Pathogenic Variants in the Myosin Chaperone UNC-45B Cause Progressive Myopathy with Eccentric Cores

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5 **Keywords**
6 UNC-45, *UNC45B*, myosin, chaperone, core myopathy, muscle, *C. elegans*
7

8 **Abstract**

9 **The myosin-directed chaperone UNC-45B is essential for sarcomeric organization**
10 **and muscle function from *Caenorhabditis elegans* to humans. The pathological**
11 **impact of UNC-45B in muscle disease remained elusive. We report ten individuals**
12 **with biallelic variants in the *UNC45B* gene, who exhibit childhood onset**
13 **progressive muscle weakness. We identified a common *UNC45B* variant which acts**
14 **as a complex hypomorph splice variant. Purified UNC-45B mutant proteins showed**
15 **changes in folding and solubility. *In situ* localization studies further demonstrated**
16 **reduced expression of mutant UNC-45B in muscle combined with abnormal**
17 **localization away from the A-band towards the Z-disk of the sarcomere. The**
18 **physiological relevance of these observation was investigated in *C. elegans* by**
19 **transgenic expression of conserved UNC-45 missense variants, which showed**
20 **impaired myosin binding and defective muscle function for one. Together, our**
21 **results demonstrate that UNC-45B impairment manifests as a chaperonopathy with**
22 **progressive muscle pathology, which discovers the previously unknown,**
23 **conserved role of UNC-45B in myofibrillar organization.**

Introduction

Muscle development and function require a complex system of structural and motor proteins organized into contractile units referred to as sarcomeres. The sarcomeric repeat is a supra-molecular dynamic structure, in which actin and myosin filaments, together with associated proteins, are arranged in a precise order. The near crystalline lattice of the sarcomere coordinates actin-myosin cross bridge cycling, which facilitates sarcomere shortening, filament gliding, and muscle contraction. The folding, stability, and organization of sarcomeric proteins into muscle filaments is governed by molecular chaperones[1]. The fundamental importance of chaperones for the development and maintenance of skeletal muscle is underscored by recent studies indicating that chaperone dysfunction is responsible for a distinct subset of hereditary myopathies. These so called chaperonopathies are characterized by pathogenic variants in genes encoding chaperones and co-chaperones of structural muscle components. For example, loss of the small heat shock protein CRYAB (*MIM:* 123590) affects folding of the muscle intermediate filament desmin, clinically manifesting as a myofibrillar myopathy known as αB-crystallinopathy[2]. Additional disease mechanisms include impaired homeostasis of chaperone-assisted selective autophagy (CASA) linked to pathogenic variants in the HSP70 co-chaperone *BAG3* (*MIM:* 603883), clinically manifesting with a progressive myofibrillar myopathy with significant cardiac as well as peripheral nerve pathologies[3; 4]. The subsequent identification of variants in *DNAJB6* (*MIM:* 611332) causing limb-girdle muscular dystrophy (LGMDD1) provided clear evidence of abnormal sacromeric aggregate pathology in these chaperonopathies[5; 6]. The clinical spectrum of chaperonopathies has expanded over the last years to include hereditary motor

1 neuropathies with or without muscle involvement, caused by *HSPB1 (MIM: 602195)*, 2 *HSPB3 (MIM: 604624)*, *HSPB8 (MIM: 608014)*, and *DNAJB2 (MIM: 604139)* gene 3 defects[7]. These examples suggest that abnormal chaperone function is an important 4 driver in neuromuscular disease, suggesting that its correction might be a valid 5 therapeutic approach[8].

6 The abovementioned chaperones are involved in the proper folding and stability of 7 aggregation prone proteins in various cell types including muscle. In contrast to the broad 8 functional spectrum of many molecular chaperones, more specialized chaperone 9 systems exist which regulate muscle contraction by folding and assembly of conventional 10 type II myosin[9]. Type II myosins are composed of two myosin heavy chains each 11 containing an identical myosin head, whose folding requires precise temporal and spatial 12 control, mediated by conserved UCS (UNC-45/CRO1/She4p) domain containing 13 proteins[10-12]. As one founding member of the UCS family, UNC-45 (MIM: 611220) was 14 first identified in *C. elegans* revealing that conditional loss-of-function mutations result in 15 abnormal myofilament assembly and *uncoordinated* locomotion defects[13]. UNC-45 16 homologs exist in all vertebrates, and various point mutations have been associated with 17 skeletal and cardiac function in *C. elegans*, *Drosophila*, zebrafish, and *Xenopus*[14; 15]. 18 UNC-45 contains four domains: an N-terminal TPR domain (TPR repeat 1–3), a 19 conserved central domain (ARM repeat 4–5), a neck domain (ARM repeat 6–9), and a C-20 terminal UCS domain (ARM repeat 10–17) (Figure 1A)[16; 17]. The N-terminal TPR domain 21 is important for binding to the chaperones Hsp70 and Hsp90, while the C-terminal UCS 22 domain associates with the head of muscle myosin[18]. Mechanistically, UNC-45 oligomers 23 have been described to serve as a multisite-docking platform, which supports precisely

1 defined collaboration with the general chaperones Hsp70 and Hsp90 in folding and
2 assembly of myosin filaments[17]. Thus, UNC-45 provides substrate specificity for the
3 partner chaperones during late stages of myofibrillogenesis (Figure 1B). The repetitive
4 arrangement of UNC-45 oligomers with myosin binding UCS domains protruding from the
5 linear protein chain serves as template that defines the periodicity of myosin organization
6 in growing sarcomeres.

7 The muscle sarcomere is a complex structure permanently challenged by
8 mechanical stress, which presupposes the dynamic coordination between protein folding
9 and degradation pathways[1; 18]. Based on its central role as myosin-directed chaperone,
10 both UNC-45 stability and localization are precisely regulated. UNC-45 protein
11 degradation is mediated by ubiquitin-dependent proteolysis, which coordinates myosin
12 folding and assembly both in *C. elegans* and human myoblasts[18; 19]. Conclusively,
13 defective degradation of UNC-45B in individuals with *VCP*-related inclusion-body
14 myopathy (MIM: 601023) is linked to disorganized myofibrils and impaired sarcomeric
15 function[19].

16 Despite the conserved role in myofibrillogenesis and evidence from multiple model
17 organisms, the relevance of muscle-specific UNC-45B dysfunction for the pathology and
18 pathogenesis of human myopathies had remained unclear[19]. Here we present
19 comprehensive findings that establish biallelic pathogenic variants in *UNC45B* (MIM:
20 611220) as a novel chaperonopathy, clinically manifesting as a progressive myopathy
21 with recognizable muscle eccentric core histology in humans.

22 **Material & Methods**

23 *Recruitment and sample collection*

1 Individuals were identified through their local neurology and genetics clinics. P3 and P4
2 were identified through GeneMatcher[20]. Written informed consent and age-appropriate
3 assent for study procedures were obtained by a qualified investigator [protocol 12-N-
4 0095 approved by the National Institute of Neurological Disorders and Stroke, National
5 Institutes of Health Institutional Review Board (IRB); Project ID: 07/N018, Research
6 Ethics Committee (REC) Ref: 07/Q0512/26 approved by the UCLH local institutional IRB;
7 Protocol 317-05 approved by the Regional Ethical Review Board in Gothenburg (317-
8 05)]. Medical history was obtained and clinical evaluations, including muscle MRI and
9 muscle biopsy, were performed as part of the standard diagnostic examination. Muscle
10 MRI included T1 axial images of the lower extremities. Muscle biopsy histology slides
11 and electron microscopy images (EM) were independently reviewed. DNA and muscle
12 biopsy samples were obtained according to standard procedures.
13
14 *Whole Exome and RNA Sequencing*
15 Whole exome sequencing (WES) was pursued by six independent teams, details are
16 provided in supplemental methods. Confirmation of individuals' variants and available
17 family members was performed by Sanger sequencing. RNA sequencing on RNA
18 extracted from muscle was pursued for P2, P3, and P4 muscle, details can be found in
19 supplemental methods.
20
21 *Western blot*
22 Skeletal muscle biopsy sections from a normal control and affected individuals P1, P2,
23 P9, and P10 were homogenized using lysis buffer contain 4 % SDS, 125 mM Tris-HCL

(pH 8.8), 40% Glycerol, 500 μM PMSF and 100 mM DTT. The lysates were sonicated on ice followed by centrifugation (14000 rpm for 15 minutes at 4°C). The protein from the supernatants was electrophoresed on NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA) under reducing condition followed by a transferring to nitrocellulose membrane (Millipore, Billerica, MA). After block with Odyssey PBS blocking buffer (LI-COR, Lincoln, NE), the membrane was incubated with primary antibody anti-UNC-45B (Sigma-Aldrich, St. Louis, MO), anti-desmin (Sigma-Aldrich, St. Louis, MO) overnight at 4°C and subsequently incubated with IRDye@ 680RD Goat anti-Rabbit IgG and IRDye@ 800CW Goat anti-Mouse IgG secondary antibodies (LI-COR, Lincoln, NE) at room temperature and imaged on the Odyssey CLx Imaging System (LI-COR, Lincoln, NE).

