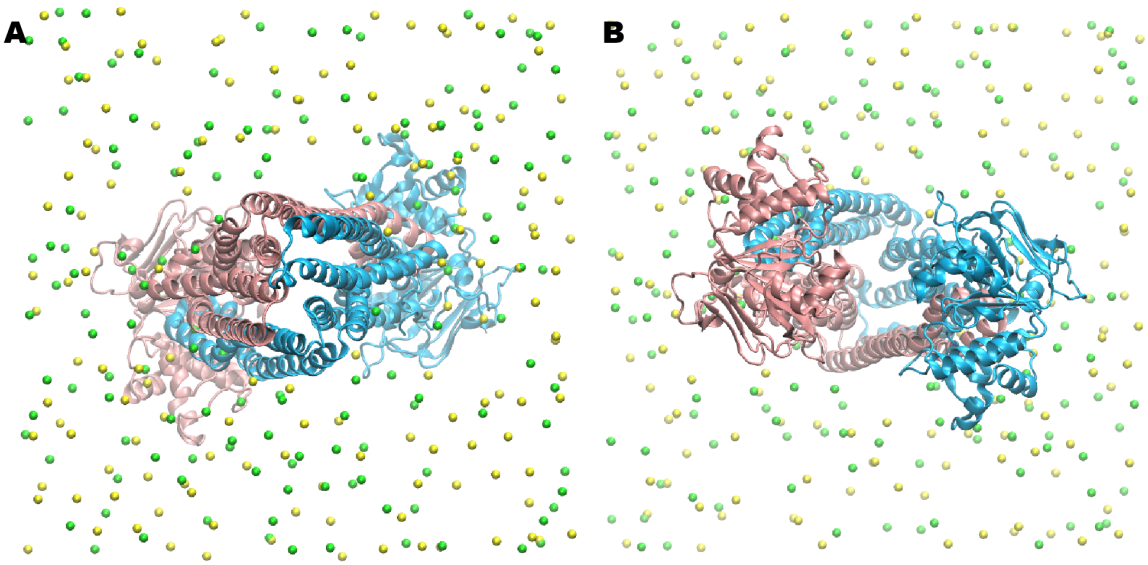

Fig. S1: Initial positions of cholesterol in a POPC:cholesterol (70:30) bilayer from the two independently prepared membrane-Pgp systems. Yellow and green beads represent the position of oxygen atoms of cholesterol molecules in the *apo* and Mg^{2+}/ATP-bound Pgp systems, respectively, indicating initial randomization of cholesterol positions between the two systems. (A) Top-down view from the extracellular side and (B) bottom-up view from the intracellular side.

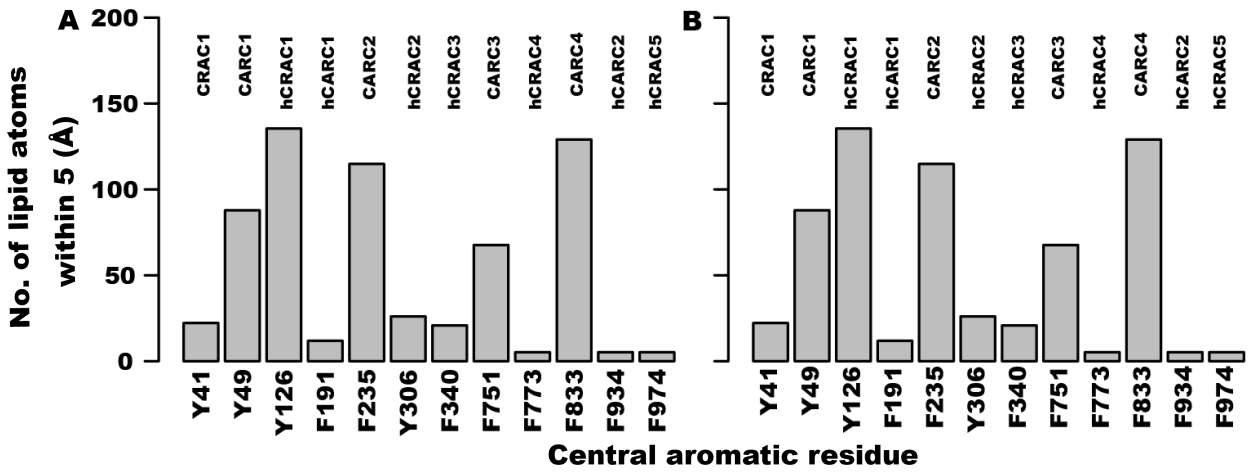

Fig. S2: Membrane accessibility of the identified cholesterol recognition motifs in (A) *apo* and (B) Mg^{2+}/ATP-bound simulations. The Tyr or Phe residue located in the middle of each recognition motif was selected for these calculations. Y-axis displays the number of lipid atoms located within 5Å of the selected residue and the x-axis displays the selected residue from each motif.

