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Frontiers of Neurogenetics and Neuroscience: Myotonic Dystrophy and RNA Stories

Misregulation of alternative splicing and microRNA processing in DM1 pathogenesis

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Abstract: Myotonic dystrophy of type I (DM1) is an autosomal dominant inherited disease caused by an unstable CTG expansion in the 3' non-coding region of the *DMPK* gene that confers to the mutant transcript a toxic RNA gain-of-function. Nuclear accumulation of *DMPK* transcripts containing expanded CUG repeats alters the activities of the splicing regulators MBNL1 and CUGBP1 resulting in alternative splicing misregulation of a numerous of transcripts in DM1 tissues. In collaboration with N. Charlet we identified a new mis-splicing event in the muscles of DM1 patients: *BIN1* exon11 splicing mis-regulation due to MBNL1 loss-of-function results in the expression of an inactive form of BIN1. Reproducing similar *BIN1* mis-splicing defect in the muscles of wild type mice is sufficient to promote T-tubule alterations and muscle strength decrease, suggesting that alteration of *BIN1* splicing contributes to DM1 muscle weakness. Interestingly, the RNA binding protein MBNL1 regulates also the processing of the microRNA miR-1 that was found mis-regulated in the heart of DM1 patients. The consequences of miR-1 mis-regulation on DM1 heart conduction defects are not fully understood yet, however this work may shed light on the alteration of this class of non-coding RNA as an additional molecular mechanisms involved in DM1 pathophysiology.

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Key words：myotonic dystrophy, CTG repeats, RNA, alternative splicing, miRNA

Myotonic dystrophy type 1 (DM1) also called Steinert disease is one of the most common muscular dystrophies encountered in adults. Progressive muscle wasting and weakness, myotonia, cardiac conduction defects, alteration in cognitive functions as well as several other multisystemic symptoms characterize this dominantly inherited disease. The DM1 mutation was identified in 1992 and this complex disease is caused by an expanding (CTG)n repeat of 50 to several thousand triplets in the 3'non-coding region of the dystrophia myotonica-protein kinase (*DMPK*) gene on chromosome 19. Unaffected individuals have fewer than 38 repeats. The size of the expansion is generally correlated with the clinical severity and the age of onset of the disease. Due to the variable clinical symptoms, several forms of the disease (asymptomatic late-onset, mild adult-onset, childhood-onset and congenital) have been described. The severe congenital form is associated with large expansions (over 1,500 CTG repeats), and affected patients have motor and mental retardation. The disease-associated repeat expansion is very unstable and the number of triplets increases across generations providing a molecular basis for the anticipation phenomenon observed in DM1 families. In addition to intergenerational instability, CTG repeat expansion is also unstable in somatic tissues throughout the lifetime of the patient.

Evidences for an RNA gain-of-function mechanism in DM1 pathogenesis came to light progressively. Both wild-type and mutant *DMPK* alleles are transcribed into mRNAs but mutant transcripts with expanded CUG repeats (CUGexpRNAs) are sequestered in the nucleus as discrete aggregates or foci leading to decrease cytoplasmic *DMPK* mRNA levels. Subsequent reduction of DMPK protein levels has been a subject of controversy but reduced DMPK levels were observed in muscles samples from DM1 patients as well as DM1 muscle cells. Possible involvement of DMPK haploinsufficiency in DM1 pathophysiology as well as reduced levels of SIX5 observed in DM1 tissues due to the *SIX homeobox 5* (*SIX5*) gene location directly downstream from the DM1 locus, were first investigated by generating mouse models. However heterozygous *Dmpk* or *Six5* knockout mice failed to reproduce DM1-like symptoms suggesting that DMPK or SIX5 haploinsufficiency are probably not responsible for the DM1 phenotype. Afterwards it has been suggested that the mutant transcripts from the expanded *DMPK* allele were pathogenic per see. Animal models were developed to inves-

