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Tag der mündlichen Prüfung: .....

# **Analysis of novel Wnt pathway components acting at the receptor level**

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## 1. Summary

The Wnt signaling pathway controls diverse cellular processes and is implicated in stem cell biology, embryonic patterning, tissue homeostasis and human diseases, notably cancer. The regulation of the pathway is complex, and some aspects remain incompletely understood. This includes the signaling mechanism of R-spondins, which potentiate Wnt signaling, and the regulation of the endocytosis of the Wnt receptors. In order to obtain a better understanding for these processes, novel Wnt pathway components regulating Wnt signaling at the receptor level were identified and characterized in this thesis.

In search for an R-spondin receptor, the leucine-rich repeat (LRR)-containing G protein-coupled receptor 5 (LGR5) was identified. This thesis provides evidence that the orphan receptor LGR5, and its homolog LGR4, act as R-spondin receptors. Functionally, LGR4 and LGR5 are required for R-spondin 3-mediated Wnt/PCP signaling during *Xenopus* development.

Moreover, in collaboration with Prof. M. Boutros, the Rab GTPase RAB8B was characterized as a novel Wnt/ $\beta$ -catenin signaling regulator that controls LRP6 activity and endocytosis. This requirement of RAB8B for Wnt/ $\beta$ -catenin signaling was confirmed in *Xenopus*.

Furthermore, angiopoietin-like 4 (ANGPTL4) was identified as a novel inhibitor of the Wnt/ $\beta$ -catenin signaling pathway. ANGPTL4 inhibits canonical Wnt signaling in mammalian cells and in *Xenopus* embryos, and is required for notochord formation during *Xenopus* development. In addition, ANGPTL4 is a mediator of TGF $\beta$  signaling and is required for mesoderm formation in *Xenopus* by regulating Activin/Nodal signaling. Similar to R-spondin 3, ANGPTL4 can bind to the syndecan family of transmembrane proteoglycans, which induce its clathrin-mediated endocytosis. The removal of ANGPTL4 increases membrane levels of LRP6, resulting in higher Wnt activity, whereas ANGPTL4 gain-of-function decreases LRP6 cell surface levels. The N-terminal domain of ANGPTL4 is sufficient to mediate this effect. These results suggest that ANGPTL4 inhibits Wnt/ $\beta$ -catenin signaling by removal of LRP6 from the plasma membrane, presumably via clathrin-mediated endocytosis.

## 2. Zusammenfassung

Der Wnt Signalweg reguliert zahlreiche zelluläre Prozesse während der Embryonalentwicklung und der Gewebshomöostase. Fehlerhafte Wnt Signalübertragung ist die Ursache verschiedener menschlicher Krankheiten, insbesondere Krebs. Die Regulation dieses Signalweges ist sehr komplex, und einige Aspekte sind noch immer weitgehend unbekannt. Um die Übertragung von Wnt Signalen zwischen Zellen besser zu verstehen, wurden in dieser Arbeit neue Moleküle charakterisiert, die den Wnt Signalweg auf Rezeptorebene regulieren.

Bei der Suche nach einem R-spondin Rezeptor wurde LGR5 als Kandidat identifiziert. Ich zeige hier, dass LGR5 und dessen Homolog LGR4 als Rezeptoren für R-spondin dienen. Die biologische Relevanz dieser Entdeckung wurde im Tiermodell belegt, wo LGR4 und LGR5 für die Aktivierung des Wnt/PCP Signalweges durch R-spondin 3 während der *Xenopus* Entwicklung benötigt werden.

Ausserdem wurde in Kollaboration mit Prof. M. Boutros ein weiterer neuer Regulator der Wnt Signalkaskade charakterisiert. Wir konnten zeigen, dass die GTPase RAB8B die Aktivität und Endozytose des Wnt Rezeptors LRP6 kontrolliert. Ähnlich wie LGR4/5 wird auch RAB8B für die Aktivierung des Wnt/ $\beta$ -catenin Signalweges während der frühen Entwicklung von *Xenopus* benötigt.

Schließlich identifiziere ich in dieser Arbeit ANGPTL4 als neuen Inhibitor des Wnt/ $\beta$ -catenin Signalweges. ANGPTL4 inhibiert den Signalweg in Säugetierzellen und in *Xenopus*, und wird für die Notochord-Entwicklung in *Xenopus* benötigt. Wie ich hier darlege, reguliert ANGPTL4 zusätzlich den TGF $\beta$  Signalweg und ist an der Mesoderm-Entwicklung in *Xenopus* beteiligt. ANGPTL4 wird nach Bindung an Syndecan-Proteine Clathrin-abhängig endozytiert. In Abwesenheit von ANGPTL4 wird LRP6 an der Zellmembran stabilisiert, wohingegen Expression von ANGPTL4 dazu führt, dass LRP6 von der Membran entfernt wird. Dieser Effekt wird durch den N-Terminus von ANGPTL4 vermittelt. Die Ergebnisse deuten darauf hin, dass ANGPTL4 den Wnt/ $\beta$ -catenin Signalweg durch Entfernung von LRP6 von der Plasmamembran inhibiert. Dies könnte durch Clathrin-abhängige Endozytose von LRP6 erfolgen.



### 3. Introduction

#### 3.1 Wnt signaling transduction cascades

The first Wnt gene, *Wnt1*, was initially discovered in 1982 as a proto-oncogene activated by mouse mammary tumor virus (MMTV) integration.<sup>1</sup> Since then, several other Wnt proteins have been identified, which now comprise a family of 19 secreted, hydrophobic glycoproteins in humans and mice. Wnt molecules are characterized by a signal peptide that mediates protein secretion, followed by a stretch of highly conserved cysteine-rich domains.<sup>2</sup>

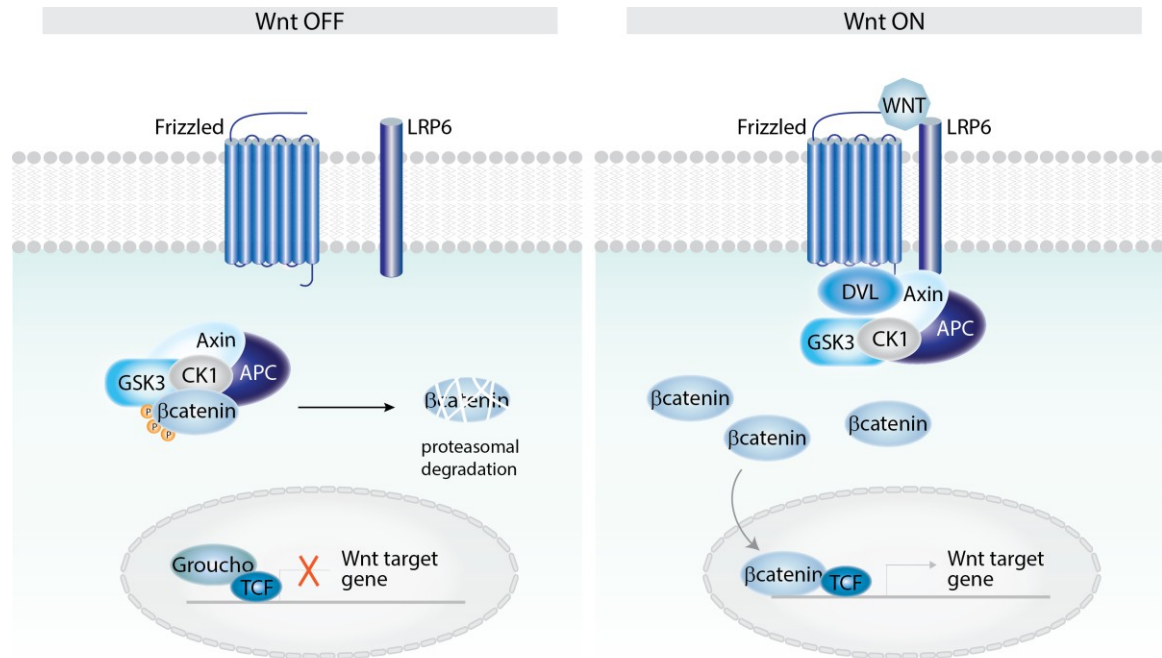
Wnts are evolutionarily conserved, and control various developmental and physiological processes.<sup>3</sup> Each Wnt ligand binds a specific combination of receptor and co-receptor, and thereby stimulates different downstream signaling events. Based on the downstream signaling output, these pathways are commonly referred to as “canonical” and “noncanonical” Wnt signaling, and individual Wnt ligands are broadly classified by their ability to activate one of these cascades. For example, canonical Wnt proteins such as WNT1, WNT3a and WNT8 are able to induce transformation of mouse mammary epithelial cells and formation of secondary axis in *Xenopus* embryos, which are prototypical biological readouts for this pathway.<sup>4,5</sup> However, this initial classification of Wnt signaling has since been extended, following the identification of several sub-branches of both signaling pathways (reviewed in Niehrs, 2012<sup>6</sup>). The induction of either of these signaling pathways depends on the cellular context and the combination of available receptors, rather than on the intrinsic property of the Wnt proteins.<sup>7</sup> In the following chapters, Wnt/ $\beta$ -catenin and Wnt/PCP signaling will be addressed in detail.

### 3.1.1 Wnt/ $\beta$ -catenin signaling

The Wnt/ $\beta$ -catenin signaling pathway controls major developmental processes such as axis formation and nervous system patterning, and is implicated in stem cell biology and human diseases, notably cancer.<sup>3,8,9</sup>

The key event in Wnt/ $\beta$ -catenin signaling is the cytoplasmic stabilization of the transcriptional co-factor  $\beta$ -catenin and its subsequent nuclear translocation. In the absence of Wnt ligands, cytoplasmic  $\beta$ -catenin levels are kept low, through continuous proteasome-mediated degradation, which is controlled by a multiprotein complex containing glycogen synthase kinase 3 (GSK3), casein kinase 1 alpha (CK1 $\alpha$ ), Axin, and adenomatous polyposis coli (APC). The scaffold proteins Axin and APC assemble the destruction complex,<sup>10-13</sup> whereas CK1 $\alpha$  and GSK3 phosphorylate  $\beta$ -catenin at conserved Ser/Thr-residues near the amino terminus.<sup>14,15</sup> Phosphorylated  $\beta$ -catenin is then recognized by the beta-transducin repeat containing E3 ubiquitin protein ligase ( $\beta$ -TrCP), which ubiquitinates  $\beta$ -catenin and thereby targets it for proteasomal degradation (Figure 1).<sup>16-20</sup>

Following Wnt binding to its receptors Frizzled (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the ternary complex undergoes clustering on dishevelled (DVL) platforms.<sup>21</sup> This in turn induces internalization of the ligand-receptor complex via endocytic vesicles, termed LRP6 signalosomes.<sup>21</sup> These vesicular structures are acidified by the vacuolar ATPase (v-ATPase), which interacts with LRP6 via the prorenin receptor (PRR).<sup>22</sup> This promotes phosphorylation of LRP5/6 by CK1 $\gamma$ <sup>21,23</sup> and GSK3<sup>24-26</sup> and leads to membrane recruitment of the negative regulator Axin together with the other components of the destruction complex.<sup>27,28</sup> Subsequently, GSK3 is inhibited, which stabilizes  $\beta$ -catenin.<sup>25</sup> Following its stabilization,  $\beta$ -catenin binds to the lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors, replacing the transcriptional co-repressor groucho, to activate Wnt target gene transcription (Figure 1).<sup>29</sup>



**Figure 1 | Model of Wnt/ $\beta$ -catenin signaling.** In the absence of Wnt,  $\beta$ -catenin is bound to the destruction complex, consisting of Axin, APC, CK1 $\alpha$  and GSK3. The latter two phosphorylate  $\beta$ -catenin, which triggers its proteasomal degradation. In the presence of Wnt, the destruction complex is recruited to the Wnt-Frizzled-LRP5/6 receptor complex and inactivated. This allows the accumulation of  $\beta$ -catenin and the activation of Wnt target gene transcription. Figure modified from Schuijers & Clevers, 2012.<sup>30</sup>

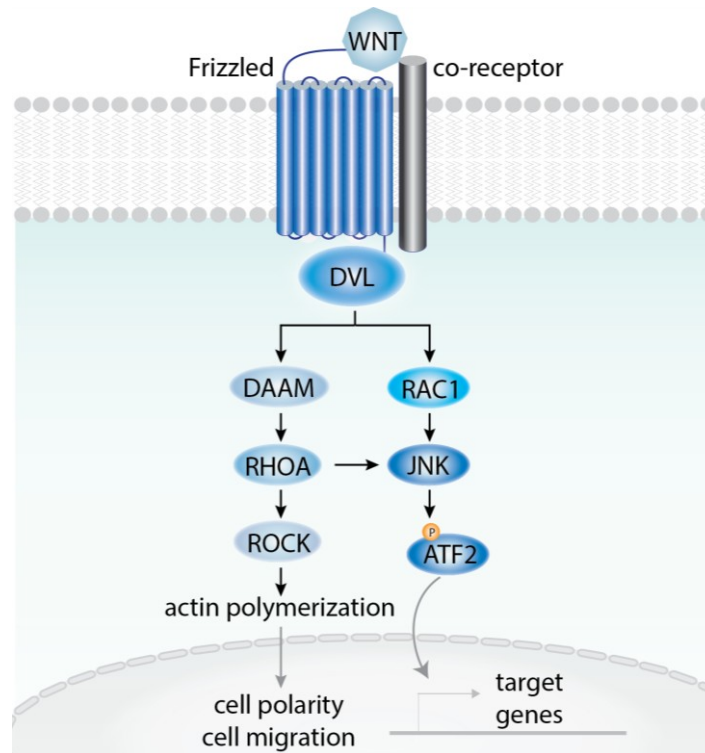
Although GSK3 activity is critical for  $\beta$ -catenin stability and thus Wnt signaling, the molecular mechanism of GSK3 inhibition remains under debate.<sup>31</sup> As a first and likely intermediate response, the phosphorylated, intracellular domain of LRP5/6 not only recruits, but also directly inhibits GSK3.<sup>32</sup> Afterwards, sustained inhibition of GSK3 is achieved by its sequestration in multivesicular bodies (MVBs).<sup>33,34</sup> Following internalization, LRP6 signalosomes are recruited to the endosomal sorting complex required for transport (ESCRT), which mediates sorting of these vesicles to late endosomes, which undergo intraluminal budding. Thereby GSK3 is sequestered in the lumen of MVBs and can no longer target its substrates, including  $\beta$ -catenin, for proteasomal degradation.<sup>33</sup> In addition, it has been observed that Axin is dephosphorylated in response to Wnt signaling. This decreases the affinity of Axin for  $\beta$ -catenin, and releases  $\beta$ -catenin from the destruction complex.<sup>35,36</sup>

### 3.1.2 Wnt/PCP signaling

The Wnt/planar cell polarity (PCP) pathway does not involve the activation of  $\beta$ -catenin/TCF-dependent transcription and is thereby distinct from canonical Wnt signaling. Wnt/PCP signaling regulates various aspects of cell migration and polarity during morphogenetic processes.<sup>2,37,38</sup> In vertebrates it mediates cell movements during gastrulation, neural tube closure, hair cell orientation and ciliogenesis.<sup>2,39,40</sup>

The PCP pathway was originally identified in *Drosophila*, where it regulates the polarity of actin-rich protrusions (trichomes) in the adult wing and abdomen.<sup>41</sup> Similar to Wnt/ $\beta$ -catenin signaling, FZD and DVL are also required for Wnt/PCP signaling. Genetic studies in *Drosophila* uncovered additional core components of the Wnt/PCP pathway. These include the four transmembrane protein Strabismus (Stbm/Vangl2), the atypical cadherin Flamingo (Fmi), and the cytoplasmic proteins Prickle (Pk) and Diego (Dgo). The atypical, cadherin-domain containing seven transmembrane protein Fmi<sup>42</sup> differentially recruits FZD and Stbm to opposite sides of the cell.<sup>43</sup> Thereby complexes of the transmembrane proteins Fmi and Stbm, together with the cytoplasmic protein Pk accumulate at the proximal side of the cell, whereas Fmi and FZD, together with DVL and Dgo, localize to the distal border. This separation allows the interaction of the two complexes at opposite sides of the cell.<sup>43,44</sup>

In contrast to *Drosophila*, the molecular basis of the interaction of Fmi with the Wnt/PCP pathway in vertebrates remains elusive.<sup>45,46</sup> Furthermore initial studies in *Drosophila* suggested that Wnt proteins are not required for signal transduction.<sup>43</sup> At least in vertebrates, however, WNT5A and WNT11 induce the Wnt/PCP signaling pathway through binding to FZD receptors.<sup>47,48</sup> This in turn recruits DVL to the plasma membrane and activates a downstream signaling cascade that involves the small GTPases RAC1 and RHOA, the Jun-N-terminal kinase (JNK), and Rho-associated coiled-coil containing protein kinase (ROCK). These kinases can phosphorylate and thereby activate transcription factors such as activating transcription factor 2 (ATF2), which induce transcription of target genes, thereby mediating cytoskeleton remodeling (Figure 2).<sup>2,39</sup>



**Figure 2| Model of Wnt/planar cell polarity (PCP) signaling.** Activation of the Wnt/PCP pathway, by binding of Wnt ligands to Frizzled and different possible co-receptors, induces recruitment of DVL and activation of the small GTPases RHOA and RAC1. These in turn activate ROCK and JNK, which phosphorylate the transcription factor ATF2. This results in actin polymerization and microtubule stabilization. Thereby Wnt/PCP signaling regulates cell polarity, motility and morphogenetic movements. Figure modified from Niehrs, 2012.<sup>6</sup>

In addition to the core components, several co-receptors for Wnt/PCP signaling have been identified. The heparan sulfate proteoglycans (HSPG) glypican and syndecan (SDC) have been shown to bind both the Wnt ligand and Frizzled and enhance Wnt signaling.<sup>49-52</sup> In the case of SDC4, direct interaction with DVL was observed.<sup>52</sup> Another class of Wnt co-receptors are the single transmembrane receptor tyrosine kinases ROR1 and ROR2. In vertebrates ROR2 binds to noncanonical Wnts, like WNT5A and WNT11, and associates with FZD receptors to mainly transduce Wnt/PCP signaling.<sup>53,54</sup>

As indicated above, Wnt/ $\beta$ -catenin and Wnt/PCP signaling are involved in a multitude of developmental processes and share several signaling components. A characteristic of Wnt/ $\beta$ -catenin and Wnt/PCP signaling is that they antagonize each other. The inhibition of one pathway will up-regulate the other branch.<sup>6</sup>

### 3.2 Wnt/ $\beta$ -catenin signaling regulation at the receptor level

Wnt signaling has to be tightly regulated to prevent excessive activation, which could induce misregulation of cell differentiation, proliferation or migration.<sup>3</sup> Aberrant regulation of Wnt signaling is associated with a variety of diseases, including bone and metabolic diseases, and cancer.<sup>8,55</sup> The majority of colorectal cancer patients carry mutations in adenomatous APC, and to a lesser extent in Axin or  $\beta$ -catenin, all resulting in activation of the Wnt/ $\beta$ -catenin signaling pathway (reviewed in Clevers & Nusse, 2012<sup>55</sup>). Therefore the Wnt signaling pathway is regulated at different levels by a wide range of effectors.<sup>3,6,56</sup> As regulation of Wnt signaling at the receptor level is the subject of this thesis, control of Wnt receptors, extracellular modifiers and different endocytic-routes are introduced below.

#### 3.2.1 Wnt receptors

Wnt/ $\beta$ -catenin signaling requires activation of the co-receptor LRP5/6, which forms a ternary complex with Wnt ligands and the FZD receptor. These receptors can be regulated at different levels, either extra- or intracellularly. Furthermore, the association of Frizzled and LRP5/6 with different co-receptors such as the syndecans can further regulate downstream signaling.

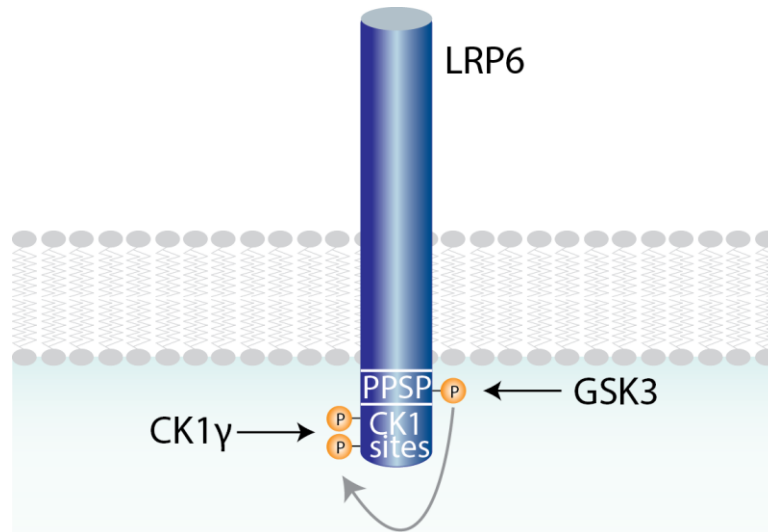
#### Frizzled

The core Wnt receptors of the Frizzled family comprise 10 members in humans.<sup>57</sup> They can associate with several different co-receptors and mediate not only Wnt/ $\beta$ -catenin, but also Wnt/PCP signaling. Frizzled proteins belong to the superfamily of G protein-coupled receptors (GPCRs) and contain seven membrane-spanning domains.<sup>58,59</sup> Frizzled proteins interact with Wnts via their highly conserved, large extracellular cysteine-rich domain (CRD).<sup>59-61</sup> Besides Wnts, Frizzled proteins bind other secreted proteins that regulate its function. For instance, FZD4 has been shown to interact with Norrin, which binds to the CRD and mediates oligomerization of FZD4, thereby activating Wnt/ $\beta$ -catenin signaling.<sup>62,63</sup> The binding of cytoplasmic proteins to the intracellular domain of FZD can further regulate the receptor. One of the best-characterized FZD interacting proteins is the scaffold protein DVL.<sup>64</sup> DVL is involved in Wnt/ $\beta$ -catenin, as well as Wnt/PCP signaling and mediates interaction of the receptor with several other

cytoplasmic proteins.<sup>2</sup> In the case of Wnt/ $\beta$ -catenin signaling, DVL contributes to the recruitment of the destruction complex, whereas in Wnt/PCP signaling it binds to atypical PKC (aPKC) or DVL-associated activator of morphogenesis 1 (DAAM1), which mediate the activation of the small GTPases RHO and RAC.<sup>65</sup>

### **LRP5 and LRP6**

LRP5, LRP6 and their *Drosophila* homolog Arrow are type-I single-pass transmembrane proteins and constitute a subfamily of the low density lipoprotein (LDL) receptor family. They are characterized by a large extracellular domain and a short cytoplasmic tail, containing five Pro-Pro-Pro-Ser/Thr-Pro (PPPSP) sites and several Ser/Thr clusters.<sup>26,66</sup> The extracellular domain (ECD) contains different Wnt binding sites and mediates the interaction with Wnt and Frizzled, resulting in the formation of a ternary signaling complex.<sup>67</sup> The regulation of LRP5/6 activity is mainly achieved by phosphorylation of the cytoplasmic domain of the protein. The PPPSP sites are required for Wnt/ $\beta$ -catenin signaling, as evidenced by the fact that an LRP6 mutant lacking those domains is inactive in signaling.<sup>28,68</sup> The phosphorylation of these PPPSP sites is mediated by proline-directed kinases, such as GSK3, upon activation of Wnt signaling (Figure 3).<sup>24</sup> Thereby, GSK3 acts as a dual regulator of Wnt signaling, as it inhibits Wnt signaling by phosphorylation of  $\beta$ -catenin and activates it by phosphorylation of LRP6.<sup>24</sup> Phosphorylation of the PPPSP sites primes LRP6 for phosphorylation by CK1 $\gamma$  at neighboring Ser residues (Figure 3). The phosphorylation of all sites promotes Axin recruitment to the plasma membrane and thereby activates Wnt signaling.<sup>23</sup>



**Figure 3 | LRP6 phosphorylation by GSK3 and CK1 $\gamma$ .** The intracellular domain of LRP6 has five PPPSP and CK1 phosphorylation sites. Phosphorylation of PPPSP sites by GSK3 primes LRP6 for CK1 $\gamma$  phosphorylation upon extracellular Wnt stimulation. Figure modified from Davidson *et al.*, 2009.<sup>69</sup>

The activity of LRP6 is also regulated by other kinases. For example, serine/threonine kinase CK2 is activated upon Wnt stimulation and phosphorylates LRP6 at a highly conserved Ser1579 site. This phosphorylation promotes binding of the endocytic adaptor disabled-2 (DAB2), which inhibits Wnt signaling by inducing clathrin-dependent LRP6 endocytosis.<sup>70</sup>

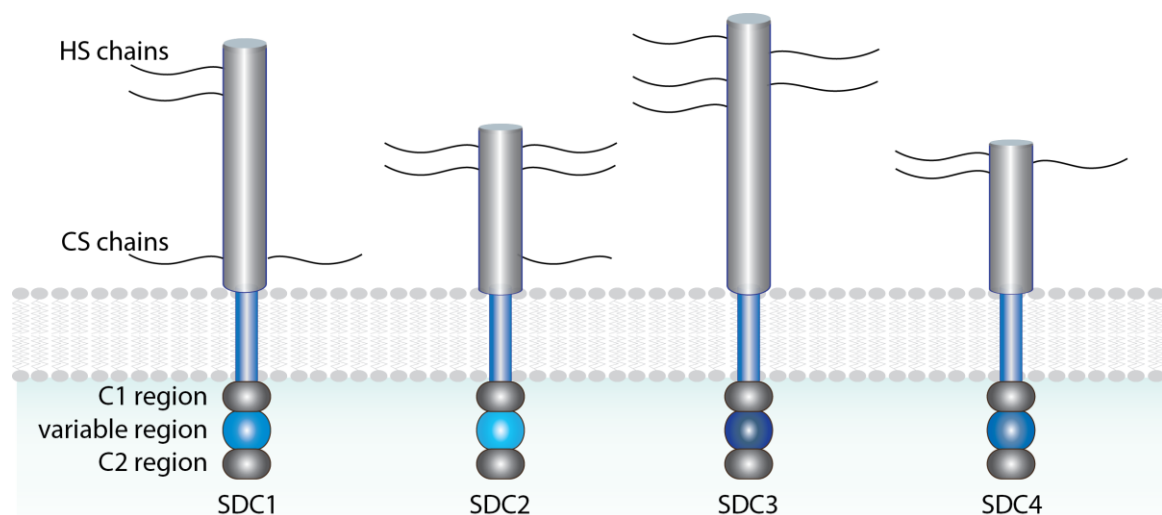
### Syndecans

Syndecans are a family of four transmembrane proteoglycans, which were described as co-receptors of Wnt signaling. Based on sequence homology, the vertebrate syndecans can be divided into two subclasses, with SDC1 and SDC3 constituting one class, and SDC2 and SDC4 making up the other.<sup>71,72</sup> Syndecans contain an extracellular domain with attachment sites for three to five heparan sulfate (HS) or chondroitin sulfate (CS) glycosaminoglycan (GAG) chains, a single-pass transmembrane domain, and a short cytoplasmic domain (Figure 4).<sup>72</sup> The HS chains mediate interaction with a variety of growth factors such as Hedgehog, FGFs, TGF $\beta$ , and Wnts.<sup>73</sup> Furthermore, interaction with several extracellular matrix proteins has been described. This mostly requires modification of the GAG chains by sulfatases, generating highly specific binding sites.<sup>74</sup> Whereas the homology of the ECD is fairly low, the transmembrane domain is highly



conserved between the four syndecans. The cytoplasmic domain can be divided into three regions, containing two highly conserved (C1, C2) and a variable (V) domain (Figure 4). These domains contain binding sites for several cytoplasmic proteins (reviewed in Tkachenko, 2005<sup>75</sup>), coupling syndecans to different downstream processes.

The four syndecan members show distinct expression patterns, which are tightly regulated during development. *SDC1* is expressed very early in development in epithelial and mesenchymal tissue, whereas *SDC3* is mainly expressed in neural and musculoskeletal tissue. *SDC2* is detected in cells of mesenchymal origin, as well as in neuronal and epithelial cells. *SDC4*, on the other hand, is ubiquitously expressed from early stages of development.<sup>75,76</sup>



**Figure 4 | Structure of syndecans.** The extracellular domain of all four syndecans with attachment sites for 3-5 heparan sulfate (HS) or chondroitin sulfate (CS) chains is shown in light grey. The transmembrane domain links the extracellular domain to the intracellular cytoplasmic part of the protein, consisting of one variable and two conserved (C1, C2) regions. The four syndecans (SDC) differ in their extracellular domain and variable region. Figure modified from Pap & Bertrand, 2013.<sup>77</sup>

Of all syndecans, the function of *SDC4* is best described. *SDC4* regulates Wnt/PCP signaling in *Xenopus* via direct interaction with FZD and DVL and recruitment of DVL to the plasma membrane.<sup>52</sup> Moreover, *SDC4* was shown to bind *RSPO3*, which also

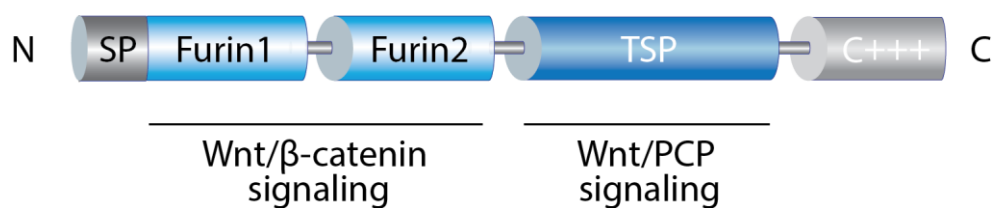
activates Wnt/PCP signaling.<sup>78</sup> In this manner syndecans add another level of regulation to the Wnt receptor complex.

### 3.2.2 Extracellular modifiers

Besides Wnt ligands, several other proteins have been identified that can interact with the Wnt (co-)receptors and thereby regulate Wnt signaling. The most prominent ones are R-spondins.

#### R-spondins

R-spondins (roof plate specific spondin, RSPO) comprise a family of four secreted proteins, which potently enhance Wnt/ $\beta$ -catenin and Wnt/PCP signaling in a Wnt-dependent manner.<sup>78-80</sup> The four R-spondin proteins consist of an N-terminal signal peptide, two furin domains which are required for Wnt/ $\beta$ -catenin signaling,<sup>79</sup> and one thrombospondin type I domain (TSP), which promotes Wnt/PCP signaling (Figure 5).<sup>78</sup> Interestingly, R-spondins enhance Wnt signaling even in the presence of low amounts of Wnt ligands. In the absence of Wnts, R-spondins are not able to induce  $\beta$ -catenin accumulation.<sup>81,82</sup>



**Figure 5| Domain organization of R-spondins.** The R-spondin family members consist of an N-terminal signal peptide (SP), mediating secretion, two cysteine-rich furin-like domains (Furin1, Furin2), a single thrombospondin type 1 repeat (TSP) and a positively charged C-terminal tail of variable length. The Furin domains are implicated in Wnt/ $\beta$ -catenin signaling and the TSP domain is required for Wnt/PCP signaling. Figure modified from Jin & Yoon, 2012.<sup>83</sup>

R-spondins have been shown to control embryonic development and differentiation,<sup>79,84-86</sup> and can stimulate the proliferation of crypt stem cells in the intestine via activation of Wnt/ $\beta$ -catenin signaling.<sup>87,88</sup> However, the molecular

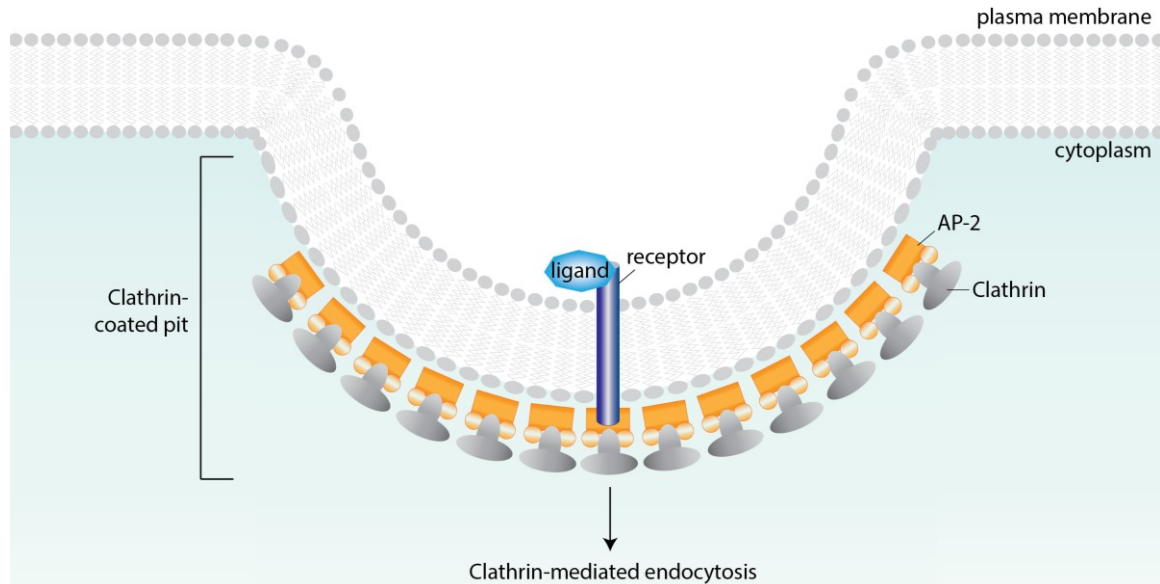
mechanism of R-spondin activity in Wnt signaling remained elusive. Several receptors were proposed as binding partners of R-spondins, including LRP6 and Kremen, which however could not be confirmed.<sup>78,81</sup> Recently, syndecans were identified as receptors for RSPO2 and RSPO3, binding to their TSP domain.<sup>78</sup> In *Xenopus*, SDC4 and RSPO3 are essential for convergent extension movements during gastrulation and head cartilage morphogenesis.<sup>78</sup> Syndecans are known to undergo internalization upon ligand binding. Consistently, RSPO3 binding induces SDC4-dependent, clathrin-mediated endocytosis of the FZD-WNT5A complex, which is essential for Wnt/PCP signaling.<sup>78</sup>

### 3.2.3 Regulation of Wnt signaling by endocytosis

There is increasing evidence that internalization of the Wnt-receptor complex can not only attenuate signal transduction, but is also required for pathway activation.<sup>89</sup> In this context, two major signaling events have been described, namely clathrin and caveolin-mediated endocytosis.

