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A homoygous FITM2 mutation causes a deafness-dystonia syndrome with motor regression and signs of ichthyosis and sensory neuropathy

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ABSTRACT

A consanguineous family from Pakistan was ascertained to have a novel deafness-dystonia syndrome with motor regression, ichthyosis-like features and signs of sensory neuropathy. By applying a combined strategy of linkage analysis and whole-exome sequencing in the presented family, a homozygous nonsense mutation, c.4G>T (p.Glu2*), in FITM2 was identified. FITM2 and its paralog FITM1 constitute an evolutionary conserved protein family involved in partitioning of triglycerides into cellular lipid droplets. Despite the role of FITM2 in neutral lipid storage and metabolism, no indications for lipodystrophy were observed in the affected individuals. In order to obtain independent evidence for the involvement of FITM2 in the human pathology, downregulation of the single Fitm ortholog, CG10671, in Drosophila melanogaster was pursued using RNA interference. Characteristics of the syndrome, including progressive locomotor impairment, hearing loss and disturbed sensory functions, were recapitulated in Drosophila, which supports the causative nature of the FITM2 mutation. Mutation-based genetic counseling can now be provided to the family and insight is obtained into the potential impact of genetic variation in FITM2.

KEY WORDS: FITM2, Lipid droplets, Drosophila, Hearing impairment, Motor development, Dystonia

INTRODUCTION

Hearing involves the transformation of sounds into electrical signals by the inner ear and the subsequent processing of these signals along the central auditory pathways. Mutations in over a hundred genes cause auditory malfunction and hearing impairment (http:// hereditaryhearingloss.org/). Defects in the proteins that function in the inner ear can give rise to hearing impairment only (non-syndromic) or, as the function of implicated proteins is often not limited to the auditory system, they can result in multisystem disorders (syndromic hearing impairment).

Deafness–dystonia syndromes are among the more than 400 syndromic forms of hearing impairment described to date (Torihalo et al., 2004; Kojovic et al., 2013a). Deafness–dystonia is clinically and etiologically heterogeneous and in many of the investigated cases the underlying causes remain elusive (Kojovic et al., 2013a,b). For some of the cases with a causative mutation identified, disruption of energy homeostasis and/or metabolism are emerging as a common theme. This is true for Mohr–Tranebjaerg syndrome (MIM# 304700, http://www.ncbi.nlm.nih.gov/omim) with mutations in TINTM8A (MIM# 300356) (Jin et al., 1996), and for a number of rare mitochondrial disorders with mutations in mitochondrial genes as well as for SUCLA2-associated disease (MIM #120737) (Carrozzo et al., 2007).

Cellular energy can be stored as neutral lipids in specialized organelles, the lipid droplets (LDs) (Walther and Farese, 2012). LDs also function in the modulation of cellular signalling, lipid metabolism, transcriptional regulation, autophagy and immunity.
Defects in genes that affect LD biogenesis and/or function can be associated with hereditary lipodystrophies or motor neuropathies without obvious effects on lipid storage and metabolism (Fujimoto and Parton, 2011). Seipin, for example, which is encoded by BSCL2 (MIM# 606158), is an endoplasmic reticulum (ER) protein involved in LD formation and maintenance as well as in adipocyte differentiation (Cui et al., 2011; Tian et al., 2011). Loss-of-function mutations in BSCL2 lead to Berardinelli–Seip congenital lipodystrophy (MIM# 269700), whereas gain-of-toxic-function mutations in BSCL2 cause a motor neuron disease (MIM# 600794) (Ito and Suzuki, 2009; Magré et al., 2001; Yagi et al., 2011).

The fat storage-inducing transmembrane (FITM) protein family consisting of two conserved proteins, FITM1 and FITM2, is involved in LD partitioning and energy metabolism (Miranda et al., 2011; Kadereit et al., 2008; Gross et al., 2010, 2011; Choudhary et al., 2015). FITM1 (MIM# 612028) is primarily expressed in skeletal muscle and, at lower levels, in heart. FITM2 (MIM# 612029) is ubiquitously expressed at low levels in brain, placenta, skeletal muscle, heart, kidney, pancreas, liver, lung, spleen and colon (Kadereit et al., 2008). Expression of FITM proteins in human adipose tissue has not been described yet. In mouse, however, Fit2 expression is demonstrated to be highest in brown and white adipose tissues (Kadereit et al., 2008). Deficiency of Fit2 in mouse adipose tissue results in progressive lipodystrophy and postnatal whole body Fit2 knockout is lethal (Miranda et al., 2014; Goh et al., 2015).

FITM2 is part of the FITM2–R3HDML–HNF4A locus that is associated with type 2 diabetes, but no phenotypes in humans have hitherto been ascribed specifically to either of the two FITM genes (Cho et al., 2012).

In this study, we identified a homozygous truncating mutation in FITM2 in a consanguineous family of Pakistani origin with Siddiqi syndrome, a novel and characteristic combination of clinical features of progressive sensorineural hearing impairment, delayed development and regression of motor skills, dystonia, low body mass index (BMI), an ichthosis-like appearance of the skin and signs of a sensory neuropathy. No indications of a lipodystrophy were present in the affected individuals. RNAi-induced gene downregulation in Drosophila melanogaster recapitulated several aspects of the human phenotype, supporting the link between the syndrome and mutations in FITM2.

RESULTS

Clinical and paraclinical evaluations of the family

Clinical observations of affected individuals

A consanguineous family was identified from the Punjab region in Pakistan with five siblings affected by syndromic hearing impairment and three healthy siblings and parents (Fig. 1A). All affected individuals had global developmental delay and subsequent neuro-regression. Sensorineural hearing impairment was the first symptom of the disease at the age of about six months, and progressed to profound in about ten years (Fig. 1B).

