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Leading Edge
Review

Wnt/β-Catenin Signaling and Disease

Hans Clevers[1,*] and Roel Nusse[2]
[1]Hubrecht Institute, KNAW and University Medical Center Utrecht, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
[2]Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
*Correspondence: h.clevers@hubrecht.eu
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The WNT signal transduction cascade controls myriad biological phenomena throughout development and adult life of all animals. In parallel, aberrant Wnt signaling underlies a wide range of pathologies in humans. In this Review, we provide an update of the core Wnt/β-catenin signaling pathway, discuss how its various components contribute to disease, and pose outstanding questions to be addressed in the future.

A Brief History of the Field

The *Wnt1* gene, originally named Int-1, was identified in 1982 as a gene activated by integration of mouse mammary tumor virus proviral DNA in virally induced breast tumors (Nusse and Varmus, 1982). The *Wnt1* proto-oncogene encodes a secreted, cysteine-rich protein. The fly *Wingless* (*wg*) gene, which controls segment polarity during larval development (Nüsslein-Volhard and Wieschaus, 1980), was later shown to be a homolog of *Wnt1* (Rijsewijk et al., 1987). By 1994, epistasis experiments examining the relationships among segment polarity mutations delineated the core of this developmental signal transduction cascade in *Drosophila* (e.g., *porcupine*, *dishevelled*, *armadillo* (β-catenin), and zeste-white 3/GSK3 gene (Noordermeer et al., 1994; Peifer et al., 1994; Siegfried et al., 1992). Injection of mouse Wnt1 mRNA into early frog embryos caused a duplication of the body axis in *Xenopus*, providing an assay to study the Wnt pathway in vertebrates (McMahon and Moon, 1989). The combined observations from *Drosophila* and *Xenopus* unveiled a highly conserved signaling pathway, commonly referred to as the canonical Wnt cascade. A few years later, major gaps in Wnt signal transduction were closed with the identification of TCF/LEF transcription factors as Wnt nuclear effectors (Behrens et al., 1996; Molenaar et al., 1996) and Frizzleds as Wnt receptors (Bhanot et al., 1996), which work together with LRPs/Arrow as coreceptors (Wehrli et al., 2000).

The first direct connection between the Wnt pathway and human disease came in the early 1990s. The adenomatous polyposis coli (APC) gene was discovered independently in a hereditary cancer syndrome termed familial adenomatous polyposis (FAP; Kinzler et al., 1991; Nishisho et al., 1991). Soon thereafter, the large cytoplasmic APC protein was found to interact with β-catenin (Rubinfeld et al., 1993; Su et al., 1993). Many additional pathway components and disease connections were uncovered over the last two decades. Below, we discuss these, taking the reader from Wnt secretion through Wnt reception and signal transduction to the nuclear response of the recipient cell.

Wnt Proteins Are Lipid Modified: Wnt Secretion Is Complex and Involves a Dedicated Machinery

Most mammalian genomes, including the human genome, harbor 19 Wnt genes, falling into 12 conserved Wnt subfamilies. At least 11 of these subfamilies occur in the genome of a Cnidaria (the sea anemone *Nematostella vectensis*), emphasizing the crucial role that Wnt proteins play in organismal patterning throughout the animal kingdom (Kusserow et al., 2005). Even sponges contain a few Wnt genes, whereas single-cell organisms do not, suggesting that Wnt signaling may have been instrumental in the evolutionary origin of multicellular animals (Petersen and Reddien, 2009), and mutations of six Wnt genes have been identified in a variety of hereditary conditions (Table 1).

Wnt proteins are ~40 kDa in size and contain many conserved cysteines (Tanaka et al., 2002). Despite the initial discovery of Wnt nearly 30 years ago, efficient production and biochemical characterization of Wnt proteins remain challenging. The first successful purification of active mouse Wnt3A revealed that Wnts are lipid modified (Willert et al., 2003). One of these is a mono-unsaturated fatty acid (palmitoleic acid) attached to a conserved serine (Takada et al., 2006).

The lipids on Wnts are required for efficient signaling and may be important for Wnt secretion (Franch-Marro et al., 2008a; Kurayoshi et al., 2007; Willert et al., 2003). Most recently, the structure of the *Xenopus* Wnt8 protein as bound to Frizzled was solved, revealing two domains on Wnt that interact with the receptor (Janda et al., 2012). Interestingly, one of these domains contains the palmitoleic acid lipid, which projects into a pocket in the Frizzled CRD, a configuration that reinforces the importance of the lipid for signaling. The role of the lipid is also reflected by the requirement for Porcupine (Porc; Figure 1), a dedicated and highly conserved component of the Wnt pathway active only in Wnt-producing cells. Porc is a multipass transmembrane O-acyltransferase in the ER that is essential for Wnt palmitoylation and maturation (Hofmann, 2000; Kadowaki et al., 1996). Loss of Porcupine leads to retention of Wnt3A in the ER (Takada et al., 2006) and a defect in Wg secretion in the *Drosophila*

Table 1. Human Diseases Associated with Mutations of Wnt Pathway Components after MacDonald et al. (2009) and the Wnt Homepage[a]

