An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice

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Supplementary Figures
l. Middle Coil
   4 weeks postnatal

m. Basal Coil
   4 weeks postnatal

n. Middle Coil
   6 weeks postnatal

o. Basal Coil
   6 weeks postnatal

p. Compound action potential

q. Endocochlear potential

r. Middle Coil

s. Basal Coil

**Organ of Corti Cell Measurements, 4 weeks postnatal**

| Genotype     | Mean  | Standard Deviation | Range       | n  |
|--------------|-------|--------------------|-------------|----|
| +/+          | 96.8mV| 9.9                | 87-110mV    | 4  |
| Dmdol+       | 116.3mV| 5.8               | 111-128mV   | 7  |
| DmdolDmdo    | 108.2mV| 7.5               | 96-113mV    | 5  |
Figure S1 | Characterisation of the diminuendo phenotype. a-k, Scanning electron micrographs of the diminuendo heterozygote (Dmdo/++; b,e, h and j), homozygote (Dmdo/Dmdo; c, f and k) and wildtype littermates (+/++; a, d, g and i) at 5 days postnatal (a-c) and at 4 weeks postnatal (d-k). At P5, heterozygotes (b) resemble the wildtypes (a), but homozygotes show misshapen hair cells (c). At 4 weeks old, in Dmdo/+ animals (e) the outer hair cell stereociliary bundle has a more rounded overall structure compared with control littermates (d) but retains its polarity (appropriately graded stereocilia and oriented bundle; compare g and h). In the heterozygotes the outer hair cells and basal turn inner hair cells (j) are also more separated than in controls (compare d with e) and in the outer hair cells there was occasionally an absence of stereocilia in the centre of the bundle (arrow in h). In Dmdo/Dmdo animals (f, k) there are virtually no hair cells visible in the organ of Corti at 4 weeks postnatal. At this stage, some pillar cells and Deiters’ cells persist although in some areas may be replaced by scar tissue as seen on the left in (k). Scale bars a-f = 10 µm; g and h = 2 µm; l, j and k = 5 µm.

I-o, Hair cell density in the cochlear duct. Hair cell counts per 100 µm of cochlear duct in the middle coil (45-50% of the distance along the length of the cochlea, l and n) and basal coil (10-15% of the distance along the length of the cochlea, m and o). 6-week old diminuendo heterozygotes (Dmdo/++; hatched bars) were found to have significantly fewer outer hair cells in the middle and to a greater extent basal regions of the cochlea compared with littermate (+/++; solid black bars) controls. P-values are indicated on the figure; they were calculated using Student's two-tailed t test or Welch's t-test, and the significance threshold set to 0.05 (4 weeks: +/- n=6; heterozygote n=5; 6 weeks: +/- n=5; heterozygote n=5). Error bars show the standard error of the mean. In contrast,
there was no significant difference in the number of inner hair cells between Dmdo/+ and +/+ littermates.

Measurement of hair cell and support cell dimensions in the adult (4 week) organ of Corti. Mean values for the span of the outer hair cell stereociliary bundle (measurement 1 in \( p \)), the diameter of the outer hair-cell apex at the widest point (measurement 2 in \( p \)) and the diameter of Dieters’ cell apex (measurement 3 in \( p \)) were determined in the middle region of the cochlear duct (i.e. 50% along the length of the cochlea). Twenty cells were examined per animal, using a Phillips XL30 scanning electron microscope, at 15kV. \( q \), the mean values for each measurement; error bars are the standard error of the mean. Both the mean span and apical diameter of outer hair cells in the Dmdo/+ mutants was significantly smaller than in littermate controls (bundle span: wildtype mean=4.73, n=99; heterozygote mean=3.86, n=95; \( \alpha = 0.05; p = 1.3 \times 10^{-26} \) (Welch’s t test), apical diameter: wildtype mean=5.46, n=97; heterozygote mean=4.71, n=84; \( \alpha = 0.05; p = 7.9 \times 10^{-30} \) (Student's t test)) and the mean diameter of the intervening supporting cell apex was larger than in controls (wildtype mean=0.52, n=75; heterozygote mean=1.40, n=84; \( \alpha = 0.05; p = 3.1 \times 10^{-38} \) (Welch's t test)). The hair cell density was no different in mutants compared with controls (wildtype, n=6, 16.47 OHCs per 100\( \mu \)m; heterozygotes, n=4, 16.95 OHCs per 100\( \mu \)m; \( \alpha = 0.05; p = 0.36 \) (Student's t test)).

Electrophysiological measurements. \( r \), Compound action potential (CAP) thresholds for 28-33 day old heterozygotes (open circles) and wildtype littermates (solid triangles). Thresholds are plotted with respect to stimulus frequency (bottom abscissa) and the position along the length of the cochlear duct (top abscissa). Controls showed typical thresholds for laboratory mice; heterozygotes showed an increase in thresholds across a range of frequencies,
while homozygotes showed no evoked responses at all up to the maximum sound pressure level used (indicated by solid squares with upward arrows). Error bars = standard error of the mean. 

**Endocochlear potentials measured from 28-33 day old wildtype, heterozygote and homozygote animals; heterozygote potentials are slightly raised (+/+ mean 96.8mV, range 87 to 110mV, n=4; Dmdo/+ mean 116.3mV, range 111 to 128mV, n=7, p=0.02; Dmdo/Dmdo mean 108.2mV, range 96 to 113mV, n=5, p=0.11).**
**Figure S2 | Mapping of the diminuendo mutation.** In order to localise the diminuendo mutation to a single chromosome, a genome scan was carried out using a panel of 60 autosomal microsatellite markers, polymorphic between C3HeB/FeJ and C57BL/6J, using the DNA of 35 mutant mice from a [C3HeB/FeJ Dmdo/ + x C57BL/6J]F1 x C3HeB/FeJ backcross. a, the percentage of backcross mice homozygous for the C3HeB/FeJ-type allele of each chromosomal marker is represented by the bars. Alternate chromosomes are indicated by different shading. Chromosome 6 (black bars) showed the highest degree of linkage between the phenotype and original (mutated) DNA background, with 94% linkage of the diminuendo phenotype with marker D6Mit138. The microsatellite markers used for the genome scan were as follows: D1Mit21 (33.89 cM); D1Mit415 (53.06 cM); D1Mit445 (75.72 cM); D1Mit353 (90.34 cM); D2Mit237 (28.71 cM); D2Mit128 (49.41 cM); D2Mit59 (60); D2Mit200 (98.36 cM); D3Mit117 (2.35 cM); D3Mit339 (29.25 cM); D3Mit199 (56.1 cM); D4Mit172 (11.23 cM); D4Mit58 (40.62 cM); D4Mit33 (67.6 cM); D5Mit345 (0 cM); D5Mit391 (18.3 cM); D5Mit115 (40.01 cM); D5Mit168 (68.65 cM); D6Mit138 (2.35 cM); D6Mit320 (22.38 cM); D6Mit366 (43.69 cM); D6Mit201 (62.94 cM); D7Mit178 (2.78 cM); D7Mit230 (22.45 cM); D7Mit253 (42.55 cM); D8Mit190 (22.69 cM); D8Mit280 (74.11 cM); D9Mit254 (20.14 cM); D9Mit214 (58.79 cM); D10Mit206 (4.47 cM); D10Mit115 (32.47 cM); D10Mit12 (52.5 cM); D10Mit180 (65.22 cM); D11Mit71 (0 cM); D11Mit140 (25.73 cM); D11Mit35 (44.74 cM); D11Mit99 (63.21 cM); D11Mit214 (79.6 cM); D12Mit69 (23.88 cM); D12Mit259 (42.92 cM); D12Nds2 (62.46 cM); D13Mit3 (8.96 cM); D13Mit9 (32.44 cM); D13Mit77 (50 cM); D14Mit99 (1.51 cM); D14Mit260 (21.21 cM); D14Mit225 (44.1 cM); D15Mit175 (5.72 cM); D15Mit43 (58.01 cM); D16Mit165 (11.17 cM); D16Mit63 (30.76 cM); D16Mit152 (48 cM); D17Mit113 (2.22 cM); D17Mit238 (30.64 cM); D17Mit155 (50.71 cM); D18Mit22 (8.97 cM);
**b.** The haplotypes of 160 heterozygote and 138 wildtype backcross animals used to refine further the mapping of the diminuendo mutation. Each column represents the marker pattern for the chromosome derived from the F1 parent, with black boxes representing the C3HeB/FeJ-like marker types and the white boxes representing the C57BL/6J-like marker types. A change vertically from black boxes to white or vice versa indicates that a recombination event has occurred during meiosis leading to the production of that chromosome in the F1 parent. The number at the bottom of each column indicates the number of chromosomes (animals) showing that particular pattern of marker types. The markers used on chromosome 6 are indicated on the left. The diminuendo mutation lies between markers D6Mit159 and D6Mit268, which is a 4.96 Mb interval.

**c.** The traces and sequence of exon 5 of the gene 2310005E10Rik, showing the C to T substitution. Although the mutation is silent, it was possible that it could affect the mRNA before it is translated, so a thorough literature search was carried out. An exonic splicing enhancer site (ESE) was predicted to be present in the wildtype but not in the mutant, using the online predictor Rescue-ESE\(^3\). In addition, a potential binding site for the miRNA miR-30a-3p was found that is present in the wildtype sequence but not in the mutant (yellow highlighting). Using the miRanda target site predictor\(^7\), the putative binding site was tested against profiles created from mouse 3' UTRs and from mouse coding regions, but the p-values, which indicate the likelihood of the binding site being functional, were 0.12 and 0.17 respectively, above the significance threshold of 0.1. The base change is at the 3' end of the binding site, far from the "seed" region which is crucial for miRNA binding and activity\(^3\). In addition, microarrays designed to detect a range of miRNAs, including miR-30a-3p, have been carried out against RNA from the inner ear, and that particular miRNA was
not detected at twofold greater than background\textsuperscript{5}. Using RTPCR, we found that the size and intensity of exon 5 does not vary between wildtype and mutant cDNA. The gel shows the bands from a PCR reaction on cDNA from three four-day-old wildtype and mutant sibling pairs. The sequences of the six animals were identical across the two exon boundaries either side of exon 5 (data not shown).
Figure a shows the luciferase activity (normalized) for several genes under different conditions. The x-axis represents different genes: Aqp5, Celar2, Myrip, Cdt2, Avil, Cbb, and Ryk. The y-axis represents the luciferase activity.

* p < 0.01  ** p < 0.005  *** p < 0.001

Figure b shows similar data as Figure a, but for different genes: Sh3kbp1, Rga3, Cdh20, Fgf3, Sox5, Avil, Cbb, and Plpn9.

* p < 0.01  ** p < 0.005  *** p < 0.001

Figure c shows the relative expression levels of several genes under different conditions. The x-axis represents different genes: Aqp5, Celar2, Cdt2, Myrip, and Ryk. The y-axis represents the relative expression level.

* p < 0.05  ** p < 0.005

Figures d to m show microscopic images of tissue sections stained for different genes under various conditions. The images indicate the presence of specific proteins or markers.
Figure S3 | Candidate target genes: Luciferase reporter assay data and expression data. 

**a**, *Aqp5*, *Celsr2*, *Myrip*, *Odf2* and *Ryk* are targeted by an siRNA mimicking miRNA-96 (siR-96). Targets were positively validated where the luciferase activity of the wildtype target construct in the presence of siR-96 (blue bars) was significantly lower than that of the disrupted target construct with siR-96 (red bars). This indicates that the disruption of the target site inhibits the ability of siR-96 to downregulate the target transcript. The observed effects were specific to siR-96, because transfection with siR-140 showed no statistically significant differences between the luciferase activity of cells co-transfected with the wildtype or disrupted target construct (data not shown). When an siRNA mimicking the Diminuendo mutation was used (siR-Dim-96), no difference in activity was observed with mutated 3' UTR binding sites. Reporter activity was normalised to the respective plasmid transfected alone, and is shown as mean ± SEM from 4 independent assays performed in triplicate for each putative target gene (n=12) using at least 2 independent plasmid preparations. P-values for the difference between wildtype 3' UTR and disrupted 3'UTR are calculated using Student's paired t-test or Welch's t-test; \( \alpha = 0.05 \) (Aqp5: siR-96 p=0.00813; siR-Dim-96 p=0.471; Celsr2: siR-96 p=0.00311; siR-Dim-96 p=0.698; MyRIP: siR-96 p=0.000163; siR-Dim-96 p= 0.150; Odf2: siR-96 p=0.00155; siR-Dim-96 p=0.595; Ryk: siR-96 p=0.000809; siR-Dim-96 p=0.281). 

**b**, *Sh3kbp1*, *Itga3*, *Cdh20*, *Fgf3*, *Sox5*, *Avil*, *Ctsb* and *Pttn9* are not targeted by an siRNA mimicking miRNA-96 (siR-96). No significant difference is visible when the putative binding sites are mutated. Although the p-value for *Avil* indicates significance, it was not considered a valid target, because there is a relatively small downregulation of the wildtype construct (the average activity of the wildtype construct with siR-96 is 73.9% that of the wildtype construct alone). The p-values for the differences between wildtype and disrupted 3'UTRs with
siR-Dim-96 for Cdh20 and Sox5 imply significance, but for both, the activity is reduced rather than increased when the disrupted UTR is used, which does not imply any function on the part of siR-Dim-96. Reporter activity was normalised to the respective plasmid transfected alone, and is shown as mean ± SEM from 4 independent assays performed in triplicate for each putative target gene (n=12) using at least 2 independent plasmid preparations. P-values for the difference between wildtype 3' UTR and disrupted 3' UTR are calculated using Student's paired t-test or Welch's t-test; \( \alpha = 0.05 \) (Sh3kbp1: siR-96 p=0.212; siR-Dim-96 p=0.919; Itga3 : siR-96 p=0.770; siR-Dim-96 p=0.324; Cdh20: siR-96 p=0.187; siR-Dim-96 p=0.011; Fgf3: siR-96 p=0.885; siR-Dim-96 p=0.265; Sox5: siR-96 p=0.607; siR-Dim-96 p=0.005; Avil: siR-96 p=0.00148; siR-Dim-96 p=0.528; CTSB: siR-96 p=0.433; siR-Dim-96 p=0.420; Ptpn9: siR-96 p=0.235; siR-Dim-96 p=0.949).

