Members of the LRRC8 family form heteromeric assemblies, which function as volume-regulated anion channels. These modular proteins consist of a transmembrane pore and cytoplasmic leucine-rich repeat (LRR) domains. Despite their known molecular architecture, the mechanism of activation and the role of the LRR domains in this process has remained elusive. Here we address this question by generating synthetic nanobodies, termed sybodies, which target the LRR domain of the obligatory subunit LRRC8A. We use these binders to investigate their interaction with homomeric LRRC8A channels by cryo-electron microscopy and the consequent effect on channel activation by electrophysiology. The five identified sybodies either inhibit or enhance activity by binding to distinct epitopes of the LRR domain, thereby altering channel conformations. In combination, our work provides a set of specific modulators of LRRC8 proteins and reveals the role of their cytoplasmic domains as regulators of channel activity by allosteric mechanisms.
In mammals, volume-regulated anion channels (VRACs) are important players in the cellular response to osmotic swelling. These anion-selective channels are closed under resting conditions but become activated upon an increase of the cell volume resulting from the influx of water under hypotonic conditions. Although the function of VRACs has been investigated for decades, their molecular identity was discovered only seven years ago, when members of the LRRC8 family were assigned as essential constituents of the channel. All family members share a high mutual conservation with pairwise sequence identities exceeding 50% between the five human paralogs and 99% between human and murine LRRC8A orthologs. In a cellular context, VRACs form heteromeric complexes all containing the obligatory LRRC8A subunit. Although the exact composition of LRRC8 heteromers is currently unknown, they are believed to constitute a diverse family of ion channels whose subunit stoichiometry determines permeation and activation properties. While the range of permeable substrates of channels containing the C-subunit is restricted to small anions, channels containing the D- or E- subunits also conduct larger molecules including osmolytes, amino acids, and anti-cancer drugs.

The general architecture of LRRC8 channels was revealed from homomeric structures of the LRRC8A and LRRC8D subunits. Although such homomeric assemblies are not observed in a cellular context, the subunits form hexamers and, in case of LRRC8A, they function as ion channels with compromised activation properties. This assumption is based on a low-resolution structure obtained from a preparation of LRRC8 oligomers containing A and C subunits. It refers to the hexameric organization of channels and their general structural features whereas differences in the molecular details and distinct conformational properties are expected to persist between homo- and heteromers. LRRC8 channels share a modular organization consisting of a membrane-inserted pore domain and cytoplasmic leucine-rich repeat (LRR) domains. In the pore domain, the ion conduction path running along the symmetry axis is constricted by a narrow extracellular region that resembles a selectivity filter, followed by pore-widening within the membrane. In contrast to the well-defined pore domain, the cytoplasmic LRR units are mobile as they are usually less well resolved in cryo-EM reconstructions. In spite of their poor definition and lack of symmetry in the majority of imaged particles, a considerable population showed a channel arrangement in which the C6 symmetry of the pore reduces to a C3 relationship within the LRR domains. Such architecture demands adjacent domains to change their respective orientation in order to maximize mutual contacts. Next to the tight interface between pairs, a second, loose interface is created at alternating positions in the hexameric channels, which is characterized by large fenestrations between interacting LRR domains. The functional relationship between the C3-symmetric channel structure and conformations with asymmetric LRR domain arrangement is still unknown. Despite the wealth of structural information, the role of the cytoplasmic LRR domains for channel function has remained elusive. This lack of knowledge is concomitant with our poor understanding of mechanisms of how VRACs sense changes in their environment and how these are converted into conformational transitions leading to channel activation.

In the present study, we are interested in the relevance of the LRR domains for the regulation of VRACs and thus investigate the effect of their interaction with proteinaceous binders on channel activity. To this end, we select synthetic nanobodies (termed sybodies) targeting the LRR domain of the obligatory LRRC8A subunit and identify proteins that either inhibit or enhance channel activity. The structural characterization of their complexes reveals the interaction-epitopes and distinct conformations in the homomeric LRRC8A channel induced by sybody binding. Together our results provide a set of specific modulators of LRRC8 channels and they emphasize the importance of the LRR domains as regulatory units that modulate channel activity by allosteric mechanisms.

**Results**

**Selection of sybodies targeting different regions of LRRC8A.** In light of the ambiguity concerning the molecular organization of LRRC8 channels and their mechanism of activation, we were interested in the characterization of these currently unknown molecular properties. We thus attempted to generate protein-based binders that specifically target different regions of the channel. For the identification of interaction partners of homomeric murine LRRC8A, we engaged in the in vitro selection of sybodies. This selection process allowed the identification of numerous unique binders from three libraries containing either a short (concave library), intermediate (loop library), or long (convex library) randomized complementary-determining region.

From the pool of sybodies enriched against the full-length LRRC8A channel, we subsequently focused on a subset of binders that target its cytoplasmic domain. For this purpose, we have expressed and purified the soluble LRR domain of LRRC8A, which is monomeric and thus does not exhibit any of the inter-subunit interactions observed in hexameric channels. Using an ELISA setup with the soluble LRR domain as target, we identified two unique sybodies of the concave library (termed Sb1 and Sb2) and Sb3, short Sb1 and Sb5), two of the loop library (termed Sb2 and Sb3) and one of the convex library (termed Sb4 and Sb5). We further characterized the interaction between the five selected sybodies and LRR domains of different paralogs, we studied the elution behavior of complexes assembled from purified components on size-exclusion chromatography and analyzed peak fractions by SDS-PAGE. In this analysis, we detected the formation of stable complexes for all five sybodies with the LRR domain of LRRC8A but no interaction with the LRR domains of LRRC8C and D. Finally, we quantified the interaction of the five sybodies to the isolated cytoplasmic LRR domains of the three paralogs by surface plasmon resonance spectroscopy. These investigations revealed that all five binders target the LRR domain of LRRC8A with dissociation constants in the nanomolar range with Sb1, Sb2, and Sb4 showing the highest, Sb5 an intermediate and Sb3 the lowest affinity (Fig. 1c). Among the five sybodies, the interaction with Sb2 is distinguished by the slowest dissociation rate k_d (with a residence time of over 300 s in the dissociation phase, Fig. 1c). As for gel-filtration experiments, no binding to the LRR domains of LRRC8C and LRRC8D was detected in the investigated concentration range (Supplementary Fig. 1c), which further emphasizes the high specificity of the interaction for the LRRC8A subunit.

**Characterization of the modular properties of LRRC8A domain binders.** After the identification of sybodies that target the cytoplasmic LRR domain of VRACs, we were interested whether any of these binders would affect the functional properties of the channel. To this end, we have studied the activation of endogenous VRAC currents in HEK293 cells by patch-clamp electrophysiology in the whole-cell configuration. HEK293 cells show a strong current response mediated by heteromeric...
channels of the LRRC8 family upon either cell swelling or the reduction of the intracellular ionic strength, although the relationship between both activation modes and the requirement of a reduction of the intracellular ionic strength, although the relation-ship between both activation modes and the requirement of a reduction of the intracellular ionic strength, although the relationship between both activation modes and the requirement of a reduction of the intracellular ionic strength, although the relationship between both activation modes and the requirement of a.

