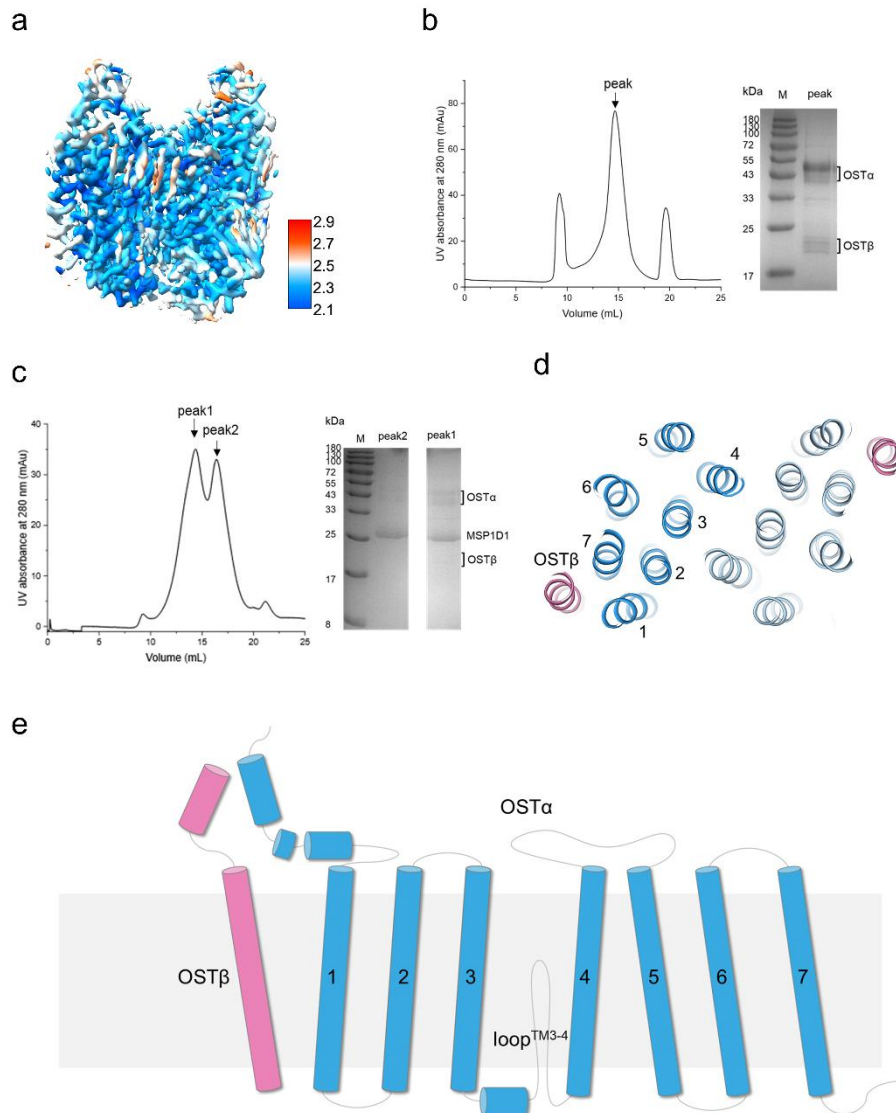




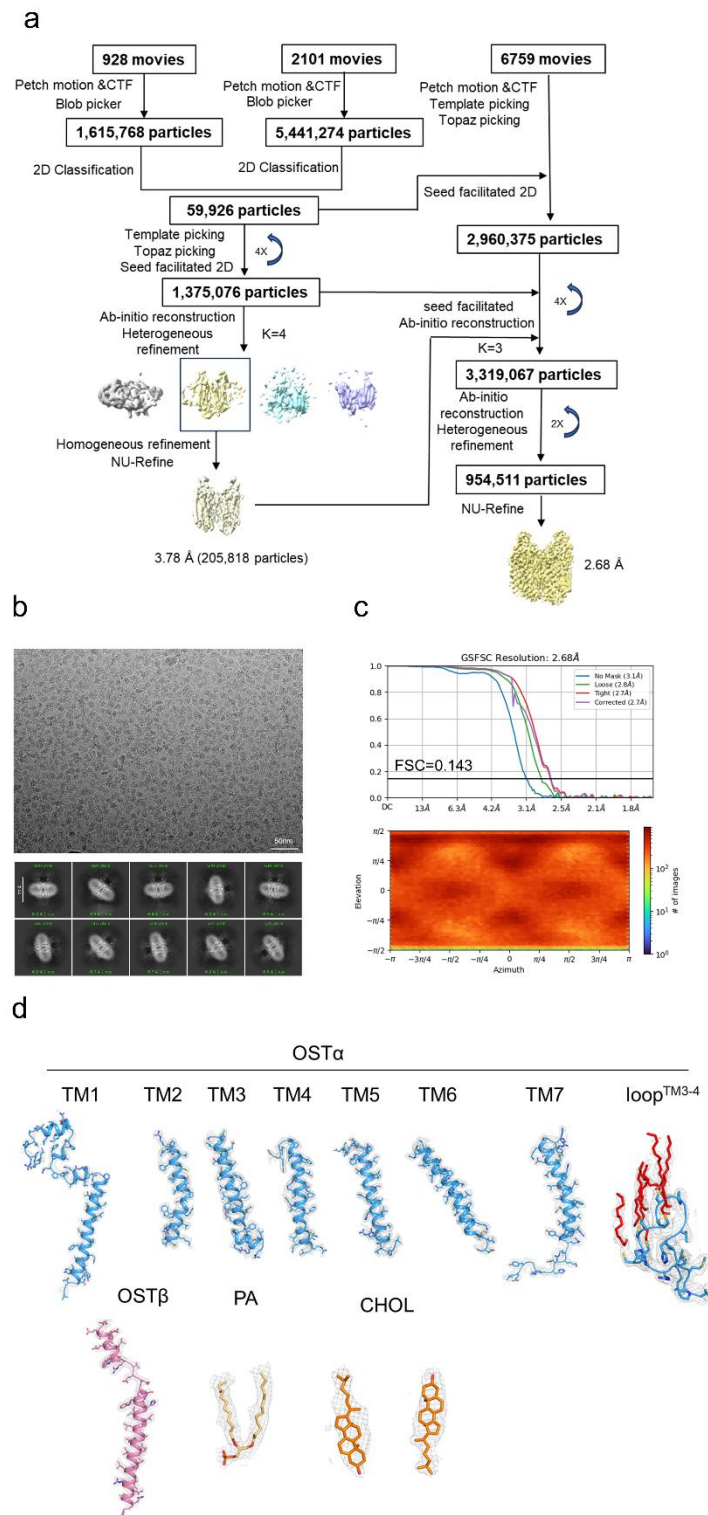
Supplementary Fig. 1 Purification and structural features of OST α/β

