LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin

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Wnt/β-catenin signaling controls various cell fates in metazoan development and is misregulated in several cancers and developmental disorders. Binding of a Wnt ligand to its transmembrane coreceptors inhibits phosphorylation and degradation of the transcriptional coactivator β-catenin, which then translocates to the nucleus to regulate target gene expression. To understand how Wnt signaling prevents β-catenin degradation, we focused on the Wnt coreceptor low-density lipoprotein receptor-related protein 6 (LRP6), which is required for signal transduction and is sufficient to activate Wnt signaling when overexpressed. LRP6 has been proposed to stabilize β-catenin by stimulating degradation of Axin, a scaffold protein required for β-catenin degradation. In certain systems, however, Wnt-mediated Axin turnover is not detected until after β-catenin has been stabilized. Thus, LRP6 may also signal through a mechanism distinct from Axin degradation. To establish a biochemically tractable system to test this hypothesis, we expressed and purified the LRP6 intracellular domain from bacteria and show that it promotes β-catenin stabilization and Axin degradation in *Xenopus* egg extract. Using an Axin mutant that does not degrade in response to LRP6, we demonstrate that LRP6 can stabilize β-catenin in the absence of Axin turnover. Through experiments in egg extract and reconstitution with purified proteins, we identify a mechanism whereby LRP6 stabilizes β-catenin independently of Axin degradation by directly inhibiting GSK3's phosphorylation of β-catenin.

Axin | GSK3 | LRP6 | Wnt

The best-characterized form of Wnt signaling is the Wnt/β-catenin, or canonical Wnt, pathway (1). During Wnt/β-catenin signaling, a Wnt ligand binds transmembrane coreceptors Frizzled (Fz) and low-density lipoprotein receptor-related proteins 5 or 6 (LRP5/6) and initiates a process that leads to stabilization and nuclear translocation of β-catenin. In the nucleus, β-catenin binds transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family and activates a Wnt/β-catenin transcriptional program.

Although the mechanism by which a Wnt ligand mediates β-catenin stabilization is poorly understood, regulation of β-catenin levels in the absence of Wnt signaling has been well characterized. In the absence of a Wnt ligand, β-catenin is marked for degradation through its interaction with a destruction complex consisting of two scaffold proteins, Axin and adenomatous polyposis coli protein (APC), and two kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α) (1). CK1α phosphorylation of β-catenin primes it for subsequent phosphorylation by GSK3, which targets β-catenin for ubiquitin-mediated proteolysis (1). It is hypothesized that Wnt signal transduction stabilizes β-catenin by inhibiting destruction complex formation or activity.

The Wnt coreceptor LRP5/6 is required for Wnt/β-catenin signaling (2–4). Although LRP6 is more potent than LRP5 in certain assays, experiments have not revealed qualitative differences in their mechanisms of action (5). Wnt signaling through LRP5/6 has been proposed to inhibit destruction complex formation by promoting degradation of the destruction complex scaffold Axin. LRP5 overexpression was initially shown to promote Axin degradation in cultured mammalian cells (6). Genetic studies in *Drosophila* indicate that activation of the Wnt pathway by Arrow, the LRP5/6 ortholog, decreases steady-state Axin levels (7). Wnt signaling through LRP6 also promotes degradation of endogenous Axin in *Xenopus* oocytes and embryos (8). Because the concentration of Axin is significantly lower than that of other destruction complex components, reduction of Axin levels represents a potentially robust mechanism for β-catenin stabilization (9). As a result, LRP5/6-mediated Axin degradation has been proposed to be a critical event in transduction of a Wnt signal (10).

Although there is strong evidence that signaling by LRP5/6 reduces Axin levels, Wnt-mediated stabilization of β-catenin in cultured mammalian cells occurs ≈2 h before substantial changes in Axin levels are detected (11–13). These data suggest that Axin degradation may not be required for initial signal transmission; alternatively, turnover of a small, localized pool of Axin may be necessary for signaling but may be undetected in these experiments. Such a mechanism has been described for β-catenin: the vast majority of β-catenin is associated with cadherins at cellular membranes, and only the small, cytoplasmic pool of β-catenin protein is stabilized in response to Wnt signaling (14, 15). Here, we address whether LRP6 can stabilize β-catenin independently of Axin degradation. We reconstituted LRP6 signaling in biochemically tractable *Xenopus* egg extract, which has been used to accurately reconstitute cytoplasmic aspects of Wnt signal transduction (16–18). We find that LRP6 can promote β-catenin stabilization in the absence of Axin degradation by directly inhibiting GSK3's phosphorylation of β-catenin.

