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Structure of a volume-regulated anion channel of the LRRC8 family

Dawid Deneka^{1,3}, Marta Sawicka^{1,3}, Andy K. M. Lam¹, Cristina Paulino^{1,2} & Raimund Dutzler^{1*}

Volume-regulated anion channels are activated in response to hypotonic stress. These channels are composed of closely related paralogues of the leucine-rich repeat-containing protein 8 (LRRC8) family that co-assemble to form hexameric complexes. Here, using cryo-electron microscopy and X-ray crystallography, we determine the structure of a homomeric channel of the obligatory subunit LRRC8A. This protein conducts ions and has properties in common with endogenous heteromeric channels. Its modular structure consists of a transmembrane pore domain followed by a cytoplasmic leucine-rich repeat domain. The transmembrane domain, which is structurally related to connexin proteins, is wide towards the cytoplasm but constricted on the outside by a structural unit that acts as a selectivity filter. An excess of basic residues in the filter and throughout the pore attracts anions by electrostatic interaction. Our work reveals the previously unknown architecture of volume-regulated anion channels and their mechanism of selective anion conduction.

Volume-regulated anion channels (VRACs) are important constituents of the cellular response to osmotic swelling¹. This process occurs as a consequence of aquaporin-mediated water influx into cells under hypotonic conditions. To counteract the volume increase and prevent swelling-induced burst, different ion transport proteins are activated; this enables a controlled efflux of ions, organic osmolytes and consequently water, and allows cells to return to their original unchallenged state². The proteins involved in such regulatory volume decrease include cation-selective ion channels, secondary-active transporters and VRACs¹. Besides their role in regulatory volume decrease, VRACs have also been proposed to participate in apoptosis³ and in the regulation of electric excitability of neurons through the release of neurotransmitters from astrocytes^{4–6}. Although investigated by electrophysiology for decades^{3,7}, the molecular identity of VRACs was unknown until 2014, when two groups independently identified LRRC8A as an essential component of the channel^{8,9}. LRRC8A is a member of the LRRC8 protein family, which is found exclusively in vertebrates. In mammals the family encompasses five paralogues (LRRC8A–E), all of which share a high degree of sequence similarity^{10–12} (Extended Data Fig. 1). The proteins consist of a transmembrane pore domain that is remotely related to pannexin proteins¹², followed by a C-terminal domain containing 15–17 predicted leucine-rich repeats (LRRD)^{11,12}. LRRC8 proteins are widely expressed and form heteromeric channels with diverse subunit stoichiometry and distinct functional properties^{8,9}. Although some assemblies are selective for small anions, others are also permeable to osmolytes, amino acids and neurotransmitters, or are involved in the uptake of antibiotics and anticancer drugs^{6,8,9,13–16}. Whereas LRRC8A is the only obligatory subunit of VRACs^{8,9}, there is at present no evidence of endogenous homomeric channels in a physiological context. However, when overexpressed on its own, LRRC8A is targeted to the plasma membrane⁹ and has been reported to form oligomers of a similar size to heteromeric assemblies composed of different paralogues, but with smaller conductance¹⁶. We therefore expect a homomeric VRAC, containing the LRRC8A subunit only, to exhibit very similar structural properties to LRRC8 heteromers, while circumventing the problem of a variable subunit stoichiometry. Here we describe the structure of the homomeric LRRC8A complex determined by cryo-electron

microscopy (cryo-EM) and the X-ray structure of its isolated cytoplasmic domain. The structure reveals the general architecture of a VRAC and, in combination with functional experiments, provides insight into the mechanism of selective anion conduction.

Structural and functional characterization

For our investigations into the molecular properties of VRACs, we have studied mouse paralogues of the LRRC8 family. To recapitulate the functional behaviour of homo- and heteromeric constructs, we have recorded currents from a human embryonic kidney (HEK) cell line that does not express endogenous LRRC8 subunits^{9,15} (*LRRC8*^{−/−}). In response to a decrease in the intracellular ion concentration, wild-type HEK cells show large anion-selective currents, which are absent in *LRRC8*^{−/−} (Extended Data Fig. 2a–e). This confirms that VRAC function is mediated by LRRC8 proteins, which are activated at low cytoplasmic ionic strength^{16,17}. As described previously⁹, the co-transfection of *LRRC8*^{−/−} cells with DNA coding for both mouse LRRC8A and LRRC8C (LRRC8A/C) restores the native-like current response (Fig. 1a, b, Extended Data Fig. 2f, g). Currents with much smaller amplitude and stronger outward rectification are evoked after transfection with LRRC8A alone despite a comparable surface expression of the proteins (Fig. 1a, c, Extended Data Fig. 2h–j, Supplementary Fig. 1). Similar to native cells and the LRRC8A/C constructs, the LRRC8A currents are also activated by a decrease in intracellular ion concentration, although with lower sensitivity (Fig. 1c, Extended Data Fig. 2h, i), which confirms that LRRC8A forms functional channels with compromised activation and seemingly poor conduction properties. For the structure determination of a VRAC, we transiently expressed LRRC8A in HEK293S GnTI[−] cells. Consistent with previous observations¹⁶, the purified homomeric protein forms hexamers, the expected oligomeric state of the family¹² (Extended Data Fig. 2k). The modularity of its organization (Extended Data Fig. 1) is highlighted by the possibility of expressing and purifying both domains as separate stable entities, with the transmembrane pore domain (LRRC8A^{PD}) retaining its oligomeric structure and the isolated cytoplasmic LRRD (LRRC8A^{LRRD}) dissociating into monomers (Extended Data Fig. 2k–m).

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Fig. 1 | Relationships between homomeric and heteromeric LRRC8 proteins. **a**, Whole-cell current response of *LRRC8*^{-/-} cells expressing either mouse LRRC8A/C (cyan, pink) or LRRC8A (orange, beige) upon a decrease of the intracellular ion concentration. The inset depicts a magnification of LRRC8A currents during the plateau and endogenous currents (purple, pink) from *LRRC8*^{-/-} for comparison. The intracellular and extracellular ion concentrations are denoted by i and o, respectively. **b**, **c**, Current-voltage relationships of *LRRC8*^{-/-} cells expressing LRRC8A/C

(**b**) and LRRC8A (**c**) at different intracellular ion concentrations. Averages of independent biological replicates are shown (LRRC8A/C: 175 mM, *n* = 7; 150 mM, *n* = 6; 100 mM, *n* = 5; LRRC8A: 100 mM, *n* = 5; 75 mM, *n* = 4; 50 mM, *n* = 5), errors are s.e.m. **a** shows representative recordings of data displayed in **b** and **c**. **d**, **e**, Cryo-EM density of LRRC8A at 4.25 Å (**d**) and LRRC8A/C at 7.94 Å (**e**) superimposed on the refined model. Left, density of the entire channel contoured at 4 σ. Right, density around the pore domain of a single subunit contoured at 6.5 σ.

Datasets of LRRC8A in the presence of 250 mM sodium chloride recorded by cryo-EM reveal the detailed structural properties of the channel (Extended Data Figs. 3, 4). 3D classification yielded reconstructions of the protein with a well-defined symmetric pore domain and a structurally heterogeneous LRRD, which indicates a higher flexibility of this region (Extended Data Figs. 3d, 4d). A major class shows a channel in which the C6 symmetry of the pore domain reduces to a three-fold relationship between pairs of LRRDs. However, even for C3-symmetric particles, the density of the cytoplasmic unit is comparably weak and of lower resolution (Extended Data Figs. 3f, 4f, 5a, b). The general structural properties of full-length LRRC8A were also observed in the 7.9 Å cryo-EM density of heteromeric channels consisting of the closely related LRRC8A and LRRC8C subunits, which confirms that the structure observed for LRRC8A hexamers is representative of the entire protein family (Fig. 1d, e, Extended Data Figs. 1, 5c–h).

The detailed molecular structure of LRRC8A was determined from a combination of different datasets. The structure of the pore domain was built into a 3.66 Å map obtained from high-resolution cryo-EM data after masking and imposing C6 symmetry (Extended Data Figs. 4d, g, h, 6a–c, Supplementary Video 1). The isolated LRRD was crystallized and its structure was determined by X-ray crystallography at 1.8 Å (Extended Data Figs. 6d, e, 7a, Supplementary Video 2). The full-length protein was finally assembled from its two parts into C3-symmetrized cryo-EM density of the entire channel at 4.25 Å, and into a second independent reconstruction at 5.0 Å with better-defined density of the cytoplasmic domains (Extended Data Figs. 3e, f, 4e, f, 5a, b, 6f, g, Supplementary Video 3). In combination, this procedure enabled an unambiguous interpretation of the protein structure by an atomic model (Fig. 2, Extended Data Fig. 7, Supplementary Video 4).

Structure of mouse LRRC8A

The LRRC8A structure shows a hexameric protein that is 180 Å long and 110 Å wide (Fig. 2). The six subunits are arranged around an axis of symmetry that defines the ion conduction path, with their N- and C termini located on the cytoplasmic side and facing the pore lumen (Figs. 2, 3a). The pore domain of each subunit contains four transmembrane helices (TM1–TM4), with extended connecting regions folding into compact structures that constitute the extra- and intracellular entrances to the pore (Fig. 3b, Extended Data Fig. 1). The relationship between the pore domain and gap junction proteins is apparent from a superposition of the membrane-inserted helices of LRRC8A with connexin^{18,19} and innexin²⁰ subunits (Fig. 3c). Similarly, the cytoplasmic LRRD shows structural resemblance to related units in other proteins²¹ (Fig. 3d). As predicted from its sequence, the LRRD consists of 16 structural repeats, each containing a β-strand followed by an α-helix

of variable length (Fig. 3e, Extended Data Fig. 1). As is characteristic for domains exhibiting this fold, the interacting strands form a parallel β-sheet on the concave side of the horseshoe-shaped protein²¹, which faces the symmetry axis in the oligomeric channel (Fig. 2a). Both terminal repeats are capped by α-helices, which connect to the pore domain

Fig. 2 | The structure of LRRC8A. **a**, Ribbon representation of the hexameric LRRC8A structure viewed from within the membrane. Membrane boundaries and molecular dimensions are indicated. **b**, LRRC8A hexamer viewed from the extracellular side.