Supplementary Figs. 2d, f and 3a, b. To characterize the modulation of VRAC activity by Sb1, we have added the sybody to the solution of the patch-clamp electrode to permit its diffusion into the cytoplasm after establishment of the whole-cell configuration. Following the activation of VRAC currents in response to a decreased (125 mM) intracellular salt concentration, we consistently observed an about fourfold reduction of current density compared to controls, thus suggest-ing that Sb1 might act as inhibitor of the channel (Fig. 2a, Supplementary Fig. 3a, b). In a next step, we investigated whether the expression of sybodies in the cytoplasm of HEK293 cells would lead to a similar inhibition of endogenous VRAC currents. We thus transfected HEK293 cells with a construct containing a C-terminal fusion of Venus-YFP. Such fusion-proteins expressed in the cytoplasm of HEK cells, termed intracellularly expressed, were shown to fold and recognize their intracellular targets.

In case of Sb1, we observed expression of the construct as judged by the strong YFP fluorescence inside the cell. To exclude the possibility that the sybody expression has perturbed the localization of VRACs within the cell, we quantified the fraction of channels at the plasma membrane by surface-biotinylation and found very similar protein levels as for non-transfected cells (Supplementary Fig. 3c, d). In recordings measured under activating conditions, we did not observe any current response, irrespectively of whether activation proceeded by swelling using a previously described protocol or exposure of the cytoplasm to 125 mM salt (in conjunction with high ATP and low Ca\(^{2+}\) and Mg\(^{2+}\) concentrations, Fig. 2a, b, Supplementary Fig. 2a–c). A small and concentration-dependent response was observed upon further reduction of the salt concentration to 100 and 75 mM (Fig. 2b). In contrast, unaltered currents compared to wild type (WT) were recorded upon transfection with a construct containing a C-terminal fusion of Venus-YFP.

**Table 1 Kinetic and dissociation constants of LRRC8A-LRR domain-sybody interactions obtained by SPR.**

| Sybody | $K_D$ (nM) | $k_{on}$ (mol\(^{-1}\)s\(^{-1}\)) | $k_{off}$ (s\(^{-1}\)) |
|--------|-----------|-------------------------------|-------------------------|
| Sb1    | 24        | 1.25E+06                      | 0.030                   |
|        | 22        | 1.66E+06                      | 0.037                   |
|        | **ave.**  | **1.5E+06**                   | **0.034**               |
| Sb2    | 1         | 8.28E+04                      | 0.003                   |
|        | 2         | 4.99E+04                      | 0.003                   |
|        | **ave.**  | **6.6E+04**                   | **0.003**               |
| Sb3    | 1         | 1.30E+05                      | 0.104                   |
|        | 2         | 2.13E+05                      | 0.113                   |
|        | **ave.**  | **1.3E+05**                   | **0.109**               |
| Sb4    | 1         | ND                             | 0.064                   |
|        | 2         | 1.72E+06                      | -                       |
|        | **ave.**  | **1.72E+06**                  | **0.221**               |

Data were fitted to a single binding site model. Table displays results of two independent biological replicates. Their average is shown in bold (**ave.**). The bold values display the average of the two biological replicates.
encoding a control sybody that was selected to target an unrelated protein (Sbn, Fig. 2b, Supplementary Fig. 2). Collectively, our results demonstrate that the presence of Sb1 in the cytoplasm of HEK cells prevents activation of VRAC channels by interacting with their LRR domains. To investigate whether other identified sybodies targeting the LRR domains would also inhibit the activity. To further investigate this property, we have recorded current at elevated (i.e. 175 mM) ionic strength where we expected the effect of potential activators to be enhanced. At such conditions, we found very low response in non-transfected cells and a pronounced, on average fivefold increase of currents in case of cells expressing Sb4 and an on average 2.3-fold increase upon expression of Sb5 (Fig. 2d, e, Supplementary Fig. 3e). Finally, we have investigated whether similar functional effects as observed for heteromeric VRACs would also be found for the homomeric channel LRRCA8A, whose activation is weak and requires very low ion concentrations. Upon coexpression of LRRCA8A with the potentiating sybodies Sb4 and Sb5 in LRRCA8A−/− cells, we recorded large, instantaneous, and anion-selective currents that are several-times increased compared to cells expressing LRRCA8A alone (Fig. 2f, g, Supplementary Fig. 3f, g). Conversely, the low currents...
of LRRC8A were further attenuated upon coexpression with the inhibitory sybody Sb3 (Fig. 2f, g, Supplementary Fig. 3f, g). Together, our data provide strong evidence that Sb1, Sb2, and Sb3 act as allosteric VRAC inhibitors and Sb4 and Sb5 as allosteric activators and that the distinct functional phenotypes of sybodies observed for LRRC8 heteromers are extended towards homomeric LRRC8A channels.

Structural basis for the interaction with the inhibitory sybody Sb1. To gain further insight into the sybody-VRAC interactions, we determined the structures of their complexes with homomeric channels composed of the obligatory subunit LRRC8A by cryo-electron microscopy (cryo-EM, Fig. 3, Supplementary Figs. 4–10, Table 2). Due to the high subunit selectivity of all five binders and their ability to target the isolated LRRC8A domain (Fig. 1, Supplementary Fig. 1), we expect these structures to also depict the binding mode of sybodies to the A-subunits in heteromeric VRAC channels.

First, we were interested in the interaction of a VRAC channel with an inhibitory sybody and hence determined the structure of the LRRC8A/Sb1 complex. The data is of high quality and allowed reconstruction of a map that extends to 3.1 Å for the entire complex and 2.7 Å for the pore domain (Supplementary Fig. 4). A large population of the particles (i.e. 26% of the particles used for 3D classification) shows a similar C3-symmetric structural arrangement as previously observed for the apo protein (Fig. 4a–c, Supplementary Fig. 4d, e). Other classes (in total encompassing 74% of the classified particles) show a well-defined pore domain but different degree of mobility of the cytoplasmic LRR domains. In the C3-symmetric structure, the densities of sybodies define the interaction of the binder with the channel at the lower part of the cytoplasmic domain towards the intracellular side (Fig. 3a, Supplementary Fig. 10a, b). In contrast to the apo protein, where the LRR domains were mobile and thus poorly defined in the cryo-EM density of the threefold symmetric channel conformation, in the LRRC8A/Sb1 complex these domains and their interacting sybodies are much better resolved (Supplementary Fig. 4d–h). The focused reconstruction on a symmetry-expanded dataset of a pair of interacting domains with bound sybodies yielded cryo-EM density at 2.8 Å, which allowed a detailed characterization of the complex (Supplementary Figs. 4h and 10a, b). In this substructure, the sybodies bind to the convex outside of the horseshoe-shaped domain (Fig. 4a–c). They target an epitope located on repeats 8–11 and bury 1420 Å² of the combined molecular surface (Fig. 4d, Supplementary Fig. 11a). As intended by the design of the concave sybody library, the interface encompasses residues from β-strands 3, 4, 5 and 8 on the flat face of the binder involving residues from all three CDRs (Figs 1a and 4e). As the epitopes on the two LRR domains are separated from each other, sybodies interact in the same manner with either domain without contacts between neighboring binders (Fig. 4a–c). On the LRR domain, the residues buried in the interface are predominantly hydrophilic, whereas on the sybody they are dominated by aromatic sidechains (Fig. 4e, f). The high-resolution map of the domain pair also defines the conformation of residues that are buried in the interface between the two LRR domains, which were not resolved in the cryo-EM reconstruction of the apo protein (Supplementary Figs. 10b and 11b–e).

