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The Wnt Pathway: From Signaling Mechanisms to Synthetic Modulators

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Abstract

The Wnt pathway is central to a host of developmental and disease-related processes. The remarkable conservation of this intercellular signaling cascade throughout metazoan lineages indicates that it coevolved with multicellularity to regulate the generation and spatial arrangement of distinct cell types. By regulating cell fate specification, mitotic activity, and cell polarity, Wnt signaling orchestrates development and tissue homeostasis, and its dysregulation is implicated in developmental defects, cancer, and degenerative disorders. We review advances in our understanding of this key pathway, from Wnt protein production and secretion to relay of the signal in the cytoplasm of the receiving cell. We discuss the evolutionary history of this pathway as well as endogenous and synthetic modulators of its activity. Finally, we highlight remaining gaps in our knowledge of Wnt signal transduction and avenues for future research.

Contents

INTRODUCTION

The transition from single-celled to multicellular organisms marked the emergence of complex life forms. With multicellularity arose the need to generate and position different cell types in development and to maintain these throughout the rest of the organism's life. The Wnt pathway is among the most ancient of the signaling pathways that regulate these physiological processes (1, 2). Conserved in all metazoans, it evolved and diversified over hundreds of millions of years as metazoans themselves did so.

The Wnt signaling pathway is an intercellular signaling cascade activated by secreted lipid-modified proteins of the Wnt family. The most fundamental form of the pathway consists of a Wnt ligand from a secreting cell, its cognate receptors on the surface of a receiving cell, and signal transducers within the receiving cell. Upon recognition of the ligand and intracellular relay of the signal, pathway activation leads to cellular responses such as mitotic activity, cell type specification, or establishment of polarity. These cellular responses, in turn, orchestrate key events in the developing organism. Wnt signaling continues to play a critical role in the adult organism as a regulator of tissue homeostasis and regeneration.

Four decades of research since the discovery of the first Wnt gene has identified components of the Wnt signaling pathway as well as their roles in numerous physiological contexts across the animal kingdom (3). Yet our picture of how molecular components of the Wnt pathway work together to transduce the signal, from ligand recognition on the cell surface to transcription of target genes in the nucleus, remains incomplete. Application of new tools in areas such as genome editing, biochemistry, and imaging has led to significant advances in our understanding of Wnt signal transduction at the molecular level. We discuss the current state of research on the mechanism and physiological function of Wnt signaling, focusing on the β-catenin-dependent, or canonical, branch. This branch of Wnt signaling depends on the signal transducer β-catenin for transcriptional activation of genes that regulate cell fate specification or cell proliferation and plays an essential role in development and tissue maintenance and repair. We highlight recent breakthroughs as well as remaining gaps in our knowledge of Wnt signal transduction and discuss avenues for further research.

A BROAD OVERVIEW OF THE WNT SIGNALING PATHWAY

In the absence of a ligand, Wnt signaling is kept off to prevent aberrant cellular response (**Figure 1*a***). This state is achieved through constant phosphorylation and degradation of the

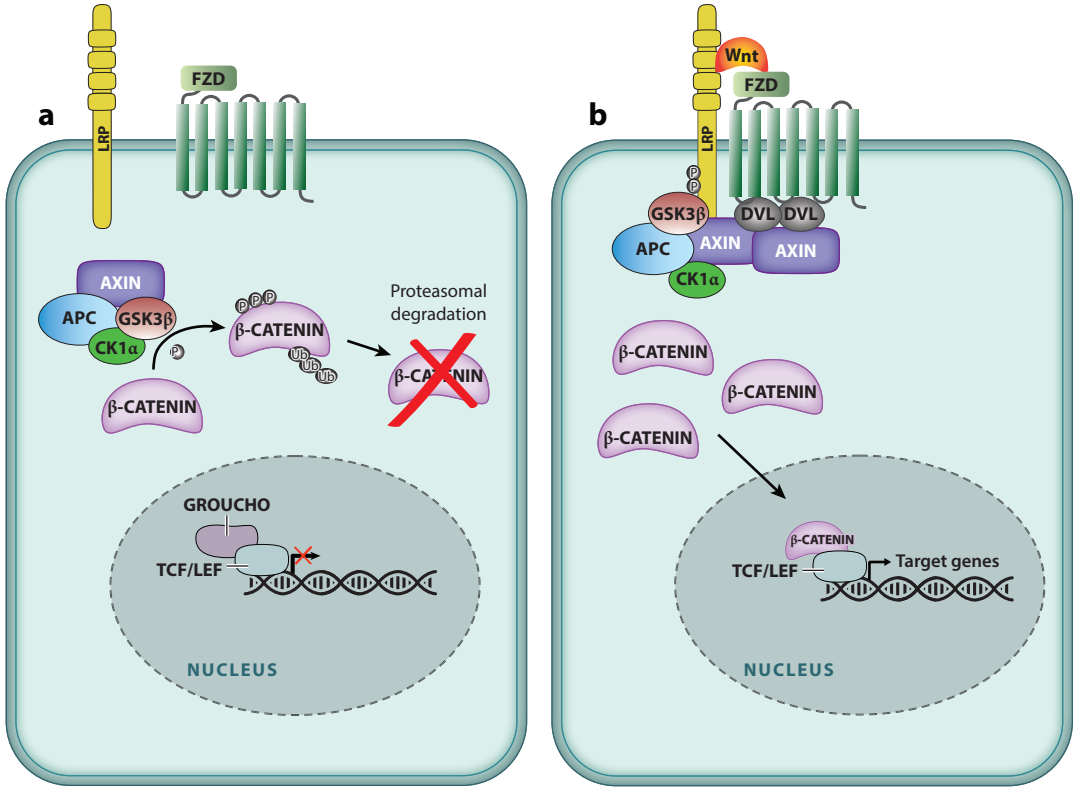

Figure 1

An overview of Wnt signal transduction. (*a*) In the absence of the Wnt signal, cytosolic β-catenin is phosphorylated by kinases CK1α and GSK3β with the help of scaffolding proteins AXIN and APC. Phosphorylation of β-catenin leads to its ubiquitylation and subsequent proteasomal degradation. With low β-catenin levels in the nucleus, transcriptional repressors prevent activation of Wnt target genes. (*b*) Extracellular Wnt signal binds coreceptors FZD and LRP5/6 on the cell surface. Subsequent phosphorylation of LRP5/6 and recruitment of signal transducers DVL and AXIN to the Wnt-bound receptors facilitate inhibition of GSK3β activity. This inhibition blocks phosphorylation and degradation of β-catenin, leading to β-catenin accumulation in the cytoplasm and translocation into the nucleus. In the nucleus, β-catenin interacts with TCF/LEF transcription factors to activate Wnt target genes. Abbreviations: APC, adenomatous polyposis coli; AXIN, axis inhibition protein; CK1α, casein kinase 1 alpha; DVL, Dishevelled; FZD, Frizzled; GSK3β, glycogen synthase kinase 3 beta; LEF, lymphoid enhancer-binding factor; LRP, low-density lipoprotein receptor–related protein; TCF, T cell factor.

central effector β-catenin. β-Catenin is phosphorylated by the serine/threonine kinase glycogen synthase kinase 3 beta (GSK3β, EC 2.7.11.26) with the help of casein kinase 1 alpha (CK1α, EC 2.7.11.1), axis inhibition protein (AXIN), and adenomatous polyposis coli (APC), which together form the so-called destruction complex. Phosphorylation of β-catenin at N-terminal serine and threonine residues leads to its ubiquitylation and subsequent proteasomal degradation (4, 5).