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tigate the role and the deleterious effects of CUGexp-RNA expression. Transgenic mice that expressed CUG repeat expansion either in the 3'UTR of the human skeletal muscle alpha actin (HSA-LR) mRNA or in its natural context within the 3'UTR of the human DMPK transcript, exhibited several DM1 features including nuclear aggregates of CUGexp-RNA, myotonia discharges and muscle abnormalities. In addition, severe muscle wasting was described in an inducible EpA 960／HSA-Cre-ER transgenic mice expressing 960 interrupted CTG repeats within the context of the DMPK exon 15 and progressive muscle atrophy was observed in mice expressing human DMPK mRNA with 550 CUG repeats. Altogether, these studies provided strong experimental supports for a key role of CUGexp-RNAs in DM1 pathogenesis. The last evidence for a RNA gain-of-function mechanism came from the identification of a myotonic dystrophy type 2 disorder (DM2) that shares similar clinical features with DM1 disease suggesting a common molecular mechanism. DM2 is caused by a (CCTG)n repeat expansion ranging from 100 to 11.000 units in the first intron of the *CCHC-type zinc finger, nucleic acid binding protein* (*CNBP* also known as *ZNF9*) gene, a non-coding region from a gene non-related to *DMPK*. The RNAs containing the expanded CCUG repeats are also retained in the nucleus and formed aggregates providing an additional support for a central role of mutant RNAs containing expanded repeats in pathophysiology of both DM1 and DM2 diseases.

The CUGexp-RNAs are not exported into the cytoplasm but are retained in the nuclear compartment as discrete aggregates or foci that are easily detected by FISH. The mutant *DMPK* mRNAs are spliced and polyadenylated but their nuclear sequestration due to expanded CUG repeats in the 3'UTR, prevents any translation. Within the nuclei, the foci of CUGexp-RNAs are localized at the periphery of the nuclear speckles, which are structures enriched in splicing snRNPs and the spliceosome assembly factor SC35 as well as many other transcription and splicing-related factors. The pathogenic *DMPK* transcripts do not enter into the speckles suggesting that their export is blocked at an early step in nucleoplasmic transport. *In vitro* studies including crystal structure, enzymatic mapping, optical melting and electron microscopy, have demonstrated that expanded CUG repeats are able to form stable hairpin structures. These double-stranded structures are defined by Watson-Crick G-C base-pairs separated by a periodic U-U mismatch. The muscleblind-like 1 (MBNL1) proteins were found to bind these expanded CUG repeats and to colocalize with nuclear foci of CUGexp-RNAs in DM1 cells. MBNL1 silencing by RNA interference significantly reduces the number of foci and restores the capacity of these pathogenic transcripts to progress through the nuclear speckles indicating that the binding of MBNL1 to the abnormal CUG repeats may promote nuclear foci formation. It should be noted that MBNL1 also colocalizes with the nuclear foci of CCUG expanded RNA in DM2 cells however the DM2 foci are not localized at the periphery of the nuclear speckles as observed for the DM1 foci. Besides the difference within the expanded nucleotide repeat (CUG vs. CCUG) between DM1 and DM2, the entrapped RNAs in DM2 may contain intronic expanded CCUG repeats only since the *CNBP* premRNA seems to be normally spliced and/or expanded CCUG repeats into abnormally spliced *CNBP* transcripts. Finally, nuclear retention of the CUGexp-RNAs participates to the pathogenic mechanism since the nuclear export of an artificial CUGexp-RNAs by inclusion of woodchuck post-transcriptional regulatory element reduces cellular defects and the expression of DM1 foci exclusively in the cytoplasmic compartment does not induce key DM1 features in a mouse model.

At the molecular level, one of the best-characterized trans-dominant effects induced by the CUGexp-RNAs in DM1 is the misregulation of alternative splicing of a subset of premRNAs. To date, more than twenty-five transcripts have been found to be mis-spliced in different tissues of DM1 patients. The misregulation of splicing events in DM1 is distinct from aberrant splicing caused by mutations in regulatory splicing sites that lead to the expression of aberrant mRNA. In DM1, mis-splicing events result from an inappropriate regulation of alternative splicing due to altered activities of splicing regulators such as MBNL1 and CELF1:

-the MBNL1 RNA binding protein has been shown to bind, in a length-dependant manner, CUGexp-RNA with high affinity and form ribonucleoprotein complexes. MBNL1 is part of a conserved MBNL family including MBNL1, 2, 3, and all members contain four CCCH zinc-finger protein domains that are structured in pairs and acted as RNA binding domains. Sequestration of MBNL1 within the nuclear aggregates of CUGexp-RNAs and the subsequent involvement of MBNL1 loss-of-function in DM1 pathogenesis has been supported by the generation of a knockout Mbnl1 mouse model that demonstrates a DM-like phenotype as well as alternative splicing misregulation. Moreover a majority of the modifications in alterative splicing observed in the HSA-LR mice expressing CUGexp-RNA can be attributed to the loss-of-function of the MBNL1 splicing factor. In addition, mis-splicing events observed in this DM1 mouse model as well as myotonia can be reversed by MBNL1 overexpression in skeletal muscles. Several reports have demonstrated the regulatory splicing function of MBNL1 on several DM1 transcripts such as *CLC-1*, *cTNT* or *IR*. Now, it is established that MBNL1 loss-of-function due to its sequestration by the

CUGexp-RNA contributes to the “spliceopathy” in DM1.