Fig. 3 | The LRRC8A subunit. **a**, Ribbon representation of the LRRC8A subunit. The pore domain and the LRRD are coloured in green and blue, respectively. **b**, Structure of the pore domain, with sections of the protein shown in unique colours and labelled. **c**, Superposition of the four transmembrane helices (as Coα-representation) of LRRC8A with the

equivalent regions of connexin Cx26 (PDB: 2ZW3) and innexin-6 (PDB: 5H1Q). **d**, Coα-representation of a superposition of the LRRD of LRRC8A and an LRRD from *L. pneumophila* (PDB: 4Q62). **e**, Ribbon representation of the LRRD of LRRC8A, with selected secondary structure elements labelled. PD, pore domain.

on the N terminus and contribute to the interaction with LRRDs of other subunits at the C terminus (Fig. 3e).

Organization of pore and LRR domains

The pore domain can be divided into three segments: a central, approximately 50 Å-long transmembrane part, and two flanking subdomains which protrude 25 Å from the membrane towards the cytoplasm and the extracellular side (Fig. 4a). Whereas the extra- and intracellular components of the pore are hydrophilic and contain several charged residues, the wide transmembrane region is dominated by hydrophobic residues (Fig. 4a). Interactions between the pore domains of neighbouring subunits bury 3,700 Å² of the combined molecular surface and are particularly tight on the outside of the membrane (Fig. 4a). The extracellular subdomain (ESD) consists of the E1 and E2 regions, which fold into an α-helix (E1H) and a three-stranded antiparallel β-sheet (E1β, E2β1 and E2β2, Fig. 3b, Extended Data Fig. 1). Part of the glycosylated loop⁹, connecting E1β and E1H, is not defined in the cryo-EM density. The ESD, which is stabilized by three disulfide bridges, harbours the narrowest part of the ion conduction path, with a radius of about 2.9 Å at its constriction (Fig. 4a–c, Extended Data Fig. 6b, c). In the membrane-inserted segment, the conical pore widens and is lined by residues of TM1 throughout its length and additionally by residues of TM2 on its intracellular half (Figs. 3a, b, 4b). Within this region, the interface between subunits is less tightly packed and contains gaps

that are probably sealed by lipids (Figs. 4a). On the cytoplasmic side, a subdomain (CSD) containing six α-helices (contributed by the cytoplasmic loop and the C-terminal region) constitutes the channel exit leading to the cytoplasmic LRRDs (Figs. 3a, b, 4a, b, Extended Data Fig. 1). The CSD also contains an unstructured loop carrying several putative phosphorylation sites^{22,23} (Extended Data Fig. 1). Within the transmembrane region and the CSD the pore is wide, extending up to 32 Å in diameter. However, as the first 14 residues of the N terminus of each subunit, located close to the intracellular membrane boundary and projecting to the pore lumen, are not defined in the structure, the dimensions of this part of the ion conduction path cannot be defined with certainty.

Below the CSD, the wide protein-enclosed exit of the pore leads into an 80 Å-long structure that is formed by the LRRDs (Figs. 2a, 3a). In the LRRC8A hexamer, these cytoplasmic units are tilted by about 30–40° with respect to the pore axis towards the plane of the membrane (Figs. 2b, 5a). The six LRRDs do not interact in the same way, but rather form a trimer of dimers. This results in a large interface between interacting pairs (referred to as l- and r subunits), which buries more than 1,603 Å² of the combined molecular surface, and a small interface of the C-terminal α-helix of the r-subunit with the l-subunit of a neighbouring domain pair burying 288 Å² (Fig. 5b–d). The tight interactions cause a change in the orientation of adjacent domains, which can be approximated by a 42° rotation relative to each other (Fig. 5a).

Fig. 4 | The pore domain. **a**, Pore domain of LRRC8A and the molecular surface of the ion conduction path viewed from within the membrane. The two front subunits are removed for clarity. The surface is coloured according to amino acid properties (polar, green; acidic, red; basic,

blue). Sections of the pore domain are indicated. **b**, The pore of LRRC8A displayed in relation to two opposite subunits with selected residues shown. **c**, The pore radius along the symmetry axis. Orientation and dimensions are as in **a**, **b**. TM, transmembrane region.

Fig. 5 | The cytoplasmic LRR domains. **a**, Co- α -trace of LRRDs of l- (green) and r- (orange) subunits after superposition of their pore domains. **b–d**, View of the intracellular part of LRRC8A. The protein is shown as a space-filling model. The relationship between views is indicated. The view

in **c** is the same as in Fig. 2a. **e**, The molecular surface of a single LRRD with polar residues coloured in green, basic residues in blue, and acidic residues in red.

This leads to a breakdown of the C6 symmetry observed for the pore region to an overall three-fold symmetry (Fig. 5d). At the intracellular end, the C-terminal α -helices of the three l-subunits engage in mutual interactions at the location of the symmetry axis (Fig. 5d). Potential access pathways for hydrated anions to the pore region are created by spacious fenestrations, up to 12 Å in size, at the small interface between domain pairs (Fig. 5b, d). The interaction interfaces bury several ionizable residues of the hydrophilic LRRDs (Fig. 5e), which suggests that the affinity of these interactions might be affected by ionic strength.

Electrostatics and selectivity filter

The homomeric channel consisting of LRRC8A subunits exhibits a strongly positive electrostatic potential throughout the ion conduction path, which is preserved in a homology model of a heteromeric channel consisting of a hypothetical arrangement of alternating A and C subunits, therefore suggesting that anions are attracted by coulombic interactions in both homomeric and heteromeric assemblies (Fig. 6a, b, Extended Data Fig. 8a, b). During permeation, the constriction of

the ESD may act as a filter, selecting for the size and charge of partially dehydrated anions. At this narrowing, located at the N terminus of α -helix E1H, the filter contains an arginine (R103), the side chain of which points towards the pore axis (Fig. 6c, d, Extended Data Fig. 6c, Supplementary Video 4). The equivalent positions in other paralogues (except for LRRC8B) are uncharged, which accounts for the lower positive potential at the extracellular pore entrance observed for the LRRC8A/C model (Fig. 6a, Extended Data Figs. 1a, 8b). LRRC8C and LRRC8E contain a leucine and LRRC8D contains a phenylalanine that, in a heteromeric channel, might widen the pore and change the structural flexibility of the filter (Extended Data Fig. 8c, d). Upstream of R103, mutations of the interacting residues K98 and D100 were found to alter the inactivation of the channel at positive voltages²⁴ (Fig. 6c, d). Towards the cytoplasmic end of the ESD, two additional basic residues (H104 and K51) contribute to the positive electrostatic environment in the narrow pore region, and close to the membrane boundary, a second narrowing that is larger in diameter exposes two threonines (T48 and T44) into the pore region (Figs. 4b, c, 6c). The mutation of T44 has previously been described to alter the selectivity between anions

Fig. 6 | Electrostatics and selectivity filter. **a**, Electrostatic potential along the pore of LRRC8A (red) and a homology model of LRRC8A/C (blue) as determined by a numerical solution of the Poisson–Boltzmann equation in the absence of mobile ions. Membrane boundaries (C, hydrophobic core; HG, lipid head groups) and the border between the pore domain and the LRRD are indicated. **b**, Ribbon representation of two opposite subunits of the model used for electrostatic calculations in relation to the membrane (box). **c**, **d**, Part of the ESD that lines the pore, viewed from within the membrane with two front subunits removed for clarity (**c**) and from the

extracellular side (**d**). The protein is displayed as a ribbon representation and selected side chains are shown as sticks. **e**, **f**, Cation/anion (**e**) and anion/anion (**f**) selectivity as a function of positive charge density in the selectivity filter recorded from the mutants LRRC8A(R103A)/C (zero), LRRC8A/C (assumed as three) and LRRC8A/C(L105R) (six). Individual measurements and averages of independent biological replicates are shown, errors are s.e.m. **g**, Schematic representation of the pore organization of a LRRC8 channel. Movements of the cytoplasmic LRRDs potentially involved in activation are indicated.

and decrease the pore conductance upon modification with negatively charged reagents⁸. Although this region is too wide for the coordination of dehydrated ions, interactions might be mediated through solvating waters (Extended Data Fig. 8e). Intracellular to T44, the pore widens further and turns predominantly hydrophobic before it becomes hydrophilic again at the CSD, where an excess of basic residues render the electrostatics attractive for anions accessing the channel from the intracellular side (Figs. 4a, 6a).

