Localization of glypican-4 in different membrane microdomains is involved in the regulation of Wnt signaling

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Accepted 2 August 2011
Journal of Cell Science 125, 449–460

doi: 10.1242/jcs.091876

Summary

Glypicans are members of the heparan sulfate proteoglycans (HSPGs) and are involved in various growth factor signaling mechanisms. Although HSPGs affect the β-catenin-dependent and -independent pathways of Wnt signaling, how they regulate distinct Wnt pathways is not clear. It has been suggested that the β-catenin-dependent pathway is initiated through receptor endocytosis in lipid raft microdomains and the independent pathway is activated through receptor endocytosis in non-lipid raft microdomains. Here, evidence is presented that glypican-4 (GPC4) is localized to both membrane microdomains and that the localization affects its ability to regulate distinct Wnt pathways. GPC4 bound to Wnt3a and Wnt5a, which activate the β-catenin-dependent and -independent pathways, respectively, and colocalized with Wnts on the cell surface. LRP6, one of Wnt3a coreceptors, was present in lipid raft microdomains, whereas Ror2, one of Wnt5a coreceptors, was localized to non-lipid raft microdomains. Expression of GPC4 enhanced the Wnt3a-dependent β-catenin pathway and the Wnt5a-dependent β-catenin-independent pathway, and knockdown of GPC4 suppressed both pathways. A GPC4 mutant that was localized to only non-lipid raft microdomains inhibited the β-catenin-dependent pathway but enhanced the β-catenin-independent pathway. These results suggest that GPC4 concentrates Wnt3a and Wnt5a to the vicinity of their specific receptors in different membrane microdomains, thereby regulating distinct Wnt signaling.

Key words: Glypican, Syndecan, Wnt, Membrane microdomain, Endocytosis

Introduction

Heparan sulfate proteoglycans (HSPGs), which are key molecules on the cell surface and in extracellular matrix (ECM), can influence various cellular functions, including growth and differentiation, by interacting with a large number of macromolecules (Bernfield et al., 1999). They consist of a core protein with covalently attached heparan sulfate chains. The glypican (GPC) and syndecan (SDC) families are two key groups among approximately 20 HSPG core proteins. GPCs (GPC1–6) have an N-terminal cyteine-rich domain and a cluster of C-terminal heparan sulfate attachment sites near the membrane anchoring site, which has a glycosylphosphatidylinositol (GPI) anchor (Bernfield et al., 1999; Mythreye and Blobe, 2009). SDCs (SDC1–4) have a region near the N-terminus that bears heparan sulfate chains and a transmembrane domain with C-terminal cytoplasmic region (Bernfield et al., 1999; Couchman, 2003; Tkachenko et al., 2005). Some SDCs are also modified with chondroitin sulfates. The HSPG family has been suggested to act as a reservoir or modulator for several growth factors and signaling molecules and have important roles in animal development (Lin, 2004).

Wnt and Wg are secreted growth factors in vertebrates and *Drosophila* that constitute a large family of cysteine-rich secreted ligands that control animal development (Logan and Nusse, 2004). There are 19 Wnt members, which exhibit unique expression patterns and distinct functions during development in humans and mice. There are at least two Wnt signaling pathways dependent on or independent of β-catenin. A group of Wnts, including Wnt1 and Wnt3a, activate the β-catenin pathway (Bienz and Clevers, 2000; MacDonald et al., 2009). In this pathway, by binding to frizzled (Fz or Fzd) and low density lipoprotein receptor-related protein 6 (LRP6), Wnt3a induces the internalization of receptors through a caveolin-dependent route, which stabilizes cytosolic β-catenin, and then β-catenin accumulating in the cytosol is translocated to the nucleus. In the nucleus β-catenin binds to and activates the transcription factors T-cell factor (Tcf) and lymphoid enhancer factor (Lef), thereby inducing the expression of various genes (Kikuchi et al., 2009; MacDonald et al., 2009). Another group of Wnts, including Wnt5a and Wnt11, activate the β-catenin-independent pathway, which primarily modulates cell movement, as initially observed during embryogenesis (Kikuchi and Yamamoto, 2008; Veeman et al., 2003). In this pathway, by binding to Fz and receptor tyrosine kinase-like orphan receptor 2 (Ror2), Wnt5a induces the internalization of receptors in a clathrin-dependent manner and activates the small G protein Rac (Kikuchi et al., 2009; Sato et al., 2010). In addition, the β-catenin-independent pathway also activates the small G protein Rho and protein kinases such as Jun N-terminal kinase (JNK), Rho-associated kinase (Rho kinase) and protein kinase C, resulting in the coordination of cell motility and polarity.

There are several reports showing that HSPGs modulate Wnt signaling (Fuerer et al., 2010). GPC3 binds to Wnt3a and Wnt7b and enhances Wnt-dependent Tcf activities in hepatocellular

carcinoma cell lines (Capurro et al., 2005b). GPC3 also binds to Wnt5a and enhances Wnt5a-dependent JNK activation in mesothelioma cells (Song et al., 2005). GPC3 knockout mice embryos exhibit a reduction in JNK activity (Song et al., 2005). Furthermore, in zebrafish Knypek (also known as GPC4 and GPC6) binds to Dickkopf1 (Dkk1), a negative regulator of Wnt signaling, and potentiates the activity of Dkk1 to activate JNK (Caneparo et al., 2007). *Xenopus* GPC4 interacts with Wnt11 and enhances Wnt-stimulated cell migration in convergent extension (Ohkawara et al., 2003). These results suggest that glypicans activate Wnt signaling. However, it has also been reported that the expression of GPC3 represses Wnt1-dependent Tcf activity and Wnt5a-dependent JNK activity in CHO cells (De Cat et al., 2003). GPC3 knockout mice embryos have elevated levels of cytoplasmic β-catenin (Song et al., 2005). Therefore, the GPC family might serve as a positive or negative regulator of both the β-catenin-dependent and -independent pathways.

A transmembrane type of HSPG, SDC, has also been reported to regulate Wnt signaling. Mice with mammary-gland-specific transgenic expression of Wnt1 develop mammary tumors, and SDC1 is required for Wnt1-induced tumorigenesis and Tcf transcriptional activation (Alexander et al., 2000; Liu et al., 2003). SDC4 has been shown to bind to Fz7 and Dvl and to be required for convergent extension in *Xenopus* (Munoz et al., 2006). Expression of SDC1 and SDC4 correlates with Wnt5a expression in melanoma cell lines, and knockdown of SDC1 and SDC4 decreases cell invasion activity, which is restored by Wnt5a (O'Connell et al., 2009). Thus, whether the action of HSPG is positive or negative for the β-catenin-dependent or -independent pathway might in part be dependent on the type of cell or tissue. However, the detailed mechanism by which HSPGs regulates distinct Wnt signaling pathways remains to be clarified.

The *GPC4* gene has been mapped to Xq26, in close proximately to *GPC3*, in the human genome, and its mRNA is expressed in most but not all adult human tissues (Veugelers et al., 1998). Functional disruption of *GPC4* orthologs in *Xenopus* and zebrafish has suggested that GPC4 is involved in convergent extension movement through the β-catenin-independent pathway (Caneparo et al., 2007; Ohkawara et al., 2003). However, it is not clear whether GPC4 affects the β-catenin-dependent pathway in mammalian cells and if so, how GPC4 regulates both pathways selectively is not known. In this study, we show that GPC4 is present in both lipid raft and non-lipid raft microdomains and that its localization in lipid raft microdomains is required for the Wnt3a–β-catenin pathway, and its presence in non-lipid raft microdomains enhances Wnt5a–β-catenin-independent pathway.

Results

GPC4 enhances the Wnt3a–β-catenin pathway

GPC mRNAs were expressed in HEK293 and HeLaS3 cells at various levels (supplementary material Fig. S1A). To examine the roles of GPCs in Wnt signaling, siRNAs for GPCs were transfected into HEK293 cells. siRNA reduced the mRNA level of each *GPC* substantially (supplementary material Fig. S1B) and did not show off-target effects on other *GPC*s (data not shown). Wnt3a-dependent phosphorylation of LRP6 at Ser1490 is essential for activation of the β-catenin pathway (MacDonald et al., 2009; Zeng et al., 2005). Knockdown of either GPC3 or GPC4 in HEK293 cells reduced Wnt3a-dependent phosphorylation of LRP6 at Ser1490 (Fig. 1A). Knockdown of GPC1, GPC5 and GPC6 did not affect the phosphorylation of LRP6 (Fig. 1A). By contrast, transient expression of haemagglutinin (HA)-tagged GPC4 (HA–GPC4) in HEK293 cells enhanced Wnt3a-dependent phosphorylation of LRP6 (Fig. 1B), demonstrating that HA–GPC4 reduces the dose of Wnt3a necessary for the phosphorylation of LRP6. Furthermore, HA–GPC4 enhanced Wnt3a- and LRP6-dependent activation of Tcf transcriptional activity in HEK293T cells (Fig. 1C). Both gain- and loss-of-function assays suggested that GPC4 is involved in activation of the β-catenin pathway in these cells.

