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FRIZZLED AS A G PROTEIN-COUPLED RECEPTOR

The role of Disheveled and G proteins for signal specification

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Karolinska Institutet

Stockholm 2013

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Cover: *The Frizzled tree – Going out on a limb to find the root.* (Acrylic painting on canvas, Kilander, M.B.C., 2013)

ISBN 978-91-7549-256-8

Till mamma och mormor

Vara är vara

Jag är
Jag är jag
Jag är det jag är
Jag är icke mer än jag är
Jag är allt det jag är
Jag är icke mindre än jag är
Jag är icke det jag icke är
Jag är helt och hållet jag

In honor of my relative; Gustaf Fröding,
acclaimed Swedish poet (1860-1911).

'Before you know, you must imagine.'
-Richard Axel

ABSTRACT

Cell communication governed and coordinated by the WNT family of lipoglycoproteins comprises essential physiological processes active throughout the life-span of metazoan organisms. In humans, severe diseases and congenital birth defects have been directly linked to disturbances in WNT signaling. In line with the general scheme of cell signaling transduction, WNT proteins bind and activate cell surface receptors in order to relay signaling to intracellular effector molecules which subsequently acts to execute changes in cell fates; such as proliferation, differentiation, migration and polarization. Thus far, a variety of structurally and functionally different proteins are identified as WNT receptors and/or co-receptors.

The ten members of the Frizzled (FZD) family of seven transmembrane-spanning proteins are the preponderant receptors of WNTs and are shown to interact with heterotrimeric G proteins and the scaffold phosphoprotein Dishevelled (DVL) to activate downstream signaling events. Due to homology in secondary structure, FZDs are classified as G protein coupled receptors (GPCRs), but display unconventional constitution and signaling properties in respect to e.g. the well-studied Class A 7TMRs. This fact generated the generally accepted notion that FZDs in fact are not GPCRs. The work presented in this thesis investigates the functional and molecular relationship between WNTs, FZDs, G proteins and DVL from a pharmacological point of view.

The link between WNTs and G proteins has been established mainly through epigenetic studies and in genetically manipulated systems. Using the [γ-^{35}S]GTP method to assay G protein activity we demonstrated that WNTs are able to activate G proteins of the PTX-sensitive $G_{i/o}$ family even at endogenous protein levels, thus providing evidence that the WNT-G protein connection indeed is not an artifact imposed by alterations in protein stochiometry.

Functional selectivity or biased agonism is a recently established feature of ligand activity at GPCRs and have provided important insights into the pharmacological aspects of 7TMR signaling. By assessment of downstream WNT signaling events and FRAP analysis of FZD_6 lateral diffusion we find that WNT isoforms are not homologous in signaling pathway activation at a specific receptor or in a defined cellular milieu. Thus, our conclusion suggests that WNTs might be able to act as endogenous biased agonists at FZDs.

Even though recent biochemical and bioinformatics data provides the definite evidence of FZDs as true GPCRs, signal transduction mechanisms and selectivity in the interactions of FZDs, G proteins and DVL are largely unknown. Here we report, using a double fluorophores FRAP and cell surface cross-linking approach, that FZD_6 protein precouples to $G\alpha_{i1}$ and $G\alpha_q$ and that the interaction is sensitive and dynamic to WNT stimulation. We also demonstrate that DVL is an essential component in the FZD_6-G protein precoupled complex. Interestingly, we find that the effect of DVL is concentration-dependent: a low as well as a high concentration of DVL destabilizes the receptor-G protein complex. Additionally, we establish that a point mutation, R511C, in the FZD_6 C-terminal region gives rise to autosomal recessive nail dysplasia and renders the receptor dysfunctional in G protein precoupling.

LIST OF PUBLICATIONS

I. **Kilander, M.B.**, Dijksterhuis, J.P., Ganji, R.S., Bryja, V., and Schulte, G. (2011). WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins. Cellular signalling 23, 550-554.

II. **Kilander, M.B.**, Halleskog, C., and Schulte, G. (2011). Recombinant WNTs differentially activate beta-catenin-dependent and -independent signalling in mouse microglia-like cells. Acta physiologica 203, 363-372.

III. **Fröjmark, A.S.**, Schuster, J., Sobol, M., Entesarian, M., Kilander, M.B., Gabrikova, D., Nawaz, S., Baig, S.M., Schulte, G., Klar, J., et al. (2011). Mutations in Frizzled 6 cause isolated autosomal-recessive nail dysplasia. American journal of human genetics 88, 852-860.

IV. **Kilander, M.B.**, Dahlström, J., Schulte, G. Assessment of FZD_6 membrane mobility reveals WNT-FZD selectivity. *Manuscript*

V. **Kilander, M.B.**, Petersen, J., Wessel Andressen, K., Ganji, S.R., Levy, F-O., Schuster, J., Dahl, N., Bryja, V., Schulte, G. Disheveled regulates precoupling of heterotrimeric G proteins to Frizzled 6. *Manuscript*

ADDITIONAL PUBLICATIONS

Related papers involving the author that are not included in the thesis:

I. Halleskog, C., Dijksterhuis, J.P., **Kilander, M.B.**, Becerril-Ortega, J., Villaescusa, J.C., Lindgren, E., Arenas, E., and Schulte, G. (2012). Heterotrimeric G protein-dependent WNT-5A signaling to ERK1/2 mediates distinct aspects of microglia proinflammatory transformation. Journal of neuroinflammation 9, 111.

II. Kříž, V., Pospíchalová, V., Mašek, J., **Kilander, M.B.**, Slavík, J., Tanneberger, K., Schulte, G., Machala, M., Kozubík, A., Behrens, J., Bryja, V. β-arrestin promotes Wnt-induced Lrp6 phosphorylation via increased membrane recruitment of Amer1. *Submitted manuscript*

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LIST OF ABBREVIATIONS

7TMR	seven transmembrane receptor
ABC	active β-catenin
AGS	activator of G protein signaling
Ala	alanine
APC	adenomatous polyposis coli
aPKC	atypical Ca^{2+}-dependent protein kinase
AR	adenosine receptor
Arg	arginine
βAR	β-adrenergic receptor
C	cystein
CaMK	calmodulin-dependent kinase
cAMP	cyclic adenosine monophosphate
CBR	cannabinoid receptor
CE	convergent extension
CFP	cyan fluorescent protein
cGMP	cyclic guanosine monophosphate
CK	casein kinase
CRD	cystein rich domain
Cys	cystein
D	aspartic acid
D4476	4-[4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide (CK1 inhibitor)
DEP	disheveled/ EGL-10/ Pleckstrin
DIX	disheveled/Axin
DNA	deoxyribonucleic acid
DVL	disheveled
ECD	extracellular domain
ECL	extracellular loop
e. g.	for example (*exempli gratia* lat.)
ERK	extracellular signal-regulated kinase
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FZD	frizzled
GAP	G protein activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HEK	human embryonic kidney
ICD	intracellular domain
ICL	intracellular loop
i. e.	that is (*id est* lat.)

IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
K	lysine
LRP	low density lipoprotein receptor-related protein
MEF	mouse embryonic fibroblasts
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	asparagine
NECA	1-(6-Amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide (adenosine receptor agonist)
NFAT	nuclear factor of activated T cells
PAGE	polyacrylamide gel electrophoresis
PCP	planar cell polarity
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDZ	postsynaptic density 95/disc-large/zona occludens-1
PIP2	phophatidylinositol bis-phosphate
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	Ca^{2+}-dependent protein kinase
PLC	phospholipase C
PP	protein phosphatase
Pro	proline
PS-DVL	phosphorylated and shifted disheveled
PTK	protein tyrosine kinase
PTX	pertussis toxin (from *Bordetella pertussis*)
QPCR	quantitative reverse transcriptase polymerase chain reaction
R	arginine
RGS	regulator of G protein signaling
RNA	ribonucleic acid
ROR	receptor tyrosine kinase-like orphan receptor
RT-PCR	reverse transcriptase polymerase chain reaction
RYK	related to receptor tyrosine kinase
S	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFRP	soluble frizzled-related protein
siRNA	small interfering ribonucleic acid
T	threonine
TCF/LEF	T cell-specific transcription factor/lymphoid enhancer factor
Thr	threonine
TSPAN	tetraspanin
Tyr	tyrosine
Vangl	vang-like
W	tryptophan
WIF	WNT inhibitory factor
WNT	*wingless/int-1*, wingless-type mouse mammary tumor virus integration site family
Y	tyrosine
YFP	yellow fluorescent protein

1 INTRODUCTION

The WNT signaling system consists of a complex and elaborate cell communication network of extracellular agonists and inhibitory molecules, receptors, co-receptors and intracellular pathway components. Physiological processes governed by WNTs range from embryonic tissue patterning, body axis formation and organogenesis to tissue homeostasis and adult stem cell renewal (Clevers, 2006; Nusse, 2012). The importance of proper WNT communication has further been appreciated since aberrant WNT signaling leads to congenital defects, cancer and neurodegenerative diseases (Holland et al., 2013; Logan and Nusse, 2004). The classification of WNTs and related signaling components is largely based on amino acid sequence similarity and biochemical properties rather than functionality (Logan and Nusse, 2004). However, as more data regarding the signaling features of WNTs are gathered, we begin to understand that current WNT categorization systems are insufficient (Gordon and Nusse, 2006). In the coming section, a general overview of signaling pathways is presented as well as a brief introduction to the molecular players involved. As the focus of this thesis is set on investigating WNT signaling events at the plasma membrane, the information in the subsequent sections will give a more thorough coverage of the central players in proximal WNT signaling, namely the seven transmembrane receptors Frizzleds (FZD), the intracellular scaffold protein Disheveled (DVL) and heterotrimeric G proteins. In the final part pharmacological aspects of WNT/FZD signaling will be commented on.

1.1 GENERAL OVERVIEW OF WNT SIGNALING PATHWAYS

Cellular communication typically occurs when a signaling molecule at the outside of the cell interacts with a transmembranous receiver molecule which has the ability to recognize and translate the signal into an intracellular message. The WNT signaling system is no exception to this fundamental biochemical process. Over the years, the knowledge of the WNT communication system has advanced mainly in the area of downstream – and endpoint signaling events as most results have been gained by performing epigenetic studies, morphogenic analysis, and measurements of gene transcription and second messengers (van Amerongen and Nusse, 2009). The information gathered from these studies allowed for the division of WNT-signaling into two distinct groups depending on the nature of the downstream outcome of the signaling pathway (Kikuchi et al., 2011; Logan and Nusse, 2004; McNeill and Woodgett, 2010).

1.1.1 The β-catenin-dependent pathway

The intracellular accumulation and stabilization of the cadherin-binding protein β-catenin was the first identified biomolecular proof of WNT action (Riggleman et al., 1990) and has since been considered the canonical and most prominent pathway of WNT signaling (Shimizu et al., 1997). β-catenin is a cytosolic protein which regulates cell adhesion and gene transcription, and under normal conditions, in the absence of WNTs, the levels of β-catenin are kept low by proteasomal degradation (Aberle et al.,

1997). β-catenin is marked for degradation by constantly being phosphorylated and ubiquitinated by a multiprotein assembly known as the destruction complex. Key components within this complex include the scaffold protein Axin, the adenomatous polyposis coli (APC) protein and the enzymes glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). WNT stimulation by the activation and interaction of WNTs with receptors and co-receptors at the plasma membrane leads to the decay of the destruction complex, an event which inactivates the phosphorylation of β-catenin by GSK3β and CK1α, and releases Axin (Clevers, 2006; Liu et al., 2002; Logan and Nusse, 2004). Active and stabilized β-catenin is able to enter into the nucleus where it functions as a co-factor to activate the T cell-specific transcription factor/lymphoid enhancer factor (TCF/LEF) transcriptional machinery by displacing the transcriptional repressor Groucho. The subsequent transcription of target genes, e.g. *c-Myc* and *cyclinD1*, determines the main role of the WNT/ β-catenin pathway as a regulator of cellular proliferation and differentiation (Hurlstone and Clevers, 2002).

1.1.2 The β-catenin-independent pathways

Research in the WNT field revealed that further effects, besides the stabilization β-catenin, transcription of cell cycle regulating genes and body axis duplication, could be mediated by this family of morphogenic proteins. It was found that cellular movement, orientation and polarization during embryonic tissue patterning were dependent of WNT communication (Heisenberg et al., 2000; Wallingford et al., 2001). Regulation of these cellular processes is performed by the two main branches within the β-catenin-independent WNT pathways; namely the WNT/planar cell polarity (PCP) and the WNT/Ca^{2+} pathways (Kikuchi et al., 2011). The PCP pathway was first identified in *D. melanogaster* where it governs movements and polarity of cells within the epithelial plane, such as the organization of hairs, bristles and ommatidia (Fanto and McNeill, 2004). Further, a pathway similar to the *D. melanogaster* PCP process is involved in convergent extension movements of the vertebrate embryo, commonly studied in *X. laevis* and zebrafish (Simons and Mlodzik, 2008). WNT/PCP regulated cellular migration and polarity is orchestrated by the downstream activation of small GTP binding proteins, Rho and Rac, and related protein kinases, such as c-Jun N-terminal kinase (JNK) and Rho-kinase (Zallen, 2007). The consequent effect leads to cytoskeleton rearrangement and coordination (Kikuchi et al., 2011). The second major branch of the β-catenin-independent pathways is represented by the WNT induced release of intracellular Ca^{2+} (Slusarski et al., 1997b) and activation of related Ca^{2+} responding effectors such as e.g. calmodulin-dependent kinase II (CaMKII) and Ca^{2+}-dependent protein kinase (PKC), eventually leading to gene transcription via nuclear factor of activated T cells (NFAT) (De, 2011; Huelsken and Behrens, 2002; Kohn and Moon, 2005; Veeman et al., 2003). Additionally, WNTs have been shown to increase Ca^{2+} by the activation of phosphodiesterases (PDEs) resulting in declining concentration of cyclic guanine monophosphate (cGMP) and consequent intracellular Ca^{2+} mobilization (Ahumada et al., 2002; Ma and Wang, 2006). The Ca^{2+} pathway is shown to activate cell migration and to have negative effects on β-catenin signaling (De, 2011; Kuhl et al., 2001). As the number of studies into WNT/β-catenin-

independent signaling currently is increasing, additional pathways have been identified; among them the activation of rap1, atypical protein kinase C (aPKC), mammalian target of rapamycin (mTOR), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K) (Semenov et al., 2007). The wide variety of effects that WNT signaling is capable of driving further highlights the necessity of researching WNT communication in a much broader perspective than only in developmental processes.

1.2 THE WNT FAMILY AND RELATED EXTRACELLULAR SIGNALING MOLECULES

The name WNT is an acronym derived from the *D. melanogaster* tissue polarity gene *wingless* and the mammalian mammary proto-oncogene int-1 (Nusse et al., 1991). This protein family, which was defined in the 1980s by the discovery that wingless and Int-1 are orthologs (Rijsewijk et al., 1987), is highly conserved throughout evolution and the mammalian genome harbors 19 different WNT isoforms, named as follows: WNT-1, -2, -3, -3A, -4, -5A, -5B, -6, -7A, -7B, -8A, -8B, -9A, -9B, -10A, -10B, -11, -16 (Nusse, 1997-2013). WNTs act as morphogens and signaling occurs both at short distances; i.e. WNTs can act in autocrine and paracrine manners, as well as being able to mediate communication over longer distances (Port and Basler, 2010). The general biochemical structure of WNTs contains an N-terminal signal sequence of approximately 20 amino acids necessary for proper secretion but which, in some cases, also is cleaved off to regulate WNT activity and function (Willert and Nusse, 2012). All WNT members contains high numbers of cysteine resides and even though analysis of primary amino acid structure predicts hydrophilic properties, WNTs typically binds tightly to extracellular matrix (ECM) structures and exhibit hydrophobic features (Clevers, 2006; Lorenowicz and Korswagen, 2009). These properties make WNT proteins particularly hard to isolate and purify and are the results of i) acetylations: palmitations at conserved cysteines and palmitoylations at serines, and ii) multiple and a highly variable number of N-linked glycosylations (Kurayoshi et al., 2007; Willert et al., 2003). These series of posttranslational modifications are important for secretion, activity and transportation; especially acetylation seems indispensable for WNT activity (Schulte et al., 2005; Willert et al., 2003). Owing their cysteine-rich and hydrophobic character, WNTs may be expected to show ordered secondary and tertiary structures. Indeed, in a recent study by Janda and colleagues (Janda et al., 2012), the crystal structure of *X. laevis* WNT-8 revealed a unique 3-dimentional structure, held together by disulfide bridges between 22 conserved cysteines, and divided into two distinct domains: an N-terminal α-helical-containing cluster (“the thumb”) and a C-terminal dual β-sheet part (“the index finger”). A linker region of nonconserved residues stretches between the two domains (“the palm”) and current results indicate that this region probably does not participate in receptor binding (Janda et al., 2012; Willert and Nusse, 2012). Possibly, this flexible part of WNTs might be subjected to genetic modification, e.g. potential site for the introduction of internal tags.

WNTs (and WNT signaling-related components) are commonly categorized according to their activity in either β-catenin-dependent or –independent pathways (McNeill and Woodgett, 2010). Assessment of β-catenin dependent pathway is fairly robust, and a battery of methods is available; such as the ability of the WNT to induce *X. laevis* embryo body axis duplication, transformation of C57MG cells, the phosphorylation of upstream effectors, activation of luciferase LCF/TCF-driven reporter gene and of course the stabilization of β-catenin itself. The analyses of β-catenin independent pathways, on the other hand, are still suffering from the lack of robust readout assays (van Amerongen, 2012). According to the current understanding, which is limited to only a subset of the different WNT-isoforms, WNTs that are considered β-catenin-dependent are: WNT-1, WNT-3, WNT-3A, WNT-7A, WNT-8A and WNT-8B, and WNTs signaling through β-catenin-independent pathways are: WNT-2, WNT-4, WNT-5A, WNT-5B, WNT-6, WNT-7B and WNT-11 (Chien et al., 2009; Kikuchi et al., 2007; McNeill and Woodgett, 2010). However, depending on the cellular context and the readout assay used, WNT-isoform specific effects, with regard to pathway outcome, can often be shown to overlap. Thus, classical β-catenin-signaling WNTs can in some situations perform β-catenin-independent signaling and vice versa (Holmen et al., 2002; Kishida et al., 2004; Slusarski et al., 1997a; van Amerongen et al., 2012).

Signaling feed-back loops are important in regulation of WNT communication. Positive and negative modification is achieved not only by combinations of different WNT-isoforms, but also by unrelated extracellular molecules (Cruciat and Niehrs, 2013; Kawano and Kypta, 2003). Some of these are true activators of WNT-signaling, such as Norrin which binds and activates FZD_4 to induce β-catenin stabilization (Huang and Klein, 2004; Xu et al., 2004). R-spondins are a group of proteins which, by acting through LGRs, sensitizes cells to WNT responses (Carmon et al., 2011; de Lau et al., 2012; Glinka et al., 2011). Dickkopfs (DKKs), Wise/Sclrostin and WNT inhibitory factors (WIFs) act to antagonize WNT signaling, often in an autocrine manner, and are shown to be upregulated in response to activation of some WNT pathways (Cruciat and Niehrs, 2013; MacDonald et al., 2009; Niehrs, 2006). Soluble Frizzled-related proteins (SFRPs) are also seen as inhibitors of WNT signaling. This group of extracellular proteins contains a FZD-like cysteine rich domain (CRD) motif which is used to bind and sequester WNTs thus competing with the considered FZD orthosteric binding site (Hsieh et al., 1999a; Jones and Jomary, 2002).

1.3 WNT RECEPTORS

In line with the principal organization of cellular communication WNTs exert their action through binding to cell surface transmembrane receptor proteins. However, WNTs are quite unique in the sense that they are able to activate a number of receptors differing greatly in structure and signaling mechanism (Kikuchi et al., 2007). With the general advances in molecular biology and the development of improved purification protocols of WNTs, the number of identified WNT receptors has increased, and the picture is getting more and more complex and complicated. Today, more than 15 different receptors and co-receptors are known to be engaged in WNT signaling

(Niehrs, 2012). The preponderance of WNT signaling communication occurs via the activation of the Frizzleds and therefore, this family of 7TM spanning proteins is considered being the chief WNT receptors (Schulte, 2010).

1.3.1 Frizzleds

FZDs were first described in 1989 by the investigation of PCP signaling genes in the *D. melanogaster frizzled* mutant fly (Vinson et al., 1989). However, FZDs were not recognized as bona fide WNT receptors until the late 1990s (Bhanot et al., 1996). Today, it is common knowledge in the WNT field, that FZDs are crucial signal transducers in most WNT communication pathways.

1.3.1.1 FZD structure and classification

Owing their seven transmembrane helices spanning (7TM) structure, all the members of the Frizzled family (FZD_{1-10}) as well as the Hedgehog pathway receptor Smoothened (SMO) are classified as G protein coupled receptors (GPCRs) in the Class Frizzled (or Class F) (Fredriksson et al., 2003; Lagerstrom and Schioth, 2008; Schulte, 2010). Class Frizzled receptors share high similarity in amino acid sequence, structure and conserved motifs; including i) the extracellular domain (ECD) with the CRD structure and the ECD linker domain, ii) the 7TM domains (common for all GPCRs), and iii) the intracellular domain (ICD) with a PDZ-ligand motif, KTxxxW (x = any amino acid), which in SMO contains an Ala between the Lys and the Thr residues (Wang et al., 2013). Based on sequence homology, Class F receptors are grouped into four distinct clusters: $FZD_{1, 2, 7}$; $FZD_{3, 6}$, SMO; $FZD_{4, 9, 10}$; $FZD_{5, 8}$ (Schulte, 2010). With the recently solved crystal structure of SMO bound to an antitumor agent (LY2940680) at hand (Wang et al., 2013), the structure of FZDs can be discussed more in detail. In the following subsections, the findings regarding the SMO protein structure will serve as a template for deduction of FZD molecular features.

