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## The evolving roles of canonical WNT signaling in stem cells and tumorigenesis: Implications in targeted cancer therapies

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### Abstract

The canonical WNT/ $\beta$ -catenin signaling pathway governs a myriad of biological processes underlying development and maintenance of adult tissue homeostasis, including regulation of stem cell self-renewal, cell proliferation, differentiation, and apoptosis. WNTs are secreted lipid-modified glycoproteins that act as short-range ligands to activate receptor-mediated signaling pathways. The hallmark of the canonical pathway is the activation of  $\beta$ -catenin mediated transcriptional activity. Canonical WNTs control the  $\beta$ -catenin dynamics as the cytoplasmic level of  $\beta$ -catenin is tightly regulated via phosphorylation by the 'destruction complex', consisting of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), casein kinase 1 $\alpha$  (CK1 $\alpha$ ), the scaffold protein AXIN, and the tumor suppressor adenomatous polyposis coli (APC). Aberrant regulation of this signaling cascade is associated with varieties of human diseases, especially cancers. Over the past decade, significant progress has been made in understanding the mechanisms of canonical WNT signaling. In this review, we focus on the current understanding of WNT signaling at the extracellular, cytoplasmic membrane, and intracellular/nuclear levels, including the emerging knowledge of crosstalk with other pathways. Recent progresses in developing novel WNT pathway-targeted therapies will also be reviewed. Thus, this review is intended to serve as a refresher of the current

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Conflict of Interest

The authors declare no conflict of interest.

understanding about the physiologic and pathogenic roles of WNT/ $\beta$ -catenin signaling pathway, and to outline potential therapeutic opportunities by targeting the canonical WNT pathway.

## INTRODUCTION

Originally identified as Int-1, the Wnt1 gene was discovered over 30 years ago as a gene activated by integration of mouse mammary tumor virus (MMTV) proviral DNA in virally induced breast tumors<sup>1, 2</sup>. An early identified fly Wingless (Wg) gene, which regulates segment polarity during larval development<sup>3</sup>, was found to be a WNT1 homolog<sup>4</sup>. In the following years, studies of *Drosophila* genetics delineating the relationships among segment polarity mutations mapped out the core of the WNT/Wg signal transduction cascade by identifying Porcupine (PORC), disheveled (DVL), armadillo ( $\beta$ -catenin), and zeste-white 3/ glycogen synthase kinase 3 (GSK3) genes<sup>5-8</sup>. A fuller image of the WNT signaling pathway emerged when T-cell factor/lymphocyte enhancer factor (TCF/LEF) transcription factors were identified as WNT nuclear effectors<sup>9, 10</sup> and Frizzleds (FZDs) were identified as WNT obligate receptors<sup>11</sup>, functioning together with co-receptors, such as low-density lipoprotein-receptor-related proteins (LRPs)/Arrow<sup>12</sup>. The first case for the involvement of WNT signaling in human cancers was made when the hereditary cancer syndrome termed familial adenomatous polyposis (FAP) gene product, adenomatous polyposis coli (APC)<sup>13, 14</sup>, was found to interact with  $\beta$ -catenin<sup>15, 16</sup>, and was later shown to play a critical role in controlling  $\beta$ -catenin protein stability. For the past two decades, numerous components of this pathway and more disease connections have been uncovered<sup>17, 27</sup>.

In most mammalian genomes, the WNT family is comprised of 19 members that are characterized by a highly conserved cysteine-rich secreted glycoproteins, which present the technical challenges in efficient production, biochemical characterization and structural analysis of WNT proteins<sup>28</sup>, although the structure of the *Xenopus* WNT8 protein as bound to Frizzled (FZD) was recently solved<sup>29</sup>. The lipid components of WNTs are required for efficient signaling, including WNT protein secretion<sup>30, 31</sup>. WNT palmitoylation is essential for WNT signaling and is carried out by PORC, a dedicated ER-localized O-acyltransferase and highly conserved component of the WNT pathway<sup>32, 33</sup>. Loss of PORC leads to retention of WNT3A in the ER<sup>34</sup>. In most cell/tissue contexts, WNTs act as short-range signaling<sup>23</sup>.

The emerging evidence indicates that WNT signaling plays an essential role in regulating many biological processes, including embryonic development, tissue homeostasis and maintenance of stem cells. Dysregulation of WNT signaling pathway is associated with various human diseases<sup>17, 27</sup>. Traditionally, WNT signaling is classified into two large categories: the canonical WNT (or  $\beta$ -catenin-dependent) and non-canonical WNT (or  $\beta$ -catenin-independent) pathways. Biologically, the canonical WNT/ $\beta$ -catenin signaling pathway usually plays crucial roles in regulating cell fate, proliferation and survival, while the non-canonical WNT signaling is more associated with differentiation, cell polarity and migration<sup>25, 27</sup>. Non-canonical WNT signaling can be initiated by WNT interaction with Frizzled receptors, or RYK and ROR receptor tyrosine kinases, and regulates small GTPases (such as RhoA, Rac and Cdc42) in DVL-dependent manner. Non-canonical WNT signaling

can also activate calcium flux and kinase cascades, including protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII) and JUN N-terminal kinase (JNK), leading to the activation of AP1- and NFAT-regulated gene expression<sup>25,27</sup>. Increasing evidence indicates that the canonical and non-canonical pathways are intersecting signaling networks that coordinately regulate complex processes, such as embryonic development, stem cell maintenance, tissue homeostasis and wound healing<sup>27</sup>. In this review, we mainly focus on the canonical WNT/ $\beta$ -catenin pathway in regulating stem cells and tumorigenesis, as well as potential anticancer therapeutic opportunities by targeting key steps of this signaling pathway.

## THE CANONICAL WNT/ $\beta$ -CATENIN SIGNALING PATHWAY

### A Simplified Overview

When specific WNT ligands are absent, cytoplasmic  $\beta$ -catenin is phosphorylated by the destruction complex formed by the three proteins APC, AXIN and GSK3 $\beta$  (**Figure 1**). Initial Casein Kinase 1 (CKI) phosphorylation occurs at Ser45 which primes the molecule for subsequent phosphorylation by GSK3 $\beta$  on Thr41, Ser37 and Ser33<sup>20, 25</sup>. Phosphorylated  $\beta$ -catenin is recognized by E3 ubiquitin ligase  $\beta$ -Trop, and degraded by ubiquitin proteasome pathway. Consequently,  $\beta$ -catenin in cytoplasm is kept at a low level. The nuclear transcription factor lymphoid enhancer-binding factor/T cell-specific factor (LEF/TCF) is associated with Groucho and histone deacetylases (HDACs), and represses the expression of WNT/ $\beta$ -catenin target genes<sup>35,37</sup>.

WNT proteins interact with the seven transmembrane receptors of FZD family and single pass transmembrane co-receptors, such as low-density lipoprotein receptor-related protein 5/6 (LRP5/6) or receptor tyrosine kinase-like orphan receptor 2 (Ror2), to induce intracellular signaling pathway. WNT ligands bind to the cysteine-rich domain of FZD, that trigger LRP5/6 phosphorylation and the formation of FZD-LRP5/6 heterotrimeric complex<sup>38</sup>. The activation of DVL protein is phosphorylated and translocate to the FZD receptor<sup>39, 40</sup>. In this context, the  $\beta$ -catenin destruction complex is disrupted which prevents  $\beta$ -catenin proteasomal degradation. Stabilized  $\beta$ -catenin accumulates in the cytoplasm and then translocated into the nucleus (**Figure 1**). Nuclear  $\beta$ -catenin displaces Groucho and forms a complex with the B-cell lymphoma 9 protein (BCL9), Pygopus, histone modifier CBP, as well as tissue-specific transcriptional activators<sup>41, 42</sup>, and binds to LEF/TCF proteins to regulate the expression of WNT target genes in a cell type specific manner<sup>43,50</sup>.

### DVL Plays an Essential Switchboard Role in Channeling WNT Signaling

The scaffold protein DVL is the key cytoplasmic partner of WNT signaling. DVL inhibits AXIN function through a direct interaction with the DIX domain of DVL, which is an important step in the activation of canonical WNT signal pathway<sup>51</sup>. DVL is involved in the formation of the FZD and LRP6 complex. FZD recruits DVL by binding to the PDZ domain of DVL. WNT promotes DVL-dependent LRP6 phosphorylation to regulate downstream gene expression<sup>38</sup>. Furthermore, DVL shuttles between the cytoplasm and the nucleus to transduce canonical WNT signaling to GSK3 $\beta$ -destruction complex of  $\beta$ -catenin, resulting in the stabilization of  $\beta$ -catenin<sup>52</sup>. The mutation in DVL nuclear localization signal domain

leads to inhibition of the WNT/ $\beta$ -catenin signaling. Nuclear DVL, c-Jun,  $\beta$ -catenin form a complex leading to stabilization of  $\beta$ -catenin/TCF interaction. Interestingly, DVL was also shown to interact with transcription factor Hipk1 to regulate the transcription of WNT/ $\beta$ -catenin target genes<sup>53</sup> Forkhead box (FOX) transcription factors, FOXK1 and FOXK2, have been recently shown to positively regulate WNT/ $\beta$ -catenin signaling by translocating DVL into the nucleus<sup>54</sup>.

