

RESEARCH ARTICLE

Glypican-3 binds to Frizzled and plays a direct role in the stimulation of canonical Wnt signaling

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ABSTRACT

Glypican-3 (GPC3) is a proteoglycan that is bound to the cell surface. It is expressed by most hepatocellular carcinomas (HCCs) but not by normal hepatocytes. GPC3 stimulates HCC growth by promoting canonical Wnt signaling. Because glypicans interact with Wnts, it has been proposed that these proteoglycans stimulate signaling by increasing the amount of Wnt at the cell membrane, thus facilitating the interaction of this growth factor with its signaling receptor, Frizzled. However, in this study, we demonstrate that GPC3 plays a more direct role in the stimulation of Wnt signaling. Specifically, we show that, in addition to interacting with Wnt, GPC3 and Frizzled interact directly through the glycosaminoglycan chains of GPC3, indicating that this glypican stimulates the formation of signaling complexes between Wnt and Frizzled. Consistent with this, we show that the binding of Wnt at the cell membrane triggers the endocytosis of a complex that includes Wnt, Frizzled and GPC3. Additional support for our model is provided by the finding that glypican-6 (GPC6) inhibits canonical Wnt signaling, despite the fact that it binds to Wnt at the cell membrane.

KEY WORDS: Glypican-3, Wnt, Proteoglycan, Hepatocellular carcinoma, Heparan sulphate, Frizzled

INTRODUCTION

Glypican-3 (GPC3) is one of six mammalian members of the glypican family of proteoglycans. Glypicans are bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor (Filmus et al., 2008; Filmus and Capurro, 2012). In addition, they can be found in the extracellular environment after being released by a lipase called Notum (Kreuger et al., 2004; Traister et al., 2008). Glypicans have been shown to display two to five long linear glycosaminoglycan (GAG) chains. In general, glypicans carry heparan sulfate chains, but glypican-5 (GPC5) can also display chains of chondroitin sulfate (Saunders et al., 1997). Another structural feature shared by the members of this family is the localization of the insertion sites for the GAG chains. These sites are located close to the C-terminus (Veugelers et al., 1999). This places the GAG chains close to the cell surface, suggesting that glypicans could interact with other cell membrane proteins (Filmus et al., 2008).

Genetic and biochemical studies have demonstrated that glypicans can regulate several signaling pathways, including

those triggered by Wnts (Song et al., 2005; Topczewski et al., 2001; Tsuda et al., 1999), Hedgehogs (Hhs) (Capurro et al., 2008; Desbordes and Sanson, 2003; Lum et al., 2003), bone morphogenetic proteins (BMPs) (Belenkaya et al., 2004; Dwivedi et al., 2013; Fujise et al., 2003) and fibroblast growth factors (FGFs) (Gutiérrez and Brandan, 2010). The glypican-mediated regulation of signaling occurs at the level of the interaction between the ligand and receptor, and glypicans can have either a stimulatory or inhibitory effect on signaling activity. The picture that is emerging from these studies is that the structural features of glypicans combine with the set of signaling molecules that are present in a given cell type to determine glypican function. In addition to their role in regulating receptor–ligand interactions, glypicans also participate in the formation of Wnt, Hh and BMP gradients in the *Drosophila* imaginal disc (Akiyama et al., 2008; Ayers et al., 2010; Gallet et al., 2008; Han et al., 2004; Wu et al., 2010).

GPC3 is widely expressed during development, but it is downregulated in most adult tissues (Pellegrini et al., 1998). We demonstrated previously that this glypican plays an important role in the regulation of cell proliferation in the developing bone (Capurro et al., 2009). Furthermore, we showed that this regulatory activity is mediated by the Hh signaling pathway (Capurro et al., 2009).

It is now well established that GPC3 is expressed by most hepatocellular carcinomas (HCCs) but not by normal hepatocytes and benign liver lesions (Capurro et al., 2003; Libbrecht et al., 2006; Yamauchi et al., 2005). Consequently, the immunohistochemical detection of GPC3 is currently being used by clinical pathologists to confirm a diagnosis of HCC (Bruix and Sherman, 2011; Marrero, 2009; Roskams and Kojiro, 2010). Work from our laboratory has also shown that, in addition to being a marker of HCC, GPC3 stimulates the growth of this malignancy by promoting canonical Wnt signaling (Capurro et al., 2005). This finding has been confirmed by others (Li et al., 2012). Studies of GPC3-null mice have also demonstrated that GPC3 regulates Wnt signaling in normal embryonic tissues (Song et al., 2005). Gain-of-function and loss-of-function studies have demonstrated that other mammalian and *Drosophila* glypicans also regulate Wnt signaling (Fico et al., 2012; Sakane et al., 2012; Yan et al., 2009). In addition, it should be noted that other proteoglycans have been shown to regulate this signaling pathway (Buraschi et al., 2010; Kamimura et al., 2013).

Activation of the canonical Wnt signaling pathway is one of the most frequent molecular events associated with the progression of HCC (Kern et al., 2002; Satoh et al., 2000; Thompson and Monga, 2007). This signaling pathway is normally triggered by the binding of Wnt to two co-receptors: Frizzled, and LRP5 or LRP6 (low-density lipoprotein receptor-related 5 or 6, hereafter LRP5/6) (Niehrs, 2012). Wnt binding induces the accumulation of the transcription factor β -catenin, which subsequently migrates to the nucleus. In the nucleus, β -catenin drives the expression of many genes, some of which are involved in the promotion of cell proliferation and survival (Clevers, 2006;

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Gordon and Nusse, 2006). It should be noted that the human genome includes 19 Wnt genes and 10 Frizzled genes, and that, depending on the cell type, only specific Wnt–Frizzled interactions have the capacity to activate the canonical pathway (Grumolato et al., 2010; Takada et al., 2005).

Based on the fact that glypicans can interact with Wnts at the cell membrane (Sakane et al., 2012; Yan et al., 2009), and that they do not have a cytoplasmic domain that could transduce signaling activity, it has been proposed that these proteoglycans stimulate Wnt signaling by increasing the concentration of Wnt in the vicinity of the signaling receptors (Sakane et al., 2012). Here, we provide experimental evidence supporting a more direct role of GPC3 in the stimulation of canonical Wnt signaling, based on the interaction of this glypican with Frizzled.

RESULTS

GPC3 stimulates Wnt3a activity at the level of signal reception

We have demonstrated previously that GPC3 stimulates canonical Wnt activity in HCC cells by using a luciferase reporter assay, and by showing that ectopic GPC3 induces the accumulation of cytoplasmic β -catenin (Capurro et al., 2005). Based on these results, and on our finding that GPC3 can interact with various Wnts (Capurro et al., 2005; Song et al., 2005), we have proposed that GPC3 stimulates canonical Wnt activity at the level of signal reception (Filmus et al., 2008). Here, to directly test this hypothesis, we first investigated whether GPC3 can stimulate Wnt3a-induced phosphorylation of LRP6. It is well established that this phosphorylation is one of the earliest events triggered by the binding of Wnt to its signaling receptors at the cell surface (Bilic et al., 2007). We transfected 293T cells with vector encoding GPC3 or with control vector, and the levels of phospho-LRP6 that were induced by different dilutions of Wnt3a-conditioned medium or control conditioned medium were assessed by using western blot analysis with an anti-phospho-LRP6 antibody. As shown in Fig. 1A, a significant increase in Wnt3a-induced phospho-LRP6 levels was observed at all the Wnt concentrations that were tested in the GPC3-transfected cells compared with cells transfected with vector control. This result provides strong support for the hypothesis that GPC3 stimulates canonical Wnt signaling by acting at the level of the cell membrane, upstream of LRP6 activation.

One possible mechanism for the GPC3-induced stimulation of canonical Wnt signaling could be based on GPC3 having the ability to increase the affinity of Wnt for the relevant Frizzled or LRP receptors that are expressed by the target cell. To directly test this hypothesis, it would be necessary to generate Scatchard plots corresponding to the interaction between Wnt and the receptor complex on intact cells. However, because cells express several Frizzled proteins and other Wnt-binding proteins, the results of such plots would be difficult to interpret. Alternatively, it is reasonable to assume that the signaling activity observed in a given cell type at increasing Wnt concentrations correlates with the overall receptor occupancy. Based on this assumption, we decided to measure the signaling activity of increasing Wnt3a concentrations in the presence and absence of GPC3 as a surrogate experiment to assess the impact of GPC3 on ligand–receptor affinity. To this end, 293T cells were transiently transfected with GPC3 and a reporter vector in which the expression of a luciferase gene was driven by a β -catenin-responsive promoter. Transfected cells were then incubated with different dilutions of Wnt3a-conditioned or control-conditioned media, and the luciferase activity was measured. As shown in

Fig. 1B, we found that GPC3 significantly increases Wnt3a-induced luciferase activity at all tested concentrations of Wnt3a. Therefore, this result provides additional support for a signal-stimulatory role of GPC3 at the level of the interaction between Wnt and Frizzled. It should also be noted that, although this experiment was not designed to measure binding affinities, the slopes of the luciferase activity curves that were obtained in the presence and absence of GPC3 were consistent with the idea that Wnt3a displays a higher binding affinity for Frizzled in the presence of this glypican.

