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WNT/FRIZZLED SIGNALING – ILLUMINATING THE ROAD TOWARDS PATHWAY SELECTIVITY

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WNT/FRIZZLED SIGNALING – ILLUMINATING THE ROAD TOWARDS PATHWAY SELECTIVITY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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För kunskap
och förståelse

“Anything worth doing, is worth doing right.”
– Hunter S. Thompson

ABSTRACT

Cells sense and respond to their environment via receptors embedded in the plasma membrane. Receptors allow flow of information from outside to the inside of the cell and are generally regulated by extracellular molecules and proteins, known as ligands. Receptors are dynamic and – when activated – change conformation to initiate signal transduction. One family of receptors are the G protein-coupled receptors (GPCRs) in which the Class F receptors comprised of Frizzleds (FZDs) and Smoothened (SMO) are found. FZDs bind their endogenous ligands called WNTs, a group of lipoglycoproteins, and interact with multiple intracellular signal transducing proteins, such as the scaffolding-protein Dishevelled (DVL) and heterotrimeric G proteins. WNT/FZD signaling is crucial for proper embryonic development and tissue homeostasis but can when dysregulated lead to diseases such as cancer. This thesis aims to illuminate the molecular mechanisms underlying WNT/FZD signal transduction and signaling specification. The findings will further the understanding of events regulated by these receptors and aid in development of therapeutics to treat FZD-related diseases.

This thesis began with the description of a molecular switch present in all Class F receptors that when mutated is a driver of cancer. It was found that the molecular switch opened in the process of receptor activation to accommodate the G protein and to initiate signaling. Mutation of the molecular switch in FZDs inhibited the receptor's ability to adopt DVL-interacting conformations, leading to increased receptor activity and enhanced WNT-induced signaling towards heterotrimeric G proteins. Furthermore, the molecular switch network was extended to include additional amino acids, including a conserved proline in FZDs. Interestingly, SMO, which binds cholesterol, harbors a phenylalanine in this position. Mutating this phenylalanine in SMO obstructed binding of cholesterol, producing a G protein signal impaired receptor. Surprisingly, mutating the conserved proline in FZDs resulted in heterogeneous signaling behavior, suggesting FZD homologue-specific signaling mechanisms. The thesis further investigated WNT/β-catenin signaling, which is a FZD-controlled signaling pathway important for cell proliferation and differentiation. DVL has a critical role in this signaling pathway, but the importance of heterotrimeric G proteins has been a long-standing debate. To that end, a series of experiments in heterotrimeric G protein knockout cells were conducted. It was concluded that heterotrimeric G proteins are not required for efficient WNT/β-catenin signaling although they still have an important regulatory role as demonstrated by earlier studies. The final part of this thesis described the development of biosensors to enable the investigation of the poorly explored area of WNT-induced FZD-DVL dynamics. It was discovered that distinctly different conformations could be adopted in WNT-induced FZD-DVL dynamics and that these conformations were WNT- and FZD-dependent.

Overall, this thesis has broadened the understanding of molecular mechanisms involved in the initiation and regulation of WNT/FZD signaling. More specifically, some molecular details of the mechanisms that determine how FZDs activate DVL- and heterotrimeric G protein-dependent signaling were clarified and, thus, this thesis has illuminated the road towards pathway selectivity.

SAMMANFATTNING

Celler känner av och svarar på sin omgivning via receptorer inbäddade i plasmamembranet. Receptorer möjliggör att information flödar från utsidan till insidan av cellen och regleras generellt av extracellulära molekyler och proteiner, så kallade. ligander. Receptorer är dynamiska i sin natur och när de aktiveras byter de konformation för att initiera signaltransduktion. En familj av receptorer är G protein-kopplade receptorer (GPCRer) och till den hör Class F av GPCRer i vilket Frizzleds (FZDs) och Smoothened (SMO) ingår. FZDs binder endogena ligander kallade WNTs, en grupp av lipoglykoprotein, och interagerar med flertalet intracellulära transduktionsproteiner så som Dishevelled (DVL) och heterotrimera G protein. WNT/FZD-signalering är vitalt för embryonal utveckling och vävnadshomeostas men leder till sjukdomar så som cancer vid okontrollerad reglering. Denna avhandlingen ämnar att belysa de molekylära mekanismer som ligger till grund för WNT/FZD signaltransduktion och signaleringsspecificering. Dessa fynd kommer bredda vår förståelse för processer reglerade av dessa receptorer och hjälpa i utvecklingen av läkemedel för FZD-relaterade sjukdomar.

Avhandlingen började med att beskriva ett molekylärt lås som är närvarande i alla Class F receptorer och som när muterat pådriver utvecklingen av cancer. Det upptäcktes att det molekylära låset öppnades i samband med receptoraktivering för att ackommodera G proteinet och initiera signalering. Mutation av det molekylära låset inhiberade receptorns möjligheter att anta DVL-interagerande konformationer vilket ledde till ökad receptoraktivitet och ökad signalering mot heterotrimera G protein. Därefter utökades det molekylära låsets nätverk till att inkludera ytterligare aminosyror, inklusive ett konserverat prolin. Intressant nog är SMO, som binder kolesterol, annorlunda och har ett fenylalanin istället för prolin. Mutation av detta fenylalanin i SMO förhindrade inbindning av kolesterol och ledde till nedsatt förmåga att signalera via G protein. Förvånande nog ledde mutation av det konserverade prolinet hos FZDs till ett heterogent signaleringsmönster, vilket föreslår att det finns FZD-homologspecifika signaleringsmekanismer. Fortsättningsvis undersökte avhandlingen WNT/β-catenin-signalering, en FZD-kontrollerad signaleringsväg viktig för cellproliferering och differentiering. DVL har en oumbärlig roll i denna signaleringsväg men vikten av heterotrimera G protein har varit kraftigt debatterad. För att finna svar på detta utfördes en serie experiment i cellinjer med utslagna heterotrimera G protein. Detta resulterade i slutsatsen att heterotrimera G protein är överflödiga för en fungerande WNT/β-catenin-signaleringsväg men att de fortfarande spelar en viktig reglerade roll vilket påvisats av tidigare studier. I den sista delen av avhandlingen beskrevs utvecklingen av biosensorer för att möjliggöra undersökningen av det outforskade området kring WNT-inducerad FZD-DVL-dynamik. Det upptäcktes att distinkt olika konformationer kunde antas i WNT-inducerad FZD-DVL-dynamik samt att dessa konformationer var WNT- och FZD-homologberoende.

Sammantaget har denna avhandling breddat förståelsen för molekylära mekanismer involverade i initiering och reglering av WNT/FZD-signalering. Mer specifikt har vissa molekylära detaljer förtydligats för mekanismer som avgör hur FZDs aktiverar DVL- och heterotrimera G protein-beroende signalvägar. Därmed har denna avhandling belyst vägen som leder till signaleringsselektivitet.

LIST OF SCIENTIFIC PAPERS

I. **A conserved molecular switch in Class F receptors regulates receptor activation and pathway selection**
Shane C. Wright*, Paweł Kozielewicz*, Maria Kowalski-Jahn, Julian Petersen, **Carl-Fredrik Bowin**, Greg Slodkowicz, Maria Marti-Solano, David Rodríguez, Belma Hot, Najeah Okashah, Katerina Strakova, Jana Valnohova, M. Madan Babu, Nevin A. Lambert, Jens Carlsson and Gunnar Schulte. *Nature Communications* 10 (2019). DOI: 10.1038/s41467-019-08630-2

II. **WNT-3A-induced β-catenin signaling does not require signaling through heterotrimeric G proteins**
Carl-Fredrik Bowin, Asuka Inoue and Gunnar Schulte. *Journal of Biological Chemistry* 294, 11677-11684 (2019). DOI: 10.1074/jbc.ac119.009412

III. **Residue 6.43 defines receptor function in Class F GPCRs**
Ainoleena Turku, Hannes Schihada*, Paweł Kozielewicz*, **Carl-Fredrik Bowin** and Gunnar Schulte. *Nature Communications* 12 (2021). DOI: 10.1038/s41467-021-24004-z

IV. **WNT-induced dynamics of Frizzled-Dishevelled interaction support an alternative ternary complex model for Frizzleds**
Carl-Fredrik Bowin, Paweł Kozielewicz, Maria Kowalski-Jahn, Hannes Schihada and Gunnar Schulte. *Manuscript*.

* These authors contributed equally.

ADDITIONAL PUBLICATIONS

I. **A NanoBRET-based binding assay for Smoothened allows for real-time analysis of ligand binding and distinction of two binding sites for BODIPY-cyclopamine**
Paweł Kozielewicz, **Carl-Fredrik Bowin**, Ainoleena Turku and Gunnar Schulte. *Molecular Pharmacology* 97 (2020). DOI: 10.1124/mol.119.118158

II. **Structural insight into small molecule action on Frizzleds**
Paweł Kozielewicz, Ainoleena Turku, **Carl-Fredrik Bowin**, Julian Petersen, Jana Valnohova, Maria Consuelo Alonso Cañizal, Yuki Ono, Asuka Inoue, Carsten Hoffmann and Gunnar Schulte. *Nature Communications* 11 (2020). DOI: 10.1038/s41467-019-14149-3

III. **Quantitative profiling of WNT-3A binding to all human Frizzled paralogues in HEK293 cells by NanoBiT/BRET assessments**
Paweł Kozielewicz¤, Rawan Shekhani, Stefanie Moser, **Carl-Fredrik Bowin**, Janine Wesslowski, Gary Davidson¤ and Gunnar Schulte¤. *ACS Pharmacology & Translational Science* 4 (2021). DOI: 10.1021/acsptsci.1c00084

IV. **Cryo-EM structure of human Frizzled 7 in complex with heterotrimeric G_s**
Lu Xu*, Bo Chen*, Hannes Schihada*, Shane C. Wright*, Ainoleena Turku, Yiran Wu, Gye-Won Han, Maria Kowalski-Jahn, Paweł Kozielewicz, **Carl-Fredrik Bowin**, Xianjun Zhang, Chao Li, Michel Bouvier, Gunnar Schulte¤ and Fei Xu¤. *Cell Research* (2021). DOI: 10.1038/s41422-021-00525-6

* These authors contributed equally.

¤ Shared corresponding authors.

CONTENTS

LIST OF ABBREVIATIONS

7TM	Seven-transmembrane
APC	Adenomatosis polyposis coli
BRET	Bioluminescence resonance energy transfer
CAMKII	Calmodulin-dependent kinase II
CD86	Cluster of differentiation 86
CELSR	Cadherin EGF LAG seven-pass G-type receptor
CG	Chorionic gonadotropin
CK1	Casein kinase 1
CK2	Casein kinase 2
CLR	Calcitonin receptor-like receptor
CM	Conditioned medium
CRD	Cysteine-rich domain
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DEP	Dishevelled, Egl-10 and Pleckstrin
DIX	Dishevelled and Axin
DKK	Dickkopf
DVL	Dishevelled
ECD	Extracellular domain
ECL	Extracellular loop
FZD	Frizzled
GEF	Guanine nucleotide exchange factor
GLP-1	Glucagon-like peptide 1
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK3	Glycogen synthase kinase 3
H8	Helix 8
HEK293	Human embryonic kidney 293
ICL	Intracellular loop
IP_3	Inositol triphosphate

KO	Knockout
LBD	Ligand-binding domain
LGR4/5	Leucine-rich repeat containing G protein-coupled receptor 4/5
LHCGR	Luteinizing hormone-choriogonadotropin receptor
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MD	Molecular dynamics
mG	Mini G protein
NEK2	Serine/threonine-protein kinase Nek2
NFAT	Nuclear factor associated T-cells
Nluc	Nanoluciferase
PA	Phosphatidic acid
PCP	Planar cell polarity
PDE	Phosphodiesterase
PDZ	Postsynaptic density protein-95, disc large, zonula occludens-1
PI	Phosphatidylinositol
PIP_2	Phosphoinositol diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
RAMP1	Receptor activity-modifying protein 1
RGS	Regulators of G protein signaling
RNF43	Ring finger 43
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1/2
RYK	Receptor tyrosine kinase
sFRP	Soluble Frizzled related protein
TCF/LEF	T-cell factor/lymphoid-enhancing factor
TMD	Transmembrane domain
VANGL	Van Gogh-like
WIF	WNT inhibitory factor
WNT	Wingless-type integration site
ZNRF3	Zinc and ring finger 3

1 INTRODUCTION

In the year 1973, a wingless *Drosophila melanogaster* mutant was reported, called *wg*^{1} (Sharma, 1973). Three years later, an article was published describing the *wg*^{1} mutation as giving mesothoracic abnormalities, suggesting it operated early during development (Sharma and Chopra, 1976). Later, studies on the mouse mammary tumor virus identified an insertion of a provirus in a gene that was named *int1* (Nusse and Varmus, 1982). However, some years later this gene was shown to be the same one identified in the *wg*^{1} *D. melanogaster* mutant. Hence, the nomenclature of the gene family became WNT — a mnemonic for wingless-type integration site (Nusse et al., 1991; Rijsewijk et al., 1987). WNTs were identified as secreted proteins and it was evident that they had an important signaling function during development. Two decades after the description of the first WNT gene, the first receptor mediating WNT signaling was identified as *frizzled2* following an experiment where *D. melanogaster* cells transfected with the receptor responded to the addition of *Wnt-1* (Bhanot et al., 1996).

1.1 G PROTEIN-COUPLED RECEPTORS

The family of G protein-coupled receptors (GPCRs) comprise a diverse set of membrane-embedded proteins designed to transfer information from extracellular stimuli into the cell, consisting of various ligands ranging from photons, ions, small molecules, peptides and large proteins (Venkatakrishnan et al., 2013; Weis and Kobilka, 2018). GPCRs comprise a single polypeptide chain beginning with the N-terminus outside the cell and continuing with seven hydrophobic transmembrane (7TM) α-helices linked by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3) ending in the amphipathic helix 8 at the C-terminus which is present on the intracellular surface. In humans, there are over 800 GPCRs and they are divided into six different classes based on sequence homology: Class A (Rhodopsin-like), Class B (secretin receptor family), Class C (metabotropic glutamate), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors) and Class F (frizzled/smoothened) (Alexander et al., 2019). In response to ligand binding, GPCRs are stabilized in certain conformations allowing for engagement with specific transducer proteins. Heterotrimeric G proteins (see section 1.1.1 “Heterotrimeric G proteins and the ternary complex model”) are one group of such transducer proteins, but also arrestins and G protein-coupled receptor kinases (GRKs) interact with GPCRs, adding to the potential selection of downstream signaling (Komolov et al., 2017; Liang et al., 2018a; Zhou et al., 2016). Furthermore, it should be mentioned that a receptor population samples multiple conformations simultaneously, favoring conformations with low free energy. Upon ligand binding and intracellular transducer protein interactions there is a shift in the free energy for these conformations via a series of microswitches in the receptor, shifting the equilibrium of which conformations are sampled by the population. Also, different ligands will stabilize different receptor conformations and therefore promote pathway selectivity. The shift in equilibrium can also be promoted by intracellular protein interactions, as is the case of constitutively active receptors that bind and activate heterotrimeric G proteins without ligand binding (Fleetwood et al., 2020; Kenakin, 2017; Weis and Kobilka, 2018; Ye et al., 2016).

