Mutations affecting the cytoplasmic functions of the co-chaperone DNAJB6 cause limb-girdle muscular dystrophy

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Limb-girdle muscular dystrophy type 1D (LGMD1D) was linked to chromosome 7q36 over a decade ago1, but its genetic cause has remained elusive. Here we studied nine LGMD-affected families from Finland, the United States and Italy and identified four dominant missense mutations leading to p.Phe93Leu or p.Phe89Ile changes in the ubiquitously expressed co-chaperone DNAJB6. Functional testing in vivo showed that the mutations have a dominant toxic effect mediated specifically by the cytoplasmic isoform of DNAJB6. In vitro studies demonstrated that the mutations increase the half-life of DNAJB6, extending this effect to the wild-type protein, and reduce its protective anti-aggregation effect. Further, we show that DNAJB6 interacts with members of the CASA complex, including the myofibrillar myopathy–causing protein BAG3. Our data identify the genetic cause of LGMD1D, suggest that its pathogenesis is mediated by defective chaperone function and highlight how mutations in a ubiquitously expressed gene can exert effects in a tissue-, isoform- and cellular compartment–specific manner.

Limb-girdle muscular dystrophies (LGMDs) are a genetically and mechanistically heterogeneous group of disorders caused by dominant or recessive mutations in a number of sarcolemmal, sarcomeric, cytoplasmic and nuclear proteins2. Here we studied the molecular cause of a dominant LGMD in five previously reported Finnish (FF1–5)3,4 and two US families (DUK1047 and DUK1701)1,5, as well as two previously unreported Italian families (IT1 and IT2) identified on the basis of clinical phenotypes and of the pattern of muscle involvement by magnetic resonance imaging. The pedigrees of the families are shown in Supplementary Figure 1; clinical findings are summarized in Supplementary Table 1.

The disorder in the two US families was originally linked to 7q36 and classified as LGMD1D. Clinical and genetic characterization of the Finnish families established linkage to the same locus and refined LGMD1D to a 3.4-Mb region containing 12 genes3,4. Sequencing of the positional candidates RNF32, UBE3C, DNAJB6 and PTPRN2 identified a c.279C>G (p.Phe93Leu) change in exon 5 of DNAJB6 in all 16 affected individuals in the Finnish families FF1–5 (nucleotide and protein numberings based on RefSeq records NM_005494.2 and NP_005485.1, respectively). The mutation was absent from 12 unaffected individuals in these families (Table 1 and Supplementary Figs. 1 and 2). Sequencing of muscle cDNA from two affected Finnish individuals confirmed that wild-type and mutant alleles were both expressed (Supplementary Fig. 2). Analysis of DNAJB6 in the two US families identified a c.267T>A (p.Phe89Ile) mutation in all 29 affected individuals (Table 1) and in two individuals who were of unknown disease status (as they were below the highest age of onset identified previously in the families). Two DNAJB6 mutations were identified in the Italian families—c.279C>A (IT1; eight cases) and c.277T>C (IT2; four cases)—both causing the same p.Phe93Leu change as the Finnish mutation (Table 1). Six available unaffected individuals in IT1 and four in IT2 had no mutations. None of the mutations were found in control chromosomes from 202 Finnish, 104 Italian or 215 US individuals. The presence of independent mutations and their segregation with the phenotype in nine families provides conclusive evidence that DNAJB6 is the causative gene for LGMD1D.

DNAJB6 (also known as mammalian relative of DnaJ (MRJ)) belongs to the J proteins (also known as the heat-shock protein 40 (Hsp40) family), a class of co-chaperones characterized by a J domain. These co-chaperones interact with chaperones of the HSPA (Hsp70)
Table 1  DNAJB6 mutation status in affected and unaffected individuals from Finnish, US and Italian LGMD1D-affected families

| Family ID | Nucleotide change (protein change) | Total | Affected | Unaffected |
|-----------|------------------------------------|-------|----------|-----------|
| FF1       | c.279C>G (p.Phe93Leu)              | 9     | 9        | 0         |
| FF2       | c.279C>G (p.Phe93Leu)              | 3     | 3        | 0         |
| FF3       | c.279C>G (p.Phe93Leu)              | 1     | 1        | 2         |
| FF4       | c.279C>G (p.Phe93Leu)              | 2     | 2        | N/A       |
| FF5       | c.279C>G (p.Phe93Leu)              | 1     | 1        | N/A       |
| DUK1047   | c.267T>A (p.Phe89Ile)              | 10    | 10       | 1^a       |
| DUK1701   | c.267T>A (p.Phe89Ile)              | 19    | 19       | 22        |
| IT1       | c.279C>G (p.Phe93Leu)              | 8     | 8        | 6         |
| IT2       | c.277T>C (p.Phe93Leu)              | 4     | 4        | 4         |