GPC3 interacts with Frizzled

How could GPC3 stimulate Wnt signaling at the level at which the signal is received? It is well established that the stimulation of FGF signaling by proteoglycans requires the assembly of a ternary complex that includes FGF, the FGF receptor and the proteoglycan (Ibrahimi et al., 2004). Based on this knowledge, we decided to investigate whether a similar mechanism is involved in the GPC3-induced stimulation of Wnt signaling. To test this hypothesis, we studied whether, in addition to interacting with Wnts, GPC3 can also bind to Frizzled. As a first approach to investigate the interaction between GPC3 and Frizzled, we performed co-immunoprecipitation studies. We transiently transfected 293T cells with expression vectors for GPC3 and FLAG-tagged Frizzled-4 (FZD4). This Frizzled protein mediates Wnt3a-induced canonical signaling in 293T cells, and it is expressed in HCC (Pan et al., 2008; Bengochea et al., 2008). Following transfection, the cells were lysed, GPC3 was immunoprecipitated and the presence of FZD4 in the precipitated material was assessed by western blot analysis. As shown in Fig. 2A, we found that FZD4 co-immunoprecipitates with GPC3. As an alternative approach to study the interaction between GPC3 and Frizzled, we performed pull-down assays. To this end, 293T cells were transfected with a FZD4 expression vector. Two days after transfection, cells were lysed, and the lysates were incubated with an anti-FLAG antibody and immunoprecipitated with Protein G beads. The beads were then incubated with a GPC3–alkaline-phosphatase (AP) fusion protein or with AP alone. After washing, the amount of AP activity that was retained by the beads was measured. As shown in Fig. 2B, GPC3–AP binds to FZD4-covered beads significantly more than does AP alone, indicating that GPC3 interacts with FZD4. We also investigated whether, in addition to FZD4, GPC3 can interact with FZD7 and FZD8, which are also expressed in HCC (Bengochea et al., 2008). As shown in Fig. 2B, we found that both FZD7 and FZD8 bind to GPC3. To confirm that the GPC3–Frizzled interaction was direct, we repeated the pull-down assay with the addition of an acid-wash step before lysing the transfected cells, to remove endogenous proteins that could be bound to FZD7. We found that the amount of GPC3–AP that was bound to the acid-washed FZD7 was similar to that observed in the absence of the acid-wash step (data not shown), indicating that GPC3 binds directly to FZD7. To further characterize the GPC3–Frizzled interaction, we investigated the role of the heparan sulfate chains. To this end, a pull-down experiment was performed by incubating the Frizzled-covered Protein G beads with GPC3 Δ GAG–AP, a fusion protein in which the GPC3 insertion sites for the heparan sulfate chains have been mutated (Gonzalez et al., 1998). No significant binding of the non-glycanated GPC3 to beads covered with any of the Frizzled proteins was observed, indicating that the heparan sulfate chains mediate the interaction (Fig. 2B). Finally, we investigated

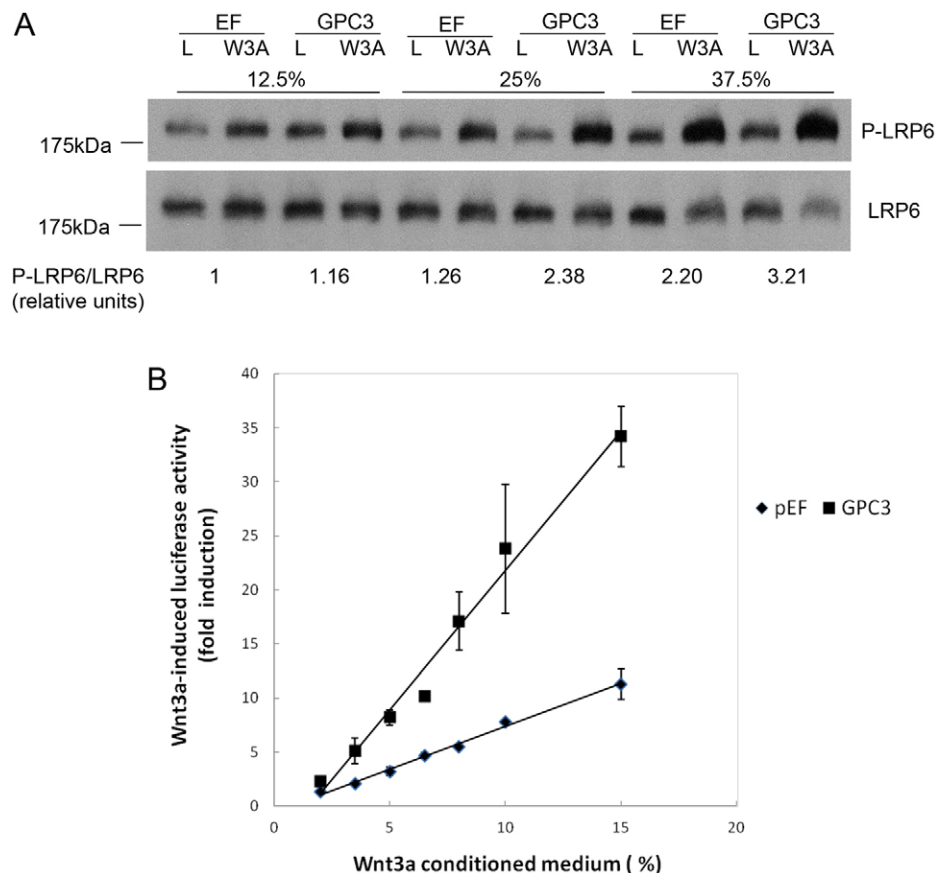


Fig. 1. GPC3 stimulates Wnt3a activity at the signal-reception level. (A) GPC3 stimulates Wnt3a-induced phosphorylation of LRP6. 293T cells were transiently transfected with GPC3 or vector control (EF). Transfected cells were stimulated with the indicated amounts of Wnt3a (W3A)-conditioned medium or L control (L)-conditioned medium for 1 h. Cells were then lysed and the levels of phospho-LRP6 (Ser¹⁴⁹⁰) (P-LRP6) or total LRP6 were assessed by western blotting. Bands were scanned and quantified using NIH ImageJ software. The ratio of P-LRP6 to total LRP6 was then calculated. The level of Wnt3a-induced LRP-6 phosphorylation of vector-control-transfected cells with the lowest Wnt3a dose was arbitrarily assigned a value of 1. Numbers at the bottom indicate the relative P-LRP6:LRP-6 ratios. This experiment was repeated three times with similar results. (B) GPC3 stimulates Wnt3a activity at various Wnt3a concentrations. 293T cells were seeded in a 6-well plate and were transfected with a Wnt-responsive luciferase reporter (500 ng), a β -galactosidase vector and GPC3 or vector control (pEF) (125 ng). At 16 h after transfection, cells were trypsinized, replated onto 24-well plates (40,000 cells/well) and incubated with various dilutions of Wnt3a-conditioned medium or L medium control for 2.5 h. Luciferase activity was then measured, normalized for transfection efficiency using the β -galactosidase activity, and the ratio between the luciferase activity in the presence and absence of Wnt3a was calculated. Each sample was performed in quadruplicate, and the data represent the mean \pm s.d. The experiment was performed twice with similar results.

whether heparin can compete with the binding of GPC3–AP to FZD7. We found that, as expected, heparin inhibits this binding in a dose-dependent manner (Fig. 2C). This result provides additional experimental evidence supporting an essential role for the heparan sulfate chains in the GPC3–Frizzled interaction.

Frizzled proteins are seven-span transmembrane receptors, and a large part of these proteins therefore cannot interact with GPC3, which is located completely outside of the cell. The N-terminal extracellular portion of Frizzled contains a cysteine-rich domain (CRD), which is known to interact with Wnts. Thus, to further characterize the Frizzled–GPC3 interaction, we investigated whether the Frizzled CRD can bind to GPC3. To this end, we performed pull-down assays by incubating beads covered with FZD4, FZD7 or FZD8 CRDs with GPC3–AP. As shown in Fig. 2D, we found that there is substantial binding of GPC3–AP to all the Frizzled CRDs that were tested. Consistent with the results observed with the full-length Frizzled, the non-glycanated GPC3 Δ GAG–AP did not interact with any of the Frizzled CRDs (Fig. 2D). These results indicate that the CRDs of Frizzled proteins mediate the interaction of these proteins with the heparan sulfate chains of GPC3.

