# Genetic Alterations of Wnt Signal Components in Cancer Cells

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## ABSTRACT

The genetics of development and cancer have converged in the identification of intra- and extra-cellular signaling pathways that are aberrantly regulated in cancer and are also central to embryonic patterning. The Wnt signaling pathway has provided an outstanding example of this. The genes for β-catenin, APC, and Axin in the Wnt signaling pathway are often mutated in human cancers. In all such cases, the common denominator is the accumulation of cytosolic and nuclear β-catenin and the activation of transcriptional factor Tcf/Lef. The resulting gene expression profile should provide a significant clue as to cancers carrying defects in the Wnt signaling pathway. In this review, the regulation of the β-catenin stability by Axin and APC, and their genetic alterations in human cancers are described.

**Key Words:** Wnt; β-catenin, APC, Axin, Tcf

## 1. Outline of the Wnt Signaling Pathway

Wnt proteins constitute a large family of cysteine-rich secreted ligands that control development in organisms ranging from nematode worms to mammals (Wodarz and Nusse, 1998). The intracellular signaling pathway of Wnt is also conserved evolutionally and regulates cellular proliferation, morphology, motility, fate, axis formation, and organ development (Wodarz and Nusse, 1998; Polakis, 2000). Wnt regulates at least three distinct pathways; the canonical β-catenin pathway, planar cell polarity pathway, and $Ca^{2+}$ pathway. It has been shown that abnormalities of the canonical β-catenin pathway lead to several human diseases including tumor formation and bone abnormalities. According to the most widely accepted current model of the β-catenin pathway (Fig. 1), casein kinase Iα (CKIα) and glycogen synthase kinase-3β GSK-3β) phosphorylate β-catenin in the Axin complex (Ikeda *et al.*, 1998; Kikuchi, 1999; Liu *et al.*, 2002). Phosphorylated β-catenin is ubiquitinated, resulting in the degradation of β-catenin by the proteasome (Kitagawa *et al.*, 1999). As a result, the cytoplasmic β-catenin level is low.

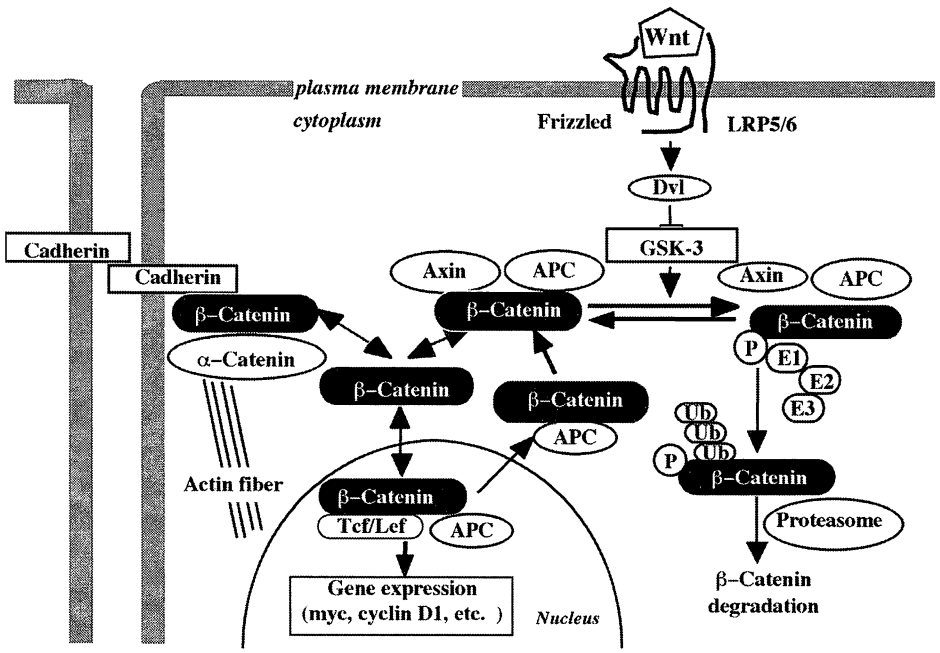


**Fig. 1.** Wnt signaling pathway. Wnt activates Tcf/Lef-dependent gene expression through stabilization of β-catenin. P, phosphorylation; Ub, ubiquitination; APC, adenomatous polyposis coli gene product.

