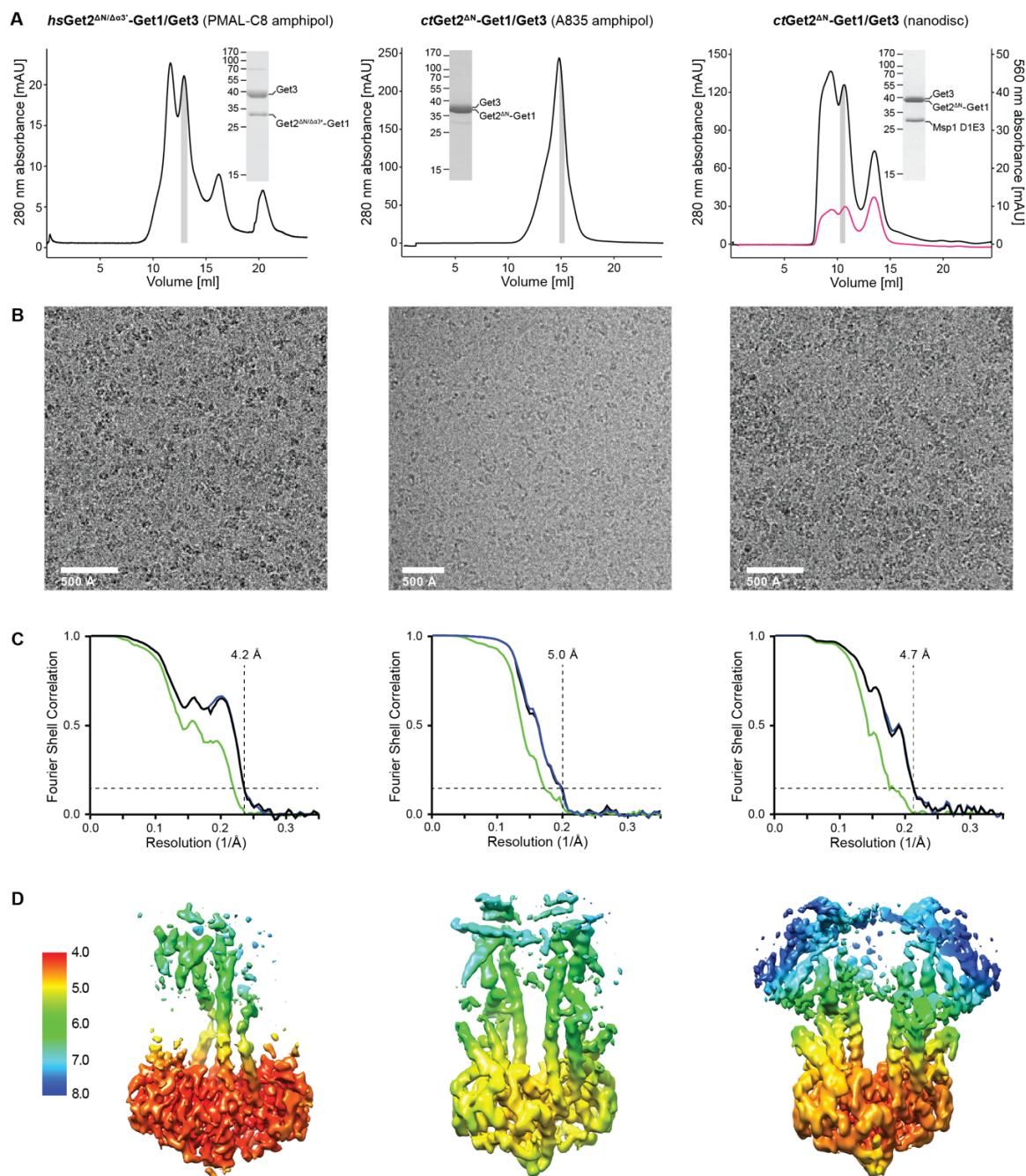
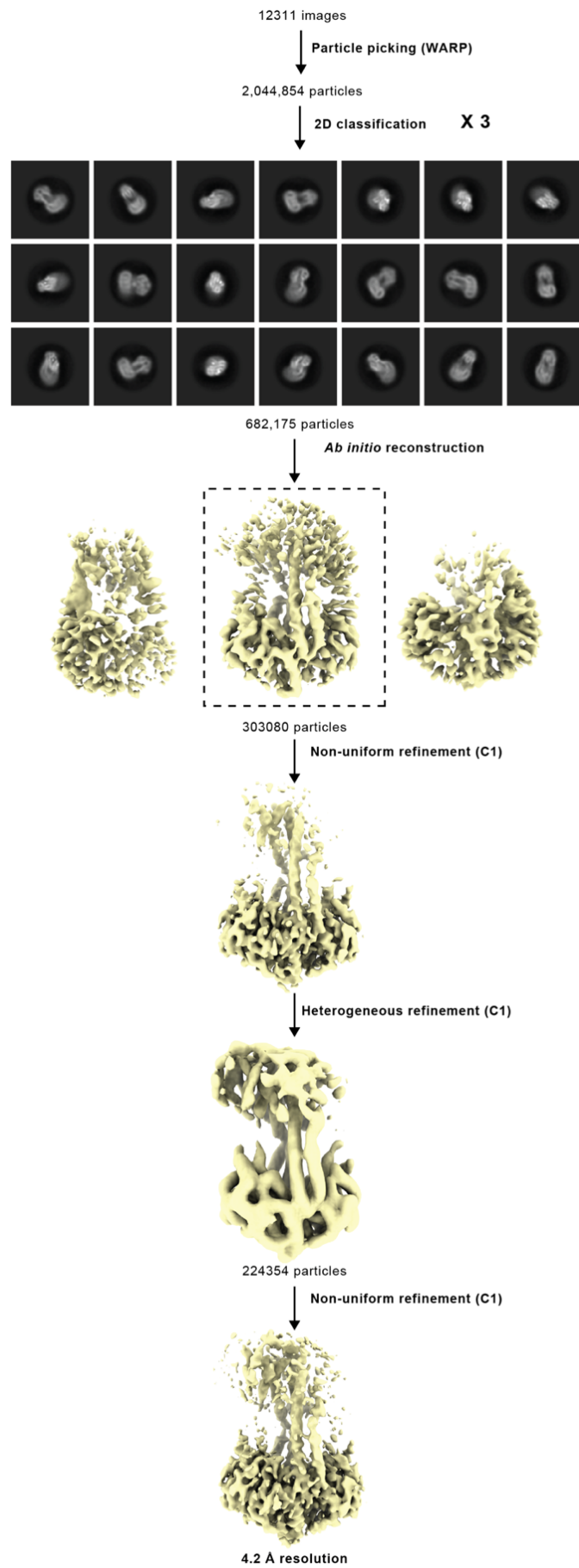


Supplementary Figures



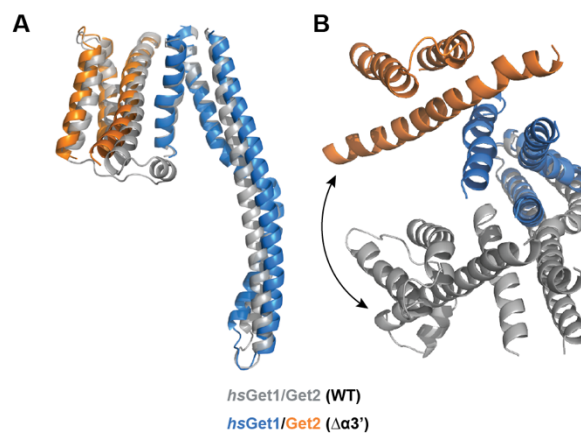
Supplementary Figure 1 – Cryo-EM sample preparation and experimental quality.

