

TMEM16 and OSCA/TMEM63 proteins share a conserved potential to permeate ions and phospholipids

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Abstract

The calcium-activated TMEM16 proteins and the mechanosensitive/osmolarity-activated OSCA/TMEM63 proteins belong to the Transmembrane Channel/Scramblase (TCS) superfamily. Within the superfamily, OSCA/TMEM63 proteins, as well as TMEM16A and TMEM16B, are thought to function solely as ion channels. However, most TMEM16 members, including TMEM16F, maintain an additional function as scramblases, rapidly exchanging phospholipids between leaflets of the membrane. Although recent studies have advanced our understanding of TCS structure-function relationships, the molecular determinants of TCS ion and lipid permeation remain unclear. Here we show that single mutations along the transmembrane helix (TM) 4/6 interface allow non-scrambling TCS members to permeate phospholipids. In particular, this study highlights the key role of TM 4 in controlling TCS ion and lipid permeation and offers novel insights into the evolution of the TCS superfamily, suggesting that, like TMEM16s, the OSCA/TMEM63 family maintains a conserved potential to permeate ions and phospholipids.

eLife Assessment

This **important** study advances our understanding of the mechanisms controlling lipid flux and ion permeation in the TMEM16 and OSCA/TMEM63 family channels. The study provides **compelling** new evidence indicating that side chains along the TM4/6 interface play a key role in gating lipid and ion fluxes in these channels. The authors suggest that the transmembrane channel/scramblase family proteins may have originally functioned as scramblases but lost this capacity over evolution.

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Introduction

Ion channels and phospholipid scramblases catalyze the passive flux of ions and phospholipids down their respective chemical gradients. Compared to ion channels, both our understanding of scramblases and the evolutionary origins of phospholipid scrambling are underdeveloped. Discoveries of two scramblase families—the TMEM16 calcium-activated phospholipid scramblases (CaPLSases) and XKR caspase-dependent phospholipid scramblases—have revealed roles for scramblases in processes such as blood coagulation^{1,2,3}, angiogenesis⁴, cell death signaling^{5,6}, phagocytosis⁶, cell migration^{7,8}, membrane repair^{9,10}, microparticle release³, cell-cell fusion^{11–13}, and viral infection^{14,15}. Among identified scramblases, TMEM16 CaPLSases are the most extensively studied at the molecular level¹⁶. The TMEM16 family (Fig. S1a) was originally classified as calcium-activated chloride channels (CaCCs) based on the first-discovered members, TMEM16A and TMEM16B^{17–19}. Most remaining members, however, exhibit CaPLSase activity, with TMEM16F representing the canonical CaPLSase²⁰ (Fig. 1a). Uniquely, CaPLSases also function as non-selective ion channels along with their non-specific permeability to phospholipids^{2,21–25}. Substrate permeation is facilitated by calcium-induced conformational changes causing the clamshell-like separation of TMs 4 and 6 to facilitate ion and phospholipid permeation^{26–28} (Fig. S1b–d). These conformational changes further catalyzed phospholipid permeation by thinning the membrane near the permeation pathway^{29,30}.

The TMEM16 family together with the OSCA/TMEM63 family and TMC family^{31–34} collectively form the Transmembrane Channel/Scramblase (TCS) superfamily¹⁶. Despite the conserved 10-TM architectural core^{35–39}, phospholipid permeability has not been experimentally demonstrated in mechanosensitive OSCA/TMEM63 or TMC proteins, raising the intriguing question of how TCS proteins discriminate between ion and ion/phospholipid substrates. Interestingly, studies have shown that the TMEM16A CaCC can be genetically modified to enable phospholipid permeability, either by substituting TMEM16F domains as small as 15 amino acids²³, or through single mutations at the hydrophobic gate²⁶. These findings suggest a modest energetic barrier for scramblase activity and lead us to hypothesize that evolutionary relatives of the TMEM16 family may maintain a conserved potential for both ion and phospholipid permeation.

To test this hypothesis, we introduced single mutations along TM 4 of TMEM16F, TMEM16A, OSCA1.2, and TMEM63A at sites near the corresponding hydrophobic gate of TMEM16F²⁶. Mutations along the TM 4/6 interface in TMEM16F and TMEM16A resulted in constitutive phospholipid scramblase activity. Strikingly, equivalent TM 4 mutations in OSCA1.2 and TMEM63A also converted these ion channels into phospholipid scramblases, which were either constitutive or activated by osmotic stimulation. Individual mutations also resulted in gain-of-function (GOF) ion channel activity, suggesting that these mutations may disrupt a conserved activation gate in these evolutionarily related proteins. Together, our findings i) advance the mechanistic understanding of gating and substrate permeation in the TMEM16 and OSCA/TMEM63 families, ii) underscore a key design principle for phospholipid scramblases, and iii) support our hypothesis that the TCS superfamily maintains a conserved potential for both ion and phospholipid permeation.

Results

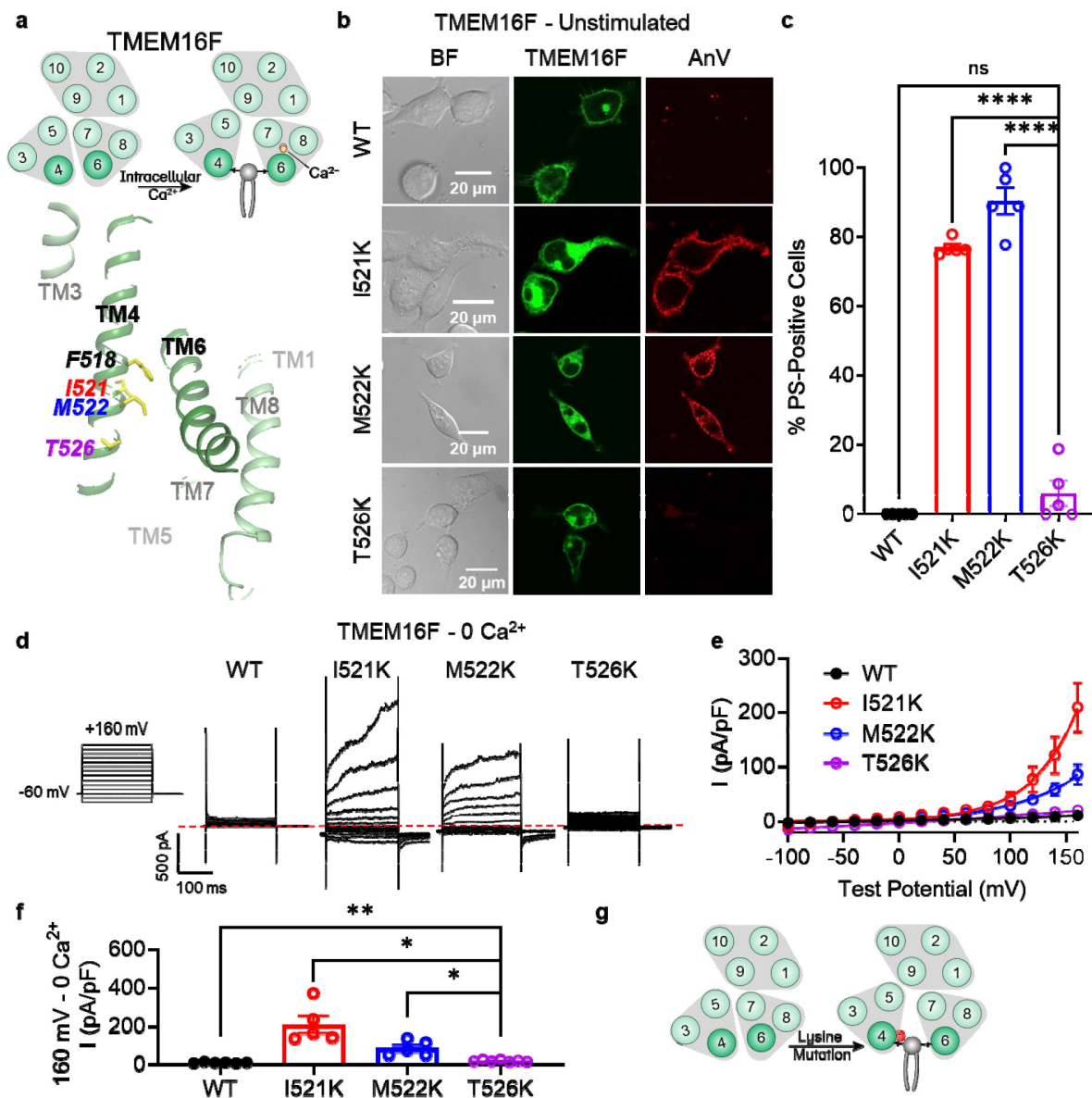


Figure 1

Lysine mutations along TM 4 enable TMEM16F channel and scramblase activities in the absence of calcium stimulation.

(a) Top: TMEM16F is a calcium-activated phospholipid scramblase. Bottom: TM 4 mutant locations mapped on TMEM16F CaPLSase structure with side chains shown as yellow sticks (PDB 6QPB). (b) Representative images of TMEM16F knockout (KO) HEK293T cells expressing eGFP-tagged TMEM16F wildtype (WT), I521K, M522K, and T526K (center column). CF 594-conjugated Annexin V (AnV, right column) labelled PS exposing cells. BF denotes bright field images (left column). (c) Quantification of the percentage of cells with AnV labelling for TMEM16F WT (n=5), I521K (n=5), M522K (n=5), and T526K (n=5) transfected cells. Values were derived from images of biological replicates, with error bars representing standard error of the mean (SEM). Statistical comparisons to T526K were done using unpaired t-tests with Welch's correction (ns: p>0.05, ****: p<0.0001). (d) Representative current recordings and (e) current-voltage (I-V) relationships of whole cell patches from TMEM16F KO HEK293T cells expressing eGFP-tagged TMEM16F WT (n=6), I521K (n=5), M522K (n=5), and T526K (n=7). Currents were elicited by the voltage protocol shown with the pipette solution containing 5 mM EGTA. Dotted line denotes zero current. (f) Quantification of current at +160 mV. Currents in (e) and (f) were normalized to cell capacitance with the mean $\pm$ SEM calculated from independent patches. Statistical comparisons to T526K were done using unpaired t-tests with Welch's correction (*: p<0.05, **: p<0.01). (g) Lysine mutations along TM 4 in TMEM16F enable spontaneous phospholipid permeation in the absence of calcium.

TM 4 mutations result in a constitutively active TMEM16F scramblase

Our previous study identified the activation gate of TMEM16F, consisting of hydrophobic residues from TMs 4, 5, and 6, which collectively govern ion and phospholipid permeation²⁶. Mutations to the activation gate result in GOF CaPLSases, some of which are constitutively activated without requiring calcium²⁶. The hydrophobic gate represents the most constricted point along the TM 4/6 interface in TMEM16F and mutations along this interface likely alter substrate permeation and gating. Structural and computational studies on the fungal *Nh*TMEM16⁴⁰ and *Aj*TMEM16²⁹ orthologs, as well as human TMEM16K⁴¹ further support a clamshell-like gating model²⁶ whereby scrambling activity is promoted by separation of the TM 4/6 interface¹⁶ (Fig. S1b-d). The conformational transition involves the N-terminal half of TM 4, which bends away from TM 6, and the C-terminal half of TM 6, which collapses onto the calcium binding sites formed together with TMs 7 and 8¹⁶. Given that the N-termini of TM 4 are largely hydrophobic among TMEM16 CaPLSases (Fig. S1f) and mutations at F518 lead to constitutive TMEM16F activity²⁶, we hypothesized that introducing a charged side chain along the TM 4/6 interface might disrupt the hydrophobic gate and result in TMEM16 scramblases with GOF scramblase activity. To test this hypothesis, we overexpressed eGFP-tagged mutant constructs in TMEM16F knockout (KO) HEK293T cells and used an established scramblase assay^{25,26,42} that detects phosphatidylserine (PS) exposure using fluorophore-tagged Annexin V (AnV) as a reporter (Fig. 1b). In the absence of calcium stimulation, PS is predominantly in the inner leaflet of the plasma membrane of TMEM16F wildtype (WT) expressing cells and is therefore not labelled by extracellular AnV. Similar to TMEM16F F518K²⁶, overexpressing the single lysine mutations TMEM16F I521K and M522K led to spontaneous, global exposure of PS on the plasma membrane without requiring calcium stimulation (Fig. 1b, c). In contrast, T526K, which is one helical turn below I521 and M522 (Fig. 1a), caused minimal spontaneous PS exposure (Fig. 1b, c). We further tested whether I521K, M522K, and T526K have GOF ion channel activity using whole-cell patch clamp. In the absence of calcium, I521K and M522K, but not WT TMEM16F or T526K, showed robust depolarization-activated outward rectifying currents (Fig. 1d-f). Our imaging and patch clamp experiments thus demonstrate that I521K and M522K are functionally expressed on the plasma membrane and their GOF phospholipid scramblase activity results in spontaneous, global PS exposure on the cell surface. Conversely, despite apparent plasma membrane localization, T526K exhibited strongly attenuated GOF ion channel and scramblase activities. The TM4/6 interaction is notably less prominent near T526 (Fig. 1a), two helical turns below the hydrophobic gate residue, F518²⁶, (Fig. 1a) and may represent a lower limit for this charge-induced effect. On the other hand, I521 and M522 are approximately one helical turn below the hydrophobic gate, (Fig. 1a) supporting the idea that charged mutations along the TM 4/6 interface promote gate opening and substrate permeation (Fig. 1g).

Our previous study also showed that glutamate, but not alanine, substitution at F518 led to spontaneous scramblase activity in TMEM16F²⁶. To assess whether alternative side chains cause spontaneous scrambling below the hydrophobic gate in TMEM16F, we tested I521A and I521E. I521E led to spontaneous, global PS exposure in all transfected cells, whereas I521A failed to cause spontaneous PS exposure (Fig. S2a, b). This result is consistent with our previous finding, providing additional support that charged TM 4 mutations disrupt the TM 4/6 interface.

