Supplemental Material

Model Building

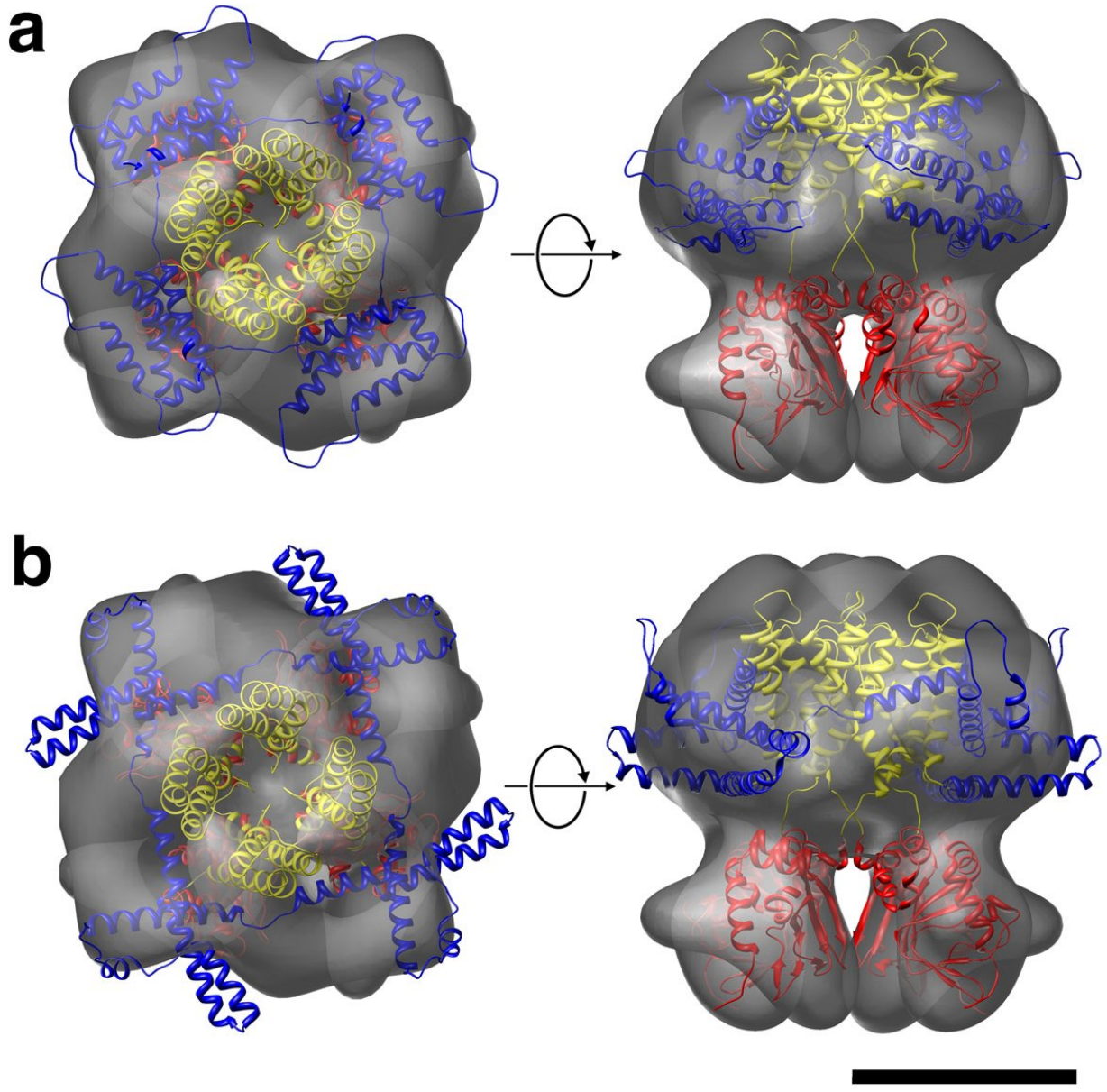

Fig. S1: Model fitting within the single particle reconstruction. (a) Top and side views of the "tilted" paddle states of MloK1 fitted to the 3D electron density from negatively stained single particles. (b) The same for the "horizontal" paddle states. S1-S4 helices, blue; S5-S6, yellow, CNBD, red. Scale bar 5 nm.