Results and Discussion

Recombinant LRP6 Intracellular Domain Protein Activates Wnt/β-Catenin Signaling in *Xenopus* Embryos. LRP5/6 is a single-span transmembrane Wnt coreceptor. Expression of the LRP5/6 intracellular domain in cultured mammalian cells accurately recapitulates LRP5/6 signal transduction, promoting β-catenin stabilization and regulating Wnt/β-catenin target gene expression (5, 19, 20). To obtain soluble LRP6 for analysis in biochemically tractable *Xenopus* egg extract, we bacterially expressed and purified recombinant polypeptide encoding the LRP6 intracel-

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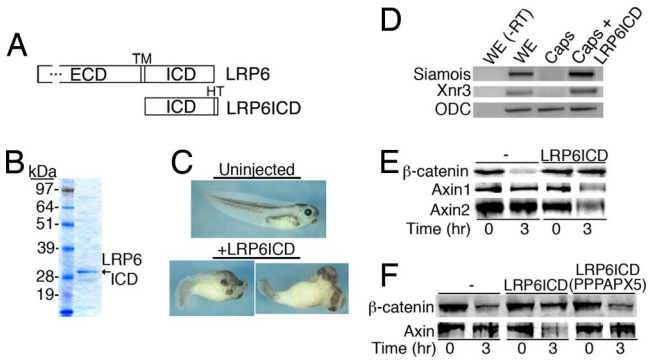

Fig. 1. Recombinant LRP6ICD activates Wnt signaling *in vivo* and in *Xenopus* egg extract. (*A*) LRP6ICD spans the intracellular domain of mouse LRP6 (amino acids 1397–1614) and does not include its transmembrane domain. ECD, extracellular domain; ICD, intracellular domain; TM, transmembrane domain; HT, 6Xhistidine tag. (*B*) Coomassie-stained gel of recombinant LRP6ICD (1 μg) purified from bacteria. (*C*) Injection of LRP6ICD protein (33 nM) into each ventral blastomere of 4-cell *Xenopus* embryos promotes development of a complete ectopic axis (*Bottom Left*, embryo side view; *Bottom Right*, embryo ventral view) in 73% of embryos ($n = 15$). A lower dose of LRP6ICD protein (20 nM) promotes axis duplication in 46% of embryos ($n = 15$). (*D*) Injection of LRP6ICD (33 nM) at the four-cell stage promotes ectopic transcription of Wnt/β-catenin targets *Xnr3* and *siamois* in animal caps as assayed by RT-PCR. WE, whole embryos; Caps, animal caps; WE-RT, no reverse transcriptase added; ODC, ornithine decarboxylase (loading control). (*E*) Addition of LRP6ICD (1.6 μM) to *Xenopus* egg extract prevents degradation of radiolabeled, IVT β-catenin and promotes degradation of radiolabeled, IVT Axin and Axin2. (*F*) Unlike LRP6ICD, LRP6ICD(PPPAPX5) (1.6 μM) does not inhibit β-catenin degradation or promote Axin degradation.

lular domain without its transmembrane domain (LRP6ICD; Fig. 1 *A* and *B*).

We first tested whether LRP6ICD activates Wnt/β-catenin signaling *in vivo*. Ventral injection of LRP6ICD protein into *Xenopus* embryos at a concentration similar to that of other pathway components (9) induces complete axis duplication and promotes transcription of Wnt/β-catenin targets, *siamois* and *Xnr3*, in ectodermal explants (Fig. 1 *C* and *D*). Our results provide phenotypic and transcriptional evidence that recombinant LRP6ICD protein purified from bacteria promotes Wnt/β-catenin signaling *in vivo*.

LRP6ICD Promotes β-Catenin Stabilization and Axin Degradation in *Xenopus* Egg Extract. To establish a cell-free system that would facilitate biochemical analysis of LRP6 signaling, we tested whether recombinant LRP6ICD, which activates Wnt signaling *in vivo*, prevents degradation of β-catenin in *Xenopus* egg extract. We find that LRP6ICD protein prevents degradation of radiolabeled, *in vitro*-translated (IVT) β-catenin in *Xenopus* egg extract (Fig. 1*E*). Consistent with a proposed mechanism for LRP6 signaling, we demonstrate that LRP6ICD also stimulates degradation of IVT Axin and Axin2 (Fig. 1*E*). We also tested whether LRP6ICD induces phosphorylation of Axin. We find that λ phosphatase reverses the LRP6ICD-mediated upward mobility shift of the Axin protein detected by SDS/PAGE, suggesting that LRP6ICD promotes Axin phosphorylation [supporting information (SI) Fig. S1]. However, in the presence of LRP6ICD, the total Axin signal is decreased even after λ phosphatase treatment, consistent with LRP6ICD mediating Axin degradation.

The ability of LRP6 to stabilize β-catenin depends on GSK3's phosphorylation of the serine residue on at least one of five Pro-Pro-Pro-Ser-Pro (PPPSP) motifs on LRP6 (21, 22). If LRP6ICD accurately reconstitutes endogenous LRP6 signaling in extract, LRP6ICD's activity should depend on intact PPPSP motifs. An LRP6 construct in which all five PPPSP motifs have been mutated to PPPAP (PPPAPX5) does not bind Axin or stabilize β-catenin in cultured cells (21). This construct also fails to activate Wnt target genes in *Xenopus* ectodermal explants (21). To test whether LRP6ICD signaling in egg extract requires intact PPPSP motifs, we expressed and purified LRP6ICD (PPPAPX5) protein from bacteria. In contrast to LRP6ICD, LRP6ICD(PPPAPX5) does not inhibit β-catenin degradation or stimulate Axin degradation in egg extract (Fig. 1*F*), even when added at a concentration 2-fold higher than that used for LRP6ICD (data not shown). We also find that LRP6ICD, but not LRP6ICD(PPPAPX5), is phosphorylated at PPPSP Ser-1490 in egg extract (21, 22) (Fig. 3*C*). Requirement of these PPPSP motifs suggests LRP6ICD in extract functions in a manner that is similar to that of LRP6 in cultured cells and *Xenopus* embryos.