1.3.1.1.1 The Class Frizzled ECD

The length of the ECD varies among the Class F receptors but all contain a short N-terminal signal sequence necessary for posttranslational trafficking and insertion in the plasma membrane. The signal sequence is followed by a number of Cys residues (10 in FZDs and 9 in SMO) which forms 5 (FZD) and 4 (SMO) disulfide bonds thus stabilizing a specialized α-helical-rich tertiary structure of approximately 120 amino acids, named the CRD (Dann et al., 2001; Schulte, 2010). In the case of SMO, the function of the CRD is unknown, but in FZDs the CRD constitutes the proposed orthosteric site for binding of ligands, i.e. WNTs, Norrin and SFRPs (Xu and Nusse, 1998). High affinity binding of a K_d of 1-10nM exist between WNTs (Carmon and Loose, 2010; Hsieh et al., 1999b; Rulifson et al., 2000) and the FZD-CRD and the recently described mouse FZD_8-CRD/*X. laevis* WNT-8 interaction complex reveals further hints of how WNTs engage in receptor binding and recognition (Janda et al., 2012). It was shown that XWNT-8 forms a pinching grip, created by hydrophobic contacts, which arches around the CRD with the N-terminal “thumb” domain and the C-terminal “index finger” part interacting with sites located on opposite sides of the

CRD (Janda et al., 2012). However, the role of the CRD in receptor conformation modulation and activation remains to be further investigated since WNT signaling still can be employed at FZDs lacking the CRD (Chen et al., 2004; Povelones and Nusse, 2005). The FZD-CRD also contains Asn residues predicted to be N-glycosylated (Wang et al., 2006), a posttranslational modification probably involved in proper signaling, maturation and compartmentalization of FZDs (Schulte, 2010; Yamamoto et al., 2005). The CRD is connected to the ECD linker domain which contains further conserved Cys residues forming disulfide bonds and intricate tertiary structures in contact with the extracellular loop regions (ECLs) (Wang et al., 2013).

1.3.1.1.2 The Class Frizzled 7TM core

A unique feature of class F receptors compared to the well-studied class A GPCRs are the long ECLs. In SMO the three ECLs forms complex structures by Cys-Cys disulfide bonds, hydrophobic forces and non-covalent interactions with the ECD linker domain (Wang et al., 2013). This extracellular ordered structure is shown to be necessary for proper regulation of SMO activity. Mutation of the conserved Cys residues in extracellular regions disrupts the inactive conformation of SMO and yields a hyperactive receptor (Carroll et al., 2012). The ECL2 seems especially important in regulation of receptor activity, since disruption of the Cys in this region renders SMO inactive (Lagerstrom and Schioth, 2008) and heterozygous mutation of the ECL2 Cys in the *D. melanogater fz* gene causes partial loss of function (Povelones et al., 2005). In the extracellular part of SMO the only secondary structure found to be shared with class A receptors is the β-hairpin of ECL2, but still there are discrepancies between SMO and the class A in the function of this structure (Wang et al., 2013). Even though the primary sequences of the ECL1-3 are highly conserved in class F receptors there are differences when it comes to the mature protein structure: e.g. FZD_4, which has a very short ECL3, cannot form a disulfide bond in the loop3 region, and the FZD_3 and $_6$ harbors a predicted N-glycosylation site in the ECL2 (MacDonald and He, 2012; Wang et al., 2013). These differences might contribute to the determination of ligand selectivity (Schulte, 2010). The transmembrane segments of class F are made up of seven α-helices (I-VII) – the hallmark structure of the GPCR superfamily (Pierce et al., 2002). On the other hand, the class F receptors are lacking the class A-conserved motifs which are critical for the interaction with heterotrimeric G proteins. Such are the DRY-motif in helix III and the NPxxY-region in helix VII, both situated at the border to the intracellular surface (Katritch et al., 2013; Rovati et al., 2007; Wess, 1998). The fact that FZDs are lacking these essential motifs provides one of the main arguments against the GPCR-thesis of FZDs. Interestingly, class F receptors contain a high number of tryptophans in the intracellular ends of helices III, IV and VII and these are shown to be involved in receptor activation (Xie et al., 1998). Moreover, in the case of helix VII, the Trp residue aligns with the NPxxY-motif of class A receptors (Wang et al., 2013).

1.3.1.1.3 The Class Frizzled ICD

Given the 7TM spanning structure, the GPCRs contain an intracellular region of 3 loops and a C-terminal tail. These structures provide an interaction platform for

intracellular binding partners and are subjected to various posttranslational modifications, e.g. phosphorylation, nitrosylation, hydroxylation and ubiquitination, determining receptor signaling dynamics and compartmentalization (DeWire et al., 2007; MacDonald and He, 2012; Xie et al., 2009; Yanfeng et al., 2006). The class F ICD tail is variable in length and contains the highly conserved PZD-ligand domain: KTxxxW (KATxxxW in SMO), responsible for binding to the PDZ domain of DVL (Gao and Chen, 2010; Umbhauer et al., 2000; Wallingford and Habas, 2005). Clues obtained from the SMO structure might further implicate a role of the PDZ-ligand domain in G protein coupling. The SMO KATxxxW domain stabilizes the formation and integrity of the short α-helix VIII, a structure positioned perpendicular to helix VII and additionally is found in bona fide GPCRs (Rasmussen et al., 2007; Wang et al., 2013; Wess et al., 2008). The formation and stabilization of helix VIII requires a number of C-terminal Cys residues and is possible in most, but not all FZDs (i.e. cluster 1 $FZD_{1, 2, 7}$) (Schulte, 2010). Moreover, charged amino acids at the beginning and the end of the ICL3 exists in class A and class F receptors alike and these residues are shown to be important in G protein coupling . Interestingly, these regions in ICL3 are further implicated in binding to the C-terminal regions of DVL, thus establishing a situation where DVL- and G protein interaction sites essentially are superimposable (Schulte, 2010; Tauriello et al., 2012). In the extreme C-terminal part FZDs hold an additional PDZ-ligand domain, however not involved in DVL-PDZ interaction. This domain has been shown to interact with class I PDZ-domains of several proteins of currently unknown function, and is experimentally confirmed in all FZDs except $FZD_{6, 9}$ and $_{10}$ (Hering and Sheng, 2002; MacDonald and He, 2012; Schulte, 2010).

1.3.1.2 FZD signal transduction

As mentioned previously, the CRD of FZDs is viewed as the primary ligand interaction site. However, it is a consequence of the fact that no other data regarding FZD-ligand binding is available. Most FZD-WNT binding studies are performed by isolating the CRD of different FZDs and thereafter measuring WNT-CRD binding affinities (Carmon and Loose, 2010; Koval and Katanaev, 2012; Schulte and Bryja, 2007). Current opinions in the field of GPCR signaling suggest that several ligand interactions sites exist at GPCRs, capable of stabilizing different active (and inactive) conformations and thus able to direct signaling into divergent pathways (Katritch et al., 2013; Kenakin, 2011; Kenakin and Christopoulos, 2013). This notion, in combination with the fact that the CRD, in some cases, seems dispensable for WNT/FZD signaling points to the existence of further sites important for ligand binding. Since WNT binding studies are further hampered by the difficulty in obtaining purified WNT proteins, WNT-FZD binding pairs are commonly deduced by investigating intracellular signaling fates. Based on this approach and WNT-CRD binding assays, some specific WNT-FZD interaction pairs have been identified. For example WNT-3A was shown to interact with $FZD_{1, 3, 4, 5}$ and $_{8}$, WNT-5A could mediate signaling via $FZD_{2, 4, 5}$ and $_{7}$, WNT-7A was shown to bind FZD_5 and $_{10}$, and WNT-11 interacted with FZD_7(Carmon and Loose, 2010; Katanaev and Buestorf, 2009; Kikuchi et al., 2011). The capacity of the FZD-CRD to form dimers with itself and other CRD-containing proteins further suggests a role of the CRD in coreceptor binding, FZD homo- and heterodimerization

as well as interaction with SFRPs (Schulte, 2010; Schulte and Bryja, 2007). In conclusion, not much is known about the mechanisms of how FZD signal transduction is activated at the extracellular surface.

FZDs, like other GPCRs, do not harbor any intrinsic enzymatic activity and are thus reliant on intercellular signaling molecules to mediate further cell communication (Dale, 1998). The ICL1-3 and the ICD are the sites of interaction of FZDs with intracellular regulatory factors and the ICD region is also the least conserved parts among FZDs (Kikuchi et al., 2007). As mentioned earlier, the FZD molecular structure provides profound evidence for the interactions with heterotrimeric G proteins and the central WNT-signal mediator DVL at overlapping regions in the ICL3 and helix VIII (Koval et al., 2011; Tauriello et al., 2012; Wang et al., 2013). Using loss- and gain of function experiments G proteins can readily be linked to developmental processes (Malbon, 2005). However, the direct link between FZDs and G proteins has proven difficult to establish. Adrenergic-FZD chimera receptors provided evidence that the intracellular region of FZD behaves similar to conventional GPCRs with regard to ligand affinity shift (Ahumada et al., 2002; Malbon, 2004). A recent study using WNT8-FZD fusion proteins expressed in yeast showed that FZDs act through G proteins to activate yeast MAPK pathway (Nichols et al., 2013), and *in vitro* direct G protein activation of reconstituted bacterially expressed $FZD_{1, 6}$ and $_7$ was shown after stimulation with WNT-3A, -5A, -5B and -7A (Katanaev and Buestorf, 2009). WNT stimulation causes hyperphosphorylation, redistribution and recruitment of cytosolic DVL to FZDs at the plasma membrane, an event which can be studied by immuno-based methods (Schulte and Bryja, 2007). Interestingly, the overexpression of FZDs yields a similar result which suggests that DVL can interact with FZDs in the absence of WNT activation (Frojmark et al., 2011; Takada et al., 2005). The KTxxxW domain of FZDs is the conventional site of interaction with DVL. However, recent results from combinatorial peptide scanning of FZD_5 and DVL1 interaction sites revealed two regions (motif I and II) in the ICL3, conserved in all FZDs except FZD_3 and FZD_6, which provides strong binding to DVL1 C-terminal parts. The interaction of this region of DVL1 with FZD_5 motif II in ICL3 was found crucial for activation of downstream WNT/β-catenin signaling (Tauriello et al., 2012). Moreover, DVL have been shown to recruit aPKC and CK1/2 to FZDs, a mechanism possibly leading to phosphorylation of conserved Ser/Thr and Tyr residues in the FZD ICLs and ICD (Malbon and Wang, 2006).

Current models of GPCR signaling describe the 7TM receptor protein as a dynamic entity, constantly switching between different states of conformational and functional arrangements (Canals et al., 2011). Apart from the obvious states of fully active and inactive conformations, the set of possible molecular arrangements includes a wide range of intermediate states with different degrees of activity (Flordellis, 2012; Venkatakrishnan et al., 2013). The stabilization, and consequently the equilibrium of these states are affected, not only by extracellular ligands, but also by ions, lipids, cholesterols, intracellular binding partners and molecular modifications of the GPCR, e.g. phosphorylation (Katritch et al., 2013). The preassembly of a GPCR-effector

molecule complex can establish a specific conformation favorable for the interaction with a specific extracellular ligand, which upon binding will stabilize a new conformational state of the receptor (Audet and Bouvier, 2012). This behavior of GPCRs is made possible by allosteric binding sites at the receptor proteins, both at intracellular and extracellular regions, and is a phenomenon described in the ternary complex model of GPCR signaling (De Lean et al., 1980). Moreover, G protein-mediated allostery provided the first evidence of G protein signaling at 7TM receptors (Lefkowitz, 2007). Using purified receptors and G proteins it was found that when depriving the system of GTP the GDP-bound G protein interacted with the GPCR and established a conformational state of high ligand affinity. The addition of GTP caused dissociation of the G protein from the 7TM protein and shifted the affinity for the ligand to a lower state (De Lean et al., 1980). In this way, allosteric modulation of FZDs by the interaction with G proteins and other intracellular regulatory components could modify selectivity in WNT binding.

1.3.1.3 FZD molecular dynamics

Protein dimerization is a frequently occurring phenomenon in nature, and has been suggested to facilitate proper maturation, localization, enzymatic activity and signal transduction of proteins (Lohse, 2010; Terrillon and Bouvier, 2004). Today there is a growing body of evidence arguing for the concepts of homo-and heterodimerization of 7TM receptors at least among the Class A receptors, the Class B secretin receptors and the Class C metabotropic glutamate receptors (Milligan, 2009; Milligan et al., 2006). In the case of FZDs it was found that FZD dimerization via the FZD-CRD was sufficient to activate WNT/β-catenin signaling (Carron et al., 2003) and the crystal structure of FZD_8-CRD demonstrated a tendency to form homodimers (Dann et al., 2001). Interestingly, FZD-FZD interaction of human FZD_4 was shown to occur via regions in the 7TM helices and thus, did not require the CRD for dimerization (Kaykas et al., 2004). However, disulfide bonds in the ECD and ECLs were shown to be important for dimerization since the use of reducing agents prevented FZD-FZD complex formation (Carron et al., 2003; Kaykas et al., 2004). The process of FZD oligomerization has in some cases also been found indispensable for proper signal transduction, receptor maturation and cell surface localization (Carron et al., 2003). Further, the crystallization of SMO revealed a dimer complex in the structure supporting the notion of dimerization among Class F receptors (Wang et al., 2013). However, the dimerization of receptors in a purified crystal structure is a common theme and can sometimes be regarded as an artificial event created by the crystallization procedure and not necessarily connected to any physiological relevance (Congreve and Marshall, 2010). The process of receptor homo- and heterodimerization can affect the ability to bind and recognize ligands and consequently to direct signaling into different pathways (Milligan et al., 2006). For example, WNT/β-catenin dependent signaling in *X. laevis* was found mediated via FZD_3 dimers (Carron et al., 2003). Forced dimerization of FZD_7 resulted in the WNT/β-catenin pathway while monomers of FZD_7 transduced WNT/β-catenin independent signaling (Carron et al., 2003). The concept of dimerization among Class F receptors is further complicated by the possibility of heteromeric complexes. Between all 10 FZD isoforms and SMO, the plausible

combinations are many. Moreover, the possibility of heterodimerization between Class F 7TM proteins and receptors of the other GPCR classes cannot be excluded (Koval et al., 2011; Parker et al., 2011; Schulte, 2010).

Internalization of GPCRs is a common mechanism to shut down and desensitize signaling and occurs both in a constitutive manner and in direct response to agonist stimulation (Freedman and Lefkowitz, 1996). Removing the receptor from the site of action and making them inaccessible to ligand binding will effectively inhibit further signal transduction. Once internalized receptors can either be recycled back to the plasma membrane or be subjected to lysosomal degradation (Ferguson, 2001). Internalization of 7TM receptors occurs via either caveolae- or clathrin-mediated processes (Liu and Shapiro, 2003). The molecular composition of the compartment in which the receptor resides determines the choice of internalization pathway. Caveolae-internalization and subsequent caveosome formation happens in so called lipid rafts, regions rich in cholesterol, sphingolipids and the transmembrane caveolin protein (Nichols, 2003). In contrast to caveolae-mediated internalization, which remains poorly understood, the clathrin-mediated process is well-investigated and results in formation of early endosomes. Plasma membrane invaginations coated with intracellular clathrin assemblies (clathrin-coated pits) are pinched off by the aid of the GTPase activity of dynamin to generate an endosomal GPCR-containing structure (McMahon and Boucrot, 2011). Several FZDs ($FZD_{1, 2, 4, 5}$ and $_{7}$) are shown to be subjected to WNT-5A or -11-induced clathrin-mediated endocytosis (Chen et al., 2009; Chen et al., 2003; Kurayoshi et al., 2007; Yu et al., 2007) and stimulation by WNT-3A was found to induce caveolae-mediated internalization of a FZD_5/LRP6 receptor complex (Yamamoto et al., 2006). Current opinions also suggest a positive role for receptor internalization in the processes of cell communication. Establishment of WNT gradients in *D. melanogaster* wing discs requires endocytosis of WNTs with related receptors and signaling components (Marois et al., 2006), and blocking caveolae-mediated endosome formation inhibits WNT-3A-activation of β-catenin dependent signaling (Sakane et al., 2010; Yamamoto et al., 2006). Since inhibition of the clathrin-mediated internalization process mainly is connected to impaired WNT/PCP signaling, the current understanding dictates a general division of WNT/β-catenin dependent signaling being associated with caveolae-mediated internalization and β-catenin independent pathways connected to clathrin-mediated endocytosis (Kikuchi et al., 2011, 2012).

1.3.2 Additional WNT receptors

As mentioned earlier, apart from FZDs, WNTs are shown to engage a variety of structurally unrelated receptors and co-receptors and some will briefly be presented here, i.e. Low-density lipoprotein receptor-related protein 5/6 (LRP5/6), Receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/2), Related to receptor tyrosine kinase (RYK), protein tyrosine kinase (PTK7), Tetraspanin (TSPAN) and Vang-like (Vangl). In many cases both β-catenin-dependent and -independent WNT signaling requires these receptors and co-receptors to work in concert (primarily with FZDs) and the different possible combinations are indeed numerous (Kikuchi et al., 2011). Some

combinations, however, favor some WNT pathways more than others and such is e.g. the case of LRP5/6.

1.3.2.1 LRP5/6

These type I single transmembrane spanning LDL receptor family proteins are important components in the WNT-FZD-mediated stabilization of β-catenin. The LRP5/6 have a large ECD which contains multiple binding sites for WNTs; e.g. WNT-3A and WNT-9B are shown to interact with different domains and in tandem (Bourhis et al., 2010). The binding of WNTs to LRP5/6 establishes a WNT-FZD-LRP signalosome responsible for mediating β-catenin-dependent signaling. The ECD of LRP5/6 also functions in an antagonistic manner to negatively regulate WNT signaling by binding DKKs. Within a few minutes after WNT activation of FZD, the short intracellular domain of LRP5/6 is phosphorylated at serines and threonines surrounding five PPSPXS repeats characteristic for these receptors (Wolf et al., 2008). The phosphorylation of LRP5/6 provides a binding platform for Axin (the scaffold protein of the destruction complex), which upon LRP5/6-mediated recruitment to the plasmamembrane causes the destruction complex to dissociate and the degradation process of β-catenin ceases (Angers and Moon, 2009).

1.3.2.2 ROR1/2, RYK and PTK7

The ROR1/2, RYK and PTK7 proteins belong to the receptor tyrosine kinase family but are atypical in the sense their enzymatic activity either is inactive or is dispensable for signal transduction (Green et al., 2008). These receptors are implicated to function in both β-catenin-dependent and –independent branches, but appears to be physiologically relevant mainly in CE cellular movements during embryonic development (Grumolato et al., 2010). ROR1/2 harbors an extracellular CRD capable of binding WNTs and can convey signaling independently of FZDs. Especially WNT-5A is shown play a prominent role in signal transduction via ROR1/2 resulting in migration and cell polarization (Minami et al., 2010). Whilst WNT-5A/β-catenin-independent ROR signaling seems to require the catalytic Tyr kinase domain (Mikels et al., 2009), β-catenin signaling by WNT-3A do not, but instead employs ROR2 as a co-receptor to FZD (Li et al., 2008). WNT-5A activation of ROR2 can also inhibit and counteract WNT-3A-induced β-catenin stabilization (Mikels et al., 2009; Mikels and Nusse, 2006).

The intracellular Tyr kinase domain of the RYK protein is catalytically inactive and thus, this receptor functions mainly as a co-receptor. In collaboration with FZD_7 and WNT-11 it acts to promote CE movements (Kim et al., 2008) and when combined with FZD_8 and WNT-1 it activates β-catenin signaling (Lu et al., 2004a). Physiologically, RYK is crucial for axon guidance and neurite outgrowth (Lu et al., 2004a). Like RYK, PTK7 has an inactive Tyr kinase domain and is viewed purely as a co-receptor (Lu et al., 2004b). Since PTK7 deletion mutants exhibit WNT-5A/PCP pathway-related defects, WNT-5A is so far thought to activate PTK7 signaling (Shnitsar and Borchers, 2008) but currently not much is known about the signaling features of these proteins.

1.3.2.3 Tetraspanins and Vangl1/2

The tetraspanin protein TSPAN12 is shown to participate in WNT signaling as a coreceptor by facilitating FZD_4 oligomerization during Norrin-induced stablilzation of β-catenin. In this case, tetraspanins are not involved in WNT/β-catenin signaling (Junge et al., 2009). Like the tetraspanins, the Vangl1/2 proteins are four-pass transmembrane proteins. Vangl proteins are implicated as key components of the PCP pathway and CE movements during neural tube closure (Lei et al., 2010; Wu and Mlodzik, 2009). Signaling mediated by WNT-5A was shown to activate CE pathway via either a Vangl2-ROR2 complex (Gao et al., 2011) or a FZD_3-Vangl2 composite (Shafer et al., 2011).

1.4 INTRACELLULAR EFFECTORS OF THE WNT SIGNALING NETWORK

The next step in the scheme of cellular signal transduction is the activation of intracellular effector proteins and in the case of WNT signaling these components include both specialized WNT communication molecules, as well as general and common cell signaling factors. Utilization of common downstream signaling molecules enables the WNT communication network to crosstalk with other cellular signaling pathways (Dalton, 2013; Jin and Esteva, 2008; Kikuchi et al., 2011). WNT signaling has e.g. been linked to autophagy via convergence with mTOR signaling, to modulate immune responses, and directly interact with synaptic signaling, e.g. cholinergic- and glutamatergic neurotransmission (Halleskog et al., 2012; Halleskog et al., 2011; Inestrosa and Arenas, 2010; Semenov et al., 2007). The FZD associated signaling effectors, i.e. DVL and heterotrimeric G proteins, provides a good example of the use of specialized versus general cell signaling components in the WNT communication system.

