Supporting Information

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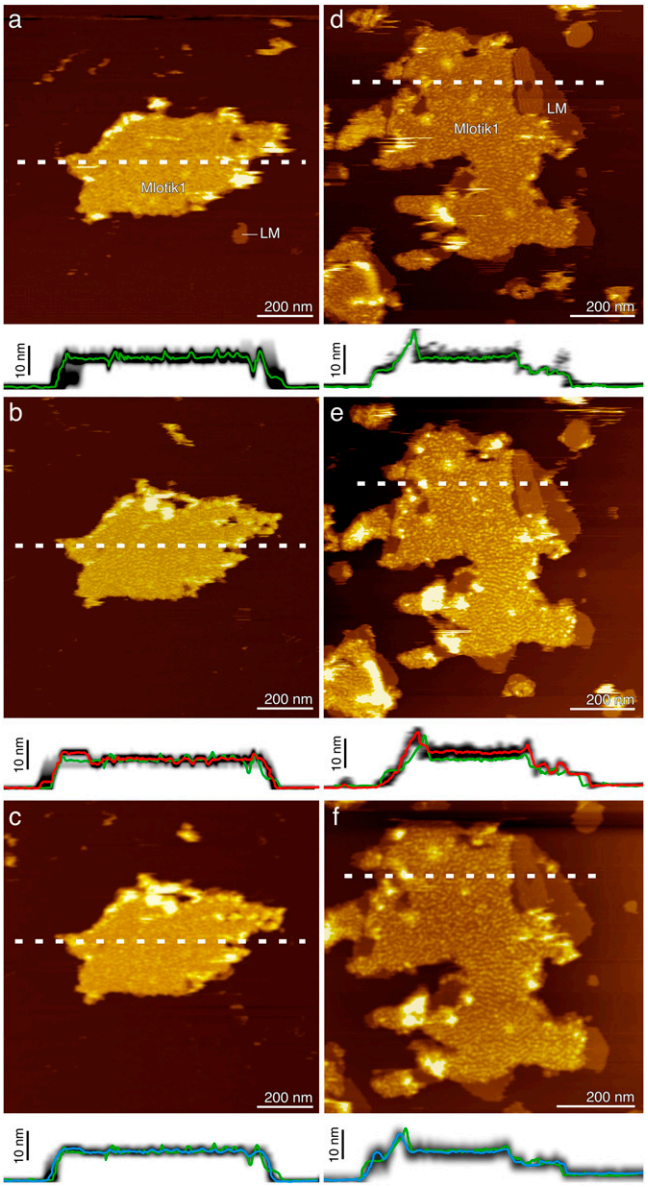

Fig. S1. Overview AFM topographs of wt and mutant R348A MlotiK1 reconstituted into membranes of *E. coli* polar lipid extract. Overviews of wt MlotiK1 channel (*a–c*) and mutant MlotiK1 channel (*d–f*) membranes prepared in the presence of cAMP (see *Materials and Methods*), and adsorbed and imaged in the presence of 200 μM cAMP (*a*) and 900 μM cAMP (*d*) after removing cAMP (*b* and e), and after the readdition of 200 μM cAMP (*c*) or 900 μM cAMP (*f*). Lipid membranes (LM) and densely packed MlotiK1 are clearly visible. Below each topograph is the height profile taken along the dashed lines. Every height profile represents the superimposition of 10 height lines and shows the height average (colored line) and height variation (gray shading). In the presence of cAMP, lipid membranes protruded 4.0 ± 0.5 nm (average ± SD; $n = 81$), wt MlotiK1 membrane patches protruded 10.2 ± 0.8 nm ($n = 176$) and mutant MlotiK1 patches protruded 10.1 ± 0.8 nm ($n = 156$) from the supporting mica. Imaging the same membranes in the absence of cAMP revealed no changes for wt MlotiK1 patches (*b*). Extending the incubation time in cAMP-free buffer solution to 4 d did not change the appearance and height (10.3 ± 0.6 nm; $n = 163$) of wt MlotiK1 membranes (compare with Fig. S4). In the absence of cAMP, mutant MlotiK1 membranes increased their height from 10.1 ± 0.8 nm (*d*) to 11.8 ± 0.8 nm ($n = 147$) (*e*). For better comparison, we superimposed the average height lines recorded in presence of cAMP (green lines) and height lines recorded in absence of cAMP (red lines) (*e*). Upon readding cAMP, wt MlotiK1 membranes showed no change in height (*c*), whereas mutant MlotiK1 membranes reduced their height back to that measured initially in the presence of cAMP (*f*). For better comparison, we superimposed the average height lines recorded in presence of readded cAMP (blue lines) and height lines recorded before depletion of cAMP (green lines) (*f*). Imaging buffers were 50 mM KCl and 20 mM Tris-HCl (pH 7.5) with cAMP concentrations as indicated. All heights for wt and mutant MlotiK1 measured in the presence of cAMP, in the absence of cAMP, and upon readding cAMP are given in Table S1. *n* gives the number of lipid membranes or MlotiK1 membranes measured.

