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**MOLECULAR ASPECTS OF
WNT/FRIZZLED
SIGNALLING IN BRAIN ANGIOGENESIS**

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MOLECULAR ASPECTS OF WNT/FRIZZLED SIGNALLING IN BRAIN ANGIOGENESIS

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*To raise new questions, new possibilities, to regard old problems from a new angle,
requires creative imagination and marks real advance in science.*

- *Albert Einstein*

ABSTRACT

The family of WNT lipoglycoproteins consists of 19 secreted proteins that are important for multiple cellular processes including cell proliferation, migration and fate. WNTs induce signalling cascades by binding the seven transmembrane receptors named Frizzleds (FZDs) classified as G protein-coupled receptors (GPCRs). Binding of WNT proteins to FZDs leads to the activation of either β -catenin-dependent and/or β -catenin-independent pathways. Mutations or misregulation in the WNT signalling components can cause severe developmental defects, and disorders such as cancer.

During embryonic development WNT/FZD signalling is one of the key regulators of vascular development in the central nervous system (CNS). Ablation of the transcriptional activator β -catenin resulted in haemorrhage throughout the entire CNS. Moreover, deletion of one of the WNT receptors, FZD₄, also exhibited vascular defects, which were mainly localised to the retina.

In this thesis we investigated the process leading to CNS haemorrhage by creating a novel mouse model where β -catenin was inhibited. By overexpressing AXIN1, one of the major components of the β -catenin destruction complex, we could conclude that inhibition of β -catenin in CNS endothelium increased vessel regression and remodelling. Furthermore, we identified an extracellular matrix protein, ADAMTS-Like Protein 2 (ADAMTSL2), to be important for proper vascularisation of the brain. In addition, RNA sequencing data of the embryonic forebrain endothelial cells provided us with the FZD expression profile revealing that FZD₄ and its close homolog FZD₁₀ are present during early blood vessels development.

Even though FZDs are classified as GPCRs, the question as to whether they can activate G proteins has been under the debate. Despite sequence homology indicating a structure similar to other classical GPCRs, the evidence that they can signal through G proteins has been limited due to a lack of pharmacological tools and robust read-out assays.

Here, we show for the first time that the two WNT receptors, FZD₄ and FZD₁₀ belonging to the same FZD homology cluster, form inactive-state complexes with the $G\alpha_{12/13}$ proteins. Moreover, we show that FZD₄, the receptor previously connected to vascular malformations is able to induce $G\alpha_{12/13}$ -dependent recruitment of p115-RHOGEF, suggesting a novel FZD₄/ $G\alpha_{12/13}$ /RHO signalling axis. In addition, we demonstrate that FZD₁₀ has a selective preference for $G\alpha_{13}$ over other G protein family members. While FZD₁₀ is able to activate β -catenin transcriptional activity in the presence of overexpressed LRP6, FZD₁₀ triggers $G\alpha_{12/13}$ -dependent transcriptional activity of Yes Associated Protein 1 (YAP) and Tafazzin (TAZ) in the absence of its co-receptor. Furthermore, we confirm by *in situ* hybridisation that as indicated in the RNA sequencing data, FZD₁₀ mRNA is indeed present in the developing CNS endothelium.

Much like WNT/ β -catenin signalling also $G\alpha_{12/13}$ dependent signalling is crucial for proper vascular development. With that in mind and with the data presented in this thesis,

I would like to propose a parallel route for WNT/FZD induced signalling during CNS vascular development, mediated through the $G\alpha_{12/13}$ proteins.

LIST OF SCIENTIFIC PAPERS

I. **The WNT/ β -catenin axis modulates early BBB development by fine-tuning TGF- β signalling through ADAMTSL2**

B. Salagic Hot, L. Dahl Jensen, D. Ramsköld, C. Yokota, S. Giatrellis, V.M. Lauschke, M.X. Li, M. Hupe, T.D. Arnold, R. Sandberg, J. Frisé, A. Martowicz, J. Wisniewska-Kruk, D. Nyqvist, R.H. Adams, J.M. Stenman and J. Kele

Manuscript

II. **WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with $G\alpha_{12/13}$**

Elisa Arthofer¹, **Belma Hot**¹, Julian Petersen, Katerina Strakova, Stefan Jäger, Manuel Grundmann, Evi Kostenis, J. Silvio Gutkind, and Gunnar Schulte

Mol Pharmacol. 2016 Oct;90(4):447-59

III. **FZD₁₀- $G\alpha_{13}$ signalling axis points to a role of FZD10 in CNS angiogenesis**

Belma Hot, Jana Valnohova, Elisa Arthofer, Katharina Simon, Jaekyung Shin, Mathias Uhlén, Evi Kostenis, Jan Mulder, Gunnar Schulte

Cell Signal. 2017 Apr;32:93-103

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LIST OF ABBREVIATIONS

ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ADAMTSL2	ADAMTS-Like Protein 2
APC	Adenomatous Polyposis Coli Protein
AXIN	Axis Inhibition Protein
BBB	blood-brain barrier
CaMK	Ca ²⁺ /Calmodulin dependent kinase
cGMP	Cyclic Guanosine 3',5-Mono-Phosphate
CK1	Casein Kinase 1
CNS	central nervous system
CRD	cysteine-rich domain
dcFRAP	double-colour FRAP
DMR	dynamic mass redistribution
DVL	Dishevelled
DKK	Dickkopf
ER	endoplasmic reticulum
ERK	Extracellular Signal-Regulated Kinase
FB	forebrain
FEVR	familial exudative vitreoretinopathy
FRAP	fluorescent recovery after photobleaching
FRET	Förster resonance energy transfer
FZD	Frizzled
GAP	GTPase-Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factor
GPCR	G protein-coupled receptor
GPR124	G protein-Coupled Receptor 124
GRK	G protein-Coupled Receptor Kinase
GSK3 β	Glycogen Synthase Kinase 3 beta
GTP	Guanosine Triphosphate
HB	hindbrain
hpf	hours post fertilization
IL3	intracellular loop 3
JNK	c-Jun-N-terminal Kinase
LEF	Lymphoid-Enhancing Factor
LPA	Lysophosphatidic Acid
LRP	Low-Density Lipoprotein (LDLR)-Related Protein
LTBP1	Latent TGF β Binding Protein1
MB	midbrain

MEF-2	Myocyte Enhancer Factor 2
MMP	Matrix Metalloproteinase
NFAT	Nuclear Factor of Activated T-cells
PAR1	Protease Activated Receptor 1
PCP	planar cell polarity
PDGF	Platelet-Derived Growth Factor
PKC	Protein Kinase C
PKE	Protein Kinase
PNVP	perineural vascular plexus
PTX	Pertussis Toxin
PVP	perivascular plexus
RAC1	Ras-related C3 Botulinum Toxin Substrate 1
RGS	Regulators of G protein Signalling
RHOA	Ras Homolog Family Member A
ROCK	Rho Associated Kinase
ROR	Receptor Tyrosine Kinase-Like Orphan Receptor
RYK	Receptor-Like Tyrosine Kinase
S1P	Sphingosine 1-Phosphophate
SFRP	Soluble Frizzled Related Protein
SMAD3	Mothers Against Decapentaplegic Homolog
SMO	Smoothened
SRF	Serum Response Transcriptional Factor
TAZ	Tafazzin
TCF	T-Cell Factor
TGF	Transforming Growth Factor
TM	transmembrane
VEGF	Vascular Endothelial cell Growth Factor
VEGFR-2	VEGF Receptor 2
WNT	Wingless/Int-1
WLS	Wntless
YAP	Yes Associated Protein 1

1 INTRODUCTION

1.1 WNT SIGNALLING PATHWAY

1.1.1 *WNT proteins*

Almost a century ago, the first members of the Wingless/Int-1 (WNT) family were described in mammals, (Bittner, 1936; Korteweg, 1936) but it took another fifty years before Roel Nusse and Harold Varmus gave the full description of the gene (Nusse and Varmus, 1982). Today, we know that the family of WNT proteins, consists of 19 secreted members able to signal both short- and long-range, serving as extracellular growth factors (Fung et al., 1985; Logan and Nusse, 2004; MacDonald et al., 2009a; van Ooyen and Nusse, 1984). The structure of WNTs enables them to act as mediators in cell to cell communication, with an N-terminal signal sequence, followed by conserved cysteines with palmitoylation sites, making them hydrophobic (Logan and Nusse, 2004; Nusse, 2005; Willert et al., 2003). Processes such as tissue patterning and homeostasis, cell migration and proliferation are all dependent on proper signalling by the WNT proteins (Nusse et al., 2008). Secreted WNTs are usually spread throughout the tissue by means of a concentration gradient, where WNT responsive cells at the same time sense the signal and respond to it, in a concentration-dependent manner (Neumann and Cohen, 1997; Zecca et al., 1996).

From synthesis to secretion and signal induction, WNT proteins undergo several modifications important for their activity (Port and Basler, 2010). In the endoplasmic reticulum (ER), the multi-pass transmembrane protein Porcupine lipid modifies WNTs (Galli et al., 2007; van den Heuvel et al., 1993; Zhai et al., 2004). With the lipid modification in place, the WNT protein is sent to the Golgi apparatus, where it binds to the seven transmembrane protein Wntless (Wls). Together with Wls, WNT is transported to the plasma membrane, before it is secreted from the cell through exocytosis (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Another post-translational modification that the WNT proteins possess is glycosylation. Glycosylation has been shown to play a crucial role both for secretion and activity of the protein (Komekado et al., 2007; Kurayoshi et al., 2007; Smolich et al., 1993).

While it is getting clearer how the WNT proteins are transported out from the cell the experimental data showing how they are transported between the cells is still poor. Lately, several hypotheses have been raised. One of these hypotheses suggests that the transfer of the WNT proteins is mediated by lipoproteins derived from body fat (Neumann et al., 2009), while another suggests extracellular transport by exosomes (Gross et al., 2012; Korkut et al., 2009).

1.1.2 Frizzleds

From neural tube closure, central nervous system (CNS) angiogenesis and blood-brain barrier (BBB) formation, to orientation of cellular structures and bone formation, the diverse roles of the main WNT receptors, Frizzleds (FZDs), clearly show their importance for proper developmental and homeostatic processes. FZDs are highly conserved among multicellular organisms and it is believed that they developed in order to allow complex tissue architecture. All FZDs, of which there are 10 in mammals, share a common structure. At the extracellular side, the receptors possess a ca. 36 amino acid long N-terminal signal sequence. Directly after the signal sequence, follows the highly conserved extracellular cysteine-rich domain (CRD) that is connected to the seven transmembrane segments by a linker region (**Fig.1**). By sequence comparison, FZDs were separated into homology clusters wherein redundancy was observed with respect to function (**Fig.2**) (Bhanot et al., 1996; Hsieh et al., 1999; Janda et al., 2012; Schenkelaars et al., 2015; Schulte, 2010; Wang et al., 2016; Xu and Nusse, 1998).

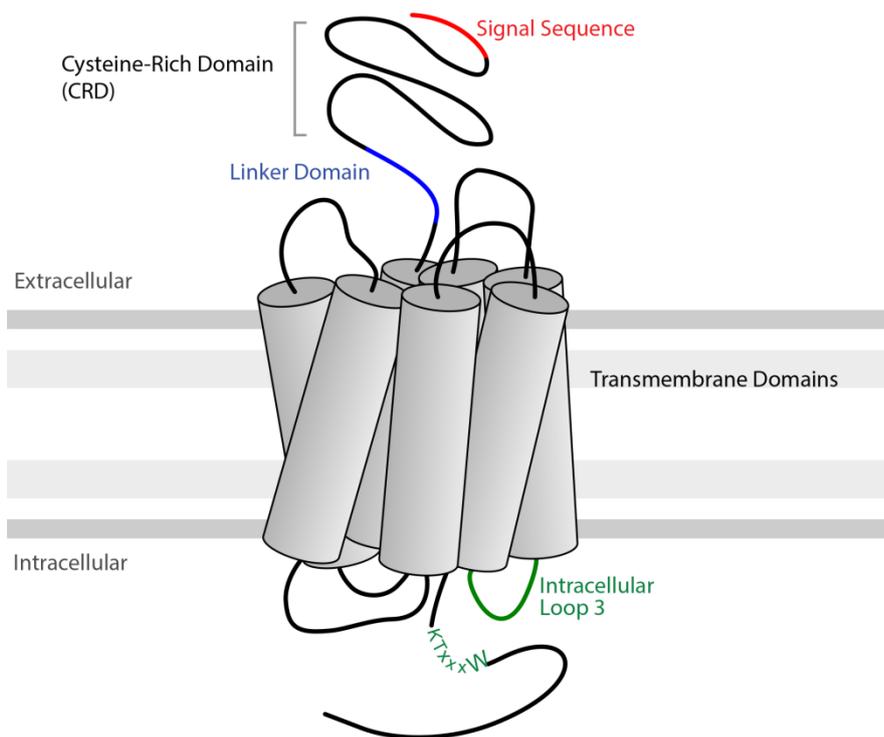


Figure 1: Illustration of the basic structure of the seven-transmembrane FZD receptor. At the extracellular N-terminus following the signal sequence is the cysteine rich domain that is in turn connected to the seven transmembrane helices by a linker region. On the intracellular side the DVL binding motif KTxxxW and intracellular loop (IL3) are visualized.

