A zebrafish Notum homolog specifically blocks the Wnt/β-catenin signaling pathway

G. Parker Flowers, Jolanta M. Topczewska and Jacek Topczewski*

SUMMARY
Multiple developmental processes require tightly controlled Wnt signaling, and its misregulation leads to congenital abnormalities and diseases. Glypicans are extracellular proteins that modulate the Wnt pathway. In addition to interacting with Wnts, these glycosphosphotidylinositol (GPI)-anchored, heparan-sulfate proteoglycans bind ligands of several other signaling pathways in both vertebrates and invertebrates. In Drosophila, Notum, a secreted α/β-hydrolase, antagonizes the signaling of the prototypical Wnt Wingless (Wg), by releasing glypicans from the cell surface. Studies of mammalian Notum indicate promiscuous target specificity in cell culture, but the role of Notum in vertebrate development has not been studied. Our work shows that zebrafish Notum 1a, an ortholog of mammalian Notum, contributes to a self-regulatory loop that restricts Wnt/β-catenin signaling. Notum 1a does not interact with Glypican 4, an essential component of the Wnt/planar cell polarity (PCP) pathway. Our results suggest a surprising specific role of Notum in the developing vertebrate embryo.

KEY WORDS: Notum, Wnt, Glypicans, Neural tube, Patterning, Zebrafish

INTRODUCTION
Wnts are members of a family of secreted signaling molecules conserved across the animal kingdom that are involved in numerous aspects of development and in the maintenance of self-renewing tissues in adulthood (Clevers, 2006). In the Wnt/β-catenin pathway, Wnt ligands bind to Frizzled and LRPS/6 co-receptors, leading to an inhibition of the activity of GSK3 kinase, a component of a β-catenin-destruction complex. This inhibition allows β-catenin to accumulate and enter the nucleus to activate target genes by interacting with LEF/TCF transcription factors (MacDonald et al., 2009). As Wnt/β-catenin signaling is crucial for numerous aspects of development, proper development requires the tight control of this pathway, and multiple regulatory mechanisms act to maintain stable Wnt pathway output (MacDonald et al., 2009). The extracellular mechanisms that affect Wnts are a growing area of investigation (Mikels and Nusse, 2006).

Glypicans are one class of molecules of particular interest because of their role in the regulation of extracellular Wnts. A glypican is a heparan-sulfate proteoglycan linked to the cell surface via a glycosphosphotidylinositol (GPI) anchor. In Drosophila, the glycans Dally and Dally-like promote the extracellular accumulation and signaling of Wg, the prototypical member of the Wnt pathway. For example, in the Drosophila wing disc, Dally-like and Frizzled 2 act to promote the long-range stability of Wg, whereas high levels of Wg repress their expression (Baeg et al., 2004; Cadigan et al., 1998). A secreted α/β hydrolase, Notum, releases glypicans at their GPI anchors to restrict the spread of the Wg ligand and to inhibit its activity (Giraldez et al., 2002; Kreuger et al., 2004). Notum expression is induced by high Wg activity (Gerlitz et al., 2002; Torisu et al., 2008); therefore, Notum acts as part of a negative-feedback mechanism that tightly controls the extracellular distribution of the Wg ligand (Piddini and Vincent, 2009). In this work, we investigate whether Notum plays a similar role as an inhibitor of Wnt/β-catenin signaling and whether it is involved in other glypican-dependent processes during vertebrate development.

In addition to regulating Wg signaling in Drosophila, glypicans have manifold developmental functions, and cleavage of glypicans by Notum could affect a variety of developmental processes. In zebrafish, the best characterized glypican, Glypican 4 (Gpc4 or Knypek), contributes to Wnt/planar cell polarity (PCP) signaling and is essential for the proper convergence and extension of cells during gastrulation (Topczewska et al., 2001). In other vertebrates, glypicans have been implicated in Wnt signaling and numerous additional pathways. Mutations of glypican 3 (GPC3) in humans result in Simpson-Golabi-Behmel Syndrome, a disorder characterized by tissue overgrowth and tumor susceptibility (Song and Filmus, 2002), while GPC6 mutations cause omodysplasia, a condition characterized by short limbs (Campos-Xavier et al., 2009). Gpc3 has been shown to have a diverse set of functions, as it can modulate the Wnt, hedgehog, bone morphogenetic protein (Bmp), and fibroblast growth factor (Fgf) signaling pathways (Filmins et al., 2008). Work in cultured cells demonstrated that mammalian Notum can induce the release of all assessed glypicans and some, but not all, GPI-anchored proteins (Traister et al., 2007). As GPI-anchored proteins are involved in a variety of processes, we were interested in whether vertebrate Notum homologues participate in a specific or broad range of developmental processes. Here, we report that a newly identified zebrafish homolog of Notum acts on a restricted set of targets to specifically inhibit the Wnt/β-catenin pathway.

MATERIALS AND METHODS
Cloning of notum 1a
Full-length notum 1a was cloned from pooled mixed-stage mRNA with the primers: 5'-AGTTTACGATCCTCAGTCCGATG-3' and 5'-AGTTGACAGAGCATGACTGAGG-3'.

