Regulation of cellular calcium in vestibular supporting cells by Otopetrin 1

Euysoo Kim, Krzysztof L. Hyrc, Judith Speck, Yunxia W. Lundberg, Felipe T. Salles, Bechara Kachar, Mark P. Goldberg, Mark E. Warchol, and David M. Ornitz

1Departments of Developmental Biology, 2Neurology, 3Otolaryngology, Washington University School of Medicine, Saint Louis, Missouri 63110, 4Department of Genetics, Boys Town National Research Hospital, Omaha, Nebraska 68131, 5Laboratory of Cell Structure and Dynamics, National Institute of Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland 20892

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Corresponding author: David M. Ornitz (Email: dornitz@wustl.edu)

Washington University School of Medicine, Department of Developmental Biology

Campus Box 8103, 660 S. Euclid Avenue, Saint Louis, MO 63110 USA

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Abstract

Otopetrin 1 (Otop1) is a multi-transmembrane domain protein, which is essential for mineralization of otoconia, the calcium carbonate biominerals required for vestibular function and the normal sensation of gravity. The mechanism driving mineralization of otoconia is poorly understood, but it has been proposed that supporting cells and a mechanism to maintain high concentrations of calcium are critical. Using Otop1 knockout mice and a utricular epithelial organ culture system, we show that Otop1 is expressed at the apex of supporting cells and functions to increase cytosolic calcium in response to purinergic agonists, such as ATP. This is achieved by blocking mobilization of calcium from intracellular stores in an extracellular calcium-dependent manner and by mediating influx of extracellular calcium. These data support a model in which Otop1 acts as a sensor of the extracellular calcium concentration near supporting cells and responds to ATP in the endolymph to increase intracellular calcium levels during otoconia mineralization.

Key words: otoconia, Otopetrin, purinergic signaling, inner ear, vestibular system
Introduction

Otoconia are calcium carbonate (CaCO$_3$) biominerals located in the extracellular space above the sensory epithelium (macula) of the utricle and saccule of the mammalian inner ear. These high density crystals are required for sensation of gravity. Otoconia forms by nucleation and growth of CaCO$_3$ crystals around an already-formed proteinaceous core composed of calcium binding and matrix proteins (Mann et al. 1983). In mice, maximal rate of mineralization occurs between E15 and E16.5, and the mineral growth persists until P7 (Anniko 1980; Lim 1973).

Nucleation of CaCO$_3$ crystals requires a high Ca$^{2+}$ concentration ([Ca$^{2+}$]), however, the endolymph bathing the sensory epithelium contains very low free [Ca$^{2+}$]. As a mechanism to maintain high [Ca$^{2+}$], a vesicular structure, called “globular substance”, is thought to be extruded from the apical surface of the maculae in the embryonic inner ear (Suzuki et al. 1995b; Tateda et al. 1998). Treatment of globular substance vesicles with ATP resulted in a 5-6 fold increase in intravesicular Ca$^{2+}$ (Suzuki et al. 1997a). This suggests that increasing the concentration of Ca$^{2+}$ in globular substance vesicles could mediate nucleation of CaCO$_3$ crystals, a process that could be regulated in vivo by ATP. The kinetics and concentration dependence of the ATP-mediated [Ca$^{2+}$] increase was most similar to that of known purinergic P2 receptors (P2Y and P2X families). P2Y receptors are metabotropic G-protein coupled receptors that mediate release of Ca$^{2+}$ from intracellular stores (Burnstock 2007). P2X family receptors are ionotropic channels that allow an influx of extracellular Ca$^{2+}$ (North 2002).

Tilted mice lack otoconia and show a head tilting behavior with inability to swim (Ornitz et al. 1998). Positional cloning identified that tilted is a mutant allele of Otopetrin 1 (Otop1) (Hurle et al. 2003). OTOP1 is predicted to have 12 transmembrane domains and three
evolutionarily conserved domains (Hughes et al. 2008). Primary structure of OTOP1 and positive
expression in the utricle and saccule raised a possibility that OTOP1 may regulate Ca\(^{2+}\) flux in
the globular substance vesicles and/or inner ear cells. Indeed, our previous studies demonstrated
that \textit{in vitro} OTOP1 is sufficient to modulate a purinergic response (Hughes et al. 2007).

Overexpression of OTOP1 in immortalized cell lines leads to a non-specific depletion of
endoplasmic reticulum Ca\(^{2+}\) stores, a specific inhibition of P2Y receptor signaling, and influx of
extracellular Ca\(^{2+}\). However, the lack of \textit{in vivo} data, which can functionally link these
biochemical activities of OTOP1 with Ca\(^{2+}\) regulation in the maculae, has yet to be resolved.

Here, we demonstrate expression of \textit{Otop1} in the extra-striolar region of the utricle and
saccule, with subcellular localization to the apical region of supporting cells. We present
evidence that OTOP1 regulates [Ca\(^{2+}\)]\textsubscript{i} in vestibular supporting cells \textit{in vivo} by inhibiting P2Y
receptor-mediated intracellular Ca\(^{2+}\) release in an extracellular Ca\(^{2+}\)-dependent manner in
response to ATP. These data support a model by which OTOP1 concentrates Ca\(^{2+}\) in supporting
cells to allow nucleation, growth and maintenance of otoconia in a low Ca\(^{2+}\) environment.

Materials and Methods

\textit{Generation of the Otop1\textsuperscript{βgal/βgal} allele.} The targeting construct was made using
recombineering methods (Liu et al. 2003). First, about 5 kb upstream and downstream of the
regions to target was retrieved from BAC clone RP24-286E11 (derived from C57BL/J6 mice)
which completely spanned the \textit{Otop1} gene. We designed a deletion of the last 62 bp of exon 2
after the \textit{Otop1c} splice acceptor site and 2.7 kb of intron 2 and inserted the \(β\)-galactosidase (\(β\)gal)
gene and the \textit{LoxP/pol-2-neo/LoxP} selectable marker (6.1 kb). This configuration created an
OTOP1\textsuperscript{βGAL} fusion protein that includes 109 amino acid residues of OTOP1a, 107 amino acid
residues of OTOP1b, or 41 amino acid residues of OTOP1c amino terminal coding sequence fused at amino acid residue 5 of βGAL. The 5' and 3' regions of homology contained a total of 8.5 kb of genomic DNA. After verifying with restriction mapping and sequencing, the targeting construct was linearized and electroporated into SCC-10 ES cells, which were derived from 129X1/SvJ mice. Electroporation was carried out in the Washington University Siteman Cancer Center Murine Embryonic Stem Cell Core facility. G418 resistant clones were screened for homologous recombination by Southern blot using 5' probes. Two positive clones were identified and homologous recombination was verified using a 3' Southern blot probe. ES clones were karyotyped and then microinjected into mouse blastocysts by the Washington University Mouse Genetics Core facility. Chimerism was determined by coat color and high percentage chimeric males were mated with C57BL6/J females. The subsequent F1 mice were intercrossed, and the litters were examined for germline transmission of the Otop1βgal allele using Southern blotting with 5' and 3' probes extrinsic to the targeting vector and by genomic PCR (Fig1. D,E). The LoxP/pol-2-neo/LoxP cassette was excised in vivo by mating to a mouse that expressed CRE recombinase in the germ line, and then the male progenies were bred to C57BL6/J females to transfer the targeted allele onto a C57BL6/J genetic background. Phenotypes that we observed in this study appeared independent of genetic background. All animal studies were carried out in accordance with the guidelines and approval (protocol number 20070220) from the Washington University Animal Studies Committee.

