

Supplementary materials for

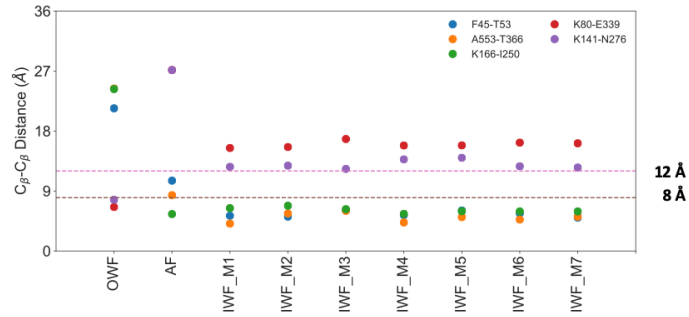
Evidence for a trap-and-flip mechanism in a proton-dependent lipid transporter

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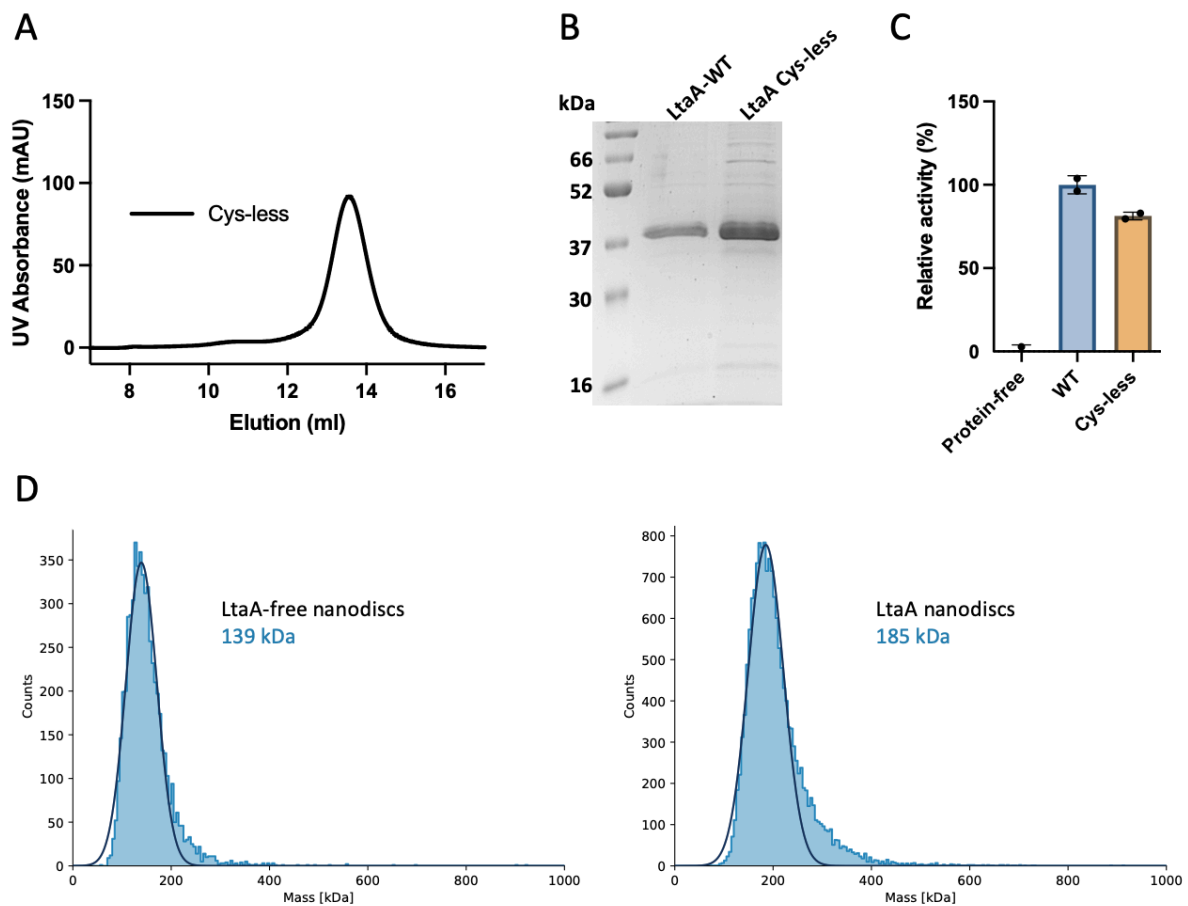
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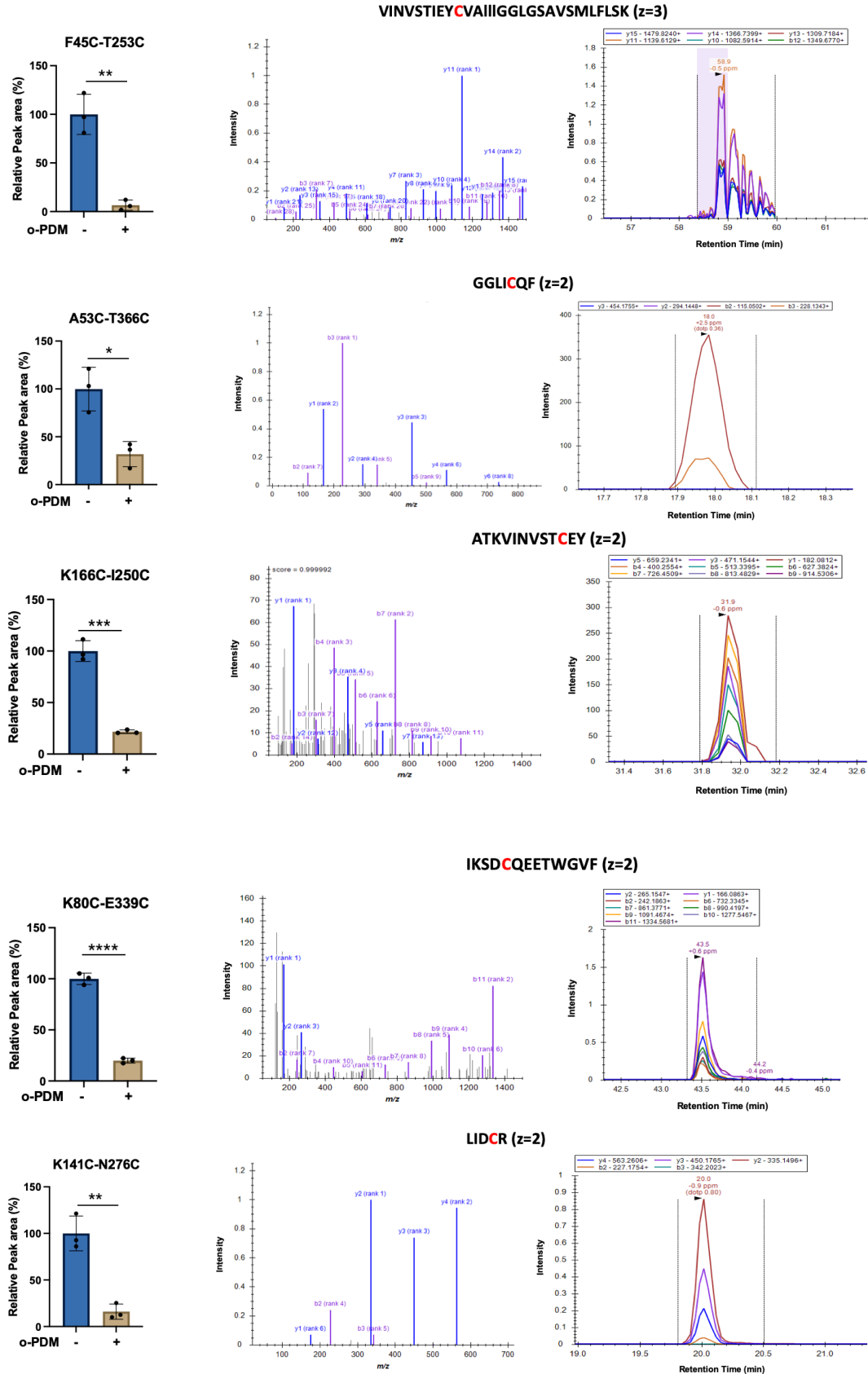


