

TMEM16 and TMEM63/OSCA 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, likely function solely as ion channels. However, the remaining 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 lysine mutations in transmembrane helix (TM) 4 allow non-scrambling TCS members to permeate phospholipids. 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 manuscript finds evidence for a latent capability in several members of the TMEM16 and OSCA/TMEM family of ion channels for lipid scramblase activity. The authors demonstrate that the introduction of lysine mutations in evolutionarily conserved areas of TM4 can confer constitutive ion conduction and scramblase activity. Although the significance and scope of the work are **important**, the strength of the evidence is **incomplete** and could be improved.

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 coagulation¹, cell death signaling², phagocytosis³, cell migration⁴, membrane repair^{5,6}, microparticle release⁷, cell-cell fusion^{8–10}, and viral infection^{11,12}. Among identified scramblases, TMEM16 CaPLSases are the most extensively studied at the molecular level¹³. The TMEM16 family (Extended Data Fig. 1a) was originally classified as calcium-activated chloride channels (CaCCs) based on the first-discovered members, TMEM16A and TMEM16B^{14–16}. The remaining members, however, exhibit CaPLSase activity, with TMEM16F representing the canonical CaPLSase¹⁷ (**Fig. 1a**). Uniquely, CaPLSases also function as non-selective ion channels along with their non-specific permeability to phospholipids. 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^{18–20} (Extended Data Fig. 1b-e). These conformational changes further catalyzed phospholipid permeation by thinning the membrane near the permeation pathway^{21,22}.

The TMEM16 family together with the TMEM63/OSCA family and TMC family^{23–26} collectively form the Transmembrane Channel/Scramblase (TCS) superfamily¹³. Despite the conserved 10-TM architectural core^{27–31}, phospholipid permeability has not been experimentally demonstrated in mechanosensitive TMEM63/OSCA 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 acids³², or through a single lysine mutation in TM 4 at the hydrophobic gate¹⁸. 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 lysine mutations along TM 4 of TMEM16F, TMEM16A, OSCA1.2, and TMEM63A at sites near the corresponding hydrophobic gate of TMEM16F¹⁸. TM 4 lysine mutations in TMEM16F and TMEM16A resulted in constitutive phospholipid scramblase activity. Strikingly, the equivalent TM 4 lysine mutations of OSCA1.2 and TMEM63A also converted these ion channels into phospholipid scramblases, which were either constitutively open or further activated by osmotic stimulation. All mutants also resulted in commensurate gain-of-function ion channel activity, suggesting that the charge in TM 4 disrupts the activation gate of these evolutionarily related proteins. Together, our findings advance the mechanistic understanding of gating and substrate permeation in the TMEM16 and OSCA/TMEM63 families, underscore a key design principle for phospholipid scramblases, and support our hypothesis that the TCS superfamily maintains a conserved potential for both ion and phospholipid permeation.

Results

TM 4 lysine 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 permeation¹⁸. Introducing polar or charged residues to the activation gate results in gain-of-function (GOF) CaPLSases, some of which are constitutively activated without requiring calcium. Structural and computational studies on the fungal *Nh*TMEM16³³ and *Af*TMEM16²¹ orthologs, as well as human TMEM16K³⁴ further support a clamshell like gating model¹⁸ whereby scrambling activity is promoted by separation of the TM 4/6 interface¹³ (Extended Data Fig. 1b-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 8¹³. Given that the N-termini of TM 4 are largely hydrophobic among TMEM16 CaPLSases (Extended

