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Molecular Pharmacology of Class F Receptor Activation

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List of non-standard abbreviations

FZD, Frizzled; GLI, glioma-associated homologue; GPCR, G protein-coupled receptor; WNT, Wingless/Int-1 lipoglycoproteins; HH, Hedgehog; SMO, Smoothened; PTCH, Patched.

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Abstract

The Class Frizzled or Class F of G protein-coupled receptors (GPCRs) consists of ten Frizzled (FZD₁₋₁₀) paralogues and Smoothened (SMO). FZDs coordinate Wingless/Int-1 (WNT) signaling and SMO mediates Hedgehog signaling. Class F receptor signaling is intrinsically important for embryonic development and its dysregulation leads to diseases, not at least diverse forms of tumours. With regard to the importance of Class F signaling in human disease, these receptors provide an attractive target for therapeutics, exemplified by the use of SMO antagonists for the treatment of basal cell carcinoma. Here, we review recent structural insights in combination with a more detailed functional understanding of Class F receptor activation, G protein coupling, conformation-based functional selectivity and mechanistic details of activating cancer mutations, which will lay the basis for further development of Class F-targeting small molecules for human therapy.

Significance statement

Stimulated by recent insights into the activation mechanisms of Class F receptors from structural and functional analysis of Frizzled and Smoothened, we aim to summarize what we know about molecular details of ligand binding, agonist-driven conformational changes and Class F receptor activation. A better understanding of receptor activation mechanisms will allow us to engage in structure- and mechanism-driven drug discovery with the potential to develop more isoform-selective and potentially pathway-selective drugs for human therapy.

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Introduction

Wingless/Int-1 (WNT) and Hedgehog (HH) signaling is essential for proper coordination of embryonal development by regulating cellular fate, orientation, positioning, polarity, proliferation and differentiation. In addition to their crucial involvement early in life, these pathways maintain importance in the adult in a plethora of process such as in the maintenance of functional stem cell niches to govern tissue renewal, for example in epithelial tissues, the hematopoietic system, bone, the nervous system or liver (Chien et al., 2009; Holland et al., 2013; Ingham, 2018; Ingham and McMahon, 2001; Steinhart and Angers, 2018; Wend et al., 2010). While tightly regulated, both WNT and HH signaling are required during embryogenesis and in the adult, and deregulation of the pathways manifests in devastating diseases ranging from developmental disorders, bone and cardiovascular disease, neurological disorders, fibrosis to various forms of cancers (Chien et al., 2009; Clevers and Nusse, 2012; De Ferrari and Moon, 2006; Foulquier et al., 2018; Freese et al., 2010; Gould and Manji, 2002; Hoeppner et al., 2009; Holland et al., 2013; Konigshoff and Eickelberg, 2010; Nusse, 2005; Polakis, 2000; Sen, 2005; Wend et al., 2010). Thus, targeting these pathways brings along a large, yet mostly untapped therapeutic potential, which needs to be balanced with an obvious risk for unwanted side effects.

Class F receptors and their ligands

While WNT effects are mediated by ten mammalian paralogues of Frizzleds (FZD₁₋₁₀), HH effects are indirectly mediated by Smoothened (SMO), all of which comprise the Class F of G protein-coupled receptors (GPCRs) (Foord et al., 2005; Schulte, 2010). WNT lipoglycoproteins are ligands of several WNT receptors, of which the seven transmembrane spanning FZDs are seen as the main component acting in concert with a series of co-receptors such as low-density lipoprotein receptor-related protein 5/6 (LRP5/6), receptor tyrosine kinase-like orphan receptor (ROR1/2), receptor tyrosine kinase (RYK), reversion-inducing cysteine-rich protein with kazal motifs (RECK), and others (Cho et al., 2017; Driehuis and Clevers, 2017; Vallon et al., 2018; Vanhollebeke et al., 2015). In the case of

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HH/SO signaling the cholesterol transporter Patched (PTCH) binds the HH protein and alters cholesterol levels in the inner leaflet of the cell membrane and cholesterol/sterol interaction with SMO are thought to regulate SMO activity (Kong et al., 2019; Kowatsch et al., 2019; Qi et al., 2018).

A short overview of molecular mechanisms of WNT and HH signaling

The complexity of WNT and HH signaling was recently reviewed elsewhere. Here we will only present basic concepts of signaling diversity referring the reader to excellent recent reviews in the field (Grainger and Willert, 2018; Kong et al., 2019; Nusse and Clevers, 2017; Steinhart and Angers, 2018; Wu et al., 2017).

WNT signaling can be divided into Dishevelled (DVL)-dependent signaling including both the β -catenin-dependent WNT/ β -catenin pathway as well as the planar-cell-polarity (PCP) pathway and DVL-independent signaling. Even though the underlying mechanisms of pathway selection are not fully understood, it is established that the WNT coreceptors LRP5/6 are essential to drive WNT signals – orchestrated by FZDs – towards the transcriptional regulator β -catenin (Macdonald et al., 2007; Nusse and Clevers, 2017). It should be mentioned that FZDs play an important role in pathway selectivity as some FZDs, such as FZD₃ and FZD₆ most likely do not mediate WNT/ β -catenin signaling (Corda and Sala, 2017; Golan et al., 2004; Wang et al., 2016). Even in the presence of LRP5/6, the WNT/PCP pathway does not involve β -catenin but also involves the phosphoprotein DVL in addition to a multitude of other so-called PCP-core proteins (Humphries and Mlodzik, 2018). While WNT/ β -catenin signaling relays transcriptional and proliferative input, PCP signaling orchestrates cytoskeletal changes translating into cellular asymmetry, migration, tissue elongation, polarity and tissue structuring information most obvious in two dimensional, planar epithelial tissues (Humphries and Mlodzik, 2018; Semenov et al., 2007). The pathways that we define here as DVL-independent signaling branches are mediated by heterotrimeric G proteins not excluding pathways that could be independent of both DVL and