I611K on TM 6 results in a constitutively active TMEM16F scramblase

We next tested whether a TM 6 mutation along the TM 4/6 interface could promote scrambling. Directly adjacent to I521 and M522 are a pair of glycine residues on TM 6 that are proposed to function as a hinge during calcium-dependent activation⁴³. We therefore chose to introduce a lysine mutation one helical turn above this site at I611, a residue adjacent to the pore-facing inner gate residue I612²⁶ (Fig. 2a). Comparable to I521K (Fig. 1b, c), I521E (Fig. S2a, b), and

M522K (Fig. 1b, c), I611K resulted in spontaneous, global PS exposure (Fig. 2b, c). Likewise, I611K-expressing cells exhibited robust, depolarization-activated outward rectifying currents in the absence of calcium (Fig. 2d-f). Together with our previous report that I612K is a GOF phospholipid scramblase and ion channel²⁶, I611K further supports that charged mutations in TM 6 near the activation gate can also promote substrate permeation in TMEM16F (Fig. 2g).

TM 4 lysine mutations convert TMEM16A into a constitutively active scramblase

TMEM16A is a CaCC^{17,19} without scramblase activity^{23,26} (Fig. 3a-c). We previously reported that a single TM 4 lysine mutation, L543K, at the hydrophobic gate of TMEM16A (Fig. 3a) allows the CaCC to permeate phospholipids spontaneously, analogous to the F518K mutation in TMEM16F²⁶. Thus, we reasoned that lysine mutations equivalent to TMEM16F I521K and M522K might also result in spontaneous phospholipid permeability. To test this hypothesis, we overexpressed eGFP-tagged TMEM16A I546K and I547K mutants in TMEM16F KO HEK293T cells and assessed their ability to expose PS on the plasma membrane with confocal microscopy. Similar to TMEM16A L543K²⁶, I546K- and I547K-expressing cells exhibit spontaneous, global PS exposure (Fig. 3b, c), analogous to the equivalent TMEM16F mutations I521K and M522K (Fig. S1f, 1b, c). Similarly, whole-cell patch clamp revealed GOF ion channel activity at depolarizing potentials, even in the absence of calcium stimulation (Fig. 3d-f). In contrast, E551K, equivalent to T526K in TMEM16F, failed to exhibit PS exposure, but showed modest ion channel activity at depolarizing potentials in the absence of calcium, perhaps reflecting the gate is open sufficiently for ion but not phospholipid permeation. In total, these results suggest that TM 4 mutations in this region destabilize interface to endow the TMEM16A CaCC with GOF ion channel activity, and, in the case of I546K and I547K, constitutive phospholipid permeability (Fig. 3g).

Ani9, a selective TMEM16A inhibitor, attenuates I546K ion and phospholipid permeation

We then tested whether Ani9, a selective, extracellular TMEM16A inhibitor⁴⁴, could attenuate phospholipid and ion permeation through I546K. Similar to our previous observation of TMEM16A L543K²⁶, chronic incubation of Ani9 at 10 μ M also prevented spontaneous PS exposure for I546K (Fig. S3d). Interestingly, 10 μ M Ani9 reversibly inhibited voltage-elicited TMEM16A I546K currents by over 50% (Fig. S3a-c). These results demonstrate that although the TM 4 GOF mutations alter TMEM16A substrate permeation, they retain their sensitivity to Ani9 inhibition (Fig. S3e). This further supports that the TM 4 GOF mutations of TMEM16A mediate the spontaneous, global PS exposure.

L438K on TM 4 converts OSCA1.2 into a constitutively active scramblase

We next applied our approach to a TMEM16 relative from the OSCA/TMEM63 family (Fig. S1a), which was first discovered in plants as a family of mechanosensitive and osmolarity-activated cation non-selective ion channels⁴⁵ (Fig. 4a). We hypothesized that analogous lysine mutations on TM 4 along the TM 4/6 interface of OSCA/TMEM63 proteins would result in GOF channels that may also permeate phospholipids. Within the family, we chose OSCA1.2 from *Arabidopsis thaliana* due to previous structural³⁵ and biophysical⁴⁶ characterization demonstrating that the channel is activated directly by membrane tension. Overexpressing eGFP-tagged OSCA1.2 WT in TMEM16F KO HEK293T cells did not induce PS exposure, demonstrating that OSCA1.2 WT lacks spontaneous phospholipid scramblase activity (Fig. 4b). We then introduced TM 4 lysine mutations at analogous sites, as identified by structural (Fig. 4a) and sequence alignment (Fig. S1f). Strikingly, a single point mutation, L438K, causes cells overexpressing the OSCA1.2 mutant to exhibit spontaneous and global PS exposure (Fig. 4b, c).

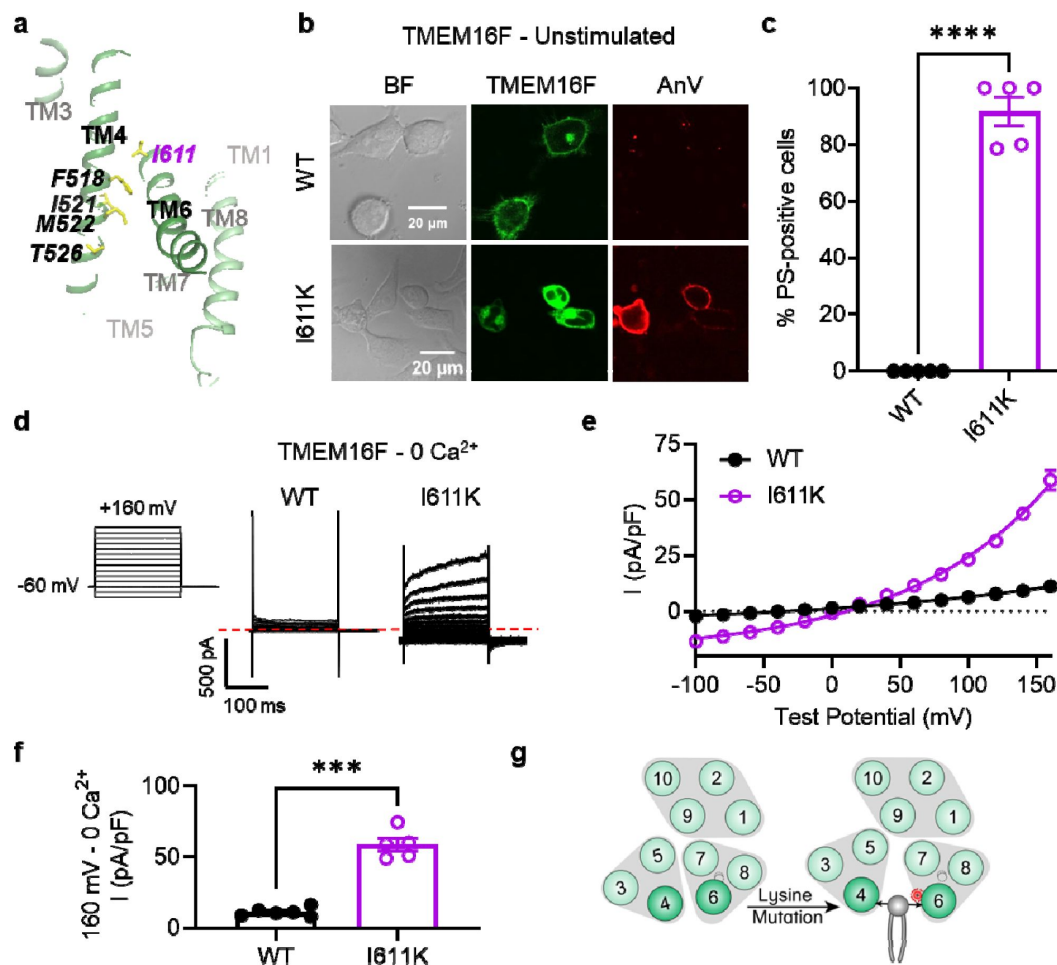


Figure 2

I611K on TM 6 enables TMEM16F channel and scramblase activities in the absence of calcium stimulation.

(a) I611 highlighted on the TMEM16F CaPLSase structure with side chains shown as yellow sticks (PDB 6QPB). (b) Representative images of TMEM16F knockout (KO) HEK293T cells expressing eGFP-tagged TMEM16F wildtype (WT) and I611K (center column). CF 594-conjugated Annexin V (AnV, right column) labelled PS exposing cells. BF denotes bright field images (left column). (c) Quantification of the percentage of cells with AnV labelling for TMEM16F WT (n=5) and I611K (n=5) transfected cells. Values were derived from images of biological replicates, with error bars representing standard error of the mean (SEM). Statistical comparison was done using an unpaired t-test with Welch's correction (****: p<0.0001). (d) Representative current recordings and (e) current-voltage (I-V) relationships of whole cell patches from TMEM16F KO HEK293T cells expressing eGFP-tagged TMEM16F WT (n=6) and I611K (n=5). Currents were elicited by the voltage protocol shown with the pipette solution containing 5 mM EGTA. Dotted line denotes zero current. (f) Quantification of current at +160 mV. Currents in (e) and (f) were normalized to cell capacitance with the mean $\pm$ SEM calculated from independent patches. Statistical comparison was done using an unpaired t-test with Welch's correction (***: p<0.001). (g) A lysine mutation on TM 6 in TMEM16F enables spontaneous phospholipid permeation in the absence of calcium.

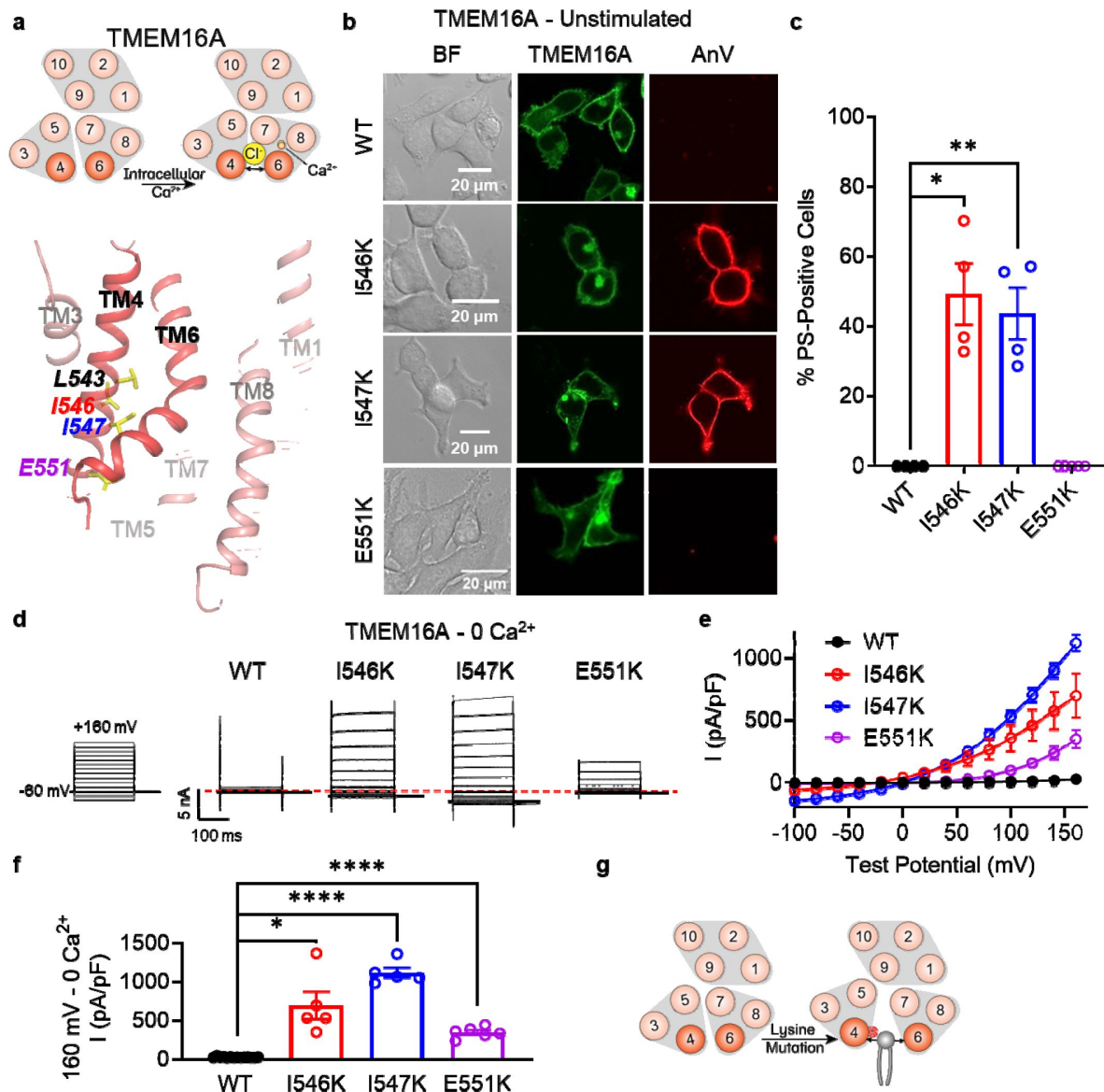


Figure 3

Lysine mutations along TM 4 enable TMEM16A channel and scramblase activities in the absence of calcium stimulation.