In the C3-symmetric LRRC8A structure, tightly interacting LRR domain pairs are denoted as left (l) and right (r) subunits according to their relative position when viewed from the outside of the channel18 (Fig. 4a, b). Their respective orientations differ by a 42° rotation around a hinge located at the boundary to the pore, which maximizes their mutual interaction and buries 1507 Å² of the combined molecular surface (Supplementary Fig. 11b). Since several of the buried residues are charged, the interaction might be dominated by electrostatic contributions (Supplementary Fig. 11c–e). In addition, the LRRC8A/Sb1 complex also reveals the conformation of the C-terminal part of a mobile loop connecting the cytosolic helices CLH1 and CLH2 on the r-subunit, which was poorly defined in the apo structure (Fig. 4b, Supplementary Figs 10b and 11f). Since this loop carries phosphorylation sites and was suggested to play a role in channel regulation10,25, the observed interaction could be of functional significance.

Structural basis for the interaction with the inhibitory sybodies Sb2 and Sb3. After identifying the binding mode of Sb1, we were interested in the structural properties of other inhibitory sybodies and thus studied the interaction of the channel with Sb2 and Sb3. The structures of the LRRC8A/Sb2 and LRRC8A/Sb3 complexes are both of high quality and define the mutual relationship between the channels and their interaction partners (Fig. 3b, c, Supplementary Figs. 5 and 6). Like in case of Sb1, the interaction of Sb3 with LRRC8A has stabilized the cytoplasmic LRR domains and thus allowed a detailed structural characterization of its binding mode (Supplementary Figs. 6d–h and 10c, d). In contrast,
the same units show increased mobility in the LRRC8A/Sb2 complex as reflected in the lower local resolution of the cryo-EM density of the LRR domains and their attached sybodies, which are both weaker and less well defined than the transmembrane part (Supplementary Figs. 5d–f and 10c, f). Consequently, we had to rely on a homology model of Sb2 to describe its interactions with the channel, which should thus be considered as tentative.

Irrespective of their sequence and their effect on the channel structure, both sybodies recognize the same epitope involving both conserved and variable residues at surface involving both conserved and variable residues. Despite their distinct location, the general interaction modes of all three inhibitory sybodies share resemblance in that they make contacts via their β-strands and adjacent loops of all three CDRs (Figs 1a, 4d, e and 5e, f, h). As opposed to Sb1, the binding interfaces of Sb2 and Sb3 contain fewer aromatic amino acids (i.e. 5 and 6 aromatic residues in Sb1) and are generally more hydrophilic (Fig. 5f, h). In light of the similar binding mode of Sb2 and Sb3, the large difference in their dissociation constants, which is primarily a consequence of the 20-times faster off-rate of

| Dataset 1 | Dataset 2 | Dataset 3 | Dataset 4 | Dataset 5 | Dataset 6 |
|----------------|----------------|----------------|----------------|----------------|----------------|
| **LRRRC8A/Sb1** | **LRRRC8A/Sb2** | **LRRRC8A/Sb3** | **LRRRC8A/Sb4** | **LRRRC8A/Sb4** | **LRRRC8A/Sb5** |
| (EMD-13202) | (EMD-13203) | (EMD-13208) | (EMD-13212) | (EMD-13213) | (EMD-13230) |
| (PDB 7P5V) | (PDB 7P5W) | (PDB 7P5Y) | (PDB 7P60) | (PDB 7P6K) | |

**Data collection and processing**

| **Microscope** | FEI Titan Krios | FEI Titan Krios | FEI Titan Krios | FEI Titan Krios | FEI Titan Krios |
|----------------|----------------|----------------|----------------|----------------|----------------|
| **Camera** | Gatan K3 GIF | Gatan K3 GIF | Gatan K3 GIF | Gatan K3 GIF | Gatan K3 GIF |
| **Magnification** | 130,000 | 130,000 | 130,000 | 130,000 | 130,000 |
| **Voltage (kV)** | 300 | 300 | 300 | 300 | 300 |
| **Electron exposure (e/Å²)** | 61 | 61 | 61 | 61 | 61 |
| **Defocus range (μm)** | −2.4 to −1.0 | −2.4 to −1.0 | −2.4 to −1.0 | −2.4 to −1.0 | −2.4 to −1.0 |
| **Pixel size (Å)** | 0.651 (0.326) | 0.651 (0.326) | 0.651 (0.326) | 0.651 (0.326) | 0.651 (0.326) |
| **Initial number of micrographs (no.)** | 5494 | 5633 | 6475 | 6416 | 4869 |
| **Initial particle images (no.)** | 579,709 | 330,072 | 756,017 | 313,757 | 507,983 |
| **Final particle images (no.)** | 59,962 | 65,959 | 76,350 | 14,718 | 38,121 |
| **Symmetry imposed** | C3 | C3 | C3 | C3 | C3 |
| **Map resolution FL, TM (Å)** | 3.1, 2.7 | 3.5, 3.0 | 3.3, 2.9 | 7.7, - | 3.8, 3.5 |
| **Map resolution range (Å)** | 2.6-6 | 2.8-12 | 2.9-6 | 7-10 | 3.1-8 |
| **Refinement** | 3.26 | 3.8 | 3.5 | 3.9 | 5.7 |
| **Model resolution (Å)** | −36 | −89 | −69 | −76 | −66 |
| **Map sharpening b-factor (Å²)** | 0.002 | 0.002 | 0.004 | 0.004 | 0.002 |
| **Non-hydrogen atoms** | 41,280 | 41,256 | 41,106 | 38,409 | 38,109 |
| **Protein residues** | 5034 | 5028 | 5028 | 4680 | 4650 |
| **B factors (Å²)** | 47.0 | 47.0 | 47.0 | 138.6 | 76.8 |
| **Protein R.m.s. deviations (Å)** | 0.004 | 0.004 | 0.004 | 0.004 | 0.002 |
| **Bond angles (*)** | 0.514 | 0.487 | 0.491 | 0.552 | 0.469 |
| **Validation MolProbity score** | 2.1 | 2.3 | 2.1 | 2.5 | 2.2 |
| **Clashscore** | 9.0 | 10.7 | 10.3 | 14.6 | 9.5 |
| **Poor rotamers (%)** | 3.5 | 3.7 | 2.6 | 5.5 | 3.9 |
| **Ramachandran plot Favor (%)** | 96.6 | 95.6 | 96.5 | 96.0 | 96.4 |
| **Allowed (%)** | 3.4 | 4.4 | 3.5 | 4.0 | 3.6 |
| **Disallowed (%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

*Values in parentheses indicate the pixel size in super-resolution.
Fig. 4 Structure of LRRC8A in complex with the inhibitory sybody Sb1. 