In situ hybridization
Whole-mount in situ hybridization was carried out as previously described (Thisse et al., 2008). Table S1 (supplementary material) provides a complete list of the probes used.
Zebrafish lines

Wild-type embryos for all experiments were of an AB × Tubingen background. The following transgenic and mutant lines were used: Tg(hsp70:tau-gfp)26. Tg(TOP:deGFP)25. Tg(hsp70:formalin)20. Tg(Red:HRAS-EGFP)419. Tg(hsp70:dn-frg-1-EGFP)41. and gpc4-6 and wnt11216. All mutant fish described in experiments were produced from crosses between heterozygous parents. To generate the Tg(hsp70:formalin)26 line, a FLAG-epitope tagged Notum 1A-FLAG was created by inverse PCR, in which a sequence encoding DYKDDDDK was inserted immediately after the predicted signal peptide cleavage site. The ORF of notum1a-flag was cloned between a hsp promoter and a polyA sequence in the pDest202CG2 vector, using the multisite Gateway system and the Tol2Kit (Kwan et al., 2007). A Tol2 transposase method of transgenesis was carried out as described previously (Kwan et al., 2007) to generate the Tg(hsp70:formalin)26 line. As the transgene backbone contains an cmic2:EGFP element, transgene-positive fish were identified by EGFP-positive hearts on the first day post-fertilization. The production of Notum 1A-FLAG in heat-shocked embryos was confirmed by in situ hybridization and overexpression phenotype. Injection of equal amounts of synthetic mRNA into one-cell embryos revealed that overexpression of notum1a-FLAG had the same effect on embryonic patterning as untagged notum1a. The genotype-phenotype association in Tg(hsp70:dn-frg-1-EGFP)41 was verified by PCR using EGFP-directed primers from DNA extracted after in situ with Tissue Lysis Solution (EZ BioResearch, St Louis, MO, USA).

Transgene induction

To induce transgene expression in Tg(hsp70:taut-gfp)26, 22 hpf embryos were heat-shocked at 42°C for 10 minutes. Prior to fixation, transgenic embryos were identified by GFP expression at 24 hpf. To induce ectopic β-catenin-GFP expression in hs:β-catenin-gfp-injected embryos (Nyholm et al., 2007), heat shock was carried out at 42°C for 10 minutes 2 hours before fixation. For LiCl experiments, Tg(hsp70:formalin)26 embryos were heat shocked for 2 hours at 37°C beginning at 50% epiboly. At 75% epiboly, heat shocked and non-heat shocked embryos were treated for 8 minutes in 0.3 M LiCl in egg water. Analysis of the cell shape was performed in the embryos injected with mcherry-HsHRAS mRNA, heat shocked at either 50% epiboly or 80% epiboly at 37°C for 2 hours and fixed at the two-somite stage. For analysis of neural-tube markers, tail markers and Fgf-target genes, Tg(hsp70:formalin)26 were heat shocked for 30 minutes at 39°C 2 hours prior to fixation.

mRNA and morpholino oligonucleotide injections

All injected mRNAs were transcribed in vitro with SP6 mMessage mMachine (Ambion, Austin, TX, USA). All mRNAs were diluted in Danieau’s buffer (Nasevicius and Ekker, 2000) so that the listed quantities were injected into the embryo in a single 1 nl drop, except when 150 ng of notum1a was injected in two 75 ng drops. All mRNAs and MOs were injected individually. Chi-square analyses were used to assess the significance of Notum 1A-mediated inhibition of Wnt1 and Wnt8 activity. Dkk1 synergy, notum1a-MO-mediated enhancement of Wnt1 activity, gpc3 MO enhancement and gpc3-mediated rescue. Each of the described multiple-injection experiments was repeated at least twice with significant results, but, owing to variability in phenotype penetrance between experiments, only one representative set of results is presented.

The dkk1 MO, with a sequence of 5'-AGAGAGCATGCGCATGATCATG-3', was obtained from Open Biosystems (Huntsville, AL, USA). All other MOs were obtained for Gene Tools (Philomath, OR, USA). Three unique MOs were obtained directed against notum1a: a 5'-UTR-directed MO, 5'-CCGAGGTTGATGCTGAAATC-3'; a start codon-directed MO, 5'-CTCCCTCTCATCCGGCAATATCGG-3'; and a splice site-directed MO, 5'-GTCAAAAGCATCACCATTACC-3'. All MOs produced similar phenotypes; however, the UTR-directed MO was generally more toxic. Unless otherwise noted, experiments used the AUG-directed MO. Notum 1a-FLAG in heat-shocked embryos was confirmed by in situ hybridization and overexpression phenotype. Injection of equal amounts of Notum 1a-FLAG in heat-shocked embryos was confirmed by in situ hybridization and overexpression phenotype. Injection of equal amounts of synthetic mRNA into one-cell embryos revealed that overexpression of notum1a-FLAG had the same effect on embryonic patterning as untagged notum1a. The genotype-phenotype association in Tg(hsp70:dn-frg-1-EGFP)41 was verified by PCR using EGFP-directed primers from DNA extracted after in situ with Tissue Lysis Solution (EZ BioResearch, St Louis, MO, USA).

Sequence alignment

Multiple sequence alignment was carried out using MultAlin (Corpet, 1988). The notum1a sequence was deposited in GenBank with Accession Number EU728672.

