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Regulation of Wnt signaling by protein-protein interaction and post-translational modifications

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Abbreviations: APC, *adenomatous polyposis coli* gene product; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CBP, CREB-binding protein; CKIα, casein kinase Iα; Gs, the oligomeric GTP-binding protein; GSK-3β, glycogen synthase kinase-3β; HMG, high mobility group; Lef, lymphoid enhancer factor; LRP5/6, lipoprotein receptor-related protein 5/6; NLK, NEMO-like kinase; PIAS, protein inhibitor of activated STAT; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SUMO, small ubiquitin-related modifier; Tcf, T-cell factor

Abstract

The Wnt signaling pathway is conserved in various species from worms to mammals, and plays important roles in cellular proliferation, differentiation, and migration. Wnt stabilizes cytoplasmic β-catenin and then the accumulated β-catenin is translocated into the nucleus, where it activates the transcriptional factor T-cell factor (Tcf)/lymphoid enhancer factor (Lef), and thereby stimulates the expression of genes including c-*myc*, c-*jun*, *fra-1*, and *cyclin D1*. Tight regulation of this response involves post-translational modifications of the components of the Wnt signaling pathway. Phosphorylation, ubiquitination, and sumoylation have been shown to affect the half-life of β-catenin and the transcriptional activity of Tcf/Lef. The precise spatio-temporal patterns of these multiple modifications determine the driving force of various cellular responses.

Keywords: beta catenin; protein interaction mapping; protein processing, post-translational; TCF transcription factors; Wnt proteins

Introduction

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 (Veeman *et al.*, 2003; Nelson and Nusse, 2004) (Figure 1). Among these intracellular cascades, the canonical β-catenin pathway has been most extensively studied. It has been shown that abnormalities of this 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, when Wnt does not act on the cells, 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) (Figure 2). 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. When Wnt acts on its cell-surface receptor consisting of Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6), β-catenin escapes from degradation in the Axin complex (He *et al.*, 2004) (Figure 2). The 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) (Figure 2). Thus, in the canonical β-catenin pathway, Wnt increases the stability of β-catenin, thereby stimulating Tcf/Lef-mediated gene expression. The details of the non-canonical pathway such as the PCP and Ca^{2+} pathways are described in other reviews (Veeman *et al.*, 2003).

The level of complexity of a particular species cannot be entirely explained by the number of genes that a particular organism possesses, a conclusion that was drawn from genome sequencing data. Additional post-translational modifications may help to explain the phenotype differences, for example, between chimpanzees and humans, whose genomic DNA sequences are so highly conserved. Post-translational modifications are defined as enzyme-

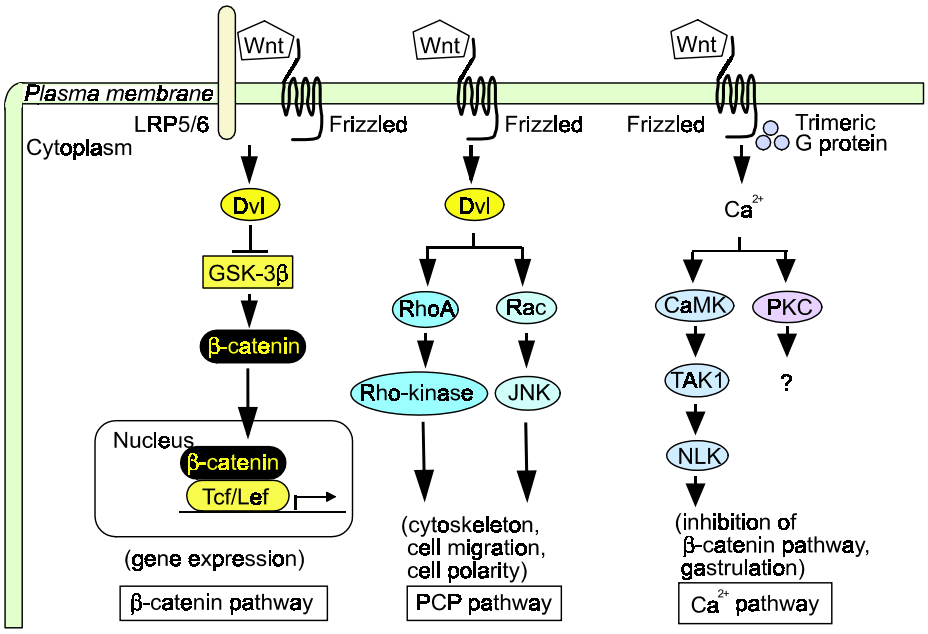

Figure 1. Wnt signaling pathways. The binding of Wnt to its receptors can stimulate at least three distinct signaling pathways: the β-catenin pathway, PCP (planar cell polarity) pathway, and Ca^{2+} pathway. The β-catenin pathway regulates gene expression through the accumulation of β-catenin. The PCP pathway activates Dvl, small G proteins (Rho or Rac), and Rho kinase or JNK, resulting in regulation of the cytoskeleton, cell migration, and cell polarity. Although the roles of the Ca^{2+} pathway are not known, it activates CaMK, PKC, TAK, and NLK, resulting in the inhibition of the β-catenin pathway and regulates gastrulation. JNK, Jun N-terminal kinase; CaMK, calmodulin-dependent protein kinase.

