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Gunnar Schulte

`gunnar.schulte@ki.se`

Karolinska Institutet <https://orcid.org/0000-0002-2700-7013>

Rawan Shekhani

Karolinska Institutet <https://orcid.org/0000-0002-4632-2131>

Magdalena Scharf

Karolinska Institutet <https://orcid.org/0000-0002-3305-3956>

Julia Kinsolving

Karolinska Institutet

Gaia Martiniani

Karolinska Institutet <https://orcid.org/0009-0008-4669-5676>

Tom Resink

Karolinska Institutet

Jan Voss

Skape Bio ApS

Pawel Kozielowicz

Karolinska Institutet <https://orcid.org/0000-0003-1414-3566>

Nele Jarnot

Karolinska Institutet

Franziska Ragaller

Karolinska Institutet <https://orcid.org/0000-0002-4148-262X>

Nini Schotman

Karolinska Institutet

Jia Yu Ho

National University of Singapore <https://orcid.org/0009-0004-5418-0585>

Fanny Peysson

IGF, Univ. Montpellier

Shi Min Tan

National University of Singapore

Allan Pang

National University of Singapore <https://orcid.org/0000-0003-4786-6445>

Jitender Kumar

CEITEC - Central European Institute of Technology, Masaryk University

Hans-Gustaf Ljunggren

Karolinska Institute <https://orcid.org/0000-0003-0908-7387>

Vítězslav Bryja

Masaryk University <https://orcid.org/0000-0002-9136-5085>

Konstantinos Tripsianes

Central European Institute of Technology, Masaryk University <https://orcid.org/0000-0003-0948-813X>

Radostin Danev

University of Tokyo <https://orcid.org/0000-0001-6406-8993>

Adnane Achour

Karolinska Institutet <https://orcid.org/0000-0003-0432-710X>

Erdinc Sezgin

Karolinska Institute <https://orcid.org/0000-0002-4915-388X>

Sebastien Granier

Institut de Génomique Fonctionnelle, CNRS INSERM Univ Montpellier <https://orcid.org/0000-0003-1550-3658>

Remy Sounier

INSERM, Institut de Genomique fonctionnelle <https://orcid.org/0000-0002-7802-2838>

Yong Zi Tan

National University of Singapore <https://orcid.org/0000-0001-6656-6320>

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

Authors: Rawan Shekhani^{1,2*}, Magdalena M Scharf^{1*}, Julia Kinsolving¹, Gaia Martiniani¹, Tom Resink³, Jan Hendrik Voss¹, Pawel Kozielowicz⁴, Nele Jarnot⁵, Franziska Ragaller⁵, Nini Schotman¹, Jia Yu Ho², Fanny Peysson⁶, Shi Min Tan², Allan H. Pang², Jitender Kumar⁷, Hans-Gustaf Ljunggren⁸, Vítězslav Bryja⁹, Konstantinos Tripsianes⁷, Radostin Danev¹⁰, Adnane Achour³, Erdinc Sezgin⁵, Sébastien Granier⁶, Rémy Sounier⁶, Yong Zi Tan^{2,11,12†}, Gunnar Schulte^{1†}

Affiliations:

¹ Karolinska Institutet, Dept. Physiology & Pharmacology, Sec. Receptor Biology & Signaling; Biomedicum, S17165 Stockholm, Sweden.

² Department of Biological Sciences, National University of Singapore, 16 Science Drive 4, Singapore 117558, Singapore.

³ Science for Life Laboratory, Department of Medicine, Division of Infectious Diseases Karolinska Institute, Solna, Sweden.

⁴ Karolinska Institutet, Dept. Physiology & Pharmacology, GPCR Pharmacology; Biomedicum, S17165 Stockholm, Sweden.

⁵ Science for Life Laboratory, Department of Women's and Children's Health, Karolinska Institutet, Solna, Sweden.

⁶ IGF, Univ. Montpellier, CNRS, INSERM; Montpellier, France.

⁷ CEITEC - Central European Institute of Technology, Masaryk University; Kamenice 5/E35/1S081, CZ-62500 Brno, Czech Republic.

⁸ Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden.

⁹ Department of Experimental Biology, Faculty of Science, Masaryk University; Brno, Czech Republic.

¹⁰ Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan.

¹¹ NUS Centre for Cancer Research (N2CR), Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore.

¹² NUS Artificial Intelligence Institute (NAII), National University of Singapore, Singapore 119391, Singapore.

* These authors contributed equally to this work.

† Corresponding authors. Email: gunnar.schulte@ki.se, yztan@nus.edu.sg.

Abstract: The WNT signaling system is essential for embryonic development, tissue homeostasis and regeneration. Upon the interaction of WNTs as secreted ligands with their transmembrane receptors from the Frizzled (FZD) family, FZDs recruit the main intracellular transducer, the Dishevelled (DVL) proteins. While the molecular details of FZD-DVL coupling are not fully understood, it is known that phosphatidyl-4,5-bisphosphate (PIP₂) increases the affinity of FZD to DVL by a bipartite protein (FZD)-protein (DVL) and protein (DVL)-membrane interaction. Using MD simulations, cryo-EM of the FZD₆-DEP complex, live cell imaging, genetically encoded biosensors and computational network analysis we extend this model by identifying a conserved PIP₂ binding site in a polar cavity of FZDs through which PIP₂ acts as a positive allosteric modulator of DVL-mediated WNT signaling.

Summary

Frizzleds (FZD) as class F G protein-coupled receptors transmit regulatory input in embryonic development and tissue homeostasis^{1,2}. FZDs recruit the scaffold protein Disheveled (DVL) as transducer to initiate WNT signaling³⁻⁵. The event of DVL membrane recruitment is central for signal propagation and is thought to involve FZDs, coreceptors and phospholipids⁶⁻⁸. However, so far, the molecular details of FZD-DVL interaction and the role of charged phospholipids such as phosphatidylinositol bisphosphate (PIP₂) are poorly defined. Here, we employ protein docking, molecular dynamics simulations, single particle cryogenic electron microscopy of a FZD-DVL complex embedded in lipid nanodiscs, mutagenesis and resonance energy transfer assays to understand the molecular underpinnings of FZD-DVL complex formation in a lipid environment and its functional consequences in living cells. Collectively, these diverse approaches identify PIP₂ as an allosteric modulator of FZDs binding a fully conserved polar cavity between helix 6 and 7. PIP₂ binding to FZDs is a prerequisite for FZD-DVL coupling and essential for signal initiation. In silico network analysis pinpoints long-ranging allosteric effects of PIP₂ binding in the receptor complex emphasizing the central regulatory role of PIP₂ at the level of FZD. Our discovery identifies a novel PIP₂-based regulatory mechanism for GPCRs and extends the understanding of the FZD-DVL interplay by the functional role of PIP₂ as a class-wide conserved positive allosteric modulator.

Main Text

The WNT signaling system is essential for regulating proliferation, differentiation and migration during embryonic development, tissue homeostasis and regeneration^{1,2}. The 19 mammalian members of the WNT family of secreted lipoglycoproteins exert their effects through ten Frizzleds (FZD₁₋₁₀), which belong to class F of G protein-coupled receptors (GPCRs)⁹. Intracellularly, the outcome of WNT action is defined by the Dishevelled proteins (DVL1, 2, 3) mediating β -catenin-dependent and -independent WNT signaling¹⁰. DVLs are the primary FZD transducer proteins and mediate some of the very first signaling steps downstream of FZDs¹¹⁻¹³. Nevertheless, the molecular details of FZD-mediated DVL activation and signal propagation remain unclear. DVLs consist of three globular domains: 1) the DIX (Dishevelled and Axin) domain, which mediates DVL and axin multimerization essential for signal amplification; 2) the PDZ (post-synaptic density protein-95, Disc large tumor suppressor, zonula occludens-1) domain, which regulates asymmetric localization of key components in the planar cell polarity pathway¹⁴; and 3) the DEP (Dishevelled, EGL-10 and pleckstrin) domain, which directly interacts with FZDs, binds phospholipids and contributes to pathway activation-dependent recruitment to the plasma membrane, an essential step for signal initiation^{4,15,16}. Furthermore, DVL proteins are highly phosphorylated in response to WNT stimulation and FZD overexpression and activation, a process catalyzed by casein kinases¹⁷⁻²¹. The exact order of events, however, from WNT-FZD binding, coreceptor engagement, FZD-DVL recruitment and interface conformational rearrangements, casein kinase regulation, to DVL phosphorylation, is poorly resolved^{15,20,22}.

FZD-DVL recruitment is mediated by the DEP domain of DVL^{4,5,15}. Through mutagenesis experiments, a topologically complex, putative FZD-DVL interface emerged. It was mapped across multiple studies to the intracellular loops 1-3 (ICL1, ICL2, ICL3), the helix H8, the lower parts of transmembrane helix 2-4 and 6 (TM2, 3, 4, 6), and the C terminus. R^{12,49}, Y^{2,39}, R/K^{6,32}, W^{7,55}, K^{8,49} (superscript denotes Ballesteros-Weinstein nomenclature²³) were consistently identified as conserved residues^{4,5,8,12,24-26}. The FZD4-DEP interface is restricted in space and defined by apolar interactions between the DEP finger loop and TM5-ICL3-TM6²⁷. However, this structure did not explain how FZD residues in ICL1, TM6, TM7 and H8, which are distant to the apolar interface, contribute to DEP-mediated DVL recruitment.

The membrane lipid phosphatidyl-4,5-bisphosphate (PIP₂) is essential for regulating WNT signaling²⁸⁻³⁰, particularly DVL membrane recruitment, where the positive charge on the α 3 helix of DEP appeared essential for membrane interaction^{5,31,32}. Furthermore, PIP₂ was important for enhancing the affinity between purified and lipid-nanodisc-embedded FZDs and DEP, suggesting a bipartite interaction of the DEP finger loop with FZD and the DEP α 3 helix with PIP₂^{7,33}. The current model for DVL recruitment is thus based on a DEP finger loop-mediated DVL-FZD interaction and a polar DVL-membrane interface^{7,27}.

Here, we identify PIP₂ as a generic allosteric modulator, directly binding a conserved cavity in FZDs and acting as an essential cofactor for FZD-DVL recruitment. This unifies prior observations by showing that key FZD residues coordinate PIP₂, shifting its role from merely facilitating DEP-membrane binding to enabling FZD-DEP interaction and engaging in intramolecular allosteric information relay.

Results

Modelling the FZD-DEP complex

While FZD residues required for DVL recruitment were mapped in several studies employing different FZD paralogs across diverse species, structural information of the FZD-DVL complex was limited. When initiating this work, AlphaFold was just emerging²⁷ and the FZD₄-DEP cryo-EM structure²⁷ was not yet published. Therefore, we used homology modelling and protein-protein docking using HADDOCK^{34,35}, guided by available experimental data to derive potential complex conformations of human FZD₅ and the DEP domain of human DVL2 (DEP2). Selected docked complexes were probed with MD simulations to identify the most stable for further investigations (**Supplementary Fig. 1**). While the final selected FZD₅-DEP complex conformation displays differences compared to AlphaFold predictions and the FZD₄-DEP cryo-EM structure²⁷, the overall orientation of DEP in relation to FZD₅ was comparable.

