 Structures of the otopetrin proton channels Otop1 and Otop3

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Otopetrins (Otop1–Otop3) comprise one of two known eukaryotic proton-selective channel families. Otop1 is required for otoconia formation and a candidate mammalian sour taste receptor. Here we report cryo-EM structures of zebrafish Otop1 and chicken Otop3 in lipid nanodiscs. The structures reveal a dimeric architecture, with each subunit forming 12 transmembrane helices divided into structurally similar amino (N) and carboxy (C) domains. Cholesterol-like molecules occupy various sites in Otop1 and Otop3 and occlude a central tunnel. In molecular dynamics simulations, hydrophilic vestibules formed by the N and C domains and in the intrasubunit interface between N and C domains form conduits for water entry into the membrane core, suggesting three potential proton conduction pathways. By mutagenesis, we tested the roles of charged residues in each putative permeation pathway. Our results provide a structural basis for understanding selective proton permeation and gating of this conserved family of proton channels.

Proton channels mediate the passage of protons across cell membranes, thereby regulating the cellular and extracellular pH as well as membrane potential1. The diverse biological roles of proton channel activity include the triggering of bioluminescence in dinoflagellates2, regulation of pH in lung epithelia3 and the detection of sour taste4–6. Knowledge of molecular mechanisms of proton conduction and selectivity is largely derived from, and limited to, the M2 proton channel of influenza7 and the eukaryotic voltage-gated Hv1 proton channel10,14. Recently, otopetrins were identified as a new family of eukaryotic proton channels15,16 with no structural similarity to M2, Hv1 or other ion channels. Mice have three related genes (Otop1, Otop2 and Otop3) that encode channels with distinct biological properties. For example, the current amplitudes of mouse Otop1 and mouse Otop3 increase linearly as a function of extracellular pH over a range 6.0–4.0, while the current of mouse Otop2 saturates at pH 5 (ref. 19). Otop1 has been demonstrated to be proton-selective, with a remarkable >2 × 105-fold selectivity for protons over Na+ (ref. 19).

Because they were only recently characterized, the physiological roles of otopetrins are just now beginning to be uncovered. Notably, Otop1 is required for proton currents in murine cells that detect sour taste and is a likely candidate for a sour taste receptor10. In addition, previous genetic studies identified Otop1 as the gene mutated in a spontaneously occurring vestibular disorder17. Otop1 is also highly expressed in brown and white adipose tissue and plays a role in insulin resistance18. Otop2 and Otop3 have been detected in the digestive tract and elsewhere in the body19,20, suggesting hitherto unappreciated roles for proton conduction in various cell types. The recent characterization of otopetrins as proton channels has opened avenues to decipher their physiological, biophysical and biochemical characteristics.

Results

Otopetrin channel function, overall structure and domain organization. To provide a starting point for mechanistic studies on otopetrins, we used fluorescence detection size exclusion chromatography (FSEC) to identify orthologs suitable for structure determination when expressed as N-terminal green fluorescent protein (GFP) fusions (see Methods). We chose zebrafish Otop1 and chicken Otop3, which are 30% identical to each other by sequence and share 44 and 59% identity with human OTO1 and OTO3, respectively (Supplementary Fig. 1). When expressed in HEK-293 cells, both zebrafish Otop1 and chicken Otop3 conduct proton currents in response to lowering of extracellular pH like their mammalian counterparts10 (Fig. 1a and Supplementary Fig. 2). Hereafter we refer to these proteins as Otop1 and Otop3, respectively.

We purified and reconstituted full-length proteins in lipid nanodiscs and determined C2-symmetric structures at overall resolutions of 3.0 Å for Otop1 and 3.3 Å for Otop3 (Fig. 1b,c, Supplementary Figs. 3 and 4 and Table 1). The quality of the cryo-EM maps was sufficient to define side chains of almost all amino acids in the transmembrane helices (Supplementary Fig. 5). Notably, there are numerous lipid-like densities surrounding the channels or embedded in an internal tunnel at the dimer interface (Fig. 1b,c). These lipids were either carried over from cells or added during purification and nanodisc reconstitution. Of these, two densities adopted distinctive shapes and were modeled as cholesterol, while an additional six densities corresponded to cholesterol hemisuccinate (CHS) in the Otop1 map, whereas two CHS molecules were built into the Otop3 map (Supplementary Fig. 5c–f).