After the first sequence description, indicating the presence of seven transmembrane helices as well as an extracellular N-terminus and an intracellular C-terminus, it was suggested that this group of receptors were G protein-coupled receptors (GPCR). Recently, this receptor family was grouped into a separate class of GPCRs together with

Smoothed (SMO) and is now included in the International Union of Basic and Clinical Pharmacology (IUPHAR) GPCR list under Class F or Class Frizzled (Foord et al., 2005; Schulte, 2010; Vinson et al., 1989).

Even though FZDs are lacking some classical GPCR motifs, (Rosenbaum et al., 2009a; Wess, 1998) they share other characteristics of GPCRs like the charged residues at the C- and N-terminal ends of intracellular loop 3, known for their importance in receptor-G protein coupling. Another proposed factor important for the G protein coupling is the helix 8 at the C-terminus. Structural analysis predicts the presence of such a stretch at the intracellular end of FZDs (Foord et al., 2005; Palczewski et al., 2000; Rasmussen et al., 2007; Rosenbaum et al., 2009a; Schulte, 2010; Wess et al., 2008). In addition, the WNT receptors share several residues and the conserved KTxxxW domain for interaction with the phosphoprotein Dishevelled (DVL), which is another important component for WNT/FZD signalling (Gammons et al., 2016; Gao and Chen, 2010; Romero et al., 2010; Umbhauer et al., 2000; Wallingford and Habas, 2005; Wong et al., 2003).

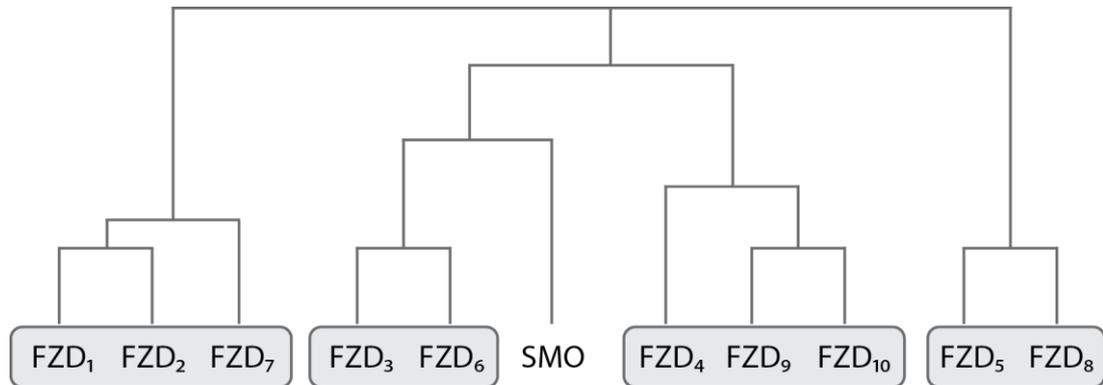


Figure 2: Schematic illustration presenting subfamilies of the class FZD GPCRs. Modified from Schulte 2010.

1.1.3 *FZD₄*

FZD₄ is the receptor that is probably best understood. In both humans and mice, the phenotypes that are caused by non-functional *FZD₄* are often connected to vascular defects. Ablation of the receptor in mice results in severe defects in retinal vasculature, but also progressive hearing loss and cerebral degeneration associated with the loss of the BBB (Wang et al., 2001, 2012; Xu et al., 2004; Ye et al., 2009). In humans, mutation in *FZD₄* cause the syndrome known as familial exudative vitreoretinopathy (FEVR), where the retina is affected by hypo-vascularization (Nikopoulos et al., 2010; Qin et al., 2005; Robitaille et al., 2002; Toomes et al., 2004).

In addition to acting as a WNT receptor, *FZD₄* is also a target receptor for the ligand Norrin. Norrin binds the *FZD₄* CRD domain and activates the downstream transcriptional activator β -catenin. Disruption in Norrin signalling is known to cause the Norrie disease in

humans. A disorder characterized by progressive hearing loss and blindness. Using Norrin knock-out (KO) mice as a model system for Norrie disease showed that the retinal phenotype observed in these animals could be rescued by stabilization of β -catenin in the developing endothelium (Luhmann et al., 2005; Rehm et al., 2002; Xu et al., 2004; Zhou et al., 2014a).

1.1.4 *FZD₁₀*

While *FZD₄* is the best studied receptor, *FZD₁₀* remains the most enigmatic despite the close homology between these two receptors. Although *FZD₁₀* is expressed widely in the CNS, no evidence has so far been reported for a *FZD₁₀* loss-of-function phenotype (Kim et al., 2001; Zhao et al., 2006). The sparse data existing for *FZD₁₀* suggest that this receptor is involved both in embryonic development and in cancer. In the developing embryo, the mRNA expression levels of *FZD₁₀* are enhanced in the CNS (Y et al., 2009). At the same time, it has been shown that the expression pattern of *FZD₁₀* mRNA in the neural tube, limb buds and Müllerian duct, overlap with the expression of *WNT-7A* (Nunnally and Parr, 2004), a FZD ligand crucial for proper development of the BBB (Stenman et al., 2008). In colorectal cancer, *FZD₁₀* has been implicated in the formation of tumours mainly through β -catenin-dependent signalling (Terasaki et al., 2002). Upregulation of *FZD₁₀* in synovial sarcoma, contrary to colorectal cancer, results in β -catenin-independent DVL-RAC-JNK signalling. Here, it plays a role in both the development and progression of synovial sarcomas (Fukukawa et al., 2009). *FZD₁₀* is also one FZD family member that has been suggested to activate heterotrimeric G proteins (Koval and Katanaev, 2011).

1.1.5 *Co-receptors and other components of the WNT pathway*

Low-Density Lipoprotein (LDLR)-Related Proteins (LRPs) are a family of single-pass transmembrane proteins of which LRP5 and LRP6 are of importance for β -catenin-dependent signalling. By forming a complex together with FZD and WNT, LRPs act as co-receptors in the regulation of WNT responsive genes (Tamai et al., 2000; Wehrli et al., 2000). Another co-receptor in the WNT signalling pathway is Receptor-Like Tyrosine Kinase (RYK). Similar to FZDs, RYK can bind to DVL and activate β -catenin-dependent signalling. Receptor Tyrosine Kinase-Like Orphan Receptor 1 and 2 (ROR1 and ROR2) are also a type of tyrosine kinase receptor involved in WNT/FZD signalling pathway. ROR2 inhibits β -catenin-dependent signalling through the binding of WNT-5A to its CRD motif (Mikels and Nusse, 2006).

Among the multiple co-receptors, there are also other regulators of WNT signalling. Soluble Frizzled Related Proteins (SFRPs) and Dickkopf (DKK) belong to the group of proteins able to inhibit the WNT signalling pathway. While the SFRPs sequester WNTs extracellularly, they have also been shown to promote signalling by stabilizing WNT proteins and facilitating their secretion and transport (Hoang et al., 1996; Logan and Nusse, 2004). The action of DKK is somewhat different to SFRPs. Here, the inhibition of

signalling is achieved by promoting internalization and inactivation of LRP5/6 (Glinka et al., 1998; Mao et al., 2002).

1.1.6 β -catenin-dependent signalling

Interestingly, β -catenin-dependent signalling is the most studied WNT signalling pathway and has historically been referred to as the "canonical" pathway. In the absence of FZD ligands, β -catenin is part of a complex named the destruction complex, consisting of Axis Inhibition Protein (AXIN), Adenomatous Polyposis Coli Protein (APC), Glycogen Synthase Kinase 3 beta (GSK-3 β) and Casein Kinase 1 (CK1). In the destruction complex, the amino terminal region of β -catenin is sequentially phosphorylated by CK1 and GSK-3 β . The phosphorylation gives rise to β -catenin ubiquitination by an E3 ubiquitin ligase subunit and subsequent degradation in the proteasome (Aberle et al., 1997; Amit et al., 2002; Yost et al., 1996).

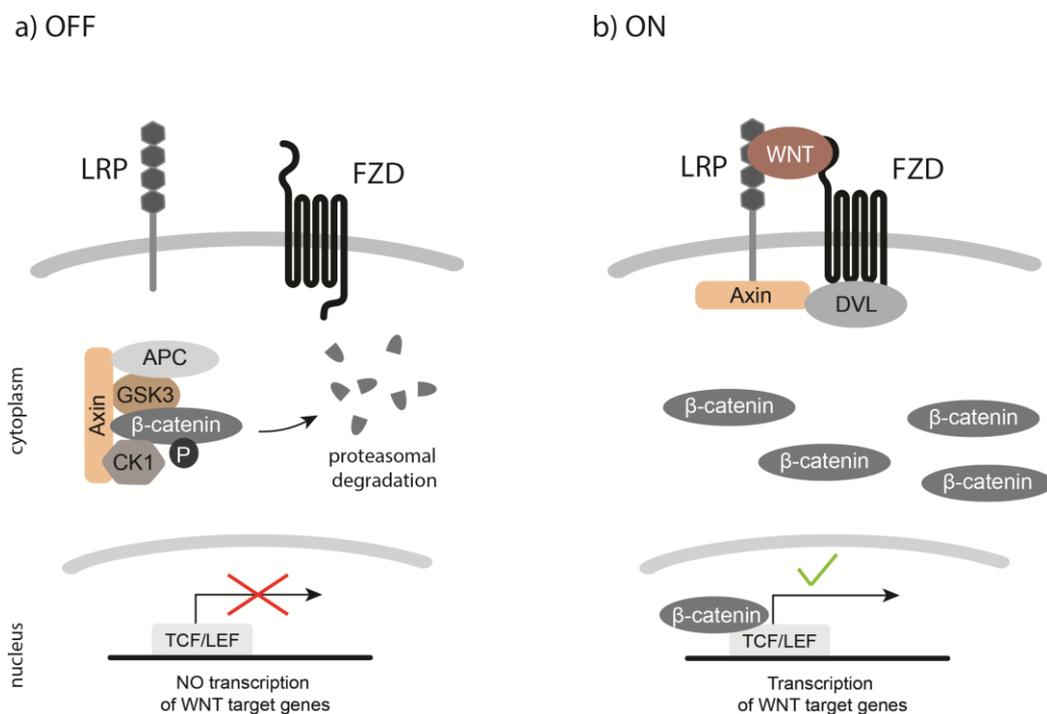


Figure 3: Schematic illustration presenting β -catenin-dependent signalling pathway. In the absence (a) of FZD ligand, β -catenin is constantly targeted for proteasomal degradation. Binding of WNT to FZD and LRP (b) allows β -catenin to translocate to the nucleus and initiate transcriptional activities.

In the presence of FZD ligands, a complex is formed between the receptor/ligand and the co-receptor LRP together with the scaffold protein DVL. This enables the phosphorylation of the co-receptor and the establishment of a docking site for AXIN to be recruited. Formation of this complex prevents degradation of β -catenin and promotes its nuclear accumulation, where β -catenin binds to the transcriptional factors T-Cell Factor (TCF) and Lymphoid-Enhancing Factor (LEF) homologs known as TCF/LEF. TCF acts as a repressor in the absence of WNT by forming a complex with Groucho/Grg/TLE proteins. β -catenin presence in the nucleus physically displaces Groucho from the

TCF/LEF sites in order to activate WNT target genes (Cavallo et al., 1998; Daniels and Weis, 2005; Roose et al., 1998). Among these genes are *cyclin D1* and *c-myc* (**Fig.3**) (He et al., 1998; Tetsu and McCormick, 1999).

