Toxic RNA in Pathogenesis of Human Neuromuscular Disorders

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Abstract In the past decade, substantial progress has been made in our understanding of the molecular pathogenesis of microsatellite expansion disorders. Some of these diseases, such as myotonic dystrophy, fragile X-associated tremor/ataxia syndrome, spinocerebellar ataxias 8, 10, 12 and Huntington's disease-like 2, are caused by an increased number of tri-, tetra- or pentanucleotide repeats in noncoding regions of the mutated genes. An RNA gain-of-function mechanism has been proposed to explain how mutations in noncoding fragments of genes lead to a disease phenotype. A number of studies demonstrated that RNA molecules harboring long repeating sequences affect the metabolism of other RNAs via deregulation of RNA processing proteins. This chapter presents the current status of knowledge related to mechanisms of pathogenesis, involvement of RNA structures and specific RNA binding proteins in seven neurodegenerative diseases associated with an RNA gain-of-function mechanism. Potential therapeutic strategies directed towards toxic RNAs are also reviewed.

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1 Introduction

Expansions of short, tandemly repeated microsatellite sequences – most frequently trinucleotide but also tetra- and pentanucleotide repeats – are responsible for more than 20 human neurological and neuromuscular disorders (Orr and Zoghbi 2007). Microsatellite expansion diseases can be divided into two categories: loss-of-function diseases resulting from silencing of gene expression (e.g., fragile X syndrome or Friedreich's ataxia) and gain-of-function disorders resulting from various changes in the properties of the mutated gene products (e.g., Huntington's disease, myotonic dystrophies or spinocerebellar ataxias) (Ranum and Cooper 2006).

Two different types of gain-of-function mechanisms can be distinguished. First, a protein gain-of-function mechanism is related to the aberrant functions of mutated proteins harboring an expanded domain of homoaminoacids. Second, an RNA gain-of-function mechanism is associated with repeat expansions within noncoding regions of the genes. Synthesis and accumulation of toxic RNAs, containing long repeating tracts, lead to complex pathological changes in cellular metabolism.

The role of toxic RNAs was initially recognized in the pathogenesis of myotonic dystrophy type 1 and type 2 (DM1 and DM2). More recently, an RNA gain-of-function mechanism was demonstrated in the fragile X-associated tremor/ataxia syndrome (FXTAS). Several lines of evidence also suggest that spinocerebellar ataxias 8, 10 and 12 (SCA8, 10 and 12) as well as Huntington's disease-like 2 (HDL2) may result from expression of toxic RNAs (Table 1) (Osborne and Thornton 2006; Ranum and Cooper 2006).

Since the RNA and protein gain-of-function mechanisms are not mutually exclusive, a disease phenotype can also result from a combination of the two mechanisms.

In this chapter, we will review recent advances in the field of RNA-mediated microsatellite expansion disorders with special emphasis on the structure of repeat containing RNAs, the molecular mechanism of the RNA toxicity, and possible therapeutic strategies targeted towards pathologic RNAs.

Table 1 Diseases associated with the RNA gain-of-function mechanism

				Repeat size		
Disease	Gene	Repeat RNA	Locus	Normal	Pathological	Location
DM1	*DMPK*	CUG	19q13	5–37	50–>4,000	3′UTR
DM2	*ZNF9*	CCUG	3q21	<27	75–11,000	Intron
FXTAS[a]	*FMR1*	CGG	Xq27.3	5–45	55–200	5′UTR
	ASFMR1	CCG				?
SCA8[a]	*ATXN8OS*	CUG	13q21	16–34	71–>1,000	3′UTR/coding
	ATXN8	CAG				
SCA10	*ATXN10*	AUUCU	22q13	10–29	280–4,500	Intron
SCA12	*PPP2R2B*	CAG	5q32	7–32	55–78	5′UTR/intron/ coding?
HDL2	*JPH3*	CUG	16q24	6–28	40–59	3′UTR/coding?

[a] The role of bidirectional expression is postulated

2 Properties of Transcripts Containing Microsatellite Repeats

2.1 RNA Structure of Repeating Microsatellites

A small group of repeat sequences – including certain tri-, tetra- and pentanucleotide microsatellites – is associated with RNA gain-of-function disorders. RNAs containing CUG, CAG, CGG, CCG, CCUG and AUUCU repeats are implicated in the pathogenesis of DM1, DM2, SCA8, SCA10, SCA12, FXTAS, and HDL2 (Table 1). Generally, these repeat tracts are located in the noncoding sequences: introns and 5′ or 3′ untranslated regions (UTR). In the case of SCA12, depending upon the site of transcription initiation, the CAG tract is located either in the 5′UTR or upstream of the promoter region, or in the intron of the *PPP2R2B* gene (Holmes et al. 1999, 2001, 2006). In HDL2, the CUG repeats can be a part of the coding sequence or the 3′UTR, depending on the alternative splicing pattern of the *JPH3* gene (Margolis et al. 2006b).

Over the past ten years, the structural properties of transcripts containing microsatellite repeats have been analyzed using several biochemical and biophysical methods (Napierala and Krzyzosiak 1997; Michalowski et al. 1999; Tian et al. 2000; Handa et al. 2003; Sobczak et al. 2003; Sobczak and Krzyzosiak 2004b; Napierala et al. 2005; Zumwalt et al. 2007). Strikingly, all of these studies documented that each repeating sequence (tri-, tetra- and pentanucleotide) associated with the RNA gain-of-function mechanism formed a stable hairpin structure in vitro. Stability of repeat-containing hairpins depends on the length of the repeat tract and its sequence composition, i.e., repeating motif, presence of sequence interruptions disturbing the homogeneity of the repeat tract, and sequence context of the microsatellite region (Napierala and Krzyzosiak 1997; Handa et al. 2003; Sobczak et al. 2003; Sobczak and Krzyzosiak 2004a, 2005; Broda et al. 2005; Napierala et al. 2005; Zumwalt et al. 2007). Hairpins formed by trinucleotide repeats are stabilized by C-G and G-C pairs separated by single mismatches. NMR and thermodynamic analyses have demonstrated the existence of hydrogen bonds between N-N (N = A, C, G or U) mismatches (Broda et al. 2005). However, the overall thermodynamic stability of the hairpin stem does not depend significantly on the sequence composition of the mismatch.

Using biochemical methods and NMR spectroscopy, three independent groups demonstrated the formation of stable hairpins by CGG RNAs (Handa et al. 2003; Napierala et al. 2005; Zumwalt et al. 2007). AGG interruptions of the pure CGG tract, naturally occurring in human *FMR1* mRNA, influence conformation of the transcript and have an impact on the thermodynamic stability of the CGG repeat-containing RNAs (Napierala et al. 2005; Zumwalt et al. 2007). It should be pointed out that formation of four-stranded RNA structures (tetraplexes) by the CGG tracts has also been postulated (Khateb et al. 2007).

Crystal structure analysis of the 18-bp antiparallel RNA duplex containing CUG repeats showed a conformation similar to the A form of RNA with the exception of U-U mismatches (Mooers et al. 2005). Mismatched bases are an important element of the RNA recognition by specific binding proteins (Sect. 2.2).

The tetranucleotide CCUG repeats that are expanded in DM2 were demonstrated to adopt stable hairpins composed of C-G and G-C base pairs separated by C-U and U-C mispairs (Sobczak et al. 2003). CCUG repeat hairpin formation has important implications in the molecular pathogenesis of DM2. The apparent similarities between DM2 and DM1 result from the same type of structure adopted by their corresponding toxic RNAs.

Unexpectedly, even the U-rich pentanucleotide sequence, AUUCU, which is expanded in SCA10 patients, is capable of forming unusual RNA hairpins. Using NMR, CD and enzymatic probing, Handa et al. proved that tracts as short as 11 repeats exist in a hairpin conformation stabilized by an equal number of hydrogen bonded A-U and U-U pairs (Handa et al. 2005).

