A Point Mutation in the Gene for Asparagine-Linked Glycosylation 10B (Alg10b) Causes Nonsyndromic Hearing Impairment in Mice (Mus musculus)

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Introduction

Congenital hearing impairment in the human (Homo sapiens) population has a newborn prevalence of 0.1–0.3% in live births in the United States [1]. Previously, about half of these cases in the developed world were believed to be genetic, while the remaining half were thought to be entirely due to other causes, such as congenital infections [2]. More recent research has suggested that genetic factors may play a role in susceptibility to hearing impairment even when the primary cause is non-genetic, such as congenital cytomegalovirus (CMV) infection [3].

Mutations in the gene for connexin-26 (GJB2, OMIM *120101) are the most common genetic causes of congenital hearing impairment (OMIM #220290) and account for up to half of the genetic cases in some populations. However, more than 120 additional human hearing loss loci have been reported, and the responsible genes have been identified for only about half of these [2,4,5].

Current treatment options for genetic hearing impairment are largely limited to hearing aids or cochlear implants [6]. Animal studies have demonstrated that adenoviral vectors can be introduced into the inner ear to regenerate lost sensorineural cells [7] and to transfer gene expression constructs to cochlear tissue [8]. An adeno-associated virus type 1 vector has recently been used to restore hearing to congenitally-deaf mice with vesicular glutamate transporter-3 deficiency (official gene symbol Slc17a8, MGI: 309629) [9]. In addition, a single systemic dose of an antisense oligonucleotide (ASO) in neonatal mice with an Ush1c (MGI: 1919338) mutation has recently been shown to rescue the mutant phenotype of deafness and blindness in these mice [10].
These studies suggest that gene-specific therapies are a viable strategy for treating genetic hearing impairment. However, before therapy can be performed, the underlying genetic defect in each individual case must be identified. Thus, a search for the remaining unknown genes responsible for hearing impairment is warranted.

Mouse mutants have long been useful tools for the identification of human hearing loss genes and for the study of the physiology of hearing [11–14]. The study of human hearing loss genes is complicated by locus heterogeneity, genetic background effects, small pedigree size, selective mating, and difficulty in performing physiological and histological studies. Each of these issues can be avoided in the mouse. Furthermore, mice can be intentionally mutagenized and screened for hearing impairment in order to generate novel mouse models of human hearing deficits [15]. Indeed, chemical mutagenesis has successfully produced a number of mouse mutants with mutations in the gene for chromodomain helicase DNA-binding protein-7 (Chd7, MGI: 2671810) mouse mutation arose in the progeny of a C57BL/6J male mouse that had been treated with the chemical supermutagen N-ethyl-N-nitrosourea (ENU). Homozygous nse5/nse5 mutant animals have a severely reduced or absent startle response to sound at weaning (3 weeks of age) and virtually no response to sound at 6 weeks of age. While many mouse hearing impairment mutants also have associated head-tossing or circling behaviors (presumably secondary to associated vestibular defects), nse5 homozygotes behave normally, implying that the mutation affects the cochlea but spares the vestibular apparatus. Mutant animals are otherwise completely normal on examination and have normal complete blood counts and blood chemistries, suggesting that the hearing impairment is nonsyndromic. A genome scan previously mapped the responsible locus to mouse chromosome 15 in a region where there were no reported hearing impairment genes, indicating that nse5 represents a novel locus for hearing impairment [18].

We report here the fine-mapping of the nse5 mutation, the identification of a leucine-serine mutation in the Alg10b gene in mutant animals, rescue of the mutant phenotype with a wildtype Alg10b transgene, and physiologic and histologic analysis of wildtype, mutant, and transgenic animals. The ALG10B protein is involved in the process of asparagine (N)-linked glycosylation. This is the first report of a mutation in this pathway resulting in nonsyndromic hearing impairment in either the mouse or in humans, and it suggests that this pathway is much more important than previously known in the establishment and maintenance of normal hearing.

**Results**

**The nse5 Mutation Maps to a 524 Kilobase (kb) Region of Mouse Chromosome 15**

Analysis of 200 N10F1 and 300 N11F1 mouse progeny (and one additional N10 mouse that was found to have a critical recombination breakpoint) localized the nse5 locus to a nonrecombinant interval between the markers D15Mit171 and rs6260777 on mouse chromosome 15 (Figure 1A). One nonrecombinant animal was initially scored as “Hearing” but was reclassified as “Deaf” when re-examined one week later. Another animal with a recombination between D15Mit241 and rs3385577 had a persistent but very subtle startle response on click-box testing. This animal was ultimately classified as “Deaf”. These two animals demonstrate the variable expressivity of the phenotype, which is a common feature of ENU-induced mutations. Given this finding, the two animals carrying the key recombinants were both backcrossed to nse5/nse5 animals, and at least five animals that inherited the recombinant chromosome were phenotyped, in order to guard against mistyping a critical animal.

