

Structure-guided mutagenesis of OSCAs reveals differential activation to mechanical stimuli

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Abstract

Abstract

The dimeric two-pore OSCA/TMEM63 family has recently been identified as mechanically activated ion channels. Previously, based on the unique features of the structure of OSCA1.2, we postulated the potential involvement of several structural elements in sensing membrane tension¹. Interestingly, while OSCA1, 2, and 3 clades are activated by membrane stretch in cell-attached patches (i.e., they are stretch-activated channels), they differ in their ability to transduce membrane deformation induced by a blunt probe (poking). In an effort to understand the domains contributing to mechanical signal transduction, we used cryo-electron microscopy to solve the structure of *Arabidopsis thaliana* (At) OSCA3.1, which, unlike AtOSCA1.2, only produced stretch- but not poke-activated currents in our initial characterization². Mutagenesis and electrophysiological assessment of conserved and divergent putative mechanosensitive features of OSCA1.2 reveal a selective disruption of the macroscopic currents elicited by poking without considerable effects on stretch-activated currents (SAC). Our results support the involvement of the amphipathic helix and lipid-interacting residues in the membrane fenestration in the response to poking. Our findings position these two structural elements as potential sources of functional diversity within the family.

eLife assessment

The manuscript "Structure Guided mutagenesis of OSCAs reveals differential activation to mechanical stimuli" seeks to dissect the molecular underpinnings of poke and stretch activation in OSCA channels. While the structural and functional experiments are well done, and the authors present some **important** data, the reviewers identified weaknesses in experimental design and interpretation that render the data **incomplete** in supporting some of the main conclusions of the paper. Nevertheless, this work will be of interest to those working in the fields of mechanosensation, sensory biology, and ion channels.

Introduction

Mechanotransduction allows organisms to gather information about their external surroundings as well as internal processes. Mechanically-activated (MA) ion channels are capable of sensing mechanical stimuli and transducing this information to the cell as electrochemical signals³. MA ion channels are diverse and capable of responding to different stimuli such as osmotic stress, membrane deformation, touch, and hearing^{3,4}.

In our previous work, we identified OSCA/TMEM63 as a conserved family of MA ion channels spanning several eukaryotic clades and having considerable diversity in the plant kingdom, where species commonly have more than one paralog. For example, *Arabidopsis thaliana* has 15 members² and *Oryza sativa* L. *japonica* (rice), 11⁵. We demonstrated these channels can respond to membrane stretch even more robustly than hypertonicity, originally thought to be the relevant stimulus, with some members (OSCA1.1 and OSCA1.2) also able to respond to poke². We used cryo-electron microscopy (cryo-EM) to solve the structure of OSCA1.2 in a closed state in both detergent (LMNG) and nanodiscs at high-resolution, identifying a new architecture for MA ion channels that is structurally homologous to TMEM16 proteins¹. Results from other groups are consistent with ours^{6,8}. Similar to the TMEM16 family, OSCAs are dimeric and each subunit has a pore lined by transmembrane helices (TMs) 3 to 7, instead of a single pore along the symmetry axes^{1,6,8}. A hydrophobic gate on the extracellular half of the pore blocks ion flow. Additionally, molecular dynamics (MD) simulations support our placement of the pore and position lipids along an opening of the pore pathway in the transmembrane domain (TMD)¹.

Based on the unique structural characteristics of OSCA1.2, we proposed that two features might be involved in sensing membrane deformation: the amphipathic helix (AH) located in the first intracellular loop, and the Beam-Like Domain (BLD), composed of the two cytoplasmic membrane-parallel helices and a hydrophobic hook that connects them and re-inserts itself into the membrane¹. Due to the close association of these two elements to the membrane, we reasoned that they could serve as sensors of mechanical stimuli and transmit the signal to the pore through interactions with the TMD. Additionally, a fenestration allows the membrane access to the pore, and MD simulations placed lipids in close association with four positively charged residues in this area suggesting a potential role in gating¹. We sought to determine whether these regions contributed to mechanotransduction.

Here, we used cryo-EM to determine a high-resolution structure of AtOSCA3.1, the first reported OSCA gene⁹, in nanodiscs. In our initial characterization, OSCA3.1 responded to stretch but not poke². Comparison of the OSCA3.1 structure reported here to previous structures of OSCA1.1⁶ and OSCA1.2¹ revealed subtle differences. Structure-function analyses revealed the involvement of the AH and the lipid-interacting residues in the pore pathway of OSCA1.2 in the ability of the channel to respond to membrane deformation by poking. Our results suggests that these features are not only important for mechanosensation in OSCAs but may serve to tune the response of OSCA/TMEM63 members to diverse stimuli.

Results

Overall architecture of OSCA3.1 is similar to OSCA1.1 and OSCA1.2

We recombinantly tagged OSCA3.1 with EGFP at its C-terminus, expressed the construct in HEK293F cells, and purified it using Lauryl Maltose Neopentyl Glycol (LMNG) supplemented with cholesteryl hemisuccinate (CHS). The EGFP was removed by PreScission Protease cleavage, leaving

a stretch of ten residues on the C-terminus. Purified protein was then reconstituted into nanodiscs and subjected to cryo-EM analysis, resulting in a 2.6 Å resolution symmetric reconstruction (**Fig. 1a**, **Supplementary Fig. 1**) sufficient to build a model of the majority of the protein (537 out of 724 residues) (**Fig. 1b-c**, **Supplementary Fig. 2**, **Supplementary Table 1**). Due to increased flexibility of the BLD relative to previous OSCA structures, we were unable to assign residue identity, and instead modeled it as two poly-Ala helices.

Overall, there is good agreement between OSCA3.1, OSCA1.1 and OSCA1.2 protomers^{1,6} (**Fig. 1c**). The pore profile of these OSCAs is maintained and the small radii towards the extracellular side suggests these channels are in a closed/non-conductive state, as suggested by previous MD simulations^{1,6} (**Fig. 1d**). Likewise, the two π -helical turns in TM5 and TM6a seen in previous structures near the neck of the pore are present at similar positions in OSCA3.1^{1,8}. We identified multiple lipid-like densities in one of our postprocessed maps (**Fig. 1d**, **Supplementary Fig. 3**). Notably, one of these densities is located at the pore fenestration, at a position similar to that observed in previous MD simulations of OSCA1.2¹, supporting the proposed occupation of the pore pathway by lipids. Recent cryo-EM structures of AtOSCA1.2¹⁰, and AtOSCA1.1 and human TMEM63A¹¹ also place a lipid at a similar position.

Previously, the structure of OSCA3.1 was solved in a digitonin micelle⁶, and more recently, in LMNG¹¹. The similarity of our nanodisc sample to these detergent structures suggests that the use of detergent did not affect the conformation or oligomerization of OSCA3.1, similar to previous results with OSCA1.2^{1,7} (**Supplementary Fig. 4a-b**). However, the inter-subunit cleft in the nanodisc structure is wider due to outward movements of innermost TMs ranging from ~4–8 Å (**Supplementary Fig. 4c**) relative to the digitonin sample, which was recently denoted as an “extended” state¹¹. Thus, our nanodisc structure represents a further “extended” state than the one found both in digitonin and LMNG micelles¹¹ (**Supplementary Fig. 4d**). Whether these differences represent biologically meaningful states, an effect of the lipid environment (detergent vs lipids), or inherent flexibility of the channel remains to be determined. Additionally, our structure enabled a higher degree of certainty in the residue assignment relative to the initial digitonin structure, particularly for TM0 where sidechains were not previously modelled⁶. Consequently, we have shifted the registry of TM0 by two residues relative to the detergent structure, in agreement with the LMNG structures¹¹.

Mutation of key residues in the amphipathic helix abrogates poke but not stretch responses in OSCA1.2

Structures of AtOSCA1.1, rice and At OSCA1.2, and OSCA3.1 (**Fig. 2a**, **Supplementary Fig. 5**), as well as sequence alignment, suggest that the AH between TM0 and TM1 of OSCAs is a conserved feature of these channels. AHs can serve as both membrane anchors and sensors of membrane deformation¹², and they are present in many MA ion channels^{1,13–15}. There is evidence for their involvement in mechanical force transduction; for example, in MscL, an AH in the cytoplasmic side of the bilayer links membrane tension to protein conformation¹⁴. Interestingly, structures of members of the TMEM16 family, structural homologs of OSCAs, have a similarly placed helix in this region; however, it is located in the cytosol and does not present amphipathic properties (**Supplementary Fig. 6**)^{16–18}. Involvement of TMEM16A in the detection of mechanical forces in myocytes and bile ducts was dependent on calcium influx (potentially caused by an upstream MA cation channel) and not a direct response to the mechanical stimulus^{19,20}. To our knowledge, characterization of TMEM16 proteins as MA ion channels has not been reported. Based on these observations, we mutated the membrane-facing residues at each end of the AH of OSCA1.2 in an effort to decrease its propensity to interact with the membrane. Single (W75K or L80E) and double (W75K/L80E) mutants in OSCA1.2 strongly abrogated the response to indentation of the membrane with a blunt glass probe (poke) (WT: 47 of 51 cells produced MA currents; W75K: 3 of 24; L80E: 2 of 16; W75K/L80E: 2 of 24; **Fig. 2b**, top panel). Moreover, in OSCA1.2_{W75K/L80E}, a larger displacement of the membrane by the probe relative to the WT

