

Autophagic degradation of nuclear components

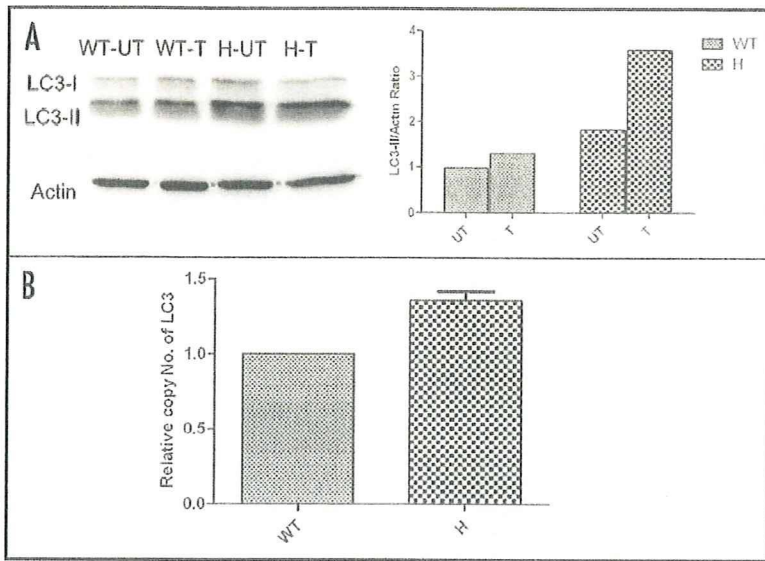
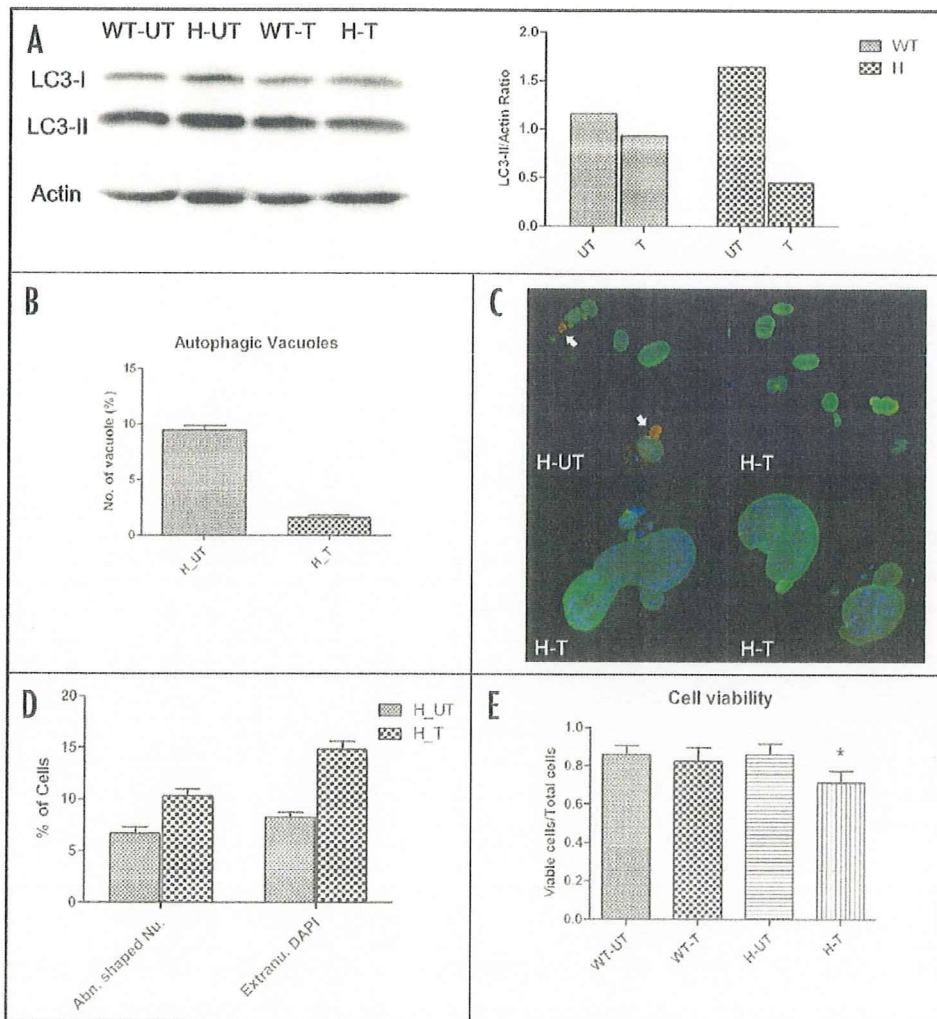


Figure 6. Immunoblotting analysis of LC3 and quantitative real-time PCR of *Maplc3b*. (A) The amount of LC3-II is highly increased in *Lmna*<sup>H222P/H222P</sup> cells, and which gets more increased with the treatment of lysosomal protease inhibitors (pepstatin A and E64d), suggesting that autophagy is markedly activated in the diseased cells. (B) By quantitative real-time PCR of *Maplc3b*, the transcriptional level of LC3 is represented to be significantly increased in *Lmna*<sup>H222P/H222P</sup> MEF compared with wild-type ( $p = 0.0141$ ). WT-UT, untreated wild-type; WT-T, treated wild-type; H-UT, untreated *Lmna*<sup>H222P/H222P</sup>; H-T, treated *Lmna*<sup>H222P/H222P</sup> cells.

reported in *S. cerevisiae*, which is induced by nutritional depletion or rapamycin stimulation. During PMN velcro-like patches are formed by the interaction between vacuolar membrane and outer nuclear membrane at nonessential portions of nuclei.<sup>42</sup> PMN is morphologically categorized as microautophagy because nuclear components are directly engulfed by vacuoles without formation of vesicular intermediates, i.e., autophagosome.<sup>43,44</sup> This concept was further supported by a recent investigation that



elucidated the core machinery on PMN.<sup>44</sup> Because detailed underlying molecular mechanism is still unclear, we could not completely exclude the possible involvement of microautophagy for degradation of nuclear blebs observed in *Lmna*<sup>H222P/H222P</sup> cells.

Figure 7. Changes in nuclear abnormalities and cell survival rate after autophagy inhibition. (A) Immunoblotting analysis of LC3 shows that LC3-II is decreased after autophagy inhibition in wild-type and *Lmna*<sup>H222P/H222P</sup> cells. (B) In treated *Lmna*<sup>H222P/H222P</sup> cells, autophagic vacuoles are significantly decreased compared with untreated cells ( $p < 0.0001$ ). (C) On immunocytochemistry of LC3 (red), lamin C (green) and DAPI (blue), the treated *Lmna*<sup>H222P/H222P</sup> cells rarely show LC3 staining whereas untreated cells frequently display perinuclear autophagosomes (arrows, upper). With autophagy inhibition, cells present severe deformation of nuclei and multiple extranuclear DAPI (lower). (D) The number of cells with markedly irregular shaped nuclei and extranuclear DAPI is much increased by autophagy inhibition ( $p = 0.0008$ ). (E) Cell viability assay represents the decreased cell survival rate in *Lmna*<sup>H222P/H222P</sup> cells by autophagy inhibition compared with wild-type cells (\* $p = 0.0029$ ). WT, wild-type; H, *Lmna*<sup>H222P/H222P</sup>; UT, untreated; T, treated.

The activation of autophagy in *Lmna*<sup>H222P/H222P</sup> cells is supported by the finding of increased amount of LC3-II on immunoblotting analysis, and the transcriptional upregulation of LC3 expression. LC3 has been previously reported to be increased in progeroid mice lacking zinc metalloproteinase STE24 or ZMPSTE24 (*Zmpste24*<sup>-/-</sup>),<sup>45</sup> an enzyme required for the maturation of lamin A. ZMPSTE24 deficiency causes accumulation of the premature form of lamin A (prolamin A) in nuclear envelope and leads to profound nuclear architecture abnormalities.<sup>46</sup> Increased LC3 was thought to be secondary to enhanced basal autophagy in skeletal and cardiac muscles due to a metabolic derangement in progeroid mice. Considering similar molecular defects of nuclear lamina in two mouse models of *Zmpste24*<sup>-/-</sup> and *Lmna*<sup>H222P/H222P</sup>, we cannot totally exclude the possibility of increased basal autophagy in *Lmna*<sup>H222P/H222P</sup> mice although they did not show comparable alterations in glucose and lipid metabolism.<sup>47</sup> In this study, the finding that GFP-LC3-positive vacuoles were consistently related to the cytoplasmic nuclear components could indicate that activated autophagy in *Lmna*<sup>H222P/H222P</sup> cells may at least in part be induced by the nuclear damage.

Reduced cell viability in *Lmna*<sup>H222P/H222P</sup> cells after autophagy inhibition probably resulted from increased frequency of nuclear abnormalities as shown in Figure 7. This result ultimately indicates that autophagy is working for the maintenance of cellular homeostasis by cleaning up nuclear wastes in *Lmna*<sup>H222P/H222P</sup> cells. With autophagic degradation of nuclear components, however, a partial loss of genetic information may be inevitable, leading to a varying degree of molecular defects on cells. Cells with bulk loss of genetic materials can be thought to eventually undergo apoptosis, but clarification of this issue is beyond the scope of this paper.

In conclusion, we have demonstrated the presence of perinuclear autophagosomes/autolysosomes in *Lmna*<sup>H222P/H222P</sup> cells. Notably, in the area of nuclear membrane interfacing with autophagosomes/autolysosomes, we could see accumulation of nuclear envelope proteins. This may suggest that autophagy could contribute to the rapid repair of the nuclear membrane, as there is a need to rescue the cells from overdegradation or to minimize the loss of nuclear components after nuclear membrane injury. This would be worth exploring in future experiments.

It is also notable that similar autophagosomes/autolysosomes containing nuclear components were found even in wild-type cells although to a much lower frequency. This implies that autophagic degradation of nuclear components is not confined to nuclear envelopathy, and can occur under other conditions that cause nuclear damage. Like other organelle-specific autophagy reported to date, the autophagy in *Lmna*<sup>H222P/H222P</sup> MEF can be proposed to be called as 'nucleophagy.' Nevertheless, the precise role of "nucleophagy" in laminopathies, and possibly in physiologic conditions, remains perplexing and a potential interest that needs to be elucidated.

## Materials and Methods

Mouse models for nuclear envelopathy and generation of *Lmna*<sup>H222P/H222P</sup>/GFP-LC3 transgenic mouse. We used H222P homozygous knock-in (*Lmna*<sup>H222P/H222P</sup>),<sup>47</sup> *Lmna* knockout

(*Lmna*<sup>-/-</sup>),<sup>48</sup> and emerin knockout (*Emd*<sup>-/-</sup>) mice<sup>25</sup> as mouse models for nuclear envelopathy in this study. The mutation of p.H222P in *LMNA* is one of the mutations causing muscular dystrophy in human, and the homozygous mouse model carrying the mutation, *Lmna*<sup>H222P/H222P</sup> reproduced the phenotype of human muscular dystrophy due to *LMNA* mutations.<sup>47</sup> *Lmna*<sup>-/-</sup> mice have shown postnatal lethality in addition to muscular dystrophy and cardiomyopathy although they were normal at birth.<sup>48</sup> *Emd*<sup>-/-</sup> mice demonstrated altered motor coordination and delayed atrioventricular conduction time in electrocardiogram, but overall they showed normal growth rate and were without obvious muscle weakness.<sup>25</sup>

*Lmna*<sup>H222P/H222P</sup> mice were crossed with GFP-LC3 transgenic mice<sup>35</sup> (kindly provided by Dr. Mizushima in Tokyo Medical and Dental University) to generate *Lmna*<sup>H222P/H222P</sup>/GFP-LC3 transgenic mice.

