# Myotonic dystrophy mouse models: towards rational therapy development

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## Abstract

DNA repeat expansions can result in the production of toxic RNA. RNA toxicity has been best characterised in the context of myotonic dystrophy. Nearly 20 mouse models have contributed significant and complementary insights into specific aspects of this novel disease mechanism. These models provide a unique resource to test pharmacological, anti-sense, and gene-therapy therapeutic strategies that target specific events of the pathobiological cascade. Further proof-of-principle concept studies and preclinical experiments require critical and thorough analysis of the multiple myotonic dystrophy transgenic lines available. This review provides in-depth assessment of the molecular and phenotypic features of these models and their contribution towards the dissection of disease mechanisms, and compares them with the human condition. More importantly, it provides critical assessment of their suitability and limitations for preclinical testing of emerging therapeutic strategies.

## Myotonic dystrophy: a paradigm of RNA toxicity

Myotonic dystrophy (dystrophia myotonica, DM) is the most common form of adult muscular dystrophy and includes at least two genetically distinct but clinically similar disease forms. DM type 1 (DM1) accounts for the majority of DM cases (traditionally >95%), but the prevalence of DM type 2 (DM2) is probably underestimated [1]. DM1 is a truly multisystem disorder, primarily affecting skeletal muscles, the heart and the central nervous system (CNS) (Box 1). The development of DM1 transgenic mice and identification of the DM2 mutation helped to elucidate a novel disease mechanism mediated by a toxic gain-of-function RNA transcript [2]. Expanded *DMPK* transcripts accumulate in the nucleus of DM1 cells [3], interfering with at least two antagonistic protein families that regulate alternative splicing throughout development: the muscleblind-like (MBNL) and CUGBP/ Elav-like family (CELF) proteins [4–6]. MBNL1 function is lost due to sequestration by ribonuclear aggregates or foci [4,6], and CELF1 (or CUG-binding protein 1, CUGBP1) is upregulated [5,7,8] through protein stabilisation that is mediated by hyperphosphorylation [9]. MBNL1 sequestration and CELF1 upregulation result in aberrant expression of embryonic splicing profiles of MBNL1- and/or CELF1-regulated transcripts in adult skeletal muscle and heart (Figure 1a) [10–12]. Similarly, toxic CCUG-containing *CNBP/ZNF9* transcripts sequester MBNL1 and disrupt splicing in DM2 [2,6]; their effect on CELF1 is

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still unclear [10,13,14]. *CLCN1* chloride channel missplicing in skeletal muscles results in myotonia (delayed muscle relaxation after initial contraction) [15–17], whereas abnormal splicing of the insulin receptor (INSR) might contribute to insulin resistance [7]. Other missplicing events have been described and are likely to play a role in disease manifestations (Table S1 in the supplementary material online). MBNL proteins can also participate in RNA transcription, processing and stability [18,19], whereas CELF1 regulates protein translation [20–23]. Therefore, it is conceivable that the DM1 mutation might have an impact beyond splicing deregulation.

RNA toxicity might also underlie spinocerebellar ataxia 8 (SCA8) and fragile X-associated tremor ataxia syndrome (FXTAS), and is likely to contribute to Huntington disease-like 2 (HDL2). In these conditions, expanded transcripts accumulate in nuclear RNA foci and sequester RNA-binding proteins, such as MBNL1, and affect downstream gene expression and/or alternative splicing [24–27]. Expanded transcripts are also found in nuclear and cytoplasmic SCA10 aggregates, supporting a *trans*-dominant role of toxic RNA repeats in disease pathogenesis [28]. Similarly, the non-coding CAG repeat expansion in SCA12 suggests a possible mechanism of RNA toxicity [29]. Missplicing has been described in spinal bulbar muscular atrophy (SBMA) knock-in mice in the absence of RNA aggregation or MBNL1 sequestration. Despite CELF1 upregulation in SBMA, altered RNA processing probably results from an effect of androgen receptor polyglutamine expansion on hormone-regulated splicing [30]. The impact of CELF1 upregulation in oculopharyngeal muscular dystrophy (OPMD) remains elusive, but it might be indicative of molecular features shared with DM1 [31].

In this review we provide in-depth assessment of the molecular and physiological phenotypes of transgenic DM1 mouse models and their contribution towards an understanding of disease mechanisms, and we compare them with the human condition. More importantly, we review their advantages and limitations for preclinical assessment of new therapies.

## Transgenic mouse models of DM: towards a methodical dissection of molecular pathogenesis

Transgenic mouse models of human neuromuscular disorders must meet genetic, molecular and physiological requirements to ensure faithful recapitulation and monitoring of the natural disease history (Box 2). DM1 transgenic lines have been developed to investigate the multisystemic pathophysiology and mechanisms of trinucleotide repeat dynamics. The mouse models available result from (i) inactivation of genes in the DM1 locus, (ii) overexpression of toxic CTG repeats, (iii) abnormal splicing regulation through *Mbnl* inactivation or *Celf* overexpression, and (iv) introduction of unstable CTG expansions (these models are described in Table S2 in the supplementary material online).

### Inactivation of genes of the human DM1 locus: Dmpk and Six5 knockout mice

Reduced cytoplasmic *DMPK* transcripts [32], as well as decreased *SIX5* mRNA levels in DM1 patients [33,34], initially suggested a role for *DMPK* and/or *SIX5* haploinsufficiency. To test this hypothesis, *Dmpk* and *Six5* knockout mice were generated. $Dmpk^{-/-}$ and $Six5^{-/-}$ mouse strains seemed to be overtly normal [35–38] and failed to reproduce the complex and multisystemic DM1 phenotype, suggesting that happloinsufficiency of the genes in the *DM1* locus is not the primary mechanism of disease. Nevertheless, *DMPK* inactivation might contribute to altered ion homeostasis in muscle and heart [35,39], whereas *SIX5* deficiency might increase susceptibility to cataracts [37,38], reduce male fertility [40] and affect cardiac dysfunction [41].

## Transgenic models of toxic RNA gain-of-function

A second generation of transgenic lines expressing toxic RNA repeats, summarised below, has greatly contributed to dissection of this novel disease mechanism, for which DM1 became the paradigm.

**Non-expanded DMPK-overexpressing mice—**To investigate the consequences of *DMPK* overexpression, transgenic lines carrying multiple copies of the human gene with a short $(CTG)_{11}$ repeat were generated (Figure 1a) [35]. The Tg26 line, with the highest copy number and *DMPK* expression levels, exhibits the most severe phenotype, including cardiomyopathy, reduced workload tolerance, myotonic skeletal myopathy associated with reduced CLCN1 staining and smooth muscle tone deficit [35,42]. The undistinguishable effects of RNA and protein overexpression in Tg26 mice confound the interpretation of results and limit their application in therapeutic development.