Quantitative real-time PCR on cDNA generated from normalised RNA from the organs of Corti of 4 day old littermates. Aqp5 and Celsr2 are significantly upregulated in the homozygote. Error bars represent standard deviation. Quantities normalised to Hprt levels; Ngfr was used as an additional control for the quantity of sensory material. Three animals were used for each genotype and DNA from each was run in triplicate. Ngfr: Wildtype, n=39, mean=1.01±0.14 (s.d.); homozygote, n=39, mean=0.94±0.23 (s.d.) Aqp5: Wildtype, n=9, mean=1.00±0.06 (s.d.); homozygote, n=9, mean=1.40±0.37 (s.d.) Celsr2: Wildtype, n=9, mean=1.01±0.14 (s.d.); homozygote, n=9, mean=1.22±0.12 (s.d.); Odf2: Wildtype, n=9, mean=1.00±0.06 (s.d.); homozygote, n=9, mean=1.10±0.13 (s.d.); Ryk: Wildtype, n=9, mean=1.00±0.07 (s.d.); homozygote, n=9, mean=0.95±0.13 (s.d). Two-tailed Student's t-test: Celsr2 p=2.78x10^{-3}; Welch's t-test: Ngfr p=0.13; Aqp5 p=0.013; Odf2 p=0.06; Ryk p=0.32; \( \alpha = 0.05 \). d, f-i show wildtypes, and e, j-m homozygous mutant siblings, at 5 days old. All are expressed in the organ of
Corti, but no difference is visible for any of the five validated targets (Aqp5: d, e; Celsr2: f, j; Myrip: g, k; Odf2: h, l; Ryk: i, m). Hair cells are indicated by arrowheads. Scale bars = 10µm.
| Discovered motif | Distance | Known motif | p-value | Motif accession number, name and potential binding factors |
|------------------|----------|-------------|---------|----------------------------------------------------------|
| k                | 0.259    | AAA         | 0.0     | M00971 Ets (Gabpa, Gabpb1, Ef1, Ets2, Ek3, Fli1, Sfp1, Ets1, Etv4) |
| l                | 0.232    | GTA         | 0.002   | M00971 Ets (Gabpa, Gabpb1, Ef1, Ets2, Ek3, Fli1, Sfp1, Ets1, Etv4) |
| m                | 0.259    | AGAA        | 0.0     | M00971 Ets (Gabpa, Gabpb1, Ef1, Ets2, Ek3, Fli1, Sfp1, Ets1, Etv4) |
| n                | 0.232    | TTCC         | 0.006   | M00971 Ets (Gabpa, Gabpb1, Ef1, Ets2, Ek3, Fli1, Sfp1, Ets1, Etv4) |
Figure S4 | Confirmation and further analysis of microarray data. a, Quantitative real-time PCR on cDNA generated from normalised RNA from the organs of Corti of 4 day old littermates. The expression changes confirm the microarray data for these thirteen genes. Of this selection, \textit{Cpne9} and \textit{Gabrb3}, which were downregulated in the mutant, bear binding sites for diminuendo miR-96, and \textit{Sdc2}, which was upregulated in the mutant, bears a binding site for wildtype miR-96. None of the others have either wildtype or mutant binding sites. Error bars represent standard deviation. Quantities normalised to \textit{Hprt1} levels; \textit{Ngfr} was used to assess the quantity of sensory material. Three animals were used for each genotype and DNA from each was run in triplicate; where mRNA was not detected and corresponding measurements were low, quantity was assumed to be 0. The animals used here for \textit{Ocm}, \textit{Slc26a5}, \textit{Gfi1}, and \textit{Pitpnm1} are different from those used for the RTPCR results shown in figure 3. \textit{Ngfr}: Wildtype, n=39, mean=1.01±0.14 (s.d.); homozygote, n=39, mean=0.94±0.23 \textit{Ocm}: Wildtype, n=9, mean=1.00±0.10 (s.d.); homozygote, n=9, mean=0.01±0.01 (s.d.) \textit{Slc26a5}: Wildtype, n=9, mean=1.00±0.08 (s.d.); homozygote, n=9, mean=0.01±0.02 (s.d.); \textit{Tuba8}: Wildtype, n=9, \textit{Cpne9}: Wildtype, n=9, mean=1.00±0.07 (s.d.); homozygote, n=9, mean=0.29±0.10 (s.d.); \textit{Kcna10}: Wildtype, n=9, mean=1.00±0.07 (s.d.); homozygote, n=9, mean=0.38±0.10 (s.d.); \textit{Ptprq}: Wildtype, n=9, mean=1.01±0.15 (s.d.); homozygote, n=9, mean=0.56±0.20 (s.d.); \textit{Gfi1}: Wildtype, n=9, mean=1.01±0.11 (s.d.); homozygote, n=9, mean=0.56±0.17 (s.d.); \textit{Gabrb3}: Wildtype, n=9, mean=1.04±0.28 (s.d.); homozygote, n=9, mean=0.56±0.39 (s.d.); \textit{Chrna9}: Wildtype, n=9, mean=1.01±0.13 (s.d.); homozygote, n=9, mean=0.62±0.28 (s.d.); \textit{Pitpnm1}: Wildtype, n=9, mean=1.03±0.25 (s.d.); homozygote, n=9, mean=0.71±0.21 (s.d.); \textit{Gm414}: Wildtype, n=9,
mean=1.00±0.11 (s.d.); homozygote, n=9, mean=0.82±0.12 (s.d.); Sdc2: Wildtype, n=9, mean=1.00±0.10 (s.d.); homozygote, n=9, mean=1.37±0.50 (s.d.); Anxa4: Wildtype, n=9, mean=1.00±0.03 (s.d.); homozygote, n=9, mean=1.90±0.19 (s.d.). Two-tailed Student's t-test: Tuba8 p=7.69x10^{-13}; Cpne9 p=7.77x10^{-12}; Ptpqr p=6.46x10^{-5}; Gfi1 p=7.98x10^{-6}; Gabrb3 p=9.63x10^{-3}; Pitpn1 p=9.41x10^{-3}; Gm414 p=2.94x10^{-3}; Welch's t-test: Ngfr p=0.13; Ocm p=1.24x10^{-8}; Slc26a5 p=6.18x10^{-11}; Kcna10 p=6.69x10^{-7}; Chrna9 p=3.20x10^{-3}; Sdc2 p=2.59x10^{-2}; Anxa4 p=3.45x10^{-7}; α=0.05). b-j, Alternative statistical analyses of the microarray data. b, c, and d show an overrepresentation analysis of seed matches for all miRNAs, plotting the log ratios for three different gene sets, corresponding with Fold Change cutoffs of >1.5, >1.2 and >1.2 respectively, for probes with uncorrected P-value < 0.05. The most enriched and depleted matches are shown, joined with a random sampling of other matches and always including the seed matches of interest (the wildtype heptamers GUGCCAA and UGCCAAA and the diminuendo heptamers AGCCAAA and GAGCCAA). e-g shows an analysis of 10,000 random shufflings of the gene list. For each miRNA seed match a Z-score is derived from the ensemble of log ratios resulting from each of the shufflings, showing the significance of the log ratio found for the gene set of the corresponding FC value. Seed matches are selected as in b-d. h-j show hypergeometric P-values computed by Sylamer\textsuperscript{23} at the same FC cutoffs, with seed matches selected as in b-d. k-n, Sequence logos displaying the matches between predicted motifs and known transcription factor binding sites. k and m show the motifs predicted from the 356 upregulated genes; l and n those predicted from the 425 downregulated genes. The comparison in k and l is against the transcription factors found among the top 1000 most significantly affected genes from the microarray, while in m and n, the predicted motifs are compared to all the
known motifs from the TRANSFAC database release 12.2\textsuperscript{33}. Distances between motifs, displayed between the predicted motifs on the left and the known motifs on the right, were calculated as described in ref. 60; only distances less than 2.5 were included. p-values are indicated to the right of the two motifs; only those motifs with p-values < 0.02 are included. On the right is the accession number of the motif, the name of the binding factor group as assigned by TRANSFAC, and, in brackets, any mouse transcription factors known to bind to that motif.
Figure S5 | Additional immunohistochemistry. a-p, Immunostaining at postnatal day 5 of oncomodulin (a-h) and prestin (i-p) in four different mutant mice; Headbanger (a, e, i, m), which shows abnormal hair cells by E18.515, Deafness (b, f, j, n), where hair cells are never functional18, Snell’s Waltzer (c, g, k, o), where the hair cells are abnormal from birth16 and Whirler (d, h, l, p), which exhibits stereocilia regression between P1 and P422. Both proteins are
present in outer hair cells in phenotypically wildtype (a-d, i-l) and mutant (e-h, m-p) animals, and similar results were observed for Beethoven, where degeneration is observed from P1534, shaker1, which has abnormal hair cells from E18.514, Headturner, where degeneration is visible by P2017, Oblivion, where the hair cells are dysfunctional by P2020 and Catweasel21 (data not shown). Hair cells are indicated by arrowheads. Scale bars = 10µm. q-y, Antibody staining for markers of hair and supporting cells. q-t show wildtype and v-y homozygous mutant newborn siblings. No difference is visible for any of the markers chosen. (q, v) Myo7A, a marker of hair cells35. (r, w) Cdkn1b, a supporting cell marker36. (s, x) Jag1, a protein secreted by supporting cells37. (t, y) Sox2, which labels supporting cells36. Hair cells are indicated by arrowheads. Scale bars = 10µm.
Supplementary methods

**Mice.** Male C3HeB/FeJ mice were injected with three doses of 100 mg/kg ENU at weekly intervals and allowed to recover for several weeks before mating to C3HeB/FeJ females\(^3\). Offspring were screened as young adults using a 20 kHz, 90 dB SPL click to trigger a Preyer reflex. The diminuendo mutant showed a reduced reflex, and subsequent breeding experiments established that it carried a new semi-dominantly-inherited mutation. The mutation was maintained on its original C3HeB/FeJ genetic background. All experiments were carried out in full compliance with UK Home Office requirements.

**Scanning electron microscopy of the inner ear sensory organs.** The inner ears of mice from several developmental stages were examined: postnatal day (P) 1 (n = 2 $Dmdo^+/+$, 1 $Dmdo/Dmdo$, 2 $+/+$), P3 (n = 4 $Dmdo^+/+$, 3 $+/+$), P4 (n = 1 $Dmdo^+/+$, 4 $Dmdo/Dmdo$, 1 $+/+$), P5 (n = 7 $Dmdo^+/+$, 4 $Dmdo/Dmdo$, 4 $+/+$), P6 (n = 10 $Dmdo^+/+$, 7 $Dmdo/Dmdo$, 4 $+/+$), P20 (n = 3 $+/+$), P21 (n = 2 $Dmdo/Dmdo$, 1 $+/+$), four weeks postnatal (n = 17 $Dmdo^+/+$, 14 $+/+$), and six weeks postnatal (n = 5 $Dmdo^+/+$, 5 $+/+$). Freshly isolated inner ears were immersed in fixative (2.5 to 4% glutaraldehyde in 0.07 to 0.1 M sodium cacodylate buffer with 0.1-0.3 mM calcium chloride, pH 7.4, depending upon age), fixed at RT for 3 to 5 hours, washed with PBS or buffer and then dissected further to expose the sensory organs. Samples were prepared using the OTOTO method (osmium tetroxide/thiocarbohydrazide\(^{38}\)), dehydrated in acetone or ethanol, critical point dried, and some were then sputter-coated with a fine gold layer. Specimens were examined in a Phillips XL30 at 15 kV, or a Hitachi FE S-4800 Scanning Electron Microscope operated at 5 kV.

**Electrophysiological measurements.** The compound action potential (CAP) and endocochlear potential (EP) were recorded from mice aged between 28 and 33 days postnatal (7 $Dmdo^+/+$, 6 $Dmdo/Dmdo$, 5 $+/+$). Animals were anaesthetised, the middle
ear opened and a silver wire recording electrode placed on the round window\textsuperscript{39}. Calibrated toneburst stimuli (15 ms duration, 1 ms rise/fall time, 100 ms interstimulus interval) were delivered through a closed sound system and responses to 200 stimuli were averaged. Thresholds for visual detection of the compound action potential, reflecting cochlear nerve activity, were established by varying intensity in 2 dB steps. After response recording, the endocochlear potential was measured by inserting a micropipette electrode filled with 150 mM KCl into the scala media through the lateral wall of the basal turn of the cochlear duct.

**Genetic mapping.** $Dmdo/^+$ mutants on a C3HeB/FeJ genetic background were outcrossed to mice from the C57BL/6J inbred strain, and $Dmdo/^+$ F1 offspring from this outcross were backcrossed to wild type (+/+) C3HeB/FeJ mice to generate backcross offspring. These were either wild type (+/+) or mutant ($Dmdo/^+$), and classification as a mutant was based upon a reduced or absent Preyer’s reflex and subtle headshaking behaviour. DNA samples from 35 backcross offspring animals were screened with 60 simple tandem repeat markers polymorphic between C3HeB/FeJ and C57BL/6J, spaced at approximately 20 cM intervals throughout the genome, selected from the Whitehead/MIT database (http://www-genome.wi.mit.edu). Genotyping was carried out by polymerase chain reaction (PCR), using standard techniques. After the initial genome scan, the crude location was refined using additional chromosome 6 markers and additional backcross mice (160 heterozygotes and 138 wildtype).

