identified as a protein binding to the 3′-UTR, but the core promoter does also contain an E box, known to be a bHLH consensus binding site. Additionally, a functional −67 A/T SNP in this promoter region has been reported to be associated with personality traits such as ADHD and bipolar disorder (Greenwood and Kelsoe 2003; Ohadi et al. 2006, 2007; Shibuya et al. 2009). HESR family proteins may also interact with this SNP. Only HESR3 increased reporter luciferase activity via the DAT core promoter. We also found that HESR1, including the Leu94Met SNP in the second helix of the bHLH domain, lacked inhibitory activity (Fuke et al. 2005). The latest study demonstrated that an SNP transformed HESR1 from an androgen receptor co-repressor to an activator (Villaronga et al. 2009).

Furthermore, HESR1 and HESR2 may differentially alter DAT expression patterns depending on VNTR alleles. Relatively strong inhibition of luciferase activity with 10r was observed with HESR1. In general, our results in these reporter assays showed a tendency for luciferase activity with 9r to be higher than that with 10r, although the difference was not statistically significant, and the highest activity was with 7r. Human HESR2, but not mouse Hesr2, diminished the difference in luciferase activity between 9r and 10r. These findings basically support our idea that different DAT expression levels can be altered by factors in each cell, depending on VNTR alleles. This may explain the discrepancies between the many previous studies described above.

10.5 Behavioral and Neurochemical Aspects of the Hesr Family

We also reported increased expression of the *DAT* gene in the brains of *Hesr1* knockout (KO) mice (Fuke et al. 2006). The KO mice showed decreased spontaneous locomotor activity, reduced exploration of novelty, and enhanced anxiety-like behavior in the open-field test and the elevated plus-maze test (Fuke et al. 2006). This is consistent with our in vitro data because HESR1 is thought to be an inhibitory factor for *DAT*. Additionally, the expression of several dopamine receptor genes, *D1*, *D2*, *D4*, and *D5*, the main targets of synaptic dopamine responsiveness, were enhanced in the *Hesr1* KO mice. Although we did not directly measure synaptic extracellular dopamine levels, decreased activity and increased dopamine transporter and receptors seem to indicate a low synaptic dopamine level in the KO mice. These phenomena are the opposite of those in *DAT* KO mice (Fig. 10.2). Mice lacking the *DAT* gene show decreased intraneural storage of dopamine, spontaneous hyperlocomotion, and down-regulation of several dopamine-related genes, such as dopamine receptor D1 and D2 (Giros et al. 1996; Caine 1998; Jaber et al. 1999; Fauchey et al. 2000; Gainetdinov et al. 2002). This indicates the importance of Hesr1 in the dopaminergic system in vivo.

We also conducted an immunohistochemical analysis to investigate the localization of Hesr family proteins in the mouse midbrain dopaminergic region (Fig. 10.3). Immunostaining for tyrosine hydroxylase (TH), a DA neuron marker, and each Hesr were conducted from the anterior (−3.04 to −3.49 relative to bregma) to the posterior part (−3.94 from bregma) of the midbrain dopaminergic regions: ventral

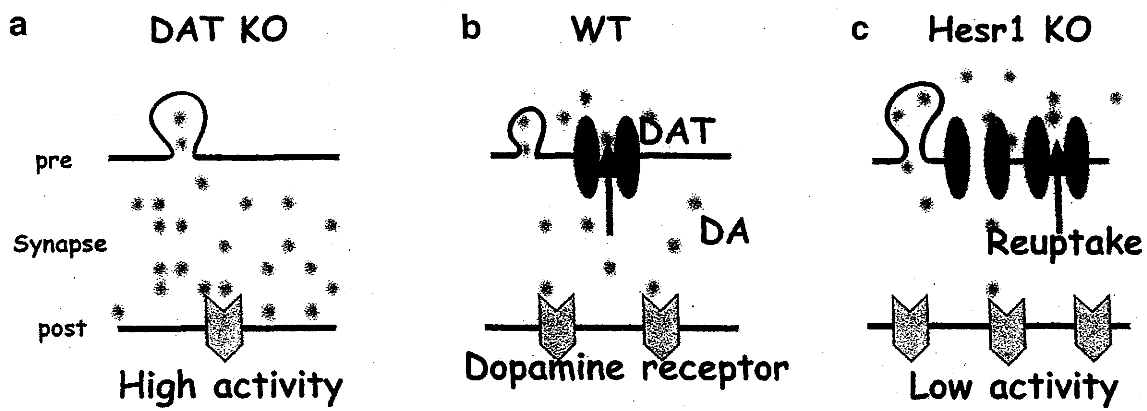


Fig. 10.2 Synapses in *DAT* or *Hesr1* knockout (*KO*) mice. (**a**) *DAT* KO mouse. This indicates increased synaptic extracellular dopamine and decreased dopamine receptors. (**b**) Wild-type mouse. This indicates the normal synaptic state. (**c**) *Hesr1* KO mouse. This indicates possibly decreased synaptic extracellular dopamine and increased dopamine receptors

Fig. 10.3 Immunohistochemistry for tyrosine hydroxylase (*TH*) and Hesr family. TH (*green*, Cy2); Hesr1/2 (*red*, Cy3); Hesr3 (*magenta*, Cy3); nucleus (*blue*, Hoechst 33342). *VTA*, ventral tegmental area, *SN*, substantia nigra; *RFF* (RFF/A8), retrorubral field and A8 DA cells; *CLi*, caudal liner nucleus of raphe. *Bars* 500 μm for immunoenzymatic staining for TH; 100 μm for immunofluorescence staining

tegmental area (VTA), substantia nigra (SN), retrorubral field and A8 DA cells (RFF/A8), caudal liner nucleus of raphe (CLi). Each Hesr was expressed in almost all dopaminergic neurons (TH-ir cells) in the mouse midbrain. Thus, Hesr family proteins may affect *DAT* gene expression, as was observed in transfected cells. Further investigation of the in vivo functions of Hesr family members, especially Hesr2 and Hesr3, in the dopaminergic system is needed.

Unique dopamine neurons have recently been found in which DAT expression is relatively low. Lammel et al. (2008) identified a type of dopaminergic neuron within the mesocorticolimbic dopamine system with unconventional fast-firing properties and low DAT/TH mRNA expression ratios that selectively projects to the prefrontal cortex and nucleus accumbens core and medial shell as well as to the basolateral amygdala. Could Hesr family proteins be involved in such a neuron, generating diversity in dopaminergic neurons? Our immunohistochemical study found differential cellular localization between the Hesr family proteins. Hesr1 and Hesr2 were primarily expressed in the nucleus, whereas Hesr3 was cytoplasmic (Fig. 10.3). Additionally, it is possible that cellular localization of Hesr1 is altered depending on the hormonal state (Belandia et al. 2005). A combination of chemical, neuroanatomical, and molecular studies is needed to understand Hesr function in the brain. Such studies may help explain conflicts in the previous in vivo neuroimaging studies (Heinz et al. 2000; Jacobsen et al. 2000; Martinez et al. 2001) and ex vivo RT-PCR analyses (Mill et al. 2002; Brookes et al. 2007).

Although it seems clear from transfection culture studies that the VNTR has a role in regulating DAT1 expression, at the same time, discrepancies have been noted in the differential effects of the various alleles. In the future, an in vivo approach using transgenic mice (e.g., *DAT-9r* or *DAT-10r* knock-in mice) may provide a clearer and more direct approach to characterizing the mechanisms of DAT transcriptional regulation. If such animals are generated, our data from luciferase assays with the mouse Hesr family can add a molecular basis to the research.

Our recent findings of HESR family function regarding *DAT* may suggest new strategies for the treatment of *DAT*-related disorders. Functional VNTR polymorphism also exists in the SERT gene located in intron 2, and two transcription factors, Y box-binding protein 1 (YB-1) and CCTC-binding factor (CTCF), were found to be responsible for the modulation of VNTR function (Klenova et al. 2004). YB-1 and CTCF are targets of lithium (LiCl), a mood stabilizer (Roberts et al. 2007). LiCl modified the levels of CTCF and YB-1 mRNA and protein. HESR proteins may also be a target of drugs.

10.6 Conclusions

Our studies and others indicate that the VNTR in the 3′-UTR of the *DAT* gene affects gene expression. Ex vivo RT-PCR studies and in vivo human neuroimaging studies have demonstrated differential DAT expression depending on the alleles, primarily focusing on 9r and 10r, although the results are conflicting.

More genetic and personality studies combined with neuroimaging should be done to clarify the relation between psychological and neurological states, especially DAT expression levels or function. Further molecular biological studies are also necessary to clarify the mechanism of modification of DAT expression and its signaling pathway, which may also help find new neuropsychological drug targets.

Acknowledgments This work was supported in part by the grants from the Ministry of Health, Labor, and Welfare, Japan, and the Human Frontier Science Program.

