Homozygous disruption of PDZD7 by reciprocal translocation in a consanguineous family: a new member of the Usher syndrome protein interactome causing congenital hearing impairment

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A homozygous reciprocal translocation, 46,XY,t(10;11),t(10;11), was detected in a boy with non-syndromic congenital sensorineural hearing impairment. Both parents and their four other children were heterozygous translocation carriers, 46,XX,t(10;11) and 46,XY,t(10;11), respectively. Fluorescence in situ hybridization of region-specific clones to patient chromosomes was used to localize the breakpoints within bacterial artificial chromosome (BAC) RP11-108L7 on chromosome 10q24.3 and within BAC CTD-2527F12 on chromosome 11q23.3. Junction fragments were cloned by vector ligation and sequenced. The chromosome 10 breakpoint was identified within the PDZ domain containing 7 (PDZD7) gene, disrupting the open reading frame of transcript PDZD7-C (without PDZ domain) and the 5′-untranslated region of transcript PDZD7-D (with one PDZ and two prolin-rich domains). The chromosome 11 breakpoint was localized in an intergenic segment. Reverse transcriptase–polymerase chain reaction analysis revealed PDZD7 expression in the human inner ear. A murine Pdzd7 transcript that is most similar in structure to human PDZD7 is known to be expressed in the adult inner ear and retina. PDZD7 shares sequence homology with the PDZ domain-containing genes, USH1C (harmonin) and DFNB31 (whirlin). Allelic mutations in harmonin and whirlin can cause both Usher syndrome (USH1C and USH2D, respectively) and congenital hearing impairment (DFNB18 and DFNB31, respectively). Protein–protein interaction assays revealed the integration of PDZD7 in the protein network related to the human Usher syndrome. Collectively, our data provide strong evidence that PDZD7 is a new autosomal-recessive deafness-causing gene and also a prime candidate gene for Usher syndrome.

INTRODUCTION

Congenital sensorineural hearing loss (SNHL) is among the most common birth defects, occurring in 1–2 of 1000 live births (1). In addition, it may arise throughout childhood and adulthood. Current research suggests that genetic defects account for at least 50–60% of cases of congenital and childhood-onset SNHL (2,3). Syndromic SNHL contributes to 20–30% of SNHL patients and includes nearly 400 different forms. Although SNHL is associated with these syndromes, it is not necessarily the predominant clinical feature. Some prevalent examples of syndromic forms of deafness are Pendred, Waardenburg 1–4, branchio-oto-renal and Usher 1–3 syndromes (4). The majority (70–80%) of the genetic SNHL has a non-syndromic etiology. As of November 2008, 57 autosomal-dominant (DFNA), 77 autosomal-recessive

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(DFNB) and 8 X-linked (DFN) loci for non-syndromic hearing loss have been listed in the Hereditary Hearing Loss Home-page (http://webh01.uu.ac.be/hhh). The causative genes at 22 DFNA, 27 DFNB and one DFN loci have already been cloned. These genes encode connexins, transcription factors, potassium channels, cytoskeletal proteins, unconventional myosins, scaffold proteins and other cellular components with auditory functions in the inner ear. Mutations in one particular gene, GJB2 (DFNB1), account for up to 50% of autosomal-recessive cases of non-syndromic SNHL and up to 30% of sporadic cases (5). Mutations in other deafness genes occur at much lower frequencies.

It is interesting to note that syndromic and non-syndromic forms of deafness can be caused by allelic mutations in the same gene. Usher syndrome is both clinically (three subtypes can be distinguished on the basis of disease onset, severity and progression) and genetically (at least 12 loci) heterogeneous and characterized by SNHL and retinitis pigmentosa (6–8). So far, eight causative genes have been identified, some of which have alleles associated with non-syndromic SNHL. Mutations in myosin VIIa cause USH1B and DFNB2 (9), in harmonin USH1C and DFNB18 (10), in cadherin 23 USH1D and DFNB12 (11) and in whirlin USH2D and DFNB31 (12). The gene products related to Usher syndrome are part of dynamic multiprotein complexes ('Usher syndrome protein interactome') that are essential for pathways in hair cells of the inner ear and photoreceptor cells of the retina (6,7).