Fig. 1. Pedigree of family W09-1008 and results of pure tone audiometry. (A) Pedigree of the family identified with Siddiqi syndrome and segregation of the c.4G>T (p.Glu2*) mutation in FITM2. (B) Pure tone audiometry of individuals II:1, II:5 and II:6. Age (years) is indicated with the symbol keys. The p95 lines indicate that 95% of individuals of 19 years old have thresholds lower than these. The arrows indicate that the thresholds are lower than 120 dB.
No intervention had been undertaken for the hearing impairment of the affected individuals, whose speech was limited to single words. Delayed motor development was evident in all five affected individuals. Four of them only walked independently at the age of three years, whereas individual II:6 never walked independently. The three oldest affected individuals displayed regression in their motor skills from six years of age, with a gradual loss of head control and the ability to sit and walk by the age of ten years. Fine motor skills were poor due to dystonic hand movements and finger deformities. Affected individuals were able to feed themselves but needed assistance in other daily living tasks. Significant dystonic limb movements were present in three cases and truncal dystonia was observed in individuals II:5 and II:8. Contractures, including pes cavus deformities, were seen in all three dystonic individuals due to long-standing immobility and dystonia.

There were no signs of spasticity. Muscle wasting of the lower limbs was observed, but given the results of neurophysiological measurements this might be more likely to result from immobility rather than from primary myopathy or motor neuropathy. All affected individuals had sensory complaints; two had non-specific pain in their joints and the remaining three experienced paraesthesia or ‘burning sensation’ in their limb peripheries, joints and trunk. Pain sensation was tested in individuals II:5 and II:6 and found to be absent in the upper limbs and face but preserved in the trunk and lower limbs. Seizures were experienced only by individual II:1 from the age of 15 years.

All five affected individuals displayed ichthyosis-like whitish scaling of the skin with more prominent abnormalities on the shin and scarring alopecia. All five individuals also failed to thrive and had low weights. They did not display dysmorphic features and their daily life behavior did not suggest severe cognitive dysfunction or visual abnormalities. The salient clinical features of the affected individuals are summarized in Table 1.

### Clinical examinations of affected individuals

Otological examination, tympanometry and pure-tone audiometry were performed in individuals II:1, II:5 and II:6 at the ages of 19, 10, and eight years, respectively. No external or middle ear abnormalities were noticed and tympanograms were normal for both ears. Pure-tone audiograms displayed bilateral, symmetric, severe or profound hearing impairment, which is sensorineural as bone conduction thresholds were in accordance with air conduction thresholds (Fig. 1B). Brainstem-evoked response audiometry (BERA) was performed for II:1 and II:5 and did not reveal any waveforms up to 90 dB, for both ears.

There were no signs of muscle damage, liver or kidney dysfunction as serum levels of glutamic oxaloacetic transaminase (SGOT), creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and aldolase were found to be within the normal range (Table 2). Fasting glucose levels were determined to be normal as well as fasting serum levels of triglycerides (Table 2).

Nerve conduction studies were performed for individual II:1 at the age of 14 years. For the left tibial nerve, small compound muscle action potentials (CMAPs) were measured with normal motor conduction velocity. Normal CMAP and motor conduction velocities were observed in the left median and ulnar nerves. Also, sensory action potentials and conduction velocities in the left median and ulnar nerves were found to be normal. Needle EMG was normal in deltoid, anterior tibial and gastrocnemius muscles. Findings were interpreted as resulting from decreased muscle bulk.

MRI of the abdomen of individuals II:5 and II:6, and of I:1 as a control did not demonstrate any signs of lipodystrophy. Values of

### Table 1. Clinical features of affected individuals with the homozygous c.4G>T (p.Glu2*) mutation in FITM2

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>II:1</th>
<th>II:3</th>
<th>II:5</th>
<th>II:6</th>
<th>II:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>26</td>
<td>Female</td>
<td>22</td>
<td>Male</td>
<td>9</td>
</tr>
<tr>
<td>Onset of hearing impairment (months)</td>
<td>6</td>
<td>Single words</td>
<td>6</td>
<td>Single words</td>
<td>6</td>
</tr>
<tr>
<td>Current speech disturbances</td>
<td>Urinary incontinence from 12 years; daily diarrhea from 16 years</td>
<td>Normal</td>
<td>Single words</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Autonomic features</td>
<td>Delayed walking at 3 years; bedridden by 10 years</td>
<td>Delayed walking at 3 years; loss of ambulation by 10 years</td>
<td>Delayed walking at 3 years; loss of ambulation by 10 years</td>
<td>Crawled at 2 years; never walked independently</td>
<td></td>
</tr>
<tr>
<td>Motor function</td>
<td>Dystonic limb movements from 2 years</td>
<td>None</td>
<td>Dystonic limb movements from 2 years</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Dystonic movements</td>
<td>None</td>
<td>None</td>
<td>Dystonic limb movements from 2 years</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Seizures</td>
<td>Daily seizures from 15 years</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Skin features</td>
<td>Ichthyosis-like features, most prominent at the shin</td>
<td>Ichthyosis-like features, most prominent at the shin</td>
<td>Ichthyosis-like features, most prominent at the shin</td>
<td>Ichthyosis-like features, most prominent at the shin</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.36</td>
<td>1.38</td>
<td>1.21</td>
<td>1.06</td>
<td>0.91</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>32.0</td>
<td>31.0</td>
<td>22.0</td>
<td>17.0</td>
<td>15.0</td>
</tr>
<tr>
<td>BMI*</td>
<td>17.2</td>
<td>16.1</td>
<td>14.8</td>
<td>15.2</td>
<td>18.1</td>
</tr>
<tr>
<td>Age at measurement (years)</td>
<td>23</td>
<td>19</td>
<td>12</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

*For comparison, the BMI of two healthy siblings (II:2 and II:7) was 23.5 and 21.0 at the ages of 22 years and 12 years, respectively.
liver fat content and subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) volumes were in the normal range. MRI of the brain with particular attention to the basal ganglia was performed for individuals II:1 and II:5 and showed no abnormalities, neither in the basal ganglia nor in other regions. A skeletal muscle biopsy (II:1) did not reveal myopathic or neurogenic changes.

In summary, the syndrome in the family is characterized by a novel combination of features: progressive hearing impairment, delayed development and subsequent regression of motor skills, dystonia and low BMI. In addition, ichthyosis-like skin changes are associated with this phenotype and there is a suggestion of small fiber neuropathy (burning sensations, non-length-dependent distribution of sensory abnormalities, normal sensory conduction studies). We propose to call the syndrome ‘Siddiqi syndrome’ after Dr Saima Siddiqi, who initiated the research in this family.

