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Altering Glypican-1 levels modulates canonical Wnt signaling during trigeminal placode development

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Abstract

Glypicans are conserved cell surface heparan sulfate proteoglycans expressed in a spatiotemporally regulated manner in many developing tissues including the nervous system. Here, we show that Glypican-1 (GPC1) is expressed by trigeminal placode cells as they ingress and contribute to trigeminal sensory neurons in the chick embryo. Either expression of full-length or truncated GPC1 in vivo causes defects in trigeminal gangliogenesis in a manner that requires heparan sulfate side chains. This leads to either abnormal placodal differentiation or organization, respectively, with near complete loss of the ophthalmic (OpV) trigeminal ganglion in the most severe cases after over-expression of full-length GPC1. Interestingly, modulating GPC1 alters levels of endogenous Wnt signaling activity in the forming trigeminal ganglion, as indicated by Wnt-reporter expression. Accordingly, GPC1 over-expression phenocopies Wnt inhibition in causing loss of OpV placodal neurons. Furthermore, increased Wnt activity rescues the effects of GPC1 over-expression. Taken together, these results suggest that appropriate levels of GPC1 are essential for proper regulation of canonical Wnt signaling during differentiation and organization of trigeminal placodal cells into ganglia.

Keywords

glypican; trigeminal ganglion; placode; Wnt

Introduction

Glypicans (GPCs) are a conserved family of cell surface heparan sulfate proteoglycans (HSPGs) that modulate major signaling pathways during embryonic development of fruit flies to mammals (Fico et al., 2007; Filmus et al., 2008). Heparan sulfate side chains, attached to the core protein at conserved serine residues near the membrane anchored glycosyl-phosphatidylinositol (GPI) linkage, are thought to facilitate binding of growth factors and ligands, including Wingless/Wnt, Dpp/BMP, Fgf and Hh, and are considered ligand carriers or co-receptors. Accordingly, functional perturbation of glypicans has been shown to cause significant defects in cell fate, cell movements, survival and proliferation in mice, *Xenopus laevis*, *Drosophila*, and zebrafish (Filmus and Song, 2000; De Cat and David, 2001; Lin, 2004; Fico et al., 2007).

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During vertebrate development, glypicans are expressed in a spatiotemporally regulated manner in the nervous system and other tissues (Niu et al., 1996; Saunders et al., 1997; Litwack et al., 1998; Luxardi et al., 2007). Their expression changes in pathological conditions, such as cancer. GPC3 and/or GPC1 are down-regulated in some ovarian cancer and mesothelioma cell lines (Lin et al., 1999; Murthy et al., 2000) while upregulated in others (e.g. pancreatic tumors) (Kleeff et al., 1998; Filmus, 2001; Matsuda et al., 2001; Su et al., 2006). Six glypican (GPC1–6) family members have been identified in mammals, two in *Drosophila melanogaster* (Dally and Dally-like), and two *in Caenorhabiditis elegans* (gpn-1 and lon-2) (Fico et al., 2007). Loss of function mutations in OCI-5/GPC3 in humans cause Simpson-Golabi-Behmel syndrome (SGBS), characterized by pre- and post-natal overgrowth, visceral and skeletal defects, and an increased risk of tumors (Pilia et al., 1996). A GPC3 deficient mouse model exhibits a similar phenotype (Cano-Gauci et al., 1999). Of the six glypicans in mammals, GPC1 is the most abundantly expressed in the developing brain, in both neuroepithelial precursors and differentiated neurons (Karthikeyan et al., 1994; Litwack et al., 1994; Litwack et al., 1998) and functions in neurogenesis of the central nervous system (Jen et al., 2009). However, its potential role in patterning and formation of the peripheral nervous system has yet to be explored.

Here, we show that Glypican-1 is expressed by ectodermal and ingressing chick trigeminal placode cells at the time they differentiate into neurons and assemble into ganglia. Modulation of GPC1 levels by either expression of full-length GPC1 or a truncated form that is thought to act in a dominant-negative fashion prevents proper placodal differentiation and formation of the trigeminal ganglion, with particularly strong effects on the ophthalmic lobe. Consistent with studies showing that Wnt signaling is important for differentiation of ophthalmic trigeminal placodes (Lassiter et al., 2007; Canning et al., 2008), we find that GPC1 regulates Wnt activity in OpV ganglion formation. The results suggest that proper levels of GPC1 are critical for appropriate modulation of canonical Wnt signaling for differentiation and assembly of trigeminal placodes into ganglia.

Materials and Methods

Embryos

Fertilized chicken (*Gallus gallus domesticus*) eggs were obtained from local commercial sources and incubated at 37°C to the desired stages.

In situ hybridization

cDNA plasmid obtained from BBSRC (ChickEST clone 418p2) was used to transcribe antisense riboprobe against chick Glypican-1. The plasmid was sequenced and found to contain the coding sequence of the chick Glypican-1 gene (NCBI accession number: XM_422590.1) corresponding to nucleotides 1233–2107. Whole mount chick in situ hybridization was performed as described (Shiau et al., 2008). Embryos were imaged and subsequently sectioned at 12 μm.

Immunohistochemistry

Primary antibodies used were: anti-TuJ1 (Covance; 1:250), anti-HNK-1 (American Type Culture; 1:3–1:5), anti-GFP to recognize GFP signal after in situ hybridization (Molecular Probes; 1:150–1:250), anti-Islet1 (DSHB, clone 40.2D6; 1:150–1:250) and anti-active-caspase-3 (Promega; 1:150–250). Appropriate secondary antibodies against the subtype of the primary antibodies were conjugated to Alexa Fluor 488, 568, or 350 dyes (Molecular Probes). Images were taken on a Zeiss Axioskop2 plus fluorescence microscope, and processed using Adobe Photoshop CS3.

In ovo electroporation of the trigeminal ectoderm

Plasmid constructs were targeted to the presumptive trigeminal placodal ectoderm at the approximate axial level between the posterior forebrain and anterior hindbrain at 5 somites stage (stage 8+) up to stage 11. Immediately after injection, platinum electrodes were placed vertically across the chick embryo delivering current pulses of 5 × 8 V in 50 ms at 100 ms intervals as described (Shiau et al., 2008). Targeting DNA to the ectoderm resulted in transfection of the trigeminal placodes in the ectoderm and subsequently placode-derived cells that detach from the ectoderm and migrate into the ganglion anlage. The operated eggs were sealed and incubated at 37°C for later analysis. Incubation times were ~16–24 hours to reach stages 13–14, ~ 24–36 hours to reach stages 15–16, ~ 40–48 hours to reach stages 17–18, and ~ 50–60 hours to reach stage 19.