a. Refined cryo-EM map of OST α/β complex structure. The cryo-EM maps are colored by UCSF ChimeraX 1.5 according to the local resolution of the cryo-EM estimated by cryoSPARC 4.7.1

b. The size exclusion chromatography profiles of human OST α/β in the detergent LMNG and **c.** in nanodiscs. The peak fractions were applied to SDS-PAGE and visualized by Coomassie blue staining.

d. Top view of OST α/β complex. Dark blue and light blue denote each OST α monomer, OST β are colored pink.

e. Topology of the OST α/β complex. OST α is colored blue and OST β is colored pink.



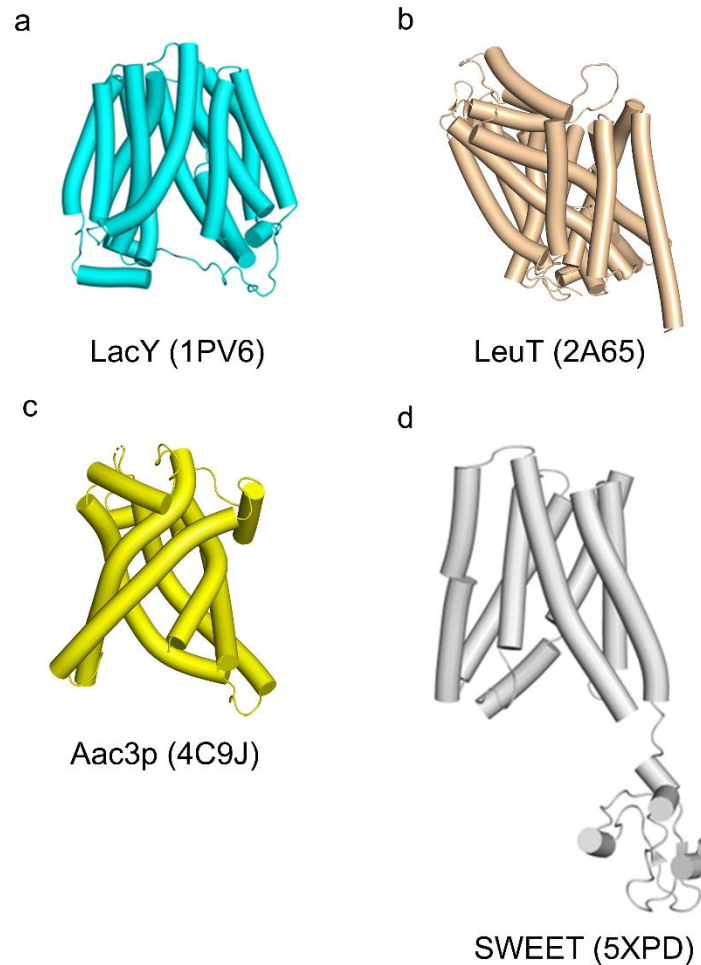
Supplementary Fig. 2 Data processing and model building of OST α / β complex.

a. Flowchart of the cryo-EM data processing by cryoSPARC v4.7.1.

b Representative cryo-EM micrograph and 2D averages. Bar: 50 nm.