FF, Finnish; DUK, US; IT, Italian LGMD1D-affected families. N/A, not available.

*Individuals of unknown disease status. At their most recent exam, these mutation carriers were younger than the oldest age of onset previously identified in their respective families (DUK1047, 55 years; DUK1701, 50 years).

DNAJB6 is expressed in all tissues, with the highest expression in brain, but its expression and localization in skeletal muscle have not been characterized previously. Immunofluorescence microscopy showed DNAJB6 primarily in Z-disks in both control (not shown) and LGMD1D muscle samples (Fig. 1a). Electron microscopy of LGMD1D patient muscle showed Z-disc myofibrillar disintegration (Fig. 1b). We also observed autophagic rimmed-vacuolar degenerative pathology by electron microscopy (Fig. 1b and Supplementary Fig. 3). LC3 immunohistochemistry and Herovici staining (Fig. 1c). Immunofluorescence microscopy of patient muscle showed DNAJB6 in protein accumulations together with its known ligands MLF1 (ref. 21) and HSPA8 (ref. 9) (Fig. 1d and Supplementary Fig. 4). We also observed myotilin, desmin, αB-crystallin and, occasionally, 

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filamin—proteins that typically aggregate in myofibrillar myopathies—in these structures. Furthermore, some accumulations stained positive for keratin 18 (KRT18), a reported client protein of DNAJB6 (ref. 9; Fig. 1d and Supplementary Fig. 4). Notably, we did not observe accumulation of thick and thin filament components such as myosins and actin (data not shown). For comparison, we examined muscle tissue from an individual with myofibrillar myopathy caused by a p.Ser60Cys alteration in myotilin, characterized by aggregation of myotilin and other proteins23-25. We found colocalization of DNAJB6, MLF1 and KRT18 with myotilin in these aggregates (Supplementary Fig. 4). In LGMD1D muscle, DNAJB6 appeared more in the periphery of the protein accumulations, in contrast to the more pronounced colocalization seen in myotilinopathy.

To gain further insights into DNAJB6 function, we examined its role in muscle integrity in zebrafish. Reciprocal BLAST identified a single zebrafish DNAJB6 ortholog (dnajb6b). Using RT-PCR, we detected its expression as early as the embryonic five-somite stage (data not shown). Next, we injected two-cell embryos with a splice-blocking morpholino (sb-MO) targeting dnaib6b. Masking scoring of embryos at 48 h after fertilization (h.p.f.) showed a reproducible muscle fiber detachment phenotype concomitant with the splice-blocking efficiency, as established by RT-PCR (Supplementary Fig. 5a). Detachment of slow fibers from their insertion sites at the vertical myoseptum was evident as early as 2 d after fertilization (d.p.f.) (Fig. 2), suggesting that the fiber termini in morphants are prone to adhesion failure soon after mechanical load is applied by the onset of strong contraction24. The phenotype was specific because it was reproduced with a second non-overlapping MO (data not shown) and rescued with co-injection of wild-type human DNAJB6b mRNA (Fig. 2). These data suggest that loss of DNAJB6 function leads to defects in muscle integrity.

To understand the mechanistic effect of LGMD1D-associated mutations, we introduced the p.Phe93Leu and p.Phe89Ile changes into human transcripts encoding each of the two isoforms and injected mutant and morphant zebrafish. (Fig. 4). The phenotype in mutant mRNA injected embryos showed enhanced severity of the muscular phenotype: in comparison to 15% of embryos injected with mutant DNAJB6b alone, 42% of co-injected embryos had severe defects in muscle integrity by 2 d.p.f. (P = 0.0004; Fig. 3a). We observed a similar effect with the p.Phe89Ile mutation (P = 0.0002; Fig. 3a). Taken together, our data indicate that the phenotype is driven by dysfunction of the cytoplasmic isoform and suggest that the LGMD-causing mutations probably exert a deleterious dominant effect on the wild-type protein. Consistent with this notion, altering the molar ratios of mutant and wild-type mRNA had the expected effects on the phenotype: an excess of mutant mRNA compared to wild-type mRNA induced lethality in embryos, whereas an excess of wild-type mRNA compared to mutant mRNA gave rise to progressively increased rescue (Supplementary Fig. 5c).