The genetic heterogeneity of SNHL renders identification of new disease genes difficult. Most currently known SNHL genes have been found by genetic linkage analyses of families with heritable hearing loss and positional cloning of candidate genes. Another promising strategy is the cytogenetic and molecular characterization of chromosome breakpoints in patients who carry apparently balanced chromosome rearrangements that are either de novo or segregating with SNHL. In these cases, the specific chromosome rearrangement that disrupts or deletes a gene(s) and/or disturbs gene regulation forms a visible bridge between the patient’s genotype and phenotype. Disease-associated chromosome rearrangements have been successfully used for the identification of causative genes underlying neurogenetic disorders (13–16). We have conducted a systematic search for balanced chromosome rearrangements in children with varying degrees of hearing impairment, who attended a specialist clinic. The offspring of a consanguineous family presented with SNHL and a homozygous reciprocal translocation, 46,XY,t(10;11),t(10;11). Here, we report on the positional cloning of PDZD7 that encodes a PDZ domain-containing scaffold protein, showing characteristics of previously identified Usher syndrome proteins.

RESULTS

Systematic search for SNHL-associated chromosome rearrangements

In more than 300 patients with congenital forms of non-syndromic hearing impairment, we found five cases with 46,XY,t(10;11)(p31.2;q25.2), 46,XX,der(2;5)(inv5)(p15.3;q23) t(2;5)(q33;p15.3), 46,XX,t(2;12)(p13;p13), 46,XY,t(10;15) (q25.3;q21.2) and 46,XY,t(10;11)(q24.3;q23.3),t(10;11) (q24.3;q23.3), respectively, in which an apparently balanced chromosome rearrangement was associated with SNHL. Because 5/300 (1.7%) are far more than the prevalence which would be expected in newborn populations (17,18), it is plausible to assume that in most of these cases the association between SNHL and chromosome rearrangement is not coincidental. Our results show that chromosome analyses in patient cohorts with genetically heterogeneous, relatively unsppecific and/or late-manifesting phenotypes are warranted, because for any disease of interest there may be a small subgroup of patients, in which chromosome rearrangements have created a dominant mutation or unmasked a recessive mutation in an underlying gene(s).

Case report

The index patient described here was introduced to the Division of Communication Disorders at the age of 4 years because of a bilateral moderate SNHL. There was no progression in the next 5 years. Physical examination revealed a normal-appearing and age-based developed boy without any anomalies and dysmophies. Routine workup included creatinine, blood urea nitrogen, complete blood count, tests for thyroid hormone levels, ophthalmological examination including funduscopy and electrocardiogram. The most common autosomal-recessive form of deafness, DFNB1, was excluded by complete sequencing of the GJB2 gene. Pedigree analysis revealed that the parents are consanguineous (Fig. 1). They have an additional two sons and two daughters and also experienced two spontaneous abortions. Chromosome banding analysis of the index patient revealed two identical balanced reciprocal translocations between the long arms of chromosomes 10 and 11, 46,XY,t(10;11)(q24.3;q23.3), t(10;11)(q24.3;q23.3) (Fig. 2A). Both of his parents (Fig. 2B and C) carry the same translocation in a heterozygous state. The mother reported a mild hearing impairment, which does not require hearing aids. The father also suffered from a mild high frequency hearing impairment; however, he works in a noisy environment. The heterozygous translocation was also found in the couple’s four other children, who all hear normally. A funduscopy of the index patient at the age of 8 years did not reveal any signs of tapetoretinal degeneration.