**Electron microscopic observation.** Soleus muscles and skin of abdomen obtained from *Lmna*<sup>H222P/H222P</sup> mice and wild-type littermates were obtained for electron microscopic observation. Tissues were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate and 0.2 M *s*-collidine for 2 hours, samples were embedded in epoxy resin.

*Lmna*<sup>H222P/H222P</sup> MEF were cultured and grown on Lab-Tek™ chambered coverglass (Nunc, Tokyo, Japan). They were fixed with 1.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for one hour. After osmification of cells with 1% OsO<sub>4</sub> in 0.1 M phosphate buffer at 4°C, they were embedded in epoxy resin.

Ultrathin sections (50 nm thickness) were stained with uranyl acetate and lead citrate, and then examined under H-600 transmission electron microscope (Hitachi, Japan) at 75 kV.

**Cell culture and immunocytochemistry.** Mouse embryonic fibroblasts (MEF) were obtained from *Lmna*<sup>H222P/H222P</sup>, *Lmna*<sup>-/-</sup>, *Lmna*<sup>H222P/H222P</sup>/GFP-LC3 transgenic mice and wild-type littermates. They were harvested in 100-mm collagen I-coated dishes with Dulbecco's modified Eagle medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS) and 1% of antibiotics, and incubated at 37°C in humidified chamber with 5% CO<sub>2</sub>.

Cultured cells were plated on collagen I-coated glass coverslips and fixed with 4% paraformaldehyde in PBS for 15 min at 4°C, permeabilized on ice with 0.25% Triton X-100/PBS for 20 min, blocked with 2% casein/PBS for 15 min at 37°C, and then immunostained with primary antibodies diluted in 2% casein/PBS for 2 hrs at 37°C. Primary antibodies used in this study are as follows: anti-lamin A (Abcam, Tokyo, Japan), lamin C,<sup>16</sup> lamin B (Santa Cruz Biotechnology Inc., CA), emerin (Novocastra Laboratories, Newcastle upon Tyne, UK), lamin-associated protein 2α (LAP2α), nesprin 1α (Abcam, Tokyo, Japan), LC3 (provided by Dr. Ueno, Juntendo university), Atg5 (Sigma-Aldrich, Tokyo, Japan), Atg9 (Biosensis, Thebarton, South Australia), Atg16L, Rab7 (Sigma-Aldrich), LAMP2 (Developmental Studies Hybridoma Bank), histone H1 (Santa Cruz Biotechnology Inc.), H2AX (Abcam). Cells were then incubated with fluorescently labeled secondary antibodies (Alexa488 or Alexa568) at room temperature

for 45 min. Coverslips were mounted together with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, CA) for nuclear localization, and then visualized under epifluorescence using Axiophoto II (Carl Zeiss).

To detect lysosomal localization, cells were incubated with PBS containing 100 nM of Lyso-Tracker<sup>®</sup> (Invitrogen) at 37°C for 1 hour. After rinsing with PBS, cells were fixed with 4% paraformaldehyde at 37°C for 20 min.

**Immunoblotting analysis of LC3.** *Lmna*<sup>H222P/H222P</sup> and wild-type MEF were grown on 100 mm-collagen I coated dishes. At 80% confluent state, cells were treated with lysosomal protease inhibitors consisting of pepstatin A (20 µg/mL, Peptide Institute, Osaka, Japan) and E64d (20 µg/mL, Peptide Institute), or with dimethylsulfoxide (DMSO) as a negative control for 4 hours. Whole cell lysates were extracted with lysis buffer (1% NP-40 cell lysis buffer supplemented with protease inhibitor). Immunoblotting analysis of LC3 was performed according to the standard method. Twenty micrograms of protein of each sample were loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was transferred to polyvinylidene (PVDF) membrane and immunostained with anti-LC3 antibody. Data was analyzed by using LAS-1000 chemiluminescence imaging system (Fujifilm, Tokyo, Japan).

**Quantitative real-time PCR.** Total RNA was extracted from *Lmna*<sup>H222P/H222P</sup> and wild-type MEF with TRIzol (Invitrogen) following manufacturer's protocol. Single-stranded cDNA was synthesized from RNA using SuperScript<sup>™</sup> III reverse transcriptase. Gene expression of LC3B was quantified by quantitative real-time PCR in Rotor-Gene<sup>™</sup> 6000 system (Corbett Life Science, NSW, Australia), using the following primers: LC3b-F; CCG AGA AGA CCT TCA AGC AG and LC3b-R; CCA TTC ACC AGG AGG AAG AA. All the results were normalized with respect to G3PDH expression.

**Autophagy inhibition.** MEF from *Lmna*<sup>H222P/H222P</sup> mice and wild-type littermates were treated with 10 mM 3-MA (Sigma-Aldrich) and 200 nM wortmannin (Sigma-Aldrich), which are also known as phosphatidylinositol-3 kinase inhibitors and autophagy inhibitors, or negative control at 80% confluent state for 2 hours as previously described.<sup>49</sup> After the treatment, the amount of LC3 was measured by immunoblotting analysis. Immunocytochemistry of LC3 and a nuclear envelope protein (lamin C) was also performed to check the changes in nuclear abnormalities with autophagy inhibition. For the calculation of cell survival rate, the cells were stained by acetoxymethyl ester of calcein (Calcein-AM, Dojindo, MD) and propidium iodide (PI, Dojindo) for 15 min at 37°C according to the manufacturer's instruction. This staining method can differentiate green-colored viable and red-colored dead cells, respectively. Cell viability was determined as the ratio of the number of viable cells per total number of cells in four groups of untreated wild-type, treated wild-type, untreated *Lmna*<sup>H222P/H222P</sup> and treated *Lmna*<sup>H222P/H222P</sup> cells.

**Statistical analyses.** To get quantitative data, three to four replicates of measurement were done for each condition. All the data were presented as mean and standard deviation. Comparisons among groups were done by using student-t test and analysis of

variance (ANOVA) as appropriate. Statistical significance was considered when p value was less than 0.05.

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#### Note

Supplementary materials can be found at: [www.landesbioscience.com/supplement/ParkAUTO5-6-Sup.pdf](http://www.landesbioscience.com/supplement/ParkAUTO5-6-Sup.pdf)

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# Human *PTRF* mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy

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Caveolae are invaginations of the plasma membrane involved in many cellular processes, including clathrin-independent endocytosis, cholesterol transport, and signal transduction. They are characterized by the presence of caveolin proteins. Mutations that cause deficiency in caveolin-3, which is expressed exclusively in skeletal and cardiac muscle, have been linked to muscular dystrophy. Polymerase I and transcript release factor (*PTRF*; also known as *cavin*) is a caveolar-associated protein suggested to play an essential role in the formation of caveolae and the stabilization of caveolins. Here, we identified *PTRF* mutations in 5 nonconsanguineous patients who presented with both generalized lipodystrophy and muscular dystrophy. Muscle hypertrophy, muscle mounding, mild metabolic complications, and elevated serum creatine kinase levels were observed in these patients. Skeletal muscle biopsies revealed chronic dystrophic changes, deficiency and mislocalization of all 3 caveolin family members, and reduction of caveolae structure. We generated expression constructs recapitulating the human mutations; upon overexpression in myoblasts, these mutations resulted in *PTRF* mislocalization and disrupted physical interaction with caveolins. Our data confirm that *PTRF* is essential for formation of caveolae and proper localization of caveolins in human cells and suggest that clinical features observed in the patients with *PTRF* mutations are associated with a secondary deficiency of caveolins.

## Introduction

Caveolae are specific invaginations of the plasma membrane characterized by the presence of the protein caveolin. To date, 3 caveolin family members have been identified. Caveolin-1 and -2 are coexpressed in many cell types, such as endothelial cells, smooth muscle cells, fibroblasts, and adipocytes, and form a hetero-oligomeric complex (1). In contrast, caveolin-3 is expressed exclusively in skeletal and cardiac muscles (2). Caveolae are involved in several important cellular processes, including clathrin-independent endocytosis, regulation and transport of cellular cholesterol, and signal transduction (3, 4).

Polymerase I and transcript release factor (*PTRF*; also known as *cavin*) is a highly abundant caveolae component and is suggested to have an essential role in caveolar formation. In both mammalian cells and zebrafish, knockdown of *PTRF* leads to a reduction in caveolae density (5). Mice lacking *PTRF* do not have morphologically detectable caveolae, in addition to a markedly diminished protein expression of all 3 caveolin isoforms (6). Interestingly, *PTRF*-knockout mice mimic lipodystrophy in humans, demonstrating considerably reduced adipose tissue mass, high circulating triglyceride levels, glucose intolerance, and hyperinsulinemia (6).

Here we report that mutations in *PTRF* (GenBank accession no. 284119) caused a disorder presenting as generalized lipodystrophy and muscular dystrophy. We demonstrate that this condition was associated with deficiency and mislocalization of all 3 caveolin family members and reduction of caveolae structure.

## Results

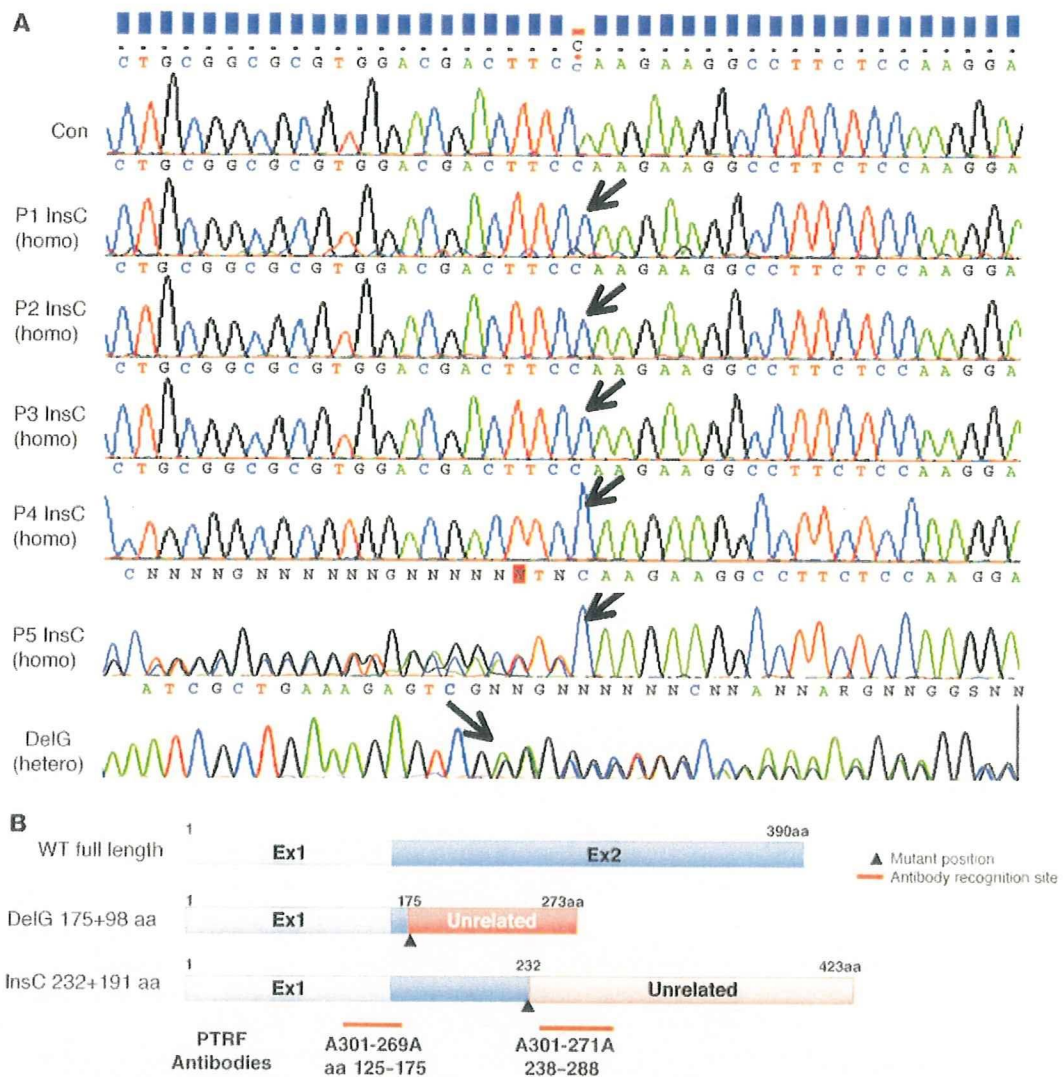
**Identification of *PTRF* mutations.** Deficiency of caveolin-3 as a result of *CAV3* gene mutations is known to cause muscular dystrophy (7). We found 5 nonconsanguineous Japanese patients whose muscle showed caveolin-3 deficiency but without *CAV3* mutation among 2,745 muscular dystrophy specimens kept in the muscle repository of the National Center of Neurology and Psychiatry. Importantly, all 5 patients also had congenital generalized lipodystrophy (CGL; also known as Berardinelli-Seip syndrome). From the findings observed in lacking cells and animal models lacking *PTRF* (5, 6), we screened for *PTRF* mutations.