Plasmid constructs

Full length chick Glypican-1 cDNA (clone CS5) was isolated from a 4– to 12– somites stage chick macroarray library (Gammill and Bronner-Fraser, 2002). To create cytopcig-FL-GPC1, the full-length coding sequence (1.65 kb) was amplified from the library clone CS5 by PCR using forward and reverse primers corresponding to the coding sequence with flanking XhoI and ClaI site respectively. Gene fragment was directionally inserted into the cytopcig vector (Shiau et al., 2008) at the XhoI/ClaI sites. Three mutant GPC1 constructs were made by a single-step or fusion PCR amplification off the full-length sequence, and modified gene fragment was directionally cloned into the XhoI and ClaI sites in the cytopcig vector. These are: 1) cytopcig-GPC1-ΔGPI which encodes the first 517 amino acids (1.55 kb) with a premature stop codon which eliminates the GPI-anchoring domain, 2) cytopcig-GPC1-ΔHS which has a 18bp deletion that spans a coding region of six residues, SGSGSG (483–488 aa), containing three tandemly positioned putative glycosaminoglycan (GAG) attachment sites (serine residues 483, 485, 487) based on sequence annotation in UniProtKB for chick GPC1 protein (accession P50593), and 3) cytopcig-GPC1- ΔGPI ΔHS which has deletions of both the GPI-anchor domain and the putative HS sites. Two versions of full-length and mutant constructs were made: one without myc-tag fusion and one with a 6x myc-tag inserted at ClaI/EcoRV sites in frame with the coding sequence at the C-terminus, which was used to validate protein expression of the constructs. Both versions were tested and determined to give the same effects on ganglion development, albeit FL-GPC1 was more potent without the myc-tag. Thus, most experiments with the full-length and mutant expressions were conducted with the construct lacking the myc-tag. Dominant active ß-catenin construct was made in the pCIG vector with IRES-nuclear localized GFP as

previously described (Megason and McMahon, 2002; Lassiter et al., 2007), and RFP-Wnt-Reporter (also named pTOP-nDSRed2) (gift from Dr. Andy Groves;(Lassiter et al., 2007)) was a modified version of the TOPGAL construct (DasGupta and Fuchs, 1999) where the reporter gene was replaced with RFP.

BrdU treatment

Electroporated embryos were screened and selected for broad GFP expression in the trigeminal region at stage 14 for both control and experimental cases. Each embryo was explanted into an individual well and treated with 0.1 mM BrdU in Ringer's solution at 37°C for 30 minutes. Embryos were then fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, incubated in 2N HCl in PBS for 30 minutes, followed by 0.1M perboric acid (H_3BO_4) for 10 minutes, washed in PBS several times, and processed for cryosectioning and immunostaining with the mouse anti-BrdU (Sigma, B2531; 1:150–1:250).

Quantification of the area of trigeminal ganglion

An outline of the ganglion as marked by TuJ1 staining was made by the freehand selection tool on whole mount grayscale fluorescent images in the ImageJ software. All images were taken with the same setup using a 5x objective on the Zeiss Axioskop2 plus microscope and at the same image size, with the entire ganglion in focus. The area of the ganglion outline was determined by the area measurement function in ImageJ with the scale calibrated to the actual length.

Results

Expression of Glypican-1 mRNA in the trigeminal placodes and other embryonic tissues during early chick development

As a first step in examining the possible developmental role of Glypican-1 (GPC1), we first characterized its mRNA expression in the chick embryo at stages 9–18 by whole mount in situ hybridization (Fig. 1 and data not shown). This corresponds to the time window of early trigeminal development starting from neural crest migration to ganglion condensation. No expression of GPC1 mRNA was noted in the trigeminal neural crest cells, derived from the midbrain and anterior hindbrain (rhombomeres 1 and 2) levels of the neural tube, from stage 9 through gangliogenesis (Fig. 1, A–C, F, L). Interestingly, we find that GPC1 is expressed by the presumptive trigeminal placodal ectoderm starting at approximately stage 12, coincident with the beginning of placodal differentiation and ingression, but not earlier (Fig. 1C, D). GPC1 expression persists later as placodal cells assemble and condense into ganglion at stages 14–16 (Fig. 1F, L) and later at stage 18 (data not shown). GPC1 is expressed by both the ophthalmic (OpV) and maxillo-mandibular (MmV) placodes that form the trigeminal ganglion (Fig. 1, F, L). To confirm that these GPC1 expressing cells are in fact placode-derived, we labeled the placodal ectoderm with GFP by in ovo electroporation prior to placodal ingression. Embryos then were collected at later stages after placodal cells have begun to delaminate from the ectoderm. GPC1 expression was detected by in situ hybridization in the GFP expressing placodal cells that had ingressed into the mesenchyme. Results show that all GFP expressing placode-derived cells and discrete regions of the placodal ectoderm express GPC1 (Fig. 1, G–J), while the interacting HNK-1 positive

trigeminal neural crest cells do not. Not all placode-derived cells are GFP-labeled as the transfection of the ectodermal region was mosaic in some cases.

In addition to placodal cells, GPC1 mRNA is expressed in other tissues. In contrast to the midbrain neural crest, GPC1 is detected in the migrating hindbrain neural crest cells from rhombomeres 4 and 6 during migration at stage 12 (Fig. 1C, E), albeit transiently, being down-regulated by stage 14 (Fig. 1M, N). In the more posterior placodes, GPC1 is expressed in the epibranchial placodal ectoderm at stages 14–16 (Fig. 1F, L–M). The otic placode expresses GPC1 by stage 12, albeit weakly; by stage 14, its expression is strong in the invaginating and forming otic vesicle (Fig. 1F, M). Furthermore, GPC1 was weakly expressed in the forming cranial neural tube and notochord throughout these stages (Fig. 1, A–M). The developing mesoderm and forming limb bud also express GPC1 (Fig. 1K). Expression is particularly dynamic in the developing somites. Through stages 9–18, the GPC1 mRNA appears to be expressed in a gradient in the presomitic mesoderm (PSM) highest at the newly forming somites and decreasing both rostrally toward the more anterior somites and caudally toward the tail (Fig. 1B, O). The expression patterns of GPC1 in the forming neural tube, somite, and limb are consistent with those described previously (Niu et al., 1996). This study analyzed developmental stages at st.7–12 and 20–25, but not the time window (st.13–18) or tissues involved in gangliogenesis as demonstrated here.

The multiple tissue specific expression patterns of GPC1 in the early developing chick embryo are consistent with possible roles for GPC1 patterning several different embryonic regions. The GPC1 expression in the trigeminal placodes at the time of neuronal differentiation and ganglion assembly, after specification, raises the intriguing possibility that GPC1 may have a role in regulating later events of trigeminal development.

Over-expression of Glypican-1 in placodes causes loss of trigeminal ganglia

Glypicans are thought to act as ligand carriers or co-receptors for several major families of signaling molecules (Wnt, FGF, BMP, Hh) (Lin, 2004; Fico et al., 2007); some of these have been implicated previously in trigeminal placode formation, most notably Wnts and Fgfs (Stark et al., 2000; Lassiter et al., 2007; Canning et al., 2008; Lassiter et al., 2009). Since modulation of glypican expression can differentially affect cellular behavior (Qiao et al., 2008) as well as the distribution and signaling of these growth factors (De Cat and David, 2001; Hufnagel et al., 2006), we asked whether changing levels of GPC1 expression in the trigeminal placodes would also affect the signaling events required for normal gangliogenesis. To test this, we expressed higher levels of GPC1 in the trigeminal placodal tissue at or just prior to its endogenous expression in the placodes. Full-length chick GPC1 expression construct (cytopcig-FL-GPC1), or the empty vector (cytopcig) as control, were introduced into the placodal ectoderm by in ovo electroporation at stages 8+ – 11 (5 – 14 ss [somites stage]) before placodal ingression and after expression of the earliest known trigeminal placode fate marker Pax3 mRNA which begins by 4ss (Stark et al., 1997). Embryos with efficient electroporation of OpV and MmV lobes were analyzed at three time points corresponding to early ganglion formation (stages 15 – 16), after condensation (stages 17 – 18), and in the mature ganglion (stage 19). Neuronal components of the ganglion were analyzed by immunostaining with the neuronal marker, ß-neurotubulin, TuJ1. Since neural

crest cells differentiate into neurons much later (beginning at embryonic day 4; ~ stages 22–24)(D'Amico-Martel and Noden, 1980), only placodal cells express this marker at the stages examined, as shown previously by the co-labeling of neuronal markers (TuJ1 and Islet1) with GFP expressed by transfected placodal cells whereas neural crest cells (Sox10 and HNK-1 positive) lack these markers (Shiau et al., 2008; Shiau, 2009).

