

# Wnt Signaling: Multiple Pathways, Multiple Receptors, and Multiple Transcription Factors\*

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Signaling pathways are an ever present force in every animal's life. During development, these pathways provide critical cell-cell communication required to coordinate the activities of vast numbers of cells. In adulthood, similar communication mechanisms are utilized to achieve tissue homeostasis and regeneration. Regulation of signaling is crucial; too much or too little activity from a given signal transduction pathway can cause devastating results such as developmental defects or, later in life, disease.

The Wnts comprise a large family of highly conserved growth factors that are responsible for important developmental and homeostatic processes throughout the animal kingdom (for a more comprehensive review see Ref. 1). Their implication in a wide array of developmental events and human diseases has made Wnts and their signaling pathways the subject of intense investigation over the last two decades. This has never been truer than in the past few years, when the association of Wnt signaling with stem cell fate has added fuel to an already active field.

Membership in the Wnt family is defined by amino acid sequence rather than functional properties. It is therefore not too surprising that Wnts have been associated with a number of different activities and downstream signaling pathways. Although the majority of work in the field to date has focused on  $\beta$ -catenin-dependent, or canonical, Wnt signaling, examples continue to accumulate in which Wnts and/or other key components of the canonical signaling cascade participate in  $\beta$ -catenin-independent processes (reviewed in Ref. 2). In this review, we will focus largely on the canonical pathway, paying particular attention to recent insights. We will then touch on some developments in  $\beta$ -catenin-independent signaling and discuss some issues that may be important to all Wnts, regardless of the signal they generate.

## Wnt Signaling through $\beta$ -Catenin

The defining event in canonical Wnt signaling is the cytoplasmic accumulation of  $\beta$ -catenin and its subsequent nuclear translocation and activity (Fig. 1). Under unstimulated conditions, a  $\beta$ -catenin destruction complex formed by proteins that

include Axin, adenomatous polyposis coli (APC),<sup>2</sup> and glycogen synthase kinase 3 $\beta$  (GSK-3) keeps cytoplasmic levels of  $\beta$ -catenin low through phosphorylation by GSK-3. Phosphorylated  $\beta$ -catenin becomes ubiquitinated and is targeted for degradation by the proteasome (3). Following Wnt binding to a receptor complex composed of members of the Frizzled (Fz) family of seven transmembrane, serpentine receptors and low density lipoprotein receptor-related protein (LRP), the Axin·APC·GSK-3 complex is inhibited, leading to a block in  $\beta$ -catenin phosphorylation by GSK-3 (Fig. 1B). Hypophosphorylated  $\beta$ -catenin accumulates in the cytoplasm and is translocated to the nucleus where it regulates target gene expression through partnerships with the T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family of transcription factors (4).

## Wnt/Receptor Interactions

The role of Fz in acting as a receptor for Wnts is long established; Fz carries an extracellular cysteine-rich domain that is sufficient to bind Wnt proteins, and addition of Fz to Wnt non-responsive cells can render signaling competence (5). However, the discovery that members of the LRP family of single pass transmembrane proteins are also required for signal transduction immediately raised the possibility that Fz and LRP function as co-receptors for Wnt proteins (6, 7). Indeed, binding has been seen between Wnt and LRP, and a ternary complex between a mouse Wnt and soluble forms of Fz and LRP extracellular domains has been reported (6). A model in which Wnt physically mediates an interaction between Fz and LRP is therefore appealing. Is such an interaction sufficient to induce signaling in the absence of Wnt binding? Early reports suggest that it is. Fusion of a heterologous ligand/receptor pair (NT3 and TrkN) to Fz and LRP, respectively, is sufficient to induce Wnt-independent signaling when the two molecules are co-overexpressed, as is a fusion of a natural LRP-binding protein, Dickkopf (DKK), to Fz (8, 9). Lastly, addition of the intracellular portion of LRP to the C terminus of Fz creates a constitutively active receptor (10). These data argue that inducing the proximity of the Fz and LRP cytoplasmic domains is the key event in Wnt signal initiation, although direct evidence of its occurrence upon Wnt ligand reception has not yet been reported.