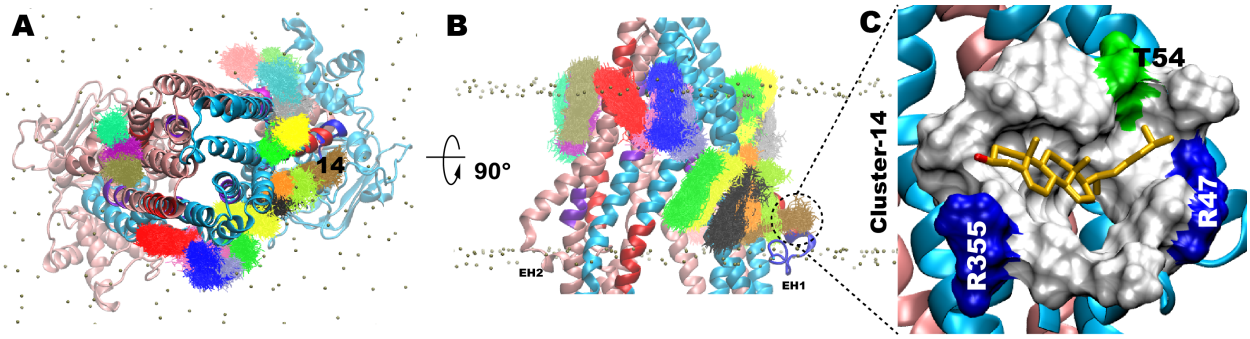

Fig. S3: Clustering analysis of cholesterol molecules located within 4 Å of Pgp obtained using *apo* and Mg^{2+}/ATP-bound simulations in POPC:cholesterol (70:30) bilayer. (A) Top 20 clusters are displayed and cluster-14 is labeled. (B) Side view of the top 20 clusters from the membrane with elbow helices (EH1 and EH2) labeled. (C) Representative binding mode from the cluster-14 showing an uncommon binding of cholesterol nearly perpendicular to the membrane normal. Residues R47, T54 (both from TM1 and part of CARC1 motif) and R355 (TM6 and located at the TM4/6 portal) are labeled.

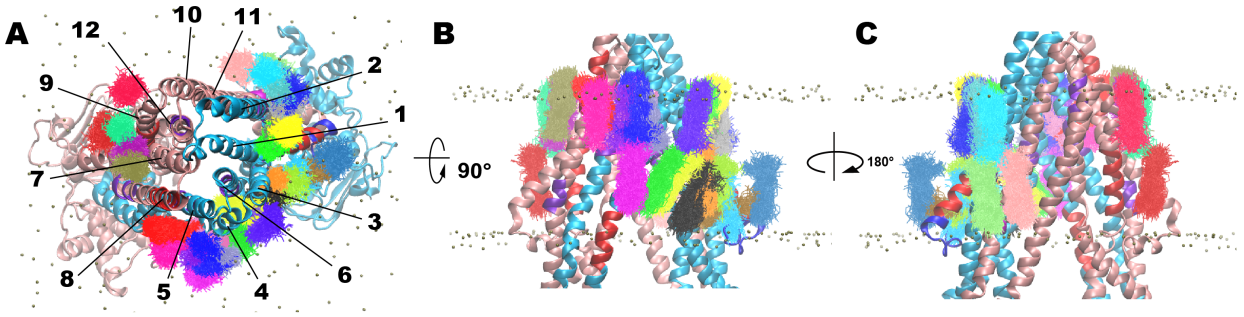

Fig. S4: Clustering analysis of cholesterol molecules located within 4 Å of Pgp obtained using *apo* and Mg^{2+}/ATP-bound simulations in POPC:cholesterol (70:30) bilayer. (A) Top 30 clusters are colored and TM helices are labeled using black lines. (B and C) Rotated side views of the top 30 clusters from the membrane.

