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Structure of the Mechanically Activated Ion Channel Piezo1

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Summary

Piezo1 and Piezo2 are mechanically activated ion channels that mediate touch perception, proprioception, and vascular development. Piezos are distinct from other ion channels and their structure remains poorly defined, impeding detailed study of their gating and ion permeation properties. Here, we report a high-resolution cryo-electron microscopy structure of the mouse Piezo1 trimer. The detergent-solubilized complex adopts a three-blade propeller shape with a curved transmembrane region containing at least 26 transmembrane helices per protomer. The flexible propeller blades can adopt distinct conformations, and consist of a series of four-transmembrane helix bundles we term ‘Piezo Repeats’. Carboxy-terminal domains line the central ion pore, and the channel is closed by constrictions in the cytosol. A kinked helical beam and anchor domain link the Piezo Repeats to the pore, and are poised to allosterically control gating. The structure provides a springboard to further dissect how Piezos are regulated by mechanical force.

Introduction

The conversion of mechanical force into cellular signals, or mechanotransduction, is an essential biological function conserved throughout evolution. Mechanically activated (MA) channels confer force sensitivity to cells and organisms by allowing the passage of ions across the membrane in response to a mechanical stimulus¹. Piezo1 and Piezo2 were identified in 2010 as a conserved class of eukaryotic MA ion channels^{2,3} involved in various mechanotransduction pathways, including touch sensation⁴, proprioception⁵, nociception⁶, vascular development⁷, and breathing⁸. In humans, gain-of-function (GOF) mutations in Piezo1 are linked to dehydrated hereditary stomatocytosis (DHS)^{9–12}, a disease in which red blood cells are dehydrated and have increased permeability to cations, and GOF Piezo2 mutations are associated with distal arthrogryposis¹³. Loss-of-function (LOF) mutations in

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Author Contributions

All authors designed experiments and analyzed data. K.S. prepared EM samples, collected and processed EM data, built the structural model, and conducted FSEC analysis. S.E.M. carried out electrophysiological experiments and data analysis. J.M.K. developed sample preparation protocols. T.W. carried out immunostaining experiments. All authors wrote and edited manuscript.

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Piezo1 and Piezo2 are linked to congenital lymphatic dysplasia¹⁴ and defective touch perception and proprioception^{15–17}, respectively. Piezos are structurally unrelated to other proteins, and are uniquely large compared to other ion channels (>2000 amino acids). Electrophysiological studies have demonstrated that Piezo1 ion channels are gated directly by changes in membrane tension^{18–20}, but the structural bases of mechanosensitivity and ion conduction in Piezo channels remain unknown. Previous crystallographic^{21,22} and medium-resolution cryo-electron microscopy (cryo-EM)²² studies have suggested the overall architecture of Piezos. However, a mechanistic understanding of Piezo function requires high-resolution structural information of the channel.

Cryo-EM sample and structure determination

A previous cryo-EM reconstruction of mouse Piezo1²² lacked amino acid side chain definitions and connectivity of resolved regions, precluding building of an accurate structural model. Here, we optimized Piezo1 sample preparation parameters to make Piezo1 amenable to high-resolution single particle cryo-EM structure determination (Extended Data Fig. 1). After computationally sorting particle heterogeneity (Extended Data Fig. 2a), we identified a subset of particles that were refined into both asymmetric and C3 symmetry-imposed maps of the mouse Piezo1 trimer, with overall resolutions of 4.5 and 3.9 Å, respectively. We conducted masked refinement of the central ‘core’ (Extended Data Fig. 2b–d) and C3 symmetry expansion followed by masked classification and refinement (Extended Data Fig. 3) to improve densities proximal to the central three-fold axis and the protein periphery, respectively. Both the 3.8 Å resolution core-masked map and a 4.1 Å resolution symmetry-expanded map (Blade Class 1) were used to build and refine a molecular model of mouse Piezo1 that spans structured regions of residues 1134–2544, including 14 TM helices per protomer and other conserved C-terminal domains (Extended Data Fig. 4, Extended Data Table 1, Supplementary Data 1).

Piezo1 core architecture

The homotrimeric Piezo1 complex exhibits a three-blade propeller shape with a extracellular ‘cap’ domain at the top of the central axis (Fig. 1), consistent with the previous medium-resolution cryo-EM reconstruction²² (Extended Data Fig 5a–c). The transmembrane region can be approximated by the weak density corresponding to the detergent micelle (Fig. 1a–b), in agreement with the hydrophobicity of the membrane-exposed amino acids in our model (Fig. 1c). Interestingly, the transmembrane region has a pronounced bend, suggesting Piezo1-induced local distortion of the membrane. Though we cannot rule out that the bending is a product of detergent solubilization, protein-induced membrane curvature has also been proposed for mechanosensitive channels of the two-pore potassium²³ and MscS^{24,25} families.

The Piezo1 core (Fig. 1d–f) defines an architecture unique from other membrane proteins of known structure. The inner and outer TM helices lie closest to the central axis, and are intervened by the extracellular cap, the structure of which was determined previously by x-ray crystallography^{21,22}. In isolation, the trimeric arrangement of inner and outer helices and cap domains bear structural resemblance to acid sensing ion channels²⁶ and P2X receptors²⁷, as noted previously²⁸. The 12 TM helices peripheral to the inner and outer

helices can be grouped into three 4-TM helix-containing bundles each with the same topology that we term 'Piezo Repeats' A, B, C lettered according to proximity to the central axis. Piezo Repeat A of one protomer is adjacent to the outer helix of a different protomer, producing a 'domain-swapped' arrangement analogous to some tetrameric ion channels^{29,30} (Fig. 1e). The 'anchor' domain forms a triangular three-helix wedge between the inner/outer helices and Piezo Repeat A. The C-terminal domain (CTD) connects to the intracellular end of the inner helices, collaring the central axis. The intracellular 'beam', is a ~64 residue (~95 Å) kinked helix that runs nearly parallel to the membrane, directly underneath Piezo Repeats A to C. While the N-terminus of each beam is tethered directly to Piezo Repeat C, its C-terminus projects towards the central axis and connects via a ~37 residue disordered linker to a short, coiled peptide we term the 'latch' that contacts the intracellular face of the CTDs. The latch then connects back to Piezo Repeat B via a relatively non-conserved ~240 residue stretch of peptide (Supplementary Data 1) not resolved in our maps. The beam and latch are positioned to transmit conformational changes from the propeller blade periphery to the central ion channel. The beam may also be influenced by a nearby domain that we term the 'clasp' (Fig. 1d,f), which was modeled without amino acid assignment due to lower resolution and discontinuity with other domains.

