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Novel mutations in DNAJB6 cause LGMD1D and distal myopathy in French families

Authors

Per H. Jonson^{1*}, PhD; Johanna Palmio^{2*}, MD, PhD; Mridul Johari¹, MSci; Sini Penttilä², MSci; Anni Evilä¹, PhD; Isabelle Nelson³, PhD; Gisèle Bonne³, PhD; Nicolas Wiart⁴, MSci; Vincent Meyer⁴, PhD; Anne Boland⁴, PhD; Jean-François Deleuze⁴, PhD; Cécile Masson⁵, PhD; Tanya Stojkovic³, MD, PhD; Françoise Chapon⁶, MD, PhD; Norma B. Romero⁷, MD, PhD; Guilhem Solé⁸, MD; Xavier Ferrer⁸, MD, PhD; Ana Ferreira⁹, MD, PhD; Peter Hackman¹, PhD; Isabelle Richard¹⁰, PhD; Bjarne Udd¹¹, MD, PhD

Full address:

1. PHJ, MJ, AE, PH: Folkhälsan Institute of Genetics and University of Helsinki, Medicum, Finland.
2. JP, SP: Neuromuscular Research Center, Tampere University Hospital and University of Tampere, Finland.
3. IN, GB, TS: Sorbonne Universités, UPMC Univ Paris 06, INSERM UMRS 974, Center of Research in Myology, Institut de Myologie, F-75013, Paris, France.
4. NW, VM, AB & J-F D: Centre National de Recherche en Génomique Humaine (CNRGH), CEA, Evry, France.
5. CM: Bioinformatics Core Facility, Université Paris Descartes - Structure Fédérative de Recherche Necker, INSERM US24/CNRS UMS3633, INSERM UMR 1163, Institut Imagine, Paris, France.
6. FC: Université de Normandie, INSERM U1075, Neuromuscular Competence Center, CHU Caen, Caen, France CHU Caen, France.
7. NBR: Unit of Neuromuscular Morphology, Institute of Myology, UPMC Paris 6, INSERM UMRS 974, Pitié-Salpêtrière Hospital, Paris, France.
8. GS, XF: Neuromuscular Reference Center, CHU Bordeaux, Bordeaux, France.
9. AF: Unité de Biologie Fonctionnelle et Adaptative, Université Paris Diderot/CNRS, France; Reference Center for Neuromuscular Disorders, Pitié-Salpêtrière Hospital, AP-HP, France.
10. IR: Généthon INSERM, U951, INTEGRARE research unit, University Paris-Saclay, Evry, F-91002, France.

11. BU: Neuromuscular Research Center, Tampere University Hospital and University of Tampere, Finland; Folkhälsan Institute of Genetics and University of Helsinki, Medicum, Finland; Department of Neurology, Vaasa Central Hospital, Vaasa, Finland.

Phone, fax, e-mail of corresponding author: Phone +358-3-3116111, fax: +358-3-35516164,

e-mail: johanna.palmio@staff.uta.fi

Corresponding author: Johanna Palmio

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*Equal contribution

Abstract

Background and purpose: To determine the genetic background of unknown muscular dystrophy in five French families.

Methods: Twelve patients with limb girdle muscular dystrophy or distal myopathy were clinically evaluated. Gene mutations were identified using targeted exon sequencing and mutated DNAJB6 was tested *in vitro*.

Results: Five patients presented with distal lower limb weakness while others had proximal presentation with variable rate of progression starting at the median age of 34.6 years. Two novel mutations (c.284A>T, p.Asn95Ile, 2 families and c.293_295delATG, p.Asp98del, 1 family) as well as the previously reported c.279C>G (p.Phe93Leu, 2 families) mutation in *DNAJB6* were identified. All showed a reduced capacity to prevent protein aggregation.

Conclusions: The mutational and phenotypical spectrum of *DNAJB6*-caused muscle disease is larger than previously reported, including also dysphagia. The originally reported

c.279C>G (p.Phe93Leu) mutation is now identified in four different populations and appears to be a mutational hotspot. Our report confirms that some *DNAJB6* mutations cause distal-onset myopathy and hence *DNAJB6* defects should be considered broadly in dominant muscular dystrophy families.