LRP6ICD Signals Independently of Disheveled in *Xenopus* Egg Extract and Embryos. Disheveled (Dsh) is a cytoplasmic protein required for signaling downstream of Fz and upstream of the β-catenin destruction complex (1). In cultured mammalian cells, overexpression of LRP6 that lacks its extracellular domain promotes Wnt signaling despite down-regulation of Dsh by RNAi or overexpression of a dominant-negative form of Dsh (23), suggesting that the intracellular domain of LRP6 can signal independently of Dsh. More recently, it was shown that Dsh is required for LRP6 oligomerization and phosphorylation (24), which are necessary for LRP6-mediated activation of Wnt/β-catenin signaling. Interestingly, LRP6 expressed without its extracellular domain bypasses this requirement for Dsh and is constitutively oligomerized and phosphorylated (24). These data suggest that LRP6ICD may mimic Dsh-activated LRP6 and circumvent the requirement for Dsh in Wnt/β-catenin signaling.

To test whether LRP6ICD signaling in *Xenopus* egg extract bypasses its requirement for Dsh, we immunodepleted endogenous Dsh from egg extract (16). Depletion of Dsh (Fig. S2*A*) did not affect the ability of LRP6ICD to stabilize β-catenin or promote Axin degradation (Fig. S2*B*). To determine whether Dsh is required for LRP6ICD signaling *in vivo*, we tested whether Xdd1 (a dominant negative form of Dsh) (25) prevents LRP6's activation of the Wnt/β-catenin pathway in *Xenopus* embryos. In mRNA coinjection experiments, Xdd1 inhibits Wnt8-induced secondary axis formation but has no effect on the ability of LRP6ICD to induce secondary axes (Fig. S2*C*). Thus, our data in *Xenopus* egg extract and embryos demonstrate that LRP6ICD signals independently of Dsh and are consistent with a model in which LRP6ICD mimics Dsh-activated LRP6 in Wnt/β-catenin signaling (24).

Axin-bound GSK3 has been suggested to play a role in phosphorylation and activation of LRP6 (26). Because phosphorylation of LRP6 is a prerequisite for its binding to Axin (21), however, the initial phosphorylation of LRP6 may occur by a pool of GSK3 that is not bound to Axin. In egg extract where Axin has been immunodepleted, we find that LRP6ICD still becomes phosphorylated at PPPSP Ser 1490 as assayed by immunoblot (data not shown), suggesting that initial LRP6 phosphorylation may occur independently of Axin.

LRP6ICD-Mediated Axin Degradation Occurs via the Ubiquitin/Proteasome Pathway and Is Distinct from GSK3-Regulated Axin Degradation. To identify the mechanism by which LRP6 promotes Axin degradation, we tested whether LRP6ICD induces Axin degradation via a ubiquitin-mediated, proteasome-dependent process. We find that LRP6ICD promotes Axin ubiquitination in *Xenopus* egg extract (Fig. 2*A*). Furthermore, we show that inhibition of the proteasome with MG132 prevents Axin degradation, leading to accumulation of a more slowly migrating form of Axin (Fig. 2*B*). Thus, our data indicate that, consistent with results