Review of the published genomic sequence of this region with the UCSC Genome Browser revealed that it is 324 kb in length and contains only two RefSeq genes, Alg10b and the 3’ end of the Copeø gene (Figure 1B) [19,20].

**A Missense Mutation in Alg10b Is Present in nse5 Mutant Mice**

Amplification and sequencing of the three coding exons and the 5’-untranslated region (UTR) of the gene for asparagine-linked glycosylation 10B (Alg10b) from an nse5/nse5 homozygous mutant animal revealed a single point mutation in this gene that was not present in the reference sequence. Analysis of DNA from both C57BL/6J and 129S6/SvEvTac inbred animals revealed that this mutation is not present in either strain (Figure 2A). The mutation converts a highly-conserved leucine residue to a serine residue in mutant animals [p.Leu389Ser, based on reference sequence NP_001028613.1, conservation shown in Figure 2B]. Leucine is a bulky, relatively non-polar amino acid, while serine is small and polar, due its hydroxyl group (Figure 2C). Analysis of this protein change with Poly-Phen2 v2.2.2r398 predicted that this change is probably damaging (score: 0.998; sensitivity: 0.27; specificity 0.99) using the HumDiv model and also probably damaging (score: 0.947; sensitivity: 0.65; specificity 0.92) using the HumVar model [21].

**A 20.4 kb Genomic Transgene Containing the Wildtype Alg10b Gene Rescues the Mutant Phenotype**

To confirm that the Alg10b mutation in nse5 animals is truly responsible for the hearing impairment phenotype, transgenic animals were generated that carry a 20.4 kb transgene derived from a bacterial artificial chromosome (BAC) containing a wildtype genomic copy of the Alg10b gene (Figures 3A & B). These animals were denoted +/+,[Tg(Alg10b)], to indicate wildtype alleles at the endogenous nse5 locus and an additional wildtype Alg10b transgene array integrated elsewhere in the genome (Figure 3C). Of the 97 fertilized FVB eggs that were injected with the transgene DNA, 97 were transferred to surrogate mothers, and ten progeny animals were born. Of these ten mice, four carried the 129Sv allele of the D15Jus1 locus within the transgene, indicating that the transgene had integrated into the genome of these four animals. (The wildtype Alg10b transgene was derived from the 129Sv mouse strain so that the transgene could be distinguished from the endogenous FVB Alg10b gene in transgenic animals, as well as from the mutant Alg10b gene, which is on a C57BL/6J background.)

These four animals were then crossed to nse5/nse5 mutant animals, and the progeny that inherited the transgene, +/+[nse5], Tg[Alg10b] mice, were backcrossed to nse5/nse5 mutant animals. The progeny of this cross are expected to be either heterozygous or homozygous for the nse5 mutation at the endogenous Alg10b locus and to either have or lack the Alg10b transgene array, leading to four possible genotypes: (1) +/+[nse5]; (2) nse5/nse5; (3) +/+[nse5], Tg[Alg10b]; and (4) nse5/nse5, Tg[Alg10b] (Figure 3C). Animals with the nse5/nse5, Tg[Alg10b] genotype were expected to be hearing-impaired, unless the Alg10b transgene is capable of rescuing the mutant phenotype. Indeed, transgene rescue of the phenotype was
A

![Genetic mapping of the neurological/sensory 5 (nse5) mutation.](image)

