Molecular Cell, Vol. 7, 801–809, April, 2001, Copyright ©2001 by Cell Press

Low-Density Lipoprotein Receptor-Related Protein-5 Binds to Axin and Regulates the Canonical Wnt Signaling Pathway

Junhao Mao,[1,4,7] Jiyong Wang,[2,7] Bo Liu,[1,4] Weijun Pan,[2] Gist H. Farr, III,[3] Christopher Flynn,[1] Huidong Yuan,[4] Shinji Takada,[5] David Kimelman,[3] Lin Li,[2,6] and Dianqing Wu[1,6]

[1]Department of Genetics and Developmental Biology
University of Connecticut
Farmington, Connecticut 06030
[2]State Key Laboratory of Molecular Biology
Institute of Biochemistry and Cell Biology
Shanghai Institutes for Biological Sciences
Chinese Academy of Sciences
Shanghai
China
[3]Department of Biochemistry
University of Washington
Seattle, Washington 98195
[4]Department of Pharmacology
University of Rochester
Rochester, New York 14627
[5]Department of Biochemistry
Setsunan University
Osaka
Japan

Summary

To understand how the Wnt coreceptor LRP-5 is involved in transducing the canonical Wnt signals, we identified Axin as a protein that interacts with the intracellular domain of LRP-5. LRP-5, when expressed in fibroblast cells, showed no effect on the canonical Wnt signaling pathway by itself, but acted synergistically with Wnt. In contrast, LRP-5 mutants lacking the extracellular domain functioned as constitutively active forms that bind Axin and that induce LEF-1 activation by destabilizing Axin and stabilizing β-catenin. Addition of Wnt caused the translocation of Axin to the membrane and enhanced the interaction between Axin and LRP-5. In addition, the LRP-5 sequences involved in interactions with Axin are required for LEF-1 activation. Thus, we conclude that the binding of Axin to LRP-5 is an important part of the Wnt signal transduction pathway.

Introduction

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules and plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate (Moon and Kimelman, 1998; Wodarz and Nusse, 1998; Peifer and Polakis, 2000). Wnt pathways are also closely linked to tumorigenesis (Barker and Clevers, 2000; Bienz and Clevers, 2000; Peifer and Polakis, 2000). Studies using *Drosophila*, *Xenopus,* and mammalian cells have established a canonical signaling pathway. Wnt proteins bind Frizzled (Fz), a membrane protein with seven transmembrane domains, and prevent Glycogen synthase kinase 3 (GSK3)-dependent phosphorylation of β-catenin, thus leading to the stabilization of β-catenin. The stabilized β-catenin interacts with transcription regulators, including leukocyte enhance factor-1 (LEF-1) and T cell factors (TCF), and activates gene transcription (Dale, 1998; Gumbiner, 1998; Wodarz and Nusse, 1998). It is still not known exactly how Wnt prevents GSK3 from phosphorylating β-catenin, although this process is likely to involve Dishevelled (Dsh/Dvl). A number of mechanisms have been proposed, including a direct inhibition of GSK3 kinase activity (Cook et al., 1996), inhibition of GSK3 by its interaction with Frat/GBP (Li et al., 1999a; Farr et al., 2000; Salic et al., 2000), and dephosphorylation and degradation of Axin (Kikuchi, 1999; Willert et al., 1999).

It is better understood how GSK3 regulates the levels of β-catenin. GSK3 forms a complex with the scaffolding protein Axin and the tumor suppressor APC. Axin binds directly to GSK3, β-catenin, and APC, while APC also directly binds to GSK3 and β-catenin. GSK3 phosphorylates β-catenin in the complex, which causes the binding of factors involved in the ubiquitination of β-catenin, targeting β-catenin for degradation by proteasomes. The exact role of APC in the complex is still uncertain, although its importance in this pathway is shown by the observation that colon cancer cell lines containing mutant forms of APC have elevated levels of β-catenin (Peifer and Polakis, 2000).

Both genetic and biochemical results have provided solid evidence indicating that Fz proteins function as Wnt receptors (Wodarz and Nusse, 1998). Recent studies on *Drosophila* Fz proteins, DFz1 and DFz2, showed that these two *Drosophila* Wnt receptors bind to Wg with different affinities (Rulifson et al., 2000) and that they showed different functional efficacies and distinctions as well (Boutros et al., 2000; Rulifson et al., 2000). Although both Dfz proteins can mediate the canonical pathway, Dfz2 appears to play a predominant role in transducing the Wg canonical signal in vivo. In addition, Dfz1, but not Dfz2, regulates planar polarity. It, however, remains to be determined whether different mammalian Fz proteins have different affinities for over 20 different mammalian Wnt proteins and whether these Fz proteins play different roles in vivo. Recently, members of the LDL receptor superfamily (LRPs) were found to also be required for the canonical Wnt signaling pathway. A *Drosophila* mutant, *arrow*, which encodes a LRP, shows phenotypes similar to the *wg* mutant (Wehrli et al., 2000). In addition, mammalian LRP-6 was shown to bind to Wnt-1 and enhance Wnt-induced developmental processes in *Xenopus* embryos (Tamai et al., 2000). Moreover, mice lacking LRP-6 exhibited developmental defects that are similar to those caused by deficiencies in various Wnt proteins (Pinson et al., 2000). Despite the evidence that the Fz and LRP proteins are receptors for the Wnt proteins, it is not known how these receptor

[6]To whom correspondence should be addressed (e-mail: dwu@neuron.uchc.edu [D. W.], lil@sunm.shcnc.ac.cn [L. L.]).
[7]These authors contributed equally to this work.

proteins transduce signals. Specifically, we do not know whether Fz, LRP, or both are directly involved in the transduction of signals to intracellular signaling mediators, because no physical links between any of the Wnt receptor proteins and any of the intracellular Wnt signal transducing proteins have been identified.

In this report, we show that LRP-5, a close homolog of LRP-6 (Hey et al., 1998), functions as a coreceptor for Wnt proteins in mammalian cells and that it can transduce the canonical Wnt signals, at least in part by binding and recruiting Axin to membranes. This establishes the first physical link from a Wnt receptor to one of Wnt intracellular signaling mediators and provides insights into the mechanism by which a Wnt receptor transduces its signals in the canonical pathway.

