


A recessive mutation in beta-IV-spectrin (*SPTBN4*) associates with congenital myopathy, neuropathy, and central deafness

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Abstract Congenital myopathies are a heterogeneous group of muscle disorders that are often genetically determined. Here, we investigated a boy with congenital myopathy, deafness, and neuropathy from a consanguineous Kurdish family by autozygosity mapping and whole exome sequencing. We found a homozygous nonsense mutation in *SPTBN4* [c.1597C>T, NM_020971.2; p.(Q533*), NP_066022.2; ClinVar SUB2292235] encoding β IV-spectrin, a non-erythrocytic member of the β -spectrin family. Western blot confirmed the absence of the full-length 288 kDa isoform in muscle and of a specific 72 kDa

isoform in fibroblasts. Clinical symptoms of the patient largely corresponded to those described for the quivering mouse, a loss-of-function animal model. Since the human phenotype of β IV-spectrin deficiency included a myopathy with incomplete congenital fiber-type disproportion, we investigated muscle of the quivering (qv4J) mouse and found complete absence of type 1 fibers (fiber-type 2 uniformity). Immunohistology confirmed expression of β IV-spectrin in normal human and mouse muscle at the sarcolemma and its absence in patient and quivering (qv4J) mouse. *SPTBN4* mRNA-expression levels in healthy skeletal muscle were found in the range of other regulatory proteins. More patients have to be described to confirm the triad of congenital myopathy, neuropathy and deafness as the defining symptom complex for β IV-spectrin deficiency.

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Introduction

Congenital myopathies are a heterogeneous group of genetic muscle disorders with hypotonia and muscle weakness from birth. About 20 genes are associated with congenital myopathies, but often the molecular pathology is unknown (Maggi et al. 2013). Histological abnormalities comprise rods, cores, caps, central nuclei, and fiber-type predominance or uniformity (North et al. 2014). Some of the congenital myopathies are characterized by congenital fiber-type disproportion (CFTD), which is an abnormality of muscle fiber size and proportion with slow-twitch type 1 fibers being consistently smaller and more numerous than fast-twitch type 2 fibers. Sometimes one may find fiber-type 1 uniformity, in unusual cases or biopsy sites only fiber-type 1 atrophy may be seen. CFTD can be inherited as an autosomal dominant (*ACTA1* MIM #255310, *MYH7* MIM #160500, *TPM3* MIM #255310, *TPM2* MIM

#609285) or recessive (*RYR1* MIM #117000, *SEPNI* MIM #255310) trait (Clarke 2011; North et al. 2014). Proteins involved in the pathophysiology of congenital myopathy play an important role in skeletal muscle contraction via their interaction with myosin. Others are involved in skeletal muscle calcium homeostasis or are located at the sarcomere of striated muscle where they associate with thin α -actinin filaments (North et al. 2014).

β IV-spectrin is a non-erythrocytic member of the β -spectrin family. Mutations in the *Sptbn4* gene (syn. *Spnb4*) of the quivering mouse disturb the axon initial segments and nodes of Ranvier, which are important domains for initiation, propagation, and modulation of action potentials (Yang et al. 2004). At the nodes of Ranvier, β IV-spectrin is required for ion channel clustering (Hedstrom and Rasband 2006; Devaux 2010). *Sptbn4* loss-of-function mutations in mice cause ataxia, neuromyotonia, myokymia, tremor, motor neuropathy, and central deafness (Parkinson et al. 2001; Devaux 2010). Humans with mutations in *SPTBN4* (MIM *606214) have not been described. Here, we report the discovery of *SPTBN4* as a novel candidate disease gene for congenital myopathy in a consanguineous Kurdish family.

Materials and methods

Human subjects

The parents of the patient provided written informed consent for all aspects of the study (Charité IRB approval EA2/107/14) and publication of the patient's photographs according to the Declaration of Helsinki. Previous gene testing had excluded spinal muscular atrophy, Prader–Willi-Syndrome, myotonic dystrophy, myotubular and centronuclear myopathy, and mutations in *COLQ*, *IGHMBP2*, *POMGNT1*, and *FKRP*. Genomic DNA was extracted from white blood cells of all investigated family members and from a chorionic villus biopsy specimen that had been obtained for prenatal testing.