(A) The nse5 mouse mutation was localized to a genomic interval using traditional intercrossing of N10 and N11 animals. All animals were genotyped at the D15Mit241 and D15Mit192 loci. Animals with discordant genotypes between these two loci were considered recombinants and genotyped at additional loci, as shown, to localize the recombination breakpoints (shown as “X”es). A brown box indicates homozygosity for the 129S6/SvEvTac allele, a black box indicates homozygosity for the C57BL/6J allele, and half-brown/half-black boxes indicate heterozygosity for the two alleles. The number of each N10F1 and N11F1 animals with each haplotype is shown, as are the phenotypes of the mice with that particular haplotype, with “Hearing” indicating a clear startle response on click-box testing, and “Deaf” indicating an absent startle response. As shown by the red box, this analysis allowed us to localize the nse5 mutation to region of mouse chromosome 15 between D15Mit171 and rs6260777. (B) This region is 524 kb in size and contains only two RefSeq genes, Alg10b and the 3’ end of the Cpona gene. An N10 animal is included here (shown in parentheses), as this animal was found to have a critical recombination breakpoint during our routine genotyping of N10 and N11 animals. This animal was classified as “Hearing”, suggesting that its genotype was +/nse5 at the nse5 locus. When backcrossed to an nse5/nse5 mouse, five progeny that had inherited the recombinant chromosome were also phenotyped as “Hearing”, confirming the +/+nse5 genotype and indicating that the nse5 locus is distal to D15Mit171. These two mice were both classified as “Hearing”. However, their genotype at the nse5 locus could only be inferred as +/+7, since they could be either +/+ or +/+nse5. Both animals were therefore backcrossed to nse5/nse5. Unfortunately, the N11F1 animal died prior to producing any progeny. Phenotyping of five progeny from the N10 animal that had inherited the recombinant chromosome revealed that all were “Deaf”, indicating that the N10 animal was +/+ at the nse5 locus and placing the nse5 locus proximal to rs6260777.

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B

seen in all four transgenic lines studied (Table 1), providing powerful evidence that the Alg10b mutation is responsible for the nse5 mutant phenotype.

Auditory Brainstem Responses (ABRs), Distortion Product Otoacoustic Emissions (DPOAEs), and Cochlear Microphonics (CM) Confront Hearing Impairment in Mutant Mice

ABRs and DPOAEs performed on six-week-old +/+ and +/+nse5, and nse5/nse5 N10F1 animals revealed a significant hearing impairment in nse5/nse5 homozygous animals. Heterozygous animals did not appear to be different from homozygous wildtype animals (Figures 4A & B). Endocochlear potential (EP) measurements performed on two +/+ and two nse5/nse5 animals were virtually identical (93.3 milliVolts [mV] & 97.7 mV versus 102.2 mV & 101.1 mV, respectively), indicating normal ion concentrations in the perilymph versus the endolymph of the cochlea in mutant animals. By contrast, EPs are substantially reduced in mice homozygous for targeted Gjb2 mutations in the cochlea [22]. CM measurements performed on four nse5/nse5 homozygous mutant animals were found to be absent at both 6 kiloHertz (kHz) and 16 kHz (Figures 4 C & D). Taken together, these results suggest that the defect in nse5/nse5 mutant animals involves the outer hair cells of the cochlea.

ABRs and DPOAEs performed on six-week-old animals from one of the four Alg10b transgenic lines (Line 6) confirmed transgene rescue of the nse5 phenotype by the wildtype Alg10b, although the auditory brainstem responses did not completely normalize in transgenic animals at higher sound frequencies (Figures 4E & F).

Immunohistochemistry Reveals Abnormalities in the Outer Hair Cells of nse5 Animals

Immunohistochemistry was performed on inner and outer hair cells from cochleas of two-month-old heterozygous and homozygous mutant animals with phalloidin (which stains filamentous actin red) and anti-prestin antibody (which stains prestin green) (Figures 3A & B). Inner hair cells were relatively intact, based on the visualization of inner hair stereocilia in both heterozygous and homozygous mutant animals. Outer hair cells, on the other hand, appeared severely abnormal in homozygous mutant animals when compared to the heterozygous controls. Numerous outer hair cells were missing completely, while many others showed a punctate pattern of prestin staining in the cytosol, as opposed to the typical staining pattern showing prestin evenly associated with the cell membrane. This suggested an outer hair cell degenerative process was occurring. A similar analysis was performed on an older (5 ½ month-old) Alg10bnse5/nse5, Tg(Alg10b) animal. Although the outer
hair cells appeared swollen, they were all present and did not demonstrate evidence of intracellular prestin particles. This indicates that the transgene rescue of the mutant phenotype inhibits outer hair cell degeneration, preventing outer hair cell death over a relatively long time interval (Figure 5C).