MD simulations to assess the role of PIP₂

In order to understand the molecular details of the FZD-DVL interaction and assess the role of PIP₂, which was postulated to assist in the formation of high affinity FZD₄-, FZD₅- and FZD₇-DEP complexes⁷, we ran MD simulations with the model of FZD₅-DEP2 selected from the protein-protein docking in membranes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), Cholesterol (Chol) and PIP₂ (SAPI24/SAPI25) (**Supplementary Fig. 2; Supplementary Table 1**). Additionally, we performed corresponding MD simulations using the cryo-EM FZD₄-DEP2 complex²⁷ (**Supplementary Fig. 2**). The PIP₂ concentration in the inner leaflet (6 PIP₂ of ~120 lipids, i.e. approximately 5%; **Supplementary Table 1**) in both simulations matched laboratory experiments employing purified FZD₅ embedded in lipid nanodiscs⁷. As expected, the conformation and orientation of DEP was more stable in the experimentally derived FZD₄-DEP complex compared to the computationally modelled FZD₅-DEP complex (**Supplementary Fig. 2**). A major difference was the closer proximity of the positively charged α 3 helix of DEP in the FZD₄-DEP complex to the plasma membrane, which led to an accumulation of PIP₂ molecules close to this charged helix (**Supplementary Fig. 3**). This orientation, which might contribute to the overall complex stability, is similar to previous descriptions of how DEP interacts with the membrane^{31,32}. Interestingly, PIP₂ reproducibly associated with a region between TM6 and TM7/H8 within the first 100 ns of the simulations in both complexes (**Fig. 1a-b; Supplementary Fig. 3**), forming polar/ionic interactions with several positively charged residues including K429^{ICL3}/K442^{ICL3}, R/K^{6,28}, K/R^{6,32} and K^{8,49} (**Supplementary Fig. 3**).

Cryo-EM of FZD₆ in lipid nanodiscs

The previously published cryo-EM structure of the FZD₄-DEP complex was obtained with detergent solubilized FZDs using NanoBiT tethering of FZD to DEP, underlining the low affinity of FZD₄ to DEP²⁷. Furthermore, no information on the functional role of PIP₂ for complex formation could be extracted in the absence of membrane lipids. To investigate the structural aspects of PIP₂-mediated regulation of FZD-DEP complexes, we used purified human FZD₆, a phosphorylated fragment of human DVL3 containing the DEP domain, and saposin-based lipid nanodiscs for complex formation and subsequent cryo-EM.

Human FZD₆ was expressed in Sf9 cells and purified using the N-terminal FLAG tag. Purified FZD₆ was assessed for complex formation with purified domains of DVL3 (**Supplementary Fig.**

4). We compared unphosphorylated DVL fragments (DEP: aa 398-496 and 389-496; PDZ-intrinsically disordered region 2-DEP (PDZ-IDR2-DEP): aa 243-496) with phosphorylated pPDZ-IDR2-DEP co-expressed with CK1 ϵ and purified from *E. coli* (**Supplementary Fig. 4**). A stable complex was formed using pPDZ-IDR2-DEP, characterized by a monodisperse peak of the complex in gel filtration (**Supplementary Fig. 4**).

We reconstituted FZD₆/pPDZ-IDR2-DEP in saposin-based lipid nanoparticles³⁶, exchanging from cymal-6 to lipid nanoparticles containing native lipids including PIP₂. After addressing substantial directional anisotropy caused by preferred orientation (**Supplementary Fig. 5-6; Supplementary Table 2**), we obtained a cryo-EM map at 2.8 Å resolution with unambiguous sidechain density for most residues (**Fig. 1c,d; Supplementary Fig. 5-7; Supplementary Table 3**). Overall, FZD₆ exhibits a typical transducer-bound GPCR-like architecture with a TM6 outward movement of 4.8 Å compared to the inactive conformation (**Supplementary Fig. 8**). However, FZDs exhibit a peculiar topology among GPCRs with an N-terminal cysteine-rich domain (CRD), the orthosteric binding site for WNTs, connected to the transmembrane domain via a flexible linker. In line with the flexibility of the CRD relative to the receptor core and in agreement with previous structures of FZDs^{26,27,37-42}, there is no interpretable electron density of the CRD in the overall density map (**Fig. 1c**). At low contour levels, the map contained additional density, presumably corresponding to the CRD. This was previously also observed for FZD₃³⁹. 3D-variability analysis allowed further visualization of the movement of the CRD and isolation of a subset of particles with improved CRD density (**Supplementary Fig. 9**). Despite this improvement, the local resolution of 6-7 Å was insufficient for model building. Therefore, we rigid body fitted residues 26-130 from the AlphaFold prediction into this low-resolution density volume which fitted the size of the AlphaFold-predicted, flexible CRD.

In addition to the seven TMs, there is clear density for the amphipathic H8, whereas the remainder of the long, flexible C-terminus of FZD₆ is unresolved. While the electron density map allowed modelling the DEP domain of DVL3 (aa 406-495) interacting with the FZD₆ core, the remaining features of pPDZ-IDR2-DEP could not be resolved.

The lipophilic FZD₆-DEP interface

The FZD₆-DEP interface is primarily characterized by hydrophobic interactions. Residues L434 and I436 in the finger loop of DEP3 interact with a hydrophobic pocket in FZD₆ involving L393^{5,69}, V396^{5,72}, V399^{5,75} and I400^{5,76} in TM5 (**Fig. 2a**). F413^{6,29} in TM6 further extends this hydrophobic pocket upward. This lipophilic interface formed between FZD₆ and DEP is the same as for the cryo-EM structure of FZD₄-DEP in detergent micelles²⁷ and agrees overall with the interface from our protein-protein docking (**Supplementary Fig. 1**).

To validate the role of key residues in the lipophilic interface of FZD₆-DEP, we combined a bioluminescence resonance energy transfer (BRET)-based FZD₆-PDZ-IDR2-DEP recruitment assay with alanine mutagenesis (**Fig. 2b**). The bystander BRET assay is based on in-cell complementation of FZD₆-SmBiT and membrane anchored CAAX-LgBiT to Nanoluciferase (Nanoluc) establishing the membrane-localized BRET donor. Venus-PDZ-IDR2-DEP serves as the BRET acceptor. BRET acceptor titration allows to quantify BRET₅₀ values as a relative measure of FZD₆-PDZ-IDR2-DEP affinity, where an increase in BRET₅₀ values monitors a drop in relative affinity between FZD₆ and PDZ-IDR2-DEP. The complementation of FZD-SmBiT with LgBiT-CAAX served as a measure of FZD cell surface expression, and we confirmed that all

tested mutants were expressed at the cell surface (**Supplementary Fig. 10**). Acceptor titration curves with FZD₆ carrying alanine mutations in the key residues L393^{5.69}A, V396^{5.72}A, V399^{5.75}A, I400^{5.76}A in the lipophilic DEP interface did not differ from wildtype FZD₆ suggesting that the overall lipophilic environment rather than single residue interactions contribute to the FZD₆-DEP interface (**Fig. 2c**). Hence, charged residues were introduced in L393R^{5.69}, V396R^{5.72}, I400R^{5.76} to interfere with DEP recruitment more drastically. While L393R^{5.69} and V396R^{5.72} mutations dramatically reduced DEP recruitment, I400R^{5.76} did not have any detectable effect, thereby validating the importance of lipophilic regions within the FZD-DEP interface (**Fig. 2d**).

The polar cavity accommodates PIP₂

Based on MD simulations of FZD₅-DEP and FZD₄-DEP in the presence of PIP₂ (**Fig. 1**), we hypothesized that FZD₆-DEP binds PIP₂ at the same position. Accordingly, the density map of the polar region between the cytosolic portion of TM6, TM7, ICL1 and the N terminal part of H8 revealed the presence of a non-proteinaceous density that could present the headgroup of a phospholipid (**Fig. 2e**) and that corresponds to the PIP₂ position obtained from the MD simulations. This region is characterized by a distinctly polar environment dominated by the basic residues R226^{12.49}, K412^{6.28}, R416^{6.32} and K498^{8.49} as well as W493^{7.55} in FZD₆ and K435 in the finger loop of the DEP domain of DVL3. Although the resolution precluded unambiguous assignment of this density to PIP₂, the strongly basic character of the cavity is consistent with accommodation of a polyanionic lipid headgroup such as PIP₂. The polar residues surrounding the non-proteinaceous density are fully conserved among human FZD paralogs and homologs in other species underlining the importance of this cofactor. Direct comparison of FZD₄-DEP with our FZD₆-DEP structure, reveals that this density is not visible in the FZD₄-DEP complex (**Supplementary Fig. 11**).

Utilizing the DEP titration BRET assay, alanine mutations of FZD₆ residues R226^{12.49}, R416^{6.32}, W493^{7.55}, K498^{8.49} surrounding the unassigned density, led to a dramatic increase in BRET₅₀ values for PDZ-IDR2-DEP recruitment indicating a loss of DEP affinity upon mutation (**Fig. 2f**). Altogether, the BRET data on constitutive PDZ-IDR2-DEP recruitment to FZD₆ emphasize the functional and topological disconnect of direct lipophilic FZD₆-DEP interaction and the importance of PIP₂-complexing residues for FZD-DEP affinity.

MD simulations corroborate a PIP₂ interaction site in the polar cavity

Because the dynamics of individual lipid-protein interactions are difficult to probe experimentally, we used MD simulations to assess overall complex stability, the capacity of FZD₆ to accommodate PIP₂ and the dynamics of FZD₆-DEP (**Fig. 3**). MD simulations were run with FZD₆-DEP embedded in a lipid environment composed of POPC and Chol (**Fig. 3a-c**), or in a POPC/Chol membrane supplemented with randomly placed PIP₂ (5% PIP₂ in the inner leaflet) (**Fig. 3d-f**), with one PIP₂ molecule prebound to the polar cavity of FZD₆ (**Fig. 3g-i**) or with randomly placed 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS; 5% POPS in the inner leaflet; **Fig. 3j-l**; **Supplementary Table 1**). Overall, the protein complex remains stable throughout all trajectories (**Supplementary Fig. 12-13**); it should, however, be noted that it is prebound in all the simulations and that simulation lengths of 500 ns might not be sufficient to reflect the full impact of the conditions on this prebound complex. PIP₂ associates with the polar cavity between TM6 and TM7/H8 within less than 100 ns and remains bound throughout the

duration of the simulation (**Supplementary Fig. 14**). Furthermore, two PIP₂ reproducibly associate with the $\alpha 3$ helix of DEP potentially contributing to DEP stabilization at the membrane (**Supplementary Fig. 15**). However, in a FZD₆-PDZ-IDR2-DEP BRET-based recruitment assay, mutation of basic residues in the $\alpha 3$ helix of DEP barely affected the apparent affinity of PDZ-IDR2-DEP to FZD₆ (**Supplementary Fig. 15d-f**), emphasizing that the major contributing force for DVL membrane recruitment is indeed the physical interaction with FZDs rather than with the plasma membrane.

In MD simulations, where a single PIP₂ was prebound to the polar cavity of FZD₆, the phospholipid remained stably bound throughout the entire simulation time (**Fig. 3g-i; Supplementary Fig. 14**). No distinct association of POPS neither with the polar cavity of FZD₆ nor with basic residues on the $\alpha 3$ helix of DEP could be observed upon replacing the randomly placed PIP₂ with the same number of POPS molecules in the inner leaflet of the membrane (**Fig. 3j-l**). These results are in line with previous data showing that phosphatidyl serine does not affect FZD-DEP affinity in a nanodisc-based reconstituted system ⁷.

