

## **Supplementary Information**

### **Native mass spectrometry and structural studies reveal modulation of MsbA-nucleotide interactions by lipids**

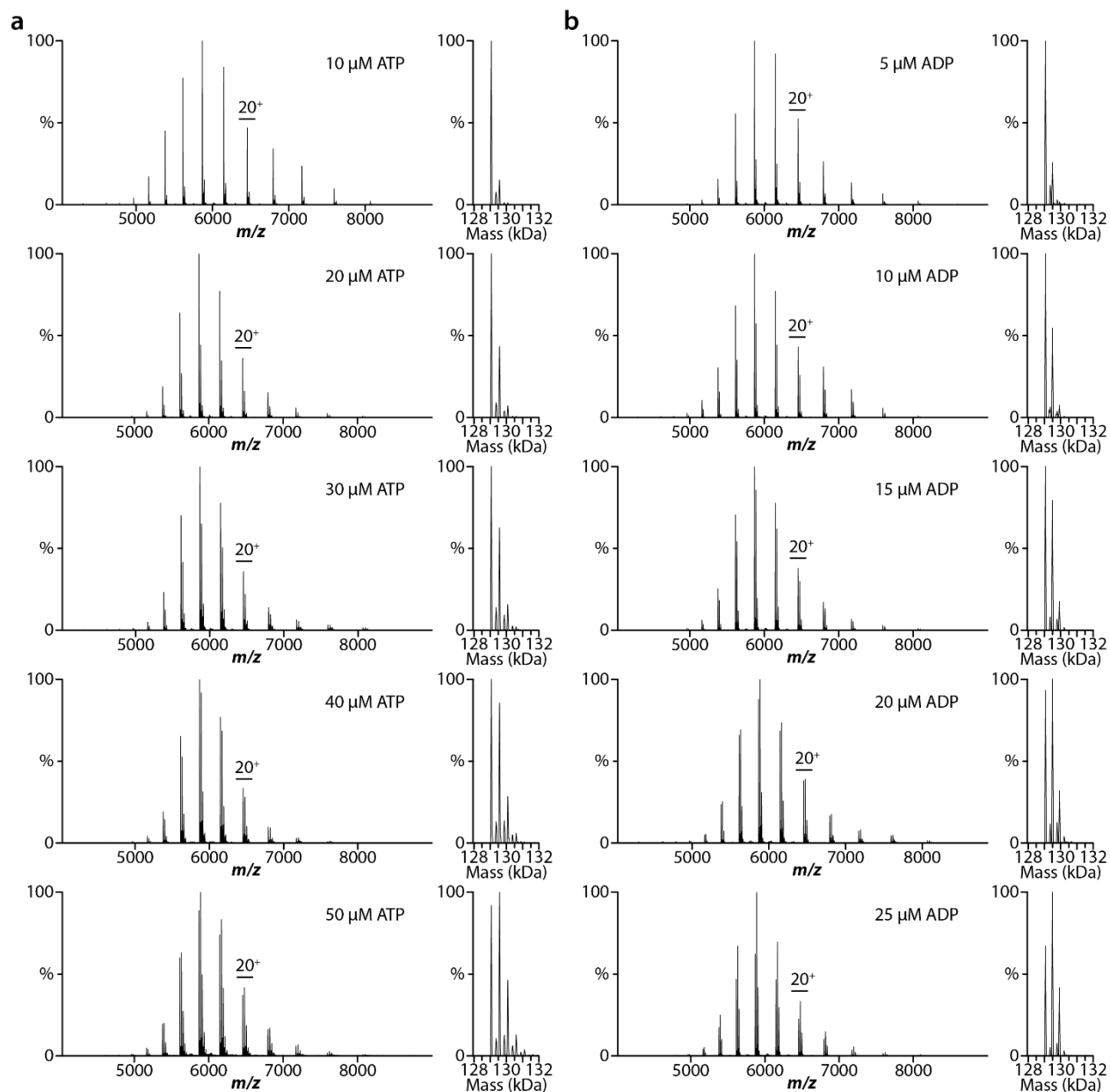
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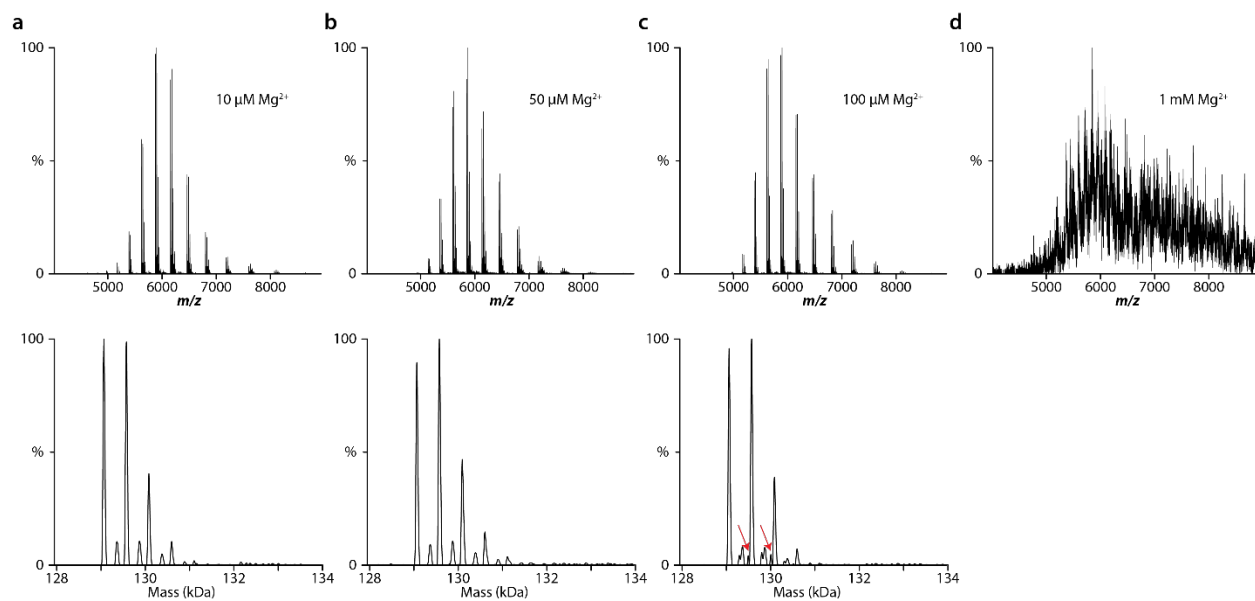
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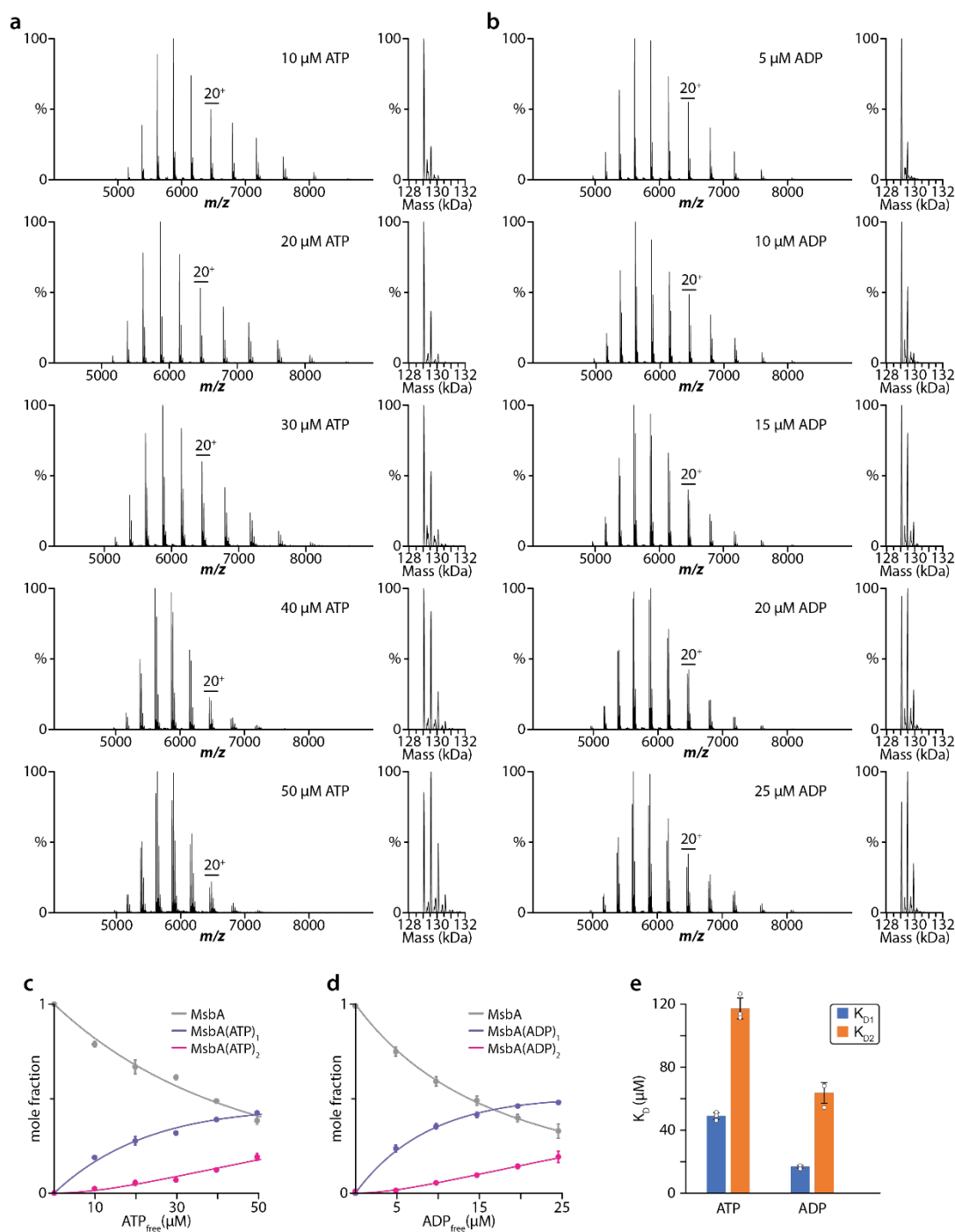
## Supplementary Figures



