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Caveolin Is Necessary for Wnt-3a-Dependent Internalization of LRP6 and Accumulation of β-Catenin

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Summary

β-catenin-mediated Wnt signaling is critical in animal development and tumor progression. The single-span transmembrane Wnt receptor, low-density lipoprotein receptor-related protein 6 (LRP6), interacts with Axin to promote the Wnt-dependent accumulation of β-catenin. However, the molecular mechanism of receptor internalization and its impact on signaling are unclear. Here, we present evidence that LRP6 is internalized with caveolin and that the components of this endocytic pathway are required not only for Wnt-3a-induced internalization of LRP6 but also for accumulation of β-catenin. Overall, our data suggest that Wnt-3a triggers the interaction of LRP6 with caveolin and promotes recruitment of Axin to LRP6 phosphorylated by glycogen synthase kinase-3β and that caveolin thereby inhibits the binding of β-catenin to Axin. Thus, caveolin plays critical roles in inducing the internalization of LRP6 and activating the Wnt/β-catenin pathway. We also discuss the idea that distinct endocytic pathways correlate with the specificity of Wnt signaling events.

Introduction

Endocytosis of cell-surface receptors is an important regulatory event in signal transduction (Conner and Schmid, 2003; Di Fiore and De Camilli, 2001). Internalization of plasma membrane proteins and lipids is mediated by the clathrin-dependent and clathrin-independent pathways, and after internalization, the intracellular itinerary of receptors regulates signal transduction. Clathrin-dependent endocytosis targets proteins to the early endosomes and is an important pathway for down-regulating many receptors. Clathrin helps to deform the overlying plasma membrane containing concentrated receptors and their ligands into a coated pit. Dynamin is a multidomain GTPase that is recruited to the necks of coated pits, where it can assemble into a spiral to mediate membrane fission and the release of clathrin-coated vesicles (Hinshaw, 2000). Clathrin-independent endocytosis through lipid rafts and a caveolin-mediated pathway has recently emerged as another important trafficking pathway (Razani et al., 2002). Lipid rafts are membrane microdomains that are enriched in cholesterol and glycosphingolipids. Caveolae are lipid-raft-enriched, flask-shaped, and caveolin-1-containing invaginations and are internalized as small vesicles. Caveolae-mediated endocytosis can be blocked by overexpressing dominant-negative mutants of dynamin (Pelkmans and Helenius, 2002). Lipid rafts and caveolae function in internalization of toxins, Simian virus 40 (SV40) virus, and glycosyl phosphatidylinositol (GPI)-anchored proteins and regulate the internalization of receptors for autocrine motility factor (AMF) and transforming growth factor-β (TGF-β) (Conner and Schmid, 2003; Di Guglielmo et al., 2003).

Wnt proteins constitute a large family of cysteine-rich secreted ligands that control development in organisms ranging from nematode worms to mammals (Wodarz and Nusse, 1998). In the current model, Wnt activates at least three intracellular signaling pathways including the β-catenin, planar cell polarity (PCP), and Ca^{2+} pathways. In the β-catenin pathway, Wnt stabilizes cytoplasmic β-catenin, and 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 (Hurlstone and Clevers, 2002). The β-catenin pathway regulates cell proliferation and differentiation, and the abnormality of this pathway leads to tumor formation. In the PCP pathway, Wnt activates small G proteins including Rho and Rac through Dvl, thereby activating Rho kinase and Jun N-terminal kinase (Adler, 2002). The PCP pathway is involved in the regulation of tissue polarity, cell migration, and cytoskeleton arrangement. Furthermore, Wnt increases the intracellular Ca^{2+} concentration probably through trimeric GTP binding proteins and activates protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase. Although the physiological roles of the Ca^{2+} pathway are not clear, this pathway seems to regulate cell proliferation and cell migration (Veeman et al., 2003).

The interaction of Wnt proteins with their receptors on the cell surface is the first step in transducing an extracellular signal into intracellular responses (Cong et al., 2004). In humans and mice, ten Frizzled (Fz) proteins, which are members of the family of seven-pass transmembrane receptors, have been identified as Wnt receptors (Wodarz and Nusse, 1998). In addition to Fz proteins, the Wnt/β-catenin pathway requires single-span transmembrane proteins that belong to a subfamily of low-density-lipoprotein (LDL) receptor related proteins (LRPs): LRP5 and LRP6 (He et al., 2004). Fly genetical study indicates that Arrow (LRP5/6 homolog) is not essential for the PCP pathway (Wehrli et al., 2000). It has been reported that Wnt-5a induces the internalization of Fz4 through the binding of Dvl to β-arrestin2 and that this internalization requires the activation of PKC (Chen et al., 2003). However, the pathway of internalization of LRP5/6 upon stimulation with Wnt remains unclear. Furthermore, how endocytosis of Wnt receptors is involved in the activation of the β-catenin pathway is not known. Here, we have investigated the mechanism responsible for the LRP6 internalization and show that caveolin is necessary for the Wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin.

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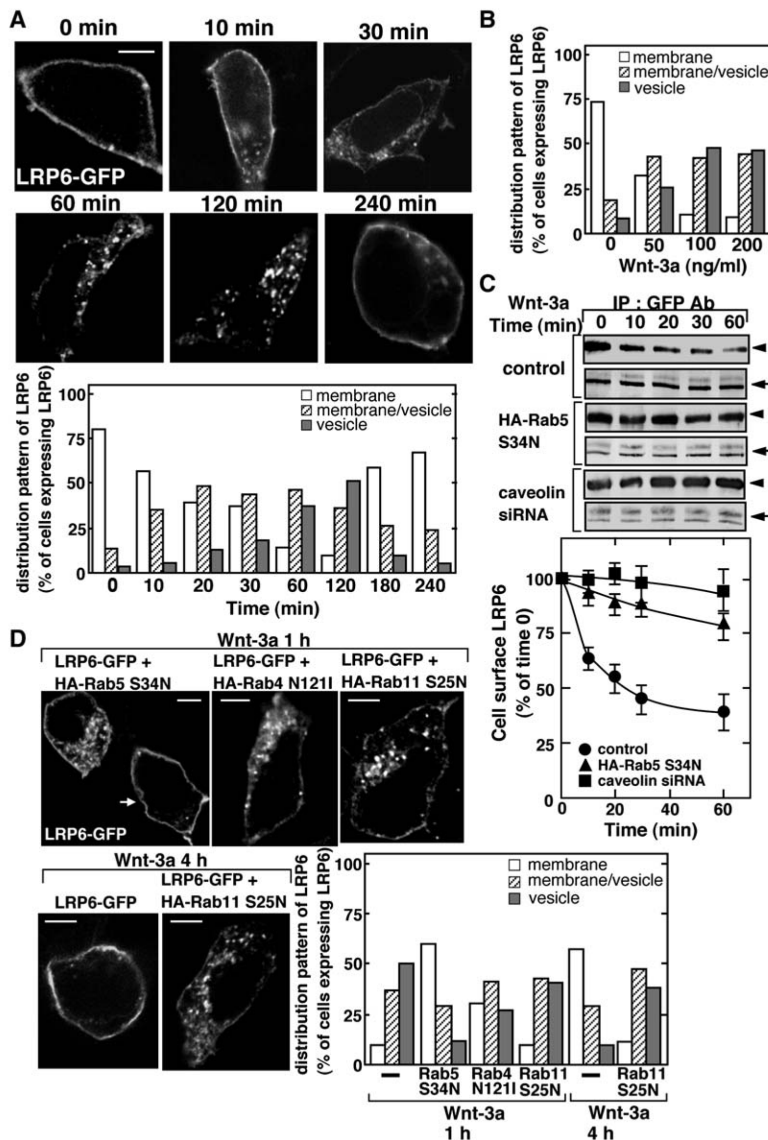

Figure 1. Wnt-3a Induces LRP6 Internalization

(A) HEK293 cells expressing LRP6-GFP were stimulated with Wnt-3a for the indicated periods of time. Upper panel, confocal images; lower panel, quantification of internalized LRP6-GFP.

