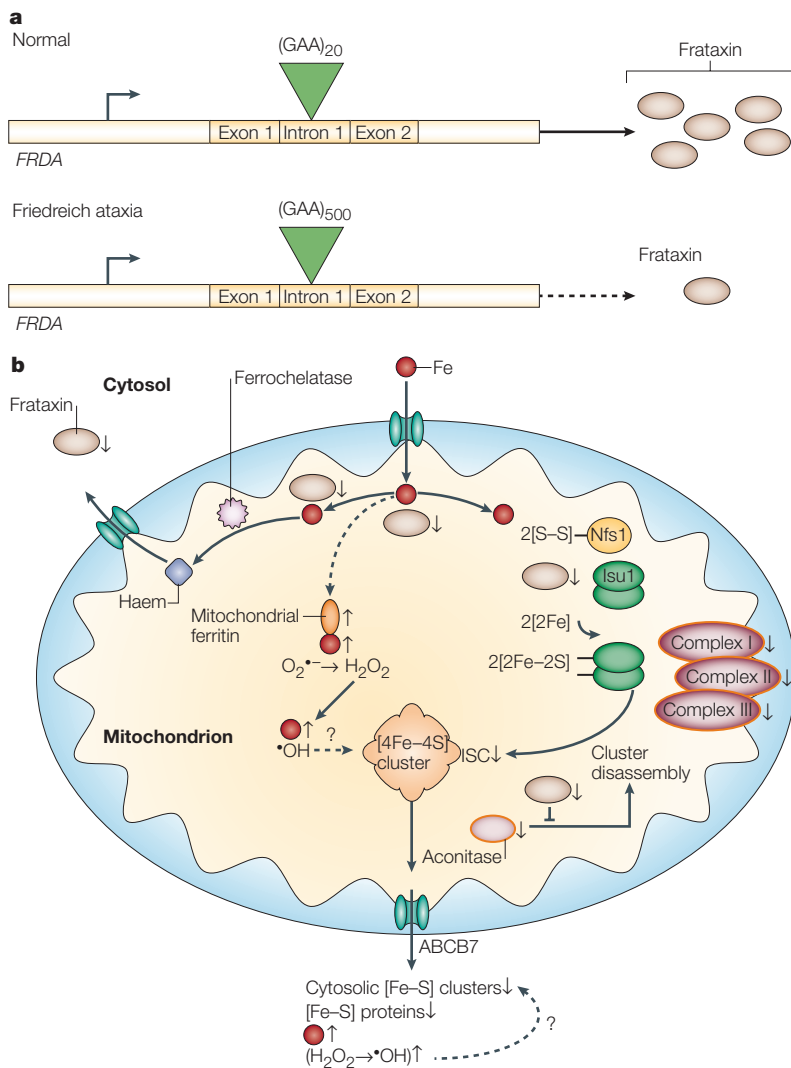


Figure 2 | Molecular and biochemical basis of Friedreich ataxia. **a** | In Friedreich ataxia (FRDA), GAA-repeat expansion in the first intron of *FRDA* results in decreased levels of frataxin owing to inhibition of transcriptional elongation. Most patients have expansions in both alleles. The longer the repeats, the lower the level of frataxin and the more severe the phenotype. **b** | Alterations in mitochondrial biochemistry that are associated with reduced frataxin levels in FRDA. Frataxin is a highly conserved nuclear-encoded mitochondrial protein for which specific functions are unknown. The proposed roles include as an iron-binding protein (carrying out a function as a mitochondrial FERRITIN); the protection and synthesis of Fe-S clusters (in yeast this is in conjunction with Nfs1, which supplies sulphur, and Isu1, which functions as a scaffold); in haem metabolism as a binding partner for ferrochelatase; or as a metabolic switch between haem metabolism and Fe-S cluster synthesis. In FRDA, when frataxin levels are low, Fe-S cluster containing proteins, such as respiratory transport complexes I-III and aconitase, are reduced. Cytoplasmic proteins that contain Fe-S clusters can also be affected. The accumulation of iron in the mitochondria leads to free-radical generation; this could feedback to further decrease the levels of Fe-S clusters, which are sensitive to the oxidative state. ABCB7, ATP-binding cassette, sub-family B, member 7 (ABC transporter 7 protein); ISC, iron-sulphur cluster.

membrane⁴⁰. Repeat expansion reduces *FXN* expression owing to inhibition of transcriptional elongation (FIG. 2a).

Studies in yeast, mice and patients have provided further insights into FRDA pathogenesis. Deletion of the yeast frataxin homologue *YFH1* results in accumulation of iron in mitochondria, increased sensitivity to

oxidative stress and diminished activity of mitochondrial proteins such as ACONITASE, which contain IRON-SULPHUR (FE-S) CLUSTERS^{41,42}. Currently, the most convincing data indicate a role of frataxin in haem biosynthesis and formation of Fe-S clusters⁴³⁻⁴⁶. Levels of mitochondrial iron are significantly elevated in fibroblasts from patients with FRDA⁴⁷, and tissues from these patients show iron deposits⁴⁸ and a generalized decrease in the activity of proteins that contain Fe-S clusters⁴⁹.