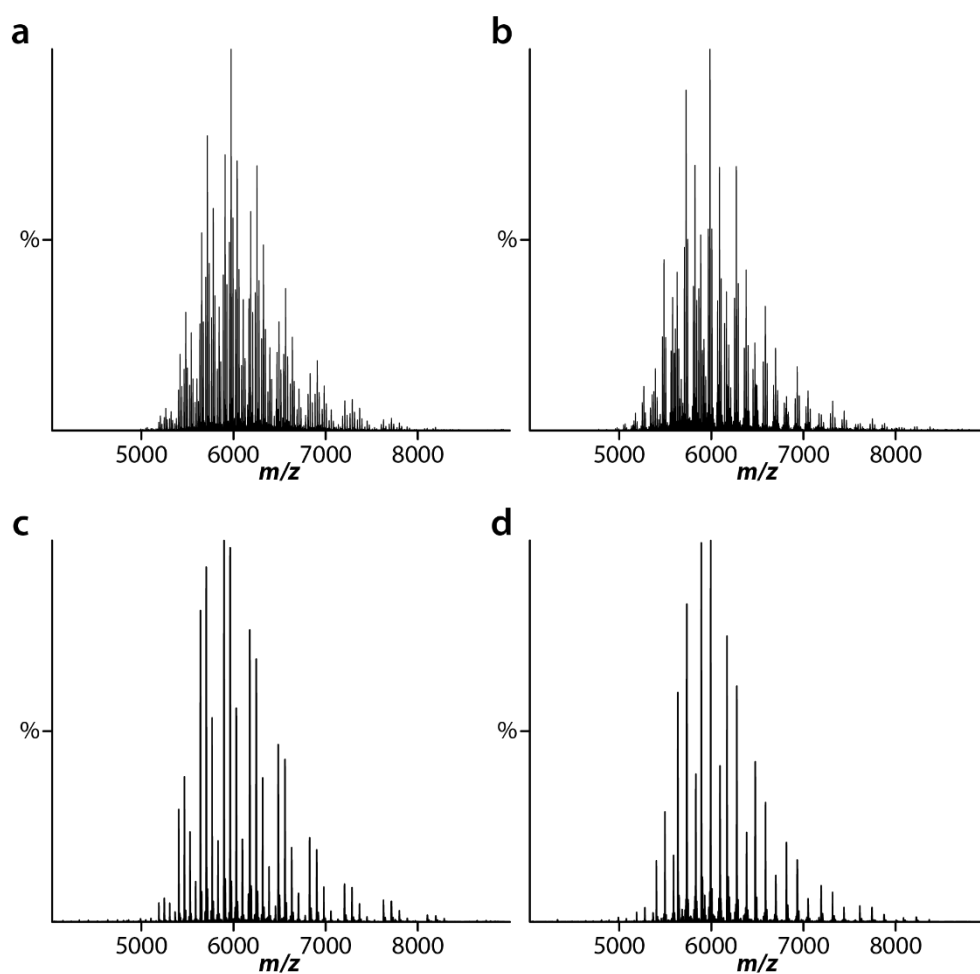
**Supplementary Fig. 1. Representative native mass spectra for nucleotide binding to MsbA.** MsbA (0.5  $\mu\text{M}$ ) was mixed with 10  $\mu\text{M}$   $\text{Mg}^{2+}$  and different concentrations of **a** ATP and **b** ADP. Deconvoluted mass spectra are shown to the right.



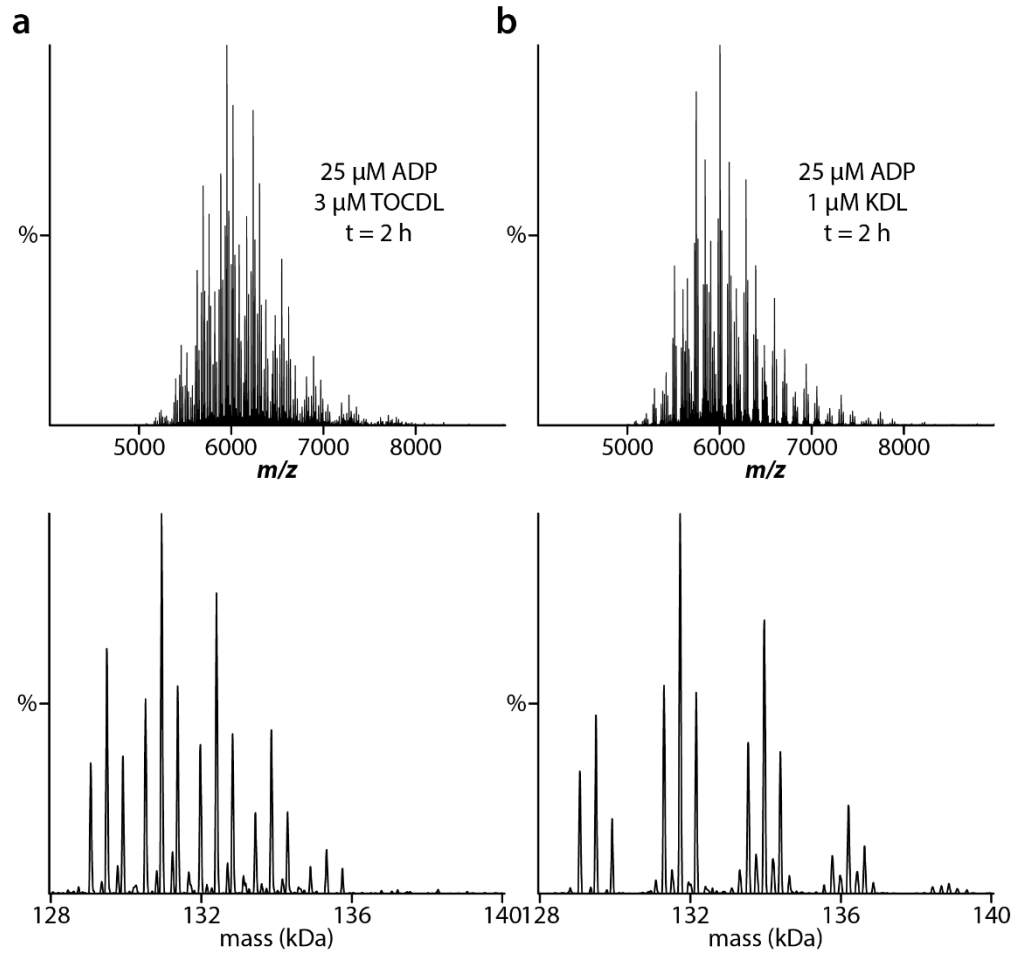
**Supplementary Fig. 2. Native mass spectra for nucleotide binding to MsbA in the presence of different concentrations of  $\text{Mg}^{2+}$ .** MsbA ( $0.5 \mu\text{M}$ ) was mixed with  $50 \mu\text{M}$  ATP and different concentrations of  $\text{Mg}^{2+}$ . Deconvoluted mass spectra are shown below with peaks corresponding to ADP binding denoted with an arrow.