Thus, the MD simulations with different membrane compositions provide in silico support for the concept that the charged phosphoinositide group of PIP₂ associates with the polar cavity of FZD₆ in the presence of DEP and that PIP₂ acts as a cofactor for FZDs.

Live cell imaging of fluorescently labelled PIP₂ by FLIM-FRET

To corroborate the in silico MD simulation data in cellulo, we established a fluorescence lifetime imaging-Förster resonance energy transfer (FLIM-FRET) assay based on red fluorescently labelled PIP₂-TMR and FZD₆ C-terminally tagged with Venus (**Fig. 4a**). The live-cell imaging assay is based on overexpression of the FRET donor FZD₆-Venus and acute incubation with the FRET acceptor PIP₂-TMR (**Fig. 4b**). In the case of the full length FZD₆-Venus (FZD₆-FL-Venus), the long C terminus (>200 amino acids) interfered with the fluorescence lifetime measurements (**Fig. 4c**), a problem that was solved by truncation (FZD₆- Δ C-Venus) reducing the distance of the FRET donor to the lipid bilayer, where the FRET acceptor PIP₂-TMR is embedded. The FZD₆- Δ C construct and respective mutants in the polar core (R^{12,49}A, K^{8,49}A) were functionally validated for the ability of the wild type to recruit PDZ-IDR2-DEP and the disruptive effect of point mutations on FZD₆- Δ C/PDZ-IDR2-DEP interaction (**Supplementary Fig. 16**). Using FLIM-FRET, we compared fluorescent lifetimes of Venus in FZD₆- Δ C-Venus in the absence or presence of PIP₂-TMR (**Fig. 4c**). The region of interest for the assessment restricted the analysis to the quantification of receptor-lipid interactions at the cell surface (**Supplementary Fig. 17**). If the fluorophores are within an efficient FRET distance/orientation, energy transfer occurs and decreases the fluorescence lifetime of the FZD₆- Δ C-Venus donor. A notable reduction of the fluorescence lifetime of the FZD₆- Δ C-Venus donor in presence of PIP₂-TMR was observed, suggesting proximity. The proximity of donor and acceptor alone, however, cannot inform about the localization of the putative PIP₂ binding site. Therefore, we substituted the conserved and presumably PIP₂-complexing residues R^{12,49} or K^{8,49} to alanine, which attenuated the PIP₂-TMR-elicited decrease in Venus fluorescence lifetime (**Fig. 4c**). Thus, the fully conserved residues R^{12,49} and K^{8,49} in the polar cavity, which are essential for DEP recruitment, contribute to FZD₆-PIP₂ association in support of our hypothesis regarding PIP₂ as a FZD₆ cofactor.

Network-based signal transmission analysis

Our data identify PIP₂ as a FZD-binding cofactor that is essential for FZD-DEP recruitment, with DEP binding serving as a proxy for the recruitment of full-length DVL, the central signaling scaffold in the WNT pathway^{3,43}. However, the question remains how the cofactor PIP₂ acts as a true positive allosteric modulator relaying information flow throughout the FZD protein and its molecular switches providing a molecular foundation for a dynamic bidirectional transmembrane allostery^{12,24,25,44-48}. Utilizing the generated MD simulation data, we therefore investigated the mechanistic role of PIP₂ in the FZD₆-DEP complex through network analyses partially based on approaches that we recently applied to other complexes⁴⁹. Networks were constructed from the estimated mutual information between the positional distributions of the FZD₆-DEP backbones and sidechains in the presence or absence of PIP₂ bound to the polar cavity of FZD₆. In these networks, nodes represented backbone (bb) and sidechain (sc), whereas edges represented the statistical dependence between their positions. The spectral decomposition of the degree-normalized symmetric graph Laplacian yielded an expectedly high similarity in overall topological connectivity within each network (**Fig. 5b; Supplementary Fig. 18**), particularly within the top 6 ranked eigenvectors (v_i). Despite this, a comparison of equivalent eigenvectors, as defined by their absolute cosine distance, suggested that dynamical differences are present in the N-terminal half of TM1 and the extracellular segment of TM6 and ECL3. These differences are both notable, given their existence amongst the top eigenpairs, and interesting, given their distance to the binding sites of DEP and the PIP₂ head group. To further explore how a signal might propagate from the DEP binding site through FZD₆, we simulated the evolution of the probability distributions from continuous time walkers starting at DEP-residue K435_{sc}. Both stochastic walkers (**Fig. 5c, Supplementary Video 1**), which only consider decaying additive contributions of eigenpairs, and coherent walkers (**Fig. 5d, Supplementary Video 2**), which allow for interference between different eigenmodes, were modeled to avoid being overly prescriptive in our choice of mechanism for allosteric communication. Regardless of mechanism, the presence of PIP₂ accelerated the signal propagation from the DEP binding site to TM1, H8, and extracellular regions of FZD₆. We also calculated the minimum cost, maximum flow paths from K435_{sc} to E194^{1.28}_{bb} and C454^{6.70}_{bb} (**Fig. 5e**), which are nodes centrally positioned in regions identified by spectral graph decomposition (**Fig. 5b**). In the presence of PIP₂, the flow to E194^{1.28}_{bb} follows a more direct path by recruiting edges at the base of TM1. On the other hand, the flow to C454^{6.70}_{bb} is partially diverted along TM1. Interpreting the combined network analyses of FZD₆-DEP (**Supplementary Data 3**), the presence of PIP₂ may lead to detectable dynamic changes towards the extracellular regions of the receptor by strengthening the dynamic coupling between the DEP binding site and these regions emphasizing the allosteric mode-of-action of PIP₂.

Discussion

Embedding the complex FZD₆-PDZ-IDR2-DEP in PIP₂-containing lipid nanodiscs for cryo-EM in combination with MD simulations, FLIM-FRET, mutagenesis and in silico network analysis enabled us to discover and validate the concept of allosteric modulation of FZDs by PIP₂. While the non-proteinaceous density in the cryo-EM structure could not be unambiguously identified, orthogonal approaches show that the polar cavity in FZDs accommodates highly

charged phospholipids such as PIP₂ to facilitate FZD-DEP recruitment. While PIP₂ is a likely candidate, we do not exclude that other PIPs such as PIP₃ (phosphatidylinositol-3,4,5-trisphosphate), which also boosts the affinity of FZD to DEP, bind the conserved cavity⁷. However, PIP (phosphatidylinositol-4-monophosphate), phosphatidylglycerol, phosphatidic acid and phosphatidyl serine did not affect DEP affinity and are unlikely ligands in the polar cavity⁷. Indeed, our MD results corroborate those wet lab findings regarding phosphatidyl serine, which did not associate with the polar cavity in the MD simulation despite its net single negative charge (Fig. 3j-l).

Historically, functional analysis of relevant FZD mutants in diverse assay formats across FZD paralogs, species and experimental models clearly emphasize that the conserved polar cavity is of utmost importance to maintain the functionality of FZD-DVL-mediated signaling as assessed by the TOPFlash luciferase reporter assay, FZD-DVL recruitment or the phosphorylation-dependent electrophoretic mobility shift of DVL. In fact, mutations of R^{12,49}, R/K^{6,32}, W^{7,55} and K^{8,49} were reported to intervene with these readouts, however, without providing a mechanistic explanation for the mutation phenotype^{4,5,8,12,24,26,50}. With our findings at hand the data can now be reinterpreted to integrate the action of PIP₂ at the level of FZD, where our network analyses demonstrate that the presence of PIP₂ leads to profound, detectable dynamical differences in the receptor dynamics presumably modulating the extent of the bidirectional communication in an allosteric manner.

FZD₆ mediates β -catenin-independent WNT pathways in contrast to for example FZD₄ and FZD₅. While it was known that FZD₄ and FZD₆ efficiently recruit DVL and the DVL DEP domain to the plasma membrane, the distinct signaling differences between these receptors suggest different FZD-DEP coupling modes^{7,51-57} corroborated by differences in WNT-induced FZD-DVL interface dynamics for FZD₄/FZD₅ compared to FZD₆^{45,47,58}. Despite these functional differences, the ligand-independent DEP binding pose appears similar in the solved FZD-DEP experimental structures and in the family-wide AlphaFold predictions (Supplementary Fig. 19). Also, PIP₂ binding to the polar cavity appears conserved throughout the FZD family, which is supported by the MD simulation data with the FZD₄-DEP and FZD₅-DEP complexes. Further studies will resolve the ternary complex of WNT/Norrin(agonist)-FZD-DEP and – in agreement with the FZD-DEP BRET biosensor data – reveal the agonist-induced rearrangement of the preformed, constitutive FZD-DEP complex representing the activated FZD-DEP complex^{45,47,58}.

In summary, our data extend our understanding of the FZD-DVL interplay by the functional role of PIP₂ as a class-wide conserved positive allosteric modulator rather than serving solely as a DEP-domain anchor in the cell membrane. Thus, our work clearly refines the role of FZDs as a novel “PIP₂ reader” and of PIP₂ in WNT signaling arguing that FZD-bound PIP₂ rather than the locally produced DEP-interacting PIP₂ is the key factor driving FZD-DVL recruitment. Nevertheless, key questions remain to be answered regarding agonist-induced FZD-DVL dynamics, the relevance and structural details of the full length DVL contributing to the formation of a functional FZD-transducer signaling unit, the detailed mechanisms that are initiated downstream of the FZD-DEP interaction and the role of FZD-DVL interaction for signal initiation and specification.

The distinct functional characteristics of the DEP interface versus the polar core harbouring PIP₂ bring along new strategies to address FZDs as drug targets. While targeting an intracellular, hydrophobic protein-protein interface with small molecule compounds poses physiochemical challenges, it might be feasible to interfere with FZD-DVL signaling by targeting the polar core in FZDs using small-molecule allosteric ligands modulating phospholipid binding. While key

residues in this site are conserved, paralog differences could provide means to reach FZD-paralog selectivity, which is desirable for the development of FZD-targeting compounds and novel therapies⁵⁹.

