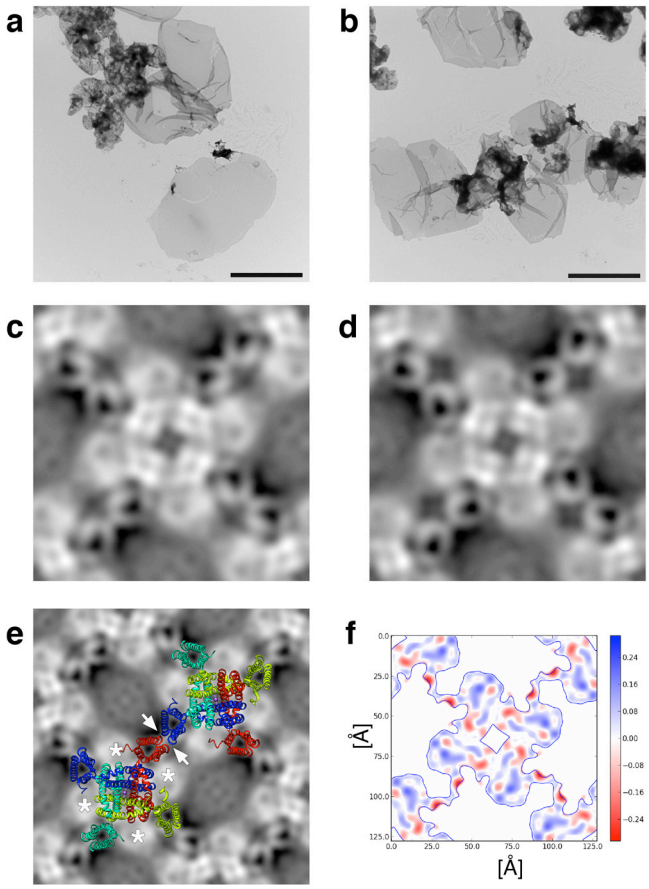
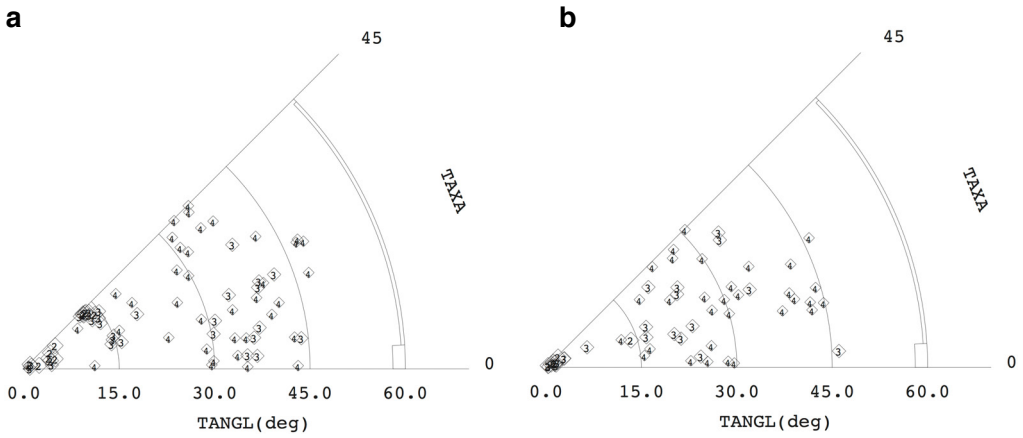
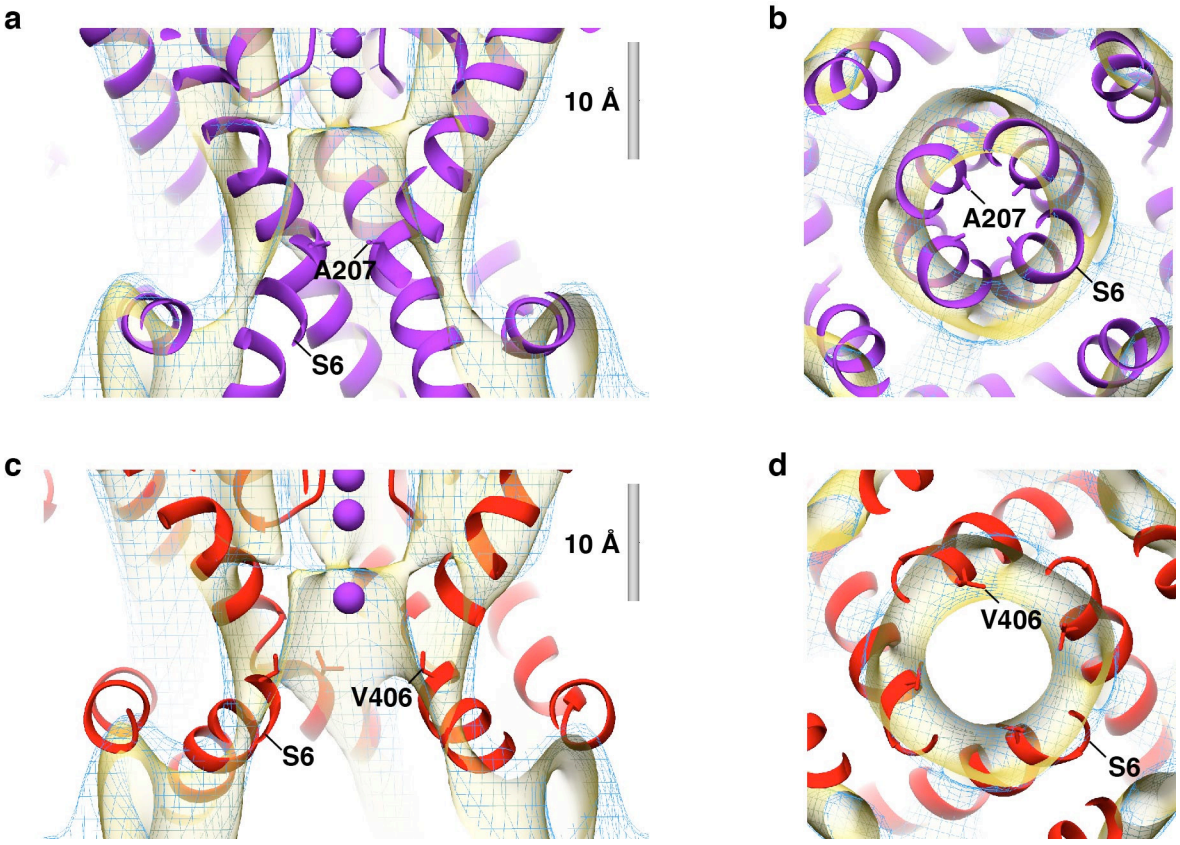