Introduction

Autosomal dominant limb-girdle muscular dystrophy type 1D (LGMD1D, OMIM 603511) caused by *DNAJB6* mutations [1,2] has so far been identified in Europe [1,3,4], Asia [5-8] and the US [1,2,9,10]. All reported mutations affect *DNAJB6*'s G/F-domain, encoded by exon 5. Classical LGMD1D is late-onset with slow progression, and most patients remain ambulatory at high age. However, some *DNAJB6* mutations can cause a much earlier onset and severe progression [3,5], and some may present with a more distal phenotype. Cardiac involvement has not been observed, but the severe p.Phe91Ile and p.Phe91Leu mutations lead to respiratory failure [3]. Muscle biopsies show myofibrillar protein accumulations and later autophagic rimmed vacuolar dystrophic pathology indicating secondary abnormal autophagic induction. The accumulations commonly contain sarcomeric Z-disc proteins such as α B-crystallin, myotilin and desmin. The rimmed vacuoles stain for the autophagic markers TDP-43, LC3 and SQSTM1, but not for lysosomal marker LAMP2, suggesting problems with autophagosome maturation and lysosome fusion [11].

We report the identification of *DNAJB6* mutations in five French families. Two novel mutations (c.284A>T; p.Asn95Ile, c.293_295delATG; p.Asp98del), in addition to the previously reported c.279C>G (p.Phe93Leu), were identified. Functional studies of the novel mutations confirm reduced anti-aggregation capacity.

Methods

Twelve patients from five French families with distal myopathy or LGMD were evaluated (Figure 1A). DNA from families 1 and 2 were sequenced using MYOcap [12] and families 3-5 with exome sequencing.

Histochemical analyses were performed on cryosections using standard methods. The anti-aggregation activity of DNAJB6 was tested using a filter trap method [1]. Further experimental details in Supplementary methods.

The study was approved by the IRB of Tampere University Hospital. All participants provided appropriate consent and the study was conducted according to the Helsinki Declaration.

Results

Clinical details and muscle investigations of the patients in Table 1. The mean age of onset was 38.5 years (range 16-55). Five patients presented with distal weakness, mainly with difficulties walking on toes, nine with proximal lower limb weakness. Three had dysphagia. CK levels were normal or slightly elevated. Electromyography findings in all patients studied (5 patients) were consistent with myopathy with occasional pseudomyotonic or myotonic discharges in three patients. Fatty degenerative involvement was observed in the hamstrings and calf muscles on muscle imaging (Figure 1B). Two patients experienced dyspnoea; none of the others had cardiac or respiratory symptoms. Muscle biopsy was available from nine patients and rimmed vacuolar pathology was a constant finding (Figure 1C).

Targeted high-throughput sequencing revealed a novel c.284A>T (p.Asn95Ile) mutation in the probands in families F1 and F2. Sanger sequencing of remaining family members confirmed the segregation. The families had no known relation, but a common haplotype showed that they are distantly related (supplement table 1).

Exome sequencing of F3:II:2 revealed a novel c.293_295delATG (p.Asp98del) mutation in *DNAJB6*. It was confirmed by Sanger sequencing and segregated in the affected. Families 4 and 5 had a typical LGMD1D phenotype with the previously reported c.279C>G (p.Phe93Leu) mutation.

Filter-trap analysis of the two novel mutation together with two earlier described Finnish mutations showed significant loss of anti-aggregation capacity (Figure 1D).

Discussion

We present the first French families with *DNAJB6* mutations. *DNAJB6* mutations were first related to LGMD1D, but this and other recent studies have shown a wider phenotype and variable severity compared to the most frequent mutation, p.Phe93Leu [1,2,6,13-15].

The novel mutations caused a larger variation in phenotype, both in presenting symptoms and disease severity. Family F1 (p.Asn95Ile) and patient F3:II:2 (p.Asp98del) presented with distal lower limb weakness, but this was not entirely consistent with mutation. Thus, we could not confirm the earlier suggestion of a genotype – phenotype correlation that C-terminal mutations in the G/F-domain correlate with a more distal phenotype [4], although some correlation cannot be excluded. Dysphagia is not uncommon and was present in three of our patients. Although *DNAJB6* is a reported cardiomyopathy susceptibility gene [16], cardiomyopathy has not been observed in any patient so far, and severe respiratory insufficiency seems very rare [17].

All described mutations affect the G/F-domain of *DNAJB6* clearly indicating an important function. The C-terminal of *DNAJB6* is alternatively spliced giving two isoforms with distinct cellular localisation. Mutations in exon 5 will affect both isoforms, but the cytoplasmic b is the pathogenic isoform in both zebrafish [1] and mouse [18]. This is probably due to the

cellular localisation as muscle phenotypes apparently arise from defect chaperonal processing of cytoplasmic proteins.