References

Bannon MJ, Michelhaugh SK, Wang J, Sacchetti P (2001) The human dopamine transporter gene: gene organization, transcriptional regulation, and potential involvement in neuropsychiatric disorder. Eur Neuropsychopharmacol 11:449–455

Belandia B, Powell SM, Garcia-Pedrero JM, Walker MM, Bevan CL, Parker MG (2005) Hey1, a mediator of notch signaling, is an androgen receptor corepressor. Mol Cell Biol 25:1425–1436

Brookes KJ, Neale BM, Sugden K, Khan N, Asherson A, D'Souza UM (2007) Relationship between VNTR polymorphisms of the human dopamine transporter gene and expression in post-mortem midbrain tissue. Am J Med Genet B Neuropsychiatr Genet 144B:1070–1078

Brunswick DJ, Amsterdam JD, Mozley PD, Newberg A (2003) Greater availability of brain dopamine transporters in major depression shown by [99m Tc] TRODAT-1 SPECT imaging. Am J Psychiatry 160:1836–1841

Caine SB (1998) Cocaine abuse: hard knocks for the dopamine hypothesis? Nature Neurosci 1:90–92

Cook EH Jr, Stein MA, Krasowski MD, Cox NJ, Olkon DM, Kieffer JE, Leventhal BL (1995) Association of attention-deficit disorder and dopamine transporter gene. Am J Hum Genet 56:993–998

D'Souza UM, Craig IW (2008) Functional genetic polymorphisms in serotonin and dopamine gene systems and their significance in behavioural disorders. Prog Brain Res 172:73–98

Fauchey V, Jaber M, Caron MG, Bloch B, Moine CL (2000) Differential regulation of the dopamine D1, D2 and D3 receptor gene expression and changes in the phenotype of the striatal neurons in mice lacking the dopamine transporter. Eur J Neurosci 12:19–26

Fuke S, Suo S, Takahashi N, Koike H, Sasagawa N, Ishiura S (2001) The VNTR polymorphism of the human dopamine transporter (DAT1) gene affects gene expression. Pharmacogenomics J 1:152–156

Fuke S, Sasagawa N, Ishiura S (2005) Identification and characterization of the Hesr1/Hey1 as a candidate trans-acting factor on gene expression through the 3′ noncoding polymorphic region of the human dopamine transporter (DAT1) gene. J Biochem 137:205–216

Fuke S, Minami N, Kokubo H, Yoshikawa A, Yasumatsu H, Sasagawa N, Saga Y, Tsukahara T, Ishiura S (2006) Hesr1 knockout mice exhibit behavioral alterations through the dopaminergic nervous system. J Neurosci Res 84:1555–1563

Gainetdinov RR, Sotnikova TD, Caron MG (2002) Monoamine transporter pharmacology and mutant mice. Trends Pharmacol Sci 23:367–373

Giros B, Caron MG (1993) Molecular characterization of the dopamine transporter. Trends Pharmacol Sci 14:43–49

Giros B, el Mestikawy S, Bertrand L, Caron MG (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. FEBS Lett 295:149–154

Giros B, el Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T, Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol 42:383–390

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:606–612
Greenwood TA, Kelsoe JR (2003) Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. Genomics 82:511–519
Heinz A, Goldman D, Jones DW, Palmour R, Hommer D, Gorey JG, Lee KS, Linnoila M, Weinberger DR (2000) Genotype influences in vivo dopamine transporter availability in human striatum. Neuropsychopharmacology 22:133–139
Henderson AM, Wang SJ, Taylor AC, Aitkenhead M, Hughes CC (2001) The basic helix–loop–helix transcription factor HESR1 regulates endothyelial cell tube formation. J Biol Chem 276:6169–6176
Inoue-Murayama M, Adachi S, Mishima N, Mitani H, Takenaka O, Terao K, Hayasaka I, Ito S, Murayama Y (2002) Variation of variable number of tandem repeat sequences in the 3′-untranslated region of primate dopamine transporter genes that affects reporter gene expression. Neurosci Lett 334:206–210
Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, Kedes L, Hamamori Y (2001) HERP, a novel heterodimer partner of HES/E (spl) in notch signaling. Mol Cell Biol 21:6080–6089
Iso T, Kedes L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194:237–255
Jaber M, Dumartin B, Sagne C, Haycock JW, Roubert C, Giros B, Bloch B, Caron MG (1999) Differential regulation of tyrosine hydroxylase in the basal ganglia of mice lacking the dopamine transporter. Eur J Neurosci 11:3499–3511
Jackson DM, Westlind-Danielson A (1994) Dopamine receptors: molecular biology, biochemistry and behavioral aspects. Pharmacol Ther 64:291–336
Jacobsen LK, Staley JK, Zoghbi SS, Seibyl JP, Kosten TR, Innis RB, Gelernter J (2000) Prediction of dopamine transporter binding availability by genotype: a preliminary report. Am J Psychiatry 157:1700–1703
Kanno K, Ishiura S (2009) Function of transcription factor HESR family on dopamine transporter expression via variable number of tandem repeat. Abstract 618.26 from 2009 Society for Neuroscience Annual Meeting, Chicago, IL
Kilty JE, Lorang D, Amara SG (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science 254:578–579
Klenova E, Scott AC, Roberts J, Shamsuddin S, Lovejoy EA, Bergmann S, Bubb VJ, Royer H-D, Quinn JP (2004) YB-1 and CTCF differentially regulate the 5-HTT polymorphic intron 2 enhancer which predisposes to a variety of neurological disorders. J Neurosci 24:5966–5973
Kokubo H, Lun Y, Johnson RL (1999) Identification and expression of novel family of bHLH cDNAs related to Drosophila hairy and enhancer of split. Biochem Biophys Res Commun 260:459–465
Kokubo H, Tomita-Miyagawa S, Hamada Y, Saga Y (2007) Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. Development 134:747–755
Krause KK, Dresel SH, Krause J, Fougere C, Ackenheil M (2003) The dopamine transporter and neuroimaging in attention deficit hyperactivity disorder. Neurosci Biobehav Rev 27:605–613
Lammel S, Hetzel A, Häckel O, Jones I, Liss B, Roeper J (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57:760–773
Leimeister C, Externbrink A, Klamt B, Gessler M (1999) Hey genes: a novel subfamily of hairy- and enhancer of split related genes specifically expressed during mouse embryogenesis. Mech Dev 85:173–177
Madras BK, Gracz LM, Fahey MA, Elmaleh D, Meltzer PC, Liang AY, Stopa EG, Babich J, Fischman AJ (1998) Altropane, a SPECT or PET imaging probe for dopamine neurons. III. Human dopamine transporter in postmortem normal and Parkinson's diseased brain. Synapse 29:116–127
Martinez D, Gelernter J, Abi-Dargham A, van Dyck CH, Kegeles L, Innis RB, Laruelle M (2001) The variable number of tandem repeats polymorphism of the dopamine transporter gene is not associated with significant change in dopamine transporter phenotype in humans. Neuropsychopharmacology 24:553–560

Michelhaugh SK, Fiskerstrand C, Lovejoy E, Bannon MJ, Quinn JP (2001) The dopamine transporter gene (SLC6A3) variable number of tandem repeats domain enhances transcription in dopamine neurons. J Neurochem 79:1033–1038

Mill J, Asherson P, Browes C, D'Souza U, Craig I (2002) Expression of the dopamine transporter gene is regulated by the 3′ UTR VNTR: evidence from brain and lymphocytes using quantitative RT-PCR. Am J Med Genet B Neuropsychiatr Genet 114B:975–979

Mill J, Asherson P, Craig I, D'Souza UM (2005) Transient expression analysis of allelic variants of a VNTR in the dopamine transporter gene (DAT1). BMC Genet 6:3

Miller GM, Madras BK (2002) Polymorphisms in the 3′-untranslated region of human and monkey dopamine transporter genes affect reporter gene expression. Mol Psychiatry 7:44–55

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189–225

Muller-Vahl KR, Berding G, Brucke T, Kolbe H, Meyer GJ, Hundeshagen H, Dengler R, Knapp WH, Emrich HM (2000) Dopamine transporter binding in Gilles de la Tourette syndrome. J Neurol 247:514–520

Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D (1999) HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somatic, and pharyngeal arch segments. Dev Biol 216:72–84

Nakagawa O, McFadden DG, Nakagawa M, Yanagisawa H, Hu T, Srivastava D, Olson EN (2000) Members of the HRT family of basic helix–loop–helix proteins act as transcriptional repressors downstream of Notch signaling. Proc Natl Acad Sci USA 97:13655–13660

Ohadi M, Shirazi E, Tehranidoosti M, Moghimi N, Keikhaee MR, Ehssani S et al (2006) Attention-deficit/hyperactivity disorder (ADHD) association with the DAT1 core promoter-67 T allele. Brain Res 1101:1–4

Ohadi M, Keikhaee MR, Javanbakht A, Sargolzaee MR, Robabeh M, Najmabadi H (2007) Gender dimorphism in the DAT1-67 T-allele homozygosity and predisposition to bipolar disorder. Brain Res 1144:142–145

Roberts J, Scott AC, Howard MR, Breen G, Bubb VJ, Klenova E, Quinn JP (2007) Differential regulation of the serotonin transporter gene by lithium is mediated by transcription factors, CCCTC binding protein and Y-box binding protein 1, through the polymorphic intron 2 variable number tandem repeat. J Neurosci 27:2793–2801

Sakamoto M, Hirata H, Ohtsuka T, Bessho Y, Kageyama R (2003) The basic helix–loop–helix genes hesr1/hey1 and hesr2/hey2 regulate maintenance of neural precursor cells in the brain. J Biol Chem 278:44808–44815

Shibuya N, Kamata M, Suzuki A, Matsumoto Y, Goto K, Otani K (2009) The −67 A/T promoter polymorphism in the dopamine transporter gene affects personality traits of Japanese healthy females. Behav Brain Res 203:23–26. doi:10.1016/j.bbr.2009.04.008

Shimada S, Kitayama S, Lin CL, Patel A, Nanthakumar E, Gregor P, Kuhar M, Uhl GR (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science 254:576–578

Steidl C, Leimeister C, Klamt B, Maier M, Nanda L, Dixon M, Clarke R, Schmid M, Gessler M (2000) Characterization of the human and mouse HEY1, HEY2, and HEYL genes: cloning, mapping, and mutation screening of a new bHLH gene family. Genomics 66:195–203

Ueno S (2003) Genetic polymorphisms of serotonin and dopamine transporters in mental disorders. J Med Invest 50:25–31

Uhl GR (2003) Dopamine transporter: basic science and human variation of a key molecule for dopaminergic function, locomotion, and Parkinsonism. Mov Disord 18:71–80

Vandenbergh DJ, Persico AM, Hawkins AL, Griffin CA, Li X, Jabs EW, Uhl GR (1992) Human dopamine transporter gene (DAT1) maps to chromosome 5p15.3 and displays a VNTR. Genomics 14:1104–1106