Chromosomal breakpoint mapping

In order to map the translocation breakpoints at higher DNA resolution, we selected region-specific bacterial artificial chromosome (BAC) clones from the database (Supplementary Material, Table S1). Individual BACs from the breakpoint regions were hybridized to the patient’s metaphase spreads. BACs RP11-108L7 at 102,666,924–102,848,009 bp and RP11-11122F15 at 102,752,043–102,905,775 bp on chromosome 10q24.3 produced distinct fluorescence in situ hybridization (FISH) signals on both derivative chromosomes (Fig. 2D), as expected for breakpoint-spanning clones. This narrowed the chromosome 10q24.3 breakpoint down to ~96 kb between 102,752,043 and 102,848,009 bp (overlapping sequence of BACs RP11-108L7 and RP11-11122F15). The reciprocal breakpoint was localized in BAC CTD-2527F12 at 115,258,420–115,437,399 bp on chromosome 11q23.3.
Database analyses (http://www.ensembl.org and http://www.ncbi.nlm.nih.gov) revealed several genes (PEO1, LZTS2, PDZD7, SEMA4G, MRPL43, SFXN3 and KAZALD) in the 10q24.3 breakpoint region, whereas no candidate gene was found in the 11q23.3 breakpoint region. However, it is interesting to note that the radixin (RDX) gene at the DFNB24 locus (19) lies 5.7 Mb proximal and the tectorin alpha (TECTA) gene at the DFNA8/12 (20) and DFNB21 loci (21), respectively, 5.2 Mb distal to the breakpoint on chromosome 11q23.3. We focused our positional cloning efforts on the 10q24.3 breakpoint region. To test whether a given candidate gene(s) is disrupted or deleted by (10;11)(q24.3;q23.3), we designed primers for the polymerase chain reaction (PCR) amplification of ~5 kb fragments from the 10q24.3 breakpoint-spanning BAC (Supplementary Material, Table S2). Fragments 10q24A, C and D could be amplified from both the patient’s genomic DNA and controls. The 5273 bp fragment 10q24B was detectable in the heterozygous translocation carriers, but not in the index patient (Fig. 3A), as expected for a breakpoint-spanning fragment. To narrow down the breakpoint region further, we subdivided the 5273 bp fragment in smaller PCR fragments. By PCR typing of the patient and his brother, the breakpoint was localized to the 564 bp fragment 10q24B4.2 (Fig. 3B) in intron 10 of the PDZ domain-containing protein 7 (PDZD7) gene (Fig. 3C).

**Breakpoint cloning**

By using a vector ligation technique, it was possible to clone a junction fragment from the derivative chromosome 11. T7 forward primer and a PDZD7 reverse primer (Supplementary Material, Table S2), which binds to the 564 bp breakpoint-containing segment on chromosome 10q24.3, produced the expected ~650 bp fragment (insert plus 90 bp of vector sequence) from control DNA and an ~1400 bp fragment from patient DNA (Fig. 4A). The heterozygous brother of the patient displayed both the 650 and the 1400 bp fragment (data not shown). Sequencing of this junction fragment 1 revealed a fusion sequence between an intergenic segment from chromosome 11q23.3 (breakpoint at 115,319,661+, Ensembl release 48) and the PDZD7 gene on chromosome 10q24.3 (102,764,759+; Ensembl release 48) (Fig. 4B). Junction fragment 1 consists of 90 bp of vector, 987 bp of chromosome 11 and 339 bp of chromosome 10 sequence and is derived from the der (11). The now available sequence information from both breakpoint regions allowed us to design a forward primer on chromosome 10q24.3 and a reverse primer on chromosome 11q23.3 (Supplementary Material, Table S2) for the amplification of the reciprocal junction fragment 2 (159 bp of chromosome 10 and 1514 bp of chromosome 11 sequence) from the der (10) (Fig. 4C). Sequencing of junction fragment 2 revealed identical breakpoint localizations on chromosomes 10 (102,764,759+) and 11 (115,319,661+). Evidently, the chromosome breakage and fusion events were not associated with DNA loss in the breakpoint regions. Bioinformatical (BioEdit) analysis (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) of the t(10;11)(q24.3;23.3) fusion sequence did not provide any evidence for the existence of a PDZD7 fusion protein.