DNAJB6 interacts [1] with proteins in the chaperone-assisted selective autophagy (CASA) pathway [19]. Other CASA proteins also have similar aggregation preventing effects [20,21] and mutations in the CASA proteins HSPB8 [22] and BAG3 [23] are known to cause neuromuscular disease. DNAJB6 has recently been shown to be important for stress granule handling via TDP-43 [24], and colocalization of DNAJB6 with the stress granule protein TIA1 in protein accumulations can be observed in patient muscle (Supplementary figure 1). DNAJB6 is also important for handling of prions in yeast [25]. The ability of DNAJB6 to recognise specific protein conformers can prevent aggregation of these proteins; and when impaired this will lead to defect handling of misfolded proteins and aggregations.

Our findings broaden the mutational spectrum of DNAJB6 with two novel mutations. Further, as p.Phe93Leu is now identified in four populations it emerges as a mutational hotspot. Our report confirms that *DNAJB6* mutations may cause distal-onset myopathy and hence that *DNAJB6* defects should be considered also in distal myopathy patients with weakness starting in the posterior compartment.

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Conflicts of interest: no

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Legends

Figure 1. Pedigree and muscle findings of the patients with novel mutations: (A) Pedigree of the families. (B) Rimmed and subsarcolemmal non-rimmed vacuolation and some myofibrillar aggregation are seen in F1 III:1. Staining with antibodies against myotilin, α B-crystallin and desmin shows strong expression in the myofibrillar lesions with aggregates. In patient F3 II:2 the findings are similar although more advanced dystrophic with increased fat and fibrosis. (C) Fatty degenerative changes are most severe in the distal lower limb muscles, soleus and medial gastrocnemius in patient F1 III:1. Less severe involvement is present in semimembranosus on the left. The posterior compartment of the calf is fatty degenerated in patient F2 III:1 with asymmetrical involvement of biceps femoris, semimembranosus and adductor magnus. Patient's F3 II:2 muscle MRI was performed at the time when she was already bedridden and consistently all muscles are severely affected. (D) Both novel mutations showed significantly impaired anti-aggregation activity (Mann-Whitney U-test). Aggregation score was calculated as a ratio of aggregated/soluble in the induced to the uninduced cells.