**Sequencing.** Genomic DNA was prepared from mice identified as homozygous mutants or wildtype by Preyer reflex or by scanning electron microscopy. Primers were designed using the primer3 program (http://frodo.wi.mit.edu/primer3/primer3_code.html\textsuperscript{40} to cover every Ensembl (http://www.ensembl.org/index.html) and VEGA (Vertebrate Genome Annotation; http://vega.sanger.ac.uk/index.html) exon within the critical region. The exon sequences were compared to identify mutations. The primers for
amplifying and sequencing exons 4-6 of 2310005E10Rik from cDNA (Fig S6) and for checking the presence of Ocm and Slc26a5 are described in Supplementary Table 4. Genes within the same region were checked against the microarray for differential expression; Pms2, Bhlha15, Lmtk2, Bri3, Eif2akl and 4921520G13Rik (Ocm), and Psmc2, Reln, Pmpcb, Dnajc2 and Armc10 (Slc26a5). PCR conditions for 2310005E10Rik: 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (58°C for 30 seconds) and extension (72°C for 30 seconds), ending with 5 minutes at 72°C. For Ocm and Slc26a5, a touchdown PCR was used: 94°C for 2 minutes, followed by 16 cycles of denaturation (94°C for 30 seconds), annealing (64°C for 45 seconds, decreasing by 0.5°C per cycle) and extension (72°C for 45 seconds), then 21 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 45 seconds) and extension (72°C for 45 seconds), ending with 7 minutes at 72°C.

**Microarrays.** RNA was amplified and labelled using the Ambion Illumina® TotalPrep™ RNA Amplification Kit (AMIL1791). 1.5µg of cRNA were applied to Illumina Mouse-6 BeadChips, following the manufacturer's instructions. Following hybridisation, washing and detection, chips were scanned using the Illumina 500GX scanner. The data was normalised using a quantile normalisation, assuming that the overall intensity distributions of the arrays should be comparable and analysed using the LUMI and LIMMA packages from Bioconductor. The P-value was adjusted for multiple tests as described in Benjamini and Hochberg (1995); the P-value cut-off was set to 0.05.

**In situ hybridisation.** In situ hybridisation was carried out using the automated Ventana Discovery systems with the Ventana reagents (Bluemap kit (cat. no. 760-120), Protease 3 (cat. no. 760-2020), Ribomap (cat. no. 760-102), ISH Red counterstain (cat. no. 780-2186), CC1 (cat. no. 950-124), EZPrep (cat. no. 950-100), LCS (cat. no. 650-010), RiboWash (cat. no. 760-105), Reaction Buffer (cat. no. 95-300), and RiboCC (cat. no. 760-
The SA-Alk Phos from the Bluemap kit was not used, and instead of the anti-Biotin, anti-DIG-AP antibody (Roche, cat. no. 11093274910) was used, diluted 1:2000 in Ribohybe (Ventana, cat. no. 760-104), which reduced the background staining. Hybridisation was carried out at 45°C, and low stringency washes at 50°C in 0.1xSSC. Probes against miR-96 (39208-05; 3'-end DIG labelled), miR-182 (39070-05; 3'-end DIG labelled), miR-183 (39071-05; 3'-end DIG labelled), and against a control sequence known to match nothing in the mouse genome (99000-05, 99003-05, 3'-end DIG labelled) were obtained from Exiqon. Probes were diluted (0.25 µl miR-96, 0.05 µl miR-182, 0.25 µl miR-183 and a variety of concentrations of the control probe) in 100 µl Ribohybe (Ventana, cat. no. 760-104). They were denatured for 10 minutes at 90°C before hybridisation. After staining, slides were dehydrated and mounted in Eukitt.

**Transfections and Luciferase assays.** For each candidate gene, a region of the 3’UTR containing the putative target site(s) was amplified from mouse genomic DNA and cloned into a modified PGL3 control vector downstream of the luciferase stop codon (Supplementary Table 5). Mutant constructs were made by replacing the putative binding site with an EcoRI site to disrupt it. Mouse NIH 3T3 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 2 mM l-glutamine and 10% foetal bovine serum (FBS) (Gibco, Invitrogen). NIH 3T3 cells (3 × 10⁴ cells/well) were transfected 18 hours after seeding with Lipofectamine 2000 (Invitrogen) with WT or mutant constructs (300 ng) either alone, with 10 nM siRNA-96 (Sigma) or siRNA-140 (Dharmacon). siRNA-140 was used as a negative control for a miRNA not expressed in the organ of Corti. siRNA-96 contained 5’-UUUGGCACUAGCACAUUUUUGCU-3’ and 5’-CAAUCAUGUGUAGUAGCCAAUAU-3’ oligonucleotides. siRNA-140 contained 5’-CAGUGGUUUUACCCUAUGGUAG-3’ and 5’-ACCAUAGGGUAAAACCACUGAG-3’ oligonucleotides. The positive control miR-96 target construct, made by inserting a synthetic double-stranded oligonucleotide which perfectly complemented the mature miR-96 into the modified pGL3 vector, was
transfected either alone, with siRNA-96 or with siRNA-140 in each transfection. Cells
-treated only with Lipofectamine served as negative controls. Transfections were carried
-out 4 times in triplicate using at least 2 independent plasmid preparations for each gene.
Luciferase assays were carried out 24 hours after transfection using the Luciferase
Reporter Gene Assay kit (Roche) according to the manufacturer’s protocol. Luciferase
activity was measured using a multilabel counter (Victor², Perkin–Elmer, MA) and
normalised to protein content measured using the BCA protein assay kit (Pierce) to
account for variability in cell number and size between wells. For the positively
validated target genes, assays were carried out using an siRNA to mimic the
Diminuendo mutation (Dim-96), in the same way as above with the following
exceptions: Cells were transfected with WT or mutant constructs (300ng) either alone or
with 10 nM Dim-96 (Sigma). Dim-96 contained 5′-
UUUGGCUCUAGCACAUUUUUUGCU-3′ and 5′-
CAAUCAUGUGUAGUGCCAUAU-3′ oligonucleotides. Relative reporter activity
for siRNA-96, Dim-96 or siRNA-140 treated cells was obtained by normalisation to the
activity of the respective WT, mutant or positive control plasmid constructs transfected
alone.

Real-time PCR. RNA from the organs of Corti of four day-old wildtype and
homozygous littermates was treated with DNase 1 (Sigma, cat.no. AMP-D1) according
to the manufacturer's instructions. In total, RNA from 11 wildtype mice and 11
homozygote mice was used for the RTPCR assays described. RNA concentration was
measured using a Nanodrop spectrophotometer (ND-1000 or ND-8000) and samples
within each pair were brought to the same concentration by dilution. cDNA was created
from the normalised RNA using the Superscript II Reverse Transcriptase kit
(Invitrogen, cat. no. 11904-018). Primers and the Taqman Master Mix were purchased
from Applied Biosystems (Ocm: MM00712881_m1, Hprt1: MM01318747_g1,
MM01545399_m1, Slc26a5, Sdc2, Anxa4, Kcnal10, Chrna9, Gm414, Pitpnml, Ptprq,
*Gfi1*: 4331348 (manual design using the Applied Biosystems program FileBuilder),

*Odf2*: MM00496679_g1, *Celsr2*: MM01199698_g1, *Aqp5*: MM01300523_m1, *Ryk*: MM01238549_m1, *Tuba8*: Mm01184204_m1, *Cpne9*: Mm01184596_m1, *Gabrb3*: Mm01324929_m1, *Ngfr*: MM00446296_m1, Master Mix: 4364340).

*Hprt1* was used as an internal control, and the quantity of sensory tissue present was checked using *Ngfr*, which is expressed in pillar cells and Hensen's cells\(^3\). cDNA was only used when *Ngfr* levels did not differ significantly between wildtype and homozygote littermates. The reaction was run on an ABI7000 machine (Applied Biosystems).

**Immunohistochemistry.** The primary antibodies used were anti-oncomodulin (Swant, cat.no OM3, diluted 1:500), anti-prestin (Santa Cruz, cat.no sc-22694), anti-Pitpnm1 (Abcam, cat. no. AB22823, diluted 1:25), anti-Gfi1 (Santa Cruz, cat.no. sc-8558), anti-Aqp5 (Sigma Aldrich, cat. no A4979, diluted 1:25), anti-Odf2/Cenexin1 (Abcam, cat. no. AB43840, diluted 1:20), anti-Ryk (GeneTex, cat.no. GTX25518, diluted 1:10), anti-Celsr2 (Abcam, cat.no. AB12957, diluted 1:400), anti-Myo7a (Proteus, cat.no 25-6790, diluted 1:50), anti-Sox2 (AbCam, cat.no ab15830, diluted 1:50), anti-Jag1 (Santa Cruz, cat.no sc-6011, diluted 1:50), and anti-Cdkn1b (Cell Signaling, cat.no 2552, diluted 1:50). The anti-Myrip antibody, which was used at a dilution of 1:150, was kindly supplied by Aziz El-Amraoui and Christine Petit and has been previously described\(^4\). The anti-Ptprq antibody, which was used at a dilution of 1:10, was a gift from Guy Richardson, and has also been previously described\(^1\). The secondary antibodies were anti-goat (Jackson ImmunoResearch, cat.no 705-065-147) and anti-rabbit (Jackson ImmunoResearch, cat.no 711-065-152), diluted 1:100. All antibodies were diluted in staining solution (10% foetal calf serum, 0.1% Triton, 2% BSA and 0.5% sodium azide in PBS).

The Ventana Discovery staining systems were used with the Ventana reagents (DABMap\(^\text{TM}\) Kit (cat.no 760-124), Hematoxylin (cat.no 760-2021), Bluing reagent
(cat.no 760-2037), CC1 (cat.no 950-124), EZPrep (cat.no 950-100), LCS (cat.no 650-010), RiboWash (cat.no 760-105), Reaction Buffer (cat.no 95-300), and RiboCC (cat.no 760-107)) to carry out the staining run. For each antibody, wildtype and homozygote sections were treated according to the same protocol.

**Binding factor analysis.** Transcription start site coordinates for the upregulated (356 genes) and downregulated (425) gene sets were retrieved from Ensembl49 mouse release 50 using Biomart50 (downloaded 29/9/2007). 500 bp long repeat-masked upstream sequences for all transcripts of these genes were retrieved using the Ensembl API51. Overlapping upstream sequences were joined. WU-BLAST dust52 was used for removing simple dinucleotide repeats, and those sequences which did not contain at least 100bp of non-degenerate positions were removed.

Overrepresented sequence motifs were discovered using NestedMICA53 allowing for motifs on either strands (-revComp) and of lengths between 6 and 12 nucleotides (-minLength 6 -maxLength 12). The number of motifs was set to 20 (-numMotifs 20) and the expectedUsageFraction parameter was set to 0.2 (specifies a prior belief that each motif is expected to be found in 20% of the input sequences). The sequence background model used was estimated from the input sequences using a Markov chain of order 1 (-order1) and 4 mosaic classes (-classes 4). Motif sets were estimated separately for the upregulated and downregulated upstream sequence set.

Distances between sequence motifs were calculated as described in ref. 60. TRANSFAC release 12.233 was compared against the upregulated by selecting the subset of TRANSFAC sequence motifs which correspond to transcription factors found among the 500 top upregulated and downregulated genes. Motifs were compared to find reciprocal closest matches between either of the NestedMICA discovered upregulated or downregulated motif sets and the mouse TRANSFAC motifs. Reciprocally matching motifs are those motif pairs that are present both in the list of
closest TRANSFAC motifs to the NestedMICA motifs and in the list of closest NestedMICA motifs to the TRANSFAC motifs. p-values were calculated by shuffling the discovered motif set 1000 times and counting the number of times the observed distance or smaller is achieved with the shuffled motifs, and dividing this by the number of shuffles; only p-values < 0.02 were included.

**Equipment and settings.** Electron microscopy: Some specimens were examined under a Phillips XL30 scanning electron microscope, at 10-15kV (Fig.1a,b, and Fig. S1a-k, p). High resolution Scanning Electron Microscopy analyses were performed using SE-4800 (Hitachi) at 5kV (Fig 1c-h). Postacquisition image analyses were performed using Adobe Photoshop.

All other images (Fig.2b-g, Fig.3b-k, Fig. S3d-m, S5) were taken using a Zeiss Axioskop 2 microscope with the Plan Neoflur 63x 1.4NA objective, a Zeiss Axiocam camera and associated Axiocam software.

Adobe Photoshop was used for processing all images and preparing figures. Minimal adjustments were made, including rotation and resizing. Where image settings were altered, both wildtype and mutant images were subjected to the same processing.