## Signal Relay in the Cytoplasm

There has been much interest in elucidating the events that bridge the activation of the Fz/LRP receptors and inhibition of the  $\beta$ -catenin destruction complex. A key intermediate in this process is Dishevelled (Dsh), a cytosolic phosphoprotein required upstream of Axin·APC·GSK-3 inhibition in both fly

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<sup>2</sup> The abbreviations used are: APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; LRP, low density lipoprotein receptor-related protein; TCF, T cell-specific transcription factor; LEF, lymphoid enhancer-binding factor 1; PCP, planar cell polarity; CE, convergence extension; RTK, receptor tyrosine kinase; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein.

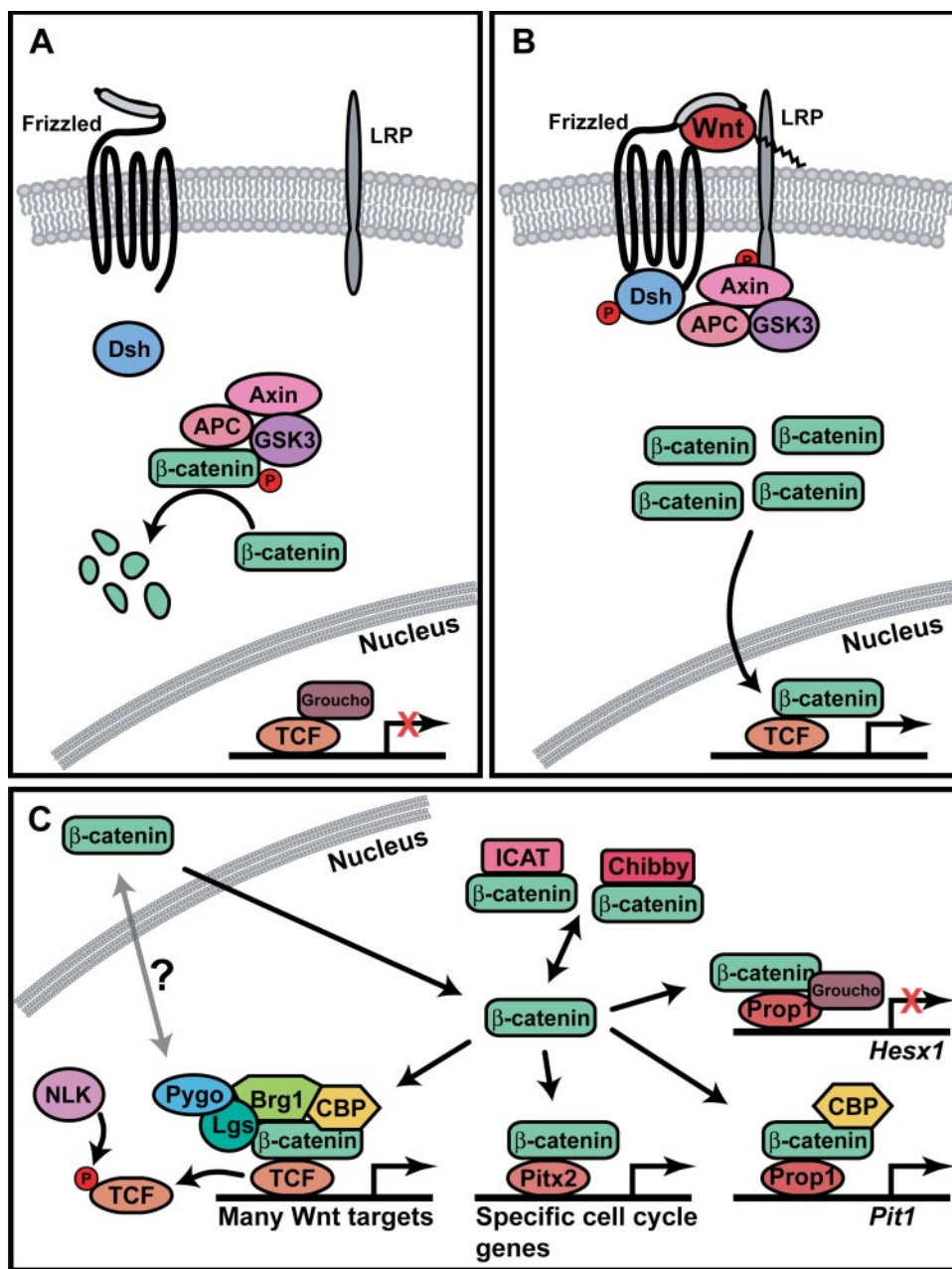
and mammalian cells. Although Dishevelled's requirement in Wnt signaling has been known for over a decade, the molecular events leading to Dsh activation by Frizzled and the manner in which Dsh transduces the Wnt signal to the inhibitory complex have remained murky. Several papers report on a physical asso-