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G proteins. The subdivision of WNT signalling into DVL-dependent and –independent pathways emerges as mechanistically likely as we will elaborate later on in this review when discussing conformational and functional selection of signaling pathways by different FZD conformations. Albeit the literature contains contradictory data (Kilander et al., 2014b; Koval et al., 2016; Liu et al., 2001), the concept emerges that simultaneous interaction of DVL and heterotrimeric G proteins with a single FZD is mutually exclusive (Bowin et al., 2019; Gammons et al., 2016; Hot et al., 2017; Kilander et al., 2014b; Schulte and Wright, 2018; Strakova et al., 2017; Wright et al., 2019). The network of heterotrimeric G protein signaling downstream of FZDs is not completely mapped although substantial progress has been made in recent years to define the FZD-G protein coupling selectivity, mechanisms and the physiological relevance of FZD-G protein signaling (Arthofer et al., 2016; Dijksterhuis et al., 2013; Halleskog et al., 2012; Halleskog and Schulte, 2013; Hot et al., 2017; Katanaev and Buestorf, 2009; Katanaev et al., 2005; Kilander et al., 2014a; Kilander et al., 2014b; Kilander et al., 2011; Koval and Katanaev, 2011; Koval and Katanaev, 2018; Luchtenborg et al., 2014; Park et al., 2015; Petersen et al., 2017; Ramirez et al., 2016; Strakova et al., 2017; von Maltzahn et al., 2012; Weivoda et al., 2016; Wright et al., 2018; Wright et al., 2019).

The release of constitutive inhibition of SMO upon HH-binding to PTCH results in the translocation of active SMO to the cilia and activation of a glioma-associated oncogene homolog (GLI)-dependent transcriptional program (Kong et al., 2019; Wu et al., 2017). This pathway was – similar to the WNT/β-catenin pathway – described as being independent of heterotrimeric G proteins. Similar to the discussion whether FZDs are GPCRs, the question whether SMO is a GPCR was a matter of intense debate (Arensdorf et al., 2016; Ayers and Therond, 2010). Interestingly, the overall picture between WNT and HH signaling diverges here substantially since there is now overwhelming evidence that HH/GLI signaling involves direct coupling of SMO to heterotrimeric G proteins (Guo et al., 2018; Manning et al., 2015; Qi et al., 2019; Riobo and Manning, 2007; Riobo et al., 2006; Shen et al., 2013), whereas WNT/β-catenin signaling most likely does not (Bowin et al., 2019). Furthermore, Classical

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GPCR-regulating entities, such as GPCR kinases and β -arrestins were identified as intrinsic elements of SMO signalling (Chen et al., 2004b; Kovacs et al., 2008).

The identification of the plant alkaloid cyclopamine as a SMO antagonist laid the foundation for a rather rich pharmacological toolbox for SMO including a wide range of small molecule ligands with varying efficacies, with important clinical applications for the treatment of basal cell carcinoma and acute myeloid leukaemia (Chen, 2016; Hoy, 2019). In contrast, hardly any small molecules that target FZDs have been identified and thoroughly validated regarding their mode of action (Generoso et al., 2015; Riccio et al., 2018; Zhang et al., 2017a) and information from the recent crystal structure of a ligand-free FZD₄ suggests that targeting FZDs with small molecules could be challenging (Yang et al., 2018).

Frizzleds: ligand-receptor interaction

WNT proteins (19 members of the family in humans) interact with the cysteine rich domain (CRD) of FZDs but not SMO involving their lipid modification and a common fold including an index finger and a thumb (Hirai et al., 2019; Janda et al., 2012; Willert and Nusse, 2012). In the published crystal structures, the lipidated thumb domain of WNT proteins binds a lipophilic groove on the CRD and an elongated index finger recognizes the CRD close to its C terminus (Hirai et al., 2019; Janda et al., 2012). This interaction, which shows affinities in the lower nanomolar range explains – at least in part – WNT-FZD selectivity (Dijksterhuis et al., 2015). However, how WNT-CRD interaction physically translates into structural rearrangements in the 7TM core of FZDs in order to establish and stabilize an active receptor conformation remains a mystery. Co-receptors of different kinds are implicated to mediate WNT-FZD and pathway selectivity (Dijksterhuis et al., 2015; Eubelen et al., 2018; Hendrickx and Leyns, 2008; Wang et al., 2016). In addition to WNTs, Norrin presents a soluble FZD₄-selective ligand, which also interacts with the FZD-CRD forming a complex with LRP5/6 in order to feed into the WNT/ β -catenin pathway (Chang et al., 2015; Ke et al., 2013; Xu et al., 2004).

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All WNT proteins, except for *Drosophila melanogaster* WNTD are lipidated at a conserved serine residue rendering them highly lipophilic (Willert et al., 2003; Willert and Nusse, 2012). Substantial evidence exists that the lipidation of WNTs is essential for their agonist activity and furthermore, the crystal structures of WNTs and FZD-CRDs suggest that the lipid moiety interacts with a conserved lipophilic groove on the CRD. This concept poses an interesting thermodynamic resolution conundrum (Deshpande et al., 2019; Janda et al., 2012; Schulte et al., 2005; Willert et al., 2003; Willert and Nusse, 2012). WNT transport from the trans Golgi network depends on the seven transmembrane spanning protein Wntless (WLS/EVI) (Banziger et al., 2006; Bartscherer et al., 2006) and it remains so far unclear how mature WNT is either embedded with the lipid moiety in the plasma membrane, how it is packaged into lipoprotein particles, carrier proteins or in exosomes (Ching and Nusse, 2006; Herr and Basler, 2012). Irrespective of the packaging state of the WNT protein, the lipid moiety is shielded from the aqueous surrounding and lipid exposure to water is energetically unfavourable. Purified WNTs are solubilized with detergents such as CHAPS and stabilized by BSA indicating that the acyl group is bound to a carrier protein (Mihara et al., 2016; Willert, 2008). The thermodynamic problem arises when the shielded fatty acid group needs to leave the lipophilic surrounding, in order to be transported through an aqueous surrounding to engage with the lipophilic groove on the CRD of the receptor. This process is intrinsically unfavourable and would therefore require energy, catalysis or as yet unidentified mechanisms to be accomplished. Thus, it remains obscure how cells handle the lipid modification of WNTs during release from the cell, extracellular transport and recognition by the receptors through CRD binding in agreement with the published crystal structures (Hirai et al., 2019; Janda et al., 2012). This aspect becomes particularly interesting in the context of the ability of FZDs to mediate CRD-independent WNT effects and the ability of non-lipidated WNTs to induce signaling (Chen et al., 2004a; Speer et al., 2019). In conclusion, CRD interactions with WNTs are essential providing affinity and proximity but other mapping additional interaction with the receptor could be important to fully understand the mode of action of WNTs as FZD agonists.

