Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance

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Abstract Wnt signaling transduces evolutionarily conserved pathways which play important roles in initiating and regulating a diverse range of cellular activities, including cell proliferation, calcium homeostasis, and cell polarity. The role of Wnt signaling in controlling cell proliferation and stem cell self-renewal is primarily carried out through the canonical pathway,
which is the best-characterized the multiple Wnt signaling branches. The past 10 years has seen a rapid expansion in our understanding of the complexity of this pathway, as many new components of Wnt signaling have been identified and linked to signaling regulation, stem cell functions, and adult tissue homeostasis. Additionally, a substantial body of evidence links Wnt signaling to tumorigenesis of cancer types and implicates it in the development of cancer drug resistance. Thus, a better understanding of the mechanisms by which dysregulation of Wnt signaling precedes the development and progression of human cancer may hasten the development of pathway inhibitors to augment current therapy. This review summarizes and synthesizes our current knowledge of the canonical Wnt pathway in development and disease. We begin with an overview of the components of the canonical Wnt signaling pathway and delve into the role this pathway has been shown to play in stemness, tumorigenesis, and cancer drug resistance. Ultimately, we hope to present an organized collection of evidence implicating Wnt signaling in tumorigenesis and chemoresistance to facilitate the pursuit of Wnt pathway modulators that may improve outcomes of cancers in which Wnt signaling contributes to aggressive disease and/or treatment resistance.

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Introduction

Wnt signaling regulates a group of evolutionarily conserved pathways which play a central role in a diverse set of cellular activities including cell proliferation, calcium homeostasis, and cell polarity.1-3 Discovery of these pathways began in 1982 when, in their study of virally-induced C3H murine breast tumors, Nusse and Varmus identified a 25 kb region named Int-1, so called for being the preferential integration site of the mouse mammary tumor virus (MMTV).6 Following sequencing of Wnt1, the Drosophila gene wingless (wg) was shown to be a homolog of Wnt1.7,8 Originally identified in a classic genetic screen by Nüsslein-Volhard & Wieschaus, wg was known to control segment polarity in larval development of Drosophila.9 Taken together, these initial results began to hint at the scope and diversity of Wnt signaling. Evolutionary conservation of Wnt signaling was established following demonstration by McMahon and Moon that injection of mouse Wnt1 mRNA in Xenopus embryos at the 4-cell stage led to duplication of the body axis — a positive indicator of Wnt signaling. Although the role of Wnt signaling in human disease, specifically cancer, was arguably hinted at by Nusse and Varmus when they discovered Int-1 as a locus for oncogenic proviral integration, an undisputed connection was established only when adenomatous polyposis coli (APC), the gene underlying the hereditary cancer syndrome familial adenomatous polyposis (FAP), was identified and subsequently found to interact with β-catenin.10-12

Historically, Wnt signaling has been classified into two large categories: the canonical Wnt (or β-catenin-dependent) and non-canonical Wnt (or β-catenin-independent) pathways. The canonical and non-canonical pathways are intersecting signaling networks (Fig. 1) that coordinately regulate complex processes, such as embryonic development, stem cell maintenance, tissue homeostasis, and wound healing. The better-understood canonical Wnt/β-catenin pathway regulates cell fate, proliferation, and survival, while non-canonical Wnt pathways are more often associated with differentiation, cell polarity, and migration. Non-canonical Wnt signaling can be initiated by Wnt interaction with Frizzled receptors (Fzs), or RYK and ROR receptor tyrosine kinases and has been shown to regulate small GTPases (such as RhoA, Rac, and Cdc42).1-3,5 Non-canonical Wnt signaling can also activate calcium flux and kinase cascades, including those of protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), and JUN N-terminal kinase (JNK), leading to the activation of gene expression mediated by the
transcription factors activating protein 1 (AP1) and nuclear factor of activated T cells (NFAT).\textsuperscript{1–3} While it is apparent that non-canonical Wnt pathways play important roles in regulating many critical cellular processes, and Wnt signaling at large exhibits extensive cross-talk with other evolutionarily conserved signaling pathways, this review will focus on the canonical Wnt pathway, highlighting its major components, the roles it plays in regulating stem cell self-renewal and fate, and its implication in the development of human diseases, particularly cancer.\textsuperscript{1–3}

**Essential components and flow of the Wnt signaling pathway**

Wnt signaling begins with secretion of one of the 19 Wnt ligands and is initiated by Wnt ligands binding extracellularly to a complex consisting of their cognate receptor, Frizzled (Fz), and a co-receptor, such as LDL-related protein receptor S/6 (LRP-5/6) in the canonical pathway (Fig. 1). Activation of Fz leads to recruitment of the cytoplasmic phosphoprotein Dishevelled (Dsh). Induction of one of multiple possible signaling cascades follows — either the canonical pathway (Fig. 2) or one of several non-canonical pathways. The two best-characterized non-canonical pathways are the planar cell polarity pathway, which regulates the polarity of epithelial cells by modifying the actin cytoskeleton (Fig. 3), and the Wnt/Ca\textsuperscript{2+} pathway, which regulates the release of intracellular Ca\textsuperscript{2+} stores (Fig. 4).\textsuperscript{13–15} Several other non-canonical pathways have been proposed with significant overlap, and are reviewed more thoroughly elsewhere.\textsuperscript{1–3} In this review, we focus on the canonical Wnt/\(\beta\)-catenin pathway, which is better-characterized and appears to play a more prominent role in human disease.

**Wnt ligands are an evolutionarily-conserved but poorly-characterized family of pleiotropic, cysteine-rich, and lipid-modified glycoproteins**

Wnt proteins are a family of 19 highly-preserved ~40 kDa secreted glycoproteins.\textsuperscript{16} They contain an N-terminal signaling sequence and a conserved set of 22–24 cysteine (Cys) residues.\textsuperscript{17,18} Sequencing data suggests the possibility of intramolecular disulfide bonds, likely promoting conformational stability. Additional post-translational modifications include N-glycosylation and lipidation (O-acylation) of conserved Cys residues.\textsuperscript{19,20} The insolubility and hydrophobicity of Wnt ligands, due to the addition of a palmitoyl adduct to conserved Cys residues, has precluded their complete biochemical characterization by confounding the ability to isolate Wnt ligands in their active form.\textsuperscript{20} Further, there is poor overlap between the primary sequences of Wnt ligands and those of commonly known protein folds and motifs.\textsuperscript{21} Beyond complicating the ability to fully characterize Wnt ligands, the role of lipid modification has yet to be fully identified. Although loss of the palmitate moiety by mutation or enzymatic removal is associated with a loss of Wnt signaling activity, a precise description of its role remains elusive.\textsuperscript{20,22} More recent experimentation demonstrates that it may interact directly with a binding site on Fz.\textsuperscript{21}

**Regulation of Wnt ligand secretion hinges on lipid modification by the O-acyltransferase Porcupine**

As is true for many secreted signaling molecules, post-translational lipid modifications play a critical role in the regulation of Wnt secretion. Loss of lipid modifications, including palmitoyl acylation, in *Drosophila* leads to...
absence of Wg on extracellular staining, demonstrating that lipid modifications are required for secretion of Wg. Palmitoyl acyl groups are normally present on both C93 and S239 residues in Wg. Tang et al demonstrated that while single mutants at either C93 or S239 can be secreted, mutation of both residues completely inhibits secretion. Further evaluation revealed that both acyl groups are required for interaction with Wntless (Wls), a cargo receptor participating in Wg secretion. However, in the same study, the authors suggest that modification at S239 plays a more important role in the regulation of Wnt signaling. Unlike Wg C93 mutants, S239
mutants demonstrated reduced binding with its cognate receptor Drosophila Frizzled 2 (Dfz2), suggesting an indispensable role for the S239 residue in the formation of a receptor-ligand complex.\textsuperscript{23} By comparison, murine Wnt3a demonstrated loss of signaling activity with no inhibition of secretion following mutation of C77.\textsuperscript{20} Loss of acylation at S209 of murine Wnt3a results in failure of ligand secretion and sequestration in the endoplasmic reticulum.\textsuperscript{24}

The O-acyltransferase Porcupine, responsible for O-acetylation of Wnt, was initially discovered in Drosophila. Porcupine is a segment polarity gene encoding a transmembrane protein localized to the ER membrane and playing a role in Wg processing.\textsuperscript{25} The purported O-acetyltransferase activity of Porcupine was supported by sequence homology with a family of known membrane-bound O-acyltransferases and confirmed experimentally.\textsuperscript{26,27} Murine, Xenopus, and human homologues were subsequently identified and found to encode multi-pass transmembrane ER proteins with similar structure and function as Porcupine.\textsuperscript{28,29} Evidence supporting a role for Wnt regulation in vertebrate species by Porcupine was reported later, after lipid-modification of Wnt1 and Wnt3a by porcupine was demonstrated in chick neural tube cells.\textsuperscript{30}

The purported role of lipid modification is to target Wnt to secretory vesicles and create a high local concentration of ligand following secretion. It has previously been hypothesized that acylation may play a role in intracellular trafficking of Wnt by targeting it to specific organelles or to intracellular lipoprotein particles.\textsuperscript{34} Zhai et al demonstrated that lipid-modification of Wg targets it to secretory vesicles, which deliver the ligand to unique microdomains at the cell surface for packaging prior to secretion.\textsuperscript{31} Inhibition of O-acyltransferase activity with 2-bromopalmitate inhibited, in a concentration-dependent manner, the incorporation of Wg into lipid rafts. Similar results were obtained using cell lysates of Porcupine-negative mice with constitutive expression of Wg relative to control animals (6.69% membrane association of DWnt1 vs 80.76% in wildtype animals).\textsuperscript{31}

Lipid modifications of ligands such as hedgehog and epidermal growth factor have been shown to enhance their association with the plasma membrane, thus allowing for achievement of a local concentration high enough for biological activity.\textsuperscript{32,33} In the case of Wnt, loss of Porcupine-mediated lipid modification has been shown to reduce the range of activity for Wnt1/3a in the chick neural tube and cause intracellular retention of Wg in Drosophila models.\textsuperscript{30} Overexpression of Wls in Drosophila embryos has been shown to overcome the absence of Porcupine.\textsuperscript{20,34} Of note, the more newly characterized Drosophila WntD has not been shown to require lipid-modification for secretion at high levels.\textsuperscript{35–37} Despite these occasionally conflicting results, it remains clear that post-translational modification plays an important role in regulating intracellular trafficking and secretion of Wnt ligands. Unlike lipid modification, current evidence does not support a critical role for N-glycosylation in the regulation of Wnt secretion. Loss of all N-glycosylations on Wg has no demonstrated effect on secretion or signaling.\textsuperscript{23}

### Secretion of Wnt is modulated by the multi-pass transmembrane protein Wntless

Secretion of Wnt proteins is mediated by the Wnt-binding protein Wntless (Wls, also known as Evi, or GPR177), which transports Wnt from the Golgi network to the cell surface for release.\textsuperscript{38} Discovered in Drosophila during a screen for suppressors of a gain-of-Wg-signaling phenotype in the Drosophila eye, Wntless is a segment-polarity gene encoding a functionally conserved Type II multipass transmembrane protein and is a transcriptional target of Wnt signaling.\textsuperscript{38–40,41(p177),42} Wls is analogous to Dispatched, a protein functioning to secrete Hedgehog (Hh) proteins.\textsuperscript{43} Homozygous loss of Wntless is associated with abnormal patterning during fetal development, suggesting that it is necessary for proper Wnt functioning.\textsuperscript{38} Wls contains seven transmembrane regions and is thought to act downstream in the cascade from Porcupine.\textsuperscript{39} Wls is hypothesized to function as a cargo receptor for Wnt, transporting it from the trans-golgi to the plasma membrane. Wls itself is then recycled back to the cis-golgi via retromer complex machinery, but separate from other retromer cargo through a morphologically distinct retrieval pathway.\textsuperscript{44}

