1 Introduction

1.1 Wnt signaling

With the emergence of multicellular organisms, the relevance of cell-cell communication increased. The Wnt pathway has emerged as one of the most important mediator of cell to cell communication in metazoans and any disturbance of Wnt signaling can cause severe developmental defects as well as diseases.

Wnt was first discovered in a *Drosophila melanogaster* mutant exhibiting reduced or absent wings and halteres (Sharma and Chopra, 1976). Based on this mutant phenotype the locus was named wingless (wg). Some years later a locus termed int-1 was identified in mouse to induce mammary tumors (Nusse et al., 1984). Competitive genomics revealed that wg and int-1 were homologs and the name Wnt emerged as a fusion of these gene nomenclatures (Nusse et al., 1991). Since then a lot of work has been done to investigate the Wnt signaling pathway. Wnt proteins are evolutionary conserved cysteine rich morphogens only present in metazoans. In humans 19 separate genes encode for Wnt proteins (Croce and McClay, 2008; Miller, 2001). It was shown that Wnt signaling has a crucial role in various biological processes in embryonic development, such as tissue patterning, cell proliferation and cell migration. In the adult, Wnt signaling regulates stem cell proliferation, which is crucial for tissue homeostasis (Logan and Nusse, 2004; Nusse et al., 2008; de Sousa e Melo and Vermeulen, 2016). Wnts bind with high affinity to the family of seven-pass transmembrane Frizzled (FZD) receptors at their extracellular cysteine-rich domain (CRD) but can also associate with various co-receptors. Indeed different downstream pathways are activated as a result of this diversity of ligand-receptor interactions within the Wnt pathway. Mutation of components functioning within the pathways can deregulate Wnt signaling, which can result in diseases such as diabetes type II, bone diseases, early coronary disease and most notably cancer (Bryan T. MacDonald; Keiko Tamai and Xi He, 2010; Clevers and Nusse, 2012; Yu et al., 2016). Considering that Wnt signaling regulates a wide variety of biological processes and can cause various diseases when deregulated, it is considered highly relevant to understand better the mechanism of how Wnt signaling is regulated. Key to gaining a comprehensive understanding of these diseases is a more complete understanding of the molecular mechanisms involved in signal transduction.

1.2 Current molecular models of Wnt signaling

Wnt proteins are secreted molecules that undergo several posttranslational modifications. On their secretory route through the endoplasmic reticulum (ER) Wnts get lipid modified by the acyl transferase porcupine which is crucial for Wnt activity as it aids binding to FZD receptors (Miura and Treisman, 2006; Takada et al., 2006; Willert et al., 2003). By the help of the p24 protein family Wnts get shuttled to the Golgi apparatus (Buechling et al., 2011; Port et al., 2011) where they undergo further modifications like acylation and N-glycosylation (Harterink and Korswagen, 2012). Both modifications are relevant during Wnt signaling and aid folding and trafficking as well as the stability of the protein (Harterink and Korswagen, 2012). To escort Wnts to the plasma membrane, the multipass transmembrane protein Wntless/Evenness interrupted (Evi) binds to the acylated Wnts (Bänziger et al., 2006; Bartscherer et al., 2006; Herr and Basler, 2012). After transferring Wnt to the plasma membrane Evi is recycled to the Golgi with the help of the retromer complex to ensure a subsequent round of Wnt secretion (Eaton, 2008).

Upon secretion, Wnts can travel several cell diameters away from the producing cell to modulate the expression of target genes at distant sites (Neumann and Cohen, 1997; Zecca et al., 1996). When Wnts reach signal-receiving / target cells they bind to their specific transmembrane receptors to activate different intracellular signal transductions classified as canonical β-catenin dependent and non-canonical β-catenin independent signaling pathways (Fig.1). It is thought that FZD receptors are globally required for Wnt signaling and that co-receptors provide signaling pathway specificity. For example, the single-pass transmembrane co-receptors LRP5/6 are generally required in combination with FZD for β-catenin dependent Wnt signaling whereas single-pass transmembrane receptors belonging to the receptor tyrosine kinase family, such as ROR1/2 and PTK1, are thought to specify β-catenin independent pathway activation in combination with FZD.