(a) Top: TMEM16A is a calcium-activated chloride channel. Bottom: TM 4 mutant locations mapped on TMEM16A CaCC structure with side chains shown as yellow sticks (PDB 5OYG). (b) Representative images of TMEM16F knockout (KO) HEK293T cells expressing eGFP-tagged TMEM16A wildtype (WT), I546K, I547K, and E551K (center column). CF 594-conjugated annexin V (AnV, right column) labelled PS exposing cells. BF denotes bright field images (left column). (c) Quantification of the percentage of cells with AnV labelling for TMEM16A WT (n=4), I546K (n=4), I547K (n=4), and E551K (n=5) transfected cells. Values were derived from images of biological replicates, with error bars representing the standard error of the mean (SEM). Statistical comparisons were done using unpaired t-tests with Welch's correction (*: p<0.05, **: p<0.01). (d) Representative whole-cell current recordings and (e) current-voltage (I-V) relationships of whole cell patches from TMEM16F KO HEK293T cells expressing eGFP-tagged TMEM16A WT (n=14), I546K (n=5), I547K (n=5), and E551K (n=6). Currents were elicited by the voltage protocol shown with the pipette containing 5 mM EGTA. Dotted line denotes zero current. (f) Quantification of current at +160 mV. Currents in (e) and (f) were normalized to cell capacitance with the mean $\pm$ SEM calculated from independent patches. Statistical comparisons were done using unpaired t-tests with Welch's correction (*: p<0.05, ****: p<0.0001). (g) Lysine mutations along TM 4 in TMEM16A enable spontaneous phospholipid permeation in the absence of calcium.

This mirrors our results with both TMEM16F (Fig. 1) and TMEM16A (Fig. 3), suggesting that the L438K mutation allows the OSCA1.2 channel to permeate phospholipids. Next, we used inside-out patch clamp to examine if L438K also enhances OSCA1.2 channel activity. We found that the mutant significantly left-shifts the conductance-voltage (G - V) relationship (Fig. 4e, f) and accelerates channel activation kinetics (Fig. 4g) compared to WT. Under -50 mmHg of pressure, L438K has a half-maximal voltage ($V_{0.5}$) of 66.7 ± 3.7 mV, while the WT $V_{0.5}$ is nearly 108.7 ± 5.6 mV (Fig. 4f), underscoring that this mutation also promotes channel gating. Together, these experiments show that, like TMEM16A, a single lysine mutation near the putative gate allows the mechanosensitive and osmolarity-activated OSCA1.2 channel to become spontaneously permeable to phospholipids (Fig. 4h).

To help visualize how L438K alters the TM 4/6 interface of OSCA1.2, we created a homology model of the mutant using the Swiss-PDB server and inserted it into a phospholipid membrane with the CHARMM-GUI webserver⁴⁷. After equilibration, we ran 900 ns atomistic molecular dynamics (MD) simulations for both WT and L438K in GROMACS, using a combination of conventional and metadynamic simulations. Remarkably, the L438K trajectory showed considerably increased hydration of the pore region compared to WT (Fig. S4b). This enhanced hydration is attributed to a modest expansion of the TM 4/6 interface throughout the simulation (Fig. S4a). Together with our functional results, these MD simulations reinforce our hypothesis that disruption of the TM 4/6 interface of OSCA1.2 promotes GOF ion and phospholipid permeation.

One helical turn above OSCA1.2 L438, at the corresponding site to the activation gate residue F518 in TMEM16F, is an endogenous lysine at position 435 (Fig. S5a). This positioning places two positively charged residues in close proximity within TM 4 of the OSCA1.2 L438K mutant. Previous studies on model transmembrane helical peptides have demonstrated that hydrophilic side chains within the hydrophobic core of the membrane promote trans-bilayer phospholipid transport and this effect is further enhanced when these hydrophilic residues are stacked within the helix⁴⁸. To explore the role of the endogenous K435 in facilitating L438K-mediated spontaneous phospholipid permeability, we assessed PS exposure in the K435L and K435L/L438K mutants. Remarkably, the double mutant K435L/L438K, which shifts the lysine to position 438, resulted in a modest level of spontaneous PS exposure (31%, Fig. S5b, c). This level is intermediate between the WT (K435) with no PS exposure and the L438K mutant (K435/K438) with 63% spontaneous PS exposure (Fig. 4c/ Fig. S5b, c). In contrast, K435L (L435/L438) did not induce significant spontaneous PS exposure (3%, Fig. S5b, c). These findings, consistent with previous studies on model transmembrane peptides⁴⁸, highlight the role of multiple charged residues in TM 4 in promoting phospholipid scrambling. Moreover, our results further support the hypothesis that the instability of the TM 4/6 interface in OSCA1.2 is crucial for controlling phospholipid and ion permeation.

A439K on TM4 converts OSCA1.2 into an osmolarity-sensing scramblase

Interestingly, mutating the neighboring amino acid (Fig. 4a), A439, to lysine resulted in minimal spontaneous PS exposure (10%, Fig. 4b, c). We thus reasoned that A439K scramblase activity may require additional stimulation to trigger scramblase activity. Given that WT OSCA1.2 ion channel activity can be induced by hypotonic treatment (Fig. S6), we acutely treated WT- and A439K-expressing cells with a hypotonic solution (120 mOsm/kg) and assessed scramblase activity using time-lapse imaging. Indeed, PS exposure was robustly induced for the A439K mutant (Fig. 5b, c) but not WT (Fig. 5a, c) in response to hypotonic stimulation. Inside-out patch clamp further demonstrated that A439K enhances OSCA1.2 ion channel activity as evidenced by the accelerated activation kinetics (Fig. 4g) and left-shifted G - V relationship (Fig. 4f). Our experiments thus indicate that A439K modestly disrupts OSCA1.2 gating and converts the osmolarity-activated ion channel (Fig. S6) into an osmolarity-sensing phospholipid scramblase (Fig. 5d).

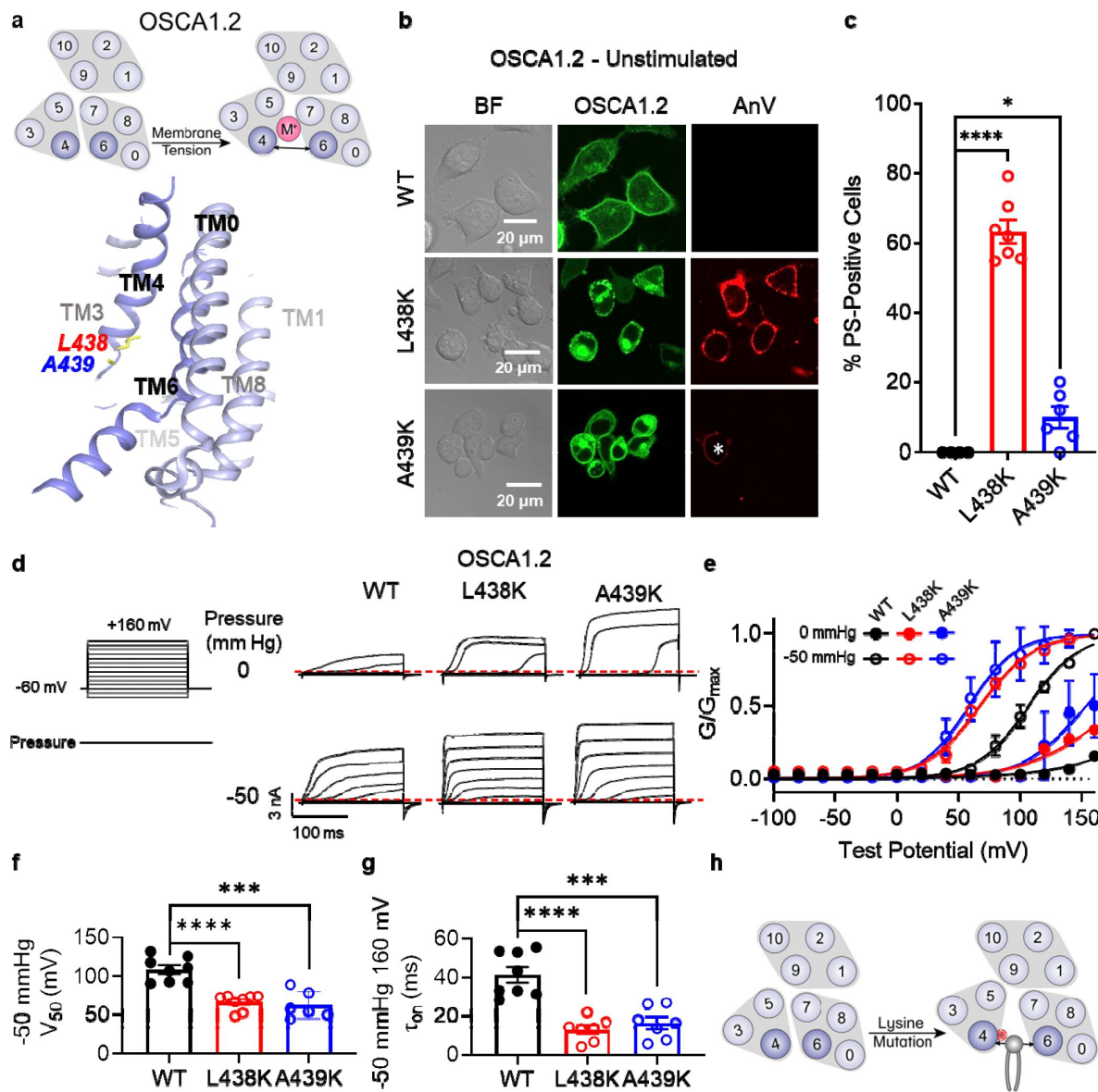


Figure 4

Lysine mutations along TM 4 enable OSCA1.2 channel and scramblase activities.

(a) Top: OSCA1.2 is a cation non-selective ion channel gated by membrane tension. Bottom: TM 4 mutant locations mapped onto the TM 4/6 interface of OSCA1.2 (PDB 6MGV) with key residues shown as yellow sticks. (b) Representative images of TMEM16F KO HEK293T cells expressing eGFP-tagged OSCA1.2 WT, L438K, or A439K mutants (center column). CF 594-conjugated AnV (right column) labels PS-exposing cells. BF denotes bright field images (left column). Asterisk highlights a PS positive cell for the A439K mutant. (c) Quantification of the percentage of cells with AnV labelling for OSCA1.2 WT (n=4), L438K (n=7), and A439K-transfected cells (n=6). Statistical comparisons were conducted with unpaired t-tests with Welch's correction (*: $p < 0.05$, ****: $p < 0.0001$). (d) Representative current recordings and (e) normalized conductance-voltage (G-V) relationships of inside-out patches from TMEM16F KO HEK293T cells expressing eGFP-tagged OSCA1.2 WT (n=8), L438K (n=8), and A439K (n=6). Currents were elicited by the voltage protocol shown next to the listed pressures. Dotted lines denote zero current. (f) Quantification of half-maximal voltage at -50 mmHg for WT (109 mV), L438K (67 mV), and A439K (63 mV). Error bars represent standard error of the mean (SEM) calculated from independent patches. Statistical comparison was conducted with unpaired t-tests with Welch's correction (***: $p < 0.001$, ****: $p < 0.0001$). (g) Quantification of activation T_{on} at -50 mmHg and 160 mV for WT (41 ms), L438K (13 ms), and A439K (16 ms). Error bars represent standard error of the mean (SEM) calculated from independent patches. Statistical comparison was conducted with unpaired t-tests with Welch's correction (***: $p < 0.001$, ****: $p < 0.0001$). (h) A lysine mutation along TM 4 converts the OSCA1.2 channel into a phospholipid scramblase with spontaneous phospholipid permeability.

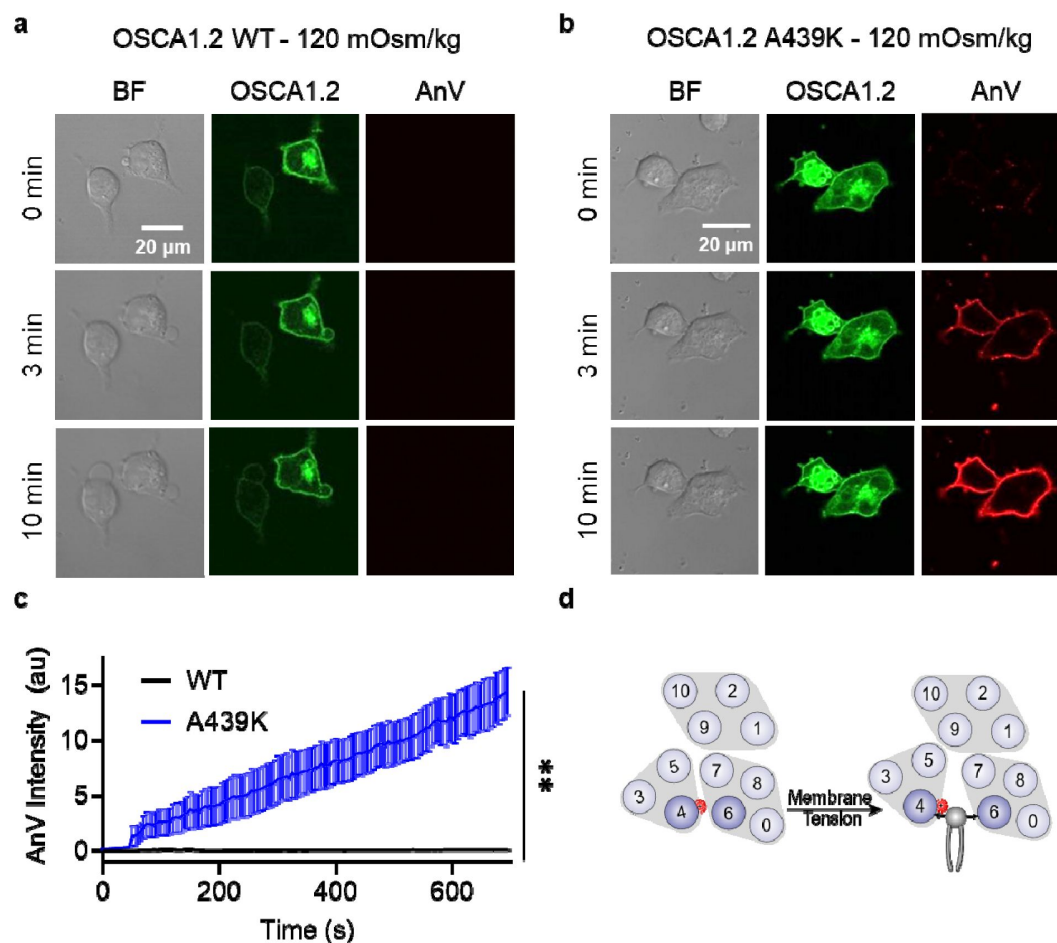


Figure 5

OSCA1.2 A439K is an osmolarity-activated scramblase.