- **a** Surface representation of the LRRC8A/Sb1 complex structure. 
- **b** Structure of the dimer of interacting domains at the tight interface with bound sybody Sb1. Left (l) and right (r) positions are indicated. 
- **c** Ribbon representation of the LRRC8A/Sb1 complex. The view is from within the membrane with membrane boundaries indicated. 
- **d** Ribbon representation of a single LRR domain with sybody Sb1 bound. Repeats contacted by Sb1 are labeled. 
- **e** View on the interaction interface of Sb1 and LRRC8A domain. The protein is shown as Cα trace with the sidechains of interacting residues displayed as sticks.

Fig. 5 Structure of LRRC8A in complex with the inhibitory sybodies Sb2 and Sb3. 

- **a** Surface representation of the LRRC8A/Sb2 complex structure. 
- **b** Structure of the dimer of interacting domains at the tight interface with bound sybody Sb2. 
- **c** Surface representation of the LRRC8A/Sb3 complex structure. 
- **d** Structure of the dimer of interacting domains at the tight interface with bound sybody Sb3. Left (l) and right (r) positions are indicated. 
- **f** Ribbon representation of a single LRR domain with bound sybodies Sb2 and Sb3. Repeats contacted by both sybodies are labeled. 
- **g** View on the interaction interface of a homology model of Sb2 based on the Sb3 structure, LRRC8A domain and Sb3. The protein is shown as Cα trace with the sidechains of interacting residues displayed as sticks.
Fig. 6 Conformational changes of LRRC8A in response to inhibitory sybody binding. a LRRC8A/Sb1 complex, b LRRC8A/Sb2 complex, c LRRC8A/Sb3 complex. a-c Shown are surface representations of domain pairs at the tight interface (top) with contact regions with bound sybodies colored and the hexameric assembly of the domains viewed from the cytoplasm with symmetry axis indicated as triangle (bottom). Insets (top right) display Cα trace of the right subunit of the respective structure, which is superimposed in b and c on the respective structure of the complex with sybody Sb1. d Surface representation of the pore domain of the LRRC8A/Sb1 (top) and the LRRC8A/Sb3 complex (bottom) viewed from within the membrane.

Sb3 compared to Sb2 (Fig. 1c, e, Table 1) is noteworthy and is presumably caused by single specific interactions of both binders. Despite the common inhibitory phenotype of the sybodies Sb1, Sb2, and Sb3, the channels in their respective C3-symmetric complex structures adopt distinct conformations that can be approximated as rigid body movements of pore and LRR domains (Fig. 6, Supplementary Video 1). In Sb2 and Sb3 complexes, these conformational changes are the consequence of the relaxation of a steric clash of the respective sybodies bound to the r-subunit with the LRR domain of the neighboring l-subunit, which would prevail in the conformation observed for the apo protein and the Sb1 complex (Fig. 5a, c and 6b, c).

In the LRRC8A/Sb2 complex, the conformational differences compared to the apo protein can be approximated by a small (7°) rotation of the LRR domain in the r- and a larger (17°) rotation of the LRR domain in the l-position around a hinge located at the respective connection to the pore domain (Fig. 6, Supplementary Video 1). The described movement causes the dissociation of contacts at the tight interface between two domains, which leads to a reduction of the buried surface area from 1390 to 447 Å² (Fig. 6a, b). The consequent opening of a gap in the center of interacting subunits leaves only few contacts at the N- and C-terminal ends of the respective LRR domains (Supplementary Fig. 11g). Due to the described movements, the three l-domains, which approach each other at the threefold axis of symmetry in the apo protein, have retracted by 16 Å to open a gap at the intracellular side (Fig. 6b, Supplementary Video 1).

In the LRRC8A/Sb3 complex, the structural rearrangements of the LRR domains are even more pronounced and they are also accompanied by changes of the pore. Whereas the conformation of the r-subunit resembles the LRRC8A/Sb2 complex, the enhanced (26°) reorientation of the LRR domain of the l-subunit results in its movement towards the membrane (Fig. 6c, Supplementary Video 1). The concurrent redistribution of interactions establishes contacts between repeats 10–16 of the l-domain and repeats 1–8 at the contacted r-domain, which bury 904 Å² of the combined molecular surface (Supplementary Fig. 11h, i). The described relocation of the LRR domains appears to be coupled to the pore where the interface between neighboring l- and r-domains is disrupted at the intracellular side and both domains are splayed open by an 8° rotation around an axis that is located close to the extracellular sub-domain, which remains unchanged in both structures (Fig. 6d, Supplementary Video 1). The resulting dissociation of contacts results in a pronounced gap at the intracellular side (Fig. 6d). The described conformational changes only affect the relationship between l- and r-subunits at the tight interface, whereas the interactions at the loose interface remain unchanged. As a result, the C6 symmetry of the pore domain observed in the apo structure is reduced to a C3 relationship relating pairs of TM domains that have retained their mutual interactions.

Thus, although Sb1, Sb2, and Sb3 share a similar inhibitory phenotype and a related mode of interaction by binding to single domains at the convex face of the LRR domain, the common epitope of Sb2 and Sb3 and the corresponding site of Sb1 are located on opposite ends of the domain. Moreover, in both the Sb2 and Sb3 complex structures, the steric clashes between interacting LRRC8A subunits and bound sybodies stabilize distinct protein conformations in the homomeric channel with currently unclear relationship to its functional state.

Structural basis for the interaction with the potentiating sybodies Sb4 and Sb5. To explore the relationship between inhibitory sybodies and binders that potentiate channel activity, we have determined structures of LRRC8A in complex with the sybodies Sb4 and Sb5. Compared to complexes with Sb1, Sb2, and Sb3, these structures show divergent features with respect to the recognized epitopes and the observed binding stoichiometry. In contrast to the three inhibitory sybodies, which target each subunit of the hexameric LRRC8A channel by binding to the convex side, the Sb4 and Sb5 sybodies target an intracellular epitope of LRRC8A, which is not accessible to the previously characterized sybodies.
face of the LRR domain, thereby stabilizing distinct conformations, the complexes with Sb4 and Sb5 display a high conformational heterogeneity where the LRR domains are poorly resolved in a majority of 3D classes (Supplementary Figs. 7 and 8). Besides the structures with high domain mobility, in both complexes we also observed C3-symmetric channel conformations, which display interactions that differ from inhibitory sybody complexes (Fig. 3d, e, Supplementary Figs. 7, 8). Whereas in these maps the pore domains are of high quality and the less well-defined LRR domains reveal the location of sybodies and their general interaction with channel epitopes, the resolution of the maps is insufficient for a detailed structural interpretation of sybody interactions (Supplementary Figs. 7g, h, 8e, f and 10g–j). The described interactions, which are based on homology models of the sybodies Sb4 and Sb5, are thus approximate.

