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# 1 Pathogenic Variants in the Myosin Chaperone UNC-45B

## 2 Cause Progressive Myopathy with Eccentric Cores

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4  
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.**

## 1 Introduction

2 Muscle development and function require a complex system of structural and motor  
3 proteins organized into contractile units referred to as sarcomeres. The sarcomeric repeat  
4 is a supra-molecular dynamic structure, in which actin and myosin filaments, together with  
5 associated proteins, are arranged in a precise order. The near crystalline lattice of the  
6 sarcomere coordinates actin-myosin cross bridge cycling, which facilitates sarcomere  
7 shortening, filament gliding, and muscle contraction. The folding, stability, and  
8 organization of sarcomeric proteins into muscle filaments is governed by molecular  
9 chaperones<sup>1</sup>. The fundamental importance of chaperones for the development and  
10 maintenance of skeletal muscle is underscored by recent studies indicating that  
11 chaperone dysfunction is responsible for a distinct subset of hereditary myopathies.  
12 These so called chaperonopathies are characterized by pathogenic variants in genes  
13 encoding chaperones and co-chaperones of structural muscle components. For example,  
14 loss of the small heat shock protein CRYAB (*MIM*: 123590) affects folding of the muscle  
15 intermediate filament desmin, clinically manifesting as a myofibrillar myopathy known as  
16  $\alpha$ B-crystallinopathy<sup>2</sup>. Additional disease mechanisms include impaired homeostasis of  
17 chaperone-assisted selective autophagy (CASA) linked to pathogenic variants in the  
18 HSP70 co-chaperone *BAG3* (*MIM*: 603883), clinically manifesting with a progressive  
19 myofibrillar myopathy with significant cardiac as well as peripheral nerve pathologies<sup>3; 4</sup>.  
20 The subsequent identification of variants in *DNAJB6* (*MIM*: 611332) causing limb-girdle  
21 muscular dystrophy (LGMDD1) provided clear evidence of abnormal sarcomeric  
22 aggregate pathology in these chaperonopathies<sup>5; 6</sup>. The clinical spectrum of  
23 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<sup>7</sup>. 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<sup>8</sup>.

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<sup>9</sup>. 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<sup>10-12</sup>. 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<sup>13</sup>. 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*<sup>14; 15</sup>.  
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)<sup>16; 17</sup>. 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<sup>18</sup>. 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<sup>17</sup>. 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<sup>1; 18</sup>. 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<sup>18; 19</sup>. 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<sup>19</sup>.

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<sup>19</sup>. 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<sup>20</sup>. 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



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

11

### 12 *Immunostaining & microscopy*

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

21

### 22 *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<sup>21</sup>. *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<sup>22</sup>. 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  $\mu$ M 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<sub>2</sub>PO<sub>4</sub> (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

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

4

#### 5 *Partial trypsin proteolysis*

6 For limited proteolysis assays, 2.4 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-  
7 UNC-45B(Arg754Gln)-6xHIS, myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-  
8 45B(Ser403Pro)-6xHIS, myc-UNC-45B(Cys514Arg)-6xHIS, myc-UNC-45-6xHIS, myc-  
9 UNC-45(Arg767Gln)-6xHIS and myc-UNC-45(Arg792Trp)-6xHIS, diluted in PBS (pH 7.4)  
10 were incubated with 20 ng of trypsin (SERVA) for 1, 2, 5, 10, 20, 40, or 60 min at room  
11 temperature (~22°C) or 37°C. A control reaction was incubated with PBS instead of  
12 trypsin for 60 min (time point 0). Reactions were stopped by adding 5x SDS sample buffer  
13 (0.25 M Tris-HCl, 10% SDS, 50% glycerol, 0.5 M DTT, 0.25% bromophenol blue) and  
14 flash freezing in liquid nitrogen. After collecting, all samples were boiled at 95°C for 5 min,  
15 run on SDS-PAGE gels and stained with Instant Blue Coomassie stain (Expedeon).  
16 Images were taken with an Odyssey CLx Imager (LI-COR Biotechnology) using the 700  
17 nm channel, intact protein bands were quantified using Image Studio Version 5.2 software  
18 and relative fluorescence signal compared to time point 0 was plotted.