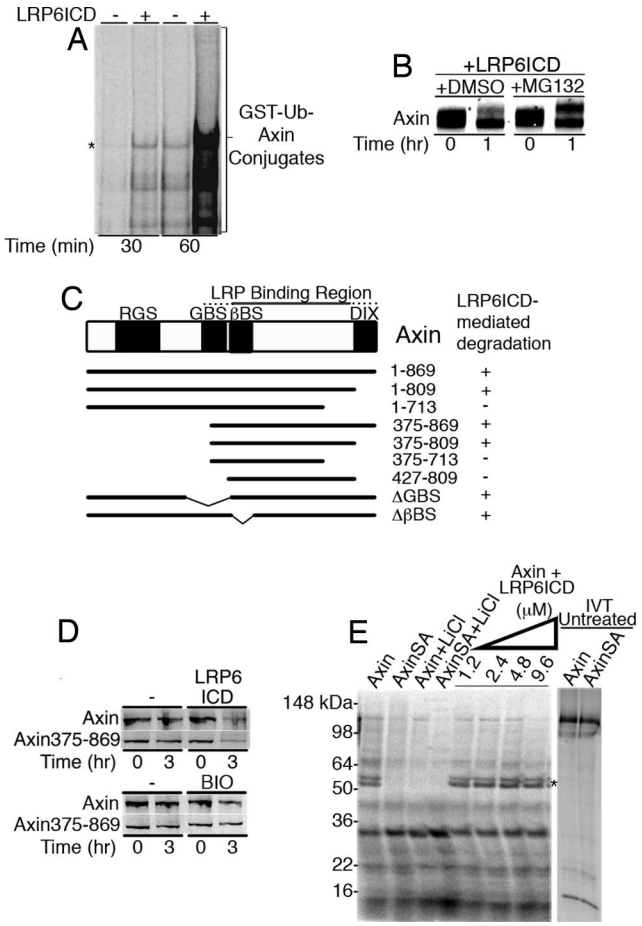

Fig. 2. LRP6ICD mediates Axin degradation independently of GSK3 inhibition. (*A*) LRP6ICD stimulates addition of GST-ubiquitin to radiolabeled, IVT Axin in egg extract. GST-ubiquitin conjugates were pulled down with glutathione beads at indicated times and analyzed by using SDS/PAGE and autoradiography. Asterisk indicates full-length Axin. (*B*) Addition of the proteasome inhibitor MG132 (1 mM) to egg extract inhibits LRP6ICD-mediated Axin degradation. (*C*) Degradation of Axin mutants in egg extract in the presence of LRP6ICD. The indicated LRP5/6-binding region on Axin is based on previous Axin-LRP5 and Axin-Arr yeast two-hybrid studies (6, 7); dotted lines represent large deletions of Axin that were not further mapped, and the borders of Axin-LRP5/6 interaction likely reside within the dotted lines. RGS, RGS domain; GBS, GSK3 binding site; βBS, β-catenin binding site; DIX, DIX domain. (*D*) LRP6ICD promotes degradation of Axin and Axin375–869, whereas the GSK3 inhibitor BIO (50 μg/ml, Calbiochem) promotes degradation of Axin but not Axin375–869. (*E*) Inhibition of GSK3-mediated Axin phosphorylation [by LiCl (50 mM) or mutagenesis (AxinSA)], but not incubation with LRP6ICD, alters the trypsin proteolysis pattern of IVT Axin after incubation in egg extract for 30 min (note bands at level of asterisk). All experiments used equal concentrations of IVT Axin. An SDS/PAGE autoradiograph of IVT Axin and AxinSA before trypsin treatment is shown at right.

from intact *Xenopus* oocytes (8), Axin degradation is proteasome dependent in egg extract.

To uncover structural elements of Axin required for its LRP6-mediated degradation, we analyzed a panel of truncated Axin polypeptides. We identified a minimal Axin fragment (Axin375–809) that degrades in response to LRP6ICD (Fig. 2*C* and Fig. S3). This minimal fragment includes the GSK3, β-catenin, and PP2A binding sites on Axin (27). However, deletion of the GSK3 or β-catenin binding domain from full-length Axin does not prevent its LRP6ICD-mediated turnover (Fig. 2*C*); thus, binding of Axin to GSK3 or β-catenin may not be required for LRP6-mediated degradation of Axin. Interestingly, amino acids 375–427 appear to be required in the large N-terminal truncation mutants (compare Axin375–809 and Axin427–809) but not in the internally truncated AxinΔGBS; we believe this may result from abnormal folding of certain truncation mutants, redundancy within Axin regarding sequences required for LRP6ICD-mediated Axin degradation, and/or dimerization of certain Axin mutants with endogenous Axin (28). Notably, we find that the region of Axin identified as binding LRP5/6 by yeast two-hybrid assays (6, 7) also appears to be required for its LRP6-mediated degradation (Fig. 2*C*). These data are consistent with a model in which LRP6/Axin binding is required for LRP6-mediated Axin degradation.

Several models for Wnt pathway activation involve inhibition of GSK3, positing global inhibition of GSK3 within the cell or specific inhibition of GSK3 within the β-catenin destruction complex. Either mechanism would allow β-catenin levels to rise because its phosphorylation, which is necessary for its degradation, is blocked. Experiments suggest an inherent feed-forward mechanism whereby GSK3 inhibition also stimulates Axin degradation by preventing phosphorylation of Axin, which is normally required for its stability (29). Thus, we tested whether LRP6ICD promotes Axin degradation by inhibiting GSK3-mediated phosphorylation of Axin.

If LRP6ICD promotes turnover of Axin by inhibiting its GSK3-mediated phosphorylation, Axin mutants that degrade in response to LRP6ICD should also be able to degrade in response to GSK3 inhibition. Alternatively, if LRP6ICD-mediated Axin turnover does not occur via GSK3 inhibition, certain Axin mutants may degrade in response to LRP6ICD but not in response to GSK3 inhibition. We find evidence in support of the latter model. Both LRP6ICD and the GSK3 inhibitor BIO (Fig. 2*D*) promote turnover of full-length Axin; in contrast, Axin375–869 degrades in response to LRP6ICD but not the GSK3 inhibitor BIO (Fig. 2*D*). Another GSK3 inhibitor, lithium (50 mM), also promotes turnover of full-length Axin but not Axin375–869 (data not shown). Furthermore, Axin mutants lacking previously identified GSK3 phosphorylation and binding sites as well as an Axin mutant (AxinSA) in which predicted GSK3 phosphorylated serines are mutated to alanines (29) degrade in response to LRP6ICD (Fig. 2*C* and Fig. S3). Together, these data indicate that LRP6 is unlikely to promote Axin degradation via a mechanism that inhibits GSK3-mediated stabilization of Axin.

