Inventory of Supplemental Items

Figure S1. Notum has minimal effects on the GPI anchor of GPC3 or GPC4. Related to Figure 1. This figure provides additional data to support Figure 1. Appear after Figure 1.

Figure S2. Characterization of Notum-TM, comparison of Notum and Tiki2, and the effect of Notum on Wnt5a. Related to Figures 2 and 3. This figure provides additional data to support Figure 2 and 3. Appear after Figure 2.

Figure S3. Evolutionary conservation of Notum proteins from human, mouse, frog, fish, fly and planarian. Related to Figure 4. This figure provides complete amino acid sequence information of *Xenopus laevis* Notum and Notum2 proteins and their homology relationship to Notum proteins from other species, supporting Figure 4. Appear before Figure 4.

Figure S4. Characterization of *Xenopus laevis Notum* and *Notum'*. Related to Figure 5. This figure provides additional data demonstrating that *Xenopus laevis* Notum and Notum' behave like mouse Notum and inhibit Wnt signaling through Wnt deacylation. Appear before Figure 5.

Figure S5. Notum depletion reduces head development and anterior neural marker expression but does not affect Organizer marker expression. Related to Figure 5.

This figure provides additional data supporting that Notum is required for anterior development without affecting Organizer formation. Appear after Figure 5.

Figure S6. Notum is required for neural induction by Noggin, but is not required for Noggin or Chordin to antagonize BMP signaling. Related to Figure 6. This figure provides additional data supporting that Notum is required for neural induction. Appear after Figure 6.

Table S1. Statistics for Figure 5F.

Table S2. Statistics for Figure S5D. Related to Figure 5.

Table S3. Statistics for Figure S5F. Related to Figure 5.

Table S4. Statistics for Figure 6A.