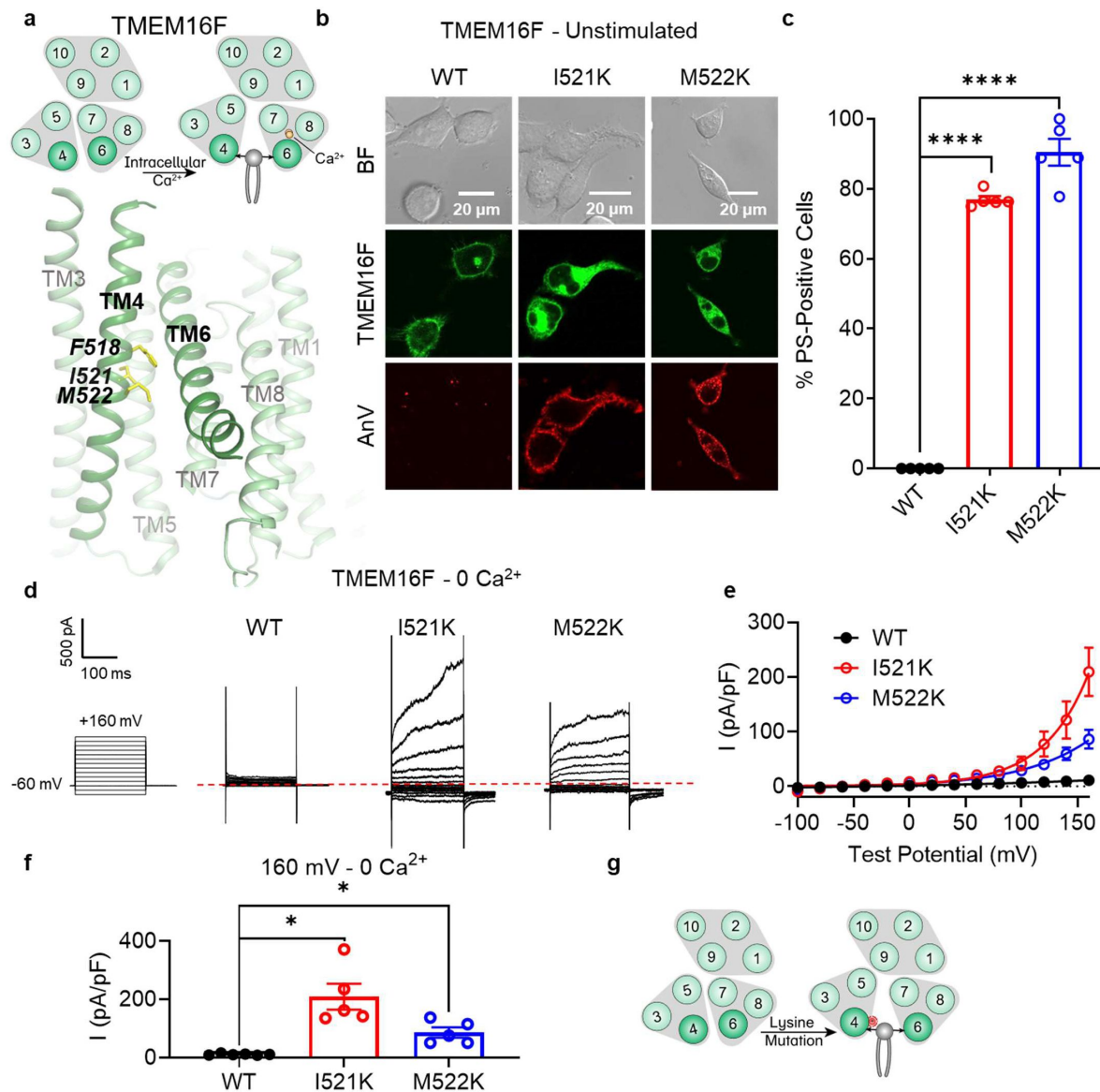


Figure 1

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 (PDB 6QPB). (b) Representative images of TMEM16F knockout (KO) HEK293T cells expressing eGFP-tagged TMEM16F wildtype (WT), I521K, and M522K (center row). CF 594-conjugated Annexin V (AnV, bottom row) labelled PS exposing cells. BF denotes bright field images (top row). (c) Quantification of the percentage of cells with AnV labelling for TMEM16F WT (n=5), I521K (n=5), and M522K (n=5) transfected cells. Values were derived from images of biological replicates, with error bars representing standard error of the mean (SEM). Statistical comparisons were done using unpaired t-tests 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), I521K (n=5), and M522K (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 comparisons were done using unpaired t-tests with Welch's correction (*: $p < 0.05$). (g) Lysine mutations along TM 4 in TMEM16F enable spontaneous phospholipid permeation.

Data Fig. 1f) and a lysine mutation at F518 leads to a constitutively open TMEM16F activation gate¹⁸, 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^{18,35,36} 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 the TMEM16F wildtype (WT) expressing cells and is therefore not labelled by extracellular AnV. Similar to TMEM16F F518K¹⁸, 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**). We further tested whether I521K and M522K have GOF ion channel activity using whole-cell patch clamp. In the absence of calcium, the mutants, but not WT TMEM16F, 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 lipid scramblase activity results in spontaneous, global PS exposure on the cell surface. I521 and M522 are approximately one helical turn below the hydrophobic gate residue, F518,¹⁸ (**Fig. 1a**) supporting the idea that a lysine mutation on TM 4 along the TM 4/6 interface promotes gate opening and substrate permeation (**Fig. 1g**).

TM 4 lysine mutations convert TMEM16A into a constitutively active scramblase

TMEM16A is a CaCC^{14–16} without scramblase activity (**Fig. 2a-c**)^{18,32}. We previously reported that a single TM 4 lysine mutation (L543K) at the hydrophobic gate of TMEM16A (**Fig. 2a**) allows the CaCC to permeate phospholipids spontaneously, analogous to the F518K mutation in TMEM16F¹⁸. 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 L543K¹⁸, I546K- and I547K-expressing cells exhibit spontaneous, global PS exposure (**Fig. 2b, c**), analogous to the equivalent TMEM16F mutations I521K and M522K (Extended Data Fig. 1f; **Fig. 1b, c**). Similarly, whole-cell patch clamp revealed GOF ion channel activity at depolarizing potentials, even in the absence of calcium stimulation (**Fig. 2d-f**). Together, these results suggest that lysine mutations in this region strongly destabilize the hydrophobic gate to endow the TMEM16A CaCC with constitutive phospholipid permeability and GOF ion channel activity (**Fig. 2g**).