Animals

Quivering mice are characterized by progressive ataxia with hind limb paralysis, deafness and tremor. They carry mutations in the murine β IV-spectrin gene (*Spnb4*) and several alleles have been described which cause phenotypes of varying severity. The study here investigated the qv4J mouse, which carries a nonsense mutation in *Spnb4* (p.Q1358*) that truncates the protein by 47% (Parkinson et al. 2001).

Autozygosity mapping

Autozygosity mapping was performed using the variant calling files (VCF) from whole exome sequencing of the patient and his parents. In these variant files, the HomozygosityMapper2012 software [<http://www.homozygosity-mapper.org>, accessed Dec 2016] (Seelow and Schuelke 2012) searched for homozygous/autozygous stretches of 300 SNPs or longer that were only homozygous in the patient but not in his parents. Homozygous stretches of >30 SNPs in the parents were excluded as candidate regions. This method yielded a total of 380 protein-coding genes that were covering 32.9 Mbp on chromosomes 7 and 19 (Fig. 2b).

Whole exome analysis

Exonic sequences were enriched from the patient and his parents using the SureSelect[®] V4 Human All Exon Kit (Agilent) and sequenced on a HiSeq 2000 machine (Illumina) as 101 bp paired-end fragments. FASTQ files were aligned to the human GRCh37.p11 (hg19) reference sequence using the BWA-MEM v0.7.1 aligner (Li 2013). The quality of the alignment is provided in Supplementary Table 2. Subsequently, variant VCF-files were generated for all exons ± 20 bp flanking regions using the GATK v3.3 software package (DePristo et al. 2011) and sent to the MutationTaster2 software [<http://www.mutationtaster.org>, accessed Dec 2016] for assessment of potential pathogenicity (Schwarz et al. 2014).

As the parents were consanguineous, variants were filtered for recessive inheritance and removed if occurring homozygously either in the ExAC database in >20 cases [<http://exac.broadinstitute.org>, accessed Dec 2016; <http://biorxiv.org/content/early/2015/10/30/030338>, accessed Dec 2016] or in the 1000 Genomes project in >10 cases [<http://www.1000genomes.org>, accessed Dec 2016]. All relevant variants were inspected visually using the Integrative Genomics Viewer (IGV) [<http://www.broadinstitute.org/igv/>, accessed Dec 2016] and their segregation was verified by Sanger sequencing using gene specific oligonucleotide primers and the BigDye[®] (Applied Biosystems) protocol on an ABI3500 Genetic Analyzer (ThermoFischer Scientific). For verification of the *SPTBN4* c.1597C>T mutation (GenBank NM_020971) we analyzed the PCR-product generated with the oligonucleotide primer pair FW: 5-CAG GGT CAC ACA GGG TCA AG-3 and REV: 5-CCC TTC CCT CTC CAT CTC CA-3.

Beyond the homozygous variant in *SPTBN4* we additionally found two potentially pathogenic variants in the autozygous region (Supplementary Table 3). The variant in the *FFAR2* gene encoding the free fatty acid receptor 2 was excluded as the cause of the described disease here,

because *FFAR2* is only expressed in blood and immune cells and *Ffar2* mutations in the mouse cause a different phenotype with exacerbating inflammations of various tissues. The homozygous variant in the *RASIP1* gene was excluded because the gene is only expressed in heart and lung and *Rasip1* mutations in the mouse cause cardiac malformations and intrauterine growth retardation and death.

Virtual gene panel and trio analysis

To exclude mutations in genes that are known to be associated with either congenital myopathies or other muscle diseases, we specifically screened the VCF-variant files of the patient for mutations therein. The composition of these virtual gene panels is provided in the Supplementary online material. Additionally, because only one child was affected in the family, we did a trio analysis for de novo mutations but did not find any in the coding regions of protein-coding genes.

