Agonist-induced dimer dissociation as a macromolecular step in G protein-coupled receptor signaling

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G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors. They can exist and act as dimers, but the requirement of dimers for agonist-induced signal initiation and structural dynamics remains largely unknown. Frizzled 6 (FZD6) is a member of Class F GPCRs, which bind WNT proteins to initiate signaling. Here, we show that FZD6 dimerizes and that the dimer interface of FZD6 is formed by the transmembrane α-helices four and five. Most importantly, we present the agonist-induced dissociation/re-association of a GPCR dimer through the use of live cell imaging techniques. Further analysis of a dimerization-impaired FZD6 mutant indicates that dimer dissociation is an integral part of FZD6 signaling to extracellular signal-regulated kinases1/2. The discovery of agonist-dependent dynamics of dimers as an intrinsic process of receptor activation extends our understanding of Class F and other dimerizing GPCRs, offering novel targets for dimer-interfering small molecules.
The superfamily of G protein-coupled receptors (GPCRs; a list of abbreviations used in this work is presented in Supplementary Note 1) mediates the effects of a plethora of endogenous and exogenous substances such as small molecules, peptides, proteins, lipids, ions, and odorants, and offers efficient targets for drug treatment\textsuperscript{1–3}. According to sequence homology, GPCRs were grouped into Classes A, B, C, F (Frizzleds), adhesion receptors, and other 7 transmembrane (TM) spanning receptors\textsuperscript{4}. In addition to the well-understood signaling unit of a monomeric GPCR\textsuperscript{5}, it has been demonstrated that GPCRs can exist as homomeric and heteromeric dimers across the different classes of the GPCR superfamily\textsuperscript{5, 6}. Even though the existence of GPCR homomeric and heterodimers is generally accepted, understanding of their role in receptor function and signal initiation is very limited\textsuperscript{6, 7}.

Class F receptors are classified as members of the GPCR superfamily based on structural and functional resemblance to class A, B, and C family receptors\textsuperscript{8}. Frizzleds (FZDs) regulate a number of processes during embryonic development, stem cell regulation, and adult tissue homeostasis. Deregression or misexpression of FZDs leads to pathogenesis, including, but not limited to, cancer and neurologic disorders; thus, making them attractive drug targets\textsuperscript{8, 9}. In mammals, there are 10 Frizzleds (FZD\textsubscript{1–10}), which are activated by the WNT family of lipoglycoproteins through interaction with the N-terminal cysteine-rich domain (CRD) of FZD\textsuperscript{10, 11}. Little is known about receptor complex constitution with regard to the stoichiometry of WNT to FZD and FZD to intracellular mediators, such as the phosphoprotein Disheveled (DVL) and heterotrimeric G proteins\textsuperscript{12, 13}. Nevertheless, it has been shown that FZD\textsubscript{1,2,3,4} dimerize and that dimerization has implications for signaling\textsuperscript{14, 15}.

Based on live cell imaging experiments, we provide evidence that FZD\textsubscript{6} dimerizes and that the dimer undergoes WNT-5A-induced dissociation and re-association. Employing mutational analysis and biochemical approaches, we map the FZD\textsubscript{6}–FZD\textsubscript{6} dimer interface to TM4 and TM5—findings that are supported by atomic resolution receptor models. In addition, expression of peptides interfering with FZD\textsubscript{6} dimerization in mouse lung epithelial (MLE-12) cells endogenously expressing FZD\textsubscript{6} reduces basal extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in like manner to cells lacking a functional copy of FZD\textsubscript{6}. This, in combination with data pointing to the enhanced signaling capacity of the FZD\textsubscript{6} dimer mutant in the absence of ligand, argues that FZD\textsubscript{6} dimers are crucial in the establishment of functional inactive-state complexes and that monomeric FZD\textsubscript{6} promotes signaling to ERK1/2. Finally, our results suggest that the FZD\textsubscript{6} monomer, and not the dimer, is the minimal signaling unit supporting the idea that receptor dissociation precedes signal initiation.

Results
FZD\textsubscript{6} forms homodimers. Previously, we reported that FZD\textsubscript{6} acts in complex with the phosphoprotein DVL and heterotrimeric G\textsubscript{\alpha}i1 or G\textsubscript{\alpha}q proteins\textsuperscript{16}. Interestingly, interaction of the heterotrimeric G proteins G\textsubscript{\alpha}i1 and G\textsubscript{\alpha}q with FZD\textsubscript{6} relied on balanced levels of DVL, which interacts with the conserved C terminal KTxxxW sequence and the third intracellular loop of FZD\textsubscript{6}\textsuperscript{8, 17, 18}. The G protein-receptor interface of many GPCRs largely coincides with the interface that mediates FZD–DVL interaction. Since simultaneous interaction of DVL and G proteins with FZD\textsubscript{6} appears competitive, we hypothesize that FZD\textsubscript{6} forms a dimeric quaternary structure, in which one protomer interacts with DVL and the other one with the heterotrimeric G protein.

To assess receptor–receptor interaction, we used dual color fluorescence recovery after photobleaching (dcFRAP)\textsuperscript{16, 19, 20}, HEK293 cells were transiently transfected with V5-FZD\textsubscript{6}-mCherry and FZD\textsubscript{6}-GFP (Fig. 1a). Surface immobilization of the V5-FZD\textsubscript{6}-mCherry was achieved with a biotinylated anti-V5 antibody and avidin\textsuperscript{19} and routinely resulted in a dramatic reduction in the recoverable fluorescence of V5-FZD\textsubscript{6}-mCherry defining the mobile fraction of the protein of interest. As predicted, the physical interaction between the V5-FZD\textsubscript{6}-mCherry and FZD\textsubscript{6}-GFP resulted in a markedly reduced mobile fraction of FZD\textsubscript{6}-GFP upon biotinylated anti-V5 antibody/avidin crosslinking. Crosslinking reduced the basal mobile fraction (80.8 ± 2.0%; mean ± standard error of the mean, s.e.m.) of FZD\textsubscript{6}-GFP to 70.7 ± 2.4% arguing for the presence of a higher order complex such as a receptor dimer (P = 0.0045; t = 2.975; df = 50; two-tailed t-test) (Fig. 1b–d). Importantly, antibody-treated cells co-expressing V5-FZD\textsubscript{6}-mCherry and cannabinoid CB\textsubscript{1}-receptor-GFP (CB\textsubscript{1}-GFP), an unrelated Class A GPCR, did not exhibit a reduction in the mobile fraction of CB\textsubscript{1}-GFP upon crosslinking, underlining the specificity of the FZD\textsubscript{6}–FZD\textsubscript{6} interaction and of this technique (Fig. 1e–g). Direct statistical comparison (one-way ANOVA) of the CL-induced differences in the mobile fraction of the respective GFP constructs in the conditions V5-FZD\textsubscript{6}-mCherry/FZD\textsubscript{6}-GFP and V5-FZD\textsubscript{6}-mCherry/CB\textsubscript{1}-GFP indicate that the observed interactions are specific (Supplementary Fig. 1a). In order to detect protein–protein interactions by dcFRAP, the relative expression levels of the crosslinked receptor population (mCherry) must be in excess relative to the mobile receptor population (GFP)\textsuperscript{21}. As a means of estimating stoichiometry, we designed a TM protein carrying an extracellular mCherry and an intracellular GFP (mCherry–TM–GFP), which we imaged with identical acquisition settings to our dcFRAP set-up over a range of fluorescence intensity levels. Values were fit to a linear regression providing a fluorescence intensity ratio of GFP to mCherry of 1.6 ± 0.1 (R\textsuperscript{2} = 0.8739; N = 54) (Supplementary Fig. 1b). Throughout the manuscript, only values with an intensity(GFP)/intensity(mCherry) <1.6 for a fixed laser ratio 488/543 were included in the analysis of dcFRAP experiments.