Frda^{-/-} mice die in embryogenesis without showing iron accumulation⁵⁰. This indicates that frataxin has an essential role in development, and that the less severe phenotype observed in patients who have FRDA is due to residual frataxin expression. Conditional frataxin-deficient mouse models recapitulate the main pathological features of the disease and show deficiency of Fe-S-containing enzymes, including respiratory transport chain complexes I-III and aconitase⁴⁰. Importantly, mitochondrial iron accumulation only occurs considerably later than the onset of pathology and changes in Fe-S-dependent enzyme activity⁵¹, indicating that this cannot be the causative pathological mechanism. According to one model, severely reduced frataxin levels result in decreased biosynthesis of proteins that contain Fe-S clusters. This leads to decreased production of cellular energy, which can trigger free-radical generation. Mitochondrial iron accumulation, which is probably a secondary event, contributes to the formation of free radicals, which might further reduce the levels of oxidative-sensitive Fe-S cluster proteins and cause increased cellular damage⁵² (FIG. 2b).

A role for oxidative stress in FRDA pathogenesis is supported by several lines of evidence. These include the increased sensitivity of *yfh1* yeast strains and patient fibroblasts to oxidative stress^{42,53}, the impaired response of patient fibroblasts to oxidative challenge⁵⁴ and increased levels of oxidative stress markers in patient samples⁵⁵. However, a recent study challenges this long-standing hypothesis, suggesting that oxidative stress has only a minor role in FRDA pathophysiology⁵⁶. This is based on the lack of improvement of cardiomyopathy in a mouse FRDA model in response to increasing antioxidant defences⁵⁶. Further studies are needed to resolve this controversy and to define the physiological role of frataxin.

Underlying principles in FRAXA, FRAXE and FRDA.

The different trinucleotide repeat expansions in FRAXA, FRAXE and FRDA mediate pathogenesis by transcriptional silencing, effectively resulting in loss of protein function. In each case, repeat length correlates with disease severity, with larger repeats resulting in greater transcriptional silencing. Loss of FMRP and FMR2 have widespread effects that interfere with neuronal development and result in cognitive deficits, whereas frataxin's mitochondrial function highlights the sensitivity of neurons and other cell types to the activity of proteins that contain Fe-S clusters. Therefore, the normal functions of the disease proteins in this class of disorders are central to the underlying

pathogenic mechanisms and clinical phenotypes, and should also be a key consideration in the development of targeted therapies.

Disorders caused by gain of function

The polyglutamine diseases constitute a class of nine genetically distinct, gain-of-function disorders that are caused by expansion of a translated CAG repeat. These include **Huntington disease** (HD), **dentatorubral-pallidoluysian atrophy** (DRPLA), SBMA and **spino-cerebellar ataxia 1, 2, 3, 6, 7 and 17** (SCA1/2/3/6/7/17) (REFS 57,58) (TABLE 2). Although the disease-causing proteins are expressed widely in the CNS, specific populations of neurons are vulnerable in each disease, resulting in characteristic patterns of neurodegeneration⁵⁷ and clinical features (TABLE 1). Human and mouse genetic data established that these diseases result mainly from a gain-of-function mechanism⁵⁷. Disease is recapitulated in transgenic and knockin mouse models that express the full-length protein with expanded repeats^{59,60}, whereas knockout models do not develop the disease phenotype⁶¹. Although the expansion might interfere with the normal function of the host protein^{62,63}, it is not yet clear whether this contributes to pathogenesis.

The role of protein aggregates in pathology. Accumulation of the mutant protein to form insoluble aggregates is a common feature of polyglutamine diseases; such accumulation is nuclear for SCA1, SCA7, and SCA17, cytoplasmic for SCA2 and SCA6, and both nuclear and cytoplasmic for HD, DRPLA, SBMA and SCA3. Aggregates are thought to be sequestered in inclusions that also include molecular chaperones and components of the UBIQUITIN-PROTEASOME SYSTEM (UPS), which indicates that the expanded polyglutamine tract alters protein conformation and recruits cellular defence mechanisms against protein misfolding and aggregation⁶⁴. This is supported by the finding that overexpression of molecular chaperones in fly and mouse models suppresses toxicity^{65,66}.

Accumulation of disease proteins has been proposed to impair the UPS⁶⁴. Eukaryotic proteasomes fail to degrade polyglutamine repeats *in vitro*⁶⁷. Although *in vitro* cell-based studies that use overexpression of truncated polyglutamine proteins are consistent with UPS impairment^{68,69}, several other *in vitro* studies that use both cell and mouse models argue against this^{70–72}. In a recent study, a ubiquitin–GFP reporter system in a knockin mouse model of SCA7 was used to provide the first *in vivo* examination of UPS activity in the context of a vulnerable population of cells⁷³. The reporter consisted of an N-terminal ubiquitin mutant in frame with GFP, targeting GFP for rapid degradation by the UPS⁷³. No evidence of UPS impairment was obtained.