**Expression of toxic CUG repeats in skeletal muscle of HSA^LR mice—**Overexpression of untranslated CUG repeats in the skeletal muscle of *HSA*^SR (short repeat length) and *HSA*^LR (long repeat length) transgenic mice (Figure 2b) provided crucial evidence for the toxicity of expanded transcripts [43]. *HSA*^LR lines expressing high levels of RNA containing 250 CUG repeats developed the most severe phenotype, including high mortality and myotonic discharges at 4 weeks of age in the absence of histological abnormalities in skeletal muscle. Myotonic discharges are characterised by high-frequency repetitive discharges evoked by insertion or movement of an EMG needle electrode. Histological signs of myopathy were detected at a later stage [43]. MBNL1 sequestration in nuclear foci disrupted the neonatal-to-adult splicing switch of multiple target transcripts (e.g. *Clcn1, Atp2a1/Serca1, Mbnl1, Ldb3/Cypher*) [10]. CELF1 is not upregulated in *HSA*^LR skeletal muscle, suggesting that sequestration of MBNL protein is sufficient to trigger missplicing [10]. Muscle-specific transgene expression restricts the application of *HSA*^LR mice to the investigation of adult skeletal muscle pathology and certain aspects of the neuromuscular junction [44]. The absence of muscle weakness or wasting represents an additional limitation, as well as the lack of *HSA*^LR expression in myogenic precursor cells.

**Expression of expanded DMPK transcripts in the context of the human DM1 locus—**Expression of expanded *DMPK* transcripts in multiple mouse tissues is driven by a large fragment of the human DM1 locus, which presumably contains the *cis* elements necessary for temporal and spatial regulation of transcription [45,46]. Mice carrying different repeat sizes have been generated (Figure 2c). Transgene expression in homozygous DM300 mice results in accumulation of ribonuclear foci in multiple tissues (e.g. skeletal muscle, heart and CNS), muscle histopathology, myotonia, progressive muscle weakness, age-dependent defects in glucose metabolism associated with *Insr* missplicing, growth retardation and high mortality [47–49]. The abnormal distribution of MAPT/tau protein isoforms in DM300 brains [47] resembles the tauopathy described in patients [50]. DM300 expansion-biased intergenerational instability led to DMSXL mice carrying 1000–1800 CTG repeats [51]. Homozygous DMSXL mice exhibit a more pronounced phenotype, including splicing abnormalities in skeletal muscle, heart and CNS that are more readily detected at 1 month of age [51]. The requirement for homozygosity, suggesting a dose-dependent effect of toxic RNA [47], increases the time and cost of DMSXL mouse breeding. Phenotypic evaluation will ascertain whether a single copy of a very large expanded transgene is sufficient to generate detectable and quantifiable phenotypes. DM300 and DMSXL mice recreate multisystemic DM1 features. Nonetheless, their phenotype is relatively mild, possibly as a result of lower expression levels of the CUG-containing transcripts compared to other mouse models [52].

**Inducible tissue-specific expression of expanded CUG repeats**—The Cre-lox system allows tissue-specific expression of large interrupted CTG repeats within *DMPK* exon 15 (Figure 2d). Expression in heart tissue resulted in severe histopathological, functional and electrophysiological features of DM1, leading to premature death within 2 weeks [53]. Transgene expression in skeletal muscle triggered myotonia, histopathology and progressive muscle impairment [54]. Acute myofibre degeneration was detected 4 weeks after tamoxifen-induced transgene expression [54]. The physiological changes detected in the heart and skeletal muscle of EpA960 mice were accompanied by sequential RNA foci accumulation, MBNL1 sequestration, CELF1 hyperphosphorylation and upregulation, and reversion to embryonic splicing profiles (Figure S1 in the supplementary material online) [9,53,54]. Whereas *HSA*[LR] mice do not show CELF1 upregulation or muscle weakness, the expression of CUG repeats within the *DMPK* 3′UTR results in increased levels of CELF1 and development of a dystrophic scenario in EpA960 mice. Hence, there is a hypothesis that the genomic context of CTG expansion and CELF1 upregulation contributes, at least in part, to muscle wasting [54].

Leaky EpA960 transgene expression introduces a confounding limitation in the dissection of tissue- and/or cellspecific mechanisms of DM1. In addition, the interrupted nature of the EpA960 repeat might trigger specific molecular events and unusual DM1 symptomatology [55,56].

**Inducible and reversible expression of the DMPK 3′UTR**—The reversible nature of RNA toxicity was demonstrated using an inducible model overexpressing the *DMPK* 3′UTR (Figure 2e). Transgene expression in hemizygous *GFP-DMPK-*(CTG)$_{200}$ mice was high enough to result in RNA foci accumulation and MBNL1 sequestration, but insufficient to generate a discernible phenotype [52]. Unlike the expanded version, the *GFP-DMPK-*(CTG)$_5$ short repeat line did not show RNA foci or MBNL1 sequestration, but exhibited profound myotonia and severe heart block on transgene expression, as well as histopathology in skeletal muscle [52]. *GFP-DMPK-*(CTG)$_5$ mice failed to model skeletal muscle atrophy, possibly because of the small repeat size or premature and sudden death from severe cardiac pathology. Modest missplicing was observed in skeletal muscle but was absent in cardiac tissue. Unlike *HSA*[LR] mice, splicing defects in *GFP-DMPK-*(CTG)$_5$ mice were associated with CELF1 upregulation [52]. An increase of the transcriptional factor NKX2-5 in both cardiac and skeletal muscle suggests ongoing transcriptional abnormalities in mice overexpressing the *DMPK* 3′UTR [57].

The CUG dose effect on myogenesis was reported in a transgenic model overexpressing the *DMPK* 3′UTR containing either 11 or 91 CTG repeats in multiple tissues under the control of the *DMPK* regulatory elements [58]. Both *DMPK-GFP-*(CTG)$_{11}$ and *DMPK-GFP-*(CTG)$_{91}$ strains displayed defective muscle differentiation, but the phenotype was exacerbated by the CTG repeat expansion in *DMPK-GFP-*(CTG)$_{91}$ mice, suggesting a cooperative role between *DMPK* 3′UTR sequences and toxic expanded repeats in the full expression of DM1-associated pathology [58]. The more severe phenotype of *GFP-DMPK-*(CTG)$_5$ mice probably reflects the very high expression levels of CUG-containing transcripts compared to other mouse models [52]. Importantly, transgene silencing in these mice resulted in phenotype reversion, providing crucial support to therapeutic neutralisation of expanded *DMPK* transcripts.

In addition to the expression of toxic CUG-expanded RNA repeats, mouse models of abnormal splicing regulation have recreated relevant features of DM1.

# Models of abnormal splicing regulators

The characterisation of the molecular and physiological phenotypes resulting from MBNL inactivation and CELF upregulation in mice, independently of repeat expansion, has illustrated the central role of these two families of splicing regulators in DM1 pathogenesis.

