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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. 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
☐	☒	An indication of 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 statistics 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
☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	EPU1.9
Data analysis	RELION 2.0, MotionCorr2.1, GCTF1.06, Coot0.8.8, ResMap1.1.4, PHENIX1.11.1, MolProbity(part of PHENIX package), PyMol1.8.6.2, Chimera1.11.2, GraphPad Prism7, Hole2.2.005, PROMALS3D

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/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research 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 list of figures that have associated raw data
- A description of any restrictions on data availability

The cryo-EM density maps of the NfMCU have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-7826 (without saposin) and EMD-7828 (with saposin, low-pass filter to 5.0 Å resolution). Atomic coordinates have been deposited in the Protein Data Bank under accession numbers 6D7W

(without saposin) and 6D80 (with saposin modeled as poly-A peptide). Source data for Fig. 4e, Fig 5b-d, Extended Data Fig. 1b and Extended Data Fig. 2 are available in Supplementary Information. The gel source data for Fig. 4e, Extended Data Fig. 1b and 1c, and Extended Data Fig. 8 are available in Supplementary Figure 1.

Field-specific reporting

Please select 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/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

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

Sample size	All fluorescent and radioactive calcium uptake assays were repeated at least 3 times. The sample size was determined based on the reproducibility of the data. As all assays yielded consistent results, the sample size was sufficient to provide accurate measurement.
Data exclusions	3-D classification yielded multiple 3-D reconstruction maps. Only the particles that gave rise to homogeneous density were selected, combined and used in the final reconstruction and refinement. Details are described in the flowchart of Extended Data Figure 3 and Methods.
Replication	All experimental findings were reliably reproduced with consistent results.
Randomization	For structure refinement and model validation, all particles were randomly split into two groups, one group was used for refinement and the other for validation. For the fluorescent calcium uptake assay, calcium measurement was performed on a population of bacteria expressing the NfMCU channel. For the radioactive calcium flux assay, calcium uptake measurement was performed on a population of purified NfMCU channels that have been reconstituted into liposomes in random orientation.
Blinding	The investigators were blinded to group allocation during data collection and analysis.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

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

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	All unique materials used are readily available from the authors.

Antibodies

Antibodies used	Mouse anti-Penta-His Antibody, Qiagen, Cat. #: 34660 Mouse anti-RNA polymerase alpha (E. coli) antibody, Santa Cruz Biotechnology, Cat. #: sc-101597 HRP conjugated sheep anti-mouse IgG antibody, GE Healthcare, Cat. #: NA931-1ML
Validation	Mouse anti-penta His antibody: Qiagen reported that the IgG antibodies were produced "in vitro" and "isolated from serum-free hybridoma cell cultures, ensuring preparations free of viruses, mycoplasma, and contaminating immunoglobulins." The specificity of the antibodies "was determined by epitope mapping using libraries of peptides synthesized directly onto nitrocellulose." (Qiagen's QIAexpress Detection and Assay Handbook: https://www.qiagen.com/us/resources/resourcedetail?id=a8d20bf6-b88c-436b-a7be-de66b5dd70fd&lang=en) HRP conjugated sheep anti-mouse antibody: GE Healthcare reported that the antibody was produced by "hyper-immunizing

sheep with purified immunoglobulin fractions from normal mouse serum" and the anti-serum was "affinity adsorbed to remove cross-reacting antibodies towards rat , human and rabbit immunoglobulins". The sheep antibodies are purified "using an affinity column of mouse IgG" and HRP was attached to the IgG "using an adaptation of the periodate oxidation technique". The antibodies were validated by Western blot. (Amersham ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody (from sheep) product specification sheet: https://www.gelifesciences.com/en/us/shop/protein-analysis/blotting-and-detection/blotting-standards-and-reagents/amersham-ecl-hrp-conjugated-antibodies-p-06260?current=NA934-1ML#related-documents)
Mouse anti-RNA polymerase alpha (E. coli) antibody: Santa Cruz Biotechnology reported that this product is a "mouse monoclonal antibody (IgG) raised against RNA polymerase alpha of E. coli origin, with epitope mapping to amino acids 209-329." The antibody is intended for Western blotting purposes and "may cross-react with most Gram-negative bacteria and with some Gram-positive bacteria." No additional information about the antibody was provided by the manufacturer. (RNA pol alpha (4RA2): sc-101597 datasheet: https://datasheets.scbt.com/sc-101597.pdf)