Flexible blades are composed of at least six 'Piezo Repeats'

Blade-focused classification and refinement of symmetry-expanded particles yielded two classes (Blade Class 1 and Blade Class 2) with improved density at the periphery (Extended Data Fig. 3). Remarkably, in each of these maps, clear density for an additional three Piezo repeats is resolved (Fig. 2a,b). Thus, a total of six Piezo repeats (which we have named A–F ordered by proximity to the central axis), or 26 TM helices per protomer (including the inner and outer helices) are apparent, in disagreement with a previous cryo-EM study²², which proposed 14 TM helices. We note the possibility of additional TM helices, and potentially Piezo Repeats, in the Piezo1 protein that were not resolved in our maps.

Although we were only able to confidently place amino acids for Piezo repeats A–C, the topology of the peripheral three repeats is resolved and indicates that all six have the same overall structure and arrangement of TM helices (Fig. 2c,d). The termini of each repeat are cytoplasmic, with a pair of short linkers connecting the N-terminal TM helices, and an extended extracellular linker connecting the third and fourth TM helices. A striking feature of the Piezo repeats is the presence of cytoplasmic membrane-parallel helices tethered to their N-termini. In addition to the Piezo Repeats, the clasp domain also contributes a membrane-parallel helix, adjacent to that of Piezo Repeat B (Fig. 2b). Amino acid assignment for these helices in Piezo repeats A, B, and C demonstrate their amphipathic nature (Extended Data Fig. 6a–d). Interestingly, amphipathic helices are thought to sense or induce membrane curvature in various proteins³¹, and have been proposed to be critical for membrane tension-induced gating in various classes of MA channels³². That the amphipathic helices dictate local membrane deformation, and perhaps mechanosensitivity of Piezo1 is an intriguing possibility. We speculate the array of amphipathic helices in each blade may work in concert to invoke a local membrane environment with continued curvature, thereby contributing to the puckered architecture.

Our blade-focused classification of the symmetry-expanded particles yielded two classes with distinct conformations demonstrating conformational flexibility of the blade. While the central regions are relatively static, the peripheral regions undergo horizontal movement in the plane of the membrane, not vertically in and out of the membrane (Fig. 2e, Extended Data Fig. 5f,g). Interestingly, associated with this lateral flexibility is a $\sim 5^\circ$ pivoting of the beam at a kink centered on a highly conserved Ala-Ser-Arg-Gly motif (A1316-G1319) (Fig. 2f, Supplementary Data 1). This pivot may be a critical structural feature that regulates how membrane tension-induced conformational changes at the periphery are transmitted through the beam to the central gating machinery.

Ion conducting pore

The extracellular cap domain, inner helix, and cytosolic CTD line the central pore axis of Piezo1. Extracellular cations could approach the pore entryway vertically through the internal cavity along the three-fold axis of the cap domain, or laterally through gaps between flexible linkers connecting the cap and inner/outer helices (Fig. 3a). The cap domain, which is flexibly linked to the TM domain, might play a role in mediating ion accessibility to the pore, as suggested previously²⁸. Indeed, a comparison of our current structure to the previous Piezo1 structure indicates distinct orientations of the cap domain and inner and outer helices (Extended Data Fig. 5d,e), illustrating the flexibility of the cap and the associated TM helices.

Below the cap, the ion conduction pathway is lined by the three inner TM helices. Notably, we find two lateral openings between the inner helices separated by a ‘seal’ formed by K2479 and F2480 (Fig. 3b). We term these openings the upper crevice (~ 11 Å wide and ~ 16 Å tall) and lower fenestration (~ 10 Å wide and ~ 12 Å tall), which may provide pore access for lipids or other hydrophobic molecules as a means to regulate ion conduction, reminiscent of the proposed force-gating mechanism of MA two-pore potassium channels²³. Molecular dynamics simulations of the homotrimeric ATP-gated P2X3 receptor³³ revealed that a lower fenestration provides a passageway for ion flow into the cytoplasm. Though the possibility that the Piezo1 lower fenestration represents an ion permeation pathway warrants further study, we assume that, like most other ion channels, the pore is coincident with the central vertical axis. The pore-facing residues of the inner helices are mainly hydrophobic (Fig. 3d). The ~ 35 Å long membrane-spanning segment of the pore is relatively wide (> 2 Å van der Waals radius) and does not house the narrowest constrictions of the conduction pathway (Fig. 3c–d). Rather, the ion conduction pathway narrows directly below the lower membrane boundary into a cytosolic ~ 11 Å long ‘neck’ (with a minimum van der Waals radius < 1 Å) that defines the closed state of the pore in the current structure.

