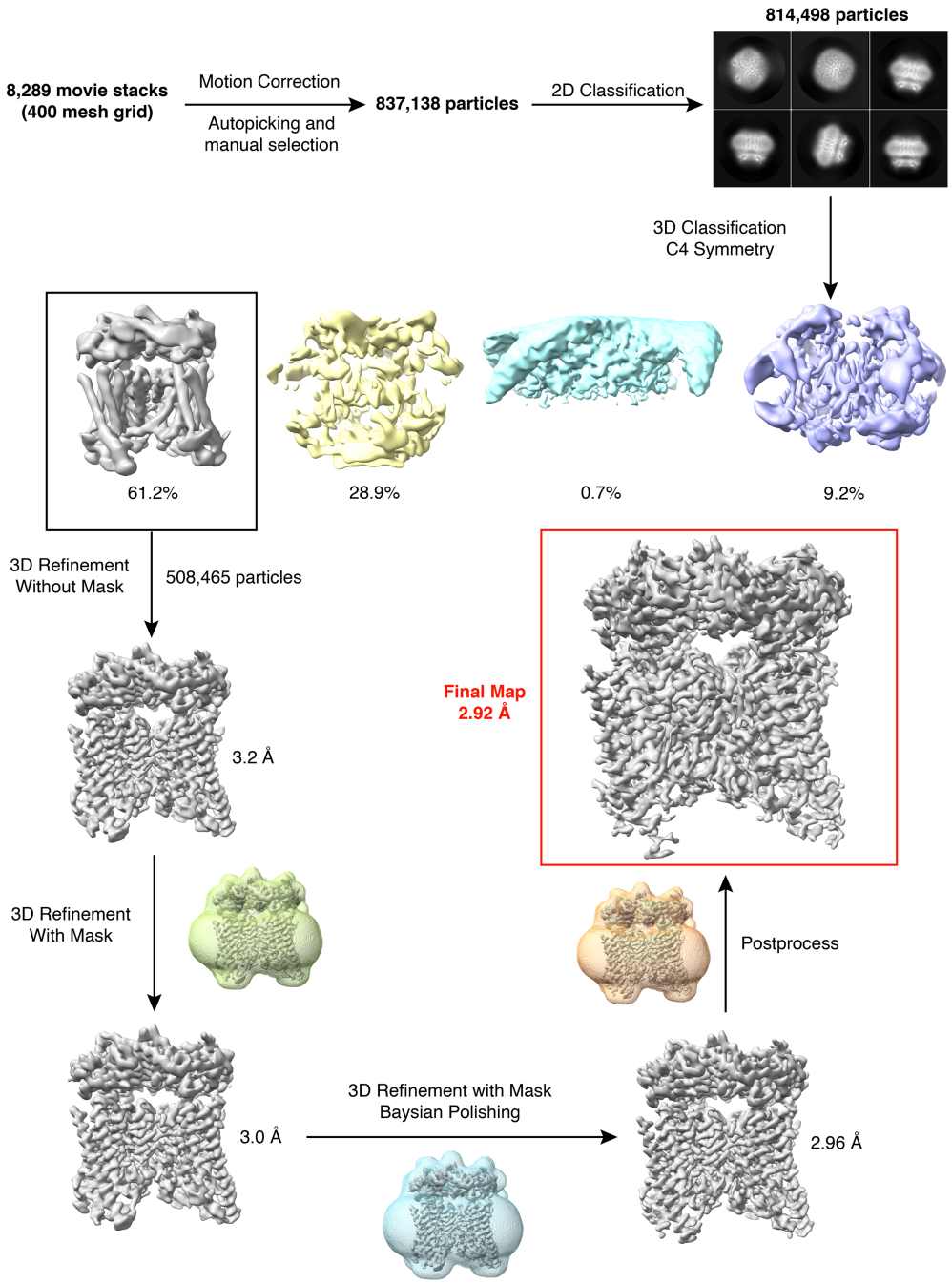

Figure S1. Processing of data acquired from 300 kV cryo-TEM, Related to Figure 1 and Table 1. The data processing workflow in RELION 3. The cryo-EM 3D classes as well as the masks used for refinement are shown. After the RELION 3 refinement, the final cryo-EM map was sharpened using post-process with a B-factor value of -130 $Å^2$.