In summary, the ability to form hairpin structures is a common property of toxic RNAs. Although RNA conformation analyses were conducted in vitro, the existence of these structures in the cellular environment is supported by the demonstration of co-localization of the expanded transcripts with structure-specific RNA binding proteins (RBPs) (Fardaei et al. 2001, 2002). Formation of hairpins containing long and stable double-stranded stems may lead to a variety of biological effects, including direct inhibition of translation, aberrant interaction with specific proteins, enzyme activation, and regulation of gene expression via the RNA interference (RNAi) pathway. Some of these processes are indeed affected by toxic RNAs (Sects. 3–5).

2.2 Proteins Binding to the Repeated Sequences in RNAs

RNA molecules that contain repeating sequences as well as hairpin structures formed by microsatellites are recognized by RNA binding proteins (Table 2). Interactions between RBPs and transcripts containing expanded repeats are believed to be the major step in the molecular pathogenesis of the RNA gain-of-function disorders.

CUG repeat binding protein 1 (CUGBP1) was the first protein identified in gel retardation assays to bind to a $(CUG)_8$ probe (Timchenko et al. 1996a,b). This protein is a member of CELF (CUGBP1 and ETR-3 Like Factors, BRUNOL) family of RNA metabolism regulators. The CELF proteins (CELF1-6) participate in control and regulation of alternative splicing, translation and deadenylation (Ladd et al. 2001; Barreau et al. 2006; Thornton et al. 2006). CUGBP1 (CELF1) shows 76% sequence identity with CUGBP2 (CELF2, ETR-3), another protein which has been demonstrated to bind CUG repeats and play a role in the pathogenesis of DM1 (Lu et al. 1999). However, recent studies have questioned the binding of the CELF proteins, especially CUGBP1, to the CUG repeats. Experiments conducted using the SELEX procedure, the yeast three-hybrid system and surface plasmon resonance (SPR) showed that CUGBP1 interacts preferentially with UG/GU-containing transcripts (Kino et al. 2004; Marquis et al. 2006; Mori et al. 2008). More importantly, CUGBP1 demonstrated preference for single-stranded RNAs, which suggests that double-stranded CUG or CCUG hairpins are unlikely targets for its binding (Kino et al. 2004).

Table 2 Proteins interacting with microsatellite-containing transcripts

	Protein	Sequence preference[a]	Conformational preference[a]	Disease
MBNL family				
	MBNL1	CYG and CYYG	dsRNA with mismatches	DM1, 2
	MBNL2 (MBLL)	CCUG, CCCG	?	DM1, 2
CELF family				
	CUGBP1 (CELF1, BRUNOL2)	UG/GU	ssRNA	DM1, 2, FXTAS
	CUGBP2 (ETR3, BRUNOL3)	UGGU	?	DM1, 2
PKR (p20)		CUG, CAG	perfect dsRNA	DM1, 2
Purα		CGG, NGG	ssRNA	FXTAS
hnRNP A2/B1		CGG, telo-eric repeats	?	FXTAS
hnRNP H		CUG[b]	dsRNA	DM1, 2

[a] The studies did not include analyses of all possible repeating sequences

[b] Efficient binding requires the presence of specific non-repeating sequences

Despite the controversies related to the CUGBP1 binding specificity, CUGBP1 is certainly involved in molecular pathogenesis of DM1, DM2 and most likely other RNA gain-of-function diseases. It has been demonstrated that expression of expanded CUG repeats increases the half-life of CUGBP1 in cultured cells (Timchenko et al. 2001). Moreover, recent studies showed that the stabilization of CUGBP1 – and consequently its increased activity – are the result of increased phosphorylation of CUGBP1 in DM1 tissues (Kuyumcu-Martinez et al. 2007).

Further studies of proteins capable of binding CUG repeats revealed a family of muscleblind-like proteins (MBNL) (Fardaei et al. 2001, 2002; Ho et al. 2004). This group of zinc finger proteins, which are homologous to the *Drosophila* muscleblind proteins, consists of three members: MBNL1 (EXP), MBNL2 (MBLL) and MBNL3 (MBXL). Although all three proteins were shown to colocalize with RNA foci in DM1 and DM2 patient cells, the majority of RNA binding studies have been conducted with MBNL1 (Fardaei et al. 2002). This protein strongly prefers double-stranded, mismatch-containing RNA helices. The consensus MBNL1 binding sequences are CYYG and CYG (Y = C or U) (Kino et al. 2004; Warf and Berglund 2007). Perfect A-RNA duplexes (e.g., CAG-CUG) almost completely abolish the MBNL1 binding. Recently, Yuan et al. showed that MBNL1 forms oligomeric complexes which are capable of binding to double-stranded CUG repeats (Yuan et al. 2007). At higher protein to RNA ratios, multiple complexes bind to a single RNA molecule. This study provided a model explaining a mechanism of MBNL1 sequestration and formation of nuclear foci by expanded CUG/CCUG repeats.

Double-stranded, repeat-containing RNAs are also binding targets for the dsRNA-dependent protein kinase, PKR (p20) (Tian et al. 2000, 2005). PKR was demonstrated to bind expanded CUG repeats. Moreover, in vitro interactions

between the CUG RNA and PKR lead to activation of PKR enzymatic activity (Tian et al. 2000, 2005). In vivo binding studies, using a yeast three-hybrid system, showed that dsRNAs lacking mismatches (e.g., CUG-CAG duplex) as well as CAG repeats interacted with PKR more efficiently than CUG tracts (Kino et al. 2004).

UV crosslinking experiments have led to the isolation of the heterogeneous nuclear ribonucleoprotein H (hnRNP H) associated with RNAs containing CUG repeats (Kim et al. 2005). This protein is known to participate in various processes related to RNA metabolism including splicing and polyadenylation (Chen et al. 1999; Arhin et al. 2002). HnRNP H is not a typical repeat binding protein. For efficient binding hnRNP H requires not only an expanded CUG tract (>46 repeats), but also a sequence containing a splice branch point. In DM1 cells upregulation of hnRNP H facilitates nuclear retention of the mutant *DMPK* transcript and stimulates aberrant splicing (Kim et al. 2005). Interestingly, hnRNP H was shown to interact in vivo with MBNL1 in an RNA-independent fashion and to form an RNA-dependent complex with the CUGBP1 (Paul et al. 2006).

None of the proteins described so far was capable of efficient binding to the CGG repeats expanded in patients with FXTAS. Recently, two groups reported the discovery of two proteins, purine-rich binding protein (Purα) and cytoplasmic fraction of hnRNP A2/B1, which interacted directly with CGG tracts, suggesting their role in molecular pathogenesis of FXTAS (Jin et al. 2007; Sofola et al. 2007b). Interestingly, CUGBP1 co-immunoprecipitated with the hnRNP A2/B1, which indicates an indirect association of the CUGBP1 with CGG repeats and a possible functional link between the molecular mechanism of DM and FXTAS (Sofola et al. 2007b).

The role of repeat binding proteins in the pathogenesis of RNA gain-of-function disorders will be presented later in this chapter (Sects. 3 and 4).

2.3 Bidirectional Expression of Genes Coding for Toxic RNAs

RNA toxicity originates from the expression of genes containing expanded CG-rich repeats. Alternatively, transcription of the gene encoded on the opposite DNA strand can lead to the accumulation of a completely different mRNA molecule containing a complementary repeating sequence. Taking into account that 61% of all human transcribed regions have a counterpart on the opposite DNA strand, the likelihood of antisense transcription is quite high (Cheng et al. 2005). Indeed, in the case of three RNA gain-of-function disorders such antisense transcripts have been detected and their putative role in molecular pathogenesis has been postulated.

In the case of DM1, an antisense transcript to the *DMPK* RNA was identified using sense-specific RT-PCR. It contained several open reading frames, but none of them encoded a polyglutamine tract. Cho et al. discovered that upon transcription of the antisense strand of the *DMPK* gene, short 21-mer RNAs can be isolated (Cho et al. 2005). These RNA fragments induced localized chromatin changes via histone H3-K9 methylation and recruitment of the heterochromatin protein 1 (HP1). The

influence of the chromatin modifications at the CTG repeat region on the disease development is unclear and requires further investigation.

SCA8 represents a very interesting case of bidirectional transcription. Two genes spanning the trinucleotide CTG repeat tract are transcribed in opposite directions. Noncoding CUG repeats are part of the *ataxin 8 opposite strand* gene (*ATXN8OS*, originally *SCA8* or *KLHL1AS*), while in the recently discovered *ataxin 8* gene (*ATXN8*) CAG repeats encode a polyglutamine domain (Moseley et al. 2006). Pathological implications of this phenomenon are described in the Sect. 5.1.