| Residues | OWF | AF | IWF_M1 | IWF_M2 | IWF_M3 | IWF_M4 | IWF_M5 | IWF_M6 | IWF_M7 |
|-----------|------|------|--------|--------|--------|--------|--------|--------|--------|
| F45-T253 | 21.4 | 10.5 | 5.3 | 5.2 | 6.2 | 5.4 | 6.1 | 5.7 | 5.0 |
| A53-T366 | 24.4 | 8.4 | 4.1 | 5.7 | 6.1 | 4.3 | 5.1 | 4.8 | 5.2 |
| K166-I250 | 24.3 | 5.5 | 6.4 | 6.8 | 6.3 | 5.7 | 6.0 | 6.0 | 6.0 |
| K80-E339 | 6.7 | 27.1 | 15.5 | 15.6 | 16.8 | 15.8 | 15.8 | 16.2 | 16.1 |
| K141-N276 | 7.6 | 27.1 | 12.7 | 12.8 | 12.4 | 13.8 | 14.0 | 12.8 | 12.5 |

Supplementary figure 1. Distances between residues selected for cross-linking in alternating conformations of LtaA. C β -C β distances between residues selected for cysteine cross-linking in the outward-facing structure (OWF), in the best inward-facing models (IWF_M), and in the AlphaFold (AF) inward-facing model. Distance thresholds considered for selection of residues pairs are shown.