-CELF1 (also known as CUGBP1) is another RNA binding protein involved in this process. This factor is a member of the CELF family that contains 6 proteins with high homology. Interestingly, CELF1 and MBNL1 are antagonistic regulators of many splicing events that are mis-regulated in DM1. CELF1 is able to bind single-strand CUG repeats but does not colocalize with the nuclear aggregates of CUGexp-RNA in DM1 cells and is not sequestered like MBNL1. In contrast the level of CELF1 is increased in DM1 tissues leading to a gain of CELF1 activity. It has been shown that the expression of CUGexp-RNA results in hyperphosphorylation and stabilization of the CELF1 protein through an inappropriate activation of the Protein Kinase C. The pathogenic role of CELF1 in DM1 was supported by the fact that transgenic mice overexpressing CELF1 reproduce splicing misregulation as well as DM1 muscle features. Furthermore, increased levels of CELF1 is also found in the DM1 mouse model expressing inducible 960 interrupted CTG repeats, which exhibits muscle wasting as well as splicing defects that are only related to CELF1 (e.g. *Capzb*, *Mfn2*, *Ank2* and *Fxr1h*) and not to MBNL1, suggesting that the elevation of CELF1 could participate to the DM1 muscle phenotype.

MBNL1 and CELF1 factors are developmental regulators of splicing events especially during the fetal to adult transition, and the modification of their activities in DM1 tissues leads to the expression of a fetal splicing pattern in adult tissues. It should be noted that altered expression of splicing factors and alternative splicing changes may also occurred during active regeneration process in degenerative muscle diseases. However no massive degeneration/regeneration was observed in DM1 muscles and altered splicing events were found in non-regenerating tissue such as DM1 cardiac tissue confirming that misregulation of alternative splicing in DM1 is more likely a primary response to the expression of CUGexp-RNAs rather than a secondary effect to robust degeneration/regeneration process. The first splicing misregulation described in DM1 cardiac muscle was the abnormal inclusion of exon 5 in *cTNT*. Since then, several other transcripts with inappropriate splicing patterns have been identified in both skeletal muscle and brain (see Table 1) including those coding for the insulin receptor (IR), the muscle specific chloride channel (CLC-1), the sarcoplasmic/endoplasmic reticulum Ca2＋ ATPase 1 and 2, the ryanodine receptor, the myotubularine-related protein 1, the tau protein and the N-methyl-alpha-aspartate receptor. Among the known missplicing events, most of them may participate to the pathologic process but very few have been directly correlated with disease symptoms. One of the exceptions is the *CLC-1* splicing defect, which has been associated with myotonia, a characteristic feature of the disease. This splicing misregulation leads to the inclusion of exon 7a and subsequently to a truncated CLC1 protein that is devoid of channel activity and is not correctly addressed to the membrane of the muscle fibers, resulting in reduced muscle chloride conductance and myotonia. Consistent with the RNA gain-of-function hypothesis that altered MBNL1 activity, both MBNL1 knockout mice and HSA-LR mice that express CUGexp-RNA showed Clc-1 splicing misregulation, loss of Clc-1 channel at the membrane and myotonia. Finally, correction of this sole splicing defect by using antisense oligonucleotide that force the skipping of exon 7a in the muscle of HSA-LR mice abolished myotonia confirming the key role of Clc-1 missplicing in the myotonic phenotype in DM1.