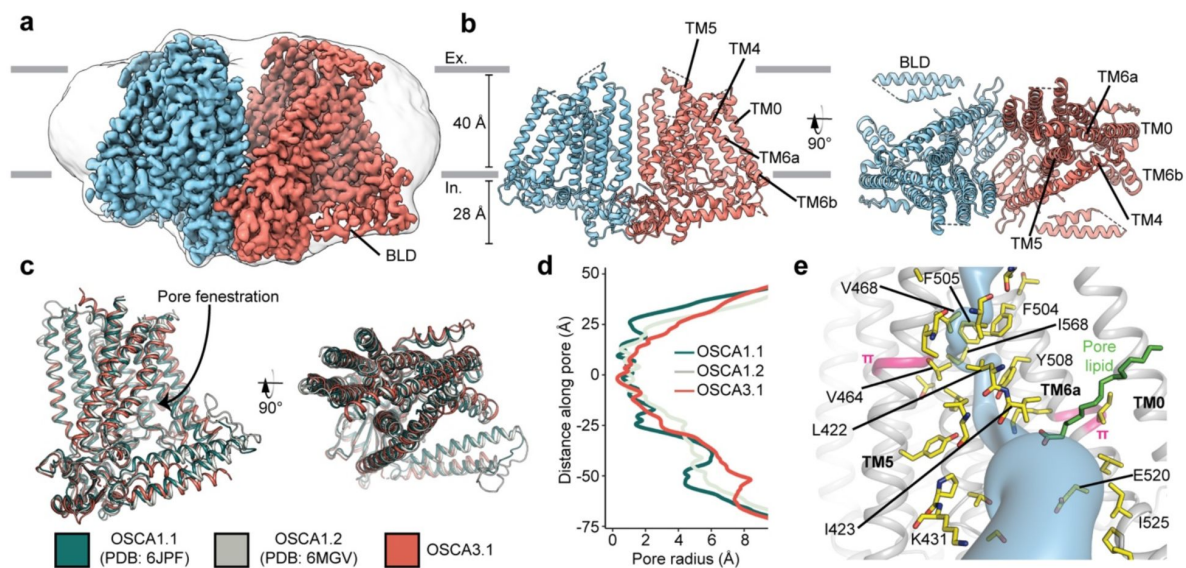


Figure 1

Cryo-EM structure and pore of OSCA3.1. **a**, Cryo-EM map of OSCA3.1 dimer colored by subunit. Nanodisc density in grey corresponds to the unsharpened map (gaussian-filtered to 1.5 σ). **b**, Front (left) and top (right) view of atomic model. Ex.: Extracellular, In.: Intracellular. **c**, Superposition of OSCA1.1, OSCA1.2 (in nanodiscs) and OSCA3.1 protomers. **d**, Pore profile of OSCAs in **c**. **e**, View of the pore pathway (blue) of OSCA3.1. Pore facing residues colored in yellow, with selected residues labeled. π -helical turns in pink. Putative pore lipid in green. Backbone of TM3 and TM4 helices hidden for clarity.

appeared to be needed to observe currents (“apparent threshold”; WT: $5.3 \pm 0.5 \mu\text{m}$ ($N = 46$; mean $\pm$ S.E.M.), W75K/L80E: $15.3 \pm 2.3 \mu\text{m}$ ($N = 2$); **Fig. 2b**, bottom panel). The insensitivity to the poking stimulus was not due to poor membrane trafficking of these constructs (see below). The loss of sensitivity of cells expressing these mutant constructs to poking suggests that the AH of OSCA1.2 plays an important role in this mechanically-activated response.

We then tested whether these mutations in the AH affected the response of OSCA1.2 to stretch. Contrary to what was observed during poking, all cell-attached patches revealed stretch-activated currents (WT: 25 of 26 cells; W75K: 3 of 3; L80E: 5 of 5; W75K/L80E: 11 of 11; **Fig. 2c**, top panel; electrode resistances were similar in all cases (see Figure legend)). Despite differences in the amplitude of macroscopic currents, which could be explained by variation in expression levels, all mutant channels were activated at similar thresholds (WT: $-48 \pm 5 \text{ mmHg}$ ($N = 25$); W75K: $-56 \pm 11 \text{ mmHg}$ ($N = 3$), L80E: $-41 \pm 6 \text{ mmHg}$ ($N = 5$), W75K/L80E: $-53 \pm 8 \text{ mmHg}$ ($N = 11$)) (**Fig. 2c**, bottom panel). In all cases, there was variability in the rate of inactivation from moderately fast to extremely slow (data not shown). Overall, the observed stretch response of OSCA1.2 was apparently not affected by the mutations we introduced.

Subsequently, we mutated the proline in the OSCA1.2 AH to the corresponding arginine in OSCA3.1 to determine whether this difference impacts the ability of OSCA3.1 to robustly transduce the poke stimulus; this change (OSCA1.2_{P77R}) had no effect on the poke response (**Supplementary Fig. 7**).

It is worth noting that in our initial characterization, OSCA3.1-expressing cells produced SAC while poke did not induce observable currents². However, in the present experiments we were able to record poke-activated currents mediated by OSCA3.1 on a few occasions (OSCA1.2_{WT}: 6 of 6 cells; OSCA3.1_{WT}: 3 of 10) (**Supplementary Fig. 8a**). The apparent threshold for OSCA3.1 activation by poke-induced membrane displacement tended to be higher than for OSCA1.2 but the difference was not statistically significant (OSCA1.2_{WT}: $10.4 \pm 1.2 \mu\text{m}$ ($N = 6$; mean $\pm$ S.E.M.); OSCA3.1_{WT}: $13.8 \pm 0.9 \mu\text{m}$ ($N = 3$)) (**Supplementary Fig. 8b**), perhaps due to the low numbers of responsive cells analyzed. These data suggest that the poking stimulus is capable of activating OSCA3.1 but the stimulus intensity required is close to the rupture point of the HEK293T-Piezo1-knockout (HEK-P1KO) cells used in our assay.

Replacing the OSCA3.1 Beam-Like Domain (BLD) in OSCA1.2 had no effect on stretch-induced currents but decreased the apparent sensitivity of the cells to poke

One of the unique features of OSCAs is the presence of the BLD, a hydrophobic hook that, by inserting itself into the membrane, may serve as an anchor that could potentially be displaced under tension^{1,7,8}. Hydrogen/deuterium exchange mass spectrometry (HDXMS) of rice OSCA1.2 indicated that the helix of the BLD closest to the membrane undergoes less deuterium exchange and potentially remains associated to a neighboring surface⁸, hinting at an important interaction with TM6b. In OSCA3.1, the BLD is poorly resolved, likely due to flexibility (**Fig. 1c**). To test whether the increased flexibility in this region affects MA responses, we replaced the BLD of OSCA1.2 with the BLD of OSCA3.1 (**Fig. 3a**). HEK-P1KO cells expressing the chimera OSCA1.2_{OSCA3.1-BLD} produced similar MA currents in both poke and stretch assays (**Fig. 3b,c**) with the exception that a slightly stronger displacement stimulus was required to activate the mutant channels (**Fig. 3b**, bottom panel). These data suggest that the BLD is not responsible on its own for the ability of the channels to be activated by poking, however, it appears to play a role. As expected for similar SAC from cells expressing OSCA1.2 and OSCA3.1, there was no obvious change in SAC when the OSCA1.2 BLD was replaced with the OSCA3.1 BLD. It should be noted that the BLD tolerates a certain degree of mutation as sequence alignment in this region shows higher variability than other secondary structures of OSCAs (**Supplementary Fig. 5**). Thus, we cannot rule out that in the OSCA1.2 background, the BLD of either channel is capable of performing a similar role.

