Structural insight into small molecule action on Frizzleds

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WNT-Frizzled (FZD) signaling plays a critical role in embryonic development, stem cell regulation and tissue homeostasis. FZDs are linked to severe human pathology and are seen as a promising target for therapy. Despite intense efforts, no small molecule drugs with distinct efficacy have emerged. Here, we identify the Smoothened agonist SAG1.3 as a partial agonist of FZD6 with limited subtype selectivity. Employing extensive in silico analysis, resonance energy transfer- and luciferase-based assays we describe the mode of action of SAG1.3. We define the ability of SAG1.3 to bind to FZD6 and to induce conformational changes in the receptor, recruitment and activation of G proteins and dynamics in FZD–Dishevelled interaction. Our results provide the proof-of-principle that FZDs are targetable by small molecules acting on their seven transmembrane spanning core. Thus, we provide a starting point for a structure-guided and mechanism-based drug discovery process to exploit the potential of FZDs as therapeutic targets.
protein-coupled receptors (GPCRs) are membrane proteins, which constitute as much as 30% of all drug targets. However, of the ~800 GPCRs in human only a small fraction is targeted by FDA-approved drugs leaving a large untapped, therapeutic potential in the remaining receptors. The Class F (Frizzled; FZD) of GPCRs, which consists of ten FZD paralogues (FZD1–10) and Smoothenoned (SMO) is critically involved involved in embryonic development, organogenesis, stem cell regulation, and in the development of diverse pathologies, such as different forms of tumors, fibrosis, bone disease, cardiovascular conditions, and neurological disease. While there are several small molecules available that target SMO as agonists (SAG1.3, SAG1.5, and purmorphamine), inverse agonists (cyclopamine-KAAD), and neutral antagonists (vismodegib and SANT–1), no small molecules with clear-cut pharmacology have been identified targeting any FZD. Given their involvement in pathology, FZDs harbor a huge therapeutic potential and therefore, drugging FZDs has attracted substantial attention. Interestingly, the crystal structure of FZD6, which presents a ligand-free receptor inferred that development of small molecules targeting the core of FZDs can be virtually impossible given the hydrophilic nature of the binding pocket, a notion that has previously been challenged. In addition, the concept of allosteric modulators has been explored with the small molecules FzM1 and FzM1.8, which were characterized as negative and ago-positive allosteric modulators, respectively, acting on the third intracellular loop (ICL3) of FZD4 with low degree of selectivity.

The WNT family of lipoglycoproteins constitutes endogenous agonists for FZDs, activating the receptor through interactions with its extracellular cysteine-rich domain (CRD). Intracellularly, FZDs interact with Dishevelled (DVL), which is a signaling hub to mediate β-catenin-dependent and planar cell polarity (PCP)-like WNT signaling. Furthermore, heterotrimeric G proteins interact with FZDs to initiate a network of G protein-dependent signaling pathways. One of the explanations for the absence of FZD-targeting small molecule compounds is the lack of high-throughput assays that monitor FZD activation more directly than the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcriptional reporter (TopFlash) assay can do. The TopFlash assay has the clear disadvantages that (i) not all FZDs, particularly not FZD3 and FZD6, mediate WNT/β-catenin signaling and (ii) it does not cover all signaling pathways that branch off from activated FZDs, such as PCP or G protein-dependent signaling pathways. Of these models, we selected the one with the best DOPe score for further studies. The selected FZD6 model (active FZD) subsequently underwent molecular dynamics (MD) simulations for 200 ns (in two independent replicas) in the ligand-free state in order to relax the structure. Consequently, SAG1.3 (Fig. 1b) was docked to the binding site in the transmembrane core of the receptor, defined by the location of the cocrystallized SAG1.5 in the SMO crystal structure (PDB ID: 4QIN, Fig. 1c). To compare SAG1.3–FZD6 interactions with those present in a SAG1.3–SMO complex, we used the SAG1.5–SMO crystal structure, in which we modified the agonist by substituting the fluorine atoms for hydrogen atoms. Subsequently, the MD simulations were run for additional 3 µs (1 µs in three independent replicas) and 600 ns (200 ns in three independent replicas) with SAG1.3–FZD6 and SAG1.3–SMO complexes, respectively (Fig. 1d, e, Supplementary Figs. 2 and 3).

Provided the recent insight into SMO activation in a ternary complex of ligand, receptor, and heterotrimeric G protein (PDB ID: 6OT0), we built also a FZD6 model based on the active SMO crystal structure, in which we modified the agonist by substituting the fluorine atoms for hydrogen atoms. Subsequently, the MD data (three independent replicas of 1 µs each) were then used for a retrospective analysis of the binding site and interactions of SAG1.3 in complex with active-like FZD6. To avoid misleading interpretations, we consider only one of the 1 µs replica of the simulations of the SAG1.3-bound inactive FZD6 in all analyses; the N-terminus and extracellular loops in the other two replicas started to undergo rapid and noisy fluctuations after 400 ns and 600 ns of the simulations, respectively (most probably due to the instability in the homology model). With the active-like FZD6 model, such behavior did not occur, and all 3 µs of data are considered in all analyses (Fig. 1d, e, Supplementary Fig. 3).

The overall binding location of SAG1.3 remained robustly similar in both studied proteins throughout different simulations (Fig. 1, Supplementary Figs. 2–4), suggesting that FZD6 has a binding site for SAG1.3 in the transmembrane core of the receptor between the TM5, TM6, TM7, and the extracellular loop 2 (ECL2), similar to SMO. When comparing this binding area to the structure of FZD6 (PDB ID: 6BD4, after 3 × 200 ns MD simulations), the extracellular portion of TM6 in FZD4 together with the ECL3 clash heavily with the suggested SAG1.3 binding

**Results**

**The SMO ligand binding pocket is similar in FZD6.** Phylogenetic analysis of Class F receptors and sequence alignment of human SMO, FZD6, and FZD4 indicate substantial homology among these receptors. However, FZD6 shows a higher degree of sequence similarity with SMO than it does with FZD1 at regions corresponding to the small-molecule binding pocket within the 7TM core (as observed in SMO; Fig. 1a and Supplementary Fig. 1), suggesting also functional similarities. Here, we chose to compare FZD6 to FZD4 and SMO because crystal structures of FZD4 and SMO allow comparison on the atomic level. Despite the differences that SMO mediates hedgehog signaling and FZD6 mediates WNT signaling, both are characterized by their ability to couple to and activate heterotrimeric G proteins, and their inability to signal via the WNT/β-catenin pathway. FZD6 and SMO are both characterized by a long TM6 extending above the plasma membrane toward the CRD, and the longest C-terminal domains across Class F receptors (SMO: 250 aa; FZD6: 211 aa). Thus, the similarities of FZD6 and SMO compared to FZD4 provided the basis of our efforts of profiling SMO agonists for FZD6.

