

Supplementary Materials for

Conserved allosteric regulation of Frizzled-Dishevelled coupling by phosphatidylinositol-bisphosphate

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Supplementary Methods

Primer sequences for the preparation of constructs used in this study

Lowercase denotes overhang. Uppercase underlined denotes stop codon. Lowercase bold denotes restriction enzyme sequence

DVL3_Fwd_243	ttatcatga TGTCACTCAACATCATCA
DVL3_Rev_496	ttg gtacc <u>TCAG</u> TCACCGAAGATGTAGTA
DEP3_R461S_fwd	GAAGGCTTCACGGA CT CGAGGGAGGCCCGCAA
DEP3_R461S_rev	TTGCGGGCCTCCCTCGAGTCCGTGAAGCCTTC
DEP3_R462S_fwd	CTTCACGGACCGGAGCGAGGCCCGCAAGTAT
DEP3_R462S_rev	ATACTTGCGGGCCTCGCTCCGGTCCGTGAAG
DEP3_R465S_fwd	GACCGGAGGGAGGCCAGCAAGTATGCCAGCA
DEP3_R465S_rev	TGCTGGCATACTTGCTGGCCTCCCTCCGGTC
DEP3_K466S_fwd	GGAGGGAGGCCCGCAGCTATGCCAGCAACCTG
DEP3_K466S_rev	CAGGTTGCTGGCATAGCTGCGGGCCTCCCTCC
DEP3_R461S_R462S_fwd	GAAGGCTTCACGGA CT CGAGCGAGGCCCGCAAGTAT
DEP3_R461S_R462S_rev	ATACTTGCGGGCCTCGCTCGAGTCCGTGAAGCCTTC
DEP3_R465S_K466S_fwd	GACCGGAGGGAGGCCAGCAGCTATGCCAGCAACCTG
DEP3_R465S_K466S_rev	CAGGTTGCTGGCATAGCTGCTGGCCTCCCTCCGGTC
DEP3_R461S_R462S_R465S_K466S_fwd	AATGTGGAAGGCTTCACGGA CT CGAGCGAGGCCAGCAGCTATGCCAGCAACCTGCTGAAA
DEP3_R461S_R462S_R465S_K466S_rev	TTTCAGCAGGTTGCTGGCATAGCTGCTGGCCTCGCTCGAGTCGTGAAGCCTTCCACATT
FZD6-R226A-fwd	TTAATTGATGTT AGAGCATT CAG ATACCCAGA
FZD6-R226A rev	TCTGGGTATCTGAATGCTCTAACATCAATTA
FZD6-E318A-fwd	GAAAATGGAGTTGTGCAGCCATCGAGCAAAA
FZD6-E318A-rev	TTTTGCTCGATGGCTGCACA ACT CCATTTTC
FZD6-L393A-fwd	GCTGGCATTATTTCCGCAAATCATGTTCGACA
FZD6-L393A-rev	TGTCGAACATGATTTGCGGAAATAATGCCAGC
FZD6-L393R-fw	GCTGGCATTATTTCCAGAAATCATGTTCGACA
FZD6-L393R-fwd	TGTCGAACATGATTTCTGGAAATAATGCCAGC
FZD6-L396A-fwd	TTTCCTTAAATCATGCTCGACAAGTCATACA
FZD6-L396A-rev	TGTATGACTTGTTCGAGCATGATTAAAGGAAA
FZD6-L396R-fwd	ATTTCCCTTAAATCATAGACGACAAGTCATACAA
FZD6-L396R-rev	TTGTATGACTTGTCTGTCTATGATTAAAGGAAAT
FZD6-E399A-fwd	ATCATGTTTCGACAAGCCATACAAACATGATGG
FZD6-E399A-rev	CCATCATGTTGTATGGCTTGTTCGAACATGAT
FZD6-I400A-fwd	CATGTTTCGACAAGTCGCACAACATGATGGCCG
FZD6-I400A-rev	CGGCCATCATGTTGTGCGACTTGTTCGAACATG
FZD6-I400R-fwd	ATGTTTCGACAAGTCAGACAACATGATGGCCG

FZD6-I400R-rev	CGGCCATCATGTTGTCTGACTTGTCGAACAT
FZD6-F413A-fwd	GAAAAACTAAAGAAAGCTATGATTCTGAATTGG
FZD6-F413A-rev	CCAATTCGAATCATAGCTTTCTTTAGTTTTTC
FZD6-R416A-fwd	AAGAAATTTATGATTGCAATTGGAGTCTTCAG
FZD6-R416A-rev	CTGAAGACTCCAATTGCAATCATAAATTTCTT
FZD6-W493A-fwd	ATCTCTGCTGTCTTCGCGGTTGGAAGCAAAAA
FZD6-W493A-rev	TTTTTGCTTCCAACCGCGAAGACAGCAGAGAT
FZD6-K498A-fwd	TGGGTTGGAAGCAAAGCAACATGCACAGAATGG
FZD6-K498A-rev	CCATTCTGTGCATGTTGCTTTGCTTCCAACCCA

Supplementary structural methods

Cloning, expression and purification of phosphorylated PDZ-IDR2-DEP

The DVL constructs cloned in pET-Z vectors were transformed into chemically competent *E. coli* BL21 (DE3) cells for gene expression using the heat shock method. Cell cultures were grown using either LB broth or M9 minimal media (containing $^{15}\text{NH}_4\text{Cl}$ 0.5g liter^{-1} as the sole nitrogen source for uniformly ^{15}N -labeled proteins). To obtain phosphorylated DVL proteins, 200 ng of DVL pET-Z vectors were co-transformed with 600 ng of CK1 ϵ pCDF vector. pET-Z vector contains an N-terminal His₆-tag, followed by a Z-tag and a TEV protease cleavage site before the inserted fragment, whereas the pCDF vector does not contain any affinity chromatography tag.

For expression of DVLs, bacterial cells were grown in LB or M9 medium supplemented with 30 $\mu\text{g/ml}$ kanamycin (and 100 $\mu\text{g/ml}$ spectinomycin for co-expression with Ck1 ϵ) at 37 °C. When OD₆₀₀ reached around 0.8, the temperature was lowered to 16 °C, cells were allowed to adapt for one hour, and gene expression was induced by adding 0.25 or 0.5 mM of isopropyl β -D-1-thiogalactopyranoside (IPTG; A1008, PanReac AppliChem). Cultures were cultivated for protein expression for 16-30 hours at 16 °C. Cells were harvested by centrifugation at 4,000 rpm using an Avanti J-26XP centrifuge for 10 minutes, washed in ice-cold lysis buffer (25 mM Tris [pH 8.0], 500 mM NaCl, 10 mM imidazole, 10% glycerol, 1% NP-40, and 300 mM sucrose), and frozen at -20 °C until use. Cell pellets were defrosted, supplemented with 1X cCompleteTM, EDTA-free Protease Inhibitor Cocktail (11873580001, Merck) and phenylmethylsulfonyl fluoride (PMSF; 329-98-6, AppliChem), and lysed by sonication (using a pulse of 5 seconds on and 10 seconds off) at 4 °C. Cell lysates were then centrifuged at 14,000 rpm for one hour at 4 °C using an Avanti J-26XP centrifuge.

For the purification of phosphorylated or nonphosphorylated DVL3 PDZ-IDR2-DEP protein, the supernatant was applied to a Ni-NTA column (HiTrapTM IMAC HP 5 ml, Cytiva), pre-equilibrated with a binding buffer (25 mM Tris [pH 8.0], 500 mM NaCl, 10 mM imidazole). Subsequently, elution was performed by applying a gradient between a binding buffer and a high imidazole buffer (25 mM Tris [pH 8.0], 500 mM NaCl, 500 mM imidazole). The His₆-tag and Z-tag were cleaved using TEV protease (purified in-house) at 4 °C overnight, and the protein solution was then dialyzed to remove the imidazole. The PDZ-IDR2-DEP protein without the tag was collected from the flow-through of the second Ni-NTA step and dialyzed to drop the salt concentration to 50 mM NaCl. The dialyzed protein was supplied to anion exchange column (HiTrapTM Q HP 5 ml, Cytiva), and the protein was eluted using a salt gradient from 0 to 1 M NaCl in a buffer of 25 mM Tris [pH 8.0] containing 1 mM EDTA. The protein was then concentrated using ultrafiltration (Vivaspin 6,

10,000 MWCO PES, Sartorius) at 4,000 rpm using Eppendorf 5810R centrifuge. In a final purification step, the PDZ-IDR2-DEP protein was loaded onto a Superdex 75 10/300 GL column (GE Healthcare) for size exclusion chromatography and eluted in 10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA for pull-down experiments.

Cryo-EM data collection FZD₆-DVL3

FZD₆ bound to pPDZ-IDR2-DEP in saposin-based lipid nanoparticles resulted in a cryo-EM acquisition and analysis yielding clear features and helices visible, as well as a clear extramembrane density, corresponding to the DVL fragment. This data set was processed and yielded a density map with high resolution features, although exhibiting strong anisotropy. To circumvent the preferred orientation, we followed multiple strategies and had improved results with tilted data acquisition at a tilt angle of 40° . This did limit the resolution we could obtain, however, likely due to increased ice thickness from tilted data collection. After combining and processing the 40° tilted dataset with the initial data set, as well as two additional datasets (at 0° and at 20° tilt angle), we obtained an improved map at 3.6 Å overall resolution (**Supplementary Fig. 5**) that although still anisotropic allowed us to build an initial model of human FZD₆ in complex with the DEP domain of DVL3 including residues 170-511 (except for residues 225-226, 263-278 and 462-467) of FZD₆ and residues 409-495 (except for 423-428) of DVL3. Through this full data collection (datasets 1-4), we noticed that there were indeed other views present, but their numbers were small. Trying another method to overcome preferred orientation and improve map quality, we decided to collect an extremely large dataset, untilted, to obtain the best signal-to-noise particles of different views. This was the last resort due to the sheer time and effort required for this collection, and was only feasible once we realised the views were indeed present from the initial four datasets.

Cryo-EM datasets 1-4 (**Supplementary Table 2**) were collected on a Titan Krios G3 (dataset 1) and Titan Krios G1 (datasets 2-4) 300 kV microscope, both equipped with a Gatan K3 detector in counting mode and correlated double sampling (CDS) mode. Dataset 1 (16,827 movies) was collected using EPU at 105,000 x magnification (0.825 Å/px, Krios G3), a slit width of 20 eV (GIF Quantum energy filter), an electron exposure of 60 e-/Å² fractionated over 60 frames, using an exposure rate of 15.5 e-/px/s, for 2.6s total exposure time. Movies were collected across a defocus range from -0.6 to -2.0 µm (0.2 µm steps). Datasets 2-4 were collected at 130,000 x magnification (0.6523 Å/px calibrated against apoferritin sample, Krios G1) across a defocus range from -0.4 µm to -1.4 µm using serialEM using a slit width of 30 eV. In dataset 2 (1,280 movies), the electron exposure was 51.754 e-/Å² (48 frames, 6.0 s total exposure time, 4.0 e-/px/s exposure rate). Dataset 3 (1,364 movies) was collected at a 20° tilt angle and electron exposure of 79.892 e-/Å² (144 frames, 18.0 s total exposure time, 2.1 e-/px/s exposure rate). Dataset 4 (3,436 movies) was collected at a 40° tilt angle, and electron exposure of 51.6 e-/Å² (48 frames, 6.0 s total exposure time, 4.0 e-/px/s exposure rate).

Data processing

Data was primarily processed in cryoSPARC (v4.2.1+). Collected movies were preprocessed using patch motion correction followed by patch CTF estimation. Exposures were curated and micrographs with CTF fit >4 Å were discarded.

Datasets 1-4

For dataset 1 (16,827 micrographs), blob picker (particle diameter 80 – 130 Å) was used to pick particles and 21,426,665 particles were extracted at 80 pixels (4x binned, 3.3 Å/px) and subjected to two rounds of 2D classification (200 classes) followed by Ab-Initio reconstruction (4 classes) and two more rounds of 2D classification (100 classes), continuously discarding bad particles. The remaining 1,653,931 particles were re-extracted at 160 pixels (2x binned, 1.65 Å/px) and iteratively subjected to Ab-Initio reconstruction (two times with 3 classes and two times with 2 classes) to further clean up the particle stack. This yielded 108,603 particles on which NU-Refinement was performed, providing an initial map that suffered from anisotropy. This particle stack was then exported to RELION 5 (using the pyem script csparc2star) and further classified using 3D classification with alignments (regularisation parameter T of 32) to select one final class of 31,392 particles. This particle set was re-imported into cryoSPARC, followed by Ab-Initio reconstruction (one class) and NU-Refinement to yield an improved but still anisotropic map.

For dataset 2 (1,280 micrographs), initially blob picker (80 – 130 Å) was used to pick particles and 402,650 particles were extracted at a pixel size of 80 px (8x binned, 5.2186 Å/px) and subjected to 2D classification (50 classes), selecting 89,691 particles which were used for Ab-Initio reconstruction (one class) followed by homogenous refinement. Particles were then re-extracted at 256 pixels (2x binned, 1.3046 Å/px) followed by homogenous refinement. Further classification with Ab-Initio reconstruction (2 classes) was used to reduce the particle stack to 54,000 particles which were subjected to NU-refinement. This was further processed with local motion correction, Ab-Initio reconstruction (2 classes), yielding 31,399 particles which were refined using NU-refinement. The corresponding volume was used to simulate data and create templates for template picking. Template picked particles were extracted at 80 pixels (6x binned, 3.9138 Å/px), underwent 2D classification (100 classes) after which good classes were selected yielding a particle stack of 84,447 particles which were further processed. First, Ab-Initio reconstruction (2 classes) was performed followed by homogenous refinement (on the separate classes, as well as the whole particle stack, but this did not yield an improvement as there was a poor separation in classes). Afterwards, the particles were re-extracted at 240 pixels (2x binned, 1.3046 Å/px), subjected to local motion correction, followed by Ab-Initio reconstruction (3 classes) and NU-refinement on the best class. This particle stack was further cleaned up using 2D classification (50 classes), followed by Ab-Initio reconstruction (twice, first with one class, then with three classes), followed by NU-refinement on the best class consisting of 23,603 particles. Reference based motion correction was applied, followed by another NU-refinement, yielding a map that was less anisotropic but with an overall resolution of 4.15 Å.