(B) HEK293 cells expressing LRP6-GFP were stimulated with the indicated concentration of purified Wnt-3a for 1 hr.

(C) LRP6-GFP was expressed in HeLaS3 cells with or without HA-Rab5^{S34N} or caveolin-1 siRNA, and the cells were stimulated with Wnt-3a for the indicated periods of time. After cell surface biotinylation, the lysates were immunoprecipitated with anti-GFP antibody, and the immunopreciptates were probed with streptavidin-HRP (arrow heads) to detect cell-surface LRP6 levels and anti-GFP antibody (arrows) to detect total LRP6. The amounts of cell surface or total LRP6 were analyzed with NIH images. The results are expressed as the ratio of cell surface LRP6 to total LRP6 (lower panel). Values at zero times are set to 100%. The results shown are means ± SE from three independent experiments.

(D) HEK293 cells coexpressing LRP6-GFP and HA-Rab5^{S34N}, HA-Rab4^{N121I}, or HA-Rab11^{S25N} were stimulated for 1 hr or 4 hr. An arrow indicates the cell expressing HA-Rab5^{S34N}. Left panel, confocal images; right panel, quantification of internalized LRP6-GFP. Scale bars, 5 μm.

Results

LRP6 Is Internalized and Recycled in Response to Wnt-3a

To visualize LRP6 in intact cells, we expressed LRP6-green fluorescence protein (GFP) together with Mesd (Hsieh et al., 2003) in human embryonic kidney 293 (HEK293) cells. In the absence of Wnt-3a, LRP6-GFP was present predominantly at the cell surface, with a few puncta in the cytosol (Figure 1A). When the cells were stimulated with Wnt-3a-conditioned medium, LRP6-GFP was internalized, and the receptor was observed in the intracellular vesicles at 10 min to 2 hr. At 3–4 hr after stimulation, LRP6-GFP was present at the cell membrane again (Figure 1A). To quantitate these observations, we fixed the cells at the indicated time and classified cells expressing LRP6-GFP into three types: (1) LRP6-GFP is dominantly present at the cell surface, (2) LRP6-GFP locates to both the cell surface and vesicles in the cytosol, (3) LRP6-GFP mainly localizes to the cytosol as vesicles (Figure 1A). Before stimulation with Wnt-3a, LRP6-GFP was present at the cell surface in 80% of the cells. When the cells were stimulated with Wnt-3a for 1 hr, cells exhibiting cell-surface localization of LRP6-GFP decreased to less than 15% and the percentage of cells in which LRP6-GFP was observed as cytoplasmic vesicles increased. At 4 hr after stimulation, 70% of the cells showed the cell surface localization of LRP6-GFP again. To exclude the possibility that something in Wnt-3a-conditioned medium causes the modulation of LRP6, we stimulated the cells with purified Wnt-3a protein for 1 hr. The purified Wnt-3a indeed induced the internalization of LRP6-GFP in a dose-dependent manner (Figure 1B). The same findings were obtained in cells treated with cycloheximide, which prevents synthesis of new proteins (data not shown). These results were confirmed by measuring the levels of LRP6 on the plasma membrane by cell-surface biotinylation. At 10 min after stimulation with Wnt-3a, cell-surface LRP6-GFP levels were reduced, indicative of receptor clearance from the cell surface membrane (Figure 1C). Therefore, LRP6 seemed to be internalized and recycled

in a Wnt-3a-dependent manner that was not dependent on protein synthesis.

Rab small GTPases exert regulatory functions in exocytotic and endocytic transport, through the recruitment of specific effector proteins to the membrane on which they are localized (Sonnichsen et al., 2000; Zerial and McBride, 2001). Rab5 organizes a membrane domain defining the site of entry into early endosomes through its effector proteins including EEA1 and Rabenosyn-5. Rab4 and Rab11 are implicated in the fast and slow recycling pathway, respectively. Rab4 is localized to separate membrane domains on early endosomes that are distinct from those harboring Rab5. At 1 hr after stimulation, the internalized LRP6-GFP colocalized with EEA1 and Rab5, and little with Rab4, indicating that the internalized LRP6 was transported to early endosomes (Figure S1). Rab11 is mainly located in the Golgi apparatus. Although LRP6-GFP did not colocalize with HA-Rab11 at 1 hr after stimulation with Wnt-3a (data not shown), colocalization of these proteins was observed at 3 hr (Figure S1). Expression of a dominant-negative form of Rab5 (HA-Rab5^{S34N}) suppressed the internalization of LRP6-GFP, but expression of a dominant-negative form of Rab4 (HA-Rab4^{N121I}) only slightly influenced it (Figure 1D). HA-Rab5^{S34N} also suppressed the clearance of LRP6 from the cell-surface membrane (Figure 1C). A dominant-negative form of Rab11 (HA-Rab11^{S25N}) did not affect the internalization of LRP6-GFP at 1 hr but delayed the reappearance of LRP6-GFP at the cell membranes at 4 hr after stimulation with Wnt-3a (Figure 1D). Thus, Rab5 and Rab11 are involved in the internalization and recycling of LRP6, respectively.

Caveolin Is Required for Wnt-3a-Induced Internalization of LRP6

To examine whether LRP6 was internalized through either of the clathrin- or caveolin-mediated pathway, we expressed LRP6-GFP in HeLaS3 cells, where caveolae are more readily observed than in HEK293 cells. When the cells were stimulated with Wnt-3a for 1 hr, LRP6-GFP colocalized mainly with caveolin-1 and hardly, if at all, with clathrin (Figure 2A). This colocalization was seen at the plasma membrane in the absence of Wnt (Figure 2B) and in intracellular puncta subsequent to stimulation with Wnt-3a (Figure 2B). Moreover, LRP6-GFP and caveolin-1 formed a complex within 20 min of Wnt-3a stimulation, but LRP6-GFP did not associate with clathrin (Figure 2C). The slight apparent lag between internalization (Figures 1A and 1C) and complex formation (Figure 2C) may be attributable to a lower sensitivity of the coimmunoprecipitation assay relative to the internalization assay.

Caveolin associates with cholesterol- and sphingolipid-rich membrane domains called lipid rafts. LRP6-GFP was present in both the lipid-raft and non-lipid-raft fractions, which contained caveolin-1 or clathrin, respectively (Figure S2A). Disruption of caveolae with Nystatin, which is known to bind to cholesterol, shifted LRP6-GFP from the lipid-raft factions into the non-lipid-raft fractions (Figure S2A), indicating that the presence of LRP6 in the low-density fraction was dependent on lipid rafts. Wnt-3a did not affect the distribution of LRP6-GFP between the lipid-raft and non-lipid-raft fractions (data not shown).

We employed a variety of methods, including small interfering RNA (siRNA)-mediated knockdowns of caveolin-1 and clathrin (Figure S2B), to examine whether caveolin is required for the internalization of LRP6-GFP. Both siRNA- and Nystatin-mediated disruption of caveolin-mediated endocytosis inhibited the internalization of LRP6-GFP induced by Wnt-3a (Figures 1C, 2D, and 2F), while analogous manipulations of clathrin-dependent pathways (siRNA and monodansylcadaverine) had no effect (Figures 2D and 2F). Monodansylcadaverine inhibits clathrin-dependent endocytosis. Furthermore, expression of caveolin-1 restored the internalization of LRP6 in the caveolin-1 knockdown cells (Figures 2E and 2F). These findings indicate that Wnt-3a internalizes LRP6, which resides in the lipid-raft compartment, and that caveolin is necessary for the internalization.

