

OTOPETRIN PROTON CHANNELS AS TARGETS FOR PHARMACEUTICAL DISCOVERY

RELATED PATENT APPLICATION

[0001] This patent application claims the benefit of Provisional Patent Application No. 62/537,900 filed on July 27, 2017, entitled " OTOPETRIN PROTON CHANNELS AS TARGETS FOR PHARMACEUTICAL DISCOVERY", naming Emily Liman as an inventor, and designated by attorney docket no. 043871-0452046. The entire content of the foregoing patent application is incorporated herein by reference, including all text, tables and drawings.

GOVERNMENT SUPPORT

[0002] This invention was made, in part, with government support under National Institutes of Health grants R01DC013741 and R21DC012747. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] Embodiments of the invention relate to compositions and methods for identifying modulators of otopetrin-mediated proton translocation.

INTRODUCTION

[0004] Lipid bilayers are impermeable to protons, whose movement into and out of cells is tightly regulated by membrane proteins, including ion channels. As presented herein, the transmembrane protein Otopetrin 1 (Otop1) and certain related transmembrane proteins are identified as proton-selective ion channels.

BRIEF DESCRIPTION OF THE FIGURES

[0005] The drawings illustrate embodiments of the technology and are not limiting. For clarity and ease of illustration, the drawings are not made to scale and, in some instances, various aspects may be shown exaggerated or enlarged to facilitate an understanding of particular embodiments.

[0006] **Fig. 1 - *Otop1* generates an acid-induced current in *Xenopus* oocytes and HEK-293 cells.** Fig. 1a shows current measured by TEVC in a *Xenopus* oocyte expressing Otop1 in response to Na⁺-free extracellular solutions of decreasing pH ($V_m = -80$ mV). Fig. 1b shows the I-V relation of the current in Fig. 1a. The voltage was ramped from -80 mV to 80 mV at 1V/s. Fig. 1c shows the average magnitude (mean $\pm$ SEM) of the evoked currents (ΔI) as a function of pH in *Xenopus* oocytes injected with Otop1 mRNA (n=4) and un-injected oocytes (grey circles; n = 4). Fig. 1d shows the current measured by whole-cell patch clamp recording in a HEK-293 cell transfected with Otop1 in Na⁺-free solution ($V_m = -80$ mV). Fig. 1e shows the I-V relation of the current evoked in an Otop1-transfected HEK-293 cells with pH_o as indicated from experiments in which the voltage was ramped (1V/s). Fig. 1f shows the average magnitude (mean $\pm$ SEM) of the evoked currents (ΔI) as a function of pH in HEK-293 cells transfected with Otop1 (squares; n = 5) and untransfected cells (grey squares; n = 3). Fig. 1g shows a fusion protein of Otop1 with YFP (YFP-Otop1) co-transfected with the cytosolic protein tag-RFP and imaged with confocal microscopy. Line scan (Fig. 1g, bottom panel) shows that YFP-Otop1 is enriched on the cell surface. Fig. 1h shows Otop1-transfected, but not sham-transfected cells, responded to a change in extracellular pH with a large change in intracellular pH, as measured with pHrodo red (mean $\pm$ SEM, n = 9-11). Similar results were obtained in 3 replicates. Average data (mean $\pm$ SEM; n = 28-29 cells) was analyzed by 2-tailed t-test. ****: $p < 0.0001$. HOAc served as a positive control.

[0007] **Fig. 2 - Ion selectivity and biophysical properties of Otop1.** Fig. 2a shows that Otop1 currents evoked in response to a low pH solution with Na⁺, Li⁺ or Cs⁺ (160 mM each) or Ca²⁺ (40 mM) replacing NMDG⁺ in the extracellular solution at the time indicated ($V_m = -$

80 mV). Average percentage change in the current is indicated. Fig. 2b shows Otop1 currents evoked in response to voltage ramps (1 V/s) at varying extracellular pH under conditions that minimized ion accumulation and allow isolation of the Otop1 current ($pH_i = 6.0$; Zn^{2+} -sensitive component is shown). Fig. 2c shows E_{rev} as a function of ΔpH ($pH_i - pH_o$) from experiments as in Fig. 2b. The red line shows the prediction for a purely H^+ -selective conductance. The data were fit by linear regression with a slope of 53 mV/ ΔpH and a Y intercept of 3.6 mV ($R^2 = 0.99$). Fig. 2d and Fig. 2e show Otop1 current (Zn^{2+} -sensitive component) measured in the presence of 140 mM NMDG⁺, 4 mM Cl⁻ ($E_{rev} = 34.4 \pm 1.0$, $n=5$); 140 mM Na⁺, 4 mM Cl⁻ ($E_{rev} = 34.4 \pm 1.0$, $n=5$); 140 mM Na⁺, 20 mM Cl⁻ ($E_{rev} = 33.8 \pm 1.0$, $n=5$); or (d) 140 mM NMDG⁺, 4 mM Cl⁻ (35.2 ± 1.2 , $n = 5$); NMDG⁺, 20 mM Cl⁻ (35.5 ± 0.5 , $n = 4$). There was no significant difference between any of the conditions, $p > 0.05$ by ANOVA. Fig. 2f shows Otop1 currents in HEK-293 cells are blocked in a dose-dependent manner by Zn^{2+} (pink bar, concentrations indicated are in mM). Fig. 2g shows average data (mean $\pm$ SEM; $n = 6-14$) was fit with a Hill equation with an IC_{50} of 0.19 mM and a Hill coefficient of 0.89.

[0008] Fig. 3 - Otopetrins encode proton channels in diverse species. Fig. 3a shows the maximum-likelihood phylogenetic tree created from the multi-sequence alignment of 13 otopetrin family proteins. Scale bar is 0.1 subs/site. Fig. 3b shows the sequence Identity in the transmembrane, cytoplasmic and extracellular domains between the 3 mouse homologs (Otop1, Otop2, and Otop3; left) and between mOtop1 and dmOTOPLc (right). TM domain 11 is highly conserved among the mammalian proteins, while TM domain 5 is most highly conserved between vertebrate and invertebrate proteins. TM domain 12 is highly conserved among all. Fig. 3c and Fig. 3e shows currents evoked in *Xenopus* oocytes expressing Otop2, Otop3 (c) or dmOTOPLc (e) in response to varying pH_o . Representative traces ($V_m = -80$ mV) and I-V relationship (from voltage ramps at 1 V/s) are shown. As expected for a proton-selective ion channel, E_{rev} shifted to the right as pH_o was lowered for Otop3 and dmOTOPLc. Otop2 showed anomalous behavior. Fig. 3d and Fig. 3f shows the average current induced (ΔI) as a function of pH for each of the channels (black circle; mean $\pm$ SEM, $n = 3-7$). Grey triangles show response from un-injected oocytes ($n = 3$, mean $\pm$ SEM). Note that the

relationship between ΔI and pH varied between channels, with mOtop3 showing the steepest relationship.

[0009] Fig. 4 - Measurement of E_{rev} under conditions that stabilize the Otop1 currents.

Fig. 4a shows the voltage and solution exchange protocol designed to stabilize Otop1 currents and reduce changes in $[H^+]_i$. Intracellular solution was adjusted to $pH_i = 6.0$ and V_m was held close to E_H (-60 mV for $pH_o = 7.0$ and +30 mV for $pH_o = 5.5$) and the voltage was ramped 1 V/s from -80 mV to +80 mV. Response of a HEK-293 cell transfected with Otop1 and an untransfected cell to a pH 5.5 solution (NMDG-based) is shown (capacitance artifacts were removed). Note that the current evoked in response to pH 5.5 in the Otop1-expressing cell remained stable throughout the duration of the recording. Fig. 4b shows the I-V curves obtained from experiments as in a. The current elicited in response to pH 5.5 following exposure to Zn^{2+} was subtracted from the current prior to Zn^{2+} exposure to derive a pure Otop1-dependent current (3-4), from which E_{rev} was measured.

[0010] Fig. 5 - Otop1 currents show only mild voltage-dependence. Fig. 5 shows Otop1 currents in *Xenopus* oocytes in response to a family of voltage steps (shown) before and during exposure to pH 5.5 solution. Right, Average data ($n = 4$) measured at the times indicated. Only for the most hyperpolarizing voltage step to -80 mV was there any time-dependent change in the current amplitude.

[0011] Fig. 6 - Otop2 and Otop3 form inwardly conducting proton channels. Fig. 6 a and Fig. 6 b shows E_{rev} as a function of pH for Otop1 and Otop2 expressed in *Xenopus* oocytes. Red line shows a linear fit of the data between pH 5 and 6 with a slope of 20.7 and 46.3 mV/log $[H^+]$ for Otop2 and Otop3, respectively. Dotted line shows E_H , assuming pH_i is 7.2. Fig. 6 b shows response of Otop2, and Otop3 to hyperpolarizing voltage steps at neutral and acidic pH in *Xenopus* oocytes shows minimal to mild voltage-dependent gating. Data are representative of $n > 3$ experiments. Note that we consistently observed an outward current in Otop2-expressing oocytes at 0 mV in pH 7.4 solution. This may be due to the outward movement of protons through the channel. Fig. 6 c shows the change in intracellular pH of HEK-293 cells transfected with Otop2 or Otop3 or sham transfected, in response to pH 5

solution without and with acetic acid monitored with the pH indicator pHrodo red (mean $\pm$ SEM). Fig. 6 d shows the average data (mean $\pm$ SEM) from experiments as in c, measured at the peak of the response). Significance determined by 2-tailed t-test. ***: $P < 0.001$. Similar results obtained in 3 replicate experiments.

[0012] Fig. 7 - Biophysical properties of Otop2 in HEK-293 cells. Fig 7a shows Otop2 currents measured with whole-cell patch clamp recording of transfected HEK-293 cells. The solution was exchanged and the membrane voltage was ramped, as indicated, from -80 mV to +160 mV (1V/s). Fig. 7b shows I-V relations from the experiment shown in (a). Fig 7c shows the average (mean $\pm$ SEM) of E_{rev} from experiment as in Figures 7a and 7b. A linear fit of the data between pH 5 and 6 with a slope of 25.3 mV/log[H⁺] is shown. A corresponding relationship for Otop2 in *Xenopus* oocytes is also shown.

[0013] Fig. 8 – Human Otop1 forms a proton channel. Fig. 8 shows further evidence that Otop1 forms a proton channel. Fig. 8a shows currents elicited in hOtop1 transfected HEK293 cells in response to varying external pH. Fig. 8b shows the average magnitude of the current from experiments as in Fig. 8a at pH 5.0 in transfected and untransfected cells. Fig. 8c and Fig. 8d show changes in intracellular pH of HEK 293 cells transfected with hOtop2 or sham transfected, as measured with the pH indicator pHrodo red (mean $\pm$ SEM), in response to a pH 5 solution with, and without acetic acid. Error bars represent SEM. Significance is calculated by ANOVA. ****: $P < 0.0001$; Similar results obtained in at least 3 replicate experiments.

[0014] Fig. 9 – Elevation of intracellular calcium in an Otop1-transfected HEK-293 cells in response to a change in extracellular pH. Fig. 9 shows intracellular calcium changes in HEK-293 cells transfected with mOtop1 or sham-transfected. Transfected cells were loaded with the calcium indicator Fura2 AM and exposed to a change in extracellular pH from 7.4 to 5.0. Fig. 9 (left panel) shows that only the Otop1 transfected cells responded (n=8 cells). Both cell types responded to HOAc, pH 5, which penetrates cell membranes and causes intracellular acidification, thereby elevating intracellular calcium by liberating it from intracellular buffers. The peak magnitude of the response is shown in the right panel. The

difference between Otop1 transfected and sham-transfected cells is significant (student's t-test. *** $p < 0.001$).

[0015] Fig. 10 - pHluorin imaging of different Otopetrins in response to various pH.

HEK-293 cells were co-transfected with pHluorin and one of the indicated otopetrin polypeptides or control (Fig. 10D, transfected with pHluorin alone). Fig. 10 shows that HEK-293 cells transfected with Otop1 (Fig. 10A)), Otop2 (Fig. 10B), and Otop3 (Fig. 10C) demonstrate a decrease in intracellular pHluorin fluorescence (which indicated that the intracellular pH was lowered), in response to applying an acidic pH solution to the cells (adjusted by HCl). A decrease in intracellular pH was not observed in the control (Fig. 10D), which was transfected with pHluorin only. However, when applying a basic solution (pH 8.5), only cells with Otop1 and Otop2 showed an increase in pHluorin fluorescence (intracellular alkalization), not Otop3. Applying the acetic acid solution at pH 5.0 (weak acid) induced intracellular acidification, which served as a control to show that pHluorin fluorescence decreased in all cells following the lowering of intracellular pH. Black line: average pHluorin fluorescence intensity. Light gray line: s.e.m. of pHluorin fluorescence intensity. Dark gray bar: different pH solutions applied (blank: pH 7.4). HOAc: Acetic acid.

SUMMARY OF THE INVENTION

[0016] In some aspects, presented herein is a method of identifying a modulator of otopetrin-mediated proton translocation activity comprising (a) contacting an otopetrin polypeptide, or functional portion thereof, with a test compound and (b) determining a proton translocation activity mediated by the otopetrin polypeptide, or the functional portion thereof, wherein an increase or decrease of at least 1% of the proton translocation activity, compared to a proton translocation activity determined in an absence of the test compound, identifies the test compound as a modulator of proton translocation activity mediated by the otopetrin polypeptide, or the functional portion thereof. In some embodiments, a proton translocation activity mediated by an otopetrin polypeptide is determined in response to a change in pH. A change in pH can be an increase or decrease in pH. In some embodiments a change in pH is a change of at least 0.1 pH units. In some embodiments, a proton translocation activity

mediated by an otopetrin polypeptide is determined in response to a change in extracellular pH. In some embodiments, the method comprises inducing a change in pH (*e.g.*, extracellular pH). In some embodiments, an otopetrin polypeptide is integrated into a lipid bilayer and the method comprises, prior to (a) or prior to (b), inducing a change in pH by adding an acid or base to one side (*e.g.*, an extracellular side) of the otopetrin polypeptide.

