Leading Edge
Review

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Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities

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The WNT signal transduction cascade is a main regulator of development throughout the animal kingdom. Wnts are also key drivers of most types of tissue stem cells in adult mammals. Unsurprisingly, mutated Wnt pathway components are causative to multiple growth-related pathologies and to cancer. Here, we describe the core Wnt/β-catenin signaling pathway, how it controls stem cells, and contributes to disease. Finally, we discuss strategies for Wnt-based therapies.

Since the initial discovery of the first member of the Wnt family 35 years ago (Nusse and Varmus, 1982), interest in Wnt signaling has steadily risen. In fields ranging from cancer and development to early animal evolution, Wnt signaling has emerged as a fundamental growth control pathway. Details about the mechanisms of Wnt signaling have been revealed, including structural information on the main molecular players. In this review, we will present an update (Clevers and Nusse, 2012) on recent insights into Wnt signaling in various contexts, during normal physiology as well as in disease.

Wnt Proteins Are Growth Factors, but What Distinguishes Wnts from Other Signals?

Wnt signaling represents one of a handful of pathways, including Notch-Delta, Hedgehog, transforming growth factor β (TGF-β)/bone morphogenetic protein (BMP) and Hippo, which are all implicated in developmental processes. Each of these signaling pathways is conserved in evolution and widespread in its activity; it could be asked what is unique about the Wnt system compared to others? What are the effects of Wnt signals on cells and why is this pathway so ubiquitously active in growing tissues? Fundamentally, Wnts are growth stimulatory factors, leading to cell proliferation (Niehrs and Acebron, 2012). In doing so, Wnt signals impact the cell cycle at various points. Compared to other growth factors, a distinctive aspect of Wnt signaling is the ability to giving shape to growing tissues while inducing cells to proliferate, acting in the process as directional growth factors (Goldstein et al., 2006; Huang and Niehrs, 2014; Schneider et al., 2015; Kitajima et al., 2013; Loh et al., 2016). Wnt signals can instruct new cells to become allocated in a way such that organized body plans rather than amorphous structures are generated (Huang and Niehrs, 2014; Wu et al., 2013; Habib et al., 2013). This morphogenetic outcome of Wnt signaling is mediated by a multitude of signal transduction steps that can be activated by Wnt, resulting in changes in gene expression but also in effects on the cytoskeleton and the mitotic spindle (Sawa, 2012). Moreover, Wnts employ receptors of different classes, generating a panoply of combinatorial Wnt signaling critical for correctly shaping tissues during development (van Amerongen and Nusse, 2009), or maintaining tissue architecture in adult life. In this overview of the field, we will mostly discuss the Wnt/β-catenin (a.k.a. "canonical") pathway, its nuclear effects, and implications for diseases, recognizing that to cover all aspects of Wnt signaling is beyond our scope.

Specificity of Wnt Signaling

There are multiple Wnt genes in any animal genome—19 in mammals for example (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/)—raising the question of specificity: do individual Wnts have unique or overlapping functions? An argument for unique roles for each Wnt comes from loss-of-function genetic data: most Wnt genes, when eliminated from the genome, have distinct phenotypes. For example, mice mutant for Wnt1 have a midbrain defect (McMahon et al., 1992) while Wnt4 mutants are compromised in the development of the kidney (Stark et al., 1994). There are numerous other unique or partially overlapping phenotypes associated with loss of Wnt genes (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/) and, not surprisingly, the morphological phenotypes correspond to where the Wnts are expressed.

In addition to these genetic arguments, a case for inherent and important differences between individual Wnt signals comes from the high vertical evolutionary conservation of Wnt proteins. Orthologs within the Wnt family can be traced throughout all animal phyla: Wnt1 in mammals is the true ortholog of Wnt1 in Hydra and Wingless in *Drosophila* (Kusserow et al., 2005). Strikingly, Hydra and other Cnidaria have a set of Wnt genes that correspond one-to-one to vertebrate counterparts (Kusserow et al., 2005). Such a high degree of conservation and evolutionary constraint would argue that intrinsic properties of different Wnts are important for their functions.

On the other hand, when it comes to biochemical signaling mechanisms or effects on target cells, different Wnts behave in a very similar way. With respect to binding of Wnts to the receptors, the Frizzleds (FZDs), there is extensive cross-reactivity (Yu et al., 2012; Dijksterhuis et al., 2015). In addition, most Wnt

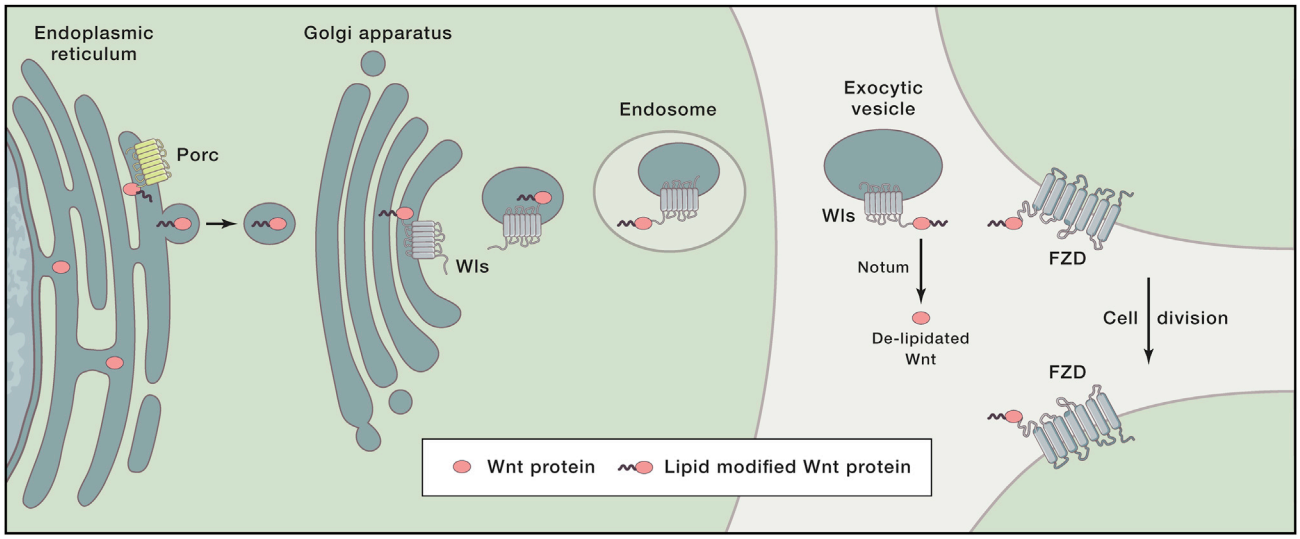

Figure 1. Model of Wnt Secretion
In the endoplasmic reticulum, Wnts are modified by Porcupine (Porc) to become lipid-bound. Transport of the lipid-modified Wnt is regulated by Wntless/Evi (Wls), possibly involving endosomes. Wnts are secreted on exocytic vesicles. Outside of cells, the Notum enzyme can act as a deacylase, removing the lipid and inactivating Wnt. After reception on the Wnt target cells by FZD and other receptors, cell-bound-Wnts may spread over tissues by cell division.

proteins will lead to elevated levels of β-catenin in cells or increases in signaling reporter activity (Alok et al., 2017). These assays however, mostly done in cell culture, may not reveal the whole spectrum of signaling activity or receptor-binding finesses of different Wnts. As we will show below, there are various co-receptors for Wnts that may modulate signaling outcome.