Figure S1

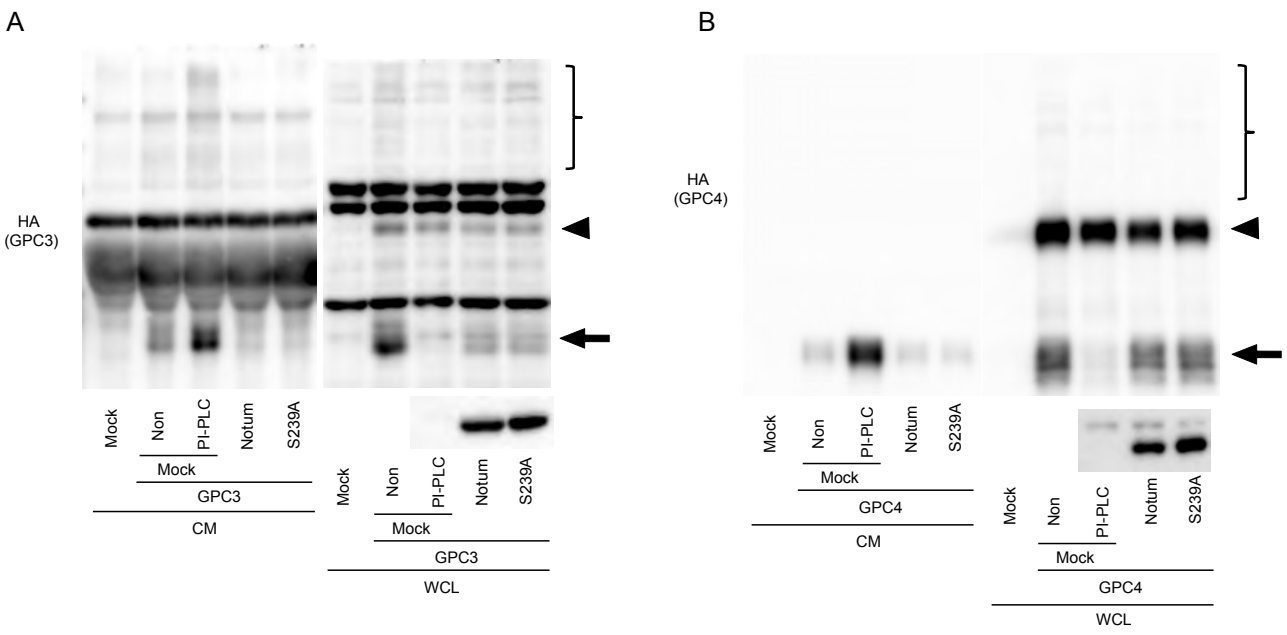

A
HA
(GPC3)
Mock
Non
PI-PLC
Notum
S239A
Mock
GPC3
CM
Mock
Non
PI-PLC
Notum
S239A
Mock
GPC3
WCL
B
HA
(GPC4)
Mock
Non
PI-PLC
Notum
S239A
Mock
GPC4
CM
Mock
Non
PI-PLC
Notum
S239A
Mock
GPC4
WCL

Figure S2

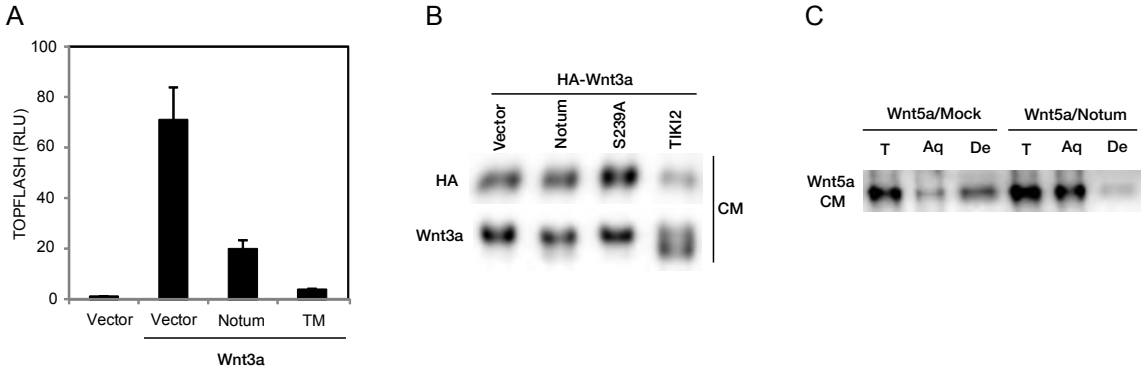
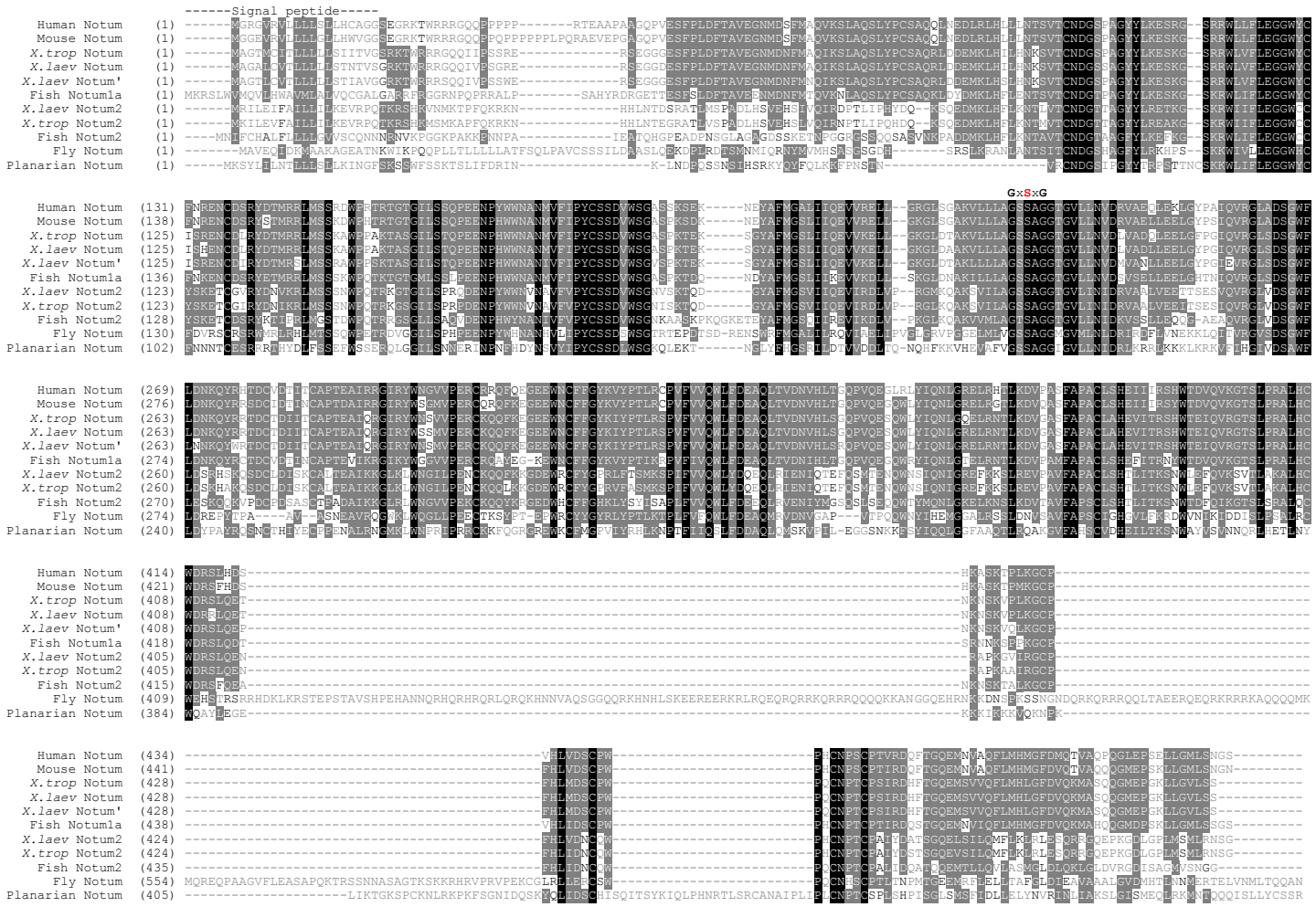