[0017] In some aspects, presented herein is a method of identifying a modulator of otopetrin-mediated proton translocation activity comprising (a) measuring a proton translocation activity mediated by an otopetrin polypeptide, or a functional portion thereof, (i) in the presence of a test compound, and (ii) in the absence of the test compound; and (b) determining a difference between the proton translocation activity measured in (a)(i) compared to the proton translocation activity measured in (a)(ii), thereby identifying the test compound as a modulator of the proton translocation activity mediated by the otopetrin polypeptide, or the functional portion thereof.

[0018] In some aspects, presented herein is a cell comprising an extracellular membrane and an otopetrin polypeptide (*e.g.*, a heterologous otopetrin polypeptide) integrated in the extracellular membrane of the cell. In some embodiments, the cell is a *Xenopus* oocyte. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a taste receptor cell. In certain embodiments, the otopetrin polypeptide is a heterologous otopetrin polypeptide (*e.g.*, heterologous to a cell that comprises the otopetrin polypeptide). For example, in some embodiments, an otopetrin polypeptide is a human otopetrin polypeptide that is expressed in a heterologous insect, reptilian or non-human mammalian cell. In some embodiments, an otopetrin polypeptide is a human otopetrin polypeptide expressed in an immortalized cell or cell-type that does not naturally express an otopetrin polypeptide.

[0019] In some embodiments, the methods described herein comprise introducing a nucleic acid into a mammalian cell or oocyte wherein the nucleic acid directs the expression of an otopetrin polypeptide. In some embodiments, a nucleic acid that directs the expression of an otopetrin polypeptide is a cDNA that encodes an otopetrin polypeptide. For example, in certain embodiments, a mammalian cell or oocyte is transfected with a cDNA encoding an

otopetrin polypeptide. Any suitable nucleic acid or cDNA that encodes an otopetrin polypeptide, or functional portion thereof, can be used for a method described herein.

[0020] In some embodiments, proton translocation activity is determined, measured, detected or assayed by a method comprising a two-electrode voltage clamping on *Xenopus laevis* oocytes, patch clamping transfected HEK-293 cells, or imaging intracellular pH indicators. For example, in certain embodiments, a nucleic acid encoding an otopetrin polypeptide protein, a functional portion thereof, or a homolog thereof is injected into *Xenopus* oocytes thereby mediating the expression and integration of an otopetrin polypeptide into the oocyte membrane. Proton translocation activity can then be assessed by measuring changes in membrane polarization, (*e.g.*, changes in membrane potential). In certain embodiments, proton translocation activity is determined by measuring pH and/or changes in pH (*e.g.*, changes in intracellular pH) with a suitable pH sensitive indicator. A pH sensitive indicator can be a pH sensitive dye (*e.g.*, pHrodo Red) or pH sensitive protein (*e.g.*, a pH sensitive green fluorescent protein (GFP), or pHluorin). In some embodiments pH and/or changes in pH can be measured with pHrodo Red. In certain embodiments, proton translocation activity is determined by measuring changes in intracellular Ca^{2+} or Zinc levels. For example, calcium levels can be measured by using fluorescent calcium indicator dyes such as Fura-2. In a typical microfluorimetry assay, a dye such as Fura-2, which undergoes a change in fluorescence upon binding a single Ca^{2+} ion, is loaded into the cytosol of Otopetrin-expressing cells. Upon exposure to a test compound, an increase in cytosolic calcium is reflected by a change in fluorescence of Fura-2 that occurs when calcium is bound.

[0021] In some embodiments, the proton translocation activity of an otopetrin polypeptide is assessed indirectly using a variety of *in vitro* and *in vivo* assays to determine functional, chemical, and physical effects, *e.g.*, measuring the binding of an otopetrin to other molecules (*e.g.*, peptide, signaling molecules, including peptides, small organic molecules, and lipids); and/or measuring increases or decreases in protein expression (*e.g.*, transcription, protein levels, etc.) in response to intracellular pH changes that result from otopetrin-dependent proton translocation. In some embodiments, proton translocation activity is determined by assessing changes in cell growth or viability where such events are mediated by otopetrin-

dependent proton translocation. In certain embodiments, proton translocation activity is determined or measured indirectly by detecting changes in amounts, or detecting modifications (*e.g.*, phosphorylation) of intracellular second messengers (*e.g.*, IP3, cGMP or cAMP), where such amounts or modifications are regulated or induced by otopetrin-dependent proton translocation.

[0022] In certain embodiment, proton translocation activity is determined using a patch clamp technique on a cell or oocytes comprising a membrane integrated otopetrin polypeptide, or functional portion thereof. In some embodiments, the presence of otopetrin-dependent proton translocation activity is determined as a net change in current of at least 0.1 pA, at least 1 pA, at least 5 pA, at least 10 pA, at least 50 pA, at least 100 pA, at least 500 pA, at least 1000 pA, at least 50 nA, at least 100 nA, at least 500 nA, at least 0.1 μ A, at least 0.2 μ A, at least 0.5 μ A, at least 1.0 μ A, or at least 2 μ A in response to pH change of at least 0.1 pH units. In some embodiments, a change in current is determined, measured or detected by measuring or detecting a corresponding change in resistance or voltage potential, which parameters are readily converted using a suitable mathematical algorithm. In some embodiments, a modulation of otopetrin-dependent proton translocation activity is a net change (*e.g.*, an increase or decrease of proton translocation activity) in otopetrin-dependent proton translocation activity of at least 10%, at least 25%, at least 50%, at least 75%, at least 2-fold, at least 5-fold or at least 10-fold. A net change can be determined by comparing an otopetrin-mediated proton translocation activity determined in the absence of a test compound to an otopetrin-mediated proton translocation activity determined in the presence of a test compound. In some embodiments, an amount of otopetrin-mediated proton translocation activity determined in the absence of a test compound is referred to as a reference level or control. For example, a decrease in proton translocation activity, in some embodiments, is determined by comparing an amount of proton translocation activity of an otopetrin polypeptide in the presence of a test compound to the proton translocation activity of an otopetrin polypeptide determined in the absence of a test compound (*e.g.*, a reference level). In certain embodiments, an increase in proton translocation activity is determined by comparing an amount of proton translocation activity of an otopetrin polypeptide in the

presence of a test compound to the proton translocation activity of an otopetrin polypeptide determined in the absence of a test compound (*e.g.*, a reference level).

DETAILED DESCRIPTION

[0023] Proton channels that can transport protons into eukaryotic cells have not been molecularly identified. An unbiased screen based on transcriptome profiling of taste cells was used to identify the transmembrane protein Otopetrin1 (Otop1) as encoding a proton-selective ion channel with novel biophysical properties. The related murine genes, Otop2 and Otop3 as well as the *Drosophila* gene dmOTOP1c also encode proton channels. It was also determined herein that Otop1 is required for Zn²⁺-sensitive proton conductance.

[0024] In some embodiments, presented herein is a method of identifying a modulator of an otopetrin-mediated proton translocation activity. In some embodiments, the method comprises contacting a test compound with an otopetrin polypeptide, or functional portion thereof, and determining a proton translocation activity mediated by an otopetrin polypeptide, or the functional portion thereof. The term “determining” as used herein in the context of “determining a proton translocation activity” means and includes, but is not limited to “measuring”, “detecting”, and/or “obtaining”.

Otopetrin polypeptides

[0025] An otopetrin polypeptide, or functional portion thereof, is a multi-pass integral membrane protein that forms a channel in a lipid bilayer. Accordingly, an otopetrin polypeptide, or functional variant thereof, as referred to herein is an otopetrin polypeptide that is integrated into a lipid bilayer (*e.g.*, a cell membrane, or synthetic lipid bilayer). When integrated into a cell membrane, an otopetrin polypeptide comprises a first side (*e.g.*, an extracellular side) and a second side (*e.g.*, an intracellular side). As shown herein, an otopetrin polypeptide provides translocation of protons from one side of the otopetrin polypeptide (*e.g.*, one side of a membrane) to the other side of the otopetrin polypeptide (*e.g.*, the other side of the membrane), often in response to a pH change (*e.g.*, an increase or decrease in protons) on one side of a membrane. In some embodiments a change in pH is

induced on one side of an otopetrin polypeptide (*e.g.*, by addition of a base or an acid) followed by detecting proton translocation through the otopetrin polypeptide.

[0026] In some embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that is obtained, expressed, or derived from any suitable biological organism, non-limiting examples of which include animals, plants, protists, cnidaria (aquatic freshwater or marine animals), arthropods, fungi, bacteria, annelids, echinoderms, chordates, and mollusks, and the like. An otopetrin polypeptide can be obtained, expressed, or derived from any suitable species. An otopetrin polypeptide, in some embodiments comprises or consists of an insect derived otopetrin polypeptide (*e.g.*, an otopetrin polypeptide, or functional portion thereof, derived from *Drosophila*). In certain embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that is obtained, expressed, or derived from a suitable mammal. In some embodiments, an otopetrin polypeptide comprises a mammal otopetrin polypeptide, non-limiting examples of which include Otopetrin-1 (Otop1), Otopetrin-2 (Otop2), and Otopetrin-3 (Otop3). In some embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that is obtained, expressed, or derived from a rodent, non-limiting examples of which include mice and rats. In certain embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that is obtained, expressed, or derived from a suitable primate. A primate can be a non-human primate or may include humans. In certain embodiments, an otopetrin polypeptide comprises, consists of, is obtained from, or is derived from a human otopetrin polypeptide, non-limiting examples of which include Otopetrin-1 (Otop1; UniProtKB - Q7RTM1; SEQ ID NO:1), Otopetrin-2 (Otop2; UniProtKB - Q7RTS6; SEQ ID NO:2), and Otopetrin-3 (Otop3; UniProtKB - Q7RTS5; SEQ ID NO:3). In certain embodiments, an otopetrin polypeptide comprises a mouse otopetrin polypeptide non-limiting examples of which include Otopetrin-1 (Otop1; UniProtKB - Q80VM9; SEQ ID NO:4), Otopetrin-2 (Otop2; UniProtKB - Q80SX5; SEQ ID NO:5), Otopetrin-3 (Otop3; UniProtKB - Q80UF9; SEQ ID NO:6), or a functional portion thereof. In certain embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that comprises, consists of, a *Drosophila* Otop polypeptide, non-limiting examples of which include *Drosophila* OTOP variant D (NM_001272325.1 (CG42492))(SEQ ID NO: 7), *Drosophila* OTOP variant A (NM_134914.3 (CG332))(SEQ

ID NO: 8), and *Drosophila* OTOP variant D (NM_001144688.3 (CG42265))(SEQ ID NO: 9). An otopetrin polypeptide can be expressed in a cell membrane of a cell using a suitable nucleic acid (*e.g.*, a cDNA) that encodes an otopetrin polypeptide. In some embodiments, a suitable cell is transfected with a cDNA encoding an otopetrin polypeptide. Non-limiting examples of a cDNA that encodes a *Drosophila* otopetrin polypeptide are provided in NCBI reference numbers NM_001272325.1 (CG42492), NM_134914.3 (CG332) and NM_001144688.3 (CG42265).

[0027] In certain embodiments an otopetrin polypeptide comprises one or more amino acid additions, deletions or substitutions. An otopetrin polypeptide can be at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to an otopetrin polypeptide described herein. In certain embodiments, an otopetrin polypeptide comprises or consists of an otopetrin polypeptide that is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to an otopetrin polypeptide sequence of any one of SEQ ID Nos: 1, 2, 3, 4, 5, or 6. In some embodiments, an otopetrin polypeptide comprises one or more amino acid analogs or one or more modified amino acids. Modified otopetrin polypeptides that comprise amino acid substitutions, amino acid deletions, amino acid additions, or amino acid analogs can be prepared by a suitable method for altering peptide sequences, non-limiting examples of which are described in *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor, or *Current Protocols in Molecular Biology*, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc. New York. An otopetrin polypeptide can also be modified and made using suitable recombinant nucleic acid technology.

[0028] The term “percent identical” or “percent identity” refers to sequence identity between two amino acid sequences. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same amino acid, then the molecules are identical at that position. When the equivalent site is occupied by the same or a similar amino acid residue (*e.g.*, similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology,

similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, WI), and can be used with, *e.g.*, default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, *e.g.*, each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

[0029] In certain embodiments, an otopetrin polypeptide described herein provides any amount of detectable proton translocation activity. In certain embodiments, an otopetrin polypeptide for use in a method described herein is naturally occurring, truncated, mutated, or genetically altered while retaining any amount of detectable proton translocation activity. In some embodiments, an otopetrin polypeptide described herein retains at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99% or more of the proton translocation activity of a wild-type otopetrin polypeptide peptide sequence. In some embodiments, an otopetrin polypeptide described herein retains at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99% or more of the proton translocation activity of an otopetrin polypeptide of any one of SEQ ID Nos: 1, 2, 3, 4, 5, 6, 7, 8 or 9.

[0030] In certain embodiments, an otopetrin polypeptide comprises a functional portion of an otopetrin polypeptide described herein. A function portion of an otopetrin polypeptide is any portion of an otopetrin polypeptide that, when integrated into a membrane, displays proton translocation activity as measured by a method described herein. A functional portion

of an otopetrin polypeptide can be made and/or expressed using a suitable recombinant method known in the arts and can be tested for proton translocation activity by a method described herein. Accordingly, one of skill in the art can readily identify a functional portion of an otopetrin polypeptide using the methods described herein. A functional portion of an otopetrin polypeptide, in certain embodiments, retains at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or 100% of the proton translocation activity of a wild type otopetrin polypeptide (*e.g.*, an otopetrin polypeptide of any one of SEQ ID Nos: 1 to 9).