Interestingly, the same study also indicated that the extent of pathology in a vulnerable population of cells was inversely related to the formation of ataxin 7 (ATXN7) nuclear inclusions, indicating a protective role of inclusions in disease⁷³. This theory is gaining increasing support. Although numerous cell-based and

post-mortem studies have produced inconsistent conclusions, arguing for either a protective or a deleterious role of inclusions, the relationship between inclusions and pathogenesis is clear in several disease models. These include SCA1 and SCA7 knockin mice and a cell-based assay^{59,60,73,74}. In these models, cells that form inclusions survive longer, whereas vulnerable cells, such as PURKINJE CELLS, are among the last to form inclusions^{59,60,73,74}. In addition, recent findings from an HD transgenic mouse model expressing the N-terminal fragment of huntingtin provide *in vivo* evidence for a lack of association between inclusions and neurodegeneration or dysfunction⁷⁵. Furthermore, inclusions of N-terminal truncated huntingtin have been shown to indirectly enhance AUTOPHAGY, a mechanism that mediates clearance of mutant huntingtin and decreases cellular toxicity in fly and mouse models of HD⁷⁶.

Whether considering the aggregated form of the mutant protein or a toxic monomeric species, changes in conformation could allow the mutant protein to recruit normal cellular proteins through a series of aberrant interactions. Some interactions could be inappropriately enhanced, whereas others might be lost or unchanged (FIG. 3a). In some cases these interactions might be mediated by the ability of the polyglutamine domain to recruit other normal proteins through non-pathogenic polyglutamine stretches found in these molecules⁷⁷. However, increasing evidence indicates that interactions mediated by many other domains of the mutant protein might be important determinants of pathogenesis⁷⁸. The cascade of pathogenic protein–protein interactions that are initiated could affect many downstream processes, leading to neuronal dysfunction and ultimately death (FIG. 3).

Transcriptional and post-transcriptional mechanisms.

The nuclear localization of the mutant protein is crucial for the pathogenesis of most polyglutamine diseases⁷⁹. In addition, transcriptional changes often precede phenotypic onset in some disease models^{60,80}. These two lines of evidence support transcriptional dysregulation as a common theme in pathogenesis. Such dysregulation might be mediated by several mechanisms. Polyglutamine proteins interact with numerous transcriptional regulators including CREB-binding protein (CBP, also known as CREBBP), TAF_{II}130 (also known as TBP-associated factor 4 (TAF4)), SP1 (Sp1 transcription factor) and tumour protein 53 (TP53; also known as p53) (REFS 81–85), some of which have been found in polyglutamine inclusions⁸⁶. However, these findings require new interpretation given the evidence for a protective role of inclusions in disease. The lack of CBP recruitment to inclusions in some polyglutamine disease models^{60,87}, and the fact that transcriptional changes often occur before inclusions are detectable⁶⁰, highlight a second mode of polyglutamine toxicity — one that stems from the soluble, non-aggregated form of the protein⁸³. Recent *in vitro* work suggests that conformational changes of soluble fragments of polyglutamine proteins facilitate their interaction with

UBIQUITIN-PROTEASOME SYSTEM

A key pathway that is involved in the degradation of intracellular proteins, especially of short-lived regulatory proteins. The system uses an enzymatic cascade that involves conjugation of multiple ubiquitin moieties to a protein substrate and degradation of the tagged protein by the 26S proteasome complex.

PURKINJE CELLS

The neurons that convey the output signals of the cerebellar cortex.

AUTOPHAGY

An intracellular pathway that leads to bulk protein degradation and involves the sequestering of cytosol into vesicles for delivery to a degradative organelle.

AXOPLASM

The intracellular fluid of the axon that facilitates vesicle transport.

CASPASE

One of a family of proteases, activated by proteolytic cleavage, that have an important role in apoptosis and inflammation.

CALPAINS

A family of highly conserved and widely expressed cysteine proteases that are activated by a rise in intracellular calcium levels. They have an important role in apoptosis and in modulating intracellular signals in response to stressful conditions.

SUMOYLATION

The post-translational modification of proteins that involves the covalent attachment of small ubiquitin-like modifier (SUMO) and regulates the interactions of those proteins with other macromolecules.

