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Regulation of Wnt/β-Catenin Signaling by Protein Kinases

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Abstract

The Wnt/β-catenin signaling pathway plays essential roles during development and adult tissue homeostasis. Inappropriate activation of the pathway can result in a variety of malignancies. Protein kinases have emerged as key regulators at multiple steps of the Wnt pathway. In this review, we present a synthesis covering the latest information on how Wnt signaling is regulated by diverse protein kinases.

Keywords

protein kinase; Wnt; β-catenin; GSK3; CKI

WNT/B-CATENIN SIGNALING

The evolutionarily conserved Wnt/Wingless (Wg) signaling pathway is involved in diverse biological processes required for embryonic development and adult homeostasis, including determination, proliferation, migration, and differentiation (reviewed in Clevers, 2006; Cadigan, 2008). Wnts are secreted extracellular proteins that trigger a wide range of cellular responses upon receptor binding and activation. Most organisms contain multiple Wnt genes, which initiate distinct intercellular pathways, namely the canonical Wnt/β-catenin cascade, the planar cell polarity pathway, or the Wnt/calcium pathway. For the purposes of this review, we will focus primarily on regulation of the canonical Wnt pathway components (Table 1; see also The Wnt Homepage).

Wnt family members are conserved throughout the animal kingdom and have been shown to be required during growth, tissue patterning, and homeostasis. In addition, inappropriate activation of Wnt-dependent gene expression in mammals can lead to numerous forms of cancer, most notably hereditary and sporadic forms of colon cancer (Kinzler et al., 1991; Nishisho et al., 1991).

Canonical Wnt signaling controls cell fate by regulating gene expression (reviewed in Cadigan and Nusse, 1997). Wnts initiate the canonical pathway by binding to the Frizzled (Fz) and LRP5/6 coreceptors (Bhanot et al., 1996; Fig. 1). Receptor complex stimulation ultimately leads to the formation of a nuclear transcription complex that contains a DNA-binding factor known as lymphocyte enhancer factor (Lef)/T-cell factor (Tcf), and the dual, signaling/adhesion protein, β-catenin. In this complex, β-catenin serves as an obligate coactivator through its ability to recruit enzymes such as CBP that promote chromatin

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remodeling and transcriptional initiation/elongation (reviewed in Willert and Jones, 2006). An elaborate series of molecular events are required to generate this transcription complex. A central aspect of Wnt signal transduction involves the inhibition of a constitutive degradation mechanism that serves to keep the cytosolic "signaling" pool of β-catenin at low levels. As a result, cytosolic β-catenin accumulates and enters the nucleus to interact with a Tcf/Lef family transcription factor to promote target gene expression (reviewed in Behrens et al., 1996; Molenaar et al., 1996; Mosimann et al., 2009). In the absence of Wnt signaling, cytosolic β-catenin is rapidly degraded through the ubiquitin–proteosome system. Removal of β-catenin from Tcf/DNA complexes allows the transcriptional corepressor Groucho to access Tcfs and repress transcription of Wnt targets (Cavallo et al., 1998; Roose et al., 1998).

At multiple steps in the Wnt pathway, protein activities, binding properties, and stability are regulated through distinct phosphorylation events. The goal of this review is to summarize these events and their molecular consequences, as well as discuss what is known about the regulation of the various kinases involved. The ubiquitous kinases, CKI and GSK3, play multiple and opposing roles in controlling Wnt signaling, revealing that the manner in which these kinases are scaffolded in the cell will be critical to understanding Wnt signal transduction and its regulation. What also emerges is an understanding of how some kinases and/or phosphorylation events serve to modulate or "fine-tune" Wnt/β-catenin signaling. An understanding of this fine-tuning will be critical for explaining how highly conserved pathways can contribute to such a variety of tissue structures, and how loss of this precise regulation contributes to a growing list of human diseases (reviewed in Chien and Moon, 2007).

Phosphorylation-Dependent Destruction of β-Catenin by an Axin–Scaffold Complex

Cytosolic and nuclear accumulation of β-catenin was one of the earliest, universal read-outs for Wnt activation (Noordermeer et al., 1992). Because excess β-catenin was observed in certain cancers, early studies were heavily focused on the molecular components that control cytosolic accumulation of β-catenin. Because this aspect of β-catenin regulation is now well-established, we will only outline key steps below. For students of the field who should seek a more historical, experimental account of this paradigm, the reader is referred to a few excellent reviews (Wodarz and Nusse, 1998; Barker and Clevers, 2000; Logan and Nusse, 2004; Clevers, 2006).

The N-terminus of cytosolic β-catenin is constitutively phosphorylated by a dual-kinase mechanism coordinated by the scaffold protein Axin (Fig. 2). Axin has binding sites for β-catenin, CK1, GSK3, as well as other factors required for Wnt-dependent signaling events (Luo and Lin, 2004). CK1 family members (including α, δ, and ε) phosphorylate β-catenin at serine 45. This priming phosphorylation is required for subsequent phosphorylations by GSK3 at residues 41, 37, and 33 (Yost et al., 1996; Liu et al., 2002). Although most studies focus on GSK3β, it is now well established that GSK3α and β are fully redundant (Doble et al., 2007). Therefore, we refer to the family members simply at GSK3, unless specifically noted. The β-catenin that is phosphorylated at residues 37 and 33 is ultimately recognized by the β-TrCP E3 ubiquitin–ligase complex, ubiquitinylated, and rapidly degraded by the 26S proteasome (Hart et al., 1999). The APC tumor suppressor gene product is also an Axin-binding partner, and is thought to remove N-terminally phosphorylated β-catenin from the Axin complex for transfer to the degradation machinery (Xing et al., 2003). Mutations in APC, Axin, or these N-terminal phosphorylation sites of β-catenin are found in multiple types of human cancers, where these mutations elevate β-catenin posttranscriptional stability and signaling (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Thus

phosphorylation-dependent degradation of β-catenin has been long considered a key step in turning-off β-catenin signals.

