

## Mutations in *B3GALT6*, which Encodes a Glycosaminoglycan Linker Region Enzyme, Cause a Spectrum of Skeletal and Connective Tissue Disorders

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Proteoglycans (PGs) are a major component of the extracellular matrix in many tissues and function as structural and regulatory molecules. PGs are composed of core proteins and glycosaminoglycan (GAG) side chains. The biosynthesis of GAGs starts with the linker region that consists of four sugar residues and is followed by repeating disaccharide units. By exome sequencing, we found that *B3GALT6* encoding an enzyme involved in the biosynthesis of the GAG linker region is responsible for a severe skeletal dysplasia, spondyloepimetaphyseal dysplasia with joint laxity type 1 (SEMD-JL1). *B3GALT6* loss-of-function mutations were found in individuals with SEMD-JL1 from seven families. In a subsequent candidate gene study based on the phenotypic similarity, we found that *B3GALT6* is also responsible for a connective tissue disease, Ehlers-Danlos syndrome (progeroid form). Recessive loss-of-function mutations in *B3GALT6* result in a spectrum of disorders affecting a broad range of skeletal and connective tissues characterized by lax skin, muscle hypotonia, joint dislocation, and spinal deformity. The pleiotropic phenotypes of the disorders indicate that *B3GALT6* plays a critical role in a wide range of biological processes in various tissues, including skin, bone, cartilage, tendon, and ligament.

Skeletal dysplasias represent a vast collection of genetic disorders of the skeleton, currently divided into 40 groups.<sup>1</sup> Spondyloepimetaphyseal dysplasia (SEMD) is one group (group 13) of skeletal dysplasia that contains more than a dozen distinctive diseases. SEMD with joint laxity (SEMD-JL) is a subgroup of SEMD that consists of type 1 (SEMD-JL1 [MIM 271640]) and type 2 (SEMD-JL2 [MIM 603546]). SEMD-JL1 or SEMD-JL Beighton type is an autosomal-recessive disorder that shows mild craniofacial dysmorphism (prominent eye, blue sclera, long upper lip, small mandible with cleft palate) and spatulate finger with short nail.<sup>2</sup> The large joints of individuals with SEMD-JL1 are variably affected with hip dislocation, elbow contracture secondary to radial head dislocation, and clubfeet. Joint laxity is particularly prominent in the hands. Skeletal changes of SEMD-JL1 are characterized by moder-

ate platyspondyly with anterior projection of the vertebral bodies, hypoplastic ilia, and mild metaphyseal flaring.<sup>3</sup> Kyphoscoliosis progresses with age, leading to a short trunk, whereas platyspondyly become less conspicuous and the vertebral bodies appear squared in shape with age. Recently, dominant kinesin family member 22 (*KIF22* [MIM 603213]) mutations have been found in SEMD-JL2;<sup>4,5</sup> however, the genetic basis of SEMD-JL1 remains unknown.

To identify the SEMD-JL1-causing mutation, we performed whole-exome sequencing experiments. We recruited seven individuals with SEMD-JL1 from five unrelated Japanese families (F1–F5) and a Singapore/Japanese family (F6) (Table 1). One family (F1) had a pair of affected sibs (P1 and P2) from nonconsanguineous parents. Genomic DNA was extracted by standard procedures

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**Table 1. Clinical and Radiographic Findings of the Individuals with B3GALT6 Mutations**

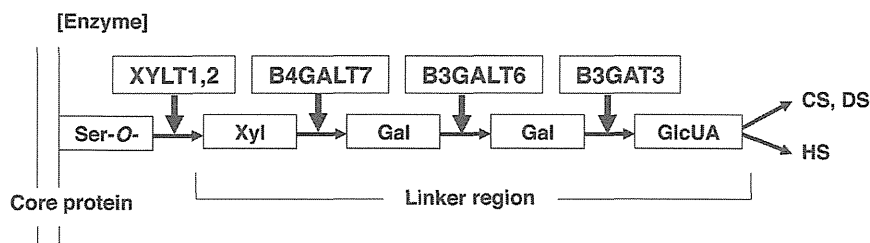
Subject ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Family ID	F1	F1	F2	F3	F4	F5	F6	F7	F8	F9	F9	F10
Clinical diagnosis	SEMD-JL1	SEMD-JL1	SEMD-JL1	SEMD-JL1	SEMD-JL1	SEMD-JL1	SEMD-JL1	SEMD-JL1	EDS-PF	EDS-PF	EDS-PF	EDS-PF
<b>General Information</b>												
Ethnicity	Japanese	Japanese	Japanese	Japanese	Japanese	Japanese	Japanese/ Singaporean	Vietnamese	Italian	Italian/ Canadian	Italian/ Canadian	Brazilian
Gender	M	M	F	M	F	F	M	M	M	F	F	F
Age	34 years	31 years	12 years, 7 months	6 years	5 years, 1 month	12 years	2 years, 9 months	34 years	8 months	7 years	1 month	5 years, 1 month
Gestational age	39 weeks, 2 days	full term	37 weeks	40 weeks, 1 day	39 weeks, 5 days	full term	39 weeks	full term	ND	36 weeks	37 weeks	39 weeks
Birth length (cm)	ND	ND	36	ND	43.1	42	43	(average)	ND	44	44	44
Birth weight (g)	ND	2,200	2,124	2,832	2,535	2,222	2,485	3,500	ND	2,097	2,790	3,300
<b>Clinical Features</b>												
Height (cm) (SD) <sup>a</sup>	127.7 (-7.4)	130 (-7.0)	88.8 (-10.7)	94 (-4.0)	90 (-4.0)	118.4 (-5.1)	78.2 (-4.0)	118 (-9.1)	66 (-1.6)	90 (-6.8)	45 (-3.7)	81 (-5.9)
Weight (kg) (SD) <sup>a</sup>	40.3 (-2.2)	36.9 (-2.5)	13.2 (-3.7)	15.4 (-1.5)	14.4 (-1.3)	23.2 (-2.0)	10.6 (-1.9)	28 (-3.3)	5.65 (-3.0)	13.9 (-2.2)	2.65 (-2.8)	8.5 (-8.4)
<b>Craniofacial</b>												
Flat face with prominent forehead	ND	ND	+	+	+	+	+	-	+	+	+	+
Prominent eyes, proptosis	ND	ND	+	-	-	+	+	-	+	+	+	+
Blue sclerae	ND	ND	+	+	+	-	+	-	+	+	+	-
Long upper lip	ND	ND	-	+	+	-	+	+	+	+	+	-
Micrognathia	ND	ND	+	+	+	+	-	+	-	-	-	-
Cleft palate	ND	ND	-	-	-	-	-	-	-	-	-	+
<b>Musculoskeletal</b>												
Kyphoscoliosis <sup>b</sup>	+ (7 months)	+ (1.2 years)	+ (8 months)	+ (infancy)	+ (2 years)	+ (3 months)	+ (8 months)	+ (1 year)	+ (6 months)	++ (prenatal)	++ (prenatal)	++ (2 years)
Spatulate finger	-	ND	+	+	+	+	-	-	+	+	+	-
Finger laxity	ND	ND	+	+	-	-	+	-	++	+	+	+
Large joint laxity	ND	ND	+	+	-	-	+	-	++	++	++	+
Restricted elbow movement	+	ND	+	+	+	-	-	+	+	+	+	+
Hand contracture	-	-	-	-	-	+	-	-	-	+	+	-

(Continued on next page)

**Table 1. Continued**

Subject ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Hip dislocation	-	-	-	+	-	+	-	-	-	+	+	+
Clubfeet	-	-	+	-	-	-	+	-	-	+	+	-
Muscular hypotonia	-	-	+	-	-	-	-	-	++	++	++	++
<b>Skin and Hair</b>												
Doughy skin	ND	ND	+	-	-	-	+	-	++	+	+	+
Hyperextensibility	ND	ND	+	-	-	-	+	-	++	+	+	-
Cutis laxa	ND	ND	-	-	-	-	-	-	+	+	-	+
Sparse hair	ND	ND	-	-	-	-	-	-	+	+	+	-
<b>Others</b>			MR, DD				camptodactyly			DD		pectus excavatum
<b>Radiological Features</b>												
Platyspondyly	+ <sup>c</sup>	+ <sup>c</sup>	+ <sup>c</sup>	+	+	+	+	+	+	+	+	+
Anterior beak of vertebral body <sup>b</sup>	+	+	- (4 years)	- (5 years)	+	+	+	-	+	+	+	+
Short ilia	+	+	+	+	+	+	+	+	+	+	+	+
Prominent lesser trochanter	+	+	+	-	+	+	+	+	+	+	+	+
Metaphyseal flaring	+	+	+	+	+	+	+	+	+	-	+	+
Epiphyseal dysplasia of femoral head	-	-	-	+	-	+	-	-	-	-	+	+
Elbow malalignment	ND	ND	+	+	+	+	+	+	+	+	+	+
Advanced carpal ossification <sup>b</sup>	- (9 years)	ND	- (12 years)	+	+	+	+	ND	+	- (7 years)	-	- (5 years)
Carpal fusion	ND	ND	+	-	-	-	-	-	-	-	-	-
Metacarpal shortening	ND	ND	+	+	+	+	+	+	-	-	+	-
Overtubulation	-	-	-	-	-	-	-	-	+	+	+	+

Abbreviations are as follows: SEMD-JL1, spondyloepimetaphyseal dysplasia with joint laxity type 1; EDS-PF, Ehlers-Danlos syndrome, progeroid form; ND, no data; MR, mitral regurgitation; DD, developmental delay.  
<sup>a</sup>At last presentation.  
<sup>b</sup>Age at medical attention provided in parentheses.  
<sup>c</sup>Absent at age 20 years in P1 and P2 and at age 12 years in P3.



**Figure 1. Enzymes Involved in Biosynthesis of the Glycosaminoglycan Linker Region and Summary Features of Diseases Caused by Their Defects Based on a Conventional Concept for the Diseases**

The biosyntheses of GAGs start with the formation of a common tetrasaccharide linker sequence covalently attached to the core protein. The linker region synthesis involves a single linear pathway composed of four successive steps catalyzed by distinctive enzymes. Abbreviations are as follows: XYLT,  $\beta$ -xylosyltransferase; B4GALT7, xylosylprotein  $\beta$ 1,4-galactosyltransferase, polypeptide 7 ( $\beta$ 1,4-galactosyltransferase-I); B3GALT6, UDP-Gal,  $\beta$ Gal  $\beta$ 1,3-galactosyltransferase polypeptide 6 ( $\beta$ 1,3-galactosyltransferase-II); B3GAT3,  $\beta$ -1,3-glucuronosyltransferase 3 (glucuronosyltransferase I); Ser-O, the serine residue of the GAG attachment site on the proteoglycan core protein;

[Disease]	EDS, progeroid form	SEMD-JL1	Larsen-like syndrome, B3GAT3 type
[Clinical feature]			
craniofacial dysmorphism	+/-	+	+
skeletal dysplasia	+	++	+/-
skin	++	(-)	(-)
heart	(-)	(-)	+
muscle	+	(-)	?

Xyl, xylose; Gal, galactose; GlcUA, D-glucuronic acid; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; EDS, Ehlers-Danlos syndrome; SEMD-JL1, spondyloepimetaphyseal dysplasia with joint laxity type 1.

from peripheral blood, saliva, or Epstein-Barr virus-immortalized lymphocyte of the individuals with SEMD-JL1 and/or their parents after informed consent. The study was approved by the ethical committee of RIKEN and participating institutions. We captured the exomes of the seven subjects as previously described.<sup>6,7</sup> In brief, we sheared genomic DNA (3  $\mu$ g) by Covaris S2 system (Covaris) and processed with a SureSelect All Exon V4 kit (Agilent Technologies). We sequenced DNAs captured by the kit with HiSeq 2000 (Illumina) with 101 base pair-end reads. We performed the image analysis and base calling by HiSeq Control Software/Real Time Analysis and CASAVA1.8.2 (Illumina) and mapped the sequences to human genome hg19 by Novoalign. We processed the aligned reads by Picard to remove PCR duplicate. The mean depth of coverage for reads was 132.8 $\times$ , and, on average, 91.0% of targeted bases had sufficient coverage (20 $\times$  coverage) and quality for variant calling (Table S1 available online). The variants were called by Genome Analysis Toolkit 1.5-21 (GATK) with the best practice variant detection with the GATK v.3 and annotated by ANNOVAR (2012 February 23).