### Stabilization and Nuclear Translocation of the $\beta$ -Catenin Protein Is the Essence of Canonical WNT Signaling

As a dual function adhesion and transcription coactivator protein,  $\beta$ -catenin is a key mediator of the canonical WNT pathway. A key step in transcriptional activation is the formation of a complex between  $\beta$ -catenin and TCF/LEF transcription factors. In the absence of  $\beta$ -catenin, TCF/LEF factors have no transcriptional activity and are bound by transcription repressors, such as Groucho (**Figure 1**). To generate a transcriptionally active complex, TCF/LEF and  $\beta$ -catenin recruit CBP or its homolog p300, as well as other components of the basal transcription machinery, to initiate transcription<sup>55</sup>. CBP and p300 proteins promote histone acetylation<sup>56</sup>. BCL9 is involved in the majority of  $\beta$ -catenin-regulated gene transcription.  $\beta$ -Catenin can bind to the HD2 domain of BCL9, which served as a specific cofactor for  $\beta$ -catenin regulated transcription.<sup>57</sup> The *Drosophila* Pygopus (Pygo) protein is shown to promote the binding of  $\beta$ -catenin to WNT responding element (WRE) and transcriptional active sites by the combination with BCL9- $\beta$ -catenin<sup>58</sup>. It has recently been shown that  $\beta$ -catenin can interact with other transcription factors (e.g. FOXOs, nuclear receptors, Sox, Smad, Oct4) and plays an important role in various cellular processes<sup>52, 59, 60</sup>. In fact, it was shown that FoxM1 can physically interact with  $\beta$ -catenin and promote  $\beta$ -catenin nuclear localization and WNT target-gene expression<sup>61</sup>. Therefore, the cell context-specific activities of canonical WNT signaling may be accomplished at least in part by differential recruitments of tissue-specific cofactors to the  $\beta$ -catenin/TCF/LEF complex to regulate the expression of different genes.

### R-Spondins Emerge as WNT Signaling Activators

R-Spondin (RSPO) family consists of four members (RSPO1-4), emerging as a group of canonical WNT signaling activators<sup>62, 63</sup>. RSPO proteins contain four major functional domains: two cysteine-rich furin-like (CR) domains, a thrombospondin type 1 (TSP1) domain and a C-terminal region containing basic amino acids. The CR domains (92–94 aa in length) of RSPO proteins are primarily responsible for activation of WNT/ $\beta$ -catenin signaling<sup>64, 65</sup>. Deletion of furin-like motifs within the CR domain of RSPO abolishes its ability to activate canonical WNT signaling. RSPO activation of canonical WNT signaling pathway may depend on the phosphorylation of LRP5/6 receptors<sup>66</sup>. It has been recently shown that Lgr proteins (or Leu-rich repeat-containing G protein-coupled receptor) can bind the furin domains of R-Spondins with high affinity to promote  $\beta$ -catenin signaling<sup>67, 68</sup>. R-spondins, acting through Lgr receptors, inhibit the transmembrane E3 ubiquitin ligases RNF43/ZNRF3 that ubiquitinate and thus degrade FZD receptors<sup>69, 70</sup>, leading to the stabilization of FZD receptors and subsequent enhancement of WNT signal. It should be pointed out that the R-Spondin (RSPO)/LGR axis is only found in vertebrate systems<sup>71</sup>.

## WNT Signaling Is Tightly Controlled by Its Naturally Occurring Secreted Antagonists

All essential pathways in mammalian cells are heavily negatively regulated. The canonical WNT signaling pathway is no exception. Numerous inhibitors of WNT signaling present outside of the cell and affect ligand–receptor interactions. The prototype antagonists, called secreted Frizzled-related proteins (SFRPs), possess FZD-like cysteine-rich domain (CRD) that competitively binds to WNT ligands and prevents the interaction between WNTs and FZD receptors<sup>72,74</sup>. Another structurally unrelated secreted inhibitor called WNT inhibitory protein (WIF) can bind WNTs, thereby blocking interactions between WNT and WNT receptors<sup>75</sup>.

Secreted inhibitors that target co-receptors LRP5/6 also exist. The Dickkopf (DKK) family of proteins can inhibit WNT/ $\beta$ -catenin signaling by competitively binding to the WNT co-receptors LRP5/6<sup>76</sup>. It was suggested that DKK1 may inhibit WNT signaling via inducing LRP6 internalization or degradation through transmembrane Kremen (Krm) proteins<sup>77</sup>, which was not however supported by recent biochemical and genetic studies<sup>78,80</sup>. WISE and SOST constitute another family of LRP5/6 ligands/antagonists<sup>81,83</sup>. SOST can disrupt WNT-induced Fz-LRP6 complex *in vitro*<sup>83</sup>. Both DKK1 and SOST are strongly implicated in human diseases<sup>22, 84, 85</sup>. It is noteworthy that none of the above naturally occurring secreted inhibitors has been identified in *Drosophila*.

## CROSSTALK BETWEEN WNT AND OTHER MAJOR SIGNALING PATHWAYS

Given the wide spectrum of WNT effects on target cells, crosstalks with other signaling pathways play an important role in fine-tuning modulation of WNT signaling during development and in maintaining tissue homeostasis. Here, we discuss several well-established cross-talks between WNT signaling and other signaling pathways.

### Some Growth Factor Signaling Pathways Can “Hijack” $\beta$ -Catenin Signaling Activity

It is conceivable that any cellular signaling that can stabilize the  $\beta$ -catenin protein should be able to activate the downstream events regulated by the  $\beta$ -catenin-TCF/LEF transcriptional complex. As the essential mediator of the canonical WNT pathway,  $\beta$ -catenin is aberrantly activated in a multitude of human cancers without any known mutations in the upstream components of the pathway or any increase in WNT expression, suggesting that non-WNT factors may be also capable of activating  $\beta$ -catenin. In fact, several growth factor and developmental signaling pathways, such as hepatocyte growth factor (HGF)<sup>86,89</sup>, epidermal growth factor (EGF)<sup>90,92</sup>, insulin-like growth factors (IGFs)<sup>93,98</sup>, vascular endothelial growth factor (VEGF)<sup>99, 100</sup>, and fibroblast growth factors (FGFs)<sup>101,106</sup>, have been found to cause the accumulation/stabilization of the  $\beta$ -catenin protein and/or to activate  $\beta$ -catenin activity.

For example, it was shown that EGFR activation involves a kinase signaling cascade that leads to the dissociation of  $\beta$ -catenin from  $\alpha$ -catenin at the adherens junction and the eventual nuclear translocation of  $\beta$ -catenin<sup>90</sup>, although EGF-induced activation of  $\beta$ -catenin may also involve histone deacetylase<sup>107</sup> or embryonic pyruvate kinase M2 (PKM2)<sup>92</sup>. The HGF receptor Met is involved in phosphorylation of  $\beta$ -catenin at tyrosine residues 654

and 670 with subsequent nuclear translocation of  $\beta$ -catenin<sup>87</sup>. A significant correlation exists between expression of c-Met and abnormal  $\beta$ -catenin expression in invasive breast carcinoma, implicating crosstalk between the two in breast cancer<sup>88</sup>. IGF1 or insulin treatment increased  $\beta$ -catenin/TCF-mediated transcription and cytoplasmic stabilization of  $\beta$ -catenin<sup>94</sup>, while IGF-1 also induced stabilization of  $\beta$ -catenin in prostate cancer and early melanoma cells<sup>97, 98</sup>. FGF19 was shown to increase  $\beta$ -catenin transcriptional activity in colon cancer cells and in transgenic mice<sup>104, 105</sup>, whereas co-activation of the WNT and FGF pathways in colorectal carcinogenesis leads to a more malignant phenotype<sup>102</sup>. Interestingly, FGFs also cooperate with Wnt1 in MMTV-induced mammary tumorigenesis<sup>101, 103, 106</sup>. Nonetheless, molecular mechanisms for these cross-talks have yet to be fully elucidated.

### Crosstalk between the WNT and TGF $\beta$ /BMP Signaling Pathways

The TGF $\beta$  family includes TGF $\beta$  and the bone morphogenetic proteins (BMPs), which share a canonical signaling cascade involving two types of receptors (type I and II) and a common set of signal transducers known as Smads<sup>108,113</sup>. Upon TGF $\beta$  or BMP binding to the type II receptor, the type I receptor is recruited to form a heterodimer complex, leading to the phosphorylation of the C-terminus of receptor-regulated Smads (R-Smads) (Smad 1, 2, 3, 5, and 8), which are then able to complex with the common Smad4. The Smads complex then translocates to the nucleus, binds to DNA, and regulates target gene expression<sup>108,113</sup>.

