Cryo-EM structure of a proton-activated chloride channel TMEM206

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TMEM206 has been recently identified as an evolutionarily conserved chloride channel that underlies ubiquitously expressed, proton-activated, outwardly rectifying anion currents. Here, we report the cryo–electron microscopy structure of pufferfish TMEM206, which forms a trimeric channel, with each subunit comprising two transmembrane segments and a large extracellular domain. An ample vestibule in the extracellular region is accessible laterally from the three side portals. The central pore contains multiple constrictions. A conserved lysine residue near the cytoplasmic end of the inner helix forms the presumed chloride ion selectivity filter. Unprece-dently, the core structure and assembly closely resemble those of the epithelial sodium channel/degenerin family of sodium channels that are unrelated in amino acid sequence and conduct cations instead of anions. Together with electrophysiology, this work provides insights into ion conduction and gating for a new class of chloride channels that is architecturally distinct from previously characterized chloride channel families.

INTRODUCTION

Chloride ions (Cl⁻) are the most abundant anions in animals; thus, Cl⁻ movement across cell membranes, mediated by Cl⁻ channels and transporters, is central to numerous cellular functions, such as regulation of cell volume, acidification of intracellular vesicles, and excitability control in muscle cells (1, 2). Widely observed in mammalian cells, the acid- or proton-activated outwardly rectifying Cl⁻ currents (I_{Cl,H⁺}, also referred to as ASOR or PAORAC) have long been recognized, but the molecular components behind these currents have remained elusive until very recently (3–12). Two independent studies using genome-wide RNA interference screen have identified TMEM206 as the underlying anion channel (13, 14). Evolutionally conserved in vertebrates, TMEM206 recapitulates biophysical characteristics of the proton-activated Cl⁻ currents, including an outwardly rectifying current-voltage (I-V) relationship and a permeability sequence of SCN⁻ > I⁻ > NO₃⁻ > Br⁻ > Cl⁻ (13, 14). The presence of I_{Cl,H⁺} in all mammalian cell types examined so far suggests that TMEM206 may be universally expressed in all tissues and play an essential role in cellular responses to extracellular acidification. The channel is activated at a highly acidic extracellular pH (~5.5 to 6.0) that may be limited to pathological conditions, such as cancer and ischemic stroke (9, 13–18) in which the I_{Cl,H⁺} currents may contribute to cell death induced by tissue acidosis (6, 19). Consistent with this notion, deletion of TMEM206 attenuated acid-induced cell death (13, 14), suggesting that pharmacological inhibition of TMEM206 potentially alleviates acidosis-associated pathologies.

Cl⁻ channels are remarkably diverse in both amino acid sequence and three-dimensional (3D) architecture, as demonstrated by the structurally and functionally characterized Cl⁻ channel families, including CLC channels (1), Bestrophin (20–22), TMEM16 (23–26), cystic fibrosis transmembrane conductance regulator (27), anion-selective Cys-loop receptors (28), and volume-regulated anion channels (29–31). The lack of signature sequences or structural motifs among these channels has posed substantial challenges with regard to the molecular identification of Cl⁻ currents, as is the case of TMEM206 (13, 14). Without discernible sequence homology with previously characterized Cl⁻ channels, TMEM206 likely represents a new class of Cl⁻ channels with distinct subunit architecture, channel assembly, and ion conduction and activation mechanisms. Here, we present a cryo–electron microscopy (cryo-EM) structure of pufferfish TMEM206, which reveals a trimeric channel architecture that is different from previously known Cl⁻ channels. In combination with electrophysiology, this work provides the first structural and functional description of an evolutionarily conserved and broadly expressed Cl⁻ channel and establishes a molecular framework for understanding Cl⁻ conduction and channel gating.

