

Repeat expansion disease: progress and puzzles in disease pathogenesis

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Abstract | Repeat expansion mutations cause at least 22 inherited neurological diseases. The complexity of repeat disease genetics and pathobiology has revealed unexpected shared themes and mechanistic pathways among the diseases, such as RNA toxicity. Also, investigation of the polyglutamine diseases has identified post-translational modification as a key step in the pathogenic cascade and has shown that the autophagy pathway has an important role in the degradation of misfolded proteins — two themes that are likely to be relevant to the entire neurodegeneration field. Insights from repeat disease research are catalysing new lines of study that should not only elucidate molecular mechanisms of disease but also highlight opportunities for therapeutic intervention for these currently untreatable disorders.

Post-translational modification

A covalent chemical modification of a protein that takes place after translation.

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A new type of human genetic disease mutation was unexpectedly discovered more than 18 years ago — expansion of a repeated microsatellite sequence. At least 22 inherited disorders, all involving the neuraxis, are now known to be caused by expanded repeats (TABLE 1). Repeat expansion diseases include some of the most common inherited diseases, such as Huntington's disease (HD) and myotonic dystrophy. In 1991, two repeat expansion mutations — for the X-linked disorders fragile X mental retardation syndrome (FMR1) and spinal and bulbar muscular atrophy (SBMA) — were reported to produce disease phenotypes by encoding proteins with expanded poly-amino acid tracts^{1,2}. We now recognize SBMA as the first member of a subcategory of repeat expansion disorders known as the 'CAG/polyglutamine' repeat diseases. Although it was initially thought that FMR1 was caused by an expanded polyarginine tract, further work indicated that the CGG repeat expansion in FMR1 is located in the 5' UTR. The expansion reduces expression of FMR1 by promoting DNA hypermethylation at the promoter³⁻⁵.

Since these seminal discoveries, various repeat expansion mutations have been identified (TABLE 1), and at least four mechanisms of disease are now known: loss of function of the gene containing the repeat; gain of function due to production of a protein containing a polyglutamine tract expansion; gain of function due to production of RNA containing an expanded CUG tract; and gain of function due to production of a protein containing a polyalanine tract expansion. Classification of the repeat expansion diseases into these four mechanistic categories

typically reflects both the sequence composition of the repeat and the location of the repeat in a gene (TABLE 1).

Despite these advances, there are still difficulties — as there were at the genesis of the field — in determining exactly how repeat expansion mutations cause inherited human diseases. Therefore, current classification schemes are at risk of becoming outdated in the future. Indeed, a number of recent findings have revealed the potential complexity of the molecular mechanisms underlying disease pathogenesis. For example, post-translational modification of disease proteins has been identified as a key step in the pathogenic cascade of CAG/polyglutamine disease, and autophagy has been implicated in the degradation of misfolded proteins. Molecular genetic studies, driven by recent advances in our understanding of the transcriptome, suggest that bidirectional transcription and chromatin structure could be involved in repeat disease pathology and genetic instability. In this Review we examine the most compelling of these paradigm-shifting advances, focusing on RNA toxicity, autophagy, post-translational modification, bidirectional transcription and genomic structure. We consider advances in these areas for the relevant subcategory of repeat expansion disease and the broader repeat disease field, and note how these findings might be translated into novel, widely applicable therapies.

RNA as a driver of toxicity

A shared mechanism for myotonic dystrophy type 1 and 2. One of the most striking developments in the repeat disease field has been the realization that expanded repeats in

Table 1 | Clinical and molecular characteristics of inherited neurological repeat expansion disorders

Disease	Main clinical features	Causal repeat (gene)	Repeat location	Mechanism or category	Comments
DM1	Muscle weakness, myotonia, cardiac-endocrine-GI disease, MR	CTG (<i>DM1</i> , also known as <i>DMPK</i>)	3' UTR	RNA GOF	A very common form of muscular dystrophy
DM2	Muscle weakness, myotonia, cardiac-endocrine-GI disease	CTG (<i>ZNF9</i> , also known as <i>CNBP</i>)	Intron	RNA GOF	A striking phenocopy of DM1
DRPLA	Seizures, choreoathetosis, ataxia, cognitive decline	CAG (<i>ATN1</i>)	Coding region	Polyglutamine GOF	Very rare, most patients are in Japan
FMR1	MR, facial dysmorphism, autism	CGG (<i>FMR1</i>)	5' UTR	Hypermethylation of promoter, LOF	Most common inherited MR
FMR2	MR, hyperactivity	GCC (<i>FMR2</i>)	5' UTR	LOF	Needs to be ruled out in X-linked MR
FRDA	Ataxia, sensory loss, weakness, diabetes mellitus, cardiomyopathy	GAA (<i>FXN</i>)	Intron	LOF, phenocopy of mitochondrial disease	Most common inherited ataxia in Caucasian ethnicity
FXTAS	Ataxia, intention tremor, parkinsonism	CGG (<i>FMR1</i>)	5' UTR	RNA GOF	Premutation carriers only
HD	Chorea, dystonia, cognitive decline, psychiatric disease	CAG (<i>HTT</i>)	Coding region	Polyglutamine GOF	One of the most common inherited diseases in humans
HDL2	Chorea, dystonia, cognitive decline	CTG (<i>JPH3</i>)	3' UTR, coding region	RNA GOF, poly-amino acid GOF and/or LOF?	A striking phenocopy of HD
Myoclonic epilepsy of Unverricht and Lundborg	Photosensitive myoclonus, tonic-clonic seizures, cerebellar degeneration	CCCCGCCCGCGG (<i>CSTB</i>)	Promoter	LOF	Rare autosomal recessive disorder found in Finland and N. Africa
OPMD	Eyelid weakness, dysphagia, proximal limb weakness	GCG (<i>PABPN1</i>)	Coding region	Polyalanine GOF	Modest expansion causes disease
SBMA	Proximal limb weakness, lower motor neuron disease	CAG (<i>AR</i>)	Coding region	Polyglutamine GOF	Phenotype includes LOF androgen insensitivity
SCA1	Ataxia, dysarthria, spasticity, ophthalmoplegia	CAG (<i>ATXN1</i>)	Coding region	Polyglutamine GOF	Accounts for 6% of all dominant ataxia
SCA2	Ataxia, slow eye movement, hyporeflexia, motor disease, occasional parkinsonism	CAG (<i>ATXN2</i>)	Coding region	Polyglutamine GOF	<i>ATXN2</i> protein may not reside in the nucleus
SCA3	Ataxia, dystonia, lower motor neuron disease	CAG (<i>ATXN3</i>)	Coding region	Polyglutamine GOF	Most common dominant ataxia
SCA6	Ataxia, dysarthria, sensory loss, occasionally episodic	CAG (<i>CACNA1A</i>)	Coding region	Polyglutamine GOF	Causal gene encodes a subunit of a P/Q-type Ca ²⁺ channel
SCA7	Ataxia, dysarthria, cone-rod dystrophy retinal disease	CAG (<i>ATXN7</i>)	Coding region	Polyglutamine GOF	Clinically distinct as patients have retinal disease
SCA8	Ataxia, dysarthria, nystagmus, spasticity	CTG/CAG (<i>ATXN8</i>)	Untranslated RNA, coding region	RNA GOF and polyglutamine GOF	Many cases of reduced penetrance
SCA10	Ataxia, dysarthria, seizures, dysphagia	ATTCT (<i>ATXN10</i>)	Intron	RNA GOF?	Huge repeats; only Mexican ancestry?
SCA12	Tremor, ataxia, spasticity, dementia	CAG (<i>PPP2R2B</i>)	Promoter, 5' UTR?	Unknown	Causal gene encodes a phosphatase
SCA17	Ataxia, dementia, chorea, seizures, dystonia	CAG (<i>TBP</i>)	Coding region	Polyglutamine GOF	Causal gene encodes a common transcription factor (TBP)
Syndromic/non-syndromic X-linked mental retardation	MR alone, with seizures or with dysarthria and dystonia	GCG (<i>ARX</i>)	Coding region	Probably LOF	Associated with West syndrome or Partington syndrome