The structures show that Otop1 and Otop3 are cuboid-shaped homodimers with dimensions of roughly 70 × 50 × 50 Å, and almost all of their ordered mass resides within the membrane (Fig. 1b,c). A single otopetrin subunit can be divided in two halves, the amino-terminal (N) and carboxy-terminal (C) domains. In the homodimeric arrangement, the N and C domains from two...
(43x57) subunits occupy four quadrants surrounding a central axis, resulting in a pseudotetrameric organization (Fig. 1b,c). A cavernous tunnel containing lipids and/or cholesterols coincides with the central twofold axis of Otop1 and Otop3. Intersubunit and intrasubunit interfaces between N and C domains appear to stabilize the overall assembly (Fig. 1b,c).
The topological organization of Otop1 (Fig. 1d) is shared by Otop3 (Supplementary Fig. 6a), and is therefore likely to be adopted among all otopetrins. Sequence conservation of otopetrins mapped onto the structural model is shown in Supplementary Fig. 6b–d. Each Otop1 subunit contains 12 transmembrane (TM) helices (TM1–TM12), of which the first six (TM1–6) form the N domain while the remaining six (TM7–TM12) form the C domain. The N and C domains are structurally similar and are related by a pseudo-twofold symmetry axis at the intrasubunit interface (Fig. 1d–f). This domain organization is reminiscent of, but distinct from, the major facilitator superfamily transporters\(^{16,19}\) and resistance–nudulation cell division family of membrane proteins\(^{20}\) (Supplementary Fig. 6e–g). The intrasubunit interface of Otop1 is mediated mainly by TM6 and TM12 and is highly conserved, suggesting a critical role in structural and functional integrity (Supplementary Figs. 6b and 7a–d). The intersubunit interface, mediated by TM1 and TM9, is less conserved between Otop1 and Otop3 and across otopetrins (Supplementary Figs. 6c and 7e–h). Two tryptophan residues (W394 and W398) in TM9 of zebrafish Otop1 are buried at the interface. FSEC analysis of the W394A and W398A mutants indicated that each expresses as monomeric species (Supplementary Fig. 7i). These monomeric mutants also resulted in loss of proton channel functionality, due to apparently impaired surface expression (Supplementary Fig. 7j–m). Therefore, bulky hydrophobic residues at the intersubunit interface stabilize dimer assembly, and cell surface trafficking probably requires dimerization.

### Lipids, including cholesterol, in Otop1 and Otop3
Facilitated by nanodisc reconstitution, numerous annular and bound lipids were captured and resolved in the structures of Otop1 and Otop3, revealing how the molecules interact with the membrane (Fig. 2). Most remarkably, the central tunnel of Otop1 harbors six robust densities that were modeled as CHS molecules (Fig. 2a,b and Supplementary Fig. 5c,d). Otop3 has a distinct arrangement of lipids in the central tunnel, including two identifiable CHS molecules (Fig. 2a,d and Supplementary Fig. 5e,f). CHS, which was added during purification of Otop1 and Otop3, greatly enhances the monodispersity of detergent-solubilized proteins as shown by FSEC (Supplementary Figs. 3a and 4a). In native bilayers, cholesterol or cholesterol-like lipids probably bind in the central tunnel to stabilize the dimeric protein assembly\(^ {21,22}\). To test this, we performed all-atom molecular dynamics simulations on both Otop1 and Otop3 with cholesterol molecules placed in the central tunnel (and at the intrasubunit interface for Otop1; see also below and Methods). The arrangement of cholesterol molecules in the tunnel is maintained throughout the sub-microsecond MD simulations, indicating that they represent stable binding poses for cholesterol-like moieties (Supplementary Fig. 8a–d). Indeed, in simulations of Otop1 from which cholesterol was omitted, we observed considerable conformational drift (Supplementary Fig. 8e–g) and unrestricted diffusion of water and ions through the wide central tunnel. Together, these data demonstrate how bound cholesterol molecules may contribute to the stability of the dimeric assembly and exclude the passage of unwanted water and solutes.

