

# A Congenital Muscular Dystrophy with Mitochondrial Structural Abnormalities Caused by Defective De Novo Phosphatidylcholine Biosynthesis

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Congenital muscular dystrophy is a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy. A number of genes harboring causative mutations have been identified, but several cases of congenital muscular dystrophy remain molecularly unresolved. We examined 15 individuals with a congenital muscular dystrophy characterized by early-onset muscle wasting, mental retardation, and peculiar enlarged mitochondria that are prevalent toward the periphery of the fibers but are sparse in the center on muscle biopsy, and we have identified homozygous or compound heterozygous mutations in the gene encoding choline kinase beta (*CHKB*). This is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. In muscle of three affected individuals with nonsense mutations, choline kinase activities were undetectable, and phosphatidylcholine levels were decreased. We identified the human disease caused by disruption of a phospholipid de novo biosynthetic pathway, demonstrating the pivotal role of phosphatidylcholine in muscle and brain.

A spontaneous mutant mouse with a neonatal-onset autosomal-recessive rostral-to-caudal muscular dystrophy (*rmd* mouse) due to a loss-of-function mutation in choline kinase beta (*Chkb*) was identified in 2006.<sup>1</sup> Interestingly, *rmd* mice exhibit a unique mitochondrial morphology in muscle fibers, which show enlarged mitochondria at the periphery of the fiber but none at the center (Figure S1). These features are similar to those seen in a congenital muscular dystrophy (CMD) that we previously reported in four Japanese individuals.<sup>2</sup> We therefore screened 15 genetically undiagnosed cases of CMD with fairly homogeneous clinical features (Table 1) for mutations in choline kinase beta (*CHKB*); we included the four cases from in our previous study in these 15 cases. Features included peculiar mitochondrial changes in muscle as well as motor delay followed by the appearance of severe mental retardation and microcephaly without structural brain abnormalities (Figure 1 and Table 1).

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethical Committee of the National Center of Neurology and Psychiatry. All mouse protocols were approved by the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychi-

atry. For muscle pathology, samples of skeletal muscle were obtained from biceps brachii or quadriceps femoris in humans and from quadriceps femoris muscle in 8-week-old *rmd* mice. Muscles were frozen and sectioned at a thickness of 10  $\mu$ m according to standard procedures, and a battery of routine histochemical stains, including hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), and Oil Red O, were analyzed. For electron microscopic analysis, muscles were fixed as previously described,<sup>3</sup> and ultra-thin sections were observed at 120kV or 80kV. All affected individuals exhibited nonspecific dystrophic features (Figure 1A). However, in mGT, NADH-TR, SDH, and COX staining, prominent mitochondria at the periphery as well as central areas devoid of mitochondria were seen (Figures 1B and 1C). Oil Red O staining was unremarkable (data not shown). Electron microscopy confirmed enlarged mitochondria (Figure 1D).

We directly sequenced all exons and their flanking intronic regions in *CHKB* (MIM 612395, NM\_005198.4, GenBank Gene ID 1120) in genomic DNA extracted from individuals' peripheral lymphocytes. All 15 individuals in three different populations (Japanese, Turkish, and British) had homozygous or compound heterozygous mutations in

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**Table 1. Summary of Clinical and Laboratory Features**