A
100
80
60
40
20
0
TOPFLASH (RLU)
Vector
Vector
Notum
TM
Wnt3a
B
HA-Wnt3a
Vector
Notum
S239A
TIKI2
HA
Wnt3a
CM
C
Wnt5a/Mock
Wnt5a/Notum
T
Aq
De
T
Aq
De
Wnt5a
CM

Figure S3

A


Signal peptide
GxSxG
Human Notum
Mouse Notum
X.trop Notum
X.laev Notum
X.laev Notum'
Fish Notum1a
X.laev Notum2
X.trop Notum2
Fish Notum2
Fly Notum
Planarian Notum

B

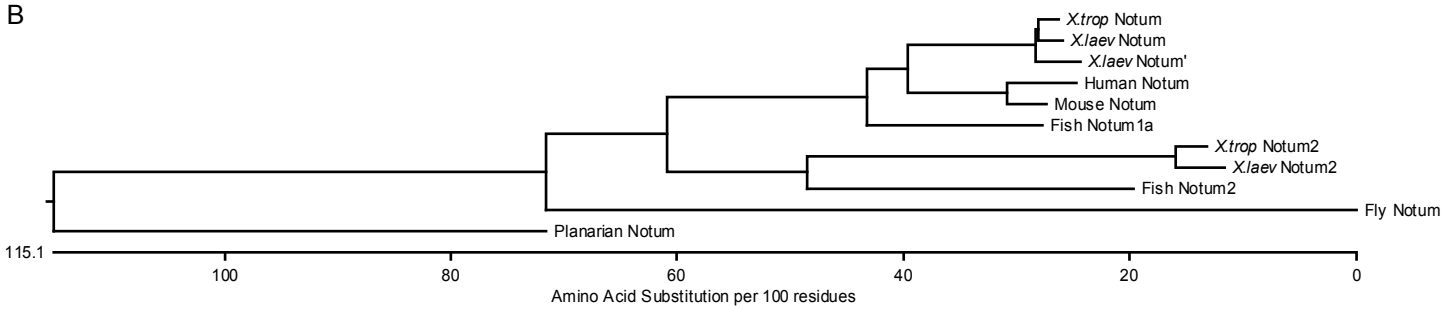

X.trop Notum
X.laev Notum
X.laev Notum'
Human Notum
Mouse Notum
Fish Notum1a
X.trop Notum2
X.laev Notum2
Fish Notum2
Fly Notum
Planarian Notum
115.1
100
80
60
40
20
0
Amino Acid Substitution per 100 residues

