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# Mechanisms of Wnt signaling and control

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## Abstract

The Wnt signaling pathway is a highly conserved system that regulates complex biological processes across all metazoan species. At the cellular level, secreted Wnt proteins serve to break symmetry and provide cells with positional information that is critical to the patterning of the entire body plan. At the organismal level, Wnt signals are employed to orchestrate fundamental developmental processes, including the specification of the anterior–posterior body axis, induction of the primitive streak and ensuing gastrulation movements, and the generation of cell and tissue diversity. Wnt functions extend into adulthood where they regulate stem cell behavior, tissue homeostasis, and damage repair. Disruption of Wnt signaling activity during embryonic development or in adults results in a spectrum of abnormalities and diseases, including cancer. The molecular mechanisms that underlie the myriad of Wnt-regulated biological effects have been the subject of intense research for over three decades. This review is intended to summarize our current understanding of how Wnt signals are generated and interpreted.

This article is categorized under:

Biological Mechanisms > Cell Signaling

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### Keywords

beta-catenin; development; frizzled; signaling; stem cells; WNT

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**CONFLICT OF INTEREST**

The authors have declared no conflicts of interest for this article.

**FURTHER READING**

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## 1 | INTRODUCTION

Since their discovery by Nusse and Varmus (1982), *Wnt* genes have captivated the attention of geneticists, protein biochemists, structural and cell biologists, embryologists, and stem and cancer cell scientists alike. The isolation and identification of the first *Wnt* gene was made possible by the insertion of the mouse mammary tumor virus, an oncogenic retrovirus, into a genomic locus then named integration site 1, or *int1*, an event that conferred a tumorigenic growth advantage on cells of the mammary gland. Low stringency hybridization of genomic DNA from multiple species with an *int1* probe hinted at a high degree of conservation of these sequences from flies to humans (Nusse, van Ooyen, Cox, Fung, & Varmus, 1984). In an unrelated line of investigation, *Drosophila* geneticists studying mutants that produced so-called segment polarity phenotypes, identified a gene called *wingless* (*wg*; Nusslein-Volhard & Wieschaus, 1980; Sharma & Chopra, 1976), and molecular cloning of the *wg* and *int1* genes revealed them to be homologs (Baker, 1987; Rijsewijk et al., 1987), an early example that underscored the close relationship between embryogenesis and tumorigenesis. With the realization that *wg* and *int1* were members of a much larger gene family (Gavin, McMahon, & McMahon, 1990), the term Wnt was coined denoting "Wingless-related integration site" (Nusse et al., 1991).

Since these early days, research on Wnt genes has radiated into virtually every organism from hydra to humans and into every discipline of the life sciences from structural biology to medicine. With thousands of publications per year on Pubmed citing "Wnt" as a keyword, the field of Wnt biology is well established with regular international meetings dedicated to this broad topic. This review alone cannot encompass the entirety of this vast field, but rather will focus on our current understanding of how Wnt signals are produced and how they influence downstream molecular signaling events. For further information on Wnt and its biology, we encourage the reader to consult the primary literature, the many books and reviews focused on this topic as well as the Wnt homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/).

The biological effects of Wnt are vast and at first glance seemingly unrelated. The thread connecting the diversity of biological effects is Wnt's property to break symmetry and establish body axes. This feature is apparent both at the cellular level, where Wnt signaling influences asymmetric cell divisions, and at the organismal level, where perturbations in Wnt signaling produce patterning defects. During adulthood, Wnt signaling plays critical roles in tissue homeostasis and wound healing, which involves the regulation and balance of tissue stem cells. Given the many essential roles of Wnt in the life of an organism, it is not surprising that alterations in Wnt signaling yield profound and often catastrophic effects, including developmental defects and cancer. This review aims to provide the reader with a foundation in the mechanisms by which Wnt signals are generated and interpreted at the cellular level.

## 2 | WNT: A UNIQUE CLASS OF SIGNALING MOLECULES

### 2.1 | Wnt proteins

Wnt genes encode secreted growth factors with short-range signaling activity. The mammalian genome contains at least 19 distinct *Wnt* genes, some of which express alternative transcripts that produce Wnts with variable activities (Bauer, Benard, Gaasterland, Willert, & Cappellen, 2013; Bunaciu, Tang, & Mao, 2008; Dichmann, Walentek, & Harland, 2015; Fear, Kelsell, Spurr, & Barnes, 2000; Katoh, Kirikoshi, Saitoh, Sagara, & Koike, 2000; Teh et al., 2007). Aside from the presence of a signal sequence, signifying entry into the secretory pathway, and a large number of invariantly spaced cysteine residues, the Wnt polypeptide sequence reveals little about its function and structure. Early biochemical experiments detected these proteins associated with the cell surface and extracellular matrix (Bradley & Brown, 1990; Brown, Papkoff, Fung, Shackleford, & Varmus, 1987; Burrus & McMahon, 1995; Papkoff, Brown, & Varmus, 1987) and, to a lesser extent, in the conditioned medium (Bradley & Brown, 1995; Shibamoto et al., 1998; van Leeuwen, Samos, & Nusse, 1994). Purification of biologically active Wnt proteins—first Wnt3a (Willert et al., 2003) and then Wnt5a (Mikels & Nusse, 2006)—revealed these proteins to be highly hydrophobic, a property afforded by a covalently attached lipid. This property still confounds isolation of other Wnt proteins, all of which exhibit slightly distinct biochemical properties. Furthermore, maintaining the solubility of purified Wnt proteins requires detergents, which are incompatible with in vivo systems, and dilution into nondetergent conditions often renders purified Wnts instantly insoluble and inactive. These obstacles have been partially overcome by preparing Wnt liposomes, which stabilize Wnt activity in the absence of detergents (Morrell et al., 2008; Tuysuz et al., 2017), though the feasibility of this process is not yet established for all Wnts.

X-ray crystallography revealed that Wnt proteins are composed of two domains that together form a structure resembling a hand (Janda, Waghray, Levin, Thomas, & Garcia, 2012). This hand-like molecule grasps the extracellular cysteine-rich domain (CRD) of Frizzled (Fzd), which is one of several receptors capable of interacting with Wnt proteins (see section “Wnt signal reception”). The amino (N)-terminal domain of Wnt, also referred to as D1, carries the lipid, which extends from a thumb-like extension, while the carboxy (C)-terminal domain, also referred to as D2, comprises an index finger-like portion (Bazan, Janda, & Garcia, 2012). Together the thumb and index finger engage the receptor with both lipid-and protein-mediated contacts. D1 resembles a saposin fold, which is a highly conserved four α-helix bundle that in many other proteins interacts with lipids. It has been proposed that over the course of evolution, the associated lipid came to be covalently attached to this fold. D2, on the other hand, resembles a cystine-knot cytokine, similar to those found in PDGF, IL17 and Noggin. The evolutionary origin of Wnt may have occurred from the fusion of these ancestral D1 and D2 precursors to create a signaling molecule unique to the metazoan lineage (Bazan et al., 2012).

### 2.2 | Wnt processing

As Wnts transit through the secretory pathway, they are modified in two important ways: acylation and glycosylation. Acylation, specifically the attachment of palmitoleic acid (PA),

a monounsaturated 16-carbon lipid, to a conserved serine (Ser) residue (Takada et al., 2006), occurs early during Wnt processing, and is essential for activity as mutation of the Ser residue produces an inactive protein. Aside from being critical for activity, the covalently attached PA renders the Wnt protein highly hydrophobic and poorly soluble in an aqueous environment (Willert et al., 2003), thereby restricting Wnt's signaling range to short distances. All Wnts tested to date (with the exception of the divergent Drosophila WntD) are lipidated at least on one site, a post-translational modification catalyzed by the endoplasmic reticulum (ER)-resident O-acyl transferase Porcupine (Porcn, Figure 1). Porcn's primary role appears to be dedicated to the processing of Wnt proteins; however, the observation that a catalytically inactive Porcn promotes a rate-limiting signaling pathway in cancer cell growth hints at an alternate function for Porcn (Covey et al., 2012). Mutation of Porcn or treatment with small molecule inhibitors of Porcn (Chen et al., 2009; Dodge et al., 2012; Proffitt et al., 2013) creates a condition akin to an all-Wnt mutant, as no active Wnt proteins are secreted from the producing cell. This natural bottleneck in Wnt processing offers a unique opportunity to explore the requirement for Wnt signaling in diverse biological settings. For example, by using cells harboring mutations in the *Porcn* gene, we demonstrated that Wnt signaling is required for reprogramming of fibroblasts to an induced pluripotent stem cell state (Ross et al., 2014). Furthermore, blocking Wnt secretion may provide an opportunity to combat Wnt-driven tumors, and Porcn inhibitors are currently in clinical trial for the treatment of advanced solid tumors.