The results show that over-expression of GPC1 in the placodal ectoderm causes a dramatic loss of the trigeminal ganglion, with nearly the entire OpV lobe missing in many cases (Fig. 2). The penetrance of this effect was categorized according to severity: “reduced” ganglia were clearly smaller by overall size in at least one lobe for all stages, whereas “severely reduced” were those that lost all or most of the OpV lobe at st. 15 – 18 (Fig. 2, A–F) or had both lobes markedly reduced at st. 19 (Fig. 2, G–L). At st. 15 – 18, 45% (n=42) of the cases had markedly “reduced” ganglia whereas ~ 10% were “severely reduced”. By st. 19, 25% (n=12) were “reduced” and 8% were “severely reduced”, while no control GFP embryos showed a reduced phenotype (n=23 at st. 15 – 18, and n= 7 at st. 19) (Fig. 2M). The slight recovery with time may be caused by dilution of the construct over time, though we cannot rule out the possibility that there also may be some compensation by other mechanisms. The striking loss of trigeminal placodal neurons in the ganglia caused by alteration in GPC1 expression suggests that appropriate regulation of GPC1 expression is essential for gangliogenesis.

GPC1 over-expression blocks proliferation and differentiation of the placodal ectoderm that leads to loss of ganglia

The severely reduced ganglia caused by GPC1 over-expression prompted us to examine whether this is mediated by defects in cell survival and/or proliferation in the placodal tissue. To analyze changes in cell death, stage-matched FL-GPC1 and control GFP embryos at stage 14 were sectioned and immunostained for active caspase-3 (casp-3), which is a robust marker for apoptotic cells. Given that we can detect a phenotype as early as stages 15–16, embryos were examined at stage 14 which allows analysis of both ingression and coalescence of placodal neurons into ganglion. Only well-transfected FL-GPC1 and control embryos were selected for analysis. We counted transfected GFP+ only and GFP+/casp-3+ double positive cells (apoptotic cells) in the placodal ectoderm as well as in the mesenchyme. The latter correspond to ingressing placode cells and those already in the forming ganglion. The percentage of dying cells was then calculated by dividing the number of GFP+/casp-3+ cells over the total number of GFP+ cells in each region (ectoderm versus mesenchyme). At least ten serial sections through the trigeminal ganglion anlage were analyzed per n. Using this assay, our data show that there is no significant difference in levels of cell death between FL-GPC1 and control embryos (in the ectoderm, 2.2 +/− 0.7 % cell death in FL-GPC1, n=4 compared with 0.85 +/− 0.3 % cell death in control cases, n=4; p-value= 0.22 using a two-tailed student's t-test, Fig. 3A). No cell death was detected in ingressing placodal cells in the mesenchyme for either control or experimental cases. Taken together, data suggest that increased cell death cannot account for the loss of the OpV lobe. Interestingly, however, we found that transfected FL-GPC1, but not control, placodal ectoderm cells tended to abnormally cluster and formed clumps of cells in the ectoderm

instead of forming a normally organized epithelial sheet from which placodes delaminate (Fig. 3B, C).

We next analyzed whether proliferation in the placodal ectoderm is affected, using the same criteria and stages as for analysis of cell death. To determine the percentage of proliferating cells in the placodal tissue at stage 14, we briefly treated electroporated embryos for 0.5 hour at the time of collection at stage 14 with bromodeoxyuridine (BrdU), an analog of thymidine that gets incorporated into dividing cells during S phase. The short treatment provided a snapshot of all proliferating cells at the time point of collection, presumably within the 0.5 hour of treatment. At least six serial sections through the trigeminal ganglion anlage were counted and analyzed per n. Interestingly, BrdU analysis shows a significant reduction in proliferation in placodal ectoderm cells of FL-GPC1 embryos (12.9 +/− 1.3% proliferation in FL-GPC1 (n=6) versus 29.7 +/− 2.7 % in control (n=4) cases; p-value = 0.00061), with relatively normal levels of proliferation in the mesenchymally located placodal cells (8.0 +/− 2.7% in FL-GPC1 and 11.2 +/− 3.8% in controls, p-value = 0.54 using a two-tailed student's t-test) (Fig. 3A, C). This difference between ectodermal and mesenchyme-residing cells suggests that GPC1 over-expression predominantly inhibits proliferation in the placodal ectoderm only.

Since proliferation was significantly blocked in the placodal ectoderm and cell death was not significantly higher, the reduced ganglia phenotype may be due to a decrease in generation of placodal neurons in the surface ectoderm and thus less of them ingressing to form ganglion. In support of this, we found significantly less ingression based on the average number of ectoderm-derived GFP+ cells in the mesenchyme of the ganglion region analyzed in FL-GPC1 (36 +/− 8 cells, n=6) compared with controls (95 +/− 17 cells, n=4; p-value = 0.015 using a two-tailed student's t-test) (Fig. 3A). However, there was no significant difference in the average number of GFP+ in the ectoderm (300 +/− 36 cells in FL-GPC1 versus 342 +/− 5 cells in control GFP cases; p-value = 0.38 using a two-tailed student's t-test)(Fig. 3A). The numbers of ingressed GFP+ cells and those in the ectoderm between control and experimental cases were counted from the same sections as those used for the BrdU analysis.

The onset of GPC1 expression at the beginning of placodal differentiation in the ectoderm (Fig. 1D) is consistent with its possible role in differentiation of placodal cells. Differentiation of neurons appears to occur in the surface placodal ectoderm prior to ingression. This observation is based on the fact that placodal cells in the ectoderm already express neuronal markers (Islet1 and TuJ1) and all, if not most, of them that have ingressed express Islet1 and TuJ1 (as determined by labeling ingressing placodal cells using ectoderm electroporation with a GFP vector and examining whether all ectoderm-derived GFP+ placodal cells express neuronal markers) ((Shiau, 2009) and data not shown). In sections through the ganglion region of experimental and control embryos, we consistently observed fewer placodal neurons in the ectoderm and in the mesenchyme in FL-GPC1 embryos, particularly in the OpV region (Fig. 3D). GPC1 over-expression significantly inhibits proliferation in the ectoderm but not in ingressed placodal cells, and subsequently leads to loss of placodal neurons. This suggests that appropriate regulation of cell division in the ectoderm is crucial for placodal differentiation.

Specification and commitment of the first placode cells to the ophthalmic (OpV) fate occurs by 8 ss (Stark et al., 1997; Baker et al., 1999). We found no difference in the effect of over-expressing GPC1 on ganglion formation at the different stages of electroporation (5–14 ss), before or after 8 ss, suggesting the effects of GPC1 over-expression occur after initial fate specification. Furthermore, no changes were noted in expression of the early OpV placode fate marker Pax3 mRNA expression in FL-GPC1 (n=5/5) embryos compared with controls (n=2) at stages 12–14 (data not shown).

