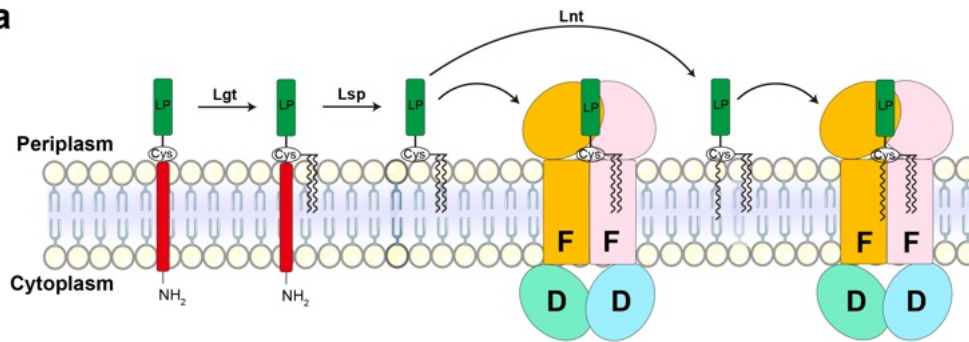
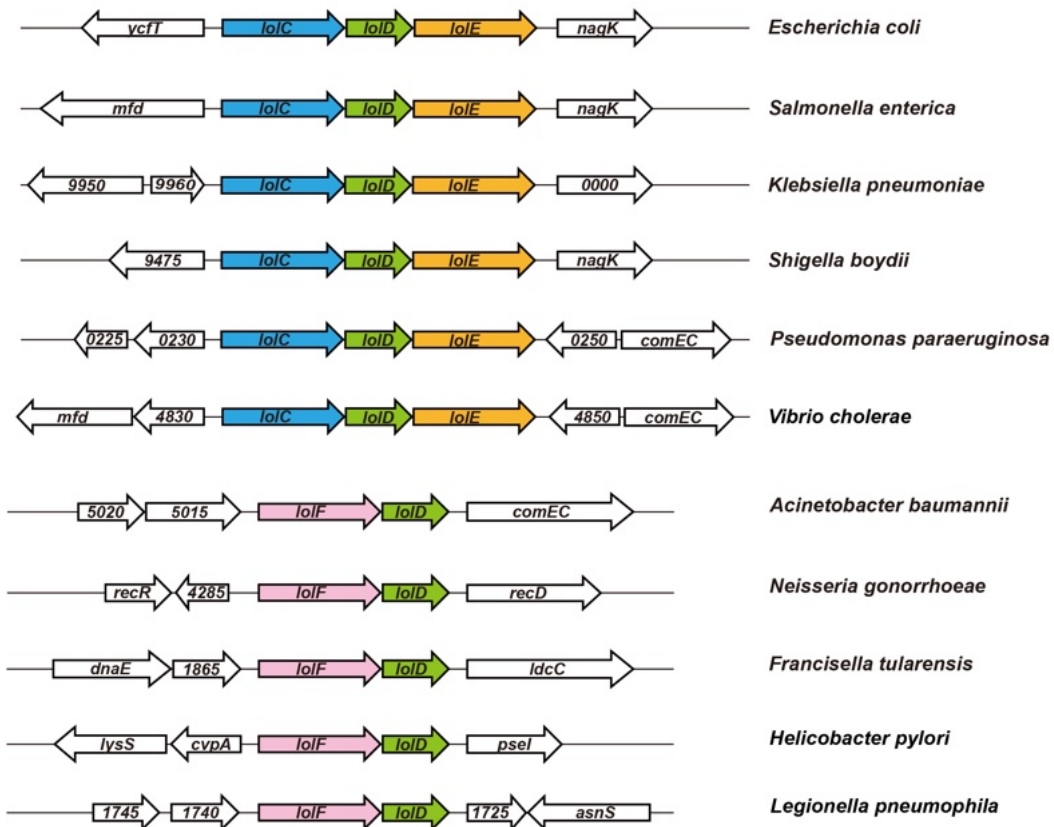
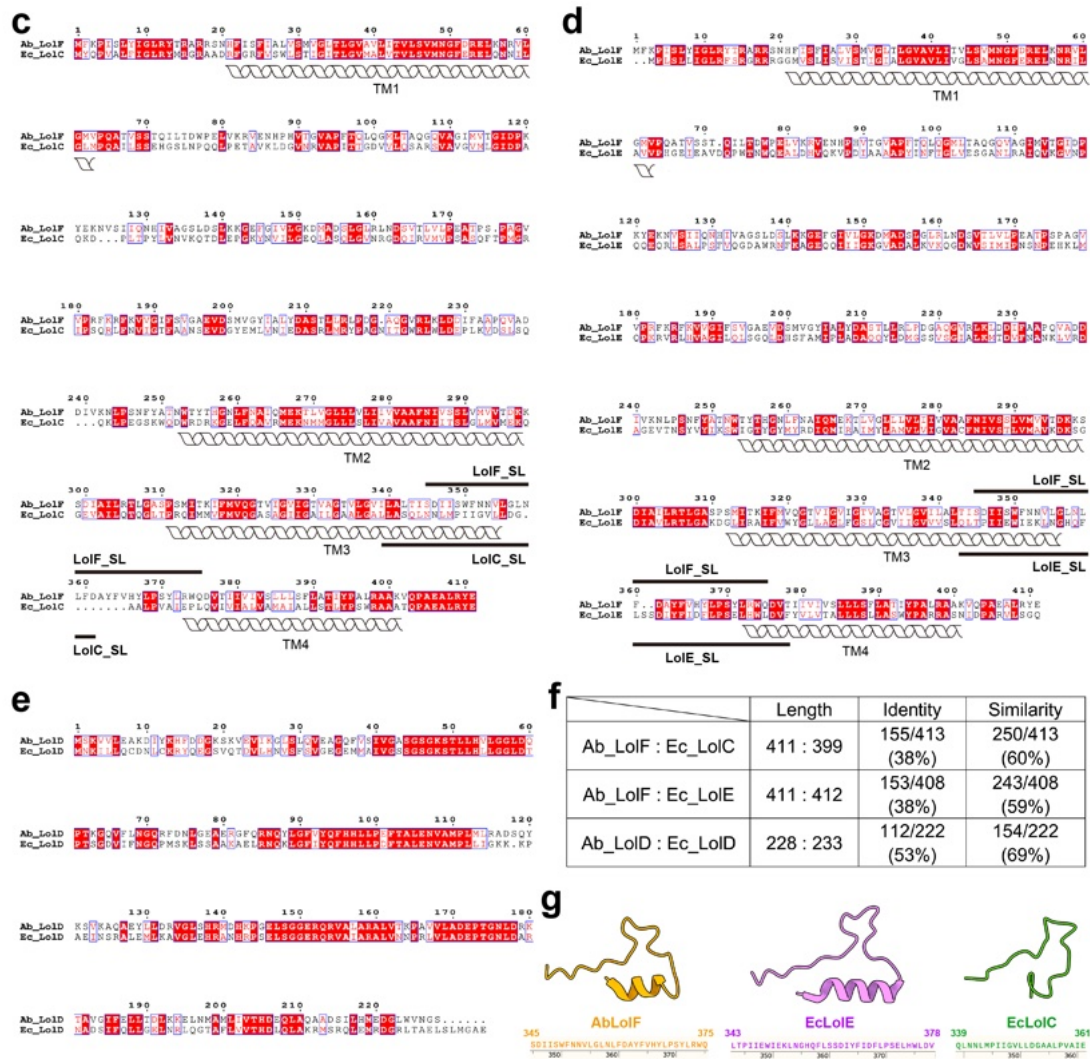