We also performed ratiometric Förster resonance energy transfer (FRET) measurements using the same C-terminally tagged FZD\textsubscript{6} constructs employed in dcFRAP experiments; however, we were not able to detect energy transfer between the fluorophores possibly due to suboptimal fluorophore orientation at the 210 aa long C-terminal tail.

Next, we set out to corroborate the FZD\textsubscript{6}–FZD\textsubscript{6} interaction by employing fluorescence cross-correlation spectroscopy (FCCS), which allowed for analysis of low receptor expression levels (from 10–1000 receptors per \textmu m\textsuperscript{2} in each examined HEK293 cell). Due to its single-molecule sensitivity, FCCS can be performed using low levels of protein\textsuperscript{22} and without the need for crosslinking. FCCS measurements were performed on HEK293 cells co-expressing FZD\textsubscript{6}-GFP and V5-FZD\textsubscript{6}-mCherry, and a prominent cross-correlation amplitude was observed (Fig. 1h–j). Analysis of crosslink-corrected FCCS data obtained in FZD\textsubscript{6}-GFP/FZD\textsubscript{6}-mCherry-transfected cells is in agreement with a model assuming that all FZD\textsubscript{6} exists as dimers (RMS = 0.12) (either as FZD\textsubscript{6}-GFP/FZD\textsubscript{6}-GFP, FZD\textsubscript{6}-GFP/V5-FZD\textsubscript{6}-mCherry or V5-FZD\textsubscript{6}-mCherry/V5-FZD\textsubscript{6}-mCherry dimers). The crosslink correction in the model assumed that CB\textsubscript{1}-mCherry was monomeric (Fig. 1j).

TM4 and TM5 form the interface for FZD\textsubscript{6} dimerization. To further support our findings of a potential FZD\textsubscript{6} dimer, we generated a FZD\textsubscript{6} homology model using the crystal structure of Smoothened (SMO), which crystallizes as a parallel homodimer and has ~30% sequence identity to FZD\textsubscript{6} in the TM region\textsuperscript{23}.
(Supplementary Fig. 2). Accordingly, the interface of the FZD₆ dimer was predicted to involve the fourth and fifth TM helices (denoted TM4 and TM5). Access to this recently determined structure allowed us to generate atomic resolution models of the FZD₆ homodimer, suggesting extensive contacts between the protomers and an interface with a buried surface area (BSA) of ~1150 Å². The principal contributing force to the interaction is predicted to originate from van der Waals contacts between the extracellular halves of TM4 and TM5 (Fig. 2a–d; Supplementary Fig. 3). To further support the likelihood of the predicted dimer interface, molecular dynamics (MD) simulations of the dimer model in a hydrated lipid bilayer were performed (Supplementary...
Fig. 2c–d). Three independent calculations were carried out for the system, totaling 300 ns of unrestrained simulation time. After an initial equilibration phase, which involved only minor rearrangements in the dimer interface, the system remained stable for the remainder of the simulation (Supplementary Fig. 2d).

In order to determine whether this interface is required for the dimerization of FZD6 in living cells, we systematically introduced a number of mutations into the extracellular parts of TM4 and TM5 (Supplementary Fig. 4). Y369S and Y369A was predicted to be in the core of the dimer interface and part of a cluster of hydrophobic residues (e.g. F337F 4.55f, V340I 4.58f, and M341 4.59f). The ionizable residues D365F 5.41f and R368F 5.44f were typically involved in interactions with N346F 4.41f and A344F 5.21f on the other protomer. Mutation of D365F 5.41f, R368F 5.44f, and Y369S 5.45f to alanine was predicted to disrupt favorable interactions in this region, thereby impairing FZD6 homodimerization. As surmised, dcFRAP experiments with the D365A, R368A, Y369A triple mutant (hereafter called the FZD6 dimer mutant) of FZD6-GFP in combination with V5-FZD6-mCherry did not result in a decrease of the mobile fraction of FZD6-GFP upon V5 antibody/avidin-mediated crosslinking, suggesting the importance of these residues for dimer formation (P = 0.1438; t = 1.482; df = 57; two-tailed t-test) (Figs. 2e and f). The absence of a CL-induced reduction in the mobile phase of the GFP-tagged dimer mutant FZD6 also supports the existence of TM4/5 interface dimers rather than higher oligomeric structures, which would in part depend on other receptor–receptor interfaces. Interestingly, exchanging these residues for chemically conserved amino acids (D365E, R368K, Y369F—hereafter called the FZD6 dimer control mutant) maintained the intrinsic ability of FZD6 to form dimers (P ≤ 0.0001; t = 4.839; df = 153; two-tailed t-test) (Figs. 2g and h). Further mutational analysis, including the Y369A mutation on TM5 and additional residues located on TM4 (F337F, M341, M345, N346), is shown in Supplementary Fig. 4. The FZD6 F337A mutant exhibited a reduced ability to dimerize, further supporting the involvement of TM4 in the dimer interface.

Based on the hypothesis that a larger proportion of the FZD6 wt population exists as dimers compared to the receptor population of FZD6 dimer mutant, we measured the diffusion of the two receptor variants in living HEK293 cells using fluorescence correlation spectroscopy (FCS) measurements. Employing GFP-tagged FZD6 and the FZD6-GFP dimer mutant, it was possible to determine the transit time (τD) of the molecules passing through the detector volume as a factor of molecular size and speed (Fig. 2i). The substantially lower values of τD in the case of wt FZD6 (52.4 ± 4.1 ms) provides strong evidence for higher order complexes in the case of wt FZD6 (P < 0.0001; t = 4.552; df = 29; two-tailed t-test). In order to reduce potential variability in diffusion speed of the receptor species caused by binding of endogenously expressed WNTs, we pretreated the cells with 5 μM C59, a porcupine inhibitor, which blocks secretion of WNT proteins.

Furthermore, we employed protein biochemical analysis of synthetic TM4 and TM5 peptides to validate the involvement of TM helix 4 and 5 of FZD6 in receptor dimerization. Analytical size–exclusion chromatography (SEC) experiments using synthetic TM4 and TM5 peptides in n-Dodecyl β-D-maltoside (DDM) showed that the complex of TM4 and TM5 eluted at a greater volume than TM4 and TM5 alone indicative of binding between the two peptides as well as the adoption of a more compact structure than either of the peptides alone. On the other hand, the combination of TM4 and TM5 (D365A, R368A, Y369A) mutant peptide (Fig. 3b) did not result in a shift to a higher elution volume suggestive of a weaker complex. Thus, we conclude that the physical interaction observed in TM4/5 peptides was lost or weakened in the D365A-, R368A-, Y369A-mutated peptide of TM5 (Fig. 3a and b) highlighting the intrinsic importance of D365F 5.41f, R368F 5.44f, Y369F 5.45f for FZD6 dimer formation. To confirm the secondary structure of the synthetic peptides in solution, circular dichroism (CD) experiments employing purified TM4 and TM5 peptides in detergent solution (DDM) revealed that the TM peptides became structured and α-helical in a lipophilic environment (Supplementary Fig. 5a–d). In addition, isotothermal titration calorimetry (ITC) showed that purified TM4 and TM5 peptides interact with micromolar affinity (Fig. 3c), whereas TM4 does not interact with the mutant TM5 (Fig. 3d). Control experiments were conducted by titrating 820 μM TM5 or TM5 mut into triple distilled water (TDW) with 0.5% DDM, and TDW, 0.5% DDM into 165 μM TM4 (Supplementary Fig. 5e–g). These experiments confirmed that the heat was produced from binding and not from the peptide dilution or conformational change. The reductionist approach of using purified TM peptides in detergent solution validates the role and contribution of TM4/5 for the receptor interface and underlines the importance of the TM4/5 interaction involving D365F 5.41f, R368F 5.44f, and Y369F 5.45f.