Besides acting as a transcriptional regulator, β -catenin also plays a major role in adherence junctions. Here, β -catenin serves as a binding partner to different cadherins (Peifer et al., 1992).

1.1.7 β -catenin-independent signalling

While WNT proteins and their receptors are capable of activating β -catenin-dependent signalling, there are also several signalling cascades that can be initiated independently of β -catenin. We refer to these cascades as β -catenin-independent signalling (Niehrs, 2012). In comparison to the β -catenin-dependent pathway, the knowledge about the independent pathways is still restricted, mostly because of poor characterization on the molecular level and a lack of robust readouts (Amerongen, 2012).

So far, the best characterized pathway known to signal independently of β -catenin is the planar cell polarity pathway (PCP). PCP pathway is mainly important for guiding cells and structures. It is important for processes like cytoskeleton rearrangements, cell polarity and gene expression through activation of small monomeric G proteins or GTPases such as Ras-Related C3 Botulinum Toxin Substrate 1 (RAC1) and Ras Homolog Family Member A (RHOA) (Axelrod, 2009; Brown et al., 2006; Devenport and Fuchs, 2008; Heasman and Ridley, 2008; Kikuchi et al., 2011; Maung and Jenny, 2011; Schulte, 2010; Simons and Mlodzik, 2008). While activation of RAC1 is known to result in the initiation of c-Jun-N-Terminal Kinase (JNK) and its stimulation of transcription factors like c-Jun and c-Fos, RHOA induction leads to the activation of the Rho Associated Kinase (ROCK) (Marlow et al., 2002; Rosso et al., 2005). Both RHOA and RAC1 have been reported to be induced by WNT proteins. For instance, cell migration induced by WNT-3A was shown to be mediated through DVL, RHOA and ROCK, while increased invasion and tumour aggressiveness was shown to be triggered by WNT/RAC signalling. In *Xenopus* gastrulae, it was demonstrated that WNT/FZD signalling regulates gastrulation through activation of either RHO or RAC. Modulating the function of these two GTPases perturbed the WNT/FZD dependent gastrulation. Moreover, inhibition of WNT-11/FZD signalling that is important for *Xenopus* gastrulation suppressed RHO and RAC signalling (Endo et al., 2005; Habas et al., 2003; Kishida et al., 2004; Kobune et al., 2007; Kurayoshi et al., 2006).

An additional β -catenin-independent pathway regulates the intracellular calcium (Ca^{2+}) levels. Overexpression of FZD₂ in *Danio rerio* leads to elevation of intracellular Ca^{2+} in a G protein-dependent manner (Slusarski et al., 1997a). Increase of intracellular Ca^{2+} leads to activation of Ca^{2+} /Calmodulin Dependent Kinases (CaMK), Protein Kinases (PKE) and Nuclear Factor of Activated T-Cells (NFAT). In mammalian cells, WNT-5A was shown to induce Ca^{2+} signalling in order to regulate processes such as cell migration (Dejmek et al., 2006; Ishitani et al., 2003; Kühl et al., 2000; O'Connell et al., 2009; Sheldahl et al., 2003; Weeraratna et al., 2002).

1.2 G PROTEIN-COUPLED RECEPTORS

1.2.1 Heterotrimeric G proteins and G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors. By responding to diverse stimuli like ions, hormones and neurotransmitters, they are able to transduce extracellular signals into intracellular responses. Some of the functions regulated by GPCRs include the sensation of smell, taste and vision (Fredriksson et al., 2003; Mahoney and Sunahara, 2016; Pierce et al., 2002). Interestingly, ca 30% of the currently marketed pharmaceutical drugs target GPCRs (Overington et al., 2006).

GPCRs are characterized by the presence of seven transmembrane alpha helices and can be divided into five classes. The division is based on sequence and structural similarity and the receptor classification is referred to as Class A, Class B, Class C, Class Frizzled and Adhesion Class GPCRs or other 7 transmembrane (TM) receptors where class A is the largest family (Alexander et al., 2013; Fredriksson et al., 2003). Even though the members within a family share similarities, individual GPCRs have their own specific signal transduction activities and complex regulatory processes (Rosenbaum et al., 2009a).

In the classical view, binding of an agonist to GPCRs leads to the activation of heterotrimeric G proteins, consisting of the $G\beta\gamma$ dimer and a $G\alpha$ subunit which is classified into four classes: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Casey and Gilman, 1988; Casey et al., 1988). By direct association between GPCRs and G proteins upon ligand stimulation, nucleotide exchange on the $G\alpha$ subunit is promoted. Bound Guanosine Diphosphate (GDP) is displaced and the binding site is instead occupied by Guanosine Triphosphate (GTP) (McKee et al., 1999). Interaction between GPCRs and $G\alpha$ has been proposed to occur through different modes. Interaction can either be achieved by an inactive state assembly between the receptor and the G protein or through collision coupling (Hein et al., 2005; Neubig, 1994; Oldham and Hamm, 2008; Qin et al., 2011). The occupancy of the free GDP site by GTP is driven by the high concentration of intracellular GTP. GTP binding induces a conformational change of the G protein subunits, where $G\alpha$ dissociates from $G\beta\gamma$, allowing them to regulate activity of specific effector proteins. For example, while $G\alpha$ interacts with proteins such as adenylyl cyclase or phospholipase C, $G\beta\gamma$ can in parallel recruit proteins such as G protein-coupled receptor kinases (GRKs) to the plasma membrane (Mahoney and Sunahara, 2016). GPCRs are in general quite dynamic. The GDP dissociation from the $G\alpha$ subunit is assumed to occur through allosteric disruption of the nucleotide binding site. According to the crystal structures of several $G\alpha$ subunits, the nucleotide is buried between the two main domains of $G\alpha$, the Ras-Homolog Domain and the Alpha-Helical Domain. It is believed that rearrangements induced by a receptor, are needed in order for GDP to exit its site. These rearrangements include the embedding of the C-terminus of $G\alpha$ into the core of the activated GPCR and interaction of the receptor with the $G\alpha$ N-terminus (Chung et al., 2011; Coleman et al., 1994; Herrmann et al., 2006; Noel et al., 1993; Rasmussen et al., 2011; Sunahara et al., 1997; Tesmer et al., 2005). GPCRs respond dynamically to

orthosteric and allosteric input by a range of conformational changes. Allosteric effects can have an influence on the interaction of the receptor with various agonists. Specific GPCR ligands are believed to stabilize specific conformations that in turn affect the selection of the receptor effectors (Kenakin, 2013; Kobilka and Deupi, 2007).

Intracellular changes of the receptor induced by the ligand are specific to the physiological system and its needs. Moreover, different ligands can have different efficacies for given GPCR, implying that the ligand interactions with the receptor are dependent on multiple interaction modes and not only occupation of the receptor binding site, in order to efficiently transfer energy between the binding pocket of the receptor and the G protein interaction site (Rosenbaum et al., 2009b). The diverse conformational changes of GPCRs can be observed if we take the example of the photoreceptor Rhodopsin and a GPCR with a diffusible agonist. Light activation of Rhodopsin by photons triggers a conformational change of the receptor leading to a full 6Å outward movement of the intracellular end of transmembrane TM6 away from TM7. For GPCRs activated by a more diffusible agonist stimulation of the receptor does not necessary lead to a full outward movement of TM6 (Altenbach et al., 2008; Choe et al., 2011; Chung et al., 2011; Farrens et al., 1996; Gether et al., 1997; Ghanouni et al., 2001; Liu et al., 2012).

1.2.2 $G\alpha_{12/13}$ signalling

Although discovered as recently as 1991, the understanding of the fourth class of G α proteins named G $\alpha_{12/13}$ has progressed slower than with other G protein families (Strathmann and Simon, 1990). As mentioned earlier, activation of GPCRs coupled to G $\alpha_{12/13}$ leads to the GDP/GTP exchange of the G α subunit, dissociation of the G $\beta\gamma$ dimer and activation of the G $\alpha_{12/13}$ -dependent downstream cellular responses (Hepler and Gilman, 1992). Shortly after their discovery, the first report on G $\alpha_{12/13}$ -mediated signalling came from experiments in *Drosophila melanogaster* where it was shown that this group of G proteins induces contraction and migration of cells in the *Drosophila* embryo (Parks and Wieschaus, 1991). This was followed by the discovery of the Guanine Exchange Factors (GEFs) of Rho GTPases named RHOGEFs important for regulation G protein signalling specifically for G $\alpha_{12/13}$ (Fukuhara et al., 2001). The main purpose of RHOGEF proteins is to act as GTPase-Activating Proteins (GAPs) in order to accelerate G protein function (Xie and Palmer, 2007; Zhong and Neubig, 2001). So far, four types of RHOGEF proteins have been shown to be regulated by G $\alpha_{12/13}$ proteins: p115-RHOGEF, PSD-95/Disc-Large/ZO-1 Homology (PDZ)-RHOGEF, Leukemia-Associated RHOGEF (LARG) and Lymphoid Blast Crisis (Lbc)-RHOGEF. Activation of G $\alpha_{12/13}$ leads to translocation of RHOGEFs from the cytosol to the plasma membrane, where they promote an active conformation of the small GTPase RHOA, by catalysing the GDP/GTP exchange. At the same time, the Regulators of G protein Signalling (RGS) domain of RHOGEF accelerates the hydrolysis of the G α -bound GTP regulating the inactivation of the G protein as a part of a negative feedback loop (Dutt et al., 2004; Fukuhara et al., 2001; Meyer et al., 2008).

RhoA regulates multiple downstream targets important for the cell cytoskeleton. By activating Rho Kinases (ROCK1 and 2), RhoA mediates processes such as actin stress fiber formation, activation of Serum Response Transcriptional Factor (SRF) and cell contractions (Narumiya et al., 1997; Riento and Ridley, 2003; Treisman et al., 1998). While RHOA activation is the most studied downstream signalling aspect, $G\alpha_{12/13}$ are also reported to regulate a range of additional proteins. Type I and type II cadherins are some of these, where it has been shown that $G\alpha_{12/13}$ can bind to the cytoplasmic tail of cadherins in order to inhibit their adhesive function and induce the release of β -catenin (Meigs et al., 2001, 2002). The most striking phenotype, showing the importance of $G\alpha_{12/13}$ family of G proteins, comes from studies performed on mice, where ablation of $G\alpha_{12/13}$ coding genes leads to severe developmental defects, mainly due to undeveloped vasculature and haemorrhage (Offermanns et al., 1997a). In addition, ablation of $G\alpha_{12/13}$ in glial and neuronal cells was shown to enhance migration of cortical neurons, while the specific ablation of $G\alpha_{13}$ in platelets increased bleeding time in mice (Moers et al., 2003, 2008).

Lately, the Hippo pathway has been suggested to be regulated by GPCRs and $G\alpha_{12/13}$. By inactivating the activity of the transcriptional co-activators Yes Associated Protein 1 (YAP) and Tafazzin (TAZ), the Hippo tumour suppressor pathway regulates processes like cell shape and polarity, cell adhesion and organ size (Piccolo et al., 2014; Yu et al., 2012a). One of the processes where YAP/TAZ activity has been shown to be important is in regulation of endothelial vessel sprouting and junctional stability during angiogenesis (Choi and Kwon, 2015; Choi et al., 2015). Recently, it was shown that Lysophosphatidic Acid (LPA) and Sphingosine 1-Phosphophate (S1P) are able to inhibit the Hippo signalling by activating YAP/TAZ transcriptional activity through activation of $G\alpha_{12/13}$. Moreover, activation of YAP through the Protease Activated Receptor 1 (PAR1) GPCR was shown to act through activation of RHOA, leading to an increased assembly of F-actin (Regué et al., 2013; Yu et al., 2012a). Interestingly, activation of the two transcriptional co-activators YAP and TAZ has also been associated to WNT signalling, where activation of the pathway by WNT-5A/B and WNT-3A leads to the de-phosphorylation of YAP/TAZ and the subsequent activation through FZD/ROR/ $G\alpha_{12/13}$ signalling, independent of β -catenin (Park et al., 2015). In addition, the two Hippo pathway components YAP and TAZ have also been suggested to be a part of the β -catenin destruction complex (Azzolin et al., 2014).