ciation between Fz and Dsh or Wnt-dependent phosphorylation of Dsh (11, 12). The kinases PAR-1 and CKII appear to directly phosphorylate Dsh, although the precise role of each in transducing the Wnt signal is not entirely clear nor is the specific activity bestowed on Dsh by phosphorylation (13).

A second component that appears to play a key role in directly linking receptor activation to inhibition of the cytoplasmic  $\beta$ -catenin destruction complex is a member of the complex itself, Axin. Once thought of as simply a scaffold protein linking together other members of the complex, Axin now appears to play a more dynamic role in signal activation by binding directly to the cytoplasmic tail of LRP in response to Wnt reception (14). The recruitment of Axin to LRP is mediated by phosphorylation of LRP on key residues by the kinases CK1 $\gamma$  and GSK-3 (15, 16). Surprisingly, overexpression of a membrane-tethered form of the LRP intracellular domain can induce  $\beta$ -catenin accumulation, even in the absence of Fz or Dsh, suggesting that membrane recruitment of Axin is sufficient to activate signaling (17). Presumably this occurs through titration of Axin away from the Axin·APC·GSK-3 complex, thereby compromising the ability of the complex to phosphorylate  $\beta$ -catenin.

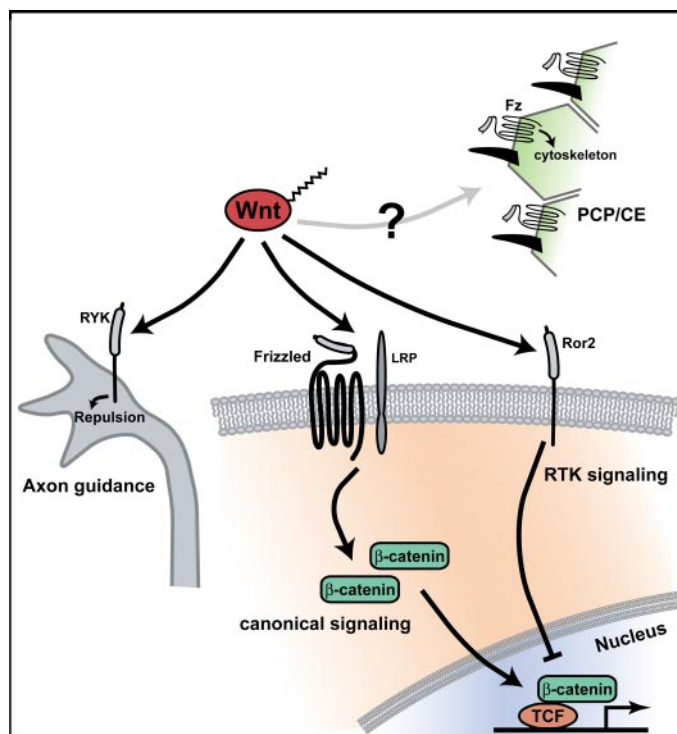
### Nuclear Activity of $\beta$ -Catenin