Role of intracellular components for FZD6 dimerization. GPCR dimer formation is seen as a receptor-intrinsic process and the requirement for intracellular receptor-binding proteins that

Fig. 1 FZD6 forms dimers. a HEK293 cells expressing V5-FZD6-mCherry and FZD6-GFP were used for dcFRAP. Arrows depict a ROI of 1.80 × 1.80 μm that was photobleached. Size bar, 10 μm. Lower panels provide fluorescence prior (left), directly after (bleach), and 100 s after (post-bleach) photobleaching. Size bar-2 μm. b, c Bar graphs provide fluorescence intensity data from dcFRAP in the absence (gray) and presence (red) of biotinylated anti-V5/avidin crosslinking (CL) for V5-FZD6-mCherry and FZD6-GFP. The CL-induced decrease in the mobile fraction of FZD6-GFP indicates receptor–receptor interaction. d Bar graph shows fluorescence intensity averages of the mobile fraction across different conditions within the time frame of 85–101 s (including 15 s pre-bleach measurements). White bars—V5-FZD6-mCherry; gray bars—FZD6-GFP; hatched—CL. N = 21 ROIs before CL; N = 31 after CL from three independent experiments. P = 0.0045; t = 2.975; df = 50. e, g Corresponding negative control dcFRAP experiments for FZD6 interaction employing V5-FZD6-mCherry and CB1-GFP. F = 0.0730; t = 3.831; df = 61. N = 29 ROIs before CL; N = 34 ROIs after CL from three independent experiments. Error bars provide standard error of the mean (s.e.m.) *P < 0.01. ns non significant (two-tailed t-test). For a statistical comparison of the FZD6/FZD6 and FZD6/CB1 data sets presented in d and g, see Supplementary Fig. 1a. Only ROIs wherein the crosslinked receptor population was in excess to the uncrosslinked were included in the data analysis (see Supplementary Fig. 1b). h HEK293 cells expressing V5-FZD6-mCherry/FZD6-GFP used for FCS. I FCS amplitudes from a representative experiment are shown. Autocorrelations in green—FZD6-GFP and red—V5-FZD6-mCherry; cross-correlation amplitude in black. G(r) provides the fluorescence correlation amplitude. r/s gives time shift per s. j The scatter plot summarizes FCS values obtained in cells co-expressing FZD6-GFP/V5-FZD6-mCherry (filled circles) or cannabinoid CB1 receptor (CB1-GFP)/V5-FZD6-mCherry (open circles). Data presentation shows the percentage of dimerization over varying numbers of green red particles. The crosses/dotted line represent a theoretical model assuming 100% FZD6 dimerization. The crossstalk correction in the model assumed that CB1-mCherry was monomeric. N = 10 cells expressing FZD6-GFP/V5-FZD6-mCherry; N = 25 cells expressing CB1-GFP/V5-FZD6-mCherry from 6 independent experiments. Data are based on two to eight 10 s acquisition periods per cell (see also Supplementary Fig. 1b).
facilitate dimerization has not been addressed. FZD₆ interacts with heterotrimeric G proteins and the phosphoprotein DVL, where DVL regulates FZD₆ interaction with Goᵢ₁/Goᵢ₆. In order to elucidate the involvement of DVL and heterotrimeric G proteins in FZD₆–FZD₆ interaction, we performed dcFRAP experiments (V5-FZD₆-mCherry/FZD₆-GFP) in cells with either decreased or increased expression levels of DVL. In contrast to previous findings where varying levels of DVL regulated FZD₆-G protein assembly, neither depletion ($P \leq 0.0001$; $t = 6.854$; df = 53; two-tailed $t$-test) nor excess of DVL ($P \leq 0.0001$; $t = 6.243$; df = 77; two-tailed $t$-test) abrogated FZD₆ dimer formation (Fig. 4a–d)¹⁶. Interestingly, the FZD₆ dimer mutant retained the ability to interact with the scaffold protein DVL, but not with Goᵢ₁ as assessed by dcFRAP between the FZD₆ dimer mutant and DVL2-GFP ($P < 0.0001$; $t = 5.173$; df = 74; two-tailed $t$-test) (Fig. 4e) or Goᵢ₁-GFP ($P = 0.5646$; $t = 0.5801$; df = 48;
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of FZD6-GFP and the dimer mutant FZD6-GFP in the presence of the porcupine inhibitor C59 (5 µM) resulted in a decrease in the FZD6-GFP mobile fraction at the cell surface (Fig. 4g). While co-expression of FZD6-mCherry and FZD6-GFP resulted in distinct cell surface expression of both receptor populations, co-expression of FZD6-R511C-GFP together with FZD6-mCherry resulted in predominant intracellular localization of both constructs. FZD6-mCherry was trapped intracellularly due to the strong mislocalization phenotype of the mutant16 and dimerization between wt and FZD6-R511C. On the other hand, the FZD6-GFP dimer mutant maintained cell surface localization despite co-expression with FZD6-R511C-mCherry. Since neither FZD6-R511C16 (able to dimerize) nor the dimer mutant of FZD6 (not able to dimerize) were able to assemble with heterotrimeric G proteins, we conclude that neither DVL nor G protein binding are required for FZD6 dimer formation (Fig. 4g).

Dynamic FZD6 dimerization. Currently, little is known about FZD complex composition, dynamics, stoichiometry, and the contribution of DVL and heterotrimeric G proteins. It was previously demonstrated that WNT-5A, an agonist for FZDs, induced the dissociation of heterotrimeric G proteins Goqi and Gq4 from the FZD inactive-state complex14. Based on these findings, the question arose if not only the receptor-G protein, but also the receptor–receptor interaction could be ligand sensitive. To this end, dcFRAP assays in cells expressing V5-FZD6-mCherry and FZD6-GFP were performed with or without WNT-5A stimulation (300 ng ml⁻¹) for 5, 10, 15, and 20 min. While interaction of V5-FZD6-mCherry and FZD6-GFP was measured at baseline (mobile fraction: 64.6 ± 2.1%), the addition of WNT-5A led to a transient loss of CL-induced decrease in the FZD6-GFP mobile fraction at the first measured time point (71.8 ± 3.2% at 5 min). Subsequently, the mobile fraction of the receptor returned to values resembling unstimulated (61.0 ± 2.2% at 20 min) (P = 0.0219; F (5, 109) = 2.757; one-way ANOVA) (Fig. 5a, b). Since these experiments, which point to a decrease of higher order complexes in the total receptor population, were performed with a relatively high degree of receptor overexpression and receptor crosslinking, we aimed to create conditions with low expression levels of FZD6 with unimpeded mobility. FCCS experiments on highly expressing HEK293 cells expressing V5-FZD6-mCherry and FZD6-GFP supported the dynamics and kinetics of the agonist-induced dissociation/re-association of the FZD6 dimer (P = 0.0042; F (7, 39) = 3.628; one-way ANOVA) (Fig. 5c–e). Cross-correlation of mCherry/GFP fluorescence indicated a stable baseline of red/green dimers, after which a transient decrease in FZD6–FZD6 interaction could be detected upon stimulation with WNT-5A (300 ng ml⁻¹) (Fig. 5c–e). Similar to the kinetics of the dcFRAP analysis, red/green correlation (Ngr/Nr values in Fig. 5c; Gc values in Fig. 5d) returned to values resembling re-association of the FZD6 promoters in the range of minutes. In order to rule out receptor internalization as a confounding factor for the dissociation observed in the FCCS experiments, we quantified the number of red (Nr) and green (Ng) fluorescent particles over the course of dynamic FCCS measurements (Fig. 5c). The transient increase in particle number and the return to values resembling prestimulation levels (values are not corrected for bleaching) support the idea of agonist-induced complex dynamics instead of agonist-induced receptor trafficking. To corroborate this finding, we assessed the degree of FZD6 internalization after exposure to WNT-5A by confocal microscopy (Fig. 5f). This was achieved by pulsing HEK293 cells expressing SNAP-FZD6 with a cell-impermeable SNAP substrate. Within the time frame of the FCCS experiments, minimal internalization of SNAP-FZD6 was observed and this was not enhanced by the addition of recombinant WNT-5A (similar to what had previously been reported using the FZD6-mCherry construct18). For the purpose of comparison, 10 µM isoproterenol stimulation of SNAP-tagged β-adrenergic receptors, a Class A GPCR known to undergo rapid receptor-mediated endocytosis, resulted in the appearance of numerous endosome-like structures after 15 min, similar to what had been described by others37. Based on dcFRAP and FCCS experiments, we propose the model shown in Fig. 5g.