**Microarray database information.** The microarray data has been deposited in the ArrayExpress database, accession number E-TABM-489.
Supplementary Tables
| Abbreviation | Molecular Function | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|-------------------|---------------|----------------|------------------|--------|-------|------------|
| 0610006I08Rik| - integral to membrane | - | 2 | RIKEN cDNA 0610006I08 gene (0610006I08Rik), mRNA | 0.0294925 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomict 32, 1000-1011 (2004). | |
| 1110008J03Rik| - | - | 1 | RIKEN cDNA 1110008J03 gene (1110008J03Rik), mRNA | 0.033144 | | |
| 1601004P12Rik| - | - | 1 | thioredoxin family Trp26 | 0.029561 | | |
| 2410160G09Rik| RNA binding | nuclear prenosome | 1 | RIKEN cDNA 2410160G09 | 0.0474408 | | |
| Pbm23 | nucleic acid binding | - | mRNA processing | 1 | Bone marrow macrophage cDNA, RIKEN full-length enriched library clone 830042K01 product Similar to S164 protein homolog (Fragment) | 0.0204618 | | |
| 2610507B11Rik| malate dehydrogenase (acceptor) | extracellular space | 1 | RIKEN cDNA 2610507B11 gene (2610507B11Rik), transcript variant 1, mRNA | 0.094795 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomict 32, 1000-1011 (2004). | |
| 2610532J11Rik| - | membrane | 1 | RIKEN cDNA 2610532J11 gene (2610532J11Rik), mRNA | 0.0222482 | | |
| 2700097D09Rik| methyltransferase | extracellular space | 1 | RIKEN cDNA 2700097D09 gene (2700097D09Rik), mRNA | 0.0547931 | | |
| Fam63a | - | - | 1 | RIKEN cDNA 4920354E16 gene (4920354E16Rik), mRNA | 0.0462638 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomict 32, 1000-1011 (2004). | |
| Fam96a | - | extracellular space | - | Protein FAA066A | 0.035599 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomict 32, 1000-1011 (2004). | |
| 6820408C78Rik| - | - | 1 | RIKEN cDNA 6820408C78 gene (6820408C78Rik), mRNA | 0.0390381 | | |
| 8430410D09Rik| - | - | 2 | RIKEN cDNA 8430410D09 gene (8430410D09Rik), mRNA | 0.03405256 | | |
| A130010J13Rik| - | - | 1 | interferon regulatory factor 6 | 0.053701 | | |
| Abbreviation | Molecular Function | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|-------------------|---------------|----------------|------------------|--------|-------|-----------|
| Rlip12       | transporter       | intracellular, membrane | transport | 1 | RIKEN cDNA A330019N05 gene (A330019N05Rik), mRNA | 0.0315487 |       |
| Afca1        | ATP binding, nucleoside triphosphatase | Golgi, plasma membrane | cholesterol transport, lipoprotein biosynthesis, phospholipid, phospholipid translocation | 1 | ATP-binding cassette, sub-family A (ABC), member 1 | 0.0629615 |       |
| Aff2         | intracellular signalling cascade | intracellular junction | - | 3 | AHNAK nucleoprotein (desmoyokin) | 0.0649064 |       |
| Ahn1a2       | retinol dehydrogenase, 3-chloroallyl aldehyde dehydrogenase | - | - | 1 | expressed sequence AI552857 | 0.0849201 |       |
| Ar           | androgen receptor, DNA/lipid/zinc ion/steroid binding | cytoplasm, nucleus | embryonic development, male gonad development, male somatic sex determination, DNA-dependent regulation of transcription | 1 | androgen receptor | 0.0413816 |       |
| Abdf         | DNA binding, | intracellular signalling cascade, DNA-dependent transcription regulation | - | 2 | ankyrin repeat and SOCS box-containing protein 6 | 0.0319869 |       |
| Abbreviation | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|---------------|----------------|------------------|--------|-------|-------------|
| Atad3a       | ATP binding, nucleoside triphosphatase | 1 | ATPase family, AAA domain containing 3A | 0.098284 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004). |
| Aof          | actin binding, calcium ion binding | 1 | actin filament organisation, barbed-end actin filament capping, response to stress, cytoskeleton organisation and biosynthesis | 0.0141104 | | |
| Btc38        | - | 1 | expressed sequence AIY124722 | 0.069329 | | |
| Evt5         | - | 1 | Riken cDNA B13050523 gene. | 0.0567175 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004). |
| BCD13529     | - | 1 | cDNA sequence BCD13529 | 0.0830296 | | |
| Nduv42       | cytochrome-c oxidase | 1 | cDNA sequence BC094511 | 0.0359728 | | |
| Byp2         | actin binding, receptor signal transducer | 1 | bromodomain and PHD finger containing 3 | 0.0659125 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004). |
| Bacb1        | - | 1 | BSD domain containing 1 | 0.0295159 | | |
| Cgaj1II      | - | 1 | cactivin resistance related protein CRIRp | 0.0508682 | | |
| Calpr1       | calcium ion binding | - | calcium binding protein 1 | 0.0559617 | | |
| Cdr20        | calcium ion binding, protein binding | 1 | cadherin 20 | 0.0043529 | | |
| Calcr2       | calcium ion binding, G-protein coupled receptor | 1 | cadherin EGF-LAG seven-pass G-type receptor 2 | 0.0768963 | | Shimaa Y. et al. Differential expression of the seven-pass transmembrane cadherin genes Calcr1-3 and distribution of the Calcr2 protein during mouse development. Dev. Dyn. 223, 321-332 (2002). |
| Chnaf        | extracellular ligand-gated ion channel, GABA-A receptor, nicotinic acetylcholine receptor | 1 | cholinergic receptor, nicotinic, alpha pentapeptide 6 | 0.0507812 | | |
| Abbreviation | Molecular Function | Gene Ontology | Number of sites | Brief Description | P-base | Notes |
|--------------|-------------------|---------------|----------------|------------------|--------|-------|
| Commd1       | RNA binding, zinc ion binding | ribonucleoprotein complex | - | 1 | 0.0152056 | |
| Cthb         | cysteine-type endopeptidase, hydrolase, peroxidase | lysosome, mitochondrion | proteolysis, regulation of catalytic activity, response to oxidative stress | 1 | cathespin B | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. GenomRes 83, 1009-1011 (2004). |
| Cugbp2       | - | nucleus, soluble fraction | mRNA splice site selection | 1 | CUG Inlet repeat. RNA binding protein 2 | Expressed in inner ear (Pompeia et al., 2004). Members of this protein family regulate pre-mRNA alternative splicing and may also be involved in mRNA editing, and translation (Ladd et al., 2001). Lastd A.N., Chantel N. and Cooper T.A. The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol. Cell. Biol. 21, 1285-1296 (2001). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. GenomRes 83, 1009-1011 (2004). |
| Dhcr24       | electron carrier, oxidoreductase acting on the CH-CH group of donors (NAD or NADP as acceptor) | endoplasmic reticulum, extracellular space, Golgi, membrane | amyloid precursor protein | 1 | 24-dehydrocholesterol reductase | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. GenomRes 83, 1009-1011 (2004). |
| Dnlf         | RNA binding | germ cell development | - | 1 | dead end homolog 1 (zebrafish) | 0.0721547 |
| Ddx32        | dsRNA binding, FAD binding, oxidoreductase | intracellular | RNA processing | 2 | dithyruondine synthase 2-like (SUMF1, S. cerevisiae) | 0.0038582 |
| Eohb1        | catalytic activity | - | metabolism | 1 | enolase, pyruvate dehydrogenase domain containing 1 | 0.005158 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. GenomRes 83, 1009-1011 (2004). |
| Elec2        | endonucleases, hydrolases, zinc ion binding | nucleus | RNA processing | 1 | elaC homolog 2 (E. coli) | 0.00778281 |
| Fbxw11       | - | - | ubiquitin cycle | 1 | F-box and WD-40 domain protein 11 | 0.0348913 |
| Fgfl2        | fibroblast growth factor receptor binding, growth factor | endoplasmic reticulum, extracellular space, nucleus | cell proliferation, organ induction, cell cycle progression regulation, signal transduction | 1 | fibroblast growth factor 3 | Fgf3 has an early function regulating Atoh1 (Millimaki et al., 2007), and is later expressed in hair and supporting cells around the time of differentiation (Wilkinson et al., 1989). Mice null for Fgf3 had malformed inner ears, and showed a reduced Phayre reflex and vestibular defects (Manessur et al., 1993). Manessur S.L., Goddard J.M. and Capeschi M.R. Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. Development 117, 13-28 (1993). Wilkinson D.G., Bhalt S. and McMahon A.P. Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. Development 105, 131-136 (1989). Millimaki B.B., Sweet E.M., Dhason M.S. and Riley B.B. Zebrafish aih1 gene: classic proneural activity in the inner ear and regulation by Fgf and Notch. Development 134, 295-305 (2007). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. GenomRes 83, 1009-1011 (2004). |
| Goa2         | glutamate decarboxylase, carbonyl-lyase | axon, membrane, synapse | carboxylic acid metabolism, neurotransmitter biosynthesis, precursor synaptic transmission | 1 | glutamic acid decarboxylase 2 | 0.058235 |
| Gm1574       | - | - | - | 1 | gene model 1574, (NCBI) | 0.00106197 |
| Abbreviation | Molecular Function | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|-------------------|---------------|----------------|------------------|--------|-------|------------|
| Gpc3         | GPI anchor binding extracellular matrix; plasma membrane | negative regulation of cell proliferation, organ morphogenesis, positive regulation of BMP signalling pathway, ureteric bud branching | 1 | glypican 3 | 0.0197913 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomik 83, 1006-1011 (2004). |
| Gpt134 | G-protein coupled receptor activity, protein binding, signal transducer activity | neuropeptide signalling pathway, signal transduction, GPCR signalling pathway | 1 | G protein-coupled receptor 134 | 0.0727916 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomik 83, 1006-1011 (2004). |
| Gpr137 | receptor | membrane | - | 2 | G protein-coupled receptor 137 | 0.0298531 |
| Gpr161 | G-protein coupled receptor, signal transducer | signal transduction, G-protein coupled receptor signalling pathway | 1 | G protein-coupled receptor 161 | 0.0294143 |
| Hspa2 | ATP and protein binding | mitochondrion | protein binding, response to heat, response to unfolded protein | 1 | heat shock protein 2 | 0.00125923 |
| Kat5 | histone acetyltransferase, chromatin/DNA/miRNA protein binding, transcription coactivator | chromatin, nucleus, transcription factor complex | chromatin assembly/disassembly, positive regulation of transcription from RNApoly2 promoter, regulation of cell growth, DNA-dependent transcription regulation | 1 | HIV-1 tat interactive protein, homolog (human) | 0.0727345 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomik 83, 1006-1011 (2004). |
| Insig2 | protein binding | membrane | response to steroid depletion | 1 | insulin induced gene 2 | 0.0725746 |
| Itga3 | G-protein coupled receptor, neuropeptide Y receptor, rhodopsin-like receptor, serotonin receptor, adrenoceptor, signal transducer | membrane | signal transduction, G-protein coupled receptor signalling pathway | 1 | 5-hydroxytryptamine (serotonin) receptor 1B | 0.09307155 |
| Irga3 | protein binding, receptor | basolateral plasma membrane, synaptosome, integrin complex | cell adhesion, integrin-mediated signalling pathway, memory, neuron migration | 1 | integrin alpha 3 | 0.0403344 | In mice, Irga3 is expressed before birth in the epithelia adjacent to the pro-sensory areas in both auditory and vestibular systems, but it is later upregulated between birth and P6 in the sensory epithelium as well (Davies and Holley, 2002). Davies D. and Holley M.C. Differential expression of alpha 3 and alpha 8 integrins in the developing mouse inner ear. J. Comp. Neurol. 445, 122-132 (2002). |
| Abbreviation | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|---------------|----------------|------------------|--------|-------|------------|
| Itpr2        | molecular function | 1              | Insoluble 1,4,5-triphosphate receptor 2 | 0.00202167 | Insoluble 1,4,5-triphosphate receptors are known to be expressed in frog vestibular canal hair cells and may be involved in signal transduction (Rossi et al., 2006). | Rossi M.L. et al. IP3 receptor in the hair cells of frog semicircular canal and its possible functional role. *Eur. J. Neurosci.* **23**, 1775-1783 (2006). |
| Lrrc33       | protein binding | -              | leucine rich repeat containing 33 | 0.0303323 |       |            |
| Lyplal1      | hydrolyase     | -              | lysophospholipase-like 1 | 0.0391469 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. *Genomics* **83**, 1000-1011 (2004). |
| Med2112      | -              | -              | mab-21-like 2 (C. elegans) | 0.0332391 |       |            |
| Mag3         | kinase         | -              | membrane associated guanylate kinase, R1P and PDZ domain containing 3 | 0.050782 |       |            |
| Mag3l7p1     | catalytic activity, enzyme activator, protein binding, TGFbeta receptor cytoplasmic mediator | -              | mitogen-activated protein kinase kinase 7 interacting protein | 0.0274843 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. *Genomics* **83**, 1000-1011 (2004). |
| Mafb5        | -              | membrane       | major facilitator superfamily domain containing 5 | 0.00778281 | PS-P12 mouse organ of Corti (MGI Accession ID: MGI:1923096). |            |
| Mof442       | -              | nucleus        | mortality factor 4-like 2 | 0.078086 | Expressed in inner ear (Pompeia et al., 2004). | Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. *Genomics* **83**, 1000-1011 (2004). |
| Mpp7         | protein binding | membrane       | membrane protein, palmitoylated | 0.0394494 |       |            |
| Myr3p         | actin, zinc ion, myosin, Rab GTPase binding | 1              | myosin VIA and Rab interacting protein | 0.0247953 |       |            |
| Abbreviation | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|---------------|----------------|------------------|--------|-------|------------|
| Nlgn2        | protein binding, serine esterase | membrane, synapse | cell adhesion, synapse organisation and biogenesis | 1 | neustiglin 2 | 0.0295928 |
| Nme4         | ATP binding, magnesium ion binding, nucleoside diphosphate kinase, transerase | extracellular space, mitochondrion | CTP/UTP/UTP biosynthetic process, nucleotide metabolic process | 1 | expressed in non-metastatic cells, protein | 0.0542175 |
| EG4234008    | - | - | - | 1 | 12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130077N03 | |
| fit4         | structural molecule activity | basement membrane, extracellular matrix, extracellular space | neuron remodelling | 1 | retin 4 | 0.0578731 |
| Otf2         | structural constituent of cytoskeleton | - | outer dense fiber of sperm tail 2 | 0.0007573 | Expressed in inner ear (Pompeia et al, 2004). | |
| Ogt          | Protein N-acetylglucosaminyltransferase | intracellular | protein amino acid O-linked glycosylation | 1 | O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) | 0.0610938 |
| Obf1361      | olfactory receptor, G-protein coupled receptor, signal transducer | membrane | G-protein coupled receptor protein signaling pathway, response to stimulus, sensory perception of smell, signal transduction | 1 | olfactory receptor 1361 | 0.0400684 |
| Olfr1361     | - | - | lipid transport, steroid metabolism | 1 | oysteroid binding protein-like 2 | 0.010257 |
| Otop2        | - | membrane | - | 1 | otopin 3 | 0.0071267 |
| Pdgf         | growth factor activity, platelet-derived growth factor receptor binding | extracellular space, membrane | cell proliferation, cellular process, regulation of peptidyl-tyrosine phosphorylation, cell cycle progression regulation | 1 | platelet-derived growth factor, D polypeptide | 0.067474 |
| Pelo         | - | nucleus | translation | 1 | pelota homolog (Drosophila) | 0.016192 |
| Pim1         | - | - | - | 1 | phosphatidylinositol 3-kinase | 0.0330777 |