Whole-exome sequencing identified a nonsense mutation in *FITM2*

Homozygosity mapping and linkage analysis of all family members revealed a single homozygous region of 8.4 Mb on chromosome 20q12–q13.2 (rs2903624–rs6096425) (Table S2). LOD score calculations using 58,023 independent SNPs genome-wide in linkage equilibrium (pairwise r^2 for each SNP <0.1) revealed a maximum LOD score of 4.00 (Fig. S1). Prolonged linkage peaks is highly unlikely as shown by the percentage of ancestral consanguinity that might reduce the significance of linkage peaks is highly unlikely as shown by the percentage of the genomes present in homozygous runs of SNPs (>1 Mb) and the pairwise checks for familial relationships (Table S3, see also Fig. S2).

The only linkage region contained 125 genes [USCS Genome Browser (https://genome.ucsc.edu/), reference sequence hg19]. Whole-exome sequencing (WES) was performed in the non-affected parents (I:1, I:2) and in two affected siblings, (II:1, II:2). The single homozygous 8.4 Mb region on chromosome 20q12–q13.2 was fully covered in the enrichment kit. In the linkage region, only non-synonymous exonic and canonical splice-site variants were selected that occurred with a frequency of less than 5% in the 1000 genomes (http://www.1000genomes.org/) and HapMap (hapmap.ncbi.nlm.nih.gov) populations, and that were homozygous in both affected siblings and heterozygous in the parents (Tables S4, S5). This revealed a single homozygous nonsense mutation in the second codon, c.4G>T (p.Glu2*, NM_001080472.1; Fig. 2), of *FITM2* that cosegregated in the family with the disease (Fig. 1A; Table S5). This *FITM2* c.4G>T variant was neither present in 274 Pakistani control alleles nor in whole-genome or exome databases (see Materials and Methods). In order to exclude other potentially causative variants, especially in genomic regions with a LOD score ≥−2, we selected all variants with a MAF (minor allele frequency) <5% that were heterozygous in the parents and compound heterozygous or homozygous in both affected siblings. These analyses did not unveil any variants potentially associated with the syndrome (Tables S5, S6, Fig. S3).

Postnatal whole-body *Fit2* knockout in mice is lethal (Goh et al., 2015), we considered the possibility that the present mutation might not lead to complete loss of *FITM2* function, potentially as a result of downstream alternative translation initiation sites. We expressed C-terminally Strep/FLAG-tagged wild-type and p.Glu2* *FITM2* in HEK293T cells to identify potential N-terminally truncated *FITM2* proteins. Even after FLAG affinity purification, no indications for significant amounts of alternative *FITM2* products were obtained (Fig. 2B). We conclude that with high likelihood the *FITM2* mutation results in a complete loss of *FITM2* function.

To further address the involvement of *FITM2* mutations in hearing impairment syndromes with characteristics overlapping those in the present family, *FITM2* was sequenced in six index individuals with deafness and a sensory polyneuropathy. Also, four index cases were tested who were suspected to be presenting with Mohr–Tranebjaerg syndrome and who did not carry *TIMM8A* mutations. Mutation analysis did not reveal biallelic variants of *FITM2* with allele frequencies <5% in the HapMap, 1000 genomes or Exome Aggregation Consortium database (ExAC; http://exac.broadinstitute.org/) databases.

**Drosophila models of *FITM2***

To gain independent support for the role of *FITM2* in the phenotype of the presented family and to dissect the underlying tissue-specific pathologies, we studied *FITM* function in *Drosophila melanogaster*. The *Drosophila* genome harbors a single, thus far uncharacterized, gene representing the human *FITM* protein family (*FITM1* and *FITM2*), CG10671, which we accordingly name *Fitm*. *FITM1* and *FITM2* share 25% amino acid sequence identity with their annotated ortholog *Fitm* (see http://www.ensembl.org/). According to ModEncode and FlyAtlas expression databases (Chintapalli et al., 2007; Graveley et al., 2011), *Fitm* is expressed widely throughout developmental stages and tissues, with highest expression in adult fat body, heart and carcass. The lack of genetic redundancy of *Fitm in Drosophila* suggests that its complete absence in a null mutant is likely to lead to lethality in early stages of development. Therefore, we decreased the expression of *Fitm* by constitutive RNA-mediated interference (RNAi) with the *UAS-GAL4* system. The efficacy of *Fitm* downregulation was determined by qRT-PCR upon ubiquitous knockdown using the *aTub84B-GAL4* driver. All RNAi lines presented comparable levels of *Fitm* downregulation with respect to the corresponding control line. The decrease in *Fitm* transcripts was 92% (P=0.003) for *Fitm* RNAi-1A, 80% (P=0.008) for *Fitm* RNAi-1B and 80% for *Fitm* RNAi-2 (P=0.008) as compared with the respective control lines (Fig. S4). Since RNAi-1A and -1B carry the same RNAi construct, we prioritized RNAi-1A and RNAi-2 lines for our experiments. For the experiments that did not give conclusive data for one of the tested conditions and allowed the use of females, we additionally investigated the X chromosome-linked RNAi line RNAi-1B.
Knockdown of Drosophila Fitm causes locomotor impairment

To address locomotor function, impaired in the presented family, we first subjected Fitm knockdown models to an explorative negative geotaxis test in which climbing capacity was visually evaluated. Flies with Fitm knockdown mediated by the αTub84B-GAL4 and Mef2-GAL4 drivers displayed a decreased climbing capability at 4, 12 and 21 days after eclosion (Movie 1). The latter driver is highly expressed in muscle cells. Upon Fitm knockdown mediated by the fat-body-specific C7-GAL4 driver, flies were severely impaired in climbing at day 21, although normal at days 4 and 12 after eclosion. This locomotion phenotype was highly consistent in both RNAi lines tested with ubiquitous, preferential skeletal muscle and fat-body promoters. Pan-neuronal knockdown of Fitm with the w; UAS-Dcr-2; elav-GAL4 driver and w, UAS-Dcr-2; n-syb-GAL4 did not lead to obvious anomalies, flies showed normal climbing behavior.