**Transcripts and protein isoforms of PDZD7**

The PDZD7 gene is 23.3 kb long and consists of 16 exons (Fig. 5A). There are four known protein-coding transcripts, which—for the sake of simplicity—are called PDZD7-A, -B, -C and -D (Fig. 5B). PDZD7-A and PDZD7-B use only exons upstream of the breakpoint and therefore may be unaffected. The corresponding protein isoforms are endowed with a tandem of two PDZ domains (Fig. 5C). PDZ1 is encoded by genomic exon 3 (7091–7657 bp) and corresponds to amino acids 86–168; PDZ2 is encoded by genomic exon 4 (8830–10 463 bp) and corresponds to amino acids 210–293. The breakpoint in intron 10 disrupts the open reading frame of transcript PDZD7-C, which encodes a protein isoform without PDZ domain, as well as the 5’-untranslated region (UTR) of transcript PDZD7-D, which encodes a proline-rich protein with one PDZ domain. The PDZ domain of PDZD7-D is encoded by genomic exons 14–16 (20 828–22 374 bp) and corresponds to amino acids 223–295.

Sequence comparisons revealed that the PDZ domains of isoforms PDZD7-A and B, which are very similar in structure,
share extensive homology with the PDZ domain-containing proteins whirlin and harmonin (Fig. 6A), which are encoded by the Usher syndrome genes *USH1C* and *DFNB31*. The PDZ domain in the isoform PDZD7-D is also homologous to whirlin and harmonin (Fig. 6B). When we sequenced the coding regions of the *PDZD7* gene in a healthy control individual, we noted an in-frame insertion of 6 nt encoding for an arginine (R) and a serine (S) in exon 14, compared with the wild-type (chromosome 10: 102,758,214–102,761,691) (Fig. 7). The insertion occurred in a stretch of seven arginine–alanine repeats in a proline-rich region of the PDZ domain-containing isoform PDZD7-D. Genotyping of 10 controls revealed the wild-type sequence in seven individuals and the insertion polymorphism, which is not yet listed in the current databases of genomic variants, in three individuals. In this context, it is interesting to note that the expanded...
repeat with eight arginine–alanine motifs corresponds to the chimpanzee and orangutan wild-type. The rhesus macaque is endowed with only six repeats. The bushbaby as a primitive primate, and the mouse as an outgroup showed shorter imperfect repeats. Whether this intra- and inter-specific insertion–deletion polymorphism affects the binding properties of PDZD7 remains to be elucidated.

Tissue expression analyses of PDZD7
According to the NCBI/UniGene/ESTProfileViewer (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.438245), the human PDZD7 gene is expressed in numerous tissues and organs including brain. However, there are no data on expression in the inner ear. We used exon-spanning primers (Supplementary Material, Table S2), which detect all four known transcripts, to study the PDZD7 expression in adult human inner ear and cortex. Primers for ACTB and GAPDH were used as controls. The expected 298 bp PDZD7 reverse transcriptase (RT)–PCR product was easily amplified from cortex total RNAs, whereas only weak bands were produced from several independent whole inner ear samples (data not shown). The corresponding genomic DNA fragment would be 822 bp long. To exclude an artifact, the weak 298 bp band from inner ear was gel-extracted, re-amplified and sequence-verified. Our data suggest that PDZD7 is weakly expressed in the adult human inner ear or, more likely, only expressed in few specialized cells of the inner ear. The murine Pdzd7 transcript NM 177605.3, which is known to be expressed in the inner ear and retina (NCBI/UniGene/EST-ProfileViewer), shares the highest structural similarity with the human PDZ domain-containing transcript PDZD7-D, which is disrupted by t(10;11)(q24.3;23.3).