**a**

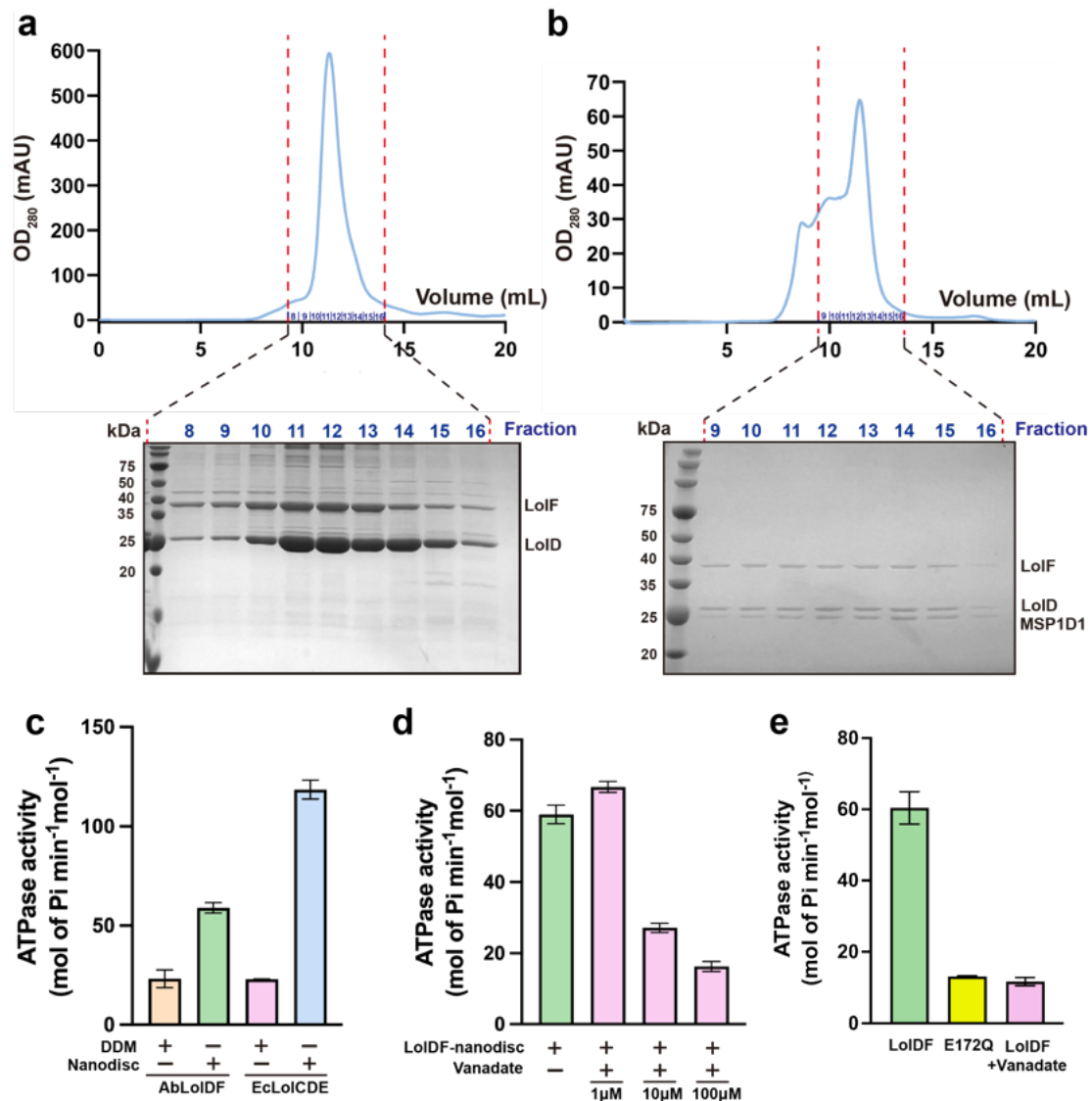


**b**

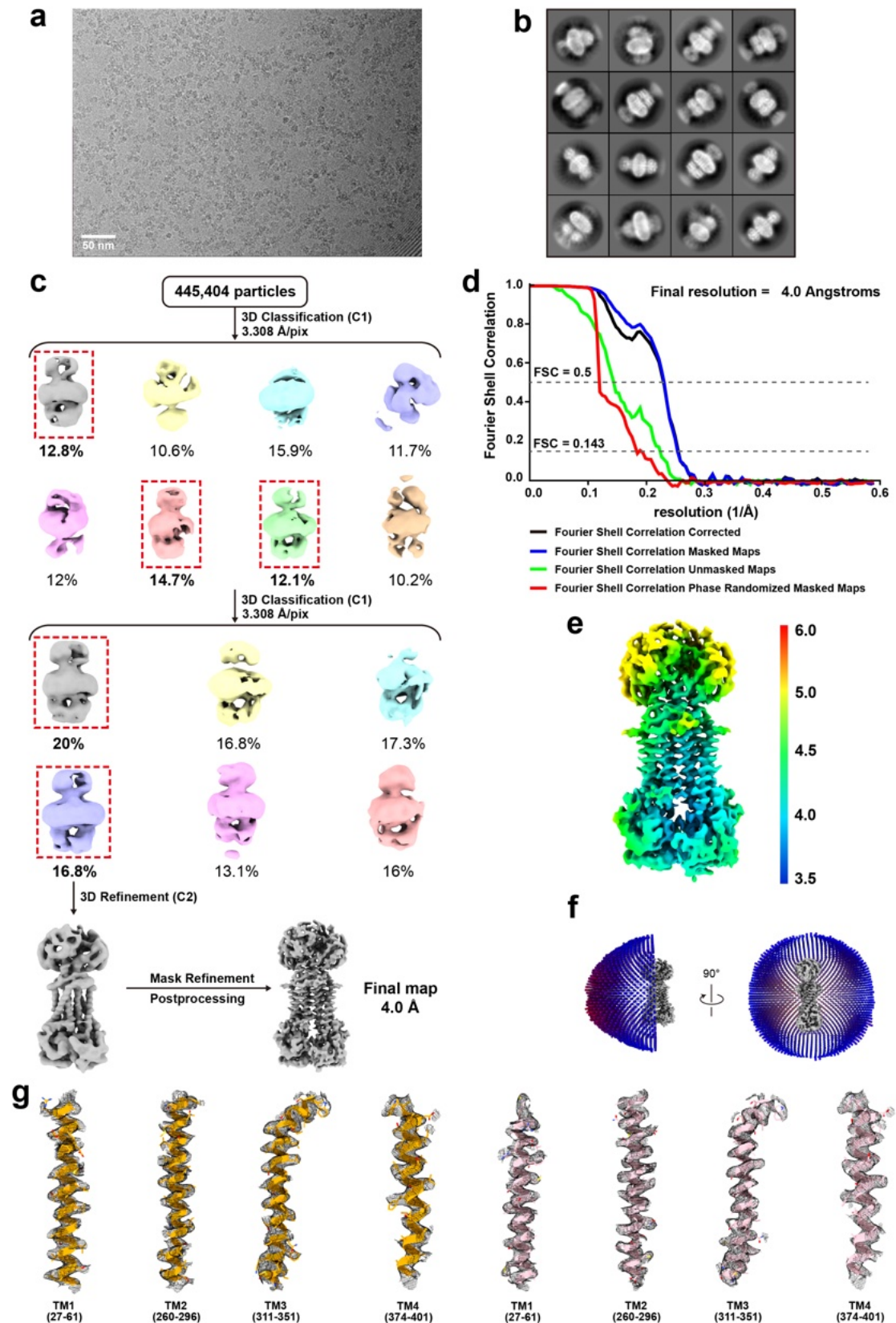




**Supplementary Figure 1 | Diagram of lipoprotein biogenesis and sequence alignment of *Lol* proteins.** **a**, Diagram of lipoprotein biogenesis in the inner membrane, including three enzymatic processing steps by prolipoprotein diacylglyceryl transferase (Lgt), lipoprotein signal peptidase (Lsp) and phospholipid/apolipoprotein transacylase (Lnt). The three lipoprotein-specific enzymes (Lgt, Lsp and Lnt) are essential for growth of the model organism, *Escherichia coli*. However, *Acinetobacter baumannii* can tolerate Lnt deletion to recognize diacylated as well as triacylated lipoproteins and to transfer either type of substrate to LolA for sorting to the OM by LolDF. **b**, Organization of *lol* genes in Gram-negative bacteria. **c**, The sequences of *E. coli* LolC and *A. baumannii* LolF were aligned, and the amino acid sequences corresponding to the four transmembrane helices were identified below. **d**, The sequences of *E. coli* LolE and *A. baumannii* LolF were aligned, and the amino acid sequences corresponding to the four transmembrane helices were identified below. **e**, Sequence alignment of LolD from *E. coli* and *A. baumannii*. **f**, Length, identity and similarity comparison of Lol proteins from *E. coli* and *A. baumannii*. **g**, Structure and sequence comparison of SL regions of *E. coli* LolC/LolE and *A. baumannii* LolF.