In both, LRRC8A/Sb4 and LRRC8A/Sb5 complexes, the sybodies bind to alternating subunits as their epitopes are only accessible in LRR domains located at the r-position of tightly interacting domain pairs whereas they are hidden in the dimer interface in the l-subunit (Fig. 7). Their interaction with residues located on the edge between the flat face and the concave inside of the LRR domain involves repeats 2–14 in case of the Sb4 complex or 2–7 in case of the Sb5 complex (Fig. 7e, Supplementary Fig. 11a). The smaller interface and lack of aromatic residues contributed by Sb5 is congruent with its lower affinity compared to Sb4 (Fig. 1e and Supplementary Fig. 1c). Both sybodies target overlapping sites, yet with different binding modes and they bridge the large fenestrations between adjacent domains at the loose interface by approaching the juxtaposed l-subunits (Fig. 7a–d). The resulting conformational changes to accommodate the binders lead to the retraction of the LRR domains from the threefold axis, although to a smaller extent than observed in the LRRC8A/Sb2 and Sb3 complexes (Fig. 6a–c, Supplementary Fig. 11j, k, Supplementary Video 2). As in the LRRC8A/Sb3 complex, the conformational rearrangement of the LRR domains is coupled to the pore domain leading to the opening of intracellular subunit contacts, akin to conformational changes observed for the Sb3 complex, though this time at the loose interface (Supplementary Fig. 11j, k, Supplementary Video 2). Together, the structures of complexes with potentiating sybodies reveal an interaction where the binders target an epitope that is buried in one subunit of interacting domain pairs. The accessibility of this site in the observed C3-symmetric channel conformation will thus depend on the relative position of LRRC8A subunits in heteromeric channels.

Discussion
In our study, we have investigated the role of the cytoplasmic LRR domains of VRACs for the regulation of their activity. These protein components form modular units of LRRC8 channels that resemble ligand-binding domains. Although their potential role as interaction platforms is evident, since structurally related units in other proteins are known to bind diverse small- and macromolecules, no ligands are currently known for this particular family of ion channels. To characterize the effect of interacting proteins on the functional properties of VRACs, we have selected nanobodies from synthetic libraries, which specifically bind the cytoplasmic domain of the LRRC8A subunit with nanomolar affinity (Fig. 1, Supplementary Fig. 1, Table 1). As shown by patch-clamp electrophysiology, these sybodies modulate the activity of endogenous VRACs in HEK293 cells (Fig. 2). Whereas three of the selected binders were found to inhibit activity, two others showed a potentiating effect, which emphasizes the importance of these domains as regulatory units of LRRC8 channels.

Insight into the structural basis of VRAC modulation was obtained from cryo-EM structures of LRRC8A-sybody complexes. Irrespective of the fact that LRRC8A homomers are not found under physiological conditions, such assemblies form functional anion channels, although with compromised activation properties. Moreover, the observed strong potentiation of LRRC8A activity by the sybodies Sb4 and Sb5 and its inhibition by Sb3 further emphasize an equivalent modular role of binders also in the context of homomeric channels (Fig. 2f, g). The respective complex structures thus likely display general properties of sybody interactions that might also extend towards heteromeric channels. Since the local concentration of the targeted A-subunit is increased compared to their heteromeric equivalents, we assume observed structural features to be even enhanced in homomeric channels. However, due to the unknown disposition of subunits in LRRC8 heteromers, we also expect unique properties of sybody interactions in endogenous heteromeric channels, which will have to be explored in future studies. In the five complexes, the sybodies show discrete binding modes, with the corresponding channel structures providing insight into accessible conformations of the LRR domains and their potential coupling to the transmembrane pore. The three inhibitory sybodies (Sb1, Sb2, and Sb3) target all subunits of the hexamer and they interact with epitopes located on two separated positions on the convex side of the LRR domain (Fig. 8a). Sybody Sb1 binds towards the C-terminus and stabilizes the threefold symmetric
channel conformation observed in the apo protein\textsuperscript{18,19} (Fig. 4a–c). The improved density of this complex now defines the previously poorly resolved conformation of residues buried in the interface (Supplementary Fig. 10a, b). The large number of ionizable residues suggest a plausible dependence of interactions between LRR domains on the ionic strength that could be weakened by the shielding of charges at higher salt concentrations and a hypothetical interaction with divalent cations, although the role of such interactions will have to be clarified in future studies (Supplementary Fig. 11c–e). Conversely, sybodies Sb2 and Sb3 recognize a common epitope that is located closer to the membrane. Their binding induces conformational changes of the LRR domains, which in both cases lead to the dissociation of the tight domain interface and which in case of Sb3 extends towards the pore (Fig. 6b–d). Collectively, these structures display a set of conformations that could be adopted as a part of the regulatory mechanism. However, since all three sybodies share a similar inhibitory phenotype, the functional correspondence of the observed conformations remains unclear and it is unlikely that any of them represents an open channel.

In contrast to the stabilizing effect of the inhibitory sybodies Sb1 and Sb3, the two potentiating sybodies Sb4 and Sb5 appear to increase the overall mobility of the LRR domains, which is reflected in the poor resolution of the respective region in both complex structures (Supplementary Figs. 7, 8 and 10g–j). In C3-symmetric channel conformations, both sybodies bind at the loose interface to a site located at the edge between the flat face and the concave side of the domain (Figs 7, 8a). In these structures, the sybodies solely target subunits on the r-position of interacting domain pairs as the epitope on the l-subunit is buried in the interface (Fig. 7a–d). The bridging to the juxtaposed domain pair causes a conformational rearrangement at the loose interface whereas the relationship between interacting LRR domains at the tight interface remains unaffected (Supplementary Fig. 11j, k). During activation, it is conceivable that the same sybodies would also target the epitope hidden in the LRR domain interface, thereby facilitating the dissociation of contacts and increasing domain mobility (Fig. 8b). The correlation between LRR domain mobility and channel activity is intriguing also in light of previous observations. C-terminal GFP fusion proteins, which might obstruct tight domain interactions were found to lead to an increased basal activity of the over-expressed channel\textsuperscript{14}. Additionally, a study using complementary fluorescent proteins acting as FRET pairs fused to different LRRC8 subunits suggested a conformational change of the LRR domains during activation\textsuperscript{28}.