1.1.1 Heterotrimeric G proteins and the ternary complex model

Heterotrimeric G proteins consist of three subunits: Gα, Gβ and Gγ. Importantly, the Gα subunit has a binding site for GDP (inactive) or GTP (active) located between the Ras and α-helical domain and harbors weak GTPase activity. They are further subdivided into four families: $G\alpha_{s}$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ which have different signaling outcomes (Milligan and Kostenis, 2006). GPCRs act as guanine nucleotide exchange factors (GEFs) and facilitate the exchange of GDP to GTP upon activation. This is achieved by the outward movement of TM6 of the GPCR, exposing a larger surface area in the cavity of the 7TM bundle that engages the

α5 helix of the Ras domain of the G protein allowing for the release of GDP (Carpenter and Tate, 2017). The high intracellular content of GTP results in rapid binding of GTP to the Gα subunit, promoting either its dissociation from the Gβγ dimer (Digby et al., 2006) or a structural rearrangement of the heterotrimer complex (Bunemann et al., 2003). This allows for the Gα and Gβγ subunits to act as effector molecules downstream in the signaling cascade. Finally, the GTPase activity of the Gα subunit, accelerated by regulators of G protein signaling (RGS), hydrolyses GTP to GDP enabling reassociation or rearrangement to the inactive heterotrimeric complex, completing the cycle (Figure 1).

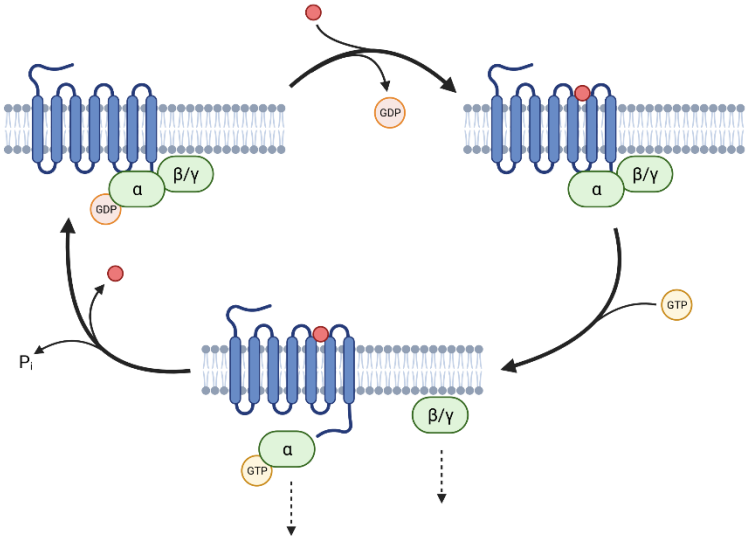

Figure 1. The heterotrimeric G protein activation cycle. The GPCR (blue) interacts with the heterotrimeric G protein (green) and the agonist (red), forming a high-affinity complex. Subsequent binding of GTP to the α-subunit activates and disassociates it, leading to further downstream signaling (dashed arrows). Hydrolysis of GTP to GDP and unbinding of the ligand turns the signaling off. Created with BioRender.

The ternary complex model (de Lean et al., 1980) explains that there is a high-affinity state composed of an agonist, receptor and heterotrimeric G protein. The receptor is stabilized in an active conformation by the bound agonist and is supported by the allosterically bound heterotrimeric G protein in its nucleotide-free state. This model has since been reinforced by structural work of active-state GPCRs bound to agonist and heterotrimeric G proteins (Draper-Joyce et al., 2018; García-Nafría et al., 2018; Liang et al., 2017; Rasmussen et al., 2011). Moreover, the ternary complex applies to intracellular proteins other than heterotrimeric G proteins, including arrestins and GRKs (Komolov et al., 2017; Liang et al., 2018a; Zhou et al., 2016). Furthermore, after the discovery of constitutive receptor activity the ternary complex model was extended to accommodate ligand-free GPCRs in the active conformation, resulting in the extended ternary complex model. This was revised further to account for pre-coupling of inactive state receptors with intracellular signaling proteins giving rise to the cubic ternary complex model (Figure 2) (Kenakin, 2017).

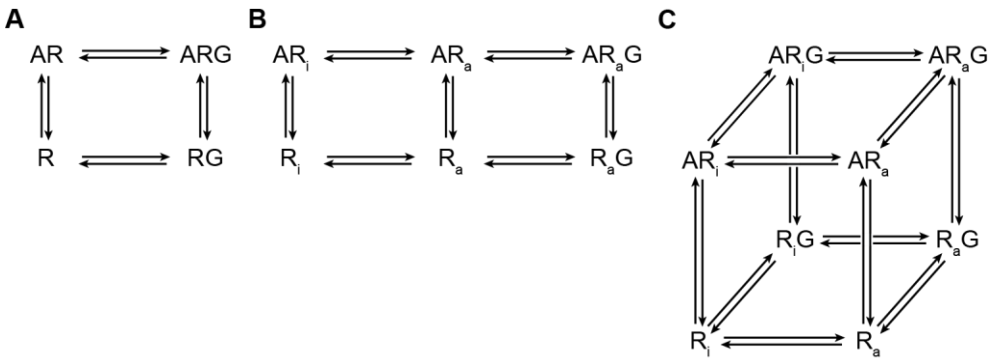

Figure 2. Different ternary complex models. (A) The first ternary complex model. A represents the agonist, R the receptor and G the heterotrimeric G protein. ARG represents the high affinity state. (B) The extended ternary complex model. R_i represents the inactive receptor state and R_a the active receptor state. (C) The cubic ternary complex model.

1.2 THE CLASS F OF GPCRS

The Class F of GPCRs in mammals is evolutionarily conserved and consists of 11 different receptors, the smallest set out of all the classes, with 10 FZD homologues (FZD_{1-10}) and SMO. FZDs are further subdivided into four homology clusters, $FZD_{1,2,7}$, $FZD_{3,6}$, $FZD_{4,9,10}$ and $FZD_{5,8}$. Structurally, Class F receptors share many similarities with other GPCRs: a 7TM core, three extracellular loops (ECL1-3), three intracellular loops (ICL1-3) and the intracellular helix 8 (H8). Moreover, Class F receptors have a large extracellular domain, called the cysteine-rich domain (CRD), and a linker region, that distinguish them substantially from Class A receptors, but place them closer to Class B and Class C receptors that also have large extracellular domains (Figure 3) (MacDonald and He, 2012; Schulte, 2010).

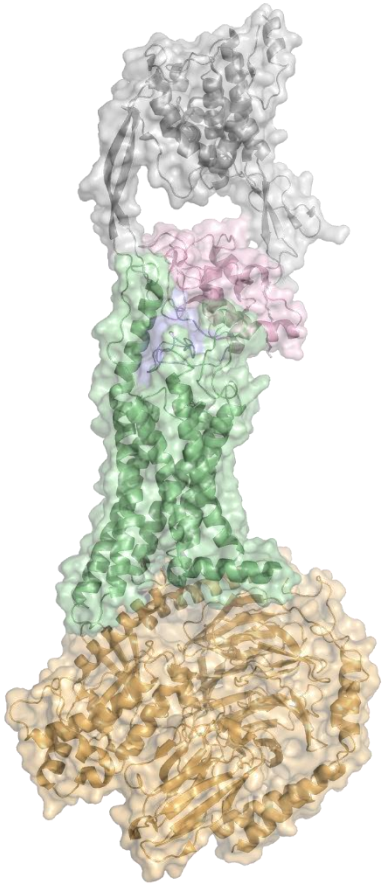

Figure 3. Model of Class F receptor. Illustrative composition of *Xenopus* WNT-8 (gray) bound to the CRD of FZD_8 (pink) (PDB: 4F0A) overlayed onto the generated model (GPCRmd ID: 12229) of human FZD_7 (green) and linker domain (blue), bound to $mG\alpha_s$, $G\beta$ and $G\gamma$ (orange) (PDB: 7EVW).

The CRD is seen as the orthosteric binding site of FZD ligands based on evidence from the structural work of the WNT-CRD interaction between *Xenopus* WNT-8 and mouse FZD_8-CRD (Janda, et al 2012) that was later also produced for human WNT-3A bound to mouse FZD_8-CRD (Hirai et al., 2019). There is also a structure of Norrin — an atypical non-WNT FZD ligand — bound to human FZD_4-CRD (Chang et al., 2015), interestingly with dimeric Norrin bound to two CRDs. Unfortunately, there is not currently any structure of a WNT bound to a full length FZD and therefore there is still a debate in the field of how WNTs exert their effect via FZDs and the location of the orthosteric binding site. In addition to WNTs binding the CRD of FZDs, the protein soluble Frizzled related protein (sFRP) – a protein that binds WNTs and therefore an inhibitor of WNT/FZD signaling – has also been associated with CRD binding. It has been suggested that the FZD-sFRP complex is an alternative model of sFRP inhibition of WNT/FZD signaling (Cruciat and Niehrs, 2013; Dann et al., 2001; Dijksterhuis et al., 2015; Janda et al., 2012; Kozielewicz et al., 2021). The CRD is dispensable for surface expression, but there are three important, conserved, cysteines in the linker domain together with a cysteine in ECL1 that are crucial for correct embedment into the plasma membrane (Valnohova et al., 2018). There is also the unanswered question as to what part the CRD plays in FZD signal transduction, where one hypothesis is that it acts as a "fishing rod" to bring WNTs closer to FZDs, as ΔCRD FZD constructs can partially rescue mutant phenotypes of *D. melanogaster* depleted of FZD_1 and FZD_2 and show activity in the transcriptional TOPFlash assay (Chen et al., 2004; Povelones and Nusse, 2005). However, it is unclear if this explanation holds up to scrutiny since WNTs potentially act via co-receptors or other FZDs in association with the ΔCRD FZD. Additionally, the CRD can regulate receptor conformation and signal transduction, as it was demonstrated that the ΔCRD of FZD_7 loses its constitutive activity to functionally couple to $G\alpha_s$ (Xu et al., 2021). Furthermore, it was observed that WNTs induce a conformational rearrangement of the CRD for full-length FZDs (Kowalski-Jahn et al., 2021), but the mechanism relating to how the CRD exerts conformational changes in the receptor remains obscure. Lastly, the core of FZDs has also been observed to play a role in the affinity of WNT-3A for the CRD, especially in the case of FZD_8 where the binding affinity to FZD_8-CRD on the core of Cluster of differentiation 86 (CD86) was higher compared to wild type

FZD_8. Likewise, the CRD of FZD_4 fused to the core of either FZD_6 or FZD_8 increased affinity for WNT-3A (Kozielewicz et al., 2021). This argues that the core of FZDs act as allosteric modulators for WNT binding affinity for the CRD in a FZD paralogue-dependent manner.

The intracellular surface of FZDs is important for interaction with both Dishevelled (DVL) (Gammons et al., 2016a; Tauriello et al., 2012) (see section 1.4 "Dishevelled") and the α-subunit of the heterotrimeric G proteins (Qi et al., 2019; Rasmussen et al., 2011; Xu et al., 2021). All FZDs except for $FZD_{3,6}$ contain a typical PDZ ligand motif at the end of the C-tail, but FZDs also have a conserved KTxxxW motif in H8 that could act as a non-typical PDZ ligand, binding to the PDZ domain of DVL (Gao and Chen, 2010; Punchihewa et al., 2009; Wallingford and Habas, 2005; Wong et al., 2003), though the *in vivo* relevance of this has been challenged (Simons et al., 2009; Tauriello et al., 2012; Yu et al., 2010). Amino acids at the bottom of TM4 and TM6, including ICL3, together with the KTxxxW motif in H8 have been defined as critical in FZDs for DEP-dependent DVL recruitment to the plasma membrane (Tauriello et al., 2012), and this was further refined to include amino acids at the bottom of TM2 (Figure 4) (Gammons et al., 2016a; Strakova et al., 2017). Moreover, H8 is involved in membrane anchoring of FZDs and is crucial for plasma membrane expression of the receptor, though the C-terminus is not (Bertalovitz et al., 2016; Gayen et al., 2013).

Although FZDs generally have been considered atypical GPCRs, they share among others the common feature of all GPCRs, activation of heterotrimeric G proteins. Indeed, GPCRs display heterogeneous activation mechanisms where all classes and even receptors in the same class display different ligand binding modes and conformational rearrangements upon activation (Ellaithy et al., 2020; Gloriam et al., 2021; Krumm and Roth, 2020; Latorraca et al., 2017). Comparing the inactive structure of FZD_4 (PDB: 6BD4) and FZD_5 (PDB: 6WW2) to the active FZD_7 structure (PDB: 7EVW), we can appreciate that TM6 swings out upon receptor activation, a classical hallmark of Class A and B activation (Tsutsumi et al., 2020; Xu et al., 2021; Yang et al., 2018). The FZD_7 structure also points towards the existence of a binding pocket in the transmembrane domain (TMD) found in other GPCRs and SMO, leading to the notion that this part of the FZDs is druggable like many other GPCRs.

Receptor cell surface expression is an important tool used by the cell for regulating receptor dependent signaling. FZD cell surface expression is regulated by the cell surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) and the homologue ring finger 43 (RNF43) via negative feedback loops (Hao et al., 2012; Koo et al., 2012). The activity of ZNRF3 is regulated by R-spondin — a secreted growth factor — requiring the leucine-rich repeat containing G protein-coupled receptor 4/5 (LGR4/5) which are receptors for R-spondin. The binding of R-spondin to ZNRF3 and LGR4 brings the two surface proteins together promoting decreased plasma membrane levels of ZNRF3 and subsequently leads to increased levels of FZD at the cell surface. Additionally, DVL is required for ZNRF3- and RNF43-dependent downregulation of FZDs although mechanistic details remain obscure (Cruciat and Niehrs, 2013; Jiang et al., 2015).