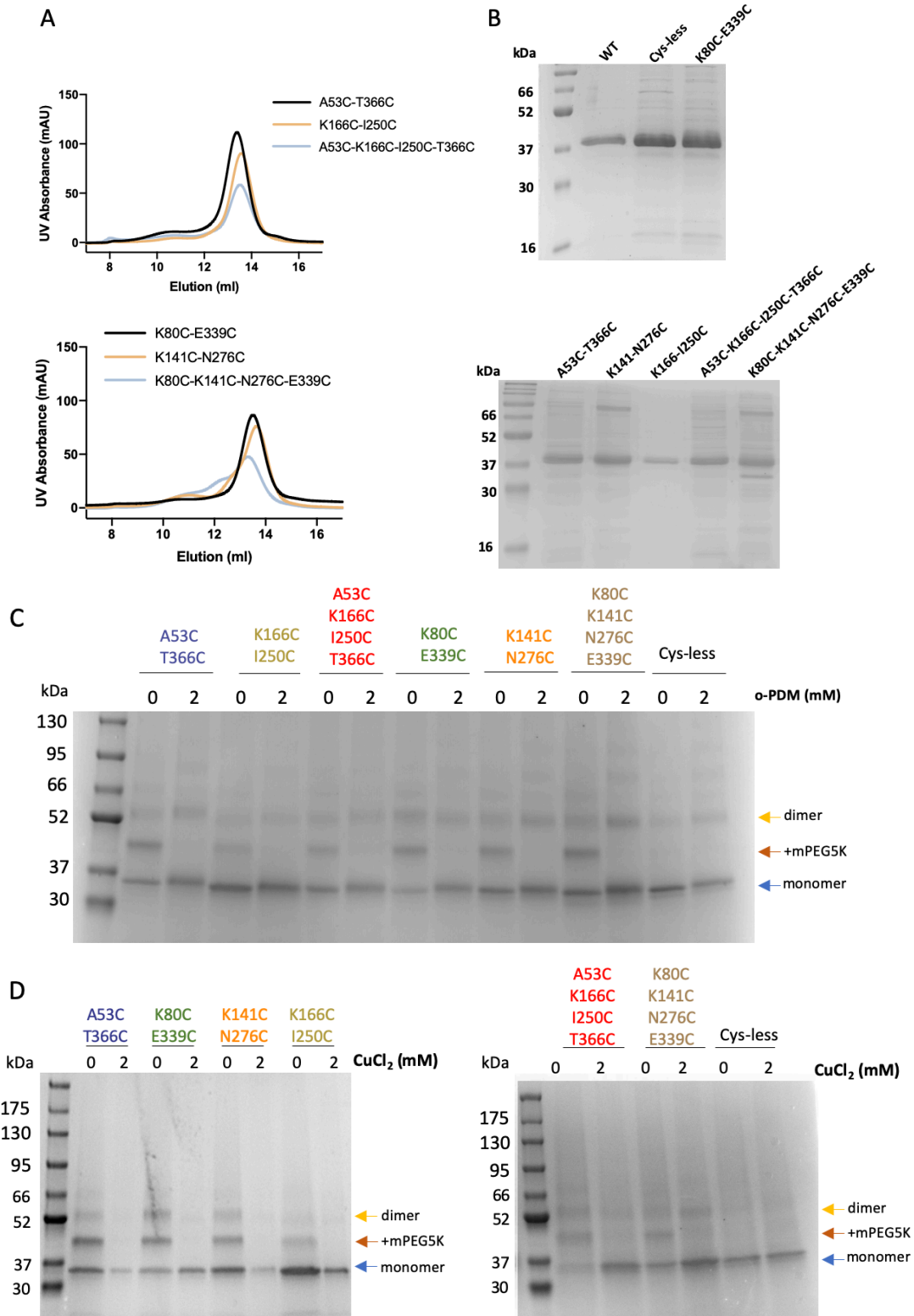


Supplementary figure 2. Analysis of LtaA wild-type and cysteine-less variant. **A.** Size Exclusion Chromatography profile of cysteine-less LtaA in a Superdex 200 Increase 10/300 GL column. **B.** SDS-PAGE of purified LtaA-WT and LtaA Cys-less. **C.** Relative flipping activity of LtaA Cys-less and LtaA-WT. Error bars show +/- standard deviation (s.d.) of technical replicates, n=3. **D.** Mass photometry analysis of nanodiscs without LtaA (*left*) and LtaA-reconstituted nanodiscs (*right*). The approximate molecular mass averages from Gaussian distributions are shown. The resulting apparent molecular mass difference (LtaA mass) is 46 kDa.