Quantitative RT-PCR analysis

Total RNA was isolated from the patient and three control fibroblast lines using the TRIzol reagent, and reversely transcribed into cDNA with SuperScript III (Invitrogen) polymerase using oligo (dT) primers. Quantitative SYBR green qPCR (Life Technologies) was performed on an ABI PRISM 7700 cycloer (Applied Biosystems) using the PCR-efficiency-corrected $-\Delta\Delta C_t$ method (Pfaffl 2001) with the *SPTBN4* (GenBank NM_020971) primer pair FW: 5-CTG GAG AAC GTG GAC AAG GC-3; REV: 5-TCA GCC GGT GAT TCC CAT C-3. Expression levels were normalized to *18S rRNA* (FW: 5-CAT TCG AAC GTC TGC CCT ATC-3; REV: 5-CTC CCT CTC CGG AAT CGA AC-3, GenBank NR_003286.2) and *GAPDH* (FW: 5-TGC ACC ACC AAC TGC TTA GC-3; REV: 5-GGC ATG GAC TGT GGT CAT GAG-3, GenBank NM_002046) as described (Relizani et al. 2014).

Muscle transcriptome analysis

Transcriptome raw data (fastq files) from four healthy male muscle samples were downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>; Project GSE56787, samples SRR1398532, SRR1398533, SRR1398534, and SRR1398535) (Yao et al. 2014) and aligned to the human reference sequence (GRCh37.75) with the STAR 2.4.0.1 aligner (Dobin et al. 2013). Resulting BAM files were investigated and normalized with the CUFFLINKS 2.2.1. pipeline (Trapnell et al. 2012), which yields the relative mRNA quantities for each transcript present in the transcriptome dataset. As the quantitative measure for gene abundance we used the FPKM value (fragments per

kilobase of transcript per million mapped reads), which is calculated by the CUFFQUANT module of the CUFFLINKS software. We depict the FPKM values from the above mentioned datasets of *SPTBN4* along with other well known muscle genes (Fig. 3).

Results

Clinical report

The male patient was born to healthy, first cousin parents. His elder sister is healthy. In the newborn period, he presented with general muscular hypotonia and facial weakness. Muscle tendon reflexes were absent, his motor development was delayed and he never gained head control. Feeding problems due to weak suck required gavage feeding from the age of 1 year. Cranial MRI at the age of 4 years was normal except for mildly enlarged CSF spaces. Serum CK-levels were never elevated; muscle biopsy at the age of 5 years demonstrated fiber-type 1 atrophy suggesting a rare congenital form of an unstructured congenital myopathy (Fig. 1f). At the age of 10 years the boy presented with myopathic facies, highly arched palate (Fig. 1a, b), severe distal muscle weakness, generalized muscle atrophy, scoliosis, ankle contractures (Fig. 1c–e), and severely retarded motor and mental development. Presently, he is not able to stand, sit, eat, or drink without support. He did not learn to speak. Early brainstem evoked potentials could not be elicited with 90 dB SPL while otoacoustic emissions were entirely normal. Neurography showed a combined axonal and demyelinating motor neuropathy with 30.9 m/s, 0.4 mV (peroneal nerve, normal >41.1 m/s, >2.6 mV).

Prenatal testing

Based on the molecular findings in the index patient, the parents asked us for prenatal testing in a further pregnancy, despite the fact that their child was the only patient described with a mutation in *SPTBN4* so far. Chorionic villus sampling was done at 12 weeks of gestation. The fetus was heterozygous for the mutation. Maternal DNA contamination of the fetal DNA was excluded by genotyping with microsatellite markers. The boy was born at term, developed normally until now (3 months of age), and did not show any signs of muscle hypotonia and weakness.

Molecular genetics

Autozygosity mapping combined with whole exome sequencing revealed an autosomal recessive nonsense mutation in *SPTBN4* (Chr19:g41,009,971C>T, GRCh37.p11 (hg19); c.1597C>T, NM_020971.2; p.(Q533*),