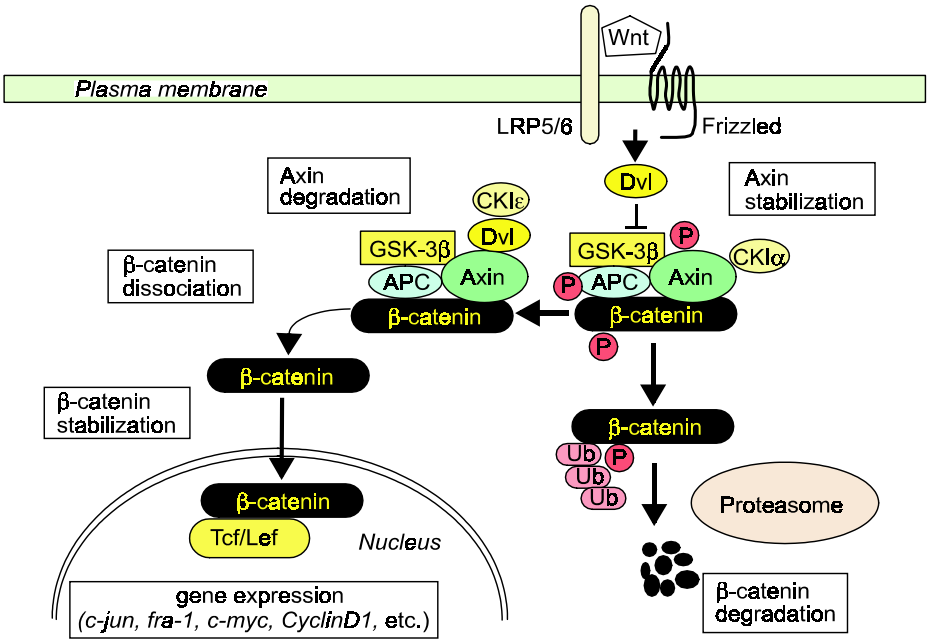

Figure 2. β-Catenin pathway. Cytoplasmic β-catenin is destabilized by a multi-protein complex containing Axin, GSK-3β, CKIα, and APC in the absence of Wnt. β-Catenin is phosphorylated by CKIα and GSK-3β efficiently in this complex, and phosphorylated β-catenin is ubiquitinated and degraded by the proteasome. When Wnt binds to its cell surface receptor consisting of Frizzled and LRP5/6, the phosphorylation of β-catenin by GSK-3β is suppressed, and consequently β-catenin is accumulated in the cytosol. The accumulated β-catenin is translocated into the nucleus, where it binds to and activates Tcf/Lef, resulting in expression of the target genes. P, phosphorylation; Ub, ubiquitin.

catalyzed changes to a protein made after it is synthesized. Various post-translational modifications have been identified, and these include phosphorylation, acetylation, methylation, ubiquitination, sumoylation, glycosylation, and lipidation (Krishna and Wold, 1993). Covalent modifications to proteins have been shown to have a profound impact at both the molecular and cellular level. For instance, the introduction of phosphoryl, glycosyl and other chemical groups to proteins can alter the activity, affect the subcellular localization, or modulate the interaction with other macromolecules. At a more global cellular level, post-translational modifications have been shown to influence cellular events such as gene expression, cell cycle progression, and programmed cell death.

The regulation of the Wnt signaling involves covalent modifications of components of this pathway.

A substantial number of excellent reviews on the biology of the Wnt signaling pathway have been published in the last few years (Hurlstone and Clevers, 2002; Veeman *et al.*, 2003; He *et al.*, 2004; Nelson and Nusse, 2004). Keeping this in mind, we have attempted to focus in particular on recent advances made in the understanding how Wnt transmits the signal specifically by the different post-translational modifications.

