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**Supplementary Table 2 – Amino acid sequences of human FLVCR variants used in this study.**

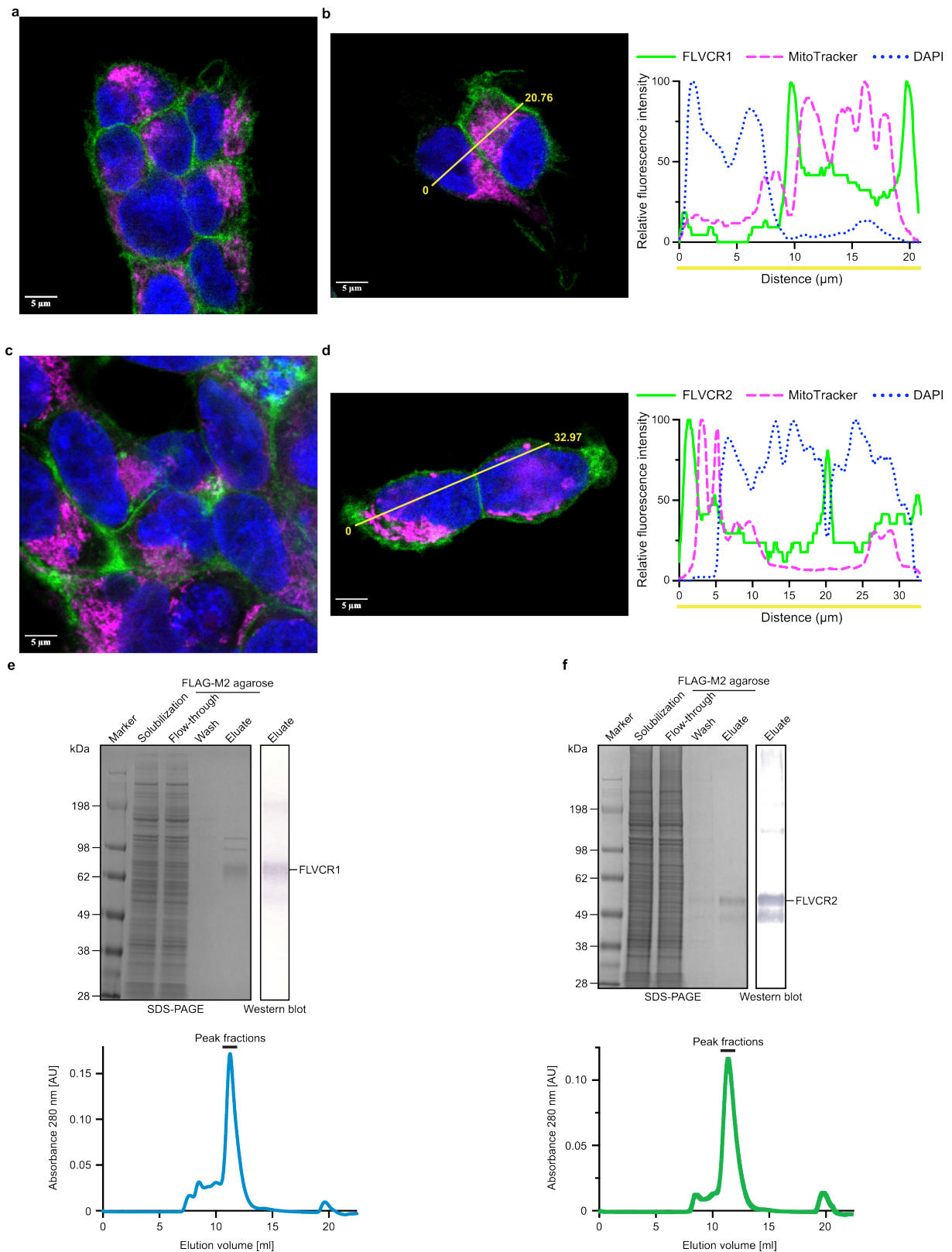
Variant	Amino acid sequence
FLVCR1a (wild-type)	MARPDEEGAAVAPGHPLAKGYLPLPRGAPVVGKESVELQNGPKAGTFPVNGAPRDSLAAASGVLGGPQTPLAPEEETQARLLP AGAGAETPGAESSPLTLTALSRRFVLLIFSLYSLVNAFQWIQYSISNVFEGFYGVTLHIDWLSMVYMLAYVPLIFPATWLLDTR GLRLTALLGSGLNCLGAWIKCGSVQQHLFWVTMLGQCLCSVAQVFILGLPSRIASVWFGPKEVSTACATAVLGNQLGTAVGFLL PPVLPNTQNDTNLLACNISTMFYGTSAVATLLFILTAIAFKEKPRYPSSQAQAALQDSPPEEYSYKKSIRNLFKNIPVLLITYGIM TGAFYSVSTLLNQMILTYEGEEVNAGRIGLTLVVAGMVGSLCGLWLDYTKYKQTTLIVYILSFIGMVIFFTLRLYIIIVFVTGG VLGFFMTGYLPLGFEFAVEITYPESEGTSSGLLNASAQIFGILFLAQGKLTSDYGPKAGNIFLCVWWMFIGIILTALIKSDLRRHNINI GITNVDVKAIPADSPTDQEPKTVMLSKQSESAIDYKDDDDK
FLVCFR2 (wild-type)	MVNEGPNQEESDDTPVPESALQADPSVSVHPSVSVHPSVSNPSVSVHPSSSAHPSALAQPSGLAHPSSSGPEDLSVIKVSRRR WAVVLVFCSCYMCNSFQWIQYGSINIFMHFYGVSAFAIDWLSMCYMLTYIPLLLPVAWVLEKFGRLTIALTGSALNCLGAWVK LGSLKPHLFPVTVVVGQLICVAQVFILGMPRIASVWFGANEVSTACSVAVFGNQLGIAIGFLVPPVLPNIEDRDELAYHISIMFYI IGGVATLLILVIVFKEKPKYPPSRAQSLSYALTSPDASYLGSARLFKNLNFVLLVITYGLNAGAFYALSTLLNRMVIWHYPGEEVN AGRIGLTIAGMLGAVISGIWLDRSKTYKETTLLVYIMTLVGMVVYFTLNLGHLWVVVITAGTMGFFMTGYLPLGFEFAVELTY PESEGSSGLLNISAQVFGIIFTISQGGIIDNYGTPGNIFLCVFLTLGAALTAFAIKADLRRQKANKETLENKLQEEEEESNTSKVPTAV SEDHLDYKDDDDK