Supplementary Figure 1: **2D crystallization and 2D projection maps of MloK1**. MloK1 was crystallized in the presence (left panels) and absence (right panels) of cAMP ligand.

(**a, b**) Large, well-ordered, vesicular 2D crystals with diameters over 5 µm and low mosaicity were obtained. Scale bars correspond to 5 µm. (**c, d**) The projection maps of plunge-frozen 2D crystals vitrified in buffer solution. Shown is one crystal unit cell $P42_12$ symmetry, where one white MloK1 protein tetramer is located at the center of each map, and a second tetramer is located in the corners of the map in opposite orientation. The unit cell dimensions were $\alpha=\beta=131$ Å, $\gamma=90°$ for crystals grown in the presence of ligand (c), and $\alpha=\beta=130$ Å, $\gamma=90°$ for ligand-free channels (d). (**e**) Projection of the X-ray structure of the MloK1 transmembrane region (PDB ID 2ZD9) superimposed on the projection map of MloK1 with ligand, (c). Each monomer is depicted in a different color. Crystal contacts are indicated by white arrows. The CNBD densities of one tetramer are indicated by white stars. (**f**) Significant differences of the map shown in (c) minus the map from (d) (see Methods section). Densities corresponding to MloK1 with ligand are in blue, and without in red. Maps (c), (d), and (f) show one crystal unit cell, map (e) shows 1.5 x 1.5 unit cells.



Supplementary Figure 2: **The tilt angle distribution plots from the 3D merging process in *2dx***. MloK1 cryo-EM data in the (**a**) presence and (**b**) absence of cAMP. The plots are produced within *2dx* and show the image distributions according to their tilt angles (TANGL) in the asymmetric triangle. Each symbol represents one image; the number in the symbols corresponds to the significance of the image in the 3D dataset[69] .