1.4.1 Dishevelled – the specialized effector and conventional WNT/FZD-signaling player

Dishevelled was first identified in *D. melanogaster* mutants displaying disruptions in hair and bristle polarity (Fahmy and Fahmy, 1959), and was later linked directly to WNT/β-catenin signaling and WNT/PCP pathway by its key functions in coordinating *D. melanogaster* embryo segment polarity and development of wing, legs and abdomen (Wallingford and Habas, 2005). Since then, DVL is found to function as a scaffold and molecular switch between β-catenin-dependent and independent pathways and is appreciated as the central mediator of most, if not all, WNT-signaling events (Axelrod et al., 1998; Boutros and Mlodzik, 1999; Malbon and Wang, 2006; Wharton, 2003). The scaffold function of DVL allows for the binding and coordination of several positive- and negative WNT-signaling components, such as Axin, WNT-signaling connected kinases and ubiquitin ligases, and Gβγ-subunits (Gao and Chen, 2010). The mammalian genome encodes three isoforms of DVL proteins, DVL1-3, with functional redundancy (Lee et al., 2008). However, the native abundance of the different DVL

isoforms differs: in HEK293 and P19 cell lines, DVL2 constitutes >80% of the total DVL pool and DVL1 and -3, combined, makes up the remaining ~20% (Lee et al., 2008; Ma et al., 2010). The ~90kDa sized DVL protein structure harbors three distinct and functionally conserved domains: the N-terminal DIX (Dishevelled/axin), the mid-region PDZ (Post-synaptic density-95/Discs-large and Zonula occludens-1), and the C-terminal DEP (Dishevelled/Egl-10/Pleckstrin) domain (Wallingford and Habas, 2005). Two additionally conserved regions of DVL proteins constitute the basic and Ser/Thr-rich region and the Pro-rich region which could provide sites for phosphorylation or protein binding. Indeed, the Pro-rich region contains a SH3 protein binding motif (Gao and Chen, 2010). Divergence in sequence homology between the different DVL isoforms is most profound in C-terminal parts of DVLs (Ma et al., 2010).

1.4.1.1 The DIX domain

The DVL-DIX domain has the capacity to mediate polymerization of DVL molecules and hetero-oligomerization with Axin via interaction with the Axin-DIX domain, albeit the assistance of additional sequences flanking the DVL-DIX is needed (Gao and Chen, 2010; Wharton, 2003). DIX-mediated multimerization was found crucial for WNT/β-catenin signaling and mutations in the DIX domain have severe effects on WNT-signaling (Schwarz-Romond et al., 2007a). However, overexpression of isolated DIX domains, as well as DEP, was found sufficient for blocking WNT-3A/β-catenin signaling (Pan et al., 2004). Conversely, deletion of DVL3-DIX was shown to inhibit WNT-5A/$Ca^{2+/}$NFAT pathway, an interesting result with regard to the fact that the majority of current data links the DIX domain to WNT/β-catenin pathway (Ma et al., 2010). On the other hand, DIX domain deletion mutation in *X. laevis* resulted in elevated Ca^{2+} signaling with active CamKII and membrane-recruited PKC (Sheldahl et al., 2003). Additionally, DVL is shown interact with actin stress fibers and vesicles through the DIX domain (Capelluto et al., 2002).

1.4.1.2 The PDZ domain

The PDZ domain of DVL serves as the docking site for the FZD KTxxxW PDZ-ligand domain and for the interaction with the C-terminal tail of RYK and PTK7 (Wong et al., 2003). Beyond its role in receptor binding the PDZ domain mediates the interaction with a vast array of intracellular signaling components (Wallingford &Habas, 2005)(Gao and Chen, 2010) and is also necessary for the binding of DVL to Axin. Owing its diversity in protein interaction, the PDZ domain has been proposed to serve as the conductor of the WNT-pathway switching function of DVL (Wallingford and Habas, 2005). DVL further binds directly to CK1ε/δ/2 via the PDZ and phosphorylation sites within the PDZ and DEP domains are thought to be recognized by CK1ε, CK2 and PAR-1b (Bernatik et al., 2011; Gao and Chen, 2010)□(Bryja et al., 2007b; Elbert et al., 2006). The PDZ was also found to be required for microtubule stabilization by inhibition of GSK3β-mediated phosphorylation of microtubule-associated proteins (Krylova et al., 2000). DVL has also been shown to bind directly to the parathyroid hormone type 1 receptor (PTH_1R) to regulate bone growth in a non-WNT-mediated β-catenin-dependent signaling pathway. The DVL-PTH1R interaction

occurred at a C-terminal tail sequence, KSxxxW, which highly resembles the FZD PDZ ligand domain (Romero et al., 2010). These results suggest that DVL might not be confined only to WNT/FZD signaling but could be employed as a more general coordinator of cell communication pathways.

1.4.1.3 The DEP domain

Recent data provided strong evidence for the essential functions of the DEP domain and the C-terminal region in interaction with motifs I and II in the FZD_5-ICL3, thus explaining previous observations of the KTxxxW-PDZ domain binding being dispensable for FZD-DVL association (Tauriello et al., 2012). A polybasic stretch of amino acids in the DEP domain further supports the anchoring of DVL to the plasma membrane by mediating interactions with negatively charged phospholipids at the inner leaflet (Wong et al., 2000). The interaction is pH-dependent and is especially important for proper WNT/PCP pathway (Simons et al., 2009). DVL has been shown to coordinate microtubule assembly during axon differentiation via DEP domain-binding to phosphorylated aPKC thus regulating the stability and activity of this kinase (Zhang et al., 2007). The DEP domain could further regulate WNT signaling by binding and inhibiting the catalytic subunit of the phosphatase PP2A, and the outcome of this interaction can have both negative and positive effects on WNT signaling (Yokoyama and Malbon, 2007). WNT-5A-induced and clathrin-mediated endocytosis of FZD_4 was shown to require DVL2-DEP interaction with a subunit of the clathrin adaptor AP-2 (Yu et al., 2007; Yu et al., 2010). The DEP domain is also responsible for the interaction of DVL with the Gβγ subunit complex, an association resulting in regulation of DVL levels and membrane localization (Egger-Adam and Katanaev, 2010).

1.4.1.4 DVL dynamics and regulation

DVL hyper-phosphorylation is a hallmark of both WNT/ β-catenin-dependent and – independent pathways and can be investigated by an electrophoretic mobility shift of DVL during migration in SDS-PAGE. However, the mechanism and significance behind this event is not completely understood and current data also suggests that polyubiqitination of DVL plays a part in regulation of DVL activity and consequent downstream WNT signaling (Angers et al., 2006; Ding et al., 2013; Gao and Chen, 2010; Sun et al., 2001; Takada et al., 2005; Tauriello et al., 2010).

DVL is dynamic with regard to its subcellular localization and different pools of DVL can be identified: cytoplasmic DVL often exists as dynamic multimers or aggregates, referred to as punctae (Gao and Chen, 2010; Schwarz-Romond et al., 2005), which upon WNT-5A-dependent and CK1ε-mediated phosphorylation are dispersed giving an even distribution of DVL molecules within the cytoplasm (Bryja et al., 2007d). On the other hand, WNT-3A-stimulated phosphorylation of DVL3 stabilized punctate distribution and formation of DVL-Axin-containing supermolecular complexes (Yokoyama et al., 2012). Furthermore, DVL punctae have in some cases also been suggested to represent vesicle-bound molecules (Capelluto et al., 2002; Park et al., 2008). Yet another pool of DVL (often in its phosphorylated state) is found at the

plasma membrane where it interacts with FZDs and other membrane-bound proteins, e.g. the Gβγ complex, to mediate further WNT-signal transduction (Axelrod et al., 1998; Egger-Adam and Katanaev, 2010; Simons et al., 2009). DVL contains nuclear export- and nuclear localization sequences and thus been suggested to translocate into the nucleus (Itoh et al., 2005; Weitzman, 2005). Once there, DVL can interact with nuclear factors to potentiate transcription of WNT/β-catenin target genes (Gan et al., 2008). However, the notion and significance of a nuclear-confined pool of DVL needs to be further evaluated.

Negative feed-back mechanisms are highly important to regulate, terminate and fine-tune WNT-signaling communication. DVL is the central hub of WNT-signaling and therefore, modulation of DVL protein stability and turnover is a powerful means of signaling adjustment (Gao and Chen, 2010; Malbon and Wang, 2006). Several proteins have been implicated in the regulation of cellular DVL levels, e.g. Dapper1/Dact1, Inversin, NEDL1, Prickle-1, KLHL12- and pVHL-E3 (Jung et al., 2009)ubiquitin ligases and Gβγ subunits (Angers et al., 2006; Cadigan and Nusse, 1997; Carreira-Barbosa et al., 2003; Chan et al., 2006; Jung et al., 2009; Miyazaki et al., 2004; Zhang et al., 2006). DVL has been shown to be degraded in the lysosomal pathway in response to a starvation- and/or rapamycin-induced autophagy-related mechanism in HEK293 (Gao et al., 2010). Additionally, hyperosmolaric sucrose-mediated inhibition of endocytosis resulted in almost complete depletion of DVL, perhaps a mechanism to compensate for non-functional internalization and desensitization of WNT cell surface components (Bryja et al., 2007a).

1.4.2 Heterotrimeric G proteins – the general effector but unconventional WNT/FZD-signaling player

The immediate downstream effectors of GPCRs are the heterotrimeric G proteins. Acting as intermediate signaling units, G proteins are essential mediators of most GPCR-evoked physiological responses, e.g. smell, taste, vision, immunity, pain perception, neurotransmission and cardiac regulation (Ahmed and Angers, 2013; Bockaert et al., 1987; Pierce et al., 2002). Initially, G proteins were not considered to participate in developmental signaling pathways, but since it later was found that deficiencies in G protein genes can cause embryonic lethality in mice and developmental diseases in humans, the important role of G proteins in development is nowadays validated (Angers and Moon, 2009; Malbon, 2005, 2011). G protein signaling activated by WNT/FZD communication is summarized in figure 1.

The heterotrimeric G protein complex consists of three subunits: α, β and γ (Spiegel, 1987; Stryer and Bourne, 1986). Isoprenylation of α- and γ-subunits anchors the G protein complex to the intracellular plasma membrane surface and keeps the trimeric complex in close proximity to receptors and other signaling components (Wedegaertner et al., 1995). The name G protein refers to the GTPase enzymatic property of the a-subunit which allows binding and conversion of GTP to GDP. General classification of heterotrimeric G protein families is based on the activity profile of the α-subunit

(Gilman, 1987). However, it is now well known that both the β- and γ-subunits also are signaling transducers in their own right (Clapham and Neer, 1997; Dupre et al., 2009; Krapivinsky et al., 1995). The human genome encodes 20 α-subunit proteins divided into four different families: $G_{s/sXL/gust/olf}$ (G_s for short), $G_{q/11/14/15/16}$ ($G_{q/11}$), $G_{i1\text{-}3/oA\text{-}B/t1\text{-}2/z}$ ($G_{i/o}$), and $G_{12/13}$. Further, there are 5 and 12 genes encoding β- and γ-subunit isoforms respectively. The signaling process of heterotrimeric G proteins can be described as a binary ON/OFF switch, established by the exchange of GDP to GTP at the a-subunit (ON signal) and the a-GTPase enzymatic breakdown of GTP to GDP (OFF signal) (Oldham and Hamm, 2007, 2008). The ON signal changes the conformation of the a-subunit leading to the dissociation of the trimeric complex and releases the β– and γ-subunits (Oldham and Hamm, 2007, 2008). β and γ remain in a dimer complex which moves on to activate GIRKs and Ca^{2+} channels, to stimulate PI_3K and PLCβ activity and to recruit GRKs2 and 3 (Morris and Malbon, 1999). Signaling relay of a-subunits involves the stimulation of adenylyl cyclases and accumulation of cAMP - G_s family, stimulation of PLCβ and breakdown of PIP_2 to IP_3 and DAG - $G_{q/11}$ family, inhibition of adenylyl cyclases and modulation of Rap1 activity - $G_{i/o}$ family, activation of PDEs – $G_{t1\text{-}2}$ and G_{gust}, and stimulation of Rho-GEFs – $G_{12/13}$ (Morris and Malbon, 1999; Stryer and Bourne, 1986). The activities of the ON and OFF signals are modulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively (Siderovski and Willard, 2005). In this sense, GPCRs can be viewed as a GEF, facilitating the exchange of GDP to GTP and activating the G protein signal, while factors with GAP activity (regulators of G protein signaling (RGS) proteins) stimulates the GTPase activity at the a-subunit thus speeding up the termination of the G protein signal (Siderovski and Willard, 2005).

1.4.2.1 $G\alpha_{i/o}$ and WNT signaling

As mentioned above, G proteins have essential functions in the process of organism development and thus are found to be mediators of WNT/β-catenin-dependent signaling and WNT/PCP/CE/Ca^{2+} pathways (Bikkavilli et al., 2008; Malbon, 2005; Malbon et al., 2001; Schulte, 2010). However, the mechanism linking WNTs to G proteins have been debated despite the fact that FZDs, being classified as GPCRs, logically could replace the missing link. Studies in *X. laevis* and *Danio rerio* using pertussis toxin (PTX, from the bacteria *Bordetella pertussis*) which selectively inhibits proteins of the $G_{i/o}$ family by cysteine ADP-ribosylation of α-subunits, showed that WNT/FZD signaling could evoke G protein complex dissociation, PLC activation and release of intracellular Ca^{2+} via βγ-subunits (Sheldahl et al., 1999; Slusarski et al., 1997a). Additional PTX-based studies have provided further evidence of WNT/FZD-induced $G_{i/o}$ protein-dependent signaling, via e.g. WNT-3A/FZD_1 (Liu et al., 2001). Interestingly, WNT-5A/FZD_2 was found to activate $G\alpha_{t2}$ proteins and downstream PDEs leading to decline in cGMP levels and Ca^{2+} signaling (Ahumada et al., 2002). Recently, WNT-5A-induced Ca^{2+} signaling and decreased cAMP levels were shown downstream of activated $G_{i/o}$ proteins in primary microglia (Halleskog et al., 2012). Further, γ-[^{35}S]-GTP-assay, performed at native protein levels, also revealed direct activation of PTX-sensitive $G_{i/o}$ by WNT-5A in the microglia primary culture

(Halleskog et al., 2012). Using co-Immunoprecipitation, $G_{i/o}$ protein dissociation from FZD was found to be dependent on WNT-3A stimulation and also involved detachment of DVL from FZD (Liu et al., 2005). WNT-3A was also found to induce activation of PTX-sensitive $G_{i/o}$ in brain tissue-derived plasma membrane fractions (Koval and Katanaev, 2011). In *D. melanogaster* G_o is recognized as a crucial component of WNT/β-catenin and WNT/PCP signaling. It was found that WNT/FZD activation of $G\alpha_o$-GTP recruited Axin from the destruction complex and that by interacted with DVL thereby co-operatively inhibiting the breakdown of β-catenin (Egger-Adam and Katanaev, 2010). Moreover, in experiments using *Escherichia coli*-expressed and reconstituted human $FZD_{1, 6}$ or $_7$, WNT-3A, -5A, -5B and -7A were all shown to evoke GDP to GTP exchange at $G\alpha_o$ proteins, albeit at different efficacies depending on the receptor subtype (Katanaev and Buestorf, 2009).

1.4.2.2 $G\alpha_s$ and WNT signaling

The role of proteins of the G_s family in WNT signaling is not as extensively studied as $G_{i/o}$. However, since overexpression of $G\alpha_s$ can promote the formation of primitive endoderm in mouse totipotent F9 teratocarcinoma cells (Gao et al., 1995) and disturbed $G\alpha_s$ gene expression is embryonic lethal, the role of G_s in development is established (Yu et al., 1998). Moreover, WNT-induced $G\alpha_s$ activation was recently shown to inhibit migratory properties of breast cancer cells. Activation of FZD_3 by WNT-5A was able to evoke adenylyl cyclase-mediated production of cAMP leading to PKA-dependent inhibition of migration (Hansen et al., 2009). The WNT-induced $G\alpha_s$-mediated cAMP/PKA pathway was also shown to activate myogenic gene expression (Chen et al., 2005) and a genetic interaction between $G\alpha_s$ and *D. melanogaster* FZD_1 was recently established by analyzing R3/R4 photoreceptor differentiation (Nichols et al., 2013).

1.4.2.3 $G\alpha_{q/11}$ and WNT signaling

Activation of $G_{q/11}$ typically leads to the activation of PLC and downstream intracellular Ca^{2+} mobilization (Morris and Malbon, 1999). Given the canonical outcome of G_q signaling, it could be expected to mainly be associated with the WNT/Ca^{2+} pathway. However, data demonstrate a clear link between $G_{q/11}$ and β-catenin pathway (Gao and Wang, 2007; Liu et al., 2001; Liu et al., 2005). It was e.g. shown that RNAi knock-down of $G\alpha_q$ effectively disrupts WNT-3A-mediated and FZD_1-dependent stabilization of β-catenin in F9 cells (Liu et al., 2001). Moreover, the role of $G_{q/11}$ in development is validated since $G_{q/11}$ double knockout mice results in embryonic lethal phenotype (Offermanns et al., 1998) and G_q is involved in the process of axon guidance in *D. melanogaster* (Ratnaparkhi et al., 2002). Also, WNT-3A-mediated regulation of bone formation was shown to be transduced via a $G\alpha_{q/11}$-PKC pathway (Tu et al., 2007).

1.4.2.4 $G\alpha_{12/13}$ and WNT signaling

The $G_{12/13}$ proteins are regulators of Rho-GTPase GEFs thereby promoting Rho-mediated cell fates, such as activation of MAPK signaling cascades and cytoskeletal rearrangements (Malbon, 2005). In *C. elegans* G_{12} mediates embryonic development and axon guidance (Yau et al., 2003) and disrupted gene expression of $G\alpha_{13}$ causes embryonic lethality in mice (Offermanns et al., 1997). However, besides the importance of $G\alpha_{12/13}$ proteins in developmental processes not much is known about the connection between this G protein family and WNT/FZD on the molecular level.

1.4.2.5 $G\beta\gamma$ and WNT signaling

The study of specific roles of Gβγ-isoforms are difficult to disipher due to high functional rendundancy within the groups of β- and γ-subunits (Malbon, 2005). In development the Gβγ complex has mainly been associated with centrosome function and spindle organisation and thus are imortant mitotic factors (Malbon, 2005; Vanderbeld and Kelly, 2000). In *X. laevis* mesodermal CE movements dependent on WNT-11/FZD_7 activation of Cdc42, Gβγ-subunits were shown to play a key regulatory role (Penzo-Mendez et al., 2003). In some WNT-coordinated cell signaling events, and on a molecular level, Gβγ is also shown to interact with and regulate DVL funcion and loclization (Angers et al., 2006; Egger-Adam and Katanaev, 2010; Jung et al., 2009). Free Gβγ-subunits have further been shown to recruit GSK-3β to the plasma membrane to coordiante subsequent LRP6 phosphorylation by GSK-3β (Jernigan et al., 2010). Recently, Halleskog et al showed that a WNT-5A-induced and PLC-mediated Ca^{2+}-regulated ERK-1/2 phosphorylation pathway in primary mouse microglia was blocked by the use of the βγ-specific inhibitor M119. Interestingly the study also showed that this signaling pathway was PS-DVL-independent since the CK1 inhibitor D4476 did not affect the activation of ERK1/2 (Halleskog et al., 2012). Moreover, it has been postulated that the impact of Gβγ in WNT/FZD signaling might be more profound than perviously thought and that FZDs perhaps employs a Gβγ-signaling mechanism similar to that of yeast pheromone receptors (Angers and Moon, 2009).

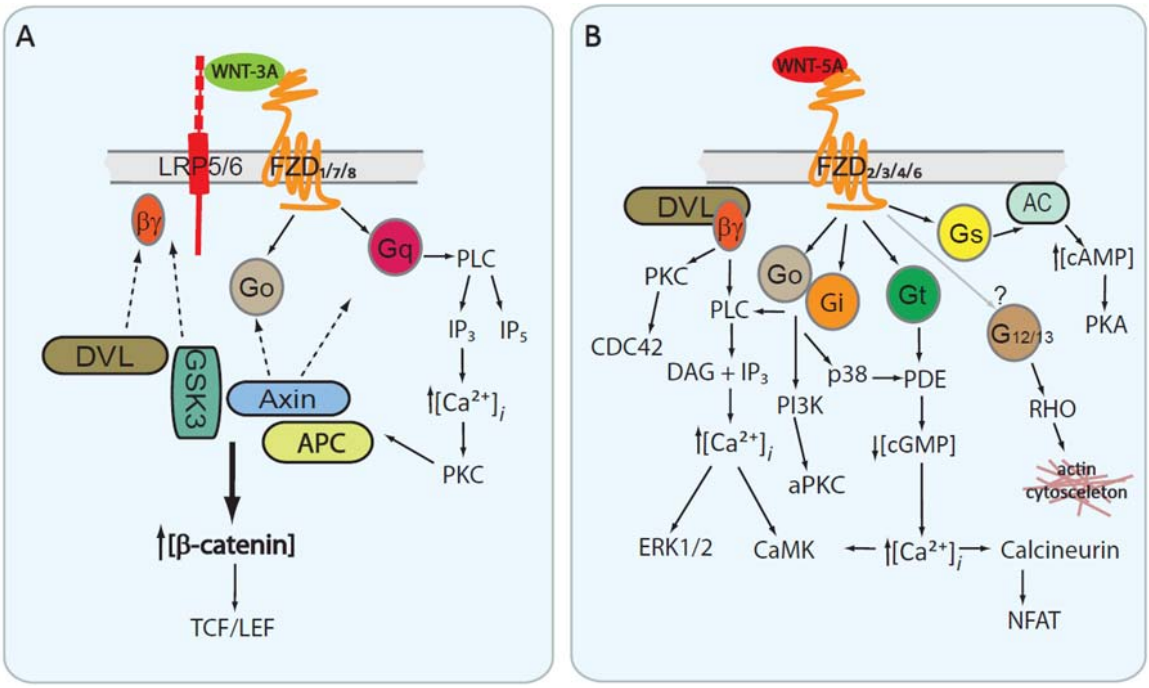

Figure 1. Summary of WNT/FZD signaling to heterotrimeric G proteins evoking either β-catenin-dependent- (**A**) or β-catenin-independent pathways (**B**).