### Following secretion, Wnt is stabilized by extracellular matrix proteins

Regulation of Wnt signaling continues after secretion, primarily via interactions with extracellular proteins. Heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) stabilize and maintain the activity of hydrophobic Wnt ligands by preventing their aggregation in the aqueous extracellular milieu. This likely occurs by direct bonding of HSPGs to Wnt, as has been demonstrated previously with glypicans-1 and XWnt8. It remains to be seen if there exists specificity in the interactions between HSPGs and Wnt ligands.\textsuperscript{45,46}

### Wnt ligands bind to the Frizzled family of receptors to initiate signaling

The Frizzled (Fz) family of receptors are the cognate receptors through which Wnt signaling primarily occurs. They are classified as a novel family of transmembrane G-protein coupled receptors (GPCRs) with ~7 transmembrane regions and an N-terminal sequence enriched with ~10 Cys residues.\textsuperscript{47–49} There is plurality in which Fz specific Wnts may bind to; a single Wnt can bind any one of multiple Frizzled (Fz) proteins.\textsuperscript{47} Encoded by the Drosophila gene family, frizzled Drosophila frizzled 2 (Dfz2) was the first member of the family discovered to be capable of activating downstream components of Wnt signaling.\textsuperscript{47} A vertebrate homolog, Rat frizzled-1 (Rfz1) was discovered soon thereafter to also induce expression of Wnt-responsive genes.\textsuperscript{50} Additional family members and vertebrate homologues have since been discovered. The International Union of Pharmacology (IUPHAR) recognizes 10 mammalian isoforms of the Fz receptor.\textsuperscript{51}

Wnt ligands interact with Fz through its N-terminal cysteine-rich domain (CRD) (Fig. 1). In early experiments, transfection with this domain alone was enough to confer...
Wnt signal transduction occurs through ligand-induced receptor dimerization and recruits the phosphoprotein Dishevelled to the intracellular membrane leaflet

The mechanism by which binding of Wnt and other ligands activates Fz and initiates intracellular signal transduction remains poorly characterized. Receptor dimerization and oligomerization, which have been demonstrated in other members of the GPCR family both immediately after synthesis and in a ligand-promoted manner, have been proposed as possible mechanisms.59–61 Crystallization of CRDs from both murine Fz8 and sFRP3 yielded homodimers with observed overlap between the Wnt-binding domain and the homodimer interface, suggesting that ligand-induced receptor dimerization may be a key step in signal transduction.62 Formation of Xfz3 dimers has been demonstrated independent of ligand-binding by Western blot and co-immunoprecipitation when overexpressed in Xenopus embryos. The truncated protein Xfz3ΔC has been shown to be capable of forming homodimers and oligomers, suggesting that only the N-terminal domain is required for dimer formation. This finding is consistent with previous observations that the N-terminal CRD is capable of homodimer formation. Of note, dimerization of Xfz3 was demonstrated to be sufficient for activation of Wnt/β-catenin signaling independent of ligand. Although Xfz7 was shown in the same study to be unable to dimerize, dimerization of Xfz7 could be observed following addition of a heterologous dimer-formation domain (see below).62

In addition to Wnt, at least 3 additional families of ligands, soluble Fz-related proteins (sFRPs), Norrin, and R-spondins, have been shown to directly bind Fz and stimulate downstream signaling (see below).53–57 Connective tissue growth factor (CTGF) binds the CRD of Fz as well, but inhibits downstream signaling, suggesting a possible role as an endogenous competitive antagonist.58

Canonical Wnt signaling stabilizes the dual-function protein β-catenin

The canonical pathway of Wnt signaling regulates the degradation of β-catenin, a protein with roles in stabilizing cell adhesion and as a transcriptional activator of cell proliferation pathways. Of the three Wnt signaling pathways, it is the best-characterized. The pathway can perhaps be best understood by contrasting its configuration in the ‘off’ state (Fig. 2A), without Wnt activation, to that in the ‘on’ state, or once it has been activated by Wnt binding to Fz (Fig. 2B).59

In the absence of Wnt signaling, β-catenin is constitutively degraded

Under physiologic conditions, the canonical Wnt signaling pathway spends most of its time in the ‘off’ configuration, where Fz is inactive and a complex of proteins known as the β-catenin destruction complex exists in the cytoplasm (Fig. 2A). The complex includes the tumor suppressors Axin, adenomatous polyposis coli (APC), the Ser/Thr kinases glycogen synthase kinase 3 (GSK-3) and casein kinase 1 (CK1), and the F-box protein β-transducin repeat-containing protein (β-TrCP).59,70 Protein phosphatase 2A (PP2A) is also known to associate with the complex and may fulfill a regulatory role.71–74 The specifics are reviewed in detail elsewhere by Stamos and Weis.69 This review will focus on the functional highlights of the complex and its role in the signaling cascade.

β-Catenin is targeted for proteolysis in a two-step process beginning with phosphorylation of an N-terminal conserved Ser/Thr-rich sequence to create a β-TrCP binding site for subsequent ubiquitination.75–78 The first step, phosphorylation of Ser45 by CK1, is facilitated by a cluster of acidic residues located seven amino acids downstream from the phosphorylation site.79,80 Successive phosphorylation of T41, S37, and S33 by GSK-3 follows; phosphorylation of each residue primes the next phosphorylation step. Together, the phosphorylated residues create a binding site for β-TrCP. Mutations in the gene encoding β-catenin, CTNNB1, which altered GSK-3β phosphorylation sites created mutant copies of β-catenin with six times higher activity than its wild-type counterpart.81 Binding of β-TrCP triggers recruitment of the protein subunits Cul1/Cdc53 and Skp1 to form the ubiquitin ligase SCFβ-TrCP.82 The ensuing
ubiquitination of β-catenin targets it for proteasome-mediated destruction.83,84

**Axin and APC play crucial scaffolding roles in facilitating destruction of β-catenin**

Although not involved directly in enzymatic modification of β-catenin, Axin and APC play crucial roles in facilitating destruction of β-catenin. Axin is a large (~96 kDa) and flexible protein containing binding sites for APC, CK1, GSK-3, and β-catenin, as well as a purported binding site for PP2A or another phosphatase.71,73,85–89 While Drosophila contains only one gene for Axin, vertebrate genomes encode two mechanistically interchangeable variants.90,91 Axin has been shown to enhance GSK-3-mediated phosphorylation of β-catenin by a factor of approximately 20,000.92 This enhancement is attributed to a “scaffold effect” created by Axin simultaneously binding β-catenin and a kinase, thereby increasing the effective concentration of enzyme and substrate.87,93 The possibility of Axin modulating kinase catalytic activity has been ruled out by analysis of crystal structures, which show Axin binding to regions of GSK-3 and CK1 far-removed from their respective catalytic sites and demonstration of unchanged enzyme kinetics following binding to Axin.92,94

APC is an even larger (~310 kDa) multi-domain protein containing short peptide motifs which bind β-catenin and Axin.95 β-Catenin binds at homologous repeats of 15–20 amino acids.96 Phosphorylation of these sites can be mediated by GSK-3 or CK1 and has been demonstrated to increase APC binding affinity for β-catenin by up to 1500-fold. Axin binds APC at ~16-amino acid conserved repeats named ‘SAMP repeats’, which are interspersed between β-catenin binding sites.85 Vertebrate APC additionally contains N-terminal dimerization domains and an armadillo repeat domain which interacts with cytoskeletal regulatory molecules and the B56 regulatory subunit of PP2A.97–102

Although the role of APC in the destruction complex remains unclear, the literature presents the following working hypotheses99:

1. **APC may promote phosphorylation of β-catenin by Axin-bound kinases.** Although structural analysis supports this hypothesis and suggests that APC is capable of scaffolding the Axin–β-catenin interaction, APC fragments containing β-catenin and Axin binding sites did not demonstrate any enhancement of β-catenin phosphorylation.103–105 Similarly, no decrease in β-catenin phosphorylation occurs when APC is mutated to produce a truncated copy lacking SAMP repeats.106 However, the literature remains conflicted. In a more recent experiment, a fragment of APC including β-catenin and Axin-binding regions was shown to directly phosphorylate β-catenin and other substrates of GSK-3.107

2. **APC may facilitate cycling of the β-catenin destruction complex.** Based on studies demonstrating that APC, when phosphorylated at 20-amino acid repeat sites, competes with Axin to bind β-catenin, Kimelman and Xu proposed a model where APC is phosphorylated by CK1 and GSK-3 after they act on β-catenin. Phosphorylated APC displaces Axin from β-catenin, thereby freeing Axin to bind a new β-catenin molecule.93,108,109 Dephosphorylation by PP2A resets the destruction complex.71,73,110 Caveats to this model of APC function include lack of supporting data from in vitro studies. Ha et al demonstrated that protein phosphatase 1 (PP1), an enzyme with a catalytic domain similar to PP2A, was unable to dephosphorylate β-catenin-bound phosphorylated APC over a span of 3 hours—a period of time considerably longer than the documented half-life of β-catenin.109,111

3. **APC may promote β-catenin ubiquitination.** Ubiquitination of β-catenin requires the presence of APC 20-mer repeat 2 (20R2), a domain that does not bind β-catenin.112–114 Inability of β-TrCP to co-precipitate β-catenin in cell lines bearing copies of APC truncated before 20R2 and restoration of co-precipitation by transfecting the cells with wild-type APC suggests that 20R2 and the associated conserved downstream sequence stabilize the interaction between β-TrCP and β-catenin.105

4. **APC may sequester β-catenin in the cytoplasmic compartment.** Sequestering β-catenin in the cytoplasm prior to proteasome-mediated destruction is necessary to prevent inappropriate activation of downstream signaling. Using fluorescence recovery after photobleaching (FRAP) to observe inter-compartmental movement of β-catenin, Kriehoff et al demonstrated that APC and Axin increased the concentration of cytoplasmic β-catenin independently of the nuclear transport protein CRM-1. However, neither elicited an effect on nuclear-cytoplasmic shuttling, suggesting they simply promote cytosolic retention of β-catenin.115 Data from multiple studies has been unable to pinpoint one β-catenin-binding repeat of APC as being essential for cytoplasmic retention.109,114 Ha et al proposed a model in which APC contains both high-affinity and low-affinity binding sites such that β-catenin can be sequestered regardless of its cytoplasmic concentration.109 However, as Stamos and Weis explain, their model does not yet explain how Axin is able to access β-catenin and act as a scaffold to promote its phosphorylation.99

5. **APC may facilitate localization of the destruction complex.** APC has been shown to localize in vertebrate cells to the basolateral cortex or to cell–cell junctions through its interactions with the microtubule and Axin cytoskeletons.103,116,117 Truncated APCs have demonstrated more efficient transit between the nucleus and cytoplasm likely due to fewer cytoskeletal interactions.118

Independent of its role in regulation of β-catenin stability, APC associates with the microtubule cytoskeleton as shown by in vivo immunofluorescence. Association occurs through the C-terminus of the full-length protein and has been shown by in vitro polymerization and immunofluorescence assays to promote assembly of microtubule arrays.119 The complexity of APC’s role in Wnt signaling is due in part to its multifunctionality.120 Although the data discussed above suggest several possible roles for APC, further
studies are needed for a complete understanding of its function with respect to β-catenin destruction.

