Molecular Cell, Vol. 13, 149–156, January 16, 2004, Copyright ©2004 by Cell Press

# A Mechanism for Wnt Coreceptor Activation

Short Article

**Keiko Tamai,[1,3] Xin Zeng,[1,3] Chunming Liu,[1,4] Xinjun Zhang,[2] Yuko Harada,[1] Zhijie Chang,[2] and Xi He[1,*]**

[1]Division of Neuroscience
Children's Hospital
Department of Neurology
Harvard Medical School
Boston, Massachusetts 02115

[2]Department of Biological Sciences and Biotechnology
Tsinghua University
Beijing 100084
China

## Summary

***L*DL receptor *r*elated *p*roteins 5 and 6 (LRP5/6) and their *Drosophila* homolog Arrow are single-span transmembrane proteins essential for Wnt/β-catenin signaling, likely via acting as Wnt coreceptors. How Wnt activates LRP5/6/Arrow to initiate signal transduction is not well defined. Here we show that a PPPSP motif, which is reiterated five times in the LRP5/6/Arrow intracellular domain, is necessary and sufficient to trigger Wnt/β-catenin signaling. A single PPPSP motif, upon transfer to the LDL receptor, fully activates the Wnt pathway, inducing complete axis duplication in *Xenopus* and TCF/β-catenin-responsive transcription in human cells. We further show that Wnt signaling stimulates, and requires, phosphorylation of the PPPSP motif, which creates an inducible docking site for Axin, a scaffolding protein controlling β-catenin stability. Our study identifies a critical signaling module and a key phosphorylation-dependent activation step of the Wnt receptor complex and reveals a unifying logic for transmembrane signaling by Wnts, growth factors, and cytokines.**

## Introduction

Signaling by the Wnt family of secreted lipoproteins plays a central role in development and disease (Wodarz and Nusse, 1998; Moon et al., 2002; van Es et al., 2003). A key Wnt transduction pathway is the canonical β-catenin signaling, which by regulating cytosolic β-catenin protein level controls the activation of Wnt-responsive genes. In the absence of Wnt stimulation, β-catenin is degraded via phosphorylation-dependent ubiquitination and proteolysis; β-catenin phosphorylation occurs in the so-called Axin complex composed of scaffolding protein Axin, tumor suppressor protein adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3), and casein kinase I (CKI) (Polakis, 2002). CKI and GSK-3 sequentially phosphorylate β-catenin (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002), thereby earmarking β-catenin for ubiquitination/degradation (Polakis, 2002). Wnt signaling inhibits the Axin complex and consequently β-catenin phosphorylation, thus stabilizes cytosolic β-catenin, which associates with the TCF/LEF (T cell factor/lymphocyte enhancer factor) family of transcription factors, and leads to Wnt responsive gene expression (Bienz and Clevers, 2003).

Wnt/β-catenin signaling requires two distinct transmembrane proteins: one is a member of the Frizzled (Fz) family of serpentine receptors (Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997), and the other is a single-span transmembrane receptor belonging to the *L*DL receptor *r*elated *p*rotein (LRP) family, the vertebrate LRP5 or LRP6 and the *Drosophila arrow* gene product (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Loss of function for *arrow* (Wehrli et al., 2000) or *Dfz1* and *Dfz2* together (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Muller et al., 1999) phenotypically copies *wingless* (*wg*, the *wnt-1* ortholog) mutation, providing genetic evidence that both Fz and Arrow functions are essential for Wg signaling. In *Xenopus* embryos, LRP6 synergizes with either Wnt or Fz to activate Wnt/β-catenin signaling, and inhibition of LRP6 function via a dominant-negative mutant blocks Wnt/Fz signaling (Tamai et al., 2000). Importantly, *lrp6* mutant mice exhibit composite phenotypes of mutations of several individual *wnt* genes (Pinson et al., 2000). The simplest model, supported by some biochemical studies in vitro (Tamai et al., 2000; Semenov et al., 2001), is that Wnt may induce the formation of a Fz and LRP5/6/Arrow complex, which triggers downstream signaling. Several lines of evidence are consistent with this model. First, several Wnt proteins have been shown to bind LRP5 and LRP6 (Tamai et al., 2000; Kato et al., 2002; Itasaki et al., 2003; Liu et al., 2003), although Wg binding to Arrow has not been demonstrated (Wu and Nusse, 2002). Secondly, forced proximity of Dfz2 and Arrow, by fusing the Arrow cytoplasmic domain with the carboxyl tail of Dfz2, initiates β-catenin signaling in the absence of Wg (Tolwinski et al., 2003). Finally, Dickkopf-1 antagonizes Wnt/β-catenin signaling by binding to LRP5/6 (Bafico et al., 2001; Mao et al., 2001a; Semenov et al., 2001), and its inhibitory activity correlates with disruption of Wnt-induced Fz-LRP5/6 complex formation (Semenov et al., 2001).

LRP5/6 plays a pivotal role in the initiation of Wnt signaling. A LRP6 mutant lacking the intracellular domain is defective in Wnt signaling, and in fact blocks Wnt and Fz signaling (Tamai et al., 2000). Conversely, LRP5/6 mutants lacking the extracellular domain (but anchored on the membrane) appear to be constitutively active (Mao et al., 2001a, 2001b). Observations that the intracellular domain of LRP5/6 and Arrow can bind and recruit Axin to the plasma membrane suggest a direct link between the Wnt coreceptor and the Axin complex

*Correspondence: xi.he@childrens.harvard.edu

[3]These authors contributed equally to this work.

[4]Present address: Department of Human Biological Chemistry and Genetics, Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-1048.

(Mao et al., 2001b; Tolwinski et al., 2003; Liu et al., 2003). It is unknown, however, how Wnt leads to LRP5/6/Arrow activation.