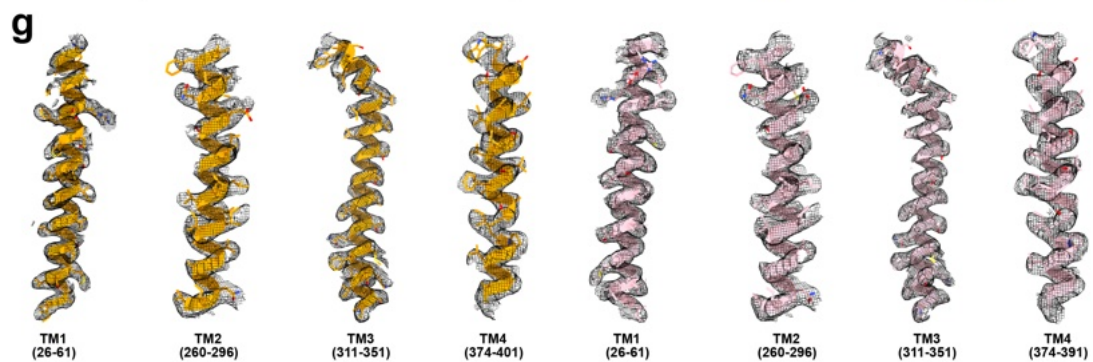
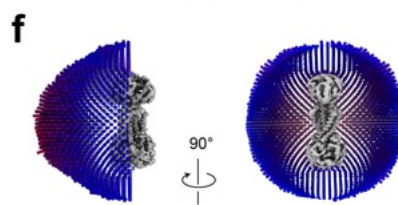
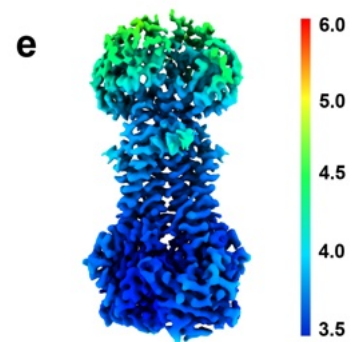
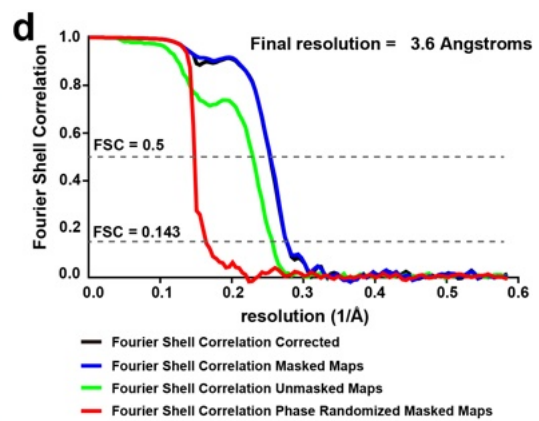
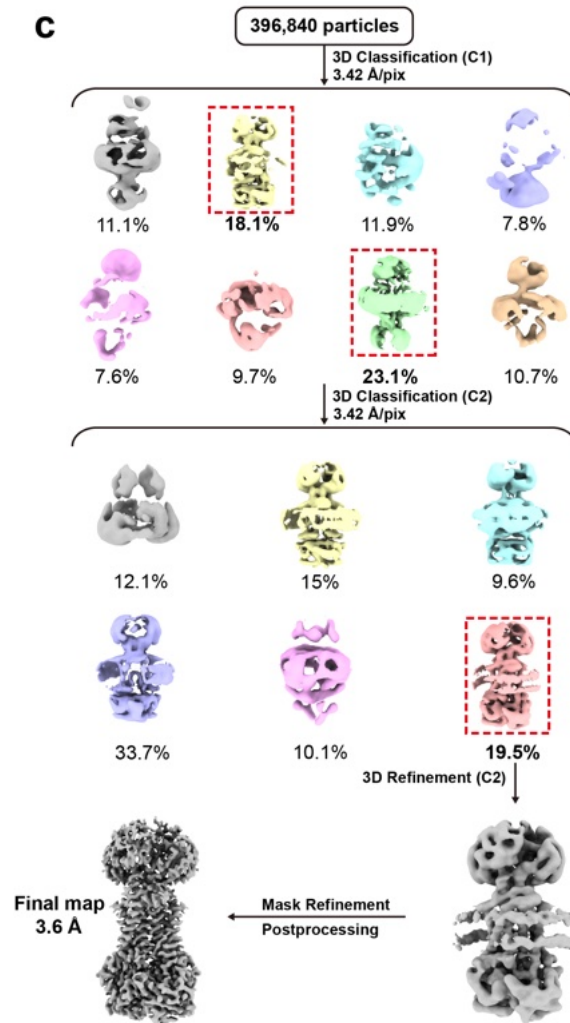
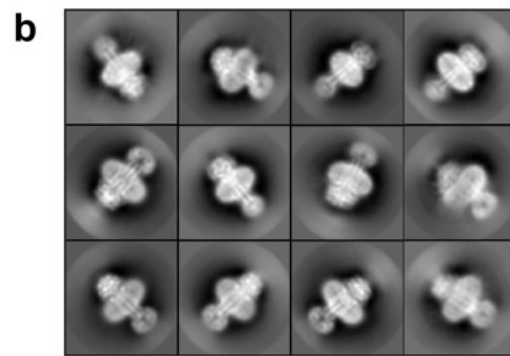
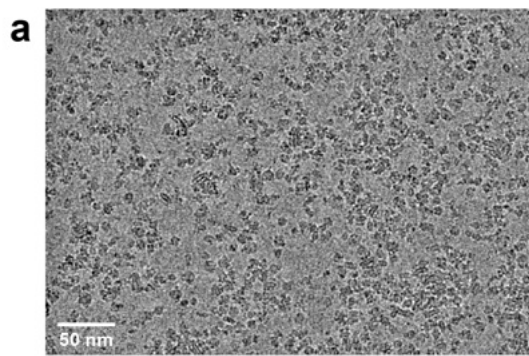


**Supplementary Figure 2 | Purification and biochemical characterization of AbLolDF.** **a**, Size-exclusion chromatography profile of AbLolDF in DDM and SDS-PAGE of the peak fraction shown in inset. **b**, Size-exclusion chromatography profile of AbLolDF in nanodiscs and SDS-PAGE of the peak fraction shown in inset. **c**, ATPase activity of AbLolDF and EcLolCDE in DDM and nanodiscs. Each data point is the average of three measurements, and error bars represent the s.d. **d**, Inhibition of ATPase activity of nanodisc-embedded LolDF with increasing concentrations of vanadate. Each data point is the average of three measurements, and error bars represent the s.d. of three measurements in one experiment. **e**, ATPase activity of LolDF in nanodiscs, the catalytically inactive E172Q mutant, and in nanodiscs with 100 μM vanadate. Each bar represents the mean of three independent measurements, and error bars represent the s.d. of three measurements in one experiment.