Immunostaining & microscopy

Pre-cooled 100% methanol fixed 8 μm muscle longitudinal sections were blocked in PBS with 10% goat serum and 0.1% Triton X-100, then incubated with primary antibodies anti-UNC-45B (Sigma-Aldrich, St. Louis, MO) and anti-myomesin (DSHB, Iowa City, IA) overnight at 4°C. The antibody labeling was detected with secondary antibodies Alexa488-conjugated goat anti-mouse IgG and Alexa568-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature. Prepared muscle sections were imaged with a Zeiss Airy microscope (Zeiss, Germany). Images were analyzed using ImageJ software (National Institutes of Health).

Human UNC-45B protein structure modelling and PyMOL structural analysis

1 For human UNC-45B protein structure modelling, the SWISS-MODEL online tool was
2 used[21]. *C. elegans* UNC-45 (PDB: 4i2z) was indicated as reference crystal structure. For
3 further structural analyses the PyMOL 2.3.3 software (Schrödinger) and proprietary
4 scripts were used. The modelled human UNC-45B protein structure was displayed and
5 single amino acid mutations inserted using the mutagenesis wizard tool choosing the
6 rotamer with the lowest root-mean-square deviation value. Steric and electrostatic
7 interactions were depicted using the show_bumps plugin and calculations of van der
8 Waals overlaps.
9
10 *Cloning, protein expression, and purification*
11 Standard molecular biology protocols were used[22]. Human UNC-45B cDNA was cloned
12 into the *E. coli* expression vector pET21a with an N-terminal myc-tag and a C-terminal
13 6xHIS-tag using the NEBuilder Master Mix (New England Biolabs). The vector was
14 mutated using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) to introduce
15 the following mutations: Arg754Gln, Arg778Trp, Ser403Pro, and Cys514Arg. A pET21a
16 vector encoding myc-UNC-45-6xHIS amplified from *C. elegans* cDNA was mutated using
17 the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) to introduce the following
18 mutations: Arg767Gln and Arg792Trp. Oligonucleotides used in this study are listed in
19 Table S2. Overexpression in *E. coli* BL21-CP was induced with 100 µM IPTG and carried
20 out at 16–18°C for 18–20 h. Subsequently, cells were harvested by centrifugation and
21 lysed by sonication in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole. The
22 tagged proteins were affinity purified by submitting the cleared bacterial lysates to Ni-NTA
23 agarose-binding (QIAGEN) according to the manufacturer's instructions. After

exchanging the buffer to 20 mM Tris (pH 8.0), 150 mM NaCl, protein amount was determined by Coomassie staining of SDS-PAGE gels with bovine serum albumin as standard.

Partial trypsin proteolysis

For limited proteolysis assays, 2.4 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-UNC-45B(Arg754Gln)-6xHIS, myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-45B(Ser403Pro)-6xHIS, myc-UNC-45B(Cys514Arg)-6xHIS, myc-UNC-45-6xHIS, myc-UNC-45(Arg767Gln)-6xHIS and myc-UNC-45(Arg792Trp)-6xHIS, diluted in PBS (pH 7.4) were incubated with 20 ng of trypsin (SERVA) for 1, 2, 5, 10, 20, 40, or 60 min at room temperature (~22°C) or 37°C. A control reaction was incubated with PBS instead of trypsin for 60 min (time point 0). Reactions were stopped by adding 5x SDS sample buffer (0.25 M Tris-HCl, 10% SDS, 50% glycerol, 0.5 M DTT, 0.25% bromophenol blue) and flash freezing in liquid nitrogen. After collecting, all samples were boiled at 95°C for 5 min, run on SDS-PAGE gels and stained with Instant Blue Coomassie stain (Expedeon). Images were taken with an Odyssey CLx Imager (LI-COR Biotechnology) using the 700 nm channel, intact protein bands were quantified using Image Studio Version 5.2 software and relative fluorescence signal compared to time point 0 was plotted.

Thermal shift assay with SYPRO Orange

10 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-UNC-45B(Arg754Gln)-6xHIS, myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-45B(Ser403Pro)-6xHIS, myc-UNC-45B(Cys514Arg)-6xHIS were diluted in ice-cold 20 mM Tris (pH 8.0), 150 mM NaCl, and

2.5x SYPRO Orange protein stain and heated in 0.5° increments from 10°C to 95°C in a CFX Real-Time PCR Cycler (Bio-Rad). Melt curves were recorded using the FRET channel, normalized to buffer-only control and fitted to Boltzmann sigmoidal curve regression in GraphPad Prism 5 software. Half maximal temperatures were read as melting temperatures.

Filter trap assay and slot blot

20 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-UNC-45B(Arg754Gln)-6xHIS, myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-45B(Ser403Pro)-6xHIS, myc-UNC-45B(Cys514Arg)-6xHIS were diluted in ice-cold 20 mM Tris (pH 8.0), 150 mM NaCl and incubated rotating at room temperature (22°C) for 1 h. Three decreasing amounts of protein solution were loaded onto a 0.2 µm cellulose acetate membrane assembled in a slot blot apparatus (Bio-Rad). The membrane was washed with PBS, 0.2% SDS and retained aggregated protein was assessed by immunoblotting for myc-tag (9E10, Roche).

C. elegans maintenance and transgenic lines

Unless stated otherwise, nematodes were grown at 15°C on nematode growth medium (NGM) plates seeded with the bacterial *E. coli* strain OP50 as a food source according to standard protocols and methods[23; 24]. The N2 Bristol strain served as wild-type. Additional strains used in this study are *unc-119(ed4)III*, *unc-45(m94)III*, and *unc-45(m94)III; hhIs84[unc-119(+); unc-54::unc-45^{FLAG}]*[17]. For the generation of transgenic rescue strains, plasmids encoding C-terminally FLAG-tagged UNC-45 under the muscle-specific promoter *unc-54*, containing the *unc-119(+)* selection marker, generated in reference[17]

1 were mutated using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). The
2 following conserved mutations were introduced: Arg767Gln, Arg792Trp, Ile422Pro, and
3 Cys523Arg. Oligonucleotides used in this study are listed in Table S2. These constructs
4 were bombarded into *unc-119(ed4)* worms as described previously[25]. Microparticle
5 bombardment was done with the Bio-Rad Biolistic PDS-1000/HE with ¼" gap distance, 9
6 mm macrocarrier to screen distance, 28 inches of Hg vacuum and a 1350 psi rupture
7 disc. Per bombardment, about 1 mg of 1 µm microcarrier gold beads were coated with 8-
8 10 µg linearized DNA. Animals were allowed to recover for 1 h at room temperature and
9 were then transferred to 90 mm NGM plates seeded with *E. coli* OP50 bacteria. After 3
10 weeks at 25°C, motile non-*unc* worms were singled and screened for homozygosity. All
11 strains that were used in this study are listed in Table S3.
12
13 *Motility assay*
14 For body bend assays, individual young adult worms grown at 25°C were placed in 1 ml
15 M9 buffer (room temperature 22°C), body bends were counted during 30 s, and doubled
16 to calculate body bend counts per minute.
17
18 *Quantification of I-Bands assembly*
19 Sarcomere assembly was monitored by labeling F-actin with phalloidin-rhodamine
20 (Invitrogen). Briefly, synchronized young adult worms were fixed in 4% (w/v)
21 paraformaldehyde solution for 20 min at room temperature. After permeabilization of the
22 cuticle in a 3% β-mercaptoethanol solution containing 1% Triton X-100, the F-actin in
23 body wall muscle sarcomeres was stained with phalloidin-rhodamine (Invitrogen). Stained

worms were mounted on glass slides and imaged using an Axio Imager.Z1 microscope (Zeiss). The number of I-bands per body wall muscle cell was counted in the same area (between pharynx and vulva).