Southern blotting and genotyping. Genomic DNA was extracted with phenol/chloroform and isopropanol precipitation. 10 μg of each sample was digested with EcoRV at 37°C overnight, and electrophoresed on a 1% agarose gel. Southern blots were probed with 5’ and 3’ probes, which were labeled with 32P (Stratagene Prime-It II Random Primer Labeling Kit, La Jolla, CA)
and purified (Amersham Biosicences MicroSpin S-200 HR columns, Pittsburgh, PA). 5’ and 3’ probes were made by amplification from RP24-286E11 BAC DNA with the following primers; 5’ probe: primer 1 (5’-CACCACGTCTAAACAAGCCA- 3’ (forward)), primer 2 (5’-GCCACACAGGACTTTCTTTT- 3’ (reverse)), 3’ probe: primer 1 (5’-GATAATTCACACTGTAAGC- 3’ (forward)), primer 2 (5’-GAGATTCAGTACCAATGATT- 3’ (reverse)). For genotyping PCR, mouse tail DNA was amplified with the following primers; primer 1 (5’-AGGGTCTCCACAAGCTTCCGGT-3’ (forward)), primer 2 (5’-TGACAGCCTACAGCCCAGGATG-3’ (Otop1+/+ reverse)), primer 3 (5’-CCATTCAGGCTGCGCAACTGT-3’ (Otop1βgal reverse)). 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 68°C for 3 min were used to amplify either 545 (Otop1+/+) or 369 (Otop1βgal) bp fragments.

H&E and X-gal staining. Temporal bones were dissected in cold PBS (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.4), and fixed in 4% paraformaldehyde at 4°C overnight. After washing in PBS, tissues were embedded in paraffin. 8 μm sections were deparaffinized and stained with hematoxylin and eosin (H&E). For X-gal staining, temporal bones were fixed at 4°C overnight in 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), 100 mM MgCl2 in PBS. Adult temporal bones were decalcified in 0.35 M EDTA in 0.1 M sodium phosphate buffer (pH 7.2-7.4) before further processing. For frozen sections, fixed temporal bones were incubated in 30% sucrose overnight and then frozen in OCT prior to cryosectioning. Whole temporal bones or frozen sections were stained for βGAL at room temperature or 4°C in the following solution: 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet-P40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% X-gal in PBS. After sufficient color development, temporal bones or sections were washed in PBS and imaged.
RNA extraction, cDNA synthesis, semi-quantitative and quantitative RT-PCR. For detection of mouse Otop1 mRNA, tissues (utricle, saccule, cochlea, lung) from either 4 adult or 8 pups were dissected and pooled. For detection of purinergic receptors, a plate of mouse utricular dissociated cultures made from 8 P3 mouse utricular epithelia was used. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and ethanol precipitation. 0.5-1 μg of RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove genomic DNA contamination prior to cDNA synthesis (Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR, Carlsbad, CA).

Mouse Otop1 mRNA levels were quantified using a TaqMan® gene expression assay (#00554705_m1, ABI inc., Foster City, CA). Taqman assays were run in a 7500 fast real-time PCR machine (ABI inc., Foster City, CA). For semi quantitative RT-PCR analysis of P2X and P2Y receptors in mouse dissociated epithelial cultures, previously designed primers and published PCR conditions were used (Hayato et al. 2007). The P value for difference in expression levels was calculated by two-tailed student’s t-test.

Production of anti-Otop1 antibody. A rabbit polyclonal antibody was made using a 16-amino acid peptide epitope (ARGSPQASGPRRGASV) derived from the N-terminus of OTOP1 (Fig 1C). The synthetic peptide was conjugated to maleimide-activated keyhole lympet hemocyanin prior to injection into the rabbit. The resulting antibody was purified on the peptide affinity column and validated by Western blotting of extracts from inner ear tissues. Further validation was performed in this study by comparing the immunostaining patterns with those of X-gal staining.

Wholemount immunohistochemistry. Temporal bones were isolated in Leibovitz’s medium L15 (Sigma, St. Louis, MO) and fixed in 4% paraformaldehyde for 1-2 hrs at room temperature. Utricular and saccular maculae were dissected from the temporal bones in cold
PBS, incubated in 0.5% Triton X-100 for 30 min at room temperature, and then washed with PBS. Samples were blocked using 4% BSA/PBS overnight at 4°C and incubated with rabbit anti-OTOP1 (1:800), mouse anti-parvalbumin (Sigma, St. Louis, MO- 1:500), rabbit anti-myosin VIIa (Proteus Biosciences, Romana, CA- 1:500), rabbit anti-βTectorin (a gift from Dr. Guy Richardson- 1:500), rabbit anti-P2Y2 receptor (Alomone Labs, Jerusalem, Israel, a gift from Dr. Tom Steinberg- 1:500), or rabbit anti-P2Y4 receptor (Alomone Labs, Jerusalem, Israel, a gift from Dr. Tom Steinberg- 1:500) for 90 min at room temperature in a humidified chamber. After washing with PBS, samples were incubated with secondary antibodies, Alexa 488 anti-rabbit IgG (Invitrogen, Carlsbad, CA - 1:600),Alexa 555 anti-mouse IgG (Invitrogen- 1:600), or Cy3 anti-rabbit IgG (Chemicon, Billerica, MA-1:200), respectively, for 45 min at room temperature. For stereocilia staining, samples were further incubated with rhodamine-phalloidin (1:200, Invitrogen, Carlsbad, CA) for 15 min at room temperature. After washing with PBS, samples were transferred to a superfrost slide, mounted with vectashield (Vector labs, Burlingame, CA), and coverslipped.

Frozen section immunohistochemistry. Frozen sections were recovered in PBS and blocked with 4% BSA, 0.3% Triton X-100 in PBS. Sections were incubated with rabbit anti-OTOP1 (1:800), chicken anti-βGAL (Abcam, Cambridge, MA- 1:1000), or mouse anti-ZO-1 (Zymed, San Francisco, CA- 1:100) antibodies overnight at 4°C. After washing with 0.3% Triton X-100 in PBS, sections were incubated with secondary antibodies (Cy3 anti-rabbit IgG – 1:200, Alexa 488 anti-chicken IgG and Alexa 488 anti-mouse – 1:600) for 2 hrs at RT. Samples were mounted with vectashield (Vector labs, Burlingame, CA).

Overexpression of EGFP-Otop1 in utricular culture cells using genegun transfection. Pieces of the utricular maculae were dissected from postnatal day 0–4 rats and attached to
coverslips previously coated with Cell-Tak (150 μg/μL; BD Biosciences, San Jose, CA). Cultures were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA) with 5–7% fetal bovine serum (FBS) and ampicillin (1.5 μg/mL; Sigma, St. Louis, MO) and kept at 37 °C and 5% CO2. For transfections, 50 μg of EGFP-Otop1 was precipitated onto 25 mg of 1 μm gold particles and loaded into Helios Gene Gun cartridges (BioRad, Hercules, CA). Tissue explants were transfected with the gene gun set at 95 psi of helium and maintained in culture for 24 hrs.