1.4.2.6 Comments on G protein – FZD interaction

Direct contact between heterotrimeric G proteins and their designated 7TMR is required in order to achieve signal transduction. Analysis of mammalian phototransduction at rhodopsin receptors provided evidence of a random collision model of receptor-G protein interaction where freely diffusing G proteins frequently and transiently make contact with receptors (Arshavsky et al., 2002). Currently, however, a growing body of data supports a second model in which the 7TMR is precoupled or preassembled with the G protein in relatively stable but still dynamic complexes (Neubig, 1994; Rebois and Hebert, 2003). The second model is attractive since 7TMRs have been found confined to microdomains of clustered receptors, co-receptors and intracellular effectors. Moreover, such a confinement should logically decrease the rate of diffusion and increase the limitation of successful collision events, thus resulting in slower signal transduction (Choquet and Triller, 2003). However, experimental data show that the speed of GPCR signaling is unaffected even if the receptor is immobilized, indicating that transduction, in some cases, not is diffusion-limited (Lober et al., 2006). The way in which FZDs contact G proteins remains to be evaluated and might differ depending on the G protein species and the FZD isoform. The work of this thesis, however, provides evidence for the precoupling model of FZD-G protein interaction.

Through mining of bioinformatics data, computational predictions of FZD-G protein interactions can be conducted. Using artificial neuronal network algorithm Koval and colleagues reported that all FZD isoforms were predicted to couple to $G_{i/o}$ proteins and

to some extent also to G_q in the case of $FZD_{4, 5}$ and $_6$. In conclusion, the *in silico*, as well as the biochemical data suggests that, out of the four different families of Gα proteins, the $G_{i/o}$ family have the most profound role in the WNT/FZD cell communication system (Halleskog et al., 2012; Kilander et al., 2011a; Kilander et al., 2011b; Koval and Katanaev, 2011; Koval et al., 2011; Wang et al., 2006).

1.4.3 Additional WNT/FZD-signaling molecules – Axin and β-arrestin

Even though many of the WNT signaling mediator molecules and their respective position in the chain of WNT communication events are known, the processes of activation, transduction and direct interactions between the components remain, in many cases, obscure (Angers and Moon, 2009; van Amerongen, 2012). The composition and regulation of the β-catenin destruction complex and the signaling events of the β-catenin dependent pathway are among the more well-studied features of WNT-communication.

1.4.3.1 Axin

As mentioned earlier, the destruction complex is the molecular machinery responsible for inhibiting accumulation of β-catenin in the absence of WNTs. Working as the scaffold of the destruction complex, Axin is a key molecule in WNT/β-catenin signaling and exert negative regulation by binding directly to GSK3β, CK1α, APC, β-catenin and additional factors of the destruction complex (Luo and Lin, 2004). Activation of FZDs by β-catenin signaling WNTs leads to relocation of Axin mediated by direct interaction with phosphorylated LRP5/6 intracellular domains (Davidson et al., 2005; Mao et al., 2001; Zeng et al., 2005), by the interaction with the DVL-DIX (via the Axin-DIX) domain (Cliffe et al., 2003; Schwarz-Romond et al., 2007b) and by direct binding to $G\alpha_o$-GTP (Egger-Adam and Katanaev, 2010). Axin contains a RGS domain which is mediating the contact with $G\alpha_o$ as well as APC. RGS domains commonly also harbor a GAP catalytic function thus accelerating the Gα GTPase activity (Natochin et al., 1998). However, this function of the Axin RGS domain is so far believed to be either lacking or to require additional factors (Schneider et al., 2012). The Axin-RGS was recently shown to have differential roles in axis induction and anteriorposterior neural tissue patterning indicating that it is the protein repertoire of a specific cellular system that determines the nature of the Axin-G protein signaling (Schneider et al., 2012). Axin has further been suggested to interact with Gα-subunits from the G_s, G_q and $G_{12/13}$ families (Castellone et al., 2005; Stemmle et al., 2006), showing a more general function of Axin to cooperate with G proteins, perhaps even outside of WNT signaling events .

1.4.3.2 β-arrestin

β-arrestin1 and -2 are highly interesting and important GPCR-associated scaffold proteins and were first identified as factors promoting clathrin-mediated rhodopsin- and adrenergic receptor desensitization, thus arresting further signaling transduction (Attramadal et al., 1992; Lohse et al., 1990). However, β-arrestins harbor additional

functions essential for localization and pathway specification of 7TM receptors. Moreover, the role of β-arrestins in pharmacological research is appreciated since it was found that exogenous ligands to β_2-adrenergic receptors (β_2AR), angiotensin II type 1 receptors, μ-opioid receptors, chemokine receptors and PTH_1R selectively activated G protein-independent signaling via β-arrestins (Drake et al., 2008; Violin and Lefkowitz, 2007). The functions of β-arrestins as signaling effectors and scaffolds as well as mediators of agonist-activated internalization are shown to be present in both β-catenin-dependent and -independent WNT/FZD signaling (Bryja et al., 2007b; Chen et al., 2001; Chen et al., 2003; Kim and Han, 2007; Kovacs et al., 2009; Schulte et al., 2010). For example, β-arrestins interacts with Axin and DVL to form a ternary complex necessary for WNT-3A/β-catenin signaling and is essential for WNT-5A stimulated CE signaling to Rho and Rac (Bryja et al., 2007b; Kim and Han, 2007). As β–arrestin1/2 double knock-out MEFs failed to execute WNT-3A-induced DVL phosphorylation, β-arrestin might have an important role in the process of DVL phosphorylation (Bryja et al., 2007b). WNT-mediated β-arrestin signaling at FZDs includes unconventional mechanistic features compared to β-arrestin signaling at other 7TM receptors. Interestingly, phosphorylated DVL is required as an adapter between FZD and β-arrestin to allow WNT-5A-induced internalization of FZD_4, again highlighting the unusual modes of signaling connected to Class F GPCRs (Chen et al., 2003).

1.5 WNT/FZD PHARMACOLOGY

1.5.1 WNT/FZD signaling and disease

The coordination of important cell fates, such as cell proliferation, differentiation, migration and apoptosis are mediated by the WNT/FZD signaling system and thus, aberrant WNT/FZD communication has been linked to severe diseased states in humans (Logan and Nusse, 2004). Dysfunctional regulation of WNT/FZD signaling is currently linked to cancer, neurodegenerative diseases, inflammatory diseases, and disorders caused by erroneous bone metabolism and endocrine function (Inestrosa et al., 2012; Marchetti and Pluchino, 2013; Regard et al., 2012; Zimmerman et al., 2012). Manifestation of WNT/FZD-related disease can be the cause of changes in expression of WNTs and secreted WNT modulators or of mutations in WNT signaling components (MacDonald et al., 2009). FZDs, established as the main WNT receptor, are directly downstream of WNTs and thus malfunctioning FZD proteins will impose severe effects on WNT-induced cell fates (Huang and Klein, 2004; Schulte, 2010). The knowledge of proximal WNT-signaling, at the level of receptor transduction, is still lacking several steps in the mechanistic and dynamic regulation of FZDs. Hence, the correlation between dysfunction in FZD signaling and disease is problematic to establish. A few pathogenic mutations and alterations in gene expression have currently been linked to the genes encoding FZD isoforms. For example, genetic connection between FZD_3 and schizophrenia (Kang et al., 2011) has been demonstrated and the correlation between mutations in the *fzd4* gene and disruption in retinal angiogenesis in familial exudative vitreoretinopathy is well-established (Robitaille et al., 2002; Wang et

al., 2012; Yang et al., 2012). Several alterations in the nucleotide sequence and in the regulation of the FZD_6 gene are linked to aberrant nail formation, development of chronic lymphocytic leukemia, neural tube defects, depression and aggressive neuroblastoma tumors (Cui et al., 2013; De Marco et al., 2012; Frojmark et al., 2011; Naz et al., 2012; Raza et al., 2013; Voleti et al., 2012; Wu et al., 2009). Also, FZD_3/FZD_6 double knockout mice show severe defects in midbrain development (Stuebner et al., 2010). In general, several human cancers can readily be found in connection with altered levels of FZD expression (Katoh, 2005; King et al., 2012; Nagayama et al., 2009; Ueno et al., 2013; Vincan, 2004).

Collectively, the data regarding WNT signaling-related diseases clearly establishes a need for drugs targeting events in the scheme of WNT communication and attractive molecular targets, from a pharmacological point of view, are proteins in the FZD family. The development of drugs requires an understanding of the pharmacological properties of WNTs and FZDs, and to date the volume of information in this area of WNT/FZD signaling is very sparse (Schulte, 2010).

1.5.2 Pharmacological aspects and druggability of FZD signaling

FZDs belong to the 7TMR superfamily which is the most common target of pharmaceutical compounds (Whalen et al., 2011). Currently, the methods developed to target WNT signaling at the FZD level involves targeting and inhibiting extracellular agonist stimulation or interfering with receptor binding to intracellular effectors (Zimmerman et al., 2012). The former approach includes the use of FZD isoform-specific antibodies (Fukukawa et al., 2008; Sen et al., 2001; Weeraratna et al., 2002), SFRPs, WNT protein mimetic compound (FOXY-5) and antagonist (BOX-5), and purified WNTs (Jenei et al., 2009; Minear et al., 2010; Nostro et al., 2008; Safholm et al., 2006; Safholm et al., 2008; Vijayaragavan et al., 2009; Willert et al., 2003), while the latter method, being more difficult to target, mainly focus on compounds blocking PDZ domain-PDZ ligand binding (Fujii et al., 2007).

In the pursuit to successfully develop novel WNT/FZD targeting drugs, the concept of receptor agonist-specific binding properties needs to be explored. As mentioned earlier, the ternary complex model is used to explain the shifts in ligand affinity of the 7TMRs due to the GTP-dependent interaction with heterotrimeric G proteins and currently the model is often extended to include additional receptor complex states and used to simulate allosteric regulation of receptors (Kenakin and Christopoulos, 2013). Several such models exists for specific Class A GPCRs, however, owing their unconventional features in G protein signal transduction, models explaining the cooperative interactions of FZD-ligand-effector are not easy to construct (Schulte, 2010). In the same manner of conventional GPCRs, which are shown to mediate effects via G proteins or β-arrestin, FZDs might be able to transduce signals via either DVL or G proteins. However, due to overlapping binding sites and to spatial limitations it seems unlikely that DVL and G proteins would make contact with FZDs at the same time. Hence, three hypothetical working models of FZD-DVL-G protein signaling are currently being discussed and proposed by our research group (fig. 2): i) the

competition model, ii) the temporal model, and iii) the compartment model (Schulte, 2010; Schulte and Bryja, 2007). The competition model assumes that DVL and G proteins are competing for the same interaction site at FZD and that signaling only occurs via one of the two effectors within one round of agonist stimulation. The temporal model suggests that signaling via G proteins might occur fast while signaling by DVL has a slower rate of transduction, however, agonist stimulation of the receptor leads in this case to activation of both effectors. The compartment model theorizes that the two effectors reside at different receptors but might cooperate in a higher order complex, in response to agonist stimulation, to execute signaling, i.e. receptor homo/heterodimerization might occur (Schulte, 2010; Schulte and Bryja, 2007). Experimental validation of these models with respect to a specific WNT and FZD isoforms would improve the chances of drug development in the WNT field.

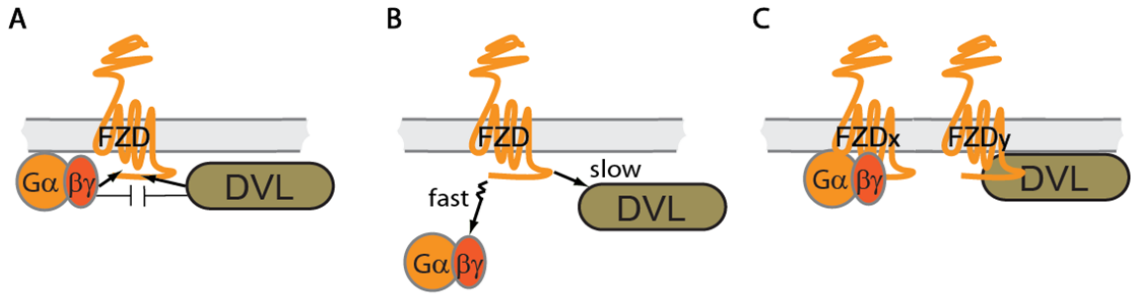

Figure 2. Schematic representation of the hypothetical models for FZD-G protein and DVL interaction: the competition model (**A**), the temporal model (**B**), and the compartment model (**C**).

Closely related to the above suggested models is the concept of functional selectivity or biased agonism. It has been shown that conventional GPCRs, such as e.g. the μ-opioid receptor, the angiotensin (AT_1) receptor, muscarinic acetylcholine (M_1) receptos and the PTH_1 receptor, can signal to different pathways (i.e. β-arrestin-mediated or G protein-dependent) depending on ligand-specific stabilization of a certain receptor conformations (Violin and Lefkowitz, 2007). Thus, it showed that the concept of correlated efficacies was insufficient to explain the many aspects of ligand-receptor signaling outcomes (Kenakin, 1995). The idea of ligand bias or functional selectivity was thereby accepted and describes receptor function and effector coupling as a consequence of the preference of ligand-bound receptors to reside at a unique subset of all possible conformational states. Conversely, for unbiased ligands, regardless of the functional outcome, the subset of conformational states stabilized are identical (Kenakin, 2005). Ligand bias can be calculated using two different approaches: the pharmacological model and the allosteric model. The former employs the concept of affinity-driven efficacy (i.e. the ability of agonists to form an agonist-receptor complex to induce a cellular response) to compare the performances in so called biased plots where an agonist's response in one assay is plotted against the response in a second assay at equimolar agonist concentration (Kenakin and Christopoulos, 2013). The

allosteric model accounts for the concepts of low and high affinity states of agonists and receptors and the formation of a ternary complex as a consequence of effector presence. Thus in the latter model the ligand efficacy is defined by the agonist's ability to induce the formation of a functional ternary complex and it is ultimately the composition of this complex which determines signaling outcome. In the pharmacological model signaling pathway selection is dependent only on the agonist (Rajagopal, 2013). As the name denotes, ligand bias is conditional with regard to the fact that it must be used in the context of another ligand. Ideally, such a standard ligand would be an endogenous agonist. However, endogenous ligands, themselves, are in most cases not devoid of biased signaling. In drug discovery research both endogenous and synthetic ligands are used as reference compounds when calculating ligand bias (Kenakin and Christopoulos, 2013).

The current goal in drug discovery is to develop ligands which selectively will activate one signaling pathway without affecting the other, thus minimizing the chances of unwanted and negative side-effects. In a clinical therapeutic context biased agonists are proven superior in the treatment of several conditions such as chronic pain, heart failure, metabolic- and psychiatric disorders and cancer (Kenakin and Christopoulos, 2013). Considering the many possible effects induced by WNTs and the growing body of data showing differential signaling properties of WNT isoforms in a specified cellular milieu, the concept of ligand bias could certainly be adopted to provide insights into WNT-pathway selectivity and aid the screening for new ways to target WNT/FZD signaling.

2 SPECIFIC AIMS

- To establish the potential of WNTs to activate heterotrimeric G proteins

- To investigate aspects of functional selectivity among WNT-isoforms using functional assays and live-cell imaging techniques in endogenous and protein expression modified cellular milieus.

- To compare pathogenic FZD_6-R511C missense mutant to wild type receptors with respect to functional and molecular properties.

- To assay WNT/FZD binding selectivity and signaling efficacy.

- To elucidate the nature of mechanistic interactions between FZDs and G proteins by using FZD_6 as a representative for Class F receptors.

- To clarify the role of DVL in the liaison between FZD_6 and G proteins.

3 MATERIALS & METHODS

This part of the thesis will mainly deal with additional comments and considerations regarding the methods and materials used. Detailed descriptions of the experimental procedures are found in the designated sections in each article.

Method/Technique	Paper	Protocol adopted/modified from ref
Cell culture propagation	I -V	(Halleskog et al., 2011; Schulte et al., 2005)
Plasma membrane fraction isolation	I-II	(Lazareno, 1997)
Cell permeabilization	I	(Lane et al., 2008; Schulz, 1990)
Molecular cloning	III, V	In-house protocol
DNA/RNAi transfection of cells	I, III, IV, V	(Bryja et al., 2007b; Gesty-Palmer et al., 2006)/in-house protocol
Inhibitor-treatment/WNT-stimulation	I-V	(Schulte et al., 2005)/in-house protocol
RT-PCR/ QPCR	I, II, III	(Halleskog et al., 2011)
γ-[^{35}S]-GTP assay	I-II	(Cooper et al., 2009)
Cell counting	I, III	In-house protocol
MTT-assay	I-II	(Mosmann, 1983)
Native-PAGE/immunoblotting	I	(Wittig et al., 2006)
SDS-PAGE/immunoblotting	I-V	(Halleskog et al., 2011)
Immunoprecipitation	I, III	(Bernatik et al., 2011)
Immunocyto- and histochemistry/X-Gal staining	III, V	(Guo et al., 2004; Schulte et al., 2005)
Genotyping/genetic linkage analysis/sequencing	III	(Entesarian et al., 2009; Klar et al., 2009)
Single fluorophore FRAP	IV	(Phair et al., 2004)
Double fluorophores FRAP/surface cross-linking	V	(Qin et al., 2011; Qin et al., 2008)
Live cell imaging	III, V	In-house protocol
Acceptor photobleaching FRET	III, V	(Goedhart et al., 2007)

Table 1. Methods used in this thesis.

3.1 METHODOLOGICAL CONSIDERATIONS

3.1.1 Propagation of primary- and cell-line cultures

The experiments comprising this thesis were mainly performed in *in vitro* cell cultures. Immortalized cell lines (HEK293T and N13) were allowed to undergo maximum 20-30 passages before being discarded. Cells were checked regularly for mycoplasma infection using RT-PCR detection (primers from Sigma) and cultures proven positive for mycoplsma were either terminated or treated with Plasmocin until tested negative.

Mycoplasma infection has been shown to severely affect cellular responses (Rottem and Barile, 1993; Uphoff et al., 2012) and we also experienced this complication first hand when trying to optimize the [γ-^{35}S]GTP assay in N13 cells (J.P. Dijksterhuis and M. Kilander unpublished observations).

3.1.2 Preparation of membrane fractions and cell permeabilization

Plasma membrane fractions were isolated from N13 using either Polytron disruption and high speed centrifugation or a commercially available plasma membrane protein extraction kit (Fermentas). The degree of "purity" of the plasma membrane fractions with respect to intracellular, soluble proteins, in particular DVL, was observed to affect the magnitude of [γ-^{35}S]GTP incorporation. Membranes contaminated with DVL performed poorly whereas fractions devoid of DVL showed high activity in the [γ-^{35}S]GTP assay. It is tempting to suggest that the presence of DVL might disturb WNT signaling to G proteins by mechanisms of competition at FZDs, however, the result observed might also be the consequence of a dilution factor, i.e. the total protein concentration of the fraction will consist of a smaller pool of receptors and membrane-associated molecules if intracellular proteins are present. Plasma membrane fractions contaminated with DVL were subjected to an additional round of isolation by centrifugation. Furthermore, formation of plasma membrane micelles will yield a random orientation of membrane-imbedded proteins; a situation in which some receptors will be positioned inside-out, in the wrong direction. Thus, just prior to addition of assay constituents, membrane preparations were briefly sonicated at low strength. Cell plasma membrane permeabilization was done using 0.00025% (w/v) saponin since this concentration was reported to not affect intracellular membranes (Schulz, 1990). By immunoblotting it was observed that cells, after being subjected to saponin permeabilization, were depleted of DVL.

3.1.3 Overexpression systems versus native conditions

The possibility to perform molecular cloning and express exogenous genes in cells and organisms has greatly improved the study of protein interaction, function, dynamics and structure. However, most cellular processes are highly fine-tuned and sensitive to changes in protein component concentration or function (Atwood et al., 2011). The plausible consequences of artificial overexpression of proteins are many; e.g. forced protein-protein interactions, impaired protein sorting, trafficking and digestion, disturbed protein localization, under/over activation of signaling pathways, and even necrosis (Adamson et al., 2011). Thus when studying cell communication the introduction of exogenous genes needs to be done with caution and is reliant on a sound set of controls. The use of native cellular conditions will circumvent these problems and results obtained from overexpression systems should ideally be validated in an endogenous milieu. Native conditions, on the other hand, require the availability of high-performing molecular tools, such as e.g. antibodies, and can often be difficult to evaluate.

