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Supplemental Information

Structural basis for no retinal binding in flotillin-associated rhodopsins

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Supplementary Materials

Table S1. Cryo-EM data collection, refinement, and validation, Related to Figure 2, Figure 3, and Figure 4.

Protein	<i>PsFAR</i>	<i>PsPR</i>	<i>PsFAR-DM</i>
PDB ID	9R21	9R22	9R23
Data collection and processing			
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	50	50	50
Defocus range (μm)	-0.5 to -2.0	-0.5 to -2.0	-0.5 to -2.0
Pixel size (Å)	0.836	0.836	0.836
Micrographs collected	4478	6112	2015
Micrographs processed	3792	4589	1678
Symmetry imposed	C5	C5	C5
Initial dataset (# of particles)	619,824	2,107,133	687,836
Final dataset (# of particles)	212,403	497,291	116,149
Map resolution (Å) FSC _{01.43}	2.56	2.48	2.81
Refinement			
Initial model used	AlphaFold2	AlphaFold2	<i>PsFAR</i> (present work)
Map-sharpening <i>B</i> factor (Å ²)	123.7	121.8	131
No. atoms			
Protein	9,230	8,895	9,194
Retinal	-	100	100
Lipid	904	543	700
Water	97	105	45
<i>B</i> -factors (Å ²)			
Protein	39.8	41.6	48.2
Retinal	-	31.8	43.0
Lipid	94.2	88.0	113.2
Water	42.3	39.4	38.7
R.m.s. deviations			
Bond lengths (Å)	2.120	0.013	0.010
Bond angles (°)	0.011	1.941	1.788
Validation			
MolProbity score	2.19	1.73	1.87
Clash score	11.88	9.09	9.33
Poor rotamers (%)	4.58	2.11	3.16
Ramachandran plot (%)			
Favored	97.52	99.02	98.61
Allowed	2.48	0.98	1.39
Outliers	0	0	0
Model to map fit CC	0.90	0.88	0.86

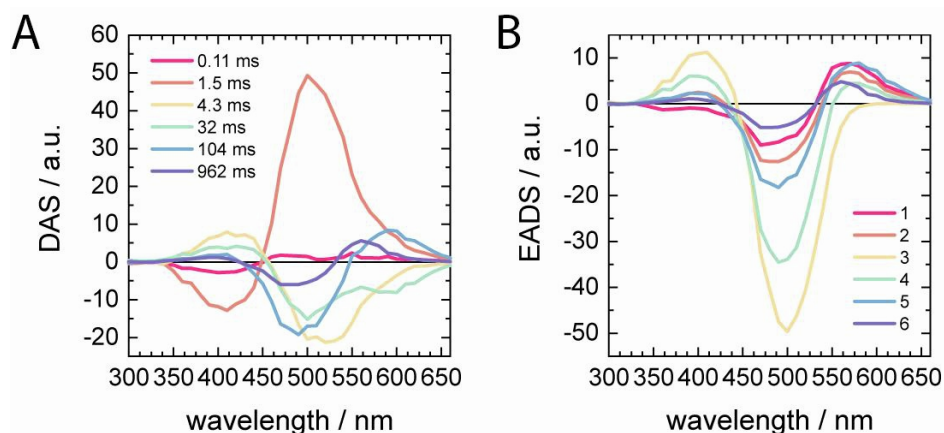


Fig. S1. Kinetic analysis of ms-dynamics of PsPR obtained by global target analysis using a sequential model, Related to Figure 1. **A.** Corresponding decay-associated spectra (DAS) highlighting the spectral changes associated with each of the six lifetimes. **B.** Evolution-associated difference spectra (EADS) displaying the spectral composition of each intermediate state. Spectral and kinetic decomposition allows the assignment of the components to construct the photocycle model following the spectral position and nomenclature originally derived for bacteriorhodopsin¹³ shown in Fig. 1E. The first two lifetimes of 0.11 ms and 1.5 ms describe the decay of the K intermediate towards the M state, spectrally characterized by a blue shift originating in the deprotonation of the RSB. M proceeds with a lifetime of 4.3 ms towards N, shifting the absorption towards the bleach. The N state decays with 32 ms and forms the red-shifted O₁ intermediate. O₁ proceeds to O₂ with a lifetime of 104 ms before the decay of O₂ recovers the ground state with a lifetime of 962 ms.