Discussion

Hearing impairment is one of the most common sensory impairments seen in the human population, and mouse models of hearing impairment have long been an avenue for the identification of human hearing impairment genes, as well as for understanding the physiology of hearing. Here, we report that a mutation in the gene for asparagine-linked glycosylation 10B (\textit{Alg10b}) is responsible for the nonsyndromic hearing impairment observed in the \textit{nse5} mouse strain. This was an unexpected finding, as the asparagine (N)-linked glycosylation pathway is highly evolutionarily conserved in eukaryotes, even in single-celled organisms such as yeast (\textit{Saccharomyces cerevisiae}), where the pathway was first characterized, and it is estimated that thousands of human proteins undergo N-linked glycosylation [23]. Mutations in other human genes in this portion of the asparagine-linked glycosylation pathway are known to cause type I congenital disorders of glycosylation (CDGs). These are generally severe multisystem disorders with significant morbidity and mortality. Hearing impairment is sometimes, but not always, associated with these diseases [24]. However, unlike most of the other human genes in the N-linked glycosylation pathway, mutations in the human \textit{ALG10B} gene have never been reported. A number of potential explanations could account for this failure.

First, \textit{ALG10B} mutations in humans may cause nonsyndromic hearing impairment, as opposed to a multisystem disease, which would explain why no \textit{ALG10B} mutations have previously been detected in a patient with a suspected CDG. However, an alternative explanation is that the \textit{ALG10B} gene appears to be duplicated in humans (which have \textit{ALG10A} and \textit{ALG10B} genes, with the \textit{ALG10A} gene sometimes being denoted as simply \textit{ALG10} in the literature) but not in mice (which only have an \textit{Alg10b} gene) [25]. Both copies of the human gene appear to be transcribed and spliced [19], and both are predicted to produce a full-length protein, though it is unclear if both proteins retain functional activity. Thus, it is possible that both copies of \textit{ALG10A} and both copies of \textit{ALG10B} would have to be mutated in humans to yield a phenotype.

\textit{ALG10A} is located in the pericentric region of the short arm of human chromosome 12, whereas \textit{ALG10B} is located in the pericentric region of the long arm of the same chromosome. Single chromosomal microdeletions involving both genes are extremely unlikely, as such a lesion would also delete the centromere of chromosome 12. However, it is possible that compound heterozygous mutations in both genes could lead to a phenotype, such as congenital hearing impairment or even age-related hearing impairment.

The link between N-linked glycosylation and nonsyndromic hearing impairment in the \textit{nse5} mouse is quite intriguing. The enzymatic step catalyzed by the \textit{ALG10B} protein during N-linked glycosylation is the addition of the last glucose molecule to the glycan “tree” inside the endoplasmic reticulum (ER), which is anchored to a dolichol molecule that is embedded in the membrane of the ER [26,27]. After this step is completed, the glycan tree is transferred to an asparagine residue on the target protein by oligosaccharyltransferase (OST), and then the glucose molecule is immediately cleaved from the glycan tree by glucosidase I (encoded by the \textit{CWH41} gene in yeast, the \textit{Mogs} gene in mice (MGI: 1929872), and the \textit{MOGS} gene in humans (OMIM *601336)) [23,28] (Figure 6).

Figure 2. Identification of a leucine-to-serine missense mutation in a highly-conserved region of \textit{Alg10b}. (A) Chromatograms from \textit{nse5}/\textit{nse5}, C57BL/6J, and 129S6/SvEvTac DNA are shown, demonstrating a novel C>T transition in \textit{nse5}/\textit{nse5} DNA, boxed in red. (B) This change converts a highly conserved leucine into a serine residue. (C) Leucine is a bulky, nonpolar amino acid, while serine is a small, polar amino acid. (Reference sequences for (B): \textit{Mus musculus}, NP_001028613.1; \textit{Rattus norvegicus}, NP_620801.1; \textit{Homo sapiens}, NP_001013642.1; \textit{Bos taurus}, NP_001069657.1; \textit{Gallus gallus}, NP_001026565.1; \textit{Xenopus laevis}, NP_001084985.1; and \textit{Xenopus tropicalis}, NP_001016192.2.)
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Given that the \textit{Alg10b}\textsuperscript{nse5} mutation is autosomal recessive, there are two possible explanations for why it is causing only nonsyndromic hearing impairment in mice, as opposed to a severe multisystem disorder. First, the \textit{Alg10b}\textsuperscript{nse5} mutation may be a hypomorphic mutation, and the mutant protein retains some residual activity. If this is the case, then the outer hair cells of the cochlea are exquisitely sensitive to a reduction in the function of the ALG10B protein, while other cells in the body are less sensitive and require less ALG10B function to maintain sufficient N-linked glycosylation of key proteins. Alternatively, the \textit{Alg10b}\textsuperscript{nse5} mutation may represent a null allele, in which case the ALG10B protein is required for the normal functioning of the outer hair cells but is dispensable in all other cell types.