How Wnts inhibit this constitutive degradative process is an area of intense investigation. Recent work has focused heavily on the molecular sequence of events that occur after ligand binding at the cell surface. While some issues remain unresolved, it is clear that the receptor complex plays an important role in assembling a platform, which ultimately serves to inactivate the Axin–scaffold phospho-destruction complex.

LRP5/6 Phosphorylation Promotes Axin Docking and Pathway Activity

Wnt ligands engage a receptor complex composed of a Frizzled serpentine receptor protein and a LRP5/6 coreceptor. LRPs are single pass transmembrane proteins belonging to the low density lipoprotein-related (LRP) receptor family represented in vertebrates by LRP5 and LRP6 and by Arrow in *Drosophila* (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Because forced clustering of Fz and LRP is sufficient to activate β-catenin signaling independently of Wnts, ligand stimulation is thought to promote the assembly of higher order LRP5/6 coreceptor complexes that in turn drive signaling (Tolwinski et al., 2003; Cong et al., 2004b; Holmen et al., 2005; Liu et al., 2005).

Upon Wnt stimulation, LRP6 is phosphorylated and recruits Axin to the plasma membrane, coincident with the inhibition of the β-catenin destruction complex (Tamai et al., 2004). Expression of LRP6 lacking the cytoplasmic domain results in a dominant-negative effect on Wnt signaling (Tamai et al., 2000), while expression of a construct lacking the extracellular domain constitutively activates the pathway activity (Tamai et al., 2004). Based on these observations, several groups have suggested that the extracellular domain exerts an autoinhibitory effect that is relieved upon Wnt stimulation (Mao et al., 2001; Liu et al., 2003).

Tamai et al. (2004) found that a proline-rich PPPSP motif is present in five copies (represented as A–E; Fig. 3) in the intracellular domain of LRP6 and is functionally required for transduction of the Wnt signal (Tamai et al., 2004). These motifs are conserved from flies to vertebrates as PPP(S/T)P residues. Mutation of the five serine residues in LRP6 to alanine renders the protein inactive in β-catenin signaling. The notion that these motifs represent protein kinase target sites was confirmed when it was found experimentally that the PPPSP motifs are phosphorylated. Once phosphorylated, these sites allow docking of Axin. Indeed, Axin will not bind to an LRP6 in which the sites have been mutated. Because it was previously shown that Wnt signaling promoted the interaction between Axin and LRP5/6, a model emerged that Wnt triggers the phosphorylation of LRP6 to induce an Axin-binding site. Tamai et al. (2004) generated a phospho-specific antibody (Ab1490) that recognizes phosphorylated serine in the first PPPSP repeat (serine 1490). Using this antibody, it was demonstrated that stimulation of cells with Wnt-conditioned medium caused rapid and pronounced phosphorylation of PPPSP residues in LRP6.

In addition to its essential role as a component of the destruction complex that targets β-catenin for degradation, glycogen synthase kinase 3 (GSK3) was shown to be involved in LRP6 phosphorylation on PPPSP (Zeng et al., 2005). Overexpression of GSK3 in vivo promoted LRP6 phosphorylation, while GSK3 inhibitors such as LiCl prevented phosphorylation. LRP6 was also not phosphorylated in mouse embryo fibroblasts harboring deletions of both *Gsk3*α and *Gsk3*β genes (Zeng et al., 2005).

To address the apparent discrepancy between the well-documented role of GSK3 as an inhibitor of Wnt signaling, and the newly identified role in promoting LRP6-Axin interactions, Zeng et al. (2005) generated membrane-tethered GSK3β. As expected, this

form of GSK3β could still phosphorylate LRP6 at the plasma membrane. This membrane-associated GSK3 was unable to participate in the well-known inhibition of β-catenin at the level of the cytoplasmic destruction complex. These observations strongly argue for two opposite roles for GSK3 in the Wnt pathway, resulting from compartmentalization of GSK3. Thus proximity of GSK3 to the plasma membrane has a positive role in Wnt signaling.

Because GSK3 typically cooperates with other kinases, the involvement of a second kinase was suspected. In 2005, two studies showed that LRP5/6 is phosphorylated by CKI (Davidson et al., 2005; Zeng et al., 2005; Fig. 3). For a detailed review of the role of CKI family members in Wnt signaling, the reader is referred to Price (2006). Two sites were identified as CKIγ target sites (Fig. 3), one named T1479 that lies upstream of the GSK3 target site (Davidson et al., 2005), while the second T1493 is situated three amino acids downstream of the GSK3 target site PPPSP (Zeng et al., 2005). The Niehrs group identified that the family member CKIγ is the primary kinase involved in mediating phosphorylation, and appears to be needed for both T1479 and T1493 sites (Davidson et al., 2005). CKIγ is an unusual CKI family member, because it is membrane-anchored through palmitoylation sites at its carboxy terminus. In contrast, cytoplasmic family members such as CKIε have no effect on LRP6 phosphorylation (Davidson et al., 2005), while they do affect Dvl activity (see below; Peters et al., 1999). Expression of dominant-negative CKIγ in HEK293T cells and *Xenopus* embryos specifically blocks LRP6 phosphorylation and Wnt signaling. The *Drosophila* CKIγ called Gilgamesh (Gish) can synergize with LRP6/Arrow, while RNAi-mediated knockdown blocks Wg signaling (Davidson et al., 2005). A phospho-specific antibody directed against a threonine in the most C-terminal CKIγ site (Tp1479) was used to demonstrate that CKIγ directly phosphorylates LRP6. CKIγ phosphorylation is essential for recruitment of Axin by LRP6. These observations that CKI played a key role in phosphorylating cluster A were extended by MacDonald and colleagues when they showed that all 5 GSK3 motifs (PPPSPxS) are followed by CKI consensus sites (PPPSPxS). All five dually phosphorylated motifs provide optimal binding sites for Axin (MacDonald et al., 2008).