Recently, an antisense transcript has been identified at the human *FMR1* locus. A novel gene, *ASFMR1* (*antisense transcript at the FMR1 locus*), overlaps with the CGG repeats of the *FMR1* gene in the antisense direction (Ladd et al. 2007). The antisense transcript is polyadenylated, has multiple splice forms, can be found in cytoplasm, and is widely expressed in human tissues. A putative open reading frame (ORF) contains a domain composed of polyproline. Since both CCG and CCU triplets code for proline, the presence of AGG interruptions in the CGG sequence (coding strand for *FMR1*) does not disrupt the purity of the putative homoaminoacid tract. Interestingly, in a pattern similar to the *FMR1* mRNA, expression of the *ASFMR1* transcript is elevated in cells harboring premutation alleles (55–200 repeats) and is silenced in the full mutation alleles (>200 repeats) (Ladd et al. 2007).

In summary, preliminary evidence suggests that bidirectional transcription may be an important aspect of toxic RNA metabolism.

3 Myotonic Dystrophy Type 1 and 2 (DM1 and DM2)

Myotonic dystrophy type 1 is the best characterized RNA gain-of-function-mediated disorder. Expansions of CTG repeats in the 3′UTR of the *DMPK* (*dystrophia myotonica protein kinase*) gene cause DM1, a multisystem disease that affects predominantly skeletal muscles, heart, eyes and endocrine system (Brook et al. 1992; Harper 2001). Like other microsatellite expansion disorders, the severity and age of onset of the DM1 correlate with the number of repeats. Shorter alleles containing 50–100 CTG repeats result in late onset cataract, alleles containing 100–500 repeats result in the classic form of DM1, while the largest expansions (over 500 CTG repeats) lead to a congenital form of the disease which manifests with infantile hypotonia and mental retardation. The classic form of DM1 is characterized by adult-onset myotonia, muscle weakness, cataract, cardiac conduction defects and endocrine abnormalities. Similar clinical features are characteristic for myotonic dystrophy type 2 (DM2), which is caused by expansions of the CCTG repeats in the first intron of the *ZNF9* (*zinc finger 9*) gene. Although the number of expanded repeats in DM2 is dramatically higher than in DM1, the general clinical presentation of DM2 is milder than DM1. Overlapping clinical features of DM1 and DM2 include myotonia, muscle weakness, cataract, cardiac arrhythmias, insulin resistance and testicular atrophy. Notably, DM2 does not present in a congenital form and is not associated with mental retardation.

3.1 Molecular Pathogenesis of Myotonic Dystrophy

How do mutations in the noncoding regions of two functionally unrelated genes lead to a similar clinical presentation? Although most of the myotonic dystrophy features can be explained by a gain-of-function effect of mRNA with expanded CUG or CCUG repeats, the molecular mechanism of myotonic dystrophy may be more complex.

3.1.1 Unlikely Role of *DMPK* and *ZNF9* Loss of Function

Both myotonic dystrophy type 1 and type 2 are inherited in a dominant mode, which can suggest a haploinsufficiency mechanism of a disease. The loss-of-function mechanism has also been suggested, based on the observation that in DM1 cells the expression of *DMPK*, as well as the adjacent *DMWD* and *SIX5* genes is decreased (Klesert et al. 1997; Thornton et al. 1997; Alwazzan et al. 1999; Eriksson et al. 1999). However, human data and mouse model studies indicate that loss-of-function is not the predominant molecular defect in myotonic dystrophy. First, no mutations in coding regions of either *DMPK* or *ZNF9* genes have been found in myotonic dystrophy patients. Secondly, mice deleted for the *Dmpk* gene do not reproduce the multisystem phenotype. Two research teams independently generated *Dmpk* knockout mice by targeted deletion of the first seven exons of the *Dmpk* gene (Jansen et al. 1996; Reddy et al. 1996). This mutation results in complete loss of the Dmpk protein in homozygous mice. *Dmpk* null mice demonstrated only mild cardiac conduction defects, late-onset myopathy (Jansen et al. 1996; Reddy et al. 1996) and insulin signaling defects (Llagostera et al. 2007). None of the *Dmpk* knockout mouse lines demonstrated the hallmark features of myotonic dystrophy such as myotonia, cataract and endocrine abnormalities. Furthermore, deficiency of the *Six5* gene in mice also failed to reproduce the features of myotonic dystrophy (Klesert et al. 2000; Sarkar et al. 2000).

ZNF9, the gene which is mutated in DM2 (Liquori et al. 2001), encodes a nucleic acid binding protein that is involved in cap-independent translation (Gerbasi and Link 2007). A recent report of *Znf9*$^{+/-}$ mice demonstrated that *Znf9* haploinsufficiency leads to heart, muscle and eye defects similar to these observed in myotonic dystrophy (Chen et al. 2007). While this is compelling evidence that a loss of the *Znf9* function leads to DM-like phenotype, human data provide evidence for a different conclusion. Analyses of *ZNF9* expression in myoblast cell lines from DM2 patients demonstrated that large CCUG expansions do not affect steady-state *ZNF9* mRNA levels (Margolis et al. 2006a), arguing against the *ZNF9* loss-of-function mechanism.

3.1.2 Evidence for the RNA Gain-of-Function Mechanism

Although deficiency of the *DMPK* or *ZNF9* genes, which are highly expressed in tissues affected in myotonic dystrophy, may contribute to the clinical manifestation of the disease, the major mechanism underlying the multisystem presentation is a

toxic effect of mRNA molecules containing expanded CUG or CCUG repeats. The RNA gain-of-function mechanism had been postulated even before the identification of the *ZNF9* gene as the second gene mutated in some cases of myotonic dystrophy (Philips et al. 1998).

The first clues for a role of mRNAs in the pathogenesis came from analyses of the intracellular localization of *DMPK* mRNA in myoblastic and fibroblastic cells from DM1 patients. Using probes specific for expanded CUG repeats, Taneja et al. demonstrated that mutant *DMPK* transcripts accumulate in nuclei in multiple discrete foci (Taneja et al. 1995). Additional observations revealed that the localization of these foci is different from the site of *DMPK* transcription, and that the mutant *DMPK* mRNA is normally spliced and polyadenylated (Davis et al. 1997). These data suggested that the mRNA containing expanded CUG repeats fails to be exported from the nucleus. The presence of nuclear foci was also demonstrated in DM1 brain and DM2 muscle cells (Mankodi et al. 2001; Jiang et al. 2004). Similar to the mutant *DMPK* mRNA, the *ZNF9* pre-mRNA containing the expanded CCUG repeats was also correctly processed. In *DMPK* mRNA the CUG repeats are a part of the mature mRNA, while in *ZNF9* transcripts the CCUG repeats are located in intronic sequence which is spliced out. Interestingly, the mutant *ZNF9* mRNA was correctly spliced and exported to the cytoplasm (Margolis et al. 2006a). The *ZNF9* RNA that was found in the nuclear foci contained only expanded CCUG repeats. These observations support the hypothesis of a *trans*-dominant effect of mutant *DMPK* and *ZNF9* transcripts, whereby expanded CUG or CCUG repeats do not disrupt the processing of repeat-bearing pre-mRNA (*cis* effect), instead, the nuclear accumulation of repeat-containing RNAs affects cellular processes leading to the disease.

3.1.3 Aberrant Splicing as the Key Molecular Feature of DM

Currently, most of the data support the following model of the molecular pathogenesis of myotonic dystrophy: expanded CUG/CCUG repeats act in *trans* affecting the processing of other RNAs via dysregulation of specific RNA binding proteins. Consistent with this hypothesis, interactions of CUG/CCUG-containing RNAs with proteins regulating RNA metabolism have been demonstrated (discussed in Sect. 2.2) (Timchenko et al. 1996a; Miller et al. 2000). Altered nuclear and cytoplasmic activities of MBNL and CELF protein families' members have been identified and linked to molecular abnormalities observed in DM1 and DM2.