Needless to say, yeast is not traditionally thought of as a model organism for hearing impairment, but the bulk of the research on \textit{ALG10B} has been carried out in yeast. \textit{ALG10B} was discovered in yeast almost two decades ago, and its function was rapidly elucidated [26,27]. However, deletion of the \textit{ALG10B} gene in yeast does not completely abolish N-linked glycosylation, suggesting that there is some redundancy in the pathway [27]. The \textit{ALG6}, \textit{ALG8}, and \textit{ALG10B} genes all appear to share a common ancestor, which could explain this redundancy [29]. Furthermore, deletion of the \textit{ALG10B} gene in yeast does not result in a detectable growth

**Table 1. Genotypes and Phenotypes of Animals from the \textit{Alg10b} Transgene Rescue.**

| Transgenic Line Number | +/nse5 | +/nse5, Tg(Alg10b) | nse5/nse5 | nse5/nse5, Tg(Alg10b) |
|------------------------|--------|------------------|------------|---------------------|
| 2                      | 7      | 8                | 11         | 14                  |
| 4                      | 9      | 9                | 7          | 15                  |
| 6                      | 40     | 45               | 39         | 36                  |
| 8                      | 9      | 11               | 10         | 10                  |

Phenotypes are based on click-box testing of animals, as described in the Materials and Methods.

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Indeed, the only phenotype that has been found in ALG10B yeast mutants is “a round cell shape and a partial unipolar distal budding pattern” in diploid yeast, suggesting a possible role in establishing and maintaining cell polarity [30]. Intriguingly, the only multicellular species for which a mutation in the ALG10B gene could be found prior to our studies is a plant—Arabidopsis thaliana. Mutant plants grow normally in Murashige and Skoog (MS) medium, but they have smaller leaves than normal when grown in soil, and root growth is hypersensitive to salt concentration relative to controls [31].

How, then, do we explain the disparate phenotypes between yeast, Arabidopsis, and mice with mutations in the gene for ALG10B? We propose that, as in yeast, the ALG10B protein has functional overlap with the ALG6 and ALG8 proteins in Arabidopsis and mice, so that mutations in the gene for ALG10B in both species reduce the efficiency of N-linked glycosylation rather than eliminating it completely. Under this hypothesis, a small subset of proteins is extremely sensitive to perturbations in the N-linked glycosylation pathway, whereas most proteins are tolerant of the defect and can function normally. This subset of ultrasensitive proteins would be very different in Arabidopsis versus mice, which accounts for the differences in mutant phenotypes. Identification of this subset of proteins in mice, which may include prestin itself, will be a rich area of study for future experiments involving the role of N-linked glycosylation in development and maintenance of normal hearing.

**Materials and Methods**

**Mice**

The nse5 mutation arose in the progeny of an inbred,ENU-treated C57BL/6J mouse and was previously mapped to mouse
chromosome 15 [18]. Mice carrying the C57BL/6J alleles of D15Mit192 and D15Mit241 were backcrossed to inbred 129S6/SvEvTac mice for at least ten generations (N10) to generate a congenic mouse strain for further studies. Animals heterozygous for the C57BL/6J alleles of D15Mit192 and D15Mit241 from the N10 and N11 generations were intercrossed to produce N10F1 and N11F1 animals, which were used to fine-map the mutation and characterize the mutant phenotype.

Tg(Alg10b) mice were generated by pronuclear injection into FVB embryos in the Baylor College of Medicine (BCM) Genetically Engineered Mouse (GEM) core, as described below.

**Ethics Statement**

All animals were housed in the Transgenic Mouse Facility at Baylor College of Medicine, a specific pathogen free (SPF) mouse facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were provided food and water ad libitum. All work was approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

**Initial Phenotyping and Fine-Mapping of the nse5 Mutation**

All N10F1 and N11F1 animals were genotyped at the D15Mit192 and D15Mit241 loci and phenotyped as either “Hearing” or “Deaf” based on the presence or absence of a “Preyer” reflex when presented with a 90 decibel (dB) sound pressure level (SPL) 20 kiloHertz (kHz) sound burst. This was performed using custom-built click-boxes obtained from Dr. Karen P. Steel. The “Preyer” reflex is a startle response consisting of backward-flicking of the ear pinnae and occasionally whole-body flinching by a hearing animal [32,33].

