

# A zebrafish Notum homolog specifically blocks the Wnt/ $\beta$ -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 glycosphosphatidylinositol (GPI)-anchored, heparan-sulfate proteoglycans bind ligands of several other signaling pathways in both vertebrates and invertebrates. In *Drosophila*, Notum, a secreted  $\alpha/\beta$ -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/ $\beta$ -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/ $\beta$ -catenin pathway, Wnt ligands bind to Frizzled and LRP5/6 co-receptors, leading to an inhibition of the activity of GSK3 kinase, a component of a  $\beta$ -catenin-destruction complex. This inhibition allows  $\beta$ -catenin to accumulate and enter the nucleus to activate target genes by interacting with LEF/TCF transcription factors (MacDonald et al., 2009). As Wnt/ $\beta$ -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 glycosphosphatidylinositol (GPI) anchor. In *Drosophila*, the glypicans 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  $\alpha/\beta$  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 and Basler, 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/ $\beta$ -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 (Topczewski 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 (Filmus 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/ $\beta$ -catenin pathway.

## MATERIALS AND METHODS

### Cloning of *notum1a*

Full-length *notum1a* was cloned from pooled mixed-stage mRNA with the primers: 5'-AGATTCTCAGCATCACCTCCGGATT-3' and 5'-AGTTGGACAGAGCATCGACTGAAGG-3'.

### In situ hybridization

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

Northwestern University Feinberg School of Medicine, Department of Pediatrics, Children's Memorial Research Center, Children's Plaza 2300, Box 204, Chicago, IL 60614, USA.

\* Author for correspondence (j-topczewski@northwestern.edu)

## Zebrafish lines

Wild-type embryos for all experiments were of an AB  $\times$  Tubingen background. The following transgenic and mutant lines were used: *Tg(hsp70l:tcf3-gfp)*<sup>w26</sup>, *Tg(TOP:dEGFP)*<sup>w25</sup>, *Tg(hsp70l:notum1a)*<sup>nu20</sup>, *Tg(Bactin:HRAS-EGFP)*<sup>nu119</sup> and *Tg(hsp70l:dn-fgfr1-EGFP)*<sup>pd1</sup>; and *gpc4*<sup>fr6</sup> and *wnt11*<sup>ts216</sup>. All mutant fish described in experiments were produced from crosses between heterozygous parents. To generate the *Tg(hsp70l:notum1a)*<sup>nu20</sup> line, a FLAG-epitope tagged Notum 1a-FLAG was created by inverted 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 *hsp70l* promoter and a *polyA* sequence in the *pDestTol2CG2* 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(hsp70l:notum1a)*<sup>nu20</sup> line. As the transgene backbone contains a *cmilc2: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(hsp70l:dn-fgfr1-EGFP)*<sup>pd</sup> 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(hsp70l:tcf3-gfp)*<sup>w26</sup>, 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  $\beta$ -catenin-GFP expression in *hs: $\beta$ -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(hsp70l:notum1a)*<sup>nu20</sup> 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(hsp70l:notum1a)*<sup>nu20</sup> 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'-AGAGAGCATGGCGATGTGCATCATG-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'-CCGGAGGTGATGCTGAGAATCT-3'; a start codon-directed MO, 5'-CTCCTCTTCATCGCGAAAAATCCG-3'; and a splice site-directed MO, 5'-GTCATAAAGCATCACACTTACCCTC-3. All MOs produced similar phenotypes; however, the UTR-directed MO was generally more toxic. Unless otherwise noted, experiments used the AUG-directed MO. The MO directed against the *gpc3* start codon was 5'-GTACAACTTCAGTCCAGGCATCATG. Zebrafish *gpc3* used for rescue was initially cloned from mixed-stage mRNA using the primers 5'-

AACTCGAGGTAGCGTTTCTGGATTGAAGAAG-3' and 5'-AAGGATCCTATTTCCGCCAAATATCTCTTGT-3'. Using this as a template, we generated a morpholino-insensitive, nine-base-mismatch construct with the primers 5'-ATGATGCCAGGTTTAAAACT-ATATGGTGGCTGATTTTGTGTGT-3', 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATGCCAGGTTTAAAACT-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAATCACTGAAGACC-CAGTGTAT-3'. This PCR produced a Gateway-cloning-compatible product that was inserted into *pCSDEST*. To make *pCS-EGFP-gpc3*, PCR was used to insert full-length *EGFP* between amino acids 29 and 30 of morpholino-insensitive *gpc3*, after the predicted signal peptide. The membrane localization of *gpc3-EGFP* was confirmed by confocal imaging of *gpc3-EGFP*-injected embryos.