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3 **Supplementary Table 3 – Cryo-EM and model data statistics.**

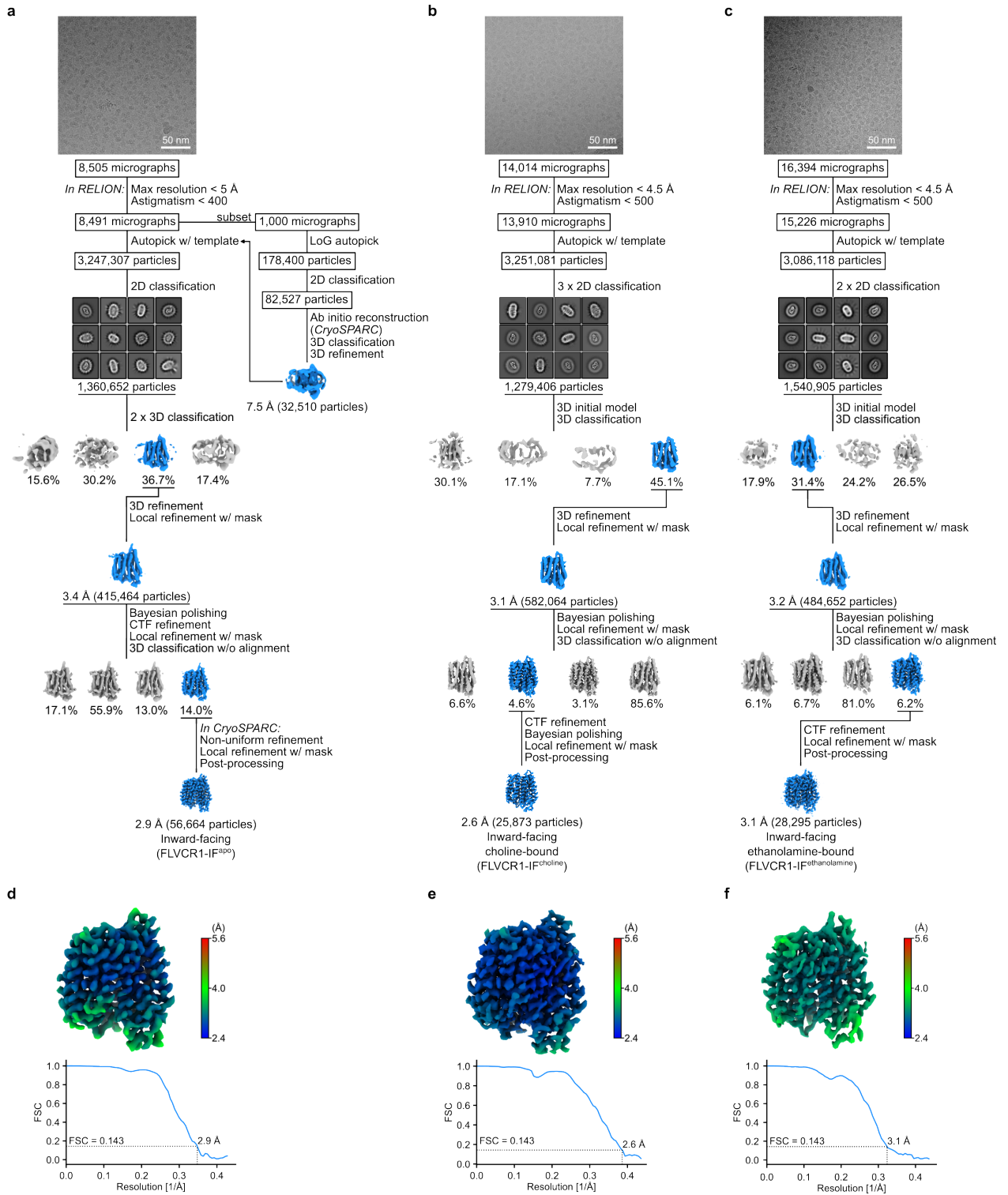
	FLVCR1-IF <sup>apo</sup> (EMD-18334; PDB 8QCS)	FLVCR1- IF <sup>choline</sup> (EMD-18335; PDB 8QCT)	FLVCR1- IF <sup>ethanolamine</sup> (EMD-19009; PDB 8R8T)	FLVCR2-IF <sup>apo</sup> (EMD-18336; PDB 8QCX)	FLVCR2-OF <sup>apo</sup> (EMD-18337; PDB 8QCY)	FLVCR2- IF <sup>choline</sup> (EMD-18339; PDB 8QD0)	FLVCR2- OF <sup>heme</sup> (EMD-18338; PDB 8QCZ)	FLVCR2- IF <sup>heme-choline</sup> (EMD-19018)
<b>Data collection and processing</b>								
Magnification	215,000	215,000	215,000	215,000	215,000	215,000	105,000	215,000
Voltage (kV)	300	300	300	300	300	300	300	300
Electron dose (e <sup>-</sup> /Å <sup>2</sup> )	55	55	55	80	80	57	80	55
Defocus range (µm)	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1	-1.1 to -2.1
Pixel size (Å)	0.573	0.573	0.573	0.573	0.573	0.573	0.837	0.573
Symmetry imposed	C1	C1	C1	C1	C1	C1	C1	C1
Initial particle images (no.)	3,247,307	3,251,081	3,086,118	3,497,517	3,497,517	4,790,154	5,419,952	1,975,710
Final particle images (no.)	56,664	25,873	28,295	50,167	49,532	51,511	51,266	37,044
Map resolution (Å)	2.9	2.6	3.1	3.1	2.9	2.8	3.1	3.1
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.4–6.5	2.6–4.9	3.0–5.3	2.8–5.5	2.7–3.8	2.6–3.8	2.7–5.8	2.7–6.0
<b>Refinement</b>								
Initial model used	AlphaFold model (AF-Q9YSY0-F1)			AlphaFold model (AF-Q9UPI3-F1)				
Model resolution (Å)	3.4	3.1	3.2	3.6	3.4	3.3	3.6	
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Model resolution range (Å)								
