A conserved molecular switch in Class F receptors regulates receptor activation and pathway selection

Shane C. Wright¹, Paweł Kozielewicz¹, Maria Kowalski-Jahn¹, Julian Petersen¹, Carl-Fredrik Bowin¹, Greg Slodkowicz², Maria Martí-Solano², David Rodríguez³, Belma Hot¹, Najeha Okashah⁴, Katerina Strakova¹, Jana Valnohova¹, M. Madan Babu², Nevin A. Lambert⁴, Jens Carlsson³ & Gunnar Schulte¹

Class F receptors are considered valuable therapeutic targets due to their role in human disease, but structural changes accompanying receptor activation remain unexplored. Employing population and cancer genomics data, structural analyses, molecular dynamics simulations, resonance energy transfer-based approaches and mutagenesis, we identify a conserved basic amino acid in TM6 in Class F receptors that acts as a molecular switch to mediate receptor activation. Across all tested Class F receptors (FZD4,5,6,7, SMO), mutation of the molecular switch confers an increased potency of agonists by stabilizing an active conformation as assessed by engineered mini G proteins as conformational sensors. Disruption of the switch abrogates the functional interaction between FZDs and the phosphoprotein Dishevelled, supporting conformational selection as a prerequisite for functional selectivity. Our studies reveal the molecular basis of a common activation mechanism conserved in all Class F receptors, which facilitates assay development and future discovery of Class F receptor-targeting drugs.
The Class F of G protein-coupled receptors (GPCRs) is evolutionarily conserved and consists of ten Frizzled paralogs (FZD$_{1-10}$) and Smoothomed (SMO) in humans. While FZDs mediate WNT signaling, SMO mediates Hedgehog signaling. Together, these receptors play key roles in embryonic development, stem cell regulation and tumorigenesis. Although Class A GPCRs contain a number of well-characterized motifs that are central to mediating receptor activation and selective interaction with heterotrimeric G proteins, similar motifs in Class F receptors are unknown. In fact, the lack of conserved E/D/R (ionic lock), toggle switch or NPxXY motifs has been described as an argument against the GPCR nature of Class F receptors.

GPCRs function as allosteric machines sampling a range of conformations spanning from inactive to agonist-bound G protein-coupled states. Active states—of which many can exist—allow receptor activation towards different effectors such as heterotrimeric G proteins, arrestins, or G protein-coupled receptor kinases. Furthermore, Class A GPCRs have been described to act as proto-oncogenes through mutations in the ionic lock that promote a ligand-independent active conformation, resulting in G protein-coupling beyond physiological constitutive activity. To make sense of the structural rearrangements that result in these overactive receptors, we need to refer to the ternary complex model to relate how the receptor-bound ligand and intracellular stopper affect one another by bidirectional allostery. To date, it is not clear what conformational rearrangements in Class F receptors lead to pathway activation as a consequence of agonist binding, irrespective of the nature of the downstream signaling route (e.g., Dishevelled (DVL)- and heterotrimeric G protein-mediated pathways). Nevertheless, there is emerging evidence that SMO and FZDs interact with their respective ligands and heterotrimeric G proteins to form a functional ternary complex reminiscent of Class A/B GPCRs. Receptor state-selective nanobodies and engineered heterotrimeric G proteins, so-called mini G (mG) proteins, have provided valuable, biotechnological tools for probing and stabilizing active Class A/B receptor conformation in living cells and offering exciting possibilities in vitro to better understand Class F receptor activation mechanisms. Although individual motifs and residues in FZDs have been identified that mediate interaction with the phosphoprotein DVL-$\alpha$, how this translates into a pathway-selective, three dimensional DVL-bound receptor conformation is currently unknown.

Here, we use a combination of population and cancer genomics data analysis, analysis of available crystal structures and computational modeling to interrogate the pathophysiological importance to the family-wide conserved residue R/K$^{6.32}$ in Class F receptors. This residue plays a central role in the formation of a ligand-receptor-G protein ternary complex as evidenced by the shift in potency of the agonist in the presence of engineered G protein upon mutation of R/K$^{6.32}$. By comparing wild type and mutant Class F receptors, we provide the proof-of-principle that we can detect the fully active, G protein-coupled Class F receptor conformation in living cells and suggest a molecular switch mechanism based on R/K$^{6.32}$ interaction with TM7. Interestingly, mutation of the molecular switch abrogates the interaction and communication with DVL, despite displaying a higher agonist potency in the mG protein recruitment assay. These findings suggest that FZDs show conformational bias towards different transducer proteins and can guide future drug discovery efforts to screen for pathway-selective drugs targeting active Class F receptors in disease.

Results

Genomic data analysis defines R$^{6.32}$ as a mutational hot spot. In order to shed light on general activation mechanisms in this class of receptors, we focused on conserved residues with putative biological function. Large scale sequence alignment of over 750 mammalian and non-mammalian FZDs and SMO revealed several positions that are conserved among the human paralogs, in mammals as well as across the animal kingdom (Supplementary Figure 1a, b and Supplementary Data). Given the role of Class F receptors in cancers, we investigated the importance of the conserved positions by analyzing which positions are significantly mutated in diverse human cancers. Investigation of the recently published data on 66,402 cancer genomes from the cBioPortal for Cancer Genomics and projection of mutation frequency onto a Class F receptor model revealed the mutational hot spots (Fig. 1a and Supplementary Figures 2a, 3a). We observed that a conserved basic residue—either an arginine (R) or a lysine (K)—at the lower part of TM6 (the residue R/K$^{6.32}$ according to the Ballesteros–Weinstein nomenclature) is significantly mutated in a series of human tumors such as colorectal adenocarcinoma in several Class F members (Supplementary Figure 3b). Focusing on FZD$_6$, it becomes obvious that R160Q$^{6.32}$ is the most prevalent variant associated with cancer in Class F receptors. In other FZD paralogs or SMO, mutation of R$^{6.32}$ to H, C, Q, and S is associated with different forms of cancer (Supplementary Figure 3b).