Activation of the canonical pathway leads to receptor dimerization, co-receptor LRP-5/6 localization, and recruitment of scaffolding proteins Dvl and Axin

Activation of the canonical pathway occurs with binding of Wnt to the extracellularly-located CRD of Fz and to the co-receptor (LRP-5/6) (Fig. 2A). LRP-5 and LRP-6 are type I single-span transmembrane proteins discovered through their homology to the LDL-receptor. Unlike the other members of the LDL-receptor (LDL-R) family, they contain four β-propeller/epidermal growth factor (PE) and three LDL-R repeats in the extracellular domain as well as Pro-rich motifs on the intracellular domain. Wnts have been shown to directly bind LRP-5/6 at PE domains.

Genetic studies comparing developmental abnormalities associated with loss of LRP-5 and LRP-6 suggest they have overlapping functions, but that loss of LRP-6 is associated with more severe defects. Combined with studies showing that overexpression of LRP-6 demonstrates stronger activity than overexpression of LRP-5 in both Xenopus and mammalian cells, it is possible that LRP-6 has a higher affinity for Wnts, a stronger signaling efficacy, or both.

Once Wnt is bound and the trimeric complex of Fz and LRP-5/6 is formed, downstream signaling is transduced through the scaffold proteins Dsh/Dvl and Axin (Fig. 2B). Dvl appears to engage the C-terminal KTxxxW motif of Fz through multiple domains.

Dvl then becomes hyper-phosphorylated after associating with kinases, including CK1-ε and CK2, although the significance of this phosphorylation step remains unclear. Subsequent recruitment and aggregation of Axin at the inner membrane leaflet via an interaction between its C-terminal DAX domain and the DIX domain of Dsh disrupts the β-catenin destruction complex as well as recruiting Axin to the cell membrane. The intracellular domain of LRP-5/6 contains five PPPSPxS motifs. Upon phosphorylation, which occurs with Wnt signaling, the motifs become docking sites for Axin (Fig. 2B). It is thought that recruitment of Axin-GSK-3 by Fz and Dvl promotes phosphorylation of PPPSPxS domains and facilitates further recruitment of Axin in a ‘feed-forward’ or self-perpetuating manner. Axin may interact with multiple PPPSPxS domains within the same LRP-5/6 co-receptor or with domains on multiple receptors. The latter has been coined the ‘initiation-amplification’ model and leads to formation of LRP-5/6 aggregates.

Two mechanistic models have been proposed for the inhibition of β-catenin destruction by activation of the Fz/LRP-5/6 receptor complex. In the first model, phosphorylated PPPSPxS directly binds and inhibits GSK-3. A newer model proposes formation of a ‘multivesicular body’ (MVB) after binding of Wnt; GSK-3 is sequestered in MVBs, thereby separating it from β-catenin. The model is based on the work of Taelman et al., who demonstrated localization of GSK-3 to acidic endosomal vesicles by fluorescence microscopy and protease protection studies.

Stable β-catenin accumulates and initiates transcription of target genes promoting cell metabolism and proliferation

Stabilization of β-catenin by Wnt signaling leads to propagation of Wnt target gene transcription. In the absence of Wnt signaling, members of the T-cell factor/lymphoid enhancing factors (TCF/LEF) family of DNA-binding factors are bound to the co-repressor protein Groucho, thereby inhibiting transcription of their target genes. Following LEF-1-facilitated nuclear translocation, β-catenin displaces Groucho to create a transcriptional activator complex. Recruitment of purported co-activators, including the histone acetyltransferases cyclic AMP response element-binding protein (CBP) and p300 and the chromatin remodeling factor Brg1, induces chromatin remodeling to favor gene transcription. Intra-nuclear regulation of Wnt signaling occurs in part via sequestration or enzymatic modification of β-catenin, TCF/LEF transcription factors, and/or co-activator molecules.

Target genes of Wnt signaling are recognized by TCF/LEF transcription factors at a highly conserved binding element composed of a consensus sequence (5'-CAGTTG[A/T][A/T]-3') in the promoter region near the transcription start site. Since the early identification of c-Myc and cyclin D1 as Wnt/β-catenin downstream targets, numerous TCF/LEF target genes have been reported. Many of these genes encode gene products capable of broadly upregulating cell proliferation and metabolism, as exemplified by the following three targets:

(1) The oncogene c-MYC, which encodes a transcription factor whose target genes regulate processes including cell proliferation (cyclin D2, CDK4, cyclin B1), metabolism (enolase A, hexokinase II, lactate dehydrogenase A, phosphofructokinase, and glucose transporter), protein synthesis (genes encoding rRNA, ribosome biogenesis proteins), cytoskeletal rearrangement (c-MYC overexpression has been shown to decrease expression of collagen and integrin genes), and apoptosis (EZH1, itself a transcription factor promoting cell cycle progression and apoptosis, and TRAP1, the tumor necrosis factor receptor associated protein). Many of these genes encode gene products capable of broadly upregulating cell proliferation and metabolism, as exemplified by the following three targets:

(2) CCND1, which encodes cyclin D1, a nuclear protein required for cell cycle progression from G0/G1 to S phase. Cyclin D1 functions as a regulatory subunit of cyclin-dependent kinase-4 and -6 (CDK4, CDK6). When bound to cyclin D1, CDK-4 and -6 phosphorylate and contribute to the inactivation of a cell cycle inhibitor, retinoblastoma protein (pRB), thereby facilitating cell cycle progression. Cyclin D1 is
synthesized rapidly during G1 phase and is degraded during S phase, lending temporality to its activity. In vitro experiments using HEK 293 T cells demonstrated a 2–3 fold increase in CCND1 expression when cells were transfected with degradation-resistant mutant N-terminal β-catenin constructs. A 10- to 15-fold increase in induction of the CCND1 promoter was observed in 293T and human neuroblastoma 2A cell lines following transfection with the same construct.

(3) AXIN2, which is present in vertebrates and encodes the Axin1 homologue Axin2. Like Axin1, Axin2 is a negative regulator of canonical Wnt signaling; unlike Axin1, it is not expressed constitutively. Its expression pattern suggests that it may play a role in regulating the duration or intensity of Wnt-initiated signals. Axin2 has additionally been shown to mediate the epithelial–mesenchymal transition (EMT) by regulating intra-nuclear activity of GSK-3β, which is primarily responsible for modulating the stability of a zinc finger transcription factor, Snail1. Snail1 is a known transcriptional repressor and a promoter of EMT when in complex with the transcriptional modulators SMA3/4. While appropriate activation of EMT facilitates critical steps in embryological development and adult tissue healing by allowing epithelial cells to change phenotype in response to developmental stimuli or stress, its inappropriate activation is involved in initiation of epithelial cancer cell metastasis.

Wnt signaling is tightly regulated by naturally-occurring extracellular antagonists

Inhibitors of Wnt signaling can be classified into two families — those that bind Wnt ligands directly and those that regulate signaling by interacting with the Fz/LRP receptor-co-receptor complex.

Direct binders sequester Wnt to inhibit Wnt signaling

Secreto Fz-related proteins (sFRPs) exhibit homology to Fz CRD and may have a biphasic effect on Wnt signaling

The discovery in mammalian cells of a family of five secreted proteins homologous to the CRD of Fz and with the ability to bind Wnt added another level of complexity to Wnt signaling. Known as secreted Fz-related proteins (sFRPs), they contain an N-terminal signal sequence, a Fz-like CRD, and a C-terminal domain of conserved hydrophilic residues. The sFRP-1 was shown to bind Wg, purportedly though the C-terminus. While the CRD may still play a role in optimizing binding capacity of sFRPs as it does for Fzs, the C-terminus has been demonstrated to be indispensable for sFRP to bind Wg. The C-terminal portion has also been demonstrated to confer heparin-binding properties, which may allow for extracellular co-localization of sFRPs with secreted Wnt ligands. Heparin was demonstrated to enhance sFRP-1-Wnt binding at optimal concentrations. There is evidence to suggest that there may be specificity to the binding interactions of sFRPs and Wnt ligands. FrzA, a sFRP highly expressed in mammalian vascular endothelium, has been shown to associate with Wnt1, but did not demonstrate a binding affinity for Wnt5a while another sFRP, Frzb-1, associated with neither Wnt1 nor Wnt5a.

These proteins have a putative negative regulatory effect on Wnt signaling. Upon binding Wnt1, FrzA was shown to inhibit Wnt-1-mediated increases in cytoplasmic β-catenin levels and transcription of a TCF/LEF reporter gene. However, in a Drosophila model, sFRP-1 demonstrated a biphasic effect on modulation of Wg signaling as measured by stabilization of Armadillo (Arm), the Drosophila homolog of β-catenin. While high concentrations of sFRP-1 (>10 μg/mL) blocked Wg activity, lower concentrations increased concentrations of Arm. These effects were not observed in the absence of Wg or in cells lacking DFz2 expression, suggesting that sFRP-1 modulates an activity of Wg dependent on its interaction with DFz2. Thus, although their role is thought to be as a competitive inhibitor of Wnt–Fz binding, the effects of sFRPs on Wnt signaling are varied and may depend on interactions with additional binding partners. This remains to be evaluated further.

Wnt-interacting factor (WIF) binds Wnt through an N-terminal WIF domain

The Wnt-interacting factor WIF-1 was first discovered in human retinal cells by Macke and colleagues and first described in the literature by Hsieh et al as a secreted protein overexpressed during Xenopus and zebrafish development to control somitogenesis by modulating spatial and temporal activity of Wnts.

The structure of WIF-1 includes five epidermal growth factor (EGF)-like repeats, a motif commonly found in extracellular matrix (ECM) proteins, suggesting that WIF-1 is a secreted component of the ECM. The EGF-like repeats are flanked by an approximately 150-residue N-terminal domain known as the WIF domain, and an approximately 45-residue C-terminal hydrophilic domain. It is thought to inhibit Wnt signaling by directly binding Wnt ligands at the WIF domain. Hsieh et al demonstrated co-precipitation of Wg and the fusion protein XWnt8-Myc (Wnt8 tagged with a C-terminal Myc epitope) by both WIF-1-IgG and WD-IgG, an IgG fusion containing only the WIF domain. XWnt8-Myc exhibits a similar binding affinity for human WIF-1 (hWIF-1) and the CRD of DFz2. A 40-fold excess of murine sFRP-3 had no effect on co-precipitation of XWnt8-Myc and WIF-1-IgG. Taken together, these results suggest that at least two Wnt proteins, Wg and XWnt8-Myc, non-covalently bind with high affinity to hWIF-1. In the case of XWnt8-Myc, these experiments suggest that hWIF-1 and DFz2-CRD bind the fusion protein at sites that either overlap or show negative cooperativity.