Distinct mechanisms underlying LRP6-mediated and GSK3 inhibition-mediated Axin degradation may induce different Axin conformations. Because changes in a protein's conformation may expose or conceal certain tryptic cleavage sites, a protein's tryptic proteolysis pattern is traditionally used to detect conformational changes (12, 30, 31). Incubation of radiolabeled, IVT Axin in egg extract followed by partial trypsin proteolysis results in a characteristic Axin digestion pattern upon analysis by SDS/PAGE and autoradiography (Fig. 2*E*). Trypsin digestion of Axin lacking GSK3 phosphorylation [either via mutation (AxinSA) or incubation with a GSK3 inhibitor (LiCl)] results in a proteolysis pattern distinct from that of wild-type Axin. Incubation of Axin with LRP6ICD, however, yields a digestion pattern that is indistinguishable from that of Axin alone. Because addition of LRP6ICD and inhibition of GSK3 phosphorylation have distinct effects on Axin conformation as assayed by trypsin digest, we propose that LRP6 signaling and GSK3 inhibition affect Axin through different mechanisms. These data are consistent with evidence in Drosophila embryos that Arrow, the *Drosophila* LRP5/6 ortholog, can promote Axin degradation in the absence of GSK3 activity (7).

LRP6ICD-Mediated β-Catenin Stabilization Does Not Require Axin Degradation. Although we hypothesize that LRP6-mediated degradation of Axin, a required component of the β-catenin destruction complex, leads to β-catenin stabilization, we wanted to

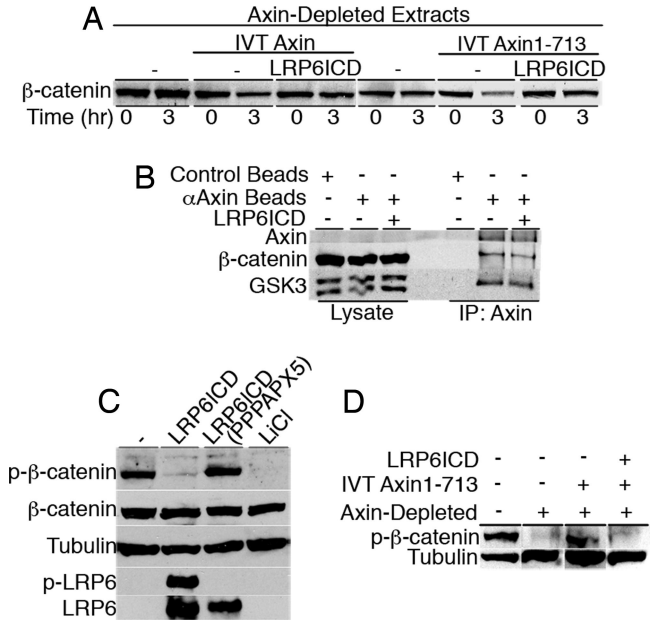

Fig. 3. LRP6ICD's inhibition of GSK3-mediated β-catenin phosphorylation is sufficient to promote β-catenin stabilization in the absence of Axin degradation. (*A*) LRP6ICD inhibits β-catenin degradation in extract where endogenous Axin is replaced by nondegradable Axin1-713. Addition of IVT Axin or Axin1-713 restores the ability of Axin-depleted extract to degrade radiolabeled β-catenin. LRP6ICD inhibits both IVT Axin and Axin1-713-induced β-catenin-degradation. (*B*) LRP6ICD does not affect the ability of Axin to bind GSK3 or β-catenin in egg extract. Endogenous Axin was immunoprecipitated from extract and immunoblotted for GSK3, β-catenin, and Axin. (*C*) Incubation of LiCl (50 mM) or LRP6ICD [but not LRP6ICD(PPPAPX5)] in egg extract (30 min) inhibits phosphorylation of endogenous β-catenin at GSK3 target sites P33/37/41. Immunoblot of LRP6ICD from the same gel reveals that LRP6ICD, but not LRP6ICD(PPPAPX5), is phosphorylated at the PPPSP Ser-1490. All samples were blotted from a single gel. (*D*) LRP6ICD inhibits GSK3-mediated β-catenin phosphorylation in extract in which endogenous Axin is replaced by nondegradable Axin1-713. Axin depletion did not affect total β-catenin levels as assayed by immunoblot (Fig. S7). Depletion of endogenous Axin prevents β-catenin P33/37/41 phosphorylation. Addition of IVT Axin1-713 restores β-catenin phosphorylation in Axin-depleted extract. LRP6ICD inhibits IVT Axin1-713-induced β-catenin phosphorylation. Extracts were analyzed after 2-h incubation. All samples were blotted from a single gel; intervening lanes were removed for clarity.

determine whether this is the only mechanism by which LRP6ICD stabilizes β-catenin. To test this model, we assessed whether LRP6ICD can stabilize β-catenin in egg extract in which endogenous Axin is replaced by a nondegradable Axin mutant, Axin1-713 (Fig. 3*A*). Axin1-713, like full-length Axin, ventralizes *Xenopus* embryos (indicative of inhibition of Wnt/β-catenin signaling) (data not shown), stimulates β-catenin degradation in egg extract (Fig. 3*A*), and binds LRP6ICD in egg extract (Fig. S4). Thus, Axin1-713 retains all measurable activities of full-length Axin except that it is not degraded in response to LRP6ICD (Fig. 2*C*). Consistent with the requirement of Axin for destruction complex formation, immunodepletion of endogenous Axin from extract (Fig. S5*A*) prevented β-catenin degradation (16) (Fig. 3*A*). Addition of IVT Axin1-713 to Axin-depleted extract restored β-catenin degradation to an extent similar to that of addition of full-length Axin. We then tested whether LRP6ICD inhibits β-catenin degradation in Axin1-713-rescued extract. As shown in Fig. 3*A*, LRP6ICD inhibits β-catenin degradation in extract where endogenous Axin is replaced by either full-length Axin or non-degradable Axin1-713. Thus, LRP6ICD can inhibit β-catenin degradation independently of Axin degradation in *Xenopus* egg extract.