The canonical β-catenin dependent pathway (Fig.1a), as the name suggests, uses cytoplasmic β-catenin as the main cytoplasmic signaling protein. β-catenin is a multifunctional protein, which is also located at the cytoplasmic face of the plasma membrane due to its association with the intracellular domain of cadherin receptor complexes. Upon activation of the pathway cytoplasmic β-catenin enters the nucleus to modulate transcription of target genes in combination with transcription factors of the Lef/Tcf family (Li et al., 2012). When the pathway is not activated, a protein complex called the destruction complex binds cytoplasmic β-catenin. Within this destruction

complex β-catenin becomes phosphorylated, which targets it for Ubiquitin-mediated degradation by the proteasome (Aberle et al., 1997; Li et al., 2012). The β-catenin dependent pathway is important for the regulation of cell differentiation and proliferation.

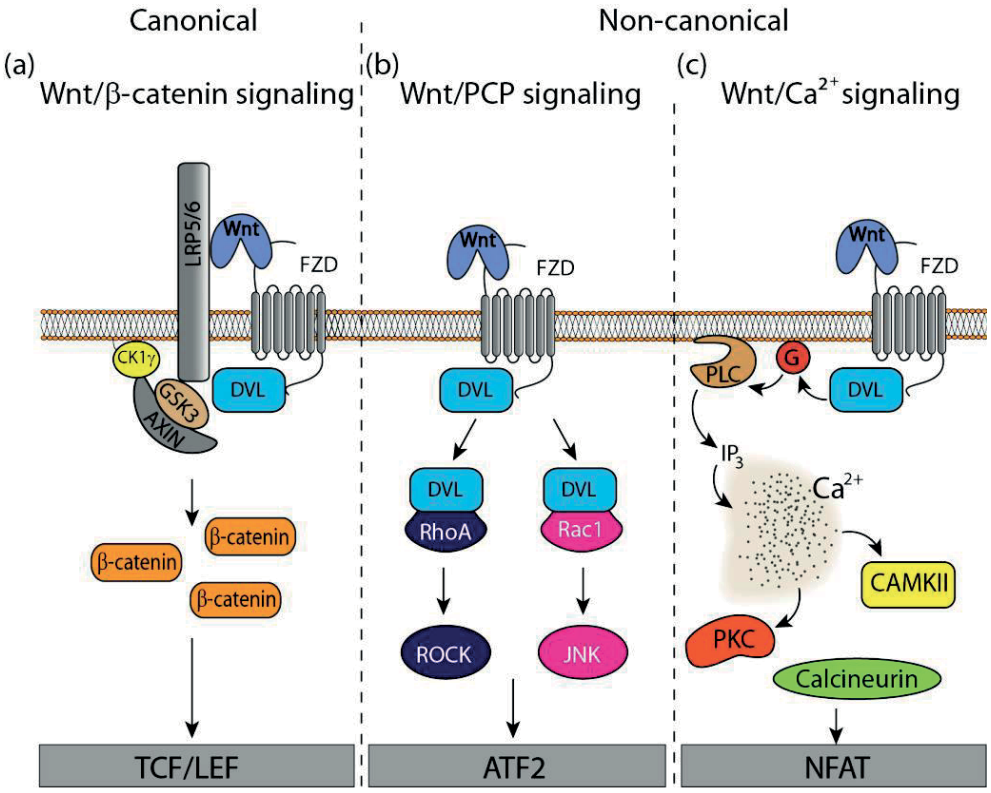

Figure 1: Wnt signaling pathways. (a) activation of canonical Wnt/β-catenin signaling starts with binding of Wnt ligand to FZD and the co-receptor LRP5/6, which induces recruitment of DVL and causes the accumulation of β-catenin in the cytosol. Free β-catenin can translocate to the nucleus where it activates together with TCF/LEF the transcription of Wnt target genes. (b) Non-canonical Wnt/PCP signaling starts with activation of DVL by the binding of Wnt ligand to FZD receptor which induces the activation of RhoA and Rac1 small GTPases and their specific effectors ROCK and JNK. (c) Non-canonical Wnt/Ca^{2+} signaling is activated by the binding of Wnt ligand to FDZ receptor which recruits DVL, thus activating the heterotrimeric G-protein and in turn PLC and IP_3. IP_3 induces the release of intracellular Ca^{2+} and activates PKC, CAMKII and Calcineurin.

Non-canonical Wnt pathways are the β-catenin independent pathways. This comprises the planar cell polarity (PCP) pathway (Fig.1b) and the Wnt/Ca^{2+} pathway (Fig.1c). PCP signaling activates a cascade of the small GTPases Rac1, RhoA and their respective effectors JUN-N-terminal kinase (JNK) and Rho-kinase (ROCK). This leads to changes in cytoskeleton properties, which can effect e.g. cell shape, polarity and migration (Mlodzik and Simons, 2008).

The Wnt/Ca^{2+} pathway triggers Ca^{2+} release from intracellular stores. Binding of Wnt to FZD stimulates heterotrimeric G-proteins that then activates phospholipase-C (PLC), thus stimulating the generation of inositol-1,4,5-triphosphate (IP_3) (Kohn and Moon, 2005; Slusarski et al., 1997a, 1997b). IP_3 triggers Ca^{2+} release from intracellular stores activating the calcium sensitive effectors calmodulin-dependent kinase II (CAMKII), phosphatase calcineurin and protein kinase-C (PKC) to regulate cell adhesion, migration and cell fate (Kohn and Moon, 2005). The Wnt/Ca^{2+} pathway is involved in cancer, inflammation and neurodegeneration (De, 2011).