Figure S4

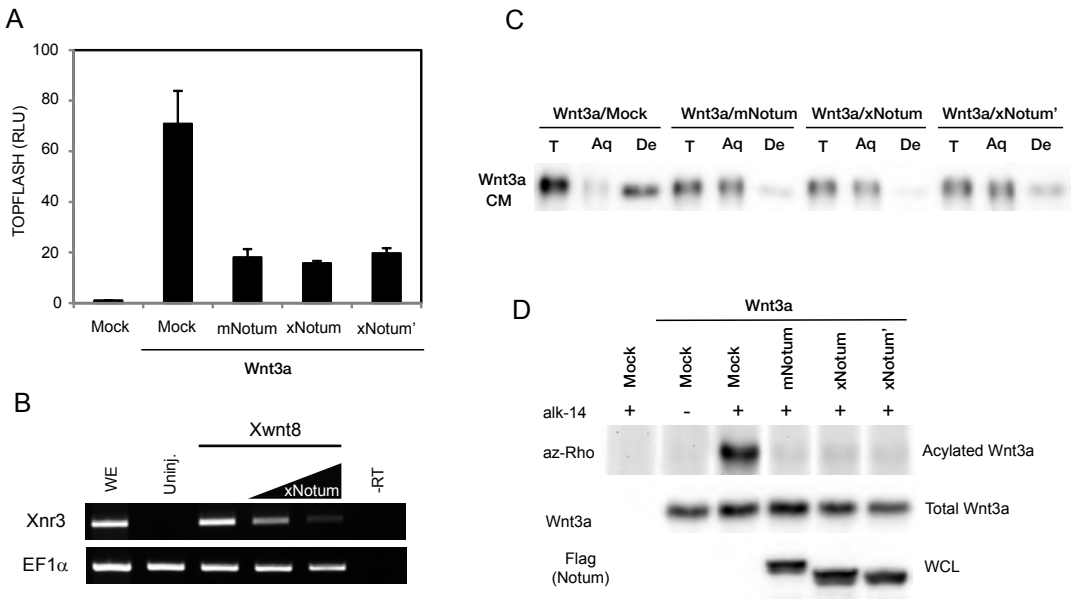

A
100
80
60
40
20
0
TOPFLASH (RLU)
Mock
Mock
mNotum
xNotum
xNotum'
Wnt3a
B
Xwnt8
WE
Uninj.
xNotum
-RT
Xnr3
EF1α
C
Wnt3a/Mock
Wnt3a/mNotum
Wnt3a/xNotum
Wnt3a/xNotum'
T
Aq
De
Wnt3a CM
D
Wnt3a
Mock
Mock
Mock
mNotum
xNotum
xNotum'
alk-14
+
-
+
+
+
+
az-Rho
Acylated Wnt3a
Wnt3a
Total Wnt3a
Flag (Notum)
WCL