Wnt-3a Induces the Internalization of Fz5

Frizzled 5 (Fz5) activates the β-catenin pathway by cooperating with LRP6 (Cong et al., 2004). When Fz5-GFP alone was expressed in HEK293 cells, Wnt-3a-conditioned medium induced the internalization of Fz5-GFP at 30 min to 2 hr, and then the receptor appeared at the cell-surface membrane at 3 to 4 hr after stimulation (Figure 3A). Purified Wnt-3a also induced the internalization of Fz5-GFP (Figure 3B). There is no significant difference between the amounts of Wnt-3a required for the internalization of LRP6 or Fz5 (see Figure 1B). At 1 hr after stimulation, this internalized Fz5-GFP clearly colocalized with EEA1 and Rab5 rather than with Rab4, and by 3 hr poststimulation, it could be found to be colocalized with HA-Rab11 (Figure S3). However, in HeLaS3 cells, only a small amount of Fz5-GFP colocalized with caveolin-1; most of the internalized Fz5-GFP colocalized with clathrin (Figure 3C). Consistent with this, Wnt-3a-induced internalization of Fz5-GFP was suppressed by clathrin siRNA or monodansylcadaverine but not by caveolin-1 siRNA or Nystatin (Figure 3D). Therefore, Fz5 may be internalized through a clathrin-dependent pathway primarily when expressed in the absence of LRP6.

However, in the Wnt/β-catenin pathway, Wnt binds to both Fz and LRP6, and this ternary complex formation is important for the accumulation of β-catenin (Cong et al., 2004). We tested the internalization of Fz5 complexed with LRP6. HeLaS3 cells coexpressing Fz5-FLAG and LRP6-GFP were stimulated with Wnt-3a for 1 hr. Fz5-FLAG was present together with LRP6-GFP in intracellular vesicles (Figure 4A), where EEA1 and caveolin-1 but not clathrin were colocalized (Figure 4B). Therefore, when both Fz5 and LRP6 are activated by Wnt, the complex may be internalized through the caveolin-mediated pathway.

The Complex of Axin and LRP6 Is Internalized

The intracellular domain of LRP6 can bind to Axin and recruit it to the plasma membrane, and the binding of LRP6 and Axin is important for the accumulation of β-catenin (Mao et al., 2001). When FLAG-Axin was overexpressed in the cells, it was observed as intracellular dots, but these dots did not colocalize with clathrin, caveolin-1, EEA1, or Rab5 (Figure S4). Expression of LRP6-GFP induced the recruitment of FLAG-Axin to the plasma membrane only when GSK-3β was further expressed (Figures 5A and 5B). Without exogenous

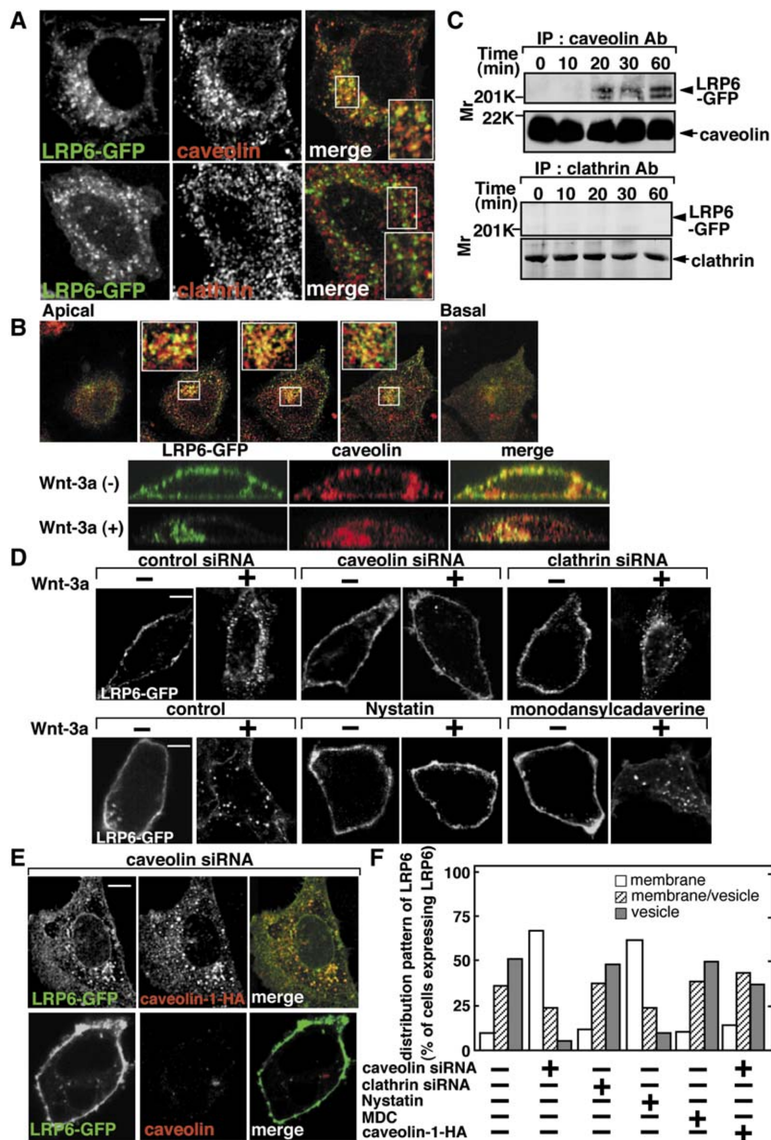

Figure 2. Caveolin Mediates Wnt-3a-Dependent Internalization of LRP6

(A) After HeLaS3 cells expressing LRP6-GFP had been stimulated with Wnt-3a for 1 hr, the cells were stained with anti-caveolin-1 or anti-clathrin antibody. In merged images, LRP6-GFP is shown in green, and caveolin-1 or clathrin is shown in red. Colocalization of LRP6-GFP and caveolin-1 appears as yellow.

(B) HeLaS3 cells expressing LRP6-GFP were left untreated or were stimulated with Wnt-3a for 1 hr, and the cells were stained with anti-caveolin-1 antibody. Upper panel, vertical section series (1 μm steps) from apical to basal in Wnt-3a-stimulated cells; lower panel, side views of the cell stimulated with or without Wnt-3a are merges of 20 vertical sections of confocal stacks.

(C) After HeLaS3 cells expressing LRP6-GFP had been stimulated with Wnt-3a for the indicated periods of time, the lysates were immunoprecipitated with anti-caveolin-1 or anti-clathrin antibody, and the immunoprecipitates were probed with the indicated antibodies.

(D) HeLaS3 cells expressing LRP6-GFP were treated with siRNA for caveolin-1 or clathrin, or cultured with Nystatin or monodansylcadaverine (MDC), and then the cells were stimulated with Wnt-3a for 1 hr.

(E) After caveolin-1 knockdown HeLaS3 cells had been transfected with caveolin-1-HA and LRP6-GFP, the cells were stimulated with Wnt-3a for 1 hr and then stained with anti-HA antibody. When caveolin-1-HA was overexpressed, the cytoplasm was diffusely stained, thereby making the margin of the nucleus clear.

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

expression of GSK-3β, stimulation of the cells with Wnt-3a for 5 min at 37°C was required for the translocation of FLAG-Axin to the plasma membrane (Figure 5B). Both proteins were internalized upon stimulation with Wnt-3a for 1 hr (Figure 5A). The complex of LRP6-GFP and FLAG-Axin colocalized with EEA1 and caveolin-1 but not with clathrin (Figure 5C).