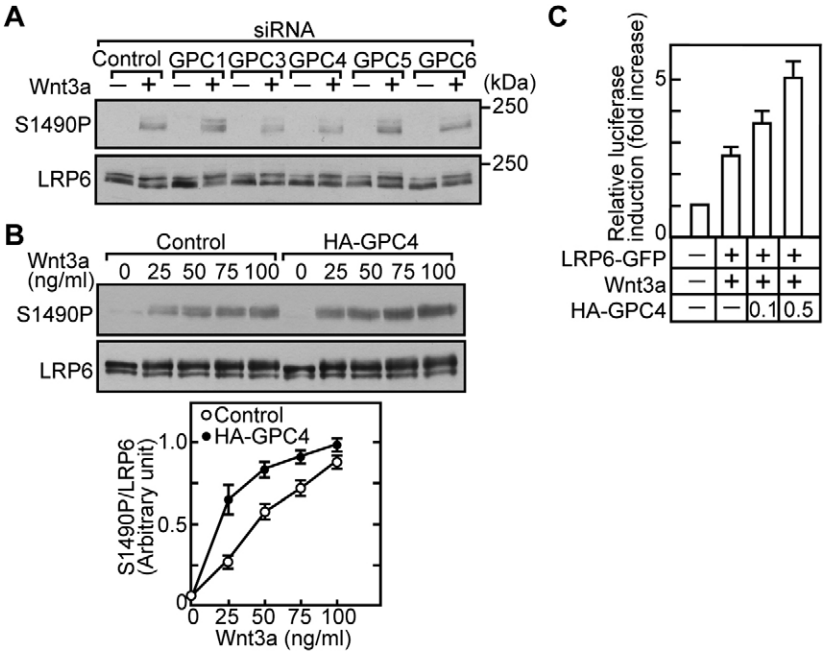

Fig. 1. GPC4 enhances the Wnt3a–β-catenin pathway. (**A**) HEK293 cells transfected with the indicated siRNAs were treated with 100 ng/ml Wnt3a for 1 hour. The lysates were probed with the indicated antibodies. S1490P, anti-phosphorylated Ser1490 LRP6 antibody. (**B**) Top: HEK293 cells transiently expressing HA–GPC4 were treated with the indicated concentration of Wnt3a for 1 hour. The lysates were probed with the indicated antibodies. Bottom: the amounts of phosphorylated LRP6 and total LRP6 were quantified using NIH Image software. The ratio of phosphorylated LRP6 to total LRP6 was calculated and expressed as arbitrary units. The results shown are means ± s.e.m. from three independent experiments. (**C**) HEK293T cells were transfected with pCS2/LRP6–GFP, pPGK-neo/Wnt3a, and 0.1 μg or 0.5 μg pCAG/HA–GPC4 as indicated. The luciferase activity was measured and expressed as the fold increase compared with the levels observed in cells transfected with TOP-*fos*-*Luc* and pEF-BOS/hTcf-4E only. The results shown are means ± s.e.m. from three independent experiments.

The chimera of GPC4 and the transmembrane domain of SDC1 suppresses the Wnt3a–β-catenin pathway

We further analyzed the roles of GPC4 in the activation of the β-catenin pathway using HeLaS3 cells stably expressing HA–GPC4 (supplementary material Fig. S2A), because HeLaS3 cells showed a low expression level of GPC4 (supplementary material Fig. S1A). It has been reported that LRP6 is present in detergent-resistant microdomains (DRMs) and non-DRMs and that Wnt3a-dependent phosphorylation of LRP6 occurs only in DRMs (Sakane et al., 2010). Lipid raft microdomains are defined as "small, highly dynamic, sterol- and sphingolipid-enriched membrane domains that compartmentalize cellular processes" (Pike, 2006). Although DRMs do not necessarily correspond to lipid raft microdomains in cells, detergent insolubility is a valuable and widely used method for identifying proteins that are localized to lipid raft microdomains and are likely to function there. In this paper, DRMs are considered to be the same as lipid raft microdomains. It is generally believed that GPCs localize to the lipid raft microdomains of the cell surface by a GPI anchor (Bernfield et al., 1999; Mythreye and Blobe, 2009). HA–GPC4 was present in both DRMs and non-DRMs (Fig. 2A). This could reflect endogenous GPC4 distribution, because endogenous GPC1 and GPC3 were also detected in both microdomains in C2H2 myoblasts (Gutierrez and Brandan, 2010) and HepG2 cells, respectively (Fig. 2B). SDCs contain a transmembrane region and SDC1–HA was observed only in non-DRMs (Fig. 2A). To change the distribution of GPC4, we generated a chimera of HA–GPC4/SDC1 in which the GPI anchor site was removed from GPC4 and the transmembrane and cytoplasmic domains of SDC1 were fused to the C-terminal end of truncated GPC4, and this chimeric protein was expressed stably in HeLaS3 cells (supplementary material Fig. S2A). As expected, HA–GPC4/SDC1 was present in non-DRMs but not in DRMs (Fig. 2A).

As in HEK293 cells, the expression of HA–GPC4 in HeLaS3 cells enhanced Wnt3a-dependent phosphorylation of LRP6 but expression of HA–GPC4/SDC1 inhibited it (Fig. 2C). HA–GPC4 also stimulated Wnt3a-dependent internalization of LRP6 (Fig. 2D and supplementary material Fig. S2B) but HA–GPC4/SDC1 suppressed it (Fig. 2D). *Axin2* is a known target gene of the β-catenin pathway, and Wnt3a increased the levels of *Axin2* mRNA in HeLaS3 cells stably expressing HA–GPC4 more efficiently than in control cells (Fig. 2E). HA–GPC4/SDC1 suppressed the expression levels of *Axin2* mRNA (Fig. 2E). These studies were done using two (HeLaS3 cells expressing HA–GPC4/SDC1) or three (HeLaS3 cells expressing HA–GPC4) different clones and similar results were obtained in each case (data not shown).

The cytoplasmic region of SDCs has been reported to interact with some signaling molecules (Shepherd et al., 2010; Tkachenko et al., 2005). To exclude the possibility that this region affects the Wnt3a–β-catenin pathway, HA–GPC4/SDC1ΔC, in which the cytoplasmic region of SDC1 was deleted, was expressed stably in HeLaS3 cells. HA–GPC4/SDC1ΔC was localized to non-DRMs only (supplementary material Fig. S3A). Similar to HA–GPC4/SDC1, expression of HA–GPC4/SDC1ΔC suppressed Wnt3a-dependent phosphorylation of LRP6 and the increase in the levels of *Axin2* mRNA (supplementary material Fig. S3B,C). These results clearly show that the effects of HA–GPC4/SDC1 are independent of the cytoplasmic region of SDC1. Taken together, GPC4 allows Wnt3a to induce the phosphorylation and internalization of LRP6 efficiently to activate the β-catenin pathway when it is located to DRMs.

Inhibitory effects of Dkk1 and Wnt5a on the Wnt3a–β-catenin pathway depend on its ability to bind to GPC4

Xenopus GPC4 and zebrafish Knypek were reported to bind to Wnt11 and Dkk1, respectively (Caneparo et al., 2007; Ohkawara et al., 2003). To analyze whether human GPC4 binds to Wnts and Dkk1, HA–GPC4 immunoprecipitated from HeLaS3 cell expressing HA–GPC4 was incubated with purified Wnt3a, Wnt5a and Dkk1. Wnt5a bound to HA–GPC4 more efficiently than Wnt3a (Fig. 3A). The binding of Dkk1 to GPC4 was undetectable under conditions where Dkk1 binds to LRP6 (Fig. 3A and supplementary material Fig. S4). HA–GPC4/SDC1 also bound to Wnt3a and Wnt5a as well as HA–GPC4 (Fig. 3A). As a control, Wnt3a and Wnt5a did not bind to the immunocomplex from wild-type HeLaS3 cells (data not shown).

When the dorsal surface of HeLaS3 cells was observed using a confocal microscopy, Wnt5a and Wnt3a appeared as small puncta on the cell surface (supplementary material Fig. S5). Coexpression of HA–GPC4 did not affect the localization of Wnt3a and Wnt5a, and HA–GPC4 was colocalized with these Wnts (Fig. 3B). In the middle section, Wnt3a and Wnt5a were detected on cell membranes where HA–GPC4 was also observed. Therefore, Wnt3a and Wnt5a bind to GPC4 on the cell surface.

Dkk1 acts as a negative regulator of Wnt–β-catenin signaling by moving LRP6 from the lipid raft microdomains to non-lipid raft microdomains and inducing the internalization of LRP6 in a clathrin-dependent manner (Niehrs, 2006; Sakane et al., 2010; Yamamoto et al., 2008). Dkk1 inhibited the Wnt3a-dependent expression of *Axin2* mRNA in wild-type HeLaS3 cells and HeLaS3 cells expressing HA–GPC4, but a higher dose of Dkk1 was required to suppress it in the latter cells (Fig. 3C). These results suggest that GPC4 binds to Wnt3a more than Dkk1 on the cell surface membrane, thereby preventing the relocation of LRP6 to non-lipid raft microdomains from lipid raft microdomains by Dkk1.

Wnt5a was shown to inhibit Wnt3a-dependent activation of the β-catenin pathway by competing with Wnt3a for binding to Fz2 (Sato et al., 2010). Wnt5a suppressed the Wnt3a-dependent expression of *Axin2* mRNA more effectively in HeLaS3 cells expressing HA–GPC4 than in control cells (Fig. 3D). The difference between the inhibitory effects of Dkk1 and Wnt5a could be based on their binding affinity to HA–GPC4.

GPC4 enhances Wnt5a–β-catenin-independent pathway

In the β-catenin-independent pathway, Wnt5a activates Rac through the internalization of Fz2 in a clathrin-dependent manner (Sato et al., 2010). Knockdown of GPC4 in HEK293 cells decreased Wnt5a-dependent activation of Rac (Fig. 4A). Wnt5a activated Rac in HeLaS3 cells stably expressing HA–GPC4 more than in wild-type cells (Fig. 4B). In contrast to Wnt3a-dependent phosphorylation of LRP6, expression of HA–GPC4/SDC1 or HA–GPC4/SDC1ΔC also enhanced Wnt5a-dependent activation of Rac as well as HA–GPC4 (Fig. 4B and supplementary material Fig. S3D).