While there is a general consensus that phosphorylation of LRP6 is essential for Wnt signaling, the molecular details are still disputed. In one model, phosphorylation by GSK3 is the primary Wnt inducible event, which then provides a priming step to allow subsequent CKI phosphorylation (Zeng et al., 2008), while a second model proposes that GSK3 phosphorylation is constitutive and that Wnt signaling promotes only CKIγ-mediated phosphorylation (Bilic et al., 2007). The latter model is based on the findings that Wnt signaling does not enhance phosphorylation of S1490 in the GSK3 consensus site, and, furthermore, that S1490 is constitutively phosphorylated (Davidson et al., 2005). In contrast, the CKIγ site is phosphorylated specifically in response to Wnt stimulation, and may not require priming by GSK3. GSK3 phosphorylation generally requires a primed substrate, as is seen with β-catenin phosphorylation on S45 by CKIα. In contrast, Davidson et al. (2005) propose that phosphorylation of both the CKI sites requires an intact PPPSP site, suggesting that GSK3 primes LRP6. As studies to date have not resolved this issue, it remains to be seen what the exact mechanism, sequence and requirements of LRP6 phosphorylation are.

LRP6 Signalosome Assembly at the Membrane

In addition to promoting phosphorylation of LRP family members, Wnt appears to stimulate the assembly of macromolecular complexes in response to ligand binding (Fig. 4). These complexes recruit cytosolic proteins to the membrane and may promote the phosphorylation of LRP as a result. Using live imaging of vertebrate cells expressing fluorescently tagged Axin and LRP6, Bilic et al. (2007) demonstrated that Wnt signaling induces plasma membrane-associated LRP6 aggregates. In unstimulated cells, Axin localizes to intracellular

punctae while LRP6 uniformly stains at the cell membrane. Wnt stimulation results in the rapid formation of LRP6 punctae, referred to as "LRP6 signalosomes." This event is followed by Axin recruitment to the aggregates. The Tp1479 antibody was used to show that the punctae are enriched for CKIγ-mediated phosphorylated LRP6, and that these structures also contain Fz8, Dvl2, GSK, and Axin (Bilic et al., 2007). Of note, the T1479 residue is not conserved in *Drosophila* Arrow, so it remains to be seen if this mechanism is evolutionarily conserved. Dvl is proposed to mediate the aggregation and phosphorylation of LRP6, based on the findings that *dsh* and *dvl* knockdown in *Drosophila* and vertebrate cells inhibits Wnt-induced LRP6 phosphorylation at T1479. In this model, Dvl would play a major role in assembling LRP6 signalosomes in response to Wnt signaling, and would stimulate LRP6 phosphorylation and Axin recruitment, followed by β-catenin stabilization. In support of this, cell lines in which the three vertebrate Dvl proteins were simultaneously reduced through knockout (*Dvl1*$^{-/-}$;*Dvl2*$^{-/-}$) and shRNA-mediated knockdown (*Dvl3*) showed reduced levels of Wnt-induced LRP6 phosphorylation (Zeng et al., 2008).

Wnt Pathway Activation Triggers Phosphorylation of Dishevelled Proteins

The Dishevelled family of proteins (Dvl1–3 in vertebrates and Dsh in flies and zebrafish) is a conserved positive regulator of canonical signaling. The modular protein also plays a key role in the noncanonical PCP pathway (reviewed in Wallingford and Habas, 2005). Dishevelled functions epistatically downstream of the Fz/LRP receptor complex. Evidence for this comes from the observation that overexpression of Dsh can activate β-catenin signaling in *Drosophila* Arrow mutants (Wehrli et al., 2000), and a constitutively active Fz2-Arrow fusion cannot transduce signaling in a *dsh* mutant background (Tolwinski et al., 2003). A likely mechanism by which Dsh functions is by means of the inhibition of Axin (Fagotto et al., 1999; Kishida et al., 1999; Li et al., 1999; Smalley et al., 1999). The translocation of Dvl to the membrane and subsequent recruitment of binding partners leads to a disruption of the destruction complex and stabilization of β-catenin as described in more detail below.

Upon Wnt activation, Dvl proteins associate with Fz and become phosphorylated (Yanagawa et al., 1995; Willert et al., 1997; Lee et al., 1999; Rothbacher et al., 2000). Several kinases are proposed to phosphorylate Dvl, including CKIε, Casein kinase 2 and PAR1 (Willert et al., 1997; Sun et al., 2001; Cong et al., 2004a; Ossipova et al., 2005). While phosphorylation of Dvl proteins is believed to be an important positive step in pathway activation, the exact mechanism of how this activates Dvl remains unresolved.