GPC3 is endocytosed along with Frizzled and Wnt3a

It is well established that the binding of Wnt3a to its cell-surface receptors Frizzled and LRP5/6 induces endocytosis of the Wnt–Frizzled–LRP complex (Blitzer and Nusse, 2006). Although the mechanism of endocytosis is still controversial, there is convincing evidence suggesting that Wnt-induced endocytosis is essential for signaling (Blitzer and Nusse, 2006; Yamamoto et al., 2008). Our results showing that GPC3 interacts with both Wnt and Frizzled strongly suggest that these three proteins form a complex. If this is the case, it is reasonable to hypothesize that GPC3 will also be present in the internalized molecular complex. To investigate this possibility, we co-transfected 293T cells with vectors encoding GPC3 and a FZD8–YFP fusion protein. One day after transfection, cells were incubated with Wnt3a-containing conditioned medium for 1 hour at 8°C. The cells were then washed and fixed, or were transferred to 37°C for 75 minutes to allow endocytosis to proceed before fixation. The cells were then immunostained for GPC3 and Wnt, and were observed by using a confocal microscope. As shown in Fig. 3, we found that, as expected, cells that expressed GPC3 and FZD8–YFP displayed

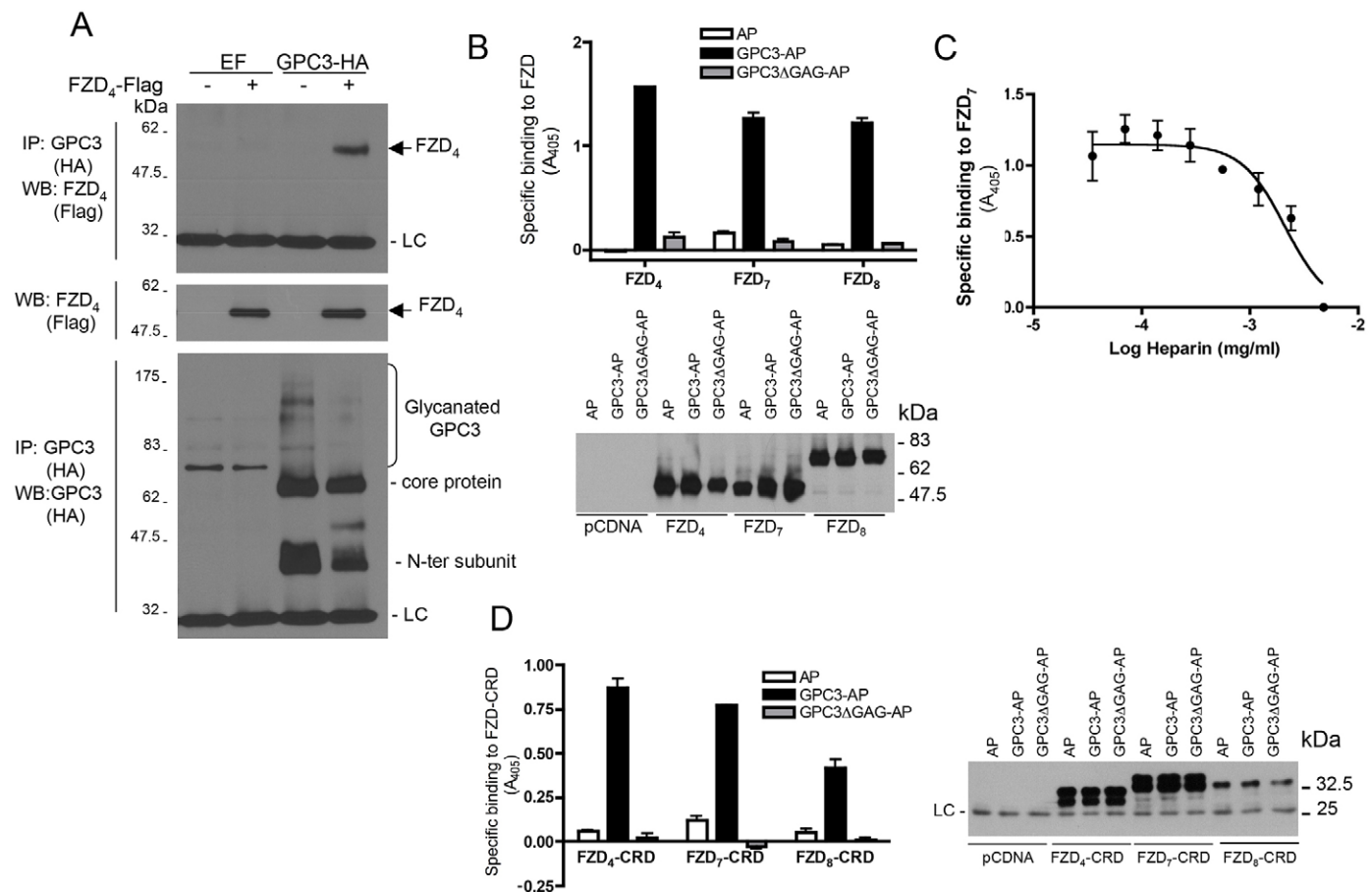


Fig. 2. GPC3 interacts with Frizzled. (A) Co-immunoprecipitation. 293T cells were transfected with HA-tagged GPC3, FLAG-tagged FZD4 (FZD₄) or the corresponding control expression vectors, and GPC3 was immunoprecipitated by using an anti-HA antibody. Upper panel, the presence of FZD4 in the precipitated material as assessed by western blotting (WB). Levels of FZD4 in whole-cell lysates (middle panel) and immunoprecipitated GPC3 (lower panel) were assessed by western blot analysis. IP, immunoprecipitation; LC, immunoglobulin light chain; HA, hemagglutinin A; EF, vector control. The positions of molecular-mass markers are indicated on the left. (B) Pull-down assays. 293T cells were transfected with FLAG-tagged FZD4, FZD7, FZD8 or vector control. After 2 days, cells were lysed and incubated with anti-FLAG antibodies and Protein G beads. The beads were then incubated with GPC3-AP, GPC3ΔGAG-AP or AP alone. After washing, the AP activity retained by the beads was measured. Bars represent the specific AP activity (mean±s.d. of triplicates) bound to the Frizzled-covered beads after subtraction of the AP activity bound to the control beads. The experiment was performed four times with similar results. In all experiments, an unpaired Student's *t*-test revealed a highly significant GPC3–Frizzled binding ($P<0.001$). Lower panel, western blot analysis confirming that similar amounts of Frizzled proteins were attached to the beads. The positions of molecular-mass markers are indicated on the right. (C) Effect of heparin on the GPC3–FZD7 interaction. A pull-down assay was performed as described above in the presence of the indicated concentrations of heparin. Results represent the mean±s.d. of triplicates. The experiment was performed three times with similar results. (D) Pull-down assays. 293T cells were transfected with Myc-tagged FZD4-CRD, FZD7-CRD, FZD8-CRD or vector control. Two days later, the cells were lysed and incubated with anti-Myc antibodies and Protein G beads. The beads were then incubated with GPC3-AP, GPC3ΔGAG-AP or AP alone. After washing, the AP activity retained by the beads was measured. Left panel, bars represent the specific AP activity (mean±s.d. of triplicates) bound to the Frizzled-CRD-covered beads after subtraction of the AP activity bound to the control beads. The experiment was performed three times with similar results. Right panel, western blot analysis confirming that the beads display similar amounts of bound Frizzled-CRDs. The positions of molecular-mass markers are indicated on the right.

detectable levels of Wnt3a bound at the cell surface (Fig. 3B). Notably, upon Wnt3a-induced endocytosis, the membrane staining mainly disappeared, and a large number of vesicles with colocalized GPC3, FZD8 and Wnt3a were observed in the cytoplasm (Fig. 3C). Endocytosis did not occur when cells were incubated in similar conditions but with control-conditioned medium (inset). This result strongly suggests that GPC3 forms a complex with Wnt3a and FZD8–YFP at the cell surface, and that this complex is internalized when endocytosis is allowed to proceed.