It has been reported that Wnt-3a induces the phosphorylation of LRP6 at Ser1490 and Thr1493, which are important for a binding to Axin (Tamai et al., 2004; Zeng et al., 2005). The LRP6 mutants in which Ser1490 or Thr1493 is mutated to Ala were generated (LRP6SA and LRP6TA). Both LRP6-GFP and LRP6SA-GFP enhanced Wnt-3a-dependent Tcf-4 activation, although the degree of the activation by LRP6SA was slightly lower than that by LRP6 (Figure S5A). Wnt-3a induced the internalization of LRP6SA-GFP (Figure S5B) and LRP6TA-GFP (data not shown) to almost the same extent as that of LRP6-GFP. It has been shown that several serine and threonine residues other than Ser1490 and Thr1493 are phosphorylated by casein kinase I (Davidson et al., 2005; Swiatek et al., 2006). Therefore, even though one phosphorylation site was impaired, the internalization of LRP6 or activation of the β-catenin pathway may not be affected.

Caveolin Induces the Dissociation of β-Catenin from Axin

Since the interaction of LRP6 with Axin is important for the regulation of downstream signaling events, we further investigated the effects of caveolin on the relevant proteins. Expression of either FLAG-Axin or HA-GSK-3β did not induce a mobility shift of LRP6-GFP, but their coexpression induced a marked shift (Figure 6A). This shift was diminished when we used LRP6SA-GFP and abolished by FLAG-Axin-(ΔGSK-3β) (an Axin mutant in which the GSK-3β binding site is deleted) (Hinoi et al., 2000) or the kinase-dead HA-GSK-3β^{K85R} (Figure 6A). The upper band of LRP6-GFP was coprecipitated with FLAG-Axin, and Wnt-3a enhanced this association, while LRP6SA-GFP acted as a hypomorph in this regard (Figure 6B). It is notable that the interaction of LRP6 with

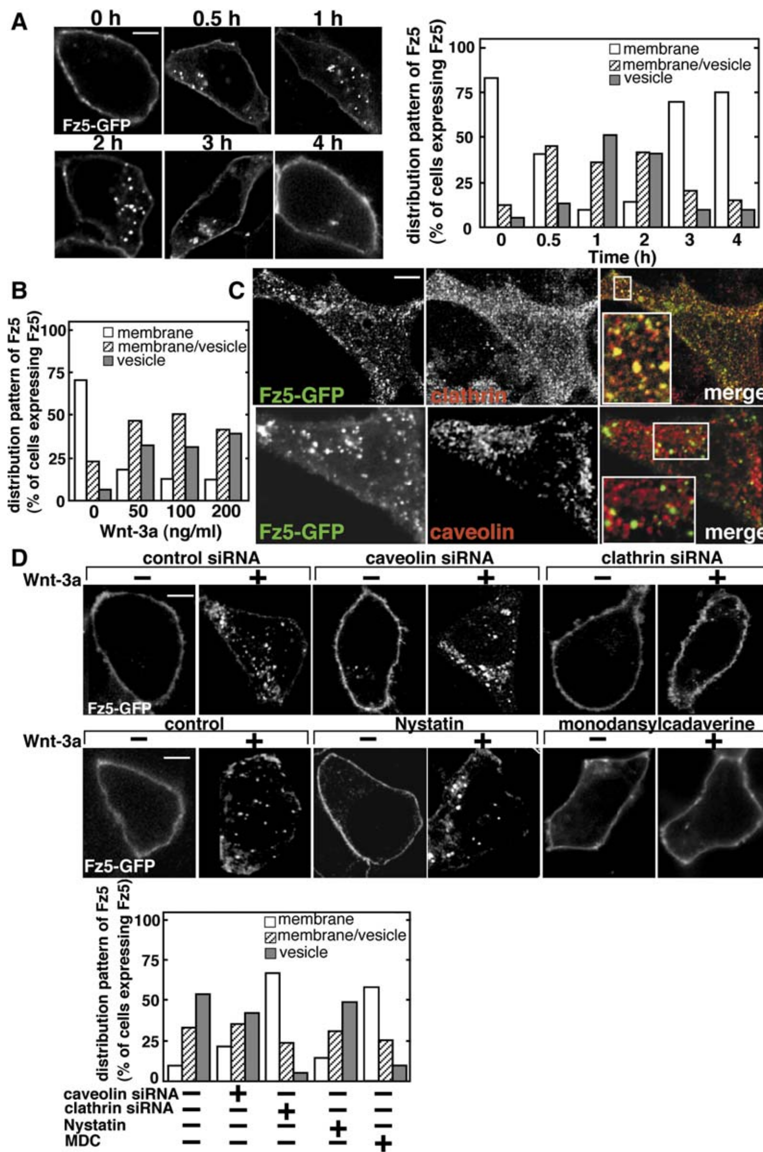

Figure 3. Wnt-3a Induces Fz5 Internalization

(A) HEK293 cells expressing Fz5-GFP alone were stimulated with Wnt-3a for the indicated times, and the fixed cells were directly processed for the microscopy. Left panel, confocal images; right panel, quantification of internalized Fz5-GFP.

(B) HEK293 cells expressing Fz5-GFP were stimulated with the indicated concentration of purified Wnt-3a for 1 hr.

(C) HeLaS3 cells expressing Fz5-GFP were stimulated with Wnt-3a for 1 hr, and then the cells were stained with anti-clathrin or anti-caveolin-1 antibody.

(D) HeLaS3 cells expressing Fz5-GFP were treated with siRNA for caveolin-1 or clathrin or cultured with Nystatin or monodansylcadaverine, and then the cells were stimulated with Wnt-3a for 1 hr. Upper panel, confocal images; lower panel, quantification of internalized Fz5-GFP at 1 hr after stimulation with Wnt-3a. Scale bars, 5 μm.

Axin was not observed without expression of GSK-3β. LRP6-GFP immunoprecipitated from the lysates of HEK293 T cells were phosphorylated in vitro; this phosphorylation was inhibited by SB216763, a GSK-3 inhibitor, and enhanced by recombinant GSK-3β (Figure 6C). These results suggest that GSK-3β bound to Axin phosphorylates at least Ser1490 of LRP6 and that the phosphorylation enhances the binding of Axin to LRP6.

When FLAG-Axin and caveolin-1-HA were expressed in HEK293 T cells, these proteins did not form a complex (Figure 6D). When LRP6-GFP was also expressed, caveolin-1-HA was detected in the FLAG-Axin immunoprecipitates, but the expression of LRP6SA-GFP led to weaker formation of the complex between FLAG-Axin and caveolin-1-HA (Figure 6D). The expression of caveolin-1-HA did not affect the binding of GSK-3β to Axin, but inhibited the binding of β-catenin to Axin (Figure 6E). Therefore, the formation of the complex between caveolin and Axin through phosphorylated LRP6 may induce the release of β-catenin from the Axin complex. This is consistent with the idea that the interaction LRP6 with Axin stablilizes β-catenin. We therefore went on to test the role of caveolin-mediated endocytosis in these events.

Caveolin Is Necessary for the Wnt-3a-Dependent Accumulation of β-Catenin

Disruption of caveolin-mediated endocytosis with siRNA, Nystatin, or Filipin III impaired the Wnt-3a-induced accumulation of β-catenin (Figures 7A and 7B). Moreover, readdition of cholesterol rescued the inhibitory effects of the lipid raft-targeting drugs Nystatin and Filipin III (Figure 7B). It has been shown that the clathrin-dependent internalization of receptors for insulin and EGF is inhibited in cells expressing the ENTH domain of Eps15 (Morinaka et al., 1999; Nakashima et al., 1999). Analogous inhibition of clathrin-mediated endocytosis (siRNA, monodansylcadaverine, or the ENTH domain) did not affect Wnt-3a-induced accumulation of β-catenin (Figures 7A and 7B).