When Wnt acts on its cell-surface receptor consisting of Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6), β-catenin is escaped from the degradation in the Axin complex although the mechanism is not clear (He *et al.*, 2004). Accumulated β-catenin is translocated to the nucleus, where it binds to the transcription factor T cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby stimulates the expression of various genes (Polakis, 2000; Hurlstone and Clevers, 2002). The broad impact of the Wnt signal on gene expression makes it a key element in regulation of cell survival, apoptosis, cell motility, cytoskeletal structure, and cell adhesion. Thus, Wnt increases the stability of β-catenin, thereby stimulating Tcf/Lef-mediated gene expression in the canonical β-catenin pathway.

Among various molecules involved in this pathway, alterations in the β-*catenin*, *APC*, and *Axin* genes have been frequently found in several human cancers. In these cancer cells β-catenin is abnormally accumulated and nucleus of these cancer cells and Tcf-mediated gene expression is increased. Since APC and Axin induce the degradation of β-catenin, it is conceivable that β-catenin functions as an oncogene product, while APC and Axin act as tumor suppressor gene products.

## 2. Regulation of degradation of β-catenin

### (1) Degradation of β-catenin through phosphorylation

Cytoplasmic β-catenin is a target for the ubiquitin-proteasome pathway and the phosphorylation by GSK-3β and CKI α is required for its ubiquitination (Fig. 2). In general, degradation of proteins by the ubiquitin-proteasome pathway involves an ubiquitin-activation enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) (Ciechanover, 1998). The ubiquitin ligase is generally thought to be directly involved in substrate recognition and consists of a multiprotein complex. An F-box protein Fbw1, which is a component of the ubiquitin ligase for β-catenin, associates with β-catenin phosphorylated by GSK-3β and CKI α and stimulates ubiquitination and degradation of β-catenin (Kitagawa *et al.*, 1999; Liu *et al.*, 2002). The amino acid sequence for the phosphorylation of β-catenin is $D^{32}SGXXSXXXTXXXS^{45}$ (D, asparatate; S, serine; G, glycine; T, threonine; X, any amino acid). CKI α -dependent phosphorylation of $S^{45}$ proceeds and the phosphorylation enhances subsequent GSK-3β-dependent phosphorylation of $T^{41}$, $S^{37}$, and $S^{33}$. $D^{32}$ and $G^{34}$ are necessary for the interaction of phosphorylated β-catenin with Fbw1. Therefore, Fbw1 directly links the phosphorylation machinery to the ubiquitination apparatus.

### (2) Roles of Axin and APC as scaffold proteins in the degradation of β-catenin

Axin binds to various components of the Wnt signaling pathway (Kikuchi, 1999). APC binds to the RGS domain (Behrens *et al.*, 1998; Kishida *et al.*, 1998). GSK-3β, β-catenin, and CKI α interact with the different sites of the central region of Axin (Ikeda *et al.*, 1998; Liu *et al.*, 2002). Dvl binds to the following C-terminal region of Axin including the DIX domain (Kishida *et al.*, 1999a). In the Axin complex, CKI α and GSK-3β phosphorylate β-catenin efficiently and phosphorylated β-catenin is ubiquitinated and degraded by the proteasome (Ikeda *et al.*, 1998; Liu *et al.*, 2002). Indeed, expression of Axin induces the downregulation of β-catenin in various cell lines (Kishida *et al.*, 1998; Kishida *et al.*, 1999a).

APC also acts as a critical component for β-catenin destruction (Polakis, 2000; Fodde *et al.*, 2001). In colon