It has been reported recently that another mammalian glypican (glypican-4) is internalized in response to Wnt3a, along with FZD2 and LRP6, through a caveolin-mediated route (Sakane et al., 2012). To investigate whether the same endocytic route is used during the Wnt3a-induced internalization of GPC3–FZD8,

the endocytosis experiment was repeated with the additional immunostaining of endogenous caveolin (Fig. 3D–F). As shown in Fig. 3D,E, we found that whereas GPC3 and FZD8 do not colocalize with caveolin when they are at the cell membrane, a significant colocalization of GPC3, FZD8 and caveolin occurs in the cytoplasmic vesicles that are formed during Wnt3a-induced endocytosis (Fig. 3F). This result indicates that GPC3 is internalized with Wnt3a and FZD8, at least in part, through a caveolin-mediated route.

GPC6 binds to Wnt3a at the cell surface, but inhibits Wnt3a activity at the signal-reception level

While we were performing studies on the function of another member of the mammalian glypican family, glypican-6 (GPC6), a

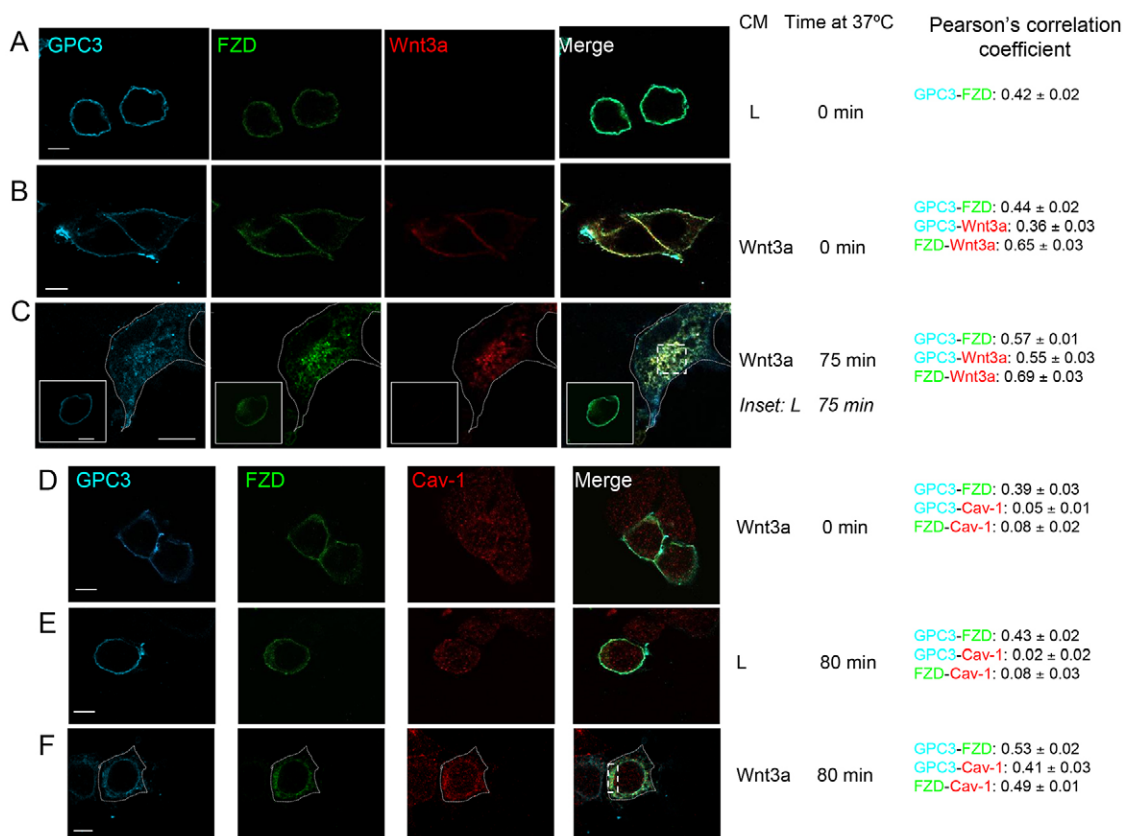


Fig. 3. Wnt3a induces the endocytosis of GPC3-FZD8-Wnt3a complexes. 293T cells were transfected with GPC3 and FZD8-YFP (FZD), and were incubated with control-conditioned medium (L) or Wnt3a-conditioned medium (Wnt3a) for 1 h at 8°C. The cells were then either fixed (A,B,D) or transferred to 37°C for 75 or 80 min to allow endocytosis to proceed (C,E,F). GPC3 (blue) and Wnt3a (red) (A–C), or GPC3 (blue) and caveolin-1 (Cav-1) (red) (D–F) were then immunostained. Yellow or white in the merged picture indicates colocalization. CM, conditioned medium. Dotted white lines mark cell boundaries, and dashed white line boxes mark examples of endocytosis regions (cytoplasmic vesicles) (C,F). Pearson's correlation coefficients for the indicated partners are included on the right (mean ± s.d. of at least 14 representative images). Scale bars: 10 μm.

luciferase reporter assay in 293T cells revealed that, in contrast to GPC3, this glypican significantly inhibited canonical Wnt signaling in a dose-dependent manner (Fig. 4A). This inhibition occurred at the level of signal reception, because GPC6 reduced Wnt3a-induced LRP6 phosphorylation (Fig. 4B).

We therefore decided to investigate whether GPC6 binds to Wnt. To this end, we first performed co-immunoprecipitation experiments. As shown in Fig. 5A, we found that Wnt3a co-immunoprecipitates with GPC6 in 293T cells transiently transfected with vectors encoding GPC6 and Wnt3a-HA. As a second approach, we investigated whether GPC6 interacts with Wnt3a in a pull-down assay. We observed that Wnt3a binds to GPC6-covered beads significantly more than to control beads (Fig. 5B). We also found that the Wnt3a-binding capacity of GPC6 is higher than that of GPC3. Interestingly, we observed that Wnt3a does not bind to a non-glycanated GPC6 (GPC6ΔGAG), indicating that the GAG chains are required for the GPC6–Wnt3a interaction (Fig. 5B). Consistent with this finding, we observed that GPC6ΔGAG is unable to inhibit canonical Wnt signaling in a luciferase reporter assay (Fig. 5C). It is highly likely that the greater Wnt3a-binding capacity of GPC6 as compared with that of GPC3 is due to the fact that, unlike GPC3, the interaction of GPC6 with Wnt3a is mediated by the heparan sulfate chains, which display multiple Wnt3a-binding sites. To definitively determine whether, like GPC3, GPC6 binds to Wnt3a at the cell

surface, we transiently transfected 293T cells with GPC6, non-glycanated GPC6 or vector control, and incubated them with Wnt3a-conditioned medium at 8°C. After washing to remove the unbound material, the binding of Wnt3a to the cells was assessed by using immunostaining. We found that Wnt3a only bound to the cells that expressed GPC6 (Fig. 5D), whereas no binding was detected in cells transfected with vector control or GPC6ΔGAG. We conclude, therefore, that GPC6 binds to Wnt3a at the cell surface through its GAG chains.

Taken together, these results show that, like GPC3, GPC6 binds to Wnt3a at the cell membrane. However, in contrast to GPC3, GPC6 inhibits Wnt3a activity at the signal-reception level. Thus, these observations indicate that increasing the amount of Wnt3a at the cell membrane is not enough to stimulate signaling.

GPC6 does not interact with Frizzled

How could GPC6 bind to Wnt3a at the cell surface, but inhibit Wnt signaling at the signal-reception level? One possibility is that GPC6, in contrast to GPC3, does not interact with Frizzled and cannot form a signaling complex with Wnt and Frizzled. As a first approach to test this hypothesis, we performed co-immunoprecipitation experiments in transiently transfected 293T cells. As shown in Fig. 6A, FZD4 does not co-immunoprecipitate with GPC6, indicating that these two proteins do not interact. To confirm this