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Figures

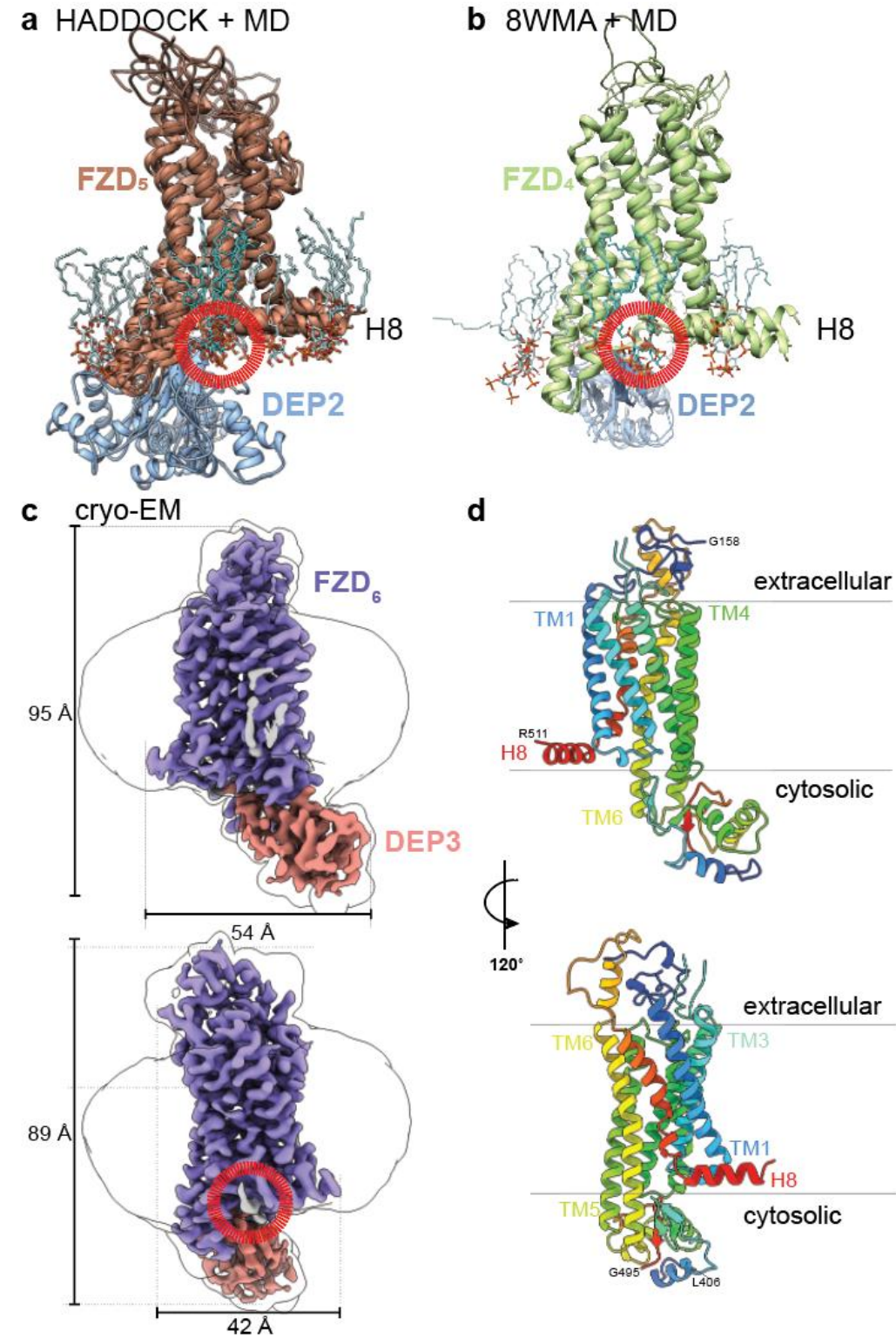


Figure 1

Fig. 1. The FZD-DEP complex in context of a lipid bilayer. (a,b) Overlay of the last frames of three independent replicas of 500 ns MD simulations of FZD-DEP complexes embedded in a bilayer of POPC/Chol with 5% PIP₂ randomly placed in the inner leaflet. PIP₂ is shown as sticks and the molecule binding in the region between TM6 and TM7/H8 is highlighted. Additional data on the PIP₂ interaction can be found in **Supplementary Fig. 3. (a)** Human FZD₅ (copper) in complex with the human DVL2-DEP domain (DEP2; skyblue). The initial complex was obtained from protein-protein docking. **(b)** FZD₄-DEP2 complex (light-green/skyblue) based on a published cryo-EM structure (PDB ID 8WMA). **(c)** Purified FZD₆ and phosphorylated PDZ-IDR2-DEP were reconstituted in saposin-based lipid nanodiscs for cryo-EM. The electron density map of FZD₆ (purple) and the DEP domain of DVL3 (salmon) embedded in the nanodisc is shown. Cryo-EM analysis allowed resolution of the structure at 2.8 Å. An unidentified, non-proteinaceous density in a similar location as PIP₂ in **(a,b)** is highlighted with a red circle. **(d)** Corresponding model of the FZD₆-DEP complex provided in ribbon style. TM - transmembrane spanning helix; H8 – helix 8. Residues 158-263 and 270-511 could be modelled for FZD₆. Residues 1-157, 264-269, 512-706 are missing from the model. From the PDZ-IDR2-DEP construct, the residues 406-495 corresponding to the DEP domain of DVL3 were modelled.

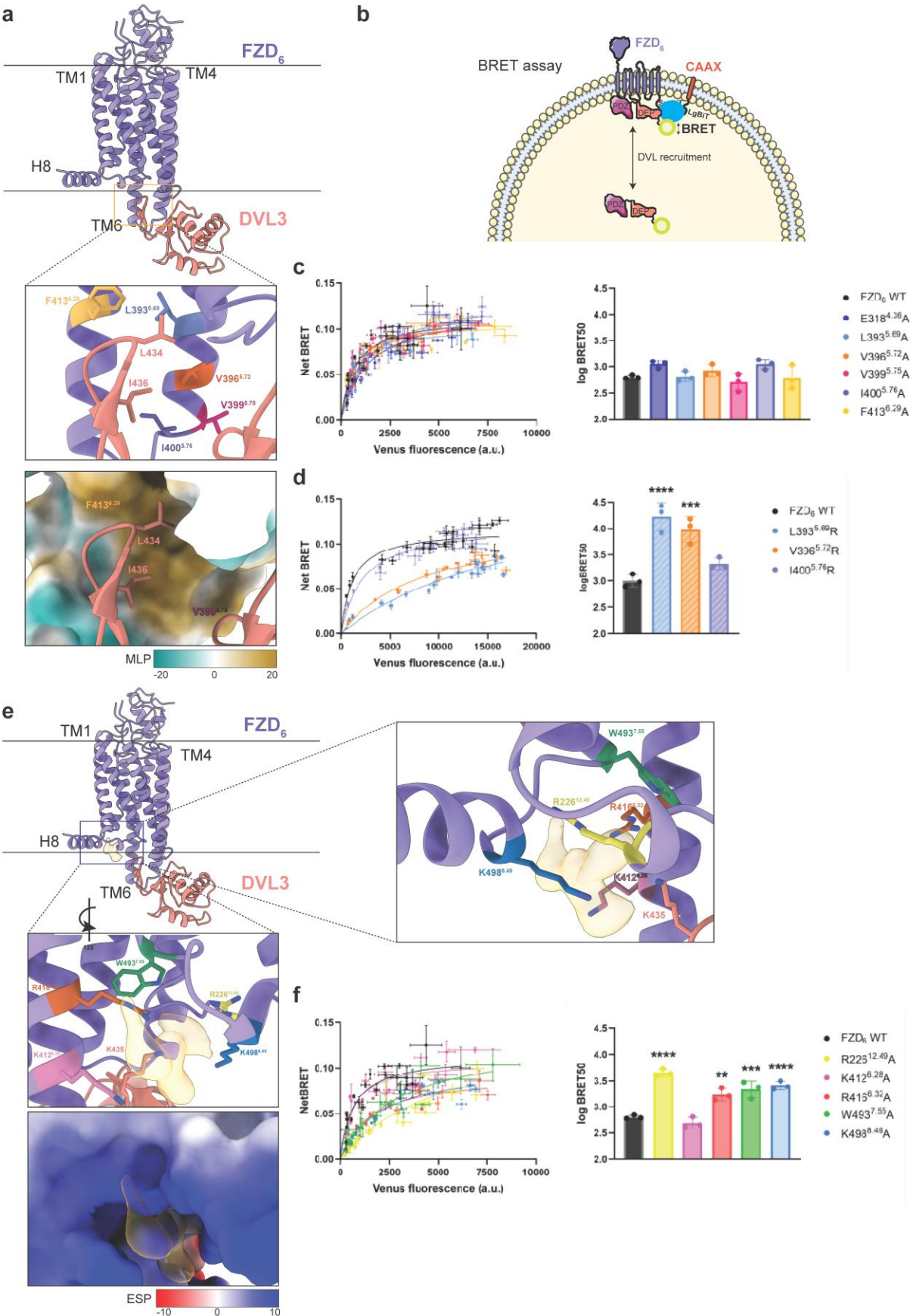


Fig. 2. The FZD₆-DEP interface. (a) The DEP domain interacts with FZD₆ through a distinct lipophilic pocket mainly composed of the intracellularly facing regions of TM5 and TM6. Main interacting residues are L393^{5.69}, V396^{5.72}, V399^{5.75} and I400^{5.76}). The insert depicts the binding domain and the residues in ribbon and stick style, and highlighting surface hydrophobicity in color (yellow – hydrophobic). (b) Schematic for the BRET-based analysis of the recruitment of PDZ-IDR2-DEP to FZD₆. 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. (c,d) Acceptor titration curves of FZD₆ carrying mutations in the lipophilic DEP interface. N=3. Values are mean $\pm$ S.D. *** p=0.0005; **** p<0.0001 (e) The region between the TM6 and TM7/H8 is occupied by an unassigned electron density (gold surface). Residues R226^{12.49}, K412^{6.28}, R416^{6.32}, W493^{7.55} and K498^{8.49} in the vicinity of the density are shown as sticks. Magnified inserts show the protein around the unassigned density in ribbon and stick style, and pseudocolored as electrostatic surface. (f) Acceptor titration curves of FZD₆ carrying mutations in the polar cavity accommodating the unassigned electron density. N=3. Values are mean $\pm$ S.D. ** p=0.0013; *** p=0.0002; **** p<0.0001 (c,d,f) LogBRET₅₀ values, providing a measure of protein-protein affinity, were extracted from the acceptor titration curves. Bars represent means $\pm$ S.D. , One way ANOVA with Dunett's post-hoc test.