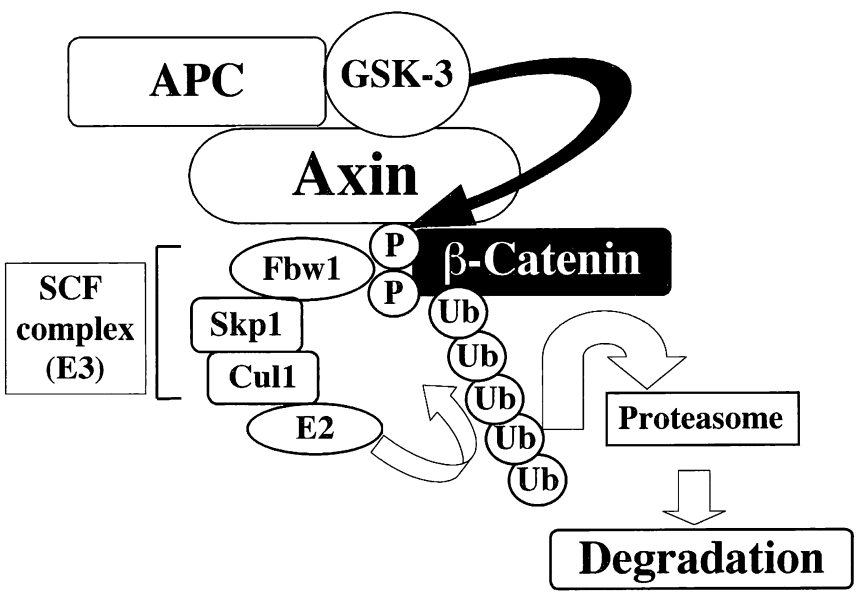


**Fig. 2.** Degradation of β-catenin in the Axin complex. Axin enhances GSK3-dependent phosphorylation and ubiquitination of β-catenin and accelerates degradation of β-catenin. P, phosphorylation; Ub, ubiquitination.

cancers, mutations of APC correlate with high levels of β-catenin and transcriptionally active Tcf/β-catenin complexes. Expression of wild-type APC in colorectal cancer cells reduces β-catenin levels, and the fragment of APC containing the 20-aa repeats is sufficient for the activity (Polakis, 2000; Fodde *et al.*, 2001). However, an APC fragment with either mutated β-catenin-binding sites or Axin-binding sites fails to induce degradation of β-catenin (Kawahara *et al.*, 2000). Therefore, the interaction of APC with both Axin and β-catenin is required for the ability of APC to degrade β-catenin. In the complex, GSK-3β bound to Axin efficiently phosphorylates APC, which enhances the binding of β-catenin to APC (Rubinfeld *et al.*, 1996; Ikeda *et al.*, 2000), and GSK-3β phosphorylates β-catenin bound to APC in addition to β-catenin bound to Axin (Hinoi *et al.*, 2000). It is likely that APC activates Axin in a manner that facilitates the phosphorylation of β-catenin by GSK-3β. Thus, Axin and APC form a core complex to degrade β-catenin.

## 3. Wnt-dependent accumulation of β-catenin

### (1) Receptor internalization in response to Wnt

The initial event of the Wnt signal is that Wnt binds its receptor consisting of Frizzzled and LRP5/6 (He *et al.*, 2004). The mechanism by which Frizzled and LRP5/6 transduce the signals remains elusive. *Drosophila* genetics have shown that expression of a dominant negative form of dynamin abolishes cuticle deposition, indicating that receptor-mediated endocytosis triggers the Wnt signaling (Moline *et al.*, 1999). Wnt-5a, which is a representative ligand that does not accumulate β-catenin, induces the internalization of Frizzled4 in cooperation with Dvl and β-arrestin2 (Chen *et al.*, 2003). In this endocytosis, Frizzled4 requires PKC-dependent phosphorylation of Dvl. Since β-arrestin2 binds to clathrin, Frizzled4 could be internalized in a clathrin-mediated pathway. However, the endocytosis of receptors such as Frizzled 5 and LRP5/6 that are involved in the canonical β-catenin pathway has not yet been clarified.

### (2) Stabilization of β-catenin in response to Wnt

Although the exact mechanism by which Wnt stabilizes β-catenin is unclear, several possible mechanisms have been proposed. The first one is based on the interaction of Dvl with Frat. Dvl binds to CKI ε and Axin (Kishida *et al.*, 1999b; Kishida *et al.*, 2001). CKI ε mediates Wnt-3a-dependent phosphorylation of Dvl and phosphorylated Dvl has a high affinity for Frat, which binds to and inhibits GSK-3 (Fig. 1) (Kishida *et al.*, 2001; Lee *et al.*, 2001; Hino *et al.*, 2003). Knockdown of CKI ε by RNA interference reduces the Wnt-3a-induced binding of Dvl to Frat and accumulation of β-catenin (Hino *et al.*, 2003). Therefore, when Wnt acts on the cells, Frat bound to Dvl phosphorylated by CKI ε may prevent GSK-3β bound to