### Mbnl1 knockout mice

Disruption of *Mbnl1* exon 3 eliminates CUG-binding isoforms and mimics the DM1 situation, in which MBNL1 is extensively sequestered and functionally unavailable [59]. *Mbnl1$^{Δ3/Δ3}$* mice exhibit overt myotonia associated with abnormal *CLCN1* splicing, reduced protein staining and chloride currents in skeletal muscle [10,59,60]. *Mbnl1$^{Δ3/Δ3}$* mice develop myopathy but no signs of muscle degeneration [59].

Loss of MBNL1 affects other tissues, resulting in typical DM1 cataracts [59], motivation deficits and apathy [61], as well as conduction defects and missplicing in heart (M. Swanson, unpublished data). Muscle weakness was not detected and might result from different mechanisms, such as CELF1 upregulation. MBNL1 has been implicated in the regulation of RNA transcription, processing and stability of downstream targets, possibly involved in DM pathogenesis [18]. *Mbnl1$^{Δ3/Δ3}$* mice provide a unique tool for identifying such targets.

### Mbnl2 knockout mice

In addition to MBNL1, MBNL2 and MBNL3 also colocalise with *DMPK* and *ZFN9* ribonuclear inclusions [6], suggesting a combinatorial loss-of-function of muscleblind-like proteins. To elucidate the role of MBNL2, two knockout lines were generated. Myopathy and myotonia associated with mild *Clcn1* missplicing were detected in one line [62], but another independent *Mbnl2*-deficient mouse was overtly normal [10]. These contradictory results raised questions about the role of *Mbnl2* in muscle physiology. *Mbnl2* knockout lines may help elucidate the impact of *Mbnl2* inactivation in other tissues and organ systems.

### CELF1 overexpression

Sequestration and functional inactivation of both MBNL1 and MBNL2 proteins in *HSA*$^{LR}$ muscle is not sufficient to recreate all muscle abnormalities, suggesting a role for additional disease intermediates. The contribution of CELF1 upregulation to DM1 pathogenesis has been confirmed by the generation of CELF1-overexpressing lines. The CUGBP1-TR line showed growth retardation, delayed myogenesis, and histological and molecular abnormalities, which correlated with the degree of CELF1 upregulation [20]. MCKCUG-BP1 mice have normally sized stillborn pups showing histological abnormalities and missplicing in skeletal muscle [63]. Both models suggest that CELF1 overexpression is sufficient to induce a developmental phenotype resembling congenital DM1. Their application is limited by high mortality and breeding difficulties. Conditional CELF1 overexpression in TRECUGBP1 mice has circumvented this limitation. Induced CELF1 expression in adult skeletal muscle recreates DM1 histopathology and functional impairment [12]. Upregulation in adult heart results in DM1-like cardiac conduction defects and histological abnormalities, leading to animal death within 2 weeks [11]. Both tissue-specific phenotypes were associated with embryonic splicing profiles.

In addition to RNA toxicity and missplicing, CTG repeat instability is another hallmark of DM1. Mouse models carrying unstable repeat expansions have been generated to investigate repeat dynamics.

### Mouse models of unstable CTG repeats

Intergenerational and somatic instability of trinucleotide repeats was recreated in *Dmt*-D transgenic mice carrying a $(CTG)_{162}$ repeat tract within the *DMPK* 3′UTR [64,65] and in DM55 and DM300 mice carrying a moderate $(CTG)_{55}$ or large $(CTG)_{300}$ expansion, respectively, in the context of the human DM1 locus [46,51,66]. Important features of intergenerational instability were reproduced in these mice, such as the effects of sex and age of the transmitting parent [46,64]. As in humans, expansion-biased somatic mosaicism was tissue-specific and age-dependent [46,65,66]. The "humanised" *Dmpk*$(CTG)_{84}$ knock-in mice exhibited very low intergenerational instability but high somatic mosaicism [67]. No overt CTG repeat instability has been reported in other DM1 transgenic mice.

Mouse models of unstable DM1 repeats have revealed *cis*- and *trans*-acting modifiers of trinucleotide dynamics [68], indicating a role for functional components of the mismatch repair system in CTG repeat expansion by mechanisms of DNA misrepair [69,70]. These lines provide suitable model systems for investigating repeat size mutation and the contribution of somatic mosaicism to phenotypic variability and disease progression.

### Transgenic mouse models of congenital DM1

It is unknown to what extent congenital DM1 shares molecular mechanisms with the adult disease form. Specific animal models of congenital DM1 are therefore required. Developmental abnormalities have been reported in DMSXL lines [47,51] and CELF1-overexpressing mice [20,63]. Further studies are required to determine whether the phenotypes detected recreate *bona fide* features of congenital DM1.

### Mouse models of DM2

Given the dissociation between foci and missplicing in transfected cells [71] and in a DM1 mouse model [52], alternative pathways of disease pathogenesis were investigated, such as inactivation of *CNBP/ZNF9* in DM2. *Zfn9*$^{+/−}$ mice exhibited myotonia, muscle wasting, defective walking, cardiac conduction defects and ocular cataracts in the absence of repeat expansion and missplicing [72], suggesting a pathogenic role for *CNBP/ZNF9* haploinsufficiency. However, contradictory reports on the effects of CCTG expansions on *CNBP/ZNF9* expression in DM2 patients challenge the relevance of *Cnbp*-deficient mice in recreating a true disease scenario [73,74].

Analogous to $HSA^{LR}$ mice, DM2-HSAtg mice express an intronic $(CCTG)_{121}$ expansion. DM2-HSAtg mice display CCUG-containing ribonuclear inclusions, CELF1 upregulation in liver and recapitulate aspects of DM2 muscle pathology, but no missplicing [14] (R. Krahe, unpublished data), in further support of the dissociation between foci accumulation and splicing defects [52,71]. Transgenic lines will be crucial to gain insight into specific aspects of DM2 and to test new therapeutic strategies.

## From molecular pathogenesis to rational therapeutic strategies

At present there is no effective treatment for DM1 or DM2. The dissection of the molecular mechanism has opened new avenues for rational therapeutic targeting of multiple disease intermediates: the DNA expansion, toxic transcripts, RNA-interacting proteins, splicing regulators and misspliced transcripts (Figure 1). As our understanding of disease mechanism improves, therapeutic targeting of pathological events could replace supportive care.

### Modulation of trinucleotide repeat dynamics

Slowing down repeat expansion may delay disease onset and symptom severity, whereas contraction to the normal repeat size range is predicted to be curative [69]. Indeed,

suppression of somatic mosaicism in a mouse model of Huntington disease resulted in delayed histopathology [75]. In addition, small compounds capable of reducing the expansion rate of the DM1 repeat were identified in cell culture [76,77]. The impact of modified repeat dynamics on DM1 manifestations can be assessed in mouse models exhibiting both somatic instability and disease phenotypes (e.g. the DMSXL model). Deciphering repeat expansion mechanisms will guide us towards rational testing of small molecules predicted to interfere with specific steps of the expansion process [69]. Importantly, whereas targeting of the downstream pathogenesis is most likely to be disease-specific, control of repeat dynamics might present general benefits for repeat expansion disorders.