Wnt ligand from a secreting cell engages two distinct receptors on the receiving cell: a Frizzled (FZD) family receptor and a member of the low-density lipoprotein receptor–related protein family, LRP5 or LRP6 (**Figure 1*b***) (6–9). Wnt ligand binding to coreceptors FZD and LRP5/6 leads to several molecular changes such as phosphorylation of LRP5/6, recruitment of the signal transducer Dishevelled (DVL) to the membrane, and subsequent oligomerization of DVL. With these changes, the destruction complex associates with the receptors and DVL to form the Wnt signalosome. As we will discuss more, although the exact sequence and dynamics of the events leading

to signalosome formation remain to be further elucidated, the key outcome of the intracellular activity is inhibition of β-catenin phosphorylation by GSK3β. This inhibition halts degradation of β-catenin and leads to its accumulation in the cytoplasm and concomitant translocation into the nucleus. In the nucleus, β-catenin interacts with the T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors to activate Wnt target genes that regulate mitotic progression and cell differentiation. Nuclear accumulation and transcriptional activity of β-catenin, therefore, are the critical functional outcomes of Wnt signaling (5, 10).

AN ANCIENT MECHANISM TO REGULATE CELL BEHAVIOR THROUGHOUT AN ORGANISM'S LIFE

Components of the Wnt pathway are conserved from the most primitive metazoan phyla to mammals, underscoring the deep history of Wnt signaling throughout animal lineages (11–14). Genomes of animals as simple as placozoans, sponges, and cnidarians encode multiple Wnts as well as main elements of the pathway such as GSK3 and β-catenin, with the mammalian genome encoding 19 members of the Wnt family defined by sequence similarity (11, 12, 14, 15).

Intriguingly, genomic analyses suggest that the Wnt pathway arose through cooption of existing signaling components by Wnt ligands. This notion is supported by the presence of homologs of the Wnt receptor FZD, GSK3, and β-catenin in the soil amoeba *Dictyostelium* in which Wnts themselves are missing (16–19). Similarly, a genomic study identified 39 animal-specific gene families by comparing the genomes of animals from sponges to mice with those of choanoflagellates, unicellular organisms considered the closest living relatives of animals. Of these, 7 gene families represented components of the Wnt pathway (including FZD, LRP, DVL, β-catenin, and TCF), whereas Wnts themselves were not identified as a gene family present in all animals analyzed (20).

The remarkable conservation of the Wnt signaling pathway in multicellular life forms reflects its indispensable role in development. Wnt signaling regulates developmental events such as establishment of the primary body axis and generation and movement of diverse cell types, and its loss leads to early lethality (21–24). For instance, restricted expression of *Wnt3* at the tip of the head establishes the oral-aboral body axis in the cnidarian hydra (12). Similarly, posterior expression of *Wnt3* and subsequent signaling through β-catenin define the anteroposterior axis in the early mouse embryo (25–27). Therefore, asymmetric activation of Wnt signaling sets up the polarity of the body axis, the first step in generating a patterned body plan in all multicellular animals. Wnt signaling continues to play a crucial role in generation and spatial arrangement of various cell types as development progresses. The organs and tissues that rely on Wnt pathway activation in development range from limb buds and internal organs such as the lungs and kidneys to brain structures and hematopoietic lineages (28–33).

In the adult organism, Wnt activity is important for maintenance of adult stem cells in various tissues. With the ability to self-renew as well as generate differentiated progeny, stem cells maintain the tissue throughout normal homeostatic turnover and repair the tissue following injury. Wnt function regulates survival and propagation of stem cells in a variety of tissues, including the intestine, stomach, skin, and mammary gland (34–38). The intestine in particular presents a paradigm of Wnt-mediated regulation of tissue stem cells, and disruption of Wnt signaling leads to loss of cycling stem cells in the mouse intestine and to death shortly after birth (35). These tissue-specific stem cells normally reside in a structured niche that provides the Wnt signal necessary for their maintenance. In the context of regeneration, apoptotic cells often serve as a source of Wnts to induce compensatory proliferation in neighboring stem cells (39–42).

Aberrant activation of the Wnt pathway, in contrast, has been implicated in diseases such as multiple types of cancer. The first Wnt gene to be cloned, *Int1/Wnt1*, was identified in a murine

mammary tumor virus integration screen for oncogenes in murine breast cancer (3). Many other Wnt pathway genes have since emerged in the human cancer context, often through mutations in negative regulators of the pathway (43–45). For example, *APC* was uncovered in families with the adenomatous polyposis syndrome and was subsequently found to be mutated in sporadic colon cancers (46, 47). Similarly, hereditary *AXIN2* mutations confer predisposition to colon cancer, while sporadic *AXIN2* mutations are occasionally seen in colon cancer (48, 49). Inactivating mutations in *AXIN1* occur in liver cancers (50). In a variety of sporadic cancers, regulatory phosphorylation sites in β-catenin's N terminus are mutated, with particularly high incidences in epithelial cancers (51–53). Pharmacological correction of aberrant Wnt pathway activation by these types of mutations has proven to be challenging, given that no obvious enzymes have emerged as drug targets at the level of β-catenin.

How Wnt signaling regulates tissue stem cells and how its dysregulation leads to various diseases have been the subjects of many reviews (28, 54–62). Altogether, these examples demonstrate the importance of the right level of Wnt activity in development and maintenance of adult tissues, as well as the need for a mechanistic understanding of Wnt signal transduction.

WNT SIGNAL PRODUCTION AND SECRETION

The Wnt signaling cascade begins with synthesis of the Wnt ligand in the secreting cell (**Figure 2**). Production and presentation of the Wnt ligand require two multipass transmembrane proteins, Porcupine (PORCN, EC 2.3.1.250) and Wntless (WLS). PORCN is a highly conserved acyltransferase that modifies Wnt with a palmitoleic acid moiety in the endoplasmic reticulum (ER) (63–68). WLS—also known as GPR177 and, in *Drosophila*, evenness interrupted (Evi)—is responsible for intracellular trafficking of Wnt from the ER through the Golgi network to the cell surface (69, 70). WLS, after delivering its Wnt cargo, is recycled through endocytosis and returns to the Golgi complex via the retromer complex (71). Consistent with the role of WLS in the secretion of all Wnt proteins, mutations that impair WLS function lead to Zaki syndrome, which involves structural birth defects affecting the brain, eyes, limbs, kidneys, and heart. These developmental defects were partially reversed by the Wnt agonist CHIR99021 in a mouse model (72).