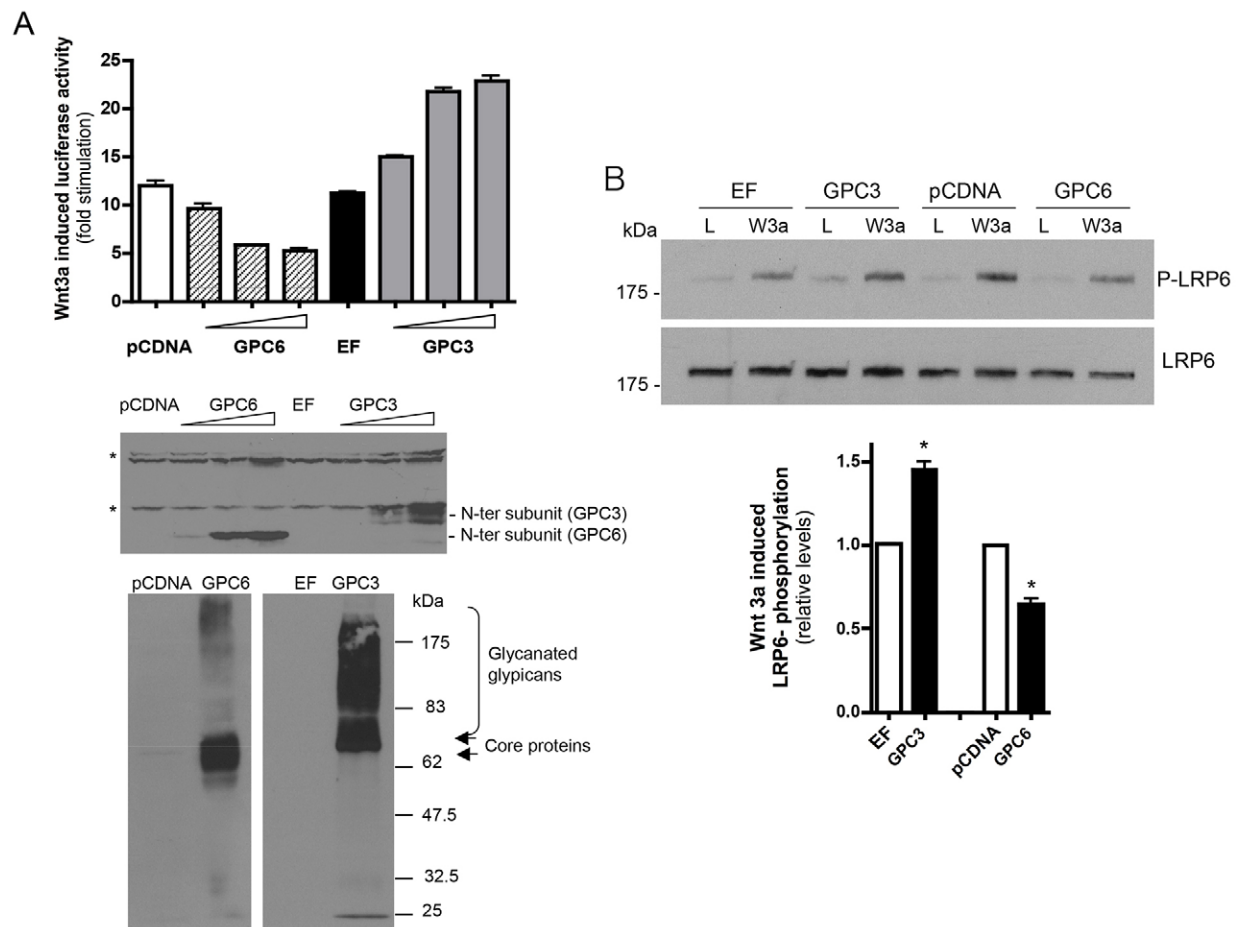


Fig. 4. GPC6 inhibits Wnt3a activity at the signal-reception level. (A) Upper panel, GPC6 inhibits Wnt3a-induced luciferase activity. 293T cells were transfected with increasing amounts of HA-tagged GPC6 or HA-tagged GPC3 expression vectors, or vector alone (pCDNA or EF), along with a luciferase reporter vector driven by a canonical Wnt responsive promoter (TOPFLASH) and β -galactosidase. Cells were then stimulated overnight with Wnt3a- or control-conditioned medium, lysed and a luciferase assay was performed. Bars represent the fold stimulation induced by Wnt3a (mean+s.d. of triplicates). The experiment was repeated four times with similar results. The unpaired Student's *t*-test revealed highly significant inhibitory and stimulatory effects of GPC6 and GPC3, respectively, at all doses tested ($P < 0.001$). Middle panel, western blot analysis that was performed in reducing conditions with an anti-HA antibody, to assess GPC6 and GPC3 levels in the cells that were transfected for the luciferase assay. Both glypicans contain an internal cleavage site that generates an N-terminal (N-ter) subunit (~30 to 40 kDa) and a C-terminal subunit, bearing the GAG chains. Under reducing conditions, the two subunits separate, and only the N-terminal subunit with the HA tag is detected. The asterisks (*) indicate non-specific bands detected by the anti-HA antibody. Lower panel, both glypicans are glycanated in 293T cells. Western blot analysis of 293T cells transfected with HA-tagged GPC3 or GPC6 and the corresponding vector controls (EF, pCDNA). Left panel, a smear corresponding to glycanated GPC6 is detected by an anti-GPC6 polyclonal antibody (directed to the full-length protein, non-reducing western blot conditions). Right panel, a smear corresponding to glycanated GPC3 is detected by the anti-GPC3 antibody 1G12 (directed to an epitope located in the C-terminus). Arrows indicate the detection of the immature non-glycanated core proteins. The positions of molecular-mass markers are indicated on the right. (B) GPC6 inhibits Wnt3a-induced phosphorylation of LRP6. 293T cells were transfected with GPC3 or GPC6 expression vectors or vector control (EF or pCDNA), and were stimulated with Wnt3a (W3a)- or control (L)-conditioned medium for 1 h. Cells were then lysed, and the levels of phospho-LRP6 (Ser1490) (P-LRP6) were assessed by western blotting. The membrane was then re-probed for total LRP6 levels (LRP6). This experiment was repeated three times with similar results. Lower panel, bands were scanned and quantified using NIH ImageJ software. The ratio of phosphorylated LRP6 to total LRP6 was then calculated. The levels of Wnt3a-induced LRP6 phosphorylation of vector-control-transfected cells were arbitrarily assigned a value of 1. Bars represent the mean+s.d. of three independent experiments. * $P = 0.005$.

result, the GPC6–FZD4 interaction was assessed in intact cells by performing a cell-binding assay. To this end, FZD4- or vector-control-transfected cells were incubated with conditioned medium containing equal activities of either GPC6–AP or AP as a control, for 2 hours at 8°C. Cells were then washed and lysed, and the amount of AP activity that remained was measured. Cells incubated with a GPC3–AP fusion protein were used as a positive control. As shown in Fig. 6B, we did not see any significant binding of GPC6–AP to the FZD4-expressing cells, indicating that these two proteins do not interact in intact cells. We also investigated whether GPC6 can interact with FZD7 and FZD8,

as was shown for GPC3. We found that none of these Frizzled proteins bound to GPC6–AP (Fig. 6B).

DISCUSSION

In this study, we provide crucial insight into the molecular mechanisms of GPC3-induced stimulation of canonical Wnt signaling, by showing that, in addition to interacting with Wnt, this glypican binds to Frizzled. Based on this, and on other results included in this paper, we propose that GPC3 can form a complex with Wnt and Frizzled at the cell membrane. This is consistent with our finding that GPC3 is internalized in complexes that also