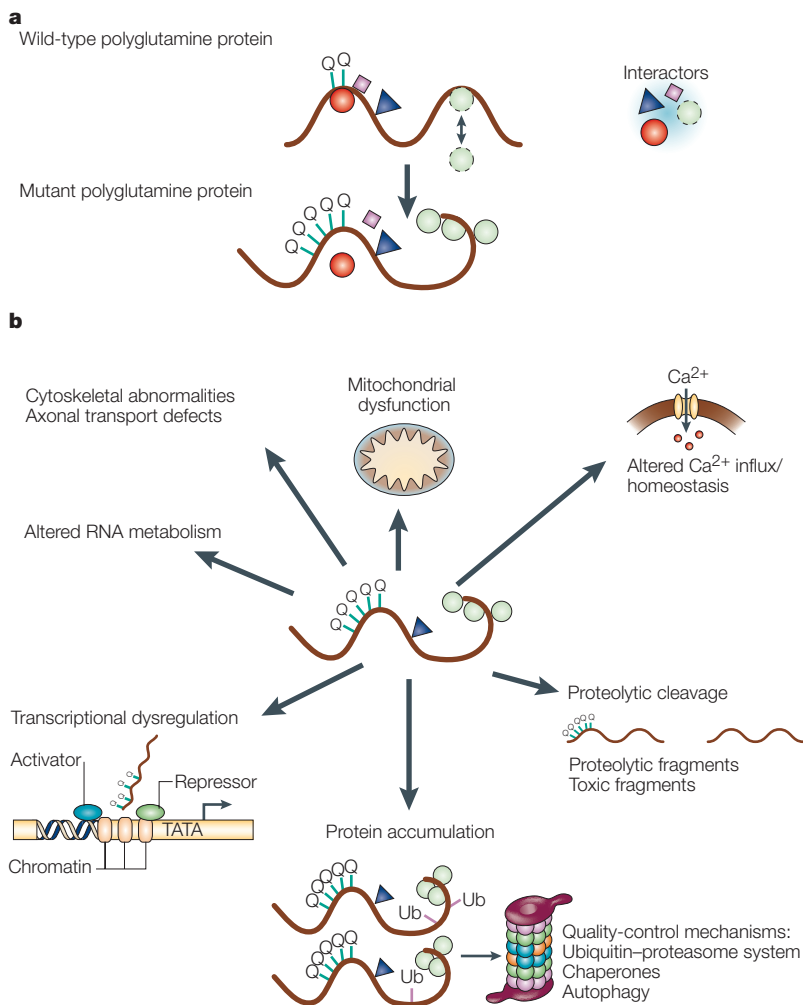


Figure 3 | Mechanisms of pathogenesis in polyglutamine diseases. **a** | A common theme among the nine polyglutamine diseases is altered protein conformation, leading to protein accumulation and aberrant interactions. The figure shows hypothetical aberrant interactions; some of these might be inappropriately enhanced, whereas others might be lost or unchanged. **b** | Expanded polyglutamine proteins might mediate pathogenesis through a range of mechanisms. Accumulation of mutant polyglutamine protein to form insoluble inclusions recruits components of the ubiquitin-proteasome system and other protein quality-control pathways, although the implications of this for pathogenesis are unclear. The full-length polyglutamine protein can also be cleaved by proteases to form fragments, which might also mediate pathogenic effects. The mutant protein affects many cellular processes, including transcription and RNA metabolism. The effects on transcription can occur through interaction of the mutant protein with transcriptional activators and repressors (including CREB-binding protein (CBP), Sp1 transcription factor (SP1) and nuclear co-repressor proteins), or through effects on chromatin. Alternatively, the mutant protein itself might serve as a transcription factor. Other cellular processes that can be affected include mitochondrial function, calcium homeostasis, and axonal transport, ultimately leading to neuronal dysfunction and death. HDACs, histone deacetylases; Q, glutamine; Ub, ubiquitin.

other benign polyglutamine proteins such as CBP and TATA box binding protein (TBP)⁸⁸.

It is also intriguing that the intrinsic functions of several of the proteins that are mutated in polyglutamine diseases have been connected to transcription. In SCA17 and SBMA, the CAG-repeat expansions occur within two transcription factors — TBP and the androgen receptor (AR), respectively^{2,58}. In addition, huntingtin might function as a transcriptional co-repressor by interacting with complexes

that contain nuclear co-repressor proteins^{85,89}, and atrophin 1, ataxin 3 and ataxin 1 have all been implicated as transcriptional regulators^{90,91}. Recent work has also established a role for wild-type ataxin 7 as a component of the STAGA/TFTC histone acetyltransferase (HAT) complex (REFS 92,93; D. Devys, personal communication). Consistent with this, effects on chromatin modifications have been highlighted as another mechanism of polyglutamine-mediated transcriptional dysregulation^{82,94}: histone deacetylase inhibitors reduce toxicity in *D. melanogaster*⁸², cell-culture and mouse models of disease⁹⁴⁻⁹⁷.

Alterations in gene expression might also occur through effects on RNA processing and stability. Several genetic modifiers in a *D. melanogaster* model of SCA1 are proteins that are involved in RNA binding and processing⁹⁸. Furthermore, ataxin 2 interacts with ataxin 2 binding protein 1 (A2BP1), which has been implicated in splicing⁹⁹.

Cytoplasmic sites of pathogenesis. Mutant polyglutamine proteins can also affect cytoplasmic processes, such as axonal transport and mitochondrial function. Both reduced levels of huntingtin and ectopic expression of huntingtin and ataxin 3 fragments that contain pathogenic repeats caused axonal blockages in *D. melanogaster*¹⁰⁰. Szebenyi *et al.* observed similar effects in isolated AXOPLASM, although here only the pathogenic expanded forms of the two polyglutamine proteins examined (an N-terminal fragment of huntingtin and a full-length form and fragment of AR) inhibited axonal transport¹⁰¹.

Morphological and functional data support a role for mitochondrial dysfunction in polyglutamine diseases¹⁰². This evidence is particularly strong for HD, where mutant huntingtin influences calcium signalling through several synergistic mechanisms, including effects on mitochondria¹⁰³. In terms of other cytoplasmic mechanisms, cleavage of expanded polyglutamine proteins by CASPASES or CALPAINS can result in the release of toxic fragments that initiate a wide range of downstream events, such as misfolding and effects on transcription.