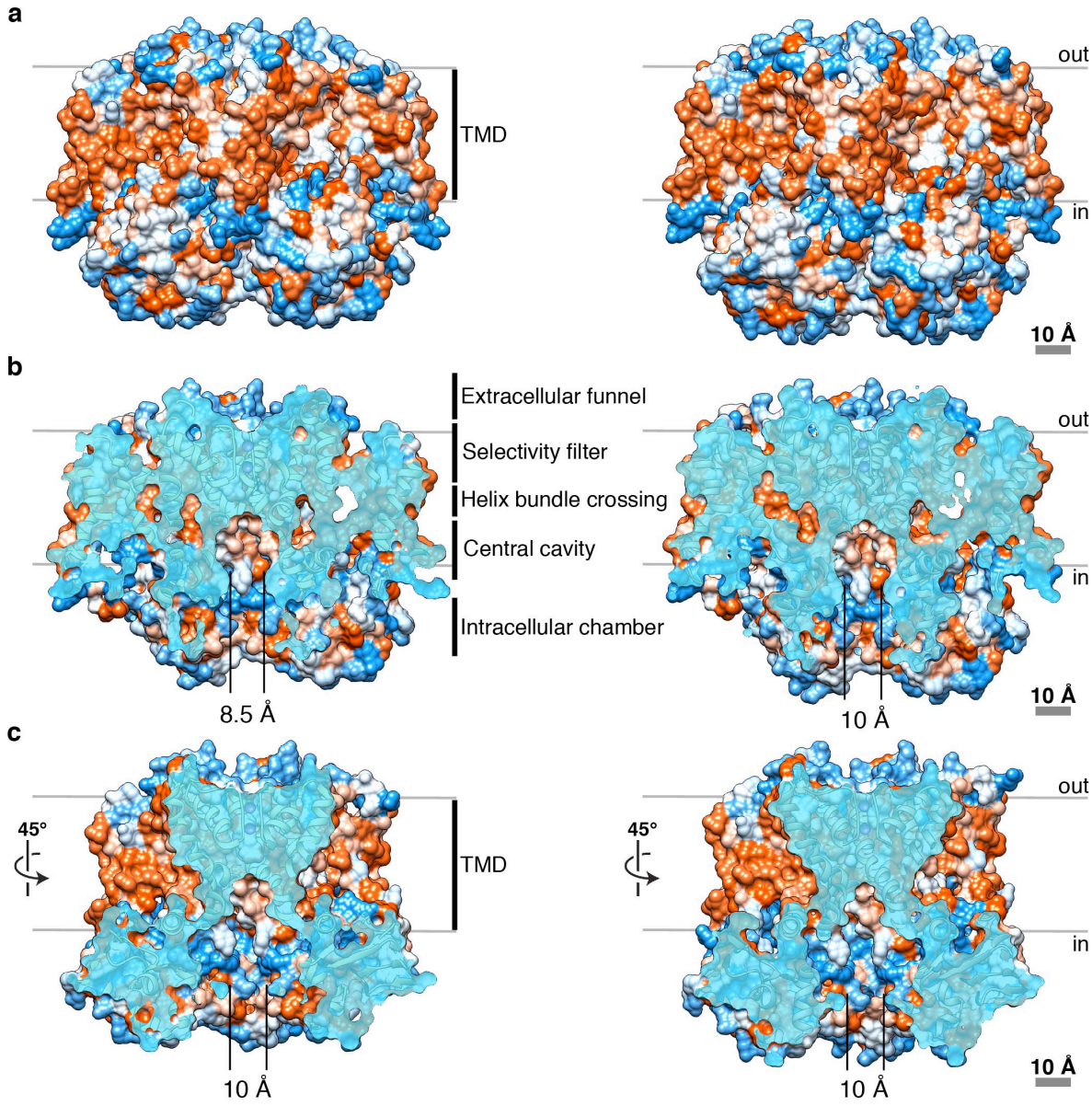
Supplementary Figure 3: X-ray structures docked into cryo-EM maps. X-ray structures of MloK1 (purple, PDB ID 2ZD9) in the closed conformation, and Shaker Kv1.2 (red, PDB ID 2A79) in the open conformation were fitted to the cryo-EM maps of cAMP-bound (mesh, blue) and cAMP-free (solid, yellow) MloK1.

(**a**) Helix S6 of the MloK1 X-ray structure is much more narrow at the helix bundle crossing area than the cryo-EM map.

(**b**) Cross-section through the helix bundle crossing region (A207) of MloK1.

(**c**) Helix S6 of Kv1.2 is wide open below the helix bundle crossing, in agreement with the cryo-EM map.

(**d**) Slice through constriction region of Kv1.2 (V406) corresponding to the A207 area in MloK1. For both MloK1 cryo-EM maps, this region has a similar diameter as in Kv1.2.