At the intrasubunit interface of Otop1, a well-resolved, cholesterol-like density is sandwiched in a pocket between the N and C domains. In contrast to those in the central tunnel, the interfacial cholesterol molecules show greater mobility and, in most cases, reposition themselves during simulations (Supplementary Fig. 8a,c). This lipid is absent in the Otop3 density map, despite high sequence conservation in this region (Supplementary Fig. 6a). Indeed, the pocket is occluded by a conserved tryptophan (W507 in Otop3) resulting in a shift of the conserved tryptophan (Supplementary Fig. 6b). This lipid is less conserved between Otop1 and Otop3 and across otopetrins (Supplementary Figs. 6c and 7e–h). Two tryptophan residues (W394 and W398) in TM9 of zebrafish Otop1 are buried at the interface. FSEC analysis of the W394A and W398A mutants indicated that each expresses as monomeric species (Supplementary Fig. 7i). These monomeric mutants also resulted in loss of proton channel functionality, due to apparently impaired surface expression (Supplementary Fig. 7j–m). Therefore, bulky hydrophobic residues at the intersubunit interface stabilize dimer assembly, and cell surface trafficking probably requires dimerization.
represents loci for proton permeation, one housed by the N-domain and the other by the C-domain (Fig. 3). The N-domain vestibule is lined by TM2–TM6 (Fig. 3a), while the C-domain vestibule is lined by TM8–TM12 (Fig. 3b). Both vestibules contain numerous polar and charged residues, many of which are conserved (Fig. 3a,b). Below the bilayer midpoint lies a region of hydrophobic residues that constrains the N- and C-domain vestibules in both Otop1 and Otop3, potentially serving as hydrophobic ‘plugs’ that regulate water or ion accessibility. Notably, hydrophobic gates are exploited across many ion channel families, including the mammalian proton channel, Hv1 (refs. 28,29). In Hv1, an arginine–aspartic acid salt bridge has been proposed to function as a ‘selectivity filter’ in the proton conduction pathway28,29. Each of the vestibules of Otop1 also contains an apparent salt bridge: R145–E215 (K149–E219 in mouse Otop1) in the N-domain vestibule (Fig. 3a) and E429–R572 (E433–R586 in mOtop1) in the C-domain vestibule (Fig. 3b). To test the functional importance of these putative salt bridges, we introduced charge reversal mutations in mOtop1 and tested their proton channel activity (Fig. 3c,d). The K149E mutant functioned similarly to wild type, while currents were greatly reduced in the E219K mutant and not ‘rescued’ in the K149E/E219K double mutant. These results argue against the functional significance for a salt bridge in the N-domain vestibule of Otop1, consistent with the observation that Q146 in Otop3, the equivalent residue to K149 in mOtop1, does not make interactions with neighboring side chains (Fig. 3b). In contrast, similar experiments provide strong support for a functional salt bridge in the C-domain; the greatly diminished current amplitude observed from each of the single mutants (E433R, R586E) was partly rescued in the double mutant (E433R/R586E). Therefore, this electrostatic interaction in the C-domain of Otop1 apparently supports a proton channel function and the exact positioning of the residues is not essential. Indeed, the equivalent residues in Otop3 (Q425, R547) interact with each other in a similar fashion via apparent hydrogen bonding (Fig. 3b), though the functional relevance of this interaction in Otop3 is unknown.

Another highly conserved feature of the putative permeation pathways within the N and C-domains of Otop1 and Otop3 is a constriction composed of glutamine–asparagine–tyrosine, which we abbreviate as the QNY triad. (Q174/N204/Y268 and Q433/N528/Y546 in Otop1 and Q175/N205/Y266 and Q429/N503/Y546 in Otop3) (Fig. 3a,b and Supplementary Fig. 1). Superposition of the domains shows that these triads occupy analogous positions (Supplementary Fig. 9a,b) and that their side chains are sufficiently close to interact directly or through intervening waters (Supplementary Fig. 9c–f). The function of this triad is uncertain, but a role in proton transfer seems possible considering it is conserved in both N and C-domains, and across otopetrin orthologs.