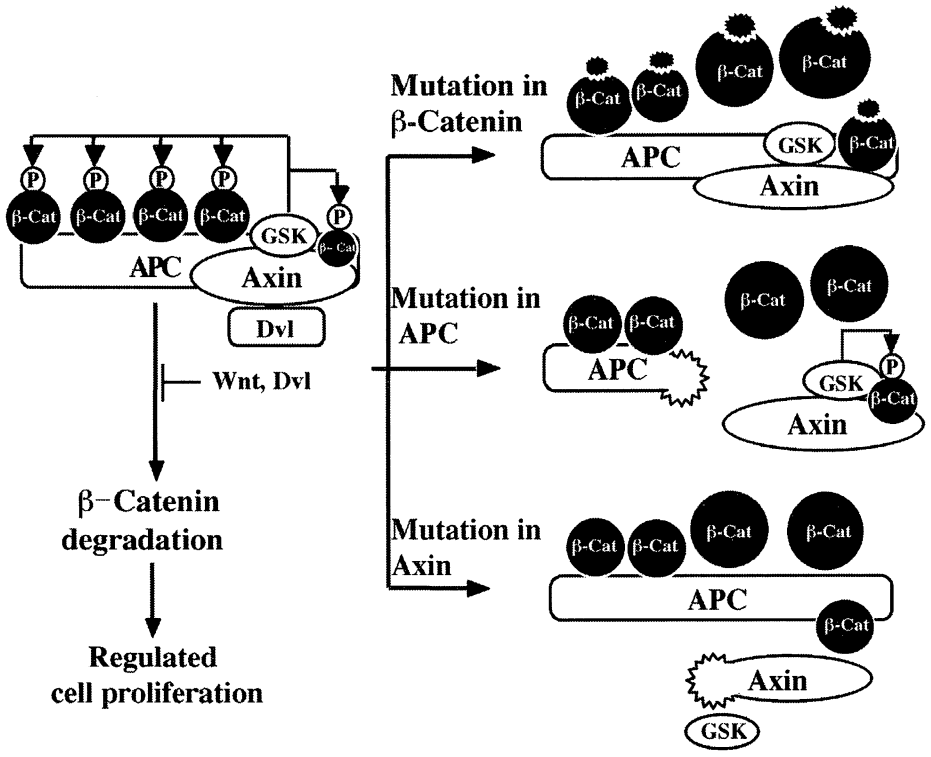


**Fig. 3.** Mutations of β-catenin, APC, and, Axin, and the mechanism of the abnormal accumulation of β-catenin. Wnt and Dvl suppress the degradation of β-catenin by antagonizing the functions of the Axin complex by unknown mechanisms. Mutations of β-catenin, APC, or Axin disturb the functions of the Axin complex, resulting in unregulated accumulation of β-catenin.

Axin from phosphorylating β-catenin, thereby stabilizing β-catenin. Taken together, Axin binds to positive and negative regulators of the Wnt signaling pathway and regulates the stability of β-catenin.

The second model is based on the interaction of Axin with LRP5/6. Wnt causes the translocation of Axin to the membrane and enhances the interaction between Axin and LRP5/6 (Mao *et al.*, 2001; Tamai *et al.*, 2004). The phosphorylation of LRP6 by an unknown kinase enhances their interaction, which is essential for the transmitting the signal to activate Lef and axis duplication in Xenopus embryos. Dvl and Axin have the DIX domain that is necessary for the binding of Dvl and Axin to intracellular vesicles and actin filaments (Capelluto *et al.*, 2002). Furthermore, disheveled (a fly Dvl homolog), is required for the recruitment of dAxin (a fly Axin homolog) to the plasma membranes in a manner dependent of wingless in fly cells (Cliffe *et al.*, 2003). However, how the interaction of Axin with LRP5/6 activates the β-catenin pathway is unknown.

