Next-Generation Sequencing Identifies Mutations of *SMPX*, which Encodes the Small Muscle Protein, X-Linked, as a Cause of Progressive Hearing Impairment

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In a Dutch family with an X-linked postlingual progressive hearing impairment, a critical linkage interval was determined to span a region of 12.9 Mb flanked by the markers DXS7108 and DXS7110. This interval overlaps with the previously described DFNX4 locus and contains 75 annotated genes. Subsequent next-generation sequencing (NGS) detected one variant within the linkage interval, a nonsense mutation in *SMPX*. *SMPX* encodes the small muscle protein, X-linked (SMPX). Further screening was performed on 26 index patients from small families for which X-linked inheritance of nonsyndromic hearing impairment (NSHI) was not excluded. We detected a frameshift mutation in *SMPX* in one of the patients. Segregation analysis of both mutations in the families in whom they were found revealed that the mutations cosegregated with hearing impairment. Although we show that *SMPX* is expressed in many different organs, including the human inner ear, no obvious symptoms other than hearing impairment were observed in the patients. *SMPX* had previously been demonstrated to be specifically expressed in striated muscle and, therefore, seemed an unlikely candidate gene for hearing impairment. We hypothesize that SMPX functions in inner ear development and/or maintenance in the IGF-1 pathway, the integrin pathway through Rac1, or both.

Hereditary nonsyndromic hearing impairment (NSHI) is genetically extremely heterogeneous, as is illustrated by the currently associated genes, numbering more than 50, and the large number of loci for which the gene harboring the causative mutation(s) is still elusive (Hereditary Hearing Loss Homepage).[1] This hampers DNA diagnostics and adequate mutation-based genetic counseling. Inheritance patterns of monogenic NSHI can be (in order of decreasing prevalence) autosomal recessive, autosomal dominant, X-linked, or mitochondrial, and digenic inheritance has also been indicated.[2,3] Age-related hearing loss is a complex disorder, although variants in genes involved in monogenic forms of NSHI have been found to be among the genetic factors.[4] Genes in which variation is associated with deafness have a wide variety of functions and have contributed significantly to our understanding of the molecular biology of hearing.[1,5] Because of this functional diversity, bioinformatic tools such as ENDEAVOUR or Prospectr have been of limited value in the positional cloning of deafness genes.[6] Currently, next-generation sequencing (NGS) is an excellent strategy for identification of disease-causing variants.[7,8] In the present study, we identified mutations in the gene encoding the small muscle protein, X-linked (SMPX [MIM 300226]) as a cause of nonsyndromic hearing impairment by using a two-step strategy of linkage analysis and NGS.

This study was approved by the medical ethics committee of the Radboud University Nijmegen Medical Centre and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects or, in case of children, from their parents. Affected subjects of a large, five-generation family (W08-1701) of Dutch origin presented with postlingual progressive hearing impairment (Figure 1). An X-linked pattern of inheritance was suggested by the absence of male-to-male transmission and the fact that hearing impairment developed earlier and was more severe in men than in women. The majority of affected family members reported bilateral, (slowly) progressive hearing impairment. Pure-tone audiometry and otoscopy were performed for all depicted individuals by standard procedures. There was no evidence for nongenetic causes of hearing impairment except for individual III.3, who reported noise exposure. Also, hearing impairment in this individual was less severe and had a late onset at about age 60. The reported age at which hearing impairment was first noticed was 2–10 years old for men (with a mean of 3.3 years old) and 3–48 years old for women (with a mean of 28.2 years old). In males, the largest increase of threshold values occurred in the first two decades, and progression to profound hearing impairment was already seen in the second decade in one of the