[0031] In some embodiments, an otopetrin polypeptide, or functional portion thereof, is linked, covalently or otherwise to another polypeptide, nucleic acid, carbohydrate, fatty acid or detectable reagent. In certain embodiments, an otopetrin polypeptide, or functional portion thereof, comprises another polypeptide. In certain embodiments, an otopetrin polypeptide or functional portion thereof, may be linked covalently to another polypeptide, or portion thereof, while retaining proton translocation activity. In some embodiments, an otopetrin polypeptide is linked to another transmembrane protein, or portion thereof.

[0032] In some embodiments, an otopetrin polypeptide is linked (covalently or non-covalently) to a distinguishable identifier. In some embodiments an otopetrin polypeptide comprises one or more distinguishable identifiers. Any suitable distinguishable identifier can be linked to or associated with an otopetrin polypeptide. In some embodiments, a distinguishable identifier is a detectable label. Non-limiting examples of a distinguishable identifier include a metallic label, a fluorescent label, a fluorescent protein (*e.g.*, green fluorescent protein (GFP)), a pH sensitive protein or pH sensitive GFP (*e.g.*, a pHluorin, or the like), any suitable fluorophore (*e.g.*, mCherry), a chromophore, a chemiluminescent label, an electro-chemiluminescent label (*e.g.*, Origen™), a phosphorescent label, a quencher (*e.g.*, a fluorophore quencher), a fluorescence resonance energy transfer (FRET) pair (*e.g.*, donor and acceptor), a protein (*e.g.*, an enzyme (*e.g.*, horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase and the like)), an antigen or part thereof, a linker, a member of a binding pair), an enzyme substrate, a small molecule (*e.g.*, biotin, avidin), a mass tag, quantum dots, nanoparticles, the like or combinations thereof. Any suitable fluorophore or

light emitting material can be used as a distinguishable identifier. A light emitting distinguishable identifier can be detected and/or quantitated by a variety of suitable techniques such as, for example, flow cytometry, gel electrophoresis, protein-chip analysis (*e.g.*, any chip methodology), microarray, mass spectrometry, cytofluorimetric analysis, fluorescence microscopy, confocal laser scanning microscopy, laser scanning cytometry, the like and combinations thereof. In some embodiments, an otopetrin polypeptide is fused to GFP. In some embodiments an otopetrin polypeptide is fused to a pHluorin.

[0033] In certain embodiments a distinguishable identifier is indirectly associated with (*e.g.*, bound to) an otopetrin polypeptide. In some embodiments a distinguishable identifier is reversibly associated with an otopetrin polypeptide. In certain embodiments a distinguishable identifier that is reversibly associated with an otopetrin polypeptide can be removed from an otopetrin polypeptide using a suitable method (*e.g.*, by increasing salt concentration, denaturing, washing, adding a suitable solvent and/or salt, adding a suitable competitor, and/or by heating).

Proton translocation activity

[0034] As described herein (*e.g.*, see Examples), an otopetrin polypeptide is a multipass transmembrane protein that when integrated into a lipid bilayer, forms an ion channel or pore that selectively allows translocation of protons from one side of the lipid bilayer to the other side. Accordingly, the phrase “proton translocation activity” refers to translocation of protons through an ion channel or pore formed by an otopetrin polypeptide, or functional portion thereof, from one side of a lipid bilayer to the other side of the lipid bilayer. Proton translocation activity can be an active or passive process. In some embodiments proton translocation activity by an otopetrin polypeptide is a passive process. Proton translocation activity of a membrane-integrated otopetrin polypeptide can be assayed or measured using a suitable *in vitro* or *in vivo* method. The translocation of protons across or through a channel or pore formed by an otopetrin polypeptide can be determined, measured, detected or assayed directly (*e.g.*, by directly measuring or detecting changes (*e.g.*, increases or decreases) in intracellular pH, current, resistance or voltage potential), or indirectly (*e.g.*, by measuring or

detecting changes (*e.g.*, increases or decreases) in intracellular calcium or zinc (*e.g.*, by imaging, *e.g.*, calcium imaging)), by detecting otopetrin-mediated cell signaling or other otopetrin-mediated events, or by measuring or detecting (*e.g.*, by imaging) light emitted from pH sensitive indicator. Non-limiting examples of a pH sensitive indicator includes pH sensitive fluorophores, pH sensitive dyes, pH sensitive proteins, the like and combinations thereof. In some embodiments a pH sensitive indicator comprises a pHluorin. In some embodiments, a pH sensitive indicator comprises pHrodo Red. The pH sensitive indicator pHrodo Red is weakly fluorescent at neutral pH but becomes increasingly fluorescent as pH decreases. This reagent can be used to quantify cellular cytosolic pH. Any suitable pH indicator can be used for a method described herein to detect changes in pH (intracellular or extracellular), non-limiting examples of which are listed in Invitrogen's Molecular Probes Handbook, *A Guide to fluorescent probes and labeling technologies*, (2010) 11th Edition, Chapter 20.

[0035] In some embodiments, proton translocation activity is determined, measured, detected or assayed by a method comprising voltage clamping (*e.g.*, a two-electrode voltage clamping on *Xenopus laevis* oocytes or patch clamping transfected HEK-293 cells), or imaging intracellular pH indicators. For example, in certain embodiments, a nucleic acid encoding an otopetrin polypeptide protein, a functional portion thereof, or a homolog thereof is injected into *Xenopus* oocytes thereby mediating the expression and integration of an otopetrin polypeptide into the oocyte membrane. Proton translocation activity can then be assessed by measuring current or by measuring changes in membrane polarization, (*e.g.*, changes in membrane potential). In certain embodiments, proton translocation activity is determined by measuring changes in intracellular pH by use of a pH indicator such as a pHluorin. In certain embodiments, proton translocation activity is determined by measuring changes in intracellular Ca^{2+} levels. For example, calcium flux can be measured by assessment of the uptake of Ca^{2+} or by using fluorescent dyes such as Fura-2. In a typical microfluorimetry assay, a dye such as Fura-2, which undergoes a change in fluorescence upon binding a single Ca^{2+} ion, is loaded into the cytosol of Otop-expressing cells. Upon exposure to a test compound, an increase in cytosolic calcium is reflected by a change in

fluorescence of Fura-2 that occurs when calcium is bound. Other suitable fluorescent calcium indicators can also be used for a method disclosed herein.

[0036] In some embodiments, the proton translocation activity of an otopetrin polypeptide is assessed indirectly using a variety of *in vitro* and *in vivo* assays to determine functional, chemical, and physical effects, *e.g.*, measuring the binding of an otopetrin to other molecules (*e.g.*, peptide, signaling molecules, including peptides, small organic molecules, and lipids); and/or measuring increases or decreases in protein expression (*e.g.*, transcription, protein levels, etc.) in response to intracellular pH changes that result from otopetrin-dependent proton translocation. In some embodiments, proton translocation activity is determined by assessing changes in cell growth or viability where such events are mediated by otopetrin-dependent proton translocation. In certain embodiments, proton translocation activity is determined or measured indirectly by detecting changes in amounts, or detecting modifications (*e.g.*, phosphorylation) of intracellular second messengers (*e.g.*, IP3, cGMP or cAMP), where such amounts or modifications are regulated or induced by otopetrin-dependent proton translocation.

[0037] In some embodiments, proton translocation activity of an otopetrin polypeptide, or functional portion thereof, is determined by measuring or detecting a change in current, resistance or voltage potential across a lipid bilayer. A lipid bilayer can be lipid bilayer of a cell or may be synthetic. In some embodiments, proton translocation activity is determined in response to a change in pH (i.e. proton concentration) on one side of a lipid bilayer. In some embodiments, proton translocation activity is induced by a change in pH (i.e. proton concentration) on one side of a lipid bilayer. A change in pH can be provided by the addition of protons (*e.g.*, an acid) to the fluid on one side of a membrane. A change in pH can be an increase or decrease in pH. In some embodiments, a change in pH is a change of at least 0.01, at least 0.05, at least 0.1, at least 0.5, or at least 1.0 pH unit. In some embodiments a change is pH is a change of at least 0.5 pH units. A change in pH can be provided on either side of a membrane. In certain embodiments, protons are added to the extracellular side of a membrane thereby providing an extracellular change in pH. Non-limiting examples of methods that can be used to determine, assay, or measure proton translocation activity

include any suitable version of a patch clamp technique (*e.g.*, cell-attached patch, inside-out patch, whole cell recording, outside-out patch, perforated patch, loose patch, a two-electrode voltage clamp and a mammalian cell patch clamp (*e.g.*, using transfected HEK-293 cells)), mammalian cell – pH imaging with a dye (*e.g.*, DFFDA BCECF, pHrodo red or Fura 2-AM (Sigma Aldrich, CA no. 108964-32-5)) or by co-transfection with a pHluorin, for example, and imaging (*e.g.*, calcium imaging), the like and combinations thereof. Additional non-limiting examples of methods that can be used to determine, assay, or measure proton translocation activity can be found in Tsien RY, *Biochemistry* (1980) 19(11):2396-404; Rink TJ, Tsien RY, Pozzan T., (1982) *J. Cell Biol.* 95(1):189-96; Grynkiewicz G, Poenie M, Tsien RY. (1985) *J Biol Chem.* 260(6):3440-5; Tsien RY, (1989) *Methods Cell Biol.* 30:127-56; Cohen-Armon M, Sokolovsky M, Dascal N., (1989) *Brain Res.* 496(1-2):197-203; Liman ER, Tytgat J, Hess P., (1992) *Neuron.* 9(5):861-71; Shimbo K, Brassard DL, Lamb RA, Pinto LH., (1996) *Biophys J.* 70(3):1335-46; and Chang RB, Waters H, Liman ER., (2010) *Proc. Natl. Acad. Sci. (USA)* 107(51):22320-5. The entire contents of the foregoing references are incorporated herein by reference.

[0038] In certain embodiments, proton translocation activity is determined using a voltage clamp technique on a cell or oocytes comprising a membrane integrated otopetrin polypeptide, or functional portion thereof. In some embodiments, the presence of otopetrin-dependent proton translocation activity is determined as a net change in current of at least 50 pA, at least 100 pA, at least 500 pA, at least 1000 pA, at least 50 nA, at least 100 nA, at least 500 nA, at least 0.1 μ A, at least 0.2 μ A, at least 0.5 μ A, at least 1.0 μ A, or at least 2 μ A in response to pH change of at least 1 pH unit.

[0039] In some embodiments, proton translocation activity is determined using a fluorescent-imaging plate reader (FLIPR). The FLIPR was a first-in-class instrument that utilized charge-coupled device imaging of a whole plate to capture fluorescent readouts (Schroeder and Neagle (1996) *J. Biomol. Screen.* 1:pp. 75-80; also see *Chemistry & Biology* (2014) 21(9):1162-1170). In certain embodiments, a system comprises a FLIPR. In some embodiments, a system is a high throughput system comprising a FLIPR.

[0040] In some embodiments, a change in current is determined, measured or detected directly. In some embodiments, a change in current is determined, measured or detected by measuring or detecting a corresponding change in resistance or voltage potential, which parameters are readily converted using a suitable mathematical algorithm. In some embodiments, a modulation of otopetrin-dependent proton translocation activity is a net change (*e.g.*, an increase or decrease of proton translocation activity) in otopetrin-dependent proton translocation activity of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, at least 2-fold, at least 5-fold or at least 10-fold. A net change can be determined by comparing an otopetrin-mediated proton translocation activity determined in the absence of a test compound to an otopetrin-mediated proton translocation activity determined in the presence of a test compound. In some embodiments, an amount of otopetrin-mediated proton translocation activity determined in the absence of a test compound is referred to as a reference level or control. For example, a decrease in proton translocation activity, in some embodiments, is determined by comparing an amount of proton translocation activity of an otopetrin polypeptide in the presence of a test compound to the proton translocation activity of an otopetrin polypeptide determined in the absence of a test compound (*e.g.*, a reference level). In certain embodiments, an increase in proton translocation activity is determined by comparing an amount of proton translocation activity of an otopetrin polypeptide in the presence of a test compound to the proton translocation activity of an otopetrin polypeptide determined in the absence of a test compound (*e.g.*, a reference level).

[0041] The term “decrease” or “reduced”, and grammatical variations thereof, as used herein, means a decrease of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, at least 2-fold, at least 5-fold or at least 10-fold as compared to a reference level or control. In certain embodiments, a test compound that induces a decrease in the proton translocation activity of an otopetrin polypeptide is identified by a method described herein as a modulator of otopetrin-mediated proton translocation activity. A test compound or modulator that decreases or reduces the otopetrin-mediated proton translocation activity of an otopetrin polypeptide is referred to herein as an

antagonist (*e.g.*, an antagonist of an otopetrin polypeptide, an antagonist of otopetrin-mediated proton translocation activity).

[0042] The term “increase” or “enhance”, and grammatical variations thereof, as used herein, means an increase of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, at least 2-fold, at least 5-fold or at least 10-fold as compared to a reference level or control. In certain embodiments, a test compound that induces an increase in the proton translocation activity of an otopetrin polypeptide is identified by a method described herein as a modulator of otopetrin-mediated proton translocation activity. A test compound or modulator that increases or enhances the otopetrin-mediated proton translocation activity of an otopetrin polypeptide is referred to herein as an agonist (*e.g.*, an agonist of an otopetrin polypeptide, an agonist of otopetrin-mediated proton translocation activity).