#### Clathrin-mediated endocytosis

In clathrin-mediated endocytosis, ligand-receptor complexes accumulate in “coated pits” at the plasma membrane (Figure 6). Clathrin-coated pits consist of cytosolic coat proteins, which assemble into a cage-like structure. This requires clathrin, the major assembly unit, which is comprised of three heavy and three light chains forming a three-legged structure, also known as triskelion.<sup>90</sup> Besides clathrin, several other proteins such as the assembly protein 2 (AP-2), are involved in the formation of the polygonal clathrin coat (Figure 6).<sup>91</sup> Other proteins like  $\beta$ -arrestin1/2 work as adaptor proteins, which on the one hand bind AP-2 and clathrin, and on the other hand interact with various activated GPCRs.<sup>92</sup> Thereby  $\beta$ -arrestins mediate the accumulation of these receptors in clathrin-coated pits.<sup>93</sup> During the late process of clathrin-coated vesicle (CCV) formation, dynamin, a GTP-binding protein, assembles at the neck of invaginating pits and mediates membrane fission.<sup>94</sup> After internalization, CCVs are uncoated and fuse with early endosomes. At this step membrane proteins can either be recycled to the plasma membrane, or transported to late endosomes and lysosomes for degradation. (reviewed in Kikuchi *et al.*, 2007<sup>95</sup>)



**Figure 6| Model of clathrin-mediated endocytosis.** Ligand-receptor complexes are concentrated in clathrin-coated pits in the plasma membrane. These structures are assembled by cytosolic coat proteins, including clathrin and assembly protein 2 (AP-2). Clathrin-coated vesicles undergo endocytosis and are uncoated after internalization, and fuse with early endosomes. Figure modified from Dobrowolski & De Robertis, 2012.<sup>96</sup>

In the case of Wnt/PCP signaling, only clathrin-mediated endocytosis seems to be essential for the induction of the signaling pathway. Wnt/PCP signaling is mainly induced by binding of Wnt ligands to their Frizzled receptors. As Frizzled receptors themselves are GPCRs,<sup>58,59</sup>  $\beta$ -arrestin is implicated in the internalization of FZD. Upon binding of FZD4 to WNT5A, FZD4 undergoes internalization, mediated by  $\beta$ -arrestin2. This requires DVL2, which recruits  $\beta$ -arrestin2 to the plasma membrane and thereby induces the internalization of FZD4.<sup>97</sup> Besides its interaction with  $\beta$ -arrestin, DVL2 also binds a subunit of AP-2. This association mediates the Wnt-dependent internalization of FZD4 and is essential for Wnt/PCP signaling in *Xenopus*.<sup>98</sup> In addition to Wnts, RSPO3 also induces clathrin-mediated endocytosis of Frizzled, which is essential for Wnt/PCP signaling.<sup>78</sup>

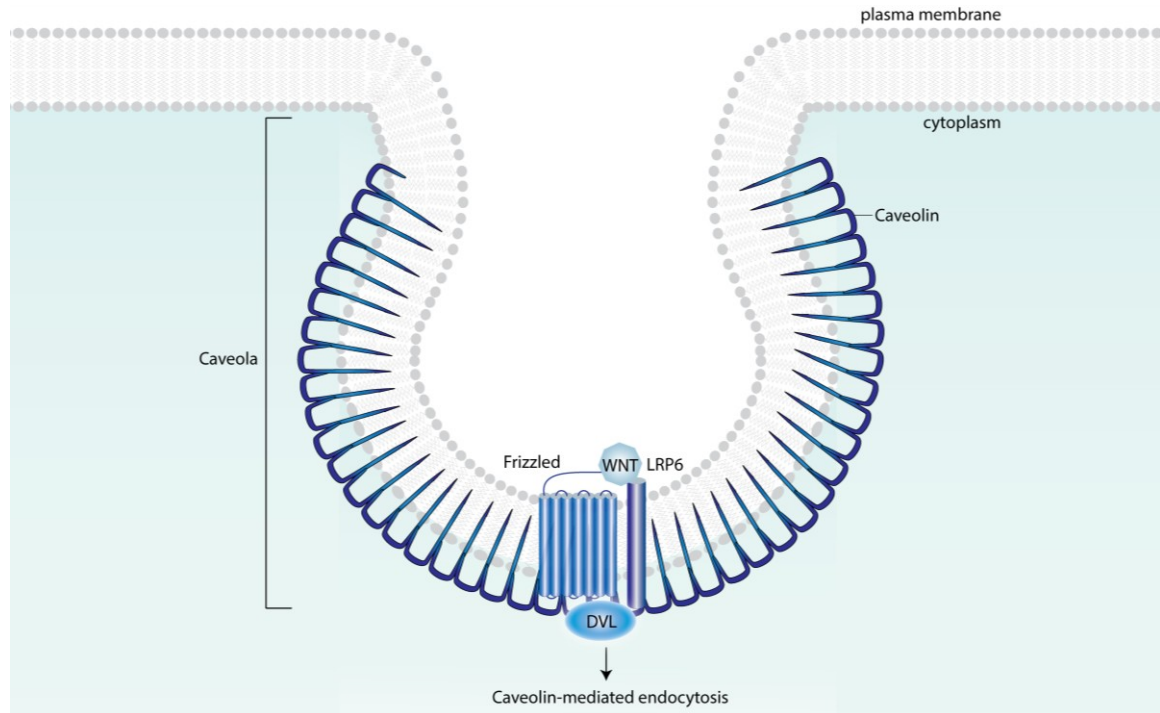
For Wnt/ $\beta$ -catenin signaling there are several reports showing the importance of caveolin-mediated endocytosis for activation of downstream signaling (see below). However, it has been proposed that clathrin-dependent internalization is also involved in the activation of  $\beta$ -catenin signaling. In murine L-cells,  $\beta$ -catenin accumulation

induced by WNT3A was suppressed upon addition of clathrin specific inhibitors.<sup>89</sup> Furthermore, inhibition of  $\beta$ -arrestin in mouse embryonic fibroblasts or *Xenopus* blocked  $\beta$ -catenin accumulation after Wnt stimulation.<sup>99</sup> In addition to activation of Wnt/ $\beta$ -catenin signaling, the internalization via clathrin-dependent routes can also inhibit the pathway. This is achieved by binding of DKK1 to LRP5/6 and Kremen. The ternary complex is rapidly internalized in a clathrin-dependent manner and attenuates Wnt signaling by removal of LRP5/6 from the plasma membrane.<sup>100</sup>

### **Caveolin-mediated endocytosis**

Caveolin-mediated endocytosis takes place in regions of the plasma membrane called caveolae. These are flask-shaped plasma membrane invaginations rich in cholesterol and sphingolipid, which concentrate many membrane transporter and signaling molecules.<sup>101,102</sup> Caveolae are formed by caveolin, which directly binds cholesterol and confers the characteristic shape and structural organization. Caveolin is an integral membrane protein and self-associates to form a striated caveolin coat on the surface of membrane invaginations (Figure 7).<sup>103,104</sup> Membrane fission of caveolae also involves dynamin, similar to clathrin-mediated endocytosis.<sup>94</sup> In contrast to CCVs, caveolin is stably associated with the plasma membrane of the internalized vesicles and caveolae are only slowly internalized<sup>102</sup> (reviewed in Lajoie & Nabi, 2010<sup>105</sup>).

Wnt/ $\beta$ -catenin signaling proceeds via caveolin-mediated endocytosis to trigger downstream signaling. In response to Wnt signaling, LRP6 has been found in intracellular aggregates that contain caveolin.<sup>21</sup> Consistently, it has been shown that LRP6 undergoes caveolin-mediated endocytosis upon Wnt stimulation, and inhibition of caveolin-dependent endocytosis blocks WNT3A-induced  $\beta$ -catenin accumulation.<sup>106,107</sup>



**Figure 7| Model of caveolin-mediated endocytosis.** Upon binding of Wnt to Frizzled and LRP6, DVL is recruited to the plasma membrane. DVL is required for the polymerization of the receptors in caveolae and endocytosis via caveolin-coated vesicles. Other components of the Wnt/ $\beta$ -catenin signaling pathway are not shown for simplicity. Figure modified from Dobrowolski & De Robertis, 2012.<sup>96</sup>

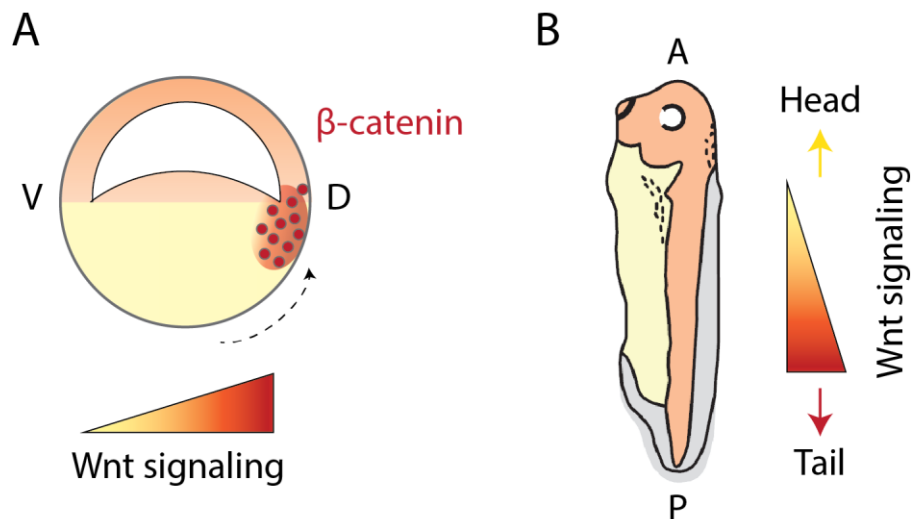
### 3.3 Wnt signaling during *Xenopus* development

*Xenopus* is an important and established model system for studying Wnt signaling in vertebrates. Major advantages are the large size of the embryos and easy accessibility, which allows microinjection experiments, short generation times and the high degree of conservation of essential cellular and molecular mechanisms, including the Wnt signaling pathway.<sup>108,109</sup>

During *Xenopus* development, Wnt/ $\beta$ -catenin signaling is first involved in the establishment of the dorsal-ventral (D-V) axis and later in the patterning of the anterior-posterior (A-P) axis. These two processes can be divided into two separate events: Maternal Wnt signaling, which is controlled by molecules inherited from the mother, and zygotic Wnt signaling, which begins after the initiation of transcription in the embryo.<sup>110</sup> D-V axis determination starts with fertilization of the egg. Sperm entry initiates rotation of the cortical cytoplasm, which relocates dorsal determinants from the

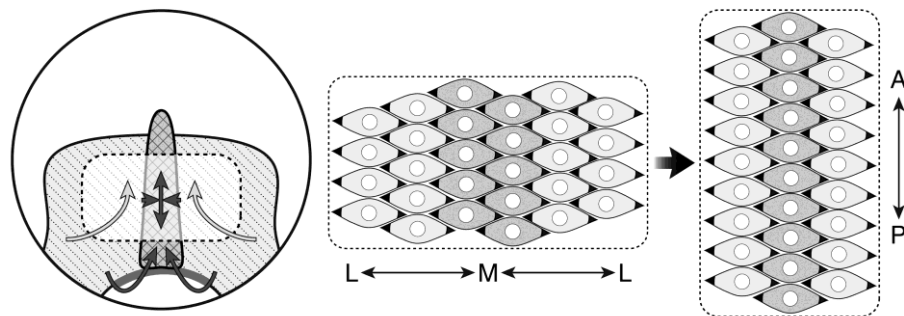
vegetal pole to the dorsal side.<sup>111-114</sup> These determinants include maternal *WNT11* mRNA,<sup>115</sup> which induces the asymmetric stabilization and nuclear translocation of  $\beta$ -catenin (Figure 8A).<sup>116-118</sup> Thereby Wnt signaling induces dorsal-specific gene expression, which is responsible for Spemann organizer formation.<sup>116,119</sup> This signaling center dorsalizes the three germ layers and is required for the formation of the A-P axis.

Zygotic Wnt/ $\beta$ -catenin signaling mediates A-P axis specification by establishing a linear signaling gradient.<sup>120</sup> Wnt/ $\beta$ -catenin signaling activity is high at the posterior pole, where Wnt signaling promotes posterior cell fates.<sup>121,122</sup> In the anterior part of the embryo, Wnt activity is low, due to secretion of Wnt antagonists (Figure 8B).<sup>123-125</sup> Accordingly, zygotic Wnt/ $\beta$ -catenin gain-of-function induces posteriorization, characterized by lack of head structures, whereas conversely, loss-of-function causes anteriorization, marked by big heads, enlarged cement glands and short trunks.



**Figure 8 | *Xenopus* axis specification by maternal and zygotic Wnt/ $\beta$ -catenin signaling. (A)** Maternal Wnt/ $\beta$ -catenin signaling establishes the dorsal (D) – ventral (V) axis. After fertilization, cortical rotation (black dotted line) induces accumulation of  $\beta$ -catenin at the future dorsal site, where it activates gene transcription leading to formation of the Spemann organizer. **(B)** Zygotic Wnt/ $\beta$ -catenin signaling is involved in anteroposterior axis specification. Secretion of Wnt antagonists in the anterior (A) region and expression of Wnt ligands in the posterior (P) region establish a Wnt gradient along the embryonic axis, which is essential for axis formation. Figure modified from Hikasa & Sokol, 2013.<sup>110</sup>

Wnt/PCP signaling also contributes to *Xenopus* development, by regulating cell movements during gastrulation. This includes convergent extension (CE) movements, which drive the elongation of the body axis. In this process, dorsal mesodermal cells align with mediolateral orientation. These cells possess protrusive activities on their lateral surfaces, which allow directed cell movements. Thereby cells intercalate and the tissue narrows (convergence) mediolaterally. At the same time, the tissue elongates along the A-P axis (extension), which results in the formation of the A-P body axis (Figure 9).<sup>126,127</sup> This process is tissue-autonomous, because explants of the dorsal marginal zone (Keller explants) undergo elongation in the absence of neighboring, mesodermal tissue.<sup>128</sup> Because Wnt/PCP signaling is essential for convergent extension, interfering with the pathway results in shortened embryonic body axis and defects in neural tube closure (spina bifida).<sup>78,129,130</sup>



**Figure 9 | Convergent extension movements during *Xenopus* gastrulation.** Schematic illustration of the dorsal vegetal region of a *Xenopus* gastrula embryo. Arrows indicate movements of cells. Mesodermal cells align mediolaterally (L $\leftrightarrow$ M $\leftrightarrow$ L) and based on their protrusive activity at the mediolateral edges, these cells intercalate. Thereby sheets of cells narrow mediolaterally (convergence) and at the same time elongate in anterior (A) posterior (P) direction (extension), which establishes formation of the A-P body axis. Figure modified from Tada & Kai, 2009.<sup>40</sup>



## 4. Aim of the thesis

The Wnt signaling pathway has been studied extensively, however several aspects of the transduction cascade, especially at the level of the receptors, remain unresolved. The aim of this thesis is to identify new regulators of the Wnt signaling pathway, which control Wnt signaling at the receptor level. These regulators should be analyzed regarding their molecular mechanism and their physiological relevance in Wnt signaling and validated *in vivo*, using *Xenopus* as a model organism. This should lead to a better understanding of the Wnt signaling pathway. Here I describe three new Wnt regulators that affect Wnt signaling at the receptor level:

**Chapter 1: LGR4 and LGR5 are R-spondin receptors mediating Wnt signaling.** R-spondins enhance both Wnt/ $\beta$ -catenin and Wnt/PCP signaling in the presence of Wnt.<sup>78-80</sup> The molecular mechanism of Wnt signal enhancement and the reason for the requirement of Wnts to enhance both signaling pathways are incompletely understood. We thus aimed to identify novel R-spondin receptors. The requirement of these receptors for R-spondin mediated Wnt/PCP signaling was analyzed in *Xenopus* embryos.

**Chapter 2: RAB8B is required for Wnt/ $\beta$ -catenin signaling in *Xenopus*.** Endocytosis plays an important role in signal transduction of the Wnt pathway,<sup>89</sup> but how endocytosis of the Wnt receptors is regulated is incompletely understood. The small GTPase RAB8B was identified as regulator of LRP6 receptor endocytosis in an RNAi screen. To analyze if the function of RAB8B on Wnt/ $\beta$ -catenin signaling is conserved, the effect of RAB8B loss-of-function was analyzed in *Xenopus*.

**Chapter 3: ANGPTL4 binds syndecans and inhibits Wnt/ $\beta$ -catenin signaling by promoting LRP6 internalization.** ANGPTL4 was identified in a genome-wide siRNA screen with the aim to discover novel molecules that regulate Wnt signaling at the receptor level. The function and mechanism of ANGPTL4 in Wnt signaling was investigated *in vitro* and the biological function was characterized during the early development of *Xenopus* embryos.

## **5. Chapter 1: LGR4 and LGR5 are R-spondin receptors mediating noncanonical Wnt signaling in *Xenopus***

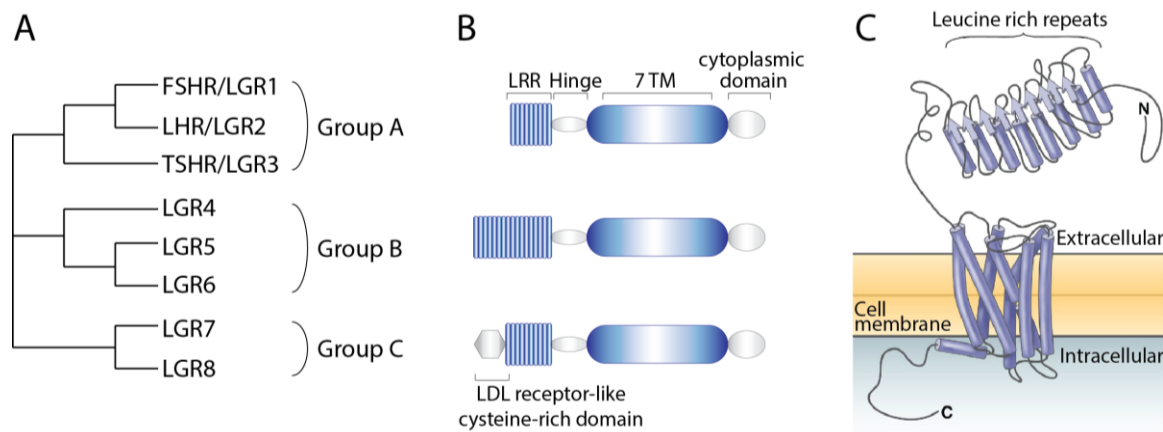
### **5.1 Introduction**

#### **5.1.1 Leucine-rich repeat-containing G protein-coupled receptor family**

The G protein-coupled receptor (GPCR) family represents one of the largest families of transmembrane proteins comprising more than 800 receptors.<sup>131</sup> The main characteristic of GPCRs is the presence of seven transmembrane domains. Through their membrane-spanning domain, these receptors convert extracellular signals into intracellular signaling responses by activation of heterotrimeric G proteins. These extracellular stimuli can be quite diverse, comprising sensory and chemical signals, including light, odor, pheromones, hormones and neurotransmitters.<sup>132</sup> The binding of the respective ligand to GPCRs induces the activation of heterotrimeric G proteins, which positively or negatively regulate the activity of effector molecules, such as enzymes and ion channels. This causes a change in ion composition, or second messenger levels, which in turn induces cellular responses.<sup>133,134</sup> Desensitization of GPCR signaling is achieved by uncoupling of GPCRs from heterotrimeric G proteins in response to phosphorylation by G protein-coupled receptor kinases (GRKs). The phosphorylation of GPCRs promotes binding of  $\beta$ -arrestin, which mediates the uncoupling process. Furthermore,  $\beta$ -arrestin binds to AP-2 and clathrin and thereby facilitates internalization of GPCRs via clathrin-mediated endocytosis. This internalization attenuates downstream signaling.<sup>92,93</sup> Based on phylogenetic analysis, the human GPCRs can be divided into five subgroups: *Glutamate*, *Rhodopsin*, *Adhesion*, *Frizzled/Taste2* and *Secretin*.<sup>131,135</sup>

The leucine-rich repeat-containing G protein-coupled receptors (LGRs) belong to the *Rhodopsin* family and can be divided into three subgroups, based on their unique domain arrangement (Figure 10A,B).<sup>136</sup> The Type A subgroup is constituted by the hormone receptors for follicle-stimulating hormone (FSHR/LGR1), luteinizing hormone (LHR/LGR2) and thyroid-stimulating hormone (TSHR/LGR3). LGR7 and LGR8 bind to peptides of the relaxin family and are classified as type C.<sup>137-139</sup> The type B receptors LGR4-6 share 50% sequence identity at the amino acid level.<sup>136</sup> The structure of the LGRs

is characterized by a large N-terminal extracellular domain containing a series of 9 to 17 leucine-rich repeats (LRR), which in the case of subgroup A and C are crucial for ligand binding (Figure 10B). In the case of LGR4, LGR5, and LGR6, the 17 LRRs are flanked by N- and C-terminal cysteine-rich domains (Figure 10C) (reviewed in Tang *et al.*, 2012<sup>135</sup>). LGRs are of ancient evolutionary origin.<sup>140</sup> Apart from vertebrates and insects, LGR homologues were found in sea anemone, mollusk, and the nematode *C.elegans*.<sup>141-145</sup>



**Figure 10| LGR subfamilies and domain organization.** (A) Phylogenetic relationship between LGR subfamilies. Group A includes follicle-stimulating (FSHR/LGR1), luteinizing (LHR/LGR2) and thyroid-stimulating (TSHR/LGR3) hormone receptors. Group B contains LGR4, LGR5 and LGR6. Group C comprises LGR7 and LGR8, receptors of the relaxin family. (B) Domain structure of the three groups. Group A contains 9 leucine-rich repeats (LRR), whereas group B contains 17 LRRs and group C 10 LRRs. Hinge, Hinge domain; 7 TM, seven transmembrane domain; LDL, low density lipoprotein. (C) Predicted structure of LGR5, consisting of a large extracellular domain of 17 LRRs, which are linked to the cytoplasmic domain via seven transmembrane helices. Panel A and B modified from Tang *et al.*, 2012<sup>135</sup>, panel C was taken from Barker & Clevers, 2010.<sup>146</sup>

The expression pattern and functions of LGR4, LGR5 and LGR6 are quite distinct.<sup>135,146</sup> LGR5 is a marker of gastrointestinal, colon, stomach and hair follicle stem cells.<sup>147,148</sup> It was shown that *LGR5* expression in mouse small intestine and colon is restricted to a very specific cell type, called crypt base columnar (CBC) cells, which are interspersed between Paneth cells at the crypt base. These LGR5 positive cells are long-lived and multipotent.<sup>147,149</sup> Similarly, the epithelial stem cells of the gastric gland in the stomach and of the follicle bulge in the hair follicle of the skin all express *LGR5*.<sup>147,148,150</sup> These cells have in common that their self-renewal and differentiation is controlled by Wnt

signaling.<sup>151-153</sup> Furthermore, it was described that Wnt signaling regulates the expression of *LGR5*.<sup>154</sup> Mutations that cause an up-regulation of *LGR5* result in the formation of colorectal cancers and tumors of the ovary, and they promote cell proliferation and tumor formation in basal cell carcinoma.<sup>155,156</sup> The knockout of *LGR5* in mice results in neonatal lethality characterized by ankyloglossia and gastrointestinal distension.<sup>157</sup>

While *LGR5* expression is mainly restricted to stem cell compartments, *LGR4* shows a wide tissue distribution, with strong expression in cartilage, kidney, stomach, heart, reproductive tracts, hair follicles and cells of the nervous system.<sup>158,159</sup> Therefore, *LGR4* knockout mice display a pleiotropic phenotype, characterized by several developmental defects, including intrauterine growth retardation associated with embryonic and perinatal lethality,<sup>159</sup> defective development of the gall bladder and cystic ducts,<sup>160</sup> dysfunctions in bone formation and remodeling,<sup>161</sup> and several other organic defects (reviewed in Barker & Clevers, 2010<sup>146</sup>). Overexpression of *LGR4* increases the invasiveness and metastasis of human colon carcinoma.<sup>162</sup> Whereas *LGR5* is a marker of several stem cells, *LGR4* rather appears to be required for the maintenance of adult stem cells in the intestine and is involved in the development of different embryonic tissues, which are of mesodermal, endodermal and ectodermal origin.<sup>146,158,163</sup>

Similar to *LGR5*, *LGR6* has been shown to be a marker of multipotent stem cells in the hair follicle of the skin, which are located directly above the bulge cells in the hair follicle.<sup>164,165</sup> The *LGR6* positive cells give rise to all lineages of the skin and are involved in wound repair in adult mice, including the formation of new hair follicles.<sup>165</sup> Similar to *LGR4* and *LGR5*, *LGR6* is also implicated in cancer. Total exon sequencing revealed that *LGR6* is often mutated in colon cancer.<sup>166</sup> Furthermore, the promoter region of *LGR6* is hypermethylated in 20-50% of human colorectal cancer samples.<sup>167,168</sup>

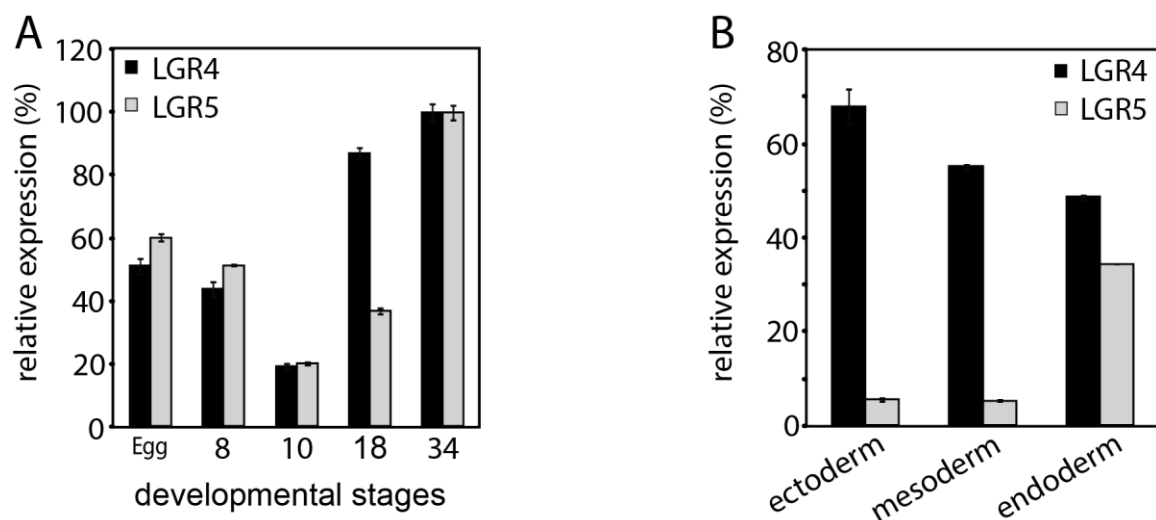
Although LGR4, LGR5 and LGR6 are all associated with cancer and are markers of various stem cells, as in the case of LGR5 and LGR6, their specific function within these cells is incompletely understood. This can be mainly attributed to the fact that LGR ligands are unknown. Here we identify R-spondins as LGR ligands. R-spondins were previously characterized as orphan ligands that enhance Wnt/ $\beta$ -catenin and Wnt/PCP signaling.<sup>79</sup> However, their mechanism of action has remained elusive. By showing direct interaction between LGRs and R-spondins, we provide evidence that Wnt signal amplification of R-spondins is regulated via LGRs.

## 5.2 Results

In search for an R-spondin receptor a genome-wide siRNA screen was performed in the Niehrs laboratory in HEK293T cells,<sup>22</sup> which led to the initial identification of LGR5 as a positive regulator of R-spondin-mediated Wnt signaling. We have shown before that RSPO3 amplifies both Wnt/ $\beta$ -catenin and Wnt/PCP signaling in *Xenopus* embryos.<sup>78</sup> Therefore we used *Xenopus* as a model organism to determine whether LGR5 could function as R-spondin receptor in Wnt/PCP signaling. Besides LGR5, LGR4 and LGR6 were also described as orphan receptors and are structurally similar to LGR5. However, *Xenopus* does not have an LGR6 orthologue, and was thus not investigated here.

### 5.2.1 LGR4 and LGR5 show distinct expression patterns in *Xenopus tropicalis*

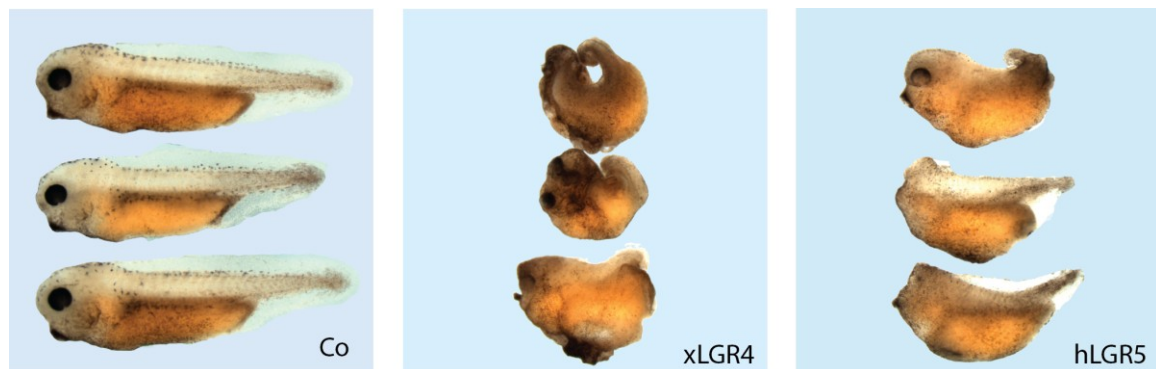
The expression of *LGR4* and *LGR5* in *Xenopus* has not been described so far. Expression analysis of *LGR4* and *LGR5* showed maternally stored mRNA, which decreased until blastula stage (stage 10). After gastrulation the zygotic transcripts increased and reached comparable levels at tailbud stage (stage 34) (Figure 11A). In the three germ layers of *Xenopus* embryos, *LGR4* showed an ubiquitous expression, whereas *LGR5* was predominantly expressed in the endoderm (Figure 11B).