Figure 1

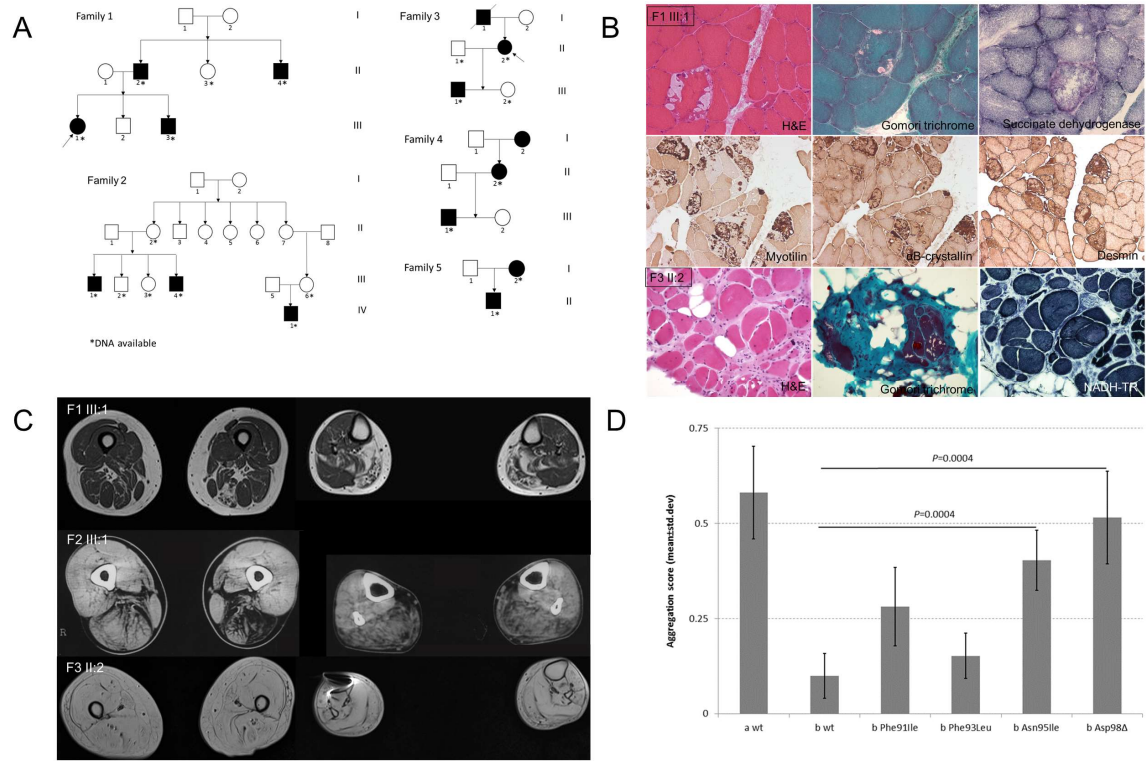


Table 1 Clinical data of the patients

| Patients | Age at last exam/ Sex | Age of onset | First symptoms | Muscle weakness (MRC, the Medical Research Council Scale) | Ambulation | Creatine kinase levels | Muscle histology | Cardiac / Respiratory symptoms |
|-----------------|----------------------------------|---------------------|----------------------------|--|----------------------|-------------------------------|--|---------------------------------------|
| F1 II:2 | 85/M | 55 | Difficulty walking on toes | Distal and proximal upper and lower limbs, dysphagia | Wheelchair (aged 85) | Normal | Rimmed vacuoles | No/ No |
| F1 II:4 | 75/M | 40 | Difficulty walking on toes | Lower limbs; tibialis (0), gastrocnemius (2), quadriceps (4), hamstrings (3) | One cane | 2 X UNL | Rimmed vacuoles | No/ No |
| F1 III:1 | 64/F | 50-54 | Difficulty walking on toes | Distal lower limbs | Ambulant | Normal | Rimmed vacuoles, myofibrillar aggregates | No/ No |

| | | | | | | | | |
|-----------------|------|----|--|--|--------------------------|---------------|----------------------------|--|
| F1 III:3 | 65/M | 54 | Difficulty walking on toes | Mild distal lower limbs; gastrocnemius (4) | Ambulant | Normal | Not done | No/ No |
| F2 III:1 | 60/M | 40 | Left lower limb weakness, difficulty climbing stairs | Scapular (4), axial (4), hyperlordosis, pelvic (3), unable to walk toes/heels | One cane 50m | Not available | Dystrophy, rimmed vacuoles | No/ No |
| F2 III:4 | 54/M | 36 | Left lower limb weakness, difficulty climbing stairs, frequent falls | Abdominal, pelvic and thigh (2), gastrocnemius (3+), tibialis (2), peroneus (3/4-), triceps/biceps (3) | Two canes 50m | 2 X UNL | Dystrophy, rimmed vacuoles | No/ exertional dyspnoea Echo: Left EF 63% / restrictive pulmonary dysfunction |
| F3 II:2 | 71/F | 16 | Distal lower limb weakness | Severe distal and proximal lower/upper limbs; dysphagia, cricopharyngeal | Wheelchair (aged 49), | Normal | Rimmed vacuoles | No/Dyspnoea Echo: aortic dilatation, no cardiomyopathy / |

| | | | | | | | | |
|-----------------|------|----|------------------------------|--|-------------------------|---------------|---------------------|--|
| | | | | myotomy; Achilles tendon surgery | bedridden (aged 65) | | | restrictive pulmonary dysfunction, VC 52 % |
| F3 III:1 | 46/M | 20 | Proximal lower limb weakness | Lower limbs (0-1), deltoid (3-), biceps (3-), triceps (2), wrist fl/ext. (4/3-), finger fl/ext (4/2) | Wheelchair | Not available | Rimmed vacuoles | No/ No Echo: normal / FVC 4,1 l (90 %) |
| F4 II:2 | 86/F | 44 | Waddling gait | Proximal and distal limbs; dysphagia | Wheelchair (aged 70) | Normal | Not available | Cardiac infarction aged 79/ No |
| F4 III:1 | 48/M | 40 | Difficulty climbing stairs | Severe in lower limbs; moderate in axial and proximal upper limbs | Rollator and wheelchair | 2 x UNL | Not available | No/ No Echo: left ventricular hypertrophy |
| F5 I:2 | 62/F | 40 | Difficulty climbing stairs | Proximal and distal lower limbs, mild scapular winging | One cane 20m | Normal | Unspecific myopathy | Not available |

| | | | | | | | | |
|----------------|------|----|-------------------------------|-------------------------------|----------|---------|------------------------|--------|
| F5 II:1 | 40/M | 25 | Difficulty climbing stairs | Proximal > distal lower limbs | Ambulant | 6 x UNL | Unspecific myopathy | No/ No |
|----------------|------|----|-------------------------------|-------------------------------|----------|---------|------------------------|--------|