Map sharpening B factor (Å <sup>2</sup> )	-80	-30	-40	-120	-100	-30	-60	
Model composition								
Non-hydrogen atoms	3,255	3,273	3,270	3283	3,273	3,290	3,316	
Protein residues	417	418	418	422	421	422	421	
Ligands	NAG: 1	NAG: 1 CHT: 1	NAG: 1 ETA: 1	-	-	CHT: 1	HEM: 1	
Average B factors (Å <sup>2</sup> )								
Protein	87.45	40.28	68.08	76.93	82.55	63.63	109.15	
Ligand	144.11	68.46	93.55	-	-	31.72	183.4	
R.m.s. deviations								
Bond lengths (Å)	0.003	0.003	0.003	0.003	0.004	0.003	0.004	
Bond angles (°)	0.570	0.512	0.594	0.512	0.592	0.641	0.631	
Validation								
MolProbity score	1.50	1.40	1.38	1.20	1.31	1.31	1.43	
Clashscore	6.82	7.22	4.07	4.20	5.71	5.68	7.24	
Poor rotamers (%)	2.5	3.1	0.6	0.6	0.0	2.8	1.4	
Ramachandran plot								
Favored (%)	97.3	98.1	96.9	99.0	98.6	99.0	98.1	
Allowed (%)	2.7	1.9	3.1	1.0	1.4	1.0	1.9	
Disallowed (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

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**Supplementary Fig. 1: Purification of human FLVCR1 and FLVCR2.** Representative immunofluorescence images of HEK293 cells overproducing FLAG-tagged wild-type human FLVCR1 (**a,b**) and FLVCR2 (**c,d**). Both FLVCRs were labelled with ANTI-FLAG® M2-FITC antibody. Mitochondria was labelled with MitoTracker™ Red CMXRos dyes. DNA was stained with DAPI. Distance trace of fluorescence intensities from these dyes within one representative image was analyzed and presented in (**b**) for FLVCR1 and (**d**) for FLVCR2. SDS-PAGE and western blot analysis of FLVCR1 (**e**) and FLVCR2 (**f**) affinity purification (left) and subsequent size-exclusion chromatography (right).