## Neutralisation or elimination of expanded CUG-containing transcripts

The reversible phenotype of *GFP-DMPK*-(CTG)$_5$ mice on transgene silencing [52] supports neutralisation of toxic RNA as an attractive therapeutic strategy. Antisense oligonucleotides targeting expanded transcripts resulted in diffusion of CUG-containing transcripts and MBNL1, splicing correction in *HSA*[LR] and DM300/DMSXL mice and reduced myotonia in *HSA*[LR] animals [78,79]. Alternatively, pharmacological inhibition of the interaction between toxic CUG-containing transcripts and MBNL1 also reduced foci accumulation, redistributed MBNL1 in cell models, and partially reversed missplicing in *HSA*[LR] skeletal muscle [80]. Because cytoplasmic aggregates seem to be innocuous [81], chemicals that increase *DMPK* transcript export out of the nucleus might be beneficial. Many of the transgenic mice described here offer a unique resource for preclinical assessment of anti-sense strategies and small molecules capable of modifying RNA toxicity in DM and other toxic RNA diseases.

## Upregulation of MBNL1 activity

Therapeutic MBNL1 overexpression is predicted to be beneficial in DM1 and DM2. MBNL1 overexpression in *HSA*[LR] skeletal muscle by adeno-associated virus (AAV) transduction overcame MBNL1 sequestration, corrected missplicing and reversed myotonia, but failed to rescue myofibre structure [82]. Efficient systemic AAV delivery in neuromuscular disorders is currently being optimised [83].

Transgenic mouse models demonstrated that MBNL1 depletion and CELF1 upregulation have overlapping, as well as independent, downstream effects [11,84]. Therefore, targeting MBNL1 alone may not fully address all DM1 symptoms.

## Downregulation of CELF1

CELF downregulation provides a window for therapeutic intervention. EpA960 mice have pointed to PKC as a potential therapeutic target [9,85]. Administration of PKC inhibitors to EpA960 transgenic mice expressing toxic expanded repeats in heart tissue reduced steady-state CELF1 protein levels, corrected CELF1-mediated splicing defects, improved mouse survival and ameliorated the cardiac phenotype [85]. Pharmacological or genetic inhibition of PKC activation might be envisaged as a cardiac therapy in DM1. The involvement of PKC-mediated CELF1 upregulation in other tissues and in DM2 is less clear. Thus, therapies based on modulation of CELF1 phosphorylation and protein levels might be limited to specific tissues and to DM1 in particular. Animal models will provide further insight.

## Reverse of spliceopathy by exon skipping

Antisense oligonucleotides can be designed to induce skipping of abnormally included exons. This highly specific strategy was used to selectively correct *Clcn1* missplicing in *HSA*[LR] and *Mbnl1*[Δ3/Δ3] transgenic mice [86]. The sustained suppression of myotonia

suggests that additional aspects of the disease might be reversed by similar strategies. To this end, different antisense oligonucleotides could be combined in a cocktail to target various misspliced transcripts. Strategies to enhance systemic delivery and uptake of antisense oligonucleotides are under development [87].

## Critical comparison of DM1 mouse models: towards an assessment of tissue-specific therapies

Given their intrinsic molecular features and phenotypes, some mouse models show greater potential than others for preclinical assessment of therapies that target specific organ systems. In the following subsections we discuss the potential of DM1 transgenic lines for therapy development in skeletal muscle, heart, CNS and other organ systems (Table 1).

### Skeletal muscle pathogenesis

Therapies targeting skeletal muscle might be primarily assessed in three mouse models: the muscle-specific $HSA^{LR}$ model [43], the inducible EpA960 line [54] and ubiquitously expressing DMSXL mice [47,51]. Myotonic discharges, histological myopathy, foci accumulation, MBNL1 sequestration and splicing abnormalities can be monitored in $HSA^{LR}$, inducible Ep960 and to some extent DMSXL mice to assess the efficiency of therapeutic approaches designed to revert the muscle phenotype. Progressive muscle weakness can be used as a quantitative indicator of muscle pathology in Ep960 mice. Strategies aimed at (i) destroying toxic transcripts, (ii) disrupting the interaction between CUG-containing RNA and MBNL proteins, (iii) increasing the levels of functional MBNL1 and (iv) correcting RNA splicing can be evaluated in skeletal muscle of the three transgenic models. Means to reduce CELF1 levels can be tested in EpA960 mice. The absence of repeat expansion and accumulation of RNA foci in $GFP\text{-}DMPK\text{-}(CTG)_5$ mice, as well as the severity of their phenotype, limits their application in therapeutic assays. Nonetheless, they offer the possibility to test means of escaping RNA toxicity and NKX2-5 activation. $Mbnl1^{\Delta3/\Delta3}$ mice display relevant, consistent and measurable myotonia and myopathic features, and can be used for testing of therapeutic strategies to circumvent the pathological consequences of MBNL1 depletion. Monitoring of motor, histopathological and molecular phenotypes in TRECUGBP1 skeletal muscle offer additional means to assess the benefits of targeting CELF1 upregulation and missplicing.

### Cardiac defects

The cardiac phenotype of EpA960 mice can be monitored by ECG recordings of systolic and diastolic dysfunction, arrhythmia and AV block. RNA foci accumulation, MBNL1 sequestration, PKC activation, CELF phosphorylation and upregulation and missplicing can be investigated by molecular methods following therapeutic intervention. Whereas both TRECUGBP1 and $GFP\text{-}DMPK\text{-}(CTG)_5$ mice provide additional means to test the cardiac response to CELF1 downregulation, only $GFP\text{-}DMPK\text{-}(CTG)_5$ mice offer the opportunity to test the benefits of NKX2-5 down-regulation. The severe phenotype and mortality of EpA960 and $GFP\text{-}DMPK\text{-}(CTG)_5$ mice represent limitations of these models. Characterisation of DMSXL and $Mbnl1^{\Delta3/\Delta3}$ cardiac function is required for future therapeutic experiments.

### CNS dysfunction

The efficacy of CNS therapeutic schemes can be assessed through analysis of nuclear RNA aggregation, missplicing and abnormal MAPT/tau protein profiles in DMSXL animals. Alternatively, amelioration of $Mbnl1^{\Delta3/\Delta3}$ motivation deficits can be monitored by behavioural phenotyping. Further characterisation of molecular and behavioural CNS

readouts in transgenic mice is required to investigate the impact of future therapies on DM1 neurological manifestations. Both inducible EpA960 and *GFP-DMPK*-(CTG)$_5$ mice offer the possibility to express toxic RNA transcripts in the CNS.