RESULTS

Fusion strategy for cryo-EM structure determination

To identify TMEM206 candidates for structural studies, we expressed multiple orthologs with a C-terminal green fluorescent protein (GFP) in yeast Pichia pastoris and analyzed channel expression and assembly profiles using fluorescence-detection size-exclusion chromatography (FSEC) (32). Subsequent large-scale purification identified pufferfish (Takifugu rubripes) TMEM206, which shares 50% sequence identity to the human channel (fig. S1), as a promising target indicated by a well-resolved oligomeric assembly on size-exclusion chromatography (fig. S2). The full-length wild-type pufferfish TMEM206 protein was purified to homogeneity and...
subjected to single-particle cryo-EM analysis. 3D reconstruction yielded a low-resolution (~6.2 Å) map that revealed a trimeric channel architecture with both transmembrane and extramembrane domains (fig. S3). The trimeric channel has a predicted molecular weight of ~120 kDa and likely contains unstructured segments. Thus, the relatively small particle size may present a challenge in high-resolution cryo-EM reconstruction. Further, particles were sparsely distributed on the cryo-EM grids (fig. S2), and preparations of grids at higher protein concentrations failed to improve the particle density because channels were increasingly inclined to aggregate upon vitrification.

To overcome these technical difficulties, we fused the channel with a C-terminal BRIL (thermostabilized apocytochrome b562 RIL), a four-helix bundle protein that has been widely used as a crystallization chaperone to improve membrane protein stability and to promote crystal formation (33, 34). To potentially facilitate particle alignment with the additional molecular mass from BRIL, we systematically shortened the extreme C terminus of pufferfish TMEM206, which is not conserved among orthologs and is most likely unstructured, to increase the overall structural rigidity between the channel and BRIL. On the basis of FSEF profiles, we selected a construct with the last four C-terminal amino acids removed (Fig. 1A and fig. S2, A to F). This construct, which we termed as TMEM206EM, contains amino acids 1 to 349 of pufferfish TMEM206 and BRIL. Purified TMEM206EM showed markedly reduced aggregation on size-exclusion chromatography and produced densely distributed particles on cryo-EM grids (fig. S2). These improvements allowed us to obtain a cryo-EM reconstruction with an overall resolution of ~3.5 Å with C3 symmetry imposed (figs. S4 and S5 and table S1). The quality of the cryo-EM density map is sufficient for de novo model building guided by bulky side chains. Portions of the N and C termini (amino acids 1 to 64 and 335 to 349) and BRIL were not resolved in the density map and were thus excluded from the model. The final atomic model, consisting of residues 65 to 159, 168 to 251, and 255 to 334, has good stereochemistry and fits well into the density (fig. S5 and table S1). The model also matches the lower-resolution map calculated from the intact wild-type channel, indicating that the BRIL fusion did not undermine the structural integrity of the channel (fig. S6). Furthermore, when expressed in TMEM206-knockout human embryonic kidney (HEK) 293T cells, TMEM206EM and the full-length wild-type pufferfish TMEM206 displayed a similar I-V relationship, pH dose response, and anion selectivity (Fig. 1, B to E, and figs. S7 and S8).

A trimeric Cl⁻ channel
TMEM206 forms a symmetric trimer, with each subunit containing a transmembrane domain (TMD) with two membrane-spanning helices (TM1 and TM2) and a large extracellular domain (ECD) enriched in β strands (Fig. 1, F and G). The outer helix TM1 and inner helix TM2 within a single subunit are arranged in an approximately antiparallel fashion, tilted by ~30° to the membrane normal. The ECD comprises an inner β-domain, consisting of TM1 and TM2, and an outer β-domain with a helix-turn-helix (HTH) insertion positioned at the periphery of the channel (Fig. 2, A to C). The outer β-domain, consisting of β1, β3, β6, and β9 to β12, is further organized into the upper and lower layers that are held together by an elongated pair of antiparallel β strands β9 and β10. β1 and β12 in the lower layer are connected to TM1 and TM2, respectively. Besides the β9-β10 pair, the upper layer contains three additional strands, β3, β6, and β11, and connects to the lower layer via a short β11-β12 linker. In contrast to a compact lower layer, the upper layer of the ECD is expanded by the peripheral outer β-domain, consisting of β2, β4, β5, β7, and β8, and the HTH insertion between β7 and β8. Extensive side-chain contacts, mainly through van der Waals interactions, are involved in the interface between the inner and outer β-domains.