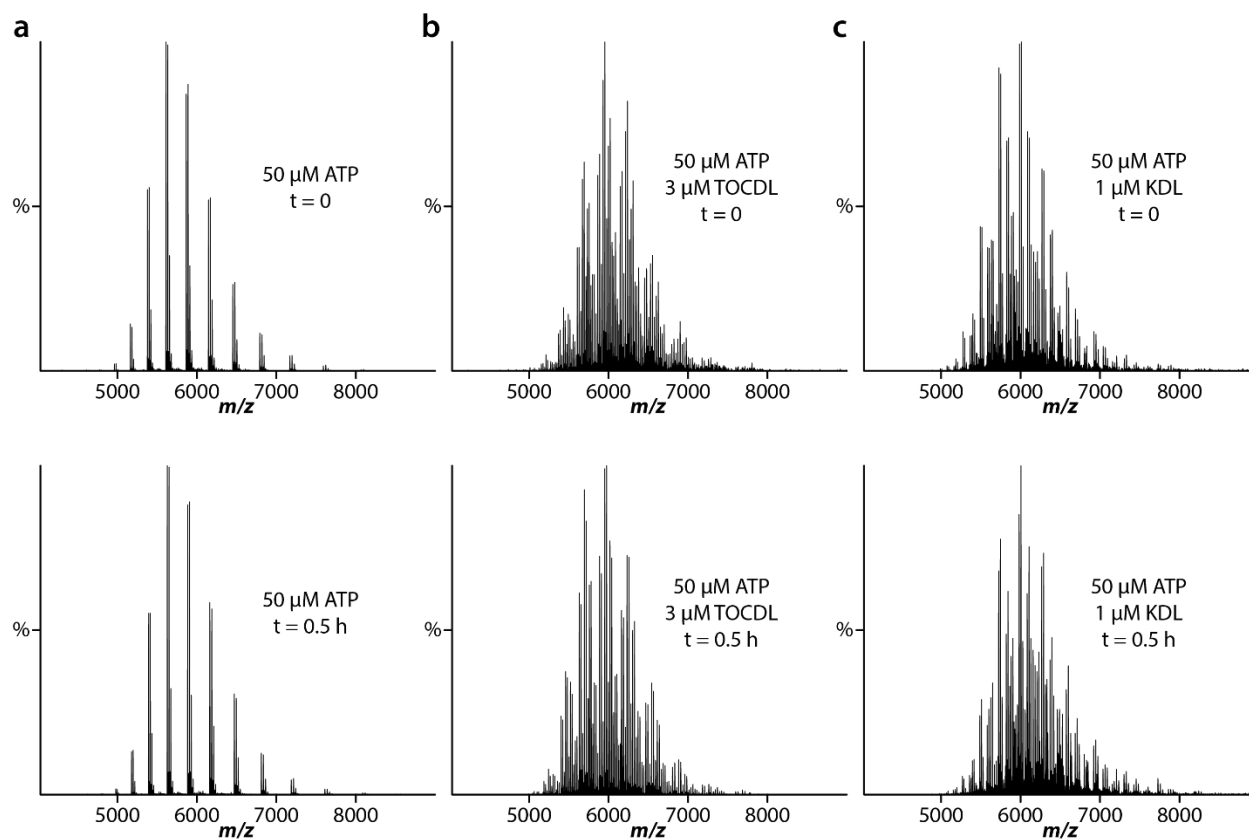
**Supplementary Fig. 3. Determination of equilibrium dissociation constants ( $K_D$ ) for individual nucleotide-binding events to MsbA in the presence of 50  $\mu\text{M}$   $\text{Mg}^{2+}$ .** Representative native mass spectra and the deconvolution for nucleotide binding to MsbA. MsbA (0.5  $\mu\text{M}$ ) was mixed with 50  $\mu\text{M}$   $\text{Mg}^{2+}$  and different concentrations of **a** ATP and **b** ADP. **c** Plot of mole fraction data for MsbA(ATP)<sub>0-2</sub> determined from the titration series (dots) and resulting fit from a sequential ligand-binding model (solid lines). **d** Plot of mole fraction data for MsbA(ADP)<sub>0-2</sub> determined as described for panel c. **e**  $K_{Dn}$  values for the  $n^{\text{th}}$  nucleotide binding to MsbA. Reported are the mean and standard deviation ( $n = 3$ , biological replicates). Source data are provided as a Source Data file.



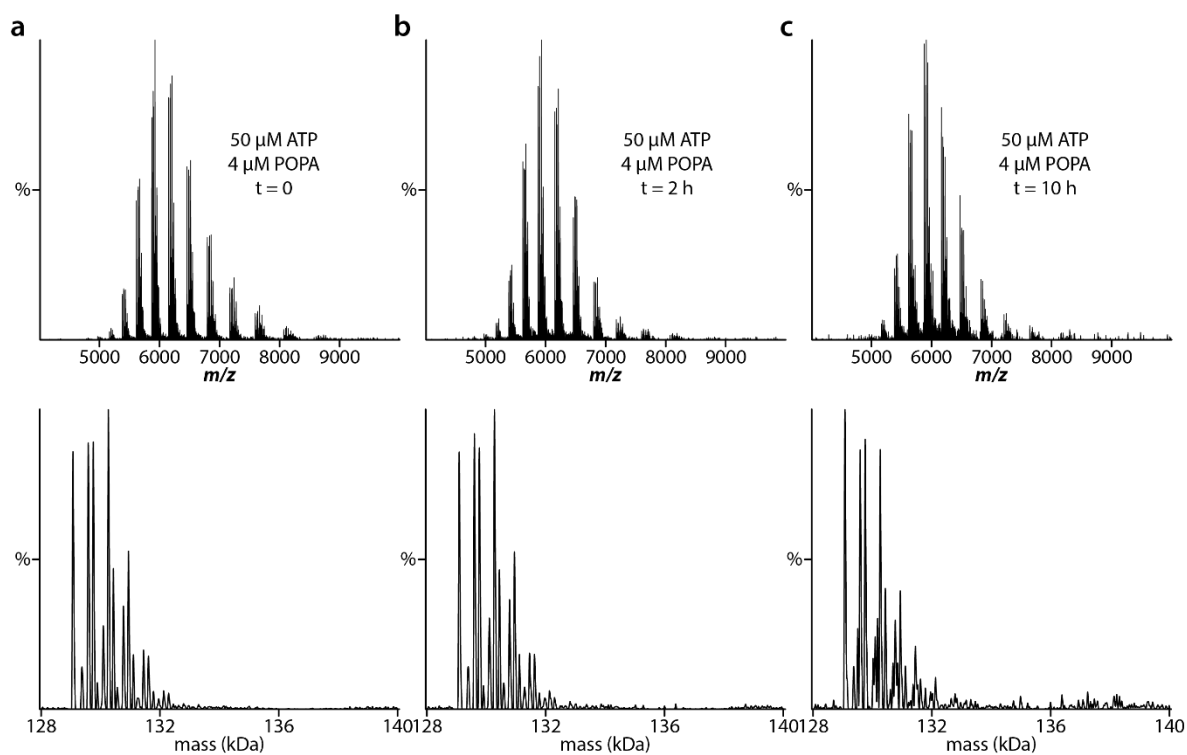
**Supplementary Fig. 4. Native mass spectra of ADP and lipid binding to MsbA.** The deconvolution of these mass spectra are shown in Figure 2.