Cerberus is an inhibitor of multiple signaling pathways, including Wnt signaling

Cerberus is another secreted Wnt inhibitor discovered in Xenopus as a transiently expressed protein with a role in dorsal axis patterning. It is structurally similar to a previously-discovered family of murine cystine-knot proteins, the can family, which act as antagonists of transforming growth factor β (TGF-β) and are known to play a role in embryonic patterning and development.
addition to its role in regulating Wnt signaling, Cerberus inhibits bone morphogenetic protein (BMP) and Nodal signaling. Binding of *Xenopus* Cerberus to *Xenopus* nodal-related-1 (Xnr1) was not affected by excess BMP in *in vitro* experiments by Piccolo et al, suggesting that Cerberus binds each ligand at an independent site.\(^{182}\) In the same study, proteolytically-processed isoforms of *Xenopus* Cerberus (XcER) retained the ability to bind Xnr1 but were unable to bind Wnt-8 and BMP-4, suggesting that proteolysis of XcER may be used to regulate Wnt antagonist activity of Cerberus.\(^{182}\)

**Direct binders of the Fz/co-receptor complex can inhibit or enhance canonical Wnt signaling**

**Dickkopf proteins bind to LRP-5/6 co-receptors to inhibit Wnt signaling**

The *dickkopf* (Dkk) family of proteins is comprised of 4 members (Dkk1-4) and was originally discovered to inhibit Wnt-induced secondary-axis induction in *Xenopus* embryos.\(^{183}\) Genetic evidence from both mammalian and *Xenopus* cells demonstrates that Dkk-1 works upstream of the destruction complex.\(^{183,184}\) Functional inhibition of Wnt signaling by Dkk-1 is independent of Fz, but decreases with increasing expression of LRP-6.\(^{183,184}\) Cross-linking experiments have demonstrated formation of a complex between Dkk-1 and LRP-6 with 1:1 stoichiometry. Taken together, these results suggest that Dkk-1 functions as a specific inhibitor of canonical Wnt signaling by blocking the functional interaction between Fz and LRP-6 required for transduction of canonical Wnt signaling.\(^{183}\) Further experimentation in *Xenopus* by Mao et al showed that this inhibition occurs by Dkk-1 or -2 binding to a domain on LRP-6 separate from those involved in the Wnt/Fz interaction.\(^{123}\)

An expression screen for cDNAs mediating binding of Dkk-1-alkaline phosphatase fusion protein identified two transmembrane proteins, later named Kremen-1 and Kremen-2. Cross-linking and co-immunoprecipitation experiments identified both as high-affinity receptors for Dkk-1 and -2, while bioluminescence resonance energy transfer (BRET) experiments demonstrated formation of a ternary complex between Dkk-1, Kremen, and LRP-6 in 293T cells. Fluorescence data of cells transfected with *krm2* revealed localization of LRP-6 from the plasma membrane to endocytic vesicles upon addition of Dkk1, indicating co-localization of the ternary complex. These experiments suggest a mechanism for Dkk- and Kremen-mediated antagonism of canonical Wnt signaling by blocking the functional interaction between Fz and LRP-6 required for transduction of canonical Wnt signaling.\(^{183}\) Further experimentation in *Xenopus* by Mao et al showed that this inhibition occurs by Dkk-1 or -2 binding to a domain on LRP-6 separate from those involved in the Wnt/Fz interaction.\(^{123}\)

Notably, DKK-1 can be induced by activation of canonical signaling via Wnt1 or ectopic expression of active β-catenin, TCF4, or LRP-6 mutants. The non-canonical Wnt ligand Wnt5A does not appear to activate DKK-1. Analysis by Northern and Western blot showed activation of the canonical pathway and increased DKK-1 RNA following co-culture of 293T cells transfected with the DKK-1 promoter and Rat2 cells stably expressing murine Wnt1 or Wnt5A. Sequence analysis of the human DKK-1 promoter reveals nine putative TCF-binding sites. Collectively, these results suggest that a negative feedback loop mediated by DKK-1 is activated by and regulates canonical Wnt signaling.\(^{186}\)

In addition, Dkk-2 has been demonstrated to function alternately as a weak agonist or as an antagonist of Wnt signaling.\(^{149,187}\) These results led to the postulate that an unknown co-factor was responsible for converting Dkk-2 from an agonist into an antagonist of Wnt signaling; this co-factor has since been demonstrated by Mao and Niehrs to be Kremen-1/2.\(^{185,188(p2)\}

**Sclerostin binds to LRP-5/6 to inhibit canonical Wnt signaling**

Sclerostin is an osteocyte-secreted antagonist of canonical Wnt signaling encoded by the gene SOST, initially identified as the gene whose loss leads to sclerosteosis, an autosomal recessive condition marked by generalized hyperostosis and sclerosis.\(^{189,190}\) SOST deficiency has also been identified in van Buchem disease, a similar sclerosing disorder also exhibiting autosomal recessive inheritance.\(^{191}\) Sclerostin inhibits BMP-mediated bone formation, but unlike classical BMP antagonists which bind BMP receptors, it inhibits Wnt signaling.\(^{190,192}\) Li et al initially reported that sclerostin-mediating antagonism of canonical Wnt signaling in human and murine cell lines could be reversed by over-expression of *LRP5*. They subsequently demonstrated that sclerostin inhibits canonical signaling by binding LRP-5 and LRP-6 at the PE domain (also referred to as the YWTD-EGF domain).\(^{193}\) Although this domain is known to also bind canonical Wnt ligands, the presence of Wnt3a did not interfere with sclerostin binding to LRP-5.\(^{193}\)

While structural characterization of sclerostin remains limited, sequencing data for the 190-residue glycoprotein predicts that it contains a cysteine-knot motif and is a member of the DAN/Cerberus protein family.\(^{194}\) Docking-based protocols used by Veverka et al identified a heparin-binding site, suggesting that sclerostin may be localized to the extracellular surface following secretion.\(^{195}\) In the same study, N- and C-terminal arms were found to be unstructured, highly flexible, and unaffected by the binding of heparin, suggesting that they may participate in stabilizing interactions with target proteins such as LRP-5/6.\(^{195}\)

**R-spondins act as facilitators of Wnt signaling**

The roof plate-specific spondins (R-spondins, RSpos) proteins are a family of four Cys-rich secreted proteins (Rspo1-4) which activate and regulate canonical Wnt signaling with varying potency.\(^{57,196}\) RSpos share 40–60% sequence homology and similar organization of a set of conserved domains; N-terminal Cys-rich furin-like domains are followed by a common thrombospondin (TSP-1) motif.\(^{197,198}\) The variation in potency of Wnt signaling activation observed across the RSpos family has been attributed to variations in furin-like domains.\(^{198}\) As with other components of Wnt signaling, RSpos also exhibit direct orthology across species.\(^{198}\)

A link between RSpos and canonical signaling was first implied by the spatial overlap observed between expression of RSpos and Wnt in murine embryogenesis.\(^{199,200}\) Since then, it has been demonstrated that RSpos compete with Dkk-1 for binding to LRP-6, thereby reducing co-receptor internalization.\(^{196,201}\) Although both antagonism of Dkk-1
and activation of Wnt signaling were subsequently mapped to furin domains, further experimentation is required to causally link Dkk-1 antagonism to activation of Wnt signaling.196

Norrin exhibits important but uncertain effects on Wnt signaling

Norrin, alternatively known as Norrie Disease Protein 2, is an atypical Wnt ligand encoded by the Norrie disease protein (NDP) gene. NDP was originally discovered by gene mapping and positional cloning as the gene underlying Norrie disease and familial exudative vitreoretinopathy (FEVR), two disorders of congenital retinal hypovascularization.202,203

Determination of Norrin’s function proved challenging, as it exhibits limited homology with proteins of known function and murine knockout models had a limited ability to phenocopy the human condition.204 However, the discovery that mutations underlying the autosomal dominant variant of FEVR included those leading to loss of function of the Wnt receptor Fz4 led to the subsequent identification of Norrin as a ligand of Fz4 capable of activating canonical Wnt signaling by binding with high affinity to Fz4-CRD.205,206 Demonstration by Nathans and colleagues that Norrin does not bind with CRDs of other Fzs, established Fz4 as a specific receptor for Norrin.207

Although LRP-5/6 is necessary for activation of canonical signaling by Norrin, earlier data from cell-binding assays showed no direct interaction between Norrin and LRP-5/6.206 Since then, however, Ke et al have demonstrated binding between Norrin and LRP-5/6 by AlphaScreen assay as well as ternary complex formation between Norrin and LRP-6.207 TSPAN12, a member of the tetraspanin family of transmembrane proteins, is also a member of the Norrin-Fz4 receptor complex and is required for Norrin-mediated Fz4 signaling. Loss of TSPAN12 was not shown to affect Wnt-mediated Fz4 signaling.208(p712)

Although Norrin is a Cys-rich protein, it exhibits limited structural similarity to Wnt ligands and is instead part of the Cys knot superfamily, exhibiting ~17% sequence homology with the growth factor TGF-β. It forms a unique dimer interface that is required for it to signal through Fz4.207

The discovery of the Norrin-Fz4 interaction places a dent in the paradigm that Fzs are dedicated receptors for Wnt ligands and creates the possibility of studying activation-related changes in Fz structure — a task made difficult in the past by the challenge of isolating active Wnt ligands.204

Wnt signaling regulates stemness and progenitor cell renewal in diverse tissue types

The Wnt signaling cascade has been identified as a regulator of self-renewal and proliferation among a variety of stem and progenitor cell populations.209 Supporting evidence demonstrating the critical role that Wnt plays in maintaining stem cell populations comes in part from studying the renewal of a range of tissue types, including intestinal epithelium, skeletal tissue, hematopoietic cells, and the hair follicle. Wnt signaling has also been shown to be capable of maintaining the transcriptional profile of pluripotent stem cells and has been found to be active in a more recently proposed class of progenitor cells implicated in tumorigenesis — cancer stem cells (CSCs).