Animals with a recombination breakpoint between D15Mit192 and D15Mit241 were identified by discordance between the genotypes at these two loci. Recombinant animals were then genotyped at the rs33858577 and D15Mit171 loci to further localize the recombination breakpoint. Animals with recombination events between rs33858577 and D15Mit171 were then genotyped at the rs33858727 locus, while animals with recombi-
nation events between \textit{D15Mit171} and \textit{D15Mit241} were geno-
typed at the \textit{rs6260777} locus. Animals carrying critical recombi-
nants (i.e., between \textit{D15Mit171} and \textit{rs6260777}) were then crossed to \textit{nse5/nse5} animals, and at least five animals that inherited the 
recombinant chromosome were phenotyped to deduce the 
genotype at the \textit{nse5} locus, in order to guard against the possibility 
of incorrectly phenotyping an animal with a critical recombinant.

Polymerease Chain Reaction (PCR)

DNA was isolated from key animals via a tail biopsy as 
described [34]. The concentration of each sample was determined 
via spectrophotometry, and then each sample was diluted to a 
concentration of 25 ng/\mu l with Tris-EDTA (TE) buffer [35].

PCR primers for the \textit{D15Mit171}, \textit{D15Mit192}, and \textit{D15Mit241} 
markers have been previously published [36]. Primers for all other 
alleles (i.e., between \textit{D15Mit192} and \textit{D15Mit241}) were designed using the Primer3 program [37] and are 
shown in Table 2. The first exon of \textit{Alg10b} was extremely difficult 
to amplify and was ultimately obtained with the GC-RICH PCR 
System (Roche Applied Science, Indianapolis, IN) with the 
to amplify and was ultimately obtained with the GC-RICH PCR 

| Primer Name       | Sequence                |
|-------------------|-------------------------|
| rs33858577_for    | 5’-GTC TAA GCC AGT CCC TGT GC-3’ |
| rs33858577_rev    | 5’-ACA GAC AGG TGC TGA TGC TG-3’ |
| rs33858727_for    | 5’-AGC ACT AGG GGG TTC TGG TT-3’ |
| rs33858727_rev    | 5’-TTT GCC GGC CTC TCT TCA TA-3’ |
| rs6260777_for     | 5’-CAA AGA ATC AAT AAA GAG CGT GTT C-3’ |
| rs6260777_rev     | 5’-TTG GTT TTC CAA CGA GTC AA-3’ |
| Alg10b_exon_1_for | 5’-CGA AGG GCT GTG GGT ATG TT-3’ |
| Alg10b_exon_1_rev | 5’-GGC CCT AAG ATC CTC CAC TC-3’ |
| Alg10b_exon_1_seq_for | 5’-CTT CCG GCC GGC CTC G-3’ |
| Alg10b_exon_1_seq_rev | 5’-CAC TAC GGA GCC CCC TTC AC-3’ |
| Alg10b_exon_2_for | 5’-TGG GAT AAC TCT TGC TGT GC-3’ |
| Alg10b_exon_2_rev | 5’-AGG TTA AAA AAA CAG ACA ACA C-3’ |
| Alg10b_exon_3_for | 5’-GGT GTG GCA AAG CCA AT-3’ |
| Alg10b_exon_3_rev | 5’-CAC GAC AAC TGC AAG CAA CT-3’ |
| D15Jus1_for       | 5’-FAM-TGG TGA ATT AAT TGC ATT GAC A-3’ |
| D15Jus1_rev       | 5’-GTG TGA ATT TCC ACG GAG GT-3’ |

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Genotyping

The \textit{D15Mit171}, \textit{D15Mit192}, and \textit{D15Mit241} markers were all 
scored visually by subjecting the PCR products to gel elecro-
phoresis on a 4% MetaPhor agarose (Lonza Rockland, Rockland, ME), 
0.5X TBE gel run at 100V. PCR products representing 
\textit{rs33858577} were digested with \textit{EcoRI} and then scored visually 
after gel electrophoresis on a standard 2% agarose, 0.5X TBE gel 
run at 100V [35]. The \textit{rs33858577} and \textit{rs6260777} markers were 
analyzed by PCR followed by dideoxynucleotide sequencing, as 
described below. The \textit{D15Mit1} marker was analyzed by running the 
products on ABI 3730xl DNA Analyzer with the GeneScan- 
1000 ROX Size Standard (Applied Biosystems, Carlsbad, CA) or 
the MapMarker 1000 X-Rhodamine ladder (BioVentures, Inc., 
Murfreesboro, TN) and analyzing the data with the Peak Scanner 
software program (Applied Biosystems, Carlsbad, CA).