## 4. Genetic alterations of β-catenin, APC, and Axin

### (1) β-catenin

Mutations in the β-catenin gene that affect specific serine and threonine residues, and amino acids adjacent to them, which are essential for the targeted degradation of β-catenin, are found in a wide variety of human cancers including colon cancers, desmoid, gastric cancer, hepatocarcinoma, medulloblastoma, melanoma, ovarian cancer, pancreatic cancer, and prostate cancer (Polakis, 2000). These mutations abrogate the phosphorylation-dependent interaction of β-catenin with Fbw1, thereby stabilizing β-catenin. The β-catenin mutations occur in $D^{32}$, $S^{33}$, $G^{34}$, $S^{37}$, $T^{41}$, and $S^{45}$ (Fig. 3). As described above, $S^{45}$ is a phosphorylation site of CKI $\alpha$, and $S^{33}$, $S^{37}$, and $T^{41}$ are phosphorylation sites of GSK-3β. $D^{32}$ and $G^{34}$ are necessary for the interaction of β-catenin with Fbw1. Therefore, the mutated β-catenin cannot bind to Fbw1, and thereby is accumulated in the nucleus and activates Tcf/Lef. Myc and cyclin D1, which are representative of genes expressed by Tcf/Lef, are clearly relevant in tumor formation, because of their roles in proliferation, apoptosis, and cell-cycle regulation. Changes in the expression of Myc and cyclin D1 are likely to increase the overall proliferating rate. The proteins of other Tcf/Lef target genes such as matrilysin, CD44, and urokinase-type plasminogen activator receptor, seem more likely to be involved in tumor proliferation, rather than initiation.

### (2) APC

Mutations in the *APC* gene are frequently identified in familial adenomatous polyposis coli (FAP) and colorectal cancers, but quite rare in other cancers (Polakis, 2000; Fodde *et al.*, 2001). Most APC mutations result in truncated proteins that lack all Axin-binding motifs and a variable number of the 20-aa repeats (Fig. 3). In FAP, germ-line mutations are scattered throughout the 5' half of the APC gene; by contrast, most somatic mutations are clustered between codons 1286 and 1513. In any case, mutations of APC relate strongly to the regulation of the stability of β-catenin. Selective pressure is directed against the presence of Axin-binding sites, because the presence of Axin-binding sites is critical to APC in the regulation of β-catenin levels. The remaining N-terminal truncated form of APC may affect cell migration by activating Asef, thereby leading to metastasis.

Importantly, the overall frequency of β-catenin mutations is quite low in colorectal cancers with APC mutations, and those with an intact APC gene contain mutations of β-catenin that alter phosphorylation sites (Polakis, 2000; Fodde *et al.*, 2001). The exclusivity of β-catenin and APC mutations in colorectal cancers is also evident from the analysis of replication error-positive tumors identified by microsatellite instability. Both the hereditary and sporadic forms of replication error-positive colorectal cancers have a relatively high frequency of β-catenin mutations, where APC mutations are relatively rare (Miyaki *et al.*, 1999).

### (3) Axin

Axin was expected to be a tumor suppressor based on its ability to downregulate β-catenin, and this has been indeed verified by documentation of its biallelic inactivation in human hepatocarcinoma (Satoh *et al.*, 2000). Importantly, these mutations of Axin are found in hepatocarcinomas with intact genes for β-catenin and APC. All of the mutations generate a truncated form of Axin eliminated the β-catenin binding site (Fig. 3). Therefore, Axin is now regarded as a tumor suppressor, and constitutes the third genetic defect in the Wnt signaling pathway.

There exists an Axin homolog, termed rat Axil (Yamamoto *et al.*, 1998), mouse conductin (Behrens *et al.*, 1998), or human Axin2. The biochemical characteristics of Axil are similar to those of Axin, and Axil is able to downregulate β-catenin under conditions of overexpression. However, this redundant protein does not suppress Axin mutations in hepatocarcinoma. Therefore, Axil/conductin/Axin2 is either not functionally equivalent to Axin or not expressed at levels sufficient to compensate for its loss in hepatocarcinoma.

## CONCLUSION

There are two complexes containing β-catenin, the Axin and Tcf complexes, in the cytoplasm and nucleus, respectively. Wnt may regulate the subcellular distribution of β-catenin between the cytoplasm and nucleus. In the cytoplasm, the amount of β-catenin is negatively regulated in the Axin complex. In the nucleus, gene expression induced by β-catenin is negatively regulated by inhibiting the complex formation of β-catenin, Tcf, and DNA. Mutations in *β-catenin, APC, and Axin* genes have been found in human cancers, and the mutations result in the accumulation of β-catenin. Since β-catenin functions as an oncogene, it is speculated that there are several mechanisms for protecting against abnormal cellular proliferation by inhibiting β-catenin signaling.

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