3.1.4 Assays of G protein activation

The investigation of direct agonist-induced G protein activation was done by measurement of [γ-^{35}S]GTP or γ-S-GTP incorporation in two ways: i) classical evaluation GTP bound Gα-subunits by counting β-particle emission, ii) quantification using antibody-recognition of GTP-$G\alpha_i$ in combination with SDS-PAGE/ immunoblotting under native conditions. The former technique is well-established and has been used for a variety of GPCRs to measure agonist activity on G proteins from at least three of the four families. However, the [γ-^{35}S]GTP assay is most suited for $G_{i/o}$ coupled 7TMRs since $G\alpha_{i/o}$ proteins, compared to Gα proteins from other families, usually are more abundant and have higher GDP to GTP exchange rates which thus yields a higher signal to noise ratio (Harrison and Traynor, 2003). The [γ-^{35}S]GTP assay is performed in the presence GDP, Mg^{2+} and Na^+. GDP is added to decrease the basal incorporation of [γ-^{35}S]GTP so that the resolution of agonist-induced G protein activity increases. The amount of GDP needed varies between experimental systems and the optimal concentration has to be titred out (Harrison and Traynor, 2003). The presence of Mg^{2+} ions, in the low mM range, is essential for GDP-GTP exchange and potentiates both basal and agonist-induced GTP binding but has a greater effect on agonist-evoked G protein activation (Birnbaumer and Zurita, 2010). Na^+ ions are not required but has been reported to have an effect on the signal to noise ratio by favoring agonist-stimulated [γ-^{35}S]GTP binding over basal. The use of immuno-based techniques to measure G protein activation is a fairly new approach and obviously requires antibodies that are able to distinguish between the active (GTP-bound) and inactive (GDP-bound) states. Recently the lab of G. Milligan managed to produce such tools and currently $G\alpha_i$-GTP antibodies, which are commercially available, are shown to perform well in immune-based assays, e.g. immunoprecipitation (Lane et al., 2008). However, caution is advised in respect to cell disruption protocols since all use of denaturizing agents will destroy the native conformation of the Gα protein thus depriving the antibody of its epitope.

3.1.5 Immuno-based assays to measure WNT-activity

Activation of WNT-signaling pathways induces a number of protein modifications and a number of these can be studied using phospho-specific antibodies. The phosphorylation of LRP5/6 is a hallmark of WNT/β-catenin signaling and good antibodies exist which can recognize this modification. However, the most common method to assay β-catenin-dependent signaling is to measure the levels of β-catenin and preferably use antibodies that only detect the active (dephosphorylated) β-catenin, so called ABC antibody. On the other hand, in cells with low basal β-catenin concentrations the use of the ABC antibody is not necessary. The detection of the electrophoretic mobility shift of DVL, referred to as PS-DVL (phosphorylated- and shifted-DVL), is a common approach to evaluate WNT-stimulation, however it is not known whether this modification is directly correlated to pathway activation or not. Moreover, the shift of DVL is usually considered the result of a hyper-phosphorylation but might also be indicative of ubiqitination. Downstream signaling events can also be evaluated using phospho-specific immuno-based analysis, such as activation of

MAPKs, e.g. ERK1/2, p38 and JNK. In particular ERK1/2 used as common readouts of PTX-sensitive $G_{i/o}$ activation. Recently, Halleskog et al showed that WNT-induced phosphorylation of ERK1/2 was mediated via $G_{i/o}$ proteins in primary microglia (Halleskog et al., 2012). Therefore, in article V, the phosphorylation of ERK1/2 was used to test WNT-5A-induced pathway functionality in HEK293.

3.1.6 MTT-assay

Classically, MTT-assay is a method developed to evaluate cell viability and is performed by measuring the switch in absorption wavelength of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) when its tetrazolium ring is cleaved by complex II (succinate dehydrogenase) in the mitochondrial respiration chain. Light absorption is read at 592 nm and corresponds to the functional state of mitochondria (Mosmann, 1983). Apart from cell viability, this assay is used to evaluate cell proliferation and was employed for this purpose in article II. In respect to proliferation, results obtained from an MTT-assay must be considered carefully since it correlates to the number of functional mitochondria and not to the number of cells in the culture. However, results of WNT-5A-induced proliferation obtained from manual cell counting (article I) and from the MTT-assay (article II), were comparable in outcome and thus we decided to use MTT-assay for evaluation of WNT-induced proliferation.

3.1.7 Fluorescence recovery after photobleaching (FRAP)

FRAP is a technique to study fluorescent particle mobility. With the discovery of fluorescent proteins and advances in molecular cloning this technique is widely used in biomolecular research. Proteins of interest are fused to fluorophores and can thus be monitored using live-cell imaging with fluorescent microscopes. Protein mobility is measured by correlating fluorescence intensities before and after high-intensity laser illumination of a region of interest. The illumination irreversibly incapacitates, bleaches, the fluorophores and perturbs the equilibrium of fluorescence intensity. If the proteins under investigation are mobile, a new equilibrium will be reestablished and the rate as well as the degree of fluorescence recovery correlates to diffusion ability in the medium the protein resides in (i.e. lipid membrane or cytoplasm), its association with subcellular structures and organelles, and its presence in protein complexes (Sprague and McNally, 2005). In this way FRAP has successfully been used to study molecular dynamic related to changes in mobility of transmembrane receptor proteins (Aguila et al., 2011; Kaczor and Selent, 2011). In article IV we employed conventional FRAP analysis to study the effect of WNT-stimulation on the mobility of FZD_6-GFP fusion proteins. In article V the FRAP approach was further developed to allow for the analysis of two fluorophore-fused proteins simultaneously. The technique, which also makes use of biotin-avidin mediated cross-linking of cell surface lysine residues and primary amines, was developed by N. Lambert and colleagues and has been used to study several aspects of GPCR and G protein molecular features. Functionality of the receptors tested was found unaffected by the cross-linking procedure and the method was validated using different G-subunits, nucleotide depletion and different rations of components (Fonseca and Lambert, 2009; Lambert, 2009; Lober et al., 2006; Qin et al.,

2011; Qin et al., 2008). Ideally, for this FRAP method to detect transient protein interactions the cross-linked and immobilized partner should be present in excess compared to the mobile component.

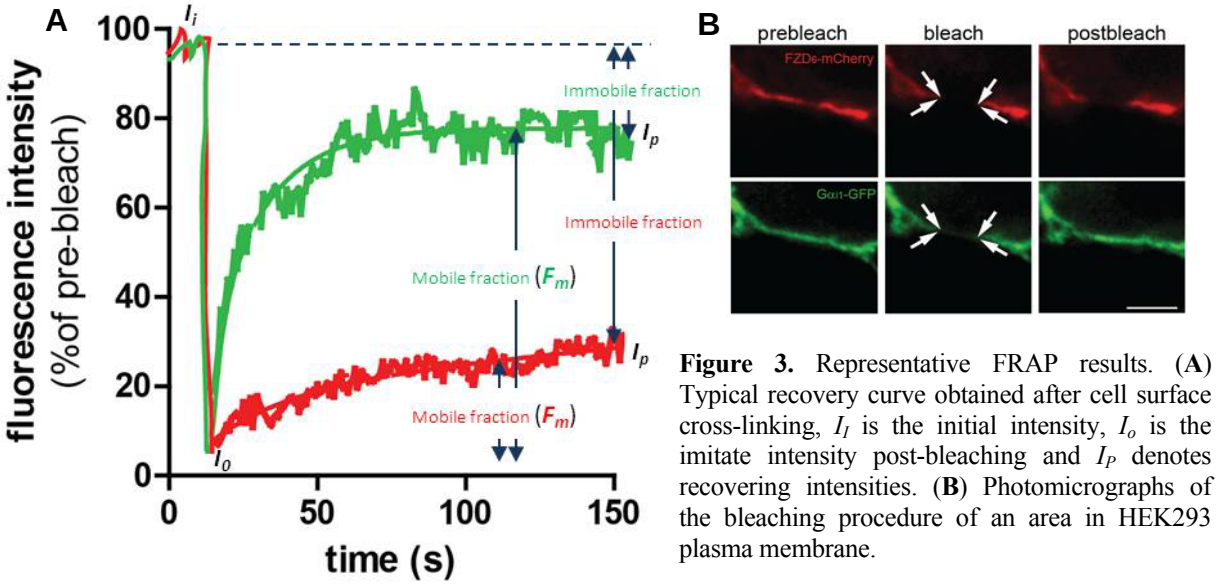

Figure 3. Representative FRAP results. (**A**) Typical recovery curve obtained after cell surface cross-linking, I_I is the initial intensity, I_o is the imitate intensity post-bleaching and I_P denotes recovering intensities. (**B**) Photomicrographs of the bleaching procedure of an area in HEK293 plasma membrane.

3.1.8 Acceptor photobleaching Förster resonance energy transfer (pbFRET)

Non-radiative energy transfer of an excited state from one fluorophore to another constitutes the basis of FRET and is today a common method to measure protein-protein interactions due to its distance-dependent features. FRET only occurs when a fluorophore pair, with appropriate spectral overlap, is situated within less than10nm of each other. A FRET pair consists of a donor fluorophore which is excited with light at the proper wavelength to transfer resonance, or vibrational, energy to the acceptor fluorophore. The process can be detected as acceptor molecule fluorescence. Through mathematical description of the FRET phenomenon the Förster radius *Ro* can be obtained and is defined as the characteristic distance between a given fluorophore pair at which FRET efficiency is 50%. The typical *Ro* distance is somewhere around 4-6 nm. The positions of the fluorophores, and their relative dipole movements towards each other, also affect the FRET efficiency. A parallel orientation is more likely to result in FRET than a perpendicular one. Thus, false negative results, in respect to protein-protein interaction, can be obtained. For example, in article V, FRET between the receptor and Gγ-subunit was performed since only a minute FRET efficiency was observed between the Gα-subunit and the receptor. The most commonly used FRET pair are the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorophores, however, some features, such as autofluorescence from CFP excitation and decreased endurance of measurement due to multiexponential decay of CFP, have led to the search for other suitable and high-performing FRET pairs. In article III and V green-red (green fluorescent protein (GFP) – mCherry) FRET pairs have been used

since i) moving excitation wavelength towards the red spectra generally decreases autofluorescence, ii) FRET efficiency usually increases for pairs at higher wavelengths (Albertazzi et al., 2009; Goedhart et al., 2007), and iii) limitations in laboratory equipment made the measurement of CFP/YFP FRET not feasible. The method of donor dequenching after acceptor photobleaching (pbFRET) is a robust, simple and well-established modification of the FRET protocol and is most commonly performed in fixed cells. Donor dequenching will increase the fluorescence of the donor if the bleached acceptor is within FRET distance. Drawbacks of this method include limitation in repeated measurements and the loss of information regarding dynamic protein interactions (Piston and Kremers, 2007).

4 RESULTS & DISCUSSION

4.1 WNT-INDUCED G PROTEIN ACTIVATION AND ISOFORM-SPECIFIC SIGNALING IN A DEFINED CELLULAR SYSTEM

As stated earlier, the mammalian WNT/FZD signaling system (consisting of 19 ligands and 10 receptors) allows for a high number of possible interaction partners able to activate a variety of different down-stream signaling pathways. Physiologically, the determination in WNT-FZD isoform pairing and in WNT pathway preferences seems to be orchestrated by the unique, local or temporal, protein expression in a specific tissue (Niehrs, 2012; van Amerongen, 2012). Moreover, WNT action occurs both at short distances in an autocrine and paracrine manners or at longer distances of up to more than 20 cell diameters (Port and Basler, 2010). This feature, in combination with stringent control of expression of signaling components, allows WNTs to perform a specific process within a defined area at a precise time point. Such precision is a prerequisite for the morphogenic role of WNTs (Gordon and Nusse, 2006; Nusse, 2012). In the first paper we analyzed the expression of WNT signaling receptors and co-receptors in a specific cell line derived from microglial linage. The analysis revealed the presence of a unique set of signaling components able to produce high response to WNT-5A stimulation. Thus, in the second article we decided to use this cellular system as a platform to further investigate WNT isoform-specific differences in signaling activation at endogenous protein stoichiometry.

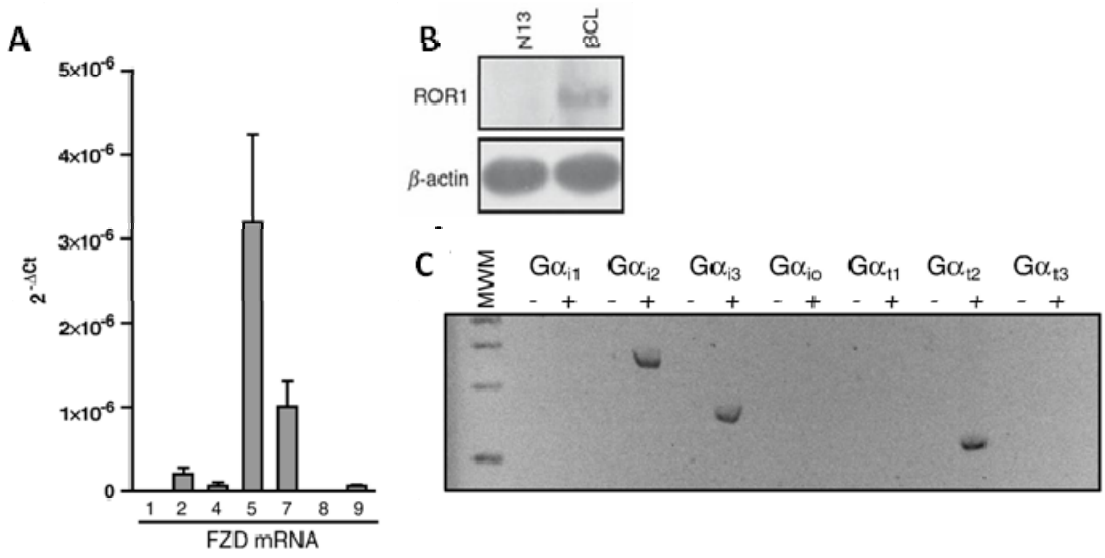

Figure 4. Assessment of WNT signaling-related components in N13. (**A**) QPCR analysis of expression levels of FZD isoforms present in N13. (**B**) Lack of ROR1 expression compared to B-cell lymphoma line (BCL), confirmed by immunoblotting. (**C**) RT-PCR screening of PTX-sensitive $G\alpha_{i/o}$ isoforms.

4.1.1 WNT-5A activates PTX sensitive heterotrimeric $G_{i2/3}$ proteins in microglia like cells (Paper I)

As discussed previously, analysis of the role of heterotrimeric G proteins in WNT signaling transduction has received relatively little attention in the WNT signaling research field. In the present paper we choose to investigate guanine nucleotide exchange by an accumulative read-out assay, [γ-^{35}S]GTP. This assay was developed in the early 1980s and has proven a robust and reliable method to study GPCR signaling and especially the activation of $G_{i/o}$ proteins. Presently, however, alternative methods have largely replaced the [γ-^{35}S]GTP assay because of the handling of radioactive emission from the [^{35}S]-isotope (Koval and Katanaev, 2012). In our hands, this assay proved to be efficient and sensitive enough to detect WNT-5A-induced exchange of GDP to GTP at heterotrimeric Gα-subunits. Hence, in this article we are able to show, for the first time, that WNTs are able to activate and relay signal transduction via G proteins in endogenous cellular systems devoid of potential artifacts created by protein overexpression.

Using isolated plasma membrane fractions from N13, a microglia-like immortalized cell line, we retain only the most proximal, plasma membrane tethered, WNT-signaling components. Thus, we are able to exclude WNT-induced effects on most cytosolic small GTPases as well as possible competition by DVL-directed signaling. The first notion that WNT-5A might activate G protein signaling was obtained from our discovery that N13 proliferative response to WNT-5A stimulation was PTX sensitive. Rapid proliferation is an important physiological feature of microglia to regulate inflammatory processes and studies in our lab have revealed microglia-modulating and immunological potentials of WNTs (Halleskog et al., 2012; Halleskog et al., 2011; Halleskog and Schulte, 2013). We therefore decided to look deeper into the effect of WNT-5A on N13 cells. WNT-5A stimulation (300ng/ml) of N13 plasma membrane fractions lead to a profound 105% increase in [γ-^{35}S]GTP incorporation compared to vehicle control (total incorporation 205 ± 27%) (fig.5A). This signal is comparable, or stronger, in magnitude to results obtained from endogenously expressed μ-opioid- and 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptors (Alper and Nelson, 1998; Traynor and Nahorski, 1995). In a related study (Halleskog et al., 2012), using primary microglia we observed a similar response, indicating that the WNT-5A activity on guanine nucleotide exchange is physiologically relevant (fig. 5B).

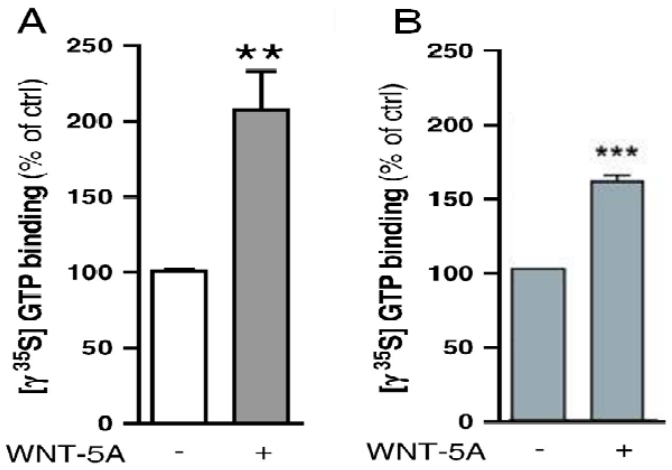

Figure 5. [γ-^{35}S]GTP assay in plasma membrane fractions from N13 (**A**) and primary microglia (**B**). Plasma membranes were incubated with either control solution or 300ng/ml WNT-5A. Error bars provide SEM.

The strength of the signal in combination with the subsequent finding that the WNT-5A effect was abolished by PTX pretreatment, strongly implicated the involvement of one or more of the 5 different subtypes (G_i, G_o, G_t, G_{gust} or G_z) in the $G_{i/o}$ protein family. However, G_z could be excluded since this subtype is not ADP-ribosylated and inhibited by PTX. Using native, non-denaturing, immunoblotting analysis in combination with an antibody recognizing the GTP-bound conformation of $G\alpha_{i1\text{-}3}$ proteins, we could subsequently confirm that the WNT-5A stimulation, in presence of [γ-^{35}S]GTP, in plasma membrane fractions and saponin permealized whole cells, indeed lead to the activation of G_i proteins. The specificity of the antibody towards $G\alpha_{i1\text{-}3}$ was established by the lack of antibody affinity towards overexpressed constitutively active (QL-mutant) $G\alpha_{i/o}$ family proteins in HEK293 cells. This result in combination with RT-PCR analysis of the expression profile of $G_{i/o}$ subtype proteins in N13 (fig. 4B) strongly indicated that the WNT-5A evoked guanine nucleotide exchange takes place at $G\alpha_{i2}$ or $_3$. With the current data at hand, however, we cannot exclude the involvement of G_t proteins totally. Further genomic analysis of WNT receptors expressed in N13 revealed that these cells contain LRP5/6 co-receptors but, on the other hand, only produce trace amounts of ROR1/2 and RYK mRNA (Halleskog et al., 2011). Moreover, using QPCR we could determine the N13 FZD protein expression profile: FZD_5>FZD_7>>FZD_2>FZD_4=FZD_9>FZD_8 (fig. 4A). Consequently, since the expression of FZD_5 was more than 3 times that of FZD_7, we could surmise that WNT-5A signaling to $G\alpha_{i2/3}$ proteins, most likely, was transduced via the activation of FZD_5. However, we could not obtain direct molecular and functional proof to support this notion. Even though FZD_5 protein abundance readily could be detected by immunoblotting, co-immunoprecipitation of FZD_5 and $G\alpha_i$ proteins was unsuccessful. Moreover, all attempts to genetically manipulate FZD_5 expression in N13 failed. Interestingly, however, by SDS-PAGE analysis we could observe an electrophoretic mobility retardation shift of FZD_5 after WNT-5A stimulation. Similar shift has previously been described at e.g. β_2ARs as a result of receptor phosphorylation following agonist

activation (Stadel et al., 1982). Of further interest was also the fact that a goat-anti-FZD_5 antibody (from R&D systems) only could recognize the receptor when a post WNT-5A-stimulation epitope was exposed by FZD_5. Perhaps, although not specified by the producer, this FZD_5 antibody is only able to detect phosphorylated receptor proteins.

In conclusion, we show in this study that WNT-5A is a direct activator of $G_{i2/3}$ proteins in cells expressing FZD_5 as the main WNT receptor. This is the first study using endogenous protein levels to detect the WNT-induced activation of G protein signaling.

4.1.2 Analysis of differential signaling by WNT isoforms in N13 cells (paper II)

In the second article we report that WNT signaling is differential in activity and pathway specificity with regard to a specific WNT isoform. The WNT signaling network holds important pharmacological potentials, however, in order to go further with WNT drug development, a thorough characterization of the specificities in receptor binding and consequent downstream events needs to be performed. Thus, we were curious to investigate how different WNT isoforms would initiate signaling in N13 cells. Since we already had established the WNT receptor profile of these cells, we could draw interesting conclusions with regard to beta-catenin-dependent and -independent signaling features of the different WNTs. 6 different commercially available (from R&D systems) recombinant WNTs; -3A, -4, -5A, -5B, -7A, -9B, were assayed using: [γ-^{35}S]GTP assay for G protein activation, immunoblotting detection of i) beta-catenin stabilization, ii) phosphorylation of LRP6 and iii) DVL electrophoretic mobility shift, and MTT-assay for cell proliferation (table 2).