Dvl is an important protein for the activation of both the β-catenin-dependent and -independent pathways (Wharton, 2003). It has been shown that Dvl is phosphorylated in the presence of Wnt3a and Wnt5a (Gonzalez-Sancho et al., 2004; Takada et al., 2005) although the physiological relevance has not been clarified. Two bands of Dvl2 were observed in the lysates of HeLaS3 cells: the upper band was a phosphorylated form, because it disappeared after treatment with alkaline phosphatase (Fig. 4C).

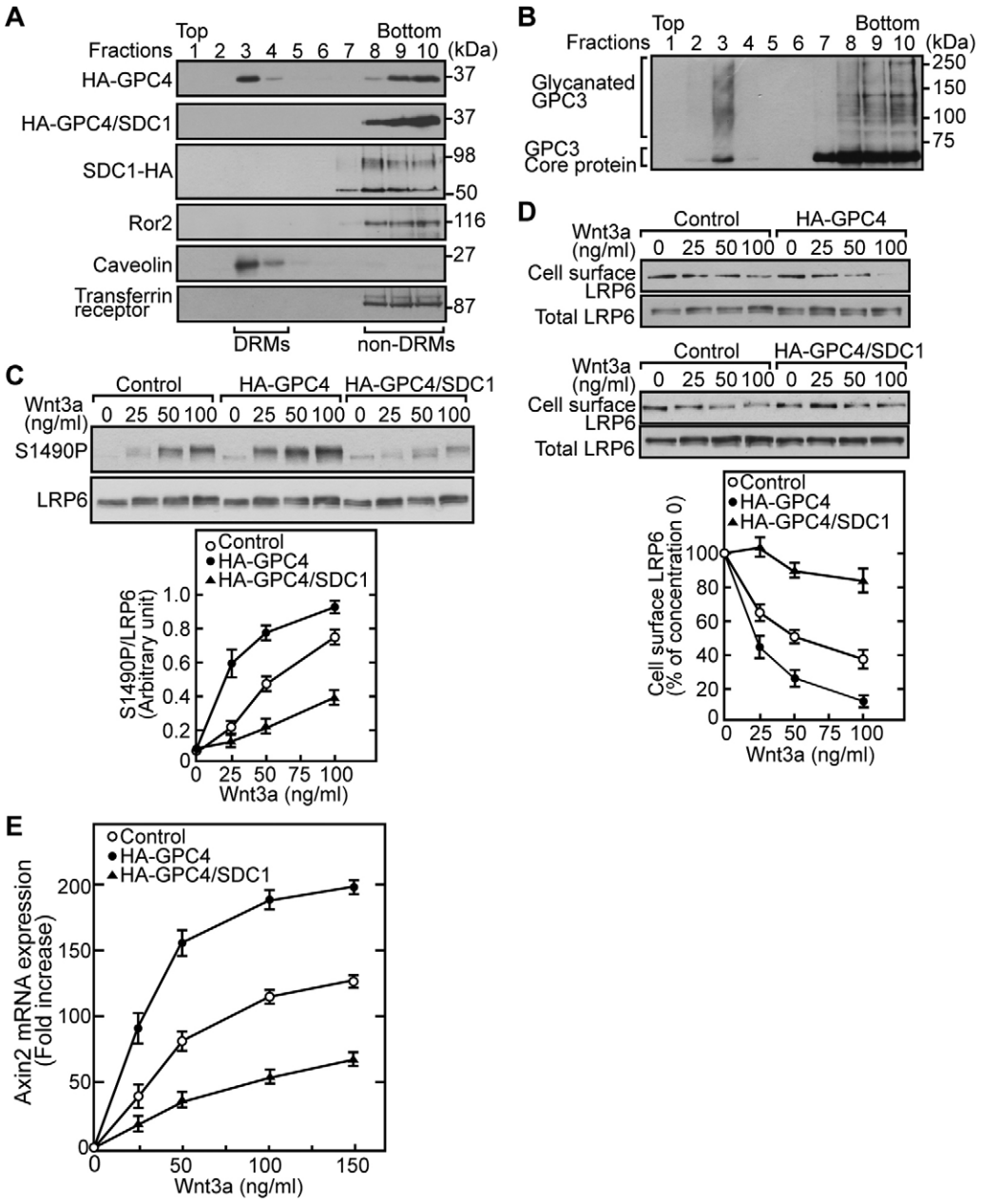

Fig. 2. The chimera of GPC4 and SDC1 suppresses the Wnt3a–β-catenin pathway. (**A**) Lysates of HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1, HEK293T cells transiently expressing SDC1–HA and control HeLaS3 cells were fractionated by sucrose density gradient centrifugation, and aliquots of the lysates were probed with anti-HA, anti-Ror2, anti-caveolin and anti-transferrin receptor antibodies. (**B**) Lysates of HepG2 cells were fractionated by sucrose density gradient centrifugation, and aliquots of the lysates were probed with the anti-GPC3 antibody. (**C**) Top: HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 and control HeLaS3 cells were stimulated with the indicated concentration of Wnt3a for 1 hour. The lysates were probed with the indicated antibodies. Bottom: the amounts of phosphorylated LRP6 and total LRP6 were quantified using NIH Image software. The ratio of phosphorylated LRP6 to total LRP6 was calculated and expressed as arbitrary units. The results shown are means ± s.e.m. from three independent experiments. (**D**) Top: HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 and control HeLaS3 cells were stimulated with the indicated concentration of Wnt3a for 1 hour. After cell-surface biotinylation using sulfo-NHS-LC-biotin, the lysates were precipitated using neutravidin-agarose beads, and the precipitates were probed with the anti-LRP6 antibody. The whole lysates were probed with the anti-LRP6 antibody to detect total LRP6. Bottom: the amounts of cell surface or total LRP6 were quantified using NIH Image software. The results are shown as the ratio of cell surface LRP6 to total LRP6. Values for control assays without Wnt3a were set to 100%. The results shown are means ± s.e.m. from three independent experiments. (**E**) HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 and control HeLaS3 cells were stimulated with the indicated concentration of Wnt3a for 8 hours. Total RNA was extracted from these cells and quantitative RT-PCR analyses for *Axin2* mRNA expression were performed. The results shown are means ± s.e.m. from three independent experiments.

Knockdown of Wnt5a reduced the level of the upper band and the addition of Wnt5a rescued this phenotype (Fig. 4C), indicating that phosphorylation of Dvl2 at endogenous levels depends on Wnt5a. The levels of the upper bands increased in HeLaS3 cells stably expressing HA–GPC4, HA–GPC4/SDC1 or HA–GPC4/SDC1ΔC (Fig. 4C and supplementary material Fig. S3E).