1.2.3 Class FZD G protein-coupled receptors

After the first cloning attempts of FZDs, it was clear that these receptors were membrane proteins comprising seven transmembrane helices and thus, belong putatively to the superfamily of GPCRs (Barnes et al., 1998; Fredriksson et al., 2003; Park et al., 1994; Vinson et al., 1989; Wang et al., 2006). The first functional indication of FZDs being GPCRs came from experiments showing that WNT/ β -catenin signalling was inhibited in F9 teratocarcinoma cells when treated with a toxin from *Bordetella pertussis*

(PTX) known to ADP-ribosylate the $G\alpha_{i/o}$ proteins (Birnbaumer et al., 1990; Liu et al., 2001; Slusarski et al., 1997a).

Following the initial studies, several groups started to report the importance of G proteins in WNT/FZD signalling. Studies performed in *Danio rerio* demonstrated that inhibition of G protein signalling blocked the changes of Ca^{2+} influx usually observed upon WNT-5A stimulation. This was followed by *in vivo* experiments demonstrating that FZDs act as GEFs for G-proteins, mediating both β -catenin and PCP signalling in *Drosophila melanogaster* (Katanaev et al., 2005; Slusarski et al., 1997b, 1997c). Adding to the proof, it was shown that FZD₂ could induce the release of Ca^{2+} . The creation of a chimeric receptor with partial segments of FZD₂ and the β_2 -adrenergic receptor, demonstrated that FZD₂ could signal through modulation of Cyclic Guanosine 3',5-Mono-Phosphate (cGMP) and that FZD₂ induced Ca^{2+} transients that could be reduced by phosphodiesterase, suggesting G protein-dependent Ca^{2+} signalling. Moreover, stable transfection of the chimera in F9 embryonic teratocarcinoma cells, showed that FZD₂-induced Ca^{2+} transients required two Pertussis Toxin-sensitive G proteins, $G\alpha_o$ and $G\alpha_t$ (Ahumada et al., 2002; Liu et al., 1999). Recently, it was shown that WNT-7B and WNT-3A regulate osteoblastogenesis by the regulation of transcriptional activity through $G\alpha_q$ and PKC δ -dependent signalling (Tu et al., 2007). Another G protein shown to be involved in WNT/FZD signalling is $G\alpha_{11}$. By mediating WNT-11/FZD₇ signalling $G\alpha_{11}$ was shown to have a function in regulation of DVL phosphorylation and plasma membrane recruitment as well as the regulation of convergent extensions in *Xenopus* (Iioka et al., 2007). Under basal conditions FZD₉ was found to be pre-coupled to $G\alpha_o$, a state from which $G\alpha_o$ could dissociate upon WNT-5A stimulation. In the postsynaptic region, this receptor was found to mediate the increase in spine density. By inhibiting G protein signalling, particularly $G\alpha_o$, WNT-5A was shown to be the controlling factor driving the process of spine density (Ramírez et al., 2016). Moreover, the PCP pathway was recently shown to be important for the repair of skeletal muscles. WNT-7A/FZD₇-induced activation of PCP leads to symmetric expansion of satellite cells enhancing the damage repair by activating the Akt/mTor pathway. $G\alpha_s$ and PI(3K) are some of the crucial components required for myotube growth induced by Akt/mTor. Both $G\alpha_s$ and PI(3K) were shown to be associated with FZD₇ in a complex (von Maltzahn et al., 2012).

Several publications from our group are supporting the growing evidence that FZDs are able to employ heterotrimeric G proteins to transduce signalling cues. In primary microglia, both WNT-3A and WNT-5A are important in the regulation of pro-inflammatory responses. These responses were mediated through PTX sensitive $G\alpha_{i/o}$ proteins, important for both β -catenin-dependent and -independent signalling (Halleskog and Schulte, 2013; Halleskog et al., 2012). WNT-5A was also shown to induce the GDP/GTP exchange of $G\alpha_{i/o}$ proteins in N13 mouse microglia-like cells (Kilander et al., 2011). Furthermore, FZD₆ was shown to be associated in an inactive state complex with $G\alpha_i$ and $G\alpha_q$. Stimulation with WNT-5A dissociated the complex and induced DVL-dependent phosphorylation of the Extracellular Signal-Regulated Kinase1/2 (ERK1/2) (Kilander et al., 2014a).

As we are just starting to understand the true nature of FZDs it is encouraging that several reports support the theory of FZDs being GPCRs. However, many questions still remain to be answered and research is limited by a lack of techniques that can be used. For that reason, it is important to continue the work on elucidating the mechanisms of these receptors with the hope that the future will bring a better and more comprehensive understanding of the WNT pathway.

1.3 VASCULAR DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

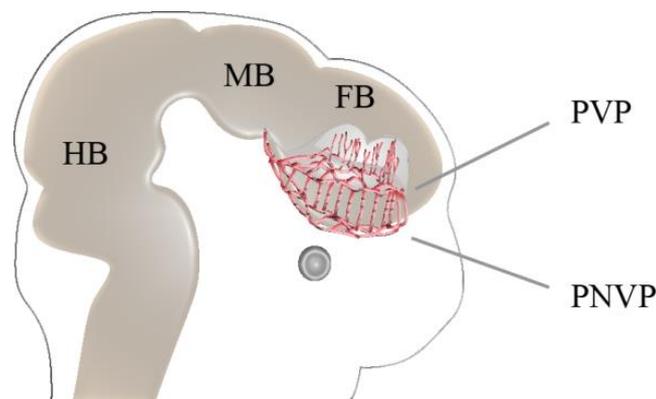
1.3.1 *Blood-brain barrier formation*

Development and homeostasis of the central nervous system (CNS) depends on the delivery of nutrients and oxygen from specialized blood vessels. Unique features of the CNS vasculature are not only the contribution to its proper function, but also serve to shield and protect the CNS from possible pathogens and toxic agents. Joined by continuous tight junctions, the endothelial cells of the blood-brain barrier (BBB) create a physical barrier between the blood and the brain parenchyma. At the same time, these unique cells express a selection of proteins like transporters and ion pumps in order to provide the brain with crucial nutrients such as glucose (Blanchette and Daneman, 2015; Engelhardt and Liebner, 2014; Paolinelli et al., 2011; Zhao et al., 2015). Tight junctions are composed of proteins like claudins and zonula occludens and create polarized cells with distinct abluminal and luminal membrane composition. Adherence junctions are found on the abluminal side of the blood vessels and are linked with tight junctions. They consist of proteins like VE-Cadherins which are important for the regulation of cell adhesion by binding the actin cytoskeleton via catenins (Brightman and Reese, 1969; Huber et al., 2001; Reese and Karnovsky, 1967; Rubin and Staddon, 1999).

Since the developing CNS originates from ectoderm germ layer that is devoid of vascular progenitor cells, it is dependent on invasion of blood vessels from the surrounding mesoderm. In the mouse, the invasive process of CNS blood vessel development begins with vasculogenesis at the embryonic stages E7.5-E8.5. Here, the mesoderm derived endothelial precursor cells named angioblasts assemble outside of the brain in order to form a simple vascular network the perineural vascular plexus (PNVP). This is followed by the sprouting process of angiogenesis at around E9.5 where new blood vessels are formed from pre-existing blood vessels by radially invading the brain in a spatio-temporal manner forming the perivascular plexus (PVP). Angiogenesis is guided by endothelial tip cells that, with the help of their numerous filopodia, sense and guide the vascular sprouts into the brain and promote fusion with adjacent endothelial cells. Following the endothelial tip cells, the stalk cells create the vascular lumen by proliferation (**Fig.4**). As a final step of CNS vascular development, the blood vessels become associated with surrounding cells like pericytes, microglia and astrocytes for stabilization and regulation of vessel perfusion (Bentley et al., 2009; Engelhardt, 2003; Franco et al., 2009; Mancuso et al., 2008; Potente et al., 2011). During all developmental

stages of the BBB, a plethora of cellular and acellular cues direct the CNS endothelial cell to acquire their characteristic BBB features (Blanchette and Daneman, 2015; Engelhardt and Liebner, 2014; Risau, 1997).

Multiple signalling pathways regulate blood vessel development. Besides the WNT pathway that will be discussed in the following section, some of the others include Vascular Endothelial cell Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor (TGF) and Notch signalling. VEGF is produced by the neural progenitors and together with its tyrosine kinase receptors VEGFR1 and VEGFR2, it is responsible for inducing the invasion of blood vessels from the PNVP and for regulating branch patterning during angiogenesis (Raab et al., 2004). While VEGF signalling is the driving regulator of sprouting and branching, Notch signalling exerts the opposite effect. Depletion of the Notch receptor and its ligand leads to enhanced sprouting, vessel branching and fusion (Adams and Alitalo, 2007). PDGF- β is produced by the migrating endothelial cells. The main responsibility of this protein is to attract pericytes to the developing vasculature. Ablation of the PDGF- β ligand or receptor leads to a lack of pericyte recruitment and lethal microaneurisms (Lindahl et al., 1997). Also ablation of the TGF- β ligand or receptor leads to a severe vascular phenotype indicating the importance of this pathway for proper development of the blood vessels (Adams and Alitalo, 2007)



Mouse embryo at E11.5

Figure 4: Simplified illustration displaying the sprouting process of mouse forebrain angiogenesis. In mice, vascularisation of the brain begins with the formation of the simple perineural vascular plexus (PNVP) outside of the brain. This is followed by the invasion of radially sprouting blood vessels into the brain that sequentially form the more complex vascular network named perivascular plexus (PVP). (forebrain (FB), midbrain (MB) and hindbrain (HB)).

1.3.2 *WNT/FZD signalling in angiogenesis*

WNT proteins are morphogens and can act in a gradient-dependent manner as long- or short-range cues deciding the fate of cells during embryonic development. WNT signalling, and in particular the β -catenin-dependent pathway, has been reported to be crucial for proper vascular development of the CNS. Important for formation,

differentiation and maintenance of the BBB, β -catenin activity has been shown to be strong in the blood vessels penetrating the brain during angiogenesis (Daneman et al., 2009; Dejana, 2010; Liebner and Plate, 2010; Stenman et al., 2008). Playing a dual role as both a structural component of adherence junctions and a transcriptional activator, β -catenin ablation causes severe haemorrhage throughout the entire CNS. Moreover, ablation of two neuroepithelial derived WNT ligands, WNT-7A and WNT-7B showed an almost identical phenotype, with low expression of the glucose transporter Glut-1 and haemorrhage indicating that these two FZD agonists were crucial for vascular development of the CNS (Daneman et al., 2009; Engelhardt, 2003; Liebner et al., 2008; Stenman et al., 2008; Zhou and Nathans, 2014; Zhou et al., 2014a). In addition to WNT-7A and WNT-7B ablation, it was recently shown that endothelial cell specific knock out of the G Protein-Coupled Receptor 124 (GPR124), believed to be a co-activator of WNT-7A and WNT-7B, resulted in the abnormal development of the blood vessels in the forebrain and spinal cord (Zhou and Nathans, 2014). FZD₄ is by far the best studied WNT receptor. Loss-of-function of this receptor is often related to vascular defects and complete ablation of FZD₄ results in increased permeability of the BBB. Strengthening the importance of this receptor in CNS blood vessel development is the phenotype observed in mice where the FZD₄-specific ligand Norrin is removed. Genetic ablation of Norrin in mice causes severe defects in the retinal vasculature and leakiness of the BBB (Junge et al., 2009; Paes et al., 2011; Smallwood et al., 2007; Wang et al., 2012; Ye et al., 2009).