## 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 10ng *pCS2-wnt1*. Cells were transfected with 500 ng of *pCS2-notum1a* or balanced to 2  $\mu$ g 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  $\mu$ g *TOPFLASH*, 10 ng of *pRL-CMV* and 500 ng *pcs2-notum1a* or 500 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 glypican expression: GPC1, 5'-TGCTTGCCTGATGACTACCTG-3' and 5'-TGAGCACAT-TTCGGCAATAG-3'; GPC3, 5'-CTCGATGAGGAAGGGTTTGA-3' and 5'-GCAGGAGGAAGAAGAAGCAC-3'; GPC4, 5'-ATGTCCCTC-GCAAATTGAAG-3' and 5'-TCAAAATCGAGATCCCCTTG-3'; GPC5, 5'-CTCCGAAGAAGTTCGGAAAC-3' and 5'-AGAATCCTG-AACCGAAGCA-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

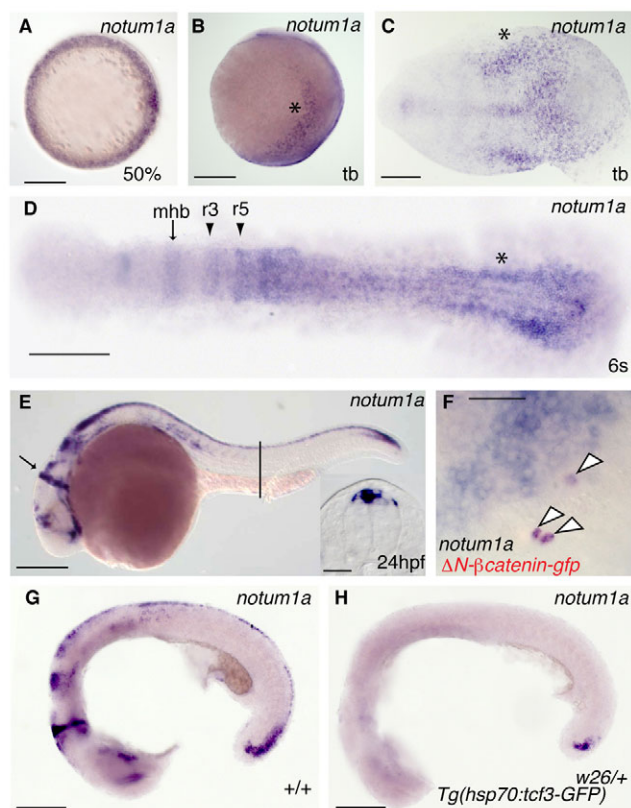
Using a bioinformatic approach, we identified three zebrafish Notum homologs, *notum1a*, *notum1b* and *notum2*, with highly conserved protein products compared with human and fly homologs. These sequences are particularly conserved surrounding the active site motifs (G-X-S-X-G) and the Ser, His, Asp catalytic triads essential for the activity of  $\alpha/\beta$  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 adjacent to the midline of the neural plate (Fig. 1D), throughout the hindbrain, with especially strong expression in rhombomeres 3 and 5 (Fig. 1D), 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/ $\beta$ -catenin signaling regulates *notum1a* expression

As *notum1a* expression overlaps with domains of high Wnt/ $\beta$ -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  $\beta$ -catenin, *hs: $\beta$ -catenin-gfp* (Nyholm et al., 2007). Mosaic induction of  $\beta$ -catenin led to ectopic expression of *notum1a* during gastrulation (Fig. 1F). Conversely, we blocked Wnt/ $\beta$ -catenin dependent signaling by heat shock-induced expression of a dominant-negative form of Tcf3 in the *Tg(hsp70l:tcf3-GFP)<sup>w26</sup>* 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  $\beta$ -catenin (Torisu et al., 2008). Our results show that, similar to *Drosophila Notum*, *notum1a* expression requires Wnt/ $\beta$ -catenin signaling.