**Supplementary Fig. 5. Characterization of ADP and lipid binding to MsbA.** Native mass spectra (top) and the deconvolution (bottom) of 0.5  $\mu\text{M}$  MsbA mixed with 10  $\mu\text{M}$   $\text{Mg}^{2+}$ , 25  $\mu\text{M}$  ADP and **a** 3  $\mu\text{M}$  TOCDL or **b** 1  $\mu\text{M}$  KDL. Data was recorded after 2-hour incubation.

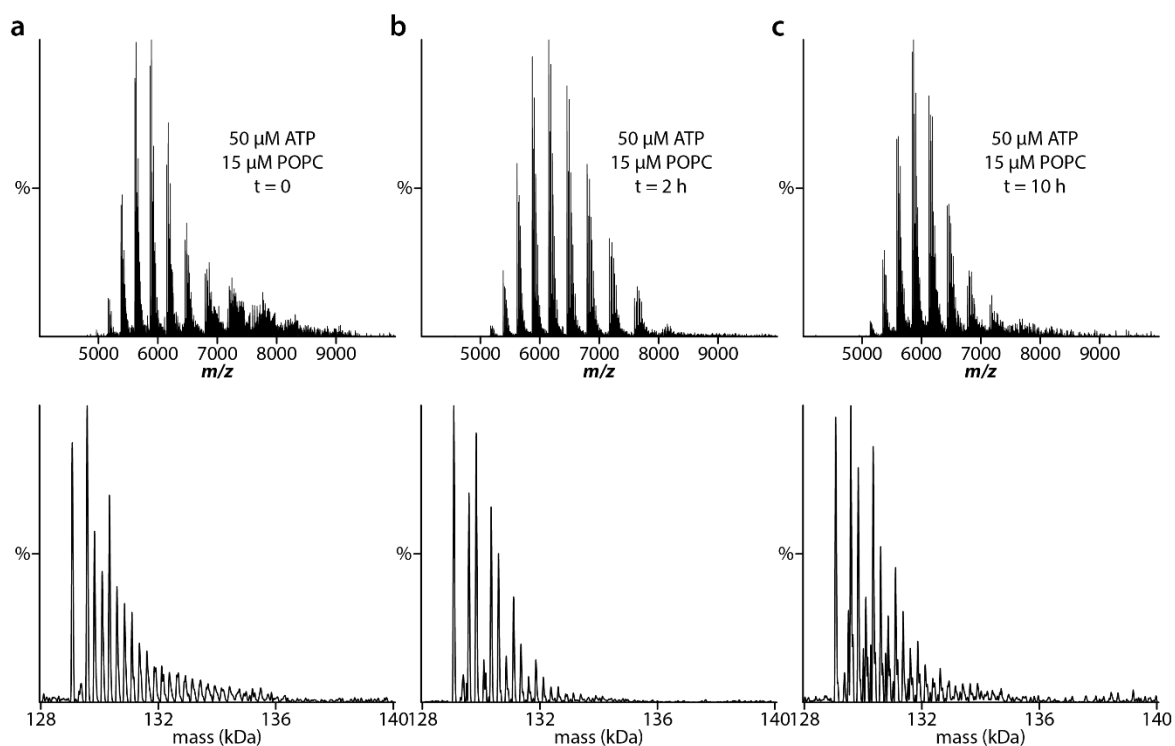


**Supplementary Fig. 6. Native mass spectra of ATP and lipid binding to MsbA.** The deconvolution of these mass spectra are shown in Figure 3.

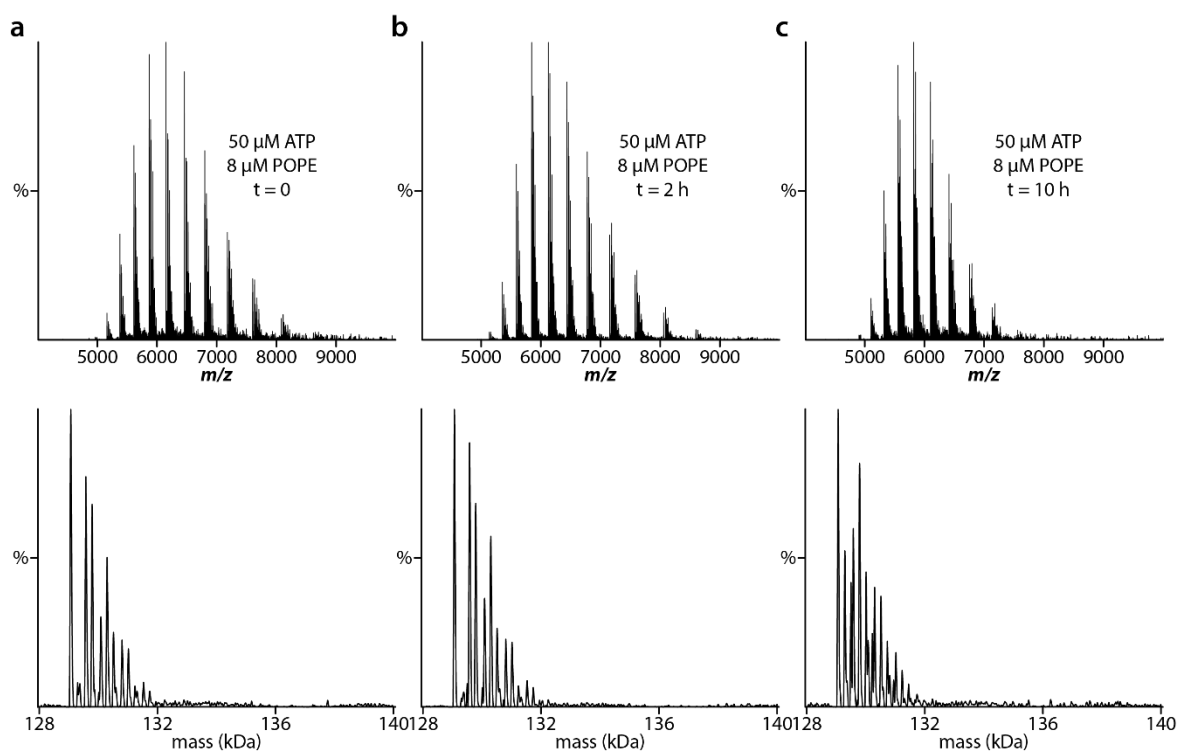


**Supplementary Fig. 7. Native mass spectra of MsbA mixed with ATP and POPA.** Mass spectra (top) and deconvolution (bottom) for 0.5  $\mu\text{M}$  MsbA mixed with 10  $\mu\text{M}$   $\text{Mg}^{2+}$ , 50  $\mu\text{M}$  ATP and 4  $\mu\text{M}$  POPA. Data was acquired **a** right after mixing and after incubation for **b** 2 and **c** 10 hours.