(a-b) Representative images of hypotonic osmolarity stimulation of TMEM16F KO HEK293T cells expressing eGFP-tagged OSCA1.2 (a) WT or (b) the A439K mutant (center columns). CF 594-conjugated AnV (right columns) labelled PS exposing cells. BF denotes bright field images (left columns). Each row of representative images corresponds to the indicated time after hypo-osmotic stimulation. (c) Quantification of AnV intensity for OSCA1.2 WT (n=5) and A439K (n=5) after hypo-osmotic stimulation. Statistical comparison was conducted with an unpaired t-test with Welch's correction (**: $p < 0.01$). (d) The A439K mutation converts OSCA1.2 to an osmolarity-activated phospholipid scramblase.

TM 4 lysine mutations convert TMEM63A into a constitutively active scramblase

Finally, we turned our attention to TMEM63A to further investigate the evolutionary conservation of our observation that TM 4 lysine mutations convert TMEM16 and OSCA members into scramblases. TMEM63s represent the mammalian members of the OSCA/TMEM63 family with three members present in humans (TMEM63A-C). Recent structural and functional characterizations indicate that TMEM63s function as mechanosensitive ion channels gated by high-threshold membrane tension and, in notable contrast to all other structurally resolved TCS superfamily members, they likely function as monomers^{49,51}. Given their structural homology to TMEM16s and OSCA1.2⁴⁹, we hypothesized that TM 4 mutations in TMEM63A would also result in GOF activity. We again identified residues near the putative gate by structural (Fig. 6a) and sequence alignment (Fig. S1f), selecting W472 (equivalent to F518 in TMEM16F, L543 in TMEM16A, and K435 in OSCA1.2), S475 (equivalent to I521 in TMEM16F, I546 in TMEM16A, and I438 in OSCA1.2), and A476 (equivalent to M522 in TMEM16F, I547 in TMEM16A, and A439 in OSCA1.2). Indeed, overexpressing eGFP-tagged mouse TMEM63A with single lysine mutations at either W472 or S475 led to spontaneous PS exposure (Fig. 6b, c), though the AnV staining revealed punctate rather than global patterns of PS exposure induced by their OSCA1.2 counterparts. Notably, A476K failed to show obvious PS exposure (Fig. 6b, c), even after 120 mOsm/kg hypotonic treatment (Fig. S7b, c), perhaps reflecting differences in mechanical pressure threshold between OSCA1.2 and TMEM63A⁴⁹. To confirm membrane localization and further probe mutant effects, we exploited TMEM63A ion channel function using the cell-attached patch clamp configuration. As TMEM63A exhibits voltage-dependent activity under high pressure, we compared mutant and WT *I-V* relationships at -80 mmHg. W472K, S475K, and A476K caused marked reductions in $V_{0.5}$ from 122.3±3.5 mV for WT to 95.9±6.3 mV, 92.1±8.6 mV, and 96.8±4.2 mV respectively (Fig. 6d-f). By comparison, mock-transfected controls failed to elicit current (Fig. 6d, e). Together, these results are consistent with our observations in TMEM16F, TMEM16A, and OSCA1.2, indicating that single lysine mutations along TM 4 of TMEM63A can facilitate both ion and phospholipid permeation (Fig. 6g).

Discussion

Mechanistically, our study improves models of TMEM16 substrate permeation and gating. We identified multiple mutations in TMEM16F and TMEM16A that promote phospholipid permeation and cause commensurate changes in ion channel activities. Our findings complement functional characterizations of TMEM16F gating mutants where gate destabilization is inversely correlated with side chain hydrophathy^{26,52}. Lysine is above only arginine on the hydrophathy index and thus likely explains why it readily destabilizes the gate. For instance, F518K exhibits spontaneous PS exposure, even when the calcium binding site is destroyed²⁶, whereas F518H does not exhibit spontaneous PS exposure⁵². Interestingly, the recent TMEM16F F518H structure (Fig. S1e) shows local membrane thinning due in part to unexpected conformational changes in TM 3⁵². Future structural studies are needed to assess whether TMEM16A or OSCA/TMEM63 mutant scramblases also promote membrane thinning and/or conformational rearrangements in TM 3. More broadly, our results highlight an increasingly appreciated design principle of scramblases where polar and charged residues often line a membrane-spanning groove. This observation has been noted for TMEM16^{53,41,54}, Xkr^{55,56}, and opsin⁵⁷ scramblases and should be a key criterion for identifying and characterizing new scramblases.

Our findings also advance our understanding of evolutionary relatives of the TCS superfamily¹⁶ and help uncover their roles in human diseases. Although OSCA/TMEM63 proteins are not known to scramble phospholipids and we did not detect obvious hypotonicity-induced PS exposure in

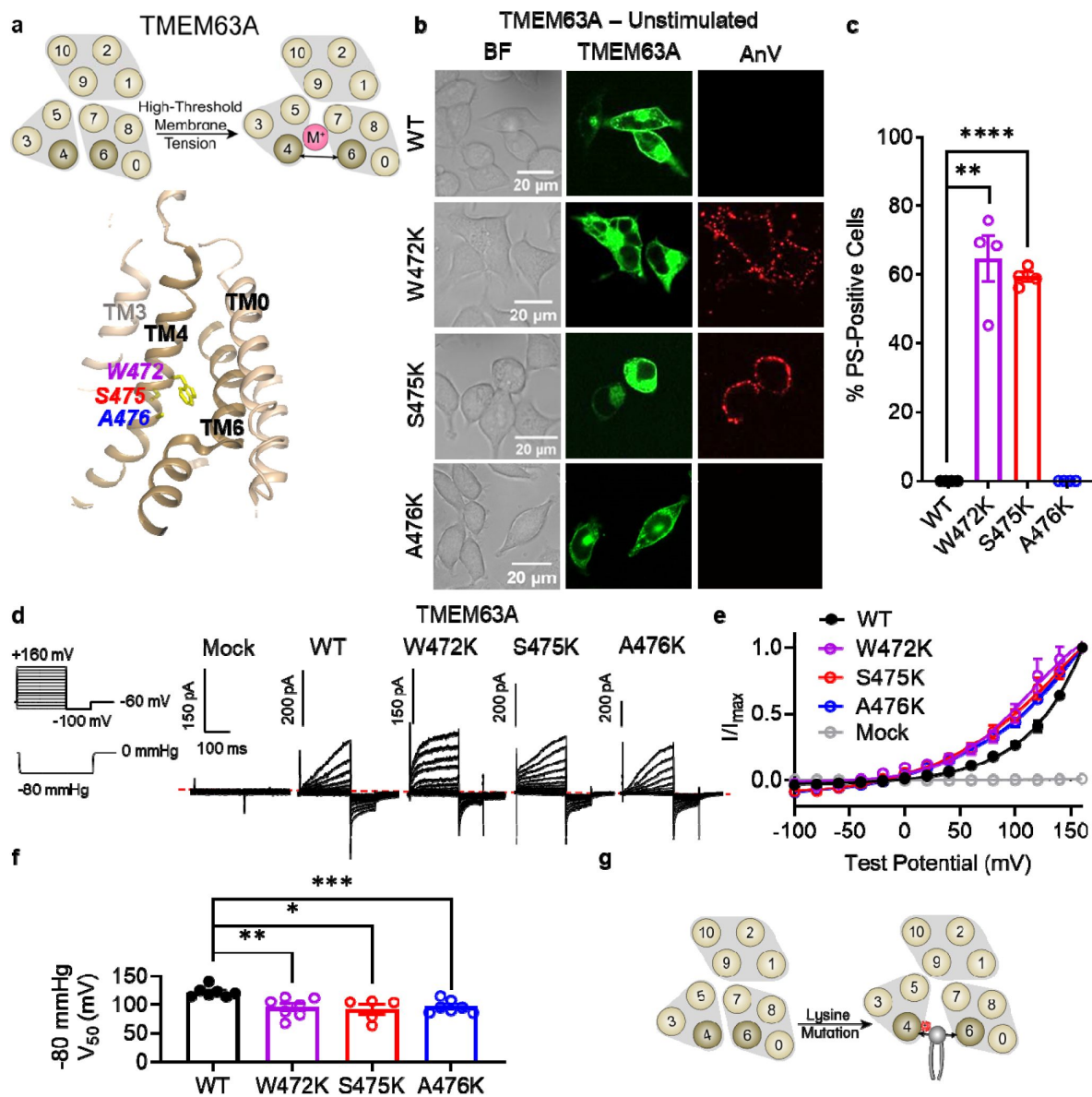


Figure 6

Lysine mutations along TM 4 enable TMEM63A channel and scramblase activities.

(a) Top: TMEM63A is an ion channel gated by high threshold membrane tension. Bottom: the TM 4/6 interface of *Hs*TMEM63A (PDB 8GRS) with key residues shown as yellow sticks using amino acid numbering corresponding to the mouse ortholog. (b) Representative images of TMEM16F KO HEK293T cells expressing eGFP-tagged TMEM63A WT, W472K, S475K, and A476K (center column). CF 594-conjugated AnV (right column) labelled PS exposing cells. BF denotes bright field images (left column). (c) Quantification of the percentage of cells with AnV labelling for TMEM63A WT (n=4), W472K (n=4), S475K (n=4), and A476K-transfected cells (n=4). Statistical comparisons were conducted with unpaired t-tests with Welch's correction (**: p<0.01, ****: p<0.0001). (d) Representative current recordings and (e) normalized conductance-voltage (I-V) relationships of cell attached patches from TMEM16F KO HEK293T cells expressing either eGFP mock-transfected (n=7) or eGFP-tagged TMEM63A WT (n=7), W472K (n=7), S475K (n=5), or A476K (n=7). Currents represent the subtraction of voltage alone from currents elicited by the voltage and pressure protocols shown. Dotted line denotes zero current. Note that the mock control was normalized to the mean maximal current elicited from WT-transfected cells. (f) Quantification of half-maximal voltage at -80 mmHg for WT (122 mV), W472K (96 mV), S475K (92 mV), and A476K (97 mV). Error bars represent standard error of the mean (SEM) calculated from independent patches. Statistical comparison was conducted with unpaired t-tests with Welch's correction (*: p<0.05, **: p<0.01, ***: p<0.001). (g) Lysine mutations along TM 4 in TMEM63A enable spontaneous phospholipid permeability.

cells overexpressing WT OSCA1.2 (Fig. 5a) or TMEM63A (Fig. S7a), we show that single mutations in TM 4 of OSCA1.2 and TMEM63A convert these osmolarity-activated and/or mechanosensitive ion channels into phospholipid scramblases, similar to our findings with TMEM16A mutants. We thus speculate that the conserved structural architecture in the transmembrane region endows TCS proteins with a potential to scramble phospholipids, though this capability may have been lost by some members during evolution. This hypothesis is especially intriguing given the recent OSCA open state structures, which detail dramatic conformational rearrangements of TMs 3-6 leading to a phospholipid-lined (or proteolipidic) pore near TMs 4 and 6⁵⁸. It will be interesting to test whether equivalent mutations can convert transmembrane channel-like (TMC) proteins—the third TCS relative of TMEM16 and OSCA/TMEM63¹⁶—into phospholipid scramblases. TMC1 is best known for its role in auditory sensation, and thus far has mostly been characterized *in vivo* due to expression difficulties in heterologous systems⁵⁹. However, recent *in vivo* characterization of mouse TMC1 M412K, known as the *Beethoven* mutation, provided an important insight⁶⁰. The deafness-associated mutation is located in TM 4 (Fig. S1f; equivalent of M522 in TMEM16F, I547 in TMEM16A, A439 in OSCA1.2, and A476 in TMEM63A) and results in constitutive PS exposure when expressed in the hair cell membranes of both heterozygous and homozygous mice⁶⁰. This raises the intriguing possibility that the *Beethoven* mutation may enable TMC1 to spontaneously permeate phospholipids, leading to a loss of membrane homeostasis and ultimately, auditory sensation⁶¹. The possibility of converting TMC proteins into phospholipid scramblases should be thoroughly investigated. Additionally, disease-associated mutations in TMEM63 proteins are present along the TM 4/6 interface, such as TMEM63B T481N⁴⁹. We speculate that introducing more hydrophobic side chains along this interface may lead to spontaneous ion and or phospholipid permeability, perhaps contributing to underlying pathophysiology.

Methods

Cloning and mutagenesis

All constructs used a pEGFP-N1 vector backbone. GFP mock controls used the empty pEGFP-N1 vector. Wild type sequence and mutation numbers correspond to NCBI: NP_780553.2 (*Mus musculus* TMEM16F) with a three amino acid (MQM) N-terminal truncation, NCBI: NP_001229278 (*Mus musculus* TMEM16A), GenBank: AIU34614.1 (*Arabidopsis thaliana* OSCA1.2), and NCBI: NP_001404481.1 (*Mus musculus* TMEM63A). AtOSCA1.2 and MmTMEM63A cDNAs were subcloned using In-Fusion Snap Assembly (Takara, #638947). Point mutants were generated by PCR site-directed mutagenesis with primers from IDT DNA Technologies. Sequences were confirmed by Sanger sequencing (Azenta).