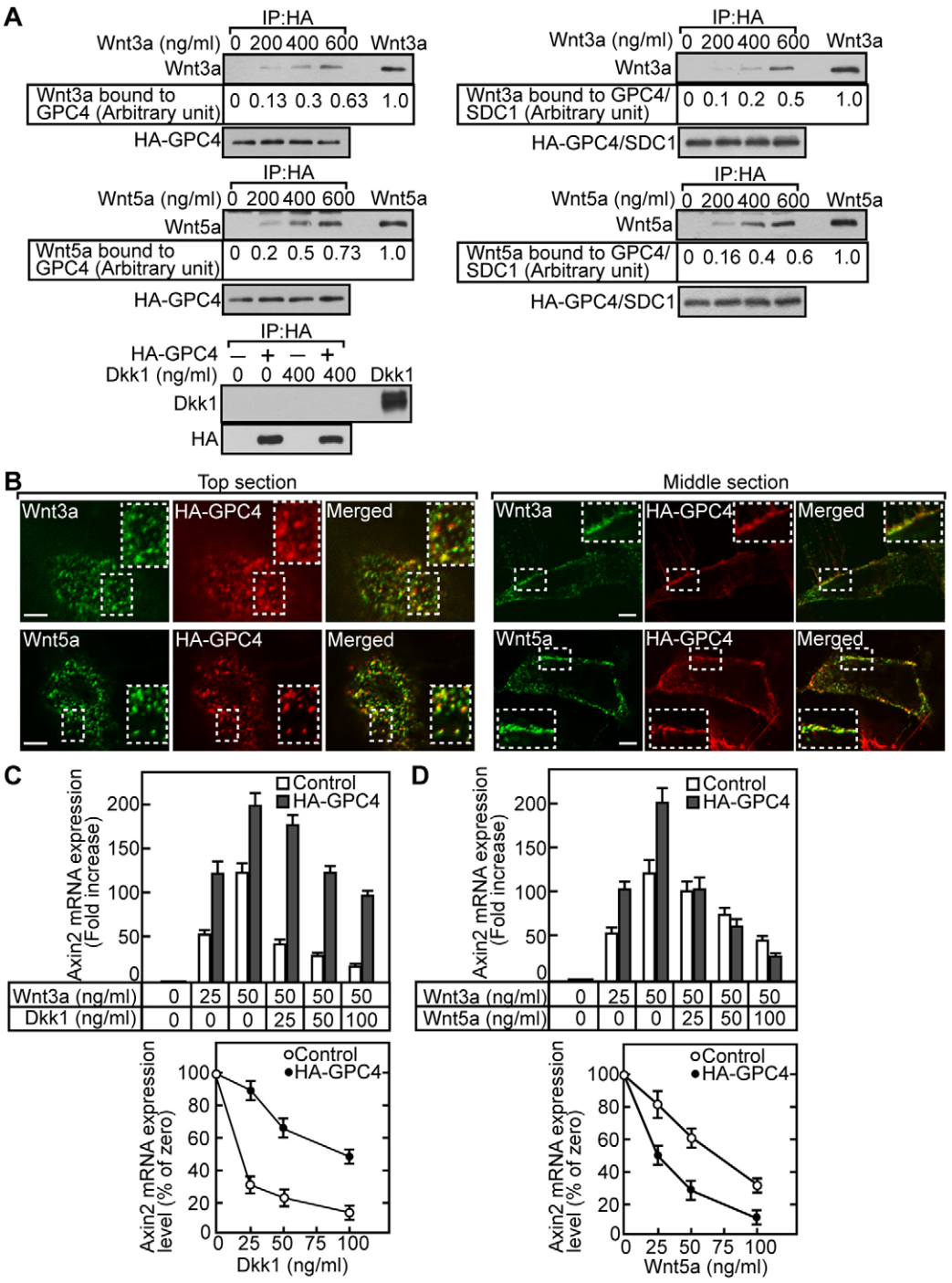

Fig. 3. GPC4 affects the inhibition of the Wnt3a–β-catenin pathway by Dkk1 and Wnt5a. (**A**) Lysates of HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1were immunoprecipitated with the anti-HA antibody. Immunoprecipitates were incubated with the indicated concentration of purified Wnt3a, Wnt5a or Dkk1–FLAG for 2 hours at 4℃ and then probed with anti-Wnt3a, anti-Wnt5a/b, anti-HA or anti-FLAG antibodies. The amount of Wnt3a or Wnt5a bound to HA–GPC4 or HA–GPC4/SDC1 was analyzed using NIH Image software. Each signal intensity was expressed as an arbitrary unit compared with that of 10 ng of purified Wnt3a, Wnt5a or Dkk1. (**B**) HeLaS3 cells were transfected with pCAG/HA-GPC4 and pCAG/Wnt3a or pCAG/Wnt5a, and then the cells were stained with anti-HA and anti-Wnt3a or anti-Wnt5a/b antibodies without permeabilization to detect HA–GPC4 and Wnts on the cell surface. The regions in the white boxes are shown enlarged in the insets. Scale bars: 5 μm. (**C**) Top: HeLaS3 cells stably expressing HA–GPC4 or control HeLaS3 cells were treated with the indicated concentration of Wnt3a and Dkk1 for 8 hours. Total RNA was extracted from these cells, and quantitative RT-PCR analyses for *Axin2* mRNA expression were performed. Bottom: relative *Axin2* mRNA expression of cells treated with 50 ng/ml Wnt3a in the presence of the indicated concentration of Dkk1. Values for assays in the absence of Dkk1 were set to 100%. The results shown are means ± s.e.m. from three independent experiments. (**D**) Top: HeLaS3 cells stably expressing HA–GPC4 or control HeLaS3 cells were treated with the indicated concentration of Wnt3a and Wnt5a for 8 hours. Total RNA was extracted from these cells, and quantitative RT-PCR analyses for *Axin2* mRNA expression were performed. Bottom: relative *Axin2* mRNA expression of the cells treated with 50 ng/ml Wnt3a in the presence of the indicated concentration of Wnt5a. Values for assays in the absence of Wnt5a were set to 100%.

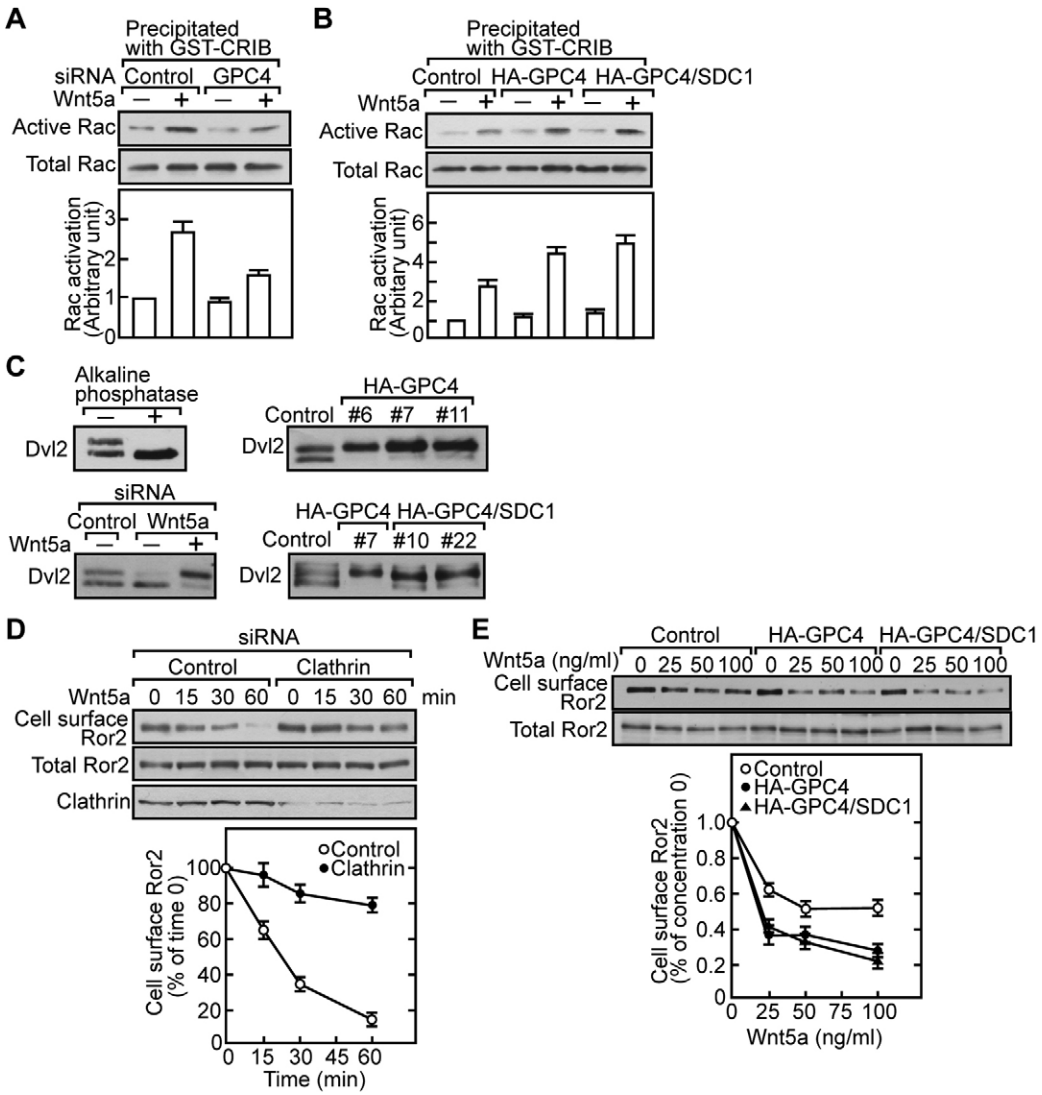

Fig. 4. GPC4 in non-lipid raft microdomains plays a role in the activation of the Wnt5a–β-catenin-independent pathway. (**A**) After HEK293 cells transfected with the indicated siRNA were treated with 50 ng/ml Wnt5a for 30 minutes, the lysates of cells were incubated with GST–CRIB immobilized on glutathione–Sepharose. Total lysates (total Rac) and precipitates (active Rac) were probed with anti-Rac1 antibody. The signals of active Rac were quantified using NIH Image software and expressed as arbitrary units compared with the signal intensity in control cells without Wnt5a stimulation. The results shown are means ± s.e.m. from three independent experiments. (**B**) HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 were treated with 50 ng/ml Wnt5a for 30 minutes, and then the cells were subjected to the Rac activation assay. (**C**) Left, top: lysates of HeLaS3 cells were immunoprecipitated with the anti-Dvl2 antibody. The immunoprecipitates were treated with alkaline phosphatase and then probed with the anti-Dvl2 antibody. Left, bottom: HeLaS3 cells transfected with the indicated siRNAs were treated with 100 ng/ml Wnt5a for 3 hours. The lysates were probed with the anti-Dvl2 antibody. Right: the lysates of HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 were probed with the anti-Dvl2 antibody. (**D**) Top: HeLaS3 cells transfected with clathrin siRNA were treated with 100 ng/ml Wnt5a for the indicated time periods. After cell-surface biotinylation using sulfo-NHS-LC-biotin, the lysates were precipitated using neutravidin–agarose beads, and the precipitates were probed with the anti-Ror2 antibody. The whole lysates were probed with the anti-Ror2 antibody to detect total Ror2. Bottom: the amounts of cell surface or total Ror2 were quantified using NIH Image software. The results are shown as the ratio of cell surface Ror2 to total Ror2. Values for assays in the absence of Wnt5a were set to 100%. The results shown are means ± s.e.m. from three independent experiments. (**E**) Top: HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1, and control HeLaS3 cells were treated with the indicated concentrations of Wnt5a for 30 minutes. After cell-surface biotinylation using sulfo-NHS-LC-biotin, the lysates were precipitated using neutravidin–agarose beads, and the precipitates were probed with the anti-Ror2 antibody. The whole lysates were probed with the anti-Ror2 antibody to detect total Ror2. Bottom: the amounts of cell surface or total Ror2 were quantified using NIH image software. The results are shown as the ratio of cell surface Ror2 to total Ror2. Values for assays in the absence of Wnt5a were set to 100%.