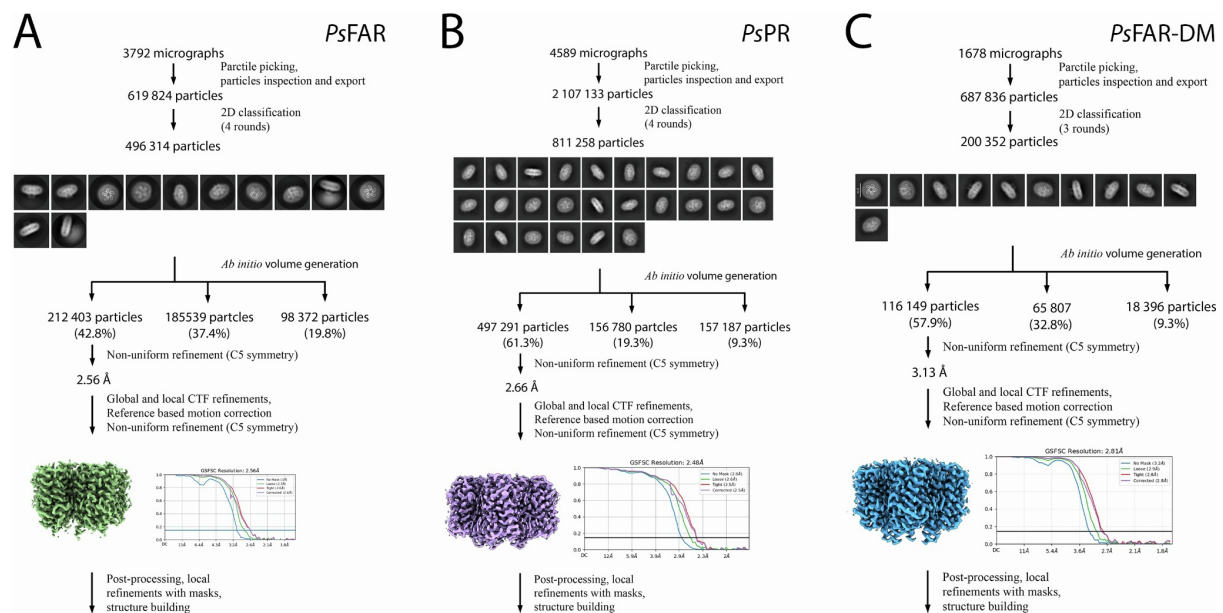


Fig. S2. Workflow for solving the structures using cryoSPARC, Related to Figure 2. A. *PsFAR*. B. *PsPR*. C. *PsFAR-DM*.