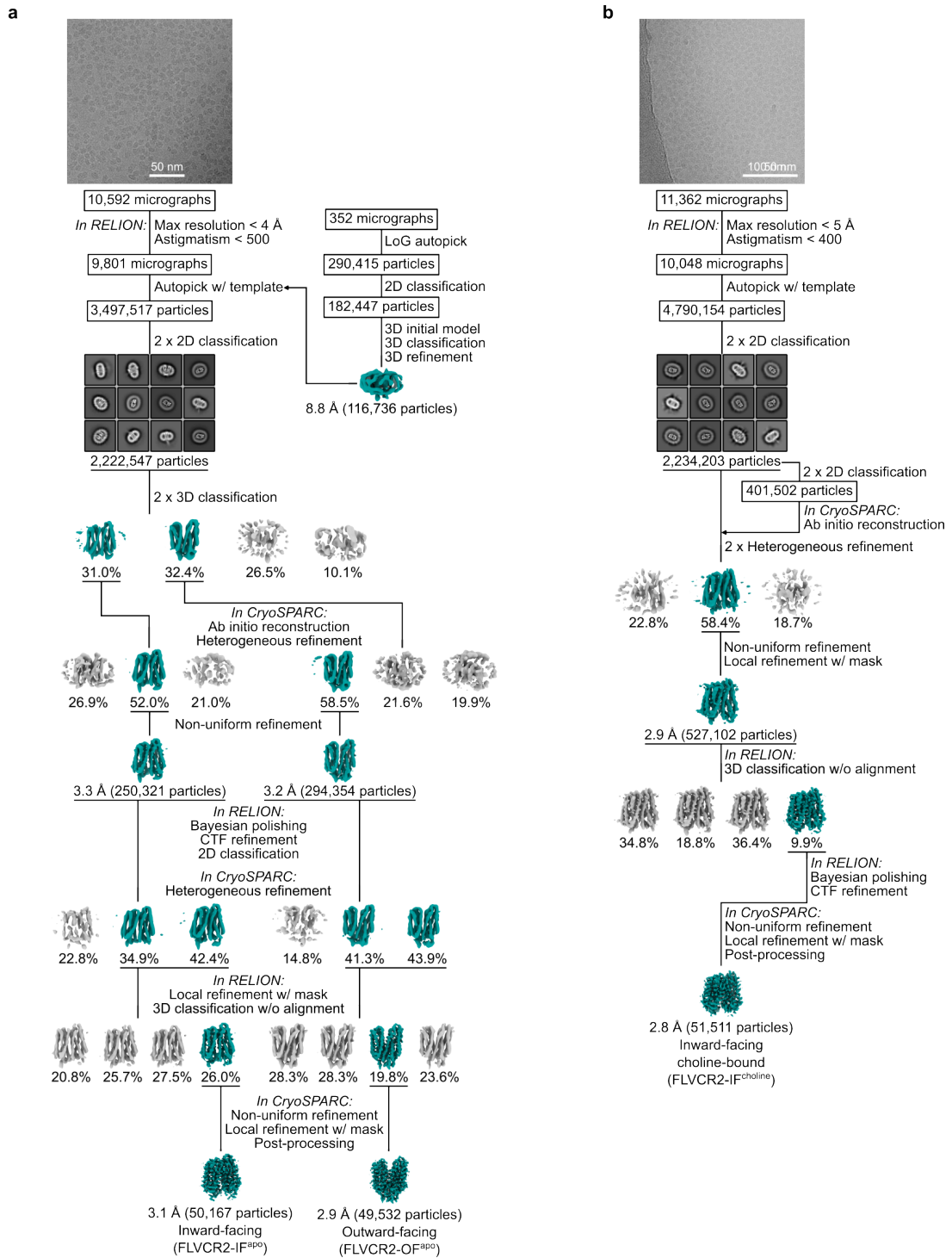
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**Supplementary Fig. 2: Single-particle cryo-EM analysis of human FLVCR1.**

**a-c**, Summary of the data processing procedure of the as-isolated FLVCR1 sample and FLVCR1 supplemented with choline. **d-f**, Local resolution estimation (left) and Fourier shell correlation (FSC) curves (right) of the final cryo-EM maps of FLVCR1-IF<sup>apo</sup> and -IF<sup>choline</sup>.

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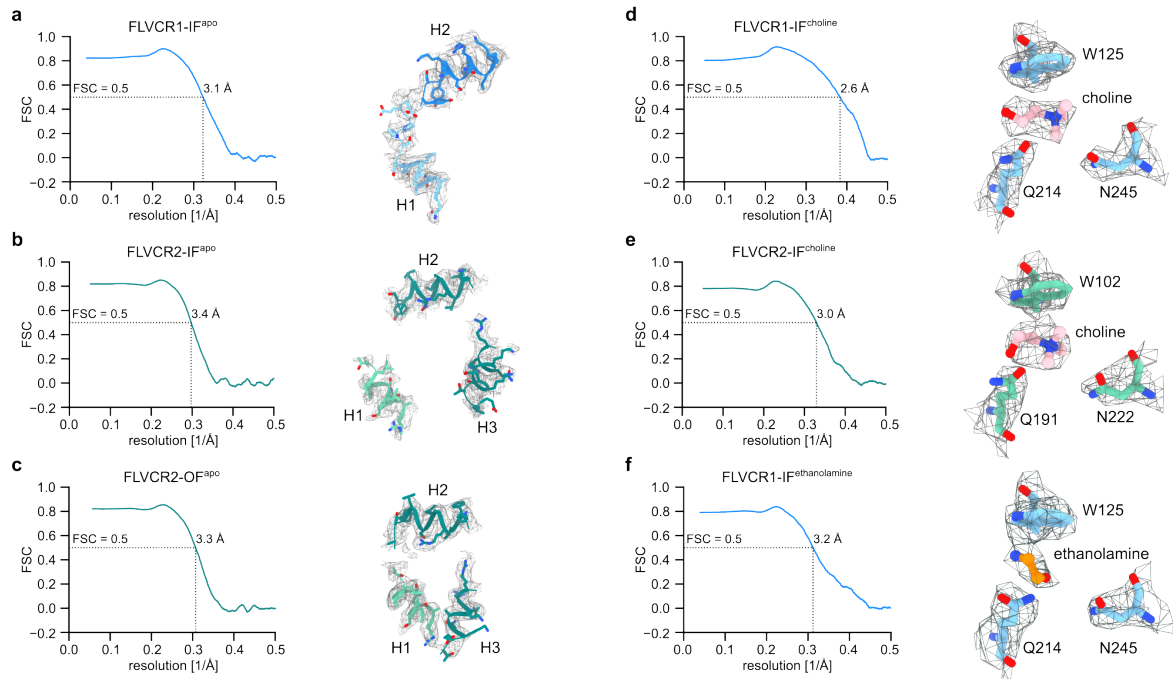