TOPFLASH assay

Human embryonic kidney HEK293 cells were maintained in DMEM with 10% FBS and antibiotics. For luciferase assays, cells were plated in six-well plates, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Each well was transfected with 995 ng TOPFLASH (Upstate Biotechnology, Lake Placid, NY, USA), 10 ng of pRL-CMV (Promega) and 10 ng pCS2-wnt1. Cells were transfected with 500 ng of pCS2-notum1a or balanced to 2 μg of total DNA with empty pGL4. Dual luciferase assays were carried out with the DLR System (Promega, Madison, WI, USA) 36 hours post-transfection. For assays in the presence of Wnt-secreting cells, cells were transfected with 10 ng pcs2-wnt1. Twenty-four hours later, another set of cells was transfected with 1 μg TOPFLASH. 10 ng of pRL-CMV and 500 ng pcs2-notum1a or 50 ng empty pGL4. Four hours after transfection, half a plate of Resuspended cells transfected with wnt1 on the previous day were added to TOPFLASH-positive cells. A Dual luciferase assay was performed 20 hours later. Each condition was repeated in triplicate and a Student’s t-test was used to assess significance.

mRNA was extracted from HEK293 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). The following primers were used for RT-PCR to determine glypicin expression: GPC1, 5'-TGCTTGTCTGTGATGCACCTG-3' and 5'-TGGACCATTTGCGCAATAG-3'; GPC3, 5'-CTCGATGGAGAAGGGTCTGTA-3' and 5'-GCAAGGAGAAAGAGAAGCAC-3'; GPC4, 5'-AGTCCCTCGCAAAAATGAG-3' and 5'-TCAAAATCGAGATCCCCTTG-3'; GPC5, 5'-CTCCGAAGAAGTTCCGAAAAAC-3' and 5'-AGAACCTCTCGACCGAAAGCA-3'.

Immunohistochemistry

Phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY, USA) staining was carried out on sections of the neural tube at the level of the yolk extension in 24 hpf embryos. Phospho-Histone H3 (1:1000) and 1:250 Cy3 Goat Anti-Rabbit (Jackson Laboratory, Bar Harbor, ME, USA) antibodies were used. Nuclei were stained with 1:10,000 Sytox Green (Molecular Probes, Eugene, OR, USA). Cells of the neural tubes were counted and mitotic indices were expressed as the percent of phospho-Histone H3 positive nuclei within the neural tube per section.

Western blot

notum1a-injected and uninjected embryos were injected with a combination of mGFP and gpc3-EGFP mRNAs. The homogenates of injected embryos were collected at 75% epiboly. The protein equivalents of five embryos per lane were separated by electrophoresis on 7.5% polyacrylamide gels and transferred to PVDF membranes. EGFP was detected using 1:500 of mouse monoclonal GFP B-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the Pierce Fast Western Kit (Thermo Fisher, Rockford, IL, USA).
RESULTS

**notum1a identification and expression**

Using a bioinformatic approach, we identified three zebrafish Notum homologs, notum1a, notum1b and notum2, with highly conserved protein products compared with human and fly homologs. These sequences are particularly conserved surrounding the active site motifs (G-X-S-X-G) and the Ser, His, Asp catalytic triads essential for the activity of α/β hydrolases (supplementary material Fig. S1A, only Notum 1a shown) (Holmquist, 2000). Of zebrafish Notum genes, notum1a shares the highest encoded peptide identity with human NOTUM, in addition to conserved genomic synteny, suggesting that these two proteins are true homologs (supplementary material Fig. S1B). notum1a and notum1b appear to be paralogs produced from the whole-genome duplication during teleost evolution (Amores et al., 1998). We selected Notum 1a for more detailed study, as in situ hybridization across the first few days of development revealed robust, dynamic gene expression that does not overlap with that of other zebrafish homologs (Fig. 1A-E). Expression of notum1a first appears around the blastoderm margin prior to the onset of gastrulation (Fig. 1A). In later gastrulation, notum1a is expressed in the lateral edges and midline of the posterior neural plate (Fig. 1B,C). During segmentation, it is expressed in stripes at the lateral edges and midline of the posterior neural plate (Fig. 1D), throughout the segmentation, it is expressed in stripes at the lateral edges and midline of the posterior neural plate (Fig. 1B,C). During gastrulation, expression is particularly pronounced in the CNS, around the midline of the neural plate (Fig. 1D), throughout the segmentation, it is expressed in stripes at the lateral edges and midline of the posterior neural plate (Fig. 1B,C). During gastrulation, expression is particularly pronounced in the CNS, and at the midbrain-hindbrain boundary (MHB; Fig. 1D). At 24 hpf, expression is particularly pronounced in the CNS, including the dorsal neural tube and the MHB (Fig. 1E).

**Wnt/β-catenin signaling regulates notum1a expression**

As notum1a expression overlaps with domains of high Wnt/β-catenin activity (Bonner et al., 2008), we asked whether Wnts regulate notum1a. To activate the Wnt pathway, we injected wild-type embryos with a DNA construct encoding a heat-shock-inducible, stabilized form of β-catenin, hs:β-catenin-gfp (Nyholm et al., 2007). Mosaic induction of β-catenin led to ectopic expression of notum1a during gastrulation (Fig. 1F). Conversely, we blocked Wnt/β-catenin dependent signaling by heat shock-induced expression of a dominant-negative form of Tcf3 in the Tg(hsp70:tc3-GFP)Δm3 transgenic line (Lewis et al., 2004). As detected by in situ hybridization, notum1a expression was dramatically reduced in all heat-shocked transgenic embryos (n=120, Fig. 1H) compared with their heat-shocked non-transgenic siblings (n=126, Fig. 1G) shortly after dominant-negative tcf3 induction. A small domain of notum1a expression persists in the tail after the Wnt blockade, suggesting either an area of increased notum1a transcript stability or Wnt-independent regulation. Interestingly, NOTUM is upregulated in human primary hepatocellular carcinomas with high levels of intracellular β-catenin (Torsis et al., 2008). Our results, that similar to Drosophila Notum, notum1a expression requires Wnt/β-catenin signaling.