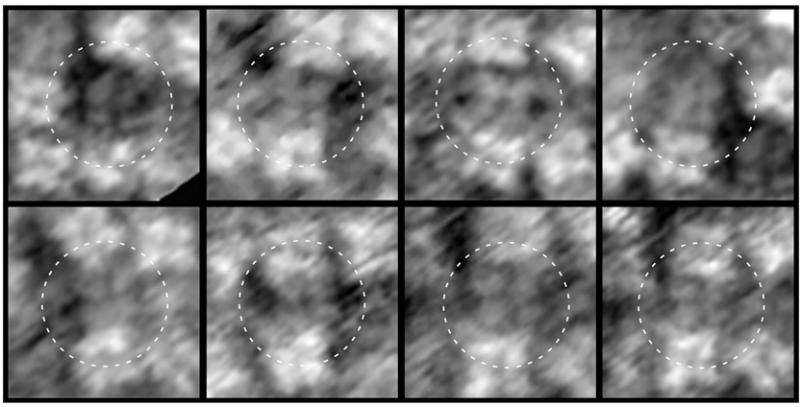

Fig. S2. Gallery of high-resolution AFM topographs of the extracellular surface of the wt MlotiK1 channel. AFM topographs revealed shallow tetrameric surfaces (outlined by dashed circles) between the highly protruding cytoplasmic surfaces of MlotiK1 channels. Different channels are shown at slightly different magnifications. The shallow tetramers protrude 1.8 ± 0.5 nm (n = 48) from the lipid bilayer, whereas the highly protruding tetramers protrude 3.2 ± 0.5 nm (n = 100) from the lipid membrane. On average, both the shallow and the highly protruding tetramers have an outer diameter of ~8–10 nm. AFM topographs show a gray level range corresponding to a vertical height scale of 4.5 nm. Imaging buffer was 50 mM KCl, 200 μM cAMP, and 20 mM Tris-HCl (pH 7.5). n gives the number of MlotiK1 tetramers measured.

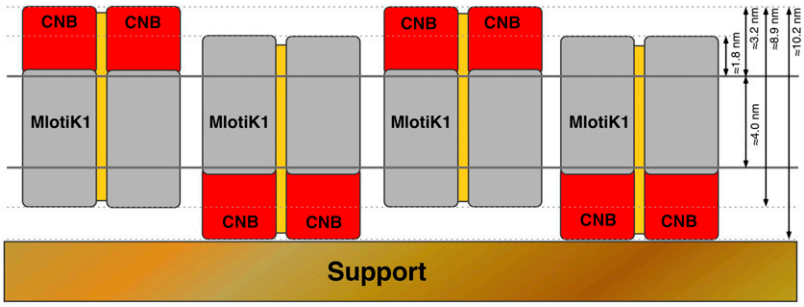

Fig. S3. Measured heights of the reconstituted wt MlotiK1 membrane. Schematic drawing showing a side view of the reconstituted MlotiK1 channels in an up-and-down orientation. Height values are average values measured from wt MlotiK1 in the presence of 50 mM KCl, 200 μM cAMP, and 20 mM Tris-HCl (pH 7.5).