For dataset 3 (1,364 micrographs, collected with 20° tilt angle), particles were picked using blob picker (80 – 130 Å); 733,053 particles were extracted at 80 pixels (6x binned, 3.9138 Å/px) and subjected to iterative 2D classification (twice; first with 200 classes, then with 50 classes). This yielded 78,547 particles which were used in Ab-Initio reconstruction (2 classes) followed by homogenous refinement on the better class with 67,658 particles. This particle stack was re-extracted at 240 pixels (2x binned, 1.3046 Å/px) and subjected to extensive, iterative Ab-Initio reconstructions (14, with 2 classes) and one 2D classification (50 classes), yielding 21,847 particles. Using the final NU-refinement volume from dataset 2 as input, NU-refinement was performed on the particle stack (21,847 particles). This particle stack was then re-extracted at 240 pixels (2x binned, 1.3046 Å/px), further subjected to iterative Ab-Initio reconstructions (five times

with 2 classes) further reducing the particle stack to 11,859 particles. This particle stack was then subjected to NU-refinement, reference-based motion correction and a final NU-refinement. This dataset exhibited an improved angular distribution; however the map was at low resolution (7.34 Å overall).

For dataset 4 (3,436 micrographs, collected with 40° tilt angle) particles were template picked with templates from dataset 2 and 3,104,406 particles were extracted at 80 pixels (6x binned, 3.9138 Å/px) and subjected to two rounds of 2D classification (100 and 50 classes). 163,614 particles were selected and used for Ab-Initio reconstruction (2 classes) followed by 2D classification (50 classes), yielding 84,427 particles that were re-extracted at 240 pixels (2x binned, 1.3046 Å/px) and used for local motion correction.

Particles from dataset 1 (a subset of 20,111 particles), dataset 2 (23,603 particles), dataset 3 (11,859 particles) and dataset 4 (84,427 particles) were extracted at 1.5003 Å/px pooled and yielded a particle stack of 139,994 particles, which were used for NU-refinement (with input volume from the final NU-refinement of dataset 2) followed sequentially by Global CTF refinement, NU-refinement, local CTF refinement and NU-refinement. At this point the 84,427 particles from dataset 4 were split from the pool and used for homogenous reconstruction, which yielded an improved map with a further improved angular distribution. This particle stack was used for reference-based motion correction (with locally motion corrected input movies) before pooling the particles again for further refinements. The particle stack of 139,994 particles was further refined by sequentially performing NU-refinement, global CTF refinement, NU-refinement, local CTF refinement, NU-refinement and finally local refinement. The pooled particle set, as well as the individual particles from datasets 1-3 and various combinations did not give better results than the particles from dataset 4. At this point, the particles from dataset 4 were again split from the pool and used for homogenous reconstruction with the alignments from the pooled refinements, which yielded a map at 3.6 Å overall resolution and reduced anisotropy.

Supplementary network methods

Dynamical network construction

Undirected, weighted networks of FZD₆-DEP in both the presence and absence of PIP₂ were constructed from the corresponding MD trajectories. To achieve this, the protein chains in each trajectory were aligned to the first frame through all-atom superposition. The center of mass (COM) of each backbone (bb) and sidechain (sc) moiety was computed from these aligned frames, yielding 3D coordinate distributions for each COM. The distances between the frame-averaged COM coordinates (d_{ij}) were also calculated. These steps were performed using functions provided by MDTraj 1.10.0 ² and NumPy 1.26.0 ³. Subsequently, the pairwise mutual information (I_{ij}) between COM distributions was estimated using the k-nearest-neighbor estimator of Kraskov, Stögbauer, and Grassberger ⁴, as implemented in infomeasure 0.6.0 ⁵. This non-parametric estimator was chosen because some of the COM distributions were observed to be multimodal, making a multivariate gaussian approximation of the marginal and joint COM distributions unsuitable ⁶. I_{ij} estimation was restricted to pairs whose average distance did not exceed 10 Å, reducing the number of comparisons needed.

To limit the inclusion of spurious edges in the final networks, percentile-based filtering was applied to prune the set of estimated I_{ij} values. Since I_{ij} was correlated to d_{ij} , this filtering was applied based on distance-adjusted I_{ij} values (I_{ij}^{adj}):

$$I_{ij}^{\text{adj}} = I_{ij} - E[I_{ij}|d_{ij}],$$

where $E[I_{ij}|d_{ij}]$ denotes the distance-dependent expected I_{ij} for a COM pair at a given d_{ij} . This expected I_{ij} was fit as a linear generalized additive model with a smooth, spline term with pyGAM 0.12.0 (DOI: <http://doi.org/10.5281/zenodo.1208723>) (**Supplementary Fig. 18a**). The I_{ij}^{adj} percentile threshold was heuristically selected to balance the retention of suitably strong edges without creating an overly sparse final network that would hinder downstream analyses and network comparisons. For this study, this threshold was set to the first quartile of I_{ij}^{adj} (**Supplementary Fig. 18b**).

These steps created a network (G) for each trajectory replicate, where the backbones and sidechains were represented by the nodes (V) and the weighted edges (E) were derived from pairwise estimations:

$$G = (V, E),$$

$$V = \{\text{nodes representing residue backbones and sidechains}\},$$

$$E_{ij} = \{I_{ij}, \text{ if } d_{ij} \leq 10\text{\AA} \text{ and } I_{ij}^{\text{adj}} \geq Q_{0.25}(I_{ij}^{\text{adj}}); 0, \text{ otherwise.}\}$$

Finally, the intersection of the edge sets of each replicate corresponding to either FZD₆-DEP in the presence or absence of PIP₂ were averaged to yield the final networks that are analyzed in this study. In addition to the other mentioned Python 3.11.14 libraries, NetworkX 3.6 (DOI: <https://doi.org/10.25080/TCWV9851>) and pandas 2.1.1 (DOI: <https://doi.org/10.25080/Majora-92bf1922-00a>) were also used.

Spectral graph analysis

To characterize the differences in topological connectivity in each network, spectral decomposition was applied to the symmetric normalized graph Laplacian (L^{sym}):

$$L^{\text{sym}} = (D^+)^{1/2} L (D^+)^{1/2} = I - (D^+)^{1/2} A (D^+)^{1/2},$$

where D^+ is the Moore-Penrose pseudoinverse of the diagonal degree matrix, A is the weighted adjacency matrix, and L is the unnormalized Laplacian matrix ($L = D - A$) {von Luxburg, 2007 #5044}. The choice of using L^{sym} instead of the unnormalized L was based on the high degree heterogeneity observed in the networks (**Supplementary Fig. 18c**). The high degree nodes are

primarily present in loop regions, partially reflecting an artifact from the rigid body alignment resulting in an overestimation of I_{ij} in the presence of large-scale collective motions, rather than a solely mechanistically interpretable observation. A similar problem has been described in the case of multi-domain protein dynamics⁷, which was addressed by independent domain alignments. We believed adopting a similar granular approach would lead to undue additional bias in our network construction through the manual selection of subdomain regions, so we opted to normalize by degree instead.

The eigenpairs (λ_i, v_i) obtained from each network were ordered by ascending eigenvalues (**Supplementary Fig. 18d**), and the eigenvectors of the first 10 non-trivial ($\lambda_i > 0$) eigenpairs were compared. An overall comparison was performed by computing the absolute cosine similarities between all pairs of eigenvectors ($|\cos(v_i, v_j)|$). Equivalent eigenvectors were then paired using average-linkage agglomerative clustering on absolute cosine distance ($1 - |\cos(v_i, v_j)|$) with the help of SciPy 1.13.1⁸.

Random walk analysis

To explore the propagation of a signal from DEP bound to FZD₆ in the presence or absence of PIP₂ based on dynamical behavior encoded by the networks, the time-dependent evolution of the probability distributions of a walker starting at K435_{sc} were generated. As it was unclear whether such a signal should travel stochastically or coherently through the receptor, both continuous-time random walks (CTRW) and quantum walks (CTQW), respectively, were modeled^{9,10}.

For the stochastic CTRW, the probability vector of observing the walker at each node at a given pseudotime ($|p(t)\rangle$) was modeled as:

$$|p(t)\rangle = e^{-Lt} |p(0)\rangle,$$

where L is the unnormalized Laplacian matrix, $|p(t)\rangle$ is an n -dimensional real vector ($|p(t)\rangle \in \mathbb{R}^n$) whose dimensionality equals the number of nodes in the network, and $|p(0)\rangle$ is the initial distribution of the walker. L is used because the evolution must satisfy $\mathbf{1}^T |p(t)\rangle = 1$, where $\mathbf{1}$ is a vector of ones, to conserve the total probability of the system, which is not the case if L^{sym} is used instead.

For the coherent CTQW, the pseudotime-dependent probability amplitude vector of observing the walker at each node ($|\psi(t)\rangle$) was described as:

$$|\psi(t)\rangle = e^{-iL_{\text{sym}}t} |\psi(0)\rangle,$$

where L_{sym} is the symmetric normalized Laplacian matrix, $|\psi(t)\rangle$ is an n -dimensional complex state vector ($|\psi(t)\rangle \in \mathbb{C}^n$) whose dimensionality equals the number of nodes in the network, and

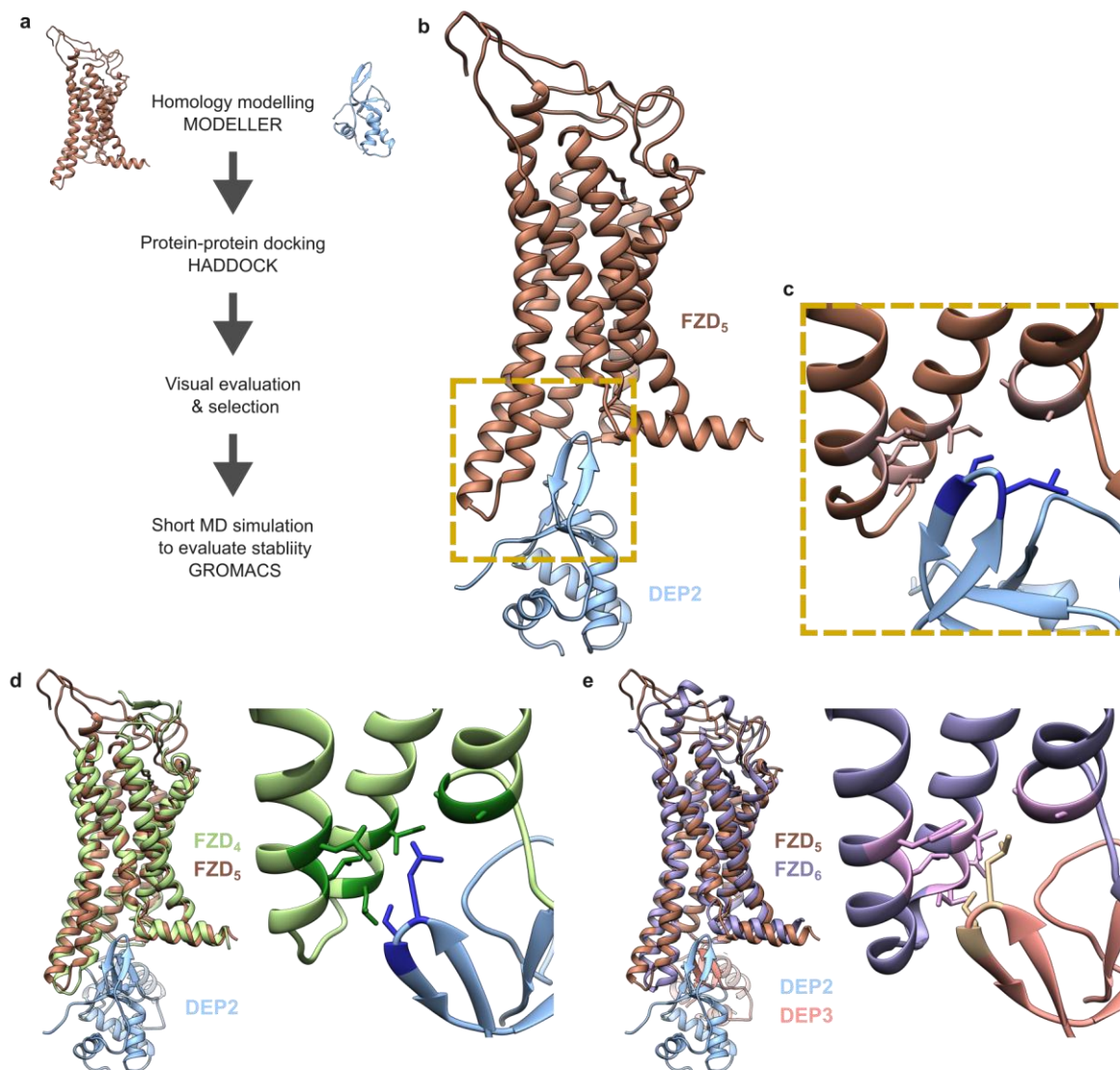
$|\psi(0)\rangle$ is the initial distribution of the walker. The probability of the walker existing at a particular node can be recovered by $p_i(t)=|\psi_i(t)|^2$. In this case, $\langle\psi(t)|\psi(t)\rangle=1$ must instead be satisfied to conserve total probability so L^{sym} can be used.

For both the stochastic and coherent walkers, the initial probability was set to 1 for node K435_{sc} and 0 elsewhere. To avoid repeated matrix exponentiation, the matrix exponentials were instead evaluated using pre-computed eigenvectors and eigenvalues. For the normalized pseudotime to maximum probability (**Fig. 5c,d**), the CTRW was sampled at logarithmically spaced time points (10 points per decade) spanning from $t_{\min}=1/(10\times\lambda_{\max})$ to $t_{\max}=2/\lambda_{\min}$, where $\lambda_{\max}$ and $\lambda_{\min}$ are the largest and smallest non-trivial eigenvalues of the Laplacian, respectively. The CTQW was sampled at linearly spaced time points for $t_{\max}=2\pi/\lambda_{\min}$ in intervals of $\Delta t=2\pi/(10\times\lambda_{\max})$, where $\lambda_{\max}$ and $\lambda_{\min}$ are the largest and smallest non-trivial eigenvalues of the symmetric normalized Laplacian, respectively. These pseudotimes were normalized through division by their corresponding $t_{\max}$. For the walker movies (**Movies S1, S2**), early probability evolution was captured with manually defined linearly spaced time points.