Figure S5

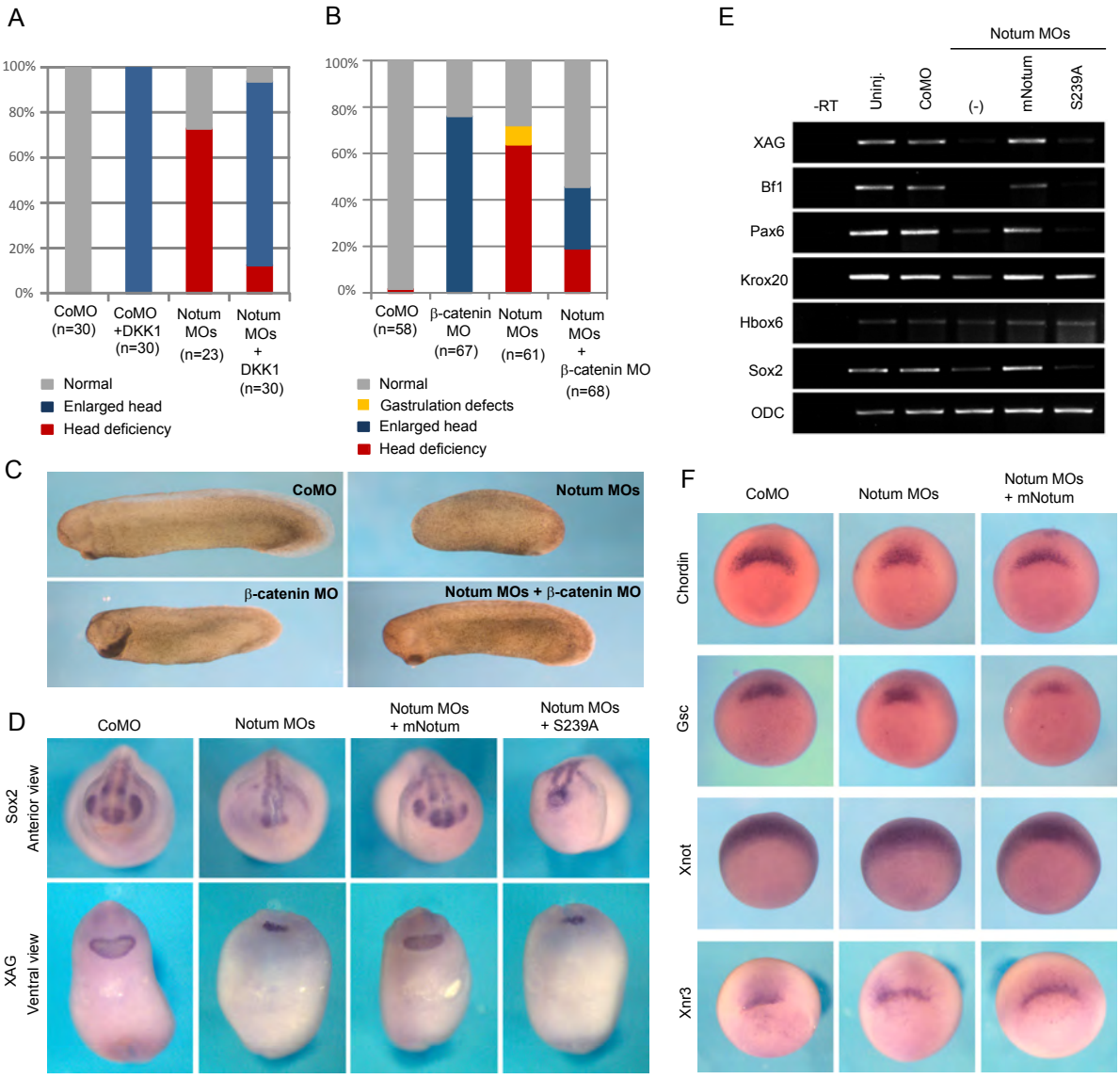
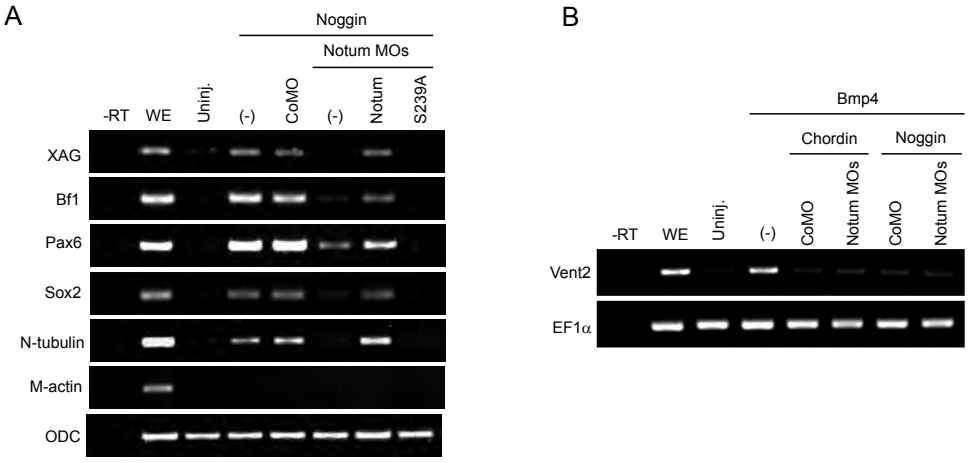