A) Size exclusion chromatography profiles for the indicated complexes. The single fraction taken for cryo-EM is indicated in grey. The insets show Coomassie stained 12.5% SDS-PAGE gels for the final samples. **B)** Representative micrographs for each complex. **C)** Gold-standard FSC curves for the final reconstruction. For each reconstruction, the no mask (green), tight mask (blue) and corrected (black) curves are shown. **D)** Local resolution estimates of the final reconstructions for each complex.



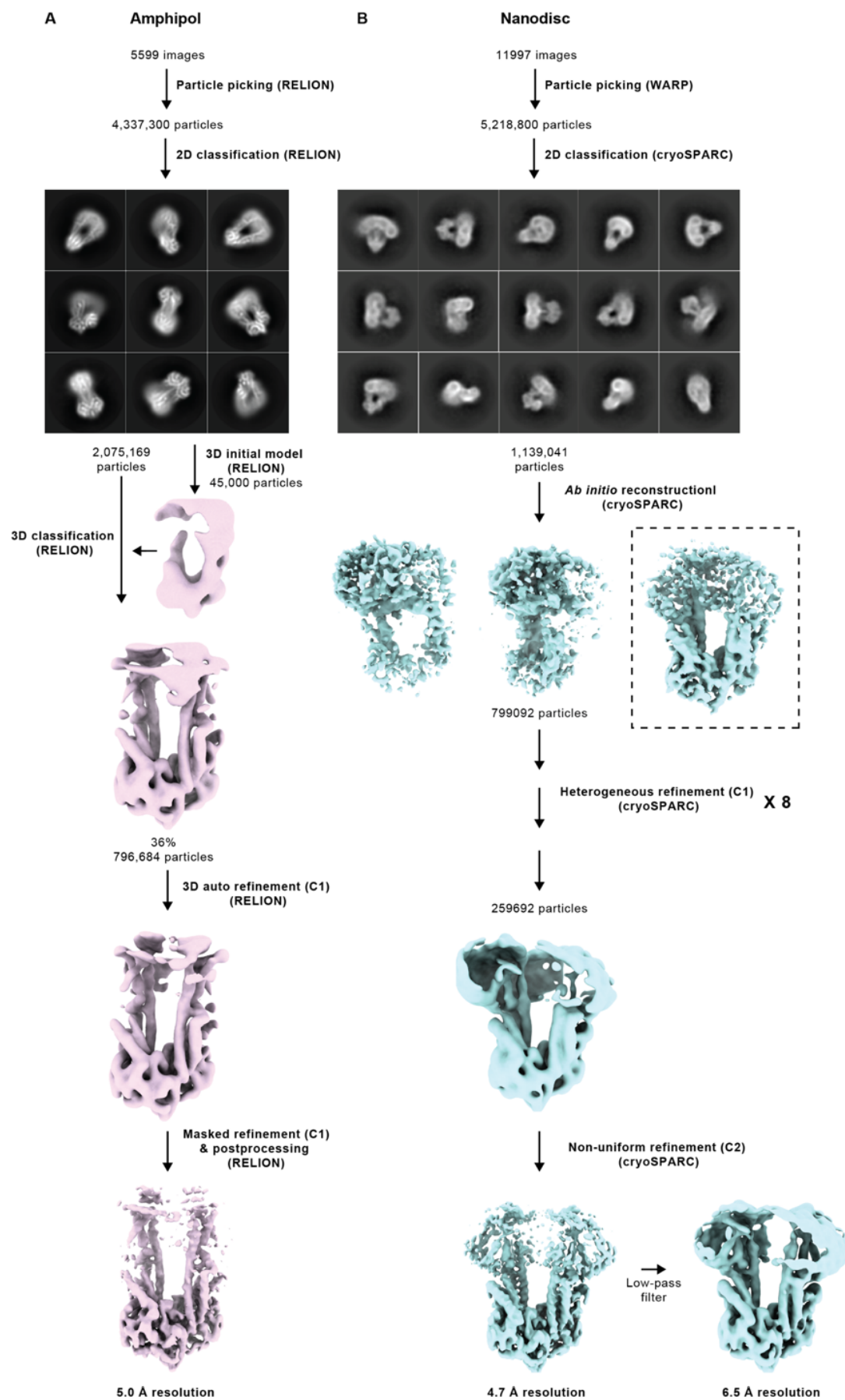
Supplementary Figure 2 – Workflow for single-particle cryo-EM data processing for *hsGet2*^{ΔN/Δα3'}-Get1/Get3 in PMAL-C8.

Particles were autopicked using WARP, then subjected to 2D classification in cryoSPARC. An initial 3D reconstruction generated *ab initio* was subjected to heterogeneous refinement and non-uniform refinement, resulting in a reconstruction with an average resolution of 4.2 Å.