In case of the inhibitory sybody Sb3 and the potentiating sybodies Sb4 and Sb5, the rearrangements of the LRR domains couple to the membrane-inserted part of the protein leading to the disruption of intracellular contacts and a breakdown of the C6 symmetry of the pore. The transition splays apart interacting subunits at the intracellular part and potentially might open the access of membrane lipids to the pore to modulate its conduction properties (Fig. 6d, Supplementary Fig. 11j, k, Supplementary Videos 1 and 2). Similar, yet less extensive features of the pore domain conformation were previously found in one of the structures obtained from LRRC8A in absence of binders\textsuperscript{20} and a moderate symmetric expansion of the pore domain was observed for a population of the channel embedded in lipid nanodiscs\textsuperscript{21}. Although not confined to a single functional phenotype of binders, these structures may illustrate a possible pathway for coupling from the LRR domain to the pore to modify a gate that impedes ion conduction in the closed conformation. The location of this gate has not yet been assigned with confidence but it might either involve the N-termini pointing towards the pore axis\textsuperscript{19,27} or the narrow pore region located at the extracellular side\textsuperscript{25,26} as mutations in both regions affect conduction and activation properties of the channel. The long-range nature of the observed transitions suggests that effects might potentially lead to changes in the conformation of the N-terminus and even extend towards the narrow extracellular filter, although its conformation appears unaltered in different structures obtained in this study.

In summary, our study has generated a diverse set of proteins that modulate VRACs by either inhibiting or potentiating their activity. The structures of their complexes have revealed the recognized epitopes and conformations of LRRC8A induced by sybody binding. While in a cellular context, the detailed interaction would depend on the currently unknown distribution of A subunits in LRRC8 heteromers, all structures display the intrinsic plasticity of the channel, which presumably underlies activation. However, in absence of a clear correlation with the modulatory phenotype of binders, the assignment of distinct conformations to functional states is at this stage ambiguous and their relevance in heteromeric channels still awaits investigation. In all cases, it is also possible that the sybodies act by preventing the binding of currently unknown interaction partners. Our data emphasize the importance of the cytoplasmic LRR domains of VRACs in modulating the activation of the pore domain by allosteric mechanisms. The generated set of interacting proteins will serve as important tools for future studies. These range from the structural characterization of heteromeric channels to their investigation in a cellular context and the development of potential therapeutic approaches aiming at the inhibition of VRAC channels in cerebral ischemia\textsuperscript{40} and their activation in certain type of cancers to facilitate the uptake of drugs\textsuperscript{41,42}. 

Fig. 8 Potential mechanisms. a Location of sybody binding sites on the LRR domain of LRRC8A. The proteins are shown as ribbon with different sybodies labeled. b Schematic depiction of a potential modulatory mechanism of channel function by sybody binding. Binding of inhibitory sybodies to the convex outside of the LRR domains (left) reduces flexibility of the domains, which stabilizes a closed channel conformation. Conversely, the binding of activating sybodies to the concave inside increases domain mobility, which is in some way transmitted to the pore region to open the ion conduction path.
Expression constructs and cloning. All constructs were generated using FX-cloning and FX-compatible vectors. The constructs encompassing full-length murine LRRC8A and C, the LRRC8A-pore domain (P) and the LRRC8A-cytosolic domain (LRR) were obtained from a previous study. The LRR domain of LRRC8A encompassed residues 398-810 of mouse LRRC8A. The boundaries for the LRR domains of LRRC8C and LRRC8D were chosen analogously to LRRC8A with constructed constructs. For periplasmic expression, full-length LRRC8A and LRRC8A-PD were cloned into a pcDX vector containing a C-terminal Rhinovirus 3C protease-cleavable linker followed by mcCherry, a myc-tag and streptavidin-binding domain (SBD) (pcDXc3MS). For phage display experiments, full-length LRRC8A and LRRC8A-PD were cloned into a pcDX vector containing a C-terminal Rhinovirus 3C protease-cleavable linker followed by mcCherry (pcDXc3MS). The LRR domains of LRRC8A, C and D were cloned into a pcDX vector containing an N-terminal SBP, a myc-tag followed by a Rhinovirus 3C protease-cleavable linker (pcDX3MS). For periplasmic expression of sybodies in mammalian cells, sybodies were cloned into a pcDX vector containing a C-terminal His-tag. For cytoplasmic expression in mammalian cells, sybodies were cloned into a pcDX vector containing a C-terminal His-tag. For cryo-EM analyses and binding tests, full-length LRRC8A and the LRRC8A-PD were subjected to size-exclusion chromatography using a Superdex 200 5/150 column (GE Healthcare), eluted with HBS supplemented with 0.25 mM DDM at a flow rate of 0.2 ml min⁻¹. Peak fractions were collected and analyzed by SDS-PAGE. For the analysis of SPR, the interaction between the LRR domain of LRRC8A and sybodies was analyzed using a Biacore T100 instrument (GE Healthcare). The chemically biotinylated LRR domain of LRRC8A was immobilized on a streptavidin-coated sensor chip (Cytiva) to obtain a maximum value of about 100 response units (RU). Measurements were performed at 20 °C at a flow rate of 30 ml min⁻¹ in 1 mM HEPES pH 7.5, 150 mM NaCl, 0.05% Tween 20. For sybody Sb1, Sb3, Sb5, Sb6 and Sb7, multi-cycle kinetics measurements with eight concentrations of the analyte were performed (1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 °C). To adsorb the analyte, the interaction with sybody Sb2 was characterized using single-cycle kinetics with injection of six concentrations of the analyte (1000, 500, 250, 125, 62.5 and 31.25 nM). Traces from a channel not containing immobilized protein and from an injection not containing an analyte were used as double reference for sensograms. Data processing and analysis were performed using BioEvaluation software. Kinetic parameters were fitted using either 1:1 or heterogenous ligand interaction models.

Surface expression analysis. For the analysis of surface expression of endogenous LRRC8 channels, HEK293T cells were cultured in high glucose DMEM (Gibco), supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U ml⁻¹ Penicillin-Streptomycin (Sigma) at 37 °C and 5% CO₂. Cells at 60% confluency were transfected with 10 µg of pcDX3VMS plasmids encoding the respective sybodies per 10 cm dish using 25 µg of 40 kDa linear polyethyleneimine (Polysciences) and grown for 24 h. For biotinylation, cells from a 10 ml culture were washed with PBS and plasma membrane proteins were labeled with 10 ml Sulfo-NHS-SS-Biotin solution with a concentration of 0.25 mg ml⁻¹ using the Pierce™ Cell Surface Protein Isolation Kit (ThermoFisher Scientific). For non-biotinylated control, cells were incubated with PBS instead. After 15 min at room temperature, biotinylation was stopped by addition of 300 µl of quenching solution and cells were harvested. Extraction and purification steps were carried out at 4 °C. After washing with cold PBS, the cell pellet was resuspended in 200 µl of the extraction buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, 1% GDN, 1x Complete EDTA-free Protease Inhibitors (Roche) and 10 µg ml⁻¹ DNase and incubated for 1 h under gentle agitation. Insoluble fractions were removed by centrifugation at 14,000 g for 15 min at 4 °C. Supernatants were analyzed by western blotting with the respective antibody as loading control (abcam, ab6528, 1:1000 dilution) and a peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, 11-
035-146, 1:10,000 dilution). The luminescent signal was developed with ECL substrate (GE Healthcare) and recorded with a Viber Fusion FX imaging system. Signal integrals were quantified using Fiji. For quantification, the signal corresponding to LRRCA8 was normalized to the signal of pan-cadherin for each sample.