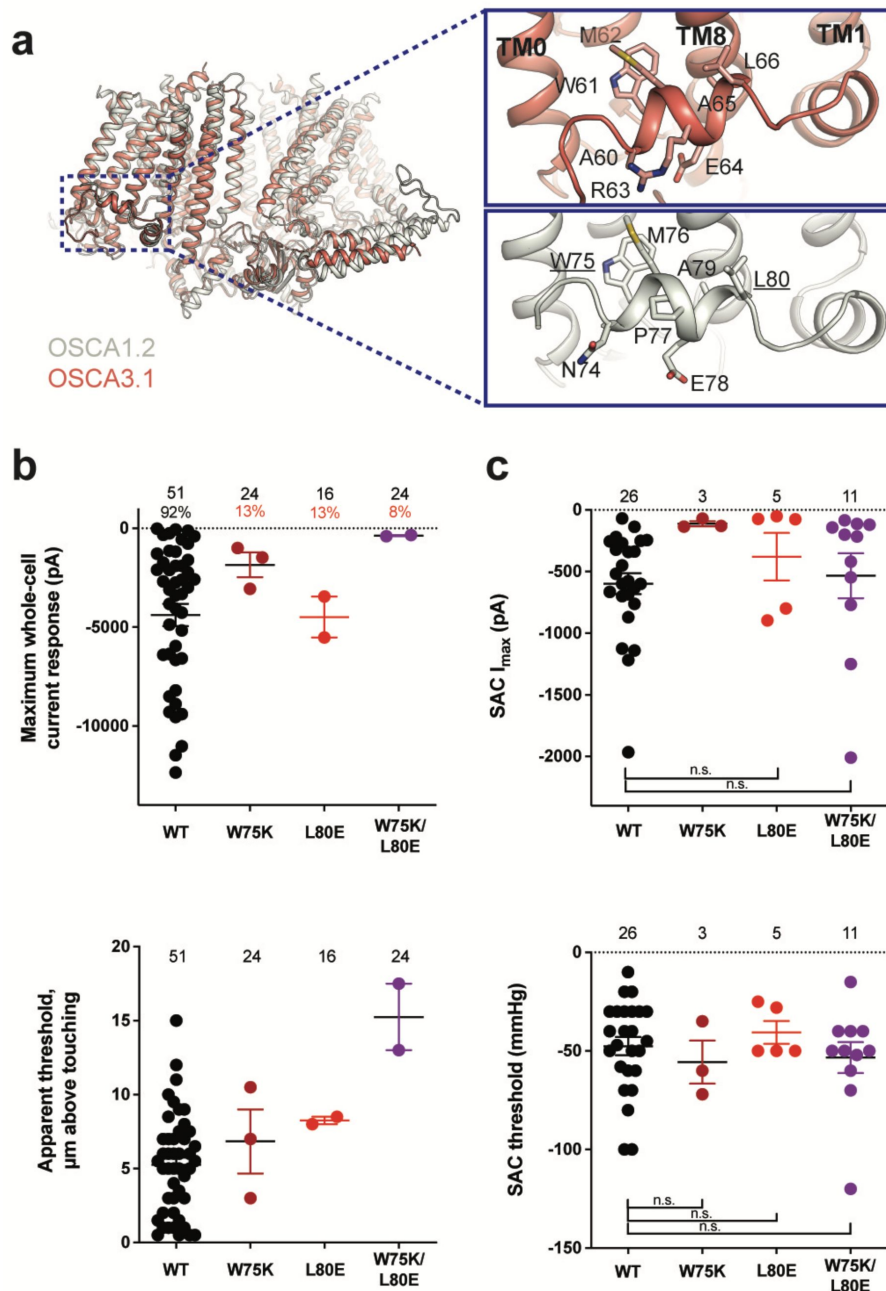


Figure 2

Amphipathic helix mutants of OSCA1.2. **a**, Superposition of OSCA1.2 (grey) and OSCA3.1 (red). Insets: close-up view of amphipathic helix. Residues substituted in OSCA1.2 for electrophysiology experiments are underlined. **b**, Few cells expressing mutations in the amphipathic helix respond to poking compared to WT controls. Top panel: Maximum poke-induced currents observed in whole-cell mode for cells exposed to displacements up to $8.9 \pm 0.6 \mu\text{m}$ ($N=51$; mean $\pm$ S.E.M.), $12.8 \pm 0.7 \mu\text{m}$ ($N=24$), $12.4 \pm 0.7 \mu\text{m}$ ($N=16$), and $12.8 \pm 0.5 \mu\text{m}$ ($N=24$) above touching for WT, OSCA1.2_{W75K}, OSCA1.2_{L80E}, and OSCA1.2_{W75K/L80E}, respectively. Bottom panel: the apparent threshold in μm above touching the cell for this cohort. **c**, SAC maximal current (I_{max}) (top) and mmHg threshold (bottom) from WT and OSCA1.2_{W75K/L80E}-expressing cells reveal no significant differences in the ability of negative pressure to activate channels in cell-attached patches (Student's t -test). Also shown are data from single mutants OSCA1.2_{W75K} and OSCA1.2_{L80E}. Too few patches were obtained for OSCA1.2_{W75K} to compare I_{max} . Electrode resistances for **c** were similar in all cases (WT: $2.3 \pm 0.1 \text{ M}\Omega$ ($N=26$); W75K: $2.1 \pm 0.4 \text{ M}\Omega$ ($N=3$), L80E: $2.2 \pm 0.1 \text{ M}\Omega$ ($N=5$), W75K/L80E: $1.8 \pm 0.1 \text{ M}\Omega$ ($N=11$)). For panels **b-c**: individual cells are represented as scatter points; mean and S.E.M. are displayed.

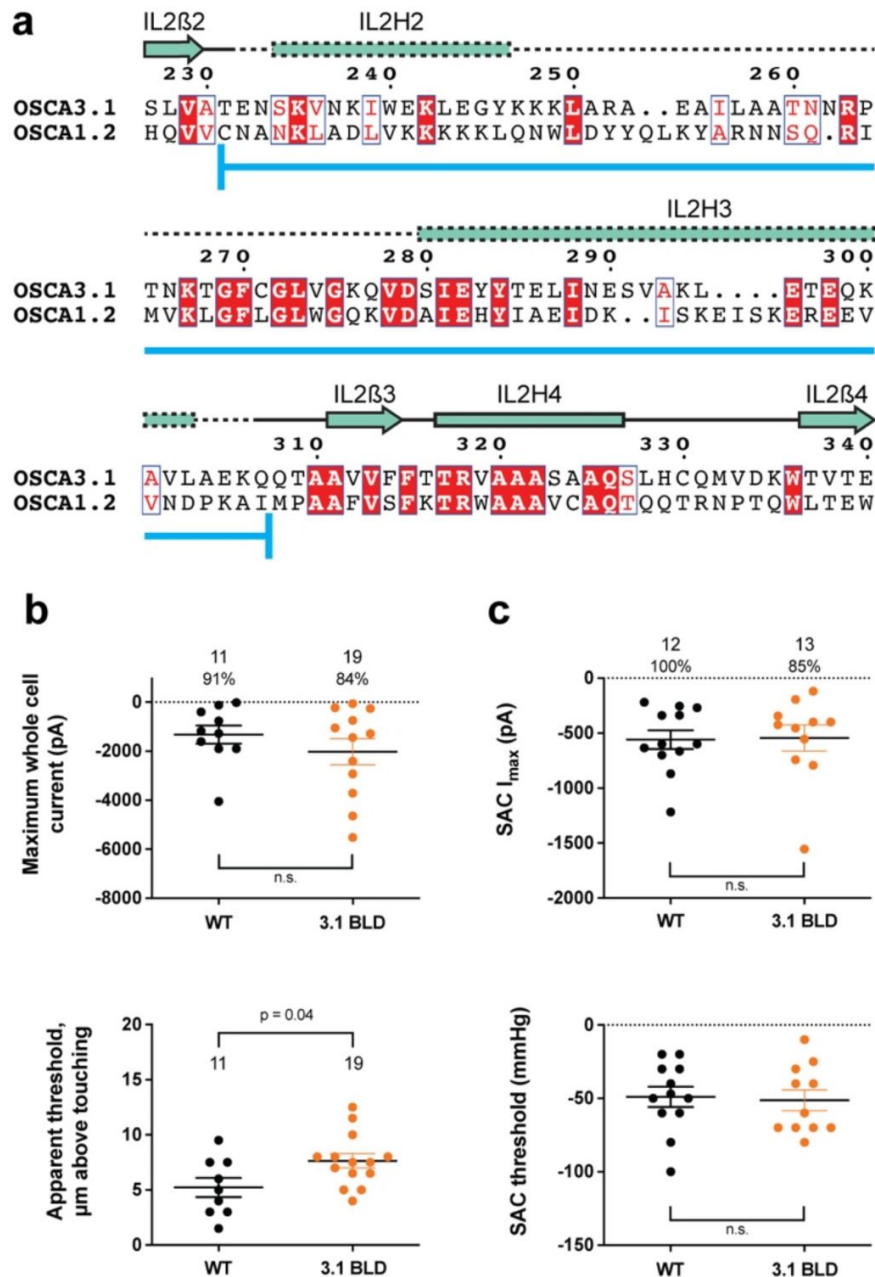


Figure 3

OSCA1.2_{OSCA3.1-BLD} chimera. a,

Amino acid sequence alignment of the BLD region of OSCA1.2 and OSCA3.1. Full alignment in **Supplementary Fig. 3**. Blue line at bottom of sequences denotes the sequence swapped in OSCA1.2_{OSCA3.1-BLD} chimera. **b**, Poke-induced responses are observed in HEK-P1KO cells expressing OSCA1.2_{3.1-BLD}. Top panel: Maximum poke-induced currents observed in whole-cell mode for cells exposed to displacements up to $9.8 \pm 0.8 \mu\text{m}$ (N=11; mean $\pm$ S.E.M.) and $11.8 \pm 0.6 \mu\text{m}$ (N=19) above touching for WT and OSCA1.2_{3.1-BLD}, respectively. The percentage of cells with responses are shown above. Bottom panel: the apparent threshold in μm above touching the cell for this cohort. **c**, SAC I_{max} (top) and mmHg threshold (bottom) from WT and OSCA1.2_{3.1-BLD}-expressing cells reveal similar activity induced by negative pressure in cell-attached patches. Data shown were obtained from the same experiments. Electrode resistances for **c** were similar in all cases (WT: $2.4 \pm 0.1 \text{ M}\Omega$ (N=12); OSCA1.2_{OSCA3.1-BLD}: $2.5 \pm 0.1 \text{ M}\Omega$ (N=13)).

Substitution of potential lipid-interacting lysine residues for isoleucine abrogates the poke response in OSCA1.2

Lipids play an important role in the stability and function of ion channels²¹. In TRAAK, a eukaryotic mechanosensitive channel, occlusion of the pore by a lipid acyl chain has been proposed as a gating mechanism^{13,22}. In the volume-regulated channel SWELL1 lipids block the pore in the closed state²³. In the bacterial mechanosensitive channel MscS, the occupation of lipid pockets may determine channel conformation^{24,25}, and in FLYC1, a Venus flytrap homolog of MscS, conformational changes may allow lipids to access the pore and occlude ion conduction²⁶. Moreover, exposure of inside-out patches from OSCA1.1-expressing HEK cells to lyso-phosphatidylcholine (LPC) enhanced channel response to stretch⁶.

Previously, we identified four lysine residues in OSCA1.2 located in TM4 and TM6b at the pore fenestration. These residues interacted with lipid phosphate head groups throughout the duration of our MD simulations¹. The OSCA3.1 structure revealed that two of these lysine residues (K435 and K536 of OSCA1.2) have been replaced by isoleucine (I423 and I525, respectively), which are expected to reduce the interaction with lipid phosphate head groups (Fig. 4a). To test for the contribution of these lipid-interacting residues to channel mechanosensitivity, we measured poke- and stretch-induced currents in the double mutant OSCA1.2_{K435I/K536I} (Fig. 4b,c). The double mutant responded poorly to poking, with only 1 out of 20 cells producing currents upon mechanical stimulation (K435I/K536I: -365 pA (N = 1)) (Fig. 4b, comparison made to WT data acquired on the same days). This finding was not due to poor expression of the double mutant in HEK-P1KO cells; the SAC $I_{\max}$ observed in cell-attached patches was similar to WT (Fig. 4c, top panel). Furthermore, the mutant could be activated by stretch at similar thresholds to WT (WT: -43 ± 10 mmHg (N = 6); K435I/K536I: -60 ± 10 mmHg (N = 7)) (Fig. 4c, bottom panel), and similar stretch-response dependence (i.e., similar pressures required to elicit half-maximal currents (P_{50}) and slope) (Fig. 4d). Although macrocurrent amplitudes in SAC recordings tended to be lower than WT tested on the same day, the ~2-fold difference unlikely accounts for the ~20-fold fewer responsive cells (Fig. 4b,c). Taken together, these results suggest that loss of positively charged sidechains in the fenestration selectively impairs poke-induced responses of OSCA1.2 under physiological conditions, but not its responsiveness to the stretch stimulus.

Discussion

Here we performed structure-function studies to determine the molecular underpinnings of mechanical activation of OSCA ion channels. We solved the structure of OSCA3.1 at a resolution of 2.6 Å in a non-conductive state and revealed a marked structural conservation to previous OSCA orthologs. The final map presented several lipid-like densities surrounding the channel, including one density close to the pore and several densities populating the inter-subunit cleft. This ion channel was reported to be activated by stretch in cell-attached patches but insensitive to a poke stimulus using a blunt glass probe². Comparisons between the structures of OSCA1.2, which is robustly activated by both poke and stretch, to OSCA3.1, which is only robustly activated by stretch, suggested 3 domains that might contribute to gating during a poke stimulus. First, a conserved short amphipathic helix (AH) which sits at the membrane interface reminiscent of a similar feature in other MA ion channels like MscL¹⁴, Piezo1¹⁵, MscS²⁷, and TRAAK²⁸. Second, side chains in TM4 and TM6b in the vicinity of the membrane fenestration may have differential interactions with lipids as indicated by MD simulations of OSCA1.2. Third, the beam-like domain (BLD) that contains 2 helices and linker that hooks around the cytoplasmic surface into the membrane in OSCA1.2 but is less well-resolved in the OSCA3.1 map presumably because it is not intimately associated with the channel/membrane.