Vandenbergh DJ, Thompson MD, Cook EH, Bendahhou E, Nguyen T, Krasowski MD, Zarrabian D, Comings D, Sellers EM, Tyndale RF, George SR, O'Dowd BF, Uhl GR (2000) Human dopamine transporter gene: coding region conservation among normal, Tourette's disorder, alcohol dependence and attention-deficit hyperactivity disorder populations. Mol Psychiatry 5:283–292

VanNess SH, Owens MJ, Kilts CD (2005) The variable number of tandem repeats element in DAT1 regulates in vitro dopamine transporter density. BMC Genet 6:55

Villaronga MA, Lavery DN, Bevan CL, Llanos S, Belandia B (2009) HEY1 Leu94Met gene polymorphism dramatically modifies its biological functions. Oncogene 29:411–420

Wang W, Campos AH, Prince CZ, Mou Y, Pollman MJ (2002) Coordinate notch3-hairy-related transcription factor pathway regulation in response to arterial injury. J Biol Chem 277:23165–23171

Yang B, Chan RCK, Jing J, Li T, Sham P, Chen RYL (2007) A meta-analysis of association studies between the 10-repeat allele of a VNTR polymorphism in the 3uUTR of dopamine transporter gene and attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet 144B:541–550

MBNL proteins regulate alternative splicing of the skeletal muscle chloride channel *CLCN1*

Shoichi Ishiura[1), Yoshihiro Kino[2), Yoko Oma[1), Noboru Sasagawa[1), Nobuyuki Nukina[2)

1) Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo
2) RIKEN Brain Science Institute, Japan

Abstract

Increased inclusion of chloride channel 1 (*CLCN-1/CLC-1*) exon 7A is associated with myotonia in myotonic dystrophy type 1 (DM1), a genetic disease caused by the expansion of a CTG repeat. In mouse models, myotonia as well as aberrant splicing of the mouse counterpart of *CLC-1, Clcn1*, can be induced by either over-expression of CUG repeat RNAs or knockout of *Mbnl1*, an RNA-binding protein sequestered by CUG repeats in DM1 cells. Here we show that MBNL and CELF proteins regulate the alternative splicing of both human *CLC-1* and mouse *Clcn1*. MBNLs were found to repress the inclusion of exon 7A. This effect was antagonized by the expression of an expanded CUG repeat or CELF4 protein, but not by CUG-BP. MBNL1, which binds directly to regions around the 5' and 3' splice sites of exon 7A, is possibly blocking splicing signals and a putative exonic splicing enhancer located in this region. These results suggest the importance of these proteins in the correct splicing of *Clcn1* and provide molecular evidence for a novel mechanism for splicing regulation.

1. Introduction

Myotonic dystrophy (*dystrophia myotonica* type 1), or DM1, is a genetic disorder with multi-systemic symptoms, such as myotonia, progressive muscle loss, cataracts, cardiac conduction defects, insulin resistance, and cognitive impairments[1]. DM1 is caused by the expansion of a CTG trinucleotide repeat in the 3' untranslated region (UTR) of the DM protein kinase (*DMPK*) gene[2-4]. Evidence suggests that the expanded CUG repeats

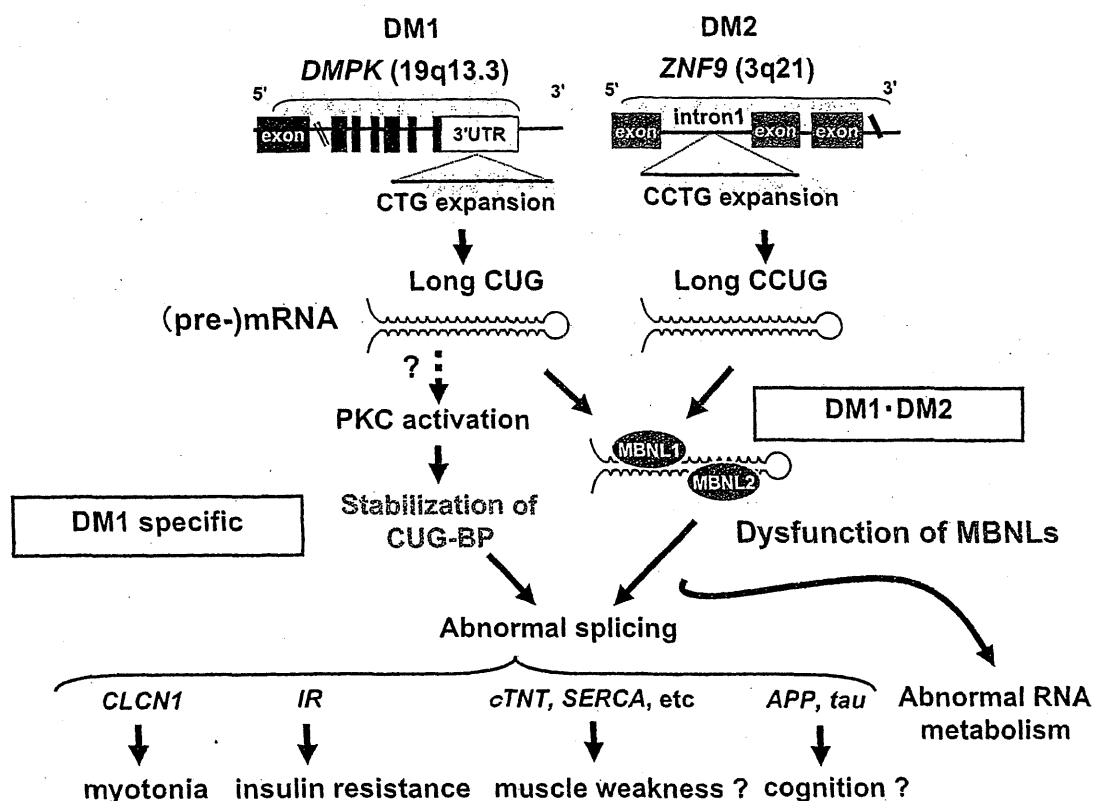


Fig. 1 RNA toxic gain-of-function model for DM

transcribed from a mutated allele cause RNA gain-of-function effects that affect the function of other cellular factors. Recently, a second locus of DM has been identified, and CCTG repeat expansion in intron 1 of the *ZNF9* gene was found to be causative of DM type 2 (DM2)[6]. Abnormalities in RNA metabolism have been found in the cells of DM patients. Splicing of certain genes is misregulated in DM1. It does not reduce the fidelity of RNA processing or weaken the recognition of constitutive exons. It selectively affects a group of exons that are normally found in fetal or neonatal tissue. These genes include cardiac troponin T (*cTNT/TNNT2*), insulin receptor (*IR*), chloride channel 1 (*CLCN1*), amyloid precursor protein (*APP*), microtubule-associated protein tau (*MAPT*), sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (*SERCA*) 1, and others (**Fig. 1**)[7-11]. The splicing patterns of some of these genes are also misregulated in DM2 patients. These results suggest that certain RNA-binding proteins that regulate pre-mRNA splicing of these genes are abnormally influenced by the mutant transcripts containing expanded CUG/CCUG repeats[12].

Two RNA-binding protein families—muscleblind-like (MBNL), and CUG-BP and ETR-3-like factor (CELF) proteins—may play major roles in the pathogenesis of DM. MBNL proteins MBNL1/EXP, MBNL2/MBLL/MLP1, and MBNL3/MBXL/CHCR are orthologs

of the *Drosophila* muscleblind protein, which is involved in the terminal differentiation of photoreceptor and muscle cells in the fly[13]. All three MBNL proteins can colocalize with RNA inclusions of expanded CUG/CCUG repeats in both DM1 and DM2 cells[14]. MBNL1 binds directly to both CUG and CCUG repeat RNA in a length-dependent manner *in vitro*[15]. Therefore, these proteins are considered to be sequestered by the expanded RNA through direct interactions, and their cellular functions can be disrupted in both types of DM. It is important to note that cellular studies have demonstrated that MBNL proteins can directly regulate the alternative splicing of the *cTNT* and *IR* genes, which are misregulated in DM1 patients[16,17]. These results strongly support the hypothesis that loss of function of MBNL proteins leads to the misregulation of splicing in DM.

CELF proteins are multi-functional proteins that play regulatory roles in translation, RNA editing, mRNA stability, as well as splicing[18]. CUG-BP regulates the alternative splicing of *cTNT* exon 5, *IR* exon 11, and *CLCN1* intron 2[7,8]. In DM1 patients, the expression of CUG-BP protein is elevated because of protein stabilization induced by PKC-mediated phosphorylation[7,19]. CUG-BP acts antagonistically against MBNL proteins in the splicing regulation of *cTNT* and *IR*[16,17] but their activities are independent, suggesting that altered CELF activities, in addition to the loss of MBNL function, can induce aberrant splicing in DM1 (see **Fig. 1**). However, the extent to which these proteins can account for splicing abnormalities and the pathogenesis of DM remains unclear.

Thus, it is important to characterize the roles of MBNL and CELF proteins in the regulation of *Clcn1* splicing to understand the mechanism of myotonia in DM. Although increased exon 7A inclusion is the most frequent abnormality of *CLCN1/Clcn1* splicing in DM[9], the mechanism of its regulation is still unclear.

We established a *Clcn1* minigene assay system and identified multiple *cis*- and *trans*-acting factors that regulate the alternative splicing of *Clcn1* exon 7A. The essential role of MBNL proteins in the normal splicing pattern of *Clcn1* was verified. Our results also highlight some CELF proteins as antagonistic regulators against MBNL proteins.

2. Materials and methods

MBNL1 and MBNL2 were amplified by PCR from a human skeletal muscle cDNA library (BD Marathon-Ready human cDNA ; Clontech). MBNL3 was amplified from a human liver cDNA library. CELF proteins were amplified from cDNA libraries of either brain or skeletal muscle of human origin[20].