PORCN adds a palmitoleic acid moiety on the conserved S209 of Wnt, a necessary step for Wnt recognition by the receptor FZD (65, 67, 73). Indeed, crystal structure analysis of the cysteine-rich domain (CRD) of FZD bound to *Xenopus* Wnt8 revealed the lipid moiety on Wnt fitting into a conserved hydrophobic groove in the FZD CRD (74). A hydrophobic cavity that accommodates the essential lipid moiety of Wnt was identified in the intracellular transporter WLS as well (75).

Interestingly, the N-terminal domain of Wnt that carries the palmitoleic acid modification resembles Saposin-like proteins, with an ancient lipid-binding function (76). It is therefore possible that Wnts arose from covalent fatty acid modification of an ancestral lipid-interacting domain. These new ligands could then have appropriated existing membrane proteins involved in sensing other molecules. Indeed, the FZD family of receptors traces back to nonmetazoan receptors that detect molecules such as cAMP, and the Wnt-interacting luminal domain of WLS resembles Seipin, a highly conserved ER membrane protein that binds phospholipids and regulates lipid droplet formation (75, 77–79). Along with these membrane proteins, existing cytoplasmic signal transducers were incorporated to constitute the Wnt pathway as we know it. This model is consistent with the presence of FZD, β-catenin, and GSK3 homologs that predate Wnts in nonmetazoan lineages (17).

Another illustration of the importance of the palmitoleic acid moiety comes from a distant corner of the prokaryotic world. *Clostridium difficile* toxin B (TcdB) can disrupt the epithelial barrier in the human colon and cause diarrhea and inflammation. Surprisingly, TcdB interacts

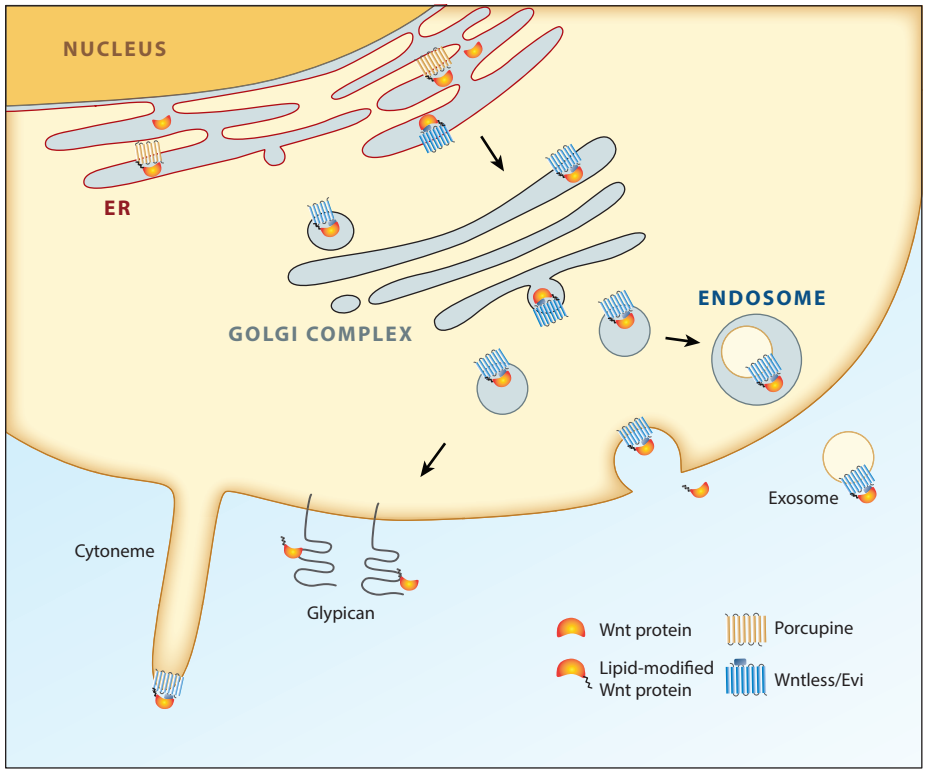

Figure 2

Wnt secretion. Newly synthesized Wnt proteins are modified in the ER by Porcupine, a transmembrane acyltransferase. Porcupine-mediated addition of a lipid moiety is essential for Wnt activity. The carrier protein Wntless, also known as Evi, is responsible for intracellular trafficking of lipid-modified Wnt proteins from the ER through the Golgi network to the cell surface. To reach neighboring cells, Wnts may rely on cytoplasmic extensions, membrane glypicans, or secreted exosomes. Abbreviations: ER, endoplasmic reticulum; Evi, evenness interrupted.

with FZD1/2/7 to mediate bacterial entry into the colon epithelium and to inhibit Wnt signaling (80). While TcdB itself is not lipid modified, a free fatty acid facilitates binding of TcdB to the CRD of FZD in an arrangement that resembles the Wnt-FZD interface (81). Consistent with the indispensable role of the palmitoleic moiety, small molecule inhibitors of PORCN potently block Wnt function, with four such molecules in clinical trials for treating cancers with dysregulated Wnt signaling (82–87). Given the well-established role of Wnts in intestine, skin, and bone homeostasis, however, it is critical to assess the effects of PORCN inhibition on these tissues prior to therapeutic application. In addition to presenting a potential treatment for Wnt-dependent cancers, PORCN inhibitors can expand our understanding of the Wnt-regulated transcriptome through assessment of gene expression changes upon global Wnt inhibition (88, 89).

While essential for signaling, the palmitoleic acid moiety renders the Wnt ligand hydrophobic. The ability of Wnts to travel multiple cell diameters away from the source, therefore, poses a conundrum of how a hydrophobic signal can spread in an aqueous environment. A number of studies on the extracellular spread of Wnt were performed in the wing imaginal disc of *Drosophila* larvae, in which the *Drosophila* Wnt, Wingless (Wg), is secreted by a strip of cells at the dorsoventral boundary and moves in both directions to instruct wing development (90).

Several analyses support spreading of cell surface–bound Wnts as a possible mechanism of intercellular transport. One such travel mechanism involves glypicans, transmembrane proteins decorated with heparan sulfate (HS) chains. Genetic and biochemical evidence indicates that glypicans facilitate Wnt movement along the cell surface. Glypicans that have been implicated range from Dally and Dally-like protein (Dlp) in the *Drosophila* wing disc to glypican-3 in human hepatocellular carcinomas (91–97). Wnts directly interact with Dlp core devoid of HS chains, and crystal structure of the Dlp core bound to a Wnt peptide revealed a binding pocket that can sequester the palmitoleic acid moiety of Wnt (98). In intestinal organoids, an epitope-tagged Wnt3 was retained on the cell surface by FZD receptors and was propagated to immediate neighbors through cell contact and division (99). These results exemplify short-range spread of cell-associated Wnts.

Advances in live-cell imaging and analysis have allowed for visualization of delicate cytoplasmic extensions, some of which serve as vehicles for Wnt trafficking. Live-cell imaging revealed fluorescent tagged Wnts, possibly in vesicles, traveling along actin-based cytonemes in the developing zebrafish neural plate and mammalian cell cultures (100–102). Intriguingly, components of the β-catenin-independent pathway, Ror2 (EC 2.7.10.1) and Vangl2, promoted the formation of such Wnt-loaded cytonemes and subsequent TCF/β-catenin activation in neighboring cells (100, 103). This marks a point of convergence of the β-catenin-dependent and the enigmatic β-catenin-independent branches of Wnt signaling.