At the top of the cytosolic neck, the side chains of M2493 and F2494, which are conserved across Piezo orthologues (Supplementary Data 1), form a hydrophobic barrier (Fig. 3e). To examine the functional role of the apparent hydrophobic barrier, we recorded MA currents (Fig. 3g,h) from HEK 293T Piezo1 knockout cells¹⁴ expressing the alanine substitution mutants M2493A, F2494A, and M2493A/F2494A. The double mutant M2493A/F2494A resulted in a nonfunctional channel despite proper trimeric assembly and surface expression (Extended Data Fig. 7a–c), demonstrating the importance of these residues. Both single

mutants expressed as functional channels, with F2494A having significantly slower inactivation kinetics than wild type for MA macroscopic currents in cell-attached and whole-cell configurations (Fig. 3g,h). Furthermore, stretch activated currents from F2494A had greater steady state currents relative to wild type (Fig. 3i, Extended Data Table 2). The increased channel activity of F2494A in these recordings is consistent with this residue playing a role in gating, possibly by stabilizing a non-conducting state of the channel. The M2493A mutant did not show altered permeation properties, while the F2494A mutant only exhibited modestly increased single channel conductance (Extended Data Fig. 7d,e) and slightly altered ion selectivity (Extended Data Fig. 7f,g, Extended Data Table 2). Our results implicate the hydrophobic barrier as an important contributor to the gating properties of Piezo1, with a minor role in determining ion conduction and selectivity. In previous studies, mutation of acidic residues (E2495 and E2496) adjacent to M2493/F2494 (Fig. 3e) affected pore properties²⁸, suggesting they might coordinate permeating cations. In the current structure these residues do not project into the pore (Fig. 3e), though structural changes associated with gating could result in this region adopting conformation(s) in which the side chains of E2495 and/or E2496 face the pore to form cation binding site(s).

Directly below the hydrophobic barrier formed by M2493 and F2494, the pore pathway narrows into a second constriction delineated by P2536 and E2537 (Fig. 3f), which are located at the turn of a helix-turn-helix motif in the distal CTD. The side chain of E2537 forms a possible hydrogen bond with Y1412 of the latch domain (Fig. 3f), which may allosterically couple movement of the latch to pore structure in this region. ~4 Å below E2537 a robust density that cannot be reliably modeled but likely originates from Piezo peptide is present (Fig 3d). This density, which forms a cytosolic ‘plug’ to the ion channel pore, could also play a role in the permeation pathway.

Gating machinery and lipid pocket

Our structure suggests that activation of Piezo1 may involve concerted movements of the inner helix, outer helix, CTD, anchor domain, latch, and beam domains. Interdomain contacts that likely govern gating movements allosterically are shown in Figure 4. The conserved anchor domain is poised to play a prominent role in gating through its interactions with the inner helix and CTD (Fig. 4b–c). In particular, anchor domain residue E2133 and R2482 of the adjacent inner helix are likely engaged in an electrostatic interaction. In previous studies, mutations at E2133 affected single channel conductance, ion selectivity, and ruthenium red block³⁴ of Piezo1, and the equivalent mutation to R2482H in human Piezo1 results in slower inactivation kinetics and is associated with DHS^{10,12}. In the context of the present structure, these data implicate the anchor domain-inner helix interaction in regulating gating and pore properties of Piezo1. The cytosolic membrane-lining helix of the anchor domain forms several contacts with the CTD (Fig. 4c), including potential electrostatic interaction(s) between R2169 and E2495/E2496, which are adjacent to pore-facing residues M2493 and F2494 (Fig. 3e). This interaction provides a possible explanation for why mutation of E2495 or E2496 affected the ion conduction properties of Piezo1²⁸. Overall, the proximity of the anchor domain membrane-lining helix to the permeation pathway’s cytosolic ‘neck’ suggests that its movement would be strongly coupled to channel gating.

We observe a robust lipid-shaped density in an apparent ligand binding pocket formed by the anchor domain on one side and Piezo Repeat A on the other (Fig. 4d). Two conserved arginines (R2035 and R2135) project into the pocket, positioned to stabilize a negatively charged species. Notably, mutation of R2135 to alanine rendered Piezo1 non-functional³⁴, suggesting that this ligand-binding pocket allosterically supports channel activity, and may therefore be a target for amphiphiles and other compounds aimed at modulating Piezo function. In addition, we note the presence of a separate density potentially representing a lipid headgroup that is sandwiched between Piezo Repeats B and C (Extended Data Fig. 6e–f).

On the cytosolic side, the CTD interacts with the latch and beam (Fig. 4e). Latch residues Y1412, L1414, and F1415 protrude into the surface of the CTD, making contacts with K2528, F2531, E2537, and W2542 (Fig. 4e). Toward the C-terminal end of the beam, a series of basic/polar residues (R1351, R1353, Q1356, and R1360) face acidic CTD residues (D2509, E2518, E2520, and E2524). These electrostatic interactions likely promote allosteric coupling between the beam and CTD. Interestingly, the equivalent mutation to R1353P, which would presumably break or kink the helical beam at this position, is found in human DHS patients and results in delayed inactivation kinetics¹⁰. Thus, the distal beam end may be a structural determinant of gating behavior, possibly through its interactions with the CTD.

Concluding remarks

The Piezo1 structure described here represents the first high-resolution view of this functionally and structurally novel class of ion channel. Importantly, a majority of known human Piezo1 and Piezo2 GOF disease mutants^{35,36} can be mapped onto the structure, which indicates that our model encompasses many of the functionally important domains, and moreover, allows a structural interpretation of disease-causing mechanisms (Fig. 5a).

A central question in the study of Piezos is how membrane tension results in channel activation. A recent study probing for mechanosensitive domains in Piezo1³⁷ implicated regions as disparate as the N-terminus (which likely resides toward the outer “tip” of the propeller blade) and the central extracellular cap. Thus, mechanical activation is likely to involve movements of various domains spanning the entirety of the protein complex. Our structure clarifies the features that contribute to this process (Fig. 5b–c). The arrayed Piezo Repeats potentially induce a remarkable degree of local membrane deformation, conceivably through their amphipathic helices. Though free energy calculations³⁸ have suggested that the energetic cost of local membrane deformation plays a key role in the mechanosensitivity of MscS²⁵ and TRAAK²³ channels, its contribution to Piezo structure and function in membrane bilayers will require further study. Nonetheless, our results allow us to speculate that membrane tension-induced conformational changes of the Piezo Repeats are transmitted through the beam and/or anchor domains to the inner helix and CTD, which in turn determine the activation state of the channel. While the nature of these conformational transitions remain unclear, the present structure of a closed state will guide further investigations of the process of Piezo mechanical activation.