[0043] In some embodiments, otopetrin-binding compounds are screened for agonistic or antagonist action in a functional assay that monitors a biological activity associated with otopetrin function such as effects upon intracellular levels of protons in a otopetrin-expressing host cell (*e.g.*, protons, calcium, zinc), pH-activated conductance, cell death (*i.e.*; receptor-mediated cell death which can be monitored using, *e.g.*, morphological assays, chemical assays, or immunological assays), depolarization of the otopetrin-expressing cells (*e.g.*, using fluorescent voltage-sensitive dyes), second messenger production which can be detected by radioimmunoassay or ELISA), calcium-induced reporter gene expression, or other readily assayable biological activity associated with otopetrin activity or inhibition of otopetrin activity. In certain embodiments, a functional assay is based upon detection of a biological activity of otopetrin that can be assayed using high-throughput screening of multiple samples simultaneously, *e.g.*, a functional assay based upon detection of a change in fluorescence which in turn is associated with a change in otopetrin activity. Such functional assays can be used to screen candidate agents for activity as either otopetrin agonists or antagonists.

[0044] In some embodiments, otopetrin-expressing cells (*e.g.*, recombinant otopetrin-expressing cells) are pre-loaded with fluorescently-labeled pH indicators (*e.g.*, pHrod Red). The Otopetrin-expressing cells are then exposed to a candidate otopetrin-binding compound and the effect of exposure to the compound is monitored. Candidate compounds that have otopetrin agonist activity are often those that, when contacted with the otopetrin-expressing cells, elicit a otopetrin-mediated change in intracellular pH or intracellular calcium relative to control cells (*e.g.*, otopetrin-expressing cells in the absence of the candidate compound, host cells without otopetrin-encoding nucleic added). Similarly, functional otopetrin assays can be used to identify candidate compounds that block activity of otopetrins (*e.g.*, block the change in intracellular pH induced by a change in extracellular pH).

[0045] In some embodiments, a method of identifying a modulator of otopetrin-mediated proton translocation activity comprises a high-throughput screening process. High-throughput screening (HTS) is a method for scientific discovery that uses robotics, data processing and control software, liquid handling devices, and sensitive detectors to screen thousands to millions of test compounds in a relatively short period of time. Any suitable High-Throughput Screening process can be used for a method described herein, non-limiting examples of which are described in U.S. Pat. Nos. 5,976,813; 6,472,144; 6,692,856; 6,824,982; and 7,091,048, the entire contents of which are herein incorporated by reference.

[0046] In some embodiments, a difference in proton translocation activity is determined. A difference in proton translocation activity can be determined by comparing a first proton translocation activity determined in the absence of a test compound, and a second proton translocation activity determined in the presence of a test compound. In certain embodiments, identifying a test compound as a modulator of an otopetrin proton translocation activity comprises determining a difference in proton translocation activity of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 20%, at least 30%, at least 50%, at least 80%, at least 90%, at least 100%, at least 2-fold, at least 5-fold or at least 10-fold.

Test compounds

[0047] Any suitable test compound can be tested by a method described herein. In some embodiments, a test compound is soluble in an aqueous medium (*e.g.*, a cell culture medium) at a concentration tested. In some embodiments, a test compound is completely soluble in an aqueous medium at a concentration tested. In some embodiments, a test compound is partially soluble in an aqueous medium at a concentration tested. In certain embodiments, a test compound is not toxic to a mammalian cell at a concentration tested. For example, in certain embodiments, a test compound does not (i) inhibit growth, (ii) reduce viability, (iii) induce necrosis or apoptosis, (iv) induce damage of DNA or RNA, (v) damage a cell membrane, or (vi) induce proteolytic cleavage of cellular proteins, at a concentration tested. In some embodiments, a test compound is not a carcinogen or co-carcinogen. In some embodiments, a test compound is not a teratogen.

[0048] As used herein, the phrase “test compound” refers to any suitable compound that can be screened for the ability to specifically modulate the proton translocation activity of an otopetrin polypeptide. Non-limiting examples of a test compound include small compounds (*e.g.*, small organic or inorganic molecules), large compounds (*e.g.*, greater than 5000 Da), polysaccharides, carbohydrates, sugars, fatty acids, lipids, biological macromolecules, (*e.g.*, peptides, polypeptides, proteins, peptide analogs and derivatives, peptidomimetics, nucleic acids, nucleotides, nucleotide analogs), naturally occurring or synthetic compounds, binding agents (*e.g.*, antibodies, or binding fragments thereof, including non-naturally occurring and synthetic binding agents (*e.g.*, TandAbs, nanobodies, aptamers, BiTEs, SMIPs, DARPins, DNLs, affibodies, Duocalins, adnectins, fynomers, Kunitz Domains Albu-dabs, DARTs, DVD-IG, Covx-bodies, peptibodies, scFv-Igs, SVD-Igs, dAb-Igs, Knob-in-Holes, triomAbs, and the like), derivatives thereof, polymers thereof, salts thereof, isomers thereof, polymorphs thereof, and combinations thereof. In some embodiments, a test compound is contained within an extract made from biological materials such as extracts of bacteria, plants, fungi, animal cells, or animal tissues. In some embodiments, a test compound is contained within a biological fluid. Accordingly, in some embodiments, a test compound comprises an extract or biological fluid. Small compounds may include molecules having a molecular weight greater than about 40 daltons (Da), but less than 5000 Da, less than 3000 Da, or less than 1000 Da. Small compounds may comprise any suitable chemical moiety or group, non-

limiting examples of which include alkanes, alkenes, alkynes, alcohols, halogens, ketones, aldehydes, carboxylic acids, ethers, esters, amines, amides, saturated, partially saturated or unsaturated ring structures, nucleotides, nucleosides, polyatomic nonmetals (*e.g.*, P, S, Se), transition metals, post-transition metals, metalloids, the like, salts thereof, and combinations thereof.

[0049] In certain embodiments, test compounds include synthetic or naturally occurring compounds of a suitable library. A multitude of small molecule libraries are known in the art, some of which are commercially available. Commercially available compound libraries can be obtained from, for example, ArQule, Pharmacopia, graffinity, Panvera, Vitas-M Lab, Biomol International and Oxford. Methods for developing small molecule, polymeric and genome based libraries are described, for example, in Ding, et al. J Am. Chem. Soc. 124: 1594-1596 (2002) and Lynn, et al., J. Am. Chem. Soc. 123: 8155-8156 (2001). Chemical compound libraries from, for example, NIH Roadmap, Molecular Libraries Screening Centers Network (MLSCN) can also be used. Any suitable method can be used to make a small compound library. A compound library can be screened using a suitable HTS screening method and/or a method described herein to identified test compounds within the library that modulate an otopenin-mediated proton translocation activity.

[0050] In certain embodiments, a test compound comprises a molecular weight of 40 to 500,000 Da, 40 to 200,000 Da, 40 to 100,000 Da, 40 to 50,000 Da, 40 to 25,000 Da, 40 to 10,000 Da, 40 to 5000 Da, or 40 to 1000 Da. In certain embodiments, a test compound comprises a molecular weight of 5000 to 500,000 Da, 10,000 to 500,000 Da, 25,000 to 500,000 Da, or 5000 to 100,000 Da.

[0051] A test compound can be tested at any suitable concentration. In some embodiments, a test compound is tested at a concentration of at least 1 pM, at least 10 pM, at least 100 pM, at least 1 nM, at least 10 nM, at least 100 nM, at least 1 μ M, at least 10 μ M, at least 100 μ M or at least 1 mM. In some embodiments, a test compound is tested at a concentration in a range of 1 pM to 100 mM, 1 pM to 10 mM, 1 pM to 1 mM, 10 pM to 100 mM, 10 pM to 10 mM, 10 pM to 1 mM, 100 pM to 100 mM, 100 pM to 10 mM, or 100 pM to 1 mM. In some

embodiments, a test compound is tested at a concentration of less than 100 mM, less than 10 mM, less than 1 mM or less than 100 nM. In some embodiments, a test compound is tested or assayed at one or more different concentrations.

[0052] In certain embodiments, a test compound comprises or consists of an electrophile. An electrophile is a compound capable of forming a covalent bound with an otopetrin polypeptide. In certain embodiments, a test compound comprises an enzyme (*e.g.*, a protease).

Cells & Membranes

[0053] In certain embodiments an otopetrin polypeptide, or functional portion thereof, is associated with a cell membrane or a synthetic membrane. The phrase “associated with a cell membrane or a synthetic membrane” means that the otopetrin polypeptide, or functional portion thereof, is integrated into the membrane, thereby forming a functional proton channel comprising proton translocation activity.

[0054] An otopetrin polypeptide can be integrated into a lipid bilayer using a suitable method. An otopetrin polypeptide can be integrated into a lipid bilayer of a suitable cell, non-limiting examples of which include an animal cell, plant cell, a protist cell, a cell of an aquatic freshwater or marine animals, an arthropod cell, a fungi cell, a bacteria cell, an annelid cell, an echinoderm cell, a chordate cell, a mollusk cell, and the like. In certain embodiments, an otopetrin polypeptide is integrated into a lipid bilayer of a mammalian cell, a primate cell, a non-human primate cell or a human cell. In certain embodiments, a cell is a primary cell or an immortal cell (*e.g.*, a tissue culture cell line), non-limiting examples of which include HeLa, HEK293, DU145, H295R, HT29, KBM-7, MCF-7, MDA-MB-468, PC3, THP-1, PC12, A549, CHO, COS, Caco-2, EL4, HEP G2, HL-60, the like, and derivatives thereof. In some embodiments, a cell is a taste receptor cell (*e.g.*, a sour taste cell). In certain embodiments, a cell is an egg cell (*e.g.*, an oocyte, *e.g.*, a *Xenopus* oocyte). In certain embodiments, a cell is a red blood cell, or derivative thereof. In certain embodiments, a cell is a ghost cell, or an anucleated cell. An otopetrin polypeptide can be integrated into a lipid bilayer of a cell using any suitable method. In certain embodiments, an

otopetrin polypeptide is integrated into a lipid bilayer of a cell by introducing a nucleic acid into the cell that directs the expression of an otopetrin polypeptide. Accordingly, in some embodiments a method described herein comprises expressing an otopetrin polypeptide, or functional portion thereof, in a cell. In some embodiments, therefore, a cell is a transfected cell, for example a cell transfected with a nucleic acid directing the expression of an otopetrin polypeptide or functional portion thereof. In some embodiments, a cell is a transduced cell, for example a cell transduced with a recombinant virus comprising a nucleic acid that directs the expression of an otopetrin polypeptide or functional portion thereof inside the transduced cell. In certain embodiments, a cell comprises an otopetrin polypeptide or functional portion thereof, integrated into the cell membrane of the cell. In certain embodiments, a cell comprises a heterologous otopetrin polypeptide, or functional portion thereof. The phrase “heterologous otopetrin polypeptide” indicates that the otopetrin polypeptide is derived from a different species than the cell that it is expressed in. In certain embodiments, a cell is *Xenopus* oocyte comprising a heterologous otopetrin polypeptide. In certain embodiments, a cell is taste receptor cell comprising a heterologous otopetrin polypeptide.

[0055] In certain embodiments, an otopetrin polypeptide is integrated into a suitable liposome, micelle, nanodisc, bilayer sheet, or bicelle for use in a method described herein. In certain embodiments, an otopetrin polypeptide is integrated into a suitable synthetic membrane for use in a method described herein. In some embodiments, an otopetrin is integrated into a suitable synthetic membrane formed in the aperture of a suitable nanopore device for use in a method described herein.

[0056] In certain embodiments, a nanopore device comprises a first fluid filled chamber and a second fluid filled chamber separated by an aperture comprising a synthetic membrane, wherein the membrane comprises one or more otopetrin polypeptides. In certain embodiments, the first fluid filled chamber comprises a first electrode and the second fluid filled chamber comprises a second electrode, where the first and second electrode are operatively linked to a means for measurement or detection of at least voltage potential, current, and/or resistance. In certain embodiments, a means for measurement or detection of voltage potential, current, and/or resistance is a device comprising an ammeter, voltmeter,

ohmmeter, oscilloscope, the like or a combination thereof. In certain embodiments, the first and or second chamber comprises one or more photocells for detection of light emitting molecular probes.

[0057] In certain embodiments, provided herein, is a system for determining proton translocation activity of an otopetrin polypeptide. In certain embodiments, provided herein, is a system for identifying a modulator of proton translocation activity of an otopetrin polypeptide (i.e., a modulator or otopetrin-mediated proton translocation activity). In certain embodiments, a system comprises one or more otopetrin polypeptides integrated into a membrane, a first fluid-filled chamber and second fluid filled chamber, wherein the first fluid-filled chamber and the second fluid-filled chamber are located on opposing sides of the membrane. In certain embodiments, an aperture comprises the membrane. For example, in some embodiments, the system comprises an aperture and the membrane is housed within the aperture. In some embodiments, the membrane is a synthetic membrane. In some embodiments, the system comprises a nanopore device.

EXAMPLES

Example 1 – Otop1 forms a proton-selective ion channel with unique biophysical properties

[0058] To characterize the function properties of Otop1, we varied pH_o and measured the evoked currents in *Xenopus* oocytes expressing Otop1. Unless otherwise noted, these and all other experiments were conducted with the large cation NMDG⁺, which is generally impermeable through ion channels, substituting for Na⁺ in the extracellular solution. Otop1 currents in *Xenopus* oocytes increased monotonically in magnitude as pH_o was lowered over a range of pH 6-4 (Fig. 1a-c) and the reversal potential (E_{rev}) of the currents shifted toward more positive voltages. Note that because in these experiments endogenous and leak currents were not subtracted, the Otop1 currents deviate from Nernstian behavior^{3,5}.