**Cryo-EM sample preparation and data collection.** Samples for structure determination of LRRCA8/sybody complexes were prepared by addition of respective sybodies to a highly concentrated stock solution of LRRCA8 without any further chromatography steps. Samples were concentrated to a final volume of 3.4 ml by centrifugation of 3.4 ml at 7,000 × g for 1 hour. The LRRCA8 monomer of 1.25–1.5. For the sample LRRCA8-Sb4_1, the sybody was added sub-stoichiometrically (at a molar ratio of sybody/LRRCA8 monomer of 0.5). For vitrification, 2.5 μl of protein samples were applied to glow-discharged holey carbon grids (Quantifoil R1.2/1.3 Au 200 mesh). Excess of the samples was removed by blotting and local refrigerations of 3–4 °C with a molar force in a controlled atmosphere (4 °C and 100% relative humidity). Grids were flash-frozen in a mixture of liquid ethane/ propane using a Vitrobot Mark IV (ThermoFisher Scientific). Samples were imaged on a 300 kV Titan Krios G3 (ThermoFisher Scientific) with a 100 μm objective aperture. All data were collected using a post-column BioQuantum electron filter (2.7 Å pixel). After initial 3D auto-croping, a previously determined map of LRRC8A was used as reference for one or two rounds of reference-free 2D classification followed by one or two rounds of 3D auto-reconstruction with masks. Particles were then subjected to 3D auto-reconstruction with masks encompassing only protein density and excluding the density of the detergent micelle, cells were transfected with 3 µg of plasmid DNA encoding sybody Sb1 and LRRC8A/Sb1 and LRRC8A/Sb3 it was also possible to unambiguously place residues 1–166 of the sybody Sb1 and 1–120 of the sybody Sb3. In data of complexes of the other three sybodies (Sb4, Sb2, and Sb5), models were refined using reference model restrained searches of previously reported LRRCA8 structures and homology models of the sybodies. In structures based on homology models, the protein-sybody interactions are tentative. Figures and movies containing molecular structures and densities were prepared with DINO (http://www.dino3d.org) and Chimera. Surfaces were generated with MMS6 (Schrödinger).

**Electrophysiology.** Cells used for electrophysiological measurements were cultured in high glucose DMEM (Gibco), supplemented with 10% FBS, 4 mM glutamine, 1 mM sodium pyruvate, and Penicillin (Sigma) at 37 °C and 5% CO2. To investigate the effect of the sybody expression on endogenous VRAC currents, HEK293T cells were gently detached from their support and seeded in 10-cm Petri dishes at 5% confluency. After a 2 h incubation step (to allow cells to adhere), cells were transfected with 5 μg of plasmid DNA encoding for the channel located in the LRR domain and residues 1–116 of the sybody Sb1 and 1–120 of the sybody Sb3. In data of complexes of the other three sybodies (Sb4, Sb2, and Sb5), models were refined using reference model restrained searches of previously reported LRRCA8 structures and homology models of the sybodies. In structures based on homology models, the protein-sybody interactions are tentative. Figures and movies containing molecular structures and densities were prepared with DINO (http://www.dino3d.org) and Chimera. Surfaces were generated with MMS6 (Schrödinger).

**Model building and refinement.** The models of full-length LRRCA8 were based on a previously determined structure (PDB entry 6G9O)18. Initially, the hexameric model was fitted into the cryo-EM density using UCSF Chimera24. Subsequently, the pore (residues 15–411) and cytosolic domains (residues 412–808) of each protomer were fitted separately as rigid bodies into the density using Coot59. Initial atomic models were further improved using rigid-body-fitting and real-space refinement in Coot and Phenix60,61. In all cases, except for the low-resolution dataset LRRCA8/Sb4, it was possible to place and refine pore-domain residues 15–68, 92–176, and 230–411 reliably. In case of datasets LRRCA8/Sb1 and LRRCA8/Sb3 it was also to unambiguously place residues 412–530 of the channel located in the LRR domain and residues 1–116 of the sybody Sb1 and 1–120 of the sybody Sb3. In data of complexes of the other three sybodies (Sb4, Sb2, and Sb5), models were refined using reference model restrained searches of previously reported LRRCA8 structures and homology models of the sybodies. In structures based on homology models, the protein-sybody interactions are tentative. Figures and movies containing molecular structures and densities were prepared with DINO (http://www.dino3d.org) and Chimera. Surfaces were generated with MMS6 (Schrödinger).
Statistics and reproducibility. Electrophysiology data were repeated multiple times from different transfections with very similar results. Conclusions of experiments were not changed upon inclusion of further data. In all cases, leaky patches were discarded.

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

Data availability. The three-dimensional cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-13320 (LRRC8A/Sb1), EMD-13323 (LRRC8A/Sb2), EMD-13268 (LRRC8A/Sb3), EMD-13212 (LRRC8A/Sb4), EMD-13213 (LRRC8A/Sb4a), and EMD-13230 (LRRC8A/Sb5). The deposition includes maps of full-length proteins, corresponding half-maps 1 and 2, the mask used for final FSC calculation as well as relevant higher resolution maps obtained after local refinement. Coordinates for the models of full-length LRRC8A/Sb1, LRRC8A/Sb2, LRRC8A/Sb3, LRRC8A/Sb4a, and LRRC8A/Sb5 have been deposited in the Protein Data Bank under accession numbers 7PSV [https://doi.org/10.2210/pdb7PSV/pdb], 7PSW [https://doi.org/10.2210/pdb7PSW/pdb], 7V6O [https://doi.org/10.2210/pdb7V60/pdb] and 7V6K [https://doi.org/10.2210/pdb7V6K/pdb], respectively. The data from electrophysiological recordings showing the effect of sybodies on LRRC8 currents have been deposited in the Dryad database (https://doi.org/10.5061/dryad.h76d9gr). Source data are provided with this paper.

Received: 5 February 2021; Accepted: 26 August 2021; Published online: 14 September 2021

References

1. Jentsch, T. J. VRACs and other ion channels and transporters in the regulation of cell volume and beyond. Nat. Rev. Mol. Cell Biol. 17, 293–307 (2016).

2. Chen, L. et al. More than just a pressure relief valve: physiological roles of volume-regulated LRCC8 anion channels. Biol. Chem. 400, 1481–1496 (2019).