The CELF proteins regulate RNA metabolism at the level of splicing, editing, translation and stability, and their activity is up-regulated by repeat-containing mRNAs (Sect. 2.2). On the other hand, the activity of MBNL proteins is decreased due to their sequestration in nuclear foci that contain long CUG and CCUG repeats (Cardani et al. 2006). CELF and MBNL proteins have been demonstrated to regulate alternative splicing by binding to specific pre-mRNA sequences and other regulatory proteins. Analyses of DM tissues and mouse models of myotonic dystrophy revealed a number of pre-mRNAs that are splicing targets of both MBNL and CELF proteins. Interestingly, MBNL and CELF act antagonistically in a process of developmentally regulated alternative splicing events. Although

both proteins are present in many tissues, CUGBP1 is mostly expressed during embryonic development, while MBNL1 is expressed postnatally (Lin et al. 2006). Therefore, CUGBP1 generates the "embryonic" mRNA isoforms, while the mRNA isoforms generated by MBNL1 are characteristic for postnatal development. As a consequence of loss of MBNL1 function due to sequestration in nuclear foci accompanied by gain of CUGBP1 function due to increased stabilization of the protein, changes of the splicing patterns of multiple mRNAs to the embryonic isoforms are observed in DM patients tissues and mouse models of myotonic dystrophy (Fig. 1).

These molecular abnormalities can be directly linked to the clinical findings in DM. Cardiac disturbances occur frequently in DM1. Conduction abnormalities range from first degree heart block to the more advanced forms including complete heart block. Hypertrophic cardiomyopathy and arrhythymias are also known to occur. Aberrant splicing of cardiac troponin T (cTNT), a protein that can regulate cardiac contractility, was the first identified example of splicing disruption in myotonic dystrophy (Philips et al. 1998). Splicing of cTNT is developmentally regulated in such a way that exon 5 is present in the fetal heart, but is excluded from the adult heart. Both CUGBP1 and MBNL1 can bind to cTNT pre-mRNA and regulate its splicing, generating either the embryonic or adult isoform (Philips et al. 1998; Ladd et al. 2005; Yuan et al. 2007). It has been demonstrated that due to increased CUGBP1 activity, an embryonic isoform of cTNT is expressed in DM1 muscles (Philips et al. 1998). While the embryonic isoform of cTNT is less sensitive to calcium, which can cause decreased contractility, the exact role of the aberrant splicing of cTNT in DM pathogenesis is not known. Missplicing of several other genes, such as *TNNT2*, *KCNAB1* and *ALP*, has also been found in DM1 hearts (Mankodi et al. 2005).

A characteristic feature of myotonic dystrophy is the occurrence of an unusual form of insulin-resistant diabetes that is due to defective insulin signaling in skeletal muscles. Misregulation of the insulin receptor (IR) splicing has been demonstrated in both DM1 and DM2 muscles (Savkur et al. 2001, 2004). It has been shown that overexpression of CUGBP1 alters the splicing in favor of a non-muscle isoform, which lacks exon 11, leading to insulin insensitivity in DM muscles. Alternatively, generation of a splice form containing exon 11 is facilitated by MBNL1. Therefore, the IR splicing events can be manipulated by changing a balance between CUGBP1 and MBNL1 activities (Dansithong et al. 2005; Ladd et al. 2005).

A classic feature of myotonic dystrophy, myotonia, is caused by aberrant splicing of the muscle-specific chloride channel 1 (CLC1) mRNA, leading to a premature termination of the message. As a consequence, loss of CLC1 protein in DM1 and DM2 skeletal muscles has been observed (Charlet et al. 2002; Mankodi et al. 2002). Reduced chloride conductance and a decreased number of functional Clc1 channels have been also demonstrated in mouse models of myotonic dystrophy (Mankodi et al. 2002; Lueck et al. 2007). Moreover, in vitro studies showed that overexpression of CUGBP1 in normal cells causes retention of intron 2 in the *Clc1* minigene, indicating that this intron is a target of CUGBP1 (Charlet et al. 2002). Subsequent studies

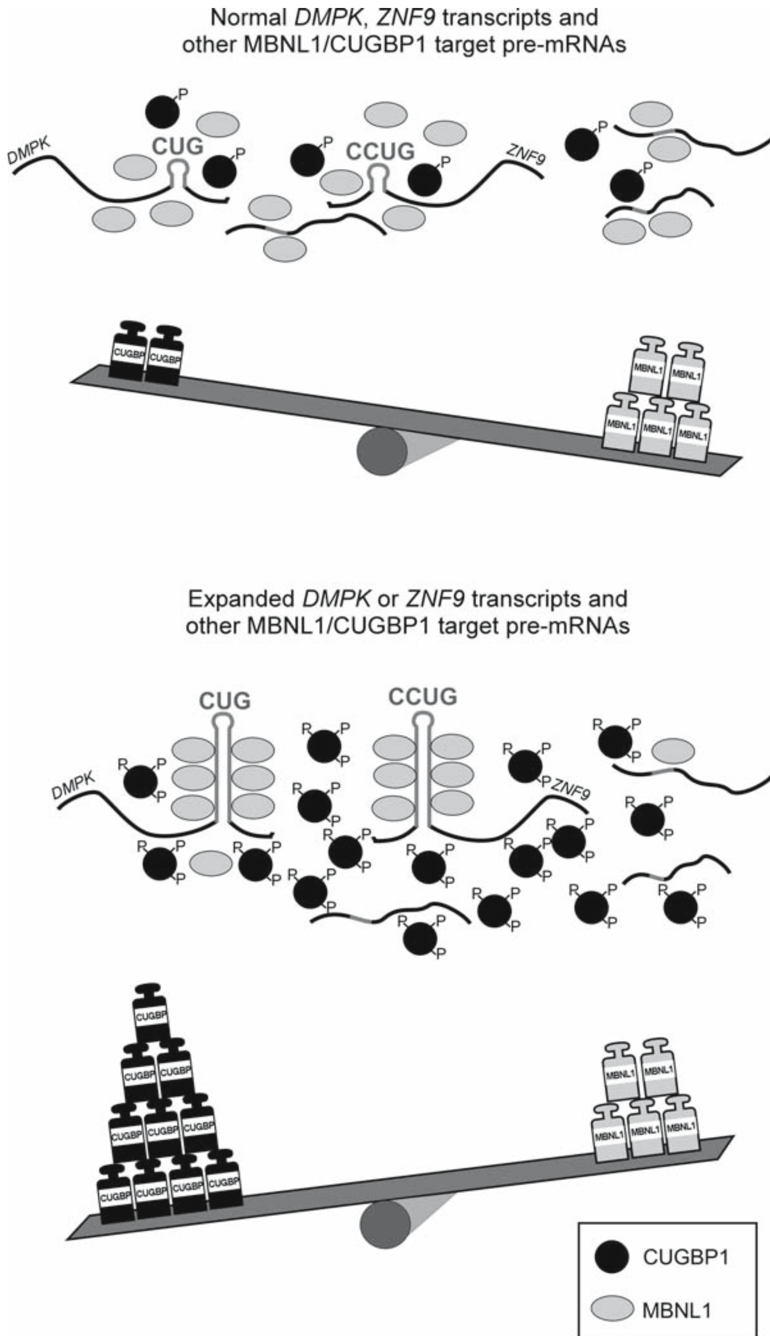

Fig. 1 Dominant negative effect of CUG/CCUG expansions. Sequestration of the *MBNL1* by expanded CUG and CCUG repeats accompanied by elevated activity (hyperphosphorylation and increased stability) of the *CUGBP1* are major steps in molecular pathogenesis of DM1 and DM2

revealed that Clc1 pre-mRNA is a target of MBNL1 as well (Kanadia et al. 2003b, 2006), again suggesting that disruption of the balance between CUGBP1 and MBNL activities leads to DM.