As shown in Fig. 2A, endogenous Ror2, a Wnt5a receptor, was observed in non-DRMs but not in DRMs in HeLaS3 cells. Wnt5a induced the internalization of Ror2 in a time- and dose-dependent manner (Fig. 4D,E). Knockdown of clathrin impaired the internalization (Fig. 4D), which was consistent with Wnt5a-dependent Fz2 internalization requiring clathrin (Sato et al., 2010). Expression of HA–GPC4 and HA–GPC4/SDC1 enhanced Wnt5a-dependent internalization of Ror2 at similar efficiency (Fig. 4E).

Taken together, these results suggest that GPC4 enhances Wnt5a signaling when it is localized to non-lipid raft microdomains.

The chimera of GPC4 and the transmembrane domain of EGF receptor affects Wnt signaling

To confirm that the localization of GPC4 on membrane microdomains is important for regulating Wnt signaling, the amino acid sequence containing the transmembrane region of epidermal growth factor receptor (EGFR) was fused to the C-terminal end of truncated GPC4 (a GPI anchor-binding motif is deleted), and this chimera protein was stably expressed in HeLaS3 cells (supplementary material Fig. S6A). It was shown that the juxtamembrane region [amino acids (aa) 581–641] of EGFR targets its transmembrane domain (aa 646–668) to DRMs and that removal of the juxtamembrane region shifts the transmembrane domain of EGFR to non-DRMs (Yamabhai and Anderson, 2002). A small proportion of GPC4/EGFR(581–668) was indeed observed in the DRMs, but most of it was detected in non-DRMs (supplementary material Fig. S6A). All of GPC4/EGFR(646–668) was present in non-DRMs. Thus, it was difficult to target GPC4 to DRMs. Consistent with the results using GPC4/SDC1, expression of GPC4/EGFR(646–668) suppressed Wnt3a-dependent phosphorylation of LRP6 and increase in the levels of *Axin2* mRNA (supplementary material Fig. S6B,C). Furthermore, expression of GPC4/EGFR(646–668) enhanced Wnt5a-dependent activation of Rac and phosphorylation of Dvl2 (supplementary material Fig. S6D,E). Taken together, these results strongly support our conclusion that localization of GPC4 to DRMs is required for its ability to enhance the Wnt3a–β-catenin pathway and that localization of GPC4 to non-DRMs is involved in the activation of the Wnt5a–β-catenin-independent pathway.

GPC4 is internalized with Wnt receptors

It has been reported that Wnt3a and Wnt5a induce the internalization of Fz2, which is required for their activation of specific signaling pathways (Sato et al., 2010). GPC3 was shown to interact with hedgehog but not with its receptor, patched, and GPC3 was internalized with hedgehog, thereby suppressing its signaling (Capurro et al., 2008). HA–GPC4 was also internalized in response to Wnt3a and Wnt5a and it was colocalized with internalized Fz2 (Fig. 5A). It was also shown that Wnt3a induces the internalization of LRP6 through a caveolin-mediated route (Yamamoto et al., 2006) and that Wnt5a induces the internalization of Ror2 through a clathrin-mediated route (Fig. 4D). At 30 minutes after Wnt3a stimulation, HA–GPC4 was colocalized with LRP6–GFP and caveolin but not with clathrin (Fig. 5B). By contrast, HA–GPC4 internalized by Wnt5a stimulation was colocalized with clathrin but not with caveolin (Fig. 5C). These results suggest that GPC4 is internalized with Wnt receptors through a ligand-specific route.

Processed GPC4 modulates Wnt signaling

It is known that the ectodomains of HSPGs are shed into the extracellular space (Bernfield et al., 1999). A soluble version of GPC4, a GPC4 ectodomain, was detected in small amounts in the conditioned medium from HEK293 or HeLaS3 cells expressing GPC4 (Fig. 6A), and the conditioned medium did not affect Wnt signaling (data not shown). We found that the GPC4 ectodomain is released more abundantly from MDCK cells stably expressing HA–GPC4 compared with HEK293 and HeLaS3 cells (Fig. 6A). GPC4 ectodomain bound to Wnt3a and Wnt5a with similar efficiency (Fig. 6B). The addition of conditioned medium, containing the HA–GPC4 ectodomain, from MDCK cells to HeLaS3 and HEK293 cells suppressed Wnt3a-dependent phosphorylation of LRP6 (Fig. 6C). The HA–GPC4 ectodomain inhibited the binding of Wnt3a to the extracellular domain of LRP6 (LRP6N; Fig. 6E). The HA–GPC4 ectodomain also suppressed Wnt5a-dependent Rac activation in HeLaS3 cells and inhibited the binding of Wnt5a to the extracellular domain of Ror2 (Ror2N; Fig. 6D,E). However, the HA–GPC4 ectodomain did not affect the binding of Wnt3a or Wnt5a to the cysteine rich domain of Fz2 (Fz2CRD; Fig. 6F). Therefore, a GPC4 ectodomain released from cells might target single-span transmembrane receptors, such as LRP6 and Ror2, and modulate Wnt signaling.

Discussion

HSPGs, such as GPCs and SDCs, can bind a variety of soluble and insoluble ligands and regulate multiple cellular functions, including morphogenesis, tissue repair and host defense (Bernfield et al., 1999; Couchman, 2003; Mythreye and Blobe, 2009; Tkachenko et al., 2005). It has also been shown that GPCs and SDCs affect distinct intracellular pathways of Wnt signaling (Alexander et al., 2000; Capurro et al., 2005a; De Cat et al., 2003; Munoz et al., 2006; O'Connell et al., 2009; Ohkawara et al., 2003). In this study, we demonstrated that GPC4 activates both the β-catenin-dependent and -independent pathway of Wnt signaling and that GPC4 ectodomain released from the cell surface membrane inhibits Wnt signaling.

Distribution of GPC4 in both lipid raft and non-lipid raft microdomains

GPCs are linked to membrane lipids without penetrating the bilayer. It is generally believed that GPI-anchored proteins associate with ordered regions of lipid raft microdomains through their saturated acyl chains (Bernfield et al., 1999). However, it has also been shown that a GPI-anchored protein can be associated with lipid raft and non-lipid raft microdomains depending on the presence of unsaturated and saturated lipids (Kinoshita et al., 2008; Maeda et al., 2007). Consistent with these findings, our results, as well as those of others, showed that GPC1, GPC3 and GPC4 localized to both the lipid raft and non-lipid raft microdomains, as assessed by solubilization using cold Triton X-100. By contrast, it has been shown that SDCs appear in non-lipid raft microdomains (Gutierrez and Brandan, 2010; Tkachenko and Simons, 2002). We also confirmed that SDC1 is located only at non-lipid raft microdomains of HeLaS3 cells and that GPC4/SDC1, a chimera of GPC4 and SDC1, appeared in non-lipid raft microdomains. Fibroblast growth factor-2 (FGF2) binds to tyrosine kinase receptors and HSPGs. It was reported that FGF2 induces clustering and redistribution of SDC4 into lipid raft microdomains (Tkachenko and Simons, 2002). In addition, GPC1 was shown to sequester FGF2 in lipid raft microdomains from FGF receptor in non-lipid raft microdomains, thereby preventing its signaling and stimulating muscle differentiation (Gutierrez and Brandan, 2010). Therefore, it is suggested that the distribution of HSPGs on the cell surface membrane is crucial for growth factor signaling.

Activation of the β-catenin pathway by GPC4

We have demonstrated that LRP6 is present in both lipid raft and non-lipid raft microdomains and that Wnt3a-dependent