Protein	Mutation Type and Associated Human Disease(s)	Key References
PORCN	LOF X-linked focal dermal hypoplasia	Grzeschik et al., 2007; Wang et al., 2007
WNT3	LOF tetra-amelia	Niemann et al., 2004
WNT4	LOF Mullerian duct regression and virilisation	Biason-Lauber et al., 2004
WNT5B	LOF? type II diabetes	Kanazawa et al., 2004
WNT7A	LOF Fuhrmann syndrome	Woods et al., 2006
WNT10A	LOF odonto-onchyo-dermal hypoplasia	Adaimy et al., 2007
WNT10B	LOF obesity	Christodoulides et al., 2006
RSPO1	LOF XX sex reversal with palmoplantar hyperkaratosis	Parma et al., 2006
RSPO4	LOF autosomal-recessive anonychia and hyponychia congenita	Blaydon et al., 2006
SOST	LOF high bone mass, sclerosteosis, Van Buchem disease	Balemans et al., 2001; Brunkow et al., 2001
Norrin (NDP)	LOF familial exudative vitreoretinopathy	Xu et al., 2004
LRP5	GOF (alternative splicing) hyperparathyroid tumors, GOF high bone mass, LOF osteoporosis-pseudoglioma, LOF eye vascular defects	Björklund et al., 2007; Boyden et al., 2002; Gong et al., 2001; Little et al., 2002; Toomes et al., 2004
LRP6	LOF early coronary disease and osteoporosis	Mani et al., 2007
FZD4	LOF familial exudative vitreoretinopathy	Robitaille et al., 2002
FZD9	LOF Williams-Beuren Syndrome	Wang et al., 1999
TSPAN12	LOF familial exudative vitreoretinopathy	Nikopoulos et al., 2010; Poulter et al., 2010
APCDD1	LOF hereditary hypothrochosis simplex	Shimomura et al., 2010
Axin1	LOF caudal duplication, cancer	Oates et al., 2006; Satoh et al., 2000
Axin2	LOF tooth agenesis, cancer	Lammi et al., 2004; Liu et al., 2000
APC	LOF familial adenomatous polyposis, cancer	Kinzler et al., 1991; Nishisho et al., 1991
WTX	LOF Wilms tumor, LOF OCTS	Jenkins et al., 2009; Major et al., 2007; Rivera et al., 2007
β-catenin	GOF cancer	Morin et al., 1997
LEF1	LOF sebaceous skin tumor	Takeda et al., 2006
TCF4	GOF type II diabetes, colon cancer	Bass et al., 2011; Grant et al., 2006

[a]Wnt homepage, http://wnt.stanford.edu.

embryo (Kadowaki et al., 1996). The human gene (PORCN) is located on the X chromosome, and mutations lead to the rare genetic disorder focal dermal hypoplasia (Table 1). This disease is characterized by skin abnormalities and other developmental defects (Grzeschik et al., 2007; Wang et al., 2007). Mutations in the X-linked PORCN gene are lethal in males, consistent with the early embryonic lethality due to gastrulation defects observed in mouse knockouts (Barrott et al., 2011; Biechele et al., 2011). Females survive with focal defects due to random X inactivation.

The seven-transmembrane Wntless (Wls) protein provides an essential though less understood function in Wnt secretion (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls localizes to the Golgi network, endosomes, and the plasma membrane and binds Wnt proteins (Figure 1). In Wls mutant cells, Wg accumulates in the Golgi (Port et al., 2008). Wls is thought to act as a sorting receptor, taking Wnt from the Golgi to the plasma membrane. Intriguingly, there is evidence for Wls- and Wg-containing secreted vesicles in the *Drosophila* neuromuscular junction, where the Wg protein is tethered to the outside of the vesicles (Korkut et al., 2009). In this configuration, the Wg protein interacts with its receptor on the receiving muscle. Whether this mode of Wg transport and signaling operates in other contexts is presently unknown.

Several studies in *C. elegans* have revealed that the retromer, an intracellular trafficking complex, is also required for Wnt signaling (Coudreuse et al., 2006; Prasad and Clark, 2006). One of the key functions of the retromer complex involves the retrograde transport of specific endocytosed transmembrane proteins back to the *trans*-Golgi network. Current evidence indicates that the retromer retrieves endosomal Wls, which is otherwise destined to be degraded in lysosomes, trafficking it to the *trans*-Golgi network by retrograde transport (Belenkaya et al., 2008; Franch-Marro et al., 2008b; Port et al., 2008; Yang et al., 2008; Figure 1).

In Most Contexts, Wnts Signal over a Short Distance

In the current literature, it is often taken for granted that Wnt signals are morphogens, molecules that exert their action across a distance in tissues. A classical morphogen forms a gradient that determines cell fate in a concentration-dependent manner. The best-known example of morphogen signaling by a Wnt is in the wing imaginal disk of *Drosophila*, where the Wingless protein (Wg) is produced by a thin line of cells. The morphogen

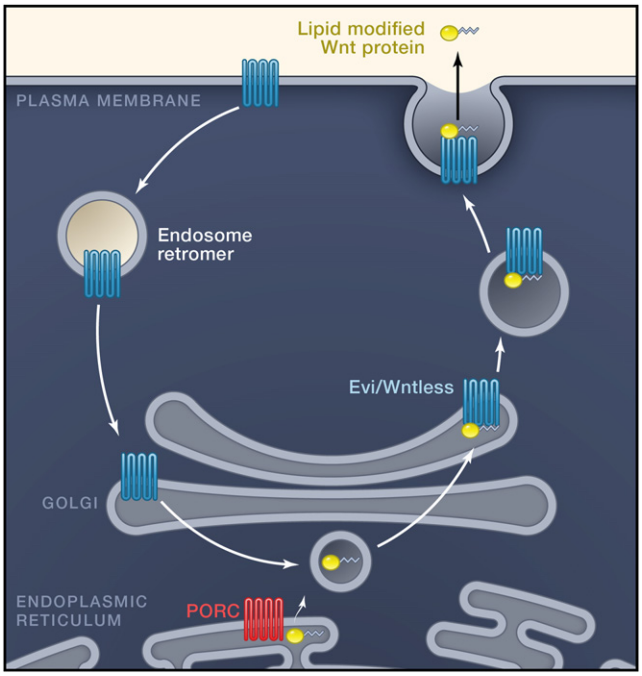

Figure 1. The Wnt Secretion Machinery
Wnt proteins become lipid modified in the endoplasmic reticulum by the Porcupine (Porc) enzyme. Further transport and secretion is dependent on the Evi/Wntless multiple pass transmembrane protein. The Retromer complex is necessary for recycling of the Evi/Wntless endosomal vesicles.

spreads out over the tissue, controlling gene expression at a distance. In this context, the Wg protein may act in association with lipoprotein particles (Panáková et al., 2005; Zecca et al., 1996). Alternatively, long-range signaling by lipid-modified Wg in the wing is facilitated by interactions with specific binding partners, such as the Swim protein (Mulligan et al., 2012).