1.3 Regulation of canonical Wnt/β-catenin signaling

In absence of Wnt signaling, β-catenin is caught by a destruction complex which is held together by the scaffold protein Axin (Fig.2a). Furthermore the destruction complex consists of the two kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α), that are bound by Axin (Dajani et al., 2003; Sobrado et al., 2005) as well as the scaffold protein adenomatous polyposis coli (APC) that binds GSK3 and Axin (Fagotto et al., 1999; Ikeda et al., 1998; Itoh et al., 1998; Rubinfeld et al., 1996). When β-catenin is entering the complex it is bound by APC and Axin resulting in rapid phosphorylation by CK1α which creates a priming site for phosphorylation by GSK3 (Amit et al., 2002; Liu et al., 2002). The phosphorylated β-catenin will then be recognized by β-TRCP, a special subunit of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex (Jiang and Struhl, 1998; Kitagawa et al., 1999; Lagna et al., 1999; Liu et al., 1999; Marikawa and Elinson, 1998) that catalyzes polyubiquitination. This modification which targets β-catenin for degradation by the proteasome and prevents Wnt target genes from transcription (Aberle et al., 1997).

To activate the Wnt/β-catenin pathway Wnt binds to FZD and LRP5/6 (Fig.2b). FZD proteins are the principal Wnt receptors, containing a large extracellular cysteine rich domain (CRD) (Bhanot et al., 1996), which mediates Wnt binding. It was shown that Wnt interacts with FZD by a two finger like structure grasping the CRD of FZD with high affinity (Janda et al., 2012). Furthermore it has been proposed that the remaining accessible surface of Wnt can simultaneously bind to the co-receptor LRP5/6 to form a ternary complex for activation. Upon Wnt binding to FZD and LRP5/6, Dishevelled (DVL) is phosphorylated and recruited to FZD at the cell surface (Chen, 2003). Additionally, DVL and Axin share DIX domains that can mediate polymerization of the respective proteins (Schwarz-Romond et al., 2007a). Interaction of the

DIX domains is required for Wnt dependent clustering of LRP6, also called signalosome formation (Bilic et al., 2007; Schwarz-Romond et al., 2005). As a result, the intracellular domain (ICD) of LRP5/6 is phosphorylated, which results in the recruitment of Axin to LRP6 (Mao et al., 2001b). GSK3 is also bound to Axin at the cell surface (Zeng et al., 2008) and is itself inhibited by direct interaction with the phosphorylated ICD of LRP6 (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). GSK3 is thus no longer able to phosphorylate β-catenin.