Lipid Kinases: PtdIns (4,5)P_2 and Wnt Receptor Complex Activation

A recent study establishes a requirement for two well-known lipid kinases for these early steps in Wnt signal transduction. Using a human kinase siRNA library, Pan et al. (2008) screened for effects on both Wnt-induced cytosolic β-catenin accumulation by an enzyme-linked immunosorbent assay, and Lrp6 phosphorylation at S1490 (the GSK3 site) by immunoblot analysis in HEK293T cells, and identified PI4KIIα and PIP5K1β. These two kinases are components of the major pathway for PtndIns(4,5)P_2 (PIP_2) production in cells (Hsuan et al., 1998; Doughman et al., 2003). Supporting a role for PIP_2 in the Wnt cascade, forced delivery of PIP_2 to the cell cytosol using a liposome delivery system, although not sufficient for phosphorylation of LRP6 at S1490, does enhance this phosphorylation in the presence of Wnt. Moreover, Wnt3a can stimulate the formation of PIP_2 in cells, and liposome-mediated delivery of PIP2 can bypass the requirement for PI5K1β. The mechanism of PIP_2 activation appears to require Dvl, which activates PIP5K activity in a dose-dependent manner, and can be found to coimmunoprecipitate with PIP5K in cell lysates. Importantly, this physical interaction requires the presence of a Wnt ligand.

PIP_2 is involved in numerous processes that involve membrane dynamics (e.g., endocytosis, exocytosis, cortical cytoskeleton regulation; Czech, 2000). Perhaps consistent with this general role, the Wnt3a-induced LRP6 aggregates appear to contain PIP_2 and are dependent on cellular PIP_2 levels for their formation (Pan et al., 2008). Elimination of PIP_2 also blocks Axin recruitment to pLRP6. Altogether, these data suggest a model where Wnt signals transmitted through Fz recruit Dvl leading to activation of PIP5K activity. This induces the local formation of PIP_2, which is required, but not sufficient for many of the activities described above, such as LRP6 aggregation, phosphorylation of LRP6 at T1479 and S1490, and Axin recruitment (Pan et al., 2008). Future studies should confirm these observations and integrate them more fully into the known membrane-proximal events in the Wnt pathway.

LRP Directly Inhibits GSK3 Activity and/or Function During Wnt Signaling

It has been long appreciated that pharmacologic inhibitors of GSK3 activity such as LiCl and BIO are sufficient to promote β-catenin stabilization and activate β-catenin/Tcf transcription (Klein and Melton, 1996; Stambolic et al., 1996; Sato et al., 2004). However, whether and how Wnts antagonize GSK3 activity has remained elusive. The regulation of GSK3 is unique for several reasons. Unlike most protein kinases involved in signal transduction, it is constitutively active and only becomes inactivated in response to a variety of signals. The range of GSK3 substrates is broad and GSK3 acts in numerous essential cellular processes, such as glycogen metabolism, insulin signaling, cell proliferation, neuronal function, and Wnt signaling (reviewed in Rayasam et al., 2009). In the insulin pathway, GSK3 activity is inhibited by Akt-mediated phosphorylation on S9 by GSK3β and S21 by GSK3α. These sites are unaffected by the status of the Wnt pathway (Ding et al., 2000; McManus et al., 2005). It has remained a mystery how phosphorylation of β-catenin by GSK3 is inhibited upon Wnt pathway activation. Three recent studies now provide compelling evidence that Wntactivated LRP6 can inhibit GSK3 function directly (Cselenyi and Lee, 2008; Piao et al., 2008; Wu et al., 2009).

Using in vitro assays that reconstitute β-catenin phosphorylation at residues S33, 37, and 41 (the former two being recognized by the E3 ubiquitin ligase, β-TrCP), these groups present evidence that a phosphorylated LRP6 PPPp**S**PXp**S** peptide directly inhibits β-catenin phosphorylation by GSK3 at S33, S37, and T41. At first glance, this result is not surprising, because it is known that the Axin–scaffold protein is also recruited to this phosphorepeat in LRP (Tamai et al., 2004). Remarkably, however, this phosphopeptide (PPPpSPXpS) - mediated inhibition of β-catenin phosphorylation can occur *independently of Axin* (Cselenyi and Lee, 2008; Wu et al., 2009). Because the PPPSPXS peptide is a GSK3 substrate itself, these studies raise the interesting possibility that this region of LRP6 directly competes with the N-terminus of β-catenin for LRP6 binding (i.e., “substrate competition model” of Wu et al., 2009). Although there are data that are incompatible with this model (see discussion in Wu et al., 2009), and others find that the phosphorylated PPP-SPXS peptide appears to be a general inhibitor of GSK3 activity (Piao et al., 2008), the “substrate competition mechanism” could enable an exquisitely local inhibition of GSK3 activity toward β-catenin’s N-terminus. This mechanism would still allow GSK3 activity toward other targets in the complex, like LRP, or important cellular targets (e.g., glycogen synthase). In other words, this mechanism might explain the paradox of GSK’s dual positive and negative roles in Wnt signaling.

Another important implication of this model is that if Wnt-mediated inhibition of β-catenin N-terminal phosphorylation occurs exclusively *at the receptor complex*, then the signaling form of β-catenin (discussed in greater detail below) may be generated at or near this complex (Hendriksen et al., 2008). This Wnt-dependent recruitment of the signaling form of

β-catenin to the membrane may have implications for understanding where some newer, signal-promoting phosphorylations in β-catenin may be acquired (Hino et al., 2005; Fang et al., 2007; Wu et al., 2008).