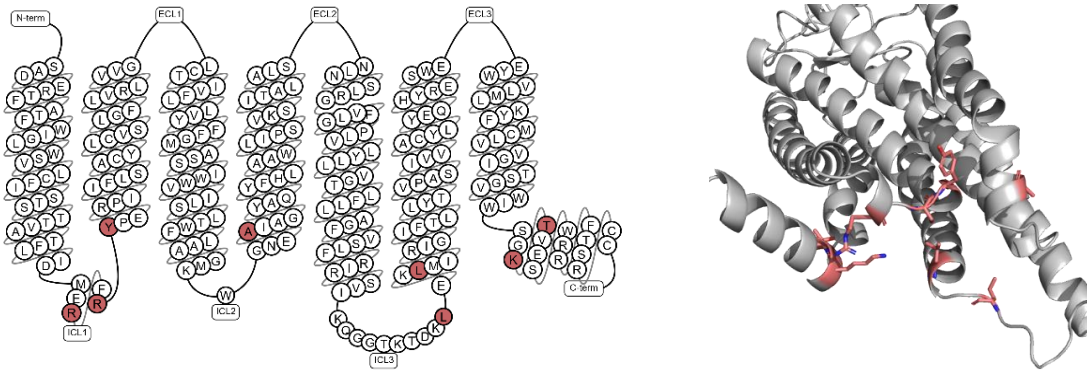

Figure 4. Amino acids in FZDs critical for DEP-dependent DVL recruitment to the plasma membrane. To the left is a snake plot of FZD_5 with the amino acids marked in red. To the right the same amino acids are highlighted (red) in the generated model (GPCRmd ID: 11849) of inactive FZD_4 (PDB: 6BD4).

1.2.1 Co-receptors

There are a number of co-receptors involved with FZDs, such as low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), receptor tyrosine kinase-like orphan receptors 1 and 2 (ROR1/2) and receptor tyrosine kinase (RYK) that also bind WNTs and regulate WNT/FZD signaling pathways (Grainger and Willert, 2018; MacDonald et al., 2007; Schulte, 2010; Semenov et al., 2007). The *D. melanogaster* counterpart of LRP5/6, Arrow, was discovered in genetic mutants showing a phenotype similar to WNT mutants (Wehrli et al., 2000). LRP5/6 are single TMD receptors and are critical in WNT/β-catenin signaling forming a FZD/LRP/DVL complex. The extracellular domain does not contain a WNT-binding CRD like FZDs but instead has β-propeller epidermal growth factor domains that bind to WNTs. Interestingly, LRP5/6 can bind multiple WNTs simultaneously but they can also bind Dickkopfs (DKKs) — secreted negative regulators of the WNT/β-catenin signaling pathway — which inhibit the formation of the signaling complex (Bourhis et al., 2010; Cheng et al., 2011; MacDonald and He, 2012). The single transmembrane receptor tyrosine kinases ROR1/2 have an N-terminal CRD for binding WNTs and they can both modulate WNT/FZD signaling. In addition, ROR2 has the ability to form WNT-dependent signaling complexes. However, in contrast to LRP5/6 they can also signal independently of FZDs and it is therefore unclear if they should be considered true co-receptors. (Green et al., 2014; Li et al., 2008; Schulte, 2010; Yamamoto et al., 2008). RYK, also a single transmembrane receptor, lacks a CRD but does instead show homology to WNT inhibitory factor (WIF), an extracellular WNT-sequestering protein inhibiting WNT/FZD signaling. RYK is unusual because of its cytoplasmic tail which has a tyrosine kinase motif, but it is inactive and instead RYK acts as a co-receptor for WNT/β-catenin signaling and FZD_7 internalization (Green et al., 2014; Kim et al., 2008; Lu et al., 2004; Schulte, 2010).

1.3 WNT/FZD SIGNALING

There are multiple proteins, too many to mention all here, that participate in different WNT/FZD signaling pathways (MacDonald et al., 2007; Schulte, 2010; Semenov et al., 2007). Two central players are DVL and heterotrimeric G proteins, both playing pivotal roles in signal transduction (Dijksterhuis et al., 2014; Gao and Chen, 2010; Schulte and Wright, 2018; Sharma et al., 2018). On the one hand, we have DVL that acts as a phospho- and scaffolding protein to regulate the WNT/β-catenin and WNT/PCP pathways. On the other hand we have heterotrimeric G proteins, involved in most WNT/FZD signaling pathways but to what degree and importance is still a matter of intense debate. One key aspect that requires better understanding is how WNT/FZD signaling pathway selectivity and modulation is achieved. Since none of these pathways exist in isolation, it is important to consider the potential crosstalk between them. A holistic view of these signaling pathways and the myriad of proteins involved therein will lead the way to a more cohesive model.

Finally, something that needs mentioning is the recruitment of DVL to FZD. There seems to be a misconception – or at least ambiguity – with this term because FZD and DVL form a pre-coupled complex in a ligand-independent manner (at least in an overexpression system) (Kilander et al., 2014; Strakova et al., 2017; Valnohova et al., 2018). Many models and descriptions of FZD signaling pathways refer to the recruitment of DVL to FZD upon WNT stimulation, but it is important to note that this most likely either refers to the already preformed complex or the additional recruitment of DVL by the formation of DVL-DVL oligomers (see section 1.4 “Dishevelled” and 1.3.1 “WNT/β-catenin signaling”).

1.3.1 WNT/β-catenin signaling

The WNT/β-catenin signaling pathway is the most studied and often referred to as the “canonical” signaling pathway (although this classification should be avoided) and is crucial for proper embryonic development and adult tissue homeostasis playing an important role in

cell proliferation, differentiation and apoptosis. This pathway has been extensively reviewed previously (Angers and Moon, 2009; Driehuis and Clevers, 2017; Grainger and Willert, 2018), but will be described here in short. In the absence of WNTs, the transcriptional regulator β-catenin is continuously degraded by the destruction complex composed of Axin, β-catenin, adenomatosis polyposis coli (APC), the glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), which continuously phosphorylate β-catenin, targeting it for ubiquitination by the E3 ligase SKP1-cullin 1-F-box, ultimately leading to proteasomal degradation. The most common working model for WNT/β-catenin signaling is the signalosome assembly, where WNT binds simultaneously to both FZD and LRP5/6 which initiates DVL/Axin oligomerization (Bilić et al., 2007; Cong et al., 2004; DeBruine et al., 2017). The mechanistic understanding is incomplete, but the formation of the signalosome complex leads to phosphorylation of LRP5/6 and DVL by GSK3 and CK1 as well as sequestering of Axin, inhibiting the destruction complex, resulting in an increase of cytosolic β-catenin and its subsequent translocation to the nucleus, where it acts as a coactivator of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) transcription factors (Dijksterhuis et al., 2014; Grainger & Willert, 2018). A common method for measuring WNT/β-catenin pathway activation is the TOPFlash assay, a luciferase-based transcriptional reporter assay for TCF/LEF activity (Korinek et al., 1997).

The investigation of WNT-FZD specificity for the WNT/β-catenin pathway is complex, partly due to there being 19 different mammalian WNTs and 10 different FZD paralogues and this is further complicated by the fact that not all WNTs are available as recombinant proteins. Furthermore, investigations are complicated by the fact that additional proteins have emerged as requirements for functional signaling via specific WNT-FZD pairs. This makes it hard to distinguish acute WNT-induced activation of FZDs from other potential effects due to co-expression. However, different WNTs and FZDs have been shown to have different preferences for this signaling cascade, where e.g. WNT-3A is a common activator of WNT/β-catenin signaling whereas WNT-5A generally is not (Driehuis and Clevers, 2017; Kikuchi et al., 2011; Shimizu et al., 1997; Topol et al., 2003). Interestingly, the FZD_4-binding ligand Norrin also activates WNT/β-catenin signaling which was discovered during the investigation of FZD_4 and abnormal retinal vascular development (Xu et al., 2004). Furthermore, FZD_3 and FZD_6 do not generally signal via the WNT/β-catenin pathway but it is unknown how they differ to allow for this pathway selectivity (Kilander et al., 2014b; MacDonald and He, 2012; Valnohova et al., 2018). It seems that the C-terminus is dispensable in this signaling bias as a mouse FZD_4/mouse FZD_3 C-terminus chimera was shown to still signal via the WNT/β-catenin pathway (Bertalovitz et al., 2016). One possibility is WNT-FZD selectivity, as FZD_3 does not bind WNT-3A and FZD_6 displays weak binding, although this is challenged by the fact that FZD_8 also presents with weak binding but is a potent activator of WNT/β-catenin signaling (Kozielewicz et al., 2021). Moreover, the expression of different co-receptors, as recently demonstrated by the involvement of Reck and GPR124 in WNT-7A-induced WNT/β-catenin signaling could offer an alternative explanation (Eubelen et al., 2018). Finally, there has been the development of WNT surrogates (see section 1.5 “Frizzled ligands”): engineered water-soluble proteins that are designed to simultaneously bind FZD-CRD and LRP5/6 to induce WNT/β-catenin signaling (Chidiac et al., 2021; Janda et al., 2017; Miao et al., 2020; Tao et al., 2019). Though the mechanistic details still are unclear, these WNT surrogates show promising results in activation of this signaling pathway and have the potential for future clinical use, such as in regenerative medicine.

1.3.2 WNT/PCP signaling

The WNT/planar cell polarity (PCP) signaling pathway results in cell asymmetry, organized in the 2D-plane of tissue. This is important for anterior-posterior body axis formation, orientation of cell division, neural tube formation and orientation of sensory hair cells. PCP results in the polarized distribution of two distinct transmembrane signaling complexes within and across adjacent cells (Butler and Wallingford, 2017; Yang and Mlodzik,

2015). In vertebrates, on the distal side of the cell, FZD forms a complex with the atypical cadherin EGF LAG seven-pass G-type receptor (CELSR), DVL and Diego and on the proximal side, the 4TM protein Van Gogh-like (VANGL) and CELSR form a complex together with Prickle. Extracellularly, FZD-VANGL and CELSR-CELSR complexes form between adjacent cells and stabilize each other in addition to propagating the signal intracellularly. WNT gradients can guide this PCP patterning, orienting the FZD complex towards them (Gao, 2012). Although the molecular details of WNT induction and regulation of PCP signaling is mostly lacking, it has been shown in mice that WNT-5A promotes a ROR2-VANGL2 complex driving phosphorylation of VANGL2 required to establish PCP (Gao et al., 2011). Furthermore, CK1 and DVL are required for this phosphorylation, but the degree of involvement of FZD is still unclear (Yang et al., 2017). Finally, there is also evidence for WNT-FZD-mediated activation of the small GTPase RHO via both DVL and the Dishevelled-associated activator of morphogenesis (DAAM) in addition to WNT-dependent FZD_4-$G\alpha_{12/13}$ activation (Arthofer et al., 2016; Habas et al., 2001).

1.3.3 WNT/Ca^{2+} signaling

The ability of FZDs to mediate Ca^{2+} signaling was first reported in *Danio rerio* after the notion that WNT overexpression in *X. leavis* embryos mimicked the effect of phosphatidylinositol (PI) modulating drugs – a Ca^{2+} driven signaling pathway. This pathway was shown to be modulated by WNT-5A and FZD_2 via phospholipase C (PLC) (Slusarski et al., 1997). Since then, two possible routes for modulating FZD-dependent WNT/Ca^{2+} signaling have been uncovered. The first route is $G\alpha_q$ protein dependent, but also regulated by $G\beta\gamma$ via $G\alpha_i$ activation, activating PLC which stimulates diacylglycerol (DAG) and inositol triphosphate (IP_3) production from phosphoinositol diphosphate (PIP_2). IP_3 in turn triggers Ca^{2+} release from intracellular storage activating further downstream proteins such as protein kinase C (PKC) and calmodulin-dependent kinase II (CAMKII) (Berridge, 1993; Kohn and Moon, 2005; Niehrs, 2012; Pfeil et al., 2020; Taciak et al., 2018; Wright et al., 2018). The second route involves the activation of cGMP-selective phosphodiesterase (PDEs) and transducin ($G\alpha_t$), leading to a decrease of intracellular cGMP concentrations that in turn mobilizes Ca^{2+} with the help of protein kinase p38 (Ahumada, 2002; Liu et al., 1999; Ma and Wang, 2007). The final effector molecules of the WNT/Ca^{2+} signaling pathway are transcription factors such as nuclear factor associated T-cells (NFAT) and cAMP response element-binding protein (CREB). Interestingly, DVL is also involved in the WNT/Ca^{2+} signaling pathway by activation of CAMKII via PKC, though the molecular mechanism is unclear (Sheldahl et al., 2003). Finally, DVL is not necessary for propagating the cGMP and p38 route of the WNT/Ca^{2+} signaling pathway as demonstrated by DVL knockdown (Ma and Wang, 2007).

1.4 DISHEVELLED

Playing a pivotal role in both WNT/β-catenin and WNT/PCP signaling as well as being involved in many other WNT-FZD signaling pathways, DVL is a phospho- and scaffold protein acting as a hub for intracellular WNT signaling (Gao and Chen, 2010; Mlodzik, 2016; Schulte, 2010; Sharma et al., 2018). Even though the importance of DVL for FZD signaling is undisputed, not much is known about how it exerts its function and distinguishes between different signaling pathways. *D. melanogaster* has one isoform of DVL while mammals express three DVL paralogues (DVL1-3), but they all share highly conserved regions and can to some extent functionally compensate for one another. DVL consists of three distinct domains: the N-terminal Dishevelled and Axin (DIX) domain, the postsynaptic density protein-95, disc large, zonula occludens-1 (PDZ) domain and the N-terminal Dishevelled, Egl-10 and Pleckstrin (DEP) domain in addition to the flexible C-terminal domain (Figure 5). At endogenous levels, plasma membrane associated DVL is primarily found in the monomeric state, but also in small quantities as dimers and trimers (Ma et al., 2020b).