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Pompeia C. et al. Genes expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004).
| Abbreviation | Gene Ontology | Number of sites | Brief Description | P-base | Notes |
|---------------|---------------|----------------|------------------|--------|-------|
| Pht10 | DNA/zinc ion/protein binding | 1 | DNA-dependent regulation of transcription | 0.0058649 | |
| Phf21a | chromatin/DNA/zinc ion/protein binding | 1 | PHD finger protein 21A | 0.0398443 | |
| Phc4 | phosphomonoester phosphatase C activity | 1 | phosphatase C, beta 4 | 0.065705 | |
| Plaklin7 | - | 1 | pleckstrin homology domain containing, family M (with RHO domain) member 1 | 0.039331 | |
| Phk1a | cAMP binding, cAMP-dependent protein kinase regulatory subunit (type I), nuclear, nucleotide binding, protein binding | 1 | protein kinase, cAMP dependent regulatory, type I, alpha | 0.0946429 | Expressed in otic vesicle (Powles et al., 2004). Powles N., Babbs C., Ficker M., Schimmang T. and Maconochie M. Identification and analysis of genes from the mouse otic vesicle and their association with development subprocesses through in situ hybridisation. Dev. Biol. 268, 24-38 (2004). |
| Phf3 | membrane | 1 | proline-rich transmembrane protein 3 | 0.0137544 | |
| Jmjd6 | receptor | 2 | phosphatidylinositol receptor | 0.0010458 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004). |
| Phn2 | prenylated/dinucleotide-binding, membrane-spanning, protein tyrosine phosphatase, hydrolase, transporter | 1 | protein tyrosine phosphatase, non-receptor type 2 | 0.0372651 | Pphp2 is expressed in the otocyst at E9 (MGI accession numbers: MGI:2032045, MGI:2032056). It’s involved with secretory vesicle fusion, and is implicated in neural tube, craniofacial and bone development (Wang et al., 2005). Wang Y. et al. Tyrosine phosphatase MEG2 modulates murine development and platelet and lymphocyte activation through secretory vesicle function. J. Exp. Med. 202, 1587-1597 (2005). |
| Rglap1 | - | 1 | recombination activating gene 1 activating protein 1 | 0.0162883 | |
| Rods2 | protein binding | 1 | regulator of chromosome condensation (RCC1 and BTB (POZ) domain containing protein 2 | 0.03898 | |
| Rhpl2 | protein binding | 1 | rhophilin, Rho GTPase binding protein 2 | 0.0291402 | Expressed in inner ear (Pompeia et al., 2004). Pompeia C. et al. Gene expression profile of the mouse organ of Corti at the onset of hearing. Genomics 83, 1000-1011 (2004). |
| Rive1 | - | 1 | RAD50-interactor 1 | 0.0407397 | |
| Abbreviation | Gene Ontology               | Number of sites | Brief Description                  | P-base      | Notes                                                                 |
|--------------|-----------------------------|-----------------|------------------------------------|-------------|----------------------------------------------------------------------|
| Rnf34        | ligase, zinc ion/protein/nucleic acid binding | nucleus, membrane | apoptosis, ubiquitin cycle | 1 | ring finger protein 34 | 0.0595252 |
| Rps6ka6      | ATP/nucleotide binding, protein serine/threonine kinase, protein tyrosine kinase, transferase | plasma membrane | - | protein amino acid phosphorylation | 1 | ribosomal protein S6 kinase polypeptide 6 | 0.0224016 |
| Ryk          | ATP/Wnt protein binding, Wnt receptor, transferase, protein tyrosine kinase | plasma membrane | - | serine/threonine kinase, protein-tyrosine kinase, transferase | 1 | receptor-like tyrosine kinase | 0.0025563 |
| Scamp3       | - | Golgi membrane | intracellular protein transport | 1 | secretary carrier membrane protein 3 | 0.0196449 |
| Sgm3p        | - | zinc ion binding | - | - | - | splicing factor, arginine/serine-rich 2, interacting protein | 0.0343134 |
| Sh3klp1      | protein binding, kinase activity | membrane, synapse, synaptosome | apoptosis, endocytosis | 1 | SH3-domain kinase binding protein 1 | 0.0162249 |
| Sic16a13     | - | membrane | transport | 1 | solute carrier family 15 (monocarboxylic acid transporters), member 13 | 0.0517607 |
| Slc25a1      | nucleotide-sugar transporter | extracellular space, Golgi membrane, membrane | carboxylate transport, nucleotide-sugar transport | 1 | solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 | 0.0510738 |
| Sdc2a1       | DNA binding, transcription factor, transcription factor complex | - | - | - | - | SRY-box containing gene 5 | 0.0562473 |
| Cytb         | - | nucleus | - | - | - | spectrin domain with coiled-coil | 0.0521244 |
| Spex1        | protein binding, extracellular matrix | extracellular space | cell adhesion, multicellular organism development | 1 | spexin 1, (Ispexin) extracellular matrix protein | 0.0420364 |

**Abbreviation**
- Rnf34: ring finger protein 34
- Rps6ka6: ribosomal protein S6 kinase polypeptide 6
- Ryk: receptor-like tyrosine kinase
- Scamp3: secretory carrier membrane protein 3
- Sgm3p: splicing factor, arginine/serine-rich 2, interacting protein
- Sh3klp1: SH3-domain kinase binding protein 1
- Sic16a13: solute carrier family 15 (monocarboxylic acid transporters), member 13
- Sdc2a1: solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1
- Cytb: spectrin domain with coiled-coil
- Spex1: spexin 1, (Ispexin) extracellular matrix protein