Water penetration of Otop1 in N and C-domains. We conducted all-atom molecular dynamics simulations of Otop1 (Fig. 4) and Otop3 (Supplementary Fig. 10) in a mixed lipid bilayer (80/20 palmitoyl oleoyl phosphatidyl choline (POPC)/cholesterol) to examine areas susceptible to water penetration, and thus to explore potential proton permeation pathways. Though simulations of both subtypes yielded similar results, we focus our discussion on the simulations of Otop1 because of the higher resolution of the starting structure. The N and C-domain vestibules both allowed water entry from the extracellular milieu into the membrane plane as well as the intrasubunit interface between the N and C-domains (Fig. 4a,b), while water permeation through the central tunnel was completely blocked by the cholesterol molecules (Fig. 4c). Close-up views of each of the water entry points are shown in Fig. 4d. We observe the stochastic formation and breaking of a water wire in both the N and C-domains (Supplementary Dataset 1). The interruption of the water wires occurs in the cytoplasmic half of the pathways, below the QNY triad, in part due to the presence of hydrophobic residues (Fig. 4d). Various hydrophilic side chains, including that of the QNY triad, contact water molecules and facilitate water penetration. The formation of a water wire during molecular dynamics simulation suggests that proton conduction could occur through a
Fig. 3 | N and C domain vestibules in Otop1 and Otop3. a, Various views of Otop1 N domain and Otop3 N domain. Top views in which TM1 is omitted (far left panels) are shown, as well as side view (middle left) of the aligned N domains of Otop1 (green) and Otop3 (purple). Middle right and far right panels show expanded views, with residues conserved between Otop1 and Otop3 colored yellow. b, Various views of Otop1 and Otop3 C domains, with panels arranged as in a. TM7 is omitted from the top views. In both a and b, residue numbering refers to zebrafish Otop1 and chicken Otop3. c, Currents measured by whole-cell patch clamp recording in HEK-293 cells expressing each mouse Otop1 mutant in response to acidic extracellular solutions, with pH indicated (pHi = 7.4, Vm = –80 mV). The currents elicited in response to pH 5.5 were inhibited by 1 mM Zn2+ (pink bars). Numbering is according to the mouse Otop1 sequence. The residues in zfOtop1 that are equivalent to those mutated in mOtop1 are bolded and marked with an asterisk in a and b (K149 in mOtop1 corresponds to R145 in zfOtop1; E219 corresponds to E215, E433 to E429 and R586 to R572). d, Averaged current magnitude in response to pH 5.0 from experiments as in the left panel. Individual data points, means and s.e.m are shown for K149E (n = 6 cells), E219K (n = 4), K149E/E219K (n = 4), E433R (n = 4), R586E (n = 4), E433R/R586E (n = 6), wild type (WT; n = 5) and untransfected (untrans; n = 4). Current magnitudes for K149E/E219K were not significantly different compared to E219K (P = 0.17), but they were significantly lower compared with K149E (P = 0.013, two-tailed Student’s t-test). Current magnitudes for E433R/R586E were significantly higher compared with E433R and R586E (P = 0.0003 and P = 0.0006, respectively, by two-tailed Student’s t-test).
water-hopping mechanism. The average (across three simulations of two subunits) distributions of water molecules along the putative pores (Fig. 4c) show a marked difference in the degree of wetting between the extracellular and cytoplasmic halves of the N and C domain pathways. An extended drier region in the cytoplasmic half would not be expected to be conducive for proton conduction in the experimentally observed conformation, suggesting this might correspond to a hydrophobic gate region. However, we also note that proton conduction may not require a fully intact water wire31.

Further, we calculated the electrostatic profile of an Otop1 subunit (Fig. 4f). The extracellular half of the N domain is highly negative throughout, while the C domain transits from negative in the extracellular side to a positive region in the membrane plane. That the N domain is overall more electronegative may suggest that it is more suitable for proton conduction than the C domain. Indeed, it has been proposed for aquaporins that an electropositive region of the permeation pathway is responsible for proton exclusion32. However, we are cautious in making conclusions from surface electrostatic potential because the exact role of electrostatics in proton conduction remains unclear, and minor movements associated with gating could have a drastic impact on the surface charge distribution.