LRP6ICD Prevents GSK3-Mediated Phosphorylation of β-Catenin. We next sought to identify the mechanism by which LRP6 stabilizes β-catenin independently of Axin degradation. It has been proposed that LRP6 might inhibit β-catenin degradation by promoting dissociation of the β-catenin destruction complex (32). To test this model, we immunoprecipitated Axin from egg extract incubated in the presence or absence of LRP6ICD and immunoblotted for GSK3 or β-catenin. As shown in Fig. 3*B* and Fig. S6, LRP6ICD (at a concentration that inhibits β-catenin degradation in *Xenopus* egg extract) does not affect Axin's ability to bind GSK3 or β-catenin. Thus, our data suggest that LRP6 does not sequester Axin from GSK3 or β-catenin.

Alternatively, LRP6 could stabilize β-catenin by directly preventing its phosphorylation within the destruction complex. CK1α phosphorylates β-catenin at Ser-45 (P45) to prime it for GSK3's phosphorylation at Ser-33/Ser-37/Thr-41 (P33/37/41), which is required for β-catenin polyubiquitination and degradation (33). Previous studies showed that Wnt signaling inhibits GSK3-mediated β-catenin phosphorylation but does not inhibit CK1α-mediated β-catenin phosphorylation (33). We therefore tested whether LRP6ICD inhibits the appearance of GSK3-phosphorylated β-catenin in egg extract. Significantly, LRP6ICD, like the GSK3 inhibitor lithium, inhibits GSK3-mediated phosphorylation of β-catenin (Fig. 3*C*). If LRP6 stabilizes β-catenin through inhibition of β-catenin phosphorylation, LRP6ICD's requirement for intact PPPSP motifs to stabilize β-catenin should extend to LRP6ICD's inhibition of β-catenin phosphorylation. Indeed, LRP6ICD(PPPAPX5), which does not inhibit degradation of β-catenin (Fig. 1*F*), does not inhibit GSK3's phosphorylation of β-catenin (Fig. 3*C*). Notably, we find that LRP6's PPPSP serine Ser-1490 is phosphorylated in extracts (Fig. 3*C*). Thus, LRP6ICD inhibits phosphorylation of β-catenin, likely through a mechanism that requires serine phosphorylated PPPSP motifs.

Phosphorylation of β-catenin by GSK3 requires its recruitment into the β-catenin destruction complex, which is mediated in part by Axin. Thus, it was possible that LRP6ICD-induced inhibition of GSK3's phosphorylation of β-catenin was a direct consequence of LRP6-mediated Axin degradation. To address this possibility, we tested whether LRP6ICD inhibits β-catenin P33/37/41 phosphorylation in egg extract in which Axin is replaced by non-degradable Axin1-713 (Fig. 3*D* and Fig. S7). Axin depletion (Fig. S5*B*) from extract inhibited GSK3's phosphorylation of β-catenin, consistent with Axin's role as a required scaffold for this phosphorylation event. Addition of non-degradable IVT Axin1-713 to Axin-depleted extract restored β-catenin P33/37/41 phosphorylation. LRP6ICD blocked this Axin1-713-induced β-catenin phosphorylation (Fig. 3*D*), demonstrating that LRP6ICD can inhibit phosphorylation of β-catenin by GSK3 independently of Axin degradation.

LRP6ICD in egg extract could specifically prevent β-catenin phosphorylation or act as a general GSK3 inhibitor (possibly by GSK3 sequestration) (19). If the former is correct, LRP6 should inhibit β-catenin phosphorylation without affecting phosphorylation of another GSK3 substrate (e.g., Tau) (Fig. 4*A*). In egg extract supplemented with exogenous GSK3, recombinant Tau is phosphorylated at its well characterized GSK3 target site Ser-396 (P396) (34). In contrast to lithium, which robustly inhibits GSK3's phosphorylation of both β-catenin and Tau, LRP6ICD inhibits phosphorylation of β-catenin but not of Tau. Thus, our data indicate that levels of LRP6ICD that stabilize β-catenin in egg extract inhibit GSK3-mediated β-catenin phosphorylation without affecting global GSK3 activity. Our finding that LRP6ICD does not act by inhibiting total GSK3 activity is also supported by our data demonstrating that LRP6ICD and lithium have distinct effects on Axin's trypsin proteolysis pattern and that LRP6 promotes Axin degradation independently of GSK3 inhibition (Figs. 2*C*, 2*D*, and 2*E*). Although previous