Results

LRP-5 Functions as a Wnt Coreceptor in Mammalian Cells

To determine if LRP-5 has any effect on the canonical Wnt signaling pathway in mammalian cells, we coexpressed LRP-5 with Wnt-1 in NIH 3T3 cells. In these cells, Wnt-1 has been shown to elevate the levels of cytosolic β-catenin and activate LEF-1-dependent transcriptional activity (Li et al., 1999a; Li et al., 1999b). Expression of LRP-5 alone had no effect on LEF-1 activity (Figure 1A), as determined using a LEF-1-dependent luciferase reporter gene assay (Hsu et al., 1998), but enhanced the effect of Wnt-1 (Figure 1A) or Wnt-3a (data not shown). To confirm that LRP-5 acted with secreted Wnt-1 protein, a paracrine coculture approach was used (Li et al., 1999a), in which LRP-5 and the reporter luciferase were transfected into one group of cells and Wnt-1 was transfected into a separate group of cells. These two groups were combined after transfection, and luciferase activities were determined after the cells were cocultured for 24 hr. Since Wnt-1 and the reporter gene were produced in different cells, any effect of Wnt-1 could be attributed only to the interaction of secreted Wnt-1 protein with cells expressing LRP-5 and containing the reporter gene. As shown in Figure 1B, LRP-5 and Wnt-1 acted synergistically in the activation of LEF-1-dependent transcription in the paracrine assay, demonstrating that LRP-5 potentiates the signaling of secreted Wnt-1. The same results were also observed for Wnt-3a in similar coculture assays (data not shown).

Tamai et al. reported that a LRP-6 mutant lacking the intracellular domain functioned as a dominant-negative mutant, presumably through sequestering Wnt proteins (Tamai et al., 2000). We also generated two LRP-5 mutants that lack the intracellular domains and transmembrane domains (Figure 1C). Consistent with their results, LRPN1, which encodes the entire extracellular domain, inhibited Wnt-1-induced, but not Dvl- or β-catenin-induced, elevation of cytosolic β-catenin levels, and LEF-1 activation in the cotransfection assay (Figures 1D and 1E). LRPN1 also inhibited Wnt-1-induced LEF-1 activation in the coculture assay (Figure 1F). In addition, we found that LRPN2, which encodes only the first YWTD spacer motif-EGF repeat domain (Figure 1C), was also able to inhibit Wnt's effects in both cotransfection and coculture assays (Figures 1D–1F). These inhibitory

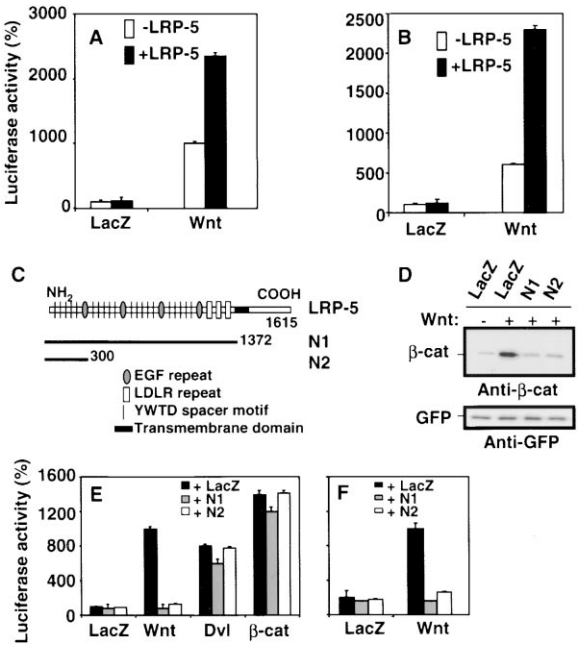

Figure 1. Involvement of LRP-5 in Wnt Signaling

(A) LRP-5 potentiates Wnt-1 in the cotransfection assay. NIH 3T3 cells in 24-well plates were transfected with 0.025 μg of a LEF-1 expression plasmid, 0.075 μg of a LEF-1 luciferase reporter plasmid, 0.15 μg of a GFP expression plasmid, and 0.15 μg of a control β-galactosidase (LacZ) or Wnt-1 (Wnt) expression plasmid in the presence or absence of 0.1 μg of a LRP-5 expression plasmid. LacZ plasmid was added to make the total amount of DNA equal (0.5 μg/transfection). One day later, cells were lysed, and the GFP levels and luciferase activities were determined. The luciferase activities presented were normalized against the levels of GFP expression. Each experiment was carried out in triplicate, and error bars represent standard deviations.

(B) LRP-5 potentiates Wnt-1 in the coculture assay. One set of 3T3 cells was transfected with a LEF-1 reporter gene, a LEF-1 expression plasmid, a GFP expression plasmid in the presence or absence of a LRP-5 expression plasmid, while the second set of cells was transfected with a plasmid expressing LacZ or Wnt-1. Three hours after transfection, cells from both sets were trypsinized. An equal number of cells from each set were mixed, and the mixed cells were reseeded into a new 24-well plate. Luciferase activity was determined after a 24 hr coculture.

(C) Schematic representation of the LRP-5 molecule and its structural features.

(D) Inhibition of Wnt-1-induced β-catenin stabilization by the N-terminal domains of LRP-5. 3T3 cells were transfected with 0.15 μg of plasmids expressing LacZ, GFP, Wnt, LRPN1 (N1), and/or LRPN2 (N2). Cells were collected 24 hr after transfection, and cytosolic fractions were prepared. Equal amounts of cytosol were analyzed by Western blotting. GFP and β-catenin (β-cat) were detected with specific antibodies.

(E and F) Inhibition of Wnt-1-induced LEF-1 activation by the N-terminal domains of LRP-5. The effect of N1 and N2 on Wnt-1 was examined in both cotransfection (E) and coculture assays (F).

effects of the LRPN mutants may also be the result of sequestering the ligand from the endogenous Wnt receptors because the extracellular domain of LRP-5, like that of LRP-6, can also bind Wnt-1. This interaction was detected by immunoprecipitation, using anti-Flag antibodies in conditioned media mixed from those containing Flag-tagged LRP-5 extracellular domain, and those containing Wnt-1 (data not shown). All these re-

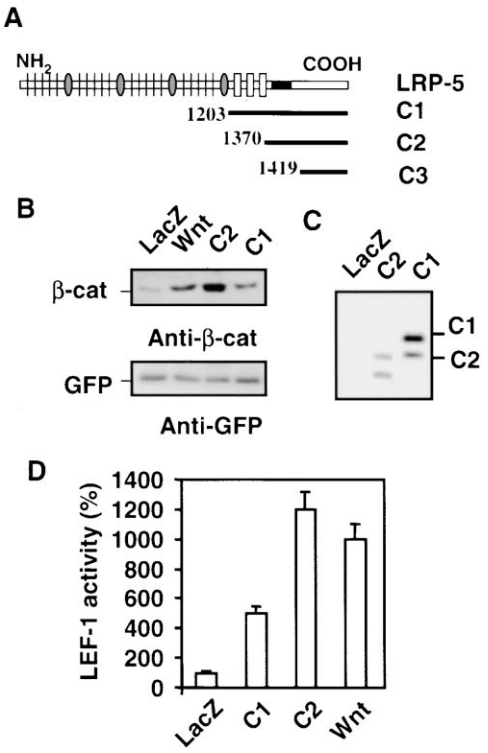

Figure 2. Removal of the Extracellular Domain of LRP-5 Leads to Constitutive Activation

(A) Schematic representation of LRP-5 mutants.

