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Wnt3a and Dkk1 Regulate Distinct Internalization Pathways of LRP6 to Tune the Activation of β-Catenin Signaling

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SUMMARY

Wnt and Dickkopf (Dkk) regulate the stabilization of β-catenin antagonistically in the Wnt signaling pathway; however, the molecular mechanism is not clear. In this study, we found that Wnt3a acts in parallel to induce the caveolin-dependent internalization of low-density-lipoprotein receptor-related protein 6 (LRP6), as well as the phosphorylation of LRP6 and the recruitment of Axin to LRP6 on the cell surface membrane. The phosphorylation and internalization of LRP6 occurred independently of one another, and both were necessary for the accumulation of β-catenin. In contrast, Dkk1, which inhibits Wnt3a-dependent stabilization of β-catenin, induced the internalization of LRP6 with clathrin. Knockdown of clathrin suppressed the Dkk1-dependent inhibition of the Wnt3a response. Furthermore, Dkk1 reduced the distribution of LRP6 in the lipid raft fraction where caveolin is associated. These results indicate that Wnt3a and Dkk1 shunt LRP6 to distinct internalization pathways in order to activate and inhibit the β-catenin signaling, respectively.

INTRODUCTION

Wnts constitute a large family of cysteine-rich secreted ligands that are essential for a wide array of developmental and physiological processes (Logan and Nusse, 2004). At least 19 Wnt members have been shown to be present in humans and mice to date. The intracellular signaling pathway activated by Wnt was originally identified as a β-catenin-dependent pathway that is highly conserved among various species. Regulation of the stability of cytoplasmic β-catenin is essential in this pathway (He et al., 2004). According to the most widely accepted current model of the β-catenin pathway, in the absence of Wnt, casein kinase 1α (CK1α) and glycogen synthase kinase-3 (GSK-3) phosphorylate β-catenin in the Axin complex (He et al., 2004; Ikeda et al., 1998; Kikuchi, 1999). Phosphorylated β-catenin is ubiquitinated, resulting in the degradation of β-catenin by the proteasome. As a result, the cytoplasmic β-catenin level is low. Thus, the mechanism of the degradation of β-catenin is well understood.

When Wnt acts on the cells, β-catenin is stabilized. The accumulated β-catenin is translocated to the nucleus, where it binds to the transcription factor T cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby stimulates the expression of various genes (Logan and Nusse, 2004). However, how β-catenin is stabilized in response to Wnt is not yet well understood. Recent evidence has shown that the phosphorylation and internalization of the Wnt receptor play important roles in the β-catenin pathway. Once Wnt binds its cell-surface receptor consisting of Frizzled (Fz) and low-density-lipoprotein receptor-related protein 6 (LRP6), the cell cytoplasmic region of LRP6 has been shown to be phosphorylated by GSK-3 and CK1γ, which enhances the binding of the Axin complex to LRP6 and is essential for the stabilization of β-catenin (Davidson et al., 2005; Zeng et al., 2005). Frizzled and Dvl are required for the process of the phosphorylation of LRP6 and the recruitment of Axin (Bilic et al., 2007; Zeng et al., 2008). This model is in accordance with the observation in *Drosophila* embryos that dishevelled is required for dAxin recruitment to the plasma membrane upon Wingless (Wg)/Armadillo signaling (Cliffe et al., 2003). Furthermore, in *Drosophila* the internalization of Wg and Arrow (a LRP6 homolog) is necessary for the Wg phenotype and Wg-dependent gene expression in the wing disc (Seto and Bellen, 2006). In mammalian cells, it has been reported that Wnt3a and Wg induce the internalization of LRP6 with clathrin, which triggers the accumulation of β-catenin (Blitzer and Nusse, 2006). In contrast, we have found that the internalization of LRP6 with caveolin is necessary for the Wnt3a-dependent accumulation of β-catenin (Yamamoto et al., 2006). Thus, there are conflicting data on the internalization pathways of LRP6 and their roles in the activation of β-catenin signaling. These differences may be attributable to distinct experimental conditions, including, for example, the concentrations used for reagents such as monodansylcadaverine. In an effort to use a more mechanistic analysis to resolve the outstanding questions in this area, we decided to investigate whether and how the phosphorylation and internalization of LRP6 are linked.

The Dickkopf (Dkk) family comprises four members (Dkk1 to Dkk4), which antagonize Wnt signaling (Niehrs, 2006). There are two possible mechanisms by which Dkk1 might inhibit the β-catenin pathway. One possibility is that Dkk1 functions by preventing Fz-LRP6 complex formation (Semënov et al., 2001). The

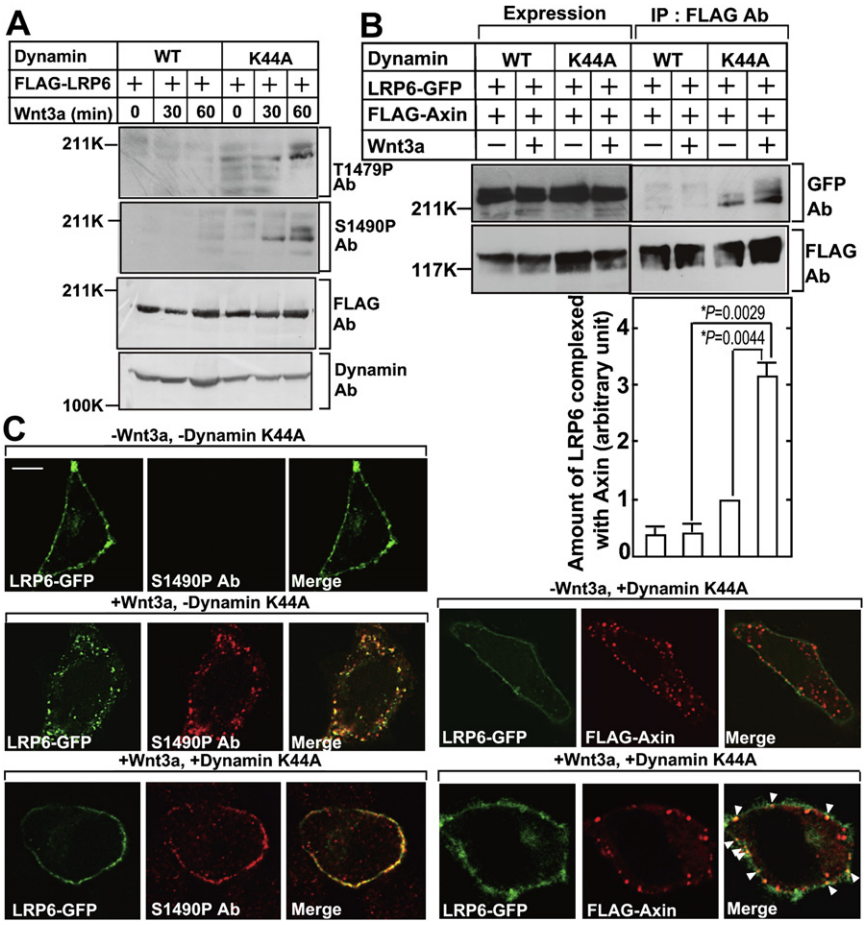


Figure 1. Wnt3a-Dependent Phosphorylation of LRP6 Occurs without Internalization

(A) HEK293T cells expressing FLAG-LRP6 and dynamin WT or K44A were stimulated with Wnt3a for the indicated periods of time. Lysates of HEK293T cells were probed with the indicated antibodies. WT, wild-type.

(B) After HEK293T cells expressing LRP6-GFP, FLAG-Axin, and dynamin WT or K44A had been stimulated with Wnt3a for 60 min, lysates were prepared and immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were probed with the indicated antibodies. IP, immunoprecipitation. The signals of LRP6-GFP complexed with FLAG-Axin were quantified using NIH Image and expressed as arbitrary units. The results shown are means ± SE from three independent experiments.

(C) After HeLa cells had been transfected with pCS2/LRP6-EGFP alone (left panels) or pCS2/LRP6-EGFP and pcDNA3/FLAG-Axin (right panels), HA-dynamin K44A was expressed by the removal of tetracycline. The cells were stimulated with Wnt3a for 30 min and then viewed directly or stained with anti-S1490P or anti-FLAG antibody. In merged images, LRP6-GFP is shown in green, and phosphorylated LRP6 or FLAG-Axin is in red. The arrowheads in the right bottom panel indicate the association of Axin with LRP6 on the membrane. Approximately 85% of the internalized LRP6 puncta reacted with anti-S1490P antibody in control cells after Wnt3a stimulation (see Table S1). Although approximately 79% of cells showed punctate cytoplasmic-Axin signals in control cells stimulated by Wnt3a, Axin signals were detected as puncta associated with the cell surface membrane in 72% of the cells expressing dynamin K44A after Wnt3a stimulation (see Table S2).

other possible mechanism is that Dkk1 interacts with another class of receptor, the single-pass transmembrane proteins Kremen1 (Krm1) and Krm2, in addition to LRP6 (Niehrs, 2006). Binding of the Dkk1/LRP6 complex to Krm results in the formation of a ternary structure, inducing rapid endocytosis and removal of LRP6 from the plasma membrane, thereby attenuating Wnt signaling. However, it is not clear whether the Dkk1-dependent internalization pathway of LRP6 is the same as that of Wnt3a-dependent internalization or not.