Taking all these observations together, we suggest that, by and large, the differences between loss-of-function Wnt phenotypes can be attributed to discrete and unique expression patterns of the Wnt genes. Because of the fact that Wnt proteins signal very close to where they are produced, it seems that the overall phenotypes caused by loss of Wnt gene function are primarily due to local expression domains of each Wnt. In addition, intrinsic differences between Wnts, their binding to receptors and co-receptors are no doubt consequential for the various developmental processes as well.

Production and Secretion of Lipid-Modified Wnts

Wnt proteins act as intercellular signals but there are several unresolved questions on the nature of the extracellular form of Wnts and the mechanisms of export. During synthesis, Wnt proteins, 40 kDa in size and rich in cysteines, are modified by attachment of a lipid, an acyl group termed palmitoleic acid (Willert et al., 2003; Rios-Esteves et al., 2014; Takada et al., 2006; Rios-Esteves and Resh, 2013). This modification is likely shared between all Wnts and is brought about by a special palmitoyl transferase: Porcupine (Rios-Esteves and Resh, 2013). The lipid functions primarily as a binding motif the Wnt receptor, FZD (see below) (Janda et al., 2012), but it also renders the Wnt protein hydrophobic and may tether it to cell membranes. The lipid may therefore contribute to restricting Wnt spreading and its range of action.

During maturation of the Wnt protein, the transmembrane protein Wntless/Evi (Wls) (Bartscherer et al., 2006; Bänziger et al., 2006) binds to the lipidated forms (Yu et al., 2014; Herr and Basler, 2012; Najdi et al., 2012) and is required for ferrying Wnts to the plasma membrane to become secreted (Figure 1). How extracellular Wnt signals are transferred to target cells remains mysterious, but available evidence suggests that the proteins are not present in a free form. More likely, Wnt proteins are incorporated into secretory vesicles or exosomes (Gross et al., 2012; Korkut et al., 2009; McGough and Vincent, 2016; Saha et al., 2016; Gross et al., 2012). These vesicles contain Wls as well as the mature Wnt signals (Korkut et al., 2009) (Figure 1),in such a form that the Wnt protein is present on the outside of the vesicle, available for binding to receptors. In another model, Wnt transfer involves direct contact between cells mediated by receptors FZD and the transmembrane E3 ligases Rnf43/Znrf3 (Farin et al., 2016) (Figure 1).

Although it is sometimes assumed that secreted Wnt signals are long-range morphogens, there is little evidence that this is the prevailing mode. In most tissues, Wnt signaling occurs between neighboring cells that contact each other. Even in the best studied example of long-range signaling by a Wnt—that is, by the Wnt ligand Wingless in *Drosophila*—recent evidence has made a case that the requirements for the gene can be largely provided by a membrane-tethered form of the protein which, in principle, cannot diffuse (Alexandre et al., 2014). While the conclusion of this result might be that Wingless does not act as a long-range morphogen, it could still be that Wingless bound to membranous vesicles or filopodia (Stanganello et al., 2015) would operate over longer distances. In support of the vesicle model, it has been shown that vesicles containing Wingless and its transporter protein Wntless/Evi are present at neuromuscular junctions in *Drosophila* and interact with FZD receptors (Korkut et al., 2009). Further alternatives to explain long-range activities by Wnts include sequential signaling between Wnt target cells and their neighbors, mediated by various Wnt family members. Indeed, Cnidaria embryos display staggered

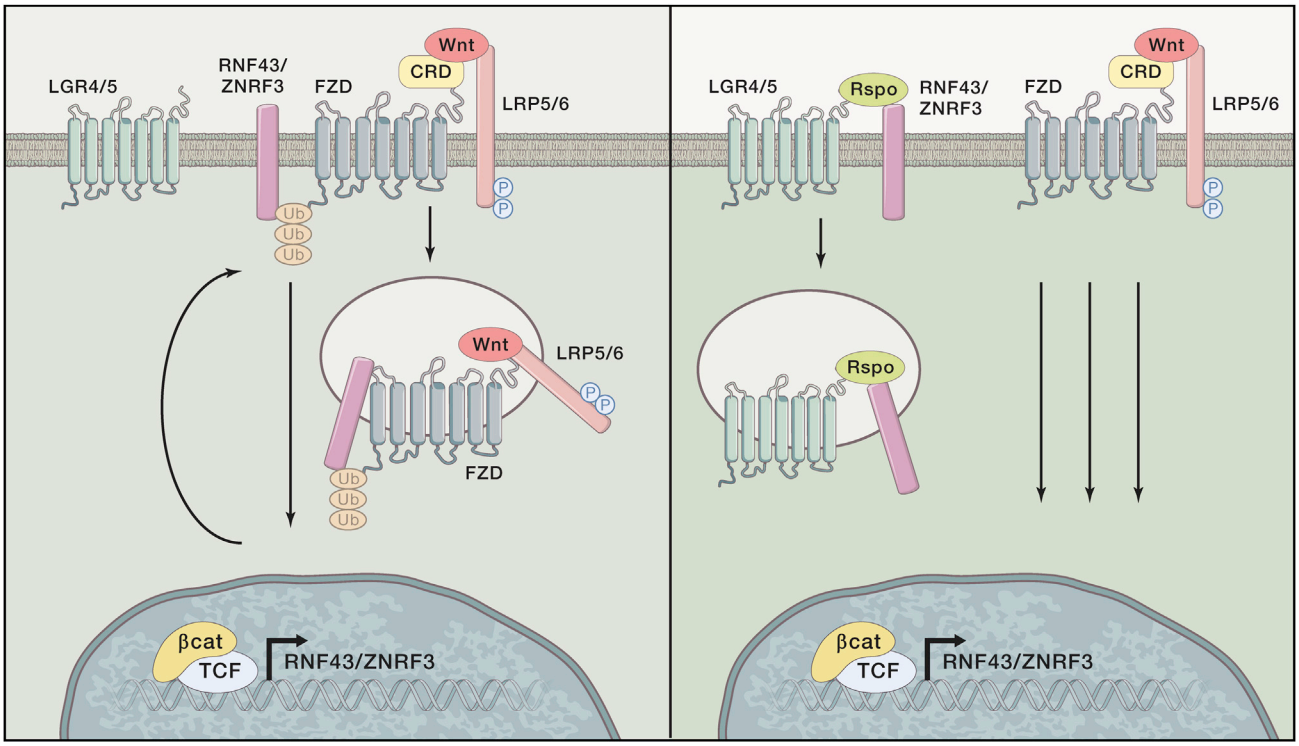

Figure 2. Wnt Receptors

FZD proteins act as the primary receptors for Wnt signals. FZD molecules have 7-transmembrane (7TM) and an extracellular N-terminal cysteine-rich domain (CRD). Wnts can bind the CRD of FZD. The co-receptors LRPs are long single-pass transmembrane proteins that are phosphorylated by several protein kinases including GSK3 and CK1. The Wnt agonists R-spondins interact on the cell surface with members of the LGR5 family to enhance Wnt signaling. ZNRF3 and RNF43 are transmembrane molecules that downregulate Wnt signaling. They have E3 ubiquitin ligase activity acting on the FZD molecules, leading to turn-over of these receptors. Binding of R-Spondin to ZNRF3 has been postulated to downregulate the activity of the ZNRF3 activity thereby enhancing Wnt signaling as the FZD receptors now become available.