**In silico analysis of SAG1.3–FZD6 interactions.** As the putative small-molecule binding pockets in the transmembrane domain of FZD6 are unknown, we built 15 homology models of FZD6 using the ΔCRD SMO–taladegib (PDB ID: 4JKV) complex as a template. Of these models, we selected the one with the best DOPe score for further studies. The selected FZD6 model (active FZD) subsequently underwent molecular dynamics (MD) simulations for 200 ns (in two independent replicas) in the ligand-free state in order to relax the structure. Subsequently, SAG1.3 (Fig. 1b) was docked to the binding site in the transmembrane core of the receptor, defined by the location of the cocrystallized SAG1.5 in the SMO crystal structure (PDB ID: 4QIN). To compare SAG1.3–FZD6 interactions with those present in a SAG1.3–SMO complex, we used the SAG1.5–SMO crystal structure, in which we modified the agonist by substituting the fluorine atoms for hydrogen atoms. Subsequently, the MD simulations were run for additional 3 µs (1 µs in three independent replicas) and 600 ns (200 ns in three independent replicas) with SAG1.3–FZD6 and SAG1.3–SMO complexes, respectively (Fig. 1d, e, Supplementary Figs. 2 and 3).
site underlying a structural basis for ligand–receptor selectivity (Fig. 1c)⁷. Sequence alignment of these receptors supports this observation, since the parts of TM6 and ECL3 that construct the binding site of SAG1.3 in FZD6 and SMO are for the most part missing from the sequence of FZD 4 (Fig. 1a, Supplementary Fig. 1).

When zooming in to the details of ligand–receptor interaction, the SAG1.5-SMO crystal structure provides two main interactions, the D473⁶.54-amine nitrogen of SAG1.5 and the N219-amide oxygen of SAG1.5 (Ballesteros-Weinstein numbering of GPCRs⁸). MD simulations (3 × 200 ns) suggest that binding of SAG1.3 to SMO is more versatile than binding of SAG1.5.
Fig. 1 The binding pocket of FZDα accommodates SAG1.3. a Sequence alignments of the binding pockets of the human SMO, FZDα, and FZDβ (Supplementary Fig. 1 and Supplementary Data file 3). Red squares indicate residues in close proximity (<4 Å) between SAG1.3 and the receptor from the SMO and FZDα molecular dynamics (MD) simulations. b Structure of SAG1.3. The bold nitrogen represents the N2 referred to in the MD simulations below. c Comparison of the SAG1.3 binding sites of SMO and FZDα (inactive model; upper panel), and SMO and FZDα (lower panel) underlining the inability of FZDα to accommodate a ligand-like SAG1.3 in this binding space because of the short TM6 (red arrow). d The last frames from the selected MD simulations of the SAG1.3-SMO (left panel) and the SAG1.3-FZDα complexes (inactive model: middle panel, active-like model: right panel) with the important residues of the binding site depicted as sticks. Different positions of SAG1.3 throughout the time of simulation are indicated by transparent SAG1.3 molecules in the binding pocket. e Distance plots over simulation time between SMO D4736.54–SAG1.3, SMO ES18.38–SAG1.3, FZDα E4386.54–SAG1.3, and R4426.58–SAG1.3 (inactive and active-like models), which are predicted to form H-bonding interactions important for stabilizing the SAG1.3 binding conformation. The dotted line (red) indicates the maximum distance (4 Å) that is still likely to allow polar interactions. Thick traces indicate the moving average smoothed over a 2 ns window and thin traces represent raw data. The origin of the y-axis for all graphs e is 0 Å.

(Supplementary Fig. 2). The only structural differences between these two ligands are two fluorine atoms at the benzo thiophene ring of SAG1.5 (Supplementary Fig. 2a), suggesting that this bulk restrains the conformational freedom of SAG1.5 in its binding site leading to a more stable binding pose to SMO (Supplementary Fig. 2b), which typically corresponds to tighter binding. This interpretation is in accordance with the previously published affinities of these two ligands. SAG1.5 showed ~2–10-fold higher affinity to SMO than SAG1.3.31,32. In the SAG1.3–SMO MD simulations the complex of N219 with the amide oxygen remained in the hydrogen bonding distance (<4 Å) throughout the simulations (Supplementary Fig. 2c), whereas the protonated amine nitrogen (labeled as N2 throughout our study) of SAG1.3 was more often interacting with E5186.38 than D4736.54 (Fig. 1d, e, Supplementary Figs. 2d, e and 4). Additionally, D384 in ECL2 remains within 4 Å distance from N2 in approximately half of the MD frames (Supplementary Fig. 4).

In complex with the inactive FZDα, N2 of SAG1.3 interacted quite robustly with E4386.54 throughout the 1 μs simulation (Fig. 1d, e, Supplementary Figs. 3a and 4). The simulation with active-like FZDα strengthens this observation further. E4386.54 remains at a hydrogen-bonding distance to N2 over 75% of all 3 μs of these MD trajectories (Fig. 1d, e, Supplementary Figs. 3d and 4). The rest of the time, N2 of SAG1.3 is interacting with D351 at ECL2 (D384 in SMO; Supplementary Fig. 3e and 4). Interestingly, the active-like FZDα simulation produced two clear clusters of binding poses, whereas in the inactive FZDα simulation the SAG1.3 poses—apart from the fact that they maintain N2–E4386.54 interaction—were notably more deviant (Fig. 1d, Supplementary Fig. 3f, g). Unlike SMO, the FZDα binding site contains only these two negatively charged amino acid residues able to interact with N2 of SAG1.3; thus SAG1.3 does not change the charge-assisted hydrogen-bonding partner to amino acid 7.38 in FZDα as it does in SMO (Fig. 1a, d, Supplementary Fig. 4).

In the active-like FZDα simulations, the average distance between N187 of FZDα (corresponding to N219 of SMO) and the amide oxygen of SAG1.3 is ~5 Å (Supplementary Figs. 3h and 4). Even though the distance is most of the time too long to suggest direct hydrogen bonding, a water-mediated hydrogen bond is highly possible (Supplementary Fig. 3i). Unlike the active-like FZDα model, the inactive model rarely poses N187 in the vicinity of the amide oxygen of SAG1.3, but R4426.58 remains there instead (Supplementary Fig. 3b, c). In the active-like model, R4426.58 interacts rather with the chlorine atom of SAG1.3 (Fig. 1d, e).

In the SAG1.3–SMO crystal structure, the corresponding MD simulations, and the SAG1.3–SMO and SAG1.3–FZDα MD simulations, the two aromatic ends of the SAG derivatives form a stacked π–π complex located at a sub-pocket lined by aromatic amino acid residues of the TM6 and the ECL2 (Fig. 1a, d, Supplementary Fig. 4). Due to the different sizes of these residues (F4846.65 in SMO vs. W4496.65 in FZDα), the aromatic end of SAG1.3 occupies a slightly different space and obtains a slightly different orientation in these two receptors (Fig. 1d). As SAG1.3 is a relatively rigid molecule (only five rotatable bonds, which contribute to its overall shape), the orientation of the aromatic part of the molecule restricts the available locations of the hydrogen-bonding functional groups of SAG1.3 at its binding site. Even though the SAG1.3–SMO (based on the MD data), SAG1.5–SMO (based on the crystal structure and the MD data), and the SAG1.3–FZDα (based on the MD data) complexes share similar hydrogen-bonding characteristics (Supplementary Figs. 2–5), the apparent fit of the aromatic ends of SAG derivatives to these receptors may be one of the factors contributing to differences in affinity to SMO and FZDα. Please see Supplementary Figs. 6 and 7, and Supplementary Data files 1–30 for the details of all the MD simulation runs.