Missplicing of other genes involved in muscle differentiation and physiology has been demonstrated both in DM patients cells as well as in mouse models of myotonic dystrophy. In muscle cells from patients with the congenital form of DM1, decreased levels of the muscle-specific isoform of myotubularin-related 1 (MTMR1) mRNA have been observed (Buj-Bello et al. 2002). MTMR1 is a target of CELF proteins, and its abnormal splicing has also been shown in skeletal muscles and heart of transgenic mice overexpressing CUGBP1 (Ho et al. 2005). Splicing patterns and mRNA levels of proteins involved in calcium homeostasis in muscles, like the ryanodine receptor 1 (RYR1) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA1 and 2), are altered due to the expansion of CUG repeats (Kimura et al. 2005). In addition, splicing alteration of a cytoskeletal protein dystrophin has been detected in skeletal muscle and heart cells from DM1 patients. Since dystrophin is highly expressed in muscle cells, and mutations in this gene cause Duchenne/Becker's muscular dystrophy, its aberrant splicing may contribute to the muscle wasting seen in DM (Nakamori et al. 2007).

Neurofibrillary degeneration observed in brains of DM1 and DM2 patients may result, at least partially, from the altered production of the microtubule-associated protein tau isoforms (MAPT) (Maurage et al. 2005). Alteration of *MAPT* exon 2 splicing has been demonstrated in brains of DM patients as well as transgenic mice expressing expanded CUG repeats (Vermersch et al. 1996; Seznec et al. 2001). In vitro studies have shown that ETR-3, a CELF family member, promotes the selective exclusion of *MAPT* exon 2 (Leroy et al. 2006), suggesting a role of CELF proteins in aberrant splicing events in brains of DM patients.

To summarize, a number of in vitro and in vivo studies provide evidence that the key molecular feature of myotonic dystrophy, both type 1 and type 2, is expression of embryonic alternative splicing patterns in adult tissues.

3.2 Mouse Models of DM

3.2.1 Mice Overexpressing CUG Repeats

Clear support for the dominant-negative effect of expanded CUG/CCUG repeats comes from the analyses of transgenic and knockout mice. First mouse models generated to elucidate molecular mechanisms of myotonic dystrophy addressed a possible gene dose effect. However, neither loss of the *Dmpk* gene nor overexpression of normal human *DMPK* gene containing a $(CTG)_{11}$ repeat tract reproduced the characteristic features of myotonic dystrophy in young animals (Jansen et al. 1996). Interestingly, the older transgenic mice (11–15 months) with five to ten fold overexpression of the human *DMPK* gene developed myotonia and hypertrophic cardiomyopathy with dysrhythmia. Thus, the continuous overexpression of the

wild type *DMPK* gene leads to accumulated distress which manifests phenotypically with age (O'Cochlain et al. 2004). To study effects of the CUG expansion in an untranslated region of a DM1-unrelated transcript, Mankodi et al. generated transgenic mouse expressing long CUG repeats within 3′UTR of actin mRNA. These mice developed early onset myotonia, suggesting that an increased number of CUG repeats alone is sufficient to induce a pathogenic effect (Mankodi et al. 2000). This notion was further confirmed by comparison of phenotypes of transgenic mice carrying the entire human *DMPK* gene with 350 CTG repeats versus mice carrying 20 CTG repeats (Seznec et al. 2001). Only mice expressing human *DMPK* mRNA with expanded repeats developed myotonia as well as histological and molecular abnormalities in skeletal muscles consistent with the myotonic dystrophy phenotype. Moreover, similar to data obtained from DM1 patients, brains of the transgenic mice bearing 350 CTG repeats demonstrated changes in the distribution of tau protein isoforms (Seznec et al. 2001).

Intriguing results were obtained using transgenic mice with inducible expression of CUG-containing mRNAs. Two groups demonstrated that severe cardiac abnormalities develop rapidly after induction of the expression of CUG-containing mRNAs in adult mice. In one of the inducible DM models, the heart-specific expression of the expanded (960 CUG) repeats resulted in 100% mortality within 2 weeks of induction (Wang et al. 2007). These mice developed physiological and molecular cardiac abnormalities similar to those observed in DM patients. Interestingly, the co-localization of MBNL1 with RNA in nuclear foci and induction CUGBP1 occurred within hours of induction, and preceded splicing changes. Surprisingly, the other mouse line developed DM-characteristic abnormalities upon induction of expression of the mRNA containing only five CUG repeats. In this case, neither MBNL1-containing nuclear foci, nor increased CUGBP1 activity were observed (Mahadevan et al. 2006).

To summarize, a number of in vivo studies conducted by different research groups provide evidence that the expression of expanded CUG/CCUG repeats, not mutations of the *DMPK* or *ZNF9* genes themselves, triggers a molecular response leading to the myotonic dystrophy phenotype.

3.2.2 CUGBP1 Transgenic and MBNL1 Knockout Mice

To test the hypothesis that increased CUGBP1 activity plays a role in the muscle pathology of myotonic dystrophy, two laboratories independently generated transgenic mice overexpressing CUGBP1 in cardiac and skeletal muscles. Consistent with the CUGBP1 gain-of-function mechanism, abnormal processing of CUGBP1-target mRNAs was observed in both transgenic mouse lines. The first group demonstrated characteristics for myotonic dystrophy splicing misregulation of the muscle-specific *CLC1*, *cTNT* and *MTMR1* mRNAs in the muscles and hearts of CUGBP1 transgenic mice (Ho et al. 2005). The second group reported that overexpression of CUGPB1 in mice increased the MEF2A and p21 proteins which resulted in delay of muscle development (Timchenko et al. 2004).

Targeted disruption of the mouse *Mbnl1* gene in such a way that only the isoforms interacting with CUG/CCUG-containing RNAs are not expressed, provided a tool to study the consequences of loss of Mbnl1's ability to process CUG/CCUG-containing RNAs. As predicted by the MBNL sequestration model, loss of this function was sufficient to cause myotonia, cataract and RNA splicing defects of cardiac troponin T and muscle-specific chloride channel similar to those seen in myotonic dystrophy (Kanadia et al. 2003a). Moreover, the overexpression of Mbnl1 in skeletal muscles of the DM mouse model carrying the human actin gene with insertion of 250 CTG repeats in 3′UTR rescued the myotonia and splicing defects (Kanadia et al. 2006). Thus, manipulations with CELF and MBNL protein activities in transgenic and knockout mice demonstrate that altered balance of opposite activities of the CUGBP1 and MBNL1 proteins underlie the aberrant splicing patterns of multiple mRNAs, which in turn leads to the multisystemic presentation of myotonic dystrophy.

Extensive studies of molecular abnormalities in DM patients' tissues and in mouse models of myotonic dystrophy provide solid evidence of the toxic RNA mechanism of the disease. Although the main stream of research is focused on the post-transcriptional RNA metabolism, there is evidence that expanded CUG/CCUG repeats also disrupt the process of transcription (Ebralidze et al. 2004). It has been demonstrated that long CUG repeats deplete basic transcription factors leading to the decreased expression of multiple genes, including *CLC1*. These unexpected findings suggest that not all features of long CUG/CCUG sequences have been revealed yet.

4 Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS)

Fragile X syndrome (FXS) is caused by large CGG repeat expansion (>200 copies) leading to the DNA methylation and silencing of the *FMR1* gene (Fig. 2) (Fu et al. 1991; Pieretti et al. 1991; Orr and Zoghbi 2007). For a long time, it was believed that premutation carriers, with 55–200 CGG repeats, were simply an asymptomatic source of the full mutation alleles. However, recently, premutation alleles have been associated with two conditions: premature ovarian failure (POF) and fragile X tremor/ataxia syndrome (FXTAS) (Hagerman et al. 2001, 2004). FXTAS is a late onset neurodegenerative disorder characterized by tremor, gait instability, cerebellar dysfunction, cognitive decline and parkinsonism. It should be emphasized that the clinical characteristics of FXTAS are entirely different from fragile X syndrome, suggesting completely distinct molecular mechanisms for these disorders (Hagerman and Hagerman 2006). Cells of the premutation carriers express *FMR1* mRNA at levels two to ten times higher than cells harboring a normal (<55) number of CGG repeats, while the level of the FMR1 protein in the premutation carriers is not elevated. This suggests that the effects of the RNA level play a significant role in FXTAS (Tassone et al. 2000a,b).