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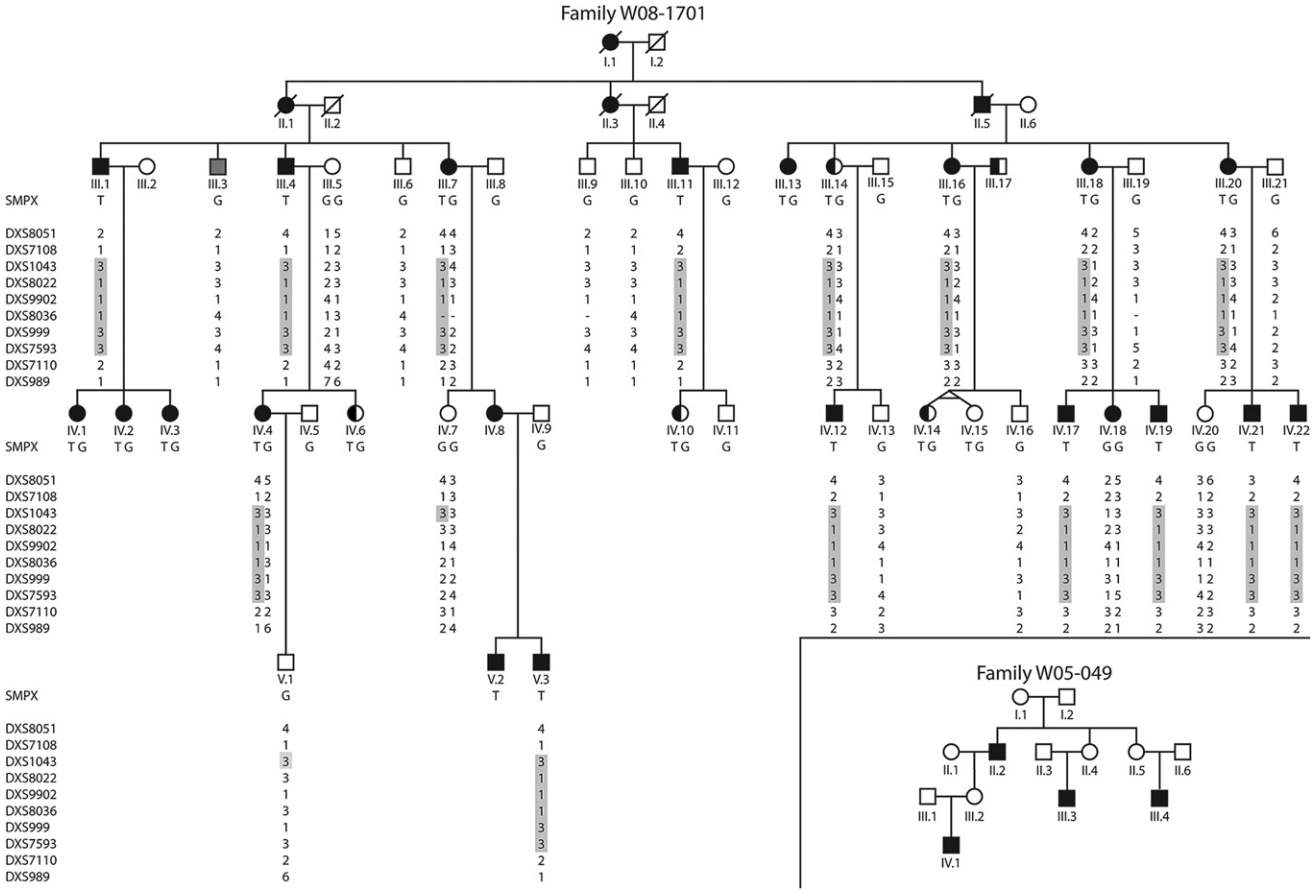

Figure 1. Pedigrees and Genetic Analyses
Only those family members of the large pedigree who were relevant for the study are depicted. The haplotype associated with the hearing impairment is indicated by the gray bar. In individual V.1, allele 3 of marker DXS1043 might be derived from the affected haplotype. The segregation of the c.214G>T is presented above the haplotypes. Family W08-1701 is of Dutch origin and family W05-049 is of Netherlands Antilles' origin. The following symbols are used: black squares, affected males; black circles, affected females; half-shaded circles, females with unilateral hearing impairment; half-shaded square, male with unilateral hearing impairment; gray shaded square, male with hearing impairment less severe than the other affected males.

affected males (Figure 2). Hearing impairment in women exhibited a large interindividual variation with regard to the severity and also with regard to interaural variation (Figure 2). Brainstem Evoked Response Audiometry (BERA) for the proband, individual V.2, revealed normal waveform responses at an intensity level of 45 dB. There was no indication of conductive hearing impairment. Furthermore, pure-tone audiometry never revealed a persistent air-bone gap or pseudoconductive hearing impairment in any of the affected family members (Figure S2, available online). A more detailed description of the audiometric evaluation of the family will be reported elsewhere.