Modulation of FZD6 dimers and downstream signaling. In order to translate our molecular and cellular findings for the importance of FZD6 dimers at the cellular level, we turned to MLE-12 cells. FZD6 is highly expressed in adult and fetal human lung tissue, but its function in the lung is mostly unknown28, 29. Immunohistochemistry data from the Human Protein Atlas indicate strong membrane-bound FZD6 staining in human bronchial epithelial cells30 (http://www.proteinatlas.org/ENSG00000146930-FZD6/tissue/bronchus). Cells derived from the distal bronchial and alveolar epithelium of SV40 transgenic mice, otherwise known as murine lung epithelial (MLE-12) cells31, express FZD6 and to a lesser extent FZD2 at the mRNA level as shown by quantitative PCR (Supplementary Fig. 6). On the protein level, FZD6 is readily detectable in cell lysates indicating that the receptor is expressed in sufficient amounts to be of functional relevance in these cells while providing a suitable model to support the role of FZD6 dimerization in endogenous receptor expression levels. Molecular validation of the cell system indicated that the addition of recombinant and purified WNT-5A (300 ng ml⁻¹) led to a time-dependent, partially pertussis toxin-sensitive phosphorylation of ERK1/2, with maximal increase in P-ERK1/2 at 5 min (Fig. 6a, b; Supporting Fig. 6). Interestingly, the kinetics of WNT-5A-induced ERK1/2 phosphorylation matched the time course of FZD6 de-
re-dimerization, suggesting a possible role for dimerization in signaling.

In order to functionally link ERK1/2 phosphorylation to FZD₆, we used CRISPR/Cas9-mediated gene editing to target FZD₆ in MLE-12 cells. Clonal analysis defined a heterozygous FZD₆⁺/− line, in which FZD₆ expression and cellular proliferation were visibly reduced (Fig. 6c; Supplementary Fig. 6). We excluded off-target effects on lysophosphatidic acid (10 μM; agonist at LPA receptors) and thrombin (10 μM; activating proteinase-activated receptors) as these are well-documented propagators of ERK1/2 signaling. We did not observe any difference in P-ERK1/2 induction in the presence of minigenes (Supplementary Fig. 7e). In short, we show that FZD₆ is required to maintain basal ERK1/2 signaling and that the dimer-interfering TM5 minigene impairs FZD₆ signaling. This raises the question as to how agonist-induced dimer dissociation and FZD₆ bound to TM5 peptide can have opposite signaling outcomes. To better understand the signaling capacity of wt and dimer mutant FZD₆, we overexpressed the two constructs in HEK293 cells and analyzed downstream ERK1/2 signaling. In serum-starved HEK293 cells, both the FZD₆ dimer mutant and FZD₆ wt increased P-ERK1/2 (Fig. 6e). Given the high degree of autocrine WNT signaling in various cell types, we used the porcine inhibitor C59 (5 μM, overnight) to determine the effect of endogenous WNTs on receptor-mediated signaling. In other words, C59 could provide a tool for addressing the role of ligand-induced receptor dynamics for signal initiation. In the presence of C59, which abrogates endogenous WNT secretion⁶⁴, FZD₆ wt-induced levels of P-ERK1/2 were reduced to basal, whereas the FZD₆ dimer mutant maintained its ability to induce ERK1/2 phosphorylation (Fig. 6f, g). Furthermore, increasing expression of either FZD₆ wt or FZD₆ dimer mutant in C59-treated HEK293 cells strengthened the finding that the dimer mutant, but not the FZD₆ wt is capable of inducing P-ERK1/2 in

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**Fig. 3** Mutation of TM5 decreases affinity between the TM4/5 peptides in DDM solution. a, b Size-exclusion chromatography experiments were performed in triple distilled water (TDW) and 0.01 % DDM. Final concentration of 500 μM from each peptide in TDW + 0.01 % DDM was injected into the size-exclusion column and their absorbance was detected as explained in the methods section. a Elution profiles of synthetic peptides TM4 and TM5 alone and in combination. b Elution profiles of TM4 and mutated TM5 (TM5 mut) peptides are shown. Helical peptide models (TM4—yellow; TM5—green) visualize the expected van der Waals interaction between TM4/5 and the lack thereof in the mutated peptide derived from the FZD₆ model presented in Fig. 2. c Isothermal titration calorimetry (ITC) experiments were performed in 0.5% DDM at 20 °C. ITC curves for the titration of 820 μM FZD₆-TM5 peptide into 165 μM FZD₆-TM4 are shown. d Titration of 820 μM FZD₆-TM5 mut peptide into 165 μM FZD₆-TM4 is shown. Fitting the binding curve to one set of sites resulted in an affinity (dissociation constant; Kₐ) in the micromolar range. The precise Kₐ value could not be determined due to high background, which is likely to be caused by the dimerization of the FZD₆-derived peptides. Control experiments with TM4, TM5, and TM5 mut peptides alone are shown in Supplementary Fig. 5e–g.

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Overexpression of TM1, the minigene that does not affect FZD₆ dimerization in HEK293 cells, did not affect basal ERK1/2 levels in MLE-12 cells. On the other hand, when expressing the dimer-interfering TM5 minigene, basal P-ERK1/2 levels dropped to 56.7 ± 11.4 % (P = 0.0091; t = 3.791; df = 6; two-tailed t-test) (Supplementary Fig. 7c, d). We excluded off-target effects on other GPCRs by transiently transfecting either empty vector, TM1 or TM5 minigenes in HEK293 cells and stimulating with lysophosphatidic acid (10 μM; agonist at LPA receptors) and thrombin (10 μM; activating proteinase-activated receptors) as these are well-documented propagators of ERK1/2 signaling. The precise Kₐ value could not be determined due to high background, which is likely to be caused by the dimerization of the FZD₆-derived peptides. Control experiments with TM4, TM5, and TM5 mut peptides alone are shown in Supplementary Fig. 5e–g.

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**Fig. 7b** Taking together the findings that the peptides are localized in the membrane, obtain the correct orientation and form helices in a lipophilic environment (Supplementary Fig. 5), supports our idea to use these minigenes as tools in cells to dissect the role of FZD₆ dimerization for ERK1/2 signaling in MLE-12 cells.