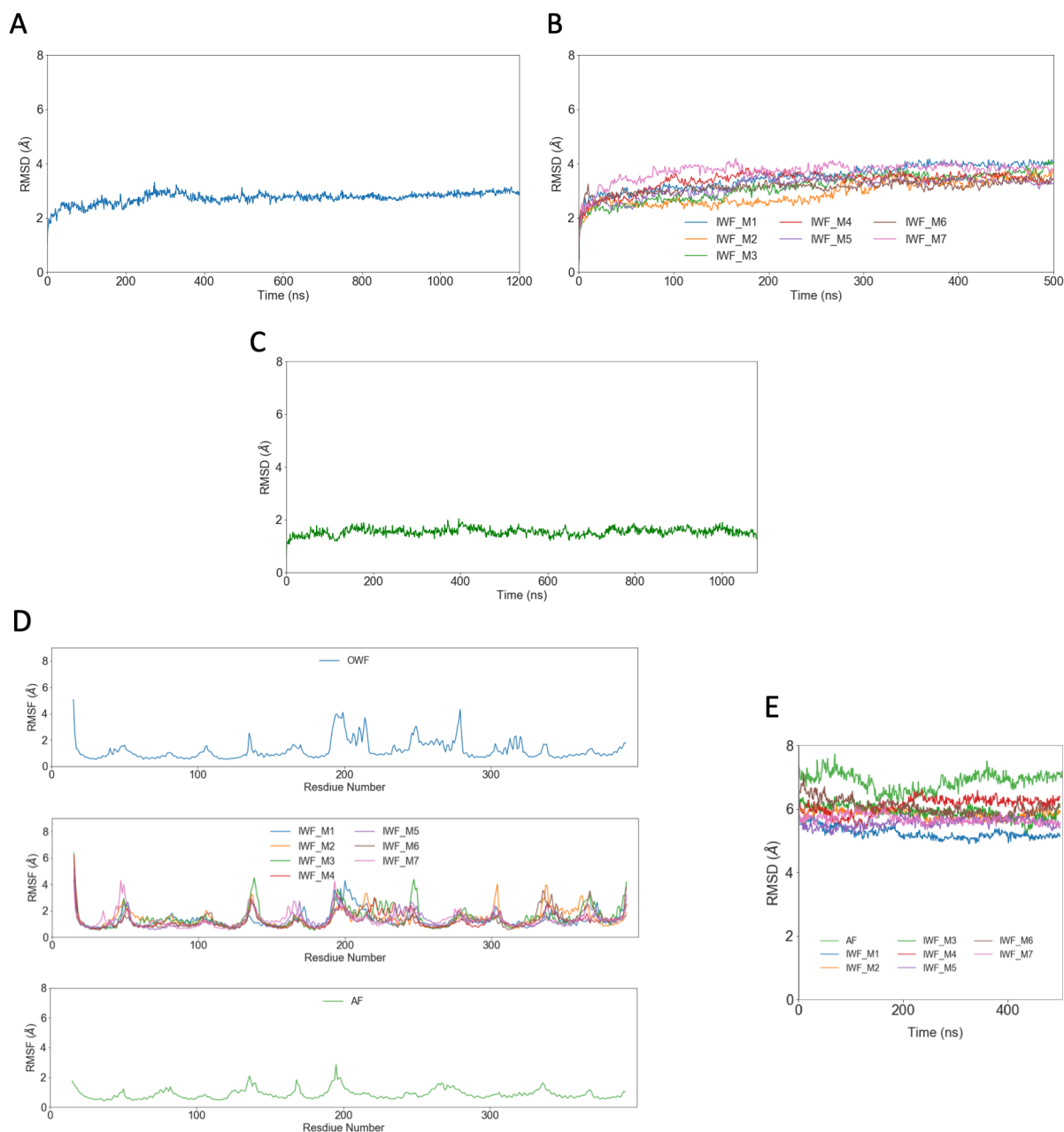


Supplementary figure 3. LC-MS analysis of cysteine cross-linking showing that LtaA adopts inward- and outward-facing states. Selected pairs of residues for cross-linking are shown in Fig. 2. Left: Relative abundance of cysteine containing peptides in absence (-) or presence (+)

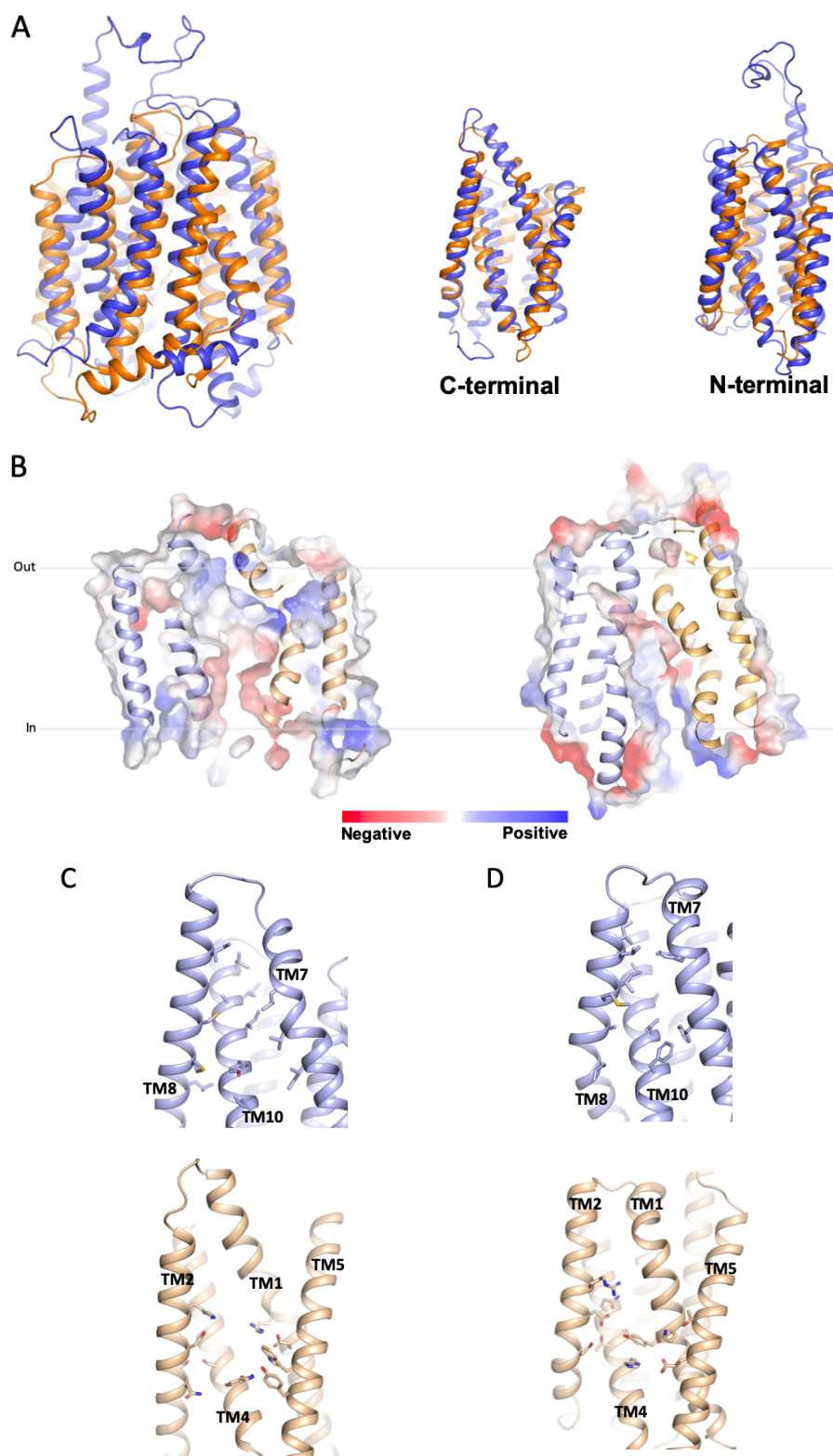
of N,N'-(o-phenylene)-dimaleimide (o-PDM). Error bars indicate +/- s.d. (n=3, biological replicates). *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. Asterisks mark the result from unpaired t-test. *Center*: Assigned Collision-Induced Dissociation (CID) spectra obtained from cysteine containing peptides in absence of o-PDM. The position of the alkylated cysteine is indicated in red and the charge state (z) of the identified peptide ion is provided. *Right*: Elution profiles of the peptide fragments (transitions) identified used for peptide quantification by targeted LC-MS analysis. Source data are provided as a Source Data file.