### Notum 1a inhibits Wnt/ $\beta$ -catenin in cultured cells

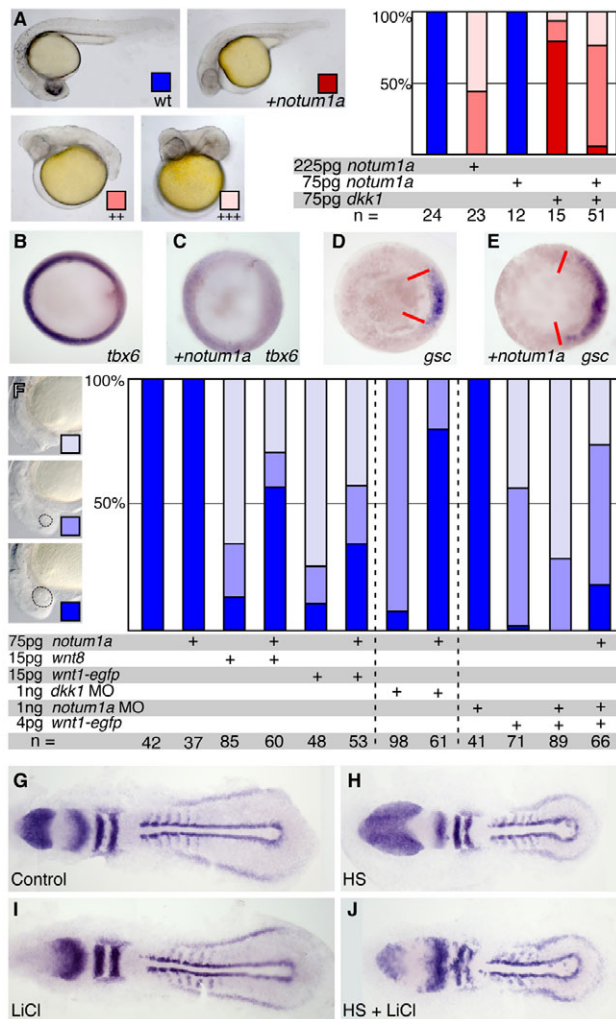
To investigate the effect of Notum 1a on Wnt signaling, we transiently transfected HEK 293 cells with the TCF-reporter plasmid *TOPFLASH*, in which Wnt-responsive elements drive the expression of luciferase (Veeman et al., 2003). Co-transfection with zebrafish *wnt1*, which, like *notum1a*, is expressed in the zebrafish roofplate (Bonner et al., 2008), caused a dramatic increase in luciferase activity compared with cells transfected with



**Fig. 1. Expression of zebrafish *notum1a*.** (A-E) Expression of zebrafish *notum1a*. (A) At 50% epiboly, *notum1a* is expressed around the entire blastoderm margin. (B) A lateral view of *notum1a* expression at tail bud reveals expression in the lateral neural plate (asterisk). (C) A flat-mount of the tail bud displays *notum1a* expression in the posterior neural plate, particularly at the lateral edges (asterisk) and midline. (D) At six somites, as neurulation proceeds, lateral neural plate *notum1a* expression becomes more medially located. More anteriorly, *notum1a* begins to appear in the midbrain-hindbrain boundary (mhb, arrow) and rhombomeres 3 and 5 (r3, r5, arrowheads). (E) At 24 hpf, *notum1a* is expressed in the mhb (arrow) and in the dorsal neural tube, as revealed in a section through the trunk at the level of the yolk extension (line, inset). Scale bar: 50  $\mu$ m. (F,G) Wnt/ $\beta$ -catenin signaling is needed for *notum1a* expression. (F) Mosaicically expressed activated  $\beta$ -catenin (red) colocalizes with ectopic *notum1a* expression (arrowheads) at 80% epiboly. Scale bar: 50  $\mu$ m. (G,H) Although normally expressed in heat-shocked non-transgenic embryos at 22 hpf (G), *notum1a* is absent in heat-shocked *Tg(hsp70l:tcf3-gfp)<sup>w26/+</sup>* embryos (H) except in small domain within the tail bud 2 hours post-heat shock. Scale bars in A-D, G, H: 200  $\mu$ m.