Dideoxynucleotide Sequencing

PCR products were either sent directly to Agencourt Biosciences 
(Beverly, MA) for purification and dideoxynucleotide sequencing 
or were gel-purified with the DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA), run through the BigDye Terminator v3.1 Cycle Sequencing kit (Applied 
Biosystems, Carlsbad, CA), followed by another purification with 
CENTRI-SEP columns (Princeton Separations, Adelphia, NJ), 
and then sent to either the Molecular Core Laboratory at Baylor 
College of Medicine (Houston, TX) or GENEWIZ (South 
Plainfield, NJ) to be run on a Genetic Analyzer. With the 
exception of exon 1 of \textit{Alg10b} (as noted in Table 2), all products 
were sequenced with the same primers that were used for the 
initial PCR. The results were visualized with the Sequencher 
software program (Gene Codes, Ann Arbor, MI).

Generation and Breeding of \textit{Alg10b} Transgenic Animals

The bMQ bacterial artificial chromosome (BAC) library is a 
genome-wide, end-sequenced BAC library derived from the 
129S7/SvEvBrd-Hprt<sup>-/-m2</sup> mouse strain [38]. Based on the 
published end-sequence data, the 78.4 kb BAC bMQ33j10 was 
determined to contain a complete genomic copy of the \textit{Alg10b} 
gene. Bacteria containing this BAC were obtained and cultured 
in overnight in 500 ml Luria broth with 20 \mu g/ml chloramphenicol 
in a 37 degree Celsius air shaker. High-quality BAC DNA was 
isolated with the NucleoBond BAC 100 kit (Clontech Laborato-
dries, Inc., Mountain View, CA), per the manufacturer’s instruc-
tions, with minor modifications. (For the “Clarification of the 
lyase step”, the filtration option was used, and the subsequent 
filtrate was not centrifuged prior to adding it to the column.) The 
DNA was resuspended in 180 \mu l ddH2O. A restriction 
digestion was set up as follows: 135 \mu l BAC bMQ33j10 DNA, 15 \mu l 10X 
buffer “O”, and 15 \mu l 10 U/\mu l BslXI (Fermentas, Inc., Glen 
Burnie, MD). This reaction was incubated at 55 degrees Celsius 
for three hours to completely digest the BAC DNA. 15 \mu l of 
loading dye was then added to the reaction, and all of it was run 
on a 0.8% agarose, 1X TAE gel at 100 V for six hours. The 
running buffer was changed at the three-hour mark. The large 
20.4 kb band was excised from the gel, and the DNA was 
extracted from this gel slice with the QIAEXII Gel Extraction Kit 
(QIAGEN, Inc., Valencia, CA), per the manufacturer’s instruc-
tions. The DNA was eluted into 60 \mu l of injection buffer [34] and 
the mixture was spun at 16,100 x g for 15 minutes to remove any 
particulate material from the suspension prior to injection into 
fertilized eggs. The DNA was estimated to be at a concentration 
of 12 ng/\mu l based on gel electrophoresis. 10 \mu l of DNA was then 
combined with 290 \mu l of injection buffer for a final concentration
of 0.4 ng/µl, and this mixture was injected into 97 fertilized FVB mouse eggs [34].

**In vivo Experiments**

Procedures performed on anesthetized mice included the measurement of ABRs, DPOAEs, EPs, and CMs. Mice were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg). Supplemental doses of anesthesia were administered to maintain areflexia to paw pinch. All methods have been fully described elsewhere [39–41] but are described briefly below.

Sine wave stimuli were generated digitally using MATLAB (Release 13, The Mathworks, Natick, MA), converted to analog signals using a digital-to-analog converter running at 200 kilo-Hertz (kHz), and then attenuated to the appropriate intensity according to our experimental design (Part numbers RP2 and PA5, Tucker-Davis Technologies, Alachua, FL) [42]. To generate the acoustic stimuli, high frequency piezoelectric speakers were used (EC1, Tucker-Davis Technologies). The speakers were connected to an ear bar inserted into the ear canal and calibrated from 4 to 95 kHz by a probe-tip microphone (type 8192, NEXUS conditioning amplifier. Bruel and Kjar, Denmark) inserted through the ear bar. The tip of the microphone was within 3 millimeters of the tympanic membrane.

The ABR signal was measured with a bioamplifier (Part number DB4, Tucker-Davis Technologies) from a needle electrode positioned at the ventral surface of the tympanic bulla referenced to an electrode placed at the vertex of the skull, as previously described [43,44]. A ground electrode was placed in the hind leg. The stimulus was a 5 millisecond (ms) sine wave tone pip of alternating polarity with cos2 envelope rise and fall times of 0.5 ms and a repetition time of 50 ms. The stimulus intensity ranged from 10 to 80 decibels (dB) SPL (sound pressure level) in 10 dB steps. The frequency range studied was 4 to 90 kHz over the 50 ms repetition time and averaged. Thresholds were calculated off-line. At each frequency, the peak-to-peak voltage of the ABR waveform was measured and the data interpolated over the range of stimulus intensities. The ABR threshold was determined at four standard deviations above the noise floor. If no ABR response was detected even at our equipment limits of 80 dB SPL, we arbitrarily defined the threshold to be 80 dB SPL.