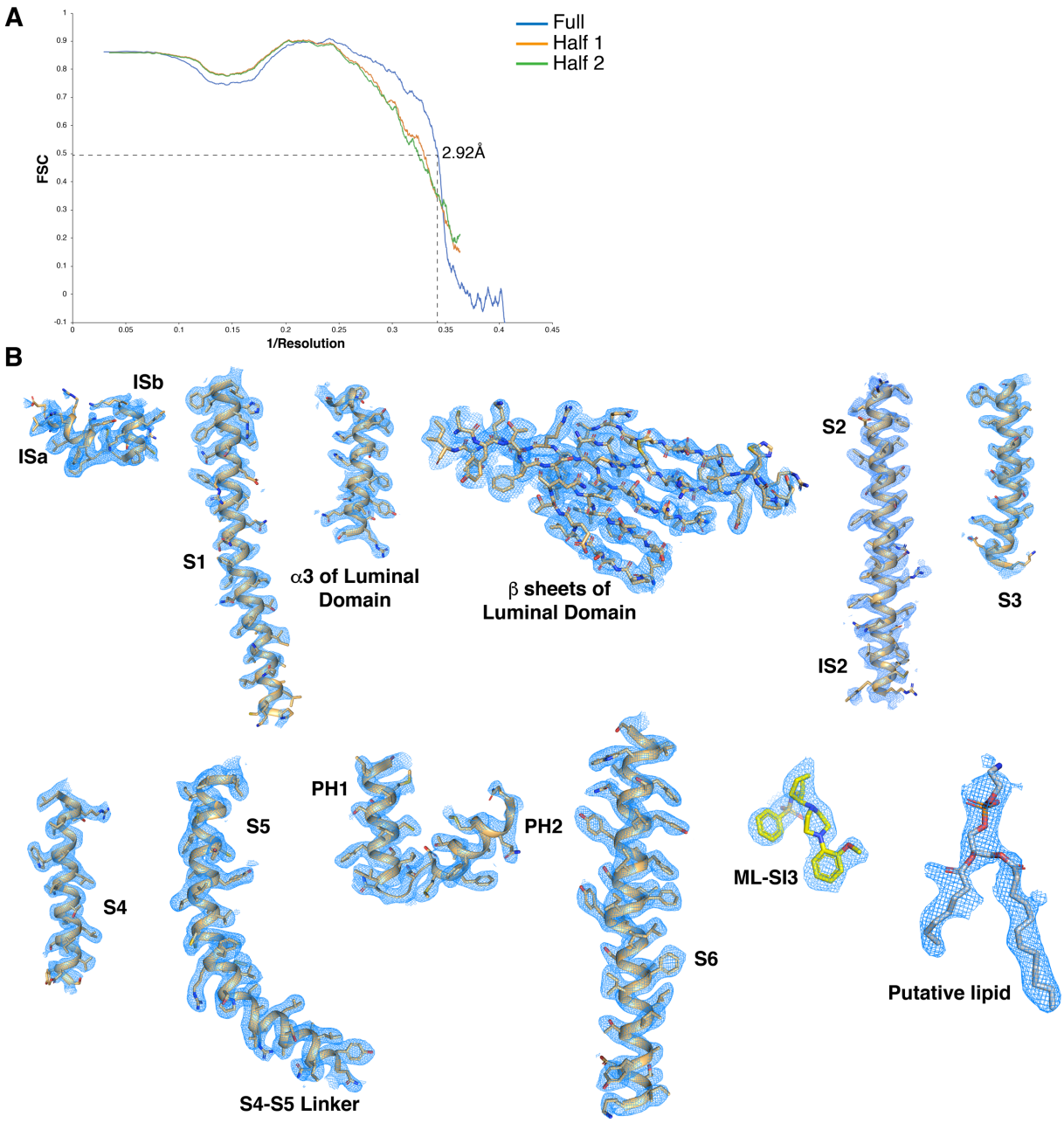

Figure S2. Cryo-EM map of the structural elements of TRPML1–ML-SI3, Related to Figure 1. (A) The FSC curves calculated between the refined structural model and the half map used for refinement (orange), the other half map (green), and the full map (blue). **(B)** The major structural features of TRPML1–ML-SI3. The density map and model are shown as mesh and cartoons, respectively. Cryo-EM maps are contoured at the 4σ level.