To further characterize the locomotor abilities of Fitm knockdown flies in a quantitative manner, the island assay (Schmidt et al., 2012) was performed using the two driver lines that resulted in impaired climbing in the negative geotaxis test. This revealed that ubiquitous Fitm knockdown leads to severe locomotor impairment, resulting in significantly higher numbers of flightless flies at days 4 and 12 after eclosion ($P<0.0001$; Fig. 3A). Upon downregulation of Fitm using the Mef2-GAL4 driver, more than 98% of the flies displayed a flightless phenotype at 4 and 12 days old ($P<0.0001$ for all analyzed conditions; Fig. 3B). The effect of fat-body-specific Fitm knockdown on locomotion was evaluated because of the evolutionary conserved role of FITM2 in LD biogenesis in adipose tissue (Kadereit et al., 2008; Miranda et al., 2014) and because of high Fitm expression in Drosophila fat bodies. In agreement with this, a progressive locomotor impairment was observed. A maximum of 13% flightless flies were observed at 4 days past eclosion and a minimum of 51% flightless flies were observed at 12 days, which further raised to more than 78% at 21 days past eclosion ($P<0.001$ for all analyzed conditions; Fig. 3C). Pan-neuronal knockdown of Fitm with the w; Dcr-2; elav-GAL4 and w, UAS-Dcr-2; n-syb-GAL4 drivers did not lead to any significant locomotor impairment in the island assay (Fig. S5).

We visually evaluated body and wing movements of the flightless flies, as sensory motor coordination is essential for flight initiation and a sensory neuropathy is part of the phenotype in the Pakistani family. At the initiation of flight, flies first raise their wings to a stable position that will be held for a few seconds before take-off (Card and Dickinson, 2008). In a subset of Fitm knockdown flies, but not in controls, these flight initiation movements were uncoordinated. The knockdown flies failed to upstroke their wings for takeoff and instead displayed fast wing movements, and uncontrolled jumping and shaking of their corpus (Movie 2). Upon pan-neuronal Fitm knockdown, flies did not display this phenotype.
In conclusion, loss of Fitm expression in Drosophila causes locomotor defects and Fitm knockdown preferentially in muscle or specifically in the fat body suffices to induce this phenotype.

Downregulation of Fitm causes abnormal dendrite branching and field coverage of Drosophila multi-dendritic sensory neurons

As signs of a sensory neuropathy are part of the syndrome caused by a nonsense mutation in FITM2, we evaluated the role of Fitm in sensory neuron development by inspecting the dorsal class IV dendritic arborization C (ddaC) neurons in third instar larvae. These nociceptive neurons show a complex, but rather stereotypic dendritic branching with a large field of coverage (Fig. 4A) that, together with other class IV dendritic arborization neurons, tile the larval body wall. Fitm expression was downregulated by RNAi in class IV dendritic arborization neurons using a combination of the 477-GAL4 and ppk-GAL4 drivers, which simultaneously induce expression of the fluorescent marker UAS-mCD8::GFP. A driver line with a combination of two GAL4 elements was used to increase the number of GAL4 molecules to bind UAS-mCD8::GFP, UAS-Fitm RNAi and UAS-Dcr-2 to enhance their expression. Knockdown of Fitm upon induction of Fitm RNAi-1A resulted in a strong reduction of the dendritic field coverage in a subset of larvae (5 of 18 analyzed), with contact with the neighboring sensory neurons being completely absent (Fig. 4B). Fig. S6 shows the obtained microscopic images of the traced neurons and representative images of untraced neurons of knockdown flies that were evaluated as normal. A dendritic field coverage defect phenotype was also observed in a Fitm RNAi-2 larva, but only occurred in one of 40 larvae analyzed (Fig. S7A-F). It was, however, never observed in any control larva, either during this or other studies (Mukhopadhyay et al., 2010; Kramer et al., 2011; Klein et al., 2015). Reduced penetrance of RNAi-induced phenotypes is a known phenomenon and could be dependent on the timing and efficiency of knockdown (Mauss et al., 2009; Godenschwege et al., 2006). In our experiment, we used genetic tools and conditions to maximize RNAi efficiency (two driver elements, UAS-Dcr-2 and a temperature of 28°C). Alternatively, reduced penetrance can also be observed in null mutants when the function of the affected gene can partially be compensated by others (Raj et al., 2010; Chalancon et al., 2012; Cooper et al., 2013).

To gain more insight in the underlying defects of the abnormal field coverage, we performed manual tracing and quantitative analysis on the control and abnormal Fitm RNAi-1A dendritic trees (Fig. 4C-G; Tables S8, S9). Sholl analysis showed that the dendritic field coverage of controls has a maximum radius of 350±21 μm, the dendritic field coverage in a subset of larvae (5 of 18 analyzed), with contact with the neighboring sensory neurons being completely absent (Fig. 4B). Fig. S6 shows the obtained microscopic images of the traced neurons and representative images of untraced neurons of knockdown flies that were evaluated as normal. A dendritic field coverage defect phenotype was also observed in a Fitm RNAi-2 larva, but only occurred in one of 40 larvae analyzed (Fig. S7A-F). It was, however, never observed in any control larva, either during this or other studies (Mukhopadhyay et al., 2010; Kramer et al., 2011; Klein et al., 2015). Reduced penetrance of RNAi-induced phenotypes is a known phenomenon and could be dependent on the timing and efficiency of knockdown (Mauss et al., 2009; Godenschwege et al., 2006). In our experiment, we used genetic tools and conditions to maximize RNAi efficiency (two driver elements, UAS-Dcr-2, and a temperature of 28°C). Alternatively, reduced penetrance can also be observed in null mutants when the function of the affected gene can partially be compensated by others (Raj et al., 2010; Chalancon et al., 2012; Cooper et al., 2013).

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Taken together, our results suggest that *Fitm* is required for normal branching and dendritic field coverage in a subset of *Drosophila* ddaC nociceptive sensory neurons.