### Other tissues and organ systems

Virtually all tissues and organ systems are affected by DM1 [88]. Therefore, the benefits of therapeutic approaches should be investigated throughout the body. Both DMSXL and *Mbnl1*$^{Δ3/Δ3}$ mice recreate multisystemic phenotypes. Further phenotypic characterisation might reveal additional DM1 features in both lines. DMSXL transgene expression under the control of the human promoter and *Mbnl1* inactivation will prove valuable in evaluating the impact of systemic therapies in different body systems. The multisystem impact of new therapies can be evaluated through analysis of DMSXL and *Mbnl1*$^{Δ3/Δ3}$ splicing profiles or accumulation of ribonuclear foci in DMSXL mice. Tissue-specific transgene expression in conditional DM1 models might further contribute to the development of therapeutic strategies that target additional tissues.

### Tissue-specific versus systemic approaches

The choice of model to be used in therapeutic experiments depends on the target tissue and the question being asked. *HSA*$^{LR}$ and inducible EpA960 and *GFP-DMPK*-(CTG)$_5$ mice recreate consistent tissue-specific phenotypes that are easily quantifiable and facilitate monitoring of the benefits and efficacy of new therapeutic strategies. Despite their milder phenotype and interindividual variability, DMSXL mice have the advantage of expressing full-length expanded *DMPK* transcripts in multiple tissues under the control of the endogenous human promoter. Therefore, they provide a unique tool for extending and refining primary tissue-specific therapeutic assays for multisystem evaluation of their benefits and efficiency.

## Concluding remarks and future perspectives: can multiple transgenic lines replace a 'perfect' model?

Complementary DM1 mouse models have recreated many molecular and phenotypic disease features, pointing to an RNA-mediated disease mechanism. Although fundamental aspects of molecular pathogenesis still deserve further investigation (Box 3), our current understanding of DM1 is sufficient to design rational therapeutic strategies that target molecular events of the pathobiological cascade. The vast collection of mouse models available offers opportunities to therapeutically target specific tissues and organ systems and investigate different levels of disease severity. Independent DM1 lines make possible proof-of-principle studies by different laboratories: parallel tests on multiple models enable replication of results, data refinement and strengthening of conclusions. Protocol standardisation for phenotype monitoring will facilitate direct comparison of independently generated results. Nonetheless, large-scale therapeutic trials on multiple transgenic lines are not practical or financially feasible. Major preclinical experiments should focus on the selected mouse model that is most relevant to the question being asked and the phenotypic readouts being measured. It might be neither necessary nor reasonable to generate a 'perfect' transgenic mouse that recreates all DM1 (and/or DM2) features. Nonetheless, there is certainly room for improvement. Whereas muscle and heart phenotypes have been reproduced and characterised in mice, investigation of CNS manifestations is lagging behind. Characterisation of the cognitive and behavioural phenotypes of the available lines is required.

Toxic RNA repeats are the central pathogenic agent behind a number of human diseases. The combined study of existing DM1, DM2, SCA8, FXTAS and SCA10 mouse models will

help to further dissect RNA toxicity and develop novel therapeutic strategies *in vivo*. The progress made so far does not bring the development and analysis of mouse models to a halt. Instead, it should be a source of great optimism for future advances.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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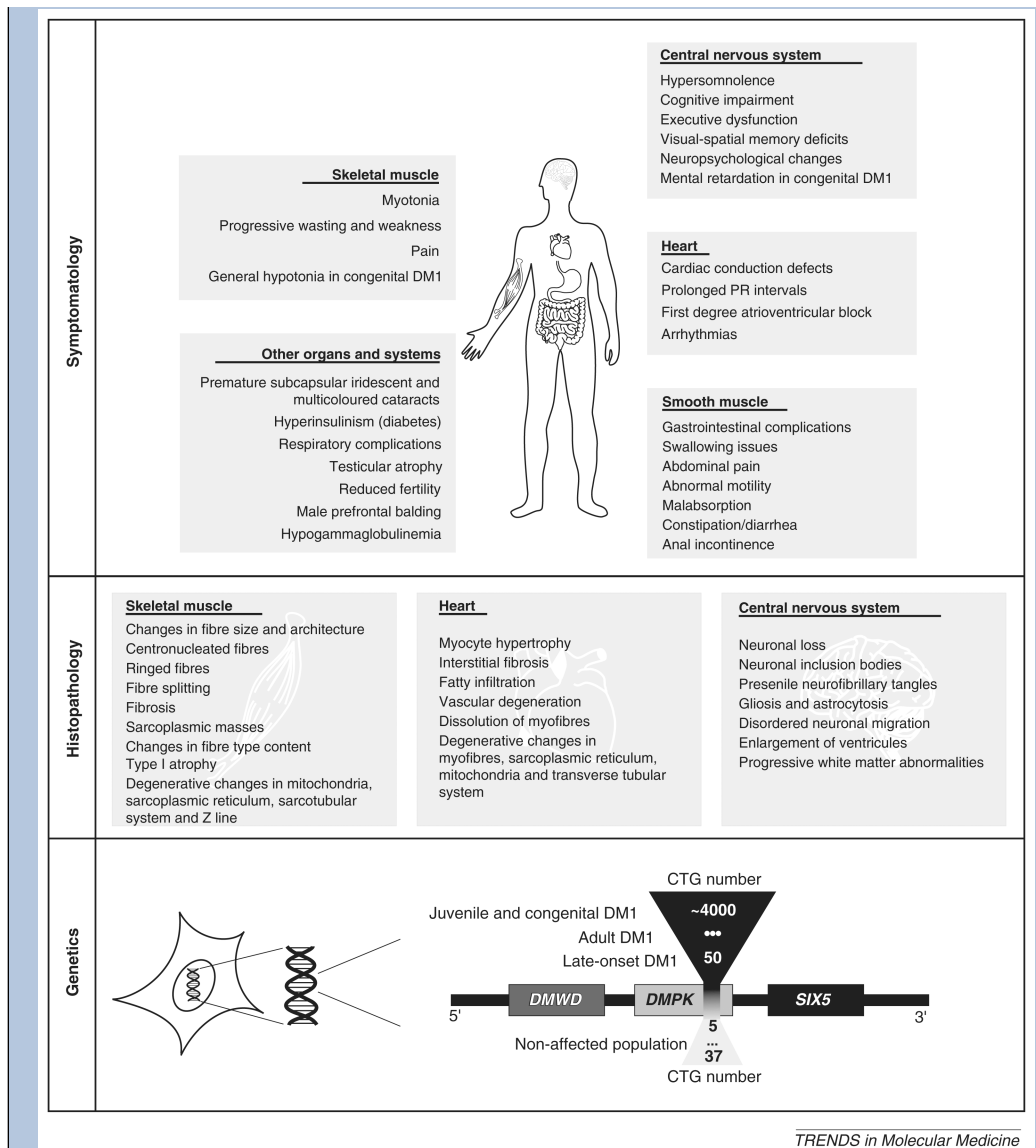
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**Box 1**

### DM1 as a multisystem disease: clinical profile, molecular genetics and trinucleotide repeat dynamics

The great variability of DM1 symptoms and age of onset results in three main clinical forms of the disease: late-onset, classical adultonset and congenital DM1.