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**Supplementary Fig. 3: Single-particle cryo-EM analysis of human FLVCR2.**

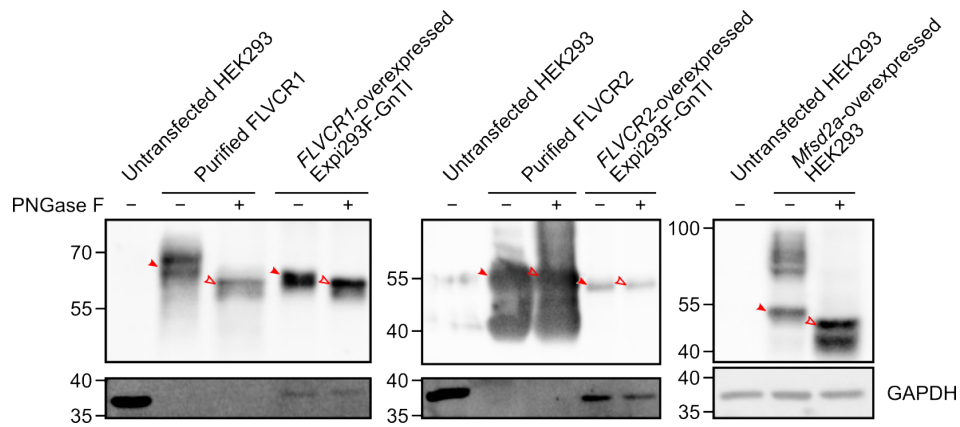
**a,b**, Summary of the data processing procedure of the as-isolated FLVCR2 and choline-supplemented FLVCR2 samples, respectively. **c-e**, Local resolution estimation (top) and FSC curves (bottom) of the final cryo-EM maps of FLVCR2-IF<sup>apo</sup>, FLVCR2-OF<sup>apo</sup>, and FLVCR2-IF<sup>choline</sup>, respectively.





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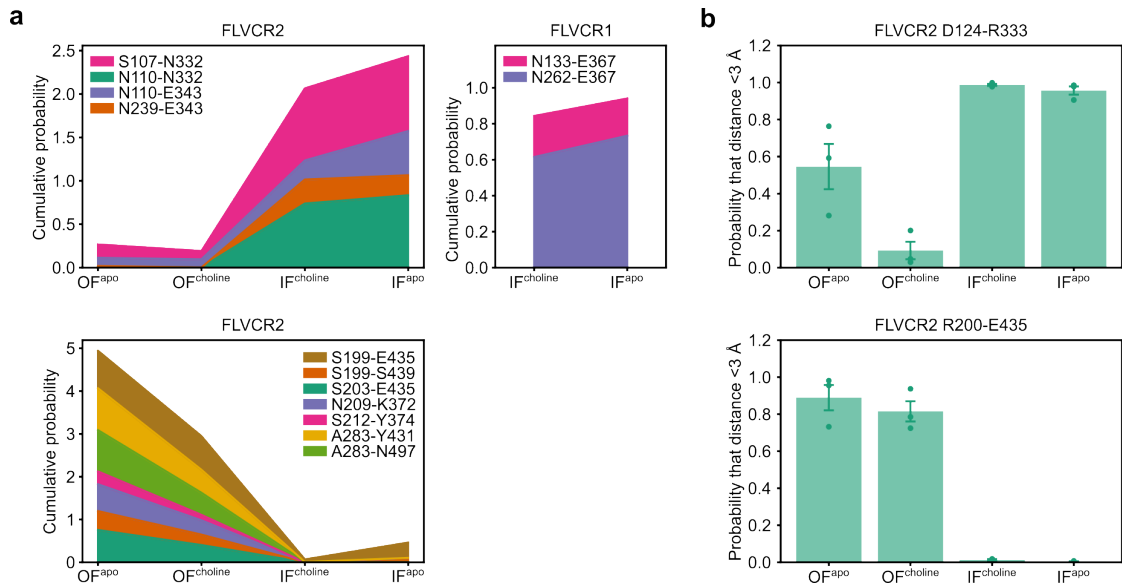
**Supplementary Fig. 4 Cryo-EM density and model fitting.** Cryo-EM map-to-model FSCs of FLVCR1-IF<sup>apo</sup> (a), FLVCR2-IF<sup>apo</sup> (b), FLVCR2-OF<sup>apo</sup> (c), FLVCR1-IF<sup>choline</sup> (d), FLVCR2-IF<sup>choline</sup> (e), and FLVCR1-IF<sup>ethanolamine</sup> (f). The maps for the intracellular helices of the apo states as well as the maps for choline/ethanolamine and the interacting residues of the ligand-bound states are shown.