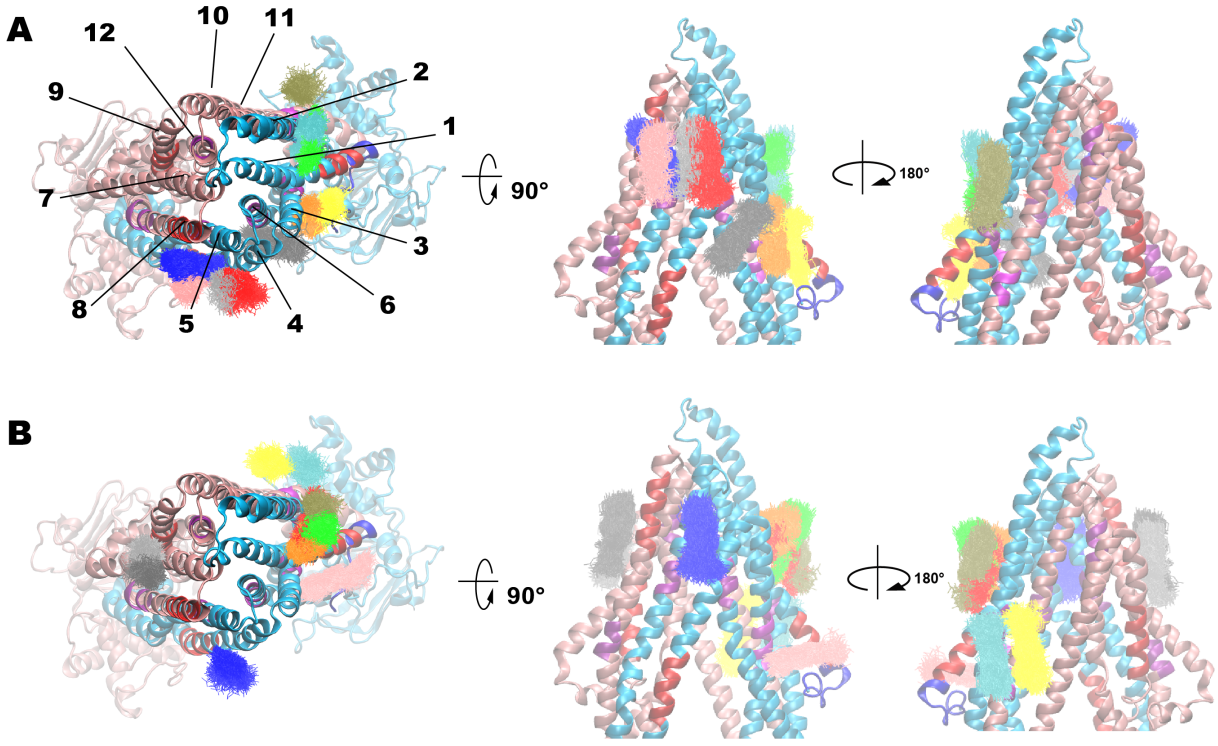

Fig. S5: Comparison of cholesterol clusters between (A) Mg^{2+}/ATP-bound and (B) *apo* systems. Top 10 clusters are shown from the extracellular side (left) and from the membrane (middle and right).