**Figure 11 | Expression analysis of *LGR4* and *LGR5* during *Xenopus* development.** (A) qPCR analysis of *LGR4* and *LGR5* in *Xenopus* embryos using different developmental stages. *ODC* was used for normalization. (B) Expression analysis of *LGR4* and *LGR5* in *Xenopus* germ layers. *Xenopus* explants of stage 10 embryos were analyzed by qPCR for *LGR4* and *LGR5* expression. *LGR4* shows expression in all three germ layers. *LGR5* expression is highest in endodermal cells. Figure from Glinka *et al.*, 2011.<sup>169</sup>

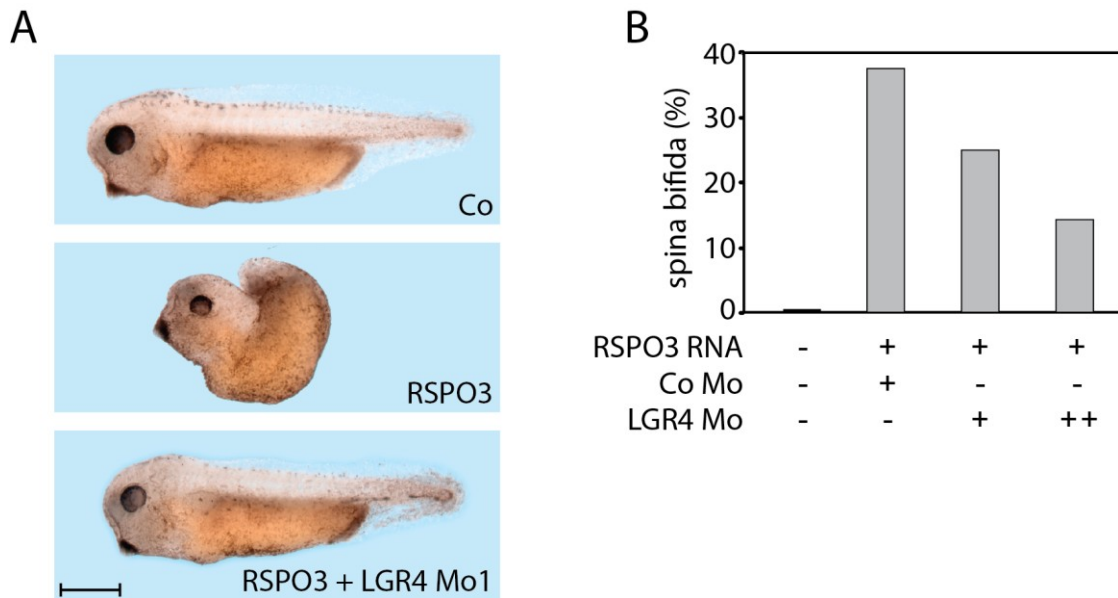
### 5.2.2 LGR4 Morpholino rescues RSPO3 induced spina bifida

Wnt/PCP signaling is a key regulator of convergent extension movements during gastrulation. Misregulation of genes involved in this process, e.g. *R-spondin 3 (RSPO3)*, results in gastrulation defects.<sup>78</sup> To investigate if LGR4 and LGR5 also affect gastrulation movements, similar to *RSPO3*, *Xenopus LGR4* and human *LGR5* messenger RNAs (mRNA) were injected into *Xenopus* embryos. Overexpression of both *LGR4* and *LGR5* induced gastrulation defects in *Xenopus* embryos (Figure 12). This phenotype is similar to the gastrulation defect and spina bifida observed after *RSPO3* mRNA injection,<sup>78</sup> and suggests a possible function of LGR4 and LGR5 in Wnt/PCP signaling in *Xenopus*.



**Figure 12 | Gain of function of *LGR4* and *LGR5* causes gastrulation defects in *Xenopus*.** Embryos were injected equatorially at 4-cell stage with messenger RNA (mRNA) for *PPL*, *Xenopus LGR4* and human *LGR5*, and phenotypes were analyzed at tailbud stage.

The spina bifida phenotype induced by *RSPO3* overexpression was rescued in a dose-dependent manner by co-injection of an antisense Morpholino oligonucleotide (Mo1) targeting the 5' untranslated region (UTR) of *LGR4* (Figure 13A,B). Co-injection of a Morpholino targeting the 5'UTR of *LGR5* (*LGR5* Mo1), was not able to rescue the *RSPO3*-induced gastrulation defect (data not shown). The results indicate a requirement of *LGR4*, but not *LGR5*, for *RSPO3*-induced spina bifida in *Xenopus* embryos.

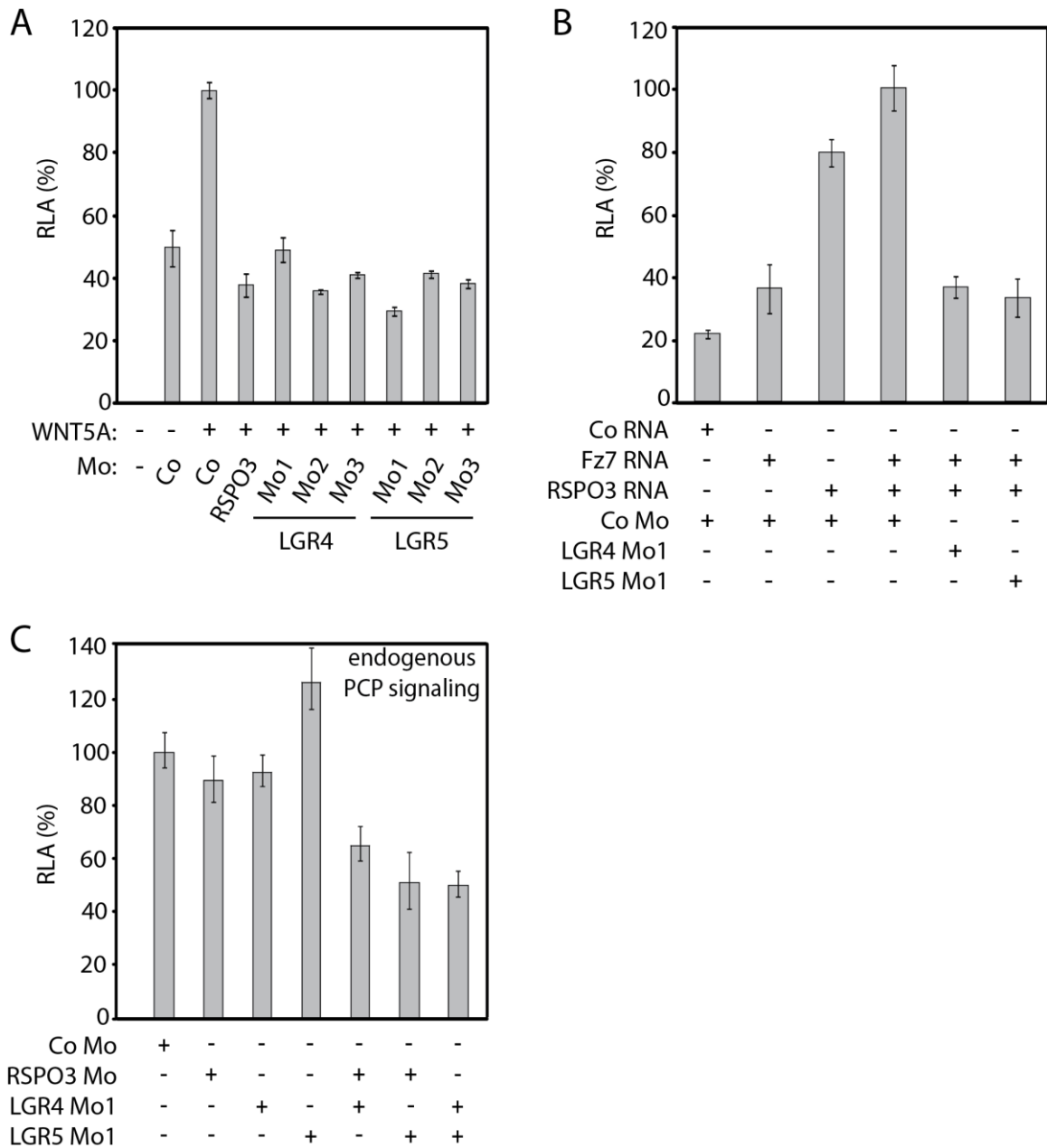


**Figure 13| RSPO3 signaling requires LGR4 to induce gastrulation defects in *Xenopus* embryos.** (A) Embryos were injected equatorially at 4-cell stage with *Xenopus RSPO3* mRNA (250 pg) and *control* or *LGR4* Mo1 (20 ng *co* Mo, 10 ng (+) or 20 ng (++) *LGR4* Mo1). Scale bar corresponds to 500  $\mu$ m. (B) Statistical analysis of the injected *Xenopus* embryos. For each sample 20 embryos were analyzed and the percentage of spina bifida phenotypes was determined at stage 25. Figure from Glinka *et al.*, 2011.<sup>169</sup>

### 5.2.3 LGR4 and LGR5 are required for RSPO3-mediated Wnt/PCP signaling in *Xenopus*

We investigated the role of LGR4 and LGR5 in RSPO3-mediated noncanonical Wnt signaling by using a specific reporter for Wnt/PCP signaling in early *Xenopus* embryos, ATF2-luciferase.<sup>170</sup> Expression of the ATF2-luciferase reporter was induced by *WNT5A* mRNA injection (Figure 14A). Stimulation of the ATF2-luciferase activity was inhibited by either *RSPO3* Mo, as described before,<sup>78</sup> or by six individual *LGR4* and *LGR5* Mos. Because the rescue of *LGR4/5* Mos proved technically difficult, six Morpholinos, targeting different sites of *LGR4* and *LGR5*, were designed to confirm Mo specificity. The same inhibitory effect was observed when PCP signaling was activated using a combination of *FZD7* and *RSPO3* mRNAs. The elevated ATF2 reporter activity was decreased to basal levels after co-injection of either *LGR4* or *LGR5* Mo1 (Figure 14B). Besides exogenous activation of the PCP signaling pathway, the effect of *LGR4/5* Mo1 on endogenous PCP signaling was investigated. For this purpose, low doses of the *RSPO3*, *LGR4* and *LGR5* Mos, which by themselves had no effect on the reporter activity, were combined. Co-injection of *RSPO3* Mo with either *LGR4*, or *LGR5* Mo1, or both *LGR* Mos together, resulted in a decrease in ATF2-luciferase activity (Figure 14C).





**Figure 14 | LGR4 and LGR5 are required for Wnt/PCP signaling in *Xenopus*.** (A-C) ATF2-luciferase activity is reduced by *LGR4/5* Mos in *Xenopus*. Embryos were injected equatorially at 4-cell stage with ATF2-luc reporter (100 pg) and Renilla reporter plasmids (25 pg) and the indicated Morpholinos (40 ng *LGR4* Mo1, 10ng *LGR4* Mo2/3, 10 ng *LGR5* Mo1, 5 ng *LGR5* Mo2, 2.5 ng *LGR5* Mo3, 10 ng *RSPO3* Mo (A, B), 20 ng *LGR4* Mo1, 5 ng *LGR5* Mo1 (C)) and mRNAs (500 pg *WNT5A*, 250 pg *FZD7*, 250 pg *RSPO3*). Luciferase reporter assays were performed from whole embryos at stage 11. Luciferase activity of embryos injected with *co* Mo was set to 100%. RLA, relative luciferase activity. Figure from Glinka *et al.*, 2011.<sup>169</sup>

These results provide evidence for a requirement of LGR4 and LGR5 for RSPO3-mediated Wnt/PCP signaling in *Xenopus* embryos.

### 5.3 Discussion

The phenotypic analysis and ATF2 reporter assays indicate that the effect of *RSPO3* on Wnt/PCP signaling in *Xenopus* embryos is mediated via LGR4 and LGR5.

#### 5.3.1 LGR4 and LGR5 are *bone fide* R-spondin receptors

In early *Xenopus* embryos, injection of *RSPO3* mRNA results in gastrulation defects, which are due to Wnt/PCP signaling pathway misregulation.<sup>78</sup> The observations that *LGR4* as well as *LGR5* mRNAs gave rise to a similar phenotype, and that *RSPO3* overexpression could be rescued by *LGR4* depletion, suggested that they may act in the same pathway as *RSPO3*. Similarly, it was shown by de Lau *et al.* that genetic deletion of *LGR4/5* in mouse intestinal crypt cultures phenocopies *RSPO1* withdrawal,<sup>171</sup> which further supports a synergistic action of R-spondins and LGRs *in vivo*. Indeed, additional experiments performed in the course of this study, including cell surface and *in vitro* binding assays, confirmed that R-spondins directly interact with LGRs.<sup>169</sup> We thus identify R-spondins as LGR ligands, which was independently confirmed by other groups.<sup>171,172</sup>

Although we observed that both LGR4 and LGR5 bind to R-spondins *in vitro*, *LGR5* depletion was not sufficient to rescue the *RSPO3* overexpression phenotype in *Xenopus* embryos. A possible explanation may be the different expression patterns of *LGR4* and *LGR5*. Whereas *LGR4* shows a ubiquitous expression in all three germ layers, *LGR5* is predominantly expressed in endodermal tissue. During gastrulation, *RSPO3* is mainly expressed in mesodermal cells, which undergo cell migration and convergent extension during gastrulation.<sup>78</sup> Because of this distinct expression pattern, it is possible that *RSPO3* and LGR4, but not LGR5, interact during gastrulation. It should be noted, however, that LGR5 may preferentially bind other R-spondin family members not investigated here, and thereby contributes to *Xenopus* development.

### 5.3.2 LGR4 and LGR5 in Wnt signaling

R-spondins were discovered in 2004 as enhancers of WNT3A-induced Wnt signaling.<sup>79</sup> Carmon *et al.* were the first to show that this potentiating effect of R-spondins is dependent on LGR4 and LGR5, whereas the maximal activity of Wnt/ $\beta$ -catenin activation is determined by the level of WNT3A and the presence of the Wnt co-receptor LRP6. Moreover, their data indicate that this potentiation is mediated by enhancing Wnt-induced LRP6 phosphorylation.<sup>172</sup> LGR4 and LGR5 belong to the GPCR superfamily, which interact with heterotrimeric G proteins and  $\beta$ -arrestin.<sup>131,132</sup> Carmon *et al.* analyzed whether LGR4 and LGR5 are coupled to heterotrimeric G proteins or  $\beta$ -arrestin to mediate their function in Wnt/ $\beta$ -catenin signaling. Although LGR4/5 show sequence homology to the *Rhodopsin* family of GPCRs,<sup>136</sup> they do not bind to G proteins or  $\beta$ -arrestins when stimulated by R-spondins. However, it cannot be excluded that LGRs bind ligands other than R-spondins, which require G proteins for signal transduction. Finally, these authors showed that LGR4 and LGR5 are constitutively internalized into large intracellular vesicles. Upon binding, R-spondins undergo co-internalization with each of the two receptors. The nature of this internalization was not analyzed further. We extended on these findings by showing that LGR4 is co-internalized with RSPO3 by clathrin-mediated endocytosis.<sup>169</sup> Thereby, RSPO3-mediated Wnt/ $\beta$ -signaling is using a different endocytic route compared to WNT3A-induced signaling, which requires caveolin-mediated endocytosis.<sup>169</sup>

In another study, de Lau *et al.* identified R-spondins as ligands for LGR4 and LGR5, by using both receptors as baits in a tandem affinity purification mass spectrometric setup.<sup>171</sup> Using this strategy, they identified LGR5, LRP6 and FZD5/7 as interaction partners of LGR4, and FZD5, LRP5 and LRP6 as LGR5 interaction partners. Hence, LGR4 and LGR5 associate with the FZD/LRP6 receptor complex. Besides binding the Wnt receptors, LGR4, LGR5 and also LGR6 were shown to bind all four R-spondins. In contrast to the members of type B family of *Rhodopsin* proteins, type A (LGR1, LGR3) and type C (LGR7, LGR8) receptors did not associate with RSPO1, showing a clear specificity of R-spondins for LGR4/5/6. The affinity of RSPO1 for LGR5 lies in the low nanomolar range and is in accordance with our results for binding of RSPO3 to LGR4 and LGR5.<sup>169</sup> Whereas we were able to show that the interaction of LGR4/5 and RSPO3 requires the two furin

domains of RSPO3,<sup>169</sup> de Lau *et al.* identified the first LRR domains at the N-terminus of LGR5 to be essential for RSPO1 binding. This is in accordance with type A homologues of the *Rhodopsin* subfamily, where binding of the glycoprotein hormones involves the N-terminal LRR ectodomain.<sup>173</sup> In addition to their cell culture studies, de Lau *et al.* performed genetic deletions of *LGR4* and *LGR5* in mouse intestinal crypt cultures, which phenocopied *RSPO1* withdrawal and induced the demise of the cultured crypt organoids. This effect was efficiently rescued by *WNT3A* overexpression, indicating that strong Wnt/ $\beta$ -catenin signal activation can overcome loss of LGR4/5 receptors.

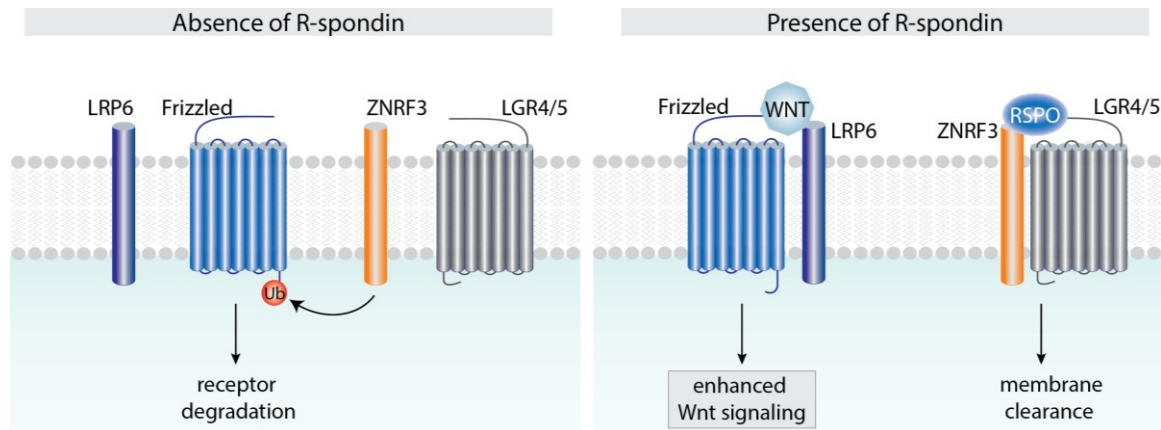
For Wnt/PCP signaling it was shown that SDC4 mediates PCP signaling and binds to the TSP1 domain of RSPO3.<sup>78</sup> As LGR4/5 bind to the Furin domains, R-spondins might bridge between syndecans and LGR4/5 in Wnt/PCP signaling. The specificity of R-spondin and LGR4/5 to signal either via the Wnt/ $\beta$ -catenin or the Wnt/PCP signaling pathway may be defined by the availability of Wnt/FZD or syndecan receptors.

### 5.3.3 Signaling mechanism of LGR4 and LGR5

Although the receptors for R-spondins were identified and their effect on Wnt/ $\beta$ -catenin and Wnt/PCP signaling was characterized, the exact mechanism of how R-spondins enhance Wnt signaling via LGR4, LGR5 and LGR6 remained to be determined.

LGR4/5/6 receptors were initially shown to interact with LRP5/6 and FZD5/7 in the Wnt signaling complex, and were thus thought to enhance receptor activity.<sup>171,174</sup> However, it was found more recently that R-spondins increase membrane levels of LRP6 and Frizzled.<sup>175,176</sup> The cell-surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) and its close homolog ring finger protein 43 (RNF43) were shown to associate with the Wnt receptor complex and promote the degradation of LRP6 and FZD, thereby inhibiting Wnt signaling. R-spondins bind to ZNRF3 and RNF43 and induce their association with LGR4/5. Because LGR4 and LGR5 are rapidly internalized, the interaction of ZNRF3/RNF43 with LGR4/5 through R-spondins thus results in their membrane clearance (Figure 15). Consequently, in the presence of R-spondins LRP6 and FZD receptors accumulate at the plasma membrane and enhance not only Wnt/ $\beta$ -catenin but also Wnt/PCP signaling.<sup>175,176</sup> This mechanism provides an elegant

explanation why R-spondins require the presence of Wnts to potentiate Wnt signaling.<sup>79,80,82</sup>



**Figure 15 | Model of ZNRF3 function in Wnt signaling and its inhibition by R-spondin.** In the absence of R-spondin, the transmembrane E3 ubiquitin ligase ZNRF3 associates with the Wnt receptor complex and ubiquitinates Frizzled. This results in degradation of Frizzled and LRP6 and inhibition of Wnt/ $\beta$ -catenin and Wnt/PCP signaling. R-spondin (RSPO) binds to LGR4/5 and ZNRF3 and thereby induces the formation of a ternary complex, which is subsequently internalized. The removal of ZNRF3 via R-spondin leads to the accumulation of Frizzled and LRP6 at the membrane level and enhances Wnt/ $\beta$ -catenin and Wnt/PCP signaling. Figure modified from Hao *et al.*, 2012.<sup>175</sup>

The structural analysis of the ternary LGR5-RSPO1-RNF43 complex confirmed the physical linkage between the three proteins.<sup>177</sup> The Cys-rich domain of RSPO1 provides two separate regions, which form distinct binding sites for LGR5 and RNF43. Whereas the first furin domain is involved in RNF43 binding, the second furin domain mediates binding to LGR5. The affinity of RNF43 and RSPO1 is increased when RSPO1 is bound by LGR5, suggesting a stabilization of RSPO1 in a configuration more favorable for RNF43 binding. Consequently, LGR5 serves as an engagement receptor, recruiting R-spondins to RNF43, whereas RNF43 acts as the effector receptor.<sup>177</sup> A similar mechanism was also described for LGR4/ZNRF3.<sup>178,179</sup>

In summary, we and others have shown that R-spondins potentiate Wnt signaling via LGR family receptors. This interaction has important implications for development, shown here, as well as stem cell maintenance. It explains for the first time why R-spondins promote the growth of stem cells, especially in the intestine,<sup>87</sup> and why addition of R-spondin is required to allow long-term culturing and organoid formation of LGR5 positive mouse intestinal crypt stem cells *in vitro*.<sup>180</sup>

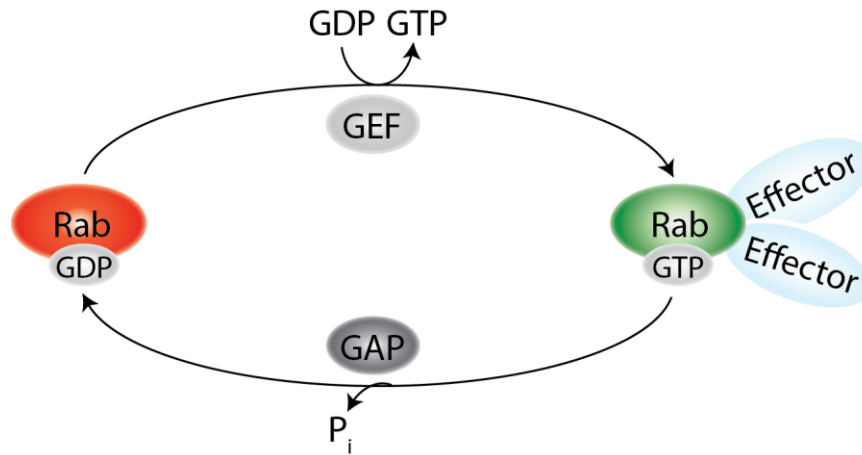
## **6. Chapter 2: RAB8B is required for Wnt/ $\beta$ -catenin signaling in *Xenopus***

### **6.1 Introduction**

#### **6.1.1 The family of Rab GTPases**

Rab proteins belong to the superfamily of Ras GTPases. They constitute the largest family of small GTPases, which act as organizers of almost all membrane trafficking processes in eukaryotic cells. For instance, Rab GTPases regulate signaling of many hormone and growth factor receptors by mediating their trafficking to lysosomes for degradation, or endosomes to relay downstream signaling.<sup>181</sup> In humans, more than 60 different members of the Rab family have been described, which differ in their intracellular membrane localization.<sup>182-184</sup> Rab GTPases control the activation and localization of various interacting molecules, including sorting adaptors, tethering factors, kinases, phosphatases, coat components and motor proteins. Characterization of approximately half of the known Rab GTPases shows that Rab GTPases, besides their known role in vesicle trafficking, are also involved in signaling, cell differentiation and proliferation (reviewed in Stenmark, 2009<sup>181</sup>).

Interaction of Rab GTPases with their effector molecules depends on the conformational state of the Rab GTPases, which alternates between a GTP-bound (active) form and a GDP-bound (inactive) form. Interaction of Rab GTPases and the different effector proteins only takes place in the active state. Through hydrolysis of the bound GTP, Rab GTPases are converted back to their inactive form. This occurs through intrinsic GTPase activity, or can be catalyzed by a GTPase-activating protein (GAP). Subsequently, Rab GDP is recognized by a guanine nucleotide exchange factor (GEF), which catalyzes the exchange of GDP by GTP. This converts the Rab GTPase back to the active state, where the cycle can start again (Figure 16). Thus, Rab GTPases act as molecular switches that impose temporal and spatial regulation on membrane transport, based on their respective state.



**Figure 16 | Activation cycle of Rab proteins.** The active, GTP-bound form of Rab GTPases is recognized by multiple effector proteins. The hydrolysis of GTP induces a conformational change and the release of inorganic phosphate ( $P_i$ ). Thereby Rab proteins are converted from an active to an inactive, GDP-bound state. This process can be catalyzed by GTPase-activating proteins (GAP). The conversion back to the active conformation requires exchange of GDP by GTP, which can be catalyzed by guanine nucleotide exchange factors (GEF). Figure modified from Stenmark, 2009.<sup>181</sup>

### 6.1.2 Rab GTPase RAB8

In mammals, two RAB8 isoforms, known as RAB8A and RAB8B have been described, which differ substantially in their C-terminal region and expression pattern.<sup>185,186</sup> Both proteins are activated by RABIN8, a GEF that seems to be specific for RAB8, as it does not activate other Rab family members.<sup>187,188</sup> The activation of RAB8A and RAB8B via RABIN8 is facilitated by another member of the Rab family, RAB11.<sup>189,190</sup> Originally, RAB8 was described to mediate the trafficking of newly synthesized proteins from the trans-Golgi network (TGN) to the plasma membrane.<sup>181</sup> Besides protein shuttling, RAB8A and RAB8B participate in various other membrane-based pathways, like exocytosis, membrane recycling, neuron differentiation and ciliogenesis.<sup>191</sup> Furthermore, RAB8 is involved in the regulation of cell migration and cell morphogenesis by connecting actin dynamics to membrane trafficking and turnover.<sup>192,193</sup> RAB8 was found to be up-regulated in mammary tumors and their lymph node metastases.<sup>194</sup> This is probably linked to the finding that RAB8 regulates polarized exocytosis of a matrix metalloproteinase involved in cell invasion.<sup>195</sup>

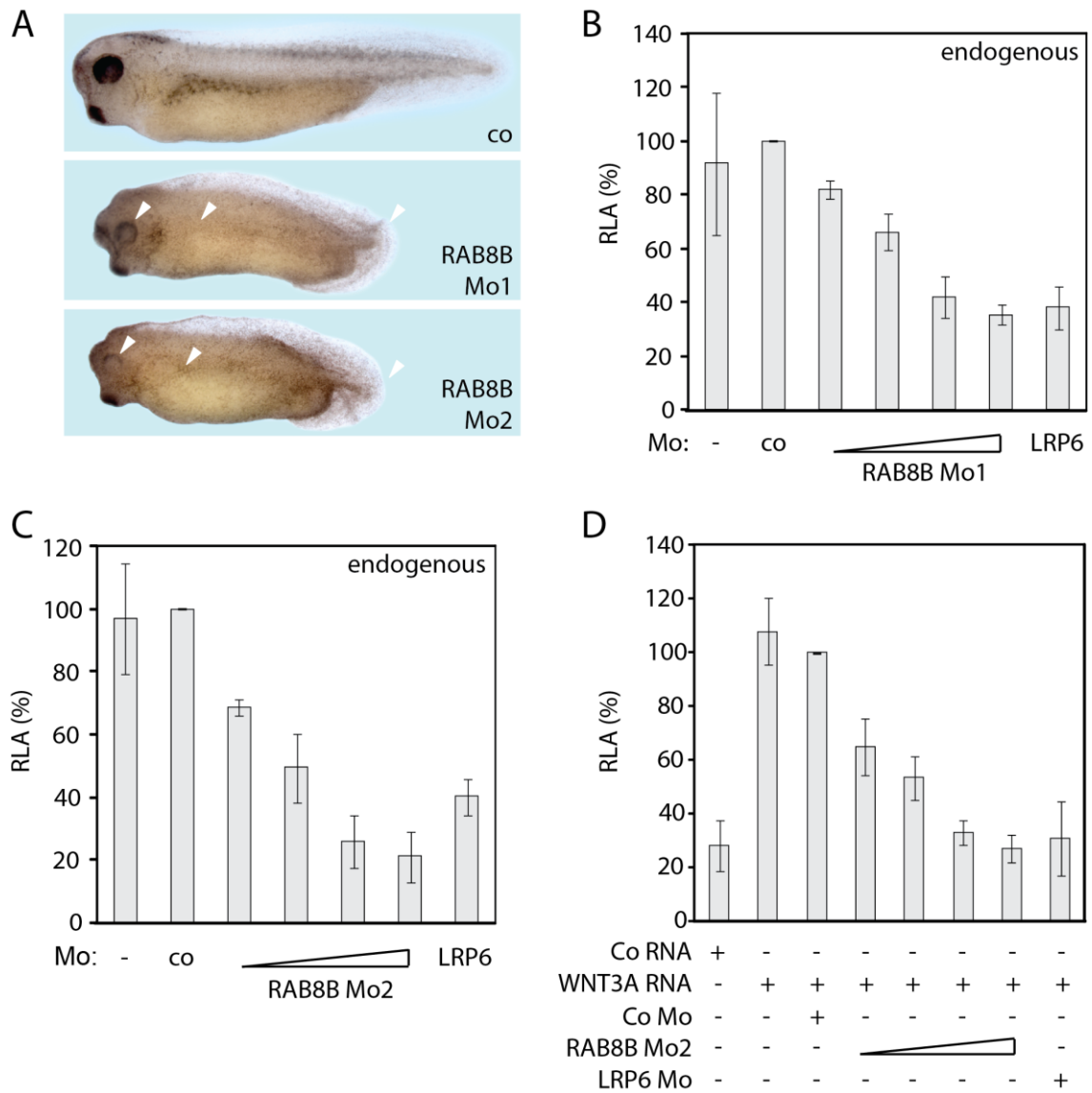
Endocytosis also plays an important role in Wnt/ $\beta$ -catenin signal transduction. After binding of Wnts, the ligand-receptor complex undergoes internalization via clathrin or caveolin-dependent routes.<sup>95</sup> However, the mechanism of Wnt signal transmission, notably how endocytosis of the Wnt receptors is regulated, remains incompletely understood. It has been suggested that the localization of LRP6 in the different microdomains of the plasma membrane could be regulated by proteins, which link LRP6 to the endocytic machinery.<sup>107</sup> The identification of proteins involved in the endocytosis process of the Wnt receptors could give new insights into the specificity of the endocytic processes and the regulation of Wnt signaling.



## 6.2 Results

In an RNAi screen aimed at identifying regulators involved in trafficking of Wnt pathway components, the small GTPase RAB8B was identified as a candidate protein in the Boutros laboratory. Based on experiments performed by Kubilay Demir in the laboratory of Prof. M. Boutros, RAB8B is required for Wnt/ $\beta$ -catenin signaling in mammalian cells. To analyze if RAB8B is also required for Wnt/ $\beta$ -catenin signaling *in vivo*, the role of RAB8B in *Xenopus* embryos was examined.