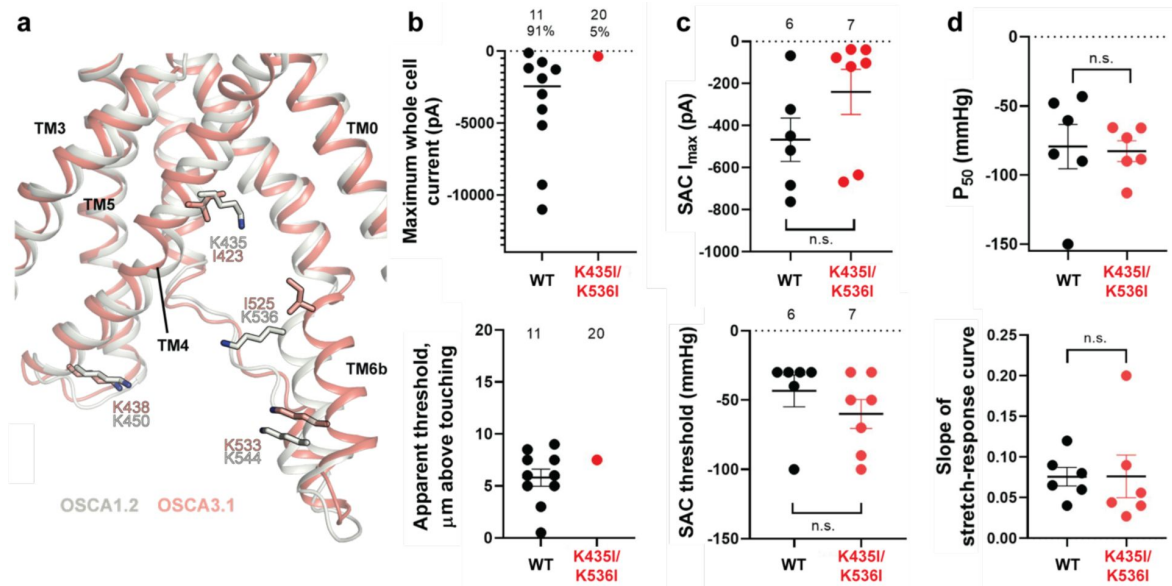


Figure 4

Probing the functional role of the potential lipid-interacting residues. a,

Superposition of OSCA1.2 and OSCA3.1 around the pore fenestration. Residues of OSCA1.2 predicted to interact with lipids and corresponding residues in OSCA3.1 are shown. **b**, Few OSCA1.2_{K435I/K536I}-expressing HEK-P1KO cells respond to the poke stimulus while nearly all cells expressing OSCA1.2 WT channels respond (percentages shown above under the N of cells tested). Top panel: Maximum poke-induced currents observed in whole-cell mode for cells exposed to displacements up to $9.7 \pm 1 \mu\text{m}$ (N=11; mean $\pm$ S.E.M.) and $12.1 \pm 0.6 \mu\text{m}$ (N=20) above touching for WT and OSCA1.2_{K435I/K536I}, respectively. Bottom panel: the apparent threshold in μm above touching the cell for this cohort. **c**, SAC I_{max} (top) and mmHg threshold (bottom) from WT and OSCA1.2_{K435I/K536I}-expressing cells reveal no significant differences in the ability of negative pressure to activate channels in cell-attached patches (Student's *t*-test). Electrode resistances were similar in all cases (WT: $2.1 \pm 0.2 \text{ M}\Omega$ (N=10); K435I/K546I: $1.7 \pm 0.3 \text{ M}\Omega$ (N=7)). Data were collected within 15 min of exposure of cells to high K^+ used in this assay. **d**, Analysis of stimulus-response relationships reveal no significant differences (Student's *t*-test) in pressure to half-maximal activation (P_{50} ; top) or the slope of the curve (bottom). Whole cell and SAC data were obtained from cells transfected at the same time.

Mutagenesis of the AH and residues at the fenestration in OSCA1.2 largely abrogated its response to poking without affecting its activation by stretch; replacing the BLD with that from OSCA3.1 only slightly impaired the response to poking, having no effect on stretch-induced activation.

Residues in the AH were mutated to either confer more of an OSCA3.1 character or tend to make the domain less likely to interact with the membrane, similar to the TMEM16 family that are not thought to be directly activated by mechanical stimuli. Swapping the divergent residue in the AH of OSCA1.2 with its equivalent in OSCA3.1 (OSCA1.2_{P77R}), had no negative impact on the sensitivity to poking. However, when mutations were incorporated to presumably destabilize the interaction of the AH domain with the membrane (OSCA1.2_{W75K/L80E}), fewer cells could be activated by poking to similar stimulus strengths while stretch-activation of channels was not impaired. Although a WT AH is critical for normal poke-induced channel opening, these mutations do not hinder gating by membrane stretch.

Substituting the BLD of OSCA1.2 for that of OSCA3.1 had little effect on poke- or stretch- activated responses. Although these results suggest that the BLD may not be involved in modulating the MA response of OSCA1.2, a recent report suggested evolutionary coupling of 3 sets of residues in the BLD and TM6b that may form electrostatic interactions between these domains¹⁰. The evolutionarily favored residue identities at these sites constitute three potential salt bridges: **R/K257-E557**, **E260-R553**, and **R/K261-E/D554** (based on OSCA1.2 numbering).

These sites in OSCA1.2_{OSCA3.1-BLD} chimera correspond to N257**K**-E557, D260**A**-K553, and Y261**R**-D554 (mutation-interacting residue), perhaps leaving intact 2 out of 3 potential salt bridges and maintaining its influence (**Supplementary Fig. 5**). It is worth noting that a similar chimera of AtOSCA1.2 with the IL2 domain of the human ortholog TMEM63A (OSCA1.2_{63A IL2}), containing most of the intracellular domain which includes the BLD as well as the dimerization domain, presented little inactivation of SAC akin to monomeric TMEM63AWT²⁹. This behavior was not due to an inability of OSCA1.2_{63A IL2} to dimerize, as the monomeric OSCA1.2_{5Mu}, obtained by disrupting the dimer interface with 5 mutations while maintaining OSCA1.2_{WT} BLD, exhibited pressure-induced SAC gating similar to OSCA1.2²⁹. This new evidence, taken together with the decrease in the apparent poking sensitivity of the OSCA1.2_{OSCA3.1-BLD} chimera in this study, suggests that the BLD plays a role in the force-induced gating of the OSCA family.

The structure of OSCA3.1 exhibits an open fenestration similar to OSCA1.2 and lipids are expected to populate this area¹, similar to lipid occlusion of the pore in other MA ion channels such as TRAAK¹³ and MscS^{24,25}. Our results suggest that TM4 and TM6b interactions with lipids may contribute to activation during poking. Modification of OSCA1.2 residues to match those of OSCA3.1 (OSCA1.2_{K435I/K536I}) produced an OSCA3.1-like phenotype (MA currents are observed in response to stretch but rarely to poke). These mutant channels are not completely insensitive to poking but appear to have a very high threshold to activate, one that is often beyond the point of cell rupture by the stimulus (similar to that seen with OSCA1.2_{W75K/L80E}). Notably, TM4 in TMEM16 lipid scramblases and TM6 in both TMEM16 scramblases and channels undergo conformational changes during activation^{16,17,30}. The loss of positive charges in OSCA3.1 may weaken the interactions between residues and lipids, similar to MscS where charge neutralization of the lipid facing residue R59 by introduction of a leucine strongly altered gating, potentially by disturbing a salt bridge with a lipid phosphate^{31,32}. A weaker interaction could decrease the tension generated by the lipids upon mechanical stimulus, hence increasing the force needed to open the channel, as seen in our poke experiments. A similar argument could be made for the AH of OSCAs: by disturbing its coupling with the lipid bilayer, a greater stimulus might be needed to activate the channel.

The phenotypes of OSCA1.2_{W75K/L80E} and OSCA1.2_{K435I/K536I} suggest a mechanism recruited by poking is no longer able to adequately activate OSCA1.2_{W75K/L80E} and OSCA1.2_{K435I/K536I} channels. Since the factors contributing to channel activation in the poking assay are not understood, we can

only speculate as to mechanisms underlying the loss of sensitivity to poking in these mutants. Our results suggest that these stimuli are sensed by different features of OSCA channels, akin to the well-studied ThermoTRP channels^{33–35}. Polymodal activation mechanisms have been recently reported for the MA channel PIEZO2 where a mutagenesis approach revealed differential effects on the ability to respond to poking and stretch³⁶. Nonetheless, the discrepancy could be due to inherent methodological differences between these two assays, as whole-cell recordings during poking involve channels in inaccessible membranes (at the cell-substrate interface) and channel interactions with extracellular and intracellular components³⁷, while the stretch assay is limited to recording channels inside the patch.

Overall, the analysis of these mutants demonstrates the involvement of the AH and residues at the membrane fenestration in the MA response of OSCAs, as we initially proposed¹. The participation of residues from TM4 and TM6 hint at further similarities with TMEM16 family, but to what extent is unknown. Perhaps the most surprising result is the selective disruption of poke-activated currents of OSCA1.2 without affecting its response to membrane stretch, which suggests that changes in these features could serve to fine-tune the response to certain mechanical stimuli and may be a source of functional diversity within this large family. The structures and characterizations presented here and in our previous work^{1,2} will prove fundamental in assessing the physiological consequences of these differences and further understanding the role of the OSCA/TMEM63 family.

Materials and methods

Expression constructs

For structural studies, human codon-optimized OSCA3.1 (UniProt ID: Q9C8G5) was cloned into the pcDNA3.1 vector used in our previous report¹. In this plasmid, the C terminus of the protein sequence is followed by a PreScission Protease cleavage site, EGFP and a FLAG tag.