While the importance of proper β -catenin signalling in blood vessel development has been studied to a larger extent, there are several reports showing the need for β -catenin-independent signalling. β -catenin-independent signalling in endothelial cells is most often associated with endothelial cell migration, cell proliferation, cell growth and vessel sprouting. In cell culture assay, inhibition of the PCP pathway resulted in disrupted processes such as cell migration, cell polarity and cell growth implicating the need for PCP signalling in vascular development and remodelling. Moreover, experiments performed on embryonic stem cells lacking WNT-5A indicated that this FZD ligand was important for both PKC and WNT/ β -catenin-dependent vascular development (Cirone et al., 2008; Yang et al., 2009).

The essential requirement for WNT signalling during blood vessel development and maintenance has been supported by several studies over the years where both β -catenin-dependent and -independent signalling play an important role. However, considering the complexity of this pathway with 19 ligand homologues and 10 receptors, more needs to be done in order to have a comprehensive view on how exactly WNT signalling is regulating the blood vessel development and BBB integrity (Cheng et al., 2008; Cirone et al., 2008; Engelhardt and Liebner, 2014; Goodwin et al., 2007; Masckauchán et al., 2006; Pinzón-Daza et al., 2014; Yang et al., 2009; Zerlin et al., 2008).

1.3.3 Role of matrix proteins during brain vascularization

While it has been established that signalling by growth factors such as WNT proteins, VEGF, TGF β and PDGF β is essential for the vascularisation (Adams and Alitalo, 2007; Engelhardt and Liebner, 2014), recent studies have focused on the need for extracellular matrix proteins in regulation of blood vessel development and maintenance. Secreted by the endothelial cells, pericytes and astrocytes extracellular matrix proteins such as collagens, laminins and fibronectin provide structural support for blood vessels and act as regulators of vessel permeability and scaffolds for growth factors (Blanchette and Daneman, 2015; Correale and Villa, 2009). Deregulation of matrix proteins has been implicated in the leakiness of the BBB as well as diverse disorders such as stroke and Alzheimer's disease (Baeten and Akassoglou, 2011; Ding et al., 2009; Engelhardt and Liebner, 2014).

An important group of matrix proteins are the extracellular metalloproteinases including Matrix Metalloproteinases (MMPs) and A Disintegrin And Metalloproteinase (ADAM) proteins. MMPs act mostly on the cell surface where they control activation of signalling molecules, but they are also crucial for the regulation of BBB permeability by acting on basal lamina and endothelial tight junctions. Families of ADAM and A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteins have been reported to be both pro- and anti-angiogenic. While ADAM proteins have been shown to support vessel sprouting, intracellular signalling and cell adhesion, they are also able to act as inhibitors by sequestering growth factors such as VEGF and TGF β (Le Goff et al., 2008a; Rodríguez-Manzaneque et al., 2015; Rosenberg, 2009).

1.3.4 $G\alpha_{12/13}$ signalling in angiogenesis

The very first studies performed on mice where $G\alpha_{12}$ and $G\alpha_{13}$ were ablated demonstrated the importance of these proteins for vascular development. While single mutants lacking $G\alpha_{12}$ are viable and appear normal, mice lacking $G\alpha_{13}$ die at E9.5 as a result of severe defects in angiogenesis. Moreover, when introducing a one allele mutation of $G\alpha_{13}$ to the viable $G\alpha_{12}$ -deficient mice, premature death occurs as a consequence *in utero*. In addition, double knock-out mice lacking both $G\alpha_{12}$ and $G\alpha_{13}$ die at the embryonic stage E8.5 as a result of impaired vascular development (Gu et al., 2002; Offermanns et al., 1997b). Studies have shown that the vascular defects observed in $G\alpha_{13}$ -deficient mice depend on the absence of the $G\alpha$ subunit specifically in endothelial cells. By cell specific ablation of $G\alpha_{13}$ in the vascular cells, Ruppel *et al.* could show that transgenic overexpression of $G\alpha_{13}$ in endothelial cells could rescue the vascular phenotype (Ruppel et al., 2005). More recent studies performed on cells derived from $G\alpha_{13}$ -deficient mice, indicate that $G\alpha_{13}$ signalling is important for the regulation of endothelial cell shape and movement given that isolated $G\alpha_{13}$ -deficient vascular cells fail to form a network and tubes in cell culture (Worzfeld et al., 2008). Another study showed that $G\alpha_{13}$ mediated angiogenesis by stimulating the transcriptional activity of Myocyte Enhancer Factor 2 (MEF-2). Downregulation of either $G\alpha_{13}$ or MEF-2 causes a decrease in endothelial cell proliferation. In cells where $G\alpha_{13}$ was downregulated, the proliferative activity could be

rescued by constitutively active MEF-2 (Liu et al., 2009). $G\alpha_{13}$ has also been shown to be important for the regulation of endothelial VEGF Receptor-2 (VEGFR-2) expression given that reduced levels of the receptor were observed in sites of angiogenesis upon cell specific ablation of $G\alpha_{13}$. Depletion of $G\alpha_{13}$ results in a reduced response to VEGF-A in endothelial cells. However, the sensitivity to VEGF-A could be restored upon normalization of the receptor levels. Here the VEGFR-2/ $G\alpha_{13}$ -mediated signalling in vascular cells is believed to be regulated through activation of RHOA and subsequent induction of NF κ B transcriptional activity (Sivaraj et al., 2013).

Understanding the mechanisms that regulate CNS vascular development opens up the possibilities for creating better treatments for severe disorders such as cancer and stroke. Due to the special characteristics of the BBB, it is not only protecting the brain from pathogens, but is also preventing administration of pharmaceuticals necessary for treatment. On the other hand, in disease states like stroke it would be of interest to regenerate the barrier features. For these reasons, understanding the underlying mechanisms of CNS blood vessel development is of crucial importance.

2 SPECIFIC AIMS

1. To develop a more in depth understanding of the role of WNT signalling in CNS-specific blood vessel development.
2. To identify the FZDs expressed during CNS angiogenesis and their role as GPCRs.
3. To determine the G protein partner(s) of FZD₄ and relevant pathways that become activated.
4. To define expression of FZD₁₀ in the developing brain, the G protein(s) it couples to and downstream signalling events.

3 MATERIAL AND METHODS

3.1 FLUORESCENT RECOVERY AFTER PHOTBLEACHING

Fluorescent recovery after photobleaching (FRAP) was developed to study binding dynamics and diffusion in live cells. Considering that more conventional binding assays are difficult to apply to the studies of FZDs, we took advantage of this technique in order to define the G protein partners of FZD₄ and FZD₁₀. The FRAP protocol used was modified as double-colour (dc)-FRAP, described previously by our group and others (Dorsch et al., 2009; Kilander et al., 2014b; Qin et al., 2008, 2011; Reits and Neefjes, 2001).

For the purpose of defining FZD/G protein partners, we used fluorescently-tagged receptors and G α subunits. The FRAP measurements are assessed by irreversible photobleaching of a small defined area, followed by measurement of the subsequent diffusion of fluorescent proteins entering the bleached region. While the bleaching is performed with a high intensity laser, the recovery of the fluorescence is measured with low laser power.

After setting the base line of physiological conditions, we chemically cross-linked the surface of the cells through the addition of sulfo-NHS-LC-LC-biotin and avidin, which efficiently immobilizes only the membrane proteins. Measuring the fluorescent recovery of FZDs and G proteins before and after cross-linking, we could identify the G α subunits with affected diffusion kinetics after immobilization of the receptor (**Fig.5**).

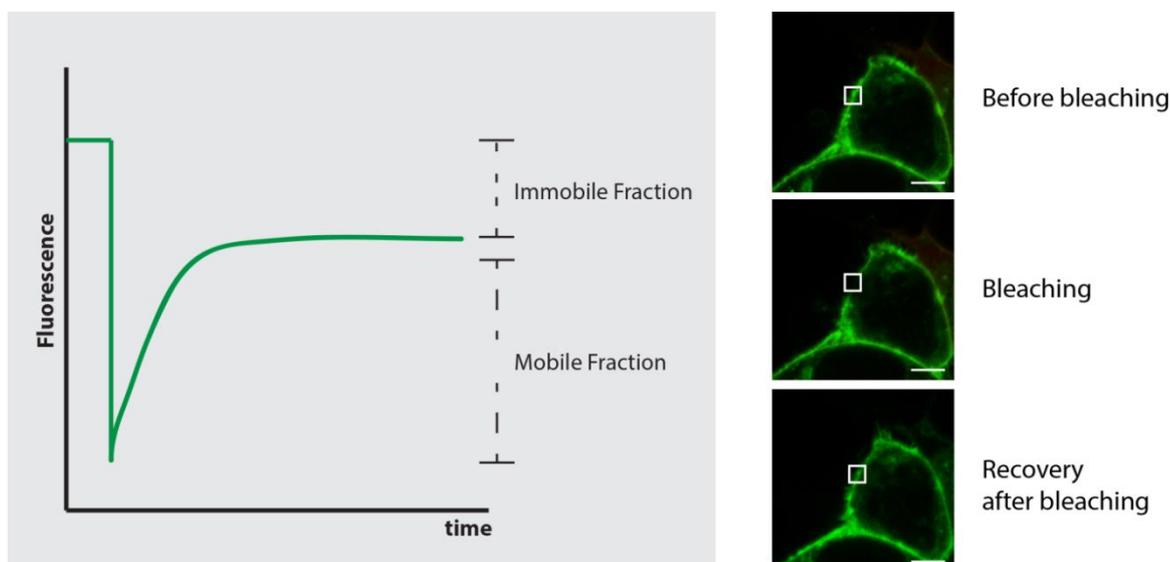


Figure 5: Illustration of FRAP measurements. A restricted area is bleached with high laser power and the fluorescence intensity is measured over time. Initially, the fluorescence decreases but recovers by the diffusion of molecules into the bleached area. By comparing the fluorescence intensity after recovery to the intensity before and just after bleaching, the mobile fraction can be calculated. Scale bar 5 μ m.

By comparing with the $G\alpha$ subunits of all four families of G proteins, we identified the group of $G\alpha_{12/13}$ as interaction partners of FZD₄ and FZD₁₀ in forming an inactive-state complex. While GPCRs are able to signal to G proteins both through collision-coupling and through inactive-state preassembly, dcFRAP can only be used for identifying proteins in the inactive-state complex (Hein et al., 2005; Neubig, 1994; Oldham and Hamm, 2008; Qin et al., 2011). In comparison to the more conventional Förster resonance energy transfer (FRET) method, dcFRAP is rather affinity based than proximity based. Thus, our data argue that the complex formed between the receptor and G protein is formed through physical interaction.

3.2 FÖRSTER RESONANCE ENERGY TRANSFER

Förster resonance energy transfer (FRET) is a technique for the determination of molecular proximity. FRET is referred to as the process where energy is transferred from one excited molecule called a donor, to another molecule called an acceptor. In order for FRET to occur, the two fluorescently tagged molecules need to interact with close proximity, with a distance less than 10nm. Also relative dipole moments between the donor and the acceptor caused by fluorophore orientation can affect FRET. A variant of FRET that is used on fixed cells, where the specimen can serve as its own control, is known as acceptor photobleaching FRET. Here, the fluorescence intensities of the donor are measured before and after bleaching, of a small limited area. Since energy transfer reduces the donor fluorescence, the intensity of the donor will increase upon bleaching of the acceptor (Fig.6) (Ishikawa-Ankerhold et al., 2012).

In Paper II, we employed acceptor photobleaching FRET in fixed cells in order to confirm that stimulation of the receptor with WNT-7A leads to dissociation of the inactive-state complex formed with $G\alpha_{12/13}$. If the $G\alpha_{12/13}$ and FZD₄ formed a complex as shown by the dcFRAP experiments, they would have a basal FRET. Stimulation of the receptor would lead to dissociation of the $G\alpha_{12/13}$ and a reduction in FRET thereby resulting in an increase of the fluorescence intensity of the donor. As seen from the results in Paper II, this indeed was the case confirming the data generated by the dcFRAP method that revealed an interaction between FZD₄ and $G\alpha_{12/13}$.