expression of various Wnt family members across the primary axis (Kusserow et al., 2005). In yet another context, stem cell niches of the intestinal crypts, Wnt protein bound to FZD receptor-expressing cells can become diluted as cells move and divide (Farin et al., 2016), a mode of Wnt transport that can also be directly visualized in intestinal organoid cultures (Figure 1). These results add to—but do not—resolve the continuing debate on the Wnt signaling landscape and the existence of morphogens.

Wnt Receptors Are FZD/LRP Heterodimers

On the surface of cells, Wnt proteins bind to a receptor complex of two molecules, FZD (FZD) and LRP5/6 (Figure 2). FZD proteins have 7-transmembrane (7TM) and an extracellular N-terminal cysteine-rich domain (CRD) (Bhanot et al., 1996). The CRD is the primary interacting module for Wnt binding with affinities in the nM range (Hsieh et al., 1999). The structure of the CRD as bound to Wnt demonstrates that there are multiple interacting surfaces, including a hydrophobic pocket in the CRD that binds to the lipid on Wnt (Janda et al., 2012). In addition, the C terminus of Wnt makes contact with the CRD (Janda et al., 2012).

During signaling, FZDs cooperate with the single-pass transmembrane molecule LRP5/6, in such a way that binding of the Wnt protein leads to dimerization of the two receptors (Figure 2) (Janda et al., 2017). This mechanism would lead to a conformational change of the receptors. As a consequence, the cytoplasmic tail of LRP, after phosphorylation by several protein kinases, recruits the scaffold protein Axin. One of these phosphorylations on LRP is mediated by GSK3 on a serine in a PPPSP motif. The same motif is found in a number of Wnt signaling components including β-catenin, Axin, and APC (Stamos et al., 2014).

While LRP has a relatively well-understood function in signaling, there is still little known about the role of FZD in Wnt reception. The cytoplasmic part of FZD can bind to Dishevelled (DVL) (Tauriello et al., 2012) (Figure 4) that would then provide a platform for the interaction between the LRP tail and Axin, through the DIX domain on DVL and Axin (Schwarz-Romond et al., 2007; Fiedler et al., 2011). Multimers of receptor-bound DVL and Axin molecules might support the formation of the LRP-FZD dimer. In line with this model, higher-order complexes containing Wnts, receptors, and DVL as well as small particles of multimerized DVL molecules have been detected in cells (Schwarz-Romond et al., 2005; Gammons et al., 2016; Jiang et al., 2015).

Wnts are not the only ligands of the FZD receptors. The cysteine-knot protein Norrin, encoded by the *NDP* gene, can also bind and activate Wnt receptors (Figure 3). In humans,

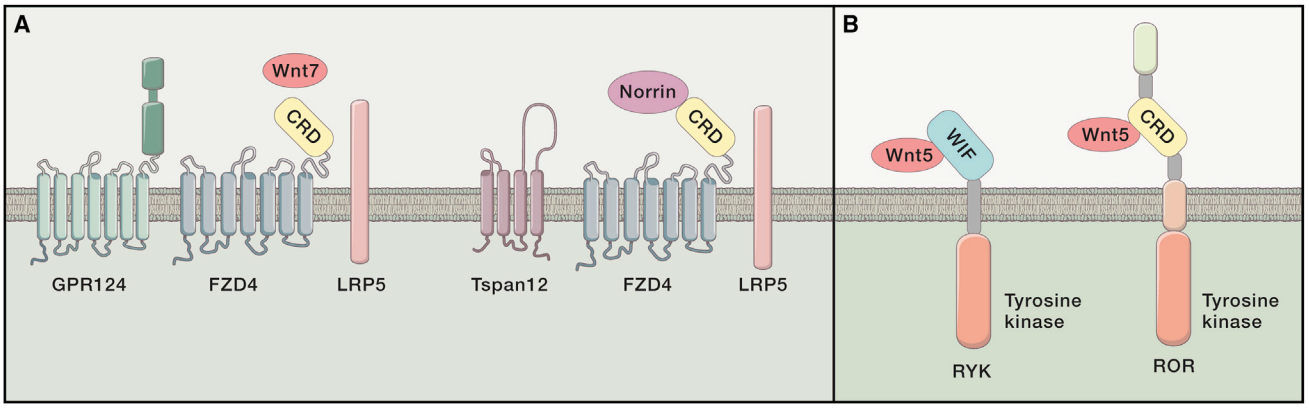

Figure 3. Alternative Wnt Receptors

(A) In regulating the blood-brain-barrier and possibly other areas of vasculature, Wnt7 can interact with not only FZD4 and LRP, but also with the multiple pass transmembrane protein Gpr124. During vascular development. Norrin can also act as a ligand for the FZD4/LRP5 complex. The tetraspanin family member, Tspan12, can be a Norrin-specific co-receptor.

(B) The RYK proteins are transmembrane tyrosine kinases. They have a Wnt binding domain similar to the WIF proteins. The ROR transmembrane tyrosine kinases can also bind to Wnts using a CRD motif similar to that of the FZDs.

NDP mutations cause Norrie disease, an X-linked disorder characterized by hypovascularization of the retina and a severe loss of visual function. Norrin binds with high affinity and specificity to FZD-4 (Ke et al., 2013; Chang et al., 2015), while coexpression of Norrin, FZD-4, and LRP5 potently activates Wnt/β-catenin signaling (Xu et al., 2004). Biochemical evidence and analyses of mice carrying mutations in the tetraspanin family member, Tspan12, provide evidence that Tspan12 is a Norrin-specific co-receptor (Figure 3) (Junge et al., 2009) that may act by forming a ternary complex with FZD4 (Ke et al., 2013).

Interestingly, FZD can also act as a receptor for the *Clostridium difficile* toxin B (TcdB) (Tao et al., 2016), a toxin known to be a critical virulence factor in causing diseases after infection by *C. difficile* infection. TcdB can bind to the CRD of FZD, with different affinities for several FZD family members. As TcdB can actually compete with Wnt for binding to FZDs and blocks Wnt signaling, the pathology underlying *C. difficile* infection could be caused by loss of Wnt signaling in the intestine, a supposition that offers hope for therapeutic intervention in *C. difficile* infections (Tao et al., 2016).