In contrast to this acylation, which occurs on at least one defined site, glycosylation of Wnt is more variable: for example, Wnt1 carries four and Wnt3a carries two N-linked glycosylations. Although mutation of individual glycosylation sites impairs Wnt's secretion (Komekado, Yamamoto, Chiba, & Kikuchi, 2007; Kurayoshi, Yamamoto, Izumi, & Kikuchi, 2007; Mason, Kitajewski, & Varmus, 1992), it is not strictly required for activity; in fact in the case of Wingless (Wg, the Drosophila homolog of Wnt1), glycosylation is entirely dispensable for its activity (Tang et al., 2012).

In the next step of its maturation, Wnt associates with Wls (also known as Gpr177 or Evi, Figure 1), a *trans*-membrane protein required for movement of Wnt from the *trans*-Golgi network to the cell surface (Banziger et al., 2006; Bartscherer, Pelte, Ingelfinger, & Boutros, 2006; Goodman et al., 2006). This association of Wnt with Wls requires Porcn-mediated acylation (Herr & Basler, 2012; Tang et al., 2012). Furthermore, interfering with Wls function leads to retrograde Golgi-to-ER transport of Wnt and to ER stress (Zhang, Zhou, Pei, Lin, & Yuan, 2016). Upon release of Wnt at the cell surface, which requires vacuolar acidification (Coombs et al., 2010), Wls is recycled via the retromer complex to the Golgi, where it can facilitate secretion of newly synthesized and acylated Wnt proteins (Belenkaya et al., 2008; Coudreuse, Roel, Betist, Destree, & Korswagen, 2006; Harterink et al., 2011; Port et al., 2008; Prasad & Clark, 2006; Yang et al., 2008; Yu et al., 2014; Zhang et al., 2011). Taken together, Wls acts as a Wnt-specific chaperone essential for the proper transit of Wnt through the secretory pathway and eventual release from the cell. Mutations in Porcn and Wls produce similar gastrulation defects in the early mouse embryo (Biechele, Cox, & Rossant, 2011; Fu, Jiang, Mirando, Yu, & Hsu, 2009), resembling those observed in Wnt3 knockouts (Barrow et al., 2007; Liu et al., 1999), which highlights the essential roles these enzymes play in Wnt maturation.

### 2.3 | Wnt secretion

Release of Wnt from a cell has been the subject of extensive research, with multiple distinct mechanisms proposed for how Wnt may contact a neighboring cell (Figure 1). Although certain Wnt proteins have been isolated in a cell-free form (Mikels & Nusse, 2006; Shibamoto et al., 1998; van Leeuwen et al., 1994; Willert et al., 2003), Wnts remain active when immobilized (Habib et al., 2013), suggesting that Wnts may signal in a manner is more akin to Notch signaling than to conventional soluble growth factors. Most importantly, flies carrying a modified *wg* gene that produces a membrane-tethered protein develop normally with only a mild proliferative effect (Alexandre, Baena-Lopez, & Vincent, 2014), suggesting that all essential Wg activity is mediated by direct cell–cell contact.

Despite their close association with cells, Wnts have been observed at greater distances from their site of synthesis. The extreme hydrophobic nature of Wnts necessitates some type of association with other molecules or subcellular structures that shield the hydrophobic moiety in a largely aqueous environment. For example, chaperones that associate with Wnt and shield the lipid moiety have been identified, including secreted Wnt interacting molecule (SWIM; Mulligan et al., 2012) and Afamin (AFM; Mihara et al., 2016). Although these proteins clearly associate with Wnt and influence its secretion and solubility, their requirement in biological processes has not been rigorously established. Within the extracellular space, Wnts have been found to interact with various types of glycans, such as syndecans, glypicans, and biglycan (Alexander et al., 2000; Berendsen et al., 2011; Capurro, Martin, Shi, & Filmus, 2014; Lin & Perrimon, 1999), which regulate Wnt distribution and signaling range. Furthermore, several studies identified a series of genes encoding enzymes involved in heparan sulfate synthesis, including *sugarless* (homolog of human *UGDH*), *sulfateless* (*NDST1*), *tout-velu* (*EXT1*), and *sister of tout-velu* (*EXT2*), all of which regulate Wnt signaling (Binari et al., 1997; Bornemann, Duncan, Staatz, Selleck, & Warrior, 2004; Hacker, Lin, & Perrimon, 1997; Haerry, Heslip, Marsh, & O'Connor, 1997; Han et al., 2004; Khare & Baumgartner, 2000; Lin & Perrimon, 1999; Takei, Ozawa, Sato, Watanabe, & Tabata, 2004). The activity of these enzymes is not absolutely required for Wnt signaling, but rather serves to modulate Wnt distribution, receptor binding, and signaling.

Another mechanism by which Wnts may reach longer distances is through actin-based membrane protrusions, variably referred to as filopodia, cytonemes, or nanotubes (Hsiung, Ramirez-Weber, Iwaki, & Kornberg, 2005; Huang & Kornberg, 2015; Stanganello et al., 2015). In this mode of distribution, Wnt would remain associated with the plasma membrane of the Wnt expressing cell. The tips of these membrane protrusions may be reached either by lateral diffusion along the plasma membrane or by molecular motors that transport Wnt-containing vesicles along actin-based structures.

Additionally, several studies have identified Wnts on extracellular particles, including exosomes (Beckett et al., 2013; Chen, Takada, Noda, Kobayashi, & Takada, 2016; Gross, Chaudhary, Bartscherer, & Boutros, 2012; Harada et al., 2017; Korkut et al., 2009; Luga et al., 2012) and lipoprotein complexes (Neumann et al., 2009; Panakova, Sprong, Marois, Thiele, & Eaton, 2005), both of which would be capable of carrying Wnts long distances in bodily fluids, such as blood and cerebrospinal fluid. It also seems possible for Wnts to traverse through several cell boundaries in vesicles called argosomes (Greco, Hannus,

& Argosomes, 2001). These various methods of Wnt protein distribution may provide a mechanism by which a membrane-tethered Wg retains biological activity (Alexandre et al., 2014). An unsolved mystery for all of these modes of Wnt transport is how Wnts are transferred from these carrier proteins and structures to their cognate receptor to initiate signaling in the target cell.

## 3 | WNT SIGNALING AT THE MEMBRANE

### 3.1 | Wnt signal reception

Several distinct Wnt receptors with signaling activities have been identified (Figure 2). The first Wnt receptor to be identified was Drosophila Frizzled 2 (Dfz2); overexpression of this cell surface protein conferred Wg binding and signaling, as monitored by stabilization of the Armadillo protein (Arm, the fly homolog of β-catenin; Bhanot et al., 1996). The role of Dfz2 in Wg signaling is functionally redundant with the activity of Fz, as only *dfz2-fz* double mutants produce the characteristic segment polarity phenotype of *wg* and *porc* (the fly homolog of Porcn) mutants (Bhanot et al., 1999; Bhat, 1998).

The mammalian genome encodes 10 Frizzled (Fzd1–10) proteins. With their seven *trans*-membrane domain spanning topology, these Wnt receptors resemble G-protein coupled receptors (GPCRs, see section “Wnt and G-proteins”). A highly conserved extracellular CRD is required for Wnt binding. The structure of this CRD indicates that it is capable of forming a dimer (Dann et al., 2001), a property that may be promoted by Wnt, with its covalently attached unsaturated lipid moiety binding in a U-shaped hydrophobic groove formed by the two CRD molecules (Nile, Mukund, Stanger, Wang, & Hannoush, 2017). Additional data indicates that Fzd dimerization is mediated by interactions of *trans*-membrane α-helices 4 and 5 (Petersen et al., 2017). The precise mechanism by which Wnt binding to the CRD activates signaling inside the cell is not well understood and will require the complete structural analysis of a Fzd molecule.

The selectivity between Wnts and Fzds remains poorly understood. Biochemical studies with Wg revealed 10-fold higher affinity of Wg to Dfz2 than to Fz (Rulifson, Wu, & Nusse, 2000). Analysis of Wnt-Fzd interaction.s is confounded in large part by the scarcity of sufficiently purified and active Wnt proteins. Kinetic binding studies between a limited number of Wnts (Wnt3a, 4, 5a and 5b) and Fzd CRDs (Fzd1, 2, 4, 5, 7 and 8) revealed strong and weak associations, with dissociation constants ($K_D$) ranging from less than 10 nM to greater than 100 nM (Dijksterhuis et al., 2015). Interestingly, Wnt3a binding to Fzd appeared to be highly promiscuous, a finding supported by Voloshanenko and colleagues who showed that Wnt3/3a coupled effectively to seven of nine Fzd receptors tested (Voloshanenko, Gmach, Winter, Kranz, & Boutros, 2017). Furthermore, Fzd5, Fzd8, and to a lesser extent Fzd4 were capable of transducing signals of multiple Wnts. Together, these studies provide somewhat limited insight into Wnt-Fzd specificities and may signify that Wnt-Fzd interactions are dictated by the tissue-specific expression patterns of these genes.