19

#### 20 *Thermal shift assay with SYPRO Orange*

21 10 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-UNC-45B(Arg754Gln)-6xHIS,  
22 myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-45B(Ser403Pro)-6xHIS, myc-UNC-  
23 45B(Cys514Arg)-6xHIS were diluted in ice-cold 20 mM Tris (pH 8.0), 150 mM NaCl, and

1 2.5x SYPRO Orange protein stain and heated in 0.5° increments from 10°C to 95°C in a  
2 CFX Real-Time PCR Cycler (Bio-Rad). Melt curves were recorded using the FRET  
3 channel, normalized to buffer-only control and fitted to Boltzmann sigmoidal curve  
4 regression in GraphPad Prism 5 software. Half maximal temperatures were read as  
5 melting temperatures.

6

#### 7 *Filter trap assay and slot blot*

8 20 µg purified proteins myc-UNC-45B(WT)-6xHIS, myc-UNC-45B(Arg754Gln)-6xHIS,  
9 myc-UNC-45B(Arg778Trp)-6xHIS, myc-UNC-45B(Ser403Pro)-6xHIS, myc-UNC-  
10 45B(Cys514Arg)-6xHIS were diluted in ice-cold 20 mM Tris (pH 8.0), 150 mM NaCl and  
11 incubated rotating at room temperature (22°C) for 1 h. Three decreasing amounts of  
12 protein solution were loaded onto a 0.2 µm cellulose acetate membrane assembled in a  
13 slot blot apparatus (Bio-Rad). The membrane was washed with PBS, 0.2% SDS and  
14 retained aggregated protein was assessed by immunoblotting for myc-tag (9E10, Roche).

15

#### 16 *C. elegans maintenance and transgenic lines*

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

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<sup>25</sup>. 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

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

4

#### 5 *Co-immunoprecipitation studies*

6 For co-immunoprecipitations, synchronized young adult wild-type worms, *unc-45(m94)*,  
7 or worms expressing transgenic, FLAG-tagged UNC-45 were sonicated in lysis buffer (50  
8 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, and protease inhibitor mix (Roche)),  
9 and immunoprecipitation was performed using the  $\mu$ MACS DYKDDDDK Isolation Kit  
10 (Miltenyi Biotec) following the manufacturer's instructions. Briefly, 150  $\mu$ g of worm lysates  
11 were incubated with 50  $\mu$ l Anti-DYKDDDDK MicroBeads for 45 min at 4°C.  
12 Immunoprecipitants were washed four times with 200  $\mu$ l Wash Buffer 1 (150 mM NaCl,  
13 1% Igepal CA-630 (formerly NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris  
14 HCl (pH 8.0)) on  $\mu$ Columns placed in the magnetic field of a  $\mu$ MACS Separator.  
15 Subsequently, immunoprecipitants were washed once in 100  $\mu$ l Wash Buffer 2 (20 mM  
16 Tris HCl (pH 7.5)) and eluted with hot 2x SDS-PAGE sample buffer. Western blotting was  
17 performed using antibodies against UNC-45, DAF-21 (gift from Richard Morimoto), UNC-  
18 54 (mAb5-8-1, DSHB) and Tubulin (T6074, Sigma). Proteins were detected by  
19 immunoblotting on PVDF membranes using Amersham ECL Prime (GE Healthcare) in a  
20 Bio-Rad ChemiDoc Imager.

21

#### 22 *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  $4.732 \times 10^{-5}$ . 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

1 paternally inherited c.2332C>T; p.(Arg778Trp) missense variant impacting the UCS  
2 domain of UNC-45B. This rare missense variant is predicted to be damaging and reported  
3 45 times in heterozygous state, and none in homozygosity, in gnomAD with an allele  
4 frequency of  $1.593 \times 10^{-4}$ . P9 was found to have the recurring p.(Arg754Gln) variant in  
5 compound heterozygosity with a rare predicted to be damaging c.1207T>C;  
6 p.(Ser403Pro) variant impacting the UNC-45B neck domain (Figure 1A). Lastly, P10 was  
7 found to be homozygous for a predicted to be damaging c.1540T>C; p.(Cys514Arg)  
8 missense variant impacting the UNC-45B neck domain. This variant is listed as a rare  
9 SNP (rs775340790) with nine reported in heterozygous state, and none in homozygosity,  
10 in gnomAD with an allele frequency of  $3.580 \times 10^{-5}$ .