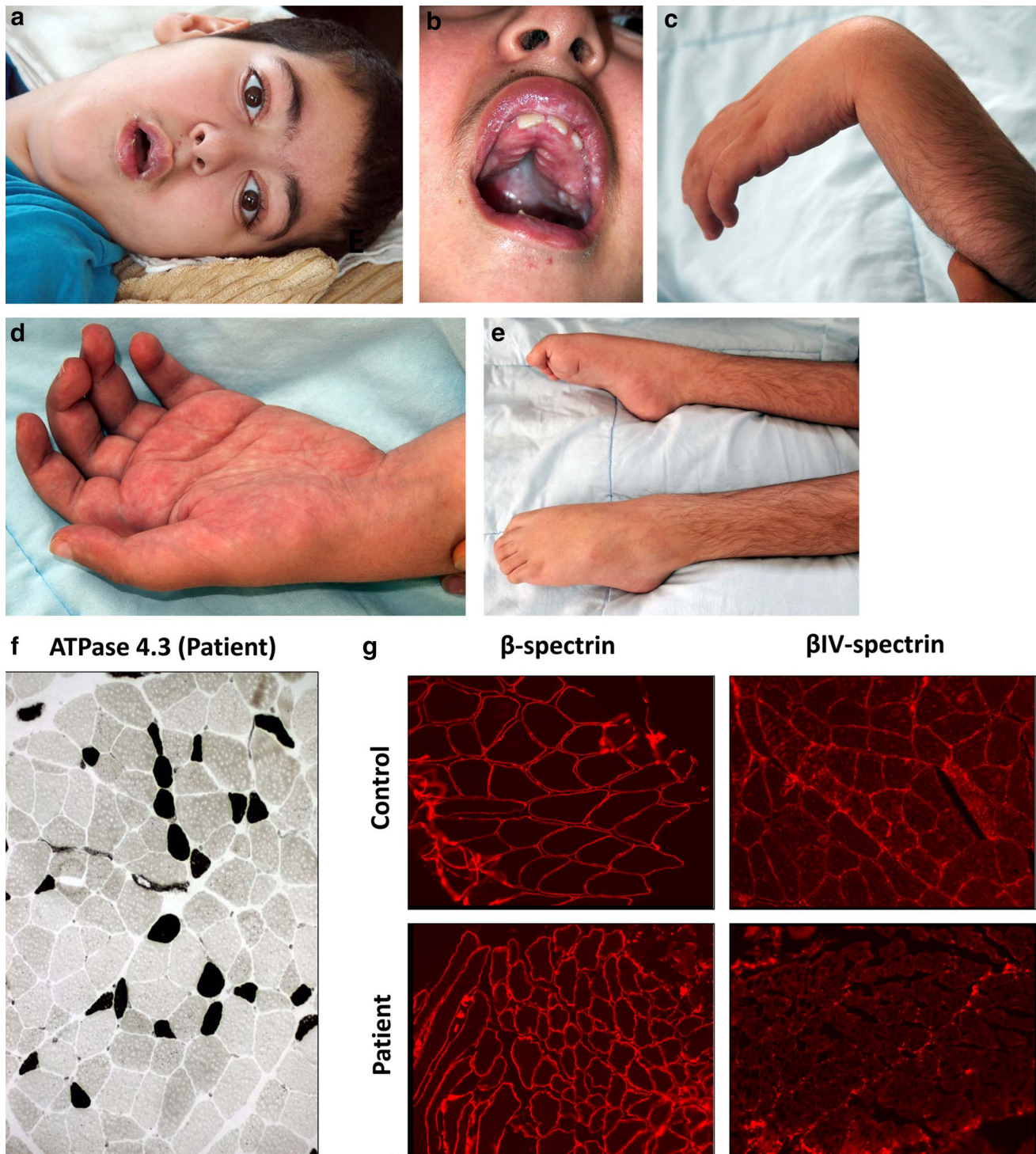


Fig. 1 a–e Clinical phenotype of the patient with a myopathic face, b highly arched palate, c distal weakness, d thenar and interdigital muscle atrophy, and e distal muscle wasting with fixed extension contractures of the feet; f The ATPase pH 4.3 stain shows fiber-type disproportion in the quadriceps muscle with reduction and atrophy of type 1 fibers (*black*) and predominance of type 2C (*dark gray*) and type 2A/B fibers (*light gray*); g Immunostaining of age-matched con-

trol and patient muscle with antibodies directed against β -spectrin (NCL SPEC1, clone RB C2/3D5, Novocastra UK; 1:100) and the non-erythrocytic β IV-spectrin (sc-368195, H-85, Santa-Cruz, 1:100), both expressed at the sarcolemma in normal muscle. Absence of anti- β IV-spectrin immunostaining at the sarcolemma of the patient; the remaining signals derive from interstitial autofluorescent material