An important insight into Wnt signaling specificity came from the finding that the *Drosophila* gene *arrow*, which encodes a single-pass *trans*-membrane protein homologous

to mammalian Lrp5 and Lrp6, was required for Wg signal reception (Wehrli et al., 2000). This finding contributed to the current model that Wnt promotes the heterodimerization of Fzd and Lrp5/6 and thereby activates downstream signaling (Pinson, Brennan, Monkley, Avery, & Skarnes, 2000; Tamai et al., 2000), which involves recruitment of intracellular signaling components, such as Axin, Disheveled (Dsh/Dvl) and glycogen synthase kinase 3 (GSK3), to the receptor complex to form the so-called signalosome (Bilic et al., 2007; Cliffe, Hamada, & Bienz, 2003; Davidson et al., 2005; Fiedler, Mendoza-Topaz, Rutherford, Mieszczanek, & Bienz, 2011; Zeng et al., 2005; Zeng et al., 2008). This mechanism of receptor heterodimerization and subsequent signal transduction was further confirmed by the use of bispecific binders, referred to as Wnt surrogates, that simultaneously bound Fzd and Lrp6 and activated signaling (Janda et al., 2017). The assignment of the terms receptor and coreceptor for Fzd and Lrp5/6, respectively, may be somewhat misleading as both components are capable of independently binding Wnt proteins (Bourhis et al., 2010). In this regard, it is noteworthy that overexpression of Fzd generally does not ectopically activate Wnt signaling whereas overexpression of Lrp5/6 does (Mao et al., 2001).

The extracellular domain of Lrp5/6 is comprised of four PE domains (YWTD β-propeller and EGF-like) that mediate interactions with several extracellular ligands, including Wnts (Bourhis et al., 2010; Mao et al., 2001; Pinson et al., 2000; Tamai et al., 2000) and the Wnt antagonists Dickkopf (Dkk; Bafico, Liu, Yaniv, Gazit, & Aaronson, 2001; Mao et al., 2001; Semenov et al., 2001) and Sclerostin (Sost; Li et al., 2005; Semenov, Tamai, & He, 2005). Analysis of the Lrp6 ectodomain using negative-stain electron microscopy revealed that each of these four PE domains forms a globular module with a flexible hinge connecting the second and third PE domain (Matoba et al., 2017), consistent with the structure solved by X-ray crystallography (Cheng et al., 2011). Binding of Dkk1 converts this flexible ectodomain into a compact conformation that likely precludes binding of Wnt, thus explaining the antagonistic activity of Dkk.

Although it is well established that a ternary complex of Wnt, Fzd and Lrp5/6 is essential to β-catenin signaling, mechanisms by which specificity is generated remain poorly understood. Recent studies on vascular development in the central nervous system shed new light on Wnt signaling specificity: the cell surface proteins Gpr124 (an orphan GPCR, gene name *Adgra2*) and Reck (Reversion-inducing-cysteine-rich protein with kazal motifs, a glycosylphosphatidylinositol [GPI]-anchored glycoprotein) act in a ternary complex with Fzd to enhance Wnt7/β-catenin signaling (Cho, Smallwood, & Nathans, 2017; Vanhollebeke et al., 2015). A distinct β-catenin signaling pathway in endothelial cells activated by Norrin (Ndp), a potent Fzd4-specific agonist, requires Tspan12 (Tetraspanin 12) for maximal signaling (Lai et al., 2017). Importantly, in this Ndp-Fzd4 context, Reck and Gpr124 have no augmenting activity. Similarly, our studies on hematopoietic stem cell development in zebrafish identified a highly specific requirement for Wnt9a: a Wnt9a morphant phenotype was only rescued by Wnt9a overexpression, but not by Wnt9b or the highly promiscuous Wnt3a (Grainger et al., 2016), suggesting the presence of additional components, such as coreceptors, that confer signaling specificity on individual Wnts. These observations hint at the potential complexity of Wnt signaling specificity: molecules distinct from Gpr124, Reck, and Tspan12 likely effect signaling specificity of other Wnt-regulated programs.

Aside from this central Fzd-Lrp5/6 complex, which stimulates Wnt/β-catenin signaling, several other Wnt receptors have been identified (Figure 2), including Ror1 and 2 (Receptor tyrosine kinase-like orphan receptors) and Ryk (Receptor-like tyrosine kinase), which promote alternate signaling cascades. The extracellular domains of Ror1/2 bear homology with the CRD of Fzd, indicating that Wnts engage these receptors in a similar manner to Fzd. Ror1 and 2 have been primarily characterized for their ability to bind Wnt5a (Fukuda et al., 2008; Liu, Rubin, Bodine, & Billiard, 2008; Mikels & Nusse, 2006; Oishi et al., 2003), however, interactions with other Wnt proteins have not been explored. Interestingly, Wnt5a is capable of mediating the heterodimerization of Ror1 and Ror2 (Yu et al., 2016), echoing Wnt's ability to promote the dimerization of the Fzd CRDs via its lipid moiety (Nile et al., 2017).

The involvement of Ryk in Wnt signaling was first suggested by the homology of its extracellular domain to the Wnt-inhibitory-factor-1 (Wif-1; Patthy, 2000), a secreted protein that binds Wnts and inhibits their activity (Hsieh et al., 1999). Studies in *Caenorhabditis elegans* vulval development (Inoue et al., 2004) and in Drosophila axon guidance (Yoshikawa, McKinnon, Kokel, & Thomas, 2003) provided genetic evidence that Ryk (LIN-18 in worms and derailed/drl in flies) interacts with and transduces Wnt signals. Mammalian Ryk was shown to interact with Wnt1 and Wnt3a via its WIF domain and form a ternary complex with Fzd (Lu, Yamamoto, Ortega, & Baltimore, 2004).

A final cell surface protein deserving mention in the context of Wnt receptors is Ptk7 (Protein tyrosine kinase 7, also known as colon carcinoma kinase-4, CCK4), a catalytically inactive receptor tyrosine kinase that interacts with multiple Wnt components both in the extra- and intracellular space to regulate cell movements and participate in the establishment of planar cell polarity (PCP; Lu et al., 2004). Several Wnts, including mouse Wnt3a, 4, 5a, and 8, interact with the extracellular domain of Ptk7 (Martinez et al., 2015; Peradziryi et al., 2011), which is comprised of seven immunoglobulin domains. Interactions of Wnt with Ptk7 require the presence of other proteins: for example, Wnt3a-Ptk7 binding requires the CRD of Fzd7 (Berger et al., 2017). In flies, the Ptk7 homologs off track (Otk) and Otk2 form complexes with Fz, Dfz2, and Wnt2 to regulate development of the genital tract (Linnemannstons et al., 2014). Although Ptk7 has a clear role in regulating tissue architecture and cell movements (so-called noncanonical Wnt processes), its role in the well-established Wnt/β-catenin pathway remains controversial. For example, in formation of the Spemann organizer, which is critical to establishment of the vertebrate body axis, *Ptk7*-activated Wnt signaling (Puppo et al., 2011). In contrast, *ptk7* mutant zebrafish showed an increase in expression of Wnt target genes, suggesting that Ptk7 acts to blunt Wnt signaling (Hayes, Naito, Daulat, Angers, & Ciruna, 2013). These controversial aspects of Wnt-Ptk7 aside, it is generally accepted that Ptk7 acts to fine-tune Wnt signaling, a process that is regulated by many other molecules as well.

### 3.2 | Tuning extracellular Wnt activity

The complexity of cell surface molecules that interact with Wnts and transduce their signals is matched by an equally diverse set of secreted and membrane bound proteins that modulate Wnt activity in the extracellular space. Wnt antagonists act at multiple levels to curtail

Wnt's signaling strength and range (Figure 3). Several secreted proteins, including secreted frizzled-related proteins (Sfrp; Finch et al., 1997; Leyns, Bouwmeester, Kim, Piccolo, & De Robertis, 1997; Rattner et al., 1997; Wang, Krinks, Lin, Luyten, & Moos Jr, 1997), Wnt-inhibitory factor 1 (Wif1; Hsieh et al., 1999) and Cerebus (Cer; Piccolo et al., 1999), act by directly binding to Wnt to block their ability to engage receptors. Sfrps carry a CRD homologous to the ligand-binding domain of Fzd, and the prevailing model has been that Sfrps antagonize Wnt signaling (Leyns et al., 1997; Wang et al., 1997). However, several studies have found that Sfrps do not always inhibit signaling (Galli et al., 2006), and at times even promote signaling (Xavier et al., 2014). Such biphasic effects may be explained by Sfrp dosage, Sfrp's ability to homo- or heteromultimerize and cellular context.