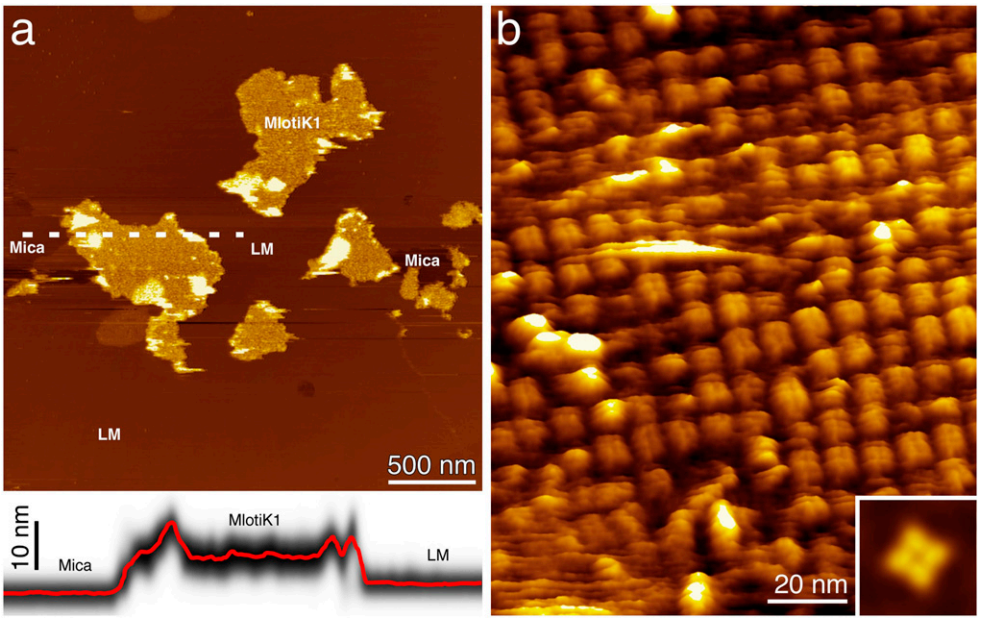

Fig. S4. AFM topographs of wt MlotiK1 membranes incubated for 4 d in cAMP-free buffer solution. (*a*) Overview topograph of wt MlotiK1 membranes. Lipid membranes (LM) embedding densely packed MlotiK1 are clearly visible. The height profile taken along the dashed line indicated in the topograph is shown at the bottom. The height profile represents the superimposition of 10 height lines and shows their average height (red line) and variation (gray shading). Lipid membranes protruded 3.6 ± 0.7 nm (n = 61), and MlotiK1 channels protruded 10.3 ± 0.6 nm (n = 163) from the support and 3.3 ± 0.5 nm (n = 117) from the lipid membrane. n gives the number of MlotiK1 membranes or channels measured. These average heights were not significantly different from average heights of wt MlotiK1 membranes recorded in the presence of cAMP (Fig. S1 *a* and *c* and Table S1). (*b*) High-resolution topograph showing wt MlotiK1 channels after incubation for 4 d in cAMP-free buffer at room temperature. The inset shows the correlation average of 117 MlotiK1 channels. Incubation and imaging buffer was 50 mM KCl and 20 mM Tris-HCl (pH 7.5). AFM topographs show full-color levels corresponding to vertical height scale of 20 nm (*a*) and 4.5 nm (*b*).