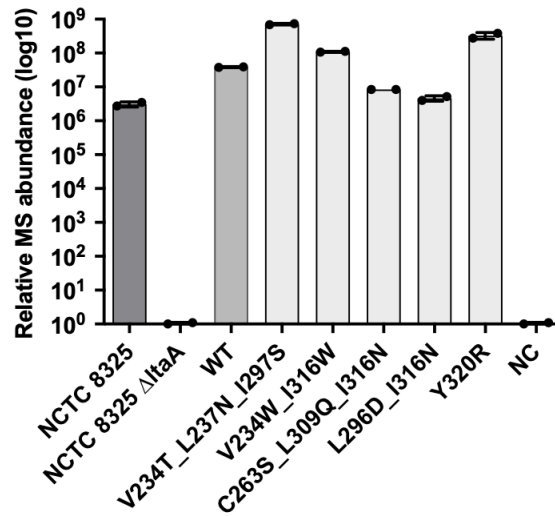
Supplementary figure 4. Purifications and cross-linking in-gel analysis of LtaA variants. **A.** Size Exclusion Chromatography profile of LtaA variants in a Superdex 200 Increase 10/300 GL column. **B.** SDS-PAGE of purified LtaA WT and variants. **C** and **D.** SDS-PAGE showing band shifts of samples treated with mPEG5K after cross-link with o-PDM or CuCl₂, respectively. SDS-PAGE experiments were independently repeated at least three times with similar results. Source data are provided as a Source Data file.



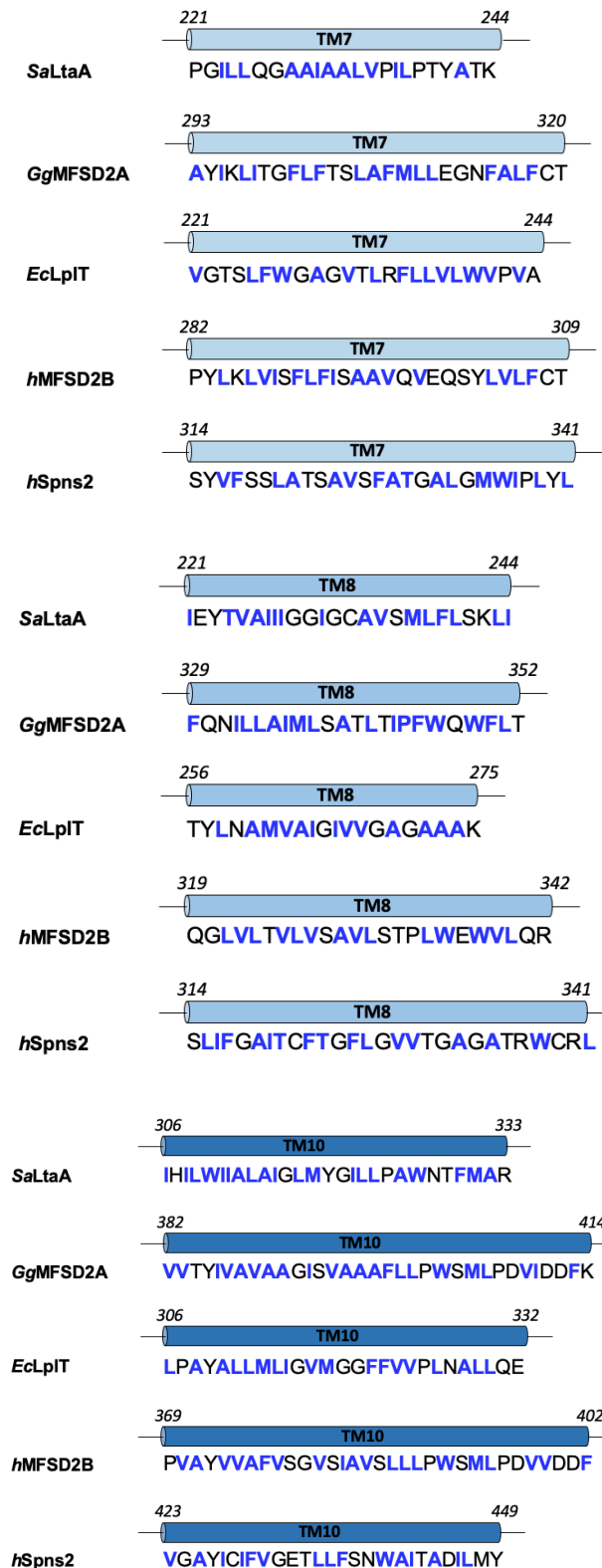
Supplementary figure 5. Stability analysis from molecular dynamics simulations of LtaA in a membrane. **A-C.** Root-mean-square-deviation (RMSD) plots of C α atoms relative to their starting points during MD simulations in a membrane composed of POG (65%), diacylglycerol (20%), cardiolipin (10%), and gentiobiosyl-diacylglycerol (5%). RMSD plots of C α atoms for outward-facing LtaA (**A**), inward-facing LtaA models obtained from the ‘repeat-swap’ approach (IWF_M) (**B**), and the inward-facing LtaA model generated by AlphaFold (AF) (**C**) are shown. **D.** Root-mean-square-fluctuation (RMSF) plot of C α atoms for outward-facing (OWF) LtaA, ‘repeat-swap’ inward-facing LtaA models, and the inward-facing LtaA model generated by AlphaFold (AF), calculated for each MD simulation based on the average structure. **E.** RMSD plot of C α atoms of ‘repeat-swap’ inward-facing LtaA models and the AF inward-facing LtaA model during MD simulations relative to the outward-facing structure (PDB ID 6S7V).