Embryonic/pluripotent stem cells

In contrast to tissue- or organ-specific multipotent stem cell populations, embryonic stem cells derived from the inner cell mass (ICM) of the blastocyst are pluripotent, retaining the ability to differentiate into any cell type (with the exception of placental tissue).210 Maintenance of pluripotency is dependent on a combination of epigenetic, transcriptional, and post-transcriptional mechanisms, including evolutionarily conserved pathways such as canonical Wnt signaling. Activation of Wnt signaling was shown by Sato et al to be sufficient for maintaining pluripotency in both murine and human embryonic stem cells (MESCs, HESCs). Expression of pluripotency-specific transcription factors Oct-3/4, Rex-1, and Nanog were maintained when both types of ESCs were treated with 6-bromoinodiribin-3'-oxide (BIO), a pharmacologic specific inhibitor of GSK-3. The effects were reversible; removal of BIO from culture medium led to physiologic differentiation in both ESCs.211 Miyabayashi et al reported similar results, demonstrating that the small molecule inhibitor IQ-1 prevents spontaneous differentiation of MESCs by activating Wnt signaling. However, their work illustrates that Wnt-based maintenance of pluripotency is dependent on activation of β-catenin/CBP-mediated transcription at the expense of β-catenin/p300-mediated transcription. IQ-1 was shown to specifically promote this transcription program by targeting a subunit of PP2A and thereby preventing, by an unknown mechanism, phosphorylation of p300 at Ser-89, a modification known to enhance its binding interaction with β-catenin.212

In addition to maintaining pluripotency, Wnt signaling has also been shown to be capable of reprogramming pluripotency in somatic cells. Although these cells, termed induced pluripotent stem (iP) cells, have previously been generated by introduction of pluripotency-promoting transcription factors Oct4, Sox2, Klf4, and c-Myc via retroviral transduction, the increased risk of tumorigenesis from infection with c-MYC retrovirus is a limitation to use of this technique in regenerative medicine.213–215 Retroviral transduction of the remaining three has not been shown to produce pluripotency with similar efficiency.216 Work by Marson et al circumvents this concern by demonstrating that soluble Wnt3a is capable of producing pluripotency in the absence of c-Myc retrovirus. Murine fibroblasts infected with doxycycline-inducible Oct4, Sox2, and Klf4 lentiviruses and grown in Wnt3a-conditioned medium (Wnt3a-CM) grew into colonies resembling the morphology of ESCs and IPS cells. When combined with results of immunocytochemistry and functional assays, Wnt3a-CM IPS cells were not discernable from ESCs.217

Intestinal stem cells

The basic unit of the intestinal epithelium is the villus—crypt complex, consisting of a villus, or finger-like
projection of tissue from the intestinal wall, surrounded by basal crypts, or depressions. Intestinal villi are lined with a specialized absorptive epithelium that is distinctive, even among epithelial tissues, for a remarkable turnover rate. A population of stem cells residing at the base of surrounding crypts generates progenitor cells to constant replace epithelial cells as they apoptose at the villus tip. Progenitor cells arrest mitosis and differentiate into intestinal epithelial cells upon reaching the crypt—villus junction.209

Current evidence suggests canonical Wnt signaling as the signal controlling differentiation of crypt stem cells into absorptive epithelial cells. Nuclear localization of b-catenin, the hallmark of canonical Wnt signaling, is observed throughout intestinal crypt cells. b-Catenin and TCF have previously been shown to inversely control expression of the cytoskeleton-modifying receptors EphB2/EphB3 and their ligand, ephrin-B1, thereby coupling cell proliferation and differentiation to movement along the crypt axis.210 Mice with homozygous loss of TCF4 have been observed to be born with normal villous epithelia, but with loss of crypt progenitor cells.211 Inhibition of Wnt signaling by transgenic expression of Dkk-1 in adult mice leads to a similar phenotype with loss of crypts. Inappropiate expression of the b-catenin/Tcf-4 complex among colorectal cancer cells lineages has been shown to maintain a progenitor cell phenotype. Disruption of b-catenin/Tcf-4 in these lineages leads to cell cycle arrest in G1 and differentiation.212

Hematopoietic stem cells

Among hematopoietic stem cells (HSCs), multiple pathways underlying key aspects of development — Notch, Hedgehog, and Wnt — are thought to play a role in self-renewal and maintenance of stem cell and progenitor cell populations.213–215 Wnt signaling in particular is thought to function in an autocrine or paracrine manner to maintain HSC phenotypes. Studies of both murine and human cell lines demonstrate expression of various Wnt ligands by hematopoietic cell lineages and by stromal cells.216 The significance of this pathway in proliferation of HSCs was demonstrated by Reyna et al, who showed that silencing of Wnt signaling by ectopic expression of a Fz ligand-binding domain or Axin inhibits in vitro growth and in vivo regeneration of HSCs.217 Willert et al showed reconstitution of HSC lineages in lethally irradiated mice injected with Wnt3A-treated HSCs.218 By contrast, in vitro functional assays demonstrate that soluble Wnt proteins synergize with the CD117 ligand steel factor in murine hematopoietic progenitor cells to inhibit differentiation and promote replication.219

A mechanism for these results is suggested by the work of Willert et al, who demonstrated that activation of Wnt signaling in hematopoietic stem and progenitor cells increases in vitro expression HoxB4 and Notch1, genes known to play a role in HSC self-renewal.220 While the canonical Wnt signaling pathway is thought to be responsible for maintenance of intestinal crypt cells, among HSCs, both canonical and non-canonical Wnt ligands have been found to control HSC renewal. Further study is required to determine which pathway predominates.209

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a multi-potent subset of non-hematopoietic cells in the stroma of bone marrow and adipose tissue capable of differentiating into a multitude of cell lineages, including osteoblasts, chondrocytes, adipocytes, tenocytes, and hematopoiesis-supporting stromal cells.220–222 Canonical Wnt signaling has been shown to play a critical role in regulating cell fate decisions of MSCs; activation of canonical Wnt signaling promotes osteogenic differentiation of MSCs by upregulating the expression of Cbfa1/Runx2, a transcription factor known to activate the program of osteoblastogenesis, and of alkaline phosphatase, a marker of osteoblastic differentiation.223,224 When combined with osteogenic bone morphogenetic proteins (BMPs), canonical Wnt/b-catenin signaling was shown to induce osteoelastic differentiation of MSCs.222 Conversely, the absence of canonical Wnt signaling has been shown to potentiate an adipogenic lineage. Activation of b-catenin via ectopic expression of Wnt1 was shown to directly suppress expression of the adipogenic transcription factor peroxisome proliferator-activated receptor γ (PPARγ) and prevent adipogenic differentiation of 3T3-L1 cells.225 Blocking Wnt/b-catenin signaling has also been shown to enhance MSC-based granulation tissue formation and peri-infarct myocardial repair.226

Musculoskeletal tissue

Skeletal tissue homeostasis relies on a balance between osteoblast-mediated bone deposition and osteoclast-mediated resorption. Changes in bone density can be explained by an imbalance of activity between the two cell types. The association of increased bone density with gain-of-function mutations in LRPS was an initial clue that Wnt signaling may play a regulatory role in bone homeostasis.227–229 Babij et al reported that transgenic mice expressing human LRPS G171V, a gain-of-function mutation associated with high bone mass in two independent human kindreds, exhibited dramatic increases in volumetric bone mineral density and osteoblast activity.230 In addition, loss of function mutations in SOST are known to lead to sclerosteosis, a disease characterized by the development of high bone mass.193,239

Glass et al reported a mechanism for the direct relationship of canonical Wnt signaling with bone density. They initially demonstrated that stabilization of b-catenin in differentiated osteoblasts leads to pathologically increase bone mass, or osteopetrosis, while loss of b-catenin leads to osteopenia. Surprisingly, histological analysis revealed that the mutations in b-catenin were influencing bone resorption rather than bone deposition. Subsequent experiments revealed that b-catenin and TCF proteins modulate expression of Osteoprotegerin, which encodes the osteoclastogenesis-inhibiting decoy receptor osteoprotegerin (OPG), was specifically identified as a target gene of the b-catenin/TCF complex.240

While the above results suggest that canonical Wnt signaling plays a pivotal role in bone homeostasis by
regulating phenotypes of skeletal precursors, canonical Wnt signaling has been shown to distinctly regulate MSCs in a biphasic manner depending on signal intensity. The proliferation and self-renewal of MSCs were promoted only under low levels of Wnt/β-catenin, whereas osteogenic differentiation was promoted under high levels of Wnt signaling.241

Hair follicles

Hair follicles are an additional site characterized by self-renewal and continuous growth containing a population of self-renewing multipotent stem cells.242 Known as ‘bulge stem cells’ for the bulge they form in a contiguous portion of the outer root sheath, these cells are located at the inferior-most permanent portion of the hair follicle.243 Following activation, bulge stem cells proliferate rapidly and form a hair germ which extends downward through the outer root sheath to surround the dermal papilla, a collection of specialized mesenchymal cells at the base of the follicle. A matrix of proliferative cells, known as transit amplifying cells, forms at the base and terminally differentiates to produce the inner root sheath and the hair shaft itself.244,245

Canonical Wnt signaling has been shown to be critical for establishment and maintenance of the hair follicle. A Lef1−/− murine model was reported to have, among other defects, a deficiency of hair follicles.246 Transgenic over-expression of Lef-1 and stabilized, ‘oncogenic’ β-catenin have been shown to induce de novo hair follicle growth, a process normally found only in embryonic skin, in adult mice.247,248 Lowry et al performed functional analyses and reported that β-catenin-mediated activation of Lef1/Tcf complexes leads to upregulation of target genes that include several that promote conversion and proliferation of transit amplifying cells into activated cells of newly-developing hair follicles.245 Their work is supported by the earlier findings of Huelsken et al, who reported that β-catenin is essential for differentiation of bulge stem cells; in its absence, stem cells adopt an epidermal phenotype rather than differentiating into follicular keratinocytes.249

Of note is that several hair keratin genes have been identified among the target genes of β-catenin and Lef1, somewhat paradoxically suggesting that in addition to controlled the expansion of precursor cells, canonical Wnt signaling plays a role in its terminal differentiation.246,247

Cancer stem cells

One of the defining characteristics of stem cells, self-renewal, can be used to draw a parallel with cancer cells. There is increasing evidence to suggest that among the array of cells occupying a tumor there exists a subset of self-renewing cells driving tumorigenesis, referred to as cancer stem cells (CSCs). These cells may derive from stem cells or rely on a similar set of pathways to drive self-renewal.250

Given the critical role that Wnt signaling has been shown to play in stem cell proliferation (reviewed above) and in oncogenesis (reviewed below), it has been proposed as a pathway critical to the development and renewal of cancer stem cells. In support of this hypothesis is the fact that many surface markers used to identify and isolate putative CSC populations from a broad range of primary tumor tissues, including LGR5/GPR49, CD44, CD24, and EpCAM, are themselves Wnt target genes.251–255 LGR5-expressing breast cancers exhibit CSC-like properties, including the formation of self-renewing spheres and high tumorigenicity, due to activation of Wnt/β-catenin signaling and are associated clinically with increased recurrence and poorer outcomes.256 Activation of canonical signaling by Wnt3a was shown to promote the self-renewal of cancer stem/progenitor cells in acute lymphocytic leukemia and prostate cancer.257,258 In the human colon cancer cell line LT97, nuclear expression of β-catenin was observed with CD44+ cells, but not in CD44− cells.259 While this evidence is certainly compelling, further studies are needed to characterize what difference, if any, there are between Wnt-mediated regulation of normal stem/progenitor cells and of CSCs.

Abnormalities in Wnt signaling play a role in select disorders of human development

As is the case for many evolutionarily conserved pathways underlying critical processes in development, growth, and metabolism, abnormal Wnt signaling has been implicated in development and progression of human disease. Broadly speaking, the two most common forms of disease associated with Wnt signaling include congenital disorders and cancer, caused by aberrant signaling during early stages of development or by inappropriate activation of signaling in adults, respectively.260–264

Loss of Wnt signaling can cause in utero developmental malformation

Murine and Drosophila Wnt knock-out models exhibit a diverse range of phenotypes due to loss of critical developmental steps. It follows that loss of Wnt function in humans would have similarly dire consequences. Niemann et al reported loss of WNT3 function by a homozygous mutation in a human infant who was the first to report the association of a WNT gene mutation with a Mendelian disorder.265 Their discovery was the first to report the association of a WNT gene mutation with a Mendelian disorder.