Utricular macular organotypic and dissociated cultures. E18.5-P3 mouse utricles were dissected in medium 199 (Gibco #12350039), and the nonsensory epithelium and otoconial layer were completely removed. After incubation in thermolysin (25 mg in 50 ml medium 1999 (Gibco #12340030)) (Sigma, St. Louis, MO) at 37°C for 50 min, the underlying stroma was removed and the remaining epithelial sheet was attached to a MatTek dish using ECM gel (Sigma, St. Louis, MO). The tissue was incubated in Medium 199 (Gibco #12340030) containing 10% fetal bovine serum (Gibco#10091-155) (growth media) overnight at 37°C. For dissociated culture, isolated epithelial sheets were treated with Trypsin/EDTA (0.05%, 0.02%) for 15 min at 37 °C. After replacing Trypsin/EDTA with Medium 199 (Gibco #12340030), cells were triturated 5 to 10 times and plated on laminin coated MatTek dishes and incubated overnight at 37°C. The next day, growth media was added.

Ratiometric calcium imaging and data analysis. All imaging experiments were carried out at room temperature in a HEPES-buffered salt solution (HCSS) containing, in mM: 140 NaCl, 5.4 KCl, 1 NaH2PO4, 1.8 CaCl2, 1 MgSO4, 12 HEPES, and 5.5 D-glucose, pH 7.4±0.1 or nominally Ca2+-free media (HCSS solution without CaCl2). Organotypic or dissociated utricular macular cultures were loaded with fura-2 by incubation for 60 min with 5-10 μM fura-2 acetoxyethyl (AM) ester (Invitrogen, Carlsbad, CA) and 0.1% Pluronic F-127 (Invitrogen,
Carlsbad, CA) in HCSS (pH=7.2) at room temperature, washed with HCSS and incubated for another 60 min to allow for ester hydrolysis. After loading, cells were imaged on an inverted microscope (Nikon Eclipse TE300, Nikon Inc., Melville, NY) equipped with a cooled CCD camera (Cooke Corp., Auburn Hill, MI) using a 20x/0.45 Plan Fluor objective (Nikon). The fluorescence excitation (75 W xenon arc lamp) was provided by band-specific filters (340 and 380 nm; Semrock, Rochester, NY) in combination with a XF73 dichroic beam splitter (Omega Optical, Brattleboro, VA). Pairs of images were collected constantly at alternate excitation wavelengths. After subtracting the matching background, the image intensities were divided by one another to yield ratio values for individual regions. For studies done with mouse macular dissociated cultures (Fig. 6), 10 μM ionomycin (EMD Biosciences, Gibbstown, NJ) was added at the end of each experiment to obtain the maximum ratio (340/380) in each region of interest. To reduce variability between experiments performed on different days, raw data were normalized as follows: Normalized Ratio = (R- R0)/(Rmax- R0), R, individual ratio value; Rmax, peak ratio after addition of ionomycin; R0, average of the prestimulation baseline ratio. Absolute baseline values were comparable between experiments. All the data in Fig. 6E-I represent normalized ratios. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. To compare the rates of decay in intracellular [Ca2+]i following the initial peak response to purinergic agonists (Fig. 6J), a single exponential decay curve (y = ye^{-λt}, y, normalized ratio; t, time; λ, decay constant) was fitted to the normalized ratio values collected within 90 sec after the initial peak. Decay constants (λ) were then averaged for individual experiments and normalized to Otop1+/+ tissues treated with ATP. To compare the rates of decay in the plateau phase (Supplemental Fig. 4) we used the same methods to analyze data points, but starting 40 sec after
the initial peak and extending until wash. Significant differences between the decay constants calculated for different experimental conditions were determined by a two-tailed Student’s t-test.

**Results**

**Otopetrin 1 (Otop1) deficiency results in otoconial agenesis**

To generate a functional null allele for *Otop1* and to create a histochemical tag to identify OTOP1-expressing cells, the *Otop1* gene was targeted by insertion of a β-galactosidase (βgal) coding sequence (Fig. 1). *Otop1* has seven exons and three identified amino terminal alternative splice variants, *Otop1a, 1b* and *1c* (Fig. 1A,B). Although *Otop1a* is the most abundant splice variant expressed in the inner ear (Hurle et al. 2003), to ensure generation of a functional null allele, the βgal cDNA was inserted in frame in exon 2 such that all known splice variants would be captured as fusion proteins with βGAL. This design expressed *Otop1βgal* transcripts under the control of *Otop1* transcriptional regulatory elements. For the *Otop1aβgal* and *Otop1bβgal* transcripts, this fusion protein includes the first two TM domains of OTOP1 (Fig. 1C). The germ-line-transmitted targeted allele was bred to homozygosity. Heterozygous *Otop1βgal/+* mice were phenotypically normal (Fig. 1F,G). *Otop1βgal/βgal* mice showed vestibular dysfunction (inability to swim) and otoconial agenesis (Fig. 1H). The sensory epithelium of the utricle and saccule were histologically normal.

**OTOP1βGAL expression in the vestibular sensory epithelium**

The spatial and temporal expression pattern of OTOP1βGAL in the inner ear was assessed by X-gal staining. βGAL activity was first apparent at E13.5 in the utricle (Fig. 2A,B), whereas expression in the saccule was first detected one day later, at E14.5 (Fig. 2C,D). By E16.5, the staining was present throughout the whole epithelium in the utricle and saccule (Fig. 2E,F). This expression pattern correlated with the onset and maximal rate of otoconial synthesis in the utricle.
and saccule (Anniko 1980; Anniko et al. 1987). Interestingly, examination of isolated whole
maculae revealed a striking absence of expression in the striolar region (Fig. 2G,H), a pattern
that persisted in the adult ears (data not shown). In addition to expression in non-striolar regions
of the macula, some transitional cells were also positive for βGAL activity (Fig. 2G,J), indicating
possible involvement of a subset of transitional cells that most likely function in the calcification
process during otoconial development. Importantly, no expression was observed in any other
inner ear organs or structures (cristae or cochlea) at any time point examined, with the exception
that, at E18.5, trace βGAL activity was occasionally detected in the endolymphatic sac (data not
shown).

Histological sections through the maculae revealed a staining pattern consistent with
expression in the maculae (black arrowheads) and transitional cells (white arrowheads) (Fig. 2J).
Inner ear sections from P12 and 6-month-old mice (Fig. 2K,L) showed that Otop1 continues to
be expressed in the adult inner ear. Depending on the orientation of the sections, we observed the
absence of X-gal staining within the central maculae, which reflected lack of expression in the
striolar region (Fig. 2G,H,K). To confirm expression in adult mice, endogenous Otop1 mRNA
was detected by quantitative RT-PCR. Consistent with the βGAL activity, Otop1 expression was
detected in both P2 and 6-month-old utricle and saccule, but not in the cochlea or lung (Fig. 2M).
These data suggest that OTOP1 could function to maintain otoconia mineralization in the adult.