Structure–function relationships

To address whether the presence of six arginine residues at the extracellular constriction of the LRRC8A pore impedes ion conduction, thereby accounting for its poor functional response, we characterized the mutant LRRC8A(R103A) and found that the truncation of this bulky side chain was insufficient to increase the current density of the homomeric channel (Extended Data Fig. 9a, b). We next examined the mutation L105R in the C subunit of the heteromeric LRRC8A/C(L105R) channel, which mimics the ring of six arginines at the narrowest constriction in the LRRC8A homomer. Consistent with the above result, we observed a very similar current response to that of wild-type channels, which provides further evidence that a high density of arginine residues at the pore constriction does not prevent ion conduction (Extended Data Fig. 9c, d). Finally, we paired the LRRC8A(R103A) subunit with the LRRC8C subunit to yield a heteromeric channel that lacks arginines at the constriction, and found decreased but still sizable currents upon lowering the intracellular ionic strength (Extended Data Fig. 9e, f). Nonetheless, in contrast to the highly anion-selective currents mediated by LRRC8A/C and LRRC8A/C(L105R), LRRC8A(R103A)/C shows measurable Na⁺ permeability (Fig. 6e, Extended Data Fig. 9g, h), which demonstrates that the presence of these constricting arginines plays an important role in determining the charge selectivity of LRRC8 channels. The general preference for anions in the mutant that does not contain any arginines at the constriction is probably conferred by other positively charged residues distributed along the pore (Figs. 4a, 6a, Extended Data Fig. 8b, f). The importance of this location for anion interactions is also reflected in the positive correlation of the arginine density with sulfate permeability, which is consistent with the presence of an anion-binding site at the position of the constriction (Fig. 6f, Extended Data Fig. 9i–n). Together, these experiments demonstrate the functional importance of the narrow extracellular region acting as an anion selectivity filter.

Discussion

We have elucidated the molecular architecture of volume-regulated anion channels of the LRRC8 family and investigated its relationship to functional properties. VRACs are hexameric proteins with a modular structure (Figs. 2, 6g). A characteristic feature of their organization is the symmetry mismatch between the six-fold symmetric pore domains and the three-fold symmetric LRRDs, akin to a break of symmetry observed in ionotropic glutamate receptors²⁵ and in the magnesium channel CorA²⁶. Although unrelated in sequence, the structure of the pore domain resembles gap junction channels, particularly with respect to the location of the transmembrane helices and the organization of the ESD (Fig. 3c, Extended Data Fig. 10a, b). Despite their general similarity, the two protein classes exhibit distinct functional features, with gap junction channels forming wide pores of poor selectivity that also permit the diffusion of larger substrates^{27,28}, and certain VRACs (such as channels composed of LRRC8A and LRRC8C subunits) being selective for smaller anions, with a negligible conductance for cations²⁹ (Fig. 6e, Extended Data Figs. 2c, 9g, h). A comparison of their structures sheds light on their distinct permeation properties (Extended Data Fig. 10). As well as the difference in the electrostatics—with a strong positive potential being a characteristic property of VRACs (Fig. 6a)—connexins^{18,19} and invertebrate innexins²⁰ form channels of large diameter throughout, whereas VRACs are constricted at their extracellular part (Fig. 4b, c, Extended Data Fig. 10c–f). In both gap junction proteins, the N terminus narrows the wide channels by

forming an α -helix that lines the pore and projects towards the extracellular direction (Extended Data Fig. 10a, b, e, f)^{18,20}. Because the equivalent part of LRRC8A channels was not defined in the cryo-EM density, its involvement in channel regulation is unclear at present.

The ESD of the VRAC pore forms a size and charge selectivity filter that interacts with at least partially dehydrated ions; this is consistent with the observed lyotropic permeability sequence of certain isoforms^{30,31}. The interaction of positively charged residues at the pore constriction with permeating anions is also supported by the appearance of sodium conductance and the decreased permeability of divalent anions observed in a mutant in which the constricting arginines were removed (Fig. 6e, f, Extended Data Fig. 9g–n). The remaining preference for anions by the mutated channel is probably conferred by positively charged residues distributed along the pore (Figs. 4a, 6a, g, Extended Data Figs. 8f, 9g, h). Although our low-resolution data of LRRC8A/C channels suggest a similar geometry of the constricted extracellular region compared to LRRC8A (Fig. 1d, e, Extended Data Fig. 5c–f), it is unclear at present whether this structural unit is rigid or whether it changes its conformation during channel opening, closing or inactivation. In contrast to the anion-selective channels composed of the LRRC8A and LRRC8C subunits, VRAC heteromers containing the LRRC8D subunit display broader substrate specificity^{13–15}. In these channels the architecture of the filter might be altered to permit conduction of larger substrates. Although the structure of the homomeric LRRC8A is representative of the family, its relation to a defined functional state is at this point ambiguous. The protein structure was determined at high salt concentration; in a cellular environment, such a condition favours a closed conformation of VRACs¹⁶. The correspondence to a closed conformation is also consistent with the apparent shift in sensitivity of activation of the homomeric LRRC8A construct towards lower ionic strength (Fig. 1c, Extended Data Fig. 2h, i). However, apart from the constricted extracellular pore entrance and the unclear conformation of the N terminus, the pore is wide and should therefore enable the passage of anions. In this respect, the role of the LRRDs and their potential structural rearrangement during activation is still unclear. Although interacting, these domains show a certain degree of flexibility, as indicated by their poorly defined structure in subpopulations of the collected particles (Extended Data Fig. 3d). Moreover, owing to the electrostatic nature of mutual interactions, their affinity could be influenced by the ion concentration and could therefore contribute to channel activation during regulatory volume decrease (Fig. 5e). Still, the definitive location of the sensor of ionic strength and the mechanism of how conformational changes are coupled to pore opening remain unknown. Other features that are unclear at present concern potential ligands of the LRRDs³², the putative regulation by oxidative stress³³ and the role of phosphorylation^{32,34} (Extended Data Fig. 1). These questions will be resolved in future studies, for which the current data provide an important foundation.

Online content

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at <https://doi.org/10.1038/s41586-018-0134-y>.

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Author contributions D.D. generated expression constructs, purified proteins and determined the X-ray structure of the cytoplasmic domain. M.S. prepared the samples for cryo-EM, collected cryo-EM data and proceeded with structure determination. A.K.M.L. recorded and analysed electrophysiology data. C. P. established data processing infrastructure and performed initial screening of conditions for cryo-EM studies. D.D., M.S., A.K.M. L. and R.D. jointly planned experiments, analysed the data and wrote the manuscript.

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METHODS

Expression constructs. The gene that encodes mouse LRRC8A (NCBI: NP_808393.1) was codon-optimized for expression in human cell lines (Integrated DNA Technologies) and synthesized by Genescript. The gene that encodes mouse LRRC8C (NCBI: NP_598658.1) was acquired from OriGene. SapI restriction sites were removed by site-directed mutagenesis. All constructs were cloned into vectors that are based on pcDNA 3.1 (Invitrogen) and that were modified to be compatible with FX cloning³⁵. Unless stated otherwise, all fusion tags were located at the C terminus and connected by a Rhinovirus 3C protease-cleavable linker. The construct containing only the pore domain of LRRC8A (LRRC8A^{PD}) consists of residues 1–428. The cytoplasmic domain construct of LRRC8A (LRRC8A^{LRRD}) consists of residues 398–810. For expression of full-length LRRC8A and LRRC8A^{PD}, the protein was fused to mCherry followed by a Myc tag and streptavidin-binding peptide (SBP)³⁶. For LRRC8A/C co-expression, LRRC8A contained an mScarlet1-His₁₀-tag³⁷ and LRRC8C contained a Venus-Myc-SBP tag. The cytoplasmic domain construct was fused at the N terminus to the Venus-Myc-SBP tag. For electrophysiology and surface expression analysis, all constructs were tagged with Myc-SBP only. The Venus-only construct used for the visualization of transfected cells for electrophysiology contained the Venus gene followed by a Myc-SBP tag.