SAG1.3 binds to FZDα Pharmacological analysis of ligand–receptor interactions is best studied using direct ligand binding experiments. Here, we employed a recently established assay format based on NanoBRET detection between a BODIPY-tagged ligand and a nanoluciferase (Nluc)–tagged receptor (Fig. 2a; summary of FZDα constructs in Supplementary Fig. 8). The interaction of BODIPY–cyclopamine with SMO allows thorough characterization of SMO-binding ligands.34 Given the similarities of SMO and FZDα in the ligand binding pocket, we used BODIPY-cyclopamine as a probe for FZDα. Moreover, in order to exclude endogenously expressed SMO as a confounding factor in the BODIPY-cyclopamine-based binding assay, we generated a ΔSMO HEK293 cell line devoid of this GPCR using CRISPR/Cas9 genome editing (Supplementary Fig. 9). BODIPY–cyclopamine binding to Nluc–FZDα resulted in monophasic and saturable concentration-dependent BRET signals (Fig. 2b; BODIPY–cyclopamine pKd ± s. d. = 6.3 ± 0.1; refer to Supplementary Fig. 10a for the assessment of cell membrane expression of the constructs and Supplementary Fig. 10b/Supplementary Data file 31 for FZDα–BODIPY–cyclopamine docking poses). Additionally, BRET was dependent on donor expression levels and acceptor-donor ratio and was not directly proportional to the acceptor levels arguing for specificity of BODIPY–cyclopamine to Nluc–FZDα binding (Supplementary Fig. 10c). In competition experiments, increasing concentrations of unlabeled SAG1.3 decreased BODIPY–cyclopamine (300 nM) binding to Nluc–FZDα in a concentration-dependent manner (Fig. 2c, SAG1.3 pKd ± s. d. = 5.6 ± 0.1). Similarly, a fixed concentration of SAG1.3 (10 μM) right shifted the BODIPY–cyclopamine binding curve (Supplementary Fig. 10d, BODIPY–cyclopamine with SAG1.3 (10 μM) pKd ± s. d. = 5.8 ± 0.2) and the BODIPY–cyclopamine binding curve was right shifted in the presence of 10 μM unlabeled cyclopamine (Supplementary Fig. 10e). Importantly, the SAG1.3–induced reduction in BODIPY–cyclopamine/Nluc–FZDα NanoBRET was not due to a nonspecific effect on luminescence or fluorescence.
(Supplementary Fig. 10f). In addition, the NanoBRET-based binding assay has the advantage of a low contribution of nonspecific binding in general as seen for other GPCRs and in particular for Class F receptors, as we recently characterized during the establishment of the assay for Nluc–SMO and BODIPY–cyclopamine binding. In conclusion, we provide here experimental data arguing that BODIPY–cyclopamine interacts with FZD₆ and that SAG1.3 interferes with BODIPY–cyclopamine interaction.

A FZD₆–FRET probe monitors SAG1.3 binding and efficacy. In order to design a functional measure for ligand–FZD₆ interactions, we designed an intramolecular FRET probe to study conformational changes of the receptor upon ligand binding. On the basis of conformational changes in active Class A and B GPCRs, the previously validated probes for other GPCRs and particularly FZD₁₆,₁₆,₃₆ and the information from the recently published active SMO structures, we created an intramolecular FZD₆–FRET probe. The probe, which was designed to monitor agonist-induced conformational changes of FZD₆ in living cells, consists of the FRET donor (TFP) at the C-terminus and the FRET acceptor FlAsH (fluorescein arsenical hairpin binder-ethanedithiol, FlAsH–EDT₂)-binding motif inserted between G404 and R405 in the ICL3 (Fig. 3b).

The FZD₆–FRET sensor was detectable on the cell surface using confocal microscopy assessing TFP fluorescence, and we therefore conclude that it is efficiently trafficked to the cell membrane (Supplementary Fig. 11a). Basal energy transfer between the fluorophores was determined as FRET efficiency of the sensor (4.4 %) by using BAL (2,3-dimercapto-1-propanol) as an antioxidant (Supplementary Fig. 11b). In order to exclude energy transfer between individual receptors, for example in a FZD₆ dimer, we assessed intermolecular FRET between FZD₆–TFP and FZD₆–FlAsH–PK and detected no measurable energy transfer (Supplementary Fig. 11c).

To quantify the efficacy of the endogenous ligand of FZD₆ in this assay, we analyzed the effect of increasing concentrations of WNT-5A using the FZD₆–FRET probe. The maximal response to WNT-5A defines the full agonist in this assay with a log₁₀ EC₅₀ ± s.d. (ng ml⁻¹) = 2.2 ± 0.1 and the maximum efficacy at 1000 ng ml⁻¹ reaching 4.9% (FRET ratio = 95.1% of basal; Fig. 3c). Since WNT-5A is not membrane permeable, the effect of ligand stimulation on the FZD₆–FRET probe further corroborates efficient trafficking of FZD₆–FlAsH–TFP to the plasma membrane. Stimulation of HEK293 cells transiently expressing the FZD₆–FRET probe with SAG1.3 resulted also in a sigmoidal, concentration-dependent decrease in the FRET ratio by 1.7 % of the basal FRET ratio with a pEC₅₀ ± s.d. (M) = 6.5 ± 0.9 (Fig. 3d).

Our data demonstrate that (i) SAG1.3 binds to FZD₆, (ii) that the polar residues D351, E438, and R442 have an important role in small-molecule binding, (iii) that agonist binding to FZD₆ evokes conformational changes that are detectable by the FZD₆–FRET sensor reminiscent of movements observed in activated Class A/B GPCRs and SMO, and (iv) that SAG1.3 acts as a FZD₆ partial agonist in this assay.