(B–D) Activation of LEF-1 and stabilization of β-catenin by LRP mutants. 3T3 cells were transfected with a LEF-1 reporter gene, a LEF-1 expression plasmid, a GFP expression plasmid, and a plasmid encoding LacZ, Wnt, LRPC1 (C1), or LRPC2 (C2). The cytosolic levels of β-catenin (B), expression levels of the LRP mutants (C), and the activity of LEF-1 were determined (D). The estimated sizes of C1 and C2 are marked. There are always fragments that appear to be about 10 kDa smaller than the estimated sizes of the LRPC mutants. These smaller bands may be proteolytic fragments.

sults are consistent with those observed in *Xenopus* (Tamai et al., 2000) and *Drosophila* (Wehrli et al., 2000), demonstrating that LRP-5 also functions as a putative coreceptor for Wnt proteins in mammalian cells.

LRP-5 Lacking the Extracellular Domain Is Constitutively Active

To determine whether LRP-5 directly participates in signal transduction, we generated several extracellular domain truncation mutants. We found that LRPC1, which includes the entire intracellular domain, the transmembrane domain, and a stretch of the extracellular domain starting at residue 1203, and LRPC2, which is the same as LRPC1 except it has a shorter extracellular domain that starts at residue 1370 (Figure 2A), are constitutive activators of the Wnt pathway. Specifically, expression of LRPC1 or LRPC2 (Figure 2C) led to increased levels of cytoplasmic β-catenin (Figure 2B), and activated LEF-1-dependent transcription in NIH 3T3 cells (Figure 2D) in a ligand-independent manner. These results indicate that removing the extracellular domain of LRP-5 leads to constitutive activation of the intracellular domain. The constitutively active nature of these two mutants also suggests that the intracellular domain of LRP-5 is actively involved in transducing Wnt signals.

LRP-5 Binds Axin

Analysis of the intracellular amino acid sequences of LRP-5 and its close homologs, including LRP-6 and Arrow, revealed that they are highly conserved, although they lack obvious enzymatic domains. Thus, we hypothesized that LRP-5 may be involved in the canonical Wnt signaling pathway through protein–protein interactions. We sought to identify proteins that interact with the intracellular domain of LRP-5 by screening a mouse fetal brain cDNA library, using the yeast two-hybrid system with the C-terminal intracellular domain of LRP-5 as bait. We screened ~4 million clones and identified one clone (Lc15) that was strongly positive and several weakly positive ones. Restriction and nucleotide sequencing analyses revealed that the Lc15 clone contained a 2.5 Kb insert that encodes the C-terminal portion of mouse Axin, starting at nucleotide 1398 of the mouse Axin sequence reported by Zeng et al. (Zeng et al., 1997). This region starts just before the GSK3 binding region (Ikeda et al., 1998) and encompasses the rest of the C-terminal portion of the mouse Axin protein (Figure 3D). To confirm the interaction between the LRP-5 intracellular domain and Axin, we coexpressed the intracellular domain of LRP-5 (LRPC3; Figure 2A) with mouse full-length Axin in COS-7 cells and found that these two proteins coimmunoprecipitated (Figures 3A and 3B). In addition, LRPC3 coimmunoprecipitated endogenous Axin (Figure 3C).

The yeast two-hybrid assay was also used to identify the Axin sequences that are required for the interaction with LRPC3. Surprisingly, we found that the full-length Axin showed a much lower apparent affinity for LRPC3 than the truncated Axin (Lc15) we obtained from the yeast two-hybrid screening (Figure 3D). The inhibitory sequences appear to be localized to the RGS domain and the sequence between the RGS domain and the GSK binding domain (Figure 3D). In addition, the Axin DIX domain appears to be essential for the binding to LRPC3, while further truncation of Lc15 from the N terminus also significantly attenuated its interaction with LRPC3 (Figure 3D), suggesting that the GSK binding domain may play a role in facilitating the interaction between Axin and LRP-5. Moreover, we examined whether LRPC3 interacted with two other known Wnt intracellular signaling factors, Dvl and GSK3, using the yeast two-hybrid assay. LRPC3 did not interact with these two proteins in this assay (Figure 3D), and these results were subsequently confirmed by immunoprecipitation (data not shown).

GSK3 Facilitates the LRP-5 and Axin Interaction

The apparent weak interaction between LRPC3 and the full-length Axin suggests that the interaction may be enhanced by posttranslational modification or the presence of accessory proteins. In fact, the interaction between LRPC3 and Axin was significantly enhanced when GSK3 was coexpressed (Figure 3E). The presence of GSK3 also enhanced the interaction of LRPC2 with Axin (Figure 3E). The kinase activity of GSK3 is required for this GSK3-mediated enhancement, because coexpres-