Based on the hypothesis that SEMD-JL1 is inherited in an autosomal-recessive fashion, we filtered variants with the script created by BITS (Tokyo, Japan) according to following conditions: (1) variants registered in ESP5400, (2) variants found in our in-house controls ( $n = 274$ ), (3) synonymous changes, (4) rare variants registered in dbSNP build 135 (MAF < 0.01), and (5) variants associated with segmental duplication. After combining variants selected by the homozygous mutation model and the compound heterozygous mutation model, we selected genes shared by individuals from three or more families. The analysis of the next-generation sequencing identified possible compound heterozygous variants in *B3GALT6* in individuals from three families (Table S2). In addition, two other subjects had possible causal heterozygous variants of *B3GALT6*.

*B3GALT6* (RefSeq accession number NM\_080605.3) is a single-exon gene on chromosome 1p36.33. It encodes UDP-Gal: $\beta$ Gal  $\beta$ 1,3-galactosyltransferase polypeptide 6 (or galactosyltransferase-II: GalT-II), an enzyme involved in the biosynthesis of the glycosaminoglycan (GAG) linker region.<sup>8</sup> The biosyntheses of dermatan sulfate (DS), chondroitin sulfate (CS), and heparin/heparan sulfate (HS) GAGs start with the formation of a tetrasaccharide linker sequence, glucuronic acid- $\beta$ 1-3-galactose- $\beta$ 1-3-galactose- $\beta$ 1-4-xylose- $\beta$ 1 (GlcUA-Gal-Gal-Xyl), which is covalently attached to the core protein. The linker region synthesis involves a single linear pathway composed of four successive steps catalyzed by distinctive enzymes (Figure 1). The first step is the addition of xylose to the hydroxy group of specific serine residues on the core protein by xylosyltransferases from UDP-Xyl, followed by two distinct galactosyltransferases (GalT-I and II) and a glucuronosyltransferase from UDP-Gal and UDP-GlcUA, respectively. The next hexosamine addition is critical because it determines which GAG (i.e., CS, DS, or HS) is assembled on the linker region. GalT-II encoded by *B3GALT6* functions in the third step of the linker formation (Figure 1).

To confirm the results obtained by the next-generation sequencing, we examined the seven subjects used for the next-generation sequencing and an additional subject from a Vietnamese family (F7) by direct sequence of the PCR products from genomic DNAs using 3730xl DNA Analyzer (Applied Biosystems). The Sanger sequencing confirmed all *B3GALT6* mutations found by the next-generation sequencing and identified additional *B3GALT6* mutations. The results indicated that *B3GALT6* mutations were found in all subjects (Tables 2 and S1). All but P4 from F3 were compound heterozygotes of missense mutations. In P4, only a heterozygous c.1A>G (p.Met1?) mutation was found, although we searched for a *B3GALT6* mutation in the entire coding region, 5' and 3' UTRs, and flanking

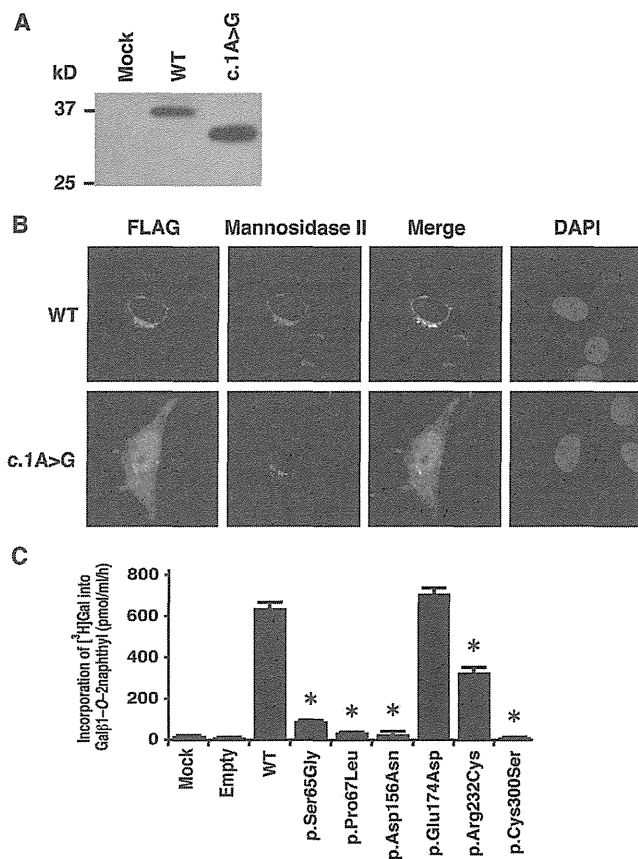
**Table 2. B3GALT6 Mutations in Spondyloepimetaphyseal Dysplasia with Joint Laxity Type 1 and Ehlers-Danlos Syndrome, Progeroid Form**

Family	Clinical Diagnosis	Nucleotide Change	Amino Acid Change
F1	SEMD-JL1	c.1A>G	p.Met1?
		c.694C>T	p.Arg232Cys
F2	SEMD-JL1	c.1A>G	p.Met1?
		c.466G>A	p.Asp156Asn
F3 <sup>a</sup>	SEMD-JL1	c.1A>G	p.Met1?
F4	SEMD-JL1	c.1A>G	p.Met1?
		c.694C>T	p.Arg232Cys
F5	SEMD-JL1	c.694C>T	p.Arg232Cys
		c.899G>C	p.Cys300Ser
F6	SEMD-JL1	c.1A>G	p.Met1?
		c.193A>G	p.Ser65Gly
F7	SEMD-JL1	c.200C>T	p.Pro67Leu
		c.694C>T	p.Arg232Cys
F8	EDS-PF	c.353delA	p.Asp118Alafs*160
		c.925T>A	p.Ser309Thr
F9	EDS-PF	c.588delG	p.Arg197Alafs*81
		c.925T>A	p.Ser309Thr
F10	EDS-PF	c.16C>T	p.Arg6Trp
		c.415_423del	p.Met139Ala141del

The nucleotide changes are shown with respect to *B3GALT6* mRNA sequence. The corresponding predicted amino acid changes are numbered from the initiating methionine residue.  
<sup>a</sup>Only a heterozygous mutation was found.

regions of *B3GALT6*. Most of the mutations are predicted to be disease causing by in silico analysis. The c.1A>G (p.Met1?) mutation was found in individuals from five of the seven families.

Although mutations affecting initiation codons have been reported to be pathogenic in several diseases,<sup>9</sup> the effects of initiation codon mutations on the encoded protein are variable among the genes. We therefore investigated the effect of the c.1A>G (p.Met1?) mutation on the protein by using C-terminally FLAG-tagged *B3GALT6* with and without the mutation expressed in HeLa cells (RIKEN Cell Bank). We detected the mutant *B3GALT6* protein with a molecular weight ~4 kD lower compared with the wild-type (WT) protein (Figure 2A). These results suggest that translation initiation at the second ATG of the coding sequence, at position c.124, would become the initiation codon because of the mutation, probably resulting in an N-terminal deletion of 41 amino acids (p.Met1\_Ala41del), in the same open reading frame that contains the transmembrane domain. We then examined the subcellular localization of the mutant *B3GALT6* protein by immunocytochemistry. The immunofluorescence for WT-*B3GALT6* was observed in a perinuclear region overlapping



**Figure 2. Analyses of B3GALT6 Missense Mutant Proteins Identified in Individuals with SEMD-JL1 In Vitro**

(A) Immunoblot analysis of lysates from HeLa cells expressing transfected wild-type (WT) and mutant (c.1A>G) *B3GALT6*. The mutant *B3GALT6* yields a shortened protein. The difference of the molecular sizes between WT and mutant proteins is approximately ~4 kD.

(B) Subcellular localization of *B3GALT6*. HeLa cells were transfected with WT and mutant (c.1A>G) *B3GALT6*. Cells were stained with anti-FLAG (green), anti- $\alpha$ -mannosidase II (red), and 4',6-diamidino-2-phenylindole (DAPI; blue). WT was expressed in the Golgi, but the mutant was found in cytoplasm and nucleus.

(C) Decreased enzyme activities of the missense mutant proteins (p.Ser65Gly, p.Pro67Lys, p.Asp156Asn, p.Arg232Cys, and p.Cys300Ser). p.Glu174Asp is a common polymorphism in the public database. The GalT-II activity is measured by incorporation of [<sup>3</sup>H]Gal into Galβ1-O-2naphthyl (pmol/ml/hr) and represents the averages of three independent experiments performed in triplicate. Empty and mock indicate the GalT-II activity obtained with the conditioned medium transfected with or without an empty vector. \*p < 0.0001 versus WT (one-way analysis of variance with Dunnett's adjustment).

with that for  $\alpha$ -mannosidase II, a marker of the Golgi as previously reported.<sup>8</sup> In contrast, the immunofluorescence for the mutant *B3GALT6* protein was observed in the nucleus and cytoplasm (Figure 2B). Therefore, the mutant protein can be considered to be functionally null because of the mislocalization.

To investigate the causality of other *B3GALT6* missense mutations, we also examined the subcellular localization of the mutant *B3GALT6* proteins by immunocytochemistry. c.193A>G (p.Ser65Gly), c.200C>T (p.Pro67Leu),

and c.694C>T (p.Arg232Cys) mutants showed mislocalization, whereas c.466G>A (p.Asp156Asn) and c.899G>C (p.Cys300Ser) mutants showed normal localization (Figure S1). To investigate whether the *B3GALT6* missense mutations affect the enzyme function, the GalT-II activities of soluble FLAG-tagged proteins for WT and mutant *B3GALT6* proteins were assayed. The GalT-II activities of p.Ser65Gly-, p.Pro67Leu-, p.Asp156Asn-, p.Arg232Cys-, and p.Cys300Ser-*B3GALT6* were significantly decreased compared with WT-*B3GALT6* (Figure 2C), indicating that these mutations resulted in a loss of enzyme function. On the other hand, there were no significant differences in the GalT-II activities between WT-*B3GALT6* and p.Glu174Asp-*B3GALT6*, a common polymorphism (rs12085009) in the public database (Figure 2C).

All SEMD-JL1 individuals with the *B3GALT6* mutation had the characteristic skeletal abnormalities, including platyspondyly, short ilia, and elbow malalignment (Table 1 and Figure S2); however, some had a range of extraskelatal and connective tissue abnormalities that overlapped with those seen in Ehlers-Danlos syndrome, progeroid form (EDS-PF [MIM 130070]). EDS-PF is an autosomal-recessive connective tissue disorder characterized by sparse hair, wrinkled skin, and defective wound healing with atrophic scars.<sup>10</sup> In addition, skeletal abnormalities so far reported in EDS-PF are limited to generalized osteopenia and radial head dislocation, which are in contrast with the severe generalized dysplasias of the axial and appendicular skeleton observed in SEMD-JL1. Thus, both disorders at first glance appear as separate clinical entities, although they share the clinical features of short stature, joint laxity and dislocation, and facial dysmorphism. In two families with individuals with EDS-PF, recessive mutations of *B4GALT7* (MIM 604327) have been found.<sup>11,12</sup> *B4GALT7* (RefSeq NM\_007255.2) encodes an enzyme, xylosylated protein  $\beta$ -1,4-galactosyltransferase, that catalyzes the second step of the GAG linker region biosynthesis (Figure 1). Therefore, we speculated that *B3GALT6* and *B4GALT7* deficiencies might show similar phenotypes. We then examined *B3GALT6* in four additional individuals (P9–P12) who had phenotypes compatible with EDS-PF (Table 1 and Figure S3) but in whom no *B4GALT7* mutations had been found. Sanger sequencing of the EDS-PF-like subjects revealed that all were compound heterozygotes for *B3GALT6* mutations (Table 2). There were two frameshift mutations and one missense mutation (c.925T>A [p.Ser309Thr]) common in two families (F8 and F9). We investigated the enzyme function of the missense mutation by using the same assay for SEMD-JL1 missense mutations. The GalT-II activities of p.Ser309Thr-*B3GALT6* were significantly decreased (Figure S4).