The intrasubunit interface is a third putative pore. Surprisingly, the intrasubunit interface flanked by the N and C domains also
In vestibular function,11,12 provides impetus for investigations of a third possible avenue for proton conduction in Otop1. That this interface is the most conserved region in otopetrins (Supplementary Fig. 1), and the E267 side chain produces an electronegative region near the bilayer midplane. The position of the cholesterol-like molecule at the intrasubunit interface does not appear to be sufficiently deep to occlude the water wire. As noted above, the interactions of the cholesterol molecules in this region are quite dynamic in our simulations, and the lipid molecules are able to diffuse away. On average the water distribution along the interface is more uniform than that of the N and C domains, without the clear break/hydrophobic gate seen in the latter.

We tested the functional role of the two conserved charged residues (E267 and H574) that are at the intrasubunit interface (Fig. 5a) in zebrafish Otop1 by expressing alanine mutants in HEK cells. Strikingly, both mutants resulted in complete loss of proton channel function (Fig. 5b–d). Therefore, E267 and H574 are critical for proper proton channel function in zebrafish Otop1. Overall, these results suggest that the intrasubunit interface presents a third possible avenue for proton conduction in Otop1. That this interface is the most conserved region in otopetrins (Supplementary Fig. 6b) further underscores its probable functional importance.

**Discussion**

Despite the broad roles of proton channels in biology, knowledge of these has lagged behind that of other types of ion channels. The recent characterization of otopetrins, previously implicated in vestibular function,11,12 provides impetus for investigations of a new proton channel family. Here we have described the cryo-EM structures of Otop1 and Otop3 in lipidic nanodiscs, elucidating the topology and dimeric organization of otopetrins in a native-like bilayer environment. We also note that a recent study33 showed that detergent-solubilized Otop3 from *Xenopus tropicalis* adopts the same overall homodimeric architecture as the structures we have described here.

Our structures and molecular dynamics simulations point to three possible routes for proton conduction per subunit: aqueous vestibules in the N and C domains, and the intrasubunit interface. At this point it remains uncertain which of these three pathways (or their combination) contributes to the proton currents recorded in electrophysiological experiments. We found mutations in each of the putative pores that result in loss of function, but it is unclear whether these phenotypes were caused by direct perturbation of a proton conduction pathway or through allosteric effects.

Despite these advances, clarification of the proton conduction pathway(s), as well as gating and selectivity mechanisms, will require further studies. Our results set the stage for such investigations on a family of proton channels involved in sour taste perception, vestibular function and other as-yet unidentified roles.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0235-9.

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Author contributions

K.S. prepared cryo-EM samples, collected and processed cryo-EM data and built structures. B.T. performed electrophysiology experiments and analyzed data. C.C.A.T. performed molecular dynamics simulation and analyzed data. W.-H.L. generated constructs and conducted FSEC experiments. Y.-H.T. designed and generated constructs and performed molecular dynamics simulation and analyzed data. J.P.K. generated constructs and performed confocal imaging. M.S.P.S. contributed equally to finalization of the manuscript. K.S. drafted a majority of the manuscript, with E.R.L. and A.B.W. supervised molecular dynamics, functional experiments and cryo-EM structure determination, respectively. K.S. is a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research. C.C.A.T. is supported by a Ray Thomas Edwards Foundation grant (to A.B.W.); funding from the NIH (No. NIDCD013741 to E.R.L.); and Wellcome (grant No. 208361/Z/17/Z), BBSRC (grant Nos. BB/N000145/1 and BB/R00126X/1) and EPSRC (grant No. EP/R004722/1; to M.S.P.S). K.S. is a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research. C.C.A.T. is supported by the Skaggs Oxford Scholarship and the Croucher Foundation.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Constructs. Zebrafish Otop1 (zfOtop1, Uniprot entry Q7ZWK8) was obtained by PCR from zebrafish whole-organ complementary DNA and confirmed by sequencing. Chicken Otop3 (chOtop3, Uniprot entry R4GK65) was synthesized and codon-optimized for expression in human cell lines. Both CDNAS were cloned into pCDNA3.1 vector with an N-terminal fusion tag consisting of an octahistidine tag followed by eGFP, a Gly-Thr-Gly-Thr linker and 3C protease cleavage site (LEVLFQGP). We call these constructs GFP-zfOtop1 and GFP-chOtop3. We note that the Uniprot entry for chicken Otop3 was used in our study in a 561-amino acid sequence that has been updated since it was published, and it synthesized to a 555-amino acid sequence. The two sequences are identical excepting a short region starting at residue 176. In our chOtop3 the sequence is 1AFFLWH35KCDIQVQHNLRT70. The corresponding sequence in the updated Uniprot entry is 1VMSQPSNLVTHSL71. In addition, alanine at position 438 in the Uniprot entry is lysine in our sequence of chicken Otop3. The sequence we used is more highly conserved and is likely to be correct. Mutations were introduced into the mouse Otop1 cDNA and sequenced as described in ref. 18.