The importance of non-polyglutamine domains in pathogenesis. It is becoming increasingly clear that protein context rather than general polyglutamine-mediated effects have the most important role in pathogenesis. Modifications outside the polyglutamine tract — such as phosphorylation of ataxin 1 at a crucial serine^{104,105} or SUMOYLATION of huntingtin¹⁰⁶ — have been shown to be important determinants of toxicity. This is supported by the recent finding that the AXH (ataxin 1/HMG box containing protein 1 (HBP1)) domain of ataxin 1 mediates neuronal degeneration through interactions with senseless (SENS) or growth factor independent 1 (GFI1) in flies and mice, respectively⁷⁸. Furthermore, expression of full-length expanded huntingtin, but not an N-terminal fragment, produces a neurodegenerative phenotype in a transgenic mouse model⁷⁵. Therefore, toxicity seems to be

mediated by the host protein as a whole rather than the glutamine tract in isolation.

Context is of particular interest when considering disease-protein function in SCA6, SCA3, SCA7 and SBMA. In the case of SCA6, the repeat expansion occurs in the context of the $\alpha 1A$ subunit of a P/Q-TYPE CALCIUM CHANNEL¹⁰⁷, which raises the question of the *in vivo* effect that the repeat expansion has on calcium homeostasis. In the case of SCA3, wild-type ataxin 3 has ubiquitin-protease activity and suppresses polyglutamine-mediated neurodegeneration in *D. melanogaster*¹⁰⁸, whereas wild-type and mutant ataxin 7 (the SCA7 disease protein) might mediate effects on transcription through their association with the STAGA/TFTC complex^{92,93} (REFS 92,93; D. Devys, personal communication). For SBMA, which involves mutation of the AR, both protein and subcellular context are important: it has been shown that ligand binding and nuclear translocation are necessary for pathogenesis. For example, women who are homozygous for the AR repeat expansion do not develop an SBMA phenotype¹⁰⁹, nor do *D. melanogaster* or mouse models in the absence of a ligand^{110,111}. So, in each of these cases, the normal function of the disease protein drives pathogenesis, and the polyglutamine tract might modulate this function by altering protein conformation and/or interactions.

Diseases caused by altered RNA function

Dystrophia myotonica 1 and 2 (DM1 and DM2). The myotonic dystrophies are multi-systemic disorders that are caused by two unstable repeat expansions. **Dystrophia myotonica 1 (DM1)** is caused by a CTG trinucleotide expansion in the 3' UTR of dystrophia myotonica protein kinase (*DMPK*)^{112–114}, whereas **dystrophia myotonica 2 (DM2)** is caused by a CCTG tetranucleotide expansion in intron 1 of zinc-finger protein 9 (*ZNF9*) (REFS 3,115) (TABLE 2). DM1 and DM2 share several clinical features¹¹⁶ (TABLE 1), but a key distinction of DM1 is the occurrence of a congenital form of the disease and distinct CNS manifestations, including mental retardation¹¹⁶.

Three hypotheses have been proposed to explain the pathogenesis of DM1: haploinsufficiency of *DMPK*, effects of the expanded allele on neighbouring genes and pathological effects of the expanded RNA. Studies of two lines of *Dmpk*^{-/-} mice argue against the haploinsufficiency hypothesis^{117,118}. These mice develop only a mild myopathy and none of the other features of DM1. Furthermore, studies using mice that are null for sine oculis-related homeobox 5 homologue (*Six5*) — a transcription factor which lies downstream of *DMPK* — failed to support the hypothesis that neighbouring genes cause the phenotype¹¹⁹. The strongest evidence supports RNA-mediated pathogenesis in these diseases¹²⁰. First, despite the high prevalence of dystrophia myotonica (DM) (1:8,000 are affected worldwide), the only causative mutations that have been identified are the CTG or CCTG expansions in non-coding regions¹¹⁶. Second, HSA^{LR} mice that carry 250 CUG repeats in the 3' UTR of the human skeletal actin gene reproduce the characteristic myotonia

and abnormal muscle histology of DM1 (REFS 121,122). Finally, DM1 and DM2 show striking clinical overlap, despite the fact that the mutations occur in different genes, on separate chromosomes and in the context of unrelated neighbouring genes. In light of this evidence, a common pathogenic mechanism has been suggested that involves aberrant binding of expanded RNAs to RNA-binding proteins, with subsequent dysregulation of protein function.

Many RNA-binding proteins that interact with these expanded RNAs regulate splicing, and abnormal splicing of specific pre-mRNA targets is thought to account for the wide range of DM phenotypes (FIG. 4). Two families of RNA-binding proteins have been implicated in DM pathogenesis on the basis of their ability to interact with RNAs that contain CUG or CCUG repeats: CUG triplet repeat RNA-binding protein 1 (CUGBP1) and ETR3-like factors (CELFs), and muscleblind-like (MBNL) proteins. Expanded CUG repeats in mutant *DMPK* transcripts form nuclear RNA foci that recruit MBNL proteins^{123,124}, and an *Mbnl* knockout mouse model develops DM-like eye and muscle phenotypes and shows defective regulation of alternative splicing¹²⁵. Although it is not recruited to the ribonuclear inclusions that form in DM, CUGBP1 function is increased in samples from patients with DM¹²⁶. Consistent with the role of CUGBP1 dysregulation in pathogenesis, two transgenic mouse models of CUGBP1 overexpression develop muscle phenotypes¹²⁷, one of which has been shown to have characteristic DM splicing alterations¹²⁷.