Once in the nucleus,  $\beta$ -catenin partners with members of the LEF/TCF family of transcription factors to activate the transcription of Wnt target genes (18, 19). TCF provides sequence-specific binding activity and, in the absence of nuclear  $\beta$ -catenin, partners with the transcriptional repressor Groucho and histone deacetylases to form a repressive complex and block transcription of Wnt target genes (20, 21). When  $\beta$ -catenin enters the nucleus, it directly replaces Groucho from its binding of TCF and converts the complex to a transcriptional activator, thereby effecting the transcription of Wnt target genes (22). Other members of this activating complex are the histone acetylase CBP/p300 and the SWI/SNF complex member Brg-1, both



**FIGURE 1. An overview of canonical Wnt signaling.** *A*, in cells not exposed to Wnt,  $\beta$ -catenin associates with and is phosphorylated by the destruction complex composed of Axin, APC, and GSK-3. Phosphorylated  $\beta$ -catenin is then targeted for degradation. At the same time, Wnt target genes are repressed by the association of TCF with Groucho. *B*, Wnt binding to the Frizzled and LRP receptors induces phosphorylation of LRP and recruitment of Axin. Dsh is also phosphorylated, and the Axin·APC·GSK-3 complex is inhibited, leading to accumulation of cytosolic  $\beta$ -catenin. Accumulated  $\beta$ -catenin then translocates to the nucleus, replaces Groucho from TCF, and activates target genes. *C*, emerging details of the complexity of nuclear  $\beta$ -catenin activity. In the classical canonical model,  $\beta$ -catenin forms a complex with TCF and the transcription factors Brg1 and CBP. Lgs and Pygo also bind to  $\beta$ -catenin, possibly driving its nuclear localization in addition to playing a direct role in transcriptional activation. Negative regulation of signaling is provided by NLK (Nemo-like kinase), which phosphorylates TCF, and ICAT (inhibitor of catenin) and Chibby, which are antagonists of  $\beta$ -catenin. In addition to TCF, two other DNA-binding proteins have been shown to associate with  $\beta$ -catenin: Pitx2 and Prop1 (*center* and *right* portion of panel). In the case of Prop1,  $\beta$ -catenin can act as a transcriptional activator or repressor of specific genes, depending on the co-factors present. The participation of any particular  $\beta$ -catenin complex in transcriptional regulation is highly cell type-dependent.





**FIGURE 2. The many flavors of Wnt signaling.** Wnts are known to associate with a number of different cell surface receptors and produce a number of cellular outcomes. Binding to Frizzled-LRP complex generates  $\beta$ -catenin signaling (center), whereas binding to the atypical RTK Ror2 can inhibit this activity (bottom right). Additionally, certain Wnts can bind Ryk, another atypical RTK, and elicit axon repulsion (left). The Wnt signaling components Frizzled and Dishevelled are involved in establishing PCP and controlling vertebrate CE. The specific involvement of Wnts in these processes is unclear (see text). Strikingly, although there are 19 Wnts in the mammalian genome, a single Wnt, Wnt5a, can participate in all of the processes shown here (excluding PCP), depending on the cell type and receptor complement present.

of which may act through the remodeling of chromatin surrounding TCF binding sites (23, 24).

There are a number of other factors that bind to the TCF- $\beta$ -catenin complex and are necessary for transcriptional activation, including Legless (Lgs), Pygopus (Pygo), and most recently discovered hyrax/parafibromin, a member of the polymerase-associated factor 1 (PAF1) complex (25–28). Lgs and Pygo are an intriguing pair of proteins that associate with  $\beta$ -catenin in the nucleus. Lgs binds directly to the N terminus of  $\beta$ -catenin and serves as an adaptor to attach Pygo to the complex (25). However, the essential function of Pygo is controversial; initially thought of as a nuclear cofactor that enhances transcriptional activation by the TCF- $\beta$ -catenin complex (25–27), a subsequent report suggested that Pygo affects transcription by recruiting otherwise cytoplasmic  $\beta$ -catenin to the nucleus (29), a claim that has since been refuted in favor of the original model (30). Additionally, the activity of TCF itself may be modulated by signaling from the mitogen-activated protein kinase cascade composed of TAK1 and NLK/Nemo (31), and TCF/LEF-independent signaling by  $\beta$ -catenin can occur through interactions with other DNA-binding proteins, including Pitx2 and Prop1 (32, 33). The latter case is further complicated by the observation that the Prop1- $\beta$ -catenin complex can act both as an activator of transcription and also as a repressor, a function mediated by binding to the co-repressor Groucho (33). In all, the