In addition to the core receptors FZD and LRP5/6, there are several other transmembrane molecules implicated in Wnt signaling. These include the ROR and RYK tyrosine kinase receptors, able to bind to Wnt ligands using a CRD or WIF domain respectively (Figure 3). Once activated, these receptors feed into other signaling pathways in cells. Each of them has also been shown to interact with DVL, leading to the phosphorylation of this common Wnt pathway component. The consequences of these DVL modifications are otherwise unknown (Ho et al., 2012; Huang et al., 2013).

Yet another receptor, GPR 124, is required for correct Wnt signaling in establishing the blood brain barrier (Zhou and Nathans, 2014; Posokhova et al., 2015). Here, Wnt7 is the locally acting ligand, working through FZD and LRP, but whether Wnt7A binds directly to the multiple pass transmembrane protein GPR124 is not clear (Figure 3) (Zhou and Nathans, 2014).

Whether all of these Wnt receptors, including ROR, RYK and GPR124 cooperate on cells, forming higher order structures, or operate independently is a major question that would require the development of new assays. Going back to the structure of the Wnt-FZD complex, it is striking that there is extensive surface left between the two separate binding domains on Wnt for FZD, suggesting that other molecules, including other receptors could participate in the complex leading to productive signaling.

Natural Wnt Inhibitors

As is commonly seen in signaling pathways, Wnt activity is regulated by extracellular proteins that antagonize the ligand. A recent example is Notum, originally discovered in *Drosophila* as an enzyme, a carboxylesterase that can remove the palmitoleate modification on Wnt (Kakugawa et al., 2015; Zhang et al., 2015) (Figure 1). As mentioned before, this palmitoleate is essential for signaling and participates in the binding of Wnt to FZD. The structure of Notum shows a large hydrophobic pocket in the protein that accommodates a palmitoleate moiety. Hydrolysis of the palmitoleate by Notum could leave an intact Wnt protein outside of cells, where it could act as a dominant interfering molecule, although this is presently unknown.

Other Wnt antagonists include proteins of the Dickkopf (DKK) and the Sclerostin/SOST families (Cruciat and Niehrs, 2013). These molecules antagonize Wnt signaling by binding LRP5/6, possibly disrupting Wnt-induced FZD-LRP6 dimerization (Cruciat and Niehrs, 2013). Wnt-interfering molecules also include the secreted FZD-related proteins (sFRPs) and Wnt inhibitory protein (WIF) proteins, both able to bind to Wnts directly. Taken altogether, a picture emerges of a complex extracellular landscape of Wnt-modifying and Wnt-binding factors, fine-tuning the strength of signaling (Niehrs, 2012).

Two highly homologous Wnt target genes, Rnf43 and Znrf3, were recently identified as potent negative-feedback regulators of Wnt signal strength. The two proteins were originally identified to be specific to the Wnt-dependent Lgr5 stem cells of crypts

(Koo et al., 2012) and to be enriched in colon cancer cells that carry activating Wnt pathway mutations (Hao et al., 2012). Like the founding member of this family, Grail, the two proteins are single-pass transmembrane E3 ligases carrying intracellular RING domains. Rnf43 and Znrf3 specifically mediate multi-ubiquitination of lysines in the cytoplasmic loops of the 7TM domain of FZDs (Figure 2) (Hao et al., 2012; Koo et al., 2012). This induces rapid endocytosis and lysosomal destruction of the Wnt receptors. The orthologous *C. elegans* PLR-1 E3 ligase A similarly abrogates FZD surface expression (Moffat et al., 2014). The structural basis for how the E3 ligases identify FZDs as their specific substrates is currently not exactly known, although it has been proposed that DVL proteins act as intermediaries in the recognition process (Jiang et al., 2015).

Loss of these two E3 ligases is predicted to result in hyper-responsiveness to endogenous Wnt signals. Indeed, co-deletion of these two Wnt modulators in murine intestinal epithelium induces an adenomatous expansion of the crypts (Koo et al., 2012), which disappears upon treatment with small molecule inhibitors of the Porcupine enzyme (required for the critical lipid modification of Wnt) (Koo et al., 2015). Mutations in Rnf43 and Znrf3 have been observed in a variety of human cancers, rendering the malignant cells dependent on much lower levels of Wnt than their healthy counterparts and sensitive to inhibition at the Wnt receptor ligand level (see below).

The Lgr5/Rnf43/R-Spondin Module of Wnt Signal Amplification

The vertebrate genome harbors four secreted R-spondin proteins, each carrying two N-terminal furin domains and a thrombospondin domain. Kazanskaya et al. (2004) first identified the R-spondins as Wnt signal enhancers in *Xenopus* embryos. R-spondin-1 was subsequently shown to potently promote Wnt-dependent intestinal crypt proliferation in vivo (Kim et al., 2005) and in vitro (Sato et al., 2009). Three members of a small family of 7-TM receptors, Lgr4, Lgr5, and Lgr6 family, bind R-spondins with high affinity and are essential for signal enhancement of low dose Wnt (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). The Lgr proteins bind R-spondins through their N-terminal ectodomain and do not appear to utilize G-proteins (Carmon et al., 2011; de Lau et al., 2011).

The prototype member of the Lgr-subfamily, *Lgr5*, was already known to mark adult stem cells in a number of actively self-renewing organs, notably of the intestine (Barker et al., 2007). A strong genetic interaction was described to exist between Lgr4 and Lgr5 in the maintenance of Wnt signal strength in crypts of double mutant mice (de Lau et al., 2011). In recent work, it was shown that Wnts by themselves are not sufficient for self-renewal of $Lgr5^+$ stem cells but instead confer competency by maintaining Rspo receptor expression and resulting stem cell expansion (Yan et al., 2017).

Lgr6 similarly marks stem cells in the skin (Füllgrabe et al., 2015; Snippert et al., 2010). Thus, the notion that the Lgr proteins act as receptors for R-spondins reinforced the intimate connection between Wnt signaling and adult stem cell biology.

But how do R-spondins and Lgrs amplify Wnt signals? Hao et al. (2012) performed a series of biochemical experiments that showed that R-spondins in an Lgr-dependent manner reversed the Rnf43/Znrf3-mediated membrane clearance of Wnt receptors. A weak yet specific interaction of R-spondin with Znrf3 was observed. A model, strengthened by X-ray crystallography (de Lau et al., 2014), was then formulated in which R-spondin's high-affinity interaction with Lgr5 through its Furin-2 repeat, allows the other Furin repeat in R-spondin to interact with Rnf43/Znrf3. This in turn would result in membrane clearance of the E3 ligases and persistence of activated Wnt/FZD/LRP receptor complexes on the plasma membrane, boosting Wnt signal strength and duration (Figure 2).