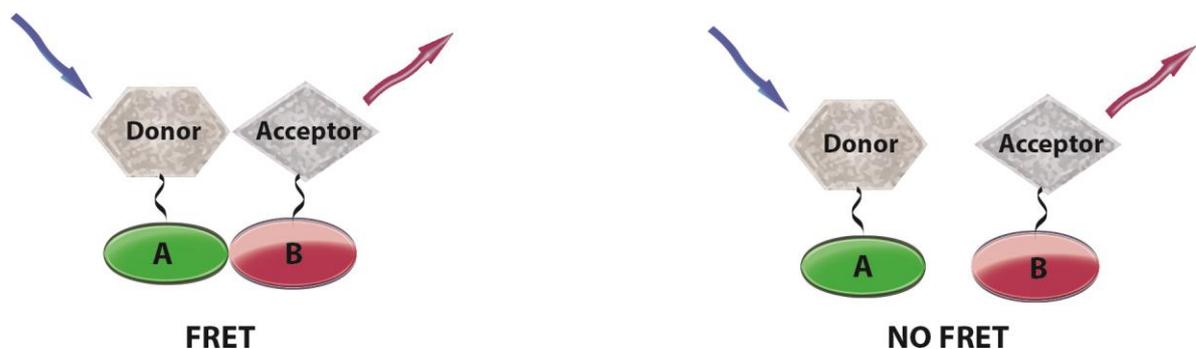


Figure 6: Illustration of FRET measurements. Only close proximity between fluorescently tagged donor and fluorescently tagged acceptor can induce FRET.

3.3 DYNAMIC MASS REDISTRIBUTION

Dynamic mass redistribution (DMR) assay is a technique developed to study ligand-induced responses as a complement to more classical end point assays giving a more comprehensive pharmacological estimate of the entire living cell. Given that DMR is a label-free, non-invasive assay, it can be applied to diverse cellular systems. Allowing for the possibility to measure various cellular responses such as receptor internalization, cytoskeletal rearrangements, adhesion and protein trafficking, DMR has been used to measure the effect of activation of receptors associated to all four G protein subfamilies, but also to detect concentration dependent cellular changes for a number of GPCRs (Antony et al., 2009; Grundmann and Kostenis, 2015; Schröder et al., 2010, 2011).

In order to measure DMR, cells are seeded on biosensor microplates and exposed to polarized broadband light that illuminates the bottom of the plate. At the bottom an optical system is formed by interacting cells and the biosensor with wavelength grating. This system spreads and reflects specific wavelength that is in resonance while the others are transmitted. Depending on the Optical Density (OD) of the cell layer covering the grating, the resonance is defined and the alterations in the reflection index can be detected. The addition of a compound like an agonist to the cells, leads to fluctuations in the OD as a result of changes in the cell shape or redistribution of intracellular molecules. This causes a shift of the outgoing wavelength in comparison to baseline. If the treatment of cells leads to an increase of the outgoing wavelength as a result of an increased OD, it is measured as a positive signal over time, while a decrease in OD and a decrease in the outgoing wavelength is measure as a negative signal over time (**Fig.7**) (Schröder et al., 2011).

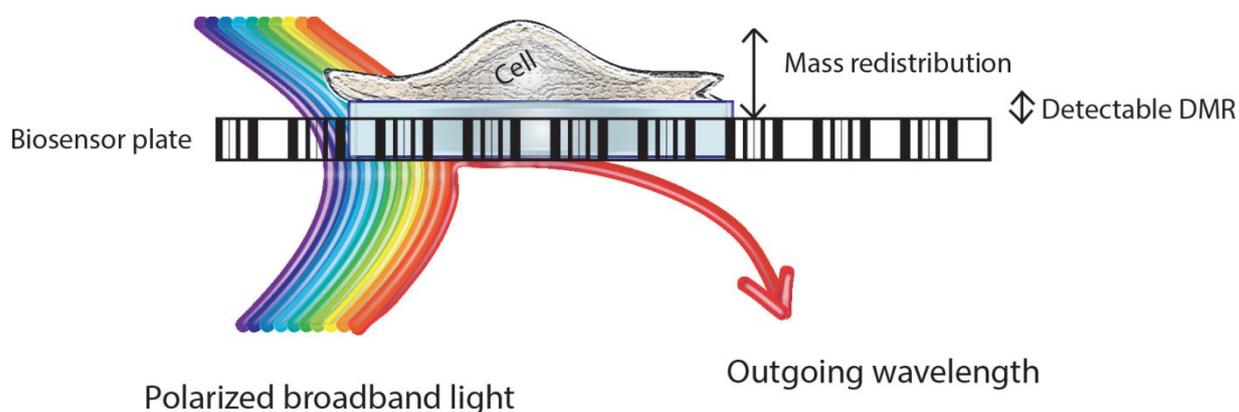


Figure 7: Illustration of DMR measurements. A cell is exposed to polarized broadband light that illuminates the bottom of the biosensor plate. Wavelength in resonance is reflected and used for calculating OD dependent reflexion index while the wavelengths that are not in resonance are transmitted.

Applying this technology on wild type and $G\alpha_{12/13}$ knock-out cells, we concluded that cells expressing FZD₄ and FZD₁₀, when stimulated with an agonist such as WNT-5A, could induce a negative DMR signal. This signal was achieved only in cells where $G\alpha_{12/13}$

were present, indicating that these two receptors provoke $G_{12/13}$ -dependent cellular changes.

3.4 P115-RHOGEF RECRUITMENT ASSAY

While studying the activation of FZDs is difficult, investigating the activation of $G\alpha_{12/13}$ is equally as challenging. Of the four families of G proteins, the subfamily of $G\alpha_{12/13}$ is the least defined and novel tools for measuring the activation of these proteins are still being developed. In order to assess whether FZD₄ and FZD₁₀ activate $G\alpha_{12/13}$ signalling we took advantage of the p115-RHOGEF recruitment assay (Meyer et al., 2008).

P115-RHOGEF has multiple roles in $G\alpha_{12/13}$ signalling. It acts as a Guanine Exchange Factor (GEF) on the small GTPases like RHOA, but it also contains domains like the pleckstrin homology (PH) domain, which is important for the subcellular localization and full exchange activity. Though p115-RHOGEF is a GEF for RHOA, it also comprises an RGS domain for the hydrolysis of the $G\alpha$ -bound GTP to GDP, inactivating the G protein.

In order to assess G protein activation of FZD₄, we used the techniques published by Mayer et al. which showed that co-expression of constitutively active $G\alpha_{12/13}$ induce a translocation of EGFP-tagged p115-RHOGEF protein from the cytosol to the membrane-anchored GTP-bound $G\alpha_{12/13}$ (**Fig.8**). By co-expressing fluorescently-tagged FZD₄ with $G\alpha_{12/13}$, p115-RHOGEF and untagged $\beta\gamma$ subunits, we could demonstrate that the presence of the receptor resulted in the translocation of p115-RHOGEF to the membrane indicating that $G\alpha_{12/13}$ subunits were activated and GTP-bound.

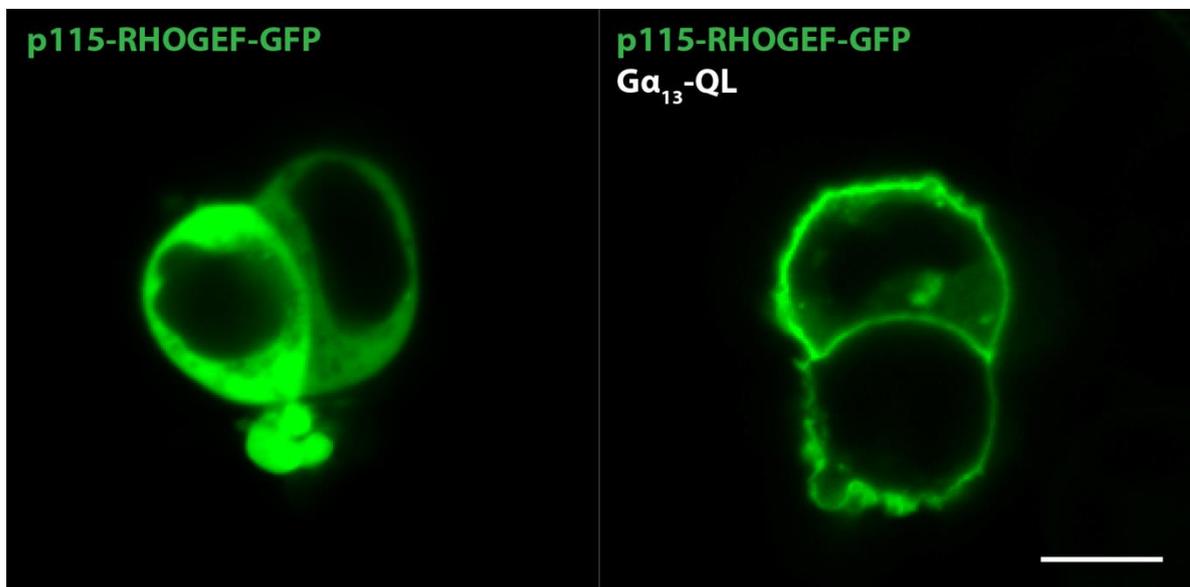


Figure 8: Transient expression of p115-RHOGEF-GFP in the absence and presence of GTP-bound, constitutively active $G\alpha_{13}$. This image shows that activation of $G\alpha_{13}$ leads to the translocation of p115-RHOGEF from the cytosol to the plasma membrane. Scale bar 10 μ m.

We also extended this technique by co-expressing the RGS domain of the p115-RHOGEF, which is catalysing the hydrolysis of the $G\alpha$ subunit and promoting its

inactivation. Addition of this component reduced the membranous location of p115-RHOGEF and confirmed that $G\alpha_{12/13}$ was indeed activated by FZD₄.

4 RESULTS AND DISCUSSION

4.1 WNT SIGNALLING IN VASCULAR DEVELOPMENT

4.1.1 *AXIN1* overexpression as a new model-system to study CNS Angiogenesis (Paper I)

The importance of β -catenin in vascular development, blood-brain barrier formation and maintenance has been well established in the past decade (Cattelino et al., 2003; Daneman et al., 2009; Stenman et al., 2008). However, a dual role for β -catenin as both a structural element of adherence junctions and as a transcriptional activator hinders the selective modulation of only one of these pools. This, in turn, has made it more difficult to dissect out if the vascular phenotype caused by β -catenin ablation is a result of mainly downregulation of its transcriptional activity or its absence in adherence junctions (Cattelino et al., 2003; Liebner and Plate, 2010; MacDonald et al., 2009b; Peifer et al., 1992). Moreover, the severe vascular phenotype observed upon deletion of β -catenin does not allow for a more detailed analysis of the cause of haemorrhage. Is the compromised BBB caused by the inability of the cells to form vascular networks, is it caused by downregulation of junctional proteins or is it caused by the inability of endothelial cells to attract supporting cells like pericytes and microglia?

By developing a new mouse model where AXIN1, the major component of the β -catenin destruction complex, is specifically expressed in endothelial cells and at specific time points by Cre-mediated recombination, we were able to conditionally modulate the β -catenin levels. Moreover, it allowed us to analyse the developing brain vasculature on both cellular and gene expression levels in detail by generating a mild overexpression of AXIN1. In addition, by targeting AXIN1, the issue of affecting the junctional β -catenin is somewhat circumvented.

Inducing endothelial-specific AXIN1 overexpression at early embryonic stages by tamoxifen leads to forebrain haemorrhage after 4 days. The embryos display a highly disorganized and immature vasculature similar to other WNT pathway mutants. Furthermore, by crossing the AXIN1 mutant with WNT reporter mice we were able to confirm downregulation of β -catenin transcriptional activity in brain endothelium at E11.5, two days after AXIN1 induction. In addition, RNA-sequencing on isolated brain endothelial cells from the same stage confirmed that AXIN1 inhibited β -catenin since known β -catenin downstream target genes like Adenomatous Polyposis Coli Downregulated 1 protein (*Apcdd1*) and Transcription Factor (*Tcf7*) were downregulated. Moreover, transporters such as Solute carrier family 2, facilitated glucose transporter member 1 (*SLC2A1* or *Glut-1*) were downregulated, while the junctional proteins like occludin (*OCLN*) and claudin5 (*CLDN5*) were differentially regulated. The RNA sequencing data also provided us with an overview of WNT receptors present in the

developing BBB at E11.5 when angiogenesis is highly ongoing (**Fig.9**), but also helped us to identify new WNT/ β -catenin downstream proteins. By analysing mouse embryonic whole-mount brain samples before the haemorrhage occurs at E11.5 and when tamoxifen was administrated at either E9.5 or E10.5, we observed that downregulation of β -catenin led to increased vascular regression and remodelling. This was illustrated, for example, by an increased number of retracted vessel sprouts, increased blood vessel diameter and decreased number of branch-points.