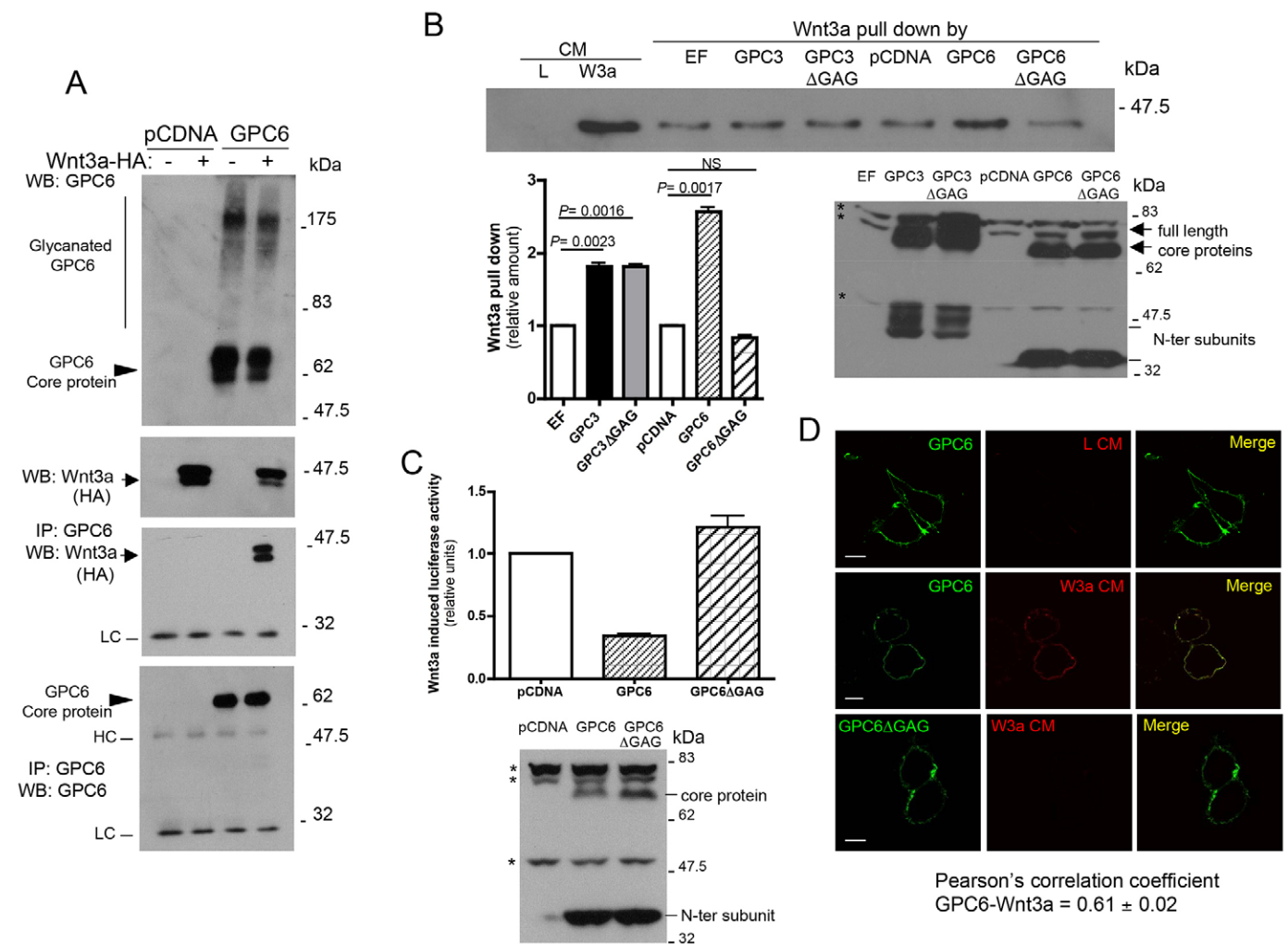


Fig. 5. GPC6 interacts with Wnt3a at the cell surface through the GAG chains. (A) Co-immunoprecipitation. 293T cells were transfected with GPC6, HA-tagged Wnt3a or the corresponding control expression vectors, and GPC6 was immunoprecipitated. The presence of Wnt3a in the precipitated material was assessed by western blotting (WB) with an anti-HA antibody (third panel). The levels of GPC6 (first panel) and Wnt3a (second panel) in whole-cell lysates, and the presence of GPC6 in the precipitated material (fourth panel) were assessed by western blotting. The assessment of GPC6 levels in whole lysates was performed under non-reducing conditions, which allow the detection of the glycanated GPC6. IP, immunoprecipitation; LC, immunoglobulin light chain; HC, immunoglobulin heavy chain; HA, hemagglutinin A. Molecular-mass markers are shown on the right. (B) Pull-down assay. Lysates of 293T cells that were transiently transfected with HA-tagged GPC3, GPC3ΔGAG, GPC6 or GPC6ΔGAG expression vectors or the corresponding control vectors were immunoprecipitated using an anti-HA antibody and Protein G beads. The beads were then incubated with equal amounts of Wnt3a (W3a)-conditioned medium (CM) for 2 h. After washing to remove the unbound material, the amount of Wnt3a that was bound to the beads was assessed by western blotting (upper panel). The blots were scanned and analyzed with ImageJ NIH software. Lower left panel, bars represent the amount of Wnt3a bound to the indicated glypican-covered beads (relative to the amount of Wnt3a bound to control beads). Data are shown as the mean±s.d. of three independent experiments (P -values are indicated above the bars; NS, not significant). Some non-specific binding of Wnt3a to EF and pCDNA control beads is detected. Lower right panel, western blot analysis performed with an anti-HA antibody assessing the levels of GPC6 and GPC3 in the lysates of the transfected cells. Asterisks (*), nonspecific bands detected by the anti-HA antibody. Molecular-mass markers are shown on the right. (C) GPC6ΔGAG does not inhibit Wnt3a-induced luciferase activity. 293T cells were transfected with the indicated expression vectors along with TOPFLASH and β-galactosidase. The cells were then stimulated overnight with Wnt3a- or L control-conditioned medium, lysed and a luciferase assay was performed. Upper panel, bars represent the fold stimulation induced by Wnt3a (mean±s.d. of triplicates). The experiment was repeated twice with similar results. Lower panel, western blot analysis of GPC6 expression levels in the transfected 293T cells. Molecular-mass markers are indicated on the right. Asterisks (*), nonspecific bands. Note that under reducing conditions the levels of the N-terminal subunit provide the best assessment of expression levels. (D) GPC6 binds to Wnt3a at the cell surface. 293T cells that were transfected with GPC6 or GPC6ΔGAG were incubated with control (L) or Wnt3a-conditioned medium for 1 h at 8°C and fixed. GPC6 (green) and Wnt3a (red) were then immunostained. Yellow in merged pictures indicates colocalization. Scale bars: 10 μm. Pearson's correlation coefficient for colocalization of GPC6 and Wnt3a is also shown (mean±s.d. of 15 representative images).

contain Wnt3a and FZD8. We also propose that the presence of GPC3 raises the affinity of the Wnt–Frizzled interaction, thus leading to an increase in the levels of signaling-productive complexes between the ligand and the receptor.

In this study, we have not assessed the role of LRP5/6 in the endocytosis of the GPC3–Wnt–Frizzled complex. However,

because it is well established that, in the presence of canonical Wnt, LRP5/6 forms a complex with this ligand and Frizzled (Niehrs, 2012), we expect that LRP5/6 would also be part of the GPC3-containing complex.

We used two different approaches to demonstrate the interaction between GPC3 and various Frizzled proteins: co-immunoprecipitation

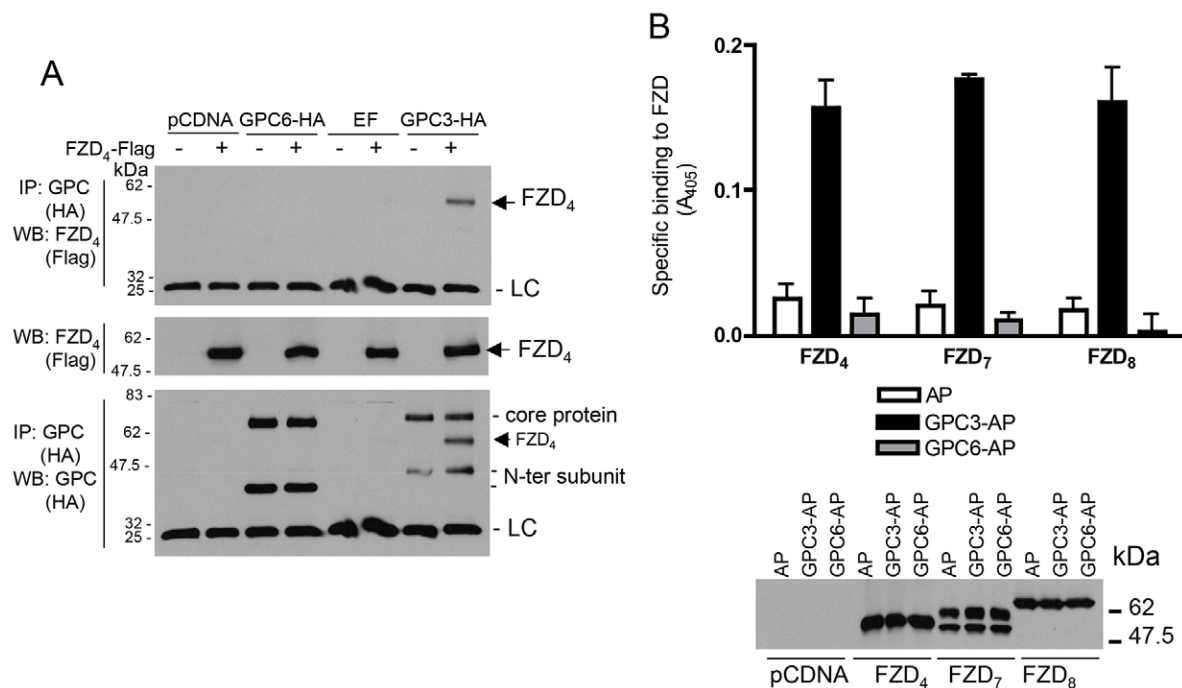


Fig. 6. GPC6 does not interact with Frizzled-4. (A) Co-immunoprecipitation. 293T cells were transfected with HA-tagged GPC6, HA-tagged GPC3, FLAG-tagged FZD4 (FZD₄) or the corresponding control expression vectors (pCDNA or EF), and GPC6 or GPC3 were immunoprecipitated using an anti-HA antibody. Upper panel, a western blot (WB) used to assess the presence of FZD4 in the precipitated material. The levels of FZD4 in whole-cell lysates (middle panel) and the levels of immunoprecipitated GPC6 or GPC3 (lower panel) were assessed by using western blotting. IP, immunoprecipitation; LC, immunoglobulin light chain; HA, hemagglutinin A. The positions of molecular-mass markers are indicated on the left. (B) Cell binding assay. 293T cells that were transfected with FLAG-tagged FZD4, FZD7, FZD8 or vector control were incubated with GPC6-AP, GPC3-AP or AP alone for 2 h at 8°C. The cells were then washed and lysed, and the AP activity of aliquots containing equal amounts of protein was determined. The background binding to cells transfected with vector control (pCDNA) (~40% of total binding) was subtracted from each measurement. Bars represent the mean+s.d. of triplicates. This experiment was performed three times with similar results. Western blot assessment of Frizzled expression levels in the different treatment groups is shown below the graph. The positions of molecular-mass markers are indicated on the right.