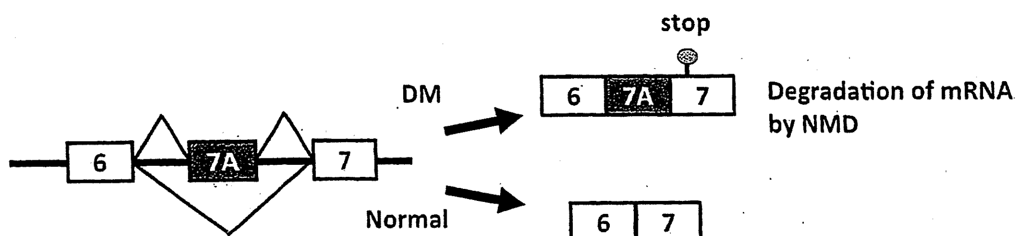


Fig. 2 Splicing regulation of *Clcn1* by MBNL and CELF proteins
Structure of chloride channel minigenes. Mouse *Clcn1* minigene was subcloned between the BglII and SalI sites of pEGFP-C1. Black boxes represent exons of the minigenes. Arrows indicate the position of primers used in the splicing assays.

Cells transfected with plasmids for the expression of a protein and a minigene were harvested 48 h post-transfection. Typically, cells were cultured in 12-well plates and transfected with 0.5 μg plasmids for protein expression (or cognate empty vector) and 0.01 μg plasmids for the expression of a minigene. Total RNA was extracted and purified using either the acidic guanidine phenol chloroform method or RNeasy Mini kit (Qiagen) including DNase treatment. Typically, 1.0 μg total RNA was reverse-transcribed using the ThermoScript RT-PCR System (Invitrogen) or Revertra Ace-α-(Toyobo) with a 1 : 1 mixture of oligo dT and random hexamer as primers. Minigene fragments were amplified by PCR using a fluorescein isothiocyanate (FITC)-labeled forward primer for the 3' region of the EGFP sequence (FITC-GFP-Fw) and a gene-specific reverse primer (Clcn1-Rv for *Clcn1* or CLCN1-Rv for *CLCN1*). PCR products were resolved by 2.0-2.5% agarose gel electrophoresis. By sampling at multiple cycles, the cycle numbers of PCR were adjusted such that the amplification was within the logarithmic phase. The fluorescence of PCR products was captured and visualized by LAS1000 or LAS3000 (Fujifilm). The intensity of band signals was quantified using Multigauge software (Fujifilm). The ratio of exon 7A inclusion in *Clcn1* and *CLCN1* was calculated as (7A inclusion)/(7A inclusion + 7A skipping) × 100.

3. Results and discussion

To examine whether the MBNL and CELF family proteins can regulate the splicing of *Clcn1*, we created a minigene covering exons 6 to 7 of the mouse *Clcn1* gene (**Fig. 2**). It is important to note that because the inclusion of exon 7A does not produce a premature termination codon in the context of our *Clcn1* minigene, the spliced products containing

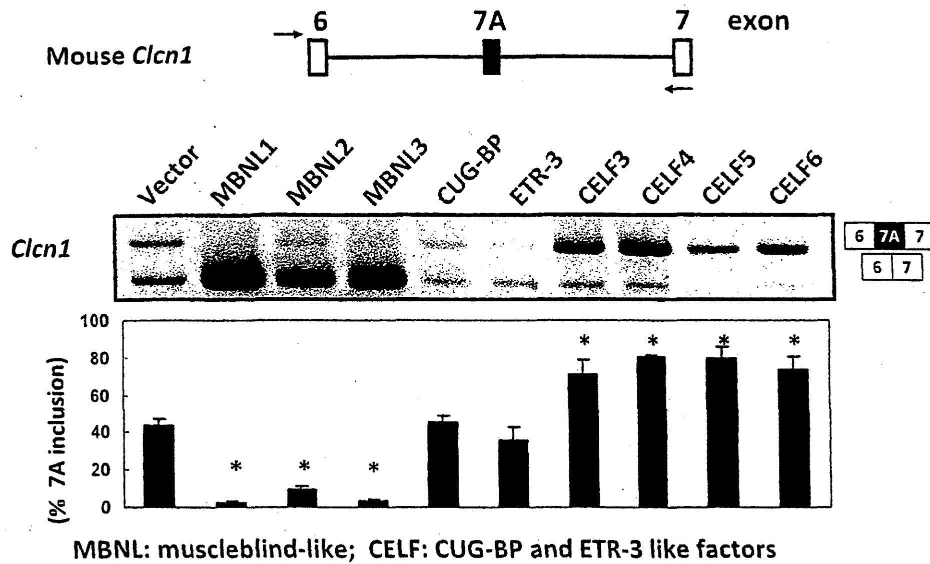


Fig. 3 Splicing regulation of MBNL and CELF proteins.
Representative results of cellular splicing assays using the *Clcn1* minigene in COS-7 cells. The upper bands correspond to a splice product containing exon 7A, whereas lower bands correspond to a splice product lacking exon 7A. Bar chart shows quantified results of exon 7A inclusion (mean ± SD, $n = 3$). Statistical significance was analyzed by analysis of variance and Dunnett's multiple comparison. All MBNL proteins and CELF proteins except for CUG-BP and ETR-3 showed significant differences ($^{*}p < 0.0001$) compared to the empty vector.

exon 7A are not substrates of nonsense-mediated mRNA decay (NMD). Thus, the minigene would provide more faithful splicing patterns compared to the endogenous *Clcn1*. We utilized non-muscle cell lines to minimize the effect of muscle-dependent backgrounds and focus on the direct effects of transgenes. When the *Clcn1* minigene was transfected into COS -7 cells, 45% of the spliced products contained exon 7A (**Fig. 3**). Next we expressed myc-tagged MBNL or CELF proteins with the *Clcn1* minigene and examined the patterns of *Clcn1* splicing. The expressions of MBNL and CELF proteins were confirmed by Western blotting using an anti-myc antibody (data not shown). All three MBNL proteins strongly repressed exon 7A inclusion (**Fig. 1B**). In contrast, CELF3, CELF4, CELF5, and CELF6 proteins significantly promoted the inclusion of 7A. Remarkably, CUG-BP (CELF1) and ETR-3 (CELF2) did not alter the ratio of exon 7A inclusion. These two proteins increased the unspliced product and reduced the spliced products with or without exon 7A (data not shown).

CLCN1/Clcn1 splicing is a key event in DM. Although the misregulation of splicing has

been well established as a characteristic abnormality of DM, few misregulated genes have a clear causal relationship to symptoms of DM. *Clcn1* misregulation can account for myotonia in DM model mice[21]. As demonstrated recently, the skipping of exon 7A induced by antisense oligonucleotide reversed the myotonic phenotype of DM model mice[22], making *CLCN1* splicing a promising target for therapeutic approaches. Understanding *Clcn1/CLCN1* splicing would aid in the design of rational strategies for correcting CLCN1 expression to perhaps prevent myotonia.

Here, we have demonstrated that the splicing regulation of *Clcn1* exon 7A by MBNL1 was observed in COS-7 as well as HeLa, and Neuro2A cell lines (**Fig. 3** and ref. 20). Thus, the regulation of exon 7A can be determined directly by the expression level of MBNL proteins. The inclusion of exon 7A was repressed by the overexpression of MBNL proteins but increased by their knockdown[20]. These results are consistent with the model that MBNL proteins directly regulate *CLCN1/Clcn1* and that the loss of MBNL function leads to *CLCN1/Clcn1* misregulation in DM.

In contrast to MBNL proteins, CELF3/4/5/6 promoted increased inclusion of exon 7A of mouse *Clcn1* (**Fig. 3**). Among these CELF proteins, CELF4 is expressed in a wide variety of tissues, including muscle[18,23]. Although mice deficient in *Celf4* have been reported to manifest a complex seizure phenotype[24], the physiological function of CELF4 is largely unclear. Although an elevation of CUG-BP and ETR-3 proteins was observed in DM1 patients, the other CELF proteins have not been well characterized. The expression level, intracellular localization, and activity of CELF4 (and CELF3/5/6) should be investigated in the context of DM. Although *Clcn1* is enriched in muscle, it is expressed in other tissues (including the brain) even at a low level. Because some CELF proteins are enriched in the brain[23], they might play a role in keeping *Clcn1* expression at a low level in tissues other than muscle through a splicing-mediated regulation of expression.

In order to understand the regulatory mechanism controlling splice site selection, it should be clarified how these RNA-binding proteins activate splicing of one substrate and repress splicing of another. Whether antagonistic regulation by MBNL and CELF is linked or not? We hope future work will help the way to treat DM.

Acknowledgments

This work was supported by research grants from the Ministry of Health, Labor and Welfare ; and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We would like to thank Dr. Yoshihide Hayashizaki for FANTOM3 clones and Dr. Gen

Matsumoto for the R-miR vector and Dr. Chika Washizu for discussion. We are also grateful to the staff of the Research Resource Center of RIKEN Brain Science Institute for DNA sequencing. Y. K. was supported by a JSPS Research Fellowship for Young Scientists and the RIKEN Special Postdoctoral Researchers Program.