It should be emphasized, however, that even in *Drosophila*, Wg rarely acts as a long-range signal in organs other than the wing. In the vast majority of the tissues, Wg mediates contact-dependent signaling. In the embryo, Wg interacts with Engrailed-positive cells that are adjacent to Wg-secreting cells (van den Heuvel et al., 1989). Likewise, Wg acts as a short-range signal in the neuromuscular junction (Korkut et al., 2009). In other animals, Wnt signaling appears to occur predominantly between cells that are close to each other, for example, in adult stem cell niches (Strand and Micchelli, 2011; Sato et al., 2010). We propose, therefore, that Wnts are not classical morphogens but signals that mediate close-range signaling.

Wnt Receptors Consist of a Heterodimeric Complex

When interacting with target cells, Wnt proteins bind a heterodimeric receptor complex, consisting of a Frizzled (Fz) and an LRP5/6 protein (Figure 2). The ten mammalian Fz proteins are seven-transmembrane (7TM) receptors and have large extracellular N-terminal cysteine-rich domains (CRD; Bhanot et al., 1996) that provide a primary platform for Wnt binding (Dann et al., 2001; Janda et al., 2012). The structure of the CRD as bound to Wnt shows multiple binding surfaces, one of them containing a hydrophobic groove that interacts with a lipid on the Wnt molecule (Janda et al., 2012). The Wnt-Fz interaction is promiscuous: a single Wnt can bind multiple Fz proteins (e.g., Bhanot et al., 1996) and vice versa, which is also borne out by the structure of the Wnt-CRD complex (Janda et al., 2012). Fzs cooperate with a single-pass transmembrane molecule of the LRP family known as Arrow in *Drosophila* (Wehrli et al., 2000) and LRP5 and -6 in vertebrates (Pinson et al., 2000; Tamai et al., 2000). A recent study describes two monoclonal antibodies against LRP6 with the unexpected ability to inhibit signaling by some Wnt proteins and enhance signaling by others. As these antibodies bind nonoverlapping regions of LRP6 protein, these findings suggest that Lrp6 contains separate binding sites for different classes of Wnt proteins (Gong et al., 2010).

Like many signal transduction pathways, signaling by dimeric Wnt receptors includes a ligand-induced conformational change of the receptors followed by phosphorylation of key target proteins. A crucial step in signaling is binding of Axin to the cytoplasmic tail of LRP6 (Mao et al., 2001; Figure 2). Axin-LRP6 binding is regulated by phosphorylation of the LRP6 tail (He et al., 2004; Tamai et al., 2004) by at least two separate kinases, GSK3 and CK1γ. GSK3 phosphorylates the serine in the PPPSP motif found in a number of Wnt signaling components, including β-catenin, Axin, APC, and potentially LRPs (Zeng et al., 2005). CK1γ is anchored in the membrane by its palmitoylated C terminus, and it phosphorylates these same proteins on residues adjacent to the PPPSP motif (Davidson et al., 2005). It is not clear whether or how Wnt activates these protein kinases, but adding Wnt protein to cells leads to CK1γ-induced phosphorylation within minutes, suggesting a direct response to the signal.

Relatively little is known on the role of Fz in Wnt reception. The cytoplasmic part of Fz interacts with Dishevelled (Dsh; Chen et al., 2003; Figure 2), facilitating interaction between the LRP tail and Axin. The DIX domain on Dsh is similar to a region in Axin, and these two DIX domains can bind each other directly (Fiedler et al., 2011; Schwarz-Romond et al., 2007). Multimers of receptor-bound Dsh and Axin molecules might encourage the formation of the LRP-Fz dimer. Higher-order complexes containing Wnts, receptors, and Dsh, as well as small particles of multimerized Dsh molecules, have been detected in cells (Schwarz-Romond et al., 2005).

Importantly, the Wnt pathway transduces signals differently than most other pathways. In many signaling cascades, protein phosphorylation amplifies the signal, as individual kinase molecules catalyze the modification of multiple substrate molecules. In contrast, Wnt-induced LRP6 phosphorylation titrates away a negative regulator, Axin, providing a stoichiometric rather than a catalytic mechanism of signal transduction. Likewise, it has been proposed that the regulation of GSK3 by Wnt is stoichiometric, caused by sequestration of the enzyme inside multivesicular endosomes (Taelman et al., 2010).

Natural Wnt Inhibitors Act in Various Ways on Wnt Receptors

Wnt/β-catenin signaling is regulated at many levels, including by secreted proteins that antagonize the ligand. Among these are secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF), both of which can bind Wnts, thereby inhibiting