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SMO: ligand-receptor interaction – a mystery

While WNTs directly interact with FZDs to induce WNT signaling, HH proteins (Desert Hedgehog DHH, Indian Hedgehog IHH, Sonic Hedgehog SHH) interact with a twelve transmembrane spanning protein called Patched (PTCH)(Alcedo et al., 1996; Stone et al., 1996). PTCH acts as cholesterol transporter and negative regulator of SMO activity by constitutively reducing cholesterol levels in the inner leaflet of the membrane (Kong et al., 2019; Kowatsch et al., 2019; Zhang et al., 2018). There is recent evidence that cholesterol and oxysterols could act as primary endogenous ligands at SMO through interaction with the SMO-CRD or the transmembrane-spanning core (Deshpande et al., 2019; Hedger et al., 2018; Huang et al., 2016; Huang et al., 2018; Luchetti et al., 2016; Nachtergael et al., 2012; Raleigh et al., 2018). However, similar to the lack of knowledge about how lipidated WNTs reach the CRD of FZDs, it remains unclear how cholesterol reaches the extracellular CRD binding pocket on SMO in living cells. In this regard, the role of a postulated intramolecular tunnel in SMO for cholesterol trafficking has neither been functionally nor experimentally confirmed so far (Deshpande et al., 2019; Hedger et al., 2018; Huang et al., 2018; Qi et al., 2019).

Similarly to the WNT/ β -catenin pathway, it remains a matter of debate under which circumstances the SMO/GLI pathway is mediated through heterotrimeric G proteins. The most recent data strongly support the concept that SMO acts as a $G_{i/o}$ and $G_{12/13}$ -coupling GPCR feeding into the SMO/GLI pathway (Manning et al., 2015; Ogden et al., 2008; Qi et al., 2019; Riobo et al., 2006; Shen et al., 2013; Wright et al., 2019). However, the role of $G_{12/13}$ proteins downstream of SMO remains less well defined (Guo et al., 2018; Wright et al., 2019).

Signal initiation – different concepts

Frizzleds. Currently, two mechanisms emerge that explain WNT/FZD-dependent signal initiation. In the WNT/ β -catenin pathway, WNT binding to the CRD of FZD results in

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signalosome formation (Bilic et al., 2007; DeBruine et al., 2017; Gammons et al., 2016). The WNT/FZD complex appears to serve as a platform – in cooperation with the co-receptors LRP5/6 – for the orchestration of a dynamic DVL recruitment, which results in the inhibition of the β -catenin destruction complex and transcriptional regulation through TCF/LEF transcription factors (Gammons and Bienz, 2018; Nusse and Clevers, 2017; Steinhart and Angers, 2018). Interestingly, in this model it remains completely unclear, what protein dynamic changes are evoked by ligand binding in FZDs to promote and regulate productive FZD-DVL interaction and signaling. While some data point to a constitutive, ligand-independent interaction between FZD and DVL, particularly in overexpressing cell systems, (Valnohova et al., 2018; Wright et al., 2019), other studies argue for a WNT-induced dissociation of a preformed FZD-DVL complex (Gammons et al., 2016) or an enhanced recruitment of DVL to FZD in response to agonist (the FZD₄-selective Norrin) (Bang et al., 2018). These discrepancies clearly underline that the dynamics in FZD-DVL interaction are not sufficiently understood. The modus of interaction between FZD and DVL proteins is complex and the functional role of FZD-DVL to mediate signalling is rather unclear and is best described as scaffolding function orchestrating interaction of signalling components, such as axin, casein kinase 1, β -arrestin, adapter protein 2 and others (Bryja et al., 2007; Schwarz-Romond et al., 2007; Strakova et al., 2018; Yu et al., 2007). Initially, a highly conserved, unconventional, internal PDZ ligand domain in the C terminus of FZDs, a KTxxxW sequence, was identified to mediate FZD-DVL contact to the PDZ domain of DVL (Umbhauer et al., 2000). Furthermore, other regions on the cytoplasmic parts of FZDs were pointed out to be important for a FZD-DVL interface. The flanking regions of the intracellular loop 3 (IL3) of FZDs provided a discontinuous docking site for DVL (Tauriello et al., 2012; Wright et al., 2018). Importantly, the DEP domain of DVL appears to have a key role for FZD interaction as well as for membrane recruitment involving electrostatic interaction between positive charges on the DEP domain and the negative head groups of the phospholipids (Gammons et al., 2016; Simons et al., 2009). Additional regions in FZDs, such as IL1, IL2 and C terminal residues distal of the KTxxxW sequence on the helix 8 of the receptor were

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identified by mutagenesis to be involved in FZD-DVL interaction (Bertalovitz et al., 2016; Pau et al., 2015; Strakova et al., 2017). However, it remains unclear if IL1 or IL2 are in fact direct interaction sites or if mutation of those residues affect the overall conformation of the receptor reducing its ability to recruit DVL. Particularly in the case of the conserved FZD₄ Y250^{2,39} (superscript numbers refer to Ballesteros Weinstein nomenclature for GPCRs; (Ballesteros and Weinstein, 1995)) in the C terminal end of IL1, we could show that an overall structural change of mutagenesis rather than direct interaction of the amino acid with DVL were the reason for reduced FZD-mediated DVL recruitment (Strakova et al., 2017).