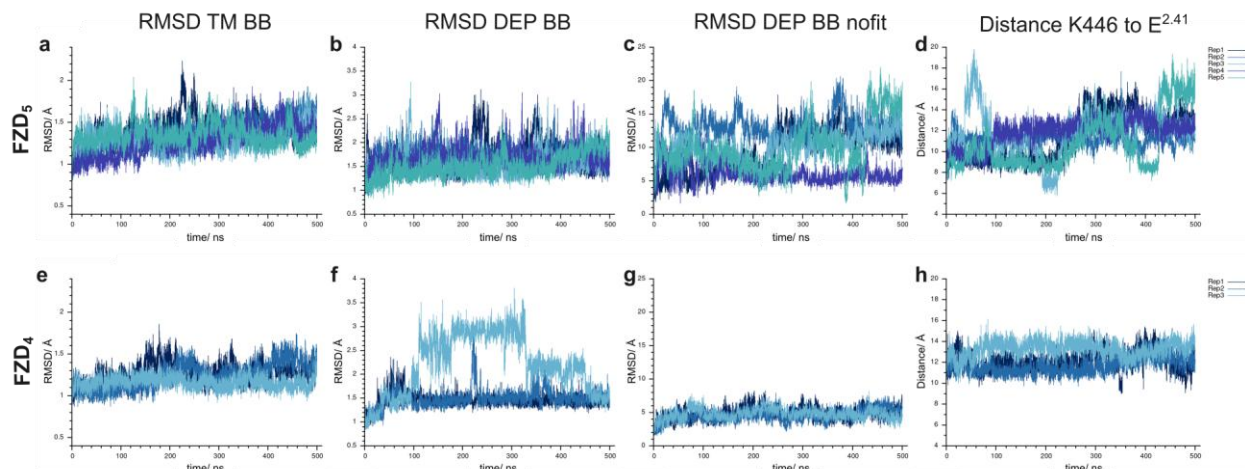
Flow analysis

The minimum-cost, maximum flow between chosen source and sink nodes in each network was calculated using NetworkX's `max_flow_min_cost` function. The capacity of each edge was set to I_{ij} and each distance was defined as its reciprocal. As with the walkers, K435_{sc} was chosen as the source node. E194^{1.28}_{bb} and C454^{6.70}_{bb} were chosen as sink nodes based on the results of the spectral graph analyses (**Fig. 5b**).

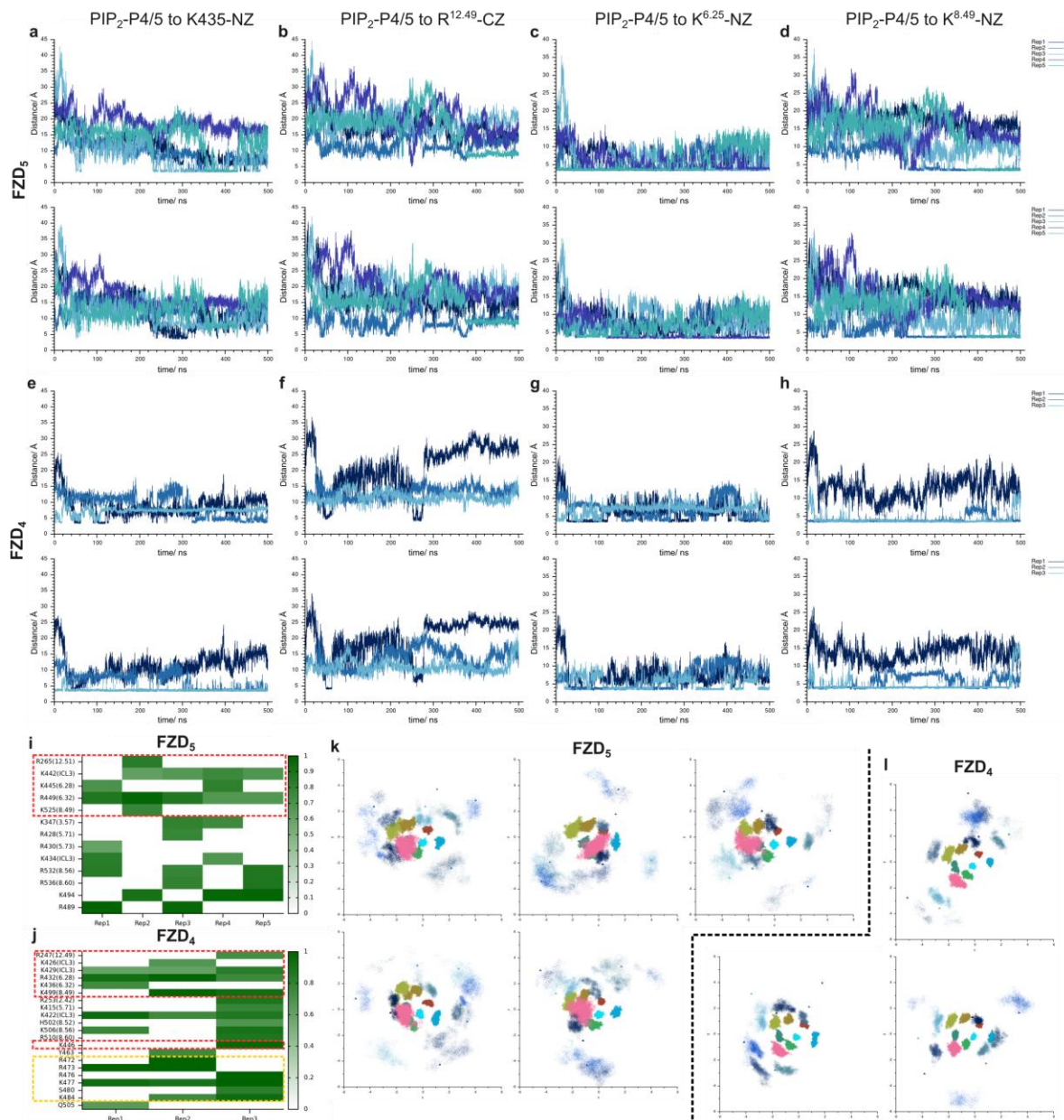
Supplementary Figures



Supplementary Fig. 1. Modelling of the FZD₅-DEP complex using protein-protein docking. (a) Schematic representation of the workflow during modeling of the FZD₅-DEP complex. (b) Side view of the final selected complex model. The yellow box indicates the area shown in (c). (c) Closeup of the apolar interaction interface between FZD₅ and DEP with selected residues shown as stick. (d) Overlay of the FZD₄-DEP cryo-EM structure with the FZD₅-DEP model. The closeup shows the interaction interface between FZD₄ and DEP as shown for FZD₅-DEP in (c). (e) Overlay of the FZD₆-DEP cryo-EM structure with the FZD₅-DEP model. The closeup shows the interaction interface between FZD₆ and DEP as shown for FZD₅-DEP in (c).

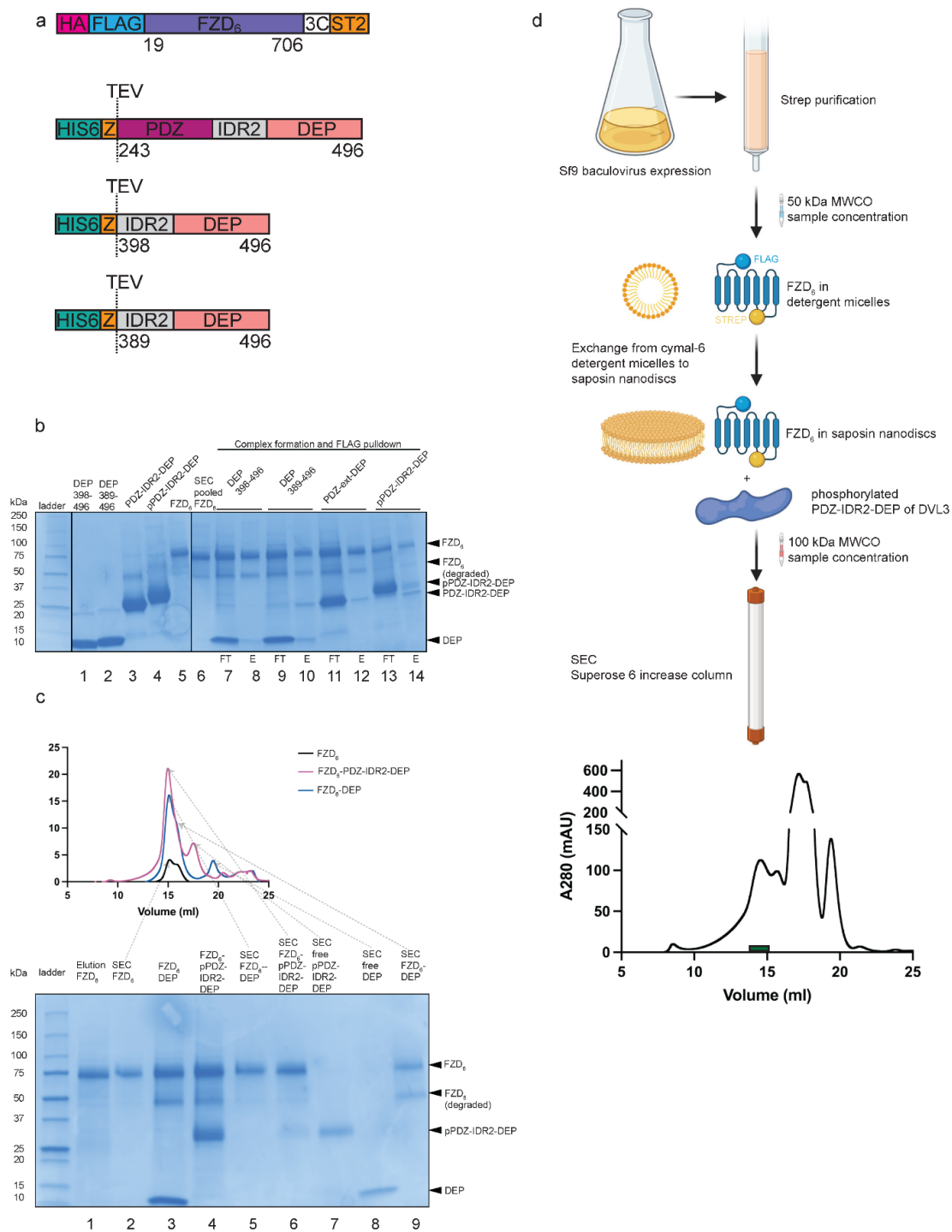


Supplementary Fig. 2. RMSD and finger-loop engagement for FZD₅-DEP and FZD₄-DEP. (a,e) RMSD of the backbone of the TM domain. (b,f) RMSD of the backbone of DEP with alignment on DEP. (c,g) RMSD of the backbone of DEP with alignment on the TM domain. (d,h) Distance between K446-C α in the DEP finger-loop and E^{2.41}-C α . Shown plots are for (a-d) FZD₅-DEP complex and (e-h) FZD₄-DEP complex. Data is plotted as time course over the entire trajectory, and replicas are colored in different shades of blue.

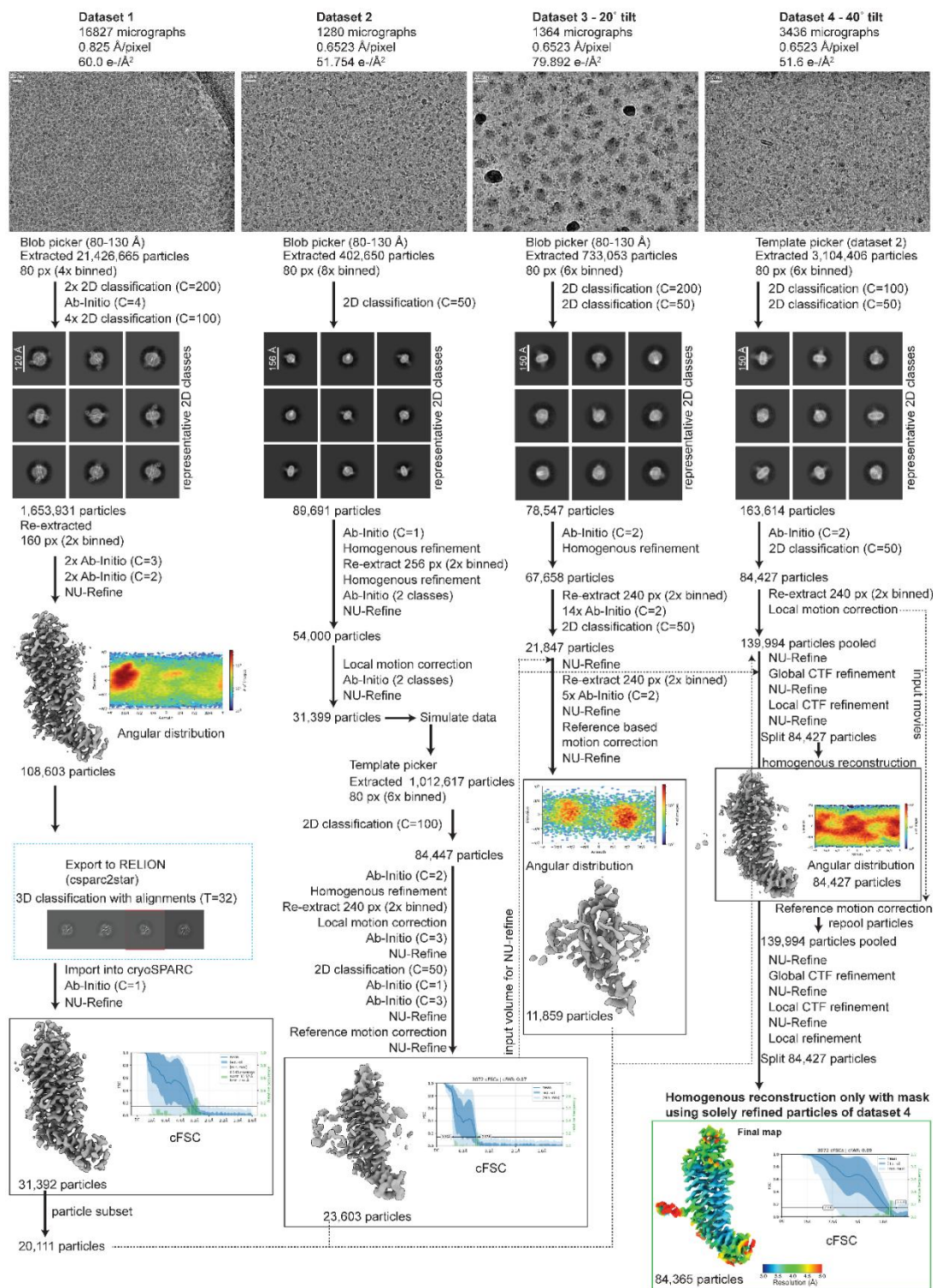


Supplementary Fig. 3. PIP₂ interactions with the polar cavity of FZD₄ and FZD₅. (a-h) Distances between Phosphates P4 and P5 of the PIP₂ binding to the polar cavity between TM6, TM7/H8 and ICL1 and selected residues of (a-d) FZD₅ and (e-h) FZD₄. Measured distances are to (a,e) NZ of DEP K435 and to FZD sidechain atoms (b,f) R^{12,49}-CZ, (c,g) K^{6,25} NZ and (d,h) K^{8,49} NZ. The different replicas are colored in shades of blue. (i,j) Heatmap displaying the frequency of polar interactions (hydrogen bonds, salt bridges) between PIP₂ and (i) FZD₄ and (j) FZD₅ as determined using getcontacts. The red box highlights interactions with residues in the polar cavity, while the yellow box highlights interactions with the α 3 helix of DEP. (k,l) Plots of the xy coordinates over the duration of the MD simulation of the center of mass of the backbone of the four most intracellular residues of each transmembrane helix (rainbow coloring), of DEP (salmon) and of the head-group of PIP₂ (shades of blue). The initial positions of PIP₂ are indicated