Collectively, 11 different mutations in individuals from 10 families were identified in *B3GALT6* by a combination of exome and targeted sequencing (Table 2 and Figure S5). None of these mutations were detected in more than 200 ethnicity-matched controls or in public databases, including the 1000 Genomes database, indicating that

they are unlikely to be polymorphisms. SEMD-JL1 and EDS-PF-like individuals had no common mutations (Table 2). The individuals with *B3GALT6* mutations were short at birth and their short stature worsened with age. Their common clinical features were a flat face with prominent forehead and kyphoscoliosis (Table 1). Kyphoscoliosis was noticed in infancy in most cases and even in utero in severe cases. Although skeletal changes were essentially the same, craniofacial and skin abnormalities, joint laxities, and muscular hypotonia were variable among the individuals with *B3GALT6* mutations. Common radiographic features were platyspondyly that becomes less conspicuous with age, short ilia, and elbow malalignment (Table 1). Prominent lesser trochanters and metaphyseal flaring were seen in most cases. No individuals showed generalized osteoporosis. The disease phenotype was very variable between families (mutations), but in two familial cases, phenotypes were similar between the pair of the sibs. As a corollary, our results indicate that EDS-PF is genetically heterogeneous, with a proportion of cases being caused by mutations in *B4GALT7* and another in *B3GALT6*.

Diseases caused by defects in enzymes involved in the biosynthesis of the GAG linker region are categorized as the GAG linkeropathy. The first member of GAG linkeropathy has been identified to arise from an EDS-PF/*B4GALT7* deficiency. *B4GALT7* mutations have been identified in homozygous c.808C>T (p.Arg270Cys)<sup>12</sup> and compound heterozygous (c.557C>A [p.Ala186Asp] and c.617T>C [p.Leu206Pro])<sup>11</sup> states. Another member of GAG linkeropathy manifests itself as Larsen-like syndrome, *B3GAT3* type (MIM 245600). A family with individuals harboring a homozygous *B3GAT3* (MIM 606374; RefSeq NM\_012200.3) mutation (c.830G>A [p.Arg227Gln]) has been identified. The clinical features of five affected individuals of the family are characterized by dislocation and laxity of joints and congenital heart defects.<sup>11</sup> The former considerably overlaps with the phenotypes of SEMD-JL1 and EDS-PF, two other GAG linkeropathies; however, the association of heart defects has critically differentiated this disease from the others (Figure 1).

Given that the linker region biosynthesis is nonparallel and that the defects in the three enzymes simply affect the amounts of the linker region available to form GAGs (CS, HS, DS), phenotypic similarities of the three diseases are quite understandable. The quantitative difference of the phenotypes (severity of the diseases) most probably results from the difference in the degree of enzyme defects resulting from mutations. On the other hand, qualitative differences of the three diseases (e.g., scoliosis caused by the *B3GALT6* mutation, heart disease caused by the *B3GAT3* mutation, etc.) suggest other explanations. Tissue expression patterns of the three genes do not entirely explain the differences. We examined their mRNA expression in various human tissues, including cartilage, bone, and connective tissues by quantitative real-time PCR (Figure S6). We detected strong expression of *B3GALT6* in

**Table 3. The Amount of GAGs in the Lymphoblastoid Cells from Individuals with Spondyloepimetaphyseal Dysplasia with Joint Laxity Type 1**

Subject	GAG (Disaccharides/mg Acetone Powder) <sup>a</sup> [pmol]			
	CS/DS	CS	DS	HS
Control	62	48	29	128
<b>SEMD-JL1</b>				
P1	313	295	118	15
P2	345	175	60	21
P3	270	162	28	20

<sup>a</sup>Calculated based on the peak area in chromatograms of digests with a mixture of chondroitinases ABC and AC-II (CS/DS), chondroitinases AC-I and AC-II (CS), chondroitinase B (DS), and heparinases I and III (HS).

cartilage and bone but only weak expression in skin, ligament, and tendon. *B4GALT7* expression was stronger in cartilage than *B3GALT6* and also weak in skin and ligament. *B3GAT3* expression was not specific to heart. The qualitative difference may result from the difference in the effects of the three genes on GAG formation.

To examine how *B3GALT6* mutations affects the products of GAGs in vivo, we measured the amounts of CS and HS chains at the surface of lymphoblastoid cells from the subjects by flow cytometry by using CS-stub and HS-stub antibodies as previously described.<sup>13–15</sup> In brief, purified GAG fractions were treated individually with a mixture of chondroitinases ABC and AC-II, a mixture of chondroitinases AC-I (EC 4.2.2.5) (Seikagaku Corp.) and AC-II (EC 4.2.2.5) (Seikagaku Corp.), chondroitinase B (EC 4.2.2.19) (IBEX Technologies), or a mixture of heparinases-I and -III (IBEX Technologies) for analyzing the disaccharide composition of CS/DS, CS, DS, and HS, respectively. The digests were labeled with a fluorophore 2-aminobenzamide (2AB) and aliquots of the 2AB derivatives of CS/DS/HS disaccharides were analyzed by anion-exchange HPLC on a PA-03 column (YMC Co.). The HS-stub antibody (3G10) showed a markedly reduced binding to the epitopes on the subjects' cells (Figure S7). The relative numbers of the HS chains presented as the mean fluorescence intensity (MFI) of the cell population stained with the antibody for P1, P2, and P3 were 26%, 56%, and 35% of the control, respectively. On the other hand, the CS-stub antibody (2B6) showed a similar binding to the epitopes on the subjects' cells relative to those of the control (Figure S7). The MFI for P1, P2, and P3 were 114%, 104%, and 106% of the control, respectively. Furthermore, we measured disaccharide of GAG chains from lymphoblastoid cells by using anion-exchange HPLC after digestion with chondroitinase and heparinase. The amounts of the disaccharide from HS chains were significantly decreased, whereas CS and DS chains were ~5 times higher than those in the control (Table 3).

Previous biochemical studies on EDS-PF with *B4GALT7* mutations show a reduction in the synthesis of DS chains.<sup>16,17</sup> The c.830G>A (p.Arg227Gln) mutation in

*B3GAT3* causes a drastic reduction in GlcAT-I activity in fibroblasts of the individual with SEMD-JL1 and numbers of CS and HS chains on the core proteins at the surface of the fibroblasts are decreased to about half of the controls.<sup>11</sup> Cultured lymphoblastoid cells from individuals with a c.419C>T (p.Pro140Leu) mutation in *B3GAT3* show that defective synthesis is more pronounced for CS than for HS.<sup>11</sup> Taken together with our results, these findings suggest that the effects of the deficiencies of the three enzymes on GAG synthesis are not identical. A possible explanation for the qualitative phenotypic differences may be that the biosynthesis of the GAG linker region is not a simple step-by-step addition but involves parallel processing and/or alternative pathways. Other glycosyltransferases may have similar biochemical functions to these three enzymes and thus complement their deficient activities to variable degrees in cell- and/or tissue-specific manners, leading to differences in the amount of GAGs in the tissues. It is known that *B3GALT6* and *B4GALT7* have several homologs.<sup>18</sup> It must be noted that all biochemical studies so far have been performed in vitro or in cultured cells, and therefore there is a severe limitation to our understanding of the pathogenesis at tissue and organ levels.

By exome sequencing, we identified loss-of-function mutations in *B3GALT6* in 12 individuals from 10 families. The mutations produced a spectrum of connective tissue disorders characterized by lax skin, muscle hypotonia, joint dislocation, and skeletal dysplasia and deformity, which include phenotypes previously known as SEMD-JL1 and EDS-PF (Figures S1 and S2). The pleiotropic phenotypes of *B3GALT6* mutations indicate that *B3GALT6* plays critical roles in development and homeostasis of various tissues, including skin, bone, cartilage, tendons, and ligaments. Biochemical studies that used lymphoblastoid cells of the individuals with *B3GALT6* mutations showed a decrease of HS and a paradoxical increase of CS and DS of the cell surface. Further clinical, genetic, and biological studies are necessary to understand the pathological mechanism of the diseases caused by enzyme defects involved in the biosynthesis of the GAG linker region and roles of the region in GAG metabolism and function.

#### Supplemental Data

Supplemental Data include seven figures and two tables and can be found with this article online at <http://www.cell.com/AJHG/>.

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## Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://browser.1000genomes.org>  
ANNOVAR, <http://www.openbioinformatics.org/annovar/>  
dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>  
GATK, <http://www.broadinstitute.org/gatk/>  
MutationTaster, <http://www.mutationtaster.org/>  
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
Novoalign, <http://www.novocraft.com/main/page.php?s=novoalign>  
Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>  
Picard, <http://picard.sourceforge.net/>  
PolyPhen, <http://www.genetics.bwh.harvard.edu/pph2/>  
RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>  
SIFT, <http://sift.bii.a-star.edu.sg/>  
UCSC Genome Browser, <http://genome.ucsc.edu>

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## *De novo* mutations in the autophagy gene *WDR45* cause static encephalopathy of childhood with neurodegeneration in adulthood

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**Static encephalopathy of childhood with neurodegeneration in adulthood (SENDA) is a recently established subtype of neurodegeneration with brain iron accumulation (NBIA)<sup>1–3</sup>. By exome sequencing, we found *de novo* heterozygous mutations in *WDR45* at Xp11.23 in two individuals with SENDA, and three additional *WDR45* mutations were identified in three other subjects by Sanger sequencing. Using lymphoblastoid cell lines (LCLs) derived from the subjects, aberrant splicing was confirmed in two, and protein expression was observed to be severely impaired in all five. *WDR45* encodes WD-repeat domain 45 (WDR45). *WDR45* (also known as WIP14) is one of the four mammalian homologs of yeast Atg18, which has an important role in autophagy<sup>4,5</sup>. Lower autophagic activity and accumulation of aberrant early autophagic structures were demonstrated in the LCLs of the affected subjects. These findings provide direct evidence that an autophagy defect is indeed associated with a neurodegenerative disorder in humans.**

NBIA is a heterogeneous group of neurodegenerative diseases that are characterized by a prominent extrapyramidal movement disorder, intellectual deterioration and deposition of iron in the basal ganglia<sup>1–3</sup>. Mutations in several genes involved in diverse cellular processes cause NBIA<sup>6</sup>. SENDA is a recently established subtype of NBIA. SENDA begins with early childhood psychomotor retardation, which remains static until adulthood. Then, during their twenties to early thirties, affected individuals develop sudden-onset progressive dystonia-parkinsonism and dementia. In addition to iron deposition in the globus pallidus and substantia nigra, individuals with SENDA have a distinct pattern on brain magnetic resonance images (MRI)

of T1-weighted signal hyperintensity of the substantia nigra, with a central band of hypointensity<sup>1–3,6,7</sup>. SENDA is always sporadic<sup>6,7</sup>, suggesting the involvement of *de novo* mutations or autosomal recessive traits. To identify *de novo* or recessive mutations, family-based exome sequencing was performed including the affected individual, an unaffected sibling and the unaffected parents.

A total of 180 and 187 rare protein-altering and splice-site variants were identified per affected subject, which were absent in dbSNP135 data and in 88 in-house control exomes (Supplementary Table 1). All genes in each subject were surveyed for *de novo* mutations and compound heterozygous or homozygous mutations that were consistent with an autosomal recessive trait in each family (Supplementary Table 2). Two *de novo* and one autosomal recessive candidate mutations were found in subject 1, and a *de novo* candidate mutation was found in subject 2. Only mutations in *WDR45* at Xp11.23, encoding *WDR45* (referred to here as WIP14), were common in the two subjects. A canonical splice-site mutation (c.439+1G>T) was found in subject 1, and a synonymous mutation located at the last base of exon 8 (c.516G>C) was found in subject 2, both of which occurred *de novo* (Fig. 1a). Sanger sequencing of *WDR45* in three other affected subjects identified one nonsense and two frameshift mutations (Fig. 1a). The c.1033\_1034dupAA mutation in subject 5 occurred *de novo*. Parental samples for the other two subjects were unavailable. None of the five mutations were found in 6,500 National Heart, Lung, and Blood Institute (NHLBI) exomes or among our 212 in-house control exomes. All subjects with a *WDR45* mutation are female.