N-Terminally Unphosphorylated β-Catenin and Signaling

While accumulation of cytosolic β-catenin is often a good indicator of Wnt/β-catenin signaling activity, studies indicate that levels of cytosolic β-catenin protein *alone* do not fully explain β-catenin/TCF transcriptional activation (Guger and Gumbiner, 2000). By generating a monoclonal antibody that was screened to recognize a peptide corresponding to β-catenin (amino acid residues 36–44), specifically when T41 and S37 are *not* phosphorylated (8E7, Upstate Biotechnology/Millipore; van Noort et al., 2002) clearly demonstrated that Wnt/β-catenin signaling is mediated through molecular forms of β-catenin that remain unphosphorylated at residues 37 and 41. Thus, the form of β-catenin recognized by monoclonal antibody 8E7 is often referred to as the signaling form of β-catenin, or Active β-Catenin (ABC). It should be noted, however, that ABC can also be readily detected at cadherin-contacts (Hendriksen et al., 2008; Maher et al., 2009), which means that the presence of ABC in cell lysates does not in and of itself reflect signaling. Moreover, it should be clarified that a similarly named monoclonal antibody, 8E4, has been incorrectly marketed as an antibody that also recognizes β-catenin “nonphosphorylated” at the N-terminal GSK sites, and has recently been shown to recognize β-catenin at a completely different epitope (van Noort et al., 2007). These issues aside, it is clear that hypophosphorylation of the N-terminal GSK sites in β-catenin constitutes a form that is intrinsically more active in signaling (Guger and Gumbiner, 2000; Staal et al., 2002). Whether phosphorylation at these residues antagonizes β-catenin signaling at the level of nuclear accumulation (Staal et al., 2002; van Noort et al., 2002), access to Lef/Tcf-bound promoters (Sadot et al., 2002) or alternate mechanisms, remains to be elucidated. Regardless of the precise mechanism, these studies strongly suggest that GSK3-mediated phosphorylation of β-catenin may not only induce degradation as suggested by the standard model, but may also directly affect transcriptional activity.

In this regard, recent studies reveal that the phosphorylation status of the N-terminus may be dynamically regulated. For example, antibodies that detect β-catenin specifically phosphorylated at S33, S37, and T41 reveal that these phosphorylations are short-lived, even in the presence of proteosome inhibitors, suggesting that this region can undergo rapid de-phosphorylation (Sadot et al., 2002). Recent evidence suggests that this de-phosphorylation is mediated by the general serine/threonine phosphatase, PP2A, and that its access to the GSK3 sites of β-catenin is somehow restricted by APC (Su et al., 2008). Using a cell-free system to study β-catenin phosphorylation, ubiquitination, and degradation, Su et al. (2008) show that APC protects the time-dependent loss of β-catenin phospho-epitopes at S33, S37, and T41 from PP2A. Because β-catenin phosphorylated at these residues comprises the recognition site for the β-TrCP ubiquitin ligase, the requirement for APC in the destruction of β-catenin may be in its ability to preserve the βTrCP recognition motif. Of interest, this shielding of p33-, p37-, and p41-β-catenin from PP2A is not observed with the common cancer-causing APC mutants, consistent with the high levels of Nterminally unphospho-β-catenin in these cancers. Given recent evidence that APC can positively impact β-catenin signals in certain contexts (Takacs et al., 2008), it will be interesting to learn whether this may occur through an ability of APC to dynamically regulate the N-terminal phosphorylation of β-catenin.

Kinase Regulation of β-Catenin Flux Through the Axin–Scaffold Phospho-Destruction Complex

The recruitment and flow of β catenin through the Axin/APC destruction complex appears to be regulated by a series of ordered phosphorylation events. Understanding this "flux" relies on an appreciation of the β-catenin structure. β-Catenin belongs to the armadillo family of proteins, which are characterized by a central domain consisting of a repeating 42 amino acid motif, termed the "arm repeat." These repeats were originally identified in the *Drosophila* β-catenin ortholog, Armadillo (Riggleman et al., 1989). X-ray crystallographic analysis of the central armadillo domain of β-catenin shows that its twelve arm repeats form a superhelix of helices that create a long, positively charged groove (Huber et al., 1997). Of interest, these positive charges are critical for β catenin binding to many of its negatively charged ligands, such as the cadherin adhesion receptor, Axin and APC degradation machinery components, or Tcf DNA binding factors (Graham et al., 2000; von Kries et al., 2000; Huber and Weis, 2001; Xing et al., 2003). An emerging theme is that phosphorylation of these binding partners introduces additional negative charges that enhance interactions with β-catenin's positively charged groove, thereby increasing binding affinity (reviewed in Daugherty and Gottardi, 2007).

These structural insights inform what is known about phosphorylations within the Axin–scaffold complex. Specifically, phosphorylation of Axin by CKI and GSK3 increases Axin binding to β-catenin (Jho et al., 1999; Willert et al., 1999; Luo et al., 2007), allowing the N-terminal region of β-catenin to be a more efficient substrate of CK1α and GSK3 (Dajani et al., 2003). Axin also promotes phosphorylation of APC by CK1ε and GSK3 (Rubinfeld et al., 1996, 2001), increasing the affinity of APC for β-catenin (Ha et al., 2004). Because phospho-APC can out-compete phospho-Axin for binding to β-catenin, it is viewed that well-coordinated phosphorylations within the Axin–APC complex (Axin first, then APC), controls the *directional* flow of β-catenin through this complex (Ha et al., 2004). Once β-catenin is bound to APC, APC ensures targeting of N-terminally phosphorylated β-catenin (pS33 and S37) to the β-TrCP ubiquitin ligase and destruction by the proteasome (Su et al., 2008).