11

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

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



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

3

4 ***UNC45B-related myopathy manifests histologically with eccentric cores.***

5 Histological analysis of the muscle biopsies was performed in seven individuals (Figure  
6 2B–K). Hematoxylin and eosin (H&E) stain showed variation in fiber size with internalized  
7 nuclei, resulting in multinucleated fibers in five individuals (Figure 2B). On Gömöri  
8 Trichrome (GT) stain, a few fibers had evidence of uneven aspect with deposit of  
9 fuchsinophilic material in three biopsies (Figure 2C), and there was evidence of apparent  
10 cytoplasmic bodies as well as rimmed vacuoles in one. On oxidative staining with succinic  
11 dehydrogenase (SDH), nicotinamide adenine dinucleotide (NADH), and cytochrome  
12 oxidase (COX), large irregular areas of oxidative defects were identified in numerous  
13 muscle fibers. In particular, in five of the seven biopsies reviewed SDH staining and COX  
14 staining revealed more defined areas of reduced oxidative activity with core-like regions  
15 often located along the periphery of fibers, consistent with eccentric cores (Figure 2D and  
16 F). In contrast, in two biopsies there were areas of reduced NADH staining alternating  
17 with areas of increased staining along the periphery of fibers. On ATPase stains, there  
18 was evidence of type I fiber predominance and areas of absence/reduction of ATPase  
19 activity, particularly in the periphery of fibers, corresponding to the areas resembling  
20 eccentric cores on the oxidative stains (Figure 2E). Electron microscopy (EM) was  
21 performed in four individuals (Figure 2G–K). In longitudinal sections, there were large  
22 areas of myofibrillar disorganization that extended the entire width of the fiber which  
23 appear devoid of mitochondria, thus resembling unstructured cores. In transverse

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

5

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

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

3

4 ***UNC-45B protein is mislocalized in UNC-45B muscle.*** Immunofluorescence  
5 localization studies were performed using myomesin, a major structural protein of the M-  
6 line, and  $\alpha$ -actinin, a Z-line protein, as markers<sup>27</sup>. Whereas in control muscle UNC-45B  
7 protein was localizing to the A-band around the M-line as expected, in affected individuals  
8 there appears to be a loss of the residual UNC-45B protein at the M-line, suggestive of  
9 mislocalization away from the A-band to the Z-disk (Figure 3A and B). Specificity of the  
10 UNC-45B antibody was confirmed in control muscle (Figure S2).

11

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

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

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

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

1 showed a significant reduction of UNC-45B total protein compared to controls (Figure  
2 4C).

3

4 **Missense variants impact conserved UNC-45B domains.** The UNC-45B amino acid  
5 sequence is highly conserved down to invertebrates, which allowed us to explore  
6 structural impacts of variants on basis of the recently elucidated structure of *C. elegans*  
7 UNC-45<sup>14; 16; 17</sup>. Both arginine residues mutated in our cohort (Arg754 and Arg778) are  
8 part of helices 1 and 3, respectively, in the armadillo (ARM) repeat 14 (Figure 5A). ARM  
9 14 is located in the hinge region, which connects the C-terminal half of the UCS domain  
10 (ARM repeats 14–17) with the N-terminal half (ARM repeats 10–13) forming part of the  
11 myosin-binding canyon. Exchanging Arg754 for a glutamine does not seem to drastically  
12 interfere with the surrounding structures according to PyMOL calculations of van der  
13 Waals overlaps. Conversely, exchanging the positively charged Arg778 for a bulky  
14 hydrophobic tryptophan would most probably sterically interfere with its surroundings, up  
15 to the possible shifting of the overall structure of the UCS domain. P9 was found to carry  
16 the recurring p.(Arg754Gln) missense in compound heterozygosity with a p.(Ser403Pro)  
17 missense located in the UNC-45 neck domain. Ser403 is conserved in zebrafish UNC-  
18 45B but position 403 appears to have a broader tolerance for other amino acids featuring  
19 a threonine in *Drosophila* and an isoleucine in *C. elegans* UNC-45. Its functional  
20 importance therefore may be restricted to vertebrates but needs to be investigated further.