As a consequence of these receptor activation events β-catenin is free to accumulate in the cytosol and translocate to the nucleus, where it interacts with the transcription factors TCF/LEF to initiate the transcription of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996). β-catenin is thought to activate gene expression by out-competing the co-repressor Groucho from TCF/LEF (Cavallo et al., 1998; Roose et al., 1998).

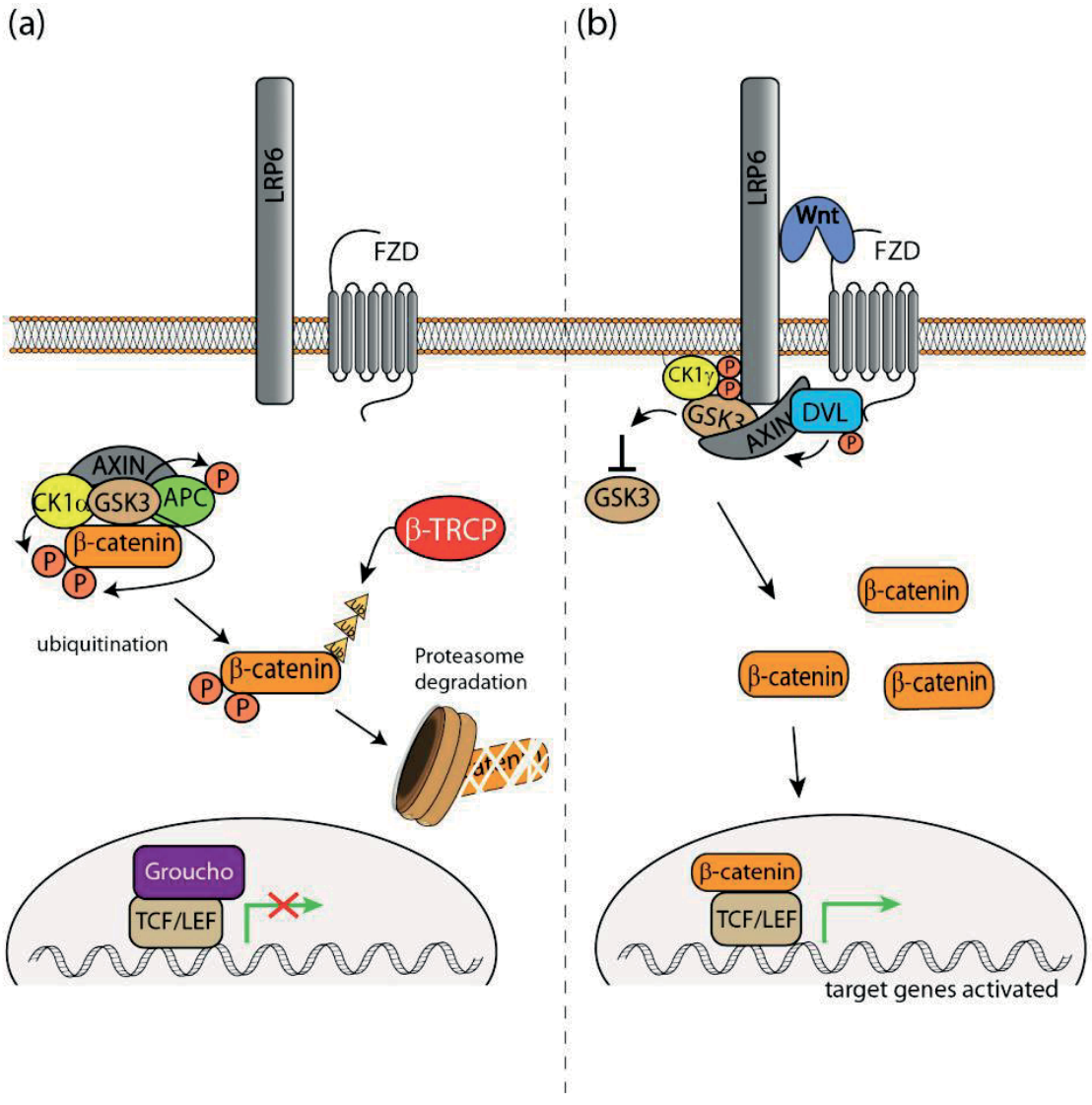

Figure 2: Canonical Wnt/β-catenin signaling. (a) In absence of the Wnt ligand β-catenin is targeted to proteolytic degradation through phosphorylation by the Axin-APC-GSK3β-CK1α destruction com-

plex and further ubiquitination by β-TRCP, a special subunit of E3 ubiquitin ligase complex. (b) Binding of Wnt to its receptors FDZ and LRP5/6 recruits DVL which inhibits the β-catenin destruction complex by recruiting Axin, GSK3β and CK1γ to the cell surface. Consequently, β-catenin is free to accumulate in the cytosol and translocate to the nucleus were it activates the transcription of Wnt target genes by replacing Groucho from TCF/LEF.

1.4 Wnt signaling antagonists and agonists

There are several secreted as well as transmembrane Wnt antagonists and agonists that can act either on the level of intracellular signal transduction or extracellularly to regulate receptor-ligand interaction (Fig.3) (Cruciat and Niehrs, 2013).

Secreted FZD related proteins (sFRPs) belong to a family that comprises five members, sFRP1-5. It has been demonstrated that sFRPs bind directly to Wnt ligands and inhibit Wnt/β-catenin signaling (Leyns et al., 1997; Lin et al., 1997; Wang et al., 1997) (Fig.3a). sFRPs however also been reported to bind to FZD, thereby inhibiting