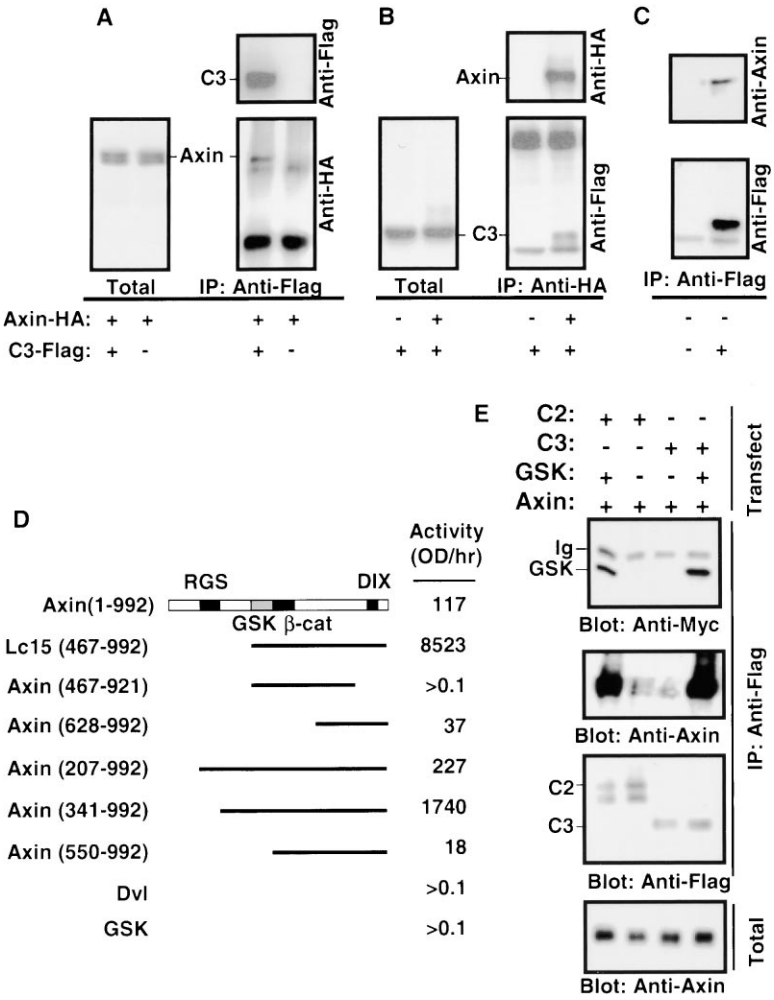

Figure 3. Interaction of the Intracellular Domain of LRP-5 with Axin

(A–C) Coimmunoprecipitation. COS-7 cells were transfected with HA-tagged full-length Axin and Flag-tagged LRPC3 (C3). Immunoprecipitation was carried out with anti-HA (B) or anti-Flag (A and C) antibodies. In panel C, the endogenous Axin was detected with an anti-Axin antibody. The levels of expression before precipitation are shown in "Total."

(D) Schematic representation of Axin and the deletion mutants used in quantification of the Axin-LRP interaction in the yeast assay. Numbers in parentheses denote amino acid residues. For the quantification experiments, a plasmid encoding LRPC3 fused to the Gal4 activation domain was cotransfected into yeast with a plasmid encoding the Gal4 DNA binding domain fused to Dvl, GSK3, full-length Axin, or mutants of Axin. The activity of the β-galactosidase reporter gene was determined.

(E) GSK3 enhances the interaction of LRP with Axin. Cos-7 cells were transfected with plasmids (LRPC2-Flag, LRPC3-Flag, GSK3-myc, and Axin), as indicated. Immunoprecipitation was carried out 24 hr after transfection. Note that GSK3 is immunoprecipitated in these experiments because it binds to Axin.

sion of a kinase-deficient mutant of GSK3 had little effect on the interaction of LRPC3 or LRPC2 with Axin (data not shown).

Wnt Stimulates the Interaction between LRP-5 and Axin

To investigate the effect of ligands on the interaction between LRP-5 and Axin, we first examined whether Wnt proteins can translocate endogenous Axin to plasma membranes. 3T3 cells were transfected with or without LRP-5 and treated with control or Wnt-3a conditioned medium. Wnt-3a conditioned medium was previously shown to be active in the canonical pathway (Shibamoto et al., 1998; Willert et al., 1999; Yamamoto et al., 1999), and we confirmed these results in our 3T3 cells (data not shown). The plasma membranes of these cells were isolated, and the levels of Axin were determined by Western analysis using an anti-Axin antibody. Wnt-3a treatment clearly increased the levels of Axin bound to the plasma membranes, and the expression of LRP-5 further potentiated the effect of Wnt-3a in Axin translocation (Figure 4A). These results indicate that Wnt proteins can translocate endogenous Axin to the membranes in a LRP-5 dependent manner.

To obtain further evidence that Wnt proteins translocate Axin to the membranes via its interaction with LRP-5, we examined whether Wnt-3a can stimulate the interaction between LRP-5 and Axin using the FRET (Fluorescence Resonance Energy Transfer) approach. FRET is a quantum mechanical process by which a fluorescent molecule, the donor, transfers energy, in a radiationless way, to an acceptor chromophore molecule in close proximity. FRET has been successfully used to detect specific intermolecular interactions. For example, CFP (cyan fluorescence protein) and YFP (yellow fluorescence protein) were previously used as a donor and an acceptor in the measurement of intracellular Ca^{2+} concentrations by detecting Ca^{2+}-dependent interaction of calmodulin-CFP and M13-YFP (M13 is the calmodulin binding domain of MLCK) (Miyawaki et al., 1997). We coexpressed a fusion protein of LRP-5 fused to YFP with a fusion protein of Axin fused to CFP in 3T3 cells and treated the cells with control medium, followed with conditioned medium containing Wnt-3a. The cells were observed using the time-lapse videomicroscopy with an excitation wavelength of 450 nm and emission wavelengths of 480 and 570 nm. If Axin-CFP is localized in close proximity to LRP-5-YFP, the transfer of energy from CFP to YFP will result in a reduction in CFP emission and an increase YFP emission. Therefore, the ratio

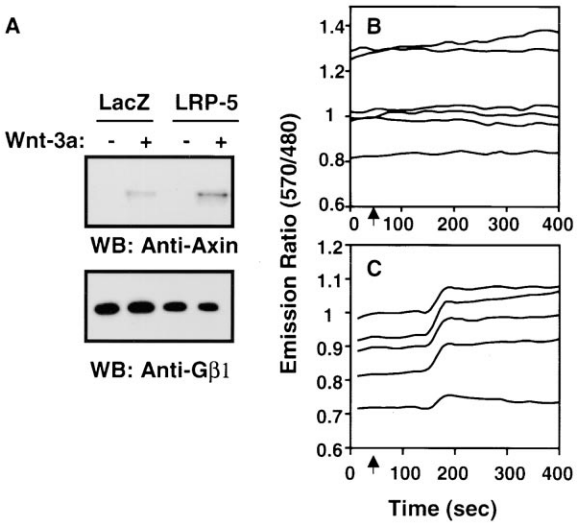

Figure 4. Effects of Ligand on the Interaction between Axin and LRP-5

(A) Membrane translocation of Axin. 3T3 cells were transfected with LacZ or LRP-5. Twenty-four hours after transfection, the cells were treated with control medium or conditioned medium containing Wnt-3a for 20 min at room temperature. Plasma membranes were prepared, and the levels of Axin and the β1 subunits of G proteins were determined by Western analysis with anti-Axin and anti-Gβ1 antibodies, respectively. Gβ1 is used as a loading control.