performed in transiently transfected 293T cells, and pull-down assays with FZD4, FZD7 and FZD8. Notably, we also showed that a non-glycanated GPC3 cannot bind to any of the Frizzled proteins in the same assay, indicating that the GAG chains mediate the interaction. In addition, we found that these GAG chains interact with the CRD of Frizzled.

As a result of recent studies on the interaction of GPC4, Wnt3a and FZD2, Sakane et al. (Sakane et al., 2012) proposed that glypicans stimulate Wnt signaling by binding to and concentrating Wnt at the cell surface, where the signaling receptors are located. The authors of this study excluded a more direct role for GPC4 in this stimulation of Wnt activity because they found that GPC4 cannot compete with Frizzled for Wnt3a binding. However, this result does not exclude the possibility that Wnt3a can simultaneously bind to Frizzled and GPC4. In fact, the observation, made in the same study, that GPC4 colocalizes with FZD2 and LRP6 on the cell membrane and in endocytic vesicles after Wnt3a-induced internalization is consistent with a model in which GPC4, FZD2 and LRP6 are part of the same protein complex.

Additional strong evidence indicating that the role of GPC3 in Wnt signaling goes beyond its ability to increase the concentration of ligand at the cell surface is provided here by our results, which show that, in the same cellular context in which GPC3 stimulates canonical Wnt signaling, GPC6 acts as an inhibitor, despite the fact that this glypican can bind Wnt3a at the cell surface. Our observation that, unlike GPC3, GPC6 does not

interact with Frizzled, suggests that this glypican cannot form a signaling complex with Wnt and Frizzled, and therefore acts as a competitive inhibitor. The fact that the GPC6-induced inhibition of canonical Wnt signaling occurs at the signal-reception level, and that the inhibitory effect is not observed with a non-glycanated GPC6 that cannot bind to Wnt3a, provides additional support to this conclusion.

Previously, Caneparo et al. (Caneparo et al., 2007) reported that Knypek, the *Xenopus* ortholog of GPC4 and GPC6, can bind to Dkk1, a secreted inhibitor of canonical Wnt signaling. It is possible therefore that the ability of GPC6 to increase the levels of Dkk1 at the cell surface could also contribute to the Wnt-inhibitory activity of this glypican.

It has been shown recently that to be able to stimulate canonical Wnt signaling, GPC4 has to be localized at lipid rafts, and that the stimulation of non-canonical Wnt signaling only occurs when GPC4 is outside of these rafts (Sakane et al., 2012). Significantly, a mutated GPC4 that is forced to localize outside of these rafts acts to inhibit canonical Wnt signaling and to stimulate non-canonical signaling. However, we consider it unlikely that localization outside of the lipid rafts could explain the inhibitory activity of GPC6 on canonical Wnt signaling described in this study, because we find that GPC6 also inhibits non-canonical signaling in the same cellular system (data not shown).

The fact that the GAG chains are essential for the interaction between GPC3 and Frizzled suggests that a non-glycanated GPC3 might not be able to stimulate Wnt activity. However, we have

shown previously that the role of the GAG chains in the GPC3-induced stimulation of canonical Wnt signaling depends on the cell context. For example, the GAG chains were required for GPC3-induced stimulation of canonical Wnt3a signaling in the HCC cell line HLF *in vitro*, but they were not required in the case of the HCC cell line PLC-PRF-5 (Capurro et al., 2005). Based on these results, we speculated that the role of the GAG chains in the GPC3-induced stimulation of canonical Wnt activity might depend on the type of Wnt or Frizzled involved, and/or on the levels of Wnt, Frizzled and GPC3 expressed by a particular cell (Capurro et al., 2005). In fact, because Wnts can interact directly with the core protein of GPC3 (Capurro et al., 2005), it is possible that, in cells expressing high levels of Frizzled, non-glycanated GPC3 could still facilitate a productive interaction of Wnt with Frizzled by binding to Wnt at the cell surface and reducing the dimensionality of ligand diffusion, thus increasing the frequency of encounters between Wnt and Frizzled (Schlessinger et al., 1995). Although GPC6 is also able to reduce the dimensionality of ligand diffusion, our observation that its interaction with Wnt3a is mediated by the GAG chains leads us to propose that, in this context, Wnt cannot form a productive interaction with Frizzled.

It has been reported previously that the GAG chains of GPC3 and Dally are not required for their interaction with Wnts (Capurro et al., 2005; Song et al., 2005; Yan et al., 2009). Interestingly, here, we show that the GAG chains are essential for the interaction of GPC3 and Frizzled. Based on this finding, we propose that, in cellular contexts with low levels of Frizzled, GPC3 might facilitate the interaction between Wnt and Frizzled by engaging the former mainly through the core protein, and the latter through the heparan sulfate chains. Notably, this mechanism is significantly different to that described for GPC5-induced stimulation of Hh signaling (Li et al., 2011). In the case of GPC5, we have shown that the heparan sulfate chains mediate the interaction of this glypican with both the ligand Sonic hedgehog (Shh) and the signaling receptor Patched (Li et al., 2011). It should be noted, however, that the protein core is necessary for the GPC5-induced stimulation of Hedgehog (Hh) signaling, because this activity could not be replaced by heparin alone (Li et al., 2011).

We have recently reported that GPC3 acts as a negative regulator of Hh signaling in the mouse embryo (Capurro et al., 2008). The inhibitory activity of GPC3 is most likely due to the fact that GPC3 binds with high affinity to Hh but does not interact with Patched, and it therefore competes with this receptor for Hh binding. Thus, GPC3 displays opposing functions with regard to the Wnt and Hh signaling pathways, at least in the cell systems in which they have been investigated.

Recently, Yan et al. (Yan et al., 2009) reported that Dally-like (Dlp), one of the two *Drosophila* glypicans, displays a biphasic regulatory activity on Wnt signaling in *Drosophila* wing disks. By using transfected cultured cells, the authors showed that Dlp stimulates Wnt signaling at low Dlp:Frizzled ratios, but that at high Dlp:Frizzled ratios the Dlp-induced stimulation is reduced. We have also observed a reduction in the Wnt-stimulatory activity at high concentrations of GPC3 (data not shown). These results are consistent with our model proposing that glypicans stimulate Wnt signaling by forming a complex with Wnts and Frizzled, because, according to this model, it would be expected that if there is more glypican than Frizzled at the cell surface, the excess glypican could act in a competitive-inhibitory manner to reduce the Wnt-stimulatory activity. There are, however, two

important differences between our results and those reported by Yan et al. (Yan et al., 2009) in *Drosophila*. First, these authors did not detect any interaction between Dlp and Frizzled in co-immunoprecipitation studies. Second, they found that the heparan sulfate chains of Dlp do not play a role in the Wnt-stimulatory activity of Dlp. Whether these differences are due to the varied experimental systems used in each study, or to species-specific features of the Wnt signaling pathway remains to be determined.

Our laboratory has recently reported that a mutated GPC3 that is secreted into the extracellular environment because it cannot be anchored to the cell membrane can inhibit canonical Wnt signaling in various HCC cell lines (Capurro et al., 2005; Zittermann et al., 2010). The results presented here are consistent with our previous report. It is reasonable to expect that a secreted GPC3 will not facilitate the interaction of Wnt and Frizzled at the cell surface. Conversely, it is expected that a secreted GPC3 will compete with Frizzled for Wnt binding, thus acting as an inhibitor of Wnt signaling.