Genetic studies in this family were initiated by linkage analysis for the known X-chromosomal NSHI loci, recently renamed DFNX1-5 (Hereditary Hearing Loss Homepage).[9–13] Twenty-eight individuals from this family were genotyped for microsatellite markers from the loci DFNX1 (MIM 304500), DFNX3 (MIM 300030), and DFNX4 (MIM 300066) (Table S1). After exclusion of DFNX1 and DFNX3, evidence of linkage with the disease was found for marker DXS8022, derived from the DFNX4 locus that was previously described as DFN6 for a family with similar audiometric features.[12] Subsequent genotyping of nine additional markers defined a critical region of 12.9 Mb flanked by the markers DXS7108 and DXS7110 (Figure 1). In this region, chrX:10,192,226-23,111,851, 75 genes have been annotated (UCSC Genome Browser, hg19). We calculated two-point LOD scores with SuperLink version 1.6 in the Easy-Linkage software package by using the genotypes of males only.[14,15] Penetrance was assumed to be 99%, and a disease allele frequency of 0.001 was employed for the calculations. Individual III.3 was included in the calculations as an individual with an unknown phenotype. A significant maximum LOD score of 4.87 ($\theta = 0.000$) was obtained for marker DXS8022; LOD scores are presented in Table S2.

Three candidate genes, *PRPS2* (MIM 311860), *SHROOM2* (MIM 300103), and *GPM6B* (MIM 300051), were selected for a mutation search by conventional Sanger sequencing because of homology with a known deafness gene or expression in the inner ear, but no pathogenic variant was identified. Subsequently, we performed targeted enrichment by using the Agilent SureSelect Human

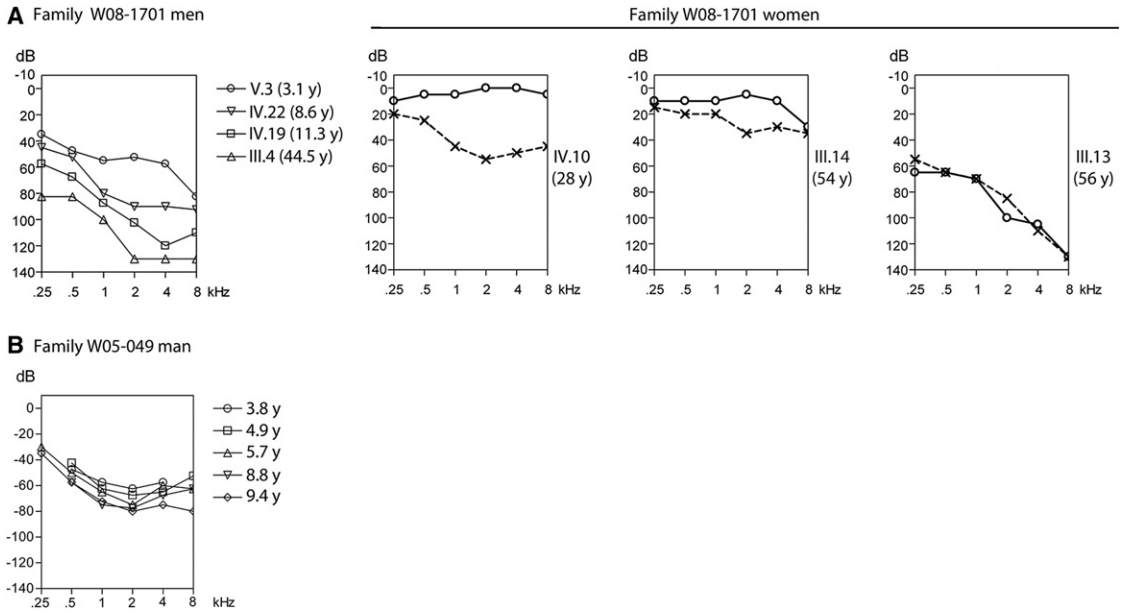

Figure 2. Audiometric Characteristics of the Families
(A) Representative audiograms (air conduction) of affected men (showing the means of thresholds of the left and right ears) and women (thresholds of left (x) and right ear (o) shown separately) of family W08-1701 demonstrate progressive hearing loss in males within the first two decades and the variability of the hearing loss in females.
(B) Representative audiograms of individual IV.1 from family W05-049 at different ages (means of thresholds of the left and right ears are shown). Pure-tone audiometry was performed in a sound-treated room according to current clinical standards. y is an abbreviation for years.