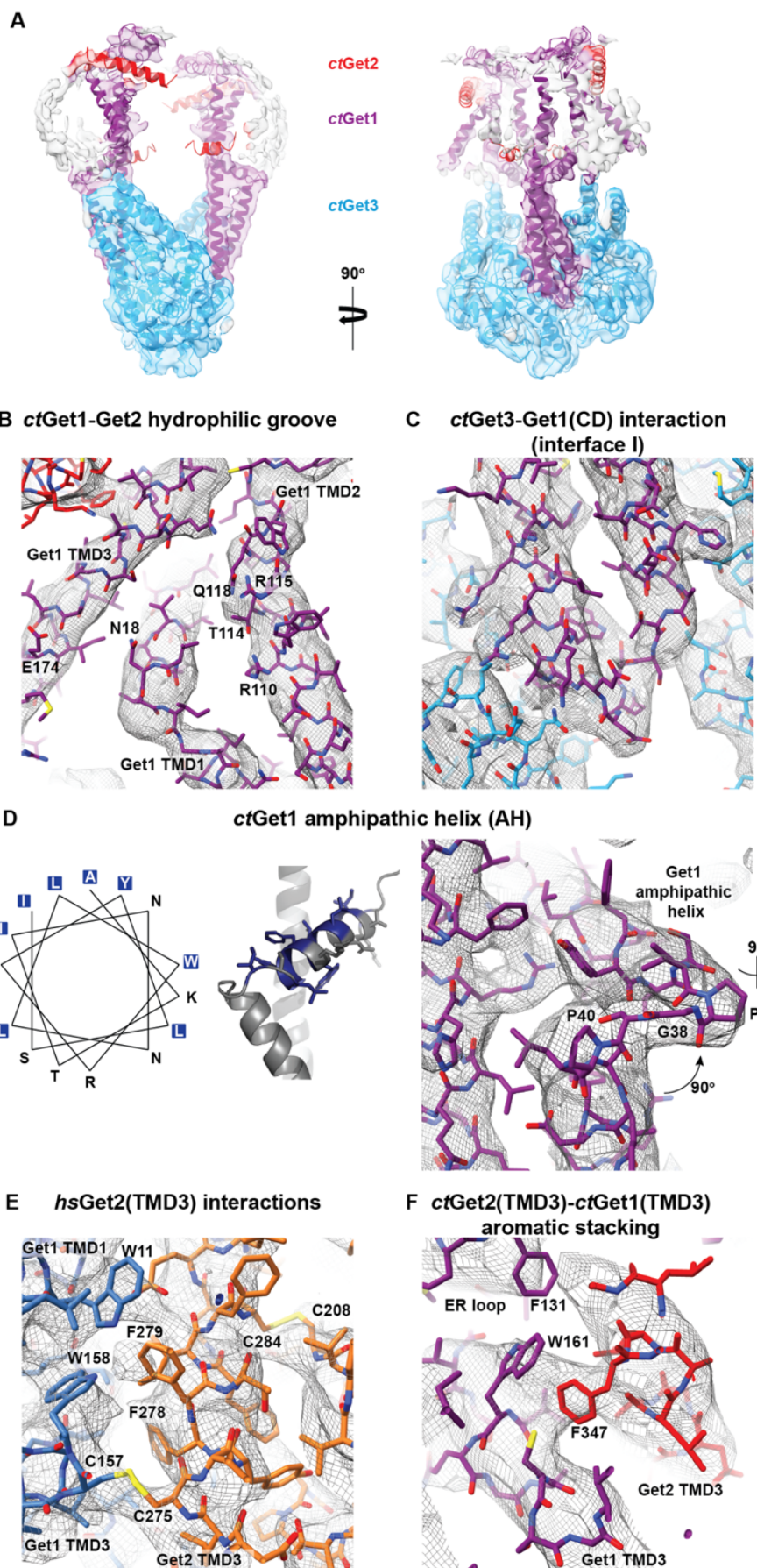
Supplementary Figure 3 – Structural features of *hsGet2*^{ΔN/Δα3'}-Get1/Get3.

A) Superimposition of the wild type (WT) and $\Delta\alpha3'$ *hsGet1/Get2* heterodimer structures (RMSD 2.93 Å over 214 C α atoms) shows a conserved fold. **B)** View of equivalent WT and $\Delta\alpha3'$ *hsGet1/Get2* heterodimers from the luminal side of the membrane after superimposition via *hsGet3* (RMSD 1.71 Å over 503 C α atoms). The arrow highlights the relative positional variation between the two heterodimers.



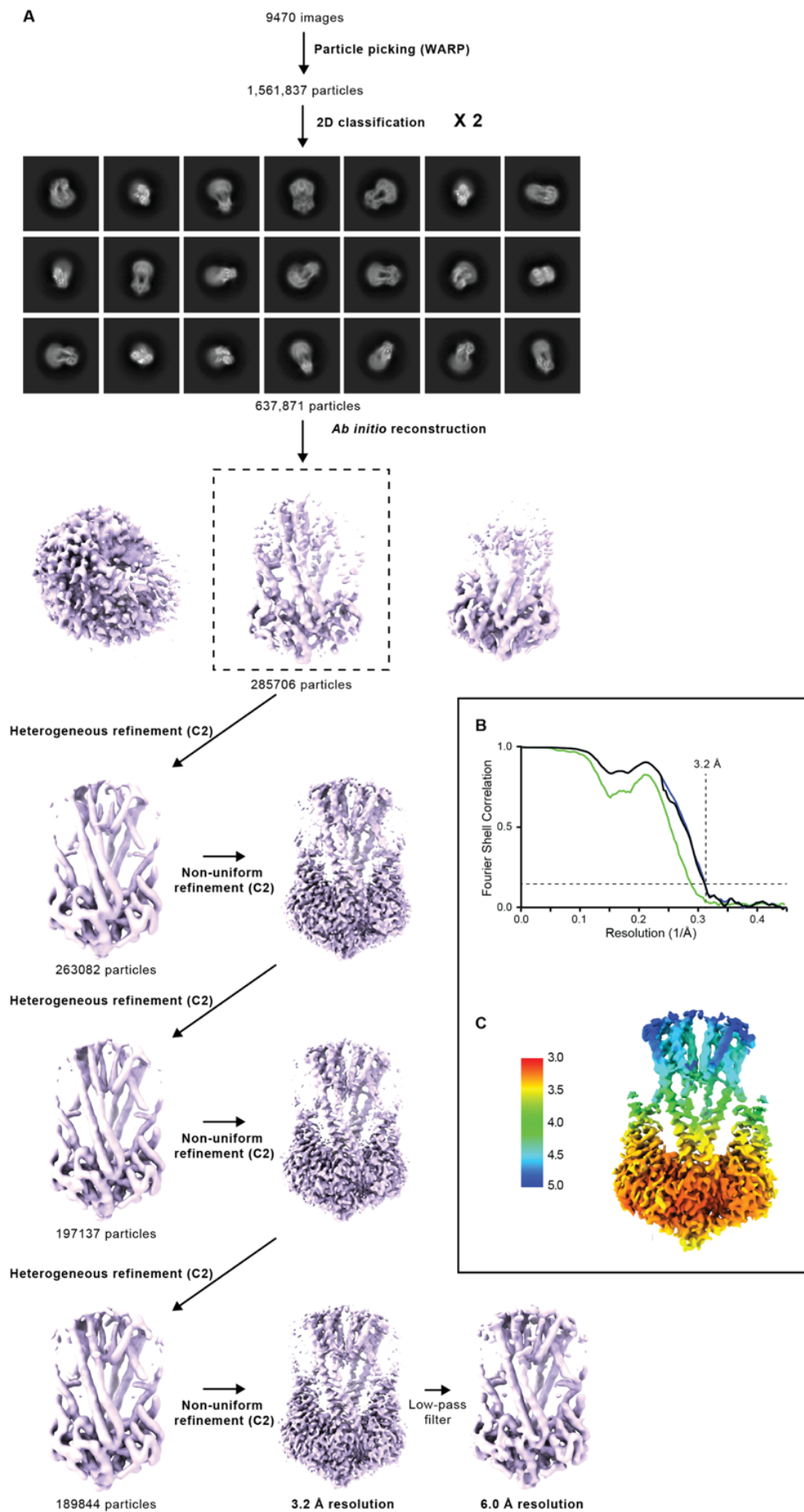
Supplementary Figure 4 – Workflow for single-particle cryo-EM data processing for α Get2^{AN}-Get1/Get3 complexes.

A) For the amphipol complex, particles were autopicked in Relion3.0, then subjected to 2D classification followed by 3D classification against a low-resolution *de novo* model. Particle images were then refined using a soft mask, resulting in a reconstruction with an average resolution of 5.0 Å. **B)** For the nanodisc complex, particles were autopicked in WARP, then subjected to 2D classification in cryoSPARC. An initial 3D reconstruction generated *ab initio* was subjected to multiple rounds of heterogeneous refinement followed by non-uniform refinement, resulting in a reconstruction with an average resolution of 4.7 Å. More complete density for low resolution regions was present in a reconstruction low-pass filtered to 6.5 Å.