Co-immunoprecipitation studies

For co-immunoprecipitations, synchronized young adult wild-type worms, *unc-45(m94)*, or worms expressing transgenic, FLAG-tagged UNC-45 were sonicated in lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, and protease inhibitor mix (Roche)), and immunoprecipitation was performed using the µMACS DYKDDDDK Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions. Briefly, 150 µg of worm lysates were incubated with 50 µl Anti-DYKDDDDK MicroBeads for 45 min at 4°C. Immunoprecipitants were washed four times with 200 µl Wash Buffer 1 (150 mM NaCl, 1% Igepal CA-630 (formerly NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl (pH 8.0)) on µColumns placed in the magnetic field of a µMACS Separator. Subsequently, immunoprecipitants were washed once in 100 µl Wash Buffer 2 (20 mM Tris HCl (pH 7.5)) and eluted with hot 2x SDS-PAGE sample buffer. Western blotting was performed using antibodies against UNC-45, DAF-21 (gift from Richard Morimoto), UNC-54 (mAb5-8-1, DSHB) and Tubulin (T6074, Sigma). Proteins were detected by immunoblotting on PVDF membranes using Amersham ECL Prime (GE Healthcare) in a Bio-Rad ChemiDoc Imager.

Statistical analysis

1 For statistical analyses the GraphPad Prism 5 software was used. Non-parametric
2 statistical tests were used according to the software's recommendations and specified in
3 the respective figure legends and descriptive text. Results are usually given as mean and
4 standard error of the mean (SEM).

5

6 **Results**

7 ***Biallelic variants in the myosin chaperone UNC-45B in individuals with muscle***
8 ***weakness.*** We report ten affected individuals from eight independent families clinically
9 manifesting with childhood onset, progressive proximal and axial muscle weakness and
10 various degrees of respiratory insufficiency. The clinical presentations are summarized in
11 Table 1. Of the identified families, there was one family with three affected siblings (P6,
12 P7, P8). The only other notable family history was for P1 who had a brother with a history
13 of nystagmus and vomiting, who was suspected to have a mitochondrial disorder of
14 unknown genetic etiology and who passed away at 3 months of age.

15 To elucidate the possible origin of the muscle disease, whole exome sequencing
16 (WES) was pursued, identifying a recurring homozygous c.2261G>A; p.(Arg754Gln)
17 missense variant in *UNC45B* (NM_173167.3) in seven affected individuals from five
18 independent families of various ethnic backgrounds (Table S1). This variant is rare with
19 nine reports in heterozygous state, and none in homozygosity, in the Genome
20 Aggregation Database (gnomAD) with an allele frequency of 4.732×10^{-5}. On the protein
21 level, this variant impacts a conserved Arginine in the UNC-45 C-terminal UCS domain,
22 which is essential for the interaction with myosin (Figure 1A). P2 was found to be
23 compound heterozygous for a maternally inherited c.2261+5G>C splice variant and a

paternally inherited c.2332C>T; p.(Arg778Trp) missense variant impacting the UCS domain of UNC-45B. This rare missense variant is predicted to be damaging and reported 45 times in heterozygous state, and none in homozygosity, in gnomAD with an allele frequency of 1.593×10^{-4}. P9 was found to have the recurring p.(Arg754Gln) variant in compound heterozygosity with a rare predicted to be damaging c.1207T>C; p.(Ser403Pro) variant impacting the UNC-45B neck domain (Figure 1A). Lastly, P10 was found to be homozygous for a predicted to be damaging c.1540T>C; p.(Cys514Arg) missense variant impacting the UNC-45B neck domain. This variant is listed as a rare SNP (rs775340790) with nine reported in heterozygous state, and none in homozygosity, in gnomAD with an allele frequency of 3.580×10^{-5}.

UNC45B variants manifest clinically as a childhood onset myopathy. Six individuals presented with first recognition of axial and proximal weakness in early childhood. These affected individuals reported slow progression of muscle weakness but remained ambulatory into adulthood. Of interest, these individuals were all homozygous for the recurring c.2261G>A; p.(Arg754Gln) variant. Three individuals with other *UNC45B* variants were reported to have a congenital onset of symptoms.

Serum creatine kinase (CK) levels were reported within normal reference ranges in all individuals. Respiratory function ranged from significantly decreased to normal with forced vital capacity (FVC) measurements ranging from 45-99% predicted. Five individuals had abnormalities on ECG, while structural cardiac abnormalities were seen in two. Lower extremity muscle MR imaging was available for five individuals (Figure 2A),

which revealed evidence of mild fat infiltration of most muscles resulting in a marbled-like appearance but without an emerging clear pattern of muscle involvement.

UNC45B-related myopathy manifests histologically with eccentric cores. Histological analysis of the muscle biopsies was performed in seven individuals (Figure 2B–K). Hematoxylin and eosin (H&E) stain showed variation in fiber size with internalized nuclei, resulting in multinucleated fibers in five individuals (Figure 2B). On Gömöri Trichrome (GT) stain, a few fibers had evidence of uneven aspect with deposit of fuchsinophilic material in three biopsies (Figure 2C), and there was evidence of apparent cytoplasmic bodies as well as rimmed vacuoles in one. On oxidative staining with succinic dehydrogenase (SDH), nicotinamide adenine dinucleotide (NADH), and cytochrome oxidase (COX), large irregular areas of oxidative defects were identified in numerous muscle fibers. In particular, in five of the seven biopsies reviewed SDH staining and COX staining revealed more defined areas of reduced oxidative activity with core-like regions often located along the periphery of fibers, consistent with eccentric cores (Figure 2D and F). In contrast, in two biopsies there were areas of reduced NADH staining alternating with areas of increased staining along the periphery of fibers. On ATPase stains, there was evidence of type I fiber predominance and areas of absence/reduction of ATPase activity, particularly in the periphery of fibers, corresponding to the areas resembling eccentric cores on the oxidative stains (Figure 2E). Electron microscopy (EM) was performed in four individuals (Figure 2G–K). In longitudinal sections, there were large areas of myofibrillar disorganization that extended the entire width of the fiber which appear devoid of mitochondria, thus resembling unstructured cores. In transverse

sections, well-demarcated unstructured cores were seen in subsarcolemmal regions. In addition, wide dark bands of diffused Z-line derived material were observed and are reminiscent of myofibrillar material. More rarely, cytoplasmic bodies (Figure 2K) and rod-like inclusions were seen, and autophagy material was observed in some fibers.