Direct binding of PDZD7 to Usher syndrome scaffold proteins
The expression profile including the inner ear and retina together with the structural and functional homology of the PDZD7 protein to harmonin and whirlin prompted us to evaluate PDZD7 in the Usher syndrome protein network (22–25). In order to validate the interaction between PDZD7 and the
Usher syndrome type 1 G (USHG1) protein SANS (scaffold protein containing ankyrin repeats and SAM domain), we adopted recombinant expressed domains of both proteins in glutathione S-transferase (GST) pull-down assays. Immobilized GST-tagged PDZD7 pulled down FLAG-tagged full-length SANS from HEK293T cell lysates. In western blot analysis with anti-FLAG, we identified a single band at ~57 kDa, the estimated molecular weight of 3xFLAG-SANS, indicating the direct binding of PDZD7 to SANS (Fig. 8A). In contrast, GST alone pulled down only a very fade band of 3xFLAG-SANS. For testing the incorporation of PDZD7 in Usher syndrome protein networks in the retina, we adopted GST-tagged PDZD7 or GST alone to protein lysates of the mouse retina and probed the western blot with anti-harmonin. Quantification of the detected bands revealed that four times more harmonin was pulled down from the retinal-expressed...
proteins with GST-tagged PDZD7 than with GST alone (Fig. 8B). All known characteristics of PDZD7 and its interaction with Usher syndrome scaffold proteins suggest PDZD7 as a further component of the Usher syndrome protein network in cochlear hair cells and retinal photoreceptor cells.

DISCUSSION

The pedigree of the consanguineous family with a homozygous reciprocal translocation carrier, 46,XY,t(10;11),t(10;11) is consistent with an autosomal-recessive form of deafness caused by disruption of the PDZD7 gene in the chromosome 10q24.3 breakpoint region. The second breakpoint on chromosome 11q23.3 lies in relatively close proximity to two other non-syndromic deafness genes, RDX (DFNB24) (19) and TECTA (DFNA8/12 and DFNB21) (20,21), although the >5 Mb distance makes a position effect on RDX and/or TECTA expression unlikely. The Usher syndrome genes CDH23 (USH1D) and PCDH15 (USH1F) lie 29.5 and 46.5 Mb, respectively, proximal to the 10q24.3 breakpoint, whereas MYO7A (USH1B) and USH1C lie 38.7 and 97.8 Mb proximal to the 11q23.3 breakpoint (http://webh01.ua.ac.be/ lhh). Expression of some genes can be influenced by
regulatory elements well outside the transcription and promoter regions (26). In a number of human developmental disorders, including X-linked deafness (misregulation of POU3F4) (13), holoprosencephaly (SHH) (27), preaxial polydactyly (SHH) (28), campomelic dysplasia (SOX9) (29,30) and Pierre–Robin sequence (SOX9 and KCNJ2) (31), it has been shown that the distance of the disease-causing chromosome breakpoint and/or microdeletion to the 3' or 5' end of the misregulated gene can be over 1 Mb. However, so far there is no evidence for position effects at >2 Mb distance from the breakpoint region.

Although this may be a chance coincidence, it is tempting to speculate that the localization of essential genes for the function of inner ear hair cell stereocilia at both breakpoint regions, PDZD7 at 10q24.3 and RDX and TECTA within a 5 Mb interval at 11q23.3, is the consequence of higher-order nuclear organization. The 'contact-first' hypothesis of the formation of chromosome rearrangements assumes that DNA repair joins ends from different DNA damage-induced double-strand breaks that were closely juxtaposed in the interphase nucleus at the time when the DNA damaging event occurred (32,33). There is a highly dynamic nuclear architecture that may have a function in the regulation of gene expression (34).

Genes on different chromosomes, i.e. PDZD7, RDX and TECTA, that are co-ordinately expressed/regulated and work together in a single biological module may be non-randomly physically associated in the nucleus (35), facilitating DNA damage-induced illegitimate recombination events.

Homogeneity for translocations is very rare. Most published cases are homogeneous Robertsonian translocations with \(2n = 44\) chromosomes, including rob(13;14), rob(13;14) (36,37) and rob(14;21), rob(14;21) (38,39). There are four reports on homozygous reciprocal translocations, namely, der(22)t(Y;22), der(22)t(Y;22) (40), t(3;16), t(3;16) (41), t(17;20), t(17;20) (42) and t(7;12), t(7;12) (43), all in the offspring of consanguineous parents. The phenotype of cortical lissencephaly with cerebellar hypoplasia in two siblings with t(7;12), t(7;12) is explained by the homozygous disruption of the reelin gene at 7q22.1. This clearly demonstrates that rare homozygous translocations can be used to identify recessive disease gene mutations.