Here we show that a PPPSP motif, which is reiterated five times in the intracellular domain of LRP5/6 and Arrow, is responsible for LRP6 activity in the Wnt/β-catenin pathway and is sufficient to transfer the LRP6 signaling function to the LDL receptor (LDLR). The PPPSP motif, upon phosphorylation, provides a docking site for Axin. Wnt stimulates LRP6 phosphoryaltion on the PPPSP motif, thus inducing the engagement of the Wnt coreceptor with Axin.

## Results

### PPPSP Motifs Are Required for LRP6 Signaling Function

We showed that LRP6ΔC, a LRP6 mutant lacking most of the intracellular domain, is a loss-of-function and in fact dominant-negative mutant (Tamai et al., 2000). We constructed a reciprocal mutant, LRP6ΔN, which lacks most of the extracellular domain and is anchored on the membrane (Figure 1A). When LRP6ΔN mRNA was injected into the ventral side of *Xenopus* embryos at 0.1 ng/embryo, dorsal axis duplication was observed in most injected embryos, a majority of which exhibit duplication of complete axes, including eyes and a cement gland (Figure 1), signifying full activation of Wnt/β-catenin signaling (Moon and Kimelman, 1998). For comparison, we have never observed a single case, among almost 1000 embryos injected, of complete axis duplication with the full-length LRP6 at 2–5 ng RNA/embryo (Tamai et al., 2000; data not shown). These results, together with findings made via TCF/β-catenin reporter assays in mammalian cells (Mao et al., 2001a, 2001b) (Figure 1C), suggest that LRP6ΔN is a constitutively active receptor. An analogous Arrow mutant lacking its extracellular domain, ArrowΔN, also induced complete axis duplication and TCF/β-catenin reporter expression (Figure 1). Together with a reciprocal observation that human LRP6 can functionally replace Arrow in *Drosophila* cells (Schweizer and Varmus, 2003), these results demonstrate a conserved signaling mechanism for Arrow/LRP5/6.

We aligned the intracellular domain of Arrow/LRP5/LRP6 and noted several regions of strong amino acid similarity, including five PPP(S/T)P motifs (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/13/1/149/DC1). A study suggested that deletion of the carboxyl 47 amino acid residues from an activated LRP5 mutant (analogous to LRP6ΔN) abolishes its ability to activate TCF/β-catenin reporter expression (Mao et al., 2001b). This 47 residue region contains three of the five PPPSP motifs (c, d, and e in Figure 1A). We were surprised and intrigued to find that a LRP6ΔN derivative without these three PPPSP motifs, ΔN1, still induced complete axis duplication and TCF/β-catenin reporter expression (Figure 1). Thus we examined whether the two residual PPPSP motifs account for this signaling activity. Indeed, ΔN2, which harbors a single PPPSP motif, also induced complete axis duplication and TCF/β-catenin reporter expression, whereas ΔN3, which lacks any PPPSP motif, was inactive in both assays (Figure 1). We also mutated the conserved S/T residues into alanine in ΔN1 (Figure 1). While mutations of either PPPSP motif in ΔN1a or ΔN1b did not abolish (but reduced) signaling, mutation of the two motifs together in ΔN1ab completely eliminated the activity in both axis duplication and TCF/β-catenin reporter assays (Figure 1). These data suggest that PPPSP motifs are critical for LRP6 function in β-catenin signaling. Indeed, a complementary LRP6ΔN variant, ΔNG, which contains the last three PPPSP motifs, is also fully active in both assays (Figure 1).

We verified the function of PPPSP motifs in the wild-type LRP6. LRP6m5, which harbors alanine substitution of the invariant S/T residue in all five PPP(S/T)P motifs in the full-length LRP6, was completely inactive in β-catenin signaling and was in fact a dominant inhibitor of Wnt function (Figure 1E). Thus LRP6m5 behaved identically to LRP6ΔC, which lacks the cytoplasmic domain (Tamai et al., 2000).

### A PPPSP Motif Activates β-Catenin Signaling When Transferred to LDLR

We next transferred one of the PPPSP motifs (a in Figure 1) to LDLRΔN, a LDLR variant lacking the extracellular domain and thus structurally analogous to LRP6ΔN. LDLRΔN is inactive in axis duplication and TCF/β-catenin reporter assays as expected (Figures 2B–2D), given that the LDLR intracellular domain is unrelated to that of LRP5/6 (Figure 2A). Strikingly, LDLRΔN-PPPSP, which harbors a single PPPSP motif in the LDLRΔN backbone (Figures 2A and 2B), induced complete axis duplication and TCF/β-catenin reporter expression (Figures 2C and 2D). Reiteration of two copies of the same PPPSP motif in LDLRΔN-PPPSPx2 further increased the activity in both assays (Figures 2B–2D). By contrast, PPPAP failed to transfer any signaling activity to LDLRΔN, either as a single copy or two copies (Figures 2B–2D). Two additional mutations that altered the surrounding proline residues of the PPPSP motif, AAASP and PPPSA, were also inactive when transferred to LDLRΔN (Figures 2B–2D). LDLRΔN-PPPSP and LRP6ΔN, but neither LDLRΔN nor LDLRΔN-PPPAP, induced β-catenin accumulation in 293T cells comparable to (or stronger than) Wnt-3a treatment (Figure 2E), corroborating the functional assays. These results demonstrated that the PPPSP motif is sufficient, upon transfer to a heterologous receptor, to initiate Wnt/β-catenin signaling, and this signaling function requires the serine and surrounding proline residues.