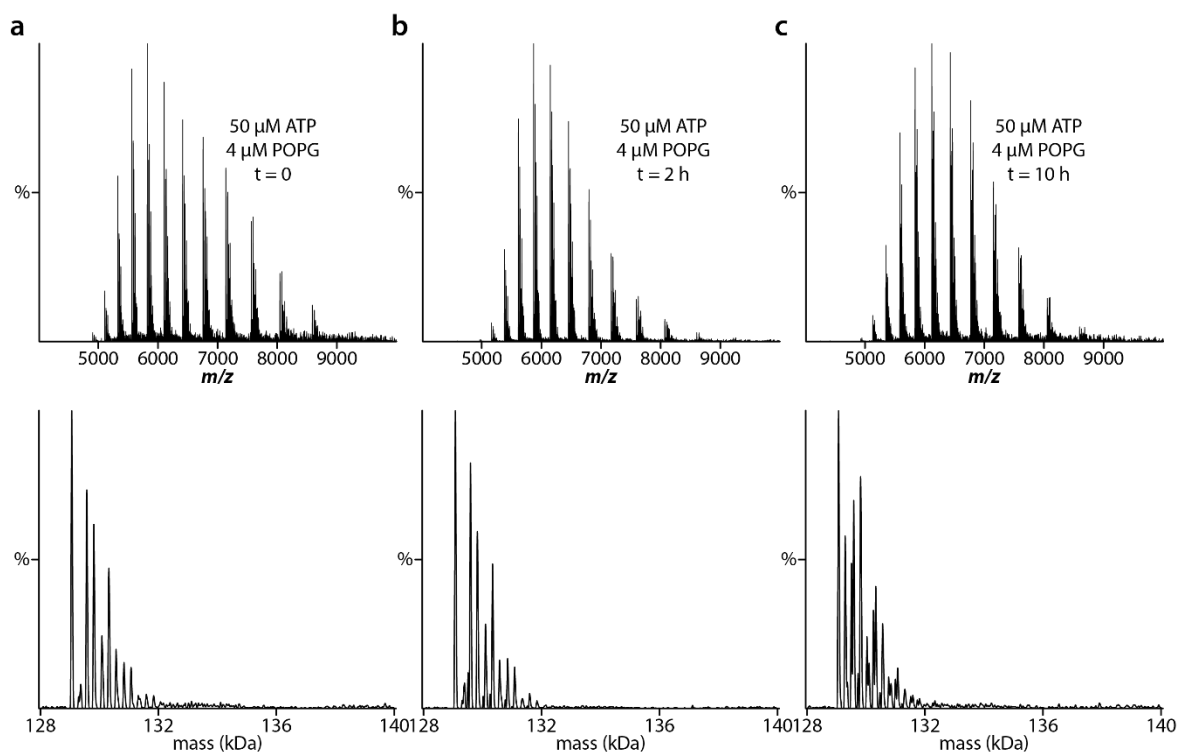




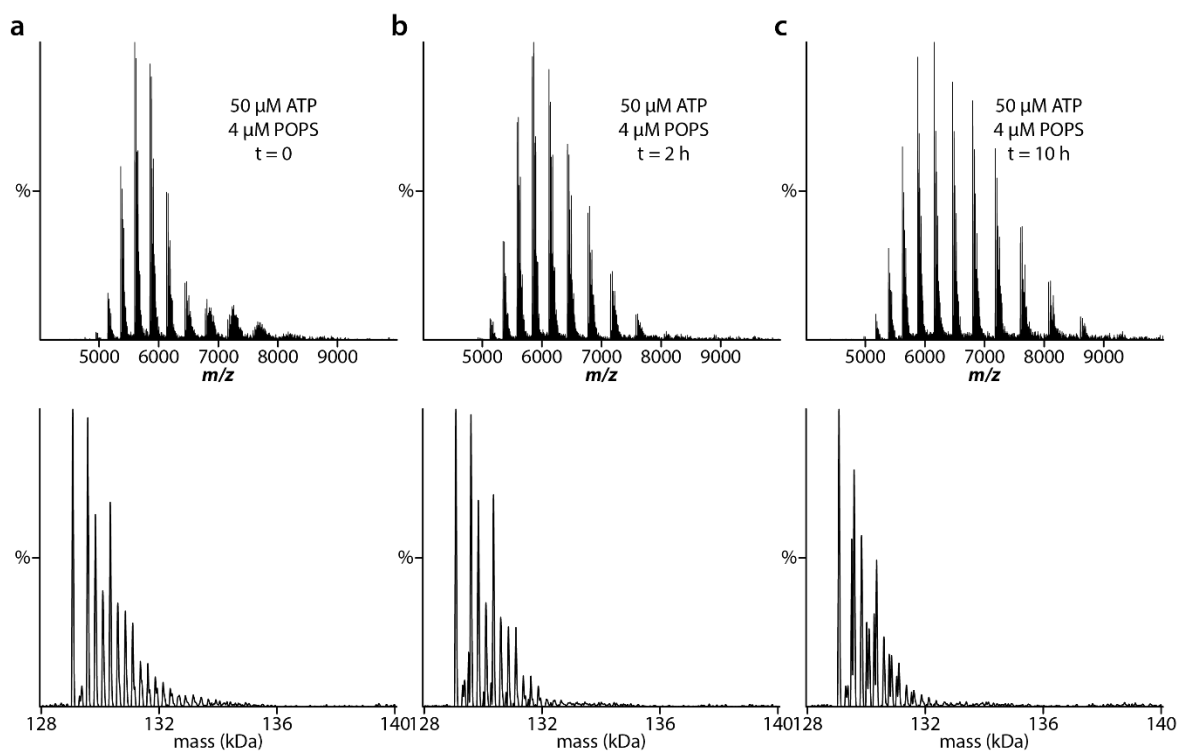
**Supplementary Fig. 8. Native mass spectra of MsbA mixed with ATP and POPC.** Mass spectra (top) and deconvolution (bottom) for 0.5  $\mu\text{M}$  MsbA mixed with 10  $\mu\text{M}$   $\text{Mg}^{2+}$ , 50  $\mu\text{M}$  ATP and 15  $\mu\text{M}$  POPC. Data was acquired **a** right after mixing and after incubation for **b** 2 and **c** 10 hours.



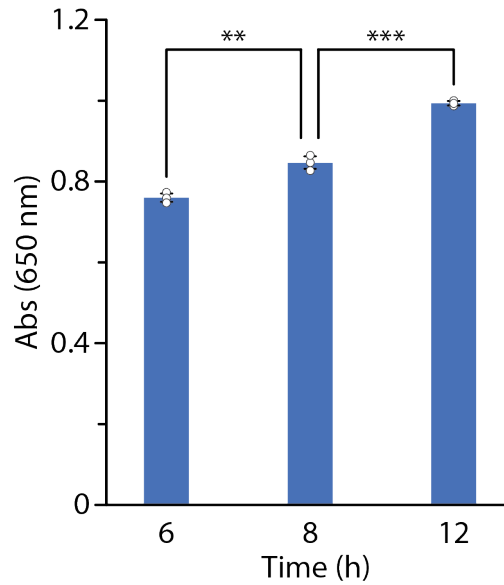
**Supplementary Fig. 9. Native mass spectra of MsbA mixed with ATP and POPE.** Mass spectra (top) and deconvolution (bottom) for 0.5  $\mu$ M MsbA mixed with 10  $\mu$ M  $\text{Mg}^{2+}$ , 50  $\mu$ M ATP and 8  $\mu$ M POPE. Data was acquired **a** right after mixing and after incubation for **b** 2 and **c** 10 hours.