**Notum 1a inhibits Wnt/β-catenin in cultured cells**

To investigate the effect of Notum 1a on Wnt signaling, we transiently transfected HEK 293 cells with the TCF-reporter plasmid TOPFLASH, in which Wnt-responsive elements drive the expression of luciferase (Veeman et al., 2003). Co-transfection with zebrafish wnt1, which, like notum1a, is expressed in the zebrafish roofplate (Bonner et al., 2008), caused a dramatic increase in luciferase activity compared with cells transfected with TOPFLASH alone. When these cells were additionally transfected with zebrafish notum1a, the effect of wnt1 on reporter activity was significantly reduced (59% reduction, P=0.0194; supplementary material Fig. S2A). Notum 1a acted as an inhibitor of Wnt signaling both when co-expressed with wnt1 and when expressed in cells in the presence of Wnt1-secreting cells (41% reduction, P=0.00002; supplementary material Fig. S2B). This indicates that Notum 1a inhibits the reception rather than the production or release of the Wnt signal. Using RT-PCR, we identified multiple glypican, GPC1, GPC3, GPC4 and GPC5, expressed in HEK 293 cells (supplementary material Fig. S2C), all of which may serve as...
targets of Notum 1a (Traister et al., 2007). Thus, in cultured cells, zebrafish Notum 1a inhibited Wnt/β-catenin signaling similarly to mammalian Notum (Traister et al., 2007).

**Notum 1a inhibits Wnt/β-catenin in the embryo**

To investigate whether Notum 1a can act as an antagonist of Wnt signaling during vertebrate development, we carried out gain-of-function experiments in the zebrafish embryo. Injection of notum1a mRNA led to a dose-dependent spectrum of phenotypes at 24 hpf, ranging from mild curling of the tail to severe truncation of the posterior body and enlargement of the eyes and forebrain (Fig. 2A). These phenotypes are remarkably similar to those observed in embryos in which the activity of multiple Wnt/β-catenin ligands is inhibited (Shimizu et al., 2005; Thorpe et al., 2005) or in which Dkk1, a secreted inhibitor of Wnt/β-catenin signaling, is overexpressed (Fig. 2A) (Shinya et al., 2000). Furthermore, when we injected embryos with a quantity of notum1a below the threshold necessary to produce any overt phenotype in combination with an amount of dkk1 that consistently induces moderate tail truncation, we found a significant synergistic effect, producing severely truncated embryos (P<0.0001; Fig. 2A). To assess the effect of Notum 1a on Wnt/β-catenin target genes, we examined the expression of tbx6, a marker of ventralateral mesoderm (Ueno et al., 2007), following notum1a overexpression. In early gastrulation (60% epiboly), the expression of tbx6 was significantly reduced in the majority of embryos (n=35/40) in which notum1a was overexpressed (Fig. 2B,C). Correspondingly, the dorsal marker gsc was expanded in notum1a-injected embryos (n=14/25) but was not affected in controls (n=0/44) in early gastrulation (shield, Fig. 2D,E). The combined expansion of gsc and depletion of tbx6 is characteristic of Wnt/β-catenin inhibition and is not characteristic of dorsalized phenotypes produced by early Bmp inhibition (Yabe et al., 2003). As notum1a overexpression reduced Wnt target gene induction, we next investigated whether notum1a could counteract phenotypes induced by elevated levels of Wnt. Injection of wnt8-encoding mRNA induced near-ubiquitous expression of sp5l, a direct transcriptional target of Wnt/β-catenin signaling (n=16/18) (Thorpe et al., 2005) at shield stage. By contrast, embryos injected with wnt8 and notum1a had a wild-type-like sp5l expression domain (n=6/12, P=0.009; supplemental material Fig. S2D-F). Wnts posteriorize the neural plate, and Wnt/β-catenin antagonism is necessary for proper forebrain formation (Wilson and Houart, 2004). We found that Notum 1a was able to inhibit the effects of wnt8 or wnt1 on anterior neural plate patterning. When wnt8 or wnt1 mRNA was injected at a level sufficient to suppress eye formation in the majority of injected embryos, co-injection of 75 pg of notum1a prevented this phenotype (P<0.0001, P=0.005, respectively; Fig. 2F). Like Wnt overexpression, knockdown of the Wnt inhibitor Dkk1, with a morpholino (MO) antisense oligonucleotide posteriorizes the neural plate. Consistent with the previous results, injection of 75 pg of notum1a mRNA suppressed this phenotype (P<0.0001; Fig. 2F). To determine whether endogenous Notum 1a negatively regulates Wnt/β-catenin activity, we tested the sensitivity of Notum 1a-depleted embryos to Wnt1 overexpression. We observed a strong enhancement of Wnt1-induced neural plate posteriorization in notum1a UTR-MO-injected embryos (P=0.0004) that was rescued by injection of MO-insensitive notum1a mRNA (P=3×10^-8; Fig. 2F), demonstrating that Notum 1a moderates Wnt/β-catenin signaling during early zebrafish development.