Protein expression and purification

Samples of OSCA3.1 in nanodisc were obtained following the same method reported previously for OSCA1.2 in nanodiscs¹, with the exception of the detergent used. Instead of beta-D-maltopyranoside (DDM), we used Lauryl Maltose Neopentyl Glycol (LMNG) due to increased yields. Thus, the detergent composition of the solubilization and wash buffers was 1%/0.1% and 0.01%/0.001% LMNG/cholesteryl hemisuccinate (CHS), respectively. Fractions from Size Exclusion Chromatography (SEC) corresponding to the OSCA3.1 in nanodiscs peak were concentrated to 3.2mg/mL (corrected using the extinction coefficient of OSCA3.1).

Cryo-EM sample preparation and data collection

3.5 μ L of OSCA3.1 at 3.2mg/mL were frozen using the same conditions as OSCA1.2 samples¹. Grids were imaged on a Titan Krios (ThermoFisher) operating at 300 kV with a K2 Summit direct electron detector (Gatan), with pixel size of 1.03Å (nominal magnification of 29,000x). A total of 38 frames were collected per movie, adding to a total dose of ~50 electrons per Å². Automated image collection was done through Leginon³⁸ using a defocus range of -0.4 to -1.5 μ m. A total of 8,375 movies were collected.

Cryo-EM image processing

During collection, movies were aligned and dose-weighted using MotionCor2³⁹. The resulting micrographs were imported into cryoSPARCv2.5⁴⁰ and CTF values were estimated through Gctf⁴¹.

~300 particles were manually picked from ~10 micrographs and subjected to 2D classification to obtain templates. Template picking was tested on 20 micrographs and particles used to generate better 2D templates. These templates were later used to pick particles on ~1600 micrographs. The resulting ~900k particles were subjected to, in order, 2D classification, *ab initio* reconstruction, 2 rounds of heterogeneous refinement, homogeneous refinement and non-uniform refinement to obtain an initial 3D reference, which reached high-resolution. Unless otherwise specified, C2 symmetry was applied to all homogeneous, non-uniform and RELION 3D refinements.

For the full dataset, 8,375 movies were imported into cryoSPARCv2 and subjected to full-frame motion correction followed by Gctf estimation. Given the large size of the dataset, and the fact that initial processing had returned a high-resolution structure, we decided to apply a 2.6 Å CTF cutoff to reduce the number of micrographs to 3,068 and speed up processing. Templates were generated based on the initial 3D reference. Template picking, followed by local motion correction and 2D classification resulted in 1,913,316 selected particles for our initial stack. *Ab initio* reconstruction produced 3 classes, and heterogeneous refinement was performed on the two worst classes. The resulting best class was combined with the best class from *ab initio* and used for two rounds of heterogeneous and homogeneous refinements. The heterogeneous refinement of the first round was done without symmetry, from that point onwards, C2 symmetry was imposed unless otherwise specified. Only particles belonging to the best class were selected for further processing (318,249). Non-uniform refinement, followed by 2 rounds of local refinement (without imposing symmetry), were performed on these particles before exporting them into RELION-3.1^{42,43}. The particle stack underwent the following process in RELION. 3D refinement, CTF refinement, 3D refinement with global angular searches, 3D refinement using SIDESPLITTER (SS)⁴⁴, and two rounds of CTF and 3D refinements using SS. Unless specified, all 3D refinements were limited to local angular searches. 3D classification without alignment (no imposed symmetry) was performed on the particle stack and the best class, comprising 197,944 particles, was subjected to a final 3D refinement with SS. The map was sharpened separately with LocalDeblur⁴⁵ through Scipion^{46–48} and DeepEMhancer using the ‘highRes’ model⁴⁹. The FSC and local resolution of the map was calculated through RELION.

Model building and refinement

A homology model for OSCA3.1 was obtained through SWISS-MODEL⁵⁰, using as template the structure of OSCA1.2 in nanodiscs (PDB: 6MGV)¹. Iterative rounds of building in Coot^{51,52} and real space refinement in Phenix^{53,54} and Rosetta⁵⁵ were used to generate the final model. SMILES codes for the ligands were used to obtain the appropriate restraints using eLBOW⁵⁶. Model validation was carried out with MolProbity⁵⁷ and EMringer⁵⁸ to the LocalDeblur map. Phenix mtriage⁵⁹ was used to calculate the map to model FSC. The final model comprises residues 2-102, 154-231, 307-388, 410-476, and 489-697, for a total of 537 out of 724 residues in OSCA3.1 sequence. A segment of the BLD was modelled as poly-A helices and registry was tentatively assigned based on sequence and structure alignment to OSCA1.2¹: residues 234-246 and 280-303 assigned as IL2H2 and IL2H3, respectively. Pore profiles were predicted by CHAP^{60,61}. Structure figures were made with PyMOL⁶², UCSF Chimera⁶³ or UCSF ChimeraX⁶⁴. Amino acid sequence alignment was obtained from Clustal omega⁶⁵ and represented with ESPript3⁶⁶. Sequences used for the alignment were the same as previous publication¹, but the display was limited to the *Arabidopsis thaliana* OSCAs with solved structure.

Generation of mutants, cell culture and transfections

A vector containing OSCA1.2 pIRES2-mCherry was used for electrophysiology experiments². The W75K substitution was introduced using Q5 site-directed mutagenesis kit (New England Biolabs, NEB). Substitutions L80E, K435I, and K536I were introduced using QuikChange multi site-directed mutagenesis kit (Agilent). For the BLD chimera, OSCA1.2 pIRES2-mCherry vector and the BLD of

OSCA3.1 were separately amplified by PCR using Q5 High-Fidelity 2X Master Mix (NEB), followed by fragment assembly using Gibson Assembly Master Mix (NEB). Kits were used according to manufacturer's instructions.

Cell culture and transfection of HEK-P1KO cells for electrophysiology experiments were conducted as previously reported¹[↗](#).

Electrophysiology

Patch-clamp experiments in transiently transfected HEK-P1KO cells were performed in standard whole-cell and cell-attached mode using a Multi-clamp700A amplifier (Axon Instruments) and followed the procedures described in our previous report²[↗](#).

Data availability

Cryo-EM maps of OSCA3.1 in nanodiscs has been deposited to the Electron Microscopy Data Bank (EMDB) under accession number EMD-41911 with the LocalDeblur map as the primary map. The corresponding atomic coordinates have been deposited to the Protein Data Bank (PDB) under ID 8U53.

Author contributions

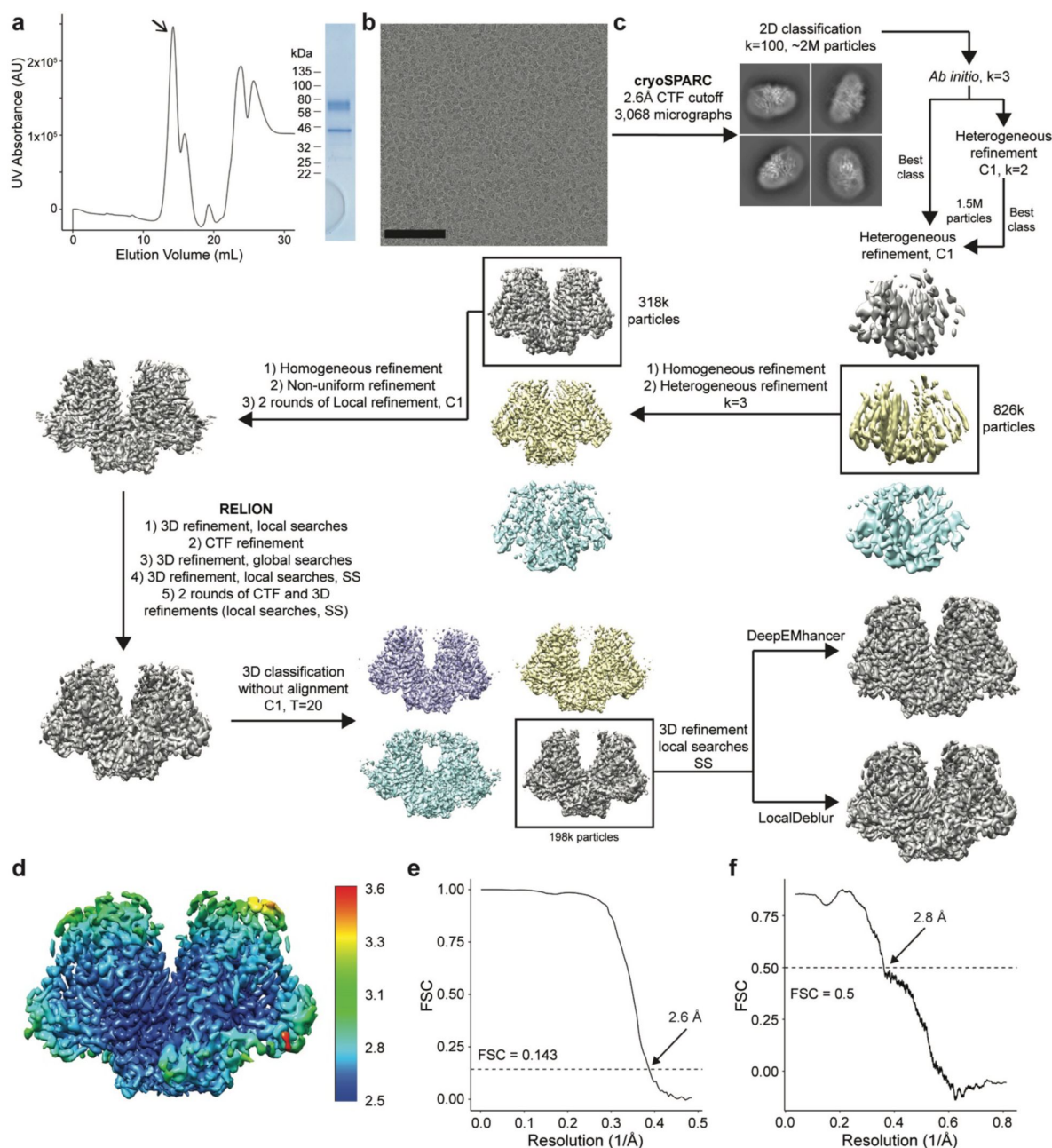
S.J.C. and W.H.L. cloned expression constructs and mutants. S.J.C. prepared protein samples and acquired cryo-EM data. S.J.C. processed data and built and refined the atomic structures. A.E.D. carried out electrophysiology experiments and analyzed data. A.B.W. supervised the project.

S.J.C. and A.E.D. drafted the manuscript, which was edited and finalized with contributions from all authors.