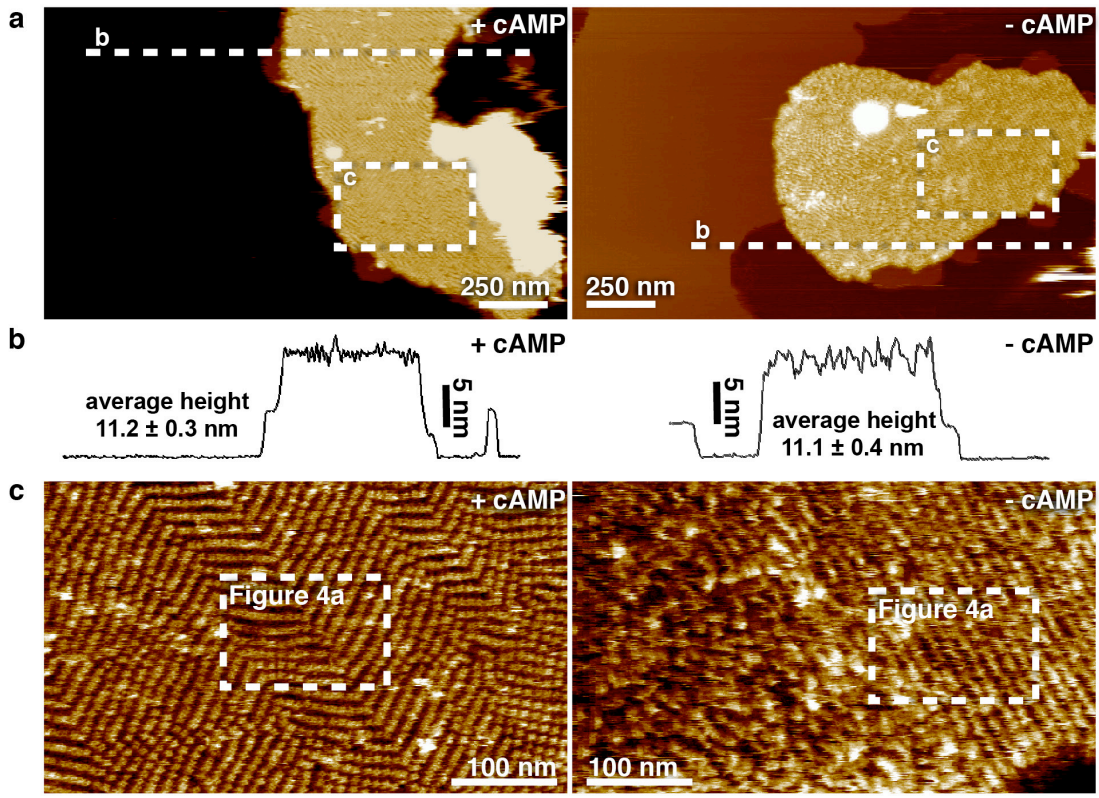


Supplementary Figure 4: **Electrostatic surface representation of liganded and ligand-free MloK1 models.**

(**a**) Surface representation of fitted MloK1 cAMP-bound (left) and cAMP-free (right) models.

(**b**) Cut-open view, revealing the central cavity, which is accessible for K^+ in both forms of the channel. All main pore elements are indicated.

(**c**) Structures from (b) rotated by 45° around the y-axis.



Supplementary Figure 5: AFM of 2D crystals of MloK1.

(**a**) Overview topographs of MloK1 channels reconstituted in lipid membranes with (left) and without (right) bound cAMP. The freshly adsorbed membranes showed MloK1 crystals of diameters up to several micrometers.

(**b**) Height profile of MloK1 membranes along the dashed lines in (a). The Cross-section analysis of MloK1 reconstitution membranes had very similar average height for the cAMP-bound 11.2 ± 0.3 nm (n = 50) (left) and cAMP-free 11.1 ± 0.4 nm (n = 50) (right) MloK1 membranes.

(**c**) Medium-resolution AFM topographs of Mlok1 in presence (left) and absence (right) of cAMP. In the +cAMP conformation single MloK1 channels could readily be observed, whereas the -cAMP conformation (right) the molecules appear as rather featureless protrusions. Different crystal forms were observed by AFM, including apparent P121 (shown here) and $P42_12$ symmetries.

Supplementary References

69. Cheng, A. & Yeager, M. A graphical representation of image quality for three-dimensional structure analysis of two-dimensional crystals. *Acta Crystallogr A* **60**, 351-4 (2004).