Protein expression and purification. Suspension cultures of HEK293S GnT1[−] cells³⁸ were grown in Pro293CDMs medium (Lonza) supplemented with 1% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U ml^{−1} penicillin–streptomycin (Sigma) at 37 °C and 5% CO₂. For protein expression, cells were transiently transfected using a high-density transfection method with linear polyethylenimine (25 kDa, Polysciences) as described previously³⁹ with minor modifications. During the transfection step, cells were resuspended in DMEM medium (with phenol red, 4,500 g l^{−1} glucose, Sigma). Plasmid DNA used for transfection was purified with a NucleoBond Xtra Maxi kit (Machery-Nagel) from *E. coli* MC1061 cultures. HEK cells were collected by centrifugation 36–48 h after transfection and washed with PBS. Pellets were flash-frozen in liquid nitrogen and stored at −20 °C for further use. The entire purification process was carried out at 4 °C. Full-length LRRC8A and LRRC8A^{PD} were purified as described. 4–8 g of cell pellet from 1–2 l of suspension culture was resuspended in 40 ml of 25 mM Tris-HCl, pH 8.5, 250 mM NaCl, 3% digitonin (AppliChem) supplemented with 50 µg ml^{−1} DNase (AppliChem), 50 µg ml^{−1} RNase A (Sigma) and protease inhibitors (cOmplete EDTA-free, Roche). The mixture was incubated for 1.5 h under gentle agitation. Lysate was centrifuged for 30 min at 10,000g and the supernatant was filtered through a 5-µm syringe filter (Minisart, Sartorius). Filtered supernatant containing the solubilized protein was mixed with 10 ml of StrepTactin Superflow resin (IBA Lifesciences) equilibrated with SEC buffer containing 25 mM Tris-HCl pH 8.5, 250 mM NaCl, 0.12% digitonin (Calbiochem). The suspension was incubated in batch for 3 h under gentle agitation. The resin was subsequently washed with 250 ml of SEC buffer. The protein was eluted with 20 ml of SEC buffer supplemented with 15 mM D-desthiobiotin (Sigma) and incubated with 0.4 mg of 3C protease for 30 min to cleave the fusion tag. The sample was subsequently concentrated using a centrifugal filter (Amicon, 100 kDa molecular weight cut-off), filtered (Millipore, 0.22 µm) and separated on a Superose 6 10/300 column (GE Healthcare) equilibrated with SEC buffer. Selected peak fractions containing the protein were pooled, concentrated and filtered. The analysis of the protein preparation by mass spectrometry revealed negligible contamination by endogenous LRRC8 subunits. For size exclusion chromatography with multi-angle light scattering (SEC–MALS) experiments, LRRC8A^{PD} was purified as described above except that buffers contained 2% of *n*-dodecyl-β-D-maltoside (DDM, Anatrace) during extraction and 0.25 mM DDM in subsequent purification steps. Heteromeric LRRC8A/C channels were expressed and purified in a similar manner to that described above with several modifications. The protein was purified from cells collected from a 4-l culture. After lysis, solubilization and centrifugation, the supernatant was supplemented with 5 mM imidazole and mixed with 10 ml of 6BCL-NTANi-500 resin (Agarose Bead Technologies) equilibrated with SEC buffer to purify channels containing the His₁₀-tagged LRRC8A subunit. The suspension was incubated for 1 h under gentle agitation and resin containing bound protein was washed with 250 ml of SEC buffer supplemented with 5 mM imidazole. The protein was subsequently eluted with 30 ml of SEC buffer supplemented with 300 mM imidazole and loaded onto a column containing 5 ml of Strep-Tactin Superflow resin (IBA Lifesciences) equilibrated with SEC buffer to bind channels containing the SBP-tagged LRRC8C subunit. The Strep-Tactin resin was washed with 50 ml of SEC buffer and the protein was eluted with SEC buffer supplemented with 15 mM D-desthiobiotin (Sigma). Further purification and concentration proceeded as described for the homomeric LRRC8A. The analysis of the preparation by mass spectrometry revealed similar ratios of LRRC8A and LRRC8C constructs and negligible contamination by endogenous subunits. The cytoplasmic domain construct LRRC8A^{LRRD} was expressed in HEK293S GnT1[−] cells and purified as described above with small modifications. The buffer used for extraction contained 10 mM Tris pH 9.4, 200 mM NaCl, 2% DDM, 50 µg ml^{−1}

DNase (AppliChem) and protease inhibitors (cOmplete EDTA-free, Roche). After batch-binding of the protein, resin was washed with SEC2 buffer (10 mM Tris pH 9.4, 200 mM NaCl, 0.1% CHAPS). The protein was released from the resin by incubating the slurry with 0.8 mg 3C protease for 30 min. Eluted protein was concentrated by filtration (Amicon, 10 kDa) and separated on a Superdex 75 10/300 column (GE Healthcare) equilibrated with SEC2 buffer. For crystallization, protein was concentrated (Amicon, 10 kDa molecular weight cut-off) and supplemented with 0.5% CHAPS and 1 mM tris(2-carboxyethyl)phosphine.

Molecular weight determination with SEC–MALS. Multi-angle light scattering experiments were performed at room temperature using an HPLC system (Agilent 1100) coupled to an Eclipse 3 system equipped with miniDAWN TREOS MALS detector and Optilab T-rEX refractometer (Wyatt Technology). 50 µl of purified LRRC8A^{PD} protein, at a concentration of 1 mg ml^{−1} was run on a Superose 6 10/300 Increase column equilibrated with buffer containing 25 mM Tris-HCl pH 8.5, 250 mM NaCl and 0.25 mM DDM. The molecular weight was calculated with the Astra software (Astra 6.1, Wyatt Technologies) on the basis of the absorption at 280 nm, light scattering, and the differential refractive index. Extinction coefficients at 280 nm (1.790 ml mg^{−1} cm^{−1}) and *dn/dc* values (0.190 mol ml g^{−2}) for LRRC8A^{PD} were estimated using sedfit⁴⁰. The *dn/dc* value for DDM was assumed⁴¹ to be 0.133 mol ml g^{−2}.

Surface expression analysis. HEK 293 LRRC8^{−/−} cells (kindly provided by T. J. Jentsch) were transfected at 60% confluency with 20 µg of DNA per 10-cm dish using Fugene 6 (Promega) and grown at 37 °C and 5% CO₂. Surface-expressed proteins were labelled with sulfo-NHS-SS-biotin 24 h post-transfection using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific) according to the manufacturer's protocol. For the non-biotinylated control, cells were treated with PBS instead of NHS-SS-biotin solution. Extraction and purification steps were carried out at 4 °C. The cell pellet obtained from two 10-cm dishes was resuspended in 1 ml of PBS supplemented with 1% DDM, protease inhibitors, 10 µg ml^{−1} DNase and 10 µg ml^{−1} RNase A and incubated for 1 h under gentle agitation. The insoluble fraction was removed by centrifugation at 16,000g for 30 min. The supernatant was mixed with 200 µl bed volume NeutrAvidin Agarose resin equilibrated with PBS and incubated for 1 h under gentle agitation. The flow-through was discarded and the resin was washed six times with 500 µl of PBS supplemented with 0.5 mM DDM. Captured proteins were eluted from the resin by incubation with SDS–PAGE loading buffer containing 50 mM fresh dithiothreitol for 1 h at 37 °C with vigorous mixing. Samples were separated by SDS–PAGE, transferred to a polyvinylidene fluoride membrane and analysed by western blotting using a mouse monoclonal anti-c-Myc primary antibody (Sigma, M 4439, Clone 9E10) and goat anti-mouse-HRP conjugated secondary antibody (Jackson ImmunoResearch, 115-035-146). The luminescent signal was developed with ECL substrate (Millipore) and recorded with a Fuji LAS3000 imaging system.

X-ray structure determination of the cytoplasmic LRR domain. The C-terminal domain construct LRRC8A^{LRRD} containing three additional residues at the N terminus (Gly-Pro-Ser) and an additional Ala at the C terminus was crystallized by vapour diffusion in sitting drops at 4 °C. Drops were prepared by mixing 0.1 µl of protein solution at a concentration of 9.2 mg ml^{−1} with 0.1 µl of precipitant solution containing 0.1 M Bis-Tris propane pH 8.5, 0.2 M sodium malonate and 20% PEG3350 (NeXtal PACT Suite, Qiagen). Crystals were collected after three weeks, cryo-protected in crystallization solution containing an additional 30% of ethylene glycol and flash-frozen in liquid nitrogen. X-ray diffraction data were collected on the X10SA beamline at Swiss Light Source of the Paul-Scherrer Institute on a Pilatus 6M detector. Native and anomalous data (collected at a wavelength of 2.06 Å) were collected from the same crystal. Data were processed with XDS⁴². Crystals are of space group P6₃ and contain one copy of the molecule in their asymmetric unit. Initial phases were obtained by molecular replacement combined with single anomalous dispersion with Phaser⁴³ implemented in the Phenix package⁴⁴ using anomalous data at a resolution of 2.5 Å. A partial search model for molecular replacement encompassing residues 644–755 of LRRC8A was constructed from a homology model generated with I-TASSER⁴⁵ on the basis of the crystal structure of a LRR protein (PDB: 4U06, Chain A). An initial structure built automatically with Phenix AutoSol⁴⁴ was used for molecular replacement against the native dataset (at a resolution of 1.8 Å). The complete structure was subsequently obtained by multiple iterations of structure building in Coot⁴⁶ and refinement with Phenix⁴⁴. *R*_{free} was calculated on the basis of 5% of reflections excluded from refinement. The final model consisting of 411 out of 416 residues, 337 waters and 4 ethylene glycols, is well refined with *R*_{work} and *R*_{free} values of 17.9% and 20.8%, respectively. Unresolved residues include the N-terminal glycine and proline and three residues (Gln-Ala-Ala) at the C terminus.

Cryo-EM sample preparation and data collection. For structure determination of LRRC8A by cryo-EM, 2.5-µl samples of purified protein at concentrations of 3.9–4.25 mg ml^{−1} were applied to glow-discharged holey carbon grids (Quantifoil R1.2/1.3 Au 200 mesh). For the structural characterization of the co-expressed LRRC8A/C assembly, 2.5-µl samples at a protein concentration of 3.5 mg ml^{−1} were

applied in a similar manner. Excess liquid was removed in a controlled environment (10 °C and 100% relative humidity) by blotting grids for 3–7 s (with a blotting force of 1 for LRRC8A and 0 for LRRC8A/C). Grids were subsequently flash-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific). LRRC8A (dataset 1) and LRRC8A/C (dataset 3) were imaged in a 300 kV Tecnai G² Polara (FEI) with a 100 μm objective aperture. A second dataset of LRRC8A (dataset 2) was recorded on a 300 kV Titan Krios (Thermo Fisher Scientific) with a 100 μm objective aperture. All data were collected using a post-column quantum energy filter (Gatan) with a 20 eV slit and a K2 Summit direct detector (Gatan) operating in super-resolution mode. Dose-fractionated micrographs were recorded in an automated manner using SerialEM⁴⁷ with a defocus range of -0.5 to -3.2 μm . Dataset 1 and dataset 3 were recorded at a nominal magnification of 37,313 corresponding to a pixel size of 1.34 Å per pixel (0.67 Å per pixel in super-resolution) with a total exposure time of 12.5 s (50 individual frames) and a dose of 1.4 electrons per square Å per frame. Dataset 2 was recorded at a nominal magnification of 46,511 corresponding to a pixel size of 1.075 Å per pixel (0.5375 Å per pixel in super-resolution) with a total exposure time of 13 s (65 individual frames) and a dose of approximately 1.1 electrons per square Å per frame. The total electron dose on the specimen level for all datasets was approximately $70\text{ e}^- \text{Å}^{-2}$.