References

1) Harper, P. S. *Myotonic Dystrophy*, 3rd edn. WB Saunders, London, 2001.
2) Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion JP, Hudson T, et al. Molecular basis of myotonic dystrophy : expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell. 1992 ; 68 : 799-808.
3) Fu YH, Friedman DL, Richards S, Pearlman JA, Gibbs RA, Pizzuti A, Ashizawa T, Perryman MB, Scarlato G, Fenwick RG, et al. Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science. 1993 ; 260 : 235-238.
4) Mahadevan M, Tsilfidis C, Sabourin L, Shutler G, Amemiya C, Jansen G, Neville C, Narang M, Barcelo J, O'Hoy K, et al. Myotonic dystrophy mutation : an unstable CTG repeat in the 3' untranslated region of the gene. Science. 1992 ; 255 : 1253-1255.
5) Mankodi A, Logigian E, Callahan L, McClain C, White R, Henderson D, Krym M, Thornton CA. Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science. 2000 ; 289 : 1769-1773.
6) Liquori CL, Ricker K, Moseley ML, Jacobsen JF, Kress W, Naylor SL, Day JW, Ranum LP. Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science. 2001 ; 293 : 864-867.
7) Savkur RS, Philips AV, Cooper TA. Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nat Genet. 2001 ; 29 : 40-47.
8) Charlet-B N, Savkur RS, Singh G, Philips AV, Grice EA, Cooper TA. Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. Mol Cell. 2002 ; 10 : 45-53.
9) Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ, Moxley RT, Cannon SC, Thornton CA. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Mol Cell. 2002 ; 10 : 35-44.
10) Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA, Esson D, Timmers AM, Hauswirth WW, Swanson MS. A muscleblind knockout model for myotonic dystrophy. Science. 2003 ; 302 : 1978-1980.
11) Kimura T, Nakamori M, Lueck JD, Pouliquin P, Aoike F, Fujimura H, Dirksen RT, Takahashi MP, Dulhunty AF, Sakoda S. Altered mRNA splicing of the skeletal muscle ryanodine receptor and sarcoplasmic/endoplasmic reticulum Ca2+-ATPase in myotonic dystrophy type 1. Hum Mol Genet. 2005 ; 14 : 2189-2200.
12) Ranum LP, Cooper TA. RNA-mediated neuromuscular disorders. Annu Rev Neurosci. 2006 ; 29 : 259-277.
13) Begemann G, Paricio N, Artero R, Kiss I, Perez-Alonso M, Mlodzik M. muscleblind, a gene required for photoreceptor differentiation in Drosophila, encodes novel nuclear Cys3His-type zinc-finger-containing proteins. Development. 1997 ; 124 : 4321-4331.
14) Fardaei M, Rogers MT, Thorpe HM, Larkin K, Hamshere MG, Harper PS, Brook JD. Three proteins,

MBNL, MBLL and MBXL, co-localize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells. Hum Mol Genet. 2002；11：805-814.

15) Kino Y, Mori D, Oma Y, Takeshita Y, Sasagawa N, Ishiura S. Muscleblind protein, MBNL1/EXP, binds specifically to CHHG repeats. Hum Mol Genet. 2004；13：495-507.

16) Ho TH, Charlet-B N, Poulos MG, Singh G, Swanson MS, Cooper TA. Muscleblind proteins regulate alternative splicing. EMBO J. 2004；23：3103-3112.

17) Dansithong W, Paul S, Comai L, Reddy S. MBNL1 is the primary determinant of focus formation and aberrant insulin receptor splicing in DM1. J Biol Chem. 2005；280：5773-5780.

18) Ladd AN, Charlet N, Cooper TA. The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol Cell Biol. 2001；21：1285-1296.

19) Kuyumcu-Martinez NM, Wang GS, Cooper TA. Increased steady-state levels of CUGBP1 in myotonic dystrophy 1 are due to PKC-mediated hyperphosphorylation. Mol Cell. 2007；28：68-78.

20) Kino Y, Washizu C, Oma Y, Onishi H, Nezu Y, Sasagawa N, Nukina N, Ishiura S. MBNL and CELF proteins regulate alternative splicing of the skeletal muscle Chloride channel *CLCN1*. Nucleic Acids Research. 2009；37：6477-6490.

21) Lueck JD, Lungu C, Mankodi A, Osborne RJ, Welle SL, Dirksen RT, Thornton CA. Chloride channelopathy in myotonic dystrophy resulting from loss of posttranscriptional regulation for CLCN1. Am J Physiol Cell Physiol. 2007；292：C1245-1247.

22) Wheeler TM, Lueck JD, Swanson MS, Dirksen RT, Thornton CA. Correction of ClC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. J Clin Invest. 2007；117：3952-3957.

23) Ladd AN, Nguyen NH, Malhotra K, Cooper TA. CELF6, a member of the CELF family of RNA-binding proteins, regulates muscle-specific splicing enhancer-dependent alternative splicing. J Biol Chem. 2004；279：17756-17764.

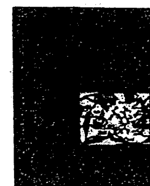
24) Yang Y, Mahaffey CL, Bérubé N, Maddatu TP, Cox GA, Frankel WN. Complex seizure disorder caused by Brunol4 deficiency in mice. PLoS Genet. 2007；3：e124.

Biochemical and Biophysical Research Communications 409 (2011) 64–69

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



Alternative splicing of *PDLIM3/ALP*, for α-actinin-associated LIM protein 3, is aberrant in persons with myotonic dystrophy

Natsumi Ohsawa [a], Michinori Koebis [a], Satoshi Suo [a], Ichizo Nishino [b], Shoichi Ishiura [a,*]

[a] *Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*
[b] *National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*

A R T I C L E I N F O

Article history:
Received 17 April 2011
Available online 28 April 2011

Keywords:
Actinin-associated LIM protein 3 (*PDLIM3/ALP*)
Alternative splicing
MBNL
Myotonic dystrophy
Sarcoplasmic/endoplasmic reticulum Ca-ATPase 1 (*SERCA1*)
ZASP-like motif

A B S T R A C T

Myotonic dystrophy type 1 (DM1) is an autosomal dominant disorder of muscular dystrophy characterized by muscle weakness and wasting. DM1 is caused by expansion of CTG repeats in the 3′-untranslated region (3′-UTR) of *DM protein kinase* (*DMPK*) gene. Since CUG-repeat RNA transcribed from the expansion of CTG repeats traps RNA-binding proteins that regulate alternative splicing, several abnormalities of alternative splicing are detected in DM1, and the abnormal splicing of important genes results in the appearance of symptoms. In this study, we identify two abnormal splicing events for actinin-associated LIM protein 3 (*PDLIM3/ALP*) and fibronectin 1 (*FN1*) in the skeletal muscles of DM1 patients. From the analysis of the abnormal *PDLIM3* splicing, we propose that ZASP-like motif-deficient PDLIM3 causes the muscular symptoms in DM. PDLIM3 binds α-actinin 2 in the Z-discs of muscle, and the ZASP-like motif is needed for this interaction. Moreover, in adult humans, PDLIM3 expression is highest in skeletal muscles, and *PDLIM3* splicing in skeletal muscles is regulated during human development.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Myotonic dystrophy (Dystrophia Myotonica; DM) is an autosomal dominant disorder and is the most common form of muscular dystrophy to affect adults [1]. Multiple systems are affected in patients with DM. The characteristic symptoms of DM are muscle hyper-excitability (myotonia), progressive muscle loss, muscle weakness, cataracts, defects in cardiac conduction, cognitive impairment, and insulin resistance [1]. Two forms of DM have been identified, DM1 and DM2. The gene that is affected in DM1 is *DM protein kinase* (*DMPK*) on chromosome 19q. This gene contains trinucleotide CTG repeats within its 3′-untranslated region (UTR) [2–4]. The expansion of this repeat triggers the pathogenesis of DM1 and, interestingly, the number of repeats is thought to correlate with symptom severity [4]. The gene that is affected in DM2 is *zinc finger protein 9* (*ZNF9*). This gene contains tetranucleotide CCTG repeats in intron 1 and, as in DM1, expansion of this repeat is believed to cause this disease [5]. There is strong evidence that the expanded repeat-containing mRNA species transcribed from the altered *DMPK* and *ZNF9* genes form foci that are retained within the nuclei of DM cells [5–7]. Since DM1 and DM2 overlap phenotypically, despite having different genetic loci, this finding suggests that the expanded repeats themselves cause DM [6].

There is evidence to suggest that the expanded CUG repeats transcribed from a mutated allele cause RNA gain-of-function effects that affect the functions of other cellular factors, leading to abnormalities in RNA splicing. The mis-spliced genes include those for chloride channel 1 (*CLCN1*), cardiac troponin T (*cTNT/TNNT2*), sarcoplasmic/endoplasmic reticulum Ca-ATPase 1 (*SERCA1*), insulin receptor (*IR*), microtubule-associated protein tau (*MAPT*), and amyloid precursor protein (*APP*) [8–13]. The splicing patterns of some of these genes are also aberrantly regulated in patients with DM2 [10,14,15]. These results suggest that certain RNA-binding proteins that regulate the pre-mRNA splicing of these genes are abnormally influenced by the mutant transcript that contains CUG/CCUG repeats [16]. The RNA-binding MBNL and CELF families of proteins have been identified, and cellular studies have demonstrated that *CLCN1*, *cTNT*, *SERCA1*, and *IR* are directly regulated by these proteins [17–20].

To determine the splicing abnormality and gene expression resulting from the expanded CUG mRNA, we used human exon arrays to compare the mRNA splicing patterns of the skeletal muscles of patients with DM1. We found remarkable perturbations of splicing, and identified more than 100 splicing events that were altered in DM1 muscles (Koebis, submitted). Among these altered splicing events, we focused on the *PDLIM3/ALP* (PDZ and LIM domain protein 3 α-actinin-associated LIM protein), PDZ and LIM domain protein 3 and the α-actinin-associated LIM proteinactinin-associated LIM protein, which binds to the spectrin repeat of α-actinin 2 *via* the PDZ domain in the Z-discs of muscles [21,22]. As Z-discs are

* Corresponding author.
E-mail addresses: cishiura@mail.ecc.u-tokyo.ac.jp, cc077725@mail.ecc.u-tokyo.ac.jp (S. Ishiura).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.04.106

essential for force transmission and muscle integrity [23], we hypothesized that abnormal *PDLIM3* splicing contributes to the symptoms of DM1.

We found that *PDLIM3* splicing was regulated during development and in a tissue-specific manner, and that the abnormal *PDLIM3* splicing was closely related with the altered splicing of *SERCA1* in each DM1 patient. We suspect that *PDLIM3* splicing is regulated by the same molecular mechanism that regulates *SERCA1*, and that abnormal splicing is developmentally regulated.

2. Materials and methods

2.1. Human skeletal muscle biopsies

Biopsies were obtained from the biceps brachii muscle or quadriceps femoris muscle of six DM1 patients and seven non-DM individuals without muscular disease (Supplementary Table). Of the non-DM individuals, three lacked histologic abnormalities, while four showed mild atrophy or atrophy of only the type 2 fibers. All the biopsies were stored at −80 °C. Clinically, all the DM1 patients had muscle weakness with myotonia. Four of the DM1 patients had congenital onset of the disease, and two experienced onset during childhood or adolescence. Pathologically, all the DM1 patients showed an immature fiber type or myopathic changes with variable fiber sizes. All biopsies were acquired with the informed consent of the patients.