The intersubunit interface, contributed by the ECD and TMD, buries ~2400 Å² of molecular surface per subunit. In the extracellular region, intimate packing interactions are limited to two regions, the top portion of the ECD and the ECD-TMD junction, leaving considerable empty spaces in the middle between subunits (Fig. 3). The three subunits come to close proximity at the very top of the ECD, with side chains of residues F238 and K267 facing the central threefold symmetry axis (Fig. 3A). On the side, loops from neighboring subunits interdigitate through a network of both van der Waals and hydrogen bonding interactions (Fig. 3B). In particular, the aromatic side chain of F198 is nestled in the hydrophobic pocket from an adjacent subunit composed of several aromatic side chains from F186, F268, and F283, and is within the distance of cation-π interaction with R239 from the neighboring subunit. The robust intersubunit interactions at the uppermost portion of the ECD may contribute to a stationary structural scaffold that supports gating transitions necessary at the distal TMD and ECD-TMD junction.

The trimeric channel assembly introduces three lateral openings (side portals) in the middle of the extracellular region (Fig. 3C). In each portal, the interior wall is predominantly lined by polar and charged side chains, likely facilitating ion and water passage. The elongated side portals extend to the ECD-TMD junction where tight packing interactions resume. The short linkers of TM1-β1 and β12-TM2 create a narrow “neck” immediately above the lipid membrane (Fig. 3D). Within the membrane, the trimer interface is primarily mediated by the inner helix TM2, which, together with the neck, restricts the central ion-conduction path. Side-chain interactions are also observed between TM2 and an adjacent TM1 on the extracellular end (Fig. 3D). Notably, this interface reveals the close proximity between L85 in TM1 and W305 in TM2. Consistent with the notion that the TM1-TM2 intersubunit interface is likely involved in regulation of channel activity, cysteine substitutions at the equivalent positions of L85 and W305 in human TMEM206 both increased I_{CLH} currents at negative voltages with the application of cysteine-modifying reagent MTSES (2-sulfonatoethyl methanethiosulfonate) (14).

Ion permeation pathway
The central ion conduction pore contains multiple constrictions that would prevent ion passage (Fig. 4, A and B), as indicated by the pore radius calculation (35). Thus, the structure represents a non-conductive conformation, which is consistent with the high pH buffer condition (pH 8.0) used for cryo-EM structure determination. The intimate assembly at the top of the ECD places side chains of F238 in close proximity, generating a constriction that separates the upper and central vestibules. The voluminous and elongated central vestibule is accessible laterally owing to the lack of protein-protein contacts in the middle of the ECD (Fig. 4C). Therefore, the narrow point at F238 might not interfere with ion conduction and could be maintained during the channel gating cycle as ions pass through the three side portals. Further, the slightly positive electrostatic...
potential of the interior walls of the central vestibule and side entryways would facilitate attraction of extracellular Cl\(^{-}\) (Fig. 4C).

At the ECD-TMD junction, the three \(\beta\) strands, connected to the outer helices, cross each other, and the three \(\beta\)12 strands move inward to join the inner helices (Fig. 4D). This arrangement generates an extracellular gate, which is constituted by V101 in \(\beta\)1 and T301 and N303 in the \(\beta\)12-TM2 linker, above the lipid bilayer (Fig. 4D). Underneath the extracellular gate and within the membrane, the ion conduction pore is lined by residues from the inner helices TM2, which cross each other at a conserved glycine residue G313 by an angle of ~60° (Fig. 4E). Consecutive constrictions at the pore-facing positions, I310, G313, and M316, appear to form a hydrophobic gate that prohibits ion conduction (Fig. 4E). Beneath the gate, side chains of a highly conserved basic residue K320 point to the upper vestibule, resulted in channel properties similar to those of the V101 mutations. Previous cysteine-scanning accessibility studies of the entire TM1 and TM2 helices of human TMEM206 revealed that the outer helix TM1 was mostly insensitive to cysteine substitutions (14). By contrast, the inner helix TM2 carried multiple cysteine substitutions that displayed either increased or decreased \(I_{\text{CL,H}}\) currents, suggesting the importance of this pore-facing position right above the presumed selectivity filter. The V101A and V101F mutations resulted in increased currents at both pH 7.3 and pH 4.6, but the folds of activation by low pH were reduced, suggesting that these mutations likely increased both basal open probability at high pH and unitary conductance. Alanine substitution of the bulky residue F238 (F238A), which forms the constriction at the upper vestibule, resulted in channel properties similar to those of the V101 mutations.