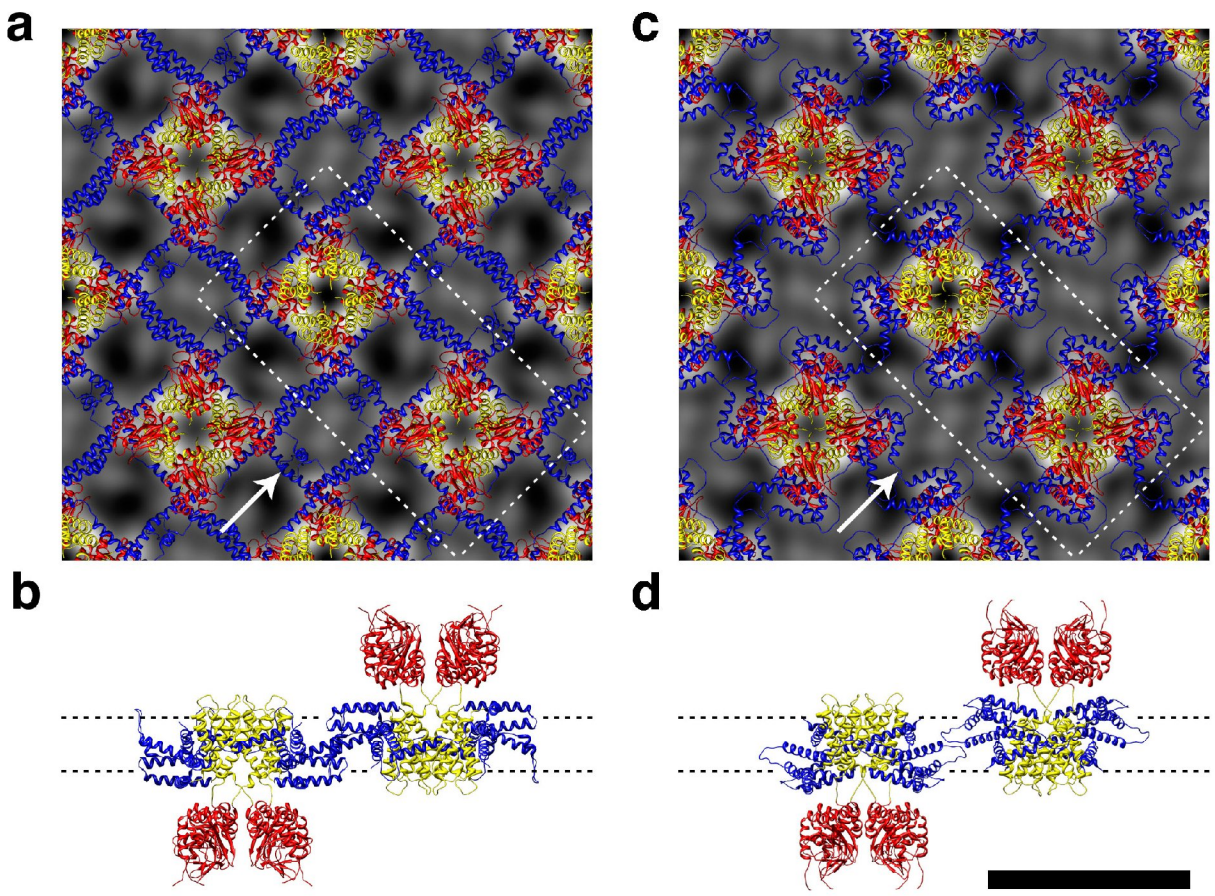

Fig. S2: Model fitting to the 2D crystal projection map. (a,b) 2x2 unit cell arrangement of the "horizontal" paddle states of MloK1, and the side-view of the area in the dotted rectangular box. (c,d) The same for the "horizontal" paddle state. Protein is white in (a,c). Scale bar 10 nm.

Maximum Likelihood Single Particle Image Processing

Crystallographic processing of a 2D crystal image in electron crystallography is usually done by computationally unbending any crystal lattice distortions, after which the resulting image is Fourier transformed, amplitudes and phases are extracted form the lattice positions, and a final reconstruction is created from these values after correction of the instrument parameters. Such processing is equivalent to averaging of the translationally aligned 2D crystal unit cells, without allowing larger rotational variations of the crystal unit cells.