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

We next applied our approach to a TMEM16 relative from the OSCA/TMEM63 family (Extended Data Fig. 1a), which was first discovered in plants as a family of mechanosensitive and osmolarity-activated cation non-selective ion channels³⁷ (**Fig. 3a**). 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 structural²⁷ and biophysical³⁸ 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 lipid scrambling activity (**Fig. 3b**). We then introduced TM 4 lysine mutations near the putative gating residues identified by structural (**Fig. 3a**) and sequence alignment (Extended Data Fig. 1e). Strikingly, a single point mutation, L438K, causes cells overexpressing the OSCA1.2 mutant to exhibit spontaneous and global PS exposure (**Fig. 3b, c**).

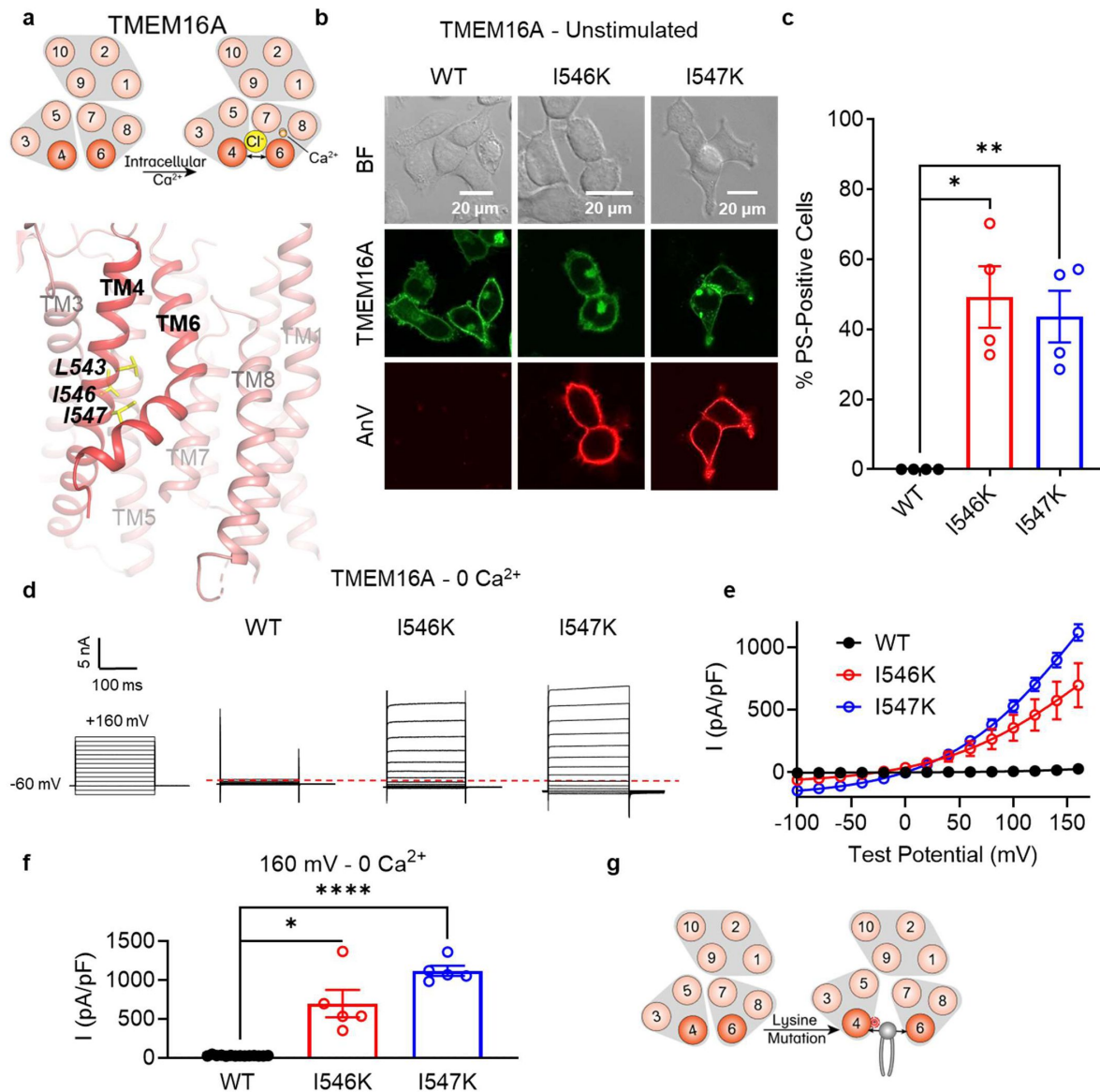


Figure 2

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 (PDB 5OYG). (b) Representative images of TMEM16F knockout (KO) HEK293T cells expressing eGFP-tagged TMEM16A wildtype (WT), I546K, and I547K (center rows). CF 594-conjugated annexin V (AnV, bottom row) labelled PS exposing cells. BF denotes bright field images (top row). (c) Quantification of the percentage of cells with AnV labelling for TMEM16A WT (n=4), I546K (n=4), and I547K (n=4) 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), and I547K (n=5). Currents were elicited by the voltage protocol shown with the pipette containing an EGTA solution. 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.