To corroborate our structural findings, we performed mutagenesis studies on key pore-lining residues (Fig. 4, G to I, and fig. S9). Substitution of the conserved basic residue K320 with alanine or acidic residues abolished acid-activated \(I_{\text{CL,H}}\) currents. In marked contrast, the arginine substitution retained channel function, further supporting the requirement of positive charges in the anion-selective filter. M316 mutations (M316A/D/E/K) also abolished \(I_{\text{CL,H}}\) currents, suggesting the importance of this pore-facing position right above the presumed selectivity filter. The V101A and V101F mutations resulted in increased currents at both pH 7.3 and pH 4.6, but the folds of activation by low pH were reduced, suggesting that these mutations likely increased both basal open probability at high pH and unitary conductance. Alanine substitution of the bulky residue F238 (F238A), which forms the constriction at the upper vestibule, resulted in channel properties similar to those of the V101 mutations.

Previous cysteine-scanning accessibility studies of the entire TM1 and TM2 helices of human TMEM206 revealed that the outer helix TM1 was mostly insensitive to cysteine substitutions (14). By contrast, the inner helix TM2 carried multiple cysteine substitutions that displayed either increased or decreased \(I_{\text{CL,H}}\) currents in response to MTSES application (14). For a total of 44 cysteine substitutions, only L309C and K319C in TM2 (corresponding to I310 and K320 in pufferfish TMEM206) failed to elicit acid-activated activation. L309C and K319C in TM2 (corresponding to I310 and K320 in pufferfish TMEM206) failed to elicit acid-activated

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**Fig. 1. Function and structure of pufferfish TMEM206.** (A) Schematic of channel constructs used for electrophysiology and single-particle cryo-EM experiments. “Thr” represents a thrombin cleavage site. (B and C) Representative whole-cell current traces activated by extracellular pH 4.6 for pufferfish TMEM206 (B) and TMEM206EM (C). Channel constructs were expressed in TMEM206 knockout human embryonic kidney (HEK) 293T cells. (D) Normalized current-to-pH relationships of pufferfish TMEM206 (n = 6 to 9 cells per data point) and TMEM206EM (n = 5 to 6 cells per data point). All currents were recorded at room temperature and normalized to pH 4.0 currents at +100 mV. (E) Anion selectivity for pufferfish TMEM206 and TMEM206EM. Data are presented as means ± SEM (n.s., not significant; Student’s t test). (F) Cryo-EM density of pufferfish TMEM206EM contoured at 7.0 \(\sigma\) and colored by individual subunits. (G) Trimeric structure of pufferfish TMEM206EM.

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Fig. 2. Subunit structure and channel assembly. (A) Structure of a single subunit, showing the transmembrane domain (red), inner β-domain (blue), outer β-domain (orange), and HTH (green). Secondary structure elements are indicated. (B) Trimeric channel assembly. Two of the subunits are shown in surface representation. (C) Orthogonal view as in (B), from the extracellular side.