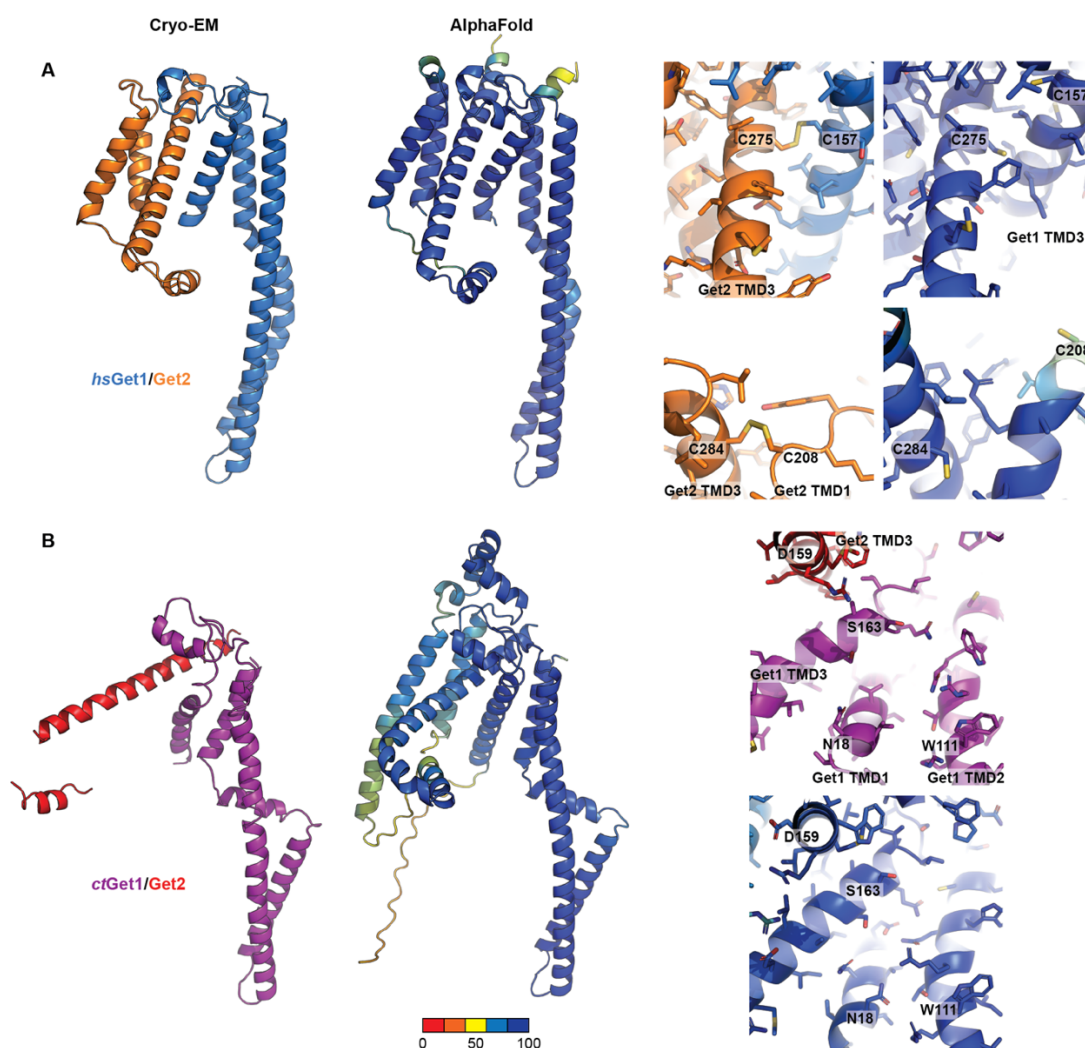
Supplementary Figure 5 – Structural features of the *ctGet2^{ΔN}*-Get1/Get3 and *hsGet2^{ΔN}*-Get1/Get3 complexes.

A) Model for *ctGet2^{ΔN}*-Get1/Get3 in nanodiscs superposed to the cryo-EM density. **B-C)** Representative density for the **B)** hydrophilic groove and **C)** *ctGet1*-CD interface with *ctGet3* in the amphipol *ctGet2^{ΔN}*-Get1/Get3 reconstruction **D)** The amphipathic helix of *ctGet1* (residues 22-36) shown in both helical wheel and structural representations. Hydrophobic residues are coloured blue. **E-F)** Representative density for **E)** *hsGet2^{ΔN}*-Get1/Get3 and **F)** amphipol *ctGet2^{ΔN}*-Get1/Get3 showing the region around the absolutely conserved tryptophan of Get1 TMD3.



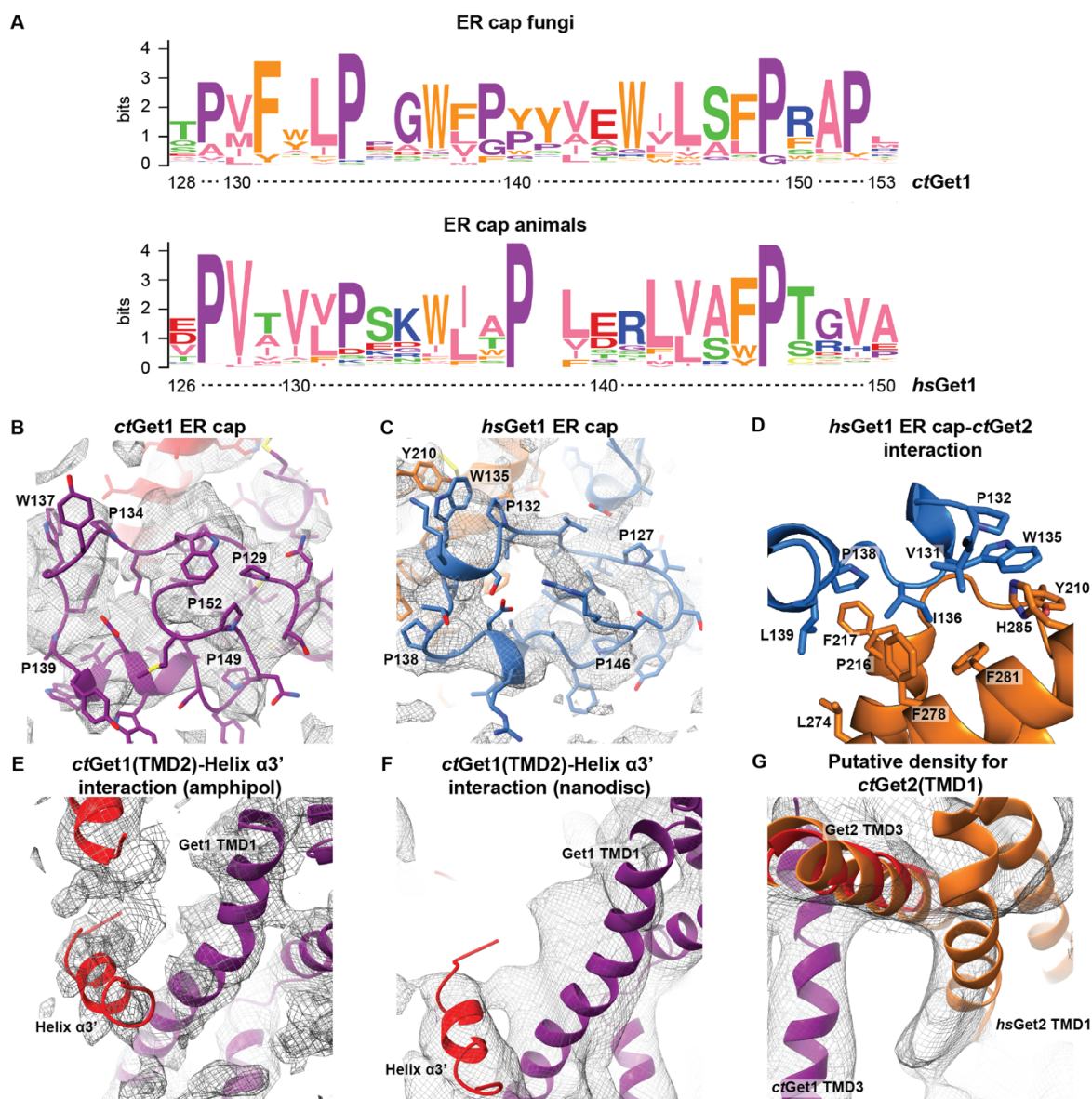
Supplementary Figure 6–Improved resolution of the *hsGet2*^{ΔN}-Get1/Get3 reconstruction.