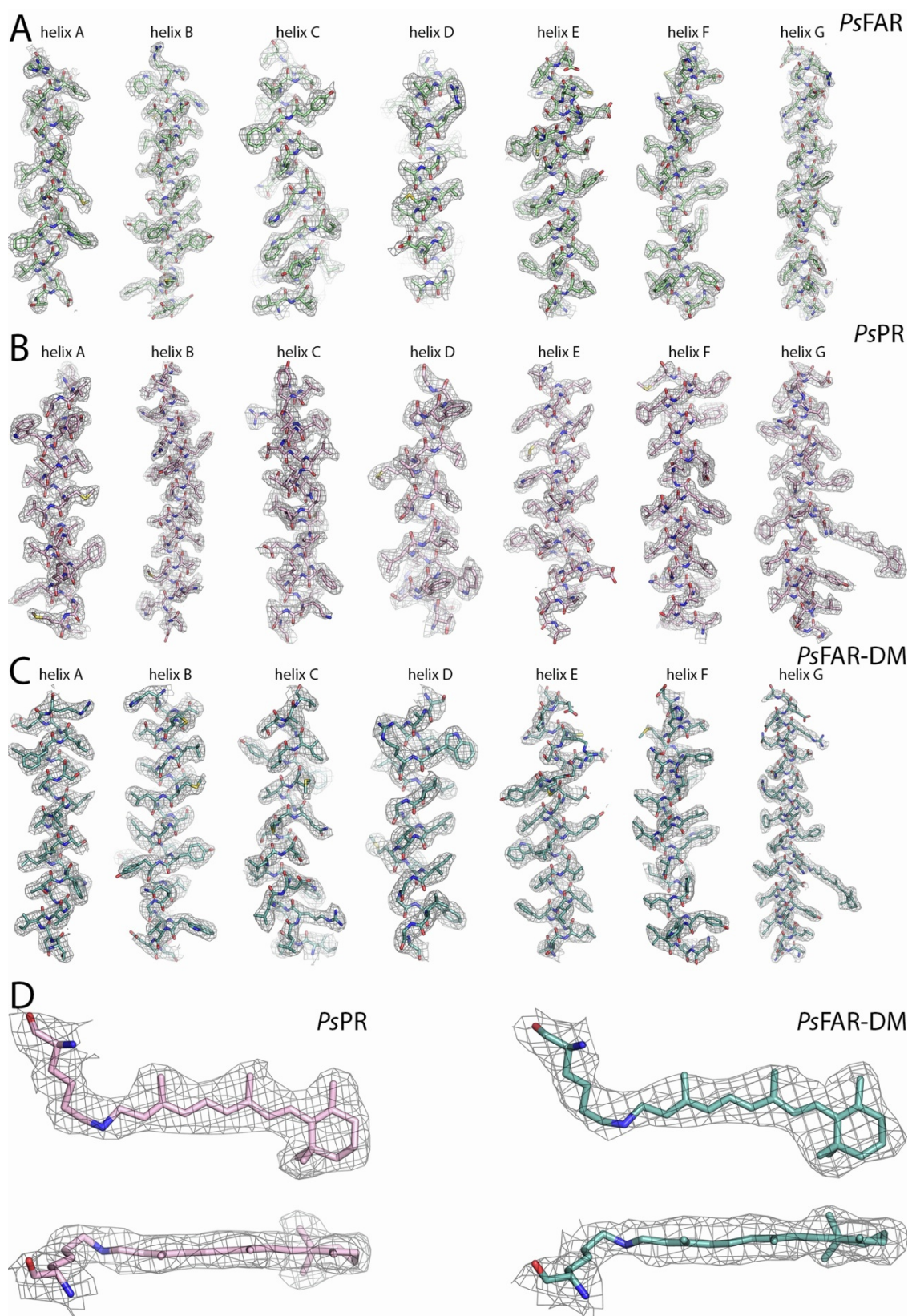


Fig. S3. Cryo-EM densities (FSC-weighted sharpened map from cryoSPARC), Related to Figure 2. A. Examples of densities around each of transmembrane helices of *PsFAR*, **B.** Examples of densities around each of transmembrane helices of *PsPR*. **C.** Examples of densities around each of transmembrane helices of *PsFAR-DM*. **D.** Densities around the retinal cofactor of *PsPR* (left panel) and *PsFAR-DM* (right panel).

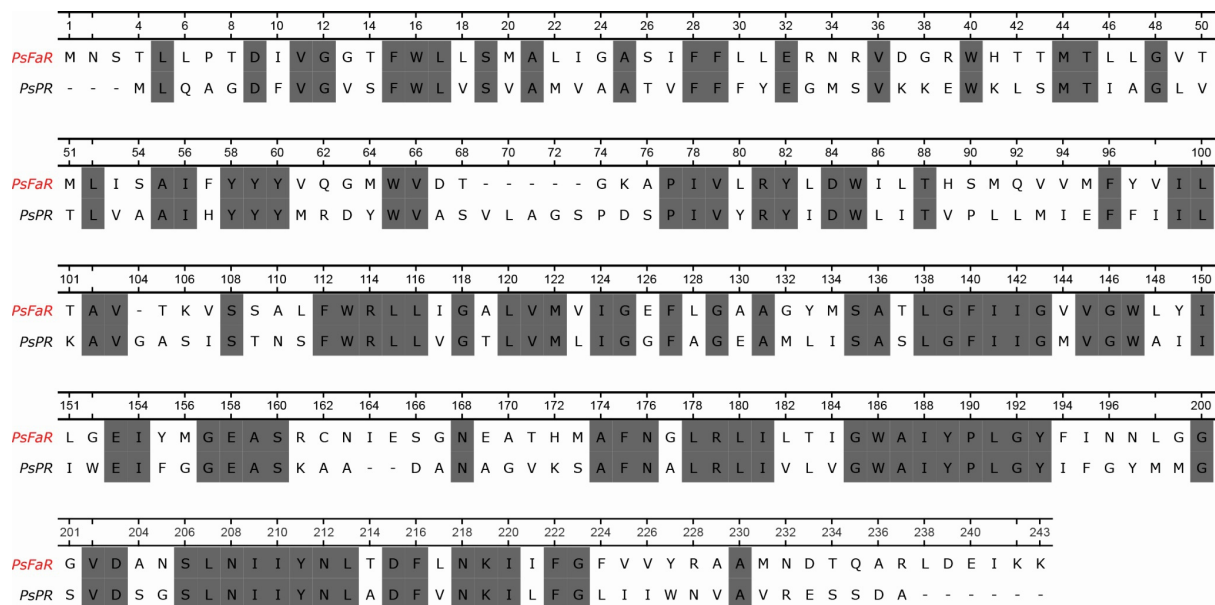


Fig. S4. Sequence alignment of *PsFaR* and *PsPR*, Related to Figure 2.