**References**
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- Ladefoged V., Brehinger R.R. and de Crombrugghe B. L-Socl, Socl and Socl control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 9, S89-101 (2004).
| Abbreviation | Molecular Function | Gene Ontology | Number of sites | Brief Description | P-base | Notes | References |
|--------------|-------------------|---------------|----------------|-----------------|--------|-------|------------|
| St3gal3      | N-acetyllactosaminyl-alpha 2,3-sialyltransferase activity; transferring glycosyl groups | extracellular space, Golgi membrane, membrane | protein amino acid glycosylation | 1 | ST3 beta-galactoside alpha-2,3-sialyltransferase 3 | 0.0239336 |
| St3gal4      | beta-galactoside alpha 2,3-sialyltransferase activity; transferring glycosyl groups | extracellular space, Golgi membrane, membrane | protein amino acid glycosylation | 1 | ST3 beta-galactoside alpha-2,3-sialyltransferase 4 | 0.0498208 Expressed in inner ear (Pompeia et al., 2004). |
| St8sia3      | alpha-N-acetyllactosaminyl-alpha 2,8-sialyltransferase activity; transferring glycosyl groups | Golgi membrane, membrane | protein amino acid glycosylation | 1 | ST8 alpha-N-acetyllactosaminyl-alpha 2,8-sialyltransferase 3 | 0.0730389 Expressed in inner ear (Pompeia et al., 2004). |
| Skf19        | ATP/magnesium ion binding, protein serine/threonine kinase, transferase | nucleus | - | 3 | serine/threonine kinase 19 | 0.0263286 Expressed in inner ear (Pompeia et al., 2004). |
| Tbr1         | DNA binding, transcription factor | nucleus | axon guidance, brain development, positive regulation of transcription from RNApol2 | 1 | T-box brain gene 1 | 0.0301581 |
| Tbr18        | DNA binding, transcription factor | nucleus | DNA-dependent regulation of transcription | 1 | T-box 15 | 0.070131 |
| Tcf7l2       | beta-catenin/DNA binding, transcription factor | nucleus, transcription factor complex | Wnt receptor signalling pathway; positive regulation of transcription from RNApol2 promoter | 1 | transcription factor 7-like 2, T-cell specific, HMG-box | 0.0247246 Expressed in inner ear (Pompeia et al., 2004). |
| Tm9sf4       | transporter | membrane | transport | 1 | transmembrane 9 superfamily protein member 4 | 0.05303937 |
| Tmem119      | - | membrane | - | 1 | transmembrane protein 119 | 0.05397672 |
| Tmem87       | - | membrane | protein targeting | 1 | transmembrane protein 81 | 0.015639 |
| Trib3        | AT/Protein/protein kinase binding, protein kinase inhibitor, protein kinase, transcription corepressor | nucleus | apoptosis, negative regulation of protein kinase activity, protein amino acid phosphorylation, DNA-dependent transcription regulation | 1 | tribbles homolog 3 (Drosophila) | 0.0729735 |
| Tsc2d3       | transcription factor | - | anti-apoptosis, DNA-dependent regulation of transcription | 1 | TSC22 domain family 3 | 0.0339536 |
| Abbreviation | Gene Ontology References | Number of sites | Brief Description | P-base | Notes |
|--------------|-------------------------|----------------|------------------|--------|-------|
| Tspan1       | -                       | 1              | tetraspanin 1    | 0.0371482 |       |
| Ubc2g1       | ligase, ubiquitin-       | 1              | protein          | 0.0472396 |       |
|              | conjugating enzyme,     |                | modification,    |        |       |
|              | ubiquitin-protein       |                | ubiquitin cycle,|        |       |
|              | ligase                  |                | ubiquitin-       |        |       |
|              | dependent               |                | protein          |        |       |
|              | catabolic process       |                |                  |        |       |
| Uck2         | uridine kinase, ATP    | 1              | uridine-         | 0.0189398 |       |
|              | binding, transmembrane |                | cytidine kinase 2 |        |       |
| Usn48        | -                       | 1              | ubiquitin specific | 0.0734992 |       |
|              | peptidase 45            |                |                  |        |       |
| Uspl1        | hydrolase               | 1              | ubiquitin specific | 0.0143279 |       |
|              | peptidase like 1        |                |                  |        |       |
| Var1         | oxidoreductase activity,| 1              | vascular        | 0.0700936 |       |
|              | zinc ion binding        |                | amine transport |        |       |
|              | protein 1 homolog (T   |                |                  |        |       |
|              | california)            |                |                  |        |       |
| XP_281294.1  | -                       | 1              | PREDICTED:       | 0.0431873 |       |
|              | similar to CG12341-PA   |                |                  |        |       |
| Xpac         | damaged DNA             | 1              | DNA repair,      | 0.0619262 |       |
|              | binding, zinc ion       |                | response to DNA  |        |       |
|              | binding                 |                | damage stimulus |        |       |
|              |                       |                | stimulus, response to oxidative stress | | |
| Zbtb22       | DNA/protein/cis-         | 1              | zinc finger and | 0.0375172 |       |
|              | ion binding             |                | BTB domain       |        |       |
|              | nucleus, intracellular  |                | containing       |        |       |
|              |                       |                | 22               |        |       |
| Zfhx6        | acetyltransferase, zinc | 1              | zinc finger,     | 0.052017 |       |
|              | ion binding             |                | DHHC domain      |        |       |
|              | membrane                |                | containing 6     |        |       |
| Zfp592       | DNA/zinc ion binding    | 1              | zinc finger protein | 0.0168374 |       |
|              | nucleus, intracellular  |                | 592              |        |       |
| Zic2         | DNA, protein and zinc   | 1              | Zinc finger      | 0.0399349 |       |
|              | ion binding             |                | protein of the   |        |       |
|              | intacellular, nucleus    |                | cerebellum       |        |       |
|              | cell differentiation,    |                | 2                 |        |       |
|              | CNS                      |                | development,      |        |       |
|              | development, neocortex  |                | closure,         |        |       |
|              | development, pigmentation |               |                  |        |       |
|              | during development       |                |                  |        |       |
| Zmynd19      | zinc ion binding        | 1              | zinc finger,     | 0.0125127 |       |
|              | cytoplasm, membrane     |                | MYND domain      |        |       |
|              | fraction, synaptolemma  |                | containing 19    |        |       |
**Table S1 | The list of 132 targets for miR-96.** miR-96 was scanned against all mouse and mammalian 3'UTRs available from Ensembl using miRanda v3.0\(^7\). This procedure computes a P-value for each potential binding site using an extreme value distribution computed from scanning mouse miR-96 sequence against all mouse 3' UTRs as a control. P-values for multiple sites on the same mouse 3' UTR were combined using poisson statistics\(^54\). For sites which were also conserved from mouse to other species, the P-values were further lowered by computing the likelihood of finding a conserved 20nt region in the orthologous 3' UTRs of that target\(^54\). This initial list of potential mouse miR-96 targets was then filtered by removing sites whose P > 0.001 and which exhibited mismatches in the 5' seed region of the miRNA (nucleotides 2-7). It was then annotated with additional functional and expression data from multiple sources including MGI (http://www.informatics.jax.org) and Ensembl (http://www.ensembl.org).
| Gene   | Ensembl ID     | No. sites | No. Cons. species | Species Conservation | Sites miRNA (3' – 5') UTR (5' – 3') | Binding Diagram |
|--------|----------------|-----------|-------------------|----------------------|-------------------------------------|-----------------|
| Aqp5   | ENSMUST00000042340 | 2         | 2                 | R. rattus            | UCGUUUUUUACACGAUC                  |                 |
| Avil   | ENSMUST0000026500  | 1         | B. taurus, C. familiaris, M. domestica | M. mulatta, H. sapiens | AGCCCAACTAGC                         |                 |
| Cdh20  | ENSMUST0000062528  | 1         | 3                 | T. rubripes, T. nigroviridis. | UCGUUUUUUACACGAUC                 |                 |
| Celsr2 | ENSMUST0000090558  | 1         | B. taurus, C. familiaris, H. sapiens, M. mulatta, R. rattus, P. troglodytes | | AGCCCAACTAGC                         |                 |
| Ctsb   | ENSMUST000006235   | 1         | 4                 | T. rubripes, X. tropicalus, R. rattus | AGCCCAACTAGC                         |                 |
| Fgf3   | ENSMUST0000033392  | 1         | 3                 | H. sapiens, R. rattus | AGCCCAACTAGC                         |                 |
| Itga3  | ENSMUST0000001548  | 1         | 3                 | C. familiaris, M. domestica | AGCCCAACTAGC                         |                 |
| Myrip  | ENSMUST0000048121  | 1         | 4                 | M. domestica, H. sapiens, M. mulatta | AGCCCAACTAGC                         |                 |
| Odf2   | ENSMUST0000028128  | 1         | 1                 | Not Conserved        | AGCCCAACTAGC                         |                 |
| Ptpn9  | ENSMUST0000034832  | 1         | B. taurus, C. familiaris, H. sapiens, M. mulatta, X. tropicalus, R. rattus | G. aculeatus | AGCCCAACTAGC                         |                 |
| Ryk    | ENSMUST0000035142  | 1         | B. taurus, C. familiaris, G. gallus, H. sapiens, R. rattus | AGCCCAACTAGC                         |                 |
| Sh3kbp1| ENSMUST0000073094  | 1         | B. taurus, T. rubripes, C. familiaris, G. gallus, H. sapiens, X. tropicalus, T. nigroviridis. | AGCCCAACTAGC                         |                 |
| Sox5   | ENSMUST0000038815  | 1         | B. taurus, G. gallus, H. sapiens, M. mulatta, R. rattus | AGCCCAACTAGC                         |                 |
Table S2 | The 13 targets selected for validation. These were chosen from the initial list of 132 putative targets. The table shows the number of sites present, the conservation, and a schematic of the predicted binding, with Mfold binding structure diagrams for the five targets which were validated.
| Gene Symbol | Fold Change | adj.P.Val | Description | GO terms | Ensembl description | Interpro description | Interpro | mRNAs sites present | Notes | References |
|-------------|-------------|-----------|-------------|----------|---------------------|---------------------|---------|---------------------|-------|-----------|
| Ocm         | 15.8847906  | 4.02E-06  | Mus musculus oncomodulin (Ocm), mRNA. | calcium ion binding; oncomodulin; calcium-binding EF-hand; Parvalbumin; | | | | | | Sakaguchi N., Henzl M.T., Thalmannc I., Thalmannc R., Schultea B.A. Oncomodulin Is Expressed Exclusively by Outer Hair Cells in the Organ of Corti. J. Histochem. Cytochem. 46, 29-40 (1998). |
| Endod1      | 1.08472873  | 7.27E-05  | Mus musculus RIKEN cDNA 210506D08 gene (210506D08RA), mRNA. | integral to membrane; nucleic acid binding; endonuclease activity | | | | | | |
| Gabrb3      | 1.31494276  | 0.000227  | Mus musculus gamma-aminobutyric acid (GABA-A) receptor, subunit beta 3 (Gabrb3), mRNA. | integral to plasma membrane; GABA-A receptor activity; receptor activity; chloride transport; gamma-aminobutyric acid signaling pathway | | | | | | Gabrb3 codes for an essential component of the GABA-A receptor, a major inhibitory neurotransmitter in the central nervous system, and knock-out mice are severely impaired in many brain regions, notably in motor function. They show hyperactivity, hypersensitivity to pain, and impaired learning and memory. They also show significant hearing dysfunction and a complete loss of hearing function by 24 weeks (Maison et al., 2006). |
| 1110017D15RA | -4.02E-06  | 0.000227  | Mus musculus RIKEN cDNA 1110017D15 gene (1110017D15Rik), mRNA. | RIKEN cDNA 1110017D15 gene; | | | | | | Homanics G.E. et al. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc.Natl.Acad.Sci.U.S.A. 94, 4143-4148 (1997). |
| Piprin1     | 1.49174927  | 0.000254  | Mus musculus RIKEN cDNA 2210413P12 gene (2210413P12Rik), mRNA. | phospholipid transfer protein; DHODH, LLS, Lysin/Netrin | | | | | | Tian D, Liu X, Trabelsi-Rahhoua H, Carron S, Lim S. N63, a novel regulator of cell morphogenesis. Mol. Cell. Biol. 22, 5256-5265 (2002). |
| Ado1b       | -1.60389233  | 0.000254  | Hypothetical SOCS family, C-terminal of STAT | intracellular signaling cascade | | | | | | |
| Scnla3      | 1.58987969  | 0.000313  | Mus musculus RIKEN cDNA 261019P12 gene (261019P12Rik), mRNA. | integral to membrane; Laminin; sodium/calcium import; selectin-L | | | | | | |
| GeneSymbol | Fold Change adj.P.Val | Description | GO terms | Essential description | mRNAs sites present | References |
|------------|-----------------------|-------------|----------|-----------------------|---------------------|------------|
| Homer1     | 1.467688172 0.000579  | human homing 1 (Desmopilin) | GTP binding; microtubule-based movement; microtubule cytoskeleton organization and biogenesis; tubulin; alpha B; tubulin; alpha A | Homo sapiens | Homo sapiens | Worley P.F., et al. Homer proteins in Ca(2+) signaling by modulator and non-modulator cells. Cell Calcium 42, 283-291 (2007). |
| Tubα1      | 1.77035729 0.000888  | Homo sapiens | Voltage-regulated potassium channel, inner segment; voltage-gated potassium channel subfamily D, member 4 (Kv); Tuba8 | Homo sapiens | Homo sapiens | Liberman M.C., et al. Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. Nature 419, 300-304 (2002). |
| Koral10    | 1.72651663 0.000888  | Homo sapiens | Sodium:dicarboxylate co-transporter family 26, member 5; Slc26a5 | Homo sapiens | Homo sapiens | Rashid S. et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc. Natl. Acad. Sci. U.S.A. 102, 5374-5379 (2005). |
| BiTul1α     | 1.47204789 0.000776  | Homo sapiens | Voltage-dependent potassium channel; Kv9 voltage-gated K+ channel; Shaker voltage-gated K+ channel; Voltage-gated K+ channel, alpha subunit (Kcna10); Voltage-gated K+ channel, alpha beta with C-terminal membrane transport; ion transport 2; Ion transport 3; Voltage-dependent anion channel 1; Voltage-dependent anion channel 2; Voltage-dependent anion channel 3; Voltage-dependent anion channel 4; Voltage-dependent anion channel 5; Voltage-dependent anion channel 6; Voltage-dependent anion channel 7; Voltage-dependent anion channel 8; Voltage-dependent anion channel 9; Voltage-dependent anion channel 10 | Homo sapiens | Homo sapiens | Rashid S. et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc. Natl. Acad. Sci. U.S.A. 102, 5374-5379 (2005). |
| BiTul1β     | 1.52305381 0.000776  | Homo sapiens | Voltage-dependent potassium channel; Kv9 voltage-gated K+ channel; Shaker voltage-gated K+ channel; Voltage-gated K+ channel, alpha subunit (Kcna10); Voltage-gated K+ channel, alpha beta with C-terminal membrane transport; ion transport 2; Ion transport 3; Voltage-dependent anion channel 1; Voltage-dependent anion channel 2; Voltage-dependent anion channel 3; Voltage-dependent anion channel 4; Voltage-dependent anion channel 5; Voltage-dependent anion channel 6; Voltage-dependent anion channel 7; Voltage-dependent anion channel 8; Voltage-dependent anion channel 9; Voltage-dependent anion channel 10 | Homo sapiens | Homo sapiens | Rashid S. et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc. Natl. Acad. Sci. U.S.A. 102, 5374-5379 (2005). |
| Pcsk9      | 1.403471726 0.000783 | Homo sapiens | Proteinase K family; Peptidase S8 and S53, subtilisin, kexin, ribonuclease A convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation; cholesterol metabolism; hydrolase activity; neuron morphogenesis; peptide hormone receptor activity; peptidase activity; proteolysis and peptidolysis; lipid metabolism; subtilase activity; subtilase activity; subtilase activity; subtilase activity | Homo sapiens | Homo sapiens | Rashid S. et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc. Natl. Acad. Sci. U.S.A. 102, 5374-5379 (2005). |
| Golm1      | -1.72651663 0.000888 | Homo sapiens | Solute carrier family 26, member 5; Slc26a5 | Homo sapiens | Homo sapiens | Rashid S. et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc. Natl. Acad. Sci. U.S.A. 102, 5374-5379 (2005). |
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Ensembl description | Interpro description | mRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|----------|---------------------|----------------------|--------------------|-------|------------|
| miR96      | 1.898684242 | 0.00106   | *Mus musculus* RIKEN cDNA 1190003J15Rik gene | Transcribed non-protein coding | RIKEN cDNA 1190003J15Rik gene | Tstrandpol; | downregulated: miR-96dm; upregulated: miR96 | | |
| Ghfr       | 1.391846392 | 0.00133   | *Mus musculus* growth hormone secretagogue receptor (Ghsr) | Growth hormone secretagogue receptor activity; Growth hormone-releasing hormone receptor activity; G-protein coupled receptor protein signaling pathway; G-protein coupled receptor activity; G-protein coupled receptor protein signaling pathway | RIKEN cDNA 1190003J15 gene | Ghsr; Transthyretin; | | | |
| Asb1       | 1.275444392 | 0.00135   | *Mus musculus* ankyrin repeat and SOCS box-containing protein 1 (Asb1) | SOCS proteins are involved in the negative regulation of cytokine signaling. | RIKEN cDNA 3100002J23 gene | Asb1 knockout mice exhibit a reduction in spermatogenesis (Kile et al., 2001). | | | |
| 9030418K01Rik | -2.056227653 | 0.00135 | *Mus musculus* Htr3a (5-hydroxytryptamine receptor 3A) | 5-hydroxytryptamine receptor 3A; Neurotransmitter-gated ion-channel; Nicotinic acetylcholine receptor; 5-hydroxytryptamine 3 receptor; 5-hydroxytryptamine 3 receptor, A subunit; Neurotransmitter-gated ion-channel ligand-binding; Neurotransmitter-gated ion-channel transmembrane region; Transport; Ion transport; Synaptic transmission | RIKEN cDNA 3100002J23 gene | Htr3a | | |
| Tmprss7    | 1.686462221 | 0.00155   | *Mus musculus* transmembrane serine protease 7 | Peptidase S1 and S6, chymotrypsin/Hap; Low density lipoprotein-receptor, class A; Peptidase S1A, chymotrypsin, BCA; | | | | | |
| Hspb3      | 1.244874235 | 0.00205   | *Mus musculus* heat shock protein 3 | Heat shock protein activity; response to heat shock protein; heat shock protein | | | | |

**Notes**

| Reference |
|-----------|
| Ghsr null mice show resistance to diet-induced obesity and altered glucose homeostasis (Zigman et al., 2005). |
| Sun Y., Wang P., Zheng H. and Smith R.G. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4679-4684 (2004). |
| Zigman J.M. et al. Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J. Clin. Invest.* 115, 3564-3572 (2005). |
| Kile B.T. et al. Functional analysis of Asb-1 using genetic modification in mice. *Mol. Cell. Biol.* 21, 6189-6197 (2001). |