A) Pipeline for re-processing of the single-particle cryo-EM dataset (EMDB-10266)²⁴. Particles were autopicked using WARP, then subjected to 2D classification in cryoSPARC. An initial 3D reconstruction generated *ab initio* was subjected to multiple rounds of heterogeneous refinement and non-uniform refinement, resulting in a reconstruction with an average resolution of 3.2 Å. More complete density for low resolution regions was present in a reconstruction low-pass filtered to 6.0 Å. **B)** Gold-standard FSC curves for the final reconstruction, showing the no mask (green), tight mask (blue) and corrected (black) curves. **C)** Local resolution estimate for the final reconstruction.



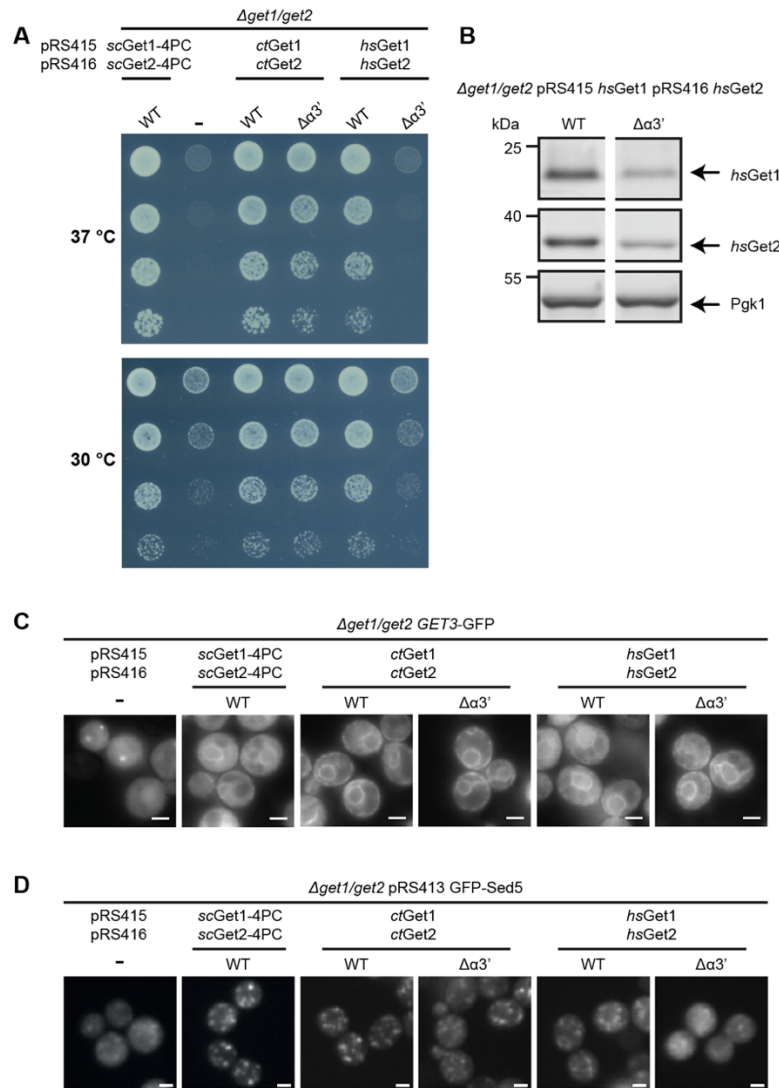
Supplementary Figure 7 – comparison of cryo-EM and AlphaFold derived models for *hsGet1/Get2* and *ctGet1/Get2*.

A) Side-by-side comparison of the updated *hsGet1/Get2* heterodimer model presented in this study with an AlphaFold model (RMSD 2.37 Å over 259 Cα atoms). Clear density for disulphide bonds (Supplementary Figure 5E) results in *hsGet1* TMD3 and *hsGet2* TMD1 being modelled with a different register in the cryo-EM reconstruction than in the AlphaFold derived model (zoomed insets). **B)** Side-by-side comparison of the amphipol *ctGet1/Get2* heterodimer model presented in this study with an AlphaFold model (RMSD 2.33 Å over 194 Cα atoms). The positioning and register of the TMDs are similar between both models (zoomed insets). Both AlphaFold models are the top ranked model from the predicted local distance difference test (pLDDT) after running the ColabFold AlphaFold2_advanced Jupyter notebook inside Google Colaboratory⁵⁰. They are coloured according to pLDDT values, where pLDDT > 90 (blue) is a high estimated accuracy of backbone and side chain positions and were used to derive models for the ER cap.