Several lines of evidence suggest that biallelic inactivation of PDZD7 can cause non-syndromic hearing impairment. In our index patient suffering from congenital SNHL, both PDZD7 alleles are disrupted by homozygous reciprocal translocation, whereas heterozygous translocation carriers either hear normally or display only mild hearing impairment as adults. Although we cannot totally exclude that environmental factors are responsible for the observed mild hearing impairment of the parents, it is well possible that PDZD7 haplinsufficiency predisposes to adulthood-onset or old age hearing impairment. The chromosome 10q24.3 breakpoint creates a loss of function mutation. One of the disrupted PDZD7 isoforms is structurally and functionally related to the PDZ domain-containing scaffold proteins harmonin and whirin, which play a central role in the Usher syndrome protein network. Genetically heterogeneous diseases such as Usher syndrome and Fanconi anemia provide good examples that mutations in functionally related genes, i.e. genes in the same multiprotein complex and/or biochemical pathway, lead to the same or similar phenotypes (6,44,45).

Pull-down experiments support the idea of an integration of PDZD7 into the protein interactome related to Usher syndrome, indicating that PDZD7 is a prime candidate for human Usher syndrome. Examination of our index patient did not show any signs of retinitis pigmentosa or vestibular dysfunction at the age of 8 years. Although we cannot exclude the possibility that he will develop additional symptoms later in life, it is more likely that homozygous disruption of intron 10 of PDZD7 in our patient causes non-syndromic hearing impairment. Similar to harmonin (USH1C) and whirin (DFNB31), more severe mutations in PDZD7 (interfering with all four known transcripts) may cause Usher syndrome. Nevertheless, the clinical phenotype (non-syndromic SNHL) caused by biallelic inactivation of PDZD7 is consistent with its relation with harmonin and whirin at the gene and protein levels.

Both scaffold proteins harmonin and whirin strongly contribute to the organization of protein networks, integrating the function of all other Usher syndrome proteins (6,22–25,46–48). In this Usher syndrome protein interactome, both proteins directly bind with their PDZ domains the motor protein myosin VIIa (USH1B), the Usher cadherins, cadherin

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**Figure 6.** Amino acid sequence similarity of PDZD7 with whirlin and harmo- nin. Comparison of the PDZ domain containing isoforms PDZD7-A (A) and PDZD7-D (B) with whirlin and harmonin. The arrows between two given PDZ domains indicate the percentage of amino acid sequence similarity.
23 (USH1D) and procadherin 15 (USH1F), the scaffold protein SANS (USH1G), the transmembrane protein USH2A and the very large G protein-coupled receptor, VLGR1b/GRP98 (USH2C). In the inner ear, this multiprotein network is assembled mainly in stereocilia and synaptic regions of hair cells. There Usher syndrome protein networks are essential for the development of stereocilia, the synaptic function and the mechano-electric transduction (6,7). In retinal photoreceptor cell networks, Usher syndrome proteins are found at synapses in the periciliary region, indicating a close relation to other ciliopathies (7,25). Based on the characteristics of PDZD7, its expression in the inner ear and interaction of the PDZD7 protein with SANS (USH1G) and with harmonin (USH1C) from retinal origin, we conclude that it is another PDZ domain-containing partner in the Usher interactome, which may participate in the development of stereocilia in hair cells and in ciliary functions in retinal photoreceptor cells. Certainly, PDZD7 is a very good candidate gene for a novel human Usher syndrome locus.

MATERIALS AND METHODS

Cytogenetic and molecular cytogenetic analyses

Metaphases were prepared from short-term phytohemagglutinin-stimulated lymphocyte cultures and analysed with classic GTG banding at the 500 band level. Region-specific BAC clones for FISH mapping were selected from the Wellcome Trust Sanger Institute Ensembl contigs (http://www.ensembl.org) and the UCSC Genome Browser (http://genome.ucsc.edu) (Supplementary Material, Table S1). Genomic BAC DNAs were labeled with fluorescein-12-dUTP or tetramethyl-rhodamine-5-dUTP (Roche Diagnostics, Mannheim, Germany) by standard nick translation. FISH was

Figure 7. Insertion–deletion polymorphism in exon 14 of PDZD7. (A) Nucleotide sequence alignment of human wild-type PDZD7 exon 14 and the identified insertion polymorphism with the chimpanzee, orangutan, rhesus macaque, bushbaby and mouse genomic reference sequences. (B) Amino acid sequence alignment of human isoform PDZD7-D, human insertion polymorphism, chimpanzee, orangutan, rhesus macaque, bushbaby and mouse.