### The PPPSP Motif Is Phosphorylated

We confirmed that LDLRΔN and derivatives, which were tagged with the VSVG epitope, were expressed at comparable levels in both axis induction and reporter assays (Figure 3A; and data not shown). Intriguingly, LDLRΔN-PPPSP and LDLRΔN-PPPSPx2 exhibited an additional band or bands above the predicted molecular weight in *Xenopus* and 293T cells (Figure 3A; and data not shown). Treatment of the embryo or cell extracts with λ phosphatase, but not with the phosphatase plus phosphatase inhibitors, converted the upper band(s) to the lower predicted molecular size (Figure 3B). By contrast, LDLRΔN-PPPAP, -PPPAPx2, -AAASP, and -PPPSA, none of which has signaling activity, exhibited only a single band of the predicted molecular weight (Figure 3A). Thus, the PPPSP motif is phosphorylated at the serine residue,

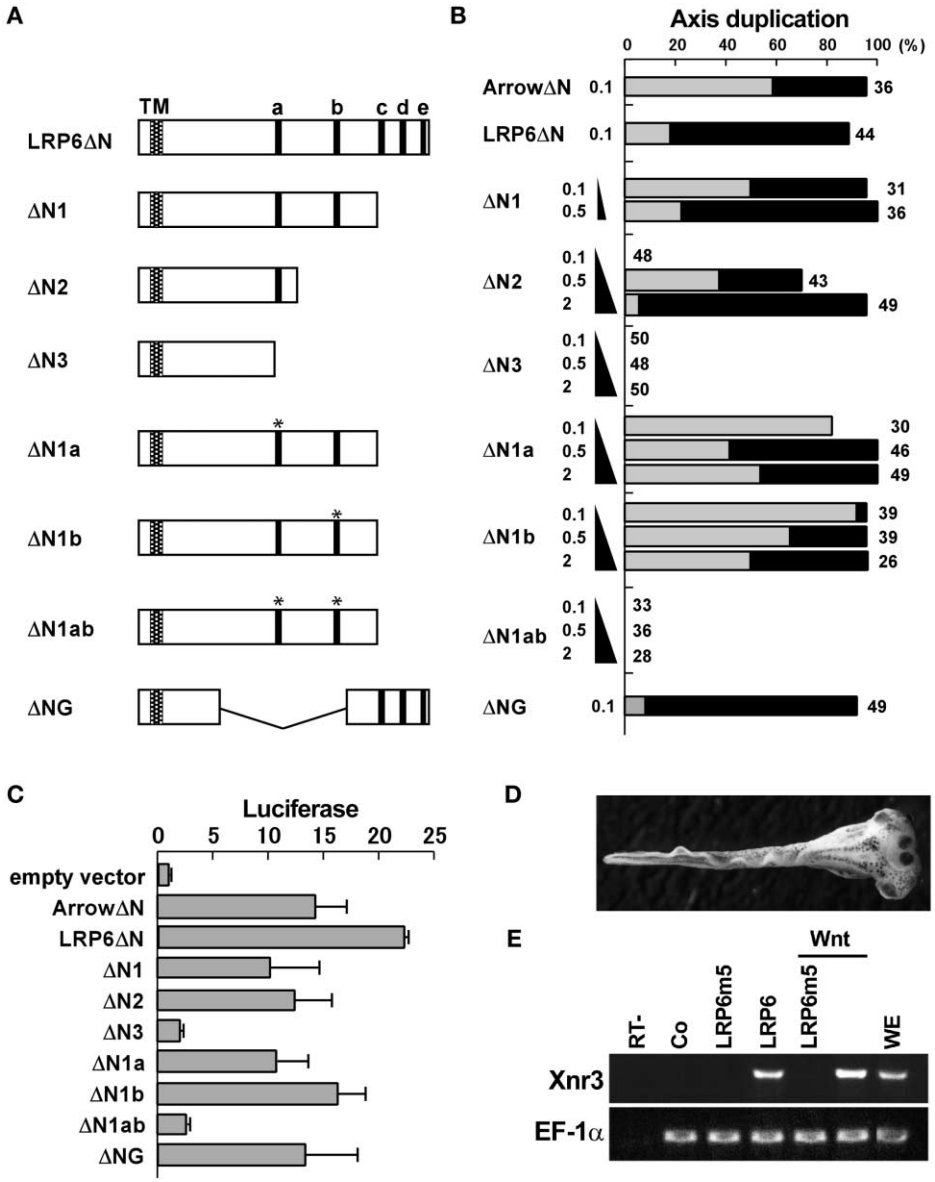


Figure 1. PPPSP Motifs Are Required for LRP6 Signaling

(A) Schematic representation of LRP6ΔN and derivatives. Five PPPSP motifs are labeled in letters a to e. TM, transmembrane region. The asterisk represents a S/T to A substitution.

(B) Axis duplication assay. The number at the bottom and the top of each bar indicates the mRNA amount injected (ng/embryo) and injected embryo number, respectively. Black bar: complete axis duplication (containing duplicated head including eyes and the cement gland); gray bar: partial axis duplication (containing duplicated trunk but no head).

(C) TCF/β-catenin reporter assay. Normalized luciferase activities are depicted in arbitrary units, with the control (transfection with the empty vector) defined as 1.

(D) An example of complete axis duplication by LRP6ΔN.

(E) Animal pole explant assay. Xnr3 is a direct target for Wnt/β-catenin signaling (Moon and Kimelman, 1998) and was induced by either LRP6 (2 ng/embryo) or Xwnt-8 (10 pg/embryo), but not by LRP6m5 (2 ng/embryo). LRP6m5 in fact blocked Xwnt-8 induction of Xnr3. Co: control injection; RT−: no reverse transcriptase added; WE: RNA from the whole embryo (PCR control); EF1α: internal control for RT-PCR and loading. The protein levels of LRP6 and LRP6m5 were comparable as detected via VSVG blotting (not shown).

and this phosphorylation requires the surrounding prolines and correlates with signaling activity.