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**Fig. 2**
HEK293 cells showed distinct co-localization predominantly in the membrane. The FZD 6-R511C mutation (predominantly localized to lysosomes and mutant showed surface expression in the presence of FZD 6-R511C. Size bar = 20 μm. ROIs before CL; ***P<0.001; t = 6.854; df = 53; N = 33 ROIs before CL; N = 22 after CL from three independent experiments) and c, d overexpressed DVL2-MYC (P<0.0001; t = 6.243; df = 77; N = 36 ROIs before CL; N = 43 after CL from three independent experiments). e, f dcFRAP experiments in combination with chemical surface crosslinking show that the FZD6 dimer mutant is still capable of assembling in an inactive state with DVL2-GFP (P<0.0001; t = 5.173; df = 74; N = 15 ROIs before CL; N = 61 after CL from three independent experiments), but not with Gαi3,1-GFP (P = 0.5646; t = 0.5801; df = 19; N = 29 ROIs before CL; N = 21 after CL from three independent experiments). Error bars give s.e.m.; ***P<0.001; ns non significant (two-tailed t-test). g V5-FZD6-mCherry and FZD6-GFP expressed in HEK293 cells showed distinct co-localization predominantly in the membrane. The FZD6-R511C mutation (predominantly localized to lysosomes and unable to interact with heterotrimeric G proteins16, 25) forced the wt FZD6 to be retained intracellularly suggestive of dimer formation. Co-expression of the FZD6-R511C mutant together with the dimer mutant of FZD6 did not result in co-localization. Due to the lack of dimerization, the FZD6 dimer mutant showed surface expression in the presence of FZD6-R511C. Size bar—10 μm

Fig. 4 Absence of a role for DVL and G proteins in FZD6 dimer formation. a–d Analogous to data shown in Fig. 1c, d, we investigated the effect of V5-FZD6-mCherry surface crosslinking (CL) on the mobility of FZD6-GFP in the presence of a, b pan-DVL siRNA (P<0.0001; t = 6.854; df = 53; N = 33 ROIs before CL; N = 22 after CL from three independent experiments) and c, d overexpressed DVL2-MYC (P<0.0001; t = 6.243; df = 77; N = 36 ROIs before CL; N = 43 after CL from three independent experiments). e, f dcFRAP experiments in combination with chemical surface crosslinking show that the FZD6 dimer mutant is still capable of assembling in an inactive state with DVL2-GFP (P<0.0001; t = 5.173; df = 74; N = 15 ROIs before CL; N = 61 after CL from three independent experiments), but not with Gαi3,1-GFP (P = 0.5646; t = 0.5801; df = 19; N = 29 ROIs before CL; N = 21 after CL from three independent experiments). Error bars give s.e.m.; ***P<0.001; ns non significant (two-tailed t-test). g V5-FZD6-mCherry and FZD6-GFP expressed in HEK293 cells showed distinct co-localization predominantly in the membrane. The FZD6-R511C mutation (predominantly localized to lysosomes and unable to interact with heterotrimeric G proteins16, 25) forced the wt FZD6 to be retained intracellularly suggestive of dimer formation. Co-expression of the FZD6-R511C mutant together with the dimer mutant of FZD6 did not result in co-localization. Due to the lack of dimerization, the FZD6 dimer mutant showed surface expression in the presence of FZD6-R511C. Size bar—10 μm

a ligand-independent manner (Fig. 6b). Taken together, the biochemical analysis of both FZD6 wt and dimer mutant with regard to their capacity to induce P-ERK1/2 in the absence of agonist reveals the overactive nature of the FZD6 dimer mutant compared to wt.

**Discussion**

The existence and functional relevance of GPCR dimers or higher order oligomers is generally accepted despite difficulties to transfer mechanistic insight from overexpression studies using recombinant receptors to endogenously expressed receptors in cell systems and in vivo. Hitherto, agonist-dependent dissociation/re-association of GPCR dimers has not yet been reported and this paradigm provides deeper insight into the potential role of dimer dynamics in the process of FZD and potentially GPCR activation and signal initiation.

Depending on the specific GPCR class, dimers vary in their receptor complex formation, signaling, trafficking and pharmacology. In the case of Class A GPCRs, it is unclear whether dimers or higher oligomers have any physiological relevance3, 6. Conversely, Class C GPCRs constitutively dimerize via rather well-understood mechanisms and with obvious functional consequences for the receptors34, 35. Class FZD receptors (FZD1–10, SMO) have also been shown to form higher order structures14, 15, 23, 36. In general, the functional importance of FZD dimerization/oligomerization remains obscure. For FZD1, it was shown that dimerization occurs in the ER and that FZD1 mutants associated with vitreoretinopathy can retain wt FZD4 in intracellular compartments14. It was also shown that FZD1, FZD2, FZD3 dimerize and that dimerization of FZD3 and FZD7 affects WNT/β-catenin signaling in Xenopus laevis14, 15. So far, FZD dimers are perceived as being static and the nature of the dimer interfaces is unknown. Due to the well-documented interaction between CRD domains of FZDs18, CRD dimerization has been proposed to form the structural basis of FZD interaction15. On the contrary, FZD1 and FZD2 were shown to form dimers independently of the CRD14 and our data are consistent with a CRD-independent interface in the TM region of FZD6. That being said, our results do not rule out the possibility for an involvement of the CRDs in supporting dimer formation. Throughout the GPCR families, various interaction interfaces have been identified in different receptor subtypes ranging from extracellular domains to basically all TM domains of the 7TM cores and the intracellular helix 83. However, the involvement of TM1 and helix 8 in FZD6 dimerization seems unlikely since the mutational analysis supports a TM4/5 interface and the C termini of either protomer point in opposing directions according to the homology model (Fig. 2a; Supplementary Fig. 2,3). The ability of FZD6 to dimerize irrespective of DVL and heterotrimeric G proteins, underscores that dimerization is receptor-intrinsic. It should be emphasized that despite all data supporting the idea that a dimer is the smallest oligomeric unit of FZD6, we cannot directly exclude the presence and dynamics of higher order receptor complexes. We base our conclusions about dimerization on the ability to define and disrupt the TM4/5 interface and on the rationale that FZD6-D365A, R368A, Y369A does not continue
Fig. 5 Agonist-dependent dynamics of FZD6 dimerization. 

a) dcFRAP in HEK293 cells expressing V5-FZD6-mCherry and FZD6-GFP allowed kinetic monitoring of receptor–receptor interactions in the absence of agonist and after 5, 10, 15, 20 min of WNT-5A (300 ng ml⁻¹) stimulation. \( P = 0.0219; F (5, 109) = 2.757 \). Before CL (20 ROIs); after CL (unstimulated = 38 ROIs, 5 min = 12 ROIs, 10 min = 18 ROIs, 15 min = 12 ROIs, 20 min = 15 ROIs). \( N = 10 \). Unstimulated (before and after CL) are from Fig. 1d and were performed in parallel with stimulation experiments. 

b) Curves depict the recovery of GFP after CL comparing unstimulated (red), 5 min (green) and 20 min (magenta) after WNT-5A stimulation.

c) Dynamic FCCS experiments in HEK293 cells expressing V5-FZD6-mCherry and FZD6-GFP from 6 different cells are summarized by normalizing the agonist-induced minimum fraction of dimers from individual cells to 0 min (\( N_{gr}/N_{r} \) represents ratio of FZD6-GFP/V5-FZD6-mCherry dimers (green/red, gr) over the total number of red molecules (red, r), representing the fraction of dimers present in the detection volume). Box plot presents data from the 25 to 75th percentile and whiskers provide min/max. \( P = 0.0093; F (7, 39) = 3.178 \). **\( P < 0.01 \) (one-way ANOVA, Fisher’s LSD post hoc analysis). Total number of \( N_{r} \) (red) and \( N_{g} \) (green) particles in the detection volume over time. Values of \( N_{r} \) or \( N_{g} \) at the first time point were set to 100.