Individual	Sex	Origin	Phenotypic Findings							Muscle Pathology						Mutations				Literature ref. on phenotype	
			Age at Last Follow-Up	Floppy at Birth	Walk Alone	Serum Creatine Kinase (IU/liter)	Head Circumference (percentile)	Mental Retardation	Seizure	Cardiomyopathy	Skin Change	Age at Muscle Biopsy	Necrotic Fiber	Regenerative Fiber	Endomyxial Fibrosis	Mitochondrial Enlargement	Status	cDNA	Consequence		Exon
1	F	Japanese	died at 13 yr	+	2 yr 6 mo	370	ND	+	-	+	-	7 yr 3 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
2	M	Japanese	died at 23 yr	+	1 yr 9 mo	190–2676	25–50	+	+	+	-	1 yr 2 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
3	F	Japanese	28 yr	+	1 yr 6 mo	502	ND	+	+	+	-	8 yr	+	+	+	+	het	c.116C>A	p.Ser39X	1	2
																	het	c.458dup	p.Leu153PhefsX57	3	2
4	M	Japanese	22 yr	+	2 yr 6 mo	230	3–10	+	+	-	-	4 yr 11 mo	+	+	+	+	het	c.116C>A	p.Ser39X	1	
																	het	c.458dup	p.Leu153PhefsX57	3	
5	M	Turkish	7 yr	-	2 yr 6 mo	843	<3	+	-	-	+	6 yr	±	+	+	+	homo	c.611_612insC	p.Thr205AsnfsX5	5	
6 <sup>a</sup>	M	Turkish	died at 2 yr 6 mo	+	no	258	<3	+	-	+	-	1 yr 3 mo	±	±	+	+	homo	c.922C>T	p.Gln308X	8	
7	F	Turkish	2 yr	-	no	368	3–10	+	-	<sup>b</sup>	-	9 mo	-	±	+	+	homo	c.847G>A	p.Glu283Lys	8	
8	M	Turkish	13 yr	ND	2 yr	1122	ND	+	-	-	-	12 yr 10 mo	±	±	+	+	homo	c.1130 G>T	p.Arg377Leu	11	
9	F	Turkish	17 yr	+	3 yr	2669	<3	+	-	ND	-	17 yr	±	±	+	+	homo	c.554_562del	p.Pro185_Trp187del	4	
10	F	Turkish	16 yr	+	3 yr	1103	<3	+	-	<sup>c</sup>	+	3 yr	-	±	+	+	homo	c.677+1G>A	ND	5	
11	F	Turkish	3 yr 3 mo	+	no	497	10–25	+	-	ND	-	3 yr	±	-	+	+	homo	c.677+1G>A	ND	5	
12	F	Turkish	5 yr	-	3 yr 6 mo	467	25–50	+	-	<sup>d</sup>	+	4 yr 6 mo	±	+	+	+	homo	c.677+1G>A	ND	5	
13	M	Turkish	3 yr 6 mo	+	no	428	<3	+	-	+	+	3 yr	+	+	+	+	homo	c.1031+1G>A	aberrant splicing	9	
14	F	Turkish	6 yr 4 mo	-	1 yr 3 mo	1606	3–10	+	-	+	-	4 yr	+	+	+	+	homo	c.1031+1G>A	ND	9	
15	M	British	died at 8 yr	-	3 yr 4 mo	607–1715	<3	+	-	+	+	2 yr 2 mo	+	-	+	+	homo	c.852_859del	p.Trp284X	8	