We show here that Wnt3a induces the phosphorylation-dependent binding of Axin to LRP6 and caveolin-mediated internalization of LRP6 as independent events and that both events are required for Wnt3a-dependent accumulation of β-catenin. In addition, we demonstrate that Dkk1 induces the internalization of LRP6 in a clathrin-dependent manner. These results indicate that these two ligands shunt LRP6 to distinct internalization pathways in order to activate and inhibit the β-catenin pathway, respectively.

RESULTS

Wnt3a-Dependent Phosphorylation of LRP6 Occurs without Internalization

First, we examined whether impairment of the internalization of LRP6 affects Wnt3a-dependent phosphorylation of LRP6. Dynamin K44A acts as a dominant-negative form, thereby suppressing both clathrin- and caveolin-dependent receptor endocytosis (Vieira et al., 1996). It has been shown that Wnt3a promotes the phosphorylation of LRP6, which is detected with anti-phospho-S1490 (anti-S1490P) and anti-phospho-T1479 (anti-T1479P) antibodies (Davidson et al., 2005; Zeng et al., 2005). Although Wnt3a induced the phosphorylation of LRP6 slightly in HEK293T cells under our conditions, transient expression of dynamin K44A in the cells enhanced the phosphorylation of LRP6 (Figure 1A). While the association of Axin and LRP6 was barely detected in the control cells even in the presence of Wnt3a, expression of dynamin K44A enhanced the basal and Wnt3a-dependent complex formation between LRP6 and Axin (Figure 1B). Under the same conditions, transient expression of dynamin K44A suppressed Wnt3a-dependent accumulation of β-catenin (see Figure S1A available online).

The effects of dynamin K44A on Wnt-3a-dependent phosphorylation of LRP6 were confirmed by immunocytochemical studies using HeLa cells, in which dynamin K44A expression was induced (Vieira et al., 1996). The addition of Wnt3a for 30 min induced the internalization of LRP6, which reacted with anti-S1490P antibody, in control HeLa cells (Figure 1C and Table S1). However, when the cells expressing dynamin K44A were stimulated with Wnt3a, most of LRP6 was retained on the cell surface membrane, and anti-S1490P antibody recognized

LRP6 on the cell surface membrane (Figure 1C and Table S1). Axin is known to present as puncta in the cytosol in the absence of Wnt stimulation. We demonstrated previously that Axin moves to the cytosol after it is translocated to the cell surface membrane in response to Wnt3a (Yamamoto et al., 2006; Figure S1B and Table S2). However, Axin signals detected as puncta associated with the cell surface membrane were increased in the cells expressing dynamin K44A after Wnt3a stimulation (Figure 1C and Table S2). Thus, Wnt3a is able to induce the phosphorylation of LRP6 without its internalization.

To exclude the possibility that overexpression of dynamin K44A gives a deleterious effect, we examined EGF signaling in the cells. Dynamin K44A enhanced EGF-dependent tyrosine phosphorylation of phospholipase Cγ and Shc (Figure S2). In addition, it has been reported that dynamin K44A increases cell growth, although it inhibits EGF-dependent MAP kinase activation (Vieira et al., 1996). Therefore, the effects of dynamin K44A on the Wnt/β-catenin pathway are not simply due to damage to the cells.

Internalization of LRP6 Occurs without Phosphorylation

Next, we asked whether the phosphorylation of LRP6 plays a role in its internalization. LRP6-(1–1505) contains two Ser and Thr clusters and one Pro-Pro-Pro-Ser/Thr-Pro (PPPS/TP) motif, which are phosphorylated by CK1γ and GSK-3, in the cytoplasmic region. Without Wnt3a, both LRP6 and LRP6-(1–1505) were localized to the cell surface membrane, and upon stimulation with Wnt3a, both receptors were internalized (Figure 2A). The ratios of internalized LRP6 and LRP6-(1–1505) were almost the same at each time point after stimulation with Wnt3a (Figure 2A). Internalized LRP6-(1–1505) was colocalized with caveolin (data not shown) but not recognized by the anti-S1490P antibody (Figure 2A). LRP6 enhanced the Wnt3a-dependent transcriptional activity of Tcf, while LRP6-(1–1505) had little effect, suggesting that the LRP6 mutant had lost activity (Figure S3). These results indicate that LRP6-(1–1505) is internalized with caveolin without phosphorylation in response to Wnt3a.

It has been reported that alkaline phosphatase, which is a GPI (glycosylphosphatidylinositol)-anchored protein, and ganglioside (GM1), which binds to cholera toxin subunit B (CTB), are located in lipid rafts and transported by caveolin-mediated endocytosis (Parton et al., 1994). Okadaic acid and CTB induce the internalization of alkaline phosphatase and GM1, respectively, together with lipid raft components. Since we found that LRP6 is localized to lipid rafts (Yamamoto et al., 2006), we asked whether these reagents also induce the internalization of LRP6. As shown in Figure 2B, LRP6 was internalized upon treatment with okadaic acid and CTB as well as Wnt3a stimulation. However, LRP6 internalized by treatment with these reagents was less phosphorylated than by Wnt3a stimulation (Figure 2B and Table S3). Furthermore, okadaic acid- or CTB-induced internalization of LRP6 was not associated with the accumulation of β-catenin (Figures 2C and 2D). Therefore, the internalization of LRP6 without phosphorylation is not sufficient for the stabilization of β-catenin.

A Constitutively Active Form of LRP6 Is Phosphorylated and Is Associated with Caveolin

It has been reported that mouse LRP5/6 and *Drosophila* Arrow mutants lacking their extracellular domain but containing their transmembrane domain (LRP6ΔN) are able to activate the β-catenin pathway without Wnt stimulation, while the intracellular domain alone (LRP6C) of LRP6 is incapable of activating it (He et al., 2004; Mao et al., 2001). Slowly migrating bands of LRP6ΔN [LRP6-(1360–1613)] that were recognized by anti-S1490P and anti-T1479P antibodies were observed, and LRP6ΔN formed a complex with Axin (Figure S4). However, LRP6C [LRP6-(1396–1613)] was neither phosphorylated nor formed a complex with Axin (Figure S4). Immunocytochemical analyses showed that LRP6ΔN is detected mainly as cytoplasmic puncta and that LRP6C is distributed throughout the cytoplasm (Figure 3A). LRP6ΔN was colocalized with caveolin but not with clathrin (Figures 3B and 3C). When the LRP6 mutants were overexpressed in HEK293T cells, LRP6ΔN was immunoprecipitated with caveolin, but LRP6C was not associated with caveolin (Figure 3D). Neither of them formed a complex with clathrin (Figure 3D). When CK1γ was expressed alone, it was observed on the cell surface membrane (see Figure S12B; Davidson et al., 2005). CK1γ was located in the cell surface membrane and cytoplasm when CK1γ and LRP6ΔN were coexpressed, and most of LRP6ΔN punctate signals were colocalized with cytoplasmic CK1γ (Figures 3E and 3F). However, LRP6ΔN was colocalized hardly, if at all, with EEA1 (Figures 3E and 3F). The accumulation of β-catenin was observed in HEK293 (Figures 3A and 4A) or L (data not shown) cells by overexpression of LRP6ΔN, but LRP6C did not induce β-catenin stability. Although caveolin itself was detected as puncta, its overexpression did not induce the stabilization of β-catenin (Figure 3A). Consistent with these observations, LRP6ΔN, but not LRP6C or caveolin-1, activated Tcf activity (Figure S5). These findings suggest that the phosphorylation of and the Axin-binding to LRP6 result in the accumulation of β-catenin when LRP6 formed a complex with caveolin and that CK1γ but not EEA1 may be localized to the vesicles containing LRP6 and caveolin in the cells stabilizing β-catenin.

Fusion of LRP6 Cytoplasmic Region and Caveolin Activates the β-Catenin Pathway

We speculated that the binding of the cytoplasmic region of LRP6 to caveolin might be able to stabilize β-catenin. To test this possibility, we generated a fusion protein of LRP6C and caveolin-1 (LRP6C-Cav) (Figure 4A). LRP6C-Cav was present as puncta, and this fusion protein was phosphorylated and associated with Axin (Figure 4B and Figure S6). Consistent with these results, LRP6C-Cav induced the stabilization of β-catenin and stimulated Tcf transcriptional activity to a similar extent as LRP6ΔN did (Figures 4A and 4B and Figure S5). Most of the LRP6C-Cav punctate signals were colocalized with CK1γ as puncta when both proteins were coexpressed; however, LRP6C-Cav was barely colocalized with EEA1 (Figure 4C). These results are similar to those observed in LRP6ΔN.