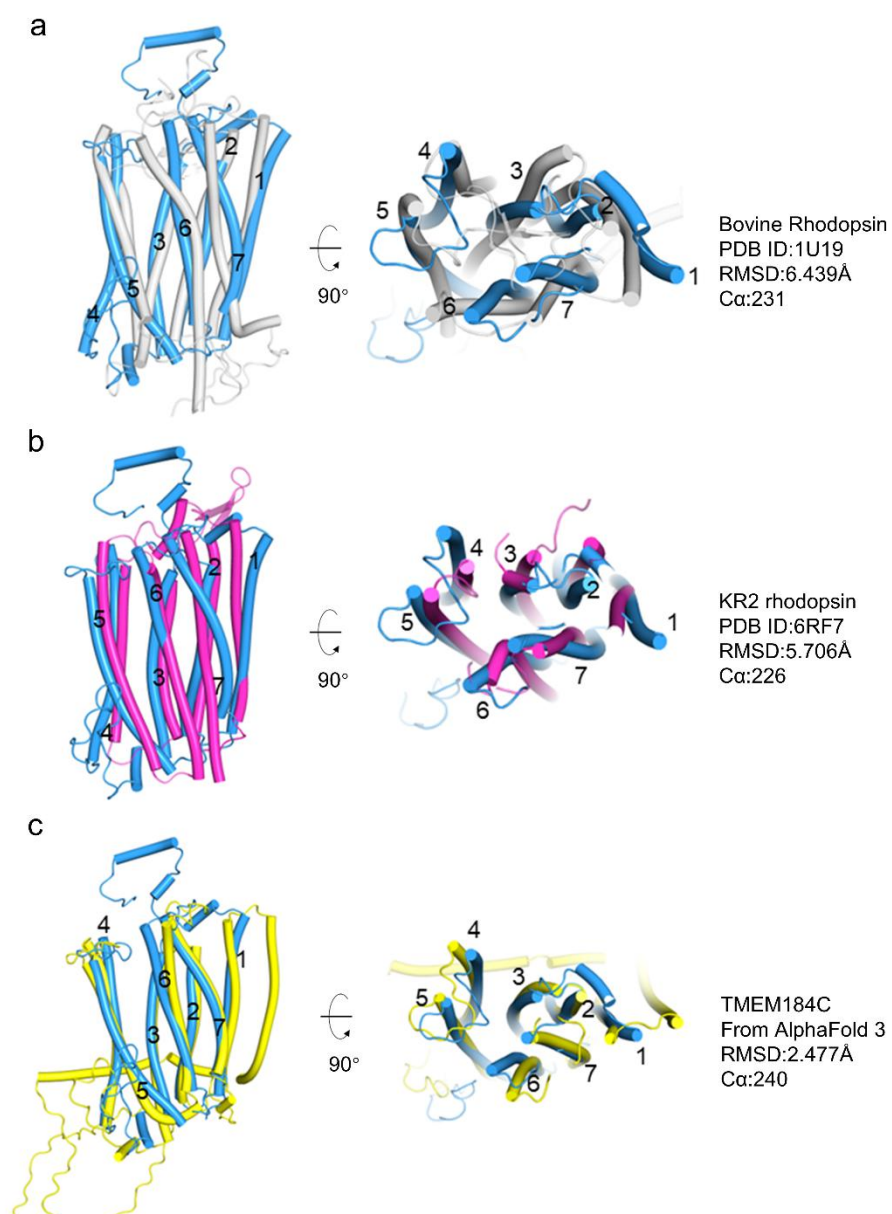
c. Fourier shell correlation (FSC) curves and Euler angle distributions of the classified particles used for the final 3D refinement of the overall maps for OST α / β complex.

d. Cryo-EM maps for representative segments of the OST α/β . The structure was reconstructed with C2 symmetry, and only half of the representative segments are thus presented. Contour levels are set at 5σ .



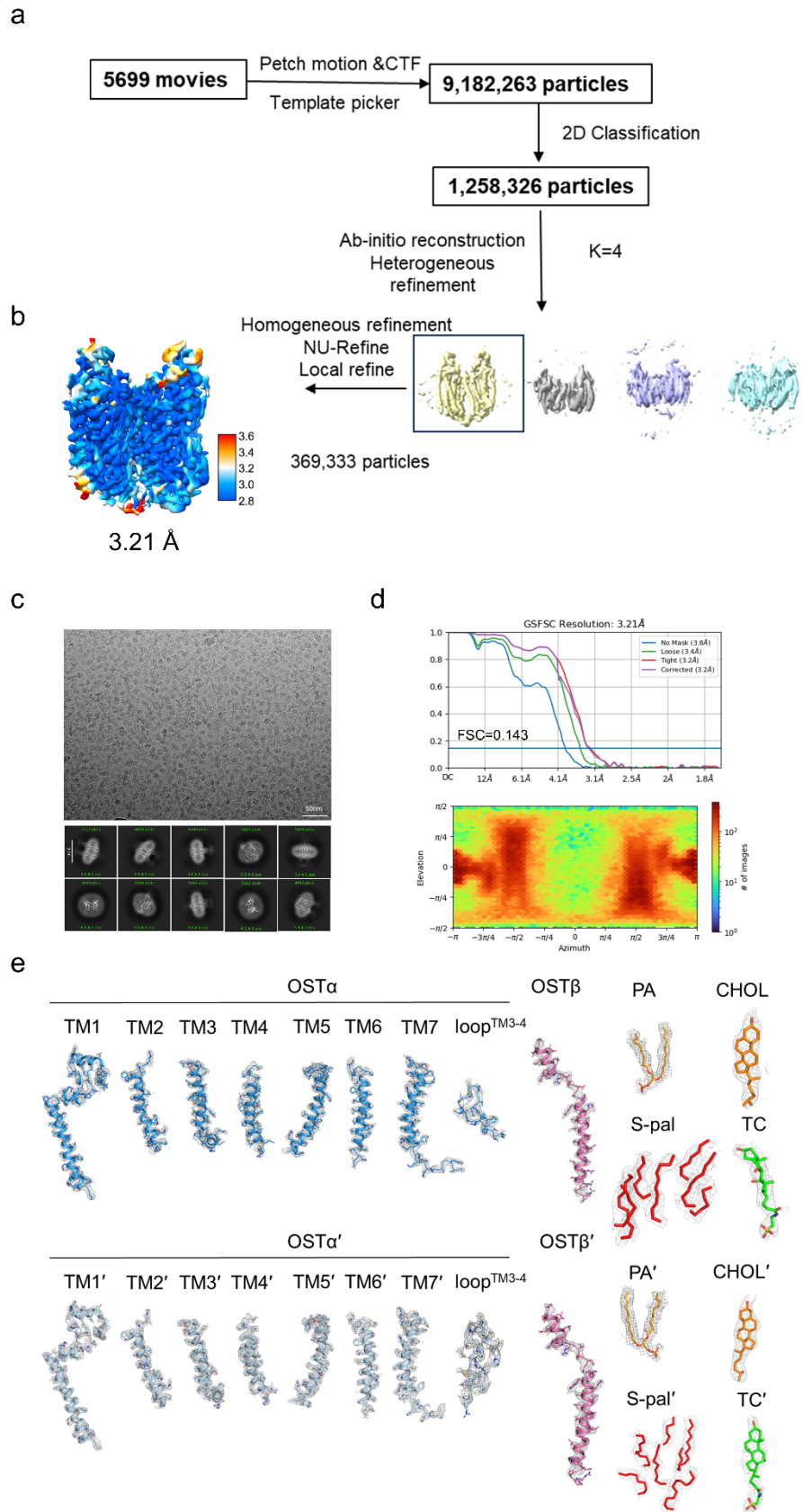
Supplementary Fig. 3 Representative structural folds of reported SLC transporters.