AR, androgen receptor; ARX, aristaless-related homeobox; *ATN1*, atrophin 1; *ATXN*, ataxin; *CACNA1A*, voltage-dependent P/Q-type calcium channel subunit α -1A; *CSTB*, cystatin B; DM, myotonic dystrophy; *DMPK*, *DRPLA*, dentatorubral-pallidolysian atrophy; *FMR1*, fragile X mental retardation syndrome; *FMR2*, fragile X E mental retardation; *FRDA*, Friedreich's ataxia; *FXN*, frataxin; *FXTAS*, fragile X tremor ataxia syndrome; GI, gastrointestinal; GOF, gain of function; HD, Huntington's disease; *HDL2*, Huntington's disease-like 2; *HTT*, huntingtin; *JPH3*, junctophilin 3; LOF, loss of function; MR, mental retardation; *OPMD*, oculopharyngeal muscular dystrophy; *PABPN1*, poly(A)-binding protein, nuclear 1; *PPP2R2B*, protein phosphatase 2 regulatory subunit B, β isoform; *SBMA*, spinal and bulbar muscular atrophy; *SCA*, spinocerebellar ataxia; *TBP*, TATA box-binding protein; *ZNF9*, zinc finger 9.

transcripts can cause cellular toxicity and neurodegeneration by altering the splicing machinery. In 1992, a CTG repeat expansion in the 3' UTR of a protein kinase gene was found to be the cause of *myotonic dystrophy type 1* (DM1), the most common form of adult muscular dystrophy^{6–8}. This discovery posed a puzzling question: how does a dominantly inherited repeat expansion in the non-coding region of a gene produce a disease phenotype that affects many different tissues? Although some evidence emerged for reduced dosage of the protein kinase DM1 (also known as *DMPK*) as the cause of certain DM1 features, haploinsufficiency could not account for most facets of DM1 pathophysiology⁹. As the *DM1* gene is located in a gene-rich region, some argued that DM1 resulted from altered expression of the DM1 protein kinase in combination with altered expression of adjacent genes and proposed that the CTG repeat expansion mimicked a contiguous gene syndrome¹⁰. This 'field theory' of DM1 pathogenesis gained support when knockout mice lacking the gene downstream of *DM1*, *SIX5*, were found to develop cataracts, which are also a feature of DM1^{11,12}.

However, the field theory of DM1 pathogenesis could not easily be reconciled with a DM1 phenocopy caused by a different genetic locus¹³. This disorder, known as *myotonic dystrophy type 2* (DM2), has a phenotype similar to DM1. A distinction is occasional congenital presentation and mental retardation in patients with DM1 who receive large CTG repeat expansions because of maternal anticipation^{14,15}. DM2 is caused by expansion of a CCTG tetranucleotide repeat in the zinc finger 9 (*ZNF9*, also known as *CNBP*) gene¹⁶, the normal function of which does not match any of the genes at the *DM1* locus. Hence, the parsimonious conclusion was that RNA transcripts containing a CUG repeat expansion — whether in a triplet repeat or a CCUG repeat — initiate a shared pathogenic cascade.

A role for RNA toxicity in DM1 was first suggested by RNA foci in cells from patients with DM1 (REF. 17), and indistinguishable RNA foci were found in samples from patients with DM2 (REF. 16). The validity of the RNA toxicity model was supported by a mouse model of myotonic dystrophy in which a 250 CTG repeat in the 3' UTR of an unrelated transgene — skeletal actin — was shown to cause myotonia and myopathy¹⁸. In another study, preceding the identification of DM2, altered splicing due to increased function of CUG-binding protein 1 (*CUGBP1*) emerged as a plausible mechanism for the RNA gain-of-function toxicity¹⁹. Subsequent work has implicated reduced function of muscleblind 1 (*MBNL1*) due to its sequestration into CUG repeat-rich foci as a contributing factor in the splicing abnormalities in DM1 and DM2 (REF. 20). *MBNL1* is one of a family of three proteins that have been shown to bind specifically to double-stranded RNA hairpins formed by CUG repeats²¹. Although increased CUG repeat-containing RNAs seem to increase *CUGBP1* levels, the formation of CUG and CCUG ribonuclear foci by expanded CUG repeat-containing RNAs results in *MBNL1* sequestration and depleted function. Therefore, the disease model is increased *CUGBP1* function combined with decreased *MBNL1* function.

Studies in *Drosophila melanogaster* showed that loss of function of *muscleblind* prevents terminal differentiation of retinal and muscle cells^{22,23}. In mammals, *MBNL1* serves an analogous function, as it favours the splicing of target genes into adult isoforms so, in DM1 and DM2, reduced *MBNL1* function allows fetal isoform production to persist in adult tissues and affects the expression level of many target transcripts²⁴. A recent study found that loss of *MBNL1* can explain the majority of splicing alterations in DM1 and identified extracellular matrix genes as a common target of expanded CUG repeat-containing RNAs, linking DM1 pathogenesis with other connective tissue diseases and muscular dystrophies²⁴. The pleiotropic phenotype of the myotonic dystrophies is therefore believed to result from the altered splicing of genes that produce proteins that function in pathways that are linked to disease features. For example, altered splicing of the chloride channel gene and insulin receptor genes are linked to myotonia and glucose intolerance, respectively.