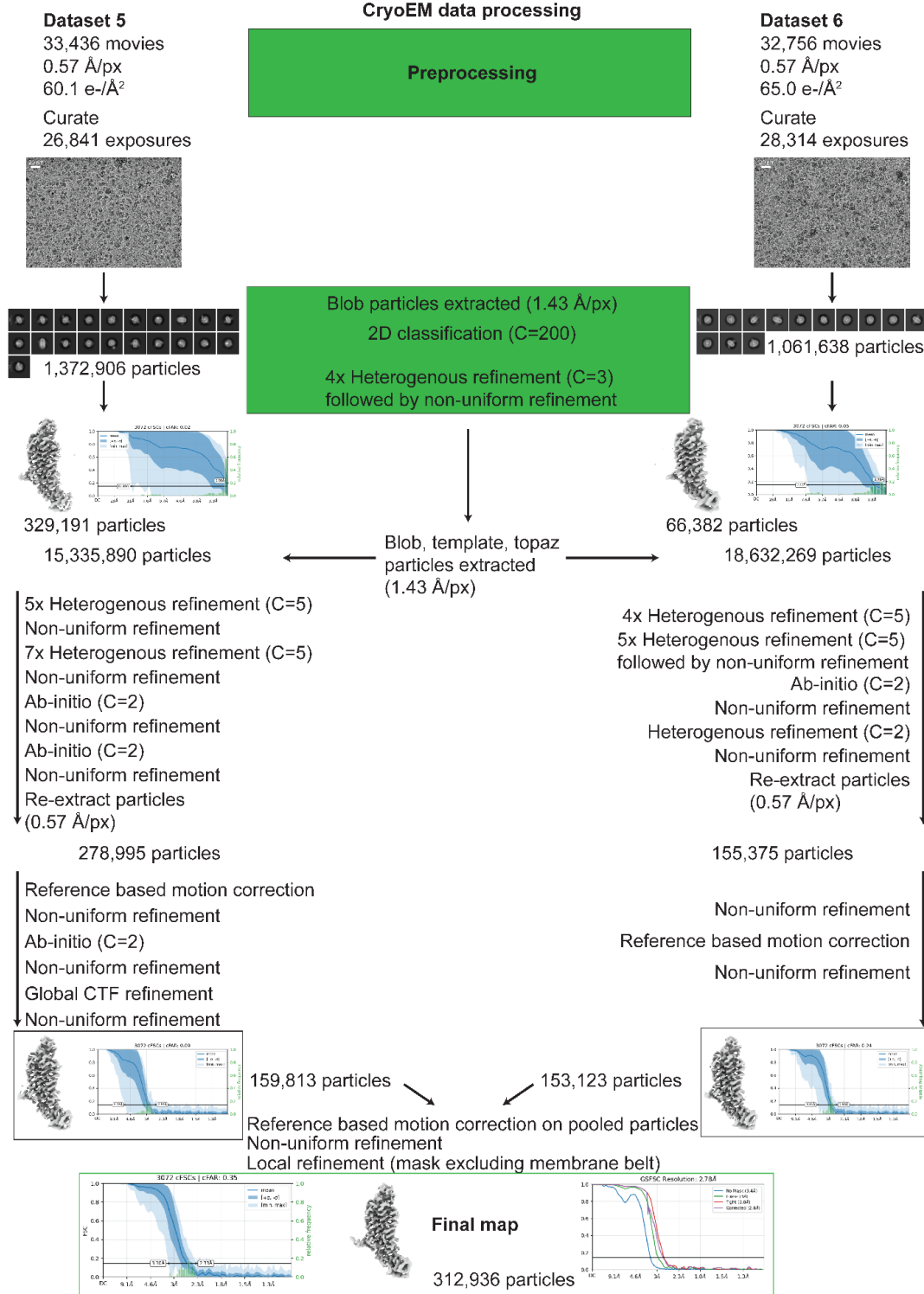
with triangles. See **Fig. 3c** for indication of TMs and DEP. Plots are shown for **(k)** FZD₄ and **(l)** FZD₅.



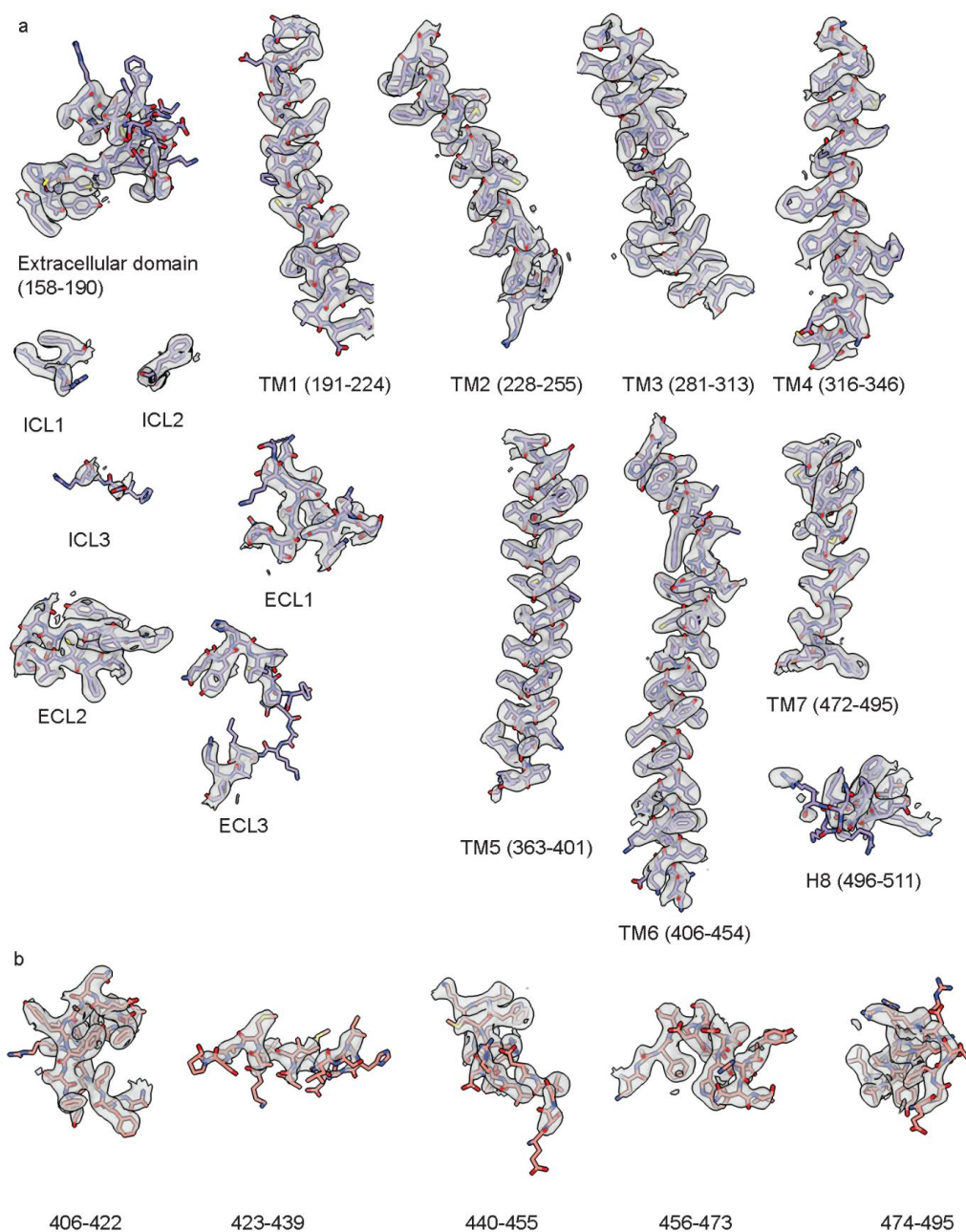
Supplementary Fig. 4. Biochemical characterization of FZD₆-PDZ-IDR2-DEP. (a) Construct design for FZD₆ and DVL3. The FZD₆ construct consists of an N-terminal hemagglutinin signal peptide (HA), followed by a FLAG tag and the FZD₆ sequence without the native signal peptide (amino acids 19-706) and flanked by a C-terminal 3C cleavage site and Twin-Strep-tag® (ST2). DVL3 constructs were flanked by an N-terminal hexahistidine-tag (HIS6), a Z-tag (Z), the DVL3 sequence corresponding to amino acids 243-496, 398-496 or 389-496. Tags were removed during purification of DVL3 (dashed line). (b) Representative SDS-PAGE result from pulldown experiments. Lane 1-5: protein controls for DVL3 and FZD₆. Lane 6: pooled fractions from purified FZD₆ after size-exclusion chromatography. Lane 7-14 show results from pulldown experiments with purified proteins. Lane 7 and 8: flowthrough (FT) and elution (E) from the FLAG pulldown of FZD₆ with DEP (amino acids 398-496). Lane 9 and 10: flowthrough (FT) and elution (E) from the FLAG pulldown of FZD₆ with DEP (amino acids 389-496). Lane 11 and 12: flowthrough (FT) and elution (E) from the FLAG pulldown of FZD₆ with PDZ-IDR2-DEP. Lane 13 and 14: flowthrough (FT) and elution (E) from the FLAG pulldown of FZD₆ with phosphorylated PDZ-IDR2-DEP. Black arrows mark the bands for FZD₆, FZD₆ (degraded), phosphorylated PDZ-IDR2-DEP, PDZ-IDR2-DEP and DEP. (c) Results from the SEC complexation experiments. SEC traces are shown for FZD₆ only (black), FZD₆ complexed with phosphorylated PDZ-IDR2-DEP (purple) and FZD₆ complexed with DEP 398-496 (blue). SDS-PAGE gel of complexation experiment before and after SEC. Lane 1: FZD₆ after elution. Lane 2: peak fraction (dashed arrow) of FZD₆. Lane 3: FZD₆-DEP before SEC. Lane 4: FZD₆-pPDZ-IDR2-DEP before SEC. Lane 5: peak fraction (dashed arrow) from FZD₆-DEP. Lane 6: peak fraction (dashed arrow) from FZD₆-pPDZ-IDR2-DEP. Lane 7: peak fraction (dashed arrow) corresponding to uncomplexed pPDZ-IDR2-DEP. Lane 8: peak fraction (dashed arrow) corresponding to uncomplexed DEP. Lane 9: peak fraction (dashed arrow) of the shoulder peak from the FZD₆-DEP experiment, corresponding to a mixture of FZD₆ and degraded FZD₆. (d) Experimental workflow for the preparation of FZD₆-pPDZ-IDR2-DEP in saposin nanodiscs for cryo-EM.



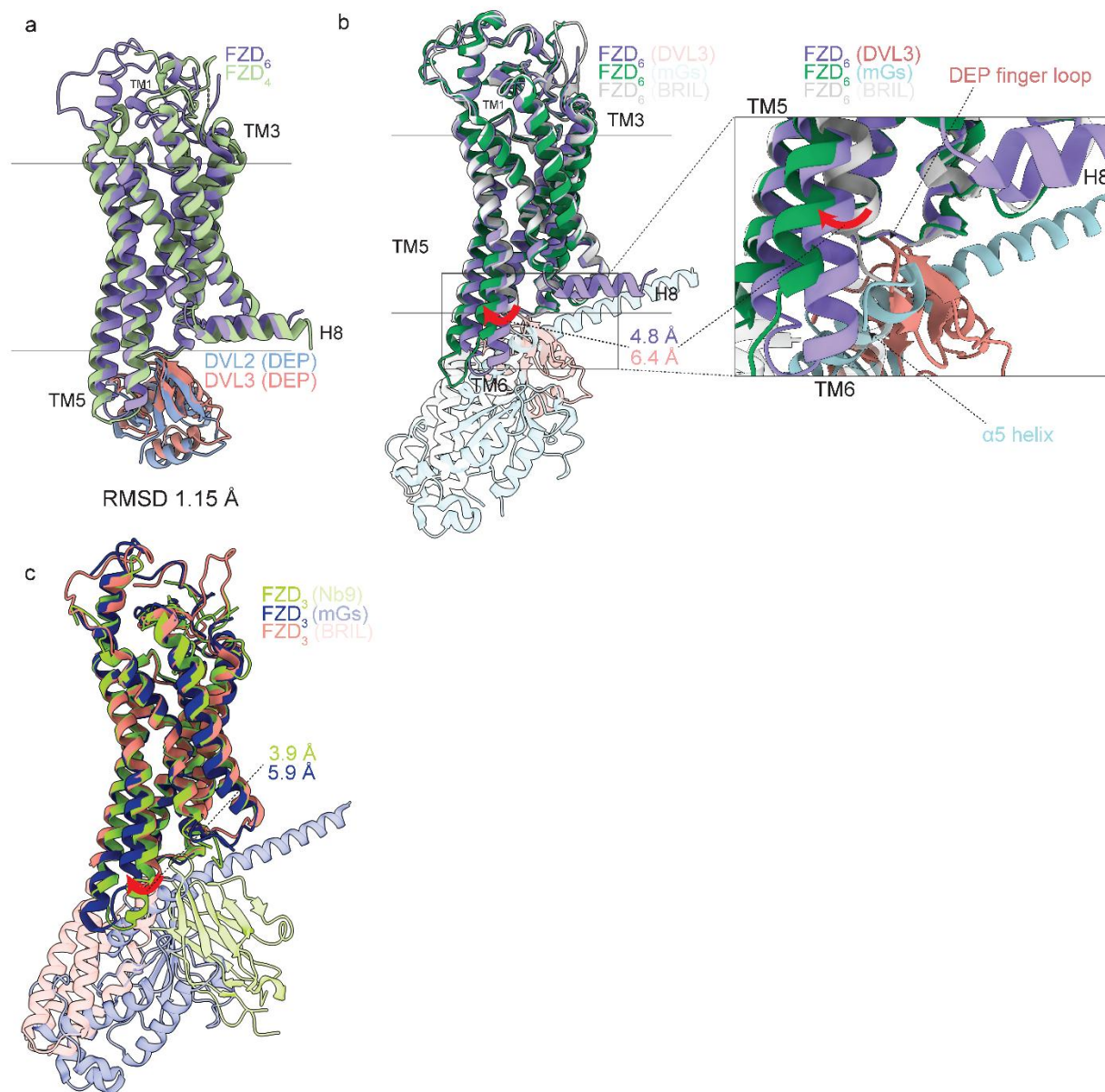
Supplementary Fig. 5. Cryo-EM data processing workflow of datasets 1-4. Workflow of individual data processing steps of datasets 1 and 2 (0° tilt), 3 (20° tilt) and 4 (40° tilt) as well as combined processing of datasets 1-4 that yielded the final map.