A
100%
80%
60%
40%
20%
0%
CoMO (n=30)
CoMO +DKK1 (n=30)
Notum MOs (n=23)
Notum MOs + DKK1 (n=30)
Normal
Enlarged head
Head deficiency
B
100%
80%
60%
40%
20%
0%
CoMO (n=58)
β-catenin MO (n=67)
Notum MOs (n=61)
Notum MOs + β-catenin MO (n=68)
Normal
Gastrulation defects
Enlarged head
Head deficiency
E
Notum MOs
-RT
Uninj.
CoMO
(-)
mNotum
S239A
XAG
Bf1
Pax6
Krox20
Hbox6
Sox2
ODC
C
CoMO
Notum MOs
β-catenin MO
Notum MOs + β-catenin MO
D
CoMO
Notum MOs
Notum MOs + mNotum
Notum MOs + S239A
Sox2 Anterior view
XAG Ventral view
F
CoMO
Notum MOs
Notum MOs + mNotum
Chordin
Gsc
Xnot
Xnr3

Figure S6


A
Noggin
Notum MOs
-RT
WE
Uninj.
(-)
CoMO
(-)
Notum
S239A
XAG
Bf1
Pax6
Sox2
N-tubulin
M-actin
ODC
B
Bmp4
Chordin
Noggin
-RT
WE
Uninj.
(-)
CoMO
Notum MOs
CoMO
Notum MOs
Vent2
EF1α

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Notum has minimal effects on the GPI anchor of GPC3 or GPC4. Related to Figure 1.

HA-GPC3 (A) or *HA-GPC4* (B) was expressed alone or with *Notum* or *Notum(S239A)* and resulting CM and WCL were analyzed. PI-PLC was added to the culture medium 20 minutes before collecting CM and WCL and used as a positive control. PI-PLC efficiently cleaved the GPI anchor, releasing GPC3 and GPC4 into CM while depleting them from WCL. Notum or Notum(S239A) had little effects on the release of GPC3 or GPC4 into CM (the protein band indicated by the arrow) when compared to PI-PLC. Glypicans have complex biosynthetic processes and are subjected to an endoproteolytic cleavage before being transported to the cell surface and the resulting two fragments remain attached to each other by disulfide bonds (De Cat et al., 2003; Filmus et al., 2008). The arrowhead indicates the un-cleaved protein core of GPC3 or GPC4 (WCL), while the arrow indicates one of the two cleaved protein fragments recognized by the HA antibody (WCL and CM) as GPC3 and GPC4 are tagged by HA at the matured amino-terminus. The broad bracket indicates glycanated but uncleaved protein species whose signals were weak: most of the mature glycanated species were cleaved but the HA tagged amino-terminal fragment was not glycanated (De Cat et al., 2003). The remaining bands in (A) were cross-reactive species: the HA tag on GPC3 is a shorter form (YDVPDYA) that was only detectable using the 12CA5 anti-HA monoclonal antibody with a higher background (Gonzalez et al., 1998). These patterns of GPC3 and GPC4 immunoblotting were identical to those described in literature (e.g., Capurro et al., 2014). The cleaved GPC3 band (arrow) in WCL was reduced by either *Notum* or *Notum(S239A)* co-expression (A), and thus this reduction likely represented a hydrolase-independent and non-specific phenomenon during GPC3 biogenesis under the overexpression condition.

Figure S2. Characterization of Notum-TM, comparison of Notum and Tiki2, and the effect of Notum on Wnt5a. Related to Figures 2 and 3.

