nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☐	☒	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	CryoEM data collection: Gatan Latitude Electrophysiological data collection: Clampex 10.7
Data analysis	CryoEM data analysis: motioncor2, Ctffind4, Relion3, ResMap Model building and analysis: Swiss-model web server, Chainsaw 2, Coot 10, Phenix 1, UCSF Chimera 1, Chimera X Molecular Dynamics simulations: VMD 1.9.2, NAMD2 All above-mentioned software used in this project was curated by SBGrid. Electrophysiological data analysis: Clampfit 10.7, Matlab2017b

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Cryo-EM density maps of Prestin have been deposited in the Electron Microscopy Data Bank under accession codes EMDB-24928 (Up), EMDB-24930 (Inhibited I), EMDB-24931 (Down I), EMDB-24932 (Down II), EMDB-24933 (Intermediate), and EMDB-24934 (Inhibited II). The atomic models of the Prestin dimer have been

deposited in the Protein Data Bank under accession code
7S8X (Up), 7S9A (Inhibited I), 7S9B (Down I), 7S9C (Down II), 7S9D (Intermediate) and 7S9E (Inhibited II), respectively.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For cryo-EM experiments, sample sizes and particle number were chosen to maximize map quality and resolution. As a result the number of movies acquired was ranged from 5,500 (Up sate) to 22,000 (Down states) movies. These are standard sample sizes for dimeric membrane proteins. For electrophysiology experiments, cell number was chosen based on convention in the field (For the electromotility tests at least 5 cells; for Salicylate blocking experiments at least 6 cells; for the NLC measurements of the mutants at least 8). These sample sizes were deemed to be sufficient to determine mean and standard error of the mean, allowing for comparison between different mutants.
Data exclusions	For cryo-EM experiments, micrographs or particles were excluded if they did not improve map quality due to astigmatism, drift and or ice contamination. This is standard practice for cryo-EM structure determination. For electrophysiological experiments, recordings were excluded from analysis if leak or endogenous currents prevented NLC analysis. This is standard practice in electrophysiology.
Replication	Structure determination was completed once, as is standard. All electrophysiological results contain data from multiple cells, ensuring reproducibility. For mutants that failed to yield NLC, at least 8 cells were measured, and at least four different transfections was done. This is standard practice in electrophysiology.
Randomization	Particles are split in two half groups in the 3D refinement step, which is a standard step in analyzing cryo-EM data. No other randomization was employed or needed, as is standard for structural and electrophysiological work.
Blinding	Blinding was not employed, as is not applicable here nor is standard for structural determination and electrophysiological work.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	HEK293S GnTI- (ATCC CRL-3022); HEK-293T (ATCC CRL-1573); Sf9 (ATCC CRL-1711).
Authentication	none
Mycoplasma contamination	not tested
Commonly misidentified lines (See ICLAC register)	not used