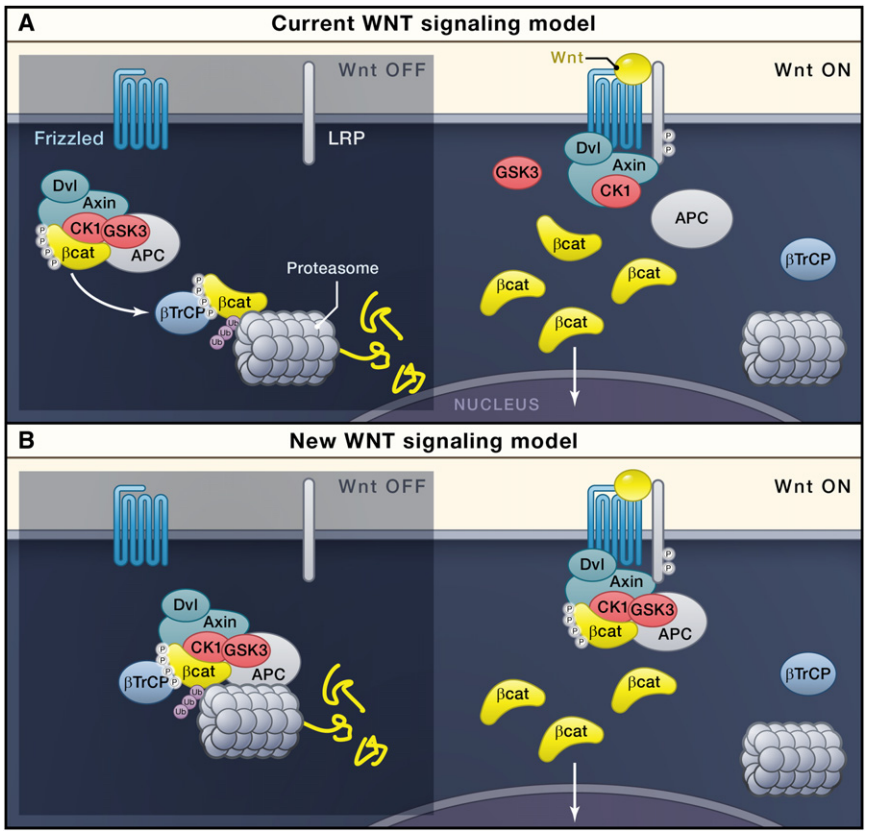

Figure 2. Wnt Signaling at the Receptor and Destruction Complex Level

(A) The current Wnt model. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds and phosphorylates β-catenin. The latter then leaves the complex to be ubiquitinated by β-TrCP (which binds to the phosphorylated "degron" motif in β-catenin) and is then degraded by the proteasome. Wnt induces the association of Axin with phosphorylated LRP. The destruction complex falls apart, and β-catenin is stabilized.

(B) A new model based on studying endogenous destruction complex components (Li et al., 2012). In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds, phosphorylates, and ubiquitinates β-catenin by β-TrCP. The proteasome recycles the complex by degrading β-catenin. Wnt induces the association of the intact complex with phosphorylated LRP. After binding to LRP, the destruction complex stills captures and phosphorylates β-catenin, but ubiquitination by β-TrCP is blocked. Newly synthesized β-catenin accumulates.

interactions between Wnt and Wnt receptors (Bovolenta et al., 2008). Other Wnt inhibitors include proteins of the Dickkopf (DKK) (Glinka et al., 1998) and the WISE/SOST families, which antagonize signaling by binding LRP5/6. Recent biochemical and genetic studies have argued that DKK1 disrupts Wnt-induced Fz-LRP6 complex formation (Ellwanger et al., 2008; Semënov et al., 2008). Like DKK1, SOST can disrupt Wnt-induced Fz-LRP6 complexes in vitro (Semënov et al., 2005). As a final example, APCDD1 is a membrane-bound glycoprotein that inhibits Wnt signaling by binding both Wnt and LRP. It is mutated in hereditary hypotrichosis simplex, a condition characterized by hair follicle miniaturization (Shimomura et al., 2010; Table 1). Of interest, no natural secreted inhibitors have been identified in flies.

Mutations in Wnt Receptors and Their Antagonists Implicate Wnt Signaling in Bone Disease

A fast-growing field connects Wnt signaling with bone biology and disease (Table 1; reviewed in Monroe et al., 2011). This link was first established by the discovery of LRP5 mutations associated with osteoporosis pseudoglioma syndrome (OPPG), a hereditary disorder characterized by low bone mass and abnormal eye vasculature (Gong et al., 2001). Subsequently, patients with distinct types of hereditary high bone mass diseases were found to carry mutations in the LRP5 extracellular domain (Boyden et al., 2002; Little et al., 2002), which render LRP5 resistant to binding of the antagonist SOST (Ellies et al., 2006; Semenov and He, 2006) and DKK1 (Ai et al., 2005; Table 1). Similarly, mutations in the SOST gene cause sclerosteosis (Balemans et al., 2001; Brunkow et al., 2001; Table 1). Additional mutations in Wnt pathway components are observed in other hereditary syndromes that display bone defects. A loss-of-function mutation in LRP6 is linked to a hereditary disorder characterized by osteoporosis, coronary artery disease, and metabolic syndrome (Table 1; Mani et al., 2007). WTX, an X-linked intracellular inhibitor of Wnt/β-catenin signaling with known tumor suppressor roles, is mutant in OSCS, a disease characterized by excessive bone deposition and hardening (Jenkins et al., 2009), whereas FZD9 is deleted in patients with Williams–Beuren syndrome, which is partially characterized by low bone density (Wang et al., 1999).

Given that Wnt activates osteoblasts and influences bone mass, secreted Wnt antagonists have become attractive targets for antibody therapy in osteoporosis. In addition, local DKK1 production by malignant plasma cells induces osteolytic bone lesions and pathological fractures, a major complication in multiple myeloma (Tian et al., 2003). Multiple reports evaluate the use of Dkk1 antibodies in animal models of this disease complication (Monroe et al., 2011), and similar efforts are ongoing using the WISE/SOST protein as a target.

Norrin and R-spondins, Secreted Agonists of the Wnt Pathway, Are Involved in Disease

Two types of proteins—Norrin and R-spondins, which are unrelated to Wnts—act through the Fz/LRP complex as Wnt agonists. The cysteine-knot protein Norrin is encoded by the *NDP* gene. In humans, *NDP* mutations cause Norrie disease, an X-linked disorder characterized by hypovascularization of the retina and a severe loss of visual function (Berger and Ropers, 2001). Severe retinal hypovascularization is also seen

in humans carrying a homozygous loss-of-function mutation in Lrp5 (Gong et al., 2001). A milder retinal hypovascularization (familial exudative vitreoretinopathy or FEVR) occurs in patients heterozygous for mutations in either Lrp5 (Robitaille et al., 2002) or Fz4 (Toomes et al., 2004; Table 1).

Norrin is a direct ligand for the Frizzled-4/Lrp5 complex. The vascular phenotypes in the retina of mouse knockout models for Norrin (Richter et al., 1998), Frizzled-4 (Wang et al., 2001), and Lrp5(Kato et al., 2002; Xia et al., 2008) resemble each other. In addition, Norrin binds with high affinity and specificity to Frizzled-4, whereas coexpression of Norrin, Frizzled-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 coreceptor (Junge et al., 2009). Indeed, several FEVR families were subsequently found to carry mutations in the TSPAN12 gene (Nikopoulos et al., 2010; Poulter et al., 2010).