In order to analyze the effect of RAB8B on Wnt/ $\beta$ -catenin signaling in *Xenopus*, two antisense Morpholino oligonucleotides (Mo) were designed, targeting distinct regions of *RAB8B*. Injection of either Morpholino gave rise to similar phenotypes in *Xenopus* tadpoles, suggesting a specific effect on RAB8B. *RAB8B* morphants were characterized by shortened body axes, smaller heads and eyes and a reduction in eye pigmentation and melanocytes (Figure 17A). This phenotype resembles conditions in which Wnt/ $\beta$ -catenin, as well as Wnt/PCP signaling are dysregulated.<sup>22,196</sup> To confirm the requirement of RAB8B for Wnt signaling in *Xenopus* on the molecular level, TOPFlash reporter assays for Wnt/ $\beta$ -catenin signaling were performed. On the endogenous level, both Morpholinos reduced the reporter activity in a dose-dependent manner, further corroborating their specificity (Figure 17B,C). This decrease in luciferase activity resembled loss of *LRP6*. Moreover, when Wnt/ $\beta$ -catenin signaling was stimulated by co-injection of *WNT3A* mRNA, increased reporter activity was fully blocked by *RAB8B* Mo2 (Figure 17C). Taken together, these results strongly suggest a positive role of RAB8B in Wnt/ $\beta$ -catenin signaling in *Xenopus* embryos.



**Figure 17 | RAB8B is required for Wnt/ $\beta$ -catenin signaling in *Xenopus*.** (A) *RAB8B* Morpholino 1 (Mo1) and Morpholino 2 (Mo2) display similar phenotypes in *X.laervis*. Embryos were injected equatorially at 4-cell stage with 40 ng Mo1 and 60 ng Mo2 and the corresponding *control* Mo (co). (B-C) TOPFlash reporter activity is reduced by *RAB8B* Mo1/2 in *Xenopus*. Embryos were injected equatorially at 4-cell stage with TOPFlash reporter (100 pg) and Renilla reporter plasmids (25 pg) and the indicated Morpholinos (5 ng, 10 ng, 20 ng, 40 ng *RAB8B* Mo1, 10 ng, 20 ng, 40 ng, 60 ng *RAB8B* Mo2, 2.5 ng *LRP6* Mo) and *WNT3A* mRNA (4 pg). Corresponding amounts of *control* Mo and *control* mRNA were injected. Luciferase reporter assays were performed from whole embryos at stage 11. Luciferase activity of embryos injected with *co* Mo was set to 100%. RLA, relative luciferase activity. Graphs show mean  $\pm$  SD,  $N=3$ . Figure from Demir *et al.*, 2013.<sup>197</sup>

## 6.3 Discussion

### 6.3.1 RAB8B is required for Wnt/ $\beta$ -catenin signaling *in vivo*

RAB8B was identified as a regulator of Wnt/ $\beta$ -catenin signaling in mammalian cells.<sup>197</sup> Because Wnt signaling plays major roles during the development of *Xenopus* embryos, this model organism is well suited to investigate putative Wnt pathway regulators *in vivo*.

In *Xenopus* embryos, *RAB8B* is already present as maternal RNA, indicating an early function during *Xenopus* development.<sup>198</sup> The loss-of-function phenotype of *RAB8B* in *Xenopus* is characterized by axis, head and pigmentation defects, which resembles Wnt/ $\beta$ -catenin and Wnt/PCP signaling defects described earlier.<sup>22,196</sup> The requirement of RAB8B for Wnt signaling *in vivo* was additionally confirmed in zebrafish embryos.<sup>197</sup> In contrast to *Xenopus*, the expression of *RAB8B* in zebrafish was first detectable in the brain of embryos at somitogenesis. Knockdown of *RAB8B* in zebrafish decreased target gene *c-myc* and reporter protein expression in transgenic Wnt/ $\beta$ -catenin reporter lines. Taken together, these results indicate a conserved regulatory role of RAB8B during vertebrate development.

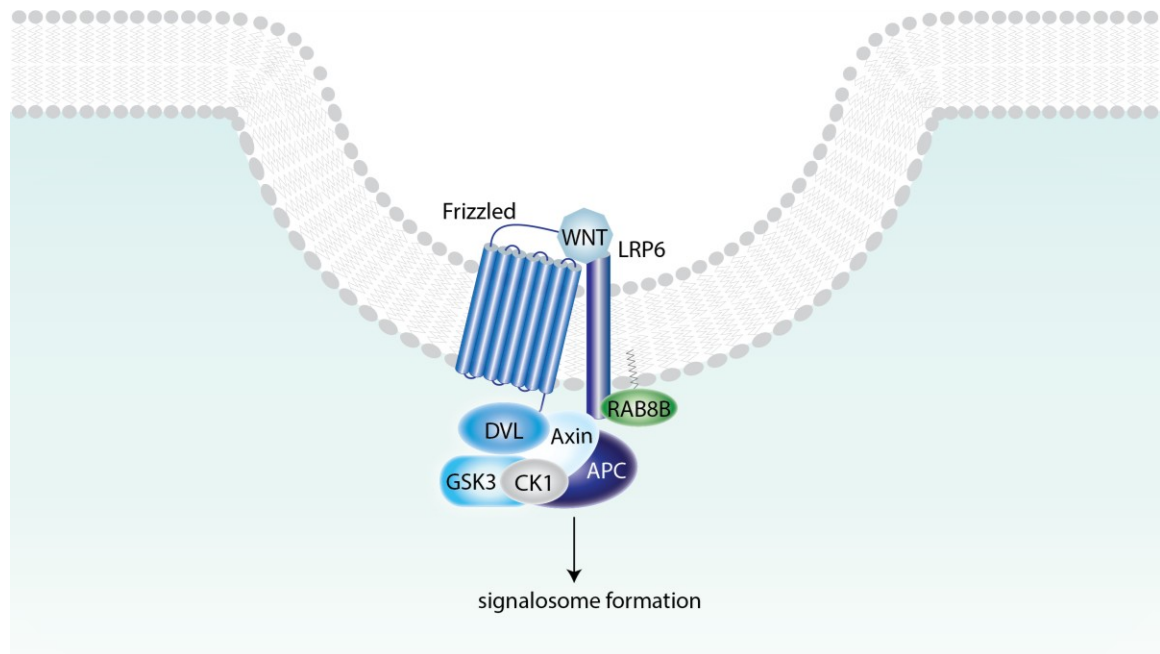
### 6.3.2 RAB8B promotes caveolar endocytosis of LRP6

To investigate the molecular mechanism of RAB8B, Demir *et al.* examined the effect of RAB8B in mammalian cells.<sup>197</sup> The effect of RAB8B is specific for Wnt/ $\beta$ -catenin signaling, as no other signaling pathways were affected, and the paralog RAB8A did not have an impact on Wnt/ $\beta$ -catenin signaling. Furthermore, these authors found that RAB8B promotes caveolin-dependent endocytosis of LRP6. In response to WNT3A, LRP6 undergoes internalization in lipid raft domains, mediated by caveolin. In contrast, DKK1 removes LRP6 from lipid raft domains and induces endocytosis based on clathrin-dependent routes.<sup>107</sup> It has been proposed that the localization of LRP6 in raft and non-raft domains could be regulated by proteins, which link LRP6 to the endocytic machinery.<sup>107</sup> The small GTPase RAB8B could thus serve as a regulator of caveolin-mediated LRP6 internalization.

RAB8B was recently shown to interact with TANK-binding kinase 1 (TBK-1) during the maturation of autophagosomes.<sup>199</sup> Because TBK-1 was shown to associate with the ESCRT-I complex,<sup>200</sup> which is involved in sorting of membrane receptors into MVBs, RAB8B may be involved in trafficking of the Wnt receptor complex to MVBs via its interaction with TBK-1. However, because the interaction of RAB8B and TBK-1 was only analyzed in the context of antimicrobial defense, this hypothesis requires further investigation.

Interestingly, Demir *et al.* additionally demonstrated that the activity of RAB8B seems to be regulated by Wnt signaling: Recruitment of DVL1 after formation of the ternary receptor complex activates RAB8B, and Wnt ligands induce the interaction of RAB8B with its activator RABIN8.<sup>197</sup> A similar mechanism was suggested for RAC1, a member of the Rho family of small GTPases.<sup>201</sup> Whereas RAC1 promotes the nuclear translocation of  $\beta$ -catenin, its activity is in turn regulated by the Wnt signaling pathway. These observations suggest that a feed-forward loop from Wnt signaling to small GTPases potentiates Wnt signaling, through regulation of receptor trafficking.

In conclusion, we have identified RAB8B as a novel agonist of Wnt/ $\beta$ -catenin signaling, which promotes Wnt activity by facilitating endocytosis of LRP6 signalosomes (Figure 18). The specific function(s) of RAB8B *in vivo* are poorly understood, but based on the results shown here it can be assumed that RAB8B-dependent Wnt signaling control is essential for development.



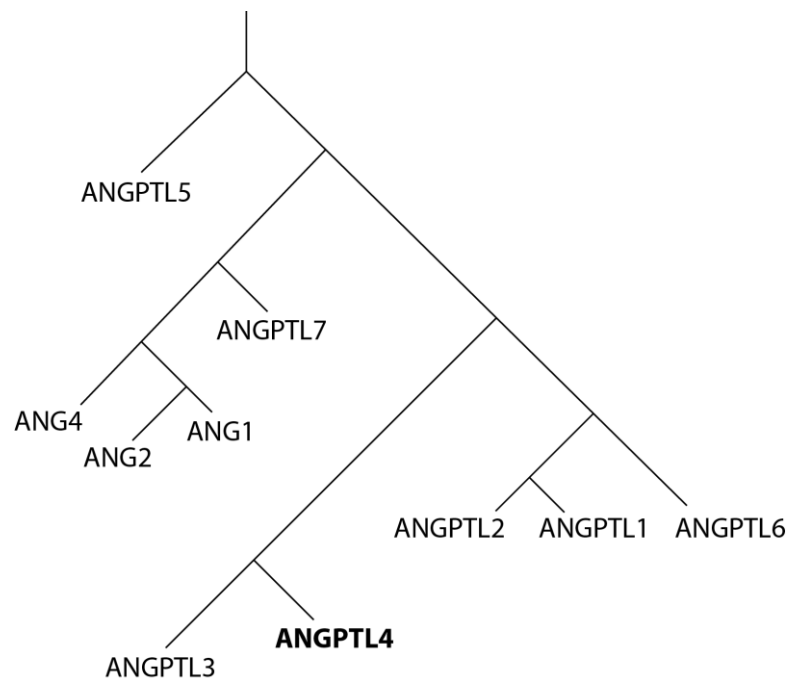
**Figure 18 | Model of RAB8B function in Wnt/ $\beta$ -catenin signaling.** After binding of Wnt to Frizzled and LRP6, the receptors accumulate in caveolae and recruit the  $\beta$ -catenin destruction complex, consisting of Axin, APC, CK1 $\alpha$ , GSK3 and DVL. At the same time, RAB8B is translocated to the membrane and activated in a Wnt- and DVL-dependent manner. RAB8B enhances signalosome formation and/or maturation and promotes LRP6 activity, which results in caveolin-dependent internalization of the receptor complex and activation of downstream signaling. Figure modified from Demir *et al.*, 2013.<sup>197</sup>

## 7. Chapter 3: ANGPTL4 binds syndecan and inhibits Wnt/ $\beta$ -catenin signaling by decreasing LRP6 levels

### 7.1 Introduction

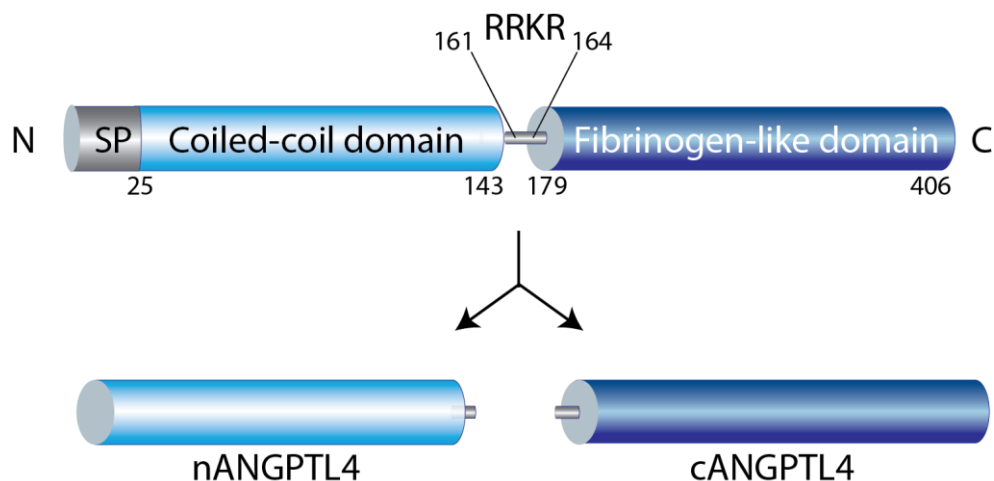
#### 7.1.1 Angiopoietin-like 4

Angiopoietin-like 4 (ANGPTL4) was initially identified as a peroxisome proliferator-activated receptor (PPAR) target gene with structural similarity to angiopoietins (ANG).<sup>202-204</sup> Besides ANGPTL4, seven other members of this family have since been discovered (ANGPTL1-8, Figure 19), most of which show little structural homology.<sup>205-207</sup> Indeed, ANGPTL4 shares just 30% sequence identity with its closest family member, ANGPTL3.<sup>208</sup>



**Figure 19 | Phylogenetic relationship of Angiopoietin-like family members.** In humans the angiopoietin-like (ANGPTL) family contains seven members, which share sequence homology to the angiopoietins (ANG), which comprise five members. The eighth member of the ANGPTL family is not shown, as it lacks the conserved fibrinogen-like domain, which is shared by ANG and ANGPTL proteins. Figure modified from Tan *et al.*, 2012.<sup>209</sup>

Most ANGPTL proteins consist of a signal peptide directing secretion, an amino-terminal coiled-coil domain (CCD), a linker region, and a large carboxy-terminal fibrinogen-like domain (FLD), which is conserved in the ANG and ANGPTL families.<sup>210</sup> An exception to this domain organization is ANGPTL8, which lacks the fibrinogen-like domain.<sup>206,207</sup> In contrast to all other members of the ANGPTL family, only ANGPTL4 and ANGPTL3 assemble into oligomeric structures.<sup>211,212</sup> The full length ANGPTL4 protein (fANGPTL4) forms dimers or tetramers prior to secretion, through disulfide bond formation in the N-terminal domain.<sup>211,213</sup> In contrast, the oligomerization of ANGPTL3 requires no intermolecular disulfide bonds.<sup>214</sup> After secretion of ANGPTL4 into the extracellular space, the full length form can bind to the extracellular matrix (ECM) via its CCD. It was shown *in vitro* that this interaction is mainly mediated by heparan and dermatan sulfates.<sup>215</sup> The release of ANGPTL4 from the ECM results in proteolytic cleavage in the linker region, which gives rise to an N-terminal (nANGPTL4) and a C-terminal (cANGPTL4) fragment (Figure 20).<sup>211,215,216</sup> This cleavage at the -RRXR- consensus cleavage site is mediated by pro-protein convertases.<sup>215,216</sup> The N-terminal CCD remains multimerized, and binds to the extracellular matrix, whereas the C-terminal FLD is released into the medium as a monomer.<sup>211,215</sup>



**Figure 20 | Domain organization of ANGPTL4.** The structure of the human full length ANGPTL4 protein consists of an amino terminal, hydrophobic signal peptide (SP), a coiled-coil domain, a linker region and a carboxy terminal fibrinogen-like domain. After secretion, the protein can undergo proteolytic processing by pro-protein convertases in the linker region at conserved basic residues (-RRKR-). This results in the formation of nANGPTL4 and cANGPTL4 fragments. Figure modified from Tan *et al.*, 2012.<sup>209</sup>

This cleavage has only been observed for ANGPTL4 and ANGPTL3, and thus distinguishes these two proteins from the other family members.<sup>211,212</sup> The cleavage of ANGPTL4 appears to be tissue and species-specific. In humans, only full length ANGPTL4 is found in adipose tissue, whereas the protein undergoes cleavage in mouse adipocytes.<sup>217</sup> Abundant ANGPTL4 cleavage was also observed in the liver, and both the full length and cleaved forms can be found in blood plasma.<sup>217</sup> The expression of ANGPTL4 can be induced by different factors. During fasting the synthesis and secretion of ANGPTL4 is increased, which is mediated by glucocorticoids. This steroid hormone is induced under fasting conditions and activates its receptor, which elevates the expression of ANGPTL4.<sup>218</sup> Similarly, chronic caloric restriction or the accumulation of free fatty acids stimulates nuclear hormone receptors of the PPAR family. These receptors bind PPAR-response elements in the promoter region of ANGPTL4 and activate its transcription.<sup>203,219-221</sup> Furthermore ANGPTL4 expression can be induced by hypoxia via hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), which binds to a recognition site upstream of the ANGPTL4 gene.<sup>222,223</sup> Moreover, it was recently shown that ANGPTL4 expression can be also regulated by the TGF $\beta$ /SMAD3 signaling pathway.<sup>224</sup>

Although ANGPTL4 is known to undergo proteolytic processing, the biological importance of this process and the function of the different fragments remain elusive.<sup>209</sup> Furthermore, the identity of ANGPTL4 binding partner(s) is still unknown. Even though ANGPTL proteins are structurally related to ANGs, they do not bind the prototypical angiopoietin receptors TIE1 and TIE2.<sup>202</sup> It was shown that cANGPTL4 can associate with integrins, whereas nANGPTL4 interacts with HSPG.<sup>215,225</sup> These components of the ECM cannot explain all molecular functions of ANGPTL4, and are likely co-receptors of ANGPTL4. Recently it was shown that ANGPTL2 and ANGPTL5 can bind the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2).<sup>226</sup> As ANGPTL4 showed no binding to this receptor, it is still considered an orphan ligand.



### 7.1.2 Physiological functions of ANGPTL4

ANGPTL4 has primarily been described as a regulator of lipid metabolism.<sup>227,228</sup> It suppresses the clearance of circulating triacylglycerols (TAGs) by inhibiting lipoprotein lipase (LPL), the enzyme which hydrolyzes TAGs in lipoprotein particles.<sup>208,229,230</sup> The N-terminal domain of ANGPTL4 binds to LPL and converts the active LPL dimer into inactive monomers.<sup>216,231</sup> This inhibitory effect is enhanced if nANGPTL4 exists as an oligomer, and indeed, mutations that prevent oligomerization severely reduce the ability of ANGPTL4 to inhibit LPL.<sup>232</sup> *ANGPTL4* expression is induced during fasting, and thus ANGPTL4 can act as a sensor for energy homeostasis by inhibiting LPL activity. Conversely, *ANGPTL4* expression is enhanced by free fatty acids, which could protect the cell from cellular lipid overload by reducing the hydrolysis of TAGs and subsequent fatty acid uptake.<sup>221</sup> Increased uptake of saturated fatty acids is typically associated with a stimulation of inflammation- and immunity-related factors, and macrophages in particular are protected from this pro-inflammatory effect via expression of *ANGPTL4*, which decreases the release of fatty acids.<sup>233</sup>

In addition to energy homeostasis and inflammation, ANGPTL4 plays a regulatory role during wound healing.<sup>225,234</sup> After skin injury, several processes are involved to facilitate wound closure, including cell migration, proliferation, and remodeling of the extracellular matrix.<sup>235</sup> It was shown that *flANGPTL4* and *cANGPTL4* are expressed by keratinocytes adjacent to the wound and coordinate cell-matrix communication during the re-epithelialization process. This involves the interaction of ANGPTL4 with vitronectin and fibronectin in the wound bed, delaying their degradation by matrix-metalloproteinases. Thereby the availability of extracellular matrix components is increased, which enhances cell-matrix interaction. In addition, *cANGPTL4* interacts with  $\beta$ 1 and  $\beta$ 5-integrins to activate integrin-mediated signaling, internalization and keratinocyte migration.<sup>225,234</sup>

### 7.1.3 ANGPTL4 in tumorigenesis and angiogenesis

Tumor tissue RNA micro-arrays revealed an increase in *ANGPTL4* expression in several human epithelial tumors.<sup>236</sup> The expression level of *ANGPTL4* correlates with tumor progression from a benign to a metastatic state.<sup>237</sup> An essential feature of tumor cells is the loss of dependence on integrin-mediated ECM contact for growth.<sup>238</sup> The interaction of cANGPTL4 with  $\beta 1$  and  $\beta 5$ -integrins on tumor cells was shown to activate the production of reactive oxygen species (ROS), which induce a pro-survival signal independent of cell-cell or cell-matrix contacts. Thus, ANGPTL4 confers anoikis resistance to tumor cells.<sup>236</sup> The inhibition of ANGPTL4 induces tumor cell apoptosis, highlighting the importance of ANGPTL4 for cancer therapy.<sup>236,239</sup>

The formation of new blood vessels through release of angiogenic factors ensures the supply of necessary nutrients and growth factors for tumor progression. Furthermore they allow dissemination of cancer cells throughout the body and the formation of metastases in distant organs.<sup>238</sup> The communication of tumor cells with the surrounding endothelium is crucial for these processes and involves the production of secreted factors such as vascular endothelial growth factor (VEGF) and ANG.<sup>240</sup>

The role of ANGPTL4 in angiogenesis and vascular permeability remains controversial. Some studies proposed an anti-angiogenic function of ANGPTL4, as it inhibits proliferation and tubule formation of endothelial cells.<sup>241</sup> In addition, ANGPTL4 decreased VEGF-induced angiogenesis and vascular leakiness and tumor growth in the dermal layer of transgenic mice.<sup>242</sup> In a hypoxic environment, ECM-bound fANGPTL4 inhibits endothelial cell motility, sprouting and tubule formation.<sup>241</sup> ANGPTL4 also prevents metastasis by inhibiting vascular permeability, as well as tumor cell motility and invasiveness.<sup>243</sup> In gastric cancers, ANGPTL4 was shown to suppress tumor formation by inhibition of angiogenesis.<sup>244</sup> Interestingly, an ANGPTL4 mutant that carries a deletion mutation in the CCD was not able to inhibit angiogenesis, indicating that this domain is required for the inhibitory effect. Accordingly, in human gastric cancers an accumulation of the deletion mutant was found.<sup>244</sup>

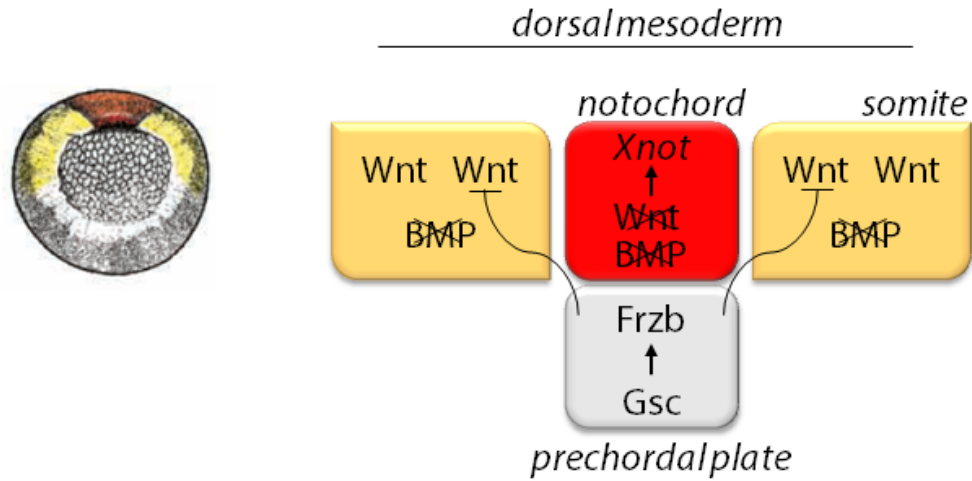
However, a pro-angiogenic function of ANGPTL4 was observed in other studies. In *in vitro* assays ANGPTL4 induced angiogenesis and rearrangement of blood vessels, and its expression was significantly increased in tumor cells from clear cell renal cell carcinomas.<sup>245</sup> Similarly, *ANGPTL4* mRNA levels are up-regulated in human gastric and colorectal cancer, where ANGPTL4 was found to promote venous invasion.<sup>237,246</sup> In Kaposi's sarcoma, a highly vascular tumor induced by human herpesvirus-8 infection, enhanced *ANGPTL4* expression is thought to facilitate angiogenesis and vascular permeability and thereby promote tumorigenesis.<sup>247</sup> In addition, ANGPTL4 was identified as one of several genes that mediate breast cancer metastasis to the lung.<sup>224,248</sup> It was shown that TGF $\beta$ , which is secreted by the tumor microenvironment under hypoxic conditions, induces the expression of *ANGPTL4* in a SMAD3-dependent manner. Tumor cell-derived ANGPTL4 disrupts vascular endothelial cell-cell junctions and increases the permeability of lung capillaries. This facilitates the trans-endothelial passage of breast tumor cells to selectively seed lung metastasis.<sup>224</sup> Mechanistically, cANGPTL4 interacts with and activates the  $\alpha 5\beta 1$ -integrin signaling pathway to weaken cell-cell contacts. Additionally, cANGPTL4 associates with vascular endothelial cadherin and claudin 5 and induces their dissociation and internalization. Both processes converge in endothelial disruption and facilitate lung metastasis in mice.<sup>249</sup>

The above observations show that the biological functions of ANGPTL4 and their underlying molecular mechanisms are incompletely understood. In particular, the specific roles of the ANGPTL4 cleavage fragments are largely unknown, and require further investigation. Moreover, to fully elucidate ANGPTL4 signaling, it will be critical to identify ANGPTL4 interactors and receptors that mediate its diverse biological effects.

#### 7.1.4 Development and regulation of notochord formation in *Xenopus*

The development of axial structures in vertebrates is induced during gastrulation by the organizer region. In amphibian embryos, the organizer can be subdivided into a head and a trunk organizer.<sup>250</sup> Whereas the head organizer induces anterior fates in the overlying ectoderm, the trunk organizer mediates neural induction in dorsal ectoderm, as well as dorsalization of equatorial mesoderm.<sup>251</sup> Besides its ability to organize surrounding tissue, the head or trunk organizers undergo differentiation and give rise to head tissue, such as the prechordal plate or the notochord, respectively. The notochord itself acts as a signaling center, which is required for patterning of surrounding tissues.<sup>252,253</sup> The development of the presumptive notochord region requires the inhibition of both BMP and Wnt/ $\beta$ -catenin signaling.<sup>254</sup>

In *Xenopus*, *wnt8* is expressed in the ventral marginal zone during gastrulation.<sup>255,256</sup> It was shown that *Xenopus* Wnt8 enhances the expression of myogenic genes, such as *myoD* and *myf5*, whereas it inhibits the expression of the notochord marker gene *Xnot*.<sup>257-259</sup> Consequently, ectopic expression of *wnt8* in dorsal mesoderm of *Xenopus* embryos changes the fate of dorsal cells (head mesoderm, notochord) to more lateral tissue (somatic muscle).<sup>257</sup> Thereby Wnt8 has a function in patterning the marginal zone, and it was proposed that Wnt8 may position the notochord/somite boundary.<sup>259</sup> This, however, requires the expression of Wnt inhibitors in the presumptive notochord region, which allow *Xnot* expression and thereby notochord formation. It was shown that the expression of *goosecoid* (*gsc*) inhibits *wnt8* expression on the transcriptional level. Furthermore *gsc* induces the expression of *frzb*, which is secreted from the prechordal plate and inhibits *wnt8* through direct binding (Figure 21). These two mechanisms exclude Wnt8 from the presumptive notochord region and allow the expression of *Xnot*.<sup>254,257</sup>



**Figure 21| Model of notochord formation during *Xenopus* development.** The induction of notochord formation requires inhibition of BMP signaling in somitic (yellow) and notochord (red) regions. In addition, Wnt signals have to be blocked in the presumptive notochord tissue. This is facilitated by secretion of Wnt inhibitors like Frzb from the prechordal plate. *Frzb* expression is regulated by goosecooid (*gsc*). The inhibition of both pathways allows the expression of the notochord marker *Xnot* and thereby the development of the notochord. Figure modified from Yasuo & Lemaire, 2001.<sup>254</sup>

Thus, maintenance of notochord development requires inhibition of Wnt/ $\beta$ -catenin signaling, through expression and secretion of specific Wnt inhibitors like Frzb.

## 7.2 Results

To identify novel Wnt pathway components regulating Wnt signaling, we performed a genome-wide small interfering RNA (siRNA) screen.<sup>22</sup> The transcription of a Wnt-responsive luciferase reporter construct was analyzed after stimulation with six different Wnt activators. In this screen, ANGPTL4 was identified as a putative negative regulator of Wnt signaling (Table 1). Compared to all other ANG and ANGPTL proteins covered by this screen, only *ANGPTL4* depletion resulted in high z-scores (>3) after stimulation of the Wnt luciferase reporter with either WNT3A conditioned medium or transfection of *WNT1*, *FZD8*, *LRP6*.

**Table 1: ANGPTL4 is a negative regulator of Wnt signaling.** Angiopoietin (ANG) and Angiopoietin-like (ANGPTL) proteins sorted according to their z-scores in the genome-wide siRNA Wnt luciferase reporter assay in HEK293T cells, stimulated by transfection of the ternary *WNT1+FZD8+LRP6* complex. The list contains only candidates with low z-score in the cell viability screen. Z-scores from Wnt luciferase reporter screens activated with WNT3A-/RSPO3-conditioned medium or by transfection with  $\beta$ -catenin, *WNT1*, *RSPO3* and *LRP6 $\Delta$ E1-4* constructs are also indicated. siRNA screen was performed by Cristina Cruciati and data analysis by Dierk Ingelfinger.<sup>22</sup> Z-scores indicate the strength of each siRNA. Strong siRNA effects are resembled by a large positive or negative z-score.<sup>260</sup>

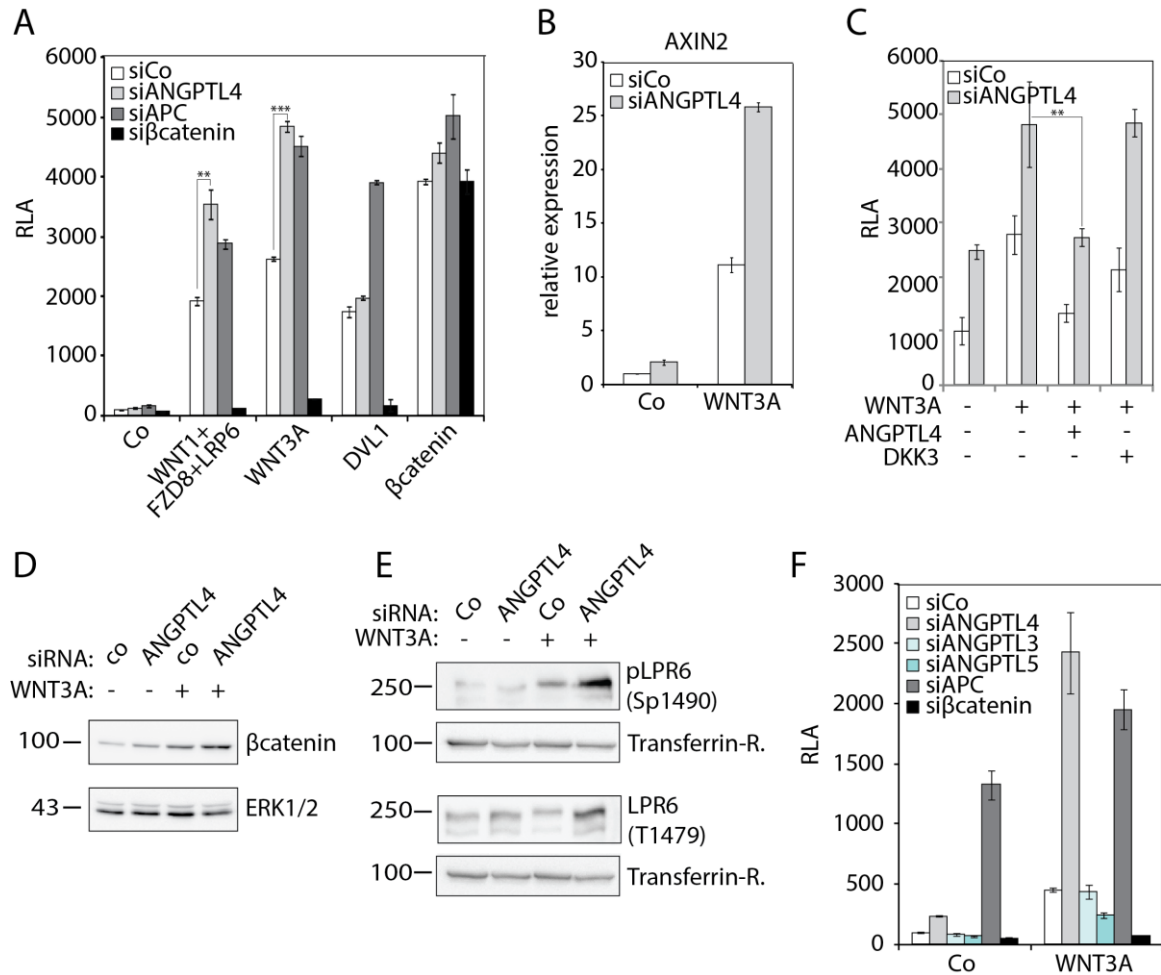
WNT3A	RSPO3	$\beta$ -catenin	Viability	<i>WNT1</i> + <i>RSPO3</i>	<i>WNT1</i> + <i>FZD8</i> + <i>LRP6</i>	<i>LRP6</i> $\Delta$ E1-4	Gene-ID	Gene Name
<b>3.58</b>	2.9	1.52	-1.31	1.65	<b>3.24</b>	<b>2.06</b>	NM_016109	<b>ANGPTL4</b>
-0.65	-0.28	1.59	0.15	0.99	1.49	0.82	NM_015985	ANG4
0.08	0.07	0.02	0.45	-0.65	1.04	0.4	NM_014495	ANGPTL3
-0.57	-0.42	0.09	0.57	0.22	0.85	0.68	NM_001147	ANG2
0.33	-0.08	1.25	-0.26	0.63	0.34	-0.18	NM_004673	ANGPTL1
-0.8	0.3	-1.86	0.12	0.96	0.14	-0.57	NM_031917	ANGPTL6
-0.08	-1.17	1.05	1.01	-0.16	-0.02	0.64	NM_001146	ANG1
-0.71	-0.49	-3.12	1.07	0.02	-0.9	-1.6	NM_178127	ANGPTL5
-0.73	-1.33	1.44	-0.22	-2.95	-2.17	-0.47	NM_012098	ANGPTL2

### 7.2.1 ANGPTL4 is a negative regulator of Wnt/ $\beta$ -catenin signaling

To validate the results of the genome-wide siRNA screen and to analyze the effect of ANGPTL4 epistatically, additional Wnt reporter assays were performed. Knock-down of *ANGPTL4* by siRNA enhanced TOPFlash reporter activity induced by a combination of *WNT1*, *FZD8* and *LRP6* or by *WNT3A* (Figure 22A). In contrast, si*ANGPTL4* did not enhance luciferase activity induced intracellularly by *DVL1* or  $\beta$ -catenin (Figure 22A). Similarly, the expression of the canonical Wnt target gene *AXIN2* was enhanced in si*ANGPTL4* transfected cells, further confirming the inhibitory role of ANGPTL4 (Figure 22B). The si*ANGPTL4* effect on TOPFlash reporter activity was rescued by the addition of ANGPTL4 conditioned medium, whereas DKK3 conditioned medium had no effect, attesting specificity (Figure 22C).