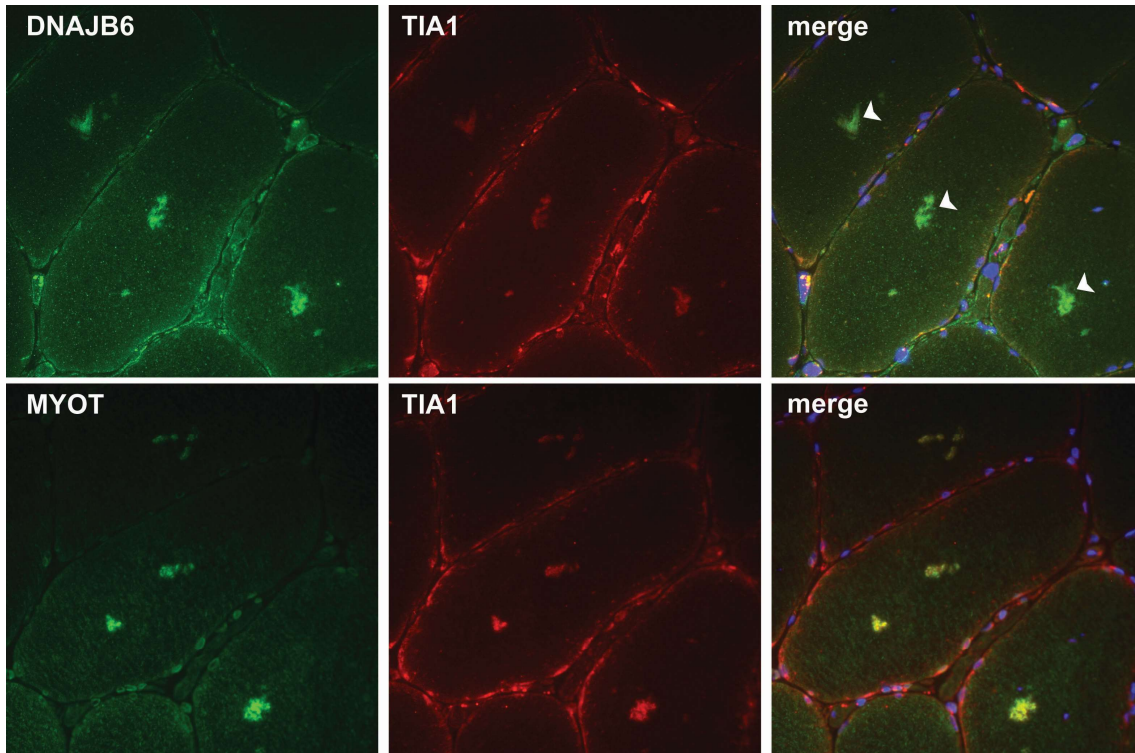
Echo, echocardiogram; FVC, forced vital capacity; UNL, upper normal limit; VC, vital capacity

Suppl. Table 1: Haplotyping of patient F1 III:1 and F2 III:4, both carrying the c.284A>T mutation, showing a common haplotype and hence indicating a distant relationship between these patients.

The tested area covers a 3.4-Mb region on chromosome 7q36.

| | Family 1 III:1 | | Family 2 III:4 | |
|--------------------|----------------|---|----------------|---|
| D7S3037 | 1 | 4 | 1 | 4 |
| D7S2465 | 1 | 3 | 3 | 3 |
| RNF32 ex9 | | | | |
| rs762861581 | C | C | C | C |
| rs7780080 | T | T | T | T |
| rs753266341 | T | T | T | T |
| D7S559 | 1 | 3 | 5 | 3 |
| DNAJB6 | A | T | A | T |
| PTPRN2 ex23 | | | | |
| rs772052329 | C | C | C | C |
| rs558686530 | C | C | C | C |
| rs537250573 | A | A | A | A |
| PTPRN2 ex11 | | | | |
| rs759458160 | G | G | G | G |
| rs772379046 | A | A | A | A |
| rs370254131 | C | C | C | C |
| D7S2423 | 1 | 3 | 3 | 3 |
| PTPRN2 ex7 | | | | |
| rs3752371 | G | C | G | C |
| rs1130499 | C | T | C | T |
| rs1130500 | A | G | A | G |
| D7S427 | 3 | 3 | 4 | 3 |
| D7S594 | 3 | 5 | 7 | 5 |

Supplementary figures



Suppl fig 1. DNAJB6 and TIA1 accumulate in muscle biopsy from a patient with DNAJB6 mutation. Immunofluorescent double staining of a Finnish patient with Phe93Leu DNAJB6 mutation. DNAJB6 and the stress granule component TIA1 (upper panel) co-localize in myofibrillar aggregates (arrowheads), together with myotilin (lower panel). Original magnification 40x.