Bioinformatics

The following sequences were obtained from UniProt and aligned using Clustal Omega: Q8BHY3-2 (MmTMEM16A), Q9NQ90 (HsTMEM16B), Q9BYT9 (HsTMEM16C), Q32M45 (HsTMEM16D), Q75V66 (HsTMEM16E), Q6P9J9 (MmTMEM16F), Q6IWH7 (HsTMEM16G), Q9HCE9 (HsTMEM16H), A1A5B4 (HsTMEM16J), Q9NW15 (HsTMEM16K), C7Z7K1 (NhTMEM16), Q4WA18 (AtTMEM16), Q9XEA1 (AtOSCA1.1), Q5XEZ5 (AtOSCA1.2), B5TYT3 (AtOSCA1.3), A0A097NUQ0 (AtOSCA1.4), A0A097NUS0 (AtOSCA1.5), A0A097NUP1 (AtOSCA1.6), A0A097NUP8 (AtOSCA1.7), A0A097NUQ2 (AtOSCA1.8), A0A097NUQ5 (AtOSCA2.1), A0A097NUS5 (AtOSCA2.2), A0A097NUP6 (AtOSCA2.3), A0A097NUQ3 (AtOSCA2.4), A0A097NUQ7 (AtOSCA2.5), Q9C8G5 (AtOSCA3.1), A0A097NUT0 (AtOSCA4.1), Q91YT8 (MmTMEM63A), Q5T3F8 (HsTMEM63B), and Q9P1W3 (HsTMEM63C), Q8R4P5 (MmTMC1), Q8R4P4 (MmTMC2), Q7TQ69 (MmTMC3), Q7TQ65 (MmTMC4), Q32NZ6 (MmTMC5), Q7TN60 (MmTMC6), Q8C428 (MmTMC7), and Q7TN58 (MmTMC8). A subset of the alignment was selected for Fig. S1. Structural models were obtained from the PDB, aligned, and visualized using Pymol (Schrödinger).

Cell culture

The HEK293T cell line was authenticated by the Duke Cell Culture Facility. The TMEM16F KO HEK293T cell line was generated by the Duke Functional Genomics Core and characterized in previous studies^{25,26}. All cells were cultured with DMEM (Gibco, #11995-065) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, # F2442) and 1% penicillin/streptomycin (Gibco, #15-140-122) at 37°C in 5% CO₂-95% air.

Transfection

Plasmids were transiently transfected into TMEM16F KO HEK 293T cells by using X-tremeGENE9 (MilliporeSigma), X-tremeGENE360 (MilliporeSigma), or Lipofectamine 2000 (Thermo Fisher). Media was replaced with calcium free DMEM (Gibco, 21068-028) 3-4 hours after transfection. The transfected cells were imaged or patched 18-24 or 18-48 hours after transfection, respectively.

Fluorescence imaging of scramblase-mediated PS Exposure

A Zeiss 780 inverted confocal microscope was used to monitor scramblase activity in live cells using the methods described in previous publications^{12,25,26}. 18-24 hours after transfection, the cells were incubated in AnV buffer (1:175 dilution of the fluorescently tagged AnV (Biotium) in Hank's balanced salt solution) immediately before and throughout the duration of the imaging experiment without a formal incubation period. Spontaneous PS positive cells were readily labeled by fluorescently tagged AnV. Results were quantified as a percentage of PS positive cells among all GFP positive cells. For osmolarity activation, 2 mM CaCl₂ in ddH₂O was added to the AnV buffer at a 2:1 ratio. The final osmolarity was ~120 mOsm/kg as measured by a micro-osmometer (Advanced Instrument). Cells overexpressing WT or mutant were treated with low osmolarity AnV buffer, and the scramblase activity was measured by recording fluorescent AnV surface accumulation at 5 (Fig. 5) or 7 (Fig. S7) second intervals. A custom MATLAB code was used to quantify AnV signal and is available at Github (https://github.com/yanghuanghe/scrambling_activity).

Electrophysiology

All electrophysiology recordings were conducted using an Axopatch 200B amplifier with the signal digitally sampled at 10 kHz using an Axon Digidata 1550A (Molecular Devices, Inc.). All electrophysiology recordings were carried out at room temperature 18-48 hours after transfection. Glass pipettes were pulled from borosilicate capillaries (Sutter Instruments) and fire-polished using a microforge (Narishige). Pipettes had resistances of 2–4 MΩ in physiological bath solution.

Inside-out patch clamp recordings

The pipette solution (external) contained 140 mM NaCl, 10 mM HEPES, 2 mM MgCl₂, adjusted to pH 7.3 (NaOH), and the bath solution (internal) contained 140 mM NaCl, 10 mM HEPES, 5 mM EGTA, adjusted to pH 7.3 (NaOH). OSCA1.2 WT and mutations were held at constant pressure administrated using a syringe calibrated with a manometer, similar to a previous study⁶². In our experience, the constant pressure and variable voltage protocol achieved more consistent measurements. Patches were held at a membrane potential of -60 mV and at the indicated pressure, then stimulated using the indicated voltage protocol, taking advantage of the larger OSCA1.2 currents elicited by depolarizing potentials.

Cell-attached patch clamp recordings

For TMEM63 recordings, cell-attach mode was used instead of inside-out to avoid breaking the giga-ohm seal under higher applied pressure and voltage. The bath solution contained (in mM): 140 KCl, 10 HEPES, 2 MgCl₂, 10 glucose, pH 7.3 adjusted with KOH. The pipette solution contained

(in mM): 130 NaCl, 5 KCl, 10 HEPES, 10 TEA-Cl, 1 CaCl₂, 1 MgCl₂, pH 7.3 (with NaOH). The mechano-activated current was evoked with a 200 ms pressure pulse at -80 mmHg using a high-speed pressure clamp system (HSPC-1, ALA Scientific Instruments, Farmingdale, NY). The membrane potential inside the patch was held at -60 mV. The voltage pulse alone was run first followed by voltage pulse with pressure. The mechanosensitive current was obtained by subtracting the voltage pulse from the voltage pulse with pressure.

Whole cell TMEM16 patch clamp recordings

The pipette solution (internal) contained 140 mM CsCl, 10 mM HEPES, 5 mM EGTA, adjusted to pH 7.3 (CsOH), and the bath solution (external) contained 140 mM NaCl, 10 mM HEPES, 5 mM EGTA, adjusted to pH 7.3 (NaOH). Patches were held at a membrane potential of -60 mV, then stimulated using the indicated voltage protocol.

Whole cell hypotonic patch clamp recordings

The pipette solution (internal) contained 140 mM Na gluconate, 10 mM HEPES, 1 mM MgCl₂, and 0.2 mM EGTA, adjusted to pH 7.3 (NaOH). Cells seeded on a small section of cover glass were placed in bath solution (external) containing 140 mM Na gluconate, 10 mM HEPES, 1 mM MgCl₂, and 0.2 mM EGTA adjusted to pH 7.3 (NaOH). Patches were held at a membrane potential of -60 mV, then stimulated using the indicated voltage protocol using a 2-second sweep protocol. After 5 sweeps, ddH₂O was slowly and gently added to the bath at a ratio of 2:1 using a hypodermic needle to induce hypotonic cell swelling. The sweep protocol continued for a minimum of 4 minutes after hypotonic stimulation.

Data analysis for electrophysiology

All data analysis was performed using Clampfit (Molecular Devices), Excel (Microsoft), MATLAB (MathWorks), and Prism softwares (GraphPad). Individual *G-V* curves were fitted with a Boltzmann function,

$$G = \frac{G_{max}}{1 + e^{\frac{-ZF(V-V_{0.5})}{RT}}} \quad (2)$$

where G_{max} denotes the fitted value for maximal conductance, $V_{0.5}$ denotes the voltage of half maximal activation of conductance, Z denotes the net charge moved across the membrane during the transition from the closed to the open state, and F denotes the Faraday constant.

Model building and MD simulations

The initial model of OSCA1.2 for atomistic MD simulations and homology modeling was constructed based on PDB: 6MGV. Missing segments were modeled using the Swiss-PDB server (<https://swissmodel.expasy.org/>). The OSCA1.2 L438K mutation structure was modeled using 6MGV as the template, and the RMSD between the mutation and the original structure of 6MGV was less than 0.5 Å. To mimic the state of transmembrane proteins in the plasma membrane, the model was inserted into a lipid membrane consisting of POPC (exoplasmic leaflet) and 1:1 POPS/POPE (cytoplasmic leaflet) using the CHARMM-GUI web server⁴⁷. To neutralize charge levels in the simulation system, 150 mM KCl solution was added. The final simulation boxes contained ~600 lipid molecules, with ~290,000 atoms. During the simulation, the CHARMM36m all-atom force field was used. The temperature was maintained at 310 K to maintain the fluidity of lipids. During the atomistic simulation, long-range electrostatic interactions were described by the Particle Mesh Ewald (PME) algorithm with a cutoff of 12 Å. Van der Waals interactions were cut off at 12 Å and the MD time step was set at 2 fs. All atomistic MD simulations were conducted using GROMACS-2022.3 software package, and enhanced sampling MD simulations were conducted using GROMACS-2022.3 with the COLVARS module. Before the production process, the system

performed 5,000 steps of energy minimization. Subsequently, all systems were equilibrated using the default equilibration parameters provided by CHARMM-GUI. After equilibration, the velocities of all atoms were randomly generated, and the product simulation system consisted of a 2-stage MD simulation. In step 1, the system used a conventional MD simulation run for 200 ns. In step 2, the system used a metadynamics simulation run for ~700 ns. During enhanced sampling MD simulations, the sampling interval of the collective variable was adjusted to adapt the lipid movement trajectory. VMD and PYMOL were used to analyze simulation trajectories and water occupancy.

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Author contributions

H.Y. and Y.Z. conceived and H.Y. supervised the project. Y.Z. and A.J.L. imaging. P.L. and A.J.L. electrophysiology. A.J.L. sequence and structure alignments. A.J.L. and Y.C.S.W. cloning and mutagenesis. Z.P. plasmids. Y.Z. MATLAB codes. M.S. MD simulations, supervised by Y.Z. A.J.L. and H.Y. drafted the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

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Editors

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Reviewer #1 (Public review):

Summary:

TMEM16, OSCA/TMEM63, and TMC belong to a large superfamily of ion channels where TMEM16 members are calcium activated lipid scramblases and chloride channels, whereas OSCA/TMEM63 and TMCs are mechanically activated ion channels. In the TMEM16 family, TMEM16F is a well characterized calcium activated lipid scramblase that play an important role in processes like blood coagulation, cell death signaling, and phagocytosis. In a previous study the group has demonstrated that lysine mutation in TM4 of TMEM16A can enable the calcium activated chloride channel to permeate phospholipids too. Based on this they

hypothesize that the energy barrier for lipid scramblase in these ion channels is low, and that modification in the hydrophobic gate region by introducing a charged side chain between TM4/6 interface in TMEM16 and OSCA/TMEM63 family can allow lipid scramblase. In this manuscript, using scramblase activity via Annexin V binding to phosphatidylserine, and electrophysiology, the authors demonstrate that lysine mutation in TM4 of TMEM16F and TMEM16A can cause constitutive lipid scramblase activity. The authors then go on to show that analogous mutations in OSCA1.2 and TMEM63A can lead to scramblase activity. The revised version does a thorough characterization of residues that form the hydrophobic gate region in TM4/6 of this superfamily of channels. Their results indicated that disrupting the TM4/6 interaction can reduce energy barrier for this channels to scramblase lipids.

Strengths:

Overall, the authors introduce an interesting concept that this large superfamily can permeate ions and lipids.

Weaknesses:

none noted in the revised version.

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Reviewer #2 (Public review):

This focused study by Lowry and colleagues that identifies a key molecular motif that controls ion permeation vs combined ion permeation and lipid transport in three families of channel/scramblase proteins, in TMEM16 channels, in the plant-expressed and stress-gated cation channel OSCA, and in the mammalian homolog and mechanosensitive cation channel, TMEM63. Between them, these three channels share low sequence similarity and have seemingly differing functions, as anion (TMEM16 channels), or stress-activated cation channels (OSCA/TMEM63). The study finds that in all three families, mutating a single hydrophobic residue in the ion permeation pathway of the channels confers lipid transport through the pores of the channels, indicating that TMEM16 and related OSCA and TMEM63 channels have a conserved potential for both ion and lipid permeation. The authors interpret the findings as revealing that these channel/scramblase proteins have a relatively low "energetic barrier for scramblase" activity. The experiments are done with a high level of rigor and the revised paper is very well written and addresses the previous concerns.

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Reviewer #3 (Public review):

This study was focused on the conserved mechanisms across the Transmembrane Channel/Scramblase superfamily, which includes members of the TMEM16, TMEM63/OSCA, and TMC families. In previous work, the authors have studied the role of the inner activation gate of these proteins. Here, the authors show that the introduction of mutations at the TM4-TM6 interface, which are close to the inactivation gate, can disrupt gating and confer scramblase activity to non-scramblases proteins.

Overall, the confocal imaging experiments, patch clamping experiments, and data analysis are performed well and in line with standard methods. The molecular dynamics simulation work is focused but adds supportive evidence to their findings. Although there could have been more extensive molecular analysis to bolster the authors' arguments on the role of the TM4-TM6 interface (e.g. evaluate effects of size/hydrophobicity, double mutants, cross-linking, more in-depth simulation data), there is adequate evidence to conclude that certain residues

at this interface is critical to ion conduction and phospholipid scramblase activity. The data presented only adds incremental depth of knowledge for each individual channel, but together, they show this to be true for conserved TM4 residues across TMEM16F, TMEM16A, OSCA1.2, and TMEM63A proteins. This breadth of data is a major strength of this paper, and provides strong evidence for a coupled pathway for ion conduction and phospholipid transport, though the underlying biophysical mechanism is still speculative and remains to be elucidated.