This mirrors our results with both TMEM16F (Fig. 1) and TMEM16A (Fig. 2), 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. 3e, f) and accelerates channel activation kinetics (Fig. 3g) compared to WT. Under -50 mmHg of pressure, L438K has half-maximal voltages ($V_{0.5}$) of 66.7 ± 3.7 mV, while the WT $V_{0.5}$ is nearly 108.7 ± 5.6 mV (Fig. 3f), underscoring that this mutation also disrupts 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. 3h).

TM 4 lysine mutation A439K converts OSCA1.2 into an osmolarity-sensing scramblase

Interestingly, mutating the neighboring amino acid (Fig. 3a), A439, to lysine resulted in minimal spontaneous PS exposure (Fig. 3b, c). We thus reasoned that A439K scramblase activity may require additional stimulation. Given that OSCA1.2 is an osmolarity-sensitive ion channel³⁸ and reduction of extracellular osmolarity does not induce PS exposure in OSCA1.2 WT expressing cells (Fig. 4a, c), we acutely treated A439K-expressing cells with a hypotonic solution (120 mOsm/kg). Indeed, PS exposure was robustly induced (Fig. 4b, c). Inside-out patch clamp further demonstrated that A439K enhances OSCA1.2 ion channel activity as evidenced by the accelerated activation kinetics (Fig. 3g) and left-shifted G - V relationship (Fig. 3f). Our experiments thus indicate that A439K disrupts OSCA1.2 gating and converts the osmolarity-activated ion channel into an osmolarity-activated phospholipid scramblase (Fig. 4d).

TM 4 lysine mutations convert TMEM63A into a constitutively active scramblase

We next 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 members, they likely function as monomers^{39–41}. Given their structural homology to TMEM16s and OSCA1.2³⁹, 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. 5a) and sequence alignment (Extended Data Fig. 1f), selecting W472 (equivalent to F518 in TMEM16F and L543 in TMEM16A) and S475 (equivalent to L438 in OSCA1.2). Indeed, overexpressing eGFP-tagged mouse TMEM63A with single lysine mutations at either W472 and S475 led to spontaneous PS exposure (Fig. 5b, c), though the AnV staining revealed punctate rather than global patterns of PS exposure. To confirm membrane localization and further probe mutant effects, we exploited TMEM63A ion channel function using cell-attached patch clamp. As TMEM63A exhibits voltage-dependent activity under high pressures, we compared mutant and WT I - V relationships at -80 mmHg. W472K and S475K caused marked reductions in $V_{0.5}$ from 122.3 ± 3.5 mV for WT to 95.9 ± 6.3 mV and 92.1 ± 8.6 mV, respectively (Fig. 5d-f). 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. 5g).