**Fitm is required for normal hearing in *Drosophila***

Affected members of the presented family displayed postnatal sensorineural hearing impairment that progressed to profound. Therefore, we tested whether *Fitm* is implicated in *Drosophila* hearing by analyzing sound-evoked mechanical and electrical responses of the antennal hearing organ (Fig. 5) upon ubiquitous or pan-neuronal knockdown. To evoke sound responses, we exposed the flies to pure tones of different intensities at the individual mechanical best frequency of their antennal sound receiver (Göpfert et al., 2006). The resulting vibrations of this receiver were measured as well as the ensuing compound action potentials (CAPs) propagated by the axonal projections of the fly’s auditory sensory neurons in the antennal nerve (Fig. 5A). In genetic background controls, sound particle velocities exceeding \( -0.05 \text{ mm s}^{-1} \) evoked CAP responses (Fig. 5B), consistent with published data on wild-type flies (Senthilan et al., 2012). As in wild-type flies, the sound-induced displacement of the antenna also scaled nonlinearly with the intensity of sound stimulation (Fig. 5A), displaying a compressive nonlinearity that, arising from motile responses of auditory sensory neurons, actively amplified the antennal displacement response to faint sounds with an amplification gain of approximately seven (Fig. 5C). Ubiquitous knockdown of *Fitm* with *Fitm* RNAi-1A significantly increased the threshold of the sound-evoked CAP responses (Fig. 5B; Table S12), documenting a loss in auditory sensitivity. Auditory sensitivity seemed uncompromised by pan-neural knockdown with *Fitm* RNAi-1A and knockdown with *Fitm* RNAi-1B or RNAi-2 (Fig. 5C), yet significant hearing impairment was detected in all three RNAi lines upon ubiquitous knockdown when we examined the nonlinear scaling of their antennal vibrations. RNAi-1A- and RNAi-1B-induced knockdown reduced this nonlinear scaling, significantly lowering the mechanical amplification gain (Fig. 5C; Table S12). Moreover, all three knockout constructs significantly increased the best frequency of the antennal sound receiver (Fig. 5D; Table S12), documenting defects in the active frequency tuning of the receiver, which is achieved through mechanical amplification.

Together, these results document that *Drosophila* auditory sensory neurons require *Fitm* for normal mechanical amplification in hearing, which is linked to auditory stimulus transduction and auditory neuron integrity (Senthilan et al., 2012).

**Fitm is important for lipid droplet size in the fat body of adult *Drosophila***

Having shown a number of parallels between human and *Drosophila* phenotypes, we finally sought to evaluate whether *Drosophila* *Fitm* functions in LD formation, as previously reported in other organisms (Kaderiet et al., 2008; Gross et al., 2011; Choudhary et al., 2015; Miranda et al., 2014). We thus knocked down *Fitm* expression in the fat body and evaluated LD size. *Fitm* RNAi-1A and RNAi-1B knockdown flies demonstrated a diminished LD size as compared with flies of the background line at 4, 12 and 21 days after eclosion (Fig. 6). The *Fitm* RNAi-2 knockdown flies exhibited a reduction in LD size that started at 12 days after eclosion (Fig. 6A). Strikingly, all RNAi lines showed a progressive phenotype; the reduction in the LD size was milder or nonexistent in young flies (4 days after eclosion) and more severe in ageing flies (12 and 21 days after eclosion) (Fig. 6A,B).
We conclude that *Fitm* function in LD formation is conserved in *Drosophila* (Kadereit et al., 2008).

**DISCUSSION**

We have described a family with a novel homozygous truncating mutation, c.4G>T (p.Glu2*), in *FITM2*. Affected individuals display Siddiqi syndrome, a novel syndrome characterized by progressive sensorineural hearing impairment, delayed motor development and subsequent regression, low BMI, ichthyosis-like skin alterations and signs of a small fiber neuropathy. Dystonia was observed in some of the affected individuals and seizures and chronic diarrhea only in the oldest affected sibling. The chronic diarrhea might well be a symptom of malabsorptive enteropathy which is seen in mouse upon postnatal *Fit2* deletion (Goh et al., 2015). The combination of the disease characteristics is novel, although observed phenotypic characteristics in the
family overlap with several known monogenic neurological conditions such as Troyer syndrome (MIM #275900) and deafness-dystonia syndromes including Mohr–Tranebjaerg syndrome (MIM #304700) and Megdel syndrome (MIM #614739). To delineate Siddiqi syndrome, further families with \(FITM2\) mutations need to be identified and evaluated clinically. Currently, it cannot be excluded that part of the phenotype results from mutations in other genes, especially the characteristics seen in only some of the cases. However, no homozygous rare variants were identified in individuals II:5 and II:6 in autozygous regions (>1 Mb) shared by II:1, II:5 and II:6 only. Also, these regions do not harbor genes known to be associated with dystonia. Similarily, regions uniquely autozygous in II:1 do not harbor potentially pathogenic heterozygous variants in both parents to explain seizures and diarrhea. Defects in known deafness genes that could explain the hearing loss only were also not identified. The causative association of the syndrome with a loss-of-function mutation in \(FITM2\) is supported by modeling of the disease in \textit{Drosophila melanogaster}, which has been proven to be a suitable model for studying conserved aspects of lipid metabolism and LD biology (Tian et al., 2011; Baker and Thummel, 2007). \(RNAi\) knockdown of the single \textit{Drosophila Fitm} ortholog recapitulated sensorineural hearing impairment, locomotor defects and abnormalities of the sensory system.

Sensorineuronal hearing impairment is the first symptom of Siddiqi syndrome. The audiemetric evaluations did not allow us to discriminate whether the hearing impairment has a cochlear or retrocochlear neuronal origin. The hearing phenotype resulting from \textit{Fitm} knockdown in \textit{Drosophila} reflected impaired auditory stimulus transduction and auditory sensory neuron function, which support a sensorineuronal hearing impairment in the human phenotype. A cochlear component might well contribute to progressive dysfunction of the auditory system in the affected individuals. LDs are prominent constituents of Hensen cells, which are highly specialized cells in the organ of Corti, and these Hensen cell LDs have been suggested to play a role in anti-inflammatory responses. Figure 6 shows the progressive decrease of lipid droplet size in RNAi flies compared to control flies over time.
In conclusion, we have described a novel deafness-dystonia syndrome that is causally related to a loss-of-function mutation in \textit{FITM2}, the phenotypic effects of which are recapitulated in a \textit{Drosophila} model. The phenotype of the affected individuals suggests that in humans, \textit{FITM2} function extends beyond its roles in neutral lipid storage and metabolism.

**MATERIALS AND METHODS**

**Patient evaluation**

Written informed consent was obtained from individuals I:1 and I:2 and included consent for themselves and for their offspring who were not able to sign and/or were younger than 18 years when the genetic studies were performed. The human subjects review boards of the Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan, the medical ethics committee of the Radboud University Medical Center, Nijmegen, the Netherlands (2010-418), and the Domain Specific Review Board for ethics of the National Healthcare Group, Singapore (2012/00295) approved the study protocol. All clinical investigations were performed according to the principles expressed in the Declaration of Helsinki.