Supplementary figure 6. Comparison of LtaA and MFSD2A fold and amphipathic cavity. A. Superposition of LtaA (orange) and MFSD2A (blue) (r.m.s.d = 4.1Å), and individual N- and C-terminal domains (r.m.s.d= 2.6Å and 2.8Å, respectively). **B.** Vacuum electrostatic surface representation of inward-facing model of LtaA (*left*) and inward-facing MFSD2A (PDB ID 7MJS) (*right*). Residues participating in formation of N- and C-terminal hydrophilic and hydrophobic pockets in LtaA (**C**) and MFSD2A (**D**). N-terminal and C-terminal domains are shown in light orange and light blue, respectively.



Supplementary figure 7. LC-MS analysis of relative abundance of LtaA-WT and variants in *S. aureus* membranes. The histogram shows relative abundances of LtaA WT and variants. Error bars show +/- s.d. of biological replicates (n=2).



Supplementary figure 8. C-terminal TM helices participating in formation of hydrophobic pockets in MFS lipid transporters. Residues in TM7, 8, and 10 as seen in the structures of *S. aureus* LtaA (PDB ID 6S7V) and *Gallus gallus* MFSD2A (PDB ID 7MJS), and residues in predicted helical segments corresponding to the same TMs in *E. coli* LpIT, human MFSD2B, and human Spns2 are shown. Hydrophobic residues are shown in blue.

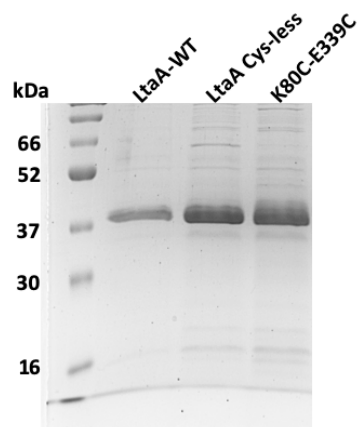
Supplementary Table 1. Validation parameters of the top seven inward-facing models.
Initial: homology models from Modeler: *SCWRL*; the refined model by *SCWRL4*; *mini*: energy minimization of the model in a bilayer using Gromacs.

| Model | Clashscore | | | MolProbity score | | | Ramachandran favoured (%) | | | Ramachandran outliers (%) | | |
|-------|------------|-------|------|------------------|-------|------|---------------------------|-------|------|---------------------------|-------|------|
| | Initial | SCWRL | mini | Initial | SCWRL | mini | Initial | SCWRL | mini | Initial | SCWRL | mini |
| 1 | 144.2 | 125.8 | 11.5 | 3.46 | 2.89 | 2.03 | 95.5 | 95.2 | 93.4 | 0.8 | 1.0 | 1.0 |
| 2 | 155.0 | 125.3 | 11.5 | 3.49 | 2.89 | 2.15 | 95.2 | 95.2 | 92.9 | 0.8 | 0.8 | 1.6 |
| 3 | 145.5 | 125.1 | 10.3 | 3.45 | 2.91 | 2.3 | 94.9 | 94.9 | 92.2 | 1.1 | 1.1 | 1.6 |
| 4 | 137.9 | 117.8 | 12.0 | 3.25 | 2.85 | 2.05 | 95.5 | 95.5 | 92.9 | 0.5 | 0.5 | 0.8 |
| 5 | 144.9 | 119.1 | 10.6 | 3.34 | 2.89 | 1.98 | 94.9 | 95.0 | 93.4 | 0.8 | 0.8 | 1.3 |
| 6 | 134.7 | 125.7 | 8.12 | 3.35 | 2.89 | 2.17 | 95.2 | 95.2 | 93.6 | 1.1 | 1.1 | 1.1 |
| 7 | 131.8 | 125.5 | 9.81 | 3.31 | 2.86 | 2.18 | 95.7 | 95.7 | 92.3 | 1.1 | 1.1 | 1.1 |

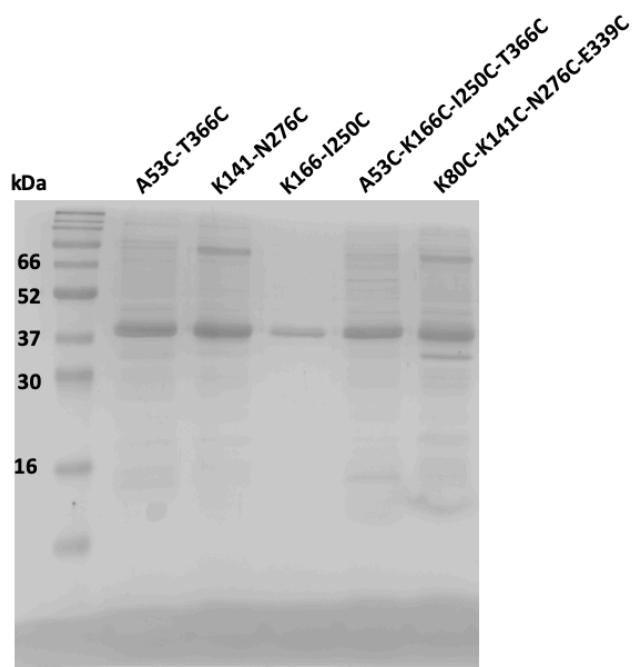
Supplementary Table 2. Oligo's used in this study. Mutagenesis is underlined