Vertebrate genomes encode four R-spondin (Rspo) proteins, small secreted proteins defined by two N-terminal furin domains and a thrombospondin domain. The first evidence that Rspo proteins potently enhance Wnt/β-catenin signals came from *Xenopus* (Kazanskaya et al., 2004). Rspo1 was subsequently found to feed into the canonical Wnt pathway, strongly promoting intestinal crypt proliferation in vivo (Kim et al., 2005) and in vitro (Sato et al., 2009). Together, these results support a crucial role for R-spondins in Wnt/β-catenin signaling. Rspo mutations have been found in two hereditary syndromes in humans (Table 1). RSPO1 is the gene disrupted in a recessive syndrome characterized by XX sex reversal, a skin abnormality called palmoplantar hyperkeratosis, and predisposition to squamous cell carcinomas (Parma et al., 2006). Mutations in the RSPO4 gene are linked to congenital anonychia, severe hypoplasia of finger- and toenails, (Blaydon et al., 2006). Mutations in RSPO2 have not been observed in humans, but rspo2 mouse mutants exhibit a variety of developmental defects involving limbs (Nam et al., 2007), lung (Bell et al., 2008), and craniofacial anatomy (Yamada et al., 2009).

Lgr Molecules are R-Spondin Receptors that Enhance Wnt Signaling

Recent studies have uncovered a small family of 7-TM receptors, the Lgr5 family, which mediate Rspo input into the canonical Wnt pathway (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). All three studies demonstrate that the Lgr receptors bind R-spondins with high affinity and are essential for signal enhancement of low-dose Wnt. Lgr4 and Lgr5 proteins physically reside within Frizzled/LRP receptor complexes (de Lau et al., 2011).

What was known previously about the Lgr proteins? Upon the cloning of these receptors, it was noted that Lgr4, Lgr5, and Lgr6 were related to the G-protein-coupled receptors (GPCRs) for thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH; Hsu et al., 2000). These receptors contain a large N-terminal extracellular leucine-rich repeat domain that binds the glycoprotein hormones. Similarly, the Lgr proteins bind R-spondins through their N-terminal ectodomain, but current evidence indicates that they do not utilize G proteins (Carmon et al., 2011; de Lau et al., 2011).

Gene knockout studies revealed that *Lgr4* as well as *Lgr5* mutant mice are neonatal lethal. Pleiotropic phenotypes were observed for *Lgr4* in male reproductive organs, eye, gall bladder, kidney, hair follicles, and a variety of other organs, whereas Lgr5 mutants displayed a single abnormality of the lower jaw and tongue (Barker and Clevers, 2010). It was subsequently found that *Lgr5* is a Wnt target gene in colon cancer and that it marks adult stem cells in a number of actively self-renewing organs, including the intestinal tract and the hair follicle (Barker et al., 2009, 2010; Jaks et al., 2008). Of note, a strong genetic interaction exists between Lgr4 and Lgr5, as seen in the gut of double-mutant mice (de Lau et al., 2011; Mustata et al., 2011). *Lgr6* similarly marks a rare, primitive stem cell that generates all lineages of the skin (Snippert et al., 2010). The finding that the Lgr proteins act as receptors for R-spondins reinforces the intimate connection between Wnt signaling and activation of adult stem cells (see below).

The Cytoplasmic APC/Axin Destruction Complex Regulates Wnt Pathway Output by Controlling β-Catenin Stability

The destruction complex regulates the stability of cytoplasmic β-catenin, playing a key role in the signaling output of the canonical Wnt cascade. The tumor suppressor protein Axin acts as the scaffold of the destruction complex, interacting with β-catenin, the tumor suppressor proteins APC and WTX, and two constitutively active serine-threonine kinases (CK1α/δ and GSK3α/β; Figure 2). APC is a large protein that interacts with both β-catenin and Axin. It 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 destruction complex function, its specific molecular activity remains unresolved. Another tumor suppressor protein involved in destruction complex function is WTX. It is mutated in some cases of Wilms tumor, a pediatric kidney cancer (Rivera et al., 2007). WTX occurs in the destruction complex in which it promotes β-catenin degradation, which would make its tumor-suppressive properties equivalent to those of Apc and Axin (Major et al., 2007). Its exact molecular functions in the Wnt pathway, however, remain in debate (Regimbald-Dumas and He, 2011).

When Fz/LRP receptors are not engaged, CK1 and GSK3 sequentially phosphorylate Axin-bound β-catenin at a series of regularly spaced N-terminal Ser/Thr residues. The phosphorylated "degron" motif is then recognized by the F box/WD repeat protein β-TrCP, part of an E3 ubiquitin ligase complex. As a consequence, β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome (Aberle et al., 1997), preventing activation of β-catenin target genes in the nucleus. Upon receptor activation by WNT ligands, Axin is recruited to the phosphorylated tail of LRP. Recent data show that, through this relocalization, the Wnt signal leads to inhibition of β-catenin ubiquitination that normally occurs within the complex. Subsequently, the complex becomes saturated by the phosphorylated form of β-catenin, leading newly synthesized β-catenin to

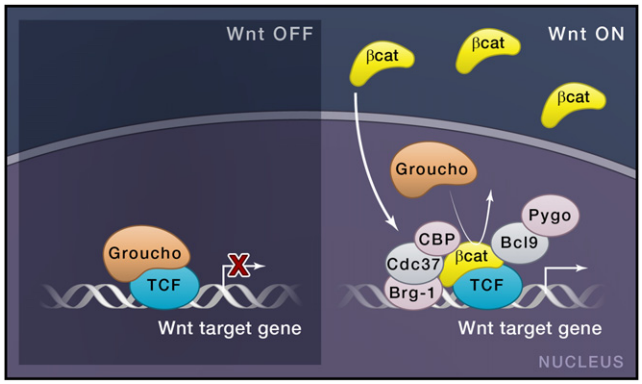

Figure 3. Wnt Signaling in the Nucleus
In the absence of Wnt signals, TCF occupies and represses its target genes, helped by transcriptional corepressors such as Groucho. Upon Wnt signaling, β-catenin replaces Groucho from TCF and recruits transcriptional coactivators and histone modifiers such as Brg1, CBP, Cdc47, Bcl9, and Pygopus to drive target gene expression.

accumulate and translocate to the nucleus to activate target genes (Figure 2 and Li et al., 2012).