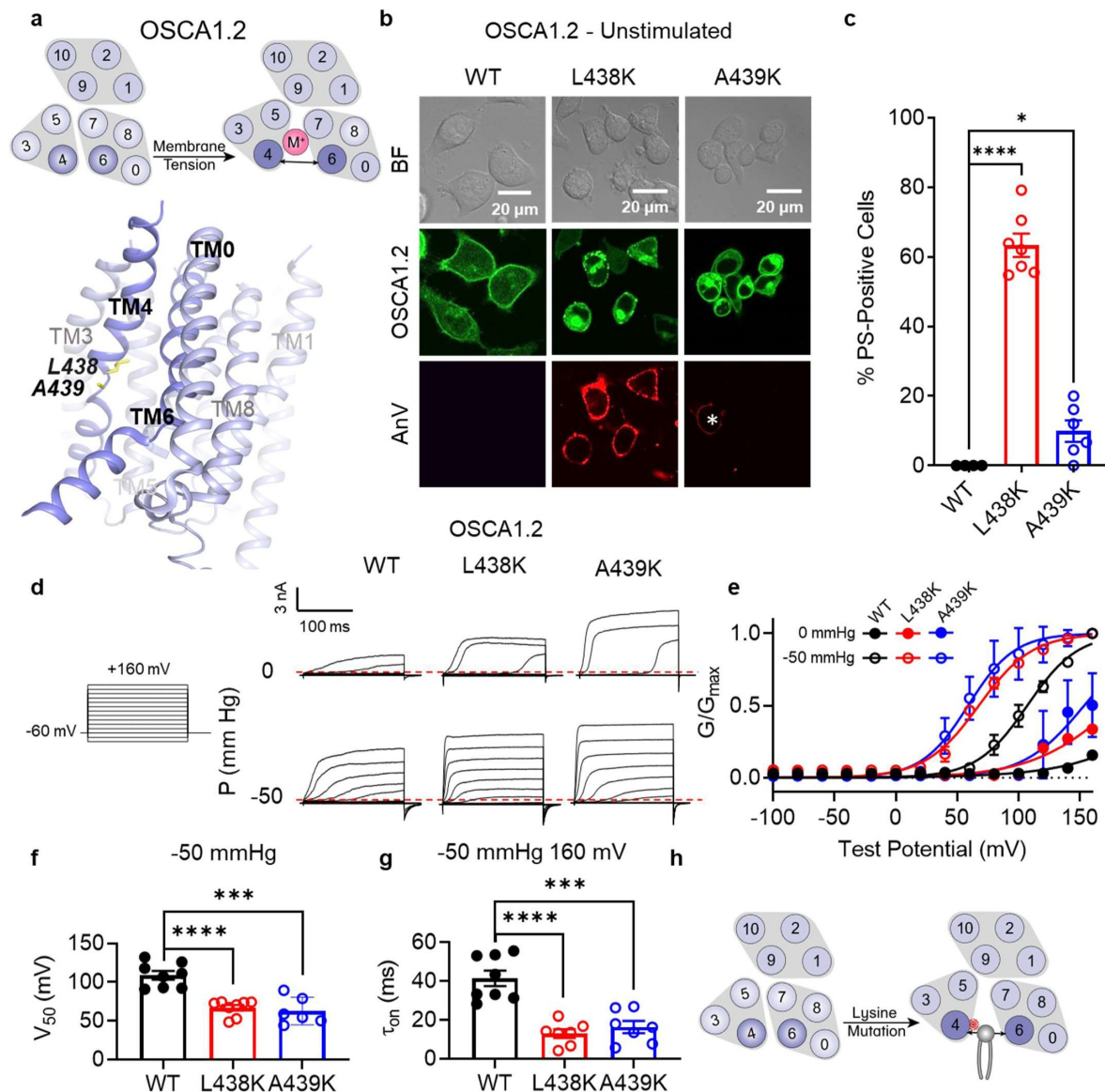


Figure 3

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

Top: OSCA1.2 is a cation non-selective ion channel gated by membrane tension. Bottom: 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 (middle row) OSCA1.2 WT (left column), L438K (middle column), or A439K mutants (right column). CF 594-conjugated AnV (bottom row) labelled PS exposing cells. BF denotes bright field images (top row). 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 an unpaired t-test 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 line denotes 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 an unpaired t-tests with Welch's correction (***: p<0.001, ****: p<0.0001). (g) Quantification of activation τ_{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 an unpaired t-tests with Welch's correction (***: p<0.001, ****: p<0.0001). (h) A lysine mutation along TM 4 converts OSCA1.2 channel into a phospholipid scramblase with spontaneous phospholipid permeability.