Taken together, the data suggest that the level of GPC1 expression is critical for the appropriate regulation of cell division in the placodal ectoderm, following their specification but prior to differentiation. GPC1 expression correlates with the onset of neuronal differentiation in the placode and its over-expression causes a specific proliferation defect in the differentiating ectoderm. This in turn leads to loss of placodal neurons, mostly those of the OpV lobe. Thus, problems with placodal differentiation likely explain the phenotype caused by GPC1 over-expression.

Altering GPC1 expression causes ganglion disorganization and both truncated and full-length forms require heparan sulfate modification

Several conserved domains of glypicans are important for their function, including the conserved heparan sulfate (HS) chains (long unbranched dissacharides) near the cell surface that attach to specific serine residues near the carboxyl terminus of the core protein and GPI membrane attachment site (De Cat and David, 2001) (see simplified schematic in Fig. 4A). Although a number of studies have highlighted the importance of HS modification for GPC function in regulation of several signaling pathways (including Wnt, Fgf, Hh, BMP) (Hacker et al., 2005; Fico et al., 2007), they are not required in all cases (Gonzalez et al., 1998; Capurro et al., 2005; Kirkpatrick et al., 2006; Williams et al., 2010). GPI anchorage may be important for cell autonomous functions of GPC, such as being co-receptors for stabilizing ligand-receptor interaction or for regulating ligand levels by endocytosis. The GPI linkage also potentially plays a role in post-translational modifications leading to cleavage of GPC to yield soluble forms of glypican that can affect distribution, spreading, or levels of ligand signaling (Fico et al., 2007; Gallet et al., 2008).

To better define the mechanism by which full-length GPC1 (FL-GPC1) affects signaling during trigeminal development, we tested the functional requirement of HS modification and membrane anchoring. Three mutant GPC1 expression constructs were designed and tested in the trigeminal placodes: cytopcig-GPC1-ΔGPI which encodes a soluble truncated form of GPC1 where the GPI anchoring domain and remaining C-terminal sequence are removed, cytopcig-GPC1-ΔHS that has a deletion of all three putative HS attachment sites near the carboxyl terminus, and cytopcig-GPC1- ΔGPI ΔHS which has deletion of both GPI anchoring and glycanation sites (Fig. 4B). We introduced these constructs into the trigeminal placodal ectoderm by in ovo electroporation during the same time window and in parallel with FL-GPC1 and control GFP as discussed above. Ganglia were scored at stages 15–19 for the reduced ganglia phenotype with only well-electroporated embryos selected for analysis.

The results show that electroporation of either the mutant with deletion of the GPI anchoring, HS attachment sites or both fail to cause reduced ganglia observed with the wild type construct (Fig. 4C). To determine the size differences, we quantified the area of the ganglion using the ImageJ area function on outlines of TuJ1 stained ganglia. As expected, the size of the ganglion increases as it continues to grow from stage 15 –16 to 19 (Supplementary Fig. 1), albeit the area value at stages 15–16 is larger due to the fact that the cells are less condensed and more spread out. Compared with controls, the ganglion area was on average decreased by 39.4 +/− 3.7 % after electroporation with FL-GPC1 over all stages. The area of FL-GPC1 electroporated embryos with phenotype (0.066 +/− 0.017 mm^2 at stages 15–16, 0.064 +/− 0.006 mm^2 at stage 17–18, 0.11 +/− 0.011 mm^2 at stage 19) was markedly reduced compared with those electroporated with control GFP constructs (0.10 +/− 0.009 mm^2 at stages 15–16, 0.11+/− 0.005 mm^2 at stages 17–18, 0.18 +/− 0.012 mm^2 at stage 19) or mutant forms of GPC1 expressing placodal ganglia at all stages (Supplementary Fig. 1). In contrast to that of FL-GPC1 (49%, n=53), expression of mutant GPC1 constructs in the trigeminal placodes resulted in significantly fewer ganglia of reduced size (GPC1-ΔGPI, 8.7% (n=23); GPC1- ΔHS, 9% (n=11); GPC1- ΔGPI ΔHS, 5.6%, n=18) (Supplementary Fig. 1). Control GFP ganglia exhibited no apparent ganglion reduction (n=30) over all stages.

Previous studies have shown that the truncated soluble form of GPC that lacks GPI membrane anchorage can act as a dominant-negative inhibitor (Zittermann et al., 2009), presumably by competing with endogenous GPC for binding to signaling factors. Consistent with this idea, we found that expression of GPC1- ΔGPI did not cause the same level of reduced ganglia phenotype as that of FL-GPC1. Instead, it caused a distinct phenotype of disrupting ganglion morphology (26.1%, n=23) at a significantly higher frequency than full-length construct (11.3 %, n=53); other mutant forms did not cause these effects (Supplementary Fig. 1). Furthermore, the subcellular localization of GPC1- ΔGPI is concentrated at or near the membrane. In contrast, the full-length construct is expressed in both the cytoplasm and on the membrane (Supplementary Fig. 2). This suggests that the GPC1- ΔGPI protein is properly processed for secretion to act in a soluble form. This is consistent with previous reports suggesting that a significant portion of secreted glypicans lacking the GPI domain remain associated with the cell membrane due to electrostatic interactions (Carey and Evans, 1989; Gonzalez et al., 1998).

These cumulative results show that both the GPI anchoring and HS GAG chains are required for the effects observed after electroporation of Glypican-1 into the placodal ectoderm. Furthermore, GPC1 may have a role in ganglion organization as expression of a putative secreted antagonist (GPC1-ΔGPI) leads to aberrant ganglia formation without reducing ganglion size. For either full-length or truncated GPC1 phenotype, we find that HS modification appears to be critical.

Manipulating levels of GPC1 alters endogenous activity of Wnt signaling

Given that glypicans interact with growth factors, it is intriguing to speculate that GPC1 may interact with Wnt signaling in the trigeminal placodes. Blocking canonical Wnt signaling inhibits placodal differentiation and OpV ganglion formation (Lassiter et al., 2007) in a

fashion reminiscent of the effects observed with GPC1 over-expression. Furthermore, Wnts have been shown to bind and interact with glypican (De Cat and David, 2001; Ohkawara et al., 2003; Baeg et al., 2004). Consistent with the possibility that Wnt signaling may be involved in various steps of trigeminal placode development, the trigeminal placode expresses Wnt receptors Frizzled-2 and -7, whereas several different Wnt ligands are expressed in the adjacent chick neural tube (Hollyday et al., 1995; Marcelle et al., 1997; Stark et al., 2000).

To address if there is a link between GPC1 and Wnt signaling in the trigeminal placodes, we tested whether increasing GPC1 expression or its mutant truncated form (GPC1-ΔGPI) would modulate endogenous levels of canonical Wnt signaling in vivo. To assay the activity of Wnt signaling, we used the RFP version of the TOPGAL Wnt-Reporter (DasGupta and Fuchs, 1999; Lassiter et al., 2007) which drives RFP expression under the control of LEF/TCF consensus binding sites. Wnt signaling leads to stabilization and nuclear localization of β-catenin which transactivates LEF/TCF transcription factors that bind to target LEF/TCF sequences to drive expression of Wnt downstream genes, and in the case of the reporter, to drive RFP expression. The Wnt-Reporter was co-electroporated with the various glypican constructs: empty GFP vector as control, FL-GPC1 to over-express GPC1, or GPC1-ΔGPI to block GPC1 at stages 9–11. Electroporated embryos with broad transfection in the trigeminal region were collected at stages 14–16 for analysis.