The accumulation of cytoplasmic  $\beta$ -catenin and the phosphorylation of LRP6 are immediate, transcription-independent read-outs for activated Wnt signaling. Consistent with results from the TOPFlash assays, knock-down of *ANGPTL4* led to an increase in  $\beta$ -catenin, phospho- and total LRP6 protein levels in unstimulated as well as in *WNT3A*-treated cells (Figure 22D, E). These results indicate an inhibitory effect of ANGPTL4 on Wnt/ $\beta$ -catenin signaling. Furthermore si*ANGPTL4* increased total LRP6 protein levels (Figure 22E), suggesting that the mechanism of ANGPTL4 in Wnt signaling could be via negative regulation of LRP6 production, maturation, trafficking or stability.

As ANGPTL4 belongs to a family of eight ANGPTL proteins, the effect of other family members on the Wnt pathway was also investigated. Knock-down of *ANGPTL3* and *ANGPTL5* using siRNA had no effect on TOPFlash reporter activity (Figure 22F), indicating that the inhibitory function on Wnt/ $\beta$ -catenin signaling is specific for ANGPTL4.

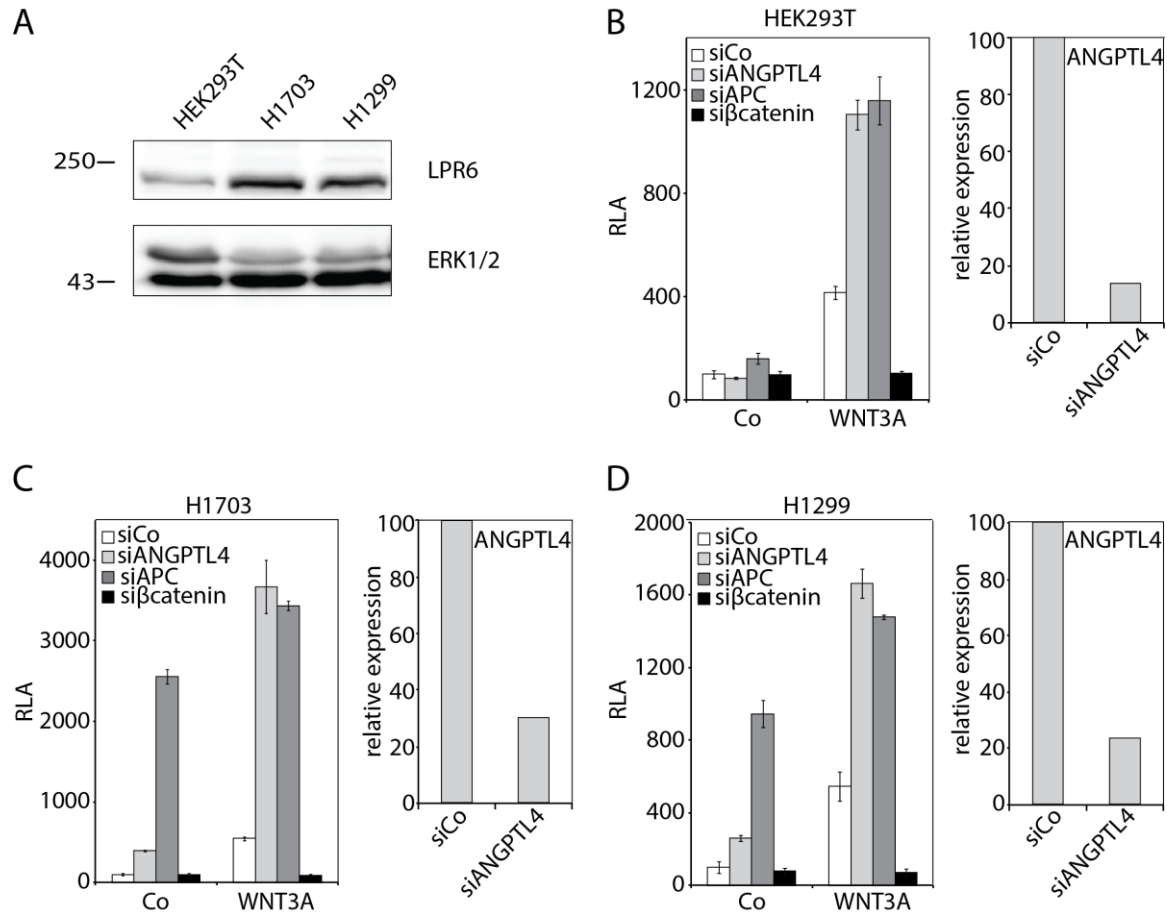


**Figure 22 | ANGPTL4 is a negative regulator of Wnt/ $\beta$ -catenin signaling.** (A) TOPFlash assay in HEK293T cells treated as indicated. Graphs show mean  $\pm$  SD,  $N=3$ . Co, control. RLA, relative luciferase activity. (B) qPCR analysis of *AXIN2* in HepG2 cells. Cells were transfected with the indicated siRNAs and stimulated with WNT3A-conditioned medium for 24h. (C) TOPFlash assay in HEK293T cells. ANGPTL4, but not, DKK3 conditioned medium rescued loss of *ANGPTL4*. (D,E) Western blot analysis of endogenous  $\beta$ -catenin, phospho (Sp1490) and total LRP6, using cytosolic (D) and membrane (E) fractions of H1703 cells. Cells were treated with control or WNT3A-conditioned medium in the presence of the indicated siRNAs. (F) TOPFlash assay in HEK293T cells transfected with the indicated siRNAs. Graphs show mean  $\pm$  SD,  $N=3$ . Co, control. RLA, relative luciferase activity.

Due to the comparatively low expression level of *ANGPTL4* in HEK293T cells, the effect of si*ANGPTL4* was also analyzed in the non-small cell lung cancer lines H1299 and H1703, which display higher protein levels of *ANGPTL4* and LRP6 and should therefore be sensitized to loss of *ANGPTL4* (Figure 23A, and data not shown). Indeed, TOPFlash reporter activity was increased 4- to 8-fold compared to 2.5-fold in HEK293T cells after depletion of *ANGPTL4*, despite comparable knock-down efficiency (Figure 23B-D). These



results indicate that ANGPTL4 is an inhibitor of Wnt/ $\beta$ -catenin signaling, acting upstream or at the level of the Wnt receptors.

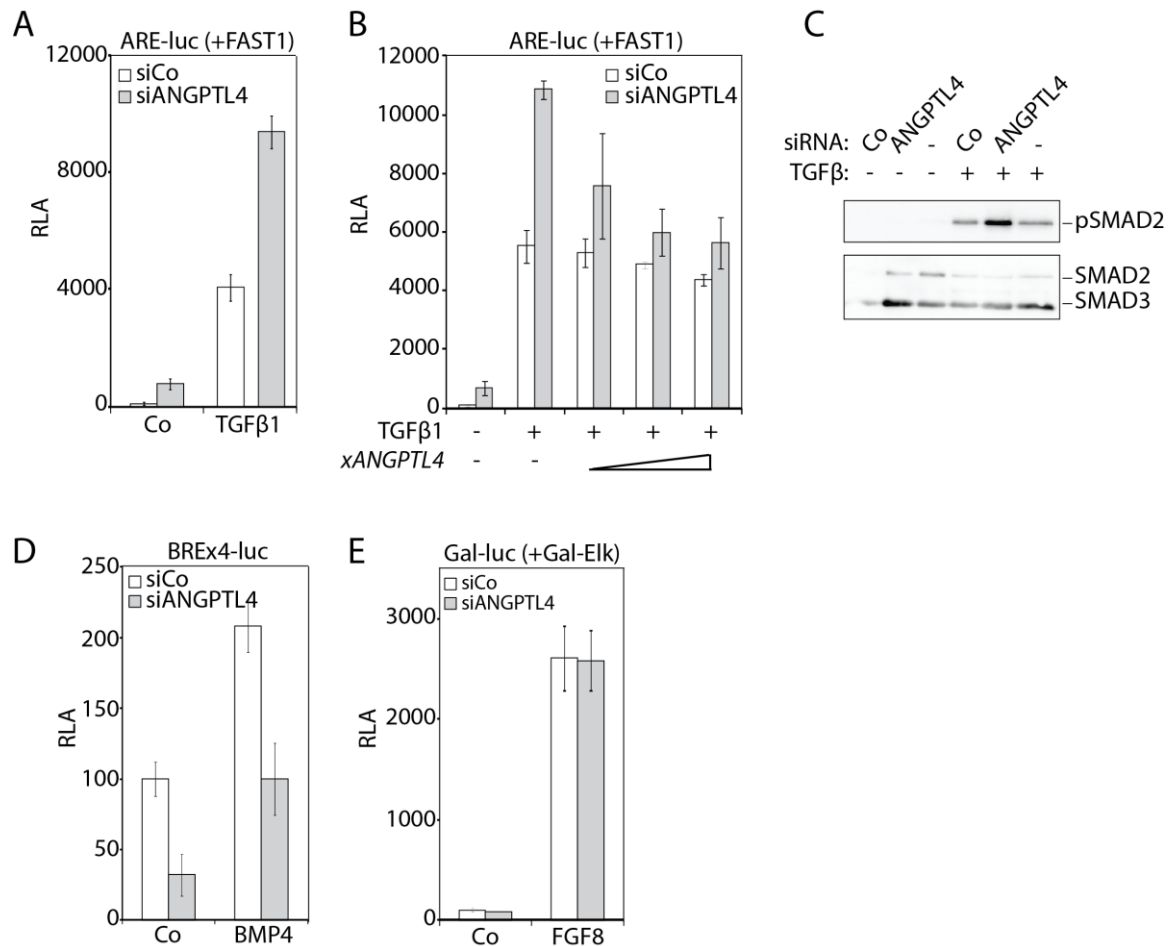


**Figure 23 | The inhibitory effect of ANGPTL4 is stronger in cells with higher LRP6 levels.** (A) Western blot analysis of endogenous LRP6 (T1479) in HEK293T, H1703 and H1299 cells. ERK1/2 was used for normalization. (B-D) Wnt luciferase reporter assays in HEK293T (B), H1703 (C) and H1299 (D) cells stimulated with WNT3A-conditioned medium, in the presence of the indicated siRNAs. Graphs show mean  $\pm$  SD,  $N=3$ . Co, control. RLA, relative luciferase activity. Knock-down efficiency was determined by qPCR, shown in the graphs on the right.

### 7.2.2 ANGPTL4 inhibits TGF $\beta$ /Smad signaling *in vitro*

To investigate whether *ANGPTL4* knock-down also affects other signaling pathways, specific reporter constructs for the TGF $\beta$ /SMAD2, BMP/SMAD1 and FGF signaling pathways were analyzed. TGF $\beta$  signaling was strongly enhanced after *ANGPTL4* siRNA transfection (Figure 24A). This siRNA effect was specific, as *Xenopus* ANGPTL4 was able to rescue the effect dose-dependently (Figure 24B). The induction of TGF $\beta$  signaling was associated with increased phosphorylation of SMAD2, as shown by Western blot analysis (Figure 24C). For BMP signaling, a decrease in BMP reporter activity was observed after *ANGPTL4* knock-down (Figure 24D). In contrast, FGF signaling was not affected by loss of *ANGPTL4* (Figure 24E). These results indicate an inhibitory function of ANGPTL4 not only on Wnt/ $\beta$ -catenin but also TGF $\beta$ /SMAD2 signaling.

To analyze whether ANGPTL4 affects Wnt/ $\beta$ -catenin and TGF $\beta$  signaling also *in vivo*, the role of ANGPTL4 in *Xenopus* was investigated.



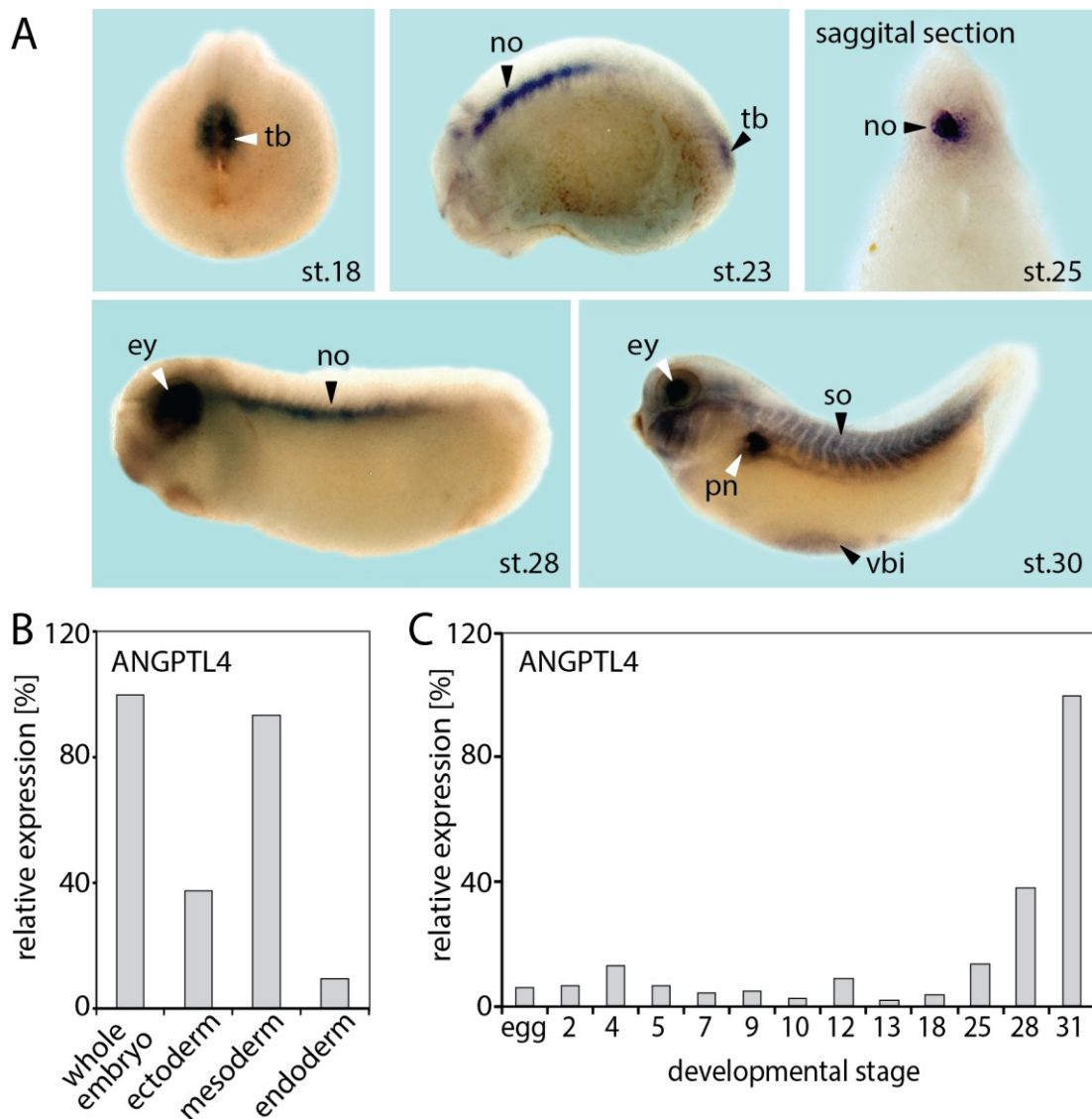
**Figure 24 | ANGPTL4 is an inhibitor of TGF $\beta$  signaling.** (A,B) TGF $\beta$ /SMAD2 luciferase reporter assays in HepG2 cells. Cells were transfected with ARE-luc reporter/FAST1, the indicated siRNAs, *xANGPTL4* DNA (B), and stimulated with recombinant human TGF $\beta$ 1 (2 ng/ml) for 2h. (C) Western blot analysis of phosphorylated SMAD2 (pSMAD2) and total SMAD2/3 after transfection with *control* or *ANGPTL4* siRNA and stimulation with recombinant TGF $\beta$ 1 (2 ng/ml) for 24h. (D) BMP/SMAD2 signaling assay using BREx4-luc reporter stimulated with recombinant human BMP4 (10 ng/ml) for 24h. (E) FGF signaling assay using Gal-luc reporter and co-transfected Gal-EIk stimulated with recombinant human FGF8 (50 ng/ml) for 24h. Error bars indicate SDs, *N*=3. RLA, relative luciferase activity.

### 7.2.3 ANGPTL4 is expressed in mesodermal tissue in *Xenopus tropicalis*

The expression and function of ANGPTL4 during *Xenopus* development is unknown. Therefore, qPCR analysis and *in situ* hybridization were performed to characterize ANGPTL4 in *Xenopus*.

In *Xenopus* embryos, *ANGPTL4* showed prominent expression in mesodermal tissues, such as the tailbud mesoderm, eye lens, somites, pronephros and ventral blood islands (Figure 25A). Furthermore, sagittal sections of stage 25 embryos showed a defined

staining in the notochord. The expression pattern determined via *in situ* hybridization was confirmed by qPCR analysis, where highest expression of *ANGPTL4* is detected in the mesodermal germ layer (Figure 25B). Furthermore it was shown that *ANGPTL4* is maternally expressed at low levels, and that the expression strongly increased after gastrula stage (Figure 25C). These results demonstrate a distinct expression profile of *ANGPTL4* in *Xenopus* embryos, with highest expression in tissues of mesodermal origin.

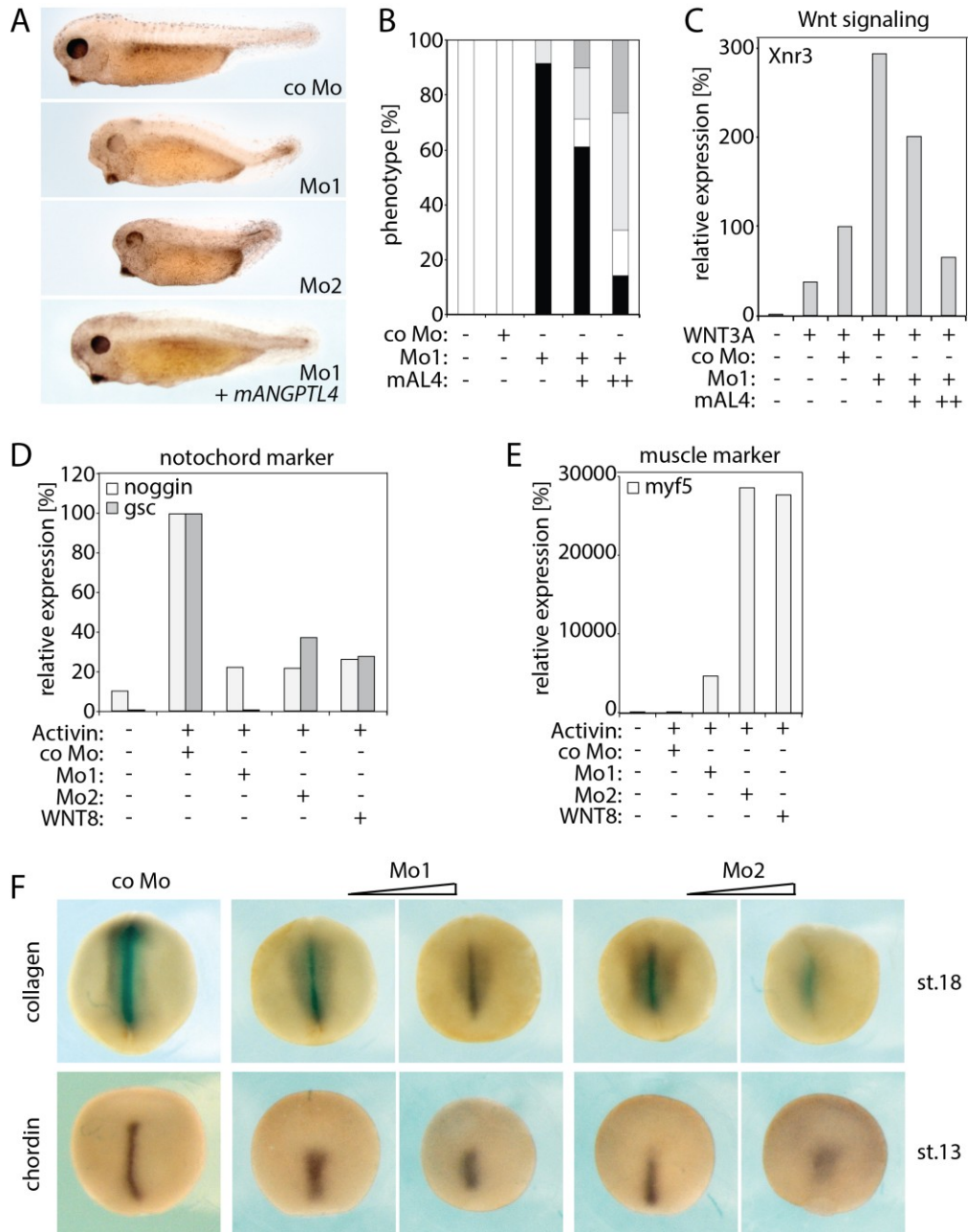


**Figure 25 | *ANGPTL4* is expressed in mesodermal tissues in *X.tropicalis* embryos.** (A) Whole-mount *in situ* hybridization analysis of *ANGPTL4* mRNA expression in *Xenopus* embryos at different stages. Cross-section of a stage-25 embryo showing *ANGPTL4* expression in the notochord. Tb, tailbud mesoderm; no, notochord; ey, eye; pn, pronephros; so, somites; vbi, ventral blood island. (B) qPCR analysis of *ANGPTL4* in *Xenopus* explants at stage 18, with strongest expression in mesodermal cells. Gene expression in whole embryos was set to 100%. (C) qPCR analysis of *ANGPTL4* in *Xenopus* embryos at different developmental stages. Ornithine decarboxylase (*odc*) was used for normalization.

#### 7.2.4 ANGPTL4 inhibits Wnt signaling in *Xenopus* and is required for notochord formation

To investigate the role of ANGPTL4 on Wnt/ $\beta$ -catenin signaling in *Xenopus*, the expression of the Wnt target genes *siamois* and *Xnr3* was analyzed. For this purpose two independent *ANGPTL4* antisense Morpholino oligonucleotides (Mo1 and Mo2), targeting different splice sites of the *ANGPTL4* pre-mRNA, were used. The injection of both Morpholinos resulted in similar phenotypes, characterized by small heads, shortened, bent body axes and defects in melanocyte and eye pigmentation (Figure 26A). This effect was specific because a mouse *ANGPTL4* construct was able to partially rescue the phenotype (Figure 26A,B). Depletion of *ANGPTL4* by Mo1 enhanced the expression of the Wnt target genes *siamois* and *Xnr3* in *Xenopus* animal caps (Figure 26C, data for *siamois* not shown). This indicates that ANGPTL4 also acts as an inhibitor of Wnt/ $\beta$ -catenin signaling in *Xenopus*. The inhibitory effect on Wnt signaling is specific, because it was rescued by co-injection of mouse *ANGPTL4* mRNA in a dose-dependent manner (Figure 26C). Interestingly, the expression of high concentrations of *ANGPTL4* resulted in a decrease of Wnt target genes, whereas the expression of low amounts of *ANGPTL4* increased *siamois* and *Xnr3* expression (data not shown). This suggests a biphasic role of ANGPTL4 in *Xenopus* and illustrates that the concentration of ANGPTL4 used in the experiments is critical.

Because the expression analysis of *ANGPTL4* shows a distinct signal in the notochord, a region where Wnt/ $\beta$ -catenin signaling has to be inhibited to ensure its development, the impact of ANGPTL4 on notochord formation was examined. Depletion of *ANGPTL4* with both Morpholinos reduced the expression of the notochord markers *noggin* and *gooseoid* (*gsc*) on the RNA level, similarly as observed after *WNT8* mRNA injection (Figure 26D). In contrast, the expression of the muscle marker *myf5* was increased (Figure 26E). These results were confirmed by *in situ* hybridization, where a reduction in the expression intensity and the expression area for *collagen* and *chordin* was detected in *ANGPTL4* morphants (Figure 26F). Taken together, these results demonstrate that ANGPTL4 is an inhibitor of Wnt/ $\beta$ -catenin signaling *in vivo* and required for notochord formation in *Xenopus*.

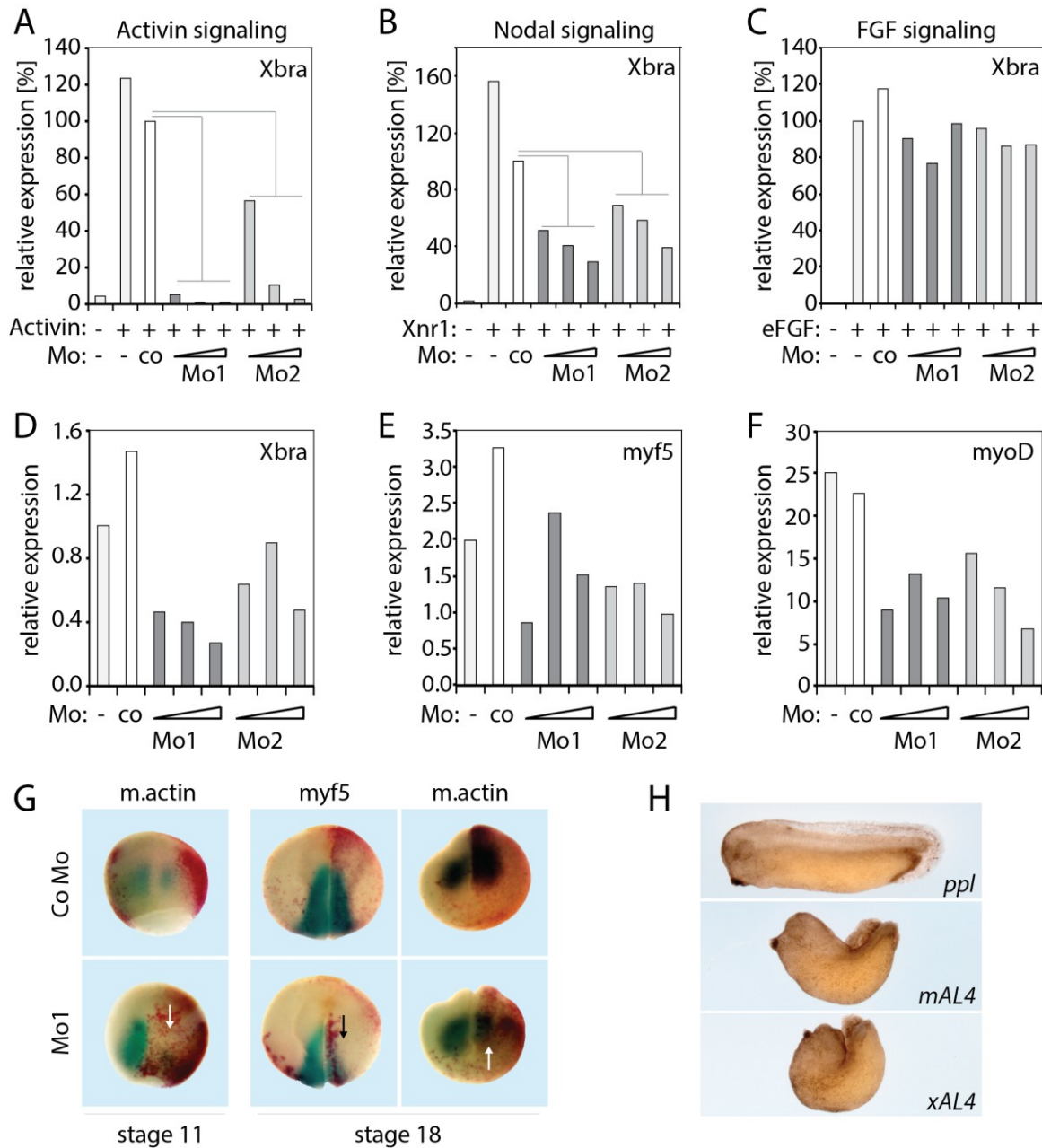


**Figure 26 | ANGPTL4 inhibits Wnt/ $\beta$ -catenin signaling and is required for notochord formation in *Xenopus*.** (A) Representative tadpole stage embryos injected equatorially at 2-cell stage with *control* (co) or two *ANGPTL4* antisense Morpholino oligonucleotides (Mo1, Mo2) in the absence or presence of mouse *ANGPTL4* mRNA. (B) Statistical analysis of the injected *Xenopus* embryos shown in panel (A). For each sample 20-60 embryos were analyzed, and the percentage of induced phenotypes was determined at stage 32. (C-E) Expression analysis in *Xenopus* animal caps by qPCR. Animal caps were excised at blastula and harvested at gastrula (C) or neurula stage (D,E). Ornithine decarboxylase (*odc*) was used for normalization. *Co Mo* was set to 100%. (C) qPCR of *Xenopus nodal-related 3* (*Xnr3*) in animal caps injected with *WNT3A* and *mANGPTL4* mRNA and *ANGPTL4* Morpholino (Mo1). (D,E) Analysis of *noggin*, *gsc* and *myf5* expression in *Xenopus* animal caps. Embryos were injected with *Activin* and *WNT8* mRNA and control (co) or two different *ANGPTL4* Morpholinos (Mo1, Mo2). *gsc*, *goosecoid*; *myf5*, *myogenic factor 5*. (F) Whole-mount *in situ* hybridization analysis of *collagen* and *chordin* mRNA expression in *Xenopus* morphants at the indicated stages. Embryos were injected with *control* (co) or two different concentrations of *ANGPTL4* Morpholino1 or 2 (Mo1, Mo2).