We next performed in vitro experiments. On the basis of evidence from the closely related DNAJB8 protein, DNAJB6 has been suggested to form oligomeric complexes2. As interference with complex formation could explain the dominant effect of the mutations, we studied oligomerization of wild-type and p.Phe93Leu DNAJB6b in COS-1 cells. In co-immunoprecipitation (co-IP) experiments, V5-tagged DNAJB6b pulled down the untagged construct, demonstrating oligomerization (Supplementary Fig. 6a), and this was unaffected by the p.Phe93Leu alteration. Likewise, in sucrose density gradient centrifugation, the distribution of wild-type and mutant proteins was similar (Supplementary Fig. 6b). Hence, the p.Phe93Leu substitution did not significantly alter the oligomerization properties of DNAJB6.

Figure 2 Muscle disintegration in DNAJB6b mutant and morphant zebrafish. (a–i) Lateral views of fish embryos 2 d.p.f. subjected to whole-mount immunofluorescence staining of slow myosin heavy chain. Injected embryos expressing wild-type (wt) DNAJB6b or DNAJB6b showed slow myofibers spanning the somite normally between adjacent myosepta (c,f) and were indistinguishable from control embryos. Injection of DNAJB6b p.Phe93Leu and p.Phe89Ile mutant mRNAs resulted in detachment of fibers from the vertical myoseptum (a,b), whereas injection of DNAJB6a mutants resulted in a normal appearance (d,e). Similar muscular disintegration was observed in dnaib6b morphant embryos injected with sb-MO against the zebrafish ortholog of DNAJB6 (g) and in embryos expressing DNAJB6b p.Phe93Ala or p.Phe93Gly (h,i). The detachment of myofibers from the myoseptum can be partial (a,b,g,h, white arrowheads) or complete (i, white asterisk). Scale bar, ~50 µm. (j) Embryos injected with the indicated constructs were categorized phenotypically based on the presence of muscle fiber detachment affecting 1–2 somites (class I, mild) or multiple somites (class II, severe; see Supplementary Figure 5 for an example). The phenotype in dnaib6b MO-injected embryos was rescued efficiently by wt human DNAJB6b mRNA (χ² test).
We next considered the possibility that LGMD1D mutations might alter DNAJB6 half-life, as we observed that cells transfected with DNAJB6 showed an increase in mutant protein abundance with no appreciable changes in mRNA levels (data not shown). To examine this, we transfected 293FT cells, blocked protein synthesis with cycloheximide (CHX) and quantified the amount of remaining protein as a function of time (Fig. 3b and Supplementary Fig. 7). Both p.Phe93Leu and p.Phe89Ile considerably decreased the turnover rate of DNAJB6. To explore whether the increased abundance of mutant DNAJB6 might be proteasome or autophagosome dependent, we treated DNAJB6-transfected cells either with a proteasome poison (lactacystin) or a lysosome inhibitor (bafilomycin A1). Suppression of the proteasome has been reported to enhance autophagy (Fig. 3c) and normalized to α-tubulin (single transfections) or HSP90 (co-transfections) intensity to obtain the relative DNAJB6 levels. In single transfections (solid lines), CHX treatment rapidly decreased wt DNAJB6 protein levels, whereas p.Phe89Ile and p.Phe93Leu mutant proteins showed reduced turnover. In co-transfections of HA- and GFP-tagged wt and p.Phe93Leu mutant constructs (dashed lines), the wt DNAJB6 level remained stable after 4 h of CHX treatment. Data from three independent experiments are presented as mean ± s.d. Relative DNAJB6 level at $t = 0$ h was set to 100 for each construct. Representative protein blots are shown in Supplementary Figure 7.