In control GFP embryos in which there is broad transfection of the entire trigeminal ganglia, we found Wnt signaling activity restricted to the OpV region. In general, few to no ganglion cells were RFP+ in the MmV (Fig. 5A). At early stage 14, Wnt-Reporter expression was observed in the dorsal ectoderm (including ectoderm overlying the dorsal neural tube) and OpV placodes at stage 14 (data not shown;(Lassiter et al., 2007)). RFP expression was restricted to the OpV lobe of the ganglion at stages 15–16 and surrounding dorsal ectoderm (generally near the neural tube and above the OpV branch) (Fig. 5A). The area of cells in the OpV ganglion that expressed the RFP Wnt-Reporter appeared to increase over time, suggesting the RFP expression is reflective of continuing addition of OpV placodal neurons to the ganglion and not merely from residual RFP expression of placodal cells from earlier stages (Fig. 5A). Over-expression of GPC1 in the trigeminal placodal ectoderm caused a reduction in Wnt signaling activity in the OpV ganglion cells, but not in the dorsal ectoderm cells external to the ganglion (22.2%, n=18, Fig. 5A,B). The knockdown of Wnt signaling by GPC1 over-expression suggests that GPC1 may negatively modulate Wnt signaling. Further support for this idea stems from the finding that GPC1 gain-of-function caused loss of OpV ganglion which is the same phenotype previously reported for inhibition of Wnt signaling (Lassiter et al., 2007).

Consistent with the possibility that GPC1 modulates Wnt signaling, the truncated GPC1 construct (GPC1-ΔGPI) has the reciprocal effect in causing some expansion of the RFP Wnt reporter expression domain in the trigeminal ganglia (36%, n=25, Fig. 5A,B). Not only was there an apparent increase in RFP expression in the OpV placodal ganglion, but, strikingly, there also was an expansion of RFP expression to the MmV domain near the border of the OpV lobe, as well as in sporadic placodal cells in the MmV ganglion distinct from controls (Fig. 5A).

These results show that manipulating GPC1 expression or function causes a change in endogenous Wnt signaling levels, suggesting a potential role for Glypican-1 as a regulator of canonical Wnt signaling in trigeminal placodes.

Activation of Wnt signaling reverses the effects of GPC1 over-expression but phenocopies effects of truncated GPC1

If the effects of GPC1 over-expression are mediated by reduced Wnt signaling, we predict that activation of Wnt signaling should rescue the ganglion loss. To test this, we used a dominant-active form of ß-catenin (DA-ßcat) in which the phosphorlyation sites required for APC-mediated degradation are mutated, thus allowing ß-catenin to constitutively activate Wnt target genes (Tetsu and McCormick, 1999; Lassiter et al., 2007). We co-electroporated DA-ßcat with FL-GPC1 or control GFP in the trigeminal placodal ectoderm at stages 9–11 and examined formation of trigeminal placodal ganglion at stages 15–18 using TuJ1 antibody staining. The results show that activation of canonical Wnt signaling in these FL-GPC1 electroporated embryos eliminated the reduced ganglia phenotype observed after GPC1 over-expression (Fig. 6A). Furthermore, constitutive activation of Wnt-signaling alone (by DA-ßcat plus GFP expression) in the placodal tissue produced disorganized ganglia (42.9%, n=7)(Fig. 6A,B), similar to the effects of expressing the GPC1- ΔGPI construct. In addition, DA-ßcat and FL-GPC1 caused ganglion disorganization (33.3%, n=6), perhaps due to abnormally high levels of Wnt signaling which not only reversed the inhibitory effects of GPC1 on Wnt signaling but also induced a gain-of-function phenotype. This effect is similar to expression of DA-ßcat alone. Thus, increased Wnt signaling through DA-ßcat expression reversed the reduced ganglia phenotype of FL-GPC1 and the effects of constitutively activated Wnt signaling phenocopied that of the truncated GPC1 that may act in a dominant-negative manner. Taken together, these data suggest a potential negative regulation of Wnt signaling by GPC1 in the trigeminal placodes. GPCs are also known to interact with other signaling pathways (Fico et al., 2007; Filmus et al., 2008), raising the possibility that GPC1 not only interact with Wnts but perhaps also with other signaling pathways functioning during placode differentiation.

Discussion

Our data provide novel insights into the expression and function of Glypican-1 as a potential modulator of Wnt signaling in the trigeminal placodes during neuronal differentiation and ganglion assembly. Wnt-glypican interactions in vivo have been best-studied in the *Drosophila* wing imaginal disc (Baeg and Perrimon, 2000; Franch-Marro et al., 2005; Gallet et al., 2008; Yan et al., 2009), but are poorly understood in vertebrate development. Dally (division abnormally delayed), orthologue of vertebrate glypican 3/5, was found in a screen for defects in cell division patterning in the forming *Drosophila* CNS (Nakato et al., 1995; Filmus and Song, 2000). Dally mutants have delayed G2–M transition in dividing cells in the eye disc and lamina as well as defects in morphogenesis of adult tissues (i.e. the eye, antenna, wing, and genitalia) and in viability. Dally appears to act as a classical co-receptor that facilitates or enhances Wingless (Wg) signaling (Franch-Marro et al., 2005). In contrast, Dally-like (Dlp), the *Drosophila* orthologue of vertebrate GPC 1/2/4/6 (Filmus et al., 2008), has the opposite effects to Dally. It inhibits local but facilitates long range Wnt/Wingless

(Wg) signaling by transporting the signal to neighboring cells (Franch-Marro et al., 2005; Gallet et al., 2008; Yan et al., 2009). Over-expression of GPC1 homologue Dlp causes reduced local Wg signaling and loss of imaginal disc tissue in a cell autonomous manner, whereas Dlp knock-down causes increased local Wg signaling (Baeg and Perrimon, 2000; Franch-Marro et al., 2005).

Similar to the effects of Dlp, we observed a decrease in Wnt signaling with GPC1 over-expression and an increase with the expression of a putative dominant-negative form of GPC1, GPC1-ΔGPI, which lacks the GPI anchorage, in the trigeminal placodal tissue. Our data show that the mutant GPC1-ΔGPI has the reciprocal effect to full-length GPC1 by causing 1) an increase in Wnt signaling, and 2) ganglion disorganization instead of a decrease in Wnt signaling and ganglion reduction as is the case after FL-GPC1 transfection. This suggests that the truncated GPC1 form acts in a dominant-negative manner to disrupt normal GPC1 function, or at least in a way that is distinct from the function of the full-length glycoprotein. In light of the alteration of endogenous Wnt signaling by manipulating GPC1 expression in the trigeminal placodes, the results suggest that GPC1 acts similarly to Dlp in that it can negatively regulate Wnt signaling. Similar negative regulation by GPC3 on canonical Wnt signaling has also been shown in mouse and culture studies (Song et al., 2005).

The effects of altering GPC1 expression on endogenous Wnt signaling in trigeminal placodes appear to occur at later times during differentiation and assembly of placodal ganglia and not during Wnt's role in early ophthalmic (OpV) fate specification (Lassiter et al., 2007; Canning et al., 2008). We found that GPC1 expression begins at ~stage 12 which is well after placode induction, and that GPC1 over-expression does not affect expression of the early placode marker Pax3, but rather blocks later steps in neuronal differentiation.