**Fig. 2. Notum 1a is an inhibitor of Wnt signaling.** (A) Overexpression of notum1a produces phenotypes resembling and synergistic with dkk1 overexpression. (Left) Representative embryos following injection of increasing amounts of notum1a. Phenotypic classes are marked by colored squares. (Right) Injection of a quantity of notum1a mRNA that does not produce overt phenotypes enhances the severity of phenotypes seen in embryos injected with dkk1. (B,C) tbx6, which is expressed around the margin at 60% in wild-type embryos (B) is eliminated following notum1a overexpression (C). (D,E) gsc expressed on the presumptive dorsal side of the embryo in wild-type shield stage embryos (D), is expanded following notum1a injection (E). (F) Notum 1a interacts with the Wnt/β-catenin pathway. Injection of wnt8 or wnt1 mRNA or dkk1 MO produces embryos lacking eyes (lightest blue) or with smaller eyes (light blue) than wild-type embryos (dark blue) at 24 hpf. Co-injection of notum1a suppressed wnt8 and wnt1 mRNA, and dkk1 MO-induced eye reduction. MO-mediated depletion of notum1a is with a splice-site-directed MO enhanced eye reduction induced by wnt1 mRNA; this enhancement was rescued by injection of notum1a mRNA. (G-J) Stimulation of Wnt signaling by LiCl partially restored wild-type forebrain n3 expression following notum1a overexpression. (G) Flat-mounted control, non-heat shocked Tg(hs:notum1a) embryo at eight somites with in situ hybridization against n3 (labeling forebrain), pax2a (midbrain-hindbrain boundary and pronephric mesoderm), egr2b (krox20) (rhombomeres three and five) and myod1 (adaxial cells and somites) (H) Tg(hs:notum1a) embryos heat shocked at 50% epiboly display expanded n3 domain. (I) Eight minutes of LiCl treatment at 75% epiboly eliminated n3 expression in non-heat-shocked embryos. (J) Heat-shock at 50% epiboly before the same LiCl treatment at 75% epiboly partially restores n3 expression.
Notum 1a induction in Tg(hsp70l:notum1a)nu20 causes Wnt/β-catenin inhibition at later stages

To investigate the action of Notum 1a in later development, we created a stable transgenic line, Tg(hsp70l:notum1a)nu20, subsequently referred to as Tg(hs:notum1a), in which notum1a expression is under the control of a heat-shock-inducible promoter. Heat-shock induction beginning at midblastula (high) stage recapitulated the phenotypes produced by mRNA injection; specifically, it produced embryos that lacked posterior structures with expanded forebrains (not shown). Similarly, induction of Notum 1a during gastrulation produced embryos with expanded expression of the forebrain marker rx3 at early segmentation (eight somites, \(n=24/31\); Fig. 2G,H). Brief treatment of embryos with LiCl, which induces Wnt targets by inhibiting Gsk3 (Klein and Melton, 1996), at 75% epiboly abolished rx3 expression at 8 somites \((n=21/22\); Fig. 2I) (Chuang et al., 1999). LiCl treatment after Notum 1a induction partially restored the endogenous rx3 domain \((n=22/41\); Fig. 2I), indicating that Notum 1a is acting to inhibit Wnt/β-catenin upstream of Gsk3. Induction of Notum 1a during segmentation continues to produce phenotypes consistent with Wnt/β-catenin inhibition. For example, txb6 is diminished in the tail bud of transgenic embryos after Notum 1a induction \((n=43/47\) compared with controls \((n=0/32\); supplementary material Fig. S2G,H). A phenotype also observed following depletion of multiple Wnt ligands with MOs (Thorpe et al., 2005). Together, these data indicate that Notum 1a continues to function as an inhibitor of Wnt/β-catenin activity across a temporally broad range and that stimulation of this pathway downstream of the ligand can overcome the effects of Notum 1a.

Notum 1a does not affect Fgf signaling

The anteriorization observed following Notum 1a overexpression also resembles phenotypes produced by Fgf inhibition (Amaya et al., 1991). As Fgfs have been shown to interact with the heparan-sulfate-modified proteoglycans, including glypicans (Song et al., 1997), we were interested in whether Notum 1a negatively regulates this pathway. Using Tg(hs:notum1a) embryos, we ectopically expressed notum1a in late segmentation using conditions similar to those that inhibit txb6 expression. We found that the two direct targets of Fgf signaling, pea3 and spry4 (Furthauer et al., 2001; Roehl and Nusslein-Volhard, 2001), were not altered following notum1a overexpression (supplementary material Fig. S3A-L). As notum1a expression overlaps with domains of Fgf signaling, such as the midbrain-hindbrain boundary (MHB), we examined the effects of altering Fgf activity in the Tg(hsp70l:dn-fgfr1-EGFP) line (Lee et al., 2005) on notum1a expression. Under conditions in which dominant-negative Fgf-receptor induction led to an elimination of spry4 expression, most domains of notum1a expression, such as the dorsal neural tube, were unaltered (supplementary material Fig. S3M-Q). We did find some specific domains in which notum1a expression was repressed and others in which it was expanded in the absence of Fgf signaling. After dn-fgfr1 induction, a domain of notum1a anterior to the choroid fissure of the eye is lost and the dorsoventral extent of notum1a expression at the MHB is reduced in transgenic embryos \((n=18\); supplementary material Fig. S3T,U). In the same embryos, notum1a expression was expanded into rhombomere 1 and broadened in its most posterior domain in the dorsal tail (supplementary material Fig. S3U,V). Although notum1a expression requires Wnt/β-catenin activity in all domains, the alterations of notum1a expression following Fgf signaling blockade indicate that the refinement of notum1a expression is a complex process integrating multiple signals. Unlike the interaction between Notum 1a and Wnt/β-catenin, the more subtle alterations of notum1a expression after loss of Fgf activity and the inability of Notum 1a to alter Fgf-target gene expression show that Notum 1a is not a part of a negative-feedback mechanism that directly regulates this pathway.