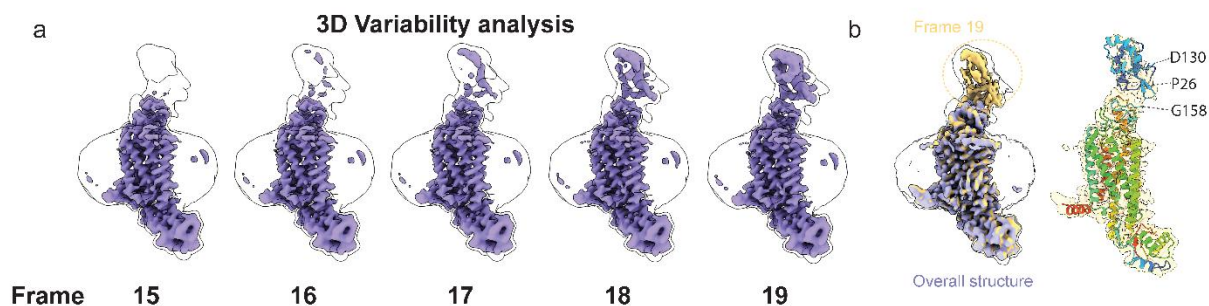
Supplementary Fig. 6. Cryo-EM data processing workflow of datasets 5 and 6. Workflow of individual data processing steps for datasets 5 and 6 as well as the final processing of the pooled particles yielding the final map.



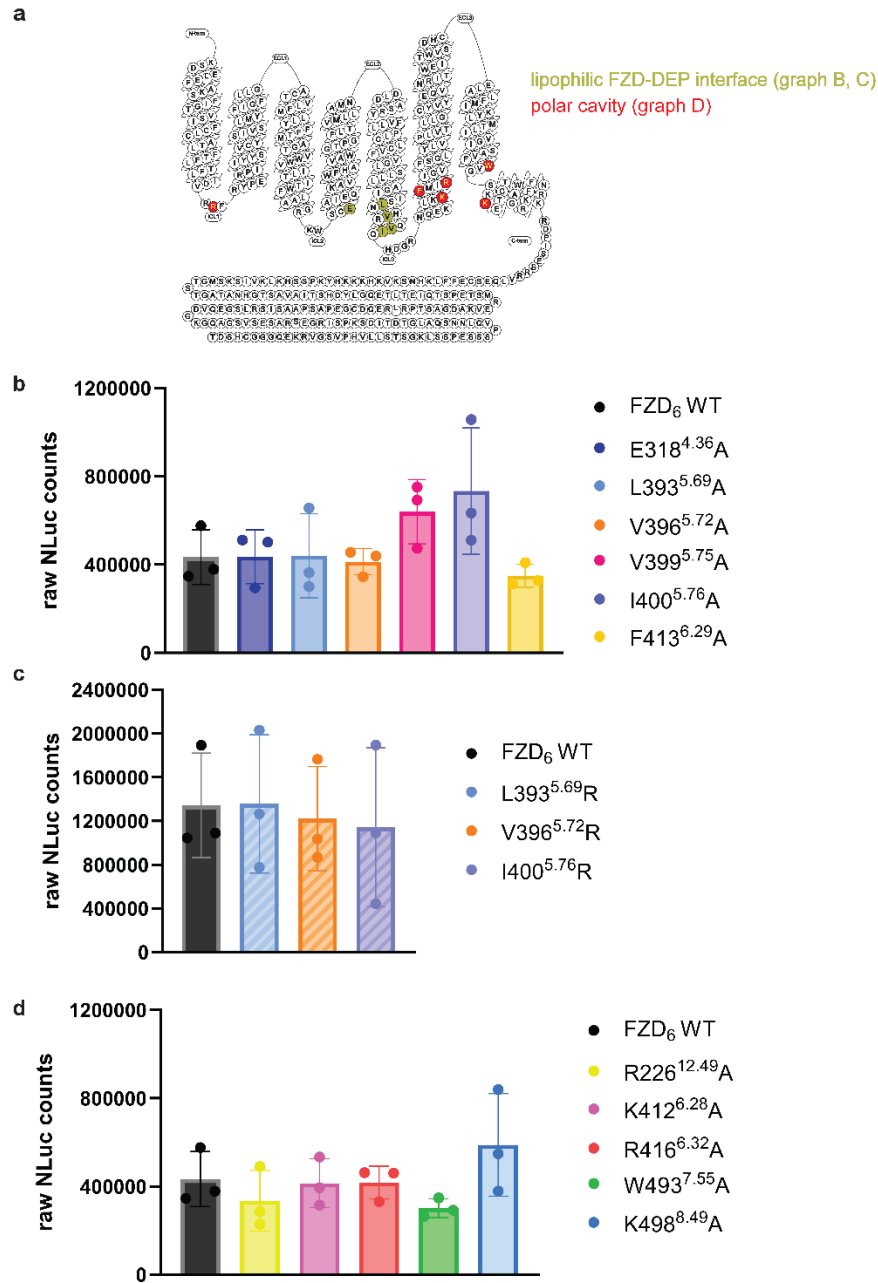
Supplementary Fig. 7. Cryo-EM maps with the model-fit of the DEP-bound FZD₆ structure. Representation of the cryo-EM map of the FZD₆-DEP structure shown in grey and contoured around (a) each respective transmembrane domain (TM1-7), helix 8 (H8) and loop of FZD₆ and (b) DVL3 (amino acids 406-495). Amino acid side chains are represented as sticks.



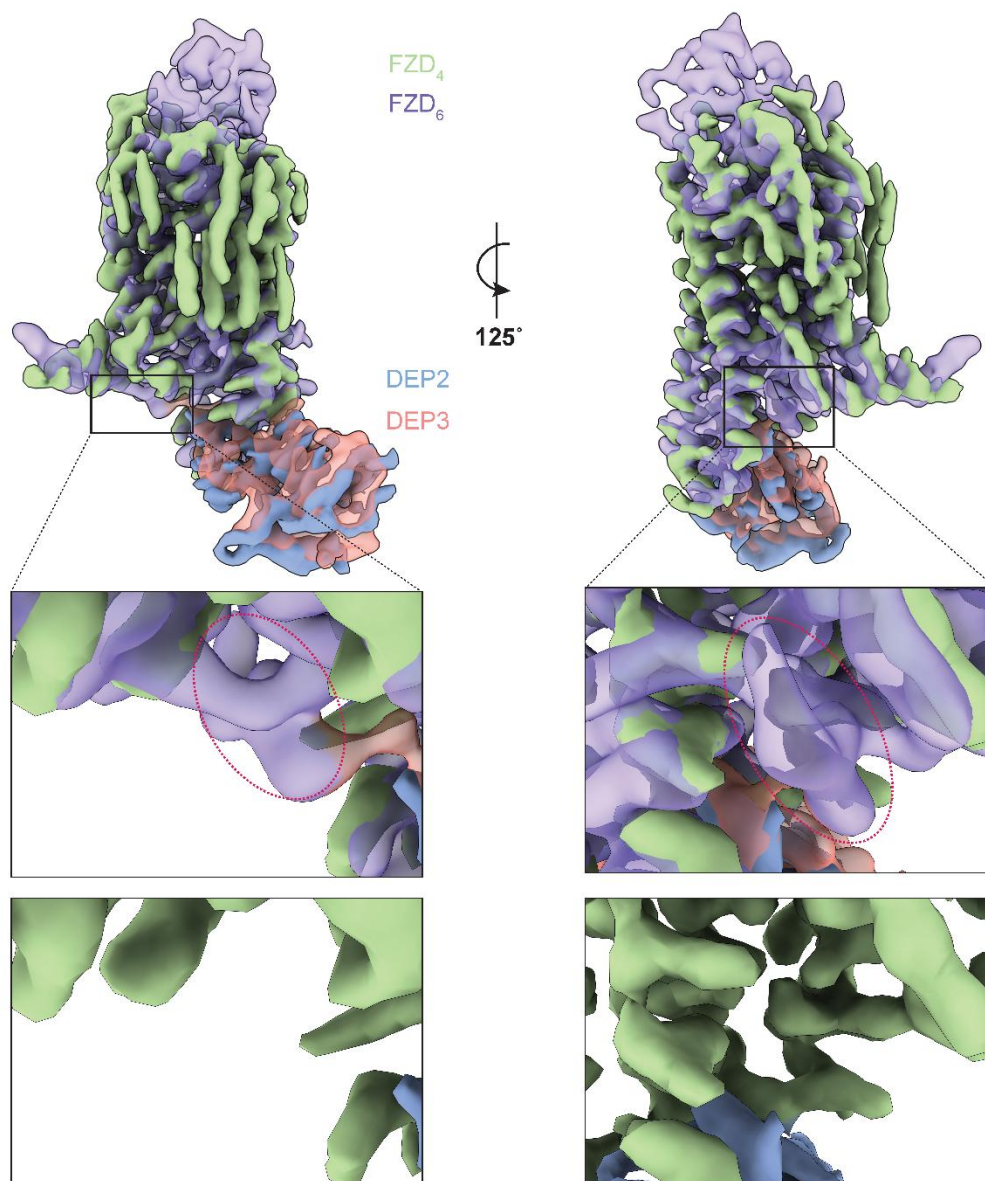
Supplementary Fig. 8. Comparisons of the FZD₆-DEP structure with available FZD structures. (a) Overlay of FZD₆-DEP structure and the published FZD₄-DEP structure (PDB: 8WMA). (b) Overlay of FZD₆-DEP structure and the published FZD₆-mGs (PDB: 8JHB) and inactive FZD₆-BRIL (PDB: 8JH7) structure. TM6 outward movement relative to the inactive structure was measured as 4.8 Å for the FZD₆-DEP structure and 6.4 Å for the FZD₆-mGs structure emphasizing the requirement for helix interruption at the conserved P^{6.43} and the presence of the proposed hinge limiter mechanisms at the molecular switch R^{6.32} and W^{7.55} 11-16. The structural overlay confirms also functional data showing that G protein and DVL binding are mutually exclusive^{17,18}. (c) Overlay of FZD₃-Nb9 (PDB: 8QW4), FZD₃-mGs (PDB: 8JHI) and inactive FZD₃-BRIL (PDB: 8JHC) structures shows a similar intermediate TM6 outward movement for the Nb9 coupled state (3.9 Å) and more pronounced movement for the G protein-coupled state (5.9 Å)^{13,19}.



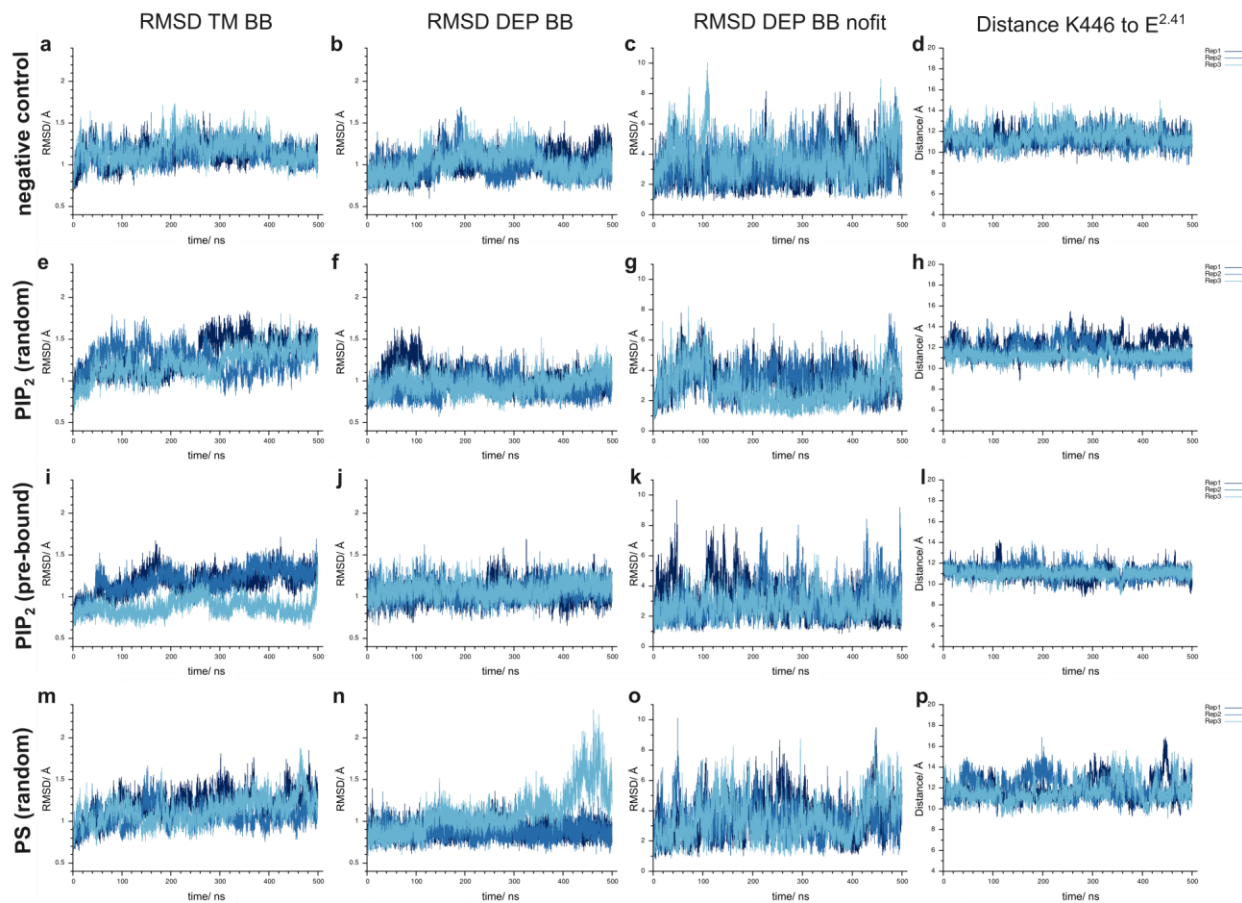
Supplementary Fig. 9. Results from 3D-variability analysis of FZD₆-DEP complex masked around the CRD region. (a) The final 5 frames, where the CRD density starts appearing, are shown. (b) Left: the maps of frame 19 with the most CRD density (yellow) and the overall map (light purple), highlighting the additional CRD density in frame 19. Right: the map of frame 19 with the CRD (26-130) rigid body fitted into the density.