FSEC and purification screening. Approximately one dozen Otopetin orthologs fused to GFP at the N-terminus were screened by FSEC as previously described17. We identified GFP-zfOtop1 and GFP-chOtop3 as promising candidates for structure determination. To conduct FSEC, HEK-293F cells suspended in Freestyle 293 expression medium were transfected with N-terminal GFP fusion constructs when they reached a cell density of ~1.5 × 10^6/ml, and incubated in an orbital shaker at 37 °C supplemented with 8% CO₂ for 2 d. One milliliter of cells was pelleted and resuspended in 200 µl of buffer containing the following: Tris pH 8.0, 150 mM NaCl, 0.5 mM n-dodecyl-β-D-maltoside, 1 mM EDTA in line with fluorescent tracking of GFP fluorescence.

Sample preparation. Similar procedures were used to prepare cryo-EM samples for zfOtop1 and chOtop3 in nanodiscs. HEK-293F cells suspended in Freestyle 293 expression medium, at a cell density of ~1.5 × 10^6/ml, were transfected with 1 mg of zfOtop1 or chOtop3 DNA and 3 mg polyethylenimines and incubated at 37 °C for 4 h, supplemented with 8% CO₂ for 48 h. Grude cell pellets were washed with cold phosphate buffered saline then resuspended in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, 1% n-dodecyl-β-D-maltoside, 0.15% cholesteryl hemisuccinate, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A and 2 µM diithiothreitol (DTT) then stirred at 4 °C for 1 h. The lysate was clarified by centrifugation in a JLA 16.25 rotor at 34,000 g for 1 h. Anti-GFP nanobody-coupled CNBr-Activated Sepharose 4B resin32 was added to the clarified lysate, and the mixture was rotated at 4 °C for 1.5 h. Resin was washed with more than ten resin volumes of wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.07% n-dodecyl-β-D-maltoside, 0.01% cholesterol hemisuccinate, 0.4 µg/ml aprotinin, 0.4 µM pepstatin A and 2 mM DTT). Washed resin was resuspended in wash buffer to make a ~40% slurry, then 50 µg PreScission Protease was added and followed by incubation at 4 °C for 5 h. Flowthrough containing cleaved zfOtop1 or chOtop3 was collected, concentrated in a 100-kDa cut-off centrifugal filter then injected into a Superose 6 increase column equilibrated with wash buffer. Peak fractions corresponding to zfOtop1 or chOtop3 were collected and concentrated to ~1 mg/ml, then mixed with MSpin22 nanodisc scaffold protein19 and soybean polar lipid extract at a molar ratio of approximately 1:124–227, 246–283, 304–366, 375–438 and 487–560. Structural figures were made from either model due to poor density. The model of zfOtop1 (586 residues in full-length) contains residues 46–113, 123–221, 248–283, 302–443 and 510–585. The model of chOtop3 (561 residues in full-length) contains residues 49–116, 124–227, 246–283, 304–366, 375–438 and 487–580. Structural figures were made in PyMOL®, UCSF Chimera® or UCSF ChimeraX®. Sequence conservation scores were calculated and mapped onto the structure of zfOtop1 using Consurf®, with –100 to +100 scores obtained from UniprotKB as input, aiming for even distribution between subtypes Otop1, Otop2 and Otop3. Sequence alignment was calculated using Clustal Omega® and represented using ESPript 3.0 (ref. 19).