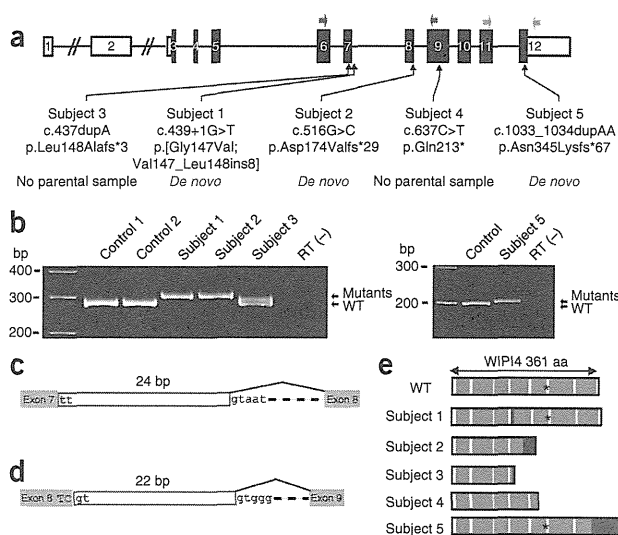
To examine the effects of the mutations on *WDR45* transcription, RT-PCR and sequencing were performed on total RNA extracted from the LCLs of subjects. The c.439+1G>T mutation in subject 1 and the c.516G>C mutation in subject 2 caused 24-bp in-frame and 22-bp

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**Figure 1** Heterozygous *WDR45* mutations in individuals with SENDA. (a) Schematic of *WDR45*, which comprises 12 exons (rectangles). The UTRs and coding region are shown in white and black, respectively. Three mutations were confirmed as *de novo*; the others were unable to be confirmed because parental samples were unavailable. Blue and green arrows indicate the locations of the two sets of primers used in mRNA analysis. (b) RT-PCR analysis using the blue primer set (left) and green primer set (right) from a. Whereas control cDNA samples showed a single product corresponding to the wild-type allele (WT), an apparently longer product was observed in subjects 1, 2 and 5, indicating that only the transcripts from the mutant allele were expressed. In subject 3, both wild-type and mutant alleles were expressed. Template without reverse transcriptase was used as a negative control, RT(-). (c) Schematic of the mutant transcript resulting from the c.439+1G>T mutation (red) in subject 1. A 24-bp insertion caused by the use of a cryptic splice donor site within intron 7 was observed, resulting in a p.Gly147Val substitution followed by an in-frame eight-amino-acid insertion (p.[Gly147Val; Val147\_Leu148ins8]). (d) Schematic of the mutant transcript resulting from the c.516G>C mutation (red) in subject 2. A 22-bp insertion from the use of a cryptic splice donor site within intron 8 was observed, leading to a frameshift (p.Asp174Valfs\*29). (e) Schematic of mutant WIPI4 proteins.  $\beta$ -propeller structures and additional residues caused by mutations are colored in blue and red, respectively. The amino-acid residues of the mutant protein predicted from cDNA sequences are shown in relation to seven- $\beta$  propeller structures<sup>13–15</sup>. An asterisk indicates the position of the FRRG motifs.



frameshift insertions, respectively (Fig. 1b–d and Supplementary Fig. 1). The c.437dupA, c.637C>T and c.1033\_1034dupAA mutations were confirmed in the transcripts (Fig. 1b and Supplementary Fig. 1). Theoretically, mutant WIPI4 would be severely truncated in subjects 2, 3 and 4 and relatively conserved in subjects 1 and 5 (Fig. 1e). As human female cells are subject to X-chromosome inactivation, subjects with a *WDR45* mutation may have two cell populations: one expressing a wild-type allele and the other expressing a mutant allele. Notably, whereas both wild-type and mutant alleles were expressed in the LCLs of subject 3, the LCLs of the other four affected subjects exclusively expressed mutant transcripts, suggesting that the wild-type alleles underwent X inactivation in most cells (Fig. 1b and Supplementary Fig. 1). In fact, X-inactivation analysis with genomic DNA from peripheral leukocytes showed a skewed pattern in subjects 2, 4 and 5 (analysis was non-informative in subject 1) (Supplementary Table 3). However, it is unknown whether the wild-type allele underwent X inactivation in brain tissues as in LCLs and leukocytes from the subjects.

The clinical features of the individuals with SENDA possessing *WDR45* mutations are summarized in Table 1 (see also the Supplementary Note). Subjects 1 and 3 have been described recently<sup>7,8</sup>. These individuals showed psychomotor developmental delay from infancy and severe intellectual disability, while their motor function gradually developed. In adulthood, severe progressive dystonia-parkinsonism and dementia developed. Four of the subjects became bedridden within a few years of onset of cognitive decline. In all subjects, blood concentrations of ceruloplasmin, copper, iron, ferritin and lactate acid were normal. Brain MRI showed T1-weighted signal hyperintensity in the substantia nigra with a central T1-weighted hypointensity band (Fig. 2a–e) and T2-weighted signal hypointensity, suggesting iron deposition in the globus pallidus and substantia nigra (Fig. 2f–h), which are characteristic of SENDA. In addition, significant cerebral atrophy was found (Fig. 2i,j). Substantial differences in the severity of clinical findings were not observed among the five subjects.

WIPI1, WIPI2, WIPI3 and WIPI4, mammalian Atg18 homologs, have an important role in the autophagy pathway<sup>4,5</sup>. Autophagy is the major intracellular degradation system by which cytoplasmic materials are enclosed by double-membrane structures called

autophagosomes and subsequently delivered to lysosomes for degradation<sup>9</sup>. More than 30 autophagy-related (ATG) genes have been identified in yeast<sup>10,11</sup>, many of which are conserved in higher eukaryotes and are essential for the formation of the autophagosome<sup>10,12</sup>. These factors include subunits of the class III phosphatidylinositol 3-kinase complex, and generation of the lipid phosphatidylinositol 3-phosphate is essential for autophagosome formation. Atg18 in yeast and WIPI subunits in mammals associate with membranes through a phosphoinositide-binding motif (FRRG) within a seven- $\beta$  propeller structure<sup>13–15</sup>. Atg18 and WIPI proteins also interact with Atg2 and its homologs in yeast and mammalian cells, respectively<sup>16,17</sup>. Autophagic activity in relation to WIPI4 expression was examined using LCLs from the subjects. Immunoblot analysis of WIPI4 showed lower expression in all five subjects compared to unaffected individuals (Fig. 3a). Although mutant WIPI4 protein sequence was relatively conserved in subjects 1 and 5, the expression of mutant WIPI4 in both subjects was severely decreased, similar to that of subjects 2, 3 and 4, in whom mutant WIPI4 was truncated. This suggests that all the mutant proteins are structurally unstable and undergo degradation. To examine the effect of the *WDR45* mutations on autophagy, an autophagic flux assay was performed using LCLs. When lysosomal degradation was blocked by the lysosomal inhibitor chloroquine, the amount of LC3-II (the membrane-bound form) was higher than in cells without the inhibitor, as for control LCLs (Fig. 3b and Supplementary Fig. 2)<sup>18</sup>. The differences in LC3-II amounts between samples with and without chloroquine represent the amount of LC3 on autophagic structures delivered to lysosomes for degradation<sup>18</sup>. In the LCLs from affected subjects, accumulation of LC3-II was observed, even under normal conditions, which was more apparent when autophagy was induced by the mTORC1 inhibitor Torin1 (Supplementary Fig. 2a–d). The increase in the LC3-II amount by concomitant chloroquine treatment was significant or tended to be suppressed in the LCLs from affected subjects, suggesting that the autophagic flux was blocked, probably incompletely, at an intermediate step of autophagosome formation (Fig. 3b and Supplementary Fig. 2e).

Consistent with the immunoblot analysis, immunofluorescence microscopy showed the accumulation of LC3-containing autophagic

**Table 1 Clinical features of subjects with SENDA with a *WDR45* mutation**

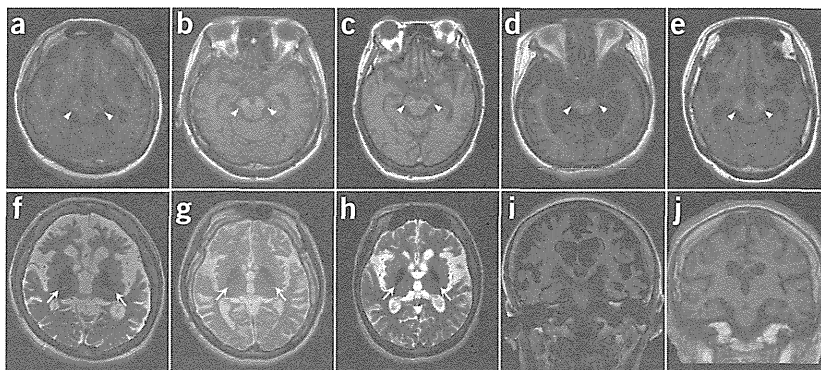
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Age	33 years	28 years	40 years	51 years	33 years
Sex	Female	Female	Female	Female	Female
Mutation	c.439+1G>T	c.516G>C	c.437dupA	c.637C>T	c.1033_1034dupAA
Protein alteration	p.[Gly147Val; Val147_Leu148ins8]	p.Asp174Valfs*29	p.Leu148Alafs*3	p.Gln213*	p.Asn345Lysfs*67
<b>Neurological symptoms</b>					
Current status	Bedridden	Wheelchair	Bedridden	Bedridden	Bedridden
Initial symptom	Psychomotor retardation	Psychomotor retardation	Psychomotor retardation	Psychomotor retardation	Psychomotor retardation
Initial walking	3 years	2 years 7 months	2 years 2 months	1 year 6 months	1 year 6 months
Speech ability	No word	One word	No word	Two-word sentences	Few words
Cognitive dysfunction during childhood	Nonprogressive	Nonprogressive	Nonprogressive	Nonprogressive	Nonprogressive
Start of cognitive decline	26 years	25 years	30 years	24 years	23 years
Period until bedridden after decline	4 years	–	3 years	1 year	6 years
Dystonia	+	+	+	+	+
Parkinsonism	Rigidity, akinesia	Rigidity, akinesia	Rigidity	Rigidity	Rigidity, tremor, impairment of postural reflex
Progressive dementia during adulthood	+	+	+	+	+
Psychiatric symptoms	Aggressive behaviors	Aggressive behaviors	None	None	Anxiety
Epileptic seizure	+	+	FS	None	+
<b>Radiological features</b>					
MRI					
Iron deposition	Globus pallidus, substantia nigra	Globus pallidus, substantia nigra	Globus pallidus, substantia nigra	Globus pallidus, substantia nigra	Globus pallidus, substantia nigra
Central band of T1 hypointensity	+	+	+	+	+
Cerebral atrophy	Moderate at 25 years, remarkable at 32 and 33 years	Moderate at 25 and 27 years	Mild at 33 years, remarkable at 39 years	Mild at 27 years, remarkable at 46 years	Remarkable at 33 years
Eye of the tiger sign	–	–	–	–	–
White matter involvement	–	–	–	–	–
Cerebellar atrophy	Mild at 25, 32 and 33 years	Mild at 25 and 27 years	Mild at 33 and 39 years	Mild at 27 and 46 years	Mild at 33 years
CT findings	High density in globus pallidus	Mild high density in substantia nigra	High density in substantia nigra	High density in ventral midbrain	High density in globus pallidus
<b>Neurophysiological examination</b>					
EEG	Bilateral frontal spike	Bilateral frontal spike, low voltage, slow wave	Low voltage	Abnormal	Abnormal
EMG	NE	NE	Dystonic pattern	Normal	NE
VEP	Normal	NE	Prolonged P100 latency	NE	Normal
ABR	Low amplitude, normal latency	NE	No response at 100 dB	NE	NE