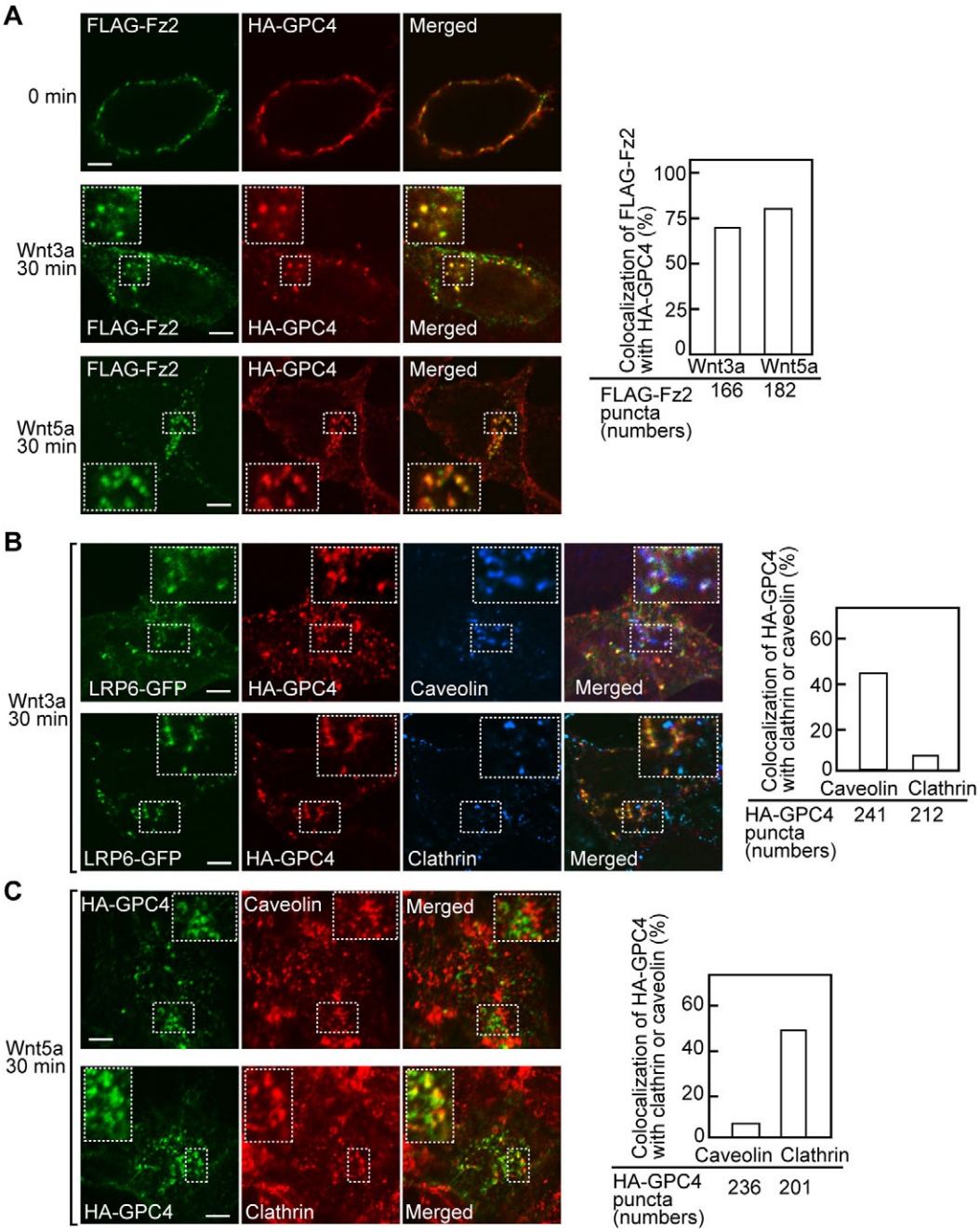

Fig. 5. Wnt3a and Wnt5a induce internalization of HA–GPC4. (**A**) Left: HeLaS3 cells expressing FLAG–Fz2 and HA–GPC4 were stimulated with 100 ng/ml Wnt3a or Wnt5a for 30 minutes, and then the cells were stained with anti-FLAG and anti-HA antibodies. Right: the percentage of FLAG–Fz2 colocalized with HA–GPC4-positive structures was quantified. Scale bars: 5 μm. (**B**) Left: HeLaS3 cells expressing LRP6–GFP, FLAG–Fz2 and HA–GPC4 were stimulated with 100 ng/ml Wnt3a for 30 minutes, and then the cells were stained with anti-HA and anti-caveolin or anti-clathrin antibodies. Right: the percentages of HA–GPC4 colocalized with clathrin- or caveolin-positive structures were quantified. Scale bars: 5 μm. (**C**) Left: HeLaS3 cells transfected with FLAG–Fz2 and HA–GPC4 were stimulated with 100 ng/ml Wnt5a for 30 minutes, and then the cells were stained with anti-HA and anti-caveolin or anti-clathrin antibodies. Right: the percentages of HA–GPC4 colocalized with clathrin- or caveolin-positive structures were quantified. The regions in the white boxes are shown enlarged in the insets. Scale bars: 5 μm.

phosphorylation of LRP6, which is essential for activation of the β-catenin pathway, occurs only in lipid raft microdomains (Sakane et al., 2010; Yamamoto et al., 2008). Expression of GPC4 enhanced Wnt3a-dependent phosphorylation of LRP6 at Ser1490 and increased *Axin2* mRNA levels in HEK293 and HeLaS3 cells. GPC4 also facilitated Wnt3a-dependent internalization of LRP6, which is necessary for activation of the β-catenin pathway (Yamamoto et al., 2006), in HeLaS3 cells. Our results clearly showed that (1) GPC4 reduces the concentration of Wnt3a required for activation of the β-catenin pathway, (2) purified Wnt3a binds to GPC4 directly in vitro, and (3) GPC4 colocalizes with Wnt3a on the cell surface of intact

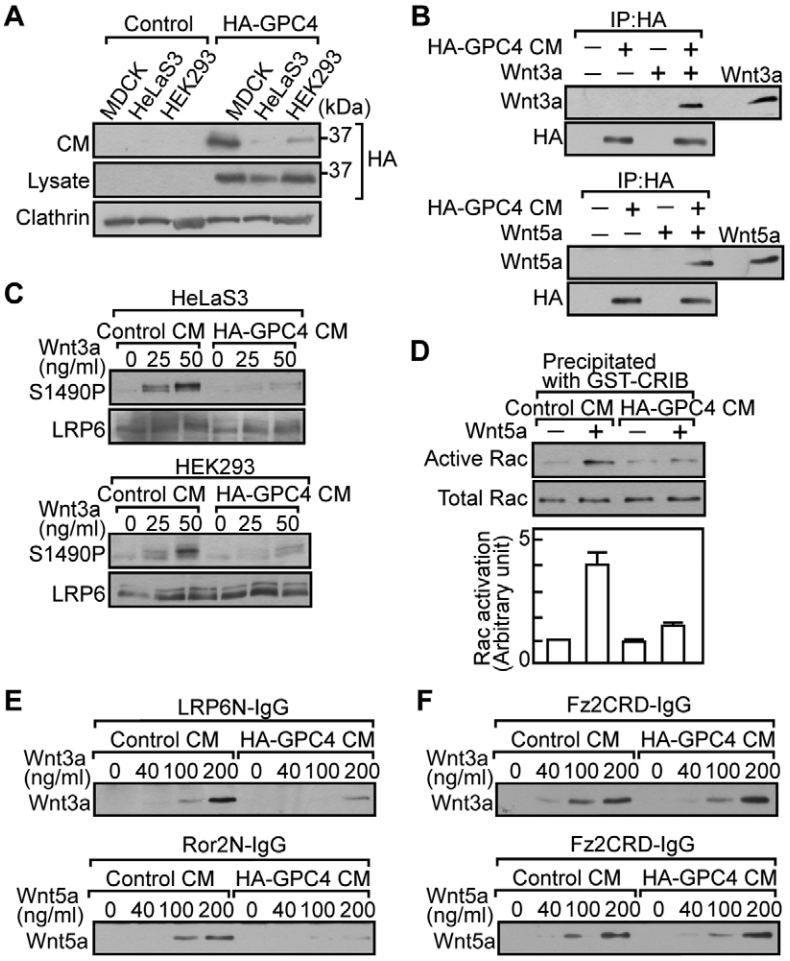

Fig. 6. GPC4 ectodomain suppresses the β-catenin-dependent and -independent pathways. (**A**) Conditioned media (CM) and lysates of MDCK and HeLaS3 cells stably expressing HA–GPC4, or HEK293 cells transiently expressing HA–GPC4 were probed with the indicated antibodies. (**B**) CM from MDCK cells stably expressing HA–GPC4 (HA-GPC4 CM) was immunoprecipitated using the anti-HA antibody. Immunoprecipitates were incubated with 400 ng/ml Wnt3a or Wnt5a for 2 hours at 4℃ and then probed with anti-Wnt3a, anti-Wnt5a/b or anti-HA antibody. 10 ng of Wnt3a or Wnt5a was applied to the right lane. (**C**) HeLaS3 or HEK293 cells were stimulated with the indicated concentration of Wnt3a for 1 hour in the presence of control CM or HA–GPC4 CM, and then the lysates were probed with the indicated antibodies. (**D**) HeLaS3 cells were treated with 50 ng/ml Wnt5a for 30 minutes in the presence of control CM or HA–GPC4 CM, and then the cells were subjected to the Rac activation assay. (**E**) The indicated concentration of Wnt3a or Wnt5a were incubated with 2 nM LRP6N-IgG or 2 nM Ror2N-IgG, respectively, in the presence of control CM or HA–GPC4 CM for 2 hours at 4℃. The precipitates were probed with anti-Wnt3a and anti-Wnt5a/b antibodies. (**F**) The indicated concentrations of Wnt3a or Wnt5a were incubated with 2 nM Fz2CRD-IgG in the presence of control CM or HA–GPC4 CM for 2 hours at 4℃. The precipitates were probed with anti-Wnt3a and anti-Wnt5a/b antibodies.

cells. Therefore, GPC4 could promote the formation of a complex between Wnt3a and its receptors by binding to Wnt3a on the cell surface. These positive effects of GPC4 on Wnt3a-dependent activation of the β-catenin pathway could require its localization to lipid raft microdomains, because GPC4/SDC1 that is present in non-lipid raft microdomains did not activate but rather suppressed Wnt3a-dependent phosphorylation and internalization of LRP6 and the increase in *Axin2* mRNA levels. Wnt3a induced the internalization of GPC4, and internalized GPC4 was colocalized with Fz2, LRP6 and caveolin, supporting our idea that the localization of GPC4 to lipid raft microdomains is important for activation of the β-catenin pathway. Therefore, GPC4/SDC1 could sequester Wnt3a to non-lipid raft microdomains away from Wnt3a receptors that are located in lipid raft microdomains, thereby inhibiting its signaling. The inhibitory effect of GPC4/SDC1 on the Wnt3a–β-catenin pathway does not depend on the cytoplasmic region of SDC1, because GPC4/SDC1ΔC and GPC4/EGFR(646–668) also suppressed this pathway.