Cryo-EM image processing. Two independent datasets of LRRC8A consisting of 9,209 (dataset 1) and 3,023 (dataset 2) micrographs were collected. The dataset of LRRC8A/C consisted of 1,545 micrographs (dataset 3). The recorded super-resolution images were down-sampled twice by Fourier cropping and all individual frames were used for correction of beam-induced movement using a dose-weighting scheme in MotionCor2 (ref. 48). The contrast transfer function parameters were estimated on movie frames using CTFFIND4 (ref. 49). Low-quality micrographs showing a considerable drift, high defocus or poor estimates of the contrast transfer function were discarded resulting in datasets of 6,231 images for the LRRC8A Polara dataset, 1,817 images for the LRRC8A Titan Krios dataset and 1,127 images of the LRRC8A/C dataset, which were subjected to further data processing using RELION2.1b1 (refs 50,51). From the LRRC8A Polara dataset, 8,208 particles were extracted with a box size of 344 pixels and subjected to an initial reference-free 2D classification. From this set, five distinctive 2D class averages were selected and used as templates for automated particle picking, which generated a starting dataset of 329,624 particles. False positives and particles of poor quality were discarded through a round of 2D classification decreasing the number of particles to 287,953. The initial 3D reconstruction, which was generated in RELION from 3,600 randomly chosen cleaned particles, was low-pass filtered to 60 Å and used as a template in a subsequent 3D classification. Independent rounds of 3D classification with a different number of classes were performed to isolate the most homogeneous subset of particles. Out of nine classes, one revealed a fully assembled reconstruction with a clear indication of a global C3 symmetry. 54,861 particles belonging to this class were subjected to auto-refinement with imposed C3 symmetry and particle polishing with a running average window of 7, a standard deviation of 1 pixel on translations and 400 pixels on particle distance. The final map was auto-refined to 5.01 Å in the presence of a soft solvent mask and sharpened using an isotropic b-factor of -173 Å^2 . This map was then low-pass filtered to 60 Å and used as a 3D reference for processing of the LRRC8A dataset collected on a Titan Krios. 105,867 particles were picked automatically using seven representative 2D class averages from the Polara dataset and subsequently subjected to 3D classification. The initial clean-up of the starting pool of particles through 2D classification was deliberately omitted so as not to discard any particles representing underpopulated views. Similarly to the Polara dataset, independent rounds of 3D classification were performed to identify the most homogeneous subset of particles. Out of five classes, one has shown the same conformation with a clear three-fold symmetry in the cytoplasmic LRR domain and a six-fold symmetry in the transmembrane domain, as observed previously for the Polara dataset. 34,674 particles belonging to this class were polished with a running average window of 7, a standard deviation of 1 pixel on translations and 500 pixels on particle distance. The resulted class was auto-refined to 4.25 Å and sharpened using an isotropic b-factor of -118 Å^2 . Despite the increase in the overall resolution, as compared to the map generated from the Polara dataset, the map from the Titan Krios dataset showed considerably weaker density in the cytoplasmic domain, indicating its intrinsic flexibility. To improve the density of the transmembrane domain a focused 3D refinement with partial signal subtraction was performed⁵². In this approach, the density corresponding to the cytoplasmic domain was subtracted from the original particles. In silico modified particles (dataset 2a) were subsequently subjected to auto-refinement with C6 symmetry imposed in the presence of a soft mask around the transmembrane domain. The final map at 3.66 Å was sharpened using an isotropic b-factor of -96 Å^2 . The initial dataset of LRRC8A/C contained 101,239 particles, which were subjected to 2D classification to discard broken particles, small aggregates and to assess sample heterogeneity. Owing to the increased flexibility in the LRR domains of heteromeric complexes, 3D refinement was performed

by either omitting or imposing C3 symmetry on 94,369 particles selected after 2D classification. Euler angles of the best matching reference to each particle from the cleaned dataset 3 were assigned using a 40 Å low-pass filtered model (obtained from dataset 1) as an initial template. Particles, which were assigned incorrect orientations, were discarded through another round of 2D classification, in which the alignment step was deliberately omitted. To segregate the remaining structural heterogeneity, particles were subjected to a side-by-side non-symmetrized and C3-symmetrized 3D classification. In the non-symmetrized case, the two highest ranking classes were selected and 59,315 particles were auto-refined imposing C3 symmetry to yield a reconstruction of 8.23 Å. For C3-symmetrized classification, the best class contained 27,777 particles and was auto-refined with C3 symmetry to yield a reconstruction at 7.94 Å. In all cases resolution was estimated in the presence of a soft solvent mask and based on the gold standard Fourier shell correlation (FSC) 0.143 criterion^{50,53–55}. High-resolution noise substitution was applied to correct FSC curves for the effect of soft masking in real space⁵³. The local resolution was estimated using BlocRes from the Bsoft package^{56,57}.

Model building and refinement. The model of the transmembrane domain of LRRC8A was built in Coot⁴⁶. The cryo-EM density of the transmembrane region generated from focused refinement with C6 symmetry applied was of high quality and enabled the unambiguous assignment of residues 15–68, 92–176 and 230–408. As reflected by the variations in local resolution, the density of the terminal helix connecting to the cytoplasmic LRR domain was less well-defined than the density of the pore and other intra- and extracellular regions. The model was improved iteratively by cycles of real-space refinement in Phenix⁴⁴ with secondary structure and six-fold non-crystallographic symmetry constraints applied, reciprocal-space refinement in REFMAC5 (refs 58,59) and manual corrections in Coot. The model of full-length LRRC8A was assembled from the transmembrane and cytoplasmic domains fitted into the 4.25 Å electron density of the C3-symmetrized hexamer using UCSF Chimera⁶⁰. The structure of the LRRD in our cryo-EM reconstruction of the full-length channel is essentially unaltered compared to the X-ray structure, and the connection between pore domain and LRRD is unambiguously defined by the overlapping α -helix CTH3, which is present in both structures. Both domains were initially defined as independent entities and refined as rigid bodies. In a later stage, the pore domain and LRRD were linked into a single polypeptide chain and refinement proceeded as described above maintaining three-fold non-crystallographic symmetry and secondary structure constraints. Validation of the refinement of the transmembrane and the full-length models was performed in REFMAC5, distributed as part of the CCP-EM suite⁶¹, and represented as Fourier shell correlations (FSC_{sum}) between the refined models and their corresponding final cryo-EM density maps. For cross-validation, the cryo-EM dataset was split into two subsets that were used to calculate two independent maps (half map 1 and half map 2). To detect possible overfitting and hence over-estimation of the resolution, random shifts—up to 0.5 Å—were applied to the coordinates of each model, which was then refined against the unfiltered half map 1. The cross-validation was performed by comparing the FSC_{work} (estimated for the shaken-refined model and half map 1) and the FSC_{free} (estimated for the shaken-refined model and half map 2, which was not used in the refinement). Pore dimensions were calculated with HOLE⁶² and MOLE⁶³ and surfaces with MSMS⁶⁴. Figures and movies containing molecular structures and densities were prepared with DINO (<http://www.dino3d.org>), PyMOL (<https://pymol.org>) and Chimera⁶⁰.

Modelling and Poisson–Boltzmann calculations. The homology model of the LRRC8C subunit was prepared with the SWISS-MODEL homology modelling server⁶⁵. Regions not defined in the cryo-EM density of LRRC8A were excluded from the model, except for the N-terminal 14 amino acids that were modelled into both the homomeric LRRC8A structure and the heteromeric LRRC8A/C structure consisting of alternating A and C subunits used for electrostatic calculations. The electrostatic potential of the LRRC8A and the LRRC8A/C hexamers was calculated by solving the linearized Poisson–Boltzmann equation in CHARMM^{66,67} on a $240 \text{ Å} \times 240 \text{ Å} \times 260 \text{ Å}$ grid (1 Å grid spacing) followed by focusing on a $160 \text{ Å} \times 160 \text{ Å} \times 200 \text{ Å}$ grid (0.5 Å grid spacing). Partial protein charges were derived from the CHARMM36 all-hydrogen atom force field. Hydrogen positions were generated in CHARMM. Histidines were deprotonated. The protein was assigned a dielectric constant (ϵ) of 2. Its transmembrane region was embedded in a 30 Å-thick slab ($\epsilon = 2$) representing the hydrophobic core of the membrane and two adjacent 15 Å-thick regions ($\epsilon = 30$) representing the head groups. The membrane region contained a 60 Å-high and 20 Å-wide aqueous cylinder ($\epsilon = 80$) around the VRAC pore and was surrounded by an aqueous environment ($\epsilon = 80$). Calculations were carried out in either 0 or 150 mM of monovalent mobile ions in the aqueous regions.