Myotonia (delayed muscle relaxation after initial contraction) and progressive wasting of distal muscles are prominent features of DM1 in skeletal muscle, and are accompanied by characteristic histopathological findings [88]. The more severe congenital form of DM1 is characterised by general muscle hypotonia and respiratory distress at birth, as well as delayed motor development. A large proportion of patients suffer from cardiac conduction blocks, detected by electrocardiogram (ECG), and cardiac histological abnormalities. Progressive cardiopathy can result in complete atrioventricular (AV) block or ventricular arrhythmias and subsequent sudden death in ∼30% of DM1 patients [88]. CNS manifestations are highly debilitating and support the view that DM1 is also a brain disorder [88,89]. DM1 neuropsychological dysfunction is accompanied by histological abnormalities, as well as brain structural changes and altered metabolism, as revealed by imaging techniques [89-91]. The impact of DM1 further affects a variety of tissues and results in presenile cataracts, abnormal glucose tolerance and hyperinsulinism, gastrointestinal dysfunction and testicular atrophy (Figure I) [88].

DM1 is caused by expansion of a CTG trinucleotide repeat in the 3′UTR of the DM protein kinase (*DMPK*) gene (Figure I) [92,93]. Repeat length is typically measured in blood samples and correlates positively with disease severity and inversely with age of onset. The expanded sequence is highly unstable, with a marked tendency to further expand during intergenerational transmissions and in somatic tissues. The mean CTG repeat size is usually larger in skeletal muscle than in blood [94]. Parent-to-child instability provides the molecular basis for anticipation, whereby the age of onset decreases and the disease severity increases in successive generations [95]. Tissue-specific, age-dependent, expansion-biased somatic mosaicism may contribute to phenotypic variability and disease progression [69]. DM2 is caused by expansion of an unstable CCTG tetranucleotide repeat within the first intron of the unrelated CCHC-type zinc finger, nucleic acid binding protein (*CNBP*) gene, previously named zinc finger protein 9 (*ZNF9*) gene [2].

**Symptomatology**

**Central nervous system**
Hypersomnolence
Cognitive impairment
Executive dysfunction
Visual-spatial memory deficits
Neuropsychological changes
Mental retardation in congenital DM1

**Skeletal muscle**
Myotonia
Progressive wasting and weakness
Pain
General hypotonia in congenital DM1

**Heart**
Cardiac conduction defects
Prolonged PR intervals
First degree atrioventricular block
Arrhythmias

**Other organs and systems**
Premature subcapsular iridescent and multicoloured cataracts
Hyperinsulinism (diabetes)
Respiratory complications
Testicular atrophy
Reduced fertility
Male prefrontal balding
Hypogammaglobulinemia

**Smooth muscle**
Gastrointestinal complications
Swallowing issues
Abdominal pain
Abnormal motility
Malabsorption
Constipation/diarrhea
Anal incontinence

**Histopathology**

**Skeletal muscle**
Changes in fibre size and architecture
Centronucleated fibres
Ringed fibres
Fibre splitting
Fibrosis
Sarcoplasmic masses
Changes in fibre type content
Type I atrophy
Degenerative changes in mitochondria, sarcoplasmic reticulum, sarcotubular system and Z line

**Heart**
Myocyte hypertrophy
Interstitial fibrosis
Fatty infiltration
Vascular degeneration
Dissolution of myofibres
Degenerative changes in myofibres, sarcoplasmic reticulum, mitochondria and transverse tubular system

**Central nervous system**
Neuronal loss
Neuronal inclusion bodies
Presenile neurofibrillary tangles
Gliosis and astrocytosis
Disordered neuronal migration
Enlargement of ventricles
Progressive white matter abnormalities

**Genetics**

CTG number
Juvenile and congenital DM1 ~4000
Adult DM1 •••
Late-onset DM1 50

5' DMWD DMPK SIX5 3'

5
...
37
Non-affected population
CTG number

*TRENDS in Molecular Medicine*

**Figure I.**
Summary of DM1 symptoms, histopathology and genetics. **Symptomatology**: disease manifestations have been reported in skeletal muscle, heart, CNS, smooth muscle, eye lens and in many other tissues and organ systems. **Histopathology**: the main histopathological findings are in the skeletal muscle, heart and CNS of DM1 patients. **Genetics**: the DM1 locus, showing the *DMWD*, *DMPK* and *SIX5* genes. The CTG repeat sequence maps within the 3′UTR of *DMPK* gene, which overlaps with the promoter region of the downstream sine oculis-related homeobox 5 (*SIX5*) gene [100]. Immediately upstream of *DMPK* lies the myotonic dystrophy WD repeat-containing *(DMWD)* gene [101]. In the unaffected population, the size of the CTG tract varies between 5 to 37 repeats. The severity of DM1 symptoms increases with the size of the repeat expansion: expansions of ~50–100 CTG repeats result in a mild and late-onset form of DM1; expansions of ~100–500 CTG repeats lead to the multisystemic adult form of DM1; expansions ~500–4000 CTG repeats often result in juvenile and congenital DM1. These boundaries are not rigid and CTG repeat sizes can overlap to some extent between clinical forms of the disease.

**Box 2**

**Ideal features of transgenic mouse models of human disease**

Genetic, molecular and phenotypic characteristics are critical if a transgenic model is to provide relevant insight into the mechanisms of the disease (Table I). Molecular, cellular and organ-based features of the human disease should be examined in transgenic mice to ensure faithful recapitulation of the disease process. A thorough, systematic and multidisciplinary approach requires close interaction between researchers, clinicians, pathologists and physiologists to ascertain the relevance of the molecular abnormalities and animal model phenotypes to the understanding of disease pathogenesis.

By contrast, mouse models used in preclinical therapeutic experiments have distinctive requirements to be predictive of the efficacy of the response in humans: *in vitro* and *in vivo* data must predict the clinical outcome (Table I). Rather than recreating every element of the human disorder, it is sufficient to develop a few clinically meaningful phenotypes (either physiological or molecular) that reflect genuine elements of disease pathogenesis. The relevant phenotypes should be strong enough to be detectable and objectively quantifiable, but not so severe as to result in early and dramatic mortality. Transgene expression determines phenotype severity and progression: whereas low expression levels might not generate discernible phenotypes, high levels of expression might result in non-specific features and high mortality. In the absence of physiological readouts, the efficiency of new therapeutic schemes can be estimated in terms using disease-associated molecular deficits.