Crosstalk between the WNT and TGF $\beta$ /BMP pathways is quite complex and occurs at multiple levels. The ligand production for the two pathways is under regulation by each other. For example, BMP4 expression in human colon cancer cells is dependent on the expression of oncogenic  $\beta$ -catenin<sup>114</sup>. Conversely, BMP2/4 is capable of regulating production of WNT8<sup>115</sup>. Smad and  $\beta$ -catenin/TCF can form a complex that binds to DNA and regulates shared target gene expression during development<sup>116, 117</sup>. WNT3a and BMP4 synergistically induce expression of common target genes, such as Id2, Msx1, and Msx2<sup>50</sup>. Many genes have been identified that harbor both Smad and TCF/LEF binding sites within their regulatory sequences, including Tbx6, Msx2, Xtwi, Emx2, Slug, c-Myc<sup>118,123</sup>. TGF $\beta$ RII knockout specifically in the stromal cells leads to increased expression of WNT3a and development of prostatic cancer<sup>124, 125</sup>. Furthermore, it has been shown that Smad7 can directly bind to  $\beta$ -catenin and induce its degradation by the recruitment of Smurf2<sup>126</sup>. Smad7 was found to physically associate with  $\beta$ -catenin, leading to an accumulation of  $\beta$ -catenin in prostate cancer cells<sup>127</sup>. Smad7 can also directly bind to AXIN, which induces disassembly of the degradation complex and subsequent stabilization of  $\beta$ -catenin at the adherens junction<sup>128</sup>. It has been recently reported that antagonism between WNT and BMP at the cytoplasmic level can be mediated by a direct interaction of DVL and phosphorylated Smad1<sup>129</sup>. Therefore, the crosstalk between TGF $\beta$ /BMP and WNT signaling pathways can be either synergistic or antagonistic depending on the cellular context<sup>130</sup>.

### Crosstalk between the WNT and Notch Signaling Pathways

Notch signaling pathway is a highly conserved mechanism of intercellular communication and essential for development, patterning, and tissue homeostasis<sup>131,133</sup>. Notch signaling is transduced through direct cell-to-cell contact and requires activation at the cell surface by



ligands of the DSL (Delta and Serrate/Jagged) family. The ligand-receptor interaction causes cleavage of the Notch receptor through intramembrane proteolysis and yields the Notch intracellular domain (NICD), which then translocates to the nucleus and activates downstream target genes through transcription factor RBPj and co-factors such as Mastermind<sup>132, 133</sup>.

Crosstalk between the Notch and WNT pathways has been observed in many developmental and cellular processes, such as somitogenesis, intestinal epithelial cell fate, and hematopoietic stem cell maintenance<sup>134, 138</sup>. Notch can repress WNT signaling during development and homeostasis by associating with  $\beta$ -catenin<sup>139</sup>, while WNT activation can antagonize Notch signaling through DVL<sup>140</sup>. Numerous reports have detailed an opposing role for the WNT and Notch pathways in tumorigenesis as the deletion or inhibition of Notch results in basal cell carcinoma<sup>141</sup>. Activation of the Delta-Notch pathway inhibits the WNT pathway in neuroblastoma cells<sup>142</sup>. Notch activation in a human tongue cancer cell line suppressed WNT signaling and led to cell cycle arrest and apoptosis<sup>143</sup>. The physical interaction between  $\beta$ -catenin and the cytoplasmic tail of membrane bound Notch resulted in degradation of  $\beta$ -catenin protein<sup>39</sup>.

Nonetheless, WNT and Notch pathways seem to work synergistically in intestinal tumorigenesis adenomas as activation of Notch in APC mutant mice hyperactive for WNT signaling accelerates adenoma development<sup>144</sup>. WNT and Notch appear to operate synergistically in other cancer types, such as liver cancer<sup>145</sup>, prostate cancer<sup>146</sup>, breast cancer<sup>147, 148</sup>, and leukemia<sup>134</sup>. Furthermore, Notch ligand Jagged1 can be transcriptionally activated by  $\beta$ -catenin<sup>149</sup>. Nonetheless, detailed mechanisms underlying the crosstalk between WNT and Notch pathways remain to be fully elucidated.

### Crosstalk between the WNT and Hedgehog Signaling Pathways

The Hedgehog pathway is essential for tissue growth, patterning, and morphogenesis<sup>150</sup>. The three mammalian Hedgehogs (Hh), Sonic, Indian, and Desert hedgehog (Shh, Ihh, and Dhh) are secreted proteins that undergo cleavage and lipid modification to become active signaling molecules<sup>150, 151</sup>. Binding of Hh ligand to its receptor, Patched, de-represses the transmembrane protein Smoothed (Smo), thereby inducing a signaling cascade which results in stabilization of Gli transcription factors<sup>152, 153</sup>, which in turn regulate the expression of target genes<sup>150, 151, 153</sup>. In basal cell carcinomas (BCC)s, elevated levels of WNT pathway components were detected in response to Hh signaling abnormalities and Gli1 expression, suggesting a requirement for ligand-driven, canonical WNT/ $\beta$ -catenin signaling for Hh pathway-driven tumorigenesis<sup>154, 155</sup>. Elevated Gli1 expression was shown to lead to accumulation of nuclear  $\beta$ -catenin in endometrial cancers<sup>156</sup>. WNT/ $\beta$ -catenin signaling induces expression of an RNA-binding protein, CRD-BP, leading to stabilization of Gli1 mRNA and increased Hedgehog signaling and survival of colorectal cancer cells<sup>157</sup>. Inhibition of Smo rescues the lethality caused by the loss of APC in mice, suggesting Hh may be activated in parallel with or downstream of WNT signaling<sup>158</sup>. Conversely, Hh signaling was shown to positively regulate the WNT pathway<sup>159</sup>. Reduced expression of Smo in APC mice suppressed  $\beta$ -catenin-dependent transcription in intestinal adenoma cells independently of the canonical Hh pathway<sup>160</sup>. Activation of the Hh pathway through Smo

or Gli2 increases WNT activity in pancreatic adenocarcinoma<sup>161</sup>. However, it was also reported that Ihh acts as an antagonist of WNT signaling in colonic epithelial cell differentiation<sup>162</sup>. WNT and Hh signaling was also found to be inversely correlated in gastric cancer specimens as overexpression of Gli1 suppressed WNT signaling in a gastric cancer cell line<sup>163</sup>. Therefore, the disparate regulations of WNT and Hh signaling pathways may be cell or tissue-specific or context-dependent and remain to be fully understood.

### Crosstalk between the WNT and Hippo/YAP/TAZ Signaling Pathways

The Hippo pathway is a potent regulator of cellular proliferation and differentiation, and has emerged as a crucial regulator of tissue development and homeostasis<sup>164,168</sup>. The Yes-associated protein (YAP)/transcriptional coactivator with a PDZ-binding domain (TAZ) are the prime mediators of the Hippo pathway<sup>166</sup>. Activation of the Hippo pathway leads to the phosphorylation and cytoplasmic retention of YAP/TAZ. While the nuclear localization of YAP/TAZ is essential for the transcriptional activities of the Hippo pathway, nonnuclear TAZ appears to be crucial for the regulation of canonical WNT signaling<sup>169</sup>. The Hippo pathway seems to restrict WNT/ $\beta$ -catenin signaling by promoting an interaction between TAZ and DVL. Cytoplasmic YAP may also counterbalance the effect of WNT signaling by limiting DVL activity<sup>170</sup>. The Hippo and WNT pathways also cooperate in the nucleus, where YAP interacts with  $\beta$ -catenin and induces the expression of canonical WNT target genes<sup>171</sup>. It has been reported that YAP and TAZ are integral components of the  $\beta$ -catenin destruction complex that serves as cytoplasmic sink for YAP/TAZ<sup>172</sup>. In WNT-ON cells, YAP/TAZ are physically dislodged from the destruction complex, allowing their nuclear accumulation and activation of WNT/YAP/TAZ-dependent biological effects<sup>172</sup>. In WNT-OFF cells, YAP/TAZ are essential for  $\beta$ -TrCP recruitment to the complex and  $\beta$ -catenin inactivation<sup>172</sup>. However, a recent study indicates that APC can regulate Hippo-YAP signaling in a  $\beta$ -catenin destruction complex-independent fashion during intestinal tumorigenesis<sup>173</sup>. In fact, activation of YAP is a general hallmark of tubular adenomas from FAP patients; and APC was shown to function as a scaffold protein that facilitates the Hippo kinase cascade by interacting with Sav1 and Lats1<sup>173</sup>. More surprisingly, a recent study suggests that YAP/TAZ may function as bona fide downstream effectors of the alternative WNT-YAP/TAZ signaling pathway as WNT5a/b and WNT3a were shown to induce YAP/TAZ activation independent of canonical WNT/ $\beta$ -catenin signaling<sup>174</sup>. The so-called “alternative WNT-YAP/TAZ signaling axis” consists of WNT-FZD/ROR-G $\alpha$ 12/13-Rho GTPases-Lats1/2 and outcomes include YAP/TAZ activation and TEAD-mediated transcription, leading to the fulfillment of YAP/TAZ-mediated biological functions of alternative WNT signaling, such as gene expression, osteogenic differentiation, cell migration, and antagonism of WNT/ $\beta$ -catenin signaling<sup>174</sup>. While these findings define a G protein-mediated pathway for WNT signaling to YAP/TAZ, it remains to be fully understood to what extent such alternative pathway transduces either canonical and/or non-canonical WNT signaling.