Expression of a dominant-negative form of dynamin (dynamin^{K44A}), which blocks both clathrin- and

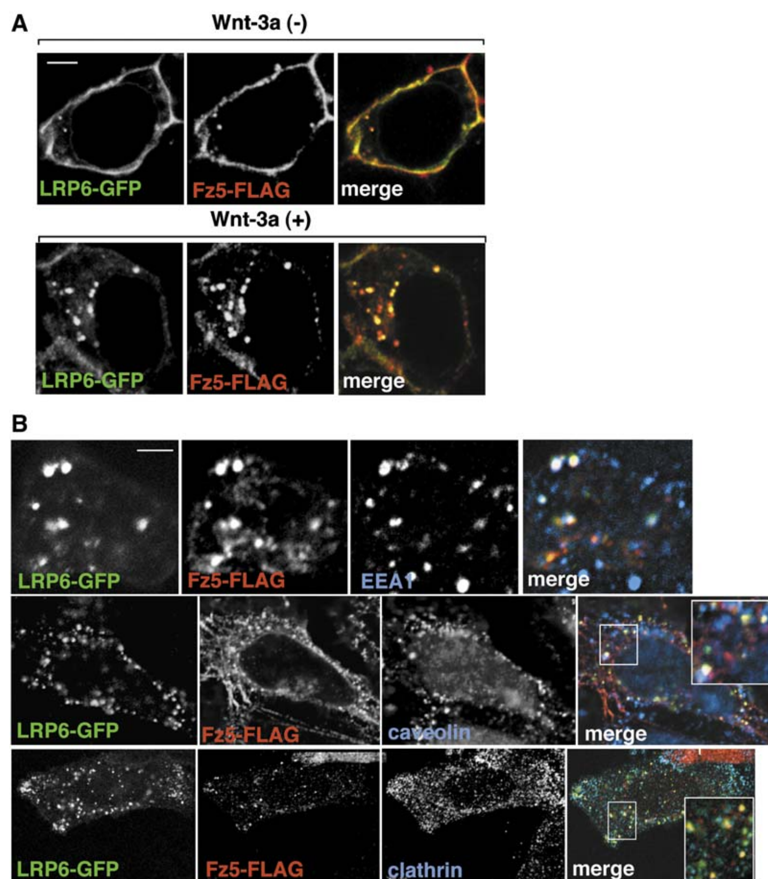

Figure 4. Fz5 Complexed with LRP6 Colocalizes with Caveolin

(A) HEK293 cells coexpressing LRP6-GFP and Fz5-FLAG were left untreated or were stimulated with Wnt-3a for 1 hr, and the cells were stained with anti-FLAG antibody.

(B) HEK293 or HeLaS3 cells coexpressing LRP6-GFP and Fz5-FLAG were stimulated with Wnt-3a for 1 hr, and the cells were stained with anti-FLAG, anti-EEA1, anti-caveolin-1, or anti-clathrin antibody. Colocalization of LRP6-GFP, Fz5-FLAG, and EEA1 or caveolin-1 appears as white. Scale bars, 5 μm.

caveolin-mediated receptor endocytosis (Pelkmans and Helenius, 2002; Sever et al., 2000), inhibited the Wnt-3a-dependent accumulation of β-catenin (Figure 7C), suggesting that at least membrane fission by dynamin is necessary for the Wnt-3a-dependent accumulation of β-catenin. This is consistent with the previously reported observations that expression of a dominant-negative form of shibire, a fly dynamin homolog, disrupts wingless (Wg)-dependent epidermal patterning (Moline et al., 1999). Expression of wild-type Rab5, Rab4, and Rab11 did not affect the Wnt-3a-dependent accumulation of β-catenin (data not shown). $Rab5^{S34N}$ suppressed the accumulation of β-catenin, but $Rab4^{N121I}$ and $Rab11^{S25N}$ had little effect (Figure 7D). These results demonstrate that caveolin- and lipid-raft-related internalization pathways play an important role in the Wnt-3a-dependent accumulation of β-catenin.

Furthermore, caveolin-1-HA stimulated the transcriptional activity of Tcf-4 and enhanced the Wnt-3a-, LRP6-, or Fz5-dependent activation of Tcf-4 (Figure 7E). LRP6 and Fz5 synergistically activated Tcf-4 in the presence of Wnt-3a, and expression of caveolin-1 further enhanced the Tcf-4 activity in the cells expressing these three proteins (Figure 7E). Knockdown of caveolin-1 suppressed Wnt-3a-induced Tcf-4 activation, and expression of caveolin-1 restored this phenotype partially (Figure 7F). Taken together, these results strongly suggest that the endocytic processes are necessary for the activation of the Wnt/β-catenin pathway.

Discussion

Internalization of LRP6 through Caveolin-Mediated Endocytic Pathway

Caveolin, a 22 KDa integral membrane protein, is a major component of caveolae and is important for the formation of caveolae invaginations and the functions of caveolae (Razani et al., 2002). Among caveolin-1, -2, and -3 family proteins, caveolin-1 has been most thoroughly examined. Caveolin-1 interacts with single span transmembrane receptors such as receptors for insulin, TGF-β, and PDGF, and these receptors are internalized through the caveolin-mediated pathway (Le Roy and Wrana, 2005; Razani et al., 2002). We showed that LRP6 is present in the lipid-raft fraction, that LRP6 is colocalized and forms a complex with caveolin-1 in response to Wnt-3a, and that caveolin-1 siRNA and Nystatin reduce the Wnt-3a-dependent internalization of LRP6. Furthermore, we showed that expression of caveolin-1 restores the internalization of LRP6 in the caveolin-1 knockdown cells. Taken together, these results demonstrate that LRP6 is internalized via the caveolin-mediated endocytic pathway.

The trafficking of caveolae to the intracellular organelles has been less extensively documented as compared with that of the clathrin-coated vesicles. SV40 and Cholera toxin (ChTx) bind to a ganglioside and are transported by caveolae to caveosomes (Orlandi and Fishman, 1998; Stang et al., 1997). SV40 is sorted from