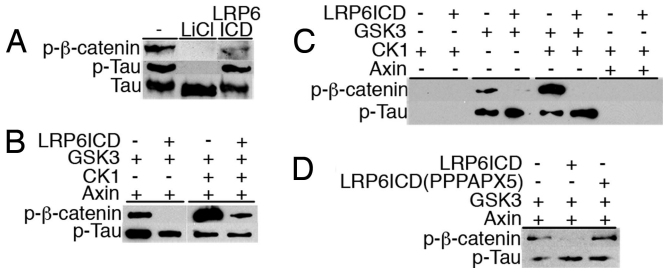

Fig. 4. LRP6ICD directly and specifically inhibits GSK3's phosphorylation of β-catenin. (*A*) LiCl (50 mM) inhibits phosphorylation of β-catenin P33/37/41 and exogenous Tau P396 by GSK3, whereas LRP6ICD inhibits phosphorylation of β-catenin, but not Tau, by GSK3. Extract was supplemented with GSK3 to enhance detection of phosphorylated Tau as previously described (32). β-catenin and Tau from the same reaction sample were immunoblotted from a single gel; intervening lanes were removed for clarity. (*B*) In an *in vitro* kinase assay containing purified, recombinant Axin (0.1 μM), GSK3 (0.79 μM), CK1 (1.37 μM), Tau (0.34 μM), and β-catenin (0.22 μM), LRP6ICD (4.1 μM) inhibits phosphorylation of β-catenin P33/37/41 by GSK3 without inhibiting the phosphorylation of Tau P396 by GSK3. (*C*) In a kinase reaction in which recombinant Axin is absent, phosphorylation of β-catenin by GSK3 is inhibited by LRP6ICD but not by LRP6ICD(PPPAPX5) (*D*). For (*B*), (*C*), and (*D*), β-catenin and Tau were incubated in the same reaction and immunoblotted from a single gel.

experiments suggested that LRP6 inhibits global GSK3 activity, the concentration of LRP6 intracellular domain in those experiments was not reported and may have been significantly greater than the concentration of LRP6ICD used in our experiments (19). Indeed, we detected inhibition of GSK3's phosphorylation of both β-catenin and Tau at higher concentrations of LRP6ICD than those required to inhibit β-catenin phosphorylation in our assays (data not shown).

LRP6ICD Directly Inhibits GSK3-Mediated β-Catenin Phosphorylation. The simplest model for LRP6 signaling is that it directly inhibits β-catenin phosphorylation by GSK3. Alternatively, LRP6-mediated inhibition of β-catenin phosphorylation may require additional components. To determine whether LRP6ICD is sufficient to inhibit GSK3-mediated β-catenin phosphorylation, we tested whether we could reconstitute LRP6 signaling with purified components.

In a kinase assay with purified, recombinant proteins, LRP6ICD inhibits GSK3-mediated phosphorylation of β-catenin at P33/37/41 without inhibiting CK1's phosphorylation of β-catenin at P45 (Figs. 4*B*, 4*C*, and Fig. S8). Importantly, the concentration of LRP6ICD tested does not inhibit GSK3's phosphorylation of Tau in the same reaction, demonstrating that inhibition of β-catenin phosphorylation by LRP6ICD is not a result of general inhibition of GSK3 activity (Figs. 4*B* and 4*C*). Thus, LRP6ICD preferentially inhibits GSK3's phosphorylation of β-catenin in a kinase assay with purified components.

The ability of GSK3 to phosphorylate β-catenin independently of Axin (albeit inefficiently) in our purified system allowed us to test whether LRP6ICD inhibits GSK3's phosphorylation of β-catenin directly or indirectly (via a conformational change of Axin upon its binding to LRP6ICD). Significantly, we find that Axin is not required for LRP6ICD's inhibition of β-catenin P33/37/41 phosphorylation (Fig. 4*C*). In addition, CK1γ's phosphorylation plays a role in LRP6 signaling *in vivo* (35), but CK1 is not required for LRP6ICD activity in our kinase assay (Fig. 4 *B* and *C*). These results demonstrate that LRP6ICD can directly inhibit GSK3-mediated phosphorylation of β-catenin and that this inhibition does not require other components.

Next, we tested whether LRP6 requires intact PPPSP motifs to inhibit GSK3's phosphorylation of β-catenin in our purified system. Unlike LRP6ICD, LRP6ICD(PPPAPX5) does not inhibit GSK3's phosphorylation of β-catenin (Fig. 4*D*), demonstrating that LRP6's PPPSP motifs are required for LRP6 to inhibit β-catenin phosphorylation *in vitro*. In a kinase assay with recombinant proteins, GSK3 phosphorylates LRP6 in a manner that requires intact PPPSP motifs (data not shown) (19). Thus, we infer that phosphorylation of PPPSP serines by GSK3 is required for LRP6's ability to inhibit β-catenin phosphorylation in our purified, reconstituted system. Our purified system exhibits specific properties that are consistent with *in vivo* and egg extract data: (*i*) requirement for PPPSP serines (21, 22), (*ii*) specificity for inhibition of β-catenin and not Tau phosphorylation (Fig. 4*A*), and (*iii*) inhibition of β-catenin phosphorylation by GSK3 but not CK1 (33). Thus, we believe these studies recapitulate distinct properties of LRP6 signaling *in vivo*.