**SAG1.3 induces mini Gₛᵣ recruitment to FZD₆.** In order to further explore the mode of action of SAG1.3 on FZD₆, we made use of Venus-tagged mini G (mG) proteins, which serve as BRET-compatible, conformational sensors of the ligand-bound, active state of GPCRs. Similar to what we have shown before for FZD₆ and other Class F receptors, we used SNAP–FZD₆–Rluc8 and FLAG–FZD₆–Nluc (BRET donor; see Supplementary Fig. 12 for analysis of membrane expression of FLAG–FZD₆–Nluc) in combination with Venus–mGₛᵣ (BRET acceptor) transiently overexpressed in HEK293 cells to monitor WNT-5A–induced Venus–mGₛᵣ recruitment to FZD₆ thereby defining the assay response with the physiological, full agonist (Fig. 4b, c). Further, we established the concentration–response relationship for both FZD₆ constructs in combination with Venus–mGₛᵣ using SAG1.3 (Fig. 4d, e). Interestingly, SAG1.3 induced a biphasic concentration–response curve similar to what was previously reported for SAG–SMO responses in the same assay format as well as in other assays. In order to exclude a functional role of the WNT-binding CRD for the SAG1.3-induced and FZD₆–mediated Venus–mGₛᵣ recruitment, we compared △CRD and full-length FLAG–FZD₆–Nluc constructs (Fig. 4e; see Supplementary Fig. 12 for analysis of membrane expression of △CRD FLAG–FZD₆–Nluc). Irrespective of the presence or absence of the CRD, SAG1.3 evoked similar, concentration-dependent Venus–mGₛᵣ recruitment confirming the location of the SAG1.3 binding site in the receptor core and the irrelevance of the CRD for receptor-activating conformational changes.
SAG1.3 action on FZD6 is independent of SMO. Since SAG1.3 was designed as SMO agonist, it appeared crucial to exclude a contribution of endogenously expressed SMO to the observed SAG1.3-induced and FZD6-mediated effects. SMO is also a Gi/Go-coupled receptor and it is expressed in HEK293 cells\(^{15,20,43}\). Thus, BRET mGsi recruitment assays were performed in the ∆SMO HEK293 cells using SNAP–FZD\(_6\)–Rluc8 and Venus–mGsi in combination with increasing concentrations of SAG1.3. A similar biphasic concentration–response curve was observed (Fig. 4f). Furthermore, we compared ∆CRD and full-length FLAG–FZD\(_6\)–Nluc with regard to their ability to recruit Venus–mGsi in ∆SMO HEK293 cells (Fig. 4g), further supporting the concept that SAG1.3 targets the receptor core. In line with the results of the indirect binding assay with the FZD6 intramolecular FRET sensor, SAG1.3 elicited a smaller maximum Venus–mGsi recruitment compared to the highest WNT-5A concentration used, underpinning the partial agonist nature of SAG1.3. In order to define subtype selectivity of SAG1.3 toward FZD\(_6\) over FZD\(_4\), we also assessed SAG1.3-induced Venus–mG recruitment to FZD\(_4\)–Nluc using Venus–mG\(_{13}\)\(^{15,44}\). In agreement with the in silico structural analysis, which suggested that SAG1.3 would not bind this FZD subtype, we did not detect any SAG1.3-induced Venus–mG13 recruitment (Fig. 4h). On the other hand, we tested FZD\(_7\), a Class F receptor from the FZD1,2,7 homology cluster, and the ability of SAG1.3 to induce Venus–mGs recruitment to SNAP–FZD\(_7\)–Rluc8\(^{15}\). SAG1.3 induced a biphasic concentration–response curve similar to what we observed for FZD\(_6\), indicating that SAG1.3 does not only act at FZD\(_6\) but also on other FZD subtypes (Supplementary Fig. 13a). Indeed, the comparison of models of FZD\(_6\) and FZD\(_7\) on the atomistic level revealed large similarities in their SAG1.3 binding site (Supplementary Fig. 13b,c), in contrast to the one of FZD\(_4\). MD simulation of FZD\(_4\) bound to SAG1.3 further underlined that the receptor–ligand interaction is stable for the time of the simulation (Supplementary Fig. 13d).

SAG1.3 promotes G protein and ERK1/2 activation. In order to further validate that SAG1.3 acts as a functional FZD\(_6\) agonist, capable of initiating downstream signaling in a G protein-dependent manner, we made use of heterotrimeric NanoBiT G proteins\(^{45}\). For this purpose, ∆SMO HEK293 cells were transfected with receptor or pcDNA, the G\(_{i1}\) and G\(_{b2}\) subunits fused to complementary parts of a modified Nluc (LgBiT and SmBiT) and the untagged Gy2 (Fig. 5a). First, we used the muscarinic M\(_3\) receptor as a prototypical G\(_i\)-coupled receptor with acetylcholine (ACh), to demonstrate that we can detect a ligand-induced decrease in Nluc luminescence (Nluc\(_{\text{fim}}\)) indicative of the dissociation of the heterotrimeric G\(_i\) protein (Supplementary Fig. 14, pEC\(_{50}\) ± s.d. (M) = 7.3 ± 0.3; in agreement with http://}

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**Fig. 3 SAG1.3 induces conformational changes in FZD6.** **a** The model of the active-like FZD\(_6\) (blue) showing a pronounced outward-motion (Δ) of the TM6 as compared to the inactive model (gray), justifying positioning of FRET acceptor and donor in the ICL3 and C-terminus, respectively. **b** The scheme depicts the FZD\(_6\)–FlAsH–TFP construct. The FlAsH-binding motif (FLNCCPGCCMEEP) was inserted into the ICL3, between G404 and R405. Receptor activation is predicted to result in a loss of FRET due to conformational rearrangement in accordance to previous data obtained for FZD\(_5\)\(^{16}\). The FRET ratio change induced by each concentration was normalized to basal FRET ratio. Data are presented as mean ± s.e.m. of total n = 5 individual experiments. **d** SAG1.3 induces a concentration-dependent decrease of the FRET ratio (FlAsH/TFP) in HEK293 cells overexpressing FZD6. The FRET ratio change induced by each concentration was normalized to basal FRET ratio. Data are presented as mean ± s.e.m. of total n = 7 individual experiments. Source data are provided as a Source Data file.
Then we used SAG1.3 to monitor its ability to induce G<sub>i</sub> heterotrimer dissociation in a FZD<sub>6</sub>-dependent manner, excluding the contribution of endogenous SMO by using ΔSMO HEK293 cells. Similar to the Venus–mGsi protein recruitment assay, SAG1.3 elicited a bell-shaped concentration response only when SNAP–FZD<sub>6</sub> was coexpressed (Fig. 5b).
GPCR-mediated activation of heterotrimeric G\textsubscript{i,o} proteins leads to phosphorylation and activation of extracellular signal-regulated kinases 1/2 (ERK1/2)\textsuperscript{46} and we have previously shown that FZD\textsubscript{6} mediates ERK1/2 phosphorylation\textsuperscript{15,25}. To further support the positive efficacy of SAG1.3 acting on FZD\textsubscript{6} resulting in G protein-dependent signaling, we quantified ERK1/2 phosphorylation in lysates of ΔSMO HEK293 cells transfected with SNAP–FZD\textsubscript{6} and stimulated with SAG1.3. These experiments were performed in the presence of endogenous G proteins. Further the autocrine stimulation by endogenously produced WNTs was blocked by pretreatment with the porcupine inhibitor C59 (5 nM). In agreement with our data so far, SAG1.3 induced a biphasic concentration-dependent ERK1/2 phosphorylation only when FZD\textsubscript{6} was transiently overexpressed in ΔSMO HEK293 cells (Fig. 5c).

**Fig. 5 SAG1.3 induces FZD\textsubscript{6}-dependent dissociation of heterotrimeric G\textsubscript{i} and phosphorylation of ERK1/2.**

**a** Schematic view of the split NanoBiT luciferase assay. Ligand stimulation of a GPCR results in dissociation of the heterotrimeric G protein and a decrease in the Nluc luminescence.

**b** SAG1.3 stimulation of SNAP–FZD\textsubscript{6} transiently overexpressed in ΔSMO HEK293 cells resulted in a concentration-dependent, biphasic decrease in basal luminescence as a measure of the dissociation of the G\textsubscript{αi1}-LgBiT, SmBiT-G\textsubscript{β5}, and G\textsubscript{γ2} complex (filled black circles). pcDNA served as no-receptor-control (open gray circles). Data are represented as mean ± s.e.m. of \( n = 6 \) individual experiments. * \( P < 0.05 \), ** \( P < 0.01 \) (one-way ANOVA). Source data are provided as a Source Data file.