[0059] To determine if otop1 can generate the pore-forming subunit of an ion channel, irrespective of cellular context, we expressed it in HEK-293 cells (Fig. 1d-f). An N-terminal

YFP-tagged channel confirmed expression at the cell surface (Fig. 1g). Large inward currents were elicited in response to lowering pH_o in Otop1-transfected cells ($I = 1,130 \pm 192$ pA for $pH_o = 5.0$, compared with 14 ± 4 pA for untransfected cells) and the current magnitude increased monotonically with pH_o (Fig. 1f), as it did in oocytes. Otop1 currents in HEK-293 cells decayed within seconds, with faster kinetics observed in response to more acidic stimuli (Fig. 1c). The decay of the currents may be due to accumulation (or depletion) of protons near the channel, which could affect the driving force for proton movement or gating of the channels^{3,5}. For example, an H^+ current of 1000 pA, such as we observe, flowing for 1 second in a cell of 10 μm diameter (524 fL volume) can be calculated to increase the total (bound + free) intracellular concentration of H^+ by up to 20 mM³. Moreover, removal of the excess H^+ can take 60-90 seconds due to the slow diffusion of H^+ , which is bound to bulky pH buffers³. Indeed, when we imaged intracellular pH using the membrane permeant dye pHrodo Red, we observed a large change in pH_i upon lowering pH_o from 7.4 to 5.0 in Otop1-transfected cells, a response not observed in mock-transfected cells (Fig. 1h).

[0060] We carried out a detailed biophysical characterization of Otop1 to determine whether it is proton selective. First, we determined whether Otop1 is permeable to other cations by measuring responses to lowering pH while varying ionic conditions. Replacing NMDG⁺ in the extracellular solution with Na⁺ (Fig. 2a) did not change the amplitude of the current elicited in response to pH 5.5 ($p > 0.05$ by paired t-test), indicating that the channel is not measurably permeable to Na⁺. Similarly, substitution of NMDG⁺ with Cs⁺, Li⁺ or Ca²⁺ caused a less than 3% increase in the current magnitude, indicating that Otop1 is also not appreciably permeable to these monovalent and divalent cations (Fig. 2a).

[0061] To determine the relative permeability of H^+ to Na⁺ and Cl⁻, we measured E_{rev} under conditions where 140 Na⁺ replaced 140 NMDG and where [Cl⁻] was increased, either in the presence or absence of Na⁺. In no case did we observe any change in E_{rev} (Fig. 2d,e). Using the Goldman-Hodgkin-Katz (GHK) equation³⁰, we calculate that a change of 5 mV, which would have been easily detectable, translates into a selectivity for H^+ over Na⁺ of $> 2 \times 10^5$ -

fold and a selectivity of H^+ over Cl^- of $>1 \times 10^5$; as we observed no change in E_{rev} , it is possible that the channel is perfectly selective for H^+ over Cl^- and Na^+ , like $Hv1^3$.

[0062] The transition metal Zn^{2+} is a potent inhibitor of $Hv1$, binding to two external histidine residues that regulate gating^{7,31,32}. It also inhibits other molecules involved in proton transport³, including the proton channel in taste cells²⁰. To gain insights into structural elements involved in ion permeation by $Otop1$, we measured its sensitivity to inhibition by Zn^{2+} . $Otop1$ currents in HEK-293 cells evoked in response to pH 5.5 were inhibited by Zn^{2+} in a dose-dependent manner with an IC_{50} of $0.19 \text{ mM} \pm 0.01$ (Fig. 2 f,g). The Hill coefficient of close to one (0.89 ± 0.06) indicates that a single Zn^{2+} ion binds to inhibit the channel.

[0063] $Otop1$ was tested to determine if it might be voltage-dependent. The response of $Otop1$ currents, evoked by pH 5.5 solution, to voltage steps between -80 to +40 mV (from a holding potential of 0 mV) was measured. The currents showed little evidence of a time-dependent change in amplitude, indicating that gating of $Otop1$ is not appreciably voltage-dependent over a range of physiologically relevant voltages (Fig. 5).

[0064] Together these data establish that $Otop1$ encodes a proton-selective ion channel with unique structural and biophysical properties.

Example 2 - Otopetrins constitute an evolutionarily conserved family of proton channels

[0065] Otopetrins are evolutionarily conserved from nematodes to humans^{28,33} (Fig. 3a). To determine if this family functions generally as proton channels, we examined some of the most distantly related members.

[0066] $Otop1$ has two murine homologs- $mOtop2$ and $mOtop3$ ²⁸ (Fig. 3a) which share 30%-34% amino acid identity with $Otop1$ (Fig. 3b). When expressed in *Xenopus* oocytes, both, $Otop2$ and $Otop3$ generated large currents upon lowering pH_o in an NMDG-based solution (Fig. 3c). $Otop3$ showed evidence of high selectivity for H^+ ; the magnitude of $Otop3$ currents increased linearly as a function of pH_o over the entire pH range tested (pH 4-6; Fig. 3c,d) and E_{rev} shifted $46.3 \text{ mV}/\log[H^+]$ (Fig. 6 a). In contrast, $Otop2$ currents behaved

anomalously; they saturated at ~pH 5 (Fig. 3c,d) and E_{rev} shifted little over a range of pH 4-6 (Fig. 6 a). These unexpected features of Otop2 are intrinsic to the channel protein as Otop2 currents measured in HEK-293 cells showed very similar properties (Fig. 7). Both Otop2 and Otop3 currents showed little evidence of voltage-dependence, like Otop1 (Fig. 6 b). When expressed in HEK-293 cells and measured with microfluorimetry, both Otop2 and Otop3 conducted protons into the cell cytosol in response to lowering pH_o (Fig. 6 c,d). Interestingly, pH_i in Otop3 transfected cells failed to recover following return to neutral pH_o , while Otop2 exhibited faster recovery as compared with Otop1, pointing to differences in H^+ conduction by the channels. Thus although Otop2 and Otop3 have distinct properties, both permeate protons.

[0067] There are three genes in the drosophila genome related to mOtop1; they encode the predicted proteins dmOTOPLa, dmOTOPLb, and dmOTOPLc (Fig. 3a), none of which has previously been characterized. dmOtopLc, a protein of 1576 amino acids, shows 14.1% amino acid identity with Otop1 over the region of homology and >30% amino acid identity in several of its transmembrane domains (Fig. 3b). When expressed in *Xenopus* oocytes dmOTOPLc produced large currents in response to lowering the extracellular pH (Fig. 3e). Like Otop1 and Otop3, dmOTOPLc currents increased as pH_o was lowered and E_{rev} shifted to more positive voltages, indicative of proton selectivity (Fig. 3e,f). Interestingly, dmOTOPLc conducted relatively more current at pH6 than Otop1 or Otop3 and the relationship between the current amplitude and pH (Fig. 3f) was shallower; this may endow the channel with a broader dynamic range.

[0068] Together these data show that highly divergent and evolutionarily distant members of the Otopetrin family form channels with distinct functional properties but a common capacity to permeate protons.

Example 3 – Currents through otopetrin channels cause changes in intracellular pH and calcium that can be measured with microfluorimetry.

[0069] To determine if proton influx through otopetrin channels, mOtop1, mOtop2, mOtop3 and hOtop1, could lead to a change in intracellular pH that could be detected with an

intracellular pH-sensitive indicator dye, we transiently expressed each channel in HEK-293 cells. Indeed, when we imaged intracellular pH from mOtop1-transfected cells using the membrane permeant pH-sensitive dye pHrodo Red and lowered the extracellular pH from 7.4 to 5.0, we observed a large change in fluorescence indicative of a large change in intracellular pH (Fig 1h). Similar results were observed (Fig. 8c and Fig. 8d) when monitoring changes in intracellular pH of HEK 293 cells transfected with hOtop1 using the pH indicator pHrodo red (mean $\pm$ SEM), in response to a pH 5 solution. In both cases, a similar response was not observed in mock-transfected cells (Fig. 1h), indicating that the change in intracellular pH is a result of proton entry through Otop1 channels. Fig 7 shows that HEK 293 cells transfected with Otop2 or Otop3 and loaded with pHrodo Red showed a large change in fluorescence upon lowering extracellular pH, indicating that both channels conduct protons into the cell cytosol in response to lowering extracellular pH (Fig. 6 c,d). Interestingly, the intracellular pH in Otop3 transfected cells failed to recover following return to neutral pH_o, while Otop2 exhibited faster recovery as compared with Otop1, pointing to differences in H⁺ conduction by the channels.

[0070] We also tested whether proton transport through Otop1 (Fig. 10A), Otop2 (Fig. 10B), and Otop3 (Fig. 10C) could be monitored with a genetically encoded pH indicator. For this we use pHluorin, which was co-transfected with each of the channels, or transfected alone (as a control). In HEK 293 cells transfected with Otop channels, large changes in fluorescent emission were detected from pHluorin when external pH was lowered (Otop1, Otop2 and Otop3) or external pH was raised (Otop 1 and Otop2). These responses were only observed in cells transfected with one of the three Otops and were not observed in untransfected cells.

Example 4. Elevation of intracellular calcium in an Otop1-transfected HEK-293 cells in response to a change in extracellular pH.

We also tested whether entry of protons through Otop channels could cause a change in intracellular calcium that could be detected by microfluorometry. Transfected cells were loaded with the calcium indicator Fura2 AM and exposed to a change in extracellular pH

from 7.4 to 5.0. Fig. 9 (left panel) shows that only the Otop1 transfected cells responded with an elevation of intracellular calcium (n=8 cells). The difference between Otop1 transfected and sham-transfected cells is significant (student's t-test. *** $p < 0.001$). As a control, we showed that both transfected and untransfected cells responded to HOAc, pH 5, which penetrates cell membranes and causes intracellular acidification, thereby elevating intracellular calcium by liberating it from intracellular buffers.

Example 5 – Material & Methods

Animals

[0071] All experimental procedures were approved by IACUC of University of Southern California. Mice in which expression of eGFP was driven by TRPM5 promoter^{22,23} and YFP was driven by the Pkd2l1 promoter(Pkd2l1-YFP)²⁰ were bred to generate mice that were positive for both GFP and YFP (Trpm5-GFP/Pkd2l1-YFP).

[0072] For expression in HEK-293 cells, cDNAs were cloned into pcDNA3 vector with In-Fusion® HD Cloning Kit (Clontech). N-terminal YFP-tagged mOtop1 was generated by eliminating the start codon and subcloning Otop1 in frame to a 5' YFP in pcDNA3. All sequences were verified by Sanger sequencing (Genewiz). In vitro transcription was performed with T7 mMESSAGE mMACHINE kit (Thermo Fisher Scientific). The mRNA were treated with TURBO DNase (37 °C for 15 min), purified with RNA Clean & Concentrator kit (Zymo), and checked for the integrity and concentration with gel electrophoresis and Nanodrop (Thermo Scientific).

Xenopus oocyte electrophysiology

[0073] *Xenopus laevis* oocytes were provided by Ecocyte Bioscience. mRNA (0.2-20 ng, 50 nL) were injected into the oocytes with Nanoject II Auto-Nanoliter Injector (Drummond) and incubated in Standard Barth's solution (SBS, Ecocyte Bioscience) at 18 °C for 1-3 days before recording.

[0074] Two-electrode voltage clamp (TEVC) was performed as previously described⁴². The borosilicate glass pipette was pulled with P-97 Flaming/Brown type micropipette puller and its resistance was within the range of 0.5-5 M Ω . The current was measured with GeneClamp 500 amplifier (Axon). Solution exchange was executed by gravity driven perfusion. For most experiments, the membrane potential was held at -80 mV, and voltage ramps were applied every second from -80 mV to +80 mV (1V/s). The oocytes were incubated in the Na⁺-free extracellular solution with 100 μ M DIDS for 2-3 min to inhibit Ca²⁺-activated Cl⁻ channels^{43,44} before the application of acids. Significance was determined by ANOVA.

Oocyte electrophysiology solutions

[0075] *Xenopus* oocytes were incubated in ND96 solution containing (in mM): 100 NaCl, 2 KCl, 1.8 CaCl₂, 2 MgCl₂, 10 HEPES, pH adjusted to 7.4 with HCl. For measuring the current change in response to a change in pH_o, sodium-free solutions were used, containing 100 N-Methyl-D-glucamine (NMDG), 2 KCl, 1.8 CaCl₂, 2 MgCl₂, buffered with either 10 mM HEPES pH 6.5-7.4 or with 2-(N-morpholino)ethanesulfonic acid (MES, 10 mM) for pH 4-6. pH was adjusted with HCl. The following chemicals were added to the sodium-free solutions as indicated in figures: 100 μ M 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), 0.03-10 ZnCl₂, 100 μ M Amantadine.

Transfection of HEK-293 Cells

[0076] Otop and GFP (5:1) were co-transfected into HEK-293 cells (CRL-1573, ATCC) using TransIT-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI). Patch clamp recording and imaging experiments on GFP-positive cells were performed at room temperature ~24-48 h after transfection.

Patch clamp electrophysiology

[0077] Whole-cell patch clamp recording was performed as previously described⁴⁹. In brief, recordings were made with an Axopatch 200A or Axopatch 200B amplifier, digitized with a Digidata 1322a 16-bit data acquisition system, acquired with pClamp 8.2, and

analyzed with Clampfit 8.2 (Molecular Devices, Palo Alto, CA). Records were sampled at 5 kHz and filtered at 1 kHz. Patch pipettes with resistance of 2–4 M Ω were fabricated from borosilicate glass, and only recordings in which a gigaohm seal was achieved were used in the analysis. For most experiments, after the whole cell configuration was achieved, the membrane potential was held at -80mV, or ramped from -80mV to +80mV (1V/s) once per second. For experiments to determine proton selectivity of Otop1 the membrane potential was held at E_H for the extracellular solution bathing the cell and ramped from -80mV to +80mV (1V/s) once per second. Solutions were changed using a linear array of microperfusion pipes (Warner Instruments, Hamden, CT).