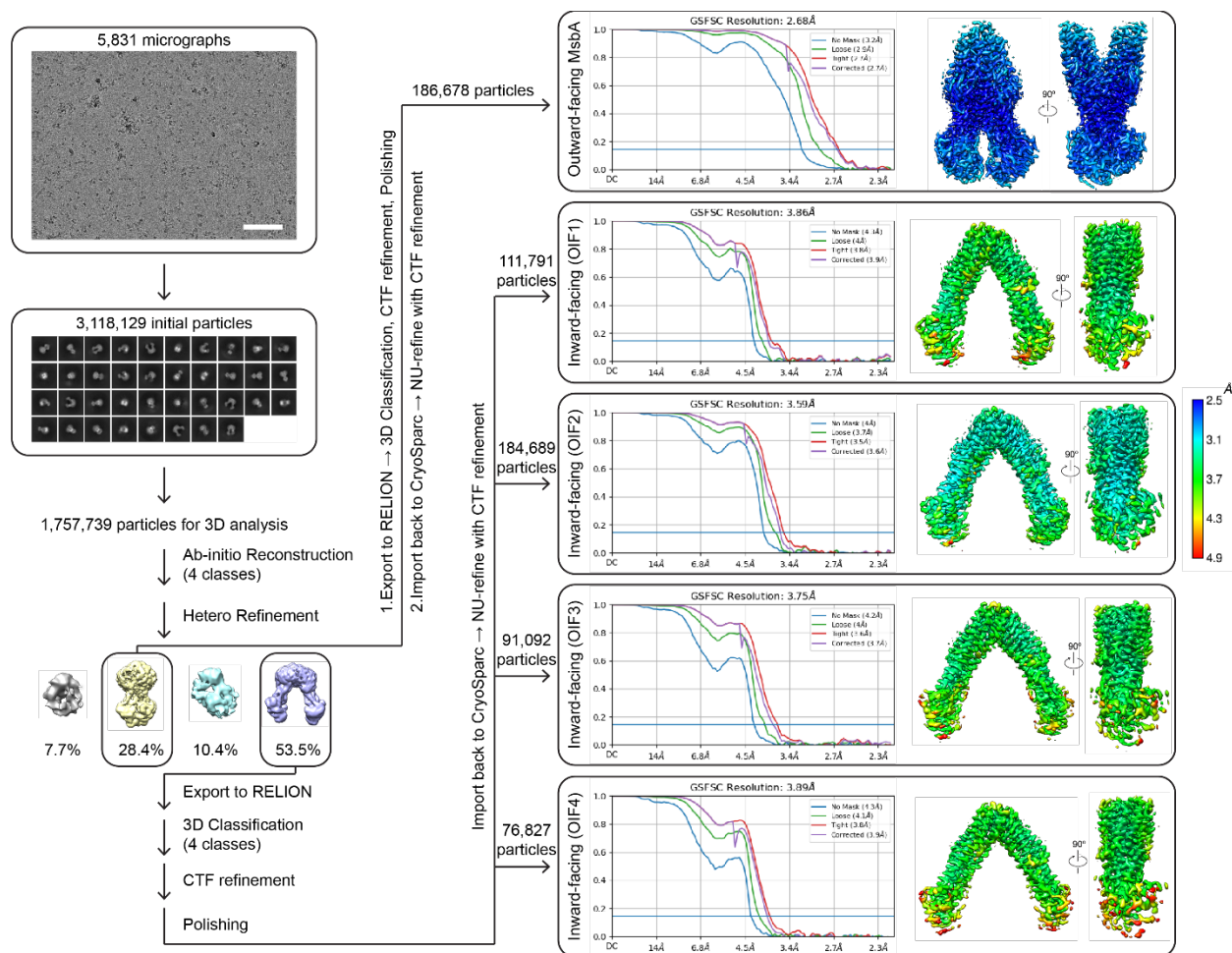
**Supplementary Fig. 10. Native mass spectra of MsbA mixed with ATP and POPG.** Mass spectra (top) and deconvolution (bottom) for 0.5  $\mu\text{M}$  MsbA mixed with 10  $\mu\text{M}$   $\text{Mg}^{2+}$ , 50  $\mu\text{M}$  ATP and 4  $\mu\text{M}$  POPG. Data was acquired **a** right after mixing and after incubation for **b** 2 and **c** 10 hours.



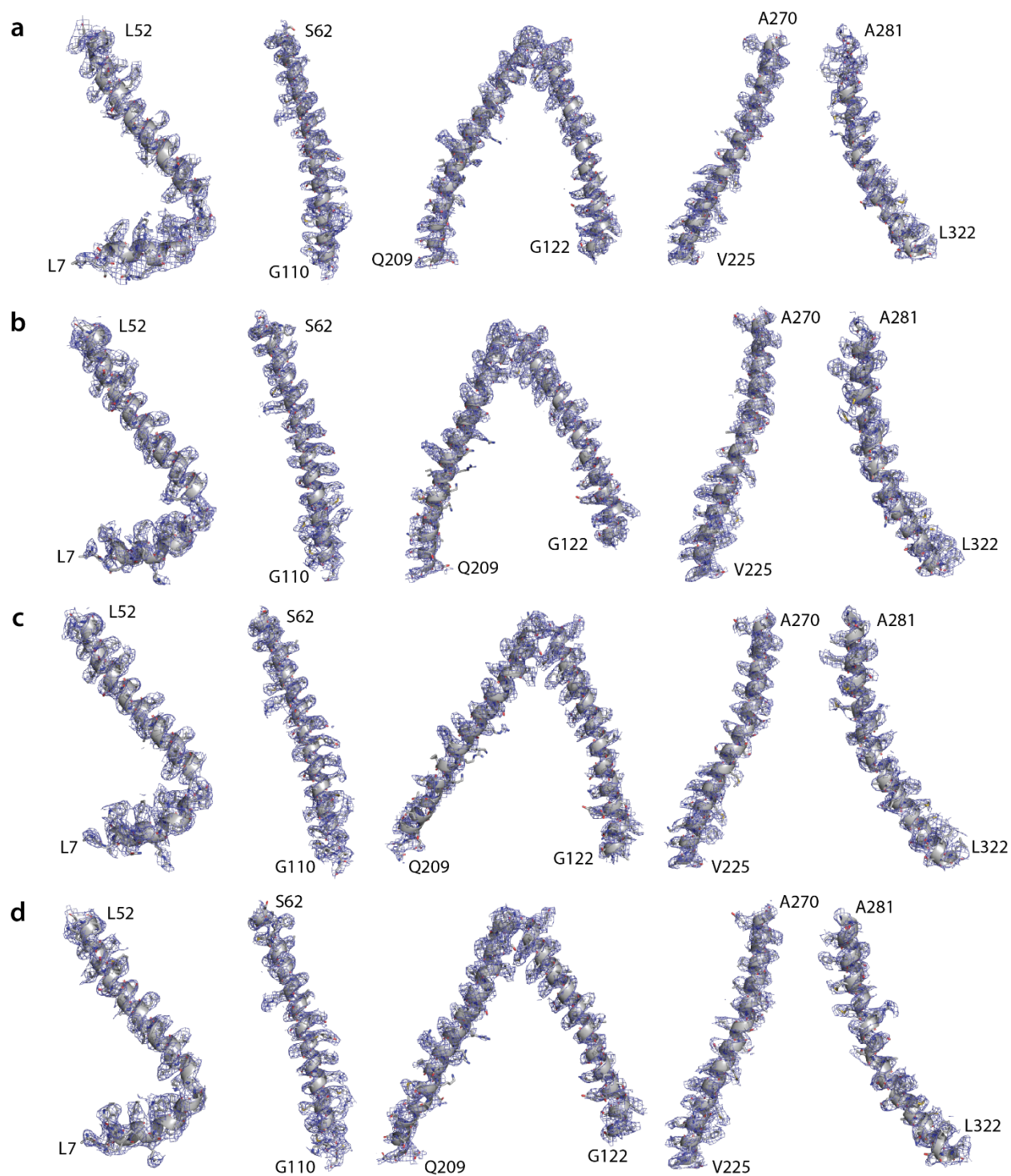
**Supplementary Fig. 11. Native mass spectra of MsbA mixed with ATP and POPS.** Mass spectra (top) and deconvolution (bottom) for 0.5  $\mu$ M MsbA mixed with 10  $\mu$ M  $\text{Mg}^{2+}$ , 50  $\mu$ M ATP and 4  $\mu$ M POPS. Data was acquired **a** right after mixing and after incubation for **b** 2 and **c** 10 hours.