both canonical and non-canonical Wnt signaling (Bovolenta et al., 2008) (Fig.3b). Most probably binding occurs through the N-terminal CRD domain of sFRP, which is highly similar to the FZD CRD and seems to be necessary and sufficient for the binding and inhibition of Wnt and FZD (Bafico et al., 1999; Lin et al., 1997). Similar to sFRPs, Wnt-inhibitory factor 1 (WIF-1) binds to Wnt ligands and prevents them from binding to their receptors. It has been shown that WIF-1 specifically binds to several Wnts, including Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11 and inhibits both canonical and non-canonical signaling (Surmann-Schmitt et al., 2009). Also Cerberus encodes for a secreted protein that has been shown to function as a multivalent growth-factor antagonist, not only binding to Wnt but also to Nodal and bone morphogenetic protein (BMP) via independent sites (Piccolo et al., 1999) (Fig.3a).

Other important classes of secreted Wnt inhibitors are the Dickkopf (DKK) family and the WISE/SOST family. The DKK family comprises four members (DKK1-4), of which DKK1, DKK2 and DKK4 inhibits Wnt signaling by binding directly to LRP5/6 (Bafico et al., 2001; Mao et al., 2001a; Semënov et al., 2001). DKK1 is the most studied member of the DKK family and was first discovered as an inducer of Spemann's organizer in *Xenopus* necessary for head induction (Glinka et al., 1998). In addition to LRP5/6, DKK1 interacts also with another class of single-pass transmembrane proteins named Kremen1 (Krm1) and Kremen2 (Krm2) (Mao et al., 2002). It was shown that DKK1 can form at ternary complex with Krm2 and LRP6 that disrupt Wnt signaling by promoting rapid endocytosis and removal of LRP6 from the cell surface (Mao