We then normalized the mutational frequency observed in somatic cancers by comparing them to the number of germ-line variants seen in the human population. To this end, we analyzed variants from over 120,000 individuals (Genome Aggregation Database, gnomAD; www.gnomad.broadinstitute.org; Fig. 1b and Supplementary Figure 2b). This analysis revealed that R$^{6.32}$ shows a relatively low amount of natural variation. Strikingly, by computing the relative variation (i.e. ratio of the frequency of somatic/cancer mutations to that of the germ-line/natural variation; see Methods) for every position, we found that R$^{6.32}$ is selectively the most often mutated position in Class F receptors in cancer genomes compared to the population-level variation (Fig. 1c and Supplementary Figure 2c). As this position is less variable among healthy individuals, but naturally found to be selectively mutated in cancer, these observations suggest that R$^{6.32}$ is likely to be important for physiological receptor activity.

Contact network between TM6/7 constitutes a molecular switch. While structural insight into Class F receptors is limited, several crystal structures of SMO provide pertinent information that can be applied to the whole receptor class. Detailed investigation into the presence of TM6/7 contacts between residues in the published SMO crystal structures, which represent inactive receptor conformations, indicates that hydrogen bonds and $\pi$-cation stacking interactions between R451$^{6.32}$ and the lower end of TM7 (T534$^{7.54}$, W535$^{7.55}$, W537$^{7.57}$) are formed in SMO structures (Fig. 2a, for all residue contact fingerprints between residues in the TM6/7 helices, see Supplementary Figure 4). In addition, the crystal structure of FZD$_4$, the high resolution FZD structure, in the absence of ligand and the extracellular cysteine-rich domain (CRD), also reveals a contact between R$^{6.32}$ and W$^{7.55}$. In the FZD$_4$ structure, an additional contact between K$^{6.32}$ and F$^{2.41}$—a negatively charged residue only conserved in FZDs—further contributes to the stabilization of this network. Despite the more detailed structural insight into this region in the inactive Class F receptors, it remains obscure what opening of a molecular lock or switch means functionally for signal activation and specification downstream of Class F receptors.

Receptors in a fully active G protein-coupling state undergo an opening of the cytoplasmic cavity of their transmembrane helix bundle to accommodate the $\alpha5$ helix of the $\alpha$ subunit allowing for guanine nucleotide exchange (GEF) activity of the receptor. Along this line, the $\pi$-cation and hydrogen bonding interactions
of the lock observed between TM6–TM7 in SMO and FZD₆ could function as a conserved molecular switch mechanism for ternary complex formation resembling the ionic lock in Class A GPCRs and the recently identified polar network in the Class B GLP-1 receptor. The analogous mechanism in Class B receptors is also based on an arginine-dependent interaction between the TM6 and TM7/H8, which is broken in the active, G protein-coupled GLP-1 receptor/Gₛ CryoEM structures (Fig. 2b). An additional homology model of FZD₆, which is based on the SMO crystal structure (PDB ID: 4JKV) was used as the basis for a FZD₆ homology model, where the conserved sites R₆.₃₂ and W₇.₅₅ are shown juxtaposed in TM6 and TM7, respectively (Fig. 2c). This model reveals hydrogen bonding of the charged R₆.₃₂ side chain to oxygen atoms of the TM7 helical backbone and π-cation interactions with the side chain of W₇.₅₅ for models of FZD₆, FZD₄ and SMO is associated with different forms of cancer (Supplementary Figure 3b). The frequent occurrence of Class F R₆.₃₂ mutations in human cancers suggests increased activity of mutant receptors similar to the increased constitutive activity of Class A GPCRs upon mutational disruption of their ionic lock or the residues involved in the structural rearrangement leading to Class A receptor activation.

To study the importance of the residue contacts mediated by R₆.₃₂ in FZD₆, a SMO crystal structure (PDB ID: 4JKV) was used as the basis for a FZD₆ homology model, where the conserved sites R₄₁₆₆.₃₂ and W₄₉₃₇.₅₅ are shown juxtaposed in TM6 and TM7, respectively (Fig. 2c). This model reveals hydrogen bonding of the charged R₄₁₆₆.₃₂ side chain to oxygen atoms of the TM7 helical backbone and π-cation interactions with the side chain of W₄₉₃₇.₅₅ for models of FZD₄, FZD₆ and SMO is associated with different forms of cancer (Supplementary Figure 3b). Further, computational mutation of position 6.₃₂ reveals that these contacts can neither be formed in the experimental R₄₁₆₆_A₅.₃₂ nor the naturally occurring R₄₁₆₆Qₖ.₃₂ mutants of FZD₆ (Fig. 2c).