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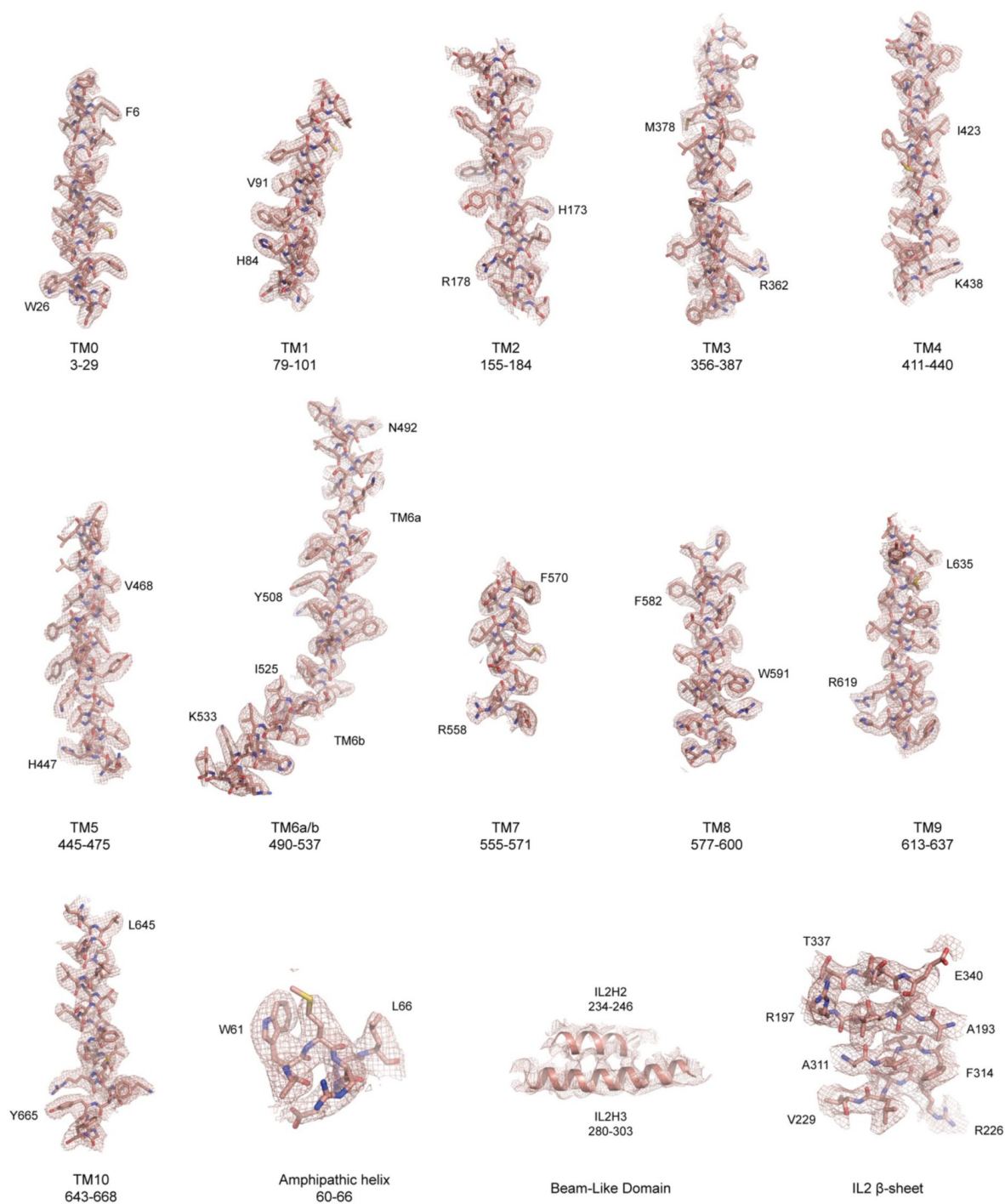
Supplemental figures



Supplementary Figure 1

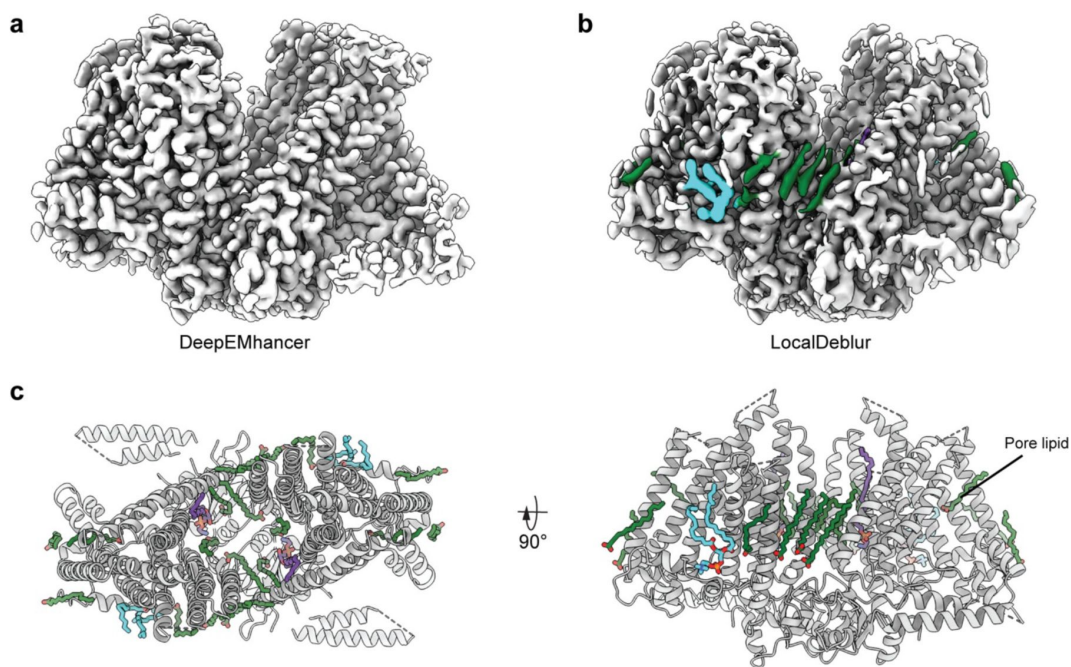
Purification and cryo-EM data processing of OSCA3.1. a,

SEC trace (left) from OSCA3.1 reconstitution in nanodiscs. Arrow points to peak corresponding to fractions pooled and ran on SDS-PAGE (right). Molecular weight of OSCA3.1 and MSP2N2 is approximately 82kDa and 43kDa, respectively. **b**, Representative cryo-EM micrograph. Black bar is 100nm. **c**, Cryo-EM processing workflow. C2 symmetry was imposed during refinements unless otherwise specified. 3D refinements in RELION were performed using the SIDESPLITTER algorithm where indicated (SS). **d**, LocalDeblur map colored by local resolution calculated in RELION. **e**, FSC plot calculated in RELION. **f**, LocalDeblur map to model FSC plot.



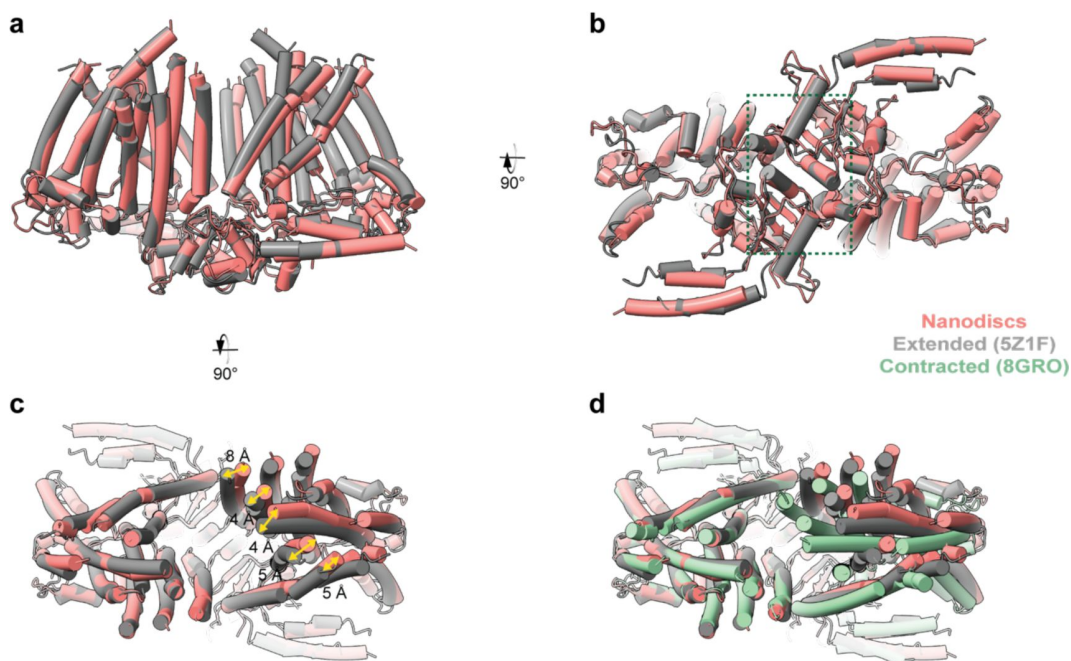
Supplementary Figure 2

Fit of OSCA3.1 model to LocalDeblur map. Fit of OSCA3.1 model to selected regions of the LocalDeblur map. Map was contoured at a threshold of 2 (for the BLD) or 4 σ .