Interestingly, the most severe effect of GPC1 over-expression was the loss of OpV placodal neurons, whereas MmV neurons were less affected. The OpV neurons are the same cells in which endogenous Wnt signaling is detected. We found that expression of the TOPGAL Wnt-Reporter was largely restricted to the OpV area, and mostly absent from the MmV. These findings are consistent with the possibility that GPC1 regulates Wnt signaling in the forming OpV lobe of the trigeminal ganglion.

How might GPC1 regulate Wnt signaling? In *Drosophila*, genetic evidence on Dally mutants clearly shows that Dally has a positive influence on Wg signaling (Lin and Perrimon, 1999; Fujise et al., 2001; Franch-Marro et al., 2005; Han et al., 2005). However, the mechanism of Dlp is more complicated and several models have been proposed to explain its negative regulation. This includes cleavage of Dlp at the GPI anchor to create secreted antagonist for Wg ligands (Kreuger et al., 2004), endocytosis of Dlp through its GPI anchor with Wg (as exchange of GPI anchor for a transmembrane domain blocks this function) (Gallet et al., 2008), functioning of Dlp as a competitor for ligand binding, and positive or negative action of Dlp based on the ratio of Wg, Wg receptor, and Dlp (Yan et al., 2009) or based on modification of Dlp by cleavage (Kreuger et al., 2004).

Here, we find that excess GPC1 expression inhibited Wnt signaling, similar to that of the Dlp, and expression of a mutant truncated GPC1 enhanced Wnt signaling. Distinct phenotypes, either loss or disorganization of the ganglion, were observed for the two types of perturbations respectively. One possibility is that GPC1 may act as a negative regulator of Wnt signaling in trigeminal placodes. Alternatively, GPC1 may act as both a negative and a positive regulator of Wnt such that different modes of GPC1 function (e.g., either as full-length or cleaved soluble form) or different levels of GPC1 expression might differentially influence Wnt signaling. For example, full-length GPC1 is capable of negatively regulating Wnt by reducing ligand levels by endocytosis via its GPI anchor whereas truncated soluble GPC1 competes with full-length for ligand binding and therefore blocks endocytosis but promotes ligand distribution. Although the detailed mechanism is not yet known, it is clear from our findings that an appropriate level of GPC1 expression is required for normal formation of trigeminal placode-derived neurons, such that elevated GPC1 levels causes dramatic ganglion loss and leads to changes to endogenous Wnt activity.

GPCs interact with several major signaling pathways in addition to Wnts. We have previously shown that interaction between Slit1 from neural crest cells and its cognate receptor Robo2 on trigeminal placodes mediates proper assembly of the trigeminal ganglion in part through regulation of N-cadherin protein distribution on placodal neurons for ganglion aggregation (Shiau et al., 2008; Shiau and Bronner-Fraser, 2009). Direct interactions of heparan sulfate proteoglycans (HSPGs) with Slit have been suggested to be important for its function (Hussain et al., 2006; Fukuhara et al., 2008; Hohenester, 2008). Consistent with this, recombinant vertebrate Glypican-1 has been shown to bind specifically to Slit1 and Slit2 in rat brain extracts (Liang et al., 1999; Ronca et al., 2001). In light of this and the effect of disorganized ganglia caused by GPC1 inhibition using the soluble truncated form of GPC1, we cannot rule out the possibility that GPC1 may also regulate aspects of Slit-Robo signaling during trigeminal gangliogenesis. Similarly, GPC1 may affect Fgf signaling, which has been implicated in differentiation and ingression of trigeminal placodes (Canning et al., 2008; Lassiter et al., 2009). Loss of Fgf signaling leads to failure of placodes to delaminate from the ectoderm and contribute to ganglion formation in the mesenchyme (Lassiter et al., 2009), similar to the effects we have shown of GPC1 over-expression. Regulation of Fgf signaling by glypicans has been demonstrated previously in other systems, including the mammalian brain and *Drosophila* tracheal morphogenesis (Su et al., 2006; Yan and Lin, 2007; Jen et al., 2009).

In summary, we identify the heparan sulfate proteoglyan, GPC1, as a novel molecular player in trigeminal gangliogenesis. It is distinctively expressed by the trigeminal placodal ectoderm and placode-derived sensory neurons. Importantly, we show that it can act as a regulator of Wnt signaling during placodal differentiation and ganglion formation, and that proper levels of GPC1 expression are essential for these processes in vivo. Our results represent an important entry point into dissecting the regulation of signaling mechanisms involved in early formation of the trigeminal sensory system in chick, potentially revealing how signaling is modulated at proper levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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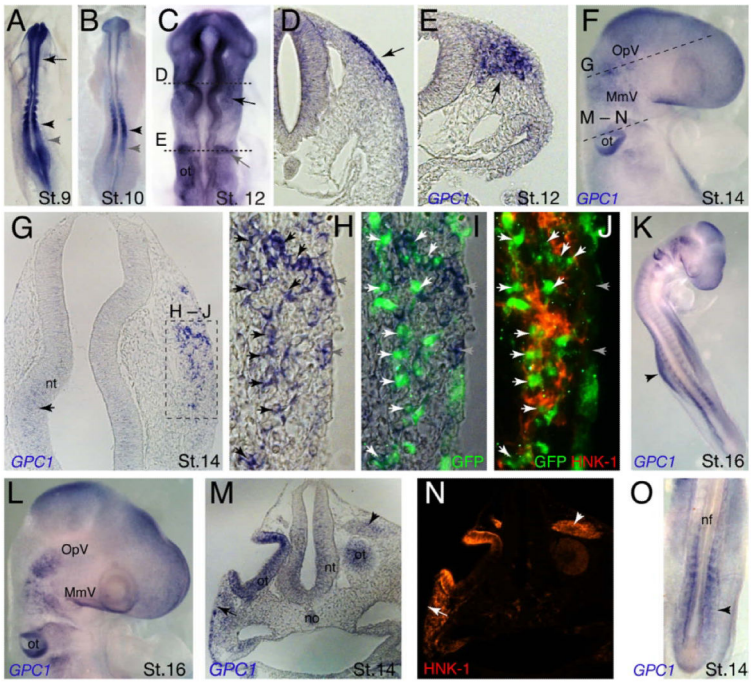

Figure 1. Expression of Glypican-1 mRNA in the early chick embryo

(A) Stage 9 and (B) stage 10 embryos showing GPC1 mRNA expression in the neural tube (arrow) but not in the migrating midbrain neural crest cells, and in the presomitic mesoderm (PSM) and the most caudal somites in a gradient fashion, high (black arrowhead) to low (gray arrowhead) expression. (C) Stage 12 embryo showing onset of GPC1 expression in the forming trigeminal placodes (arrow) and migrating hindbrain neural crest cells at rhombomere 4 (r4) (gray arrow). Cross sections as indicated in C showing GPC1 expression in (D) placodal ectoderm and spurs of ingressing placodal cells (arrow), (E) and hindbrain migratory neural crest cells (arrow). (F) GPC1 expression in the ophthalmic (OpV) and maxillo-mandibular (MmV) placodes of the forming trigeminal ganglion and in the otic vesicle at stage 14. (G) Cross section through the OpV region of F showing GPC1 expression in the placodal ectoderm and ingressing placodes (dotted box), and weakly in the neural tube. (H–J) Higher magnification of the dotted box in G showing GFP-labeled ingressing trigeminal placodal cells express GPC1 (arrows) but HNK-1 expressing neural crest cells do not. (K) Stage 16 embryo showing expression in the forming limb bud (arrowhead). (L) GPC1 expression persists in the OpV and MmV placodes and the otic vesicle. (M–N) Cross section of F showing GPC1 expression in the otic placode, epibranchial ectoderm (arrow), and r4 HNK-1 expressing hindbrain neural crest cells (arrowhead). (O) GPC1 expression persists in the PSM and newly formed somites at stage 14 (arrowhead). OpV, ophthalmic; MmV, maxillo-mandibular; nt, neural tube; ot, otic; no, notochord; nf, neural folds.