The DIX domain is indispensable for WNT/β-catenin signaling, forming dynamic DVL-DVL and DVL-Axin oligomers crucial for inhibition of the β-catenin destruction complex (see section 1.3.1 "WNT/β-catenin signaling") but is dispensable for the WNT/PCP signaling pathway. Additionally, DIX-DIX oligomerization leads to – especially in overexpression systems – cytosolic DVL puncta (Bryja et al., 2007; Kishida et al., 1999; Schwarz-Romond et al., 2007). Formation of these puncta can be prevented by introducing a

Figure 5. **The domains of DVL.** The three domains of DVL, DIX, PDZ and DEP are shown together with the C-terminus with the beginning and end amino acid of each domain annotated

mutation in the DIX domain of DVL (M2/M4) (Schwarz-Romond et al., 2007), but also by co-expression of FZDs (Boutros et al., 2000; Bryja et al., 2007a; Valnohova et al., 2018). Upon WNT-3A stimulation, the DIX domain is responsible for an increased DVL density and DVL-DVL oligomer size at the plasma membrane (Ma et al., 2020b). The PDZ domain was long thought to be the main mediator of FZD interaction and crucial for WNT/β-catenin signal transduction via the KTxxxW motif in H8 of FZDs (Punchihewa et al., 2009; Wong et al., 2003), but this has been refuted by later studies instead pointing towards DEP being the crucial mediator of the FZD-DVL interaction and signal transduction (Gammons et al., 2016a; Ma et al., 2020b; Paclíková et al., 2017; Tauriello et al., 2012). The DEP domain is ~11 kDa and consists of three α-helices, a β-hairpin and two β-sheets with a finger loop containing two amino acids at the tip (L445 and K446 in DVL2) important for FZD-DVL interaction and indispensable for functional WNT/β-catenin signaling (Figure 6) (Gammons et al., 2016b). However, the molecular function of these amino acids and what role they play in FZD-DVL dynamics is still unknown. One potential explanation is that these amino acids play an important role in basal recruitment to FZDs recognizing a specific subset of FZD conformations, but this has to be further investigated (Schulte and Wright, 2018). Furthermore, removal of the DEP domain reduces DVL membrane association to the same levels as in FZD null cells and abolishes any response in the transcriptional assay TOPFlash (Ma et al., 2020b; Paclíková et al., 2017; Rothbächer et al., 2000), probably due to diminished DEP dependent FZD-DVL interaction but this needs further validation. The DEP domain can also form dimers important for WNT/β-catenin signal transduction, although mechanistic details remain unclear with regard to protein dynamics and when the dimers form during signal transduction (Gammons et al., 2016a). Moreover, the DEP domain can interact electrostatically with phosphatidic acids (PAs) of the plasma membrane, more specifically via basic amino acids located at H3 neighboring the DEP finger loop (Capelluto et al., 2014; Simons et al., 2009). As mentioned above, the DIX domain forms DIX-DIX interactions leading to puncta, but interestingly removal of the DEP domain also abolishes puncta formation in the same way as the polymerization mutant M2/M4 does. These phenomena need further investigation but could be explained by its inability to form DEP dimers. To summarize, the DEP domain of DVL plays an important role in both FZD-dependent plasma membrane recruitment and WNT/FZD-mediated signaling.

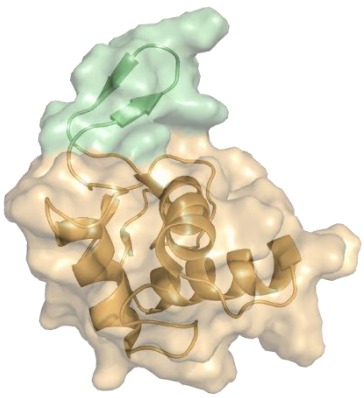

Figure 6. The DEP domain of DVL. The structure of the DEP domain (orange) of mouse DVL1 with the finger loop highlighted (green) (PDB: 1FSH).

DVL plasma membrane recruitment is not only modulated by FZDs as demonstrated by the GPR125-mediated recruitment of DVL to the plasma membrane, a process involved in WNT/PCP signaling (Li et al., 2013). Moreover, DVL is dynamically phosphorylated and

becomes heavily phosphorylated in response to WNT stimulation. Therefore, measuring DVL phosphorylation by immunoblotting – the phosphorylation-dependent DVL mobility shift – is a common way to measure the response to WNTs, but it is unspecific and does not provide any information on pathway selectivity. This phosphorylation is orchestrated by multiple kinases, including CK1 (Bryja et al., 2007a; Peters et al., 1999), CK2 (Willert et al., 1997), PKC (Kinoshita et al., 2003) and serine/threonine-protein kinase Nek2 (NEK2) (Cervenka et al., 2016). Also, recent studies have attempted to decipher how phosphorylation patterns regulate signaling and changes between different DVL conformations (Beitia et al., 2021; Hanáková et al., 2019; Harnoš et al., 2019; Jurásek et al., 2021; Lee et al., 2015). Indeed, the C-terminus of DVL can interact with the PDZ domain, forming a "closed" conformation that is regulated by CK1 activity which phosphorylates the PDZ domain promoting the "open" conformation. Furthermore, WNT stimulation activates the CK1-dependent phosphorylation of DVL and therefore promotes the open conformation. Additionally, the closed conformation of DVL is to a greater extent associated with puncta formation and the open conformation with FZD-dependent plasma membrane recruitment (Harnoš et al., 2019; Lee et al., 2015). Finally, the phosphorylation of DVL has also been associated with preventing DEP domain-swapping dependent dimer formation of DVL (Beitia et al., 2021).

1.5 FRIZZLED LIGANDS

The 19 mammalian WNTs (Wodarz and Nusse, 1998) are approximately 40 kDa cysteine-rich lipoglycoproteins that most commonly have palmitoleic acid modifications making them hydrophobic (Driehuis & Clevers, 2017; Willert et al., 2003). It should be noted that this property makes WNTs difficult to purify and work with, resulting in the widespread usage of conditioned medium (CM) instead of purified recombinant protein. One drawback with conditioned medium is of course the potential presence of other secreted proteins which can be partially solved by purification but also the challenge in determining the WNT concentration. WNT binds to FZDs by pinching the CRD like a "thumb" and "index finger" with the palmitoleic acid buried in a hydrophobic groove (Figure 7) (Hirai et al., 2019; Janda et al., 2012). The palmitoleic acid has been thought of as crucial for WNT-FZD activity (Driehuis and Clevers, 2017; Grainger and Willert, 2018), but recently this was challenged by the discovery of non-acylated WNTs that both retain their expression and activity but with less efficacy (Speer et al., 2019), arguing for different binding modes between WNTs and FZDs. This is further strengthened by the small WNT-5A-derived hexapeptide Foxy-5, an agonist which impairs migration and invasion of breast cancer cells (Säfholm et al., 2006, 2008). However, it is not yet known how this small peptide binds and exert its effects on FZDs. Of interesting note is that the CRD of FZD_6 neither interacts with WNT-3A nor WNT-5A (Sato et al., 2010), even though there is ample evidence that WNT-5A can act via FZD_6 (Corda and Sala, 2017; Kilander et al., 2014b; Petersen et al., 2017). Put in the context of the WNT-5A-derived peptide Foxy-5, this advocates for alternative – non-CRD – binding modes of WNT-FZD, though it can only be speculated as to where and how this binding would occur,

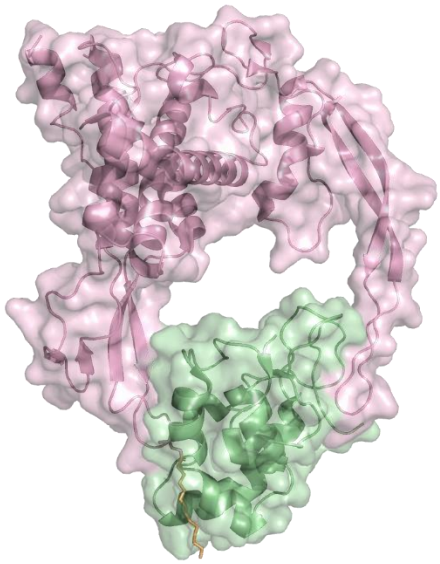

Figure 7. WNT-CRD structure. The structure of *Xenopus* WNT-8 (pink) bound to the CRD of mouse FZD_8 (green) with the thumb and palmitoleic acid (orange) on the left side and index finger on the right side (PDB: 4F0A).

presuming it binds FZDs in the first place and does not act via co-receptors in a FZD-dependent manner.

Expectedly, WNTs also have varying affinities for different FZD CRDs (Dijksterhuis et al., 2014, 2015; Grainger and Willert, 2018; Kozielewicz et al., 2021). It was recently described that WNT-3A can bind to all FZDs except FZD_3 and FZD_9 to varying degrees (Kozielewicz et al., 2021), but it should be mentioned that binding to FZD_6 and FZD_8 was weak. There are also differences among WNTs in their ability to activate different FZD paralogues (Schihada et al., 2021), but what consequences this has for pathway selectivity and downstream signaling still requires further investigation. In addition to binding the CRD of FZDs, WNTs also bind other proteins and one such protein is the sFRP. sFRPs have a CRD homologous to the CRD of FZDs and they are considered inhibitors of WNT/FZD signaling, despite being able to potentiate WNT/β-catenin signaling depending on the cellular context (Grainger and Willert, 2018; Xavier et al., 2014). Moreover, WNTs can bind to WIFs but the cellular mechanism for how WIFs regulate WNT signaling is still not understood (Malinauskas et al., 2011).

Furthermore, there has also been development of so-called WNT surrogates, polypeptides composed of FZD-CRD binding motifs and the LRP5/6 binding domain of DKK1 (Chidiac et al., 2021; Janda et al., 2017; Miao et al., 2020; Tao et al., 2019), designed to specifically activate the WNT/β-catenin signaling pathway. They do so by forming FZD-LRP heterodimers but the molecular mechanism for signal initiation remains elusive. Unfortunately, there has been almost no development in the area of small molecules targeting FZDs, despite recent evidence for a binding pocket in the 7TM core as seen in Class A and B GPCRs (Xu et al., 2021) and SMO structures (Deshpande et al., 2019; Qi et al., 2019, 2020; Wang et al., 2013). This is in part due to the notion that FZD ligands exclusively exert their effect via the CRD but also due to the lack of efficient screening methods that monitor receptor activation. A recent study demonstrated that the small molecule SAG1.3 (Figure 8) – a SMO agonist – could bind the core of FZD_6 as well as to induce mini G_i protein (mG_i) recruitment (Kozielewicz et al., 2020a). This finding lends support to the concept that the core of FZDs is druggable by small molecules, which contradicts what was previously concluded based on the FZD_4 structure (Yang et al., 2018), and it shows promise for the future development of small molecule drugs targeting FZDs.

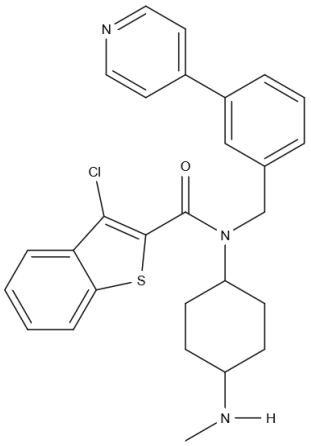

Figure 8. Chemical structure of SAG1.3.

2 SPECIFIC AIMS

The overarching objective of this thesis is to further the understanding of WNT/FZD signaling and pathway selectivity. The specific aims are to:

- Develop assays to measure FZD-DVL interaction.
- Examine the role of heterotrimeric G proteins in FZD signaling pathways.
- Define FZD conformational changes involved in signaling.
- Investigate how pathway specificity is achieved in the context of FZD activation and intracellular transducer proteins.
- Gain mechanistic insight into WNT-induced FZD-DVL dynamics.
- Aim to create a more holistic view of WNT/FZD signaling.

3 MATERIALS AND METHODS

The materials and methods employed in the thesis are well-described in each respective paper and I will therefore herein provide a short and general description of a selection of the materials and methods used throughout the thesis. Furthermore, I will discuss different advantages and disadvantages related to the aforementioned selection in addition to certain considerations to be made when interpreting the generated data.

3.1 KNOCKOUT CELL LINES

Throughout the thesis, HEK293 (Human Embryonic Kidney) cells, originally developed in the 1970s, were used as a model system for investigations (Russell et al., 1977). These cells are advantageous for multiple reasons: ease of transfectability, high levels of recombinant protein expression, sturdy and easy to culture and fast growing to mention a few. One drawback is that these cells represent a highly artificial system with less physiological relevance but that is also what makes them great for molecular interaction and signaling pathway studies. With time, there has been further developments of the HEK293 cell line and in the FZD field one important milestone was the advent of CRISPR/Cas9 gene editing that enabled the creation of two knockout (KO) cell lines: $FZD_{1,2,4,5,7,8}$ KOs (Voloshanenko et al., 2017) and FZD_{1-10} KOs (ΔFZD_{1-10}) (Eubelen et al., 2018), the latter being extensively used in this thesis. Since there is ubiquitous expression of the 19 different WNTs and 10 FZDs across cell types, including HEK293 cells, studying FZD signaling was difficult due to the background signaling from endogenous receptors. Having a FZD null system meant that only the specific FZD of interest could be re-introduced and studied. Moreover, in **Paper II**, two different heterotrimeric G protein KO HEK293 cell lines were used for investigating the involvement of said proteins, more specifically $G\alpha_{s/olf,q/11,12/13,z}$ null cells and full $G\alpha$-depleted cells where $G\alpha_{i/o}$ was also knocked out (Grundmann et al., 2018; Hisano et al., 2019). Again, having a full KO system of a specific category of proteins can help in determining the role of a given protein and its importance in different cell signaling systems. Finally, another important cell line tool is the HEK293 DVL1-3 KO (ΔDVL1-3) cells (Cervenka et al., 2016; Gammons et al., 2016b) which can help in dissecting the role of DVL as an intracellular protein hub for FZD signaling. It is impressive that these KO cell lines survive and is a proof of how spectacular and adaptable life is, but it also raises the question of what changes these cell lines have undergone in order to survive. Recently, there was a comparison of the effects on cell rewiring in either siRNA-mediated or CRISPR/Cas9 gene knockout of β-arrestin1/2 in HEK293 cells. Interestingly, siRNA-mediated knockout produced a more consistent result while different CRISPR/Cas9 clones had different rewiring of the signaling pathways and responded differently to reintroduction of the knocked-out proteins (Luttrell et al., 2018). While not too surprising – since the cell needs to find a way to survive and keep a viable equilibrium and there are numerous ways to achieve this – this is an important caveat that one has to keep in mind when working with KO cell lines. That being said, these cell lines offer many advantages and are a substantial tool for understanding and dissecting signaling pathways.

3.2 PHARMACOLOGICAL TOOLS AND WNTS

Pharmacological tools are, when available, a viable option or sometimes better compared to gene editing for modulating protein expression and activity, and one such tool are Porcupine inhibitors (Liu et al., 2013; Proffitt et al., 2013). The autocrine secretion of WNTs could be a potential source of FZD activation creating potential problems with readouts, especially in heavily amplified systems. Since WNTs generally are palmitoylated before being transported for secretion, inhibiting this system would disrupt WNT release (Herr and Basler, 2012). Thus, by inhibiting Porcupine, a membrane-bound O-acyltransferase, it is possible to disrupt the autocrine secretion of WNTs. Unfortunately, this does not fully inhibit the secretion

of all WNTs, since WNT-8 (also known as *WntD*) in *D. melanogaster* does not depend on lipid modifications for secretion (Ching et al., 2008) and some non-acylated mammalian WNTs still retain signaling capabilities in *Xenopus* embryos (Speer et al., 2019), although it is unclear if this is the case for mammalians. Even so, Porcupine inhibitors are useful tools for minimizing the influence of autocrine WNTs in systems where they may influence signaling readouts, such as the measurement of constitutive activity, but it is imperative to keep in mind that some WNT secreting capabilities and signaling might be retained.