**c** SAG1.3 (10 min) induced phosphorylation of ERK1/2 (P-ERK1/2) in a biphasic manner only in SNAP–FZD\textsubscript{6}-transfected ΔSMO HEK293 cells. Serum starved cells were pretreated with C59 (5 nM; overnight). Representative immunoblots are shown. Data are presented as mean ± s.e.m. of \( n = 4 \) individual experiments; \( F(4,14) = 3.141 \). * \( P < 0.05 \), ** \( P < 0.01 \) (one-way ANOVA). Source data are provided as a Source Data file.
colocalization and recruitment of cytosolic DVL present in punctate aggregates to membrane-expressed FZDΔs. However, quantification of recruitment and measurement of ligand-induced dynamics were not possible. Employing direct BRET, it was recently shown that the FZDΔ-selective agonist Norrin enhances FZDΔ-DVL interaction. In order to assess agonist-induced effects on FZDΔ-DVL interactions, we used WNT-5A and SAG1.3 in two different experimental paradigms of BRET-based assays. First, we assessed the proximity of Nluc–DVL2 to SNAP–FZDΔ or FLAG–FZDΔ–His indirectly in a bystander BRET assay, where the authors used YFP–DVL2 (BRET acceptor) and FZDΔ–Rluc (BRET donor). However, it is envisaged that fusing the BRET acceptor to FZD circumscribes the potential outcomes arising from DVL polymerization at high expression levels required for validation of the assay by a saturation curve. In order to assess ligand-induced effects on DVL–FZD BRET, we chose an acceptor:donor ratio corresponding to the plateau part of the saturation curves for both setups. In addition, we did not treat the cells with the porcine inhibitor C59 to block secretion of endogenous WNTs as their presence had no significant effect on the basal recruitment of the overexpressed DVL2 to the overexpressed FZDΔ as recently reported and presented in Supplementary Fig. 15a, b. To avoid any input of endogenous FZDs or SMO, we used ΔFZD1,10 HEK293 cells to study WNT-5A-induced effects and ΔSMO HEK293 cells to study SAG1.3-induced effects. As shown in Fig. 6b, c, f, g, both ligands increased BRET between Nluc–DVL2 and Venus–KRas or FZDΔ–Venus in a concentration-dependent manner. Interestingly, SAG1.3 evoked FZDΔ–DVL2 BRET changes with lower potency than SAG1.3-induced G protein-related events. Moreover, SAG1.3 did not show the bell-shaped concentration–response when monitoring FZD–DVL recruitment. SAG1.3 also displayed a positive efficacy on ΔCRD FZDΔ (Fig. 6d, g). Further, we have validated the assays and SAG1.3-selectivity using pcDNA- and SNAP–FZDΔ-transfected ΔSMO HEK293 cells (Fig. 6h). Biochemically, we were able to detect a SAG1.3-induced electrophoretic mobility shift of the endogenous DVL2 indicative of its phosphorylation and activation in the SNAP–FZDΔ but not control-transfected ΔSMO HEK293 cells (Supplementary Fig. 15c). Finally, we confirmed that SAG1.3 (10 μM) does not activate FZDΔ-specific TopFlash activity in ΔFZD1,10 HEK293 cells (Fig. 6i), arguing for the subtype selectivity of this small-molecule ligand.

WNT and SAG1.3 stimulation increased BRET in both experimental paradigms. However, given the ratio of receptor–DVL expression (Supplementary Fig. 14) and the nature of BRET as a readout, we cannot differentiate clearly between an increase in FZD–DVL recruitment in a 1:1 ratio, DVL polymerization in close proximity to the receptor or a rearrangement of the FZD–DVL complex in response to agonist affecting distance or dipole orientation. Nevertheless, a change in FZD–DVL BRET can serve as a functional readout of FZD ligands keeping the caveats of this technique in mind.

**Mutational analysis of the SAG1.3 binding site.** Having established a diverse set of functional readouts for FZDΔ activation by SAG1.3 allowed now a mutagenesis analysis of residues involved in SAG1.3 interactions. The MD simulations in SAG1.5- and SAG1.3-bound SMO, and SAG1.3-bound FZDΔ using the inactive- and active-like models provided detailed insight into the engagement of residues in SAG–derivative interactions over time (Fig. 1d, Supplementary Fig. 4). In this analysis, D351, E438Δ5, K479Δ1, and R442Δ5,8 emerged as the most relevant, polar residues, which were included in a mutagenesis approach (Supplementary Fig. 16). SNAP-tagged receptor mutants were tested for their cellular and membranous expression in comparison to pcDNA- and SNAP–FZDΔ-transfected ΔSMO HEK293 cells (Supplementary Fig. 16a). While these proteins are indeed translated, mutation of these residues dramatically affects receptor maturation and cell surface expression. Only E438DΔ5, K442Δ5,8, and R442Δ5,8 were detectable at the membrane albeit at lower levels compared to wild-type SNAP–FZDΔ (Supplementary Fig. 16a). Nevertheless, surface expression mirrored the ability of the receptor mutants to recruit Nluc–DVL2 to the membrane assessed by bystander BRET using Nluc–DVL2 and Venus–KRas (Supplementary Fig. 16b). In order to provide biologically sound and meaningful data, we only used three mutants showing surface expression and DVL recruitment to assess SAG1.3-induced effects. Mutation of the SAG1.3 binding site in FZDΔ affected the ability of SAG1.3 to induce mGsi and Nluc–DVL2 recruitment and G protein activation (Supplementary Fig. 16c, d, e). In general, SAG1.3-induced responses of E438DΔ5,54 and R442KΔ5,8 mutants showed lower efficacy and potency when compared with the wild-type receptor (Supplementary Figs. 16c, d, e and 17). Furthermore, SAG1.3 stimulation of the R442AΔ5,8 mutant resulted in hardly detectable responses corroborating even weaker SAG1.3 interactions. Thus, mutating residues to their chemically conserved counterparts allows preserving a somewhat functional SAG1.3 binding site, whereas alanine mutation does not. While functional assays are directly affected by the fraction of the receptor protein that is trafficked to the cell membrane, we reasoned that assessment of ligand affinity could be a suitable complement to quantify the direct involvement of key residues in the FZDΔ binding site. We focused on FZDΔ, R442Δ5,8 and its alanine mutation comparing the affinity of BODIPY–cyclopamine to FZDΔ and FZDΔ, R442AΔ5,8 in the presence and absence of 10 μM SAG1.3 (Supplementary Fig. 18). Since the presence of SAG1.3 right shifted BODIPY–cyclopamine binding only in the case of wild-type FZDΔ, we concluded that R442AΔ5,8—in agreement with the in silico predictions and functional assay—is a key component of the SAG1.3 binding site.

In order to further support our findings concerning the importance of N2–E438Δ5,54 interaction, we provide MD data investigating the likelihood of interaction for inactive FZDΔ with a mock SAG1.3 (mock ligand), where we introduced a carbon (C7) instead of the N2 nitrogen (Supplementary Fig. 19a). MD simulations of FZDΔ with SAG1.3 in comparison to mock ligand (3 × 200 ns) and distance plots between the E438Δ5,54 and either N2 of SAG1.3 or the C7 of the mock ligand indeed argue that SAG1.3 binds closer to E438Δ5,54 than the mock ligand over the time course of the simulation (Supplementary Fig. 19b).