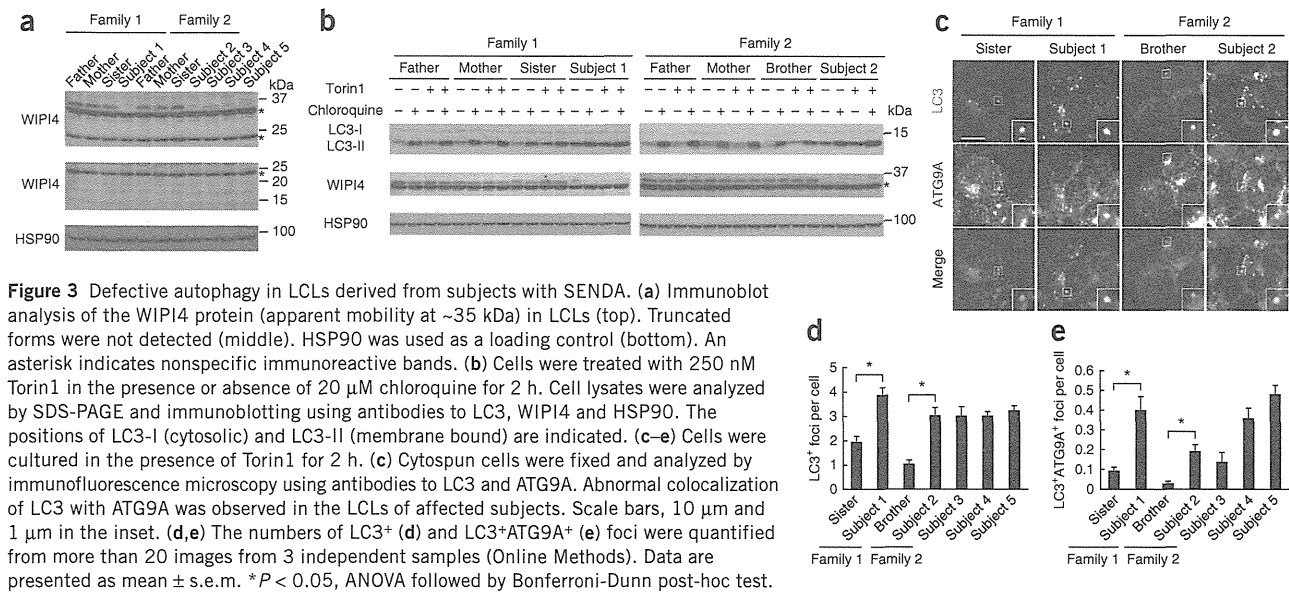
FS, febrile seizure; EEG, electroencephalogram; EMG, electromyogram; VEP, visual evoked potential; ABR, auditory brainstem response; NE, not examined.

structures in the LCLs from affected subjects, some of which were abnormally enlarged compared with those observed in control LCLs (Fig. 3c,d). Therefore, we examined whether these LC3-positive

structures in fact included premature or abnormal autophagic structures. A recent study showed that knockdown of *Wdr45* in rat kidney cells and mutation in *epg-6* (encoding a WIPI4 homolog)

**Figure 2** Brain MRIs at 3.0 T and 1.5 T. (a–e) T1-weighted imaging shows hyperintensity of the substantia nigra with a central band of T1-weighted hypointensity (arrowheads). Images are shown for subject 1 at 33 years (a), subject 2 at 25 years (b), subject 3 at 39 years (c), subject 4 at 46 years (d) and subject 5 at 33 years (e). (f–h) T2-weighted imaging shows marked hypointensity of the globus pallidus (arrows), suggesting iron deposition. Cerebral atrophy and mild cerebellar atrophy are also seen. Images are shown for subject 1 (f), subject 2 (g) and subject 3 (h). (i,j) The fluid attenuated inversion recovery (FLAIR) image of subject 1 (i) and the T1-weighted FLAIR coronal image of subject 2 (j) also show cerebral atrophy.





in *Caenorhabditis elegans* cause accumulation of early autophagic structures<sup>5</sup>. One supposed function of WIPI4 (Epg-6) is to regulate the distribution of ATG9A-marked vesicles<sup>5</sup>, which transiently localize to the autophagosome formation site and induce autophagosome formation<sup>19,20</sup>. ATG9A is absent from completed autophagosomes in mammalian cells; therefore, colocalization of ATG9A and LC3 is rare. However, enlarged structures positive for both ATG9A and LC3 accumulated in LCLs from all five subjects (Fig. 3c,e), indicating improper autophagosome formation.

The importance of the housekeeping activity of autophagy in neurons, as well as the ubiquitin-proteasome system, has been demonstrated in mice. Mice lacking autophagy in the central nervous system developed progressive motor and behavioral deficits<sup>21,22</sup>. Histologically, inclusion bodies containing polyubiquitinated proteins were observed in neurons, and their size and number increased with age<sup>21,22</sup>. Neuronal cell death was observed in subsets of neurons<sup>21,22</sup>, implying that the impairment of autophagy contributes to the pathogenesis of neurodegenerative disorders. Indeed, dysregulation of autophagy has been suggested in various neurodegenerative disorders in humans<sup>23</sup>. In addition, mutations in *PARK2* and *PINK1*, both of which cause familial Parkinson's disease<sup>24,25</sup>, impair the selective autophagic degradation of damaged mitochondria, called mitophagy (*PARK2*, also called Parkin, is recruited to damaged mitochondria in a *PINK1*-dependent manner)<sup>26,27</sup>. However, a direct link between the core autophagy machinery and human neurodegenerative disorders has not been reported. Here, we showed that mutations in *WDR45*, a core autophagy gene, result in a neurodegenerative disorder. Notably, the autophagy defects were partial, implying that some autophagic activity could be maintained in the neurons of affected subjects. We hypothesize that this might be a possible explanation of why childhood intellectual disability in individuals with SENDA remains static until adulthood, unlike in other forms of NBIA<sup>1–3</sup>. In contrast to heterozygous *WDR45* mutations in females, hemizygous germline mutations in males, leading to the expression of mutant *WDR45* in all cells, possibly cause lethal phenotypes from complete loss of *WDR45* function, as mice defective in autophagy die shortly after birth<sup>28–32</sup>. While this paper was under review, Haack *et al.* reported *WDR45* mutations in 20 subjects, including 3 males, 1 of whom possessed

a mutation that was somatic mosaic, supporting the idea that male germline mutations could be lethal<sup>33</sup>.

*WDR45* is widely expressed in human tissues, with the highest expression found in skeletal muscle<sup>34</sup>. Nevertheless, SENDA phenotypes seem to be limited to the brain. These facts may reflect cell type-dependent differences: autophagy could be more important in neurons (non-dividing, terminally differentiated cells) than in LCLs (rapidly dividing cells). In addition, it is possible that the other WIPI homologs (*WIPI1*, *WIPI2* and *WIPI3*) could compensate for the deficiency in *WIPI4* in a cell type-dependent manner, and the relative contribution of *WIPI4* among *WIPI* factors may be high in neurons.

In conclusion, heterozygous mutations of X-linked *WDR45*, a core autophagy gene, were identified in SENDA, providing direct evidence that an autophagy defect is indeed associated with a neurodegenerative disorder in humans.

**URLs.** NHLBI Exome Sequencing Project, <http://evs.gs.washington.edu/EVS/>; Picard, <http://picard.sourceforge.net/>; SAMtools, <http://samtools.sourceforge.net/>; dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Reference sequences are available from GenBank for *Homo sapiens WDR45* transcript variant 1 mRNA (NM\_007075.3) and *WIPI4* isoform 1 (NP\_009006.2).

*Note: Supplementary information is available in the online version of the paper.*

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#### AUTHOR CONTRIBUTIONS

H.S., N. Mizushima and N. Matsumoto designed and directed the study. H.S., T.N., K.M., N. Mizushima and N. Matsumoto wrote the manuscript. K.M., S.K., K.S., E.K.-Y., N.S., H.N., A.H., F.R., S.Y., H.A. and M.K. collected samples and provided the subjects' clinical information. H.S., H.K., K.N., Y.T., M.N. and N. Miyake performed exome sequencing and Sanger sequencing. H.S. and K.N. performed the RNA analysis. Y.K. performed the X-inactivation analysis. T.N. and N. Mizushima analyzed protein expression and autophagic activity.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Subjects.** We analyzed five Japanese individuals with SENDA. Diagnosis was made on the basis of clinical features, including psychomotor retardation at early childhood that was static for decades and severe progressive dystonia-parkinsonism and dementia after several decades, as well as characteristic findings on brain MRI scans. Genomic DNA was isolated from blood leukocytes according to standard protocols. The Institutional Review Board of Yokohama City University approved the experimental protocols. Informed consent was obtained for all individuals included in this study in agreement with the requirements of Japanese regulations. Clinical information on the subjects with a *WDR45* mutation is presented in **Table 1** and in the **Supplementary Note**.

**Mutation screening.** Mutation screening of exons 3–12 covering the *WDR45* coding region (of transcript variant 1, GenBank accession NM\_007075.3) was performed by direct sequencing. PCR was performed in a 20- $\mu$ l mixture containing 1  $\mu$ l of genomic DNA, 1 $\times$  PCR Buffer for KOD FX NEO, 0.4 mM of each dNTP, 0.3  $\mu$ M of each primer and 0.4 U of KOD FX NEO polymerase (Toyobo). Details on PCR conditions and primer sequences are given in **Supplementary Table 4**.

**Exome sequencing.** Genomic DNA was captured using the SureSelect Human All Exon v4 kit (51 Mb; Agilent Technologies) and sequenced with four samples per lane on an Illumina HiSeq2000 with 101-bp paired-end reads. Image analysis and base calling were performed by sequence control software real-time analysis and CASAVA software v1.8 (Illumina). Reads were aligned to GRCh37 with Novoalign (Novocraft Technologies). Duplicate reads were marked using Picard (see URLs) and excluded from downstream analysis. After merging the BAM files of all members in each family using SAMtools, local realignments around indels and base quality score recalibration were performed with the Genome Analysis Toolkit (GATK)<sup>35</sup>. Single-nucleotide variants and small indels were identified using the GATK UnifiedGenotyper and filtered according to the Broad Institute's best-practice guidelines (version 3). Variants registered in dbSNP135, which were not flagged as clinically associated, were excluded. Variants that passed the filters were annotated using ANNOVAR<sup>36</sup>.

**RNA analysis.** LCLs were established from five affected subjects and their family members. RT-PCR using total RNA extracted from LCLs was performed as previously described<sup>37</sup>. Briefly, 4  $\mu$ g of total RNA extracted with an RNeasy Plus Mini kit (Qiagen) was subjected to reverse transcription, and 2  $\mu$ l of cDNA was used for PCR. Details on primer sequences and PCR conditions are given in **Supplementary Table 4**. PCR products were electrophoresed in a 10% polyacrylamide gel and sequenced.

**X-inactivation analysis.** The X-inactivation pattern was studied using the human androgen receptor (HUMARA) assay and a fragile X mental retardation (*FRAXA*) locus methylation assay as previously described<sup>38–40</sup>. Briefly, genomic DNA from the subjects, a control male and a control female was digested with two methylation-sensitive enzymes, HpaII and HhaI. Details on PCR conditions and primer sequences are given in **Supplementary Table 4**. Fluorescently labeled products were analyzed on an ABI PRISM 3500 Genetic Analyzer with GeneMapper Software version 4.0 (Applied Biosystems). X-inactivation ratios of less than or equal to 80:20 were considered to represent a random pattern, ratios greater than 80:20 were considered to represent a

skewed pattern, and ratios greater than 90:10 were considered to represent a markedly skewed pattern<sup>38</sup>.

**Cell culture.** LCLs were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, tylosin and antibiotic-antimycotic solution in a 5% CO<sub>2</sub> incubator.

**Immunoblotting.** An affinity-purified rabbit polyclonal antibody against WIPI4 peptide antigen (CFPDNPRKLFEDTRDNP, amino acids 129–145) was generated by Medical & Biological Laboratories. The specificity of the antibody was tested using lysate from HeLa cells in which *WDR45* was knocked down. For immunoblot analysis, cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride and a protease inhibitor cocktail (Complete EDTA-free protease inhibitor, Roche)). Cell lysates were clarified by centrifugation at 12,000g for 20 min and analyzed by SDS-PAGE and immunoblotting using antibodies to WIPI4, LC3 (ref. 41) and HSP90 (BD Transduction Laboratories, 610418). Signal intensities were analyzed using a LAS-3000 mini imaging analyzer and Multi Gauge software version 3.0 (Fujifilm). Contrast and brightness adjustments were applied to the images using Photoshop 7.0.1 (Adobe Systems).

**Fluorescence microscopy.** LCLs were spun onto a glass slide at 500 RPM (28g) for 1 min in a Shandon Cytospin 4 cytofuge (Thermo Electron). Cells were fixed with 4% paraformaldehyde, permeabilized using 50  $\mu$ g/ml digitonin and then stained with antibodies to LC3 (clone 1703, Cosmo Bio) and Atg9A<sup>19</sup>. Cells were observed with a confocal laser microscope (FV1000D IX81, Olympus) using a 60 $\times$  PlanApoN oil immersion lens (1.42 numerical aperture (N.A.), Olympus). For final output, images were processed using Adobe Photoshop 7.0.1 software. The number of staining foci was determined as follows: foci were extracted using the top hat operation (parameter of 300  $\times$  300 pixel area), and a binary image was created. Small foci (with an area of less than 10  $\times$  10 pixels) were removed using an open operation. The number of foci was counted using the integrated morphometry analysis program. False foci were removed by comparison with the original image.