![Image](328x61 to 423x135)

**Figure 3** Dominant effect of mutant DNAJB6 proteins. (a) Co-injection of wt DNAJB6 mRNA with p.Phe93Leu or p.Phe89Ile mutant mRNA in zebrafish embryos led to a more severe muscle phenotype, with a statistically significant increase in the number of class II embryos ($\chi^2$ test). (b) DNAJB6 constructs were expressed in 293FT cells, and protein synthesis was blocked by cycloheximide (CHX). Whole-cell extracts were obtained at the indicated time points and analyzed by protein blotting. DNAJB6 band intensities were quantified and normalized to α-tubulin (single transfections) or HSP90 (co-transfections) intensity to obtain the relative DNAJB6 levels. In single transfections (solid lines), CHX treatment rapidly decreased wt DNAJB6 protein levels, whereas p.Phe89Ile and p.Phe93Leu mutant proteins showed reduced turnover. In co-transfections of HA- and GFP-tagged wt and p.Phe93Leu mutant constructs (dashed lines), the wt DNAJB6 level remained stable after 4 h of CHX treatment. Data from three independent experiments are presented as mean ± s.d. Relative DNAJB6 level at $t = 0$ h was set to 100 for each construct. Representative protein blots are shown in Supplementary Figure 7.

A possible explanation for the increased abundance of mutant–wild type protein complexes would be formation of aggregates. However, we did not detect any increased aggregation propensity of the mutants in cell fractionation, sucrose density gradient centrifugation or immunofluorescence microscopy of transfected cells (data not shown). Hence, primary aggregation of DNAJB6 is unlikely to underlie the pathogenesis of LGMD1D. This is in line with the muscle pathology, which showed DNAJB6 in the periphery of the protein accumulations rather than in the center.

DNAJB6 has been shown to suppress aggregation of various proteins in cell culture models. To investigate the effect of the mutations on the anti-aggregation function, we tested the ability of wild-type and mutant DNAJB6 to inhibit aggregation of polyglutamine-containing huntingtin (pEGFP/HD-120Q) in a filter-trap assay. The nuclear DNAJB6 isoform, known to be inefficient in inhibiting cytoplasmic huntingtin aggregation, served as negative control. Wild-type DNAJB6 efficiently suppressed huntingtin aggregation, as demonstrated by a decrease in the aggregate amount and a concomitant increase in SDS-soluble huntingtin (Fig. 4 and Supplementary Fig. 8). In contrast, mutant constructs showed significantly impaired anti-aggregation function, although they still retained some activity. This compromised function could impair protein quality control in LGMD1D muscle, leading to protein accumulation.

Chaperone-assisted selective autophagy (CASA), mediated by a complex containing HSPA8, HSPB8 (also known as Hsp22), and the α-interacting protein (CHIP), is important for Z-disk maintenance of thin filaments and regulatory complexes (Fig. 5). Several pieces of evidence prompted us to investigate the link between DNAJB6 and CASA. First, DNAJB6 is a known co-chaperone of HSPA8.
The interaction with DNAJB6 suggested a possible role for BAG3 in LGMD1D muscle. Second, both DNAJB6 and the CASA complex localize to the Z-disk. Finally, mutations in BAG3 cause a myofibrillar myopathy with protein accumulations and autophagic pathology. To study a possible association of DNAJB6 with the CASA complex, we performed co-IP studies of endogenous and transfected proteins in COS-1 cells and observed interaction of DNAJB6b with BAG3, HSPB8 and STUB1. Negative control experiments were performed with DNAJB6 or BAG3 antibodies alone. Each image shows a representative maximum-intensity projection through a z stack of 6.4 μm, with the PLA signal (red) superimposed on the phalloidin counterstain (gray). Scale bar, 5 μm.

The four LGMD1D mutations affect two highly conserved, closely spaced phenylalanine residues in the G/F domain. The mutations cause a dominant, late-onset muscle phenotype. Although the mutations alter the sequence of DNAJB6, they affect muscle pathology by impairing binding of DNAJB6 to the CASA complex.

This protein has been implicated in a variety of degenerative disorders such as Parkinson’s and Huntington’s diseases, as well as cancer. Notably, the DNAJB6 mutations we identified cause a tissue-specific disease (muscular dystrophy); despite high expression of DNAJB6 in brain, there are no indications of neurological involvement.