In addition to the formation of a heterogenous signaling platform consisting of FZDs, LRP5/6, additional co-receptors and DVL, recent reports point at important dynamic changes in FZD-FZD interactions (DeBruine et al., 2017b). It has been known for some time that FZDs can form dimers or oligomers, even though their function and relevance for signal initiation was more unclear (Kaykas et al., 2004). The FZD₄-selective agonist Norrin acts as a homodimeric peptide and is capable of binding two CRD of FZD₄ (Ke et al., 2013). Furthermore, it was shown that unsaturated fatty acids can dimerize CRDs of FZDs implicating that the lipid modification of WNTs could bridge two CRDs resulting in ligand-induced receptor dimerization or oligomerization (DeBruine et al., 2017a; Nile et al., 2017). In contrast to the model that ligand binding brings receptors together, we reported that FZD₆ dimers dissociate upon WNT-5A stimulation in order to promote signaling towards ERK1/2 (Petersen et al., 2017). Thus, the dimeric form of FZD₆ constitutes the ligand-interpreting receptor species, whereas the monomeric form presents the active species initiating signaling. Re-association of FZD₆ dimers in the range of minutes after WNT addition correlated with signal termination further supporting the concept of dynamic dimerization. The argument that a monomeric GPCR presents the minimal signaling unit is established for many receptors that show ligand-dependent G protein coupling when solubilized in detergent micelles at monodispersion or embedded in HDL particles or nanodiscs (Draper-Joyce et al., 2018; Garcia-Nafria et al., 2018a; Garcia-Nafria et al., 2018b; Krishna Kumar et al., 2018; Liang et

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al., 2017; Whorton et al., 2007; Zhang et al., 2017c). Furthermore, inactive GPCRs frequently crystallize as dimers (see also SMO (Wang et al., 2013)), whereas not a single active, G protein-bound GPCR structure was reported as dimer.

The idea of dimer dissociation upon receptor activation also merges well with the second concept of Class F receptor activation, which is reminiscent of what is known for the activation of Class A and B GPCRs: the agonist, the receptor and the heterotrimeric G proteins act in a ternary complex to induce GDP release and subsequent GTP binding of the $G\alpha$ subunit as the primary output of the ligand-receptor interaction (De Lean et al., 1980; Schulte and Wright, 2018). Decades of structural analysis have identified a common theme in GPCR activation generally involving a swing out of the helix 6 of the receptor firmly accommodating the G protein in the active state of the receptor (Fig. 1) (Gether and Kobilka, 1998; Latorraca et al., 2017; Weis and Kobilka, 2018). FZD-FRET probes designed to monitor agonist-induced dynamics especially in TM6 of the receptor relative to the C terminus support the idea that agonist stimulation of FZDs induces an active state similar to those observed in Class A and B receptors (Schulte and Wright, 2018; Wright et al., 2018). In fact, the recently identified molecular switch between the lower ends of TM6 and TM7, which is highly conserved among Class F receptors, is very similar to a polar network essential for Class B receptor activation and G protein coupling (Liang et al., 2018b). Its functional relevance is further underlined by the oncogenic potential of mutations in the residues participating in the molecular switch, such as the cancer-associated SMOM2 mutant in W^{7.55} (Wright et al., 2019).

Smoothened. Similar to FZDs, different conceptual perceptions of receptor activation mechanisms have emerged. On one hand, SMO translocation to the cilium has served as a measure of receptor and pathway activation (Milenkovic et al., 2009). While this physical relocation of SMO coincides with pathway activation, it could be misleading regarding the biophysical and pharmacological understanding of receptor activation manifesting as a conformational change in the receptor molecule. As an example, the plant alkaloid

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cyclopamine, which binds SMO without (antagonist) or with negative (inverse agonist) efficacy, affects ciliary translocation of SMO, without promoting conformational changes resulting in receptor activation (Chen et al., 2002a; Incardona et al., 1998; Schulte and Kozielewicz, 2019; Wang et al., 2009; Weierstall et al., 2014; Wright et al., 2019). The availability of diverse SMO ligands ranging from the natural compound cyclopamine, smoothed agonists (SAG series of compounds), antagonists (SANT series of compounds) and diverse sterols have been so far helpful to define small molecule driven SMO activation and signal initiation (Chen, 2016; Chen et al., 2002b; Frank-Kamenetsky et al., 2002; Kozielewicz et al., 2019).

SMO in ternary complex with heterotrimeric G_i or nanobody NbSmo8

Over the years, detailed structural and mechanistic insight into the activation of Class F receptors was lacking, and the concept of Class F receptors coupling to heterotrimeric G proteins was – despite substantial experimental evidence – met with scepticism (Malbon, 2011; Schulte, 2010; Schulte and Bryja, 2007; Schulte and Kozielewicz, 2019). The large amount of SMO structures in inactive states as well as the first crystal structure of a FZD in the apo (ligand-free) state were recently complemented with two mammalian SMO structures in the active state. Despite the different experimental approaches used to resolve SMO activation by (i) CryoEM imaging of a sterol ligand-bound human SMO in complex with heterotrimeric G_i (PDB ID: 6OT0 (Qi et al., 2019)) and (ii) X-ray crystallography of an agonist (SAG21k)-bound human SMO in complex with a stabilizing nanobody (PDB ID: 6O3C (Deshpande et al., 2019)), both structures support a similar concept of receptor activation, the involvement of a common molecular switch mechanism and overall similar structural rearrangements. In comparison with active, G protein-bound Class A and B receptors, the active SMO structures also present an outward movement of TM6 (Fig. 1) (Deshpande et al., 2019; Hua et al., 2016; Krishna Kumar et al., 2018; Liang et al., 2018a; Liang et al., 2018b; Qi et al., 2019; Rasmussen et al., 2011; Song et al., 2017; Wacker et al., 2010; Yang et al.,

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2018; Zhang et al., 2017c). This outward movement in the active state is a consequence of ligand-induced and G protein-stabilized structural rearrangements breaking interactions in conserved motifs, such as the D(E)RY motif in Class A and a polar network in Class B receptors. However, the mode of conformational change observed in active SMO is slightly different compared to Class A/B receptors. In particular, there is a notable 7-8 Å outward movement of TM6 and a 4-5 Å inward movement of TM5. The larger changes on the intracellular facing part of the receptor are mirrored by minor rearrangements in the extracellular part. The active, NbSmo8-bound SMO presents with an upward displacement of TM6 and ECL3 and a minor tilt in the CRD compared with inactive SMO (Deshpande et al., 2019). Similarly, the G_i-bound SMO presents with a TM6 rearrangement manifesting in an outward movement and an upward shift of TM6 (Qi et al., 2019). Interestingly, the TM6 appears to link CRD and the intracellular regions that undergo larger conformational changes physically, thereby potentially explaining complex allosteric modulation of receptor activity by the CRD itself and molecules interacting with the CRD and lower binding pockets. As mentioned above, the structures of the active state of SMO confirm the concept of a common molecular switch in Class F receptors, which we identified recently (Wright et al., 2019). The polar interactions between the conserved basic residue R/K^{6.32} and the backbone of TM7 that are common in inactive Class F receptor structures are broken in the active SMO structures as a result of the outward movement of TM6 (Deshpande et al., 2019; Qi et al., 2019; Wright et al., 2019). Furthermore, the distance between the R/K^{6.32} and the aromatic electron system of the W^{7.55} is increased indicating that also the π-cation interactions as part of the molecular switch are weakened (Deshpande et al., 2019; Qi et al., 2019).