- a.** Lactose permease LacY from *E. coli*. LacY contains 12 TMs, with the N-terminal and C-terminal six TMs forming two distinct helical bundles connected by a long loop between TM 6 and 7. The two 6-TM domains share the same topology and exhibit an approximate two-fold symmetry¹.
- b.** Sodium-coupled leucine transporter LeuT from *Aquifex aeolicus*. LeuT contains 12 TMs, with an internal structural repeat in the first 10 TMs: TM1–TM5 and TM6–TM10 exhibit 176.5° rotational symmetry around a pseudo-two-fold axis within the membrane plane².
- c.** ADP/ATP carriers Aac3p from *Saccharomyces cerevisiae*, Aac3p contains 6 TMs, tilted at approximately 45° relative to the membrane plane. The odd-numbered TMs are kinked at proline or serine residues, forming a barrel-like structure with threefold pseudosymmetry³.
- d.** Sugar transporter SWEET from *Arabidopsis thaliana*. Each SWEET comprises 7 TMs. The N-terminal and C-terminal 3 TMs form 6-TM THB domains, as an inversion linker, TM4 spans the membrane and orients THB2 parallel to THB1⁴.



Supplementary Fig. 4 Superposition of OST α/β complex with GPCR.

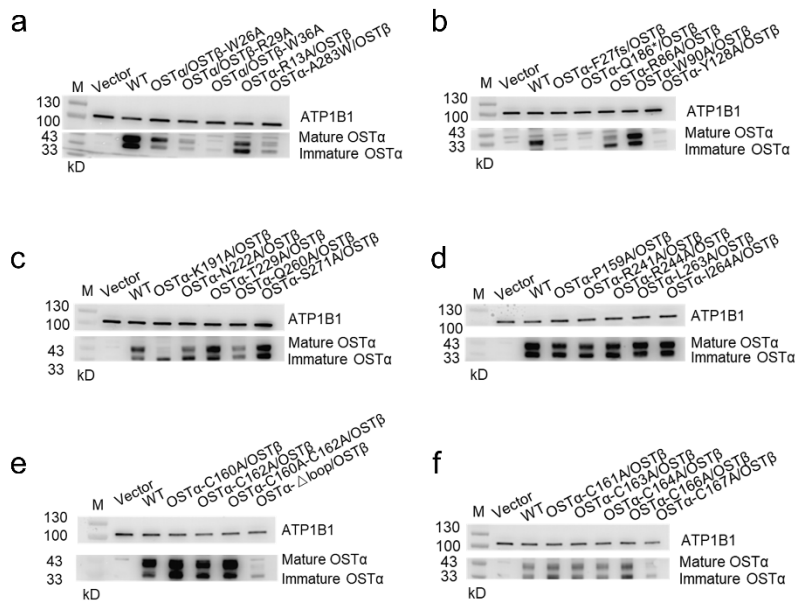
Superposition of OST α/β complex with bovine rhodopsin(**a**)(PDB code: 1U19), KR2(**b**)(PDB code:6RF7), TMEM184C(**c**)(predicted structure use AlphaFold3).



Supplementary Fig. 5 Data processing and model building of TC bound OST α/β complex.

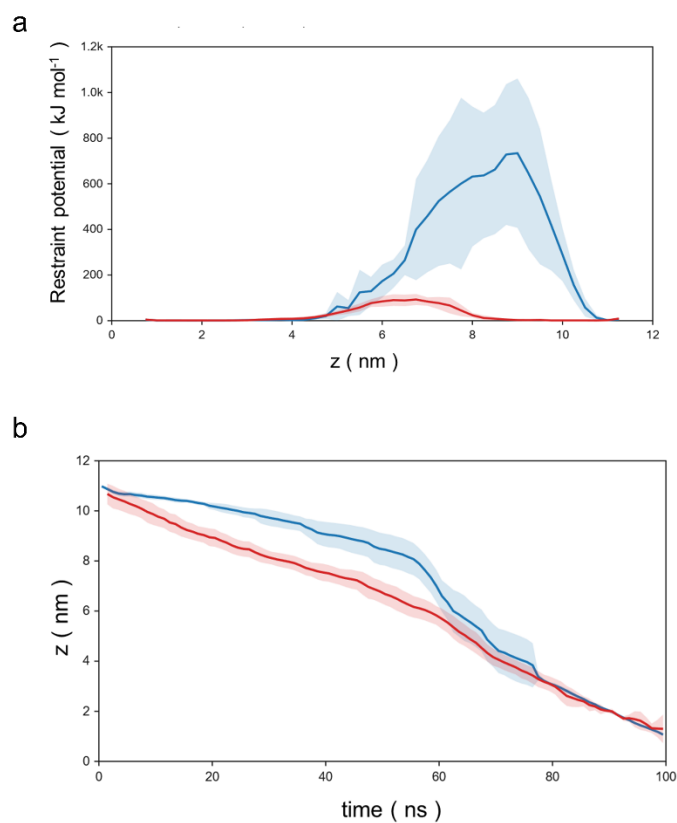
a. Flowchart of the cryo-EM data processing by cryoSPARC v4.7.1.