Patch clamp electrophysiology solutions

[0078] Tyrode's solution contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20mM dextrose, 10mM HEPES (pH 7.4 with NaOH). Pipette solutions contained 120 mM Cs-aspartate, 15 mM CsCl, 2 mM Mg-ATP, 5 mM EGTA, 2.4 mM CaCl₂ (100nM free Ca²⁺), and 10mM HEPES (pH 7.3 with CsOH). For experiments in which pH_o was varied (Fig. 1d-f), extracellular solutions contained 160mM NMDG, 2mM CaCl₂, and either 10mM HEPES (for pH 7.4), 10 mM MES (for pH 6-5.5), or 10 mM HomoPIPES (for pH 5-4), pH adjusted with HCl. For ion substitution experiment (Fig. 2a), solutions contained 160mM NMDG-Cl was replaced by equimolar concentrations of NaCl, LiCl, or CsCl and 200 μ M amiloride was added to block endogenous ENaC channels. For high calcium solutions, 60 mM NMDG was replaced by 40 mM CaCl₂ to maintain consistent osmolality. In the experiments shown in Fig. 1d-f, 3f-g, and Fig. 7, 100 μ M DIDS was added to block endogenous Cl⁻ currents⁴⁵. DIDS was omitted in all other experiments.

[0079] For measurement of proton selectivity (Fig. 2b-e), the pipette solution contained: 130 mM TMA- methane sulfonate, 5 mM TEA-Cl, 2 mM MgATP, 5 mM EGTA, 2.4 mM CaCl₂ and 80 mM MES titrated to (6.0) with ~15 mM TMA-OH and adjusted to 305 mOsm with dH₂O. NMDG based (Na-free) external solution contained: 130 mM NMDG-methane sulfonate, 2 mM CaCl₂, 100 mM HEPES (pH7.0 – 7.4), or 100 mM MES (pH5.5 – 6.5), or 100 mM HomoPIPES (pH4.5 – 5.25), pH adjusted with ~10 – 15mM NMDG-OH and

adjusted to 305 mOsm with dH₂O. Na-containing solution contained: 130 mM Na-methane sulfonate, 2mM CaCl₂, 100 mM HEPES (pH7.0), or 100 mM MES (pH5.5), pH adjusted with NaOH. Where indicated, ZnCl₂ (10 mM, final concentration), NMDG-Cl (20 mM), NaCl (20 mM) or MgCl₂ (10 mM) were added to the Na-free or Na-containing solution and osmolarity was adjusted with dH₂O. The pH of each solution was measured before use and adjusted if necessary. Note that the measurements were limited in accuracy by small variations in pH on the order of +/- 0.01 pH units or ~+/-3% change in [H⁺] at pH 5.5; this could cause small changes in current magnitude when measuring response in the presences of different ions such as those shown observed in Fig. 2a. Liquid junction potentials of 5 mV were measured between the pipette solution and the bath solution (Tyrode's) and were corrected posthoc. To minimize junction potentials between solutions delivered by sewer pipes and the bath solution, a 1 M KCl agar bridge was utilized and the bath solution was adjusted to contain a similar composition of ions as the sewer pipe in use⁴⁶. Junction potentials of <2 mV were not corrected.

pH Imaging

[0080] HEK-293 cells co-transfected with Otop and GFP were mixed with HEK-293 cells transfected with CFP and plated on protamine coated coverslips at 37°C. After at least one hour, cells were loaded with the intracellular pH indicator pHrodo Red AM, using PowerLoad concentrate according to the manufacturer's instructions (Molecular Probes). Cells co-transfected with Otop and GFP were identified using a U-MNIBA2 GFP filter cube (Olympus) while "sham" transfected cells expressing CFP were identified using a U-N31044v2 CFP filter cube (Olympus) –or by absence of fluorescence. pH imaging optics and image acquisition were the same as previously described^{23 21}. pHrodo Red fluorescence intensity for each cell was measured in response to pH 5.0 solutions buffered with MES (150 mM NaCl, 10 mM MES, 2 mM CaCl₂) or with acetic acid (150 mM NaCl, 10 mM acetic acid, 2 mM CaCl₂). The fluorophore was excited with 560 nm light, and emission at 630 nm was detected by a Hamamatsu digital CCD camera attached to an Olympus IX71 microscope using a U-N31004 Texas Red/Cy3.5 filter cube (Chroma Technologies). The pHrodo Red

fluorescence intensity of each cell was normalized to its baseline fluorescence in pH 7.4 solution (150 mM NaCl, 10 mM HEPES, 2 mM CaCl₂) before the first acid application.

Data analysis and statistics

[0081] All data are presented as mean $\pm$ SEM, unless otherwise noted. Statistical analyses (ANOVA or student's t-test) were performed using Graphpad Prism (Graphpad Software Inc). Sample sizes in the present study are similar to those reported in the literature for similar studies. Representative data shown in the figures was in some cases decimated 10-fold before exporting into the graphics programs Origin (Microcal) and Coreldraw (Corel).

[0082] For Fig. 3, The maximum-likelihood phylogenetic tree was created from the multi-sequence alignment of 13 otopenin family proteins using Align-X (Invitrogen) and NJplot⁴⁸. Accession numbers are as follows: Human, NP_819056; Cow, NP_001193713; Mouse, NP_766297; Dog, XP_545943; Chicken, XP_015141351; Frog, XP_012811170; Zebrafish, NP_942098; m_OTOP2, NP_766389; m_OTOP3, NP_081408; dm_OTOPLa, AAF46050; dm_OTOPLb, AAN10385; dm_OTOPLc, ACL82893; ce_OTOP1, CCD61337. See Hughes et al.²⁸ Transmembrane topology was predicted using the TMHMM server 2.0 (On the internet <URL:<http://www.cbs.dtu.dk/services/TMHMM/>> accessed April 24, 2017).

Example 6 – Elevation of intracellular calcium in an Otop1-transfected HEK-293 cells in response to a change in extracellular pH.

[0083] Calcium Imaging was used to assay for intracellular calcium changes in HEK-293 cells co-transfected with Otop and GFP, and HEK-293 cells transfected with CFP, that were cultured on protamine coated coverslips at 37°C (Fig. 9). After at least one hour, cells were loaded with the intracellular calcium indicator fura-2 AM, using PowerLoad concentrate according to the manufacturer's instructions (Molecular Probes). Cells co-transfected with Otop and GFP were identified using a U-MNIBA2 GFP filter cube (Olympus) while "sham" transfected cells expressed either CFP –identified using a U-N31044v2 CFP filter cube (Olympus) –or no fluorescence. Calcium imaging optics and image acquisition were the same as described (Chang et al., 2010). Fura-2 excitation ratios, for each cell was measured

in response to pH 5.0 solutions buffered with MES (150mM NaCl, 10mM MES, 2mM CaCl₂) or with Acetic acid (150mM NaCl, 10mM acetic acid, 2mM CaCl₂).

Example 7 – References

The following references are incorporated by reference in their entirety.

1. Hille, B. *Ionic channels of excitable membranes* (Sinauer Associates Inc., Sunderland, MA, 2001).
2. Casey, J. R., Grinstein, S. & Orlowski, J. Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol* **11**, 50-61 (2010).
3. Decoursey, T. E. Voltage-gated proton channels and other proton transfer pathways. *Physiol Rev* **83**, 475-579 (2003).
4. Pinto, L. H., Holsinger, L. J. & Lamb, R. A. Influenza virus M2 protein has ion channel activity. *Cell* **69**, 517-28 (1992).
5. Mould, J. A. et al. Permeation and activation of the M2 ion channel of influenza A virus. *J Biol Chem* **275**, 31038-50 (2000).
6. Takeda, M., Pekosz, A., Shuck, K., Pinto, L. H. & Lamb, R. A. Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture. *J Virol* **76**, 1391-9 (2002).
7. Ramsey, I. S., Moran, M. M., Chong, J. A. & Clapham, D. E. A voltage-gated proton-selective channel lacking the pore domain. *Nature* **440**, 1213-6 (2006).
8. Sasaki, M., Takagi, M. & Okamura, Y. A voltage sensor-domain protein is a voltage-gated proton channel. *Science* **312**, 589-92 (2006).
9. Thomas, R. C. & Meech, R. W. Hydrogen ion currents and intracellular pH in depolarized voltage-clamped snail neurones. *Nature* **299**, 826-8 (1982).
10. Byerly, L., Meech, R. & Moody, W., Jr. Rapidly activating hydrogen ion currents in perfused neurones of the snail, *Lymnaea stagnalis*. *J Physiol* **351**, 199-216 (1984).
11. DeCoursey, T. E. Hydrogen ion currents in rat alveolar epithelial cells. *Biophys J* **60**, 1243-53 (1991).
12. Capasso, M., DeCoursey, T. E. & Dyer, M. J. pH regulation and beyond: unanticipated functions for the voltage-gated proton channel, HVCN1. *Trends Cell Biol* **21**, 20-8 (2013).
13. Morgan, D. et al. Voltage-gated proton channels maintain pH in human neutrophils during phagocytosis. *Proc Natl Acad Sci U S A* **106**, 18022-7 (2009).

14. Lishko, P. V., Botchkina, I. L., Fedorenko, A. & Kirichok, Y. Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell* **140**, 327-37 (2010).
15. Wu, L. J. et al. The voltage-gated proton channel Hv1 enhances brain damage from ischemic stroke. *Nat Neurosci* **15**, 565-73 (2012).
16. DeCoursey, T. E. Voltage-gated proton channels: molecular biology, physiology, and pathophysiology of the H(V) family. *Physiol Rev* **93**, 599-652 (2013).
17. Ramsey, I. S., Ruchti, E., Kaczmarek, J. S. & Clapham, D. E. Hv1 proton channels are required for high-level NADPH oxidase-dependent superoxide production during the phagocyte respiratory burst. *Proc Natl Acad Sci U S A* **106**, 7642-7 (2009).
18. DeCoursey, T. E. & Hosler, J. Philosophy of voltage-gated proton channels. *J R Soc Interface* **11**, 20130799 (2014).
19. Smith, S. M. et al. Voltage-gated proton channel in a dinoflagellate. *Proc Natl Acad Sci U S A* **108**, 18162-7 (2011).
20. Chang, R. B., Waters, H. & Liman, E. R. A proton current drives action potentials in genetically identified sour taste cells. *Proc Natl Acad Sci U S A* **107**, 22320-5 (2010).
21. Bushman, J. D., Ye, W. & Liman, E. R. A proton current associated with sour taste: distribution and functional properties. *FASEB J* **29**, 3014-26 (2015).
22. Clapp, T. R., Medler, K. F., Damak, S., Margolskee, R. F. & Kinnamon, S. C. Mouse taste cells with G protein-coupled taste receptors lack voltage-gated calcium channels and SNAP-25. *BMC Biol* **4**, 7 (2006).
23. Zhang, Z., Zhao, Z., Margolskee, R. & Liman, E. The transduction channel TRPM5 is gated by intracellular calcium in taste cells. *J Neurosci* **27**, 5777-86 (2007).
24. Tang, F. et al. RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nat Protoc* **5**, 516-35 (2010).
25. Zhou, T., Chien, M. S., Kaleem, S. & Matsunami, H. Single cell transcriptome analysis of mouse carotid body glomus cells. *J Physiol* **594**, 4225-51 (2016).
26. Chaudhari, N. & Roper, S. D. The cell biology of taste. *J Cell Biol* **190**, 285-96 (2010).
27. Picelli, S. et al. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* **9**, 171-81 (2014).
28. Hughes, I. et al. Identification of the Otopetrin Domain, a conserved domain in vertebrate otopetrins and invertebrate otopetrin-like family members. *BMC Evol Biol* **8**, 41 (2008).

29. Kim, E. et al. Regulation of cellular calcium in vestibular supporting cells by otopetrin 1. *J Neurophysiol* **104**, 3439-50 (2010).
30. Chizhnikov, I. V. et al. Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. *J Physiol* **494** (Pt 2), 329-36 (1996).
31. Cherny, V. V. & DeCoursey, T. E. pH-dependent inhibition of voltage-gated H(+) currents in rat alveolar epithelial cells by Zn(2+) and other divalent cations. *J Gen Physiol* **114**, 819-38 (1999).
32. Takeshita, K. et al. X-ray crystal structure of voltage-gated proton channel. *Nat Struct Mol Biol* **21**, 352-7 (2014).
33. Hurle, B. et al. Non-syndromic vestibular disorder with otoconial agenesis in tilted/mergulador mice caused by mutations in otopetrin 1. *Hum Mol Genet* **12**, 777-89 (2003).
34. Kim, E. et al. Missense mutations in Otopetrin 1 affect subcellular localization and inhibition of purinergic signaling in vestibular supporting cells. *Mol Cell Neurosci* **46**, 655-61 (2011).
35. Wu, C. et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* **10**, R130 (2009).
36. Wang, G. X. et al. Otopetrin 1 protects mice from obesity-associated metabolic dysfunction through attenuating adipose tissue inflammation. *Diabetes* **63**, 1340-52 (2014).
37. Cichy, A. et al. Extracellular pH regulates excitability of vomeronasal sensory neurons. *J Neurosci* **35**, 4025-39 (2015).
38. Wang, T. M., Holzhausen, L. C. & Kramer, R. H. Imaging an optogenetic pH sensor reveals that protons mediate lateral inhibition in the retina. *Nat Neurosci* **17**, 262-8 (2014).
39. Walther, L. E., Blodow, A., Buder, J. & Kniep, R. Principles of calcite dissolution in human and artificial otoconia. *PLoS One* **9**, e102516 (2014).
40. Lundberg, Y. W., Xu, Y., Thiessen, K. D. & Kramer, K. L. Mechanisms of otoconia and otolith development. *Dev Dyn* **244**, 239-53 (2015).
41. Graze, R. M. et al. Allelic imbalance in Drosophila hybrid heads: exons, isoforms, and evolution. *Mol Biol Evol* **29**, 1521-32 (2012).
42. Liman, E. R., Tytgat, J. & Hess, P. Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron* **9**, 861-71 (1992).