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

1 missense variant, which impacts the neck domain, and is therefore the only individual in  
2 this series without an UNC-45 UCS domain allele. Given that the UNC-45 neck domain  
3 confers flexibility and allows exact positioning of the UCS and the TPR domains to one  
4 another, structural interference in this region is likely to impact protein function. The  
5 affected cysteine residue is buried in the inner structure of ARM 9 in the neck domain of  
6 UNC-45. Exchanging a cysteine for a positively charged arginine would most probably  
7 lead to steric hindrance and electrostatic interference within the protein structure (Figure  
8 5A).

9  
10 ***Recombinant UNC-45B protein variants are prone to aggregation.*** To further  
11 elucidate structural impacts of the *UNC45B* variants, we recombinantly expressed the  
12 UNC-45B mutant proteins in *E. coli* and subjected them to time-dependent partial trypsin  
13 proteolysis experiments at room temperature (22°C). All mutant variants seemed to be  
14 less susceptible to proteolysis than the wild-type with the p.(Ser403Pro) variant showing  
15 the most pronounced difference (Figure S4A and B). Repeating the partial trypsin  
16 proteolysis assay at the physiological temperature 37°C corroborated the result that  
17 trypsin proteolysis was delayed in the mutant variants with the p.(Ser403Pro) variant  
18 being significantly different in non-parametric Friedman test (Figure 5B and C, Table S4).  
19 In addition, we performed thermal shift assays (TSA) using the protein stain SYPRO  
20 Orange to determine melting temperatures of the recombinant proteins in solution. The  
21 stain intercalates with gradually exposed hydrophobic residues on the protein's surface  
22 allowing for melting temperature estimation based on the half-maximal SYPRO  
23 fluorescence in a Boltzmann approximation. The assay revealed that all variant proteins

1 have a lower melting temperature (Arg754Gln:  $38.18 \pm 0.05145^\circ\text{C}$ , Arg778Trp:  
2  $37.85 \pm 0.04907^\circ\text{C}$ , Ser403Pro:  $37.88 \pm 0.1241^\circ\text{C}$ , Cys514Arg:  $36.90 \pm 0.03901^\circ\text{C}$ )  
3 compared to wild-type ( $43.16 \pm 0.04358^\circ\text{C}$ ) in the Tris-based buffer (Figure 5D, Tables S5  
4 and S6). Exposed hydrophobic residues at a lower temperature might suggest  
5 aggregation tendency. On these grounds, we subjected the recombinant mutant variants  
6 to a filter trap assay to search for aggregates after incubation at room temperature ( $22^\circ\text{C}$ ).  
7 A subsequent slot blot confirmed the suspected higher aggregation of the mutant variants  
8 compared to the wild-type to a similar degree as the absolute SYPRO fluorescence at  
9  $22^\circ\text{C}$  indicated (Figure 5E and F). Further increased aggregation propensity of the mutant  
10 variants around the melting temperature of  $37^\circ\text{C}$  could also explain the observed delay in  
11 *in vitro* proteolysis due to a fraction of the substrate being sequestered in trypsin-  
12 inaccessible aggregates. Together, biochemical analyses of the recombinant UNC-45B  
13 mutant variants suggest that amino acid substitutions at the here reported positions will  
14 likely lead to structural changes in the UNC-45B protein.