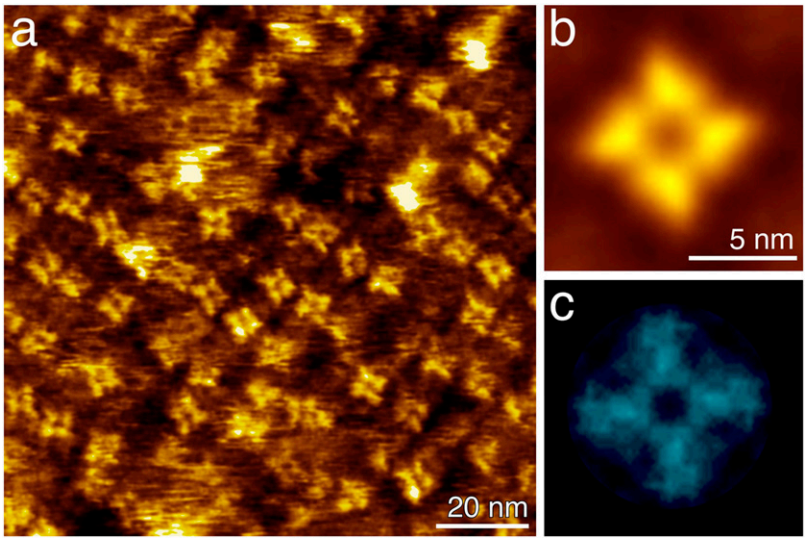

Fig. S5. High-resolution AFM topograph of wt MlotiK1 incubated for 12 h in cAMP-free buffer solution at room temperature. (*a*) Topograph showing single MlotiK1 channels with clearly visible CNB domains. Correlation average of 54 MlotiK1 tetramers showing the arrangement of the four CNB domains (*b*) and corresponding SD map showing the structural variability of the domains (*c*). As revealed from the AFM topographs, the appearance of wt MlotiK1 did not change when removing cAMP (compare Fig. 1 and Fig. S4). In absence of cAMP, the wt MlotiK1 channels protruded 3.2 ± 0.5 nm (n = 54) from the lipid bilayer. n gives the number of MlotiK1 channels measured. Images have full-color levels corresponding to vertical height scales of 5 nm (*a*), 4.5 nm (*b*), and 0.6 nm (*c*). Imaging buffer was 50 mM KCl and 20 mM Tris-HCl (pH 7.5).