Wif1 is composed of a WIF domain (also found in Ryk/Drl) followed by five EGF-like domains. Binding studies revealed that Wif1 is capable of interacting with multiple Wnts (Hsieh et al., 1999; Surmann-Schmitt et al., 2009), and structural analysis indicates that both the WIF domain and EGF-like domains synergize to bind Wnt proteins (Malinauskas, Aricescu, Lu, Siebold, & Jones, 2011).

Cer, a secreted cystine-knot domain-containing protein, is highly expressed during gastrulation and is a potent inducer of the embryonic axis. It largely acts as an inhibitor of TGF-β signaling by binding to Nodal and BMP. Interestingly, in Xenopus Cer binds Wnt (Piccolo et al., 1999), an interaction that may not be conserved in other vertebrates, such as mice (Belo et al., 2000).

Aside from sequestering Wnt proteins through interactions with such molecules as Sfrp, Wif1, and Cer, Wnt proteins can also be inactivated enzymatically. *Notum*, which suppresses Wnt signaling in a wide variety of settings (Flowers, Topczewska, & Topczewski, 2012; Giraldez, Copley, & Cohen, 2002; Petersen & Reddien, 2011), encodes an extracellular deacylase that cleaves the essential PA moiety from Wnt, thereby rendering it inactive (Kakugawa et al., 2015; Zhang et al., 2015). In contrast, *Tiki1* and *Tiki2* (gene names *Trabd2a* and *Trabd2b*) encode *trans*-membrane Wnt-specific metalloproteases that cleave the N-termini of Wnts to inactivate them (Zhang et al., 2012; Zhang et al., 2016).

Wnt signaling activity is furthermore regulated by a host of cell surface molecules that regulate receptor availability. For example, the *trans*-membrane E3 ubiquitin ligases Znrf3 and Rnf43 ubiquitinate Fzd receptors and promote their internalization and subsequent degradation, thus desensitizing a cell to extracellular Wnt ligands (Hao et al., 2012; Koo et al., 2012). Importantly, binding of R-spondin (Rspo1–4), a secreted Wnt agonist, to the stem cell marker Lgr4/5/6, downregulates these ubiquitin ligases and thereby leads to an increase in Fzd proteins on the cell surface. Therefore, Rspo acts to augment or uncover Wnt signaling activity by increasing receptor availability, however, it should be noted that Rspo has no Wnt signaling activity in itself.

As mentioned above, other secreted proteins, including Dkk and Sost, directly bind to Lrp5/6 to preclude Wnt-mediated heterodimeriztion between Lrp5/6 and Fzd. In addition, Kremen1 and 2 interact with Dkk to form a ternary complex with Lrp6 and promote the rapid internalization of Lrp6 (Davidson, Mao, del Barco Barrantes, & Niehrs, 2002; Mao

et al., 2002). Acting similarly, the extracellular portion of Apcdd1 directly interacts with Wnt3a and Lrp5 to preclude formation of a productive Wnt-Fzd-Lrp5/6 receptor complex (Shimomura et al., 2010).

A feature common to all signal transduction pathways is the activation of negative feedback loops, which serve to desensitize cells to the initial signaling input. Likewise, many of the above-mentioned Wnt antagonists are direct targets of Wnt signaling, including Dkk1 (Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004), Sfrp2 (Lescher, Haenig, & Kispert, 1998), Notum (Gerlitz & Basler, 2002; Torisu et al., 2008), Cer (Huggins et al., 2017), Apcdd1 (Takahashi et al., 2002), Znrf3, and Rnf43 (Hao et al., 2012; Van der Flier et al., 2007).

### 3.3 | Wnt and G-proteins

The seven-span *trans*-membrane topology of Fzd predicted that these Wnt receptors are GPCRs. Experimental evidence of G-protein involvement in Wnt signaling first surfaced in the study of noncanonical Wnt signaling where Wnt5a and Fzd synergized to increase the frequency of intracellular $Ca^{2+}$ transients in zebrafish embryos (Slusarski, Corces, & Moon, 1997; Slusarski, Yang-Snyder, Busa, & Moon, 1997). Using a chimeric receptor that incorporated the ligand binding domains of the $\beta_2$-adrenergic receptor into Fzd, Liu and colleagues showed that the β-adrenergic agonist isoproterenol was able to promote the stabilization of β-catenin, an effect that was blocked by addition of pertussis toxin and by depletion of Gαq and Gαo (Liu et al., 2001). Furthermore, Wnt5a binding to rat Fzd3 led to decreases in cyclic guanosine monophosphate (cGMP) levels and increases in inositol triphosphate (IP3) and diacylglycerol (DAG), both well-known downstream messengers of G-protein signaling (Ahumada et al., 2002). However, it should be noted that Wnt5a acts primarily through the Ror family of receptor tyrosine kinases as simultaneous disruption of Ror1 and Ror2 in mice cause embryonic defects that phenocopy loss of Wnt5a (Ho et al., 2012). A well-established in vitro assay for Wnt5a signaling is inhibition of Wnt/β-catenin signaling (Ishitani et al., 1999; Mikels & Nusse, 2006), and this pathway requires Ror expression, is pertussis toxin insensitive and has no effect on $Ca^{2+}$ levels (Mikels & Nusse, 2006), observations inconsistent with the involvement of G-proteins downstream of Wnt5a-Ror signaling.

Nonetheless, several recent lines of research provide evidence for roles of G-proteins downstream of Wnt signaling. For example, genetic experiments in Drosophila showed that the Gαo subunit mediates signaling downstream of Fzd receptors (Katanaev, Ponzielli, Semeriva, & Tomlinson, 2005). Furthermore, in rat brain membranes and cultured cells, Wnt3a triggered Fzd-dependent guanine-nucleotide exchange in a Wnt antagonist and pertussis toxin sensitive manner (Koval & Katanaev, 2011). Nichols, Floyd, Bruinsma, Narzinski, and Baranski (2013) suggested that Fzd may act as nontraditional GPCRs by preferentially coupling to Gαs heterotrimeric G-proteins. Using fluorescence recovery after photobleaching (FRAP) and Förster resonance energy transfer (FRET) technology, Schulte and colleagues showed that Fzd complexes with Gαi and Gαq dissociated upon Wnt5a stimulation (Kilander et al., 2014; Kilander, Dahlstrom, & Schulte, 2014). Finally, a recent study demonstrated that Daple, a Dvl-binding protein, bridges Fzd-Gαi interactions

to mediate G-protein activation by Wnt (Aznar et al., 2015). Taken together, mounting evidence points to a role of G-proteins downstream of Wnt in a manner somewhat distinct from traditional GPCRs.

## 4 | WNT SIGNALLING IN THE CYTOPLASM

Once a Wnt signal has been received and transduced across the membrane, a variety of signaling pathways may be activated. Most prominent among the various pathways is Wnt/β-catenin signaling, commonly referred to as “canonical” or “cell fate” Wnt signaling (Loh, van Amerongen, & Nusse, 2016). In addition to this pathway, which culminates in the nucleus to activate expression of target genes, several other cytoplasmic pathways and effectors, collectively termed “noncanonical,” “β-catenin independent” or “cell polarity” signals, have been described. Although these alternative signaling pathways likely comprise independent signaling events, they are not necessarily mutually exclusive from each other, a finding that is only beginning to be uncovered. For example, in the lung, the classic beta-catenin independent Wnt, *Wnt5a* acts upstream of *Axin2*, a beta-catenin target gene (Nabhan, Brownfield, Harbury, Krasnow, & Desai, 2018). In addition, particular Wnt-Fzd combinations may be able to activate multiple signaling cascades, though this is poorly understood. These noncanonical branches of Wnt signaling are beyond the scope of this review and will not be further discussed here, and have been reviewed elsewhere (van Amerongen, 2012).

### 4.1 | β-catenin: Adherens junction component versus transcriptional regulator

β-catenin acts as the primary mediator of Wnt signaling by either translocating to the nucleus to activate target gene expression, or being tagged and degraded constitutively. β-catenin’s critical role in Wnt signaling was first appreciated in *Drosophila*, where Armadillo (the fly homolog of β-catenin) acted in the same pathway as Wingless (a fly Wnt) to regulate segment polarity (Nusslein-Volhard & Wieschaus, 1980). Years later, a role for β-catenin in cell junctions was identified in *Xenopus* (McCrea, Turck, & Gumbiner, 1991). Therefore, there are two pools of β-catenin in the cell, one associated with adherens junctions, and another associated with the Wnt pathway, either in the cytosol, or the nucleus.