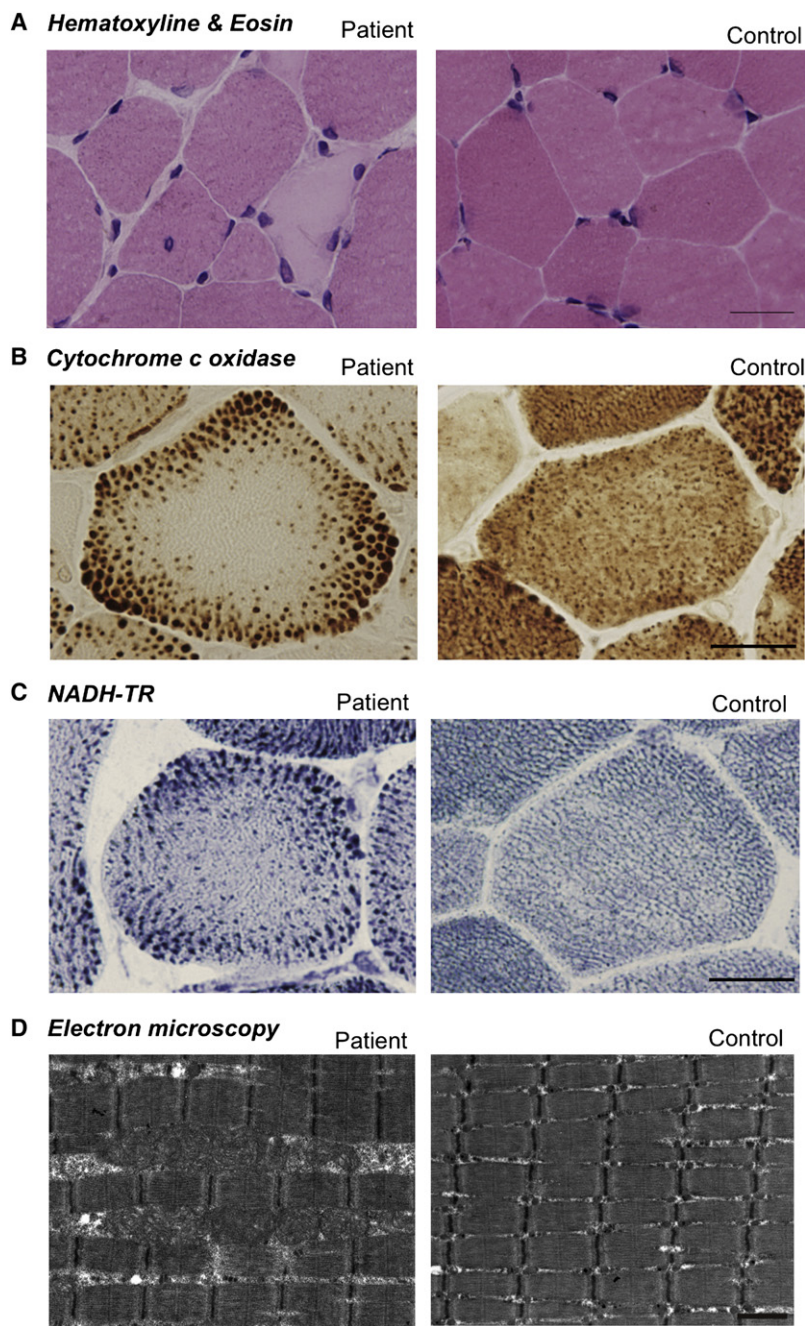
Detailed clinical information for individual 1 to 4 was previously described (2). Eleven CHKB mutations were identified in 15 affected individuals. All exhibited generalized muscle hypotonia and weakness from early infancy. Ambulation was delayed, and gait in those who achieved walking was limited. In addition, all displayed marked mental retardation, and most never acquired meaningful language. Microcephaly with head circumferences at or below the 3<sup>rd</sup> to 10th percentile was observed in most cases. Cranial magnetic resonance imaging showed no developmental brain defects. Six individuals had dilated cardiomyopathy, and two had cardiac anomaly. Individuals 1, 2, 6, and 15 died from cardiomyopathy at ages 13 yr, 23 yr, 2 yr 6 mo, and 8 yr, respectively. No one had respiratory insufficiency. Ichthyosiform skin changes were frequent. All showed mildly to moderately elevated serum creatine kinase (CK) levels. Individuals 7 and 9 also had homozygous single-nucleotide substitutions, c.902C>T (p.Thr301Ile) and c.983A>G (p.Gln328Arg), respectively. CHK activities of recombinant CHK-β proteins with p.Thr301Ile and p.Gln328Arg were only mildly decreased (Figure S2), suggesting these are likely to be neutral polymorphisms or only mildly hypomorphic mutations. Individuals 10, 11, and 12, who have same c.677+1G>A mutation, and individuals 13 and 14, who have same c.1031+1G>A mutation, are not siblings. Abbreviations are as follows: ND, not determined; p, percentile; F, female; and M, male.

<sup>a</sup> An affected sibling had ichthyosis and died at age 6 years with cardiomyopathy.

<sup>b</sup> Patent ductus arteriosus.

<sup>c</sup> Atrial septal defect.

<sup>d</sup> Mitral valve prolapse.



**Figure 1. Muscle Pathology of the Affected Individuals**

Cross-sections of muscle fiber from a human control and individual 4.

(A) On H&E staining, nonspecific dystrophic features with necrotic and regenerating fibers, internalized nuclei, and endomysial fibrosis are seen. The scale bar represents 25  $\mu$ m.