So far, 13 pre-mRNA targets have been shown to be altered in DM, including chloride channel 1 (CLC1), cardiac troponin T and insulin receptor transcripts^{122,125,128–131}. Decreased CLC1 function could cause the myotonia in DM. In addition, splicing alterations that lead to aberrant expression of an embryonic isoform of cardiac troponin T and a non-muscle-type insulin-receptor isoform in skeletal muscle could contribute to the cardiac manifestations and insulin insensitivity, respectively, that are associated with DM. Recent evidence indicates that RNA-mediated pathogenesis also extends to the CNS. Splicing alterations that affect the *N*-methyl-D-aspartate (NMDA) receptor NR1 subunit and the neuronal microtubule-associated protein Tau, as well as a more subtle effect for the APP (amyloid precursor protein) transcript, were observed in post-mortem brain tissue from patients with DM¹³¹. Importantly, many of the splicing alterations observed in DM indicate that CELF and MBNL proteins might function antagonistically to regulate common pre-mRNA targets. Of the 13 misregulated splicing events observed in DM, at least 4 are consistent with the loss of MBNL and/or gain of CELF activity^{125,126,128–130,132}.

These findings provide substantial evidence for a *trans*-dominant RNA-mediated pathogenic mechanism in DM1 and DM2, which poses important questions. Do other RNA-binding proteins have a role in the misregulation of alternative splicing? Do the mutant transcripts have other effects *in vivo*, such as on

P/Q-TYPE CALCIUM CHANNEL

A high voltage activated calcium channel found in cerebellar Purkinje cells that is responsible for most of the calcium current in these neurons.