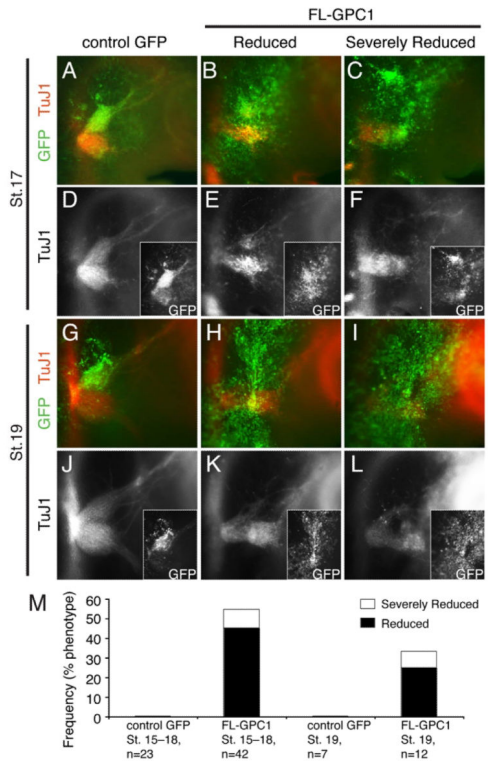

Figure 2. Increased Glypican-1 expression in the placodal ectoderm leads to significant loss of placodal ganglia

Ectoderm-electroporated embryos analyzed at stage 17 showing (A) normal trigeminal ganglion formation with control GFP and (B–C) reduced or severely reduced ganglia with full-length GPC1 (FL-GPC1). Color overlay images show area of transfection by GFP expression in green and placodal neurons by TuJ1 antibody staining in red. (D–F) Single channel images of TuJ1 of the above overlay images in A–C; insets, GFP expression only. Ganglia assessed at stage 19 showing (G) normal ganglion in controls but (H–I) markedly reduced ganglia in FL-GPC1 embryos. (J–L) Single channel images of TuJ1 of G–I and insets show GFP expression of the color overlay. (M) Histogram showing frequency of reduced and severely reduced ganglia phenotypes. n represents the number of ganglia analyzed.

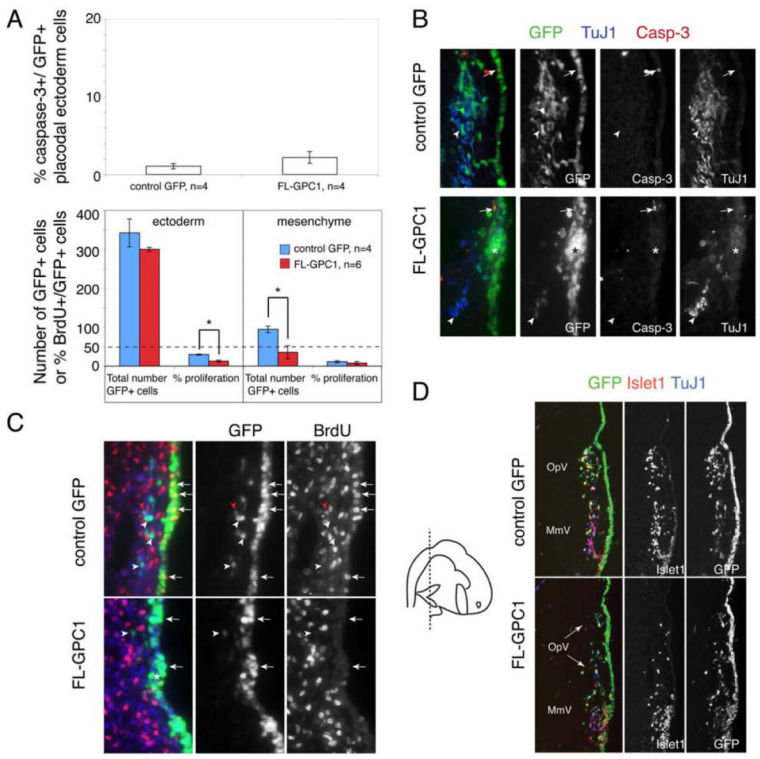

Figure 3. GPC1 over-expression blocks cell proliferation and differentiation of the placodal ectoderm, but does not induce significant cell death

(A) Top, histogram showing no significant difference in cell death level between control GFP and FL-GPC1 in the ectoderm. Bottom, graph showing compared with controls, FL-GPC1 embryos had significantly reduced percentage of proliferation in the placodal ectoderm and decreased total number of ingressing placodal cells. Dotted line marks the 50% level. Bars indicate s.e.m. (B) Cross sections through the OpV region at stage 15 showing few caspase-3 positive transfected cells in the ectoderm (arrow) and none in the mesenchyme (arrowhead, which are ingressed placodal cells) in both control GFP and FL-GPC1 embryos. FL-GPC1 transfected ganglia had regions of strikingly aberrant clustering of cells in the ectoderm distinct from controls (asterisk). (C) Electroporated embryos collected at stage 14 were treated with a short half hour pulse of BrdU to detect S-phase mitotic cells at the time of analysis. Frontal plane sections at stage 14 showing (top panels) many proliferating cells in the ectoderm in control (BrdU +, arrows). By contrast, (bottom panels) most FL-GPC1 transfected ectodermal cells are non-mitotic (BrdU − and GFP +, arrows) and tend to cluster (asterisk). Most ingressed placodal cells in both control and FL-GPC1 transfections were not mitotic (BrdU −, arrowheads) though occasionally a very few number of them are (red arrowhead, top panel). (D) Frontal plane sections through the ganglion as shown by the dotted line in the schematic (left) showing strikingly less placodal neurons (Islet1+/TuJ1+) particularly in the OpV region in FL-GPC1 embryo (arrows) compared with control. OpV, ophthalmic; MmV, maxillo-mandibular.