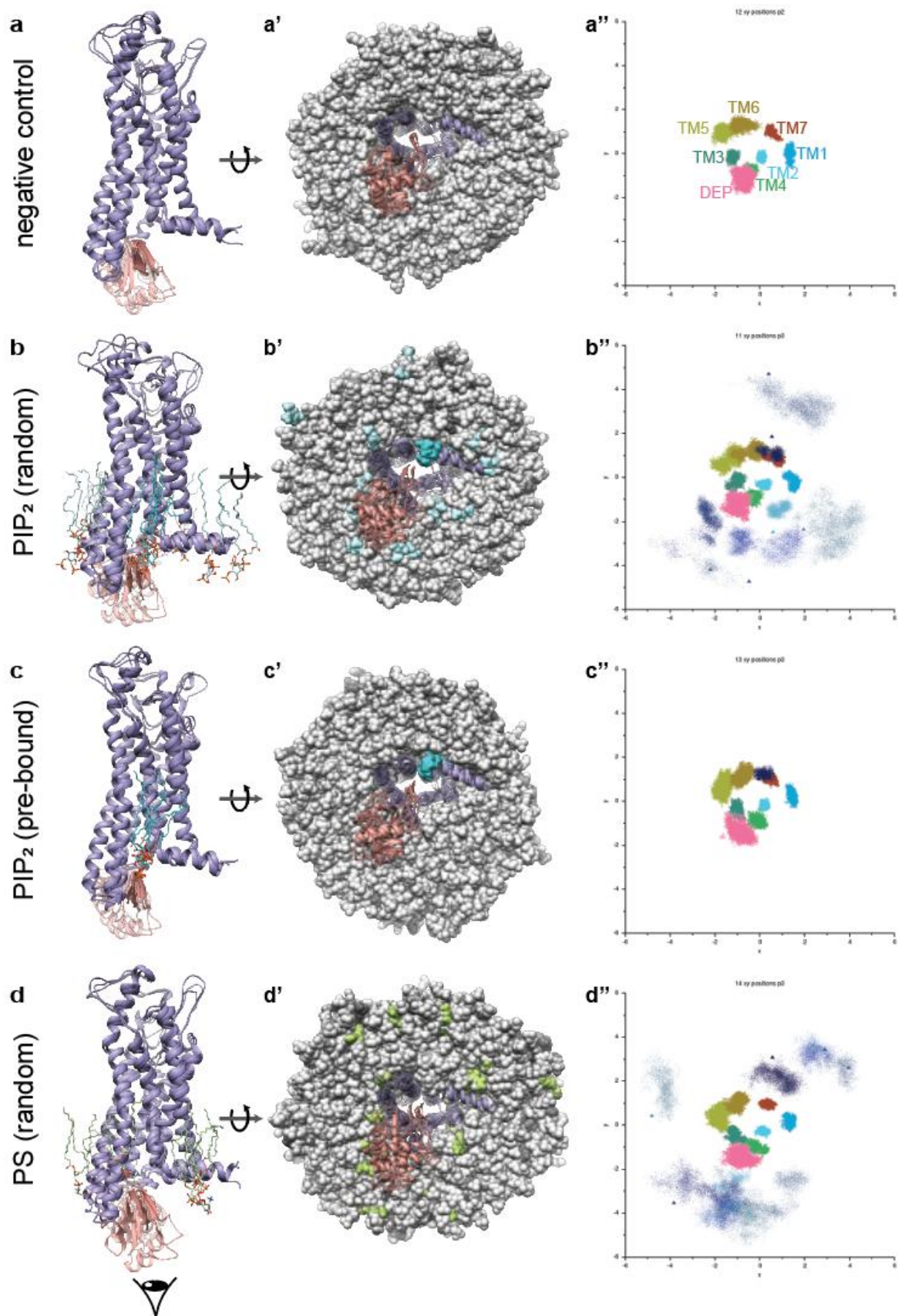


Figure 3

Fig. 3. MD simulations reveal PIP₂ interaction with the polar cavity and the α 3 helix of DEP. Overlay of the last frames of three independent replicas of 500 ns MD simulations of the 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**). (**a'**, **b'**, **c'**, **d'**) View of the same snapshots from the intracellular side of the FZD₆-DEP complex. The membrane lipids are shown in sphere representation (POPC: light grey; Chol: dark grey; PIP₂: cyan; POPS: light green). (**a''**, **b''**, **c''**, **d''**) 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, 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. One representative replicate is shown, for additional replicates see **Supplementary Fig. 13**. The regions TM1, TM2, TM3, TM4, TM6, TM7 and DEP are indicated in **c**. The same color code applies in the following graphs.

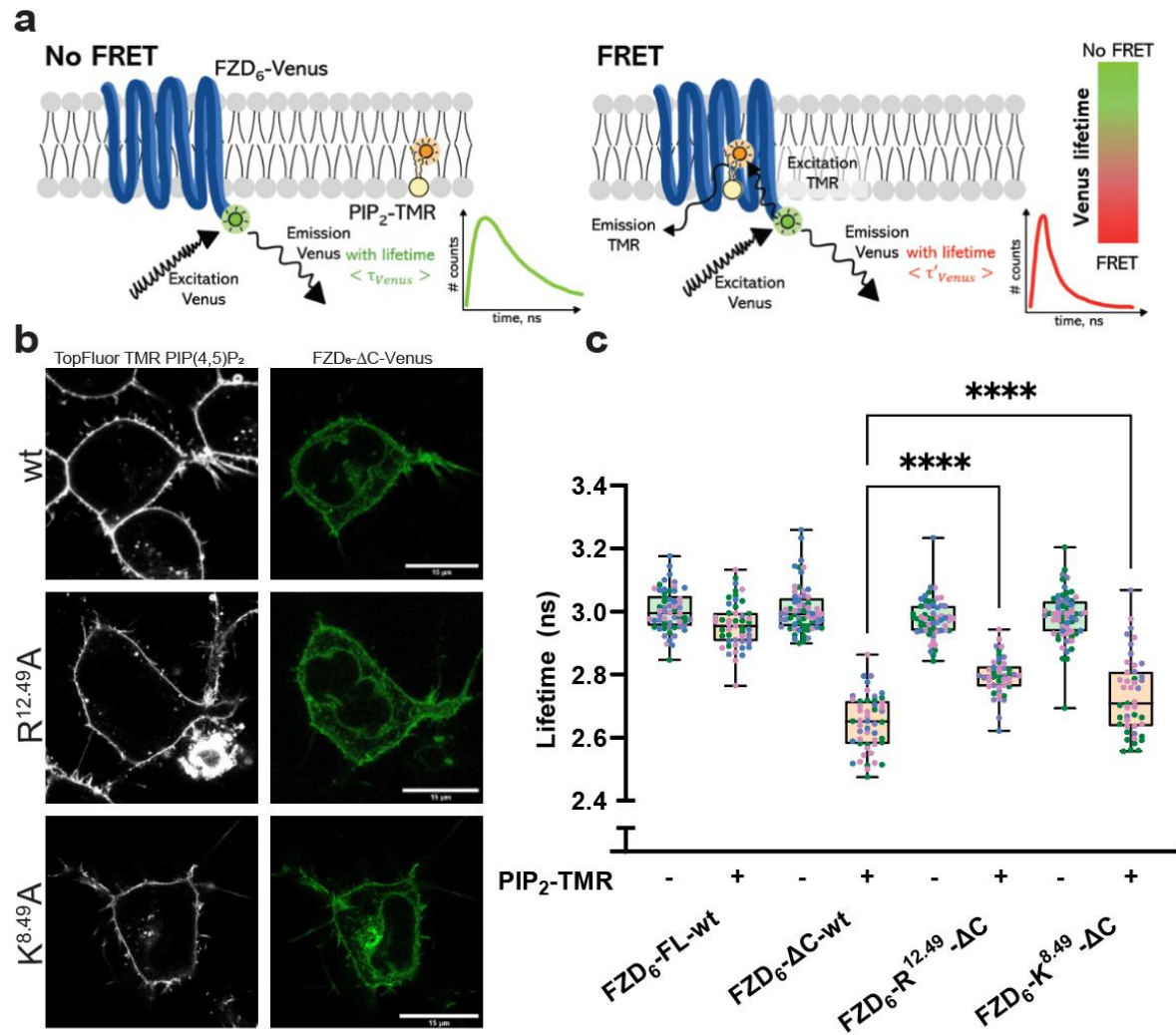


Fig. 4. FLIM-FRET analysis supports PIP₂ interaction with FZD₆. (a) Schematic depiction of the experimental FLIM-FRET live cell imaging paradigm. (b) Subcellular localization of FZD₆ ΔC-Venus (green) and fluorescently labelled PIP₂ (white). (c) FLIM FRET analysis reporting on FZD₆-FL-Venus and FZD₆ ΔC-Venus fluorescent lifetime changes in the presence and absence of TMR-labelled PIP₂ (0.5 ug/mL (0.4 μM); PIP₂-TMR) in wild type and mutant (R^{12.49}A; K^{8.49}A) FZD₆. Data are based on 3-4 independent experiments (replicate 1 in purple, replicate 2 in green, replicate 3 in pink, and replicate 4 in blue). Box and whisker plots represent points representing individual cells with quartile ranges shown by box. ****p<0.0001, one-way ANOVA with Sidak's test.

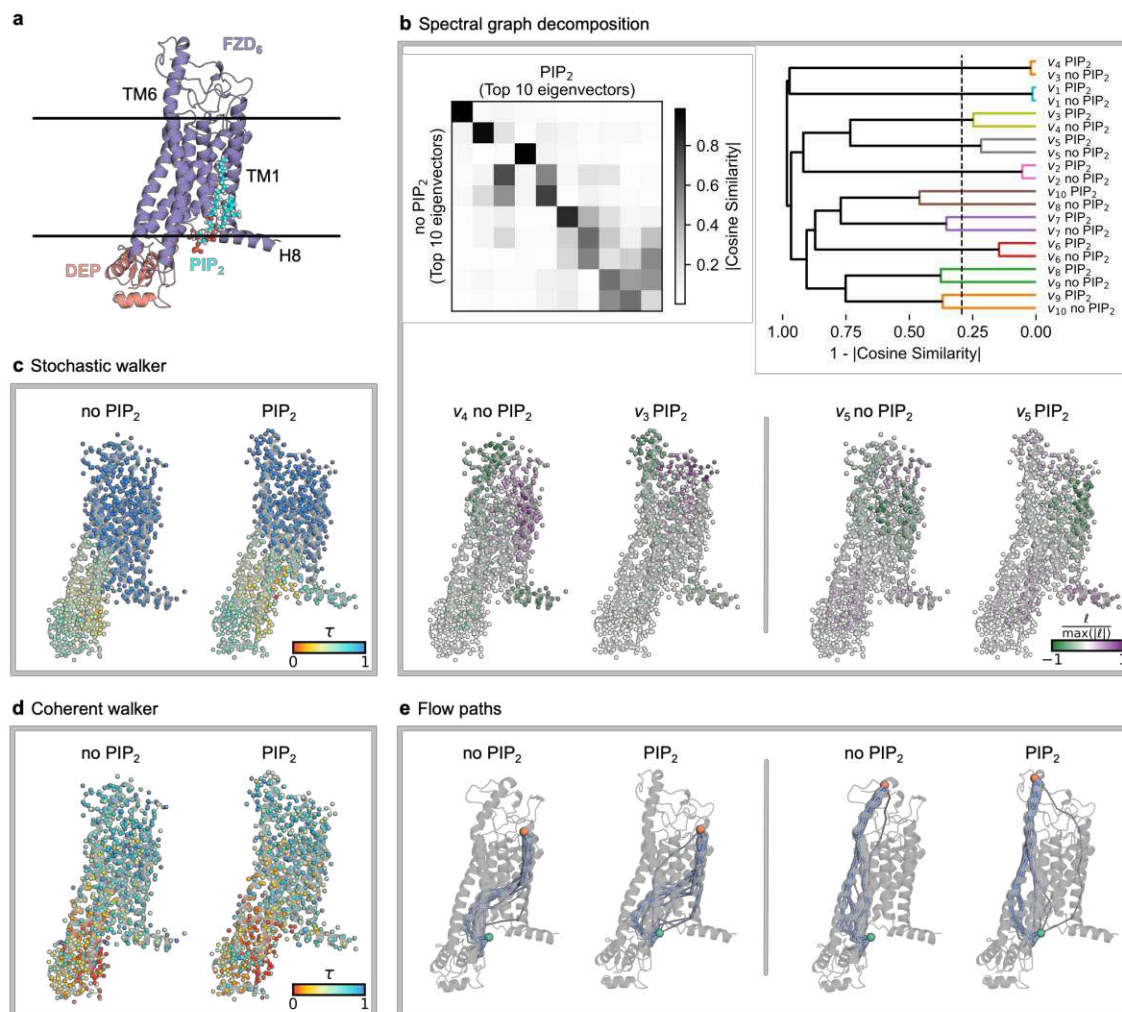


Fig. 5. Network analyses highlight the allosteric connectivity of the PIP₂ interface. (a) Structural overview of the FZD₆-DEP-PIP₂ complex. The orientation matches those in all other subfigures. (b) Spectral decomposition of the degree-normalized symmetric graph Laplacians of the FZD₆-DEP networks in the presence and absence of PIP₂. The absolute cosine similarity (top left) was used to hierarchically pair equivalent eigenvectors (top right). Comparing the loadings (ℓ) of select eigenvectors (bottom) demonstrates differences in the dynamics of the FZD₆-DEP complex depending on the presence of PIP₂. The loadings were independently normalized by the largest magnitude loading for each eigenvector. The normalized pseudotime to maximum probability (τ) for each node was computed for (c) stochastic and (d) coherent walkers starting from K435_{sc} to visualize how quickly a signal propagates from this node through the networks. (e) Minimum-cost, maximum flow paths from K435_{sc} to E194^{1.28}_{bb} (left) and C454^{6.70}_{bb} (right). The presence of PIP₂ profoundly alters how communication between these residues may travel through the receptor. These sink nodes were chosen based on the regions whose loadings differed in b.