| Oligo name | 5'-3' sequence |
|---------------------|--|
| Sa-Sall-FW | GAGAGTCGACGGTCATTCATCACAACC |
| Sa-V234T-L237N-FW | GGTGCCGCAATTGCTGCCCTAACCCCTATAAAATCCAACATATGCTACTAAGGTTAT |
| Sa-V234T-L237N-RV | ATAACCTTAGTAGCATATGTTGGATTATAGGGGTTAGGGCAGCAATTGCGGCACC |
| Sa-V234W-FW | GTGCCGCAATTGCTGCCCTATGGCCTATATTACCAACATATGCTAC |
| Sa-V234W-RV | GTAGCATATGTTGGTAATATAGGCCATAGGGCAGCAATTGCGGCACC |
| Sa-C263S-FW | CATTATTGGTGGTATCGGCTCTGCAGTTTCGATGCTATTT |
| Sa-C263S-RV | AAATAGCATCGAACTGCAGAGCCGATACCACCAATAATG |
| Sa-L296D-FW | TCTAAGCGGATTTATTTTATACATGATAGATATTTTACTCTATCTATGATTGTTAATATTCACA |
| Sa-L296D-RV | TGTGAATATTAACAATCATAGATAGAGTAAAAATATCTATCATGTATAAAAATAAATCCGCTTAGA |
| Sa-I297S-FW | GATTTATTTTATACATGATATTAAGTTTTACTCTATCTATGATTGTTAATATTC |
| Sa-I297S-RV | GAATATTAACAATCATAGATAGAGTAAAACTTAATATCATGTATAAAAATAAATC |
| Sa-L309Q-I316N-FW | CTATGATTGTTAATATTCACATCCAATGGATTATCGCTTTAGCTAATGGTCTAATGTATGGCATCTT ATTAC |
| Sa-L309Q-I316N-RV | GTAATAAGATGCCATACATTAGACCATTAGCTAAAGCGATAATCCATTGGATGTGAATATTAACA ATCATAG |
| Sa-I316N-FW | CTTGTGGATTATCGCTTTAGCTAATGGTCTAATGTATGGCATCTTATTAC |
| Sa-I316N-RV | GTAATAAGATGCCATACATTAGACCATTAGCTAAAGCGATAATCCACAAG |
| Sa-I316W-FW | CTTGTGGATTATCGCTTTAGCTTGGGGTCTAATGTATGGCATCTTATTAC |
| Sa-I316W-RV | GTAATAAGATGCCATACATTAGACCCAAGCTAAAGCGATAATCCACAAG |
| Sa-Y320R-FW | TTAGCTATCGGTCTAATGCGTGGCATCTTATTACCAGCATG |
| Sa-Y320R-RV | CATGCTGGTAATAAGATGCCACGCATTAGACCGATAGCTAA |
| LtaA-NotI-RV | GAGAGCGGCCGCAATAGTATTGTTAATCGTAGTATGTTTGAATTAATAAGA |
| F45C-FW | GAGCTACATTAAGTGTCTGCCACCG |
| F45C-RV | CGGTGGCAGACAGTTAATGTAGCTC |
| A53C-FW | CCGTGACATCGATTGTGTGCTATTACATC |
| A53C-RV | GATGTAATAGCGACACAAATCGATGTCACGG |
| K80C-FW | CGTTATCGGCTTCTTATTATGTAAGTTTGGAACTAAGATC |
| K80C-RV | GATCTTAGTTCCAACTTACATAATAAGAAGCCGATAACG |
| K141C-FW | GAGGATAAACCGGGCTGTCAAATGGGCTACGTG |
| K141C-RV | CACGTAGCCATTTGACAGCCGCTTATCCTC |
| K166C-FW | CTTTATGAATTTGCTGATCTGIGTTATCCTACTCGTTCCG |
| K166C-RV | CGAAGCGAGTAGGATGAACACAGATCAGCAAATTCATAAAG |
| I250C-FW | CAAAAGTCATTAATGTATCAACGTGTGAATATACCGTGGCTATTATC |
| I250C-RV | GATAATAGCCACGGTATATTCAACGTTGATACATTAAATGACTTTTG |
| T253C-FW | GTATCAACGATTGAATATTGTGTGGCTATTATCATCGGCGG |
| T253C-RV | CCGCCGATGATAATAGCCACACAATATTCAATCGTTGATAC |
| C263S-FW | CGGCCGATTGGATCGGCCGTTAGTATGTTG |
| C263S-RV | CAACATACTAACGGCCGATCCAATGCCGCCG |
| N276C-FW | CTTAGTAAGTTGATCGACTGCCGTTTCGCGTAACTTTATGTAC |
| N276C-RV | GTACATAAAGTTACGCGAACGGCAGTCGATCAACTTACTAAG |
| E339C-FW | GCTTCATCAAGAGCGACTGTCAAGAGGAGACCTGG |
| E339C-RV | CCAGGTCTCCTCTTGACAGTCGCTCTTGATGAAGC |
| T366C-FW | GTTTGGCGGATTGATTGCCAGTTCACCAAC |
| T366C-RV | GTTGGTGAAGTGGCAAATCAATCCGCCAAAC |
| LtaA-V234T-L237N-FW | GATTGCGGCTTTGACTCCTATCAACCCTACGTATGCCACA |
| LtaA-V234T-L237N-RV | TGTGGCATACGTAGGGTTGATAGGAGTCAAAGCCGCAATC |