While Rnf43/Znrf3 homologs exist in invertebrates, the R-spondin/Lgr5/Rnf43 module is a recent evolutionary "add-on" seen only in vertebrates and by-and-large dedicated to adult stem cells. The evolutionary emergence of sophisticated stem cell/transit amplifying cell compartments in vertebrates coinciding with the general increase in vertebrate body size may have been facilitated by this novel mechanism of Wnt signal amplification.

The Cytoplasmic APC/Axin Destruction Complex Controls β-Catenin Stability

The key switch in the canonical Wnt pathway is the cytoplasmic protein β-catenin (Figure 4). Its stability is controlled by the destruction complex (DC). In this complex, the tumor suppressor protein Axin acts as the scaffold, interacting with β-catenin, the tumor suppressor protein APC, and two constitutively active serine-threonine kinases (CK1α/δ and GSK3α/β). The large APC protein contains three Axin-binding motifs that are interspersed between a series of 15- and 20-amino acid repeats that bind β-catenin. Although it is clear from studies on colorectal cancer that APC is essential for DC function, its specific molecular activity has only partially been resolved (Figure 4).

When Fz/LRP receptors are not engaged by ligands, CK1 and GSK3 sequentially phosphorylate Axin-bound β-catenin at a series of regularly spaced N-terminal Ser/Thr moieties: β-catenin is first phosphorylated by CK1 at Ser45, followed by GSK3 phosphorylation at Thr41, Ser37, and Ser33 residues (Liu et al., 2002). The phosphorylated "degron"-motif acts as a docking site for the F-box-containing protein E3 ubiquitin ligase β-TrCP inducing ubiquitination and subsequent proteasomal degradation of β-catenin (Aberle et al., 1997; Kitagawa et al., 1999) (Figure 4). Receptor engagement relocalizes the DC to the cell membrane and interferes with its activity such that free β-catenin levels rapidly increase. At the same time, the phosphorylated LRP receptor may act to inhibit GSK-3 directly and thereby promote β-catenin stabilization (Stamos et al., 2014). Biochemical scrutiny of the endogenous DC has revealed that ubiquitination of phosphorylated β-catenin is blocked within the intact complex. As a consequence, the complex becomes saturated by the phosphorylated form of β-catenin, leading to accumulation of newly synthesized β-catenin, free to translocate to the nucleus and to activate target genes (Li et al., 2012; Azzolin et al., 2014). As an alternative mechanism, it has been proposed that Wnt receptor engagement models result in the dynamic regulation of β-catenin phosphorylation (Hernández et al., 2012) and phosphorylation-regulated Axin-complex disassembly (Kim et al., 2013). Several studies have identified a highly conserved

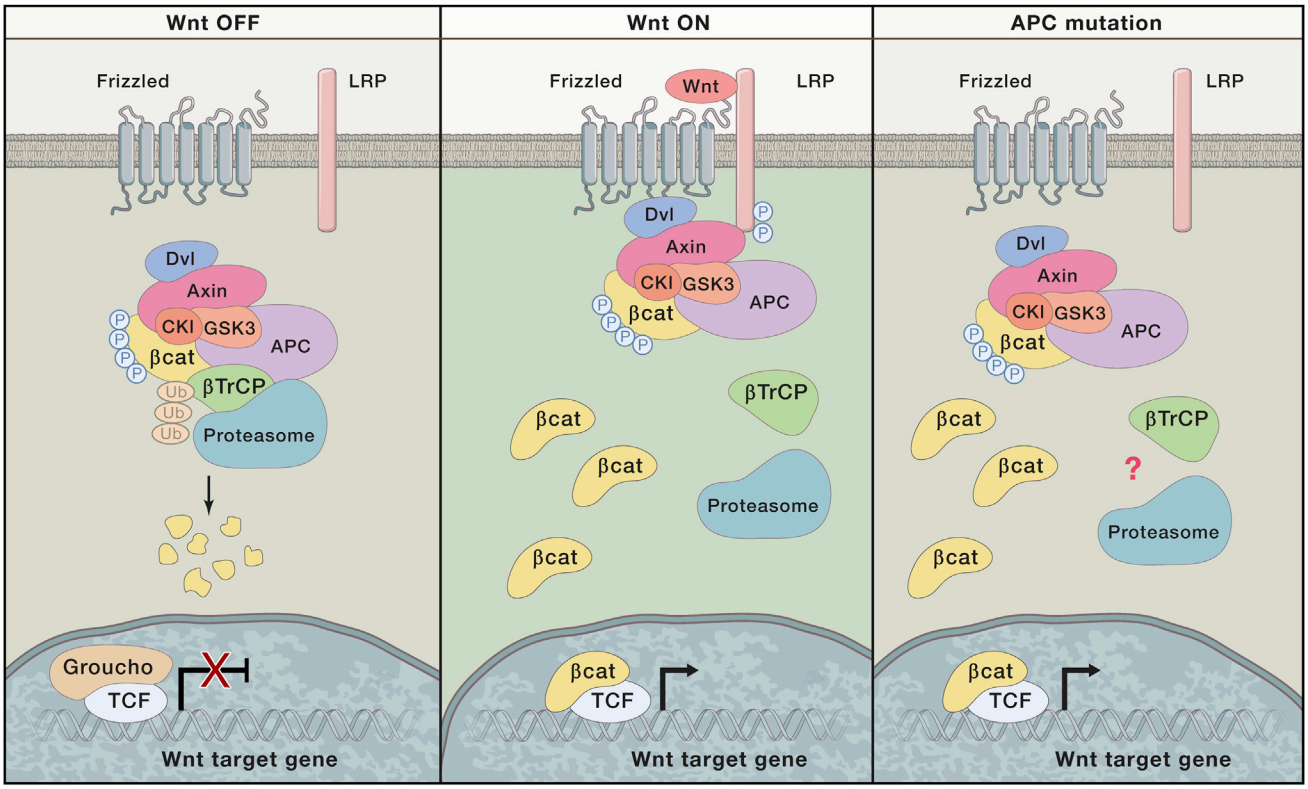

Figure 4. Wnt Signaling in Cells

Left: In the absence of a Wnt signal, β-catenin is degraded by a complex of proteins including Axin, APC, the Ser/Thr kinases GSK-3 and CK1, protein phosphatase 2A (PP2A), and the E3-ubiquitin ligase β-TrCP. The complex specifies a β-TrCP recognition site on β-catenin by phosphorylation of a conserved Ser/Thr-rich sequence near the amino terminus. Phosphorylation requires scaffolding of GSK-3 and CK1 and β-catenin by Axin. After phosphorylation and ubiquitination, β-catenin is degraded by the proteasome. SCF, the Skp1/cullin/F-box complex. Dvl (Disheveled) is required for activating the pathway as well. In the nucleus, T cell factor (TCF) is in an inactive state as the consequence of binding to the repressor Groucho.