Wnt signaling defects can cause neurodevelopmental abnormalities

As part of its role in controlling aspects of embryological development such as body patterning, Wnt signaling is increasingly implicated in key aspects of neural development.266 Although reviewed more thoroughly elsewhere by Freese et al, we present here a brief review of neurodevelopmental abnormalities in humans which have been linked to abnormalities in Wnt signaling.266
Neural tube defects (NTD) have been associated with multiple components of Wnt signaling.\textsuperscript{266} Dvl2 was shown to be necessary for neural tube closure in murine models by Hamblet et al. Mice with homozygous null mutations in Dvl2 developed thoracic spina bifida, while those with loss of both Dvl1 and Dvl2 presented with a more severe disease phenotype.\textsuperscript{267} Murine models have also shown that mutations in axin and LRP6 can lead to NTD, with the latter also being folate-responsive.\textsuperscript{90,268}

Finally, accruing evidence suggests a role for Wnts in the development of Williams Syndrome, a neurodevelopmental disorder characterized by a lowered seizure threshold, impaired spatial cognition, enhanced language abilities, effusive personality, and preserved social function.\textsuperscript{269} Williams Syndrome is caused by a heterozygous microdeletion of approximately 20 genes on chromosome 7, including the gene for Fz9.\textsuperscript{270} It is likely that some cognitive defects in Williams Syndrome are due to loss of Fz9. Zhao et al reported that mice with null mutations of Fzd9 exhibit neurendedevational defects, including decreased visuospatial memory and a lowered seizure threshold, while those with loss of one allele have an intermediate presentation similar to Williams Syndrome.\textsuperscript{271}

Abnormalities in canonical Wnt signaling are frequently implicated in tumorigenesis

Abnormalities in canonical signaling leading to tumorigenesis are the most commonly-reported link between Wnt signaling and disease. The discussion will be grouped into Wnt ligand-independent and Wnt ligand-dependent mechanisms for abnormal activation of the canonical pathway leading to tumorigenesis.

Wnt ligand-independent pathways for tumorigenesis are most commonly found in gastrointestinal carcinomas

Increased expression of Fz receptors is found in select gastrointestinal carcinomas

Increased expression of Fz has been demonstrated in gastrointestinal carcinomas. Uregulation of Fz understandably leads to increased activation of Wnt signaling but has been demonstrated in only select tumor types, primarily gastrointestinal carcinomas. Human FzE3 was identified as a gene expressed specifically in human esophageal carcinoma cell lines, especially those with poorly-differentiated histology, and has since been identified in cases of gastric carcinoma as well.\textsuperscript{272,273} Expression of FzE3 in cases of gastric carcinoma was associated with accumulation of $\beta$-catenin or with upregulation of CCND1 expression.\textsuperscript{273} Human Fzd7 shares 98.8% sequence identity with FzE3 and was found by Kirikoshi et al to be overexpressed in one gastric cancer cell line (MKN7) and one of six gastric cancer samples tested.\textsuperscript{274} Finally, overexpression of Fzd10 has been demonstrated in 2 cases of primary colon cancer by Terasaki et al.\textsuperscript{275} In the same study, they demonstrated that Fz10 works synergistically with select classes of Wnt ligands in upregulating canonical signaling.\textsuperscript{275}

Loss of APC function by nonsense mutation or promoter hypermethylation is commonly implicated in tumorigenesis, especially of colorectal adenocarcinomas

Although the gene for Wnt1 was first discovered as the target of an oncogenic retrovirus in a murine breast cancer model, the connection between dysregulation of canonical Wnt signaling and development of cancer was first established ~10 years later. In their landmark work which led to proposal of the adenoma-carcinoma sequence for stepwise accumulation of genetic alterations facilitating colorectal tumorigenesis, Vogelstein et al proposed biallelic loss of APC as the first step in the development of inherited (e.g., through familial adenomatous polyposis) and up to 85% of sporadic cases of colorectal cancers.\textsuperscript{11,12} Loss of APC function leads to unregulated proliferation of intestinal epithelial cells and formation of an adenoma, a benign lesion.\textsuperscript{276} Stepwise accumulation of mutations in genes including KRAS and TP53 is required for malignant transformation.\textsuperscript{277}

Point mutations in APC are seldom sufficient for causing loss of APC function. Development of a cancerous phenotype requires loss of entire domains and the functionality they confer upon the full-length protein. While most tumorigenic APC nonsense mutations are located in the 5’ half of the sequence, the spread of mutation loci varies between hereditary and sporadic colorectal cancers. Nagase and Nakamura demonstrated that germ-line nonsense mutations in APC are distributed throughout the 5’ region of the gene, while approximately two-thirds of sporadic mutations map to a narrow region termed the Mutation Cluster Region (MCR), spanning 8% of the coding sequence (codons 1286-1513).\textsuperscript{278,279} The position at which APC nonsense mutations occur and, hence, the size of the truncated protein, can be correlated to disease severity. Yang et al have demonstrated that truncations of APC which retain the three 15-amino acid repeats and one or two of the 20-amino acid repeats are associated with a more aggressive tumor phenotype; more attenuated tumor phenotypes develop with loss of all $\beta$-catenin regulating domains.\textsuperscript{106} Nonetheless, an attenuated FAP phenotype has been reported in association with a chain-terminating mutation in the 3’ end of APC.\textsuperscript{106,280}

To explain these and similar results, Propping and colleagues proposed a model linking the size of the APC mutant to disease severity in which mutations occurring 5’ to codon 157 or 3’ to codon 1597 are associated with an attenuated FAP phenotype.\textsuperscript{281} These mutations fail to preclude the formation of stable dimers with preserved binding interactions. Mutations occurring 5’ to codon 157 create a fragment too short for stable dimerization, thereby allowing a relatively large amount of stable, wild-type homodimer to be formed. When the mutation occurs 3’ to codon 1597, the fragment is capable of forming stable heterodimers with full-length protein. These heterodimers maintain correct conformation at the $\beta$-catenin binding site. In both cases leading to the attenuated phenotype, a so-called ‘second hit’, or mutation in the second copy of APC, is required for polyp formation. This follows with Knudson’s two-hit hypothesis requiring biallelic inactivation for tumor suppressor genes such as APC to lose their function.\textsuperscript{282,283} Mutations occurring between codons 168 and 1578 lead to a more severe disease phenotype, or a typical
case of FAP. These fragments exert a dominant negative mutant effect; they are able to form a stable heterodimer, but do not retain the ability to correctly associate with β-catenin. The loss of APC function occurs simultaneously and across all epithelial cells. These mutations do not require a second hit for expression of the disease phenotype.

The adenoma-carcinoma sequence is the pathway underlying development of traditional (non-serrated) adenomas (TAs), the precursor lesions for approximately 60% of colorectal adenomas. Another 35% arise from sessile serrated adenomas (SSAs), which develop via the ‘serrated pathway’, a separate set of stepwise alterations that are often characterized by microsatellite instability. However, abnormal Wnt signaling has more recently been implicated in their development as well. Yachida et al demonstrated nuclear accumulation of β-catenin in 35 of 54 (67%) SSAs and in all 27 (100%) SSAs with evidence of epithelial dysplasia. Although mutations in CTNNB1 capable of causing abnormal β-catenin accumulation were not identified, earlier work by Fu et al suggests a possible mechanism. In a subset of the SSAs they analyzed (5/12, 41.7%) the canonical Wnt pathway was moderately activated by aberrant hypermethylation of APC promoter 1A. Thus, Wnt activation apparently plays a role in development of both TAs and SSAs, albeit by different mechanisms.

Although APC-inactivating mutations are most commonly associated with colorectal tumors, they have been reported among other tumor types as well. Despite being the most common cancer worldwide, carcinogenesis of gastric cancer remains poorly understood. Given that some gastric cancers are thought to arise from intestinal metaplasia, Nakatsuru and Horii evaluated gastric cancers for the presence of nonsense mutations in APC. Upon examination of a region corresponding to 30% of the APC coding sequence, including the MCR, in 57 sporadic gastric cancers, they identified mutations in 41% of very well-differentiated adenocarcinomas, 11% of well- or moderately-differentiated adenocarcinomas, and 30% of signet-ring cell carcinomas. Mutations were not found in poorly-differentiated adenocarcinomas. Likewise, Ebert et al reported finding APC mutations at a higher rate among intestinal-type gastric cancers than in diffuse-type gastric cancers. The results of both studies suggest that mutation of APC may contribute to tumorigenesis of intestinal-type gastric cancers.

Among endometrial carcinomas, mutations of APC and β-catenin have primarily been studied among the most common type, endometrioid endometrial carcinoma (EEC). Although nuclear accumulation of β-catenin has been detected among EECs, it is in association with missense mutations in β-catenin (see below), not with truncating mutations of APC. Palacios and colleagues did, however, report methylation of the APC promoter 1A occurring more frequently among EECs relative to NEECs. Mutations in APC have been shown to occur on occasion in breast adenocarcinoma as well. Schlosshauer et al screened twenty-four human breast cancer cell lines and found a novel APC mutation, E1577stop, in one cell line (DU 4475). The mutation was associated with cytosolic accumulation of β-catenin. Jönsson et al found a 13% rate of β-catenin accumulation among 54 primary breast tumors screened for abnormalities in APC/β-catenin signaling. APC was altered in 6% of tumors, and no samples contained mutations in the N-terminal region of CTNNB1. While 9% of tumors demonstrated upregulation of c-MYC, it did not correlate with β-catenin accumulation. Taken together, these results suggest that dysfunction of APC likely plays a limited role in breast carcinogenesis.

Loss of Axin function is found in hepatocellular and colorectal cancers

Loss of Axin has been demonstrated in cell lines and patient samples from hepatocellular and colorectal cancers. Mutations in AXIN1 were demonstrated by Satoh et al in 3 of 4 cell lines and 5 of 87 primary hepatocellular carcinoma (HCC). Cells with AXIN1 mutations additionally demonstrated cytosolic accumulation of β-catenin on IHC and increased nuclear binding of β-catenin-associated TCF transcription factors to DNA. AXIN mutations appear to play a role in development of a subset of colorectal cancers, specifically those with defects in mismatch repair. Liu et al demonstrated frameshift mutations in AXIN2 and corresponding loss of Axin function in 11/45 colorectal cancer tumors with defective mismatch repair. All 11 mutations were found to disrupt the DIX domain, which is necessary for homo-oligomerization in the AXIN2 homologue Axin. An alternate mechanism by which AXIN mutations may preclude β-catenin destruction was demonstrated by Webster et al who identified two heterozygous AXIN1 sequence variants. One of these mutations, L396M, was found in multiple colorectal cell lines and was shown by gene mapping as well as biochemical and functional studies to interfere with the ability of Axin to bind GSK-3β.

Prostaglandin E2 is a potent mitogen in colon cancer cell lines with homozygous loss of APC. PGE2 has been shown to stimulate β-catenin-mediated transcription by disrupting the Axin-GSK-3β interaction. Upon binding to its GPCR, EP2, PEG2 activates a signaling route involving direct association of the G protein αs subunit with Axin and subsequent release of GSK-3β from the β-catenin destruction complex.