Methods

Cloning, baculovirus production and recombinant protein expression in Sf9 cells

Human FZD₆ (amino acids 19-706) lacking the native signal peptide was cloned into the pFastBac1 vector flanked N-terminally by a hemagglutinin signal peptide and FLAG tag and C-terminally by a 3C cleavage site followed by a Twin-Strep-tag®. This expression vector was transformed in MAX Efficiency™ DH10Bac Competent Cells (Gibco) according to the manufacturer's protocol, diluted 10-fold with SOC media and recovered for 4 hours (37 °C, 220 rpm) after which 50-100 µl was spread on LB agar plates containing Bluogal (100 µg/ml), IPTG (40 µg/ml), kanamycin (50 µg/ml), gentamycin (7 µg/ml) and tetracycline (10 µg/ml). After 24-48 hours, white colonies were picked, replated and grown for 24 hours before picking 2 single, white colonies for 3 ml overnight cultures grown in LB media supplemented with kanamycin, gentamycin and tetracycline at the concentrations stated above (37 °C, 220 rpm). Cells were pelleted by centrifuging at 3,400 x g for 10 min and either used directly or frozen and stored at -20 °C until bacmid extraction. For bacmid extraction, cells were resuspended in 250 µl resuspension buffer (P1, Qiagen), lysed in 250 µl lysis buffer (P2, Qiagen) and neutralized with 250 µl neutralization buffer (P3, Qiagen). This mixture was centrifuged at 21 000 x g for 5 min at 4 °C. The supernatant was aspirated and transferred to another tube with 800 µl isopropanol and mixed before centrifuging at 21,000 x g for 5 min at 4 °C. The supernatant was decanted, and the pellet was washed twice with 700 µl 70% ethanol, and centrifuged at 21 000 x g for 5 min. Finally, the purified bacmid was air dried and dissolved in 30 µl of Opti-MEM™ (Gibco).

Initial virus generation (P0) was performed by transfection of Sf9 TriEx cells (Novagen). Three mL of Sf9 cells from a suspension culture were seeded on a 6-well plate (Corning) at a concentration of 0.25×10^6 cells/ml and incubated at 27 °C (0% humidity, 0% CO₂) for 30 minutes to make adherent. For the transfection mixture, 7.5 µl of X-tremegene HP DNA (Roche) transfection reagent was mixed with 125 µl of Opti-MEM, added to the 30 µl of bacmid DNA, and incubated for 20 min at room temperature. Subsequently, the transfection mixture was added dropwise to the Sf9 cells and the cells were incubated for 60-72 hours at 27 °C, with the 6-well plate sealed with parafilm to avoid media evaporation. Afterwards, the P0 virus was harvested by carefully aspirating and transferring the supernatant to a sterile 15 ml falcon tube. This P0 virus was amplified to generate P1 virus by transfecting 25 to 50 ml of Sf9 cells at 1.5×10^6 cells/ml with 250 to 500 µl of P0 virus. Cell doubling and viability was monitored daily and the cells were diluted to 1.5×10^6 cells/ml, until the cells stopped doubling, at which point they were incubated for another 24 hours before harvesting the P1 virus by spinning down the cell cultures at 3,400 x g for 10 minutes and transferring the supernatant to a sterile 50 ml falcon tube. For large scale expression, 1 L of cells were prepared per 2.8 L flask (Nalgene) at a concentration of 3.5 to 4×10^6 cells/ml and transfected with 50 ml of P1 virus. Cells were grown for 48 hours and pelleted by centrifugation at 3,000 x g for 10 minutes, resuspended in a smaller volume of the supernatant (25 ml per liter of pelleted cells), and spun down at 3,400 x g for 30 minutes at 4 °C. The supernatant was discarded; dry cell pellets (15-20 g per L of culture) were flash frozen and stored at -80 °C.

FZD₆ purification in detergent micelles

Sf9 cell pellets expressing FZD₆ were thawed and resuspended in lysis buffer (20 mM TRIS pH 7.5, 1 mM EDTA) at a 6:1 v/w ratio, supplemented with protease inhibitors (PMSF 1 mg/ml,

Benzamidine HCl 1 mg/ml and leupeptin 0.05 mg/ml) and iodoacetamide (2 mg/ml), and homogenized by stirring on a magnetic stirrer for 20 min at 4 °C. The solution was spun down at 38,400 x g for 10 min at 4 °C, after which the supernatant was discarded and the pellet was resuspended in solubilization buffer (50 mM TRIS pH 7.5, 200 mM NaCl) at a 3:1 v/w ratio supplemented with the same concentrations of protease inhibitors and iodoacetamide, dounce homogenized with 20 strokes with douncer pestle A (Kimble). Detergent stock solutions were added at a final detergent concentration of 1% v/v n-Dodecyl- β -D-Maltopyranoside (DDM)/0.1% cholesteryl hemisuccinate (CHS) (for FZD₆ DDM/CHS purification) or 1% v/v 6-Cyclohexyl-1-Hexyl- β -D-Maltoside (cymal-6) (for FZD₆ cymal-6 purification). Samples were solubilized for 90 min at 4 °C while stirring (on a magnetic stirrer, for volumes >50 ml) or rotating (on a carousel, for volumes <50 ml). Solubilisate was spun down for 30 min at 104,000 x g, 4 °C, and the supernatant was transferred to 50 ml falcon tubes. Clarified solubilisate was batch incubated with M2 FLAG resin (Sigma) for 2 hours at 4 °C while rotating with 1 ml of FLAG resin per 50 ml solubilisate. After batch incubation, it was spun down at 3,400 x g for 15 min and the supernatant was decanted and the M2 FLAG resin was transferred to Econo-Columns® and washed with 10 resin volumes of solubilization buffer (50 mM TRIS pH 8.0, 200 mM NaCl, with protease inhibitors and detergent (0.02% DDM/0.002% CHS or 0.002% LMNG/0.0002% CHS or 0.04% cymal-6), followed by 30 resin volumes of wash buffer (20 mM TRIS pH 7.5, 100 mM NaCl, with protease inhibitors and detergent as for the previous wash). The protein was eluted with 5 resin volumes of wash buffer supplemented with 0.4 mg/ml FLAG peptide and concentrated in a 50 kDa MWCO concentrator (Amicon) to a volume <500 μ l and injected on a Superdex 200 increase 10/300 column (Cytiva) equilibrated in 20 mM TRIS pH 7.5, 100 mM NaCl, with 0.02% DDM/0.002% CHS (for DDM/CHS purification) or with 0.04% cymal-6 (for cymal-6 purification). Peak fractions of FZD₆ were collected and used immediately to form complexes.

FZD₆-DVL3 pulldown experiments

Purified DEP (amino acids 389-496 and 398-496), PDZ-IDR2-DEP and phosphorylated pPDZ-IDR2-DEP (12.5 μ M) were each separately co-incubated with purified FZD₆ (6 μ M) in buffer (50 mM TRIS pH 7.5, 100 mM NaCl, 0.02% DDM, 0.002% CHS) and kept on ice for one hour (total volume 300 μ l). Subsequently, the samples were incubated with 50 μ l of M2 FLAG resin inside a 1.5 mL tube and incubated for one hour while gently rotating in the cold room. After incubating, the tube was centrifuged (5,000 x g, 4 °C), and the supernatant was discarded. The resin was washed with 10 resin volumes of buffer, before eluting in batch in 5 resin volumes of buffer supplemented with 0.4 mg/ml FLAG peptide. Elutions were pooled and loaded on Mini-PROTEAN TGX stain-free precast gels (BioRad) for SDS-PAGE, and imaged stain-free as well as with Coomassie blue staining (InstantBlue®) on a ChemiDoc™ MP imaging system (Bio-Rad).

FZD₆-DVL3 SEC complexation experiments

Purified DEP (amino acids 398-496) and phosphorylated PDZ-IDR2-DEP at a final concentration of 6.25 μ M were each co-incubated with an equimolar amount of purified FZD₆ in a final reaction volume of 120 μ l (in 10 mM TRIS pH 7.5, 100 mM NaCl, 0.02% DDM and 0.002% CHS). The mixture was incubated for one hour on ice, before being injected on a pre-equilibrated Superdex 200 Increase 10/300 column, equilibrated with the same buffer. Peak fractions were collected and loaded on Mini-PROTEAN TGX stain-free precast gels for SDS-PAGE, and imaged stainfree as well as with Coomassie blue staining (InstantBlue®) on a ChemiDoc™ MP imaging system (Bio-Rad).

FZD₆ purification, FZD₆-DVL3 reconstitution in saposin nanodiscs and complex formation

FZD₆ was purified in cymal-6 in the same way as described above. Before size-exclusion chromatography, the purified receptor was exchanged into saposin nanodiscs based on the published protocol³⁶. Briefly, the FLAG purified receptor was incubated for 5 min at 37 °C. Subsequently, a 1.2-fold molar excess of diC8 PIP₂ (Avanti Polar Lipids 850185, dissolved as 100 µM stock in milliQ water or buffer) and 0.1121 µg brain extract from bovine brain (Sigma B1502, dissolved in a 1% DDM solution) per picomole of receptor was added and incubated for 5 minutes at 37 °C. Afterwards, 0.1121 µg saposin was added per picomole of receptor, and incubated for another 5 minutes at 37 °C. Finally, the FZD₆-pPDZ-IDR2-DEP complex was formed by adding 1.2-fold molar excess of purified pPDZ-IDR2-DEP, incubated for 60 min at room temperature and injected on a Superose 6 increase 10/300 or 3.2/300 column (Cytiva) equilibrated with buffer (20 mM TRIS pH 7.5, 100 mM NaCl). Peak fractions were pooled and concentrated in a 100 kDa MWCO concentrator to ±2-6 mg/ml for cryo-EM sample preparation.

Grid preparation

For dataset 1, Quantifoil Au R 1.2/1.3, 300 mesh grids were glow-discharged for 40 s at 20 mA on a GloQube Plus glow discharger. Subsequently, 3 µl of purified FZD₆-pPDZ-IDR2-DEP (±6 mg/ml) supplemented with 0.0001% LMNG/0.00001% CHS shortly before vitrification was applied to the grid and blotted for 3 s (force 0) and plunge frozen in liquid ethane on a ThermoFisher Scientific Vitrobot MKIV maintained at 100% humidity, 4 °C.