Notum 1a interacts with Gpc3

Previous studies demonstrate that the release of glypicans by Notum can antagonize Wnt/β-catenin signaling (Kreuger et al., 2004; Traister et al., 2007). In cell culture, mammalian Notum is able to release multiple glypicans and some GPI-anchored proteins (Traister et al., 2007). However, the specific phenotypes produced by Notum 1a overexpression suggest that it may act on a smaller set of targets. We speculated that if Notum 1a has specific targets, then depletion of one or more such targets would potentiate Notum 1a activity. The established participation of Gpc3 in Wnt signaling (Song et al., 2005) and the strong expression of zebrafish gpc3 in early development (De Cat et al., 2003) made this glypican a good candidate for investigation. We used a MO directed against gpc3 at a concentration that did not produce any overt phenotype alone; however, this dose of gpc3 MO synergized with notum1a mRNA to produce severely anteriorized embryos \((P=0.003\); Fig. 3A). This enhancement was rescued by the injection of MO-insensitive gpc3 mRNA \((P=8\times10^{-7}\); Fig. 3A). To address whether Notum 1a cleaves Gpc3, we generated an EGFP-tagged form of Gpc3. By western blot analysis, although we detected no alteration in total Gpc3-EGFP protein level following Notum overexpression, we found that Gpc3-EGFP from Notum 1a-overexpressing embryos migrates faster than that from control embryos, an observation consistent with the loss of the GPI anchor (Fig. 3B). Together, these data suggest that Notum 1a-induced Wnt inhibition is at least partially mediated by its action on Gpc3.

Notum 1a does not interact with Gpc4

Glypicans and GPI-anchored proteins are crucial for a wide variety of signaling pathways contributing to numerous developmental processes. For example, zebrafish Gpc4 is required for proper Wnt/PCP signaling (Topczewski et al., 2001). Perturbation of Wnt/PCP signaling impairs convergence and extension cell movements during gastrulation and segmentation. In particular, the cells of the notochord of gpc4m6 mutant embryos fail to elongate along the mediolateral axis (Fig. 3C,D). Such elongation defects are never observed in notum1a-overexpressing embryos (Fig. 3F), including those in which the posterior body is severely truncated. Furthermore, notum1a overexpression did not inhibit the ability of gpc4 mRNA to rescue the gpc4 mutant phenotype \((P=0.565\); Fig. 3G). In addition, the shortening of the body in heat shocked Tg(hs:notum1a) embryos was not enhanced by a loss of a functional copy of the gpc4 gene \((gpc4^{+/+}}=1206±96.6 \, \mu m, n=7\); gpc4m6 =1190±96.0 \, \mu m, \(n=9\); \(P=0.38\). Furthermore, we do not see the narrow laterally extended somites that are characteristic of gpc4 mutants in notum1a-overexpressing embryos (Fig. 2H) (Topczewski et al., 2001). Gpc4 potentiates the signaling of Wnt11 to mediate cell movement and elongation (Topczewski et al., 2001). To assess whether Notum 1a could inhibit Wnt11 activity, we used the wnt11m2216 mutants that lack functional Wnt11 and display cyclopia arising from impaired extension of axial tissues (Heisenberg et al., 2000). This cyclopia can be efficiently rescued by injection of wnt11 mRNA (Heisenberg et al., 2000). We found that injection of a quantity of notum1a, which is sufficient to inhibit the activity of wnt8, did not reduce the ability of wnt11 to rescue.
cyclopia in slh<sup>216</sup> mutants (P=0.95; Fig. 3H). Unlike with Gpc3, we did not see any alterations by western blot in Gpc4-EGFP migration or levels following Notum 1a overexpression (not shown). These data indicate surprising specificity of Notum 1a, which is capable of inhibiting Wnt/β-catenin activity but does not act as an inhibitor of Wnt/PCP signaling.

**Notum 1a does not interact with other GPI-anchored proteins**

Additionally, we assessed whether *notum1a* overexpression produces phenotypes resembling loss of GPI-anchored proteins other than glypicans. Elimination of One-eyed pinhead (Oep), a GPI-anchored EGF-CFC co-factor required for proper Nodal signaling, results in embryos with deficient mesoderm and endoderm formation (Schier et al., 1997). However, in embryos in which *notum1a* was overexpressed, we observed no reductions in endoderm, as assessed by *sox32* expression (supplementary material Fig. S4A,B) (Kikuchi et al., 2001) or in the prechordal plate, as assessed by *gsc* expression (Schier et al., 1997) during gastrulation. Furthermore, *notum1a* overexpression failed to induce any abnormalities in rhombomeric boundary formation similar to those observed in the absence of the GPI-anchored Ephrins A (not shown) (Cooke et al., 2005). Together, the inability of *notum1a* overexpression to mimic the loss of several GPI-anchored proteins, including some glypicans, indicates that Notum 1a acts on a limited set of targets to inhibit Wnt/β-catenin in the zebrafish embryo.