Patients of the presented family (W09-1008; Fig. 1A) were evaluated by medical specialists in pediatrics, otorhinolaryngology, and neurology. Tympanometry, pure-tone audiometry, brainstem-evoked response audiometry (BERA), magnetic resonance imaging (MRI) of the brain, measurements of fasting levels of glucose and triglycerides and of other molecules in serum and neurophysiological evaluations were performed according to standard protocols. Muscle tissue derived from a muscle vastus lateralis biopsy was embedded in paraffin and stained with hematoxylin-eosin according to standard protocols.

**MRI of abdomen**

The abdominal MR images were acquired from a 3T MR scanner (Tim Trio Siemens) using two-point Dixon sequence repetition time (TR)=5.28 ms, echo time (TE)1=2.45 ms, TE2=3.68 ms, FA=9°, bandwidth1=500 Hz Px\(^{-1}\), bandwidth2=780 Hz Px\(^{-1}\) and Siemens body matrix coil after anatomical localization. For the parent (I:1), 80 axial slices with 3 mm thickness, 0.6 mm interslice gap and in-plane resolution of 1.25×1.25 mm were acquired and 52 slices with in-plane resolution of 1.02×1.02 mm were acquired for two affected children (II:5 and II:6). A fully automated segmentation technique was employed to segment and quantify the abdominal fat volumes between the first (L1) and fifth (L5) lumbar vertebrae (Sadananthan et al., 2015). First, the fat tissues were separated from non-fat tissues by intensity thresholding. The extracted fat tissues were then classified into subcutaneous (SAT) and visceral (VAT) adipose tissues using graph theoretic segmentation.

**MR spectroscopy of the liver**

Fat content in the liver was determined using \(^1\)H magnetic resonance spectroscopy (MRS). The liver spectra were obtained from a 2×2×2 cm\(^3\) voxel from two locations (right and left lobes) using a point-resolved spectroscopy (PRESS) sequence (TE=30 ms, TR=2000 ms) and a Siemens body matrix coil. The acquired spectra were fitted using the linear combination of model spectra (LCModel) (Sadananthan et al., 2015; Provencher, 1993). The liver fat was determined from the concentration of methyl and methylene groups of lipids and the unsuppressed water signal and corrected for T2 losses (Cowan et al., 2008).

**Genetic analyses**

**SNP genotyping**

Genomic DNA was isolated from peripheral blood lymphocytes by standard procedures. All family members were genotyped employing the HumanOmniExpress BeadChip v1.1 (Illumina) arrays with 719,659 SNPs. Homozygosity mapping using 696,513 autosomal SNPs with genotype calls in all samples (364,151 polymorphic) using PLINK v1.07 was performed (Purcell et al., 2007). Overlapping homozygous regions >5 Mb in size present in all affected and absent in the unaffected individuals were selected. Family relationships among genotyped individuals using identity-by-descent checks were performed. We further conducted a genome-wide linkage scan using \textsc{Merlin} 1.1.2 on a pruned subset of 53,028 independent SNPs (defined as pair-wise \(r^2<0.1\), as the inclusion of SNPs in strong linkage disequilibrium is known to result in inflation of linkage tests, based on inheritance of the same ancestral mutant allele (0.001) from both parents (coded as first cousins) under a recessive model (Abeecasis et al., 2002).

We confirmed the reported familial relationships among genotyped samples using PLINK identity by descent (IBD) analysis (~genome), with...
parent-offspring pairs sharing ~50% of alleles IBD and ~100% of loci sharing 1 out of 2 alleles IBD, and full sibling pairs sharing ~50% alleles IBD with the expected ~25% of loci sharing 0 alleles IBD, ~50% sharing 1 allele IBD and ~25% sharing 2 alleles IBD.

Sequence analysis; WES and Sanger sequencing
Whole-exome sequencing was performed in the non-affected parents, I-1 and I-2, and in two affected siblings, II-5 and II-6, using the Nimblegen SeqCap EZ exome v3 kit and protocol (Roche). The captured libraries were barcoded, pooled and sequenced on a single lane in a multiplexed 2×101 bp Illumina HiSeq 2000 sequencing run. Reads were mapped against the UCSC Genome Browser Hg19 assembly (build 37) using BWA v1.7 and variants were called using the Genome Analysis Toolkit (GATK) v2 (Broad Institute) following the recommended guidelines. Mean sequence depth was 79.5× with >96% of the exome covered by ≥10 reads. Identified variants were evaluated with the SIFT tool (http://sift.jcvi.org/) and checked against public databases of exomic or genomic variants [1000 genomes, HapMap and ExAC (65,000 exomes). The variant was not predicted to be pathogenic.

Amplification by PCR was performed on 40 ng of genomic DNA with Taq DNA polymerase (Roche) or AmpliTaq (Life Technologies). Primer sequences are provided in Table S1. PCR fragments were purified with NucleoFast 96 PCR plates (Clontech) in accordance with the manufacturer’s protocol. Sequence analysis was performed with the ABI PRISM 3730 DNA analyzer (Applied Biosystems).

Expression analysis of FITM2
Wild-type and c.4G>T FITM2 cDNA were cloned in an expression vector containing a C-terminal Streptavidin-FLAG-tag (SF-TAP) (Gloeckner et al., 2007) using Gateway Cloning technology (Life Technologies) according to the manufacturer’s instructions. Only the protein-coding FITM2 sequences are represented in the construct (NM_001080472.1). HEK293T cells were cultured in high-glucose DMEM with 10% FCS, 1% penicillin/streptomycin and 1 mM sodium pyruvate. For DNA transfections, HEK293T cells were seeded in 6-well plates, grown overnight, and transfected with 2 µg of plasmid using PEI transfection reagent (Merck Millipore). Twenty-four hours after transfection cells were washed with PBS and lysed on ice in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100 supplemented with complete protease inhibitor cocktail (Roche)]. Affinity purification of SF-TAP-tagged proteins was performed on cleared lysates using anti-FLAG M2 affinity gel (Sigma Aldrich). Lysates were incubated for four hours at 4°C and subsequently precipitated by centrifugation and washed three times in lysis buffer. Protein lysates and affinity purified samples were analyzed on western blots of NuPAGE Novex 12% Bis-Tris Protein Gels (Life Technologies) and imaged using the Odyssey Infrared Imaging System (LI-COR). Tagged molecules were detected by anti-FLAG antibodies (1:1000; Sigma Aldrich, F7425) and IRDye680 goat-anti-rabbit IgG (1:10,000; LI-COR, 926-32211) as described (Roosig et al., 2014).