In addition to its role in Wnt pathway, β-catenin performs a second, unrelated role in simple 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). Though 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 two different β-catenin homologs (Korswagen et al., 2000).

Wnt Target Genes Are Controlled by the TCF/β-Catenin Complex

The ultimate outcome of the Wnt signal is shaped by those genes whose activity is controlled through β-catenin and TCF. Upon Wnt pathway activation, β-catenin accumulates in the cytoplasm and then enters the nucleus, where it engages DNA-bound TCF transcription factors (Behrens et al., 1996; Molenaar et al., 1996). The consensus TCF cognate motif for vertebrate and *Drosophila* TCF is AGATCAAAGG (van de Wetering et al., 1997), and the widely used Wnt/TCF reporters such as pTOPflash (Korinek et al., 1997) contain concatamers of this motif. In the Wnt "off" state, Tcfs interact with Groucho transcriptional repressors (Figure 3; Cavallo et al., 1998; Roose et al., 1998), preventing gene transcription. In the Wnt "on" state, the association with β-catenin transiently converts TCF into a transcriptional activator of its target genes, with additional modulation of TCF coming from phosphorylation (Hikasa et al., 2010; Lee et al., 2009). Whereas most Wnt target genes are tissue or developmental stage specific, the *Axin2* gene is generally regarded as a global transcriptional target and therefore a general indicator of Wnt pathway activity (Lustig et al., 2002). In *Drosophila*, a TCF "helper" site may explain some of the specificity of TCF interaction with DNA (Chang et al., 2008).

Two other components of the TCF/β-catenin complex, Bcl9/Legless and Pygopus, were first identified in *Drosophila* (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), and Bcl9 has been proposed to bridge Pygopus to the N terminus of β-catenin. Although most Wnt signaling events in *Drosophila* appear to depend on Bcl9 and Pygopus, knockout studies in mice suggest that these proteins are largely dispensable in mammals (Brack et al., 2009; Schwab et al., 2007).

β-catenin influences gene transcription in several ways. Its C terminus acts as a transcriptional activation domain (van de Wetering et al., 1997). It binds histone modifiers such as CBP and Brg-1 (reviewed in Städeli et al., 2006) and Parafibromin/Hyrax, homologs of yeast Cdc73 (Mosimann et al., 2006).

The details of how β-catenin shuttles between the cytoplasm and the nucleus are unclear, although recent evidence suggests a role for microtubules and active transport (Sugioka et al., 2011). In some instances, active Wnt signaling may increase the overall level of β-catenin without any detectable amount of the protein in the nucleus. Measuring and modeling β-catenin-dependent effects indicate that fold change rather than absolute levels are critical, suggesting that even low levels of β-catenin would be sufficient for transcriptional changes (Goentoro and Kirschner, 2009).

Wnt Signaling and Stem Cell Self-Renewal

Within the wide spectrum of Wnt effects on target cells, the role of Wnts in maintaining stem cells has drawn particular interest. Stem cells are defined as having the capacity to self-renew while also producing specialized cells. These choices are primarily dictated by extrinsic signaling factors. These extrinsic signals and the cells that produce them comprise a niche, or signaling center, with a short range of action capable of limiting the number of stem cells (Losick et al., 2011). Although other niche signals are known, such as proteins from the BMP, Hedgehog, and Delta/Notch families, Wnt signals stand out because of their widespread activity.

An early finding showed that mutations in mouse TCF4 lead to loss of intestinal stem cells and subsequent breakdown of the tissue (Korinek et al., 1998). Subsequent studies showed the Wnt pathway to be required for a variety of specific stem cell niches. In the hair follicle, Wnt signaling plays multiple roles in the biology of stem cells and progenitors (DasGupta and Fuchs, 1999). Blocking Wnt signaling by overexpression of Dkk eliminates hair follicles and other skin appendages, such as the mammary gland, in a way that suggests that the tissue-specific stem cells were primarily affected (Andl et al., 2002). In the hematopoietic system, overexpressing Axin lowers the numbers of transplantable stem cells (Reya et al., 2003). Conversely, activating Wnt signaling by the use of mutant forms of β-catenin can lead to the expansion of stem cells in both hematopoietic and hair follicle systems (Gat et al., 1998). 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). Similarly, Wnt protein can expand the number of clonogenic cells from the mammary gland, with retention of the developmental potential of the cells when transplanted back into animals (Zeng and Nusse, 2010). The Wnt agonist R-Spondin can maintain stem cells in a self-renewing state that are able to produce differentiated cells of the intestinal

epithelium (Sato et al., 2010). And Wnt can mediate maintenance of pluripotency in mouse embryonic stem cells (Ten Berge et al., 2011). Despite all of these examples, it should be mentioned that there are clear cases in which there is no role for Wnts in stem cell maintenance, particularly in the male and female germlines of *Drosophila* (Losick et al., 2011).

In parallel, powerful genetic tools and lineage tracing have provided further evidence for Wnts as critical stem cell signals. These experiments have been based on identifying Wnt target genes such as LGR5 and Axin2 in tissues, some of them being expressed in putative stem cells (Snippert and Clevers, 2011). Labeled stem cells have been detected in organs ranging from the intestine to the stomach, the skin, the hair follicle, and the mammary gland (Barker et al., 2007; Barker et al., 2010; van Amerongen et al., 2012). Combined with the cell culture and genetic data described above, the lineage tracing experiments make a compelling case for Wnts as self-renewal signals for many different varieties of adult stem cells.