- b** Refined cryo-EM map of OST α/β complex structure. The cryo-EM maps are colored by UCSF ChimeraX 1.5 according to the local resolution of the cryo-EM estimated by cryoSPARC 4.7.1
- c.** Representative cryo-EM micrograph and 2D averages. Bar: 50 nm.
- d.** Fourier shell correlation (FSC) curves and Euler angle distributions of the classified particles used for the final 3D refinement of the overall maps for TC bound OST α/β complex.
- e.** Cryo-EM maps for representative segments of the TC bound OST α/β complex. The structure was reconstructed with C1 symmetry, and all the representative segments are thus presented. Contour levels are set at 5σ except S-palmitoyl modification on the cysteine side chain which is at 3σ .



Supplementary Fig. 6 Western blot analysis of wild-type and mutant OSTα/β protein expression.

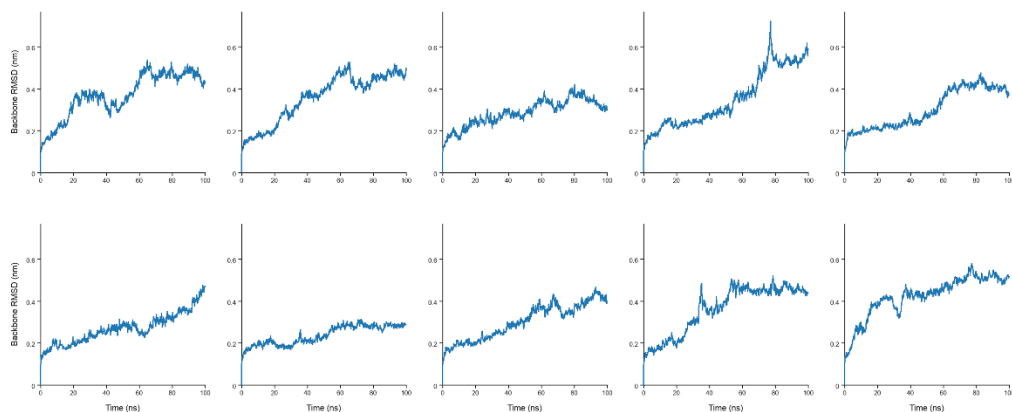
a-f. Western blot analysis assessing expression of wild-type OSTα/β and indicated mutant constructs. PVDF membranes were cut at the 72 kDa molecular weight marker; the upper portion was probed with mouse anti-ATP1A1 antibody as membrane loading control, and the lower portion was incubated with anti-FLAG antibody to detect FLAG-tagged OST variants.



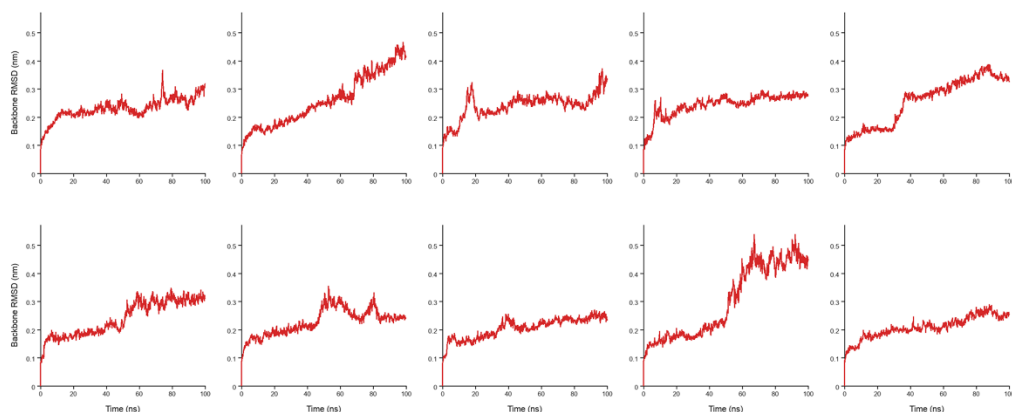
Supplementary Fig. 7 Molecular dynamics simulations validate the lateral transport of substrates.

- a.** Plumbed restraint-potential profiles for a TC molecule traversing the central cavity or lateral surface groove. The restraint potential was calculated as $U = \frac{1}{2}k(z - z_{\text{target}})^2$, and the corresponding response force was obtained from its derivative, $dU/dz = k(z - z_{\text{target}}) = -F_z$.
- b.** z-coordinate trajectories of TC during SMD traversal through the central cavity or lateral surface groove.