**OTOP1 is localized towards the apex of supporting cells**

Otoconia are formed within the gelatinous membrane above the utricular and saccular
sensory epithelia. We hypothesized that OTOP1 protein would be localized at a subcellular site
consistent with a role in the biosynthesis and/or maintenance of otoconia. Polyclonal anti-
OTOP1 antibody was generated to an epitope located in the amino terminus of OTOP1, and
because this epitope is maintained in the OTOP1^{βGAL} fusion protein (Fig. 1C), immunostaining of Otop1^{βgal/βgal} tissues revealed the location of the chimeric protein. Co-localization of anti-OTOP1 and anti-βGAL signals on Otop1^{βgal/βgal} sections (Fig. 3D-F) confirmed the specificity of the anti-OTOP1 antibody.

In Otop1^{+/+} macula, endogenous OTOP1 immunoreactivity was concentrated toward the apical surface of the epithelium (Fig. 3A-C, 4A-C). Co-localization of OTOP1 with zona occludens 1 (ZO-1) protein showed that some OTOP1 protein is localized at the apical cell surface (Fig. 4A-C). In Otop1^{βgal/+} and Otop1^{βgal/βgal} utricles, one or two copies of the targeted allele resulted in formation of large inclusions, with Otop1^{βgal/βgal} tissues showing a more intense signal (Supplemental Fig. 1). Immunostaining of wholemounts of Otop1^{+/+} and Otop1^{βgal/βgal} utricle and saccule with the anti-OTOP1 antibody was used to identify cell-specific expression of the endogenous OTOP1 protein. Co-staining with either phalloidin (to mark stereocilia) (Fig. 4D, E) or α-parvalbumin (to mark the hair cell body) (Fig. 4F) showed no co-localization of either endogenous OTOP1 and phalloidin-stained stereocilia or OTOP1^{βGAL} intracellular aggregates and α-parvalbumin, indicating exclusive localization of OTOP1 in supporting cells. Additionally, biolistic transfection of EGFP-tagged OTOP1 (EGFP-OTOP1) in macular epithelium showed protein localization to apical microvilli in supporting cells (Fig. 4G-I). Optical sections through the middle of the sensory epithelium showed some expression in intracellular vesicles (Fig. 4J-L). Optical sections through the basal end of the sensory epithelium showed no EGFP-OTOP1 expression (Fig. 4M-O). Transfected hair cells also showed localization of transfected EGFP-OTOP1 to the stereocilia bundles (data not shown), demonstrating that apical targeting of the OTOP1 protein occurs independent of cell type.
In summary, X-gal staining and immunohistochemical analysis showed that OTOP1 is expressed in the supporting cells in the extra-striolar regions of the utricle and saccule, suggesting that this population of supporting cells is crucial for development of otoconia. To examine whether deletion of *Otop1* could affect development of hair cells or the striola, immunostaining with Myosin VIIa or βTectorin was performed, respectively (Supplemental Fig. 2). Normal expression and localization of these markers suggested that otoconia agenesis and lack of *Otop1* in *Otop1*βgal/βgal inner ears is unlikely to affect hair cell development or formation of the striola.

**OTOP1 modulates purinergic response in the utricular maculae**

Previous in vitro studies (Hughes et al. 2007) identified a potential role for OTOP1 in modulating cytosolic free calcium ion concentration ([Ca^{2+}]_i) in response to purinergic signals, such as ATP. Because endolymph is known to contain ATP and purinergic receptors have been identified in several cell types within the inner ear (Housley 2000; Lee and Marcus 2008), we hypothesized that OTOP1 may regulate purinergic signaling in vestibular supporting cells. To determine if endogenous OTOP1 is necessary to regulate a purinergic response in mouse utricular epithelium, organotypic utricular macular cultures from *Otop1*+/+ and *Otop1*βgal/βgal inner ears were loaded with the ratiometric Ca^{2+} indicator, fura-2, and imaged to monitor [Ca^{2+}]_i before, during, and after stimulation with 100 μM ATP (Carter et al. 1988; Tsien et al. 1985). [Ca^{2+}]_i was examined in multiple areas within each organotypic culture (Fig. 5A-C). Comparable baseline ratio values for *Otop1*+/+ and *Otop1*βgal/βgal cultures showed that absence of *Otop1* does not affect resting [Ca^{2+}]_i. In *Otop1*+/+ cultures, addition of 100 μM ATP resulted in an increase in [Ca^{2+}]_i, characterized by an elevated plateau that persisted until removal of the agonist (Fig. 5D, black symbols). The shape of this response curve resembled that of the globular substance.
vesicle response to ATP observed by Suzuki et al. (Suzuki et al. 1997a). In contrast, in

$Otop1^{\beta gal/\beta gal}$ maculae, adding ATP resulted in a rapid increase in $[\text{Ca}^{2+}]_i$, followed by a slow
decline in $[\text{Ca}^{2+}]_i$ to an elevated plateau (Fig. 5D, red symbols). These observations demonstrated
that OTOP1 modulates purinergic signaling in the utricular maculae.

To further understand the mechanism by which OTOP1 regulates purinergic receptor
functions, dissociated cultures from $Otop1^{+/+}$, $Otop1^{\beta gal/+}$, and $Otop1^{\beta gal/\beta gal}$ maculae were used.
This allowed more efficient use of isolated macular epithelia. Attempts to dissociate mouse
maculae resulted in cultures that contained regions of epithelial sheets and clusters of cells that
formed globule-like structures in all genotypes examined (Fig. 6A, asterisk and arrow).
Interestingly, $\beta$GAL activity in $Otop1^{\beta gal/+}$ or $Otop1^{\beta gal/\beta gal}$ cultures was present in cells
associated with these globule-like structures, and was rarely found in the adjacent epithelial
sheets (Fig. 6D, arrow, asterisk). Therefore, the regions near the globule-like structures were
selected for imaging (Fig. 6B,C). Similar to the response seen in organotypic cultures (Fig. 5D),
addition of ATP to $Otop1^{\beta gal/\beta gal}$ dissociated macular cells resulted in a higher peak and increased
rate of decay than that observed in $Otop1^{+/+}$ dissociated cultures (Fig. 6E,J). This result
confirmed that dissociated cultures behave similarly to whole maculae and can therefore be used
to further characterize the function of OTOP1. Importantly, this differential response between
$Otop1^{+/+}$ and $Otop1^{\beta gal/\beta gal}$ cultures was no longer observed when the response to ATP was
compared from regions of the cultures that did not express OTOP1 (in $Otop1^{+/+}$ cultures) or
OTOP1$^{\beta GAL}$ (in $Otop1^{\beta gal/\beta gal}$ cultures) (Fig. 6F), confirming the specificity of OTOP1 activity.
Notably, $Otop1^{+/+}$ and $Otop1^{\beta gal/+}$ cultures showed a similar response to ATP (Fig. 6E),
demonstrating that a single $Otop1$ allele is sufficient for OTOP1-specific modulation of the
purinergic response, and is consistent with the presence of normal otoconia in $Otop1^{βgal/+}$ mice (Fig. 1G).