Hipk Can Promote β-Catenin Stabilization and Target Gene Expression

The Homeodomain-interacting protein serine/threonine kinases (Hipks) have recently been found to play roles in regulating Wnt signaling. Studies of *Drosophila* Hipk, murine Hipk2, and *Xenopus* Hipk1 have reported kinase-dependent effects on Wnt target gene expression (Lee et al., 2009; Louie et al., 2009). *Drosophila* Hipk promotes stabilization of Armadillo both in vitro and in vivo and reductions in *hipk* lead to decreased Arm levels. Elevated Hipk and Hipk2 expression promotes accumulation of β-catenin and increased Wnt target gene expression in vitro and in vivo. In *Xenopus* assays, modulation of Hipk1 can both promote and antagonize Wnt target genes in a context-specific manner (Louie et al., 2009). The effect of Hipk/Hipk2 on Tcf-dependent gene expression and Arm stabilization is dependent on an intact kinase domain. Hipk can associate with Arm and Tcf and can phosphorylate Arm at both the N and C termini, although the exact sites have not been determined (Lee et al., 2009). While the functional significance of this modification has yet to be determined, it is clear that Hipk/Hipk2 acts to promote Arm stabilization. Hipk might facilitate the interactions between Arm and its transcriptional cofactors, as Hipk2 phosphorylation has been shown to affect gene regulation by modifying the composition of various transcriptional complexes (reviewed in Calzado et al., 2007). Hipk may also enhance the formation of the β-catenin/Tcf transcriptional complex by inducing a conformational change and/or reducing the affinity of possible inhibitors for β-catenin through the phosphorylation of β-catenin.

The role of Hipks in Wnt signaling seems to be complex, as family members have been found to have molecular interactions with several proteins associated with β-catenin. *Xenopus* Hipk1 was identified in a two-hybrid screen for Dsh-interacting partners, and subsequently shown to also bind Tcf3 (Louie et al., 2009). Several studies have physically linked murine Hipk2 to Axin (Rui et al., 2004; Li et al., 2007). It is tempting to speculate that Hipks may interact with the destruction complex and prevent either phosphorylation by GSK3 or CKI, or Hipk proteins may act to block release from the Axin-based destruction complex and subsequent proteasomal degradation.

β-Catenin Phosphorylations That Enhance Signaling Activity

Although the N-terminal CKI/GSK3-phosphorylation sites in β-catenin are the best-defined, recent studies reveal that other phosphorylations positively contribute to β-catenin signaling activities (Fig. 2). For example, phosphorylation of S675 by PKA may enhance β-catenin transcriptional activity by promoting β-catenin stability (Hino et al., 2005) and association with Creb Binding Protein (CBP) (Taurin et al., 2006). The latter finding is consistent with previous domain-mapping studies that place S675 within the transcriptional activator region of β-catenin (Hecht et al., 1999). Whether and how this region impacts β-catenin flux through the destruction complex remains to be clarified. Moreover, as canonical Wnts are not generally found to activate PKA, phosphorylation at S675 is likely mediated by upstream signals that “cross-talk” with β-catenin signaling at this site.

Phosphorylation of β-catenin at S552 by AKT has also been found to enhance β-catenin protein levels and nuclear signaling by standard reporter assays (Tian et al., 2004; Fang et al., 2007), although the precise mechanism remains unclear. Lastly, serines 191 and 605 were recently identified as Rac-activated JNK2 sites, and mutations of these residues appears to reduce the nuclear accumulation of β-catenin in a murine bone marrow-derived stromal cell line, ST2 (Wu et al., 2008). Whether this mechanism is a universal feature of Wnt pathway activation, or plays a more prominent role in ST2 cells remains to be determined. Given strong evidence that β-catenin nuclear/cytoplasmic distribution is largely determined by the availability of nuclear versus cytoplasmic binding partners (Krieghoff et al., 2006), it will be important to learn whether these sites regulate nuclear import per se, or affect binding to cytoplasmic-localized β-catenin ligands.

Phosphorylation of Tcf/Lef by Nlk and Casein Kinases

In the past decade, the Nemo-like kinase (Nlk) family of protein kinases has been shown to play roles in several Wnt-regulated processes (Ishitani et al., 1999, 2003; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999; Behrens, 2000; Golan et al., 2004; Kanei-Ishii et al., 2004; Thorpe and Moon, 2004; Zeng and Verheyen, 2004). Nlk inhibits Wnt-dependent gene expression at the level of the β-catenin/TCF transcription complex (Ishitani et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). In vertebrates, Nlk phosphorylates human Tcf4 on two sites in its central domain, T178 and T189 (and the corresponding sites T155 and S166 of human Lef-1), and inhibits the DNA-binding ability of the Tcf/β-catenin complex (Ishitani et al., 1999, 2003; Fig. 5). Ectopic expression of Nlk in *Xenopus* can block axis duplication induced by β-catenin but not by expression of Tcf targets, showing that Nlk acts at the level of Tcf/β-catenin (Ishitani et al., 1999; Hyodo-Miura et al., 2002). Evidence that Wg signaling in flies induces Nemo expression indicates that Nlk is a negative feedback regulator of β-catenin/TCF transcription (Zeng and Verheyen, 2004).