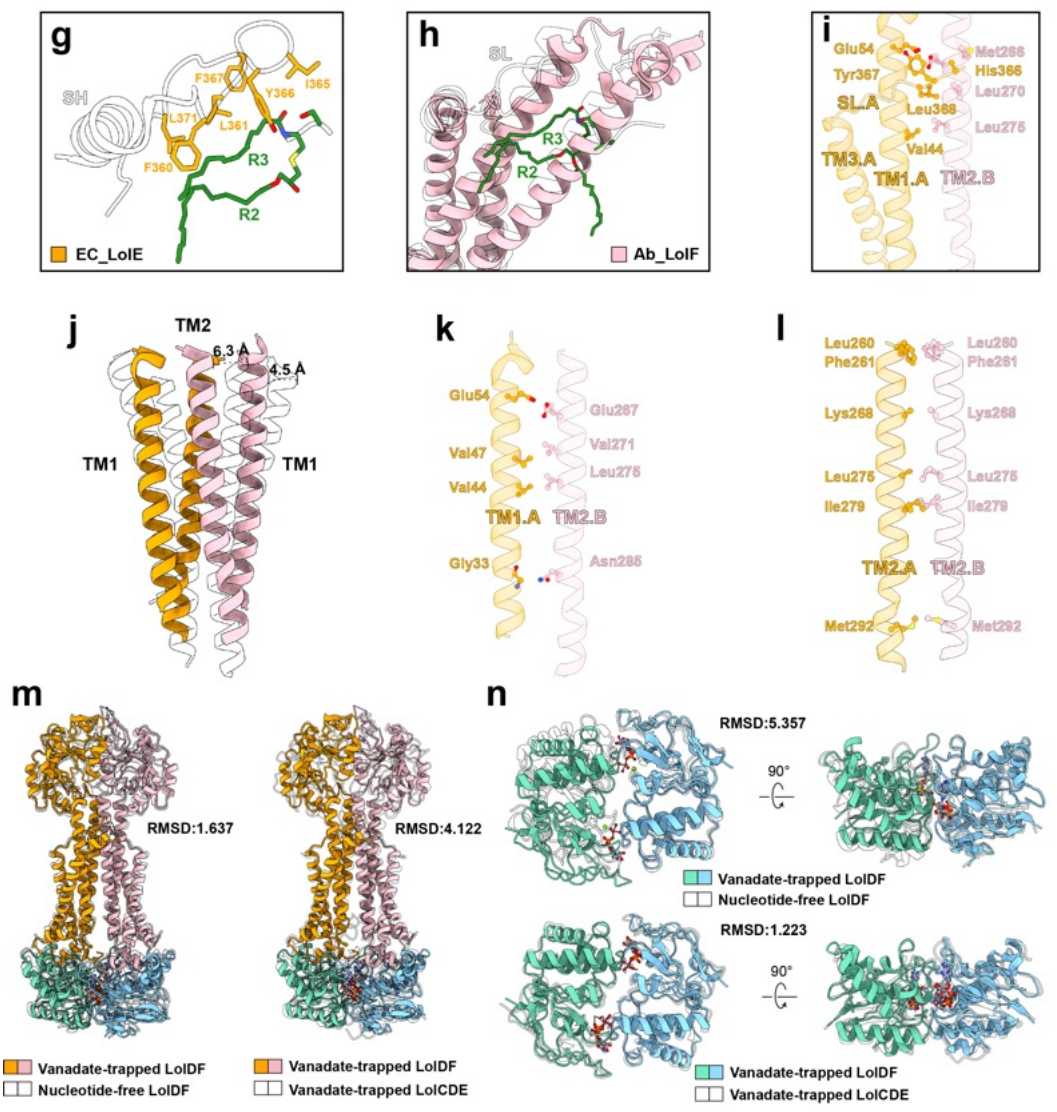
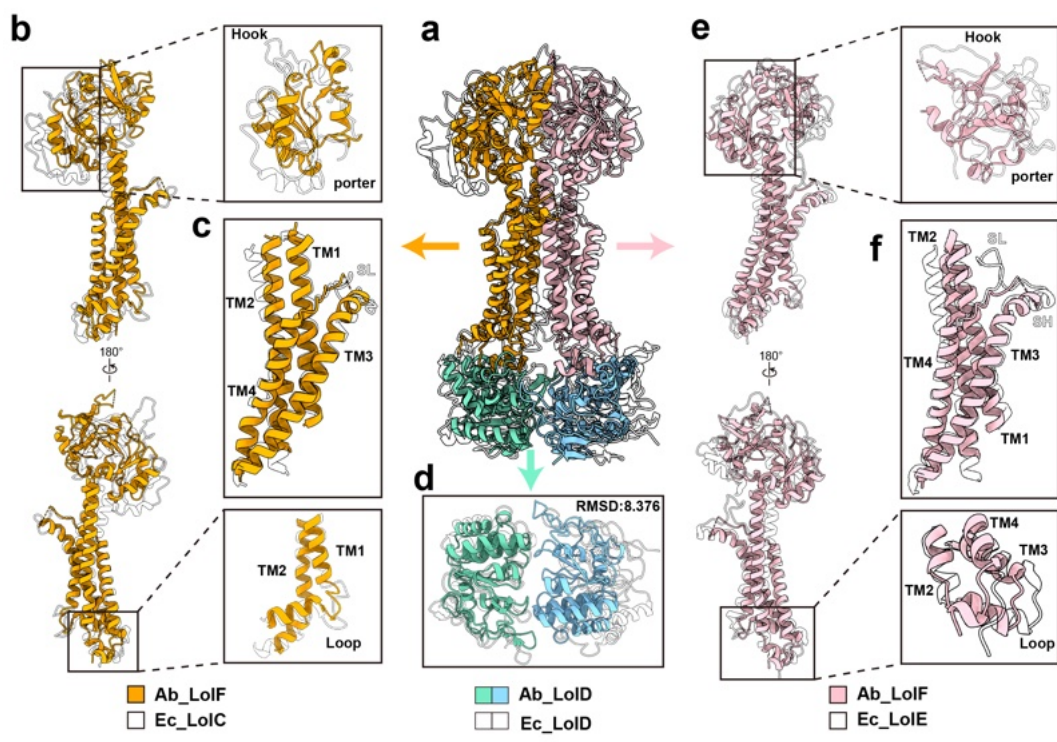


**Supplementary Figure 3 | Single-particle cryo-EM study of nucleotide-free LolDF in nanodiscs.** **a**, Representative cryo-EM image of nucleotide-free LolDF. **b**, Two-dimensional class averages of cryo-EM particle images. Box size is 256 Å. **c**, Three-dimensional

classification and refinement of cryo-EM particle images. **d**, Fourier shell correlation (FSC) curves: Corrected FSC curve (black), masked FSC curve (blue), unmasked FSC curve (green), and phase-randomized masked FSC curve (red). The final overall resolution is estimated based on the gold-standard FSC = 0.143 criterion. **e**, Local resolution of the final cryo-EM map. **f**, Angular distribution of particle images included in the final 3D reconstruction. **g**, Superimposition of cryo-EM densities and the model of selected regions.