X Chromosome Kit and single-read 76 nt NGS on the Illumina GAII sequencer for individual III.4.[16] In total, 28,363,277 reads were obtained, of which 23,339,533 could be mapped, and 95.1% of the targeted bases were covered at least 10-fold. After analysis of the sequence data with in-house-developed tools and filtering of the predicted sequence variants against dbSNP, the 1000 Genome Project, and 200 Danish control individuals,[17] two variants remained, chrX:117960412T>G and chrX:21755734C>A (base-pair positions according to the NCBI37/hg19 assembly of the human genome), and only the latter was located within the critical region. This variant, c.214G>T, is located in exon 4 of *SMPX* (NM_014332.1) and introduces a premature stop codon predicted to result in a truncated protein after residue 71 (p.Glu72X). The presence of this candidate disease-causing variant was confirmed by Sanger sequencing in the affected males III.1, III.4, and V.2 and a female carrier, IV.10 (Figure 3).

The c.214G>T transversion removes a restriction site for *Hpy188I*. Therefore, we performed restriction digestion of exon 4 amplicons according to the manufacturer's protocol (New England Biolabs) to test all family members and ethnically matched controls for the presence of the mutation. DNA fragments were analyzed on 2% agarose gels (Figure S1). None of the 172 control individuals carried the mutation, whereas in the family, the mutation was found to fully cosegregate with hearing impairment in males, as expected from the linkage analysis, and individual III.3 indeed did not carry the mutation (Figure 1). Therefore, his hearing impairment might well be caused by the reported noise exposure. For females the mutation also coincides with the mutation-carrying haplotype, indicating that individual IV.18 is either a phenocopy or a genocopy. She has a mild increase in thresholds in the pure-tone audiogram for the frequencies 0.25–1 kHz. On the other hand, individual IV.15 shows no signs of hearing impairment but does carry the mutation as does her twin sister (monozygotic). The latter exhibits unilateral hearing impairment. Both sisters were 25 years old at the last visit in the clinic, and because the age at onset for females from this family is variable (3–48 years), individual IV.15 could well develop hearing impairment in the coming years.

To investigate the involvement of *SMPX* in other families with hearing impairment, we performed Sanger sequencing of the three protein-coding exons (2–4) and the flanking intronic sequences in 26 index patients of small families for which X-linked inheritance of NSHI was not excluded. Sequence analysis was performed as described,[18] and primer sequences and conditions for PCR amplification are provided in Table S3. In one of the index patients (individual VI.1 of family W05-049, Figure 1), a deletion of a single base pair, c.130delG, was found in exon 3. This variant leads to a frameshift and a premature stop codon, p.Glu44ArgfsX37. The patient's mother (III.2 in Figure 1) was found to carry the deletion as well. No DNA samples from other family members were available for testing. The mutation was not detected in 129 Dutch control individuals who are not ethnicity matched because the family is of Netherlands Antilles' origin. Audiograms of the index patient are presented in