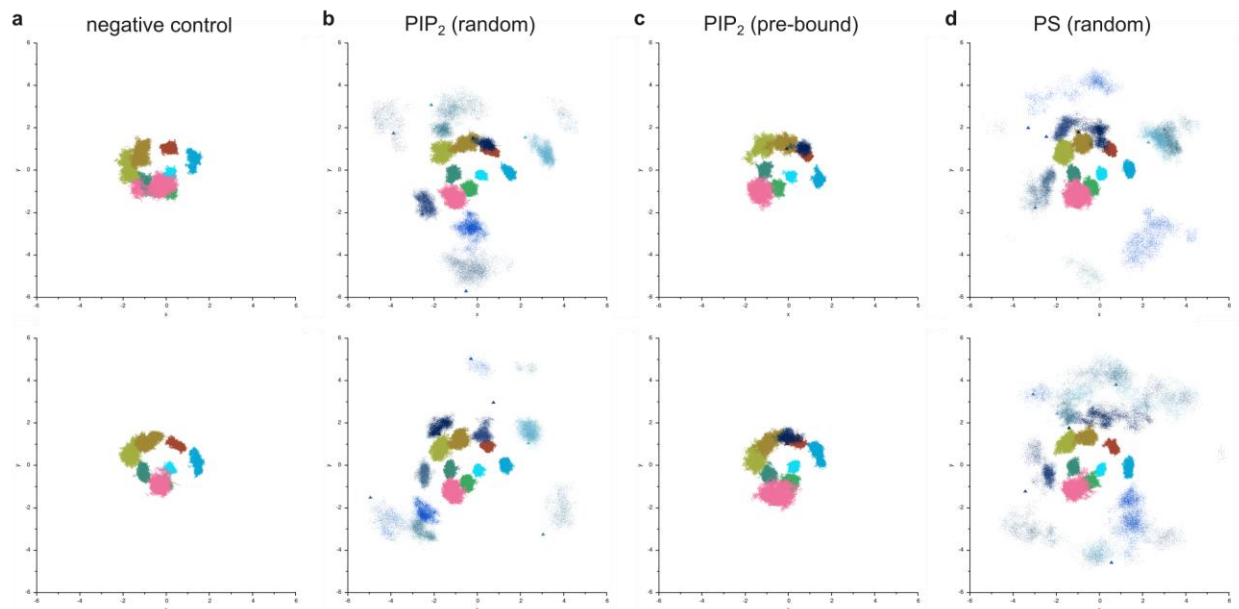
Supplementary Fig. 10. Validation of FZD₆ mutants in the DEP interface and the polar cavity. (a) Snake plot for human FZD₆ (created using the GPCR database at https://gpcrdb.org/protein/fzd6_human/) shows residues in the lipophilic DEP interface (beige) and the polar cavity (red). Surface expression of the mutants in the lipophilic FZD-DEP interface (b, c) and the polar cavity (d) was assessed by luminescence counts emerging from the reconstitution of the FZD₆-SmBiT with the CAAX-HiBiT to Nanoluc (NLuc). Errors bars show standard error of the mean (SEM) of 3 independent experiments performed in triplicates.



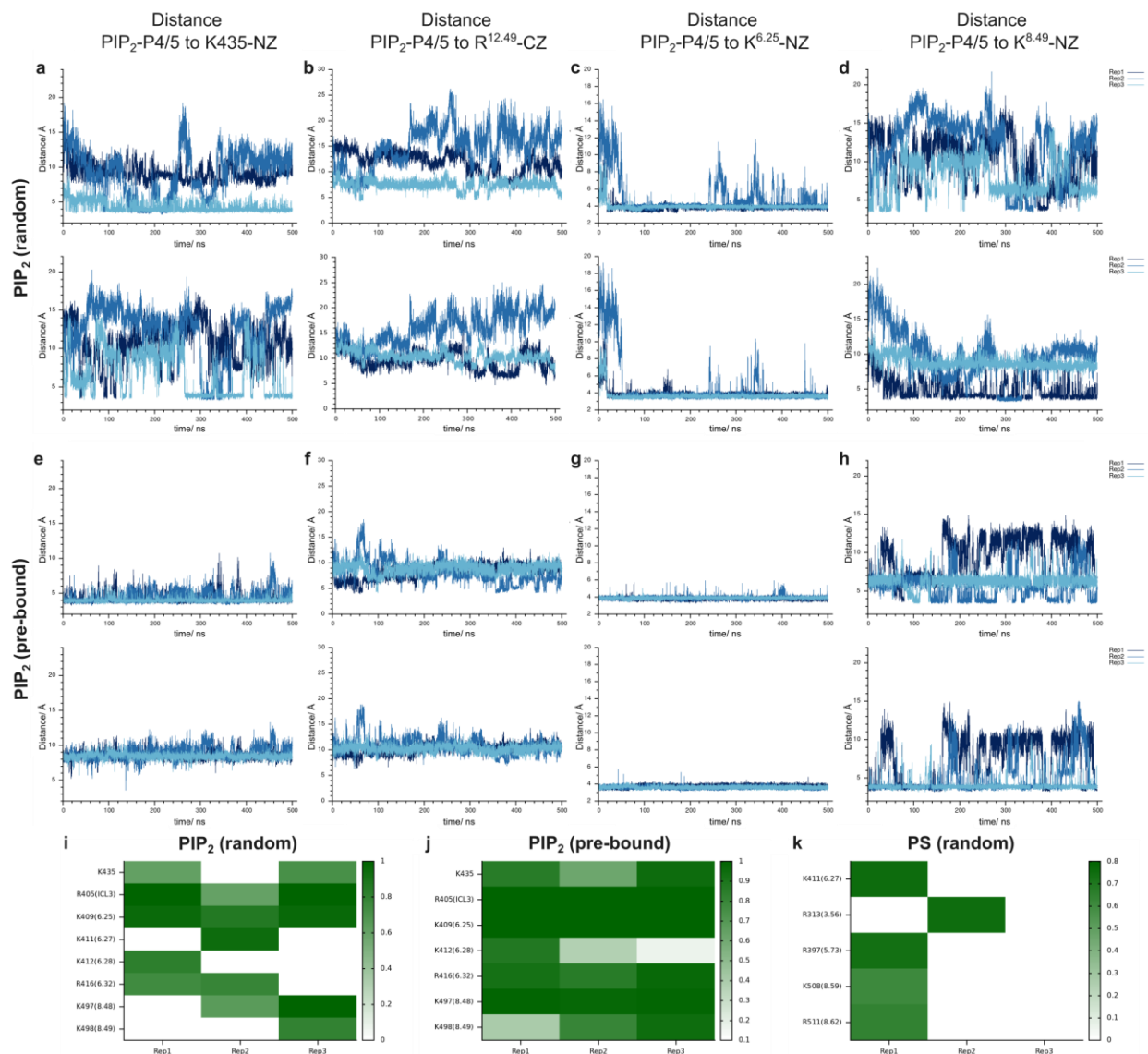
Supplementary Fig. 11. Comparison of detergent-solubilized FZD₄-DEP with nanodisc-embedded FZD₆-DEP complexes. In the magnified inserts with corresponding color coding, the FZD₆-associated density is encircled in red (upper inserts). This density is absent on the detergent solubilized FZD₄-DEP complex (lower inserts; PDB ID 8WMA).



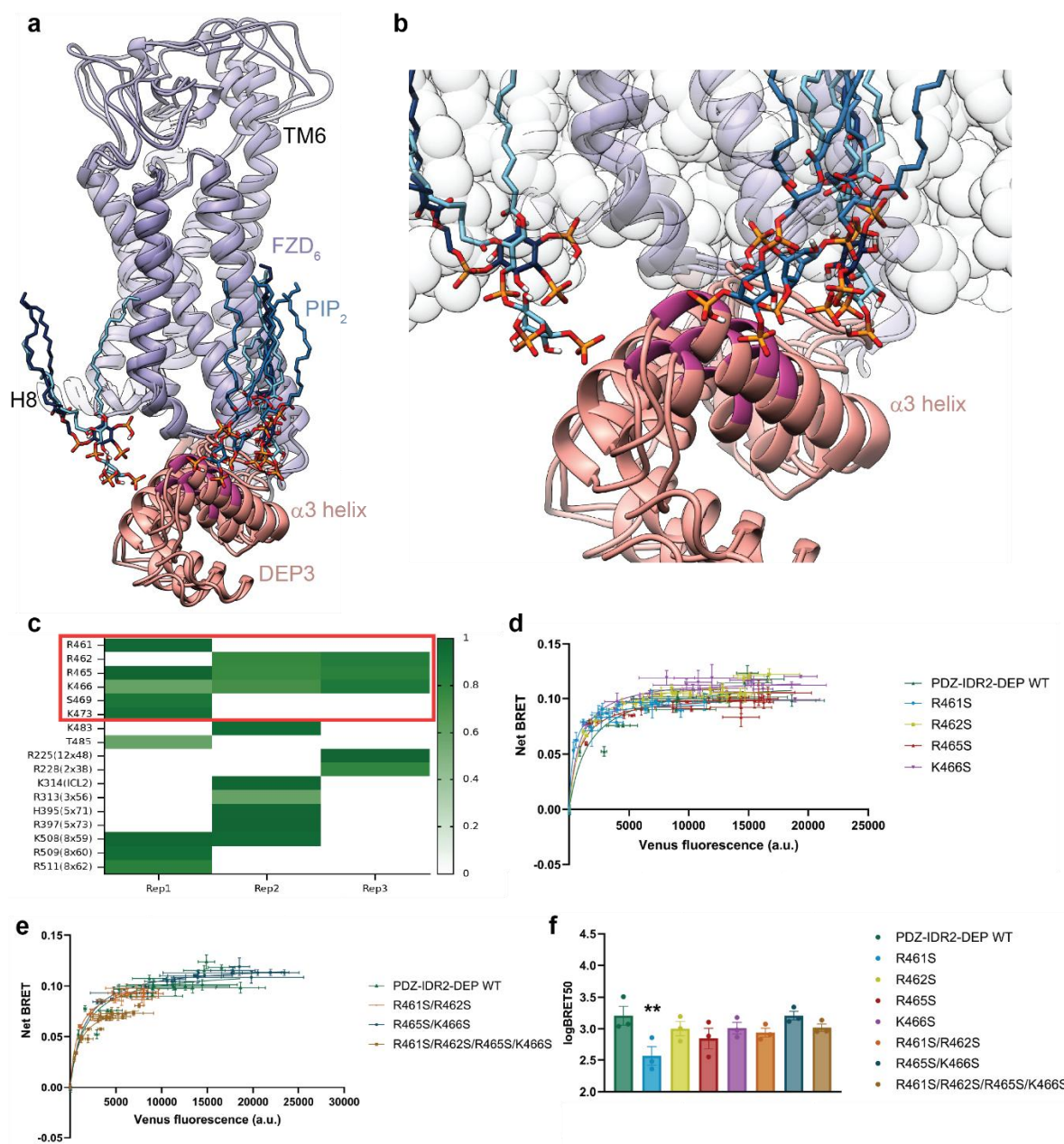
Supplementary Fig. 12. RMSD and finger-loop engagement for FZD₆-DEP MD simulations. (a,e) RMSD of the backbone of the TM domain. (b,f) RMSD of the backbone of DEP with alignment on DEP. (c,g) RMSD of the backbone of DEP with alignment on the TM domain. (d,h) Distance between K446-C α in the DEP finger-loop and E^{2.41}-C α . Shown plots are for the FZD₆-DEP complex embedded in a membrane consisting of (a-d) POPC/Chol, (e-h) POPC/Chol with 5% randomly placed PIP₂ in the inner leaflet, (i-l) POPC/Chol with one PIP₂ molecule pre-bound to the polar cavity between TM6/TM7/H8 of FZD₆ and (m-p) POPC/Chol with 5% randomly placed POPS in the inner leaflet. Data is plotted as time course over the entire trajectory, and replicas are colored in different shades of blue.



Supplementary Fig. 13. Coordinates in xy for FZD₆-DEP MDs. Plot of the xy coordinates over the duration of the MD simulation of the center of mass of the backbone of the four most intracellular residues of each transmembrane helix (rainbow coloring), of DEP (salmon) and of the head-group of PIP₂ or POPS molecules (if present; shades of blue). The initial positions of PIP₂/POPS are indicated with triangles. See **Fig. 3c** for indication of TMs and DEP. Plots for FZD₆-DEP complex embedded in a membrane composed of POPC/Chol (**a**), POPC/Chol and 5% PIP₂ in the inner leaflet (**b**), POPC/Chol with one PIP₂ pre-bound to the polar cavity (**c**) or POPC/Chol and 5% POPS in the inner leaflet (**d**) are shown. The plots for the third replica can be found in **Fig. 3**.