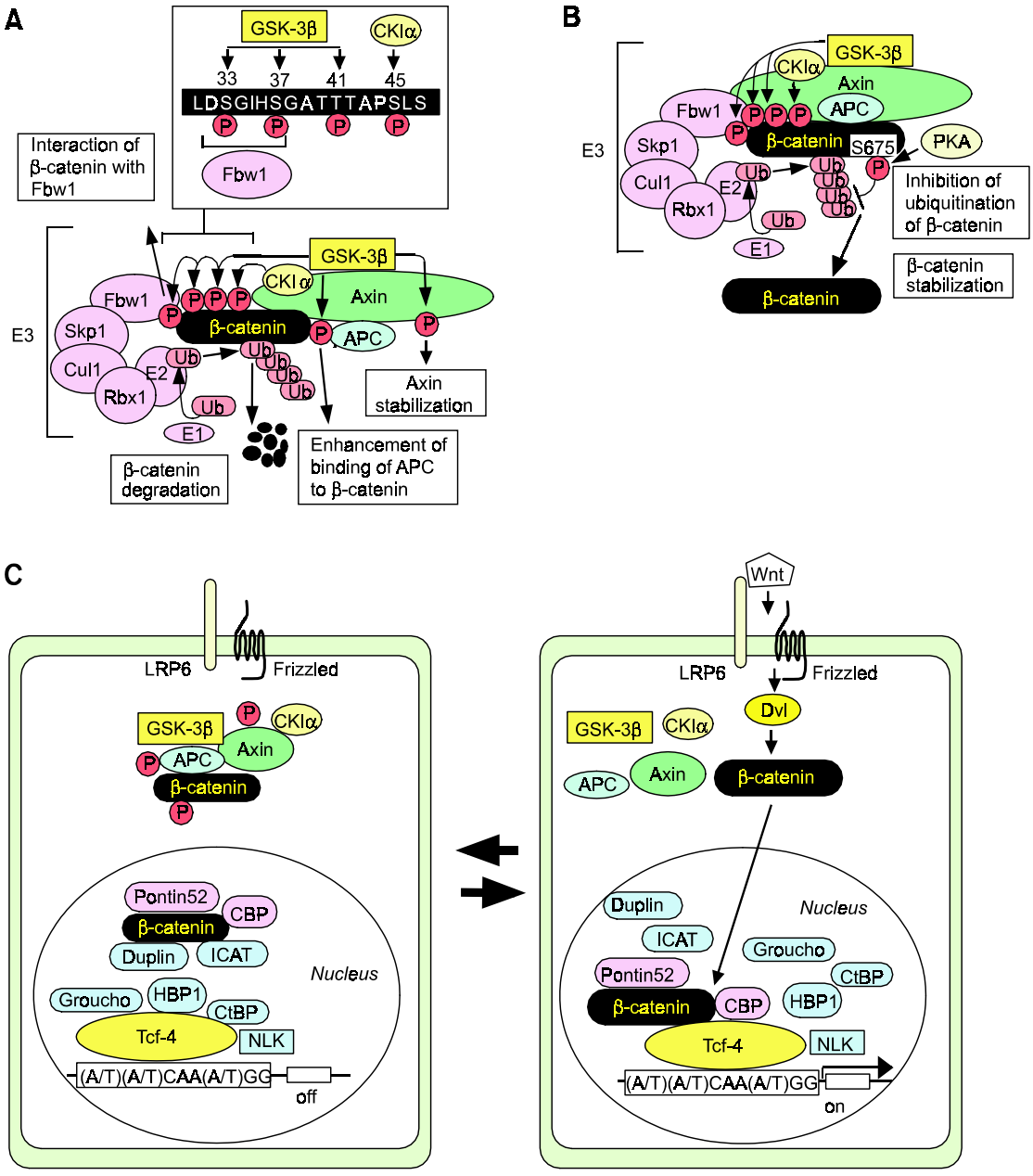

Figure 3. Regulation of β-catenin stability and Tcf-4 transcriptional activity. (A) β-Catenin degradation in the Axin complex. β-Catenin is phosphorylated by CKIα and GSK-3β efficiently in the Axin complex. Phosphorylated β-catenin is recognized by Fbw1 and ubiquitinated. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-ligase. (B) Stabilization of β-catenin by PKA. PKA-dependent phosphorylation of β-catenin at Ser^{675} inhibits the ubiquitination of β-catenin, although GSK-3β -dependent phosphorylation of β-catenin is not affected. (C) Tcf-4 activation. In the absence of Wnt, Tcf-4 acts as a repressor. The transcriptional activity of Tcf-4 is regulated by the binding of several proteins. β-Catenin accumulated in response to Wnt translocates to the nucleus and binds to Tcf-4. The β-catenin acts as an activator of Tcf-4.