We showed previously that Dkk1 and Wnt5a suppress Wnt3a-dependent activation of the β-catenin pathway by distinct mechanisms (Sakane et al., 2010; Sato et al., 2010; Yamamoto et al., 2008). Dkk1 inhibits the β-catenin pathway by moving LRP6 from lipid raft to non-lipid raft microdomains and induces the clathrin-dependent internalization of LRP6, whereas Wnt5a inhibits the pathway by competing with Wnt3a for the binding to Fz2. In vitro binding studies showed that the binding of Dkk1 to GPC4 is weak and that its binding activity is far less than that of Wnt3a to GPC4. The binding activity of Wnt5a to GPC4 was stronger than that of Wnt3a. Consistent with these findings, GPC4 weakened the inhibitory activity of Dkk1 on Wnt3a-dependent activation of the β-catenin pathway and strengthened that of Wnt5a. Taken together, these results suggest that GPC4 is involved in positive and negative regulation of the β-catenin pathway. However, it was also reported that Wnt5a suppresses the β-catenin pathway by inhibiting the accumulation of β-catenin or Tcf transcriptional activity, probably through Ror2 (Ishitani et al., 2003; Mikels and Nusse, 2006; Topol et al., 2003). As discussed below, GPC4 enhanced the internalization of Ror2 and facilitated Wnt5a signaling. Therefore, it is also possible that the Wnt5a inhibition of the β-catenin pathway overcomes Wnt3a-dependent activation of the β-catenin pathway by promoting the formation of a complex between Wnt5a, Fz and Ror2 in the presence of GPC4.

Activation of the β-catenin-independent pathway by GPC4

We have shown that Wnt5a activates Rac in the β-catenin-independent pathway, which requires clathrin-mediated internalization of Fz2 (Sato et al., 2010). The present results demonstrated that Wnt5a also induces clathrin-dependent internalization of endogenous Ror2, and that GPC4 enhances receptor-mediated endocytosis in response to Wnt5a. In addition,

both HA–GPC4 and HA–GPC4/SDC1 enhanced Wnt5a-dependent Rac activation and the phosphorylation of Dvl2. Therefore, GPC4 could concentrate Wnt5a on the cell surface to place it to the vicinity of Fz and Ror2 when it is located to non-lipid raft microdomains. GPC4/SDC1 acted on the β-catenin-dependent and -independent pathways in an opposing manner, indicating that GPC4/SDC1 interacts with Wnt3a or Wnt5a, thereby sequestering Wnt3a to non-lipid raft microdomains away from Fz2 and LRP6 in lipid raft microdomains, while enhancing the formation of a complex between Wnt5a and its receptors, Fz2 and Ror2. These results support our model that membrane microdomains and receptor-mediated endocytosis play a crucial role in the selective activation of the β-catenin-dependent or -independent pathway of Wnt signaling. The cytoplasmic domain of SDC1 has been shown to interact with Tiam1, which is a GDP–GTP exchange protein for Rac (Shepherd et al., 2010). Therefore, it might be also possible that GPC4/SDC1 activates Rac through the binding to Tiam1. However, this possibility is unlikely, because GPC4/SDC1ΔC and GPC4/EGFR(646–668) were able to activate the β-catenin-independent pathway.

It has been reported recently that R-spondin 3 binds to SDC4 and induces the internalization of SDC4 and Fz7 and that Wnt5a and R-spondin 3 are required for gastrulation and activation of the β-catenin-independent pathway in *Xenopus* embryos (Ohkawara et al., 2011). These results are consistent with our finding that GPC4 is internalized in a clathrin-dependent manner to activate the Wnt5a-dependent and β-catenin-independent pathway.

Blockade of the β-catenin-dependent and -independent pathways by GPC4 ectodomain

GPCs can be released from the cell surface membrane by the action of phosphatidylinositol-specific phospholipase C (Bernfield et al., 1999). Although it is not known whether the generation of GPC ectodomain is a regulated or physiological process, GPC ectodomains are found in cell culture media and they are good tools for understanding of the roles of GPCs in cellular functions. We found that MDCK cells release a soluble form of GPC4 into the medium more abundantly than HeLaS3 and HEK293 cells. In contrast to the effects of membrane-anchored GPC4, soluble GPC4 ectodomain suppressed both the β-catenin-dependent and -independent pathways. This inhibitory action could be due to inhibition of the binding of Wnt3a and Wnt5a to LRP6 and Ror2. These are consistent with the findings that SDC1 ectodomain inhibits cell proliferation probably through the binding to FGF2 (Elenius et al., 2004). However, SDC1 ectodomain was also shown to activate the β-catenin pathway in cultured cells (Alexander et al., 2000). Currently, we do not know why membrane-anchored GPC4 and soluble GPC4 have different effects on Wnt signaling. Although direct evidence has not yet been obtained, anchored GPC4 would promote the binding of Wnt3a and Wnt5a to their receptors.

It was reported that GPC3 is subjected to endoproteolytic processing by convertases in CHO cells, and that processed GPC3 modulates both the β-catenin-dependent and -independent pathways (De Cat et al., 2003). Expressed GPC4 was also subjected to processing in HeLaS3, HEK293 and MDCK cells, because a major protein band was detected at ~37 kDa on SDS-PAGE under reducing condition, as reported previously (see Fig. 2A) (Watanabe et al., 1995). However, we did not examine whether proteolytic processing of GPC4 is important for its action, because it was also reported that processing by covertases is not required for the Wnt3a-dependent activation of Tcf activity in hepatocarcinoma cells (Capurro et al., 2005a).

The interactions of Wnts with their receptors and other extracellular proteins on cells surface membranes are highly complex. The association of GPC4 with unknown proteins in addition to Wnt on the cell surface could determine the specific functions of GPC4 for Wnt signaling. Taken together, the localization (lipid raft or non-lipid raft microdomains) and form (membrane-anchored structure or soluble structure) of GPC4 could be important for the regulation of not only Wnt signaling but also other growth factor signaling pathways.

Materials and Methods

Materials and chemicals

pCS2/LRP6-GFP, pCMV-FLAG/Mesd and pCS2/*Xenopus* Dkk1-FLAG were kindly provided by Christof Niehrs (Division of Molecular Embryology, DKFZ, Heidelberg, Germany) (Davidson et al., 2005). pCS2/rat Fz2, pGEX-aPAK-CRIB, pEGFP-N3/Ror2 and pRK5/LRP6N-IgG were from Shinji Takada (National Institutes of Natural Sciences, Okazaki, Japan), Kozo Kaibuchi (Nagoya University, Nagoya, Japan), Yasuhiro Minami (Kobe University, Kobe, Japan) and Xi He (Harvard Medical School, Boston, MA, USA), respectively. cDNA of GPC4 and SDC1 were obtained from a human liver cDNA library. These cDNA fragments were first cloned into a pCRII vector (Invitrogen, Life Technologies, Inc., Carlsbad, MA). The *GPC4* cDNA fragment was inserted into the *Xho*I and *Not*I sites of a pCAG vector, and then the nucleotide sequence corresponding to the amino acid sequence of the HA epitope, YPYDVPDYA, was introduced into the N-terminal *Sac*I site of the *GPC4* cDNA fragment. For the construction of the HA–GPC4/SDC1 chimera, the 1.58-kb fragment encoding HA–GPC4 (aa 1–528; excluding the GPI anchorage signal) with *Xho*I and *Xba*I sites, and the 0.18-kb fragment encoding SDC1 (aa 250–311; including the transmembrane domain and cytoplasmic domain) with *Xba*I and *Not*I sites were digested with *Xho*I plus *Xba*I, and *Xba*I plus *Not*I, respectively. Then, these fragments were inserted into the *Xho*I and *Not*I sites of a pCAG vector, simultaneously. For the construction of HA–GPC4/SDC1ΔC, the 87-base fragment encoding the SDC1ΔC fragment (aa 250–274; including the transmembrane domain) with *Xba*I and *Not*I sites was digested with *Xba*I plus *Not*I. This fragment and the HA–GPC4(1–528) fragment, which was digested with *Xho*I plus *Xba*I, were inserted into the *Xho*I and *Not*I sites of a pCAG vector, simultaneously. For the construction of HA–GPC4/EGFR(581–668) and HA–GPC4/EGFR(646–668), EGFR fragments (aa 581–668; including the juxtamembrane region and the transmembrane domain and aa 646–668; including the transmembrane domain) were digested with *Xba*I plus *Not*I. These fragments were inserted into the *Xba*I and *Not*I sites of a pCAG vector containing the HA–GPC4(1–528) fragment. Standard recombinant DNA techniques were used to construct pCAG/Wnt3a, pCAG/Wnt5a and pRK5/Ror2N(1–400)-IgG. pCS2/FLAG-Fz2 and pRK5/Fz2-CRD-IgG were constructed as described previously (Sato et al., 2010). The restriction enzymes were from TaKaRa Bio (Otsu, Japan) or Toyobo (Osaka, Japan).

Wnt3a, Wnt5a, and Dkk1–FLAG were purified from conditioned media as described previously (Bafico et al., 2001; Komekado et al., 2007; Kurayoshi et al., 2007; Sato et al., 2010). An anti-Wnt3a polyclonal antibody was prepared as described previously (Kishida et al., 2004). For the isolation of HeLaS3 or MDCK cells stably expressing HA–GPC4, HA–GPC4/SDC1, HA–GPC4/SDC1ΔC, HA–GPC4/EGFR(581–668), or HA–GPC4/EGFR(646–668), pCAG/HA–GPC4, pCAG/HA–GPC4/SDC1, HA–GPC4/SDC1ΔC, HA–GPC4/EGFR(581–668) or HA–GPC4/EGFR(646–668) was transfected into HeLaS3 cells or MDCK cells with pPGK-neo. Transfectants of HeLaS3 cells or MDCK cells were selected with 800 μg/ml or 1000 μg/ml G418, respectively, and the colonies of resistant cells were isolated.