*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 glypicans, *GPC1*, *GPC3*, *GPC4* and *GPC5*, expressed in HEK 293 cells (supplementary material Fig. S2C), all of which may serve as





**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/ $\beta$ -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* 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 *rx3* expression following *notum1a* overexpression. (G) Flat-mounted control, non-heat shocked *Tg(hs:notum1a)* embryo at eight somites with in situ hybridization against *rx3* (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 *rx3* domain. (I) Eight minutes of LiCl treatment at 75% epiboly eliminated *rx3* expression in non-heat-shocked embryos. (J) Heat-shock at 50% epiboly before the same LiCl treatment at 75% epiboly partially restores *rx3* expression.

targets of Notum 1a (Traister et al., 2007). Thus, in cultured cells, zebrafish Notum 1a inhibited Wnt/ $\beta$ -catenin signaling similarly to mammalian Notum (Traister et al., 2007).

### Notum 1a inhibits Wnt/ $\beta$ -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/ $\beta$ -catenin ligands is inhibited (Shimizu et al., 2005; Thorpe et al., 2005) or in which Dkk1, a secreted inhibitor of Wnt/ $\beta$ -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/ $\beta$ -catenin target genes, we examined the expression of *tbx6*, a marker of ventrolateral 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/ $\beta$ -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/ $\beta$ -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 ( $n=6/12$ ,  $P=0.009$ ; supplementary material Fig. S2D-F). Wnts posteriorize the neural plate, and Wnt/ $\beta$ -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/ $\beta$ -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 ( $P=3\times 10^{-8}$ ; Fig. 2F), demonstrating that Notum 1a moderates Wnt/ $\beta$ -catenin signaling during early zebrafish development.

### Notum 1a induction in Tg(*hsp70l:notum1a*)<sup>nu20</sup> causes Wnt/ $\beta$ -catenin inhibition at later stages

To investigate the action of Notum 1a in later development, we created a stable transgenic line, Tg(*hsp70l:notum1a*)<sup>nu20</sup>, 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. 2J), indicating that Notum 1a is acting to inhibit Wnt/ $\beta$ -catenin upstream of Gsk3. Induction of Notum 1a during segmentation continues to produce phenotypes consistent with Wnt/ $\beta$ -catenin inhibition. For example, *tbx6* 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/ $\beta$ -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 *tbx6* 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/ $\beta$ -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/ $\beta$ -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.