Regulation of degradation of β-catenin

Degradation of β-catenin through phosphorylation

Cytoplasmic β-catenin is a target for the ubiquitin-proteasome pathway, and phosphorylation by GSK-3β and CKIα is required for β-catenin ubiquitination. In general, the degradation of the proteins by the ubiquitin-proteasome pathway involves a ubiquitin-activation enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a 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 CKIα and stimulates the ubiquitination and degradation of β-catenin (Kitagawa *et al.*, 1999; Liu *et al.*, 2002) (Figures 2 and 3A). The amino acid sequence specifying the phosphorylation of β-catenin is $D^{32}SGXXSXXXTXXXS^{45}$ (D, asparatate; S, serine; G, glycine; T, threonine; X, any amino acid). CKIα-dependent phosphorylation of Ser^{45} proceeds and the phosphorylation induces subsequent GSK-3β-dependent phosphorylation of Thr^{41}, Ser^{37}, and Ser^{33}. Asp^{32} and Gly^{34} are necessary for the interaction of phosphorylated β-catenin with Fbw1. Therefore, Fbw1 directly links the phosphorylation machinery to the ubiquitination apparatus.

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) (Figures 3A and 4A). *Adenomatous polyposis coli* gene product (APC) binds to the RGS domain of Axin (Behrens *et al.*, 1998; Kishida *et al.*, 1998). GSK-3β, β-catenin, and CKIα 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, CKIα and GSK-3β phosphorylate β-catenin efficiently and phosphorylated β-catenin is ubiquitinated and degraded by the proteasome (Ikeda *et al.*, 1998; Kitagawa *et al.*, 1999; Liu *et al.*, 2002). Indeed, the expression of Axin induces the downregulation of β-catenin in various cell lines (Kishida *et al.*, 1998; 1999a).

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

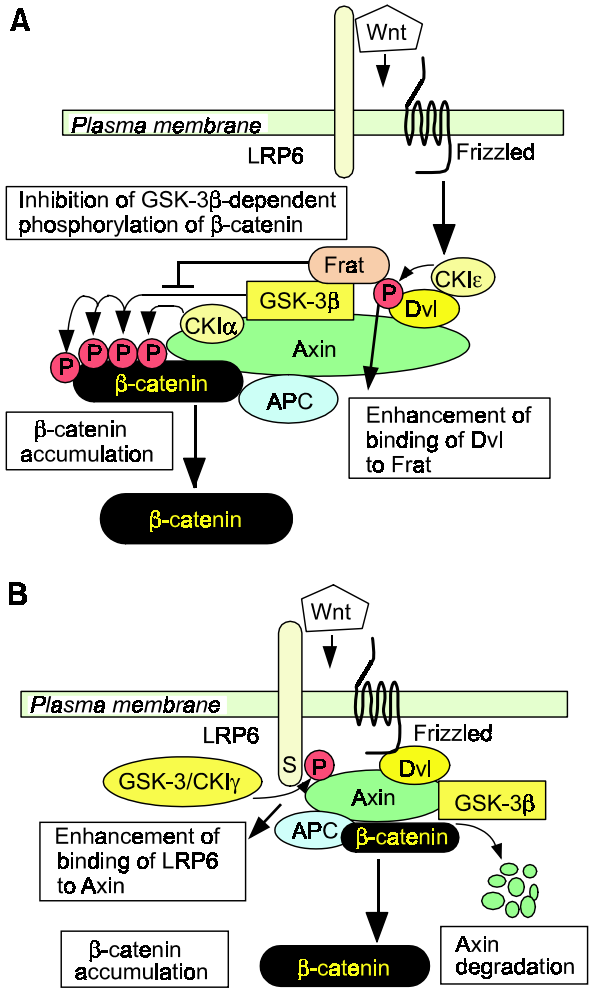

Figure 4. Wnt-dependent stabilization of β-catenin. (A) Involvement of the Dvl/Frat complex. Wnt triggers CKIε-dependent phosphorylation of Dvl and enhances the binding of Dvl to Frat-1. Frat-1 binds to GSK-3β in the Axin complex, resulting in a reduction of the phosphorylation of β-catenin by GSK-3β. (B) Involvement of the Axin and LRP6 complex. Wnt triggers the phosphorylation of LRP6 by CK1γ and GSK-3β, which provides a docking site for Axin and recruits it to the plasma membrane. Wnt also enhances the binding of Dvl to Frizzled, and Dvl bound to Frizzled is necessary for the formation of a complex between Axin and LRP6, resulting in reduced phosphorylation of β-catenin and Axin by GSK-3β.

the 20-amino acids repeats is sufficient for this activity (Polakis, 2000; Fodde *et al.*, 2001). However, an APC fragment with either a mutated β-catenin-binding site or Axin-binding site fails to induce the 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 phosphory-

lates 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.