**Purmorphamine is also a FZDΔ agonist.** In addition to SAG derivatives, purmorphamine presents another, structurally unrelated SMO agonist that is surmountable by the inverse agonist cyclopamine–KAAD (Supplementary Fig. 20a)54,55. In order to support the broader applicability of our findings, we also examined purmorphamine–FZDΔ interaction by in silico docking and performed a pharmacological characterization of purmorphamine activity using two key assays presented in this work. In
silico docking indicated that the purmorphamine binding site overlaps substantially with that of SAG1.3 (Supplementary Fig. 20b), in agreement with previous pharmacological characterization \(^{55}\). Compared to SAG1.3 purmorphamine promoted a similar, concentration-dependent recruitment of Venus–mGsi to SMO–Rluc8 and SNAP–FZD\(_6\)–Rluc8, albeit with lower potency and without the distinct bell-shaped pattern (Supplementary Fig. 20c, d). Since the mGsi protein serves as sensor of the active FZD\(_6\) conformation feeding into heterotrimeric G protein signaling, we conclude that purmorphamine binding to

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Fig. 6 SAG1.3 modifies the interactions between FZD₆ and DVL2. a Schematic illustration of the bystander BRET setup to detect SNAP-FZD₆-induced recruitment of Nluc–DVL2 to membrane bound Venus–KRas. b WNT-5A stimulation of SNAP-FZD₆-transfected ΔFZD₁₀ HEK293 cells increased the bystander BRET ratio in a concentration-dependent manner (filled black circles; total n = 5 individual experiments). WNT-5A did not affect BRET in cells transfected with pcDNA (open gray circles; total n = 3 individual experiments). c Bystander BRET ratio changes (Nluc–DVL2 and Venus–KRas) assessed in ΔSMO HEK293 cells in response to increasing concentrations of SAG1.3 in the presence of SNAP-FZD₆ (black filled circles; total n = 9 individual experiments) or pcDNA (open gray circles; total n = 5 individual experiments). d Bystander BRET ratio changes (Nluc–DVL2 and Venus–KRas) assessed in ΔSMO HEK293 cells in response to increasing concentrations of SAG1.3 in the presence of FLAG–FZD₆–His (total n = 6 individual experiments) or ΔCRD FLAG–FZD₆–His (total n = 6 individual experiments) in the ΔSMO HEK293 cells. e The scheme illustrating the direct BRET setup in which the signal is detected between FLAG–FZD₆–Venus and Nluc–DVL2. f WNT-5A induced BRET ratio indicative of closer interactions between FLAG–FZD₆–Venus and Nluc–DVL2 (total n = 6 individual experiments) in the ΔFZD₁₀ HEK293 cells. g SAG1.3 induced BRET indicative of closer interactions between FLAG–FZD₆–Venus (total n = 11 individual experiments) or ΔCRD FLAG–FZD₆–Venus (total n = 6 individual experiments) and Nluc-DVL2 in the ΔSMO HEK293 cells. h SAG1.3 did not induce the bystander BRET (n = 4 individual experiments) or TOPFlash reporter activity in the ΔSMO HEK293 cells with transiently overexpressed SNAP–FZD₆ (WNT-3A used as a positive control; n = 3 individual experiments; F(2,12) = 88.69. ***p < 0.0001, two-way ANOVA). Data are presented as mean ± s.e.m. Source data are provided as a Source Data file.

FZD₆ results in receptor activation and G protein activation. On the other hand, purmorphamine did not affect FZD₆–DVL2 dynamics (Supplementary Fig. 20e) arguing for a distinct functional selectivity of this ligand.

Discussion

Here, we provide the proof-of-principle that FZDs are druggable with small-molecule ligands targeting the 7TM core of the receptors. This stands in stark contrast to previous claims that the FZD binding pocket might be unfavorable for accommodating small-molecule ligands⁷. Our discovery opens the door for the development of FZD-targeting small molecules interacting with the receptor at a site reminiscent of that of Class A GPCRs and SMO ligands. Based on the data monitoring receptor binding, FZD₆ conformational changes, mG protein association as conformational sensors of the active GPCR state of FZD₆, heterotrimeric G protein dissociation, and FZD₆–DVL2 recruitment, we argue that SAG1.3 acts as a partial agonist with functional selectivity toward G proteins over DVL. SAG1.3-induced effects on FZD₆ were generally moderate but statistically significant calling for medicinal chemistry efforts to expand on our proof-of-principle study (Supplementary Fig. 21). We also provide evidence that SAG1.3 acts at FZD₆ but not FZD₄ and that purmorphamine acts through FZD₆ albeit with lower potency, indicating that different scaffolds exist to initiate a medicinal chemistry optimization. Furthermore, we use BODIPY–cyclcopamine for assessing ligand binding introducing another, sterol-based moiety interacting with FZD₆. While small-molecule agonists will provide an exciting tool to understand FZD activation mechanisms and receptor pharmacology, ligands with negative or no efficacy would be more suitable for anticancer therapy. Inverse agonists or neutral antagonists could provide a useful therapeutic approach in tumors that are driven by high levels of WNTs or constitutively signaling. This is supported by the finding that an active SAG1.3–FZD₆–mGsi complex is stabilized at lower nanomolar concentrations, whereas micromolar SAG1.3 concentrations are required to affect FZD₆–DVL2 interaction. Additional support of this concept is provided by the purmorphamine data, showing a positive efficacy toward mGsi protein but not DVL. Furthermore, SAG1.3 is not merely an allosteric modulator of FZD₆ amplifying basal WNT input as it significantly increases the phosphorylation of ERK1/2 in the absence of endogenous WNTs.

In line with what we have previously proposed⁵,¹⁵ our data suggest that SAG1.3 can stabilize at least two distinct FZD₆ conformations feeding into FZD–G protein and FZD–DVL signaling. This is supported by the finding that an active SAG1.3–FZD₆–mGsi complex is stabilized at lower nanomolar concentrations, whereas micromolar SAG1.3 concentrations are required to affect FZD₆–DVL2 interaction. Additional support of this concept is provided by the purmorphamine data, showing a positive efficacy toward mGsi protein but not DVL. Furthermore, SAG1.3 is not merely an allosteric modulator of FZD₆ amplifying basal WNT input as it significantly increases the phosphorylation of ERK1/2 in the absence of endogenous WNTs.

Employing in silico analysis, we predict that D351, E438⁵,⁶,⁴¹, and R442⁵,⁶,⁴¹ in FZD₆ are key residues involved in polar interactions with SAG1.3 (Fig. 1d). We then generated mutants of these residues (together with K479⁵,⁴¹) and assessed their cellular and membranous expression as well as SAG1.3-induced effects. SAG1.3-induced and receptor-mediated effects were reduced as shown by mGsi and Nluc–DVL2 BRET recruitment and G NanoBiT assays, however, only few mutants are folded and trafficked to the cell membrane albeit not as efficiently as the wild-type FZD₆. This obviously needs to be taken into consideration when interpreting these mutagenesis experiments. BRET experiments are ratiometric, i.e., they do not rely on expression levels per se because the signal is detected only when donor and acceptor come into close proximity (up to 100 Å). Thus, BRET experiments can be used to evaluate and compare ligand-induced effects on non-equally expressed receptors. However, in the case of G protein dissociation measured by the NanoBiT assay, it cannot be ruled out that—given the cell permeability of SAG1.3—events from inner membranes contributes to the response.