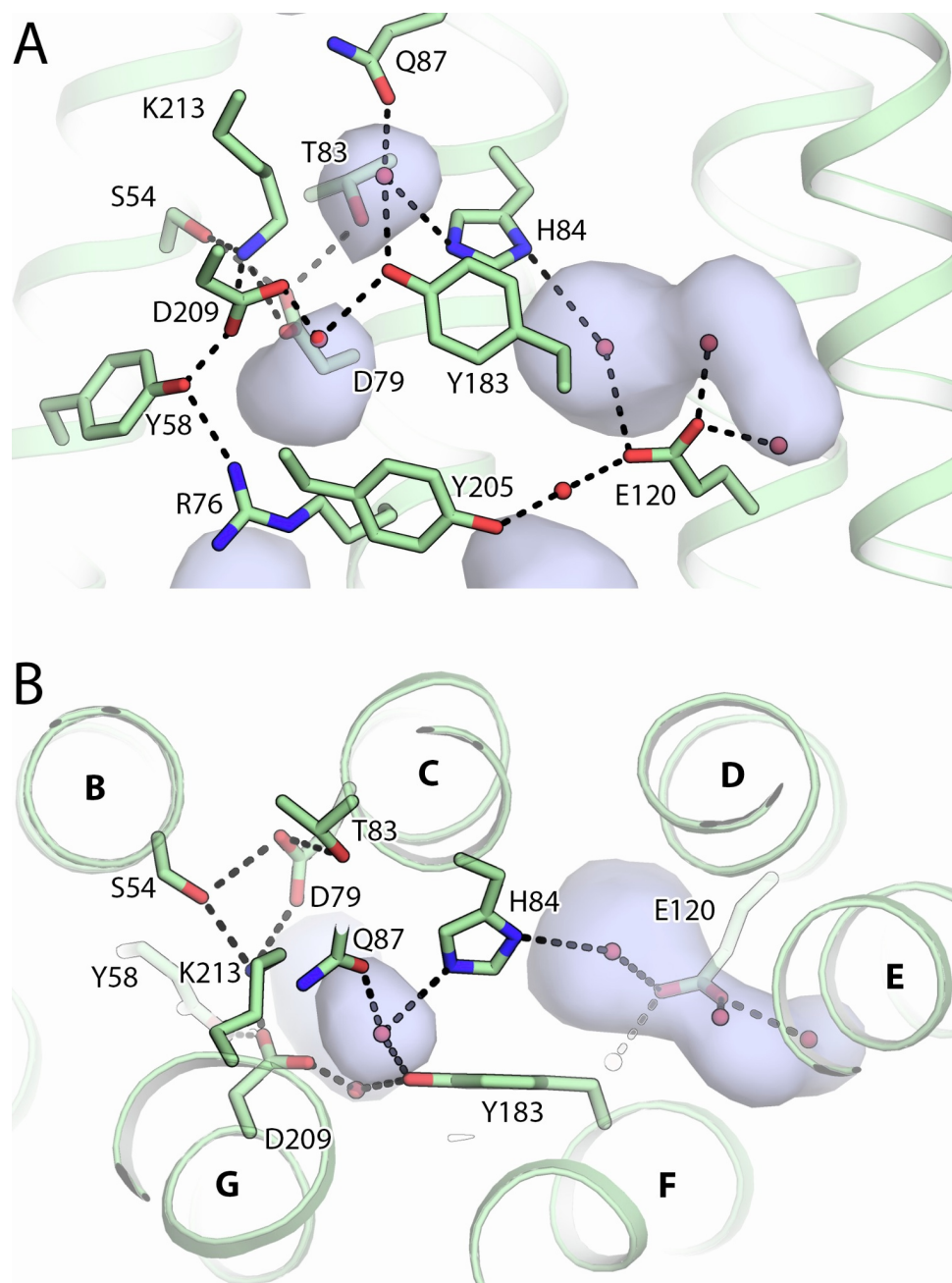


Fig. S5. Hydrogen bond network in the central region of *PsFAR*, Related to Figure 2. A. Side view. Helices F and G are not shown for clarity. **B.** Section view from the cytoplasmic side.