On the other side of signal transmission, cytoplasmic projections were observed in Wnt-receiving cells, such as *Drosophila* myoblasts accepting Wg from wing disc cells and mouse embryonic stem cells interacting with Wnt3a-coated beads and Wnt-secreting trophoblasts (104–106). These results support the role of cytonemes in Wnt signal transport and reception, although the pleiotropic effects of disrupting such structures pose a challenge to studying their broader significance.

Further research will determine whether the various vehicles for Wnt transport identified thus far function in specific biological contexts or whether one or a combination of them constitutes a predominant mechanism. For instance, glypicans enhance cytoneme stability, and Wnt ligands could enter or exit cytonemes in exosomes, presenting points of intersection among these modes of transport (107, 108). Other reviews focused on intercellular transport of Wnts offer comprehensive overviews on the subject (109–113).

WNT RECOGNITION AND PATHWAY INITIATION

Upon reaching the receiving cell, the Wnt ligand simultaneously engages FZD and LRP coreceptors. Some Wnts exhibit preferential binding to different extracellular domains of LRP6 (114, 115). The ligand-receptor interaction preference, however, relies predominantly on the identity of the FZD receptor (116–118). While some Wnt-FZD binding preferences have been identified through biochemical and cell-based assays, comprehensive mapping of the Wnt-FZD interactome remains a challenge, with 19 Wnt and 10 FZD paralogous genes in the mammalian genome. In one analysis, CRISPR targeting of multiple FZD receptors in HEK293T cells generated a FZD loss-of-function background (119). Individual FZD receptors and Wnts were then reintroduced to assess the signaling activity of each pair. This approach confirmed that FZDs can be grouped into four main clusters that share higher sequence identity and Wnt preference profiles. The FZD5 and FZD8 cluster showed the highest degree of ligand promiscuity, a finding in line with the critical role of FZD5 and FZD8 in multiple tumor cell types with hyperactive Wnt signaling (120, 121).

Despite some degree of FZD binding preferences, individual Wnts display significant cross-reactivity to FZD subtypes. Context-dependent coreceptors have emerged as an endogenous means to further restrict cellular response to a subset of Wnt ligands. GPR124 and Reck are

two such transmembrane receptors that interact specifically with Wnt7a and Wnt7b. Wnt7a/b signaling is required for proper endothelial junction formation in the central nervous system (122–125). While Reck alone is capable of binding Wnt7a/b even in the absence of all 10 FZDs, signaling activation depends on GPR124-mediated recruitment of Reck-Wnt7a/b to the FZD–LRP receptor complex (126–128). These results support a model in which Reck and GPR124 associate with FZD and LRP in a Wnt ligand–dependent manner to activate signaling and regulate the development of central nervous system endothelium.

Epidermal growth factor receptor (EGFR, EC 2.7.10.1) was identified as another context-specific coreceptor for β-catenin-dependent Wnt signaling. Hematopoietic stem and progenitor cell (HSPC) development specifically requires Wnt9a-FZD9b signaling (129). As a proximal interactor of FZD9b, EGFR is indispensable for specific recognition of Wnt9a and pathway activation in zebrafish HSPCs (130). GPR124/Reck and EGFR illustrate how atypical coreceptors can confer additional Wnt ligand specificity in developmental contexts. Identification of additional coreceptors will reveal how components of the Wnt pathway and other signaling cascades may cooperate in tighter control of cellular behavior.

The typical signaling core at the cell surface, however, consists of the Wnt ligand and FZD and LRP receptors in a ternary complex. The N- and C-terminal domains of Wnt grasp FZD, with the linker between the two domains projected to interact with LRP (114, 131, 132). Structural analyses suggest that multiple units of this complex may cluster for pathway activation, potentially through FZD dimer formation (132–134). There is a debate around receptor complex internalization upon Wnt ligand binding via endocytosis and its role, if any, in signal transduction. For example, endocytic activity has been observed following Wnt pathway activation (135–138). Due to the pleiotropic effects of endosomal pathway disruption, however, it has been difficult to resolve whether receptor internalization is part of signal transduction or mainly a mechanism for downregulating Wnt receptor abundance on the cell surface (139–144).

Regardless, the unequivocal importance of LRP-FZD association in Wnt pathway activation has guided the development of a suite of agonists (**Figure 3*a***). Experiments with chimeric proteins made up of FZD and the endogenous LRP6 inhibitor Dickkopf1 (DKK1) pioneered the notion that molecules that induce LRP-FZD hetero-oligomer formation can bypass a requirement for Wnt ligand (145). Building on this notion, one set of agonists induced receptor heterodimerization by connecting a LRP6-binding module from DKK1 with an engineered FZD-binding module (146). These molecules exhibited strong signaling activity, eliciting downstream responses such as expansion of primary organoid cultures and maintenance of intestine stem cells in vivo in the absence of endogenous Wnt secretion (147, 148). These bivalent agonists, when further improved with a module with a more extensive FZD-binding interface, induced oligomerization of FZD and LRP (147, 149, 150). Other groups sought to prescribe receptor oligomerization through multivalent agonists. Their tetravalent agonists consisted of two binding sites for FZD and LRP and bridged together two molecules of each receptor. These surrogates exhibited potent Wnt signaling activity, from β-catenin accumulation to mesoderm fate induction in cultured pluripotent stem cells and intestinal organoid growth (151–153).

The bivalent and tetravalent agonists are superior to natural Wnt ligands in certain research and therapeutic applications. These agonists are water soluble and scalable and can be designed to activate signaling in a FZD subtype–specific manner, avoiding the effects of indiscriminate Wnt pathway activation. For instance, selective activation of Wnt signaling through recruitment of FZD7-LRP6 or FZD4-LRP5 was achieved through specific antibody agonists. These selective FZD7-LRP6 and FZD4-LRP5 agonists induced mesendodermal differentiation of human pluripotent stem cells and restored vascular development and barrier function in a mouse retinopathy model, respectively (154, 155). Fruits of cumulative insight from structural and