**Depletion of Notum 1a results in expanded Wnt/β-catenin signaling**

To address the role of Notum 1a in normal development, we used multiple MOs to deplete embryos of endogenous Notum 1a, all showing similar effects. On the whole, *notum1a*-MO-injected embryos were morphologically normal up to 24 hpf, although we observed slight bending of the posterior tail and delay of anterior brain development that becomes more pronounced after the first day of development (supplementary material Fig. SSA-D). To assess directly whether domains of Wnt activity are expanded in *notum1a*-overexpressing embryos, we employed the Tg(TOP:dEGFP)<sup>26</sup> line in which a Wnt/β-catenin-responsive reporter induces destabilized EGF expression (Dorsky et al., 2002). Injection of 2 ng MO leads to expanded dEGFP expression at 24 hpf, especially in the tail bud (supplementary material Fig. S5A-D). To address the role of Notum 1a in normal development, we used multiple MOs to deplete embryos of endogenous Notum 1a, all showing similar effects. On the whole, *notum1a*-MO-injected embryos were morphologically normal up to 24 hpf, although we observed slight bending of the posterior tail and delay of anterior brain development that becomes more pronounced after the first day of development (supplementary material Fig. SSA-D). To assess directly whether domains of Wnt activity are expanded in *notum1a*-overexpressing embryos, we employed the Tg(TOP:dEGFP)<sup>26</sup> line in which a Wnt/β-catenin-responsive reporter induces destabilized EGF expression (Dorsky et al., 2002). Injection of 2 ng MO leads to expanded dEGFP expression at 24 hpf, especially in the tail bud (supplementary material Fig. S5A-D).
motoneurons in the ventral neural tube, such as domains of markers of differentiated oligodendrocytes and (supplementary material Fig. S6E–H,K,L) (Dirksen and Jamrich, 1991). Additionally, the (supplementary material Fig. S6E–H,K,L) (Dirksen and Jamrich, 1991). Furthermore, we examined the consequence of loss of notum1a on cell proliferation and found no difference in the mitotic indices, as assessed by phospho-Histone H3 staining at 24 hpf (control=1.9±0.6%, n=8 embryos; MO=1.9±1.0%, n=8 embryos; P=0.99) or in the total cell counts (control=61.1±1.2 cells/section/embryo, n=8 embryos, 2565 cells; MO=61.6±1.2, n=8 embryos, 5853 cells, P=0.73) between the neural tubes of control and notum1a-MO-injected embryos. Furthermore, we did not see alterations in the activity or expression of components of other signaling pathways that may contribute to neural tube patterning in morphant embryos, such as bmp4 (Hammerschmidt et al., 1996). These data indicate that the depletion of Notum 1a results in an expansion in the domains of dorsally expressed, Wnt-dependent markers located near the source of Wnt signals, such as msxc and notum1a itself, more ventrally expressed markers or overall cell proliferation within the neural tube are not affected. The enhancement of the domains of expression of Wnt-reporter genes, including notum1a itself, following Notum 1a loss of function reveals that it contributes to an autoregulatory loop restricting Wnt signaling close to its source within the dorsal neural tube. Manipulation of Notum 1a activity provides a novel means with which to misregulate Wnts locally and to investigate their role in neural tube development.

**dkk1 is ectopically expressed following loss of Notum 1a**

Although we did not observe alterations in markers of neural tube patterning following loss of Notum 1a outside of the dorsal domain, we found ectopic expression of dkk1, a transcriptional target of Wnt/β-catenin signaling (Chamarro et al., 2005), in notum1a MO-injected embryos. In wild-type embryos at 24 hpf, dkk1 is expressed in the epidermis dorsal to the neural tube but is not detectable by in situ hybridization within the neural tube at the level of the yolk extension (n=38; Fig. 4G–J). However, in the majority of Notum 1a-depleted embryos, a distinct band of dkk1 expression was detected three to four cell diameters ventral to the roof plate (n=37/48; Fig. 4G–J). This ectopic induction of dkk1 in Notum 1a morphants may arise from an expanded range of Wnt signaling within the neural tube and implies the initiation of auto-regulatory mechanisms to normalize Wnt output. Although the ectopic induction of dkk1 in Notum 1a-depleted embryos suggests that upregulation of additional Wnt inhibitors may compensate for loss of Notum 1a, we did not detect changes in pax3 expression or a further expansion of msxc in embryos injected with moderate amounts of both dkk1 and notum1a MOs. Thus, in addition to the complex network of intracellular molecules maintaining proper Wnt/β-catenin activity, the observed upregulation of Wnt inhibitors in Notum-depleted embryos indicates that redundant feedback mechanisms act to maintain proper neural tube patterning.

**DISCUSSION**

Studies in Drosophila and in mammalian cell culture have demonstrated that Notum induces the release of all examined glypicans (Gerlitz and Basler, 2002; Giraldéz et al., 2002; Kreuger et al., 2004; Piddini and Vincent, 2009; Traister et al., 2007). Glypicans have broad functions conferred by the low affinity binding properties of heparan-sulfate side chains, as well as more
specialized functions conferred by the core proteins (Williams et al., 2010). The number of glycan genes expands from one in C. elegans and two in Drosophila to six in mammals (Gumienny et al., 2007). Work in planarians and Drosophila indicate a role for Notum as part of an auto-regulatory loop that restricts Wnt/β-catenin signaling (Petersen and Reddien, 2011; Gerlitz and Basler, 2002; Giraldez et al., 2002), whereas the biochemical data from mammalian cells suggest that Notum could have much broader activity in vertebrates. We present evidence that, during zebrafish development, a Notum homolog, Notum 1a, has a very restricted function. While Notum 1a inhibits Wnt/β-catenin signaling by acting in part through Gpc3, it is not interacting with the Gpc4-mediated Wnt/PCP pathway (Topczewski et al., 2001).