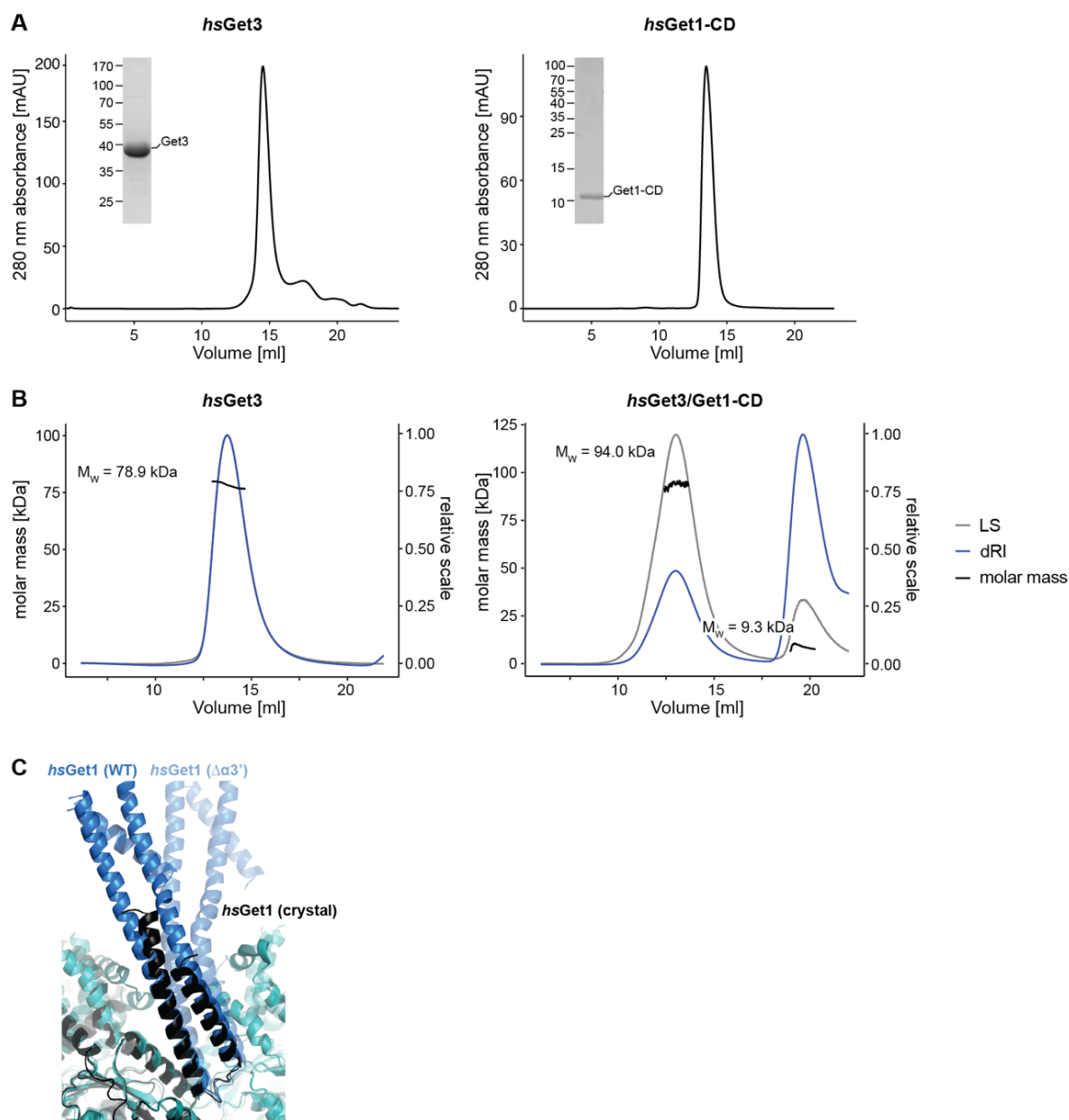
Supplementary Figure 8 – Conserved structural features between the *ctGet2*^{ΔN}-Get1/Get3 and *hsGet2*^{ΔN}-Get1/Get3 complexes.

A) Sequence conservation within the ER cap of 453 fungal and 445 animal Get1 sequences. Residues in the sequence logos are numbered according to the residues in *ctGet1* and *hsGet1*. B-G) Structural features within the *ctGet2*^{ΔN}-Get1/Get3 and *hsGet2*^{ΔN}-Get1/Get3 reconstructions. G) Superimposition of *ctGet2*^{ΔN}-Get1 and *hsGet2*^{ΔN}-Get1 as shown in Figure 2C, showing the density from the *ctGet2*^{ΔN}-Get1/Get3 nanodisc reconstruction.



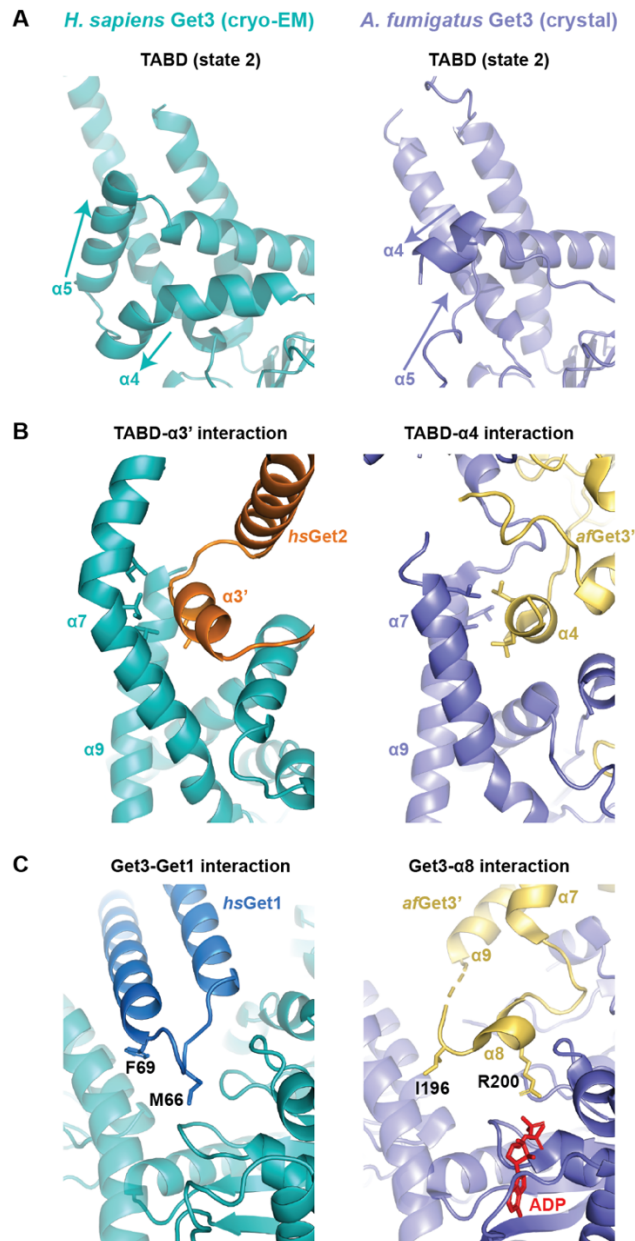
Supplementary Figure 9 – The functional importance of Get2 helix α3'.

scGet1-4PC/Get2-4PC, *ctGet1/Get2* or *hsGet1/Get2* were co-expressed from the indicated plasmids in *Δget1/get2* yeast strains for wild type (WT) and mutant sequences. Transformants with the empty vector were taken as a negative control (-). **A)** Serial dilutions of *Δget1/get2* yeast cells expressing *scGet1/Get2*, *ctGet1/Get2* or *hsGet1/Get2* constructs spotted on synthetic dropout plates at 37°C and 30°C. The spotting assays are representative of n=3. **B)** Western blot of *hsGet1/Get2* expression in *Δget1/get2* yeast cells. Pgk1 was analysed as a loading control. The immunoblots are representative of n=3. **C)** Representative images from fluorescence microscopy showing the subcellular localisation of *scGet3-GFP* in *Δget1/get2* yeast cells expressing genomically tagged Get3 and *scGet1/Get2*, *ctGet1/Get2* or *hsGet1/Get2* constructs. **D)** Representative fluorescence microscopy images of *Δget1/get2* cells expressing GFP-Sed5 and *scGet1/Get2*, *ctGet1/Get2* or *hsGet1/Get2* constructs from separate plasmids. All scale bars represent 2 μM.