We next analyzed the stability of the residue contacts by performing molecular dynamics simulations employing the FZD₆ model (Supplementary Figure 5c). In order to more closely characterize the observed changes between wild-type FZD₆ and R₄₁₆₆A₅.₃₂, we quantified the distance between TM3-TM6 regions that undergo large conformational changes in Class A GPCRs upon activation. Comparing the distribution of distances between TM3-TM6, the minimum observed distance was smaller in FZD₆ than in the R₄₁₆₆A₅.₃₂ mutant. This suggests a higher capability of the wild-type receptor to form a more closed, inactive conformation and the mutant to form a more open, active-like conformation (Fig. 2d). Due to the fact that the MD simulations were carried out in the absence of G protein, the dynamics refer to an intermediate and not fully active state. An additional homology model of FZD₆, which is based on the inactive SMO crystal structure fused with the lower part of TM6 modeled according to the active bovine opsin crystal structure in complex with the C₅ α-helix of transducin, allowed us to study an active-state conformation including an outward movement of TM6. In this model, the conformational change prevents interactions between R₄₁₆₆₆.₃₂ and TM7—a finding that is consistent with its role as an activation switch (Supplementary Figure 5b). These calculations suggest that mutation of R₆.₃₂ may facilitate the receptor to sample the active-like conformation more frequently and may confer constitutive basal activation of the receptor in the absence of agonist, but in the presence of the intracellular transducer.

**Mutation of R₆.₃₂ in FZD₆ affects basal receptor activity.**

Constitutive activity of GPCRs is traditionally assessed with inverse agonists, where the negative efficacy reduces basal activity in the absence of orthosteric agonist. Due to the inexistence of inverse agonists targeting FZDs, we employed pharmacological inhibitors to create conditions that were free of endogenously secreted WNT proteins in the presence of overexpressed wild type or FZD₆ R₄₁₆₆₆.₃₂ as a means of measuring the ligand-independent, receptor-intrinsic activity. In order to test whether
the R416A,6.32 mutation could also confer ligand-independent constitutive activity of exogenously expressed FZD6, we monitored basal phosphorylation of extracellular-signal regulated kinases 1/2 (ERK1/2)—similar to what we have previously shown.44 Inhibition of Porcupine—the enzyme that is required for WNT acylation and secretion—blunts endogenous WNT secretion.47 While HEK293 cells stably expressing FZD6 exhibited higher basal ERK1/2 phosphorylation compared to control cells, expression of FZD6 R416A,6.32 was accompanied by a more pronounced ERK1/2 phosphorylation. Incubation with the Porcupine inhibitor C59 reduced both FZD6- and FZD6 R416A,6.32-induced ERK1/2 phosphorylation. Whereas the wild-type FZD6 showed a tendency for constitutive activity, FZD6 R416A,6.32 exhibited a more pronounced constitutive activity in the absence of endogenous WNTs and in the presence of endogenous G proteins (Fig. 3b). These results collectively suggest that mutation of this position confers a higher constitutive activation of the receptor in a ligand-independent manner initiating a cellular response.

The molecular switch defines functional selectivity of FZDs. Despite the apparent constitutive activity for the G protein-dependent pathway to ERK1/2,14,44,48, signaling through the phosphoprotein DVL—a central mediator of WNT/FZD signaling—was negatively affected by disruption of the molecular switch. Both the experimental FZD6 R416A,6.32 and the naturally occurring cancer mutants of the molecular switch R416Q,6.32 and W493L,7.55 were impaired in the ability to recruit DVL to the membrane and to induce the electrophoretic mobility shift associated with DVL activation (Fig. 4a–c and Supplementary Figure 7).50,51 Recruitment of DVL to the plasma membrane was quantified by bystander bioluminescence resonance energy transfer (BRET) employing the Venus-tagged CAAX domain of kras as a membrane marker in combination with an N-terminally Nluc-tagged DVL2. Contrary to the wild-type receptor, all tested mutants of FZD6 were incapable of recruiting DVL to the membrane as referenced by the negative control, the β2 adrenergic receptor (Fig. 4a). Furthermore, we took advantage of the recently described phospho-specific antibody detecting the C-terminal, phosphorylated S648 of FZD6, which is indicatively of functional casein kinase 1 (CK1) targeting and DVL recruitment.52 While FZD6 is significantly phosphorylated in the presence of coexpressed CK1s and DVL2, disruption of the molecular switch in all three mutants impaired S648 phosphorylation, leaving FZD6 W493L,7.55 with residual S648 phosphorylation (Fig. 4d, e).