**Notum 1a restricts the range of Wnt/β-catenin signaling**

Notum 1a is both a target and inhibitor of Wnt/β-catenin signaling, analogous to the situation observed in the Drosophila. This observation indicates a conserved role for Notum as a component of a Wnt/β-catenin self-inhibitory mechanism. Interestingly, ectopic expression of the Wnt/β-catenin inhibitor dkk1 in the neural tube of Notum 1a-depleted embryos, several cells away from the roof plate (the source of Wnt1 and Wnt3a), suggests that Dkk1 may function as a longer-range inhibitory signal in this self-inhibitory mechanism. In Drosophila, Notum was shown to restrict the spread of Wg by acting upon glypicans that normally promote its distribution (Giraldez et al., 2002). Similarly, in notum1a morphants, we found that the neural tube domains of msxc and notum1a were ventrally expanded, suggesting an increased range of Wnt activity in the neural tube following Notum 1a depletion. Previous authors have proposed that Notum acts to sharpen the Wg/Wnt morphogen gradient in Drosophila (Giraldez et al., 2002; Kreuger et al., 2004); however, whether a gradient of Wnts is necessary for proper neural tube patterning in vertebrates is unclear. Our work indicates that the Notum 1a is needed to restrict the most dorsal Wnt-dependent fates in the neural tube. Modulation of Notum 1a activity may provide a tool to investigate the significance of Wnt gradients in neural tube development.

**Notum 1a overexpression phenotypes suggest a requirement for glypicans in Wnt/β-catenin signaling**

In vitro and in vivo studies have demonstrated differing and cell-type-specific roles for several glypicans in Wnt/β-catenin signaling. For example, tissues from Gpc3 knockout mice exhibit enhanced Wnt/β-catenin signaling (Song et al., 2005), overexpression of Gpc3 inhibits Wnt/β-catenin in some cell lines (De Cat et al., 2003), whereas Gpc3 stimulates this pathway in hepatocellular carcinoma lines (Capurro et al., 2005). Furthermore, overexpression of Gpc1 results in depleted Wnt/β-catenin signaling in the chick, while expression of a soluble Gpc1 lacking a GPI anchor results in enhanced Wnt/β-catenin signaling (Shiau et al., 2010). Despite the different effects on the Wnt/β-catenin pathway observed with individual vertebrate glypicans, we observe a surprisingly specific effect of the glycan-cleaving enzyme Notum 1a on Wnt/β-catenin inhibition.

The discrepancy between the inhibitory effect of Notum 1a on Wnt/β-catenin shown in our studies and the previously described effects of individual glypicans is probably due to several factors. Work in Drosophila has demonstrated that whether the glycan Dally-like functions as an activator or repressor of Wg signaling depends on the ratio of Dally-like to the receptor Frizzled 2 (Yan et al., 2009). If this same principle applies to vertebrate glypicans involved in Wnt/β-catenin signaling, whether such glycan function as inhibitors or activators of this pathway varies with concentration and between tissues. Additionally, as there are six mammalian glypicans and several more in zebrafish (Films et al., 2008; Hubbard et al., 2009) there is probably some functional overlap among glypicans that limits the utility of a knockdown approach as a means of elucidating redundant roles of individual glypicans. The observation that we were able to modulate Notum 1a activity by depleting and elevating Gpc3 levels does not indicate that Gpc3 is the only target of Notum 1a; rather, this suggests that Gpc3 is one of several targets, as depletion of Gpc3 or other glypicans alone does not phenocopy Notum 1a overexpression (not shown). Thus, overexpression of Notum 1a may reveal a more complete role of glycan function in Wnt/β-catenin signaling than can be inferred from manipulation of individual glypicans.

**Notum 1a does not inhibit other glypican-dependent pathways**

In contrast to Wnt/β-catenin, we did not observe any effect of Notum 1a on Fgf, Bmp or Wnt/PCP pathways, all of which have been shown to interact with glypicans. Although no specific glypicans have been shown to be essential for Fgf and Bmp signaling in zebrafish, Gpc4 is required for Wnt/PCP signaling. In Drosophila, no ligand involved in Wnt/PCP signaling has been identified, and roles of glypicans and Notum in this pathway have not been reported. Work in mammalian cell culture has demonstrated that mouse Notum can induce the release of several glypicans, including Gpc4, and other GPI-anchored proteins (Traister et al., 2007). However, our observations suggest that Notum 1a has a much smaller set of targets, as overexpression does not inhibit Wnt/PCP signaling, reduce Gpc4 activity or affect Gpc4 processing. Notum 1a specificity may be a result of particular glypican modifications found in the developing embryo and not present cell culture conditions. For example, GPI anchors may undergo a variety of modifications, the functional consequences of which are not well understood (Orlean and Menon, 2007), some of which may confer resistance to cleavage by Notum. Another possibility is that, as there are multiple zebrafish Notum homologs and only one mammalian Notum, zebrafish Notum genes have undergone subfunctionalization. However, our expression analysis and preliminary loss- and gain-of-function experiments with the remaining zebrafish Notum homologs indicate that they are not acting on Wnt/PCP or Wnt/β-catenin signaling (not shown). Notum 1a, rather than affecting all glypican-dependent processes, has retained the role of ancestral Notum as an inhibitor of Wnt/β-catenin signaling at multiple steps of zebrafish development. Whether the difference in specificity arises from biological constraints on Notum activity in the developing embryo or species-specific specialization is a subject of current investigation. Understanding the source of Notum target specificity, as well as the complete set of Notum targets will be of great value in the study of Wnt/β-catenin signaling.

**Acknowledgements**

We thank A. Picker for sharing unpublished data and C. B. Chien for providing the Tol2Kit. We thank the zebrafish community for sharing probes and reagents. We thank A. Sam for technical help with early experiments. We greatly appreciate the fish care provided by A. Hoekstra and C. Hunter.

**Funding**

This work was supported in part by the National Institutes of Health/National Institute of Dental and Caranofacial Research [R01DE016678 to J.T.]. Deposited in PMC for release after 12 months.