2.2. RNA extraction and reverse transcription (RT)

Total RNA samples were isolated from the biopsies using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol but without DNase treatment, and purified by phenol–chloroform extraction and isopropanol precipitation. Total RNA samples from other tissues were taken from the Human Total RNA Master Panel II (Clontech, Mountain View, CA). All total RNA samples were stored at −80 °C.

The cDNA samples were synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan) in a total volume of 10 μl using the oligo(dT) primers and the total RNA samples (0.5 μg for biopsies; 1.0 μg for other tissues). The cDNA of fetal skeletal muscle (BioChain, Hayward, CA) was synthesized using the total RNA sample from a male, 20-week-old donor. All the cDNA samples were stored at −20 °C.

2.3. Polymerase chain reaction (PCR)

PCR was performed using ExTaq DNA polymerase (TaKaRa Bio), according to the manufacturer's protocol. The primer sequences, annealing temperatures, and cycle numbers used are listed in Table 1. The following conditions were used for the PCR: initial denaturation at 96 °C for 2 min, followed by quantitative cycles (96 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min), and a final extension step (72 °C for 5 min). The numbers of cycles were adjusted such that the amplification occurred within the logarithmic phase.

The PCR products were resolved by electrophoresis on an 8% polyacrylamide gel or a 1% agarose gel. The gels were strained with ethidium bromide and analyzed using LAS-3000 imaging system (Fujifilm, Tokyo, Japan). The intensity of the band signals was quantified using the Multigauge software (Fujifilm). The splicing percentages of *PDLIM3* were calculated as (PDLIM3b band)/(All isoform' bands), those of *SERCA1* were calculated as (SERCA1b band)/(All isoform' bands). The mean values are shown, and the *P*-values were determined using the Student's *t*-test. The correlation of the splicing percentages for *PDLIM3* and *SERCA1* for every DM1 patient is represented by the Pearson product-moment correlation coefficient. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced.

3. Results

3.1. Aberrant splicing in patients with DM1

To identify aberrant alternative splicing in DM1, we performed RT-PCR on the biopsies of non-DM1 individuals and DM1 patients (Supplementary Table). From the exon array results, we selected the following six candidate exons (Table 1): *PDLIM3* exon 4; *FN1* exon 25 and exon 33; *PKP2* exon 6; *TTN* exon 45; and *EGLN2* exon 4. These genes are highly expressed in skeletal muscles or these exons are alternative exons. As a positive control (PC), we used *SERCA1*, which is known to undergo abnormal splicing in DM1 patients and DM1 model (HSA^{LR}) mice [12]. Assuming that the percentage of exon inclusion or exclusion relative to the total number of transcripts changes significantly, as for *SERCA1* exon 22, the same physiologic abnormality with abnormal splicing should occur in DM1 muscle. Using RT-PCR, we detected aberrant splicing for *PDLIM3* exon 4 (Fig. 1A and B; $P = 0.0015$) and *FN1* exon 33 (data not shown; $P = 0.0051$), as well as for *SERCA1* exon 22 (Fig. 1C;

Table 1
Primers used in RT-PCR.

Gene	Accession number	Exon	DM1 isoform[a]	Primer name	Primer sequence (5′–3′)	Annealing	Cycle[b]
PDLIM3	NM_001114107	ex4	Ex4 + ex5,6−	PDLIM3_ex4_Fw PDLIM3_ex4_Rv	CAGCTCACCAGCTGTGTCTC GAGCCATCGTCCACCATTCC	66 °C	27
FN1	NM_002026	ex25	-	FN1_ex25_Fw FN1_ex25_Rv	ATGGACAGGAAAGAGATGCG AAAAGTCAATGCCAGTTGGG	66 °C	30
		ex33	ex33+	FN1_ex33_Fw FN1_ex33_Rv	CCTGGGAGCAAGTCTACAGC TAGCATCTGTCACACGAGCC	66 °C	31
PKP2	NM_001005242	ex6	-	PKP2_ex6_Fw PKP2_ex6_Rv	TCCAGGTGCTGAAGCAAACC TCGCTTTTCTCCCATCAGCG	66 °C	32
TTN	NM_003319	ex45	-	TTN_ex45_Fw TTN_ex45_Rv	AGCACAGCCAACCTGAGTCT CCGGTTCACCCTCTAAAACA	54 °C	31
EGLN2	NM_053046	ex4	-	EGLN2_ex4_Fw EGLN2_ex4_Rv	CTGGGCAGCTATGTCATCAA TGGACACCTTTCTGTCCTGA	64 °C	30
SERCA1 (PC)	NM_004320	ex22	ex22−	SERCA1_ex22_Fw SERCA1_ex22_Rv	ATCTTCAAGCTCCGGGCCCT CAGCTCTGCCTGAAGATGTG	63.5 °C	25

[a] The DM1 isoform predominates in the skeletal muscles of patients with DM1.
[b] 'Cycle' refers to a quantitative cycle of RT-PCR for biopsies.

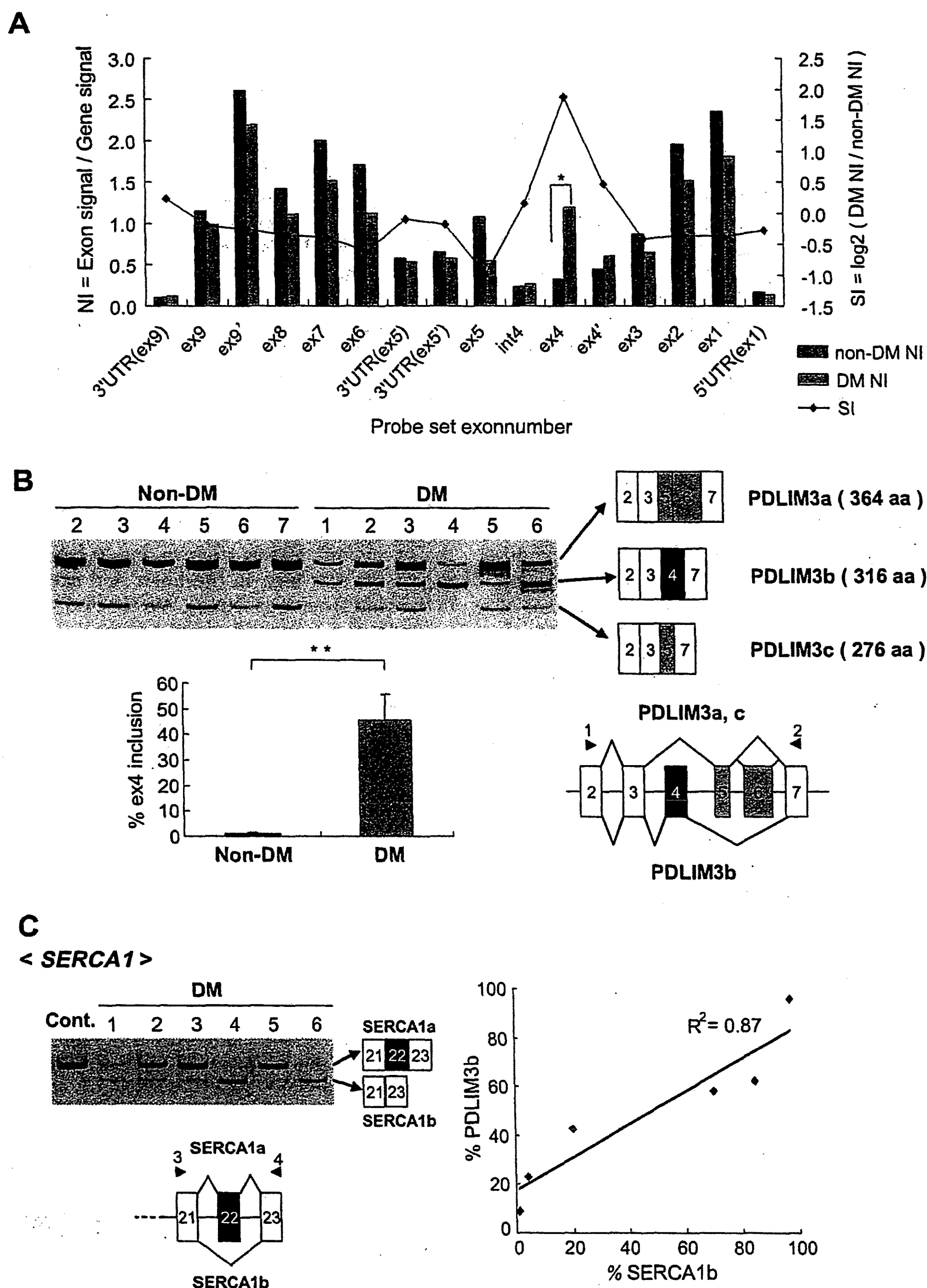


Fig. 1. *PDLIM3* splicing is abnormal in patients with DM1. (A) Exon array analysis of *PDLIM3* in four non-DM1 and three DM1 biopsies. NI, normalized exon intensity (NI = exon level signal/gene level signal); SI, splicing index (SI = $\log_2$ $NI_{DM}/NI_{non\text{-}DM}$). Statistical significance was analyzed using the Student's *t*-test for $NI_{non\text{-}DM}$ and NI_{DM}; $^*P < 0.05$. (B) The level of PDLIM3b (exon 4 inclusion isoform) is increased in DM1 muscles. RT-PCR of endogenous *PDLIM3* in DM1 skeletal muscles ($n = 6$; Nos. 1, 2, 3, 4, 5, and 6) and non-DM1's ($n = 6$; Nos. 2, 3, 4, 5, 6, and 7) was performed using the primer set (arrowhead 1, 2). The lower panel shows the percentages of exon 4 inclusion isoform relative to the total level of transcripts (means ± SD). Statistical significance was analyzed by the Student's *t*-test ($^{**}P < 0.0015$). (C) *PDLIM3* splicing correlates with *SERCA1* splicing in each DM patient. RT-PCR of endogenous *SERCA1* in DM1 skeletal muscles ($n = 6$; Nos. 1, 2, 3, 4, 5, and 6) and non-DM1 skeletal muscles (Cont.) using the primer set (arrowhead 3, 4). The right panel shows the correlation between the percentage of *PDLIM3* exon 4 inclusion isoform (% PDLIM3b) and *SERCA1* exon 22 exclusion isoform (% SERCA1b) relative to the total level of transcripts. R^2 is the Pearson product-moment correlation coefficient, and the correlation is significant at $R^2 > 0.87$, $P = 0.0064$.