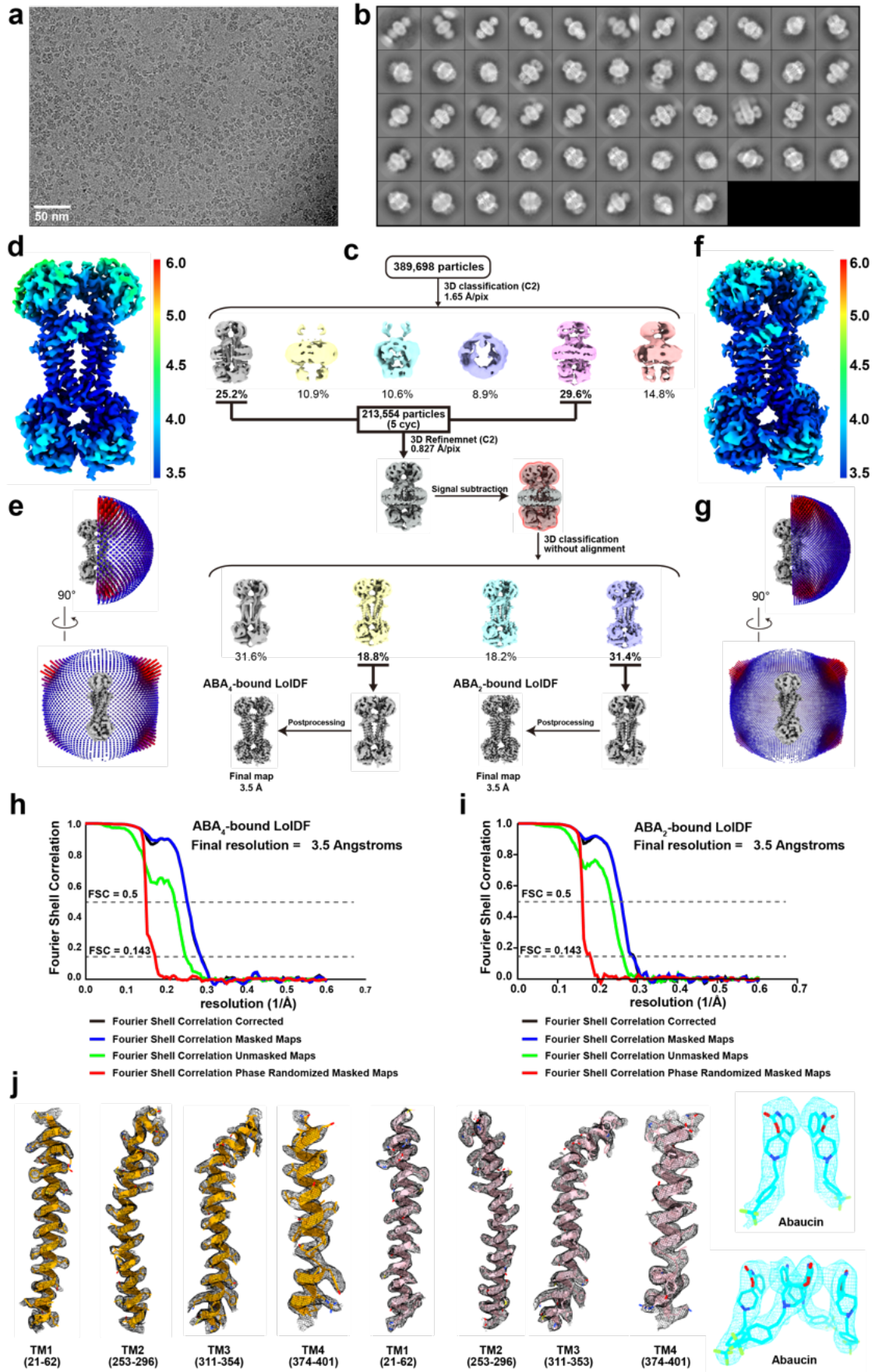


**Supplementary Figure 4 | Single-particle cryo-EM study of vanadate-trapped LolDF in nanodiscs.** **a**, Representative cryo-EM image of vanadate-trapped LolDF. **b**, Two-dimensional class averages of cryo-EM particle images. Box size is 256 Å. **c**, Three-dimensional classification and refinement of cryo-EM particle images. **d**, Fourier shell correlation (FSC) curves: Corrected FSC curve (black), masked FSC curve (blue), unmasked FSC curve (green), and phase-randomized masked FSC curve (red). The final overall resolution is estimated based on the gold-standard FSC = 0.143 criterion. **e**, Local resolution of the final cryo-EM map. **f**, Angular distribution of particle images included in the final 3D reconstruction. **g**, Superimposition of cryo-EM densities and the model of selected regions.