Fig. 3. Intersubunit interface. (A) Trimeric interface at the apex of the ECD. Side chains of K267 and F238 are highlighted. (B) Side view of the intersubunit interface at the top layer of the ECD. Residues involved in the interface are shown in stick representation. (C) Side portal in the middle of the ECD between two neighboring subunits. Surface and residues lining the wall are illustrated. (D) TM1-TM2 intersubunit interface.
I\text{Cl,\textsubscript{H}} currents (14). In light of our structure, these experiments underscore the essential role of these two pore-lining residues. Specifically, I310 is a critical component of the transmembrane gate, and K320 forms the anion selectivity filter (Fig. 4, E and F). G313 and M316 are the remaining two residues that constitute the transmembrane gate in pufferfish TMEM206. The corresponding cysteine substitutions in human TMEM206 (G312C and L315C) showed a pronounced MTSES-dependent increase in I\text{Cl,\textsubscript{H}} currents (14). In addition, the introduction of an acidic residue at position 315 (L315D), which is one helical turn above the anion selectivity filter that is composed of lysine residues (Fig. 4E), rendered the channel nonselective with permeability to both cations and anions likely because of charge neutralization in the filter region (14). Collectively, key pore-lining residues with functional implications in gating and selectivity identified by the systematic cysteine substitution experiments are in accordance with our structural and electrophysiological findings. Further, these data support structural conservation between the human and pufferfish orthologs and that our TMEM206EM structure represents a physiologically relevant model for the entire family of Cl\textsuperscript{−} channels.

**Structural convergence of cation and anion channels**

Surprisingly, the topology, structure, and assembly of TMEM206 are reminiscent of those of the epithelial sodium channel (ENaC)/degenerin
superfamily of ion channels, including acid-sensing ion channels (ASICs) and ENaCs (Fig. 5, A to D), despite a lack of discernible amino acid sequence homology (36–38). These trimeric channels, selective for oppositely charged ions Na\(^+\) and Cl\(^-\), share a common core structure composed of a 12-stranded β-domain flanked by two transmembrane helices. The β-domains in these channels have the same topology and align well with each other (Fig. 5D). TMEM206 contains a simple HTH insertion to the outer β-domain, whereas ASICs and ENaCs are furnished with more complex structural elements surrounding the outer β-domain (Fig. 5, A to D). Structures of ASIC1a reveal a central ion permeation path analogous to that of TMEM206, including the upper, central, and cytoplasmic vestibules and multiple constrictions (Fig. 5, E and F). In addition, ASIC1a contains an extracellular vestibule and fenestrations, which enlarge upon channel activation to allow ion conduction, leaving the extracellular constrictions along the central path largely unaltered (Fig. 5F) (36, 37). By contrast, TMEM206 lacks the additional structural elements involved in formation of the acidic pocket as in ASIC1a, suggesting a distinct acid-sensing mechanism. Nevertheless, conserved titratable amino acids in the extracellular region cluster at the intersubunit interface (Figs. S1 and S10), raising an interesting possibility that the “acid sensor” in TMEM206 may be positioned at the subunit interface. Notably, in ASIC1a, L414 and N415 from the β11-β12 linker, which demarcates the upper and lower β-domains, serve as a molecular “clutch” by swapping side-chain orientations to promote channel desensitization upon sustained exposure to protons (37). In TMEM206, conserved titratable residues (R289 and D290 in pufferfish TMEM206) from the β11-β12 linker may play a comparable role

![Fig. 5. Structural comparison with ASIC and ENaC.](http://advances.sciencemag.org/)
during channel gating (figs. S1 and S10). Conserved structural features between TMEM206 and ASICs further suggest the likelihood of analogous gating conformational changes, such as a relatively stationary structural scaffold at the upper ECD, expansion of the lower β-domain, and an iris-like opening of the gate in the membrane.