43. Faria, D. et al. The calcium-activated chloride channel Anoctamin 1 contributes to the regulation of renal function. *Kidney Int* **85**, 1369-81 (2014).
44. Schroeder, B. C., Cheng, T., Jan, Y. N. & Jan, L. Y. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* **134**, 1019-29 (2008).
45. Zhu, G., Zhang, Y., Xu, H. & Jiang, C. Identification of endogenous outward currents in the human embryonic kidney (HEK 293) cell line. *J Neurosci Methods* **81**, 73-83 (1998).
46. Neher, E. Correction for liquid junction potentials in patch clamp experiments. *Methods Enzymol* **207**, 123-31 (1992).
47. Ye, W. et al. The K⁺ channel KIR2.1 functions in tandem with proton influx to mediate sour taste transduction. *Proc Natl Acad Sci U S A* **113**, E229-38 (2016).
48. Perriere, G. & Gouy, M. WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* **78**, 364-9 (1996).
49. Wang, Y. Y., Chang, R. B., Waters, H. N., McKemy, D. D. & Liman, E. R. The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions. *J Biol Chem* **283**, 32691-703 (2008).

Example 8 – Otopetrin Sequences

[0084] Human – OTOF1 (SEQ ID NO:1):

MLEGLGSPASPRAAASASVAGSSGPAACSPSSAPRSPESPAPRRGGVRASVPQKLA
 EMLSSQYGLIVFVAGLLLLLAWAVHAAGVSKSDLLCFLTALMLLQLLWMLWYVGR
 SSAHRRLFRLKDTAGAGWLRGSITLFAVITVILGCLKIGYFIGFSECLSATEGVFPVT
 HSVHTLLQVYFLWGHAKDIIQSFKTLERFGVIHSVFTNLLWANGVLNESKHQLNEH
 KERLITLGFGNITTVLDDHTPQCNCPTLCTAISHGIYYLYPFNIEYQILASTMLYVL
 WKNIGRKVDSSHQHQKMQFKSDGVMVGAVLGLTVLAATIAVVVVYLIHIGRSKTKS
 ESALIMFYLYAITLLMLMGAAGLAGIRIYRIDEKSLDESKNPARKLDSDLLVGTSAGS
 WLISWGSILAILCAEGHPRYTWYNLPYSILAIVEKYIQNLFIFESIHREPEKLSEDIQTLR
 VVTVCNGNTMPLASSCPKSGGVARDVAPQGKDMPPAANGNVCMRESHDKKEEEKQE
 ESSWGGSPSPVRLPRFLQGNARKKVLNRNIAAFLFLCNISLWIPPAFGCRPEYDNGLEEI
 VFGFEPWIIVVNLAMPFSIFYRMHAAASLFEVYCKI

[0085] Human – OTOP2 (SEQ ID NO:2):

MSEELAQGPKESPAPRAGPREVWKKGGRLLSVLLAVNVLLLA
CTLISGGAFNKVA
VYD TDV FALLTAMMLLATLWILFYLLRTVRCPCAVPYRDAHAGPIWLRGGLVLF
GI
CTLIMDVFKTGYYSFFECQSAIKILHPLIQAVFVIIQTYFLWVSAKDCVHVHLDLTW
CGLMFTLTNLAIWMAAVVDES VHQSHSYSSSHSNASHARLISDQHADNPVGGDSC
LCSTAVCQIFQQGYFYLYPFNIEYSLFASTMLYVMWKNVGRFLASTPGHSHTPTPVS
LFRETFFAGPVLGLLLFVVGLAVFIIYEVQVSGDGSRTTRQALVIYYSFNIVCLGLTTLV
SLSGSIYRFDRRAMDHHKNPTRTLDVALLMGAALGQY AISYYSIVAVVAGTPQDLL
AGLNLTHALLMIAQHTFQNMFIIESLHRGPPGAEPHSTHPKEPCQDLTFTNLDAHLTL
SACPPNPGLVSPSPSDQREAV AIVSTPRSQWRRQCLKDISLFLLCNVILWIMPAFGAR
PHFSNTVEVD FYGYS LWAVIVNICLPFGIFYRMHAVSSLLEVYVLS

[0086] Human – OTOP3 (SEQ ID NO:3):

MGRGAAAAQSRWGRASRASVSPGRTIRSAPAVGEAQETEAPEKENRVDVGAE
ERAAATRPRQKSWLVRHFSLLLRRDRQAQKAGQLFSGLLALNVVFLGGAFICSMIFN
KVAVTLGDVWILLATLKVLSLLWLLYYVASTTRRPHAVLYQDPHAGPLWVRGSLV
LFGSCTFCLNIFRVGYDVSHIRCKSQLDLVFSVIEMVFIGVQTWVLWKHCKDCVRVQ
TNFTRCGLMLTLATNLLLWVLAVTND SMHREIEAELGILMEKSTGNETNTCLCLNAT
ACEAFRRGFLMLYPFSTEYCLICCAVL FVMWKNVGRHVAPHMGAHPATAPFHLHG
AIFGP LLGLLVLLAGVCVFLVFQIEASGPAIACQYFTLYYAFYVAVLPTMSLACLAGT
AIHGLEERELDTVKNPTRSLDVLLMGAALGQMGIAYFSIVAIVAKRPHELLNRLILA
YSLLLILQHIAQNLFII EGLHRRPLWETVPEGLAGKQEAEP PRGS LLELGQGLQRASL
AYIHSYSHLNWKRRALKEISLFLILCNITLWMMPAFGIHPEFENGLEKDFYGYQIWFA
IVNFGLPLGVFYRMH SVGGLVEVYLGA

[0087] Mouse – OTOP1 (SEQ ID NO:4):

MPGGPGAPSSPAASSGSSRAAPSGIAACPLSPPLARGSPQASGPRRGASVPQKLAET
LSSQYGLNVFVAGLLFLLAWAVHATGVGKSDLLCVLTALMLLQLLWMLWYVGRS
YMQRRLIRPKDTHAGARWLRGSITLFAFITVVLGCLKVAYFIGFSECLSATEGVFPVT
HAVHTLLQVYFLWGHAKDIIMSFKTLERFGVIHSVFTNLLLWANSVLNESKHQLNEH
KERLITLGFGNITIVLDDHTPQCNC TPPALCSALSHGIYYLYPFNIEYQILASTMLYVL

WKNIGRRVDSSQHQMQRFDGVLVGSVLGLTVLAATIAVVVVYMIHIGRSKSKSE
SALIMFYLYAITVLLLMGAAGLVGSWIYRVDEKSLDESKNPARKLDVDLLVATASGS
WLLSWGSI LAIACAETRPPTWYNLPYSVLVIVEKYVQNIFIHESVHLEPEGVPEDVRT
LRVVTVCSSEAAALAASTLGSQGMADGSPAVNGNLCLQQRCGKEDQESGWEGAT
GTTRCLDFLQGGMKRRLRNITAFLLCNISLWIPPAFGCRPEYDNGLEEIVFGFEPWI
IVVNLAMPFSIFYRMHAAAALFEVYCKI

[0088] Mouse – OTOP2 (SEQ ID NO:5):

MSEELVPHPNESLPGPRASPREVWKKGGRLLSVLLAVNVLLLACTLISGGAFNKVAV
YDTDV FALLTTMMLLAALWIVFYLLRTARCPDAVPYRDAHAGPIWLRGGLVLFGIC
TLVMDVFKTGYYSFFECQSAIKILHPHIIQAVFVIVQTYFLWISAKDCIHTHLDLTRCG
LMFTLATNLAIWMAAVVDES VHQAHSYSGSHGNTSHTRLNPDSKRAGGAAEEDPC
LCSTAICQIFQQGYFYLYPFNIEYSLFASTMLYVMWKNVGRLLASTHGHGHTPSRVS
LFRETFAGPVLGLLLFVVG LAVFILYEYVQVSGERGHTRQALVIYYSFNIVCLGLMTL
VSLSGSVIYRFDRRAMDHHKNPTRTLTDVALLMGAALGQY AISYYSIVAVVVGSPRD
LQGALNLSHALLMIAQHTFQNVFIHESLHRGPPGAEPREMPPEPCQGITFANLDAIRT
LPSCPPTPRLVIPNLESPQEA VAIISAPRCHWRRRCLKDISLFLLCNVILWIMPAFGAR
PHFSNTVEVDIFYGYSLWAAIVNICLPFGIFYRMHAVSSLLEVYVLS

[0089] Mouse – OTOP3 (SEQ ID NO:6):

MASQTSAPAEPAPMPSPEAKTTEGASSYDQADMETKHAGSPCPPKQKSWLARHFSL
LLRRDRQAQKAGQLFSGLLALNVVFLGGAFICSMIFNKVSVTLGDVWILL AALKVLS
LLWLLYYTVGTTRKPHAVLYRDPHAGPIWVRGSLVLFGSCTVCLNIFRMGYDVSHI
HCKSEVELIFPAIEIVFMIIQTWVLWRHCKDCVQVQTNFTRCGLMLTLATNLLMWVL
AVTNDSMHREIEAELDALMEKFSGNGTNTCMCLNTTVCEVFRKGYLMLYPFSTEYC
LICC AVL FVMWKNVSRSLAAHTGAHPNRSPFRLHGTIFGPLLGLLALVAGVCVFVLF
QIEASGPDIARQYFTLYYAFYVAVLPTMSLACLAGTAIHGLEERELDTLKNPTRSLDV
VLLMGAALGQMGIAYFSIV AIVATQHELLNQLILAYSLLLILQHITQNLFIIEGLHRRP
LWEP AVSGVMEKQDVELPRRGS LRELQDLRRASRAYIHSFSLNWKRRMLKEISL
FLILCNITLWMMPAFGIHPEFENGLEKDFYGYRTWFTIVNFGLPLGVFYRMH SVGGL
VEVYLG A

[0090] Drosophila – OTOP variant D (CG42492), (SEQ ID NO:7):

MQRCPYIHEMRERLLDQPRETLQLENMERANLLDNRQSASESNQLQGDGYHTSPA
H QRTPLVPHDLGEDFNLDFFDDFPIDARRPKNANDIHPAVLTRPQQRTSLFIVTSLVYAI
LLIVVCIAYVISDVTTTHRLPVLYYETFFTYLYGVLSILFLLYVFCFLQESSCCNGGNGG
SKPKPQPKEKSKKAKNADPADSKDAKSGKDSGKAAKGAAYQHTLAKFLEAPVDA
EVAVTPKNVRKRKTTHSDLTHGSFFLRVGAIAFGLGAMIYIGLEFGSFFEIPFDSPCHH
ILIGVNPLLQMIFTFMQMYFIFMNARTRPPFQLNIHRFKVIARFGLMHV VATNICVWI
RTL VKESLLEITIYHQKNEPEAGASSIAHSIRQH ALRHAGTVLRTHAGPNSEFEVLDG
EDILPKDVYKSDNVLSKLV RNTVDGISKSLGMGGDQALDSSTSSSTTTTTRAPFTTP
NYQWHSTT MARKLKKFITSATTAATTAAGSSSTASSTTTISPTISSTTIPSTTISSTISS
TTFSPFSPSTTTTTTTTAAALNLETSGSESPFGGLQRILSSAAPPSLAPVDGFGSASAAT
PTSGSGAGSFVDSFLASTLSPASSTEGSASIMNNLFGQGP MENSFQTYTDLGHEEATG
LVSFENLES LDNIYPAALSSNIGTLNSTACGRIDIMGTIVYDSAPYLYPFIIEYSLIGAVV
LYVMWKHIGRYPGRMNDEDLEHRLEVMLSRRAVAMAQQARSGRVDCVGSSKGLF
FGLLLVGALICLILFFVLVRHQQFSL LAIYLADASHCILMAFAILAIIVGFIRVKNLKF
RCEEQSNLNDILLRISAFGLFTYSVFSIIAGSLKVLESEPSLLVTTTGGVAVFQVILQLL
FIADVSRRRVHLPEHDRSKPGRQIVTFLLICNVAMFAIYTFEAQKVFANPVSRYVQLE
FYGFVPWSIIQRITLPLCIFHRFHS AVTLAEIWKTTYKARLE

[0091] Drosophila – OTOP variant A (SEQ ID NO:8):

MSLINLKS KDMYDEPINLWRTKQRVHYHHDVISKGNDSHRSSDACEYITISAPKKSG
SFSRSPSSLPTSVPGT PRHSVAATSNQVIRYARTSCDHC GHHSIPVMSPHPISPLAKSG
TNLDLVEHGSQRQALLPLPTVGMHHEDSACTLQVSRRPSLLLQEILTQRPPLFGRRD
GNGFLSPRTAKNGNLQGTASGSTATINFQSGATSARNGSTAFFDNGAKSFQAKQQK
DKNRRTGND AISSALSATYCKLLVLLGVCLPITEVISDQIPTYVYQGFYVYLYVGSILF
VIFLYISAFRNRSLFNALKDYHEKNSNVHLKHKVTHFGSFYLRVGAIAFAIGTMVYS
GLEFGQFFELNGHPGCRDV FVAITPICRMVLCIAQVQFIFLNTTYMDMARHKVTSRF
GLMHMVATNLCEWLYVLVEETKHEIFHISQHEVDP AHDLVIHNSSMSRTDWA AVNE
SLHQHHHHHALNNTLVANVSSVIVNMTITPSPTPAAFSGCSRTTIMGALVQQLSPFLF
PCTIEYSLICAVILFEMWKTVKSIPDIDKTRKNSVKPAAAKPAHHFSVDCSQSHKGLF
FGILIIVM TIISMIMYFVLYTQPGYELVATQEVTLWETFMYFMCAA AVITGMILMRDL