Fiber contractile function is normal in P1 UNC-45B muscle. Muscle contraction is affected by the cyclic interaction between the myosin cross-bridges and actin as an adenosine triphosphate (ATP) hydrolysis dependent process[26]. The maximum force that a muscle fiber can generate is dependent on the rate of myosin cross bridge cycling kinetics. To evaluate whether force generation in UNC-45B muscle was impaired in this assay, we compared the mechanics of permeabilized single muscle fibers from P1 (homozygous c.2261G>A) to controls. Rate of tension redevelopment (K_{tr}) was used as a parameter reflecting the rate of both cross-bridge attachment and reattachment, while tension cost reflects the rate of myosin cross bridge detachment from actin determined by measuring the ATP utilization during force generation. P1 muscle expressed predominately myosin heavy chain (MHC) type I fibers. Contractile properties depend on MHC composition, and therefore, we compared contractile data to controls in whom MHC type I and type II fibers were separated out. Our data showed normal fiber contractile performance, with no difference in maximal tension generated by UNC-45B fibers compared to controls (Figure S1A). The calcium sensitivity of force, the Ca^{2+} concentration needed for 50% of maximal force generation, was also normal in P1 muscle compared to control (Figure S1B). Overall, single fiber mechanics in P1 muscle

tissue showed normal cross-bridge cycling kinetics, with normal tension costs compared to control (Figure S1C and D).

UNC-45B protein is mislocalized in* UNC-45B *muscle. Immunofluorescence localization studies were performed using myomesin, a major structural protein of the M-line, and α-actinin, a Z-line protein, as markers[27]. Whereas in control muscle UNC-45B protein was localizing to the A-band around the M-line as expected, in affected individuals there appears to be a loss of the residual UNC-45B protein at the M-line, suggestive of mislocalization away from the A-band to the Z-disk (Figure 3A and B). Specificity of the UNC-45B antibody was confirmed in control muscle (Figure S2).

The recurring UNC45B c.2261G>A variant acts as a complex hypomorph splice variant. *UNC45B* appears to be intolerant to complete biallelic loss of function, which is in accordance with the essential role of UNC-45 in *C. elegans*[28-30]. Therefore, we did not observe individuals with biallelic null variants, and no homozygous null variants are listed in gnomAD[31]. Haploinsufficiency of *UNC45B,* however, does not appear to cause a severe or early myopathy, as individuals heterozygous for a loss of function *UNC45B* variant are reported in gnomAD. The recurring c.2261G>A *UNC45B* variant affects the last base pair (G) of exon 17 and is adjacent to the splice donor site thus potentially interfering with normal splicing (Figure 4A)[32; 33]. To investigate this possibility, muscle RNA sequencing, available for two individuals (P3 and P4, both homozygous for the c.2261G>A *UNC45B* variant) was analyzed (Figure S3A). This transcript analysis revealed a five-fold reduction of *UNC45B* transcripts compared to controls and

furthermore indicated that two detectable *UNC45B* splice products were generated from the mutant allele (Figure S3B). The first detectable transcript encodes the full-length protein containing the Arg754Gln missense variant. The second transcript results from altered splicing due to interruption of the normal splice donor and subsequent activation of a nearby intronic cryptic splice donor site c.2261+10 (Figure 4B). The resulting splice product extends exon 17 into the intron, causing the inclusion of an in-frame STOP codon. This nonsense transcript is unstable and most likely subject to nonsense-mediated decay. A low level of the elongated nonsense transcript escaped mRNA decay and was therefore detectable on muscle RNA sequencing. From this we conclude that the c.2261G>A *UNC45B* variant results in a hypomorphic splice variant. The effect is a situation in which the majority of transcript is mis-spliced to include a premature termination codon with predominant degradation before translation occurs, while any residual correctly spliced full-length transcript will result in a protein containing the Arg754Gln missense variant.

RNA sequencing and reverse transcription of mRNA with subsequent cDNA sequencing in P2 muscle extracts revealed allelic imbalance towards the p.(Arg778Trp) variant, while the c.2261+5G>C splice variant resulted in activation of the same nearby intronic cryptic donor site as seen for the c.2261G>A variant, and is thus expected to lead to the same nonsense mediated decay (Figure S3C–H). In summary, the c.2261G>A and the c.2261+5G>C *UNC45B* variants both impact the proper splicing of the mRNA and thus the stability of the *UNC45B* mRNA.

To investigate the impact of the *UNC45B* variants on the protein level, we performed western blot on muscle extracts from P1, P2, P9, and P10. All four individuals

showed a significant reduction of UNC-45B total protein compared to controls (Figure 4C).

Missense variants impact conserved UNC-45B domains. The UNC-45B amino acid sequence is highly conserved down to invertebrates, which allowed us to explore structural impacts of variants on basis of the recently elucidated structure of *C. elegans* UNC-45[14; 16; 17]. Both arginine residues mutated in our cohort (Arg754 and Arg778) are part of helices 1 and 3, respectively, in the armadillo (ARM) repeat 14 (Figure 5A). ARM 14 is located in the hinge region, which connects the C-terminal half of the UCS domain (ARM repeats 14–17) with the N-terminal half (ARM repeats 10–13) forming part of the myosin-binding canyon. Exchanging Arg754 for a glutamine does not seem to drastically interfere with the surrounding structures according to PyMOL calculations of van der Waals overlaps. Conversely, exchanging the positively charged Arg778 for a bulky hydrophobic tryptophan would most probably sterically interfere with its surroundings, up to the possible shifting of the overall structure of the UCS domain. P9 was found to carry the recurring p.(Arg754Gln) missense in compound heterozygosity with a p.(Ser403Pro) missense located in the UNC-45 neck domain. Ser403 is conserved in zebrafish UNC-45B but position 403 appears to have a broader tolerance for other amino acids featuring a threonine in *Drosophila* and an isoleucine in *C. elegans* UNC-45. Its functional importance therefore may be restricted to vertebrates but needs to be investigated further.

Close cooperation of the chaperones Hsp90 and Hsp70 with UNC-45 allows for the precise temporal and spatial control of the incorporation of myosin into contractile muscle thick filaments[1; 17; 34]. P10 was found to be homozygous for a p.(Cys514Arg)

missense variant, which impacts the neck domain, and is therefore the only individual in this series without an UNC-45 UCS domain allele. Given that the UNC-45 neck domain confers flexibility and allows exact positioning of the UCS and the TPR domains to one another, structural interference in this region is likely to impact protein function. The affected cysteine residue is buried in the inner structure of ARM 9 in the neck domain of UNC-45. Exchanging a cysteine for a positively charged arginine would most probably lead to steric hindrance and electrostatic interference within the protein structure (Figure 5A).

Recombinant UNC-45B protein variants are prone to aggregation. To further elucidate structural impacts of the *UNC45B* variants, we recombinantly expressed the UNC-45B mutant proteins in *E. coli* and subjected them to time-dependent partial trypsin proteolysis experiments at room temperature (22°C). All mutant variants seemed to be less susceptible to proteolysis than the wild-type with the p.(Ser403Pro) variant showing the most pronounced difference (Figure S4A and B). Repeating the partial trypsin proteolysis assay at the physiological temperature 37°C corroborated the result that trypsin proteolysis was delayed in the mutant variants with the p.(Ser403Pro) variant being significantly different in non-parametric Friedman test (Figure 5B and C, Table S4). In addition, we performed thermal shift assays (TSA) using the protein stain SYPRO Orange to determine melting temperatures of the recombinant proteins in solution. The stain intercalates with gradually exposed hydrophobic residues on the protein's surface allowing for melting temperature estimation based on the half-maximal SYPRO fluorescence in a Boltzmann approximation. The assay revealed that all variant proteins

have a lower melting temperature (Arg754Gln: 38.18±0.05145°C, Arg778Trp: 37.85±0.04907°C, Ser403Pro: 37.88±0.1241°C, Cys514Arg: 36.90±0.03901°C) compared to wild-type (43.16±0.04358°C) in the Tris-based buffer (Figure 5D, Tables S5 and S6). Exposed hydrophobic residues at a lower temperature might suggest aggregation tendency. On these grounds, we subjected the recombinant mutant variants to a filter trap assay to search for aggregates after incubation at room temperature (22°C). A subsequent slot blot confirmed the suspected higher aggregation of the mutant variants compared to the wild-type to a similar degree as the absolute SYPRO fluorescence at 22°C indicated (Figure 5E and F). Further increased aggregation propensity of the mutant variants around the melting temperature of 37°C could also explain the observed delay in *in vitro* proteolysis due to a fraction of the substrate being sequestered in trypsin-inaccessible aggregates. Together, biochemical analyses of the recombinant UNC-45B mutant variants suggest that amino acid substitutions at the here reported positions will likely lead to structural changes in the UNC-45B protein.