Methods

Sample Preparation

Protocols to purify mouse Piezo1 were adapted from previous study²² with modifications. Suspension-adapted HEK 293F cells (purchased and authenticated from ATCC, tested negative for mycoplasma) were grown at 37° C to a cell density of ~1.5 million/mL and transfected with a pcDNA3.1-IRES-GFP plasmid²² containing mouse Piezo1 (Uniprot KB accession number E2JF22) fused to Prescission protease cleavage site (LEVLFQGP) followed by GST coding sequence (this plasmid was a kind gift from Bailong Xiao). 300 µg of DNA and 900 µg of polyethylenimine were used per liter of transfected HEK cells. 4 mM sodium butyrate was added to the cells 12–24 hours after transfection. ~48 hours after transfection, cells were harvested and resuspended in buffer containing 25 mM PIPES pH 7.2, 150 mM NaCl, 1% CHAPS, 0.5% soy phosphatidylcholine, 0.1% C₁₂E₉, 2 mM DTT, and cOmplete protease inhibitor cocktail (Roche). The lysate was stirred for 1 hour at 4C, clarified by centrifugation, and incubated with Glutathione Sepharose 4B resin (GE Healthcare) for 3 hours. The resin was washed extensively with buffer A (25 mM HEPES pH 8.0, 150 mM NaCl, 100 µM glyco-diosgenin, 2mM DTT). Prescission protease was added to a ~30% slurry of resin in buffer A at a ratio of ~100 µg per 1 mL resin and incubated at 4C overnight. The flowthrough was concentrated and subjected to size exclusion chromatography on a Superose 6 increase column equilibrated to buffer A. The peak corresponding to Piezo1 trimer was collected and concentrated to ~5 mg/mL. 3.5 µL of protein was applied to a freshly plasma cleaned UltrAuFoil R1.2/1.3 grid, blotted for 3.5 seconds with a blot force of 0, and plunge-frozen in liquid ethane using a Vitrobot Mark IV (FEI).

Cryo-EM Data collection

An initial data set of mouse Piezo1 was in a Talos Arctica electron microscope (FEI) equipped with a K2 direct detector camera (Gatan) operated at 200 kV and having a nominal pixel size of 1.15 Å. Subsequent data were collected on a Titan Krios electron microscope (FEI) equipped with a K2 direct detector camera (Gatan) operated at 300 kV and having a nominal pixel size of 1.03 Å pixel size. Micrographs were automatically collected using Legion software³⁹. The total accumulated dose was ~60 e⁻/Å² and the target defocus range was 0.8–2.2 µm. Micrograph movies frames were dose-weighted and aligned using MotionCor2⁴⁰. CTF values were estimated using Gctf⁴¹.

Image Processing

Template-based particle picking, 2D and 3D classification, and 3D refinements were carried out in Relion 2.0⁴². Unwanted particle picks due to ice contaminations or the grid hole edges were removed using EM Hole Punch⁴³. An initial set of 527 micrographs was collected on the Talos Arctica microscope, from which ~96,000 particle images were extracted. After reference-free 2D classification and 3D classification using the published medium-resolution Piezo1 reconstruction²² as an initial reference (low pass filtered to 60 Å), a ~26,000 particle subset was identified that refined to a ~5 Å resolution reconstruction with C3 symmetry applied.

Two separate data collection sessions were conducted on the Titan Krios microscope. 1978 micrographs were collected in session 1, yielding ~281,000 particles. This particle stack was subjected to 2D classification to remove classes not displaying well-defined features of the Piezo1 trimer, resulting in ~266,000 particles. In session 2, 1086 micrographs were collected, yielding ~144,000 particles from which ~112,000 particles were chosen after 2D classification. The post-2D classification particle stacks from sessions 1 and 2 were combined into a stack with ~378,000 particles. This stack was subjected to 3D classification requesting 3 classes, using a 60 Å low pass-filtered map of mouse Piezo1 generated from the Talos Arctica data set as an initial reference. The best class contained ~106,000 particles, which were subjected to a round of 3D classification without alignment requesting six classes. The most populated class from this round of refinement contained ~73,000 particles. Auto-refinement of these particles with and without C3 symmetry imposed resulted in reconstructions at 3.9 and 4.5 Å resolution, respectively. C3 symmetry imposed refinement with a soft mask around the Piezo1 core (cap, inner and outer helices, anchor, CTD, Piezo Repeats A–C, latch, and beam domains) requesting local searches resulted in a 3.8 Å resolution map with improved features.

Inspection of the asymmetrically refined map indicated a progressive break of symmetry toward the blade periphery (Extended Data Fig. 3a). To better resolve the peripheral regions of the blade, *relion_particle_symmetry_expand* was used to artificially enlarge the particle stack three-fold, and add 120° or 240° to the first Euler angle determined in the initial C1 refinement. Alignment-free 3D classification of these stack of ~220,000 “expanded” particles requesting six classes with a soft mask around a single blade yielded two highly populated classes containing ~102,000 (Blade Class 1) and ~74,000 expanded particles (Blade Class 2) with distinct blade conformations. Refinement with a soft mask around a single blade and the most symmetric parts of the molecule (cap, anchor, Piezo Repeat A, inner and outer helices) and requesting local angular searches resulted in reconstructions at 4.1 (Blade Class 1) and 4.5 Å (Blade Class 2) resolution, respectively.