| | |
|---------------|--|
| LtaA-V234W-FW | GATTGCGGCTTTGTGGCCTATCCTGCCTAC |
| LtaA-V234W-RV | GTAGGCAGGATAGGCCACAAAGCCGCAATC |
| LtaA-C263S-FW | GCGGCATTGGATCGGCCGTTAGTATG |
| LtaA-C263S-RV | CATACTAACGGCCGATCCAATGCCGC |
| LtaA-L296D-FW | GGTTTTATTCTGTATATGATTGATATCTTCACGTTGTCTATG |
| LtaA-L296D-RV | CATAGACAACGTGAAGATATCAATCATATACAGAATAAAACC |
| LtaA-I297S-FW | TTATTCTGTATATGATTCTTAGTTTCACGTTGTCTATGATTGTC |
| LtaA-I297S-RV | GACAATCATAGACAACGTGAAACTAAGAATCATATACAGAATAA |
| LtaA-L309Q-FW | GTCAACATCCATATCCAATGGATTATTGCCTTGG |
| LtaA-L309Q-RV | CCAAGGCAATAATCCATTGGATATGGATGTTGAC |
| LtaA-I316N-FW | TGCCTTGGCGAACGGCCTGATG |
| LtaA-I316N-RV | CATCAGGCCGTTCCGAAGGCA |
| LtaA-I316W-FW | ATTATTGCCTTGGCGTGGGGCCTGATGTAT |
| LtaA-I316W-RV | ATACATCAGGCCCCACGCCAAGGCAATAAT |
| LtaA-Y320R-FW | GATCGGCCTGATGCGTGGGATTCTGTTGCCA |
| LtaA-Y320R-RV | GATCGGCCTGATGCGTGGGATTCTGTTGCCA |



Suppl. Fig. 2B and 4B



Suppl. Fig. 4B

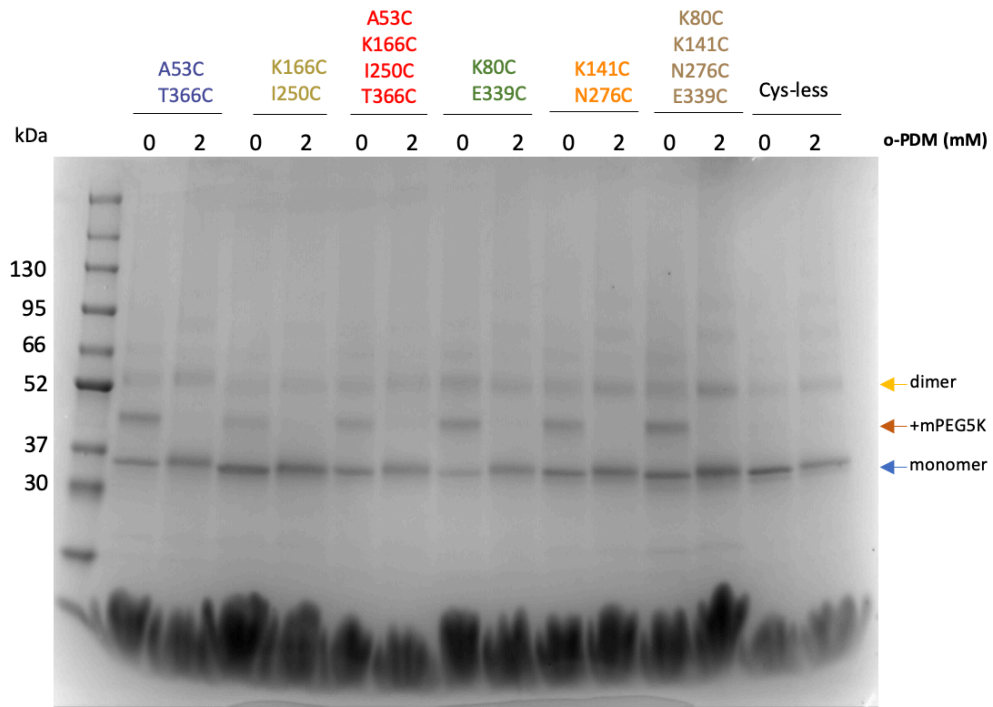


Fig. 2B and Suppl. Fig. 4C

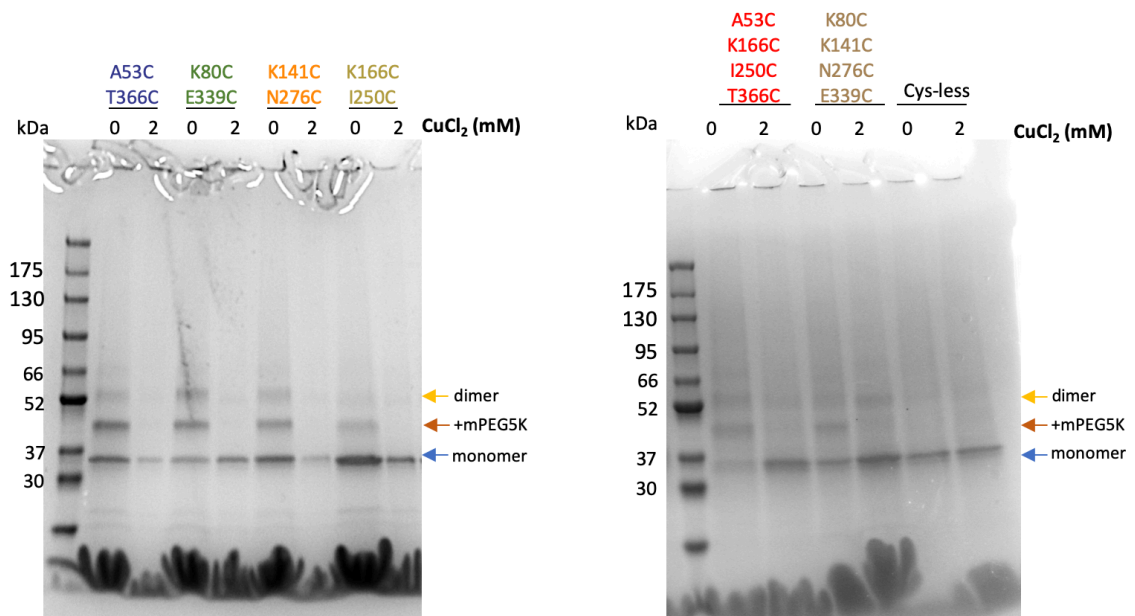


Fig. 4D and Suppl. Fig. 4D