Interestingly, the only isoform truly capable of beta-catenin-dependent signaling was WNT-3A which showed strong phosphorylation of LRP6, stabilization of beta-catenin and activation of DVL3. Although all WNTs tested did show activity in inducing guanine nucleotide exchange, only WNTs -5A and -9B were able to induce significant increases in proliferation. This result also correlated to the efficacy of G protein activation as WNT-5A and -9B were able to induce [γ-^{35}S]GTP incorporation at 49 and 40%, respectively, above control level. The fact that activity in the [γ-^{35}S]GTP assay was observed for all the WNTs, even for the -7A isoform which performed poorly in all other experimental methods used, prompted us to test of the purity of the WNT preparations, although endotoxin levels were reported very low. To make sure that no WNT unrelated activities by contaminants were responsible for the results obtained (as shown by Cajanek et al in 2010 (Cajanek et al., 2010)), we performed WNT signal blocking by SFRP-1 sequestering. The pre-incubation of SFRP-1 with WNT isoforms successfully blocked all downstream PS-DVL3 formation, except in the case of WNT-9B. However, both WNT-5A- and -9B-induced proliferation was abolished by the presence of SFRP-1. With these results in hand, we could conclude that the observed effects were WNT-related. Studies have previously shown that combinatorial stimulation with WNT-3A and -5A could lower the -3A-induced beta-catenin

dependent signaling response by competition on LRP6 or FZD_2 or by ROR-related signaling transduction (Bryja et al., 2007c; Mikels and Nusse, 2006; Topol et al., 2003). Since we had established that FZD_2 and ROR1/2 mRNA levels were low in N13 and that ROR1 protein expression was hardly detectable in these cells, we expected to find no effect of WNT-5A on -3A-induced beta-catenin stabilization. As hypothesized, no reductions in P-LRP6 or beta-catenin levels were observed when adding increasing concentrations of WNT-5A to WNT-3A stimulated cells. However, there seemed to be an additive effect on the formation of PS-DVL3 induced by the combination of WNT-3A and -5A stimulation. For each of the WNTs investigated in this article, several different FZDs have been reported as binding partners. FZD_5, being the most abundant WNT receptor in N13, is of course interesting to consider as the primary target of WNT signaling transduction. WNTs -5A, -7A and -9B have been reported to interact with and induce signaling via FZD_5 but perhaps, due to the lack of ROR1/2 and RYK co-receptors, more of the WNT isoforms are able to communicate via FZD_5. Of the other FZD proteins present in N13, FZD_2 and FZD_9 were reported to be associated to WNT-4 and -7A signaling respectively.

WNT	Cat#	Species	Transformation of C57MG cells	Expression in the brain	P-LRP6	β-catenin stabilization	PS-DVL3	G protein activation	Proliferation
WNT-3A	1324-WN	Mouse	+++	-	+++	+++	+++	+	-
WNT -4	475-WN	Mouse	-	+	-	-	+++	+	-
WNT-5A	645-WN	Mouse	-	+	-	-	++	+++	++
WNT-5B	3006-WN	Mouse	-	+	-	-	+	++	+
WNT-7A	3008-WN	Human	+	+	-	-	+	+	-
WNT -9B	3669-WN	Mouse	+	+	-	-	+	+++	++

Table 2. Summary of the properties and activities of the WNTs used in the study. Preparations are commercially available from R&D systems and column 2 provides the catalogue number. The WNT's capacity to transform C57MG cells and their expression in the brain was summarized from the literature (for references, see paper II). Data is described by: -, no activation/expression; +, weak activation/expression; ++, intermediate activation/expression; +++, strong activation/expression.

The potential in using an immortalized cell line as a read-out system for physiological responses is of course limited. However, the results shown in this study, somewhat correlates with WNT-induced proinflammatory responses observed in primary microglia cultures (Halleskog et al., 2012; Halleskog et al., 2011; Halleskog and Schulte, 2013). Moreover, all of the WNTs tested, except WNT-3A, are expressed in the healthy adult brain, indicating an important role of these signaling molecules in maintaining and regulating normal neuro-pathophysiological processes.

4.2 CHARACTERIZATION OF DYSFUNCTIONAL WNT/FZD SIGNALING IN MUTATED FZD_6 PROTEINS (PAPER III)

The role of WNT/FZD signaling pathways in organism development has been appreciated since the early days of their discovery. Moreover, mutations in either the ligands or the receptors are often found to cause lethality in embryonic stages. Perhaps, due to the severe effects of mutations in WNT signaling-related genes, only very few pathogenic mutations have been found in human FZD genes, e.g. mutated FZD_4 protein can be directly linked to the development of familial exudative vitreoretinopathy/advanced retinopathy of prematurity. In the third article we identify and investigate two novel pathogenic mutations in the human FZD_6 gene giving rise to autosomal recessive nail dysplasia.

Proper nail development involves the communication and interaction between mesenchymal cells of the nail plate and epidermal cells of the nail bed. Phenotypic evaluation of individuals suffering from nail dysplasia indicated that the disease was caused by improper formation and attachment of the nail plate. Moreover, analysis of nail development in mice FZD_6/FZD_3 double mutants reveled perturbed nail development in 50% of the male animals and investigation of FZD_6 expression during embryonic development showed a transient FZD_6 mRNA expression peak at E14.5 in epidermal cells of digital tips. The results thus establish that FZD_6 is expressed in the developing nail and important for the formation of the nail bed.

Further genetic evaluation of four individuals in each of two different families (F1 and F2) suffering from autosomal recessive nail dysplasia were shown to be homozygous for a nonsense mutation (c.1750G>T; p.E584X) and a missense mutation (c.1531C>T; p.R511C) respectively. Notably, the individuals in F2, carrying the missense mutations, showed a more severe disease phenotype than the members of F1. Primary fibroblasts from disease affected F1-individuals revealed that the nonsense mutation lead to improper protein expression; only 13% mRNA was found in these cells compared to wild-type (WT) fibroblasts. Further, plasmid DNA transfection in HEK293 with the nonsense mutated FZD_6 gene did not result in any protein product. Therefore, only the missense FZD_6-R511C mutant was analyzed with regard to subcellular localization. However, since primary fibroblasts only were obtained from F1 members, downstream WNT-beta-catenin-dependent and –independent signaling potentials were analyzed in these cells. WNT-3A and WNT-5A stimulation of the F1-primary fibroblasts revealed absence of β-catenin stabilization and no increased expression of DKK1 or the transcription factor MSX1 respectively. WNT/ β-catenin-dependent and –independent signaling pathways were thus shown to be impaired in primary fibroblasts lacking the FZD_6 protein. Moreover, we here demonstrate that FZD_6 is able to mediate β-catenin stabilization, an interesting result regarding the fact that FZD_6 is considered as a PCP/CE-signaling transducer and shown to affect β-catenin signaling in an antagonistic manner (Golan et al., 2004).

Analysis of subcellular localization of missense-mutated FZD_6 proteins expressed in HEK293 revealed that the receptor was confined to intracellular vesicular compartments to a much greater degree than WT FZD_6 (fig. 6). Thus we could observe that the missense mutation caused FZD_6 to display improper plasma membrane localization. We then investigated the nature of the vesicles containing FZD_6 R511C and our results clearly showed that the consequence of the missense mutation further caused the receptor to be trapped in lysosomal compartments. However, we were unable to determine the events leading to this outcome, i.e. if FZD_6 R511C proteins are inserted but removed from the plasma membrane more extensively than WT receptors, or if the missense mutants trapped in lysosomes never were inserted in the membrane.

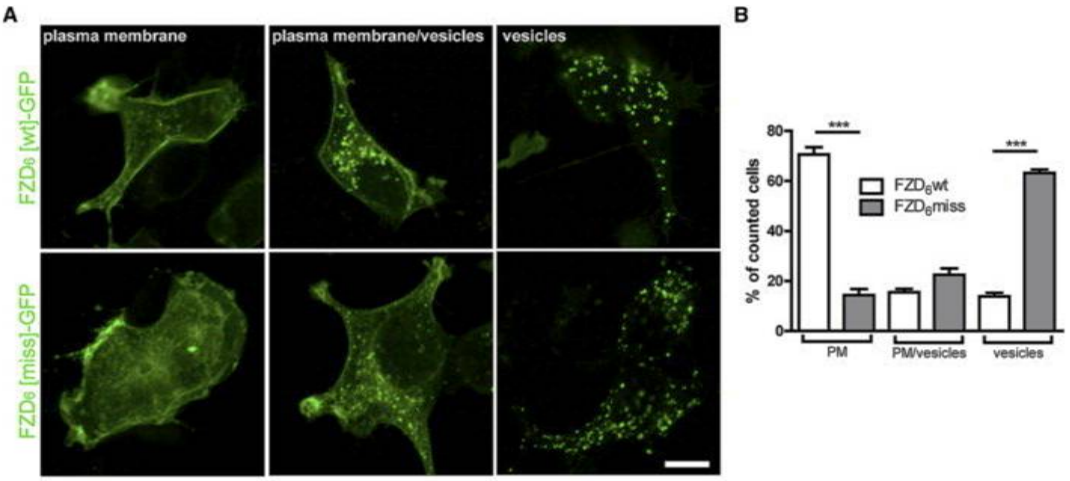

Figure 6. Subcellular localization of WT FZD_6 and FZD_6-R511C. (**A**) Represenative pictures of the different receptor localization patterns observed. (**B**) Quantification of localization patterns.The mean values ± standard deviation (SD) were 70.7 ± 2.8 (FZD_6[wt]membr); 14.3 ± 2.4 (FZD_6 [missense]membr), 15.5 ± 1.3 (FZD6[wt]membr to vesic), 22.5 ± 2.6 (FZD6[missense]membr to vesic), 13.8 ± 1.5 (FZD_6[wt]vesic),and 63.2 ± 1.4 (FZD_6[missense]vesic).

Interestingly, the R511C point mutation is situated 7 amino acids downstream of the KTCTE PDZ-lignad domain and could therefore have an impact on the interaction of FZD_6 with DVL proteins. We examined the ability of the FZD_6 R511C to associate with DVL1 using immunocytochemical evaluation of FZD-mediated membrane recruitment of DVL, as well as measuring acceptor photobleaching FRET between GFP-fused FZD_6 proteins and Cy3-antibody tagged DVL1. Our results showed that despite the proximity of the mutation to the PDZ-ligand domain, FZD_6 R511C was still able to associate with DVL1, albeit not as efficient as FZD6wt. This further suggests that additional regions of FZD_6, other than the KTCTEW, might contribute to biding to DVL1, much like the interaction between FZD_5 and DVL1. Also, the fact that FZD_6 R511C can associate with DVL1 opens up the possibility that missense-mutated receptors at the plasma membrane still could mediate signaling. Collectively, our results suggest that loss of FZD_6 protein and consequent WNT/FZD_6 signaling is causing the nail dysplasia phenotype. The fact that F2 individuals with missense-mutated FZD_6 shows a more severe phenotype than nonsense-FZD_6-carrying F1 members do, moreover suggests that additional disturbances in signaling created by an impaired but still present receptor might contribute to further negative effects in the WNT-regulated process of nail tissue maintenance.

4.3 FRAP-BASED ASSESSMENTS OF FZD_6 SIGNALING PROPERTIES

In order to further investigate WNT signaling events at the receptor level we decided to employ a live-cell imaging approach; i.e. Fluorescence Recovery After Photobleaching (FRAP). This technique has been used to successfully establish e.g. the oligomerization of β_2ARs, the dynamic compartmentalization of neurokinin- and μ-opioid receptors and the association of G proteins with 5-HT_{1A} receptors (Cezanne et al., 2004; Dorsch et al., 2009; Pucadyil et al., 2004; Sauliere-Nzeh Ndong et al., 2010). Thus in the fourth and the fifth article we introduce and establish FRAP-based techniques as novel tools to assay WNT actions on defined FZD protein molecules and intracellular effectors.

4.3.1 Analysis of WNT isoform-specific effects on FZD_6 lateral mobility (paper IV)

In article four mono-color fluorophore FRAP was performed to assess WNT stimulation-induced changes in FZD_6-EGFP protein lateral diffusion in HEK293 plasma membranes. Surprisingly, WNT stimulation did not lead to retardation in receptor mobility, rather, after 15 minutes of WNT-5A incubation FZD_6 rate of diffusion was significantly increased as observed by the elevation of the fluorescence recovery curve plateau. Control experiment using NECA-induced elevation of adenosine 1 receptors (A_1R) mobility confirmed that the result was not caused by the association-dissociation of ligand to-from the receptor since the molecular size of NECA is much smaller than that of WNT-5A. We therefore began to suspect the involvement of intracellular effector molecules. Overnight pretreatment with PTX as a means to uncouple/inhibit $G_{i/o}$ protein–receptor association resulted in up-shifted recovery curve plateaus, i.e. increased receptor mobility in the plasma membrane. This outcome highly resembled the WNT-5A stimulation-induced behavior of the FZD_6. Moreover, PTX pretreatment effectively abolished any further WNT-5A-promoted increase in receptor mobility. Collectively, these results strongly pointed to the participation of heterotrimeric $G_{i/o}$ proteins in the WNT-5A-evoked effect on FZD_6. To further test this hypothesis, HEK293 was transfected with PTX resistant $G\alpha_{i2}$C352I mutant proteins and the subsequent discovery showed that in the presence of PTX, this action was sufficient for the recovery and re-establishment of FZD_6 low basal mobility and sensitivity towards WNT-5A stimulation. Altogether, these results support the notions of: i) in the basal state FZD_6 consists of a precoupled receptor-G_i protein complex, and ii) the complex dissociates upon WNT-5A stimulation ultimately leading to an elevation in lateral diffusion capacity of the receptor. Thus, the results provide evidence of a WNT/FZD/G protein interaction in living cells.

In article three we showed that the FZD_6-R511C protein, even though harboring a mutation in close proximity to the KTCTEW domain, still is able to interact with and recruit DVL to the plasma membrane. However, we did not look into the mutant receptor's capacity to associate to G proteins. As was concluded in the study, the individuals homozygous for the missense mutation displayed a more severe phenotype than the FZD_6 nonsense mutated patients, indicating that, besides the lysosomal

compartmentalization, there might be additional pathogenic features of the missense mutation. In order to gain further knowledge regarding this notion, we subjected FZD_6-R511C-GFP transfected HEK293 cells to FRAP analysis. Interestingly, we found that the FZD_6-R511C protein was non-responsive to WNT-5A stimulation with regard to increased lateral mobility effects. Moreover, pretreatment with PTX neither caused any increase in basal, non-stimulated, state mobility nor lead to any change in the insensitivity of the mutated receptor towards WNT-5A. Collectively, the results suggest that the missense mutant FZD_6-R511C might be deficient in G protein coupling and consequently unable to relay WNT-induced activation of heterotrimeric G proteins, a cellular process which might otherwise be necessary for maintaining proper nail physiology.

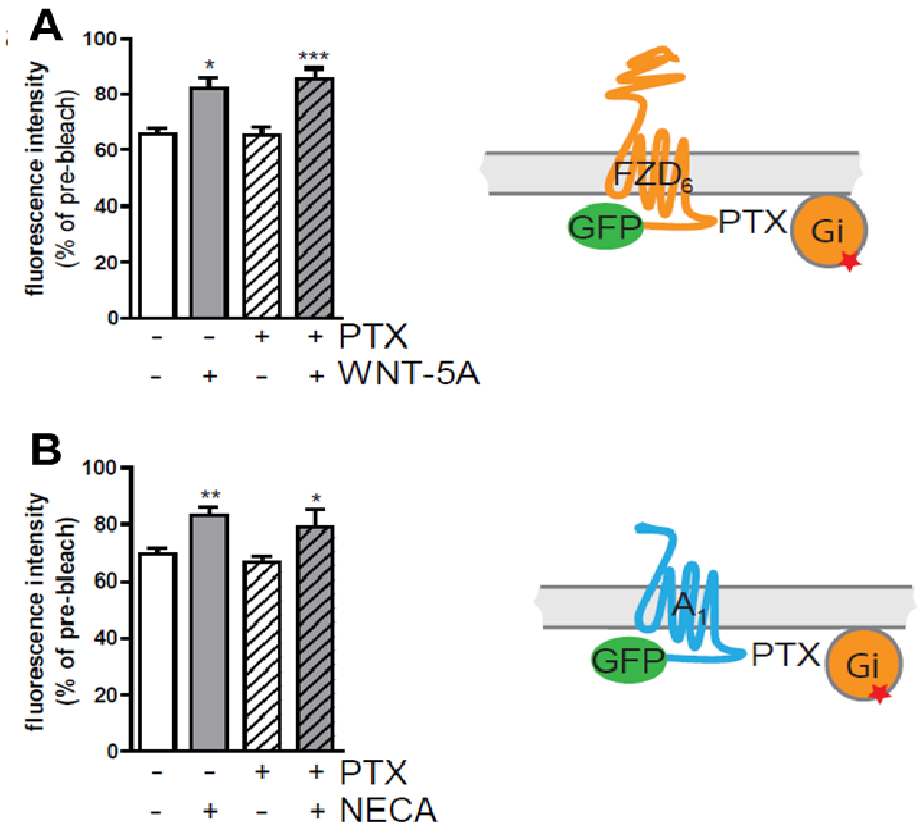

Figure 7. PTX-resistant mutant $G\alpha_{i2}$-C352I rescues agonist-induced effects on receptor lateral mobility in presence of PTX. (**A**) FZD_6-GFP fluorescence recovery in HEK293 co-transfected with untagged $G\alpha_{i2}$-C352I at 0 and 15 min of agonist stimulation in absence (open bars) and in presence (hatched bars) of overnight PTX pretreatment. (**B**) Presentation of A_1R-GFP mobile fractions using the same set up as in A.

In current pharmaceutical research there is an increasing interest in the concept of functional selectivity or signaling bias, not only mediated by drugs and synthetic ligands but also by endogenous agonists. For example, recent studies have revealed that the actions of endomorphins, on μ-opioid receptors seem to exhibit pathway-selective, biased, signal transduction (Rivero et al., 2012). In the second article we showed that WNTs might act on FZDs in a similar way to steer cell communication into different directions. To analyze this concept at the receptor level, we used the reductionistic FRAP approach to measure WNT-isoform-specific abilities in eliciting changes in FZD_6 molecular mobility. 10 different recombinant WNTs (WNT-1, -2B, 3A, -4, -5A, -5B, -7A, -9B, -10B and -11) were used and FZD_6 mobility was measured at 0, 5, 15 and 30 min of stimulation. Interestingly, the sensitivity of the assay proved sufficient enough to detect differences among the WNTs. As was seen in article II, WNTs -3A and -4 showed similar efficacies and dynamics in evoking GDP/GTP exchange and again in the FRAP-assay these two WNTs produced similar time-dependent patterns in their FZD_6 mobility induction activity. The result is a bit surprising since WNT-3A is classified as a β-catenin dependent WNT while WNT-4 is performing mainly β-catenin independent signaling. On the other hand, although both WNT-1 and WNT-3A are categorized as classical β-catenin dependent signaling WNTs, their activation profiles in the present assay differs. While WNT-3A shows a slow and increasing effect on FZD_6 diffusion, WNT-1 has a quicker and more transient effect indicating different abilities in receptor conformation stabilization. Moreover, the fact that these two β-catenin-dependent WNTs shows activity at FZD_6 is interesting since FZD_6 mainly is viewed as a mediator of PCP signaling and can act as a repressor of β-catenin dependent WNT signaling. WNT-7A, which previously has been proven a binding partner for FZD_6, shows a sharp, very transient action in changing receptor diffusion rate and a similar, but less efficient, result was obtained for WNT-2B. By analysis of gene expression in differentiated gut epithelial cells, FZD_6 expression was found in close proximity to WNT-2B-producing cells in a region devoid of β-catenin related signaling events (Gregorieff et al., 2005). These results indicate a rapid and transient FZD_6 mobility change, perhaps connected to a β-catenin-independent signaling pathway activated by WNT-2B. The activities of WNT-5A and WNT-10B are similar in kinetics but the response maximum of WNT-10B is almost 10% stronger than WNT-5A. Although both WNT-7A and WNT-10B seems to have the same abilities in total receptor binding, they appear to have different functions in stabilizing FZD_6 conformation; binding of WNT-7A results in the formation of a less stable agonist-induced receptor conformation than the interaction of WNT-10B with FZD_6 does. The FRAP-based measurements of WNT activity further showed that WNTs -5B and -11 most likely are very weak agonists at FZD_6, since no significant increase in FZD_6 mobility was observed when stimulating the receptor with these WNTs. WNT-9B activity also proved moderate in comparison to WNT-1, -5A, -7A and -10B, suggesting that WNT-9B, like WNTs -5B and -11, is a low affinity agonist at FZD_6. Using the data at hand, four different groups can be distinguished: group I) fast and transient: WNT-2B and WNT-7A; group II) intermediate and transient: WNT-1, WNT-5A, WNT-9B and WNT-10B; group III) slow and persistent: WNT-3A and WNT-4; and group IV) no/low activity: WNT-5B, (WNT-9B) and WNT-11 (fig. 8).

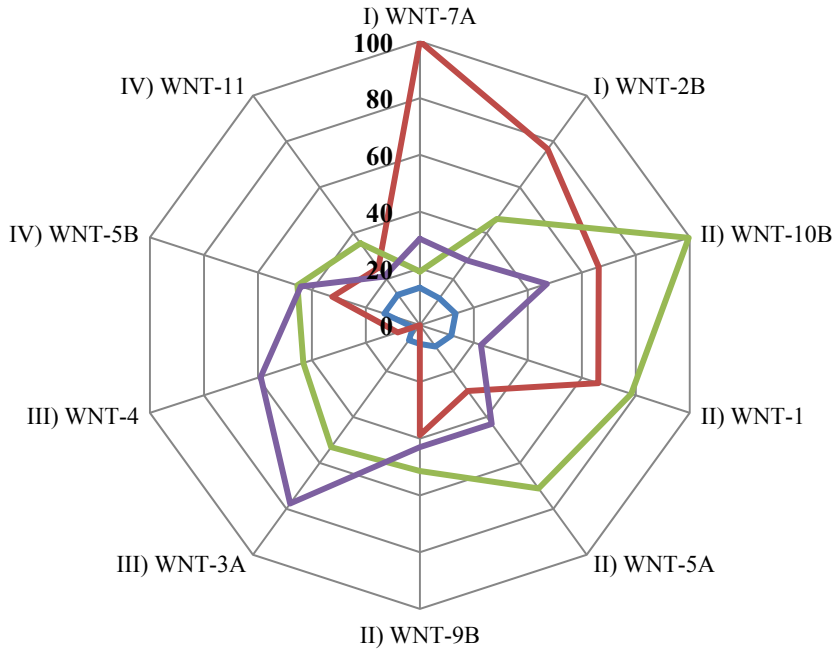

Figure 8. Web diagram summarizing the efficacies of WNTs presented as changes in FZD_6-GFP protein mobility over agonist stimulation time period. Blue line: 0 min stimulation, red: 5 min, green: 15 min and purple: 30 min. WNT efficacies are normalized to the lowest and highest values. Results based on at least 3 independent experiments per WNT.