Model Building and Refinement

The 3.8 Å resolution map of the Piezo1 core sharpened with a B factor of –118 automatically determined using *relion_postprocess* was primarily used for model building and refinement. Initially, the crystal structure of the extracellular cap domain²² (PDB ID: 4RAX) was docked as a rigid body, and a poly-alanine model was fit into the rest of the Piezo1 core. Rough sequence assignment was ascertained from the position of the docked cap domain, as well as transmembrane topology prediction and previous immunostaining and mass spectrometry data³⁴ demonstrating the positions of extracellular loops and intracellular phosphorylation sites, respectively. We were able to model amino acids for 14 of the 26 TM helices observed in our EM maps. We note here that the previous membrane topology analysis³⁴ approximates the presence of 21 TM helices in addition to the 14 we have modeled. The N- and C- termini of the channel are intracellular³⁴, necessitating an even number of TM segments. This would suggest a putative total of 34 or 36 TM helices, and up to 8 Piezo repeats per protomer. Nonetheless, additional studies are needed to clarify the exact membrane topology of Piezo proteins.

Precise sequence registry assignment was determined by locating bulky Phe, Arg, Tyr, and Trp side chains, as well as the absence of side chain density for Gly. Side chain densities were not well resolved for many Asp and Glu side chains, likely due to radiation damage⁴⁴. Some portions of the beam and Piezo Repeats B and C were built using the Blade Class 1 map (sharpened to an automatically determined B-factor of -152), then symmetrized three-fold to fit into the C3 symmetry core map. Iterative manual model building and automatic refinement were carried out in Coot⁴⁵, and Phenix.real_space_refine⁴⁶, respectively. Final structural refinement was carried out in the C3 symmetry core map, with strong restraints applied to portions built using the Blade Class 1 map, as well as non-crystallographic symmetry restraints for the entire model. Model geometry and fit to map were validated using MolProbity⁴⁷ and EMRinger⁴⁸. Electrostatic potential surfaces were calculated using APBS⁴⁹. Structural figures were made in PyMOL⁵⁰ or UCSF Chimera⁵¹.

Generation of mutants

mPiezo1(M2493A), mPiezo1(F2494A) and mPiezo1(M2493A, F2494A) mutants were generated using QuickChange II XL site-directed mutagenesis kit according to the manufacturer's instruction and confirmed by full-length DNA sequencing. All mPiezo1 constructs were inserted into an IRES-GFP pcDNA3.1 vector.

Cell culture and transfections

Wild-type and mutant mouse Piezo1 were transfected and tested in Piezo1 deficient Human embryonic kidney 293T (HEK293T P1KO) cells. HEK293T P1KO cells were generated using CRISPR/Cas9 nuclease genome editing technique as described previously¹⁴ and not tested for mycoplasma contamination nor further authenticated. Cells were grown in Dulbecco's Modified Eagle Medium containing 4.5 mg ml^{-1} glucose, 10% fetal bovine serum, 50 units ml^{-1} penicillin and $50 \mu\text{g ml}^{-1}$ streptomycin. Cells were plated onto 12-mm round glass poly-D-lysine coated coverslips placed in 24-well plates and transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. All plasmids were transfected at a concentration of 600 ng ml^{-1} . Cells were recorded from 24 to 36 hours post transfection.

Electrophysiology

Patch-clamp experiments were performed in standard whole-cell or cell-attached mode using Axopatch 200B amplifier (Axon Instruments). Macroscopic currents were filtered at 2 kHz and sampled at 20 kHz. Leak currents before mechanical stimulations were subtracted off-line from the current traces. Voltages were not corrected for a liquid junction potential except for ion selectivity experiments. Liquid junction potential was calculated using Clampex 10.3 software. All experiments were done at room temperature.

Mechanical stimulation—For whole-cell recordings, mechanical stimulation was achieved using a fire-polished glass pipette (tip diameter $3\text{--}4 \mu\text{m}$) positioned at an angle of 80° relative to the cell being recorded. Downward displacement of the probe towards the cell was driven by Clampex-controlled piezoelectric crystal microstage (E625 LVPZT Controller/Amplifier; Physik Instrumente). The probe had a velocity of $1 \mu\text{m ms}^{-1}$ during the ramp phase of the command for forward movement and the stimulus was applied for 150

or 300 ms. To assess the mechanical sensitivity of a cell, a series of mechanical steps in 0.5 or 1 μm increments was applied every 20 s. For I–V relationship recordings, voltage steps were applied 700 ms before mechanical stimulation (150 ms) from a holding potential of -60 mV . Voltage steps were given from -80 mV to $+80$ in 20 mV increments.

Stretch-activated currents were recorded in the cell-attached patch-clamp configuration. Membrane patches were stimulated with a 5 s prepulse duration of $+5$ or 0 mmHg followed by 500 ms negative pressure pulses through the recording electrode using Clampex-controlled pressure clamp HSPC-1 device (ALA-scientific)¹⁹. Since the single-channel amplitude is independent of the pressure intensity, the most optimal pressure stimulation was used to elicit responses that allowed single-channel amplitude measurements. These stimulation values were largely dependent on the number of channels in a given patch of the recording cell. Single-channel amplitude at a given potential was measured from trace histograms of 5 to 10 repeated recordings. Histograms were fitted with Gaussian equations using Clampfit 10.3 software. Single-channel slope conductance for each individual cell was calculated from linear regression curve fit to single-channel I–V plots.

Solutions—For whole-cell patch-clamp recordings, recording electrodes had a resistance of 2–3 M Ω when filled with internal solution composed of (in mM) 133 CsCl, 1 CaCl₂, 1 MgCl₂, 5 EGTA, 10 HEPES (pH 7.3 with CsOH), 4 MgATP and 0.4 Na₂GTP. The extracellular solution was composed of (in mM) 133 NaCl, 3 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.3 with NaOH) and 10 glucose. For ion-selectivity experiments, internal solution used was (in mM) 150 CsCl, 10 HEPES (pH 7.3 with CsOH) and extracellular solution consisted of (in mM) 100 CaCl₂ and 10 HEPES (pH 7.3 with CsOH).