Fig. 2 Interactions between R/K,6.32 and helix 7 allow for a molecular switch mechanism. a General receptor and magnified view centered on residue 6.32 of a structural overlay of all available SMO and FZD4 crystal structures (PDB IDs SMO: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4JKV, 4QIM, 4QIN, 6D32, 6D35; FZD4: 6BD4). Residue 6.32 and its interacting residues are shown in orange stick representation. The bottom, left inset shows contact fingerprints for all interactions measured using the Protein Contact Atlas between residue 6.32 and residues in TM7 and TM2 (an orange box indicates that the contact is present in that structure, a white box indicates absence of the contact). All structures present inactive structures in the absence of heterotrimeric G protein. b Representation of the equivalent receptor region in the previous panel in GLP-1 receptors. Inactive (PDB IDs: 5VEW and 5VEX, gray), intermediate (PDB ID: 5NX2, orange), and active/G protein-bound (PDB IDs: 5VAI and 6B3J, green) structures are shown. The proposed TM6–TM7–H8 switch residues are shown as sticks. c Left panel: computational model of FZD6 based on the SMO crystal structure (PDB ID: 4JKV). R416Q,6.32 on TM6 of SMO + SANT1 (4N4W) and SMO + Cyclopamine (4O9R). R416A,6.32 on TM6 and W493L,7.55 on TM7 are highlighted in orange. Middle panel: representation of the naturally occurring cancer mutant FZD6 R416Q,6.32. Right panel: representation of the experimental R416A,6.32 mutant. d Analysis of the frequency of the of TM3–TM6 (W,5.30–G6.34) distance distributions over MD simulation time for FZD6 and FZD6 R416A,6.32. Threshold values are compared using unpaired t test; n = 3 (FZD6) n = 4 (FZD6 R416A,6.32); P = 0.0281; t = 3.060; df = 5. *P < 0.05 (two-tailed t-test).
Because FZD6 is more restrictive in its pathway selectivity and is not known to mediate WNT/β-catenin signaling, we extended our studies to FZD5, which is known to mediate both G protein- and WNT/β-catenin-dependent signaling. Similar to FZD6, mutation of the molecular switch in FZD5 abolished DVL recruitment to the membrane to levels comparable to the β2 adrenergic receptor (Fig. 4f). In agreement with a loss in FZD-DVL interaction, FZD5 R449A induced WNT-3A-induced T cell factor (TCF)/lymphocyte enhancer factor (LEF)-dependent transcriptional activity as monitored by the TOPFlash assay in cells devoid of endogenous FZD1-10 expression (Fig. 4g). Given the lack of endogenous FZDs, WNT-3A stimulation did not evoke a response in control-transfected cells. While FZD3 expression dramatically enhanced the TCF/LEF transcriptional activity in response to WNT-3A compared to the empty vector control, FZD5 R449A did not. In order to exclude the possibility that the absence of a response in cells transfected with the mutant receptor might be due to poor membrane expression of SNAP-FZD3 R449A, we optimized transfection conditions that yielded similar surface levels validated by flow cytometry (Supplementary Figure 6a) using a cell impermeable, fluorescent SNAP substrate in parallel to the TOPFlash experiments. Transfection conditions that yielded similar surface expression of the receptor in HEK293 cells were compared for the ability to mediate WNT-3A-induced TCF/LEF transcriptional activity in the cells lacking FZD1-10, clearly underlining the inability of the SNAP-FZD3 R449A to mediate WNT/β-catenin signaling.

Collectively, these findings with FZD3 and FZD5 merge well with the current understanding of the existence of different ternary complexes defined by the nature of the intracellular transducer and the concept of functional selectivity or signaling bias. The FZD6 R416A mutation preferentially accommodates G protein binding over DVL interaction as evidenced by the ability of the mutant receptor to induce P-ERK1/2 and its inability to induce PS-DVL or to recruit DVL to the membrane (Fig. 4a–d and Supplementary Figure 7). Conversely, our data suggest that wild-type FZD6 could be biased towards interaction with DVL over heterotrimeric G protein—a process that could be affected by local differences in transducer concentrations. In this context, the inability of FZD5 R449A to recruit DVL and to mediate WNT/β-catenin signaling supports this model. Previous studies on FZD5 identified a mutation at the lower end of TM2, at the evolutionary conserved Y250F, which negatively affects DVL interaction while maintaining its ability to functionally interact with heterotrimeric G12/13 proteins. In contrast, the FZD6 R511C nail dysplasia mutant maintained interaction with DVL, but lost its ability to associate with G1 or Gq. Together with our current findings, these data collectively support the existence of distinct conformational states that selectively feed into either DVL or heterotrimeric G protein signaling (Fig. 4h).

mG sensors detect a fully active Class F receptor state. In order to better understand the mechanism of action of the R/K6.32 mutations present in Class F receptors and given the absence of a high resolution ternary complex structure, we made use of recently developed conformational sensors of GPCR activation—so-called mG proteins. These mG proteins have served to detect the active state conformation of GPCRs in living cells and to stabilize active, purified receptors for crystallization and CryoEM studies. Based on emerging evidence that Class F receptors function as bona fide GPCRs, we postulated that agonist stimulation of Class F receptors would lead to the recruitment of the mG protein to the receptor.