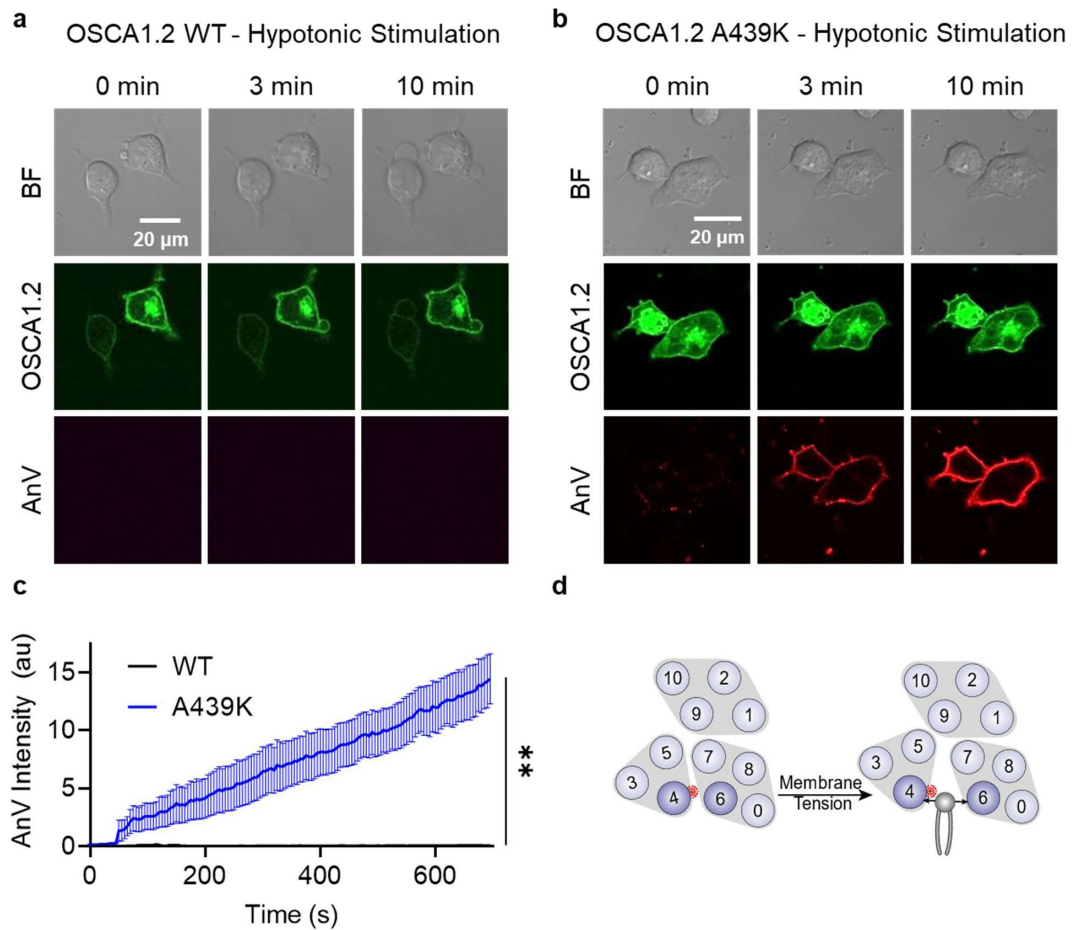


Figure 4

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 rows). CF 594-conjugated AnV (bottom rows) labelled PS exposing cells. BF denotes bright field images (top rows). Each column 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-tests with Welch's correction (**: $p < 0.01$). (d) The A439K mutation converts OSCA1.2 to an osmolarity-activated phospholipid scramblase.