We identified 2 different frameshift mutations in all 5 patients examined: patients 1–4 (P1–P4) had the same homozygous c.696\_697insC (p.K233fs) mutation in exon 2, and P5 harbored a compound heterozygous mutation of the same c.696\_697insC and c.525delG (p.E176fs) in exon 2 (Figure 1A). The c.525delG mutation changes the last 275 amino acids to an unrelated 98-amino acid sequence, whereas c.696\_697insC substitutes the last 158 amino acids with an unrelated 191-amino acid sequence (Figure 1B). Both mutations were not identified in the chromosomes of 200 Japanese control subjects.

In order to determine whether the common c.696\_697insC mutation has the same haplotype, we examined 6 sets of single nucleotide polymorphisms (SNPs) within *PTRF*: rs2062213, rs8070945, rs963988, rs963987, rs963986, and rs9252. All 5 patients had the same haplotype for all 6 SNPs, which occurred homozygously (Table 1). During mutation screening, we found a novel 9-bp insertion polymorphism in the 3' noncoding region

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**Figure 1**

Mutations in *PTRF*. (A) All 5 patients had a homozygous or compound heterozygous mutation in *PTRF* (shown by arrows). P1–P4 had the same homozygous insertion mutation of c.696\_697insC (InsC) in exon 2, whereas P5 had a compound heterozygous mutation of the same c.696\_697insC insertion mutation and a deletion mutation of c.525delG (DelG) in exon 2. (B) Schema of the position of mutations in *PTRF*, putative proteins produced by mutations, and antibody recognition sites. The c.525delG mutant changes the last 275 amino acids to an unrelated 98–amino acid sequence, while the c.696\_697insC mutant substitutes the last 158 amino acids with an unrelated 191–amino acid sequence.

of *PTRF* (c.1235\_1236insTCTCGGCTC). This 9-bp insertion was found heterozygously in 26% and homozygously in 2% of Japanese control individuals. In P1–P5, none had this 9-bp insertion. We also examined 2 microsatellite markers (STS-W93348 and D17S1185) close to *PTRF* and found heterozygosity in the patients (Table 1). From these results, a founder effect may not be likely, although we could not completely rule out the possibility.

**Mutation screening of the other genes associated with lipodystrophy and muscular dystrophy.** From the clinical and pathological findings, we performed mutation screening for the genes associated with muscular dystrophy and lipodystrophy, including *CAV3*, *LMNA*, *AGPAT2*, *BSCL2*, *CAVI*, *PPARG*, *AKT2*, and *ZMPSTE24*. We found a heterozygous nucleotide change of c.1138G>A (p.D380N) in *BSCL2* in P1. This substitution was also identified heterozygously

in 16% of Japanese control individuals, and we believe this to be a novel nonsynonymous SNP. For all the other genes examined, no other mutation was identified in P1–P5.

**Clinical features of the patients with *PTRF* mutations.** Clinical information for P1–P5 is summarized in Table 2. Common to all patients was the presence of muscular dystrophy and generalized lipodystrophy. However, despite having the same mutation, the patients' additional symptoms were variable. Generalized loss of subcutaneous adipose tissue in several areas, including the face, was noticed in infancy or early childhood. Hepatosplenomegaly, acromegaloid features, and umbilical prominence were often observed in the patients. No patient showed intellectual deficit or acanthosis nigricans. Patients presented with mild muscle weakness, but with hypertrophy of muscles (Figure 2A). Electrically silent percussion-induced

**Table 1**  
Haplotype analysis

	P1	P2	P3	P4	P5	Control <sup>A</sup>
Intron 1, rs2062213	C/C	C/C	C/C	C/C	C/C	C/C (53%)
Intron 1, rs8070945	C/C	C/C	C/C	C/C	C/C	C/C (78%)
Intron 1, rs963988	G/G	G/G	G/G	G/G	G/G	G/G (33%)
Intron 1, rs963987	G/G	G/G	G/G	G/G	G/G	G/G (31%)
Intron 1, rs963986	G/G	G/G	G/G	G/G	G/G	G/G (34%)
Exon 2, 9-bp insertion <sup>B</sup>	no/no	no/no	no/no	no/no	no/no	no/no (72%)
Exon 2, rs9252 <sup>B</sup>	C/C	C/C	C/C	C/C	C/C	C/C (78%)
STS-W93348 (bp)	251/253	251/253	251/253	251/253	251/253	251/253/264
D17S1185 (bp)	219/219	170/219	170/219	170/170	170/203	170/203/215/219/225/237

<sup>A</sup>Percentages denote the frequency of the haplotype in the HapMap JPT population. <sup>B</sup>Exon 2 is 3' noncoding.

muscle mounding was characteristic. Cardiac arrhythmia, transient immunodeficiency, recurrent pneumonia, constipation, and chaliasia were variably seen. Available laboratory data in the patients are summarized in Table 3. Metabolic complication was mild, and none of our patients showed marked elevation of fasting glucose levels. The result of oral glucose tolerance tests revealed moderate fasting hyperinsulinemia in P1 and P2 associated with glucose intolerance in P2, but normal levels in P4 (Table 4). High triglyceridemia was seen in P4 and P5. Serum creatine kinase (CK) levels were moderately elevated in all patients. Abdominal CT images of P4 revealed marked loss of subcutaneous and intra-abdominal fat (Figure 2, B and C). In addition, his body fat ratio, as determined by whole body dual energy X-ray absorptiometry, was 7.1% (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI38660DS1), while head fat was relatively preserved.

*Clinical features of the heterozygous parents.* There was no family history of muscular dystrophy or lipodystrophy in P1–P5. Genetic

analysis revealed a heterozygous c.696\_698insC mutation in both parents of P4. Clinically, both father and mother had hypertension requiring medication, whereas P4 was normotensive. Mild lipid metabolism abnormality and borderline glucose intolerance was also seen (Supplemental Table 2). DNA samples from the other parents were not available.

*Loss of PTRF with deficiency or mislocalization of caveolins in skeletal muscle.* Biopsied skeletal muscles from P1–P5 showed consistent findings, with chronic dystrophic changes

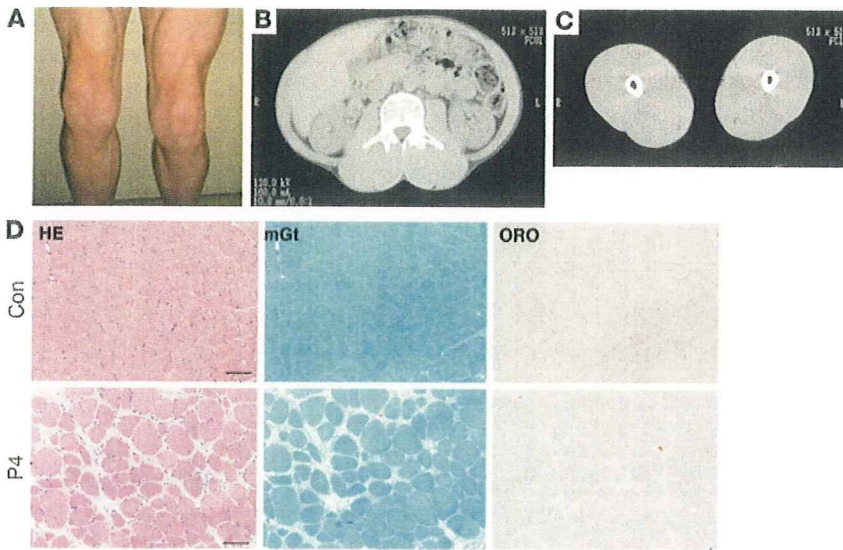
including marked variation in muscle fiber size, increased number of fibers containing internalized nuclei, a few necrotic and regenerating fibers, and increased interstitial fibrosis (Figure 2D and Supplemental Figure 1). Intramuscular lipid droplets, as visualized by oil red O staining, were not increased (Figure 2D).

Immunohistochemistry demonstrated that the PTRF antibodies A301-269A and A301-271A (which recognize the N- and C-terminal regions of the protein, respectively; Figure 1B) showed sarcolemmal membrane staining of muscle fibers, with stronger immunoreaction at intramuscular blood vessels in control muscles (Figure 3A). Caveolin-3 was clearly observed at sarcolemma, whereas caveolin-1 and -2 were present only in blood vessels. In contrast, muscles from P1–P5 showed barely detectable immunoreaction to both PTRF antibodies (Figure 3A). Caveolin-3 immunoreactivity was greatly reduced in the sarcolemma, but cytoplasmic staining was remarkably increased. This caveolin-3 staining pattern was similar to that seen in the patients with muscular dystrophy

**Table 2**  
Clinical summary

	P1	P2	P3	P4	P5
Age/sex	8-yr-old female	14-yr-old female	10-yr-old male	27-yr-old male	24-yr-old male
Height, body weight	124 cm, 21.3 kg	149 cm, 40.5 kg	NA	164 cm, 49.0 kg	152 cm, 40 kg
Lipodystrophy	Generalized	Generalized	Generalized	Generalized	Generalized
Mental retardation	No	No	No	No	No
Acanthosis nigricans	No	No	No	No	No
Liver/spleen	Hepatosplenomegaly	Fatty liver	NA	Hepatosplenomegaly	No
Endocrine abnormalities	Reduced growth hormone secretion	NA	NA	Accelerated bone age, acromegaloid features, no androgynism	Acromegaloid features, no androgynism
Muscle weakness	Distal dominant	No	No	Generalized	Distal dominant
Muscle mounding	Positive	NA	NA	Positive	Positive
Other muscle symptoms	Muscle hypertrophy	Myalgia, muscle stiffness	NA	Muscle hypertrophy	Muscle hypertrophy
Cardiac symptoms	Arrhythmia	No	No	Atrial fibrillation	No
Skeletal abnormalities	Lordosis, Contractures (ankles, shoulders, fingers)	No	No	Scoliosis, contractures (ankles)	Scoliosis
Other symptoms	Constipation	Transient IgA deficiency, recurrent pneumonia	Nephrosis	Umbilical prominence, renal stones	Recurrent pneumonia, chaliasia, constipation

NA, not available.



**Figure 2** Muscle hypertrophy and dystrophic changes. (A) Prominent musculature feature of legs in P5. (B and C) CT images from P4 showed hypertrophy of paravertebral and thigh muscles with minimal subcutaneous and intra-abdominal fat tissue. (D) H&E stain of biopsied skeletal muscle from P4 showed dystrophic changes, including marked variation in fiber size, enlarged fibers with internalized nuclei, endomysial fibrosis, and few necrotic and regenerating fibers. Intramuscular lipid droplets were not increased compared with control. mGt, modified Gomori trichrome; ORO, oil red O. Scale bars: 50  $\mu$ m.

caused by *CAV3* mutations (data not shown). Similarly, dysferlin was decreased in the sarcolemma and mislocalized into the cytoplasm (data not shown), and the same pattern is also seen in muscles of individuals with *CAV3* mutations (8). Immunoreactivity to caveolin-1 and caveolin-2 in blood vessels was barely detectable in P1–P5 (Figure 3A). Other antibodies related to muscular dystrophy, including dystrophin, sarcoglycans, dystroglycans, emerin, merosin, and collagen VI, showed normal immunostaining patterns (data not shown).