Genetic analyses in *Caenorhabditis elegans* revealed that the Nlk homolog, Lit-1, controls polarity and cell fate decisions, two processes regulated by distinct Wnt pathways (Kaletta et al., 1997; Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999). The most well-studied role for *lit-1* is in promoting the nuclear export of worm Tcf Pop-1 during

asymmetric cell division. In this *variant* Wnt pathway Lit-1 requires Wrm-1, a β-catenin homolog, to activate Lit-1/Nlk, which ultimately leads to inhibition of Pop-1 and subsequent activation of gene expression (Rocheleau et al., 1999; Lo et al., 2004). Such a role for Nlk in overcoming Tcf-mediated repression has also been described in zebrafish (Thorpe and Moon, 2004).

Lastly, it should be pointed out that Lef/Tcfs also appear to be substrates of various general kinases. Specifically, phosphorylation of Tcf3 by CK1 enhances, while GSK inhibits Tcf3 binding to β-catenin (Lee et al., 2001). Moreover, phosphorylation of human Lef-1 by CK2 at S42 and S61 increases the affinity of β-catenin for Lef-bound chromatinized templates and enhances gene transcription (Wang and Jones, 2006). Paradoxically, another group mapped the murine S40 residue (corresponding to human S42) as a target site of CKIδ phosphorylation involved in disruption of the β-catenin-Lef-1 complex (Hammerlein et al., 2005). Thus, local modulation of kinases on Lef/Tcf-regulated promoters can impact gene-specific β-catenin recruitment and transcriptional activation.

FUTURE DIRECTIONS

Overall the regulatory phosphorylation events described in this review act together at multiple steps in the pathway to ensure a tightly regulated and precise level of Wnt signaling. The developmental and pathological consequences of misregulation of Wnt are well known (reviewed in Clevers, 2006). Likely as a consequence, several safeguards have evolved to fine-tune the activities of pathway components, most notably the receptor associated complex and the β-catenin/Tcf nuclear complex. Future studies will surely uncover further refinements of the pathway, as well as uncover tissue- and organism-specific modulators.

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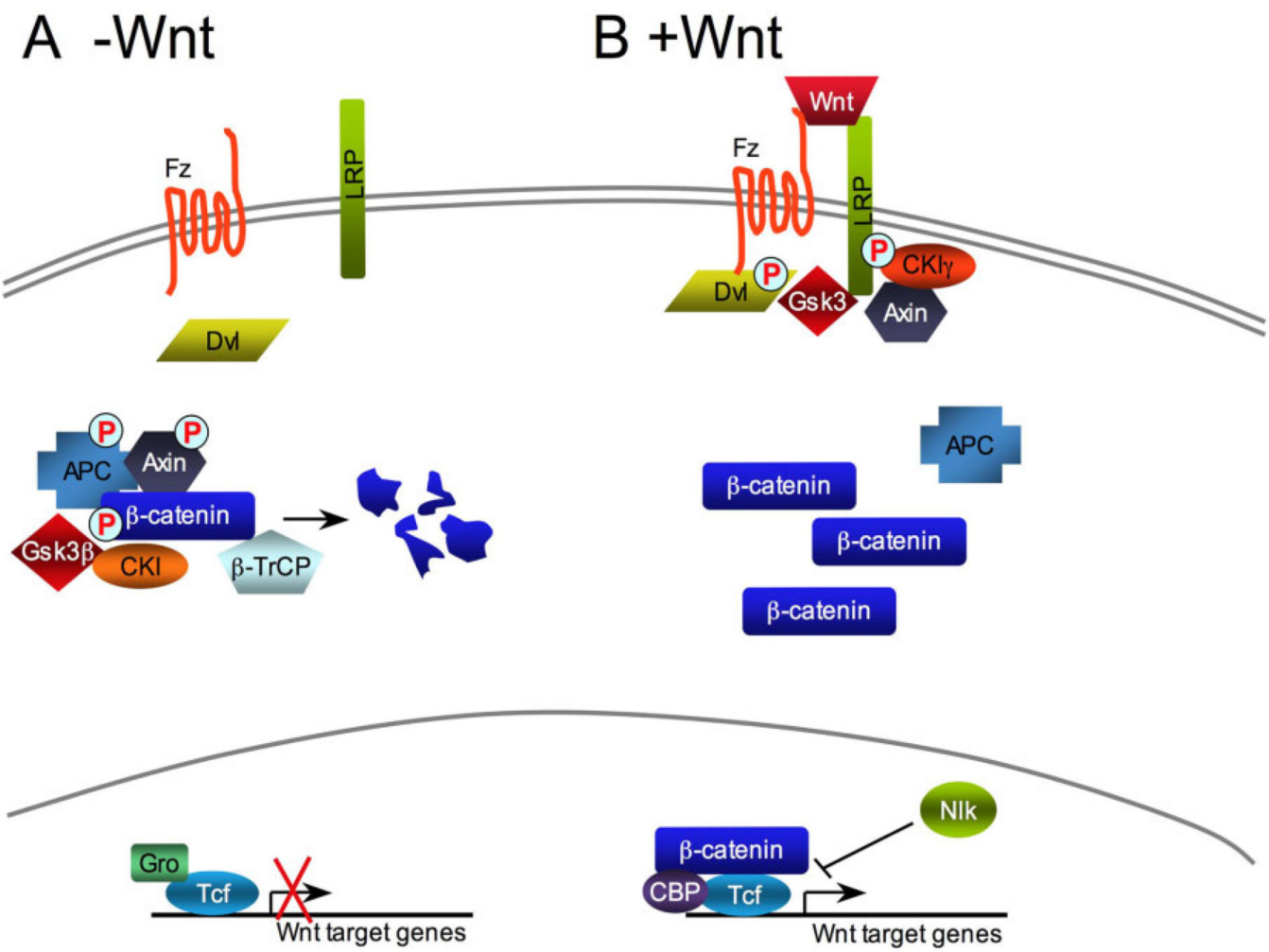

Fig. 1.