15

16 **Myopathy-related UNC-45B mutant missense proteins cannot rescue a conditional**  
17 **loss-of-function allele.** To test for the functional performance of the disease associated  
18 UNC-45B UCS variant proteins in myosin assembly, we made use of an *in vivo* rescue  
19 approach in *C. elegans* described previously<sup>17</sup>. Temperature-sensitive (*ts*) *unc-45(m94)*  
20 mutant worms exhibit a severe movement defect and disarrangement of the otherwise  
21 highly conserved sarcomere organization when grown at the non-permissive temperature  
22 of  $25^\circ\text{C}$ <sup>34</sup>. By expressing the corresponding *C. elegans* UNC-45 variants in conditional  
23 *loss-of-function* worms, we were able to analyze to what degree muscle function of the

1 *unc-45 ts* mutant could be restored. When transferred to liquid medium, worms swim by  
2 thrashing their bodies sideways. This agile movement is almost completely abolished in  
3 the *m94* allele containing mutant worms grown at 25°C. Integrated transgene expressing  
4 the orthologue protein of the p.(Arg778Trp) variant UNC-45(Arg792Trp), of the  
5 p.(Ser403Pro) variant UNC-45(Ile422Pro), and of the p.(Cys514Arg) variant UNC-  
6 45(Cys532Arg) were unable to rescue the movement phenotype of the *unc-45(m94)*  
7 strain. Body bend/thrashing counts of young adult worms in liquid medium were only  
8 slightly improved compared to wild-type levels for the UNC-45(Arg792Trp) and UNC-  
9 45(Ile422Pro) transgenes, whereas worms expressing the UNC-45(Cys532Arg)  
10 transgene were as impaired as *unc-45(m94)* without transgenic expression (Figure 6A).  
11 Assessing population motility of 60 worms using an ARENA WMicrotracker (NemaMatrix)  
12 reproduced the motility defect on a solid agar surface (Figure 6C). These data suggest  
13 that the c.2332C>T; p.(Arg778Trp), the c.1207T>C; p.(Ser403Pro), and the c.1540T>C;  
14 p.(Cys514Arg) variants very likely affect UNC-45B protein function. In contrast,  
15 expressing the orthologue protein of the p.(Arg754Gln) variant UNC-45(Arg767Gln) in the  
16 *unc-45(m94)* background was able to rescue the defect in movement (Figure 6A and C).  
17 Although not possessing polynuclear myofibers, sarcomere organization and  
18 components in *C. elegans* body wall muscle cells are highly conserved: thick filaments  
19 are formed by myosin heavy chains A and B, thin filaments are formed by actin fibers,  
20 and Z-disk-equivalent dense bodies are formed by  $\alpha$ -actinin and integrins<sup>35</sup>. Sarcomere  
21 numbers in body wall muscle cells can be assessed by established staining methods,  
22 which allow counting periodically organized sarcomeric components. In accordance with  
23 motility assays and contrary to the UNC-45(Arg767Gln) transgenic rescue, the UNC-



1 45(Arg792Trp), the UNC-45(Ile422Pro), and the UNC-45(Cys532Arg) transgenic rescues  
2 showed no improvement of the sarcomeres of the *m94 ts*-allele in phalloidin-staining of  
3 filamentous F-actin-containing I-bands (Figure 6D and E).

4         Based on the known mechanistic role of the UCS domain<sup>16; 17; 34</sup>, the corresponding  
5 arginine residues in *C. elegans* can be precisely linked to myosin binding. Whereas  
6 FLAG-tagged UNC-45(Arg767Gln) was able to bind to the substrate myosin heavy chain  
7 B (MHC B/UNC-54) in co-immunoprecipitation experiments (Figure 6F), UNC-  
8 45(Arg792Trp) was not able to pull down MHC B from worm lysates, suggesting  
9 conformational changes of the myosin binding canyon in the UCS domain for the variant  
10 protein. Conceivably, binding to *C. elegans* Hsp90 orthologue DAF-21 via the TPR  
11 domain was not impaired in both variant proteins.