Degradation of β-catenin in phosphorylation-independent manner

Although CKIα- and GSK-3β-dependent phosphorylation is essential for the degradation of β-catenin, a phosphorylation-independent pathway has also been reported (Liu *et al.*, 2001; Matsuzawa and Reed, 2001). Siah binds to the ubiquitin-conjugating enzyme with its N-terminal RING domain and forms a complex with Ebi through SIP and Skp1. Ebi is an F-box protein that binds to β-catenin. The complex of SIP, Skp1, and Ebi functions as a ubiquitin ligase. Since Siah downregulates a β-catenin mutant in which the serine and threonine residues between codons 33 and 45 are mutated, and a dominant-negative form of Fbw1 does not block Siah-mediated downregulation of β-catenin, Siah is likely to promote the degradation of β-catenin through a mechanism independent of GSK-3β-mediated phosphorylation and the Fbw1-mediated proteasome pathway. Interestingly, Siah binds to the C-terminal region of APC, and the downregulation of β-catenin is dependent on APC. Another interesting aspect of this pathway is that Siah is a p53-inducible regulator of cell cycle arrest and apoptosis. Indeed, Siah mediates the p53-inducible degradation of β-catenin. Although it is clear that there is a novel pathway by which Siah and APC mediate β-catenin degradation linked to the p53 response, the physiological role of this pathway in the regulation of β-catenin stability is not fully understood.

Wnt-dependent accumulation of β-catenin

Receptor internalization in response to Wnt

The initial event of the Wnt signal is that Wnt binds its receptor consisting of Frizzled and LRP5/6 (He *et al.*, 2004). There are 19 *Wnt* genes, 10 *Frizzled* genes, and 2 *LRP5/6* genes in the human and mouse genome. The basis of the specificity between the ligand (Wnt) and the receptor (Frizzled/LRP5/6) is totally unknown. Furthermore, the mechanism by which Frizzled and LRP5/6 transduce the signals remains elusive. *Drosophila* genetics have shown that in the fly, a dominant-negative form of dynamin abolishes cuticle deposition, indicating that receptor-mediated endocytosis is necessary for the activation of the β-catenin pathway (Moline *et al.*, 1999). Wnt-5a, which does not induce the accumulation of β-catenin, triggers the internalization of Frizzled4 in cooperation with Dvl and β-arrestin2 (Chen *et al.*, 2003). This endocytosis of Frizzled4 requires protein kinase C (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 β-catenin pathway has not yet been examined.

Stabilization of β-catenin in response to Wnt

Although the exact mechanism by which Wnt stabilizes β-catenin is not clear, several possible mechanisms have been proposed. The first one is based on the interaction of Dvl with Frat (Figure 4A). Dvl binds to CKIε and Axin (Kishida *et al.*, 1999b; Kishida *et al.*, 2001). CKIε mediates Wnt-3a-dependent phosphorylation of Dvl, and phosphorylated Dvl has a high affinity for Frat, which binds to and inhibits GSK-3 (Kishida *et al.*, 2001; Lee *et al.*, 2001; Hino *et al.*, 2003). Knockdown of CKIε by RNA interference reduces the Wnt-3a-induced binding of Dvl to Frat and the accumulation of β-catenin (Hino *et al.*, 2003). Therefore, when Wnt acts on the cells, Frat bound to Dvl phosphorylated by CKIε may prevent GSK-3β that has interacted with Axin from phosphorylating β-catenin, thereby stabilizing β-catenin. Axin is also phosphorylated by GSK-3β, and this phosphorylation stabilizes Axin, in contrast to the effect on β-catenin (Yamamoto *et al.*, 1999) (Figure 2). Wnt-3a inhibits the GSK-3β-dependent phosphorylation of β-catenin (Liu *et al.*, 2002; Yanagawa *et al.*, 2002) and induces the downregulation of Axin (Yamamoto *et al.*, 1999; Tolwinski *et al.*, 2003). Taken together, these facts indicate that 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 (Figure 4B). 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 direct phosphorylation of LRP6 by CK1γ and GSK-3 enhances the interaction between Axin and LRP6, which is essential for the transmitting the signal to activate Lef and axis duplication in *Xenopus* embryos (Davidson *et al.*, 2005; Zeng *et al.*, 2005). In this phosphorylation, the membrane localization of CK1γ and GSK-3α/β is important. Dvl and Axin interact with each other and this binding is necessary for the stabilization of β-catenin (Kishida *et al.*, 1999b). Dvl binds to Frizzled, and this binding is also essential for the Wnt signaling (Wong *et al.*, 2003; Cong *et al.*, 2004). Furthermore, disheveled is required for the recrui-

tment of dAxin to the plasma membranes in a manner dependent on *wingless* in fly cells (Cliffe *et al.*, 2003). These possible mechanisms are not exclusive, and a combination of the various models would provide a clear understanding of the overall stabilization mechanism in response to Wnt.