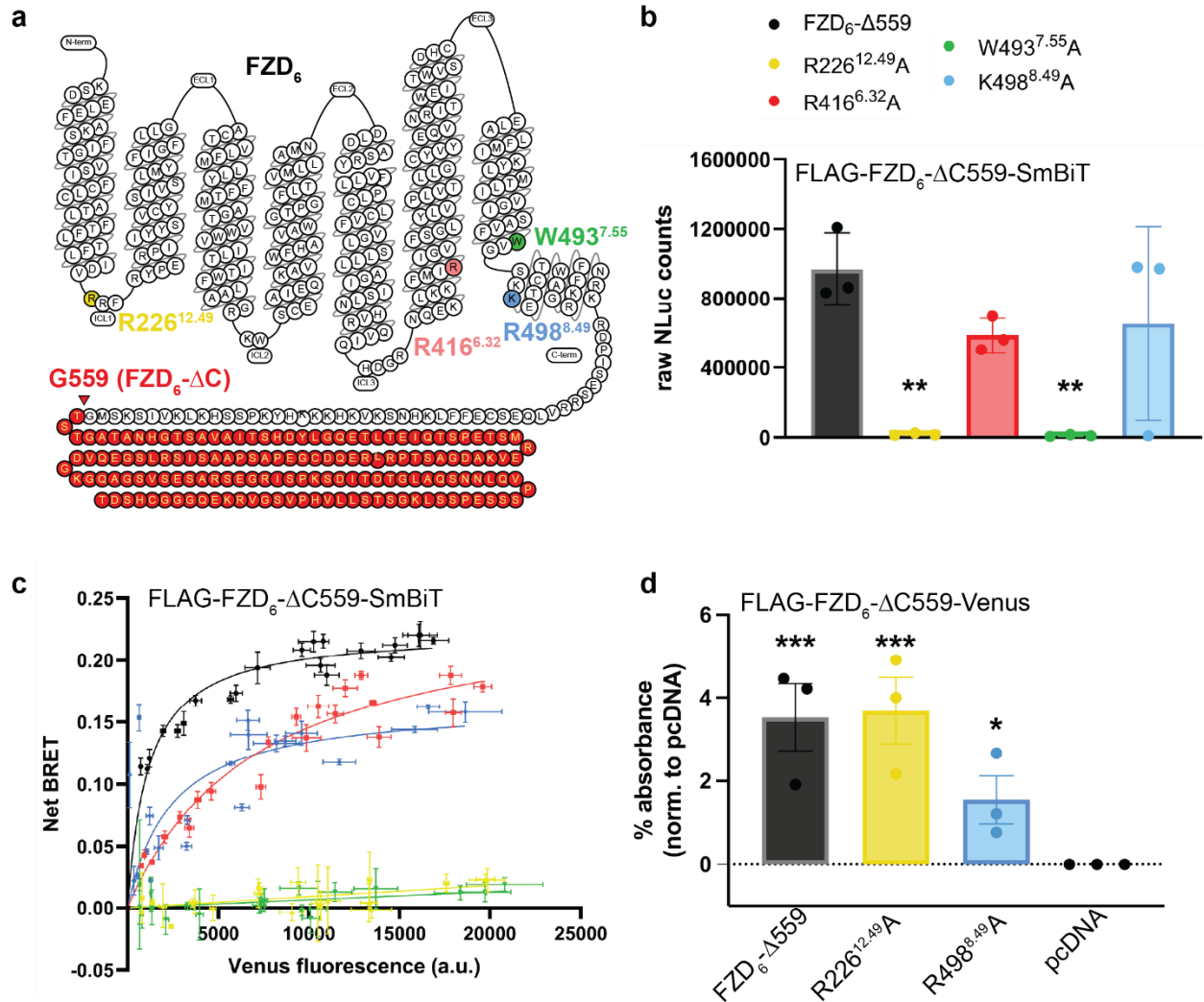


Supplementary Fig. 14. PIP₂ interactions with the polar cavity of FZD₆. (a-h) Distances between Phosphates P4 and P5 of the PIP₂ binding to the polar cavity between TM6, TM7/H8 and ICL1 and selected residues of FZD₆ embedded in a membrane of POPC/Chol with (a-d) 5% randomly distributed PIP₂ in the inner leaflet and (e-h) PIP₂ pre-bound to the polar cavity. Measured distances are to (a,e) NZ of DEP K435 and to FZD sidechain atoms (b,f) R^{12,49}-CZ, (c,g) K^{6,25} NZ and (d,h) K^{8,49} NZ. The different replicas are colored in shades of blue. (i-k) Heatmaps displaying the frequency of polar interactions (hydrogen bonds, salt bridges) as determined using getcontacts between (i) FZD₆ polar cavity residues and PIP₂ when randomly placed in the membrane, (j) FZD₆ polar cavity residues and PIP₂ when pre-bound and (k) FZD₆ and POPS when randomly placed in the membrane.



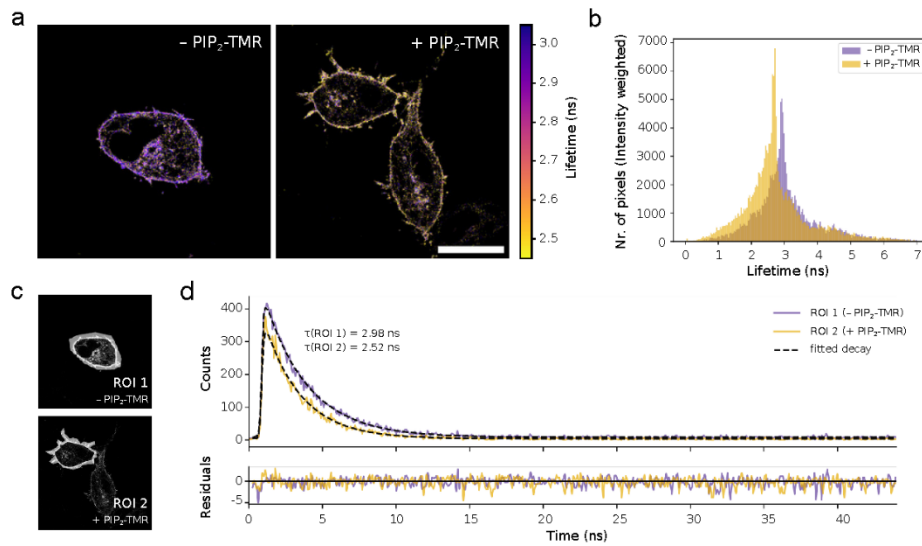
Supplementary Fig. 15. Mutational analysis of basic residues in the $\alpha 3$ helix of DEP3 and the effect on FZD₆-mediated PDZ-IDR2-DEP fragment membrane recruitment. (a) Overlay of the last frames of three independent replicas of 500 ns MD simulations of the FZD₆-DEP complex embedded in a bilayer of POPC/Chol with 5% PIP₂ randomly placed in the inner leaflet. PIP₂ is shown as sticks (different shades of blue for the different MD replica). Residues in the $\alpha 3$ helix of the DEP3 domain predominantly interacting with PIP₂ are highlighted in pink (from left to right: R461, R462, R465, R466). (b) Magnified view of the $\alpha 3$ helix-PIP₂ contacts with same coloring as in (a). POPC location in replica 1 is shown as transparent spheres as a reference for the membrane location. (c) The H-bond/salt-bridge interactions between protein and PIP₂ were calculated based on geometric orientation of amino acid side chains using getcontacts (<https://getcontacts.github.io/>). All interactions with an interaction frequency above 60 % over the

trajectory duration of at least one of the simulated systems were included into the fingerprint heatmaps and colored according to their frequency. The red frame highlights the interactions of the $\alpha 3$ helix residues with PIP₂ molecules. FZD₆-PIP₂ interactions in the polar cavity are shown in **Fig. S14**. **(d-e)** The BRET donor (Nanoluc) is reconstituted by FZD₆-SmBiT and the membrane-anchored CAAX-LgBiT. The BRET acceptor Venus is fused to the PDZ-IDR2-DEP fragment of DVL3. BRET experiments were performed as acceptor titration using single, double or quadruple mutants (R or K to S) of the residues R461, R462, R465 and K466. Mutation to S and not to A was chosen to remove the positive charge while maintaining a certain polarity to help avoid problems with protein folding. **(c)** Acceptor titration curves of single mutants. **(d)** Acceptor titration curves of double and quadruple mutants. N=3 independent experiments performed in triplicates. Values are mean $\pm$ S.D. **(f)** LogBRET₅₀ values, providing a proxy of FZD₆-PDZ-IDR2-DEP affinity, were extracted from the acceptor titration curves. Bars represent means $\pm$ S.D.; ** p=0.0065, One way ANOVA with Dunett's post-hoc test.

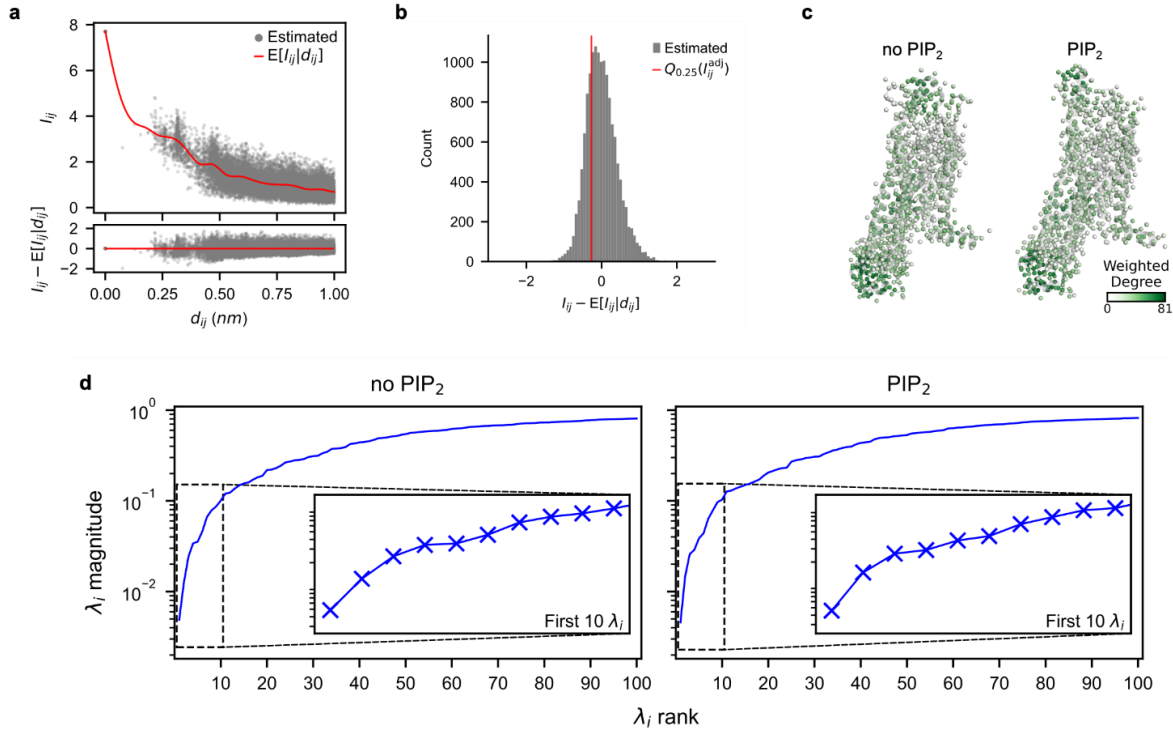


Supplementary Fig. 16. Characterization of the FZD₆-ΔC mutants used for FLIM-FRET analysis. For the FLIM-FRET analysis of TMR-PIP₂ proximity to FZD₆-Venus, the long C terminus (>200 aa) needed to be shortened to optimize the FRET yield. As seen in Fig. 4C, the full length, Venus-tagged FZD₆-FL did not show a reduced lifetime in the presence of TMR-PIP₂. (a) In order to bring Venus closer to the membrane and thereby into an efficient Förster distance, we shortened the C terminus at G559. The mutants R226^{12.49}A, R416^{6.32}A, W493^{7.55}A and K498^{8.49}A are highlighted in green. The snake plot for human FZD₆ was created using the GPCR database at https://gpcrdb.org/protein/fzd6_human/. (b) The FZD₆-ΔC constructs and its mutants were expressed at the cell surface as assessed by quantifying Nluc complementation with CAAX-LgBiT. Despite a large drop in NLuc counts both R226^{12.49}A and W493^{7.55}A provided still NLuc counts clearly above background. Errors bars show standard error of the mean (SEM) of 3 independent experiments performed in triplicates. ** p=0.0049 for W493^{7.55}A and p=0.0051 for R226^{12.49}A (c) In the DEP recruitment based on the bystander BRET assay, where the BRET donor (Nanoluc) is reconstituted by FZD₆-ΔC-SmBiT and the membrane-anchored CAAX-LgBiT and the BRET acceptor is the Venus-tagged PDZ-IDR2-DEP fragment of DVL3, FZD₆-ΔC shoes a high affinity to PDZ-IDR2-DEP. N=3. Values are mean ± S.D. The affinity to the mutants R226^{12.49}A and R416^{6.32}A was substantially reduced and abolished for the W493^{7.55}A and

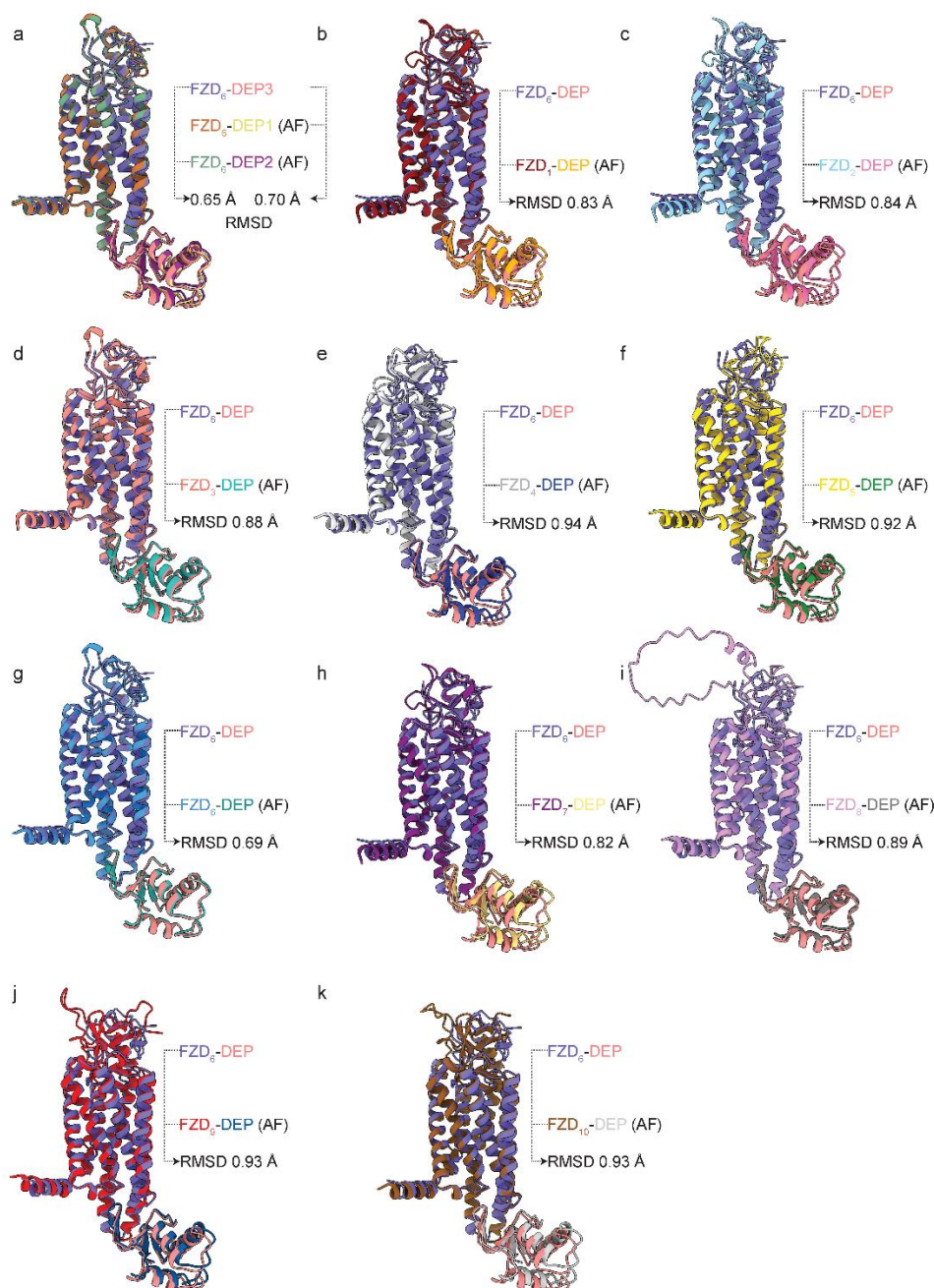
K498^{8.49}A. Only R226^{12.49}A and K498^{8.49}A as representatives of the two populations regarding affinity reduction and cell surface expression were used for FLIM-FRET experiments. The FZD₆-ΔC-SmBiT R^{12.49}A and K^{8.49}A were incapable of recruiting the Venus-tagged PDZ-IDR2-DEP fragment of DVL3. However, surface expression of these constructs was also substantially affected. Since these experiments were performed with FLAG-FZD₆-ΔC-SmBiT aim to validate the use FLAG-FZD₆-ΔC-Venus wt, R^{12.49}A and K^{8.49}A for FLIM-FRET, we also used FLAG-FZD₆-ΔC-Venus wt, R^{12.49}A and K^{8.49}A for surface ELISA (**d**). The constructs with an N terminal FLAG tag and a C-terminal Venus tagged were expressed at the cell surface and therefore suitable for the FLIM-FRET experiments. Cell surface expression in HEK293A cells, transiently transfected with the different receptor constructs, or pcDNA3.1 was quantified by whole-cell ELISA using an antibody directed against the N-terminal FLAG tag. Data show mean ± SEM of three independent experiments performed in triplicate and mean values were normalized to pcDNA3.1 expression. Statistical differences between the constructs and pcDNA were assessed using ordinary one-way ANOVA, followed by an uncorrected Fisher's LSD post-hoc test. *** p=0.0001 for R226^{12.49}A; *** p=0.0002 for Δ559; * p=0.0412 for K498^{8.49}A.