In the here presented project, this process could lead to an averaging of differently oriented MloK1 2D crystal unit cells. If the crystal were composed of a unit cell that were only two-fold symmetric but would be either "north-south" oriented, or "east-west" oriented, the averaging process could still produce an apparently four-fold symmetric result. A dimer-of-dimer arrangement of the CNBDs of the tetrameric MloK1 particles could thereby be masked in the image processing, and appear as four-fold symmetric average structure.

To investigate the possibility of a non-crystallographic orientation of two-fold symmetric particles within a square crystal arrangement, we have subjected the MloK1 crystal image used in Fig. 4b to a single particle processing procedure, applying maximum likelihood (ML) weighted alignment and allowing a 90º rotation for the individual tetrameric MloK1 particles: The 2D crystal image was windowed into 1900 unit cell particle images, which each were centered onto either the bright strongly stained CNBD side of the MloK1 particles, or the darker weakly stained extracellular side of the MloK1 particles. These particle images were then aligned, while allowing for each particle to contribute in either the original orientation, or in the 90º rotated orientation, using the ML weighting scheme. The usage of a maximum likelihood processing reduced any reference bias and resulted in a better reconstruction. Cross-correlational alignment produced the similar result at lower resolution. Processing was done with the *2dx* software package, available at *2dx.org*.

The resulting maximum-likelihood reconstruction showed that 1096 particles preferred the original orientation, while 804 particles showed a stronger correlation with the iteratively established average structure when rotated by 90 degrees.

The resulting maps are shown in Fig. S1, for the processing of the particles centered on either of the two MloK1 tetramers within the 2D crystal unit cell. These results confirm that even when allowing individual MloK1 tetramers to choose any of the 90º rotated orientations, the densities attributed to the CNBDs remained in clearly four-fold symmetric arrangement, separated by gaps.

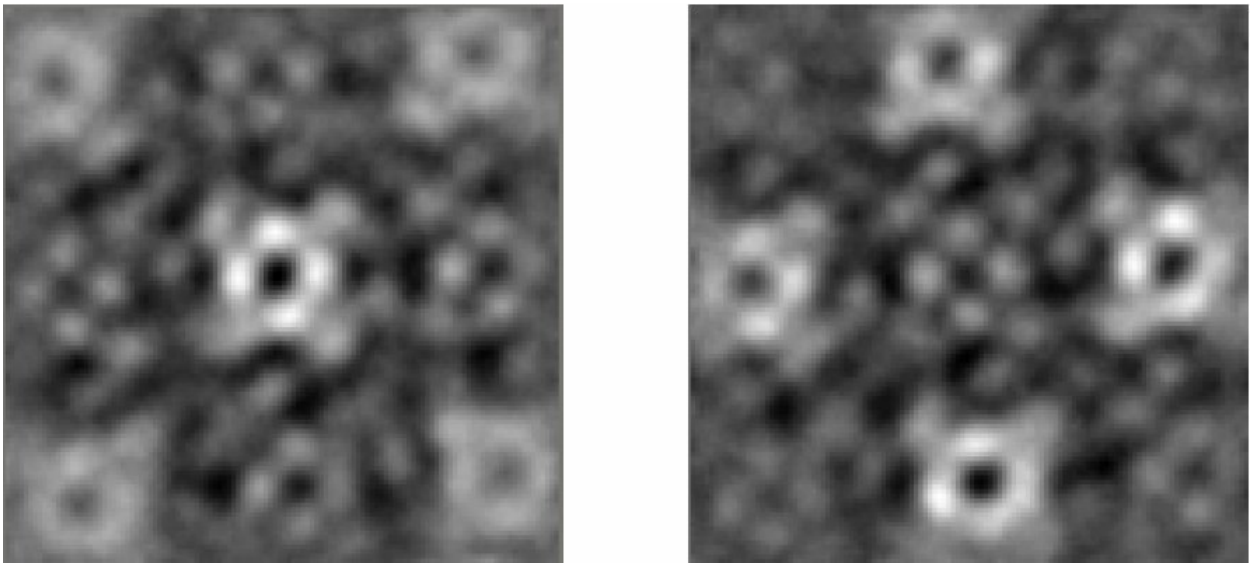

Fig. S3: The result of maximum likelihood single particle processing of a 2D crystal image of MloK1. Left: centered on the MloK1 tetramer with deeply stained CNBDs. Right: centered on MloK1 tetramers with deep stain on the extracellular side. The central four densities attributed to the CNBDs remain clearly separated and in four-fold symmetric arrangement.