A

B

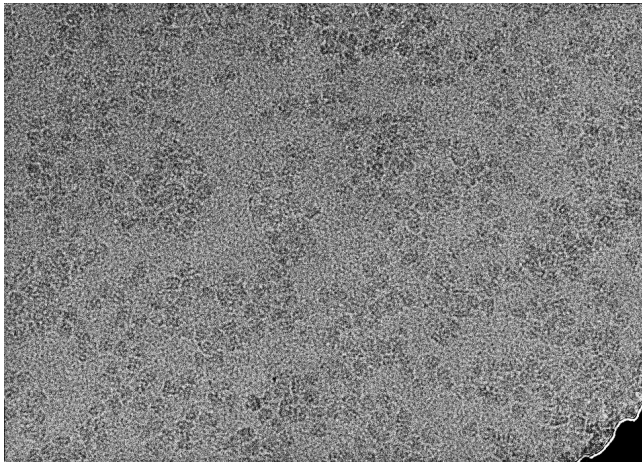**C** GSFSC Resolution: 2.96Å

No Mask (3.8Å)
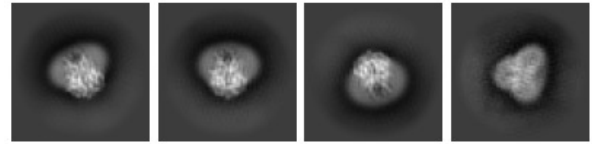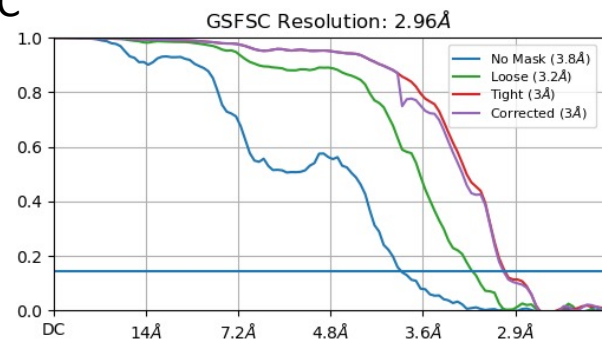Loose (3.2Å)
Tight (3Å)
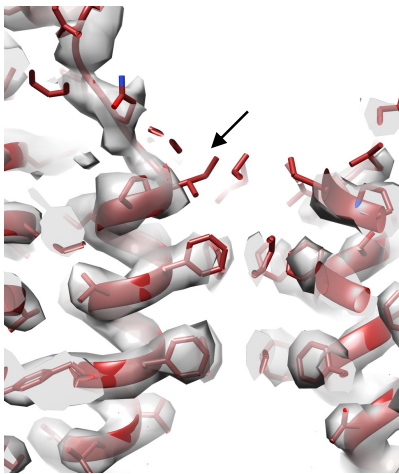Corrected (3Å)

Consensus on-the-fly refinements
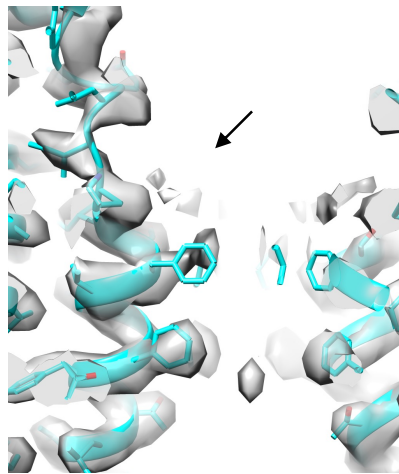Hydrophobic gate (neck)

D Negative (closed)
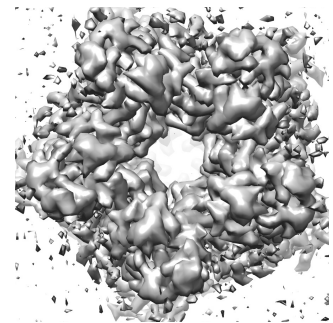
E Positive (intermediate open)
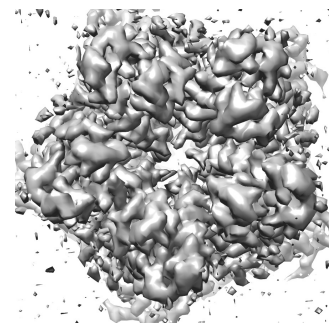
F **3D classification**

Open
41,771 particles (18%)

G **Closed**
189,238 particles (82%)

A) Representative micrograph from Krios
B) Representative 2D classes
C) FSC curve for the 3D consensus refinement of a "positive hit" processed on-the-fly
D) Example of negative consensus refinement
E) Example of positive hit consensus refinement demonstrating heterogeneity within the neck gate
F) 3D classification separates the first open state (18%) of a wild-type human Best1 channel
G) 3D classification separates the 82% closed class