3. Okada, Y. et al. Roles of volume-regulatory anion channels, VSOR and Maxi-C1, in apoptosis, cisplatin resistance, necrosis, ischemic cell death, stroke. Microvasc. Res. 83, 205–283 (2015).

4. Nilius, B. et al. Properties of volume-regulated anion channels in mammalian cells. Prog. Biophys. Mol. Biol. 68, 69–119 (1997).

5. Strange, K., Emma, F. & Jackson, P. S. Cellular and molecular physiology of cell volume and beyond. Nat. Rev. Mol. Cell Biol. 291, 1704–1705 (2016).

6. Zhou, P., Polovitcikaya, M. M. & Jentsch, T. J. LRCC8 N terminus influence pore properties and gating of volume-regulated anion channels (VRACs). J. Biol. Chem. 293, 13440–13451 (2018).

7. Konig, B., Hao, Y., Schwartz, S., Plesdet, A. J. & Stauber, T. J. A FRET sensor of C-terminal movement reveals VRAC activation by plasma membrane DAG signaling rather than ionic strength. Elife 8, https://doi.org/10.7554/ elife.45421 (2019).

8. Voets, T., Droogmans, G., Raskin, G., Eggermont, J. & Nilius, B. Reduced intracellular ionic strength as the initial trigger for activation of endothelial volume-regulated anion channels. Proc. Natl Acad. Sci. USA 96, 5298–5303 (1999).

9. Schenck, S. et al. Generation and characterization of anti-VGLUT nanobodies acting as inhibitors of transport. Biochemistry 56, 3962–3971 (2017).

10. Moutel, S. et al. NaLi-H1: a universal synthetic library of humanized fluorescent nanobodies. Elife 8, https://doi.org/10.7554/ elife.34317 (2019).

11. Rottenberg, S., Disler, C. & Perego, P. The rediscovery of platinum-based anticancer drugs. J. Natl. Cancer Inst. 109, 369–381 (2017).

12. Geertsma, E. R. & Dutzler, R. A versatile and efficient cloning tool for structural biology. Proteins 88, 1–11 (2020).

13. Matsushima, N., Takatsuka, S., Miyashita, H. & Kretsinger, R. H. Leucine rich proteo-proteins derived from Discosoma sp. red fluorescent proteins. Proteins 119, 72–83 (2015).

14. Xu, R., Wang, X. & Shi, C. Volume-regulated anion channel as a novel cancer therapeutic target. Int. J. Biol. Macromol. 159, 570–576 (2020).

15. Geertsma, E. R. & Dutzler, R. A versatile and efficient cloning tool for structural biology. Proc. Natl Acad. Sci. USA 116, 6157–6163 (2019).

16. Xue, C., Wilson, D. S., Seelig, B. & Szostak, J. W. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SFP-Tag. Protein Expr. Purif. 23, 440–446 (2001).
46. Rekas, A., Alattia, J. R., Nagai, T., Miyawaki, A. & Ikura, M. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. J. Biol. Chem. 277, 50573–50578 (2002).

47. Reeves, P. J., Callewaert, N., Conterras, R. & Khorana, H. G. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylgalactosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc. Natl Acad. Sci. USA 99, 8353–8358 (2002).

48. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

49. Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. Elife 7, https://doi.org/10.7554/eLife.41666 (2018).

50. Zivanov, J., Nakane, T. & Scheres, S. H. W. Estimation of high-order aberrations and anisotropic magnification from cryo-EM data sets in RELION-3.1. IUCrJ 7, 253–267 (2020).

51. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).

52. Rohou, A. & Grigorieff, N. CTFFIND4: Fast and accurate defocus estimation from electron micrographs. J. Struct. Biol. 192, 216–221 (2015).

53. Zivanov, J., Nakane, T. & Scheres, S. H. W. A Bayesian approach to beam-induced motion correction in cryo-EM single-particle analysis. IUCrJ 6, 5–17 (2019).

54. Chen, S. et al. High-resolution noise substitution to measure overfitting and validate resolution in 3D structure determination by single electron cryomicroscopy. Ultramicroscopy 135, 24–35 (2013).

55. Rosenthal, P. B. & Henderson, R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J. Mol. Biol. 333, 721–745 (2003).

56. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 (2012).

57. Scheres, S. H. & Chen, S. Prevention of overfitting in cryo-EM structure determination. Nat. Methods 9, 835–841 (2012).

58. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput Chem. 25, 1605–1612 (2004).

59. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr D. Biol. Crystallogr 60, 2126–2132 (2004).

60. Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46, W296–W303 (2018).

61. Kirchhofer, A. et al. Modulation of protein properties in living cells using nanobodies. Nat. Struct. Mol. Biol. 17, 133–138 (2010).

62. Rasmussen, S. G. et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. Nature 479, 449–555 (2011).

63. De Genst, E. et al. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc. Natl Acad. Sci. USA 103, 4586–4591 (2006).

64. Adams, P. D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr D. Biol. Crystallogr 58, 1948–1954 (2002).

65. Afonine, P. V. et al. Real-space refinement in PHENIX for cryo-EM and crystallography. Acta Crystallogr D. Struct. Biol. 74, 531–544 (2018).

66. Sanner, M. F., Olson, A. J. & Spehner, J. C. Reduced surface: an efficient way to compute molecular surfaces. Biopolymers 38, 305–320 (1996).

Acknowledgements

This research was supported by grants from the Swiss National Science Foundation (No. 310030B_182828 to R.D. and No. 310030_188817 to M.A.S.). We thank Simona Sorrentino and the Center for Microscopy and Image Analysis (ZMB) of the University of Zurich for the support and access to the electron microscopes, Iwan Zimmermann for help in syboby selection, Jens Sobek from the Functional Genomics Center of the UZH/ETH Zurich for help with surface plasmon resonance experiments and T. J. Jentsch for providing the LRRC8+/− HEK cell-line and an aliquot of an Anti-LRRC8A antibody used for preliminary studies. All members of the Dutzler lab are acknowledged for their help at various stages of the project. The cryo-electron microscope and K3-camera were acquired with support of the Baugarten and Schwyzer-Winiker foundations and a Requip grant of the Swiss National Science Foundation (No. 316030_183382).

Author contributions

D.D. and S.R. generated expression constructs and purified proteins. D.D. and C.A.I.H. selected sybodies and M.A.S. supervised sybody selection. S.R. carried out surface-biotinylation experiments. M.S. oversaw cryo-EM experiments, M.S. and D.D. and S.R. prepared the samples for cryo-EM and collected cryo-EM data. D.D., S.R., and M.S. proceeded with structure determination and refinement. D.D. recorded and analyzed electrophysiology data. D.D., S.R., M.S., and R.D. jointly planned experiments, analyzed the data and wrote the manuscript. M.A.S. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25742-w.

Correspondence and requests for materials should be addressed to Marta Sawicka or Raimund Dutzler.

Peer review information Nature Communications thanks Seok-Yong Lee and other anonymous, reviewers for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.