Furthermore, the SMO-G_i structure revealed a different arrangement of the α5 helix of the G_i protein that leads to a less pronounced TM6 outswing compared to G_i-coupled Class A receptor (e.g. cannabinoid CB₁ receptor; PDB ID: 6N4B (Krishna Kumar et al., 2018)). Interestingly, the SAG21k- and cholesterol-bound SMO (PDB ID: 6O3C (Deshpande et al., 2019)) represents a unique ligand-bound receptor structure in which three ligands are bound

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simultaneously to a single receptor. The SAG21k occupies the upper ligand binding site in the 7TM core just on top of a cholesterol bound to the lower binding site in the 7TM core of SMO. Furthermore, a second cholesterol occupies the lipophilic binding groove in the CRD (Fig. 2). So far, the majority of the cyclopamine- or sterol-bound SMO structures solved until now (Byrne et al., 2016; Huang et al., 2018; Wang et al., 2014; Wang et al., 2013; Weierstall et al., 2014; Zhang et al., 2017b) differ substantially with regards to ligand binding poses as these small molecules have been reported to bind the CRD only, the CRD and the upper 7TM core site, the CRD and the lower 7TM core site or only the upper 7TM core site (Fig. 2). It cannot be ruled out that these differences reflect different purification and reconstitution approaches used in the various studies, but it also underlines the high complexity of SMO receptor activation mechanisms (Hedger et al., 2018; Luchetti et al., 2016; Myers et al., 2017). As opposed to other GPCRs, it remains still obscure, which binding sites on SMO present orthosteric or allosteric sites, and what the functional role of the CRD could be as an allosteric modulator (Byrne et al., 2016; Kozielewicz et al., 2019). Interestingly, additional sites have been implicated, such as a cytoplasmic binding pocket, comprised of cytoplasmic facing portions of TM1, TM3, TM6 and TM7, that was identified by docking of oxysterols to human SMO (Raleigh et al., 2018). Along these lines, the nature of endogenous SMO ligands, cholesterol and naturally occurring oxysterols, is also not fully understood as these molecules may serve as orthosteric agonists or allosteric modulators. Interestingly, there is a possibility that under certain cellular conditions with sufficiently high cholesterol levels, SMO signaling may occur, in fact, in the absence HH stimulation.

The CRD appeared flexible in the SMO-G_i complex resulting in poor resolution by CryoEM not allowing secure conclusions regarding its position in the ternary complex with heterotrimeric G protein (Qi et al., 2019). On the other hand, the CRD of human SMO is well resolved in the crystal structure of the SMO-NbSmo8 complex indicating that the previously reported reorientation of the CRD in *Xenopus laevis* SMO does not accompany receptor activation (Deshpande et al., 2019; Huang et al., 2018; Schulte and Kozielewicz, 2019).

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Pathway selective conformations

Identification of a conserved molecular switch in Class F receptors provided a molecular mechanism for constitutive and ligand-induced activation of Class F receptors in agreement with what is known from other GPCRs (Trzaskowski et al., 2012; Wright et al., 2019). It is known that WNTs can induce WNT/β-catenin in parallel to β-catenin-independent signaling for example via WNT/ERK1/2 signaling in mouse, primary microglia cells (Halleskog and Schulte, 2013) and in other biological systems (Samarzija et al., 2009; Schlange et al., 2007). Employing genome editing to create a cell system deficient in signaling through heterotrimeric G proteins, we have recently shown that WNT-3A maintains its ability to induce WNT/β-catenin signaling efficiently in the complete absence of heterotrimeric G protein signaling (Bowin et al., 2019). While these findings strongly argue against an involvement of heterotrimeric G proteins in the WNT-induced WNT/β-catenin signaling they further support the concept of conformation-driven, functional selectivity (Fig. 3). It is impossible for FZDs that carry a mutation in the conserved R/K^{6,32} molecular switch, which presents with enhanced potency towards G protein coupling, to interact with DVL and to mediate DVL-dependent signaling pathways such as the WNT/β-catenin branch (Wright et al., 2019). Along the same line, we have previously described FZD mutations that exhibit selective DVL over G protein preference or vice versa (Kilander et al., 2014b; Strakova et al., 2017), underlining that pathway selection between G protein-dependent signaling and DVL-dependent pathways originates in a conformational selection in agreement with the recently developed concept of GPCR bias or functional selectivity (Kenakin, 2019). Especially, the Y250F^{2,39} mutation in FZD₄, which presents with an impaired ability to recruit DVL is conceptually of interest. This residue is engaged in a polar network with H348^{4,46} and W352^{4,50} stabilizing an overall receptor conformation, which is essential for effective FZD-DVL interaction (Strakova et al., 2017; Yang et al., 2018). While mutation of this residue interferes with FZD-DVL interaction, FZD₄ Y250F^{2,39} maintains its ability to interact with heterotrimeric G proteins underlining that distinct conformations are required for complex

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formation with either DVL or heterotrimeric G protein (Arthofer et al., 2016; Strakova et al., 2017). This argumentation and the similarity between the SMO-NbSm08 and the SMO-G_i complex structure also further support the implication by Deshpande et al. that the NbSm08 indeed stabilizes the active, G protein-bound SMO. Looking forward, it will now be interesting to explore if there are alternative active conformations for SMO for example in complex with β-arrestin or GPCR kinases (Chen et al., 2004b; Schulte and Wright, 2018; Zhao et al., 2016). Excitingly, the concept of conformational selection comes with opportunities for biased ligands to target Class F receptors in a pathway selective manner similar to what has been successful in the case of Class A GPCRs (Kenakin, 2019).