For cell-attached patch-clamp recordings, external solution used to zero the membrane potential consisted of (in mM) 140 KCl, 1 MgCl₂, 10 glucose and 10 HEPES (pH 7.3 with KOH). Recording pipettes were of 1–3 M Ω resistance when filled with standard solution composed of (in mM) 130 mM NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 TEA-Cl and 10 HEPES (pH 7.3 with NaOH).

Surface immunostaining

Surface immunostaining of mPiezo1 was carried out as described previously³⁴, with slight modifications. Briefly, pcDNA3.1 IRES-GFP vector encoding wildtype or mutant mPiezo1 constructs containing myc-tag (EQKLISEEDL) inserted at a previously determined extracellular amino acid position 897³⁴ were expressed in HEK293T P1KO cells on poly-D-lysine treated glass coverslips. 2 days after transfection, labeling of non-permeabilized cells was carried out by incubating the cells with 9E11 anti-Myc antibody (1:50; Santa Cruz Biotechnology). After six washes with warm medium, cells were incubated with secondary antibodies conjugated to Alexa Fluor 568 (1:200; Life Technologies) for 10 min at room temperature. Cells were washed six times with warm media and once with PBS, and then fixed with 4% PFA/PBS for 30 min. For permeabilization, cells were fixed with 4% PFA for 10 min and then treated with 0.3% Triton X-100 and blocked with 10% normal goat serum in PBS prior to incubation with antibodies (primary: 1:200 for 2 hr, secondary: 1:400 for 1 hr, in block solution). Cells were imaged with a Nikon C2 confocal microscope with 40 $\times$

oil immersion objective by illumination with the 543 nm laser. The live labeling and permeabilized staining were repeated in three separate experiments to confirm results.

Fluorescence Detection Size Exclusion Chromatography (FSEC)

Suspension-adapted HEK293F cells were transfected with pcDNA3.1 vector containing mPiezo1 or mPiezo1-M2493A F2494A double mutant fused to tdTomato at the C-terminus. 48 hours post-transfection, 1 mL of the culture was pelleted and resuspended in 200 μ L of buffer containing 25 mM HEPES pH 8.0, 150 mM NaCl, 1% CHAPS, 0.5% soy phosphatidylcholine, 1 mM PMSF, 2 μ M pepstatin A, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin. 100 μ L of clarified supernatant was injected into a Superose 6 increase column in line with a fluorimeter tracking tdTomato fluorescence (excitation at 554 nm, emission at 581 nm).

Data Availability

EM Maps were deposited into the Electron Microscopy Data Bank (EMDB) under accession ID 7128. Structural model was deposited to Protein Data Bank (PDB) under accession ID 6BPZ. All other data can be obtained upon request to the corresponding authors.

Extended Data

Extended Data Figure 1. Purification and EM analysis of mPiezo1

a, Preparative gel filtration chromatogram of mPiezo1 after affinity purification and proteolytic removal of GST tag. **b**, SDS-PAGE analysis of mPiezo1 following affinity purification (Pre-SEC) and after subsequent SEC step (SEC Fractions). Purifications of mPiezo1 have been repeated more than three times with similar results. **c**, aligned micrograph of purified mPiezo1 embedded in a thin layer of vitrified ice. Scale bar represents 100 nm. **d**, Representative 2D classes of Piezo1 showing different particle orientations.

Extended Data Figure 2. Classification and refinement Piezo1 core

a, Data processing flow chart. **b**, local resolution maps calculated by the locres program in RELION 2.0. Color key for local resolution (in Å) is shown. **c**, angular distributions of the particles after the final step of refinement in RELION. The radius of the sphere is proportional to the number of particles with a given orientation. Only one third of the sphere is shown due to applied C3 symmetry. **d**, FSC plots calculated using relion_postprocess for unmasked maps and maps with a soft mask applied to remove the contribution of scattered detergent density.

Extended Data Figure 3. Asymmetry of Piezo1 and blade-focused classification and refinement
a, Duplicate unsharpened maps of Piezo1 refined without symmetry imposed, with the blue map rotated $\sim 120^\circ$ around the central axis of pseudosymmetry relative to the pink map, then superimposed with the “fit in map” function of UCSF chimera. Left panel shows top view, while right panel shows a horizontal slice through the transmembrane region. **b**, flow chart of propeller blade focused classification and refinement procedure. **c**, local resolution maps calculated by the locres program in RELION 2.0. Color key for local resolution (in Å) is shown and is the same as Extended Data Fig 2b. **d**, angular distributions of the particles after the final step of refinement in RELION. The radius of the sphere is proportional to the number of particles with a given orientation **e**, FSC plots calculated using relion_postprocess for unmasked maps and maps with a soft mask applied to remove the contribution of scattered detergent density.

Extended Data Figure 4. Fit of molecular model to electron density

Select regions of the molecular model are shown as yellow cartoon, with superimposed electron density as blue mesh. The density is derived from the C3 symmetry core masked map for the top two rows, and derived from Blade Class 1 for the bottom row.

Extended Data Figure 5. Structural comparisons of current and previous (EMDB 6343) mPiezo1 structures

For each panel, “fit in map” function of UCSF Chimera was used to align uncropped maps. In **a–e**, unsharpened map of C3 refinement is shown. **a–c**, top (**a**), bottom (**b**) and side (**c**) views of current mPiezo1 (green) and EMDB 6343 (gray) structurally aligned. **d** shows expanded top view of the cap domain to illustrate a $\sim 15^\circ$ rotation of the cap between the two maps. **e** shows expanded sliced top view of pore-proximal transmembrane region. Subtle conformational rearrangements are present between the current and previous structures in the inner and outer helices. **f–g**, top and side views of EMDB 6343 (gray), Blade Class 1 (blue), and Blade Class 2 (orange). Coarsely, the trajectories of the propeller blades are similar across the maps in both the membrane-parallel and membrane-normal directions.