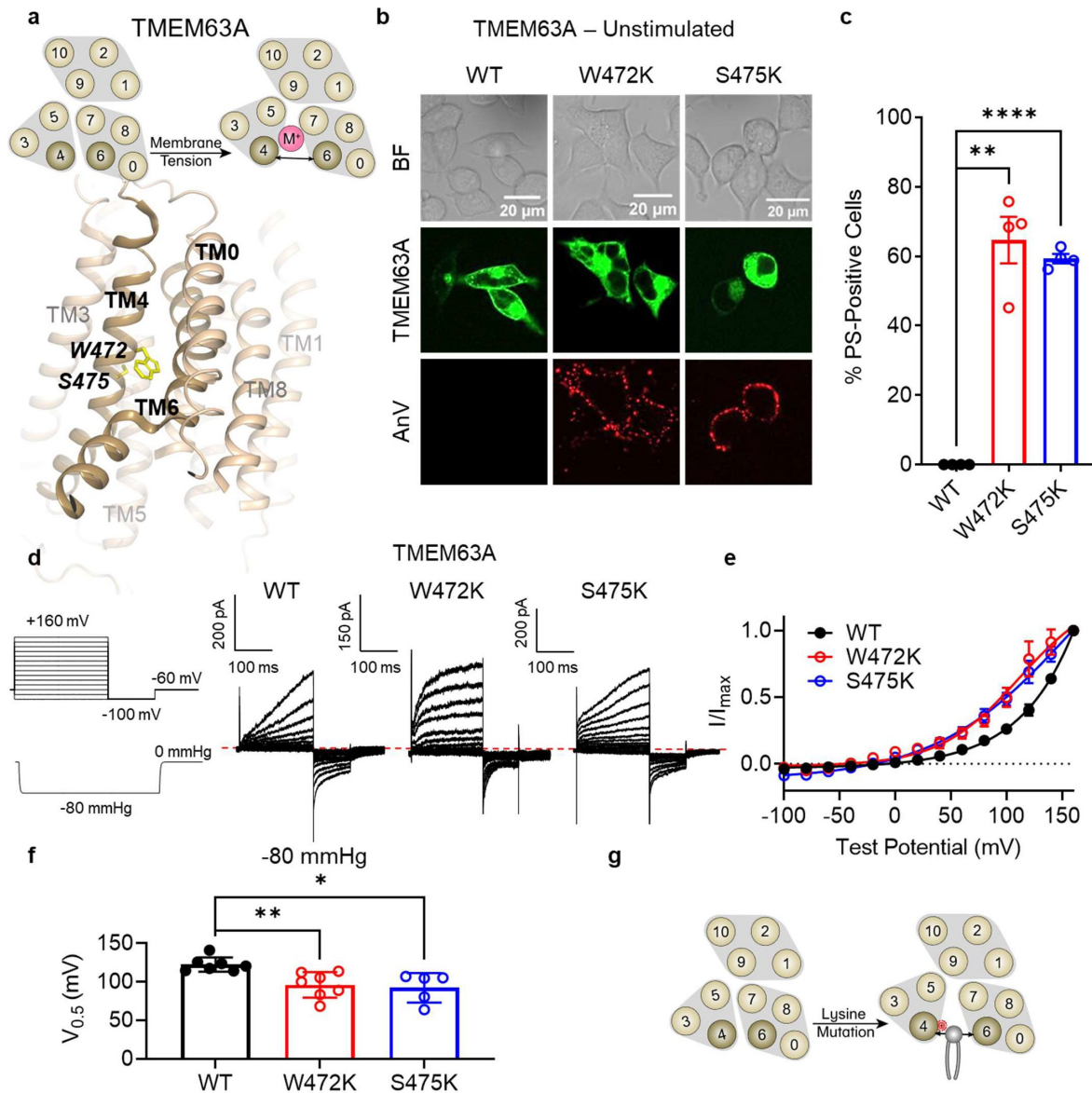


Figure 5

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

Top: TMEM63A is a cation non-selective ion channel gated by 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 (middle row) TMEM63A WT (left column), W472K (middle column), or S475K mutants (right column). CF 594-conjugated AnV (bottom row) labelled PS exposing cells. BF denotes bright field images (top row). (c) Quantification of the percentage of cells with AnV labelling for TMEM63A WT (n = 4), W472K (n = 4), and S475K-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 eGFP-tagged TMEM63A WT (n = 7), W472K (n = 7), and S475K (n = 5). Currents represent the subtraction of voltage alone from currents elicited by the voltage and pressure protocols shown. Dotted line denotes zero current. (f) Quantification of half-maximal voltage at -80 mmHg for WT (122 mV), W472K (96 mV), and S475K (92 mV). Error bars represent standard error of the mean (SEM) calculated from independent patches. Statistical comparison was conducted with an unpaired t-tests with Welch's correction (*: p<0.05, **: p<0.01). (g) Lysine mutations along TM 4 in TMEM63A enable spontaneous phospholipid permeability.

Discussion

Mechanistically, our study improves models of TMEM16 substrate permeation and gating. We identified multiple lysine 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^{18,42}. 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 destroyed¹⁸, whereas F518H does not exhibit spontaneous PS exposure⁴². Interestingly, the recent TMEM16F F518H structure (Extended Data Fig. 1d) shows local membrane thinning due in part to unexpected conformational changes in TM 3⁴². 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^{43,34,44}, Xkr^{45,46}, and opsin⁴⁷ 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 superfamily¹³ and help uncover their roles in human diseases. Although OSCA/TMEM63 proteins are not known to scramble phospholipids, we show that TM 4 lysine mutations in OSCA1.2 and TMEM63A convert these osmolarity-activated and mechanosensitive ion channels into phospholipid scramblases, analogous to our findings with the equivalent TMEM16A mutations. 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 during evolution. It will be interesting to test whether equivalent TM 4 lysine mutations can convert transmembrane channel-like (TMC) proteins—the third TCS relative of TMEM16 and OSCA/TMEM63¹³—into phospholipid scramblases. TMC1 and TMC2 are best known for their roles in auditory sensation, and thus far have mostly been characterized *in vivo* due to expression difficulties in heterologous systems⁴⁸. However, recent *in vivo* characterization of mouse TMC1 M412K, known as the *Beethoven* mutation, provided an important insight⁴⁹. The deafness-associated mutation is located in TM 4 (Extended Data Fig. 1f; equivalent of M522 in TMEM16F, I547 in TMEM16A, and A439 in OSCA1.2) and results in constitutive PS exposure when expressed in the hair cell membranes of both heterozygous and homozygous mice⁴⁹. 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 sensation⁵⁰. 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 use a pEGFP-N1 vector backbone. 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 (*AjTMEM16*), 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 Extended Data Fig. 1. 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^{18,35}. 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 or X-tremeGENE360 transfection reagents (MilliporeSigma). 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^{9,18,35}. Briefly, 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) before imaging. Spontaneous PS positive cells were labeled by fluorescently tagged AnV and imaged. 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 OSCA1.2 were treated with low osmolarity AnV buffer, and the scramblase activity was measured by recording fluorescent AnV surface accumulation at 5 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 2–3 MΩ in 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 study⁵⁴. Patches were held at a membrane potential of -60 mV and at the indicated pressure, then stimulated using the indicated voltage protocol.

Cell-attached patch clamp recordings

For TMEM63 recordings, cell-attach mode was used. 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 with pressure pulse.

Whole cell 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.

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.

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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. 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. 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 plays an important role in processes like blood coagulation, cell death signaling, and phagocytosis. In a previous study, the group 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 the 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.

Strengths:

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

Weaknesses:

The electrophysiology data does not entirely support their claims.