RNAi and phenotypic analyses in Drosophila melanogaster
Drosophila melanogaster stocks and maintenance
We modeled loss of human FITM2 by constitutive knockdown in Drosophila, exploiting two independent, inducible RNAi constructs against both isoforms encoded by Cg10671, Cg10671-RA and Cg10671-RB, and the UAS-GAL4 system (Brand and Perrimon, 1993; Dietzl et al., 2007). Experiments were replicated in multiple stocks, two from the GD RNAi library (v44433 and v44435 harboring the RNAi construct GD3580; referred to as Fitm RNAi-1A and Fitm RNAi-1B, respectively) with the corresponding genetic background control (v60000; Control-1) and one from the KK RNAi library (v109895 with the RNAi construct KK107999; Fitm RNAi-2) with the corresponding genetic background control (v60100; Control-2). The stocks were obtained from the Vienna Drosophila RNAi Centre (VDRC; http://stockcenter.vdrc.at/control/main) (Dietzl et al., 2007).

RNAi expression was induced by a variety of GAL4 driver lines, which carry a tissue-specific promoter driving the expression of GAL4. The w; C7-GAL4; UAS-Dcr-2 (fat body expression) line was kindly provided by Marek Xudim (Rynes et al., 2012). The GAL4-PRISM BigDye Terminator Cycle Sequencing 1.2 Ready Reaction kit was analyzed with the ABI PRISM 3730 DNA analyzer (Applied Biosystems).

Presence of the FITM2 c.4G>T transversion was determined in 137 ethnically matched healthy controls by restriction analysis of amplicons of CG10671. The c.4G>T variant was also verified in the Nijmegen Wild-type and c.4G>T variant was also verified in the Nijmegen Institute (following the recommended guidelines. Mean sequence depth was 79.5× with >96% of the exome covered by ≥10 reads. Identified variants were evaluated with the SIFT tool (http://sift.jcvi.org/) and checked against public databases of exomic or genomic variants [1000 genomes, HapMap and ExAC (65,000 exomes). The variant was not predicted to be pathogenic.

Confirmation of Fitm knockdown by qRT-PCR
In order to evaluate the efficiency of RNAi-induced knockdown, Fitm RNAi-1A, Fitm RNAi-1B and Fitm RNAi-2 lines were crossed to the attB84B-GAL4 driver (ubiquitous). One-day-old males of the appropriate genotype were selected for qRT-PCR evaluation of Fitm RNAi-1A and RNAi-2 lines, one-day-old females for evaluation of knockdown using the RNAi-1B line. The RNAI-1B and RNAI-2 lines were crossed to the transgenic lines w; UAS-Dcr-2; UAS-α-GAL4 (32079), both from BDSC. Crosses were maintained according to standard procedures at 25°C.

Expression analysis of FITM2 in HEK293T cells
Wild-type and c.4G>T FITM2 cDNA were cloned in an expression vector containing a C-terminal Streptavidin-FLAG-tag (SF-TAP) (Gloeckner et al., 2007) using Gateway Cloning technology (Life Technologies) according to the manufacturer’s instructions. Only the protein-coding FITM2 sequences are represented in the construct (NM_001080472.1). HEK293T cells were cultured in high-glucose DMEM with 10% FCS, 1% penicillin/streptomycin and 1 mM sodium pyruvate. For DNA transfections, HEK293T cells were seeded in 6-well plates, grown overnight, and transfected with 2 µg of plasmid using PEI transfection reagent (Merck Millipore). Twenty-four hours after transfection cells were washed with PBS and lysed on ice in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100 supplemented with complete protease inhibitor cocktail (Roche)]. Affinity purification of SF-TAP-tagged proteins was performed on cleared lysates using anti-FLAG M2 affinity gel (Sigma Aldrich). Lysates were incubated for four hours at 4°C and subsequently precipitated by centrifugation and washed three times in lysis buffer. Protein lysates and affinity purified samples were analyzed on western blots of NuPAGE Novex 12% Bis-Tris Protein Gels (Life Technologies) and imaged using the Odyssey Infrared Imaging System (LI-COR). Tagged molecules were detected by anti-FLAG antibodies (1:1000; Sigma Aldrich, F7425) and IRDye680 goat-anti-rabbit IgG (1:10,000; LI-COR, 926-32211) as described (Roosig et al., 2014).

Negative geotaxis assay
Fitm RNAi lines and the corresponding genetic background control lines were crossed to the attB84B-GAL4, Mel2-GAL4 and the C7-GAL4 driver lines. Female and male progeny of the appropriate genotypes and age were subjected to the negative geotaxis assay (Benzer, 1967; Ali et al., 2011). Locomotor climbing abilities were observed after tapping down the flies in the vials. The natural response of flies is to climb up the vials after tapping. In case of locomotor impairment, flies exhibit slower climbing behavior or non-ability to climb. All behavioral tests were performed at room temperature under standard light conditions. Aged flies were transferred to fresh food vials every three to four days.

Island assay
Fitm RNAI lines and the corresponding genetic background control lines were crossed to the attB84B-GAL4 (ubiquitous), the Mel2-GAL4 (preferentially expressed in skeletal muscle) and the C7-GAL4 (fat body) driver lines. Female and male progeny of the appropriate genotypes and age were subjected to the island assay (Schmidt et al., 2012). In brief, flies were simultaneously released onto a platform in the middle of a soapy bath and their escape response was videotaped. Flies remaining on the platform after 10 s were manually counted. Flight ability, wing and leg movements were visually evaluated (Lee et al., 2009). If an abnormal locomotion behavior
was found, at least one additional experiment was performed to confirm the observed behavioral defects. The SPSS statistics 20 package (IBM) was used for the ANOVA statistical comparisons.

All behavioral experiments were performed at room temperature under standard light conditions. Aged flies were transferred to fresh food vials every three to four days.