Activation of P2Y receptors results in release of Ca$^{2+}$ from inositol-1,4,5-trisphosphate (IP3)-sensitive intracellular stores, whereas the P2X receptor family acts as a channel for Ca$^{2+}$ influx from the extracellular space. To determine which purinergic receptor family is regulated by OTOP1 in vivo, we sought to block P2Y or P2X mediated Ca$^{2+}$ signaling by restricting Ca$^{2+}$ influx into the cytosol from intracellular stores or the extracellular space, respectively. Bis-phenol inhibits the function of intracellular Ca$^{2+}$-ATPases, consequently emptying intracellular Ca$^{2+}$ stores and uncoupling P2Y receptor signaling (Brown et al. 1994; Harper et al. 2005). In the presence of bis-phenol, the response of $Otop1^{+/+}$ cultures to ATP did not significantly change (Fig. 6E,G). However, $Otop1^{βgal/βgal}$ cultures no longer showed the high peak after addition of ATP and responded in a similar manner with a peak and rate of decay comparable to that of $Otop1^{+/+}$ cultures (Fig. 6E,G, J). This suggests that the high peak originally shown in $Otop1^{βgal/βgal}$ cultures (Fig. 6E) is indicative of P2Y mediated Ca$^{2+}$ release and that OTOP1 normally interferes with this process. Our macular epithelial cultures express all seven P2Y isoforms (Supplemental Fig. 3), and each isoform has distinct agonist sensitivity (Burnstock 2007; Ralevic and Burnstock 1998). To identify which P2Y receptor function is inhibited by OTOP1, we additionally performed ratiometric Ca$^{2+}$ imaging with three other major purinergic ligands, UTP, ADP, and UDP. Since the initial response after addition of the ligand indicates an increase in [Ca$^{2+}$], by P2Y-mediated signaling, we looked for ligands which would result in a significant difference in the initial peak level between $Otop1^{+/+}$ and $Otop1^{βgal/βgal}$ cultures. Treating cultures with UTP, a P2Y-specific ligand (Supplemental Fig. 4) (Burnstock 2007; Ralevic and Burnstock 1998), allowed examination of the effect of OTOP1 exclusively on P2Y
receptors and not on P2X receptors. Notably, similar to what was observed with ATP, in response to UTP, [Ca$^{2+}$] in Otop1$^{βgal/βgal}$ cultures increased to a higher level when compared to that in Otop1$^{+/+}$ cultures (Fig. 6H). However, significant differences in the peak value were not observed following treatment with either ADP or UDP (Supplemental Fig. 5). This suggested that OTOP1 might preferentially inhibit the function of P2Y receptor subtypes featuring high affinity to ATP and UTP, such as P2Y2 and P2Y4 (Dubyak 2003; Ralevic and Burnstock 1998). Other purinergic receptors expressed in the macular epithelium, that are not regulated by OTOP1, may also contribute to the observed increase in [Ca$^{2+}$], in response to ADP or UDP. Alternatively, the inhibitory function of OTOP1 may require ATP or UTP but not ADP or UDP.

Next, we investigated the source of Ca$^{2+}$ that contributes to elevated [Ca$^{2+}$], (plateau phase) in response to ATP in the presence of OTOP1. If OTOP1 inhibits all P2Y-mediated Ca$^{2+}$ release and wholly relies on extracellular Ca$^{2+}$ for increased [Ca$^{2+}$], in response to ATP, we would not see any increase in [Ca$^{2+}$], in Otop1$^{+/+}$ cultures in Ca$^{2+}$-free media. In support of this model, a similar response to ATP was observed when imaging in either “nominally” Ca$^{2+}$ free media (containing 10-20 µM Ca$^{2+}$, determined with a Ca$^{2+}$-selective electrode and fura-2 fluorometry) (Fig. 6I) or Ca$^{2+}$-free media (normal media with 10 mM EGTA which contains less than 1 nM Ca$^{2+}$, data not shown). Notably, similar results were also obtained when the imaging was performed in nominally Ca$^{2+}$-free media (Fig. 6I) or continuously in the same dish, first with normal media and second following a change to nominally Ca$^{2+}$-free media (Supplemental Fig. 6). Interestingly, Otop1$^{+/+}$ cultures in nominally Ca$^{2+}$-free media did show a response to ATP with similar peak levels to that shown in the presence of extracellular Ca$^{2+}$, but the following plateau phase was much reduced, resulting in an increased rate of decay (Fig. 6E,I,J). This suggests that OTOP1 interferes with P2Y receptor function (possibly P2Y2 and/or P2Y4).
and promotes extracellular Ca\(^{2+}\) influx. \textit{Otop1}\(^{\beta\text{gal}/\beta\text{gal}}\) cultures also showed a reduction in the plateau phase, but most interestingly, the difference in the initial peak value between \textit{Otop1}\(^{+/+}\) and \textit{Otop1}\(^{\beta\text{gal}/\beta\text{gal}}\) cultures was no longer observed when extracellular Ca\(^{2+}\) was removed. A possible explanation for this observation is that the function of OTOP1, to inhibit P2Y activity, may depend on extracellular Ca\(^{2+}\) (at concentrations higher than that in nominally Ca\(^{2+}\) free media). These data support a model in which OTOP1 inhibits P2Y receptor-mediated Ca\(^{2+}\) release in macular epithelial cells in a Ca\(^{2+}\)-dependent manner, and promotes an influx of Ca\(^{2+}\) in response to ATP.

**Discussion**

\textit{Otop1} is essential for otoconia formation and thus for the normal physiological function of the gravity receptor organs in the inner ear. Several missense alleles in \textit{Otop1} have been identified (\textit{tilted, mergulhador, backstroke}) and all lead to otoconial agenesis in the mouse or otolith agenesis in the zebrafish (Hurle et al. 2003; Sollner et al. 2004). The biochemical function(s) of OTOP1 is poorly understood and the biochemical consequences of these missense mutations are not known. To interpret phenotype in relation to the biochemical function of \textit{Otop1} alleles requires knowledge of the genetic null allele and its phenotype. To this end, we have created a functional null for \textit{Otop1}, tagged with \(\beta\text{-galactosidase (Otop1}\(^{\beta\text{gal}})\). Homozygous \textit{Otop1}\(^{\beta\text{gal}/\beta\text{gal}}\) mice have otoconial agenesis, similar to that seen in \textit{tilted} and \textit{mergulhador} mice.

The \textit{Otop1}\(^{\beta\text{gal}}\) allele permitted a highly sensitive assay to identify spatial and temporal patterns of \textit{Otop1} expression and to validate an antibody directed against OTOP1. Staining whole maculae for \(\beta\text{-galactosidase activity revealed several novel features of \textit{Otop1} expression. Most remarkably, \textit{Otop1} is excluded from the striola in both the utricle and saccule, while intensely expressed in the remaining regions of the maculae. Otoconia size range from 0.1 to 25
μm in mammals (Mann et al. 1983; Ross and Pote 1984), and the striola region of the utricle is
known to have fewer and smaller otoconia. In the saccular striola, otoconia are again smaller, but
are greater in number forming a ridge in the otoconial layer (Lim 1973; Lindeman 1973). Thus,
otoconia formed above the site where Otop1 is expressed are likely to be larger in size in the
extra-striolar areas, correlating Otop1 expression with its proposed role in the regulation of
otoconia growth or calcification. We hypothesize that otoconia found above the striola originated
in adjacent regions expressing Otop1 and ceased growing once removed from regions above
Otop1 expression. Similarly, in other sites in the inner ear that do not express Otop1 (ampula and
cochlea), otoconia are not formed even though the major otoconia core protein, Otoconin 90, is
ubiquitously expressed throughout the inner ear (Verpy et al. 1999; Wang et al. 1998).