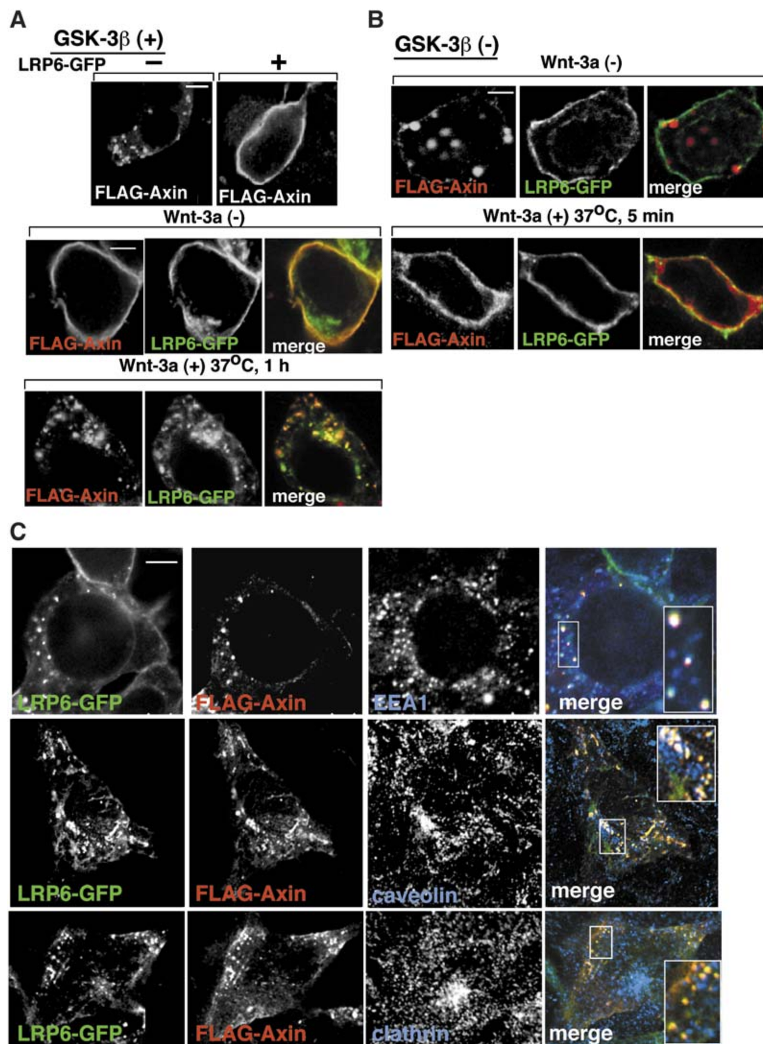

Figure 5. The Complex of Axin to LRP6 Is Internalized

(A) HEK293 cells expressing FLAG-Axin and HA-GSK-3β with or without LRP6-GFP were stained with anti-FLAG antibody. After HEK293 cells expressing FLAG-Axin, HA-GSK-3β, and LRP6-GFP were left untreated or were stimulated with Wnt-3a for 1 hr, the cells were stained with anti-FLAG antibody.

(B) HEK293 cells expressing FLAG-Axin and LRP6-GFP were left untreated or were stimulated with Wnt-3a for 5 min, and the cells were stained with anti-FLAG antibody.

(C) HEK293 or HeLaS3 cells expressing FLAG-Axin, HA-GSK-3β, and LRP6-GFP were stimulated with Wnt-3a for 1 hr, and then the cells were stained with anti-FLAG, anti-EEA1, anti-caveolin-1, or anti-clathrin antibody. Colocalization of LRP6-GFP, FLAG-Axin, and EEA1 or caveolin-1 appears as white. Scale bars, 5 μm.

caveosomes into vesicular and tubular membrane structure that travel to the smooth ER, while ChTx passes through early endosomes and accumulates in the Golgi complex (Pelkmans et al., 2001; Richards et al., 2002). The composition and morphology of early endosomes, where most incoming ligands encounter as the first sorting compartment, reflect the broad functional diversity of endocytic organelles (Zerial and McBride, 2001). Rab5 serves as one of the central organizers of early endosomes and enhances the fusion between endocytic vescicles and early endosomes, and it has in fact been shown that Rab5 is involved in the process of the trafficking of caveolar vesicles from caveosomes to early endosomes (Pelkmans et al., 2004).

We found that LRP6 colocalizes with Rab5 and EEA1 and that a dominant-negative form of Rab5 inhibits the Wnt-3a-dependent internalization of LRP6. These results suggest that caveolar vesicles containing LRP6 move to the early endosomes via a Rab5-dependent process in response to Wnt-3a. However, at present, the molecular mechanism by which Rab5 regulates the trafficking of caveolar vesicles containing LRP6 is not clear. Rab11 is involved in the regulation of the recycling pathway (Sonnichsen et al., 2000). We also showed that internalized LRP6 appears at the cell-surface membrane 4 hr after stimulation with Wnt-3a and that a dominant-negative form of Rab11 blocks this process, suggesting that LRP6 can be recycled at least through a Rab11-dependent recycling pathway. TGF-β receptor internalizes through caveolae associated with rapid receptor degradation (Di Guglielmo et al., 2003). Therefore, we cannot exclude the possibility that a part of LRP6 is transported to lysozome or proteasome and subsequently degraded.

It has been reported that Wnt-5a induces the internalization of Fz4 in a β-arrestin2-dependent manner (Chen et al., 2003). Since β-arrestin2 plays a role in clathrin-dependent endocytosis (Lefkowitz and Shenoy, 2005), Fz4 could be internalized via clathrin-coated vesicles. Consistent with these observations, we found that Wnt-3a induces the internalization of Fz5, which requires clathrin, when Fz5 is expressed without coexpression with LRP6. Although the Wnt-5a-dependent internalization of Fz4 required activation of PKC (Chen et al., 2003), the Wnt-3a-dependent internalization of Fz5 was not dependent on PKC. Therefore, the different Fz receptor proteins may have distinct regulation of endocytosis. However, Fz5 complexed with LRP6 colocalized with caveolin-1 when both proteins were expressed together. Fz5 is known to activate the β-catenin pathway, and LRP6 is essential for the activation of this pathway.

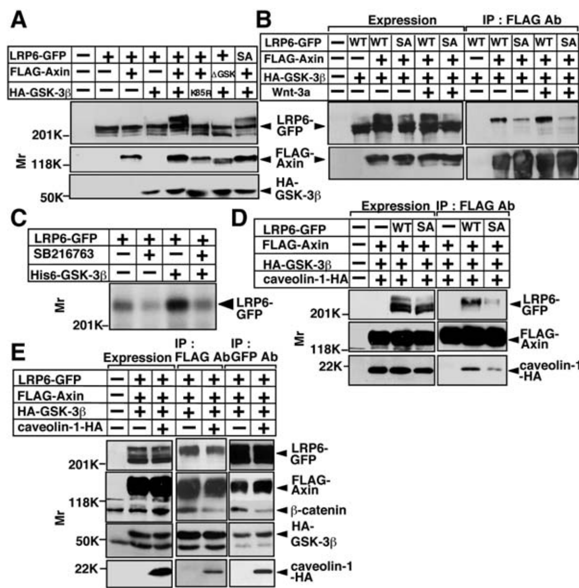

Figure 6. Formation of a Complex between Axin and Caveolin Affects the Axin Complex

(A) The lysates of HEK293 T cells expressing FLAG-Axin, FLAG-Axin (ΔGSK-3β), HA-GSK-3β, HA-GSK-3β^{K85R}, LRP6-GFP, or LRP6SA-GFP as indicated were probed with anti-GFP, anti-FLAG, and anti-HA antibodies. ΔGSK, Axin-(ΔGSK-3β); K85R, GSK-3β^{K85R}.

(B) HEK293 T cells expressing FLAG-Axin, HA-GSK-3β, LRP6-GFP, or LRP6SA-GFP as indicated were left untreated or were stimulated with Wnt-3a for 1 hr. The lysates were probed with the indicated antibody. The same lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were probed with the indicated antibodies. WT, wild-type LRP6; SA, LRP6S1490A.

(C) The lysates of HEK293 T cells expressing LRP6-GFP were immunoprecipitated with anti-GFP antibody. The immunoprecipitates were incubated with or without His6-GSK-3β or SB216763 as indicated in the kinase reaction mixture. The samples were subjected to SDS-PAGE followed by autoradiography.

(D) The lysates of HEK293 T cells expressing FLAG-Axin, HA-GSK-3β, caveolin-1-HA, LRP6-GFP, or LRP6SA-GFP as indicated were probed with the indicated antibody. The same lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were probed with the indicated antibodies.

(E) The lysates of HEK293 T cells expressing FLAG-Axin, HA-GSK-3β, caveolin-1-HA, or LRP6-GFP as indicated were probed with the indicated antibody. The same lysates were immunoprecipitated with anti-FLAG or anti-GFP antibody, and the immunoprecipitates were probed with the indicated antibodies.