### WNT/ $\beta$ -CATENIN SIGNALING AND STEM CELLS SELF-RENEWAL

Emerging evidence has established the important and wide-range roles of canonical WNT signaling in stem cell self-renewal and/or lineage-specific differentiation in diverse tissues and



cell types *in vivo*<sup>27</sup>. Given the short-range feature of the signaling gradient, WNT signals can function as ideal stem cell niche factors, which may control immediately adjacent stem cell, leading to the parsimonious control of progenitor cell fate<sup>27, 175</sup>. Here, we discuss the roles of canonical WNT signaling in regulating several well-characterized stem cell systems.

### WNT/ $\beta$ -Catenin Signaling in Embryonic and Pluripotent Stem Cells

Embryonic stem cells (ESCs) are generated from the inner cell mass (ICM) of the blastocyst, and possess the pluripotent capacity to retain their ability to make all cell types within the organism<sup>176</sup>. WNT/ $\beta$ -catenin pathway is required for the establishment and self-renewal of ESCs cells<sup>27, 175, 177</sup>. WNT3a or inhibitor of GSK3 $\beta$  was shown to promote the formation of ESC-like colonies<sup>178</sup>. WNT/ $\beta$ -catenin signaling stimulates self-renewal by inhibiting the repressor activity of endogenously expressed TCF3, while WNT/ $\beta$ -catenin activation may also result in differentiation<sup>27, 175</sup>. The effects of APC mutation or endogenous GSK3 $\beta$  preventing activation of WNT/ $\beta$ -catenin signaling result in unable to differentiate normally in either embryoid body (EB) or teratoma differentiation assays<sup>179</sup>. WNT/ $\beta$ -catenin activation and GSK3 $\beta$  inhibitors can enhance somatic cell reprogramming and iPSC formation<sup>180</sup>. The efficiency of WNT/ $\beta$ -catenin-stimulated reprogramming appears to be stage-dependent; and TCF3/4 and LEF1/TCF1 act temporally in this process. Pluripotency can be maintained as long as conditions favor expression of the core transcription factors Oct4, Sox2, and Nanog.  $\beta$ -Catenin was shown to interact with reprogramming factors Klf4, Oct4, and Sox2, further enhancing the expression of pluripotency related genes<sup>27, 175, 181</sup>.

### WNT/ $\beta$ -Catenin Signaling in Mesenchymal Stem Cells

Mesenchymal stromal cells (MSCs) derived from stroma of bone marrow (BM), adipose tissue, or placental tissue, have the potential to differentiate into multiple cell types<sup>22, 111, 112, 182</sup>. Canonical WNT signaling plays a critical role in regulating cell fate decisions of MSCs. The activation of canonical WNT pathway can promote the osteogenic differentiation of MSCs by upregulating the expression of Cbfa1/Runx2 and alkaline phosphatase (ALP)<sup>85, 183</sup>. Canonical WNT/ $\beta$ -catenin signaling was shown to induce overlapping target genes and to act synergistically with osteogenic BMPs in inducing osteogenic differentiation of MSCs<sup>184, 188</sup>. The adipogenic differentiation is enhanced in the absence of WNT signaling as activation of  $\beta$ -catenin via ectopic expression of WNT1 was shown to directly suppress PPAR $\gamma$  expression and prevent 3T3-L1 adipogenic differentiation<sup>189</sup>. GSK3 $\beta$  mediates WNT inhibition of adipogenesis interfering with PPAR $\gamma$  transcriptional activation<sup>190</sup>. In the cardiac differentiation process of MSCs, the WNT/ $\beta$ -catenin signaling pathway had been inhibited<sup>191</sup>. Blocking the WNT/ $\beta$ -catenin signaling can enhance MSC-based granulation tissue formation and myocardial repair<sup>192</sup>. Canonical WNT signals distinctively 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/ $\beta$ -catenin, whereas osteogenic differentiation was promoted under high levels of WNT signaling<sup>193</sup>.

### WNT/ $\beta$ -Catenin Signaling in Intestinal Stem Cells

Intestinal stem cells have been demonstrated to be divided into two populations: those located at the base of the crypt and those at the position 4 from the base of the crypt<sup>27, 194</sup>.

The position 4 cells characterized by the stem cell marker *Bmi1* are thought to be quiescent, slow cycling and apparently activated only during injury<sup>194,196</sup>. The crypt base columnar (CBC) cells residing at the base of the crypt are rapid cycling and responsible for sustained tissue homeostasis, which can be identified by the expression of *Lgr5*<sup>196,197</sup>. WNT/ $\beta$ -catenin signaling is required for proper stem cell maintenance and differentiation in the intestine<sup>197</sup>. The expressions of several WNT ligands and receptors (*WNT3*, 6, 9b, *FZD* 4, 6, 7, *LRP5*, *SFRP5*) are detected in epithelial cells of the intestinal crypt<sup>198</sup>. WNT antagonist *SFRP5* highly expressing in +4 cell surrounding area has been associated with the regulation of the stem cell niche in the intestine<sup>199</sup>. Numerous target genes of the WNT/ $\beta$ -catenin pathway have been identified in the intestine. *Sox9* as both a transcriptional target and a regulator of the WNT pathway has been shown to be required for paneth cell differentiation<sup>200</sup>. The transcription factor achaete scute-like 2 (*Ascl2*) acts as an R-spondin/WNT-responsive gene and regulates the expression of the genes essential to the stem cell state together with  $\beta$ -catenin /TCF<sup>201</sup>. *EphB2/3* is required for the correct positioning of cells in the intestinal epithelium controlled by  $\beta$ -catenin and TCF<sup>202</sup>. Notch and WNT signaling are required both for stem cell maintenance and for a proper balance of differentiation between secretory and absorptive cell lineages<sup>194</sup>. In the absence of Notch signaling, stem cells preferentially generate secretory cells at the expense of absorptive cells as blocking Notch signaling disturbs the normal function of the intestine stem cells and lead to the mis-expression of prosecretory genes by inhibiting the WNT signaling pathway<sup>194,203</sup>.

### WNT/ $\beta$ -Catenin Signaling in Hematopoietic Stem Cells

WNT signaling pathway plays a key role in the early stage of hematopoiesis<sup>23,175</sup>. WNT/ $\beta$ -catenin signaling is indispensable in the formation of vascular endothelial cells to hematopoietic stem cell (HSC) transformation process on early stage of hematopoiesis<sup>21,27</sup>. The WNT signaling is activated in the early stage of embryonic formation of red blood cells. Activation of the WNT/ $\beta$ -catenin pathway can increase the specific markers of pronormoblast and induce the formation of hematopoietic progenitors (MPP)<sup>204</sup>. Knockout of *WNT3a* in mouse decreased the hematopoietic stem/progenitor cells in the fetal liver<sup>205</sup>. Overexpression of activated N-terminal truncation  $\beta$ -catenin promoted the expansion of HSC<sup>206</sup>. WNT/ $\beta$ -catenin inhibitors, *DKK1* and *Wif1*, can disrupt the quiescent state of HSCs and result in the loss of HSC self-renewal and decrease hematopoietic reconstitution<sup>207,208</sup>. Survivin expression, which is regulated by WNT/CBP/ $\beta$ -catenin, is important during hematopoiesis and is prominently up-regulated in CD34+ hematopoietic stem/progenitor cells upon growth factor treatment, as survivin-deficient hematopoietic progenitors were shown to have defects in erythroid and megakaryocytic lineage formation<sup>209,210</sup>. WNT/ $\beta$ -catenin signaling, together with other pathways, such as Notch, PGE2, and BMPs, is important for maintaining the hematopoietic lineage balance. It was shown in zebrafish that the timed WNT to Notch relay signaling serves as an early upstream mechanism in HSC specification<sup>211,212</sup>. After the formation of mesoderm, the BMP signaling activates WNT signaling pathway and *Cdx-Hox* to promote hematopoiesis<sup>211,212</sup>. The BMP and WNT signaling pathways were shown to regulate hematopoiesis related genes and erythroid differentiation through the transcription factors *Smad1* and TCF after acute injury of hematopoietic system<sup>213</sup>.