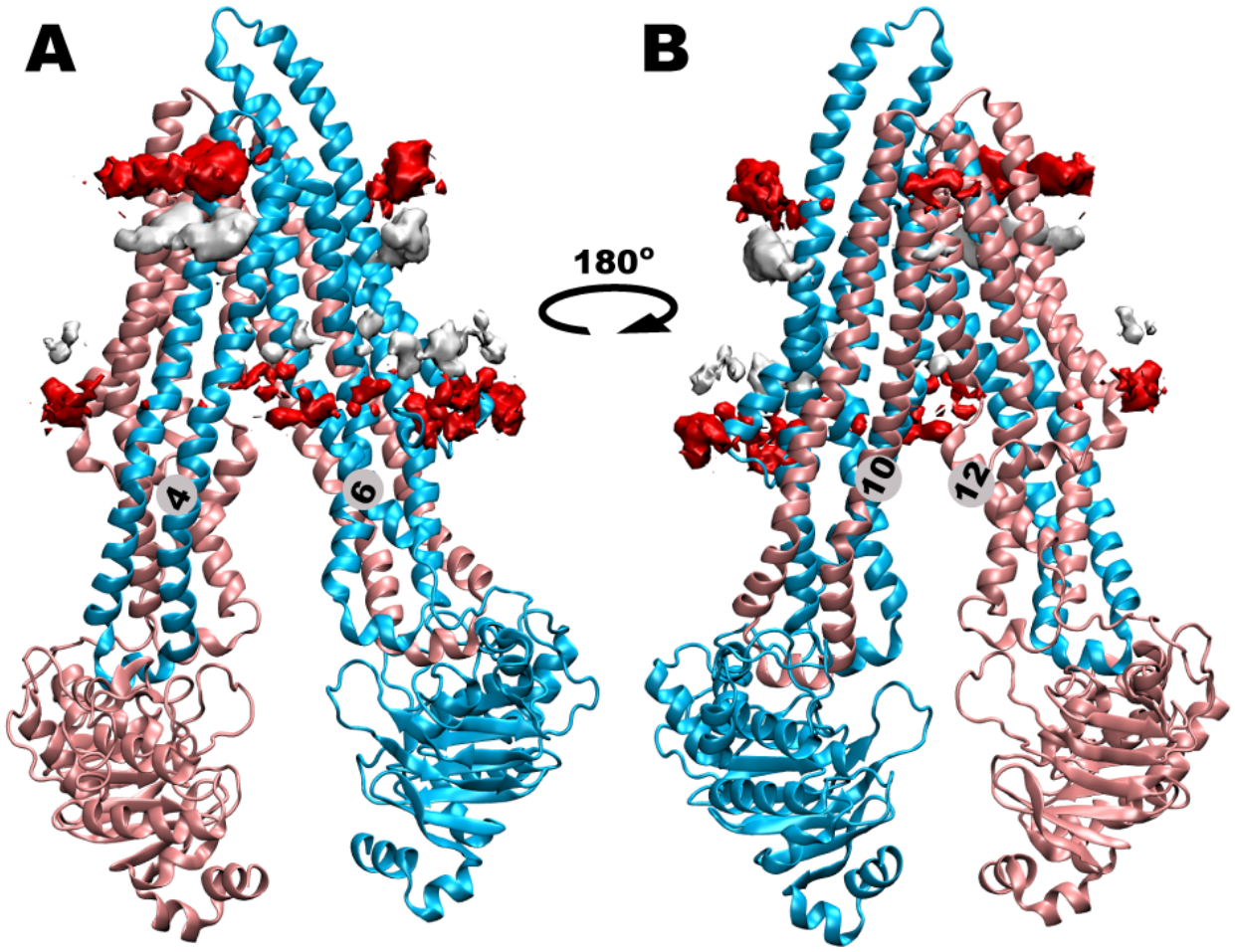

Fig. S6: Occupancy maps of cholesterol molecules. (A) Front and (B) back views of Pgp with occupancy maps of cholesterol molecules calculated using the last 50 ns of *apo* and Mg^{2+}/ATP-bound simulations in POPC:cholesterol (70:30) bilayer. O3 and C17 atoms of cholesterol were used in occupancy maps calculations and shown as red and white surfaces, respectively (see Fig. 1C for atom numbering). Key TM helices (4, 6, 10, 12) are numbered to enable comparison of occupancy densities.