Anti-LRP6 monoclonal, anti-S1490P polyclonal, anti-Dvl2 polyclonal and anti-Wnt5a/b monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, CA). Anti-caveolin polyclonal, anti-clathrin monoclonal (for immunoblotting) and anti-Rac1 monoclonal antibodies were from BD Biosciences (San Jose, CA, USA). Anti-clathrin monoclonal (for immunocytochemistry), anti-FLAG M2 monoclonal, anti-HA monoclonal (16B12) and anti-transferrin receptor antibodies were from Calbiochem (San Diego, CA), Sigma-Aldrich (St. Louis, MO), Covance (Princeton, NJ) and Zymed Laboratories (South San Francisco, CA), respectively. Anti-GPC3 monoclonal (1G12) and anti-Ror2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN), respectively.

Preparation of DRM fractions

HeLaS3, HEK293T or HepG2 cells were lysed in 0.5 ml 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 cell lysates were further homogenized using a Dounce homogenizer (40 strokes) and subsequent passage through a 25-gauge needle (Brown and Rose, 1992). Lysates (0.5 ml) were mixed with 0.5 ml 80% (w/v) sucrose in TNE and overlayered with 2 ml 35% sucrose in TNE, followed by 1 ml 5% sucrose in TNE. The gradients were centrifuged at 190,000 ***g*** for 18 hours at 4℃ in an RPS56T rotor (Hitachi, Tokyo, Japan). From the top of the gradient 400-µl fractions were removed. Aliquots were probed with the indicated antibodies.

Rac activation assay

Activation of Rac was assayed using glutathione *S*-transferase (GST)–CRIB (Benard and Bokoch, 2002). After HeLaS3 and HEK293 cells were cultured (60-mm diameter dish) in serum-free medium containing 0.1% bovine serum albumin (BSA) for 24 hours and 18 hours, respectively, the cells were stimulated with 50 ng/ml Wnt5a for 30 minutes. Cells were lysed in 200 µl of buffer A [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol and protease inhibitors (2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µM phenylmethylsulfonyl fluoride)] containing 30 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS and 20 µg of GST–CRIB (Sato et al., 2010). After the lysates were centrifuged at 20,000 ***g*** for 10 minutes, the supernatants were incubated with glutathione–Sepharose (20 µl each) for 2 hours at 4℃. Glutathione–Sepharose was precipitated by centrifugation, and the bound proteins were probed with the anti-Rac1 antibody.

Biotinylation internalization assay

HeLaS3 cells were treated with Wnt3a or Wnt5a, and then the cells were incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) at 4℃ for 30 minutes (Yamamoto et al., 2006). After quenching of excess biotin with 50 mM NH_4Cl, the cells were lysed in 0.2 ml of TNE buffer containing 1% Triton X-100 and 0.4% sodium deoxycholate instead of 0.4% Triton X-100 as above. The lysates were precipitated with neutravidin-agarose beads (Pierce), and the precipitates were probed with anti-LRP6 or anti-Ror2 antibodies. The whole lysates were probed with anti-LRP6 and anti-Ror2 antibodies to detect total LRP6 and Ror2, respectively.

Binding of purified proteins to HA–GPC4 and HA–GPC4/SDC1

Lysates of HeLaS3 cells stably expressing HA–GPC4 or HA–GPC4/SDC1 were immunoprecipitated using the anti-HA antibody, and the immunoprecipitates were washed with PBS containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) three times. Immunoprecipitated HA–GPC4 or HA–GPC4/SDC1 was incubated with various concentrations of Wnt3a, Wnt5a or Dkk1 in 200 µl of PBS containing 0.5% CHAPS for 2 hours, and then washed with PBS containing 0.3% CHAPS three times. The immunoprecipitates were probed with the indicated antibodies.

Receptor binding assay

Conditioned medium (CM) from HEK293 cells expressing Fz2CRD-IgG, human LRP6N-IgG or Ror2N-IgG was incubated with protein-A–Sepharose for 2 hours. Precipitates were washed three times with PBS containing 0.5% CHAPS. For the binding assay, 2 nM Fz2CRD-IgG, human LRP6N-IgG or Ror2N-IgG was incubated with the indicated concentration of Wnt3a or Wnt5a in the presence of CM from control MDCK cells or CM from MDCK cells expressing HA–GPC4 stably for 2 hours at 4℃. The precipitates were washed with 0.1% CHAPS three times and then probed with anti-Wnt3a and anti-Wnt5a/b antibodies.

Internalization of LRP6–GFP, FLAG–Fz2 and HA–GPC4

HeLaS3 cells were used for internalization assays as described previously (Yamamoto et al., 2006; Yamamoto et al., 2008). The cells were seeded onto 18 mm glass coverslips coated with poly-D-lysine (Sigma, St. Louis, MO, USA), and pCS2/FLAG-Fz2, pCAG/HA-GPC4, pCS2/LRP6-GFP and pCMV-Myc/Mesd were transfected into the cells using Lipofectamine LTX (Invitrogen, Life Technologies, Inc., Carlsbad, MA, USA). At 24–48 hours after transfection, the cells were incubated with ice-cold binding medium [DMEM containing 20 mM HEPES–NaOH (pH 7.5) and 0.1% BSA] for 30 minutes and treated with 100 ng/ml Wnt3a or Wnt5a for 1 hour at 4℃. To observe cell surface FLAG–Fz2 or HA–GPC4, anti-FLAG or anti-HA antibody was also added to the medium. After unbound Wnt3a, Wnt5a, anti-FLAG antibody or anti-HA antibody was removed by washing with cold PBS, internalization was initiated by adding warm DMEM at 37℃. To observe the internalization of FLAG–Fz2, HA-GPC4 and LRP6-GFP, the cells were washed with cold PBS to stop internalization. After the cells were fixed and permeabilized, they were probed with the secondary antibody. To observe LRP6–GFP, the cells were viewed directly using a confocal microscope (LSM510, Carl-Zeiss, Jena, Germany). The images shown are representative of 100 microscopic fields in five independent experiments.

Immunocytochemistry

Cells grown on glass coverslips were fixed for 15 minutes in PBS containing 4% (w/v) paraformaldehyde and then permeabilized with PBS containing 0.2% (w/v) Triton X-100 and 2 mg/ml BSA for 30 minutes. The cells were stained with anti-caveolin or anti-clathrin antibody before confocal microscopy was performed. The images shown are representative of 100 microscopic fields of at least three independent experiments.

To observe cell surface Wnt3a or Wnt5a, HeLaS3 cells were transfected with pCAG/Wnt3a or pCAG/Wnt5a in the presence or absence of pCAG/HA-GPC4. At 24 hours after transfection, anti-Wnt3a, anti-Wnt5a/b and anti-HA antibodies were added to the medium and incubated for 1 hour. Then cells were fixed for 15 minutes in PBS containing 4% (w/v) paraformaldehyde and probed with secondary antibody without permeabilization.

RT-PCR

The cells were treated with Wnt3a in DMEM containing 10% FBS for 8 hours, and then quantitative RT-PCR was performed (Hino et al., 2005). Forward and reverse primers were as follows: human *Axin2*, 5′-CTGGCTCCAGAAGATCACAAAG-3′ and 5′-CATCCTCCCAGATCTCCTCAAA-3′; human *GAPDH*, 5′-CCTGTTC-GACAGTCAGCCG-3′ and 5′-CGACCAAATCCGTTGACTCC-3′; human *GPC1*, 5′-TCGCTCCTTTGTGCAG-3′ and 5′-GAGGTTCCTCCACTCG-3′; human *GPC2*, 5′-GGAGTTGTGCAGAGACC-3′ and 5′-GTGAACCAGAAAGGAGCC-3′; human *GPC3*, 5′-GGCAAGTTATGTGCCC-3′ and 5′-GTGTCGTTTTCC-GCCA-3′; human *GPC4*, 5′-AAGTTGCTCGGAAGTG-3′ and 5′-CACTGTTC-GCTGACCA-3′; human *GPC5*, 5′-TTTGCAGTACCTACAGGAAC-3′ and 5′-TTCTGAGTATTCCAGGGAAC-3′; human *GPC6*, 5′-TTTGTACGGACCTA-TGGC-3′ and 5′-GCTGGTCAGTGTATTTGC-3′.

siRNA

In analysis with siRNAs, the following target sequences were used. Randomized control, 5′-CAGTCGCGTTTGCGACTGG-3′; *GPC1*, 5′-GGAGAAGCTGGTC-TCTGAA-3′; *GPC3*, 5′-GGCTCTGAATCTTGGAATT-3′; *GPC4*, 5′-TGCA-GGATAATAGTGTTCA-3′; *GPC5*, 5′-GCTGATCAGCTTTGTGCTA-3′; *GPC-6*, 5′-TGGAAGACAAGTTAAGCCA-3′.

Statistical analysis

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

Other methods

Western blotting data were representative of at least four independent experiments. Tcf-4E transcriptional activity was measured using TOP-*fos*-*Luc* and pEF-BOS/hTCF-4E as described previously (Yamamoto et al., 2006).

Acknowledgements

We thank C. Niehrs, S. Takada, K. Kaibuchi, Y. Minami and X. He for donating cells and plasmids.

Funding

This work was supported by the Ministry of Education, Science, Culture of Japan by Grants-in-Aid for Scientific Research [grant number A212490170 to A.K.], for Scientific Research on priority areas (2008~2010) [grant number 17014066 to A.K.], and for Young Scientists (B) (2010) [grant number 22790316 to H.S.]; and a Grant from the Kobayashi Cancer Foundation (2010) [grant number J550703178 to A.K.].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.091876/-/DC1

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