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

21  
22  
23  
24

## 1 Discussion

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

17 Early-onset myopathies are a clinical and genetic heterogeneous group of  
18 disorders of variable severity in which approximately half of individuals do not have a  
19 confirmed genetic etiology<sup>39; 40</sup>. Significant extraocular and facial weakness were notably  
20 absent in the *UNC45B* individuals reported here, which is in contrast to the core  
21 myopathies due to pathogenic variants in *RYR1* (MIM: 180901), *CACNA1S* (MIM:  
22 114208), or *SPEG* (MIM: 615950) in which involvement of the extraocular and facial  
23 muscles are typically seen<sup>41-43</sup>. Of the ten individuals reported here, tachycardia was  
24 noted in two, while two individuals presented with structural cardiac changes (VSD and

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

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

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

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

1 Previous work on the highly conserved UNC-45 in various models including *C.*  
2 *elegans*, zebrafish and mouse, has shown that loss of UNC-45 is embryonically lethal<sup>29</sup>;  
3 <sup>30</sup>; <sup>53</sup>. In this context it is notable that we have not observed individuals with biallelic null  
4 variants, and no homozygous null variants are listed in GnomAD, suggesting that this  
5 situation in the human may also be either not tolerated or be associated with a  
6 considerably more severe phenotype. We have shown that the recurring c.2261G>A  
7 variant is a complex splice allele, that creates a hypomorph scenario, with the residual  
8 protein containing the p.(Arg754Gln) variant. This conclusion is supported by analysis of  
9 available UNC-45B muscle biopsies, as all analyzed samples had a reduction in UNC-  
10 45B protein by western blot, which was most significant in the homozygous c.2261G>A  
11 biopsy (Figure 4C). Thus, we suspect that the disease mechanisms for this variant are  
12 largely driven by the protein deficiency, with additional contribution from a functionally  
13 impaired residual protein. Recombinant UNC-45B(Arg754Gln) variant protein indeed  
14 exhibited a lower melting temperature in thermal shift assays and aggregation tendency  
15 (Figure 5D and E). On the other hand, the ability of the corresponding *C. elegans* variant  
16 UNC-45(Arg767Gln) to rescue a temperature-sensitive mutant allele in transgenic rescue  
17 assays corroborates the notion, that the reduction in overall protein amount is the cause  
18 for the disease phenotype rather than the missense and possibly impaired residual  
19 protein itself (Figure 6).

20 In supplemental *C. elegans* biochemical assays, no changes in UNC-45  
21 ubiquitylation were detected (Figure S4C), although both UCS domain missense variants  
22 were slightly more susceptible to trypsin proteolysis (Figure S4D and E). The UNC-45B  
23 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<sup>8</sup>. 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

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

10

#### 11 **Supplemental Data**

12 Supplemental Data includes: Supplemental Methods, Supplemental Tables S1-6

13 Supplemental Figures S1-4.

14

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21

## 22 **Declaration of Interests**

23 The authors declare no competing interests.



1

## 2 **Web Resources**

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

7

## 8 **Data Availability**

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

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## 9 **Figure Titles and Legends**

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

22

23 **Figure 2. Muscle MRI and histological findings in individuals with *UNC45B*-related**  
24 **myopathy.** (A) Lower extremity muscle MR imaging was available for five (P1-P5)  
25 individuals and showed mild, fat infiltration evident in all lower extremity muscles resulting  
26 in a marbled-like appearance. In individuals P1, P4, and P5 there is abnormal (increased)  
27 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  $\mu\text{m}$ , orange scale bar  
14 corresponds to 5  $\mu\text{m}$ .

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  $\alpha$ -actinin (green) revealing colocalization

1 of UNC-45B at the Z-line. Overlay of intensity profile of UNC-45B and  $\alpha$ -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)<sup>17</sup>. 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