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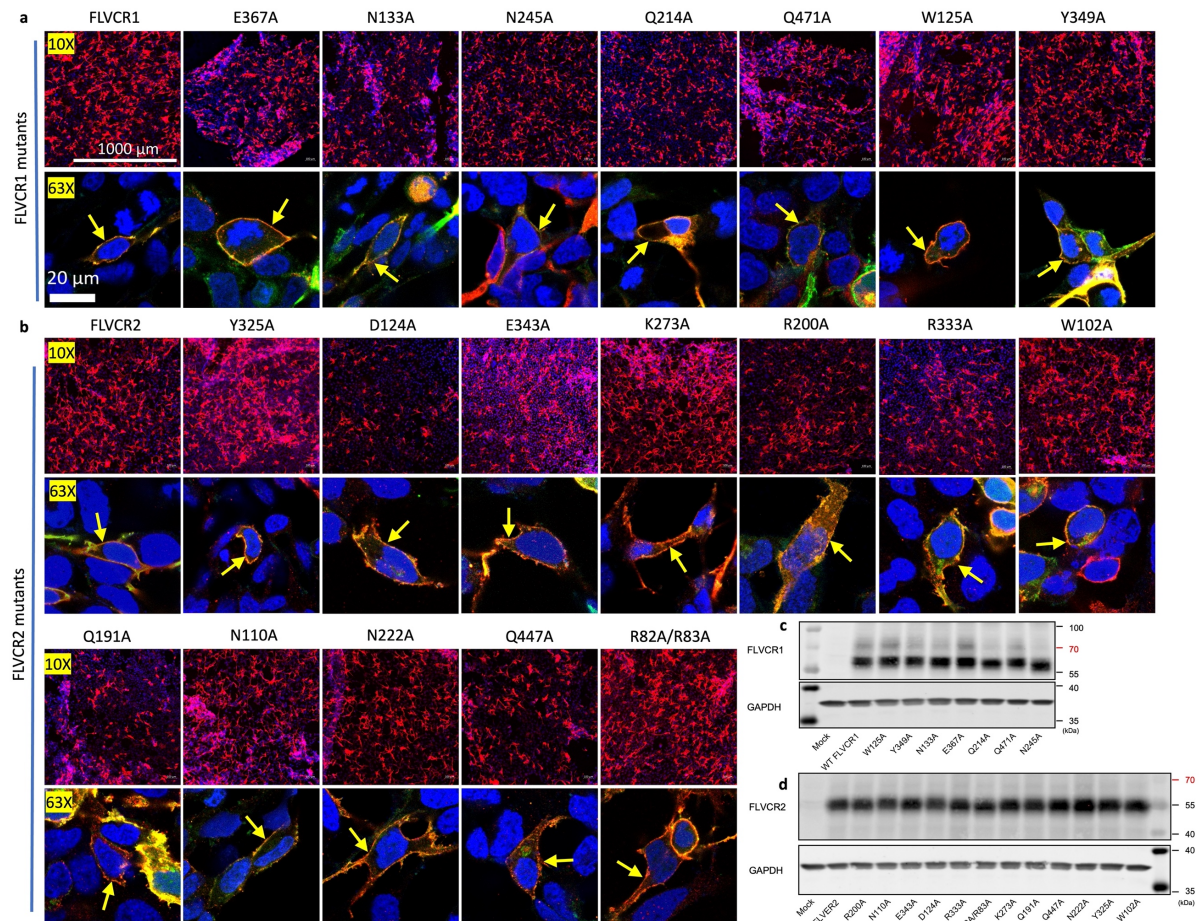
**Supplementary Fig. 5: FLVCR1 deglycosylation assay.**

Western blots of PNGase F treated (+) and untreated (-) samples from cell lysates of Expi293-GnTI cells overexpressing FLVCRs or from the purified proteins for cryo-EM analysis. *Mfsd2a*-overexpressed HEK293 cell lysate was used as a control. Filled arrows represent the molecular weight of proteins before deglycosylation and empty arrows represent the molecular weight after deglycosylation.



**Supplementary Fig. 6: Persistence of critical interactions for stabilization of FLVCR1 and FLVCR2 in MD simulations.** **a**, Cumulative probability of inter-domain hydrogen bond formation at the external gate (top) and the internal gate (bottom) with or without choline in the binding cavity. For each pair of residues, the probability was calculated as the fraction of the trajectory in which the distance between the residues remained below 3 Å. **b**, Stability of salt bridges within the external gate (top) and internal gate (bottom) of FLVCR2 in MD simulations. The displayed data represents the mean values obtained from three independent replicas, with error bars representing standard errors of the mean (s.e.m.).