Stabilization of β-catenin by cyclic AMP-dependent protein kinase (PKA)

The protein product of the Alzheimer's disease-linked gene *presenilin1* forms a complex with GSK-3β, β-catenin, and the catalytic domain of PKA (Soriano *et al.*, 2001; Kang *et al.*, 2002). In the presenilin1 complex, PKA phosphorylates Ser^{45} and enhances the GSK-3β-dependent phosphorylation of β-catenin (Kang *et al.*, 2002), suggesting that PKA and presenilin1 induce the downregulation of β-catenin independent of the Wnt-controlled Axin complex. Indeed, nuclear accumulation of β-catenin is observed in the epidermis of *presenilin1*-deficient mice. Several lines of evidence support the concept that presenilin1 is an important negative regulator of β-catenin. In a genetic screen, *Drosophila presenilin* was identified as a negative regulator of the wingless/Wnt signaling pathway (Cox *et al.*, 2000), and *Drosophila presenilin* deficiency resulted in cytoplasmic accumulation of Armadillo, a *Drosophila* β-catenin homolog (Noll *et al.*, 2000).

In contrast to the findings that PKA might function as a negative regulator of β-catenin stability, however, it has been shown that stimulation with prostaglandin E2, which activates PKA, increases the transcriptional activity of Tcf in HEK-293 cells probably through direct phosphorylation and inhibition of GSK-3β by PKA (Fujino *et al.*, 2002). In addition, the activation of PKA has been demonstrated to increase the cytoplasmic and nuclear β-catenin protein level, and to stimulate Tcf-dependent transcription through β-catenin (Hino *et al.*, 2005) (Figure 3B). Direct phosphorylation of Ser^{675} of β-catenin by PKA inhibits the ubiquitination of β-catenin in intact cells and *in vitro*. Thus, PKA inhibits the ubiquitination of β-catenin by phosphorylating β-catenin, thereby causing β-catenin to accumulate. PKA is able to act as a positive regulator of the β-catenin pathway under certain conditions.

The activation of PKA suppresses the proliferation of most types of cells, whereas it stimulates the cellular growth of endocrine cells. Mutations of the α-subunit of oligomeric GTP-binding proteins that activate adenylate cyclase were found in human pituitary tumors producing growth hormone (Landis *et al.*, 1989) and in thyroid adenoma (Lyons *et al.*, 1990). Therefore, constitutive activation of PKA may stabilize β-catenin and activate Tcf, resulting in abnormal cell growth of these tumor cells.

Another model has demonstrated that prostaglandin E2 activates the oligomeric stimulatory G protein (Gs) and that the association of the α subunit of Gs directly binds to the RGS domain of Axin, resulting in the dissociation of GSK-3β form the Axin complex and the accumulation of β-catenin (Castellone *et al.*, 2005). In this model, the activation of PKA by prostaglandin E2 is not required for the accumulation of β-catenin. To clarify the molecular mechanism of the crosstalk between the Wnt/β-catenin and PKA pathways provides a new clue to understand how cyclooxygenase-2 and its inflammatory metabolite prostaglandin E2 enhance colon cancer progression.

Regulation of Tcf transcriptional activity

Activation of Tcf

Tcf and Lef bind directly to DNA through their high mobility group (HMG) domains, but they cannot activate gene expression; rather, they suppress it (Hurlstone and Clevers, 2002) (Figure 3C). The repression of gene expression by Tcf may be due to its direct interaction with co-repressors such as Groucho/TLE, CtBP, and HBP1 (Hurlstone and Clevers, 2002). Since β-catenin activates Tcf/Lef by binding to the N-terminal region of Tcf/Lef and thereby stimulates gene expression, it is thought that β-catenin supplies additional cofactors required for transcriptional activation and that this might involve additional proteins that bridge the Tcf and β-catenin complex to the basal transcriptional machinery. Candidates may be Pontin52 (Bauer *et al.*, 2000) and *Drosophila* teashirt (Gallet *et al.*, 1999). The DNA-binding HMG domains of the Tcf/Lef family proteins have a high affinity for the DNA sequence (A/T)(A/T)CAA(A/T)GG. Tcf/Lef activated by β-catenin stimulates the expression of various genes, including c-*myc, cyclin D1, fra1,* c-*jun, peroxisome proliferator-activated receptor δ, matrilysin, CD44,* and *urokinase-type plasminogen activator receptor* (Polakis, 2000; Hurlstone and Clevers, 2002), which contain Tcf/Lef binding sites in their promoters.