(B) On cytochrome c oxidase staining, enlarged mitochondria at the periphery and central areas devoid of mitochondria were seen. The scale bar represents 20  $\mu$ m.

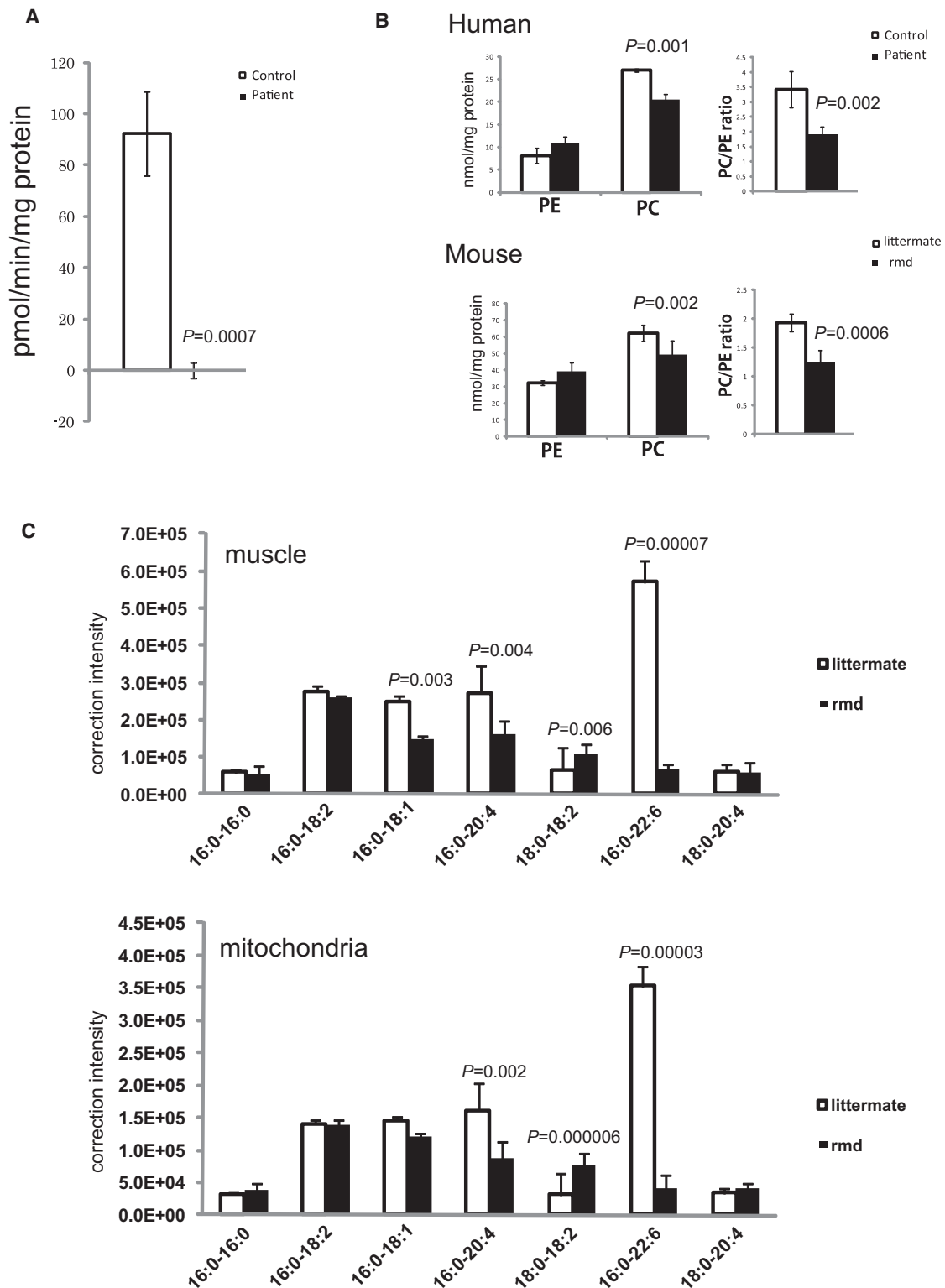
(C) On NADH-TR staining, the intermyofibrillar network was preserved even in the central areas that are devoid of mitochondria, suggesting the presence of myofibrils and only absence of mitochondria. The scale bar represents 20  $\mu$ m.