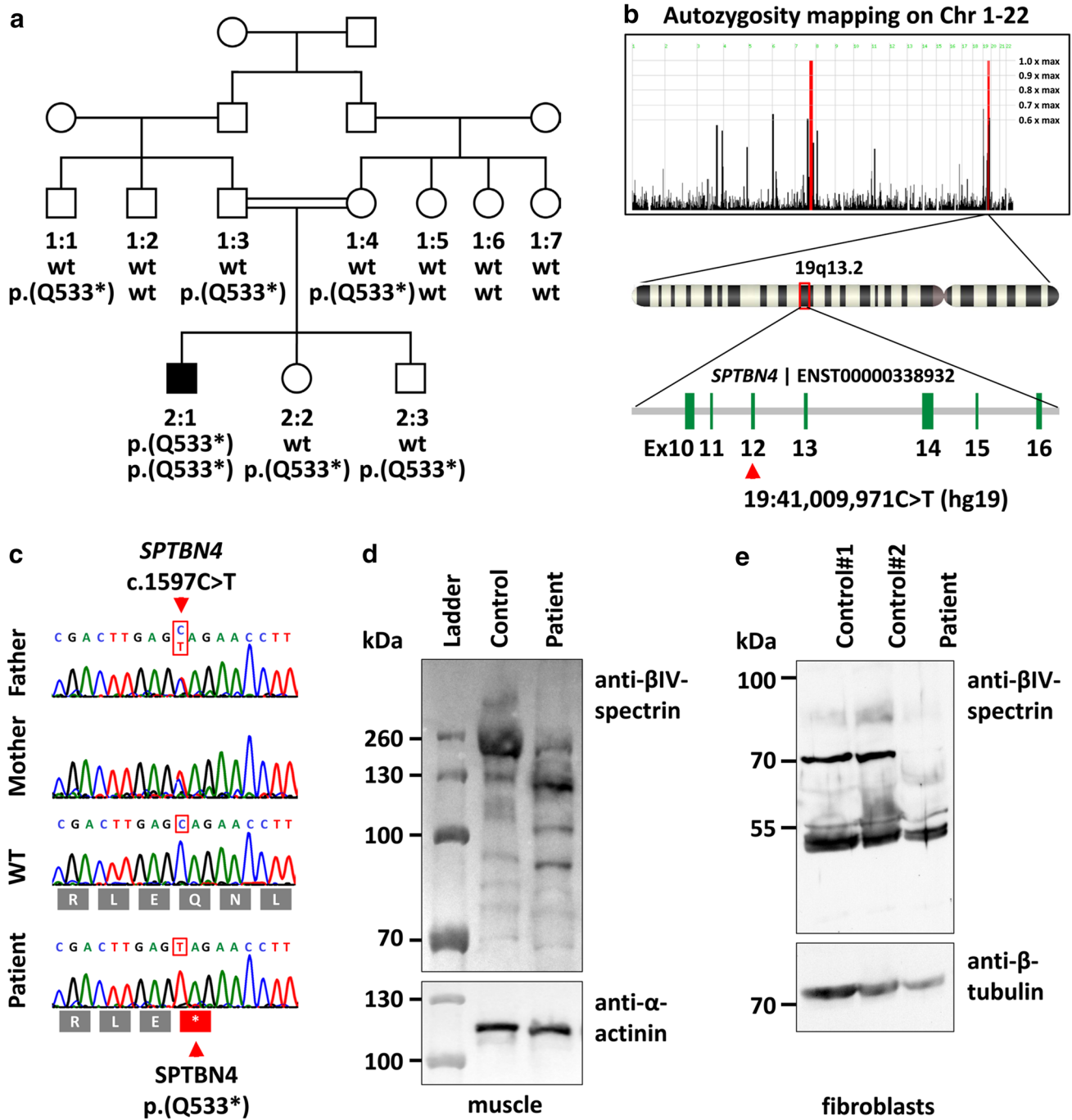


Fig. 2 **a** Pedigree of the consanguineous family with segregation of the *SPTBN4* mutation; **b** autozygous regions stretching over 300 SNPs that only occurred in the patient, but not in his healthy parents. The graph has been generated with our HomozygosityMapper2012 software. The red bars mark the two autozygous regions. **c** Sequence electropherograms of the c.1597C>T nonsense mutation in control, parents, and patient; **d** Western blot of patient and control muscle