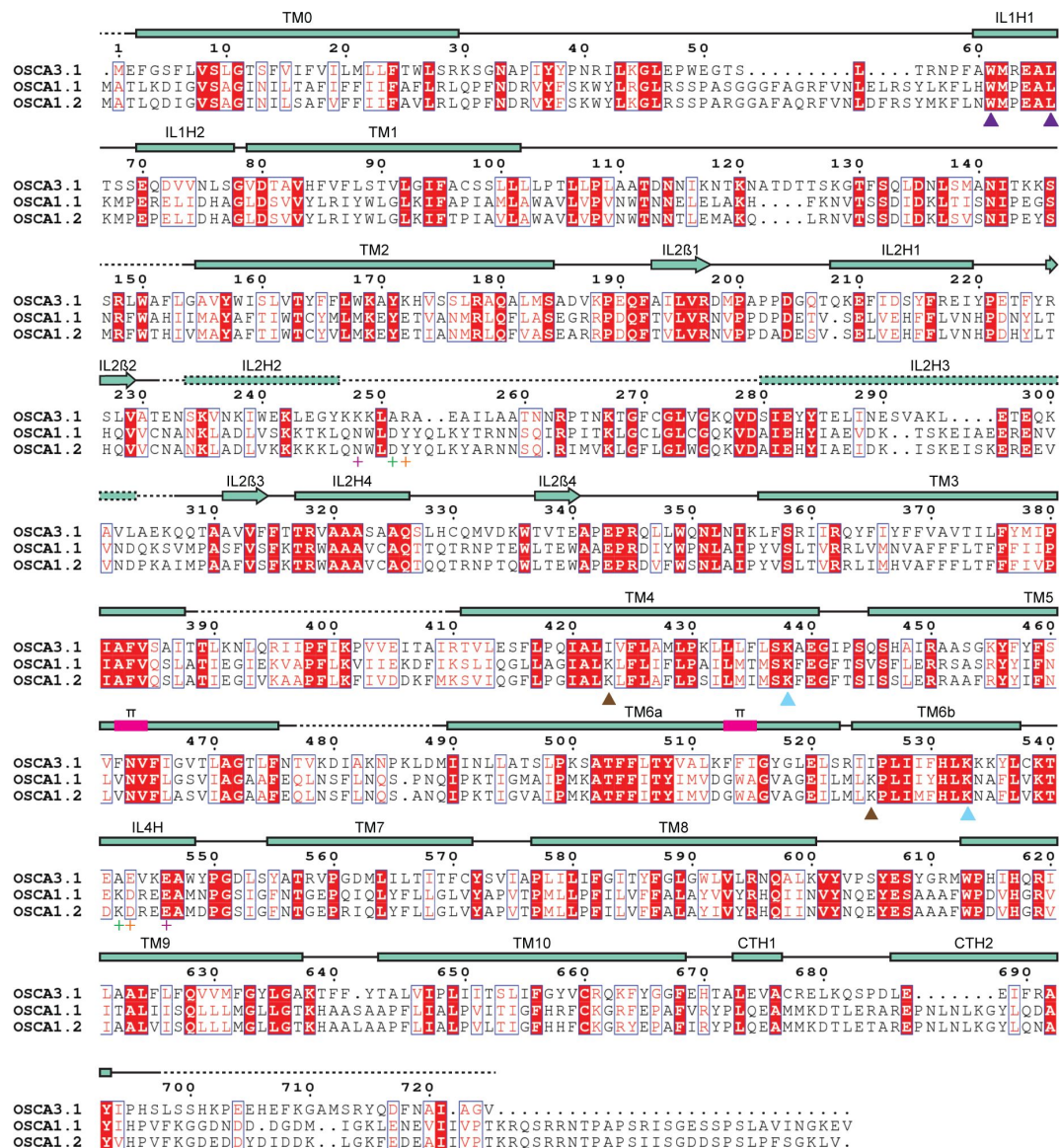
Supplementary Figure 3

Comparison of DeepEMhancer and LocalDeblur postprocessed maps. **a**, DeepEMhancer map. **b**, LocalDeblur map with lipid-like densities colored. **c**, Top (left) and front (right) view of OSCA3.1 model with modelled lipids. Lipids were tentatively modelled as palmitic acid (green), phosphatidylcholine (cyan), and lyso-phosphatidylcholine (purple).



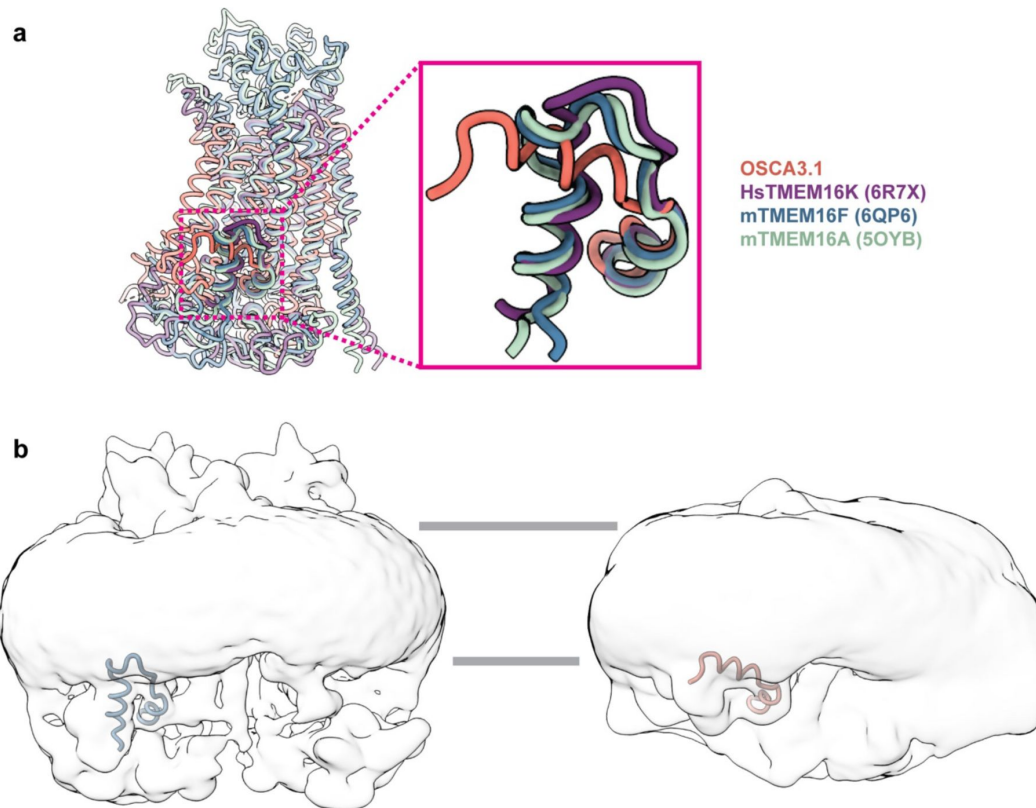
Supplementary Figure 4

Comparison of OSCA3.1 in nanodiscs and detergent states. **a**, Front view of model superposition of OSCA3.1 in nanodiscs and extended state in digitonin (PDB: 5Z1F). Alignment done on left protomer. **b**, Intracellular view. Dimerization domain is enclosed in a green square. **c**, Extracellular view illustrating shifts of the nanodisc sample towards the outer edges, and increasing the distance between the two protomers in the inter-subunit cleft. **d**, Extracellular view of the OSCA3.1 in nanodiscs, extended state in digitonin, and contracted state in LMNG (PDB: 8GRO).



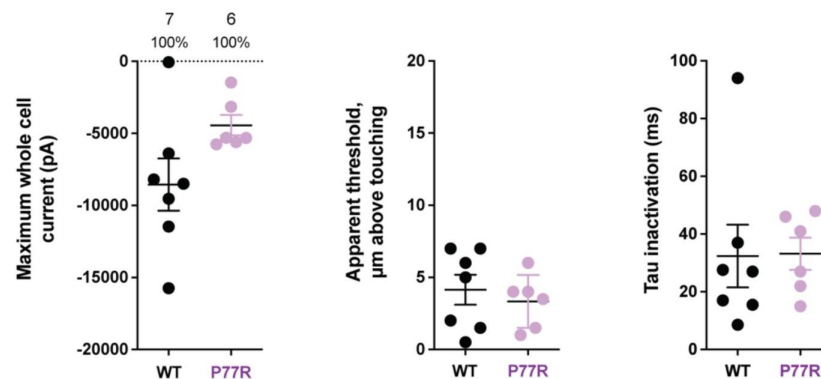
Supplementary Figure 5

Protein sequence alignment of OSCA3.1. Amino acid sequence alignment of *Arabidopsis thaliana* OSCA3.1, OSCA1.1 and OSCA1.2. Secondary structure of OSCA3.1 represented on top, where rectangles represent a helices and arrows β strands. Dashed lines correspond to regions not modelled. π -helical turns labeled in magenta. IL2H2 and IL2H3 were modelled as poly- Ala and the registry was assigned tentatively. Purple arrowheads point to residues in the amphipathic helix that were mutated in OSCA1.2 for electrophysiology experiments. Predicted lipid-interacting residues denoted by light blue (conserved between OSCA1.2 and OSCA3.1) or brown (not conserved) arrowheads. Evolutionarily coupled residues marked by plus sign (+), with each pair having the same color. TM: Transmembrane; IL: Intracellular Loop; CT: C-terminal; H: Helix; β : β -strand.



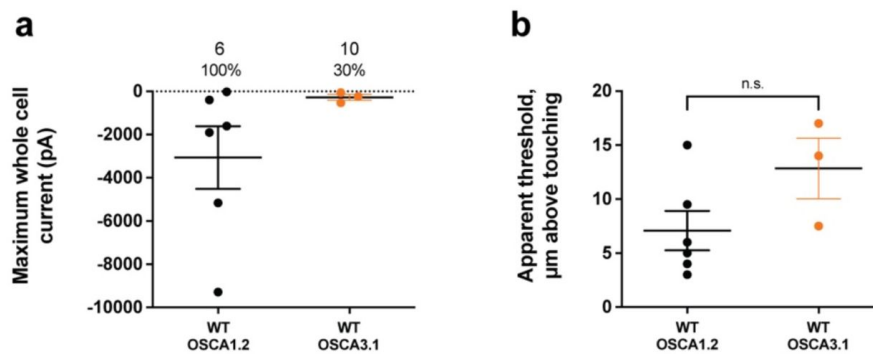
Supplementary Figure 6

Comparison of amphipathic helix of OSCA3.1 with corresponding helices in TMEM16 structures. **a**, Superposition of one protomer of OSCA3.1 and selected members of the homologous TMEM16 family. Inset: zoom-in view of amphipathic helix of OSCA3.1 and homologous intracellular helix of TMEM16 homologs. Hs: *Homo sapiens*; m: mouse. **b**, EM density of mTMEM16F (left, EMDB: 4611) and unsharpened density of OSCA3.1 (right) showing position of respective helices shown in **a**. A gaussian-filter of 2σ was applied to the densities. Horizontal gray lines denote the approximate position of the membrane bilayer.