### The Phosphorylated PPPSP Motif Is a Docking Site for Axin

The transferable nature of the PPPSP motif suggests that it is a docking site for an intracellular signaling component, and phosphorylation may be a docking signal. Previous studies showed that LRP5/6 and Arrow can bind to Axin (Mao et al., 2001b; Liu et al., 2003; Tolwinski et al., 2003). Indeed Axin coimmunoprecipitated (IPed) LDLRΔN-PPPSP and -PPPSPx2, but not LDLRΔN, LDLRΔN-PPPAP, or -PPPAPx2 (Figure 3C). Most interestingly, Axin co-IPed only the phosphorylated,

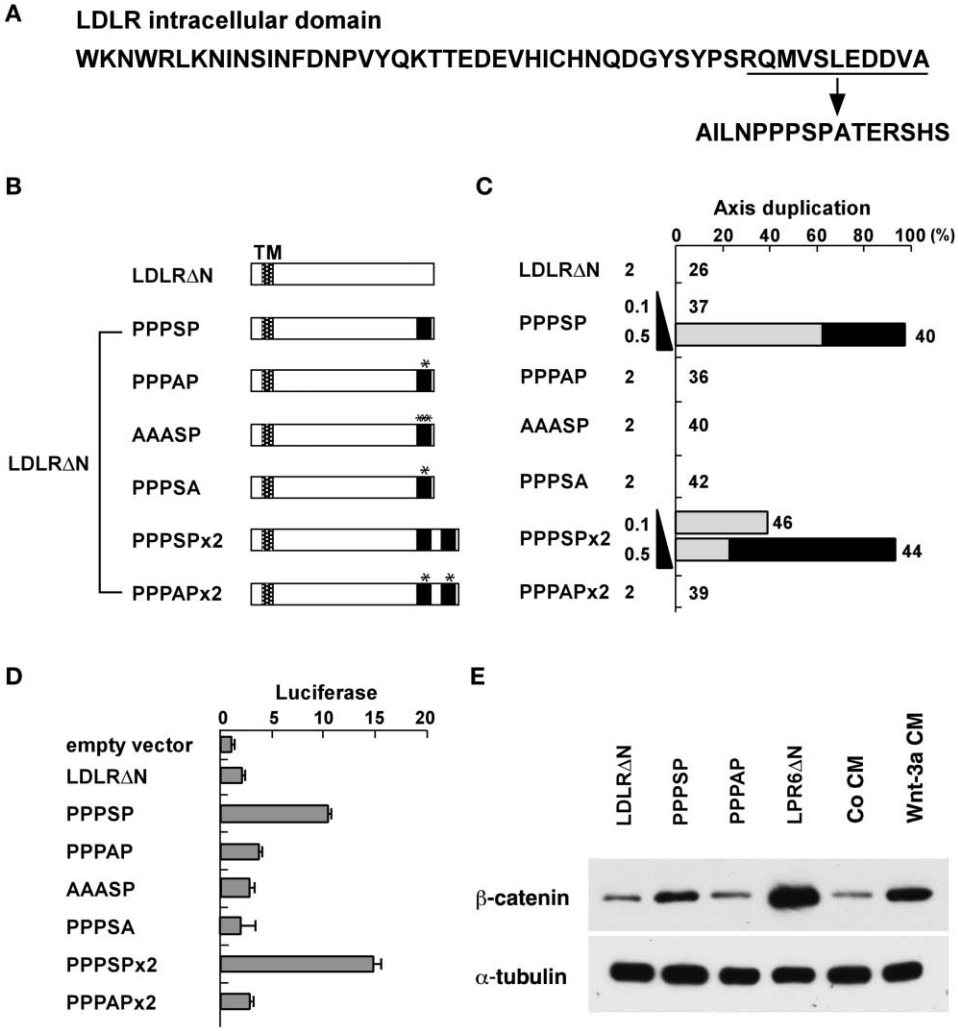


Figure 2. A Single PPPSP Motif Is Sufficient to Transfer β-Catenin Signaling Activity to LDLRΔN

(A) LDLR intracellular domain. The underlined sequence is replaced by a PPPSP motif with flanking amino acid residues indicated, generating LDLRΔN-PPPSP.

(B) Schematic representation of LDLRΔN and derivatives. Black boxes indicate the PPPSP motif and its mutant variants (*).

(C) Axis duplication assay. None of the PPPSP mutants exhibited any activity when injected at 0.1, 0.5, or 2 ng (RNA/embryo).

(D) TCF/β-catenin reporter assay.

(E) Stabilization of cytosolic β-catenin. Wnt-3a CM treatment served as a positive control. Co CM: control CM; α-tubulin: a loading control.

but not the unphosphorylated, form of LDLRΔN-PPPSP and -PPPSPx2 (Figure 3C). Thus, the phosphorylated PPPSP motif is a docking site for Axin.

LRP6ΔN and derivatives also exhibited higher molecular weight species in immunoblotting, possibly due to phosphorylation, but the appearance/intensity of such upper bands was somewhat variable among experiments (data not shown). We suspected that phosphorylation of LRP6ΔN and derivatives may be labile and subjected to rapid dephosphorylation in cells or during extract preparation. Indeed, IP of Axin resulted in coprecipitations of predominantly upper bands of LRP6ΔN (Figure 3D), which represented the phosphorylated LRP6ΔN since λ phosphatase converted them into the expected molecular size, i.e., the unphosphorylated form (Figure 3D). By the same criteria, phosphorylated ΔN1, ΔN2, and ΔNG, which have two, one, and three different PPPSP motifs, respectively (Figure 1), were also co-IPed with Axin (Figures 3D and 3E). By contrast, ΔN3, which does not have any PPPSP motif (Figure 1), and LRP6ΔNm5, which harbors an alanine substitution of the S/T residue in all five PPP(S/T)P motifs in LRP6ΔN and is severely defective in signaling (data not shown), were poorly co-IPed with Axin (Figure 3D and 3F). Thus, PPPSP phosphorylation of LRP6ΔN and derivatives confers Axin binding.