Furthermore, we have identified a pan network of interactions involving ECL2, TM6, and TM7 that is part of the proposed FZD₆ ligand binding site. According to our mutagenesis data, this network is crucial to maintain proper protein folding and cell surface trafficking of FZD₆ (Supplementary Fig. 19c)⁴,⁵,⁶,⁵⁷.

The pharmacological profiles of SAG1.3 as an agonist on FZD₆ differ depending on the experimental readout. While SAG1.3-induced conformational changes in the FZD₆–FRET probe and FZD₆–DVL2 interaction assays follow a sigmoidal concentration–response relationship with an EC₅₀ in the lower micromolar range, all G protein-related readouts follow a bell-shaped pattern with higher potency. Remarkably, the shape of the concentration–response curves is similar to what has been reported for SAG1.3-induced and SMO-mediated effects on Gli-1 reporter, mG siRNA, and inositol phosphate accumulation assays⁵,⁰,⁴⁰,⁴¹. However, it remains obscure what the descending part of these SAG1.3 concentration–response curves represents in the context of SMO and FZD₆ signaling⁴⁰,⁴⁸, and interestingly, purmorphamine, which occupies a similar binding site, does not exert a bell-shaped concentration–response curve as SAG1.3 in the G protein-dependent assays. Similarly to what we demonstrate for SAG1.3–FZD₆, it can also be seen for SMO that SAG1.3 potencies differ depending on the assay type and the cell line used (Supplementary Fig. 22). While this could merely represent the differential functionality and sensitivity of the engineered assay probes, it seems more likely that they underline the functional selectivity or ligand bias of the agonist. Similar discrepancies in the pharmacological profiles have been reported for the β₂ adrenergic receptor⁵⁹.

The subtype selectivity of SAG1.3 toward FZD₆ and FZD₄ over FZD₁ is mostly determined by the length of TM6, which in case of a shorter connection to TM7 in FZD₄ traverses through the SAG1.3 binding pocket. While more experiments are necessary to
map the SAG1.3 selectivity for all FZD1–10, it is likely that FZDs with a long TM6 could accommodate this ligand, whereas the homology cluster FZD3,9,10 might not.

In summary, repurposing of a SMO agonist led to the identification of a partial agonist of FZD9, that acts in the 7TM core of the receptor. Medicinal chemistry efforts are now required to define structure–activity relationships of FZD small-molecule ligands to better understand subtype selectivity, and to promote the discovery of diverse pharmacological probes for FZDs as a starting point for the development of therapeutic compounds.

Methods

Computational modelling and MD simulations. The homology modeling of the inactive FZD3 has been described previously[2]. Briefly, the taladepg-bound structure of SMo was used as a template (PDB ID: 4JVK)[65] and the sequence of FZD3 (UniProt ID: O66353) was aligned to that of SMO (UniProt ID: Q99885) with ClustalX[66]. The N- and C-termini were excluded due to a lack of suitable template, and the alignment was manually edited to ensure the proper alignment of transmembrane domains and conserved motifs present in Class F GPCRs. Fifteen homology models were generated with MODELLER 9.11[15] and a representative model was selected based on DOPE score and visual inspection. Active–24,25(S)-epoxysterol cholesterol and G, bound—SMO structure (PDB ID: 6TOI)[25], was published during the revision of our study. To implement these new structural data, we built 20 new FZD3 and FZD7 models with MODELLER 9.11[15] and 4QIN29, respectively) using GROMACS66. The missing residues in the SMO parametrized with AmberTools18 package (University of California San Francisco) docking complex was also used for initiating MD simulations. SAG1.3 was ARTICL...
were from Madelon M. Maurice (University Medical Center Utrecht, Utrecht, The Netherlands), pNluc–N1, Venus–Kras, Venus–mGส, Venus–mGsf, Venus–mG13, SMO–Ruc8 (coding for mouse SMO), and FZD4–Nluc were from Nevin A. Lambert (Augusta University, Georgia, USA). Venus–N1 was from Addgene (#27793). In order to generate Nluc–FZD4, FZD4–coding sequence from SNAP–FZD4 was subcloned into an empty N-terminally tagged Nluc vector containing the 5-HT3A signal peptide (from Nluc-A3) using BamHI and XbaI restriction enzymes. First, SNAP–FZD4 was reengineered site-directed mutagenesis (GeneArt, Thermo Fisher Scientific). Next, the FZD4 sequence was cloned in-frame into the Nluc vector. FLAG–FZD4–Venus and ACRD FLAG–FZD4–Venus were subcloned from FLAG–FZD4–His and ACRD FLAG–FZD4–His, respectively, into pNluc–N1 with BglII and AgeI. SNAP–FZD4–Ruc8, SNAP–FZD4–Ruc8, SMO–Ruc8, Nluc–DLC2, FLAG–FZD4–His, ACRD FLAG–FZD4–His, and FZD4–Nluc were generated and validated in our previous study. 

The SNAP–FZD4, D351A, D351E, E438A–54, E438D–54, E438N–54, E438C–54, R442A–58, R442E–58, and K479N–741 mutants were made using the GeneArt site-directed mutagenesis kit (Thermo Fisher Scientific). In order to create FRET-based sensors, the FZD4–coding sequence from FZD4–GFP54 was subcloned into pcDNA–3TP1 (Alike Biotechnology and Pharmaceuticals) between the HindIII and EcoRI restriction sites. Subsequently, the FAMA binding sequence (FLNC PCGC MECGEP) was inserted between G404 and R405 of the FZD4, ICL3 using GeneArt site-directed mutagenesis kit (Thermo Fisher Scientific). The SNAP–FZD4–FAM–PK was generated by subcloning the FZD4 insert from FZD4–GFP into PK vector (DiscoverX) with BglII and HindIII, and subsequently the FAM binding sequence was inserted as above. These constructs can be seen in the Supplementary Fig. 23. All the constructs were confirmed by sequencing (GATC-Eurofins, Konstanz, Germany).