$P = 0.022$). The remaining four exons did not show significant mis-splicing.

The three isoforms of *PDLIM3* splicing were observed. The normal isoforms are PDLIM3a ("exons 5 and 6 inclusion and exon 4 exclusion" isoform) and PDLIM3c ("exon 5 inclusion and exons 4 and 6 exclusion" isoform), which predominate in non-DM1 muscles, whereas the DM1 muscles contained the PDLIM3b isoform ("exon 4 inclusion and exons 5 and 6 exclusion" isoform). The pattern of *FN1* splicing revealed that the exon 33 exclusion isoform was more common than the exon 33 inclusion isoform in non-DM1 muscles, whereas the exon 33 inclusion isoform predominated in DM1 muscles.

To gain insight into the factors that regulate the splicing of *PDLIM3*, we compared the percentages of splicing of *SERCA1* and *PDLIM3* for each patient with DM1. *PDLIM3* splicing showed a statistically significant correlation with *SERCA1* splicing (Fig. 1C; R^2 = 0.87; P = 0.0064). However, there was also a correlation between the splicing of *SERCA1* and *FN1* (R^2 = 0.82; P = 0.032; data not shown). Nevertheless, we focused on the correlation between *PDLIM3* and *SERCA1*, since this correlation was stronger than that between *FN1* and *SERCA1*, and the expression of *PDLIM3* is high in skeletal muscles.

We considered that *PDLIM3* splicing might also be regulated by MBNL family proteins, such as MBNL1, 2, and 3, as *SERCA1* splicing is regulated by MBNL1 [19,24]. SERCA1b (exon 22 exclusion isoform) is seen in DM1 skeletal muscle and DM1 model mice: HSA^{LR} [12]. During the development of fast-twitch fibers, SERCA1b is expressed in the fetal and neonatal stages but it is completely replaced by SERCA1a (exon 22 inclusion isoform) in adult muscle fibers [25,26]. Therefore, we performed a cellular splicing assay for *PDLIM3* in HEK-293, HeLa, and SH-SY5Y cells. The overexpression of MBNL1, 2, and 3 resulted in the shifting of *SERCA1* splicing from SERCA1b (exon 22 exclusion isoform) to SERCA1a (exon 22 inclusion isoform), whereas the shifting of *PDLIM3* splicing from PDLIM3b to PDLIM3a or PDLIM3c was negligible (data not shown).

Furthermore, the overexpression under the same conditions of CELF family proteins, such as CUGBP1, ETR-3, CELF3, 4, 5, and 6, showed that CUGBP1 and CELF3 increased SERCA1b (exon 22 exclusion isoform), although this result was not statistically significant. *PDLIM3* splicing was not regulated by either CUGBP1 or CELF3.

3.2. *PDLIM3 splicing during skeletal muscle development*

Using RT-PCR, we investigated whether the shift in isoforms occurred during the development of skeletal muscle (Fig. 2A). The detection of an isoform shift would indicate that *PDLIM3* splicing is regulated by factors that change according to developmental stage. In addition, this might suggest that alteration of the physiologic properties of *PDLIM3* is related to DM1 pathogenesis.

PDLIM3b was mainly expressed in fetal skeletal muscles (Fetus; 20 weeks) (Fig. 2A), whereas PDLIM3a and PDLIM3c were predominantly detected after birth (Cont.; 6 months of age). *PDLIM3* splicing changed between 20 weeks (Fetus) and 6 months of age (infant), albeit not in the brain or liver. The change in *PDLIM3* splicing was specific for skeletal muscle. PDLIM3b was expressed mainly in DM1 skeletal muscles, but also in fetal muscles and other tissues. Thus, *PDLIM3* splicing is fetal-type in DM1, and it is thought that the condition of the DM1 muscle resembles that of fetal muscle.

To examine how *PDLIM3* splicing and expression are regulated in each tissue we performed RT-PCR on various adult tissues (Fig. 2B). *PDLIM3* splicing could be categorized into two tissue groups: muscle and other tissues. In muscle (heart and skeletal muscles), PDLIM3a and PDLIM3c were expressed predominantly, while in other tissues, the main product was PDLIM3b. In glands, low-level expression of PDLIM3a was observed. These results suggest that *PDLIM3* splicing is regulated in a muscle-specific manner. Furthermore, we detected *PDLIM3* expression in all tissues, with the exceptions of the kidneys and spleen. The level of *PDLIM3* expression was high in the heart and skeletal muscles, and low in the central nervous tissues. We conclude that *PDLIM3* expression is regulated in a muscle-specific manner.

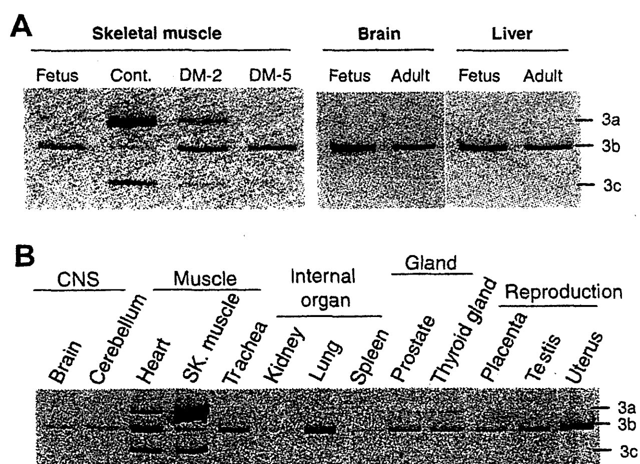


Fig. 2. Patterns of *PDLIM3* splicing during the development of skeletal muscles and various tissues. (A) RT-PCR of endogenous *PDLIM3* in fetal, non-DM, and DM1 skeletal muscles and other tissues. The fetal skeletal muscle isoform is PDLIM3b (exon 4 inclusion isoform), which is the same as that in the DM1 skeletal muscles (DM-2, DM-5), brain, and liver but not the same as that in the adult skeletal muscle (Cont.). PDLIM3b does not change to other isoforms during the development of the brain (fetus, 26–40 weeks; adult, 43 years old) or liver (fetus, 22–40 weeks; adult, 51 years old), except in the skeletal muscles. (B) Endogenous *PDLIM3* splicing in various tissues. The intensities of the bands obtained after 28 cycles of PCR for the exon 4 exclusion isoforms (PDLIM3a and PDLIM3c) are greater in the heart, skeletal muscle, and gland tissues. Reverse transcription of all the tissue samples was performed using 1.0 μg of total RNA.

4. Discussion

In the present study, we show that the splicing of *PDLIM3* exon 4 and *FN1* exon 33 occurs aberrantly in patients with DM1 (Fig. 1B). Aberrant *FN1* splicing was originally identified in patients with DM1, although it has also been reported in an array analysis of DM1 model ($MBNL^{\Delta3/\Delta3}$) mice [27]. The splicing changes of *FN1* have also been observed during heart development in wild-type mice [27]. Aberrant *PDLIM3* splicing has already been reported [15], although it has not been fully analyzed in patients with DM1.

In the present study, we show that *PDLIM3* splicing produces three isoforms of exons 4, 5 or 6, and that in patients with DM1, PDLIM3b ("exon 4 inclusion, exons 5 and 6 exclusion" isoform) predominates. PDLIM3 binds to α-actinin 2 via its PDZ domain [21], and the ZASP-like motif (encoded by exon 6) is necessary for this interaction [28,29]. Therefore, it is possible that the PDLIM3b proteins are unable to bind sufficiently to α-actinin 2, resulting in the symptoms of DM1 muscle. Furthermore, some mutations of *PDLIM3* have been reported in dilated cardiomyopathy (DCM) [30] and hypertrophic cardiomyopathy [31]. In addition, $PDLIM3^{-/-}$ mice develop cardiomyopathy that resembles human arrhythmogenic right ventricular cardiomyopathy (ARVD/C) with mild left ventricular involvement [32]. Therefore, *PDLIM3* may be necessary for the physiologic functions of heart muscle. However, skeletal muscle functions and development are normal in *PDLIM3*-deficient mice [22]. We propose that abnormal *PDLIM3* splicing affects the heart more than the skeletal muscles in patients with DM1.

PDLIM3 is in the same family as *Cypher/ZASP/LDB3* [33], and abnormal *Cypher* splicing has been observed in DM1and DM2 muscles [15,34]. Moreover, *Cypher* has been linked to cardiomyopathy in mice and humans [34–36]. Moreover, *Cypher*-knockout mice die prenatally of severe congenital myopathy [34], and human *Cypher* mutations have been linked to a novel autosomal dominant muscular dystrophy [36]. Therefore, it seems that two abnormal splicings of *PDLIM3* and *Cypher* are related to the symptoms observed for DM1 muscles.