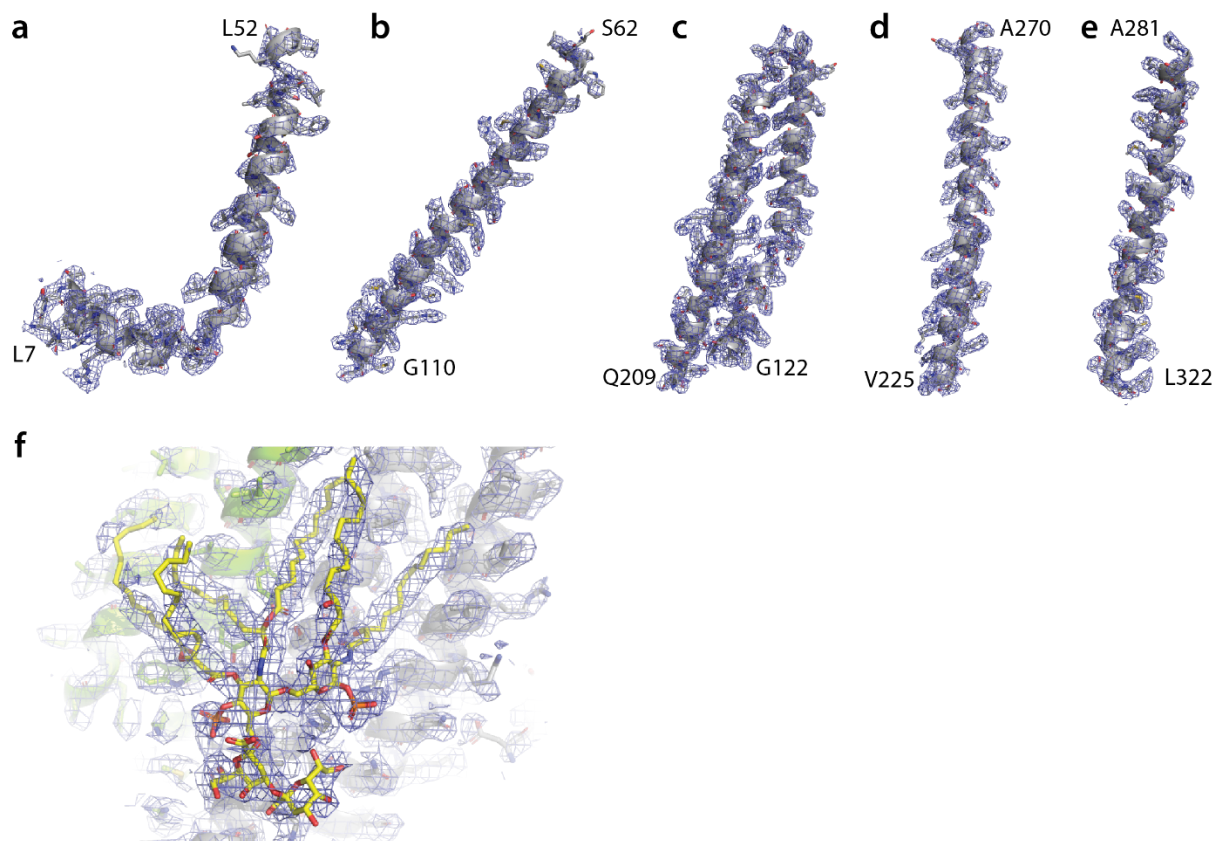
**Supplementary Fig. 12. ATPase activity assay of MsbA sample under conditions used for cryoEM.** MsbA samples were frozen at a 6-hour time point and the transporter was still active, hydrolyzing ATP. Reported are the mean and standard deviation of the absorbance from a malachite green assay ( $n = 3$ , biological replicates). A student's  $t$ -test (\*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ) was performed to compare different time points. Source data are provided as a Source Data file.



**Supplementary Fig. 13. Single-particle cryoEM analysis workflow for MsbA.** A representative motion-corrected micrograph is shown along with a 90-nm scale bar. Representative 2D class averages are shown, with the edges of the bounding boxes corresponding to  $\sim 273$  Å. Fourier shell correlation curves of the final reconstructions are presented. The final sharpened maps, colored with local resolutions, are shown in two distinct orientations. The outward-facing map is contoured using a threshold of 1.4. The inward-facing maps are contoured using a threshold of 0.4. The resolutions reported in this study were determined using the FSC=0.143 criterion.

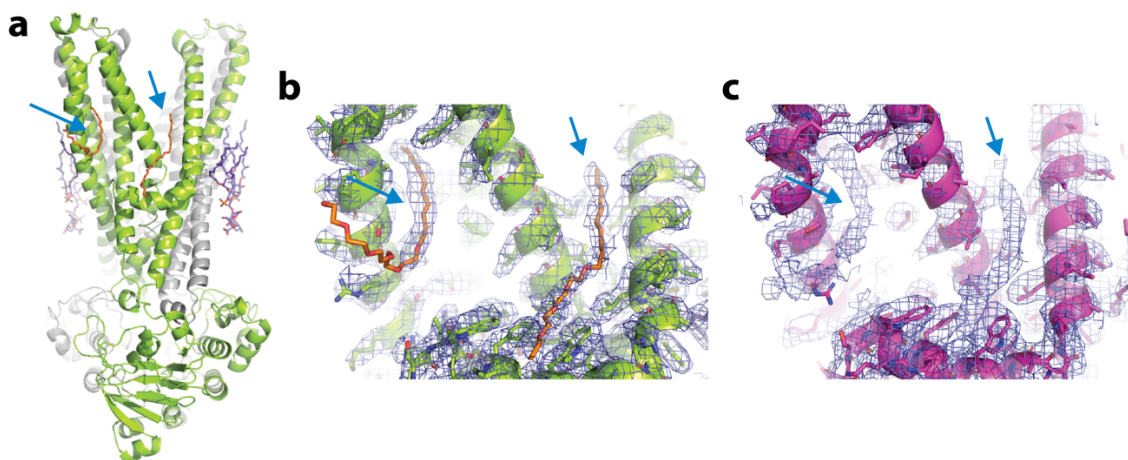


**Supplementary Fig. 14. CryoEM density of the inward-facing MsbA structures.** Density (contoured at 6 sigma) and atomic model for six transmembrane helices of **a** OIF1, **b** OIF2, **c** OIF3 and **d** OIF4 MsbA structures.



**Supplementary Fig. 15. CryoEM density of the open, outward-facing MsbA structure. a-e** Density (contoured at 6 sigma) and atomic model for six transmembrane helices. **f** Density (contoured at 6 sigma) for the bound KDL.





**Supplementary Fig. 16. Tube-like density in open, outward-facing MsbA nucleotide-free and bound to ADP and vanadate.** **a** Structure of nucleotide-free MsbA in open, outward-facing conformation shown in cartoon representation. Bound KDL (purple) and C<sub>10</sub>E<sub>5</sub> (orange) are shown in stick representation. The modeled C<sub>10</sub>E<sub>5</sub> detergents are highlighted by a blue arrow. **b** View of the tube-like densities (contoured at 6 sigma) in transmembrane region. The left penetrates a hydrophobic pocket formed by TM5 and TM6. The right is located between TM6 and TM1. **c** Similar view as b but for the open, outward-facing MsbA structure bound to KDL, ADP and vanadate. The density is shown as well (contoured at 4 sigma).