The ten FZDs are subdivided into four evolutionarily-related clusters consisting of FZD1-2, 7–9, FZD3, 6, FZD5-8, and FZD9, 10 (Fig. 5b). With the aim of investigating the generality of the presented mechanism, we assessed mG protein interaction with one representative of each FZD homology cluster and SMO. Based on what is known about FZD-G protein selectivity, we focused on FZD3-G41, FZD3-G3, FZD5-G14, FZD7-G62, and SMO-G17, 18, 63. BRET assays were performed in transiently transfected HEK293 cells using recombinant, purified WNT-5A (FZDs) and SAG (SMO) as agonists. Concentration-response curves were produced comparing the potency of agonist at the wild-type receptor with the R/K6.32 to alanine mutants. A dramatic left shift in the agonist potency was detectable for all tested Class F mutants compared to the respective wild-type receptors at similar surface expression levels (Fig. 5c–g; Supplementary Figure 6b, c). In addition to the experimental R/K6.32 to alanine mutants, we have also performed mG BRET experiments using the naturally occurring cancer mutants FZD6 R416Q and SMO R455H, as well as FZD6 W493L and SMO W539L (Supplementary Figures 6d, 8a, b). In short, these experiments confirmed that: (1) the validated mG proteins act as conformational sensors, detecting and binding to the active conformation of the respective Class F receptors, (2) the mutation of R/K6.32 or W7.55 increases the potency of agonists by being able to bind better to the cognate G proteins and (3) the naturally occurring cancer mutants in the molecular switch mechanistically phenocopy the experimental alanine mutants. In order to further complement our conclusions, we ran MD simulations of SMO and its naturally occurring cancer mutants R6.32 to H6.32 and W7.55 to L7.55 based on the crystal structure of human SMO in the absence of the extracellular CRD and without a crystallization scaffold in IL3 (PDB 4JKV; Supplementary Figure 8c–f). For the time of the MD simulation (150 ns, 3 replicates), the positioning of the residues was more stable and TM6/7 interactions in the molecular switch region were more
Fig. 4 Mutation of the molecular switch confers functional selectivity. a To quantify DVL2 membrane recruitment, bystander BRET between Venus-kras and Nluc-DVL2 was assessed over a range of acceptor/donor ratios in the presence of FZD5, FZD6 R416A6.32, FZD6 R416Q6.32, FZD6 W493L7.55 or the β2-adrenergic receptor as negative control. net BRET values are presented as mean ± standard deviation (s.d.) of n = 3 independent experiments. b, c HEK293 cells transfected with empty vector (control), FZD5, FZD6 R416A6.32, FZD6 R416Q6.32, or FZD6 W493L7.55 were analyzed by immunoblotting using antibodies to SNAP, -DVL2, and -GAPDH (loading control) antibodies. Bar graphs for the ratio of PS-DVL2 (upper band) to DVL2 (lower band) summarize densitometry data. Experiments were performed in the presence of 5 nM C59 (overnight). Data are presented as mean ± s.e.m. of 3 independent experiments; F (4, 15) = 17.71. **P < 0.01 (one-way ANOVA). See also Supplementary Figure 7. d, e HEK293 cells cotransfected with empty vector, FZD6, FZD6 R416A6.32, FZD6 R416Q6.32, or FZD6 W493L7.55 and DVL2/CXK were analyzed by immunoblotting using anti-phospho-S648 GAPDH anti-SNAP, and anti-GAPDH antibodies. The P-S648 signal was quantified by densitometry and summarized in a bar graph. Data are presented as mean ± s.e.m. of n = 3 independent experiments; F (4, 15) = 83.78. ****P < 0.0001, **P < 0.01 (one-way ANOVA). f In a similar setup to a bystander BRET was measured between Venus-kras and Nluc-DVL2 in the presence of FZD5, FZD6 R449A6.32 or the β2-adrenergic receptor (data points for β2-adrenergic receptor are identical to a). net BRET values are presented as mean ± s.d. of n = 3 independent experiments. g HEK293TΔFZD10 cells were transfected with Renilla and Firefly luciferase together with empty vector (control), FZD5 or FZD5 R449A6.32 and stimulated with 300 ng ml⁻¹ recombinant WNT-3A overnight. The luciferase signal was normalized to the average of unstimulated control values. Data are represented as mean ± s.e.m. of n = 3 independent experiments. P = 0.0065. F, (2, 6) = 13.12. **P < 0.01 (one-way ANOVA). h Schematic presentation of the concept of conformation-driven signaling bias of wild-type FZD and molecular switch mutant FZD. FZD models were produced in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) long-lived in the wild type than in the mutant receptors. These in silico observations support the concept of a molecular switch in receptor activation allowing association of an intracellular transducer—the heterotrimeric G protein.

Due to the general lack of well-characterized small molecule drugs targeting FZDs, we employed additional compounds acting at SMO to characterize the mode of action of the R/K6.32 molecular switch. Similar to what was previously observed with SAG in a luciferase-based reporter assay20,44, the agonist presented a bell-shaped concentration-response curve in the mG protein recruitment assay. It was suggested that SAG acts on an off-target site at higher concentrations and this is supported by the finding that cyclopamine-KAAD, an orthosteric inverse agonist, solely affects the SAG concentration-response curve on the ascending part of the bell-shaped curve20,64. In the SMO-mGsi BRET assay, the SAG concentration-response curves in wild type and R455A6.32 SMO appear similarly biphasic. Incubation with 100 nM cyclopamine-KAAD reversed the R455A6.32 mutation.
phenotype in SMO, shifting the curve rightward comparable to the wild-type SMO without affecting the descending segment of the curve (Fig. 5g).

Given the distinct differences between wild type and the R/K6.32 or W7.35 mutants, it could be possible that mutation of the molecular switch region conveys the ability to couple to heterotrimeric G proteins promiscuously. In order to exclude this possibility, we examined the G protein-coupling profile of wild-type SMO in a nucleotide depletion assay allowing to directly assess the formation of a ternary complex by BRET in the absence of nucleotides. Constitutive activity or ligand-independent G protein-coupling cannot be measured with mG proteins and so we made use of full heterotrimeric G proteins, which are nucleotide sensitive in order to de