Conclusions and future perspectives

This review aims to summarize the state of the art of our understanding of Class F receptor activation fuelled by the recent structural and functional dissection of active SMO in a ternary complex (Deshpande et al., 2019; Qi et al., 2019; Wright et al., 2019). The implications of this more detailed insight into SMO and FZD activation for understanding Class F receptor biology, disease-associated mutations in Class F receptors and future efforts to develop more selective, potentially pathway-biased Class F receptor-targeting drugs are exciting and promising. While some general concepts and molecular mechanisms of receptor activation emerge, several resulting key questions remain. How is it possible to assess the contribution of the different small molecule ligand binding pockets on SMO (Fig. 2) for receptor activation and how can that knowledge be exploited to target SMO therapeutically potentially avoiding resistance-prone mutations? How are the different sterol-targeted binding sites driving receptor activation *in vivo* and how does that knowledge translate to FZDs regarding the localization and function of the lipid modification of WNTs for ligand binding and receptor activation? How does WNT binding to the CRD drive receptor activation? How does the CRD contribute to agonist-induced and constitutive receptor activation? How does the accessibility and local concentration of intracellular binding partners contribute to ligand-induced pathway selection? With the advances of structural analysis of transmembrane receptor complexes

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using for example CryoEM, gene editing technology and the optimization of sensitive resonance energy transfer assays to assay protein-protein interactions and receptor dynamics in living cells, future work will shed even more light on the activation mechanisms defining this enigmatic receptor family. The hope is that the new knowledge will not only result in better understanding of the biology of Class F receptors but also in assay development for innovative drug screening technologies, efficient drug discovery of Class F-targeting and potentially pathway-biased compounds.

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Footnotes

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Figure Legends:

Fig. 1: Comparison of the intracellular re-arrangements between the active and inactive states of Class A, B and F GPCRs. In Class A, interactions between TM3 and TM6 stabilize the inactive receptors conformations, whereas in Classes B and F similar interactions are observed between TM6 and TM7/8 and to lesser extent TM2. In each of these receptor classes, outward movement of TM6, manifesting the receptor activation, breaks the observed interaction pattern. Currently, active structures of Class C GPCRs remain unsolved, and thus, Class C is excluded from the comparison. Receptors are shown as a range of grey and green cartoon (inactive and active structures, respectively) and the key residues as corresponding sticks. Black dashes indicate possible hydrogen bonds. View is from the intracellular side of the receptors. PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used for visualization.

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Fig. 2: SMO contains (at least) three small molecule binding pockets. The pharmacology of SMO is complex. The receptor model was derived from the active, NbSmo8-bound crystal structure of human SMO (PDB ID: 6O3C) bound to two cholesterol molecules and a small molecule agonist called SAG21k (Deshpande et al., 2019). The full length receptor is shown as cartoon with round helices and opaque surface. The N terminal CRD is shown in red. The structured linker domain is shown in green. Ligands are shown as spheres in yellow and the three main ligand-binding sites are encircled in bright yellow. The grey part of the receptor presents the transmembrane domain. A clear distinction of orthosteric and allosteric sites is so far not possible because the endogenous ligand of SMO and its primary binding site are not identified. In addition, cooperativity between binding sites remains obscure. PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used for visualization.

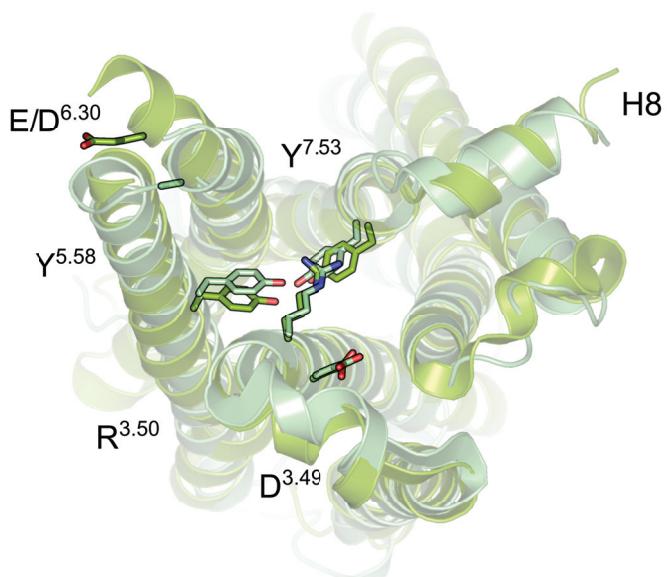
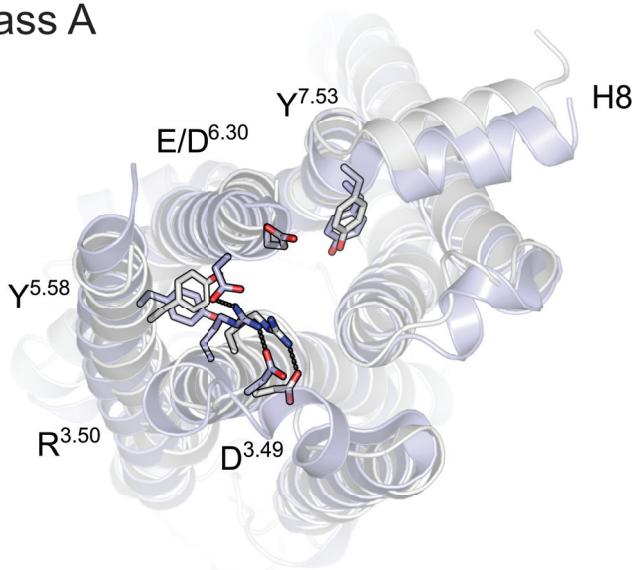
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Fig. 3: The concept of conformational selection and functional selectivity. In accordance with what is known for Class A and B receptors (Kenakin, 2019; Latorraca et al., 2017), we present here how conformational selection either driven by endogenous ligands of the receptors, by selective Class F receptor-targeting drugs, intrinsic constitutive activity and/or intracellular binding partners can contribute to pathway selective signal initiation. Colours highlight receptor conformations that are stabilized by interaction for example with heterotrimeric G proteins, DVL or arrestins (Schulte and Wright, 2018). Other, yet unidentified binding partners could stabilize additional active states of Class F receptors. The theoretical number of active conformations is unlimited. FZD₆ models (Wright et al., 2019) were modified using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The CRD is not shown.