The ability of LRP6C-Cav to activate the β-catenin pathway was confirmed in *Xenopus* embryos. Ventral injection of *LRP6C-Cav* mRNA induced axis duplication in a dose-dependent manner (Figure S7A). Embryos expressing LRP6C had a normal axis. LRP6C-Cav-induced axis duplication was suppressed by injection of β-catenin morpholino oligo (Figure S7A). Furthermore, animal cap cells injected with LRP6C-Cav mRNA showed elevated expression levels of *siamois* and *Xnr3*, which are target genes of the β-catenin pathway, and the expression

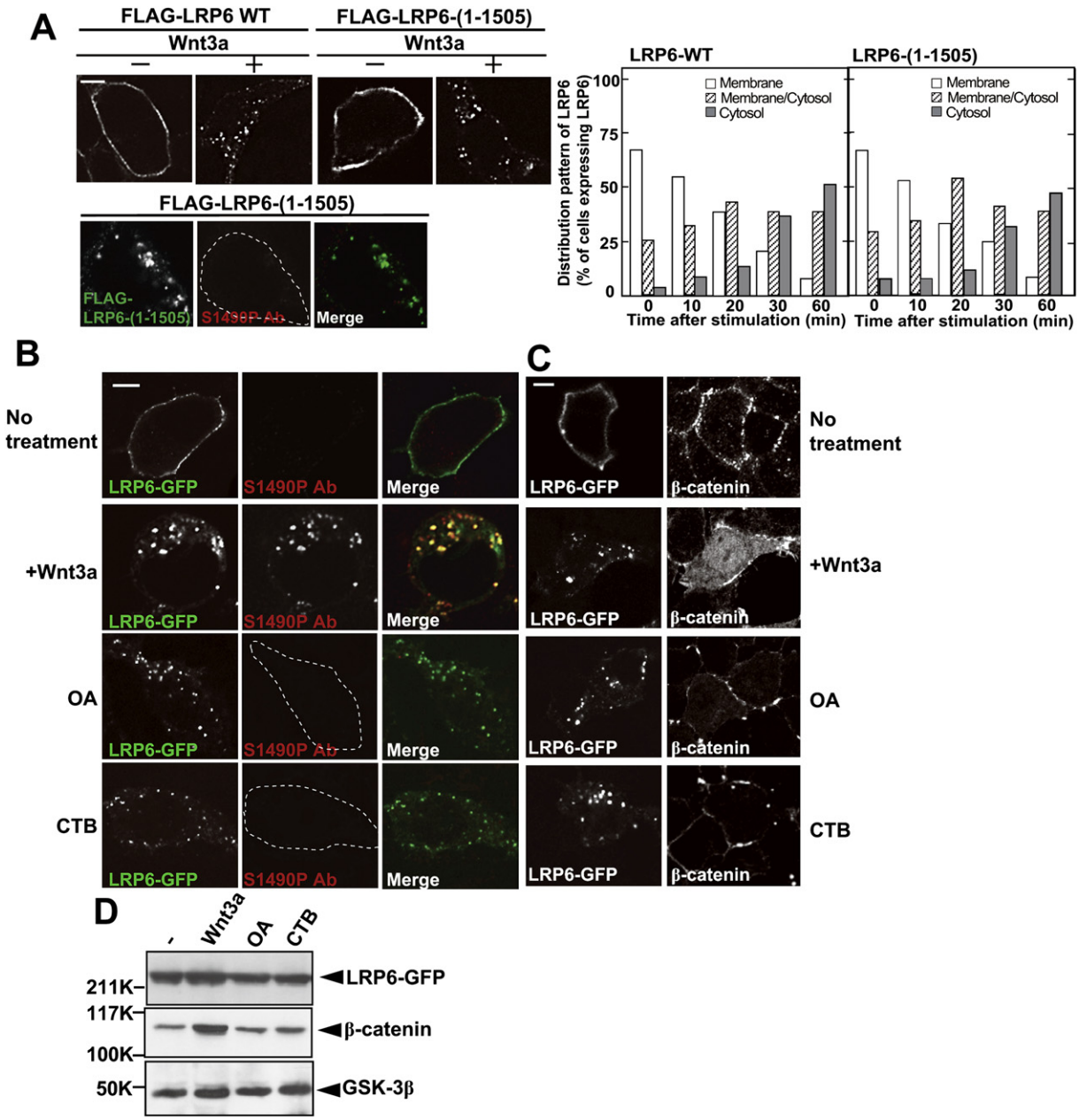


Figure 2. Internalization of LRP6 Occurs without Phosphorylation

(A) HEK293 cells expressing FLAG-LRP6 or FLAG-LRP6-(1–1505) were stimulated with Wnt3a for 1 hr, and the fixed cells were stained with anti-FLAG or anti-S1490P antibody. The percentages of internalized FLAG-LRP6 or FLAG-LRP6-(1–1505) were quantified in the right panels.

(B and C) HEK293 cells expressing LRP6-GFP were treated with Wnt3a, okadaic acid (OA), or cholera toxin subunit B (CTB) for 1 hr. Then the cells were viewed directly or stained with anti-S1490P (B) or anti-β-catenin (C) antibody (see Table S3). In merged images, LRP6-GFP is shown in green and phosphorylated LRP6 in red. Scale bars, 5 μm.

(D) After HeLaS3 cells expressing LRP6-GFP were stimulated with Wnt3a, OA, or CTB for 2 hr, the cytosolic fraction of HeLaS3 cells was probed with anti-β-catenin antibody.

was inhibited by β-catenin morpholino oligo (Figure S7B). These results suggest that the formation of a complex between the cytoplasmic region of LRP6 and caveolin is sufficient for the accumulation of β-catenin.

Caveolins possess a 33-residue central hydrophobic region (102–134 aa, membrane-spanning domain, MSD), which might form a hairpin in the lipid bilayer, flanked by cytoplasmically exposed N- and C-terminal domains (Figure 4A) (Parton et al., 2006). Caveolins interact with lipid rafts or caveolae through this region. LRP6C-Cav-(1–140) and LRP6C-Cav-(96–178) were detected as cytoplasmic puncta like LRP6C-Cav, and LRP6C-Cav-(96–140) showed a diffuse cytoplasmic distribution

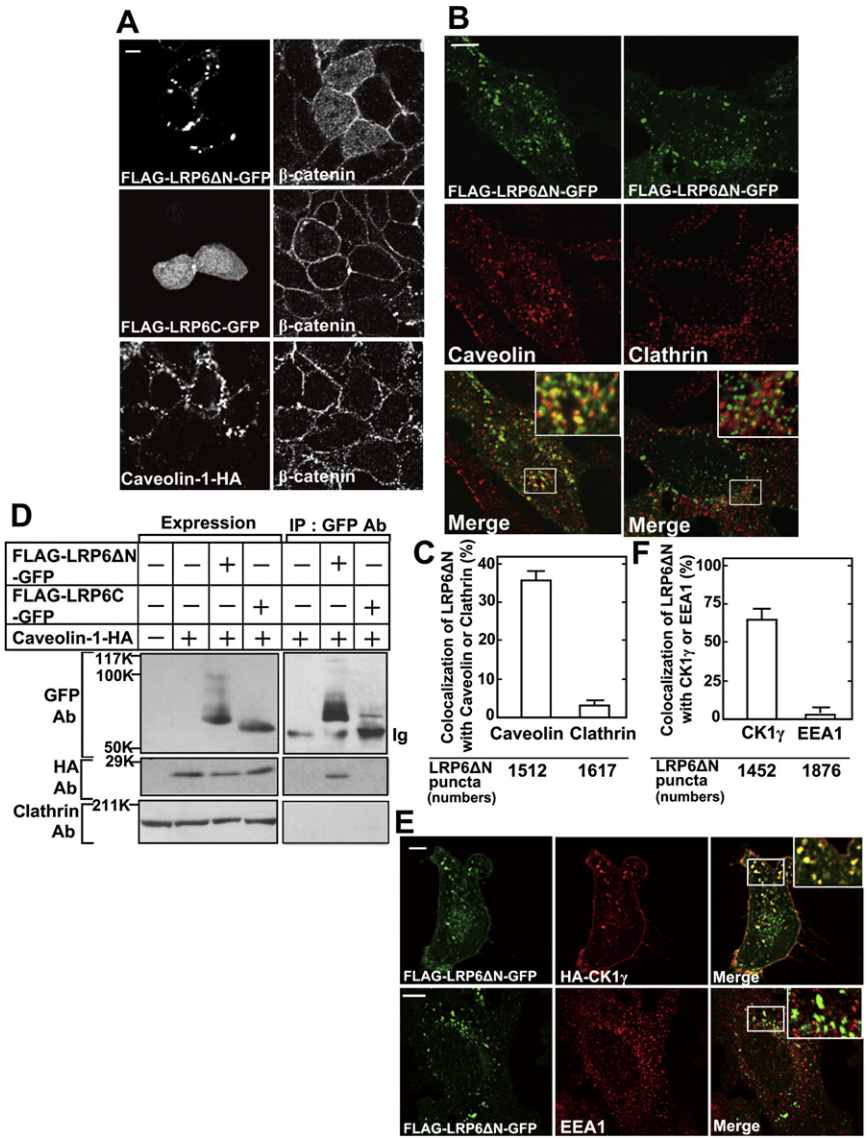


Figure 3. A Constitutively Active Form of LRP6 Is Phosphorylated and Is Associated with Caveolin

(A) HEK293 cells expressing FLAG-LRP6ΔN-GFP, FLAG-LRP6C-GFP, or caveolin-1-HA were viewed directly or stained with anti-β-catenin antibody. Caveolin-1-HA was detected by anti-HA antibody.