MATERIALS AND METHODS

Cell lines, plasmids and transfections

The cell lines 293T, L and L-Wnt3a were obtained from the ATCC (Manassas, VA). All cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Expression vectors for GPC3, GPC3ΔGAG, GPC3-AP and GPC3ΔGAG-AP inserted in the pEF plasmid, have been described previously (Capurro et al., 2008; Gonzalez et al., 1998). FLAG-tagged Frizzled vectors were provided by Liliana Attisano (University of Toronto, Canada), and the Frizzled-8-YFP vector was provided by Christof Niehrs (DKFZ, Heidelberg, Germany). Myc-tagged FZD-CRD vectors were a gift from Jeremy Nathans (The Johns Hopkins University, Baltimore, MD). The full-length human GPC6 DNA inserted in the pCDNA plasmid was obtained from Alex Tokar (Harvard Medical School, Boston, MA). A non-glycanated mutant of GPC6 (GPC6ΔGAG) was generated by mutating four serine residues (Ser⁴⁹³, Ser⁴⁹⁵, Ser⁴⁹⁷ and Ser⁴⁹⁹) to threonine (Thr), alanine (Ala), Ala and Thr, respectively, by site-directed mutagenesis. Mutations were verified by DNA sequencing. GPC6-AP was generated by inserting the human GPC6 cDNA into the *BspEI* site of the pAP-Tag2 vector (Gene-Hunter Corporation, Nashville, TN). Transfection of 293T cells was performed by using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). Conditioned media containing AP fusion proteins were generated by transfecting 293T cells with the indicated expression vectors, and were collected 48 h after transfection in serum-free conditions. L- and Wnt3a-conditioned media were collected after growing the L mouse fibroblast (L) or Wnt3a-stably transfected L cells (Wnt3a) at high confluence for 4 days in DMEM containing 2% FBS. After collecting the conditioned medium, cell debris was removed by centrifugation. Conditioned media were stored at −80 °C.

Assessment of LRP6 phosphorylation

293T cells were transiently transfected with a GPC3 or GPC6 expression vector, or respective control vectors. The following day, the medium was replaced with fresh medium containing 1% FBS. After an overnight incubation, L- or Wnt3a-conditioned medium diluted 1:3 with serum-free medium was added for 1 h. Cells were then lysed, and the levels of phosphorylated LRP6 (P-LRP6) and total LRP6 were assessed by western blot analysis. When indicated, other dilutions of L- and Wnt3a-conditioned medium were added. The antibody against P-LRP6 (Ser1490) was from Cell Signaling Technology (Danvers, CA), and the antibody against total LRP6 was from Santa Cruz Biotechnology (sc-25317, Santa Cruz, CA).

Luciferase assay

293T cells were seeded in 24-well plates and were co-transfected with a luciferase reporter vector [in which luciferase expression is driven by a β -catenin-responsive promoter (TOPFLASH)], β -galactosidase and the

indicated amounts of GPC3 or GPC6 expression vectors or control vectors. One day after transfection, cells were incubated overnight with L- or Wnt3a-conditioned medium, lysed, and luciferase activity [Luciferase Assay System, Promega (Madison, WI)] and β -galactosidase activity were determined. Each luciferase value was normalized for transfection efficiency using β -galactosidase activity.

Co-immunoprecipitation

Transfected 293T cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS), and the cell lysates were precleared with protein-G-Sepharose for 1 h at 4°C. GPC3 or GPC6 were then immunoprecipitated by incubating the cell lysates overnight with the anti-HA 12CA5 monoclonal antibody (Roche, Laval, QC, Canada) or the anti-human GPC6 antibody AF2845 (R&D Systems, Minneapolis, MN). The presence of FLAG-tagged FZD4 or HA-tagged Wnt3a in the precipitated material was assessed by western blotting with anti-FLAG M2 (Sigma-Aldrich, St Louis, MO) or 12CA5 antibodies, respectively.

Pull-down assay

For the GPC3–Frizzled pull-down assay, 293T cells transfected with the indicated vectors were lysed in RIPA buffer, and the cell lysates were first incubated overnight with an anti-FLAG M2 antibody to detect Frizzled or with an anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology) to detect Myc-tagged Frizzled-CRD, and then with protein-G-Sepharose for 90 min at 4°C. Beads were then washed four times with RIPA buffer and were blocked for 2 h with 5% BSA in PBS containing 0.1% Triton X-100. Aliquots with equal amount of beads were then incubated for 1 h with GPC3–AP, GPC3 Δ GAG–AP or AP-conditioned medium containing the same amount of AP activity. Beads were then washed four times with 20 mM HEPES pH 7.4, 150 mM NaCl and 0.25% Tween 20. The AP activity that was bound to the beads was measured by using *p*-nitrophenyl phosphate disodium hexahydrate (Sigma-Aldrich) as the substrate. When indicated, different concentrations of heparin were added to the GPC3–AP or control AP-conditioned medium. For the Wnt3a–glypican pull-down assay, lysates of 293T cells that were transfected with HA-tagged GPC3, GPC3 Δ GAG, GPC6 or GPC6 Δ GAG, or the corresponding control vectors (EF or pCDNA) were immunoprecipitated using the anti-HA antibody 12CA5 and protein G-Sepharose beads. The beads were then washed three times with PBS containing 0.5% CHAPS, and were incubated with 1 ml of Wnt3a-conditioned medium for 2 h. After three more washes with 0.3% CHAPS in PBS, the beads were resuspended in sample buffer, and the amount of Wnt3a that was bound to the beads was detected by western blot with an anti-Wnt3a antibody (2391, Cell Signaling Technology).

Cell-surface binding and endocytosis assay

293T cells transfected with GPC3 and FZD8–YFP expression vectors were trypsinized and plated onto poly-L-lysine-treated coverslips. At 24 h after transfection, the cells were incubated with ice-cold DMEM containing 20 mM HEPES pH 7.4 and 0.1% BSA for 30 min at 8°C, and were treated with Wnt3a- or control L-conditioned medium for another hour at the same temperature. To assess cell surface binding, unbound ligand was removed by washing three times with ice-cold PBS, and the cells were fixed with 4% paraformaldehyde in PBS for 15 min. Alternatively, after removal of unbound ligand, warm DMEM was added and the cells were transferred to 37°C for 75 or 80 min to allow endocytosis to proceed. Cells were then washed with PBS and were fixed as described above. For immunostaining, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min, and were blocked with 5% non-fat dry milk in PBS (blocking buffer) for 30 min. All incubations with primary and secondary antibodies were performed in blocking buffer for 1 h at room temperature. The antibodies used were: mouse anti-GPC3 1G12 monoclonal antibody, rat anti-Wnt3a monoclonal antibody (MAB1324, R&D Systems), rabbit anti-caveolin-1 polyclonal antibody (clone N-20, Santa Cruz Biotechnology), Alexa-Fluor-647-conjugated donkey anti-mouse-IgG (Invitrogen) and Cy3-conjugated donkey anti-rat-IgG (Jackson ImmunoResearch, Bar Harbor, ME). To study GPC6–Wnt3a cell-surface binding, 293T cells transfected

with HA-tagged GPC6 or GPC6 Δ GAG, or pCDNA were plated onto coverslips, and incubated with L- or Wnt3a-conditioned medium. For immunostaining, GPC6 was detected with mouse anti-HA 12CA5 monoclonal antibody and Alexa-Fluor-488-conjugated donkey anti-mouse-IgG (Invitrogen). Confocal Images were generated using a scanning laser microscope LSM 510 v3.2 SP2 (Carl Zeiss Inc., Pickering, ON, Canada) and Zeiss LSM Image Browser. Colocalization between the indicated proteins was quantified by using Pearson's correlation coefficient, using a minimum of 14 representative cells from at least three independent experiments. Only the caveolin immunostaining experiment was performed twice.

Cell-binding assay

293T cells were transfected with FLAG-tagged FZD4, FZD7, FZD8 or with pCDNA as a control. At 1 day after transfection, cells were transferred to 8°C, and GPC6–AP-, GPC3–AP- or AP-conditioned media containing the same amount of AP activity were added to the cells for 2 h. After unbound ligand was removed by four washes with PBS, the cells were lysed in 10 mM Tris-HCl pH 8 containing 1% NP40. Lysate aliquots with equal amount of proteins were heated to 65°C for 10 min to inactivate the cellular phosphatases, and the AP activity was then measured with a Sigma fast *p*-nitrophenyl phosphate tablet set (Sigma-Aldrich).

Competing interests

The authors declare no competing interests.

Author contributions

M.C., W.S. and T.M. performed the experiments, M.C. and J.F. wrote the manuscript.

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