(B and C) Detection of the interaction between LRP-5 and Axin by FRET. 3T3 cells were transfected with LRP-5-YFP, Axin-CFP, and GSK3β. One day later, cells were observed using a time-lapsed fluorescence videomicroscope at 5 s intervals. Arrow indicates the addition of conditioned media. The same cells were first treated with control medium (B), and then treated with Wnt-3a-conditioned medium (C) (see Experimental Procedures). Each trace represents the fluorescence emission ratio of 570 nm to 480 nm of an individual cell expressing both CFP and YFP. There are five such cells in this view field. The increases in the ratio shortly after the addition of Wnt-3a-conditioned medium indicate FRET, which is the result of the interaction between Axin-CFP and LRP-5-YFP. These changes in the ratio did not occur in cells treated with control-conditioned medium.

of YFP emission to CFP emission provides an optimal gauge for changes in the interactions between Axin-CFP and LRP-5-YFP. If Wnt proteins enhance the interactions, there should be an increase in the ratio of YFP emission to CFP emission. As shown in Figure 4, addition of Wnt-3a conditioned medium (Figure 4C), but not the control medium (Figure 4B) led to increases in the ratio of YFP emission to CFP emission. The change occurred only a couple of minutes after the addition of Wnt-3a conditioned medium, suggesting that Wnt-3a induced a quick interaction between LRP-5 and Axin.

The Interaction of LRP-5 with Axin Is Required for Its Activity

Knowing that the intracellular domain of LRP-5 is capable of binding to Axin and transducing the canonical Wnt signals, an important question is whether the interaction between LRP-5 and Axin is required for the signal transduction process. To address this question, we wanted to identify the LRP-5 sequences that are required for the interaction with Axin and determine if the LRP binding sequences are required for the activation of LEF-1. We generated a number of LRP mutants starting with LRPC2 as depicted in Figures 5A and 5D. The ability of these mutants to activate LEF-1 and bind to Axin was determined. LRPC2 mutants Δ47 and Δ78, which lack the last 47 and 78 amino acids, respectively, could not activate LEF-1 (Figure 5B) and did not bind to Axin (Figure 5C), demonstrating that the C-terminal portion of LRPC2 is important for both LEF-1 activation and Axin binding. Mutants lacking the last 28 and 36 amino acids showed attenuated LEF-1 activation and Axin binding (Figures 5B and 5C), whereas truncation of the C-terminal 11 amino acids had little effect on either transcriptional activation or Axin binding (data not shown). Since there is a strong correlation between LEF-1 activation and Axin interaction, we suggest that the activation of LEF-1 by LRPC2 is mediated by its interaction with Axin.

Translocation of Axin by LRP-5 Plays a Role in Signal Transduction

While LRPC3 has an apparent affinity for Axin that is similar to that of LRPC2 (Figure 3E), LRPC3 is much less active than LRPC2 in stimulating LEF-1-dependent transcription (Figure 6A), even though both proteins are expressed at comparable levels (data not shown). Thus, the interaction with Axin, although it is required for LRP-5 to activate transcription, does not appear to be sufficient to achieve the full activity. We found that expression of LRPC2, but not LRPC3 or Δ47, led to reduced levels of Axin, especially the hyperphosphorylated form of Axin (Figure 6C). Interestingly, Wnt has previously been shown to induce the degradation of Axin (Willert et al., 1999; Yamamoto et al., 1999). Because the difference between LRPC2 and LRPC3 is the presence of the transmembrane domain in LRPC2 (Figure 6B), our results suggest that the recruitment of Axin to the membrane is important for its degradation. To determine if the transmembrane domain plays any role other than in anchoring the intracellular domain of LRP-5 on the membranes, we expressed LRPC3 carrying a myristylation signal derived from the Src kinase. The myristylated LRPC3 showed marked increases in its ability to activate LEF-1 transcription (Figure 6, mC3 compared to C3). Taking all these results together, we propose that both the membrane localization and Axin interaction are involved in the LRP-5-mediated activation of LEF-1 and that ligand-bound LRP-5 translocates Axin to membranes and leads to Axin destabilization.

Discussion

Three previous reports have provided solid evidence for the involvement of LRP-6 and its *Drosophila* homolog Arrow in the canonical Wnt signaling pathway in *Drosophila*, *Xenopus,* and mice (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Although these reports demonstrated that LRP proteins are required and that the full-length LRP-6 can enhance Wnt signaling, it still remains unclear how LRP and/or Fz are directly involved in transducing signals to the intracellular signaling components of the Wnt pathway. In this report we demonstrate that LRP-5 can transduce canonical Wnt signals via an interaction with Axin. Thus, we have revealed a

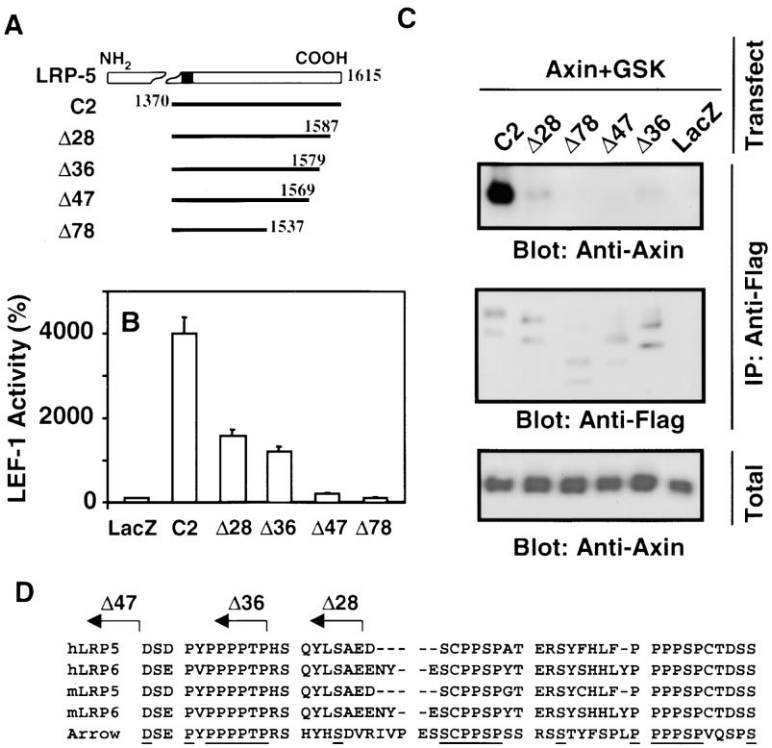

Figure 5. Characterization of LRP-5 Sequences Required for Interaction with Axin and Activation of LEF-1

(A) Schematic representation of LRP-5 mutants: LRPC2 and its deletion mutants, which are all Flag-tagged. The number after the Δ indicates the number of amino acids truncated from the C terminus.