Jin et al. demonstrated, in *Drosophila*, that expression of the premutation length $(CGG)_{90}$ RNA, even in the absence of the entire *FMR1* gene, leads to

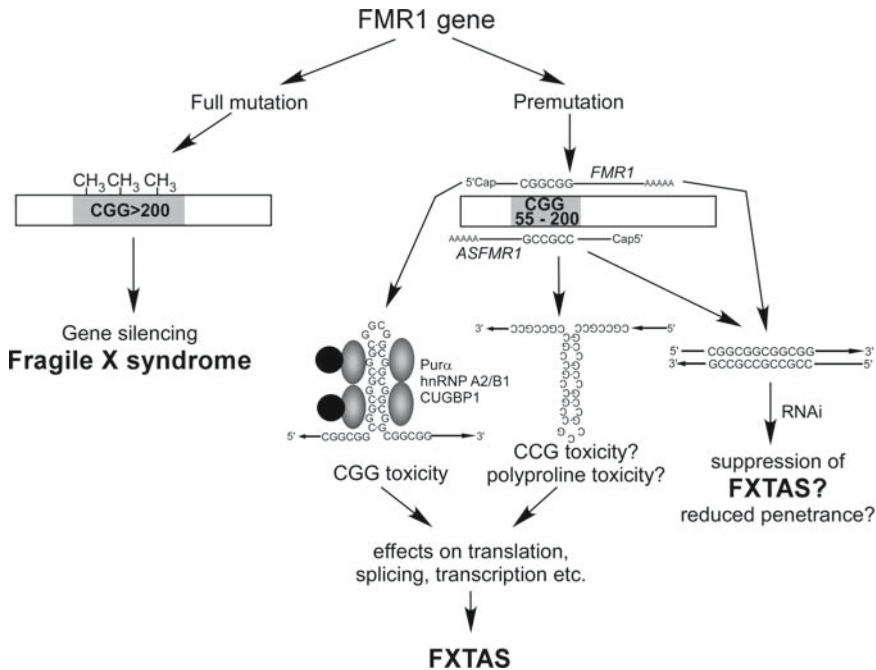

Fig. 2 Molecular pathogenesis of fragile X syndrome and FXTAS. Bidirectional transcription of the *FMR1* locus results in formation of transcripts containing CGG (*FMR1* mRNA) and CCG repeats (*ASFMR1* mRNA). Repeat regions in both types of RNAs can form hairpins and induce RNA toxicity. It is not known whether translation of the *ASFMR1* triggers protein toxicity. Toxic effects of CGG and CCG RNAs can potentially be eliminated by RNAi-mediated degradation of complementary transcripts

neurodegeneration (Jin et al. 2003). Neuropathologically – similar to the DM1 and DM2 – FXTAS is characterized by the presence of ubiquitin-positive, RNA-containing nuclear inclusions in both neurons and astrocytes (Greco et al. 2002). Inclusions were also present in the knock-in mice containing approximately 100 CGG repeats in the mouse *Fmr1* gene (Willemsen et al. 2003). The increase in number as well as in size of the inclusions was observed over the course of the lifetime of the mouse, corresponding to the progressive character of FXTAS. The inclusions isolated from post-mortem brains of FXTAS patients contain, in addition to the *FMR1* mRNA molecules, more than 20 different proteins, none of which contributed to more than 7% of total proteins detected in the inclusions (Tassone et al. 2004; Iwahashi et al. 2006). Two of the identified proteins, MBNL1 and hnRNP A2/B1, are well characterized RNA binding proteins, as discussed above (Sect. 2.2). The inclusions also contained intermediate filament (IF) proteins, which could potentially contribute to the RNA toxicity and neurodegeneration by regulating RNA synthesis and processing (Hutchison and Worman 2004; Iwahashi et al. 2006).

Recently, another RNA binding protein, Purα, has been detected in FXTAS inclusions (Jin et al. 2007). This protein, in concert with hnRNP A2/B1, is essential for the neurodegeneration observed in the *Drosophila* model of FXTAS. Over-expression of either of these proteins alleviates the neurodegenerative phenotype in the fly model providing evidence that sequestering and inhibition of Purα and hnRNP A2/B1 play a role in molecular pathogenesis of FXTAS (Jin et al. 2007; Sofola et al. 2007b). Although both Purα and hnRNP A2/B1 are widely expressed and involved in various aspects of RNA metabolism, their exact functions in development of FXTAS have not yet been elucidated.

In summary, substantial evidence has accumulated to support the following model for RNA toxicity in FXTAS (Fig. 2). RNA hairpins containing CGG repeats are targets for RNA binding proteins including MBNL1, Purα and hnRNP A2/B1. Additionally, the cellular level of CUGBP1 is affected by its interactions with hnRNP A2/B1. Binding of these proteins influences their downstream targets and functions, leading to the clinical phenotype of FXTAS. In parallel, expression of the antisense gene *ASFMR1* can stimulate RNA toxicity via CCG hairpins and/or protein toxicity via the putative polyproline-containing *ASFMR1* gene product as discussed in Sect. 2.3 (Fig. 2).

In a separate disease process, expanded CCG repeats (>200) located in the *FMR2* gene are associated with non-syndromic X-linked mental retardation (FRAXE) (Knight et al. 1993). Large expansion of these repeats induces transcriptional silencing of the *FMR2* gene. However, no neurodegenerative phenotype has been associated with premutation alleles of *FMR2*.

Interestingly, expression of premutation length CCG repeats (complementary to FXTAS CGG tract) can also trigger an RNA mediated neurodegenerative phenotype in a *Drosophila* model (Sofola et al. 2007a). Hence, independent expression of premutation size CGG or CCG transcripts leads to a neurodegenerative phenotype in transgenic *Drosophila*. Surprisingly, co-expression of both complementary RNAs in the fly model alleviates their independent neuronal toxicity (Sofola et al. 2007a). The rescue depends on the RNAi pathway suggesting a possible avenue for the future therapeutic intervention in toxic RNA-mediated disorders (Sect. 6). In humans, *FMR1* premutation carriers express high levels of both the *FMR1* mRNA and its antisense counterpart *ASFMR1* containing CCG repeats. Approximately one-third of the male premutation carriers will develop FXTAS (Jacquemont et al. 2004), implicating involvement of other factors, perhaps the expression of the complementary transcript, in the suppression of the disease (Fig. 2).

5 Other Disorders Associated with Toxic RNAs

5.1 Spinocerebellar Ataxias (SCAs)

Autosomal dominant spinocerebellar ataxias are a group of clinically and genetically heterogeneous neurodegenerative disorders. Approximately one-third of roughly

30 distinct SCAs are caused by expansion of microsatellite sequences (Duenas et al. 2006). The molecular pathogenesis of three diseases, SCA8, 10 and 12, is thought to be associated with RNA effects.

5.1.1 SCA8

The most characteristic features of SCA8 are dramatic instability of the expanded CTG repeats and extremely reduced penetrance (Moseley et al. 2000). Normal alleles harbor 16–34 repeats, while disease alleles contain from 71 to over 1,000 repeats in two bidirectionally transcribed genes: *ATXN8* and *ATXN8OS* (formerly *SCA8*) (Koob et al. 1999; Moseley et al. 2006; Ikeda et al. 2007). Since the discovery of bidirectional expression at the SCA8 disease locus, it has become apparent that the polymorphic CTG repeats are part of these two different genes that utilize opposite DNA strands as templates for transcription.

Initially, SCA8 was considered an RNA gain-of-function disease due to the CUG repeat expansion in the noncoding region of the *ATXN8OS* gene, making toxic RNA the most likely mechanism of the SCA8 pathogenesis. Expression of the expanded (112 CUG repeats) as well as normal (nine CUG repeats) transcripts in a *Drosophila* SCA8 model led to the neurodegeneration. Using this neurodegeneration phenotype in *Drosophila* retina, Mutsuddi et al. discovered four proteins that modify (enhance or suppress) the SCA8-induced pathogenesis (Mutsuddi et al. 2004). All of them encoded neuronally expressed RNA binding proteins: staufen, muscleblind, split ends and CG3249. The influence of these *Drosophila* SCA8 phenotype modifiers depended on the size of the CUG repeat (Mutsuddi et al. 2004).