(B) HeLa cells expressing FLAG-LRP6ΔN-GFP were stained with anti-caveolin-1 or anti-clathrin antibody. In merged images, FLAG-LRP6ΔN-GFP is shown in green, and caveolin-1 or clathrin is in red. Colocalization of FLAG-LRP6ΔN-GFP and caveolin-1 appears as yellow.

(C) The percentages of LRP6ΔN colocalized with caveolin-1- or clathrin-positive vesicles were quantified. The results shown are means ± SE from three independent experiments. Approximately 36% of 1512 LRP6ΔN puncta were colocalized with caveolin-1-positive dots.

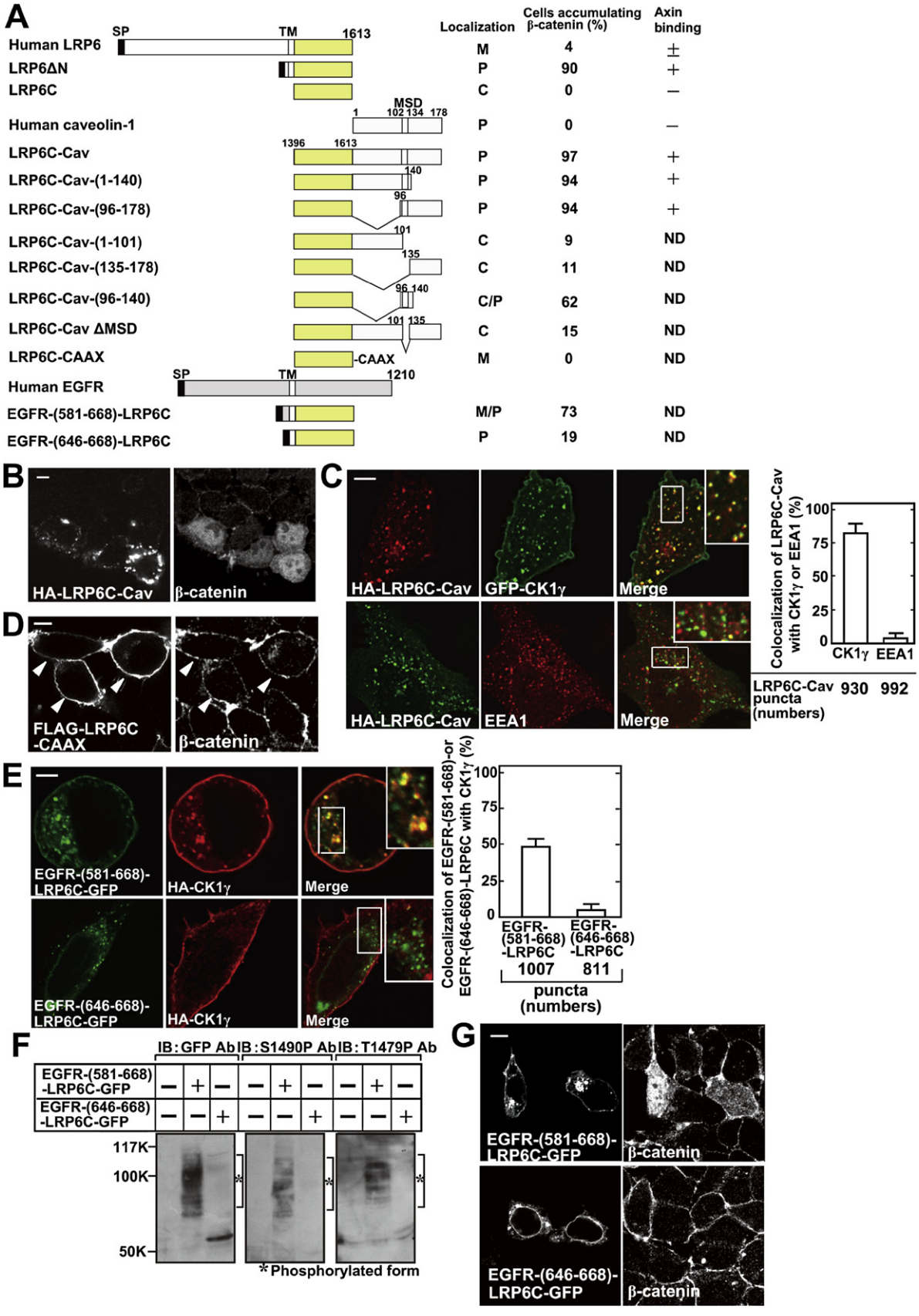
(D) Lysates of HEK293T cells expressing FLAG-LRP6ΔN-GFP or FLAG-LRP6C-GFP and caveolin-1-HA were probed with the indicated antibodies (left panel). The same lysates were immunoprecipitated with anti-GFP antibody, and the immunoprecipitates were probed with the indicated antibodies (right panel).

(E) HeLa cells coexpressing FLAG-LRP6ΔN-GFP with or without HA-CK1γ were viewed directly or stained with anti-HA or anti-EEA1 antibody. In merged images, FLAG-LRP6ΔN-GFP is shown in green, and HA-CK1γ or EEA1 is in red. Scale bars, 5 μm.

(F) The percentages of LRP6ΔN colocalized with CK1γ- or EEA1-positive vesicles were quantified. The results shown are means ± SE from three independent experiments. Approximately 70% of 1452 LRP6ΔN puncta were colocalized with CK1γ-positive dots.

with clear puncta (Figure S8). These mutants induced the stabilization of β-catenin (Figure 4A and Figure S8). However, LRP6C-Cav-(1–101), LRP6C-Cav-(135–178), and LRP6C-Cav-ΔMSD were distributed throughout the cytoplasm and induced the accumulation of β-catenin slightly (Figure 4A and Figure S8). Thus, the membrane-spanning domain of caveolin is important for LRP6C to stabilize β-catenin.

To examine whether membrane localization of LRP6C is sufficient for β-catenin signaling, we generated LRP6C-CAAX, in which the C-terminal CAAX domain of Ras was fused to the C terminus of LRP6C. Ras is localized to the plasma membrane through this motif (Hancock et al., 1990). LRP6C-CAAX was indeed localized to the cell surface membrane but did not induce the accumulation of β-catenin (Figures 4A and 4D). LRP6C-CAAX was neither phosphorylated nor associated with Axin (data not shown). We further examined whether the specific localization of LRP6 is necessary for the stabilization of β-catenin. It was shown that the juxtamembrane region (aa 581–641) of the EGF receptor (EGFR) is sufficient to target its transmembrane (aa 646–668) and cytoplasmic domains to the lipid raft fraction and that removal of the juxtamembrane region shifts the transmembrane domain of EGFR to the nonlipid raft fraction (Yamabhai and Anderson, 2002). We generated EGFR-(581–668)-LRP6C and EGFR-(646–668)-LRP6C (Figure 4A). The former construct was located at the cytoplasmic puncta and cell surface membrane and colocalized with CK1γ like LRP6ΔN and LRP6C-Cav, while the latter construct was mainly present as cytoplasmic puncta but not colocalized with CK1γ (Figure 4E). In addition, slowly migrating bands of EGFR-(581–668)-LRP6C on SDS-PAGE were recognized by the anti-S1490P and anti-T1479P antibodies (Figure 4F). However, EGFR-(646–668)-LRP6C was not phosphorylated. Consistent with these results, expression of EGFR-(581–668)-LRP6C induced the accumulation of β-catenin well, but that of EGFR-(646–668)-LRP6C showed a small effect on it (Figures 4A and 4G). Taken together, these results suggest that the simple membrane localization of LRP6C is not sufficient


A
Localization
Cells accumulating β-catenin (%)
Axin binding
Human LRP6
LRP6ΔN
LRP6C
Human caveolin-1
LRP6C-Cav
LRP6C-Cav-(1-140)
LRP6C-Cav-(96-178)
LRP6C-Cav-(1-101)
LRP6C-Cav-(135-178)
LRP6C-Cav-(96-140)
LRP6C-Cav ΔMSD
LRP6C-CAAX
Human EGFR
EGFR-(581-668)-LRP6C
EGFR-(646-668)-LRP6C
SP
TM
MSD
B
HA-LRP6C-Cav
β-catenin
C
HA-LRP6C-Cav
GFP-CK1γ
Merge
HA-LRP6C-Cav
EEA1
Merge
Colocalization of LRP6C-Cav with CK1γ or EEA1 (%)
CK1γ
EEA1
LRP6C-Cav puncta (numbers)
930
992
D
FLAG-LRP6C -CAAX
β-catenin
E
EGFR-(581-668)-LRP6C-GFP
HA-CK1γ
Merge
EGFR-(646-668)-LRP6C-GFP
HA-CK1γ
Merge
Colocalization of EGFR-(581-668)-or EGFR-(646-668)-LRP6C with CK1γ (%)
EGFR-(581-668) -LRP6C
EGFR-(646-668) -LRP6C
1007
811
puncta (numbers)
F
IB: GFP Ab
IB: S1490P Ab
IB: T1479P Ab
EGFR-(581-668) -LRP6C-GFP
EGFR-(646-668) -LRP6C-GFP
117K
100K
50K
*Phosphorylated form
G
EGFR-(581-668)-LRP6C-GFP
β-catenin
EGFR-(646-668)-LRP6C-GFP
β-catenin

for the stabilization of β-catenin and that the localization to the specific area, probably lipid rafts, might be important for it.