## Supplementary Tables

**Supplementary Table 1. Theoretical and experimental masses.** Reported are the mean and standard deviation of centroid masses.

	Theoretical (Da)	Experimental (Da)
<b>MsbA<sub>2</sub></b>	129,074	129,067 ± 2
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(ATP)<sub>1</sub></b>	129,581	129,573 ± 1
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(ATP)<sub>2</sub></b>	130,088	130,085 ± 2
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(ADP)<sub>1</sub></b>	129,501	129,494 ± 0
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(ADP)<sub>2</sub></b>	129,928	129,922 ± 1
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(KDL)<sub>1</sub>(ATP)<sub>2</sub></b>	132,323	132,324 ± 1
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(KDL)<sub>2</sub>(ATP)<sub>2</sub></b>	134,558	134,559 ± 2
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(KDL)<sub>3</sub>(ATP)<sub>2</sub></b>	136,793	136,796 ± 1
<b>MsbA<sub>2</sub>(Cu)<sub>2</sub>(KDL)<sub>4</sub>(ATP)<sub>2</sub></b>	139,028	139,035 ± 2

**Supplementary Table 2. Equilibrium dissociation constants of MsbA-nucleotide interactions determined by native MS.**  $K_D$  was obtained from fitting a sequential ligand binding model to mole fraction data from titration. Reported are the mean and standard deviation ( $n = 3$ , biological replicates).

$Mg^{2+}$ ( $\mu M$ )	$K_{D1}$ (ATP, $\mu M$ )	$K_{D2}$ (ATP, $\mu M$ )	$K_{D1}$ (ADP, $\mu M$ )	$K_{D2}$ (ADP, $\mu M$ )
<b>10</b>	47.8 $\pm$ 2.5	124.4 $\pm$ 6.2	17.8 $\pm$ 1.3	62.3 $\pm$ 4.7
<b>50</b>	49.0 $\pm$ 2.1	117.4 $\pm$ 6.7	16.7 $\pm$ 0.9	63.7 $\pm$ 6.6

**Supplementary Table 3. Distance between NBDs of open, inward-facing MsbA structures.** The reported distance corresponds to the distance between T561 C $\alpha$  to T561 C $\alpha$  of the neighboring subunit.

	Distance (Å)
OIF4 (8TSR)	91.7
OIF3 (8TSS)	89.9
3B5W	85.1
8DMO	79.3
OIF2 (8TSQ) and 6BL6	75.9
OIF1 (8TSP)	64.8

**Supplementary Table 4. Statistics of cryoEM data collection and processing.**

<b>Microscope</b>	Krios (University of Chicago)				
<b>Magnification</b>	81,000				
<b>Voltage (kV)</b>	300				
<b>Spherical aberration (mm)</b>	2.7				
<b>Detector</b>	K3				
<b>Camera mode</b>	Super resolution counting				
<b>Exposure rate (e<sup>-</sup>/pixel/s)</b>	15				
<b>Total exposure (e<sup>-</sup>/Å<sup>2</sup>)</b>	50				
<b>Defocus range (μm)</b>	-1.0 to -2.5				
<b>Pixel size (Å)</b>	0.5325 (1.065 physical)				
<b>Mode of data collection</b>	Image shift				
<b>Energy filter</b>	20 eV slit				
<b>Software for data collection</b>	EPU				
<b>Number of micrographs</b>	5,831				
<b>Symmetry imposed</b>	C2				
<b>Box size (pixel)</b>	256				
<b>Initial particle images (no.)</b>	3,118,129				
<b>Particle images for 3D (no.)</b>	1,757,739				
	Open, outward-facing MsbA	Open, inward-facing (OIF1)	Open, inward-facing (OIF2)	Open, inward-facing (OIF3)	Open, inward-facing (OIF4)
<b>Final particle images (no.)</b>	186,678	111,791	184,689	91,092	76,827
<b>Map resolution, unmasked (Å)</b>	3.2	4.3	4.0	4.2	4.3
<b>Map resolution, masked (Å)</b>	2.7	3.9	3.6	3.7	3.9
<b>B-factor used for sharpening (Å<sup>2</sup>)</b>	108.7	168.7	153.1	155.9	151.5
<b>EMD accession code</b>	EMD-41596	EMD-41597	EMD-41598	EMD-41560	EMD-41599

Supplementary Table 5. Statistics of cryoEM model refinement and geometry for five MsbA structures.

Model	Open, outward-facing MsbA	OIF1	OIF2	OIF3	OIF4
PDB accession code	8TSO	8TSP	8TSQ	8TSS	8TSR
Composition (#)					
Chains	4	2	2	2	2
Atoms	9298 (Hydrogens: 0)	8888 (Hydrogens: 0)	8918 (Hydrogens: 0)	8918 (Hydrogens: 0)	8888 (Hydrogens: 0)
Residues	Protein: 1146 Nucleotide: 0	Protein: 1146 Nucleotide: 0	Protein: 1150 Nucleotide: 0	Protein: 1150 Nucleotide: 0	Protein: 1146 Nucleotide: 0
Water	0	0	0	0	0
Ligands	KDL: 2 CXE: 4	0	0	0	0
Bonds (RMSD)					
Length (Å) (# > 4 $\sigma$ )	0.004 (0)	0.004 (0)	0.005 (0)	0.004 (0)	0.005 (0)
Angles (°) (# > 4 $\sigma$ )	0.626 (0)	0.752 (2)	0.655 (0)	0.750 (0)	0.832 (0)
MolProbity score	1.34	1.70	1.82	1.91	1.76
Clash score	5.75	10.09	10.06	11.94	11.20
Ramachandran plot (%)					
Outliers	0.00	0.00	0.00	0.09	0.00
Allowed	2.10	2.98	4.28	4.45	3.15
Favored	97.90	97.02	95.72	95.46	96.85
Rotamer outliers (%)	0.00	0.72	0.51	0.41	0.51
C $\beta$ outliers (%)	0.00	0.00	0.00	0.00	0.00
Peptide plane (%)					
Cis proline/general	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
Twisted proline/general	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	7.7/0.0
CaBLAM outliers (%)	1.41	1.41	2.71	2.36	1.67
ADP (B-factors)					
Iso/Aniso (#)	9298/0	8888/0	8918/0	8918/0	8888/0
Protein	52.90	40.94	14.99	56.07	59.21
Ligand	47.17	/	/	/	/
Data					
Box					

<b>Lengths (Å)</b>	90.53, 75.62, 136.32	67.10, 122.48, 136.32	75.62, 133.13, 135.26	67.10, 135.26, 136.32	67.10, 139.52, 135.26
<b>Angles (°)</b>	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
<b>Supplied Resolution (Å)</b>	2.7	3.9	3.6	3.8	3.9
<b>Resolution Estimates, Masked (Å)</b>					
<b>d model</b>	3.0	4.2	3.9	4.1	4.2
<b>d FSC model (0/0.143/0.5)</b>	2.6/2.6/2.8	3.7/3.8/4.1	3.3/3.5/3.9	3.6/3.7/4.1	3.7/3.8/4.1
<b>Map min/max/mean</b>	-6.34/11.07/0.10	-1.73/2.41/0.04	-2.44/3.34/0.04	-1.72/2.30/0.03	-1.53/1.88/0.04
<b>Model vs. Data</b>					
<b>CC (mask)</b>	0.80	0.74	0.72	0.74	0.76
<b>CC (box)</b>	0.64	0.68	0.67	0.67	0.71
<b>CC (peaks)</b>	0.63	0.62	0.62	0.60	0.63
<b>CC (volume)</b>	0.76	0.71	0.69	0.70	0.72