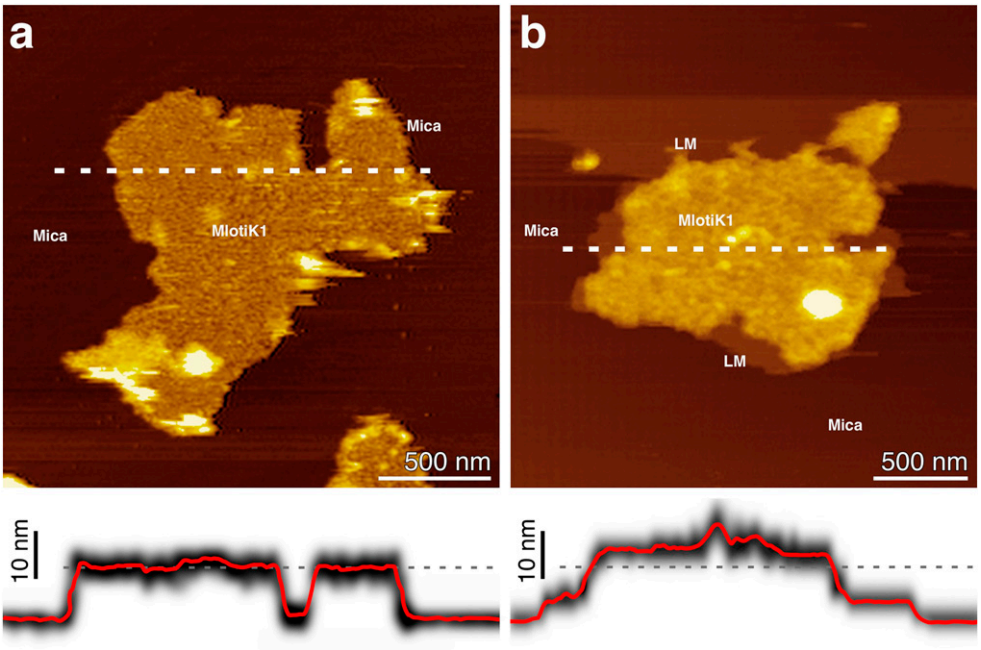

Fig. S6. AFM topographs of wt and mutant R348A MlotiK1 membranes prepared, adsorbed, and imaged in cAMP-free buffer solution. (*a*) Overview topograph of wt MlotiK1 membranes. The average height determined from 105 membranes was 10.0 ± 0.7 nm. (*b*) Overview topograph of a mutant MlotiK1 membrane. Lipid membranes (LM) embedding densely packed MlotiK1 are clearly visible. Lipid membranes protruded 3.9 ± 0.6 nm (n = 47) and MlotiK1 membranes protruded 13.5 ± 0.7 nm (n = 153) from the support. Below each topograph is the height profile taken along the dashed line. Each height profile is a superimposition of 10 height lines and shows their average (red line) and variation (gray shading). The gray dashed line indicates a height of 10 nm. Before adsorption to the supporting mica, the MlotiK1 membranes underwent a long dialysis step (9 d, with several dialysis-buffer changes) against cAMP-free buffer (see *Materials and Methods*). After dialysis the membranes were adsorbed to the mica and imaged by AFM using cAMP-free buffer solution [50 mM KCl and 20 mM Tris-HCl (pH 7.5)]. n gives the number of membranes measured.