Extended Data Figure 6. Structural features of Piezo repeats

a, top view of Piezo1 depicted as cartoon, with amphipathic helices from Piezo Repeats A, B, C shown in red. Extracellular cap domain is omitted for clarity. **b**, **c**, **d**, ribbon models of amphipathic helices from Piezo Repeat A (**b**), Piezo Repeat B (**c**), and Piezo Repeat C (**d**). Cα positions are shown as spheres and hydrophobic amino acids are colored gray, basic amino acids are colored blue, acidic amino acids are colored red, and polar uncharged amino acids are colored yellow. **e**, cartoon model of a portion of the Piezo1 propeller blade, with Piezo Repeats colored separately. A strong density peak putatively representing a lipid head group is shown as pink mesh. **f**, Expanded view of the putative lipid binding site sandwiched between Piezo repeats B and C (helices B4 and C1). Electron density (blue mesh) is superimposed onto the molecular model, and the putative lipid head group is shown as pink mesh. Side chains contributing to the binding pocket are shown as yellow sticks and labeled.

Extended Data Figure 7. Expression and functional properties of Piezo1 mutants

a, HEK293T P1KO cells expressing mPiezo1 M2493A/F2494A do not show discernible currents activated by indentation with blunt pipette (left) or stretch (right). **b**, FSEC traces of untransfected HEK293F cells (gray) or cells expressing C-terminal tdTomato fusions of wild-type mPiezo1 or mPiezo1 M2493A/F2494A injected into Superose 6 increase column. Similar to wild-type, the double mutant displays a single dominant peak eluting shortly after void volume, indicating proper trimeric expression. Data from one independent experiment. **c**, Representative images of Myc labeling in mPiezo1 IRES-GFP (top row), mPiezo1-myc IRES-GFP (middle row) or mPiezo1-myc M2493A/F2494A IRES-GFP (bottom row) transfected HEK293T P1KO cells. Myc tags were inserted at position 897. Immunostaining was performed before (left panels) or after (right panels) cell permeabilization. mPiezo1-myc M2493A/F2494A is labeled at the surface in live cells similar to mPiezo1-myc, indicating that surface expression is preserved in the nonfunctional mutant. Scale bar, 10 μ m. Experiments were repeated 3 times with reproducible results. **d**, Left, representative traces of stretch activated single channel currents recorded (-80 mV) from HEK293T P1KO cells expressing wild type, M2493A or F2494A mPiezo1 channels (traces displayed after applying 1kHz digital filter). The corresponding pressure stimulus used to elicit the response is illustrated above the current trace. For each condition, the amplitude histogram for the corresponding trace is depicted on the right. The Gaussian fit for the closed (black curve) and open (red curve) components is overlaid on the histograms. **e**, Left, average I-V relationship of stretch activated single channel currents from wild type (N=4), M2493A (N=5) or F2494A (N=4) mPiezo1 channels. Amplitude was measured as a difference in Gaussian fits of full-trace histograms. Right, average unitary conductance calculated from the slope of linear regression line fit to individual cells in each condition. ** $P < 0.01$ One-way ANOVA with Dunn's comparison relative to mPiezo1. **f**, Average I-V relationship curves of MA currents recorded from wild type (N=6), M2493A (N=8) or F2494A (N=9) mPiezo1 channels with 150mM CsCl-based intracellular solution and 100mM CaCl₂-based extracellular solution. Currents were elicited from -69.6 to 50.4 mV (± 20 mV). Inset, expanded view of curves between 5 and 15mV. Values are mean $\pm$ s.e.m. **g**, Average reversal

potential from individual cells; mPiezo1: 8.8 ± 1.3 mV (n=6), M2493A: 10.2 ± 1.2 mV (n=8) and F2494A: 13.5 ± 0.5 mV (n=9). **P=0.0063 One-way ANOVA with Dunn's comparison relative to mPiezo1. N=X individual cells.

Extended Data Table 1

EM data collection, data processing, model refinement and validation.

	mPiezo1 Core (EMDB-7128) (PDB 6BPZ)	mPiezo1 Blade Class 1 (EMDB-7128)
Data collection and processing		
Magnification	29,000	29,000
Voltage (kV)	300	300
Electron exposure (e-/Å ²)	60	60
Defocus range (μm)	0.8–2.2	0.8–2.2
Pixel size (Å)	1.03	1.03
Symmetry imposed	C3	C1 [*]
Initial particle images (no.)	425,103	425,103
Final particle images (no.)	72,627	102,289 [*]
Map resolution (Å)	3.8	4.1
FSC threshold	0.143	0.143
Refinement		
Map sharpening B factor (Å ²)	–118	–152
Model composition		
Protein residues	2700	
Ligands	0	
R.m.s. deviations		
Bond lengths (Å)	0.01	
Bond angles (°)	1.52	
Validation		
MolProbity score	1.68	
Clashscore	4.1	
EMRinger score	1.62 2.54 [†]	
Poor rotamers (%)	0.27	
Ramachandran plot		
Favored (%)	91.8	
Allowed (%)	8.0	
Disallowed (%)	0.2	

^{*} Particles are C3 symmetry expanded (triplicated, adding 120° or 240° to first euler angle from unmasked asymmetric refinement, then classified with mask around single blade before refinement).

[†] EMRinger score calculated for “inner core”, which includes Piezo Repeat A, anchor, inner helix, outer helix, CTD, and latch domains.

Extended Data Table 2

MA current properties of Piezo1 constructs.