(D) Electron microscopy confirmed enlarged mitochondria. The scale bar represents 1  $\mu$ m.

members of individual 1 and 2 were not available. Individuals 3 and 4 (siblings, Japanese) had the same compound heterozygous mutation c.116C>A (p.Ser39X) and c.458dup (p.Leu153PhefsX57). Both parents were healthy, and the father was heterozygous for mutation c.116C>A (p.Ser39X), whereas the mother was heterozygous for mutation c.458dup (p.Leu153-PhefsX57), thus confirming a recessive inheritance pattern. These mutations cosegregated with the disease phenotype in all family members tested.

We therefore measured CHK activity in biopsied muscle. For all biochemical analyses, because of the limiting amounts of remaining tissue, biopsied muscle samples were available only from individuals 2, 3, and 4. Biopsied muscle samples from these three individuals were homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.5), 154 mM KCl, and 1 mM phenylmethanesulfonyl fluoride with a sonicator (MISONIX), and supernatant fractions (105,000  $\times$  g, 60 min) were prepared and analyzed for CHK activity as previously described.<sup>6</sup> Similar to muscles of *rmd* mice,<sup>1</sup> muscles from individuals 2, 3, and 4, who carried homozygous or compound heterozygous nonsense mutations, did not have any detectable CHK activity (Figure 2A). Individuals 7, 8, and 9 had homozygous missense mutations c.847G>A (p.Glu283Lys) and c.1130 G>T (p.Arg377Leu) and a homozygous 3 amino acid deletion, c.554\_562 del (p.Pro185\_Trp187del), respectively. We screened 210 control chromosomes for the identified missense mutations and small in-frame deletion by direct sequencing or single-strand conformation polymorphism (SSCP) analysis. SSCP was performed with Gene Gel Excel (GE Healthcare) as previously described.<sup>7</sup> These missense mutations and this small in-frame deletion were not identified in control

*CHKB* (Table 1). Among a total of 11 mutations identified, six were nonsense, two were missense, one was a 3 amino acid deletion, and two were splice-site mutations. The six nonsense mutations, c.116C>A (p.Ser39X), c.458dup (p.Leu153PhefsX57), c.611\_612insC (p.Thr205AsnfsX5), c.810T>A (p.Tyr270X), c.852\_859del (p.Trp284X), and c.922C>T (p.Gln308X), were predicted to truncate the protein and eliminate highly conserved domains of CHK.<sup>4,5</sup> Individuals 1 and 2 (unrelated, Japanese) had the same homozygous nonsense mutation of c.810T>A (p.Tyr270X). Individual 2's mother, who was healthy, had the heterozygous c.810T>A (p.Tyr270X) mutation. Unfortunately, a DNA sample from the father of individual 2 was not available. DNA samples from other family



**Figure 2. Choline Kinase Activity and Phospholipid Analyses**

(A) In muscle tissue from individuals 2, 3, and 4, CHK activity cannot be detected ( $n = 3$ ). Data represent the mean of three individuals. (B) PC and PE content in frozen biopsied muscle tissues from individuals 2, 3, and 4 and hindlimb muscles from 8-week-old *rmd* mice ( $n = 4$ ) and control littermates ( $n = 5$ ) were analyzed by thin-layer chromatography followed by phosphorus analysis. PC and the PC/PE ratio are significantly decreased in affected individuals and *rmd* mice ( $n = 3$  for humans,  $n = 4$  for *rmd* mice,  $n = 5$  for littermates). (C) Fatty acid composition of PC molecular species in muscles and isolated mitochondria from hindlimb muscles of *rmd* mice are determined by electrospray ionization mass spectrometry (ESI-MS). We observed that 34:1-PC (16:0-18:1), 36:4-PC (16:0-20:4), and 38:6-PC (16:0-22:6) species are significantly decreased, whereas 36:2-PC (18:0-18:2) is increased in *rmd* muscle. Similarly, in isolated mitochondria from hindlimb muscle, 36:4-PC (16:0-20:4) and 38:6-PC (16:0-22:6) species are decreased, whereas 36:2-PC (18:0-18:2) is increased.