The generally accepted RNA mechanism of SCA8 pathogenesis was re-evaluated upon the discovery of the *ATXN8* gene, which encodes a polyglutamine domain containing protein (Moseley et al. 2006). This gene is transcribed in the opposite direction to *ATXN8OS*. Translation of the *ATXN8* transcript results in formation of 1C2 positive intranuclear inclusions. This hallmark feature of the polyglutamine disorders was detected in cerebellar Purkinje and brainstem neurons in both SCA8 transgenic mice and human autopsy tissue.

In conclusion, simultaneous expression of the CUG (*ATXN8OS* gene) and CAG (*ATXN8* gene) expanded repeat-containing transcripts suggests that SCA8 may be the first known disease caused by both toxic RNA and protein gain-of-function.

5.1.2 SCA10

Spinocerebellar ataxia type 10 (SCA10) is a dominantly inherited ataxia caused by the expansion of the pentanucleotide repeat sequence ATTCT. SCA10 patients demonstrate progressive cerebellar dysfunction which manifests as limb and gait ataxia, ocular movement abnormalities, and dysarthria (Lin and Ashizawa 2005). The expansions occur within intron 9 of the *ATXN10* gene and are among the largest observed in the repeat expansion disorders, ranging from 280 to 4,500 repeats (Matsuura et al. 2000; Lin and Ashizawa 2005). ATTCT sequences can act as DNA

unwinding elements (DUEs) – DNA sequences that are frequently associated with replication origins (Potaman et al. 2003, 2006). Aberrant initiation of DNA replication is likely to be responsible for dramatic expansions observed at the SCA10 locus (Liu et al. 2007).

In SCA10 patients, both normal and expanded alleles are expressed at a similar level thus arguing against the role of haploinsufficiency in the etiology of this disease (Ashizawa 2006). Thus far, two lines of evidence indicate a possibility of the involvement of toxic RNAs in the pathogenesis of SCA10. First, AUUCU transcripts can adopt hairpin structures in vitro (Handa et al. 2005). Secondly, over-expression of uninterrupted ATTCT repeats leads to the accumulation of the AUUCU transcript and the formation of RNA inclusions (Lin and Ashizawa 2005). However, proteins targeting AUUCU repeats and the downstream processes affected in the SCA10 cells have not been identified.

5.1.3 SCA12

Spinocerebellar ataxia type 12 is caused by a unique, among dominant ataxias, expansion of the CAG repeat in the noncoding region of the *PPP2R2B* gene. In unaffected individuals, the number of CAG repeats varies from 7 to 32, while pathogenic expanded alleles have 55–78 repeats (Holmes et al. 1999, 2001, 2006). The *PPP2R2B* gene encodes a regulatory subunit (Bβ) of a ubiquitous serine/threonine phosphatase PP2A (PP2), which is involved in the regulation of many cellular processes, including cell growth, apoptosis, differentiation, channel function and DNA replication (Price and Mumby 1999; Virshup 2000).

The structure of the *PPP2R2B* gene, and its splice variants, is quite complex. The predominant protein isoform, Bβ1, is derived from the exon 7 promoter with the CAG repeats located upstream of transcription start site or within the 5′UTR. Among several possible reading frames, only one putative variant, called Bβ7, predicts a polyserine tract at the N-terminus of the protein (Holmes et al. 2006). No evidence supporting polyglutamine toxicity in SCA12 has been discovered. Current data suggest that changes in the expression levels of one or more isoforms of the *PPP2R2B*, which result in alterations in PP2A activity, rather than RNA toxicity, are crucial in the development of SCA12 (Holmes et al. 2006). The role of toxic CAG RNAs cannot be completely ruled out since these repeats are capable of forming stable hairpin structures which are potential targets for MBNL1 and PKR binding (Sobczak et al. 2003; Kino et al. 2004; Michlewski and Krzyzosiak 2004; Sobczak and Krzyzosiak 2005).

5.2 Huntington's Disease-Like 2 (HDL2)

HDL2 is an autosomal dominant progressive neurodegenerative disease that is clinically and pathologically similar to Huntington's disease (HD). This disease is caused

by expansion of the CTG repeat tract located in the variably spliced exon 2A of the *Junctophilin-3* (*JPH3*) gene on chromosome 16q24.3. Unaffected individuals carry 6–28 trinucleotide repeats while patients with HDL2 have 40–59 repeats. Depending on the alternative splicing, the CUG repeats may encode polyalanine or polyleucine tracts, or they may be a part of the 3′UTR (Margolis et al. 2006b).

Since no apparent evidence of protein toxicity could be detected in HDL2, an RNA gain-of-function mechanism of pathogenesis was considered. Rudnicki et al. detected RNA inclusions in the brains of HDL2 patients. These inclusions contained different variants of the *JPH3* mRNA, including pre-mRNA, and co-localized with the MBNL1 protein (Rudnicki et al. 2007). Additionally, overexpression of an untranslatable form of the *JPH3* gene, which contained an expanded CUG repeat, in HEK293 and HT22 cell lines led to the formation of inclusions and cellular toxicity. Importantly, abnormalities in splicing of microtubule-associated protein tau and amyloid-β precursor protein (APP) were found, suggesting direct involvement of the impaired function of MBNL1 in HDL2 development (Rudnicki et al. 2007). Although it would be premature to entirely exclude the possible contribution of a toxic protein (containing polyalanine, polyleucine or even polyglutamine, considering bidirectional transcription), results gathered so far strongly implicate the involvement of CUG RNA in the pathogenesis of HDL2.

6 Toxic RNAs as Therapeutic Targets

The first microsatellite sequence expansion responsible for a human disease was discovered in 1991 (La Spada et al. 1991). Although much progress has been made in understanding the molecular mechanisms of the repeat expansion disorders, effective treatments have not been developed thus far. Analyses of mechanisms of different genetic diseases suggest that the challenges faced in the case of toxic RNA-mediated disorders can be circumvented. Since in all of the noncoding repeat diseases structurally normal proteins are synthesized, the major therapeutic goal would be to eliminate the toxic RNA molecules.

In this section, we present current research in development of therapeutic strategies for RNA gain-of-function disorders with a focus on the toxic RNAs as targets for therapeutic intervention. Since the majority of studies related to RNA-targeted therapy have been conducted using DM1 as a model, we will concentrate predominantly on the strategies developed towards therapies for this disease. Selective inhibition of transcription of the allele harboring long repeating tract, blocking the toxic RNA interaction with RBPs, degradation-mediated removal of toxic RNAs by antisense, ribozymes and siRNAs can potentially alleviate the pathogenic effects of the alleles with expanded repeat tracts (Fig. 3).

Attempts have been made to target the mutant *DMPK* transcript using a retroviral vector expressing a 149-nucleotides-long antisense RNA that is complementary to 13 CUG repeats and 110 nucleotides of the unique *DMPK* sequence flanking the repeat region (Furling et al. 2003). DM1 myoblasts infected with this construct

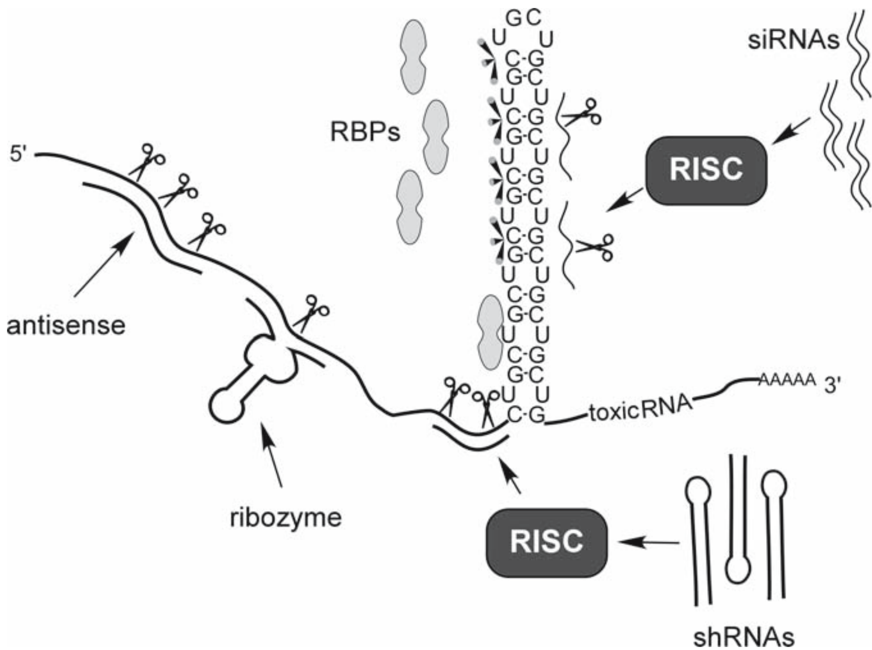

Fig. 3 Therapeutic approaches targeted directly towards toxic RNAs. RNA toxicity can be suppressed by eliminating RNA via ribozyme, antisense RNA or RNAi mediated cleavage. Both small interfering RNAs and short hairpin RNAs are processed by *RISC* complex and may be designed for unique RNA sequences as well as repeating regions. Interactions between RBPs and RNA can also be altered by specific small molecules

showed differential inhibition of expanded and normal transcripts at the levels of 80 and 50%, respectively. The expression of the antisense RNA effectively restored physiological functions of the DM1 myoblasts and lowered the intracellular levels of CUGBP1.