Staining histological sections of the maculae for βGAL activity and
immunohistochemical detection of endogenous OTOP1 revealed that OTOP1 is expressed in the
supporting and a subset of transitional cells of the mouse utricle and saccule, but not in hair cells.
In contrast, zebrafish Otop1 mRNA was localized to hair cells during otolith growth (Sollner et
al. 2004). This disparity between mouse and zebrafish might reflect differences in the cell types
that are critical for otolith and otoconia formation. Importantly, it has been shown that hair cells
in mice (Bermingham et al. 1999; Hughes et al. 2006; Lundberg et al. 2006) and supporting cells
in zebrafish (Haddon et al. 1999; Hughes et al. 2006; Lundberg et al. 2006) are not required for
otoconia/otolith formation.

Otoconia are formed during embryonic and perinatal development and Otop1 is
expressed throughout this time. Once formed, the initial complement of otoconia is thought to
last throughout the life of a mammalian organism, with only limited amounts of Ca^{2+} turnover
(Balsamo et al. 2000; Preston et al. 1975). The observation that Otop1 expression persists in the
adult (mice) suggests a possible role for OTOP1 in the maintenance and/or repair of otoconia mineralization. Demonstration of such a role for OTOP1 will require conditional inactivation of *Otop1* at a time after otoconial development is complete. In humans, otoconia are prone to degeneration and displacement, leading to benign positional vertigo and progressive loss of balance (House and Honrubia 2003; Ross et al. 1976; Welling et al. 1997). It is not known whether, in humans, *Otop1* is expressed throughout life and whether OTOP1 is necessary to maintain or repair otoconia. Analysis of *Otop1* expression in human utricle and saccule maculae could provide a clue as to whether OTOP1 has a conserved role in the maintenance of otoconia in different species. It will also be interesting to investigate whether deformation and fragmentation of otoconia, a frequent occurrence in old animals (Jang et al. 2006) and humans (Walther and Westhofen 2007), results from diminished expression or activity of OTOP1.

Previous studies showed that OTOP1 expression in heterologous cells could modify the cellular response to purinergic signals, resulting in altered $[\text{Ca}^{2+}]_i$ (Hughes et al. 2007). To determine whether a similar OTOP1-dependent biochemical activity exists in inner ear supporting cells *in vivo*, we examined OTOP1 function in mouse utricular epithelial cultures. Comparing purinergic response in *Otop1*+/+ and *Otop1*βgal/βgal mouse utricular macular epithelial cultures identified distinct functions by which OTOP1 modulates purinergic signaling *in vivo*. It is important to note that the observed modulation of purinergic signaling caused by the absence of OTOP1 could be an indirect consequence on the expression or localization of another critical protein that normally interacts with OTOP1. Most importantly, these studies show that endogenous OTOP1 functions to inhibit P2Y receptor activity and subsequent release of $\text{Ca}^{2+}$ from intracellular stores in response to purinergic stimuli *in vivo*. This function was closely phenocopied by depleting intracellular $\text{Ca}^{2+}$ stores by application of bis-phenol, an inhibitor of all
microsomal Ca\(^{2+}\) ATPases that effectively uncouples Ca\(^{2+}\) release from P2Y signaling (Brown et al. 1994; Harper et al. 2005). Interestingly, thapsigargin (TG), which functions similarly to bisphenol by inhibiting Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase, did not have any effect on macular epithelial cultures (Data not shown). Considering several previous studies which have identified TG-resistant cells (Tanaka and Tashjian 1993; Waldron et al. 1995; Watson et al. 2003), it is possible that macular epithelial cells may contain unique TG-resistant intracellular Ca\(^{2+}\) stores, possibly identifying a unique cellular compartment in these cells.

The function of P2Y signaling during otoconia formation is not known. However, recent studies have identified a role for P2Y receptors in bone mineralization, which share a number of common features with otoconia mineralization (Hughes et al. 2006; Orriss et al. 2007). In osteoblasts, P2Y2 receptor activation by ATP and UTP inhibits mineralization \textit{in vitro} and P2Y\(^{-}\)/mice show increased bone mineral density (Hoebertz et al. 2002; Orriss et al. 2007). In the inner ear, multiple P2Y receptors are expressed in the sensory (Supplemental Fig. 3) and nonsensory epithelium (Piazza et al. 2007; Sage and Marcus 2002; Vlajkovic et al. 2007), and could function to inhibit ectopic mineralization in the inner ear. Suppressing ectopic mineralization would be essential because ectopic mineral deposits or otoconia fragments in the semicircular canals (cupulolithiasis) is thought to be a major cause of positional vertigo (Marom et al. 2009; Parnes and McClure 1992) and ectopic mineralization in the cochlea could impair auditory function. Inhibition of P2Y receptor signaling by OTOPI in the macular epithelium could serve to locally suppress P2Y activity and thus be permissive to localized mineralization of otoconia. An important long-term goal would be to produce \textit{P2Y} (most preferentially \textit{P2Y2} or \textit{P2Y4}) and \textit{Otop1} double null mice to see if the loss of \textit{P2Y} function rescues the \textit{Otop1} mutant phenotype.
An additional passive mechanism that could prevent ectopic mineralization in the inner ear is the very low concentration of Ca\(^{2+}\) ions normally found in endolymph (10-100 μM) (Anniko 1980; Salt et al. 1989). However, a consequence of low endolymph [Ca\(^{2+}\)] is the requirement to locally increase [Ca\(^{2+}\)] at the site and time of otoconia nucleation and growth. This could be achieved by nucleating otoconia within Ca\(^{2+}\)-rich vesicles released from supporting cells and/or by maintaining local high concentrations of Ca\(^{2+}\) in the otoconial membrane. The presence of globular substance vesicles (Suzuki et al. 1997b; 1995a) and Ca\(^{2+}\) ATPase activity in the apical end of the supporting cells (Yoshihara et al. 1987) support both mechanisms. The observation that endogenous OTOP1 promotes a P2X receptor-like influx of extracellular Ca\(^{2+}\) suggests a possible role for OTOP1 in sequestration or trafficking of Ca\(^{2+}\) in the maculae. As a transmembrane domain protein, OTOP1 could function as a novel regulator of Ca\(^{2+}\) flux, which does not have homology to any known multi-transmembrane domain proteins, or it may induce activity of other P2X-like channels. It is possible that OTOP1 regulated flux of Ca\(^{2+}\) in supporting cells is a prerequisite for locally increasing [Ca\(^{2+}\)], either directly in the extracellular space of the otoconial membrane or within globular substance vesicles. Notably, the characteristics of the ATP-induced increase in [Ca\(^{2+}\)] by OTOP1 was very similar to the atypical purinergic response described by Suzuki et al in isolated globular substance vesicles (Suzuki et al. 1997a), suggesting that OTOP1 may be responsible for the ATP-mediated increases in intravesicular [Ca\(^{2+}\)], seen in these studies. Differences in the observed response to UTP may be due to presence of different proteins in globular substance vesicles and supporting cells or due to species-specific differences. In addition to OTOP1, Plasma Membrane Ca\(^{2+}\)-ATPase 2 (PMCA2) is expressed on the apical surface of sensory hair cells (Dumont et al. 2001) and Pmca2\(^{-/-}\) mice show agenesis of otoconia (Kozel et al. 1998). Notably, [Ca\(^{2+}\)] is lower in Deafwaddler (Pmca2
frameshift mutation) endolymph compared to controls (Wood et al. 2004). Thus, both OTOP1 and PMCA2 may cooperate to regulate local concentrations of Ca^{2+} above the macular epithelium.