1 14 is located in the hinge region, which connects the C-terminal half of the UCS domain  
2 (ARM repeats 14–17) with the N-terminal half (ARM repeats 10–13) and forms part of the  
3 myosin-binding canyon. The p.(Ser403Pro) (pink, P9) and p.(Cys514Arg) (dark green,  
4 P10) variant impact the UNC-45B neck domain. the structure of the human UNC-45B  
5 protein was inferred from the *C. elegans* UNC-45 structure<sup>17</sup> (PDB ID: 4i2z) using Swiss-  
6 Model<sup>21</sup> and corresponding residues were mutated in PyMOL to display the reported  
7 variants. Van der Waals overlaps of the newly incorporated amino acid residues with the  
8 unmodified protein structure are depicted as red disks. (B) Purified UNC-45B<sup>WT</sup>, UNC-  
9 45B<sup>Arg754Gln</sup>, UNC-45B<sup>Arg778Trp</sup>, UNC-45B<sup>Ser403Pro</sup>, and UNC-45B<sup>Cys514Arg</sup> buffered solutions  
10 were subjected to partial proteolysis with trypsin at 37°C and samples were loaded on  
11 SDS-PAGE gels for separation. Coomassie-stained gels of one of two repetitions are  
12 shown. (C) Quantification of full-length protein (FL, ~109 kDa) compared to time point 0  
13 at 37°C of two repetitions. Mean and SD can be found in Table S4. (D) Purified UNC-  
14 45B<sup>WT</sup>, UNC-45B<sup>Arg754Gln</sup>, UNC-45B<sup>Arg778Trp</sup>, UNC-45B<sup>Ser403Pro</sup>, and UNC-45B<sup>Cys514Arg</sup>  
15 buffered solutions were slowly heated from 10°C to 95°C in the presence of SYPRO  
16 Orange protein stain. Boltzmann sigmoidal curves were fit to normalized combined melt  
17 curves of three experiments. Half maximal temperatures indicate melting temperatures in  
18 the Tris-based buffer. Mean and SD can be found in Table S5 (E) Slot blot of purified  
19 UNC-45B<sup>WT</sup>, UNC-45B<sup>Arg754Gln</sup>, UNC-45B<sup>Arg778Trp</sup>, UNC-45B<sup>Ser403Pro</sup>, and UNC-  
20 45B<sup>Cys514Arg</sup> Tris-buffered solutions incubated for 1 h at room temperature filtered through  
21 a 0.2 µm acetyl-cellulose membrane. Three decreasing amounts of protein solution were  
22 blotted for comparison. (F) Baseline-subtracted SYPRO fluorescence values at 22°C  
23 obtained in two experiments in (D) are plotted for each protein solution. No significant



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

3

4 **Figure 6. Various missense UNC-45 mutant proteins cannot rescue a conditional**

5 **loss-of-function allele.** Transgenic UNC-45<sup>Arg792Trp</sup> mutant protein is unable to rescue

6 the motility defect of *unc-45(m94)* worms grown at the restrictive temperature of 25°C,

7 whereas UNC-45<sup>Arg767Gln</sup> rescues the *m94* motility defect. (A) Body bends of 30 young

8 adult *unc-45(m94)* worms expressing the indicated UNC-45 variants were counted in at

9 least three different experiments. Values are mean ± SEM; \*: p<0.0001 compared to

10 control in non-parametric Kruskal-Wallis test. (B) Expression levels of UNC-45-FLAG

11 transgenes in young adult *unc-45(m94)* worms. (C) Population motility of 60 young adult

12 *unc-45(m94)* worms expressing the indicated UNC-45 variants on a 24 well plate filled

13 with NGM and seeded with OP50 was measured with the ARENA WMicrotracker system

14 (NemaMetrix) at 25°C for 24 hours after reaching adulthood. (D) Phalloidin-staining of F-

15 actin-containing I-bands in *unc-45(m94)* mutant worms expressing the indicated UNC-45

16 variants. (E) The number of I-bands is given per body wall muscle cell (indicated by

17 dashed white line in D), with all 12 analyzed cells located in the same area between

18 pharynx and vulva. Values are mean ± SEM; \*: p<0.005 compared to control in non-

19 parametric Kruskal-Wallis test. (F) Co-immunoprecipitation of myosin heavy chain B

20 (MHC B) and Hsp90 (DAF-21) from cell lysates of *unc-45(m94)* mutant worms expressing

21 the indicated FLAG-tagged UNC-45 variants grown at 25°C. Representative result of one

22 of three experiments is shown.

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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