Immunoblotting showed detection of PTRF as an approximately 50-kDa band in control muscles and 3T3 cells, which were used as a positive control. No band was detected in the muscle of P1–P5 (Figure 3B). Caveolin-3 was detected in all samples examined, but relative protein amount, determined using densitometry and normalized by myosin heavy chain (MHC), decreased in P1–P5 compared with control subjects (Figure 3C). The band for caveolin-2 was observed in control muscles and 3T3 cells, but was barely detectable in the muscles of P1–P5 (Figure 3B).

In order to determine mRNA expression of PTRF, RT-PCR was performed using total RNA extracted from biopsied skeletal muscles. Using primers designed to amplify whole coding region of mRNA, PTRF was amplified as a single transcript in control muscles. In contrast, no PCR product was amplified in P1–P5 (Figure 4A). To compare mRNA levels for caveolins, we performed quantitative RT-PCR and normalized results to GAPDH expression. The mRNA amounts of all 3 caveolin families in the patients' muscles were variable, but not markedly decreased, compared with control muscles (Figure 4, B and C). Preserved mRNA levels, but decreased protein amounts of caveolins, suggested destabilization of caveolin proteins when PTRF is lacking, as previously reported (9).

*Loss of PTRF causes reduced caveolae formation in human muscles.* Greatly reduced caveolae formation was previously reported in PTRF knockdown

mammalian cells, zebrafish, and knockout mice (5, 9). Decreased caveolae number was also reported in skeletal muscle from limb girdle muscular dystrophy type 1C (LGMD1C) patients with *CAV3* mutations (10). We therefore examined muscle caveolae in P2 and P3 using electron microscopy. Plasma membrane of muscle fibers from both patients was nearly flat, and caveolae density was notably reduced, compared with control muscle (Figure 5). Caveolae formation in the intramuscular vascular smooth muscle cells was also remarkably reduced (data not shown).

*Altered localization of mutant PTRF and reduced interaction with caveolins in transfected cells.* In order to determine the intracellular localization of mutant PTRF, FLAG-tagged WT or 2 mutants (c.525delG and c.696\_697insC) and T7-tagged caveolin-3 or -1 were cotransfected in C2C12 myoblasts and COS-7 cells. In C2C12 cells, WT PTRF was detected at the cell membrane and colocalized with caveolin-3 (Figure 6A). Interestingly, c.525delG was detected as intranuclear aggregations and was not observed at the cell membrane (Figure 6, A and B). Caveolin-3 was present only in cytoplasm, and did not merge with PTRF (Figure 6A). The c.696\_697insC mutant was observed as microtubular filament network in cytoplasm and colocalized with  $\beta$ -tubulin (Figure 6B). This finding is consistent with the localization of the truncated PTRF<sub>1–322</sub>, as described previously (9). Similar mislocalization and/or aggregation of transfected mutant PTRF was observed in COS-7 cells (data not shown).

**Table 3**  
Laboratory data

Measurement	Reference range	P1	P2	P3	P4	P5
CK (IU/l)	56–244	1,374	542–2,253	2,000	554–1,545	645–2,630
Fasting glucose (mg/dl)	70–109	75	99	NA	93–116	102
HbA1c (%)	4.3–5.8	NA	NA	NA	5.0–5.4	NA
Total cholesterol (mg/dl)	130–220	164	NA	NA	185–267	218
Triglyceride (mg/dl)	50–150	93	NA	NA	143–450	359
LDL-C (mg/dl)	70–139	NA	NA	NA	188	NA
Leptin (ng/ml)	0.9–13.0	NA	NA	NA	0.6	NA
Adiponectin ( $\mu$ g/ml)	None	NA	NA	NA	1.05	NA

NA, not available.



**Table 4**  
Oral glucose tolerance test of P1, P2, and P4

	Pre	30 min	60 min	120 min
<b>P1</b>				
Glucose (mg/dl)	75	98	69	62
IRI ( $\mu$ U/ml)	22.8	141.6	64.7	23.8
<b>P2</b>				
Glucose (mg/dl)	99	127	160	172
IRI ( $\mu$ U/ml)	20	53	65	80
<b>P4</b>				
Glucose (mg/dl)	93	124	140	70
CPR (ng/ml) <sup>A</sup>	2.8	5.9	8.3	5.5
IRI ( $\mu$ U/ml)	1.0	22.3	32.9	6.2

IRI, immunoreactive insulin; CPR, C-peptide immunoreactivity. <sup>A</sup>Reference range, 0.7–2.2 ng/ml.

We performed immunoprecipitation assay in order to examine the binding ability of PTRF and caveolins. WT PTRF was coimmunoprecipitated by anti-T7 antibody, and vice versa (Figure 6, C and D). The c.525delG mutant showed smaller molecular weight (estimated 30 kDa; Figure 1B), and no immunoprecipitated protein was detected by FLAG and T7 antibodies. The c.696\_697insC mutant showed slightly larger molecular weight, and coimmunoprecipitated proteins were greatly reduced (Figure 6, C and D). These results suggest that mutant PTRFs cannot localize properly and lose their binding ability to caveolins even if they are produced.

**Activation of myostatin and Akt signaling pathways in PTRF-deficient skeletal muscles.** Caveolin-3 is suggested to have an important role for suppression of myostatin-mediated signaling in skeletal muscle (11). In order to determine the functions of mislocalized caveolin-3 in PTRF mutant cells, we performed quantitative RT-PCR for myostatin and immunoblotting analysis to examine phosphorylation status of Mad homolog 2/3 (p-Smad2/3), an intracellular effector of myostatin in skeletal muscles. In P1–P5, increased amounts of p-Smad2/3<sup>S423/425</sup> were observed in skeletal muscles, while myostatin mRNA levels were variable (Figure 7, A–C). Positive immunoreaction to p-Smad2/3 was detected in few myonuclei from muscle of patients with PTRF or CAV3 mutations, but not in those from muscle of control subjects (data not shown). These results suggest that myostatin signaling is also activated in P1–P5.

Despite the activation of myostatin, a negative regulator of muscle growth, the patients showed hypertrophy of muscles. Since Akt (also known as protein kinase B) is known as the key molecule to regulate muscle mass (12), we examined p-Akt<sup>T308</sup> and p-Akt<sup>S473</sup> by immunoblotting analysis. p-Akt was elevated in the muscle of P1–P5 compared with controls, except for p-Akt<sup>S473</sup> in P2 (Figure 7, D–F). This result suggests that Akt pathway is activated, probably through an as-yet-undefined mechanism, and could contribute to the muscle hypertrophy observed in P1–P5.

**Neuronal NOS activity is variable and mildly increased in PTRF-deficient skeletal muscles.** Caveolin-3 is known to interact with and negatively regulate the catalytic activity of neuronal NOS (nNOS) in skeletal muscle (13); this notion is supported by the finding of increased nNOS activity in muscle of transgenic mice expressing mutant caveolin-3 (14). We thus examined nNOS expression and its activity in muscles from patients with mutations in PTRF or CAV3 compared with those from age-matched controls. The immunoreactivity of nNOS was seen in sarcolemma and cytoplasm of

each muscle fiber with variable intensity, but no obvious difference was seen between patients and controls (Figure 3A). Immunoblotting analysis also revealed comparable amounts of nNOS (Figure 3, B and D). In order to examine nNOS activity of each muscle fiber, we performed NADPH diaphorase (NDP) activity assay. The intensity of NDP staining appeared variable among muscle fibers and was slightly increased in patients with mutations in PTRF or CAV3 compared with age-matched controls (Figure 8).

## Discussion

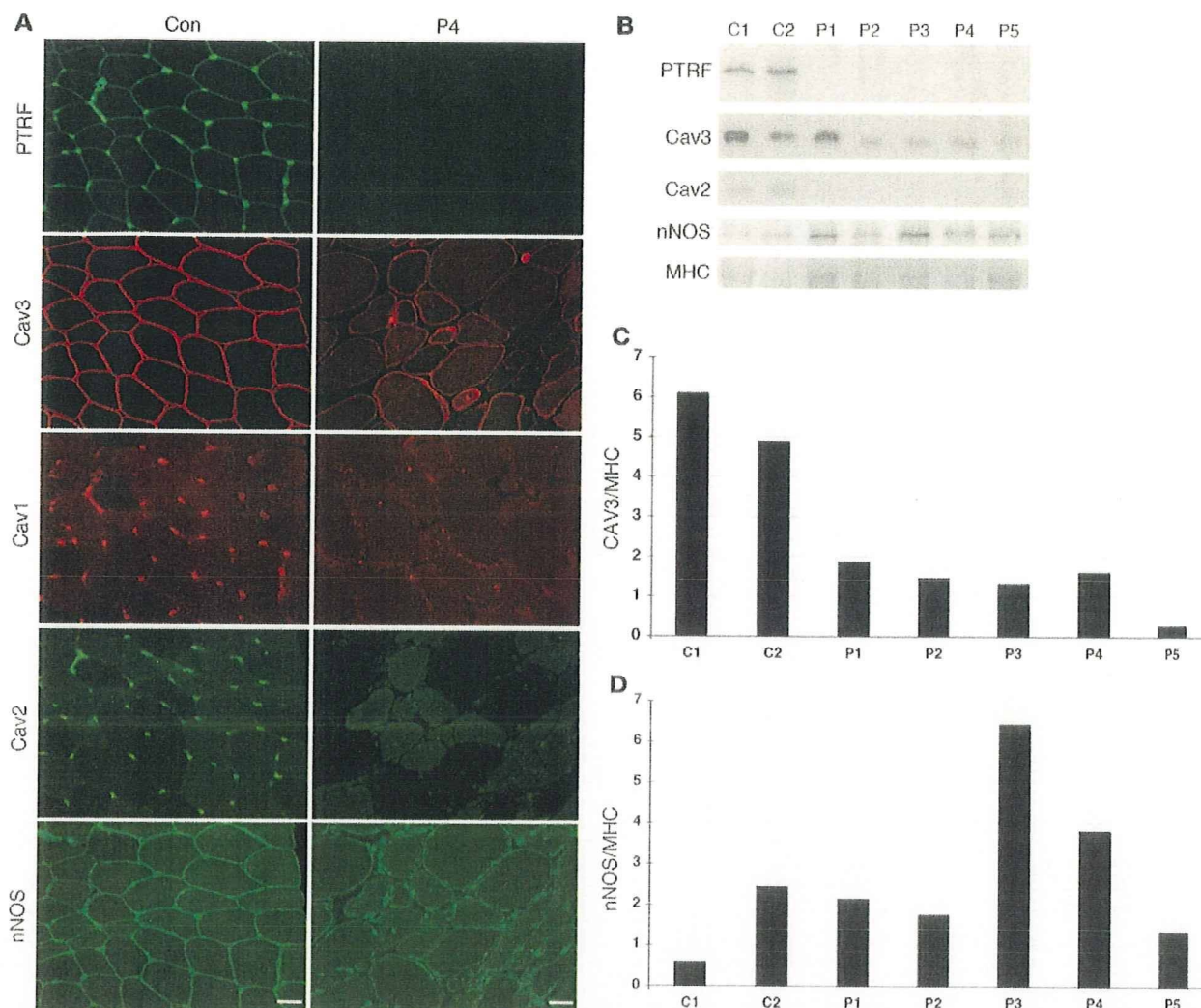
Lipodystrophy is a heterogeneous group of disorders characterized by loss of adipose tissue from the body. The degree of fat loss varies from small areas to near-complete absence of adipose tissue. The extent of fat loss usually determines clinical severity and metabolic complications, such as insulin resistance and high levels of serum triglycerides.