<https://doi.org/10.7554/eLife.96957.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Recommendations For The Authors):

Figures 1 and 2. How do the authors know that the lysine mutations are specific to constitutive activity and not because it is causing the channel to be now voltage sensitive?

As shown in the revised Figs. 1b, S2a, and 3b, TMEM16F I521K/M522K, TMEM16F I521E, and TMEM16A I546K/I547K spontaneously expose PS, respectively. Neither membrane depolarization nor calcium stimulation was introduced under these conditions and the cells were grown in calcium-free media after transfection to limit calcium-dependent activation. Our new experiments further demonstrate that TMEM16F T526K (Fig. 1b) and TMEM16A E551K (Fig. 3b), which are further away from the activation gate, exhibit either strongly attenuated or lack spontaneous lipid scrambling activity. According to these results, the gain-of-function mutants (TMEM16F

I521K/M522K/I521E and TMEM16A I546K/I547K) are indeed constitutively active. This constitutive scramblase activity is not due to a gain of voltage sensitivity as ion channel activity is also minimal around the resting membrane potential of a HEK cell (Fig. 1d, e and Fig. 3d, e).

The authors see very large currents of 5 -10 nA in their electrophysiology experiments in Figures 2D and 3D. I understand that Figure 2D are whole-cell recordings but are the authors confident that the currents that they are recordings from the mutants are indeed specific to TMEM16A. More importantly, in Figure 3D they see 3-5nA currents in insideout patches, which is huge. They have no added divalent in their bath solution, which could lead to larger single-channel amplitudes, but 3-5nA seems excessive. Some control to demonstrate that these are indeed OSCA1.2 currents is important.

TMEM16A and TMEM16F are well-known for their high cell surface expression. Therefore, the current amplitude is usually huge even in excised inside-out or outside-out patches—please see our previous publications for details: 1) 10.1016/j.cell.2012.07.036, 2) 10.7554/eLife.02772, 3) 10.1038/s41467-019-11784-8, 4) 10.1038/s41467-019-09778-7, 5) 10.1016/j.celrep.2020.108570, 6) 10.1085/jgp.202012704, and 7) 10.1085/jgp.202313460.

HEK293 cells do not have endogenous TMEM16A (<https://doi.org/10.1038/nature07313>, 10.1016/j.cell.2008.09.003 , DOI: 10.1126/science.1163518). It therefore serves as a widely used cell line for studying TMEM16A biophysics. As overexpressing the WT control barely elicited any obvious current in 0 Ca²⁺ (Fig. 3d), there is no doubt that the large outward-rectifying current (hallmark of CaCC) in the revised Fig. 3d (previous Fig. 2D) was elicited from the mutant TMEM16A channels. The strong outward rectification also rules out the possibility of this being leak current.

Regarding Fig. 4d (previous Fig. 3D), OSCA1.2 has excellent surface expression as shown in Fig. 4b. OSCA1.2 also has much higher single channel conductance (121.8 ± 3.4 pS, 10.7554/eLife.41844) than TMEM16A (~3-8 pS) and TMEM16F (<1 pS). Therefore, recording nA OSCA1.2 current from excised patches is normal given larger OSCA1.2 current at depolarized voltages than the current recorded at hyperpolarized voltages (please see our explanation in the next response). As the reviewer pointed out, lack of divalent ions in our experimental conditions may also partially contribute to the large conductance. To further verify, we conducted mock transfection recordings (please see Author response image 1 below). WT- but not mock (GFP)transfected cells gave rise to large current, further supporting that the recorded current was indeed through OSCA1.2.

Author response image 1.

Representative inside-out currents for mock (GFP)- and OSCA1.2 WT-transfected cells. OSCA1.2 is responsible for nA currents elicited by the pressure and voltage protocols shown.

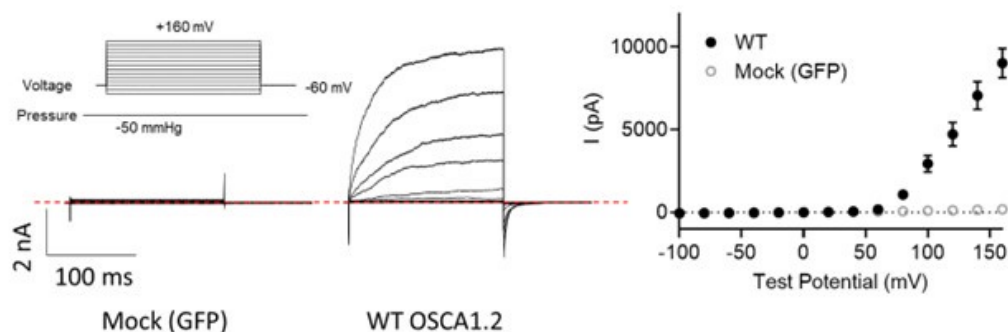
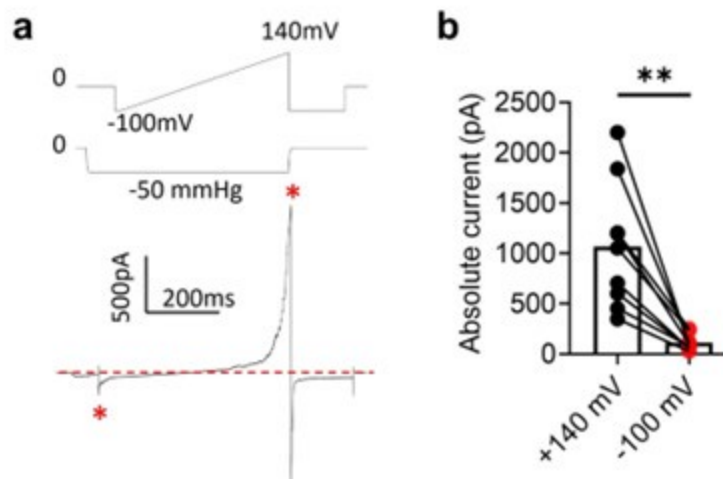


Figure 3D and 5D. Most of the traces and current quantification is done at positive potentials and is outward current. Do the authors observe inward currents? It is difficult to judge by the figures since currents are so large. OSCA/TMEM63s are cationic channels and all published data on these channels have demonstrated robust inward currents at negative, physiologically relevant potentials. The lack of inward currents but only large outward currents suggests that these mutations could be doing something else to the channel.

Yes. We indeed observe inward current at negative holding potentials under pressure clamp (Author response image 2). However, mechanosensitive OSCA and TMEM63A channels are also voltage dependent. Their outward current is an order of magnitude larger at depolarized voltages (e.g., Author response image 2, also 10.7554/eLife.41844, see Fig. 1H).

Author response image 2.

Voltage-dependent rectification of OSCA1.2 current. a. Representative OSCA1.2 trace (bottom) elicited by a voltage-ramp under -50 mmHg (top). b. The difference in inward and outward current amplitudes.



We found that quantifying the OSCA1.2 outward current has advantages over the inward current. Usually, using the gold standard pressure clamp protocol at negative holding voltages, peak inward current amplitude is quantified. However, OSCA inward current quickly inactivates (10.7554/eLife.41844, see Fig. 1C). This makes robust quantification and comparison with mutant channels difficult. Holding the membrane at a constant pressure and measuring OSCA1.2 G-V overcomes these issues associated with the classical inward current measurements. The large depolarization-driven outward current does not inactivate, and robust tail current (Response Fig. 1, 2) allows us to construct G-V relationships. We found quantifying mutants' voltage dependence at constant pressure is more consistent than quantifying pressure dependence at constant voltage. These advantages make our new protocol preferable to the commonly used gold standard pressure clamp protocol for characterizing and comparing the gating mutations identified in this manuscript.

Figure 3 and 5. Why are mechanically activated currents being recorded at random pressure stimuli (-50 mmHg for OSCA) and (-80 mmHg for Tmem63a)? The gold standard in the field is to run an entire pressure response curve. Given that only outward currents are observed at membrane potentials +120mV and above at 0mmHg, this questions whether they are indeed constitutively active.

As we explained in the previous response, both voltage and membrane stretch activate OSCA/TMEM63A channels. We found measuring voltage dependence under constant pressure provided more consistent quantification than the gold standard pressure response protocol. This may be due to the variability of applied membrane tension under repeated stretches versus the more consistent applied voltage. Additionally, we chose -50 mmHg and -80 mmHg to reflect the reported differences in half-maximal pressures between OSCA1.2 and TMEM63A (e.g., P50 ~55 mmHg for 1.2 and ~61 mmHg for 63A in 10.7554/eLife.41844 versus ~86 mmHg for 1.2 and -123 mmHg for 63A in 10.1016/j.neuron.2023.07.006).

We also used higher pressure in cell attached mode to increase TMEM63A current amplitudes, which are usually tiny. We have updated our method section (Lines 329334) to further clarify why we used these protocols.

Please note that in TMEM16 proteins, ions and lipids might not always co-transport.

This means that under certain conditions, only one type of substrate may go through. For instance, in WT TMEM16F, Ca²⁺ stimulation can easily trigger PS exposure at resting membrane potential. No ionic currents are elicited until strong depolarization is applied. Similarly, the TMEM16F GOF mutations spontaneously transport lipids, leading to loss of lipid

asymmetry (Fig. 1b, c). However, in 0 Ca²⁺, these TMEM16F mutant channels still need strong depolarization for ion conduction (Fig. 1d, e). Although the detailed mechanism still needs to be further investigated, the OSCA1.2 and TMEM63A GOF mutations share similar features with TMEM16 proteins, exhibiting ion conduction under high pressures and depolarizing voltages, yet constitutively active scrambling.

Some clarity is needed for their choice of residues. I understand that a lot of this is also informed by the structures of these ion channels. According to the alignment shown in Supplementary Figure 1, they chose LA for OSCA1.2, which is in line with the IM (TMEM16F) and II(TMEM16A) residues but for Tmem63a they chose the hydrophobic gate residue W and S. Was the A476 tested? Also, OSCA1.2 already has a K in the hydrophobic gating residue region. How do the authors reconcile this with their model?

We appreciate this critical comment. We have included the characterization of TMEM63A A476K (Fig. 6, corresponding to M522 in 16F, I547 in 16A, and A439 in OSCA1.2). Interestingly, A476K transfected cells did not show obvious spontaneous PS exposure yet exhibited a modest shift in V₅₀ comparable to W472K and S475K. These differences may reflect the high-tension activated nature of the TMEM63 proteins (10.1016/j.neuron.2023.07.006) as compared to OSCA1.2, where the corresponding mutation (A439K, Fig. 4b, c) showed very little spontaneous activity and required hypotonic stimulation to promote more robust PS exposure (Fig. 5).

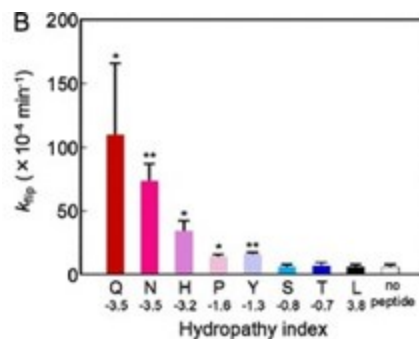
Furthermore, as we showed in Figs. 1b-c and 3b-c, there is a lower limit (towards the Cterminus) of the TM 4 lysine mutation effect, which becomes insufficient to cause a constitutively open pore for spontaneous lipid scrambling. It is possible that TMEM63A A476K represents the lower limit of TM 4 mutations that can convert TMEM63A into a spontaneous lipid scramblase.

Regarding OSCA1.2 K435 and TMEM63A W472, these sites correspond to the hydrophobic gate residues on TM 4 in TMEM16F (F518, Fig. 1a) and TMEM16A (L543, Fig. 3a) so it is unsurprising to us that a lysine mutation at this site causes constitutive scramblase activity in TMEM63A (Fig. 6b, c). For OSCA1.2, it is more intriguing since this residue is already a lysine (K435). In Supplementary Fig. 5 our new experiments show that neutralizing K435 with leucine (K435L) in the background of L438K significantly attenuates spontaneous PS exposure from ~63% PS positive for L438K alone (two lysine residues) to ~31% for K435L/L438K (one lysine). On the other hand, the K435L mutation by itself is also insufficient to induce PS exposure. Therefore, the endogenous lysine at residue 435 has an additive effect on the spontaneous scramblase activity of L438K. We believe the explanation for this result lies in experiments conducted in model transmembrane helices, which have shown that stacking hydrophilic side chains within the membrane interior promotes trans-bilayer lipid flipping (see 10.1248/cpb.c22-00133).

These same studies also support our observation (10.1038/s41467-019-09778-7) that highly hydrophilic side chains (such as lysine or glutamic acid) accelerate trans-bilayer lipid flipping more effectively than hydrophobic side chains such as isoleucine or alanine (Author response image 3, see also 10.1021/acs.jpcb.8b00298).

Author response image 3.

Trans-bilayer lipid flipping rates (kflip) accelerate with increasing side chain hydropathy for a residue placed in the center of a model transmembrane helical peptide



How do the authors know that osmotic shock is indeed activating OSCA1.2 and TMEM63A? If they can record from the channels then electrophysiology data that confirms activation of the channel in the presence of hypoosmotic shock will strengthen the osmolarity active scramblase activity demonstrated in Figure 4. So far, there is conclusive data showing that they are mechanically activated but conclusive electrophysiological data for OSCA/TMEM63 osmolarity activation is not described yet, including the reference (38) they indicate in line 132. Although osmotic shock can perturb mechanical properties of the membrane it can also activate volume-regulated anion channels, which are also present in HEK cells.