**References**

*Proc. Natl. Acad. Sci. U.S.A.* 101, 4679-4684 (2004).
| Gene Symbol | Fold Change | adj.P.Val | Description | GO terms | mRNAs sites present | Notes | References |
|-------------|-------------|-----------|-------------|----------|---------------------|-------|------------|
| Fadd        | -1.250271991| 0.00211   | Mus musculus Fas (TNFRSF6)-associated via death domain (Fadd), vIFRA | apoptosis regulator activity; signal transduction; apoptosis; apoptosis program |  |     |             |
| OBV1        | 1.0823982929| 0.00556   | Mus musculus mRNA for pancratin, complete cds. |          |                     |      |             |
| Topp        | 1.2880068886| 0.00386   | Mus musculus RIKEN cDNA 2900041A09 gene (2900041A09Rik), mRNA |          |                     |      |             |
| Lin25       | 1.792323369 | 0.00446   | Mus musculus ADP-ribosylation factor 3 (Arf2), vIFRA | GTPase activity; GTP binding; transporter activity; GTPase activity; protein transporter activity; intracellular protein transport; small GTPase mediated signal transduction |  |     |             |
| AK2         | -1.371845265| 0.00446   | Mus musculus AQP-3 (aquaporin 3), vIFRA | GTPase activity, GTP binding; transporter activity; GTPase activity; protein transporter activity; intracellular protein transport; small GTPase mediated signal transduction |  |     |             |
| Chrna9      | 1.911890635 | 0.00446   | Mus musculus cholinergic receptor, nicotinic, alpha polypeptide 9 (Chrna9), mRNA | cholinergic receptor, nicotinic, alpha polypeptide 9; Neurotransmitter-gated ion-channel ligand-binding; Neurotransmitter-gated ion-channel transmembrane region; Neurotransmitter-gated ion-channel ligand-binding; Neurotransmitter-gated ion-channel transmembrane region |  |     | (He et al., 2004) |
| Cnp9        | 2.496661098 | 0.00446   | Mus musculus RIKEN cDNA A730016F12 gene (A730016F12Rik), transcript variant 1 | copine family member IX; C2 calcium-dependent membrane targeting; Copine; von Willebrand factor, type A |  |     |             |
| Gene Symbol | Fold Change | adj.P.Val | Description | GO terms | Ensembl description | Interpro description | mRNA site present | Notes | References |
|-------------|-------------|-----------|-------------|----------|---------------------|----------------------|-------------------|-------|------------|
| miR96 sites present | downregulated: miR-96dm | upregulated: miR96 | | | | | |
| 1700024G13Rik | -1.396678532 | 0.00444 | miR-96dm, up-regulated: miR96 | Protease inhibitor II, leucine; Proteinase inhibitor II, serpin; | | | | |
| SerpinA3 | -1.636938363 | 0.00481 | Serpin3 | Proteinase inhibitor I4, leucerpin 2; Proteinas e inhibitor I4, serpin; | | | | |
| Wdr16 | -1.556170353 | 0.00575 | Wdr16 | WD repeat domain 16; Regulator of chromosome condensation, RCC1; WD-40 repeat; | | | | Hirschner W. et al. Biosynthesis of Wdr16, a marker protein for kinocilium-bearing cells, starts at the time of kinocilia formation in rat, and wdr16 gene knockdown causes hydrocephalus in zebrafish. J. Neurochem. 101, 274-88 (2007). |
| 2310046K01Rik | -2.411615655 | 0.00575 | 2310046K01 | Integral to membrane; RIKEN cDNA 2310046K01 gene; Protein of unknown function DUF1011; | | | | |
| Gfi1 | 1.310393404 | 0.00575 | Gfi1 | Nucleic acid binding; DNA binding; ATP binding; inhibition of growth factor independent 1; Zinc finger (C2H2-type); Zinc finger, C2H2-subtype; Gfi1 is upregulated by Pou4f3 (Hertzano et al. 2004) and repressed by itself and Gfi1b (Doan et al. 2004). Gfi1-null mice have disorganised hair cells, which degenerate with age (Wallis et al. 2003). | | | | Hertzano R. et al. Transcription profiling of inner ears from Pou4f3(ddl/ddl) identifies Gfi1 as a target of the Pou4f3 deafness gene. Hum Mol Genet. 13, 2143-53 (2004). Doan L.L. et al. Targeted transcriptional repression of Gfi1 by GFI1 and GFI1B in lymphoid cells. Nucleic Acids Res. 32, 2508-19 (2004). Wallis D. et al. The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. Develop. 130, 221-32 (2003). |
| Hsd17b7 | 2.203810232 | 0.00575 | Hsd17b7 | Integral to membrane; Extracellular space; Endoplasmic reticulum; Oxidoreductase activity; 3-keto sterol reductase activity; Steroid biosynthesis; Metabolism; Cholesterol biosynthesis; Hsd17b7 is a catalytic enzyme found in the ovaries of pregnant rodents, and is thought to be responsible for the final step in the biosynthesis of E2 in the corpus luteum (Nokelainen et al. 1998). | | | | Nokelainen P., Peltoketo H., Vihko R. and Vihko P. Expression Cloning of a Novel Estrogenic Mouse 17b-Hydroxysteroid Dehydrogenase/17-Ketosteroid Reductase (m17HSD7), Previously Described as a Prolactin Receptor-Associated Protein (PRAP) in Rat. Mol. Endocrinol. 12, 1048-1059 (1998). |
| Gm414 | 1.744725412 | 0.00589 | Gm414 | Complement C1r; C1s, Collagen triple helix repeat | | | | |
| Cdh1 | 1.087020889 | 0.00527 | Cdh1 | Cadherin (C1q domain); Cdh1 is a cadherin family member | | | | |
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Ensembl description | Interpro description | mRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|-----------|---------------------|----------------------|---------------------|-------|------------|
| Psat1      | 1.662091723 | 0.00627   | Mus musculus phosphoserine aminotransferase 1 (Psat1), mRNA. | phosphoserine aminotransferase 1; Aminotransferase, class VI; | | | | |
| 1700009P17Rik | 1.818816504 | 0.00759   | Mus musculus RIKEN cDNA 1700009P17 gene (1700009P17), mRNA. | RIKEN cDNA 1700009P17 gene; | | | | |
| D娜p15     | 1.286732315 | 0.00799   | Mus musculus RIKEN cDNA 1700009P17 gene (1700009P17), mRNA. | Dnak (heat); heat shock protein subfamily C; number 16; | Heat shock protein (Dna.) | | | |
| 4011603B11Rik | 1.235185223 | 0.00799   | Mus musculus RIKEN cDNA 4011603B11 gene (4011603B11), mRNA. | | | | | |
| D娜p15     | 1.204972315 | 0.00759   | Mus musculus RIKEN cDNA 4732437J24 gene (4732437J24), mRNA. | DnaJ (Hsp40) homolog, subfamily C, member 16; | Heat shock protein (Dna.) | | | |
| Mup1       | 1.634670657 | 0.00771   | Mus musculus major urinary protein 1 (Mup1), mRNA. | Major urinary protein 1; major urinary protein 2; major urinary protein 2; major urinary protein 1; | Lipocalin; Betelkopalizin; Cuticular protein; Golocalin; Major urinary protein 2; Major urinary protein 1; Major urinary protein 2; Major urinary protein 1; Allergen; Prostaglandin D synthase; Neutrophil gelatinase-associated lipocalin; Lipocalin-related protein and Bos/Can allergen; | | |
| Cpne9      | 1.236679867 | 0.00771   | Mus musculus copine family member IX (Cpne9), mRNA. | Copine family member IX; | | | | |
| Samd9l     | 1.242976261 | 0.01163   | Mus musculus EST AA171628 (AA171628), mRNA. | | | | | |
| Serca9     | 1.242293632 | 0.00974   | Mus musculus ZFT AAC17256 (AAC17256), mRNA. | Sarcoplasmic reticulum calcium-transporting ATPase 9; | | | | |
| Gene Symbol | Fold Change | adj.P.Val | Description | GO terms | Interpro description | mIRNA sites present | Notes | References |
|-------------|-------------|-----------|-------------|----------|----------------------|---------------------|-------|------------|
| Sdc2        | -1.418140036| 0.00948   | Mus musculus syndecan 2 (Sdc2), mRNA. | membrane; integral to membrane; cytoskeletal protein binding; syndecan 2; | Synthet | miR-96 dm; up-regulated: miR-96 | Human Syndecan 2 and protein 4.1 (EP400) are found by Coi, which is thought to mediate a link between the extracellular matrix and the actin cytoskeleton via its interaction with syndecan and protein 4.1. Syndecan 2 and protein 4.1 (Sdc2) are present in the mouse brain (Wollum et al., 2008), as in 3,2 (Humphrey et al., 2006). Cohen A.R. et al. Human CASK/LIN-2 binds syndecan 2 and protein 4.1 and localises to the basolateral membrane of epithelial cells. J. Cell Biol. 142, 129-138 (1998). Wollum P. et al. White complexes with p300 at the interactions by differing fetal development. Hum. Mol. Genet. 21, 87-97 (2002). | | |
| Kalrn       | 1.366987452 | 0.00948   | Mus musculus RIKEN cDNA 2210407G14, mRNA. | guanyl-nucleotide exchange factor activity | GTPase | n/a | Probe sequence maps to intergenic region between Tct and alx1 (X22 12) | | |
| Huak2       | -1.204972315| 0.00948   | Mus musculus RIKEN cDNA 1200013B22, mRNA. | ATP binding; transferase activity; protein serine/threonine kinase activity; kinase activity | NUAK family, DUF781-bearing kinase, 2; Protein kinase; Serine/threonine protein kinase; active site; Tyrosine protein kinase; RIO kinase | miR-96 dm; up-regulated: miR-96 | Kalirin is a cytosolic protein with specific GTP/GDP exchange factor activity. It is also expressed in the CNS and its overexpression in cells affects cytoskeletal organisation and secretion of ACTH-related peptides (Mains et al., 1999). Mains R.E. et al. Kalirin, a multifunctional PAM COOH-terminal domain interactor protein, affects cytoskeletal organisation and ACTH secretion from AtT-20 cells. J. Biol. Chem. 274, 2929-2937 (1999). | | |
| Cat1        | -1.362177193| 0.00948   | Mus musculus cytokine receptor-like factor 1 (CRLF1), mRNA. | extracellular space; receptor activity; cytokine receptor-like factor 1; | Fibroblast, type III | miR-96 dm; up-regulated: miR-96 | | |
| 08103792RRA | 1.278367475 | 0.0107    | | | | | | |
| 1705203801R | -1.611048582| 0.0108    | Mus musculus RIKEN C3HR gene (1705203801), mRNA. | | | | | |
| A330021E22R | -1.384676532| 0.0113    | Mus musculus RIKEN A330021E22 gene (A330021E22), mRNA. | | | | | |
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Ensembl description | Interpro description | mRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|----------|----------------------|----------------------|---------------------|-------|------------|
| Pkg        | 1.486694868 | 0.0128    | Mus musculus protein kinase inhibitor, gamma (PKIG), mRNA | cAMP-dependent protein kinase activity, kinase activity, protein kinase inhibitor activity; negative regulation of protein kinase activity | | | | | |
| Pkg        | 1.486694868 | 0.0128    | Mus musculus protein kinase inhibitor, gamma (PKIG), mRNA | cAMP-dependent protein kinase activity, kinase activity, protein kinase inhibitor activity; negative regulation of protein kinase activity | | | | | |
| Ifit172    | -1.227013270 | 0.0133    | Mus musculus intraflagellar transport 172 (IFT172), mRNA | intraflagellar transport 172; WD-40 repeat | | | | | |
| Slc34a3    | 1.224336392 | 0.0133    | Mus musculus solute carrier family 34 (sodium phosphate), member 3 (SLC34A3), mRNA | brush border; apical plasma membrane; sodium-dependent phosphate transporter activity; phosphate transport solute carrier family 34 (sodium phosphate), member 3; Sodium:dicarboxylate symporter; Na+/Pi-cotransporter; | | | | |
| Fadd       | -1.278099363 | 0.0133    | Mus musculus Fas (TNFRSF6)-associated via death domain (FADD), mRNA | apoptosis regulator activity; signal transduction; apoptosis; apoptotic program; Fas (TNFRSF6)-associated via death domain; Death effector; Death; | | | | |
| Ppp1r3d    | 1.53368266  | 0.0146    | Mus musculus RASD family, member 3 (PPP1R3D), mRNA | | | | | |
| Ras2       | 1.316766922 | 0.0146    | Mus musculus Fukuyama type congenital muscular dystrophy homolog (human) (FSDKD), mRNA | | | | | |
| Fktn       | 1.316766922 | 0.0146    | Mus musculus Fukuyama type congenital muscular dystrophy homolog (human) (FKTN), mRNA | | | | | |
| 2703000C16Rk | 1.045905690 | 0.0146    | Mus musculus RIKEN cDNA 2700000G10Rik (2700000G10Rik), mRNA | | | | | |
| Gene Symbol | Fold | adj.P.Val | Description | GO terms | Interpro description | mRNA sites present | Notes | References |
|-------------|------|-----------|-------------|----------|----------------------|-------------------|-------|------------|
| Spnq        | 1.22 | 0.015     |             |          |                      |                   |       |            |
|             |      |           | protein tyrosine phosphatase receptor type 1q |         |                      |                   |       |            |
| Mitga       | 1.63 | 0.017     |             |          |                      |                   |       |            |
|             |      |           | extracellular matrix integral to membrane, extracellular tyrosine protein kinase activity, transmembrane protein activity, actin binding, protein kinase domain |         |                      |                   |       |            |
|             |      |           | Hemagglutinin, globular zinc finger domain; Hemagglutinin-related protein; Hemagglutinin-related protein, variant 2, mRNA |         |                      |                   |       |            |
|             | -0.19| 0.017    |             |          |                      |                   |       |            |
|             |      |           | extracellular space integral to membrane |         |                      |                   |       |            |
| Cbn1        | 1.34 | 0.019     |             |          |                      |                   |       |            |
|             |      |           | endoplasmic reticulum complex, kinesin complex, extracellular space, development |         |                      |                   |       |            |
|             |      |           | Olfactomedin |         |                      |                   |       |            |
|             |      |           | Endoplasmic reticulum targeting sequence; Latrophilin receptor; Olfactomedin-like |         |                      |                   |       |            |
|             | -1.66| 0.021     |             |          |                      |                   |       |            |
|             |      |           | RIKEN cDNA 1700027A23 gene |         |                      |                   |       |            |
|             | -1.30| 0.021     |             |          |                      |                   |       |            |
|             |      |           | RIKEN cDNA 1700027A23 gene |         |                      |                   |       |            |
|             | 1.21 | 0.021     |             |          |                      |                   |       |            |
|             |      |           | short chain dehydrogenase/reductase SDR; Glucose-6-phosphate dehydrogenase; 2,3-dihydroxy-2,3-dihydroxybenzoate dehydrogenase |         |                      |                   |       |            |
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Essential description | Interpre description | miRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|-----------|-----------------------|---------------------|--------------------|-------|------------|
| Myo3a      | -1.321380899| 0.0091    | Myo3a       | ATP binding, transmembrane activity | myosin VI | Myosin Biken; Rho GTPase activating protein; Myosin VI; Myosin VI; Myosin VI | downregulated: miR-96dm; upregulated: miR-96 | Myosin VI has been implicated in sarcoplasmic reticulum calcium release and is involved in skeletal muscle function. | Schneider-M Mi et al. A new compartment at stereocilia tips defined by spatial and temporal patterns of neuronal activity. J Neurosci. 26, 10291-10302 (2006) |
| Cyp4f39    | 1.271000729 | 0.026     | Cyp4f39     | integral to membrane, membrane | RIKEN cDNA | Cytochrome P450; Cytochrome P450; Cytochrome P450; Cytochrome P450; Cytochrome P450; Cytochrome P450; Cytochrome P450 | integral to membrane, membrane | AnxA4 has been shown to regulate chloride channel activity and inhibit chloride conductance. | Chan H.C., Kaetzel M.A., Gotter A.L., Dedman J.R., and Nelson D.J. Annexin IV inhibits calmodulin-dependent protein kinase II-activated chloride conductance. A novel mechanism for ion channel regulation. J. Biol. Chem. 269, 32464-32468 (1994) |
| Chrna1     | 1.626758396 | 0.0285    | Chrna1      | membrane, integral to membrane, extracellular region, transmembrane region | Cholinergic receptor, nicotinic, alpha polypeptide | Neurotransmitter-gated ion-channel; Nicotinic acetylcholine receptor; Neurotransmitter-gated ion-channel ligand-binding; Neurotransmitter-gated ion-channel transmembrane region | chrna1 is expressed in the hair cells until P7, and is activated by Atoh1 | Scheffer D. et al. The alpha1 subunit of nicotinic acetylcholine receptors in the inner ear: transcriptional regulation by ATOH1 and co-expression with the gamma subunit in hair cells. J. Neurochem. 103, 2651-2664 (2007) |
| D19Ertd652e| -1.25962998 | 0.0289    | D19Ertd652e | nuclear, nuclear matrix, nuclear binding | expressed sequence | | | | |
| Anp32a     | 1.265109468 | 0.0289    | Anp32a      | nuclear, nuclear matrix, nuclear binding | expressed sequence | | | | |
| Pmk211     | -1.329677199| 0.0289    | Pmk211      | extracellular space | polyubiquitin family, ubiquitin domain 1 (Pum1211), Pum211 | polyubiquitin family, ubiquitin domain 1 (Pum1211), Pum211 | Polyubiquitin substrate; Ubiquitin domain; Ubiquitin domain; Ubiquitin domain; Ubiquitin domain; Ubiquitin domain; Ubiquitin domain; Ubiquitin domain | | |
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Interpro description | mRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|----------|----------------------|-------------------|-------|------------|
| Odf3b      | -1.479387509 | 0.03      |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 20180311.22 gene | Protein of unknown function DUF 1386 |                   |       |            |
| Galnt13    | 1.151089491  | 0.0351    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13; Glycosyl transferase, Family 2; Ricin B lectin |                   |       |            |
| Pwnt1      | 1.189149988  | 0.0351    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | Protein-L-isoaspartate (D-aspartate) O-methyltransferase activity; S-adenosylmethionine-dependent methyltransferase activity; transferase activity; methyltransferase activity; protein modification; protein amino acid methylation |                   |       |            |
| Reep1      | 1.520874907  | 0.0387    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | Protein-L-isoaspartate (D-aspartate) O-methyltransferase; Methyltransferase type 11 |                   |       |            |
| Slc33a2    | -1.479902431 | 0.0387    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | Integral to membrane; Gafl immunoreceptor tyrosine kinase; alpha 2, 8-sialyltransferase; alpha-N-acetylglucosaminyltransferase; alpha-N-acetylneuraminyltransferase |                   |       |            |
| Bhlhe40    | 1.314031627  | 0.0387    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | Basic helix-loop-helix domain containing, class B2; Orange; Transcriptional repressor. May play a key role in regulating pathways that lead to growth arrest and terminal differentiation |                   |       |            |
| Mrps25     | 1.160703914  | 0.0387    |             |          |                      |                   |       |            |
|            |              |           |             | RIKEN Cdna 2018041L15 gene | Mitochondrial ribosomal protein S25 |                   |       |            |