Myopathy-related UNC-45B mutant missense proteins cannot rescue a conditional loss-of-function allele. To test for the functional performance of the disease associated UNC-45B UCS variant proteins in myosin assembly, we made use of an *in vivo* rescue approach in *C. elegans* described previously[17]. Temperature-sensitive (*ts*) *unc-45(m94)* mutant worms exhibit a severe movement defect and disarrangement of the otherwise highly conserved sarcomere organization when grown at the non-permissive temperature of 25°C[34]. By expressing the corresponding *C. elegans* UNC-45 variants in conditional *loss-of-function* worms, we were able to analyze to what degree muscle function of the

unc-45 ts mutant could be restored. When transferred to liquid medium, worms swim by thrashing their bodies sideways. This agile movement is almost completely abolished in the *m94* allele containing mutant worms grown at 25°C. Integrated transgene expressing the orthologue protein of the p.(Arg778Trp) variant UNC-45(Arg792Trp), of the p.(Ser403Pro) variant UNC-45(Ile422Pro), and of the p.(Cys514Arg) variant UNC-45(Cys532Arg) were unable to rescue the movement phenotype of the *unc-45(m94)* strain. Body bend/thrashing counts of young adult worms in liquid medium were only slightly improved compared to wild-type levels for the UNC-45(Arg792Trp) and UNC-45(Ile422Pro) transgenes, whereas worms expressing the UNC-45(Cys532Arg) transgene were as impaired as *unc-45(m94)* without transgenic expression (Figure 6A). Assessing population motility of 60 worms using an ARENA WMicrotracker (NemaMetrix) reproduced the motility defect on a solid agar surface (Figure 6C). These data suggest that the c.2332C>T; p.(Arg778Trp), the c.1207T>C; p.(Ser403Pro), and the c.1540T>C; p.(Cys514Arg) variants very likely affect UNC-45B protein function. In contrast, expressing the orthologue protein of the p.(Arg754Gln) variant UNC-45(Arg767Gln) in the *unc-45(m94)* background was able to rescue the defect in movement (Figure 6A and C). Although not possessing polynuclear myofibers, sarcomere organization and components in *C. elegans* body wall muscle cells are highly conserved: thick filaments are formed by myosin heavy chains A and B, thin filaments are formed by actin fibers, and Z-disk-equivalent dense bodies are formed by α-actinin and integrins[35]. Sarcomere numbers in body wall muscle cells can be assessed by established staining methods, which allow counting periodically organized sarcomeric components. In accordance with motility assays and contrary to the UNC-45(Arg767Gln) transgenic rescue, the UNC-

45(Arg792Trp), the UNC-45(Ile422Pro), and the UNC-45(Cys532Arg) transgenic rescues showed no improvement of the sarcomeres of the *m94 ts*-allele in phalloidin-staining of filamentous F-actin-containing I-bands (Figure 6D and E).

Based on the known mechanistic role of the UCS domain[16; 17; 34], the corresponding arginine residues in *C. elegans* can be precisely linked to myosin binding. Whereas FLAG-tagged UNC-45(Arg767Gln) was able to bind to the substrate myosin heavy chain B (MHC B/UNC-54) in co-immunoprecipitation experiments (Figure 6F), UNC-45(Arg792Trp) was not able to pull down MHC B from worm lysates, suggesting conformational changes of the myosin binding canyon in the UCS domain for the variant protein. Conceivably, binding to *C. elegans* Hsp90 orthologue DAF-21 via the TPR domain was not impaired in both variant proteins.

It is noteworthy however that in these transgenic rescue experiments we are not able to control for the additional hypomorphic deficiency situation of the p.(Arg754Gln)/(Arg767Gln) variant. Although the Arg767Gln transgene is expressed at a slightly lower levels than the wild-type transgene in these experiments (Figure 6F), it is still able to rescue the muscle phenotype and bind to MHC B. Reduced levels of UNC-45 have been shown to be detrimental for muscle development[29; 36]. Our results therefore suggest, that the *UNC45B* p.(Arg754Gln) missense is not the primary cause of pathogenicity in those individuals, and the disease is most likely driven by the reduction in total UNC-45B protein due to degradation of the majority of mis-spliced transcripts.

Discussion

We report ten individuals from eight independent families with a largely consistent clinical phenotype of early onset, slowly progressive muscle weakness manifesting with axial and proximal weakness and respiratory involvement with a muscle histotype findings including eccentric and unstructured cores as well as ultrastructual findings suggstive of an accumulations of myofibrillar material. All individuals were found to have biallelic variants in *UNC45B,* which encodes a highly conserved myosin-specific chaperone that is involved in assembly, function, and maintenance of type II myosin, facilitating assembly and function of striated muscle contraction. UNC-45, together with the general chaperones Hsp70 and Hsp90, forms a transient anchoring chain that organizes a properly spaced assembly line, locking the myosin head into an actin bound confirmation by facilitating hydrolysis[17]. This repeating unit stabilizes the thick filament and inhibits the myosin power stroke[37]. A single individual with myopathy who was homozygous for the c.2261G>A variant was previously reported, suggesting a tentative disease association; however, the precise interaction of UNC-45B with myosin in muscle function and disease remained poorly understood[38].

Early-onset myopathies are a clinical and genetic heterogeneous group of disorders of variable severity in which approximately half of individuals do not have a confirmed genetic etiology[39; 40]. Significant extraocular and facial weakness were notably absent in the *UNC45B* individuals reported here, which is in contrast to the core myopathies due to pathogenic variants in *RYR1* (MIM: 180901)*, CACNA1S* (MIM: 114208), or *SPEG* (MIM: 615950) in which involvement of the extraocular and facial muscles are typically seen[41-43]*.* Of the ten individuals reported here, tachycardia was noted in two, while two individuals presented with structural cardiac changes (VSD and

aortic coarctation). UNC-45B is highly expressed in human cardiac tissue and has an important evolutionary role in cardiac contractility[28; 44; 45]. Thus, the lack of more significant functional cardiac involvement in our cohort is noteworthy; however, it remains unclear whether a more severe cardiac phenotype might evolve over time.

Pathogenic variants in various myosin chains including *MYH2* (MIM: 160740) and *MYH7* (MIM: 160760) are a known cause of myopathies of variable severities, with clinical and histological findings similar to our *UNC45B* individuals[46; 47]. Various genetically defined core myopathies with clinical overlap, including *RYR1* and *MYH7*-related myopathies can present with a characteristic and recognizable pattern of muscle involvement and sparing when assessed by muscle imaging[48-50]. A selective and potentially diagnostic pattern of muscle involvement, however, was thus far not evident on muscle imaging in our *UNC45B* cohort. Instead, imaging revealed a fairly uniform involvement of all muscles with a possible hint of relative sparing of the semimembranosus muscle of the hamstring group. In contrast to a dystrophic process, which is characterized by uniform fatty infiltration, or to a neurogenic process in which we typically see a coarse "moth-eaten" appearance on MRI, the muscle of *UNC45B* individuals had a characteristic "marbled-like" appearance on fat sensitive T1 MRI sequences, which could potentially be a characteristic and thus diagnostically helpful finding[49]. Given the potential developmental role of UNC-45B[14; 15], it is of note that the myofibrillar apparatus in the individuals reported here appears to be normal at baseline with normal *in vitro* contractile performance and myosin cross bridge cycling. Therefore, we hypothesize that with ongoing use and stress on the muscle fibers over time, the sarcomere is inadequately maintained due to reduction in myosin chaperone availability

and/or functioning. Repair capacity may be overloaded, and sarcomeres start disintegrating, thereby giving rise to the unstructured cores and the accumulation of Z-line/myofibrillar material consistently seen in the UNC-45B muscle biopsies.