a

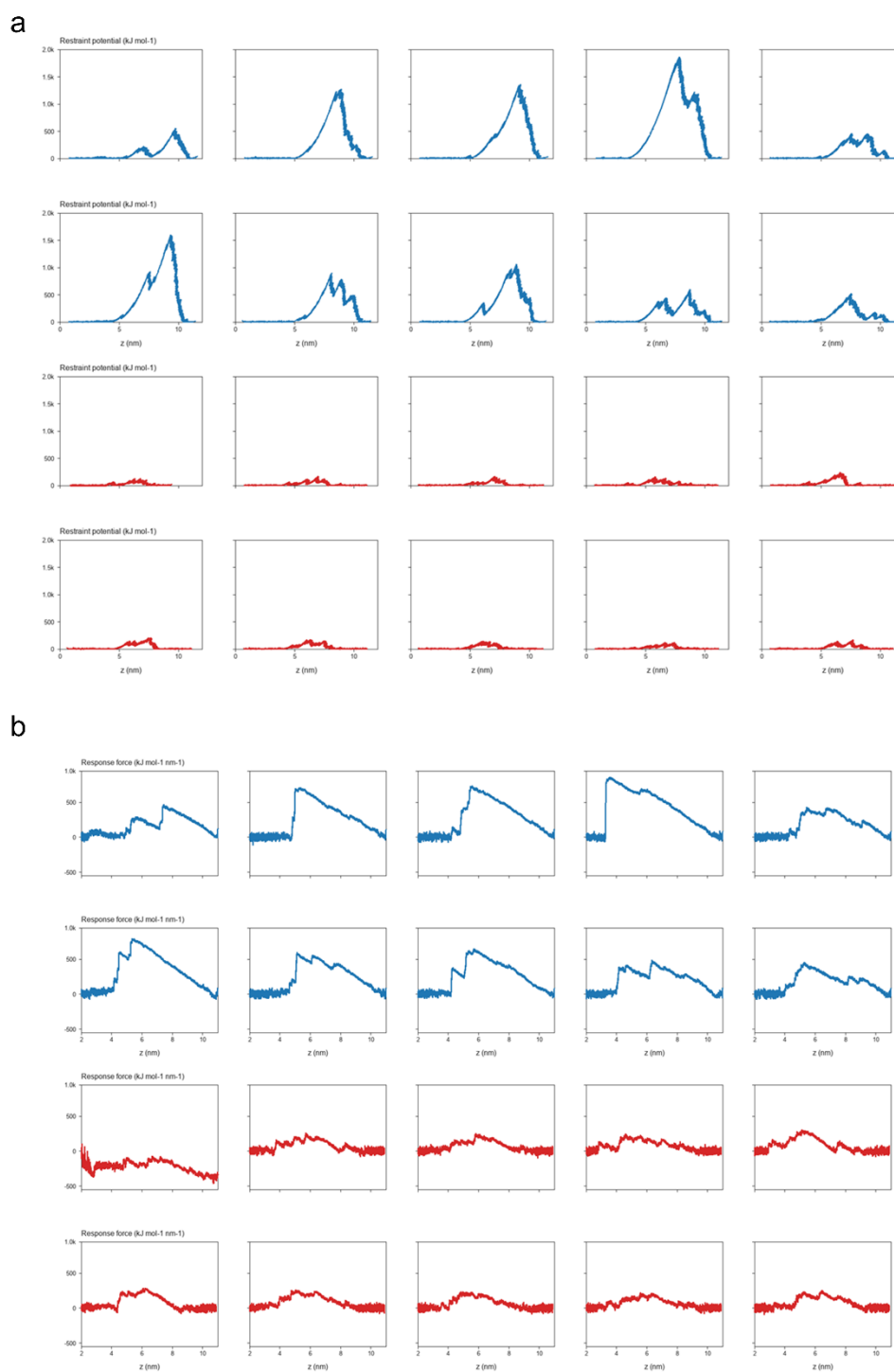


b



Supplementary Fig. 8 Backbone RMSD profiles during the SMD simulation for the 20 independent trajectories.

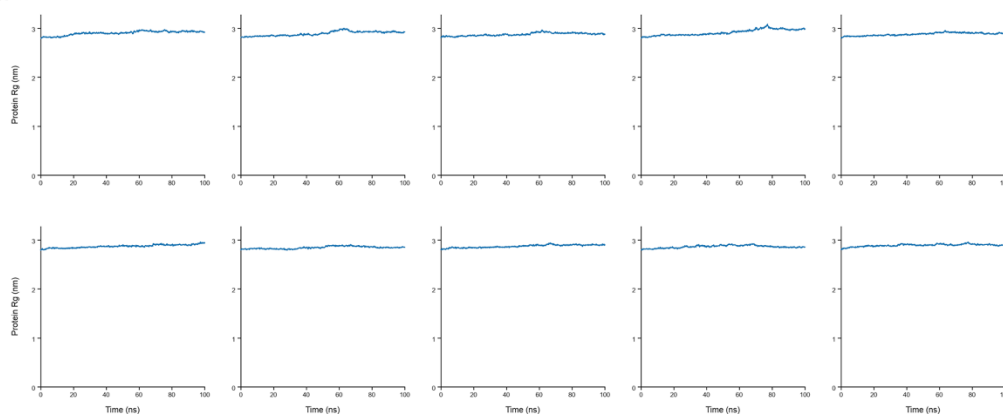
Backbone RMSD profiles during the SMD simulation for the 20 independent trajectories, comprising 10 central-cavity(**a**) and 10 lateral-groove(**b**) replicates. Each panel represents one replicate and is used to assess structural stability before the SMD pulling phase.



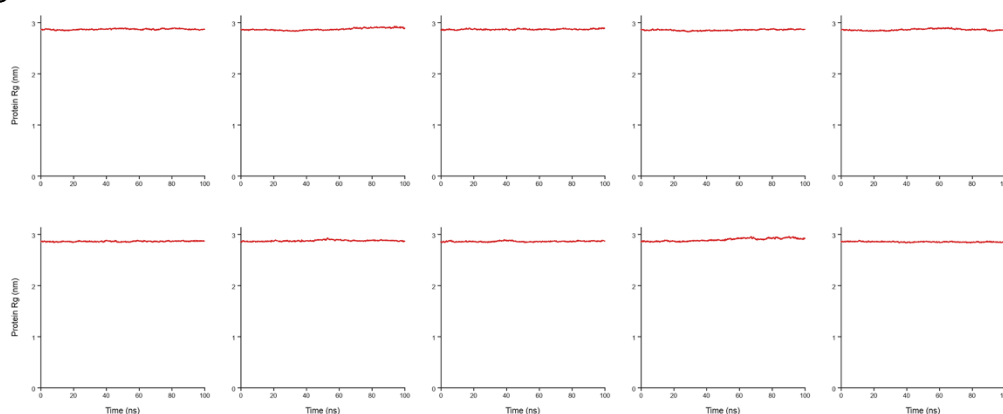
Supplementary Fig. 9 Restraint-potential profiles and Response-force profiles for the 20 independent SMD trajectories.