## WNT/ $\beta$ -Catenin Signaling in Hair Follicle Stem Cells

Hair follicle stem cells residing in the hair follicle (HF) are quiescent when the follicle is resting, but rapidly expand and differentiate in response to hair periodical regeneration, maintenance of adult skin homeostasis and wound repair<sup>214, 215</sup>. WNT/ $\beta$ -catenin signaling is required for embryonic HF morphogenesis<sup>215</sup>. Forced activated  $\beta$ -catenin signaling converts embryonic ectoderm to hair follicle fate. The expression of nuclear  $\beta$ -catenin is described in hair germ progenitor cells at anagen onset, in hair follicle precursor cells during anagen, but undetectable in telogen HFs<sup>216</sup>. Conditional loss of  $\beta$ -catenin in skin epithelia leads to hair follicle stem cells depletion, whereas hair follicle stem cells-specific ablation inhibits the proliferation of hair germ progenitor cells and fate specification of bulge stem cells<sup>217</sup>. TCF3 and TCF4 are present in quiescent stem cells, where WNT/ $\beta$ -catenin activity is silent<sup>218</sup>. Elevation of WNT/ $\beta$ -catenin depresses TCF3/TCF4/TLE-bound target genes, including chromatin-repressed genes, and then activates LEF1 to drive the progenitor cells along the hair differentiation lineage<sup>219</sup>. Pygo2 was shown to function as an important regulator of WNT/ $\beta$ -catenin function in skin epithelia and  $\beta$ -catenin-induced activation of hair follicle stem/early progenitor cells<sup>220</sup>. Furthermore, it is well established that canonical WNT signaling regulates the fate of hair follicle stem cells in concert with other signaling pathways in their niches. Notch ligand Jagged-1 was shown to be a WNT/ $\beta$ -catenin target gene in hair follicle formation of the adult epidermis<sup>149</sup>. The antagonistic competition between BMP and WNT signaling balances hair follicle stem cell activity, as reduced BMP signaling and increased WNT signaling activated hair follicle stem cell toward hair fate and hair follicle cycle<sup>221</sup>. WNT7b as a putative target of canonical BMP signaling serves as a key component required for normal hair follicle stem cells activation during the telogen-anagen transition<sup>222</sup>.

## WNT/ $\beta$ -CATENIN SIGNALING AND TUMORIGENESIS

Given the important roles and pleiotropic effects of canonical WNT signaling in virtually every organ system in normal tissue homeostasis and tissue injury repair, it is expected that dysregulation of this signaling pathway would be associated with a large array of human diseases, including neurological diseases, inflammatory and fibrotic disease, and disorders of endocrine function and bone metabolism in adults<sup>22, 23, 27, 85</sup>. Here, we focus on the consequences of aberrant regulations of WNT/ $\beta$ -catenin signaling in the development of human cancers.

### Aberrant Activation of WNT/ $\beta$ -Catenin and Tumorigenesis

WNT1 was initially discovered as a potential oncogene in mouse mammary glands, which was further substantiated by the fact that WNT1 transgenic mice developed mammary tumors<sup>1, 223</sup>. These early studies strongly suggest a causative role for WNT1 in mammary tumorigenesis. Later studies demonstrated a pivotal relation between hyperactivated WNT/ $\beta$ -catenin signaling pathway and the initiation of colorectal cancer<sup>224, 225</sup>. The high frequency of mutations in various components of WNT pathway in many types of human cancers further highlights the importance of activation of WNT/ $\beta$ -catenin signaling in tumorigenesis<sup>226, 227</sup>. Germline inactivating mutations in *APC*, resulting in nuclear accumulation of  $\beta$ -catenin stability, are found in patients with familial adenomatous

polyposis (FAP)<sup>13, 228</sup>, while a nonsense mutation in the coding region of the APC gene causes multiple intestinal neoplasia (Min) phenotype in mice<sup>229</sup>.

Dysregulation of the WNT/ $\beta$ -catenin pathway has also been widely found in non-colorectal cancers<sup>18</sup>. For example, it was reported that up to 44% and 25% of hepatocellular carcinoma (HCC) tumors contain mutations of  $\beta$ -catenin in exon 3 or mutations in AXIN1, respectively<sup>230, 231</sup>. Oncogenic mutations of  $\beta$ -catenin are commonly found in human skin cancers, including melanoma<sup>232</sup>. Increasing evidence indicates that WNT/ $\beta$ -catenin signaling is involved in pancreatic ductal adenocarcinoma (PDAC) tumorigenesis<sup>161, 233, 234</sup>. The results of pancreatic circulating tumor cell RNA studies implicated that WNT2 expression was up-regulated, suggesting WNT2 may be associated with PDAC metastasis<sup>235</sup>. It is noteworthy that many types of human cancers exhibit nuclear and/or cytoplasmic  $\beta$ -catenin accumulation, indicating the activation of the canonical WNT pathway without any identifiable mutations in APC, AXINs,  $\beta$ -catenin, or other components of the canonical WNT pathway<sup>232, 236</sup>. Furthermore, as one of the hallmarks of tumorigenesis telomerase is regulated by  $\beta$ -catenin<sup>237</sup>. Conversely, WIF1, a component of the WNT pathway and a competitive inhibitor of WNT pathway, was down-regulated in prostate, breast, lung, bladder cancer and osteosarcoma<sup>238, 239</sup>.

As discussed earlier, Yes-associated protein 1 (YAP1) was shown to be essential to the survival and transformation of  $\beta$ -catenin-active cancer cell lines, and YAP is induced by  $\beta$ -catenin in colorectal cancer cells and is up-regulated in APC mutant colorectal cancer cells<sup>240, 241</sup>. The role of YAP and TAZ as mediators of WNT signaling is further supported by the findings from an animal model in which showed that both YAP and TAZ were required for loss of APC-induced crypt hyperplasia<sup>172</sup>. Nonetheless, a recent study revealed that YAP is required for the development of APC-deficient adenomas, but APC functions as a scaffold protein to facilitate the Hippo kinase cascade by interacting with Sav1 and Lats1, which is independent from its involvement in the  $\beta$ -catenin destruction complex<sup>173</sup>. These findings indicate that while the causative role of aberrantly activated WNT/ $\beta$ -catenin signaling in human cancer development is well established, the detailed molecular mechanisms underlying WNT/ $\beta$ -catenin signaling in tumorigenesis are far from being clearly understood.

### WNT/ $\beta$ -Catenin Signaling and Cancer Metastasis

Cancer metastasis is a complex multistep process involving breaking through the extracellular matrix and basement membrane at the primary tumor sites<sup>242, 243</sup>. WNT/ $\beta$ -catenin pathway related gene and target gene is associated with tumor invasion and metastasis, such as matrix metalloproteinase (MMP) 7, CD44, vascular endothelial growth factor, E-cadherin<sup>244, 245</sup>. E-cadherin/ $\beta$ -catenin complex mediated cell adhesion is to establish and maintain normal polarity and cell tight junction of epithelial cells<sup>244</sup>. Epithelial to mesenchymal transition (EMT) are known about the epithelial plasticity that are important in cancer metastasis<sup>246, 248</sup>. Activation of WNT/ $\beta$ -catenin signaling leads to the nuclear translocation of  $\beta$ -catenin to disturb the E-cadherin/ $\beta$ -catenin complex, contributing to the EMT process and cancer metastasis<sup>246, 248</sup>. WNT/ $\beta$ -catenin activity usually up-regulates the expression of EMT-promoting genes, including SNAIL1/Snail 1, SNAIL2/Snail 2

(also known as Slug), ZEB1, ZEB2, E47 and KLF8<sup>247, 248</sup>. Activation of the canonical WNT/TCF pathway through LEF1 and HOXB9 was also identified as a determinant of metastasis to brain and bone during lung adenocarcinoma progression<sup>249</sup>. Furthermore, the WNT/ $\beta$ -catenin signaling can up-regulate the expression of cyclooxygenase-2 (Cox2) to promote tumor angiogenesis, which subsequently promotes tumor metastasis. It was also reported that the metastasis-stroma interaction in human breast cancer metastasis was regulated by the HGF/nuclear Met/phospho-c-Src/ $\beta$ -catenin-TCF/WNT pathway<sup>250</sup>.

### WNT/ $\beta$ -Catenin Signaling and Cancer Stem Cells

The WNT/ $\beta$ -catenin pathway is also involved in the regulation of cancer stem cells (CSCs) from many tissue types<sup>23, 175, 194, 237</sup>. Many of the cancer stem cell surface markers, such as LGR5/GPR49, CD44, CD24, and Epcam, which are used to identify and isolate putative cancer stem cell populations in a variety of tissues, are WNT target genes<sup>23, 175, 194</sup>. In breast cancer LGR5-expressing cells exhibit cancer stem cell-like properties, including the formation of self-renewing spheres and high tumorigenicity by activating WNT/ $\beta$ -catenin signaling<sup>251</sup>. WNT3a can promote the self-renewal of cancer stem/progenitor cells in acute lymphocytic leukemia (ALL) and prostate cancer<sup>252, 253</sup>. CD44 is closely associated with tumor growth, invasion, metastasis as an important tumor stem cell marker<sup>254</sup>. In human colon cancer cell line LT97, CD44 positive cells were detected with the expression of nuclear  $\beta$ -catenin, while CD44 negative cells exhibited no nuclear  $\beta$ -catenin<sup>255</sup>. However, it remains to be fully elucidated if WNT/ $\beta$ -catenin signaling regulates normal stem cells/progenitor cells versus cancer stem cells.