**Table I**
**Ideal features of transgenic mouse models generated to understand the molecular pathogenesis of disease or to develop preclinical assays**

| | Requirements for mouse models of human neuromuscular disease |
|---|---|
| Genetic features | Same genetic defect as the human condition |
| | Homogeneous and stable background |
| | Easy colony maintenance and mouse breeding |
| | Low interindividual variability |
| Molecular phenotype | Faithful reproduction of the pathobiological cascade |
| Physiological phenotype | Consistent, robust and quantifiable phenotypes |
| | Sufficient complexity to recreate the human disease |
| Ideal features for utility in preclinical assays | Meaningful biochemical defects |
| | At least one robust, consistent and quantifiable phenotype |
| | Phenotype development within a reasonable time frame |
| | Reversible phenotype and responsiveness to treatments |
| | Prediction of the human response to treatment |

**Box 3**

### Outstanding questions

Molecular pathogenesis

- To what extent does the DM1 (and DM2) spliceopathy explain the full disease spectrum? What is the pathogenic role of deregulated gene expression [18,19,57,96,97] and/or cell stress-induced effects on protein translation [22]?

- What is the degree of molecular pathogenesis overlap between adult and congenital DM1? Do they share the same pathobiology?

- It has been shown that the mouse strain affects repeat dynamics [67], but a detailed study of the effects of strain background and/or genetic drift on disease manifestation and progression has not been reported. To what extent is the DM phenotype influenced by genetic modifiers in humans and in mice?

- Tissue-specific expression of toxic RNA in an individual tissue is sufficient to induce relevant DM1 features. However, it is conceivable that non-expressing tissues might exhibit phenotypes secondary to integral dysfunction of body physiology. What is the boundary between the primary and secondary impacts of DM1 expansion? Monitoring of secondary phenotypes in tissue-specific transgenic lines will shed light on this question, despite the confounding effects of possibly leaky transgene expression.

  Therapeutic strategies

- What are the most relevant phenotypic readouts for assessing the efficiency of therapeutic schemes in the CNS of transgenic mouse models?

- What is the best strategy for systemic treatments that target multiple tissues and organs affected by DM?

- Is it possible to develop pharmacological approaches based on non-toxic chemical compounds capable of reversing DM-associated spliceopathy?
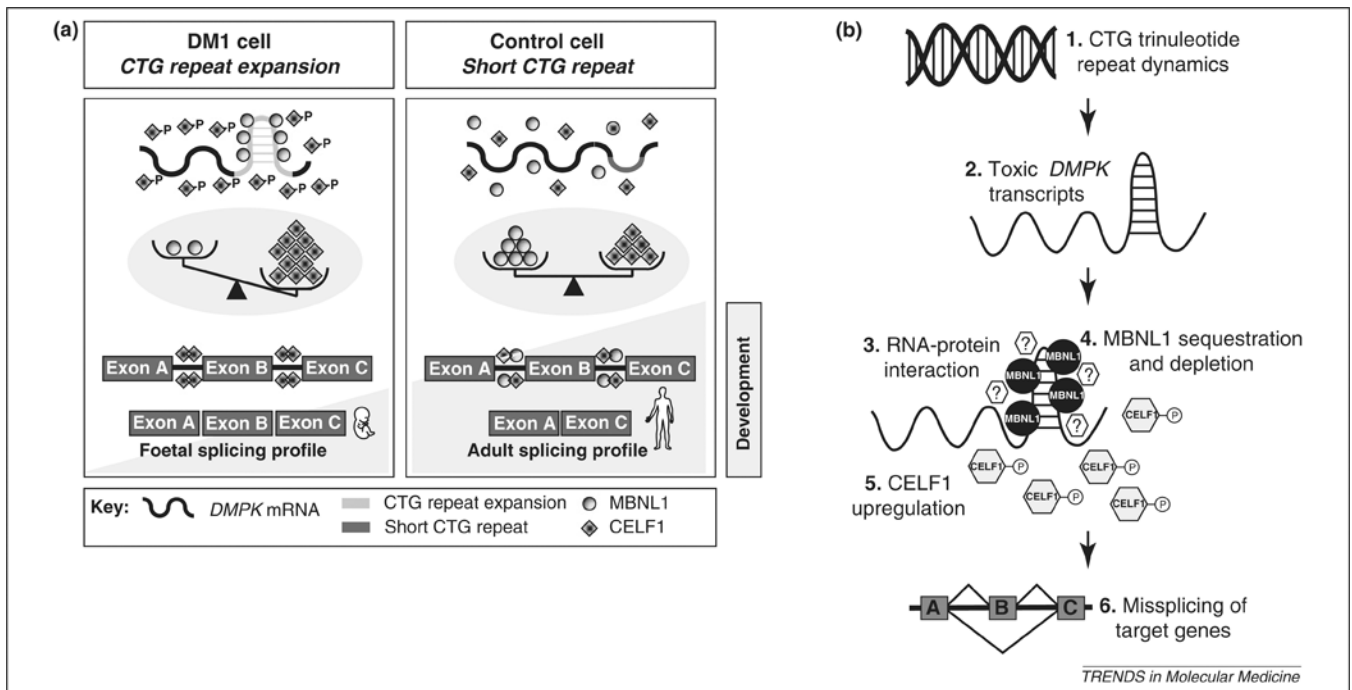
**Figure 1.**
The toxic RNA gain-of-function model of DM1 molecular pathogenesis and molecular targets for rational DM1 therapies model. (a) Unaffected cells (right panel), carrying short CTG sequences, show a functional equilibrium between two antagonistic splicing regulators: muscleblind-like 1 (MBNL1) and CUGBP/Elav-like family member 1 (CELF1). The balance between MBNL1 and CELF1 plays a central role in the establishment of adult splicing profiles for a series of developmentally regulated genes. The CTG repeat expansion observed in a DM1 cell (left panel) forms an imperfect double-stranded structure that has at least two pathogenic consequences: MBNL1 sequestration by nuclear RNA foci and protein kinase C (PKC)-mediated CELF1 hyperphosphorylation and stabilisation. As a result of MBNL1 sequestration and CELF1 upregulation, the functional balance between these two splicing regulators is disturbed and the alternative splicing of a series of developmentally regulated genes reverts to a foetal profile. The abnormal expression of foetal splicing isoforms in adult skeletal muscle and heart is likely to contribute to the onset of DM1 disease symptoms. (b) Dissection of the molecular pathobiology of DM1 has identified molecular targets and potential strategies of therapeutic intervention. (1) Expansion-biased CTG trinucleotide repeat dynamics can be modified genetically or pharmacologically to stabilise the repeat or induce repeat contractions. (2) Antisense approaches can eliminate expanded myotonic dystrophy protein kinase (*DMPK*) transcripts. (3) Pharmacological inhibition of the interaction of transcripts with MBNL1 and other as yet unidentified proteins (question marks) or induced export to the cytoplasm can neutralise some of the pathogenic effects of the transcribed repeat. (4) MBNL1 overexpression or replacement techniques may restore levels of functional MBNL1, which is sequestered and depleted from the nucleoplasm. (5) CELF1 downregulation strategies, such as inactivation of PKC, may prove useful in reducing the levels of this splicing regulator. (6) Targeting of downstream genes and correction of alternative splicing can eliminate specific disease symptoms.
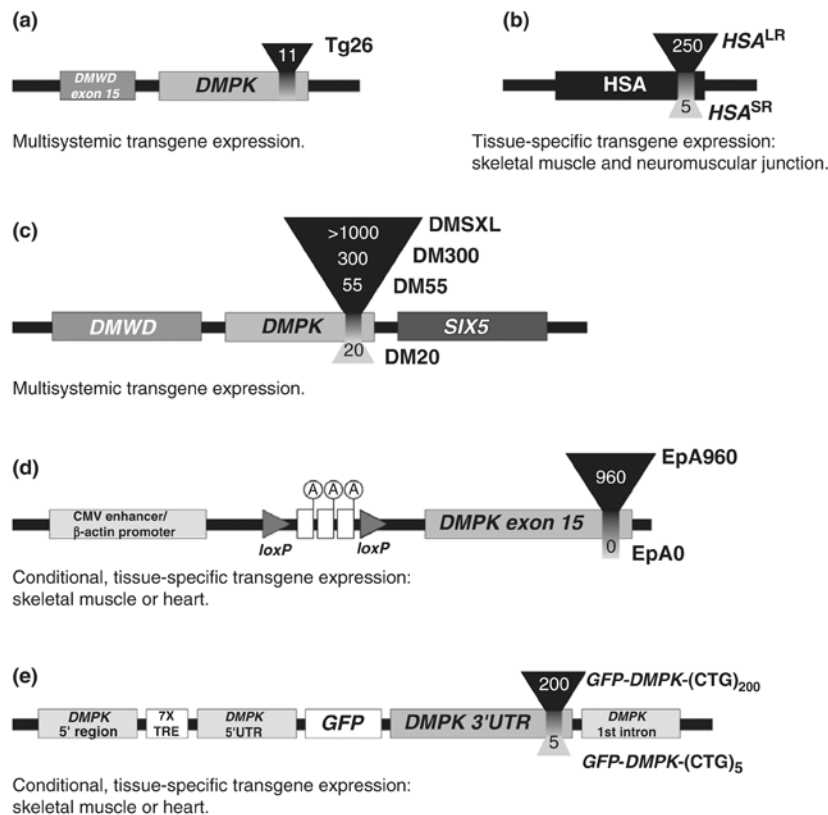
**Figure 2.**
Schematic representation of CUG-expressing transgene constructs comprising multiple genetic elements. A brief description of the spatial profile of transgene expression is included for each insert. (a) Tg26 mice carry ∼25 copies of a fragment of human genomic DNA containing the *DMPK* gene sequence with 11 CTG repeats and the last *DMWD* exon. (b) *HSA*[SR] and *HSA*[LR] transgenic mice carry a fragment of human skeletal actin (HSA) gene, containing a short 5-CTG or an expanded 250-CTG repeat sequence inserted in its final exon, respectively. *HSA*[SR] lines carry 1-20 copies of the transgene, whereas *HSA*[LR] lines have 1-12. (c) DM20, DM55, DM300 and DMSXL transgenic mice carry one to three copies of a large fragment of human genomic DNA containing the three genes of the DM1 locus and a variable number of CTG repeats: 20, 55, ∼300–600 and ∼1000–1800, respectively. (d) Inducible EpA mice carry the human version of *DMPK* exon 15, either without repeats (EpA0 line) or with an interrupted 960-CTG expansion (EpA960 lines). Transgene expression is driven by the cytomegalovirus (CMV) enhancer and (β-actin promoter. The polyadenylation cassette located between the two *loxP* sites prevents *DMPK* exon 15 expression prior to recombination. Tamoxifen administration induces tissue-specific Cre-mediated recombination, removes the transcriptional stop and results in tissue-specific expression of control and expanded transcripts. (e) *GFP-DMPK*-(CTG)$_n$ mice carry a tetracycline-responsive version of the *DMPK* promoter to express a *GFP* transcript fused to the *DMPK* 3′UTR containing either 5 or 200 CTG repeats. These animals also express the reverse tetracycline transactivator constitutively. In the presence of doxycycline, the transactivator binds to the tetracycline response elements (TRE) and activates expression of the *GFP-DMPK*-(CTG)$_n$ transgene.