On a different note, WNTs have a reputation of being hard to purify. Hence, the revelation that WNTs are lipid modified led to the purification of recombinant WNTs in the early 2000s (Schulte et al., 2005; Willert et al., 2003) and the possibility to replace the previously used CM. Purified recombinant WNTs (henceforth referred to as recombinant WNTs) have many advantages since CM could contain co-factors and other proteins or molecules producing confounding effects. Similarly, recombinant WNTs also contain impurities and are usually offered with >75% purity. Furthermore, the concentration of WNTs in CM is often low and hard to determine creating problems for pharmacological studies. Even so, CM is still used by many laboratories since it has the advantage of cheaper production, being detergent-free and offers the possibility to produce every WNT, whereas not all WNTs are available as purified recombinant proteins. Nonetheless, recombinant WNTs, which are of a higher purity and lyophilized, offer significant advantages and have made it possible to dissect FZD signaling with a higher degree of detail.

3.3 BIOLUMINESCENCE RESONANCE ENERGY TRANSFER

Bioluminescence resonance energy transfer (BRET) was first described more than two decades ago for investigating protein-protein interactions (Xu et al., 1999). It relies on the naturally occurring phenomenon of Förster resonance energy transfer between the light-emitting protein luciferase[1] (donor) and accepting fluorophore (acceptor), depending on the overlap between donor emission and acceptor excitation spectra as well as the distance and dipole orientation between donor and acceptor. Light is separately collected from donor and acceptor spectra using a microplate reader and the signal is expressed as the ratio between the two (acceptor/donor). This ratio is robust and minimizes experiment-to-experiment variation (e.g. protein expression and cell density). With time, methodological advancements were made, increasing the energy transfer efficiency, brightness and optimizing spectral overlap, subsequently creating a powerful toolbox for the investigation of protein-protein interactions, protein trafficking and protein conformational changes in living cells (Dacres et al., 2012; Galés et al., 2005; Hall et al., 2012; Machleidt et al., 2015; Nagai et al., 2002; Namkung et al., 2016; Schwinn et al., 2018; Weihs et al., 2020). Today, there are many different BRET donor-acceptor pairs available and with them the choices of different luciferase substrates, consequently with their own advantages and disadvantages. I will not go into detail about it here, but one should be aware of reasons for why different BRET pairs and substrates could be preferred depending on practical and cost-efficiency perspectives.

As mentioned, one often used application of BRET is to observe protein-protein interactions – or rather proximity – because the energy transfer is proportional to the distance between the acceptor-donor pair and only occurs for distances < 100 Å (Xu et al., 1999). Additionally, BRET depends upon the dipole orientation of the two proteins, something that is

[1] Poetically, the protein name luciferase is derived from the Latin word for the morning star, *lucifer* (light-bearing or light-bringing) – a name for the planet Venus. The fluorescent protein and BRET acceptor Venus is also named after the planet: '*Venus is the brightest object in our nighttime sky except for the moon, and thus we call the SEYFP-F46L variant "Venus"'* (Nagai et al, 2002; Lee, 2008).

overlooked in many cases. When determining the interaction between two proteins with BRET, the BRET ratio is plotted against the measured fluorescence of the acceptor (Figure 9), but it is imperative that proper controls are set in place to avoid misinterpretations or overstatements, since the BRET ratio can be affected by changes in the stoichiometry of donor and acceptor proteins (Lan et al., 2015; Szalai et al., 2014). Thus, two non-interacting proteins will present with a linear increase and two interacting proteins will present with a hyperbolic increase in relation to increasing fluorescence (acceptor protein). Furthermore, BRET sensors have been developed for measuring the activation of GPCRs (Zhou et al., 2021), such as the mG sensors that are recruited to GPCRs in their active state (Wan et al., 2018) or sensors that detect the activation of the heterotrimeric G protein subunits (Avet et al., 2020; Maziarz et al., 2020; Olsen et al., 2020). Moreover, by applying fluorescent ligands with N-terminally luciferase-tagged receptors, BRET has been adapted for use in receptor-ligand binding assays in living cells (Kozielewicz et al., 2020b, 2021; Stoddart et al., 2015, 2018; Wesslowski et al., 2020; White et al., 2019). Despite the disadvantages brought by modifying the ligand with a fluorophore, BRET ligand binding brings many advantages such as practicality, cost-efficiency, low non-specific signal, live cell measurements and reduced usage of radioligand binding assays. Furthermore, the change in energy transfer efficiency upon reorientation of the dipoles can be harnessed for measuring protein dynamics in response to receptor stimulation. This can be applied to create intramolecular BRET sensors that detect conformational changes within the protein of interest (Charest et al., 2005; Schihada et al., 2018) but it can also be applied to investigate intermolecular protein dynamics as in **Paper IV**. A general issue with BRET is – in many cases – the requirement of tagging the protein of interest and its potential interference with protein function, especially when changes are made to the middle of proteins, more specifically sites of protein-protein interactions or catalytic activity, instead of tagging the N- or C-termini. One exception is a new generation of BRET biosensors, sensitive enough to detect the activation of endogenous receptors (Avet et al., 2020; Maziarz et al., 2020). Furthermore, one has to take into account the unspecific signal resulting from the random collision between two non-interacting proteins, so-called bystander BRET. However, this can also be utilized to monitor protein trafficking by tagging the acceptor protein with compartment-specific markers (e.g. anchors of the plasma membrane or early endosomes) (Namkung et al., 2016), something that was exploited in **Paper I** and **Paper III**. The hurdles of BRET can be overcome by implementing proper controls and the usage of orthogonal assays when applicable, but one still has to be cautious in interpretating the data and be humble in light of the blind spots of the technique.

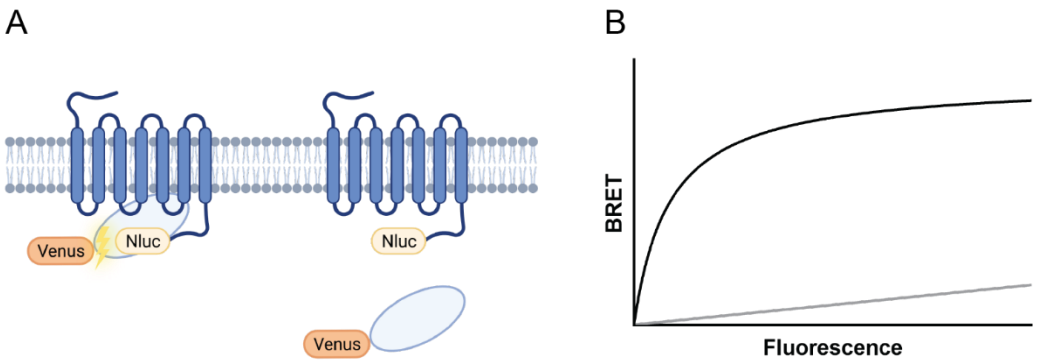

Figure 9. The principle of BRET. (A) To the left, two interacting proteins are in close proximity allowing for energy transfer between the donor (Nluc) and acceptor (Venus). To the right, two non-interacting proteins does not allow for energy transfer between the donor and acceptor. (B) Two interacting proteins generate a hyperbolic curve (black) and two non-interacting proteins generate a linear line (grey) when the BRET ratio is plotted against the fluorescence. The fluorescence is proportional to the number of acceptor molecules. Created with BioRender.

4 RESULTS AND DISCUSSION

With the work in this thesis, I have illuminated parts of the black box of WNT/FZD signaling. I have highlighted important residues for receptor function and activation, investigated the complex relationship between FZDs, heterotrimeric G proteins and DVL and increased the understanding of the FZD-DVL interaction and WNT-induced FZD-DVL dynamics.

4.1 RESIDUES SIGNIFICANT FOR CLASS F RECEPTOR FUNCTION AND ACTIVATION

Class A, B and C GPCRs all contain conserved motifs (keeping TM6 and TM7 in a closed conformation) that are disrupted upon receptor activation, referred to as the ionic lock in Class A and C (Ballesteros et al., 2001; Doré et al., 2014) or polar network in Class B (Liang et al., 2018a). Since FZDs and SMO are involved in cancer, we had in **Paper I**, via population and cancer genomics data identified a conserved arginine or lysine (R/K$^{6.32}$) (Ballesteros-Weinstein nomenclature (Ballesteros and Weinstein, 1995)) and tryptophan (W$^{7.55}$) forming a hydrogen bond and π-cation interaction acting as a molecular switch. Molecular dynamics (MD) simulations of a mutation to an alanine in the R/K$^{6.32}$ position in FZD_6 revealed that the hydrogen bond and π-cation interaction was broken, resulting in a more open receptor conformation. Since FZDs are known to interact with both heterotrimeric G proteins and DVL, we applied multiple different assays to monitor these pathways in the mutated receptor. First, we observed an increased constitutive activity of FZD_6 R$^{6.32}$A as monitored by the heterotrimeric G protein-dependent phosphorylation of ERK1/2. Furthermore, to monitor the WNT-induced and heterotrimeric G protein-mediated activation of FZDs, we employed a set of BRET sensors known as mG proteins, which are recruited to GPCRs in their active state (Carpenter et al., 2016; Wan et al., 2018). With these, we could observe a potency shift for the molecular switch mutants after stimulation of recombinant WNT-5A for representative receptors of all Class F homology cluster molecular switch mutants. Additionally, both the FZD_5 R$^{6.32}$A and FZD_6 R$^{6.32}$A mutants were impaired in their ability to induce the phosphorylation of DVL in addition to FZD_5 R$^{6.32}$A being impaired in its ability to signal via the transcriptional assay TOPFlash (Figure 10A). Moreover, to monitor FZD-dependent plasma membrane recruitment of DVL, we employed a bystander BRET setup with the Venus-tagged CAAX domain of KRas (Venus-KRas) together with N-terminally Nluc-tagged DVL2 (Figure 10B). Contrary to their wild type receptors, both the FZD_5 R$^{6.32}$A and FZD_6 R$^{6.32}$A mutants were impaired in DVL recruitment to the plasma membrane as was also observed for the negative control, β_2 adrenergic receptor (β_2AR). This explains the mutants' inability to

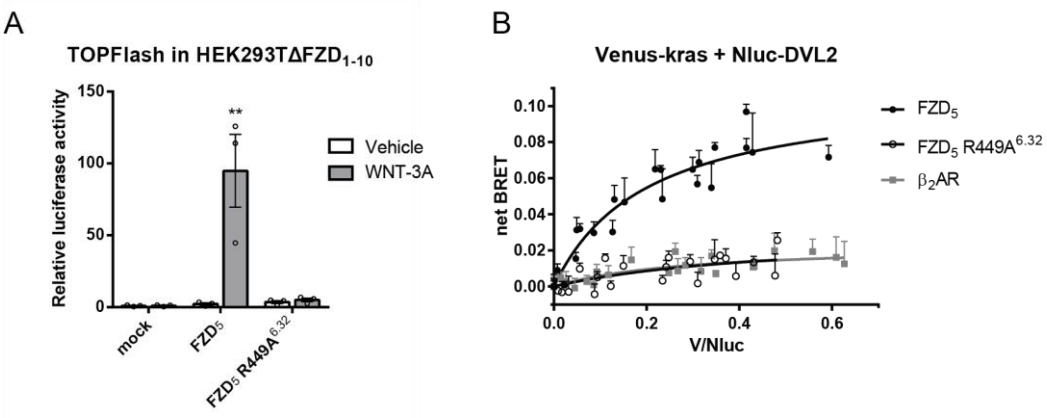

Figure 10. Molecular switch mutant is impaired in FZD-DVL interaction. (A) FZD_5 R$^{6.32}$A was impaired in its ability to activate the WNT/β-catenin signaling pathway. (B) FZD_5 R$^{6.32}$A molecular switch mutants was incapable of recruiting DVL to the plasma membrane. β_2AR was used as a negative control. Adapted from **Paper I**.

signal via TOPFlash, since it requires functional FZD-DVL interaction. In conclusion, these data demonstrate that the Class F molecular switch (R/K$^{6.32}$ and W$^{7.55}$) act as an important regulator of pathway selectivity between heterotrimeric G proteins and DVL via distinctly different receptor conformations.

Another conserved residue in Class F is P$^{6.43}$, where SMO is the only outlier having a phenylalanine. This proline is highly conserved in Class A GPCRs (P$^{6.50}$) and is involved in the outward movement of TM6 upon receptor activation (Venkatakrishnan et al., 2014; Weis and Kobilka, 2018). In **Paper III,** P$^{6.43}$ was hypothesized to have an important role in kinking TM6 in FZDs and that the F$^{6.43}$ in SMO was important to keep TM6 straighter to allow for cholesterol binding. We applied MD simulations and receptor mutagenesis together with ligand binding and downstream signaling assays to investigate this.

The introduction of the F$^{6.43}$P mutation in SMO resulted in dramatically reduced binding affinity of BODIPY-cyclopamine when comparing N-terminally HiBiT-tagged SMO (HiBiT-SMO) to HiBiT-SMO F$^{6.43}$P. SMO has two binding pockets in the 7TM bundle, where the lower of the two pockets is the high-affinity binding site of BODIPY-cyclopamine (Kozielewicz et al., 2020b). The F$^{6.43}$P mutation kinked TM6 and led to a reduced volume of the lower binding pocket, as analyzed by MD, explaining the observed reduction in affinity. Furthermore, the same analyses on the reverse P$^{6.43}$F mutation in FZD$_6$ showed no change in BODIPY-cyclopamine binding affinity. As expected, the mutation resulted in a straighter TM6 but it did not make any significant difference to the binding pocket of FZD$_6$, since it lacks the lower binding pocket found in SMO. Furthermore, the F$^{6.43}$P mutation in SMO abrogated recruitment of mG$_i$ (the same BRET sensor used in **Paper I**) in addition to NbSmo2 (a SMO active state sensor) upon SAG1.3 stimulation (Arveseth et al., 2021). This determined that SMO F$^{6.43}$P samples a different conformational landscape compared to the wild type receptor. Moreover, in accordance with our BODIPY-cyclopamine binding data, FZD$_6$ P$^{6.43}$F stimulated with SAG1.3 did not show any change in its ability to recruit mG$_i$. Since FZDs, but not SMO, also recruit DVL we employed the same bystander BRET setup as in **Paper I** to investigate the effect of the P$^{6.43}$F mutant in all homology clusters (FZD$_{4,5,6,7)}$. The FZD$_5$ P$^{6.43}$F and especially the FZD$_6$ P$^{6.43}$F mutant presented with a reduced ability to recruit DVL2 to the plasma membrane compared to their respective wild type receptors (Figure 11A). Interestingly, subsequent analysis via TOPFlash exhibited a reduced signal not only for FZD$_5$ but also for FZD$_4$, even though it did not display a reduced basal recruitment of DVL2 to the plasma membrane (Figure 11B). This suggests that FZD$_4$ P$^{6.43}$F samples a different conformational landscape in response to WNT stimulation compared to wild type FZD$_4$, though further investigation is needed. Since FZD$_6$ P$^{6.43}$F exhibited the most pronounced difference in DVL2 membrane recruitment and does not signal via the WNT/β-catenin pathway, we further investigated its ability to recruit DEP, the primary FZD interacting domain of DVL (Gammons et al., 2016a; Tauriello et al., 2012), to the plasma membrane. This setup was similar to the previous bystander BRET but with Nluc-DEP instead of Nluc-DVL2 together with Venus-KRas. We also measured the surface expression of the N-terminally SNAP-tagged FZD$_6$ receptors with a membrane impermeable SNAP dye. This allowed us to plot the bystander BRET signal against receptor surface expression, revealing a reduced ability for the FZD$_6$ P$^{6.32}$F mutant to recruit DEP to the plasma membrane, indicative of a change in the conformational landscape similar to what was found for SMO (Figure 11C).