Several genes responsible for inherited lipodystrophy have been identified. CGL is an autosomal-recessive disorder, with most patients presenting soon after birth with severe insulin resistance and elevated serum triglycerides. CGL1 is caused by mutations in *AGPAT2* on chromosome 9q34, which encodes 1-acylglycerol-3-phosphate-O-acyltransferase 2, an enzyme involved in the biosynthesis of triacylglycerol and glycerophospholipids (15). CGL2 is caused by mutations in *BSC12* on chromosome 11q13, which encodes a functionally unknown protein named seipin (16). Recently, mutations in *CAVI* on chromosome 7q31 have been reported to cause generalized (i.e., CGL3) and partial lipodystrophy (17, 18).

Several causative genes for autosomal-dominant familial partial lipodystrophy are known: *LMNA* on chromosome 1q21 (19), *ZMPSTE24* on chromosome 1p34 (20), *AKT2* on chromosome 19q13 (21), *PPARG* on chromosome 3p25 (22), and *LMNB2* on chromosome 19q1 (23). Nevertheless, many patients clinically diagnosed with lipodystrophy carry no mutation in the known genes, suggesting the presence of other causative genes.

Here we conclude that PTRF mutations can cause CGL. In our series, patients showed generalized loss of adipose tissue from infancy or early childhood. Because PTRF is reported to colocalize with hormone-sensitive lipase and translocate to the nucleus in the presence of insulin in adipocytes (24), it could be surmised that PTRF plays an important role in lipid metabolism and insulin-regulated gene expression. Interestingly, metabolic complications were milder in patients with PTRF mutations than in patients with CGL1 and CGL2, and these were observed only in the elder patients. Although we could not examine the status of caveolae and caveolins in adipose tissues, the secondary deficiency of caveolins might have an important role in the process of lipodystrophy, since *CAVI* mutation can cause lipodystrophy in both humans and mice (17, 18, 25). Notably, the heterozygous parents had mild metabolic disorders, but a robust conclusion could not be reached, as a limited number of the heterozygous carriers of the PTRF mutation were available to us. Further investigation is needed to determine the effect of haploinsufficiency of PTRF.

Skeletal muscle symptoms with serum CK elevation represent another common symptom in patients with PTRF mutations. The clinical and pathological findings are very similar to those observed in patients with CAV3 mutation (7, 26–28), although P1–P5 had no CAV3 mutations. The secondary loss of caveolin-3 in the sarcolemma may contribute to the muscle phenotype. Moreover, serum CK elevation may be a good laboratory marker for diagnosis of lipodystrophy patients with PTRF mutations.



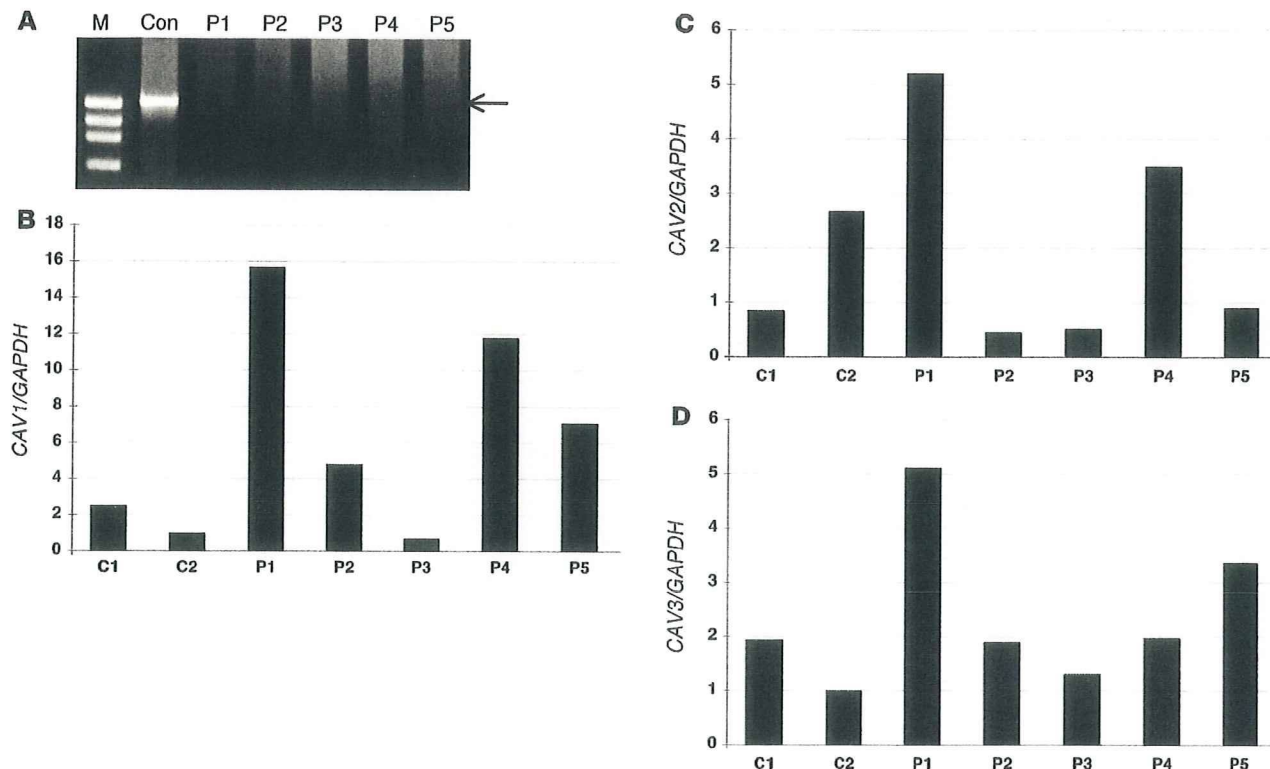
**Figure 3**

Loss of PTRF is associated with deficiency and mislocalization of caveolins in muscle. (A) In control muscle, PTRF was clearly seen in sarcolemma as strongly staining blood vessels. Caveolin-3 (Cav3) was clearly visible at sarcolemma, and caveolin-1 and -2 stained intramuscular blood vessels. The muscle of P4 was negative for PTRF. Membrane staining of caveolin-3 was reduced with increased cytoplasmic staining, and caveolin-1 and -2 were barely detectable. Immunoreactivity of nNOS varied between muscle fibers, but was not markedly different between control and patient muscle. Scale bar: 50  $\mu$ m. (B) Immunoblotting analysis of skeletal muscles. 3T3 cells were used as a positive control. PTRF and caveolin-2 were seen only in the muscles of 2 control subjects and in 3T3 cells, and were barely detectable in the muscles of P1–P5. The bands for caveolin-3 and nNOS were variably seen. (C and D) Quantification of immunoreactive bands was performed by densitometric analysis and normalized with MHC. In P1–P5, relative amounts of caveolin-3 decreased compared with control subjects (C), whereas nNOS amounts varied (D).

Caveolin-3 was previously reported to have an important role in inhibition of myostatin signaling by suppressing activation of its type I receptor. In mutant *Cav3* transgenic mice, loss of caveolin-3 causes muscular atrophy with increased p-Smad2, and this muscle atrophy can be rescued by myostatin inhibition (11). Consistent with the secondary reduction of caveolin-3, skeletal muscles from P1–P5 showed increased amounts of p-Smad2/3. Unexpectedly, however, muscle hypertrophy was seen in these patients.

The Akt pathway, when activated, is known to promote protein synthesis, stimulate muscle hypertrophy, and inhibit atrophy-related gene expression by phosphorylating FoxO transcription factors (12). This pathway is also known to play a pivotal role

in the regulation of glucose transport and glycogen synthesis in skeletal muscle cells. Akt is activated by insulin, various growth factors, nutrients, and exercise, whereas it is negatively regulated by myostatin and cytokines. Akt is phosphorylated at T308 by phosphoinositide-dependent kinase and at S473 by mammalian target of rapamycin in association with rictor. The increase in phosphorylated Akt in the muscle of P1–P5 may explain, at least in part, the muscle hypertrophy observed. Akt pathway activation might be associated with the metabolic complications observed in P1–P5. However, the upregulation of myostatin observed is contradictory to the established knowledge on muscle hypertrophy. This would be worthwhile to investigate in future studies, in order to



**Figure 4** mRNA expression of PTRF in skeletal muscle, and quantitative RT-PCR of mRNAs for caveolins. (A) RT-PCR analysis revealed a single band for *PTRF* mRNA (arrow) in a control subject, but no detectable product was seen in P1–P5. M, marker. (B–D) By quantitative RT-PCR, mRNA for *CAV1*, *CAV2*, and *CAV3* normalized with *GAPDH* expression was not decreased in P1–P5.

elucidate the role of PTRF deficiency in muscle hypertrophy and related signaling pathway.

In addition to lipodystrophy and muscular dystrophy, P1–P5 had various other symptoms, whose association to *PTRF* mutation might be difficult to ascertain at this time. For example, 2 of 5 patients had arrhythmia. Although we could not examine the expression of caveolins in cardiac muscle, this cardiac abnormality may be caused by secondary deficiency of caveolins in heart, as cardiac involvement was previously reported in patients with *CAV3* mutations and in mutant mice with double knockout of *Cav1* and *Cav3* (29–33).

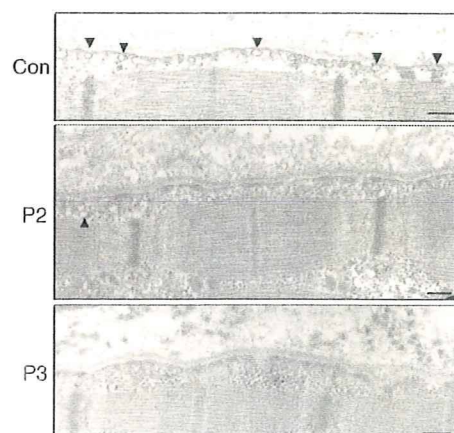
Remarkable reduction in expression of caveolin-1 and -2 with decreased caveolae density was observed in vascular endothelial cells in P1–P5. There was no obvious symptom related to vascular endothelial blood vessels in the patients; however, further careful investigation is necessary in order to determine the involvement of endothelial cells, which was observed in *Cav1* knockout mice (34). The severe constipation and esophageal dilatation observed in the patients might be associated with dysfunction of caveolin-1 in smooth muscle cells, as *Cav1* knockout mice had alteration of