shows a reduction of the full length βIV-spectrin protein abundance in the patient. Staining with anti-α-actinin served as loading control. **e** Western blot of patient and control fibroblasts shows absence of the 72 kDa βIV-spectrin band in the patient, an unspecific band at ≈55 kDa and anti-β-tubulin (ab6046, Abcam, 1:10,000) serving as loading controls

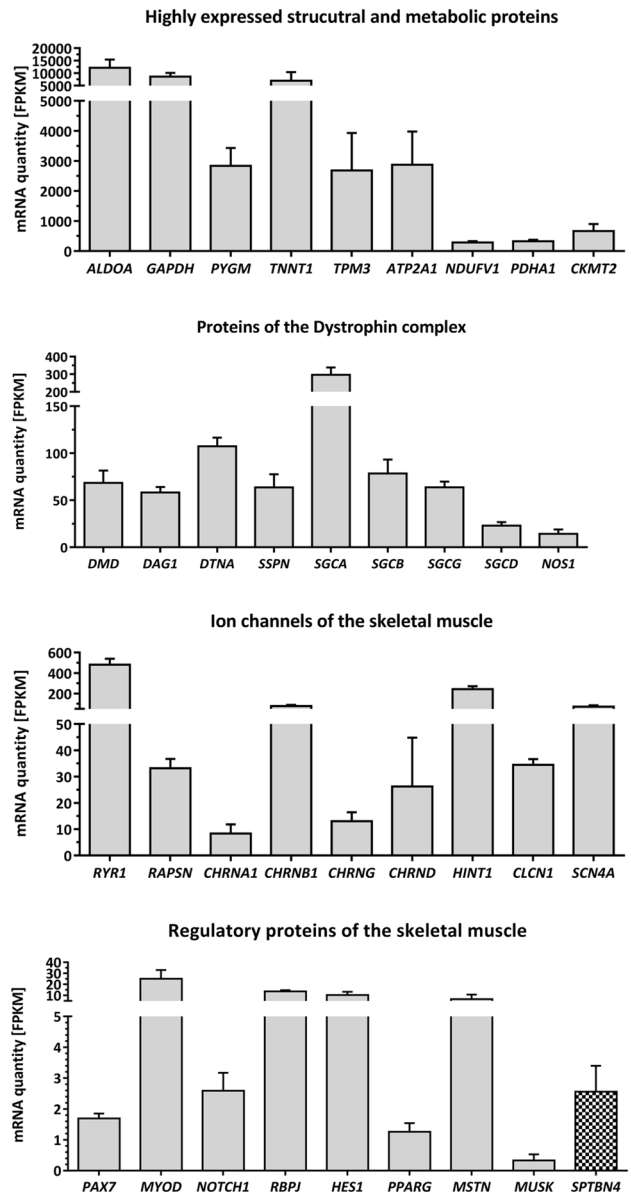
NP_066022.2, ClinVar SUB2292235). This variant was neither listed in the 1000 Genomes, the Exome Variant Server, dbSNP, or the ExAC database. Additionally, the variant was absent from our local whole exome database (>150 exomes).

To exclude mutations in genes that are known to be associated with either congenital myopathies or with other muscle diseases, we specifically screened the VCF-variant files of the patient for mutations therein (Supplementary