**Dendritic morphology of class IV dendritic arborization neurons**

Male third instar larvae were dissected following a ventral midline incision for imaging of the dorsal class IV ddaC neurons. The *Fitm RNAl-1A and *Fitm RNAl-2 lines and the corresponding controls were crossed to w, UAS-Dcr-2; 477-GAL4, UAS-mCD8::GFP; yuk-GAL4 driver line. Dendritic neurons were stained with rat anti-mouse CD8a (1:100; Thermo Fisher Scientific, MCD0800) and goat anti-rat Alexa Fluor 488 (1:200; Thermo Fisher Scientific, A-11006). Z-stack images were taken at a Zeiss LSM 510 confocal microscope with a 20× objective. Z-stacks were imported into NeuronStudio (version 0.9.92; Wearne et al., 2005; http://research.mssm.edu/ncic/tools-ns.html) for generation of neuronal reconstructions and Sholl analyses (10 μm interval). Tracings files were analyzed with L-Measure (version 5.2; Scorcioni et al., 2008) and statistical significance was analyzed using the one-sample t-test in GraphPad Prism. Data was collected from larvae selected from two independent experiments.

**Hearing test**

To assess sound responses of the *Drosophila* Johnston’s organ (JO), antennal vibrations and ensuing antennal nerve potentials were measured in adult flies three days after eclosion as previously described (Göpfert et al., 2006). The *Fitm RNAl-1A, *Fitm RNAl-1B and *Fitm RNAl-2 lines and the corresponding controls were crossed to the *elav-GAL4 and aTube84B-GAL4 drivers. In the *Fitm RNAl-1B line, the RNAI construct was located in the X chromosome and therefore only female progeny were evaluated of this condition and its control. When using the lines *Fitm RNAl-1A, Control-1, *Fitm RNAl-2 and Control-2, both females and males were selected. In brief, antennal vibrations were monitored at the tip of the antennal arista using a PSV-400 scanning laser Doppler vibrometer (LDV) with an OFV-500 close-up unit (Polytec GmbH). Pure tones adjusted to the mechanical best frequency of the antenna were used as sound stimuli. The resulting sound particle velocity was measured with an Emkay NR3158 pressure gradient microphone (distributed by Knowles Electronics, Inc.) at the position of the fly. In line with previous reports (Göpfert et al., 2006), the individual best frequency of each antenna was determined from the power spectrum of its mechanical free fluctuations in the absence of sound stimulation, and tone-evoked antennal vibration amplitudes were measured as Fourier amplitudes at the frequency of sound stimulation. Ensuing nerve potentials were measured in the form of compound action potentials (CAPs) from the axonal projections of JO neurons in the antennal nerve via an electrolytically tapered tungsten electrode inserted between the antenna and the head (Effertz et al., 2011; Senthilanan et al., 2012). A tungsten wire inserted into the thorax served as indifferent electrode. CAP amplitudes were plotted against the corresponding sound particle velocities. Hill fits were used to determine the sound particle velocity threshold of the CAPs, whereby the particle velocity corresponding to 10% of the maximum amplitude approached by the fit was used as the threshold criterion. To quantify the amplification gain exerted by motile responses of JO neurons, the antenna’s mechanical sensitivity, measured as antennal displacement amplitudes normalized to the corresponding sound particle velocities, was plotted against the particle velocity of the stimulus tones. The amplification gain was then measured as the ratio between the antenna’s mechanical sensitivity in the low and high intensity regimes (Göpfert et al., 2006; Senthilanan et al., 2012). Data analysis was performed using Polytec-VIB (Polytec GmbH), Spike 2 (Cambridge Electronic Design), Excel 2007 (Microsoft), SigmaPlot 10 (Systat Software) and Prism (GraphPad).

**Fat body analysis**

*Fitm RNAl lines and the corresponding controls were crossed to the C7-GAL4 driver line (fat body). Progeny were transferred to vials with fresh food every two days. Fat bodies of female flies were dissected at the indicated adult age, fixed in PBS with 3.7% paraformaldehyde for 20 min, rinsed with PBS, stained with Bodipy (1:2000; Life Technologies, C3922) for 20 min at room temperature, and mounted in Vectashield with DAPI (Vector Laboratories). Pictures were obtained using a Zeiss Axio Imager Z1 fluorescence microscope (Zeiss). Data was collected from flies selected from two independent experiments. LD area was assessed using Fiji (NIH). A minimum of two random regions of interest (ROI) of 35.5 μm² were created from each image and the area of the LDs contained in the ROI was retrieved and analyzed. Statistical significance was calculated by using ANOVA with the Tukey correction for multiple testing incorporated in GraphPad Prism.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

C.Z.S.: Island assay and fat body analysis and data analysis of island assay, fat body analysis and type IV neurons, writing the manuscript. A.C.-N.: Island assay and fat body analysis, preparation of the manuscript. S.-H.N.: Performance of hearing test in flies, preparation of manuscript. M.S. and C.C.K.: Genetic analyses. J.N.F.: Genetic analyses, preparation of the manuscript. M.v.d.V.: Type IV neuron analysis, preparation of the manuscript. S.S.V.: Supervised the analysis of fat distribution in the patients. B.N.: Data analyses, preparation of the manuscript. J.O.: WES, Sanger sequencing and FITM2 cDNA cloning into an expression vector. E.d.V.: Expression analysis of FITM2 in HEK293T cells, preparation of the manuscript. R.K.: Hearing tests in flies. A.M., S.S. and S.A.R.K.: Patient evaluation. M.H., R.S. and M.O.: Candidate gene evaluation for the linkage interval. L.T.: Tested TIMM8A in patient DNA and contributed patient DNA of TIMM8A-negative cases. E.v.W.: was involved in the methodology and support on imaging, supervision and editing of the manuscript. J.M.: D.: Visiopharma preparations for further analyses. J.B. and P.d.J.: Pretested patient DNA for polyneuropathy genes and provided DNA of negative cases. S.A.S.: Analysis of fat distribution in the patients. B.P.v.d.W.: Evaluated the clinical data, preparation of the manuscript. M.C.G.: Supervised the hearing tests in flies, preparation of the manuscript. R.Q.: Supervised the collection and clinical evaluation of the family, preparation of the manuscript. A.S.: Conceived and supervised the experiments performed in *Drosophila*, preparation of the manuscript. H.K.: Conceived and supervised the study, writing of this paper. S.S.: Ascertained the family, collection and management of the clinical data, performed genetic analyses, preparation of the manuscript.

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**Supplementary information**

Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.026476.supplemental

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