**DISCUSSION**

Single-particle cryo-EM has facilitated structure determination of integral membrane proteins that are unattainable using traditional x-ray crystallography. However, achieving near-atomic resolution for membrane proteins of small size remains a major technical challenge because of low contrast and signal-to-noise ratios (39). In this study, we obtained a 3.5-Å-resolution structure of a channel with an ordered portion of only ~90 kDa. This was made possible by fusion with a small crystallization chaperone BRIL, which has proven to be useful in improving stability of otherwise suboptimal membrane proteins and in promoting crystal packing (33). The improvement in resolution was enabled by markedly increased particle density on the cryo-EM grids as a result of enhanced homogeneity and reduced aggregation, rather than a gain of molecular mass as BRIL was invisible in the density map. Perhaps owing to the flexible connection between the channel and BRIL, the structural and functional integrity is maintained in the fusion construct TMEM206EM. Analogous to crystallography, the BRIL fusion strategy may be broadly applicable to single-particle cryo-EM studies of small-sized and intrinsically unstable membrane proteins.

This work now defines the structure and assembly of a new class of Cl− channels TMEM206 and establishes a framework for further functional and mechanistic investigation. To provide structural insights into proton activation, we attempted to determine structures of TMEM206EM at low pH (~5.0) but were unable to obtain meaningful cryo-EM reconstructions. Notably, the structure of the TMEM206 Cl− channel closely resembles those of Na+ -selective channels ASICs and ENaCs, which are unrelated in amino acid sequence and conduct positively, instead of negatively, charged ions (36–38). The conserved core structure suggests that these channels, selective for either cations or anions, may experience similar gating conformational changes.

During the revision of our manuscript, cryo-EM structures of human TMEM206 in nonconducting states at both high and low pH were published (40). The overall structures of the human and pufferfish channels in the high-pH resting states are similar, suggesting conservation of structure and potentially activation mechanisms among family members. Consistent with our proposal that the conserved lysine residue in TM2 (K320 in pufferfish TMEM206) is important for ion selectivity, mutation of the equivalent lysine residue in human TMEM206 (K319E) rendered the channel cation selective (40). Together, these studies provide initial insights into the structure and function of the TMEM206 family of channels. Additional structures representing the open, conductive conformations are necessary to understand acid activation mechanisms.

**MATERIALS AND METHODS**

**Protein expression and purification**

The codon-optimized DNA fragments encoding 16 TMEM206 orthologs were synthesized (Bio Basic Inc.). For overexpression in yeast *P. pastoris*, DNA fragments were transferred into a modified pPICZ-B vector with a PreScission protease cleavage site followed by a C-terminal GFP-His10 tag. The pufferfish TMEM206 was identified as a promising candidate for structural studies, as evaluated by FSEC. Initial cryo-EM analysis using the full-length wild-type protein showed that purified channels were prone to aggregation on cryo-EM grids, precluding structure determination to high resolution. To improve the biochemical stability of the channel, BRIL (thermostabilized apocytochrome b562 RIL) was fused to the C terminus of pufferfish TMEM206. To increase the structural rigidity of the fusion construct, the last four C-terminal residues of TMEM206 were further removed. The expression construct TMEM206EM includes residues 1 to 349 of pufferfish TMEM206 and a C-terminal BRIL followed by the PreScission protease cleavage site and GFP-His10 tag. For electrophysiological recordings, DNA fragments were ligated into a modified pCEU vector containing a C-terminal GFP-His6 tag. Mutations used in this study were generated by site-directed mutagenesis.

Yeast cells expressing the full-length wild-type pufferfish TMEM206 were disrupted by milling (Retsch MM400) and resuspended in buffer containing 50 mM tris (pH 8.0) and 150 mM NaCl supplemented with deoxyribonuclease I and protease inhibitors including leupetin (2.5 μg/ml), pepstatin A (1 μg/ml), 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (100 μg/ml), aprotonin (3 μg/ml), 1 mM benzamidine, and 200 μM phenylmethylsulfon-nyl fluoride. The cell mixture was extracted with 1% (w/v) lauryl maltose neopentyl glycol (Anatrace) for 2 hours with stirring at 4°C and then centrifuged for 1 hour at 30,000g. The supernatant was collected and incubated with 3 ml of cobalt-charged resin (G-Biosciences) for 3 hours at 4°C. Resin was then collected and washed with 30 ml of buffer 20 mM tris (pH 8.0), 150 mM NaCl, 20 mM imidazole, and 85 μM glyco-diosgenin (GDN; Anatrace). The channel protein was eluted with 200 mM imidazole and digested with PreScission protease at 4°C overnight to remove the C-terminal GFP-His10 tag. Further purification was performed on a Superose 6 Increase 10/300 gel filtration column (GE Healthcare Life Sciences) in 20 mM tris (pH 8.0), 150 mM NaCl, and 40 μM GDN. Peak fractions containing channel protein were concentrated to ~6 mg/ml and used immediately for cryo-EM grid preparations. Purification of TMEM206EM followed the same procedure as that for the wild type, except that soybean polar lipid extract (0.05 mg/ml; Avanti Polar Lipids Inc.) was included in the wash, elution, and gel filtration buffers.