### 7.2.5 ANGPTL4 is required for mesoderm formation in *Xenopus* through Activin/Nodal signaling

To investigate if ANGPTL4 also affects TGF $\beta$  signaling in *Xenopus*, the expression of target genes was analyzed. In animal cap explants, *Xbra* expression can be induced by different signaling pathways. The effect of ANGPTL4 on inducers of TGF $\beta$  (Nodal/Activin) and FGF signaling pathways was examined. Co-injection of *ANGPTL4* Mo1 and Mo2 decreased *Xbra* expression induced by *Activin* and *Xnr1* (Figure 27A, B). In contrast, depletion of *ANGPTL4*, by Mo1 or Mo2, had no effect on *Xbra* expression induced by *eFGF* RNA (Figure 27C). This indicates that ANGPTL4 has no effect on FGF signaling, confirming the *in vitro* results. These results suggest that ANGPTL4 is required for Activin/Nodal, but not FGF signaling in *Xenopus*.

Among other processes, Activin/Nodal signaling is involved in mesoderm formation during *Xenopus* development. Because ANGPTL is required for Activin/Nodal signaling in *Xenopus*, it is possible that ANGPTL4 is also involved in this process. To address this question, mesodermal markers were analyzed. The expression of the pan-mesodermal marker *brachyury* (*Xbra*) was decreased in *ANGPTL4* morphants as shown by qPCR analysis (Figure 27D). Accordingly, the expression of the muscle markers *myf5* and *myoD*, which develop from the mesodermal germ layer, was reduced (Figure 27E, F). This was confirmed by *in situ* hybridization of unilateral injected embryos, which showed a decrease in *muscle actin* and *myf5* staining, upon knock-down of *ANGPTL4* (Figure 27G). The misregulation of critical components, required for mesoderm development, results in gastrulation defects in *Xenopus* embryos. A similar phenotype was observed after overexpression of either mouse or *Xenopus* *ANGPTL4* (Figure 27H). The phenotype of these tadpoles is characterized by shortened embryonic axis, defects in neural tube closure and small heads. This indicates that ANGPTL4 is required for mesoderm formation in *Xenopus* through its effect on Activin/Nodal signaling.

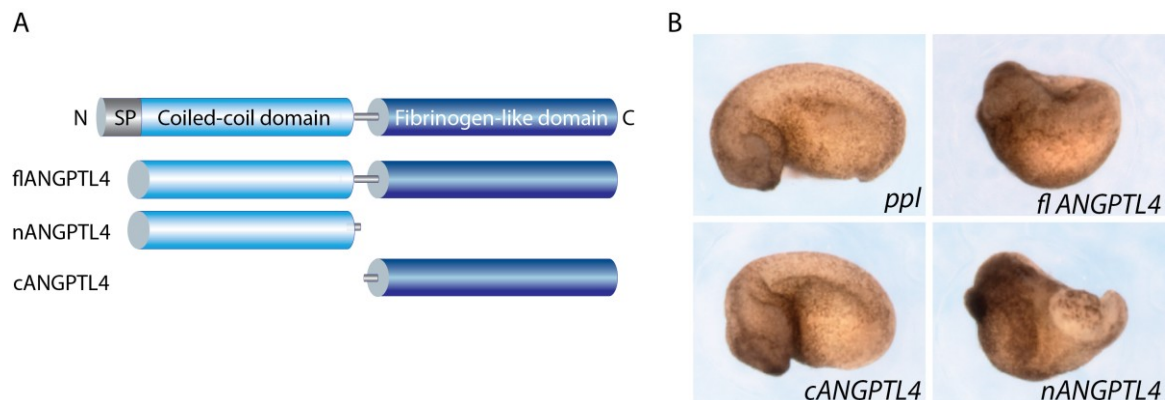


**Figure 27 | ANGPTL4 is required for Activin/Nodal signaling and mesoderm formation in *Xenopus*.** (A-C) Expression analysis of *Xbra* in *Xenopus* animal caps by qPCR. *Xbra* expression was induced by injection of *Activin* (A), *Xnr1* (B) or *eFGF* mRNA (C). Embryos were co-injected with control (co) or ANGPTL4 Morpholinos (Mo1, Mo2) using three different concentrations (10ng, 20ng, 40ng). Animal caps were excised at blastula and harvested at gastrula stage. *Ornithine decarboxylase* (*odc*) was used for normalization. Co Mo was set to 100%. (D-F) Expression analysis of *Xbra* (D), *myf5* (E), and *myoD* (F) in *Xenopus* whole embryos by qPCR. Embryos were injected with control (co) or ANGPTL4 Morpholinos (Mo1, Mo2) as in (A-C). (G) Whole-mount *in situ* hybridization analysis of *m.actin* and *myf5* mRNA expression in *Xenopus* morphants at different stages. Embryos were injected unilaterally with control (co) or ANGPTL4 Morpholino1 (Mo1) in combination with the lineage tracer  $\beta$ -galactosidase. Staining of  $\beta$ -galactosidase (red) indicates injected site. (H) Representative tadpole stage embryos injected equatorially at 4-cell stage with control (*ppl*), mouse (*mAL4*) or *Xenopus* (*xAL4*) ANGPTL4 mRNAs. *Xbra*, *Xenopus brachyury*; *Xnr1*, *Xenopus nodal-related 1*; *eFGF*, *embryonic fibroblast growth factor*; *myf5*, *myogenic factor 5*; *myoD*, *myogenic differentiation 1*; *m.actin*, *muscle actin*.



Full length ANGPTL4 is proteolytically processed after secretion, and gives rise to an N-terminal (nANGPTL4) and a C-terminal (cANGPTL4) fragment. To analyze which part of ANGPTL4 is required for mesoderm induction during *Xenopus* development, different deletion constructs were cloned (Figure 28A). In gain-of-function experiments, nANGPTL4 was able to induce spina bifida similar to the full length protein (Figure 28B). The cANGPTL4 construct, on the other hand, did not induce this phenotype (Figure 28B), suggesting that the C-terminal part is dispensable for the effect of ANGPTL4 on mesoderm formation.

In contrast to its inhibitory effect on TGF $\beta$  signaling *in vitro*, ANGPTL4 is required for Activin/Nodal signaling and mesoderm formation in *Xenopus*, and this function is likely mediated by the N-terminal CCD domain.



**Figure 28 | N-terminal domain of ANGPTL4 is sufficient to induce spina bifida in *Xenopus*.** (A) Domain organization of ANGPTL4. Based on the structure of ANGPTL4 a full length (flANGPTL4), N-terminal (nANGPTL4) and C-terminal (cANGPTL4) construct was cloned. (B) Embryos were injected equatorially at 4-cell stage with control (ppl), flANGPTL4, nANGPTL4 or cANGPTL4 mRNA. Phenotypes were analyzed at tadpole stage.

### 7.2.6 ANGPTL4 binds to syndecans

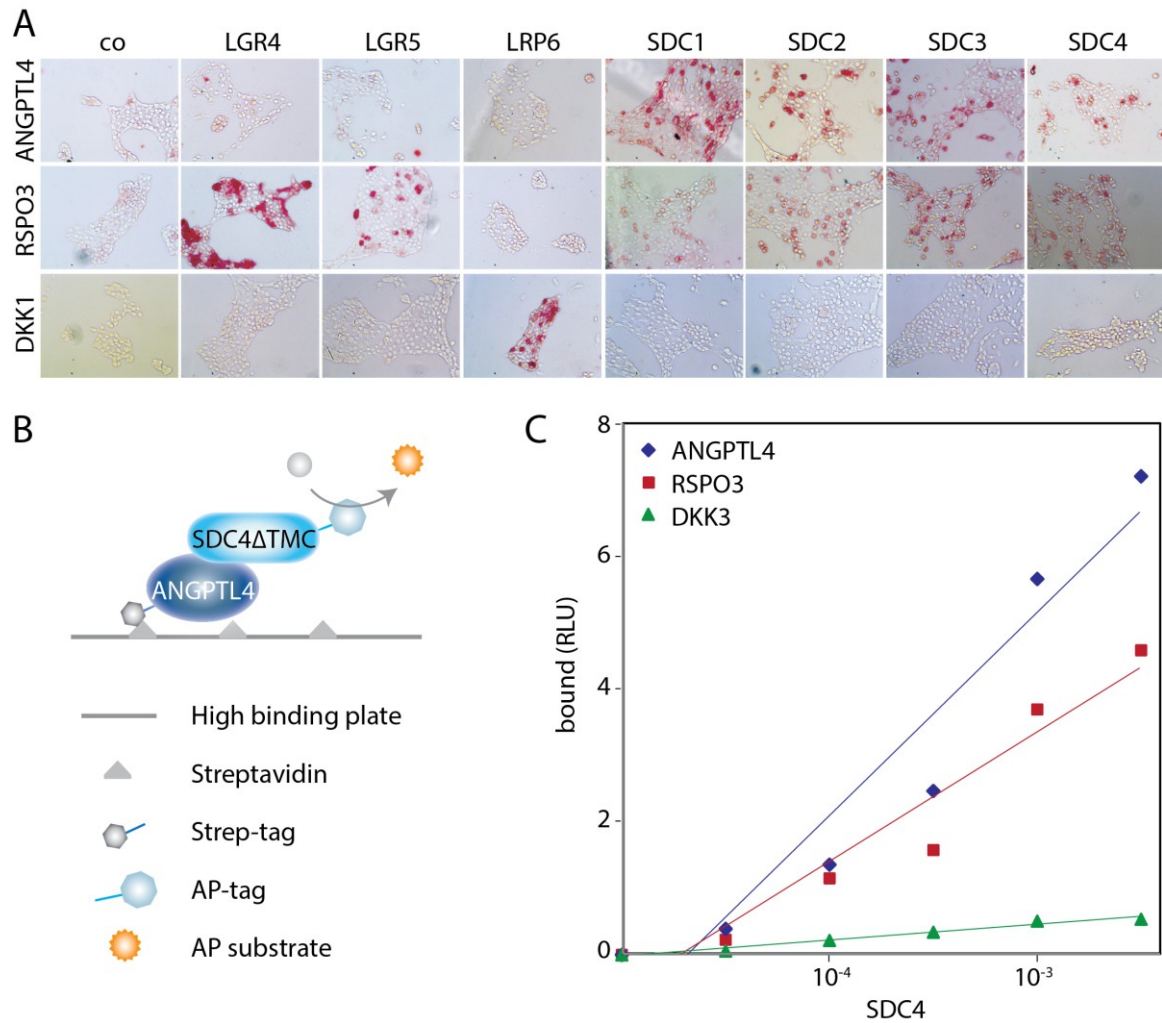
As shown above, ANGPTL4 regulates Wnt/ $\beta$ -catenin and TGF $\beta$  signaling *in vitro* and *in vivo*. To investigate the molecular mechanism by which ANGPTL4 regulates both signaling pathways, the identity of the interaction partner was investigated. For this purpose, co-immunoprecipitation (co-IP) with subsequent mass spectrometric analysis was performed. A total of six membrane-associated proteins were identified, including components of the extracellular matrix and three members of the syndecan family of transmembrane proteoglycans (Table 2).

**Table 2: Transmembrane or membrane-associated proteins identified via mass spectrometry after ANGPTL4 co-IP.** ANGPTL4 IP was performed in HepG2 cells after addition of HRP-Str.-ProteinA-ANGPTL4 conditioned medium for 1h. After a two-step purification process, IP samples were separated by SDS-PAGE and proteins were stained with Coomassie Blue. Co-precipitated proteins were analyzed by mass spectrometric analysis (ESI-MS/MS). Transmembrane or extracellular matrix proteins were identified based on gene ontology (bioDBnet). These proteins were sorted according to the number of unique peptides detected during MS analysis.

Accession No.	Identified protein	#unique peptides
ZO2_HUMAN	Tight junction protein ZO-2	17
FINC_HUMAN	Fibronectin	10
SDC4_HUMAN	Syndecan-4	4
LEG8_HUMAN	Galectin-8	5
SDC1_HUMAN	Syndecan-1	5
SDC2_HUMAN	Syndecan-2	3

As syndecans (SDC) are transmembrane proteins, and ANGPTL4 was shown to act epistatically at the Wnt receptor level (Figure 22), a possible direct interaction of ANGPTL4 and SDC was tested. In cell surface binding assays, an alkaline-phosphatase (AP) fusion protein of ANGPTL4 bound to cells expressing SDC1, SDC2, SDC3 and SDC4 (Figure 29A). In contrast, no binding of ANGPTL4 to cells transfected with LGR4/5 or LRP6 was detected, which however bound their respective ligands, RSPO3 and DKK1 (Figure 29A). To confirm the direct binding between ANGPTL4 and SDC, an ELISA-based binding assay was used. Here Strep-tagged ANGPTL4 was coupled to Streptavidin-coated plates and different concentrations of AP-tagged, soluble SDC4 were applied (Figure 29B). In the tested concentration range, a near-linear correlation between bound and free SDC4 was observed for ANGPTL4 and the known syndecan ligand RSPO3. In

contrast, no binding of DKK3 to SDC4 was detected (Figure 29C). Together these results show that ANGPTL4 can bind all four syndecans.

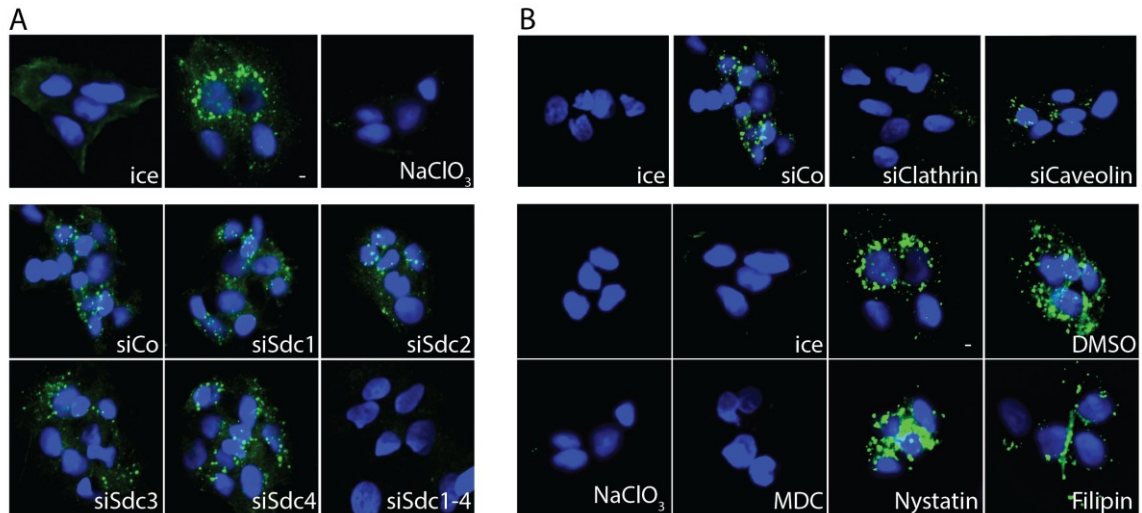


**Figure 29 | ANGPTL4 binds syndecans.** (A) Cell surface binding assay. HEK293T cells were transfected with the indicated plasmids and subjected to binding assays with conditioned medium containing alkaline-phosphatase (AP) fusion proteins of ANGPTL4, R-spondin3- $\Delta$ C (RSPO3), or DKK1. Bound ligands were detected with AP substrate (red). (B) Schematic model of ELISA-based *in vitro* binding assay. High binding affinity plates were coated with Streptavidin, and Strep-tagged ANGPTL4 was bound to the Streptavidin-coated plate. After washing, different concentrations of alkaline-phosphatase (AP) fusion protein of SDC4 $\Delta$ TMC was applied. Binding of SDC4 was detected by AP measurement. Besides ANGPTL4, RSPO3 $\Delta$ C and DKK3 were used as controls. (C) ELISA-based *in vitro* binding assay using ANGPTL4 (blue), RSPO3 $\Delta$ C (red) and DKK3 (green). As binding was still in the linear phase, linear fitting was applied.

### 7.2.7 Syndecans mediate clathrin-dependent internalization of ANGPTL4

A characteristic feature of syndecans is their ability to induce endocytosis upon ligand binding.<sup>261-264</sup> This is essential for signal transduction, for example in FGF signaling.<sup>264</sup> To investigate if ANGPTL4 undergoes endocytosis, internalization assays were performed. For this purpose a horseradish peroxidase (HRP) fusion protein of ANGPTL4 was used, which can be visualized by tyramide signal amplification. Incubation of HepG2 cells with HRP-ANGPTL4 at 37°C induced internalization of ANGPTL4 within 40 min. In contrast, no internalization was observed when cells were incubated on ice (Figure 30A). The treatment of cells with chlorate inhibits the sulfation of heparan sulphate chains, which is required for proper function of HSPGs like syndecans.<sup>265</sup> The incubation of cells with chlorate completely abrogates ANGPTL4 endocytosis (Figure 30A), indicating that HSPGs are required for ANGPTL4 internalization. The depletion of all four syndecans, using siRNA mediated knock-down, also reduced ANGPTL4 internalization. Interestingly, the depletion of only one member of the syndecan family had no effect (Figure 30A), indicating that they can compensate each other.

To determine the nature of this endocytosis process, cells were treated with inhibitors of clathrin and caveolin-dependent endocytosis. The clathrin inhibitor monodansylcadaverine (MDC) blocked ANGPTL4 internalization, unlike inhibitors of caveolin-mediated endocytosis, including nystatin and filipin (Figure 30B). Consistent with the drug treatment, depletion of *clathrin* by siRNA reduced ANGPTL4 cytoplasmic punctae, whereas siRNA targeting *caveolin* had no effect (Figure 30B). Conclusively, the data show that syndecans are required for clathrin-mediated endocytosis of ANGPTL4.

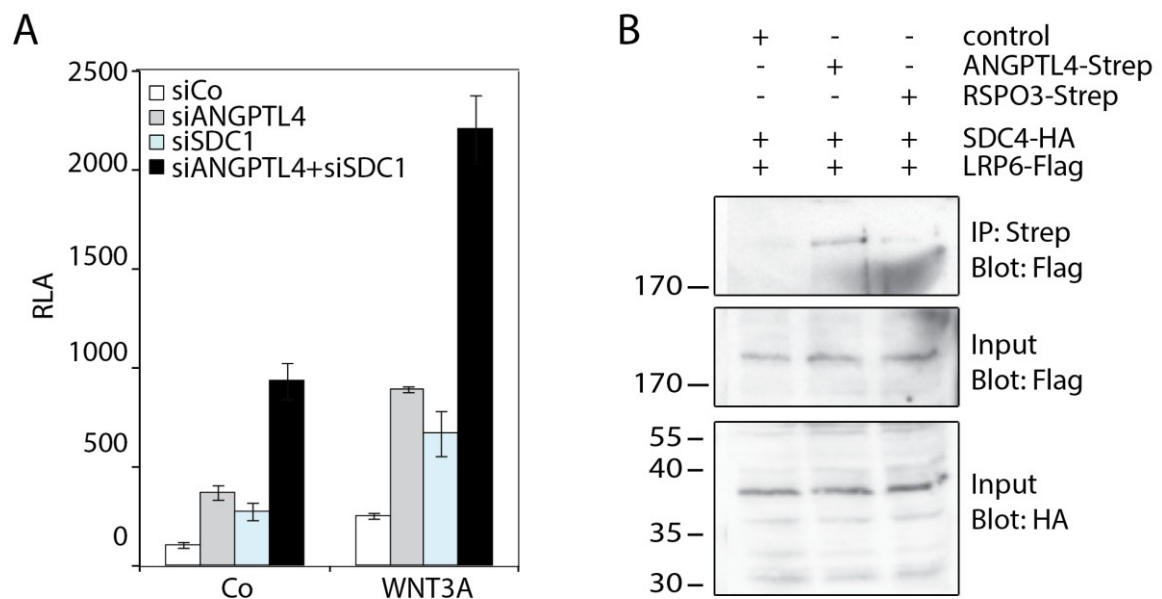


**Figure 30 | Syndecans mediate clathrin-dependent internalization of ANGPTL4.** (A,B) Internalization assay in HepG2 cells treated for 40 min with horseradish peroxidase (HRP)-tagged ANGPTL4 on ice or at 37°C. HRP-tagged ANGPTL4 was visualized by tyramide signal amplification (green). Nuclei were counterstained with Hoechst-33342 (blue). Where indicated cells were pretreated with siRNAs for 3 days or with 25 mM sodium chlorate for 48 h. (B) Internalization of ANGPTL4 in the presence of different clathrin (MDC) or caveolin (Nystatin, Filipin) specific inhibitors. Cells were treated for 1 h with the indicated inhibitors. DMSO was used as a control. MDC, monodansyl-cadaverin.

### 7.2.8 ANGPTL4 binds to LRP6 likely via syndecan

Endocytosis is an essential mechanism of receptor mediated signaling. In the case of Wnt signaling, the internalization of the Wnt-receptor complex is an obligatory step in activating downstream signaling. Syndecans were not only characterized as efficient inducers of endocytosis, but were also described as co-receptors of Wnt signaling in different organisms.<sup>78,266</sup> Recently it was shown that syndecan 4 does not only promote noncanonical, but also inhibits canonical Wnt signaling.<sup>267</sup> Syndecans could therefore mediate the inhibitory effect of ANGPTL4 on Wnt/ $\beta$ -catenin signaling. To analyze if syndecans have a similar function as ANGPTL4, TOPFlash reporter assays were performed. The reporter activity was enhanced after depletion of *SDC1*, similar to *siANGPTL4* (Figure 31A). The combination of siRNA targeting *ANGPTL4* and *SDC1* further enhanced reporter activity, indicating that both cooperate in Wnt pathway inhibition.

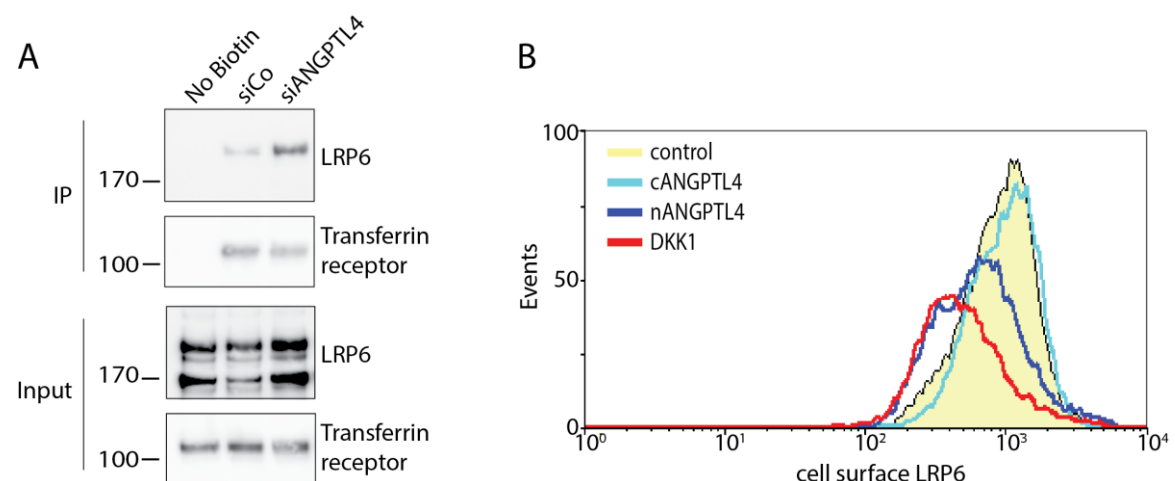
Based on the synergistic effect of ANGPTL4 and syndecan, and the role of syndecans as Wnt co-receptors, it was investigated if ANGPTL4 binds to Wnt receptors via syndecans. Indeed, Flag-tagged LRP6 and Strep-tagged ANGPTL4, which was added as conditioned medium, can be immunoprecipitated in the presence of co-expressed haemagglutinin-tagged SDC4 (SDC4-HA). In contrast, no interaction was observed, when control or Strep-tagged RSPO3 conditioned media were added (Figure 31B). These results suggest that ANGPTL4 and syndecans synergize in Wnt/ $\beta$ -catenin pathway inhibition and ANGPTL4 interacts with LRP6 in the presence of syndecan.



**Figure 31 | ANGPTL4 synergizes with syndecan in Wnt/ $\beta$ -catenin signaling and binds to LRP6.** (A) Wnt luciferase reporter assay stimulated with WNT3A-conditioned medium in the presence of the indicated siRNAs. Co, control medium. RLA, relative luciferase activity. (B) Western blot analysis shows binding of ANGPTL4 to LRP6 in the presence of SDC4. HEK293T cells were transfected with SDC4-HA and LRP6-Flag, and Strep-tagged ANGPTL4, RSPO3 or control conditioned medium was added to cells for 1 h. After lysis of cells, Strep-tagged proteins were precipitated using streptavidin beads, and interacting proteins were examined by Western blot analysis. SDC4 and LRP6 are equally expressed in all conditions. Panel B was provided by Stefan Koch.

### 7.2.9 ANGPTL4 inhibits Wnt/ $\beta$ -catenin signaling by decreasing LRP6 levels

Based on the interaction of ANGPTL4 with syndecans and LRP6, and the increase in total LRP6 levels detected after ANGPTL4 depletion (Figure 22E), ANGPTL4 may inhibit Wnt/ $\beta$ -catenin signaling by affecting LRP6 levels. Biochemical assays were conducted to test this hypothesis. A cell-surface protein biotinylation assay was performed and LRP6 levels were detected by Western blot after Streptavidin-IP. Treatment with *ANGPTL4* siRNA increased levels of cell surface LRP6, without affecting other transmembrane proteins, like transferrin receptor (Figure 32A). This indicates that ANGPTL4 affects LRP6 cell surface levels. This was confirmed by gain-of-function studies using a flow cytometry based approach.<sup>268</sup> The detection of cell surface LRP6 revealed a decrease in LRP6 surface levels after addition of ANGPTL4 conditioned medium (data not shown). To determine which part of ANGPTL4 is required for this effect, deletion constructs were generated, containing either the N-terminal or C-terminal domain. Interestingly, the addition of nANGPTL4 decreased cell surface LRP6, whereas cANGPTL4 had no effect (Figure 32B). Taken together, these results indicate that ANGPTL4 inhibits Wnt/ $\beta$ -catenin signaling by reducing LRP6 membrane levels, which requires only the N-terminal domain of ANGPTL4.



**Figure 32 | ANGPTL4 decreases LRP6 membrane levels.** (A) *ANGPTL4* loss-of-function increased LRP6 cell surface levels as indicated by cell surface biotinylation assay. Western blot analysis of LRP6 after cell surface biotinylation, cell lysis (Input) and pull down of biotinylated proteins (IP) in the presence of the indicated siRNAs. (B) nANGPTL4 gain-of-function decreased cell surface LRP6 in FACS analysis. Flow cytometric analysis of membrane LRP6 in cells treated with control, C-terminal ANGPTL4 (cANGPTL4), N-terminal ANGPTL4 (nANGPTL4) or DKK1 conditioned medium for 1 h. Figure was provided by Stefan Koch.

## 7.3 Discussion

### 7.3.1 ANGPTL4 is a regulator of mesodermal differentiation in *Xenopus*

ANGPTL4 has previously been described as an integral regulator of lipid metabolism,<sup>227,228</sup> cell migration,<sup>225,234</sup> and angiogenesis.<sup>242,244,246</sup> Here I outline a new function of ANGPTL4 in *Xenopus* development. In *Xenopus* embryos, *ANGPTL4* expression was mainly detected in mesodermal tissue, including the notochord. The development of the notochord is strictly regulated and requires co-inhibition of BMP and Wnt signaling.<sup>254</sup> It was shown that inhibition of Wnt/ $\beta$ -catenin signaling is essential for normal specification of axial (notochord) versus lateral (somatic) mesoderm.<sup>251,269</sup> Because *ANGPTL4* shows prominent expression in the notochord, it was investigated whether depletion of *ANGPTL4* affects on notochord formation. The analysis of different notochord markers, using both qPCR and *in situ* hybridization, confirmed this hypothesis. The knockdown of *ANGPTL4* resembled overexpression of *WNT8*,<sup>258</sup> which suggests that ANGPTL4 is an inhibitor of Wnt signaling *in vivo*. In contrast to the reduction in notochord marker gene expression, mesodermal markers were increased upon depletion of *ANGPTL4*. These data are in accordance with the observation that overactivation of Wnt signaling results in re-specification of the notochord as somitic mesoderm.<sup>257</sup> These results demonstrate that the inhibitory effect of ANGPTL4 is of physiological relevance, as ANGPTL4 promotes notochord development in *Xenopus*, presumably by inhibiting Wnt signaling. It should be noted, however, that *ANGPTL4* knock-out mice are viable, although they are born at lower frequency than heterozygous litter mates.<sup>227</sup> Similarly, hemizygous loss of *ANGPTL4* in humans affects lipid metabolism, but does not result in overt developmental defects.<sup>270</sup> It has recently been suggested that deleterious genetic mutations may not manifest phenotypically due to compensatory gene regulation.<sup>271</sup> The striking effect of acute Morpholino-mediated knock-down on *Xenopus* notochord differentiation may therefore reflect more faithfully the developmental function of ANGPTL4.



### **7.3.2 ANGPTL4 inhibits Wnt/ $\beta$ -catenin signaling at the receptor level.**

The above results strongly suggest that ANGPTL4 regulates mesodermal differentiation primarily by inhibiting Wnt/ $\beta$ -catenin signaling. Indeed, it was shown previously that ectopic over-expression of the prototypical Wnt antagonist *DKK1* in zebrafish is sufficient to induce notochord enlargement.<sup>272</sup> Similarly, it has been suggested that Frzb antagonizes WNT8 during *Xenopus* development, thereby ensuring notochord differentiation.<sup>254</sup> Consistent with the concept that ANGPTL4 acts as a secreted Wnt inhibitor, knock-down of *ANGPTL4* *in vitro* strongly induced Wnt signaling, and epistasis assays revealed that ANGPTL4 inhibits Wnt signaling at the receptor level. This function is unique for ANGPTL4, as no such effect was observed for ANGPTL3 and ANGPTL5.

### **7.3.3 ANGPTL4 is a mediator of TGF $\beta$ signaling and regulates mesoderm formation in *Xenopus***

Besides being a negative regulator of Wnt/ $\beta$ -catenin signaling, ANGPTL4 is also involved in the regulation of TGF $\beta$  signaling. TGF $\beta$  reporter activity was strongly enhanced in cells treated with *ANGPTL4* siRNA. This effect is likely mediated by the SMAD2/3 pathway, as *ANGPTL4* depletion increased SMAD2 phosphorylation. In contrast to the inhibitory effect of ANGPTL4 *in vitro*, *in vivo* results revealed a requirement of ANGPTL4 for Activin/Xnr1 induced *Xbra* expression. Based on preliminary studies, it appears that different domains of ANGPTL4 exert these divergent effects. In *Xenopus* embryos the N-terminal part of ANGPTL4 is sufficient to induce spina bifida formation. It is possible, that TGF $\beta$  signaling in mammalian cell lines requires a different part of ANGPTL4. Comparatively, the *in vitro* experiments were performed using TGF $\beta$ 1, whereas the *Xenopus* effects were investigated using Activin and Nodal ligands. Therefore another explanation could be that different ligands of the TGF $\beta$  family mediate the interaction of ANGPTL4 with different effector molecules, which induce distinct effects. Besides the effect on TGF $\beta$  signaling in *Xenopus*, ANGPTL4 seems to be required for mesoderm formation, as depletion reduced mesodermal markers. In the process of mesoderm development several pathways converge to allow formation of the three germ layers (reviewed in Kimelman *et al.*, 1992<sup>273</sup>). Whereas ANGPTL4 has no effect on FGF signaling, it strongly affects TGF $\beta$  signaling. Therefore in *Xenopus* development the

requirement of ANGPTL4 for the process of mesoderm formation is likely mediated by the positive effect of ANGPTL4 on TGF $\beta$  signaling.

Taken together, the data indicate that ANGPTL4 is not only an inhibitor of Wnt/ $\beta$ -catenin, but also a mediator of TGF $\beta$  signaling *in vitro* and in *Xenopus*.