## TARGETING WNT/ $\beta$ -CATENIN SIGNALING FOR CANCER TREATMENT

The broad involvement and pleiotropism of WNT signaling in stem cells and human diseases has attracted extraordinary amounts of interests in the development of novel strategies targeting this signaling pathway<sup>21, 26, 256, 260</sup>. One of the earliest such efforts involved the reintroduction of wild type APC into human colorectal cancer cell lines, which induced growth inhibition and apoptosis of the cancer cells<sup>261</sup>. Similarly, expression of AXIN I also promoted apoptosis in cancer cell lines containing mutations in either  $\beta$ -catenin, APC or AXIN I<sup>231</sup>. These experiments strongly suggest that therapeutic intervention targeting WNT signaling can be developed for anticancer therapies.

For the past decade, significant progresses have been made for the past decade in identifying the druggable targets of the WNT pathway and/or in developing novel small molecules that specifically target WNT/ $\beta$ -catenin signaling<sup>26, 257, 260</sup>. Although most of these drugs have not yet progressed to evaluation in clinical trials (**Table 1**), current genomics and proteomics studies enable more targeted approaches for high-throughput screening of the WNT/ $\beta$ -catenin pathway, which is expected to deliver clinical drugs in the coming decade. Here, we primarily focus on the recent development of potential anticancer therapies by targeting the WNT signaling pathway. As the WNT pathway lends itself ample targeting nodal points for drug development, numerous efforts have been devoted to targeting WNT signaling at different regulatory levels of the signaling cascade.

## Targeting WNT Signaling at Extracellular Level

Directly targeting WNT ligands may prove to be an attractive strategy for targeting WNT signaling preferentially in cancer cells that exhibit aberrantly overexpressed WNTs. Several WNT-blocking antibodies were developed and shown to inhibit proliferation and induce apoptosis in different cancers<sup>21, 262, 265</sup>. Intraperitoneal injections of WNT3A-neutralizing antibodies decrease proliferation and induce apoptosis in a mouse model of prostate cancer<sup>125</sup>.

FZD receptors are another class of logic targets for developing WNT-targeting biologics. One such agent OMP18R5 was developed by OncoMed Pharmaceuticals and is a humanized monoclonal antibody that binds to FZD1, FZD2, FZD5, FZD7 and FZD8<sup>26</sup>. OMP18R5 recently completed the Phase Ia clinical trial in patients with advanced solid tumors<sup>26</sup>. A total of 18 patients were treated and, the most common drug-related adverse events included fatigue, vomiting, abdominal pain, constipation, diarrhea and nausea. There were three cases of prolonged stable disease in patients with neuroendocrine tumors<sup>26</sup>.

The naturally occurring FZD receptor antagonists SFRPs are logic agents to target WNT signaling. These factors are extracellular inhibitors that bind directly to WNT ligands or to Frizzled receptors. The SFRP1 or SFRP1-derived peptides were shown to delay HCT116 xenograft tumor formation in nude mice and reduced the proportion of mitotic<sup>266</sup>. WNT inhibitory factors (WIFs) are also secreted proteins that competitively displace certain WNT ligands from their receptors. Overexpression of WIF1 was shown to inhibit osteosarcoma cell growth in soft agar assays and in xenograft assays<sup>267</sup>. It's noteworthy that SFRPs may regulate cell proliferation of some cancer cells, such as prostate cancer cells, in a context-dependent manner, as the overexpression of SFRP4 or SFRP3 decreases the proliferation of human PC3 cells<sup>268</sup>, whereas the overexpression of SFRP1 promotes the growth of BPH1 prostate cancer cells<sup>269</sup>. As SFRPs and WIFs are associated with multiple WNTs, it's conceivable that altering SFRP and WIF levels may have pleiotropic effects on cancer cell proliferation.

Alternatively, a competitive inhibition of WNT signaling can be achieved by overexpression of the secreted forms of FZD receptors. In fact, it was reported that administration of a fusion protein consisting of the Fc region of IgG fused to the extracellular domain of FZD8 (FZD8CRD) inhibited the formation of tumor xenografts by two non-engineered cancer cell lines, the N-TERA2 human testicular cancer line and the PA1 human ovarian cancer cell line<sup>270</sup>. It was also shown that soluble FZD7 can inhibit WNT signaling and sensitize hepatocellular carcinoma (HCC) cells towards doxorubicin<sup>271</sup>. Interestingly, the FZD7 peptides derived from the domains that interact with DVL effectively inhibited the growth of HCC cells<sup>272</sup>.

Furthermore, since the SFRP genes are usually silenced by hypermethylation, it is conceivable drugs that affect the DNA methylation can be used to alter the methylation status of SFRP gene promoters and hence re-activate the expression of SFRPs. It was reported that aberrant epigenetic modification of SFRP gene was one of the major mechanisms by which WNT signaling is activated in human gastric cancer cells, and sodium



butyrate can modulate the SFRP1/2 expression through histone modification and promoter demethylation, causing anti-tumor effects<sup>273</sup>.

### Targeting WNT Signaling at Cytoplasmic Membrane Level

DVL is an essential mediator in the WNT signaling pathway and transduces extracellular WNT signals to downstream components. DVL utilizes its PDZ domain to bind to the carboxyl-terminal region of the FZD receptors. Thus, binders to the PDZ domain of DVL proteins may disrupt the WNT signaling cascade. Three compounds, namely NSC 668036, FJ9 and Compound 3289–8625, were identified through in silico screening and nuclear magnetic resonance (NMR) spectroscopy approaches, and were shown to block WNT signaling *in vivo*<sup>274,276</sup>. The inhibitor NSC668036 provided a basis for rational design of high-affinity inhibitors of the PDZ domain and can block WNT signaling by interrupting the FZD-DVL interaction<sup>274</sup>. The inhibitor FJ9 can disrupt the interaction between FZD7 and PDZ domain of DVL<sup>275</sup>. The Compounds 3289-8625 was identified as a small molecule inhibitor of PDZ domain of DVL, and was shown to suppress the growth of prostate cancer PC-3 cells<sup>276</sup>. These results strongly suggest that blocking the PDZ domain of DVL may offer ample opportunities for developing effective and specific inhibitors of the WNT signaling pathway.

### Targeting WNT Signaling Intracellular and Nuclear Levels

**Targeting PORC and TANKS**—Recent studies have demonstrated that porcupine (PORC) and tankyrases (TANKs) may serve promising drug targets of the WNT signaling pathway. PORC is a member of the membrane-bound O-acyltransferase family and adds a palmitoyl group to WNT proteins, which is essential to their signaling ability and is required for WNT secretion<sup>34</sup>. Tankyrase 1 (TANK1) and tankyrase 2 (TANK2) are members of the larger family of poly(ADP-ribose) polymerase (PARP) enzymes. TANKs interact with a highly conserved domain of AXIN and promote its ubiquitylation and degradation<sup>277</sup>. Chen et al identified and characterized two classes of several small molecules called IWRs (inhibitors of WNT response, such as IWR-1) that stabilize the protein AXIN and IWP (inhibitors of WNT production, such as IWP-2) that inhibit the PORC acyltransferase activity<sup>278</sup>. The IWP inhibitors can efficiently inhibit WNT pathway by disrupting the WNT ligand in colon cancer cell line, while IWR-1 was confirmed as a TANK inhibitor<sup>278</sup>. Huang et al also identified another class of TANK inhibitors XAV939, which was shown to induce the stabilization of AXIN<sup>277</sup>. Another tankyrase inhibitor JW55 was shown to decrease canonical Wnt signaling in colon carcinoma cells and to reduce tumor growth in conditional APC mutant mice<sup>279</sup>. Similarly, the compound G007-LK displayed favorable pharmacokinetic properties and inhibited in vivo tumor growth in a subset of APC-mutant colorectal cancer xenograft models<sup>280</sup>. Recent efforts have been devoted to the development of more potent and selective second-generation of TANK inhibitors<sup>281</sup>. Meanwhile, a new PORC inhibitor LGK974 was shown to potently inhibit WNT signaling and exhibit strong efficacy in rodent tumor models, yet well-tolerated<sup>282</sup>, which has entered a Phase I trial by Novartis<sup>259</sup>. Another PORC inhibitor WNT C59 was shown to have 10-fold higher than the therapeutic dose to cause extensive loss of intestinal proliferation<sup>283</sup>.