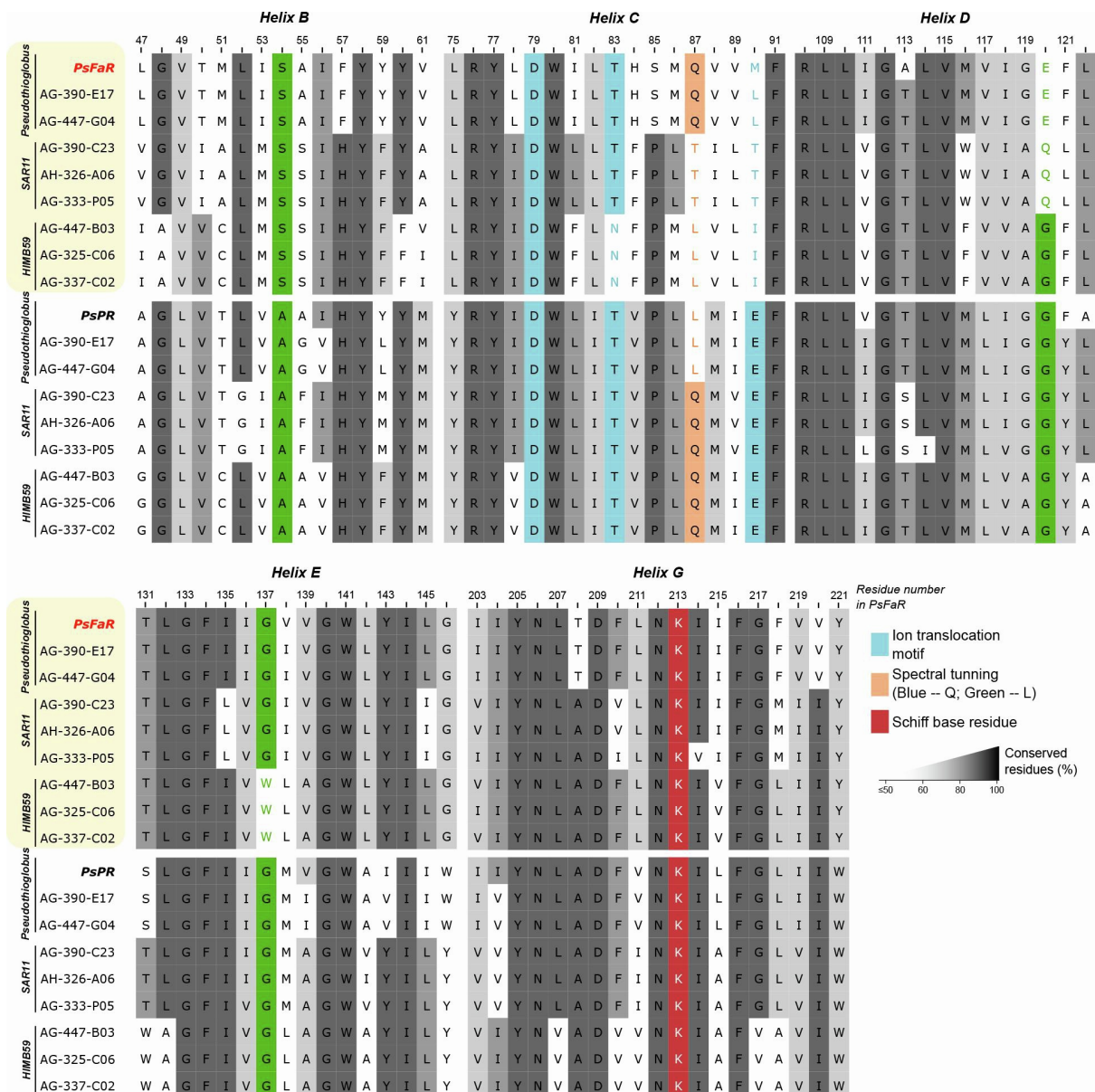


Fig. S6. Sequence alignment of representative paralog FARs and PRs, Related to Figure 2. Position of S54 in *PsFaR* is indicated in green to highlight conservativity of the residue within FARs but not PRs.

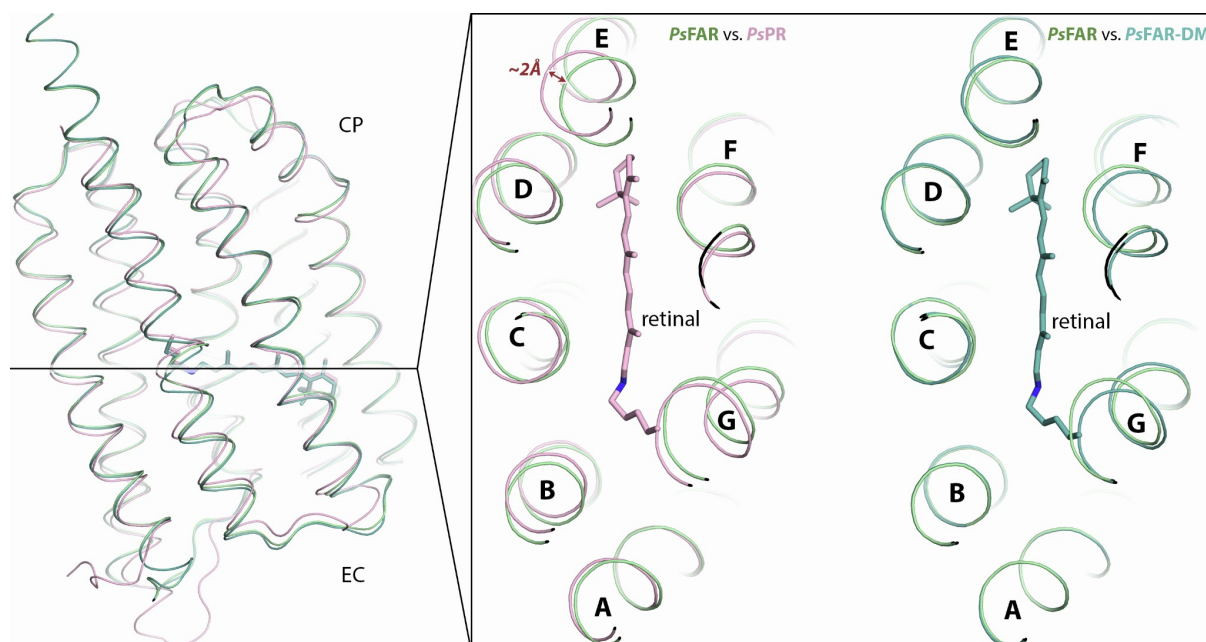


Fig. S7. Comparison of helices positions in the central region of *PsFAR*, *PsPR*, and *PsFAR-DM*, Related to Figure 2 and Figure 4. Side view (left panel) and section view (right panel) of the structural alignment of the *PsFAR* (green), *PsPR* (pink), and *PsFAR-DM* (deep cyan) protomers. The most pronounced shift of helix E in *PsPR* and *PsFAR* is indicated with a red arrow.