**Statistical analysis.** Differences were analyzed statistically using unpaired *t* tests or analysis of variance (ANOVA) with a Bonferroni-Dunn post-hoc test.

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# Mitochondrial Complex III Deficiency Caused by a Homozygous *UQCRC2* Mutation Presenting with Neonatal-Onset Recurrent Metabolic Decompensation

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**ABSTRACT:** Mitochondrial complex III (CIII) deficiency is a relatively rare disease with high clinical and genetic heterogeneity. CIII comprises 11 subunits encoded by one mitochondrial and 10 nuclear genes. Abnormalities of the nuclear genes such as *BCS1L* and *TTC19* encoding mitochondrial assembly factors are well known, but an explanation of the majority of CIII deficiency remains elusive. Here, we report three patients from a consanguineous Mexican family presenting with neonatal onset of hypoglycemia, lactic acidosis, ketosis, and hyperammonemia. We found a homozygous missense mutation in *UQCRC2* that encodes mitochondrial ubiquinol-cytochrome *c* reductase core protein II by whole-exome sequencing combined with linkage analysis. On the basis of structural modeling, the mutation (p.Arg183Trp) was predicted to destabilize the hydrophobic core at the subunit interface of the core protein II homodimer. In vitro studies using fibroblasts from the index patient clearly indicated CIII deficiency, as well as impaired assembly of the supercomplex formed from complexes I, III, and IV. This is the

first described human disease caused by a core protein abnormality in mitochondrial CIII.

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**KEY WORDS:** mitochondrial complex III (CIII); *UQCRC2*; whole exome sequence; supercomplex

## Introduction

The mitochondrial respiratory chain generates energy as ATP by means of the electron-transport chain and the oxidative-phosphorylation system. The mitochondrial respiratory chain, located in the inner mitochondrial membrane, is composed of five multimeric protein complexes: I, II, III, IV, and V. Among them, the complex III (CIII) (bc1 complex or ubiquinol-cytochrome *c* reductase; EC1.10.2.2) monomer is composed of 11 proteins [Iwata et al., 1998]. One protein is encoded by mitochondrial DNA (*MTCYB*) and the other 10 are encoded by nuclear DNA. The latter are categorized into three groups: (1) core proteins (encoded by *UQCRC1* and *UQCRC2*), (2) respiratory proteins (*CYC1* and *UQCRFS1*), and (3) low-molecular-weight proteins (*UQCRH*, *UQCRB*, *UQCRCQ*, *UCRC*, *UQCRI1*, and *UQCRFS1*). In its native state, the CIII monomer is quickly converted into a catalytically active homodimer that is incorporated into a supercomplex (respirasome) with complexes I and IV, and this supercomplex functions as a single enzyme [Schagger and Pfeiffer, 2000].

Mitochondrial CIII enzyme deficiency (CIII deficiency; MIM# 124000) is a relatively rare disease with clinical and genetic heterogeneity. Until now, mutations in four genes have been known to cause autosomal recessive CIII deficiencies: *UQCRB* (NM\_006294), *UQCRCQ* (NM\_014402), *BCS1L* (NM\_004328), and *TTC19* (NM\_017775). *UQCRB* and *UQCRCQ* encode components of CIII itself, whereas *BCS1L* and *TTC19* produce mitochondrial assembly factors. Although recessive *BCS1L* mutations are the most frequent cause of CIII deficiency, the majority of the genetic causes of CIII deficiency remain unknown [Benit et al., 2009; de Lonlay et al.,

Additional Supporting Information may be found in the online version of this article.

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2001; DiMauro and Schon, 2003; Fernandez-Vizarrá et al., 2007; Hinson et al., 2007; Visapaa et al., 2002]. Interestingly, *BCS1L* mutations cause variable clinical presentations: Bjornstad syndrome (MIM# 262000), which is characterized by sensorineural hearing loss and pili torti [Hinson et al., 2007]; GRACILE syndrome (MIM# 603358), which presents with fetal growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death [Visapaa et al., 2002]; and Leigh syndrome (MIM# 256000) [de Lonlay et al., 2001]. A homozygous mutation of *TTC19* causes a progressive neurodegenerative disorder [Ghezzi et al., 2011]. A homozygous 4-bp deletion of *UQCRB* causes hypoglycemia and lactic acidosis [Haut et al., 2003] and a homozygous missense mutation of *UQCRQ* results in severe psychomotor retardation, extrapyramidal signs, and dementia [Barel et al., 2008].

Here, we describe the first human mutation of *UQCRC2* encoding core protein 2 of CIII, utilizing linkage analysis and whole-exome sequencing.

## Materials and Methods

### DNA Preparation

DNAs from family members and fibroblasts from patients were collected after obtaining informed consent. DNA was extracted from blood leukocytes using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) or QuickGene-610L (Fujifilm, Tokyo, Japan), according to the manufacturers' instructions. DNAs from 80 Mexican control subjects were purchased from the Coriell Institute for Medical Research (Camden, New Jersey). The experimental protocols were approved by the institutional review board of Yokohama City University.

### Linkage Analysis

SNP typing was performed using an Affymetrix Human Mapping SNP 10K Xba I 142 2.0 array (Affymetrix, Santa Clara, California), according to the manufacturer's instructions. A multipoint linkage analysis was performed using Allegro version 2.0 [Gudbjartsson et al., 2005]. An autosomal recessive mode of inheritance with complete penetrance and a disease allele frequency of 0.005 was used.

### Exome Sequence

Briefly, 3 µg of genomic DNA was sheared and captured using a NimbleGen SeqCap EZ Exome Library SR (Roche NimbleGen, Inc., Madison, New Jersey), according to the manufacturer's instructions. The captured sample was sequenced on a GAIIx instrument (Illumina, Inc., San Diego, California) using 76-bp paired-end reads. Image analysis and base calling were performed by sequence-control software real-time analysis (Illumina, Inc.) and CASAVA software v1.7 (Illumina, Inc.). The quality-controlled (path-filtered) reads were mapped to human genome reference hg19 with Mapping and Assembly with Qualities (MAQ; <http://maq.sourceforge.net/>) and NextGENe software v2.00 (SoftGenetics, State College, Pennsylvania). The variants from MAQ were annotated by SeattleSeq annotation 131 (<http://snp.gs.washington.edu/SeattleSeqAnnotation131>). The priority scheme of the variants was described previously [Tsurusaki et al., 2011]. The nucleotide numbering of the variants reflects the cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, accord-

ing to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)). The initiation codon is codon 1.

### Expression Vector Preparation

For construction of a mammalian expression vector, full-length *UQCRC2* (NM\_003366.2) was amplified from a cDNA library from a multiple-tissue cDNA (MTC) panel (Clontech, Mountain View, California) using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR product was cloned into the entry vector (pDONR<sup>TM</sup>221) of the gateway system (Invitrogen, Carlsbad, California). Each of the two missense mutations was independently introduced into the entry clone using a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, California). Each insert was cloned into pcDNA-DEST40 (C-terminal V5 and 6xHis tag) by LR recombination. All the clones were verified by direct sequencing. In addition, full-length *UQCRC2* (wild type, mutant, or SNP [rs4850: c.548G>A, p.Arg183Gln]) and AcGFP constructs were cloned into multiple cloning sites A and B of the pIRES vector (Clontech).

### Intracellular Localization

Each mammalian expression construct (200 ng) was transfected into COS-1 cells using FuGENE6 (Roche Diagnostics, Indianapolis, Indiana). After 24 hr of transfection, MitoTracker Red CMXRos (Invitrogen) was added and incubated for 30 min. The cells were then fixed with 4% paraformaldehyde for 20 min at room temperature. After permeabilization with 0.1% Triton/1× PBS for 10 min, C-terminally V5-6xHis-tagged *UQCRC2* protein was stained with a mouse anti-V5 antibody (1:1,000) (Invitrogen) and an Alexa Fluor 488-conjugated goat antimouse IgG secondary antibody (1:1,000) (Molecular Probes, Carlsbad, California). Confocal images were taken with a FLUOVIEW FV1000-D microscope (Olympus, Tokyo, Japan).

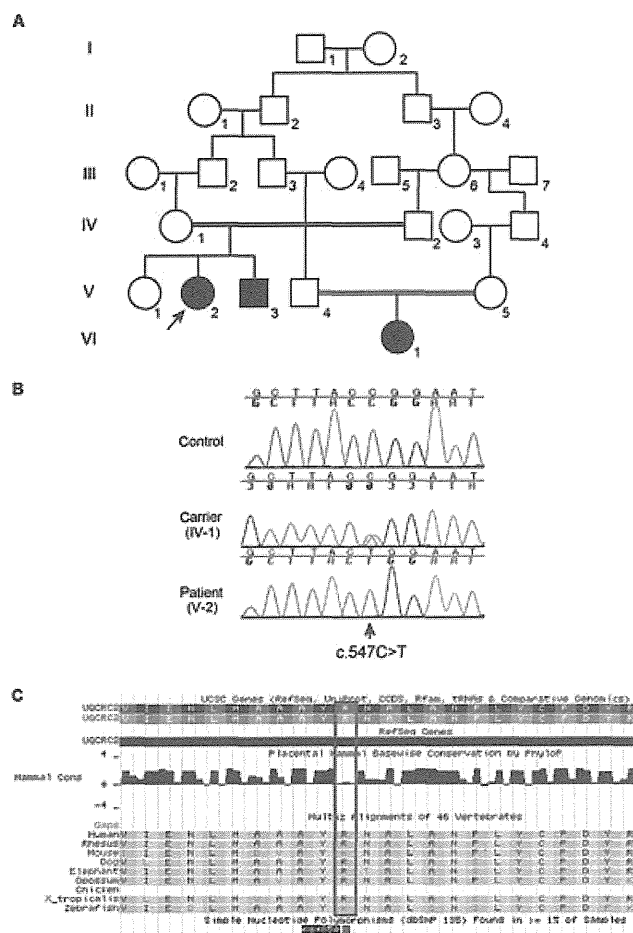
### Mitochondrial Enzyme Activity Assay

Mitochondrial enzyme activities were measured using a previously reported method [Trounce et al., 1996], with slight modifications. The complex I activity is indicated as the rotenone-sensitive NADH-CoQ1 reductase activity. In control assays, the activity was decreased to 20% by rotenone.

### Western Blotting

Mitochondrial enzyme activity and supercomplex formation were analyzed by western blotting. The enzyme activities of the mitochondrial respiratory chain complexes were measured using mitochondrial fractions prepared from primary fibroblasts derived from patient 1 ( $n = 3$ ) and control subjects ( $n = 10$ ). Each measurement was basically performed in triplicate (if the available materials allowed). The values were normalized to complex II or citrate synthase. Immunoblot detection of each respiratory chain complex was performed using mitochondria solubilized with 0.5% *n*-dodecyl- $\beta$ -D-maltoside (DDM). The same amount of pooled mitochondrial protein from control subjects ( $n = 10$ ) was used as the control. The primary antibodies used were as follows: 2 µg/ml anti-NDUFA9 (complex I; Invitrogen), 0.02 µg/ml anti-SDHA (complex II; Invitrogen), 2 µg/ml anti-UQCR1 (CIII; Abcam, Cambridge, Massachusetts), 0.2 µg/ml anti-MTCO1 (complex IV; Invitrogen), and





**Figure 1.** Identification of a *UQCRC2* mutation in a consanguineous Mexican family. **A:** Pedigree of the reported family. The arrow indicates the proband. **B:** Electropherogram of the c.547C>T *UQCRC2* mutation. All three patients (1, 2, and 3) showed a homozygous change, whereas the parents and an unaffected sibling of patients 1 and 2 were heterozygous carriers. The arrow indicates c.547C>T. **C:** Evolutionary conservation of p.Arg183 in *UQCRC2* outlined in red. rs4850 (chr 16: 21976762, G>A, p.Arg183Gln) is a common SNP based on the dbSNP135 database.

2  $\mu\text{g/ml}$  anti-ATP5B (complex V; Invitrogen). Immunoblot detection of the respiratory supercomplex was performed using mitochondria solubilized with 1% (w/v) digitonin. The same amount of pooled mitochondrial protein from control subjects ( $n = 10$ ) was used as the control. The primary antibodies used were as follows: 0.02  $\mu\text{g/ml}$  anti-SDHA (complex II; Invitrogen) and 2  $\mu\text{g/ml}$  anti-UQCRC1 (CIII; Abcam). The band intensity of the supercomplex was estimated by densitometry and normalized to that of complex II. The data were obtained by three independent assays.