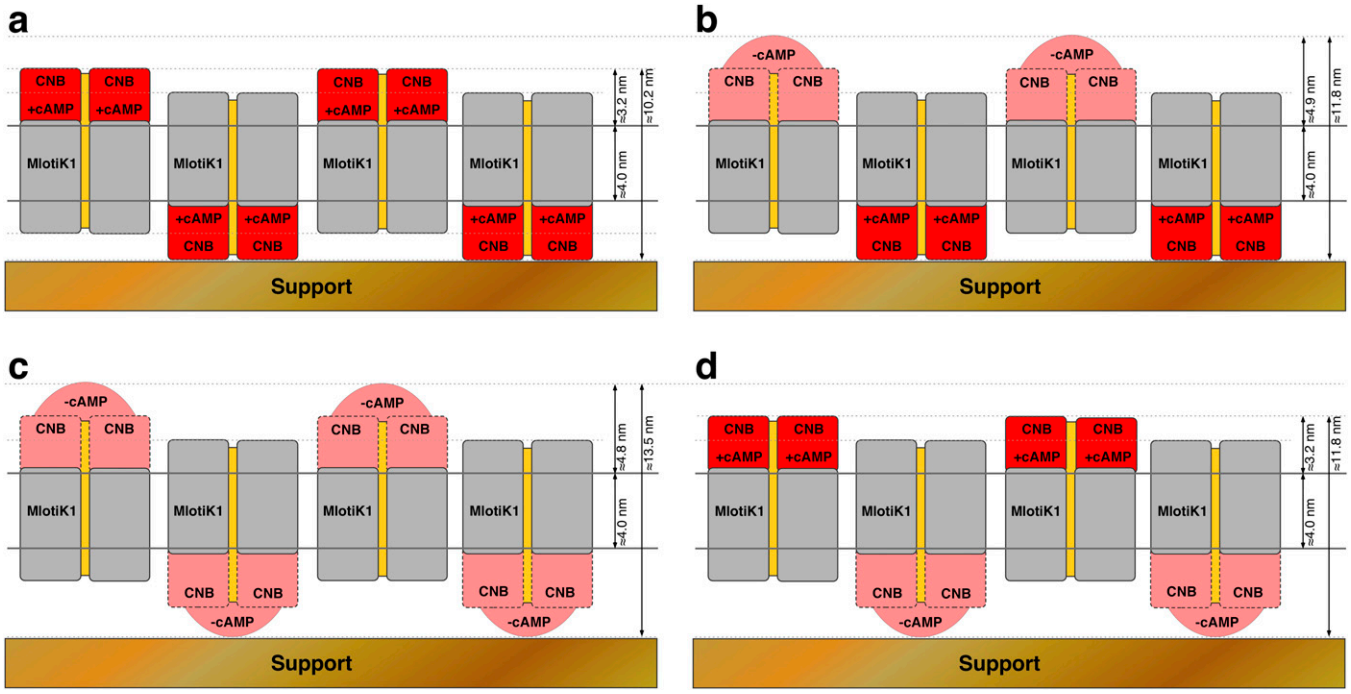

Fig. S7. Heights measured for reconstituted wt and mutant R348A MlotiK1 in the presence and absence of cAMP. (*a*) When prepared, adsorbed, and imaged in the presence of cAMP, wt and mutant MlotiK1 show the same heights within the errors of the AFM measurements (see Figs. S1 *a* and *d* and S3 and Table S1). The heights of wt MlotiK1 do not change after incubation and imaging in cAMP-free buffer (Figs. S1 *a*–*c*, S4, S5, and S6*a* and Table S1). (*b*) Mutant MlotiK1 membranes prepared and adsorbed in the presence of saturating cAMP concentrations but imaged in the absence of cAMP show an increased height of ∼11.8 nm (Fig. S1e and Table S1). This height change is attributable to conformational changes of the CNB domains facing the buffer solution (Fig. 3*D* and Figs. S8 and S9 and Table S1). Reincubating the cAMP-free mutant MlotiK1 (*b*) with cAMP reduces the membrane height to that measured for mutant and wt MlotiK1 membranes in the presence of cAMP (Figs. 3 *A* and *G*, Figs. S1*f* and S8 and Table S1). (*c*) When prepared, adsorbed, and imaged in the absence of cAMP, mutant MlotiK1 membranes show a height of ∼13.5 nm (Fig. S6*b* and Table S1). (*d*) Upon incubating the cAMP-free mutant MlotiK1 membranes (*c*) with saturating cAMP concentrations, the membranes show a height of ∼11.8 nm.

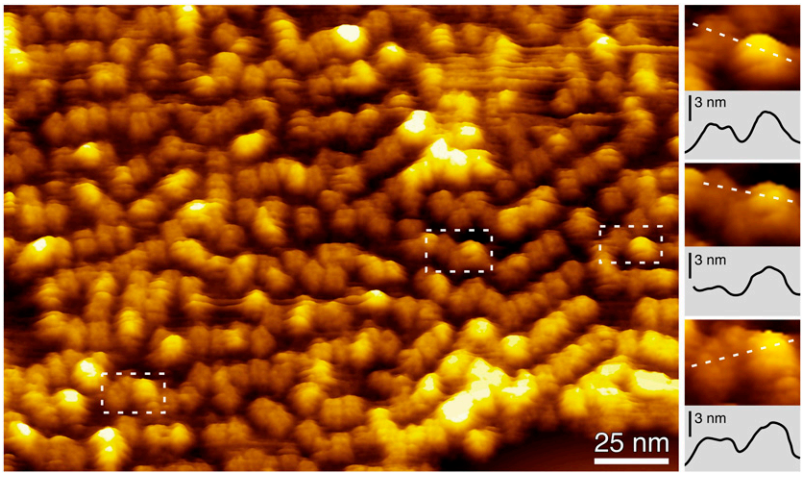

Fig. S8. High-resolution AFM topograph of mutant R348A MlotiK1 recorded after readdition of 600 μM cAMP. After adsorption to the supporting mica, cAMP-bound mutant MlotiK1 membranes were incubated in cAMP-free buffer solution [50 mM KCl and 20 mM Tris-HCl (pH 7.5)] for several hours. In the absence of cAMP, the MlotiK1 tetramers undergo a conformational change and increase height (Fig. 3*D*). After this, the membranes were incubated with 600 μM cAMP that was insufficient to saturate mutant MlotiK1. The AFM topograph shows an overview of MlotiK1 tetramers that coexist in two different conformational states. The lower protruding MlotiK1 tetramers (compare Figs. 1 and 3*A*) represent the cAMP-bound conformations, whereas the higher protrusions having the diameter of approximately one MlotiK1 tetramer (∼8–10 nm) represent the cAMP-unbound conformations. At fully saturating cAMP concentrations, all MlotiK1 tetramers (∼95%) are in the cAMP-bound conformation (Fig. 3*G*). The image on the right shows selected regions of the topograph at larger scale. These regions were taken from dashed rectangles indicated in the topograph. Below every selected region, we show a height profile (noise-filtered) that has been taken along the dashed line indicated. The height difference between lower-protruding (cAMP-bound) and higher-protruding (cAMP-unbound) MlotiK1 channels corresponds to ∼1.7 nm. The topographs show a full-color level corresponding to vertical height scale of 6 nm. The height analysis of cAMB-bound and cAMP-unbound mutant MlotiK1 channels is shown in Table S2. Imaging was 50 mM KCl, 600 μM cAMP, and 20 mM Tris-HCl (pH 7.5).