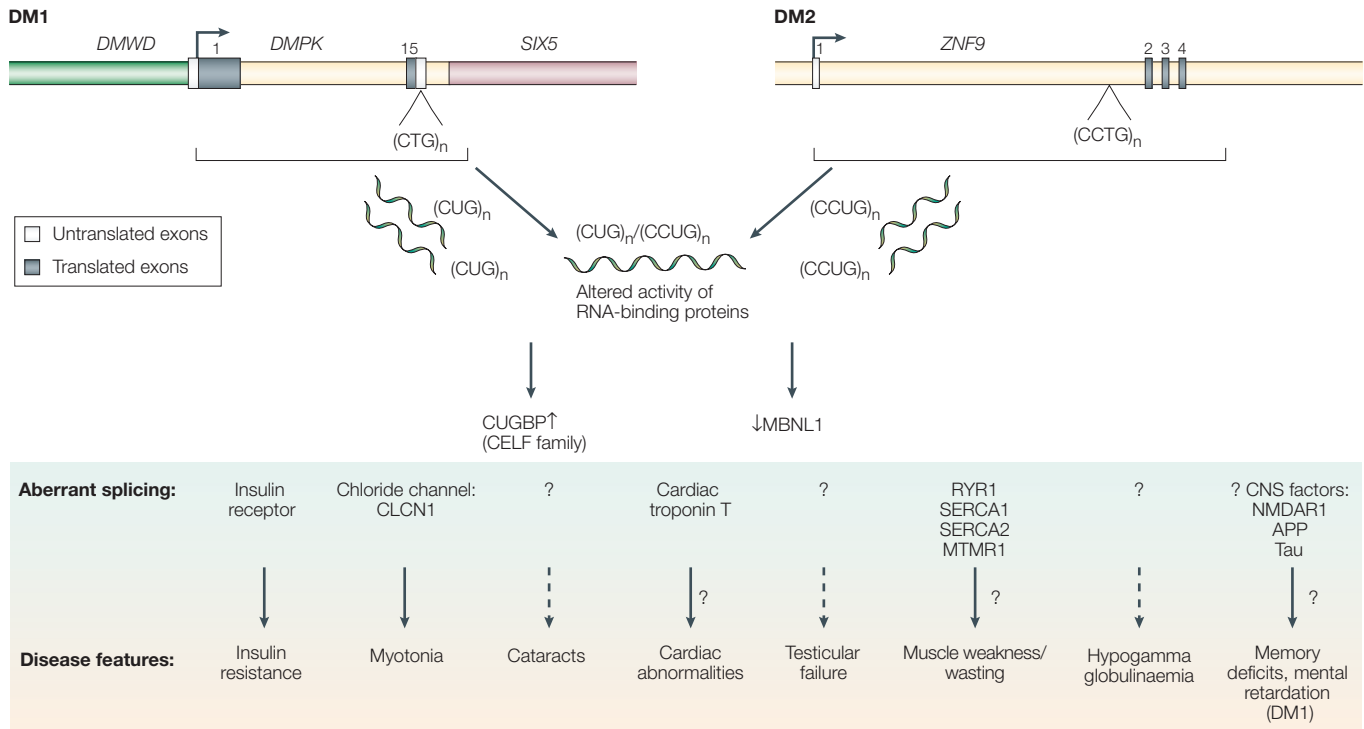


Figure 4 | **Mechanisms of pathogenesis in dystrophy myotonia 1 and 2.** In dystrophy myotonia 1 (DM1) pathogenesis is caused by the expansion of a CTG repeat in the dystrophy myotonia protein kinase (*DMPK*) gene to produce RNAs with long CUG tracts. In dystrophy myotonia 2 (DM2) a similar mechanism that involves the zinc-finger protein 9 (*ZNF9*) gene occurs, producing RNAs with expanded CCUG tracts. These abnormal RNAs bind to and alter the activity of RNA-binding proteins, including CUGBP1 and ETR3-like factors (CELFs), and muscleblind-like (MBNL) proteins. Although a negative effect is exerted on MBNL1, the activity of its antagonist, CUGBP1, is upregulated. These effects lead to aberrant splicing of several transcripts that are important in a wide range of physiological processes. The proteins that are affected in this way and the proposed effects of their aberrant splicing in the disease phenotype are shown in the lower panel. APP, amyloid precursor protein; CLCN1, chloride channel 1; *DMWD*, dystrophy myotonia-containing WD repeat motif; NMDAR1, *N*-methyl-D-aspartate receptor 1; RyR1, ryanodine receptor 1; SERCA, sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase; *SIX5*, sine oculis-related homeobox 5 homologue; Tau, microtubule-associated protein tau.

specific transcription factors that have been shown to redistribute to ribonuclear inclusions *in vitro*¹³³? And what role do the ribonuclear inclusions, which have been found to co-localize with specific proteasome components in one study¹³¹, have in pathogenesis? Addressing these questions will aid in the development of targeted therapies¹³⁴.

Fragile X-associated tremor/ataxia syndrome (FXTAS). Carriers of premutation alleles (60–200 CGG repeats) in the *FMR1* gene can develop two clinical phenotypes distinct from fragile X syndrome. One of these is fragile X tremor/ataxia syndrome (FXTAS), a neurodegenerative disorder that affects older male premutation carriers^{8,135} (TABLE 1). A pathological hallmark of the disease is the presence of eosinophilic, ubiquitin-positive intranuclear inclusions in neurons and astrocytes throughout the brain¹³⁶.

Several findings indicate an RNA-mediated pathogenesis for this disease. The main molecular abnormality in FXTAS is the 2–5 fold increase in *FMR1* mRNA^{137,138}, although FMRP expression is also decreased owing to reduced translational efficiency¹³⁹. FXTAS is never observed in older males with full mutations who have both decreased *FMR1* mRNA and FMRP, indicating

that FMRP deficiency cannot be the primary cause of pathogenesis. Furthermore, a CGG knockin mouse model, generated by replacing the normal CGG element in the *Fmr1* gene with 100 CGG repeats¹⁴⁰, shows increased levels of *Fmr1* mRNA and develops neuronal intranuclear inclusions, despite normal levels of FMRP¹⁴⁰. These mice do not develop astrocytic inclusions and show no evidence of neurodegeneration up to 72 weeks, but a recent report suggests that there are subtle age-dependent deficits in visual-spatial learning¹⁴¹. The lack of overt neurodegeneration could reflect strain-specific genetic modifiers or be due to the relatively short lifespan of the mouse.

One indication of how *FMR1* RNA that contains an expanded repeat might cause pathogenesis in FXTAS comes from the results of overexpressing a repeat tract of 90 CGGs that is transcribed but not translated in *D. melanogaster*. This is sufficient to cause inclusion formation and progressive degeneration in the *D. melanogaster* eye¹⁴². Although these inclusions are predominantly cytoplasmic, they are immunoreactive for ubiquitin and heat shock protein 70 (HSP70) (REF. 142). This link to the proteasome degradation pathway implicates protein misfolding in the pathogenesis of FXTAS.

Common mechanisms for DM and FXTAS. Although DM and FXTAS are caused by different trinucleotide repeat expansions, a general model of RNA-mediated pathogenesis may apply to both. Interaction of expanded RNA species with specific sets of RNA-binding proteins may lead to dysregulation of the function and/or conformation of such proteins. Furthermore, the inclusions present in both DM and FXTAS, which might be of different overall composition, co-localize with components of the protein degradation and quality-control machinery^{131,140}. These findings indicate that protein misfolding and degradation could be another shared mechanism. Although either or both of these mechanisms could contribute to the progressive dysfunction in these disorders, the evidence indicates that, at least in DM, inclusion formation itself might not be a primary event in pathogenesis¹⁴³. Identification of CGG-binding proteins and downstream events in FXTAS will provide an insight into how similar or different it is from other RNA-mediated disorders.

Diseases of unknown pathogenic mechanism

SCA8, caused by a CTG expansion in the 3' UTR of an untranslated RNA¹⁴⁴, shows variable penetrance that has complicated the determination of normal and pathogenic repeat sizes (TABLE 2). One theory is that the SCA8 transcript functions as an endogenous antisense RNA that partially overlaps with the kelch-like 1 gene (*KLHL1*) (REF. 145); repeat expansion could lead to misregulation of *KLHL1*, which encodes an actin-binding protein. Alternatively, pathogenesis could be mediated through toxic effects of the expanded CUG RNA, similar to DM. Supporting this second hypothesis, progressive retinal degeneration is seen in *D. melanogaster* on expression of both expanded and normal human SCA8 RNA¹⁴⁶. Interestingly, four modifiers of pathogenesis included neuronally expressed RNA-binding proteins: *staufen*, *muscleblind*, *split ends* and *CG3249* (REF. 146). This indicates that SCA8 pathogenesis, like DM1 and DM2, could be RNA-mediated, involving the recruitment and subsequent dysregulation of cellular proteins by the expanded RNA.