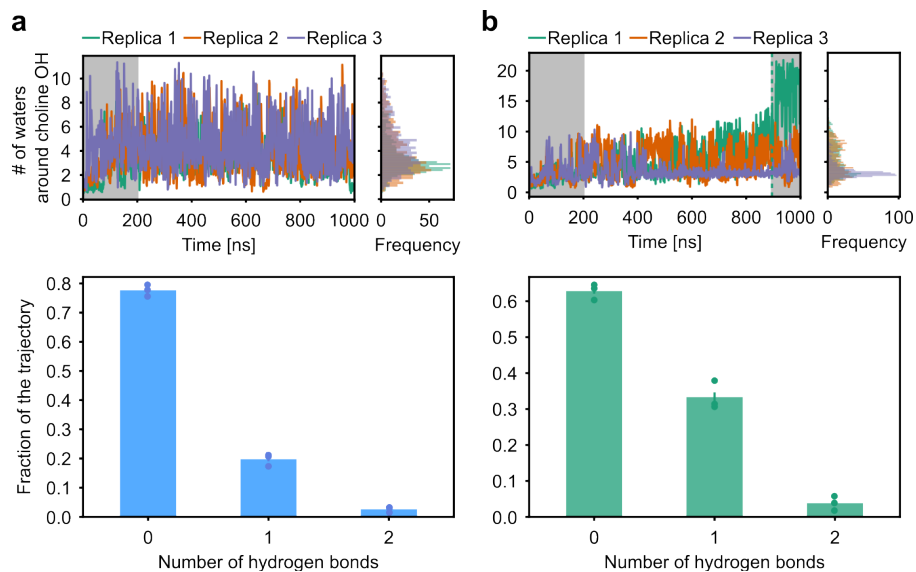
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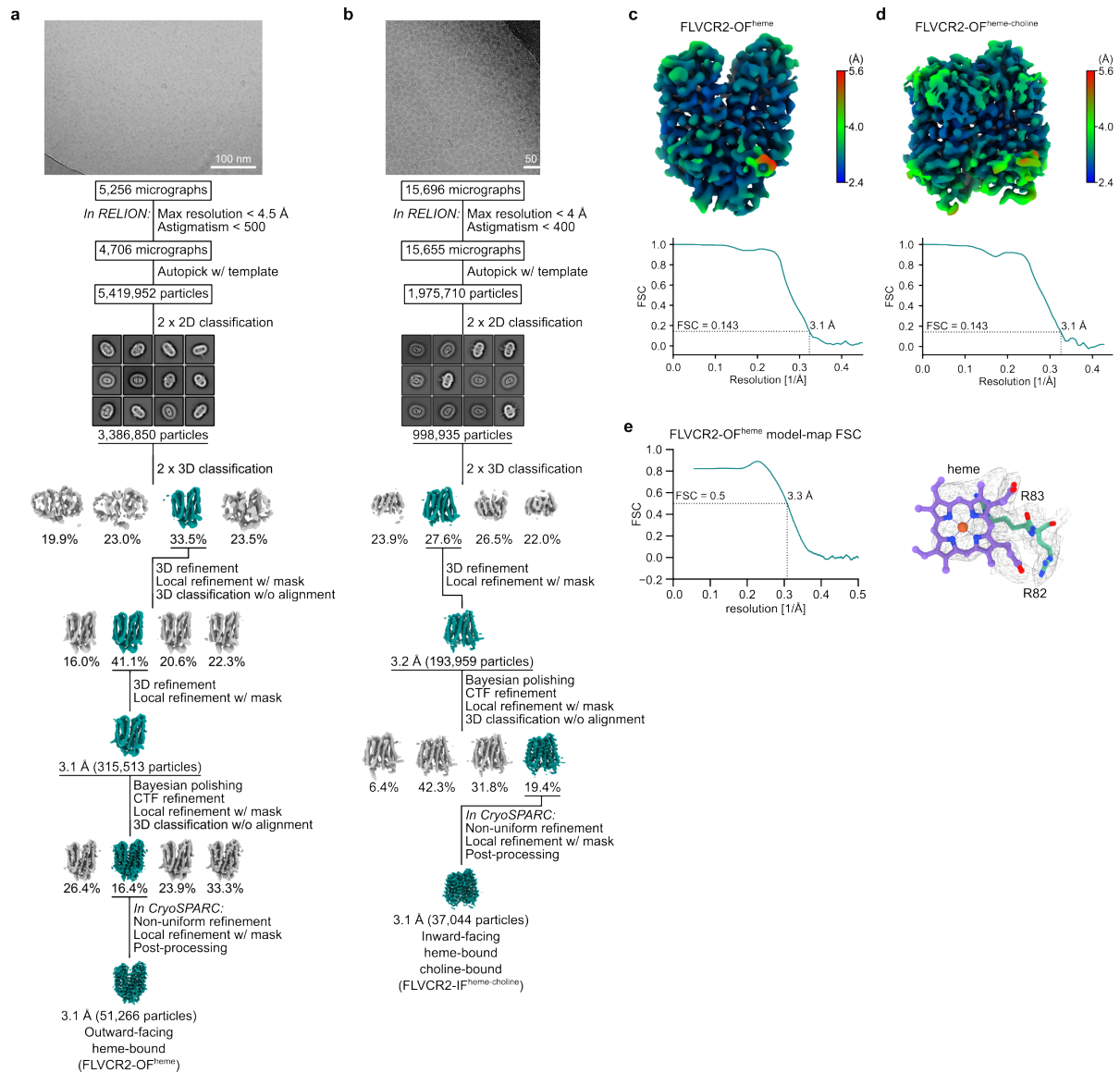
**Supplementary Fig. 7: Immunofluorescent images and western blots of FLVCR mutants.**