The factors affecting whether β-catenin acts as a transcriptional regulator, or in adherens junctions are poorly understood, but seem to involve a balance with other cadherins. It is possible that β-catenin exists in two pools, one involved in Wnt signaling, as a monomer, and one bound to α-catenin and existing as dimers in adherens junctions; the regulation of which form may be regulated by phosphorylation and conformational changes, although this is not entirely clear (Gottardi & Gumbiner, 2004). Overexpression of cadherins in *Xenopus* or *Drosophila* phenocopy loss of the Wnt/β-catenin signal, and can be rescued by β-catenin, but not by Xwnt-8, suggesting that cadherins negatively regulate the Wnt signal at the level of β-catenin (Heasman et al., 1994; Sanson, White, & Vincent, 1996). β-catenin’s interactions with the nuclear DNA-binding proteins encoded by the Tcf/Lef family seem to similarly compete with cadherins in the context of human cancer cell lines, as well as other tissue culture models (Gottardi, Wong, & Gumbiner, 2001; Kuphal & Behrens, 2006; Shtutman et al., 1999; Stockinger, Eger, Wolf, Beug, & Foisner, 2001). In addition, releasing

β-catenin from adherens junctions with proteases, such as ADAM10, is sufficient to drive nuclear translocation of β-catenin and an increase in the Wnt signal (Maretzky et al., 2005; Reiss et al., 2005; Uemura et al., 2006), further supporting the notion that β-catenin exists in two pools, and shuttling between these regulates, at least in part, the Wnt signal. However, it is worthy of note that a loss of E-cadherin is not sufficient to affect the Wnt signal (Herzig, Savarese, Novatchkova, Semb, & Christofori, 2007); this may be due to compensatory loss of β-catenin, mediated for example, by the destruction complex.

### 4.2 | The β-catenin destruction complex

Evidence for how the Wnt signal is transduced from the membrane to the nucleus came from studies of Drosophila segmentation, and the Drosophila homolog *wingless* (*wg*) and its requirement for maintaining expression of the segment polarity gene *engrailed* (*en*; DiNardo, Sher, Heemskerk-Jongens, Kassis, & O'Farrell, 1988). The β-catenin homolog, *armadillo (arm)*, was linked to *wg* since flies deficient for either *wg* or *arm* were phenotypically similar, and both transcripts are expressed in similar domains (Peifer, Rauskolb, Williams, Riggleman, & Wieschaus, 1991). In a similar study, it was observed that loss of *zesty-white3* (*zw3*), the homolog to *GSK3*, phenocopies the loss of Wg, and leads to an activation *engrailed*, in a Wg-dependent manner (Siegfried, Chou, & Perrimon, 1992), providing the first mechanistic insight into β-catenin (*arm*) regulation. These studies built the foundation for our understanding of the most well-studied (canonical, or β-catenin-dependent) arm of the Wnt signaling cascade, where β-catenin operates as an inductive switch; its nuclear translocation results in the activation of target gene transcription (discussed below). To achieve this, the buildup of β-catenin in the cytoplasm, resulting in its nuclear translocation is tightly regulated by a group of proteins commonly called the "destruction complex."

The destruction complex is comprised of several components, including Axin, adenomatous polyposis coli (APC), the serine/threonine kinases GSK3 and Casein kinase 1 (CK1), protein phosphatase 2A (PP2A), and beta-transducin repeat-containing E3 ubiquitin-protein ligase (βTrCP); together, these function to sequester and target β-catenin for degradation in the proteasome, downregulating the Wnt signal (Figure 4). Another component acting upstream of the destruction complex, called Disheveled (Dvl in mouse, Dsh in flies), was first identified in *Drosophila* as a segment polarity gene (Klingensmith, Nusse, & Perrimon, 1994), required for transmitting the Wg signal (Noordermeer, Klingensmith, Perrimon, & Nusse, 1994; Theisen et al., 1994). Dsh/Dvl is a phosphorylated protein present in the cytosol, which becomes hyperphosphorylated and associated with the membrane in response to Wnt signaling (Yanagawa, van Leeuwen, Wodarz, Klingensmith, & Nusse, 1995). In mice, this is more complicated, since there are three homologs to Dvl (Klingensmith et al., 1996; Sussman et al., 1994; Tsang et al., 1996; Willert, Brink, Wodarz, Varmus, & Nusse, 1997), which display some degree of functional overlap in some developmental contexts (Etheridge et al., 2008). Surprisingly, mice deficient for two of the three Dvl homologs have normal Wnt/β-catenin reporter activity, which is only abolished in triple mutants (Etheridge et al., 2008), confirming a requirement for Dvl in the Wnt signal in vertebrates, but suggesting considerable functional overlap as well as functional variation. Dvl interacts with Fzd at the C-terminal tail (Tauriello et al., 2012), and operates in both β-catenin dependent and independent pathways, and seems to play a role in determining whether or

not the receptor complex is internalized (Jiang, Charlat, Zamponi, Yang, & Cong, 2015; Yu et al., 2007). The mechanism of regulation and differences in Dvl specificity are poorly understood, and may relate to different interacting partners, as reviewed in Mlodzik (2016). In the β-catenin signaling cascade, Dvl binds to Axin, Fzd and LRP5/6 in response to Wnt, to form signalosomes, which are necessary for Wnt signaling (Bilic et al., 2007; Zeng et al., 2008), although the mechanism of action beyond this is incompletely understood.

It is thought that one of the earliest steps in dissociation of the destruction complex is Axin association with Lrp5/6 (Mao et al., 2001; Tolwinski et al., 2003). Axin acts as a scaffolding anchor to the destruction complex; consistent with this, it is known to interact directly with APC, GSK3β, and β-catenin (Ikeda et al., 1998; Itoh, Krupnik, & Sokol, 1998; Kishida et al., 1998; Rubinfeld et al., 1996). Axin is thought to be the rate-limiting component (Lee, Salic, Kruger, Heinrich, & Kirschner, 2003), and its levels are tightly controlled by GSK-mediated phosphorylation (Yamamoto et al., 1999). Upon Wnt signal reception and formation of the signalosome, Axin is dephosphorylated by protein phosphatase 1 (PP1), reducing its association with β-catenin and consequently inhibiting β-catenin phosphorylation (Kim et al., 2013; Willert, Shibamoto, & Nusse, 1999), which leads to β-catenin stabilization and nuclear translocation. Axin levels are also regulated by SUMOylations at the C-terminus (Kim, Chia, & Costantini, 2008), which prevent its ubiquitin-mediated proteasomal degradation. On the other hand, this ubiquitination is balanced by the ubiquitin specific protease 34 (Lui et al., 2011). Additionally, Lrp5/6 regulates Axin levels: Lrp6 loss leads to an accumulation of Axin, suggesting that Lrp6 induces Axin degradation (Kofron et al., 2007), which is mediated, at least in part, by the E3 ubiquitin ligase Smurf (Smad ubiquitin regulatory factor; Fei et al., 2014; Kim & Jho, 2010). Finally, the poly-ADP ribosylating (PARsylating) enzyme Tankyrase promotes the degradation of Axin (Huang et al., 2009), through the E3 ubiquitin ligase RNF146 (Callow et al., 2011; Zhang et al., 2011). More recently, it has been demonstrated that there are likely two pools of Axin, with some residing near the membrane and being targeted for Tankyrase-dependent degradation, while another pool associates cytoplasmically with the β-catenin destruction complex; the localization seems to depend on the levels of APC, adding a further level of control to steady-state Axin levels (Wang, Tacchelly-Benites, Yang, & Ahmed, 2016). Altogether, these studies indicate that the tight control of Axin is a critical step in regulating the destruction complex and in controlling the Wnt signal output.