As mentioned earlier, GPCRs are continuously sampling many different conformations and the equilibrium changes depending on the free energy landscape (Weis and Kobilka, 2018). The molecular switch between R/K$^{6.32}$ and W$^{7.55}$ from **Paper I** is broken and therefore continuously open when mutated leading to heterotrimeric G protein activation bias, both constitutively and after WNT-5A stimulation. The molecular switch was also confirmed open in the active structures of SMO and FZD$_7$-mG$_s$ complex (Deshpande et al., 2019; Qi et al., 2019; Xu et al., 2021). Interestingly, the constitutive activity of FZD$_7$ was dependent on the CRD, as the ΔCRD FZD$_7$ was inactive, hinting that WNTs might modulate FZD-G protein

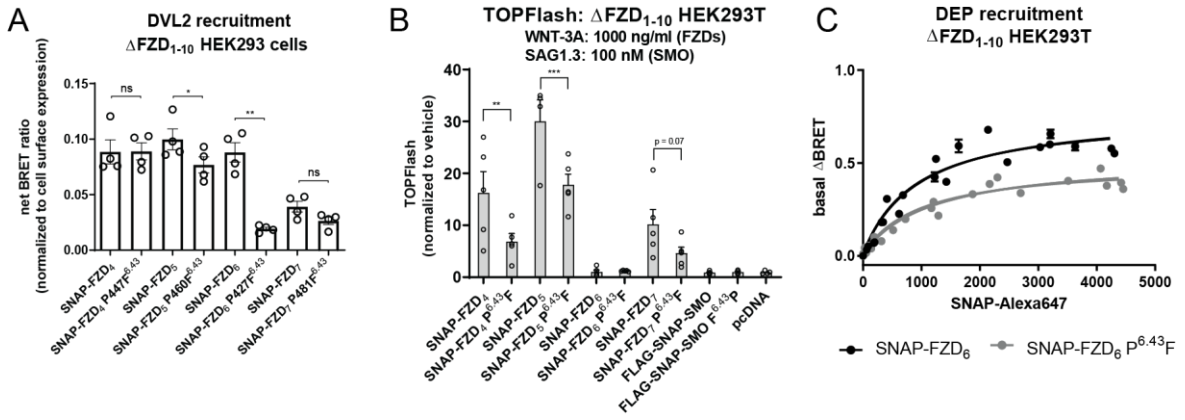

Figure 11. FZD $P^{6.43}F$ mutants show heterogeneity in DVL recruitment. (A) Bystander BRET with Nluc-DVL2 and Venus-KRas. (B) TOPFlash response after stimulation with WNT-3A. (C) Bystander BRET with DEP-Nluc and Venus-KRas plotted against the surface expression of the receptor. Adapted from **Paper III**.

activation via the CRD. This is well in line with the previously published WNT-CRD structures (Hirai et al., 2019; Janda et al., 2012) but details for how the CRD modulates conformational stability and opening of the molecular switch still requires investigation since the CRD was unresolved in the active FZD_7 structure. Evidence for conformational flexibility also comes from intramolecular BRET sensor experiments that revealed WNT-3A- and WNT-5A-dependent movement of the CRDs of FZD_5 and FZD_6. This event precedes the intracellular conformational rearrangement of FZDs in response to WNT stimulation, but how these events are linked remains to be investigated further (Schihada et al., 2021). Furthermore, even though the current explanation for WNT surrogate induced β-catenin signaling excludes conformational changes (Tsutsumi et al., 2020) – corroborated by the observation that it does not induce a conformational change in the CRD or ICL3 of FZDs (Kowalski-Jahn et al., 2021) – the FZD_5 $R^{6.32}A$ mutant is incapable of signaling via the transcriptional assay TOPFlash (Tsutsumi et al., 2020). This could be explained by the inability of mutated receptors to recruit DVL to the membrane, hampering their ability to form a functional complex required for the WNT/β-catenin signaling pathway. Likewise, another viable explanation could be the inability of these mutated receptors to accommodate the necessary conformation(s) needed for functional WNT/β-catenin signaling. Further investigation would be needed to understand this phenomenon. Overall, the data in **Paper I** and **Paper III** further demonstrates and corroborates the finding that FZDs undergo conformational rearrangements upon agonist stimulation (Schulte and Wright, 2018). They also pinpoint the involvement and importance of molecular switch networks which help dictate the different conformational states and guide the signaling output of the receptor. Disruption of this network by mutations in the conserved $P^{6.43}$ ($F^{6.43}$ in SMO) changes the receptor's ability to couple to transducer proteins and ability of further downstream signaling.

4.2 DVL OR HETEROTRIMERIC G PROTEINS?

The WNT-FZD signaling network has a diverse interaction profile with other proteins. Not only does it involve different co-receptors such as LRP5/6, ROR1/2 and RYK but also intracellular signal transducer proteins such as DVL and heterotrimeric G proteins. (MacDonald et al., 2007; Schulte, 2010; Semenov et al., 2007). DVL has been seen as the primary FZD-interacting intracellular protein that is responsible for conveying downstream signaling, whereas heterotrimeric G proteins have been given a minor role or even seen as irrelevant, even though there is overwhelming data supporting their direct involvement (Schulte and Wright, 2018). This became especially true in light of the recent structures of G_i-bound SMO and mG_s-bound FZD_7 (Qi et al., 2019, 2020; Xu et al., 2021). As discussed above with regards to **Paper I**, we identified a molecular switch in TM6/7 important for both FZD-DVL and heterotrimeric G protein interaction and signaling, suggestive of functional

selectivity dependent on distinct receptor conformations and structural rearrangements. Moreover, DVL has been implicated in the regulation of heterotrimeric G protein pre-coupling to FZD_6 and FZD_{10} (Hot et al., 2017; Kilander et al., 2014a), but not FZD_4 (Arthofer et al., 2016), advocating that FZD activity is fine-tuned by the cellular context and concentrations of signal transducers, a concept applied to many other GPCRs (Ritter and Hall, 2009; Schulte and Wright, 2018).

With this intricate relationship in mind, we wanted to investigate WNT/β-catenin signaling in the aforementioned context. WNT/β-catenin signaling can be disrupted by mutating or knocking down DVL (Paclíková et al., 2017; Tolwinski et al., 2003) – since these proteins play a vital role in signal transduction (Gao and Chen, 2010; Sharma et al., 2018) – but it has not been clear to which degree heterotrimeric G proteins are involved or necessary in this process. By inhibiting $G\alpha_{i/o}$ or $G\alpha_q$, studies have implicated heterotrimeric G proteins in WNT/β-catenin signaling (Halleskog and Schulte, 2013; Koval et al., 2016; Liu et al., 2005). Even so, neither of these studies completely suppressed signaling and the question remained if heterotrimeric G proteins are needed for functional WNT/β-catenin signaling. To that end, in **Paper II** we made use of HEK293A cells devoid of all Gα proteins except $G\alpha_{i/o}$ (ΔG7). Additionally, by treating these cells with pertussis toxin (PTX) – a catalyzer of $G\alpha_{i/o}$ ADP ribosylation (Sunyer et al., 1989) – we could inhibit $G\alpha_{i/o}$ activity and create a system devoid of functional Gα proteins. Furthermore, to remove the effect of endogenous WNTs, we inhibited the autocrine WNT secretion of the cells by treatment with C59, a porcupine inhibitor (Proffitt et al., 2013). Using a system depleted of Gα proteins, we stimulated endogenous FZDs with recombinant WNT-3A, a potent activator of WNT/β-catenin signaling (Bryja et al., 2007b; Halleskog and Schulte, 2013; Liu et al., 2005), and still detected important hallmarks of pathway activation via western blotting. These hallmarks included phosphorylation of LRP6, the phosphorylation-dependent electrophoretic mobility shift of DVL2 and the increase and dephosphorylation of β-catenin. In addition, recombinant WNT-3A-induced transcriptional TOPFlash activity showed full functionality in PTX-treated ΔG7 cells and with similar EC_{50} values, albeit with an increased signal amplitude compared to the parental cell line. Finally, to completely abrogate any possibility of Gα proteins influencing the signaling, we complemented our data with experiments from a full Gα protein knockout cell line. As expected, these cells displayed the same hallmarks of WNT/β-catenin pathway activation as the ΔG7 cells.

Taken together, these data clearly demonstrated that heterotrimeric G proteins are superfluous for WNT-3A-induced WNT/β-catenin signaling. However, the absolute levels of activation via TOPFlash were different between the parental and knockout cell lines, with the latter showing about twice the absolute signal. We tried re-introducing different Gα proteins but could not make any definite conclusions as to why these cells differ in their signal amplitude. A potential explanation is the clone-specific signal rewiring and protein expression compensation as a consequence of knocking out the heterotrimeric G proteins (Luttrell et al., 2018). Furthermore, there is a possibility that certain FZDs rely on heterotrimeric G proteins for WNT/β-catenin signaling, even though this was impossible to determine from **Paper II** because we were investigating FZDs at endogenous levels in HEK293 cells, which express multiple different FZD homologues (Atwood et al., 2011; Paclíková et al., 2021). Nevertheless, heterotrimeric G proteins undoubtedly play a modulatory role as demonstrated by reduced levels of WNT/β-catenin activity in PTX-treated cells (Halleskog and Schulte, 2013; Koval et al., 2016) and it is indeed possible that certain FZD-heterotrimeric G protein combinations directly activate WNT/β-catenin signaling, similar to what is understood for other GPCRs (Nag et al., 2018).

Naturally, a central question is how FZD interaction with DVL or heterotrimeric G proteins is modulated. As observed in **Paper I**, the molecular switch mutant in FZDs hampers plasma membrane recruitment of DVL and increases potency for Gα protein activation.

Previous studies on FZD_4 identified the conserved amino acid $Y^{2.39}$, which if mutated negatively affects DVL recruitment while maintaining $G\alpha_{12/13}$ interaction (Strakova et al., 2017). Additionally, R511C in FZD_6, a naturally occurring mutation causing nail dysplasia (Fröjmark et al., 2011), is selectively impaired in Gα protein pre-coupling but not DVL recruitment (Kilander et al., 2014a). Furthermore, DVL concentration in the cell affects the ability of FZDs to pre-couple to heterotrimeric G proteins (Hot et al., 2017; Kilander et al., 2014a). Surprisingly, in the case of FZD_6, conditions where DVL was depleted or overexpressed negatively affected FZD_6-$G\alpha_i$ pre-coupling, but in the case of FZD_{10}, only high levels of DVL disrupted pre-coupling of the G protein. Of note, DVL was depleted with siRNA in the FZD_6 case but ΔDVL1-3 cells were used in the FZD_{10} case, suggesting cell rewiring as a potential explanation for the discrepancy observed. Interestingly, FZD_4 is not affected by DVL depletion or overexpression in its ability to pre-couple to $G\alpha_{12/13}$ (Arthofer et al., 2016). This is further complicated by the fact that the Gβγ protein subunits interact with DVL (Angers et al., 2006; Seitz et al., 2014). In short, the relationship between FZD, DVL and heterotrimeric G proteins is complex making it difficult to construct a universal model.

4.3 MEASURING FZD-DVL INTERACTION AND DYNAMICS

In the FZD field, assays measuring DVL-dependent receptor activation are lacking, especially compared to the different biosensors existing for heterotrimeric G proteins (e.g. the mGs used in **Paper I**). Since DVL is involved in many different FZD signaling pathways (MacDonald et al., 2007; Schulte, 2010; Semenov et al., 2007; Sharma et al., 2018) measuring DVL activity has been of importance to the FZD field. Unfortunately, there are limitations with the currently used assays for measuring WNT-induced DVL-dependent FZD activation. The commonly used transcriptional assay TOPFlash, which is heavily amplified and at the end of the signaling cascade, could have many potential factors feeding into it making it challenging to distinguish the influence from different signaling branches. Furthermore, the immunoblotting assay DVL shift is semi-quantitative and unspecific in what response it measures besides general FZD activation. Additionally, confocal microscopy has been employed to determine FZD-DVL association (e.g. when evaluating FZD or DVL mutants for plasma membrane recruitment) (Bertalovitz et al., 2016; Boutros et al., 2000; Rothbächer et al., 2000; Valnohova et al., 2018), but this is limited in its sensitivity and throughput. There have also been attempts to observe membrane association of DEP after WNT stimulation with confocal microscopy (Gammons et al., 2016a), but any conclusions drawn from such experiments are highly uncertain because of the non-quantitative nature of the assay, and as such should be met with skepticism. Besides, a hurdle when looking at the FZD-DVL interaction and dynamics is the constitutively formed FZD-DVL complex (Gammons, Rutherford, et al., 2016; Valnohova et al., 2018), which makes it challenging to detect any WNT-induced changes in this preformed complex. This is further complicated by the observation that DVL continues to be associated with FZDs upon WNT stimulation (Ma et al., 2020b), most likely only changing its conformation. Recently though, there has been progress in this area with the use of TIRF (Total Internal Reflection Fluorescence) microscopy, measuring WNT-3A-induced DVL2 oligomerization at and recruitment to the plasma membrane (Ma et al., 2020b).