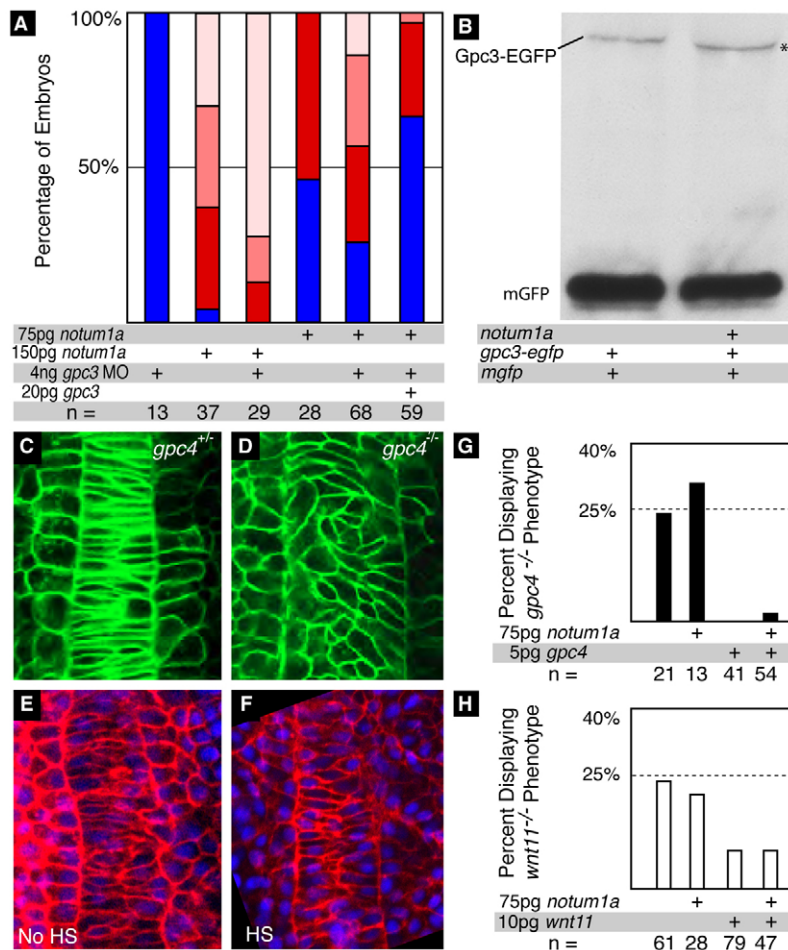
### Notum 1a interacts with Gpc3

Previous studies demonstrate that the release of glypicans by Notum can antagonize Wnt/ $\beta$ -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 ( $P=8 \times 10^{-7}$ ; 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 *gpc4*<sup>fr6</sup> 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*<sup>+/+</sup>=1206±96.6  $\mu$ m,  $n=7$ ; *gpc4*<sup>fr6/+</sup>=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 *wnt11*<sup>z216</sup> 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





**Fig. 3. Notum 1a interacts with specific glypicans.**

(A) Inhibition of posterior body in *notum1a*-overexpressing embryos is enhanced by depletion of *gpc3*. Although co-injection of *gpc3* MO increases the number of embryos with severe inhibition of posterior body ( $P=0.003$ ), co-injection of *gpc3* mRNA reduces the number of embryos displaying any posterior body impairment compared with *notum1a*-injected ( $P=0.05$ ) and *notum1a* mRNA- and *gpc3* MO-injected ( $P=0.002$ ) embryos. The phenotypic classes are the same as depicted in Fig. 2A. (B) Notum 1a overexpression produces a faster migrating form of Gpc3-EGFP. A western blot of lysates from mGFP/*gpc3*-EGFP-injected and mGFP/*gpc3*-EGFP/*notum1a*-injected embryos probed with an anti-GFP antibody reveals that the *gpc3*-EGFP band migrates faster in lysates from *notum1a*-injected embryos. (C-F) Notum 1a overexpression does not mimic the Gpc4 loss-of-function phenotype. (C,D) Confocal images of the notochords of *gpc4*<sup>tr6/tr6</sup> (C) and *gpc4*<sup>tr6/tr6</sup> *Tg(Bactin:HRAS-EGFP)*<sup>vu119</sup> embryos at five somites. Although the cells of the notochords of *gpc4*<sup>tr6/tr6</sup> embryos display normal mediolateral elongation and orientation, the cells are disorganized in the homozygous mutants. (E,F) Confocal images of three somite embryos injected with *mCherry-HsHRAS* RNA non-heat shocked and *Tg(hs:notum1a)* embryos heat shocked at 50% epiboly. Both control (E) and *notum1a*-overexpressing (F) embryos display normal cell elongation and orientation. (G) *notum1a* does not interact with Gpc4 or Wnt11. An incross of *gpc4*<sup>tr6/tr6</sup> results in a Mendelian ratio of embryos displaying a mutant phenotype at 24 hpf. Injection of *gpc4* mRNA efficiently rescues this phenotype. Co-injection of *notum1a* with *gpc4* mRNA does not impair the rescue of the mutant phenotype ( $P=0.565$ ). (H) Similarly, *wnt11*<sup>tz216/tz216</sup> fish were incrossed to produce a Mendelian ratio of mutant embryos displaying some degree of cyclopia. This phenotype can be suppressed by injection of *wnt11* mRNA, and co-injection of *notum1a* did not inhibit this effect ( $P=0.95$ ).

cyclopia in *slb*<sup>tz216</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/ $\beta$ -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/ $\beta$ -catenin in the zebrafish embryo.