Fragile X tremor ataxia syndrome: two mechanisms at the *FMR1* locus. Advances in our understanding of myotonic dystrophy pathogenesis have set the stage for the characterization of other RNA gain-of-function repeat diseases. One of the most intriguing of these is fragile X tremor ataxia syndrome (FXTAS)^{25,26}. This disorder occurs primarily in male carriers of the *FMR1* premutation allele and seems to result from an entirely different molecular mechanism from *FMR1*. CGG expansions exceeding ~200 repeats produce a mental retardation phenotype by reducing expression of *FMRI*, whereas CGG expansions of 55–200 repeats result in higher expression of a transcript containing the CGG tracts. The RNA molecules with the expanded CGG tract initiate a cascade of events that culminate in central nervous system (CNS) neurodegeneration, which is characterized by ubiquitin-positive inclusions in the nuclei of neurons and glia²⁷. Studies in *D. melanogaster* showed that CGG repeat expansions are sufficient to produce neurodegeneration²⁸, and features of FXTAS histopathology were also recapitulated in a knock-in mouse model²⁹. In light of the *CUGBP1* model of myotonic dystrophy, researchers investigated the function of CGG RNA-binding proteins. Through a combination of biochemical and genetic approaches, mainly in flies, three proteins were found that bind CGG repeats and have reduced function in the disease models: purine-rich binding protein- α (*PURA*), heterogeneous nuclear ribonucleoproteins A2/B1 (*HNRNPA2B1*) and *CUGBP1* (REFS 30,31). The extensive work on myotonic dystrophy and FXTAS, which combined molecular approaches with model organism studies and proteomics, has emphasized that mutant transcripts can produce neuronal dysfunction by disturbing the balance and availability of RNA-binding proteins. Altered function of these proteins seems to be the crux of the molecular pathology in these diseases (FIG. 1).

Toxic RNAs in CAG/polyglutamine diseases? If uninterrupted CUG repeat expansions in RNA can produce neurotoxicity, is it possible that other types of repeat

Haploinsufficiency

A condition in a diploid organism in which a single functional copy of a gene results in a phenotype, such as a disease.

Contiguous gene syndrome

A multi-symptom disorder caused by the deletion of a large sequence of DNA that encodes several genes.

Phenocopy

A phenotype that is closely similar to a phenotype determined by a different gene.

Anticipation

The tendency of certain diseases to have an earlier age of onset and increasing severity in successive generations.

Myotonia

The failure of muscle to relax immediately after voluntary contraction has stopped.

Myopathy

A disease of the muscle.

Premutation

An unstable mutation that has no phenotypic effect but that is highly likely to mutate to a full mutation during transmission through the germ line, as is seen with some expanding trinucleotide repeats.

Inclusions

Accumulations of proteins and other materials that are visualized as discrete entities at the light microscope level, often after the application of special stains or antibodies.

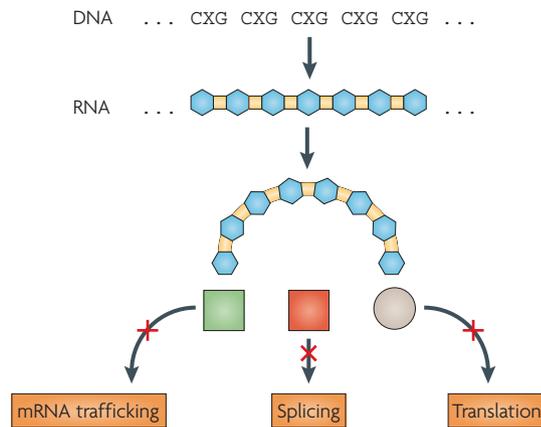


Figure 1 | RNA toxicity in repeat expansion disease. An important mechanism that is now well established for many repeat expansion diseases is the toxicity of RNAs that contain expanded repeat sequences. In these diseases, the RNAs that contain expanded repeats interact with different RNA-binding proteins (coloured shapes) to produce disease. This is a ‘trans-dominant’ model of RNA toxicity: the interaction of mutant RNA with RNA-binding proteins is envisioned to interfere with the functions of the interacting proteins, which leads to abnormalities in the pathways regulated by the RNA-binding proteins.

expansions also exert their toxic effects at the RNA level? In the case of the CAG/polyglutamine repeat diseases (TABLE 1), extensive work has shown that protein toxicity rather than RNA toxicity is principally responsible for the disease. Evidence against CAG RNA toxicity includes studies of spinocerebellar ataxia type 1 (SCA1) and SBMA transgenic mice in which production of mutant protein and mutant RNA did not yield a neurodegenerative phenotype when the mutant protein could not enter the nucleus (because of mutation in the nuclear localization signal or lack of ligand binding)^{30–33}. Furthermore, in *D. melanogaster* no evidence for general CAG RNA toxicity was found³⁴. However, the recent identification of *muscleblind* as a modifier of disease toxicity in the SCA3 fly model prompted Li *et al.* to re-examine this question and, contrary to earlier work, they showed that untranslated RNAs containing tracts of 100 or 250 CAGs can cause retinal degeneration and neuronal dysfunction in *D. melanogaster*, although interrupted CAGCA tracts were non-pathogenic³⁵. These researchers did not find evidence for altered splicing involving *Muscleblind*, so the basis of CAG RNA toxicity and of the modifier effect remains unclear. Therefore, whether CAG RNA toxicity contributes to any, or all, of the nine canonical polyglutamine repeat diseases remains highly controversial. At the same time, considerable progress has been made in understanding the mechanistic basis of polyglutamine proteotoxicity, and sophisticated models of disease pathogenesis, involving protein modification and interaction, have now been proposed.

Autophagy in polyglutamine disease

‘Autophagy’, literally ‘self-eating’, is a catabolic process in which cell constituents, such as organelles and proteins,