## Results

Patient 1 (V:2 in Fig. 1A) is a Hispanic female born to a 26-year-old healthy female (G2P2Ab0) and a 28-year-old healthy male who are second cousins. She was delivered at 37 weeks by Cesarean section because of a pathological cardiotocogram. At birth, she weighed 2,329 g (5–10th percentile) with a length of 46 cm (5–10th percentile), and her occipitofrontal circumference was 34 cm (25–50th percentile). Her Apgar scores were 8, 9, and 9 at 1, 5, and 10 min, respectively. She developed a severe metabolic acidosis

(pH 7.1, with a base excess of  $-24.6$  mEq/l) within 1 day, requiring admission to a neonatal intensive care unit. Blood lactate and pyruvate on admission were 25.5 mM (reference range:  $<2.2$  mM) and 0.436 mM (reference range:  $<0.16$  mM), respectively (lactate to pyruvate ratio = 58.48). Clinical examination revealed tachypnea (47 breaths/min), tachycardia (181 beats/min), mild subcostal retractions, Levine II/VI systolic cardiac murmur, no organomegaly, and poor sucking reflex. Blood ammonia was 126  $\mu\text{M}$  (reference range in neonates:  $<80$   $\mu\text{M}$ ). The patient responded promptly to supportive therapy, with intravenous glucose infusion providing 10 mg/kg/min and a sodium bicarbonate drip improving the blood lactate level down to 12.7 mM within 24 hr. The lactate and pyruvate levels further improved to 3.1 and 0.125 mM within 3 days, respectively. Urine organic acid analysis on admission was remarkable for massive lactic and pyruvic aciduria, as well as ketonuria. Plasma amino acids were remarkable for a high alanine level (1,519  $\mu\text{M}$ ; reference range: 200–600  $\mu\text{M}$ ). Magnetic resonance imaging (MRI) of the brain revealed small right parietal and temporal infarcts.

She recovered without sequelae and was discharged on full oral feeds with a high-carbohydrate, reduced-fat formula (60% of calories from carbohydrate, 30% of calories from fat) after 1.5 months of hospitalization. She was also diagnosed with an atrial septal defect and renal tubular acidosis. After the initial hospitalization, she was hospitalized more than 10 times because of episodic metabolic decompensation with lactic acidosis (highest value was 10.8 mM at the age of 3 years and 10 months), hyperammonemia (highest value was 346  $\mu\text{M}$  at the age of 3 years and 3 months), ketosis, and hypoglycemia, which were triggered by intercurrent illnesses including fevers, vomiting, and diarrhea. The patient is now 5 years of age, with normal growth and no signs of intellectual disability. The frequency of hospitalization has decreased, although she still requires urgent medical treatment with intravenous glucose infusion to prevent metabolic decompensation during intercurrent illnesses.

Patient 2 (V:3 in Fig. 1A) is a younger full sibling of patient 1. He was born at 39 weeks of gestation by repeat Cesarean section. At birth, he weighed 2,658 g (5–10th percentile) with a length of 49 cm (25–50th percentile), and his occipitofrontal circumference was 34.3 cm (25th percentile). His Apgar scores were 8 and 9 at 1 and 5 min, respectively. He developed tachypnea, grunting, and poor feeding within 1 day because of lactic acidemia. The initial capillary blood gas showed a pH of 7.05,  $p\text{CO}_2$  of 25 mmHg, bicarbonate of 5.8 mmol/l, and a base excess of  $-22$  mEq/l. He was intubated for 2 days and treated with intravenous glucose infusion and a bicarbonate drip to correct the metabolic acidosis. Feeding with a high-carbohydrate, reduced-fat formula was started in 10 days. His initial hospitalization was 1-month long, during which he was diagnosed with congenital lactic acidemia and persistent hypoglycemia of unknown etiology. He was treated with corticosteroid replacement therapy owing to adrenal insufficiency for 4 months until a normal adrenocorticotrophic hormone stimulation test was obtained. At the age of 8 months, he was found unresponsive after 6 hr of fasting owing to decreased appetite associated with a 2-day mild upper-respiratory-tract infection. At a local emergency room, metabolic acidosis (pH 7.23), hypoglycemia (3 mg/dl; reference range:  $>60$  mg/dl), and hyperammonemia (463  $\mu\text{M}$ ), as well as ketosis (blood and urine), were noted. He had five episodes of generalized seizure associated with this episode. Following treatment with levetiracetam, he has been seizure free. Brain MRI findings at the age of 8 months were unremarkable. He was hospitalized for 1 month and discharged without sequelae, and had more than 10 hospitalizations because of similar episodes of lactic acidosis, hypoglycemia, hyperammonemia, and ketosis triggered by intercurrent illnesses. Developmental delay was noted once at 4 months of age. Following

physical and speech therapy, his development was later evaluated as normal at 3 years of age. He is now 4 years of age, with normal growth and no signs of intellectual disability. Physical examination revealed neither dysmorphic features nor abnormal focal neurological signs. He has been fed with a reduced-fat, high-carbohydrate diet and fasting precautions. The frequency of hospitalization has decreased, although he continues to require urgent medical treatment with intravenous glucose infusion to prevent metabolic decompensation during intercurrent illnesses. Laboratory study data obtained in the acute severe metabolic decompensation stage at 16 months of age were remarkable, which are as follows: pH 7.19 capillary blood gas, 11 mg/dl glucose, 348  $\mu$ M blood ammonia, and 6.8 mM blood lactate. Urine organic acid analysis showed markedly elevated 3-hydroxybutyrate and acetoacetate indicating severe ketosis, markedly elevated lactate and pyruvate indicating lactic acidosis, markedly elevated dicarboxylic acids (adipic acid, 1,194 mmol/mol Cr [reference range: <15 mmol/mol Cr], suberic acid, 122 mmol/mol Cr [reference range: <7 mmol/mol Cr], sebacic acid, 288 mmol/mol Cr [reference range: <2 mmol/mol Cr]) indicating hyperactive fatty acid beta oxidation, and moderately elevated tricarboxylic acid cycle intermediates including malate, fumarate, and 2-oxoglutarate. Plasma amino acids showed elevated alanine at 440  $\mu$ M (reference range: 23–410  $\mu$ M). Acylcarnitine profiles obtained at 19 months of age in mild decompensation showed marked elevation of C2 (48 nmol/ml [reference range: 2.6–15.5 nmol/ml]) and moderate elevation of 3-hydroxyacylcarnitines (C12–C18).

Patient 3 (VI:1 in Fig. 1A) is a girl born to consanguineous parents within the same pedigree as patients 1 and 2, but in a different branch. She was small for gestational age and was born vaginally to a 23-year-old mother after a full-term gestation. Her birth weight was 2,200 g. Initially, she had mild respiratory distress and required 1 additional day of monitoring. By 18 months of age, she had undergone four hospitalizations for vomiting, dehydration, and hypoglycemia. An initial blood examination at 18 months of age showed that her blood glucose was 17 mg/dl, bicarbonate was 8 mmol/l, and anion gap was 30 mmol/l. The simultaneous blood lactate and pyruvate levels were 26.3 mg/dl (reference range: <16.0 mg/dl) and 1.5 mg/dl (reference range: <1.5 mg/dl), respectively. She responded quickly to intravenous dextrose with correction of the hypoglycemia and metabolic acidosis. She had developmental delay and microcephaly (second percentile) that led to a brain MRI, but this was interpreted as normal. At 18 months, she spoke only two words but could follow two-part commands. She walked at 15 months of age and had low body weight until starting occupational therapy at 14 months of age. She was not dysmorphic. Her muscle strength and tone were normal when she was in good health, allowing her to climb, hop, and jump in a manner appropriate for her age.

Considering the consanguinity in this family, we hypothesized that the disease was inherited in an autosomal recessive fashion. Linkage analysis using two patients (1 and 2) and three unaffected family members (IV:1, IV:2, and V:1) indicated that homozygous regions totaling 36-Mb were shared by the two affected individuals with logarithm of the odds scores  $\geq 2.0$ , as calculated by Allegro version 2 [Gudbjartsson et al., 2005] (Supp. Table S1). We then performed whole-exome sequencing of DNA from patient 1. Two homozygous variants within the 36-Mb homozygous regions were identified: c.547C>T, p.Arg183Trp in *UQCRC2* (NM\_003366) and c.1675A>G, p.Met559Val in *TNRC6A* (NM\_014494). Sanger sequencing confirmed the two variants in patient 1. The Polyphen-2 program (<http://genetics.bwh.harvard.edu/pph2/>) predicted that p.Arg183Trp in *UQCRC2* and p.Met559Val in *TNRC6A* were probably damaging and benign, respectively (Table 1). *TNRC6A* was

**Table 1. Prediction of Mutational Effects in UQCRC2**

Mutation	Alteration	Type	Grantham score <sup>a</sup>	Polyphen-2	Energy ddG <sup>b</sup>
c.547C>T	p.Arg183Trp	Mutant	101	0.998	10.02
c.548G>A	p.Arg183Gln	SNP	43	0.177	2.19
c.547_548CG>AA	p.Arg183Lys	Ortholog	26	0.001	1.74

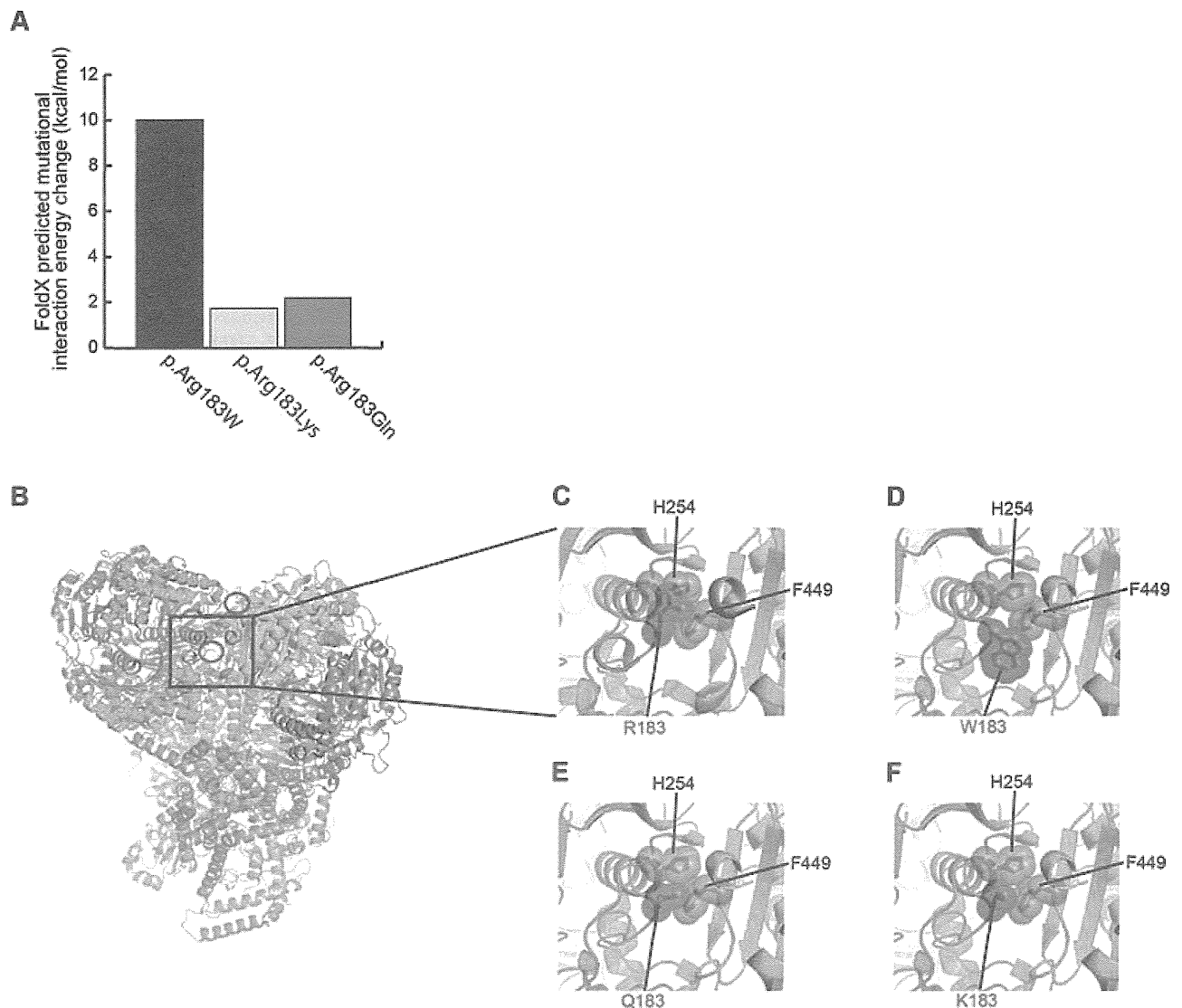
<sup>a</sup>Grantham score indicates the chemical dissimilarity caused by codon replacements.  
<sup>b</sup>The corrected average interaction energy ddG of each altered amino acid is calculated by FoldX as homozygous mutation.

ruled out as a candidate because the heterozygous *TNRC6A* change was found in patient 3. *UQCRC2* encodes ubiquinol-cytochrome *c* reductase core protein II (*UQCRC2*; MIM# 191329), a core protein of CIII. All three patients possessed the homozygous p.Arg183Trp change in *UQCRC2*, whereas the father (IV-2), mother (IV-1), and sister (V-1) (all unaffected) were heterozygous (Fig. 1B). This change was not observed among 80 Mexican control alleles or 750 Japanese control alleles.