Fig. 5 Switch mutations at position 6.32 of Class F receptors increase agonist potency. a Illustration depicts the experimental setup wherein luciferase-tagged Class F receptors (R) are expressed at the plasma membrane and validated Venus-tagged mG proteins are localized to the cytosol24. Relying on excitation from proximity to the luciferase (donor), the Venus-tagged mG protein (acceptor) fluoresces only when the receptor is in its active conformation. b Maximum-likelihood phylogenetic tree of human FZD and SMO paralogs, with the four major FZD clusters and SMO color-coded. Branch lengths are given in amino-acid substitutions per site. c–g BRET experiments in HEK293 cells transiently expressing representatives of Class F with mG proteins: c FZD$_4$/mG13 (n = 6), d FZD$_6$/mGs (n = 5), e FZD$_6$/mGs (wild type n = 7; R416A$_{6.32}$ n = 6), f FZD$_7$/mGs (n = 8), and g SMO/mGsi (n = 6). Wild-type receptor (filled circle) and molecular switch mutants (open circle) were compared in parallel and receptor surface expression was measured by bystander BRET and flow cytometry (Supplementary Figure 6). Cells were stimulated with the indicated concentrations of recombinant WNT-5A or SAG and the normalized BRET ratio of Venus to luc8/Nluc was measured. In g, effects of SAG alone were compared to increasing concentrations of SAG in the presence of the inverse agonist cyclopamine-KAAD (100 nM; red open circle). Data are represented as mean ± s.e.m. h Summary scheme illustrating the activation states of Class F receptors in the absence and presence of receptor-activating ligands, mG protein and the R/K$_{6.32}$ mutation. Only the combination of agonist and mG protein can stabilize a fully active state. The receptor models in the active state are a fusion of the full-length SMO structure (PDB ID 6D35) and the lower end of TM6 of the adenosine A$_{2A}$ receptor in complex with a mG protein (PDB ID 5G53$_{20}$).
have a higher affinity for the receptor by removing GDP and GTP through apyrase treatment in permeabilized cells. We then promoted the dissociation of the heterotrimERIC G protein through the addition of the inverse agonist cyclopamine. The difference, reflected by the decrease in BRET between luciferase-tagged wild-type SMO, Venus-tagged Gp3y, and untagged Gαi2, as well as the presence or absence of cyclopamine revealed that SMO couples to Gαi and G12, but not to Gq or Gs (Supplementary Figure 9a)—in agreement with previously published results13,16,65. Using mG proteins to control for the G protein specificity of SMO R455A-32, we confirmed that mutation of the molecular switch does not render the receptor promiscuous (Supplementary Figure 9b).

Discussion

Our data identify a conserved network of interactions in TM6/TM7, which serves as a molecular switch required for the full activation of G protein-bound Class F receptors. These findings contribute to a better understanding of Class F receptor activation mechanisms connecting structural indications34 with functional selectivity to different transducer proteins, such as heterotrimeric G proteins and the phosphoprotein DVL to distinct receptor complexes that depend on biased receptor conformational states. This concept is well-established in the field of GPCR pharmacology, where exciting opportunities for the development of biased ligands promise improved selectivity and reduced unwanted side effects66. More work needs to be done to structurally define the distinct receptor conformations and structural features in Class F receptors that define coupling selectivity to different transducer proteins, such as heterotrimERIC G proteins and DVL. However, these findings merge well with previous data showing that overexpression of DVL negatively impacts FZD-G protein interaction and signaling4,16. Based on different signaling profiles of purified WNTs in FZD-expressing mouse microglia-like N13 cells or FZD-free 32D cells stably expressing individual FZD isoforms, we had proposed that WNTs could act as biased ligands of FZDs distinguishing G protein over DVL signaling, even though this interpretation still needs to be pharmacologically and quantitatively validated47,68.

Mutations in W755 in SMO, a residue that we define here as part of the Class F molecular switch, were previously identified as oncogenic drivers40,41. Despite the fact that the R632 is the most frequently mutated residue in FZDs in human cancers, it remains obscure if and how mutations in the molecular switch (Supplementary Figure 3) render FZDs oncogenic. While the mutated molecular switch in FZDs apparently does not provide input to the DVL-dependent WNT/β-catenin pathway, enhanced FZD-induced activation of heterotrimERIC G proteins could provide tumor-promoting signals8. Since the present study explores cancer and population genomics solely to identify residues of mechanistic importance for receptor activation, further studies are required to define the contribution to and the underlying mechanisms of molecular switch mutations in Class F receptors found in human cancer.

In summary, our findings open the door for the development of high-throughput-compatible screening assays directly monitoring Class F receptor activation on a structural level instead of using signal amplified transcriptional reporter assays that are prone to deliver off-target hits69. Moreover, our data are directly applicable to mechanism-based drug discovery and the potential development of biased compounds targeting abnormal Class F receptor-mediated G protein signaling in cancer. Drugs such as cyclopamine-KAAD that target oncogenic mutants of SMO display an effect on the R632 molecular switch providing the proof-of-principle that FZDs may also be targeted in a similar way to combat diseases associated with upregulated WNT/FZD signaling80.

Methods

Computational modeling and molecular dynamics simulation. The homology models of inactive FZD3,4 were generated using a structure of SMO as a template (PDB ID: 5L7D). The sequence of FZD3 (UniProt ID: Q9ULV1), FZD4 (UniProt ID: Q13432), FZD5 (UniProt ID: Q9ULV1), FZD6 (UniProt ID: Q13467), FZD7 (UniProt ID: O60553), FZD8 (UniProt ID: O75084), FZD9 (UniProt ID: Q9H461), FZD10 (UniProt ID: U00144), and FZD11 (UniProt ID: Q9ULV1) were aligned to that of SMO (UniProt ID: Q9H835) with ClustalX2. 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. In order to generate an active-like FZD3 model, we used the crystal structure of rhodopsin, which is also a Gt-coupled receptor, in its G protein-bound conformation as a template (PDB ID: 3DQB). Residues 408-427 (19-36 in 3DQB) from TM6 of FZDs were modeled using corresponding residues from TM6 (A6, A6, A6) of rhodopsin. Fifteen homology models of each FZD receptor were generated with MODELLER 9.19 and the representative ones were selected based on DOPE score and visual inspection. R1632 of FZD3 was mutated to A41632 in UCSF Chimera 1.12.6.