More recently, the newly identified *BIN1* splicing defect has been associated with muscle weakness, another hallmark of DM1 (1). This splicing defect was identified in collaboration with N. Charlet by using a whole genome approach (Affymetrix exon array). New as well as previously described splicing alteration events were identified in congenital DM1 muscle cells containing large CTG expansion and confirmed in skeletal muscles from DM patients. We focus our attention on BIN1 exon 11 splicing misregulation since mutation in this gene leads to Centronuclear Myopathy that share some similar features with the severe congenital form of DM1. BIN1 is a protein specialized in membrane curvature, whose function is regulated by alternative splicing. In skeletal muscles, inclusion of the muscle-specific exon 11, which encodes a phosphoinositide-binding (PI) domain, generates an isoform of BIN1 that induces tubular invaginations of membranes and is implicated in T-tubules biogenesis. The muscle T-tubule network is a specialized membrane structure fundamental for excitation-contraction (E-C) coupling, and the disruption of *BIN1* in Drosophila leads to severely disorganized T-tubules and defects of the E-C coupling machinery. We demonstrate that MBNL1 binds to *BIN1* pre-mRNA and regulates its alternative splicing. *BIN1* splicing misregulation results in expression of an inactive form of BIN1 deprived of PtdIns5P-binding and membrane-tubulating activities. Consistent with a defect of BIN1, muscle T-tubules are altered in DM patients and membrane structures are restored upon expression of the normal splicing form of BIN1 in DM1 muscle cells. In non-affected muscles, BIN1 is organized in transversal projections and co-localized with the L-type calcium channel CACNA1S. In DM1 muscles, BIN1 was disorganized and presented a more diffuse localization. Ultrastructural analysis confirmed alterations of the T-tubule network with presence of irregular and longitudinally orientated T-tubules. To test the contribution of *BIN1* splicing alteration for DM phenotype, we artificially forced exon 11 skipping in mouse

Table 1

Tisues	Pre-mRNA	exon/intron deregulation	Inclusion/exclusion	Ref.
Skeletal Muscle	Insulin receptor (INSR)	Exon 11	Exclusion	Savkur & al., 2001
	Chloride channel (CLCN1)	Intron 2	Inclusion	Charlet & al., 2002 Mankodi & al., 2002
		Exon 7A	Inclusion	Lueck & al., 2006
	BIN1 (Amphyphisine 2)	Exon 11	Exclusion	Fugier & al., 2011
	Calcium channel (Ca (V)1.1)	Exon 29	Exclusion	Tang & al., 2012
	Skeletal Troponin T (TNNT3)	Exon fœtal	Inclusion	Kanadia & al., 2003
	Ryanodine recptor (RyR1)	Exon 70	Exclusion	Kimura & al., 2005
	Sarcoplasmic/endoplasmic reticulum Ca2+ATPase 1 (SERCA1)	Exon 22	Exclusion	
	Sarcoplasmic/endoplasmic réticulum Ca2+ATPase 2 (SERCA2)	Intron 19	Inclusion	
	LIM domain inding 3 (LB, ZASP)	11	Inclusion	Lin & al., 2006
	Titin (TTN)	Zr4	Inclusion	
		Zr5	Inclusion	
	Nebulin-related anchoring protein (NRAP)	12	Inclusion	
	Calpaïn 3 (CAPN3)	16	Exclusion	
	Attractin-like (ATRNL1, ALP)	5a et 5b	Inclusion	
	Forming homology 2 domain containing 1 (FHOD1)	11a	Exclusion	
	Glutamine-fructose-6-phosphate transaminase 1 (GFPT1)	10	Exclusion	
	MBNL1	7	Inclusion	
	MBNL2	7	Inclusion	
	SET and MYND domain containing 1 (SMYD1)	39	Inclusion	Du & al., 2010
	Sperm associated antigen 9	39	Inclusion	
	Myotubularin-related protein 1 (MTMR1)	Exon 2.1	Exclusion	Buj-Bello & al., 2002 Ho & al., 2005
		Exon 2.3	Exclusion	
	Alpha-dystrobrevin (DTNA)	Exon 11a	Inclusion	Nakamori & al., 2008
		Exon 12	Inclusion	
Brain	Tau (MAPT)	Exon 2	Exclusion	Sergean & al., 2001
		Exon 3	Exclusion	
		Exon 6	Exclusion exon 6c Inclusion exon 6d	Leroy & al., 2006
		Exon 10	Exclusion	Sergeant & al., 2001 Jiang & al., 2004
	Récepteur-N-methyl-D-aspartate (NMDAR1)	Exon 5	Inclusion	Jiang & al., 2004
	Amyloid precursor protein (APP)	Exon 7	Exclusion	
	MBNL1	Exon 5	Inclusion	Dhaenens & al., 2010