Supplementary Figure 7

Poke-induced response of OSCA1.2_{P77R}. Introduction of arginine from OSCA3.1 into the OSCA1.2 background did not alter the poke-induced response. Left panel: Maximum poke-induced whole-cell currents observed for cells exposed to displacements up to $7.2 \pm 1.4\ \mu\text{m}$ ($N=7$; mean $\pm$ S.E.M.) and $6.5 \pm 0.6\ \mu\text{m}$ ($N=6$) above touching for WT and OSCA1.2_{P77R}, respectively. Center panel: the apparent threshold in μm above touching for the cells in this cohort. Right panel: Time constant of inactivation of currents elicited in the cells in this cohort.



Supplementary Figure 8

OSCA3.1 may be poke-sensitive at high thresholds.

Poke-induced whole-cell current responses are observed in HEK-P1KO cells expressing OSCA3.1. **a**, Maximum poke-induced currents observed in whole-cell mode for cells exposed to displacements up to $10.4 \pm 1.2 \mu\text{m}$ ($N=6$; mean $\pm$ S.E.M.) and $13.8 \pm 0.9 \mu\text{m}$ ($N=3$) above touching for OSCA1.2_{WT} and OSCA3.1_{WT}, respectively. Although 6 of 6 OSCA1.2 expressing cells responded to poking, only 3 of 10 showed a response before the disruption of the recording. **b**, The apparent threshold in μm above touching the cell for this cohort.

OSCA3.1	
Data collection and processing	
Magnification	29000
Voltage (kV)	300
Electron exposure (e ⁻ /Å ²)	50
Defocus range (μm)	-0.4 to -1.5
Pixel size (Å)	1.03
Initial particle images (no.)	1,913,316
Symmetry imposed	C2
Final particle images (no.)	197,944
Map resolution (Å)	2.6
FSC threshold	0.143
Map sharpening B factor (Å ²)	-64
Model	
Composition	
Peptide chains	2
Protein residues	1148
Ligands	24
R.m.s. deviations	
Bond lengths (Å)	0.023
Bond angles (°)	1.677
Validation	
MolProbity score	0.74
Clashscore	0.74
EMRinger score	4.12
Poor rotamers (%)	0.00
Ramachandran plot	
Favored (%)	98.93
Allowed (%)	1.07
Disallowed (%)	0.00
Deposition ID	
EMDB	41911
PDB	8U53

Supplementary Table 1.

Data collection, processing, model refinement and validation.

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Editors

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Reviewer #1 (Public Review):

Summary:

The OSCA/TMEM63 channels have recently been identified as mechanosensitive channels. In a previous study, the authors found that OSCA subtypes (1, 2, and 3) respond differently to stretch and poke stimuli. For example, OSCA1.2 is activated by both poke and stretch, while OSCA3.1, responds strongly to stretch but poorly to poke stimuli. In this study, the authors use cryo-EM, mutagenesis, and electrophysiology to dissect the mechanistic determinants that underlie the channels' ability to respond to poke and stretch stimuli.

The starting hypothesis of the study is that the mechanical activation of OSCA channels relies on the interactions between the protein and the lipid bilayer and that the differential responses to poke and stretch might stem from variations in the lipid-interacting regions of OSCA proteins. The authors specifically identify the amphipathic helix (AH), the fenestration, and the Beam Like Domain (BLD) as elements that might play a role in mechanosensing.

The strength of this paper lies in the technically sound data - the structural work and electrophysiology are both very well done. For example, the authors produce a high-resolution OSCA3.1 structure which will be a useful tool for many future studies. Also, the study identifies several interesting mutants that seemingly uncouple the OSCA1.2 poke and stretch responses. These might be valuable in future studies of OSCA mechanosensation.

However, the experimental approach employed by the authors to dissect the molecular mechanisms of poke and stretch falls short of enabling meaningful mechanistic conclusions. For example, we are left with several unanswered questions surrounding the role of AH and the fenestration lipids in mechanosensation: Is the AH really important for the poke response if mutating residues conserved between OSCA1.2 and OSCA3.1 disrupts the OSCA1.2 ability to respond to poke but mutating the OSCA1.2 AH to resemble that of OSCA3.1 results in no change to its "pokability"? Similar questions arise in response to the study of the fenestration-lining residues.

Reviewer #2 (Public Review):

Summary:

Jojoa-Cruz et al. determined a high-resolution cryo-EM structure in the *Arabidopsis thaliana* (At) OSCA3.1 channel. Based on a structural comparison between OSCA3.1 and OSCA1.2 and the difference between these two paralogs in their mechanosensitivity to poking and membrane stretch, the authors performed structural-guided mutagenesis and tested the roles of three structural domains, including an amphipathic helix, a beam-like domain, and a lipid fenestration site at the pore domain, for mechanosensation of OSCA channels.

Strengths:

The authors successfully determined a structure of the AtOSCA3.1 channel reconstituted in lipid nanodiscs by cryo-EM to a high resolution of 2.6 Å. The high-resolution EM map enabled the authors to observe putative lipid EM densities at various sites where lipid molecules are associated with the channel. Overall, the structural data provides the information for comparison with other OSCA paralogs.

In addition, the authors identified OSCA1.2 mutants that exhibit differential responses to mechanical stimulation by poking and membrane stretch (i.e., impaired response to poke assay but intact response to membrane stretch). This interesting behavior will be useful for further study on differentiating the mechanisms of OSCA activation by distinct mechanical stimuli.

Major weakness:

1. The major weaknesses of this study are the mutagenesis design and the functional characterization of the three structural domains - an amphipathic helix (AH), a beam-like domain (BLD), and the fenestration site at the pore, in OSCA mechanosensation.

1. First of all, it is confusing to the reviewer, whether the authors set out to test these structural domains as a direct sensor(s) of mechanical stimuli or as a coupling domain(s) for downstream channel opening and closing (gating). The data interpretations are vague in this regard as the authors tend to interpret the effects of mutations on the channel 'sensitivity' to different mechanical stimuli (poking or membrane stretch). The authors ought to dissect the molecular bases of sensing mechanical force and opening/closing (gating) the channel pore domain for the structural elements that they want to study.

Furthermore, the authors relied on the functional discrepancies between OSCA1.2 (sensitive to both membrane poking and stretch) and OSCA3.1 (little or weak sensitivity to poking but sensitive to membrane stretch). But the experimental data presented in the study are not clear to address the mechanisms of channel activation by poking vs. by stretch, and why the channels behave differently.

1. The reviewer questions if the "apparent threshold" of poke-induced membrane displacement and the threshold of membrane stretch are good measures of the change in the channel sensitivity to the different mechanical stimuli.
2. Overall, the mutagenesis design in the various structural domains lacks logical coherence and the interpretation of the functional data is not sufficient to support the authors' hypothesis. Essentially the authors mutated several residues on the hotspot domains, observed some effects on the channel response to poking and membrane stretch, then interpreted the mutated residues/regions are critical for OSCA mechanosensation. Examples are as follows.

In the section "Mutation of key residues in the amphipathic helix", the authors mutated W75 and L80, which are located on the N- and C-terminal of the AH in OSCA1.2, and mutated Pro in the OSCA1.2 AH to Arg at the equivalent position in OSCA3.1 AH. W75 and L80 are conserved between OSCA 1.2 and OSCA3.1. Mutations of W75 and/or L80 impaired OSCA1.2 activation by poking, but not by membrane stretch. In comparison, the wildtype OSCA3.1 which contains W and L at the equivalent position of its AH exhibits little or weak response to poking. The loss of response to poking in the OSCA1.2 W/L mutants does not indicate their roles in poking-induced activation.

Besides, the P2R mutation on OSCA1.2 AH showed no effect on the channel activation by poking, suggesting Arg in OSCA3.1 AH is not responsible for its weak response to poking. Together the mutagenesis of W75, L80, and P2R on OSCA1.2 AH does not support the hypothesis of the role of AH involved in OSCA mechanosensation.

In the section "Replacing the OSCA3.1 BLD in OSCA1.2", the authors replaced the BLD in OSCA 1.2 with that from OSCA3.1, and only observed slightly stronger displacement by poking

stimuli. The authors still suggest that BLD "appears to play a role" in the channel sensitivity to poke despite the evidence not being strong.

OSCA1.2 has four Lys residues in TM4 and TM6b at the pore fenestration site, which were shown to interact with the lipid phosphate head group, whereas two of the equivalent residues in OSCA3.1 are Ile. In the section "Substitution of potential lipid-interacting lysine residues", the authors made K435I/K536I double mutant for OSCA1.2 to mimic OSCA3.1 and observed poor response to poking but an intact response to stretch. Did the authors mutate the Ile residues in OSCA3.1 to Lys, and did the mutation confer channel sensitivity to poking stimuli resembling OSCA1.2? The reviewer thinks it is necessary to perform such an experiment, to thoroughly suggest the importance of the four Lys residues in lipid interaction for channel mechanoactivation.

Reviewer #3 (Public Review):

Summary:

Jojoa-Cruz et al provide a new structure of At-OSCA3.1. The structure of OSCA 3.1 is similar to previous OSCA cryo-em structures of both OSCA3.1 and other homologues validating the new structure. Using the novel structure of OSCA3.1 as a guide they created several point mutations to investigate two different mechanosensitive modalities: poking and stretching. To investigate the ability of OSCA channels to gate in response to poking they created point mutations in OSCA1.2 to reduce sensitivity to poking based on the differences between the OSCA1.2 and 3.1 structures. Their results suggest that two separate regions are responsible for gating in response to poking and stretching.

Strengths:

Through a detailed structure-based analysis, the authors identified structural differences between OSCA3.1 and OSCA1.2. These subtle structural changes identify regions in the amphipathic helix and near the pore that are essential for the gating of OSCA1.2 in response to poking and stretching. The use of point mutations to understand how these regions are involved in mechanosensation clearly shows the role of these residues in mechanosensation.

Weaknesses:

In general, the point mutations selected all show significant alterations to the inherent mechanosensitive regions. This often suggests that any mutation would disrupt the function of the region, additional mutations that are similar in function to the WT channel would support the claims in the manuscript. Mutations in the amphipathic helix at W75 and L80 show reduced gating in response to poking stimuli. The gating observed occurs at poking depths similar to cellular rupture, the similarity in depths suggests that these mutations could be a complete loss of function. For example, a mutation to L80I or L80Q would show that the addition of the negative charge is responsible for this disruption not just a change in the steric space of the residue in an essential region.