Information about Class F receptor mutations in human tumor samples was extracted from the cbioPortal for Cancer Genomics73. In order to systematically characterize contacts of residue 632 in all SMO crystal structures (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to filter contacts between consecutive residues, we disregarded all contacts that were 4 or less amino acids apart in the receptor sequence. The GPCRdb was then used to annotate the detected interactions according to Ballesteros—Weinstein numbering. For a complete list of all calculated interaction fingerprints (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to systematically characterize contacts of residue 632 in all SMO crystal structures (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to filter contacts between consecutive residues, we disregarded all contacts that were 4 or less amino acids apart in the receptor sequence. The GPCRdb was then used to annotate the detected interactions according to Ballesteros—Weinstein numbering. For a complete list of all calculated interaction fingerprints (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to filter contacts between consecutive residues, we disregarded all contacts that were 4 or less amino acids apart in the receptor sequence. The GPCRdb was then used to annotate the detected interactions according to Ballesteros—Weinstein numbering. For a complete list of all calculated interaction fingerprints (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to filter contacts between consecutive residues, we disregarded all contacts that were 4 or less amino acids apart in the receptor sequence. The GPCRdb was then used to annotate the detected interactions according to Ballesteros—Weinstein numbering. For a complete list of all calculated interaction fingerprints (PDB IDs: 5L7D, 5L7I, 5V56, 5V57, 4O9R, 4N4W, 4JKV, 4QIM, and 4QIN), we retrieved all contacts using the Protein Contact Atlas with default conditions74. In order to filter contacts between consecutive residues, we disregarded all contacts that were 4 or less amino acids apart in the receptor sequence. The GPCRdb was then used to annotate the detected interactions according to Ballesteros—Weinstein numbering.
van der Waals interactions were used. All the bonds between hydrogen and other atoms were constrained using the LINCS algorithm. The data files were saved every 100 ps. The MD simulation data (~3 µs combined) were analyzed using VMD and PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Cell culture and transfections. HEK293 cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 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. Pharmacological inhibition of SMO was accomplished with cyclopamine-KAAD (Abcam). C59 (2-[4-(2-Methylpyridin-4-yl)phenyl]-N-[4-(pyridin-3-yl)phenyl]acetamide) was used to inhibit Porcuple to abrogate endogenous secretion of WNTs. For stimulation, recombinant WNT-5A (645-WN; R&D Systems/Biotechnie) and SAG (N-Methyl-N’-[3-pyridinylbenezyl]-N’-[3-chlorobenzoyl]b[biophen-2-carbonyl)-1,4-diaminocyclohexane; Abcam) were used.

In order to get all SNAP-tagged cell lines stably expressing SNAP-FZD5 and SNAP-FZD6, HEK293 cells were transfected with SNAP-FZD5 or SNAP-FZD6, R416A6.32 constructs using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions. About 24 h post transfection cells were passaged at 1:10 and 48 h post transfection medium was supplemented with 300 µg ml−1 zeocin (Thermo Fisher Scientific). The medium was replaced every two days to select the cells transfected with the plasmids. The cells were maintained in the presence of the antibiotic for a period of 4 weeks until the stable culture was established. Monoclonal cell populations were isolated by limiting dilution. HEK293 control cells underwent the same selection procedure. The stability of protein expression and homogeneity of cell population were verified by immunoblotting and flow cytometry. The stable cell lines were maintained in complete D Mell medium in the presence of 150 µg ml−1 zeocin. Absence of mycoplasma contamination was routinely confirmed by PCR using 5′-ggc gaa tgg agg agc agc-3′ and 5′-cgg atc aag cgg cgg ggc gtc gg-3′ primers detecting 16S ribosomal RNA of mycoplasma in the media after 2–3 days of cell exposure.

Cloning of receptor constructs and mutagenesis. FLAG-SNAP-FzAR was from Davide Calabro (University of Birmingham, UK). hFZD4-Nluc was subcloned from the pEGFP-N1 (Robert J. Lekowitz, Duke University, USA) into pNluc-N1 with BamHI and Nhel. The mouse SNAP coding sequence was amplified from pEGFP-mSmoe (Addgene plasmid #25395) with primers incorporating a 5′ HindIII site and a 3′ EcoRI site, and subcloned into pBluescript-8. Mouse SMO forward primer: 5′-gtc ccc gct gtc ttc ctg gtt gga agc aaa aa-3′. Cells were seeded onto 48-well plates and transfected using Lipofectamine 2000 according to the manufacturer’s instructions. Protein lysates were obtained using urea lysis buffer (0.5% NP-40, 2% glucose, 0.5 mM CaCl2, and 0.5 mM MgCl2) and maintained in the same buffer. The stability of protein expression and homogeneity of cell population were maintained in complete DMEM medium in the presence of 150 µg ml−1 zeocin.