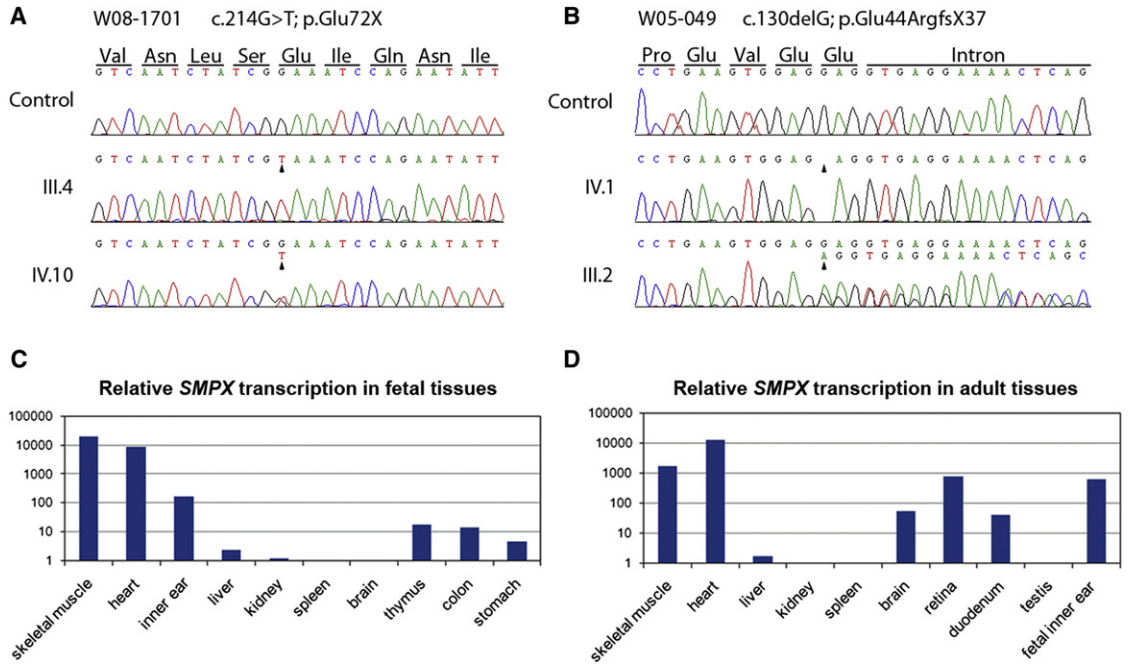

Figure 3. Mutation and Expression Analysis of *SMPX*

(A and B) Partial *SMPX* sequence chromatograms are shown for normal controls (upper panels), affected males (middle panels), and female carriers (bottom panels). The predicted amino acid changes and the surrounding amino acids are indicated above the sequence. Mutated nucleotides are marked by an arrowhead. As a reference, we employed sequence NM_014332.1 by using the first ATG translation initiation codon for numbering of the nucleotide change.

(C and D) Relative *SMPX* mRNA expression as determined by quantitative PCR in fetal (C) and adult (D) human tissues. Because this was performed for adult and fetal tissues in two separate experiments, fetal inner ear was included in both for comparison.

Figure 2B. Hearing impairment was first suspected around the age of 4 and has progressed since then (Figure 2). No air-bone gap was detected (Figure S2), and BERA revealed normal waveform responses at 65 dB. The proband's mother (III.2 in Figure 1) did not report any signs of hearing impairment at her last visit at clinic at the age of 38. Also, no hearing impairment was reported for obligate female carriers in the previous generations. In an independent study of two additional families with X-linked hearing impairment, two different truncating mutations have been detected in *SMPX*. The family in which the DFNX4 locus was determined was one of these.[12] The results are presented in the accompanying paper by Huebner et al. in this issue.[19]

Because of its high and preferential expression in striated muscle, *SMPX* was not an obvious candidate for NSHI.[20,21] As a first step to identify a function of SMPX in the inner ear, we analyzed *SMPX* transcription by RT-PCR on RNA isolated from human inner ear of an embryo at 8 weeks gestation. Indeed, *SMPX* mRNA could easily be amplified and sequence verified (data not shown). Further evidence for *SMPX* expression in the inner ear is provided by RNA in situ hybridization in the mouse embryos at 14.5 days of gestation (Eurexpress database assay 006968 and Genepaint assay DC27). In addition to being present in developing muscle, Smpx transcripts are present in a region that presumably corresponds to the developing sensory epithelium of the vestibular organs. Immunohistochemistry with an Smpx antibody on sections of an adult mouse's organ of Corti revealed staining in different cell types, including Böttcher cells, root cells, pillar cells, and interdental cells of the limbus spiralis. Low levels of staining were detected in hair cells.[19] Transcription of *SMPX* was further addressed by quantitative PCR (qPCR) on cDNA derived from various fetal and adult human tissues as described previously (Table S3).[18] In Figure 3 the relative amounts of *SMPX* transcripts in the tissues as compared to that in the spleen (set to 1) are depicted. The housekeeping gene *GUSB* (MIM 611499) was used as a reference gene. *SMPX* mRNA levels were highest in both fetal and adult skeletal muscle and heart, which is in agreement with previous studies.[20,22] No transcripts were detected in fetal brain or in adult kidney and spleen tissues. Importantly, relatively high *SMPX* transcript levels are detected in fetal inner ears, which supports the involvement of *SMPX* in X-linked NSHI. Retinas derived from adult humans also exhibits a relatively high level of *SMPX* transcripts. Despite the indications for significant expression levels of *SMPX* in heart skeletal muscles and retinas, no clear symptoms indicating an adverse effect of a truncating *SMPX* mutation in these tissues are reported by affected individuals from family W08-1701. Although one of the males reported mild muscle injury upon heavy exercise, a causative link to a defective SMPX remains to be determined by detailed testing of muscle function. No information is available for family W05-049. For heart and skeletal

muscle, functional redundancy was already indicated by studies of a conditional knock-out allele of *Smpx* in mice.[22] This conditional knockout allele appeared to be null because immunoblot analysis revealed no detectable Smpx protein. However, the knockout mice displayed no overt developmental or structural deficits in their skeletal muscles or hearts, suggesting a genetic or functional redundancy.