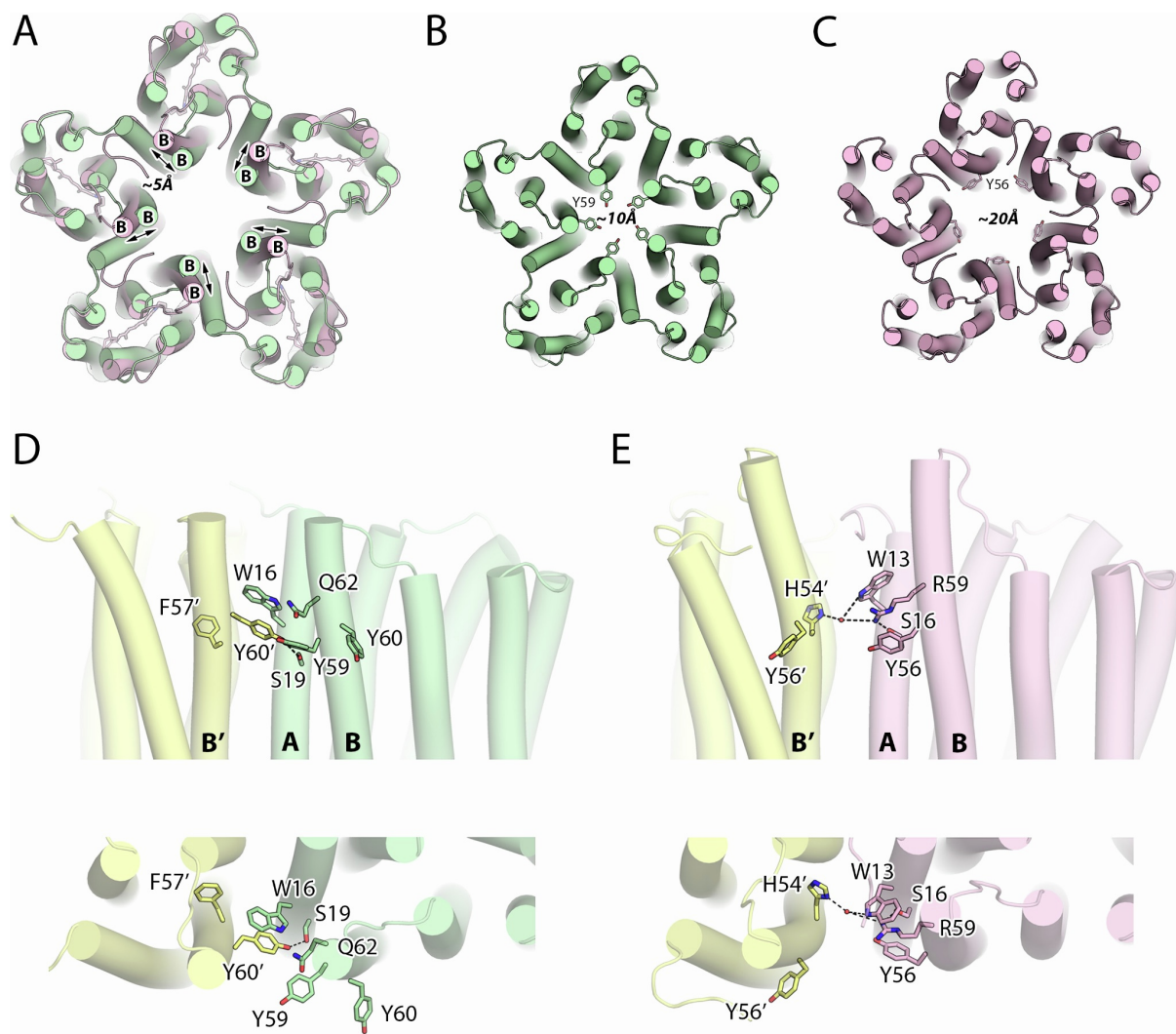


Fig. S8. Comparison of interprotomeric contacts in *PsFAR* and *PsPR*, Related to Figure 3. **A.** View from the extracellular side on the structural alignment of *PsFAR* and *PsPR*. The difference in the tip of helix B position is indicated with black arrow. **B.** Y59 arrangement in *PsFAR*. **C.** Y56 arrangement in *PsPR*. The approximate diameters of the narrowest section of the central region are given in Å. **D.** Detailed view of the interprotomeric contacts at the extracellular side of the *PsFAR* pentamer. Views from the central part of the pentamer (top panel) and from the extracellular side (bottom panel) are shown. **E.** Detailed view of the interprotomeric contacts at the extracellular side of the *PsPR* pentamer. Views from the central part of the pentamer (top panel) and from the extracellular side (bottom panel) are shown. Hydrogen bonds mediating the contacts are shown with black dashed lines. Neighboring protomer is colored yellow.