(B) LEF-1 activity. 3T3 cells were transfected with the reporter genes, a GFP expression plasmid, and a plasmid encoding LacZ, LRPC2, or its mutants. The luciferase activity was determined 24 hr after transfection.

(C) Interactions with Axin. Cos-7 cells were transfected with plasmids as indicated, and immunoprecipitation was carried out 24 hr after transfection.

(D) Amino acid sequence comparison of the C-termini of mouse LRP-5,6, human LRP-5,6, and the *Drosophila* homolog Arrow. Identical amino acids are underlined, and the last amino acids of Δ28, Δ36, and Δ47 are denoted.

direct link, for the first time, between a Wnt receptor and one of the intracellular signaling components of the Wnt pathway.

Using cultured fibroblasts, we show that LRP-5 acts as a coreceptor for the Wnt signal transduction pathway, consistent with previous results from other systems. We find that expression of the extracellular domains block the ability of Wnt to stimulate LEF-1 transcription, as was also observed in *Xenopus* (Tamai et al., 2000). The inhibition of Wnt signaling by the extracellular domain might occur by blocking Wnt from interacting with Fz and/or with the endogenous LRP coreceptor.

Importantly, we find that expression of the cytoplasmic domain of LRP-5, with or without the transmembrane domain, acts as a constitutive activator of the Wnt pathway, activating LEF-1 transcription and stabilizing β-catenin. This result suggests that the extracellular domain inhibits the function of the intracellular domain. Binding of a Wnt ligand presumably overcomes this inhibition, perhaps by inducing a conformational change in LRP-5 or through interactions with the Frizzled receptor. Intriguingly, a construct containing the intracellular domain and the transmembrane region (LRPC2) was significantly more active than one containing just the intracellular domain (LRPC3; Figure 6), indicating that recruitment of Axin to the membrane is important for the activation of the Wnt pathway by LRP-5. The finding that a LRPC3 variant containing a myristylation signal showed an activity similar to LRPC2 further supports this idea. One of the roles that the translocation plays is Axin destabilization, which was previously shown to be induced by Wnt (Willert et al., 1999; Yamamoto et al., 1999), because expression of LRPC2, but not LRPC3, caused Axin degradation (Figure 6C). Together with our results showing that Wnt stimulation causes Axin to be recruited to the membrane via LRP-5 (Figure 4), we propose that Wnt induces Axin destabilization at least in part by stimulating the interaction of LRP-5 with Axin. Although the precise mechanism by which activated LRP-5 causes the destabilization of Axin is not clear, it is reasonable to conclude that Axin destabilization contributes to the stabilization of β-catenin (Zeng et al., 1997; Hamada et al., 1999).

As previously proposed, the degradation of Axin is not the only means of inhibiting its function (Willert et al., 1999). This is consistent with our results that a construct that does not degrade Axin (LRPC3) can still stimulate LEF-1 transcription. Thus, part of the mechanism by which the intracellular domain of LRP-5 inhibits the function of Axin may simply involve the binding of this region to Axin, thus preventing it from participating in the degradation of β-catenin. The region necessary for binding Axin and for stimulating LEF-1 transcription was narrowed down to 40 C-terminal amino acids in LRP-5. This region contains three copies of a motif PPT/SP, which is conserved in human and mouse LRP-5 and LRP-6 and *Drosophila* Arrow (Figure 5D). Although removal of the very C-terminal motif showed little effect, elimination of two of these motifs reduced both Axin binding and LEF-1 stimulation (Figure 5) but still retained some activity. Truncation of an additional 10 amino acids, including the third motif, abolished Axin binding and transcriptional activation. We note that this repeated motif could function as a phosphorylation site for a serine/threonine kinase. In this light, it is interesting that the wild-type GSK3 strongly stimulated the binding of Axin to LRP-5, whereas a kinase-dead GSK3 did not. While the intracellular domain of LRP-5 cannot be phosphorylated by GSK3 when immunoprecipitated LRPC2 and GSK were tested in an in vitro kinase assay (data

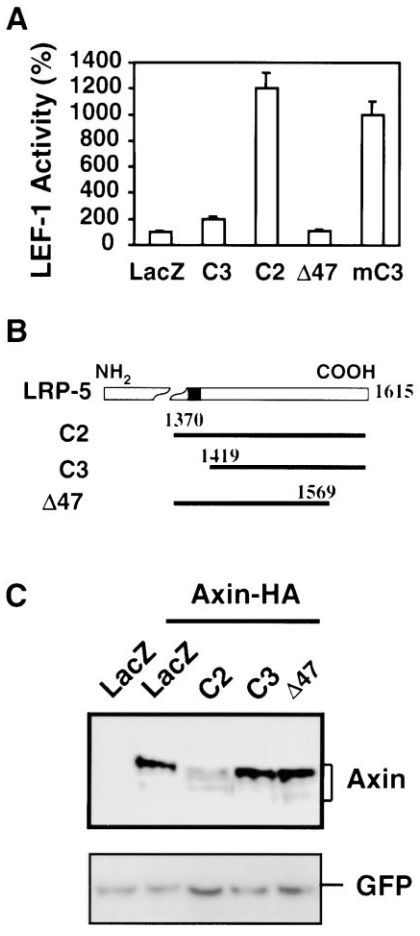

Figure 6. Role of the LRP-5 Transmembrane Domain in Signal Transduction

(A) LEF-1 activity. 3T3 cells were transfected with a reporter gene, a GFP expression plasmid, and a plasmid encoding LacZ, C2, C3, Δ47, or a C3 mutant carrying a myristylation signal (mC3). The luciferase activity was determined 24 hr after transfection.

(B) Schematic representation of C2, C3, and Δ47. The transmembrane domain is shown as a black box.