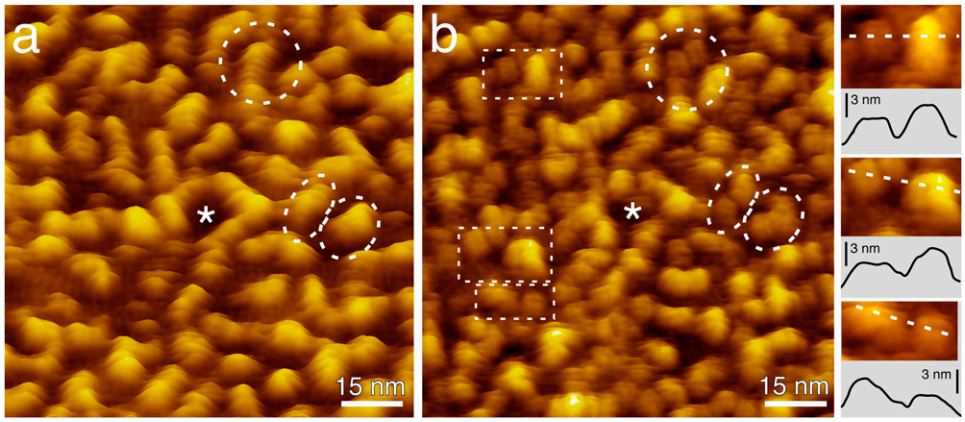

Fig. S9. Time-lapse AFM topographs of mutant R348A MlotiK1 recorded in the absence and presence of cAMP. (*a*) Mutant MlotiK1 channels recorded in cAMP-free buffer solution. After an incubation time of ~3 h all channels increased height by ~1.7 nm (see Fig. 3*D* and Table S2). (*b*) The same mutant MlotiK1 channels as in *a* recorded ~150 min after readdition of 600 μM cAMP. The asterisk highlights a reference area, and the dashed circles and ellipses highlight conformational changes of the same membrane area imaged in absence and presence of cAMP. The lower-protruding MlotiK1 tetramers represent the cAMP-bound conformations (compare Figs. 1 and 3*A* and Fig. S8), whereas the higher protrusions having the diameter of about one MlotiK1 tetramer represent the cAMP-unbound conformations (compare Fig. 3D and Fig. S8). Because mutant MlotiK1 was not fully saturated at 600 μM cAMP, not all channels have re-adopted the cAMP-bound conformation. The image on the right shows selected regions of the MlotiK1 membrane at larger scale. These regions were taken from dashed rectangles indicated in the topograph *b*. Below every selected region, we show a height profile (noise-filtered) that has been taken along the dashed line indicated. The height difference between lower-protruding (cAMP-bound) and higher-protruding (cAMP-unbound) MlotiK1 channels corresponds to ~1.7 nm. Topographs have full-color levels corresponding to a vertical height scale of 6 nm. Imaging buffer was 50 mM KCl, no cAMP (*a*), 600 μM cAMP (*b*), and 20 mM Tris-HCl (pH 7.5).

Table S1. Heights measured for wt and mutant R348A MlotiK1 membranes protruding from the mica support in the presence and absence of cAMP

Preparation/imaging	wt MlotiK1	Mutant MlotiK1
+cAMP/+cAMP/+cAMP	10.2 ± 0.8 nm (*n* = 176)	10.1 ± 0.8 nm (*n* = 156)
+cAMP/+cAMP/−cAMP	10.3 ± 0.6 nm (*n* = 163)*	11.8 ± 0.8 nm (*n* = 147)†
−cAMP/−cAMP/−cAMP	10.0 ± 0.7 nm (*n* = 105)*	13.5 ± 0.7 nm (*n* = 153)†
−cAMP/−cAMP/+cAMP	—	12.1 ± 0.8 nm (*n* = 39)†

Average heights and SDs measured from a number (*n*) of MlotiK1 membranes imaged by AFM. Sample preparation, adsorption, and imaging conditions were as follows:

+cAMP/+cAMP/+cAMP: MlotiK1 membranes were prepared in the presence of cAMP (see *Materials and Methods*), adsorbed in cAMP-containing buffer, and imaged in cAMP-containing buffer [200 μM cAMP (wt MlotiK1) or 900 μM cAMP (mutant MlotiK1)].