Inactive

Active

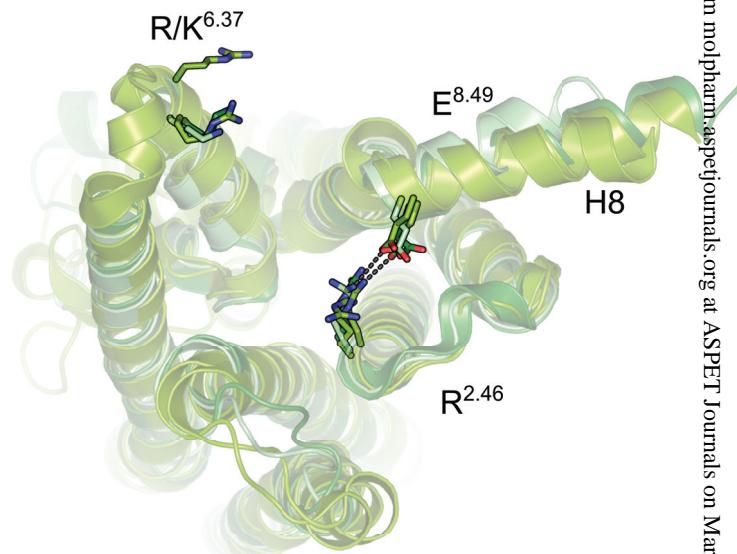
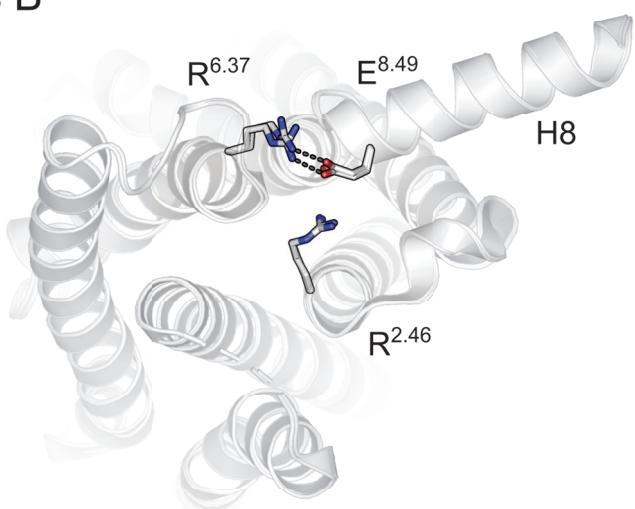
Class A



β_2 adrenoceptor (PDB ID: 3NYA)
CB₁ cannabinoid receptor (5TGZ)

β_2 adrenoceptor (3SN6)
CB₁ cannabinoid receptor (6N4B)

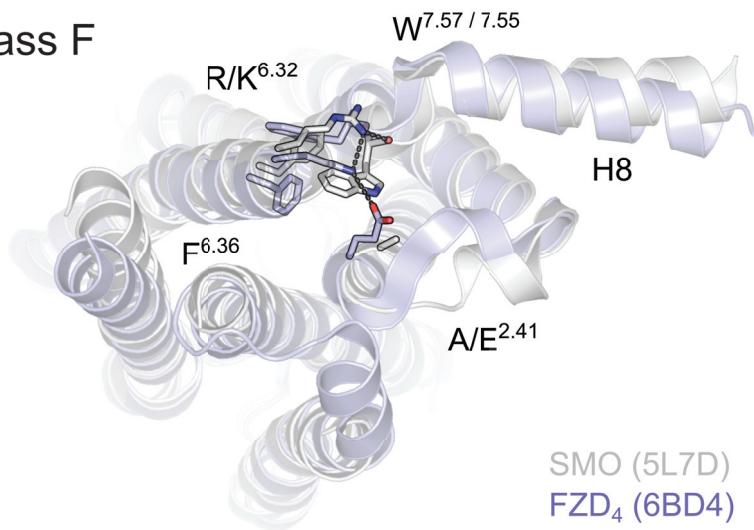
Class B



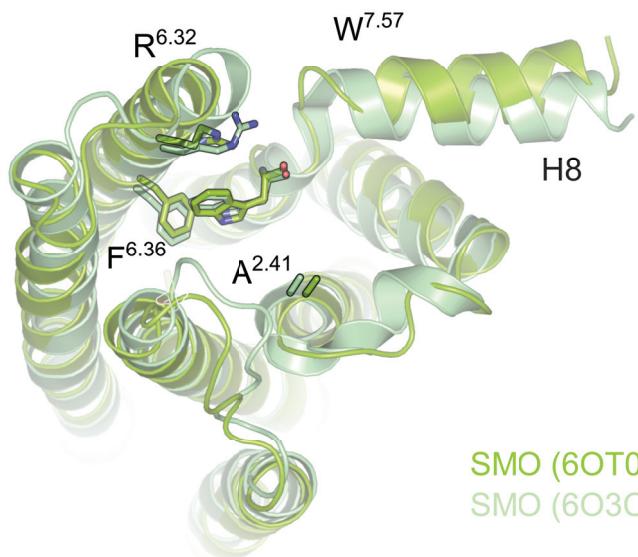
GLP-1 receptor (5VEW, 5VEX)

GLP-1 receptor (5VAI, 6B3J)
CLR receptor (6E3Y)
CT receptor (6NIY)

Class F



SMO (5L7D)
FZD₄ (6BD4)



SMO (6OT0)
SMO (6O3C)