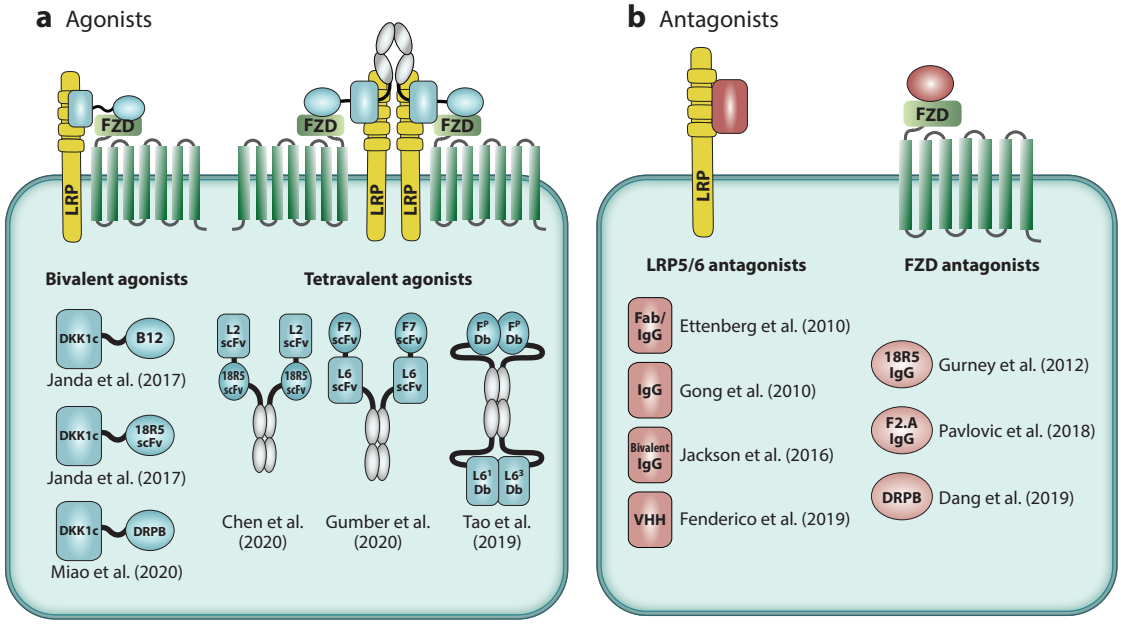

Figure 3

Synthetic agonists and antagonists of Wnt receptors. (*a*) Soluble agonists that activate Wnt signaling are designed to bring together one molecule of FZD and LRP in the case of bivalent agonists (146, 147) and two molecules of FZD and LRP in the case of tetravalent agonists (151, 153, 155). (*b*) Soluble antagonists that bind extracellular epitopes of FZD or LRP inhibit Wnt signaling through steric blocking of Wnt ligand interaction with receptors. Synthetic modulators of Wnt receptor activity and references thereof are shown (149, 161–166). These antagonists and agonists can be designed to inhibit or activate Wnt signaling in a FZD subtype–specific manner. Abbreviations: Db, diabody; DKK1c, C-terminal domain of the human Wnt antagonist Dickkopf1; DRPB, designed repeat protein binder; Fab, antigen-binding fragment; FZD, Frizzled; IgG, immunoglobulin G; scFv, single-chain variable fragment; LRP, low-density lipoprotein receptor–related protein; VHH, variable heavy chain domain of a heavy chain antibody.

cellular studies in the field, these agonists not only will allow us to modulate Wnt activity with increasing specificity but also will help reveal the composition and stoichiometry requirements of the Wnt receptor complex.

ADDITIONAL LAYERS OF WNT PATHWAY REGULATION

Whereas the Wnt ligand and its agonists engage FZD and LRP coreceptors simultaneously to activate the pathway, ligands that bind FZD or LRP alone tend to antagonize signaling activity (**Figure 3*b***). DKK and Sclerostin, for instance, are endogenous secreted inhibitors that compete with Wnts for LRP5/6 binding (156–160). Similarly, antibodies designed to bind extracellular epitopes of FZD or LRP can inhibit signaling through steric blocking of Wnt ligand interaction with receptors (149, 161–166). Just as agonists that bind specific FZD receptors along with LRP allow for selective Wnt activation, FZD binders with extensive interfaces can achieve FZD subtype–specific inhibition (149). Furthermore, FZD subtype–specific binding reagents can be used in targeted therapies against cancer types with elevated FZD levels. For instance, an antibody-drug conjugate composed of an antibody to human FZD7 and a microtubule inhibitor selectively killed FZD7-expressing ovarian cancer cells in vitro and induced regression of ovarian tumors in murine xenograft models (167).

Other secreted antagonists reduce the level of free Wnts available for signaling. Secreted Frizzled-related proteins (sFRPs), Wnt inhibitory factor 1 (WIF1), and Notum (EC 3.1.1.98)

fall into this category of endogenous Wnt inhibitors (168–173). While sFRPs and WIF1 bind and inhibit Wnts, Notum is a secreted deacylase that reduces Wnt activity by removing its palmitoleic acid moiety (174, 175).

An intricate network at the cell membrane provides another layer of Wnt signaling modulation. R-spondins were discovered as potent amplifiers of Wnt signaling strength, yet their mode of action remained obscure for some time (176). This state changed with the identification of two families of surface receptors: the transmembrane ubiquitin ligases ring finger 43 (RNF43, EC 2.3.2.27) and zinc and ring finger 3 (ZNRF3, EC 2.3.2.27) and their antagonists, the transmembrane receptors LGR4, LGR5, and LGR6 (leucine-rich repeat–containing G protein–coupled receptors 4, 5, and 6) (**Figure 4**) (177). In the absence of R-spondin, RNF43 and ZNRF3 function as negative regulators of Wnt signaling through ubiquitylation and subsequent degradation of the Wnt receptors FZD and LRP (178, 179). As transcriptional targets of the Wnt pathway, RNF43 and ZNRF3 provide negative feedback on signal strength along with AXIN2 and Notum, other Wnt targets that downregulate Wnt activity (180). Once R-spondin engages an LGR and RNF43/ZNRF3, the R-spondin–LGR–RNF43/ZNRF3 complex is internalized (181–185). This progression antagonizes RNF43/ZNRF3-mediated downregulation of cell surface Wnt receptors, enhancing cellular response to Wnt. R-spondins, therefore, are natural potentiators of Wnt signaling, and expression of LGRs is associated with elevated signaling and stem cell identity in tissues that undergo constant self-renewal (34, 186–188). Accordingly, many types of organoids require Wnts, R-spondins, or a combination of both in culture media to maintain a self-renewing cell population (189).

The R-spondin–RNF43/ZNRF3 module can also be a target of Wnt-activating mutations. *RNF43* mutations were originally reported in pancreatic cancer, while *ZNRF3* mutations were first seen in adrenocortical carcinoma (190, 191). Gene fusions involving R-spondin 2 or R-spondin 3 occur with low frequencies in colon cancers (192). These *RNF43* and *ZNRF3* mutations and R-spondin fusions allow cancer cells to proliferate even with low levels of Wnt. Unlike cancer cells harboring mutations in cytoplasmic signal transducers such as APC and β-catenin, cells with *RNF43* and *ZNRF3* mutations and R-spondin fusions require an exogenous Wnt source. The latter group of cells should be, in principle, treatable with small molecule inhibitors of the Wnt acyltransferase PORCN (82–84, 86, 193). Blocking the activity of R-spondin fusion proteins with antibodies or using the extracellular domains of FZD or LRP5/6 as a Wnt sink is another potential avenue (194).

Additional complexities in the function of R-spondins continue to be unveiled. A subset of R-spondins augment Wnt signaling in the absence of LGRs, interacting with HS proteoglycans rather than LGRs to neutralize RNF43/ZNRF3 activity (195–197). Moreover, in addition to enhancing Wnt signaling, R-spondins may antagonize bone morphogenetic protein (BMP) signaling through ZNRF3-mediated downregulation of BMP receptors (198). This finding presents an intriguing intersection of two signaling cascades with crucial roles in development and tissue homeostasis, particularly in light of the widespread use of R-spondins in stem cell and organoid culture.