To investigate the requirement for extracellular Ca^{2+} for OTOP1 activity, wild type and Otop1^{βgal/βgal} macular epithelial cultures were assayed for purinergic responses in “nominally” Ca^{2+} free media and Ca^{2+} free media (with 10 mM EGTA). The [Ca^{2+}] in “nominally” Ca^{2+} free media is similar to the [Ca^{2+}] in endolymph (10-20 μM). The observation that OTOP1 could no longer inhibit P2Y function in “nominally” Ca^{2+} free media (or in the presence of 10 mM EGTA) suggests that OTOP1 is very sensitive to extracellular [Ca^{2+}]. In future studies, it will be important to test whether OTOP1 can bind Ca^{2+} and act as a sensor for extracellular [Ca^{2+}] or interact with other Ca^{2+} sensor molecules to form a feedback signal to regulate [Ca^{2+}] above the macular sensory epithelium.

The studies presented here demonstrate that one function of OTOP1 is to modulate purinergic signaling in macular supporting cells (Fig. 7). This raises the important question of the role of ATP in endolymph. ATP is thought to be stored and released from vesicles in the marginal cells of the stria vascularis. In the vestibular system non-sensory epithelium, dark cells (which are functionally similar to strial marginal cells) are a possible source of ATP (Lee and Marcus 2008). Because OTOP1 responds to ATP in a dose-dependent manner (Hughes et al. 2007), it may serve as an apical sensor of local ATP concentrations. For example, during otoconia formation, release of ATP containing vesicles might increase and maintain active OTOP1 and active otoconial growth. Alternatively, ATP may function to activate purinergic receptors throughout the inner ear (to suppress ectopic mineralization) and, through OTOP1 P2Y inhibitory activity, permit mineralization only in the otoconial membrane above the maculae.
Lastly, it remains possible that the OTOP1 response to purinergic signals and the modulation of cellular or extracellular Ca\(^{2+}\) may not be the only role for OTOP1 during otoconia formation. For example, in zebrafish, OTOP1 was shown to regulate the trafficking of *starmaker* in the sensory epithelium, a protein required for otolith formation (Sollner et al. 2004).
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References


Figure Legends

Figure 1. Targeting the *Otopetrin 1* (*Otop1*) gene. A, *Otop1* spans over 28 kb of genomic DNA on mouse chromosome 5 and contains seven exons. B, *Otop1a* coding sequence starts within exon 2 and is the major splice form of *Otop1*. *Otop1b* and *1c* are transcribed from exon 1 and splice to different sites in exon 2. In the targeting vector, the β-galactosidase (*βgal*) gene and *LoxP/pol-2-neo/LoxP* cassette were inserted in frame with exon 2 after the *Otop1c* splice site. C, The targeted allele, *Otop1*\[^βgal\]*, created a fusion protein with the first two transmembrane (TM) domains of OTOPI at the N-terminus of βGAL. The epitope to which OTOPI antibody was made is located within the N-terminal tail (red line, arrow). D, Southern blot after digesting tail genomic DNA with EcoRV. The wild type allele produces a 17 kb fragment with both 5’ and 3’ probes. Targeted allele produces 8 kb (5’) and 9 kb (3’) restriction fragments, respectively. E, PCR genotyping with tail genomic DNA. Wild type allele, 545 bp; Targeted allele, 369 bp. F-H, Histological sections through the utricle and saccule. Otoconia are normally formed above the sensory epithelia (SE) of *Otop1*\[^+/+\]* and *Otop1*\[^βgal/+\]* utricle (upper) and saccule (lower) (*F, G*). No otoconia are observed in *Otop1*\[^βgal/βgal\]* otolithic organs (*H*). Scale bar, 100 μm.

Figure 2. Spatial and temporal expression of OTOPI\[^βGAL\]* in *Otop1*\[^βgal/βgal\]* inner ears. A, B, At E13.5, βGAL activity is observed only in the utricle. C, D, The saccule starts to express OTOPI\[^βGAL\] at E14.5. White dotted regions (*B, D*) indicate location of the saccule. E, F, At E16.5, both the utricle and saccule are positive for βGAL activity. No staining is observed in the cochlea or cristae. G-H, X-gal staining of P0 *Otop1*\[^βgal/βgal\]* wholemount maculae. OTOPI\[^βGAL\] expression is observed in the macular sensory epithelium but is excluded from the striola region (S) in the utricle (*G*) and saccule (*H*). I, High magnification of the boxed region in (*G*) showing
OTOP1\textsuperscript{βGAL} expression in some transitional epithelial cells (TE). \textbf{J-L}, Histological sections through the maculae of the utricle. Localization of OTOP1\textsuperscript{βGAL} in the sensory epithelium (SE, black arrowheads) and transitional epithelium (TE, white arrowheads) is apparent at P0 (\textit{J}). Similar expression patterns are maintained at P12 (\textit{K}) and in adult tissue (\textit{L}). In some sections (\textit{K}), the striola (S) can be identified by the absence of X-gal staining. \textbf{M}, Quantitative RT-PCR showing endogenous positive \textit{Otop1} expression in the utricles/saccules from P2 and 6 month-old inner ear, but not in lung or cochlea. Error bars are standard deviations of three replicates. Scale bars, (\textit{A, C, E}) 200 μm; (\textit{B, D, F, G, H}) 100 μm; (\textit{I-L}) 20 μm.

**Figure 3.** Validation of an anti-OTOP1 antibody. \textbf{A-F}, Frozen sections of E16.5 \textit{Otop1}\textsuperscript{+/+} (\textit{A-C}) and \textit{Otop1}\textsuperscript{βgal/βgal} (\textit{D-F}) utricles stained with antibodies against OTOP1 (red) and βGAL (green). \textbf{A-C}, Endogenous OTOP1 is concentrated near the apical surface of the sensory epithelium. \textbf{D-F}, The \textit{Otop1}\textsuperscript{βGAL} chimeric protein is localized in an intracellular vesicular pattern. Anti-βGAL and anti-OTOP1 signals colocalize (\textit{F}). Scale bar, 20 μm.

**Figure 4.** OTOP1 is localized towards the apex of supporting cells. \textbf{A-C}, Frozen sections of E16.5 \textit{Otop1}\textsuperscript{+/+} utricles stained with antibodies against OTOP1 (red) and zona occludens 1 (ZO-1) (green). OTOP1 is localized at the apex (arrowheads) and intracellularly near the apex. \textbf{D-F}, Confocal Z-stacked images of P0 \textit{Otop1}\textsuperscript{+/+} (\textit{D, E}) and \textit{Otop1}\textsuperscript{βgal/βgal} (\textit{F}) wholemount utricular macula. \textbf{D}, Staining of the wild type utricle with phalloidin (red) and OTOP1 (green). Phalloidin marks the stereocilia bundles of hair cells and the unstained area beneath the stereocila identifies the hair cell body. \textbf{E}, Same image as in (\textit{D}), showing only the anti-OTOP1 channel. The asterisk indicates the absence of OTOP1 signals in hair cells. \textbf{F}, Staining of
OTOP1βgal/βgal utricle with α-parvalbumin (red) and anti-OTOP1 (green) showing that the
OTOP1βGAL chimeric protein does not colocalize with the hair cell-specific marker α-
parvalbumin. G-O, Confocal Z-stacked images of supporting cells transfected with EGFP-Otop1
(green). Phalloidin (red) marks the cell membrane. G-I, Optical sections through the apical
region showing EGFP signals present in the microvilli. J-L, EGFP signals are found in several
vesicular structures within the supporting cell body. M-O, The basal side of supporting cells
shows minimal EGFP fluorescence. Scale bars, (C) 10 µm; (E) 14µm; (O) 3µm.