Center: Binding of Wnt to its receptors induces the association of Axin with phosphorylated lipoprotein receptor-related protein (LRP). The destruction complex falls apart, and β-catenin is stabilized, subsequently binding TCF in the nucleus to upregulate target genes.

Right: Mutations in APC disrupt the degradation complex and thereby lead to activation of the pathway.

regulatory domain in APC, the β-catenin inhibitory domain (CID), located between the second and the third 20-amino acid repeats (Kohler et al., 2009; Roberts et al., 2011). The CID is believed to be essential for downregulating β-catenin levels and Wnt transcriptional activity. In agreement, the CID is located right at the mutation cluster region, the site of common truncation of APC in cancer. The CID has been proposed to promote β-catenin ubiquitination by stabilizing the association with APC as well as to repress β-catenin/TCF transcription in the nucleus (Choi et al., 2013). A more recent study proposed another model: GSK3-mediated phosphorylation around the CID region induces a conformational change in the APC protein that allows accessibility of the E3-ligase to phospho-β-catenin (Figure 4) (Pronobis et al., 2015).

Complicating its analysis, β-catenin plays a second, major role in epithelia. It is an essential binding partner for the cytoplasmic tail of various cadherins, such as E-cadherin in adhesion junctions (Peifer et al., 1992). While the half-life of the signaling pool of β-catenin is in the order of minutes, the adherens junction-pool is highly stable. The adhesive and signaling properties of β-catenin are most likely independent. Indeed, in *C. elegans* the two functions of β-catenin are performed by distinct homologs (Korswagen et al., 2000).

TCFs Are the Effectors of the Wnt Cascade

Canonical Wnt signaling leads to a defined cellular response through the activation of β-catenin/TCF target genes (Figure 4). Upon Wnt pathway activation, β-catenin accumulates in the cytoplasm and nucleus, where it engages DNA-bound TCF transcription factors (Behrens et al., 1996; Molenaar et al., 1996). The cognate TCF binding motif is 5′-AGATCAAAGG-3′ (van de Wetering et al., 1997). Widely used Wnt/TCF reporters such as pTOPflash (Korinek et al., 1997) contain multimers of this motif. In the Wnt "off" state, Tcfs interact with Groucho proteins to mediate transcriptional repression (Cavallo et al., 1998; Roose et al., 1998). In the Wnt "on" state, engagement of β-catenin transiently converts TCF into a transcriptional activator (Figure 4). While most Wnt target genes are cell-type- and developmental stage-specific, the *Axin2* gene represents a generic transcriptional target gene, often used as indicator of canonical Wnt pathway activity (Lustig et al., 2002). Active Wnt signaling may involve an increase in overall β-catenin levels without any

detectable nuclear accumulation. It has been suggested that fold-change rather than absolute β-catenin levels are critical, implying that, indeed, low levels of nuclear β-catenin suffice for target gene activation (Goentoro and Kirschner, 2009). Multiple non-TCF transcription factors have been implied as alternative transcriptional effectors. These studies typically await independent confirmation. Contrasting with these studies, recent genome-wide approaches in mammalian cells (Schuijers et al., 2014) and *Drosophila* (Franz et al., 2017) imply that all direct activation of β-catenin target genes involves TCFs as final effectors. β-catenin, once recruited to promoter and enhancer elements, activates gene transcription through its C-terminal transcriptional activation domain (van de Wetering et al., 1997). It binds chromatin modifiers such as CBP and Brg-1 (reviewed in (Städeli et al., 2006) and Parafibromin/Hyrax, homologs of yeast Cdc73 (Mosimann et al., 2006).

Wnt Signals Control Stem Cell Biology and Growth

Wnts exert a wide variety of effects on target cells during development. Arguably, the hottest focus of the Wnt field involves its role in healthy stem cells and in cancer. Stem cells—be it embryonic stem (ES) cells or adult stem cells—display the defining capacity to self-renew, while also producing specialized cells. Stem cell fate and behavior are primarily dictated by extrinsic, short-range signals, which typically emanate from the stem cell niche (Losick et al., 2011).

As first proof of the involvement of Wnt in adult stem cell biology, gene disruption of mouse TCF4 lead to loss of intestinal stem cells and the subsequent breakdown of the epithelium (Korinek et al., 1998). Since then, the Wnt pathway has been found to be required for most if not all stem cell types. Thus, the ES phenotype can be maintained in culture by just two small molecules, one being the Wnt activating GSK3 inhibitor CHIR (Silva et al., 2008). Indeed, purified Wnt protein maintains pluripotency of ES cells as well (ten Berge et al., 2011).

In the hair follicle, Wnt signaling plays multiple roles in the biology of stem cells and progenitors (DasGupta and Fuchs, 1999; Lim et al., 2016). Blocking Wnt signaling by overexpression of Dkk eliminates hair follicles and other skin appendages, such as the mammary gland (Andl et al., 2002). In the hematopoietic system, overexpressing Axin1 lowers the numbers of transplantable stem cells (Reya et al., 2003). In another approach, treatment of hematopoietic stem cells with isolated Wnt3a protein increases self-renewal, as measured by clonogenic assays and long-term reconstitution in irradiated mice (Willert et al., 2003).

LGR5 and Axin2 (two stem cell-specific Wnt target genes, themselves encoding Wnt pathway components) have allowed the creation of powerful genetic tools for lineage tracing of a multitude of known and novel adult stem cells. Lgr5 is expressed in small, cycling cells at the base of small intestinal crypts that were observed originally by Paneth (1887) and were later postulated (Cheng and Leblond, 1974) to represent the intestinal stem cells. An Lgr5 locus-specific CreERT2 mouse demonstrated by lineage tracing that the constantly cycling Lgr5^{+} stem cells are long-lived, multipotent adult stem cells (Barker et al., 2007). Using the same lineage-tracing strategy, Lgr5 was subsequently demonstrated to mark stem cells in many other organs and tissues, including the hair follicle (Jaks et al., 2008), stomach (Barker et al., 2010), pancreas (Huch et al., 2013a), liver (Huch et al., 2013b), kidney (Barker et al., 2012), ovarial epithelium (Ng et al., 2014), inner ear (Chai et al., 2012; Shi et al., 2012), taste buds (Yee et al., 2013), and mammary gland (de Visser et al., 2012; Plaks et al., 2013). In agreement, lineage tracing approaches based on Axin2-CreERT2 and other genes have revealed Wnt-responsive adult stem cell function in the mammary gland (van Amerongen et al., 2012), the interfollicular epidermis (Lim et al., 2013), the quiescent bulge of telogen hair follicles (Lim et al., 2016), the nail (Takeo et al., 2013), and the pericentral region of liver lobules (Wang et al., 2015).