Supplementary Fig. 17. Fluorescent lifetime images of cells expressing FZD₆-ΔC-Venus and selection of fitting regions. (a) Lifetime images of FZD₆-ΔC-Venus-expressing cells in the absence (left) and presence of PIP₂-TMR (0.5 ug/mL which corresponds to 0.384 μM; right). The lifetime maps were generated from a full lifetime image fit (2 x 2 pixel binning) using the Leica Application Suite LAS X FLIM/FCS software. Lifetime values were pseudocoloured according to the indicated colour scale and pixel brightness was weighted by the corresponding fluorescence intensity to suppress low-signal background. Scale bar: 20 μm. (b) Histograms showing the lifetime distribution across all pixels in the full images shown in A (FZD₆-ΔC-Venus without PIP₂-TMR in purple, with PIP₂-TMR in orange). (c) For lifetime comparisons only the membrane signal was selected. For each cell, regions of interest (ROI) were manually picked where the FZD₆-ΔC-Venus membrane signal could be clearly identified. All intracellular FZD₆-ΔC-Venus signal was excluded from the analysis. Indicated are ROI 1 and ROI 2 selected from the FZD₆-ΔC-Venus images without and with PIP₂-TMR added, respectively, as shown in A. (d) The average fluorescence lifetime of each ROI was determined by mono-exponential fitting over a time range of 0.2–44 ns using the Leica software. Depicted are the fluorescence decay curves of ROI 1 (purple) and ROI 2 (orange) and their corresponding fits (black). The residual counts are shown below. For the example ROIs, the average lifetime values τ for ROI 1 (without PIP₂-TMR) and ROI 2 (with PIP₂-TMR) derived from the fit, were 2.98 ns and 2.52 ns, respectively.



Supplementary Fig. 18. Construction of networks and select properties. (a) Distance-dependence of I_{ij} for an example trajectory. $E[I_{ij}|d_{ij}]$ (red line) was fit against the estimated I_{ij} values of a trajectory (gray dots). (b) $I_{ij} - E[I_{ij}|d_{ij}]$ histogram for the same example trajectory. The first quartile (red line) was chosen as the minimum edge inclusion threshold. (c) Nodes of the FZD₆-DEP networks in the presence and absence of PIP₂ colored by their respective weighted degree. The high degree regions correspond to loop regions and DEP. (d) Eigenvalue spectra of the first 100 eigenvalues for the FZD₆-DEP networks. The first 10 eigenpairs of each network were compared (Fig. 5b), therefore these eigenvalues are shown in greater detail (insets).



Supplementary Fig. 19. Comparison of AlphaFold-based FZD-DEP complexes for all human paralogs. Models of the determined FZD₆-DEP structure are directly compared with AlphaFold (AF)-generated models of FZD-DEP complexes for all ten FZD paralogs and DEP domains of DVL1, 2, 3. **(a)** The experimental FZD₆-DEP (DEP3) structure is similar to AlphaFold predictions of FZD₆ in complex with the DEP domains of DVL1 (DEP1) and DVL2 (DEP2), as expected based on the overall conservation of the DEP domain. **(b-k)** The structural conservation is also found across FZDs with AlphaFold-predicted FZD-DEP structures showing an overall RMSD < 1.0 Å (as measured across pruned residues) compared to the FZD₆-DEP structure.

Supplementary Table 1. Summary of simulated MD systems and their setup.

	FZD ₅ PIP ₂ (random)	FZD ₄ PIP ₂ (random)	FZD ₆ (neg control)	FZD ₆ PIP ₂ (random)	FZD ₆ PIP ₂ (pre-bound)	FZD ₆ PS (random)
Complex	FZD ₅ -DEP2 Protein-protein docking	FZD ₄ -DEP2 Cryo-EM ²⁰	FZD ₆ -DEP3	FZD ₆ -DEP3	FZD ₆ -DEP3	FZD ₆ -DEP3
Lipid composition (no. of lipids)						
Top layer	POPC:Chol 97:24	POPC:Chol 98:24	POPC:Chol 99:24	POPC:Chol 98:24	POPC:Chol 101:24	POPC:Chol 98:24
Bottom layer	POPC:Chol: SAPI24:SAPI25 90:24:3:3	POPC:Chol: SAPI24:SAPI25 92:24:3:3	POPC:Chol 97:24	POPC:Chol: SAPI24:SAPI25 90:24:3:3	POPC:Chol 97:24	POPC:Chol:POPS 90:24:6
No. of rep.	5	3	3	3	3	3
Simulation length per replica [ns]						
Equil. NPT	0.25	0.25	0.25	0.25	0.25	0.25
Equil. NVT	26.75	26.75	26.75	26.75	26.75	26.75
Production	500	500	500	500	500	500
Box dimensions [Å]	100.5x100.5 x157.8	100.9x100.9 x142.4	101.3x101.3 x142.4	100.9x100.9 x142.4	101.8x101.8 x144.3	100.9x100.9 x142.4
Total no. of atoms	128775; 128820; 128793; 128919; 128916	114855; 114987; 114915	117496; 117463; 117442	116406; 116289; 116316	120068; 120128; 120161	116224; 116092; 116188
No. of water molecules	30641; 30656; 30647; 30689; 30688	26003; 26047; 26023	26777; 26766; 26759	26475; 26436; 26445	27495; 27515; 27526	26459; 26415; 26447
NaCl conc. [mM]	150	150	150	150	150	150

Supplementary Table 2. Cryo-EM data collection, refinement, and validation statistics of datasets 1, 2, 3 and 4.

	Dataset 1	Dataset 2	Dataset 3	Dataset 4
<i>Data collection and processing</i>				
Microscope	TFS Titan Krios G3		TFS Titan Krios G1	
Detector	Gatan K3 BioQuantum		Gatan K3 BioQuantum	
Magnification	105,000x		130,000x	
Voltage (kV)	300		300	
Total exposure (e-/Å ²)	60.0	51.8	79.9	51.6
Exposure rate (e-/pixel/s)	15.5	4.0	2.1	4.0
Exposure time (s)	2.6	6.0	18.0	6.0
Number of frames	60	48	144	48
Energy filter slit width (eV)	20		30	
Defocus range (µm)	-0.6 to -2.0		-0.4 to -1.4	
Pixel size (Å)	0.825		0.6523	
Automation software	EPU		SerialEM	
Mode	Counting (CDS)		Counting (CDS)	
Tilt Angle (°)	0	0	20	40
Number of micrographs	16,827	1,280	1,364	3,436
Number of initial particles	21,426,665	402,650	733,053	3,104,406
EMPIAR ID				
Number of particles (in final map)	-	-	-	84,365
Symmetry	-	-	-	C1
Resolution (global) (Å)	-	-	-	3.6
Resolution range (Å)	-	-	-	3.4-8.5
Directional resolution range (Å)	-	-	-	3.3-7.1
3DFSC Sphericity value	-	-	-	0.84
SCF Value*	-	-	-	0.96
cFAR	-	-	-	0.09
EMDB accession number	-	-	-	EMD-82483

*The sampling compensation factor (SCF) value is calculated as described²¹ and assumes that all orientations have been determined accurately

Supplementary Table 3. Cryo-EM data collection, refinement, and validation statistics of datasets 5 and 6.

	Dataset 5	Dataset 6
<i>Data collection and processing</i>		
Microscope	JEOL CRYO ARM 200 II	
Detector	Gatan K3 BioQuantum	
Magnification	80,000x	
Voltage (kV)	200	
Total exposure (e-/Å ²)	60.1	65.0
Exposure rate (e-/pixel/s)	7.0	
Exposure time (s)	3.0	
Number of frames	80	
Energy filter slit width (eV)	20	
Defocus range (μm)	-1.2 to -1.5	
Pixel size (Å)	0.571	
Automation software	SerialEM	
Mode	Counting (CDS)	
Tilt Angle (°)	0	
Number of micrographs	32,756	33,436
Number of initial particles	1,372,906	1,061,638
EMPIAR ID	TBC	
Number of particles (in final map)	312,936	
Symmetry	C1	
Resolution (global) (Å)	2.8	
Resolution range (Å)	2.4-5.1	
Directional resolution range (Å)	2.3-3.3	
3DFSC Sphericity value	0.97	
SCF Value*	0.82	
cFAR	0.35	
<i>Refinement</i>		
Initial model used	ModelAngelo	
Model resolution (Å)	2.9	
FSC threshold	0.5	
Map sharpening B factor (Å ²)	93.5	
<i>Model composition</i>		
Non-hydrogen atoms	3522	
Protein	438	
<i>B factors (Å²) (min/max/mean)</i>		
Protein	36.60/119.91/69.79	
<i>R.m.s deviations</i>		
Bond lengths (Å)	0.003	

Bond angles (°)	0.582
<i>Validation</i>	
MolProbity score	1.06
Clashscore	2.69
Poor rotamers (%)	0.85
<i>Ramachandran plot</i>	
Favored (%)	98.15
Allowed (%)	1.85
Outliers (%)	0.00
EMDB accession number	EMD-82424
PDB code	43ZR
PDB extended code	pdb_000043ZR

*The sampling compensation factor (SCF) value is calculated as described ²¹ and assumes that all orientations have been determined accurately.

Supplementary Table 4. Dynamics IDs of the trajectories deposited in the GPCRmd (<https://gpcrmd.org>). For technical reasons, individual replicas were deposited for some MD systems, while all trajectories are submitted to a single entry for other MD systems.

	Dynamics ID	All replicas as
FZD ₅ -DEP (PIP ₂ random)	3300, 3301, 3302, 3303, 3304	Individual entries
FZD ₄ -DEP (PIP ₂ random)	3346, 3347, 3348	Individual entries
FZD ₆ -DEP (negative control)	3277	Single entry
FZD ₆ -DEP (PIP ₂ random)	3296, 3297, 3298	Individual entries
FZD ₆ -DEP (PIP ₂ pre-bound)	3278	Single entry
FZD ₆ -DEP (PS random)	3279	Single entry

Supplementary Video 1. Early time-dependent probability evolution of a stochastic walker starting from K435_{sc}. Nodes are colored according to the probability of observing the walker at that node at a particular time. Time is given in units of unnormalized pseudotime. The dashed line in the color bar indicates the expected probability if the walker were uniformly distributed across all nodes.

Supplementary Video 2. Early time-dependent probability evolution of a coherent walker starting from K435_{sc}. Nodes are colored according to the probability of observing the walker at that node at a particular time. Time is given in units of unnormalized pseudotime. The dashed line in the color bar indicates the expected probability if the walker were uniformly distributed across all nodes.

Supplementary Data 1. (separate file). Density map (data sets 5-6) of the FZD₆-pPDZ-IDR2-DEP complex embedded in saposin-based lipid nanodiscs, at 2.8 Å resolution,

Supplementary Data 2. (separate file). Model (data sets 5-6) of the FZD₆-pPDZ-IDR2-DEP complex embedded in saposin-based lipid nanodiscs.

Supplementary Data 3. (separate file). PyMOL session mapping the results of the network analyses onto models of FZD₆-DEP. The scene has been arranged so that all objects pertaining to FZD₆-DEP in the presence or absence of PIP₂ are shown on the right and left respectively. Each analysis has been organized into a separate group for convenience. The *references* group contains a FZD₆-DEP-PIP₂ model (*binding_ref*; **Fig. 5a**), an aligned PIP₂ molecule (*pip2*), as well as two groups displaying reference structures and nodes for FZD₆-DEP in the absence (*no_ref*) and presence (*pip_ref*) of PIP₂. The compared eigenvectors of each network (**Fig. 5b**) are available in *eigenvectors*. Similarly, both the stochastic walker (**_classic*; **Fig. 5c**) and coherent walker (**_quantum*; **Fig. 5d**) results are stored in *walkers*. The minimum-cost, maximum flow paths (**Fig. 5e**) from K435_{sc} to E194^{1,28}_{bb} (**_TMI*) and C454^{6,70}_{bb} (**_TM6*) are in *flows*. Objects with nodes colored by their weighted degree (**Supplementary Fig. 18c**) are found in *degree*.

Supplementary Data 4. (separate file). Density map (data sets 1-4) of the FZD₆-pPDZ-IDR2-DEP complex embedded in saposin-based lipid nanodiscs, at 40° tilt and 3.6 Å resolution, still with directional anisotropy.

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