In contrast, ICAT and Duplin interfere with the formation of a complex between β-catenin and Tcf (Sakamoto *et al.*, 2000; Tago *et al.*, 2000; Kobayashi *et al.*, 2002) (Figure 3C). Both proteins directly bind to β-catenin and inhibit β-catenin-dependent gene expression. ICAT and Duplin may function to establish a threshold to prevent premature and inappropriate signaling events and accumulation of β-catenin would compete with them for binding to Tcf/Lef.

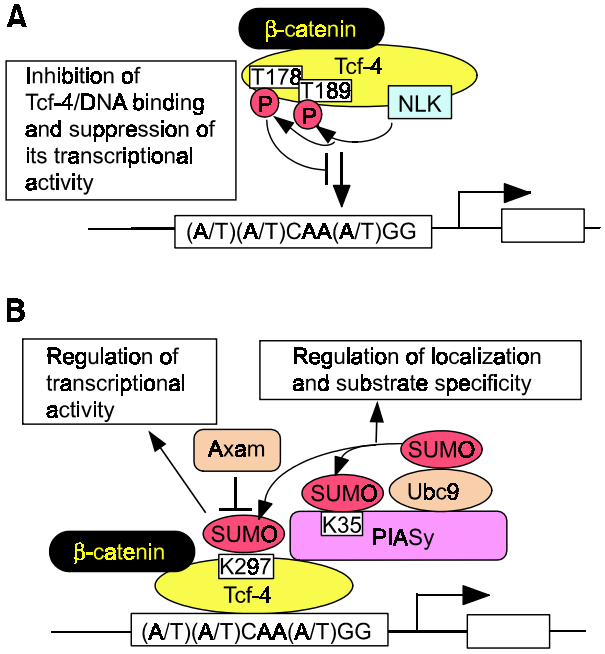

Figure 5. Regulation of Tcf-4 by post-translational modifications. (A) Phosphorylation of Tcf-4. Phosphorylation of Tcf-4 by NLK at Thr^{178} and Thr^{189} inhibits the interaction of the β-catenin and Tcf-4 complex with DNA. (B) Sumoylation of Tcf-4. PIASy enhances the sumoylation of Tcf-4 at Lys^{297} and its transcriptional activity. Sumoylation of Lys^{35} in PIASy determines the subnuclear localization of PIASy and is necessary for PIASy-dependent sumoylation and transcriptional activation of Tcf-4. Axam, a desumoylation enzyme, inhibits the sumoylation and transcriptional activity of Tcf-4.

Phosphorylation of Tcf

NEMO-like kinase (NLK), which acts downstream of TAK1 kinase activated by TGF-β, associates with and phosphorylates Lef-1 and Tcf-4 (Ishitani *et al.*, 1999). The phosphorylation of Tcf-4 by NLK inhibits the binding of the β-catenin and Tcf-4 complex to DNA, thereby suppressing the Wnt signaling pathway (Figures 1 and 5A). Wnt-5a is representative of the Wnt ligands that do not activate the β-catenin pathway, and it rather inhibits this pathway (Miller *et al.*, 1999; Weidinger and Moon, 2003). Wnt-5a has been shown to increase the intracellular Ca^{2+} concentrations and thereby activate PKC and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Ishitani *et al.*, 2003). CaMK is able to activate NLK through TAK1, thereby inhibiting the Tcf/Lef activity. This may be one of the possible inhibitory mechanisms by which Wnt-5a inhibits the β-catenin pathway.

Sumoylation of Tcf

The small ubiquitin-related modifier (SUMO) modification (sumoylation) pathway resembles the ubiquitin conjugation pathway, but the enzymes involved in these two processes are distinct (Hochstrasser, 2000; Yeh *et al.*, 2000; Müller *et al.*, 2001). SUMO is activated for conjugation by the E1 enzyme Aos/Uba2, subsequently transferred to the E2 conjugation enzyme Ubc9, and finally conjugated to target proteins by the E3 ligases: protein inhibitor of activated STAT (PIAS) or RanBP2 (Jackson, 2001). Known target proteins of sumoylation include p53, MDM2, PML, RanGAP1, IκBα, and c-Jun. Modification of these proteins by sumoylation changes their subcellular localization, function, and stability. Sumoylation is reversible, and there are at least seven mammalian SUMO-specific proteases, which are designated the SENP family proteins (Hochstrasser, 2000; Yeh *et al.*, 2000; Müller *et al.*, 2001).