Mutations stabilizing β-catenin can substitute for upstream aberrations of Wnt signaling

Increased levels of free β-catenin can occur directly from stabilizing, or activating, mutations in CTNNB1. Exon 3, specifically the NH2-terminal regulatory domain, encodes a sequence of conserved Ser/Thr residues and has proven to be a mutation hotspot. Mutations in this location activate constitutive Wnt signaling by precluding GSK-3β-mediated phosphorylation of the Ser/Thr residues, thereby preventing formation of a β-TrCP binding site. These mutations have been identified frequently across tumor types. In the case of colon adenocarcinoma, although the overwhelming majority of cases develop following mutation of APC (reviewed above), Sparks et al report that approximately half (48%) of colon adenocarcinomas with wild-type APC have an activating mutation in CTNNB1 and that mutations in APC and CTNNB1 are mutually exclusive. The latter finding supports the two sets of mutations having equivalent effects on β-catenin stability, suggesting that CTNNB1 activating mutations may substitute for loss of APC function in colon carcinogenesis.
Among other gastrointestinal carcinomas, CTNNB1 activating mutations have been reported most frequently in association with hepatocellular carcinoma (HCC). Miyoshi et al found somatic CTNNB1 mutations in 14 samples from a screen of 75 primary HCC samples. Of the 14, eight were either deletions in exon 3 or missense mutations altering the regulatory Ser/Thr residues. The remaining tumors contained mutations at codons neighboring a conserved Ser residue. In their analysis of human HCC samples, de la Coste et al report a similar rate; eight of 31 samples (26%) contained point mutations or deletions in CTNNB1 leading to dysregulation of β-catenin. Of note is that a lower rate of has been seen in cases of aflatoxin-associated HCC, as reported by Devereux et al. Although 20 of the 62 HCC tumors from individuals exposed to aflatoxin B1 showed strong staining on IHC for β-catenin, CTNNB1 mutations were identified in only 5 (12.2%), suggesting that the observed β-catenin accumulation in aflatoxin-associated HCC may be due to mutation in another component of Wnt signaling.

Among ovarian carcinomas, activating mutations of CTNNB1 have been reported among the endometrioid subtype. In a study of 40 stage I and stage II ovarian borderline tumors and carcinomas, five out of six endometrioid lesions exhibited nuclear localization of β-catenin on IHC. Three of the endometrioid lesions, including one borderline tumor and two carcinomas, were heterozygous for missense mutations in CTNNB1 and were confirmed to have abnormal β-catenin expression. The results suggest that CTNNB1 mutations may play a role in ovarian tumorigenesis and may characteristically give rise to the endometrioid phenotype. Their results are supported by later work of Wright et al, who reported finding activating mutations of β-catenin among 10 of 63 (16%) of endometrioid ovarian tumors analyzed. All mutations were missense mutations in the GSK-3β consensus site and were confirmed by IHC to be associated with nuclear localization of β-catenin.

Abnormal localization of β-catenin has been reported in thyroid carcinomas on multiple occasions and even associated with the aggressive behavior of the anaplastic subtype. In a study of 145 thyroid tumor samples collected from 127 patients, reduced membrane localization of β-catenin was observed among all 115 thyroid carcinomas analyzed and found to be associated with progressive loss of tumor differentiation, a known indicator of poor prognosis. Mutational analysis revealed that mutations in CTNNB1 were found exclusively in poor-to-undifferentiated tumors; 25% of poorly-differentiated and 65.5% of undifferentiated carcinomas contained exon 3 mutations. In addition to identifying β-catenin dysregulation as a pathway in development or progression of thyroid carcinomas, these findings suggest that β-catenin expression patterns and mutational analysis may be a clinically-relevant tool for subtyping thyroid carcinomas and for predicting disease outcomes.

Mutations in exon 3 of CTNNB1 have also been found in the cribriform-morular variant of papillary thyroid carcinoma (CMV-PTC), a rare subtype of PTC. Although most commonly diagnosed as an extra-colonic manifestation of FAP, CMV-PTC can occur sporadically as well. Xu et al evaluated five CMV-PTC tumors, including both sporadic and FAP-associated cases. Nuclear accumulation of β-catenin on IHC and somatic mutations in exon 3 of CTNNB1 were observed in all five cases. Although germline APC mutation was observed in one of the two FAP-associated cases, loss of heterozygosity was not observed.

**Epigenetic silencing of Wnt signaling inhibitors**

Upregulation of Wnt signaling in tumorigenesis has also been shown to occur secondary to downregulation of Wnt signal inhibitors, most commonly by epigenetic modifications. These changes have also been found, in select cancers, to be prognostic indicators. Screening tests for these changes in said cancers may therefore be a clinically-relevant prognostic tool.

sFRPs are epigenetically silenced in some human cancers

Promoter hypermethylation of SFRP1 in human colorectal cancer was first reported by Suzuki et al following a screen of 10,000 candidate genes by cDNA microarray analysis. Their microarray strategy combined gene expression status with epigenetic regulation to identify genes with hypermethylated promoters in a colorectal cancer cell line, RKO. Three other genes belonging to the same family as SFRP1, SEZ6L, KIAA0786, and CXX1, also demonstrated promoter hypermethylation in RKO cells. Similar results were obtained in primary colorectal cancer tissues. Promoter hypermethylation of all four genes was subsequently evaluated in other colorectal cancer cell lines, as well as in cell lines of gastric, breast, lung, liver, and prostate cancers. Promoter hypermethylation of these genes was largely observed only in colorectal and gastric cancer cell lines, suggesting that comprehensive marker panels with these genes may have diagnostic utility in identifying colorectal and gastric cancers.

Suzuki et al also investigated methylation and expression of other SFRP family members, (SFRP2-5) and found that three are frequently hypermethylated in colorectal cancer cell lines. These three, SFRP2, 4, and 5, were also found to be methylated in primary colorectal tumors. Of the 124 patients samples, 30 (24.1%) demonstrated promoter methylation of four SFRP genes (1, 2, 4, and 5).

Nojima et al investigated silencing of the SFRP gene family among gastric cancer cell lines and primary tumor samples. They reported that promoter hypermethylation of SFRP1, 2, and 5 occurs frequently among gastric cancer cell lines and primary tumor samples. Promoter hypermethylation of SFRP1, 2, and 5 was observed in 16 (100%), 16 (100%), and 13 (81%) cell lines, as well as 42 (91%), 44 (96%), and 30 (65%) primary tumor samples. Ectopic expression of SFRP1, 2, and 5, suppressed TCF/LEF activity in three gastric cancer cell lines with constitutively active Wnt signaling. Changes in gene expression profiles by overexpression of SFRPs were evaluated using SFRP2 and the colon cancer cell line SNu638, which has high TCF/LEF activity. Four Wnt target genes, LEF1, MMP7, cyclin D1, and CD44 were found to be downregulated by ectopic SFRP2 expression. These results support SFRP silencing as a common event in gastric cancer and as a potential therapeutic target.

Although infection with human papilloma virus (HPV) is most significant risk factor associated with cervical cancer,
it is not a requirement for cervical carcinogenesis. Increased cytoplasmic and nuclear localization of β-catenin have been observed in samples of invasive cervical carcinomas, indicating that there may be a role for canonical Wnt signaling in disease progression. Chung et al report a possible mechanism for the observed dysregulation of canonical Wnt signaling; they identified promoter hypermethylation of SFRP1, 2, and 4 in cervical lesions, with increased frequency in tumors relative to in situ lesions. Their results support the potential use of these genes as a screening tool for cervical carcinogenesis in precursor lesions. Of note, promoter methylation of SFRP5 was significantly decreased in patients without lymph node metastases.

In the case of bladder cancer, SFRP promoter hypermethylation has been found to be a predictor of invasive disease and, thus, a prognostic indicator. In a population-based study of 355 individuals, Marsit et al found a linear relationship between the number of methylated SFRP genes and risk for invasive cancer. Promoter methylation of any SFRP gene was independently associated with overall poorer survival (hazard ratio 1.78). When SFRP methylation was present along with mutation of the tumor suppressor gene TP53, the risk for invasive disease was greater than 30-fold. These results suggest that promoter methylation of SFRP genes may be a powerful tool for predicting the development of an invasive phenotype in bladder cancer.

Down-regulation of WIF is found in some human cancers

Down-regulation of genes encoding WIF across multiple tumor types was first demonstrated by Wissmann et al in a study designed to detect novel Wnt pathway genes involved in tumorigenesis. Chip hybridization of microdissected matched pairs from 94 primary prostate carcinomas identified multiple Wnt pathway genes with differential expression. Of these, WIF1 and SFRP4 showed the most significant aberrant expression — expression of WIF1 was down-regulated in 64% of samples while expression of SFRP4 was up-regulated in 81%. IHC of primary tissues showed a strong reduction in WIF-1 protein expression among 23% of prostate carcinomas, 60% of breast adenocarcinomas, 75% of non-small cell lung (NSCLC) cancers, and 26% of bladder cancers. Although down-regulation of WIF1 was correlated to higher tumor staging among urinary bladder tumors, there was no significant correlation observed with breast, prostate, or non-small cell lung carcinomas. Later work by Mazieres et al demonstrated CpG island hypermethylation in the WIF-1 promoter of human lung cancer cell lines and in 15 of 18 (83%) resected NSCLC tumors. Treatment of cell lines with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine restored WIF-1 expression.

Epigenetic inactivation of WIF-1 expression has since been shown to be a common mechanism for WIF-1 down-regulation across multiple tumor types. Hypermethylation of the WIF1 promoter was correlated to loss of WIF-1 expression by Batra et al in four out of four malignant pleural mesothelioma cell lines, and seven of eight primary tissue samples. Demonstration of similar results in cell lines and primary tumor samples of osteosarcoma, breast adenocarcinoma, nasopharyngeal carcinoma, esophageal carcinoma, and urinary tract transitional-cell carcinoma suggests that WIF-1 promoter hypermethylation may be a common mechanism for Wnt-mediated tumorigenesis and a potential therapeutic target for multiple cancers. To this end, experimentation by Hoang and colleagues has demonstrated that overexpression of WIF-1 in the highly metastatic osteosarcoma cell line, 143B, led to statistically significant inhibition of primary tumor growth and decreased the frequency of pulmonary metastases in a murine xenograft model. This work lends promise to the therapeutic benefits of targeting Wnt signaling in a broad range of tumor types. However, it specifically is a step in the right direction towards development of novel therapies for osteosarcoma, a disease characterized by inter-tumor variability in its molecular signature and clinical outcomes which have remained largely stagnant despite optimization of conventional surgical and cytotoxic treatments.

Epigenetic silencing of DKKs is found in some human cancers

Downregulation of DKK-1, indicating loss of the Dkk-1-mediated negative feedback loop regulating canonical Wnt signaling, has been reported in colon adenocarcinomas. This downregulation was found to be a result of CpG island promoter hypermethylation in the DKK-1 promoter. Promoter hypermethylation was found in nine of 54 (17%) of primary colorectal tumors analyzed and, relative to promoters SFRP-1 and WIF-1, selectively hypermethylated in advanced neoplasms, defined as Dukes’ C and D tumors. In their analysis of colon cancer lines and primary tumor samples, Yu et al report a higher incidence of promoter hypermethylation for DKK-3. They observed hypermethylation in 3 of 9 cell lines (33.3%) and 67 of 128 primary tumors (52.3%). Although DKK-3 silencing was correlated to poor outcomes with gastric cancer (see below), no correlation was observed with colorectal cancer.