<https://doi.org/10.7554/eLife.96957.1.sa2>

Reviewer #2 (Public Review):

This concise and focused study by Lowry and colleagues identifies a motif in the pores of three families of channel/scramblase proteins that regulate exclusive ion permeation and lipid transport. These three ion channel families, which include the TMEM16s, the plant-expressed and stress-gated cation channel OSCA, and the mammalian homolog and mechanosensitive cation channel, TMEM63 share low sequence similarity between them and have seemingly differing functions, as anion (TMEM16s), 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 the 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 themselves seem to be done with a high level of rigor and the paper is well written. A weakness is the limited scope of the experiments which, if fixed, could open up a new line of inquiry.

<https://doi.org/10.7554/eLife.96957.1.sa1>

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. The authors show that the introduction of lysine residues at the TM4-TM6 interface can disrupt gating and confer scramblase activity to non-scramblase proteins. Specifically, 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 the paper and provides strong evidence for an underlying linked mechanism for ion conduction

and phospholipid transport. Overall, the confocal imaging experiments, patch clamping experiments, and data analysis are performed well.

However, there are several concerns regarding the scope of experiments supporting some claims in the paper. Although the authors propose that the TM4/TM6 interface is critical to ion conduction and phospholipid scramblase activity, in each case, there is very narrow evidence of support consisting of 1-3 lysine substitutions at specific residues on TM4. 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. 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.

<https://doi.org/10.7554/eLife.96957.1.sa0>

Author response:

Overall recommendations.

A brief summary of the main reviewers' recommendations that should be prioritized is listed below. Detailed recommendations as suggested by each individual reviewer are also included.

-Better justification of the choice of the substitutions for the mutations should be added. In addition, authors should strongly consider adding more mutations to enable mechanistic tests of the proposed model for lipid conduction.

We will characterize more mutations to the key residues at the TM4-TM6 interface. In addition to the TM4 lysine mutations shown in the original manuscript, we will include mutations to alanine and glutamate, and justify our choice of the substitutions in the revised manuscript. Furthermore, we will also test if introducing lysine mutations in TM6 will convert the ion channels into lipid scramblases. These additional experiments will greatly strengthen our conclusion.

-Blockers to validate the concern that the recorded currents indeed arise from TMEM16A or OSCA/TMEM63 channels should be tested. Do the pore blockers also block scramblase activity in the gating mutants?

TMEM16A and OSCA1.2 are readily expressed on cell surface. OSCA1.2 also has large conductance. This is the reason why we can record huge current even with inside-out patches. We will include TMEM16A inhibitor Ani9 and a non-specific inhibitor of OSCA channels to further validate. We have reported that Ani9 can inhibit a TMEM16A-derived lipid scramblase (L543K in TM4) in our previous publication (PMID: 31015464). We will test if Ani9 can also inhibit other TMEM16A scramblases reported in this study. We will also examine if Gd3+ is capable of blocking lipid scrambling of the OSCA1.2 gating mutations.

-Include details of missing experimental conditions for scramblase activity.

We will conduct a thorough revision to include detailed experimental conditions for scramblase activity measurement.

-Additional mutants above and below the putative lysine gate as suggested by reviewer 3 to better assess the model.

As we explained in Response #1, we will extend our mutations around the putative activation gate.

-Concern about whether osmolarity changes are in fact activating OSC and TMEM63. As suggested by reviewers 1 and 3. This could be addressed by assessing scramblase activity and currents at different osmolarity levels.

We will test the engineered OSCA1.2 scramblases in response to solutions with different osmolarity.

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 plays an important role in processes like blood coagulation, cell death signaling, and phagocytosis. In a previous study, the group 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 the 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.

Strengths:

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

Weaknesses:

The electrophysiology data does not entirely support their claims.

We appreciate your positive comments. We will conduct more experiments including more electrophysiology characterizations as suggested.

Reviewer #2 (Public Review):

This concise and focused study by Lowry and colleagues identifies a motif in the pores of three families of channel/scramblase proteins that regulate exclusive ion permeation and lipid transport. These three ion channel families, which include the TMEM16s, the plant-expressed and stress-gated cation channel OSCA, and the mammalian homolog and mechanosensitive cation channel, TMEM63 share low sequence similarity between them and have seemingly differing functions, as anion (TMEM16s), or stress-activated

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We appreciate the positive comments from the reviewer. We will conduct more experiments listed in our responses to the Overall Recommendations to improve the scope and quality of our study.

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. The authors show that the introduction of lysine residues at the TM4-TM6 interface can disrupt gating and confer scramblase activity to non-scramblase proteins. Specifically, 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 the paper and provides strong evidence for an underlying linked mechanism for ion conduction and phospholipid transport. Overall, the confocal imaging experiments, patch clamping experiments, and data analysis are performed well.

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We appreciate the positive and constructive comments from the reviewer. As we outlined in our responses to the Overall Recommendations, we will include more mutations at the TM4 and TM6 interface to further strengthen our conclusion.