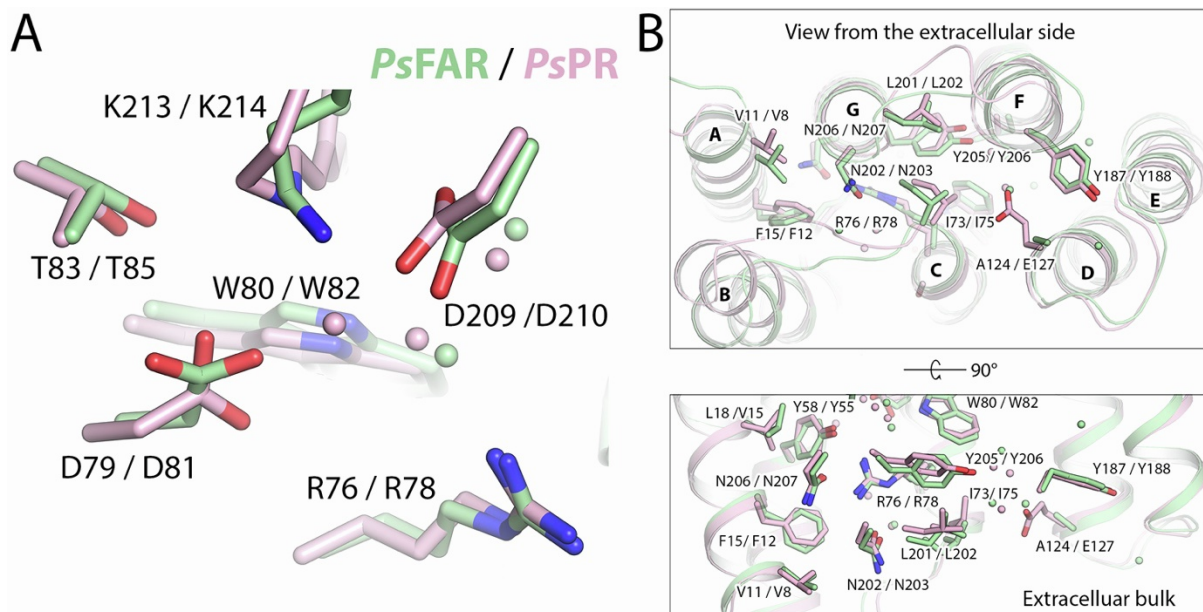


Fig. S9. Structural alignment of *PsFAR* and *PsPR*, Related to Figure 2. A. The RSB region. B. The extracellular side between R76 / R78 (*PsFAR* / *PsPR*, respectively) and extracellular bulk.

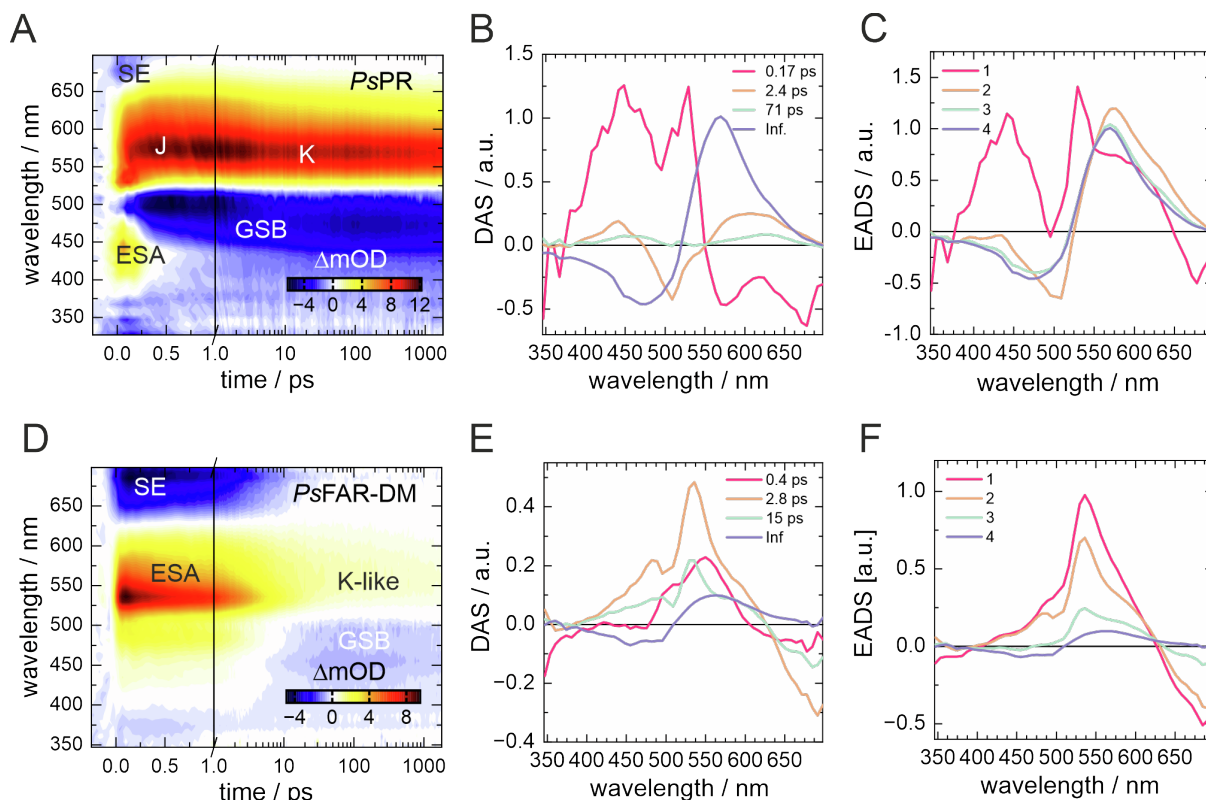


Fig. S10. Ultrafast spectroscopy of *PsPR* and *PsFAR-DM*, Related to Figure 4. **A.** 2D-contour plot of a fs-TA measurement of *PsPR* (pH 9.1) excited at 510 nm. **B.** Corresponding decay-associated spectra (DAS) and **C.** evolution-associated difference spectra (EADS) obtained by global target analysis of *PsPR*. **D.** 2D-contour plot of a fs-TA measurement of *PsFAR-DM* (pH 8.0) excited at 490 nm. **E.** DAS and **F.** EADS obtained by global target analysis of *PsFAR-DM*. The timescale of the transient maps is linear until 1 ps and logarithmic afterwards. In the dynamics of *PsPR* the first lifetime of 170 fs is assigned to the excited state decay and the formation of the J intermediate. Within 2.4 ps J decays forming the K intermediate, which persists beyond the observation limit of 1.8 ns. In contrast, *PsFAR-DM* required three time constants of 0.4 ps, 2.8 ps and 15 ps to describe the excited state decay adequately. The decay of the excited state results in the formation of a K-like intermediate, outlasting the observed timescale.