The interaction of BAG3 with DNAJB6 and its aggravating effect on the zebrafish phenotype suggest a role for BAG3 in the pathomechanism. The myofibrillar disintegration with protein accumulations in LGMD1D muscle may reflect impaired function of the CASA machinery, leading to insufficient maintenance of sarcomeric structures or defective clearance of misfolded sarcomeric proteins. The DNAJB6 mutations cause a dominant, late-onset muscle disease characterized by abnormal protein accumulations and autophagic pathology. Although the mutations alter the sequence of both nuclear and cytoplasmic DNAJB6, they affect muscle pathology exclusively through the cytoplasmic isoform. This is reminiscent of the mechanism proposed for Lafora progressive myoclonus epilepsy, in which mutations in PMP22 are expressed in both the nucleus and the cytoplasm but seem to interfere specifically with its cytoplasmic phosphatase activity.

The literature contains few other such

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The interaction with DNAJB6 suggested a possible role for BAG3 in LGMD1D muscle and implied a pathomechanistic link between LGMD1D and the BAG3 myopathy. We therefore asked whether BAG3 could modulate the phenotype caused by mutant DNAJB6b in zebrafish. Injection of wild-type DNAJB6b mRNA alone caused no observable phenotype, whereas its co-injection with DNAJB6b p.Phe93Leu mRNA and wild-type (wt) or p.Pro209Leu mutant BAG3 mRNA in indicated combinations. Co-injection of wild-type BAG3 with mutant DNAJB6b resulted in a more severe phenotype than mutant DNAJB6b alone, reflected by a significant increase in class II embryos. In contrast, co-injection of p.Pro209Leu BAG3 with mutant DNAJB6b did not alter the phenotype distribution. Statistical significance (χ² test) is denoted by P value or n.s. (not significant). Scale bar, 5 μm.

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Subjects and muscle biopsies. The Finnish (FF1–5) and US families (DUK1047 and DUK1701) have been described. Primary muscle biopsies were obtained from the Finnish families. Previous diagnostic muscle biopsies from the DUK families were reviewed. All examinations were performed with informed consent according to the Declaration of Helsinki. Studies were approved by local ethics committees.

Sequencing. PCR and Sanger sequencing were performed with standard methods. Sequences were analyzed with Sequencher 4.8 (Gene Codes Corporation).

RNA isolation and RT-PCR. RNA was isolated from muscle biopsies using the Dr. P kit (BioChain Institute, Inc.) and reverse-transcribed using SuperScript II (Invitrogen). Target sequences were amplified with DreamTaq (Fermentas GmbH) and sequenced.

Primary antibodies. Commercial primary antibodies were from Abcam plc (HSPA8, HSPB8, KRT18, MLF1, α-tubulin); Abnova (DNAJB6); Calbiochem, Merck Chemicals (STUB1); Cell Signaling Technology (HSPPB); DSHB (myosin heavy chain); Epitomics, Inc. (desmin); Invitrogen (V5); Novocastro, Leica Microsystems GmbH (αβ-crystallin); Novus Biologicals, Inc. (LC3); Proteintech Group, Inc. (BAG3); Sigma-Aldrich (filamin, GFP, HA, α-tubulin); and Santa Cruz Biotechnology, Inc. (GFP, HSP90). Myotilin antibodies were a gift from O. Carpén. For product details and antibody dilutions, see Supplementary Table 2.

Electron microscopy. Muscle specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer overnight at 4 °C, washed in 0.1 M phosphate buffer, dehydrated, embedded in Epon blocks, sectioned and imaged by transmission electron microscopy.

Immunofluorescence staining and microscopy. Muscles were embedded in Tissue-Tek O.C.T. medium (Sakura Finetek Europe B.V.), frozen in liquid nitrogen–chilled isopentane and sectioned. For immunofluorescence, the sections were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 5% BSA in PBS for 30 min and immunostained with antibodies in 1% BSA in PBS. Immunohistochemical and histological stainings were performed according to standard procedures. For whole-mount immunofluorescence, zebrafish embryos were fixed in 4% PFA overnight and stored in 35%–70% methanol at −20 °C. After rehydration in PBS, embryos were washed in 0.1% Tween-20, 1% BSA in PBS for 10 min at room temperature, incubated in blocking buffer (10% PBS, 1% BSA in PBS) for 1 h at room temperature and immunostained with antibodies in blocking buffer. Primary antibody incubations were 1–2 h at room temperature (sections) or overnight at 4–8 °C (zebrafish embryos). Alexa Fluor–labeled secondary antibodies were obtained from Molecular Probes, Invitrogen.