**Table 1**

**Preclinical assessment of therapeutic strategies in DM1 mouse models**

| Mouse model[a] | Therapeutic strategies[b] | Quantifiable phenotypes in target tissues | | | | Limitations | Refs |
|---|---|---|---|---|---|---|---|
| | | Skeletal muscle | Heart | CNS | Others | | |
| HSA[LR] | Elimination of toxic RNA, disruption of RNA–MBNL protein interactions, MBNL upregulation, restoration of missplicing | Myotonia, myopathy, foci accumulation, MBNL1 sequestration, missplicing events | Not applicable | Not applicable | Not applicable | No muscle weakness, normal CELF1 levels | [43] |
| EpA960 | Elimination of toxic RNA, disruption of RNA–MBNL protein interactions, MBNL upregulation, CELF1 downregulation, PKC inactivation | Myotonia, motor function, muscle wasting, myopathy, foci accumulation, MBNL1 sequestration, missplicing events | Cardiac conduction defects, cardiomyopathy, foci accumulation, MBNL1 sequestration, missplicing events | Not reported | Not reported | Leakage of transgene expression in skeletal muscle, high mortality due to cardiac complications | [9,53,54] |
| DM300 and/or DMSXL | Elimination of toxic RNA, disruption of RNA–MBNL protein interactions, MBNL upregulation, restoration of missplicing, modification of triplet repeat dynamics | Myotonia, myopathy, foci accumulation, muscle strength, missplicing events | Foci accumulation, missplicing events | Foci accumulation, missplicing events, tauopathy | Foci accumulation, missplicing events | Mild splicing abnormalities, interindividual variability, time-consuming breeding strategies | [47,49,51, 98,99] |
| DMPK-GFP-(CTG)$_5$ | Elimination of toxic RNA, NKX2-5 downregulation | Myotonia, myopathy, missplicing events. | Cardiac conduction defects | Not reported | Not reported | No muscle weakness, no RNA foci, short repeat sequence, high mortality | [52] |
| Mbnl1[Δ3/Δ3] | MBNL upregulation or replacement | Myotonia, myopathy, missplicing events | Cardiomyopathy | Motivation and apathy deficits | Not reported | No muscle weakness, normal CELF1 levels | [59,61] |
| TRECUGBP1 | CELF1 downregulation, restoration of missplicing | Motor function, myopathy, muscle weight, missplicing events | Cardiac conduction defects, cardiomyopathy | Not reported | Not reported | High mortality | [11,12] |

[a] Different mouse lines have different advantages and limitations in assessing the benefits and efficacy of new therapeutic schemes in specific tissues and organ systems.

[b] Possible therapeutic approaches are presented for mouse models that replicate key events in the biochemical cascade of RNA toxicity and develop relevant DM1 phenotypes.