emerging complexity of  $\beta$ -catenin nuclear activity may speak to one of the fundamental questions in the field, which is how different cell types respond to Wnt signaling by transcribing different (although often overlapping) sets of target genes.

### $\beta$ -Catenin-independent Signaling

As its name denotes, the first *fz* gene described was not discovered for its role in Wnt signaling, but rather its function in organizing the bristles and hairs of the adult *Drosophila* cuticle (34). In flies mutant for *fz*, the normally uniform direction of hairs and bristles on the wings and thorax are perturbed, a result of disrupting what is now known as the planar cell polarity (PCP) pathway (reviewed in Ref. 35). In addition to Fz, another Wnt pathway component, Dsh, is also required for this process, and genetic analysis of the fly *dsh* gene has revealed mutations that separate its function in PCP and canonical Wnt signal transduction (36). Interestingly, mutations in the Dsh DEP (Dishevelled-Egl20-Pleckstrin) domain, which is absolutely required for PCP but dispensable for  $\beta$ -catenin signaling, have also been found to affect the process of convergence extension (CE) in vertebrates (37, 38). CE is also disrupted by loss of function in vertebrate homologs of the dedicated PCP genes *strabismus*, *flamingo*, and *prickle*, suggesting that CE and PCP are controlled by homologous pathways (39).

All evidence in flies and vertebrates points toward a complete separation of  $\beta$ -catenin signaling and PCP signaling downstream of Dsh. However, there is a long outstanding question of what lies upstream of Fz in PCP, and more specifically, whether it is a Wnt. There are no known Wnt gene mutations in the fly that disrupt PCP but mutations in the zebrafish *wnt5* and/or *wnt11* genes cause CE phenotypes, suggesting that Wnts do act as ligands in this system (37, 40). However, there is no direct evidence that Wnts act as directional cues in CE, rather than simply permissive signals. This is not to say that Wnts cannot act as directional cues; during the development of the vertebrate and invertebrate central nervous system, sources or gradients of Wnt protein can provide directional guidance to extending axons by transducing a signal through Ryk, an atypical member of the receptor tyrosine kinase (RTK) family (41–43).

Just as PCP is most likely an example of Wnt-independent Fz function, the association of Wnt and RTKs may illustrate a Fz-independent function for Wnt (Fig. 2). Certain atypical members of the RTK family have an extracellular Wnt binding domain, cysteine-rich domains in the case of Ror1 and Ror2 and a WIF (Wnt inhibitory factor) domain in the case of Ryk (44). The *Drosophila* homolog of Ryk, Derailed, is an axon guidance receptor that mediates repulsion from Wnt5 during embryonic central nervous system patterning, a process that apparently does not involve Dsh or downstream canonical signaling components (41). Similarly, a number of Wnts, including Wnt5a (a mammalian ortholog of *Drosophila* Wnt5), have been shown to act through Ryk as axon repellents during mammalian central nervous system development (42). Whether the fly and mouse processes are homologous is not known, and an answer will require more detailed knowledge of the other protein components mediating these signals. Moreover, Wnt5a can block canonical signaling in a process that depends on another RTK,

Ror2 (45). Clearly, there is much work still to be done in defining the signal transduction pathways downstream of Wnt-binding RTKs and sorting out the specificities of each ligand/receptor(s) interaction in modulating axon guidance or  $\beta$ -catenin stability.