APC was first identified as the gene causative for familial adenomatous polyposis, a disease characterized by the formation of colorectal tumors (Kinzler et al., 1991; Nishisho et al., 1991). Screens for APC interactors identified β-catenin, which was thought at the time to be associated with a role for this pair in cell adhesion and migration (Rubinfeld et al., 1993; Su, Vogelstein, & Kinzler, 1993). It was not until later that mutational analysis of APC revealed that its loss leads to an accumulation of nuclear β-catenin, a driving mutation of colorectal tumorigenesis (Korinek et al., 1997; Morin et al., 1997; Rubinfeld, Albert, Porfiri, Munemitsu, & Polakis, 1997). APC is thought to operate as a scaffolding protein in the destruction complex and like Axin, APC is found in two distinct pools in the cell, either at the microtubules at the distal end of cell extensions (Nathke, Adams, Polakis, Sellin, & Nelson, 1996), or in distinct locations in the destruction complex (Penman, Leung, & Nathke, 2005). Axin recruits APC to the destruction complex in response to Wnt, and the

Axin-binding domain is required for this redistribution of APC (Faux et al., 2008). Axin also plays a role in regulating APC levels in response to Wnt (Choi, Park, Costantini, Jho, & Joo, 2004). Although APC is traditionally thought to have a negative effect on the Wnt signal, it has also been shown to be a positive regulator in certain contexts (Brauburger et al., 2014; Takacs et al., 2008). Interestingly, some truncations in Axin and APC can be overcome, at least in vitro, and complemented by each other (Pronobis, Deuitch, Posham, Mimori-Kiyosue, & Peifer, 2017; Xu, Liu, Xu, Zhu, & Gao, 2017). Nevertheless, truncating mutations in APC that are common in cancer lead to an increase in nuclear β-catenin, leading to an increase in Wnt signal output, suggesting that these truncations are difficult to overcome in vivo.

β-catenin is targeted for degradation first by phosphorylation events, initially by the priming kinase CK1 at Serine 45, followed by GSK3 at Serines 33 and 37, and Threonine 41 (Amit et al., 2002; Peifer, Pai, & Casey, 1994; Yost et al., 1996); kinases are therefore important components of the destruction complex. There are at least two kinases, GSK3 and CKIα that are associated with the destruction complex. As discussed above, *GSK3* (*zw3*) was first identified as a component of the *wg* signaling cascade in Drosophila (Siegfried et al., 1992). Further studies indicated that *zw3* and *wg* have opposite effects on the distribution of the β-catenin homolog armadillo (Peifer, Sweeton, Casey, & Wieschaus, 1994), and that *zw3* is required for armadillo phosphorylation (Peifer, Pai, & Casey, 1994), the first suggestions that destruction complex-mediated β-catenin phosphorylation leads to a decreased Wnt signal. There are two mammalian GSK3 homologs (α and β); these are functionally redundant, as shown by systematic allelic complementation in embryonic stem cells, where only double mutants affected kinase or Wnt activity (Doble, Patel, Wood, Kockeritz, & Woodgett, 2007). However, a recent study in mouse embryonic stem cells (mESCs) provided evidence for distinct roles for the two GSK3 homologs, with GSK3β maintaining mESC self-renewal and GSK3α promoting neural differentiation (Chen et al., 2017), indicating a greater complexity than previously appreciated.

APC, Axin, and LRP6 are also phosphorylated by GSK3, leading to a variety of cellular responses (Rubinfeld et al., 1996; Willert et al., 1999; Zeng et al., 2005). For example, GSK3-mediated phosphorylation of APC leads to enhanced binding to β-catenin (Rubinfeld et al., 1996), which is reversed by the phosphatase PP2A (Seeling et al., 1999). Axin phosphorylation by GSK3 increases its affinity for β-catenin (Willert et al., 1999). These phosphorylations can also be influenced by other members of the Wnt cascade; for example, LRP6 can directly inhibit GSK3 phosphorylation of β-catenin (Cselenyi et al., 2008). Inhibitors of GSK3 kinase activity, such as lithium, BIO (6-bromoindirubin-3′-oxime), CHIR98014, and CHIR99021, have proven to be potent activators of Wnt/β-catenin signaling. In fact, the mode of action of lithium in bipolar disorder may in part be mediated through its inhibition of GSK3 (Klein & Melton, 1996; Sato, Meijer, Skaltsounis, Greengard, & Brivanlou, 2004).

Another class of serine/threonine kinases is the casein kinase 1 (CK1) family, which encompasses several isoforms (alpha, beta, gamma 1–3 delta, epsilon), that play a myriad of roles in many cell types and have been reviewed elsewhere (Jiang, 2017). Of these, CK1 alpha, gamma, and delta have been implicated in Wnt signaling. Dvl, LRP5, TCF/LEF,

Axin, β-catenin, and APC have all been shown to be phosphorylated by CK1, though the outcomes on the Wnt signal are varied (Cong, Schweizer, & Varmus, 2004; Gao, Seeling, Hill, Yochum, & Virshup, 2002; Kishida et al., 2001; Lee, Salic, & Kirschner, 2001; Liu et al., 2002; Peters, McKay, McKay, & Graff, 1999; Rubinfeld, Tice, & Polakis, 2001; Yanagawa et al., 1995; Zeng et al., 2005; Zhang et al., 2006). For example, overexpression of a dominant-negative CK1 results in a phenotype similar to loss of Wnt (Peters et al., 1999), indicating that CK1 is a positive regulator of the Wnt signal. CK1 associates with Dvl, and coexpression of these is sufficient to drive nuclear β-catenin accumulation (Kishida et al., 2001; Peters et al., 1999). CK1 expression leads to a decrease in β-catenin degradation, suggesting that it functions to destabilize the destruction complex, possibly through recruitment of PP2A (Gao et al., 2002). This is consistent with the finding that XWnt-8 or Wnt-3a induce CK1 activity (Swiatek et al., 2004). CK1 has been shown to be the “priming” kinase for GSK3 on β-catenin, leading to it being targeted for degradation (Liu et al., 2002). CK1 has also been implicated in negatively regulating Wnt signaling, by phosphorylating LEF-1, and inhibiting its interaction with β-catenin (Hammerlein, Weiske, & Huber, 2005). Altogether, these data indicate the importance of phosphorylation events in regulation of the destruction complex, and the Wnt signal.

In the absence of a Wnt ligand, β-catenin is targeted for degradation by the destruction complex (described below) in concert with a member of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, SCF-β-TRCP (Winston et al., 1999). This occurs in a series of events. First, β-catenin binds to Axin, which positions it for serial phosphorylations by CK1 and GSK3 (Amit et al., 2002; Liu et al., 2002). Following this, SCF-β-TRCP mediates the poly-ubiquitination of β-catenin, leading eventually to its degradation in the proteasome (Kitagawa et al., 1999; Lagna, Carnevali, Marchioni, & Hemmati-Brivanlou, 1999; Liu et al., 1999; Marikawa & Elinson, 1998). It is thought that these interactions between β-catenin and the destruction complex allow for carefully tuned control of Wnt-mediated transcription.

Since the destruction complex plays such an important role in regulating the Wnt signal, it is an attractive therapeutic target to treat diseases that have upregulated Wnt signaling levels, such as colorectal cancers. For instance, Tankyrase inhibitors such as IWR-1, JW74, and XAV939 stabilize the level of the rate-limiting member Axin, and reduce the Wnt signal (Chen et al., 2009; Huang et al., 2009; Stratford et al., 2014). CK1α has also been shown to be activated by pyrvinium, leading to an increase in β-catenin degradation, and a resultant loss of the Wnt signal (Thorne et al., 2010). A recent small molecule screen has identified a novel mechanism of Wnt regulation: Peptidylarginine deiminase 2 (PAD2) deaminates arginines to citrullines on β-catenin, which leads to its degradation. PAD2 activity can be increased by the small molecule nitazoxanide; Wnt signaling can therefore be blocked independent of APC (Qu et al., 2017). This line of targeting is downstream of the frequently mutated APC and β-catenin, and is therefore an attractive chemotherapeutic target.

## 5 | SIGNAL SIGNALING IN THE NUCLEUS

### 5.1 | β-catenin shuttling

In the presence of a Wnt ligand, the destruction complex is thought to be dissociated, allowing for the cytoplasmic buildup of β-catenin, followed by its import into the nucleus;

how this import happens is poorly understood, since β-catenin does not have a nuclear import or export signal sequence. Assays conducted in vitro indicate that β-catenin docks directly to the nuclear pore machinery to facilitate its entry to the nucleus, independently of energy requirements (Fagotto, Gluck, & Gumbiner, 1998; Yokoya, Imamoto, Tachibana, & Yoneda, 1999). It is likely that β-catenin is sequestered in the cytoplasm, and allowed to move into the nucleus upon dissociation of the destruction complex, since loss of the structural components important for nuclear pore binding results in constitutive nuclear localization, and cadherins and Axin have both been shown to sequester β-catenin away from the nucleus (Fagotto, Funayama, Gluck, & Gumbiner, 1996; Gottardi et al., 2001; Heasman et al., 1994; Orsulic & Peifer, 1996; Sadot, Simcha, Shtutman, Ben-Ze'ev, & Geiger, 1998; Sanson et al., 1996; Shtutman et al., 1999; Tolwinski & Wieschaus, 2001). Upon entry into the nucleus, β-catenin can act as a transcriptional activator.