Figure 8. Validation of the PDZD7 integration into the Usher syndrome protein network. (A) Validation of the SANS–PDZD7 interaction by GST pull-down analyses. The lysate of HEK293T cells transiently transfected with 3xFLAG-SANS (lane 1, 10% of input) was incubated with GST-PDZD7 (lane 2) or GST alone (lane 3). Anti-FLAG western blot analysis revealed a prominent band at ~57 kDa, resembling the ascertained molecular weight of 3xFLAG-SANS (arrow), pulled down by GST-PDZD7. In contrast, a very fade band was observed in pull downs with GST alone. (B) PDZD7 interaction with harmonin in the mouse retina validated by GST pull-down assay. Murine retina extract (lane 1, 15% of input) was incubated with immobilized full GST-PDZD7 (lane 2), GST (lane 3) or sepharose beads alone (lane 4). Anti-harmonin revealed a band of ~72 kDa (lane 2), the ascertained molecular weight of the abundant splice variant harmonin a1 (arrow), pulled down by GST-PDZD7. A 4-fold weaker band is detected in the control incubated with GST alone (lane 3). No signal was observed in the sepharose bead control (lane 4). For quantification, intensities of the 72 kDa band were assigned by an Odyssey infrared imaging system: lane 1, 0.36; lane 2, 3.95; lane 3, 0.99.
performed on the patient’s metaphase spreads, as described previously (14,49).

PCR analyses

All PCRs were performed according to standard protocols. The reaction mixture consisted of 2.5 μl 10× PCR buffer, 2.5 μl 50 mM MgCl₂, 2.5 μl 10 mM dNTP mix, 1.0 μl (100 ng) of each forward and reverse primer, 0.5 μl (2.5 U) Taq polymerase, 14 μl PCR-grade water and 100 ng template DNA. PCR amplifications were carried out with an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 30 s, primer-specific annealing temperature for 30 s, 72°C for 60 s and a final extension step at 72°C for 10 min. In order to amplify larger (~5 kb) fragments from the 10q24.3 breakpoint region, the Expand Long Template PCR System (Roche Diagnostics) was used according to the recommendations of the manufacturer with a series of primer pairs (Supplementary Material, Table S2) chosen from the genomic sequence of breakpoint-spanning BAC clone RP11-108L7. The PCR reaction contained 10 μl 5× Expand Long Range Buffer with 12.5 mM MgCl₂, 2.4 μl nucleotide mix, 1.5 μl (150 ng) each of forward primer and reverse primer, 0.7 μl (3.5 U) Expand Long Range Enzyme mix, 100 ng template DNA and 33 μl water. Annealing temperatures and elongation times were optimized for each primer pair.

Vector ligation

A new vector ligation technique was used to clone a junction fragment of the t(10;11)(q24.3;23.3) translocation. Genomic DNA of the index patient with homozygous translocation, of his brother with heterozygous translocation and a control person with normal karyotype was digested with PstI. This restriction enzyme was chosen because it cuts nearby but not within the chromosome 10q24.3 breakpoint-containing 564 bp segment. Because there is a PstI restriction site approximately every 1000 bp in the human genome sequence, the PstI-digested junction fragment can be expected to be 1000–2000 bp in size. Two micrograms of genomic DNA each and 2.0 μl pBluescriptII phagemid vector DNA were digested in a 40 μl reaction containing 1.5 μl PstI (New England Biolabs, Frankfurt/Main, Germany), 4 μl 10× NEBuffer 3 and PCR-grade water. Following incubation at 37°C overnight, the enzyme was inactivated at 80°C for 20 min. To remove 5′-phosphate residues, the plasmid vector was treated with calf intestinal alkaline phosphatase (New England Biolabs). Digested genomic DNA and vector DNA were ligated together at 16°C overnight using 1 μl plasmid vector, 10 μl (~500 ng) target DNA, 12.5 μl T4 DNA ligase 2× rapid ligation buffer and 1.5 μl T4 DNA ligase (Promega, Mannheim, Germany). The reaction was stopped by incubation at 75°C for 20 min. The ligation products were cleaned with DNA Clean & Concentrator 5 (Zymo Research, Orange, CA, USA). The pBluescriptII vector contains T7 and M13 primer-binding sites. Different combinations of vector and insert (breakpoint-spanning fragment) primers (Supplementary Material, Table S2) were used to amplify a junction fragment from the plasmid library.