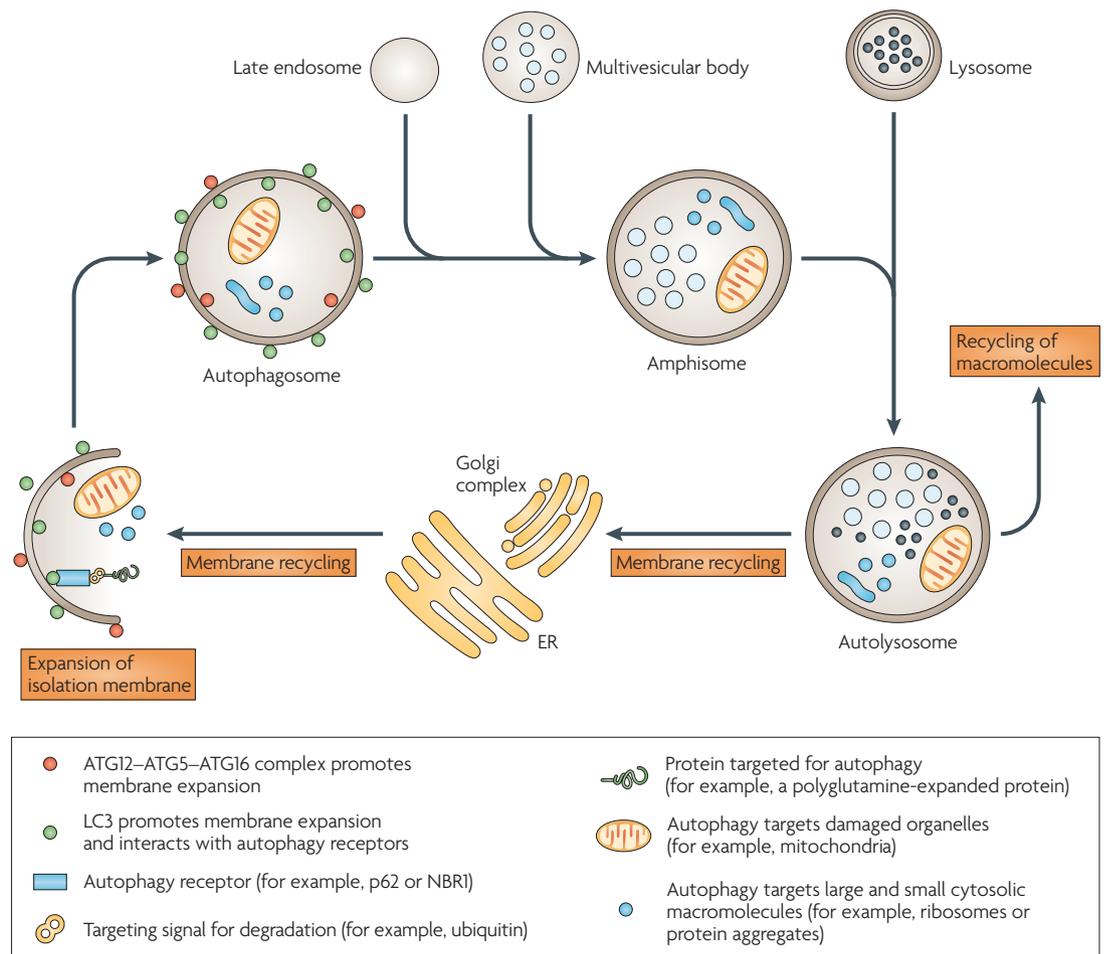
are delivered to the lysosomal compartment for degradation (BOX 1). Defects in autophagy genes underlie an array of human diseases, ranging from cancer to autoimmune disease to neurodegeneration³⁶. It has recently become evident that autophagy has an important role in polyglutamine disease pathogenesis, and possibly in the pathogenesis of other repeat expansion diseases as well. This has garnered substantial interest as the process of autophagy is amenable to pharmacologic manipulation, therefore creating optimism about the possibility of targeting autophagy for therapy. But is autophagy activated or impaired in polyglutamine disease? And should the aim be to activate autophagy or to suppress it? Answers to these questions have evolved as the role of autophagy in disease has been illuminated.

The association between autophagy and the repeat expansion diseases was first made during the study of brains from patients with HD in which mutant huntingtin was found to be associated with accumulated autophagic vacuoles. This finding was corroborated by the examination of lymphoblasts from patients with HD and in a mouse model of HD^{37–40}. Hallmarks of autophagy have now been reported in animal models of other repeat expansion diseases, including SCA1, SCA7 and SBMA^{41–43}. Initially, the accumulation of autophagic vacuoles in dying neurons in HD and related diseases was interpreted as evidence that autophagy could contribute to cell death⁴⁴. This hypothesis was supported by evidence that autophagy provides a non-apoptotic mode of programmed cell death — termed type II programmed cell death in some literature — in some circumstances. For example, autophagy is reported to mediate cell death in the Lurcher mouse model of cerebellar degeneration⁴⁵. Subsequently, the role of autophagy has been extensively examined in animal models of polyglutamine disease, and most empirical evidence suggests that autophagy is usually neuroprotective in these settings⁴⁶.

Neuroprotective autophagy. Autophagy might provide neuroprotection through accelerated turnover of misfolded disease proteins. Indeed, it has been shown that poly(A)-binding protein 2 with an expanded alanine tract (the disease protein in oculopharyngeal muscular dystrophy) and at least some polyglutamine-expanded proteins are delivered to autophagic vacuoles^{38,47} and degraded by autophagy *in vitro*^{48,49}. Further support for this view comes from studies in *D. melanogaster* models of SBMA and HD, in which genetic ablation of autophagy leads to greater accumulation of polyglutamine disease proteins and increased neurodegeneration^{50,51}. Moreover, pharmacological augmentation of autophagy using small molecules, such as rapamycin, lithium or trehalose, or genetic augmentation by overexpression of histone deacetylase 6 (HDAC6) results in accelerated turnover of expanded polyglutamine protein and reduced neurodegeneration in *D. melanogaster* and mouse models of polyglutamine disease^{52–57}.

A caveat to these studies is that virtually all have been performed in models based on overexpression of exogenous mutant protein, which is precisely the scenario in which autophagy would be expected to have

Box 1 | **Autophagy**



Autophagy refers to a set of biological processes in which cell constituents, such as organelles and proteins, are delivered to the lysosomal compartment for degradation. Autophagy can be broadly divided into three types: microautophagy, which involves the direct engulfment of small volumes of cytosol by lysosomes; chaperone-mediated autophagy, which involves receptor-mediated translocation of proteins into the lysosomal lumen; and macroautophagy, which is described below. Autophagy is an evolutionarily conserved process for rapid mobilization of macromolecules when nutrient availability is limited¹³³. Amino acid starvation, for example, induces autophagy and results in increased degradation of non-essential proteins to provide amino acids for the synthesis of essential proteins. In lower organisms autophagy is not essential when nutrients are abundant, but in mammals the role of autophagy has broadened to include additional functions beyond adapting to starvation. These include essential roles in development, immunity and tumour suppression, among others³⁶.

The first step in macroautophagy involves the expansion of an isolation membrane (see figure) that engulfs a portion of the cell. This membrane eventually fuses to form a new double-membraned structure known as an autophagosome. The source of the membrane is not clear, but it might arise from endoplasmic reticulum (ER) or the Golgi complex. The process of autophagy is controlled by parallel activation cascades that involve ubiquitin-like protein modification and are strikingly similar to the activation cascade that regulates the ubiquitin proteasome system. The first arm of the cascade produces a large (~350 kDa) multimeric complex (ATG12-ATG5-ATG16) that is thought to act as a structural support for membrane expansion. A second arm of the cascade conjugates the microtubule-associated protein light chain 3 α (LC3) with the phospholipid phosphatidylethanolamine (PE). As PE is a component of the autophagosomal membrane, the lipidation reaction results in studding of the inner and outer membranes of autophagosomes with LC3. LC3 also contributes to membrane expansion, but an additional important function is its ability to bind autophagy receptors, such as sequestosome 1 (SQSTM1, also known as p62) or next to BRCA1 gene 1 (NBR1), which permits selective autophagy.