### 5.2 | LEF/TCF (lymphoid-enhancing factor/T-cell factor) transcription

The LEF/TCF family of transcription factors is encoded by four genes in mammals, *LEF1*, *TCF7*, *TCF7L1*, and *TCF7L2* (formerly *TCF1*, *TCF3*, and *TCF4*, respectively; Korinek et al., 1997; Molenaar et al., 1996; Radler-Pohl, Pfeuffer, Karin, & Serfling, 1990; Travis, Amsterdam, Belanger, & Grosschedl, 1991). These bind to a plethora of degenerate Wnt responsive elements (WREs) on the DNA, through their HMG box DNA-binding domains (Badis et al., 2009). In the absence of a Wnt signal, LEF/TCFs are associated with transcriptional repressors, such as Groucho and transducing-like enhancer (Cavallo et al., 1998; Roose et al., 1998). In addition to these, LEF/TCFs are associated with chromatin modifiers that repress transcription, such as C-terminal-binding protein (CtBP1) and the Polycomb (PcG) complex (reviewed in (Chinnadurai, 2002). With the exception of TCF7L1, which seems to operate solely as a repressor (Dorsky, Itoh, Moon, & Chitnis, 2003; Kim et al., 2000; Liu, van den Broek, Destree, & Hoppler, 2005), upon Wnt activation, β-catenin interacts with these directly to transduce the Wnt signal through target gene activation (Behrens et al., 1996; Brunner, Peter, Schweizer, & Basler, 1997; Huber et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). This interaction can be disrupted by the inhibitor of β-catenin and Tcf-4 (ICAT), a 9-kDa protein that sterically inhibits this interaction and antagonizes the Wnt signal (Tago et al., 2000). Target gene specificity among LEF/TCFs seems to be at least partially reliant on a domain adjacent to the HMG box, called the C-clamp, which has been reviewed elsewhere (Ramakrishnan & Cadigan, 2017).

Like other members of the Wnt cascade, LEF/TCFs are subject to post-translational modifications regulating their function. For example, the Nemo-like kinase (NLK) has been shown to phosphorylate TCF, leading to a decrease in the affinity of the TCF/β-catenin complex for DNA (Ishitani et al., 1999; Smit et al., 2004), a finding that has been also observed in multiple species, and with multiple kinases (Hammerlein et al., 2005; Hikasa et al., 2010; Hikasa & Sokol, 2011; Lee et al., 2001; Lo, Gay, Odom, Shi, & Lin, 2004). These observations highlight the complex nature of Wnt target gene regulation.

There are other nuclear factors required for the Wnt signal. For example, pygopus (pygo) and legless (lgs; BCL9 in vertebrates) were first identified in a *Drosophila* genetic screen for segment polarity genes (Kramps et al., 2002; Parker, Jemison, & Cadigan, 2002). These are

required for transmission of the Wg signal throughout development; lgs recruits pygo to the TCF/β-catenin transcriptional complex, where it functions as a transcriptional coactivator (Kramps et al., 2002). In vertebrates, these are similarly required for β-catenin-mediated transcription (Belenkaya et al., 2002; Hoffmans & Basler, 2007; Thompson, Townsley, Rosin-Arbesfeld, Musisi, & Bienz, 2002), though some of these effects are context specific (Song et al., 2007; Sustmann, Flach, Ebert, Eastman, & Grosschedl, 2008). Lgs and Pygo are also implicated in the nuclear localization of β-catenin (Brembeck et al., 2004; Townsley, Cliffe, & Bienz, 2004). In addition to these, components of chromatin modifying complexes, such as polymerase-associated factor 1 (PAF1), are required for the Wnt transcriptional machinery (Mosimann, Hausmann, & Basler, 2006). TCF/β-catenin has also been shown to bind to the DNA helicase TIP49a/Pontin52, and members of the chromatin remodeling complex Brg-1, among others (Barker et al., 2001; Bauer et al., 2000). Further understanding of these interactions will allow us to target Wnt transcription. For example, Indocyanine Green (ICG)-001 is a small molecule inhibitor of the TCF/β-catenin complex, which is able to treat a variety of Wnt-mediated pathologies in vivo in mice, such as pulmonary fibrosis, sepsis following myocardial injury, and smooth muscle remodeling in asthma (Eguchi, Nguyen, Lee, & Kahn, 2005; Henderson Jr et al., 2010; Yousif, Hadi, & Hassan, 2017; Koopmans et al., 2016).

### 5.3 | Molecular targets of the Wnt signal

A variety Wnt target genes have been reported in a diverse set of biological processes, including stem cells self-renewal, maintenance, and proliferation. Many Wnt target genes are induced in a tissue and context-specific manner, although like other signaling cascades, Wnt signaling induces expression of negative regulators, such as *SP5, Nkd1, and Axin2* (Huggins et al., 2017; Jho et al., 2002; Larraguibel et al., 2015; Yan et al., 2001). Wnt stimulation of cell lines, followed by mRNA analyses have uncovered several transcripts induced by Wnt (Gorrepati et al., 2015; Huggins et al., 2017; Jackson, Abete-Luzi, Krause, & Eisenmann, 2014; Maubant et al., 2015; Willert, Epping, Pollack, Brown, & Nusse, 2002). How these are regulated, however, remains elusive; a recent study found that only a subset of β-catenin-bound genomic loci are transcriptionally regulated by Wnt signaling, implying a further requirement for inputs to activate transcription (Nakamura, de Paiva Alves, Veenstra, & Hoppler, 2016).

Over the past decade, one of the best characterized context-specific target genes has been *Leucine-rich containing G-protein coupled receptor 5* (*Lgr5*), originally identified in a screen for genes upregulated in the intestinal crypt in response to Wnt activation in APC-mutant mice (Barker et al., 2007). This GPCR has since been shown to be a marker of a variety of Wnt-responsive epithelial stem cell pools, including the small and large intestine, the stomach, the hair follicle and the mammary gland, among others (Barker et al., 2007, 2008, 2010; Barker & Clevers, 2010; de Visser et al., 2012; Jaks et al., 2008; Sato et al., 2009). In addition to the normal stem cell niche, *Lgr5* is also a marker of cancer stem cells (Barker et al., 2009; Hirsch et al., 2014; Kemper et al., 2012; Merlos-Suarez et al., 2011; Schepers et al., 2012). These studies indicate the importance of the Wnt signal in stem cells and cancer stem cells, which has also been shown in other contexts. For example, Wnt signaling through the FZD7 receptor is required to maintain human embryonic stem

cell pluripotency (Fernandez et al., 2014), and also for generating induced pluripotent stem cells (Ross et al., 2014). This is by no means an exhaustive list, as there are many examples of how Wnt regulates these process, reviewed elsewhere (Clevers & Nusse, 2012). A more comprehensive and continuously updated list of Wnt target genes can be found on the Wnt homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/). Improving our understanding of which targets Wnt regulates will improve our ability to target and treat Wnt-mediated diseases such as cancers.

## 6 | CONCLUSION

The intense research on the mechanisms of Wnt signal initiation and transduction, as summarized in this overview, has opened many new avenues of research and provided many opportunities for therapeutic intervention in currently incurable diseases, including neurodegeneration and cancer. Novel drugs that target specific points of Wnt signaling will likely be of significant clinical value: inhibition of Wnt signaling shows promise in the treatment of cancers, whereas activation of Wnt signaling will be useful in tissue engineering and regenerative medicine. However, as highlighted in this review, many key questions remain unanswered and await further probing. For example, with the large number of Wnt ligands, Fzd receptors and additional Wnt binding proteins, it remains unclear how signaling specificity is established. Recent studies hint at a much greater complexity in how specific Wnt signals are received and interpreted than previously appreciated. Critical to advancing, our understanding of this complex signaling module will be the development of new tools and assays, such as strategies to activate specific arms of the pathway and trigger desired biological outcomes.