Growing Organoids from Adult Stem Cells by Driving Wnt Signaling

An organoid can be defined as a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organizes through cell sorting and spatially restricted lineage commitment. Purified Wnt protein was shown to expand the number of clonogenic cells from mammary gland adult stem cells, while retaining the developmental potential of the cells upon transplantation (Zeng and Nusse, 2010). More complete organoids were observed when growth factors cocktails were refined. Based on the observation that the Wnt-dependent Lgr5 crypt stem cells divide 1,000s of times in vivo, a culture system was established that allows growth of epithelial organoids ("mini-guts") from a single Lgr5 stem cell (Sato et al., 2009). The stem cells are suspended in Matrigel and are stimulated with R-spondin1, complemented with EGF and the BMP inhibitor Noggin. The organoids grow as a simple highly polarized and fully differentiated epithelium, tightly closing off a central lumen, from which crypt-like structures project outward. All cell types of the gut epithelium are represented at normal ratios (Grün et al., 2015; Sato et al., 2009). The organoids can be grown for years and are remarkably stable, both genetically and phenotypically. As proof of this stability, organoids grown from a single murine Lgr5 colon stem cell were transplanted into multiple mice with experimental colitis. The integrated organoids persisted long-term as functional epithelial patches, indiscernible from the surrounding host epithelium (Yui et al., 2012). Addition of small molecule inhibitors of Alk and p38 allowed long-term culture of human small intestine and colon organoids (Jung et al., 2011; Sato et al., 2011). Similar cultures that additionally contained mesenchymal elements could be started from induced pluripotent stem cells (iPSCs) (Spence et al., 2011)

This culture system has since been adapted to grow organoids from Wnt-dependent adult stem cells from the epithelial compartments of a growing number of mouse and human tissues of ecto-, meso-, and endodermal origin. The essential components are a potent source of Wnt, a potent activator of tyrosine kinase receptor signaling (such as EGF), inhibition of BMP/TGF-β signals, and Matrigel. Thus, organoid protocols have been reported for mouse and human stomach (Barker et al., 2010; Bartfeld et al., 2015; McCracken et al., 2014), liver (Huch et al., 2013b, 2015), pancreas (Boj et al., 2015; Huch et al., 2013a), prostate (Boj et al., 2015; Chua et al., 2014; Huch et al., 2013a; Karthaus et al., 2014), taste buds (Ren et al., 2014), inner ear (McLean et al., 2017), esophagus (DeWard et al., 2014), fallopian tube epithelium (Kessler et al., 2015),

Table 1. Diseases Associated with Wnt Signaling Components

Disease	Gene	Reference
Bone density defects	LRP5	Gong et al., 2001; Little et al., 2002; Boyden et al., 2002
	LGR4	Styrkarsdottir et al., 2013
	SOST	Brunkow et al., 2001; Balemans et al., 2001
	WNT16	Zheng et al., 2012
	WNT1	Pyott et al., 2013
	WTX	Jenkins et al., 2009
Familial exudative vitreoretinopathy	LRP5	Toomes et al., 2004
	FZD4	Robitaille et al., 2002
	Norrin	Xu et al., 2004
	TSPAN12	Poulter et al., 2010
Robinow syndrome	WNT5A	Person et al., 2010
	DVL1	White et al., 2015
	ROR2	van Bokhoven et al., 2000
Tooth development defects	LRP6	Massink et al., 2015
	WNT10A	Adaimy et al., 2007
	WNT10B	Yu et al., 2016
	AXIN2	Lammi et al., 2004

Based on http://web.stanford.edu/group/nusselab/cgi-bin/wnt/human_genetic_diseases (selected for diseases with multiple pathway components implicated).

mammary gland (Jamieson et al., 2016), and salivary gland (Maimets et al., 2016; Nanduri et al., 2014).

The development of potent "surrogate" Wnt proteins greatly facilitates the activation of Wnt receptors in organoid cultures, as the surrogates are not lipid-modified and therefore do not require serum-derived carrier proteins (Janda et al., 2017). Another technical improvement involves the replacement of Matrigel by a synthetic hydrogel (Gjorevski et al., 2016). It currently appears that most, if not all, mammalian epithelia utilize Wnt-dependent Axin2/Lgr5$^+$ stem cells for their homeostatic self-renewal and damage repair, and this, likely in all cases, allows the establishment of culture conditions for long-term organoid growth.

Wnt Signaling, Diseases, and Therapies

Cancer

Since Wnt signals are crucial for the activity of epithelial stem cells, it is not surprising that Wnt pathway mutations are frequently observed in carcinomas. The APC gene was first identified by being mutated in a hereditary colon cancer syndrome termed familiar adenomatous polyposis (Kinzler et al., 1991; Nishisho et al., 1991). Similarly, most cases of sporadic colorectal cancer result from loss of both APC alleles (Kinzler and Vogelstein, 1996; Wood et al., 2007). Loss of APC function leads to the inappropriate stabilization of β-catenin (Rubinfeld et al., 1996) and the formation of constitutive complexes between β-catenin and the intestinal TCF family member TCF7l2/TCF4 (Korinek et al., 1997).

A growing series of activating mutations in other Wnt pathway components has been reported since in a variety of cancers. Patients with hereditary Axin2 mutations display a predisposition to colon cancer (Lammi et al., 2004). In rare cases of colorectal cancers that are wild-type for APC, the same *Axin2* gene is mutated (Liu et al., 2000). Axin1 mutations were first noted in hepatocellular carcinomas (Satoh et al., 2000). In a small, distinct set of colon cancer cases, activating point mutations in β-catenin remove the regulatory N-terminal Ser/Thr residues (Morin et al., 1997). Similar β-catenin mutations were reported in melanoma (Rubinfeld et al., 1997) and have since been observed in a variety of other carcinomas.

Most recently, inactivating mutations were first reported in the E3 ligase genes Rnf43 in pancreas cancer (Wu et al., 2011) and Znrf3 in adrenocortical carcinoma (Assié et al., 2014) and subsequently seen in multiple other cancers, adding these two genes to the list of Wnt pathway tumor suppressors. Gene fusions involving R-spondin2 or R-spondin3 are observed in yet another class of rare APC wild-type (WT) colon cancers (Seshagiri et al., 2012). These latter mutations and fusions render the cancer cells highly sensitive to low levels of Wnt, yet (unlike APC, Axin1/2, or β-catenin mutants) are still ultimately dependent on exogenous Wnts and have been implied to be treatable with inhibitors of Wnt secretion or of the FZD/LRP receptor complex (see below).

The link between Wnt-driven stem cells and carcinogenesis is reinforced by reports that demonstrate a link between Wnt signal strength, stem cell signature, and colon cancer stem cell behavior (Merlos-Suárez et al., 2011; Vermeulen et al., 2010; Tammela et al., 2017).

Degenerative Diseases

There are many degenerative genetic diseases caused by mutations in Wnt signaling components, either at the somatic cell level or with an inherited component. Table 1 lists various diseases and the Wnt pathway-associated genes that are mutated. Among these are genetic cases where multiple different Wnt signaling components are involved in the same disease, including abnormalities in bone density, tooth development, and the retina. The best-known disorders are mutations in the SOST and LRP6 genes causing sclerosteosis and hereditary osteoporosis (Baron and Kneissel, 2013). Another example of the involvement of multiple Wnt components comes from the retina, where disorders such as familial exudative vitreoretinopathy can be caused by mutations in LRP5, FZD4, or Norrin (Table 1).