**Cryo-EM grid preparation and imaging**

Cryo-EM grids were prepared by applying freshly purified channel protein (~3.5 μl) to glow-discharged copper Quantifoil R2/2 holey carbon grids (Quantifoil), which were then blotted for 2 s with ~100% humidity and flash frozen in liquid ethane using FEI Vitrobot Mark IV (FEI). Images were collected using a Titan Krios (FEI) electron microscope operating at 300 kV with a Gatan K2 Summit (Gatan) detector and Gatan Imaging Filter Quantum energy filter with a slit width of 20 eV. Data collection was performed using EPU software (https://fei.com/software/epu-automated-single-particles-software-for-life-sciences/) in the super-resolution mode with a pixel size of 0.55 Å and a nominal defocus range of ~1.0 to ~2.5 μm. With a dose of ~7.8 electrons/Å2 per second, each micrograph was recorded for 8 s in 40 frames (an accumulated dose of ~62 electrons/Å2).

**Data processing and map calculation**

Recorded micrographs were motion corrected and dose weighted using MotionCor2 (41). Dose-weighted micrographs were subjected
temperature using an Axon 700B amplifier (Molecular Devices). Pipettes were pulled from borosilicate glass (BF 150-86-10; Sutter Instrument) with a Sutter P-1000 pipette and filled with the intracellular solution containing 135 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 5 mM EGTA, 4 mM MgATP (280 to 290 mOsm/kg; pH 7.2 with CsOH). The external solution contained 145 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM Hepes, 10 mM glucose (300 mOsm/kg; pH 7.3 with NaOH). To make different acidic pH solutions, 5 mM Na₃-citrate was used as buffer, and the pH was adjusted using citric acid. Holding at 0 mV, voltage ramp from −100 to +100 mV for 500 ms was used to record whole-cell currents. Currents of pufferfish TMEM206 and mutants were normalized by cell capacitance to calculate current densities. For anionic selectivity experiments, bath solutions were replaced with 145 mM NaX/5 mM Na₃-citrate (pH 4.6 was adjusted with citric acid), where X was Cl⁻, Br⁻, and I⁻, respectively. Data were acquired using Clampex 10.4 software (Molecular Devices). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices). The concentration-response curve was fitted with the logistic equation

\[ Y = \frac{Y_{\text{max}}}{1 + 10^{\left(\log pK_d - X\right) \times \text{Hill slope}}} \]

where \( Y \) is the response at a given pH, \( Y_{\text{max}} \) and \( Y_{\text{min}} \) are the maximum and minimum responses, \( X \) is the logarithmic value of the pH, and Hill slope is the slope factor of the curve. \( pK_d \) is the pH value that gives a response halfway between \( Y_{\text{max}} \) and \( Y_{\text{min}} \). The permeability ratios were calculated from shifts in reversal potential with the Goldman-Hodgkin-Katz equation. All data are presented as means ± SEM.

**Immunofluorescence staining**

The TMEM206-knockout HEK293T cells were transfected with GFP-tagged pufferfish TMEM206 and mutants. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde for 30 min and washed twice with phosphate-buffered saline (PBS) buffer. To stain the cell membranes, cells were incubated with a wheat germ agglutinin (WGA; 1:400, Biotium) lectin tagged with wheat germ agglutinin (WGA; 1:400, Biotium) lectin tagged with CF 594 (5 µg/ml) for 10 min at 37°C according to the manufacturer’s instruction. After washing with PBS, cells were mounted with 4',6-diamidino-2-phenylindole Fluromount-G (SouthernBiotech) for 10 min. Immunofluorescence staining was performed using the program HOLE (35). Structural figures were rendered using PyMol (pymol.org).