Wnt-3a activates not only the β-catenin pathway but also the noncanonical pathway such as the PCP pathway (Habas et al., 2003; Kishida et al., 2004). Therefore, it is intriguing to speculate that when Wnt-3a binds to the Fz5 and LRP6 complex, caveolin is necessary for the internalization of this complex to activate the β-catenin pathway, and that when Wnt-3a binds to Fz5 alone, clathrin is required for the internalization of this complex to activate the noncanonical pathway such as the PCP and Ca^{2+} pathways. It remains to be clarified whether the distinct pathways of endocytosis of the Wnt receptors result in segregation of the different intracellular signaling pathways of Wnt.

Internalization of LRP6 and Its Impact on Wnt/β-Catenin Signaling

Axin is an important protein in the degradation of β-catenin (Ikeda et al., 1998; Kikuchi, 1999; Kishida et al., 1998) and recruited to LRP5/6 in response to Wnt-3a (Mao et al., 2001). In fly embryos, Wg-induced Axin recruitment to the membrane requires disheveled, and in *Xenopus* embryos, the binding of Axin to LRP6 induces the axis duplication (Cliffe et al., 2003; Tamai et al., 2004). The phosphorylation of the cytoplasmic region of LRP6 is important for the binding of Axin to LRP6 (Tamai et al., 2004). It has been reported that GSK-3 and casein kinase I phosphorylate LRP6 and that the membrane association of these protein kinases is important for the activation of the β-catenin pathway (Davidson et al., 2005; Zeng et al., 2005). We also found that GSK-3β bound to Axin phosphorylates LRP6. Since expression of GSK-3β was required for the translocation of Axin to the plasma membrane where LRP6 was present in the absence of Wnt-3a, Wnt-3a may induce the recruitment of Axin through the activation of GSK-3β.

Our results demonstrated that the phosphorylation of LRP6 at Ser1490 or Thr1493 is not essential for the Wnt-3a-induced internalization of LRP6. However, these results do not always mean that the phosphorylation of LRP6 is not important for its internalization, because there are multiple phosphorylation sites other than Ser1490 and Thr1493 (Davidson et al., 2005; Swiatek et al., 2006). Furthermore, since Axin still bound to LRP6SA, although the degree was partially decreased as compared with wild-type LRP6, we cannot exclude the possibility that this interaction of LRP6 with Axin is sufficient for the internalization. Therefore, identification of the phosphorylation sites of LRP6 that are required for the binding to Axin would be necessary to understand how the association of Axin with phosphorylated LRP6 regulates the internalization of LRP6.

We also showed that caveolin-1 siRNA, Nystatin, or a dominant-negative form of Rab5 or dynamin prevents the Wnt-3a-dependent accumulation of β-catenin, indicating that caveolin and lipid rafts are important for the stabilization of β-catenin. Since these treatments inhibited the internalization of LRP6, the results suggest that endocytic processes of LRP6 are important for the Wnt-3a-dependent accumulation of β-catenin. However, these treatments may disrupt Wnt-3a-dependent formation of a complex between Axin and LRP6. At present we cannot conclude that the internalization of LRP6 itself is essential for the Wnt-3a-dependent accumulation of β-catenin. To analyze time-dependent complex formation of Axin, caveolin, and LRP6 at each step of the internalization of LRP6 will clarify how the internalization of LRP6 is involved in the accumulation of β-catenin.

It was reported that Rab5 suppresses the Wnt-3a-responsive reporter activity in HEK293 T cells (DasGupta et al., 2005). Expression of Rab5 indeed partially suppressed the Wnt-3a-dependent activation of Tcf-4, and knockdown of Rab5 enhanced it slightly (Figures S6A and S6B). Furthermore, expression of Rab5 restored this phenotype in the Rab5 knockdown cells (Figure S6B). In addition, expression of Rab5 inhibited β-catenin-induced activation of Tcf-4 (Figure S6A). Rab5 is suggested to regulate gene expression by cycling APPL (*a*daptor protein *P*H domain, *P*TB domain, and *l*eucine zipper motif), a member of the nucleosome remodeling partner, between cytosol and nucleus (Miaczynska et al., 2004). Therefore, Rab5 may regulate the Wnt pathway at two distinct steps, namely, endocytosis of LRP6

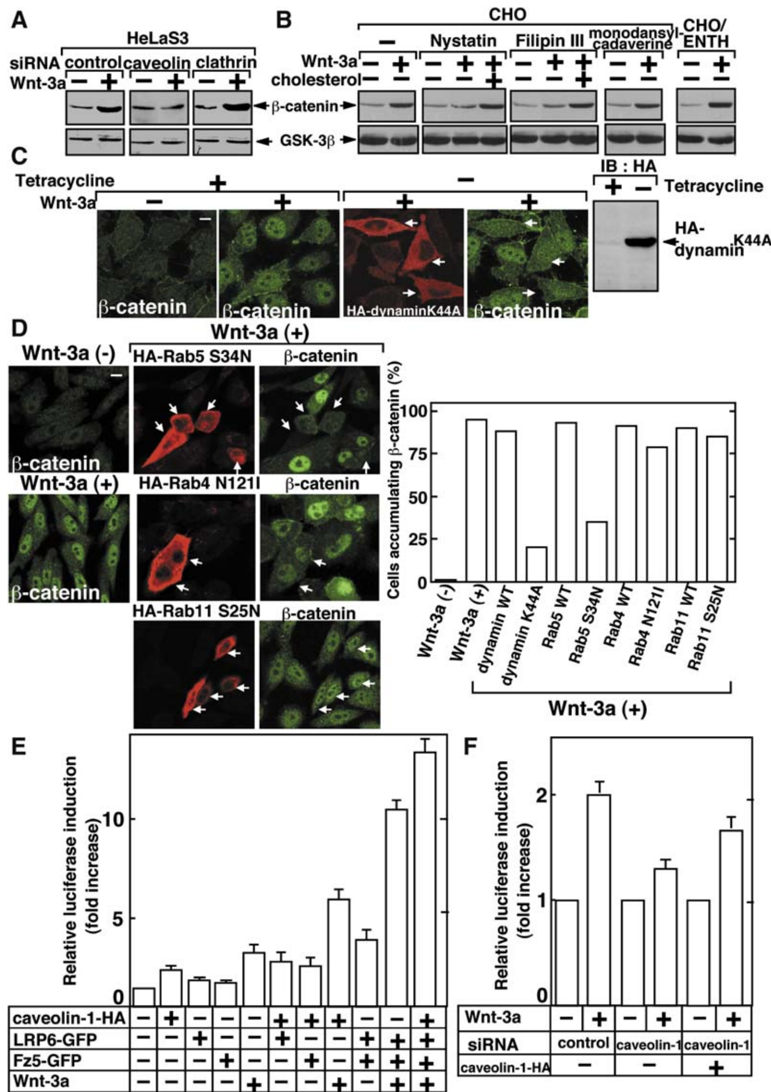

Figure 7. LRP6 Internalization Is Required for Wnt-3a-Dependent Accumulation of β-Catenin

(A) HeLaS3 cells treated with siRNA for caveolin-1 or clathrin were stimulated with Wnt-3a for 1 hr, and the lysates were probed with the indicated antibodies.

(B) CHO cells cultured with Nystatin, Filipin III, or monodansylcadaverine, or CHO cells expressing Myc-ENTH, were stimulated with Wnt-3a for 1 hr. Where indicated, 0.1 mg/ml cholesterol was added to the medium.

(C) After HA-dynamin^{K44A} was expressed in HeLa cells by the removal of tetracycline, the cells were stimulated with Wnt-3a for 1 hr and stained with the indicated antibodies (left panel) or the lysates were probed with anti-HA antibody to detect HA-dynamin^{K44A} (right panel). Arrows indicate the cells expressing HA-dynamin^{K44A}.