Electrophysiology. LRRC8^{−/−} HEK293 cells were transfected with the constructs of interest at 3 μg total DNA per 6-cm Petri dish using the calcium phosphate co-precipitation method. LRRC8 constructs not containing fluorescent protein tags were co-transfected with a Venus-only construct, at an LRRC8:Venus ratio of 2:1, to aid identification of transfected cells. Cells were used the day after

transfection. LRRC8 constructs were transfected at a 1:1 ratio. Whole-cell patch-clamp recordings were performed on single LRRC8^{-/-} HEK293 cells expressing the LRRC8 constructs of interest. Seal resistance was typically 4 GΩ or higher before the establishment of the whole-cell configuration. Patch pipettes were pulled from borosilicate glass capillaries with an outer diameter of 1.5 mm and inner diameter of 0.86 mm (Sutter) and were fire-polished before use. Pipette resistance was typically 6–12 MΩ when filled with the iso-osmotic low ionic strength solution (see below) required to activate the LRRC8 channel. Series resistance was compensated by 60–80% and was typically between 2–8 MΩ after compensation. Voltage-clamp recordings were performed using Axopatch 200B and Digidata 1550 (Molecular Devices). Analogue signals were filtered at 5 kHz using the in-built 4-pole Bessel filter and were digitized at 10–20 kHz. Data acquisition was performed using Clampex 10.6 (Molecular Devices). Local perfusion was achieved using a gravity-fed system through a double-barrelled theta glass pipette. Liquid junction potential, calculated in Clampex, was corrected when it was larger than 2 mV. All experiments were performed at 20 °C. All recording solutions used in this study were approximately iso-osmotic with an osmolality of 308–330 mmol kg⁻¹ as measured using a vapour pressure osmometer (VAPRO). The solutions were also designed to create a minimal junction potential (less than 2 mV) when used as local perfusates. Unless otherwise indicated, recordings were performed under symmetrical ionic conditions. For anion permeability experiments, bi-ionic conditions were used. Pipette solution with a standard sub-physiological ionic strength contained 100 mM NMDG-Cl, 100 mM D-mannitol, 1 mM EGTA, 3 mM Na₂ATP and 10 mM HEPES at pH 7.4. When lower ionic strength was required, the salt/mannitol ratio was adjusted accordingly to maintain an approximately equal osmolality. When supra-physiological intracellular ionic strength was required, additional NMDG-Cl was reconstituted from a 1-M stock prepared from NMDG and HCl, and the extracellular solution contained equi-osmotic D-mannitol reconstituted from a 0.5-M stock to maintain an iso-osmotic condition. The extracellular solution contained 100 mM NMDG-Cl, 100 mM D-mannitol, 1.99 mM Ca(OH)₂, 1 mM EGTA and 10 mM HEPES at pH 7.4. When SO₄²⁻ was used as a charge carrier, the extracellular solution contained 100 mM (NMDG)₂SO₄, 1.99 mM Ca(OH)₂, 1 mM EGTA and 10 mM HEPES at pH 7.4. In cation permeability experiments, the extracellular solution contained 150 mM NaCl, 1.99 mM Ca(OH)₂, 1 mM EGTA and 10 mM HEPES at pH 7.4. The use of these solutions enabled us to record LRRC8-mediated currents with defined ionic composition on both the extracellular and intracellular sides with minimal interference by osmosis. This is crucial for the rigorous examination and interpretation of both activation and current–voltage characteristics of LRRC8 channels. Activation of LRRC8 proteins by iso-osmotic pipette solutions with sub-physiological ionic strength was monitored using a ramp protocol delivered every 2–4 s. Conduction and permeation characteristics of LRRC8 proteins were examined through their current–voltage (*I*–*V*) relationships constructed from instantaneous current responses following a voltage jump in a step protocol from –100 mV to +120 mV with a 20 mV increment per step. Instantaneous *I*–*V* relationships were corrected for activity changes during the step protocol by normalizing to the current amplitudes during a –40 mV pre-pulse. Membrane potential was held close to the Nernst potential of Cl⁻ when Cl⁻ was the only charge carrier or the reversal potential when bi-ionic or asymmetrical conditions were used to minimize asymmetrical changes of ion concentration in the extracellular and intracellular compartments. In permeability experiments, voltage steps with a shorter duration, typically of 300 ms, were used to minimize changes of ion concentration on either side of the membrane. Relative permeability for Na⁺ was calculated using

$$\frac{P_{\text{Na}}}{P_{\text{Cl}}} = \frac{[\text{Cl}^-]_o e^{\frac{FE_{\text{rev}}}{RT}} - [\text{Cl}^-]_i}{[\text{Na}^+]_o}$$

and for SO₄²⁻ using

$$\frac{P_{\text{SO}_4}}{P_{\text{Cl}}} = \frac{[\text{Cl}^-]_i}{4[\text{SO}_4^{2-}]_o} \left(1 + e^{-\frac{FE_{\text{rev}}}{RT}} \right) e^{-\frac{FE_{\text{rev}}}{RT}}$$

where E_{rev} is the reversal potential, $\frac{P_x}{P_{\text{Cl}}}$ is the permeability of the ionic species of

interest relative to that of Cl⁻, and *R*, *T* and *F* have their usual meanings. Data were analysed using Clampfit 10.6 (Molecular Devices), Excel (Microsoft) and Prism 5 or 6 (GraphPad), and are presented as mean ± s.e.m.

Statistics and reproducibility. Electrophysiology data were repeated several times from different transfections with very similar results. The conclusions of experiments were not changed upon inclusion of further data. In all cases, leaky patches were discarded.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. The cryo-EM density maps of full-length homomeric mouse LRRC8A generated independently from datasets 1 and 2, the pore domain of LRRC8A (dataset 2a) and full-length heteromeric LRRC8A/C assembly have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-4366, EMD-4367, EMD-4362 and EMD-4361, respectively. Coordinates for the atomic models of LRRC8A refined against 5.01 Å and 4.25 Å cryo-EM densities, the LRRC8A pore domain and the LRRC8A LRR domain have been deposited in the Protein Data Bank under accession numbers 6G9L, 6G9O, 6G8Z and 6FNW, respectively. Data are available from the corresponding author upon reasonable request.

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Extended Data Fig. 1 | Sequence alignment and topology.

a, Multiple sequence alignment of mouse paralogs of LRRC8. Shown are the sequences of LRR8A (NCBI: NP_808393), LRR8B (NCBI: NP_001028722), LRR8C (NCBI: NP_598658), LRR8D (NCBI: NP_001116240) and LRR8E (NCBI: NP_082451). Residues strictly conserved in all subunits are coloured in blue. Segments of LRR8A that are not defined in the cryo-EM density are highlighted in grey. Secondary structure elements of LRR8A are shown below (pore domain, green; LRRD, blue; LRβ16 and LRα16 correspond to a less conserved non-canonical repeat). Numbering corresponds to LRR8A. The coloured

symbols represent the following: Red circles, two threonine residues facing the pore at the extracellular membrane boundary; blue triangles, basic residues of the ESD of LRR8A facing the pore; yellow inverted triangles, cysteine residues involved in disulfide bridges; green inverted triangles, residues at the extracellular side that have a role in desensitization; black squares, annotated phosphorylation sites (Uniprot: Q80WG5). The two paralogs LRR8A and LRR8C investigated in this study are of similar length and share 58% of identical and 72% of homologous residues.

b, Topology of the pore domain with secondary structure elements indicated.

Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Electrophysiology and biochemical

characterization. **a**, Time-dependent whole-cell currents of non-transfected HEK293T cells recorded at 100 mV (dark blue) and -100 mV (light blue) evoked in response to low intracellular ion concentration (100 mM NMDG-Cl). **b**, Representative current response of activated channels at different voltages at a symmetric Cl^- concentration of 100 mM. **c**, Current-voltage relationships in symmetric (left, $n=6$) and asymmetric (right, $n=4$) ionic conditions. The negative reversal potential corresponding to the Nernst potential of Cl^- in the asymmetric condition is indicative of an anion-selective current. **a** and **b** show a representative recording of data shown in **c**, left. **d**, **e**, Time-dependent response (**d**) and representative traces and current-voltage relationships (**e**) of the LRRC8 knockout cell line $\text{LRRC8}^{-/-}$ at the indicated ion concentrations ($100_o/100_i$, $n=5$; $100_o/50_i$, $n=5$). **d** and **e**, left show representative recordings of data shown in **e**, right. **f**, **g**, Time-dependent response (**f**) and current response (**g**) of $\text{LRRC8}^{-/-}$ cells expressing LRRC8A/C at the indicated ion concentrations. **f** and **g** show representative recordings of data shown in Fig. 1b. **h**, **i**, Time-dependent response (**h**) and current response (**i**) of $\text{LRRC8}^{-/-}$ cells expressing LRRC8A at the indicated ion concentrations. **h** and **i** show representative recordings of data shown in Fig. 1c. Dashed lines show the corresponding responses of $\text{LRRC8}^{-/-}$ cells at the indicated ion concentrations. Data in **a–i** were recorded under voltage-clamp conditions in the whole-cell configuration. Current-voltage relationships show averages of the indicated number of independent experiments, errors are s.e.m. In **a**, **d**, **f**, **h**, the black triangle indicates the point of break-in. In **b**, **e**, **g**, **i**, traces show representative data recorded

at the plateau at the indicated ion concentrations. **j**, Plasma membrane expression of LRRC8 constructs probed by surface biotinylation. Representative western blot ($n=3$) shows avidin-purified chemically biotinylated proteins at the cell surface, which are detected by an anti-Myc antibody recognizing a fusion tag on the overexpressed LRRC8 constructs. Lanes show M, marker with molecular weights indicated; NT, non-transfected biotinylated $\text{LRRC8}^{-/-}$ cells; NB, LRRC8A/C transfected, non-biotinylated $\text{LRRC8}^{-/-}$ cells; A, LRRC8A transfected, biotinylated $\text{LRRC8}^{-/-}$ cells; AC, LRRC8A/C transfected, biotinylated $\text{LRRC8}^{-/-}$ cells. The blot shows comparable surface expression of LRRC8A and LRRC8A/C constructs, which is in agreement with previous reports⁹. The experiment was repeated three times as independent biological replicates with similar results. **k**, Elution profile of LRRC8A (red) and $\text{LRRC8A}^{\text{PD}}$ (blue) from a Superose 6 10/300 column. The first peak between 13 and 14 ml corresponds to a hexamer of LRRC8A, the smaller peak at around 17 ml corresponds to the cleaved fluorescent fusion protein. **l**, Elution profile and derived molecular weight for $\text{LRRC8A}^{\text{PD}}$ purified in DDM. The continuous black line shows normalized absorbance at 280nm. The calculated molecular weight of the entire complex and its protein and detergent components based on MALS experiments are shown in purple, blue and red respectively. The calculated values are consistent with a hexameric assembly of $\text{LRRC8A}^{\text{PD}}$. **m**, Elution profile of $\text{LRRC8A}^{\text{LRRD}}$ from a Superdex 75 10/300 column. The main peak around 12 ml corresponds to a monomer of the cytoplasmic domain. In **k**, **m**, arrows indicate the void and bed volume.