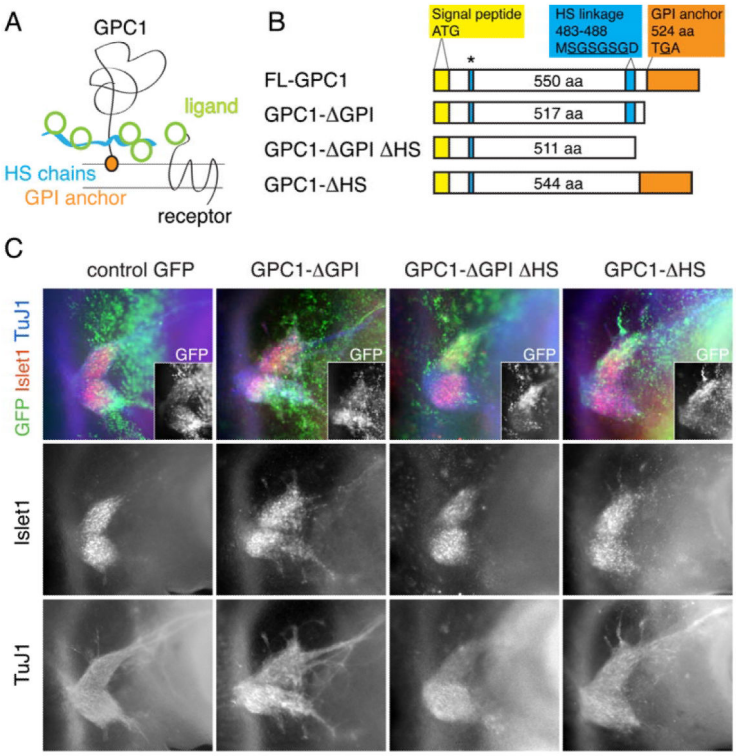

Figure 4. Truncated form of GPC1 causes disorganization of ganglion distinct from full-length, and both full-length and truncated forms require the putative heparan sulfate glycosaminoglycan (GAG) attachment sites for function

(A) Simplified schematic of glypican shows its heparan sulfate (HS) side chains near the cell surface and GPI membrane anchor acting as a potential co-receptor or ligand carrier for signaling receptors. (B) Diagram showing the domains of the full-length GPC1 (FL-GPC1) and the modifications made on the mutant forms of GPC1. Since HS attachment sites are known to localize near the membrane anchor of glypicans, all predicted HS attachment sites at the C-terminus for chick GPC1 in the UniProtKB database were excised in HS deletion constructs; there remains only one predicted site located near the N-terminus (asterisk). (C) Representative images showing stage 18 placodal ganglia (Islet1+/TuJ1+) electroporated with mutant GPC1 constructs with deletions of HS or both GPI and HS domains that were generally normal and similar to controls. By contrast, GPC1-ΔGPI transfected placodal ganglia were normally sized but disorganized.

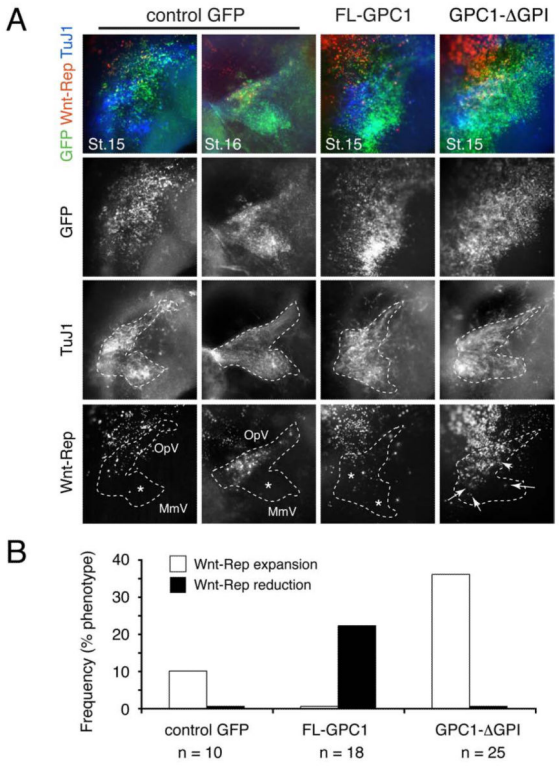

Figure 5. Changes to GPC1 expression alters endogenous Wnt signaling activity

(A) Top row, color overlay images showing trigeminal ganglia in whole mount chick embryos at stages 15–16 after electroporation with control GFP, FL-GPC1, or GPC1-ΔGPI constructs. GFP in green showing area of transfection (also shown in second row panels). TuJ1 in blue showing placodal neurons (also shown in third row panels). RFP version of TOPGAL Wnt-reporter expression in red showing endogenous activity of Wnt signaling (also shown in fourth row panels). Outline of the trigeminal ganglion is demarcated by the dotted lines. Fourth row panels, controls show Wnt-Reporter expression restricted to the OpV region at stages 15 and 16 with generally absence of reporter in the MmV region (asterisk), albeit occasionally one or a few cells were Wnt-Rep+. Reporter expression in the OpV appears to increase over time. By contrast, FL-GPC1 expressing placodal ganglia had markedly reduced Wnt-Reporter expression in the OpV region, and similar to controls, had generally little to no reporter expression in the MmV region (lower asterisk). Conversely, expression of GPC1-ΔGPI led to more expression of Wnt-Reporter in the OpV (arrowhead) and expansion of reporter expression into the MmV region in more cells, though sparsely (arrows). (B) Histogram showing percentages of cases of reduction of Wnt-Reporter expression in the OpV and of expansion of Wnt-Reporter expression, which means an increase in reporter expression in OpV as well as expression in more number of cells in MmV, after electroporation with control GFP, FL-GPC1, or GPC1-ΔGPI. n shows number of ganglia analyzed. Wnt-Rep, Wnt-Reporter. OpV, ophthalmic; MmV, maxillo-mandibular.

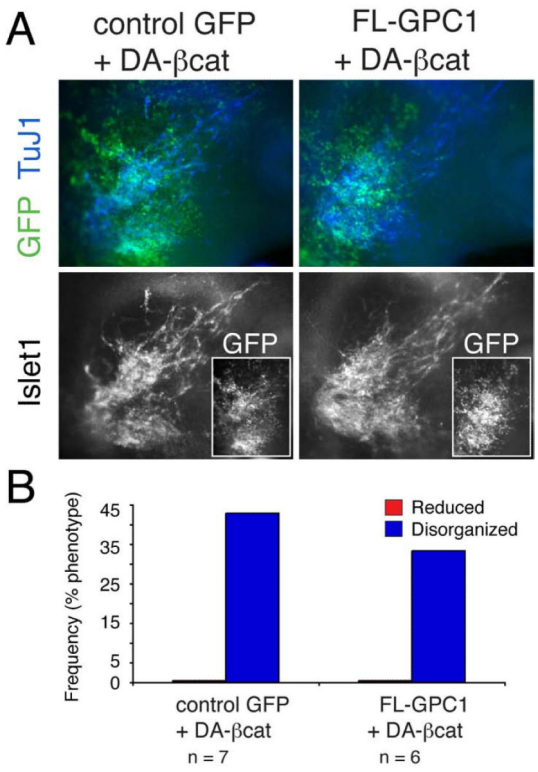

Figure 6. Induced activation of Wnt signaling phenocopies the effects of truncated GPC1 and reverses the effects of GPC1 over-expression

(A) Co-expression of a dominant-active form of ß-catenin (DA-ßcat) with FL-GPC1 in the placodal tissue suppresses the reduced ganglia phenotype. Both control and experimental expressions of DA-ßcat with control GFP or FL-GPC1, respectively, led to some disorganized integration of placodal cells into ganglia. GFP in green showing area of transfection and TuJ1 in blue showing placodal neurons. (B) Histogram showing frequency of phenotypes: “reduced” means decreased in overall ganglion size and “disorganized” means aberrant integration of placodal cells. n represents number of ganglia analyzed.