In conclusion, these results indicate that the WNTs tested have different affinities for FZD_6 and upon binding are able to establish different receptor conformations with different stabilities over time. Also, the observed results could be dependent on different FZD-FZD homo/heterodimers as well as FZD-coreceptor complexes which differs in affinity for the tested WNTs and might have varying degrees of complex stability upon WNT stimulation. The observed isoform-specific activation patterns could further suggest that depending on the WNT binding, signaling will be relayed via different combinations of intracellular effectors which ultimately determine the nature/magnitude of FZD_6 lateral diffusion in the plasma membrane. However, it should be stressed that the FRAP-based evaluation of agonist-receptor activity only accounts for changes in FZD_6 protein mobility and does not cover other possible outcomes of WNT signaling action. Therefore, further assays needs to be performed in order to fully understand how and which WNT-FZD_6 interactions truly occur.

4.3.2 Analysis of FZD_6 signaling complex composition (paper V)

In this study a dual fluorophores approach was used for FRAP analysis of the FZD_6 signaling complex. FZD_6 fused to mCherry or GFP and Gα subunits tagged with either GFP ($G\alpha_{i1}$ and $G\alpha_s$), Venus ($G\alpha_{oA}$ and $G\alpha_q$), or mCherry ($G\alpha_{12}$) were coexpressed in HEK293 in combination with untagged βγ subunits. FRAP measurements were obtained simultaneously for both fluorophore-fused proteins and receptor lateral mobility was modulated by employing extracellular surface Sulfo-NHS-LC-LC-biotin

and avidin mediated cross-linking. The NHS-group reacts with lysines and primary amines protruding from the outer leaflet and establishes a surface of covalently bound biotin moieties. The addition and binding of avidin to biotin creates a tight mesh of cross-linked molecules which immobilizes all proteins extending out from the cell surface. This modified FRAP approach has been used and validated for the analysis of receptor oligomerization, receptor-G protein precoupling and heterotrimeric G protein complex stability (Digby et al., 2006; Fonseca and Lambert, 2009; Lober et al., 2006; Qin et al., 2011; Qin et al., 2008). Here, we investigate precoupling of FZD_6 to different Gα subunits representing the four G protein families: G_s, $G_{q/11}$, $G_{i/o}$ and$G_{12/13}$. Additionally, using the modified dual color FRAP technique, the interaction of DVL with FZD_6 was examined as well as the role of DVL in the process of FZD_6-G protein assembly.

Analysis of protein localization, determined by immunocytochemistry, placed FZD_6, $G\alpha_{i1}$ and DVL at the plasma membrane. Thus, even though G proteins are overexpressed, FZD_6 is still able to recruit DVL, indicating a possible molecular interaction and assembly consisting of FZD_6, G proteins and DVL. Using cell surface cross-linking and dual color FRAP we found that FZD_6 was precoupled to $G\alpha_{i1}$ and G_q with about 20% of the total pool of tagged Gα subunits, but unable to form a stable complex with $G\alpha_{oA}$, $G\alpha_s$ and G_{12} (fig. 9). The interaction of FZD_6 with either $G\alpha_{i1}$ or G_q was further confirmed by acceptor photobleaching FRET between FZD_6-mCherry and Venus-tagged $G\gamma_2$. $G\alpha_{i1}$-FZD_6 precoupling was also shown to be PTX-sensitive, a result which agrees with the data observed in paper IV. The effect of PTX on receptor-G protein precoupling is indicative of constitutive activity, since PTX only is able to ribosylate the Cys moiety at Gα subunits during the phase of nucleotide exchange. The results thus suggest that FZD_6 has constitutive activity or perhaps that endogenous auto/paracrine WNT stimulation in the cell culture will activate FZD_6. Since PTX treatment is performed overnight, both scenarios provide a plausible explanation for the observed complete abolishment of Gαi1-FZD_6 precoupling in the presence of PTX. Next, the precoupling of FZD_6 to $G\alpha_{i1/q}$ was investigated in the presence of WNT-5A. Results showed that the interaction between FZD_6 and $G\alpha_q$ was dynamic since coupling was lost after 5 minutes of WNT-5A stimulation but reestablished again after 15 min. The dissociation of $G\alpha_{i1}$ proteins from FZD_6 was somewhat more persistent, or perhaps slower, since no pbFRET or FRAP-measured mobility retardation could be observed after 15 min of WNT-5A stimulation. Compared to conventional G protein activation and dissociation kinetics, the presently observed WNT-5A-induced dissociation of $G\alpha_{i1}$ is rather slow. However, lack of dissociation in the early steps of activation is a known feature of $G\alpha_{i1}$ proteins (Gales et al., 2006) and thus could contribute to the observed results. In conclusion, these results show that FZD_6 selectively precouples to G_{i1} and G_q heterotrimeric Gα subunits and that WNT-5A is able to evoke signaling at FZD_6 leading to the uncoupling of G proteins from the receptor. Interestingly, our FRAP analysis also revealed that the FZD_6-R511C mutant is not able to form stable receptor-G protein complexes, consolidating and confirming the results obtained in paper IV. The result provides further indication that aberrant WNT-

G protein signaling might be an important factor in the development of the nail dysplasia phenotype.

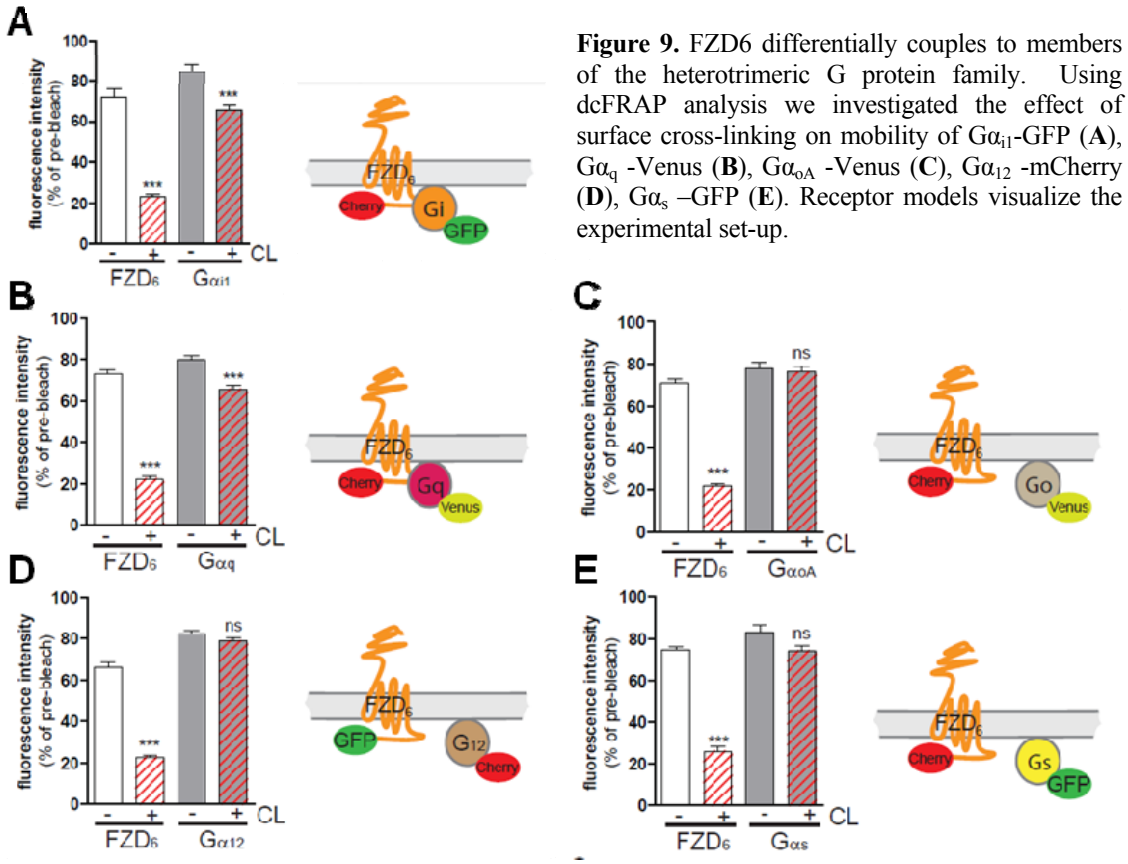

Figure 9. FZD6 differentially couples to members of the heterotrimeric G protein family. Using dcFRAP analysis we investigated the effect of surface cross-linking on mobility of $G\alpha_{i1}$-GFP (**A**), $G\alpha_q$ -Venus (**B**), $G\alpha_{oA}$ -Venus (**C**), $G\alpha_{12}$ -mCherry (**D**), $G\alpha_s$ –GFP (**E**). Receptor models visualize the experimental set-up.

The R511C point mutation is located eight amino acids from the PDZ-ligand domain in the C-terminal direction and in a part resembling a basic region conserved in several Class A 7TM receptors. The presence of an additional Cys in this particular region might cause distortions in secondary and tertiary structures required for G protein coupling at FZDs. The direct molecular interaction of FZD with DVL has been studied intensely and current results using peptide interaction scanning methods shows that apart from the PDZ-ligand domain in the ICD, DVL-DEP domain also engages a discontinuous motif in ICL3 for interaction with FZDs. Moreover, DVL membrane localization is stabilized by pH-dependent electrochemical interactions between positively charged moieties in the DVL C-term and negative charges on membranous acidic lipid molecules. Interaction with electronegative plasma membrane was also found to be a requirement of M_3R-$G\alpha_q$ preassembly, showing that localization patterns of both G proteins and DVL are sensitive to changes in pH. Despite the well-established fact that molecular interaction between FZDs and DVL exists, the interaction is difficult to quantify. For example, pull down of FZD and mass spectrometric analysis of FZD-associated proteins have so far been unsuccessful in detecting DVL. Thus, in the present study, we used the dcFRAP technique to circumvent disruption of the native cellular milieu. Biotin-avidin-mediated immobilization of FZD_6-mCherry in HEK293 cells coexpressed with DVL2-GFP

revealed that approximately 40% of fluorescently tagged DVL molecules are affected by the cross-linking of FZD_6, thus indicating a strong association between these two proteins. Further, our FRAP data showed that the FZD_6-R511C protein retains the ability to interact with DVL, an interesting result with regard to the close proximity of the Arg to Cys mutation to the KTCTEW domain of FZD_6. The fact that the mutated receptor is unable to couple to G proteins, but on the other hand can associate to DVL raises the question of whether it is the disturbance in the balance between different signaling branches or the total impairment of signaling ability due to the loss of a crucial signaling component that ultimately results in a diseased phenotype. Moreover, with the present data at hand the redundancy in the requirement of specific protein-protein interaction domains at DVL and FZDs can be questioned. Computational predictions and biochemical experiments have identified the FZD region which interacts with G proteins and DVL. The intriguing fact that these interaction sites virtually are overlapping pushed us to investigate the role of DVL in the FZD_6-$G\alpha_{i1}$ and FZD_6-$G\alpha_q$ protein interactions. A few models of the FZD-DVL-G protein molecular interplay have been postulated, such as e.g. a DVL-G protein competition model; a DVL-G protein temporal model (of fast G protein signaling and slow DVL communication) and a DVL-G protein compartment model (G proteins and DVL resides in different regions/receptors at the plasma membrane). According to or dcFRAP results either panDVL RNAi silencing or overexpression of DVL1-3 caused severe impairment of G protein coupling, indicating a crucial role of DVL in the FZD-G protein precoupling process. Not only was DVL found to be required but, moreover, the balance of DVL protein concentration is an essential factor (fig. 10). Such a molecular relationship has not been described earlier and might explain the difficulty in observing WNT/G protein signaling when working with DVL-overexpression systems. Control experiment using the cannabinoid 1 receptor (CB_1R)-$G\alpha_{i1}$ precoupling pair confirmed that the DVL effect indeed was receptor-specific for FZD_6. The DVL protein contains three distinct domains: the N-terminal DIX domain involved in DVL (or Axin) oligomerization; the PDZ domain interacting with the FZD KTxxxW region; and the C-terminal DEP domain responsible for plasmamembrane and FZD-ICL3 engagement. Previous studies have shown a possible interaction between the DVL-DEP domain and the $G\beta\gamma$ complex perhaps suggesting a role for DVL as an AGS class III signaling factor. We thus investigated the requirement of the different DVL domains in the stability of the FZD-G protein complex. Using DVL3 deletion mutants we observed impaired FZD_6-$G\alpha_{i1}$/$G\alpha_q$ precoupling only in the experiments with DIX domain-containing mutants. This result is interesting since the DIX domain, thus far, mainly has been implicated in β-catenin dependent pathways where DVL oligomerization was found to be required for proper signaling. Moreover, since the DIX domain was found dispensable for FZD_5-DVL1 interaction, the DIX-mediated effects on FZD_6-$G_{i/q}$ precoupling is probably not reliant on direct receptor binding. On the other hand we can see a clear membrane recruitment of DVL3-DIX mutant in the presence of overexpressed FZD_6. Alternatively, the overexpression of DIX domains capable of protein multimerization might generate an intracellular mesh of DIX domain-endogenous DVL molecules at the plasmamembrane, thereby creating distortions in the spatial requirements for FZD_6-G protein precoupling. The DIX domain is also shown to

mediate LRP6 clustering via activation and interaction with PIP5K. Induced clustering of LRP6 could potentially disturb FZD_6 complex arrangement and G protein precoupling. Yet, in order to fully understand the role of DVL and the DIX domain in FZD_6-G protein interactions more studies need to be performed analyzing stoichiometry and dynamics between the molecules.

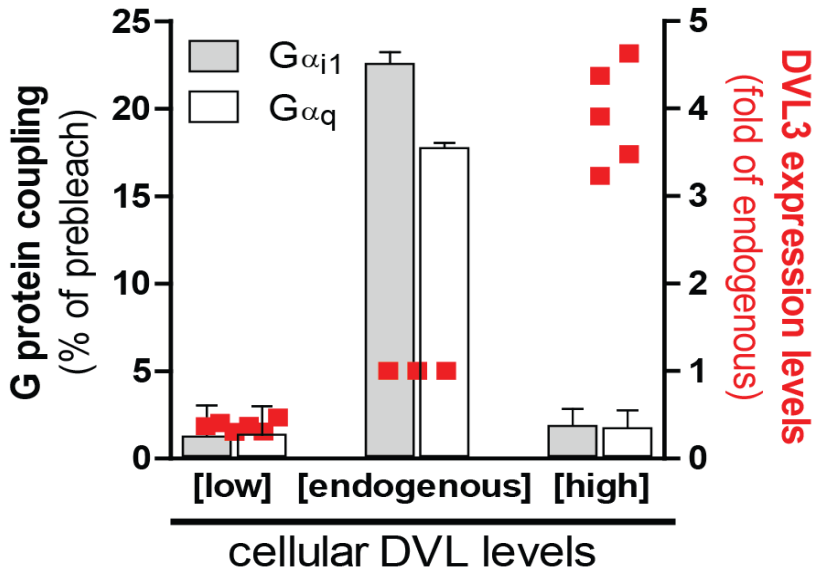

Figure 10. FZD_6-$G\alpha_{i1}$/$G\alpha_q$ precoupling is affected by changes in DVL protein levels. Receptor-G protein coupling efficiencies were evaluated using dcFRAP analysis of cells with high DVL concentration (cotransfected with DVL3-FLAG), endogenous concentration (no cotransfection) and low concentration (pan DVL siRNA transfection).

In order to establish the functional outcome of DVL concentration-dependent FZD_6-G protein coupling we measured WNT-5A-induced ERK1/2 activation. Even though precoupling was perturbed at low/high DVL levels, the collision coupling mode of GPCR-G protein interaction and activation might be functional. However, as we could deduce from our results, cell subjected to either PTX treatment, DVL RNAi silencing or DVL1 overexpression did not induce ERK1/2 phosphorylation when stimulated with WNT-5A. Collectively, the results show that precoupling of receptors and G proteins, rather than collision coupling, is the limiting factor in signaling transduction at FZD_6.

5 GENERAL DISCUSSION

WNT signaling governs important processes throughout the lifespan of metazoans, such as embryonic development and tissue homeostasis and its role in human disease is now well established. Thus the WNT signaling system holds valuable therapeutic potentials. Moreover, FZDs belong to the GPCR protein superfamily and the 7TM receptors are the most common targets of drugs today on the market. Developing compounds to target specific levels of and events in the WNT signaling pathways would be desirable. However, the study of WNT/FZD signaling pharmacology is cumbersome to perform due to several factors:

i) Agonist complexity - the WNT proteins are complex molecules with high degree of hydrophobicity and unspecific interactions with the ECM. Hence, in a physiological context, the concentrations at which WNTs exert their activities are difficult to deduce. Additionally, owing acylations and glycosylations, WNTs are difficult to purify and isolate in active and functional form. Typically, in a given preparation of recombinant WNT there is currently no possibility of knowing the exact concentration of active WNT. Thus, measurements of affinity, efficacy and potency using endogenous WNT proteins at the cognate receptors are not possible to conduct at this stage.

ii) Receptor complexity – the diversity of WNT signal activation at the plasmamembrane is a fact: currently 15 different proteins have been identified as receptors or co-receptors in WNTs signaling pathways. Since experimental results indicate that various combinations of WNT receptors/co-receptors are able to respond to a specific WNT isoform, the receptor profile of the cell/tissue under investigation must be established in order to deduce which components to target. Additionally, transmembrane proteins are difficult to isolate in their native and active state and thus the investigation of WNT to receptor signal transduction is a slow process. Knowledge concerning which pairs of receptors and co-receptors are needed for WNT activation is indeed limited.

iii) FZDs are unconventional GPCRs – despite the 7TM receptor structure, conserved and essential G protein-binding motifs present in Class A receptors are lacking in FZDs. Attempts to obtain FZD crystal structures or isolated FZD proteins has so far been unsuccessful. Moreover, G protein activation downstream of FZDs can happen in non-classical manners, such as Go-mediated β-catenin signaling, in which DVL and Axin participate in G protein interaction. Assessment of FZD isoform-specific activities is a cumbersome process since the vast majority of known cell types endogenously express several FZD isoforms.

Considering the above factors, it is clear that current and common pharmacological methods and tools, in most cases cannot be employed. The study of WNT/FZD/G protein signaling requires the use and development of alternative approaches. In the work comprising this thesis both classical methods, such as [γ-^{35}S]GTP assay, as well

as novel techniques, e.g. dcFRAP and receptor immobilization, were utilized in order to gain new knowledge into the properties of FZD signal transduction.

As mentioned earlier, the notion of the receptors of Class F being bona fide GPCRs have been the source of a long-standing debate within the WNT field even though sound and compelling data strongly pointed out the connection between FZDs and G proteins (Ahumada et al., 2002; Liu et al., 2001; Liu et al., 1999; Sheldahl et al., 1999; Slusarski et al., 1997a). Recent work of other research groups as well as the results presented in this study, further provides direct evidence of WNT-induced and FZD-mediated G protein signaling, thus confirming previous observations and establishing FZDs as true GPCRs. It is, however, evident that FZD signal transduction is different compared to the conventional pattern of signaling outlined for Class A 7TMRs. The recently solved SMO crystal structure suggests that, even though the motifs essential for G protein signaling in Class A receptors are missing, alternative primary and secondary structures can compensate to mediate G protein interaction. Thus, we begin to realize that the structure-function features of GPCRs are much more diverse than was previously thought. FZDs, as stated earlier, have unique surfaces for protein interaction. The intracellular part contains specific motifs shown to interact with DVL and these sites overlap with the predicted points of G protein contact. Our present results show that $G\alpha_{i2/3}$ signaling, presumably via FZD_5, is disturbed by the presence of DVL as observed by "contaminated" plasma membrane fractions and by the addition of recombinant DVL (fig.11). Since it was shown that FZD_5 engages DVL by motifs in the third ICL as well as by the C-terminal KTxxxW, it is reasonable to suggest that competition between DVL and G_i for the interaction with FZD5 exist. However, this model might not be true for other FZD isoforms. E.g. FZD6 and FZD3 lack the DVL1-DEP-C domain-binding motif I in the ICL3 which is otherwise conserved among Class F receptors. Thus, it was postulated that the loss of motif I in FZD_6 and $_3$ could result in weakened binding or alterations the position of DVL at these receptors. Since FZD_6 and FZD_3 both are strongly connected with PCP/CE signaling, this less rigid DVL interaction might favor β-catenin-independent signaling over the β-catenin-dependent pathway. Moreover, a flexible binding of DVL to FZD, as seems to be the case of FZD_6, could perhaps allow simultaneous DVL and G protein binding. In the light of our finding that DVL presence is needed, albeit only at an endogenous concentration, in order for FZD_6-$G_{i/q}$ precoupling to occur, it can be concluded that different FZD isoforms might employ dissimilar models of DVL/G-protein signal transduction. Applying the previously postulated models of FZD-G protein-DVL signaling mechanisms, one can speculate that FZD_5 belongs to a category of FZDs which employs a DVL-G protein competition type of signaling model, while FZD_6 might form DVL-G protein signalosomes in which the binding of DVL will confer a stabilizing effect on FZD-G protein precoupling. Consequently, the results could be indicative of a compartment model of FZD_6 signaling complex assembly, i.e. dimers of FZD_6 are formed in which one receptor couples to G proteins and the other to DVL. Functional signaling and G protein precoupling would then be dependent on an intracellular and DVL-mediated dimerization of FZD receptors. In order to confirm this notion, more information regarding the molecular stoichiometry of the involved

proteins needs to be gathered. Interestingly, the fact that the FZD_6-R511C mutant can interact with DVL but is deficient in G protein precoupling could further suggest that $G\alpha_{i/q}$ proteins preferentially engages FZD_6 at the C-terminal helix VIII, while DVL association mainly is connected to motif II in ICL3.