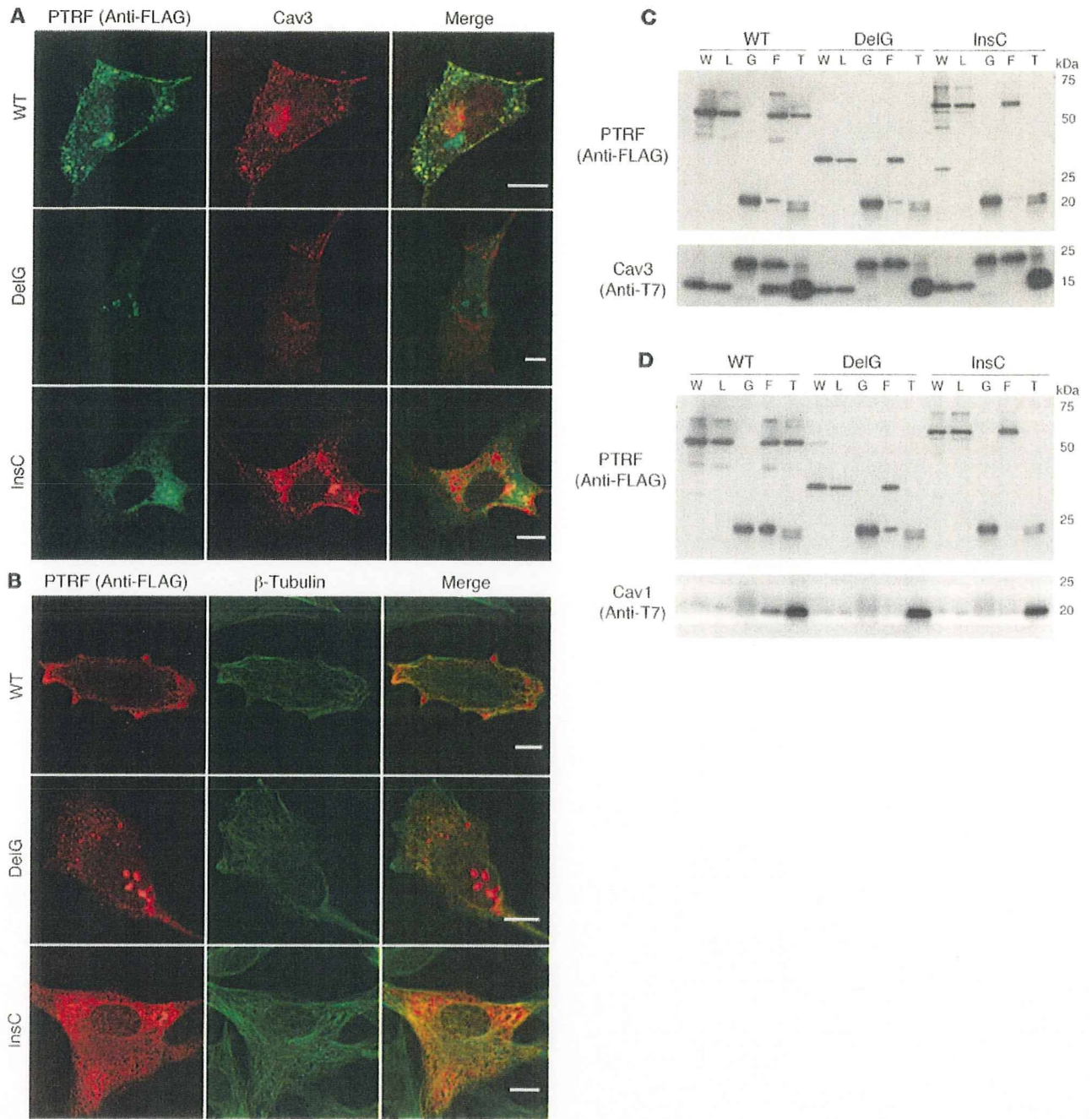
smooth muscles and interstitial cells of Cajal, the pacemaker cells of the muscle layers of the gastrointestinal tract (35).

Caveolae was previously suggested to have a role in the internalization of growth hormone in vitro (36). The acromegaloid features, accelerated bone age, or abnormal growth hormone activity observed in 3 patients in the present study might be associated with reduced caveolae formation. Recurrent pneumonia and transient immunodeficiency observed in 2 patients were also noted, although the pathomechanisms are still unknown. Further detailed studies are needed to elucidate the roles of PTRF; however,

**Figure 5**

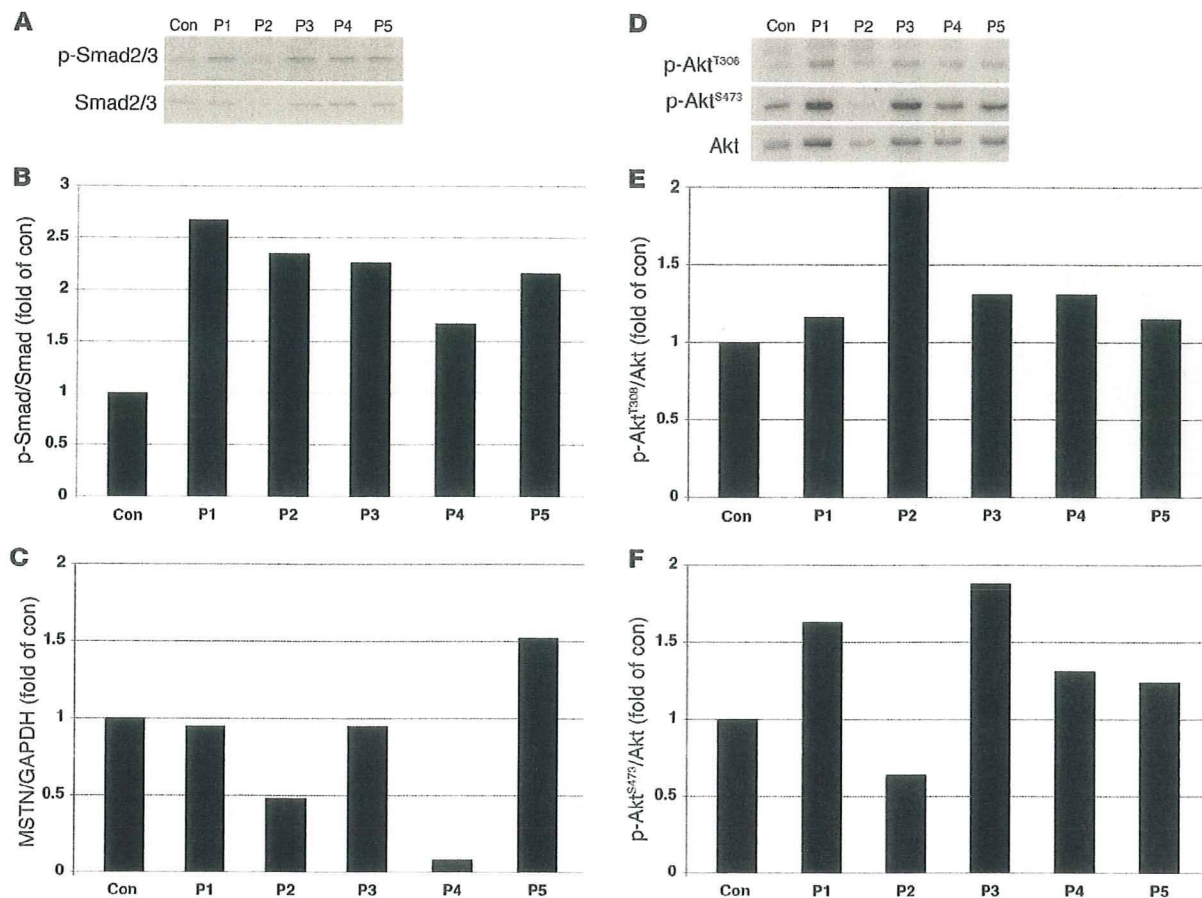
Reduced caveolae formation in skeletal muscle, as assessed by electron microscopy. In control muscle, an abundance of caveolae (arrowheads) was observed close to the plasma membrane. Plasma membrane of muscle fibers from P2 and P3 was nearly flat, and caveolae density was greatly reduced compared with that of control muscle. Only a few caveolae were seen in P2. Scale bars: 200 nm.





**Figure 6**

Altered localization of mutant PTRF in C2C12 cells and reduced binding ability to caveolins. C2C12 myoblasts were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696\_697insC) PTRF cDNA and T7-tagged human caveolin-3. (A and B) WT PTRF stained by anti-FLAG antibody colocalized with caveolin-3 at the cell membrane. The deletion mutant accumulated in the nucleus, and the insertion mutant was seen in cytoplasm. (A) Membrane staining of caveolin-3 was decreased and was not colocalized with mutant PTRF. (B) The PTRF insertion mutant clearly colocalized with  $\beta$ -tubulin. Scale bars: 10  $\mu$ m. (C and D) COS-7 cells were cotransfected with FLAG-tagged WT or mutant PTRF cDNA and T7-tagged human caveolin-3 (C) or caveolin-1 (D). The PTRF deletion mutant showed smaller molecular weight (estimated 30 kDa), and no immunoprecipitated protein was detected for FLAG or T7 antibodies. The PTRF insertion mutant showed slightly larger molecular weight, and amounts of coimmunoprecipitated proteins were greatly reduced. W, whole homogenate; L, cell lysate, G, control IgG; F, anti-FLAG; T, anti-T7.



**Figure 7**

Increased p-Smad2 and p-Akt in P1–P5 skeletal muscle. (A–C) Immunoblotting analysis of Smad2/3 and p-Smad2/3<sup>S423/425</sup> (A) and densitometric analysis (B) showed increased p-Smad2/3 in P1–P5 compared with control muscle, with variable mRNA expression levels of myostatin (MSTN; C). (D–F) Immunoblotting analysis of p-Akt<sup>T308</sup> and p-Akt<sup>S473</sup>. Total Akt (D) and densitometric analysis (E and F) showed increased amounts of p-Akt in all patients except for p-Akt<sup>S473</sup> in P2.

most clinical features observed in P1–P5 are likely to be explained by secondary reduction of caveolae and deficiency of caveolins.

Previously, Rajab et al. reported 10 of 17 patients with congenital generalized lipodystrophy unlinked to the loci of known CGL genes (37). The patients showed reduced exercise tolerance, percussion myoedema, cardiac hypertrophy, and arrhythmias. None of these patients had insulin resistance or early endocrine abnormalities (37). Ghanem also reported myoedema in a patient with Berardinelli-Seip lipodystrophy (38). Very recently, Simha et al. described CGL patients with muscle weakness and cervical spine instability (39). Because muscle involvement of these patients is similar to that of P1–P5 in the present study, *PTRF* mutations may not be rare in CGL patients.

This entity of generalized lipodystrophy with muscular dystrophy – which we believe to be novel – seems to represent a complicated disorder, as the occurrence of other symptoms could not readily be explained. Collection of detailed clinical information would therefore be essential in order to understand the precise function of *PTRF*.

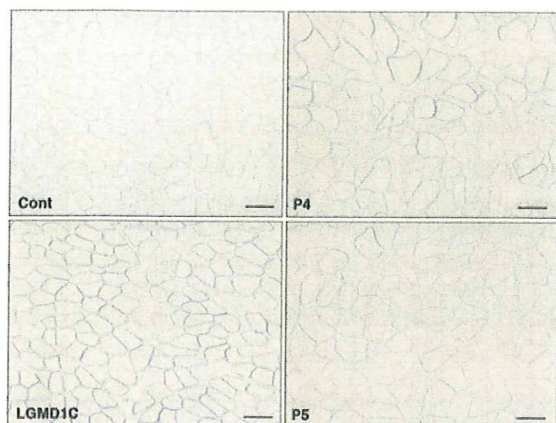
## Methods

**Clinical materials.** All clinical materials used in this study were obtained for diagnostic purposes and with informed consent. Subjects were

selected from 2,745 muscular dystrophy specimens kept in the muscle repository of the National Center of Neurology and Psychiatry. The present studies were approved by the Ethical Committee at National Center of Neurology and Psychiatry.

**Mutation screening and haplotype analysis.** Genomic DNA was isolated from peripheral lymphocytes or muscles using standard techniques. All exons and their flanking intronic regions of *PTRF*, *CAV3*, *LMNA*, *AGPAT2*, *BSCL2*, *CAVI*, *PPARG*, *AKT2*, and *ZMPSTE24* were directly sequenced using genomic DNA from all patients using an ABI PRISM 3100 automated sequencer (Applied Biosystems). Primer sequences are listed in Supplemental Table 3. To confirm the compound heterozygosity in P5, the PCR product was cloned and sequenced. In order to determine the frequency of the mutations in *PTRF*, we performed enzyme digestion of PCR products from 200 Japanese control subjects using *Hpy*188III (New England Biolabs) for c.696\_697insC and *Taq*I (New England Biolabs) for c.525delG. *Mbo*II (New England Biolabs) was used for enzyme digestion of PCR products to detect the c.1138G>A substitution in *BSCL2*.