Another approach used to eliminate toxic repeat-containing RNAs was to target *DMPK* transcripts with ribozymes (Langlois et al. 2003). Ribozymes are small, catalytic RNA molecules capable of cleavage, in *trans*, of a specific phosphodiester bond in the RNA substrate. Transiently expressed hammerhead ribozyme, directed towards the 3′UTR of the *DMPK* mRNA downregulated the normal and expanded transcripts by 50–63%. The reduction of *DMPK* mRNA in human DM1 primary myoblasts was accompanied by significant reduction in the number and intensity of nuclear foci as well as partial restoration of expression of the insulin receptor isoform B (Langlois et al. 2003). The reasons for preferential reduction of the expanded *DMPK* transcript in both studies were not clear.

Recently, an adeno-associated virus expressing ribozyme was intramuscularly injected into the DM1 transgenic mice containing 350 CUG repeats. One month after the single injection into the tibialis anterior (TA) muscle, a significant, 55–60%,

decrease of the *DMPK* mRNA level was detected, together with a 30% increase in the TA muscle volume (Doucet et al. 2007).

Downregulation of *DMPK* mRNAs, both normal and expanded, was also accomplished using lentivirus-delivered short hairpin RNAs (shRNAs) directed towards a non-repeating sequence of the 3′UTR (Langlois et al. 2005). These shRNAs were processed by the RNA interference pathway leading to specific knockdown of the *DMPK* transcripts due to their cleavage by Dicer. Surprisingly, this approach downregulated not only cytoplasmic, normal *DMPK* transcript containing 18 CUG repeats, but also the expanded transcripts, harboring approximately 3,200 repeats, which were retained in the nucleus of the DM1 cells. Although RNAi is thought to be primarily a cytoplasmic process, these results demonstrated that shRNAs can act both in the cytoplasm and in the nucleus. On the other hand, the cytoplasmic localization of the mutant *DMPK* transcript has also been reported, leaving the possibility that only the cytoplasmic fraction of the expanded *DMPK* mRNA was downregulated (Taneja et al. 1995).

Repeat regions of toxic RNAs adopt stable hairpin structures, but frequently short normal alleles, containing only a few repeats, are incapable of forming of stable hairpins. These structural differences proved to be useful in the design of allele-specific therapeutic strategies. Krol et al. demonstrated that the ribonuclease Dicer, involved in RNAi pathway, controls the level of expanded transcripts containing CUG or CAG repeats (Krol et al. 2007). Short repeat fragments cleaved out from long repeat hairpins subsequently act as siRNAs and further induce downstream silencing of the expanded transcripts. Similarly, transfection of short synthetic oligoribonucleotides containing $(CAG)_7$ or $(CUG)_7$ repeats into cells led to the silencing of the expanded transcripts. Interestingly, in the HeLa cells treated by the CUG or CAG siRNAs only longer transcripts, harboring 70 and 200 repeats, were affected (Krol et al. 2007). These results show that siRNA induced downregulation of CUG or CAG RNAs is, in fact, dependent on the length of the repeats thus establishing a rationale for the future allele-specific therapy.

Intriguingly, it is also possible that the RNAi pathway, induced by the dsRNA that results from bidirectional transcription, is involved in the natural regulation of the repeat-containing transcripts. This process may explain the reduced penetrance observed in SCA8 and FXTAS. In conclusion, the RNAi pathway becomes undoubtedly one of the most promising approaches for the future treatment of several incurable neurodegenerative disorders (Denovan-Wright and Davidson 2006; Paulson 2006; Rodriguez-Lebron and Paulson 2006).

Expanded RNA repeats, via sequestering some RNA binding proteins and activation of others, deregulate a delicate balance between these proteins and influence downstream processes leading to neurodegeneration. Restoration of this balance, by correcting the expression levels of appropriate proteins, can be therapeutically beneficial. Three groups, almost simultaneously, reported therapeutic effects of modifying the expression of MBNL1, CUGBP1 and hnRNP H in different models of DM1. Reversal of aberrant splicing and myotonia was observed in transgenic mice harboring 250 CUG repeats after AAV-mediated overexpression of *Mbnl1* (Kanadia et al. 2006). However, the normal structure of

myofibers was not restored, most likely due to the low expression of *Mbnl1* relative to the CUG repeat-containing transcript. These results showed that either overexpression of MBNL1 (perhaps together with MBNL2) or suppression of interactions between CUG transcripts and the RBPs may represent a successful strategy for treatment of DM1 and DM2.

On the other hand, results of overexpression of the green fluorescent protein (*GFP*) gene containing a 3′UTR derived from the normal *DMPK* allele (five CUG repeats) suggested that binding of MBNL1 to the CUG repeats might be necessary for neutralizing repeat-induced toxicity (Mahadevan et al. 2006). In these circumstances, protein binding plays a protective role. Therefore, abolishing CUG RNA–MBNL1 interactions may be potentially deleterious to DM1 cells. An alternative therapeutic approach could involve the control of *DMPK* expression or a correction of the pathological effects of the increased CUGBP1 levels.

Recent data on the aberrant splicing of the insulin receptor as a model for DM1-specific spliceopathy demonstrated that siRNA-mediated knockdown of CUGBP1 and hnRNP H did not rescue aberrant IR splicing (Paul et al. 2006). Also, overexpression of MBNL1 only partially alleviated the splicing abnormalities. However, simultaneous increase of the MBNL1 and decrease of hnRNP H further stimulated correction of the IR splicing. Taken together, these results suggest that fine tuning of several elements is required to restore proper physiology of DM1 cells.

A significant disadvantage of therapeutic approaches that rely on expression of ribozymes, siRNAs, antisense RNAs or entire proteins is the necessity for the proper delivery of relatively large vectors into the specific cells. This task is further complicated by the necessity to cross the blood–brain barrier and evade the immunological response of the organism. Therefore, small molecules that specifically recognize and alter functions of toxic RNAs could present an alternative solution to these problems (Fig. 3). Future development of high-throughput screens (HTS), using both in vitro and cell-based assays, may result in identification of compounds capable of ameliorating the toxic activity of expanded RNAs. A cell-based assay, utilizing the PC12 neuronal cell line stably transfected with a luciferase gene containing 250 CUG repeats in the 3′UTR, was used to screen a library of 235 bioflavonoids and other chemicals structurally resembling flavonoids (Furuya et al. 2005). Some of the analyzed compounds, applied in micromolar concentrations, reduced CUG induced cytotoxicity, presumably by interfering with the affinity of protein binding to the toxic RNAs. Nevertheless, biochemical assays for specific inhibitors of RNA–protein interactions remain to be developed. Potentially, small molecules could also selectively target expression of the expanded allele by altering epigenetic modifications frequently associated with DNA regions containing long repeating sequences (Wang et al. 1996; Coffee et al. 2002; Herman et al. 2006).

In summary, although there has been tremendous progress in the understanding of the molecular mechanisms of RNA gain-of-function, many questions related to the pathogenesis of these disorders remain to be answered. Attempts of therapeutic intervention, such as those described above, indicate that RNA toxicity can be reversed.

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