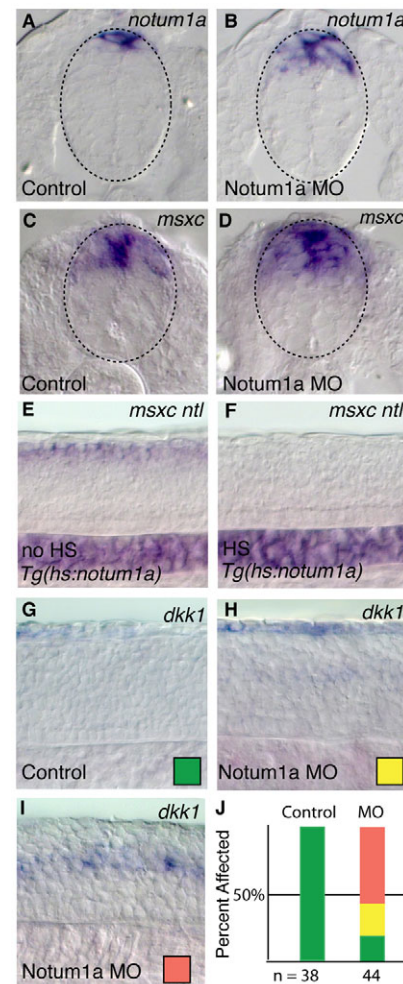
### Depletion of Notum 1a results in expanded Wnt/ $\beta$ -catenin signaling

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. S5A-D). To assess directly whether domains of Wnt activity are expanded in *notum1a* MO-injected embryos, we employed the *Tg(TOP:dEGFP)*<sup>w26</sup> line in which a Wnt/ $\beta$ -catenin-responsive reporter induces destabilized EGFP 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. S6A,B), a domain of high *notum1a* expression. Multiple studies have demonstrated a role of Wnt signaling in controlling both cell proliferation and patterning, particularly within the dorsal domain of the developing neural tube, with recent work proposing temporally independent roles for Wnt/ $\beta$ -catenin signaling in these two processes in the zebrafish neural tube (Bonner et al., 2008). As *notum1a* expression in the roof plate is controlled by Wnt/ $\beta$ -catenin activity, we investigated the consequences of Notum 1a knockdown on its own expression. In situ hybridization reveals that the domain of *notum1a* expression, expressed as a percentage of the total dorsoventral height of the neural tube, was significantly ventrally expanded within the dorsal neural tube in MO-injected embryos

(control=15.7±1.9%,  $n=10$ ; MO=23.5±2.3%,  $n=12$ ,  $P=7.8 \times 10^{-9}$ ; Fig. 4A,B). Similarly, the expression of *msxc* is ventrally expanded in Notum 1a-depleted embryos (control=27.1±1.0%,  $n=8$ ; MO=33.9±0.9%;  $P=2.0 \times 10^{-4}$ ; Fig. 4C,D), which is consistent with a requirement for Wnt/ $\beta$ -catenin signaling for the proper expression of this gene in the neural tube (Bonner et al., 2008). Conversely, induction of ectopic *notum1a* in transgenic embryos eliminates *msxc* expression in the dorsal neural tube ( $n=25/28$ ), while such a loss of *msxc* expression was never observed in heat-shocked non-transgenic embryos (Fig. 4E,F). Depletion of Notum 1a had effects limited to the most dorsal domains of zebrafish neural tube. No alterations in the expression of markers of ventral or intermediate neural progenitors such as *pax3a*, *pax6a* or *foxa* were detected in Notum 1a-depleted embryos (supplementary material Fig. S6E-H,K,L) (Dirksen and Jamrich, 1995; Seo et al., 1998; Krauss et al., 1991). Additionally, the domains of markers of differentiated oligodendrocytes and motoneurons in the ventral neural tube, such as *lhx3*, were not affected in *notum1a* MO-injected embryos (supplementary material Fig. S6I,J) (Appel et al., 1995). We also 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 although 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/ $\beta$ -catenin signaling (Chamorro 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 *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