Wide-field fluorescence microscopy was performed using Zeiss Axioplan 2 (Carl Zeiss MicroImaging GmbH) or AZ100 (Nikon Instruments Inc.). Confocal microscopy was performed using LSM 510 Meta (Carl Zeiss Microimaging) with an x63, numerical aperture (NA)-1.4 objective lens. Image processing and analysis were done using the LSM 510 Meta 3.2 software (Carl Zeiss MicroImaging), Adobe Photoshop CS4 11.0.2 (Adobe Systems Inc.) and ImageJ 1.41o (US National Institutes of Health).

SDS-PAGE and protein blotting. SDS-PAGE and protein blotting were performed according to standard methods. Blots were visualized with chemiluminescence using film or a ChemiDoc XRS+ system (Bio-Rad Laboratories) or by fluorescence detection with the Odyssey system (LI-COR Biosciences). Molecular Probes, Invitrogen.

Electrophoresis, zebrafish embryos were fixed in 4% PFA overnight and stored in 35%–70% methanol at −20 °C. After rehydration in PBS, embryos were washed in 0.1% Tween-20, 1% BSA in PBS for 10 min at room temperature, incubated in blocking buffer (10% PBS, 1% BSA in PBS) for 1 h at room temperature and immunostained with antibodies in blocking buffer. Primary antibody incubations were 1–2 h at room temperature (sections) or overnight at 4–8 °C (zebrafish embryos). Alexa Fluor–labeled secondary antibodies were obtained from Molecular Probes, Invitrogen.

Co-immunoprecipitation (co-IP). COS-1 cells were collected 1–2 d after transfection, lysed with ice-cold 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1× Complete and incubated on ice for 10 min. NaCl was added to 150 mM. The lysates were incubated on ice for 5 min and centrifuged 15 min at 16,000g at 8 °C. After removing total protein samples, the lysates were rotated with anti-V5 beads (Novus Biologicals) overnight at 8 °C. The beads were washed 4–6 times with ice-cold 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Triton X-100.

Alternative, for experiments with cross-linking, cells were lysed in ice-cold PBS containing 0.5% Triton X-100, 1× Complete by trituration through a 27G needle, and centrifuged for 15 min at 16,000g at 8 °C. The lysates were cross-linked with 1 mM dithiobis(succinimidy) propionate (Pierce, Thermo Fisher; from 20 mM stock in DMSO) for 30 min on ice. Cross-linking was stopped with 20 mM Tris–HCl, pH 7.4 for 15 min on ice. After removing total protein samples, the lysates were rotated with anti-V5 beads three times with ice-cold RIPA (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS).

Bound proteins were eluted into 2× SDS sample buffer with 10% 2-mercaptoethanol at 95 °C for 5 min.

Density gradient centrifugation. The density gradient centrifugation protocol was adapted from Hageman et al. COS-1 cells were transfected with wild-type or p.Phe93Leu pEF6-DNAJB6b. After 24 h, cells were lysed with 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 3% glycerol, 1× Complete and centrifuged for 15 min at 300g. Supernatants were loaded on 10–80% sucrose gradients in 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA and centrifuged for 18–20 h at 100,000g at 8 °C. The gradients were fractionated and proteins precipitated with TCA essentially as described.

Cell cultures and transfections. COS-1 (ATCC), 293FT (Invitrogen) and T-REx 293 cells (Invitrogen) were cultured at 37 °C, 5% CO₂ in DMEM with 10% FCS and l-glutamine. Media were supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (for COS-1 and T-REx 293) and 5 μg/ml blasticidin S (for T-REx 293). Transfections were done with FuGENE 6 (Roche Applied Science).