What is the mechanism by which Wnt signals maintain stemness? Is there a common underlying mode of action? Although we cannot yet definitively answer this question, we can invoke the fundamental concept that stem cells are intrinsically destined to differentiate. It seems, therefore, that Wnt signals could block the default step, the differentiation of cells, possibly by suppressing differentiation-specific genes. There are indeed a few examples of genes that are downregulated rather than activated by Wnt signals, and gene suppression could be part of a global mechanism of regulation controlled by Wnts. It is likely that epigenetic control lies at the root of the mechanism for influencing gene expression, enabling the stable propagation of the state of the cells. At the same time, a conditional signal-dependent state is required to make cells continuously dependent on the signal to maintain stemness. Combined with the limited range of the lipid-modified Wnt proteins (Willert et al., 2003), this fits with an essential hallmark of the niche, a constrained environment sustaining a small number of stem cells.

There are clear implications of the connection between Wnt and stem cells, including the use of Wnt proteins or Wnt pathway agonists to maintain stem cells in culture. Though stem cells are difficult to expand in a self-renewing state, adding Wnts or R-Spondins has overcome some of these problems (Sato et al., 2010; Zeng and Nusse, 2010; Ten Berge et al., 2011). Likewise, Wnt reagents may have clinical applications for the treatment of degenerative diseases.

Wnt Signaling in Cancer

Given the importance of Wnt signaling for adult stem cell biology, it is not surprising that Wnt pathway mutations are frequently observed in cancer, most notably of tissues that normally depend on Wnt for self-renewal or repair. Germline mutations in the *APC* gene cause a hereditary cancer syndrome termed familiar adenomatous polyposis (FAP; Kinzler et al., 1991; Nishisho et al., 1991). FAP patients carry heterozygous *APC* mutations. The second allele is frequently lost in individual cells, which grow into colon adenomas, polyps, in early adulthood. Additional mutations in genes like *kras*, *tp53*, and *smad4* induce some of these polyps to progress toward malignancy. Most cases of sporadic colorectal cancer result from loss of both APC alleles (Kinzler and Vogelstein, 1996). Loss of APC function leads to the inappropriate stabilization of β-catenin and the formation of constitutive complexes between β-catenin and the intestinal TCF family member Tcf4 (Korinek et al., 1997). In rare cases of colorectal cancers wild-type for APC, *Axin2* is mutated (Liu et al., 2000), or activating point mutations in β-catenin remove the regulatory N-terminal Ser/Thr residues (Morin et al., 1997). Patients with hereditary Axin2 mutations display a predisposition to colon cancer in addition to tooth agenesis (Lammi et al., 2004). Recent global exome-sequencing efforts have confirmed that the overwhelming majority of colorectal cancers carry inactivating APC mutations (Wood et al., 2007). In addition, this approach has revealed rare but recurrent fusions between *VTI1A* and *Tcf7l2*, the gene encoding Tcf4, adding yet another Wnt pathway member to the list of colon cancer genes (Bass et al., 2011).

Activating Wnt Pathway Mutations Are Not Restricted to Cancer of the Intestine

Loss-of-function mutations in Axin have also been found in hepatocellular carcinomas, whereas oncogenic β-catenin mutations, first described in colon cancer and melanoma (Rubinfeld et al., 1997), occur in a wide variety of solid tumors (reviewed in Reya and Clevers, 2005). Inactivating mutations in the TCF family member Lef1 occur in sebaceous skin tumors (Takeda et al., 2006). The link between stem cell biology and colon cancer is further reinforced by several recent reports demonstrating a link between Wnt signal strength, stem cell signature, and cancer stem cell behavior (Merlos-Suárez et al., 2011; Vermeulen et al., 2010).

Wnt Signaling Components and Metabolic Diseases

A rapidly emerging field involves the link between Wnt signaling and metabolism. Increased risk for type II diabetes has been linked to specific SNPs in WNT5B (Kanazawa et al., 2004), WNT10B (Christodoulides et al., 2006), and TCF7L2 (Grant et al., 2006). The Tcf7l association occurs in multiple ethnic groups, and changes in Tcf7l2 define the strongest genetic risk determinant for this disease (Tong et al., 2009). Although the molecular and physiological mechanism underlying this link remains a matter of debate, a recent transgenic study in mice suggests that metabolic risk is directly proportional to Tcf7l2 gene expression (Savic et al., 2011).

Small-Molecule Wnt Modulators

The broad involvement of Wnt signaling in disease, as outlined in this Review, has driven extensive research efforts at targeting the Wnt pathway with small molecules. Table 2 lists many of the published molecules. From the perspective of blocking Wnt signaling in cancer, the most effective target would be the complex between TCF and β-catenin, as it is a critical junction in signaling and operates downstream in the pathway. Disappointingly, this complex has proven to be an elusive target. Its structure reveals a large binding surface that is not readily dislodged by chemical entities (Huber et al., 1997). Indeed, though several compounds, such as PKF115-854 and CGP049090, have been suggested to target Wnt signaling at this level (Lepourcelet et al., 2004), their specificity and efficacy remain to be

Table 2. Small-Molecule Wnt Pathway Modifiers

Small Molecule	Molecular Target	Function	Effect on Wnt Pathway Output	Reference
IWP	Porcupine	inhibitor	inhibits	(Lu et al., 2009)
XAV939	tankyrase 1/Axin	activates Axin	inhibits	(Huang et al., 2009)
IWR	Axin	activates Axin	inhibits	(Lu et al., 2009)
Pyrvinium	CK1	inhibitor	inhibits	(Thorne et al., 2010)
SB-216763	GSK3	inhibitor	activates	(Coghlan et al., 2000)
BIO(6-bromoindirubin-3′-oxime)	GSK3	inhibitor	activates	(Sato et al., 2004)
ICG-001	CREB-binding protein	inhibitor	inhibits	(Emami et al., 2004)
PKF115-584 (and several other compounds)	TCF/β-catenin	inhibitor	inhibits	(Lepourcelet et al., 2004)

established firmly, and clinical screens of these compounds have not been initiated.