et al., 2002) (Fig.3e). Moreover, studies in *Xenopus* indicate that Krm1/2 inhibit Wnt signaling during early anteroposterior patterning of the central nervous system (Davidson et al., 2002). It was recently reported that Dkk1 can also associate with CKAP4 to regulate cell proliferation (Kimura et al., 2016). WISE and SOST belong to subfamily of cysteine knot-containing proteins (Avsian-Kretchmer and Hsueh, 2004). Like DKK1, SOST bind to LRP5/6 to disrupt Wnt1-induced FZD-LRP6 complex, thus inhibiting canonical Wnt signaling (Semënov et al., 2005) (Fig.3b). WISE was shown to block Wnt1, Wnt3a and Wnt8 activity (Blish et al., 2008; Itasaki et al., 2003; Yanagita et al., 2004) and to interact with LRP6 through one of the three loops formed by the cysteine knot (Lintern et al., 2009). In addition to modulating IGF signaling, insulin-like growth-factor binding protein-4 (IGFBP-4) was also shown to physically interact with LRP6 and FZD8 to inhibit the binding of Wnt3a to its receptors (Fig.3c).

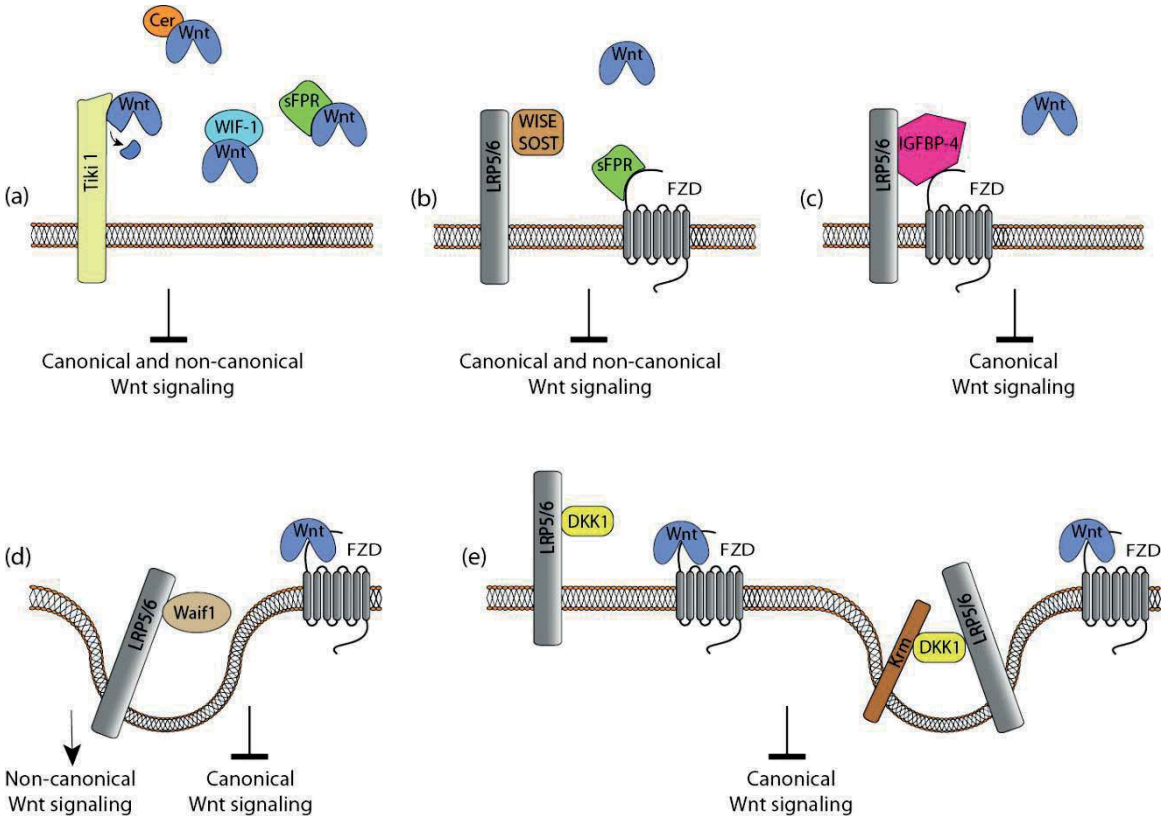

Figure 3: Wnt signaling antagonists. (a) Binding of sFRPs, WIF-1 and Cerberus to Wnt results in inhibition of canonical and non-canonical Wnt signaling. Tiki1 is inhibiting Wnt signaling by removing several amino-terminal residues from Wnt, this happens on the secretory pathway of Wnt as well as at the cell surface. (b) sFRPs can also inhibit canonical and non-canonical Wnt signaling by binding to FZD. WISE/SOST binding to LRP6 blocks Wnt induced FZD-LRP6 complex formation thus inhibiting canonical Wnt signaling. (c) IGFBP-4 is forming a complex with LRP6 and FZD thereby inhibiting signal transduction by Wnt.(d) Waif1 binding to LRP6 results in internalization of LRP6-Waif1 complex

which on one hand inhibits canonical Wnt signaling and on the other hand promotes non-canonical Wnt signaling. (e) Binding of DKK1 to LRP6 blocks Wnt induced FZD-LRP6 complex formation and/or induces LRP6 endocytosis in the presence of Kremen which inhibits canonical Wnt signaling. Figure is adapted from Cruciat and Niehrs, 2013.