Thank you for raising this important question. While reference 38, (now reference 39) shows direct electrophysiological evidence of hypertonicity-induced current (e.g., Fig. 4 f, g, i, and j in 10.1038/nature13593), direct electrophysiological evidence that OSCA/TMEM63 can be activated by hypotonic stimulation is still missing. To address this question, we conducted whole-cell patch clamp experiments on mocktransfected and OSCA1.2 WT-transfected cells stimulated with 120 mOsm/kg hypotonic solution, comparable to the same conditions as hypotonic-induced scrambling shown in Fig. 5. As shown in Supplementary Fig. 6, our whole-cell recording detected a slowly evolving yet robust outward rectifying current in OSCA1.2-transfected cells, which was not observed in mock transfected cells.

To avoid the contamination from endogenous SWELL osmo-/volume-regulated chloride channels, our new experiment used 140 mM Na gluconate to replace NaCl in both the pipette and the bath solution. Because SWELL/VRAC channels are minimally permeable to gluconate anions (e.g., 10.1007/BF00374290), we conclude that hypotonic stimulation can indeed activate OSCA1.2 albeit with perhaps lower efficiency compared to mechanical stimulation.

Minor comments

*What is the timeline for the scramblase assay for all the experiments (except Figure 4)?
How long is the AnnexinV incubated before imaging?*

Thank you for pointing out this point where we have not provided sufficient detail. Cells were imaged in the scramblase assay (including in Fig. 4, now revised Fig. 5) in AnnexinV-containing buffer immediately and without a formal incubation period because AnnexinV binding to exposed PS proceeds rapidly. We have included additional detail in the methods section to eliminate any confusion (Lines 310-312).

In some places of the document, it says OSCA/TMEM63, and in other places, it is denoted as TMEM63/OSCA. The literature so far has always called the family OSCA/TMEM63- please stay consistent with the field.

Thank you for pointing this out, we have corrected these instances to be consistent with the field.

Reviewer #2 (Recommendations For The Authors):

(1) The authors' statement that the channel/scramblase family members have a relatively low "energetic barrier for scramblase" activity needs further support. While mutating the hydrophobic channel gate certainly could destabilize ion conduction to cause a GOF effect on channel activity, it is still not clear why scramblase activity, which is tantamount to altered permeation, happens in the mutant channels. Are permeation and channel gating (opening) coupled in these channels? If so, what is the basis for the coupling? Is scramblase activity only observed when the gating is destabilized or are they separable?

We appreciate these great questions. For the question about the 'energetic barrier' statement, please see our response to point (3) where we have carried out MD simulations of the OSCA1.2 WT and L438K mutant to provide insight into how the permeation pathway is altered by these mutations.

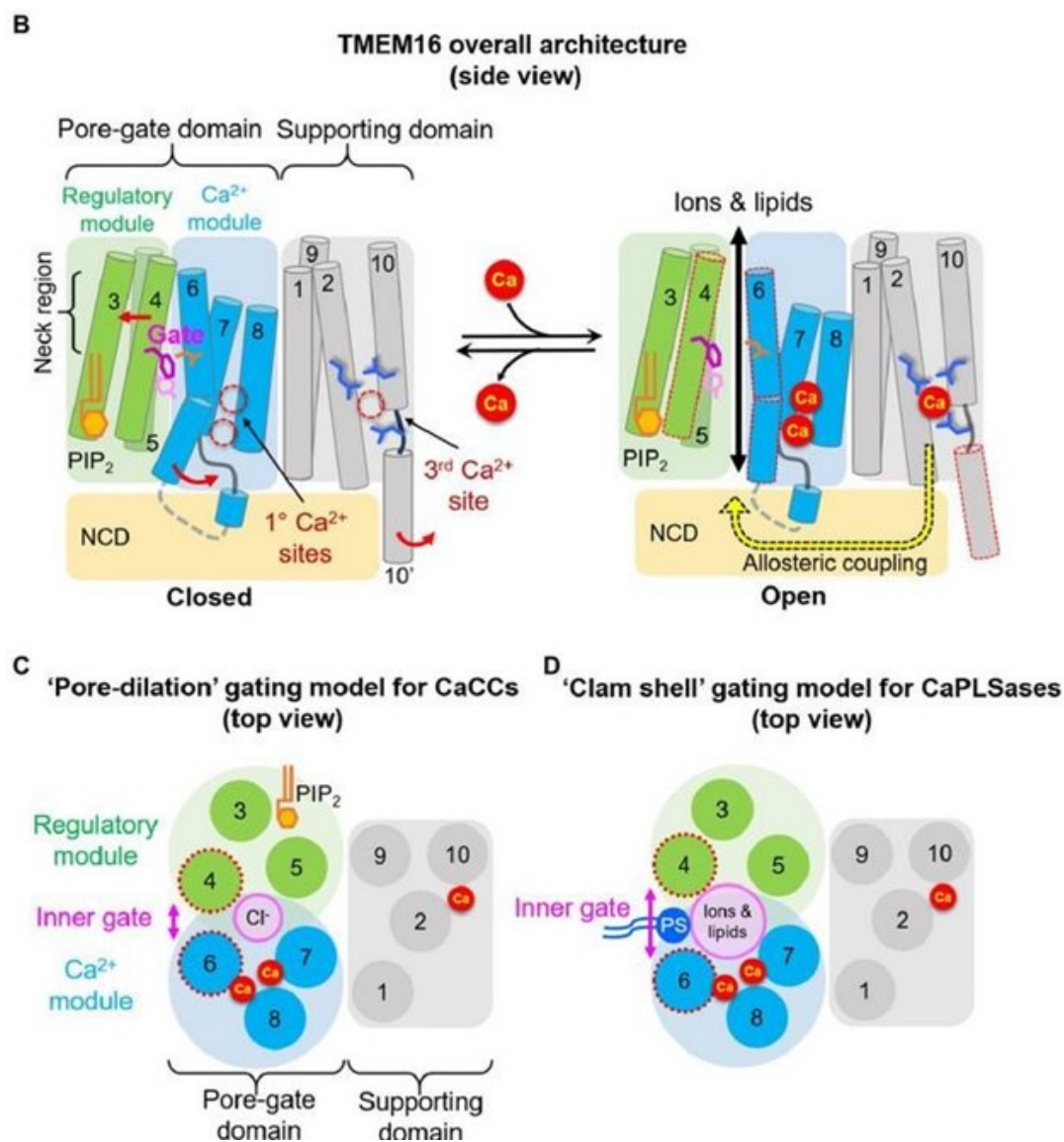
Regarding why TMEM16A can be converted into a scramblase, we use the extensively studied TMEM16 proteins as examples to improve our current understanding of OSCA/TMEM63 proteins. For further details please see our original paper (10.1038/s41467-019-09778-7) and our review (10.3389/fphys.2021.787773), which are summarized as follows:

- (1) The "neck region", consisting of the exofacial halves of TMs 3-6, form the poregate region for both ion and lipid permeation (Author response image 4B). In the closed state, the neck region is constricted and TMs 4 and 6 interact with each other, preventing substrate permeation. The hydrophobic inner activation gate that we identified (10.1038/s41467-019-09778-7) resides right underneath the inner mouth of the neck region, controlling both ion and lipid permeation scrambling.
- (2) Based on our functional observations and the available scramblase structures of TMEM16 proteins in multiple conformations, we proposed a clamshell-like gating model to describe TMEM16 lipid scrambling (Author response image 4D). According to this model, Ca²⁺-induced conformational changes weaken the TM 4/6 interface. This promotes the separation of the two transmembrane segments, analogous to the opening of a clam shell, allowing a membrane-spanning groove to facilitate permeation of the lipid headgroup.
- (3) For the CaCC, TMEM16A, Ca²⁺ binding dilates the pore. However, the binding energy likely cannot open the TM 4/6 interface at the neck region so, in the absence of groove formation, only Cl⁻ ions but not lipids can permeate. (Pore dilation model, Author response image 4C).
- (4) Introducing charged residues near the inner activation gate disrupts the neck region, potentially by weakening the hydrophobic interactions between TMs 4 and 6. This mutational effect results in constitutively active TMEM16F scramblases and enables spontaneous lipid permeation in the TMEM16A CaCC.
- (5) In our revision, we tested additional mutations with different side chain properties (Supplementary Fig. 2), validating previous findings by us (10.1038/s41467-01909778-7) and others (10.1038/s41467-022-34497-x) that gate disruption increases with the side chain hydrophathy of the mutation.
- (6) We further extended lysine mutations to two helical turns below the inner activation gate on TM 4 and identified a lower limit for mutation-induced spontaneous scramblase activity in TMEM16F and TMEM16A (Figs. 1b, c and 3b, c, respectively). Together, all these points lend additional support to our proposed gating models for TMEM16 proteins, which we postulate

may also relate to the OSCA/TMEM63 family based on the evidence provided in our manuscript.

Author response image 4

Model of gating (and regulatory) mechanisms in the TMEM16 family. (B) overall architecture and proposed modules, (C) pore-dilation gating model for CaCCs, (D) Clamshell gating model for CaPLSases.



Regarding the relationship between ion and lipid permeation through TMEM16 scramblases, the following is the summary of our current understanding:

(1) Functionally, ion and lipid permeation are not necessarily obligatory to each other. This is evidenced by our previous biophysical characterizations of TMEM16F ion channel and lipid scramblase activities. Ca^{2+} can trigger TMEM16F lipid scrambling at resting membrane potentials, however, Ca^{2+} alone is insufficient to record TMEM16F current. Strong membrane depolarization synergistically with elevated intracellular Ca^{2+} is required to activate ion permeation. Based on these observations, we postulate that ions and lipids may have different extracellular gates, despite sharing an inner activation gate (10.1038/s41467-019-

09778-7). Ca^{2+} alone may sufficiently open the inner gate (and extracellular gate) for lipids, whereas depolarization is likely required to open the extracellular gate and allow ion flux. Further structure-function studies are needed to test this hypothesis.

(2) Structurally, the open conformation of TMEM16 scramblases such as the fungal orthologs and human TMEM16K (Supplementary Fig. 1 b-d) are widely open, which allows lipid and ion co-transport. Ion and lipid co-transport has also been demonstrated in various MD simulations (e.g., 10.7554/eLife.28671, 10.3389/fmolb.2022.903972, and 10.1038/s41467-021-22724-w)

(3) Functionally, we (10.1085/jgp.202012704) and others (10.7554/eLife.06901.001) have measured dual recording of channel and scramblase activities, also demonstrating that ions and lipids are co-transported simultaneously when the proteins are fully activated.

(4) In this manuscript, we also provide multiple examples (TMEM16F in Fig. 1, TMEM16A in Fig. 3, OSCA1.2 in Fig. 4, and TMEM63A in Fig. 6) of mutations showing spontaneous phospholipid scramblase activities, yet their channel activities require strong depolarization or, in the case of TMEM63A, high pressures to be elicited.

Together, this new evidence further supports our hypothesis that there might be multiple gates for ion and lipid permeation, in addition to the shared inner gate we previously identified. We hope these detailed explanations help convey the intricacy of these intriguing questions. Of course, future studies are needed to test our hypothesis and elucidate the complex relationship between ion and lipid permeation of these proteins.

(2) One weakness in the experimental approach is the very limited number of substitutions used to infer the conclusion regarding the energetic barrier and other conclusions relating to scramblase activity. Additional substitutions of charged and polar amino acids at the hydrophobic gate would be helpful in illuminating the molecular determinants of the GOF phenotype and also reveal varying patterns of lipid permeation which could be enormously informative. These additional mutations for analysis of TMEM16F and OSCA should be added to the study.

We appreciate these great suggestions which were shared by multiple reviewers. We have included our duplicated response below.

“Response to reviewers 2 & 3: In our 2019 paper (10.1038/s41467-019-09778-7), we have systematically tested the side chain properties at the inner activation gate of TMEM16F on lipid scrambling activity (Response Fig. 6) and, since then, these results have been supplemented by others as well (10.1038/s41467-022-34497-x). In summary, mutating the inner activation gate residues to polar or charged residues generally results in constitutively activated scramblases without requiring Ca^{2+} (Fig 5a in 10.1038/s41467-019-09778-7). Because these residues form a hydrophobic gate, introducing smaller side chains via alanine substitution are also gain-of-function with the Y563A mutant as well as the F518A/Y563A/I612A variant being constitutively active (Fig. 3a in 10.1038/s41467-019-09778-7). Meanwhile, mutating these gate residues to hydrophobic amino acids causes no change for I612W, a slight gain-of-function for F518W, slight loss-of-function of F518L, and complete loss-of-function for Y563W (Fig. 4b in 10.1038/s41467-019-09778-7). These findings clearly demonstrate that the side-chain properties are critical for regulating the gate opening. Charged mutations including lysine and glutamic acid are the most effective to promote gate opening (Fig 5a in 10.1038/s41467-019-09778-7).

Similarly, others have observed that side chain hydropathy at the F518 site in TMEM16F correlates with shifts in the Ca^{2+} EC50 (Fig. 2 of 10.1038/s41467-022-34497-x). Note that this publication resolved the structure of the TMEM16F F518H mutant, revealing a previously unseen conformation that we have highlighted in Supplementary Fig. 1e and discussed in

lines 235-238. Please also see our response to Reviewer #1 above, where we discuss discoveries in model transmembrane helical peptide systems showing that transbilayer lipid flipping rates correlate with side chain hydrophathy (Author response image 3), distance between stacked hydrophathic residues (schematic in 10.1248/cpb.c22-00133), and even helical angle between stacked side chains (not show).

Following the reviewers' suggestions, we have tested additional mutations in alternative locations and with different side chains.

(1) We have added data for TMEM16F I521A and I521E to demonstrate a similar effect of alternative side chains to what has previously been reported by us and others. We found that I521A failed to show spontaneous scrambling activity (Supplementary Fig. 2), yet I521E (Supplementary Fig. 2) is a constitutively active lipid scramblase, similar to I521K (Fig. 1). This further demonstrates that gate disruption correlates with the side chain hydrophathy and that this site lines a critical gating interface.