d) FCCS curves present cross-correlation of V5-FZD6-mCherry and FZD6-GFP from a representative cell prior to WNT-5A stimulation (red), at maximal dimer dissociation (green) and upon dimer re-association (magenta). When normalized to the green auto-correlation amplitude, the amplitudes of the three \( G_{cc} \) curves revealed that 11, 6, and 21% of red molecules were also green, respectively.

e) Representative example of \( N_{gr}/N_{r} \) after WNT-5A stimulation for a cell expressing wt FZD6-GFP and wt FZD6-mCherry. \( t = 0 \) refers to first measurement after the addition of WNT-5A. f) HEK293 cells expressing SNAP-FZD6 or SNAP-β2-AR were pulsed with a cell-impermeable SNAP substrate to monitor internalization after WNT-5A (300 ng ml⁻¹, 15 min) or isoproterenol (10 μM). Arrows indicate internalized receptors. Scale bar = 5 μm.

f) Model of FZD6 de- and re-dimerization upon WNT-5A stimulation. Error bar provides s.e.m
to interact with FZD₆ wt in dFRAP experiments—suggesting that other interaction modes enabling higher order complex formation do not exist. This conclusion is further corroborated by the analysis of the FCCS data, where the data fits with a model that assumes receptor dimerization.

The agonist-induced dynamic dissociation/re-association of the FZD₆ complex is clearly different from the rather stochastic, agonist-independent and transient interactions of Class A GPCRs measured by single-particle tracking in living cells^{38-40}. Recent data report antagonist-induced changes in the oligomeric organization of serotonin 5-HT₃A receptors, where treatment with 5-HT₃A-selective antagonists reduced the complexity of higher oligomeric receptor assemblies assessed by spatial intensity distribution analysis^{41}. Furthermore, agonist-induced rearrangements in the receptor-G protein complex composition of a calcitonin CTR receptor in relation to G protein association, coupling and activation support the idea of receptor dimer dynamics exemplified by a Class B GPCR^{42}. Our findings extend...
beyond the pre-existing concepts of constitutive, transient, or stable GPCR dimerization by introducing the idea that FZD₆ dimers dissociate and re-associate in a ligand- and receptor-activity-dependent manner.

In the current study, we map the dimer interface of FZD₆ to TM4/5 through mutational analysis, homology modeling and MD simulations. A similar interface has recently been described in the constitutive dimer of metabotropic glutamate receptor 2 (mGluR2), where agonist stimulation resulted in a structural rearrangement towards a smaller interface at TM6 35. These findings were further corroborated in another FRET-based study investigating conformational dynamics of Class C GPCRs 43. In the case of Class C receptors, protomer dissociation is prevented by covalent interaction at the N-terminus. When it comes to FZD₆, however, a structural rearrangement resembling that of mGlur2 results in dissociation because the receptor protomers are not covalently linked. The similarity between mGlur2 and FZD₆ dimer dynamics upon agonist stimulation supports the idea that dimer dynamics are integral to receptor activation and that this phenomenon might represent a more general principle than previously appreciated 15, 43. Similar to our observations with FZD₆, the chemokine CXCR4 receptor dimer structure 44 is also based on a TM4/5 interface localized to the upper regions of the helices with an interaction area of 850 Å². Although the molecular and structural details of Class FZD receptor activation are still unknown, extrapolation from the conformational changes observed for the β₃-adrenergic receptor bound to G protein or a nanobody surrogate suggests that activation could involve structural changes in TM5 45, 46, and thereby influence the homodimer interface of FZD₆. Interestingly, D3655.41f, R3685.45f, and Y3695.45f in FZD₆, which upon mutation influence dimerization, are also in close proximity to the region in TM5 that undergoes a conformational change upon activation of the β₃-adrenergic receptor.

Present and previous dcFRAP experiments on FZD₆ dimer and G protein dissociation, respectively, argue that both processes follow similar kinetics 16. In addition, we employ the TM1 and TM5 minigenes to elucidate dimer formation and the connection between dimerization and signaling (Supplementary Fig. 7). On the level of endogenous receptor expression in MLE-12 cells, we suggest that FZD₆ expression is required to maintain basal ERK1/2 phosphorylation—a pathway that is intrinsically linked to cell proliferation, survival, and repair 47. In combination with the strong FZD₆ membrane expression in bronchial epithelium recently reported by the Human Protein Atlas project (http://www.proteinatlas.org/ENSG00000164930-FZD6/tissue/bronchus), these data argue that FZD₆ dimers could be important for the maintenance of a functional epithelium in the respiratory tract.

The detection, visualization, and analysis of GPCR dimers in cells endogenously expressing the receptors present a true challenge, which is not easily overcome. Employing both dcFRAP and FCCS with high and low expression of tagged receptors, we depict agonist-induced dimer dynamics in living cells. Translation of our findings to MLE-12 cells is possible using the validated dimer-interfering peptides. The TM5 minigene-mediated reduction in P-ERK1/2 in MLE-12 cells (Supplementary Fig. 7) indicates that peptide-induced FZD₆ dimer disruption is neither equivalent with agonist-induced dissociation nor is it sufficient to evoke signaling. One possible explanation is that the TM5 minigene disrupts the dimer through interaction with the FZD₆ TM4 and correlates the individual protomers in a negative allosteric modular manner to maintain the protomers in an inactive conformation (Supplementary Fig. 7). This hypothesis is further corroborated by the finding that the dimer mutant confers constitutive activity to the ERK1/2 pathway. Building on this idea, this could imply that the two protomers in a FZD₆ dimer maintain an inactive state in a bidirectional, negative allosteric fashion—a model that requires further experimental validation. Here, we conclude that the dimeric complex represents the inactive, ligand-responsive form of FZD₆ whereas the agonist-induced appearance of monomers or the increase of monomers by dimer-interfering mutations evokes downstream signaling (Fig. 6). While wt FZD₆ forms an inactive-state complex with DVL and Gαq/Gβ proteins 16, 26, it is not possible to detect the assembly of the FZD₆-mCherry dimer mutant with Gαq-GFP by dcFRAP (Fig. 5f). Nevertheless, the FZD₆ dimer mutant still interacts with DVL (Fig. 5e) and signals to ERK1/2 in an agonist-independent manner when over-expressed in HEK293 cells (Fig. 6g). This apparent conundrum can be explained by the switch from an inactive-state receptor/G protein assembly to a collision-coupling mechanism in the predominantly monomeric dimer mutant as it would be expected from the ternary complex model 48. In a complementary way, calcitonin GPCR (CTR) complex dynamics support our proposed model for FZD₆ because (i) the dimeric CTR appears to be the agonist-receptor species and (ii) that increasing G protein interaction/coupling in the presence of agonist resulted in the loss of dimeric CTR 42.

Our data present a new kinetic perspective for the activation of Class FZD receptors where agonist stimulation leads to the dissociation and re-association of the receptor dimer. Here, we argue that the FZD₆ wild-type (wt) dimer is relevant in living cells and that constitutive signaling to ERK1/2 can be affected by modulating the dimeric status of FZD₆. Most importantly, we provide evidence that the monomeric rather than the dimeric form of FZD₆ is the active signal initiating unit of the receptor complex. This is in agreement with several lines of evidence supporting that the active conformation observed in GPCRs is associated with the monomeric receptor rather than a dimer 4, 42, 46, 49, 50.

FZDs present an attractive target for drug development, even though no small compounds targeting these receptors have been reported so far 8-10. However, targeting the dimer interface to impede with receptor activation might offer novel therapeutic possibilities. Further studies will be required to fully understand the function of dynamic FZD dimerization for the agonist-induced initiation of different signaling avenues. Also, the phenomenon of dynamic and agonist-sensitive dimerization might not be restricted to Class F receptors, but could provide a more general concept of GPCR signal transduction.