Primary antibodies: rabbit polyclonal anti-DNAJB6 (ab96539, Abcam), goat polyclonal anti-TIA1 (ab61700, Abcam), and anti-myotilin rabbit polyclonal (10731-1-AP, ProteinTech).

Supplementary methods

Genetics

DNA samples from families 1 and 2 were analysed by targeted high-throughput sequencing as previously described [12] using version 2 of the MYOcap gene panel, which is targeted to the exons of 236 genes known or predicted to cause muscular dystrophy or myopathy.

DNA from families 3-5 were analysed by exome sequencing by the National Centre for Genotyping (CNG, Evry, France). DNA capture and enrichment was done with the Agilent "AllExonV5" kit and the DNA samples were sequenced on an Illumina platform. Sequence alignment was done against the hg19 human genome. Variants were then annotated and filtered out with an in-house-developed software system, (PolyWeb) [26]. We focused our analyses on nonsynonymous variants, splice-acceptor and donor-site variants, and coding indels, not considered benign according to the prediction softwares SIFT and Polyphen2.

The results were verified by Sanger sequencing of exon 5 of *DNAJB6*. Primer sequences are available on request.

Haplotyping of Families 1 & 2

Patient III:1 from Family 1 and III:4 from Family 2 were genotyped for 6 microsatellite markers (D7S3037, D7S2465, D7S559, D7S2423, D7S427 and D7S594) and 12 SNPs (rs762861581, rs7780080, rs753266341, rs772052329, rs558686530, rs537250573, rs759458160, rs772379046, rs370254131, rs3752371, rs1130499, rs1130500) in the linked area covering a 3.4-Mb region on chromosome 7q36. Primer list is available on request.

PCR reactions were performed using 20-40 ng of genomic DNA. Fluorescently labelled PCR products were analysed using ABI3730xl (Applied Biosystems) with a G5 dye set and GeneMapper 4.0 software (Applied Biosystems). PCR products for SNP analysis were sequenced using the BigDye v3.1 termination reaction (Applied Biosystems) and ABI3730xl DNA Analyzer. Sequences were analysed using Sequencher v5.0 software (Genes Codes Corporation).

Muscle pathology

Histochemical analysis were performed on cryosections using standard methods with haematoxylin and eosin, Gomori trichrome, reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), and ATPase at pH 10.4, pH 4.6, and succinate dehydrogenase (SDH). For immunohistochemistry antibodies against desmin, myotilin, α B-crystallin, spectrin, caveolin, sarcoglycans, and dysferlin were applied. Ventana Benchmark automated immunostainer with DAB-detection was used for immunohistochemistry.

Plasmid constructs

The DNAJB6a wild-type, DNAJB6b wild-type, DNAJB6b p.Phe91Ile, DNAJB6b p.Phe93Leu and pEGFP/HD-120Q constructs have been described earlier [1,3,27]. The novel p.Asn95Ile and p.Asp98del mutations were introduced to wild-type pcDNA5/TO-DNAJB6b constructs by site-directed mutagenesis and verified by Sanger sequencing.

Filter Trap

The distribution of soluble and aggregated polyQ-huntingtin (120Q-HTT) was followed using a filter trap method essentially as described [1,3]. In short, T-REx 293 (Invitrogen) cells were transfected with 120Q-HTT and tetracyclin inducible pcDNA5/TO-DNAJB6 constructs, induced with tetracyclin after 4 hours and harvested 48 h post transfection. The soluble 120Q-HTT fraction was measured with western blotting and the aggregates trapped in a 0.2 μ m cellulose acetate filter (Whatman GmbH). The membranes were scanned and analysed using an Odyssey scanner and ImageStudio v. 3.1.4 (LI-COR). The aggregation score was calculated as: aggregation score = $(\text{aggregated/soluble})_{\text{induced}} / (\text{aggregated/soluble})_{\text{uninduced}}$. Statistical significance was calculated in Excel using a two-sided Mann-Whitney U-test.

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