### Wnt Family History

By reading even a cursory review such as this one, it is easy to appreciate the enormous complexity of Wnt signaling. The large number and diversity of components utilized in transduction of Wnt signals is staggering and at least partially underlies the specificity seen in the way a particular cell responds to a given Wnt. How did Wnt signaling acquire such diversity and complexity? Part of the answer may come from the recent discovery that each Wnt gene has had a surprisingly large amount of time to evolve independently of the others. Mammals have 19 Wnt genes that, through phylogenetic analysis, can be placed into 12 subfamilies (46, 47). The surprising observation is that these subfamilies are not the result of any recent evolutionary diversification; at least 11 of these subfamilies are present in Cnidaria (specifically, the sea anemone *Nematostella vectensis*), a group that split from the last common ancestor of Bilaterians (chordates, flies, worms, etc.) very early in metazoan evolution (48). This indicates that the acquisition of the Wnt subfamilies was an early development in the evolution of metazoa and likely occurred about 650 million years ago (48). What was the reason for early expansion of the Wnt family and its maintenance along multiple disparate lineages over many millions of years?

The Wnt family's long evolutionary history raises another question: whether, aside from amino acid sequence similarity, there are any universal properties of Wnts. The recent discovery that Wnt proteins can be purified to homogeneity allows for some unique opportunities to address this issue and has already raised one possible universal feature (49). Long known to be poorly soluble in aqueous solution, it was discovered that Wnts are covalently modified by the attachment of a palmitoyl group, making them more hydrophobic than analysis of the primary sequence would predict (49). This observation provided a potential link to the protein Porcupine, which was known to be required for secretion of Wnt from cells (50). It is thought that Porcupine, which resembles an acetyltransferase by sequence, may be the enzyme responsible for addition of the palmitate to Wnt and that this modification is required for secretion. Similarly, a second gene required for the secretion of Wnts has recently been discovered called *wntless/evi* (51, 52). The molecular details of how Wnts are recognized and then modified and/or targeted for secretion by *Wntless/Evi* are unknown but could provide more insight into structural features that may be universal to Wnts.

### Concluding Remarks

Interest in the Wnt signaling pathway continues to expand rapidly. Discovered nearly 20 years ago, Wnts are mentioned in nearly 5000 journal articles listed on PubMed, over half of which were published in the past 3 years. Driving such intense interest are a number of factors. First, the complexities of the Wnt family and the Wnt signaling pathways have provided plentiful fruit for genetic, genomic, and biochemical dissection.

Second, evidence that mis-regulation of Wnt signaling is a contributing factor in a number of human diseases continues to accumulate. The most famous example is that of APC, mutation of which causes familial adenomatous polyposis, a condition that inevitably leads to colorectal cancer (see Ref. 53). However, mutations in Wnts or Wnt signaling components have been associated with diseases that cover a wide spectrum of afflictions, from arthritis to schizophrenia (see Ref. 54). Lastly, Wnts appear able to expand, or perhaps maintain, certain undifferentiated stem cell populations (49). This observation has made Wnts more than just targets for understanding and potentially treating disease; Wnt proteins hold potential as agents to manipulate multipotent cells *in vitro* and could provide a key element in developing stem cell-derived tissue replacement therapies.

Even as our understanding of the Wnt pathway continues to expand, there are a number of important questions that remain. In terms of signal transduction, the details of how signaling is initiated upon Wnt binding to Fz and LRP need to be addressed further, as does the mechanism by which the  $\beta$ -catenin destruction complex is regulated. More daunting perhaps is the question of how specificity is achieved in the nuclear activity of  $\beta$ -catenin and its regulation of target genes: How does cell identity and the integration of other signals influence the set of genes transcribed upon Wnt signal activation? Manipulating this specificity using small molecules that target the proteins involved could hold promise in treating specific disease processes. Additionally, as interest in using Wnt ligands in more therapeutic settings increases, it will be important to understand more thoroughly the biochemical characteristics of these proteins and the distinguishing characteristics of each family member. Finally, it will be interesting to see whether we can integrate the large amount of information we have on canonical Wnt signaling with the increasing number of examples of  $\beta$ -catenin-independent signaling. Are there features that are universal to the activities of all Wnts? Only a more complete understanding of this enormously complex family of signaling proteins will lead us to the answer.

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