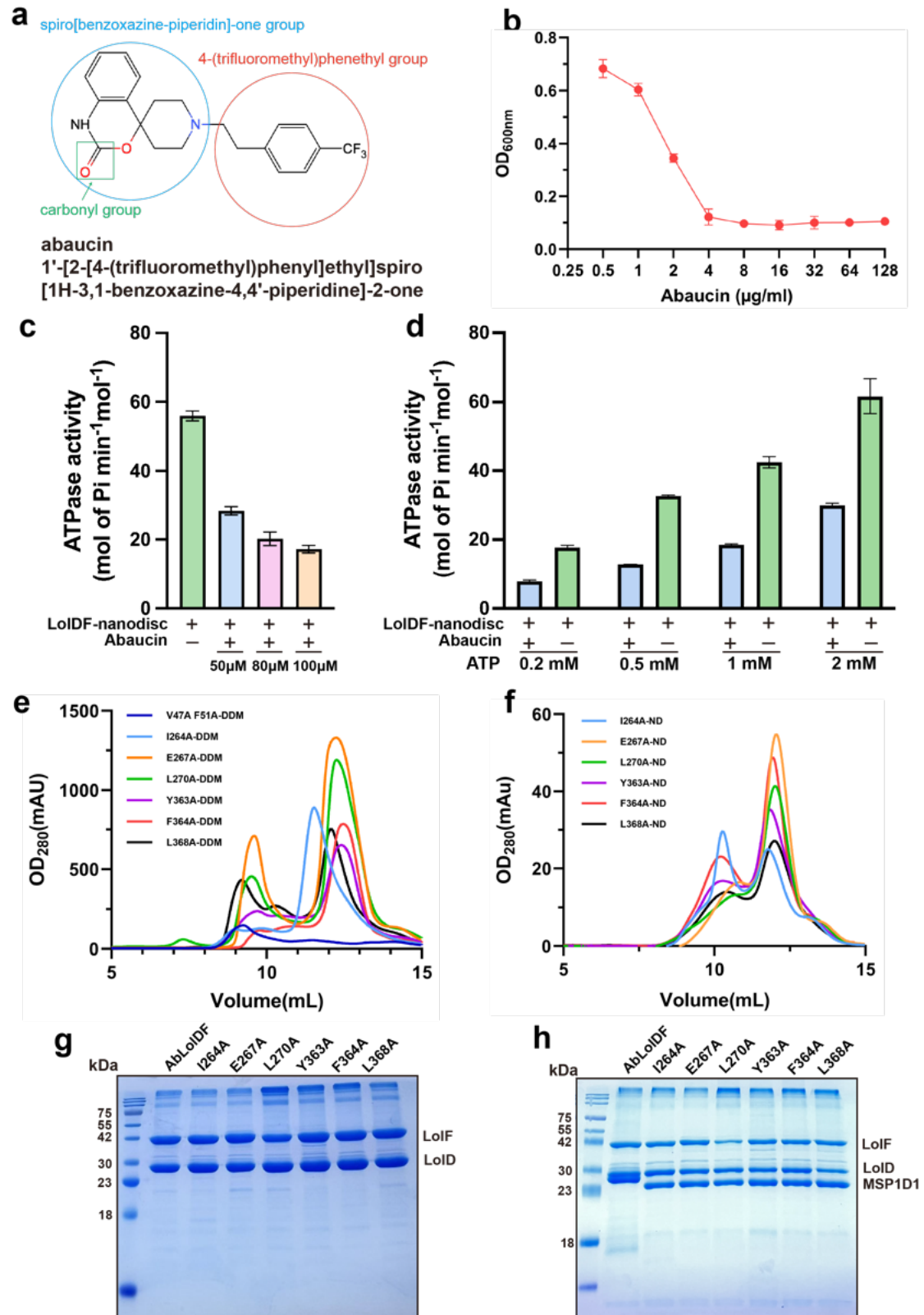


**Supplementary Figure 5 | Comparison of structure between EcLolCDE and AbLolDF. a,**

Comparison of the overall structure of nucleotide-free EcLolCDE and AbLolDF. AbLolDF is represented by colors, and EcLolCDE is shown as transparent outlines. **b,** Comparison of the structure of EcLolC and AbLolF. AbLolF is represented in orange, and EcLolC is shown as transparent contours. The expanded solid wireframe represents the comparison of PD domain between EcLolC and AbLolF. **c,** TMD comparison of EcLolC and AbLolF. **d,** NBD comparison of EcLolD and AbLolD. **e,** Comparison of the overall structure of EcLolE and AbLolF. AbLolF is represented in pink, and EcLolE is represented in transparent contours. The expanded solid wireframe represents the comparison of PD domain between EcLolE and AbLolF. **f,** TMD comparison of EcLolE and AbLolF. **g,** The shoulder loop (SL) interactions with lipoprotein in the structure of EcLolE. **h,** Comparison of the AbLolF and EcLolE in the region of the shoulder loop (SL), as indicated in (f), predicted binding with lipoprotein. **i,** The inter-subunit interaction between TM2 and opposing TM2/TM3 in nucleotide-free LolDF. The side chains of the subunits involved in hydrophobic interactions are displayed in ball & stick representation. Subunit names are labeled in the same color as their respective TMs. **j,** Comparison of the TM1 and TM2 of AbLolDF and EcLolCDE, the TM1 and TM2 of LolF shift by  $\sim 4.5$  Å and  $\sim 6.3$  Å, respectively. **k,** The inter-subunit interaction between two TM2 in nucleotide-free LolDF. **l,** The inter-subunit interaction between TM1 and opposing TM2 in nucleotide-free LolDF. **m,** Comparison of the overall structures of vanadate-trapped LolDF, nucleotide-free LolDF and vanadate-trapped LolCDE, vanadate-trapped LolDF is represented by colors, while nucleotide-free LolDF and vanadate-trapped LolCDE are shown as transparent outlines. **n,** Comparison of the NBDs of vanadate-trapped LolDF, nucleotide-free LolDF and vanadate-trapped LolCDE, the NBD of vanadate-trapped LolDF is represented by colors, while the NBDs of nucleotide-free LolDF and vanadate-trapped LolCDE are shown as transparent outlines.

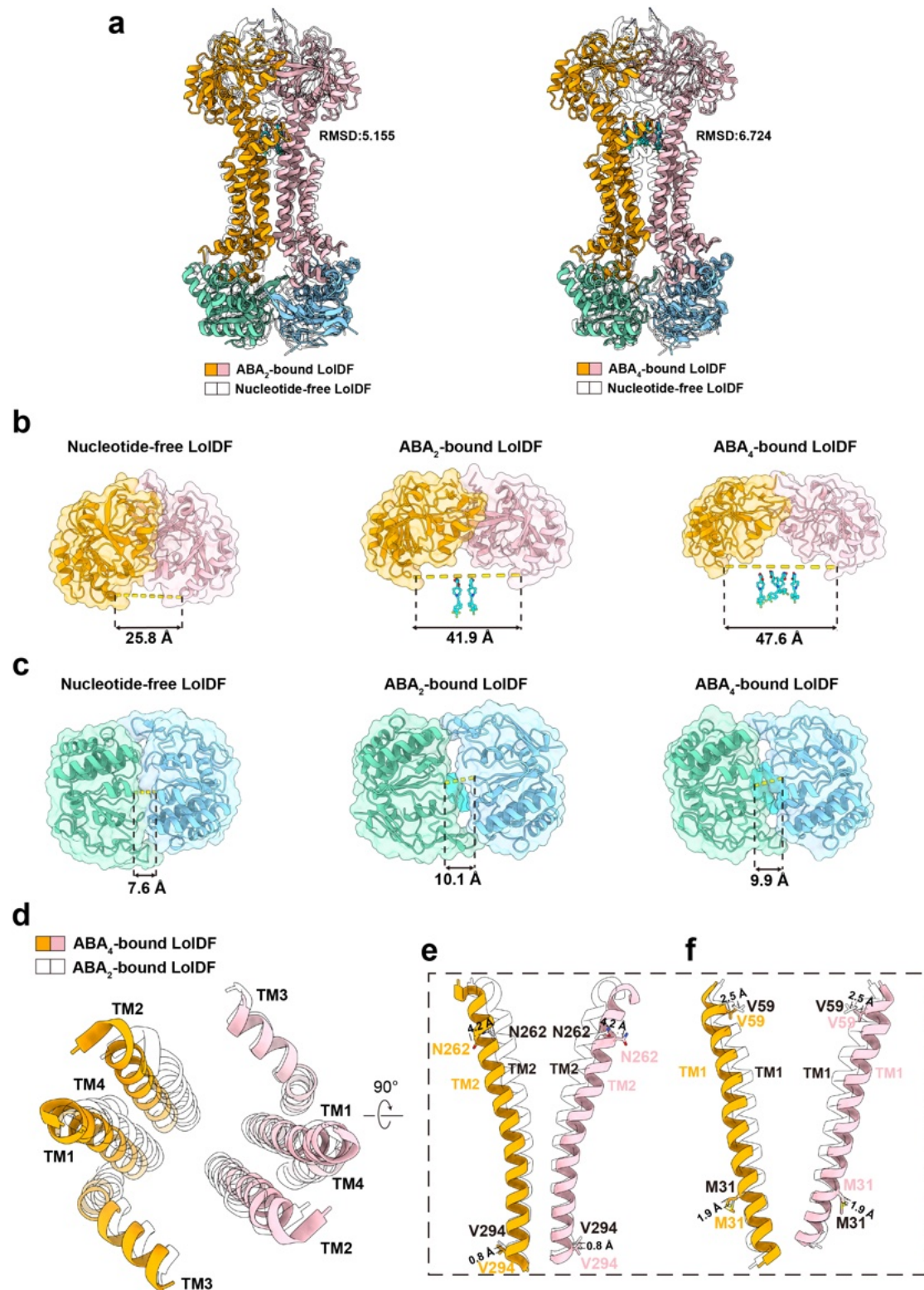


**Supplementary Figure 6 | Single-particle cryo-EM study of ABA-bound LolDF in nanodiscs.** **a**, Representative cryo-EM image of ABA-bound LolDF. **b**, Two-dimensional class averages of cryo-EM particle images. Box size is 256 Å. **c**, Three-dimensional classification and refinement of cryo-EM particle images. **d**, Local resolution of the ABA<sub>2</sub>-bound LolDF final cryo-EM map. **e**, Angular distribution of particle images included in the final 3D reconstruction of ABA<sub>2</sub>-bound LolDF. **f**, Local resolution of the ABA<sub>4</sub>-bound LolDF final cryo-EM map. **g**, Angular distribution of particle images included in the final 3D reconstruction of ABA<sub>4</sub>-bound LolDF. **h**, Fourier shell correlation (FSC) curves of ABA<sub>2</sub>-bound LolDF: Corrected FSC curve (black), masked FSC curve (blue), unmasked FSC curve (green), and phase-randomized masked FSC curve (red). The final overall resolution is estimated based on the gold-standard FSC = 0.143 criterion. **i**, Fourier shell correlation (FSC) curves of ABA<sub>4</sub>-bound LolDF: Corrected FSC curve (black), masked FSC curve (blue), unmasked FSC curve (green), and phase-randomized masked FSC curve (red). The final overall resolution is estimated based on the gold-standard FSC = 0.143 criterion. **j**, Superimposition of cryo-EM densities and the model of ABA<sub>2</sub>-bound LolDF and ABA<sub>4</sub>-bound LolDF.