Consistent with an abnormal function of UNC-45B in relation to the sarcomere, muscle immunofluorescence findings performed in three biopsies reveal abnormal localization of the residual UNC-45B protein away from the M-line centered A-band where the myosin heads are located in need of maintenance and repair, instead accumulating in the Z-disc region (Figure 3A and B). In zebrafish it has been shown that the Z-line holds a “reservoir” of UNC-45B which shuttles to the myosin-containing A-band of the muscle sarcomere in response to eccentric exercise or induced damage to the myofiber[51; 52]. This abnormal localization was observed in all three analyzed samples and thus seems to be independent of the underlying variant and UNC-45B domain impacted. Even though a biopsy provides only a static image, the abnormal localization is consistent with the assumption that the dynamic shuttling process might be impaired as well. Lack of proper UNC-45B localization and function under conditions of continued sarcomere use and stress could then conceivably lead to the multifocal disruption and ultimate disarray of the myofibrillar apparatus, corresponding to the histologic and ultrastructural findings of eccentric and unstructured cores seen in our affected individuals. This might also help explain why the myopathic phenotype seen in *UNC45B*-related disease shows a progressive course, distinct from the typical more static clinical course of congenital myopathies. Further *in vitro* testing will be required to determine whether the mislocalized UNC-45B observed in affected individual’s muscle impairs the recurring myosin cross bridge cycling needed for repeated contraction.

Previous work on the highly conserved UNC-45 in various models including *C. elegans*, zebrafish and mouse, has shown that loss of UNC-45 is embryonically lethal[29; 30; 53]. In this context it is notable that we have not observed individuals with biallelic null variants, and no homozygous null variants are listed in GnomAD, suggesting that this situation in the human may also be either not tolerated or be associated with a considerably more severe phenotype. We have shown that the recurring c.2261G>A variant is a complex splice allele, that creates a hypomorph scenario, with the residual protein containing the p.(Arg754Gln) variant. This conclusion is supported by analysis of available UNC-45B muscle biopsies, as all analyzed samples had a reduction in UNC-45B protein by western blot, which was most significant in the homozygous c.2261G>A biopsy (Figure 4C). Thus, we suspect that the disease mechanisms for this variant are largely driven by the protein deficiency, with additional contribution from a functionally impaired residual protein. Recombinant UNC-45B(Arg754Gln) variant protein indeed exhibited a lower melting temperature in thermal shift assays and aggregation tendency (Figure 5D and E). On the other hand, the ability of the corresponding *C. elegans* variant UNC-45(Arg767Gln) to rescue a temperature-sensitive mutant allele in transgenic rescue assays corroborates the notion, that the reduction in overall protein amount is the cause for the disease phenotype rather than the missense and possibly impaired residual protein itself (Figure 6).

In supplemental *C. elegans* biochemical assays, no changes in UNC-45 ubiquitylation were detected (Figure S4C), although both UCS domain missense variants were slightly more susceptible to trypsin proteolysis (Figure S4D and E). The UNC-45B transgenes of p.(Arg778Trp) (*C. elegans* Arg792Trp), of p.(Ser403Pro) (*C. elegans*

1 Ile422Pro), and of p.(Cys514Arg) (*C. elegans* Cys532Arg) were found to have a more
2 detrimental effect on function, with an inadequate rescue of the paralyzed phenotype and
3 impaired binding to myosin for p.(Arg778Trp). These more severe and obvious functional
4 consequences in the *C. elegans* rescue assay are consistent with the fact that the variants
5 cause disease in the human situation. Therefore, we propose that *UNC45B* variants
6 impact UNC-45B chaperone activity through reduction in overall protein levels
7 (c.2261G>A), impaired normal myosin-binding (c.2332C>T; p.Arg778Trp), or a
8 combination of both.

9 *UNC45B*-related disease can be classified as both a chaperonopathy resulting in
10 a secondary myosinopathy as well as a myofibrillar dystrophy, given the progressive
11 disintegration of the myofibrillar apparatus. The continued dissolution of the myofibrillar
12 structure which clinically manifests as progressive muscle weakness is characteristic of
13 the chaperone dysfunction in forms of childhood onset dystrophy[8]. Pathologically, this
14 disorder could be considered a myofibrillar dystrophy, as the excessive nuclear
15 centralization seen on histology is a marker of an activated regenerative process. This is
16 in contrast to a sarcolemmal dystrophic process, which typically leads to a more
17 inflammatory and fibrotic picture, leading to excessive matrix and fat proliferation. In
18 *UNC45B*-related disease we suspect a “dystrophic process internal to the myofiber”
19 causing fibers to disappear focally with less of matrix reaction, hence the marbled
20 appearance on muscle MRI. Biallelic variants in *UNC45B* should therefore be considered
21 in individuals presenting with a myopathy specifically in the presence of core histology
22 and ultrastructural findings including cytoplasmic bodies, rods and autophagic lesions.
23 Additional research is needed to further elucidate the exact pathogenic *UNC45B*-related

disease mechanism and to find therapeutic strategies aimed towards restoring sarcomeric homeostasis through modulating chaperone activity. This has shown great promise for neurodegenerative disorders, with specific interest in the finding that overexpression of UNC-45 in a *Drosophila* model of Huntington-induced cardiac amyloidosis resulted in reduced poly-glutamine aggregation and myofibrillar disorganization[54]. Taken together, our data solidifies the role of UNC-45B as a key regulator of myofibril maintenance and function, a tightly regulated pathway that is conserved from human to yeast, with impaired UNC-45B function resulting in recognizable muscle pathology clinically manifesting with myopathy.

Supplemental Data

Supplemental Data includes: Supplemental Methods, Supplemental Tables S1-6 Supplemental Figures S1-4.

Acknowledgments

We thank the individuals and their families for participating in our research study and Christopher Mendoza, Christine Jones, Gilberto ("Mike") Averion, Rupleen Kaur, Kia Brooks, and Janelle Hauserman for their help in clinic and the laboratory. We would like to thank CHUGA Molecular Biology facility platform for their help with exome sequencing. We thank Y. Kohara, the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resources), the Dana-Farber Cancer Institute, Addgene and Geneservice Ltd for plasmids, cDNAs, and worm strains. We thank the CECAD Imaging facility for support with confocal microscopy. In particular, we thank Vishnu Balaji for purification of LET-70-HIS and VSV-CHN-1-HIS proteins and help with

in vitro studies. This research was supported (in part) by the Intramural Research Program of the NIH, NINDS to C.G.B. and by the Deutsche Forschungsgemeinschaft (DFG) (FOR2743 and CECAD) to T.H. Sequencing and analysis were provided by the Broad Institute of MIT and Harvard Center for Mendelian Genomics (Broad CMG) and was funded by the National Human Genome Research Institute, the National Eye Institute, and the National Heart, Lung and Blood Institute grant UM1 HG008900 and in part by National Human Genome Research Institute grant R01 HG009141. Family (P9) was collected as part of the SYNaPS Study Group collaboration funded by The Wellcome Trust and strategic award (Synaptopathies) funding (WT093205 MA and WT104033AIA). This research was conducted as part of the Queen Square Genomics group at University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. This study was partially supported by the Intramural Research Grant (29-4 to NI; 30-9 to SN, AI) for Neurological and Psychiatric Disorders of NCNP; and by AMED under Grant Numbers JP19ek0109285h0003 and Joint Usage and Joint Research Programs, the Institute of Advanced Medical Sciences, Tokushima University (2019, A9 to AI). This work was supported by the France Génomique National infrastructure, funded as part of the « Investissements d'Avenir » program managed by the Agence Nationale pour la Recherche (ANR-10-INBS-09), and by Fondation Maladies Rares whithin the frame of the "Myocapture" sequencing project.

Declaration of Interests

The authors declare no competing interests.

Web Resources

For Genotype Tissue Expression (GTEx) Project, see http://www.gtexportal.org/; for UCSC Genome Browser, see http://genome.ucsc.edu/; for UniProt database, see http://www.uniprot.org/; for GnomAD, see http://gnomad.broadinstitute.org/; for Online Mendelian Inheritance in Man, see http://www.omim.org.