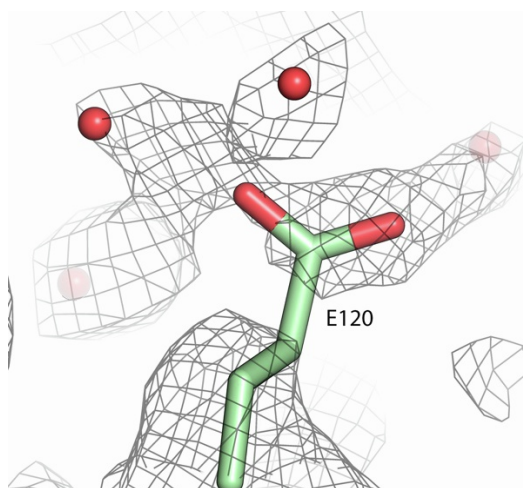


Fig. S11. Example of fragmented cryo-EM densities around E120 of *PsFAR*, Related to Figure 2.

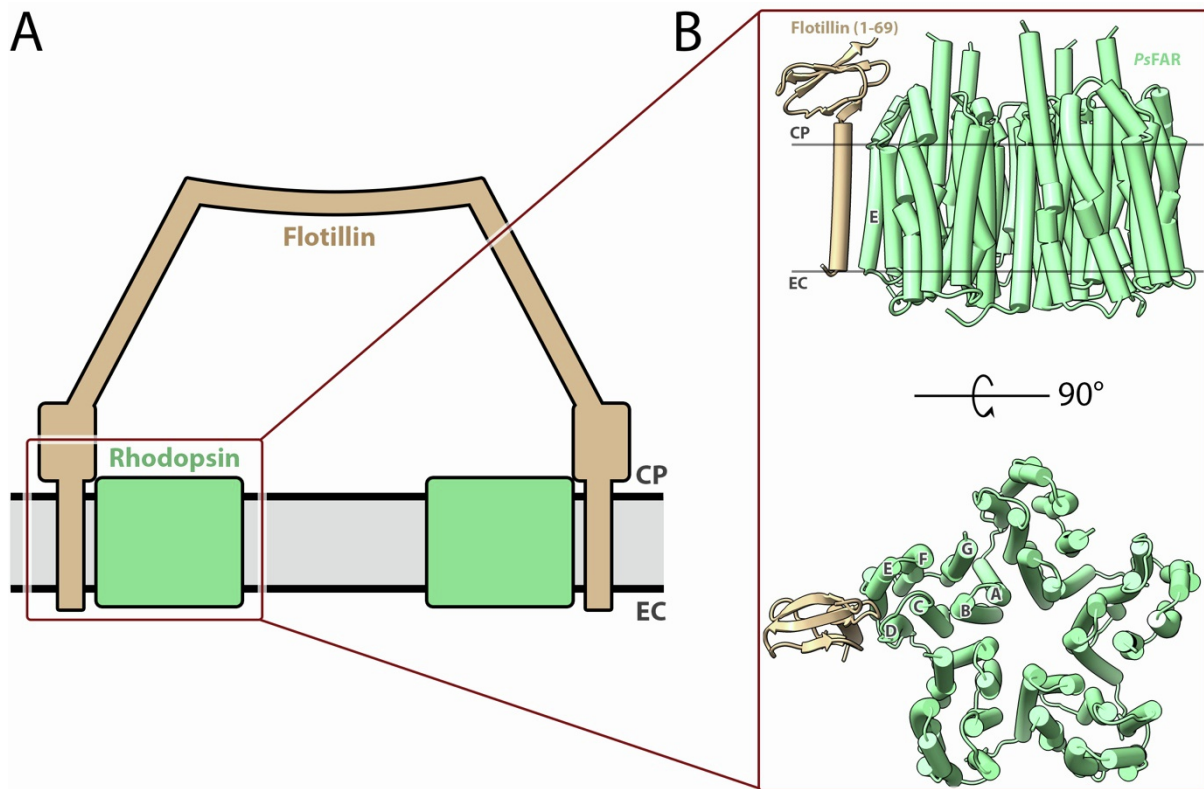


Fig. S12. Hypothetical model of the flotillin interaction to the *PsPAR* pentamer, Related to Figure 3. **A.** Schematic representation of the flotillin (beige) and rhodopsin (green) molecules. Membrane is shown in gray. Flotillins delimit a large area of the cell membrane (over 30 nm as shown in the case of human flotillins), within which several rhodopsin pentamers (with a lateral size of less than 9 nm) can be located. Thus, the interaction may occur “tangentially”. **B.** Detailed view of the hypothetic tangential interactions of *PsFAR* pentamer (this work) and membrane-associated N-terminal domain of flotillin (AF3 prediction). The proposed interactions are a speculation and are only schematically shown to visualize the hypothesis, and the interactions have not been observed experimentally or computationally.