For haplotype analysis, we used 6 SNPs (rs2062213, rs8070945, rs963988, rs963987, rs963986, and rs9252) within *PTRF*. PCR products were analyzed by direct sequencing or enzyme digestion using *Mae*III (Boehringer Mannheim). We also identified a novel 9-bp insertion polymorphism at the



**Figure 8**  
NDP activity assay. NDP activity was variable between muscle fibers, and was slightly increased in the muscle of P4, P5, and a LGMD1C patient with *CAV3* mutation compared with an age-matched control subject. Scale bars: 100  $\mu$ m.

3' noncoding region, and its frequency was calculated by PCR amplification using 50 Japanese control individuals. We also examined 2 microsatellite markers, STS-W93348 and D17S1185, the closest markers to *PTRF*. PCR product size was analyzed by GeneMapper using ABI 310 automated sequencer (Applied Biosystems).

**Histochemical analysis.** Biopsied muscle specimens were flash frozen with isopentane cooled in liquid nitrogen. Serial 10- $\mu$ m-thick frozen sections were analyzed with 20 kinds of histochemical staining, including H&E, modified Gomori trichrome, NADH-tetrazolium reductase, and oil red O. The NDP activity assay was performed to examine nNOS activity of each muscle fiber, as described previously (40). In brief, 10- $\mu$ m-thick frozen sections were fixed with 4% paraformaldehyde in PBS for 2 hours at 4°C. After a brief rinse with PBS, sections were incubated with 0.2% Triton X-100 in PBS for 20 minutes at 37°C. The reaction was performed for 1 hour in a dark, humidified chamber at 37°C in 0.2% Triton X-100, 0.2 mM NADPH, and 0.16 mg/ml nitro blue tetrazolium. The reaction was terminated by washing with water. We examined 6 age-matched controls and 2 LGMD1C patients with *CAV3* mutations (p.R27G and p.E33K).

**Immunohistochemical analysis.** Immunostaining was performed using standard methods. Serial 6- $\mu$ m-thick frozen muscle sections were fixed in cold acetone for 5 minutes. After blocking with normal goat serum, sections were incubated with the primary antibodies for 2 hours at 37°C. We used antibodies against PTRF (A301-269A and A301-271A; BETHYL Laboratories), caveolin-1 (BD Biosciences), caveolin-2 (Sigma-Aldrich), caveolin-3 (BD Biosciences), and nNOS (BD Biosciences). Rabbit anti-PTRF antibody of A301-269A recognizes residue from 125 to 175, and A301-271A was raised against residue 238 and 288 of human PTRF (Figure 1B). In order to exclude other diagnosable muscular dystrophies, we used antibodies for dystrophin (DYS1, DYS2, and DYS3; Novocastra);  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans (Novocastra);  $\alpha$ -dystroglycan (Upstate Biotech);  $\beta$ -dystroglycan (Novocastra); dysferlin (Novocastra); emerin (Novocastra); merosin (Chemicon); and collagen VI (ICN Biomedicals). After 6 rinses with PBS, sections were incubated with secondary antibodies of Alexa Fluor 488- or Alexa Fluor 568-labeled goat anti-mouse or -rabbit antibodies at room temperature for 45 minutes.

**Immunoblotting analysis.** Immunoblotting analysis was performed according to standard methods. Frozen muscle specimens were homogenized in SDS sample buffer and centrifuged at 15,000 g for 5 minutes. Protein (20  $\mu$ g) from each sample was loaded on 12% SDS-polyacrylamide gels and transferred to

PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in PBS and immunoreacted with antibodies to PTRF (A301-269A and A301-271A), caveolin-2, caveolin-3, nNOS, Smad2/3 (Cell Signaling Technology), p-Smad2/3<sup>S423/425</sup> (Santa Cruz Biotechnology Inc.), Akt (Cell Signaling Technology), p-Akt<sup>T308</sup> (Cell Signaling Technology), and p-Akt<sup>S473</sup> (Cell Signaling Technology) overnight at 4°C. After washing in PBS containing 0.1% Tween-20, the membrane was incubated with horseradish peroxidase-labeled secondary antibody and visualized with ECL (Amersham Pharmacia Biotech). Data were analyzed using LAS-1000 chemiluminescence imaging system (Fujifilm). Quantification of immunoreactive bands was performed by densitometric analysis using Quantity One (PDI), and protein amounts for caveolin-3 and nNOS were normalized by the intensity of MHC. The ratio of p-Smad2/3 and p-Akt, to Smad and Akt, respectively, was also calculated.

**Electron microscopy.** Muscle specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 hours, samples were embedded in epoxy resin. Semithin sections (1  $\mu$ m thick) were stained with toluidine blue. Ultrathin sections 50 nm thick were stained with uranyl acetate and lead citrate, then examined under H-600 transmission electron microscope (Hitachi) at 75 kV.

**RT-PCR.** Total RNA was extracted from biopsied skeletal muscles using TRIzol (Invitrogen), and RT-PCR was performed using SuperScript III (Invitrogen) with random hexamer according to the manufacturer's instructions. Primers for each gene were located on different exons or directly spanning exon-exon boundaries of the genomic sequence in order to minimize amplification from any contaminating genomic DNA. After performing preliminary gradient PCR assays, the optimal annealing temperature for all the primer pairs was determined in order to generate the lowest Ct value as well as a sharp melting peak, with no amplification of nonspecific products or primer-dimer artifacts. Quantitative RT-PCR was performed to compare the mRNA expression of caveolin-1, caveolin-2, caveolin-3, and myostatin using Rotor-Gene 6000 according to the manufacturer's instructions (Corbett Life Science). The reactions were performed in reference to the GAPDH. We used 4 points consisting of 10-fold serial dilution using each primer set to build the standard curve. The PCR reaction (50 cycles) was followed by a melting curve analysis, ranging from 72°C to 95°C, with temperature increasing steps of 0.5°C every 10 seconds. Baseline and threshold values were automatically determined and analyzed.  $R^2$  values exceeded 0.97. The 2-standard curve method was used to determine the relative expression ratio of the target gene in the patient samples versus the control sample, with reference to GAPDH expression.

**Cell culture and transfection.** COS-7 and C2C12 cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum. Full-length PTRF and caveolin-1 and -3 were amplified using total RNA from control human muscle and cloned into the pGEM-T-easy vector (Promega). The PTRF mutants c.525delG and c.696\_697insC were generated using appropriate primers. All primer sequences are shown in Supplemental Table 3.

**Immunocytochemistry.** COS-7 and C2C12 myoblasts were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696\_697insC) PTRF cDNA and T7-tagged human caveolin-3 using FuGENE6 (Roche). Transfectants were fixed for 30 minutes in 2% paraformaldehyde or 100% methanol, then permeabilized in 0.1% Triton X-100 for 10 minutes. Polyclonal antibodies to FLAG (Sigma-Aldrich) with caveolin-3 (BD Biosciences) or FLAG with  $\beta$ -tubulin (Calbiochem) were applied for double staining.

**Immunoprecipitation.** COS-7 cells were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696\_697insC) PTRF cDNA and T7-tagged human caveolin-1 or caveolin-3 using FuGENE6 (Roche). The sequences of all constructs were verified with DNA sequencing using ABI PRISM 310 (Applied Biosystems). After 48 hours, the lysates from transfectants were



solubilized with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and Complete-mini EDTA-free proteinase inhibitors (Roche) (9). The solubilized lysate precleared with Protein G Sepharose (GE Healthcare) was incubated with anti-FLAG (M2; Sigma-Aldrich) and anti-T7 (Novagen) antibodies. Immunoprecipitated proteins were dissociated from beads by boiling in sample buffer and were resolved by SDS-PAGE. Immunoblotting was performed using standard techniques.

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Case report

## A novel *POMT2* mutation causes mild congenital muscular dystrophy with normal brain MRI

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### Abstract

We report a patient harboring a novel homozygous mutation of c.604T > G (p.F202V) in *POMT2*. He showed delayed psychomotor development but acquired the ability to walk at the age of 3 years and 10 months. His brain MRI was normal. No ocular abnormalities were seen. Biopsied skeletal muscle revealed markedly decreased but still detectable glycosylated forms of alpha-dystroglycan ( $\alpha$ -DG). Our results indicate that mutations in *POMT2* can cause a wide spectrum of clinical phenotypes as observed in other genes associated with  $\alpha$ -dystroglycanopathy. Presence of small amounts of partly glycosylated  $\alpha$ -DG may have a role in reducing the clinical symptoms of  $\alpha$ -dystroglycanopathy.

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**Keywords:** *POMT2*;  $\alpha$ -Dystroglycan;  $\alpha$ -Dystroglycanopathy; Congenital muscular dystrophy; Limb girdle muscular dystrophy; Brain MRI

### 1. Introduction

Alpha-dystroglycan ( $\alpha$ -DG) is a surface membrane protein that links extracellular basal lamina and intracellular cytoskeleton.  $\alpha$ -DG is a highly glycosylated protein mainly composed of unique *O*-mannosyl glycans. Reduced/altered glycosylation of  $\alpha$ -DG causes a wide variety of muscular dystrophies including Walker–Warburg syndrome (WWS), muscle-eye-brain disease (MEB), Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophies type 1C and type 1D, and limb girdle muscular dystro-

phies (LGMD) type 2I, 2K to 2N. They are collectively called alpha-dystroglycanopathies ( $\alpha$ -DGP). So far, six causative genes for  $\alpha$ -DGP have been identified including *protein-O-mannosyl transferase 1 and 2 (POMT1 and POMT2)*, *protein O-mannose  $\beta$ -1,2-N-acetylglucosaminyltransferase (POMGnT1)*, *fukutin (FKTN)*, *fukutin-related protein (FKRP)*, and *acetylglucosaminyl transferase-like protein (LARGE)*. Here we report a mild congenital muscular dystrophy patient associated with a novel homozygous mutation in *POMT2*.

### 2. Case report

A 4-year-old Japanese boy, the only child from healthy consanguineous parents, was delivered uneventfully at full term. During few days after birth, he was

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low spirited and showed sucking weakness. Floppiness was not prominent but serum CK levels were markedly elevated up to 33,000 IU/l (normal < 70). His condition was improved within 2 weeks, but serum CK levels were persistently higher than 1000 IU/l. His motor milestones were delayed and he could control his head at 5 months of age. At 6-month-old, he could not sit without support, and muscle weakness and atrophy were noticed in lower limbs. Deep tendon reflexes were normal. No high arched palate or macroglossia were seen. Enjoji Scale of Infant Analytical Development (ESID) at his age of 7 months revealed mild delay in body movement (developmental age was 4 months, expression of language: 5 months), and his DQ was 83. Brain computed tomography (CT) revealed no definite abnormalities. Nerve conduction study was normal. His motor functions developed gradually and he was able to walk without support at 3 years and 10 months old. Gowers' sign was positive. Mild calf hypertrophy was seen with no joint contractures (Fig. 1A). Deep tendon reflexes were normal except for diminished Achilles tendon reflexes. ESID performed at his age of 3 years and 11 months showed general developmental delay (body movement:

15 months, hand movement: 24 months, activity of daily living: 27 months, personal relations: 24 months, expression of language: 18 months, and comprehension of language: 24 months), and his DQ was 47. Brain magnetic resonance imaging at 4 years and 1-month-old revealed no notable anomaly or cortical dysplasia (Fig. 1B). Detailed ophthalmological examinations revealed no abnormalities. No cardiac involvement was detected by chest X-ray, electrocardiogram, and echocardiography.