Fig. 3 Transcriptome analysis of $n = 4$ healthy individuals by RNA sequencing places *SPTBN4* mRNA-expression levels in the low range, typically occupied by other regulatory proteins and transcription factors. Transcript abundance is depicted as FPKM values (fragments per kilobase of transcript per million mapped reads). The figure gives an overview of the relative mRNA-expression levels of genes coding for various muscle proteins. Muscle enzymes: *ALDOA* aldolase A, *GPDH* glyceraldehyde-3-phosphate dehydrogenase, *PYGM* glycogen phosphorylase; Components of the contractile apparatus: *TNNT1* troponin T1, *TPM3* tropomyosin 3; Components of the energy metabolism: *ATP2A1* sarcoplasmic reticulum type Ca⁺⁺-transporting ATPase, *NDUFB1* NADH:ubiquinone oxidoreductase core subunit VI, *PDHA1* pyruvate dehydrogenase α -subunit 1, *CKMT2* mitochondrial creatine kinase 2; Proteins of the dystrophin complex: *DMD* dystrophin, *DAG1* dystroglycan 1, *DTNA* α -dystrobrevin, *SSPN* sarcospan, *SGCA/B/G/D* sarcoglycan $\alpha/\beta/\gamma/\delta$, *NOS1* nitric oxide synthase 1; Ion channels of the muscle: *RYR1* ryanodine receptor 1, *RAPSN* receptor associated protein of the synapse, *CHRNA1/B1/G/D* nicotinic cholinergic receptor subunits $\alpha/1/\beta/1/\gamma/\delta$, *CLCN1* voltage-gated chloride channel 1, *SCN4A* voltage-gated sodium channel α -subunit 4; Regulatory proteins of the muscle: *PAX7* paired box factor 7, *MYOD* myogenic differentiation factor 1, *NOTCH1* Notch 1, *RBPJ* recombination signaling binding protein for immunoglobulin κ J region, *HES1* Hes family BHLH transcription factor 1, *PPARG* peroxysome proliferator-activated receptor γ , *MSTN* myostatin, *MUSK* muscle associated receptor tyrosine kinase, *SPTBN4* non-erythrocytic β IV-spectrin

online material). Additionally, because only one child was affected in the family, we did a trio analysis for de novo mutations, but did not find any in the coding regions of protein-coding genes. Sanger sequencing confirmed autosomal recessive inheritance (Fig. 2a, c).

To explore the effect of the mutation on the protein level, we first investigated cultured skin fibroblasts (Fig. 2e). *SPTBN4* encodes a minor full-length isoform of 2564 amino acids with a predicted molecular weight of 289 kDa and a truncated major isoform of 72 kDa comprising 678 amino acids starting from amino acid position 1335 of the minor isoform (Tse et al. 2001). In two control fibroblast lines, the 72 kDa band was clearly present, while it was absent from the patient fibroblasts. As the antibody was raised against a peptide encoding AA #1661–1745 of β IV-spectrin, it would be able to bind to the truncated isoform, which does not include the premature termination codon at AA #533. The absence of the short isoform protein band in the patient indicated that nonsense-mediated mRNA decay of the full-length mRNA had occurred before or during splicing. Second, we performed Western blot analysis in human skeletal muscle from the patient and a control. We found a strong band at \approx 290 kDa in control tissue corresponding to the full-length isoform of β IV-spectrin and a clear reduction of this band in the patient (Fig. 2d). Immunostaining of muscle cryosections from healthy humans located β IV-spectrin at the sarcolemma and in the muscle capillaries, while the sarcolemmal signal was clearly absent in the muscle of the patient (Fig. 1g).



qPCR with two primer pairs, one product located in the 5'-region and the other one in the 3'-region of the large mRNA that had been isolated from skin fibroblasts (Supplementary online material) demonstrated a \approx 85% reduction of *SPTBN4* mRNA copy numbers (patient 0.03 ± 0.01 *SPTBN4*/10⁶ 18S RNA, controls ($n = 3$) 0.18 ± 0.07). This finding would be consistent with nonsense-mediated mRNA messenger decay.

Histological investigation of mouse muscle

Anti- β IV-Spectrin staining of mouse muscle from controls revealed a strong signal at the sarcolemma and the interstitial tissue, which was absent in the muscle cryosections from the quivering mouse (qv4J) that carries a nonsense

mutation in the murine *Sptbn4* gene. More severe as seen in the human muscle, type 1 muscle fibers were entirely absent from the quivering mouse *tibialis anterior* muscle as demonstrated by the ATPase pH 4.3 staining (Supplementary Fig. 3).