These transmembrane proteins and secreted ligands are examples of endogenous modulators that refine the range and amplitude of Wnt signaling. Further research will reveal additional regulators that intervene at different levels of signal transduction, as well as intricacies in the function of known regulators.

INTRACELLULAR SIGNAL TRANSDUCTION

In the absence of the Wnt signal, β-catenin is sequentially phosphorylated, first by CK1α at residue S45 and then by GSK3β at residues S33, S37, and T41 (4). Such phosphorylation requires the scaffolding proteins AXIN and APC, which constitute the β-catenin destruction complex along

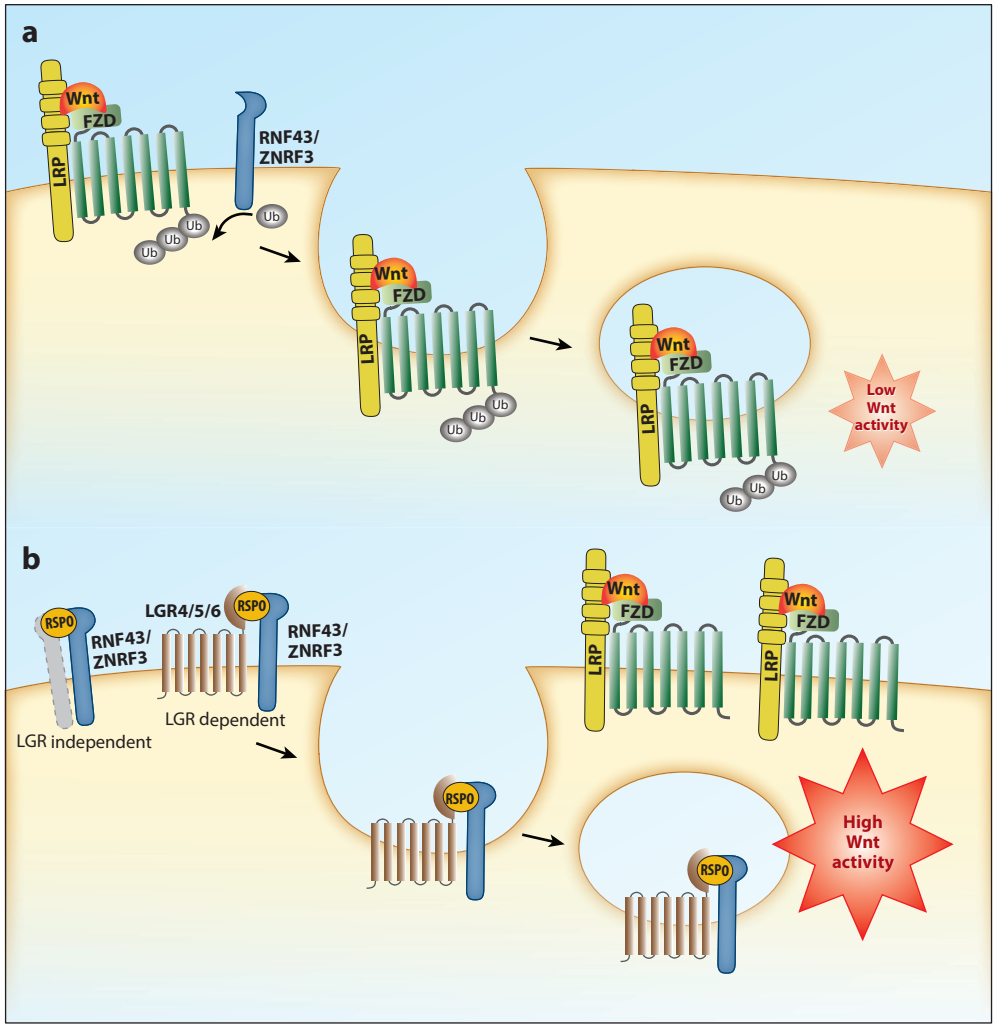

Figure 4

Modulation of Wnt activity by RNF43 and ZNRF3. One mechanism of Wnt signaling modulation relies on the transmembrane ubiquitin ligases RNF43 and ZNRF3. Ubiquitylation of Wnt receptors by RNF43/ZNRF3 downregulates receptor availability on the cell surface. Binding of the secreted protein RSPO to RNF43/ZNRF3 and LGR4/5/6 or alternative coreceptors inhibits the ubiquitin ligase activity of RNF43/ZNRF3. This inhibition increases Wnt receptor abundance on the cell surface and enhances Wnt signaling. Abbreviations: FZD, Frizzled; LGR, leucine-rich repeat–containing G protein–coupled receptor; LRP, low-density lipoprotein receptor–related protein; RNF43, ring finger protein 43; RSPO, R-spondin; ZNRF3, zinc and ring finger 3.

with the kinases. GSK3β-mediated phosphorylation of β-catenin leads to its ubiquitylation by beta transducin repeat-containing protein (β-TrCP) and Skp1–Cullin–F-box (SCF) E3 ubiquitin ligases and its subsequent proteasomal degradation (4, 5, 199).

At the core of AXIN and APC function in β-catenin degradation is the ability of AXIN and APC to form a multiprotein complex that brings β-catenin and GSK3β together. The APC-truncating mutations common in colorectal cancers impair its interaction with AXIN and β-catenin recruitment to the destruction complex, leading to erroneous activation of Wnt signaling (47, 200). Mutations in the C-terminal DIX domain of AXIN similarly compromise β-catenin recruitment to

the destruction complex, as polymerization of AXIN mediated through its DIX domain is crucial to its function (201, 202). The destruction complex may contain tens to hundreds of AXIN molecules in cytoplasmic puncta formed through aggregation and phase separation, even at near-endogenous AXIN levels (203–206).

Once the Wnt ligand is recognized by receptors, intracellular signal transducers undergo molecular changes to relay the signal, ultimately inhibiting GSK3β-mediated β-catenin phosphorylation. GSK3β is a promiscuous kinase that phosphorylates numerous targets besides β-catenin, including its namesake glycogen synthase as part of insulin signaling (207). Inhibition of GSK3β activity through LiCl or the small molecule CHIR99021, often used to mimic Wnt pathway activation, affects phosphorylation of many other targets of GSK3β and should be employed with caution (208, 209). In the presence of the Wnt ligand, however, signal transducers of the pathway appear to block GSK3β activity on a subset of its targets, including β-catenin (210, 211). Interestingly, cells in G2 and M phases of the cell cycle exhibit elevated Wnt signaling in a phenomenon known as Wnt-dependent stabilization of proteins, or Wnt/STOP. Mitotic Wnt activation is associated with stabilization of a multitude of GSK3β phosphorylation targets, including the cell cycle regulator c-Myc, and with increased protein content and cell size (212–214).