Once formed, new autophagosomes move through a stepwise maturation process that results in acidification and delivery of lysosomal hydrolases, permitting degradation of the luminal contents. In mammals, autophagosomes first fuse with endosomes and multivesicular bodies to form amphisomes, which subsequently fuse with lysosomes to create degradative vacuoles termed autolysosomes. Finally, the breakdown products from the autolysosome are translocated back across the lysosomal membrane for reuse in metabolic processes in the cytosol, or in some cases are extruded from the cell, and the membrane is recycled.

the greatest effect. It remains important to examine the role of autophagy further in animal models that more faithfully recapitulate endogenous protein expression levels and patterns, using knock-in or bacterial artificial chromosome (BAC) transgenic approaches, for example. Nevertheless, the view of autophagy as a neuroprotective process is consistent with the emerging appreciation that autophagy is cytoprotective in numerous contexts — such as under conditions of oxidative stress, growth-factor deficiency or nutrient limitation — through accelerated turnover of damaged organelles and maintenance of metabolic homeostasis by mobilization of intracellular energy stores^{58,59}.

Is autophagy a disease target? Despite compelling evidence that autophagy affords neuroprotection by degrading disease proteins, the question remains: why is this protection incomplete? Is it possible that autophagy is not only a modifier of disease but also a target of disease? It has been suggested that the increased numbers of neuronal autophagic vacuoles in some repeat expansion diseases may reflect a defect in autophagy flux rather than autophagy induction⁶⁰. It is now appreciated that there is significant basal autophagy in many mammalian tissues. The demand for basal autophagy differs among tissues; it is particularly important in the liver and in post-mitotic cells, such as neurons and myocytes^{61–64}. Indeed, neurons are especially vulnerable to perturbations in the autophagy–lysosomal system — not only because they are highly metabolically active but also because of their unique cellular architecture. In neurons, lysosomes are concentrated in the soma adjacent to the nucleus. Therefore, autophagosomes produced in dendrites, axons or synaptic terminal regions must be transported substantial distances to enable fusion with lysosomes, which makes autophagy in neurons particularly vulnerable to defects in vesicular trafficking⁶⁶. The importance of basal autophagy in the CNS was shown by conditional knockout of key autophagy genes, which resulted in neurodegeneration with the accumulation of ubiquitin-positive inclusions similar to those seen in human neurodegenerative diseases (including many repeat expansion diseases)^{66,67}. The increased vulnerability of neurons to impairment of the autophagy–lysosomal system might account for the high frequency of neurological phenotypes produced by mutations that target the endosomal–lysosomal system⁶⁸.

It has yet to be shown convincingly that autophagy is impaired in repeat expansion disease. Given the broad range of cellular abnormalities associated with polyglutamine diseases and other repeat expansion diseases, it would not be surprising to find impairment of autophagy. Impaired autophagy — a sort of ‘cellular indigestion’ — could result from overburdening the autophagy–lysosomal system with misfolded, aggregated proteins that are difficult to degrade. Alternatively, autophagy could be directly impaired if a disease protein is important for the process of autophagy. As noted above, huntingtin associates with components of the endosomal–lysosomal system and is trafficked with autophagosomes in axons, which suggests that huntingtin might normally regulate

autophagy^{8,69}. Recently, it was shown *in vitro* and in a mouse knock-in model of HD that polyglutamine expansion in huntingtin impairs the activity of the GTPase Ras-related protein 11A (RAB11A) and leads to a defect in endosome recycling^{70,71}. Therefore it is possible that polyglutamine expansion impairs a normal function of huntingtin and contributes to impaired autophagy? This would be consistent with the emerging theme that altered native protein function underlies polyglutamine disease pathogenesis.

Post-translational modification

Non-polyglutamine determinants of disease. A striking feature of polyglutamine diseases is the selective vulnerability of the CNS despite widespread expression of many polyglutamine disease proteins in non-neural cell types. However, there is striking divergence in clinical phenotypes among the polyglutamine diseases: neurologists can easily distinguish the movement disorder of HD from the weakness in SBMA or the ataxia in SCA1. The disease-specific features reflect selective loss of different populations of neurons: despite wide expression within the CNS, polyglutamine expansion in huntingtin selectively affects striatal neurons and cortical neurons, whereas the same genetic mutation in the *androgen receptor* or *ataxin 1* targets motor neurons or Purkinje neurons, respectively. On the basis of these observations it was predicted that features other than polyglutamine, unique to each disease protein, must influence pathogenesis⁷². This prediction has been borne out in recent advances that have highlighted the importance of host protein context in polyglutamine disease pathogenesis. Principal among these have been insights into the influence of post-translational modification in pathogenesis and, in at least one case, in determining cell-type specificity. Here, we summarize some important insights into the role of post-translational phosphorylation, acetylation and sumoylation in polyglutamine disease pathogenesis (TABLE 2).

Phosphorylation of ataxin 1 maintains a balance.

The importance of post-translational modification in polyglutamine disease was first shown by the Orr and Zoghbi laboratories in a series of papers examining ataxin 1 phosphorylation. Orr and colleagues detected polyglutamine length-dependent phosphorylation of ataxin 1 that mapped to serine 776 (S776)⁷³. Their interest was heightened after observing that an antibody specific for phospho-S776-ataxin 1 preferentially stained pathological, nuclear-localized ataxin 1 in their SCA1 mouse model. They generated transgenic mice expressing polyglutamine-expanded ataxin 1 with an alanine substituted for serine at position 776 (S776A) to prevent phosphorylation. The mice expressing polyglutamine-expanded ataxin 1-S776A had minimal behavioural and histopathological abnormalities, showing the importance of this phosphorylation in pathogenesis.

Phosphorylation alters the conformation of a target protein and can profoundly influence target-protein function, often by regulating protein–protein interactions⁷⁴. Ataxin 1 interacts with two discrete heterotypic protein

Table 2 | **Post-translational modifications in repeat expansion disease**

Disease	Protein	Sumoylation	Acetylation	Phosphorylation
HD	Huntingtin	K6*, K9*, K15*, K91* (REFS 102, 103)	K444* (REF. 101)	S421*, S434*, S536*, S1181*, S1201*, S2076, S2653, S2657 (REF. 93), T3* (REF. 92)
SBMA	Androgen receptor	K385*, K511* (REF. 134)	K630*, K632*, K633* (REF. 135)	S215*, S792* (REF. 136)
DRPLA	Atrophin 1	Unknown lysine(s)* (REF. 137)	†	S734
SCA1	Ataxin 1	K16, K194, K610, K697, K746	†	S776* (REFS 73, 75), S239, T236
SCA2	Ataxin 2	†	†	S860, S864
SCA3	Ataxin 3	Unknown lysines(s)* (REF. 138)	†	S256
SCA6	CACNA1A	†	†	†
SCA7	Ataxin 7	†	K257* (REF. 99)	S840, S849, T854
SCA17	TBP	§	§	§