**Targeting  $\beta$ -Catenin/TCF Transcription Complex**—Effectively disrupting the protein-protein interaction between TCF/LEF and  $\beta$ -catenin via small molecules is attractive but technically challenging. Nonetheless, an early high-throughput ELISA-based screening assay of approximately 7,000 natural products and 45,000 synthetic compounds, which was confirmed by the bioassay for axis duplication in *Xenopus laevis* embryos, identified two structurally related compounds, PKF115-584 and CGP049090<sup>284</sup>. Interestingly, both PKF115-584 and CGP049090 were shown to disrupt  $\beta$ -catenin-APC interaction as well<sup>284</sup>. While these compounds have not advanced to clinical trials, they indeed show anti-WNT efficacy in preclinical models of hepatocellular cancers<sup>285</sup> and hematologic cancers<sup>286</sup>. Another high-throughput screen of a large compound library, 2,4-diamino-quinazoline was identified as an inhibitor lead of the  $\beta$ -catenin-TCF4 pathway<sup>287</sup>. A cell-based high-throughput screening in *D. melanogaster* cells with a WNT-responsive luciferase reporter was carried out to screen 14,977 compounds and identified three candidates, namely iCRT3, iCRT5 and iCRT14, which were shown to disrupt the  $\beta$ -catenin-TCF interaction in vitro and to inhibit the expression of WNT target genes with cytotoxicity in colorectal cancer cells<sup>288</sup>. A combination of virtual and biophysical screening identified the synthetic compound PNU-74654 as a druglike  $\beta$ -catenin-TCF antagonist<sup>289</sup>. A virtual screen of the 1,990 small-molecule diversity set of the US National Cancer Institute identified the organo-copper complex BC21 as the top-ranked compound that can bind to the armadillo repeat<sup>290</sup>. AV-65 was identified by screening from a library of more than 100,000 small-molecule chemical compounds for novel Wnt/ $\beta$ -catenin signaling inhibitors and was shown to diminish  $\beta$ -catenin protein levels and TCF transcriptional activity, as well as to prolong the survival of multiple myeloma-bearing mice<sup>291</sup>. More recently a stapled peptide approach was used to identify potent inhibitors to target the  $\beta$ -catenin-BCL9 interface<sup>292</sup>, and the  $\beta$ -catenin-TCF4 interface<sup>293</sup>. While many of the above inhibitors possess high translational potential, their biological activity profiles and/or mechanisms of action remain to be fully defined.

**Targeting  $\beta$ -Catenin/TCF Co-Activators**— $\beta$ -Catenin/TCF complex needs to recruit the transcriptional co-activator CBP or p300 to regulate the expression of downstream target genes. A small molecule ICG-001 was identified to specifically bind to the co-activator CBP, but not p300, with high affinity<sup>294, 295</sup>. Subsequently, several small molecules (IQ-1 and ID-8), which selectively block the p300- $\beta$ -catenin interaction, were also identified<sup>296, 298</sup>. The therapeutic potential of ICG-001 was examined in several preclinical tumor models and was shown to safely eliminate drug-resistant tumor-initiating cells<sup>299, 301</sup>. Another specific CBP/ $\beta$ -catenin interaction inhibitor PRI-724 was developed by Prism Pharma and partnered with Eisai Pharmaceuticals and entered an open-label Phase Ia safety study in individuals with solid tumors<sup>259, 302</sup>.

### Targeting WNT Signaling by Nonspecific Inhibitors and Repurposed Drugs

Given the pleiotropic effects of the canonical WNT signaling pathway, it is conceivable many anticancer drugs and/or small molecule inhibitors may target WNT signaling as a part of their mode of action. For example, non-steroidal anti-inflammatory drugs (NSAIDs) and the selective COX2 inhibitor celecoxib were shown to inhibit  $\beta$ -catenin dependent transcription in colorectal cancer cells<sup>303, 304</sup>. Other molecules, including CCT036477, CCT070535 and CCT031374, also showed their inhibitory abilities in the SW480 and

HCT116 colorectal cell lines<sup>26</sup>. The tyrosine kinase inhibitor imatinib (Gleevec; Novartis) and c-MET inhibitor PHA665752 were shown to inhibit WNT/ $\beta$ -catenin signaling<sup>305,306</sup>. It was shown that inhibitors of phosphatidylinositol 3-kinase (PI3K)/AKT signaling can inhibit WNT/ $\beta$ -catenin signaling cross-talk as PDK1 inhibitor OSU03012 suppressed the growth of established medulloblastoma xenograft tumors in a dose-dependent manner and augmented the antitumor effects of mammalian target of rapamycin (mTOR) inhibitor CCI-779<sup>307</sup>. Inhibitor IQ-1 of the protein phosphatase 2A (PP2A) regulatory subunits PR72 and PR130 was shown to maintain pluripotency in murine embryonic stem cells in a WNT-dependent manner<sup>296</sup>, whereas the inhibitor ID-8 of the dual specificity YAK1-related kinases (DYRKs) was shown to allow for long-term WNT-mediated maintenance of human embryonic stem cells<sup>297</sup>. It has been reported that nuclear receptor ligands retinoic acids may induce Disabled homolog 2 (DAB2), whereas vitamin D may induce DKK1 and DKK4<sup>308</sup>.

Derivatives for some natural products, such as quercetin, epigallocatechin-3-gallate (EGCG), curcumin, resveratrol, ginsenoside Rg3, and tetrandrine, have been reported as potential WNT signaling inhibitors<sup>308,314</sup>. Silibinin, a natural compound isolated from milk thistle seed extracts, was shown to inhibit WNT/ $\beta$ -catenin signaling by suppressing LRP6 expression in human prostate and breast cancer cells<sup>315</sup>. Another natural plant polyphenol, Rottlerin, was shown to induce LRP6 degradation and suppress both WNT/ $\beta$ -catenin and mTORC1 signaling pathways in prostate and breast cancer cells<sup>316</sup>.

Several inhibitors of WNT signaling have been identified by drug-repurposing screening of the libraries of FDA-approved drugs. For example, the anti-helminthic drug pyrvinium was identified as an agent that potentiates CK1 $\alpha$  activity and thus promotes degradation of  $\beta$ -catenin and the co-activator Pygopus, leading to a reduction in WNT/ $\beta$ -catenin signaling<sup>317</sup>. Another anti-helminthic niclosamide was shown to promote FZD1 endocytosis, down-regulate DVL2 protein, and inhibit WNT3A-stimulated  $\beta$ -catenin stabilization and LEF/TCF activity<sup>318</sup>. Niclosamide was also shown to suppress cancer cell growth by inducing LRP6 degradation and inhibiting the Wnt/ $\beta$ -catenin pathway<sup>319</sup>, and niclosamide can inhibit tumor growth in human colon cancer xenograft model<sup>320</sup>. More recently, niclosamide was shown to inhibit cell proliferation and/or tumor growth in ovarian cancers, breast cancer, prostate cancer, and osteosarcoma cells<sup>321,325</sup>, although niclosamide's anticancer activity may be also mediated by inhibiting other signaling pathways<sup>322,325</sup>. The antibiotic potassium ionophores salinomycin and nigericin were shown to block the phosphorylation of LRP6 and induce its degradation, thereby down-regulating WNT signaling<sup>326</sup>. Interestingly, another polyether ionophore antibiotic, monensin, was shown to be a potent blocker of WNT-induced transcription in the cells stimulated with WNTs or GSK3 inhibitors and to inhibit the progression of intestinal tumors without any sign of toxicity on normal mucosa<sup>327</sup>. These findings suggest that many small molecule inhibitors may function as WNT signaling modulators.

## CONCLUSIONS AND FUTURE DIRECTIONS

It has been three decades since the ground-breaking discovery of WNT signaling as a fundamental and evolutionarily conserved pathway. For the past decade there has been a

rapid expansion in our understanding about the regulatory circuitry and complexity of this pathway although many details involved in the essential aspects of WNT signaling mechanisms remain to be fully elucidated. Numerous new components of Wnt/ $\beta$ -catenin signaling, such as RSPOs, LGRs, NZRF3/RNF43, PORC, and TANKS, have been identified and linked to signaling regulation, stem cell functions and tissue homeostasis. It has been well-established that WNT signaling plays important roles in regulating cell self-renewal and differentiation in many types of stem cells and cancer stem cells. The lipid-modified WNT signals act primarily over short ranges to control stem cell behavior within the spatial confines of the niche, which implies that in particular tissues WNT-dependent stem cells are spatially restricted to the vicinity of the WNT-producing niche, physically delimiting the stem cell compartment and preventing uncontrolled stem cell expansion. The short-range action feature of WNT signaling may also account for the context-dependent nature of this pathway as emerging evidence suggests that WNT/ $\beta$ -catenin signaling, as well as  $\beta$ -catenin-independent WNT signaling pathway, can either promote or inhibit cancer progression in a context-dependent manner. Our better understanding of WNT signaling has opened numerous avenues and drawn significant interests for developing novel and effective drugs that may specifically target distinct steps of the WNT signaling pathway although their efficacy and toxicity remain to be fully evaluated.

Nonetheless, the detailed mechanisms underlying WNT signaling under physiological and pathological conditions are far from clearly understood. Future directions should be directed to address the following questions: How is the specificity of individual WNT ligand's interaction with FZDs and co-receptors determined? Through what mechanisms do WNTs interact with co-receptors, such as RORs and RYK? What are the upstream regulatory signals of WNT signaling? How extensively does WNT signaling cross-talk with other major signaling pathways and/or act through what detailed mechanisms? How many downstream target genes are regulated by individual WNTs and/or how these target genes are different in different cell/tissue types? How is canonical or non-canonical WNT signaling determined and at what level(s) of the pathway? How differently do the secreted antagonists interact with individual WNT ligands in determining to transduce canonical or non-canonical signaling? Can components, such as GSK3 $\beta$ , of WNT signaling be used or hijacked by other signaling pathways? How is  $\beta$ -catenin transported into the nucleus? Can we identify any bona fide, safe and effective WNT inhibitors and eventually move them to treat human diseases? With the rapid technological advances in genomics and systems biology, we expect to get satisfactory answers for many of the above questions in next 5-10 years.