### Wnt Induces LRP6 Phosphorylation

Wnt/Wg signaling appears to promote LRP5/6/Arrow to bind and recruit Axin to the plasma membrane (Mao et al., 2001b; Liu et al., 2003; Cliffe et al., 2003). Our results thus suggested a model that Wnt stimulates LRP5/6 phosphorylation on PPPSP motifs, thereby creating docking sites for Axin. We generated an antiserum specific for a phosphorylated PPPSP motif (a in Figure 1A). Ab1490 (for phosphorylation at serine 1490 of LRP6)

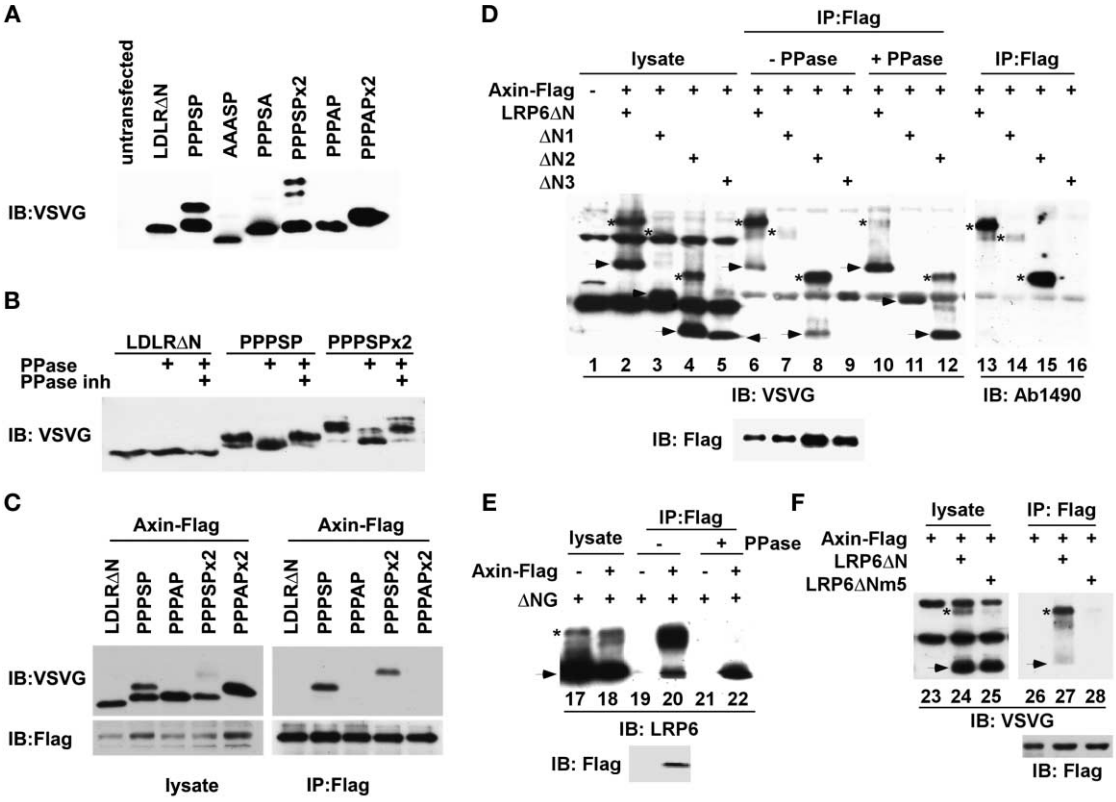


Figure 3. The PPPSP Motif Is Phosphorylated and Binds Axin upon Phosphorylation

(A) Immunoblotting (IB) of lysates from cells expressing LDLRΔN and derivatives (VSVG-tagged).

(B) Immunoblotting of the same cell lysates treated with or without λ phosphatase, or with λ phosphatase plus its inhibitors (inh), NaF plus $Na_3VO_4$.

(C) Co-IP of Axin (Flag) and LDLRΔN derivatives, which were coexpressed. Immunoblotting of cell lysates (left) and Axin immunoprecipitates (right) for LDLRΔN and derivatives (VSVG, top) and Axin (Flag, bottom).

(D–F) LRP6ΔN and derivatives were phosphorylated and bound Axin upon phosphorylation. Axin and LRP6ΔN derivatives (VSVG) were coexpressed. Phosphorylated (*) and unphosphorylated (arrow) forms are highlighted. Lanes 1–5: lysates; lanes 6–12: Axin immunoprecipitates either untreated (lanes 6–9) or treated with λ phosphatase (lanes 10–12). Lanes 1–12 were blotted for LRP6ΔN derivatives. For reasons not clear, phosphorylated ΔN1 level is low (lane 3), and thus Axin co-IP of ΔN1 is proportionally weak (lane 7). One possibility is that ΔN1 was particularly prone to phosphatases in cells or during cell extract preparation. Lanes 13–16: Axin immunoprecipitates blotted by Ab1490 for phosphorylated LRP6ΔN derivatives. Lanes 17 and 18: lysates; lanes 19–22: Axin immunoprecipitates either untreated (lanes 19 and 20) or treated with λ phosphatase (lanes 21 and 22). Lanes 17–22 were blotted for ΔNG (using an Ab for LRP6). Lanes 23–25: lysates; lanes 26–28: Axin immunoprecipitates. Lanes 23–28 were blotted for LRP6ΔN or LRP6ΔNm5 (VSVG). The Axin level after IP is shown in lanes 6–9, 19–20, and 26–28 (bottom). We note that some Axin precipitates contained a small amount of unphosphorylated LRP6ΔN or derivatives (lanes 6, 8, 20, and 27), possibly due either to low-affinity association with Axin or to rapid dephosphorylation of LRP6ΔN derivatives during/after IP. Longer exposures revealed weak co-IP between Axin and LRP6ΔNm5 (lane 28), possibly due to a combined effect of weak Axin binding by five mutated PPPAP motifs or to indirect Axin-LRP6 association via other (unknown) proteins.