*Post-translational modifications for which empirical evidence indicates a role in pathogenesis. †There are no data pertaining to this post-translational modification. ‡This post-translational modification occurs, but there are no data pertaining to its role in disease pathogenesis. CACNA1A, voltage-dependent P/Q-type calcium channel subunit α -1A; DRPLA, dentatorubral-pallidolusian atrophy; HD, Huntington's disease; K, lysine; S, serine; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; T, threonine; TBP, TATA box-binding protein.

complexes, one defined by the transcription factor capicua and the other defined by the RNA-binding protein RBM17. Phosphorylation of ataxin 1 regulates the balance of ataxin 1 association with these distinct functional complexes. The evidence suggests that polyglutamine expansion upsets this balance and drives pathogenesis. Specifically, polyglutamine expansion promotes phosphorylation of S776, which favours association with the RBM17 complex and so might contribute to SCA1 neuropathology through a gain-of-function mechanism. Concomitantly, polyglutamine expansion attenuates the formation and function of the capicua complex, contributing to SCA1 through a partial loss-of-function mechanism^{75,76}. This discovery has important implications, as this model suggests that polyglutamine disease pathogenesis might involve subtle alteration of native protein function rather than an entirely novel gain of function. This insight highlights the importance of understanding the normal interactions and functions of each disease protein to understand the pathogenesis of each polyglutamine disease.

Phosphorylation and huntingtin trafficking. Huntingtin is subject to phosphorylation at multiple serines, and phosphorylation of S421 has emerged as a particularly important determinant of HD pathogenesis. Phosphorylation of S421 has been reported to be carried out by multiple kinases, including the serine/threonine kinase AKT1 and serum/glucocorticoid-induced kinase 1 (SGK1)⁷⁷⁻⁷⁹. Significant levels of phospho-S421-huntingtin are present in normal human and mouse striatum but are reduced by polyglutamine expansion. The basis of reduced phosphorylation is unclear but is presumed to be due to altered huntingtin conformation with polyglutamine expansion. Through the use of phosphorylation-site mutants and manipulation of the relevant kinases, phosphorylation of S421 has been shown to substantially reduce toxicity in cultured striatal neurons and in a rodent model of HD⁷⁷⁻⁸⁰. What is the basis for this? Huntingtin normally associates with

vesicle membranes and microtubules and contributes to endocytosis and transport of endocytic vesicles⁸¹⁻⁸⁶. Indeed, huntingtin might mediate retrograde transport of signalling endosomes that carry brain-derived neurotrophic factor (BDNF)⁸⁶. This is crucial, as BDNF signalling supplied by cortico-striatal projection neurons to the striatum — the brain region most severely affected in HD — is an important survival factor^{87,88}. Although it has been known for some time that polyglutamine expansion impairs the ability of huntingtin to support BDNF signalling, the basis for this was unclear⁸⁶. However, an explanation is offered by the recent findings that the phosphorylation status of S421 modulates the recruitment of motor proteins to endocytic vesicles, is a key determinant of huntingtin regulation of vesicular transport and is altered by polyglutamine expansion. Indeed, constitutive phosphorylation of S421 can overcome the BDNF signalling defect caused by polyglutamine expansion and can suppress toxicity in HD models^{89,90}. These data show that the alteration of post-translational modification by repeat expansion can affect the ratio of native protein-protein interactions. Therefore, as in the case of SCA1, the pathogenesis of HD seems to be mediated, at least in part, by the alteration of native protein function.

Additional phosphorylation sites in huntingtin have been implicated as modifiers of disease, although less is known about how they are influenced by polyglutamine expansion or the mechanisms by which they influence pathogenesis. For example, phosphorylation at S343 and S536 is associated with reduced cleavage of huntingtin, and phosphorylation at threonine 3 is associated with reduced aggregation of huntingtin amino-terminal fragments⁹¹⁻⁹³. Notably, the phosphorylation statuses of S13 and S16 have recently been implicated as important determinants of HD pathogenesis. Gu and colleagues generated transgenic mice that express full-length mutant huntingtin with S13 and S16 mutated to either aspartate (which mimics constitutive phosphorylation) or alanine (which is resistant to phosphorylation)⁹⁴. Both mutant proteins retain normal huntingtin function, as

Endocytosis

The process whereby cells engulf extracellular material through invagination of the plasma membrane to create an endocytic vesicle.

Neurotrophic factor

A small protein that promotes the growth and/or survival of neurons.

shown by their ability to rescue huntingtin knockout phenotypes. Mice expressing the phosphorylation-resistant protein exhibited typical neurodegeneration with associated motor and behavioural phenotypes. By contrast, mice expressing the phosphomimetic protein did not exhibit neurodegeneration, showing the dramatic impact of this post-translational modification on toxicity. How might phosphorylation of S13 and S16 influence pathogenesis? Thompson and colleagues recently found that these serines are phosphorylated by the I κ B kinase (IKK) complex. Through promoting modification of adjacent lysine residues, phosphorylation by IKK targets huntingtin for degradation by a process that requires both the proteasome and lysosome⁹⁵. Phosphorylation has also been implicated in influencing the toxicity of SCA3, dentatorubral-pallidoluysian atrophy (DRPLA) and SBMA. For example, phosphorylation of ataxin 3 at S256 by glycogen synthase kinase 3 β reduces aggregation of polyglutamine-expanded ataxin 3 (REF. 96), phosphorylation of polyglutamine-expanded androgen receptor by mitogen-activated protein kinase at S516 is associated with increased cleavage and toxicity in cell models of SBMA⁹⁷, and S734 of atrophin 1 is phosphorylated by Jun N-terminal kinase, although the significance of this in the pathogenesis of DRPLA has not yet been examined.

Acetylation as a determinant of stability. Post-translational protein acetylation takes place on the ϵ -amino group of lysines and prevents this group from becoming positively charged, therefore affecting the electrostatic properties of the protein⁹⁹. Acetylation has diverse consequences, including altered protein–DNA and protein–protein interactions. Acetylation can compete with ubiquitylation at lysine residues, such that acetylation increases protein stability⁹⁸. For example, acetylation of ataxin 7 at lysine 257 (K257) was recently found to stabilize the protein and promote accumulation of the ataxin 7 N-terminal truncation product, which is believed to be the toxic, disease-causing species⁹⁹. Acetylation can also, occasionally, reduce protein stability¹⁰⁰.