**Notes and References:**

- Odf3b
  - Mus musculus (mRNA: DQ770121.21 gene)
- Galnt13
  - Mus musculus (mRNA: DQ770121.21 gene)
- Pwnt1
  - Mus musculus (mRNA: DQ770121.21 gene)
- Reep1
  - Mus musculus (mRNA: DQ770121.21 gene)
- Slc33a2
  - Mus musculus (mRNA: DQ770121.21 gene)
- Bhlhe40
  - Mus musculus (mRNA: DQ770121.21 gene)
- Mrps25
  - Mus musculus (mRNA: DQ770121.21 gene)

**References:**

- Sun H. and Taneja R. Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. J Cell Sci 2000.
- Sun H. and Taneja R. Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. J Cell Sci 2000.
| GeneSymbol | Fold Change | adj.P.Val | Description                                                                 | GO terms                                                                 |
|------------|-------------|-----------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Chrna1     | 1.258757174 | 0.0402    | Mus musculus cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)    | component; membrane; extracellular space; integral to membrane; extracellular space; extracellular ligand-gated ion channel activity; neurotransmitter receptor activity; GABA-A receptor activity; neurotransmitter-gated ion-channel activity; transport ion transport; synaptic transmission |
| miR96 sites present | (downregulated: miR-96dm; up-regulated: miR96) |          |                                                                              |                                                                          |
| Chrna1     | 1.274560627 | 0.0437    | Mus musculus similar to Carboxylesterase 2 (LOC234668), mRNA.                | component; membrane; integral to membrane; extracellular space; Golgi apparatus; plasma membrane; neurotransmitter-gated ion channel activity; transport ion transport; synaptic transmission |
| Rshl3      | -1.20163605 | 0.0433    | Radial spokehead-like 3; Radial spokehead-like protein;                      |                                                                           |
| Ubxn1      | -1.313121125| 0.0443    | Probe maps to intergenic region between Setdb1 and Amt (Chr 3)              |
| Leprotl1   | 1.165541198 | 0.0443    | Mus musculus leptin receptor overlapping transcript-like 1 (Leprotl1), mRNA. | integral to membrane; extracellular space; leptin receptor overlapping transcript-like 1; Vacuolar protein sorting 55; MORN motif; |
| Morn5      | -1.293248932| 0.0455    | Mus musculus RIKEN cDNA 1700010A17 gene (1700010A17Rik), mRNA.              |                                                                           |
| Morn5      | -1.293248932| 0.0455    | Radial spokehead-like 3; Radial spokehead-like protein;                      |                                                                           |

Notes: Different probe for the same gene as above.
| GeneSymbol | Fold Change | adj.P.Val | Description | GO terms | Interpro description | mRNA sites present | Notes | References |
|------------|-------------|-----------|-------------|----------|-----------------------|--------------------|-------|------------|
| Meig1      | -1.78977/0.020 | 0.0561   | Meix1 musculus meiosis expressed gene1 (Meig1), mRNA | nuclear | nuclear expressed (gene 1) | miR-96 (downregulated; miR-666) |       |            |
| Smpx       | 1.3456/0.019   | 0.0471   | Mus musculus small muscle protein, X-linked (Smpx), mRNA | nucleus; cytoplasm; striated muscle contractile | small muscle protein, X-linked | miR-96 (up-regulated) |       |            |
| Nup210     | 1.3877/0.048   | 0.048    | Mus musculus nucleoporin 210 (Nup210), mRNA | nuclear pore; integral to membrane; nucleus; integral to membrane; extracellular matrix membrane pore activity | nucleoporin 210; Bacterial Ig-like group 2 | miR-96 (up-regulated) |       |            |
| Mns1       | -1.9319/0.048  | 0.048    | Meix1 musculus meiosis specific nuclear structural protein 1 (Mns1), mRNA | nuclear membrane; intermediate filament | nuclear membrane; intermediate filament | miR-96 (downregulated) |       |            |
| Ppp1r15a   | 1.2075/0.035   | 0.039    | Mus musculus myeloid differentiation primary response gene 116 (Myd116), mRNA | cell differentiation | myeloid differentiation primary response gene 116; Bacterial Ig-like group 2 | miR-96 (up-regulated) |       |            |
Table S3 | The 96 transcripts with significant changes in expression, from the microarray ($\alpha=0.05$). Downregulation in the mutant is indicated by a positive fold change value; upregulation by a negative fold change value. Further information has been added for a number of genes, in particular those which were considered to be potential candidates for affecting hair cell function. Potential miR-96 binding sites are indicated; wildtype binding sites for genes which are upregulated in the mutant, and diminuendo mutant binding sites for those downregulated. Red: 9nt match; orange: 8nt match; yellow: 7nt match; green: 6nt match. The position indicates whether the match includes the 5' A base, which is not in the seed region; miRNA targets are often flanked by adenosines\textsuperscript{4}. Each site is unique; if an 8nt site is present, the 7nt and 6nt sites which are included in that 8nt are not listed.

|          | Forward         | Reverse                        |
|----------|-----------------|-------------------------------|
| 2310005E10Rik | ATACGTTCTTGATGCTTGG | GGGACTGTAGGCTGTGATGG. |
| Slc26a5  | CACCTGGACAGTGAAGTGGA | TGCTGGATAAGGGCAGTCAG |
| Ocm      | GGTGGTGAAGGACCATCTGC | ACCACAGATCCCTTTGGAAA |

Table S4 | Primer details. Forward and reverse primers used for checking the sequence of the gene 2310005E10Rik and for the presence of the first exon of Ocm and Slc26a5.
| Gene symbol | Gene name                                      | Ensembl Transcript ID | Chromosome | Region amplified          | Summary of function                                                                 |
|-------------|-----------------------------------------------|-----------------------|------------|---------------------------|-------------------------------------------------------------------------------------|
| Aqp5        | Aquaporin 5                                   | 00000088200           | 15         | 99,422,383-99,422,825     | Water transporter, required for inner ear maturation                               |
| Avil        | Advillin                                      | 00000026500           | 10         | 126,423,536-126,423,937   | Actin filament organisation                                                        |
| Cdh20       | Cadherin 20                                   | 00000062528           | 1          | 106,821,953-106,822,885   | Calcium ion binding, cell adhesion                                                 |
| Celsr2      | Cadherin EGF LAG seven-pass G-type receptor 2 | 00000090558           | 3          | 108,520,528-108,520,750   | Calcium ion binding, cell adhesion                                                 |
| Ctsb        | Cathepsin B                                   | 0000006235            | 14         | 62,096,564-62,097,250     | Cysteine-type endopeptidase                                                       |
| Fgf3        | Fibroblast Growth Factor 3                    | 00000033392           | 7          | 144,652,383-144,652,724   | Cell proliferation, signal transduction                                         |
| Itga3       | Integrin alpha 3                              | 0000001548            | 11         | 94,860,572-94,861,829     | Cell adhesion                                                                      |
| Myrip       | Myosin VIIa and Rab interacting protein        | 0000048121            | 9          | 120322901-120323532       | Actin cytoskeleton organisation                                                   |
| Odf2        | Outer dense fiber of sperm tails 2            | 0000028128            | 2          | 29744444-29744593         | Structural constituent of cytoskeleton                                           |
| Ptpn9       | Prenylated/non-membrane spanning protein tyrosine phosphatase | 0000034832 | 9          | 56859317-56860191         | Intracellular transporter                                                          |
| Ryk         | Receptor-like tyrosine kinase                 | 0000035142            | 9          | 102765112-102765567       | ATP/Wnt protein binding                                                            |
| Sh3kbp1     | SH3-domain kinase binding protein 1           | 0000073094            | X          | 155317287-155318086       | Protein binding, kinase activity                                                  |
| Sox5        | SRY-box containing gene                       | 0000038815            | 6          | 143791158-143791666       | May regulate transcription of L-type Ca(2+) channel alpha(1S)                      |
Table S5 | Luciferase construct data for the 13 candidate genes. PCR amplification of constructs from mouse genomic DNA was carried out using 3'UTR sequences obtained from Ensembl Transcript database. The genomic nucleotide position of the region amplified was obtained from the Ensembl database (http://www.ensembl.org). Predicted target site(s) were identified using the miRanda algorithm⁷.
**Supplementary notes**

**Statistical tests.** All the datasets where t-tests were used were tested for normality using the Kolmogorov-Smirnov test and for homoscedasticity using the F test. In all cases for the Kolmogorov-Smirnov test, p>0.05, indicating that they showed a normal distribution. Where the F test indicated equal variance, Student's t-test was suitable to determine the significance of the differences between control and experimental sets. Where the F test indicated heteroscedasticity, Welch's t-test was used instead.

**Use of Sylamer to analyse the microarray data.** In Figure S14 we compare the overrepresentation analysis by Sylamer with a simpler approach using simple log ratios of word counts. To this end we used three Fold Change cutoffs of >1.5, >1.2, and >1.1 respectively for probes with uncorrected P-value < 0.05 and upregulated in the mutant. It is seen in the first row that the log ratio score (i.e. the overrepresentation of a word in a selected set of sequences compared to a background set of sequences) for the pertinent wild-type heptamer seed match (GUGCCAA) is not the highest ranked according to this criterion in any of the FC cutoffs. A trend can be observed that the ranking of the GUGCCAA log ratio score improves as the FC cutoff is lowered and the size of the associated gene set increases. In the second row we compute a Z-score for the observed log ratio scores of all the miRNA seed matches, by comparing them to an ensemble of log ratio scores computed by randomly shuffling the gene list 10,000 times and considering random gene sets of the same size (as derived from the respective FC cutoffs). It is now seen that the Z-score for the wild-type heptamer is the most significant for respectively the 1.2 and 1.1 FC cutoffs. These combined pictures indicate that the effect of the mutant microRNA extends to genes found only in low FC cutoff values, but paint a somewhat blurred picture. This can be attributed to the fact
that the log ratio scores constitute a simple measure that is volatile for small gene sets and difficult to equip with statistical power. In the last row Sylamer P-values are shown for enrichment of the wild type seed in the same gene sets as before. These P-values are clearly highly significant for the >1.2 and >1.1 FC cutoffs. It can be observed that these P-values correlate with the P-values obtained from Figure 4 by inspecting the pertinent curve for GUGCCAA at the appropriate FC cutoffs. They are not identical as the gene sets in this analysis were filtered for P-values < 0.05, whereas the gene FC ranking in Figure 4 was not submitted to such a filtering.

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