recognizes a synthetic PPPSP peptide in which the serine residue is phosphorylated, but not a control peptide that has identical residues except for an unphosphorylated serine (see Experimental Procedures). Indeed, Ab1490 specifically detected the upper phosphorylated, but not the lower unphosphorylated, LDLRΔN-PPPSP (Figure 4A) and LRP6ΔN (Figure 3D; and data not shown). Remarkably, stimulation of 293T cells with Wnt-3a-conditioned medium (CM) induced robust and rapid LRP6 phosphorylation at the PPPSP motif (Figure 4B), demonstrating a key biochemical alteration of a Wnt receptor component upon activation.

## Discussion

Our study has uncovered an activation mechanism for Wnt coreceptor LRP6, and by extension, LRP5 and Arrow. We demonstrated that a PPPSP motif, which is reiterated five times in the cytoplasmic domain of LRP5/6/Arrow, is necessary and sufficient for Wnt/β-catenin signaling. We further showed that phosphorylation of the PPPSP motif is critical for its signaling activity and Axin binding, and that Wnt stimulates LRP6 phosphorylation in vivo. These results suggest that Wnt activates transmembrane signaling by inducing LRP5/6/Arrow phosphorylation at the PPPSP motifs, which provide phosphorylation-dependent docking sites for and recruit Axin to the plasma membrane, thereby regulating the Axin complex and thus β-catenin phosphorylation and degradation (Figure 4C).

Because a PPPSP motif represents a minimal functional module that can bind Axin and fully activate β-catenin signaling, LRP5/6/Arrow appears to operate primarily by engaging Axin, although our study does not rule out the possibility that LRP5/6/Arrow may also bind

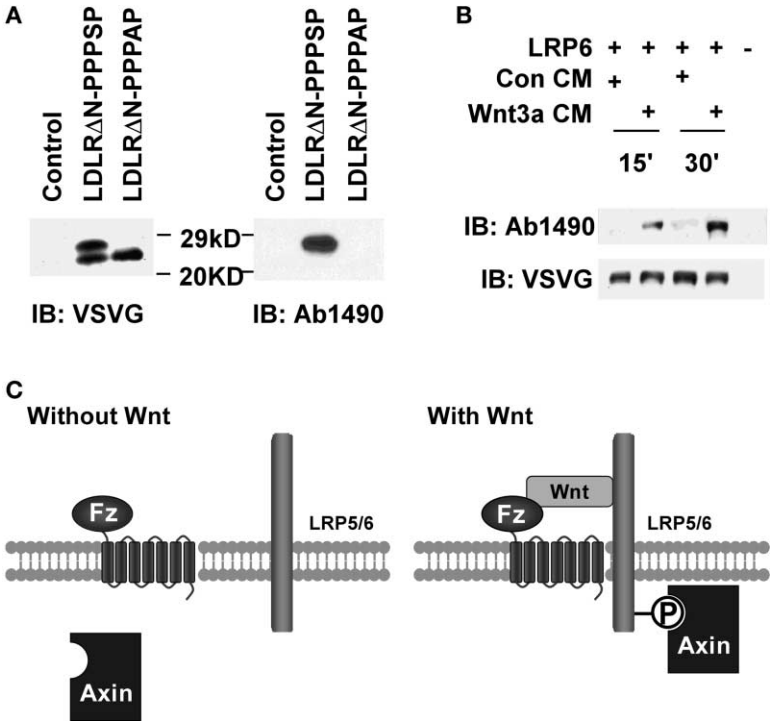


Figure 4. Wnt Induces LRP6 PPPSP Phosphorylation

(A) Ab1490 specifically recognized phosphorylated LDLRΔN-PPPSP, but neither unphosphorylated LDLRΔN-PPPSP nor LDLRΔN-PPPAP. Lysates from untransfected cells, or cells transfected with LDLRΔN-PPPSP or –PPPAP, were blotted by a VSVG Ab (left) or Ab1490 (right). Control: untransfected cells.

(B) Cells transfected with the wild-type LRP6 (VSVG-tagged) were treated with control or Wnt-3a CM for 15 and 30 min. Cell lysates were blotted by a VGVG Ab (bottom) or Ab1490 (top). We cotransfected LRP6 with Mesd and Axin for technical considerations. Mesd is an endoplasmic reticulum chaperone and facilitates the folding and transport of LRP5/6 to the plasma membrane (Hsieh et al., 2003), whereas Axin binding appears to protect PPPSP motifs from dephosphorylation.

(C) A working model for Wnt receptor complex activation and phosphorylation-induced engagement of Axin.

other proteins that exert regulatory function. The serine and surrounding proline residues of the PPPSP motif are required for phosphorylation, Axin binding, and signaling (Figures 2 and 3), and it is possible that other flanking residues may play some roles as well. It remains to be understood how LRP5/6/Arrow recruitment of Axin results in β-catenin pathway activation. Prolonged Wnt signaling, either by Wnt treatment (Yamamoto et al., 1999; Willert et al., 1999) or overexpression (Tolwinski et al., 2003), or by overexpression of an activated LRP5 (Mao et al., 2001b), promotes Axin degradation (but see Cliffe et al., 2003), which probably leads to β-catenin stabilization. However, β-catenin accumulation induced by Wnt treatment can occur before an obvious reduction of endogenous Axin protein level (Willert et al., 1999), implying that inhibiting the activity of the Axin complex may play a pivotal role as well.