The causative mutation in SCA10 is an unstable ATTCT pentanucleotide repeat expansion near the 3' end of a large intron in a gene of unknown function⁴. Potential pathogenic mechanisms include transcriptional silencing of this gene and/or neighbouring genes, or alterations that are mediated at the RNA level. Examining affected tissues for RNA accumulation, in addition to determining the expression levels of SCA10 and neighbouring genes, will be important in distinguishing between these mechanisms.

In SCA12, the CAG expansion occurs in the non-coding 5' region of protein phosphatase 2 (formerly 2A) regulatory subunit B (*PPP2R2B*) (REF. 147), a brain-specific regulatory subunit of protein phosphatase 2A (PP2A). Because of the complexity of the 5' region of the *PPP2R2B* transcript, the exact relationship of the CAG expansion to the promoter and the 5' UTR, and therefore its role in pathogenesis, are unclear. CAG expansion in the promoter could

result in altered *PPP2R2B* expression, and increased expression has been reported in some cell types¹⁴⁸. Alternatively, expansion in the 5' UTR could involve an RNA-mediated mechanism.

Huntington disease-like 2 (HDL2) is a rare neurodegenerative disease with clinical manifestations that are similar to HD (TABLE 1). The causative mutation is a CTG expansion in *JPH3*, which encodes junctophilin 3 (REF. 149), a brain-specific protein that is involved in forming complexes between the plasma membrane and endoplasmic reticulum (ER). The repeat is located in an alternatively spliced exon that has multiple splice-acceptor sites¹⁴⁹. Depending on the site used, the repeat is either in the 3' UTR (consistent with an RNA mechanism), or is in frame with the coding region¹⁴⁹.

Animal models of these disorders will be invaluable in differentiating between RNA and protein-mediated mechanisms. Questions to be addressed include the function of the disease gene product (in the case of SCA10), expression levels on expansion, and whether RNA or RNA/protein accumulation occurs in vulnerable neurons. Such studies will lead to a better understanding of RNA or protein-mediated mechanisms and might provide an insight into even more complex modes of pathogenesis.

Conclusions

The general pathogenic mechanisms in unstable repeat disorders include loss of protein function, gain of function (enhanced or novel protein function), or altered function that is due to aberrant RNA-protein interactions. In diseases that are caused by a loss-of-function mechanism, progress is being made in understanding how loss of normal protein activity accounts for the phenotypes observed. In the case of polyglutamine diseases, which are caused by a gain-of-function mechanism, significant emphasis has been placed on the polyglutamine tract as the toxic moiety over the past decade. However, evidence now supports protein context as a crucial determinant in pathogenesis; the polyglutamine tract seems to modulate toxicity that is largely driven by the normal function of the full-length protein. Therefore, for both classes of disorders that are mediated at the protein level, the normal function of the disease protein is central to pathogenesis.

The role of protein misfolding, altered function and accumulation — initially recognized in polyglutamine diseases — is now emerging as a point of overlap with RNA-mediated disorders. This convergence indicates that repeat expansions, whether affecting RNA or protein, might elicit a common cellular response mechanism. In polyglutamine disorders, altered conformation of the mutant protein triggers aberrant interactions that lead to aggregation, but in the case of RNA-mediated disorders, the proteins that bind the mutant RNA might undergo a conformational change that alters their function, interactions and/or clearance. Given this overlap, it will be of interest to determine whether further parallels emerge between RNA-mediated and protein-mediated neurodegenerative disorders.

In the case of *FMRI*, expansions within a single gene can give rise to either a neurodevelopmental disorder (loss-of-function disorder) or a neurodegenerative disorder (RNA gain-of-function disorder). This begs the question of whether dual pathogenic mechanisms could exist in other repeat diseases. As a corollary, could expansions that are mediated at the protein level also have an RNA component? Increased understanding of RNA and protein interactions and cellular contexts will be crucial in contemplating such questions.

Finally, in many of the neurodegenerative repeat expansion disorders there is late onset of symptoms and delayed cell loss. Coupled with the ability to partially reverse disease phenotypes by halting mutant protein expression, as observed in SCA1 and Huntington

disease conditional mouse models^{150,151}, this provides a crucial window of opportunity for therapeutic intervention. For neurodevelopmental repeat expansion disorders, it has been suggested that early therapeutic intervention might be needed to confer benefit. However, recent studies in a *Drosophila* model of fragile X syndrome showed improvement of behavioural phenotypes even when treatment was withheld until adulthood¹⁷. A window of therapeutic benefit might therefore also exist for these disorders, although further evaluation in mammalian systems is needed to confirm this. Altogether, the large body of data that has been compiled about pathogenic mechanisms in unstable repeat expansion disorders over the past 15 years is now providing the framework for the development of new treatment strategies.

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Competing interests statement

The authors declare no competing financial interests.

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