Further Wnt antagonists are the transmembrane proteins Shisa, Wnt-activated inhibitory factor 1 (Waif1/5T4) and Tiki1. Shisa functions cell autonomously in the ER where it interacts with immature FZD and prevent it from reaching the cell surface, thereby inhibiting Wnt signaling (Yamamoto et al., 2005). Waif1 has a dual function; on the one hand it was shown to inhibit Wnt/β-catenin signaling by binding to LRP6 and thereby regulating its subcellular localization, on the other hand Waif1 activates non-canonical Wnt signaling (Kagermeier-Schenk et al., 2011) (Fig.3d). Tiki1 was identified as a transmembrane metalloprotease that inhibits Wnt signaling by removing eight amino-terminal residues from Wnt, which promotes formation of oxidized Wnt oligomers that exhibit normal secretion but impaired receptor-binding capability (Zhang et al., 2012) (Fig.3a).

In contrast to the inhibiting proteins mentioned above, Norrin and R-spondin (Rspo) are Wnt/β-catenin signaling agonists that are apparently unrelated to Wnt ligands (Fig.4). Norrin is a specific ligand for FZD4 thus inducing FZD4 and LRP5/6 dependent activation of the canonical pathway during retinal vascularization (Xu et al., 2004). Rspo however synergizes with Wnt, FZD and LRP6 to activate Wnt pathway signaling more generally (Kazanskaya et al., 2004; Kim, 2005; Nam et al., 2006; Wei et al., 2007). The leucine-rich repeat containing G-protein-coupled receptor 4/5 (LGR4/5) are receptors for Rspo and are required for Rspo dependent Wnt/β-catenin signaling (Carmon et al., 2011; Glinka et al., 2011). Moreover Glinka et al. showed that Rspo/LGR4 triggered signaling requires clathrin mediated endocytosis, whereas Wnt3a mediated signaling requires caveolin mediated endocytosis, suggesting internalization as a mechanism of Rspo signaling (Glinka et al., 2011). Indeed it was shown that Rspo together with LGR4 internalize the transmembrane E3 ubiquitin ligase zinc ring finger 3 (ZNRF3) to enhance Wnt signaling via a de-repression mechanism (Hao et al., 2012). In the absence of Rspo, ZNRF3 inhibits Wnt signaling by promoting the turnover of FZD and LRP6 (Hao et al., 2012).