We hypothesized that *PDLIM3* splicing is regulated by MBNL family proteins, as well as *SERCA1* splicing, since a significant

correlation between *SERCA1* and *PDLIM3* splicing was detected in each patient with DM1 (Fig. 1C). However, in the cellular splicing assay, we were unable to demonstrate that MBNL or CELF family proteins regulate *PDLIM3* splicing (data not shown). In the same assay, *SERCA1* splicing was found to be regulated by MBNL. Although we carried out the splicing assay with HEK-293, HeLa, and SH-SY5Y cells, we did not detect the factors that regulate *PDLIM3* splicing. Possible reasons for this outcome are: (1) our splicing assay could not detect a minor splicing event; (2) some factor that acts with MBNL is necessary for the regulation of *PDLIM3* splicing; and (3) factors other than MBNL regulate *PDLIM3* splicing. If the amount of transfected vector that encodes each factor was increased, we might resolve issue (1) above. For issues (2) and (3), splicing factors other than MBNL might be abnormal in DM1. Currently, we are unable to conclude which of the above possibilities is the one most likely to be true.

Abnormally spliced exons in DM1 can be divided into two groups: (1) that in which the splicings become muscle-specific during development and (2) that in which the splicings change after birth [15]. *PDLIM3* exon 4 is in the former category, as the splicing pattern changed from the fetus at 20 weeks to the infant at 6 months of age (Fig. 2A). The *SERCA1* exon 22 is in the latter category [15,19]. The former group contains many gene exons that have developmental functions. Therefore, *PDLIM3* may be associated with muscle development. The developmental abnormality of *PDLIM3*$^{-/-}$ mice was observed in the heart [32], not in the skeletal muscles [22]. Moreover, *PDLIM3* may regulate muscle differentiation, since disruption of *PDLIM3* expression affects the expression of myogenin and MyoD [37].

In each human tissue, *PDLIM3* splicing was regulated in a tissue-specific manner (Fig. 2B). PDLIM3a (exon 6 inclusion isoform) was detected only in skeletal muscles and the heart. Therefore, exon 6 may have a muscle-specific function in mature muscles. As PDLIM3b (exon 4 inclusion isoform) was detected in the other tissues, exon 4 may have functions other than those it executes in skeletal muscles. Since *PDLIM3* expression was much higher in the heart and skeletal muscles than in other tissues, the roles of *PDLIM3* in other tissues may be minor.

Acknowledgments

We would like to thank Dr. Noboru Sasagawa and Yoko Oma, Ms. Tamami Kiyatake, Zhao Yimeng, Mr. Kosuke Oana for valuable discussions. This work was supported in part by an intramural research grant (20B-13) for Neurological and Psychiatric Disorders from the NCNP, Ministry of Health, Labor and Welfare, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.106.

References

[1] P. Harper, Myotonic Dystrophy, third ed., W.B. Saunders, London, 2001.

[2] J.D. Brook, M.E. McCurrach, H.G. Harley, A.J. Buckler, D. Church, H. Aburatani, K. Hunter, V.P. Stanton, J.P. Thirion, T. Hudson, et al., Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member, Cell 69 (1992) 385.

[3] Y.H. Fu, D.L. Friedman, S. Richards, J.A. Pearlman, R.A. Gibbs, A. Pizzuti, T. Ashizawa, M.B. Perryman, G. Scarlato, R.G. Fenwick Jr., et al., Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy, Science 260 (1993) 235–238.

[4] M. Mahadevan, C. Tsilfidis, L. Sabourin, G. Shutler, C. Amemiya, G. Jansen, C. Neville, M. Narang, J. Barcelo, K. O'Hoy, et al., Myotonic dystrophy mutation: an unstable CTG repeat in the 3′ untranslated region of the gene, Science 255 (1992) 1253–1255.

[5] C.L. Liquori, K. Ricker, M.L. Moseley, J.F. Jacobsen, W. Kress, S.L. Naylor, J.W. Day, L.P. Ranum, Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9, Science 293 (2001) 864–867.

[6] A. Mankodi, E. Logigian, L. Callahan, C. McClain, R. White, D. Henderson, M. Krym, C.A. Thornton, Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat, Science 289 (2000) 1769–1773.

[7] K.L. Taneja, M. McCurrach, M. Schalling, D. Housman, R.H. Singer, Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues, J. Cell Biol. 128 (1995) 995–1002.

[8] R.N. Kanadia, K.A. Johnstone, A. Mankodi, C. Lungu, C.A. Thornton, D. Esson, A.M. Timmers, W.W. Hauswirth, M.S. Swanson, A muscleblind knockout model for myotonic dystrophy, Science 302 (2003) 1978–1980.

[9] B.N. Charlet, R.S. Savkur, G. Singh, A.V. Philips, E.A. Grice, T.A. Cooper, Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing, Mol. Cell 10 (2002) 45–53.

[10] A. Mankodi, M.P. Takahashi, H. Jiang, C.L. Beck, W.J. Bowers, R.T. Moxley, S.C. Cannon, C.A. Thornton, Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy, Mol. Cell 10 (2002) 35–44.

[11] A.V. Philips, L.T. Timchenko, T.A. Cooper, Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy, Science 280 (1998) 737–741.

[12] T. Kimura, M. Nakamori, J.D. Lueck, P. Pouliquin, F. Aoike, H. Fujimura, R.T. Dirksen, M.P. Takahashi, A.F. Dulhunty, S. Sakoda, Altered mRNA splicing of the skeletal muscle ryanodine receptor and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase in myotonic dystrophy type 1, Hum. Mol. Genet. 14 (2005) 2189–2200.

[13] R.S. Savkur, A.V. Philips, T.A. Cooper, Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy, Nat. Genet. 29 (2001) 40–47.

[14] R.S. Savkur, A.V. Philips, T.A. Cooper, J.C. Dalton, M.L. Moseley, L.P. Ranum, J.W. Day, Insulin receptor splicing alteration in myotonic dystrophy type 2, Am. J. Hum. Genet. 74 (2004) 1309–1313.

[15] X. Lin, J.W. Miller, A. Mankodi, R.N. Kanadia, Y. Yuan, R.T. Moxley, M.S. Swanson, C.A. Thornton, Failure of MBNL1-dependent post-natal splicing transitions in myotonic dystrophy, Hum. Mol. Genet. 15 (2006) 2087–2097.

[16] L.P. Ranum, T.A. Cooper, RNA-mediated neuromuscular disorders, Annu. Rev. Neurosci. 29 (2006) 259–277.

[17] Y. Kino, C. Washizu, Y. Oma, H. Onishi, Y. Nezu, N. Sasagawa, N. Nukina, S. Ishiura, MBNL and CELF proteins regulate alternative splicing of the skeletal muscle chloride channel CLCN1, Nucleic Acids Res. 37 (2009) 6477–6490.

[18] T.H. Ho, B.N. Charlet, M.G. Poulos, G. Singh, M.S. Swanson, T.A. Cooper, Muscleblind proteins regulate alternative splicing, EMBO J. 23 (2004) 3103–3112.

[19] S. Hino, S. Kondo, H. Sekiya, A. Saito, S. Kanemoto, T. Murakami, K. Chihara, Y. Aoki, M. Nakamori, M.P. Takahashi, K. Imaizumi, Molecular mechanisms responsible for aberrant splicing of SERCA1 in myotonic dystrophy type 1, Hum. Mol. Genet. 16 (2007) 2834–2843.

[20] W. Dansithong, S. Paul, L. Comai, S. Reddy, MBNL1 is the primary determinant of focus formation and aberrant insulin receptor splicing in DM1, J. Biol. Chem. 280 (2005) 5773–5780.

[21] H. Xia, S.T. Winokur, W.L. Kuo, M.R. Altherr, D.S. Bredt, Actinin-associated LIM protein: identification of a domain interaction between PDZ and spectrin-like repeat motifs, J. Cell Biol. 139 (1997) 507–515.

[22] K. Jo, B. Rutten, R.C. Bunn, D.S. Bredt, Actinin-associated LIM protein-deficient mice maintain normal development and structure of skeletal muscle, Mol. Cell. Biol. 21 (2001) 1682–1687.

[23] D. Frank, C. Kuhn, H.A. Katus, N. Frey, The sarcomeric Z-disc: a nodal point in signalling and disease, J. Mol. Med. 84 (2006) 446–468.

[24] R.N. Kanadia, J. Shin, Y. Yuan, S.G. Beattie, T.M. Wheeler, C.A. Thornton, M.S. Swanson, Reversal of RNA missplicing and myotonia after muscleblind overexpression in a mouse poly(CUG) model for myotonic dystrophy, Proc. Natl. Acad. Sci. USA 103 (2006) 11748–11753.

[25] C.J. Brandl, N.M. Green, B. Korczak, D.H. MacLennan, Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences, Cell 44 (1986) 597–607.

[26] C.J. Brandl, S. deLeon, D.R. Martin, D.H. MacLennan, Adult forms of the Ca^{2+}ATPase of sarcoplasmic reticulum. Expression in developing skeletal muscle, J. Biol. Chem. 262 (1987) 3768–3774.

[27] A. Kalsotra, X. Xiao, A.J. Ward, J.C. Castle, J.M. Johnson, C.B. Burge, T.A. Cooper, A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart, Proc. Natl. Acad. Sci. USA 105 (2008) 20333–20338.

[28] T. Klaavuniemi, A. Kelloniemi, J. Ylanne, The ZASP-like motif in actinin-associated LIM protein is required for interaction with the alpha-actinin rod and for targeting to the muscle Z-line, J. Biol. Chem. 279 (2004) 26402–26410.

[29] T. Klaavuniemi, N. Alho, P. Hotulainen, A. Kelloniemi, H. Havukainen, P. Permi, S. Mattila, J. Ylanne, Characterization of the interaction between actinin-associated LIM protein (ALP) and the rod domain of alpha-actinin, BMC Cell Biol. 10 (2009) 22.

[30] A.M. Arola, X. Sanchez, R.T. Murphy, E. Hasle, H. Li, P.M. Elliott, W.J. McKenna, J.A. Towbin, N.E. Bowles, Mutations in PDLIM3 and MYOZ1 encoding myocyte Z line proteins are infrequently found in idiopathic dilated cardiomyopathy, Mol. Genet. Metab. 90 (2007) 435–440.

[31] R.D. Bagnall, L. Yeates, C. Semsarian, Analysis of the Z-disc genes PDLIM3 and MYPN in patients with hypertrophic cardiomyopathy, Int. J. Cardiol. 145 (2010) 601–602.