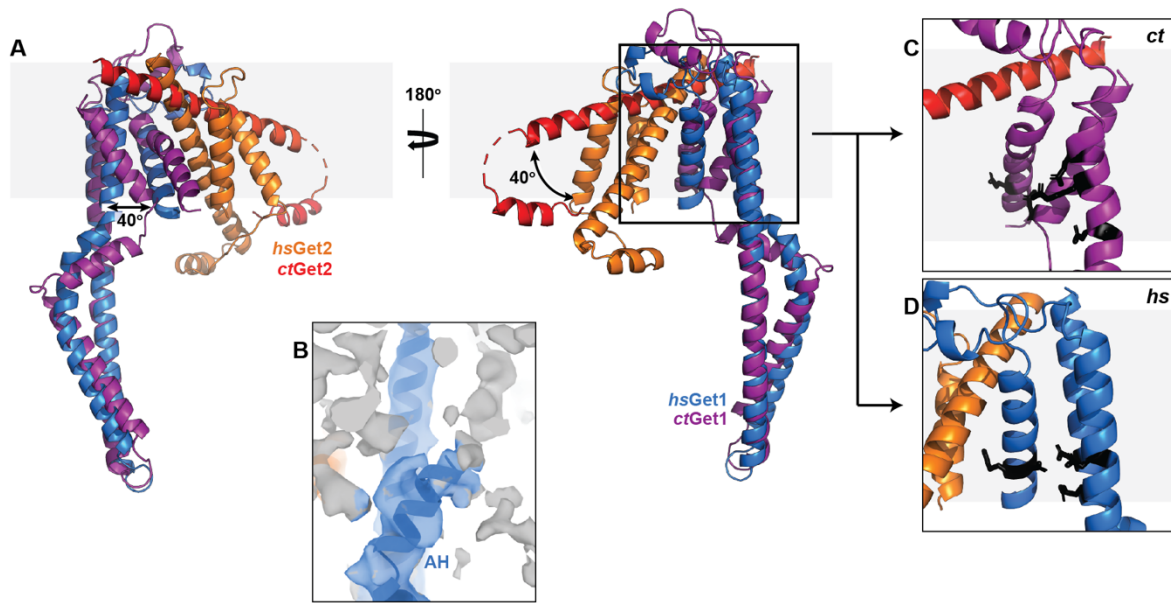
Supplementary Figure 10 – Analysis of the *hsGet3/Get1-CD* complex.

A) Size exclusion chromatography profiles for *hsGet3* and *hsGet1-CD*. The insets show Coomassie stained SDS-PAGE gels for the final samples. **B)** Analysis of the oligomeric state of *hsGet3* (expected 80.2 kDa for dimer) and reconstituted *hsGet3/Get1-CD* (expected 96.6 kDa for 2:2 complex, 8.2 kDa for Get1-CD alone) by size exclusion chromatography and in-line MALS. The left axis represents the molecular weight at any given point in the chromatogram. **C)** Superposition of Get3 within the *hsGet3/Get1-CD* crystal structure (black) with Get3 chain A within the WT *hsGet2^{ΔN}-Get1/Get3* (RMSD 1.48 Å over 237 Cα atoms), which is shown overlaid with *hsGet2^{ΔN/Δα3'}-Get1/Get3* as in Figure 1C.



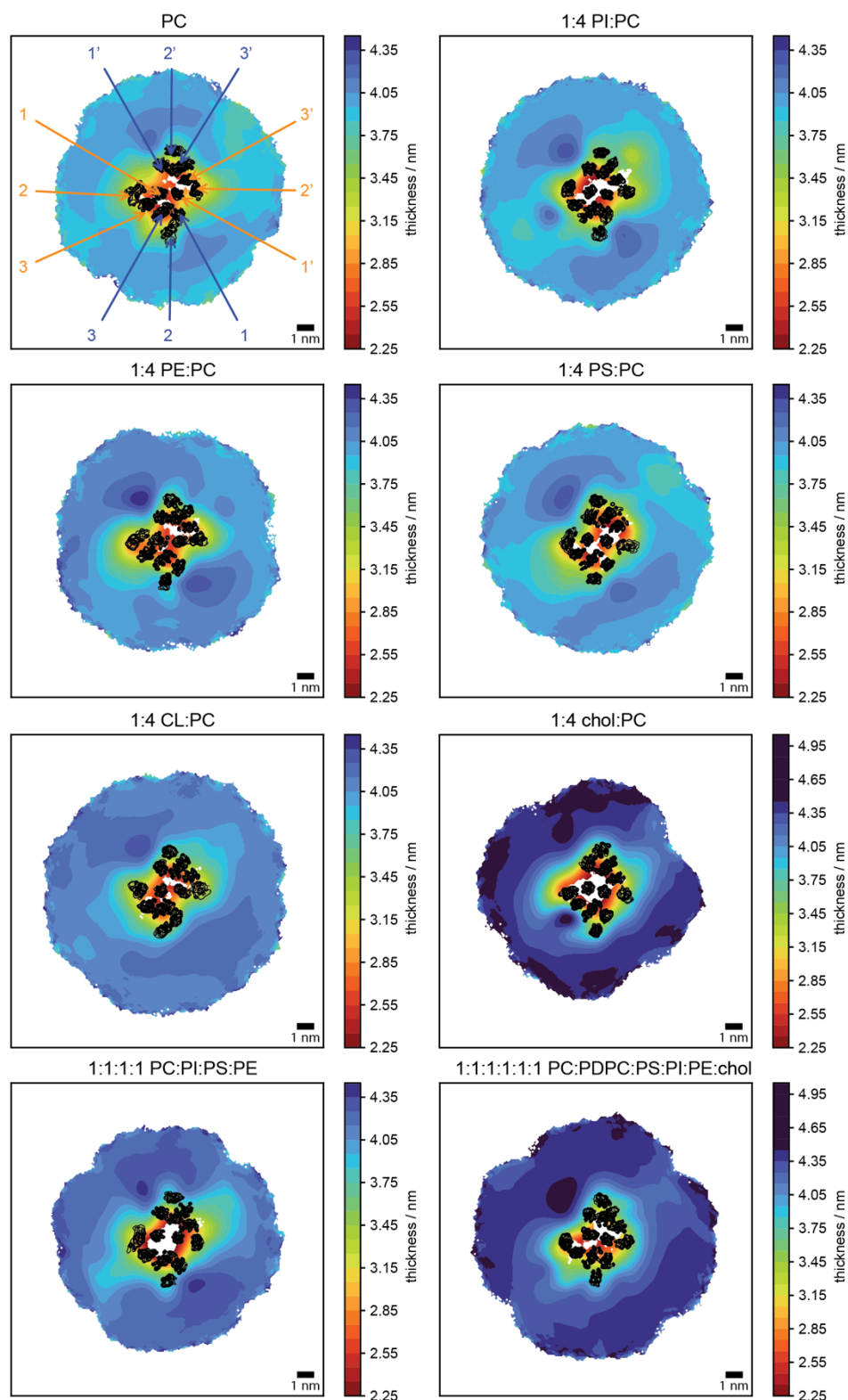
Supplementary Figure 11 – Comparison of the wild type *hsGet2*^{ΔN}-Get1/Get3 structure with the *afGet3* crystal structure.