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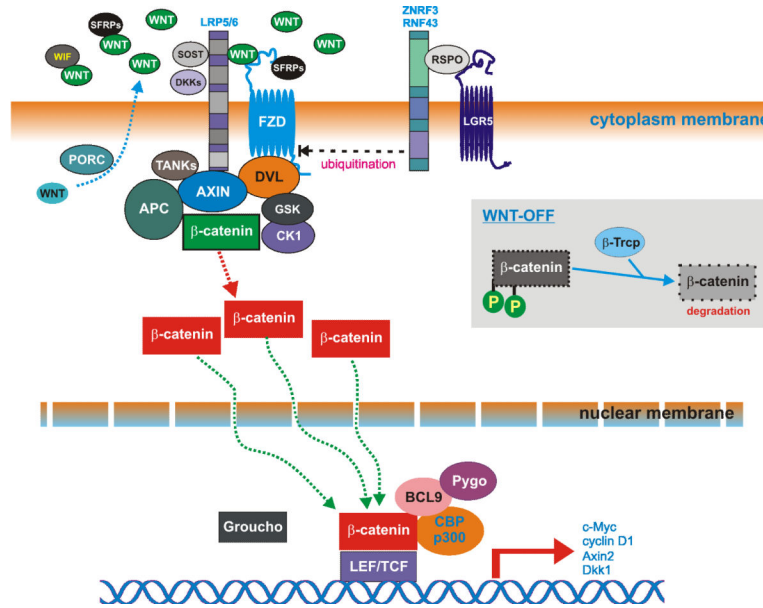


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**Figure 1. Schematic depiction of the canonical WNT signaling pathway**

WNT ligands are post-translationally modified in ER with the participation of porcupine (PORC) and secreted into the extraextracellular space, where WNTs interact with receptors FZDs and co-receptors LRP5/6. Antagonists SFRPs and WIF can bind to WNT ligands while SFRPs can also interact with FZDs. Antagonists SOST and DKKs bind to LRP5/6 to compete with WNT ligands. WNT binding to the receptors initiates the disassembly of the destruction complex consisting of DVL/AXIN/APC/GSK3 $\beta$ /CK1, resulting in the release of the stabilized  $\beta$ -catenin to cytoplasm, which is subsequently translocated into cell nucleus and regulates WNT target genes in concert with co-activators BCL9, Pygo and CBP/p300. TANKs interact with AXIN and promote its degradation. RSPOs interact with LRG5 and membrane-bound E3 ubiquitin ligases ZNRF3/RNF43 and inhibit the ubiquitination-mediated degradation of FZDs. In WNT-OFF cells (the inserted box), the destruction complex is assembled, and the  $\beta$ -catenin protein is phosphorylated by CK1 and GSK3 $\beta$ , and doomed for proteasome-mediated protein degradation.

**Table 1**

## Currently Known Inhibitors of the Canonical WNT Signaling Pathway

Molecular Targets	Inhibitors	Anti-WNT and Anticancer Activities	Stage of Development	References
WNTs	Antibodies	WNT-blocking antibodies were developed and shown to inhibit proliferation and induce apoptosis in different cancers	preclinical	21, 262, 265
	SFRPs/WIF	overexpress naturally occurring antagonists of WNT ligands		266, 268
	SFRP peptides	SFRP1 and SFRP1-derived peptides can delay HCT116 xenograft tumor formation		266
	DNA demethylation agents	use DNA demethylation agents to reverse hypermethylation of SFRP promoters		273
FZDs	OMP18R5	humanized monoclonal antibody that binds to FZD1, FZD2, FZD5, FZD7 and FZD8	Phase Ia	28
	FZD8CRD	fusion protein consisting of the Fc region of IgG fused to the extracellular domain of FZD8	preclinical	270, 271
DVL	NSC668036	inhibits the DVL PDZ domain, not reported in cancer	Preclinical	274, 276
	3289-8625	inhibits the growth of prostate cancer PC-3 cells		
	FJ9	disrupts the interaction between FZD7 and the PDZ domain of DVL, induces apoptosis and inhibits H460 lung cancer growth		
	sulindac	inhibits proliferation of lung cancer A549 cells	FDA Approved	300, 301
TANKs	XAV-939	inhibits colony formation of $\beta$ -catenin-dependent DLD-1 cells	Preclinical	277
	JW55	decreases canonical Wnt signaling in SW480 and HCT-15 colon carcinoma cell lines; reduces cell cycle progression and proliferation in SW480 cells in vitro		279
	G007-LK	suppresses APC mutation-driven colorectal tumor growth		280
	IWR-1	inhibits L-cells expressing Wnt3A		278
PORC	IWP	inhibits colorectal cancer cells invasion by WISP2	Preclinical	278
	LGK-974	inhibits growth of Mouse MMTV-WNT1 tumor model and human head and neck squamous cell carcinoma model	Phase I clinical trial	259, 282
	WNT C59	PORC inhibitor with 10-fold therapeutic dose over toxic dose	Preclinical	283
Activation of CK1 $\alpha$ to promote $\beta$ -catenin degradation	Pyrrvinium	Pyrrvinium synergizes with 5-fluorouracil in mediating the apoptosis of SW620 colorectal cancer cells and inhibits the	Preclinical	317

Molecular Targets	Inhibitors	Anti-WNT and Anticancer Activities	Stage of Development	References
		proliferation of SW480 and HCT116 cells.		
β-catenin/TCF interaction	iCRT3, iCRT5, and iCRT14	reduced the growth of colorectal cancer cells	Preclinical	288
	PKF115-584, CGP049090 and PKF118-310	inhibit the growth of HCC cells in xenografts		284, 286
	2,4 diamino-quinazoline	inhibitor lead of the β-catenin-TCF4 pathway		287
	PNU-74654	a druglike β-catenin-TCF antagonist		289
	BC21	an organo-copper complex as the top-ranked compound that can bind to the armadillo repeat		290
	AV-65	inhibits progression of multiple myeloma in a mouse model		291
	stapled peptides	potent inhibitors to target the β-catenin-BCL9 interface and the β-catenin-TCF4 interface		292, 293
β-catenin/CBP interaction	ICG-001	decreases xenograft growth of SW620 colon carcinoma cells	Preclinical	294, 295
	PRI-724	downregulates survivin (BIRC5) expression in circulating tumour cells, suppresses growth of refractory pancreatic cancer	Phase Ia	259, 302
β-catenin/TCF-regulated transcription; signal crosstalk; non-specific or overlapping targeting	CCT036477, CCT070535, and CCT031374	Inhibits the growth of SW480 and HCT116 colorectal cell lines	Preclinical	26
	OSU03012	PDK1 inhibitor OSU03012 inhibits the growth of various medulloblastoma cell lines		307
	Celecoxib	induces apoptosis in cervical cancer cells	FDA Approved	303, 304
	Imatinib	tyrosine kinase inhibitor that inhibits TCF/β-catenin activity	FDA Approved	305
	PHA665752	c-MET inhibitor shown to inhibit WNT/β-catenin signaling	Preclinical	306
	OSU03012	PDK1 inhibitor to suppress medulloblastoma xenograft tumors		307
	IQ-1	protein phosphatase 2A (PP2A) regulatory subunits PR72 and PR130 shown to maintain pluripotency in murine ES cells in a WNT-dependent manner		296
	ID-8	dual specificity YAK1 related kinases (DYRKs) shown to allow for long-term WNT-mediated maintenance of human ES cells		297
	Retinoic acids	induces Disabled homolog 2 (DAB2)		308
	Vitamin D	induces DKK1 and DKK4		308
	Natural products	quercetin, epigallocatechin-3-gallate (EGCG), curcumin, resveratrol, ginsenoside Rg3, and tetrandrine		308, 314

Molecular Targets	Inhibitors	Anti-WNT and Anticancer Activities	Stage of Development	References
	Silibinin	suppressing LRP6 expression in human prostate and breast cancer cells		315
	Rottlerin	shown to induce LRP6 degradation and suppress both WNT/ $\beta$ -catenin and mTORC1 signaling pathways in prostate and breast cancer cells		316
	Nicosamide	promoting FZD1 endocytosis, down-regulating DVL2 protein, and inhibiting WNT3A-stimulated $\beta$ -catenin stabilization and LEF/TCF activity; anticancer activity in WNT-independent manner as well	FDA Approved	319_325
	Salinomycin	blocking the phosphorylation of LRP6 and induce its degradation	Preclinical	326
	Monensin	a potent blocker of WNT-induced transcription and to inhibit the progression of intestinal tumors without any sign of toxicity on normal mucosa		327

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