Combining different techniques and developing a novel mouse model enabled us to study the role of β -catenin in the formation of BBB in more detail. While it could be assumed that the haemorrhage of the CNS vasculature occurs as a result of a disrupted BBB, leaking vessels and reduced recruitment of other cell types, our analysis showed that the main reason is due to immediate retraction of newly formed vessel sprouts.

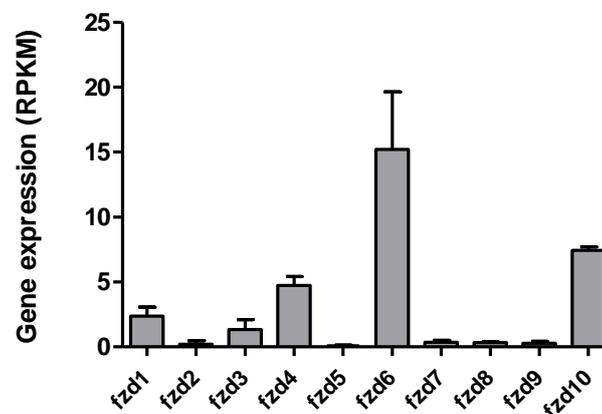


Figure 9: Graph illustrating gene expression levels of all ten FZDs in brain endothelial cells at E11.5.

4.1.2 Interplay between WNT/FZD signalling and other pathways in BBB development (Paper I)

After the identification of the process leading to haemorrhage, we investigated our RNA sequencing data in order to identify possible candidates that might be acting downstream of β -catenin. Extracellular matrix proteins are a group of proteins that are less studied in the context of BBB development, but where more evidence is accumulating, demonstrating their role in proper vascular development. Some of the processes where extracellular matrix proteins play a role are blood vessel remodelling and maturation (Baeten and Akassoglou, 2011). Among the genes in the RNA sequencing data, ADAMTS-Like Protein 2 (ADAMTSL2) was one such extracellular matrix protein identified as the best candidate with high expression levels under normal conditions and strong downregulation upon AXIN1 overexpression. In contrast to other ADAMTS proteins, ADAMTSL2 lacks protease activity, but still preserves its adhesive function. As a secreted extracellular matrix protein ADAMTSL2 regulates the bioavailability of TGF β

through binding to Latent TGF β Binding Protein1 (LTBP1) (Koo et al., 2007; Le Goff et al., 2008b).

In order to study the role of ADAMTSL2, we used the zebrafish model system where we started with identifying the presence of ADAMTSL2 through antibody detection. Immunohistochemistry showed us that ADAMTSL2 readily expressed in the zebrafish embryo 24 hours post fertilization (hpf) and that the expression became more pronounced in the head at 72 hpf. Injection of AXIN1 mRNA led to reduced protein levels of ADAMTSL2. Moreover, AXIN1 overexpression induced a mild defect of the head and a dramatic reduction in central artery density at 72 hpf. Both β -catenin and ADAMTSL2 could rescue the vascular phenotype caused by AXIN1. In addition, downregulation of ADAMTSL2 with morpholino led to reduced ADAMTSL2 protein levels and vascular defects that were rescued by co-injection with ADAMTSL2 mRNA.

Previously, it was shown that ADAMTSL2 regulates the level of TGF β (Le Goff et al., 2008a). We tested whether ADAMTSL2 knock-down in endothelial cells would lead to increased TGF β signalling. Indeed, downregulating of ADAMTSL2 in human umbilical vein endothelial cells (HUVECs) resulted in a significant increase in phosphorylation of Mothers Against Decapentaplegic Homolog 3 (SMAD3), the transcriptional activator of TGF β signalling. To test the hypothesis that the CNS vascular malformation depends on increased TGF β signalling, we administrated small molecule inhibitors of this pathway to AXIN1 overexpressing mice. This treatment partially reduced the vascular phenotype where the main effect was seen in decreased levels of dilated vessels. On the other hand, the vascular phenotype caused by ADAMTSL2 knock out in zebrafish was completely rescued by treatment with TGF β inhibitors.

In conclusion, in Paper I, we show that β -catenin regulates CNS blood vessel development by controlling the vascular remodelling. Moreover, we identify ADAMTSL2 as a β -catenin downstream protein mediating the crosstalk between WNT/ β -catenin and TGF β signalling, during BBB formation and maturation.

4.2 FRIZZLEDS AS G PROTEIN-COUPLED RECEPTORS

Ever since the discovery of FZDs, their role as GPCRs has been questioned. However, accumulating structural and mechanistic data is supporting their classification in the class Frizzled GPCRs (Foord et al., 2005; Halleskog et al., 2012; Kilander et al., 2011, 2014a; Lagerström and Schiöth, 2008; Schulte, 2010) together with Smoothed. As a consequence of the long debate, the field of β -catenin-independent and G protein-mediated WNT signalling has developed with slower haste. In addition, the development of necessary tools to study WNT-induced G protein signalling is lagging behind. The fact that numerous combinations of interactions between the ten receptors and 19 ligands exists and that majority of cells express several FZDs makes it even more complex to study G protein activation of one selective receptor. The situation becomes more complex when considering that no pharmacologically defined ligands or small molecules exist that could be used to study WNT signalling in a FZD-isoform-selective manner, while the

commercially available recombinant WNTs suffer from instability and potential impurities with signal activity (Cajane et al., 2010).

4.2.1 *FZD₄ forms an inactive state complex with G α _{12/13} (Paper II)*

While it is well established that FZD₄ can signal to β -catenin and that it is important for vascular development (Wang et al., 2012; Zhou et al., 2014b), little is known about the role of FZD₄ as a GPCR. In Paper II, we used the double-colour FRAP method in combination with surface crosslinking of the receptor, in order to show that FZD₄ is able to form an inactive state complex with G α ₁₂ and G α ₁₃, but no other representatives of the G α subfamilies. This was illustrated by a reduction of the mobile fraction of G proteins, caused only by a physical interaction between the crosslinked receptor and the G protein. Moreover, we could demonstrate that stimulation of the receptor with WNT-3A, WNT-5A, WNT-7A and WNT-10B could dissociate the complex with G α ₁₂ or G α ₁₃. In line with the dcFRAP data, our results using FRET measurements show that WNT-7A stimulation leads to decreased energy transfer between FZD₄ and G α _{12/13} compared to baseline. Furthermore, by using the pan DVL siRNA and overexpressing the three different DVL isoforms, we could confirm that the formation of the inactive-state complex between FZD₄ and G α _{12/13} was independent of DVL.

In conclusion, in this study we identify for the first time that a WNT receptor is able to form a complex with the proteins of the G α _{12/13} subfamily. In addition, we show that this complex formation is independent of the phospho-protein DVL and that stimulation by WNTs dissociates the G α _{12/13} from FZD₄.

4.2.2 *FZD₄-mediated G protein signalling (Paper II)*

In order to dissect out if the inactive-state assembly and dissociation leads to G α _{12/13} activation, we employed DMR a technique that is able to detect ligand-induced cell changes in live cells. In cells, DMR can be triggered by cellular processes like cytoskeletal rearrangements and adhesion known to be connected to G α _{12/13} signalling (Schröder et al., 2011; Worzfeld et al., 2008). Using DMR measurements on wild type and G α _{12/13} knock-out cells, we conclude that that stimulation of wild type cells with WNT-5A induced negative DMR when FZD₄ was over-expressed. In addition, the negative DMR was absent in cells where G α _{12/13} was ablated. Therefore, we were able to show that the DMR response observed in wild-type cells depends on both FZD₄ and G α _{12/13}.

To further assess the FZD₄-dependent and G α _{12/13}-mediated downstream events and to confirm that FZD₄ initiate the GDP/GTP exchange of G α _{12/13} we performed the p115-RHOGEF recruitment assay. P115-RHOGEF is an RGS for the G α _{12/13} group of G proteins, binding specifically to the GTP form of the G α subunit. Activation of G α _{12/13} leads to recruitment of p115-RHOGEF to the plasma membrane and the GTP-bound protein (Hart et al., 1998; Meyer et al., 2008). Overexpression of fluorescently-tagged FZD₄, G α _{12/13} and p115-RHOGEF illustrated that FZD₄ induced the translocation of p115-

RHOGEF to the membrane, to the same extent as the Lysophosphatidic Acid 1 receptor (LPA₁ receptor) known to signal through G $\alpha_{12/13}$ (Mutoh et al., 2012). This indicates that this receptor not only binds to G $\alpha_{12/13}$, but also induces the GDP/GTP exchange and G protein activation. In contrast, the p115-RHOGEF membranous localization was not present in samples where G $\alpha_{12/13}$ or FZD₄ was absent. In cell culture, endogenous secretion of WNTs is constantly present. In order to evaluate the ligand-dependent p115-RHOGEF recruitment, we treated our cells with the porcupine inhibitor C59. This treatment leads to reduction of WNT protein production and endogenous WNT signalling (Dodge et al., 2012; Galli et al., 2007). In concert with the observed data of inhibited LPA₁ signalling with the LPA₁ receptor-specific antagonist Ki16425 (Ohta et al., 2003), treatment of FZD₄/G $\alpha_{12/13}$ overexpressing cells led to reduced recruitment of p115-RHOGEF.

Finally, to confirm the functionality of FZD₄/G $\alpha_{12/13}$ -mediated signalling to p115-RHOGEF, we introduced the RGS-domain of p115-RHOGEF. p115-RHOGEF binds to the active GTP-bound G $\alpha_{12/13}$ and utilizes RGS activity on the G α subunit as a feedback loop inactivating the signalling (Kozasa et al., 1998). By co-transfecting the domain of p115-RHOGEF accelerating the hydrolysis of GTP back to GDP, the RGS-domain, in combination with FZD₄, G $\alpha_{12/13}$ and p115-RHOGEF, we could decrease the recruitment of p115-RHOGEF to the plasma membrane.

Altogether, the findings in Paper II show for the first time that FZD₄ is a GPCR capable of mediating signalling through the G $\alpha_{12/13}$ /p115-RHOGEF axis.

4.2.3 FZD₁₀ selectively interacts with G α_{13} (Paper III)

While FZD₄ was able to form an inactive-state complex with both G α_{12} and G α_{13} , FZD₁₀ was only capable of interacting with G α_{13} . Isoform selective signalling by the family of G $\alpha_{12/13}$ proteins has been observed before, for example, in the case of stress fiber formation (Gohla et al., 1999). Moreover, stimulation of the inactive-state complex with WNT-5A and WNT-7A dissociated G α_{13} from FZD₁₀ as seen by dcFRAP measurements. On the contrary, stimulation with WNT-3A did not lead to an increase in the mobile fraction of the complex. These data indicate that FZD₁₀ has a selective preference for specific G proteins and specific FZD ligands.

In the case of FZD₄, we showed that DVL was dispensable for FZD₄-mediated G $\alpha_{12/13}$ signalling, while in the case of FZD₆, the G protein interaction was sensitive to both low and high DVL levels (Arthofer et al., 2016; Kilander et al., 2014a). Assessing the importance of DVL for FZD₁₀-mediated signalling, we performed dcFRAP experiments in the presence and absence of the phospho-protein. Data obtained from dcFRAP measurements in DVL1/2/3 knock-out cells clearly show that the absence of DVL does not affect the inactive-state complex between FZD₁₀ and G α_{13} . In contrast to DVL ablation, overexpression of DVL2 abrogated the formation of the complex, indicating that although DVL is not essential for FZD₁₀/G α_{13} -mediated signalling, it can negatively regulate this signalling axis.