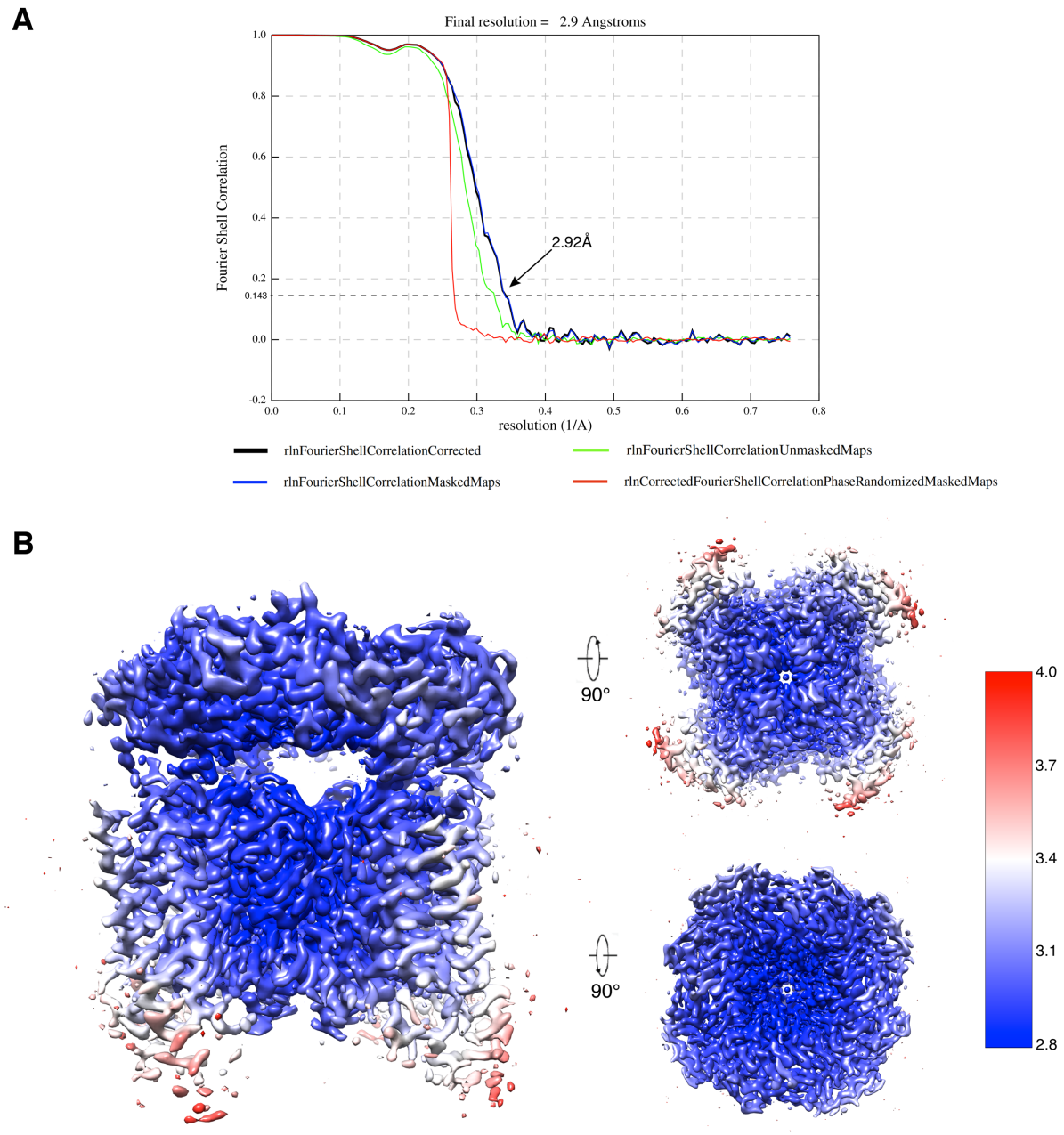

Figure S3. FSC curve and estimation of the local resolution, Related to Figure 1. (A) FSC curve as a function of resolution using RELION 3 output. **(B)** Density maps of the structure of TRPML1–ML-SI3, colored by local resolution estimation using RELION 3.

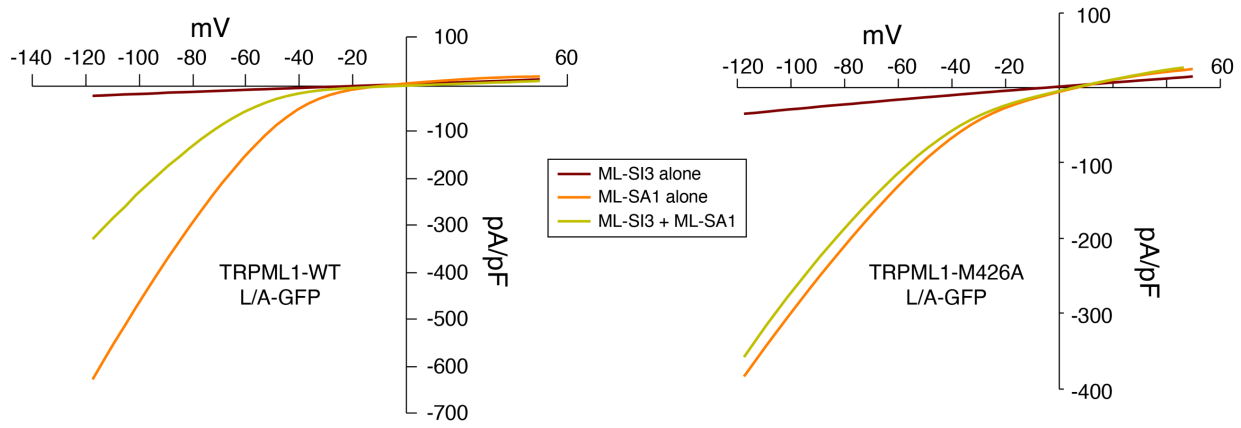

Figure S4. Current Voltage relationship of TRPML1 'WT' and 'M426A-mutant' L/A-GFP cells, Related to Figure 3. Typical inward rectification of TRPML1 currents with agonist ML-SA1 (10 μM) are reduced during co-application of antagonist ML-SI3 (10 μM) in TRPML1 'WT' L/A-GFP cells, left. Cells expressing TRPML1 L/A-GFP containing the M426A mutation respond similarly to agonist treatment, but antagonist treatment fails to significantly reduce inward rectification, right. Maximal response determined at -100 mV with 10 μM ML-SA1.