A) Equivalent views of the Get3 TABD after superimposition via chain A of *hsGet3* and *afGet3* (PDB ID 3IBG; RMSD 1.27 Å over 246 C α atoms). The arrows show the directionality of the helices. **B)** Equivalent views of the Get3 TABD after superimposition via chain A helices $\alpha 7$ - $\alpha 9$ of *hsGet3* (residues 174-237) and *afGet3* (residues 168-231; RMSD 2.61 Å over 262 total atoms). The interaction between *hsGet3* and *hsGet2* helix $\alpha 3'$ is similar to that between *afGet3* and *afGet3* helix $\alpha 4$ from an adjacent dimer. **C)** Equivalent views of the Get3 dimer interface after superimposition as in A. The interaction between *hsGet3* and the tip of the *hsGet1*-CD is similar to that between *afGet3* and *afGet3* helix $\alpha 8$ from an adjacent dimer.



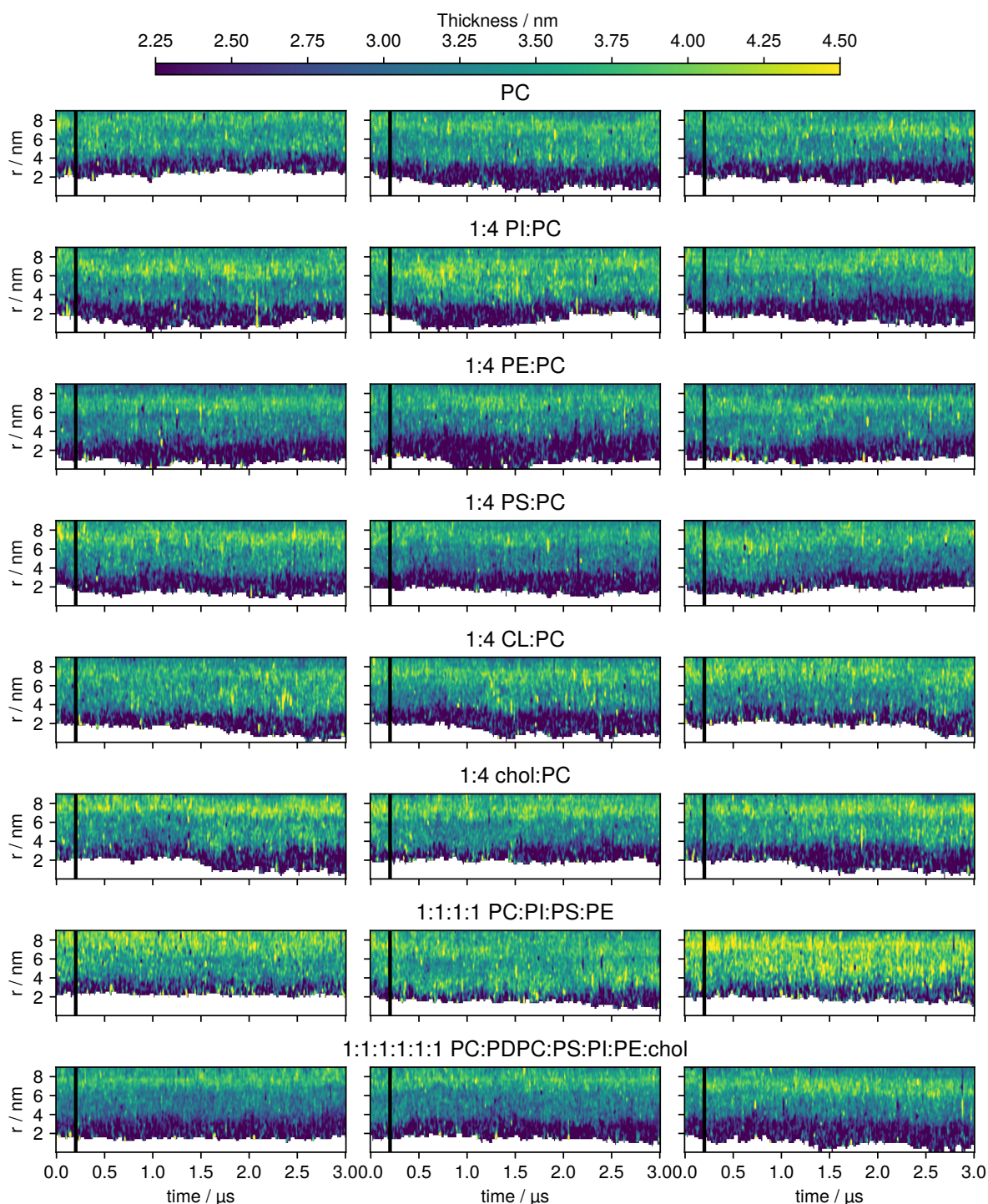
Supplementary Figure 12 – Positioning of the *ct*Get1/Get2 and *hs*Get1/Get2 heterodimers in the membrane plane.

A) Superimposition of the *hs*Get1-CD with the *ct*Get1-CD (RMSD 2.18 Å over 79 Cα atoms). The 40° change in tilt of *ct*Get1 TMD1 and *ct*Get2 TMD3 is shown. Membrane plane defined perpendicular to the Get3 symmetry axis with boundaries inferred from the location of the TMDs. **B)** Model for *hs*Get1 superimposed with the *hs*Get2^{ΔN/Δα3⁻}-Get1/Get3 cryo-EM density contoured to 0.17. Within the poorly resolved *hs*Get1/Get2 heterodimer, the density beyond the AH is of insufficient quality to model *hs*Get1 TMD1 but indicates TMD1 and TMD2 are no longer arranged in parallel. **C-D)** Zoomed insets of the boxed region in **A** showing the position of the hydrophilic groove formed by **C)** *ct*Get1/Get2 TMDs and **D)** *hs*Get1/Get2 TMDs relative to the membrane plane. Hydrophilic residues pointing into the groove are shown as black sticks.



Supplementary Figure 13 – Membrane thinning by the GET insertase is independent of bilayer composition.

Membrane thickness maps, calculated as described in the Methods section, for each lipid composition studied (Supplementary Table 2).



Supplementary Figure 14 – Temporal and spatial evolution of membrane thickness.

The average membrane thickness from the center of mass of the protein transmembrane domain as a function of radial lateral distance calculated for each simulation frame. Each row corresponds to the simulation system mentioned above it, and each column is an independent repeat. The vertical black lines indicate the 200 ns time point, after which the data are used for other analyses. The membrane gets thinner close to the protein, and the results are consistent throughout the simulation and across the three simulation repeats of each system.