The strength of β-catenin pathway activation appears to correlate, to some degree, with the number of PPPSP motifs present (Figures 1 and 2). It is conceivable that, upon Wnt activation, five PPPSP motifs reiterated in Arrow/LRP5/6 provide simultaneous docking sites for multiple Axin molecules; or, they function together to provide a high local concentration of Axin binding sites to ensure a tight Axin-LRP5/6 association. Indeed, while a single PPPSP motif upon phosphoryaltion is sufficient to bind Axin, two copies of this same PPPSP motif in tandem exhibit more effective or stronger Axin binding (Figure 3C). The modular nature of LRP6 signaling and its phosphorylation-induced engagement to Axin are unexpected, but bear striking similarity to signaling by well-characterized tyrosine kinase receptors and cytokine receptors (Pawson and Scott, 1997), thereby elucidating a unifying logic for receptor activation by Wnts, growth factors, and cytokines. Our results further imply the existence of two additional components in controlling LRP5/6 activation: a kinase that phosphorylates the PPPSP motif and a phosphatase that dephosphorylates it. Wnt may regulate the kinase or phosphatase activity or their access to LRP5/6.

The full active nature of LDLRΔN-PPPSP in β-catenin signaling raises questions about the function of Fz, which probably acts, together with Wnt, to activate Arrow/LRP5/6 signaling. It is intriguing that the Fz cytoplasmic regions, and thus its signaling function, are required for β-catenin signaling (Umbhauer et al., 2000; and our unpublished data) and that another intracellular scaffolding protein, Dishevelled, functions downstream of Fz and probably Arrow (Wehrli et al., 2000; Tolwinski et al., 2003) and is required for Wg-induced recruitment of Axin to the plasma membrane (Cliffe et al., 2003). Whether and how Fz and Dishevelled might regulate Arrow/LRP5/6 phosphorylation and/or Arrow/LRP5/6 interaction with Axin deserve investigation. As loss-of-function and gain-of-function mutations in human LRP5 are associated with familial osteoporosis and high bone density syndromes, respectively (Patel and Karsenty, 2002), understanding of LRP5/6 phosphorylation and the kinase and phosphatase involved may have therapeutic significance.

## Experimental Procedures

### Plasmids

All LRP6 and LDLR constructs were tagged with the VSVG epitope and subcloned in pCS2+. The VSVG-tag was inserted after LRP6 signal peptide at residue 25, introducing a BspEI site in the corresponding cDNA. To generate LRP6ΔN, a primer with BspEI site was used to amplify cDNA corresponding to amino acid residues 1370–1613. ΔN1, ΔN2, and ΔN3 delete the carboxyl 49, 107, and 127 residues, respectively. ΔNG deletes amino acid residues 1442–

1533 of LRP6 from LRP6ΔN. LDLRΔN (residues 781–860) and ArrowΔN (residues 1445–1678) were generated by fusing the LRP6 signal peptide and the VSVG-tag with the transmembrane and intracellular domains of LDLR and Arrow, respectively. To generate LDLRΔN-PPPSP, the last 11 amino acids were substituted by a PPPSP motif with several amino acids flanking each side (Figure 2A), using an internal XhoI site in LDLR. LRP6m5 and LRP6ΔNm5 were generated via sequential mutagenesis using the QuickChange Kit (Stratagene). Details of the plasmids are available upon request.

### *Xenopus laevis* Embryo Manipulations

Synthetic RNAs were injected at the four-cell stage. Procedures for embryo staging, injection, and animal pole explants were done as described (Tamai et al., 2000).

### Antibodies, Immunoprecipitation, and Immunoblotting

Ab1490 was generated in rabbits (Convance, Denver, PA) using synthetic phospho-peptide CLNPPPS*PATER (S*: phospho-S) as immunogen. Bleeds were passed through the phosphopeptide affinity column and washed with 0.1 M Tris (pH 8.0). Bound antibodies were eluted with 0.2 M Glycine (pH 2.5) and neutralized with 1 M Tris (pH 8.0). The specificity of Ab1490 was verified by its immunoreactivity to the phosphopeptide but not to the nonphosphorylated control peptide via dot-blotting (data not shown), and to the phosphorylated LDLRΔN-PPPSP (Figure 4A). A polyclonal LRP6 antiserum was generated by immunizing rabbits with a 6×His tag fusion protein containing amino acids 1409–1613 of human LRP6. Other antibodies were used according to manufacturers' instructions: anti-β-catenin (C2206, Sigma), anti-VSVG (V4888, Sigma), and anti-α-tubulin (sc8053, Santa Cruz). Immunoprecipitation, SDS-PAGE, and immunoblotting were done as described (Liu et al., 2002).

### Mammalian Cell Transfection, Luciferase Assay, Extracts, and Phosphatase Treatment

Transfections were done with Effectene (Qiagen) on 293T cells in 6-well plates. Luciferase assays were performed using DualLuciferase reporter system (Promega). LRP6ΔN, LDLRΔN, or derivatives (1 μg/well) were transfected together with the TOPFLASH-luciferase (1 μg/well) and the *Renilla* luciferase pRL-CMV (0.1 μg/well). Relative luciferase units (RLU) were measured and normalized against *Renilla* luciferase activity 48 hr after transfection. For co-IPs, LRP6ΔN, LDLRΔN, or derivatives and Axin were transfected at 1 μg/well each. For Wnt stimulation of LRP6 phosphorylation, LRP6, Mesd, and Axin were cotransfected at 0.67 μg/well each. Wnt3a CM and control CM were added to cells 48 hr after transfection.