How do Wnt signal transducers achieve specific inhibition of GSK3β? This inhibition depends on the interaction of Wnt-activated receptors with cytoplasmic pathway components. Within minutes of Wnt stimulation, LRP6 is phosphorylated at its intracellular proline- and serine-rich motifs. LRP6 phosphorylation is both an early hallmark and an essential step in Wnt signal transduction, as phosphorylated LRP6 can recruit and directly inhibit GSK3β (215–217). Structural and biochemical data support that phosphorylated LRP6 motifs bind GSK3β, inhibiting its activity on β-catenin (218, 219). Of note, an alternative mechanism has been proposed in which GSK3β activity on β-catenin remains unchanged upon Wnt signal reception. Specifically, a study by Li and colleagues showed that phosphorylated β-catenin escapes degradation and saturates the APC-AXIN complexes in Wnt-activated cells (220).

GSK3β inhibition depends on DVL, an indispensable intermediary that facilitates the interaction between Wnt-bound receptors and cytoplasmic pathway components. The role of DVL in Wnt signal transduction depends on two key attributes: its ability to associate with FZD and its ability to form dynamic oligomers. Upon pathway activation, DVL is recruited via its DEP domain to the cytoplasmic interface of FZD (221–224). Indeed, single-molecule imaging of endogenous tagged DVL2 revealed its increased dwell time on the membrane upon Wnt addition in a manner dependent on the DEP domain, supporting the idea that DVL associates with the receptor complex via its interaction with FZD (225). Along with DVL, AXIN and GSK3β relocate to Wnt-bound receptors on the plasma membrane (204, 226–228).

DVL also oligomerizes through its DIX domain, which is homologous to the DIX domain of AXIN, in response to Wnt stimulation (229–231). Due to the self-associating nature of the DIX domain, overexpression of DVL leads to formation of large ectopic puncta (232, 233). At endogenous expression levels, however, DVL exhibits limited oligomerization. Live imaging of DVL2 with a knock-in tag showed that most Wnt-induced DVL complexes consist of fewer than five molecules (225, 234).

The DVL DIX domain associates not only with itself but also with the DIX domain present in AXIN to facilitate DVL-AXIN interaction upon pathway activation, although the extent to which endogenous DVL and AXIN hetero-oligomerize remains to be determined (201, 222, 229, 235, 236). Indeed, the key to the switch from the Wnt-inactive to the Wnt-active state is the transition of AXIN from associating with the destruction complex to associating with DVL in the signalosome, an event that in part hinges on the relative levels of AXIN and DVL. Elevated AXIN

levels favor the Wnt-inactive state, whereas elevated DVL levels induce the Wnt-active state (204). A feedback regulator at the crux of this AXIN-DVL balancing act is naked cuticle (Naked) and its vertebrate orthologs, NKD1/2 (237). Conserved in all animals, Naked antagonizes Wnt signaling by binding and destabilizing DVL in short-term Wnt activation but may promote Wnt signaling by destabilizing AXIN during prolonged Wnt stimulation (238).

Interestingly, while Wnts are not found in the plant kingdom, a protein family containing a DIX domain exists in plants (239, 240). Termed SOSEKI proteins, they establish cell polarity in plants through asymmetric localization that depends on their DIX-mediated oligomerization. Remarkably, swapping the DIX domains of human DVL and plant SOSEKI revealed their functional equivalence (239). DVL has long been known to regulate epithelial cell polarity in a β-catenin-independent manner in animal systems (232, 241). Therefore, identification of plant SOSEKI proteins sheds light on the ancient role of DVL in cell polarity regulation that predates its function in Wnt signaling.

The molecular events that take place in the Wnt-receiving cell, taken together, illustrate a model of Wnt signal transduction that integrates changes in oligomerization and localization of pathway components. In the absence of the Wnt signal, AXIN-bound GSK3β phosphorylates β-catenin with help from CK1α and APC. Once the Wnt ligand engages LRP/FZD, receptor complexes form and DVL associates with FZD on the cell membrane, as well as with other molecules of DVL and AXIN. Limited yet rapid oligomerization of receptors and intracellular signal transducers, and the bridging of these two groups through recruitment of intracellular components to the cell membrane, would increase the local concentration of Wnt pathway components that might not have high affinity for each other as individual molecules. Enhanced avidity, ultimately, would promote the interaction between activated, phosphorylated LRP with AXIN-bound GSK3β and would lead to inhibition of GSK3β-mediated degradation of β-catenin. This model ensures that, among the pool of cytoplasmic GSK3β, AXIN-bound GSK3β molecules responsible for β-catenin phosphorylation are selectively inhibited by Wnt pathway activation.

NUCLEAR FUNCTION OF TRANSCRIPTIONAL EFFECTORS

The central effector of Wnt signaling, β-catenin, functions as a transcription activator. Upon pathway activation, β-catenin escapes GSK3β-mediated phosphorylation and accumulates in the cytoplasm and the nucleus. As β-catenin alone does not have DNA-binding and transcription-activating abilities, interaction with nuclear partners is crucial to its function. In the nucleus, the Armadillo repeat domain of β-catenin mediates its interaction with DNA-binding proteins such as the TCF/LEF family of transcription factors (242–245). β-Catenin replaces Groucho family transcription repressors, which associate with TCF/LEF in the absence of the Wnt signal, and induces transcription of TCF/LEF-bound sites (246–248). Targets of TCF/LEF/β-catenin regulate cellular behavior such as cell cycle progression and stem cell self-renewal (249). Wnt transcription activity can be measured through reporters such as TOPFLASH, which contains multimerized TCF/LEF-binding motifs (250, 251). Negative feedback regulators of Wnt signaling such as *AXIN2* are general Wnt target genes and serve as a faithful readout of Wnt pathway activation through mRNA quantification or visualization of *AXIN2*-driven reporters (38, 252–255).

An array of other transcription factors cooperate with β-catenin to fine-tune target gene expression in a developmental stage– and tissue-specific manner (245, 256–258). TCF/LEF and β-catenin can interact with BCL9 and its paralog, BCL9L (259, 260). BCL9 and BCL9L in turn engage Pygopus in contexts ranging from *Drosophila* embryonic development to vertebrate heart formation and engage Tbx3 in forelimb development, regulating different sets of genes as dictated by these cofactors (259, 261–264). Further examples of context-specific interactors of β-catenin

include SOX17 in the dorsal endoderm of the *Xenopus* embryo and MyoD in differentiating myoblasts (265–267).

Once at target sites, β-catenin interacts with chromatin modifiers and transcription regulators to achieve target gene activation. For instance, β-catenin can regulate chromatin structure through its interaction with the histone acetyltransferases CBP (CREB-binding protein) and p300 (EC 2.3.1.48) and the nucleosome remodeler BRG1 (Brahma-related gene 1) (268–270). The full range of nuclear proteins that cooperate with β-catenin and the physiological processes they regulate await further examination.

While β-catenin may rely on cofactors, its central role in Wnt signal transduction is indubitable, as illustrated by grave consequences of mutations that alter its activity. Mutations that block β-catenin phosphorylation at serine and threonine residues, which lead to constitutive accumulation of β-catenin, are found in many cancers with hyperactive Wnt signaling (51, 200, 271). As a proof of principle, transcript-level single-nucleotide substitution at a phosphorylation site of β-catenin by the RNA-targeting CRISPR effector Cas13 led to enhanced Wnt signaling and cell proliferation (272).