SPTBN4 gene expression in skeletal muscle

Transcriptome analysis of human skeletal muscle by RNA sequencing revealed comparatively low *SPTBN4* mRNA expression levels in the range of transcription factors (*PAX7*, *MYOD*, *PPARG*), proteins of the Notch pathway (*NOTCH1*, *RBPJ*, *HES1*), muscle growth factors (*MSTN*), and proteins important for the regulation of neuromuscular transmission (*MUSK*).

Discussion

We describe the first human patient with a loss-of-function mutation of β IV-spectrin. He presented with congenital myopathy, neuropathy, and deafness. The clinical features in a human thus largely correspond to those of the quivering mouse. Loss of β IV-spectrin in mice was associated with progressive ataxia and a hearing defect that was attributed to mislocation of voltage-gated channels at the axon initial segments and nodes of Ranvier (Parkinson et al. 2001; Komada and Soriano 2002). Alterations in the location of sodium and potassium channels in myelinated nerves slow the propagation and desynchronize action potentials. Changes in axonal membrane potential can lead to neuropathy and the alteration of membrane excitability, which might be an explanation for the severe combined axonal and demyelinating neuropathy of our patient.

As in the quivering mouse, we detected a disturbed auditory function. Otoacoustic emissions assist differentiating between pathologies located at cochlear versus higher (e.g., cochlear nerve, brainstem) levels. While the inner ear function appeared undisturbed, the brainstem conduction was severely impaired in our patient. These results exactly resemble the phenotype of the quivering mouse where cochlear morphology and function were normal, while the transmission of auditory potentials through the brainstem nuclei was abnormal thereby verifying central deafness in the quivering mouse as opposed to the most frequent causes of deafness originating from the cochlea (Bock and Steel 1983; Deol et al. 1983).

In addition to the originally described quivering mouse, the human phenotype of β IV-spectrin deficiency includes congenital myopathy with incomplete congenital fiber-type disproportion. So far, the muscle of the quivering mouse had not been evaluated in detail and we

were now able to demonstrate a complete absence of type 1 fibers (fiber-type 2 uniformity) in the *tibialis anterior* muscle of the quivering (qv4J) mouse (Supplementary Fig. 3). Western blot analysis of human skeletal muscle from the patient and a control demonstrated the presence of β IV-spectrin in muscle tissue and a clear reduction of the protein amount in the mutated muscle specimen. We found a predominantly sarcolemmal distribution of β IV-spectrin in human and in mouse skeletal muscle. Immunohistochemical analysis of the trans-sarcolemmal protein β -dystroglycan confirmed the integrity of the cell membrane (Supplementary Figure 1), and the absence of α -laminin V staining at the sarcolemma largely rules out a congenital muscular dystrophy (Supplementary Fig. 2).

Hund et al. found β IV-spectrin to associate with critical structural and regulatory proteins in excitable cells not only in the brain, but also in the heart (Hund et al. 2010). The authors postulate that β IV-spectrin may associate with key membrane domains of other tissues where it modulates local signaling pathways and alters cell excitability, e.g., the flow of sodium ions through the postsynaptic membrane at the neuromuscular junction. This might explain the early developmental defect of the muscle and the comparatively low *SPTBN4* mRNA-expression levels in the range of other regulatory proteins of the skeletal muscle (Fig. 3).

In conclusion, the abrogation of β IV-spectrin in muscle cells leads to a distinct form of congenital myopathy associated with neuropathy and central deafness. However, more patients have to be found to verify this triad as the defining symptom complex for β IV-spectrin deficiency. Replication will be the ultimate proof that this gene is indeed involved in congenital myopathies, and will give an idea of its frequency and presence in other cohorts.

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Authors' contributions MS, EK phenotyped the patient and gathered clinical information and material; SMG performed cell culture experiments; EG, FS performed molecular genetics experiments; WS performed muscle histology; SDU, TJH provided and prepared the muscle samples of the quivering (qv4J) mouse; MS performed homozygosity mapping, sequence alignment, and bioinformatic analysis of the WES data. EK analyzed and validated the results of the WES data. EK, MS co-authored the first draft of the manuscript and contributed funding. All authors read the final version of the manuscript for intellectual content and gave their permission for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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