Several screens have yielded compounds exerting an effect on more upstream components, including Axin. The stability of Axin is regulated, in part, by ADP ribosylation, catalyzed by Tankyrase. Inhibiting Tankyrase with molecules such as IWR (Lu et al., 2009) and XAV939 (Huang et al., 2009) increases Axin levels, which, in turn, destabilizes β-catenin to inhibit Wnt signaling. These molecules have been used only in laboratory experiments thus far. As mentioned before, the stability of β-catenin is also controlled by phosphorylation, in part regulated by CK1 gamma. This enzyme appears to be activated by the compound Pyrvinium, resulting in inhibition of the Wnt pathway and possibly providing another reagent to interfere specifically with the pathway (Thorne et al., 2010).

Somewhat surprisingly, there are no effective small-molecule inhibitors of the receptors for Wnt. The notion that Fzs signal through a G protein is not universally accepted, and whether G protein inhibitors affect Wnt signaling remains an open question. Likewise, although LRPs can be blocked by specific antibodies and protein ligands such as Dkk and SOST (Gong et al., 2010), these receptors remain difficult to target pharmacologically. At the cytoplasmic tail of LRP, GSK3 and CK1-γ are required for critical phosphorylation events, which are associated with activation of signaling. Whereas protein kinases have proven to be tractable drug targets in cancer (exemplified by the BRAF and Abl kinases), specific inhibitors of CK1-γ have yet to be identified. GSK3 has pleiotropic roles in Wnt signaling—promoting pathway activation at the receptor level while also acting as a negative regulator of β-catenin—and this makes it a problematic target.

By far, the most specific small-molecule inhibitors in the Wnt pathway block Porc, the enzyme promoting acylation of Wnt proteins. The compound IWP2 can inactivate Porc with a high degree of selectivity (Lu et al., 2009). As Wnt proteins lacking lipids are not secreted, the IWP2 compound specifically blocks Wnt production. Applications of the drug have been limited to cell culture experiments and have demonstrated Wnt dependency of stem cells in self-renewal assays (Sato et al., 2010; Ten Berge et al., 2011). Although the hydrophobicity of the IWP molecules precludes in vivo delivery and clinical use, ongoing screens for variants and derivatives may overcome this hurdle. Evidence is being unearthed that Wnt signals contribute in a paracrine mode to the growth of cancer cells or cancer stem cells (Malanchi and Huelsken, 2009), and because metastasizing tumor cells bear the hallmarks of Wnt signaling (Nguyen et al., 2009), targeting Porc could be clinically important.

Can Wnt signaling be activated by small molecules? The most widely used strategy has been to inactivate the GSK3 enzyme, using compounds ranging from LiCl to Bio, CHIR, and SB-216763 (references listed in Table 2). This approach certainly can induce Wnt target gene expression and lead to biological effects, but the results need to be carefully interpreted. GSK3 is involved in numerous signaling events in cells, and its inactivation has pleiotropic effects. The inhibitory effect of GSK3 inhibition on the Wnt signal owes to the direct coupling of GSK3 to the Wnt receptors by the scaffold proteins APC and Axin. There are various reports on other chemical compounds that promote Wnt signaling, but most of these elevate an existing Wnt signal rather than activate the pathway in a ligand-independent manner.

To sum up, a small set of chemical compounds interfere with Wnt signaling. Though these tools are limited, screens for such molecules are ongoing. Moreover, our understanding of the Wnt pathway is incomplete, leaving room for the discovery of more components. For example, protein kinases involved in TCF phosphorylation may provide good targets for drugs, just like the protein kinases that act in other signaling pathways.

Ten Outstanding Questions in the Wnt Field

By reviewing the current status of Wnt signaling, we realized how many questions in this field remain unanswered. We highlight ten of those questions, hoping to inspire future research.

(1) What is the evolutionary origin of Wnt signals? Wnt genes and signaling components are found in all metazoan animals, including sponges. Given that Wnts are intercellular signals, it is not surprising that they are not present in unicellular organisms. Although the recently established Wnt-Fz structure has not yet pointed to a particular evolutionary origin, one can speculate that Wnts have a more ancient origin, possibly derived from enzymes secreted by prokaryotes.

(2) What is the nature of Wnt as a signal? Is the protein active by itself, or is it packaged in membranes, together with possible cofactors? The importance of "Wnt delivery" needs to be understood.

(3) Where does Wnt signaling take place in cells? Whereas Hedgehog signaling is located at the primary cilium, the

subcellular location of Wnt signaling events remains unknown, though the endosomal compartment has been implicated as a signaling center.

(4) Besides the well-studied Frizzled and LRP receptors, there are other mechanisms for Wnt reception that involve the tyrosine kinases ROR and RYK. There is very little insight into the mechanisms of action of these receptors, and they deserve more intense study.

(5) How is the stabilized form of β-catenin ferried into the nucleus? Is there a role for active microtubule-based transport?

(6) How does Wnt signaling coordinate cell fate changes with changes in cell shape and polarity? This is a key question in many developmental contexts of Wnt signaling, including stem cells.

(7) Many different kinds of stem cells are controlled by Wnts, in such a way that self-renewal and the developmental potential of the cells are preserved by the Wnt signal. Is there a universal ''stemness'' property conferred to cells by Wnts?

(8) How much of the genome is Wnt controlled across various cell types? Given the broad effects of Wnt signaling and many Wnt target cells, the total number of Wnt-controlled genes could be significant.

(9) Are cancer stem cell behaviors controlled by Wnt signaling? The definition of cancer stem cells as being able to self-renew the tumorigenic state but also able to differentiate suggests that their behavior is dependent on external signals, such as Wnts.

(10) Can we identify bona fide and effective Wnt inhibitors? Though several molecules have been described to inhibit Wnt signaling in cells, there is a great need for additional reagents interfering with the Wnt pathway.

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