Turnover assays and drug treatments. 293FT cells were transfected at 70% confluence with wild-type or mutant HA- or GFP-tagged DNAJB6. 48 h after transfection, the medium was replaced with fresh medium containing 50 μg/ml cycloheximide (Sigma-Aldrich) and optionally 20 μM lactacystin (EMD Chemicals Inc.) or 100 nM bafilomycin A1 (Sigma-Aldrich). At indicated time points, cells were washed twice with PBS and lysed with RIPA supplemented with Complete Protease Inhibitor Cocktail (Complete; Roche Applied Science). Lysates were centrifuged for 10 min at 15,800g at 4 °C, and supernatants were analyzed by protein blotting. Bands were quantified with Quantity One (Bio-Rad). The ratios of DNAJB6 constructs to α-tubulin or HSP90 were plotted against time. For time-series experiments, the ratio at t = 0 h was set to 100% for each construct.

Co-immunoprecipitation (co-IP). COS-1 cells were collected 1–2 d after transfection, lysed with ice-cold 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1× Complete and incubated on ice for 10 min. NaCl was added to 150 mM. The lysates were incubated on ice for 5 min and centrifuged 15 min at 16,000g at 8 °C. After removing total protein samples, the lysates were rotated with anti-V5 beads (Novus Biologicals) overnight at 8 °C. The beads were washed 4–6 times with ice-cold 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Triton X-100.

Alternatively, for experiments with cross-linking, cells were lysed in ice-cold PBS containing 0.5% Triton X-100, 1× Complete by trituration through a 27G needle, and centrifuged for 15 min at 16,000g at 8 °C. The lysates were cross-linked with 1 mM dithiobis(succinimidy) propionate (Pierce, Thermo Fisher; from 20 mM stock in DMSO) for 30 min on ice. Cross-linking was stopped with 20 mM Tris–HCl, pH 7.4 for 15 min on ice. After removing total protein samples, the lysates were rotated with anti-V5 beads three times with ice-cold RIPA (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS).

Bound proteins were eluted into 2× SDS sample buffer with 10% 2-mercaptoethanol at 95 °C for 5 min.

Density gradient centrifugation. The density gradient centrifugation protocol was adapted from Hageman et al. COS-1 cells were transfected with wild-type or p.Phe93Leu pEF6-DNAJB6b. After 24 h, cells were lysed with 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 3% glycerol, 1× Complete and centrifuged for 15 min at 300g. Supernatants were loaded on 10–80% sucrose gradients in 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA and centrifuged for 18–20 h at 100,000g at 8 °C. The gradients were fractionated and proteins precipitated with TCA essentially as described.
Filter-trap assay (FTA). T-REx 293 cells were plated on collagen-coated 6-well plates at 150,000 cells per well and co-transfected with 875 ng of pcDNA5/TO-DNAJB6 and 125 ng of pEGFP/HD-120Q. Each transfection was done in duplicate, and after 7 h, DNAJB6 expression was induced in one of the duplicates with 1 µg/ml tetracycline. 48 h after transfection, FTA was performed according to a protocol adapted from Hageman et al.\textsuperscript{7}. The cells were lysed with 750 µl of FTA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM dithiothreitol) containing 2% SDS and 1× Complete, triturated through a 27-G needle, sonicated for 1 min at room temperature using a Lotzonic bath sonicators and heated to 98 °C. Samples for SDS-PAGE analysis of soluble huntingtin were removed, and 100–150 µl of the remaining lysates were filtered through a cellulose acetate membrane (pore size 0.2 µm, Whatman GmbH), followed by three washes with 300 µl FTA buffer containing 0.1% SDS.

FTA membranes and western blots of SDS-soluble huntingtin were immunostained with GFP antibody, visualized using fluorescent detection and quantified with the Odyssey software (LI-COR). Aggregation scores ([aggr./sol.]\textsubscript{induced}/[aggr./sol.]\textsubscript{uninduced}) were calculated from the amounts of aggregated (aggr.) and soluble (sol.) huntingtin in the uninduced and induced cells in each transfection pair. Each construct was tested in three quadruplicate experiments.

In situ proximity ligation assay (PLA). PLA was performed on rat gastrocnemius muscle sections using the Duolink kit (Olink Bioscience). The samples were prepared and PLA performed essentially as described\textsuperscript{37}.

Statistical analysis. Results were analyzed with two-tailed Mann-Whitney U-test with Bonferroni correction (filter-trap assay) or with the $\chi^2$ test (zebrafish phenotype distributions).

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