Nozaki et al report similar results among NSCLC. In their analysis of 57 surgically-resected tumors, 36 (63%) had reduced expression of Dkk-3. Downregulation was frequently observed in poorly-differentiated adenocarcinomas and squamous cell carcinoma. The authors propose that reduced expression of DKK-3 may explain rapid cell proliferation in NSCLCs.

In studies of gastric cancer, epigenetic silencing of DKK-3 has been found to be a common event and one of prognostic significance. Yu et al reported promoter hypermethylation of DKK-3 in 12 of 17 gastric cancer cell lines (70.6%) and in 117 of 173 (67.6%) primary gastric tumors. Multivariate analysis revealed that DKK-3 methylation was significantly associated with poor disease survival (relative risk, 2.534). Kaplan—Meier survival curves demonstrated that gastric cancer patients with DKK-3 methylation had significantly shorter survival (median of 0.76 years) than their counterparts without DKK-3 methylation (median of 2.68 years).

Wnt ligand-dependent pathways for tumorigenesis remain uncommon and are not fully understood

While ligand-independent pathways, primarily secondary to mutations in APC or CTNNB1, remain the most commonly described link between canonical Wnt signaling and tumorigenesis, there are reports of tumors with overexpression of Wnt ligands.
Redundant expression of canonical Wnt ligands has been reported in human breast cancer cell lines. Since the discovery of Wnt1/Int1 as a virally-induced oncogene for MMTV, the role of Wnt in breast carcinogenesis has been a topic of study. Abnormal expression of β-catenin and cyclin D1 has been found frequently among breast cancer cell lines and patient samples. Although increased expression of cyclin D1 has previously been reported in approximately half of all breast cancers, gene amplification accounts for less than 20% of cases. In an analysis of 123 patient samples, Lin et al found expression of β-catenin and cyclin D1 to be associated with significantly poorer patient prognosis and decreased survival. However, despite the wealth of evidence suggesting a role for abnormal Wnt signaling in breast carcinogenesis, genetic abnormalities in common mutational target genes such as APC and AXIN have been identified only on rare occasion in malignant breast tumors.

In the face of this evidence, Benhaj et al evaluated expression of several Wnt signaling components, including the 19 Wnt ligands and 10 known Fz, among six breast cancer cell lines. They reported an increased, redundant expression of six canonical Wnt ligands (Wnt3A, Wnt4, Wnt6, Wnt8B, Wnt9A, Wnt10B) and decreased expression of WNT5A and WNT5B, which encode non-canonical Wnts, among breast cancer cell lines. They did not observe differential expression of any Fzs between malignant and non-malignant cell lines. Overexpression of canonical Wnt ligands in the malignant cell lines was correlated with nuclear accumulation of β-catenin and induction of CCND1 expression. These results suggest that ligand-dependent induction of Wnt signaling may play a significant role in the development of breast cancer and presents a new class of potential therapeutic targets in the treatment of this disease. Epigenetic silencing of Wnt antagonists in breast cancer has since been reported (reviewed above), and likely works in concert with direct activation of canonical Wnt signaling.

**Dysregulation of Wnt signaling is implicated in cancer drug resistance**

In addition its clear role in carcinogenesis, a growing body of evidence suggests that unregulated activation of β-catenin may play a significant role in the development of resistance to traditional cytotoxic chemotherapy. Perhaps more importantly, several of these studies demonstrate reversal of resistance following inhibition or silencing of canonical Wnt signaling.

**Wnt/β-catenin-mediated chemoresistance is characterized by increased Wnt pathway activity in tumors and is reversible with inhibition of Wnt signaling**

Chemoresistance of HCC to cisplatin, used extensively in the treatment of unresectable cases, presents challenges to treatment of HCC and remains poorly understood. Abnormal activation of the canonical pathway, known to play a role in the development of HCC (see prior sections), has been proposed as a likely mechanism for this resistance. Studies supporting β-catenin-mediated resistance to cisplatin in HCC include work on adult hepatic progenitor (oval) cells. Increased proliferation of oval cells, known to give rise to HCC, was initially observed following forced expression of a constitutively-active β-catenin mutant. Activation of Wnt signaling led to enrichment of HCC OV6+ cells, a progenitor-like subpopulation with known chemoresistance, in HCC cell lines and tumor samples. Resistance of OV6 cells to cisplatin was reversible with lentiviral microRNA (miRNA)-mediated knockdown of CTNNB1. In addition to identifying a specific subpopulation of cells responsible for the chemoresistance of HCC, this work highlights the potential of canonical Wnt signaling as a druggable target for reversing this resistance.

These results have since been added to by the work of Wei et al, who modified Huh7 cells to exhibit cisplatin-resistance and found that they demonstrated nuclear accumulation of β-catenin, a sign of abnormal canonical signaling. Sorafenib, a kinase inhibitor drug known to improve median survival in unresectable or advanced HCC, was shown to sensitize these cisplatin-resistant Huh7 cells to cisplatin both in vitro and in vivo. These effects were abrogated by addition of the canonical ligand Wnt3a. Although not used as extensively as cisplatin, combination therapy with interferon α and the cytotoxic pyrimidine analog 5-fluorouracil (5-FU) is used to good effect in the treatment of HCC tumors with expression of Type I interferon receptor type 2 (IFNAR2). In IFNAR2+ patients with poor response to therapy, chemoresistance has been shown to be associated with increased canonical Wnt signaling by Noda et al, suggesting that this pathway may be implicated broadly in chemoresistance of HCC.

β-Catenin-mediated chemoresistance to the cytotoxic drug doxorubicin has been reported in neuroblastoma, a primarily pediatric cancer and the most common extra-cranial tumor occurring in childhood. Cells from the neuroblastoma cell line SK-N-SH expressing CD133, a known CSC marker, are known to be more resistant to cytotoxic therapies such as doxorubicin than their CD133− counterparts. Work published by Vangipuram et al in 2012 implicates canonical Wnt signaling in this resistance. Overall, Wnt pathway activity was higher in CD133+ cells than in CD133−, both with and without doxorubicin. In response to treatment with doxorubicin (100 ng/mL over 48 hours), overall Wnt activity was suppressed in CD133− SK-N-SH cells as shown by Wnt PCR Array. By comparison, Wnt activity was upregulated in doxorubicin-treated CD133+ cells. As expected, a higher percentage of CD133+ cells survived doxorubicin treatment. Combined treatment with doxorubicin and one of the Wnt pathway inhibitors XAV-939 and ICG-001 led to statistically similar rates of cell death in both groups. When treated with only ICG-001, CD133− had a significantly higher percentage of cell survival.

Chemoresistance is the perhaps the greatest challenge in treatment of OS, a disease whose standard therapy includes multi-drug regimens which, despite optimization, have failed to produce improvements in overall survival for the past three decades. In their study of the human OS cell line SaOS2, Ma et al demonstrated upregulation of the Wnt signaling components Wnt3a, β-catenin,
and Lef1 in SaOS2 relative to human fetal osteoblasts. Sensitivity to the cytotoxic drug methotrexate, which in combination with adriamycin and doxorubicin is the most commonly-used multi-drug regimen in the treatment of OS, was enhanced by knockdown of β-catenin. Sensitivity increased further when both inhibitors of β-catenin and the Notch signaling pathway were used, suggesting that multiple pathways may be responsible for the extensive drug resistance seen in OS. 347

Increased Wnt pathway activity underlying cancer drug resistance occurs due to dysregulation at multiple levels

As with tumorigenesis, current evidence suggests multiple mechanisms by which abnormal Wnt signaling contributes to chemoresistance in cancer.

Increased signaling due to upregulation of Wnt ligands, specifically Wnt5a, has been shown to contribute to poor clinical outcomes and increased drug resistance in ovarian carcinoma. Peng et al reported that expression of Wnt5a was higher in malignant ovarian tumors than in benign or normal tissue and that increasing expression was correlated with a decrease in both overall and disease-free survival. Overexpression of Wnt5a in ovarian carcinoma cell lines decreased chemosensitivity, while miRNA-mediated silencing of WNT5A led to a significant increase in chemosensitivity. 348

Increased expression of Fz as a cause of chemoresistance has been reported in studies of neuroblastoma. Comparison of gene expression between two parental neuroblastoma cell lines and their doxorubicin-resistant derivative cell lines revealed FZD1 as the most differentially expressed gene. Silencing of FZD1 restored the doxorubicin-sensitive phenotype and, interestingly, parallel down-regulation of MDR1, a β-catenin target gene encoding a multi-drug transporter and a known mediator of chemoresistance. Upregulation of FZD1 and/or MDR1 were found in post-treatment tumor samples as well. Of note, β-catenin target genes encoding cell cycle regulators such as Cyclin-D1 and IGF-2 were differentially upregulated as well in the doxorubicin-resistant cell lines. Taken together, these results suggest that FZD1-mediated chemoresistance is likely due to upregulation of MDR1, as well an array of proliferative and cell survival characteristics. 349

Emerging evidence suggests that changes in levels of miRNAs, potent regulators of gene expression, may play a large role in tumorigenesis and may be responsible for several examples of cancer drug resistance. 350(p1),351 In the case of HCC, Xu et al discovered increased levels of miR-130a in HCC patients following treatment with cisplatin-based chemotherapy. They demonstrated a decrease in cisplatin-induced inhibition of proliferation in Huh7 cells with forced expression of miR-130a, and increased inhibition following knockdown of miR-130a. Huh7 cells resistant to cisplatin (Huh7-R) exhibited nuclear accumulation of β-catenin, while Huh7 cells did not. Further studies aimed at elucidating the mechanism for these observations revealed runt-related transcription factor 3 (RUNX3) as a target of miR-130a. Inhibition of RUNX3 by siRNA led to an increase in Wnt signaling. Taken together, these results confirm that inhibition of RUNX3 by increasing levels of miR-130a upregulates Wnt signaling and contributes to cisplatin resistance in HCC. 352

Conclusions and future directions

The evolutionarily conserved Wnt signaling pathway plays an indispensable role in regulating a diverse range of cellular activities. The past 10 years have seen a rapid expansion in our understanding of the regulatory circuitry and the complexity of this pathway as new components of Wnt signaling, especially those related to signaling regulation, stem cell renewal, and tissue homeostasis, have been identified and characterized.

However, the exact mechanisms underlying Wnt signaling under physiological and pathological conditions remain far from fully understood. Many critical questions remain to be fully explored, including the specificity of the interaction between Wnt ligands, Fz receptors and co-receptors, the level of signaling at which canonical or non-canonical Wnt signaling is determined, and further study of upstream regulatory signals of Wnt signaling. The relationship of individual Wnt ligands with downstream target genes in varying tissue or cell types and the nature of their interaction with secreted antagonists remain to be fully explored as well. It is also critical to understand how extensively Wnt signaling cross-talks with other major signaling pathways. With rapid technological advances in genomics and systems biology, however, we remain hopeful that satisfactory answers to these questions will continue to be pursued and may be discovered as soon as in the next decade.

Accompanying these advances in our understanding of Wnt signaling regulation and complexity is an increasing awareness of its role not only in physiological development, but in the development of human pathology, especially cancers, when regulatory aspects of the pathway go awry. While allowing us to unravel the pathophysiology of several cancers, many of which have heretofore proven challenging to cure, these new discoveries present a new class of potential therapeutic targets in the management of malignant and non-malignant Wnt-associated diseases.

Conflicts of interest

The authors declare no conflict of interest.

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