Restraint-potential profiles(**a**) and Response-force profiles(**b**) for the 20 independent SMD trajectories, comprising 10 central-cavity(blue) and 10 lateral-groove(red) replicates.

a

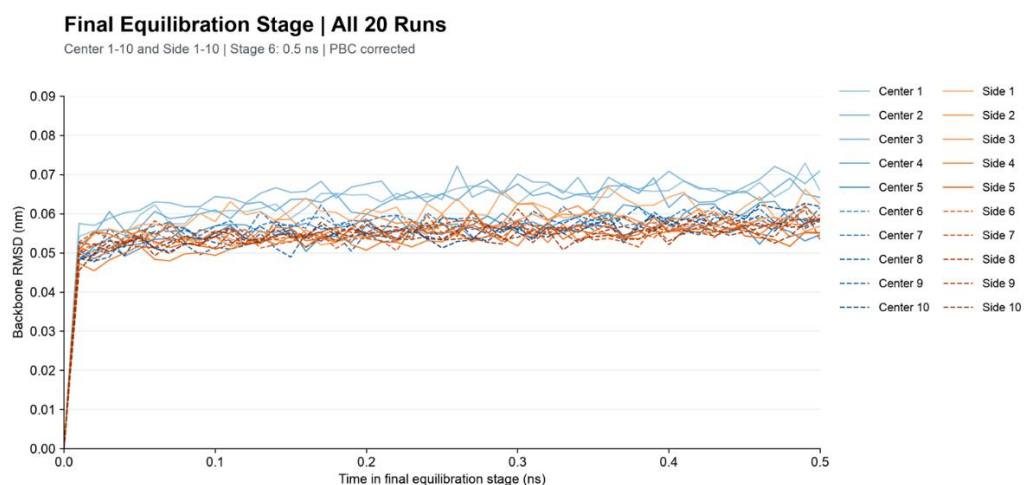


b

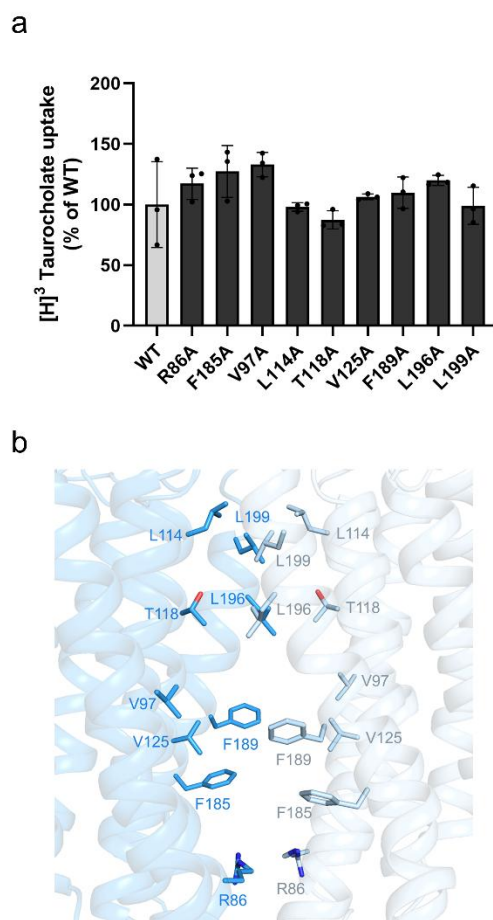


Supplementary Fig. 10 Radius-of-gyration profiles during the SMD simulation for the 20 independent trajectories.

Radius-of-gyration profiles during the SMD simulation for the 20 independent trajectories, comprising 10 central-cavity(**a**) and 10 lateral-groove(**b**) replicates. The Rg trajectories were used to monitor the overall compactness of the protein system and to evaluate its structural stability before SMD.



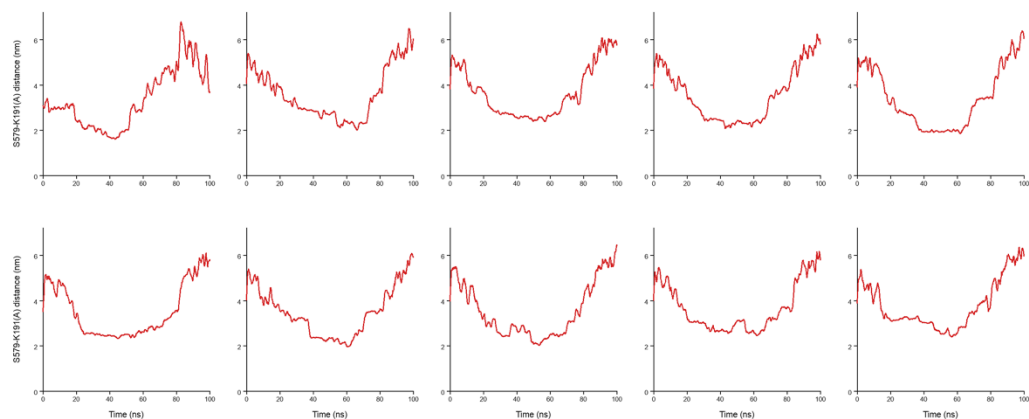
Supplementary Fig. 11 Backbone RMSD during the final 0.5 ns of equilibration for 10 center and 10 side replicates. Each trajectory was aligned to its own first frame after PBC correction. All recorded frames are shown without smoothing.