Immunofluorescent images of cells overexpressing mutants of *FLVCR1* (a) or *FLVCR2* (b). Arrows indicate the localization of FLVCRs. Scale bars represent 1000  $\mu\text{m}$  for 10X, and 20  $\mu\text{m}$  for 63X magnification. Western blot analysis of mutated proteins from overexpression in HEK293 cell lysates of *FLVCR1* (c) or *FLVCR2* (d).



**Supplementary Fig. 8: Hydration of choline in MD simulations.** Interactions between choline and water in the binding site of inward-facing FLVCR1 (a) and FLVCR2 (b). The upper panels indicate the number of water molecules in the proximity of the hydroxyl group of choline with a cutoff of 5 Å in the  $O_{\text{choline}}-O_{\text{water}}$  distance. The first 200 ns of simulations were considered as equilibration and thus not included in the histograms. The last 105 ns of replica 1 in the FLVCR2 simulation were discarded as well due to choline release. The lower panels represent the hydrogen bond formation between waters and the hydroxyl group of choline in the simulations with a distance and angle cut-off of 3 Å and 20°, respectively. Error bars represent s.e.m.

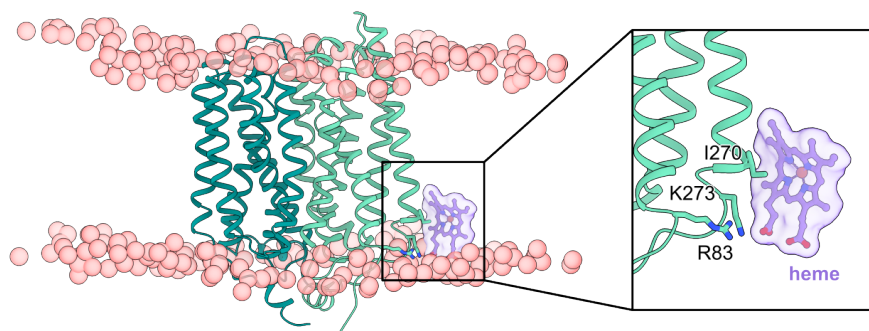
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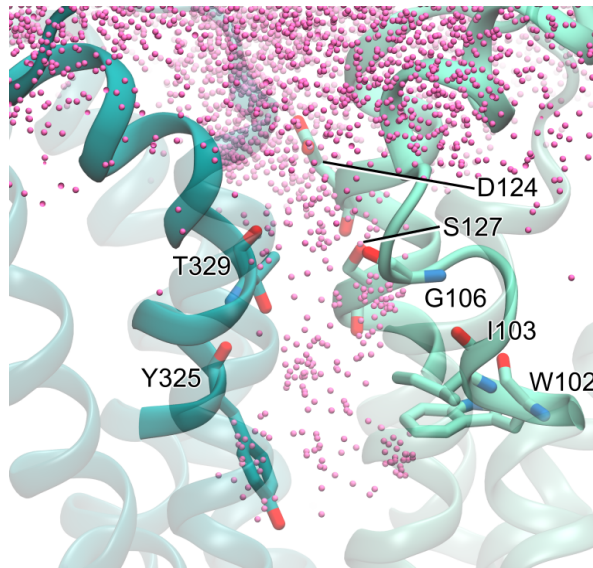
**Supplementary Fig. 9: Single-particle cryo-EM analysis of human FLVCR2 in the presence of heme.**

**a,b**, Summary of the data processing procedure of heme-supplemented, and heme-choline-supplemented FLVCR2 samples, respectively. **c,d**, Local resolution estimation (top) and FSC curves (bottom) of the final cryo-EM maps of FLVCR2-OF<sup>heme</sup>, and FLVCR2-IF<sup>heme-choline</sup>, respectively. **e**, Cryo-EM map-to-model FSCs of FLVCR2-OF<sup>heme</sup> and the map for the bound heme molecule.



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**Supplementary Fig. 10: Snapshot of new, stable interactions formed between heme and FLVCR2 within the lipid bilayer in MD simulations.** The heme molecule is shown in ball-and-stick representation and the phosphates of POPE/POPG are shown as red spheres. The right panel shows the close-up view of the heme molecule and the interacting residues of FLVCR2.



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**Supplementary Fig. 11: Choline entry from solution into the outward-facing cavity of FLVCR2 in MD simulations.** Residues that interact with choline frequently are shown as sticks. Pink dots represent choline positions in all frames of one 1- $\mu$ s trajectory.