Cell culture and transfection for electrophysiology and confocal microscopy. HEK-293 cells (CRL-1573, ATCC) were cultured in DMEM containing 10% fetal calf serum and 50 µg/ml gentamycin. Cells were transfected in 35-mm Petri dishes, with approximately 600–750 ng DNA and 2–4 µM were fabricated from borosilicate glass, and only recordings in which a Records were sampled at 5 kHz and filtered at 1 kHz. Patch pipettes of resistance 0.143 by postprocessing in RELION-3.0 (ref. 39). Subsequent masked refinement of these particles resulted in a 3.07-Å resolution map. The refinement parameters and reconstruction from this refinement run were used as inputs for Bayesian polishing in RELION-3.0 (ref. 39). After Bayesian polishing, masked refinement yielded a 3.02-Å resolution reconstruction. The polished particles were then subjected to a final round of three-dimensional classification without alignment (k = 3, tau fudge = 8). Masked refinement of 67,425 particles from the highest-resolution class resulted in the final 2.98-Å resolution map. For all refinements and classification jobs, C2 symmetry was applied unless otherwise noted and stated resolutions were calculated using Fourier shell correlation (FSC) = 0.143 by postprocessing in RELION using a soft mask around the zfOtop1 protein and nanodisc belt.

Cryo-EM data collection. Images were collected at 300 K using a Titan Krios coupled with a K2 Summit direct electron detector (Gatan) at a nominal magnification of x29,000 with a pixel size of 1.03 Å. Fifty-three frames (for zfOtop1) or 45 frames (for chOtop3) of 250-ms exposure time were collected per movie, resulting in a total accumulated dose of ~50 electrons Å⁻². Automated micrograph collection was performed using Leginon software52 with a target defocus range of 0.6–1.8 µm.

Cryo-EM data processing. Micrographs were aligned and dose-weighted using MotionCor2 (ref. 16). Contrast transfer function (CTF) parameters were obtained with Gctf®. Data processing for zfOtop1 and chOtop3 was carried out similarly. The following processing procedure was used for zfOtop1, and the procedure for chOtop3
Tyrode's solution containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, pH adjusted at the concentrations indicated. Before recording, cells were bathed in Atomistic MD simulations. Insufficient to prevent the flow of water via the central tunnel in our simulations. Were added back to the zfOtop1 system, in accordance with the structure. For structures were added back to the sets of simulations in the mixed membrane, after conversion to the atomistic system via manual alignment to the original models. Any water molecules, ions or lipid molecules (one POPC molecule usually ends up in the central tunnel in coarse-grained self-assembly) that resulted in steric clashes in the atomistic system (within 6 Å) were removed. Eight cholesterol molecules were added back to the zfOtop1 system, in accordance with the structure. For chOtop3, however, having two cholesterol molecules in the central tunnel was insufficient to prevent the flow of water via the central tunnel in our simulations. We therefore placed six cholesterols in the central tunnel of chOtop3 in the same starting position as zfOtop1 as an approximation. We note, however, that the distribution of cholesterols and other lipid-like densities in our EM maps of zfOtop1 and chOtop3 varies.

**Atomic MD simulations.** The system with the added cholesterol molecules was energy minimized to maximum force 1,000 kJ mol⁻¹ nm⁻², before running a 1 ns simulation (time-step, 1 fs) with the protein backbone atoms position restrained (force constant, 2,000 kJ mol⁻¹ nm⁻²). Three repeats of 100-ns production run (time-step, 2 fs) were performed based on the final snapshot of the short simulations without position restraints. Here, distance restraints were applied between endings of the missing loops (force constant, 1,000 kJ mol⁻¹ nm⁻²); lower and upper bound values were ±1 Å of the starting distances. The AT simulations were performed as NPT ensembles held at 1 bar and 310 K. A semi-isotropic Parrinello-Rahman thermostat was used (coupling constant, 4.5 × 10⁻⁴ ps⁻¹) and a velocity-scaling thermostat (coupling constant, 0.1 ps⁻¹) were used. All solvent bonds were constrained using the LINCS algorithm. Electrostatics were modeled with a Particle Mesh Ewald model, and van der Waals interactions were modeled using a cutoff scheme, both with cutoff distance at 10 Å.