(D) CHO cells expressing Rab5^{S34N}, Rab4^{N121I}, or Rab11^{S25N} were left untreated or were stimulated with Wnt-3a for 1 hr, and then the cells were stained with anti-β-catenin and anti-HA antibodies. Right panel, quantification of β-catenin-positive cells in the nucleus in (C) and (D). Arrows indicate the cells expressing Rab small GTPases.

(E) HEK293 T cells were transfected with pcDNA3/caveolin-1-HA, pCS2/LRP6-EGFP, pCS2/Fz5-EGFP, or pPGK-neo/Wnt-3a as indicated and the Tcf-4 activity was measured.

(F) After HeLaS3 cells had been treated with siRNA for caveolin-1, the cells were transfected with pPGK-neo/Wnt-3a with or without pcDNA3/caveolin-1-HA, and then the Tcf-4 activity was measured. The results shown are means ± SE from three independent experiments.

and activity of Tcf-4 downstream of β-catenin. During the course of the review and revision process for this manuscript, two papers describing the relationship between the internalization of Wg and its signaling in fly were published (Rives et al., 2006; Seto and Bellen, 2006). One paper suggested that the internalization of Wg and Arrow (LRP6 homolog) is necessary for the Wg signaling and Wg-dependent gene expression in wing disc, while the other showed that siRNA for Rab5, which inhibits endocytosis of Arrow, enhances Wg-dependent gene expression in cultured cells. The reason why the results vary may be due to the differences of the experiments in which particular parts of the body or cells of a tissue are used. However, our data suggest that Rab5 may have multiple Wnt-related functions in a single cell, e.g., promoting signaling at the level of the internalization of LRP6, while antagonizing it at the level of the complex formation between β-catenin and Tcf or its activity. Thus, we suggest that tissue-specific effects of Rab5 may be attributable to differential reliance on these distinct functions.

Taken together, our results suggest the following model. Wnt-3a triggers the recruitment of the Axin complex to LRP6 and the internalization of LRP6 in a manner dependent on caveolin. GSK-3β bound to Axin enhances the phosphorylation of LRP6, which further increases the binding of Axin to LRP6. On the plasma membrane or in the process of the transport of the LRP6-containing vesicles to the early endosomes, caveolin acts on Axin to inhibit the binding of β-catenin to Axin, thereby leading to the accumulation of β-catenin. Thus, caveolin seems to have critical roles in the activation of the β-catenin pathway in the Wnt signaling. However, mice lacking each of caveolin-1, caveolin-2, or caveolin-3 displayed no overt phenotypic abnormalities (Razani et al., 2002), suggesting that the β-catenin pathway signaling during development does not critically depend on caveolin. These are different from the phenotypes of Wnt-3a or β-catenin knockout mice. Wnt may activate the β-catenin pathway through different endocytic pathway during development. Otherwise, although we showed that caveolin is necessary for Wnt-3a-dependent stabilization of β-catenin, other Wnt ligands that are able to stabilize β-catenin may use the different process. The mechanism that we found may be applied to cell type in which caveolin stimulates the stabilization of β-catenin. Elucidating the exact role of caveolin in

Wnt signaling is therefore an important goal for future work.

Experimental Procedures

Internalization of LRP6-GFP and Fz5-FLAG

HEK293 or HeLaS3 cells were used for the internalization assay. The cells were seeded onto 18 mm glass coverslips coated with poly-D-lysine (Sigma, St. Louis, MO), and pCS2/LRP6-EGFP and pCMV-FLAG/Mesd or pCS2/Fz5-EGFP were transfected into the cells with Lipofectamine 2000 (Invitrogen, Life Technologies, Inc.). At 24–48 hr after transfection, 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 stimulated with Wnt-3a-conditioned medium (100 ng/ml Wnt-3a) (Kishida et al., 1999) for 1 hr at 4°C. We used Wnt-3a conditioned medium in the experiments except Figures 1B and 3B. Purified Wnt-3a proteins (Kishida et al., 2004) were used in Figures 1B and 3B. After unbound Wnt-3a was removed by washing with cold PBS three times, internalization was initiated by adding warm DMEM medium and the dishes were transferred to a heated chamber (37°C, 5% CO_2). When the internalization of LRP6-GFP or Fz5-GFP was observed, the cells were washed three times with cold PBS to stop endocytosis. To examine the effects of cholesterol depletion or clathrin inhibitor on the internalization of LRP6-GFP and Fz5-GFP, HEK293 cells were pretreated with 25 μg/ml Nystatin or 3 μg/ml Filipin III or 100 μM monodansylcadaverine for 1 hr at 37°C before Wnt-3a stimulation.

As another internalization assay, cell surface biotinylation was carried out on HeLaS3 cells (in 35 mm diameter dishes) expressing LRP6-GFP with 0.5 mg/ml sulpho-NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer. After quenching of excess biotin with 50 mM NH_4Cl, the cells were lysed in 0.2 ml TNE buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA-NaOH [pH 8.5]) containing 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 antibody, and the immunoprecipitates were analyzed by immunoblot with anti-GFP antibody (total LRP6) or streptavidin-HRP (cell-surface LRP6).

Quantification of Internalized LRP6 and Fz5

To quantify the internalization of LRP6-GFP and Fz5-GFP, the appearance of the intracellular localization of LRP6 and Fz5 was classified into three types with regard to the distribution of these proteins and the number of puncta in the cytosol. The first type showed clear localization at the cell surface, with a few puncta in the cytosol. The second type showed localization to both the cell surface and puncta in the cytosol. The third type showed the disappearance of the cell surface distribution, with more than 20 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 viewed directly with a confocal microscope (LSM510, Carl-Zeiss, Jena, Germany) to examine LRP6-GFP and Fz5-GFP or incubated with anti-GFP, anti-FLAG, anti-HA, anti-caveolin-1, anti-clathrin, anti-EEA1, anti-Rab5, anti-Rab4, or anti-Axin antibody before confocal microscopy was performed.

Preparation of Lipid-Raft Membranes

HEK293 T cells (in 100 mm diameter dishes) expressing LRP6-GFP were lysed in 0.5 ml of ice-cold TNE buffer containing 1% Triton X-100, 2 μg/ml leupeptin, and 2 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and homogenized with a Dounce homogenizer (40 strokes) and by passage through a 25 gauge needle (Brown and Rose, 1992). Each lysate was mixed with 0.5 ml of 80% (w/v) sucrose in TNE and over layered 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.

siRNA

The following RNA duplexes were used: human clathrin (sense), 5′-CCUGCGGUCUGGAGUCAACTT-3′; human caveolin-1 (sense), 5′-GAGAAGCAAGUGUACGACGTT-3′ and 5′-CCUGAUUGAGAUUCAGUGCTT-3′; human Rab5 (sense), 5′-AAGGCCGACCUAGCAAAUAAA-3′; randomized control (sense), 5′-CAGUCGCGUUUGCGACUGGTT-3′. Double-stranded RNA oligonucleotides were annealed in vitro before transfection. Two RNA duplexes for caveolin-1 were pooled and used. To examine the function of caveolin-1 in caveolin-1 knockdown cells, the caveolin-1 rescue construct was prepared with PCR-based techniques by introducing silent substitutions in the target site of siRNA.

Assay of Tcf-4 Activity

pPGK-neo/Wnt-3a, pCS2/LRP6-EGFP, pCS2/Fz5-EGFP, pUC/EF-1α/β-catenin^{SA} (a constitutively active form of β-catenin), pCI-neo-HA-Rab5, or pcDNA3/caveolin-1-HA was transfected into HEK293 T 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).

Supplemental Data

The Supplemental Data include Experimental Procedures and six figures and are available online at http://www.developmentalcell.com/cgi/content/full/11/2/213/DC1/.

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