**Electrophysiology**

Knockout of the human TMEM206 gene in the HEK293T cell line was conducted using CRISPR-Cas9–mediated gene disruption (48). The TMEM206 gene was targeted using the reported guide RNA (gRNA; 5’-GGACCGAGAAGACGTTCTTC-3’, negative strand) sequence (13). The gRNA was inserted into PX459 V2.0 plasmid (Addgene, catalog no. 62988) and transfected into HEK293T cells using FuGENE HD transfection reagent (Promega). After 24 hours, cells were transferred to fresh medium with puromycin (5 µg/ml) for an additional 7 days. Single colonies were then isolated using limiting dilution. The knockout cell line was determined through genotyping analysis of frameshift mutations by target site–specific polymerase chain reaction and cloning followed by Sanger sequencing.

The TMEM206 knockout cell line was used for electrophysiology experiments. The C-terminal GFP-tagged wild-type pufferfish TMEM206 channel or each mutant channel was transfected. Whole-cell patch-clamp recordings were performed at room temperature using an Axon 700B amplifier (Molecular Devices). Pipettes were pulled from borosilicate glass (BF 150-86-10; Sutter Instrument) with a Sutter P-1000 pipette and filled with the intracellular solution containing 135 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 5 mM EGTA, 4 mM MgATP (280 to 290 mOsm/kg; pH 7.2 with CsOH). The external solution contained 145 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM Hepes, 10 mM glucose (300 mOsm/kg; pH 7.3 with NaOH). To make different acidic pH solutions, 5 mM Na₃-citrate was used as buffer, and the pH was adjusted using citric acid. Holding at 0 mV, voltage ramp from −100 to +100 mV for 500 ms was used to record whole-cell currents. Currents of pufferfish TMEM206 and mutants were normalized by cell capacitance to calculate current densities. For anionic selectivity experiments, bath solutions were replaced with 145 mM NaX/5 mM Na₃-citrate (pH 4.6 was adjusted with citric acid), where X was Cl⁻, Br⁻, and I⁻, respectively. Data were acquired using Clampex 10.4 software (Molecular Devices). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices). The concentration-response curve was fitted with the logistic equation

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The TMEM206-knockout HEK293T cells were transfected with GFP-tagged pufferfish TMEM206 and mutants. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde for 30 min and washed twice with phosphate-buffered saline (PBS) buffer. To stain the cell membranes, cells were incubated with a wheat germ agglutinin (WGA; 1:400, Biotium) lectin tagged with CF 594 (5 µg/ml) in Hanks’ balanced salt solution without phenol red for 10 min at 37°C according to the manufacturer’s instruction. After washing with PBS, cells were mounted with 4’,6-diamidino-2-phenylindole Fluromount-G (SouthernBiotech) and analyzed using a Nikon confocal microscope. For colocalization analyses, 50 cells for each channel construct from at least three different coverslips were selected. The colocalization ratios of GFP⁺ area in WGA⁺ area, analyzed by ImageJ, represent the expression levels on the plasma membrane. All the data were collected and analyzed using GraphPad prism 7.0.

**Supplementary Materials**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/9/eabe5983/DC1

View/request a protocol for this paper from Bio-protocol.

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project. Z.D., Y.Z., J.F., H.H., and P.Y. analyzed the results and prepared the manuscript, with input from all authors. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The cryo-EM maps have been deposited to the Electron Microscopy Data Bank with accession codes EMD-22342 and EMD-22343. Atomic coordinates have been deposited to the Protein Data Bank (PDB) with accession code 7JI3. Correspondence and requests for materials should be addressed to P.Y. (yuanp@wustl.edu).

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