For datasets 2-5, UltrAUfoil R 1.2/1.3, 300 mesh grids were glow-discharged for 25 s at 15 mA on a PELCO easiGlow™ glow discharger. Then, 1.2 to 2 µl of purified FZD₆-pPDZ-IDR2-DEP (±2-6 mg/ml) was applied and blotted for 2 s (front blotting) before being plunge frozen in liquid ethane on a Leica EM-GP2 (maintained at >80% humidity, 4 °C).

For dataset 6, UltrAUfoil R 1.2/1.3, 300 mesh grids were glow discharged for 45 seconds on each side on a PDC-32G plasma cleaner with the RF power level set to “hi”. Subsequently, 3 µl of purified FZD₆-pPDZ-IDR2-DEP (±2-6 mg/ml) was applied to the grid and blotted for 10 s (force 19) and plunge frozen in liquid ethane on a ThermoFisher Scientific Vitrobot MKIV maintained at 100% humidity, 4 °C.

Cryo-EM data collection FZD₆-DVL3

Data collection for cryo-EM datasets 1-4 is described in more detail in the Supplementary Methods. Cryo-EM datasets 5-6 (**Supplementary Table 3**) were collected on a CRYO ARM 200 II (JEM-Z200CA) 200 kV microscope using similar procedure as published here⁶⁰. Movies (33,436 for dataset 5 and 32,756 for dataset 6) were acquired using SerialEM for automated data collection and recorded on a Gatan K3 detector in counting mode and CDS mode at a nominal magnification of 80,000 (0.571 Å/px), with an energy slit width of 20 eV and a 150 µm objective lens aperture diameter. The defocus range was set between -1.5 to -1.2 µm and the electron exposure was 60.1 e-/Å² (dataset 5) and 65.0 e-/Å² (dataset 6), fractionated over 80 frames, using an exposure rate of 7.0 e-/px/s, for 3.0s total exposure time

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. Processing for Datasets 1-4 is described in the Supplementary methods.

Datasets 5-6

Curated micrographs (26,841) from dataset 5 were used for particle picking using blob picker and 7,893,792 particles were extracted at 160 pixels (2.5x binned, 1.43 Å/px). 2D classification was performed and 1 372 906 particles were selected and subjected to four rounds of heterogenous refinement with 3 classes (1 good class, 2 junk classes) followed by NU-refinement. This yielded an initial, anisotropic map with 329,191 particles that was used for template and Topaz picking. Combining blob, template and Topaz picked particles, yielded 15,335 890 particles after removing duplicates. This particle stack was used for 5 rounds of heterogenous refinement with 5 classes (1 good class, 4 junk classes), followed by NU-refinement, another seven rounds of heterogenous refinement followed by another NU-refinement. This particle stack was then used for Ab-initio reconstruction with 2 classes, and the good class was subjected to NU-refinement. This was repeated once, before re-extracting 278,995 particles at 400 pixels (0.57 Å/px). This particle set was subjected to reference-based motion correction, followed by NU-refinement. Ab-initio (two classes) was performed, and the best class with 159,813 particles was subjected to NU-refinement, global CTF refinement and another, final NU-refinement to yield a map at 3.0 Å overall resolution (cFAR = 0.08, SCF = 0.82).

Curated micrographs (28 314) from dataset 6 were used for particle picking using blob picker and 10,188,003 particles were extracted at 160 pixels (2.5x binned, 1.43 Å/px). 2D classification was performed and 1,061,638 particles were selected and subjected to four rounds of heterogenous refinement with 3 classes (1 good class, 2 junk classes) followed by NU-refinement. This yielded an initial, anisotropic map from 66,382 particles that was used for template and Topaz picking. Combining blob, template and Topaz picked particles, yielded 18,632,269 particles after removing duplicates. This particle stack was used for four rounds of heterogenous refinement with 5 classes (1 good class, 4 junk classes), followed by NU-refinement, another five rounds of heterogenous refinement followed by another NU-refinement. This particle stack was then used for Ab-initio reconstruction with 2 classes, and the best class was subjected to NU-refinement. Subsequently, heterogenous refinement with 2 classes (based on the previous Ab-initio job) was performed and the best class was subjected to NU-refinement, followed by re-extracting 155,375 particles at 400 pixels (0.57 Å/px). This particle stack was subjected to non-uniform refinement, reference-based motion correction and another non-uniform refinement, yielding a more directionally isotropic (cFAR = 0.26, SCF = 0.83) map at 3.0 Å overall resolution.

The particle stacks from dataset 5 (159,813 particles) and 6 (153,123 particles) were pooled and subjected to reference-based motion correction, followed by NU-refinement. Finally, a local refinement was performed with a mask that excluded the membrane belt, yielding a final map at 2.8 Å overall resolution (cFAR = 0.35, SCF = 0.82).

Protein modeling

The final electron density map was sharpened using Phenix Autosharpen and enhanced using DeepEMhancer⁶¹, and the three maps were used for subsequent model building and refinement using Coot 9.8.92⁶² and ChimeraX 1.5⁶³ with Isolde⁶⁴ For the initial model in the map from datasets 1-4, the AlphaFold 2 predicted structure of FZD₆-DVL3-DEP was used as a template for model building and rigid-body fitted in the density. The initial model in the final map from datasets 5-6 was obtained from ModelAngelo (using the raw map as input) and used to build the final model.

In Coot, the different regions were fitted and refined in real space. After the initial model building in Coot, the model was refined using real-space-refine from Phenix⁶⁵. Suboptimal areas were manually inspected and further refined interactively, correcting rotamer outliers, fixing clashes and Ramachandran outliers. Additionally, the model was further improved in ChimeraX with Isolde, especially focusing on rotamer outliers. This was done iteratively, between Coot and Isolde for interactive improvements and Phenix for refinements. A final model refinement with real-space-refine and validation was performed in Phenix. Model statistics of the final model can be found in **Supplementary Table 3**.

Cell culture and transfection

Human embryonic kidney cells (HEK293A, Thermo Fisher Scientific) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 1% penicillin/streptomycin (Gibco, #151-40122) and 10% fetal bovine serum (FBS, Gibco), in a humidified 5% CO₂ incubator at 37°C. Cell culture plastics are from Sarstedt or VWR and transfections used either Lipofectamine 2000 (Invitrogen) or PEI MAX (Polysciences, Warrington, PA, stock solution 1 mg/mL) at a ratio of 2:1 or 3:1 of PEI (μL): DNA (μg). The absence of mycoplasma contamination was routinely confirmed by a strip test kit (InVivoGen, Toulouse, France).

FZD₆ and DVL3 PDZ-IDR2-DEP BRET acceptor titration assays

The bystander BRET assay was performed in HEK293 cells, where in-cell complementation of FZD₆-SmBiT and membrane anchored CAAX-LgBiT to Nanoluciferase (Nanoluc) establishes the membrane-localized FZD₆-SmBiT as the BRET donor, whereas Venus-PDZ-IDR2-DEP serves as the BRET acceptor. BRET acceptor titration allows to quantify BRET₅₀ values as a relative measure of FZD₆-PDZ-IDR2-DEP affinity. Thus, an increase in BRET₅₀ values monitors a drop in affinity between FZD₆ and PDZ-IDR2-DEP as a result of a FZD mutation. The relative affinity between FZD₆ mutants and DVL3 was assessed by acceptor titration experiments using a fixed concentration of FLAG-FZD₆-smBiT (50 ng cDNA per 350,000 cells) and a truncated, N-terminally monomeric Venus-tagged version of human DVL3 wt and mutants starting from its PDZ domain (Venus-DVL3-PDZ-IDR2-DEP; 10-700 ng cDNA per 350,000 cells). Mutagenesis of FLAG-FZD₆-smBiT and Venus-DVL3-PDZ-IDR2-DEP constructs was performed using the GeneArt site directed mutagenesis system (ThermoFisher) according to the manufacturer's instructions. We co-expressed a plasma membrane-tethered LgBiT (LgBiT-CAAX; 200 ng cDNA) to exclusively monitor FZD-DVL interactions at the plasma membrane. On the first day of the experiment, HEK293A cells were transfected in suspension with cDNA as indicated above using chemical transfection with polyethylenimine (PEI MAX®, Polysciences). A total of 1,000 ng cDNA was used for transfection; the remaining amount was complemented by empty pcDNA3.1(+) vector. A total of 100 μl cells at a density of 350,000-400,000 cells per ml were seeded into a 96-well white bottom plate (Greiner) and incubated for 40-48 h at 37°C and 5% CO₂. On the assay day, the media was aspirated, cells were washed in Hank's balanced salt solution (HBSS), and 90 μl of HBSS were added to the cells. Plates were read on a Spark multimode plate reader (Tecan). Initially, Venus fluorescence was measured three times (excitation 485/20 nm; emission 535/25 nm). Then, 10 μl of furimazine (Promega) were added to each well, and after 6 minutes we measured Nanoluciferase and Venus emission at 445-480 nm or 460-500 nm, and 520-560 nm, respectively, three times each. Net BRET values were obtained by subtracting the BRET ratio (Venus/Nluc emission) of a negative control not expressing any Venus-DVL3-PDZ-IDR2-DEP) from each BRET ratio. Net BRET values were plotted against Venus fluorescence and data

was fit using a one site – specific binding model in GraphPad PRISM 10 to obtain $BRET_{max}$ and $\log BRET_{50}$ values. Receptor surface expression was analyzed by the obtained Nluc reads.

Fluorescence Lifetime Imaging Förster Resonance Energy Transfer (FLIM-FRET) Microscopy

To evaluate the proximity between FZD₆ with PIP₂, 750 ng of FLAG-FZD₆-ΔC-Venus constructs and 250 ng of empty pcDNA3.1(+) vector were transfected into HEK293A cells with Lipofectamine 2000 and seeded at 60,000 cells/well in μ-slides (8-well glass bottom dishes, ibidi). The following day, cells were washed once with PBS and prepared for imaging. 1-oleoyl-2-(6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl)amino)hexanoyl)-sn-glycero-3-phosphoinositol-4,5-bisphosphate (ammonium salt) (TopFluor TMR-PI(4,5)P₂; Avanti Research A81384) was prepared in DMSO at a working concentration of 1 mg/ml and diluted in PBS to a final concentration of 0.5 μg/ml. Control cells were imaged in PBS, whereas cells labelled with TopFluor TMR-PI(4,5)P₂ were incubated with the probe for 30–60 min at room temperature prior to imaging. Fluorescence lifetime measurements were performed on a Leica SP8 3X STED with a FALCON FLIM/FCS utilizing a STED white HC PL APO CS2 100x/1.40 oil objective. Excitation output power was set to 70% of a pulsed white-light laser (pulse duration: ~ 100–150 ps). Excitation occurred at 488 nm (at 3% laser power), and emission was collected using a SMD HyD detector set to photon counting mode (10% internal gain) within a 495–535 nm window at scan speed of 400 Hz. Power settings for FLIM ensured a maximum count rate of 0.5 photons per laser pulse, and all measurements were taken at 20 MHz repetition rate. Besides lifetime images, fluorescence intensity images were acquired with the same excitation and emission collection as above for the various FZD₆-Venus constructs. PIP₂-TMR was excited at 561 nm (at 2 % laser power) and emission was collected within a 600–650 nm window. Lifetime and intensity images were acquired using a line average of four repetitions.