DNA sequencing

Following Exo/SAP digestion, dye terminator cycle sequencing of the PCR products was carried out using the CEQ DTCS Quick Start Kit (Beckman Coulter, Krefeld, Germany). Sequencing products were separated on a Beckman Coulter CEQ 8000 Genetic Analysis System. The BLAST program (http://www.ensembl.org/Homo_sapiens/blastview and http://www.ncbi.nlm.nih.gov/BLAST) was used to analyze the sequencing data.

Expression analysis

Total RNAs were extracted from adult inner ear and cortex using TRIZOL reagent (Invitrogen, Karsruhe, Germany). Tissue samples were obtained from male persons between 20 and 45 years within 2 days post-mortem. The samples were frozen immediately after dissection and stored in liquid nitrogen. RNA quality and quantity were determined with a NanoDrop spectrophotometer (Peglab, Erlangen, Germany). cDNA was synthesized by reverse transcription of total RNA with SuperscriptIII transcriptase (Invitrogen), using the Invitrogen protocol for first strand synthesis. Two microliters of this cDNA was then used as a template in a PCR reaction with primers spanning PDZD7 exons 6 and 7 (Supplementary Material, Table S2).

GST pull-down assays

Constructs encoding full-length murine Pdzd7 cDNA (FANTOM 3 clone 9130207N01, imaGenes) were cloned into pDEST15 vector (Gateway Cloning System, Invitrogen). GST-fusion proteins were generated by transforming Escherichia coli BL21AI cells with pDEST15-Pdzd7. Cells were incubated at 30°C with 0.5 mM isopropyl-β-D-thiogalactopyranoside overnight and subsequently lysed with STE buffer [1% Sarkosyl, 1% Triton X-100, 5 mM dithiothreitol (DTT)], supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Lysates were incubated with glutathione–sepharose 4B beads (Amersham Biosciences, Freiburg, Germany). The GST-fusion proteins bound to the beads were washed with lysis buffer and TBSTD (TBS with 1% Triton X-100 and 2 mM DTT). The amount of bound GST-fusion protein was verified on a NUPAGE Novex 4–12% bis–tris sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) gel and stained with Simply Blue Safe Stain (Invitrogen). FLAG-tagged human SANS full-length protein was produced by transfection of HEK293T cells with the appropriate vectors, using a combination of Lipofectamine LTX and PLUS reagent (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection cells were washed with phosphate-buffered saline and subsequently lysed on ice in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100). Retina lysate of mature C57BL/6J mice was prepared in HNTG buffer (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 6 mM EDTA, 10% glycerol, pH 7.4). Cell extracts or retinal extracts were incubated overnight at 4°C with equal amounts of beads preincubated either with GST or with GST fusion protein.
Beads were washed, and precipitated protein complexes were eluted with SDS sample buffer and subjected to SDS–PAGE and western blot analysis.

**Western blot analyses**

For western blot analyses, samples of GST pull-downs were mixed with SDS–PAGE sample Laemmlli buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, 1 mM EDTA and 0.025 mM Bromphenol blue) and separated on 12% polyacrylamide gels. Subsequently, separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany) on a semidry blotter (BioRad Laboratories, Munich, Germany). As a molecular marker, a pre-stained ladder (Sigma-Aldrich) was used, ranging from 11 to 170 kDa.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement**. None declared.

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