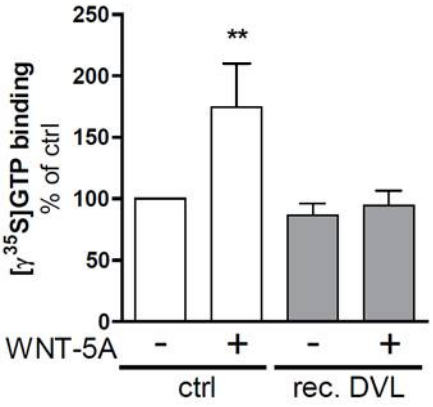

Figure 11. WNT-5A induced incorporation of [γ-^{35}S]GTP in N13 plasma membrane fractions is blocked by the addition of recombinant DVL.

Current data dictates a preponderant role of $G_{i/o}$ in WNT/FZD signaling. However, in accordance with *in silico* prediction of FZD-G protein interactions, we also observe $G\alpha_q$ precoupling at FZD_6. The kinetics of heterotrimeric G protein complex dissociation has been reported to occur faster at $G\alpha_q\beta\gamma$ complexes than at $G\alpha_i$, and if this is the case at FZD_6 proteins, it could provide an interesting feature to investigate in respect to WNT functional selectivity. Considering WNT isoform-specific abilities in FZD_6 mobility induction, it could be postulated that fast performing WNTs (i.e. WNT-2B and WNT-7A) might favor $G\alpha_q$ activation over $G\alpha_i$. The connection between $G\alpha_q$ and WNT-7A could further be established by the fact that $G\alpha_q$ activation readily is linked to β-catenin signaling, which also is the pathway typically induced by WNT-7A. However, the finding that $G\alpha_o$ proteins are activated downstream of WNT-7A-induced FZD_6 signal transduction when FZDs are ectopically expressed in E. coli, points out the demand for additional data in order to understand the nature of WNT isoform-specific G protein subtype bias.

In the concept of biased agonism, receptor phosphorylation patterns by GRK activity at intracellular regions seem to play an important part in dictating pathway preference, especially with regard to β-arrestin functional activity (Liggett, 2011; Nobles et al., 2011). It was found that upon agonist stimulation β_2-adrenergic receptors (β_2AR) were targeted by GRK2 or GRK6 to induce changes in β-arrestin conformation leading to either receptor internalization or ERK1/2 activation respectively. The phosphorylation pattern or barcode seems dependent on the nature of the ligand since it was shown that β-arrestin biased ligands induced distinctly different phospho-barcoding of β2ARs compared to unbiased agonists (Nobles et al., 2011). Moreover the type of GRK also dictates the magnitude and duration of receptor- β-arrestin interaction. The receptor phosphorylation-related mechanisms of biased ligand hold important pharmacological properties and are currently under intense investigation, albeit primarily for class A 7TMRs. Analyzing phosphorylation barcodes in the unconventional mode of

WNT/FZD-induced and DVL-dependent signaling to β-arrestins and G-proteins would be highly interesting. Since FZD internalization seems ligand- as well as FZD-subtype-dependent, e.g. WNT-5A induces FZD_4 internalization while endocytosis of FZD_6 never was observed regardless of the WNT used, it would be valuable to investigate how FZDs interact with specific GRKs. Moreover, will a specific FZD phosphorylation pattern affect DVL conformation and functional activity in the same way that β_2-AR phosphor-barcode directs β-arrestin signaling?

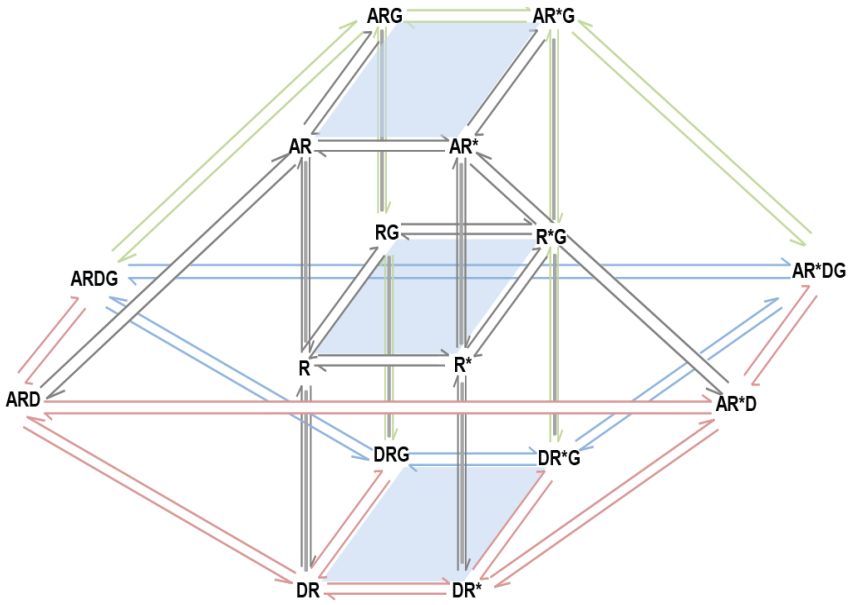

Figure 12. Hypothetical quaternary complex model representing the different states of FZD: R activation and molecular interactions with WNTs: A, G proteins: G and DVL: D. Dimerization states have been omitted. Illustration by J. Petersen.

Collectively, the current data investigating signal transduction at FZD proteins clearly describes the presence of a mechanistic interplay between FZDs, G proteins and DVL, and further that the relationship is dynamic in response to WNT-stimulation. Therefore, we propose that a quaternary complex model (fig. 12) of FZD signal transduction should be used as a platform for future investigation of WNT signaling kinetics, affinities and efficacies, and by extension in order to establish the mechanisms of biased agonism in the WNT/FZD signaling system.

6 SUMMARY & CONCULSIONS

Paper I

WNT-5A mediated signal trafficking leads to GDP to GTP exchange at the Gα subunit of PTX-sensitive heterotrimeric G_i proteins in microglia-like cells at endogenous protein stoichiometry.

Receptor/G protein repertoire in N13, as assessed by PCR, marks FZD_5-$G\alpha_{i2/3}$ as the signaling components most likely responsible for transducing the WNT-5A effect.

PaperII

Assessment of WNT signaling profiles of 6 different recombinant WNTs using N13, a defined cell type with a unique set up of WNT-signaling components, as a readout platform reveals isoform-specific signaling features:

-Only WNT-3A was able to signal in β-catenin dependent manner.

-All WNTs activated G proteins; however the WNTs most efficacious in G protein activation (-5A and -9B) also caused cell proliferation.

-All WNTs tested caused phosphorylation of DVL, showing the central importance of this protein in WNT-mediated cellular signaling.

Taken together, these results suggest that WNTs could act as endogenous biased agonists modulating cellular responses according to the respective ability of the isoforms to favor a specific subset of signaling components.

PaperIII

Two mutations in FZD_6, E581X and R511C, were found to be pathogenic and responsible for the isolated autosomal-recessive nail dysplasia disease phenotype.

The FZD_6 mutant receptors were deficient in β-catenin-dependent and –independent signaling but retained the ability to recruit DVL despite the proximity of the R511C missense mutation to the PDZ-ligand domain.

The aberrant WNT signaling is most likely a consequence of the severely disturbed subcellular distribution pattern. Compared to wild-type FZD_6, the R511C mutant shows strong tendency to be confined to lysosomal vesicular compartments.

PaperIV

Using FRAP to monitor FZD_6-GFP lateral diffusion, we were able to detect direct WNT-induced effects on the mobility of the receptor protein. Thus, we establish a novel approach to investigate proximal WNT-signaling events.

FRAP assessment revealed that receptor mobility was increased upon WNT-5A stimulation. The effect was PTX-sensitive but could be rescued by expression of the

$G\alpha_{i2}$C352I PTX-resistant mutant. Collectively, results suggest the involvement of $G_{i/o}$ proteins in the WNT-5A-induced FZD_6 mobility change.

FRAP-based FZD_6 mobility analysis also exposed WNT isoform-specific abilities in receptor mobility induction, showing that the assay is sensitive enough to detect differential signaling patterns of WNTs.

PaperV

FRAP analysis using a dual fluorophores- and cell surface cross-linking-approach reveals specificity in FZD_6 precoupling to heterotrimeric G proteins. G_i and G_q were precoupled but not G_o, G_s and G_{12}.

WNT-5A stimulation as well as PTX-pretreatment effectively causes dissociation of the FZD_6-Gi complexes. WNT-5A stimulation also leads to dynamic changes in FZD_6-G_q molecular complexes.

Both the wild type FZD_6 and the FZD_6-R511C mutant show strong DVL interaction, but the missense mutated receptor is deficient in G protein precoupling.

DVL is a master regulator of FZD-G protein coupling since both RNAi silencing and DNA plasmid overexpression of DVL severely disrupts the G protein-FZD_6 interaction. Thus, there is a fine-tuned mechanistic relationship between FZD/G protein- and FZD/DVL-signaling.

Thesis conclusion

Collectively, the work of this thesis shows that WNTs activate heterotrimeric G proteins both in overexpression systems and under native conditions. Further, it establishes FZD as the intermediate signaling molecule responsible for relaying WNT signals to G proteins. Moreover, the studies comprising this thesis point to a novel model of mechanistic relationship between 7TMRs and G proteins in which the precoupling complex is reliant on the local concentrations of a third party molecular component. Thus, with the present data at hand a quartenary complex model, consisting of WNT-FZD-G protein-DVL, is purposed in order to explain the interactions observed. However, the presence of a FZD dimer within this complex can also be suggested. This thesis work additionally shows the possibility of biased agonism in the WNT signaling system, a property which holds great opportunities in the pursuit to develop new drugs to target human disease.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Cell till cell kommunikation, även kallad cellulär signalering, är grunden till allt multicelluärt liv och bygger på tre huvudsakliga steg. Steg ett: en meddelande- eller signalmolekyl, t ex ett hormon eller en neurotransmittor, utsöndras från en cell för att sedan påverka samma cell och/eller andra celler runt om i kroppen. Steg två: vid cellens yta interagerar signalmolekylen med en mottagarmolekyl som sitter inbäddad över cellens hölje, det s k plasmamembranet. Mottagarmolekylen, vilken kallas receptor, översätter signalmolekylens meddelande och i och med detta kan nästa steg initieras. Steg tre: receptorn för vidare meddelandet genom att aktivera verkställande effektormolekyler inuti cellen vilket ofta leder till förändringar i cellens mognad eller funktion, till celldelning eller cellvandring, samt även ibland till celldöd. Farmakologiskt är steg två i den cellulära signaleringsvägen av störst betydelse och de flesta läkemedlen på marknaden idag verkar på receptorns förmåga att översätta signalmolekylens meddelande.

WNT-signalering är en typ utav cellulär signalering som är mycket viktig vid fosterutveckling och vävnadsbalans i vuxna individer. På grund av detta kan felaktig WNT-signalering leda till allvarliga sjukdomar, så som cancer och neurodegenerativa tillstånd. Steg ett i WNT-signalering utförs av en grupp av 19 närbesläktade proteiner som går under namnet WNT. I steg två interagerar WNT främst med en klass av receptorer som kallas Frizzled (FZD). Klassen FZD består av 10 proteiner som i steg tre interagerar med och för över WNT-meddelandet till två typer av effektorproteiner: 1) heterotrimera G proteiner – ett vanligt förekommande signalkomplex bestående utav tre olika delproteiner (α, β och γ), och 2) Disheveled (DVL) – ett stort multifunktionellt stödstruktursprotein främst knuten till WNT signalering. På grund av likheter i strukturellt utseende kategoriseras klassen FZD som G protein kopplade receptorer (GPCRs), en proteinfamilj som, vilket namnet antyder, utmärks av deras förmåga att kommunicera med heterotrimera G proteiner. Trots deras klassificering finns det en debatt kring huruvida FZDs verkligen är sanna GPCR proteiner eftersom biokemiska bevis som stödjer en GPCR-hypotes länge har saknas.

Arbetet i denna avhandling har haft som mål att undersöka kopplingen mellan FZD och heterotrimera G proteiner och att föra fram nya bevis som kan understödja idén om att FZD fungerar som klassiska GPCRer, som t ex adrenalin- och serotoninreceptorer. Resultaten från avhandlingens studier visade att WNT aktiverar G proteiner och att FZD_6 kan binda G proteiner, men endast vissa subtyper av G proteiner. Som nämnts tidigare, utför FZD cellulär signalering genom både G proteiner och DVL, ett faktum som är svårtförklarat då receptorns kontaktytor för dessa två effektorproteiner nästan helt överlappar varandra. Analys av det molekylära och funktionella förhållandet mellan FZD_6, DVL och G proteiner visade att kopplingen mellan FZD_6 och G proteiner, samt WNT-signaleringen genom FZD_6, krävde närvaron av DVL. Dessa resultat poängterar att klassen FZD har vissa egenskaper som skiljer sig från konventionella GPCRer och påvisar förekomsten av en tidigare okänd cellär signalöverföringsmekanism. Slutligen har arbetet i denna avhandling identifierat en mutation i FZD_6 proteinet som ger upphov till en ärftlig nagelsjukdom kallad Autosomal recessiv nageldysplasi. Intressant nog visade det sig att muterade FZD_6 proteiner kunde binda till DVL men var oförmögna att koppla till G proteiner.

8 ACKNOWLEDGEMENTS

The work comprising this thesis would not have been possible without the support and companionship that I have been given during my time at the Department of Physiology and Pharmacology, Karolinska Institutet, Sweden. I would like to extend my most sincere appreciation to all the people who have contributed and helped me throughout the process of completing this thesis. Especially I would like to thank:

My supervisor **Dr. Gunnar Schulte**, your scientific knowledge and sharp mind has really been most inspiring to me during my doctoral studies. Your never-ending enthusiasm for research in general is very contagious! Thank you for all the support and commitment you have given me and my project, for giving me the liberty to work independently, for believing in my abilities when I have been in doubt, for giving me the opportunity to work and experience the international science scene and for introducing me to the research community. I am truly grateful to have had you as my supervisor.

My co-supervisor **Dr. Vitězslav Bryja**, for your profound skill in research and your keen understanding of the scientific process. Thank you for successful collaborations and your always insightful comments. Most of all I would like to thank you for welcoming me into your lab in the Czech Republic, it was truly a memorable experience!

My co-supervisor **Prof. Rune Toftgård**, for your deep and robust experience in science and for supporting me in my scientific career.

My mentor **Dr. Mia Göransson Emgård**, you are in a way responsible for my current situation… ☺. You really inspired me to go out and do research! Thank you for all the valuable advice and for teaching me the foundations of scientific work.

Prof. Bertil B Fredholm, your expertise in science is truly an asset and your dedication to science is a source of great inspiration. Thank you for always providing valuable advice.

The head of the Department of Physiology and Pharmacology **Prof. Stefan Eriksson**, for providing a creative, secure and good working environment.

A big Thank you goes out to all the amazing past and present members of the Schulte group: **Carina Halleskog**, we have been through a lot together and I thank you for being such a good friend and wonderful person. I really appreciate your warmth! **Jacomijn Dijksterhuis**, you have been a great friend and colleague, always encouraging and insightful. We've sure had some fun and memorable times in and outside of the lab, thank you! And, Carina and Jacomijn, I'll always think of us three as "Schulte's Angels"… ☺. **Eva Lindgren**, I cannot express how much I appreciate your

support during stressful times. I will never forget your understanding nature and your concern. You are a great colleague and friend. **Julian Petersen**, your laugh is contagious! Thank you for a great collaboration, fun traveling and for being such a positive and intense person, I think you really will accomplish great things in research. **Elisa Arthofer**, I really have enjoyed your company and your humor. Thank you for fun discussions and I must say that you are great with the video camera! **Jenny Dahlström**, thank you for fruitful collaborations and for being a great companion in work-related situations and in out-side-the-lab activities! I truly believe that you'll be a very accomplished MD/PhD! **Tilman, Tobias, Natália**, thank you for making the lab a fun place, you all have given me many great memories.

A big thank you to my collaborators: **Niklas Dahl, Ann-Sofie Fröjmark, Jens Schuster, Maria Sobol, Miriam Entesarian, Dana Gabrikova, Sadia Nawaz,, Shahid Baig, Schulte, Joakim Klar, Kjetil Wessel Andressen and Finn-Olav Levy.**

Dr. Andrei Chagin and the members of his group, **Karuna**, **Phillip** and **Thibault**, you are truly good corridor neighbors and thank you for providing a very positive working atmosphere.

To past and present PhD-students, co-workers and friends at FyFa: **Sara, Torun, Anna, Amanda, Magdalena, Aki, Maria, Louise, Sandra, Carl, Lars, Åsa, Cecilia, Olivia, Marlene, Daniel, Markus, Klas, Ellinor, Jens, Frank, Martin, Therese, Olga, Björn, Tianle, Mingmei, Jangning, Yinxing, Ina, Andreas, Michael, Sabina, Katrin, Guo, Mi-Young, Devesh, Gustaf, Petra, Eva-Karin, Susanna, Ebba, Inger, Meta, Annika and Caina,** thank you for your support, for sharing equipment and for making this department a fantastic place to work at. Also, I will always remember the fun parties, enjoyable running events, dinners and beautiful Lucia performances…

I am also very grateful for all the sympathy, support, help and patience I always have received from the administrative staff at FyFa: **Camilla F-H, Eva G, Monica P-S, Freddie H, Ylva H, Sarah L, Rene R, Sofia P and Ulla W**. And a huge thank you to **Micke E, Peter W, Renee A and Eva N,** for keeping the wheels of this department turning, without your help my work would not have been possible to complete.

Past and present members of the Bryja lab: **Ondra, Marki, Ranjani, Alena, Igor and Janek,** thank you for introducing me to Brno and the Czech way of life, for beers, climbing, slack-lining, Czech punk music and parties. I will never forget the anarchistic atmosphere at Řečkovice, it was great times!

All the great people I had the opportunity to meet at SU, especially: **Petra, Mia, Stefan, Hans G, Sara, Miche, Shavon, Robert, Roger, Milli, Jeanette and Gustavo.** "Grodorna" at Gula villan/Greens villa, in particular: **Lina, Lisa, Ylva, Lena, André, Linus, Malin, Simon, Karin, Sarah, Vera, Olle, Pavve, Anna, Lillan, Disa, Malin, Klas, Rickard and Fredrik** studying have never been more fun with all of you around. Thank you for some crazy good memories!

The study mates at Forskarskolan, especially: **Katta, Amanda, Pablo, Kalle, Olle, Christoffer, Sara, Annika, Martina and Érik,** it has been a blast getting to know you, to travel with you and to share the pride and joy of being a science nerd with you.

Carro, thank you for coming to Sweden! It was great working with you in the lab, you have given me lots of memorable moments. I really had fun listening to rock, drinking beer and making jokes about Swedish men with you.

Thank you **Teres and Sonny**, for showing me the great benefits of creative anachronism (and anarchism), for great times at Långholmen, Visby and Högalidsgatan.

My dear friends from Viktor Rydbergs Gymnasium: **Emelie**, I'm happy that we and our families have shared many great moments (and cats!) together, you are a fantastic "sambo" and diving-buddy, so grateful for your friendship! Wish you and **Olov** all happiness, you really are good together! **Rebecka**, I am forever a big fan of you, Lebecca you're clazy…☺, I will never forget our back-packing in Asia, looking forward to many more marathon races with you! **Hedvig**, you are such a great friend, you always put me in a good mood, although I had no chance beating you at MMA, guess **Filip** is a better match for you (hihi)...

My beautiful **Beatrice**, you are an amazing woman! I'm glad we found each other in the dungeon at MF, working late at the parties and the pubs was a blast with you around. I cherish all the memories you have given me.

Daniel, you crazy ninja! I'm happy for your friendship; I hope you know how much it means to me. Thank you for long discussions about life and nonsense… Long live the incredible Ulk!

Jenny, you are my role model, I have so much to thank you for, I don't know where to start and finish… I can truthfully say that I wouldn't have been the same person without you.

Sifu Yang Chungui, du är en underbar vän och mentor. Din syn på livet och din värme har varit ett stort stöd för mig. Tack för allt du gör för mig och min familj!

Ylva, min gudmor, thank you for your warmth and for the times we spent together, hope for many more in the future.

To my family, all my cousins: **Anna, Johan, Kalle, Klaes, Henrik och Amanda**, thank you for all the happy memories and I am so glad that we still stay together. My uncles and aunt, **Larsa, Uffe and Pärlan** and aunts in-law, **Lottie and Pajsan**, I thank you for always supporting me and providing me with a strong sense of family.

Min fina **mormor**, kan inte säga hur mycket du har givit mig genom åren, du betyder så mycket för oss alla. Älskar dig!

My brothers and my sister, **Erik, Alex and Stephanie**, you are simply the best siblings, I could never wish for any better. All the fun we've had, you are my foundation and I cannot imagine life without you. To **Karin and Malte**, I'm so happy that you are a part of my life and I hope we get to see each other very frequently in the future!

Pappa, thank you for always being there for me, supporting and helping me to grow. I have the deepest appreciation for you.

Mamma, my dearest, thank you for everything you have given me, you have taught me so much in life and I cannot find any words that ever could describe my love for you. Älskar dig alltid!

Javier, I'm so happy that we found each other, thank you for always being you, for your warm and humoristic mind and for putting up with me and my peculiarities… te amo!

And those of you who are not forgotten, but not mentioned, you know that I appreciate all the support you have given me… man tackar!

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