Construct	Whole-cell/Poke				Cell-attached/Stretch				
	I _{max} (pA)	Threshold (μm)	Tau Inactivation (ms)	E _{rev} (mV)	I _{max} (pA)	P ₅₀ (mmHg)	Tau Inactivation* (ms)	%SS*	Unitary Conductance (pS)
mPiezo1	2398 ± 728 (7)	3.8 ± 0.31 (7)	13 ± 1 (7)	8.7 ± 1.3 (6)	77 ± 18 (6)	30.7 ± 5.5 (5)	37.6 ± 6 (6)	10.4 ± 1.8 (6)	27.3 ± 0.3 (4)
M2493A	3120 ± 607 (8)	3.6 ± 0.3 (8)	19.6 ± 1.2 (8)	10.2 ± 1.2 (8)	232 ± 73 (7)	47.2 ± 8.7 (7)	68.6 ± 6.3 (7)	11.5 ± 1.3 (7)	29.0 ± 0.4 (5)
F2494A	4172 ± 806 (8)	3.4 ± 0.3 (8)	48.8 ± 6 (8)	13.5 ± 0.5 (9)	122 ± 29 (7)	35.0 ± 1.4 (6)	95.5 ± 15 (7)	30.4 ± 3.6 (7)	32.8 ± 0.7 (4)
M2493A, F2494A	Nonfunctional (11)				Nonfunctional (10)				

* Tau inactivation and percent steady state current (%SS) measured at –60 or –70 mmHg and –60 mmHg, respectively. %SS was measured as the ratio of I_{max} to residual current at the end of the 500 ms pressure stimulus. Values indicate mean ± s.e.m. Numbers in parenthesis indicate Ns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Architecture and domain arrangement of the Piezo1 core

Side (a) and top (b) views of the Piezo1 cryo-EM map refined with C3 symmetry imposed. Density corresponding to modeled regions is colored green. Less resolved density that could not be modeled is colored yellow. At lower thresholds (transparent gray map), scattered density likely originating from detergent micelle can be observed. c, surface electrostatics of the Piezo1 core model. Note the bent hydrophobic stripe representing the transmembrane region. Dotted lines approximate the extracellular (black) and cytosolic (yellow) membrane boundaries. d, e, side (d) and top (e) views of the Piezo1 core in cartoon representation, with each domain colored differently. In e, the cap domain is removed to highlight domain swapping between Piezo Repeat A and outer helix. f, schematic of the Piezo1 core domain arrangement. Dotted lines represent flexible regions that were not clearly resolved in the density maps.

Figure 2. Propeller blade composition and conformational heterogeneity

a, b, side (**a**) and top (**b**) views of Blade Class1 EM map, with individual domains segmented and colored uniquely. In **b**, the map is sliced along the dotted line in **a** to highlight the positions of the TM helices. **c**, schematic of Piezo repeat topology, which includes a cytosolic membrane parallel helix and four TM helices. **d**, Aligned models for Piezo Repeats A (yellow), B (cyan), and C (pink). **e**, Sagittal slice of EM density corresponding to the transmembrane region of Blade Class 1 (blue) and Blade Class 2 (orange). Approximate trajectory of the beam is shown as pink dotted line. Individual Piezo Repeats are circled by black dotted lines and labeled A–F. Red double arrow depicts direction of conformational change between the two classes. **f**, Superposed EM maps and cartoon models corresponding to beam domains of Blade Class 1 (blue) and Blade Class 2 (orange). Ca positions of the conserved Ala-Ser-Arg-Gly motif at the beam pivot are shown as spheres.

Figure 3. Ion pore structure and electrophysiological characterization of M2493A and F2494A
a, cartoon model of Piezo1, with inner helix and CTD colored blue and purple, respectively. Flexible linkers between the cap domain and the transmembrane region are depicted as dotted lines. Green spheres represent ions, with possible entryways into the pore shown as black arrows. **b**, surface representation of transmembrane region of Piezo1 surrounding the central axis, highlighting lateral membrane openings to the pore pathway. **c**, van der Waals radii of the pore, plotted against distance along the pore axis. The radial distance between the pore axis and the protein surface is shown as cyan surface in **a**, **b**, and **d**. **d**, expanded view of the Piezo1 pore, with residues lining the pore pathway shown in yellow and labeled. EM density corresponding to a ‘cytosolic plug’ is shown as yellow mesh. **e**, **f**, expanded top-down views of constrictions formed by M2493 and F2494 (**e**) and P2536 and E2537 (**f**). Residues mentioned in the text are also labeled. **g**, Left, representative traces of probe displacement and MA whole-cell currents recorded (-80 mV) from one cell expressing wild type, M2493A or F2494A mPiezo1 channels. Right, inactivation time constant for individual cells across different conditions (mPiezo1 N=7 cells, M2493A N=8 cells and F2494A N=8 cells). **h**, Left, representative traces of stretch activated macroscopic currents recorded (-80 mV) in response to -60 mmHg pressure from one cell expressing wild type, M2493A or F2494A mPiezo1 channels. Right, inactivation time constant (at -60 mmHg) for individual cells across different conditions. **i**, Percent steady state current (at -60 mmHg) for individual cells across different conditions. **h–i**, mPiezo1 N=7 cells, M2493A N=8 cells and F2494A N=8 cells. **g–i**, whiskers represent mean $\pm$ s.e.m.; One-way analysis of variance (ANOVA) with Dunn’s multiple comparison relative to mPiezo1. **g** *** $P=0.0001$, **h** * $P=0.008$ and **i** * $P=0.012$).

Figure 4. Interdomain interactions and lipid pocket

a shows cartoon model of Piezo1, with color-coded boxes around regions expanded in **b–e**. In **a** and **d**, red mesh shows EM density corresponding to putative lipid. In **a–e**, residues contributing to interdomain contacts (**b**, **c**, **e**) or putative lipid binding (**d**) are shown as sticks and colored according to domain.

Figure 5. Mapping of disease mutants and schematic diagram of Piezo1 structure and conformational flexibility

a, Model of the Piezo1 core, with Ca positions of Piezo1 and Piezo2 human GOF disease mutants shown as red spheres. **b–d**, schematic diagrams of the various Piezo1 structural elements that contribute to its function as a membrane-tension gated channel. In **c** and **d**, bottom-up (**c**) and top-down (**d**) views of a single Piezo1 propeller blade are shown, with dotted lines and arrows depicting lateral conformational flexibility, which may be involved in gating.