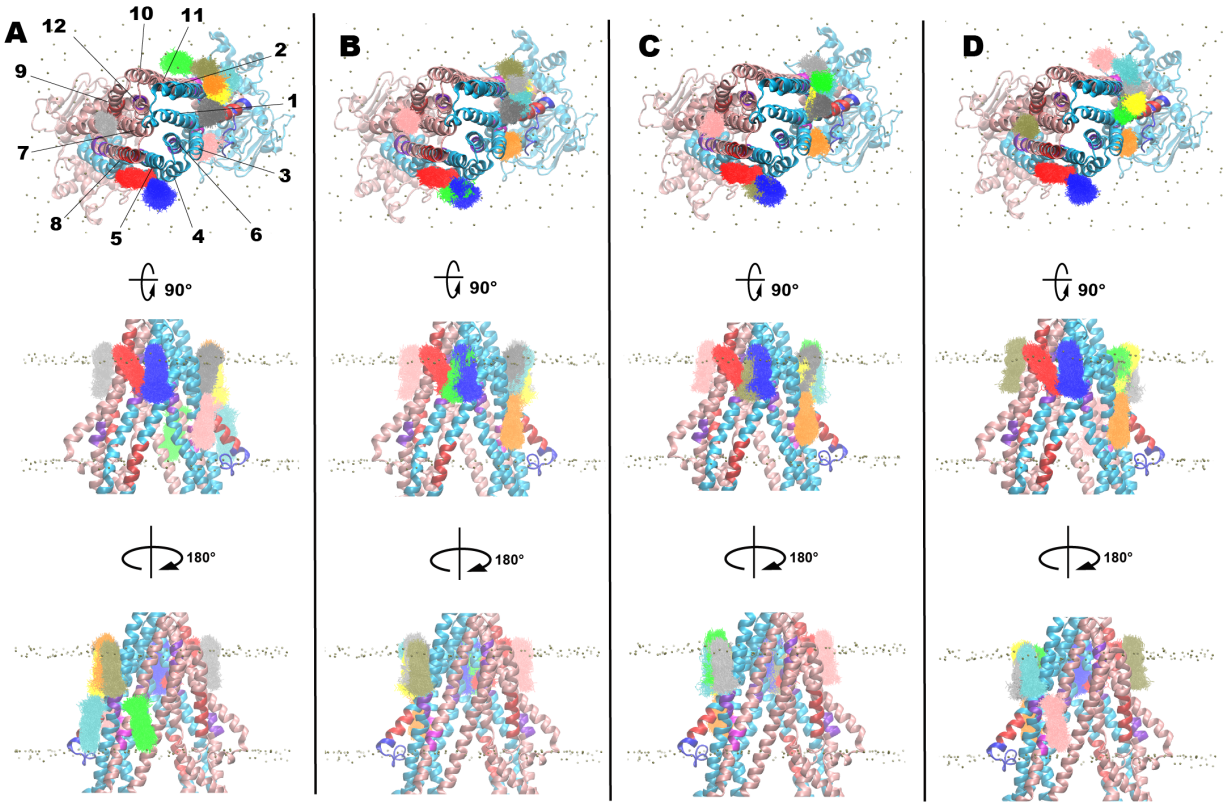

Fig. S7: Convergence analysis of cholesterol molecules located within 4 Å around Pgp obtained using 4 time segments of 200 ns trajectories (each 50 ns). (A-D) A top-down view from the extracellular side showing the top 10 clusters obtained using 4 separate segments of data, with their corresponding rotated side views shown in the middle and bottom rows, respectively. (A) 1-50 ns; (B) 51-100 ns; (C) 101-150 ns; and (D) 151-200 ns time segments of 200 ns trajectories. Each cluster is colored differently and TM helices are labeled using black lines.