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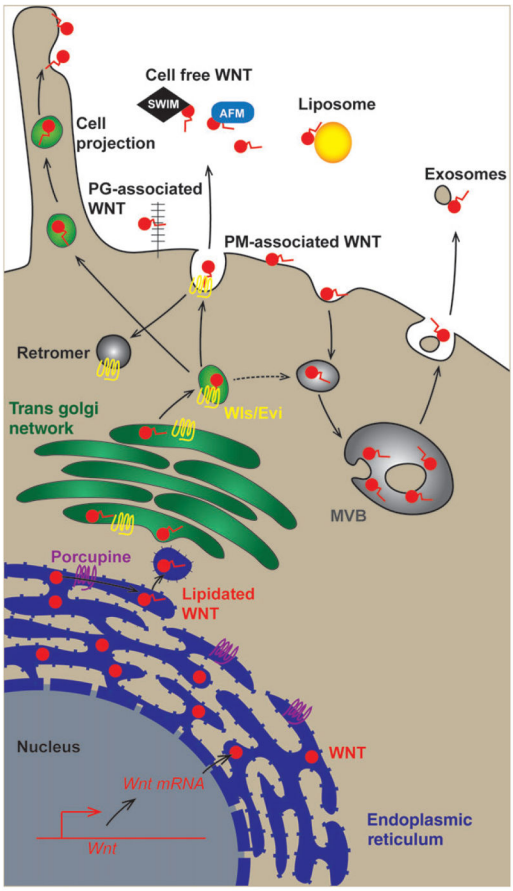


**FIGURE 1.**

Wnt secretion. Wnt proteins are translated into the endoplasmic reticulum where they are acylated by Porcupine (PORCN). Wntless (Wls/Evi) escorts acylated Wnt to the cell surface where it may associate with the plasma membrane (PM-associated Wnt) or with proteoglycans (PG-associated Wnt). Carrier proteins, such as SWIM and AFM, facilitate solubility of cell free Wnt proteins. Wnt proteins can signal at greater distances via cell projections (also known as filopodia, cytonemes, and nanotubes) or by associating with exosomes or liposomes

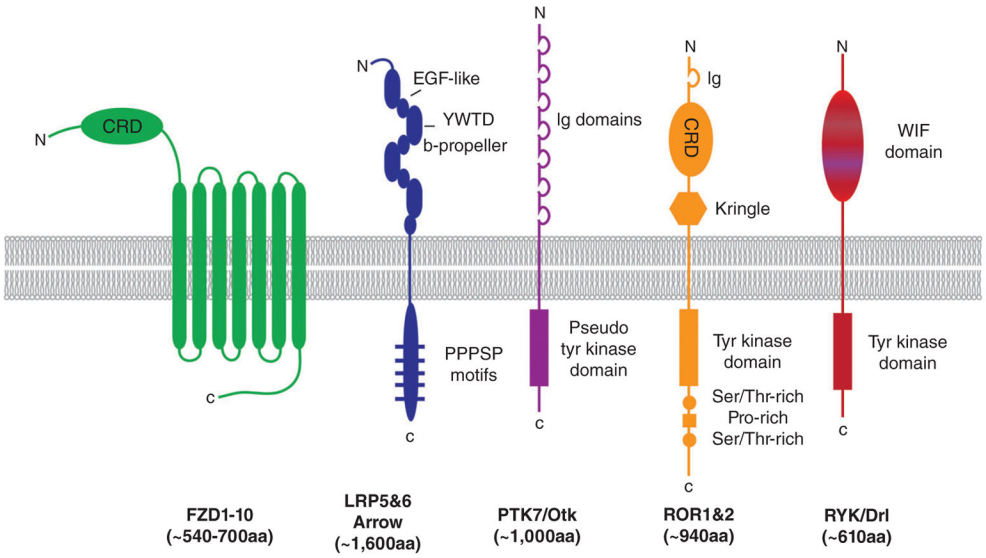


**FIGURE 2.**

Wnt receptors. Several cell surface proteins directly bind Wnt proteins, including Frizzled (FZD), LRP5 & 6 (fly homolog is Arrow), PTK7 (fly homolog is Otk), ROR1 & 2, and RYK (fly homolog is Drl). Abbreviations: aa = amino acids, CRD = cysteine-rich domain, C = carboxy terminus, Ig = immunoglobulin domain, N = amino terminus, WIF = Wnt inhibitory factor

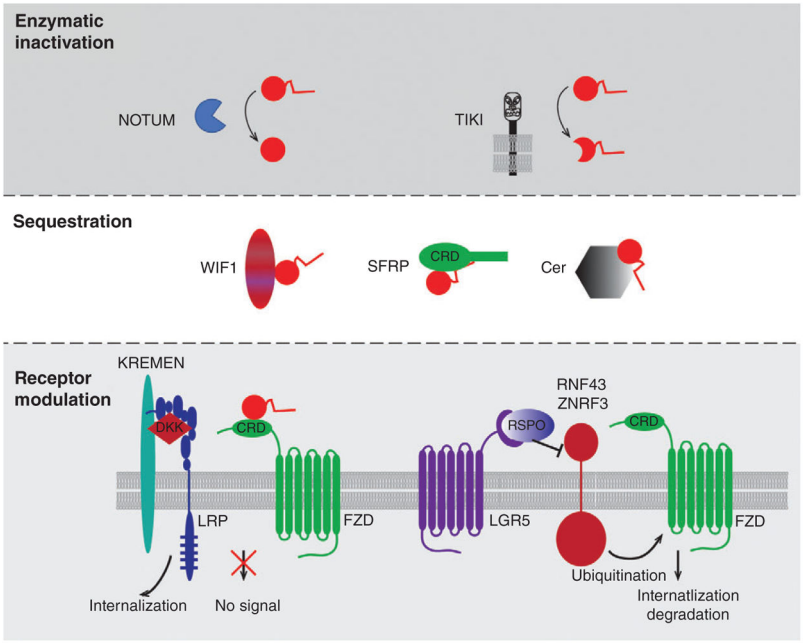


**FIGURE 3.**

Regulating extracellular Wnt activity. Wnt signaling activity is modulated by three main mechanisms: (a) enzymatic inactivation by NOTUM, which cleaves off the essential lipid moiety, or by TIKI, which removes a portion of the amino terminus, (b) sequestration of Wnt by proteins such as WIF, SFRP, and Cer, and (C) receptor modulation by DKK, which binds LRP and Kremen to prevent the formation of a Wnt-FZD-LRP5/6 receptor complex, and by the ubiquitin ligases ZNRF3 and RNF43, which act to down regulate cell surface expression of FZD. Binding of the secreted protein RSPO to LGR5 interferes with the ability of these ubiquitin ligases to target FZD protein for degradation, thereby increasing Wnt receptor availability on the cell surface

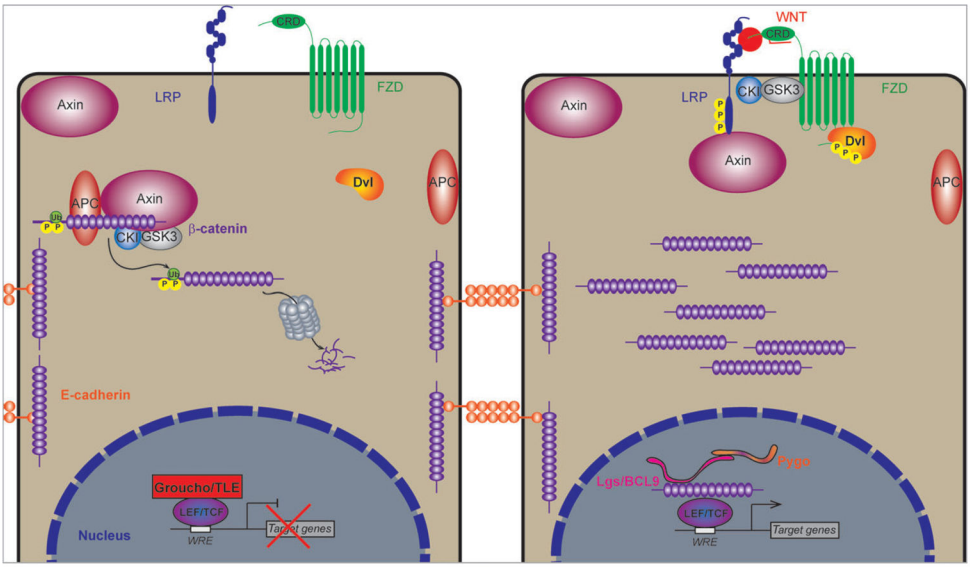


**FIGURE 4.**

β-catenin mediated Wnt signaling. In the absence of a Wnt ligand, β-catenin is sequestered by the multiprotein "destruction complex," where it is phosphorylated and ubiquitinated, and targeted for proteasomal degradation. In the nucleus, LEF/TCF (lymphoid-enhancing factor/T-cell factor) transcription factors are resident on Wnt responsive elements (WREs), and recruit corepressors, such as Groucho/TLE. Upon Wnt-Fzd interaction, LRP oligomerizes with the receptor-ligand complex, and the destruction complex is dissociated. This leads to an accumulation of β-catenin in the cytosol, and eventual translocation to the nucleus, where it interacts directly with LEF/TCF transcription factors, and other transcriptional coactivators to initiate target gene expression