**Figure 5.** Purinergic response of organotypic macular cultures from the utricles of E18.5-
P3 mice. A, Bright field image of a utricular macula in culture for 24 hr. Scale bar, 200µm. B,
Ratio (340/380) image of a culture loaded with fura-2 before stimulation with ATP. Circles show
areas selected for analysis. C, Addition of 100 µM ATP results in an increase in [Ca^{2+}], as
indicated by change in color (red: high, purple: low). D, The baseline ratios for Otop1^{+/+} and
Otop1βgal/βgal are comparable. Following addition of 100 µM ATP, Otop1^{+/+} maculae respond
with an increase in [Ca^{2+}], to an elevated plateau, whereas Otop1βgal/βgal maculae show a higher
increase in [Ca^{2+}], followed by a reduction to an elevated plateau. [Ca^{2+}], returns to baseline after
removal of agonist (wash). Error bars indicated standard errors (n= 79 and 81 independent
sampling regions from two Otop1^{+/+} and two Otop1βgal/βgal maculae, respectively).

**Figure 6.** Purinergic response of dissociated macular cultures from the utricles of E18.5-
P3 mice. A, Bright field view of dissociated utricular epithelial cells after 24 hr in culture. Note
regions of flattened epithelial-like cells (*) and clumps of cells associated with globule-like
Circles show areas selected for analysis. C, Addition of 100 μM ATP results in an increase in [Ca\textsuperscript{2+}] as indicated by change in color (red: high, purple: low). D, Otop1 expressing cells, identified in heterozygous and homozygous cultures (marked by X-gal staining), are predominantly associated with globule-like structures (arrow). These cultures also contain Otop1 negative regions (*). E, Otop1\textsuperscript{+/-} (n=57) and Otop1\textsuperscript{βgal/+} (n=31) cultures respond to 100 μM ATP by increasing [Ca\textsuperscript{2+}], to an elevated plateau. Otop1\textsuperscript{βgal/βgal} (n=36) cultures respond to 100 μM ATP by increasing [Ca\textsuperscript{2+}], to an elevated peak followed by a faster rate of decay compared to the Otop1\textsuperscript{+/-} cells. F, Change in [Ca\textsuperscript{2+}], was recorded in regions of dissociated cultures which do not normally express OTOPI (negative for βGAL activity and not associated with globule-like structures, see panel D) (Otop1\textsuperscript{+/-} (n=15), Otop1\textsuperscript{βgal/βgal} (n=12)). These cells respond to ATP but the characteristics and kinetics of the response are different from that shown in panel (E). In addition, no difference in response between Otop1\textsuperscript{+/-} and Otop1\textsuperscript{βgal/βgal} cultures is observed. G, Cultures were treated with 40 μM bis-phenol to deplete Ca\textsuperscript{2+} from intracellular stores. After incubation with bis-phenol, Otop1\textsuperscript{βgal/βgal} (n=20) cultures no longer showed a higher initial peak or differences in the rate of decay compared to Otop1\textsuperscript{+/-} (n=13) in response to 100 μM ATP. H, Dissociated cultures stimulated with the P2Y receptor-specific agonist UTP show a faster decay rate (UTP does not activate P2X receptors in dissociated mouse cultures, see supplemental fig. 4) in all genotypes compared to stimulation with ATP (E). Note that Otop1\textsuperscript{+/-} (n=18) and Otop1\textsuperscript{βgal/+} (n=43) cultured cells still show a decreased peak compared to the Otop1\textsuperscript{βgal/βgal} (n=26) cultures. I, In nominally Ca\textsuperscript{2+} free media, the plateau phase of the response is greatly reduced in both Otop1\textsuperscript{+/-} (n=49) and Otop1\textsuperscript{βgal/βgal} (n=50) cultures in response to 100 μM ATP, resulting in an increased rate of decay compared to normal media (E). Note that the difference in initial peak value between Otop1\textsuperscript{+/-} and Otop1\textsuperscript{βgal/βgal} cultures observed in normal media (E) is no longer
observed in the absence of extracellular Ca\textsuperscript{2+}. \textbf{J}, Comparison of normalized decay constants of \textit{Otop1\textsuperscript{+/+}} and \textit{Otop1\textsuperscript{βgal/βgal}} cultures in different experimental conditions. X-axis indicates letters corresponding to experimental graphs in panels (\textit{E-I}). *, \(P < 0.001\) for indicated comparisons (t-test). The decay constants between \textit{Otop1\textsuperscript{+/+}} and \textit{Otop1\textsuperscript{βgal/βgal}} were not significantly different in experiments \textit{F, G, H, I}). The data in (\textit{E-J}, mean ± SEM) are the combined results of at least five independent experiments.

\textbf{Figure 7.} Model for possible function(s) of OTOP1 in supporting cells of the vestibular sensory epithelium. OTOP1 is hypothesized to 1) localize at and/or near the apical membrane, 2) inhibit P2Y receptors, which blocks release of Ca\textsuperscript{2+} from intracellular stores, 3) in response to ATP, OTOP1 itself and/or by interacting with P2X receptors or other proteins induces influx of extracellular Ca\textsuperscript{2+}. These proposed functions of OTOP1 seem to be dependent on the presence (or concentration) of extracellular Ca\textsuperscript{2+} and result in increasing Ca\textsuperscript{2+} concentration in the cytosol and intracellular stores, which could be important for otoconia mineralization. See the text for detailed description of possible functions of OTOP1.
Figure 1

A

B

C

D

E

F

G

H
Figure 2
Figure 3

$Otop^{1+/-}$  $Otop^{\beta gal/\beta gal}$

(A) OTOP1  (D) OTOP1

(B) $\beta$GAL  (E) $\beta$GAL

(C) Merge  (F) Merge
Figure 4

A: OTOP1 Merge
B: OTOP1 DAPI
C: OTOP1 ZO-1

D: Merge
E: Phalloidin
F: OTOP1 Merge

G: OTOP1 PhalloidinMerge
H: Otop1+/+ Otop1
I: Otop1βgal/βgal

J: Otop1 Merge
K: apical
L: mid
M: basal
N: Otop1+/+ Otop1
O: Otop1βgal/βgal

Scale bar: 50 μm
Figure 5

(A) Image of a cell. (B) Time course of ATP wash. (C) Graph showing the ratio of 340/380 over time. (D) Graph showing the effect of ATP on Otop1+/+ and Otop1βgal/βgal.
Figure 6
Figure 7

Apical

Supporting cell

OTOP1

Ca²⁺ Ca²⁺

P2XR

Ca²⁺

P2YR

Ca²⁺ Ca²⁺

ATP

Ca²⁺

Basal