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

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ABSTRACT

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.

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

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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	CAGCTCACCAGCTGTGTCTC	66 °C	27
				PDLIM3_ex4_Rv	GAGCCATCGTCCACCATTCC		
FN1	NM_002026	ex25	−	FN1_ex25_Fw	ATGGACAGGAAAGAGATGCG	66 °C	30
				FN1_ex25_Rv	AAAAGTCAATGCCAGTTGGG		
		ex33	ex33+	FN1_ex33_Fw	CCTGGGAGCAAGTCTACAGC	66 °C	31
				FN1_ex33_Rv	TAGCATCTGTCACACGAGCC		
PKP2	NM_001005242	ex6	−	PKP2_ex6_Fw	TCCAGGTGCTGAAGCAAACC	66 °C	32
				PKP2_ex6_Rv	TCGCTTTTCTCCCATCAGCG		
TTN	NM_003319	ex45	−	TTN_ex45_Fw	AGCACAGCCAACCTGAGTCT	54 °C	31
				TTN_ex45_Rv	CCGGTTCACCCTCTAAAACA		
EGLN2	NM_053046	ex4	−	EGLN2_ex4_Fw	CTGGGCAGCTATGTCATCAA	64 °C	30
				EGLN2_ex4_Rv	TGGACACCTTTCTGTCCTGA		
SERCA1 (PC)	NM_004320	ex22	ex22−	SERCA1_ex22_Fw	ATCTTCAAGCTCCGGGCCCT	63.5 °C	25
				SERCA1_ex22_Rv	CAGCTCTGCCTGAAGATGTG		

[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.

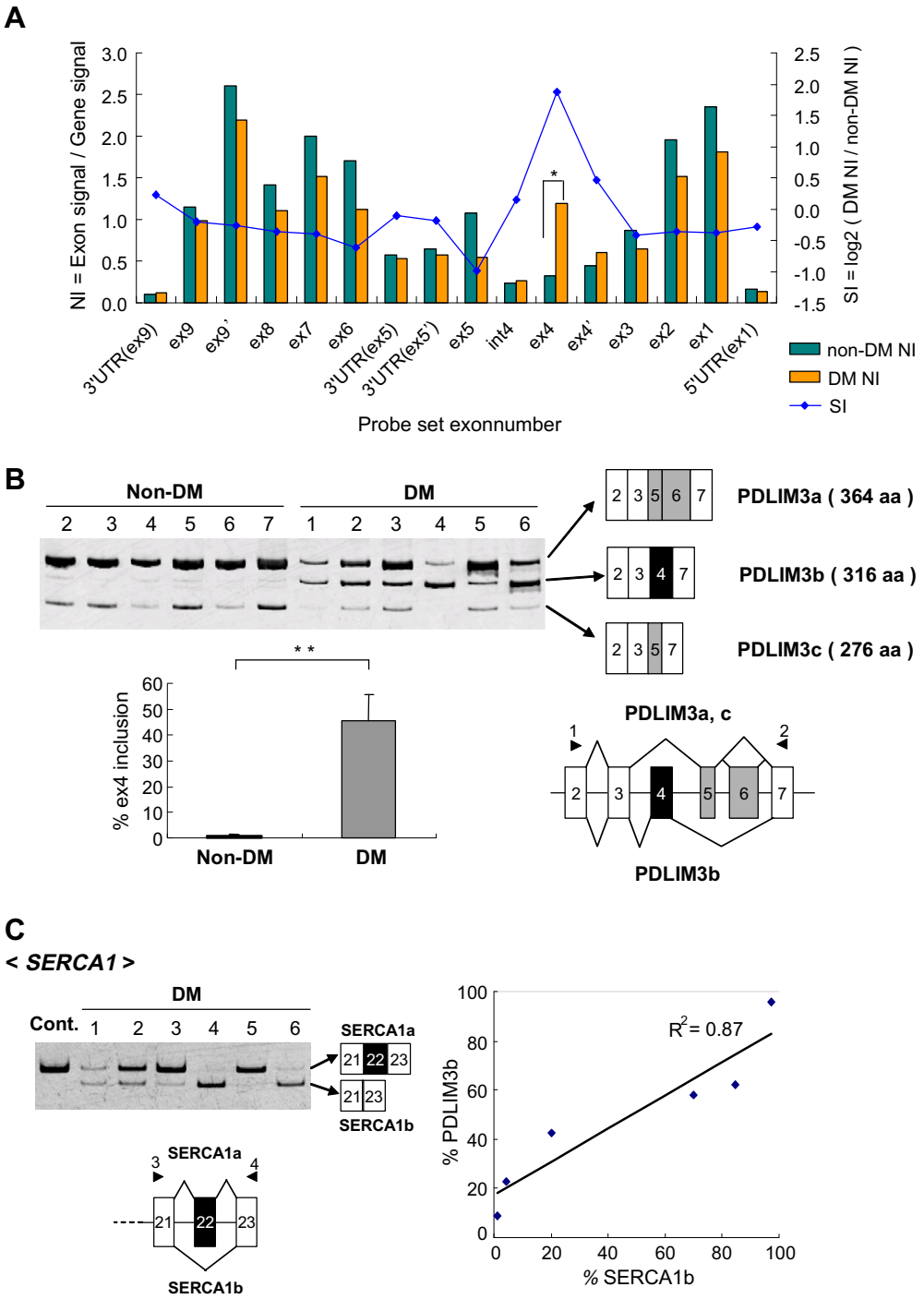

Fig. 1. *PDLIM3* splicing is abnormal in patients with DM1. (A) Exon array analysis of *PDLIM3* in four non-DM1 and three DM1 biopsies. NI, normalized exon intensity (NI = exon level signal/gene level signal); SI, splicing index ($SI = \log_2 NI_{DM}/NI_{non\text{-}DM}$). Statistical significance was 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.

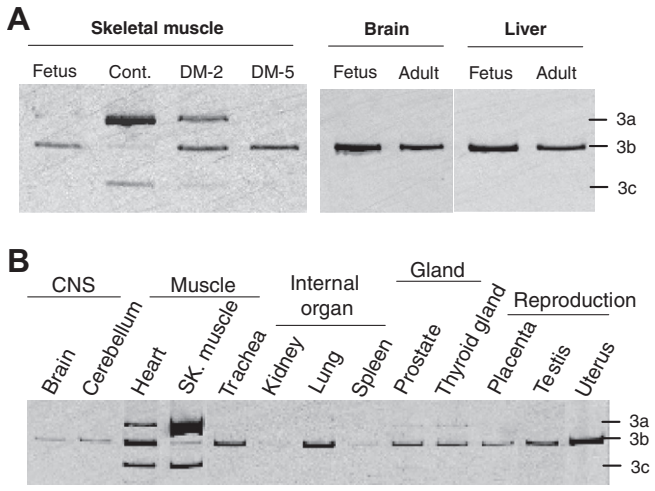

Fig. 2. Patterns of *PDLIM3* splicing during the development of skeletal muscles and various tissues. (A) RT-PCR of endogenous *PDLIM3* in fetal, non-DM, and DM1 skeletal muscles and other tissues. The fetal skeletal muscle isoform is PDLIM3b (exon 4 inclusion isoform), which is the same as that in the 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.

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.

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 ($MBNL^{\Delta 3/\Delta 3}$) 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.

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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.

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