Data Availability

Sequence data that support the findings of this study have been deposited in dbGaP (http://www.ncbi.nlm.nih.gov/gap). All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request

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Figure Titles and Legends

Figure 1. Biallelic variants in the myosin-directed chaperone UNC-45B. (A) UNC-45 contains four domains: an N-terminal TPR domain (light green), a conserved central domain (dark grey), a neck domain (light grey), and a C-terminal UCS domain (orange). The N-terminal TPR domain is important for the interaction with Hsp90 and Hsp70 chaperones, and the C-terminal UCS domain binds directly to the myosin head domain. The recurring *UNC45B* variant p.Arg754Gln (dark blue) and the p.Arg778Trp (light blue) variant impact conserved arginines in the myosin-binding UCS domain. Variants p.Ser403Pro (pink) and p.Cys514Arg (dark green) are located in the UNC-45B neck domain. (B) Overview of the muscle sarcomere. UNC-45 is involved in myofibrillogenesis cooperating with Hsp90 to fold and to incorporate myosin into the thick filament. In adulthood UNC-45 is stored at the Z-disk. Upon injury to the muscle fiber, UNC-45 shuttles to the A-band to help refold damaged myosin.

Figure 2. Muscle MRI and histological findings in individuals with *UNC45B*-related myopathy. (A) Lower extremity muscle MR imaging was available for five (P1-P5) individuals and showed mild, fat infiltration evident in all lower extremity muscles resulting in a marbled-like appearance. In individuals P1, P4, and P5 there is abnormal (increased) T1 signal without an apparent pattern of muscle involvement except for relative sparing

1 of the semimembranosus muscle in individuals P1 and P5. P1 and P3 had evidence of
2 mild, generalized muscle atrophy, while in P2, P4 and P5 muscle bulk appeared normal
3 (B-K). *UNC45B*-related myopathy manifests histologically with unstructured cores. On
4 histological analyses there are findings of: (B) increased internalized nuclei with
5 numerous multinucleated fibers seen on H&E staining (P1); (C) uneven deposits of
6 fuchsinophilic material is seen on GT staining (P4); (D) core-like regions often located
7 along the periphery of fibers and consistent with eccentric cores are seen on SDH staining
8 (P3); (E) areas of increased staining along the periphery of fibers with decreased staining
9 centrally is seen on NADH staining (P6); and (F) large irregular areas of decreased
10 staining seen on COX staining (P4). On EM there are findings of: (G) myofibrillar disarray
11 (P3); (H) diffusion of Z-line material (P4); (I) autophagy lesions (P3); (J) wide bands of
12 diffusion of the Z-line material which have spread in the disorganized areas (P3); and (K)
13 a cytoplasmic body (P4). White scale bar corresponds to 50 μm, orange scale bar
14 corresponds to 5 μm.
15
16 **Figure 3. Misclocalized UNC-45B in affected individual's muscle.** (A) Longitudinal
17 sections of muscle biopsies of control and P1, P9, and P10 stained for UNC-45B (red)
18 and M-line protein myomesin (green) (top row). UNC-45B is reduced in the M-line and
19 mislocalized from the A-band, around the M-line, to the Z-disc in these three individuals
20 compared to control. Overlay of intensity profile of UNC-45B and myomesin (bottom
21 row) from the cropped area (middle row) shows mislocalization of UNC-45B away from
22 the A-band to the Z-disk. (B) Longitudinal sections of muscle biopsies of control and P1
23 stained for UNC-45B (red) and Z-disk protein α-actinin (green) revealing colocalization

1 of UNC-45B at the Z-line. Overlay of intensity profile of UNC-45B and α-actinin in P1
2 from the cropped area (middle row) shows loss of co-location of UNC-45B with the M-
3 line.
4
5 **Figure 4. *UNC45B* variant c.2261G>A creates a complex hypomorph splice variant.**
6 (A) The last base pair of exon 17 in *UNC45B* c.2261G, orange) is adjacent to the splice
7 donor site of intron 17 (bold black). The c.2261G>A transition, located in the exonic part
8 of the 5' recognition sequence (G-▼-G-U-G-A-G-U), leads to the activation of a nearby
9 cryptic splice donor site (blue). The resulting spliced mRNA transcript is elongated by 9
10 additional bases including an in-frame STOP codon (blue frame). (B) Schematics of
11 normal *UNC45B* exon 17–18 splicing (1) and of the two splice products seen in *UNC45B*
12 c.2261G>A muscle: (2) full length product including the Arg754Gln substitution and (3)
13 activation of the nearby cryptic splice donor site generating an elongated splice product
14 that includes an in-frame STOP codon. (C) Western blot analysis of UNC-45B in muscle
15 extracts from P1, P2, P9, and P10 compared to control. Quantification shows a significant
16 reduction of UNC-45B in P1, P2, P9, while levels in P10 were slightly reduced. Mouse
17 monoclonal anti-desmin was used as a loading control.
18
19 **Figure 5. UNC-45B mutant proteins are prone to aggregation.** A) Structure of human
20 UNC-45B based on the *C. elegans* UNC-45 3D structure (PDB ID: 4i2z)[17]. The recurring
21 p.(Arg754Gln) (dark blue) and the p.(Arg778Trp) (P2) *UNC45B* variant (light blue) impact
22 conserved arginine residues in the myosin binding UCS domain (orange) and can be
23 precisely mapped to helices 1 and 3, respectively, in the armadillo (ARM) repeat 14. ARM

14 is located in the hinge region, which connects the C-terminal half of the UCS domain (ARM repeats 14–17) with the N-terminal half (ARM repeats 10–13) and forms part of the myosin-binding canyon. The p.(Ser403Pro) (pink, P9) and p.(Cys514Arg) (dark green, P10) variant impact the UNC-45B neck domain. the structure of the human UNC-45B protein was inferred from the *C. elegans* UNC-45 structure[17] (PDB ID: 4i2z) using Swiss-Model[21] and corresponding residues were mutated in PyMOL to display the reported variants. Van der Waals overlaps of the newly incorporated amino acid residues with the unmodified protein structure are depicted as red disks. (B) Purified UNC-45B$^{\text{WT}}$, UNC-45B$^{\text{Arg754Gln}}$, UNC-45B$^{\text{Arg778Trp}}$, UNC-45B$^{\text{Ser403Pro}}$, and UNC-45B$^{\text{Cys514Arg}}$ buffered solutions were subjected to partial proteolysis with trypsin at 37°C and samples were loaded on SDS-PAGE gels for separation. Coomassie-stained gels of one of two repetitions are shown. (C) Quantification of full-length protein (FL, ~109 kDa) compared to time point 0 at 37°C of two repetitions. Mean and SD can be found in Table S4. (D) Purified UNC-45B$^{\text{WT}}$, UNC-45B$^{\text{Arg754Gln}}$, UNC-45B$^{\text{Arg778Trp}}$, UNC-45B$^{\text{Ser403Pro}}$, and UNC-45B$^{\text{Cys514Arg}}$ buffered solutions were slowly heated from 10°C to 95°C in the presence of SYPRO Orange protein stain. Boltzmann sigmoidal curves were fit to normalized combined melt curves of three experiments. Half maximal temperatures indicate melting temperatures in the Tris-based buffer. Mean and SD can be found in Table S5 (E) Slot blot of purified UNC-45B$^{\text{WT}}$, UNC-45B$^{\text{Arg754Gln}}$, UNC-45B$^{\text{Arg778Trp}}$, UNC-45B$^{\text{Ser403Pro}}$, and UNC-45B$^{\text{Cys514Arg}}$ Tris-buffered solutions incubated for 1 h at room temperature filtered through a 0.2 µm acetyl-cellulose membrane. Three decreasing amounts of protein solution were blotted for comparison. (F) Baseline-subtracted SYPRO fluorescence values at 22°C obtained in two experiments in (D) are plotted for each protein solution. No significant

differences between WT and mutant proteins were found in non-parametric Kruskal-Wallis test.