(2) We also added lysine mutations two helical turns below the conserved inner activation gate for TMEM16F T526 (Fig. 1), TMEM16A E551 (Fig. 3). We found that there is indeed a lower limit for the observed effect in TMEM16, where lysine mutations no longer induce spontaneous lipid scrambling activity. This indicates that when TM 4/6 interaction is weaker toward intracellular side (Figs. 1a, 3a), the TM 4 lysine mutation loses the ability to promoting lipid scrambling by disrupting the TM 4/6 interface to enable clamshell-like opening of the permeation pathway.

(3) We added a TMEM16F lysine mutation on TM 6 at residue I611 (Fig. 2). Similar to I612K (Response Fig. 6), I611K also leads to spontaneous lipid scrambling and enhanced channel activity in the absence of calcium (Fig. 2). This shows that charged mutations along TM 6 can also promote lipid scrambling, strengthening our model that hydrophobic interactions along the TM 4/6 interface are critical for gating and lipid permeation."

(3) Related to the above point, it would be enormously useful to perform even limited computational modelling to support the "energetic barrier" statement. Specifically, can the authors model waters in the putative pore to examine water occupancy in the WT and mutant channels to better understand how the barrier for ions and lipids is altered in the TMEM16?

We appreciate this suggestion and have now conducted atomistic MD simulations of OSCA1.2 WT and L438K mutant for ~1 μ s (Supplementary Fig. 4). The simulations revealed, elevated water occupancy in the pore region of the L438K mutant, likely due to a widening at the TM 4/6 interface. Conversely, the WT interface remained constricted, largely disallowing water occupancy. These computational results support our previously proposed clamshell-like gating model for TMEM16 scramblases and provide strong support that the L438K mutation is disrupting the interaction of the TM 4/6 interface, in turn reducing the energetic barrier for both ion and lipid permeation.

(4) I am puzzled about the ability of OSCA and the TMEM63 proteins which are cation channels to conduct negatively charged lipids. How can the pore be selective for cations and yet permeate negatively charged molecules when lipids are presented?

This is a great question. TMEM16 scramblase (as well as other known scramblases, such as the Xkr and Opsin families) are surprisingly non-selective to phospholipids (all major phospholipid species, not just anionic lipids like PS). It is still debated whether lipid headgroups indeed insert into an open pore or hydrophilic groove (Response Fig. 5), or if they may traverse the bilayer by the so-called 'out-of-groove' model. Regardless of the model, the consensus is that Ca²⁺-induced conformational changes catalyze lipid permeation and the

mutations we have introduced are designed to mimic these conformational changes by separating the TM 4/6 interface.

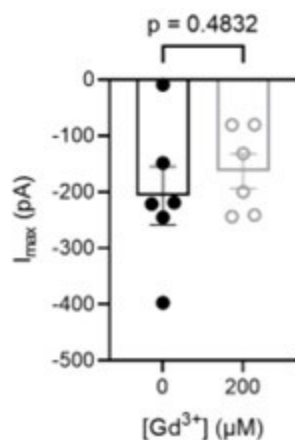
Additionally, TMEM16F channel activity was first characterized as cation non-selective (10.1016/j.cell.2012.07.036), similar to OSCA/TMEM63s, which may even exhibit some chloride permeability (10.7554/eLife.41844.001). Thus, it appears as though scramblase activity is agnostic to headgroup charge and compatible with both a mutant anion channel (TMEM16A) and mutant cation channels (TMEM16F, OSCA1.2, and TMEM63A), however, more detailed structural, functional, and computational studies are needed to further clarify ion and lipid co-transport mechanisms.

(5) Do pore blockers like Gd^{3+} which block permeation also inhibit the scramblase activity of the mutant channels? This should be tested for the mutant channels.

While extracellular Gd^{3+} has been previously reported as an inhibitor of OSCA1.2 (10.7554/eLife.41844.001), we did not observe this effect (Author response image 5), but instead saw inhibition by intracellular Gd^{3+} (Author response image 6). Given this discrepancy, we did not test Gd^{3+} inhibition of the OSCA1.2 scramblases, but instead tested Ani9, a paralog-specific inhibitor of TMEM16A, on the TMEM16A I546K gain-of-function and found it attenuated both ion channel and phospholipid scramblase activities (Supplementary Fig. 3).

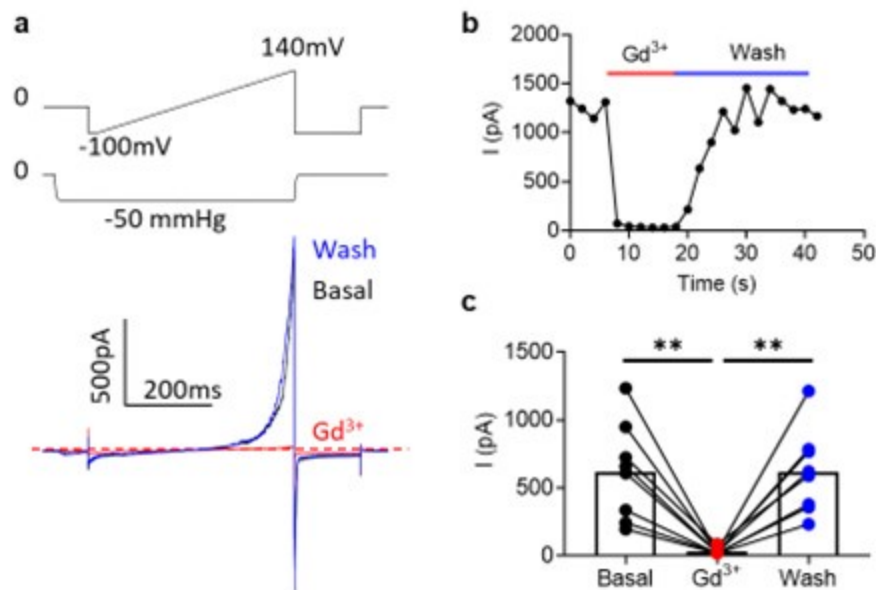
Author response image 5.

200 μM Gd^{3+} ext fails to inhibit OSCA1.2 currents in cell-attached patches. Pressure-elicited peak currents ($n=6$ each). Statistical test is an unpaired Student's t-test.



Author response image 6.

200 μM Gd^{3+} int completely inhibits OSCA1.2 currents in inside-out patches. (a) representative traces in before (black), during (red), and after (blue) Gd^{3+} application. (b) Representative application timecourse. (c) Quantification of peak currents ($n=8$ each). Statistical test is one-way ANOVA.



Minor:

- Some of the current amplitudes shown in Figures 2 and 3 are enormous. Is liquid junction potential corrected in these experiments? If not, it would be preferable to correct this to avoid voltage errors.

Thanks for the question. The large current amplitude is due to 1) great surface expression of the proteins; 2) large single channel conductance of OSCA channels, 3) much larger current at positive voltages for OSCA channels. Our control experiment showed that WT TMEM16A at 0 Ca^{2+} did not give rise to any current (Fig. 3d), further demonstrating that the large current was not due to liquid junction potential. For the OSCA recordings, we also did not observe current in mock-transfected cells, further excluding the possible interference of liquid junction potential (Response Fig. 1)

- Related, authors could consider adding some evidence using selective pharmacology to support the conclusions that the observed currents arise from TMEM or OSCA channels.

Thanks for the suggestion. As mentioned above, we have added experiments with Ani9, a specific inhibitor of TMEM16A, in Supplementary Fig. 3. We found that Ani9 robustly attenuated both ion channel and phospholipid scramblase activities for the TMEM16A I546K gain-of-function mutant. This is also consistent with our previous publication (10.1038/s41467-019-09778-7), where Ani9 efficiently inhibited the TMEM16A L534K mutant scramblases. Additionally, we have provided mock controls (Response Fig. 1, Fig. 6d, e) to show that the observed currents are indeed attributable to OSCA1.2 and TMEM63A.

Reviewer #3 (Recommendations For The Authors):

Given that the authors postulate that the introduction of a positive charge via the lysine side chain is essential to the constitutive activity of these proteins, additional mutation controls for side chain size (e.g. glutamine/methionine) or negative charge (e.g. glutamic acid), or a different positive charge (i.e. arginine) would have strengthened their argument. To more comprehensively understand the TM4/TM6 interface, mutations at locations one turn above and one turn below could be studied until there is no phenotype. In addition, the equivalent mutations on the TM6 side should be explored to

rule out the effects of conformational changes that arise from mutating TM4 and to increase the strength of evidence for the importance of side-chain interactions at the TM6 interface.

We appreciate these great suggestions which were shared by multiple reviewers. We have included our previous responses below.

“Response to reviewers 2 & 3: In our 2019 paper (10.1038/s41467-019-09778-7), we have systematically tested the side chain properties at the inner activation gate of TMEM16F on lipid scrambling activity (Response Fig. 6) and, since then, these results have been supplemented by others as well (10.1038/s41467-022-34497-x). In summary, mutating the inner activation gate residues to polar or charged residues generally results in constitutively activated scramblases without requiring Ca^{2+} (Fig 5a in 10.1038/s41467-019-09778-7). Because these residues form a hydrophobic gate, introducing smaller side chains via alanine substitution are also gain-of-function with the Y563A mutant as well as the F518A/Y563A/I612A variant being constitutively active (Fig. 3a in 10.1038/s41467-019-09778-7). Meanwhile, mutating these gate residues to hydrophobic amino acids causes no change for I612W, a slight gain-of-function for F518W, slight loss-of-function of F518L, and complete loss-of-function for Y563W (Fig. 4b in 10.1038/s41467-019-09778-7). These findings clearly demonstrate that the side-chain properties are critical for regulating the gate opening. Charged mutations including lysine and glutamic acid are the most effective to promote gate opening (Fig 5a in 10.1038/s41467-019-09778-7).

Similarly, others have observed that side chain hydrophathy at the F518 site in TMEM16F correlates with shifts in the Ca^{2+} EC50 (Fig. 2 of 10.1038/s41467-022-34497-x). Note that this publication resolved the structure of the TMEM16F F518H mutant, revealing a previously unseen conformation that we have highlighted in Supplementary Fig. 1e and discussed in lines 235-238. Please also see our response to Reviewer #1 above, where we discuss discoveries in model transmembrane helical peptide systems showing that transbilayer lipid flipping rates correlate with side chain hydrophathy (Author response image 3), distance between stacked hydrophathic residues (schematic in 10.1248/cpb.c22-00133), and even helical angle between stacked side chains (not show).

Following the reviewers' suggestions, we have tested additional mutations in alternative locations and with different side chains.

(1) We have added data for TMEM16F I521A and I521E to demonstrate a similar effect of alternative side chains to what has previously been reported by us and others. We found that I521A failed to show spontaneous scrambling activity (Supplementary Fig. 2), yet I521E (Supplementary Fig. 2) is a constitutively active lipid scramblase, similar to I521K (Fig. 1). This further demonstrates that gate disruption correlates with the side chain hydrophathy and that this site lines a critical gating interface.

(2) We also added lysine mutations two helical turns below the conserved inner activation gate for TMEM16F T526 (Fig. 1), TMEM16A E551 (Fig. 3). We found that there is indeed a lower limit for the observed effect in TMEM16, where lysine mutations no longer induce spontaneous lipid scrambling activity. This indicates that when TM 4/6 interaction is weaker toward intracellular side (Figs. 1a, 3a), the TM 4 lysine mutation loses the ability to promoting lipid scrambling by disrupting the TM 4/6 interface to enable clamshell-like opening of the permeation pathway.

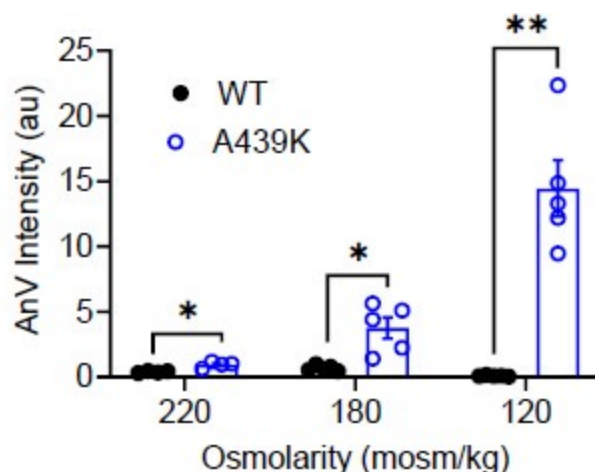
(3) We added a TMEM16F lysine mutation on TM 6 at residue I611 (Fig. 2). Similar to I612K (Response Fig. 6), I611K also leads to spontaneous lipid scrambling and enhanced channel activity in the absence of calcium (Fig. 2). This shows that charged mutations along TM 6 can also promote lipid scrambling, strengthening our model that hydrophobic interactions along the TM 4/6 interface are critical for gating and lipid permeation.”

The experiments for OSCA1.2 osmolarity effects on gating and scramblase in Figure 4 could be improved by adding different levels of osmolarity in addition to time in the hypotonic solution.

We thank the reviewer for this excellent suggestion. We extensively tested this idea and found evidence (Response Fig. 10) that intermediate osmolarity (220 and 180 mOsm/kg) also can enhance the scramblase activity of the A439K mutant, albeit to a milder extent compared to 120 mOsm/kg stimulation. This suggests that swelling-induced membrane stretch may proportionally induce A439K activation and lipid scrambling. Due to the relatively mild sensitivity of OSCA to osmolarity and the variations induced by the experimental conditions, we believe it is better to not include this data to avoid overclaiming. We hope the reviewer would agree.

Author response image 7.

AnV intensities of WT- and A439K-transfected cells after 10 minutes of hypotonic stimulation at the listed osmolarities.



Some confocal images appear to be rotated relative to each other (e.g. Figures 2b and 3b).

Thank you for identifying these errors, they are corrected in the revision.

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