With this in mind, BRET is a useful tool for investigating FZD-DVL interaction and dynamics. It allows for relatively good spatial and temporal resolution and is able to detect conformational changes with the right setup and controls (Figure 12). As first shown in **Paper I**, we can observe the pre-coupled FZD-DVL state with the help of bystander BRET deploying an N-terminally nanoluciferase (Nluc) (Hall et al., 2012) tagged DVL2 (Nluc-DVL2) and a plasma membrane-anchored Venus (Venus-KRas). This is in many aspects superior to confocal microscopy, as it allows for a more quantitative analysis and a less biased signal, averaged over many cells. This was further developed, and in **Paper III** we used both the bystander BRET

with Nluc-DVL2 and Venus-KRas together with a more refined bystander BRET using the C-terminally Nluc-tagged DEP domain of DVL, DEP-Nluc, together with Venus-KRas in combination with a cell membrane impermeable SNAP dye to look at surface expression of the transiently transfected SNAP-tagged FZD_6 receptor. This allowed us to plot the BRET response against the surface expression of the receptor and offered additional information about the interaction, allowing for a clearer distinction between the wild type receptor and the mutant which cannot be surmised from only one FZD and DVL concentration. One major drawback with the bystander BRET setup is that it is not suitable for investigation of specific protein conformations, but rather a tool for looking at spatial distribution of a protein which can infer information about protein-protein interaction and conformation. Therefore, in **Paper IV**, by instead C-terminally tagging the receptor with Nluc and pairing that with an N-terminally Venus-tagged DVL2 (Venus-DVL2) we could investigate WNT-induced FZD-DVL dynamics. This allowed for a more detailed investigation not available with previous methods.

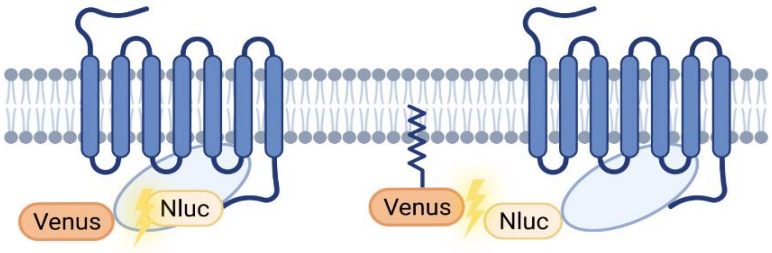

Figure 12. Comparison of direct BRET and bystander BRET. In direct BRET (left) the two proteins of interest are tagged with either acceptor or donor protein, respectively. A specific BRET signal then occurs when the two proteins are in close proximity which usually is interpreted as protein-protein interaction. In bystander BRET (right) the spatial distribution of a protein can be monitored by having a compartment specific (e.g. plasma membrane anchored) fluorescent protein (acceptor) expressed together with the luciferase (donor) tagged protein of interest and an untagged interactor (e.g. receptor) of that protein of interest. Created with BioRender.

4.4 WNT-INDUCED FZD-DVL DYNAMICS

With the right tools in place, it was possible to investigate the molecular dynamics of FZD-DVL interaction. As has been mentioned, it is still not clear how FZDs transduce WNT binding downstream, even though many important proteins have been identified for functional signal transduction. In **Paper IV**, we hypothesized that the FZD-DVL complex undergoes a conformational change upon WNT stimulation that allows for further signal transduction. First, we confirmed that Venus-DVL2 was readily recruited to C-terminally Nluc-tagged FZD_5 or FZD_6 by measuring basal BRET (i.e. the BRET signal without stimulation). Thereafter, we investigated and confirmed that the FZD-DVL BRET pairs responded to both WNT-3A and WNT-5A stimulation, demonstrated by the change in the BRET signal (Figure 13A-B). In an attempt to better understand what the WNT-induced response constituted, we turned to DVL oligomerization since this is an important aspect of WNT/β-catenin signaling. We demonstrated with an oligomerization-deficient DVL mutant (DVL2-M2/M4) that the WNT-induced response was – at least in part – independent of DIX-DIX oligomerization, in line with our hypothesis of a conformational change. Furthermore, we looked at LRP5/6 since they also are crucial in the transduction of WNT-3A-induced WNT/β-catenin signaling (MacDonald and He, 2012) and could potentially result in conformational changes in FZDs upon association. By using either DKK1 or LRP5/6 HEK293 knockout (ΔLRP5/6) cells, we demonstrated that the FZD-DVL dynamics measured by this assay are independent of LRP5/6. Moreover, considering that the DEP domain of DVL is crucial for FZD-DVL interaction (Gammons et al., 2016a; Tauriello et al., 2012), we continued building upon the notion that the DEP domain of DVL could serve as a more minimal conformational sensor of FZD-DVL dynamics (Schulte and Wright, 2018). Simultaneously, we could also reduce issues most likely stemming from DVL puncta formation due to DIX-DIX polymerization (i.e., sequestering of DVL in other parts of the cell and competitive binding reducing the interaction with FZDs) (Yang-Snyder et al., 1996). First, we confirmed that DVL2 lacking the DEP domain was deficient in its ability to interact with FZDs, corroborating previous results (Gammons et al., 2016b; Tauriello et al.,

2012). Thereafter, with the cloning of a C-terminally Venus-tagged DEP domain (DEP-Venus), we created a sensor with properties that produced a specific and efficient basal BRET signal with FZD_5- or FZD_6-Nluc. Furthermore, expression of saturating amounts of Venus-DEP together with a small amount of FZD-Nluc resulted in a large change in BRET in response to both WNT-3A and WNT-5A stimulation. These data further substantiated the fact that the observed dynamics were due to a conformational change between FZD and the DEP domain (Figure 13C-D).

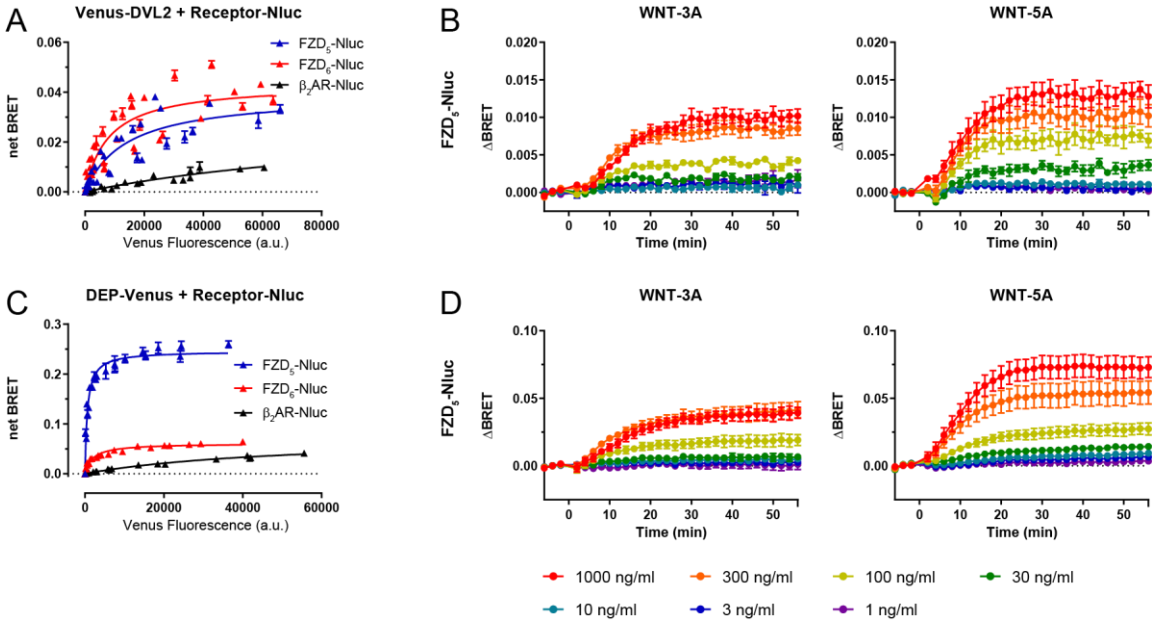

Figure 13. WNT-induced FZD-DVL dynamics. (A) Venus-DVL2 was recruited to FZD-Nluc but not β_2AR-Nluc without WNT stimulation. (B) WNTs induced a measurable and concentration dependent increase in BRET between FZD_5 and DVL2. (C) DEP-Venus was recruited to FZD-Nluc but not β_2AR -Nluc. (D) DEP-Venus recapitulates the response observed for Venus-DVL2. Adapted from **Paper IV**.

Interestingly, FZD_5 and FZD_6 displayed two different signaling profiles in response to WNT stimulation, the former with a stable increase and the latter a transient decrease. As mentioned before, the C-termini of FZDs are of varying lengths. In particular, FZD_6 has the longest C-tail and does not possess the PDZ ligand domain in contrast to other FZDs. To exclude the C-terminal differences as a potential source for the observed signaling differences, we created two chimeric receptors with swapped C-termini (i.e., FZD_5 was engineered to have the C-terminus of FZD_6 and vice versa). As expected, the two chimeric receptors displayed the same signaling profile albeit with opposite signal intensities. This clearly argues that the C-terminus is not involved in the observed FZD-DVL dynamics but that the length of the C-terminus will affect the signal amplitude, most likely because the energy transfer efficiency in BRET depends on the distance between the acceptor and donor (Xu et al., 1999).

Another interesting observation was the difference in amplitude between WNT-3A and WNT-5A-induced FZD_5-DEP dynamics. This difference is likely a reflection of the receptor population either sampling different conformations or sampling the same conformations with different probabilities (see section 1.1 “G protein-coupled receptors”) (Weis and Kobilka, 2018). Thus, to further understand what was happening on the molecular level, we dissected the WNT-induced response with the help of different DEP mutants. There are three DEP domain mutants of DVL2 described in the literature that we found interesting, G436P, L445E and K446M (Gammons et al., 2016b, 2016a). Specifically, the G436P mutant is hampered in its ability to form DEP dimers and the L445E and K446M mutants are impaired in FZD_5-dependent plasma membrane recruitment (assessed by confocal microscopy). The G436P and K446M mutants were both recruited to FZD_5 and FZD_6 in a concentration-dependent manner albeit with reduced affinity and maximum BRET signal. As mentioned, the K446M mutant

was not recruited to the plasma membrane as measured by confocal microscopy which is in disagreement with the BRET data. This is best explained by the more quantitative nature of BRET compared to previous methods. Finally, as expected, the L445E mutant did not show any specific interaction. Intriguingly, the WNT-induced FZD_6-DEP dynamics of the G436P and K446M DEP mutants did not show any obvious differences compared to wild type DEP. The WNT-induced response profile was similar and could not be inhibited or modified by either DKK1, LRP5/6 removal, FZD_6 C-terminal change or mutations of the DEP domain. Intriguing as this may be, more experiments are needed to make further conclusions. Interestingly though, WNT-induced FZD_5-DEP dynamics did show an apparent difference in the mutants when compared to wild type DEP (Figure 14). Foremost, it is important to underline that one should be careful when comparing different constructs with this specific BRET setup but there are some interesting observations that can be discussed. First, with regards to the DEP K446M mutant, there was no difference in the observed BRET signal between WNT-3A or WNT-5A stimulation, in stark contrast to the substantial difference observed for wild type DEP. Second, when comparing WNT-5A-induced FZD-DEP dynamics with the DEP K446M mutant to the wild type DEP experiments, the BRET increase was considerably lower. This observation could be explained – at least in part – by the lower basal BRET observed between FZD_5-Nluc and DEP-Venus K446M, but it could also be explained by a change in FZD_5-DEP conformations. Taken together, K446 is seemingly important for mediating WNT-induced FZD_5-DEP dynamics and especially those invoked by WNT-5A. Second, DEP G436P displayed a surprising difference where WNT-3A resulted in a larger BRET increase than WNT-5A, opposite of what was observed for the wild type DEP. In addition, the WNT-3A-induced dynamics between FZD_5 and the DEP G436P mutant presented with a larger BRET increase compared to that of wild type DEP, despite the basal BRET being lower for the DEP G436P mutant. Interestingly, it was previously reported that the DEP domain can undergo dimerization and that this is vital for functional WNT/β-catenin signaling (Gammons et al., 2016a). In the homodimer state, the DEP domain can no longer interact with FZD but since the G436P mutant is unable to form dimers, this could be a reflection of the larger BRET response.

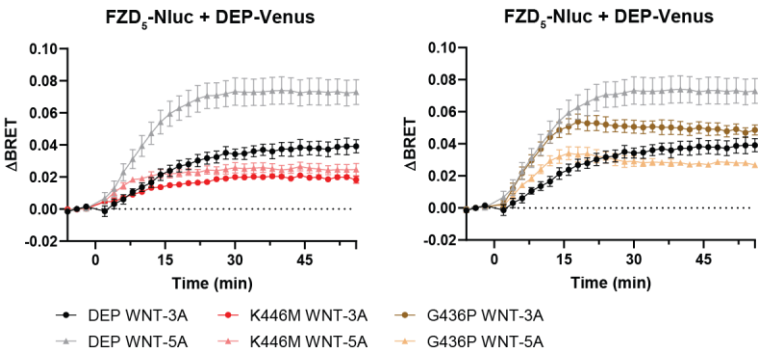

Figure 14. DEP mutants show apparent differences in WNT-induced FZD_5-DEP dynamics. Stimulation with 1 µg/ml of either WNT-3A (circle) and WNT-5A (triangle). The response of the DEP wild type (black and grey) was compared to the response of the K446M mutant (red and pink) and G436P mutant (gold and wheat). Data was adapted from **Paper IV**.

Another question is whether the FZD_5-DVL dynamic response could be linked to WNT/β-catenin signaling. It was demonstrated that both WNT-3A and WNT-5A stimulation induced movement of the CRD and ICL3 of FZD_5 but a WNT surrogate did not (Schihada et al., 2021). This argues that the WNT/β-catenin signaling pathway relies on proximity with LRP5/6 and not on conformational changes in the CRD or ICL3 of the receptor. This could indicate that the observed WNT-induced FZD_5-DVL dynamics reflect something other than activation of the WNT/β-catenin signaling pathway. Howbeit, the mechanistic details for how the WNT surrogate asserts its effect is unknown and therefore WNT-induced β-catenin signaling could behave differently in terms of WNT-induced FZD_5-DVL dynamics. Finally, individual WNTs are likely able to activate multiple signaling pathways when they bind FZDs (Schulte, 2015) and the FZD-DVL dynamics observed in **Paper IV** could represent multiple

conformations since we are unable to distinguish between them due to the nature of the BRET assay. This seems especially likely when taking into account the response of the DEP mutants.