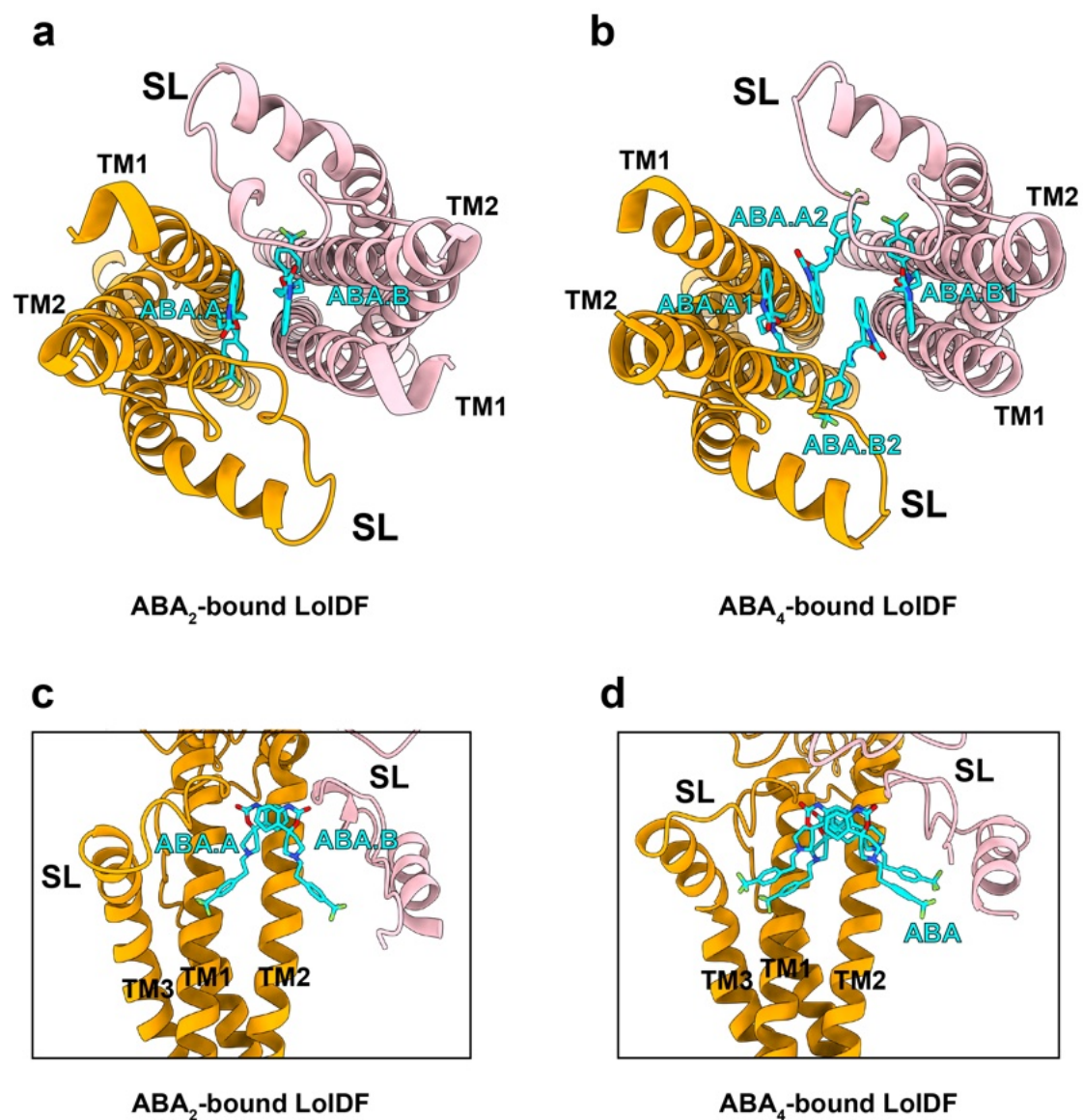
**Supplementary Figure 7 | Biochemical characterization of LolDF mutants.** **a**, The chemical structure of abaucin. **b**, Growth inhibitory effect of abaucin in *A. baumannii*. Each point represents mean  $\pm$  s.d. of three separate measurements. **c**, ATPase activities of LolDF in the

presence of abaucin. Each point represents mean  $\pm$  s.d. of three separate measurements. **d**, ATPase activities of LolDF with 50  $\mu$ M abaucin and different concentrations of ATP. Each point represents mean  $\pm$  s.d. of three separate measurements. **e**, Size-exclusion chromatography profile of LolDF mutants in DDM. This experiment was repeated three times independently with similar results. **f**, Size-exclusion chromatography profile of LolDF mutants in nanodiscs. This experiment was repeated three times independently with similar results. **g**, SDS-PAGE of LolDF mutants in DDM. **h**, SDS-PAGE of LolDF mutants in nanodiscs.