Flow cytometry. HEK293 cells transiently transfected with SNAP tagged FZD constructs and mG constructs or stably expressing SNAP-FZD5 and SNAP-FZD6, R416A6.32 were grown in a 6-well plate. On the day of the experiment, the cells were detached with ice-cold 10 mM EDTA/PBS and then centrifuged at 400 g for 5 min in complete DMEM medium. The cells were resuspended in ice-cold 0.5% BSA/PBS, counted and transferred (3 × 105 cells) to a round-bottom 96-well plate that was then centrifuged 3 min at 300 × g. Subsequently, 5× 104 cells were incubated with SNAP-substrate: either SNAP-Surface Alexa Fluor 488 (NEB #9912S), SNAP-Surface Alexa Fluor 647 (NEB #59136S) or SNAP-Cell 647-Sir (NEB #9102S) at 1:200 dilution in complete DMEM medium for 30 min at 37 °C. The plate was centrifuged twice, cells were resuspended in ice-cold 0.5% BSA/PBS, and assayed immediately on an ADP Cyan flow cytometer. The median fluorescence intensity (MFI) data were analyzed using FlowJo V10 (Tree Star).

Immunoblotting. HEK293 cells were plated in 12 or 24-well plates. After 24 h, cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. Protein lysates were obtained using urea lysis buffer (0.5% NP-40, 2% glucose, 0.5 mM CaCl2, and 0.5 mM MgCl2) and maintained in the same buffer. Protein lysates were obtained using urea lysis buffer (0.5% NP-40, 2% glucose, 0.5 mM CaCl2, and 0.5 mM MgCl2) and maintained in the same buffer.

AlphaScreen quantification of ERK1/2 and P-ERK1/2 levels. Cells were seeded into a transparent 96-well plate at a density of 5 × 103 cells/well and allowed to adhere for over 6 h. The medium was then replaced and the cells were incubated with different concentrations of C59 or vehicle (DMSO) in serum-free DMEM at 37 °C overnight. The levels of ERK1/2 and P-ERK1/2 were assessed using the AlphaScreen Ultra Multiplex assay kit (PerkinElmer) according to the manufacturer’s instructions. Briefly, cells were lysed in 100 µl of SureFireUltra lysis buffer and 10 µl of 1:10 diluted lysate was added to a test of a 384-well light plate (Perkin-

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according to manufacturer’s instructions in white 96-well plates with the following modifications: cells were lysed in 50 µl Passive Lysis Buffer, Stop & Glo reagent was used at 0.5X and 25 µl of LABII and Stop & Glo Reagent were used for each well. Luminescence was measured using a Synergy2 microplate reader (BioTek).

**Live cell imaging.** HEK293 cells were seeded on 35 mm ECM gel-coated (1:300, Sigma-Aldrich) glass bottom dishes (Greiner Bio One 4 compartment 35 mm glass bottom dishes) at a density of 10^5 cells/well. After 24 h, cells were transiently transfected using Lipofectamine 2000 according to the manufacturer’s instructions with DWL2-GFP and either SNAP-FZD6 or SNAP-FZD6, R416A. About 24 h post transfection, medium was removed and cells were incubated with SNAP-Cell 647-SIR (1:500) in BE buffer for 15 min, subsequently washed twice and imaged using a Zeiss LSM 710 confocal microscope.

**Relative variation score.** Site wise relative variation score at each position i was calculated as:

\[
\text{RelVar}_i = \log \left( \frac{CV_i}{\text{max}(CV)} + 1 \right)
\]

where CV, is the number of cancer variants at position i, max(CV) is the maximum number of cancer mutations at any position, and NV, is the number of naturally occurring variants at position i.

**Phylogenetic analysis.** Phylogenetic tree for human FZD1-10 and SMO was obtained by first aligning protein-coding sequences with the MAFFT aligner ran in the G-INS-i mode and then performing phylogeny reconstruction in RAxML using the PROTGAMMALG substitution model.

**Multiple sequence alignment of Class F homologs.** Sequences for one-to-one orthologs for each Class F receptor in human were downloaded from Ensembl for all species with the exception of *S. cerevisiae* using the BiomaRt package. Orthologs with homology confidence 1 were retained and corresponding sequences were aligned using MAFFT in the G-INS-i mode (Supplementary Figure 1b and Supplementary Information).

**Statistical analysis.** Statistical and graphical analysis were performed using Graph Pad Prism software. Data were analyzed by two-tailed t-test or one-way ANOVA with Fisher’s least significant difference (LSD) post-hoc analysis. Concentration-response curves of BRET data were fit using three, four parameter or bell-shaped non-linear regression. Significance levels are given as: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data points throughout the manuscript are indicated as either the mean ± standard error of the mean (s.e.m) or the mean ± standard deviation (s.d.).

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file.

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Author contributions

S.C.W., P.K., C.-F.B., M.K.-J. and N.O. performed wet lab experiments presented in the report. P.K., M.M.-S., D.R. and J.C. generated receptor models, structural alignments, and molecular dynamics simulation data. M.K.-J., I.P., S.C.W., P.K., C.-F.B., K.S., B.H. and J.V. prepared receptor constructs, mutants and performed validation of cellular receptor expression and function. Database analysis was done by Greg.S., M.M.S., Gunnar.S., M.M.B., S.C.W., P.K., M.M.-S., Greg.S. and Gunnar.S. prepared figures for publication. N.A.L provided tagged mGFRs, FZD3, FZD7, MLO-8, RU58, Venus-kras, and expertise for setting up receptor-mG and bystander BRET. Gunnar.S., S.C.W., P.K. and M.M.B. wrote the manuscript with input from M.M.S., Greg.S., J.C. N.A.L., Gunnar.S. supervised and coordinated the project.
Additional information

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