Krainc and colleagues found that huntingtin stability is regulated by acetylation at K444 (REF. 101). Interestingly, they found that acetylation facilitates trafficking of mutant huntingtin into autophagosomes, therefore serving as a signal for degradation by the autophagy–lysosomal system rather than influencing proteasomal degradation. Acetylation accelerates clearance of the mutant huntingtin by autophagy and reverses its toxic effects in mouse primary striatal and cortical neurons and in a *Caenorhabditis elegans* model of HD. When polyglutamine-expanded huntingtin was rendered resistant to acetylation, the protein steadily accumulated and increased degeneration in cultured neurons and in mouse brain. This study provided the first evidence that acetylation can target proteins for autophagic degradation. However, the mechanism of targeting for degradation remains to be determined, as does the extent to which proteins other than huntingtin use this mechanism.

Sumoylation as a determinant of cell-type vulnerability. Sumoylation is a reversible post-translational modification in which a small ubiquitin-like modifier (SUMO) is covalently conjugated to a lysine residue in a target protein. It provides an efficient way to modulate the subcellular localization, activity and stability of a wide variety of substrates. A role for sumoylation in polyglutamine disease was first suggested because of SUMO immunoreactivity in pathological inclusions characteristic of HD, SCA3 and DRPLA. Thompson and colleagues reported that sumoylation of huntingtin at K6 and K9 stabilizes N-terminal fragments of huntingtin, reduces its ability to form aggregates and promotes its capacity to repress transcription¹⁰². They also noted that sumoylation of a polyglutamine-expanded huntingtin fragment exacerbated neurodegeneration in a *D. melanogaster* model of HD.

Recently, huntingtin sumoylation was found to depend upon the small guanine nucleotide-binding protein RHES by a mechanism that is independent of RHES GTPase activity¹⁰³. RHES preferentially binds polyglutamine-expanded huntingtin and promotes sumoylation at various lysines, including K9, K15 and K91. Sumoylation of mutant huntingtin at K15 and K91 correlated with reduced aggregation and increased cytotoxicity. RHES is expressed only in the striatum, and endogenous RHES copurified with huntingtin in extracts from transgenic mice, suggesting that the interaction of these proteins takes place under physiological conditions. Therefore, RHES–huntingtin interactions may account for the localized neuropathology of HD, which is largely restricted to the corpus striatum. Other polyglutamine disease proteins — including ataxin 1, androgen receptor, TATA box-binding protein (TBP) and atrophin 1 — are sumoylated under physiological conditions, and in some cases this has been correlated with increased toxicity in cell culture disease models. However, the extent to which sumoylation influences pathogenesis in these diseases is yet to be explored.

Bidirectional transcription

The two faces of spinocerebellar ataxia type 8. SCA8 is a progressive, dominantly inherited disease characterized by ataxia in combination with signs of corticospinal tract dysfunction¹⁰⁴. SCA8 was found to be caused by expansion of a CTG repeat in a gene that is transcribed and not translated¹⁰⁵. Therefore the researchers proposed that expression of the expanded repeat was neurotoxic in the form of RNA analogous to the mechanism observed in DM1 and DM2. Initially, this hypothesis was not widely accepted, but *D. melanogaster* and mouse models were generated that showed that RNA with the CTG expansion was indeed toxic^{106,107}.

However, further analysis of the SCA8 BAC transgenic mice revealed an unexpected finding — 1C2-positive intranuclear inclusions of the type that would be detected in polyglutamine expansion diseases¹⁰⁶. CTG is read as CAG on the opposite strand, and re-examination of the antisense sequence indicated the potential translation of a protein that consists almost

entirely of polyglutamine. Examination of samples from patients with SCA8, SCA8 transgenic mice and cell culture showed that a transcript (*ATXN8*) encodes the predicted polyglutamine-rich protein — now called ataxin 8 (REF. 106). The authors proposed that polyglutamine pathology and RNA gain-of-function toxicity combine to cause the SCA8 phenotype. Recently this group has shown that the ataxin 8 antisense transcript (ataxin 8 opposite strand (*ATXN8OS*)) might sequester the MBNL1 protein¹⁰⁸. MBNL1 sequestration was shown to alter the splicing of *Gabt4* (also known as *Slc6a11*), which encodes a transporter of the inhibitory neurotransmitter GABA (γ -aminobutyric acid). Altered splicing of *Gabt4* resulted in increased GABT4 protein and correlated with the predicted loss of GABAergic inhibition in the cerebellar granule layer of SCA8 mice. The discovery of MBNL1 sequestration and a splicing defect in this SCA8 model reveals unexpected similarity to the pathogenic mechanism responsible for DM1 and DM2. A caveat to this study is that so far the splicing of only a limited number of genes has been examined in detail. As the splicing defect and the relationship to MBNL1 deficiency are more fully explored it will be interesting to learn the extent to which the splicing defect underlies the neurodegenerative phenotype. For example, to what extent will restoration of MBNL1 levels in SCA8 mice rescue the neurodegenerative phenotype?

Multiple mechanisms in Huntington’s disease-like 2?

Another repeat disease that is difficult to classify by pathogenic mechanism is Huntington’s disease-like 2 (HDL2), a phenocopy of HD¹⁰⁹. HDL2 is caused by a CTG repeat expansion in the junctophilin 3 (*JPH3*) gene; affected individuals have 40 or more CTGs¹⁰⁹. JPH3 belongs to a family of proteins that link plasma membrane voltage sensors with intracellular ion channels¹¹⁰. It has been a daunting challenge to determine how the *JPH3* CTG repeat expansion produces molecular pathology, as it lies in the variably spliced exon 2A. Three splice variants have been reported: one places the CTG repeat in a polyglutamine translational frame, one places it in a polyalanine translational frame and one places it in the 3’ UTR¹¹¹.

Database queries do not show evidence for transcription of the opposite, CAG repeat-containing strand. Nevertheless, ubiquitin-positive neuronal intranuclear inclusions (that incidentally do not contain huntingtin) are observed, which suggests the deposition of misfolded protein due to polyglutamine or some other poly-amino acid tract. RNA foci containing MBNL1 have been detected in HDL2 brain, and the expression of CUG-expanded *JPH3* in cell culture also causes RNA foci containing MBNL1 (REF. 111). Furthermore, altered splicing of genes encoding tau and amyloid precursor protein occurs in HDL2 frontal cortex. Therefore, it seems that CUG RNA toxicity, together with some form of proteotoxicity, may combine in HDL2 to produce neurodegeneration. Hence, SCA8 and HDL2 seem to belong to a category of diseases that involve simultaneous RNA gain-of-function and protein gain-of-function molecular pathology (FIG. 2).