**Molecular dynamics simulation trajectory analysis.** Protein–cholesterol distances were calculated with the distance tool in GROMACS based on their centers of geometry (Supplementary Fig. 8c,d). Root-mean-square deviation measurements were calculated using the RMSD trajectory tool in VMD. Alignments and calculations were based on the backbone atoms of transmembrane residues (Supplementary Fig. 6e–g). For the extraction of water trajectories (Supplementary Dataset 1), the trajectories were aligned either at the domain level (for the selection in the N and C domains) or at the subunit level (for the selection at the interface). Water molecules within the N domain pathway were selected based on their proximity (within 5 Å) to the domain excluding TM1 (starting from V81 for zfOtop1, A81 for chOtop3), while excluding those found within 6 Å of lipid molecules or within 8 Å of the other three domains. A similar method was used to select waters in the C domain pathway (within 5 Å of the entire domain) and with the same exclusions as the N domain. A cuboid selection measuring 10 × 12 × 42 Å was used to select water molecules along the intrasubunit interface, which covers the outer half of the interface. Molecular dynamics-related figures were rendered using PyMOL. Electrostatic profiles (Fig. 4f and Supplementary Fig. 10h) were generated using the APBS Electrostatic plugin in PyMOL. When estimating water density along the putative pathways (Supplementary Dataset 1 and Fig. 4e), kernel density estimations were performed on the z-position of oxygen atoms of water molecules using the density function in R⁸. Gaussian kernels were used with the bandwidths chosen using the method of Sheather and Jones⁸.

**Data availability**
Cryo-EM maps and atomic coordinates have been deposited at the Electron Microscopy Data Bank and PDB, respectively, with accession codes EMDB-9360 and PDB 6N4 (zebrafish Otop1) and EMD-9361 and PDB 6NF (chicken Otop3). All other data are available upon reasonable request to the corresponding authors.

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Software and code

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Data collection
- Cryo-EM data was collected using Leginon. Electrophysiology data were acquired with pClamp 8.2

Data analysis
- Cryo-EM data were analyzed with gctf v1.06, Relion 2.1, Relion 3.0 and cryoSPARC v0.6.5. Structures were built, refined, and analyzed in coot version 0.8.9., phenix version 1.14, rosetta, pymol 2.0, chimera x, and chimera 1.11.2. Electrophysiology data were analyzed with Clampfit 8.2. Molecular dynamics simulations were performed using GROMACS 5.0.2, MemProtMD and analyzed with VMD.

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Cryo-EM maps of zebrafish Otop1 and chicken Otop3 in nanodiscs have been deposited to the Electron Microscopy Data Bank under accession codes 9360 and 9361, respectively. Atomic coordinates of zebrafish Otop1 and chicken Otop3 have been deposited to the PDB under IDs 6NF4 and 6NF6, respectively. All other data are available upon reasonable request to the corresponding authors.
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Sample size  For electrophysiology and confocal imaging, sample sizes represent the number of cells used for recordings and analysis. The sizes were chosen based on consistency and quality of data across conditions and multiple experiments.

Data exclusions  For patch clamp recordings, no data was excluded unless the recording quality was poor due to factors such as large noise or instability. For confocal imaging, cells were selected that showed moderate levels of GFP and RFP.

Replication  Electrophysiological experiments and confocal images were reproduced according to the sample size as indicated in each figure or the text.

Randomization  For patch clamp recordings and confocal imaging, samples were grouped based on the genes of interests transfected into the cells.

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Mycoplasma contamination  The HEK293F and HEK293T cell lines tested negative for mycoplasma contamination.

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