The nature of these mutations not only illuminates the relevance of the pathways for human health, it also sheds light on the mechanisms of signaling. For example, patients with hereditary abnormal high bone mass carry specific mutations in the LRP5 extracellular domain (Boyden et al., 2002), that generate the receptor refractory to binding of the antagonists SOST and DKK1 (Ellies et al., 2006; Chu et al., 2013). In this case, mapping the sites of the mutations suggested locations of protein interactions. A striking example of how genetics inform Wnt pathway understanding comes from Robinow syndrome. This inherited disease, affecting the skeleton in addition to other parts of the body, is associated with mutations in three different Wnt signaling components: Wnt5a, ROR2 (van Bokhoven et al., 2000), and DVL1 (Table 1).

Wnt Modulators in the Clinic

What can we learn from these disease implications, and can therapies be designed based on Wnt signaling mechanisms?

Table 2. Small Molecules to Activate or Inhibit Wnt Signaling

Compound	Target	Inhibitor/Activator of the Target	Effect on Wnt Signaling	Reference
IWP	Porcupine	inhibitor	inhibits	Chen et al., 2009
LGK974	Porcupine	inhibitor	inhibits	Kulak et al., 2015
C59	Porcupine	inhibitor	inhibits	Proffitt et al., 2013
Apicularen and bafilomycin	vacuolar ATPase	inhibitor	inhibits	Cruciat et al., 2010
XAV939	tankyrase Axin	activates Axin	inhibits	Huang et al., 2009
IWR	tankyrase, Axin	activates Axin	inhibits	Kulak et al., 2015
G007-LK, G244-LM	tankyrase, Axin	activates Axin	inhibits	Lau et al., 2013
IQ1	PP2A	activator	activates	Miyabayashi et al., 2007
QS11	ARFGAP1	activator	activates	Zhang et al., 2007
SB-216763	GSK3	inhibitor	activates	Coghlan et al., 2000
CHIR99021	GSK3	inhibitor	activates	Ying et al., 2008
BIO (6-bromoindirubin-3′-oxime)	GSK3	inhibitor	activates	Sato et al., 2004
L807mts	GSK3	inhibitor	activates	Licht-Murava et al., 2016
LY2090314	GSK3	inhibitor	activates	Atkinson et al., 2015
ICG-001	CREB-binding protein	inhibitor	inhibits	Emami et al., 2004

Based on http://web.stanford.edu/group/nusselab/cgi-bin/wnt/smallmolecules.

In considering a widely used pathway such as Wnt as a target for intervention, a concern arises from the predicted side effects of drugs (Kahn, 2014). In the case of Wnt signaling, however, one of the components, SOST, provides a unique target in bone diseases, including osteoporosis. SOST, one of the negative regulators of Wnt, is expressed in the bone only and has phenotypes limited to the bone tissue. This suggests that blocking SOST would only impact on bone (Jawad et al., 2013), and indeed, an antibody targeting SOST, known under the brand name Romosozumab, has yielded encouraging results in clinical trials (Cosman et al., 2016).

With respect to other possible targets, extensive efforts have been made to block Wnt signaling with small molecules, facilitated by sensitive and quantitative Wnt reporters (Table 2). The most effective target in the case of cancer would be the complex between TCF and β-catenin, as it mediates signaling at a downstream node in the pathway, but despite numerous efforts, this target has proven to be elusive. The screens have, however, led to compounds that impact the stability of Axin, which is regulated by tankyrase-mediated ADP-ribosylation. Molecules such as IWR (Lu et al., 2009) and XAV939 (Huang et al., 2009) inhibit tankyrase, thereby increasing Axin levels and lowering β-catenin to inhibit Wnt signaling (Kulak et al., 2015). At other levels of Wnt signaling, very specific and useful inhibitors have been found to block Porcupine, the enzyme catalyzing the acylation of Wnt proteins (Table 2). These molecules include IWP2, C59, and LGK974, all inhibiting Porcupine and thereby leading to a block in Wnt secretion, as acylation is required for Wnt transport (Lu et al., 2009). In cancers that are the consequence of β-catenin/APC mutations, it is unlikely that interfering with Wnt would have a significant effect on the growth of the tumors. On the other hand, there is substantial evidence that the outgrowth of metastatic lesions and cancer stem cells is promoted by Wnts themselves (Malladi et al., 2016; Nguyen et al., 2009; Yu et al., 2013; Tammela et al., 2017), suggesting that Porcupine targeting drugs could be beneficial. In promising experiments, some of these drugs have been shown to inhibit the growth of transplanted or even autochthonous tumors in mouse models (Madan et al., 2016; Tammela et al., 2017) and a clinical trial for the Porcupine inhibitor LGK974, in patients with several forms of cancer, is ongoing (https://clinicaltrials.gov/ct2/show/NCT01351103).

The possible requirement for Wnt ligands in cancer cell proliferation also boosts hope for intervention at the receptor level, and there are promising leads in this area. A recent example comes from genetic screens for mutations that sensitize pancreatic tumor cells that are mutant for RNF43 (RNF43 mutations make these cells dependent on Wnt ligand). Among the mutation suppressing the RNF43 growth phenotype were several in FZD5, indicating that the tumor cells are dependent on Wnt-FZD signaling. As a follow-up, it was shown that the growth of these tumors was attenuated by antibodies directed at FZDs (Steinhart et al., 2017). Similarly, a monoclonal antibody (OMP-18R5) that binds to several FZD family members inhibits the growth of several tumors in xenograft studies, while antibodies that are raised against R-spondins cause the differentiation of colon tumor cells and loss of stem cell function (Storm et al., 2016).

In addition to blocking Wnt signaling, clinical value could also emerge from stimulating the pathway for tissue regeneration. The Wnt protein itself is problematic for use as a drug because of its hydrophobicity and because of complications in producing significant quantities. Recently, however, soluble Wnt protein agonists have been shown to activate Wnt signaling in vivo (Janda et al., 2017). In addition, several small molecule compounds (L807mts, Bio, CHIR, and SB-216763) (Licht-Murava et al., 2016) interfere with GSK3 and thus induce Wnt target gene expression. There is hope that these drugs are of use in treating neurodegenerative disorders, including Alzheimer's disease (Licht-Murava et al., 2016). Mechanistically, the effect of GSK3 inhibitors in the CNS could be mediated by the Wnt target gene REST, which acts as a is a repressor of neuronal genes

during embryonic development and has been shown to be protective in Alzheimer's disease (Lu et al., 2014).

In conclusion, we are now at a point in the history of Wnt signaling where the implications of this pathway for understanding disease are coming into focus. The efforts in finding ways to interfere with Wnt signaling are still at an early stage, but there are promising leads that hopefully will translate soon into real therapies.

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