Fluorescence Lifetime Analysis

Lifetime analysis was performed using the Leica Application Suite LAS X FLIM/FCS software (version 4.5.0). For each imaged cell, regions of interest (ROIs) were defined where FZD₆-Venus exhibited clear membrane localization (**Supplementary Fig. 17**). Fluorescent aggregates and intracellular FZD₆-Venus signal were excluded from the analysis. To determine the average fluorescence lifetime of each ROI, fluorescence decay curves were fitted using mono-exponential reconvolution with the instrument response function (IRF) calculated by FALCON FLIM software. Fitting was performed over a time range of 0.2–44 ns.

Whole-cell ELISA

To quantify cell-surface expression of FLAG-tagged FZD₆ constructs used for FLIM-FRET experiments, HEK293A cells were transfected with 750 ng of the different constructs and 250 ng of pcDNA3.1(+) vector with Lipofectamine 2000. Cells were plated (30,000 cells/well) in transparent 96-well plates precoated with poly-D-lysine (PDL, Sigma Aldrich). After 48 h, media was aspirated and cells were incubated an anti-FLAG-M2 mouse antibody (Sigma Aldrich, #F1804) at 1:1,000 in 1% BSA/DPBS supplemented with CaCl₂ and MgCl₂ with for 1 h at 4°C. After, cells were washed five times with 0.5% BSA/DPBS supplemented with CaCl₂ and MgCl₂

and then incubated with horseradish-peroxidase conjugated goat anti-mouse antibody (1:4,000) prepared in 1% BSA/DPBS supplemented with CaCl_2 and MgCl_2 . Cells were washed again five times and peroxidase substrate 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, T8665) was added and incubated for 30 min at room temperature in the dark. Acidification was performed using equal volumes of 2M HCl and the absorbance was read at 450 nm using a Spark multimode plate reader (Tecan).

Protein-protein docking

The protein structure of DVL2-DEP (S418-G510) and FZD₅ (G180-C539) in an active conformation was modelled using MODELLER10.1 (**Supplementary Fig. 1**)⁶⁶. The following templates were used for modelling: human DEP2: PDB IDs 1FSH (410-499), 3ML6 (423-510), 5LNP (455-508) and 5SUY/5SUZ (455-509); FZD₅: 7EVW (FZD₇), 6XBM (SMO), 6BD4 (FZD₄; 475-513), 6WW2 (FZD₅; 500-536) as well as AlphaFold predictions (linker domain:180-226; ECL3: 479-501). To obtain the FZD₅-DEP complex, protein-protein docking calculations using the HADDOCK webserver (v2.4) were conducted^{34,35}. To guide the docking, information on reported mutations that affect DEP recruitment was included as Ambiguous Interaction Restraints as active residues (directly involved in interaction; DEP: R442, W444, L445, I447; FZD₅: T253^{1,50}, T256^{1,53}, R263^{12,49}, R265^{12,51}, Y266^{2,39}, L443^{ICL3}, L446^{6,29}, R449^{6,32}, W520^{7,55}, K525^{8,49}, T526^{8,50}) and passive residues (solvent accessible surface neighbors of active residues; DEP: E438, V439, R440, D441, M443, K446, T448, I449, Y502; FZD₅: I259^{1,56}, D260^{1,57}, M261^{12,47}, E262^{12,48}, P267^{2,40}, E268^{2,41}, R269^{2,42}, N350^{4,35}, A352^{4,37}, A354^{4,39}, I429^{5,72}, I433^{5,76}, D441^{ICL3}, K442^{ICL3}, E444^{6,27}, K445^{6,28}, M447^{6,30}, I450^{6,33}, S517^{7,52}, I521^{7,56}, S523^{8,47}, E528^{8,52}, S529^{8,53})^{4,12}. Of these restraints, 50% were randomly excluded during the docking runs. The finger-loop of DEP (440-452) and FZD₅-ICL1 (260-266) were kept flexible during docking. The obtained complex models were then inspected visually to exclude models with no or few FZD-DEP interactions or with unphysical orientations (e.g. polar and apolar regions oriented towards each other). Selected complex models were embedded in a lipid bilayer like the one described in **Supplementary Table 1** and subjected to a brief MD simulation of 200 ns. The complex model that appeared the most stable during this brief MD simulation was finally selected and prepared for MD simulations as described below.

Structure preparation for MD simulations

The FZD₅-DEP complex model was used as obtained from the protein-protein docking calculations.

For the MD simulations of the FZD₄-DEP complex, the published cryo-EM structure with PDB ID 8WMA was prepared²⁷. Missing side chains as well as single point mutations in the sequence were fixed using UCSF Chimera and the Dunbrack rotamer library^{67,68}. Missing residues in the N-terminus (including one half of the conserved disulfide bond) and in ECL1 were added/replaced with the corresponding regions of FZD₄ structure 6BD4 (N-terminus: C181-C200; ECL1: C282-E289)⁶⁹.

For the FZD₆-DEP MD simulations, the missing residues in ECL1 were added/replaced by inserting the corresponding region from the AlphaFold predicted FZD₆ model (K262-L269), while the rest of the cryo-EM structure was used as is. For the MD simulations with the pre-bound PIP₂, the complex of FZD₆-DEP and PIP₂ bound to the polar cavity of FZD₆ was extracted from the last

frame of one of the replicas of the MD simulations with randomly distributed PIP₂ in the inner leaflet and used without further modifications.

MD system setup

The MD simulation systems and input were generated using the CHARMM-GUI bilayer builder⁷⁰⁻⁷². To orient the models in the membrane, the receptor was aligned to the entry for the FZD₅ structure with PDB ID 6WW2 in the Orientations of Proteins in Membranes database using UCSF Chimera (v.1.16)^{73,74}. Histidine protonation was assigned manually based on local environment. All disulfide bonds were assigned as predefined (FZD₅: C182-C486, C190-C484, C192-C218, C222-C298 and C315-C390; FZD₆: C161-C181, C185-C260, C282-C357 and C454-C461; FZD₄: C181-C200, C204-C282 and C302-C377; no disulfide bonds in DEP). Protein termini were patched by acetylation and methylamidation. The protein complex was placed in a hexagonal box with the receptor embedded in a bilayer of the composition as indicated in **Supplementary Table 1**. The system was neutralised by the addition of Na⁺ and Cl⁻ up to a concentration of 150 mM and solvated using TIP3P water. For each replica, a separate MD system with randomly placed lipids in the bilayer was set up.

MD simulations

All MD simulations were run using GROMACS2021 and the CHARMM36 and CHARMM36m force field⁷⁵⁻⁷⁷. After an initial energy minimization with positional restraints using steepest descent until the maximum force was below 1000 kJmol⁻¹ nm⁻¹, the MD systems were equilibrated for 250 ps in an NVT ensemble and for 1750 ps in an NPT ensemble while stepwise loosening positional restraints on atoms in the system. The initial velocities were assigned randomly based on the Maxwell distribution at 310 K. The last step of the equilibration placed only light positional restraints (50 kJ mol) on protein backbone and was run for 25 ns. After equilibration, production runs of 500 ns (timestep: 2 fs) were performed using the velocities from the last equilibration step. Equilibration and production were run in independent replicas as indicated in **Supplementary Table 1**.

The temperature of 310 K and the pressure of 1 bar were maintained using the v-rescale and c-rescale algorithms, respectively, during both equilibration and production^{78,79}. Bonds to hydrogen atoms were constrained using the LINCS algorithm⁸⁰. Long-range non-bonded interactions were cut off using a force-switch between 10 and 12 Å and electrostatic interactions were calculated using the Particle-Mesh Ewald algorithm with a cutoff of 12 Å^{81,78,79}. Bonds to hydrogen atoms were constrained using the LINCS algorithm⁸⁰. Long-range non-bonded interactions were cut off using a force-switch between 10 and 12 Å and electrostatic interactions were calculated using the Particle-Mesh Ewald algorithm with a cutoff of 12 Å⁸¹.

MD analysis

The production trajectories were centered and aligned using GROMACS2021 and AmberTools 24 cpptraj⁸². The entire 500 ns of each trajectory were included for the analyses. Analyses were conducted using cpptraj. Interactions were computed using getcontacts (<https://getcontacts.github.io/>). Pylipid was run as an unbiased control analysis of lipid binding sites⁸³. All MD trajectories are deposited on GPCRmd (<https://gpcrmd.org>)⁸⁴. Plots were created with gnuplot (<http://gnuplot.info/>).

Network analyses of the MD trajectories

Undirected, weighted networks of FZD₆-DEP-PIP₂ and FZD₆-DEP were derived from the MD trajectories. Nodes were defined as the center of mass of each backbone (bb) and sidechain (sc). Edges were defined by as the estimated mutual information (MI) between pairs of nodes within 10 Å of each other and filtered by magnitude to only include those within the top 75% of the distance-adjusted MI distribution (Supplementary methods). MI was estimated using a k-nearest-neighbor approach⁸⁵. The differences in topological connectivity in the presence and absence of PIP₂ were analyzed by comparing the top eigenvectors obtained from the spectral decomposition of the symmetric degree-normalized graph Laplacian. To similarly compare the propagation of a signal through the receptor, the evolution of the probability distributions of a continuous-time stochastic, random walker and a coherent, quantum walker starting from K435_{sc} were modeled. Based on the results of the spectral graph analysis, the maximum-flow, minimum-cost paths from K435_{sc} to E194^{1,28}_{bb} and C454^{6,70}_{bb} were also calculated.

Data visualization

Data were visualized using Graphpad Prism 10 (for B105RET data visualization), Jalview (for sequence alignment), and UCSF Chimera v1.16 and UCSF ChimeraX v1.5 (for structure visualization). The visualizations of all network analyses were prepared with Matplotlib 3.8.0 (DOI: <https://doi.org/10.1109/MCSE.2007.55>) and open-source PyMOL 3.1.0.

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Author contributions:

Each author's contribution(s) to the paper is listed in alphabetical order.

Conceptualization: AA, GS, MMS, RS, TR, YZT

Methodology: AA, ES, FR, GS, MMS, NJ, RS, TR, YZT

Software: AA, MMS, TR

Investigation: AHP, FP, FR, GM, GS, JHV, JK, JKu, JYH, KT, MMS, NJ, NS, PK, RD, RS, SMT, TR, VB, YZT

Visualization: AA, ES, FR, GM, GS, JK, MMS, NJ, RS, TR, YZT

Funding acquisition: AA, GS, KT, MMS, RD, RS, VB, YZT

Project administration: GS

Supervision: AA, ES, GS, HGL, JK, KT, RD, RSo, SG, VB, YZT

Resources: AA, GS, HGL, KT, MMS, RD, RSo, SG, VB, YZT

Writing – original draft: AA, ES, GS, MMS, NJ, RS, TR, YZT

Writing – review & editing: GS, JK, MMS, RSo, RS, TR, VB, YZT

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Materials & Correspondence. Materials requests can be addressed to the corresponding authors Gunnar Schulte (gunnar.schulte@ki.se) or Yong Zi Tan (yztan@nus.edu.sg).

Data, code, and materials availability: All data are available in the main text or the supplementary materials. Materials, such as expression vectors, are available upon request. All code associated with the network analyses are available from the Zenodo repository (available to the reviewer from the editor; persistent DOI will be provided upon acceptance).

MD simulations are made available in the GPCRmd database via publication link <https://gpcrmd.org/dynadb/publications/2537/> or the individual Dynamics IDs as indicated in Supplementary Table 4. Structures are deposited at the RCSB PDB (PDB ID 43ZR, Extended PDB ID pdb_000043ZR) and EMDB (EMD-82424).

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