**Methods**

**Cell culture and transfections.** HEK293 and MLE-12 cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine (all from Invitrogen Technologies) in a humidified CO₂ incubator at 37 °C. All cell culture plastics were from Sarstedt, unless otherwise specified. HEK293 cells were used because of their advantageous transfectability, their ability to grow with and their appearance making them amenable to live cell imaging experiments. For live cell imaging experiments, cells were seeded on 35 mm ECM gel-coated (1:300, Sigma-Aldrich) glass bottom dishes (MatTec or Greiner Bio One 4 compartment 35 mm glass bottom dishes). Cells were transfected with Lipofectamine 2000 24 h prior to analysis. Treatment with the porcupine inhibitor C59 (2-[(4-1H-2-Methylpyridin-4-yl)phenyl]-N-[4-(pyridin-3-yl)phenyl]acetamide) was performed at 5 μM overnight 24. Treatment with pan-DVL siRNA was done as previously reported 16. Absence of mycoplasma contamination is routinely confirmed by PCR using 5'-ggc gaa tgg tgt agt acg acg-3' and 5'-cgg ata acg ctt gcg act atg-3' primers detecting 16 S ribosomal RNA of mycoplasma in the media after 2-3 days of cell exposure. FZD₆-GFP and FZD₆-R511C-GFP were from Niklas Dahl (Uppsala University, Uppsala, Sweden) 26. GFP-tagged human FZD₆ and FZD₆-R511C were re-cloned into the pmCherry-N1 vector using the restriction enzymes BgIII and AgeI. The following signal peptide (5'-ctg tgg cag cgg tgg cag cgg tgg cag cgg cgg cgg cgg cgg-3') was cloned into the pmCherry-N1 plasmid using HindIII and KpnI restriction sites. V5-FZD₆ was cloned using the following primers and inserted with KpnI and AgeI restriction sites into the aforementioned
pmCherry-N1 construct. Forward primer: 5′-acc gct gcg aaa cgg att ccc acc ccg ctt ggc gtt gat aag acct ctc acc ctg tga-3′. Reverse primer: 5′-gac cgg tgg aag tga aag ccc acc acc aac ccg ctg ctg ctt ggc gct cgt ggg ccc agg acg ccg ggg-3′. SNAP-Fzd2 was cloned into the pCMV-N1-phi vector using HindIII and KpnI restriction sites. vS-Fzd2, was cloned using the first primers and inserted with KpnI and AgeI restriction sites into the pcmCherry-N1 construct. Forward primer: 5′-acc gct gcg aaa cgg att ccc acc ccg ctt ggc gtt gat aag acct ctc acc ctg tga-3′. Reverse primer: 5′-gac cgg tgg aag tga aag ccc acc acc aac ccg ctg ctg ctt ggc gct cgt ggg ccc agg acg ccg ggg-3′. SNAP-Fzd2 was a gift from M.M. Maurice (Utrecht University Medical Center, The Netherlands). SNAP-β-arrestin receptor was from Davide Calebiro (University of Würzburg, Germany). CB,R- GFP was from Zolt Lenkei (Ecole Supérieure de Physique et Chimie Industrielle–Paristex, Paris, France); DVL2-MYC was from S.A. Yanagawa (Kyoto University, Kyoto, Japan). Gαt, -GFP was from Mark M Rasenick (University of Illinois, Chicago, USA). Plasmids encoding β and γ subunits were from www.cdna.org. The mCherry–TM–FGF stoichiometry was synthesized as a gBlock Gene Fragment by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) and was composed of an Nhel restriction site, a Kozak sequence, the Igc signal sequence, a 6×His and a 3×Gly linker, an EcoRI restriction site, the coding sequence for mCherry association of FZD (Fig. 5c–d), and their interacting complex, when detected in the cell surface membrane, as judged by visual inspection in the wide-field and confocal mode. Auto-fluorescence, as well as fluorescence from both GFP and mCherry in the intracellular region was negligible. The number of fluorescent receptors assessed in each experiment varied from 10–1000 receptors per μm² in each HEK293 cell.

Dynamic FCCS measurements of WNT-dependent dissociation and re-association of Fzd (Figs 5c–e) were performed on an FCS/FCCS-equipped Zeiss 780 confocal microscope (Zeiss, Jena, Germany). The instrument details were the same as those of the Confocor 3 described above, with the exception that 561 nm excitation was used for excitation of mCherry, that GFP emission was detected from 500 nm, and that mCherry emission was detected at 600 nm.

Experiments on cells were performed as described above, with each measurement point consisting of 3–6 measurements of 10 s each. In contrast to dFRAP, FCS experiments allowed assessment of receptor dynamics over time in single cells.

**FCCS analysis.** Analysis of initial FCS measurements confirming the presence of Fzd2-FGFR5-V5-Fzd2-mCherry dimers (Fig 1h–j). In FCS/FCCS measurements, fluctuations in the detected fluorescence intensity I(t), are typically generated as molecules diffuse in and out of a focused laser beam. These fluctuations, I(t), are autocorrelated according to G(τ) = (δI(t)δI(t+τ))/I(0)², or in case of two wavelength separated fluorescence intensities F1(t) and F2(t), cross-correlated according to G(τ) = (δF1(t)δF2(t+τ))/F1(0)F2(0). In the case of two fluorophore-labeled species, with concentration c1 and c2, and their interacting complex, with concentration c12, which are diffusing on a two-dimensional surface, the following equations hold:[12]

\[
G_{c12}(τ) = 1 - \frac{\text{Diff}_{c12}^{G}(τ)}{\text{Diff}_{c12}^{B}(τ)} = 1 - \left(1 + \frac{4D_{c12}^{G}}{R_{1}^{2}}\right)^{-1} \left(1 + \frac{4D_{c12}^{B}}{R_{1}^{2}}\right)^{-1}
\]

\[
\frac{\text{Diff}_{c12}^{G}(τ)}{\text{Diff}_{c12}^{B}(τ)} = \left(1 + \frac{4D_{c12}^{G}}{R_{1}^{2}}\right)^{-1} \left(1 + \frac{4D_{c12}^{B}}{R_{1}^{2}}\right)^{-1}
\]

Here, A2 = c0c2A0, A1 = c0c1A0 and A5 = c50 + c50/2 are the effective detection areas, respectively, where c0 and c50 are the radii of the detected fluorescence brightness distribution of the two excitation lasers and \(\text{Displ}(t) = 2\text{π}^{2}t^{2}/(ht_{\text{ave}}^{2} + \text{K}^{2}t_{\text{ave}}^{2})\) is the displacement function related to a non-perfect overlap between the two excitation lasers. \(D_{c12}\) is the diffusion coefficient of species \(c_{1}\) and \(c_{2}\), which is the crossstalk parameter, defined as the brightness ratio of the two frequencies at the center of the red channel. \(B_{G}\) and \(B_{R}\) are the background fluorescence in the green and the red channel, respectively, when both lasers are on. The terms \(x_{g}\) and \(x_{r}\) are the
absorption cross-section ratios of the g and the gr species and the r and the gr species, respectively. The expression in Eq. 1 was globally absorbed to evaluate the structural stability of the selected model, three independent MD measurements and ωfl of one data point/cell was 20 kHz/molecule and the cross-correlation amplitude ωfl of one 10 s measurement, which are summarized in the presentation of 10/25 cells in Fig. 1.