Dkk1 Induces the Internalization of LRP6 in a Clathrin-Dependent Manner

It has been shown that LRP6 is internalized with Kremen in response to Dkk1, which acts as a negative regulator of the Wnt/β-catenin pathway, but the detailed route of endocytosis is not known (Niehrs, 2006). Consistent with previous observations, when HEK293 cells expressing LRP6-GFP were stimulated with Dkk1, LRP6 was internalized and the receptor was observed in intracellular vesicles at 10 min to 1 hr (Figure S9).

Half of the internalized LRP6 was colocalized with clathrin but not with caveolin in HeLaS3 cells (Figure 5A). Clathrin siRNA but not caveolin-1 siRNA inhibited the internalization of LRP6 induced by Dkk1 (Figures 5B and 5C). Furthermore, monodansylcadaverine, chlorpromazine, and sucrose, which are known to block clathrin-dependent endocytosis (Okamoto et al., 2000), inhibited Dkk1-induced internalization of LRP6, while disruption of lipid rafts using nystatin, which inhibits caveolin-dependent endocytosis (Rothberg et al., 1990), had little effect (Figures 5D and 5E and Figure S10A).

Clathrin-Dependent Internalization of LRP6 Is Required for the Ability of Dkk1 to Inhibit the Accumulation of β-Catenin

Next, we asked whether clathrin-mediated internalization of LRP6 is functionally important for the action of Dkk1. Wnt3a induced the nuclear accumulation of β-catenin in HeLaS3 cells, and Dkk1 reduced the cells responding to Wnt3a (Figure 6A). Since LRP6ΔN and LRP6C-Cav lack the Dkk1-binding site, Dkk1 did not inhibit LRP6ΔN- and LRP6C-Cav-dependent stabilization of β-catenin (Figure S11). When clathrin was knocked down, Wnt3a-dependent β-catenin accumulation was not affected. However, Dkk1 could not suppress Wnt3a-dependent accumulation of β-catenin in clathrin knockdown cells (Figure 6A). When clathrin-mediated endocytosis was disrupted using monodansylcadaverine, chlorpromazine, or sucrose, Dkk1 did not inhibit Wnt3a-dependent accumulation of β-catenin (Figure 6B and Figure S10B). The present findings make it clear that LRP6 is internalized via distinct endocytic routes depending on different extracellular ligands and that Dkk1-dependent internalization of LRP6 through clathrin is required for the inhibitory action of Dkk1 on the β-catenin pathway.

Redistribution of LRP6 between the Lipid Raft and Nonlipid Raft Fractions in Response to Dkk1

To examine how Dkk1 induces the internalization of LRP6 via a route different from Wnt3a, we fractionated the lipid raft and nonlipid raft fractions by sucrose density gradient centrifugation. The detergent-resistant membranes including caveolin-1 (Figure 7A, fractions 3 and 4) were obtained in lower density fractions than the soluble membranous and nonmembranous materials (Figure 7A, fractions 8–10). We previously demonstrated that overexpressed LRP6 is present in both the lipid raft and nonlipid raft fractions (Yamamoto et al., 2006). Kremen2 and GSK-3β were located in the nonlipid raft fraction, while CK1γ was mainly present in the lipid raft fraction (Figure 7A). The CK1γ mutant, in which the C-terminal region including the membrane localization signal was deleted [CK1γ-(1–403)], was observed in the nonlipid raft fraction, but this mutant showed the same phosphorylation activity as wild-type in vitro (Figure S12A). In addition, immunocytochemical analyses showed that wild-type CK1γ is located to the cell surface membrane, while CKIγ-(1–403) was distributed throughout the cytoplasm (Figure S12B). The transferrin receptor, which is known to be internalized in a clathrin-dependent manner (Hopkins and Trowbridge, 1983), was detected in fractions 8–10 but not in fractions 3 and 4. Therefore, it is conceivable that LRP6 and CK1γ in the lower density fractions were mainly present in the lipid raft fraction with caveolin. When LRP6 and CK1γ were coexpressed, LRP6 in the lipid raft fraction showed a mobility shift (Figure 7B), as detected with anti-T1479P antibody (data not shown). In contrast, CK1γ-(1–403) did not promote the phosphorylation of LRP6 in either the lipid raft or nonlipid raft fraction (Figure 7B). Therefore, CK1γ located in the lipid raft fraction was responsible for phosphorylating LRP6.

When HEK293T cells were treated with Dkk1, the proportion of LRP6 present with caveolin in the lipid raft fraction was reduced (Figure 7C). In addition, Dkk1 caused a similar reduction of LRP6 in the lipid raft fraction in monodansylcadaverine- or chlorpromazine-treated cells (Figure 7C and Figure S10C). Thus, Dkk1 may remove LRP6 from the lipid raft fraction independently of

Figure 4. Fusion of LRP6 Cytoplasmic Region and Caveolin Activates the β-Catenin Pathway

(A) Fusion constructs of LRP6 cytoplasmic portion and caveolin used in this study. SP, signal peptide; TM, transmembrane domain; MSD, membrane-spanning domain; M, plasma membrane; C, diffusely in cytoplasm; P, cytoplasmic puncta; ND, not determined. The percentages of cells with β-catenin accumulation in the cells expressing LRP6 or its mutants are indicated.

(B) HEK293 cells expressing HA-LRP6C-Cav were stained with anti-HA and anti-β-catenin antibodies.

(C) HeLa cells coexpressing HA-LRP6C-Cav with or without GFP-CK1γ were viewed directly or stained with anti-HA and anti-EEA1 antibodies. In merged images, HA-LRP6C-Cav is shown in red or green, GFP-CK1γ is in green, and EEA1 in red. The percentages of LRP6C-Cav colocalized with CK1γ- or EEA1-positive vesicles were quantified in the right panel. The results shown are means ± SE from three independent experiments. Approximately 80% of 930 LRP6C-Cav puncta were colocalized with CK1γ-positive dots.

(D) HEK293 cells expressing FLAG-LRP6C-CAAX were stained with anti-FLAG and anti-β-catenin antibodies. The arrowheads indicate cells expressing FLAG-LRP6C-CAAX.

(E) HeLa cells coexpressing EGFR-(581–668)-LRP6C-GFP or EGFR-(646–668)-LRP6C-GFP with HA-CK1γ were viewed directly or stained with anti-HA antibody. In merged images, LRP6 mutants are shown in green, and HA-CK1γ is in red. The percentages of LRP6 mutants colocalized with CK1γ-positive vesicles were quantified in the right panel. The results shown are means ± SE from three independent experiments. Approximately 50% of 1007 EGFR-(581–668)-LRP6C puncta were colocalized with CK1γ-positive dots.

(F) HEK293T cells expressing EGFR-(581–668)-LRP6C-GFP or EGFR-(646–668)-LRP6C-GFP were probed with the indicated antibodies. *, phosphorylated forms of EGFR-(581–668)-LRP6C-GFP.

(G) HEK293 cells expressing EGFR-(581–668)-LRP6C-GFP or EGFR-(646–668)-LRP6C-GFP were viewed directly or stained with anti-β-catenin antibody. Scale bars, 5 μm.

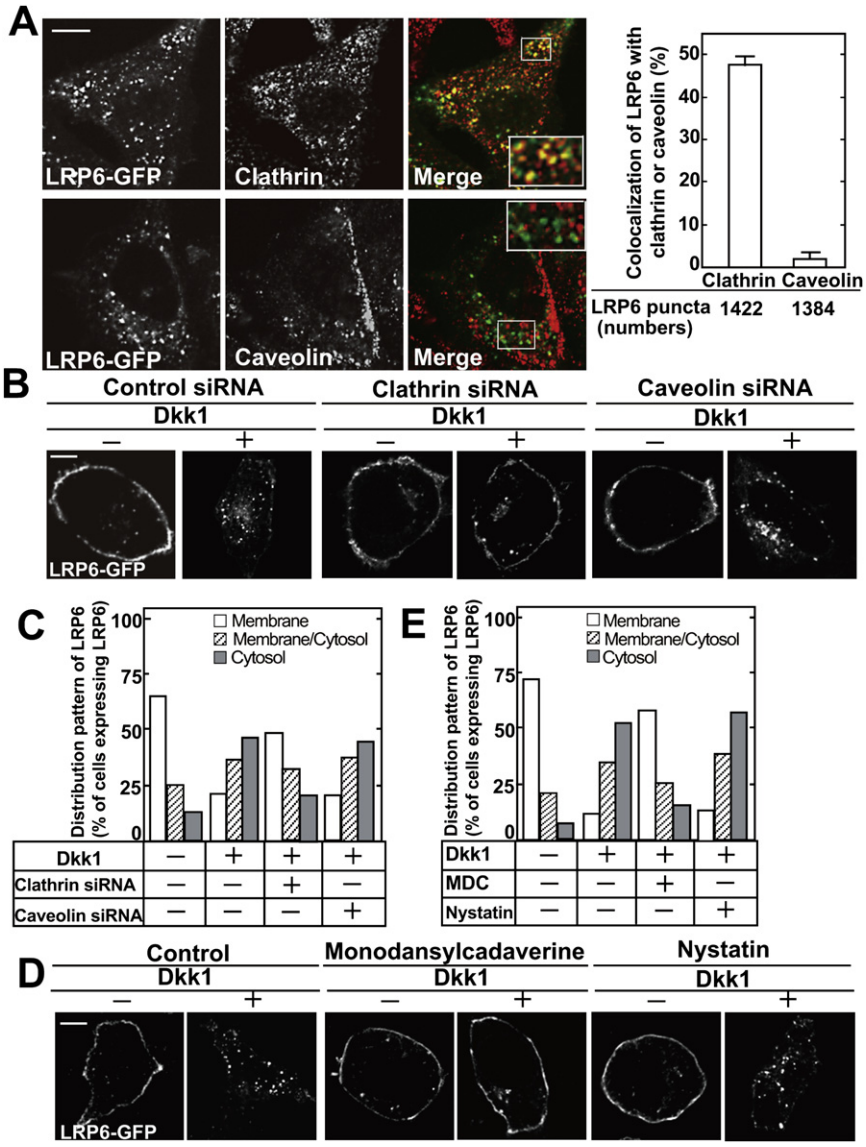


Figure 5. Dkk1 Induces the Internalization of LRP6 in a Clathrin-Dependent Manner