In addition to functioning as a transcriptional activator along with cofactors, β-catenin is found at cellular locations as diverse as cell-cell junctions and centrosomes, where it interacts with E-cadherin to mediate cell adhesion and regulates centrosome separation in mitosis, respectively (273–277). The jack-of-all-trades quality of β-catenin, while making it a fascinating protein, poses a challenge to quantitative analysis of β-catenin dynamics in the context of Wnt signal transduction. Nonetheless, elegant biochemical studies unveiled important aspects of the behavior of β-catenin as a signal transducer (10, 278). These studies demonstrated that the fold change in the level of β-catenin upon inhibition of its phosphorylation, rather than the final absolute level of β-catenin, dictates Wnt transcriptional output. Moreover, β-catenin fold change is a robust parameter buffered from genetic and pharmacological perturbations of other pathway components.

High-resolution imaging-based studies of β-catenin dynamics substantiated these findings (143, 279–281). Single-cell-resolution live imaging of endogenous β-catenin revealed significant cell-to-cell variability in the rate and extent of β-catenin accumulation but consistent fold increase in nuclear β-catenin at the cell population level. One study integrated β-catenin dynamics with transcriptional response at the single-cell level through mRNA tagging of the Wnt target gene *Cyclin D1* (281). This analysis showed an increase in the number of cells transcribing the target gene as early as 15 min following Wnt addition and a strong correlation between the rate of nuclear β-catenin increase and transcription induction. Altogether, these results point to the rate of change in nuclear β-catenin as the main determinant of downstream transcriptional activity. Such a mechanism would confer robustness to the biological outcome of signaling despite cell-to-cell variability in biochemical parameters. These studies also highlight the agility of a signaling network with a central effector poised to escape degradation and bind cofactors already occupying target transcription sites.

One enigmatic aspect of β-catenin behavior is the mechanism of its translocation into the nucleus. Imaging studies revealed rapid nuclear accumulation of β-catenin in response to Wnt, implicating other mechanisms for subcellular movement in addition to diffusion (143, 280–282). Partners interacting with β-catenin may play a role; destruction complex components APC and AXIN may promote cytoplasmic retention of β-catenin, while transcription activators may promote its nuclear retention (283). Alternatively, although β-catenin lacks an obvious nuclear import or export signal, several transport proteins, including the intraflagellar transport protein IFT140, the guanine nucleotide exchange factor RAPGEF5, and the nuclear importin IPO11, have been implicated (284–286). Whether there is a universal mechanism for active nuclear transport of β-catenin remains to be resolved.

CONCLUSION AND PERSPECTIVES

The range of physiological processes modulated by Wnt signaling and layers of regulation thereof underscore the long history of a signaling pathway that evolved along with all metazoans. Biochemical, cell biological, and genetic experiments have identified key signal transducers of this multifaceted pathway as well as molecular changes they undergo upon pathway activation. However, we are left with an incomplete picture of the intracellular activities that connect Wnt ligand-receptor binding to transcription of target genes. What is the sequence of changes in localization, posttranslational modification, and intermolecular interaction of Wnt pathway components? Importantly, what is the functional significance of the changes in signal transduction, if any?

A new set of tools offers an opportunity to gain more mechanistic insight into Wnt signal transduction and to address these outstanding questions. Molecular tools now at our disposal can be applied to the field of Wnt signaling in two broad ways: observing endogenous Wnt pathway components at work and altering their behavior with a high level of specificity and control.

The first approach is simply to monitor the behavior of Wnt signal transducers upon pathway activation with unprecedented spatiotemporal resolution. High-resolution live-imaging techniques allow us to visualize localization, movement, and aggregation of Wnt pathway components as well as target transcription upon signaling initiation at single-cell or even single-molecule resolution (225, 281). In parallel, genome engineering permits precise tagging of endogenous components to reduce unexpected effects on signaling caused by overexpressed proteins. Studies mentioned above analyzed dynamic behavior of endogenous DVL2 or β-catenin through knock-in fluorescent tagging coupled with live-cell imaging (143, 225, 279, 280).

Proximity labeling enzymes offer a proteomic tool that can also be coupled with genome engineering to capture interactors of endogenous signal transducers. Engineered enzymes such as BioID, TurboID, and APEX can identify proximal interactors of a protein of interest through biotin tagging (287–289). Notably, the ability of APEX to capture transient or weak interactions with a temporal resolution of seconds is suitable for studying a signaling cascade as dynamic as the Wnt pathway (290, 291). APEX labeling has identified known and novel proximal interactors of Wnt receptors upon pathway activation (130, 292). In addition to imaging or proteomic mapping of individual Wnt signal transducers, split tags can be attached to pairs of them such that complemented bioluminescence, fluorescence, or biotin labeling activity will report the pairs' interaction (293–296). Experimental investigations of Wnt pathway proteins and their interactions could be aided by neural network–based protein structure predictions (297, 298). While these programs need further optimization to accurately predict protein complexes or oligomerization states, recent advances in their performance indicate their potential to provide new insights into, for instance, interaction interfaces or stoichiometry of Wnt receptor or intracellular complexes.

The second approach is to identify the minimum molecular requirements for Wnt signal transduction through biochemical reconstitution of pathway components or controlled modulation of pathway component behavior. In an extraordinary effort, Ranes and colleagues reconstituted the destruction complex in vitro, complete with full-length AXIN1, APC, CK1α, GSK3β, and β-catenin (202). This reductionist approach shed light on the roles of the scaffolding proteins APC and AXIN at different steps of destruction complex function, from β-catenin recruitment to ubiquitylation, as well as the effects of several APC and AXIN functional domain mutations on each step.

Another avenue for mechanistic investigation of the Wnt pathway utilizes light- or drug-controlled protein modules. Such modules can change the localization or oligomerization of signal transducers to achieve ligand-independent activation of Wnt signaling and to identify the molecular requirements for signal transduction. For instance, LRP6 was tagged with a

blue-light photoreceptor module to induce optogenetic oligomerization of LRP6 and subsequent Wnt pathway activation (299, 300). Light- or drug-controlled domains can induce a range of molecular changes beyond oligomerization, such as recruitment to another protein or a cellular compartment, and can allow us to investigate which of these changes are sufficient for Wnt signal transduction (301). Lastly, the synthetic agonists described above, with their modular nature and ability to bind specific receptor subtypes, will be valuable tools in resolving how Wnt receptor combinations and their stoichiometries determine pathway activation and signaling amplitude.

With higher-resolution pictures of Wnt pathway dynamics from these multiple angles, we will be able to determine the precise order and functional significance of molecular events in Wnt signal transduction. Better structural understanding of the Wnt ligand-receptor interface has led to informed design of Wnt agonists and antagonists. Insight into the critical role of the lipid moiety on Wnts has led to pharmacological lipidation inhibitors that effectively block Wnt signaling. All the biological components are there for us to build first a more complete understanding of their function in Wnt signaling and then ways to modulate this ancient pathway with greater confidence.

DISCLOSURE STATEMENT

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