+cAMP/+cAMP/−cAMP: MlotiK1 membranes were prepared in the presence of cAMP (see *Materials and Methods*), adsorbed in cAMP-containing buffer [200 μM cAMP (wt MlotiK1) or 900 μM cAMP (mutant MlotiK1)], and imaged in cAMP-free buffer.

−cAMP/−cAMP/−cAMP: MlotiK1 membranes were prepared in the absence of cAMP (see *Materials and Methods*), adsorbed in cAMP-free buffer, and imaged in cAMP-free buffer.

−cAMP/−cAMP/+cAMP: MlotiK1 membranes were prepared in the absence of cAMP (see *Materials and Methods*), adsorbed in cAMP-free buffer, and imaged in cAMP-containing buffer (900 μM cAMP for mutant MlotiK1).

For every experimental condition listed, the statistical height analysis of MlotiK1 membranes was taken from membranes imaged in at least 12 independent experiments. For each experiment between 20 and 60 AFM, topographs of membranes have been recorded.

*Indicates that the average height is not significantly different (Student *t* test) from the average height shown at the top of the row. Adsorption buffer: 200 mM KCl, 20 mM Tris-HCl (pH 7.5), and cAMP concentrations as indicated. Imaging buffer: 50 mM KCl, 20 mM Tris-HCl (pH 7.5), and cAMP concentrations as indicated.

†Indicates that the average height is significantly different (Student *t* test) from the average height shown at the top of the row (+cAMP/+cAMP/+cAMP).

Table S2. Heights measured for wt and mutant R348A MlotiK1 channels protruding with their CNB domains from the lipid bilayer

Preparation/imaging	wt MlotiK1	Mutant MlotiK1
+cAMP/+cAMP/+cAMP	3.2 ± 0.5 nm (*n* = 100)	3.1 ± 0.5 nm (*n* = 57)
+cAMP/+cAMP/−cAMP	3.3 ± 0.5 nm (*n* = 117)*	4.9 ± 0.6 nm (*n* = 148)†
−cAMP/−cAMP/−cAMP	3.4 ± 0.6 nm (*n* = 78)*	4.9 ± 0.6 nm (*n* = 123)†
−cAMP/−cAMP/+cAMP	—	3.2 ± 0.5 nm (*n* = 45)*

Average heights and SDs measured from a number (*n*) of MlotiK1 channels imaged by AFM. Sample preparation, adsorption, and imaging conditions were as follows:

+cAMP/+cAMP/+cAMP: MlotiK1 membranes were prepared in the presence of cAMP (see *Materials and Methods*), adsorbed in cAMP-containing buffer, and imaged in cAMP-containing buffer [200 μM cAMP (wt MlotiK1) or 900 μM cAMP (mutant MlotiK1)].

+cAMP/+cAMP/−cAMP: MlotiK1 membranes were prepared in the presence of cAMP (see *Materials and Methods*), adsorbed in cAMP-containing buffer [200 μM cAMP (wt MlotiK1) or 900 μM cAMP (mutant MlotiK1)], and imaged in cAMP-free buffer.

−cAMP/−cAMP/−cAMP: MlotiK1 membranes were prepared in the absence of cAMP (see *Materials and Methods*), adsorbed in cAMP-free buffer, and imaged in cAMP-free buffer.

−cAMP/−cAMP/+cAMP: MlotiK1 membranes were prepared in the absence of cAMP (see *Materials and Methods*), adsorbed in cAMP-free buffer, and imaged in cAMP-containing buffer (900 μM cAMP for mutant MlotiK1).

*Indicates that the average height is not significantly different (Student *t* test) from the average height shown at the top of the row. Adsorption buffer: 200 mM KCl, 20 mM Tris-HCl (pH 7.5), and cAMP concentrations as indicated. Imaging buffer: 50 mM KCl, 20 mM Tris-HCl (pH 7.5), and cAMP concentrations as indicated.

†Indicates that the average height is significantly different (Student *t* test) from the average height shown at the top of the row (+cAMP/+cAMP/+cAMP).