(C) Western analysis of Axin. 3T3 cells were transfected with GFP, Axin, and plasmids encoding LacZ and C2, C3, or Δ47. Cells were lysed in SDS sample buffer thirty hours after transfection, and proteins were separated on a 5% SDS gel. After blotting, Axin and GFP were detected with an anti-Axin antibody and an anti-GFP antibody, respectively. The multiple-band migration pattern of Axin represents different levels of phosphorylation. GFP was used as a control for protein expression levels.

not shown), GSK3 may stimulate an additional kinase. Alternatively, the phosphorylation of Axin by GSK3 might cause it to bind LRP-5 more effectively. For instance, the phosphorylation of Axin by GSK3 enhances its ability to bind β-catenin and to bind GSK3 itself (Ikeda et al., 1998; Willert et al., 1999). Given that GSK3 promotes β-catenin degradation, while the LRP/Axin interaction has the opposite result, the stimulatory effect of GSK3 on the interaction between Axin and LRP-5 is somewhat surprising. However, such an effect may allow LRP-5 to interact only with the Axin molecules that are associated with GSK3. In this way, LRP-5 could specifically direct the degradation of only those (GSK3-associated) Axin molecules that would otherwise participate in the degradation of β-catenin.

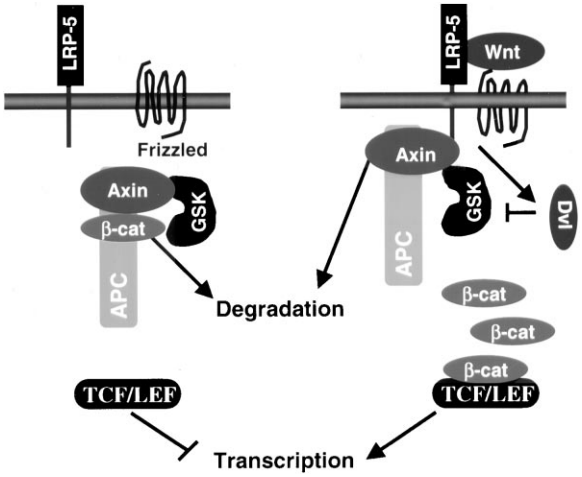

Figure 7. A Model for the Involvement of LRP-5 in the Canonical Wnt Signaling Pathway

In the absence of Wnt proteins, Axin, APC, β-catenin, and GSK3 form a complex, in which β-catenin is phosphorylated by GSK3, leading to β-catenin degradation. When Wnt proteins bind to Frizzled (Fz) and LRP-5, Axin is recruited to the membranes. The interaction between Axin and LRP-5 may prevent Axin from participating in the degradation of β-catenin, at least in part by destabilizing Axin. However, this is probably not the sole mechanism by which Wnt proteins transduce the canonical signal. Dvl may also receive signals from the Fz/LRP complex, resulting in the inhibition of GSK3.

Wnt-3a-induced binding of Axin to LRP-5 occurs within 4 min of the addition of ligand as detected by FRET (Figure 4). This ligand-induced process occurs much sooner than the other processes that have thus far been reported. Wnt proteins were shown to induce the dephosphorylation of Axin and Dvl (Willert et al., 1999; Yanagawa et al., 1995) within 30 min, and the degradation of Axin starting at approximately 2 hr (Yamamoto et al., 1999). Thus, the rapid stimulation of the interaction of LRP-5 and Axin by Wnt-3a suggests that the interaction between LRP-5 and Axin might be one of the first events in the Wnt canonical pathway.

In summary, on the basis of the observations made by us and others, we propose a model as depicted in Figure 7 describing the involvement of LRP proteins in the canonical Wnt signaling pathway. In this model, we suggest that LRP, when activated by Wnt proteins, recruits Axin to the membranes. The translocation of Axin to the membrane prevents it from participating in the degradation of β-catenin and plays an important role in the destabilization of Axin. LRP-6, a close homolog of LRP-5, may act in the same way as LRP-5, because a LRP-6 mutant that is equivalent to LRPC2 can also bind Axin and constitutively activates LEF-1 (Mao et al., unpublished data). Thus, binding to Axin and activation of the canonical Wnt signaling pathway may occur with other LRP-5 homologs including Arrow.

Experimental Procedures

Yeast Two-Hybrid Screening and Quantification Assay

The ProQuest two-hybrid system and the mouse fetal brain cDNA library were purchased from Life Technologies (MD). Two-hybrid screening and yeast two-hybrid quantification assays were carried out as suggested by the manufacturer. The bait for library screening

was the intracellular domain of LRP-5 (equivalent to LRPC3, Figure 2A).

Cell Culture, Transfection, and Luciferase Assay

COS-7 and NIH 3T3 cells were maintained and transfected as previously described (Li et al., 1999b). For luciferase assays, NIH 3T3 cells in 24-well plates were seeded at 5×10^4 cells/well and transfected with 0.5 μg DNA/well using Lipofectamine Plus (Life Technologies, MD), as suggested by the manufacturer. For immunoprecipitation, COS-7 cells in 6-well plates were seeded at 2×10^5 cells/well and transfected with 1 μg DNA/well. Transfection is usually stopped by switching to normal growth medium after 3 hr. Cell extracts were collected 24 hr later for luciferase assays, immunoprecipitation, and Western analysis. Luciferase assays were performed as previously described (Li et al., 1999b; Yuan et al., 1999). Luminescence intensity was normalized against fluorescence intensity of GFP.

Construction of Expression Plasmids and Mutagenesis

The wild-type and mutant forms of human LRP-5, mouse Wnt-1, Dvl1, and Axin were generated by PCR using the high fidelity thermostable DNA polymerase, *Pfu* (Stratagene, CA). Myc, HA, or Flag epitope tags were introduced to the C-termini of the full-length and mutant molecules. The expression of these molecules was driven by a CMV promoter (Li et al., 1999a; Li et al., 1999b; Yuan et al., 1999). All constructs were verified by DNA sequencing. The LEF-1 reporter gene constructs were kindly provided by Dr. Grosschedl (Hsu et al., 1998).