(A) HeLaS3 cells coexpressing LRP6-GFP and FLAG-Kremen2 were incubated with Dkk1 for 1 hr, and then the cells were viewed directly or stained with anti-clathrin or anti-caveolin-1 antibody. In merged images, LRP6-GFP is shown in green, and caveolin-1 or clathrin is in red. Colocalization of LRP6-GFP and clathrin appears as yellow. The percentages of internalized LRP6-GFP colocalized with caveolin-1- or clathrin-positive vesicles were quantified in 50 cells in the right panel. The results shown are means ± SE from three independent experiments. Approximately 48% of internalized 1422 LRP6 puncta were colocalized with clathrin-positive vesicles.

(B) After HeLaS3 cells expressing LRP6-GFP had been treated with siRNA for clathrin or caveolin-1, they were incubated with Dkk1 for 1 hr.

(C) The cells showing internalized LRP6-GFP in (B) were quantified as described in Experimental Procedures.

(D) After HEK293 cells expressing LRP6-GFP were treated with monodansylcadaverine or nystatin, the cells were incubated with Dkk1 for 1 hr.

(E) The cells showing internalized LRP6-GFP in (D) were quantified. Scale bars, 5 μm.

clathrin-mediated endocytosis. Although Wnt3a did not affect the distribution of LRP6 between the lipid raft and nonlipid raft factions in the absence of Dkk1, Wnt3a reversed the reduction of LRP6 in the lipid raft fraction induced by Dkk1 when clathrin-dependent endocytosis was blocked (Figure 7C). Therefore, Wnt3a and Dkk1 may regulate the localization of LRP6 on the cell surface membrane.

DISCUSSION

Our work focused on the relationship between the phosphorylation and internalization of LRP6 in response to Wnt3a stimulation, and the differences between the effects of Wnt3a and Dkk1 on LRP6. Our conclusions are that Wnt3a regulates the phosphorylation and caveolin-dependent internalization of LRP6 independently and that both effects on LRP6 are necessary for the activation of the β-catenin pathway. In addition, we found that Dkk1 induces the internalization of LRP6 with clathrin, which is a different route from Wnt3a-induced internalization, thereby suppressing the β-catenin pathway. We think it likely that the internalization of Wnt receptors determines the specific activation of the different intracellular cascades in Wnt signaling.

Both Phosphorylation and Internalization of LRP6 Are Necessary to Induce Accumulation of β-Catenin

We found that expression of dynamin K44A keeps LRP6 located in the plasma membrane after Wnt3a stimulation and enhances Wnt3a-dependent phosphorylation of LRP6. Therefore, dephosphorylation of LRP6 may be coupled to its internalization. The phosphorylated LRP6 in the plasma membrane recruited Axin in the cells expressing dynamin K44A, but Wnt3a did not stabilize β-catenin. These results suggest that the phosphorylation of LRP6 and the binding of Axin to LRP6 are not sufficient for Wnt3a-dependent stabilization of β-catenin.

Even when the C-terminal four PPPS/TP motifs were removed, the LRP6 mutant [LRP6-(1–1505)] was still internalized in response to Wnt3a. However, the internalization of this LRP6 mutant was not associated with the phosphorylation at T1479 and S1490 or the activation of Tcf transcriptional activity. These results indicate that the internalization of LRP6 occurs without its phosphorylation and that the phosphorylation of LRP6 at T1479 and S1490 requires the phosphorylation of other PPPS/TP motifs. It has been reported that the extracellular domain-truncated form of LRP6 containing a single PPPS/TP motif is

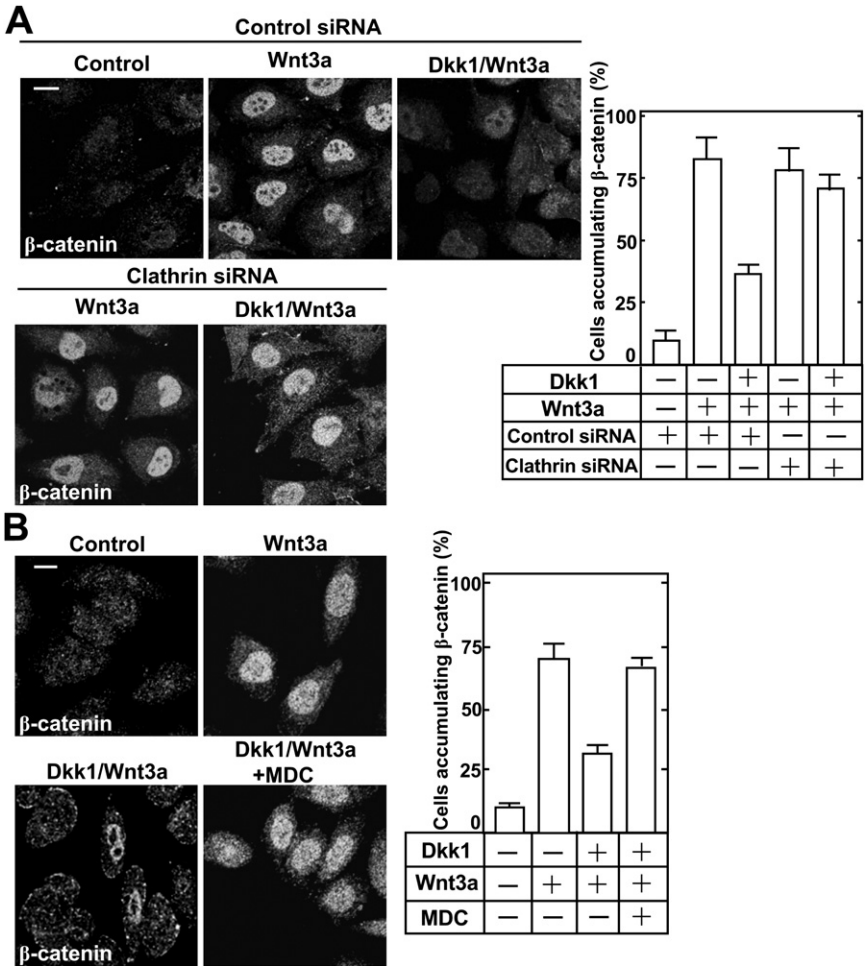


Figure 6. Clathrin-Dependent Internalization of LRP6 Is Required for the Ability of Dkk1 to Inhibit the Accumulation of β-Catenin

(A) After HeLaS3 cells had been treated with control or clathrin siRNA, the cells were incubated with Dkk1 for 1 hr, followed by stimulation with Wnt3a for 1 hr. The cells were stained with anti-β-catenin antibody in the left panels, and cells accumulating β-catenin were quantified in the right panel. More than 100 cells were counted. The results shown are means ± SE from three independent experiments.

(B) After CHO cells had been treated with monodansylcadaverine (MDC) for 30 min, the cells were incubated with Dkk1 for 1 hr in the presence of MDC, followed by stimulation with Wnt3a for 1 hr. The cells were stained with anti-β-catenin antibody in the left panels, and cells accumulating β-catenin were quantified in the right panel. The results shown are means ± SE from three independent experiments. Scale bars, 5 μm.

active (He et al., 2004). The difference between our and previous reports is the presence or absence of the extracellular Wnt-binding site of LRP6. By removing the Wnt-binding site, a single PPPS/TP motif may be sufficient for LRP6 to be converted into an active form.

CTB and okadaic acid induce caveolin-mediated endocytosis (Parton et al., 1994). We showed that CTB and okadaic acid induce the internalization of LRP6, consistent with our previous observation that LRP6 is localized to lipid rafts (Yamamoto et al., 2006). However, the internalized LRP6 was not phosphorylated and did not stabilize β-catenin. These results suggest that the internalization of LRP6 without phosphorylation is not able to induce the stabilization of β-catenin. Taken together, these facts suggest that neither the phosphorylation nor the internalization of LRP6 by itself is sufficient for the activation of the β-catenin-dependent pathway and that both are necessary for Wnt3a-dependent stabilization of β-catenin.