Figure 6. Various missense UNC-45 mutant proteins cannot rescue a conditional loss-of-function allele. Transgenic UNC-45^{Arg792Trp} mutant protein is unable to rescue the motility defect of *unc-45(m94)* worms grown at the restrictive temperature of 25°C, whereas UNC-45^{Arg767Gln} rescues the *m94* motility defect. (A) Body bends of 30 young adult *unc-45(m94)* worms expressing the indicated UNC-45 variants were counted in at least three different experiments. Values are mean ± SEM; *: $p<0.0001$ compared to control in non-parametric Kruskal-Wallis test. (B) Expression levels of UNC-45-FLAG transgenes in young adult *unc-45(m94)* worms. (C) Population motility of 60 young adult *unc-45(m94)* worms expressing the indicated UNC-45 variants on a 24 well plate filled with NGM and seeded with OP50 was measured with the ARENA WMicrotracker system (NemaMetrix) at 25°C for 24 hours after reaching adulthood. (D) Phalloidin-staining of F-actin-containing I-bands in *unc-45(m94)* mutant worms expressing the indicated UNC-45 variants. (E) The number of I-bands is given per body wall muscle cell (indicated by dashed white line in D), with all 12 analyzed cells located in the same area between pharynx and vulva. Values are mean ± SEM; *: $p<0.005$ compared to control in non-parametric Kruskal-Wallis test. (F) Co-immunoprecipitation of myosin heavy chain B (MHC B) and Hsp90 (DAF-21) from cell lysates of *unc-45(m94)* mutant worms expressing the indicated FLAG-tagged UNC-45 variants grown at 25°C. Representative result of one of three experiments is shown.

1 **Table Title and Legend**

2 **Table 1. Clinical characteristics and *UNC45B* variants identified.** CK, creatine kinase;
3 ECG, electrocardiogram; EM, electron microscopy; EMG, electromyography; F, female;
4 FVC, forced vital capacity; IVC, inferior vena cava; m, maternal; M, male; mo, months;
5 NA, not available; NADH, Nicotinamide adenine dinucleotide; p, paternal; R, right; s/p,
6 status post; VSD, ventricular septal defect; yrs, years.

Individual	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
UNC45b mutation	Homozygous c.2261G>A p. (Arg754Gln)	c.2332C>T p.(Arg778Trp) (p) c.2261+5G>C (m)	Homozygous c.2261G>A p. (Arg754Gln)	Homozygous c.2261G>A p. (Arg754Gln)	Homozygous c.2261G>A p. (Arg754Gln)	Homozygous c.2261G>A p. (Arg754Gln)	Homozygous c.2261G>A p. (Arg754Gln)	Homozygous c.2261G>A p. (Arg754Gln)	c.2261G>A p. (Arg754Gln) (p) c.1207T>C p.(Ser403Pro) (m)	Homozygous: c.1540T>C p. (Cys514Arg)
Sex / Age at last examination (yrs)	F /18	F / 19	M / 27	M / 31	M / 19	M / 53	M / 55	M / 52	F / 6	M / 10
Ethnicity / Consanguinity	Hispanic / No	Hispanic / No	Turkish / Yes	Turkish / Yes	Turkish / Yes	Thai / No	Thai / No	Thai / No	Sicilian / No	Swedish / No
First symptoms (age)	Proximal muscle weakness (6 yrs)	Progressive scoliosis, poor weight gain (7 mo)	Delayed motor milestones: walked at 2 yrs	Delayed motor milestones: walked at 2 yrs	Proximal muscle weakness (4 yrs)	Proximal muscle weakness (6 yrs)	Proximal muscle weakness (5 yrs)	Muscle weakness (6 yrs)	Congenital hypotonia, weak cry, feeding difficulties. Delayed motor milestones: walked at 3 yrs	Congenital hypotonia, knee contractures, feeding and respiratory difficulties
Distribution of weakness	Slowly progressive axial and proximal weakness	Axial and proximal weakness	Slowly progressive proximal weakness	Slowly progressive proximal weakness	Slowly progressive proximal weakness	Slowly progressive childhood onset proximal weakness	Slowly progressive childhood onset proximal weakness	Slowly progressive proximal muscle weakness	Proximal weakness	Axial weakness
Muscle bulk	Calf hypertrophy	Calf hypertrophy	-	Calf hypertrophy	Calf hypertrophy; Atrophy of shoulder girdle	-	-	Calf hypertrophy	-	Moderate generalized atrophy
CK (U/L) [reference range]	130 [26-192]	Normal	220 [140-200]	137 (140-200)	56	149 [1-190]	95 [1-190]	241 [1-190]	35 [1-150]	Normal
EMG	Mildly neurogenic	Myopathic	Myopathic	Myopathic	Not myopathic	Myopathic	NA	NA	NA	Myopathic
Muscle biopsied (age)	Vastus lateralis (18 yrs)	Quadriceps (5yrs)	Deltoid (26 yrs)	Deltoid (31 yrs)	Quadriceps (13yrs)	Biceps (43 yrs)	NA	NA	Vastus lateralis (5 yrs)	Vastus lateralis (10 yrs)
Histologic findings	Moderate variation in fiber size with rounded and elongated atrophic fibers and numerous internalized nuclei. Areas devoid of oxidative staining, suggestive of cores. Uniform type 1 fiber predominance.	Slight variation in fiber size with increased number of internalized nuclei. Areas devoid of oxidative staining, consistent with eccentric cores. Uniform type 1 fiber predominance	Large, irregular areas of oxidative defects and myofibrillar disorganization. Evidence of fuschinophilic inclusions on trichrome stain. Type 1 fiber predominance.	Large, irregular areas of oxidative defects and myofibrillar disorganization. Evidence of fuschinophilic inclusions on trichrome stain. Type 1 fiber predominance.	Severe fatty replacement. Increased number of internalized nuclei in the remaining muscle fibers.	Moderate variation in fiber size with increased number of internalized nuclei. Evidence of ring fibers, moth-eaten and core-like fibers on NADH stain. Ring and Necklace-like cytoplasmic bodies and rimmed vacuoles on trichrome stain. Type 1 fiber predominance.	NA	NA	Variation in fiber size with increased number of internalized nuclei. Evidence of fuschinophilic inclusions on trichrome stain. Large, irregular areas of oxidative defects and myofibrillar disorganization.	Type 1 fiber predominance. Occasional internalized nuclei. Occasional fibers with areas devoid of oxidative staining.
Ultrastructural findings (on EM)	Large areas of disorganization & some diffusion of the Z-line material	Large areas of disorganization & some diffusion of the Z-line material	Cytoplasmic bodies and granulo-filamentous aggregates	Cytoplasmic bodies and granulo-filamentous aggregates	NA	Non-diagnostic	NA	NA	NA	NA
FVC (% predicted)	62% (18 yrs)	45% (19 yrs)	60% (26 yrs)	81% (31 yrs)	80% (19 yrs)	NA	NA	NA	NA	99% (13 yrs)
Cardiac evaluations	Echocardiogram: normal; ECG: normal (18 yrs)	Aortic coarctation and IVC s/p surgery	Echocardiogram: normal; ECG: normal (26 yrs)	Echocardiogram: normal; ECG: tachycardia (31 yrs)	Echocardiogram: normal; ECG: normal (15 yrs)	Echocardiogram: normal; ECG: incomplete R bundle branch block (53 yrs)	ECG: left ventricular hypertrophy (55 yrs)	ECG: complete R bundle branch block, tachycardia (50 yrs)	ECG: Normal (5 yrs)	Aortic coarctation and VSD s/p surgery
Other	Dysphagia, fatigue	Episode of supraventricular tachycardia, primary amenorrhea	Dysphagia	Dysphagia	Fatigue	-	Episode of pneumonia with respiratory failure, difficulty weaning of ventilator; nighttime BiPAP (55 yrs)	-	Ankle contractures and prominent calcaneus.	Mild ophthalmoplegia, joint hypermobility, premature adrenarche

1