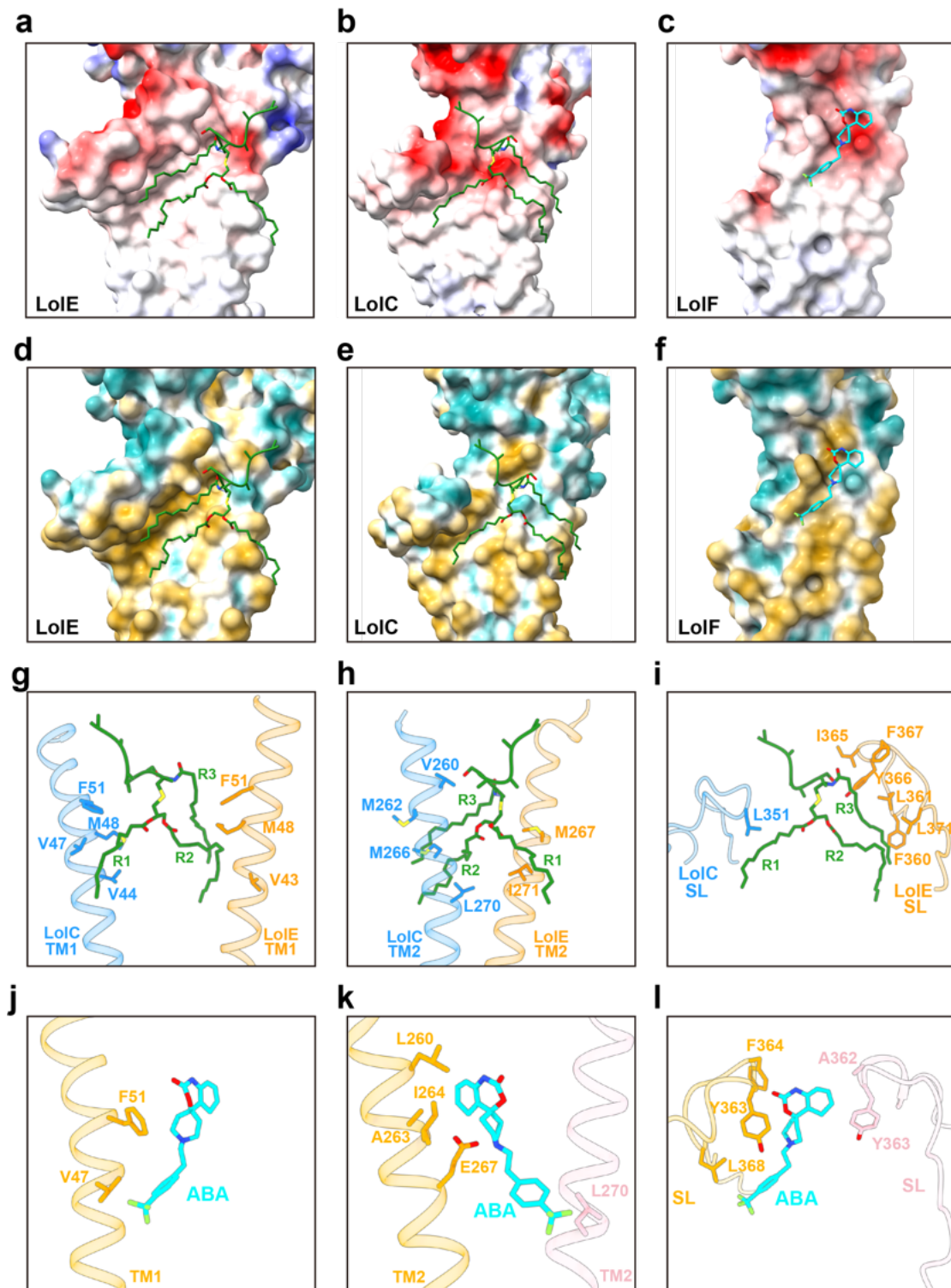


**Supplementary Figure 8 | Overall comparison of AbLolDF structures.** **a**, Overall comparisons of ABA-bound LolDF with nucleotide-free LolDF. Nucleotide-free LolDF is uniformly represented by transparent ribbons, ABA<sub>2</sub>-bound LolDF and ABA<sub>4</sub>-bound LolDF are displayed in the same color scheme. **b**, Rotation and movement of the periplasmic domain

(PD) of LolDF, and the distance between the two LolDs is determined based on the distance between the two Phe231. **c**, Rotation and movement of the nucleotide binding domains (NBDs) of LolDF, and the distance between the two LolDs is determined based on the distance between the two His205. **d**, Cross-sectional view perpendicular to the membrane plane of ABA<sub>4</sub>-bound LolDF and ABA<sub>2</sub>-bound LolDF. ABA<sub>2</sub>-bound LolDF is uniformly represented by transparent ribbons, ABA<sub>4</sub>-bound LolDF is displayed in the color scheme. **e**, Comparison of TM2 from ABA<sub>4</sub>-bound LolDF and ABA<sub>2</sub>-bound LolDF. ABA<sub>2</sub>-bound LolDF is uniformly represented by transparent ribbons, ABA<sub>4</sub>-bound LolDF is displayed in the color scheme. **f**, Comparison of TM1 from ABA<sub>4</sub>-bound LolDF and ABA<sub>2</sub>-bound LolDF. ABA<sub>2</sub>-bound LolDF is uniformly represented by transparent ribbons, ABA<sub>4</sub>-bound LolDF is displayed in the color scheme.



**Supplementary Figure 9 | Comparison of AbLolDF structures.** **a-b**, Cross-sectional view comparisons of the inhibitor-bound pocket between the structures of ABA<sub>2</sub>-bound LolDF (**a**) and ABA<sub>4</sub>-bound LolDF (**b**). **c-d**, Vertical-sectional view comparisons of inhibitor-bound pockets between the structures of ABA<sub>2</sub>-bound LolDF (**c**) and ABA<sub>4</sub>-bound LolDF (**d**).



**Supplementary Figure 10 | Structural comparison of the AbLolDF abaucin-binding pocket and the EcLolCDE lipoprotein-binding pocket. a-c,** Electrostatic potential analysis of binding pockets between AbLolF and EcLolC/E, indicating areas of positive (blue) and negative (red) charge. **d-f,** Hydrophobic surface analysis of binding pockets between AbLolF and EcLolC/E. The hydrophilic and hydrophobic surfaces are shown in turquoise and goldenrod, respectively. **g-i,** Close-up views of the lipoprotein binding pocket in EcLolCDE,

with the TM1 (**g**), TM2 (**h**), and SL (**i**) regions shown separately. **j-l**, Close-up views of the abaucin binding pocket in AbLolDF, with the TM1 (**j**), TM2 (**k**), and SL (**l**) regions shown separately.

**Supplementary Table 1 | Statistics of structural analysis of AbLoIDF Cryo-EM maps**

|                                                    | Nucleotide-free<br>LoIDF<br>9W36 | Vanadate-trapped<br>LoIDF<br>9W37 | ABA <sub>2</sub> -bound<br>LoIDF<br>9W2W | ABA <sub>4</sub> -bound<br>LoIDF<br>9W35 |
|----------------------------------------------------|----------------------------------|-----------------------------------|------------------------------------------|------------------------------------------|
| <b>Data collection and processing</b>              |                                  |                                   |                                          |                                          |
| Magnification                                      | 105,000 ×                        | 105,000 ×                         | 105,000 ×                                | 105,000 ×                                |
| Voltage (kV)                                       | 300                              | 300                               | 300                                      | 300                                      |
| Electron exposure(e <sup>-</sup> /Å <sup>2</sup> ) | 50                               | 50                                | 50                                       | 50                                       |
| Defocus range (μm)                                 | -1.0 to -2.0                     | -1.0 to -2.0                      | -1.0 to -2.0                             | -1.0 to -2.0                             |
| Pixel size (Å)                                     | 0.827                            | 0.855                             | 0.827                                    | 0.827                                    |
| Symmetry imposed                                   | C2                               | C2                                | C2                                       | C2                                       |
| Initial particle images (no.)                      | 445,404                          | 396,840                           | 389,698                                  | 389,698                                  |
| Final particle images (no.)                        | 45,074                           | 48,502                            | 67,055                                   | 40,148                                   |
| Map resolution (Å)                                 | 4.0                              | 3.6                               | 3.5                                      | 3.5                                      |
| FSC threshold                                      | 0.143                            | 0.143                             | 0.143                                    | 0.143                                    |
| Map resolution range (Å)                           | 3.8-4.2                          | 3.1-3.7                           | 3.3-3.6                                  | 3.2-3.5                                  |
| <b>Refinement</b>                                  |                                  |                                   |                                          |                                          |
| Initial model used (PDB code)                      | 7MDX                             | 7MDY                              | 9W36                                     | 9W2W                                     |
| Model resolution (Å)                               | 4.2                              | 3.7                               | 3.6                                      | 3.5                                      |
| FSC threshold                                      | 0.143                            | 0.143                             | 0.143                                    | 0.143                                    |
| Map sharpening B factor (Å <sup>2</sup> )          | -189                             | -142                              | -164                                     | -130                                     |
| Model composition                                  |                                  |                                   |                                          |                                          |
| Non-hydrogen atoms                                 | 8,044                            | 8,636                             | 7,947                                    | 7,582                                    |
| Protein residues                                   | 1,220                            | 1,230                             | 1,245                                    | 1,247                                    |
| Ligands                                            | 0                                | 4                                 | 2                                        | 4                                        |
| B factors (Å <sup>2</sup> )                        |                                  |                                   |                                          |                                          |
| Protein                                            | 82.78                            | 55.82                             | 37.91                                    | 51.29                                    |
| Ligand                                             | N/A                              | 36.98                             | 29.51                                    | 48.84                                    |
| R.m.s deviations                                   |                                  |                                   |                                          |                                          |
| Bond lengths (Å)                                   | 0.002                            | 0.004                             | 0.002                                    | 0.005                                    |
| Bond angles (°)                                    | 0.630                            | 0.860                             | 0.513                                    | 0.639                                    |
| Validation                                         |                                  |                                   |                                          |                                          |
| MolProbity score                                   | 1.68                             | 1.90                              | 1.85                                     | 2.04                                     |
| Clash score                                        | 4.96                             | 6.81                              | 9.25                                     | 9.17                                     |
| Poor rotamers (%)                                  | 0                                | 0                                 | 0.20                                     | 0.53                                     |
| Ramachandran plot                                  |                                  |                                   |                                          |                                          |
| Favored (%)                                        | 93.60                            | 90.94                             | 94.81                                    | 90.04                                    |
| Allowed (%)                                        | 6.06                             | 8.73                              | 4.95                                     | 9.80                                     |
| Disallowed (%)                                     | 0.33                             | 0.33                              | 0.24                                     | 0.16                                     |