Muscle biopsy taken at 7 months of age with informed consent showed dystrophic changes with scattered necrotic and regenerating fibers and mild endomyxial fibrosis (Fig. 2A). No inflammatory changes were seen. On immunohistochemistry, glycosylated forms of  $\alpha$ -DG detected by VIA4-1 antibody (Upstate Biotechnology, NY) was markedly reduced in the sarcolemma, while immunoreactions for the core region of  $\alpha$ -DG using GT20ADG antibody [1] (data not shown) and for  $\beta$ -DG (43DAG1/8D5; Novocastra Laboratories, UK) was well preserved (Fig. 2A). On immunoblotting analysis, faint, broad band of around 140 kDa in size was detected by VIA4-1, whereas GT20ADG recognized a band of around 90 kDa in size. Laminin overlay assay showed barely detectable binding product (Fig. 2B). These results suggested altered glycosylation of  $\alpha$ -DG in the muscle.

We performed mutation screening in all six causative genes for  $\alpha$ -DGP. Genomic DNA was extracted from peripheral lymphocytes using standard technique after informed consent. Primer sequences we used are available on request. All exons and their flanking intronic regions were directly sequenced by ABI PRISM 3100 (PE Applied Biosystems, CA). We identified a homozygous missense mutation of c.604T > G (p.F202V) in exon 5 of *POMT2* (Fig. 1C), which is not described in previous publications [3–8] and the mutation database (<http://www.dmd.nl/>).

The protein *O*-mannosyltransferase (*POMT*) activity was measured as previously described [2]. Mutant *POMT2* (F202V) co-expressed with *POMT1* in COS cells showed barely detectable *POMT* activity (data not shown).

### 3. Discussion

*POMT2* is the gene encoding an enzyme for protein *O*-mannosylation, and it is required to form a complex with *POMT1* for the enzyme activity [2]. Recently, some patients with mutations in *POMT2* have been reported [3–8]. Most patients showed floppiness at birth, delayed psychomotor development, congenital muscular dystrophy, and severe mental retardation with or without ocular involvement. Brain anomalies are prominent including hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, and cerebel-

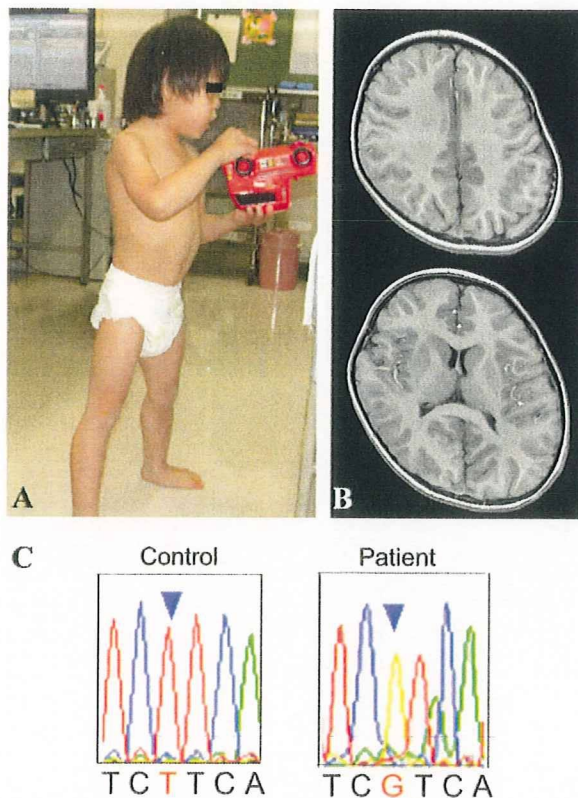


Fig. 1. (A) The patient can stand and walk with no support. Minimal calf hypertrophy is seen. (B) T2 weighted brain magnetic resonance imaging shows no obvious brain anomaly, cortical dysplasia, or white matter changes. (C) Sequence analysis of *POMT2* revealed a homozygous mutation at c.604T > G in exon 5.

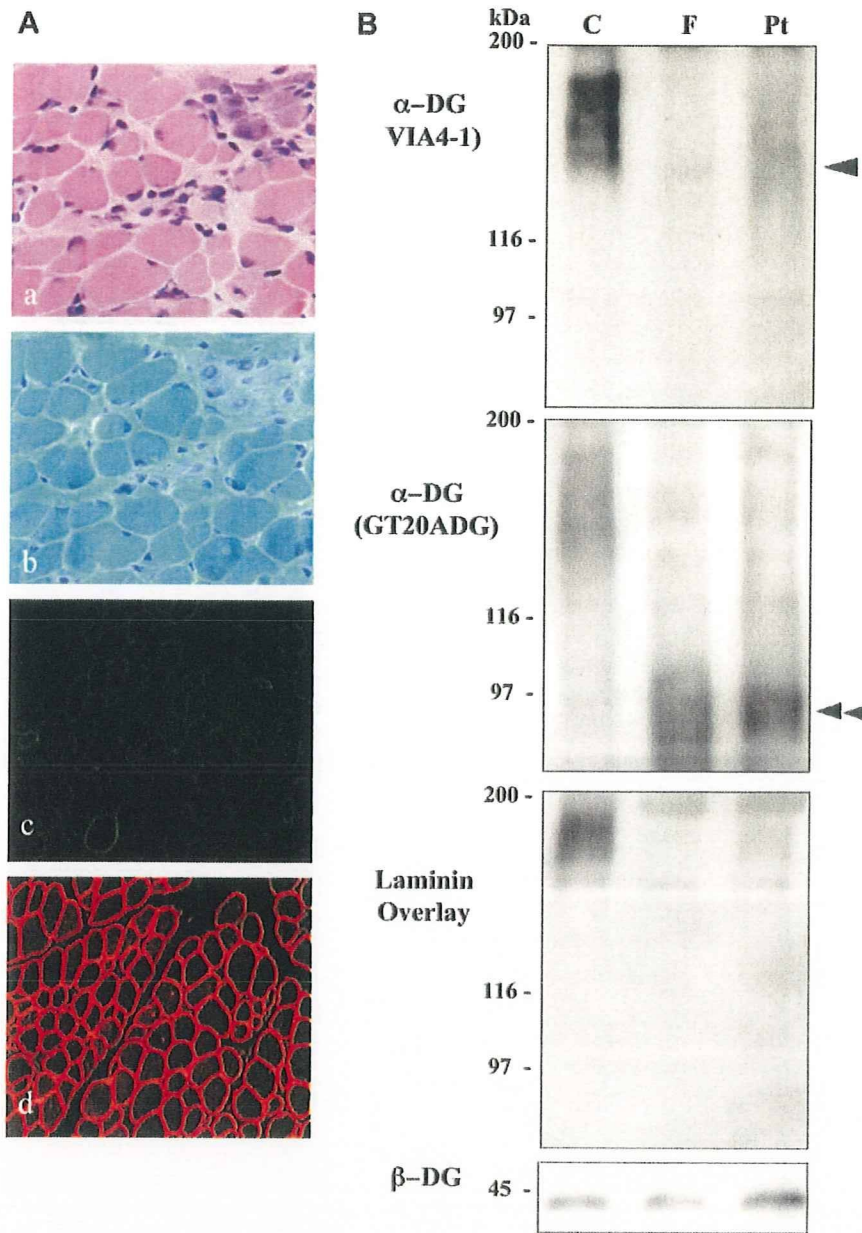


Fig. 2. (A) Histological analysis. On Hematoxylin and eosin (a) and modified Gomori-trichrome (b) staining, variation in fiber size and scattered necrotic and regenerating fibers are seen. Immunohistochemical analysis using antibodies VIA4-1 (c), which recognize heavily glycosylated form of  $\alpha$ -dystroglycan ( $\alpha$ -DG), showed greatly reduced sarcolemmal staining in patient, but well preserved immunoreactivities of  $\beta$ -DG (d) is seen. Bar = 50  $\mu$ m. (B) Immunoblotting analysis. Immunoblotting analysis using antibodies of VIA4-1, GT20ADG for  $\alpha$ -dystroglycan ( $\alpha$ -DG) and laminin overlay assay are performed using skeletal muscle from control (C), Fukuyama-type congenital muscular dystrophy (FCMD; F), and the patient (Pt). VIA4-1 recognizes a broad band about 156 kDa in size in control, and approximately 90 kDa in FCMD. In the patient muscle, reduced in size and amount compared with control was observed. GT20ADG revealed bands at approximately 90 kDa in both the patient and FCMD muscles. Laminin overlay assay shows barely detectable band in both the patient and FCMD.

lar hypoplasia [3–5]. In contrast, the patient reported here shows milder clinical features. Although his psychomotor milestones were delayed, he achieved independent ambulation with no marked brain malformation and ocular involvement. His clinical phenotype was intermediate between congenital muscular dystrophy

and limb girdle muscular dystrophy. Milder clinical features with mutations in *POMT2* have been recently reported and designated as limb girdle muscular dystrophy type 2N [6,7]. Mutations in *POMT2* can cause wide spectrum of clinical phenotypes from Walker–Warburg syndrome to limb girdle muscular dystrophy (LGMD),

as demonstrated in patients with *FKRP*, *FKTN*, or *POMT1* mutations.

Pathological changes of skeletal muscle also showed mild dystrophic changes consistent with clinical findings. Clinical and pathological severity may not be always correlated to the molecular mass of  $\alpha$ -DG [9]. However, some clinically milder patients with  $\alpha$ -DGP show reduced but positive glycosylated forms of  $\alpha$ -DG detected by the VIA4-1 antibody [10]. Preservation of partly glycosylated forms of  $\alpha$ -DG could contribute to the milder clinical phenotype of this patient.

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## Congenital myotonic dystrophy can show congenital fiber type disproportion pathology

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**Abstract** Congenital myotonic dystrophy (CDM) is associated with markedly expanded CTG repeats in *DMPK*. The presence of numerous immature fibers with peripheral halo is a characteristic feature of CDM muscles together with hypotrophy of type I fibers. Smaller type I fibers with no structural abnormality are a definitive criterion of congenital fiber type disproportion (CFTD). Nonetheless, we recently came across a patient who was genetically confirmed as CDM, but had been earlier diagnosed as CFTD when he was an infant. In this study, we performed clinical, pathological, and genetic analyses in infantile patients pathologically diagnosed as CFTD to evaluate CDM patients indistinguishable from CFTD. We examined CTG repeat expansion in *DMPK* in 28 infantile patients pathologically diagnosed as CFTD. Mutation screening of *ACTA1* and *TPM3* was performed, and we compared clinical and pathological findings of 20 CDM patients with those of the other cohorts. We identified four (14%) patients with CTG expansion in *DMPK*. *ACTA1* mutation was

identified in four (14%), and *TPM3* mutation was found in two (7%) patients. Fiber size disproportion was more prominent in patients with *ACTA1* or *TPM3* mutations as compared to CFTD patients with CTG expansion. A further three patients among 20 CDM patients showed pathological findings similar to CFTD. From our results, CDM should be excluded in CFTD patients.

**Keywords** Congenital myotonic dystrophy (CDM) · Congenital fiber type disproportion (CFTD) · *DMPK* · CTG expansion · *ACTA1* · *TPM3*

### Introduction

Congenital myotonic dystrophy (CDM; OMIM 160900) is caused by marked expansion of trinucleotide (CTG) repeat in the 3' untranslated region of the dystrophin myotonia protein kinase gene (*DMPK*; OMIM 605377) on chromosome 19q [1, 5, 9]. The CTG repeat in normal individuals varies from 5 to 35, whereas it expands to more than 1,000 repeats in CDM [7]. Typically, the mothers of CDM patients show clinical features of myotonic dystrophy which makes the diagnosis of CDM easier. Clinically, CDM patients show hypotrophy at birth, tented upper lip, facial muscle weakness, and neonatal respiratory insufficiency. Mental retardation becomes evident in later life. On muscle pathology, the presence of numerous immature fibers with peripheral halo is a characteristic feature together with increased number of fibers with centrally placed nuclei and hypotrophy of type I fibers, mimicking myotubular myopathy [6].

Smaller sized type I fibers as compared to type 2 fibers are a characteristic pathological feature of congenital fiber type disproportion (CFTD; OMIM 255310). CFTD is a

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