5 GENERAL DISCUSSION AND CONCLUSIONS

Cells are continuously exposed to a multitude of extracellular signaling molecules influencing their behavior via membrane-anchored receptors transducing the signal into the cell. FZDs are a group of receptors which are involved in important functions during both development and tissue homeostasis and in diseases such as cancer (Clevers & Nusse, 2012). Their signaling pathways involve a plethora of proteins, including 19 different endogenous ligands, a great number of different co-receptors and transducer and effector proteins. Although we have begun to understand the molecular events unfolding upon WNT-FZD binding, a great amount is left to be discovered for a more detailed picture that would benefit our understanding of health and disease. Additionally, it would aid the development of drugs and treatments for FZD-related diseases. This thesis aims to bring more knowledge on the topic of WNT/FZD signaling and does so by investigating the molecular events, protein-protein interactions and receptor dynamics involved. By understanding the FZD protein interactome and how it is modulated, we can shine light on the road to functional selectivity and signal transduction.

FZDs consist of motifs and switches that stabilize the receptor in certain conformations, allowing it to dynamically respond to ligand stimulation by rearrangement of these amino acid networks (Gloriam et al., 2021). One such network is the molecular switch found in **Paper I**, a mutation found in some cancers. It opens up to allow for the accommodation of the Gα protein and further signal transduction as corroborated by the active FZD_7 and SMO structures (Deshpande et al., 2019; Qi et al., 2019, 2020; Xu et al., 2021). Additionally, mutation of the molecular switch produces a heterotrimeric G protein-biased receptor that is (in the case of FZD_6) more constitutively active and unable to efficiently couple to DVL. This signaling bias highlights the nature of functional selectivity of FZDs, but how it is utilized and molecularly modulated by the cell is unclear. In **Paper III**, we observed that the network of the molecular switch was extended upwards in the receptor by aromatic π-π interactions. Interestingly, SMO is different compared to FZDs, with a straight TM6 due to having a $F^{6.43}$ instead of a $P^{6.43}$. The straight TM6 is important to allow for the accommodation of cholesterol, which is crucial for $G\alpha_i$ activation of SMO as attested by the $F^{6.43}P$ mutation. This is not the case for FZDs, where the $P^{6.43}F$ mutation resulting in a straighter TM6 did not have the same drastic impact on receptor activity. Rather, there was a discrepancy between the different FZDs tested: neither the FZD_6 nor FZD_7 $P^{6.43}F$ mutants showed any dramatic difference in $G\alpha_i$ and $G\alpha_s$ protein activation, respectively, compared to wild type. However, the ability to recruit DVL to the plasma membrane was drastically reduced for the FZD_6 $P^{6.43}F$ mutant, but not for the FZD_7 $P^{6.43}F$ mutant. The FZD_5 mutant also showed a reduction in DVL plasma membrane recruitment, but FZD_4 did not, whereas both of them displayed reduced WNT/β-catenin signaling capabilities. Furthermore, we showed in **Paper II** that WNT/β-catenin signaling is fully functional without any Gα proteins in the cell, although heterotrimeric G proteins do regulate WNT/β-catenin signaling depending on cellular context, as demonstrated by previous studies (Halleskog and Schulte, 2013; Jernigan et al., 2010; Koval et al., 2016; Liu et al., 2005). Hence, the integration of heterotrimeric G proteins into the WNT/β-catenin signaling pathway is an exciting future question.

The FZD-DVL interaction has been studied intensely, but one of the major road blocks in understanding the underlying molecular dynamics has been the lack of proper and accessible tools to investigate this relationship. In **Paper IV**, we developed BRET-based tools for this purpose. We established that WNT stimulation induces dynamic conformational changes in the FZD-DVL interaction that can vary between different WNT and FZD combinations. Additionally, we developed a miniaturized sensor consisting of the DEP domain of DVL2 that recapitulates FZD-DVL dynamics and corroborated the findings that DEP is the primary FZD-interacting domain. Moreover, mutations in this DEP sensor allowed us to further investigate the involvement and importance of certain amino acids deemed to be significant for the FZD-DEP interface in DVL2: K446 at the tip of the DEP finger loop is important for sensing certain

WNT-induced FZD conformations while G436 appears to affect the FZD-DEP dynamics in a more WNT-selective manner. Taken together, these data support the notion of an alternative WNT-FZD-DVL ternary complex where DVL is able to adapt multiple conformations depending on the signaling context. Furthermore, the DEP sensor/BRET setup could be further developed for future drug screens of small molecule compounds and even used to search for heterotrimeric G protein- and DVL pathway-biased ligands. The development of potent small molecule ligands for FZDs would be an additional great milestone for the field and could serve not only as new research tools but also be developed into new drugs targeting FZDs for the treatment of diseases.

In summary, conserved structures in the FZD family can have different homologue-dependent effects and no FZD family-wide model as of now seems apparent to explain these observed phenomena. Additionally, the findings within this thesis highlight how FZDs can sample different conformations in cooperation with intracellular signal transducer proteins to achieve functional selectivity.

FZDs can signal via a myriad of different signaling pathways, but it requires additional efforts to fully understand how pathway selectivity is achieved. It is known that a battery of co-receptors and intracellular proteins are involved in different WNT/FZD signaling pathways, but understanding how they regulate and conduct pathway selectivity by predisposing different FZD conformations is important for this complex signaling network (Grainger and Willert, 2018; MacDonald et al., 2007; Niehrs, 2012; Schulte and Wright, 2018; Semenov et al., 2007). Therefore, one important aspect is to further identify the microswitches and intramolecular changes in FZDs underlying the mechanisms of signal initiation. This will improve our understanding of pathway selectivity and how it would be possible to modulate this system with biased ligands. Hence, the recent publication of the active FZD_7 structure (Xu et al., 2021) was an important step in understanding mechanisms of signal initiation. For the future, an active WNT-bound FZD structure would extend this progress with additional understanding of FZDs in general and pathway selectivity in particular. Moreover, a FZD-DVL or FZD-DEP structure would help in understanding molecular and atomic details of the FZD-DVL interface and identify the differences and similarities between DVL and heterotrimeric G protein pathway selectivity. Interestingly, post-translational modifications are involved in regulating receptor and heterotrimeric G protein interaction efficacy (Patwardhan et al., 2021) and this concept could also be applied to DVL since it as well is heavily post-translationally modified (Beitia et al., 2021; Hanáková et al., 2019; Sharma et al., 2018). Indeed, phosphorylation of DVL is associated with promoting different DVL conformations that could in part explain pathway selectivity (Beitia et al., 2021; Harnoš et al., 2019; Lee et al., 2015). Therefore, a viable option to understand this selectivity would be to create specific pathway-biased DVL or DEP sensors, which could be used for mapping WNT-FZD signaling specificity. Likewise, a common approach for understanding GPCR signaling, and used throughout this thesis, is the overexpression of proteins in immortalized cell lines. It is an adequate and sometimes preferred solution in many cases, but another attractive approach is the investigation of FZD dynamics on endogenous protein expression levels, especially in light of data demonstrating that DVL concentrations in the cell affects Gα protein pre-coupling (Hot et al., 2017; Kilander et al., 2014a). This allows for the system to more closely resemble the *in vivo* environment and reduce potential overexpression artifacts. Excitingly, this strategy is feasible with today's gene editing capabilities brought forth by CRISPR/Cas9 technology and sensitive assays based upon split luciferase and BRET. This approach has already been applied to other GPCRs to investigate ligand binding and protein-protein interactions (Kilpatrick et al., 2019; Soave et al., 2021; White et al., 2019, 2020).

The cubic ternary complex model explains the interchangeability of multiple receptor complexes where the equilibrium shifts depending on the free energy landscape, which is regulated by ligands and intracellular proteins (Kenakin, 2017). FZDs form complexes with, among others, heterotrimeric G proteins and DVL. Hence, an important question emerges how WNTs achieve conformational rearrangement of the receptor to allow for activation, functional selectivity and signal initiation. Therefore, drawing inspiration from mechanism of other GPCRs could help in imagining activation mechanisms for FZDs. Class B receptors have peptide ligands that bind the TMD. They also comprise a flexible extracellular domain (ECD) that in most structures is unresolved for this specific reason (Krumm and Roth, 2020; Liang et al., 2017, 2020; Ma et al., 2020a). However, Class B receptors demonstrate heterogeneity in their binding mode, where the Glucagon-like peptide 1 (GLP-1) receptor rigidly binds to GLP-1 partially via the ECD (Zhang et al., 2017). Interestingly, there is a two-step binding mode for Class B GPCRs, where an initial fast recognition by the ECD is followed by a kinetically slower recognition by the TMD (Ma et al, 2020). Similarly, this could be one explanation for the somewhat slow kinetics observed with agonist stimulation and binding to FZDs (Kozielewicz et al., 2021; Wesslowski et al., 2020; Wright et al., 2018) and would fit with the previously mentioned "fishing rod" hypothesis. Moreover, Class C GPCRs have a large ECD composed of both a CRD and a ligand-binding domain (LBD) that holds the orthosteric ligand binding site. These receptors are found predominantly as constitutive dimers formed via their LBD and in part the TMD. Upon agonist stimulation, Class C receptors do not show the otherwise characteristic movement of TM6 seen in many other GPCRs. Instead, there is a rearrangement of the inter-TMD interaction bringing about intra-TMD conformational changes allowing for heterotrimeric G protein activation. Moreover, it is proposed that agonist-induced conformational changes in the LBD are conveyed via interdomain disulfide bonds present between the inter-CRD dimers. Furthermore, the signal is transferred to the TMD via interactions between the CRD or linker domain and ECL2 (Ellaithy et al., 2020). Another arguably more relevant dimer is the one consisting of the Class B receptor calcitonin receptor-like receptor (CLR) and the single TMD receptor activity-modifying protein 1 (RAMP1). This heterodimer promotes the active and ligand bound conformation by stabilizing the ECD, TMD and ECL2 of CLR (Liang et al., 2018b). Inspired by these activation models, it is attractive to think of a WNT-induced heterodimer forming between FZD and LRP5/6 that allows for TMD interactions and conformational change. This in turn would elicit FZD-DVL dynamics to initiate signaling via the WNT/β-catenin signaling pathway. Furthermore, it is important to underline the unlikeliness of a signalosome composed of rigid proteins that has been proposed for WNT/β-catenin signaling (DeBruine et al., 2017; Tsutsumi et al., 2020). To induce change, movement is necessary and conformational changes in the receptor accomplishes this by different means and involvement of different parts of the receptor. Hence, stabilization of distinct receptor conformations allows for selective engagement of distinct transducer proteins. Therefore, the above-mentioned dynamic model can help explain the somewhat paradoxical observation that the WNT surrogate – which binds LRP5/6 and the CRD of FZDs – does not induce conformational change in the CRD or ICL3 but still initiates WNT/β-catenin signaling (Kowalski-Jahn et al., 2021). Interestingly, MD stimulations demonstrate that the CLR-RAMP1 heterodimer does not affect the mobility of ICL3 in CLR (Liang et al., 2018b). This denotes the concept that receptors can undergo conformational change locally and establishes the possibility for FZD conformational change without movement in ICL3.

Since WNTs are known to bind the CRD (Hirai et al., 2019; Janda et al., 2012; Kozielewicz et al., 2021), but this domain is unresolved in all full-length FZD structures, we can only speculate on how information flow from ligand binding to effector activation is achieved. Nonetheless, a recent publication of the active Class A luteinizing hormone-choriogonadotropin receptor (LHCGR) bound to the endogenous ligand chorionic gonadotropin (CG) (Duan et al., 2021) could help understand an activation mechanism for FZDs. This receptor, like FZDs, has a large ECD containing a ligand binding site. In the paper,

the authors describe how the ECD of the inactive LHCGR is tilted towards the plasma membrane, but upon ligand binding the ECD is "pushed" and "pulled" into a more perpendicular position to accommodate the ligand that would otherwise clash with the plasma membrane. Interestingly, this concept is supported for FZDs by the recent observation that the CRD of FZDs move upon WNT stimulation and where MD simulations predicted that the available receptor conformations are restricted due to the WNT clashing with the plasma membrane (Kowalski-Jahn et al., 2021). Furthermore, the hinge loop (located at the C-terminal end of the ECD) of the active CG-bound LGCGR acts as an agonist by binding to the TMD of the receptor, inducing a conformational change, which is an active receptor conformation. Something similar could be imagined for FZDs as they also have a hinge (linker) domain between the CRD and TMD and this could be one explanation for how WNT-dependent functional selectivity and signal transduction is achieved. Furthermore, this activation mechanism is reminiscent of what is proposed for Class C GPCRs. Hence, it is attractive to imagine an activation mechanism where the hinge loop of FZDs rearranges upon binding of an agonist to the CRD, which in turn changes the interaction and conformation of the TMD. However, it should be noted that the linker domain of FZDs is stabilized by two cysteine bridges and is therefore expected to be rather rigid. Interestingly, there was a recent paper describing activation mechanisms and microswitches across all GPCR classes (Gloriam et al., 2021). There, it is described how a contact between $F^{3.29}$ at the top of TM3 and $Y^{45.51}$ and $V^{45.52}$ in ECL2 acts as an activator, suggesting that ECL2 plays an important role in FZD signal transduction. Additionally, the authors also corroborate our findings in **Paper I** and **Paper III** where the extended molecular switch network is an inactivator of the receptor and acts as an important gatekeeper of heterotrimeric G protein activation.

To understand DVL a comparison with other intracellular transducer proteins such as β-arrestin is reasonable. Arrestins can relay signaling via different pathways and it was recently understood that β-arrestin does this by adopting different conformations when bound to the receptor (Cahill et al., 2017; Kumari et al., 2016, 2017). Furthermore, it was demonstrated that two non-heterotrimeric G protein-engaging 7TM receptors interact with β-arrestin in distinctly different conformations compared to prototypical GPCRs (Pandey et al., 2021). Applied to FZD-DVL dynamics, this is in line with what was observed in **Paper IV** where the DEP mutants suggest that there are different FZD-DEP conformations induced by different WNT-FZD pairs, suggesting a model for how FZDs can signal via different pathways with DVL at the crossroads, but it needs further experimental validation and detailed understanding.

In summary, one should appreciate that there are differences throughout the FZD family, especially when considering the diversity found among other GPCRs. This diversity is not only mirrored by the differential ability of FZD subtypes to activate WNT/β-catenin signaling, but also by diverse Gα protein-coupling profiles. Certainly, there are Class F-wide activation mechanisms as demonstrated by the molecular switch identified in **Paper I**, but this is most likely not the case for all receptor conformations and signaling pathways. Indeed, the observations in **Paper III** and **Paper IV** support the notion that there are specific FZD homologue differences with regards to transducer protein interactions and activation mechanisms. In light of what has been discussed, there are multiple different plausible models for WNT/FZD signaling and pathway selectivity that could be explored and could explain the observed heterogeneity. More work is needed to fully understand WNT/FZD signaling, but the body of work in this thesis has moved the field forward with knowledge and understanding of WNT/FZD signaling and through the development of new tools enabling further investigations.

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