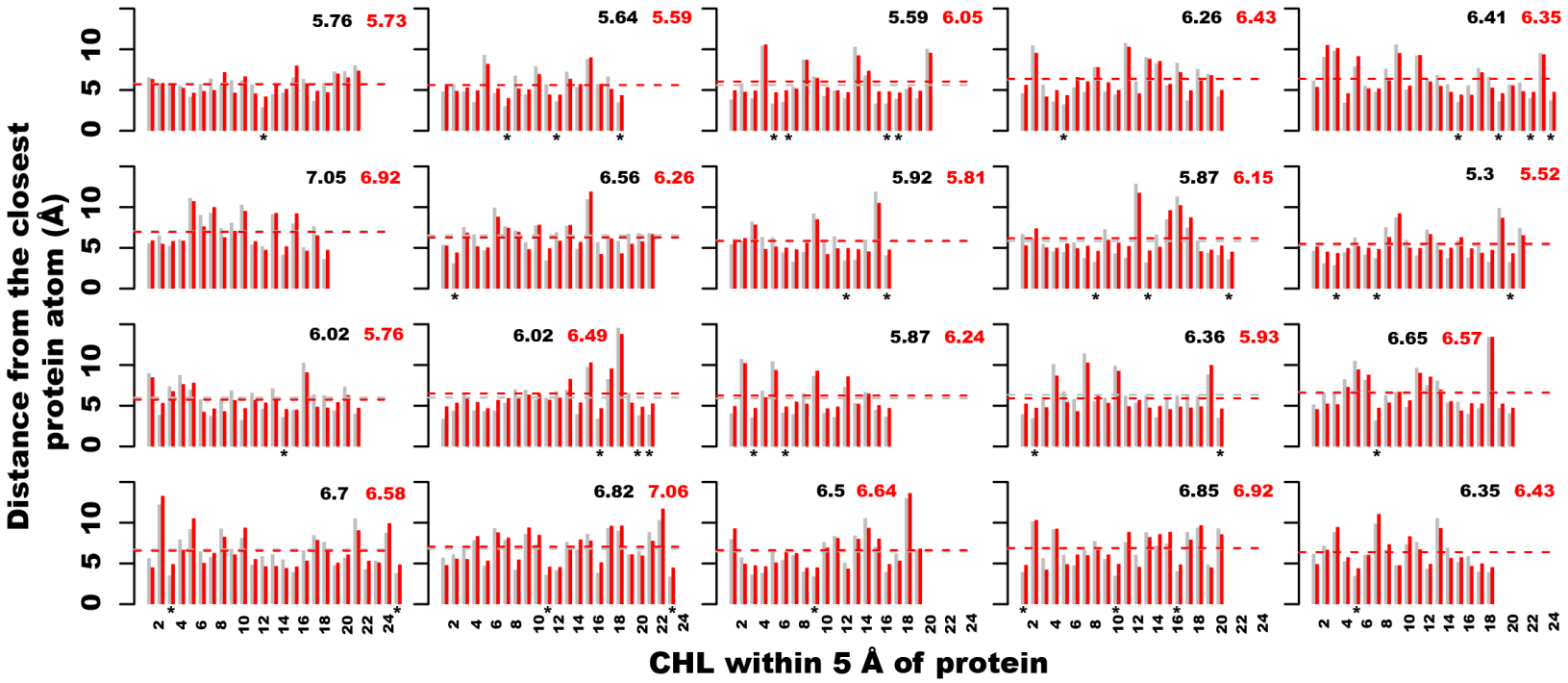

Fig. S8: Binding preference between rough and smooth faces of cholesterol molecules to Pgp in its *apo* form. Calculated center-of-mass distance profiles between rough and smooth faces of cholesterol and the closest atom of Pgp are shown in gray and red color bars, respectively (see Fig. 4A). X-axis lists the number of cholesterols present within 5Å of the protein and selected for this analysis. Mean distance values are provided in black and red for the rough and smooth faces, respectively. Asterisks denote the sandwiched cholesterols between protein atoms.

Table S1: Results of the clustering analysis showing number of cholesterols present in each of the top 30 clusters and their membrane locations, along with the nearby recognition motifs for these clusters. Last 50 ns of simulation data of *apo* and Mg^{2+}/ATP-bound systems in POPC:cholesterol (70:30) bilayer are used in these calculations.

Cluster No.	**# of cholesterols**	**TM helices**	**Leaflet**	**Recognition Motif**
1	915	4, 5	Extra	hCRAC2
2	805	4, 5, 8	Extra	CARC3, hCRAC2
3	502	1, 2	Intra	CARC1, hCRAC1
4	379	1, 3, 6	Extra	hCARC1
5	348	1, 2	Extra	hCARC1
6	332	7, 8, 9	Extra	CARC3, CARC4
7	326	1, 2	Extra	hCRAC1
8	315	1, 2	Extra	hCRAC1
9	276	10, 11	Intra	hCARC2
10	273	2, 11	Extra	hCRAC1
11	255	7, 8, 9	Extra	CARC3, CARC4
12	247	2, 11	Intra	hCRAC1
13	245	5, 6	Extra	hCRAC2
14	241	1, 3, 6	Intra	CRAC1, CARC1, hCARC1
15	234	4, 5	Extra	hCRAC2
16	226	3, 6	Intra	hCARC1
17	205	4, 6	Intra	CARC2, hCRAC3
18	193	1, 3, 6	Intra	CRAC1, CARC1, hCARC1
19	190	4, 6	Intra	CARC2, hCRAC3
20	190	7, 9	Extra	CARC4
21	181	2, 11	Extra	hCRAC1
22	178	1, 3, 6	Intra	CRAC1, CARC1, hCARC1
23	171	1	Intra	CRAC1, CARC1
24	171	2	Extra	hCRAC1
25	161	3, 4, 6	Extra	hCRAC3
26	160	4, 5	Extra	hCRAC2
27	155	4	Intra	CARC2
28	153	5, 8	Extra	hCRAC2, CARC3
29	147	9, 10	Intra	CARC4
30	147	7, 9	Intra	CARC4