FRET Experiments

CYF and YFP were fused at the C-termini of Axin and LRP-5, respectively. 3T3 cells on coverslips in 35 mm dishes were cotransfected with 0.1 μg of Axin-CFP and GSK3β, 0.05 μg of LRP-5-YFP, and 1.75 μg of LacZ. The coverslip was mounted in a observation cuvette (Molecular Probes, OR) containing 200 μl culture medium. The control-conditioned medium (400 μl) was added after a short baseline was recorded. After the control experiment, medium (400 μl) were taken out from the cuvette, and a new recording followed with Wnt-3a-conditioned media (400 μl). Cells were observed using time-lapse fluorescence confocal videomicroscopy (Zeiss LSM510) at 5 s intervals with an excitation wavelength of 450 nm and emission wavelengths of 480 ± 20 nm and 530 ± 20 nm. Individual cells that express both CFP and YFP were traced. Data were processed with the manufacturer's software. The experiments were repeated five times.

Preparation of Plasma Membranes

The plasma membranes were prepared essentially as previously described (Fagotto et al., 1999). Cells in 10 cm dishes were washed once with ice-cold PBS and scraped into homogenization buffer containing 20 mM HEPES, (pH 7.4), 2 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl, 40 μM pyrophosphate, 25 mM NaF, 1 mM $NaVO_3$, 0.4 μM microcystin, and a cocktail of proteinase inhibitors. Cells were homogenized in a Dounce homogenizer, and cell debris and organelles were precipitated by low-speed centrifugation at 500 g. The supernatant was then subject to high-speed centrifugation at 100,000 g in a bench-top ultracentrifuge for 1 hr. The pellets were dissolved in SDS sample buffer and analyzed by Western blotting using anti-Axin (Willert et al., 1999) and anti-Gβ antibodies, respectively.

Acknowledgments

We thank R. Nusse for anti-Axin antibodies and control and Wnt-3a-expressing L1 cells, D. H. Kim, A. McMahon, R. Grosschedl, F. Costantini, and D. Sussman for plasmids and Zhong Li and other members of the labs for their help. This work is supported by grants to D.W. from the NIH (GM53162, GM54167, and CA85420) and American Cancer Society; to L. L. from NNSF of China (39990600 and 39928008), Chinese Academy of Sciences (KSCX2-2-06), and Shanghai Research Center for Life Sciences; and to D. K. from the NIH (HD27262). D. W. is an Established Investigator of American Heart Association.

Received December 5, 2000; revised January 23, 2001.

References

Barker, N., and Clevers, H. (2000). Catenins, Wnt signaling and cancer. Bioessays *22*, 961–965.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311–320.

Boutros, M., Mihaly, J., Bouwmeester, T., and Mlodzik, M. (2000). Signaling specificity by Frizzled receptors in Drosophila. Science *288*, 1825–1828.

Cook, D., Fry, M.J., Hughes, K., Woodgetts, J.R., and Dale, T. (1996). Wingless inactivates glycogen synthase kinase 3 via an intracellular signaling pathway which involves a protein kiasne C. EMBO J. *15*, 4526–4536.

Dale, T.C. (1998). Signal transduction by the Wnt family of ligands. Biochem. J. *329*, 209–223.

Fagotto, F., Jho, E.H., Zeng, L., Kurth, T., Joos, T., Kaufmann, C., and Costantini, F. (1999). Domains of Axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. J. Cell Biol. *145*, 741–756.

Farr, G.H., 3rd, Ferkey, D.M., Yost, C., Pierce, S.B., Weaver, C., and Kimelman, D. (2000). Interaction among GSK-3, GBP, axin, and APC in Xenopus axis specification. J. Cell Biol. *148*, 691–702.

Gumbiner, B.M. (1998). Propagation and localization of Wnt signaling. Curr. Opin. Gen. Devel. *8*, 430–435.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N., and Akiyama, T. (1999). Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin. Science *283*, 1739–1742.

Hey, P.J., Twells, R.C., Phillips, M.S., Yusuke, N., Brown, S.D., Kawaguchi, Y., Cox, R., Guochun, X., Dugan, V., Hammond, H., et al. (1998). Cloning of a novel member of the low-density lipoprotein receptor family. Gene *216*, 103–111.

Hsu, S.C., Galceran, J., and Grosschedl, R. (1998). Modulation of Transcriptional Regulation By Lef-1 in Response to Wnt-1 Signaling and Association With Beta-Catenin. Mol. Cell. Biol. *18*, 4807–4818.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with Gsk-3-beta and beta-catenin and promotes Gsk-3-beta-dependent phosphorylation of beta-catenin. EMBO J. *17*, 1371–1384.

Kikuchi, A. (1999). Roles of Axin in the Wnt signalling pathway. Cell. Sig. *11*, 777–788.

Li, L., Yuan, H., Weaver, C., Mao, J., Farr, G.H., III, Sussman, D.J., Jonkers, J., Kimelman, D., and Wu, D. (1999a). Axin and Frat-1 interact with Dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J. *18*, 4233–4240.

Li, L., Yuan, H., Xie, W., Mao, J., McMahon, E., Sussman, D., and Wu, D. (1999b). Dishevelled proteins lead to two different signaling pathways; regulation of the JNK and b-catenin pathways. J. Biol. Chem. *274*, 129–134.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. Nature *388*, 882–887.

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.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science *287*, 1606–1609.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C. (2000). An LDL receptor-related protein mediates Wnt singaling in mice. Nature *407*, 535–538.

Rulifson, E.J., Wu, C.H., and Nusse, R. (2000). Pathway specificity

by the bifunctional receptor frizzled is determined by affinity for wingless. Mol. Cell. *6*, 117–126.

Salic, A., Lee, E., Mayer, L., and Kirschner, M.W. (2000). Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. Mol. Cell. *5*, 523–532.

Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M., and Takada, S. (1998). Cytoskeletal reorganization by soluble Wnt-3a protein signalling. Genes Cells *3*, 659–670.

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.

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.

Wong, H.C., Mao, J., Nguyen, J.T., Srinivas, S., Zhang, W., Liu, B., Li, L., Wu, D.Q., and Zheng, J. (2000). Structural basis of the recognition of the dishevelled DEP domain in the Wnt signaling pathway. Nat. Struct. Biol., in press.

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 3b regulates its stability. J. Biol. Chem. *274*, 10681–10684.

Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J., and Nusse, R. (1995). The dishevelled protein is modified by wingless signaling in Drosophila. Genes Dev. *9*, 1087–1097.

Yuan, H., Mao, J., Li, L., and Wu, D. (1999). Regulation of GSK and LEF-1 by Wnt, Frat, and Akt; suppression of GSK kinase activity is not sufficient for LEF-1 activation. J. Biol. Chem. *274*, 30419–30423.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L.R., Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell *90*, 181–192.