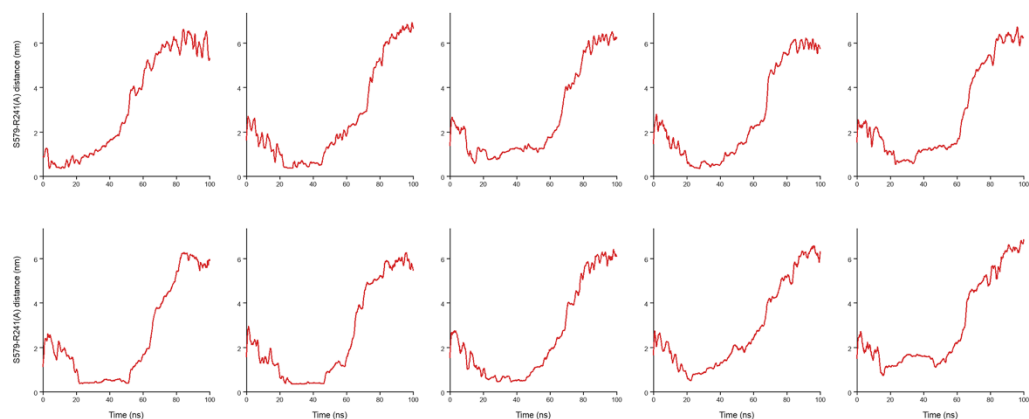
Supplementary Fig. 12 Transport activities of wild-type OST α/β complex and OST α/β mutants at central channel residues.

a. Transport activities of [³H] taurocholic acid mediated by the wild-type OST α/β complex and OST α/β mutants at central channel residues. The activity of the wild type was set to 100%. Each bar represents the average of three independent experiments ($n = 3$), and the error bars represent the means $\pm$ standard deviations (SDs). One - way analysis of variance (ANOVA) is used for the comparison of statistical significance of wild-type OST α/β (WT) with its mutants. The P values of < 0.05 , 0.01 , and 0.001 are indicated with *, **, and ***, respectively. **b.** Residue positions in the central cavity chosen for site-directed mutagenesis and functional transport assays.

a



b



Supplementary Fig. 13 Distance profiles between the sulfur atom of the PLUMED-manipulated TCH579 molecule (S579) and the nitrogen of side chain of K191 and the nitrogen of side chain of R241 during TC transport along the lateral surface groove.

Distance profiles between the sulfur atom of the PLUMED-manipulated TCH579 molecule (S579) and the nitrogen of side chain of K191(**a**) and the nitrogen of side chain of R241(**b**) during TC transport along the lateral surface groove. The profiles were calculated for the 10 side replicates and show the time-dependent changes in these local contacts as TCH579 progresses through the groove.

Supplementary Table 1 Cryo-EM data collection, refinement and validation statistics.

	apo OST α/β	TC bound OST α/β
Magnification	105,000	105,000
Voltage (kV)	300	300
Electron exposure (e-/Å ²)	55	55
Defocus range (μm)	-2.3 to -1.3	-2.3 to -1.3
Pixel size (Å)	0.82	0.82
Symmetry imposed	C2	C1
Initial particle images (no.)	16,533,781	9,182,263
Final particle images (no.)	954,511	208,996
Map resolution (Å)	2.68	3.21
FSC threshold	0.143	0.143
Map sharpening B factor (Å ²)	-129.7	-117.7
Refinement		
Initial model used (PDB code)	AlphaFold 3	
Model resolution (Å)	2.68	3.21
FSC threshold	0.143	0.143
Model composition		
Nonhydrogen atoms	5728	5702
Protein residues	734	722
Ligands	None	TC
<i>B</i> factors (Å ²)		
Protein	42.695	65.22
Ligand	None	20.00
R.m.s. deviations		
Bond lengths (Å)	0.005	0.007
Bond angles (°)	0.983	1.105
Validation		
MolProbity score	1.98	1.87
Clashscore	10.75	13.92
Poor rotamers (%)	3.91	1.11
Ramachandran plot		
Favored (%)	96.83	96.92
Allowed (%)	3.17	3.08
Disallowed (%)	0	0

Supplementary Table 2 Composition and box dimensions of the center and side pathway systems

Parameter	Center pathway	Side pathway
Initial box dimensions (nm) ^a	16.401 × 16.401 × 14.124	16.886 × 16.886 × 12.591
Box geometry	Orthorhombic	Orthorhombic
Total number of atoms	358,758	337,450
Water model	TIP3P	TIP3P
Number of water molecules	83,668	74,492
Nominal salt concentration ^b	0.15 M KCl	0.15 M KCl
Potassium ions	229	204
Chloride ions	246	220
POPC molecules	572	616
Cholesterol molecules	242	246
DLPA molecules	2	2
Total lipid molecules including cholesterol	816	864
Taurocholic acid	1	2
Net system charge (elementary charges)	0	0
Box x = y after re-equilibration (nm) ^c	15.891-15.934	16.277-16.305
Box z after re-equilibration (nm) ^c	13.755-13.863	12.282-12.325

^a Box lengths are reported as x × y × z before equilibration.

^b Nominal KCl concentration; ion counts include ions added for charge neutralization.

^c Ranges across the final equilibration frames of seven newly re-equilibrated replicas per channel, not the historical SMD trajectories.

All counts are per simulation box. POPC, palmitoyloleoyl phosphatidylcholine; DLPA, dilauroyl phosphatidic acid.

References

1. Abramson, J. et al. Structure and mechanism of the lactose permease of Escherichia coli. *Science* **301**, 610-5 (2003).
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3. Ruprecht, J.J. et al. Structures of yeast mitochondrial ADP/ATP carriers support a domain-based alternating-access transport mechanism. *Proc Natl Acad Sci U S A* **111**, E426-34 (2014).
4. Han, L. et al. Molecular mechanism of substrate recognition and transport by the AtSWEET13 sugar transporter. *Proc Natl Acad Sci U S A* **114**, 10089-10094 (2017).