**Fig. 4. Notum 1a is required for proper neural tube patterning.**

(A,B) Compared with uninjected controls at 24 hpf (A), *notum1a* expression is expanded in the neural tubes of Notum 1a-depleted embryos ( $P=7.8 \times 10^{-9}$ ) (B). (C,D) Similarly, compared with uninjected embryos at 24 hpf (C), *msxc* is expanded in *notum1a* MO-injected embryos ( $P=2.0 \times 10^{-4}$ ) (D). (E,F) *msxc* expression is lost in the dorsal neural tube of heat-shocked *Tg(hs:notum1a)* embryos at 24 hpf (F). Note the normal expression of *ntl* in the notochord. (G-J) Loss of *notum1a* leads to ectopic *dkk1* expression in the neural tube. Although *dkk1* is not observed in the neural tube of uninjected embryos at 24 hpf (G), it was faintly (I) or intensely (H) expressed within the neural tube in the majority of *notum1a* MO-injected embryos (J).

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/ $\beta$ -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; Giraldez 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 glypican 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/ $\beta$ -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/ $\beta$ -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/ $\beta$ -catenin signaling

Notum 1a is both a target and inhibitor of Wnt/ $\beta$ -catenin signaling, analogous to the situation observed in the *Drosophila*. This observation indicates a conserved role for Notum as a component of a Wnt/ $\beta$ -catenin self-inhibitory mechanism. Interestingly, ectopic expression of the Wnt/ $\beta$ -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/ $\beta$ -catenin signaling

In vitro and in vivo studies have demonstrated differing and cell-type-specific roles for several glypicans in Wnt/ $\beta$ -catenin signaling. For example, tissues from Gpc3 knockout mice exhibit enhanced Wnt/ $\beta$ -catenin signaling (Song et al., 2005), overexpression of Gpc3 inhibits Wnt/ $\beta$ -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/ $\beta$ -catenin signaling in the chick, while expression of a soluble Gpc1 lacking a GPI anchor results in enhanced Wnt/ $\beta$ -catenin signaling (Shiau et al., 2010). Despite the different effects on the Wnt/ $\beta$ -catenin pathway observed with individual vertebrate glypicans, we observe a surprisingly specific effect of the glypican-cleaving enzyme Notum 1a on Wnt/ $\beta$ -catenin inhibition.

The discrepancy between the inhibitory effect of Notum 1a on Wnt/ $\beta$ -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 glypican 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/ $\beta$ -catenin signaling, whether such glypicans 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 (Filmus 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 glypican function in Wnt/ $\beta$ -catenin signaling than can be inferred from manipulation of individual glypicans.

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

In contrast to Wnt/ $\beta$ -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/ $\beta$ -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/ $\beta$ -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/ $\beta$ -catenin signaling.

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## Competing interests statement

The authors declare no competing financial interests.

## Supplementary material

Supplementary material available online at

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**Table S1.** The following probes were used for in situ hybridization

<b>Gene/probe</b>	<b>ZFIN ID Number</b>	<b>Accession Number</b>
<i>notum1a</i>		EU728672
<i>tbx6</i>	ZDB-GENE-980526-171	NM 131052
<i>gsc</i>	ZDB-GENE-980528-2060	NM 131017
<i>sp5l</i>	ZDB-GENE-030131-2981	NM 194371
<i>rx3</i>	ZDB-GENE-990415-238	NM 131277
<i>pax2a</i>	ZDB-GENE-990415-8	NM 131184
<i>egr2b (krox20)</i>	ZDB-GENE-980526-283	NM 130997
<i>myod1</i>	ZDB-GENE-980526-561	NM 131262
<i>sox32</i>	ZDB-GENE-011026-1	NM 131851
<i>msxc</i>	ZDB-GENE-980526-306	NM 131272
<i>bmp4</i>	ZDB-GENE-980528-2059	NM 131342
<i>foxa</i>	ZDB-GENE-990415-76	NM 131282
<i>pax3a</i>	ZDB-GENE-980526-52	NM 131277
<i>pax6a</i>	ZDB-GENE-990415-200	NM 131304
<i>lhx3</i>	ZDB-GENE-980526-131	NM 131208
<i>dkk1b</i>	ZDB-GENE-990708-5	NM 131003
<i>pea3</i>	ZDB-GENE-990415-71	NM 131425
<i>spry4</i>	ZDB-GENE-010803-2	NM 131826