For extracts, cells were lysed 48 hr after transfection in a buffer containing 50 mM HEPES (pH 7.4), 1.5 mM EDTA, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM $Na_3VO_4$, 0.5 mM DTT, and a cocktail of protease inhibitors. For phosphatase treatment, lysates or immunoprecipitates, which were washed in lysis buffer without NaF and $Na_3VO_4$, were treated with λ phosphatase (New England Biolabs) at 37°C for 1 hr. The β-catenin protein stabilization assay was described (Liu et al., 2002).

### Acknowledgments

We thank B. Holdener for Mesd cDNA, X. Chen for technical help, and M. Semenov and other lab members for discussion. C.M.L. is supported by a B.H. Campbell & A.F. Hall postdoctoral fellowship from The Medical Foundation. Y.H. is supported in part by a postdoctoral fellowship from Naito Foundation (Japan). Z.C. is supported by a grant (3022807) from NSFC (China). X.H. is supported by a grant from NIH, and is a W.M. Keck Foundation Distinguished Young Scholar.

Received: September 25, 2003
Accepted: November 11, 2003
Published: January 15, 2004

### References

Amit, S., Hatzubai, A., Birman, Y., Andersen, J.S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev. *16*, 1066–1076.

Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S.A. (2001). Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat. Cell Biol. *3*, 683–686.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225–230.

Bhanot, P., Fish, M., Jemison, J.A., Nusse, R., Nathans, J., and Cadigan, K.M. (1999). Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during *Drosophila* embryonic development. Development *126*, 4175–4186.

Bhat, K.M. (1998). *frizzled* and *frizzled 2* play a partially redundant role in wingless signaling and have similar requirements to wingless in neurogenesis. Cell *95*, 1027–1036.

Bienz, M., and Clevers, H. (2003). Armadillo/beta-catenin signals in the nucleus—proof beyond a reasonable doubt? Nat. Cell Biol. *5*, 179–182.

Cliffe, A., Hamada, F., and Bienz, M. (2003). A role of Dishevelled in relocating Axin to the plasma membrane during Wingless signaling. Curr. Biol. *13*, 960–966.

He, X., Saint-Jeannet, J.P., Wang, Y., Nathans, J., Dawid, I., and Varmus, H. (1997). A member of the Frizzled protein family mediating axis induction by Wnt-5A. Science *275*, 1652–1654.

Hsieh, J.C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M.E., Rosenquist, T., and Holdener, B.C. (2003). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell *112*, 355–367.

Itasaki, N., Jones, C.M., Mercurio, S., Rowe, A., Domingos, P.M., Smith, J.C., and Krumlauf, R. (2003). Wise, a context-dependent activator and inhibitor of Wnt signalling. Development *130*, 4295–4305.

Kato, M., Patel, M.S., Levasseur, R., Lobov, I., Chang, B.H., Glass, D.A., 2nd, Hartmann, C., Li, L., Hwang, T.H., Brayton, C.F., et al. (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J. Cell Biol. *157*, 303–314.

Kennerdell, J.R., and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell *95*, 1017–1026.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell *108*, 837–847.

Liu, G., Bafico, A., Harris, V.K., and Aaronson, S.A. (2003). A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. Mol. Cell. Biol. *23*, 5825–5835.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001a). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature *411*, 321–325.

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001b). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell *7*, 801–809.

Moon, R.T., and Kimelman, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. Bioessays *20*, 536–545.

Moon, R.T., Bowerman, B., Boutros, M., and Perrimon, N. (2002). The promise and perils of Wnt signaling through beta-catenin. Science *296*, 1644–1646.

Muller, H., Samanta, R., and Wieschaus, E. (1999). Wingless signaling in the Drosophila embryo: zygotic requirements and the role of the frizzled genes. Development *126*, 577–586.

Patel, M.S., and Karsenty, G. (2002). Regulation of bone formation and vision by LRP5. N. Engl. J. Med. *346*, 1572–1574.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075–2080.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C.

(2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature *407*, 535–538.

Polakis, P. (2002). Casein kinase 1: a Wnt'er of disconnect. Curr. Biol. *12*, R499–R501.

Schweizer, L., and Varmus, H. (2003). Wnt/Wingless signaling through beta-catenin requires the function of both LRP/Arrow and frizzled classes of receptors. BMC Cell Biol. *4*, 4.

Semenov, M.V., Tamai, K., Brott, B.K., Kuhl, M., Sokol, S., and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr. Biol. *11*, 951–961.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J.P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature *407*, 530–535.

Tolwinski, N.S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S., and Wieschaus, E. (2003). Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity. Dev. Cell *4*, 407–418.

Umbhauer, M., Djiane, A., Goisset, C., Penzo-Mendez, A., Riou, J.F., Boucaut, J.C., and Shi, D.L. (2000). The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. EMBO J. *19*, 4944–4954.

van Es, J.H., Barker, N., and Clevers, H. (2003). You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. Curr. Opin. Genet. Dev. *13*, 28–33.

Wehrli, M., Dougan, S.T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000). *arrow* encodes an LDL-receptor-related protein essential for Wingless signalling. Nature *407*, 527–530.

Willert, K., Shibamoto, S., and Nusse, R. (1999). Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. Genes Dev. *13*, 1768–1773.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

Wu, C.H., and Nusse, R. (2002). Ligand receptor interactions in the Wnt signaling pathway in *Drosophila*. J. Biol. Chem. *277*, 41762–41769.

Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J. Biol. Chem. *274*, 10681–10684.

Yanagawa, S., Matsuda, Y., Lee, J.S., Matsubayashi, H., Sese, S., Kadowaki, T., and Ishimoto, A. (2002). Casein kinase I phosphorylates the Armadillo protein and induces its degradation in *Drosophila*. EMBO J. *21*, 1733–1742.

Yang-Snyder, J., Miller, J.R., Brown, J.D., Lai, C.J., and Moon, R.T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr. Biol. *6*, 1302–1306.