FIAH labeling and FRET efficiency measurements. HEK293 cells were seeded onto coverslips. Cells were transfected 18–20 h later using Effectene (Qiagen), according to the manufacturer’s instructions. Cell culture medium was replaced 24 h later and the analysis was done 48 h after transfection. For analysis of the cellular expression and FRET efficiency determination, the cells were transfected with 0.5 µg per well of the corresponding receptor construct, either FZD6 or FZD6–ΔR405 of the FZD6 ICL3 using GeneArt site-directed mutagenesis kit (Thermo Fisher Scientifi). 0.5 µg per well of the corresponding receptor construct, either FZD6 or FZD6–ΔR405 of the FZD6 ICL3 using GeneArt site-directed mutagenesis kit (Thermo Fisher Scientific). Analysis of the cells was done 48 h after transfecting/seeding 100 ng of pcDNA–SmBiT (Lambert (Augusta University, Georgia, USA). Venus–N1 was from Addgene (Netherlands). pNluc

Live-cell imaging. Confocal microscopy experiments were performed on a Leica TCS SF2 system, equipped with a HCX PL APO CS 63× 1.32 oil objective. Cells with desired constructs were transfected with a total amount of 1000 ng of plasmid DNA using Nluc–FZD4: 10 ng or 100 ng for low donor condition or 1000 ng for high donor condition, and the remaining plasmid amount of pcDNA. The cells (100 µl) were seeded onto a PDL-coated black 96-well cell culture plate with solid flat bottom (Greiner Bio-One). Forty eight hours post transfection, cells were washed with complete DMEM/HBSS (HyClone) and maintained in the same buffer. BRET was performed 24 h post transfection. In the saturation experiments, the cells were incubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine. 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the saturation experiments, the cells were incubated with different concentrations of BODIPY–cylopamine (5 µM final concentration, 10 µl; Biosynth #C-7004) for 6 min prior to the BRET measurement. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection. In the competition experiments, the cells were either preincubated with different concentrations of SAG1.3 (70 µM) for 30 min at 37 °C followed by the addition of BODIPY–cylopamine/10 µM cyclopamine, 100 ng of pcDNA was used and the experiments were performed 24 h post transfection.
Immunoblotting. ΔSMO HEK293 cells were transfected in suspension using Lipofectamine 2000. For the experiments, 4 × 10^5 cells ml^-1 were transfected with 500 ng of plasmid DNA and 500 ng of pCMV-Snail. The cells (500 µl) were seeded onto a 24-well plate. Twenty-four hours later, the medium was changed and the cells were serum-starved overnight in the presence of C59 (5 nM). For the ERK1/2 phosphorylation experiments, the cells were stimulated with the indicated concentrations of SAG1.3 for 10 min at 37 °C and lysed immediately. For the mobility shift experiments, the cells were stimulated with SAG1.3 (10 µM) for 2 h at 37 °C and lysed immediately. The lysates were obtained using urea lysis buffer (final composition: 0.5% NP-40, 2% SDS, 75 mM NaCl, 88 mM Tris/HCl, 4.5 M urea, 10% β-mercaptoethanol, 10% glycerol, pH = 7.4), initially by lysing the cells in ice-cold 1% NP-40. Lysates were sonicated and analyzed by 7.5, 10, or 4–20% 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 in TBS-T, membranes were incubated with primary antibodies in blocking buffer: rabbit anti-GAPDH (1:1000; Cell Signaling Technology #2118), rabbit anti-DVL2 (1:1000; Cell Signaling Technology #3216), rabbit anti-P-ERK1/2 (1:1000; Cell Signalling Technology #9101S), rabbit anti-ERK1/2 (1:1000; Cell Signalling Technology #9102), mouse anti-SMO (1:100; Santa Cruz Biotechnology #sc-166685), and rabbit anti-SNAP tag (1:1000, New England Biolabs #P93105) overnight at 4 °C. Proteins were detected with horseradish peroxidase-conjugated secondary antibody (1:4000, goat anti-rabbit; Thermo Fisher Scientific #31460 or 1:2000, goat anti-mouse; Thermo Fisher Scientific #31430) and Clarity Western ECL Blotting Substrate (Bio-Rad). For each experiment, the phosphorylated and shifted (PS-DVL2) to unshifted DVL2 ratios, and SNAP-surface Alexa Fluor 647 staining of LARII and Stop & Glo reagent were used for each well. The analysis was made using the BIORAD Gel Doc XR+ gel documentation system (Bio-Rad). For the DVL2 mobility shift experiments, the cells were stimulated with SAG1.3, changed and the cells were serum-starved overnight in the presence of C59 (5 nM). After 14 days in HBSS and the DMEM medium for 30 min at 37 °C. Subsequently, the cells were washed three times in HBSS and the fluorescence (excitation 625–30 nm, emission 680–30 nm) was read with a CLARIOstar microplate reader (BMG). The data were analyzed using GraphPad Prism 6.

TopFlash luciferase assay. ΔΔZD11, HEK293 cells were seeded onto a 48-well plate at 2 x 10^5 cells per well and the next day they were transfected using Lipofectamine 2000 with M50 Super 8x TopFlash (Addgene #12456), pRL-TK Luc (Promega #E2241), SNAP–FZD5, and pcDNA plasmids to a total of 250 ng per well (with the ratio of 2:1:1:6, 4 h post transfection, medium was changed to starvation medium together with vehicle, WNT-3A (300 ng ml^-1) or SAG1.3 (10 µM). Twenty four hours after transfection, cells were analyzed by the Dual-Luciferase Reporter Assay System (Promega #E1910) according to manufacturer’s instructions in a white 96-well plate with solid flat bottom (Greiner Bio-One) with the following modifications: cells were lysed in 50 µl Passive Lysis Buffer, and 25 µl of LARII and Stop & Glo reagent were used for each well. The analysis was made on a CLARIOstar microplate reader (BMG) reading 580–80 nm for Firefly and 480–80 nm for Renilla.

SNAP-surface Alexa Fluor 647 staining. For quantification of cell surface expression of N-terminally SNAP-tagged receptors, ΔSMO HEK293 cells at the density of 10^5 cells ml^-1 were transfected in suspension using Lipofectamine 2000 with 500 ng of the indicated receptor plasmid DNA and 500 ng of the pcDNA from Alexa Fluor 647 (New England Biolabs #A9936S) in complete DMEM medium for 30 min at 37 °C. Subsequently, the cells were washed three times in HBSS and the fluorescence (excitation 625–30 nm, emission 680–30 nm) was read with a CLARIOstar microplate reader (BMG). The data were analyzed using GraphPad Prism 6.

Statistical analysis. Statistical analysis and graphical analysis were performed using GraphPad Prism 6 software. Data were analyzed for differences by two-tailed unpaired t-test, two-tailed one sample t-test, two-tailed paired t-test, and one- or two-way analysis of variance (ANOVA) with Fisher’s least significant difference (LSD) post hoc analysis. Concentration–response and binding curves were fit using three, four parameters or bell-shaped nonlinear regression, and represented as a mean ± s.e.m. Specifically, the 5000-10000 pM binding curves was done using extra-sum-of-squares F test (P < 0.05). All the other data points throughout the manuscript represent the mean ± s.e.m. of maximum n individual experiments (biological replicates) performed typically in triplicates (technical replicates) unless stated otherwise. Statistical significance are given as: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. No experimental datasets were excluded from the analysis.
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Author contributions
P.K. and G.S.—conceived and designed the study. A.T. and P.K.—performed the in silico work. P.K., C.-F.B. and M.C.A.C.—performed the wet lab experiments. A.I., Y.O., P.K., M.C.A.C., J.V., J.P. and C.H.—contributed important tools and control experiments. P.K., A.T. and G.S.—prepared the figures with the input from C.H., and M.C.A.C. P.K., A.T. and G.S.—wrote the manuscript. G.S.—supervised, financed, and coordinated the project.

Competing interests
The authors declare no competing interests.

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