Although we analyzed only the role of LRP6 and Axin in the stabilization of β-catenin in this study, Frizzled and Dvl are essential for the Wnt/β-catenin pathway (He et al., 2004; Logan and Nusse, 2004). It has been reported that Frizzled5 and Dvl are required for the recruitment of Axin to the plasma membrane and the phosphorylation of LRP6 (Bilic et al., 2007; Zeng et al., 2008). These results were consistent with our previous observations that Frizzled5 and LRP6 are internalized simultaneously and colocalized with caveolin in response to Wnt3a (Yamamoto et al., 2006).

Impact of Caveolin on Activation of the Wnt/β-Catenin Pathway

We found that LRP6ΔN and LRP6C-Cav exhibit similar characteristics. LRP6ΔN, which is known to activate the β-catenin pathway, was present as puncta in the cytoplasm. Furthermore, LRP6ΔN colocalized with caveolin and CK1γ but not with clathrin. LRP6C-Cav, which was also observed as puncta colocalized with CK1γ, induced the accumulation of β-catenin. Both LRP6ΔN and LRP6C-Cav were phosphorylated and associated with Axin. *Xenopus* embryos expressing LRP6C-Cav showed axis duplication and increased mRNA levels of *siamois* and *Xnr3*, and these were suppressed by the β-catenin morpholino oligo. Expression of LRP6ΔN in *Xenopus* embryos caused similar effects (He et al., 2004). Therefore, formation of a complex between LRP6 and caveolin is able to activate the β-catenin pathway.

Caveolin is essential for caveolae formation. The minimal region of caveolin that is required for the activation of LRP6C is its membrane-spanning domain, which inserts into the lipid bilayer of the membrane and mediates the formation of caveolin oligomers (Parton et al., 2006). Therefore, the association of LRP6 with caveolin oligomers, proteins, and/or lipids in the caveolin-containing membrane fractions may be necessary for the stabilization of β-catenin. In addition, EGFR-(581–668)-LRP6C, which was colocalized with CK1γ and phosphorylated, induced the accumulation of β-catenin. Although LRP6C-CAAX and EGFR-(646–668)-LRP6C were present in the cell surface membrane and intracellular vesicles, respectively, these mutants failed to stabilize β-catenin. Therefore, the simple membrane localization of LRP6C is not sufficient for the stabilization of β-catenin, but

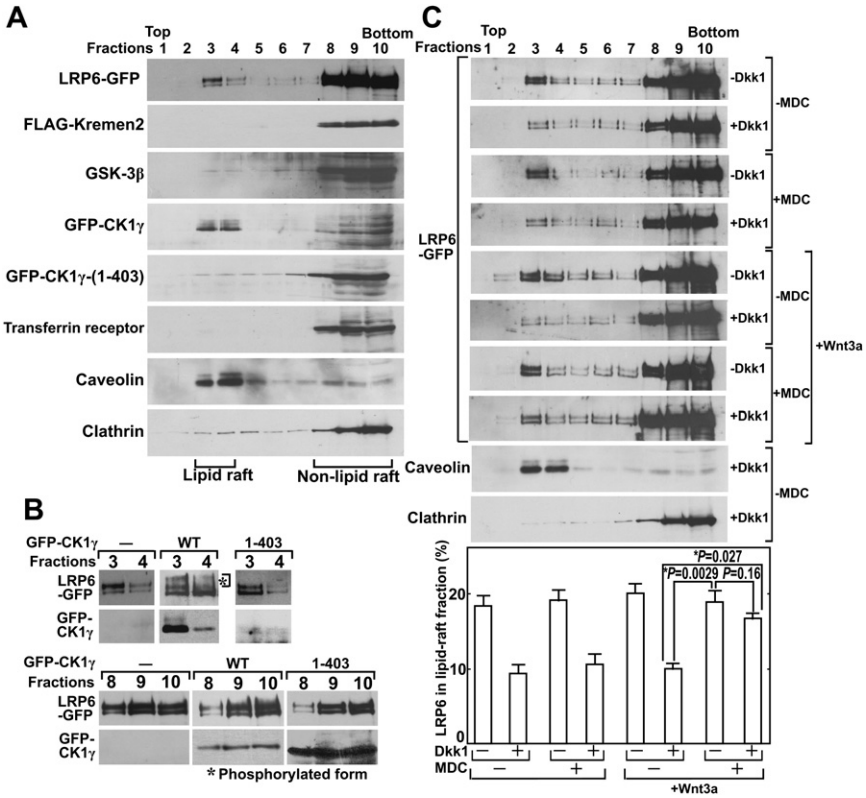


Figure 7. Redistribution of LRP6 between the Lipid Raft and Nonlipid Raft Fractions in Response to Dkk1

(A) Lysates of HEK293T cells expressing LRP6-GFP, FLAG-Kremen2, GFP-CK1γ, or GFP-CK1γ-(1–403) were fractionated by sucrose density gradient centrifugation, and aliquots were probed with anti-GFP or anti-FLAG antibody. Endogenous GSK-3β, transferrin receptor, caveolin-1, and clathrin were detected using their specific antibodies. Endogenous caveolin-1 and clathrin indicate the positions of the lipid raft and nonlipid raft fractions, respectively.

(B) Lysates of HEK293T cells coexpressing LRP6-GFP with GFP-CK1γ or GFP-CK1γ-(1–403) were fractionated, and the aliquots of fractions 3, 4, 8, 9, and 10 were probed with anti-GFP antibody. *, Phosphorylated form of LRP6-GFP.

(C) After HEK293T cells coexpressing LRP6-GFP and FLAG-Kremen2 were preincubated with or without MDC for 30 min, the cells were treated with Dkk1, Wnt3a, or control CM for 1 hr. Then lysates of the cells were fractionated, and the aliquots were probed with anti-GFP, anti-caveolin, and anti-clathrin antibodies. The band intensity of LRP6-GFP in the lipid raft and nonlipid raft fractions was quantified using NIH image, and the percentage of LRP6-GFP in the lipid raft fraction was calculated as (lipid raft/(lipid raft + nonlipid raft)) × 100. The results shown are means ± SE from four independent experiments.

the localization to the specific area, probably lipid rafts, might be important for it.

We do not know whether cytoplasmic LRP6ΔN- and LRP6C-Cav-containing vesicles were formed due to the internalization of lipid rafts of the cell surface membrane. Caveolin is synthesized on the rough endoplasmic reticulum and appears to travel along the secretory pathway to the plasma membrane through the Golgi complex (Parton et al., 2006). At some stage in the secretory pathway, caveolin associates with the lipid raft domain. Therefore, it is possible that LRP6ΔN and LRP6C-Cav on the secretory vesicles containing caveolin and CK1γ are constitutively phosphorylated by CK1γ, probably due to their lack of the N-terminal Wnt-binding region, and that these LRP6 mutants are associated with the Axin complex including GSK-3β, thereby stabilizing β-catenin. Given that LRP6ΔN and LRP6-Cav are barely colocalized with EEA1, it is conceivable that LRP6ΔN and LRP6-Cav are not always internalized from the plasma membrane and that fusion of vesicles containing LRP6ΔN and LRP6-Cav with early endosomes is not essential for the activation of the β-catenin pathway. This may explain why siRNA for Dvl does not suppress LRP6ΔN-dependent LEF1 activation (Li et al., 2002). Thus, to recruit the Axin complex to certain regions containing lipid rafts may be critically important in the Wnt/β-catenin pathway.

The working model of how Wnt signaling stabilizes β-catenin under physiological conditions is as follows. The binding of Wnt3a to LRP6 in lipid rafts triggers a conformational change of LRP6 that promotes its phosphorylation and association with Axin. These processes require Fz and Dvl (Bilic et al., 2007; Cliffe et al., 2003; Zeng et al., 2008). The vesicles containing phosphorylated LRP6, the Axin complex, some proteins including caveolin, and lipids are severed by dynamin from the membrane. By forming the vesicle, the complex between proteins and/or lipids on the vesicles may be altered, resulting in the inhibition of GSK-3 (Mi et al., 2006) and dissociation of β-catenin from Axin (Yamamoto et al., 2006) and thereby stabilizing β-catenin.

Distinct Routes of Endocytosis of LRP6 by Wnt3a and Dkk1

We found that Dkk1 internalizes LRP6 with clathrin but not with caveolin. Disruption of clathrin-mediated endocytosis inhibited the internalization of LRP6 induced by Dkk1 and attenuated Dkk1-dependent suppression of β-catenin stabilization by Wnt3a. Furthermore, Dkk1 reduced the distribution of LRP6 in the lipid raft fraction, and Wnt3a reversed removal of LRP6 from the lipid raft fraction induced by Dkk1 when clathrin-dependent endocytosis was blocked. These results indicate that Dkk1 internalizes LRP6 through a different endocytic route from that induced by Wnt3a, thereby attenuating the β-catenin pathway by removing LRP6 from the cell surface membrane, and that Wnt3a and Dkk1 determine the distribution of LRP6 in the lipid raft microdomains containing caveolin on the cell surface membrane.