#### 7.3.4 ANGPTL4 binds all four syndecans

In search for an ANGPTL4 receptor, which mediates the effects of ANGPTL4 on Wnt/ $\beta$ -catenin and TGF $\beta$  signaling, syndecans were identified as a putative candidate. Besides syndecans, the intracellular tight junction protein ZO-2, as well as the extracellular matrix components fibronectin and galectin 8 were detected by mass spectrometry. Binding of ANGPTL4 to these proteins is likely indirect via integrins or syndecans, for which interaction has already been described.<sup>274-276</sup>

By independent lines of evidence, it was shown that syndecans act as receptors or co-receptors for ANGPTL4, including co-IP, cell surface binding, ELISA-based binding assays and functional cooperation. Syndecans comprise a family of four transmembrane proteoglycans, which act as co-receptors for different growth factors and are involved in cell proliferation, differentiation, adhesion, and migration.<sup>277-279</sup>

Recent studies suggest a regulatory function of SDC2 in TGF $\beta$  signaling. It was shown that SDC2 impacts TGF $\beta$  signaling via direct binding of the TGF $\beta$  ligand and regulation of receptor expression.<sup>280,281</sup> However, another report observed an inhibitory effect of SDC2 on TGF $\beta$  signaling in alveolar epithelial cells. In this background SDC2 promotes caveolin-dependent internalization of ligand (TGF $\beta$ ) and receptor (TGF $\beta$ RI), thereby inhibiting TGF $\beta$  signaling.<sup>282</sup> These different effects of SDC2 on TGF $\beta$  signaling are in line with a biphasic effect of SDC1 on BMP signaling in *Xenopus* embryos.<sup>283</sup> Based on the regulatory role of syndecan in TGF $\beta$  signaling, it is possible that ANGPTL4 exerts its effect on TGF $\beta$  signaling via syndecans. The requirement of the N-terminal domain of ANGPTL4 for spina bifida induction in *Xenopus* is in line with this, as interaction of ANGPTL4 with HSPG is mediated by a binding motif in the N-terminal domain of the protein.<sup>215</sup> Furthermore the contradictory results of SDC2 on TGF $\beta$  signaling could provide an explanation for the different effects of ANGPTL4, observed *in vitro* and *in vivo*. The effect

of ANGPTL4 on TGF $\beta$  signaling via syndecans could be tissue specific or dependent on the respective microenvironment.

Syndecans also function in Wnt signaling. SDC1, for example, was described as a modulator of Wnt signaling and is required for WNT1 induced mammary tumorigenesis in mice.<sup>266</sup> Furthermore SDC4 was shown to act as a co-receptor for RSPO3 in Wnt/PCP signaling in *Xenopus*, and mediates clathrin-dependent internalization of RSPO3.<sup>52,78</sup> Additionally, it has been shown that besides noncanonical Wnt signaling, SDC4 also has an effect on canonical Wnt signaling, as it inhibits Wnt/ $\beta$ -catenin signaling in mammalian cell lines and *Xenopus*.<sup>267</sup> Therefore the observed effect of ANGPTL4 on Wnt/ $\beta$ -catenin signaling could be mediated by syndecans. This is further supported by the observation that SDC1 synergizes with ANGPTL4 in Wnt/ $\beta$ -catenin inhibition. These results indicate that ANGPTL4 and syndecans cooperate in Wnt signal inhibition.

In contrast to the observed effect of ANGPTL4 on mesoderm development, SDC4 however, has no effect on *Xbra* expression.<sup>78</sup> This could be due to redundant effects of other SDC members, which could mediate the effect of ANGPTL4 during mesoderm formation. It is also possible that other receptors transmit ANGPTL4 signals in this process. A close examination of ANGPTL4 and syndecans reveals a number of overlapping functions during development. Similar to *ANGPTL4* knockout mice, *SDC1*, *SDC3* or *SDC4* deficient mice are viable, fertile and have no overt phenotypic abnormalities.<sup>228,266,284-286</sup> On the molecular level, however, ANGPTL4 and syndecans are implicated in several overlapping biological mechanisms in vertebrates. For *SDC1* deficient mice a very slow re-epithelization during wound healing was described, similar to *ANGPTL4* knockout mice.<sup>234,287</sup> Furthermore SDC1 plays an important role in regulating inflammation, similar to ANGPTL4.<sup>233,288</sup> Both ANGPTL4 and SDC2 are required for angiogenesis.<sup>245,289</sup> Additionally, *SDC4* deficiency results in delayed wound healing and defective angiogenesis, a process in which ANGPTL4 is also required.<sup>225,234,286</sup> This shows that ANGPTL4 and syndecans are involved in the same biological processes, and suggests that syndecans may mediate the effects of ANGPTL4.

### 7.3.5 Syndecans mediate the internalization of ANGPTL4

A characteristic of syndecans is their ability to induce endocytosis upon ligand binding.<sup>261-264</sup> It was shown that ANGPTL4 is internalized via clathrin-dependent endocytosis, which requires syndecans and sulfation of their GAG chains. This is in accordance with binding of other extracellular proteins.<sup>78,278,279</sup> Similarly, binding of RSPO3 to SDC4 also requires GAGs and induces clathrin-mediated endocytosis.<sup>78</sup>

Whether the internalization of ANGPTL4 is required for its inhibitory effect on Wnt/ $\beta$ -catenin signaling is unknown. In the context of Wnt signaling, internalization of the receptor complex is an essential step in the initiation of signal transduction.<sup>89,106,290</sup> In vertebrates, Wnt ligands induce caveolin-dependent receptor endocytosis, whereas DKK1 promotes clathrin-dependent endocytosis of LRP5/6, leading to inhibition of the pathway.<sup>95,106,107</sup> Because ANGPTL4 acts as an inhibitor of Wnt signaling and is internalized via clathrin-dependent endocytosis, similar to DKK1, it is feasible that its internalization plays an important role with regard to Wnt signaling.

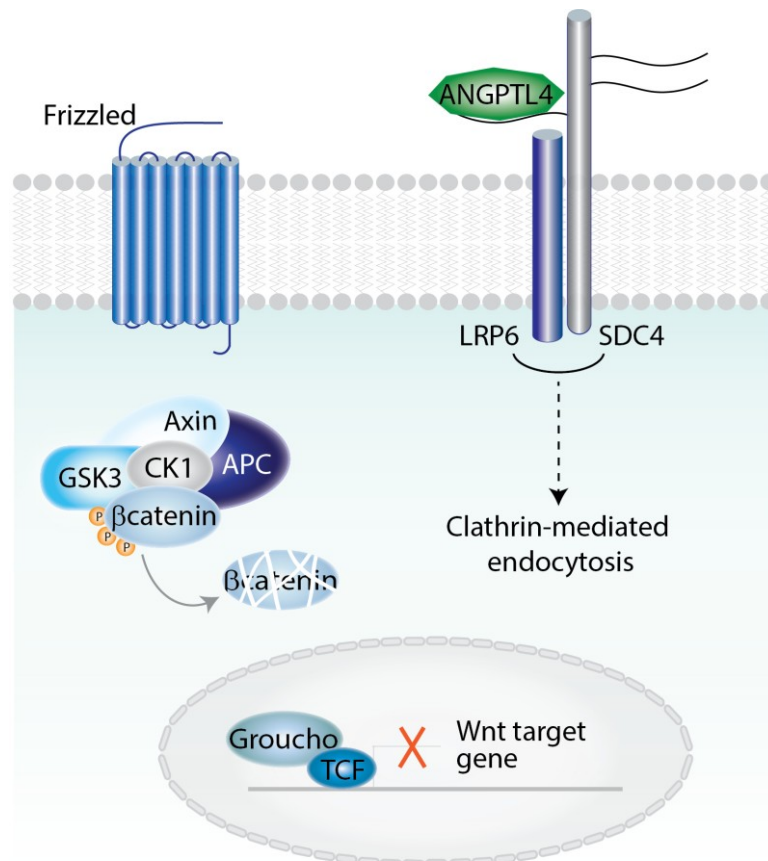
### 7.3.6 ANGPTL4 reduces LRP6 at the plasma membrane level

The similarity of ANGPTL4 internalization to that of DKK1 and RSPO3, and the fact that ANGPTL4 and syndecans synergize in Wnt/ $\beta$ -catenin inhibition, raised the possibility that ANGPTL4 affects the Wnt receptor complex via syndecans. Indeed, in the presence of syndecan, ANGPTL4 interacts with LRP6. Via this interaction ANGPTL4 regulates cell surface levels of LRP6, as shown by gain- and loss-of-function studies. It can be hypothesized that ANGPTL4 removes LRP5/6 from the cell surface via clathrin-dependent endocytosis, thereby attenuating Wnt signaling. Interestingly, it was found that the N-terminal CCD of ANGPTL4 is sufficient to decrease LRP6 membrane levels, whereas the C-terminal FLD is dispensable. As the CCD was already characterized to harbor the binding site for HSPG<sup>215</sup>, it further underlines that syndecans mediate the observed effects of ANGPTL4.

### 7.3.7 Model of ANGPTL4 inhibition of Wnt/ $\beta$ -catenin signaling

Collectively the data shown here support a model, in which ANGPTL4 binds to syndecans, which likely mediate the interaction of ANGPTL4 with LRP6. This interaction results in the membrane clearance of LRP6, likely by clathrin-mediated endocytosis of the receptor complex, which in turn inhibits Wnt/ $\beta$ -catenin signaling (Figure 33). The N-terminal domain of ANGPTL4 is sufficient to affect LRP6 membrane levels.

Clathrin-mediated endocytosis targets membrane proteins to early endosomes, where they can be either recycled or transported to late endosomes and lysosomes for degradation.<sup>95</sup> As also total LRP6 levels are affected by ANGPTL4, it is possible that LRP6 is undergoing lysosomal degradation, after ANGPTL4-induced removal from the plasma membrane. Further investigation is required to proof this hypothesis.



**Figure 33 | Model for the inhibitory effect of ANGPTL4 on Wnt/ $\beta$ -catenin signaling.** ANGPTL4 binds to syndecans via its N-terminal coiled-coil domain. This requires sulfation of the glycosaminoglycan chains of syndecans. Thereby ANGPTL4 associates with LRP6 and likely induces clathrin-mediated endocytosis of this complex. This results in a decrease of LRP6 membrane levels, leading to the inhibition of Wnt/ $\beta$ -catenin signaling.

In general, syndecans do not act as primary receptors but rather as co-receptors.<sup>277</sup> Therefore it is possible that ANGPTL4 interacts with another transmembrane protein, which mediates the interaction with the Wnt receptors. This is supported by the fact that ANGPTL4 has no effect on FGF signaling, a signaling pathway in which syndecans have also been implicated.<sup>264,278,279</sup> The identification of this additional factor could provide an explanation for the specificity of ANGPTL4 for Wnt/ $\beta$ -catenin and TGF $\beta$  signaling.

### 7.3.8 Outlook

This study led to the identification of a novel antagonist of the Wnt/ $\beta$ -catenin signaling pathway. The inhibitory effect of ANGPTL4 is of physiological relevance, as it promotes notochord formation during *Xenopus* development. Because both ANGPTL4 and Wnt signaling are implicated in cancer development and progression, it will be very interesting to see if the anti-angiogenic function of ANGPTL4 is mediated via inhibition of Wnt/ $\beta$ -catenin signaling. In accordance with this hypothesis is the finding that *ANGPTL4* is frequently silenced by methylation in human gastric cancers,<sup>244,291</sup> where Wnt signaling is aberrantly activated.<sup>292</sup> Interestingly, the tumor suppressive effect of ANGPTL4 is mediated by the N-terminal CCD,<sup>244</sup> which is in line with our results, showing that the N-terminal part of ANGPTL4 is sufficient to decrease LRP6 membrane levels.

The finding that syndecans are (co-)receptors for the orphan ligand ANGPTL4 opens new directions for studies of ANGPTL4 functions. Based on the number of overlapping biological roles of ANGPTL4 and syndecans and the implication in cancer and tumor progression, it will be interesting to analyze the functional interaction in these processes.

## 8. Materials and Methods

### 8.1 Equipment and reagents

#### Equipment

Agarose gel chambers (Biozym), Balances (Sartorius, Kern), Blotting apparatus Trans-Blot SD Semi-Dry Transfer Cell (Biorad), Centrifuges (Heraeus), Electroporator (BioRad), Fluorescence microscope (Nikon), Homogenizers (Wheaton), Imaging System LAS3000 (Fujifilm), Incubators (Labotect, Heraeus), Laminar flow hoods (Labotect), LightCycler 480 (Roche), Light Source KL1500 (Schott), Luminometer (Fluoroskan Ascent FL, Thermo), Magnetic stirrer (CAT), Microcentrifuges (Heraeus), Microinjector (Harvard Apparatus), Microwave (Sharp), Multiscan RC plate-reader (Labsystems), Multi-channel pipettes (Transferpette), PAGE minigel chambers (Biorad), PCR thermocyclers (MJ Research, BioRad), pH meter (Sartorius), Power supplies (Biorad), Rotators (CAT, Kisker), Shakers (Thermo, EB), Spectrophotometer NanoDrop 2000 (Thermo), Stereomicroscopes MZ8 (Leica), Stereomicroscope Discovery.V20 (Zeiss), Thermoshaker (Eppendorf), Thermostat cabinet (Aqua Lytic), Ultrapure water purification system (Millipore), UV photodocumentation system (Intas), Vortexer (Scientific industries), Water baths (Julabo, GFL).

#### Chemicals

Acetic Acid (Merck), Acrylamide (Roth), Agarose (Biozym), Ampicillin (Sigma), Biotin (Pierce, Thermo), Blocking Reagent (Roche), BM Purple AP substrate (Roche), Bromophenol blue (Merck, Serva), BSA Grade V (Roche),  $\beta$ -mercaptoethanol (Roth),  $\text{CaCl}_2$  (Merck), CHAPS (Serva), Chloroform (Roth), complete protease inhibitor cocktail (Roche), L-cysteine (Sigma), DMSO (Sigma), dNTPs (Thermo, Fermentas), DTT (Roche, Sigma), Diethylpyrocarbonate (DEPC) (Roth), EDTA (Gerbu), EGTA (Sigma), Ethanol (Sigma), Ethidium bromide (Roth), Fluoromount-G (SouthernBiotech), Freon (Riedel-de Haën), Formaldehyde (J.T. Baker), Formamide (Merck), Formamide deionized (AppliChem), Glycogen (Fermentas), Glycerol (Roth, Sigma), Heparin (MP), human chorionic gonadotropin (HCG) (Sigma, Aska), HCl (Sigma), Hydrogen peroxide (Merck), HEPES (GERBU), Hoechst (Sigma), Iodacetamide (Sigma), Isopropanol (Sigma), KCl (Fluka,

Roth, Sigma), LB agar (Fluka), Maleic acid (Serva), Methanol (Sigma), MgCl<sub>2</sub> (Merck), MgSO<sub>4</sub> (Merck), Monodansylcadaverine (MDC) (Sigma), MOPS (Sigma), NaOH (Fluka), NaCl (Sigma, VWR), NaF (Sigma), Na<sub>2</sub>HPO<sub>4</sub> (Merck), NaH<sub>2</sub>PO<sub>4</sub> (Roth), Na<sub>3</sub>VO<sub>4</sub> (Sigma), NaOAc (Fluka, Merck), Nonidet NP-40 (Sigma), Phenol (Roth), Phenol/Chloroform (Ambion), Paraformaldehyde (PFA) (Merck, Sigma), PMSF (Sigma), Proteinase K (Roche), Random Primers (Invitrogen), Restore Western Blot Stripping Buffer (Thermo), RNA cap structure analog (NEB), RNase-free H<sub>2</sub>O (Qiagen), Rose-Gal (Genaxxon), Roti-Phenol (Phenol/Chloroform/Isoamylalcohol-25/24/1) (Roth), SDS (Roth), Sephadex beads C-25 (GE Healthcare) and G-50 (Sigma), Sodium pyrophosphate (Sigma), Streptavidin (Vector, SA-5000), TEMED (Serva), Trypsin (Sigma), Triton X-100 (Gerbü), Triethanolamine (Sigma), Trizol (Ambion), Tris base (Sigma), Trypan blue (VWR), Tween-20 (Gerbü), Yeast RNA (Boehringer Mannheim).

#### **Agarose beads**

Anti-Flag M2 affinity gel (Sigma, A2220), CH-IgG rabbit beads (17.6 mg/ml, homemade), Heparin-Agarose (Sigma, H6508), Protein A-Agarose (Sigma, P3476), Streptavidin Agarose (Thermo, 20359).

#### **Enzymes, reagents and kits for molecular biology**

4-Dinitrophenylphosphate disodium salt hexahydrate (Sigma, N9389), CIAP (Fermentas, EF0341), Clarity Western ECL substrate (BioRad, 1705060), DNA Orange loading dye (6x) (Thermo, R0631), DTT 0.1 M (Invitrogen, 00147), Dual-Luciferase reporter assay system (Promega, E1960), Gene Ruler DNA ladder 1 kb (Thermo, SM1163), Gene Ruler DNA ladder 100 bp (Thermo, SM0241), LDS sample buffer NuPage (Life Technologies, NP0008), LightCycler 480 Probes master (Roche, 04887301001), MEGAscript *in vitro* transcription kits SP6/T7/T3 (Ambion, AM1330/AM1334/AM1338), Page Ruler Prestained Protein Ladder (Thermo, 26616), PCR buffer (10x) (Life Technologies, N8080129), Poly-L-Lysin (Sigma, P5899), ProSieve QuadColor Protein Marker 4.6-300 kDa (Lonza, 00193837), QIAprep Spin Miniprep Kit (Qiagen, 27106), QIAquick Gel extraction kit (Qiagen, 28706), QIAquick PCR purification kit (Qiagen, 28104), QuantaBlu Fluorogenic Peroxidase substrate (Pierce, 15169), RiboLock RNase Inhibitor (Thermo, EO0381), RiboRuler High Range RNA ladder (Thermo, SM1823), RiboRuler Low Range



RNA ladder (Thermo, SM1833), RNA guard (Thermo, EO0381), RNA loading dye (2x) (Thermo, R0641), RNase A (Thermo, EN0531), RNase-free DNase Set (Qiagen, 79254), SEAP reporter gene assay (Roche, 11779842001), Super signal West Pico Chemiluminescence substrate (Thermo, 34078), Superscript II Reverse Transcriptase (Invitrogen, 18064-014), UPL probes (Roche), TEV protease (Life Technologies, 12575-015).

### **Cell culture reagents and cell lines**

DharmaFECT (Dharmacon), DMEM (Lonza), FCS (Gibco), H1299 cells (American Type Culture Collection (ATCC), CRL-5803), H1703 cells (ATCC, CRL-5889), Hanks Balanced Salt Solution (Hank's BSS) (Sigma, H6648), HEK293T cells (GenHunter, WAK-Chemie, Q401), HepG2 (ATCC, HB-8065), L cells with/out stably transfected with mouse Wnt3a (ATCC, CRL-2647/-2648) L-glutamine (Sigma), Opti-MEM (Gibco), Penicillin-Streptomycin (Lonza), RPMI (Lonza), Sodium pyruvate (Sigma), Trypsin-EDTA (Gibco), X-treme Gene 9 DNA transfection reagent (Roche).

### **Buffers and solutions**

AP substrate solution: 2 M Diethanolamine pH 9.8, 1 mM MgCl<sub>2</sub>, 2 tablets 4-Dinitrophenylphosphate disodium salt hexahydrate (Sigma).

Barth solution (10x): 880 mM NaCl, 10 mM KCl, 24 mM NaHCO<sub>3</sub>, 8.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 3.3 mM Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 4.1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 100 mM HEPES, pH 7.6.

DEPC-H<sub>2</sub>O: 0.01% (v/v) DEPC/ddH<sub>2</sub>O, mixed for 12 h at room temperature and autoclaved.

Bicarbonate buffer: 50 mM NaHCO<sub>3</sub> pH 9.6.

Bleaching solution (for ISH): 5% Formamide, 0.5% SSC (20x), 3% H<sub>2</sub>O<sub>2</sub> (30%) in ddH<sub>2</sub>O (Formamide has to be diluted in H<sub>2</sub>O before adding H<sub>2</sub>O<sub>2</sub>, otherwise an exothermic reaction occurs).

Boehringer Block (10x): 127 g Tris-HCl, 23.6 g Tris Base, 58.44 g NaCl, 50 g Boehringer reagent (Boehringer), add ddH<sub>2</sub>O up to 1 l, adjust pH to 7.5, dissolves when autoclaved and store at -20°C.

dNTP mix: 2 or 5 mM dATP, dCTP, dGTP, dUTP, store at -20 °C.

Ethanol/NaOAc mix: 100 mM sodium acetate in 100% ethanol.

Hybridization buffer (500 ml): 5 g Boehringer Block, 250 ml deionized Formamide, 125 ml SSC (20x), dissolve for 1 h at 65°C, add 50 ml yeast RNA (10 mg/ml), 1 ml Heparin (50 mg/ml), 2.5 ml Tween (20%), 5 ml CHAPS (10%), 5 ml EDTA (0.5 M), DEPC-H<sub>2</sub>O, store at -20°C.

IP lysis buffer: 1% Triton X-100, 2 mM β-mercaptoethanol, 1 mM MgCl<sub>2</sub>, 1x complete protease inhibitor cocktail (Roche), 1x TBS, adjust pH to 7.5.

IP wash buffer 1: 1% Triton X-100, 2 mM β-mercaptoethanol, 1x TBS, adjust pH to 7.5.

IP wash buffer 2: 0.1% Triton X-100, 2 mM β-mercaptoethanol, 1x TBS, adjust pH to 7.5.

Lämmli loading buffer (4x): 5 ml solution C for SDS-PAGE, 2 ml glycerol (100%), 2 ml SDS (20%), 1 ml β-mercaptoethanol (14 M), 200 μl bromophenol blue (0.5%).

Lämmli running buffer (10x): 0.25 M Tris, 1.92 M glycerol, 1% SDS, add up to 1 l deionized water, pH ~8.3.

LB (1x): 10 g tryptone, 5 g yeast, 10 g NaCl.

Maleic acid buffer (MAB): 100 mM maleic acid, 150 mM NaCl, pH 7.5.

Modified Ringer solution (MR): 0.1 M NaCl, 1.8 mM KCl, 2.0 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 5.0 mM HEPES-NaOH, pH 7.6, autoclaved.

MEM buffer (10x): 1 M MOPS pH 7.4, 20 mM EGTA, 10 mM MgSO<sub>4</sub>.

MS222 (1 l): 1.5 g MS222 (Sigma), 1.4 g NaHCO<sub>3</sub>, pH 7.

NP-40 buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1x complete protease inhibitor cocktail (Roche), 1% NP-40, 0.1% SDS, 5 mM β-mercaptoethanol, 5 mM glycerophosphate.

PTW buffer: 1x PBS, 0.1% Tween 20.

Phosphate-buffered saline (PBS, 10x): 1.36 M NaCl, 26.8 mM KCl, 14.7 mM KH<sub>2</sub>PO<sub>4</sub>, 162.9 mM Na<sub>2</sub>HPO<sub>4</sub>, adjusted to 1 l with ddH<sub>2</sub>O, autoclaved.

Ringer solution: 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES pH 7.4.

Saponin buffer: 0.05% Saponin, 1 mM MgCl<sub>2</sub>, 2 mM β-mercaptoethanol, 10 mM NaF, 5 mM glycerophosphate, 0.1 mM PMSF, 1x complete protease inhibitor cocktail (Roche), 1x TBS.

SDS-PAGE solutions: Solution B: 1.5 M Tris, 0.4% SDS in 1 l ddH<sub>2</sub>O pH 8.8; Solution C: 0.5 M Tris, 0.4% SDS in 1 l ddH<sub>2</sub>O pH 6.8.

TBE buffer (10x, for agarose gel-electrophoresis): 540 g Tris Base, 275 g Boric Acid, 200 ml EDTA (0.5 M) pH 8.0, in 5 l ddH<sub>2</sub>O.

TBE buffer (10x, for semidry blotting): 108 g Tris Base, 55 g Boric Acid, 40 ml EDTA (0.5 M), add up to 1 l ddH<sub>2</sub>O, adjust pH to 8.3.

TEV buffer: 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 20 mM Tris pH 8.0.

Tris-buffered saline (TBS, 20x): 1 M Tris pH 7.4, 3 M NaCl, 54 mM KCl, add up to 1 l ddH<sub>2</sub>O, autoclaved.

TBS-T (1x): 1x TBS, 0.05% Tween.

TE buffer: 10 mM Tris-HCl pH 7.4, 1 mM EDTA, autoclaved.

Transferbuffer (10x): 58.2 g Tris Base, 29.3 g Glycin, 200 ml EDTA (0.5 M) pH 8.0, add up to 1 l ddH<sub>2</sub>O (for 1x add 200 ml Methanol/l).

### Antibodies

Mouse anti- $\beta$ -catenin (BD, 610154), Mouse anti-FLAG (Sigma, F-3165), Mouse anti-MAPK (ERK1+ERK2) (Sigma, M8159), Mouse anti-myc (DSHB, 9E10), Mouse anti-SMAD2/3 (BD, 610842), Mouse anti-Tubulin (Sigma, T5168), Mouse anti-V5 (Invitrogen, 46-0705), Rabbit anti-AP (Zymed, 18-0099), Rabbit anti-HRP (Acris, R1107), Rabbit anti-LRP6 (T1479) homemade antibody, Rabbit anti-phospho-LRP6 (Sp1490) (Cell Signaling), Rabbit anti-phospho-LRP6 (Sp1490) homemade antibody, Rabbit anti-phospho-SMAD2 (Ser465/467) (Cell Signaling, 138D4), Rabbit anti-phospho-SMAD3 (Ser423/425) (Cell Signaling, C25A9), Rabbit anti-Transferrin receptor (Cell Signaling, 13113P), Rat anti-HA (Roche, 1867423).

### Plasmids

Human and mouse *ANGPTL4* was received from imaGenes (Table 3).

**Table 3 | Human and mouse ANGPTL4 cDNA clones obtained from imaGenes.**

Gene	Clone ID	IMAGE ID	vector
Human ANGPTL4	IRAU p969A0880D	5088323	pOTB7
Mouse Angptl4	IRAV p968A0472D	5137159	pCMV-SPORT6

*Xenopus angptl4* was cloned by Christine Dolde from st.40 *X. tropicalis* embryos using the following primers: ATGAAGCTGTTACTTGCAAGTATAACT (forward primer), CACAGTAAGGTCTGTATCAACAGG (reverse primer). The cloned *angptl4* was ligated into pCS2<sup>+</sup> vector (EcoRI, BamHI) and pBluescript KS<sup>+</sup> (BamHI, XbaI).

Using the human and mouse ANGPTL4 cDNA clones from imaGenes, different constructs were cloned for the experiments performed in this thesis (Table 4).

**Table 4 | ANGPTL4 constructs cloned for this thesis.** AP, alkaline phosphatase; NT, N-terminal domain of ANGPTL4; CT, C-terminal domain of ANGPTL4; SP, signal peptide for secretion; Prot.A<sub>x2</sub>, two times Protein A-tag; TEV, cleavage site for protease from Tobacco Etch Virus; Str., Strep-tag; HRP, horseradish peroxidase; myc, myc-tag; h, human; m, mouse.

Construct	Insert	Vector	Cloning Sites
hANGPTL4-AP	Full length hANGPTL4	pCDNA3	EcoRI, BglII
hANGPTL4-AP (NT)	N-terminal part of hANGPTL4	pCDNA3	EcoRI, BglII
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-hANGPTL4	Full length hANGPTL4	pCS2 <sup>+</sup>	BspEI, XbaI
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-hANGPTL4 (NT)	N-terminal part of hANGPTL4	pCS2 <sup>+</sup>	BspEI, XbaI
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-hANGPTL4 (CT)	C-terminal part of hANGPTL4	pCS2 <sup>+</sup>	BspEI, XbaI
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-mAngptl4	Full length mAngptl4	pCS2 <sup>+</sup>	BspEI, XbaI
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-mAngptl4 (NT)	N-terminal part of mAngptl4	pCS2 <sup>+</sup>	BspEI, XbaI
SP-Prot.A <sub>x2</sub> -TEV-Str.-HRP-mAngptl4 (CT)	C-terminal part of mAngptl4	pCS2 <sup>+</sup>	BspEI, XbaI
myc-mAngptl4	Full length mAngptl4	pCS2 <sup>+</sup>	BglII, XhoI
myc-mAngptl4 (NT)	N-terminal part of mAngptl4	pCS2 <sup>+</sup>	BglII, XhoI
myc-mAngptl4 (CT)	C-terminal part of mAngptl4	pCS2 <sup>+</sup>	BglII, XhoI

## 8.2 Molecular Biology

General molecular biology methods, like preparation of XL1-blue competent cells, transformation of plasmid DNA, amplification and quantification of DNA and RNA samples, PCR amplification and DNA restriction digest were performed as described.<sup>293</sup> For isolation of plasmid DNA from bacteria, isolation of RNA from cells, and gel extraction of PCR fragments Kits from Qiagen were used, according to manufacturer's instructions. DNA oligonucleotides were synthesized by Sigma Aldrich. DNA samples were sequenced by GATC Biotech.

### Bioinformatic tools

Genomic sequences were obtained from the public databases NCBI ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)) and Ensembl Genome Browser ([www.ensembl.org](http://www.ensembl.org)). Sequence searches and comparisons were performed with BLAST ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). DNA restriction and translation maps were analyzed with the service of DKFZ-HUSAR ([www.genome.dkfz-heidelberg.de/husar](http://www.genome.dkfz-heidelberg.de/husar)). Real time PCR primers and corresponding UPL probes were designed using the Universal ProbeLibrary Assay Design Center from Roche ([www.roche-applied-science.com/](http://www.roche-applied-science.com/)) choosing intron spanning assays. For literature searches PubMed was used ([www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)).

### RNA isolation

RNA from mammalian cells was isolated using the RNeasy Mini Kit (Qiagen), according to manufacturer's instructions. The RNA of *Xenopus* embryos/explants was isolated with Trizol reagent (Ambion). Samples were homogenized in 1 ml Trizol, and 200 µl chloroform was added. Samples were vortexed for 30 sec and then centrifuged at 13.000 rpm for 15 min and 4°C. The upper aqueous phase was transferred to a fresh tube, mixed 1:1 with isopropanol and precipitated at -80°C for at least 1 h, or overnight in the case of explants. RNA was pelleted by centrifugation at 13.000 rpm for 30 min. RNA pellets were washed with 70% ethanol, again centrifuged, briefly dried and dissolved in RNase-free H<sub>2</sub>O. RNA concentration was quantified by measuring the absorbance at 260 nm in a spectrophotometer.

**cDNA synthesis**

cDNA was synthesized from total RNA using SuperScript II reverse transcriptase. Up to 1 µg of total RNA was mixed with 1 µl random primers (0.5 µg/µl) and 2 µl dNTPs (5 mM) in a total volume of 12 µl and heated to 65°C for 5 min. Samples were cooled on ice and 2 µl First strand buffer (5x), 1 µl DTT (0.1 M) and 1 µl RNase inhibitor were added. Samples were mixed and 0.5 µl SuperScript II reverse transcriptase (100 U) was added. Reverse transcription was carried out in a PCR-cycler at 25°C for 5 min, and 42°C for 90 min. The reaction was stopped by heat-inactivation at 70°C for 15 min. The cDNA samples were diluted 1:5 with RNase-free H<sub>2</sub>O and used for quantification of gene expression by qPCR.

**Quantitative RT-PCR (qPCR)**

Quantitative real-time PCR was used to examine and compare gene expression levels using cDNA templates. The measurements were performed with the Roche Lightcycler 480, using UPL mono-color hydrolysis probes. Primer sequences and UPL probes, which were used in this thesis are indicated in Table 5.

For qPCR 11 µl reactions were used, containing 5.5 µl PROBES master (2x) (Roche), 0.5 µl primer and probes mix (11 µM of each primer, 2.2 µM UPL probe), and 5 µl cDNA template. For the detection of mono-color hydrolysis probes, a program, consisting of a pre-incubation step, 55 amplification cycles, and a cooling step, was used. The annealing temperature during the amplification cycles was set to 60°C (1 sec hold). All samples were analyzed in duplicates. Relative expression levels for marker gene analysis were obtained by normalizing the expression to the housekeeping gene *odc* (*Xenopus*) or *GAPDH* (human). For each gene a standard curve was generated by usage of a dilution series of the respective cDNA.

**Table 5| Primer and UPL-probes used for qPCR analysis of *Xenopus* and human genes.** Primers were ordered from Sigma and UPL probes from Roche.