Smpx, previously also called Csl, is proline-rich and was described to contain a nuclear localization signal, two casein kinase II phosphorylation sites, and a proline, glutamic acid, serine, and threonine-rich (PEST) sequence that suggests Smpx undergoes rapid degradation.[20] From late-fetal to neonatal stages of murine cardiac- and skeletal-muscle development onward, Smpx becomes associated with the costameres.[22] Costameres are subsarcolemmal protein assemblies at the sarcomere-sarcolemma attachment sites.[23] Three actin-associated costameric protein complexes have been distinguished: the focal adhesion-type complexes, the spectrin-based complex, and the dystrophin-based complex, all of which tether molecules that control, among other processes, mechanoreception and cytoskeletal remodeling.[24] Smpx is likely to be part of the actin-associated complex because it was found, upon expression in mouse myoblasts, to influence actin turnover and to induce lamellipodia in a Rac1-dependent manner.[22,25] Furthermore, Smpx colocalizes with focal adhesion proteins at the membrane of these lamellipodia, suggesting a link to integrin signaling.[25] Interestingly, both Rac1 and integrins ($\alpha 8\beta 1$) are essential for normal cochlear development and function.[26,27] Conditional inactivation of *Rac1* (MIM 602048) leads to a shortened cochlea and abnormal cellular organization of the sensory epithelium. Furthermore, planar cell polarity of cochlear hair cells and the morphogenesis of the hair bundle are affected.[26] Integrin (type $\alpha 8\beta 1$) was found to be essential for normal hair-bundle development and/or maintenance and colocalizes with its ligand fibronectin and the integrin-regulated focal adhesion kinase in the apical region of developing vestibular hair cells.[27] On the basis of all these data, we hypothesize that Smpx functions in the development and/or maintenance of the sensory hair cells.

A second link between SMPX and cochlea development and function is provided by IGF-1 (MIM 147440). Smpx modifies cell shape and promotes myocyte fusion when expressed in C2C12 mouse myogenic cells in an IGF-1 dependent manner.[22] *Igf-1*-deficient mice have multiple cochlear abnormalities, including an abnormal differentiation, a reduced survival of spiral ganglia neurons, and an abnormal tectorial membrane.[28,29] *MEF2* (MIM 600663) has been indicated to be a target gene of IGF-1 in the mouse cochlea in both the sensory cells and the spiral ganglia neurons. Interestingly, the IGF-1-mediated increase of MEF2 activity in myoblasts is augmented by Smpx.[22] Furthermore, the consensus sequence for MEF2 binding is present twice in the highly conserved 5′ upstream region of *SMPX*.[20] *IGF1* mutations in humans cause syndromic, severe to profound, and congenital or very early-onset sensorineural hearing impairment (MIM 608747).[30–32] This inner ear phenotype is more severe than that in the families included in this study.

In-depth studies are required for the discernment of which cell types and pathways in the cochlea are affected by mutations in *SMPX*. Fast deterioration of hearing in the first decades of life, as seen in family W08-1701, has been reported previously for patients with mutations in a number of genes, including *ACTG1* (MIM 102560) encoding the cytoskeletal γ-1-actin.[33,34] Interestingly, this is thought to be the major cytoskeletal actin in costameres.[35]

In conclusion, this study identifies *SMPX* as a gene in which variation is associated with X-linked deafness and illustrates that NGS is instrumental in the efficient identification of disease-causing variants in unexpected genes. Our results will contribute to adequate mutation-based genetic counseling of patients and their relatives. Because females can be affected, although generally not in childhood, *SMPX* should also be considered in small pedigrees with dominantly inherited hearing impairment and those pedigrees in which X-linked inheritance cannot be excluded. Further analysis of the function of SMPX will provide insights into inner ear development and function, and SMPX might well be a player in the regulation of stereocilia development and/or maintenance.

Supplemental Data

Supplemental Data include two figures and three tables and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genome Project, http://www.1000genomes.org/
Eurexpress, A Transcriptome Atlas Database for Mouse Embryo, http://www.eurexpress.org/ee/
Genepaint, a Digital Atlas of Gene Expression Patterns in the Mouse, http://www.genepaint.org

Hereditary Hearing Loss Homepage, http://hereditaryhearingloss.org/

Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim

UCSC Human Genome Browser, Build hg19, March 2006, http://www.genome.ucsc.edu

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