To predict the effect of the missense mutation (c.547C>T, p.Arg183Trp) on the structural stability of CIII, we calculated the free-energy change of interactions between the core protein monomers (encoded by *UQCRC2*) with and without the mutation using FoldX software (version 3.0) [Guerois et al., 2002; Khan and Vihinen, 2010]. For this calculation, we used the crystal structure of bovine CIII (PDB code 2A06) as a structural model because no crystal structure is available for human *UQCRC2*. Amino acid position 183 of *UQCRC2* is a highly conserved basic amino acid among species from zebrafish to humans (e.g., Arg in humans and cows, Lys in mice; Fig. 1C) and is reported to be substituted for Gln as a nonsynonymous human SNP (rs4850 [c.548G>A, p.Arg183Gln]) (Fig. 1C). Therefore, we also calculated the interaction-energy change upon replacement of Arg183 with Lys or Gln, in addition to the Trp found in the patient. The calculated interaction-energy change caused by replacement of Arg183 with Trp was estimated as 10 kcal/mol, whereas those caused by replacement with Lys or Gln were no more than 2 kcal/mol (Fig. 2A, Table 1). The molecular structure of the wild-type core protein homodimer indicated that the methylene part of the Arg183 side chain of one subunit forms a hydrophobic core with the side chains of His254 and Phe449 of the other subunit at the homodimer interface (Fig. 2B and C). When the Arg183 of the core protein was replaced by Trp, the introduced Trp183 side chain flipped outward from the original side-chain position because of steric hindrance (Fig. 2D). In contrast, when Arg183 was replaced by Lys or Gln, each side chain occupied the original position to maintain a hydrophobic core with the methylene part of Lys or Gln (Fig. 2E and F). This indicates that the Arg183Trp mutation in *UQCRC2* would disrupt the hydrophobic core formed at the interface of the *UQCRC2*-containing complex, resulting in destabilization of CIII. In vitro experiments showing that the exogenous and endogenous expressions of the *UQCRC2* mutant were significantly reduced (Supp. Figs. S1 and S2) may support the protein instability.

To test whether this mutation alters *UQCRC2* localization at the mitochondrial inner membrane, we created mammalian full-length wild-type, mutant, and SNP (rs4850) constructs; transiently overexpressed them in COS1 cells; and observed their localization microscopically. The mutant protein colocalized with mitochondria, similar to the wild-type and SNP proteins (Supp. Fig. S3). This indicates that the p.Arg183Trp mutation probably does not alter the intracellular localization.

To evaluate mitochondrial function in vitro, we measured the enzyme activities of the mitochondrial respiratory chain complexes



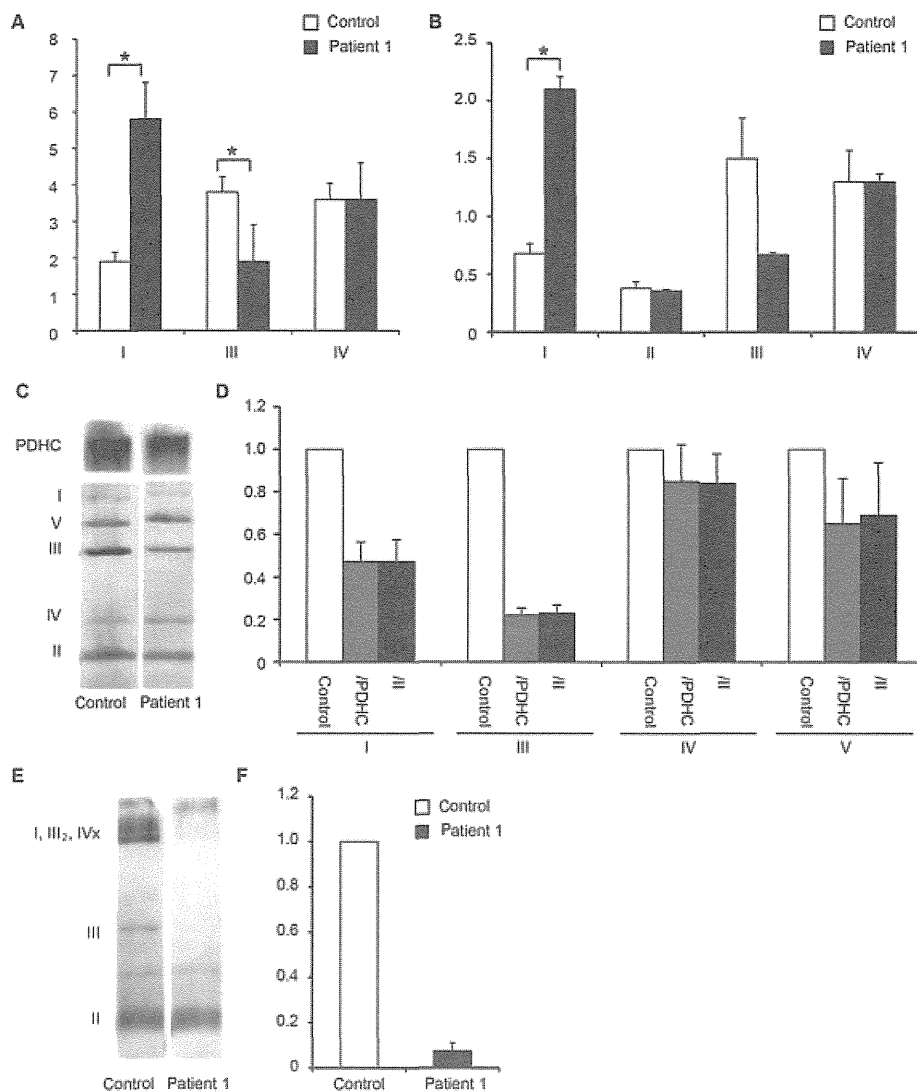
**Figure 2.** Molecular structural consideration of the effect on dimerization of amino acid replacement at residue position 183 in the core protein. **A:** Calculated interaction-energy change of the core protein homodimer upon replacement by the indicated amino acids at residue 183 using FoldX software. **B:** Overview of the crystal structure of the bovine mitochondrial bc1 (CIIII) complex (PDB code 2A06). The core protein monomers are colored green and cyan; the other components are shown in gray. The helices, strands, and loops are shown as ribbons, arrows, and threads, respectively. The red circle indicates residue 183 in the core protein. The box corresponds to the enlarged areas shown in parts (C)–(F). **C–F:** Detailed views of the core protein homodimerization interface in the wild-type (C) and mutated, polymorphic, orthologous (p.Arg183Trp/Gln/Lys) (D, E, F, respectively) complex structures. The residues at amino acid 183 of one subunit (red), and His254 and Phe449 of the other subunit (orange) are shown as sticks with Connolly surfaces. All graphics were drawn using PyMOL ([www.pymol.com](http://www.pymol.com)).

using mitochondrial fractions prepared from primary fibroblasts derived from patient 1. With normalization to complex II activity, the CIII activity of patient 1 was decreased to 50% of that in the control subjects ( $n = 10$ ), whereas complex I activity increased by threefold and complex IV activity remained at the same level as in the control subjects (Fig. 3A). Similar results were obtained using normalization to citrate synthase activity (Fig. 3B). We also investigated the steady-state level of the respiratory complexes by blue-native polyacrylamide gel electrophoresis (BN-PAGE) using the same mitochondrial fraction used for the enzyme activity measurements. For analysis of individual complexes, mitochondria were solubilized with 0.5% (w/v) DDM. For analysis of the supercomplex (complexes I, III, and IV), mitochondria were solubilized with 1% (w/v) digitonin. After BN-PAGE, we performed immunoblotting with specific antibodies for the respi-

ratory complexes (Fig. 3C–E, Supp. Notes, and Supp. Fig. S4). In the patient's fibroblasts, we found that CIII and supercomplex assembly were decreased to 18%–20%  $\gg$  22%–23% (Fig. 3C and D) and 4%  $\gg$  7.5% (Fig. 3E and F) of the levels in pooled control samples, respectively. These data indicate that a homozygous missense mutation (c.547C>T, p.Arg183Trp) in *UQCRC2* causes moderately impaired CIII function and severely decreased amounts of CIII and supercomplex, which would be the primary molecular pathogenesis in the patients.

## Discussion

Among the genes known to cause CIII deficiency, impairment of *UQCRC2*, as found in our patients, leads to a similar clinical course to that reported for *UQCRB* defects with recurrent crises of



**Figure 3.** Mitochondrial enzyme activity and supercomplex formation. **A, B:** Enzyme activities of the mitochondrial respiratory chain complexes using mitochondrial fractions prepared from primary fibroblasts derived from patient 1 ( $n = 3$ ) and control subjects ( $n = 10$ ). Each measurement was performed in triplicate. The values were normalized to complex II (**A**) or citrate synthase (**B**). Error bars represent the SEM. **C, D:** Immunoblot detection of each respiratory chain complex using mitochondria solubilized with 0.5% DDM. The same amount of pooled mitochondrial protein from control subjects ( $n = 10$ ) was loaded into the control lane. The band intensity of each respiratory complex was estimated by densitometry and normalized to that of PDHC (gray bar) or complex II (black bar). The data were obtained by three independent assays and the error bars in (**D**) represent the SEM. **E, F:** Immunoblot detection of the respiratory supercomplex using mitochondria solubilized with 1% (w/v) digitonin. The same amount of pooled mitochondrial protein from control subjects ( $n = 10$ ) was loaded into the control lane. The band intensity of the supercomplex was estimated by densitometry and normalized to that of complex II (black bar). The data were obtained by three independent assays and the error bars in (**F**) represent the SEM.

hypoglycemia, lactic acidosis, and ketosis, although the latter did not show hyperammonemia. In contrast, impairment of BCS1L, TTC19, and UQCRC2 leads to rather severe complications such as intrauterine growth retardation, liver failure, tubulopathy, sensorineural hearing loss, and abnormalities on brain MRI. The normal development in our patients, despite frequent metabolic crises, may suggest that the UQCRC2 phenotype in our family is milder than disorders of the CIII genes and that this UQCRC2 abnormality does not primarily affect the brain. However, patients 2 and 3 showed epilepsy, and developmental delay was noted in patient 3. It remains to be seen whether this clinical variability is caused by variable expressivity, unknown modifiers, or secondary to the severity of the acute metabolic crises. Interestingly, our patients showed hyperammonemia, highly abnormal urine organic acids indicative

of mitochondrial dysfunction, and highly elevated plasma hydroxyl fatty acids during their crises, whereas patients with the other reported CIII impairment disorders did not [Barel et al., 2008; de Lonlay et al., 2001; Ghezzi et al., 2011; Haut et al., 2003; Hinson et al., 2007; Visapaa et al., 2002]. These observations may imply that UQCRC2 mutations have secondary effects in other metabolic pathways including the Krebs cycle, beta oxidation, and urea cycle.

## Conclusion

We have identified, for the first time, a homozygous mutation in human UQCRC2 encoding a core protein of mitochondrial CIII. Further studies of additional patients with UQCRC2 abnormalities are necessary to fully understand human CIII disorders.