Target	Primer sequences	UPL probe
<i>Xenopus ANGPTL4</i>	forward: TCTGATTTTAACTGCGCCAAA reverse: ATGGCCGCATGAGCTAAA	UPL probe #88
<i>Xenopus brachyury</i>	forward: TTCAAGGAGCTACCAATGA reverse: CGACACGCTCACCTTTAGAA	UPL probe #97
<i>Xenopus chordin</i>	forward: AGGAGCCCCTCCAATCTAAG reverse: GATGCCATGAATCCTCCAGA	UPL probe #31
<i>Xenopus gsc</i>	forward: GAAACCAAGTACCCAGACGTG reverse: CCTCCACTTTGCTCTTCGAT	UPL probe #126
<i>Xenopus myf5</i>	forward: AGCTGCTCAGATGGCATGA reverse: AGCTGCTGTTCTCCAGAC	UPL probe #66
<i>Xenopus myoD</i>	forward: GGTCCAAGTCTCCGATG reverse: CTGCTGTCGTAGCTGTTCTT	UPL probe #1
<i>Xenopus noggin</i>	forward: TGGGGAGTTGGATCTCCTT reverse: TTTGATTTCTGCTGGCATTG	UPL probe #29
<i>Xenopus odc</i>	forward: TTTGGTGCCACCCTTAAAC reverse: CCACTGCCAACATGGAAAC	UPL probe #50
<i>Xenopus siamois</i>	forward: TTGACCCCTAGTCAACAGC reverse: ACCAGCGCCTCTTACATT	UPL probe #101
<i>Xenopus Xnot2</i>	forward: ACAACAGCAGCCAATGAGG reverse: GGCAATGGGAGTAGGGTAAAC	UPL probe #96
<i>Xenopus Xnr3</i>	forward: CCAAAGCTTCATCGCTAAAAG reverse: AAAAGAAGGGAGGCAAATACG	UPL probe #102
Human <i>ANGPTL4</i>	forward: GTTGACCCGGCTCACAAT reverse: TGGCGCCTCTGAATTACTG	UPL probe #75
Human <i>AXIN2</i>	forward: CCACACCCTTCTCCAATCC reverse: TGCCAGTTTTCTTTGGCTCTT	UPL probe #36
Human <i>GAPDH</i>	forward: GCATCCTGGGCTACACTGAG reverse: AGGTGGAGGAGTGGGTGTC	UPL probe #82

### 8.3 Cell culture and transfection

#### Cell lines and culture condition

HEK293T and HepG2 cell lines were maintained in DMEM, 10% FCS, 1% penicillin/streptomycin (10.000 U/ml), and 1% L-glutamine (200 mM) at 37°C in a humidified atmosphere with 10% CO<sub>2</sub>. H1703 and H1299 cell lines were grown in RPMI, 10% FCS, 1% penicillin/streptomycin (50 µg/ml), 1% L-glutamine (200 mM) and 1% sodium pyruvate (11 mg/ml) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

#### Preparation of the WNT3A conditioned medium

WNT3A conditioned medium was produced from mouse L-cells stably transfected with mouse WNT3A (ATCC, CRL-2647) and the control conditioned medium was received from non-transfected L-cells (ATCC, CRL-2648).<sup>294</sup> To obtain conditioned medium, cells were split 1:10 and grown for two to three days until confluence. Cells were supplemented with fresh medium and 2 days later conditioned medium was harvested. The medium was centrifuged at 2500 rpm for 5 min at 4°C, to remove residual cells, and stored in glass bottles at 4°C.

#### Preparation of ANGPTL4, R-spondin and DKK conditioned medium

HEK293T cells were seeded in 15 cm-dishes one day before transfection that they reached 80% confluence at the following day. For transfection 10 µg of the respective Plasmid-DNA was used. The cells were transfected using X-treme Gene9 (Roche) transfection reagent according to manufacturer's instructions. On day after transfection medium was changed and after 48 h - 72 h medium was harvested. The conditioned medium was centrifuged at 2500 rpm for 5 min and 4°C. The medium was stored in glass bottles at 4°C. In total 3 harvests of conditioned medium were collected.

#### Transfection of DNA

For DNA transfection, cells were seeded one day before. Transfection of DNA was carried out using X-treme Gene9 (Roche) according to the manufacture's instruction. The cells were harvested for analysis after one to three days.

For cell surface binding assays, HEK293T cells were seeded one day before transfection on Poly-L-Lysine-treated 24-well plates, that they reached 50% confluence at the day of



transfection. For immunofluorescence, cells were seeded one day before transfection on glass coverslips in 24-well plates. On day of transfection cells reached 60-70% confluence.

### Transfection of siRNA

Cells were transfected with siRNA using Dharmafect (Dharmacon) transfection reagent according to manufacturers' instruction. In general, cells were cultured for two to three days for RNAi effect to occur.

### Luciferase reporter assay

Luciferase assays were carried out in 96-well plates in triplicates. Cells were transfected with different constructs, including Firefly (TOPFlash reporter plasmid, which contains TCF/LEF binding sites in the promoter driving the expression of a Firefly luciferase construct) and Renilla luciferases. For the transfection of one 96-well the following DNA concentrations were used: 10 ng TOPFlash, 5 ng pTK-Renilla, 10 ng *mWnt1*, 2 ng *mFzd8*, 12 ng *hLRP6*, 0.2 ng *Xenopus*  $\beta$ -catenin, 40 ng *hDVL*. pCS2<sup>+</sup> DNA was used to adjust total DNA amount to 100 ng per well of a 96-well plate.

For monitoring TGF $\beta$ , BMP and FGF signaling different luciferase reporters were used (Table 6).

**Table 6 | Luciferase reporter constructs used to monitor TGF $\beta$ , BMP and FGF signaling.**

Signaling pathway	Luciferase reporter construct	Concentration per well	Reference
TGF $\beta$ /SMAD2 signaling	ARE-luc reporter, FAST1	10 ng, 1 ng	295
BMP/SMAD1 signaling	BREx4-luc reporter	10 ng	296
FGF signaling	Gal-Elk/Gal-luc	2 ng / 10 ng	297,298

After 24 to 48 hours of transfection, cells were lysed in 50  $\mu$ l passive lysis buffer (1x, Promega) for 15 min at RT with continuous shaking. 15  $\mu$ l of the lysates was transferred to 96-well white Microtiter Plates (Thermo Electron Corporation). The dual Luciferase Assay Kit (Promega) was used to measure Firefly and Renilla luciferase activities using a Fluoroskan Ascent FL luminometer (Labsystems) according to manufacturer's instruction. The activity of Firefly luciferase was normalized to Renilla luciferase activity.

#### 8.4 *Xenopus* methods

*In vitro* fertilization, culturing of embryos, staging and microinjection of *X. laevis* and *X. tropicalis* embryos were generally carried out as described.<sup>299-302</sup> Morpholinos were ordered from Gene Tools.

##### Priming, fertilization and microinjection

*X. laevis* females were injected with 600 U of human chorionic gonadotropin (HCG, Sigma, Aska) to induce ovulation. *X. tropicalis* females were pre-primed with 10 U of HCG in the evening before the experiment. On the next morning, females were injected with 200 U of HCG. About 4 h later females started laying eggs, which were *in vitro* fertilized by mixing them with a piece of testis minced in 1x Ringer's solution. After 3 min, eggs were covered with 0.1x Barth solution to promote sperm activation. After 20-25 min, embryos were de-jellied in 2% cysteine in dH<sub>2</sub>O, pH 7.6. Embryos were washed three times in 0.1x Barth solution followed by 2 washes and cultivation in 1/9x Modified Ringer. Microinjections were performed in 1% agarose (dissolved in 1/9x Modified Ringer) dishes containing 1/9x Modified Ringer and 2% Ficoll 400, using calibrated glass capillary needles. Embryos were injected at 1- to 8-cell stage with a total volume of 5 to 10 nl per embryo. After injection embryos were cultured in 1/18x Modified Ringer in plastic dishes at temperatures between 18°C and 25°C.

##### Morpholino injections

Morpholinos were designed according to the general guidelines suggested by Gene Tools, LLC. All obtained Mos were dissolved in RNase-free H<sub>2</sub>O to a final concentration of 20 µg/µl and stored at RT. Mos, which were used in this thesis are indicated in Table 7. Before injection, Mos were heated up to 70°C for 3 min, chilled on ice for 5 min and then kept at RT during injection.

**Table 7 | Morpholinos with their respective sequences used in this thesis.** Morpholinos were ordered from Gene Tools and concentration was adjusted to 10 µg/µl in RNase-free H<sub>2</sub>O.

Gene	Morpholino sequence	Target site	Reference
<i>LGR4</i> Mo1	CAACAGCTTGACGGTCCGACACCT	5'UTR	169
<i>LGR4</i> Mo2	CACAACCTGCACTTTATTTGGCCGC	5'UTR	169
<i>LGR4</i> Mo3	GCCATACTTACAGCGAGTGGGTGAA	splice site	169
<i>LGR5</i> Mo1	AGGTGTCCATGGTGCCGATCAGATC	ATG	169
<i>LGR5</i> Mo2	CGCTGCTCTAATGGTGACGGCTAAA	5'UTR	169
<i>LGR5</i> Mo3	GCTTGAAAGGTCCTAGGGAGAAAAG	splice site	169
<i>RSPO3</i> Mo	ATGCAATTGCGACTGCTTTCTCTGT	ATG	86
<i>RAB8B</i> Mo1	GGTAGTCGTAAGTCTTCGCCATCTT	ATG	197
<i>RAB8B</i> Mo2	AAGTCTTCGCCATCTTTAGTCCTCC	ATG	197
<i>LRP6</i> Mo	CCCCGGCTTCTCCGCTCCGACCCCT	5'UTR	303
<i>ANGPTL4</i> Mo1	TTTATCTGACCTGAAAGGTGTTTGG	splice site	-
<i>ANGPTL4</i> Mo2	TGTCGGCCACTCACTTGAAACAATA	splice site	-

### Explants

Explants were generally performed as described.<sup>304</sup> In brief, excision of explants was performed in dishes covered with a layer of 1% agarose (dissolved in 0.5x Barth) and 0.5x Barth solution. The vitelline membrane of embryos was removed and explants were cut using two watchman forceps (Dumont number 5). Explants were cultured in agarose-coated dishes in 0.5x Barth until the desired stage.

### Luciferase reporter assay in *Xenopus* embryos

For Wnt reporter assays in *Xenopus* embryos 80 pg TOPFlash DNA, containing three copies of the TCF-binding site, together with 25 pg pRLTK (Renilla) DNA for normalization, was injected into *Xenopus* embryos together with Mos and mRNAs. At the desired stage, embryos were collected and lysed in 1x passive lysis buffer (6.7 µl lysis buffer/embryo for *X. tropicalis* and 15 µl lysis buffer/embryo for *X. laevis*). Lysates were vortexed 10 min and centrifuged at 13,000 rpm for 10 min at 4°C to remove yolk. Firefly and Renilla luciferase reporter activity was measured in triplicates using the dual

luciferase assay system (Promega) and a Fluoroskan Ascent FL luminometer (Labsystems). The activity of Firefly luciferase was normalized to Renilla luciferase activity.

### Preparation of mRNA for embryo injections

For mRNA synthesis linearized DNA template was used. The synthesis was performed using the MEGAscript *in vitro* transcription kits from Ambion. RNA cap structure analog was added to increase stability and translation efficiency of the mRNA. Since free cap analog is a potent translation inhibitor and toxic to cells, mRNA was purified after synthesis.

#### *Linearization of DNA template*

Plasmid DNA (5 µg) was linearized by restriction digest for 2 h at 37°C. After linearization the DNA was purified by phenol-chloroform extraction. The upper phase (100 µl) was precipitated with 200 µl ethanol (100%), 10 µl NaOAc (3 M) and 2 µl glycogen (4 mg/ml) at -80°C for at least 1 h. Precipitate was pelleted at 13.000 rpm for 20 min, washed with 70% ethanol, dried and resuspended in 10 µl ddH<sub>2</sub>O.

#### *Synthesis of mRNA*

For mRNA synthesis 1 µg of linearized DNA was used for a 20 µl reaction:

2 µl	10x buffer (Ambion MEGAscript kit)
2 µl	ATP solution (75 mM for T3/T7, 50 mM for SP6)
2 µl	CTP solution (75 mM for T3/T7, 50 mM for SP6)
2 µl	UTP solution (75 mM for T3/T7, 50 mM for SP6)
0.4 µl	GTP solution (75 mM for T3/T7, 50 mM for SP6)
3.75 µl	RNA cap structure analog (40 mM)
2 µl	T7 / T3 / SP6 RNA polymerase (Ambion MEGAscript kit)
2 µl	DNA template (1 µg)
3.85 µl	RNase-free H <sub>2</sub> O (Qiagen)
<hr/>	
20 µl	

Samples were incubated at 37°C for 2 h, and subsequently treated with 1 µl DNase I (RNase free, Roche) at 37°C for 15 min. After the synthesis 10 volume-% of EDTA (0.5 M) was added.

*Purification of RNA transcript*

For the purification of the synthesized mRNA self-made columns were used. Tips (200  $\mu$ l) were filled with glass wool, and 300  $\mu$ l G50-sephadex beads and 100  $\mu$ l C25-sephadex beads in G+E buffer were added. Columns were washed two times with 300  $\mu$ l G+E buffer at 1500 rpm and 4°C. The mRNA was applied to the columns and centrifuged for 2 min at 1500 rpm and 4°C. Concentration was determined photometrically and quality of the mRNA was analyzed on an agarose gel.

**DIG-labeled RNA probes for *in situ* hybridization**

Synthesis of DIG-labeled RNA probes was performed using linearized template DNA, RNA polymerase from the *in vitro* transcription kit from Ambion (Ambion MEGAscript Kit), and DIG-labeled dNTPs. Linearization of Plasmid-DNA and purification was conducted as described before for mRNA synthesis.

For a 20  $\mu$ l reaction volume 1  $\mu$ g of linearized template DNA was mixed with the following components:

2 $\mu$ l	10x buffer (Ambion MEGAscript kit)
4 $\mu$ l	DIG-labeled dNTPs (Roche)
2 $\mu$ l	T7 / T3 / SP6 RNA polymerase (Ambion MEGAscript kit)
3 $\mu$ l	DNA template (1 $\mu$ g)
9 $\mu$ l	RNase-free H <sub>2</sub> O (Qiagen)
<hr/>	
20 $\mu$ l	

DIG-labeled RNA probes were synthesized for 2 h at 37°C. After addition of 1  $\mu$ l DNase I (Ambion MEGAscript kit) for 15 min at 37°C the reaction was stopped by adding 10 volume-% of EDTA (0.5 M). The purification was performed as described for the mRNA transcript but only G50-sephadex beads were used. The concentration was determined photometrically and quality of the DIG-labeled RNA probe was analyzed on an agarose gel.

### **Lineage tracing in *Xenopus* embryos**

For lineage tracing experiments in *Xenopus* embryos, *lacZ* mRNA (200 pg per half of the embryo) was unilaterally co-injected and  $\beta$ -galactosidase staining was performed as described,<sup>302</sup> using Rose-Gal substrate. After staining of the embryos the protocol for *in situ* hybridization was followed.

### **Whole mount *in situ* hybridization (WMISH)**

WMISH of *Xenopus* embryos was carried out as described in the following.

#### *Preparation of embryos (Day 0)*

Embryos are collected at the desired stage in 5 ml glass vials and fixed in freshly prepared MEMFA (1x MEM, 3.7% formaldehyde, in DEPC-H<sub>2</sub>O) at RT, for 1 h. After fixation embryos were dehydrated using different dilutions of methanol in DEPC-H<sub>2</sub>O (25%, 50%, 75% and four times 100%) for 5 min, each on a roller. Dehydrated embryos were stored in 100% methanol at -20°C until hybridization.

#### *Hybridization (Day 1)*

Washes and incubations were performed in 3 ml for 5 min, if not stated otherwise. Fixed embryos were rehydrated through a reverse series of methanol in DEPC-H<sub>2</sub>O (100%, 75%, 50%, 25%), and then washed four times with PTW buffer. Embryos were treated with proteinase K (10  $\mu$ g/ml in PTW) to increase the permeability of the tissue for hybridization probes and antibodies. Embryos of all stages were treated with proteinase K for 7 min. Vials were not shaken or rolled during the incubation. To stop the proteinase K digest, embryos were washed three times with PTW, followed by two washes in 0.1 M triethanolamine. To neutralize positive charges, which would otherwise bind nucleic acids by ionic bonding, resulting in high background staining, acetic anhydride treatment was included. Embryos were incubated in 3 ml triethanolamine with 7.5  $\mu$ l acetic anhydride for 5 min on a roller. Additional 7.5  $\mu$ l of acetic anhydride was added and embryos were rolled again for 5 min. Embryos were washed three times in PTW for 5 min. Embryos were re-fixed for 20 min in freshly prepared 4% formaldehyde in PTW. Embryos were rinsed five times in PTW. For pre-hybridization, all but 1 ml of PTW was removed and 250  $\mu$ l hybridization buffer was added. After embryos have settled, the buffer was replaced by 500  $\mu$ l hybridization buffer. Again embryos

were allowed to settle, before hybridization mix was replaced. Embryos were incubated in hybridization buffer for 30 min at 65°C with gentle shaking in a water bath. Hybridization buffer was replaced and embryos were pre-hybridized for 6 h with gentle shaking in a water bath. At this point, embryos were either processed directly or stored at -20°C.

Pre-hybridized embryos were incubated at 65°C in a water bath for at least 10 min. DIG-labeled RNA probes were diluted in hybridization buffer to a concentration of 200-750 ng/ml for *angptl4*, *myf5*, *muscle actin*, *collagen* and *chordin*. The RNA probes were denatured by heating up for 3 min at 95°C. Then probes were immediately cooled down on ice for 5 min and afterwards heated to 70°C for 10 min. The hybridization buffer of the embryos was replaced by 500 µl of the respective probe. Hybridization was carried out over night at 60°C in a water bath with gentle rocking. Probe dilutions were recovered and re-used, resulting in less background after several rounds of hybridization.

Solutions for washing steps were pre-warmed at 65°C. RNA probes were replaced by 500 µl hybridization buffer and after 10 min, 500 µl of 2x SSC was added to the embryos and incubated for 20 min. Embryos were washed three times 20 min in 2x SSC. Embryos were treated with 10 µg/ml RNase A for 30 min at 37°C. RNase was removed by washing of the embryos with 2x SSC for 10 min at RT, followed by two washes for 30 min in 0.2x SSC at 65°C. Embryos were incubated in freshly prepared MAB on a roller for two times 15 min at RT. Embryos were washed for 15 min in 2% Boehringer Block reagent in MAB, followed by blocking for 1 h in 2% Boehringer Block reagent and 10% goat serum in MAB rolling at RT. The blocking solution was replaced by a 1:5.000 dilution of anti-Digoxigenin-AP Fab fragments (Roche) in 2% Boehringer Blocking reagent and 10% goat serum in MAB. After 4 h with gentle rocking at RT, embryos were washed 2x with MAB for 30 min. The washing was continued over night at 4°C on a roller. On the following day embryos were again washed eight times 10 min at RT, following two times 5 min in water.

*Color reaction*

Water was replaced with BM Purple AP substrate (Boehringer Mannheim) and staining was proceeded in the dark with gentle shaking. Staining was analyzed regularly. If staining had to be proceeded for several days, the staining solution was replaced every morning and evening and incubation occurred at 4°C at night. After staining reached the desired intensity, embryos were washed once in water for 5 min, followed by 100% methanol for 2-3 min and 50% methanol for 5 min, to remove unspecific background staining. Methanol was removed by washing again with water and embryos were stored in 1x MEMFA.

*Bleaching of Xenopus embryos*

*Xenopus* embryos were bleached to remove the natural pigmentation, as this might mask the hybridization signals. Embryos, stored in MEMFA, were washed once in 1x SSC for 5-10 min at RT. The washing solution was replaced by bleaching solution and the glass vials were rolled in the cold room (4°C) under direct light. The incubation at 4°C prevents the embryos from turning black during the exothermic reaction. Embryos were incubated until pigmentation was removed (approximately 1-2 h). Embryos were rinsed two times with PBS and fixed and stored in 1x MEMFA until pictures were taken.



## 8.5 Biochemical methods

### Membrane and cytosolic cell extraction

The Western blots for LPR6 and  $\beta$ -catenin were performed using membrane and cytosolic extracts, respectively. Medium of cells was removed and 1 ml ice cold Hank's BSS was added. Cells were scraped from plate and centrifuged at 1500 rpm and 4°C for 5 min. Cell pellets were stored at -80°C until extraction was performed.

The cell pellets were resuspended in 50-100  $\mu$ l Saponin buffer, and incubated on ice for 30 min. After centrifugation at 13.500 rpm and 4°C for 5 min, supernatant containing the cytosolic fraction was mixed with Lämmli loading buffer, boiled at 95°C for 3 min and stored at -20°C until Western blot analysis.

The pellet, containing the membrane fraction, was resuspended in 50  $\mu$ l NP-40 Lysis buffer and after 30 min incubation on ice, samples were centrifuged at 10.500 rpm and 4°C for 3 min. The supernatant was mixed with LDS sample buffer (4x) and 10 mM DTT, heated up to 70°C for 10 min and samples were stored at -20°C until Western blot analysis.

### SDS-PAGE and Western blot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed according to standard protocols.<sup>293</sup> The signals were detected with the SuperSignal® West Pico Chemiluminescent substrate (Pierce) or for weak signals with the Clarity Western ECL substrate (BioRad). Chemiluminescence was detected with the luminescent image analyzer LAS3000 (Fujifilm).

### AP activity assay

For the AP-tagged constructs, produced as conditioned medium, the AP activity was determined. As a reference for the AP activity, a dilution series of calf intestine alkaline phosphatase (CIAP, 1000 U/ml) (Fermentas) was prepared in DMEM. The measurement was performed at 405 nm in 96-well plates using 50  $\mu$ l AP substrate solution per well. After addition of 1  $\mu$ l conditioned medium or different dilutions of CIAP, the absorption at 405 nm was measured. The CIAP standard curve was used to determine the AP activity of the different AP-tagged constructs.

**HRP activity assay**

The HRP activity of the HRP-tagged constructs, produced as conditioned medium, was measured using the QuantaBlu Fluorogenic peroxidase substrate kit (Pierce). The measurement was performed according to manufacturer's instructions. For one well of a 96-well plate 100  $\mu$ l substrate solution (9x QuantaBlu substrate solution, 1x QuantaBlu stable peroxide solution) was mixed with 1  $\mu$ l of the conditioned medium containing the HRP-tagged protein.

**Tyramide signal amplification of HRP-tagged proteins**

HepG2 cells, seeded on glass cover slips, were incubated for 40 min with conditioned medium containing 50 U/ $\mu$ l of the respective HRP-tagged protein. Afterwards cells were washed three times with ice cold Hank's BSS and fixed with 0.5 mM DSP in Hank's BSS with 10 mM HEPES pH 7.2 for 15 min on ice and additional 30 min at RT. Fixation solution was removed and cells were permeabilized with 0.1% saponin in TSA buffer (100 mM Tris, 10 mM imidazole pH 8.8) for 15 min at RT. The TSA reaction was performed for 30 min in the dark in a humidified chamber using 3  $\mu$ M Rhodamine-tyramide and 0.003% H<sub>2</sub>O<sub>2</sub> in TSA buffer. After staining of the HRP-tagged proteins, cells were washed three times 10 min at RT with PBS containing 0.1% saponin. In the second wash step a 1:10.000 dilution of Hoechst was added to stain nuclear DNA of the cells. Cover slips were briefly rinsed with ddH<sub>2</sub>O before mounted with the Fluoromount-G (SouthernBiotech) on a glass slide (Roth).

The treatment of cells with different endocytic inhibitors and siClathrin or siCaveolin was performed as described previously.<sup>169</sup>

**Cell surface binding assay**

For cell surface binding assays, HEK293T cells were seeded on Poly-L-Lysin treated plates. For this purpose Poly-L-Lysin was diluted 1:20 in ddH<sub>2</sub>O and 1 ml was added to each well of a 24-well plate for 1 h at RT. Afterwards plates were washed three times with ddH<sub>2</sub>O and UV-treated for 30 min. Plates were directly used for seeding of cells or stored at RT up to 6 months. One day after seeding, cells were transfected with DNA (200 ng/well) using X-treme Gene 9 (Roche), according to manufacturer's instructions. The AP-tagged proteins were produced as conditioned medium as described in 8.2 *Cell*

*culture methods, preparation of ANGPTL4 conditioned medium.* Cell surface binding assay was performed like described before.<sup>78</sup>

### **ELISA based binding assay**

For the ELISA-based binding assay, white 96-well plates with high binding affinity (Greiner) were used. Plates were coated with 2 µg/ml streptavidin in bicarbonate buffer over night at 4°C. On following day, plate was washed three times with TBS-T and blocked with 5% BSA in TBS-T for 1 h at RT. After four additional washing steps with TBS-T, Strep-tagged ANGPTL4 / RSPO3 / DKK3 conditioned medium was added in 5% BSA in TBS-T. Binding was performed over night at 4°C and plate was washed three times with 150 µl and four times with 300 µl TBS-T on next day. Soluble AP-tagged SDC4 was added in different dilutions in 5% BSA in TBS-T and 1 mM MgCl<sub>2</sub> for 2 h at RT with gentle shaking. Unbound SDC4 was removed by washing three times with 150 µl and four times with 300 µl TBS-T. For the detection of bound AP-tagged SDC4, the SEAP reporter gene assay kit (Roche) was used. For each well 50 µl substrate solution (50 µl SCPD, 950 µl substrate buffer) was added, incubated for 10 min and chemiluminescence was measured.

### **Co-immunoprecipitation for identification of ANGPTL4 interaction partners**

HepG2 cells were seeded in 10x 15 cm-dishes and medium was changed after 24 h. Two days after seeding of cells, 15 ml of SP-Prot.A<sub>x2</sub>-TEV-Str.-HRP-hANGPTL4 conditioned medium (50 U/µl) was added to the cells. After 30 min at 37°C, medium was removed and cells were washed three times on ice with ice cold Hank's BSS. Cells were lysed in 5 ml ice cold IP lysis buffer for 10 min on ice. Cell lysis was increased using a dounce homogenizer. Non-disrupted cells were removed by centrifugation at 13.000 rpm and 4°C for 3 min. The supernatant was supplemented with 1 mM EDTA and EGTA to avoid precipitation of the proteins.

#### *1<sup>st</sup> purification step using IgG beads*

After a pre-clearing step using sephadex beads, lysates were incubated with 100 µl IgG beads over night with rotation at 4°C. On the following day, beads were washed three times with IP wash buffer 1 on ice, followed by three washes using 1x TBS and 1% Triton-X100. To inhibit proteases, beads were washed two times with a buffer containing 1x

TBS, 1% Triton-X100, 1 mM PMSF, 0.5 mM EDTA, 1x Benzamidine and 2 mM iodacetamide (freshly prepared). After washing once again with 1x TBS and 1% Triton-X100, beads were washed four times with TEV buffer at 4°C with rotation. To elute bound ANGPTL4 protein from the beads, 150 µl of TEV buffer, 1 mM β-mercaptoethanol and 3 µl TEV enzyme was added to the beads. Elution was performed over night at 4°C with rotation.

#### *2<sup>nd</sup> purification step using Streptavidin beads*

After centrifugation, new TEV buffer and 2 µl TEV enzyme was added to the beads and re-elution was performed 4 times. The different elutions were combined and mixed with 20 µl Streptavidin beads (Thermo). Binding to beads was proceeded over night at 4°C with rotation. The next day, beads were washed three times in IP wash buffer 2. The elution of the protein was accomplished by addition of 2 mM Biotin in IP wash buffer 2. Four elutions à 30 µl were obtained and analyzed via Western blot. The first two elutions were combined and processed for mass spectrometric analysis by the core facility for mass spectrometry and proteomics at the “Zentrum für Molekulare Biologie der Universität Heidelberg” (ZMBH).

## 9. Abbreviations

ANG	Angiopoietin
ANGPTL	Angiopoietin-like
AP	Alkaline phosphatase
A-P	Anterior-posterior
AP-2	Assembly protein 2
APC	Adenomatous polyposis coli
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
Bp	Base pairs
BSA	Bovine serum albumin
cANGPTL4	C-terminal domain of ANGPTL4
cDNA	Complementary DNA
CCD	Coiled-coil domain
CCV	Clathrin-coated vesicle
CE	Convergent extension
CK1	Casein kinase 1
Co	Control
co-IP	Co-Immunoprecipitation
CRD	Cysteine-rich domain
Cys	Cysteine
DEPC	Diethylpyrocarbonate
Dgo	Diego
DKK	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
D-V	Dorsal-ventral
DVL	Dishevelled
ECD	Extracellular domain
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetate
EGTA	Ethylene glycol tetraacetate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
flANGPTL4	Full length ANGPTL4
FLD	Fibrinogen-like domain

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Fmi	Flamingo
FZD	Frizzled
GAG	Glycosaminoglycan
GEF	Guanine exchange factor
GPCR	G protein-coupled receptor
gsc	Goosecoid
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
h	Hours
H1299	Human non-small cell lung carcinoma cell line
H1703	Human non-small cell lung carcinoma cell line
HA	Hemagglutinin
HCG	Human chorionic gonadotropin
HEK293T	Human embryonic kidney cell 293 with SV-40 large T-antigen
HepG2	Human hepatocellular liver carcinoma cell line
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans
IP	Immunoprecipitation
ISH	<i>In situ</i> hybridization
JNK	Jun-N-terminal kinase
kDa	Kilodaltons
LDL	Low density lipoprotein
LEF	Lymphoid enhancer factor
LGR	Leucine-rich repeat-containing G protein-coupled receptor
LILRB2	Leukocyte immunoglobulin-like receptor, subfamily B, member 2
LPL	Lipoprotein lipase
LRP	Low-density lipoprotein receptor-related protein
LRR	Leucine-rich repeat
min	Minutes
Mo	Morpholino
mRNA	Messenger RNA
MVB	Multivesicular body
myf5	Myogenic factor 5
myoD	Myogenic differentiation 1
nANGPTL4	N-terminal domain of ANGPTL4
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
Pk	Prickle
PPAR	Peroxisome proliferator-activated receptor
Pro	Proline
PRR	Prorenin receptor

qPCR	Quantitative PCR
RNA	Ribonucleic Acid
RNAi	RNA interference
RNF43	Ring finger protein 43
rpm	Revolutions per minute
RSPO	R(oof plate-specific)-spondin
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SDC	Syndecan
SDS	Sodium dodecylsulfate
Sec	Seconds
Ser	Serine
siRNA	Small interfering RNA
St	Stage
Stbm	Strabismus
TAG	Triacylglycerols
TCF	T-cell factor
TEMED	Tetramethyl ethylene diamine
TGF	Transforming growth factor
Thr	Threonine
UPL	Universal probe library
wt	Wildtype
Xbra	<i>Xenopus</i> brachyury
Xnot	<i>Xenopus</i> notochord
Xnr	<i>Xenopus</i> nodal related protein
ZNRF3	E3 ubiquitin ligase zinc and ring finger 3

For simplicity the human nomenclature was used for all genes and proteins within this thesis. Appropriate nomenclature is indicated below with LGR as an example.

Species	Gene symbol	Protein symbol
Human	<i>LGR</i>	LGR
<i>Xenopus</i>	<i>lgr</i>	Lgr
Mouse	<i>Lgr</i>	Lgr

## 10. Publications derived from this thesis

Glinka A\*, Dolde C\*, **Kirsch N**\*, Huang YL\*, Kazanskaya O, Ingelfinger D, Boutros M, Cruciat CM, Niehrs C (2011) LGR4 and LGR5 are R-spondin receptors mediating Wnt/ $\beta$ -catenin and Wnt/PCP signaling. *EMBO Rep.* 12, 1055.

Demir K, **Kirsch N**, Beretta CA, Erdmann G, Ingelfinger D, Moro E, Argenton F, Niehrs C, Boutros M (2013) RAB8B is required for activity and caveolar endocytosis of LRP6. *Cell Rep.* 4, 1224.

**Kirsch N**, Koch S, Dolde C, Niehrs C. ANGPTL4 binds syndecans and inhibits Wnt/ $\beta$ -catenin signaling by promoting LRP6 internalization. *Manuscript in preparation.*

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