Wnt/β-catenin signaling pathway. **A:** In the absence of Wnt ligand, cytoplasmic b-catenin is targeted for destruction by the Axin–scaffolded protein complex containing Axin, APC, GSK3, and CKI. Association of DNA-bound Tcf with Gro and other transcriptional corepressors inhibits Wnt target genes. **B:** Upon ligand binding, the destruction complex is inhibited and cytoplasmic β-catenin accumulates and enters the nucleus where it can activate gene expression through Tcf and CBP binding.

Fig. 2.

Phosphorylation sites on β-catenin. β-Catenin is phosphorylated on its N-terminus by GSK3 (shown in orange) and CKI (shown in orange). These phosphorylations target β-catenin for ubiquitination and subsequent proteasomal degradation. β-Catenin is also phosphorylated on the indicated sites by the diverse kinases PKA, AKT, and JNK2. These events are believed to promote β-catenin activity.

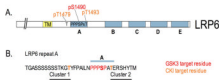

Fig. 3.

Phosphorylation sites in the cytoplasmic domain of LRP6. **A:** The cytoplasmic domain of LRP6 is shown, indicating the sites of GSK3 (red) and CKIγ (orange) phosphorylation. Five clusters of GSK3 sites followed by CKIγ sites are shown as blue boxes labeled A–E. TM, transmembrane domain. **B:** The amino acid sequence of LRP6 repeat A (adapted from Davidson et al., 2005). Cluster 1 contains the T1479 CKIγ target site and Cluster 2 follows the GSK3 site and contains a second CKIγ site (T1493).

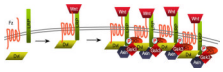

Fig. 4.
LRP6 signalosome assembly. Wnt ligand binding triggers assembly of LRP6-associated complexes termed signalosomes. Ligand association results in receptor association and recruitment of Dvl. Subsequently, GSK3 and Axin are recruited to the complex, along with membrane associated CKIγ, and LRP6 is phosphorylated.

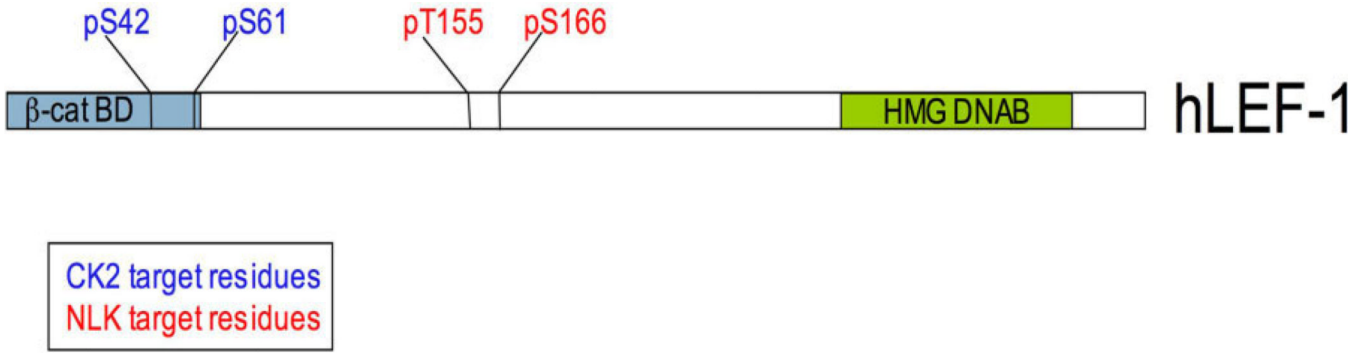

Fig. 5.

Phosphorylation sites on Lef-1. Lef/Tcf family members can be phosphorylated by Nlk, CKI, and CK2. CK2 phosphorylates human Lef-1 at residues S42 and S61 within the β-catenin binding domain (β-cat BD). Nlk can phosphorylate Lef-1 at residues T155 and S166, as well as in the corresponding T178 and T189 in Tcf4. The HMG DNA-binding domain (DNAB) is at the C-terminus of the protein.

TABLE 1

Wnt Pathway Components and Regulators

Mouse/Human	**Drosophila**	***C. elegans***
Wnt	Wingless (Wg)	Wnt
Frizzled (Fz)	Frizzled (Fz)	Frizzled (Fz)
LRP5/6	Arrow	LRP5/6
Dishevelled (Dvl)	Dishevelled (Dsh)	Mig-5
GSK3	Zw3	Gsk-3
CKI	Gilgamesh	Kin-19
Axin	Axin	Pry-1
APC	APC	Apr-1
β-catenin	Armadillo (Arm)	Bar-1, Sys-1, Wrm-1, Hmp-2
Tcf/Lef	dTcf/Pangolin	Pop-1
Nlk	Nemo	Lit-1
Hipk2	Hipk	