Extended Data Fig. 3 | Cryo-EM reconstruction of LRRC8A at 5.01 Å

a, Representative cryo-EM micrograph of dataset 1 acquired with an FEI Tecnai G² Polara microscope. The image shows well-dispersed single particles with occasional micro-aggregates. **b**, 2D class averages of LRRC8A. **c**, Angular distribution plot of all particles included in the final C3-symmetrized reconstruction showing uniform orientations. The length and the colour of cylinders correspond to the number of particles with respective Euler angles. **d**, The data-processing workflow. Seven out of nine classes generated during 3D classification reveal a well-defined, C6-symmetric pore domain and structural heterogeneity in the LRRCs, which reflects their intrinsic mobility. The second class from

the left shows additional density representing a part of the cytoplasmic subunits of a neighbouring particle. Particles assigned to the boxed class were used in further refinement. The distribution of all particles (%) and the resolution of each class is indicated. **e**, FSC plot of the final refined unmasked (orange), masked (pink), phase-randomized (green) and corrected for mask convolution effects (blue) cryo-EM density map of LRRC8A. The resolution at which the FSC curve drops below the 0.143 threshold is indicated. The inset shows the atomic model within the mask that was applied for calculations of the resolution estimates. **f**, Final 3D reconstruction coloured according to local resolution.

Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Cryo-EM reconstruction of LRRC8A at 4.25 Å and its pore domain at 3.66 Å. **a**, Representative cryo-EM micrograph of dataset 2 obtained at an FEI Titan Krios. **b**, 2D class averages of LRRC8A. **c**, Angular distribution plots of all particles included in the final C3-symmetrized full-length reconstruction (upper panel) and the final C6-symmetrized pore domain reconstruction from focused refinement (lower panel) show uniform orientations. The length and colour of cylinders correspond to the number of particles with respective Euler angles. **d**, The data-processing workflow. 3D classification was performed directly on the initial set of particles to include less-populated orientations, which could be otherwise excluded if subjected to prior 2D classification. One of two predominant classes (boxed) revealed a subset of homogeneous particles, which were used in further processing. The distribution of

all particles (percentage) and the resolution of each class is indicated. The focused refinement with C6 symmetry improved the resolution of the pore domain. The inset shows the mask applied to the map during refinement, in which the particle signal outside the mask, corresponding to the LRRDs, was subtracted **e**, FSC plot of the final refined unmasked (orange), masked (pink), phase-randomized (green) and corrected for mask convolution effects (blue) cryo-EM density map of C3-symmetric LRRC8A. The resolution at which the FSC curve drops below the 0.143 threshold is indicated. The inset shows the atomic model within the mask that was applied for calculations of the resolution estimates. **f**, Final 3D reconstruction of full-length LRRC8A coloured according to its local resolution. **g**, as in **e**, but for the 3D reconstruction of the C6-symmetric pore domain. **h**, as in **f**, but for the 3D reconstruction of the pore domain.

Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Comparison of cryo-EM 3D reconstructions of LRRC8A and LRRC8A/C. **a**, C α -representation of the refined model of LRRC8A with cryo-EM density at 4.25 Å at low (4.0 σ , left) and high (6.0 σ , right) contour threshold superimposed. **b**, C α -representation of the refined model of LRRC8A with cryo-EM density at 5.01 Å at low (7.0 σ , left) and high (9.7 σ , right) contour threshold superimposed. **c**, Representative cryo-EM micrograph of dataset 3 acquired with an FEI Tecnai G² Polara microscope. **d**, 2D class averages of the LRRC8A/C assembly. LRRC8A/C heteromers were obtained from the co-expression of the two constructs in HEK293 cells followed by a tandem affinity purification to select channels containing both subunits. **e**, The angular distribution plot of all particles included in the final C3-symmetrized reconstruction shows uniform orientations. The length and the colour of cylinders correspond to the number of particles with respective Euler angles. **f**, The data-processing workflow for LRRC8A/C. After 3D classification with either C1 or C3 symmetry imposed, the three predominant classes show a symmetric pore domain and LRRDs with higher flexibility. Cryo-EM density represents an average of LRRC8A

and C subunits which, owing to their strong sequence relationship, are essentially identical in structure at the current resolution of the data. The final refined C3-symmetrized reconstructions reveal the overall architecture of the hexameric protein, which exhibits very similar features to the LRRC8A homo-hexamer. Although the final C3-symmetric model generated from non-symmetrized 3D classes contains twice the number of particles, its resolution is lower compared to the model calculated from the predominant symmetrized 3D class. We therefore used the latter for structural descriptions. The distribution of all particles (percentage) and the resolution of each class is indicated. **g**, FSC plot of the final refined unmasked (orange), masked (pink), phase-randomized (green) and corrected for mask convolution effects (blue) cryo-EM density map of LRRC8A/C. The resolution at which the FSC curve drops below the 0.143 threshold is indicated. The inset shows the cryo-EM density within the mask that was applied for calculations of the resolution estimates. **h**, Final 3D reconstruction at high (left) and low (right) thresholds, coloured according to local resolution.

Extended Data Fig. 6 | Cryo-EM and X-ray electron density. **a**, Sections of the cryo-EM density (grey, $6.5\ \sigma$) superimposed on the refined structure of the pore domain. Secondary structure elements are labelled. **b**, Cryo-EM density (grey, $6.0\ \sigma$) around disulfide bonds in the ESD. **c**, Stereo view of cryo-EM density ($6.5\ \sigma$) of the pore region in the ESD with front subunits removed for clarity. **a–c** show cryo-EM density of the C6-symmetrized pore domain of LRRC8A at $3.66\ \text{\AA}$. **d**, **e**, Section (**d**) and

stereo (**e**) views of the isolated LRRD of LRRC8A. Electron density ($1.0\ \sigma$) of the LRRD determined by X-ray crystallography at $1.8\ \text{\AA}$ is shown superimposed on the model. **f**, **g**, Section (**f**) and stereo (**g**) views of the LRRD in the full-length LRRC8A channel. C3-symmetrized cryo-EM density ($4.0\ \sigma$) is shown superimposed on the model. Although the resolution of the entire reconstruction is $4.25\ \text{\AA}$, the local resolution of the LRRDs is lower. In **a–g**, structures are shown as sticks.

a X-ray data collection, phasing and refinement statistics			b Cryo-EM data collection, refinement and validation statistics				
	Anomalous dataset	Native dataset (PDB 6FNW)		Dataset 1 LRRRC8A ^{FL} (EMD-4366) (PDB 6G9L)	Dataset 2 LRRRC8A ^{FL} (EMD-4367) (PDB 6G9O)	Dataset 2a LRRRC8A ^{PD} (EMD-4362) (PDB 6G8Z)	Dataset 3 LRRRC8A/C (EMD-4361)
Data collection			Data collection and processing				
Space group	P6 ₃ (170)	P6 ₃ (170)	Microscope	FEI Tecnai G ²	FEI Titan		FEI Tecnai G ²
Cell dimensions			Camera	Polara	Krios		Polara
<i>a, b, c</i> (Å)	140.3, 140.3, 41.9	140.1, 140.1, 42.3		Gatan K2	Gatan K2		Gatan K2
α, β, γ (°)	90, 90, 120	90, 90, 120	Magnification	Summit + GIF	Summit + GIF		Summit + GIF
Resolution (Å)	40.45-2.5	40.45-1.8		37,313	46,511		37,313
<i>R</i> _{merge}	9.1 (67.9)	6.5 (158.3)	Voltage (kV)	300	300		300
<i>I</i> / σ	79.9 (13.4)	27.1 (2.3)	Electron exposure (e ⁻ /Å ²)	70	70		70
Completeness (%)	99.3 (93.7)	100.0 (99.9)	Defocus range (μm)	-0.5 to -3.2	-0.5 to -3.2		-0.5 to -3.2
Redundancy	154.2 (123.3)	20.2 (19.8)	Pixel size (Å)	1.34(0.67)	1.075(0.5375)		1.34(0.67)
Refinement			Initial particle images (no.)	329,624	105,867		101,239
Resolution (Å)		1.8	Final particle images (no.)	54,861	34,674		27,777
No. reflections		43,155	Symmetry imposed	C3	C3	C6	C3
<i>R</i> _{work} / <i>R</i> _{free}		17.9 (20.8)	Map resolution (Å)	5.01	4.25	3.66	7.94
No. atoms			FSC threshold 0.143				
Protein		3,426	Map resolution range (Å)	3.0-7.0	3.0-6.0	3.0-4.5	5.0-9.5
Ligand/ion		4	Refinement				
Water		337	Model resolution (Å)	5.3	4.3	3.7	
<i>B</i> -factors			FSC threshold 0.5				
Protein		47.5	Model resolution range (Å)	194-5.01	206-4.25	129.0-3.66	
Ligand/ion		73	Map sharpening b-factor (Å ²)	-173	-118	-92	
Water		57.5	Model composition				
R.m.s deviations			Non-hydrogen atoms	35,532	35,532	15,966	
Bond lengths (Å)		0.013	Protein residues	4,308	4,308	1,908	
Bond angles (°)		1.27	<i>B</i> factors (Å ²)				
*Values in parentheses are for highest-resolution shell.			Protein	292	155	99	
			Refrmac FSC _{avg} /Rfactor	0.805/0.261	0.717/0.384	0.857/0.301	
			R.m.s. deviations				
			Bond lengths (Å)	0.004	0.005	0.006	
			Bond angles (°)	0.793	0.798	1.033	
			Validation				
			MolProbity score	1.71	1.66	0.85	
			Clashscore	6.04	5.15	0.22	
			Poor rotamers (%)	0.08	0.08	0	
			Ramachandran plot				
			Favored (%)	94.52	94.36	96.15	
			Allowed (%)	5.48	5.64	3.85	
			Disallowed (%)	0	0	0	

*Values in parentheses indicate the pixel size in super-resolution.