chromosomes. To elucidate the pathogenesis of these substitutions, we measured CHK activity in recombinant proteins with mutations. We cloned the open reading frame of *CHKB* into pGEM-T easy (Promega), then subcloned it into pET15b (Novagen) to make His-tagged CHK- $\beta$ .<sup>8</sup> Each mutation was induced by site-directed mutagenesis.<sup>7</sup> Plasmids were transformed into *Escherichia coli* strain BL21 (DE3) and inoculated at 20°C to an OD<sub>600</sub> of approximately 0.5, and the addition of 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside induced expression. The His-tagged CHK- $\beta$  proteins were subjected to affinity purification on a nickel column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 300 mM imidazol, and 1 mM phenylmethanesulfonyl fluoride, and 25 ng protein was analyzed for CHK activity. CHK activity of recombinant proteins with these mutations decreased to less than 30% of wild-type CHK activity, suggesting that these mutations are causative in these individuals (Figure S2). For individual 13, who had a mutation at the splice site of the exon-intron border after exon 9 (c.1031+1G>A), we also analyzed cDNA sequences. Exons 4 through 10 were amplified from the first-strand cDNAs, and direct sequencing followed. cDNA analysis of *CHKB* in skeletal muscle from individual 13 showed four splicing variants, all of which remove consensus domains for *CHKB* (Figure S3). This suggests the same loss-of-function mechanism in humans and *rmd* mice.

Because phosphorylation of choline by CHK is the first enzymatic step for phosphatidylcholine (PC) biosynthesis,<sup>9</sup> we anticipated that PC content should be altered in affected individuals' muscles. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and total phospholipid amounts were measured in biopsied muscles from individuals 2, 3, and 4 and in leg muscles from 8-week-old *rmd* mice by either one-dimensional or two-dimensional thin-layer chromatography (TLC) followed by phosphorus analysis.<sup>10,11</sup> As expected, PC levels decreased in affected individuals' skeletal muscle (Figure 2B), as they did in *rmd* mice (Figure 2B and Sher et al.<sup>1</sup>), suggesting that the CMDs due to *CHKB* mutations in humans and *rmd* mice are not only pathologically but also pathomechanistically similar.

PC is present in all tissues and accounts for around 50% of phospholipids in biological membranes in eukaryotes. Selective tissue involvement can be explained by the different tissue distribution of CHK isoforms. There are two CHK isoforms: CHK- $\alpha$  and CHK- $\beta$ , encoded by distinct genes, *CHKA* (MIM 118491) and *CHKB*, respectively. They

are known to form both homodimers and heterodimers, with differential tissue distribution.<sup>12</sup> In mice, disruption of *Chka* causes embryonic lethality,<sup>13</sup> suggesting the importance of CHK- $\alpha$  in embryonic development. In skeletal muscles from *rmd* mice, CHK activity is absent, and PC levels are decreased.<sup>1</sup> In other tissues, however, CHK activity is only mildly decreased, PC levels are not altered, and no obvious pathological change is seen.<sup>1</sup> CHK activity in skeletal muscle from individuals 2, 3, and 4 is barely detectable, and PC levels are significantly decreased, suggesting that CHK- $\beta$  is the major isoform in human skeletal muscle. In support of this notion, CHK- $\alpha$  was not detected in human muscle (Figure S4). These results suggest that muscular dystrophy in affected individuals and *rmd* mice is caused by a defect in muscle PC biosynthesis. In addition, in *rmd* mice, hindlimb muscles are more significantly affected than forelimb muscles.<sup>1</sup> This is most likely explained by the fact that CHK activity is detected, though decreased, in forelimb muscles in *rmd* mice as a result of the continued post-natal expression of *Chka*.<sup>14</sup> This indicates that the severity of muscle involvement is determined by the degree of deficiency of CHK activity.