In *Drosophila*, Wg and Arrow (LRP6 homolog) are trafficked through the endocytic pathway to the lysosome (Rives et al., 2006), and knockdown of Shibire (dynamin homolog) and Rab5 causes reduced Wg-dependent transcriptional activity (Seto and Bellen, 2006), suggesting that internalization facilitates Wg signaling. Since there is no caveolin homolog in *Drosophila*, certain proteins may substitute for caveolin to activate the Wg

pathway. Among the proteins in lipid rafts, flotillin belongs to a larger class of integral membrane proteins that carry an evolutionarily conserved domain called the prohibitin homology (PHB) domain (Morrow and Parton, 2005). Flotillin is expressed in a wide variety of species from mammals to *Drosophila*. In addition, flotillin is highly expressed in cells such as neurons which lack caveolin, suggesting that it may represent a functional analog of caveolin in caveolin-negative cells. Therefore, it will be of interest to examine whether flotillin or PHB domain proteins are involved in the internalization of LRP6 and stabilization of β-catenin.

Although evidence has been accumulating that a receptor internalizes with clathrin or caveolin depending on the circumstances, the molecular mechanism by which Wnt3a and Dkk1 regulate the functions of LRP6 by determining the endocytic routes is not known at present. The possible model is as follows. After Wnt3a binds to LRP6, LRP6 would be linked to molecules that reside in lipid rafts and internalized with caveolin. In contrast, by stimulation with Dkk1, LRP6 would be moved to nonlipid rafts, associated with molecules there, and then internalized with clathrin. To identify these molecules that link LRP6 to the endocytic machinery would provide a clue to understand the mechanism.

EXPERIMENTAL PROCEDURES

Internalization of LRP6-GFP by Wnt3a and Dkk1

HEK293 or HeLaS3 cells were used for the internalization assay as described (Yamamoto et al., 2006). At 24–48 hr after transfection with pCS2/LRP6-EGFP or other pCS2-derived vectors and pCMV-FLAG/Mesd or pCS2/FLAG-Kremen2, the cells were incubated with ice-cold binding medium (DMEM, 20 mM HEPES/NaOH [pH 7.5], 0.1% bovine serum albumin) for 30 min and treated with 1 ml of Wnt3a-conditioned medium (CM) or Dkk1 CM, 1 μM okadaic acid, or 1 μg/ml CTB for 1 hr at 4°C. Internalization was initiated by adding warm DMEM medium, and the dishes were transferred to a heated chamber (37°C, 5% CO_2). For observing the internalization of LRP6-GFP, the cells were washed three times with cold PBS to stop endocytosis. The cells were viewed directly with a confocal microscope (LSM510, Carl-Zeiss, Jena, Germany) to observe LRP6-GFP or stained with anti-FLAG antibody to detect FLAG-LRP6 and FLAG-LRP6-(1–1505). When necessary, HEK293 cells were pretreated with 25 μg/ml nystatin, 50 μM monodansylcadaverine, 10 μg/ml chlorpromazine, or 0.4 M sucrose for 30 min at 37°C before Dkk1 and/or Wnt3a treatment. The images shown are representative of 100 microscopic fields in five independent experiments.

Quantification of Signals in Fixed Cells

To quantify the distribution of FLAG-LRP6 or FLAG-LRP6-(1–1505), the appearance of their localization was classified into three types with regard to the distribution of these proteins and the number of puncta in the cytosol. The "membrane" type showed clear localization to the cell surface, with a few puncta in the cytosol. The "membrane/cytosol" type showed both localization of puncta to the cell surface and in the cytosol. The "cytosol" type showed the disappearance of the cell surface distribution, with more than 20 puncta in the cytosol. When the distribution of FLAG-Axin was quantified, the localization of Axin was classified into two types. The "membrane-associated" type showed the localization of most Axin puncta immediately beneath the cell surface membrane. The "cytosol" type showed more than 20 Axin puncta in the cytosol. More than 100 cells were evaluated in each experiment.

Immunocytochemistry

Cells grown on glass coverslips were fixed for 15 min in PBS containing 4% (w/v) paraformaldehyde and then permeabilized with PBS containing 0.2% (w/v) Triton X-100 and 2 mg/ml bovine serum albumin for 20 min. The cells were stained with anti-S1490P, anti-HA, anti-FLAG, anti-caveolin-1, anti-clathrin, or anti-EEA1 antibody before confocal microscopy was performed. The images shown are representative of 100 microscopic fields of at least three independent experiments.

Complex Formation and Immunoprecipitation

To show the complex formation of LRP6 and its mutants with Axin or caveolin, HEK293T cells (60 mm diameter dish) were lysed in 200 μl of lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA-NaOH [pH 8.5], 1% Triton X-100, 60 mM octylglucoside, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride). The lysates were immunoprecipitated with anti-GFP, anti-FLAG, or anti-HA antibody, and the immunoprecipitates were probed with the indicated antibodies. The results shown are representative of at least three independent experiments.

Accumulation of β-Catenin

To visualize the accumulation of β-catenin by confocal microscopy, HEK293, CHO, or HeLaS3 cells were seeded onto 18 mm glass coverslips coated with poly-D-lysine in 35 mm diameter dishes. HEK293 cells (35 mm diameter dishes) expressing LRP6-GFP were stimulated by the addition of 1 ml of Wnt3a CM, 1 μM okadaic acid, or 1 mg/ml CTB for 1 hr at 37°C. CHO cells cultured with 50 μM monodansylcadaverine, 10 μg/ml chlorpromazine, or 0.4 M sucrose for 30 min at 37°C were treated with 1 ml of F12 containing 200 μl of Dkk1 CM for 1 hr in the continuous presence of monodansylcadaverine or chlorpromazine, and then the cells were stimulated by the addition of 100 μl of Wnt3a CM for 1 hr at 37°C. HeLaS3 cells treated with siRNA for clathrin were treated with 1 ml of DMEM containing 200 μl of Dkk1 CM for 1 hr, and then the cells were stimulated by the addition of 100 μl of Wnt3a CM for 1 hr at 37°C. These cells were stained with anti-β-catenin antibody. The images are representative among 50 microscopic fields of four independent experiments. To observe the accumulation of β-catenin by immunoblotting, the cytosolic fraction of HeLaS3 cells was prepared and probed with anti-β-catenin antibody. The results shown are representative of at least three independent experiments.

Assay of Tcf-4 Activity

pPGK-neo/Wnt3a, pCS2/FLAG-LRP6, pCS2/FLAG-LRP6-(1–1505), pCS2/FLAG-LRP6ΔN-EGFP, pcDNA3/caveolin-1-HA, pCGN/LRP6C, or pCGN/LRP6C-Cav was transfected into HEK293T cells (in 35 mm diameter dishes) with TOP-*fos*-Luc, pME18S/lacZ, and pEF-BOS/hTcf-4E. At 46 hr after transfection, the cells were lysed, and the luciferase activity was measured as described (Yamamoto et al., 2003).

Xenopus Injection and Analyses of Phenotypes

For in vitro transcription for microinjection, an mMESSAGE mMACHINE SP6 kit (Ambion, USA) was used. After artificial fertilization and dejellying, embryos were injected animally at the 2 cell (for cDNA synthesis) or ventrally at the 4–8 cell stage (for phenotype observation) in 5% Ficoll 400 solution. For cDNA synthesis, the animal cap was dissected at stage 9 and dissolved in ISOGEN (NipponGene, Japan), and total RNA was isolated. For superficial observation, injected embryos were cultivated in 0.1% Steinberg's solution until the 3 day tadpole stage. Morpholino antisense oligo against β-catenin was used as described (Heasman et al., 2000).

Preparation of Lipid Raft Membranes

HEK293T cells (in 100 mm diameter dishes) expressing LRP6-GFP were incubated with ice-cold binding medium for 30 min and then stimulated with Wnt3a CM and/or Dkk1 CM. When necessary, FLAG-Kremen2, GFP-CK1γ, or GFP-CK1γ-(1–403) was expressed in HEK293T cells. The cells were lysed in 0.5 ml of ice-cold TNE buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 5 mM EDTA-NaOH [pH 8.5]) containing 0.4% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride, and then the cell lysates were further homogenized with a Dounce homogenizer (40 strokes) and subsequent passage through a 25-gauge needle (Brown and Rose, 1992). Each lysate (0.5 ml) was mixed with 0.5 ml of 80% (w/v) sucrose in TNE and overlayered with 2 ml of 35% sucrose in TNE, followed by 1 ml of 5% sucrose in TNE. The gradients were centrifuged at 190,000 × g for 18 hr at 4°C in an RPS56T rotor (Hitachi, Tokyo, Japan). Four hundred microliter fractions were harvested from the top of the gradient. Aliquots were probed

with the indicated antibodies. The results shown are representative of five independent experiments.

Kinase Assay

HEK293T cells (60 mm diameter dish) expressing GFP-CK1γ or GFP-CK1γ-(1–403) were lysed in 200 μl of lysis buffer. The lysates were immunoprecipitated with anti-GFP antibody, and the immunoprecipitates were incubated with 0.3 μM casein in 30 μl of kinase reaction mixture (50 mM HEPES-NaOH [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μM [γ-^{32}P]ATP [500–1000 cpm/pmol]) for the indicated periods of time at 30°C. The samples were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography, and the radioactivities of phosphorylated casein were counted.

Statistical Analysis

The experiments were performed at least three times, and the results were expressed as means ± SE. Statistical analysis was performed using StatView software (SAS Institute, Inc). Differences between the data were tested for statistical significance by the t test. *P* values less than 0.05 were considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include twelve figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/15/1/37/DC1/.

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