RYIKNTSDEHHSMDLDNLLLIVAQGTGVYLYGMFSILGSYFAKWDTVPDRVEGIIAEV
 FGVVQTSLQTMFILHSSHRRRCRGTNQVRRKPGREIITFLLVANIAIWVFNLIKGRAVF
 RESHLEFFGVWGWTHIITHISMPLAIFYRFHSTICLFEVWKITYKAKAH

[0092] Drosophila – OTOP variant D (CG42265) (SEQ ID NO:9):

MDSSPDLSLKLRRGSSDSRDNFYMDFAQGIDSDIEEVDNTANNQEAGEVPPPPLPTVS
 LAEEVLLLVAAPPPPPSLLGQPLPTLTETDDIPPTPTPPQKDDDEGDEDEREEPVE
 QDQGAPAAPSPPGSPINSVLELELIPPPPLSPMDDAGLRTDDDGEGETDDAEVAAIP
 PPHEMLDIESNPDEEEEEEEQEQASQEDTPKEEDEEEDDDKSTPPPPPLPLPSNFSYVQ
 GHNLGQVTPPLTKSPSNSPSPVTPPPCPNLSRMVSPPAQHISQIPPLTPSDESEGEAE
 SQPNSPPLRLDAEQPPDMDQPEPEDQPPEPENEPEPEPEPEPEPEPVSGAREDYSRSL
 DNEDESTTITPPSNGYSASSIAPPPEHFAELDEDRGFIPPPLEQEPEEEVEEEEEEEEE
 ELTKETDEISVDRESLQDQGGSISSPRPASILTGSISTSVGGGAGGSPKPESRGPSRSG
 SQRSQLRSGSQQGSIAESRGGSRIGSRTGSVASAQAAGVLSQASLKSQTSIRSQQQA
 GVRSPAGSIKSGSQRMQSPQAGEGAPAMSPPLMRSPPELARQMHSPPRITPPRVC
 SPPLVSSPPKLAESAAAVGVAATVKEQIGSSSSTAEPLEPSKPEPLKPPIATVSYQDE
 QKPSPPPTAAAPAVTTTAATTAVTSQPRSHFTSSHHHYHLPHQFQHPHHQNHHTHS
 VRVPTPTVPSSYAPPPPPDSGSSSSPVDRRRLFMAGVAPPIAAGAGSLMAMPAEPAVA
 ISPGRVSARSGSQHHVTIDESSLP SHKGNIQETPGPSGLIIGGGDGDGDRDIGGGGGPD
 SSDPPSSPGSSSQPALSGSQADGQLALMYHSHQLTNYPVLPAIKRTHRPSFVYPPMP
 RVKAGDALATLFSALYGKLLVVMGIAFPMAEVISTYIPPSFYEVYYLYLYIGSMIFLL
 FMYATLIWGRPKLPVPIASPSKSAKASGTDSMDESDDTDSNSVHHRLPPPIPVRRPSLL
 SPLGRRDAHYGsfYLRMGAVAFGIGSMIYSGLEFGQYFELNPDTKCHNVLLALTPAT
 RMAFIFIQMYFIFLNNEQIKVYRYKIIARFGLMHMIGTNLAVWLVLIQETKHEILTFY
 NPENRTLRIHRIPGHSRGHAIHQHDPTAHLRVPRGLKGPYQIFECRRTNIIGTLVQDAS
 PFLFPCTIEYSLICAILYVMWRSISRPTPTPQRPDMISSPMKRSPHHYSVDCARAHK
 GLFVGILILVLTIIISLIIFVLISRPEFVAMAVTEVTICELLIYGTATIATLVGMIQIRHLQ
 YDAYRSFSLDDILLVGAQTGSFLYNIFTVIAGHFTLRSDDMLVPINALASIVQTACQT
 MFILDASRRQAVSPEHLRKKPGREIVTFMLVVNLAMWAISTLEKSRAESHPIQLNFY
 GLWAWTHIITHVSMPLAIFYRFHSTVCLCEIWKRAYKLKPTYMXEFARSRIQSIAQQQ

QFCEDLKTNLSYCYCSTTLAGGELETVEEVDSGESNSAEDAGAGAGSGGSRGSGGG
AGAAEAGEAGEEGQQGGDSSCGLKAPIRALSPQSLNTEKAFCPVYVINGE

* * *

[0100] The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented here. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, and designed in a wide variety of different configurations, all of which are explicitly contemplated and make part of this disclosure.

[0101] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

[0102] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (*e.g.*, bodies of the appended claims) are generally intended as “open” terms (*e.g.*, the term “including” should be interpreted as “including but not limited to,” the term “having” should be interpreted as “having at least,” the term “includes” should be interpreted as “includes but is not limited to,” etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases “at least one” and “one or more” to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles “a” or “an” limits any particular claim containing such introduced claim recitation to inventions containing only one such

recitation, even when the same claim includes the introductory phrases “one or more” or “at least one” and indefinite articles such as “a” or “an” (*e.g.*, “a” and/or “an” should typically be interpreted to mean “at least one” or “one or more”); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should typically be interpreted to mean at least the recited number (*e.g.*, the bare recitation of “two recitations,” without other modifiers, typically means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to “at least one of A, B, and C, etc.” is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (*e.g.*, “a system having at least one of A, B, and C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to “at least one of A, B, or C, etc.” is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (*e.g.*, “a system having at least one of A, B, or C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase “A or B” will be understood to include the possibilities of “A” or “B” or “A and B.”

[0103] It will be understood by those of skill in the art that numerous and various modifications can be made without departing from the spirit of the technology. Therefore, it should be clearly understood that the forms of the technology are illustrative only and are not intended to limit the scope of the technology.

[0104] All references cited herein are hereby incorporated by reference in their entirety.

CLAIMS

1. A method of identifying a modulator of otopetrin-mediated proton translocation activity comprising:
 - (a) contacting an otopetrin polypeptide, or host cell expressing said polypeptide with a test compound; and
 - (b) determining a proton translocation activity mediated by the otopetrin polypeptide; wherein an increase or decrease of at least 1% of the proton translocation activity, compared to an amount of proton translocation activity determined in an absence of the test compound, identifies the test compound as a modulator of the proton translocation activity mediated by the otopetrin polypeptide; and wherein determining the proton translocation activity comprises: measuring conductivity, current, resistance, voltage potential across a membrane comprising the otopetrin polypeptide; or intracellular pH or intracellular calcium within a host cell expressing an otopetrin polypeptide.
2. The method of claim 1, wherein:
 - the otopetrin polypeptide comprises a mammalian otopetrin polypeptide, wherein the mammalian otopetrin polypeptide comprises a polypeptide sequence selected from Otop1, Otop2, Otop3;
 - the otopetrin polypeptide comprises an insect otopetrin polypeptide;
 - the otopetrin polypeptide is associated with a cell membrane or synthetic membrane;and/or
 - the otopetrin polypeptide is: at least 90%; at least 95%; at least 96%; at least 97%; at least 98%; at least 99%; or 100%; identical to an otopetrin polypeptide sequence of any one of SEQ ID Nos:1-9.
3. The method of claim 1 or 2, wherein determining the proton translocation activity comprises expressing the otopetrin polypeptide, wherein:
 - the cell is a mammalian cell;
 - the cell is an oocyte; and/or
 - the cell is a HEK-293 cell or a CHO cell.

4. The method of any one of claims 1 to 3, wherein the test compound is selected from the group consisting of a small organic molecule, small inorganic molecule, electrophile, polysaccharide, peptide, protein, antibody, nucleic acid, and an extract, wherein the extract is from a biological material is selected from a bacteria, plant, fungi, animal cell, and animal tissue.

5. A method of identifying a modulator of otopetrin-mediated proton translocation activity comprising:

(a) measuring a proton translocation activity mediated by an otopetrin polypeptide, (i) in the presence of a test compound, and (ii) in the absence of the test compound; and

(b) determining a difference between the proton translocation activity measured in (a)(i) compared to the proton translocation activity measured in (a)(ii), thereby identifying the test compound as a modulator of the proton translocation activity mediated by the otopetrin polypeptide;

wherein measuring the proton translocation activity comprises measuring conductivity, current, voltage potential or resistance across a membrane comprising the otopetrin polypeptide; and/or

wherein measuring the proton translocation activity comprises measuring conductivity, current, intracellular pH, intracellular calcium, or voltage potential or resistance across a membrane comprising the otopetrin polypeptide in response to a change in pH.

6. The method of claim 5, wherein:

the otopetrin polypeptide comprises a mammalian otopetrin polypeptide;

the mammalian otopetrin polypeptide comprises a polypeptide sequence selected from a murine or human Otop1, Otop2, Otop3;

the otopetrin polypeptide comprises an insect otopetrin polypeptide;

the otopetrin polypeptide is associated with, or integrated in a cell membrane or synthetic membrane; and/or

the otopetrin polypeptide is: at least 90%; at least 95%; at least 96%; at least 97%; at least 98%; at least 99%; or 100%; identical to an otopetrin polypeptide sequence of any one of SEQ ID Nos:1-9.

7. The method of claim 5 or 6, the method comprising inducing a change in pH on one side of the otopetrin polypeptide, wherein:

the change in pH is an extracellular change in pH;

the change in pH is an increase in pH;

the change in pH is a decrease in pH; and/or

the change in pH is at least 0.1 pH units.

8. The method of any one of claims 5 to 7, wherein:

the heterologous or homologous cell is a mammalian cell; and/or

the heterologous or homologous cell is an oocyte.

9. The method of any one of claims 5 to 8, wherein the test compound is selected from the group consisting of a small organic molecule, small inorganic molecule, polysaccharide, peptide, protein, antibody, nucleic acid, and an extract, wherein the extract is from a biological material is selected from a bacteria, plant, fungi, animal cell, and animal tissue.

10. The method of claim 1, wherein:

the method comprises comparing the proton translocation activity determined in (b) to a proton translocation activity determined for the otopetrin polypeptide, in the absence of the test compound;

an increase of at least 1% of the proton translocation activity indicates the test compound is an agonist of the proton translocation activity mediated by the otopetrin polypeptide; or

a decrease of at least 1% of the proton translocation activity indicates the test compound is an antagonist of the proton translocation activity mediated by the otopetrin polypeptide.

11. The method of claim 1, wherein:

the conductivity, voltage potential, current, resistance, intracellular pH, intracellular calcium, or the is measured in response to a change in pH;

determining the proton translocation activity comprises a patch clamp technique, voltage clamp technique, measurement of whole cell current, or use of a pH sensitive indicator or calcium-sensitive indicator; or

the pH sensitive indicator comprises pHluorin or pHrodo Red, DFFDA, and BCECF;
the calcium-sensitive indicator comprises Fura-2, or a fluorescent calcium indicator;
the change in pH is an extracellular change in pH;
the change in pH is a change in intracellular pH;
the change in pH is an increase in pH;
the change in pH is a decrease in pH; and/or
the change in pH is at least 0.1 pH units.

12. The method of claim 4, wherein:

the test compound has a molecular weight in a range of 50 to 500,000 Daltons;
the test compound is at a concentration in a range of: 1 pM to 100 mM; 1 pM to 10 mM;
1 pM to 1 mM; 10 pM to 100 mM; 10 pM to 10 mM; 10 pM to 1 mM; 100 pM to 100 mM; 100
pM to 10 mM; or 100 pM to 1 mM; or
the test compound is at a concentration of: at least 1 pM; at least 10 pM; at least 100 pM;
at least 1 nM; at least 10 nM; at least 100 nM; at least 1 μ M; at least 10 μ M; at least 100 μ M; or
at least 1 mM.

13. The method of claim 5, wherein:

the difference is at least a difference of 1%;
the proton translocation activity measured in (a)(i) is at least 1% greater than the
proton translocation activity measured in (a)(ii) and the test compound is identified in (b) as an
agonist of the proton translocation activity mediated by the otopetrin polypeptide;
the proton translocation activity measured in (a)(i) is at least 1% less than the proton
translocation activity measured in (a)(ii) and the test compound is identified in (b) as an
antagonist of the proton translocation activity mediated by the otopetrin polypeptide;
measuring the proton translocation activity comprises a patch clamp technique,
measurement of whole cell currents, two-electrode voltage clamping, or a fluorescence assay
using a voltage-sensitive dye; or
measuring the proton translocation activity comprises expressing the otopetrin
polypeptide in a heterologous or homologous cell.

14. The method of claim 9, wherein:

the test compound has a molecular weight in a range of 50 to 500,000 Daltons;

the test compound is at a concentration in a range of: 1 pM to 100 mM; 1 pM to 10 mM; 1 pM to 1 mM; 10 pM to 100 mM; 10 pM to 10 mM; 10 pM to 1 mM; 100 pM to 100 mM; 100 pM to 10 mM; or 100 pM to 1 mM; or

the test compound is at a concentration of: at least 1 pM; at least 10 pM; at least 100 pM; at least 1 nM; at least 10 nM; at least 100 nM; at least 1 μ M; at least 10 μ M; at least 100 μ M; or at least 1 mM.