Extended Data Fig. 7 | X-ray crystallography and cryo-EM statistics and model validation. **a**, Statistics of X-ray data collection, phasing and refinement. **b**, Statistics of cryo-EM data collection, processing and model refinement. **c, d**, FSC plots of refined atomic models against the cryo-EM density maps of the full-length protein at 4.25 Å (**c**, dataset 2) and the pore domain of LRRRC8A at 3.66 Å (**d**, focus-refined dataset 2a). The *FSC*_{sum}

(orange) is calculated for the full masked map and the model refined against the complete dataset, *FSC*_{work} (blue) is calculated for the masked half-map 1 and the shaken model refined against the dataset comprising the half map 1, *FSC*_{free} (pink) is calculated for the masked half-map 2 and the shaken model refined against the half map 1. FSC thresholds at 0.5 and 0.143 were used for *FSC*_{sum} and *FSC*_{work}/*FSC*_{free}, respectively.

Extended Data Fig. 8 | Homology model and electrostatics. **a**, Model of LRRC8A used for electrostatic calculations. The high sequence conservation between paralogues and the structural similarity between homo- and heteromeric channels has enabled the construction of a homology model of LRRC8A/C. Whereas in this model the general features of LRRC8C are reliably represented, the alternate arrangement of A and C subunits in the hexameric channel, although plausible, is at this stage speculative. In both structures, the missing residues at the respective N termini were modelled in such a way that they do not obstruct the pore. Membrane boundaries (C, hydrophobic core and HG, head-group regions) are indicated. **b**, Electrostatic potentials along the pores of LRRC8A (red) and LRRC8A/C (blue), as determined by a numerical solution of the Poisson–Boltzmann equation at 150 mM of monovalent ions in the solvent and the pore region. **c**, **d**, Part of the ESD of the LRRC8A/C model that lines the pore, viewed from within the membrane with front subunits

removed for clarity (**c**) and from the extracellular side (**d**). The protein is displayed as a ribbon representation, with selected side chains as shown as sticks. Subunits A and C are shown in green and orange, respectively. Although most of the interaction interface is conserved in the pore domain of this model, differences in pore-lining residues alter the electrostatic properties of the ion conduction path. **e**, Residual density (5.0σ) in the pore region of the C3-symmetrized cryo-EM map of LRRC8A. The region around T44 and T48 is highlighted, with front subunits removed for clarity. The view is as in **c** and the protein is displayed as sticks. **f**, The electrostatic potential along the pore of the LRRC8A(R103A)/C mutant (green), as determined by a numerical solution of the Poisson–Boltzmann equation in the absence (left) and at 150 mM (right) of monovalent ions in the solvent and the pore region. In **b**, **f**, the membrane boundaries and the border between the pore domain and the LRRD are indicated.

Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Electrophysiology of selectivity filter mutants. **a, b**, Time-dependent response (**a**) and representative traces and current-voltage relationship (**b**) of *LRRC8*^{-/-} cells expressing LRRC8A(R103A) (*n* = 4). **c, d**, Time-dependent response (**c**) and representative traces and current-voltage relationships (**d**) of *LRRC8*^{-/-} cells expressing LRRC8A/C(L105R) (*n* = 7). **e, f**, Time-dependent response (**e**) and representative traces and current-voltage relationships (**f**) of *LRRC8*^{-/-} cells expressing LRRC8A(R103A)/C (*n* = 4). In **a, c, e**, the black triangle indicates the point of break-in. In **b, d, f**, traces show representative data recorded at the plateau at the indicated symmetric Cl⁻ concentrations. Current-voltage relationships show averages of the indicated number of independent experiments, errors are s.e.m. **g**, Current-voltage relationships of LRRC8A(R103A)/C (*n* = 5), LRRC8A/C (*n* = 4) and LRRC8A/C(L105R) (*n* = 5) at the indicated ionic gradient. **h**, Reversal potentials measured from the current-voltage relationship shown in **g**.

In **g, h**, the Nernst potential of Cl⁻ is indicated. **i–k**, Current-voltage relationships of the indicated constructs with different extracellular anions (LRRC8A(R103A)/C (**i**), *n* = 4; LRRC8A/C (**j**), *n* = 5; LRRC8A/C(L105R) (**k**), *n* = 6). **l**, Reversal potentials measured from the current-voltage relationship shown in **i, j** and **k**. **m**, Ratio of the outward (SO₄²⁻) and inward (Cl⁻) current at ±100 mV measured from the current-voltage relationship shown in **i, j** and **k**. **n**, Relative inward current amplitude used to scale the normalized current-voltage relationship recorded in the presence of extracellular SO₄²⁻ in **i, j** and **k**. The shift in the reversal potential under the bi-ionic SO₄²⁻/Cl⁻ condition and the increase in relative SO₄²⁻ current reflects the increase in the relative permeability of the divalent anion as the number of positive charges in the selectivity filter increases. In **g–n**, the data show averages of the indicated number of independent experiments, errors are s.e.m. Data in **a–n** were recorded under voltage-clamp conditions in the whole-cell configuration.

Extended Data Fig. 10 | Comparison with gap junction proteins.

a, Superposition of subunits of LRRC8A (green) and connexin Cx26 (PDB: 2ZW3, red, left) and expanded view of the ESD (right). Selected secondary structure elements are indicated. **b**, Superposition of subunits of LRRC8A (green) and innexin-6 (PDB: 5H1Q, blue, left) and expanded view of the ESD (right). **a**, **b**, Proteins are displayed as ribbons; selected secondary structure elements are indicated. **c**, Pore radius along the symmetry axis. The dashed green line corresponds to the structure of LRRC8A containing the modelled N terminus used for electrostatic calculations. In

the case of connexin Cx26, the difference in pore geometry arises from a shortening of helix E1H, and in *C. elegans* innexin-6 the difference arises from its larger, octameric assembly. **d–f**, Pore domains of LRRC8A with modelled N-terminal residues (**d**) connexin Cx26 (PDB: 2ZW3) (**e**) and innexin-6 (PDB: 5H1Q) (**f**), viewed from the extracellular side (top), and two opposing subunits of the respective channels shown from within the membrane with N termini indicated (bottom). In **d–f**, the proteins are shown as space-filling models.

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► Experimental design

1. Sample size

Describe how sample size was determined.

No statistical method was used to determine sample size but addition of more data did not change conclusions.

2. Data exclusions

Describe any data exclusions.

Obvious outliers and data with poor signal to noise ratio were excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Electrophysiology data show the mean of specified number of biological replicates. Experimental findings were reliably reproduced.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

The study did not involve animals or human research participants thus samples were not randomized for the experiments.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

The study did not involve animals or human research participants thus thus no blinding was used.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☒ ☐ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☒ ☐ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☒ ☐ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

For data cryo-EM data processing and validation and X-ray crystallography the following software was used: Motioncor2, CTFFIND4.1, RELION, BlocRes, Chimera, XDS, CCP4, CCPEM, Coot, Phenix and Refmac; For electrophysiology data, Clampfit 10.6, Excel and GraphPad Prism were used. Multi-angle light scattering data were analyzed with the Astra and Sedfit software. Pore geometry was analyzed with the HOLE and MOLE program, surfaces were calculated with MSMD, and electrostatics with the CHARMM program suite. Figures were prepared with DINO, Pymol and Chimera.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restriction on availability of material used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibodies used for Western-blotting include a mouse monoclonal anti-c-myc primary antibody (Sigma, M 4439, Clone 9E10) and goat anti-mouse-HRP conjugated secondary antibody (Jackson ImmunoResearch, 115-035-146).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Cell-lines used in the studies either originate from commercial sources unless otherwise indicated. For electrophysiology experiments transient transfection was used and the cell line obtained from ATCC (293 [HEK-293] (ATCC® CRL-1573™). The LRRC8 knock-out cell line was provided by Prof. T. J. Jentsch, the HEK293S GnTI⁻ cells by Prof. P. J. Reeves.

b. Describe the method of cell line authentication used.

No further authentication was performed for commercially available cell lines and for HEK293S GnTI⁻. The HEK LRRC8^{-/-} cell line was tested by electrophysiology and did not show any VRAC mediated currents.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were tested and are free from mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

Not applicable.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used in this study.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.