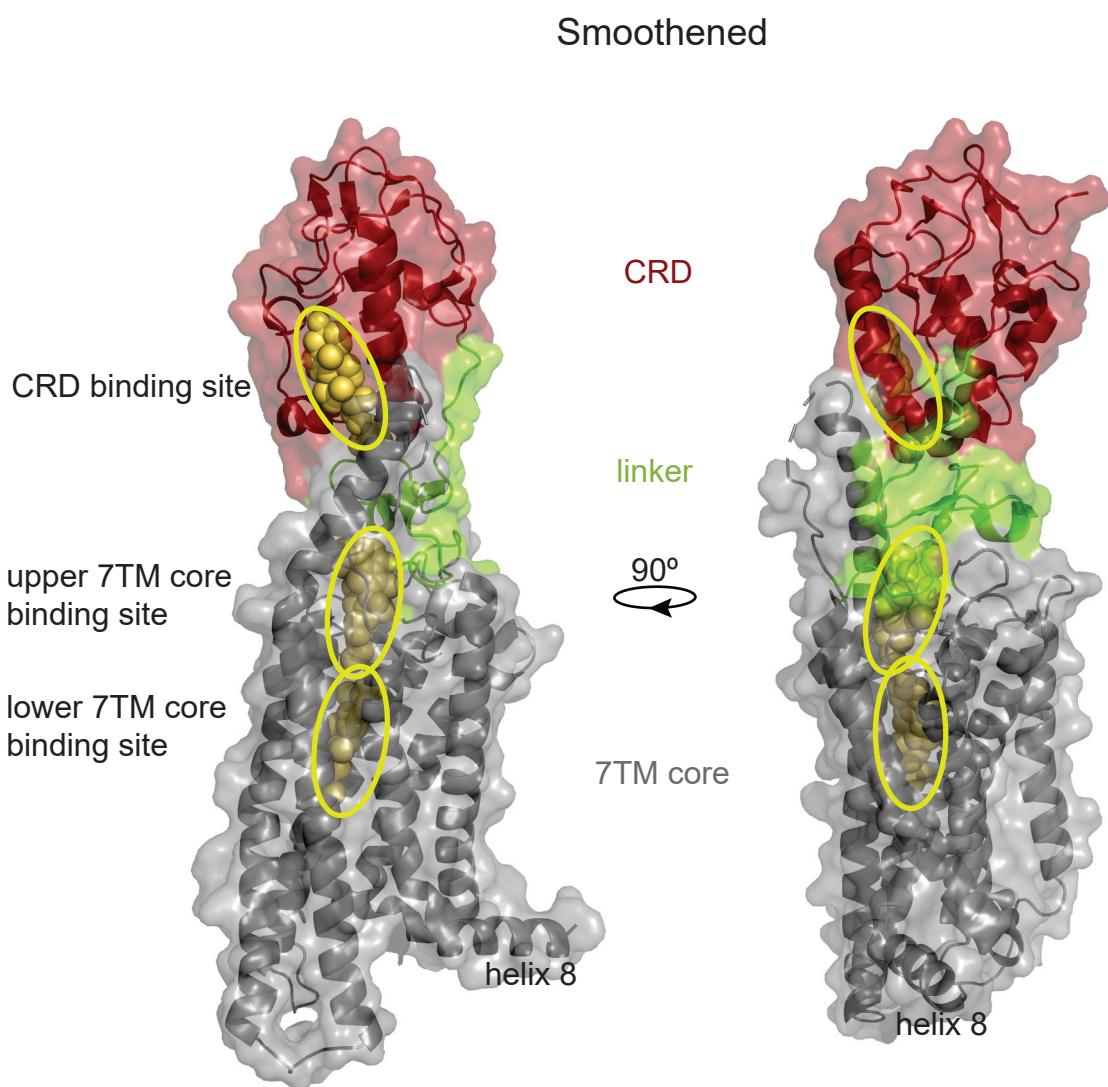


Figure 2

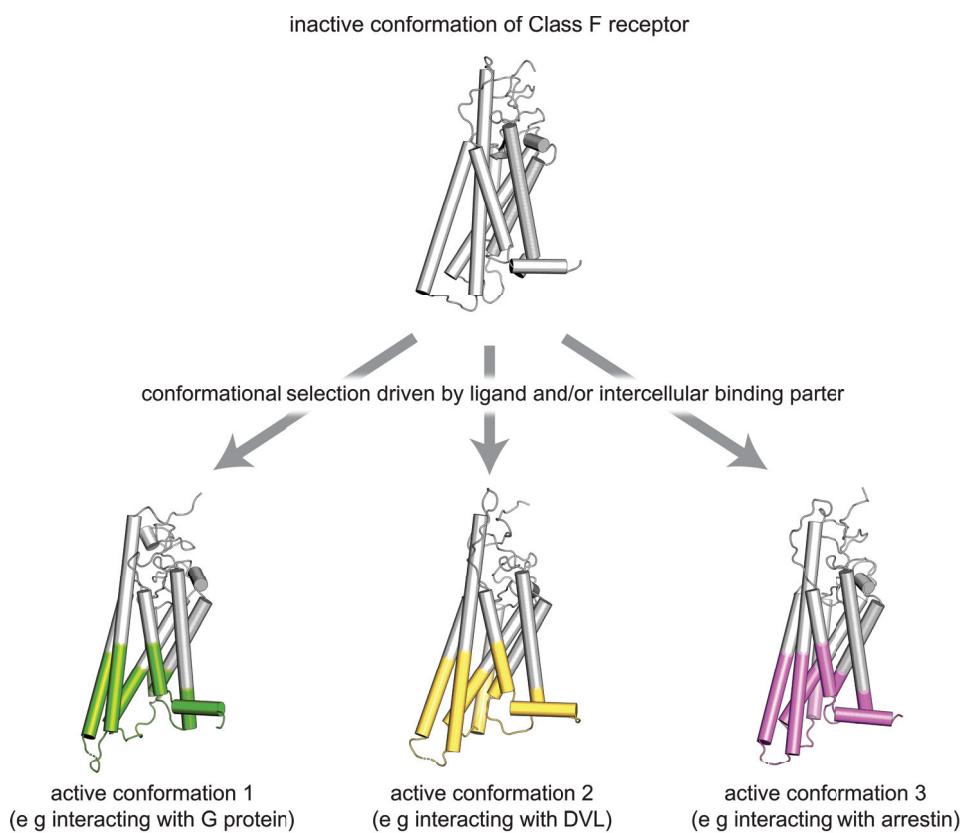


Figure 3