DMR is a live cell assay capable of capturing receptor activation linked to G proteins including $G\alpha_{12/13}$ -dependent activation (Schröder et al., 2010). Similar to experiments performed on FZD₄, we employed DMR measurements to dissect out FZD₁₀-mediated $G\alpha_{12/13}$ activation. Stimulation with the two FZD ligands, WNT-5A and WNT-7A, evoked a negative DMR caused by global mass redistribution change of the wild-type cells when FZD₁₀ was transiently expressed. In contrast, WNT-7A and WNT-5A were not able to induce the same changes in cells with genetic ablation of $G\alpha_{12}$ and $G\alpha_{13}$. These data supports the dcFRAP data where WNT-5A and WNT-7A could induce dissociation of the inactive-state complex and further strengthens the proof that FZD₁₀ is able to interact with $G\alpha_{13}$ and activate downstream signalling events. However, in order to state with certainty that the response from FZD₁₀ is exclusively from $G\alpha_{13}$, one would need to repeat the experiment in $G\alpha_{13}$ single knock-out cells in comparison with $G\alpha_{12}$ knock-out cells.

4.2.4 Activation of β -catenin-dependent and -independent signalling by FZD₁₀ (Paper III)

Recently, the WNT signalling pathway has been connected to the hippo pathway and activation of the two nuclear effectors YAP and TAZ. In addition to the WNT involvement in the signalling of this pathway, the family of $G\alpha_{12/13}$ proteins has also been shown to regulate the activation of YAP/TAZ (Azzolin et al., 2014; Park et al., 2015; Yu et al., 2012b). In order to assess downstream signalling of FZD₁₀/ $G\alpha_{13}$, we employed a 8xGTIIIC-luciferase reporter assay to measure FZD₁₀-dependent and $G\alpha_{13}$ -mediated YAP/TAZ activation. This assay confirmed that FZD₁₀ was able to induce activation of YAP/TAZ through $G\alpha_{13}$. Moreover, to prove that activation of $G\alpha_{13}$ was necessary for the induction of YAP/TAZ activity, we once again took advantage of the RGS-domain of p115-RHOGEF, the GAP domain of p115-RHOGEF accelerating the hydrolysis of GTP to GDP on the $G\alpha_{12/13}$ subunit. Indeed the RGS-domain completely abolished the FZD₁₀-induced YAP/TAZ activity showing that FZD₁₀ activates $G\alpha_{13}$ that in turn induce YAP/TAZ transcriptional activity.

Previously, it has been reported that FZD₁₀ is able to activate β -catenin-dependent signalling (Terasaki et al., 2002; Wang et al., 2005). To confirm this and to dissect out the difference between YAP/TAZ and β -catenin activation, we performed the TOPFlash luciferase reporter assay. Indeed, FZD₁₀ was able to activate β -catenin-dependent transcriptional activation. However, in contrast to YAP/TAZ activation FZD₁₀-dependent β -catenin activation required LRP6 coexpression.

The selective preference of FZD₁₀ to particular ligands and G protein isoforms as well as its diverse downstream signalling, illustrates the complexity of the WNT signalling pathway. In Paper III, we add to our knowledge that FZD₁₀ is also able of activating G proteins namely $G\alpha_{13}$. While it does not exclude the potential to signal through β -catenin, depending on the context, FZD₁₀ has the capability of activating $G\alpha_{13}$ downstream effects such as YAP/TAZ-mediated transcriptional activation.

4.2.5 Possible roles of FZD₄/FZD₁₀ mediated Gα_{12/13} signalling in angiogenesis (Paper II & Paper III)

FZD₄ is known to be important for the vascular development, in particular in the formation of the blood-retina barrier (Wang et al., 2012; Xu et al., 2004; Ye et al., 2009; Zhou et al., 2014b). The FZD₄-dependent signalling has so far been associated with the activation of β-catenin. In Paper II, we identify an additional FZD₄/Gα_{12/13}/p115-RHOGEF-mediated signalling axis. With the knowledge that Gα_{12/13} dependent signalling is crucial for proper blood vessel development our findings further support the role of this receptor in vascularisation, both through WNT/β-catenin dependent- and independent signalling (Offermanns et al., 1997b; Ruppel et al., 2005; Sivaraj et al., 2013).

Similar to FZD₄, we identified another receptor homolog, FZD₁₀, which mediates Gα₁₃-dependent signalling to YAP/TAZ. Also YAP/TAZ activation is vital for proper blood vessel development (Choi and Kwon, 2015; Choi et al., 2015; Offermanns et al., 1997b). In addition, in Paper III, we present RNA expression and protein expression data for FZD₁₀ in the developing CNS vasculature by employing *in situ* hybridization and immunohistochemistry. Keeping in mind that FZD₄ and FZD₁₀ belong to the same subfamily of WNT receptors (Schulte, 2010) and that both are expressed during early embryonic development, one could speculate that these two receptors work in a coordinated fashion to provide the necessary signalling cues to the developing vasculature.

5 GENERAL DISCUSSION AND CONCLUSION

Without blood vessels, the organs of our body would not be functional. Our vasculature serves as the main supplier of oxygen and nutrients to our organs, while providing a passage for the immune surveillance from pathogens and toxic agents (Engelhardt and Liebner, 2014). In order to develop better treatments for severe malignancies like stroke and cancer, it is of the utmost importance that we expand our knowledge about the vasculature and its development.

For years, it has been known that WNT signalling is important for the development of blood vessels; in particular, the development of CNS vasculature and the BBB (Cattelino et al., 2003; Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008). WNT signalling and its many components are crucial during development, homeostasis and tissue regeneration. Perturbation of this pathway is associated with developmental defects and severe malignancies including vascular disorders like FEVR and Norrie disease (Luhmann et al., 2005; Nikopoulos et al., 2010; Qin et al., 2005; Rehm et al., 2002; Robitaille et al., 2002; Toomes et al., 2004). In addition, elevated levels or mutation of the components of the pathway are often seen in tumours. While the field has moved forward since the discovery of WNT proteins we are still only scratching the surface when it comes to understanding and drugging the diverse signalling pathways. With this thesis, I hope to broaden the knowledge of WNT signalling and its role in CNS vascular development with the intention that this will contribute to better treatments for the associated diseases.

Despite the wealth of data supporting the inclusion of FZDs as GPCRs, there is still a clear divide in the field and the question is open for debate. FZDs exhibit clear differences from classical GPCRs, but are in many ways similar to their distant family members (Foord et al., 2005; Schulte, 2010). Modifying multiple proteins of the WNT pathway has provided us an insight into how important WNT signalling is in controlling numerous cellular processes like proliferation, migration and adhesion (MacDonald et al., 2009b). Unfortunately, less emphasis has been placed on the basic pharmacological understanding of the structure and mechanism of WNT receptors. The lack of pharmacological tools has introduced roadblocks when it comes to studying FZDs. Not only do ten isoforms of the receptor exist in mammals, but there are also 19 different ligands that are capable of activating the FZDs. Moreover, FZDs are ubiquitously expressed in most cell types. The fact that cells often express several isoforms makes it difficult to study WNT activity both *in vivo* and *in vitro*. In addition, redundancy among FZD isoforms when studying ligand-receptor specificity or loss-of-function mutations adds to the complexity (Hua et al., 2014; Yu et al., 2012c). Lastly, the presence of endogenous FZD ligands and the lack of pharmacological tools like synthetic agonists or small peptides that are able to modulate WNT signalling are other obstacles slowing down progress in the field.

In this thesis, we demonstrate that FZD₄ and FZD₁₀ two receptors that are in the cluster of FZD₄, FZD₁₀ and FZD₉ share ca. 65% homology (Fredriksson et al., 2003) and are both able to activate signalling through the heterotrimeric G protein subfamily of G $\alpha_{12/13}$. With these results, we support the role of FZDs being GPCRs and activating G protein-

dependent signalling cascades. While both of these receptors have been associated to the β -catenin dependent pathway, activation of G proteins by these receptors does not exclude parallel or subsequent activation of β -catenin. It is important to mention that while activation of G proteins takes place within seconds or minutes of ligand stimulation, activation of β -catenin takes up to an hour and remains persistent over longer time. Considering the fact that WNT signalling is important for embryonic development where cellular changes occur from second to second and minute to minute, it is important to consider that events such as the activation of heterotrimeric G proteins might take place before activation of β -catenin regulating more rapid cellular responses (Schulte and Bryja, 2007). The multiple data existing on WNT signalling pathways surely confirms that in the case of the activation of this pathway, the outcome is context dependent.

While FZD₄ has previously been associated with vascular development, its close homolog FZD₁₀ has remained unexplored. Both receptors are expressed during early embryonic development and although FZD₁₀ has not previously been associated with development of the CNS blood vessels, it is worth mentioning that the expression of this receptor is strongly dynamic with highest expression levels during the embryonic stages when angiogenesis is ongoing. Interestingly, with the RNA sequencing experiments, *in situ* hybridization and immunohistochemistry of embryonic tissue, we show with our data that FZD₁₀ mRNA and protein is definitely present in CNS endothelium. Moreover, we show that mRNA expression of FZD₁₀ in endothelial cells is declining towards the end of angiogenesis. The knowledge that these two receptors belong to the same homology cluster and that both can be associated with CNS endothelial cells makes it even more interesting that they are both able to activate β -catenin signalling and to form an inactive-state complex with $G\alpha_{12/13}$. Mutants lacking both of these proteins show severe vascular defects (Cattelino et al., 2003; Daneman et al., 2009; Offermanns et al., 1997b; Stenman et al., 2008). In the case of $G\alpha_{12/13}$ mutants, ablation of $G\alpha_{13}$ shows a strong vascular phenotype while the defects are further amplified when depleting both $G\alpha_{12}$ and $G\alpha_{13}$. In addition, it is worth noting that dissociation of $G\alpha_{12/13}$ from the inactive-state complex formed with either of these two receptors is induced by stimulation with WNT7A one of the two WNT isoforms essential for proper CNS vascular development (Daneman et al., 2009; Stenman et al., 2008). Considering that no phenotype has been reported to belong to perturbed FZD₁₀ signalling and that FZD₄ mutants are exhibiting strongest defects in retina it is possible that these two receptors are employing a compensatory effect considering that both are able to activate the same or similar downstream effectors. For this reason it would be desirable to create a double-knockout mouse depleting both of these receptors in endothelial cells in order to dissect out their role in vascular development.

In order to improve our knowledge in WNT signalling it is important to gain knowledge in the pharmacology of FZDs. The Class FZD GPCRs is some of the least studied GPCR families and to be able to take additional steps forward it is important that we develop new tools to study this group of unconventional receptors. The lack of read-out tools and molecules to modify the receptors with is making it further difficult to

explore the basic structure and mechanism of these receptors. Though we know that receptors such as FZD₁₀ and FZD₄ are able to activate β -catenin- and $G\alpha_{12/13}$ signalling we do not know how the selection between these two routes takes place. Does the activation of either molecule depend on the context and additional proteins such as LRP5/6, does it depend on the ligand or does it simply depend on the induced conformation depending on the binding site of the ligand. We do know that WNTs can bind to the CRD domain of FZDs but so far it has not been demonstrated whether WNTs also bind at the hypothetical binding pockets in the receptor core. Different receptor conformations can be also induced by binding of ligands to allosteric sites on the receptor (Kobilka and Deupi, 2007). If FZDs contain such sites remains to be elucidated but in order to answer these questions we need to continue working on developing correct tools and modifying the existing ones like DMR, dcFRAP, FRET and p115-RHOGEF recruitment assay. From a pharmacological point of view targeting receptors with drugs is far more desirable than targeting proteins like β -catenin, given that modification of for instance β -catenin is opening up for an array of off target effects. For this reason we need to improve our knowledge about FZDs in order to better understand and in order to be able to better modify the WNT signalling pathway.

My hope is that I with this thesis have contributed to better understanding of the WNT signalling pathway. Although it has been known for years that WNT signalling is important for CNS vascular development in this thesis we show that formation of blood vessels in the brain depends on a crosstalk between multiple molecules and pathways. Moreover we open up the possibility that the WNT receptors expressed by the developing endothelium are able of activating other proteins such as heterotrimeric $G\alpha_{12/13}$ in order to regulate proper vascular development. With this I conclude that in order to understand this complex pathway we need to work on developing tools and we need to take advantage of the basic pharmacological approaches when studying this pathway. Finally with the hope that future research will lead to better treatment for patients we need to keep our mind open for the unexpected and not dismiss a hypothesis because we do not yet have the possibility or knowledge to test it.

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