Assuming that M is FZD2-GFP (green) and N is FZD2-mCherry (red) and that all FZD2 form dimers, then the probability of finding a green-red FZD2 dimer is

\[ p = \frac{2xM(N+M-N-1)}{2(N+M)}, \]

where \( x \) is the corresponding concentrations of the only FZD2 dimers, the red and green FZD2 dimers and the only red FZD2 dimers. Given a population of M + N FZD2 molecules, we define the probability to pick one green as M/(M + N), followed by picking one red as N/(M + N) + 1 or to pick one red as N/(M + N) followed by picking one green as M/(M + N) + 1. The concentration of dimers consisting of a red and a green FZD2 is then \( p_{\text{gr}} = (M + N)/(2(N + M + N)/2) \) (there are \( (M + N) \) pairs). The corresponding concentration of dimers consisting of green-red FZD2 is then \( p_{\text{rg}} = (M + N)/(2(N + M + N)/2) \). From the experiments, we determine the concentrations for \( p_{\text{gr}}, p_{\text{rg}}, \) and \( c_{\text{rg}} \) and compare the concentrations \( p_{\text{gr}}, p_{\text{rg}} \) for each cell to get an estimation of how close the model is to experimental data:

\[ c_{\text{gr}} = 2c_{\text{gr}} - \frac{c_{\text{gr}}}{c_{\text{rg}}} \frac{c_{\text{rg}} + c_{\text{rg}}}{c_{\text{rg}} + c_{\text{rg}}} \frac{c_{\text{rg}} + c_{\text{rg}}}{c_{\text{rg}} + c_{\text{rg}}} \frac{c_{\text{rg}} + c_{\text{rg}}}{c_{\text{rg}} + c_{\text{rg}}} \]

Analysis of the dynamic FCSS measurements of WNT-dependent dissociation and re-association of FZD2. Auto-correlation and cross-correlation curves were analyzed as described above and the amplitudes \( G_{\text{rr}}(0)-1, G_{\text{gr}}(0)-1, G_{\text{rg}}(0)-1 \) from each of the more than 300 measurements of 10 s duration were estimated by fitting to theoretical models. The non-perfect overlap between the red and green detection volumes was not taken into account since the dynamic FCSS measurements focused on the relations in fractional binding. The fraction of red diffusing units carrying also a green label, \( N_{\text{rg}}/N_{\text{rr}} \), was estimated from the ratio \( (G_{\text{gr}}(0)-1)/(G_{\text{rr}}(0)-1) \) for FCS/FCSS measurements, about 20 % of the measurements were deselected due to membrane movements or low molecular brightness (≤0.2 CPM per second (cpm)). Also, cells where the intensity ratio between the channels \( I_{\text{r}}/I_{\text{g}} \) were deselected.

With the settings described above for detection of emission, the crossstalk from the GFP channel to the mCherry channel, defined as the intensity detected in the mCherry channel divided by the intensity detected in the GFP channel, \( I_{\text{g}}/I_{\text{r}} \), for a cell expressing only FZD2-GFP, was 0.051 or 5.1 %. The cross-correlation amplitude \( G_{\text{rr}} \) was therefore corrected for crossstalk before calculating the relative cross-correlation amplitude \( G_{\text{rr}}/G_{\text{rr}} \).

Molecular modeling and molecular dynamics simulations. The structure of SMO crystallized in dimeric form (PDB code 4IKV) was used as a template to model the FZD3 homodimer. The sequence of FZD3 (Uniport ID O60353) was aligned to that of SMO with ClustalW2.45 Termi residues corresponding to those not resolved in the SMO structure were omitted and the alignment was manually edited to ensure the proper alignment of conserved motifs among Class F GPCRs. In the final sequence alignment, FZD3 and SMO shared a 30% sequence identity in the TM region. Fifteen homology models of the FZD3 homodimer were generated with MODELLER 9.115 and were further analyzed by manual inspection. In order to evaluate the structural stability of the selected model, three independent MD simulations were performed using GROMACS 4.6.7.2. As described previously, the dimer was inserted into a pre-equilibrated hydrated 1-Palmitoyl-2-oleoylphosphatidylcholine bilayer. Data were then collected in 100 s production runs of unrestrained MD, resulting in more than 300 ns of simulation data. Simulations were conducted using the OPLSAA force field for the protein with TIP3P waters for which bond lengths and angles were constrained using the SETTLE algorithm, while the LINCS algorithm was used to constrain bond length in the protein and lipid. The double-pairlist-harmonic method was used to enable the compatibility of Berger parameters. The lipids. In all the simulations, the system was simulated in a hexagonal prism-shaped simulation box using periodic boundary conditions. A time-step of 2 fs was used and neighbor lists were updated every 10 fs for non-bonded interactions within 12 Å whereas the Particle Mesh Ewald (PME) method was used for the treatment of Coulomb interactions beyond the cutoff. All simulations were carried out at 310 K using the Nose–Hoover thermostat and at constant pressure of 1 bar using the semisotropic Parinello–Rahman barostat with a coupling constant of 2 ps and an isothermal compressibility constant of 4.5 x 10⁻⁵ bar⁻¹.
Cells were stimulated (24 h post-transfection) with 300 ng ml\(^{-1}\) presentation providing the sum of independent observations. Significant plate at a density of 100,000 or 50,000 cells/well, respectively. After 24 h, cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were stimulated (24 h post-transfection) with 300 ng ml\(^{-1}\) recombinant carrier-free WNT-5A (CF WNT-5A; R&D Systems). Stimulation was stopped by the addition of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris/HCl pH 8). Lysates were sonicated and analyzed by 7.5 or 10% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes using the Trans-Blot Turbo system (Bio-Rad). After blocking with 5% milk or BSA in TBS-T, membranes were incubated with primary antibodies in blocking buffer: mouse anti-β-actin (1:30,000; Sigma #A5491), rabbit anti-FZD (1:1000; NovusBio #NB1-100830), rabbit anti-Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000; Cell Signaling Technology #9101-L), rabbit anti-p44/42 MAPK (ERK1/2) (1:1000; Cell Signaling Technology #9102), rabbit anti-Phospho-C-RAF (Ser338) (1:1000; Cell Signaling Technology #566A), rabbit anti-SNAP tag (1:1000, New England Biolabs #P93105), and rabbit anti-HA (1:4000, abcam #ab9110) overnight at 4°C. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse (Pierce)) and Clarity Western ECL Blotting Substrate (Bio-Rad). All uncropped immunoblots can be found in the Supplementary Information.

**Statistical analysis.** Statistical and graphical analysis was performed using Graph Pad Prism 5 software. Data were analyzed by two-tailed t-test or one-way ANOVA with Fisher’s least significant difference post hoc analysis. Curve fitting of FRA data was done along with the association nonlinear function using the least square fit. Data for receptor titration in the presence of C59 and output to P-ERK1/2 in Fig. 6h was fit to a linear regression model. All experiments were repeated at least three times in independent experiments (not representing technical replicates). For single cell analysis (dCFRAP), the number of the individual ROIs obtained from several cells in at least three independent experiments is provided in the figure legends. Data from individual ROIs are summarized for data presentation providing the sum of independent observations. Significance levels are given as: *P < 0.05; **P < 0.1; ***P < 0.001. Data in FRAP curves and bar graphs (FRAP) are presented as mean ± s.e.m.

**Data availability.** The data that support the findings of this study are presented within the article and its Supplementary Information file and from the corresponding author upon reasonable request. All constructs originally described in this study can be obtained and used without limitations for non-commercial purposes on request from the corresponding author.

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J.P., S.C.W., D.R., P.M., J.C., S.W., J.S., N.L., and A.V. performed experiments, produced data for presentation. J.P., S.C.W., D.R., P.M., J.C., S.W., N.L., A.V., A.F., and G.S. planned experiments, analyzed data, and compiled figures. S.C.W., J.P., and G.S. wrote the paper.

Additional information

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