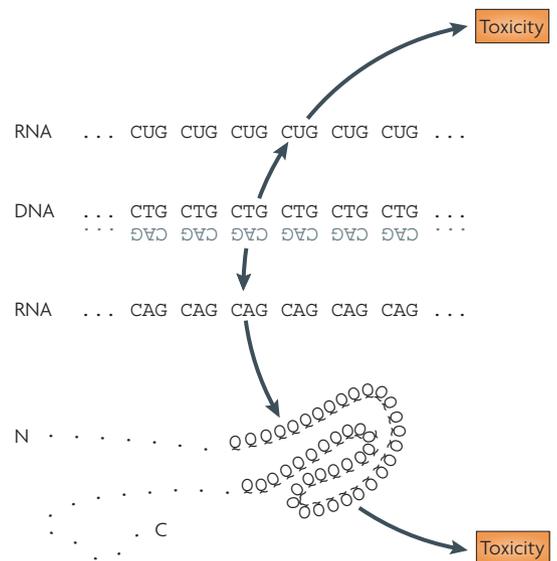


Figure 2 | Sense and antisense toxicity. For a subset of repeat expansion diseases, sense and antisense transcripts are produced from the repeat region. In these cases, one transcript may cause toxicity through an RNA gain-of-function pathway and the other transcript, which is generated in opposite orientation, may yield a toxic poly-amino acid tract-containing protein. In the example shown here for spinocerebellar ataxia type 8 (SCA8), a transcript containing a CUG repeat expansion is suggested to produce toxicity, and a polyglutamine protein encoded from an antisense transcript is predicted to simultaneously produce toxicity. Even for diseases, such as SCA3, that do not exhibit bidirectional overlapping transcription, concomitant RNA toxicity from the RNA transcript containing the CAG repeat expansion has been proposed. Therefore the potential toxic contribution of different gene products (RNA and proteins) from the sense strand must be considered in addition to the potential toxic contribution of different gene products from antisense overlapping transcription. C, carboxyl terminus; N, amino terminus.

In the case of HDL2, a third possible simultaneous mechanism is partial loss of function of JPH3, as *Jph3*-null mice and heterozygous-null mice show impaired coordination and shortened lifespan¹¹², and patients with HDL2 express reduced levels of JPH3 (REF. 111). However, knockout of *Jph3* in mice does not cause RNA foci or ubiquitin-positive inclusions, suggesting that *Jph3* haploinsufficiency alone does not account for HDL2.

Genomic structure and antisense transcription

Up to this point, we have considered the potential contribution of the transcriptional and translational gene products to disease pathogenesis. However, unique aspects of the repeat disease loci are also emerging at the level of DNA sequence and chromatin organization. Most repeat expansion diseases involve gene loci with highly polymorphic repeats in normal individuals; both the median and mode of repeat size are quite large, even though the repeats are non-pathogenic. CTG repeats are potent nucleosome positioning elements¹¹³, so it seems

Nucleosome
The basic unit of chromatin. A nucleosome contains approximately 146 bp of DNA wrapped around a histone octamer.

Heterochromatin

Parts of chromosomes with an unusual degree of contraction and that consequently have different staining properties from euchromatin at nuclear divisions. Largely composed of repetitive DNA, heterochromatin forms dark bands after Giemsa staining.

plausible that expanded repeats could influence the chromatin organization. Filippova *et al.* identified binding sites for the CCCTC-binding factor *CTCF* — an evolutionarily conserved zinc finger DNA-binding protein with activity in chromatin insulation, transcription regulation and genomic imprinting¹¹⁴ — adjacent to and/or flanking some triplet repeat loci¹¹⁵. *CTCF*-binding sites can now be identified by directed approaches (such as electrophoretic mobility shift assays) and discovery-driven approaches (such as chromatin immunoprecipitation followed by microarray (ChIP–chip))^{116–118}, and evidence for *CTCF* binding in close proximity to the repeat sequences is emerging. At the *DM1* locus, *CTCF* binding prevents the spread of heterochromatin into the *DM1* gene by blocking antisense transcription¹¹⁹. Hyperexpansion of the CTG repeat — as in congenital DM1 — correlates with loss of *CTCF* binding and expansion of the region of antisense transcription, which results in heterochromatinization of DM1 and adjacent genes. In some cases, *CTCF* binding seems to be associated with antisense transcription^{119,120}, but in DM1 and Friedreich's ataxia *CTCF* depletion may permit production of an antisense transcript, resulting in heterochromatin formation and gene silencing^{119,121}. *CTCF* has also been implicated in genetic instability, which occurs in most repeat expansion diseases¹²². Abrogation of *CTCF* binding at the *SCA7* locus by point mutation or methylation of the binding site promotes CAG repeat instability in *SCA7* transgenic mice, both in the germ line and in somatic tissues¹²³.

Surveys of mammalian transcriptomes are uncovering tremendous numbers and varieties of non-coding RNAs, including antisense transcripts at the start sites of sense transcription and in adjacent 5' promoter regions^{124–127}, and this phenomenon seems to be prominent at repeat disease loci. For example, at the *FMR1* locus, an antisense transcript overlapping the CGG repeat in opposite orientation has been identified¹²⁸. This transcript (antisense fragile X mental retardation 1 (*ASFMR1*)) is upregulated in patients carrying premutation alleles and seems to be silenced in full mutation carriers. Altered antisense transcription might be a characteristic of some repeat disease loci and raises important questions about the relationship between non-coding and coding transcription and how it is influenced by changes in repeat length and chromatin structure. *CTCF*- and

non-coding-transcript-mediated regulation of genomic architecture could have an important role in transcription dysregulation in many repeat expansion diseases, especially as evidence of altered epigenetic regulatory processes in certain of the polyglutamine diseases is increasing^{129,130}. We expect that a considerable amount of information on this topic will emerge over the next few years.

Conclusions

Enormous strides have been made since the initial discovery of a novel, esoteric type of mutation as the cause of SBMA and FMR1. Repeat expansion research has revealed unexpected complexities, including toxicity mediated at the protein, RNA and genomic levels. Perturbations of numerous cellular functions have been implicated in disease pathogenesis — for example, transcription, splicing, translation, mitochondrial function and protein quality control, in addition to specific neuronal functions, such as axonal transport and synaptic activity. This complexity has attracted a diverse group of scientists and has catalysed the development of interdisciplinary approaches for elucidating the mechanisms of disease. As a result, research in the repeat expansion field has often been at the vanguard of key advances in molecular genetics. For example, repeat disease research has a leading role in elucidating the basis of defective RNA metabolism and autophagy in human disease.

A nagging question for the repeat disease field is why the development of meaningful treatments has not kept pace with the remarkable trajectory of advances in the study of mechanisms and pathways. Although this disconnect applies to most neurological disorders, the repeat diseases offer a wonderful opportunity for rational therapy development because presymptomatic definitive diagnosis based on repeat allele expansion can normally be made. Discoveries over the past few years have yielded a promising crop of candidate targets and pathways and have led to some very encouraging therapeutic developments, including the use of oligonucleotide and morpholino antisense repeats targeted against the DM1 CTG expansion and the use of small molecules to augment autophagy^{131–133}. There can be no doubt that the successful application of one or more of these strategies as a therapy for a repeat expansion disease would be the most crucial advance that could come out of this field.

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Competing interests statement

The authors declare no competing financial interests.

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