DPOAE thresholds were measured as previously described [41]. Briefly, the stimuli for eliciting DPOAEs were two sine wave tones of differing frequencies (F1 and F2; F2 = 1.2F1) of one-second duration with F2 ranging from 4 to 90 kHz. The two tones were presented at identical intensities, which ranged from 20 to 80 dB SPL in 10 dB increments. The acoustic signal detected by the microphone in the ear bar was digitized at 200 kHz and the magnitude of the 2F1-F2 distortion product determined by FFT (Fast Fourier Transform). The surrounding noise floor was also measured from the ball-ended tip of a Teflon-coated silver wire (0.003 inch diameter, A-M Systems, Carlsborg, WA) advanced onto the round window membrane with a micromanipulator. The signal was referenced to a silver wire inserted under the skin near the vertex of the skull. The ground electrode was placed in the hind leg. A bioamplifier was used (Part number DB4, Tucker Davis Technologies) to amplify the signals 100 times, and no filtering was used. The stimulus was a 30 ms 6 kHz tone, repeated every 1 s, with an intensity range from 10–100 dB SPL. By measuring the speaker output with the probe tip microphone in the ear bar, FFT analysis demonstrated that all stimulus harmonics and noise at all other frequencies were at least 50 dB below the primary signal at all stimulus intensities. The CM signal measured by the bioamplifier was digitized at 200 kHz, and the magnitude of the response at 6 kHz and 16 kHz determined by FFT.

**Whole-mount preparations to view the hair cell epithelium**

Mice were anesthetized and sacrificed by cervical dislocation. Their cochleae were removed and immersed in a 313 mOsm/kg artificial perilymph solution containing 150 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 10 mM HEPES, and 10 mM glucose at pH 7.35. Under a dissecting microscope (SteREO Discover.V8, Zeiss, Germany), the vestibular structures and ossicles were carefully removed. The cochleae were then fixed in 4% paraformaldehyde at room temperature for two hours and then glued upright into custom-built chambers. Once the cochleae were secured, the otic capsule over the basal turn was removed with a fine knife. The outer hair cell epithelium was exposed after Reissner’s membrane and the tympanic membrane were removed with a pick.

The cochleae were permeabilized with 0.1% Triton X-100 in PBS and simultaneously stained with Alexa Fluor 568 Phallolidin (Invitrogen; 1:200 in phosphate-buffered solution) for 15 minutes at room temperature. Immunolabeling was performed by first blocking the organs of Corti with 4% donkey serum (017-000-121, Jackson Immuno Research Laboratories, West Grove, PA) in PBST for 1 hour at room temperature and then incubating with the primary antibody at 4°C overnight. Specimens were washed three times with PBST and then incubated with the secondary antibody at room temperature for 1 hour. The primary antibody was goat anti-prestin N-20 (1:500; SC-22692, Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody was Alexa Fluor 488 donkey anti-goat (1:500; Invitrogen, Grand Island, NY).

Labeled cochleae were imaged using a custom-built two-photon microscope [45]. Briefly, the microscope consisted of a moveable objective microscope (MOM; Sutter) fitted with a 20x water-immersion objective (NA 0.95, XLUMPlanFl, Olympus America,
A femtosecond Ti:sapphire laser (Chameleon, Coherent, Santa Clara, CA) tuned to 800 nanometers (nm) provided two-photon excitation. The emitted fluorescence was detected by two photomultiplier tubes after optical filtering. Lateral scanning was achieved by two galvanometer-actuated mirrors controlling the laser beam. Axial scanning was achieved by a separate actuator that controlled the objective lens. A modified version of ScanImage open source software [46] was used to control the hardware.

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Author Contributions
Conceived and designed the experiments: FJP RRC DdG II IC AX JSO MJJ. Performed the experiments: FJP RRC DdG APS II SSG IC AX JSO MJJ. Analyzed the data: FJP RRC SS IC AX JSO MJJ. Contributed reagents/materials/analysis tools: FJP RRC DdG II SS IC AX JSO MJJ. Wrote the paper: FJP MJJ.

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