LRP6ICD Associates with β-Catenin *in Vivo*. Given that LRP6ICD is sufficient to inhibit GSK3-mediated β-catenin phosphorylation in a kinase assay with purified proteins, we hypothesized that LRP6ICD may directly interact with β-catenin to prevent its GSK3-mediated phosphorylation. To determine whether β-catenin and LRP6ICD can interact in cultured mammalian cells, we performed bimolecular fluorescence complementation (BiFC) between β-catenin and LRP6ICD (36). In this assay, interacting proteins that are fused to N- and C-terminal halves, respectively, of YFP bring the two halves of YFP in close enough association to produce a functional, fluorescent YFP molecule. BiFC-mediated fluorescence requires a relatively stable protein–protein interaction in the range of several seconds and detects direct or very close interactions within protein complexes (36). Similar to fluorescence energy transfer (FRET), BiFC indicates a potential for physical interaction in a cell. As a positive control, cells transfected with N- and C-terminal halves of YFP fused to separate glutathione *S*-transferase (GST) proteins (which have been shown to oligomerize) produce cytoplasmic YFP fluorescence in ≈50% of cells (Fig. S9). In contrast, none of the cells transfected with N-and C-terminal halves of YFP fused to LRP6ICD and GST, respectively, or fused to GST and β-catenin, respectively, produce any detectable fluorescent signal (Fig. S9). In addition, the individual fusion proteins, when expressed in cells alone, do not produce fluorescence (data not shown). Importantly, cells transfected with N- and C-terminal halves of YFP fused to LRP6ICD and β-catenin, respectively, produce functional, fluorescent YFP in ≈15% of cells (Fig. S9). These results indicate that LRP6ICD and β-catenin form a stable interaction *in vivo* (likely within the Axin complex).

In conclusion, we provide evidence that LRP6 can promote β-catenin stabilization independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin. This mechanism is consistent with cultured cell experiments demonstrating Wnt-mediated stabilization of β-catenin in the absence of Axin degradation (12). Intriguingly, we find that LRP6 directly and specifically inhibits GSK3's phosphorylation of β-catenin *in vitro*, independently of Axin. It has been previously shown that addition of Wnt ligand to cultured mammalian cells rapidly induces recruitment of Axin to LRP5/6 (6). We propose that this interaction between LRP5/6 and Axin serves to bring LRP5/6 in close proximity to β-catenin and GSK3, allowing for inhibition of β-catenin phosphorylation. Consistent with this hypothesis, we find that LRP6ICD and β-catenin can interact in cultured cells. We also find that LRP6 requires intact PPPSP motifs to directly inhibit GSK3's phosphorylation of β-catenin. Thus, we propose the following working model. A Wnt signal induces GSK3's and CK1γ's phosphorylation of LRP5/6, which promotes the binding of Axin to LRP5/6 (22, 35). Axin thereby brings β-catenin and GSK3 in close proximity to LRP5/6 where its phosphorylated PPPSP motifs are involved in mediating inhibition of GSK3's phosphorylation of β-catenin. Analysis of the

molecular details of this interaction may help elucidate the mechanism by which LRP6 prevents β-catenin phosphorylation.

Because Axin is the limiting factor in β-catenin destruction complex formation, we predict that Axin degradation (although not required for all aspects of β-catenin stabilization) plays an important role in LRP5/6-mediated Wnt signal transduction (9). Thus, we suggest that both LRP5/6-mediated inhibition of β-catenin phosphorylation and stimulation of Axin degradation contribute significantly to Wnt/β-catenin signaling. The existence of two mechanisms by which LRP5/6 mediates β-catenin stabilization may allow for more robust transduction of a Wnt signal. Furthermore, these two mechanisms are fundamentally different and could lead to distinct downstream responses. Regulation of the relative contributions of both mechanisms for stabilizing β-catenin would allow an organism to fine-tune sensitivity to Wnt signals for precise temporal and spatial control of tissue patterning. Moreover, it is likely that additional mechanisms not described here further contribute to the robustness and regulation of Wnt-mediated β-catenin stabilization (12).

Methods

Ubiquitination Assay. Radiolabeled IVT Axin (1 μl) was incubated at room temperature (RT) with 17.5 μl of egg extract supplemented with GST-ubiquitin (50 μg/ml) in the presence or absence of LRP6ICD. At indicated times, the reaction was diluted with Buffer A and applied to 5 μl of glutathione-Sepharose beads. After 2-h shaking at 4°C, the beads were washed and eluted with sample buffer for analysis.

Trypsin Digest. Egg extract (3 μl) was incubated with IVT, radiolabeled Axin (0.5 μl), and GSK3 (15 μg/ml) for 30 min. Bovine pancreatic trypsin (0.38 mg/ml) (Sigma) was added, and samples were incubated at RT for 80 sec. Soybean trypsin inhibitor and sample buffer were then added for analysis.

Complete details regarding materials and methods are described in *SI Methods*.

Note Added in Proof. Hendriksen *et al*. (37) recently reported that dephosphorylated β-catenin accumulates at activated, phosphorylated LRP6 in response to canonical Wnt signaling in cultured mammalian cells. This finding further supports our model in which Wnt-activated LRP6 directly inhibits GSK3's phosphorylation of β-catenin within the destruction complex at the plasma membrane.

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