skeletal muscle using an U7-snRNA exon-skipping strategy. Artificial skipping of *Bin1* exon 11 promotes Bin1 mislocalization but no major atrophy or degeneration of muscle fibers. However -30% of T-tubules were abnormal in *Bin1* exon 11 skipped muscles, with longitudinally orientated, disorganized and irregular structures suggesting that alteration of the T-tubule network. No significant muscle mass loss was observed but isometric strength measurement showed that skipping of *Bin1* exon 11 induced a -28% decreased of specific muscle *strength*. Our results suggest that splicing misregulation of *BIN1* and of other pre-mRNAs involved in E-C coupling ultimately results in muscle weakness in DM patients. Interestingly, a recent report proposed that missplicing of the Cav1.1 that altered the function of this calcium channel is also associated with muscle weakness and may exacerbate DM1 myopathy. Altogether, these data suggest

that a common mechanism, involving BIN1 and alteration of the calcium homeostasis coupled to the excitation-contraction process, may underlie muscle weakness in DM1.

Over the years, the RNA gain-of-function hypothesis has progressively emerged as a pathogenic mechanism for the complex DM1 disease. Alternative splicing misregulation of several pre-mRNAs due to the altered activities of MBNL1 and CELF1 RNA binding proteins by CUGexp-RNAs, contributes to the DM1 pathophysiology. However it seems unlike that it can explain the wide spectrum of DM1 clinical symptoms. The CUGexp-RNAs have effects in *trans* and may alter other processes at both post-transcriptional and transcriptional levels. Indeed, altered activities of the MBNL1 and CELF1 may affect other RNA-processing events regulated by these RNA binding proteins. Thus, the activity of CELF1 varies depending on its cellular localization. In the nucleus, CELF1 acts as a splicing regulator whereas in the cytoplasm, it can regulate the translational activity of proteins like p21 and MEF2A, which are involved in muscle cell differentiation (Iakova and others 2004; Timchenko and others 2004). A concomitant translational deregulation of CELF1 targets and associated functions indicate that other post-transcriptional mechanisms could also be altered by the CUGexp-RNAs. Unlike CELF1, no effect on translation has been described for MBNL1 yet, even though MBNL1 is also present in the cytoplasmic compartment. Alternative splicing results in the production of several isoforms of *MBNL1* and the associated protein isoforms have been shown to have either a nuclear or a nucleo-cytoplasmic localization (Tran and others 2011). It should be noted that the splicing of MBNL1 it-self is altered in DM1 leading to increased levels of exclusively the nuclear isoforms. However the impact of such alterations on the activities of MBNL1 is still not clearly understood.

More recently, N. Charlet's team has described a novel function of the RNA binding protein MBNL1 as a regulator of the micro-RNA miR-1 biogenesis (2). MBNL1 is localized both in the nucleus and the cytoplasm suggesting a distinct cytoplasmic function for this splicing factor and/or a shuttling function for this RNA binding factor. Predictive bioinformatic analysis indicates that pre-miR-1 have potential MBNL1 binding site. Based on this observation, a miRNome analysis performed on human muscle cells isolated from DM1 and non-affected individuals showed a significant alteration of miR-1 expression in DM1 cells. At the molecular level, MBNL1 binds to a UGC motif located within the loop of pre-miR-1 and competes for the binding of LIN28, which promotes pre-miR-1 uridylation by TUT4 and blocks Dicer processing. Because inactivation of *miR-1-2* in mice did not result in an overt phenotype in skeletal muscle but cause cardiac dysfunction, miR-1 expression was analyzed in heart samples from DM1 patients. The expression of miR-1 is specifically altered in the DM1 heart samples when compared to unaffected individuals. As a consequence of miR-1 loss, expression of connexin 43 and CACNA1C, which are targets of miR-1, are also increased in DM1 hearts. CACNA1C and connexin 43 encode the main calcium-and gap-junction channels in heart, respectively, and their misregulation could contribute to the cardiac dysfunctions such as conduction defect observed in the DM1 patients. Interestingly, other miRNA were also deregulated in muscles of DM1 patients suggesting that the deregulation of this species of small non-coding RNA could have an impact on DM1 pathology. By interfering with RNA metabolism of either coding or non-coding RNAs, the CUGexp-RNAs may act on the expression of various proteins in a tissue-specific manner and participate to the complex and multisystemic DM1 phenotype.

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