Generally, phospholipids have saturated or monounsaturated fatty acids at the *sn*-1 position and polyunsaturated fatty acids at the *sn*-2 position of glycerol backbone.<sup>15</sup> It has been shown that phospholipids have tissue-specific fatty acid composition.<sup>15</sup> For example, heart PC and muscle PC mainly contain docosahexaenoic acid (22:6) (Nakanishi et al.<sup>15</sup> and Figure 2C), but liver PC includes various fatty acids.<sup>15</sup> NanoESI-MS analyses of PC molecular species in muscle and isolated mitochondria were performed with a 4000Q TRAP (AB SCIEX, Foster City, CA, USA) and a chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA).<sup>16</sup> Quadriceps femoris (hindlimb) and Triceps (forelimb) muscle from affected *rmd* mice and littermate controls were frozen with liquid nitrogen, and total lipid was extracted by the Bligh and Dyer method.<sup>10</sup> The ion spray voltage was set at -1.25kV, gas pressure at 0.3 pound per square inch (psi), and flow rates at 200 nl/min. The scan range was set at *m/z* 400~1200, declustering potential at -100V, collision energies at -35~-45V, and resolutions at Q1 and Q3 "unit." The mobile phase composition was chloroform:methanol (1/2) containing 5 mM ammonium formate and was normalized to the muscle weight. The total lipids were directly subjected by flow injection, and selectivity was analyzed by neutral loss scanning of the polar head

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In muscle and isolated mitochondria, the 38:6-PC molecular species is profoundly decreased (n = 6 for muscle, n = 5 for isolated mitochondria).

Mitochondria from skeletal muscles of whole hindlimbs of *rmd* mice were isolated by the differential centrifugation method. Fresh muscle was minced and homogenized with a motor-driven Teflon pestle homogenizer with ice-cold mitochondrial isolation buffer (10 mM Tris-HCl [pH 7.2], 320 mM sucrose, 1mM EDTA, 1mM DTT, 1 mM PMSF, 1 mg/ml BSA, and protease inhibitor cocktail [Roche]) and centrifuged at 1,500  $\times$  g for 5 min. The supernatant fraction was centrifuged at 15,000  $\times$  g for 20 min, the pellet was resuspended in mitochondrial isolation buffer, and the centrifugation/resuspension was repeated twice more.

All data are presented as means  $\pm$  standard deviation (SD). Means were compared by analysis with a two-tailed t test via R software version 2.11.0.

group for PC in negative-ion mode.<sup>17</sup> Interestingly, there was a 10-fold decrease (9.8%) in the 16:0-22:6-PC levels versus the control in *rmd* hindlimb muscle and also in muscle mitochondria (Figure 2C), indicating the importance of the PC de novo synthesis pathway for maintaining not only PC levels but also fatty acid composition of PC molecular species. Similarly, in forelimb muscle 16:0-22:6 PC levels were also decreased in comparison to the control, but to a milder extent (18.2%), suggesting an association between severity of muscle damage and fatty acid composition alteration of PC (data not shown). In *rmd* mice, it has been shown that muscle PC can be delivered from plasma lipoprotein,<sup>18</sup> suggesting that non-decreased PC molecular species might be derived from the plasma, whereas 16:0-22:6 PC might be synthesized only in muscle (and possibly in brain). However, confirmation of this requires further studies.

Individuals with *CHKB* mutations have severe mental retardation in addition to the muscular dystrophy. Interestingly, polymorphisms near the *CHKB* locus and decreased *CHKB* expression have been associated with narcolepsy with cataplexy, suggesting a link between *CHK-β* activity and the maintenance of normal brain function in humans.<sup>19</sup> Furthermore, brain damage in pneumococcal infection has been attributed to the inhibition of de novo PC synthesis, suggesting the importance of PC synthesis for the brain.<sup>20</sup> Our data provide evidence that altered phospholipid biosynthesis is a causative agent for a human congenital muscular dystrophy, and further studies will elucidate the detailed molecular mechanisms of the disease in both muscle and brain.

### Supplemental Data

Supplemental Data include four figures 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:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>

R software version 2.11.0, <http://www.r-project.org/>

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