It has been suggested that PIASy (an E3 ligase) and Axam (a desumoylation enzyme, SENP2) are involved in the Wnt signaling pathway (Figure 5B). PIASy interacts with Lef-1, resulting in the sumoylation of Lef-1, and inhibits the transcriptional activity of Lef-1 (Sachdev *et al.*, 2001). Tcf-4 is also sumoylated at Lys^{297} and PIASy enhances the sumoylation of Tcf-4 (Yamamoto *et al.*, 2003). In contrast to the effect on Lef-1, sumoylation of Tcf-4 enhances its transcriptional activity. Axam has been identified as an Axin-binding protein that regulates the Wnt signaling pathway negatively by inducing the degradation of β-catenin (Kadoya *et al.*, 2002). Axam inhibits the sumoylation and activity of Tcf-4 (Yamamoto *et al.*, 2003).

PIASy itself is also modified with SUMO-1 at Lys^{35} (Figure 5B). $PIASy^{K35R}$, in which Lys^{35} is mutated to Arg, neither enhances the sumoylation of Tcf-4 nor stimulates the transcriptional activity of Tcf-4 (Ihara *et al.*, 2005). Wild-type PIASy and $PIASy^{K35R}$ show a distinct distribution in the nucleus, although both are colocalized with Tcf-4. Therefore, sumoylation of Lys^{35} in PIASy determines the subnuclear localization of PIASy and is necessary for PIASy-dependent sumoylation and transcriptional activation of Tcf-4.

Although the precise molecular mechanism by which sumoylation and desumoylation regulate the transcriptional activity of Tcf is not known, it is likely that sumoylation, like phosphorylation and ubiquitination, is involved in the Wnt signaling pathway. Tcf-4 complexed with sumoylated PIASy would be efficiently modified with SUMO-1. When PIASy is sumoylated and present with Tcf-4 at an appropriate place in the nucleus, desumoylation enzymes may not act on sumoylated Tcf-4.

Acetylation of Tcf

Nuclear histone acetyltransferases play important

roles in gene regulation (Workman and Kingston, 1998). They catalyze the reversible acetylation of histones to alleviate chromatin-mediated restrictions imposed on promoter activation. Adenovirus E1A-associated p300 and the closely related CREB-binding protein (CBP) are well-characterized acetyltransferases. CBP and p300 can form larger protein complexes that include other acetylases and serve as coactivators of numerous transcriptional factors. They can accomplish their task not only by utilizing their intrinsic histone acetyltransferase activity but also as adaptor proteins that link activators to the basal transcriptional machinery.

Drosophila CBP (dCBP) has been shown to regulate *wingless* signaling negatively by acetylating Tcf (Waltzer and Bienz, 1998). The acetylation inhibits the binding of Tcf to Armadillo in *Drosophila*. In contrast, p300 and CBP serve as coactivators for β-catenin in vertebrates (Hecht *et al.*, 2000). Although dCBP and mammalian orthologs may be functionally different from each other, acetylation may be involved in the Wnt signaling pathway.

Closing remarks

Signaling pathways initiate a series of post-translational modifications that regulate the intracellular localization of proteins, protein-protein interactions, and protein stability. Combinations of several protein-protein interactions and post-translational modifications are involved in the regulation of the Wnt signal pathway. In the cytosol, GSK-3β, CKIα, β-catenin, and APC form a complex with Axin, and β-catenin is phosphorylated efficiently and ubiquitinated in the Axin complex, resulting in its rapid degradation by proteasomes. Wnt induces the CK1γ- and GSK-3-dependent phosphorylation of LRP6 and this phosphorylation is important for the binding of the Axin complex with LRP6 and the subsequent degradation of β-catenin. The PKA-dependent phosphorylation of β-catenin stabilizes β-catenin by inhibiting its ubiquitination. In the nucleus, several β-catenin-binding proteins and Tcf/Lef-binding proteins regulate the transcriptional activity of Tcf/Lef by affecting the formation of a complex between β-catenin, Tcf/Lef, and DNA. The phosphorylation of Tcf/Lef by NLK decreases its affinity for DNA, and the sumoylation and desumoylation of Tcf/Lef modulates its transcriptional activity.

Genetic alterations of the *β-catenin*, *APC*, and *Axin* genes are often identified in human malignant tumors, including colorectal, hepatocellular, and thyroid carcinomas. In all such cases, the common denominator is the abnormal accumulation of β-catenin and the abnormal expression of Tcf/Lef target genes. Although we do not pay much attention on the non-canonical pathways in this review article, they determine cell motility, adhesion, and polarity. The abnormalities of the non-canonical pathway are involved in the metastasis and invasion of malignant tumors. Therefore, to clarify how all the pathways of Wnt signaling is regulated by protein-protein interactions and post-translational modifications would provide new ideas to develop novel cancer therapies.

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