(A) Expression of either *Notum-TM* or *Notum* inhibited *Wnt3a*-induced TOPFLASH. Error bars represent SD of triplicated experiments. Notum-TM was more potent than Notum in this assay, while the converse was observed in Figure 2C. These differences were reproducible, and might be explained by differences of the two experimental conditions. Under the condition of this figure Notum-TM was tethered to and concentrated on the cell surface, possibly making it more effective in inactivating Wnt3a near the plasma membrane (where signaling occurs) than Notum, which was diffusible in the CM. On the other hand, under the condition of Figure 2C, Wnt3a CM taken from Notum-expressing cells contained Notum, which likely continued to exert its action after the Wnt3a CM was harvested and applied to responding cells for the duration of the TOPFLASH assay. However Wnt3a CM taken from Notum-TM-expressing cells did not contained Notum (i.e., Notum-TM), and inactivation of Wnt3a ceased when the CM was harvested, potentially explaining the difference.

(B) Notum did not cleave the amino terminal HA tag of HA-Wnt3a, while TIKI2 did. Note that Wnt3a mobility alteration by Notum was much more subtle than that by Tiki2.

(C) Coexpression of *Wnt5a* and *Notum* resulted in loss of hydrophobicity of the secreted Wnt5a protein, as shown by the Triton X-114 detergent-aqueous phase separation assay, suggesting that Wnt5a is a Notum substrate.

Figure S3. Evolutionary conservation of Notum proteins from human, mouse, frog, fish, fly and planarian. Related to Figure 4.

(A) ClustalW alignment of Notum proteins computed by AlignX (VectorNTI). Black, grey, and light grey represent identical, conservative, and similar amino acid residues, respectively. Position of the Notum S239A catalytic mutant is shown above the

alignment with a red S in the GxSxG motif. Sources for alignment: Human (*Homo sapiens*) NP_848588.3; Mouse (*Mus musculus*) NP_780472.3; *X.trop* (*Xenopus (Silurana) tropicalis*) Notum NP_001120228.2 and Notum2 XP_002932265.2; Fish (Zebrafish *Danio rerio*) Notum1a NP_001155126.1 and Notum2 XP_694400.2; Fly (*Drosophila melanogaster*) NP_730096.2 and Planarian (*Schmidtea mediterranea*) AEF01556.1. Annotated full-length Notum sequences were unavailable for *X.laev* (*Xenopus laevis*) and were deduced from the *Xenopus laevis* v7.2 genome assembly: Notum (Scaffolds 179377 and 182780), Notum' (Scaffold 122810) and Notum2 (Scaffold 9994). Fish (Zebrafish *Danio rerio*) Notum1b NP_001002644.1 encodes a truncated 139 amino acid protein lacking the catalytic residues for Notum function and was omitted from the alignment. The gene duplication producing Notum1a and Notum1b is restricted to fish lineages and is separate from the *Xenopus laevis* allopolyploidization event that produced Notum and Notum'. At this time only the *Xenopus* Notum gene is annotated on Xenbase: X. *tropicalis* XB-GENE-996986 and X. *laevis* XB-GENE-996989.

(B) Notum protein phylogenetic tree generated by MegAlign (DNAstar Lasergene). Straight branched cladogram distances reflect the amino acid substitutions from the ClustalW alignment.

Figure S4. Characterization of *Xenopus laevis Notum* and *Notum'*. Related to Figure 5.

(A) *Xenopus laevis Notum* (*xNotum*) and *Notum'* (*xNotum'*), like mouse *Notum* (*mNotum*), inhibited *Wnt3a*-induced TOPFLASH. Error bars represent SD of triplicated experiments.

(B) *xNotum* inhibited *Xnr3* induction by *Wnt8* in animal pole explants. Two doses of *xNotum* mRNA were used.

(C) The Wnt3a protein modified by mouse Notum or xNotum or xNotum' became hydrophilic in the Triton X-114 detergent-aqueous phase separation assay.

(D) xNotum and xNotum', like mNotum, diminished Wnt3a acylation when each was coexpressed with *Wnt3a* in HEK293T cells.

Figure S5. Notum depletion reduces head development and anterior neural marker expression but does not affect Organizer marker expression. Related to Figure 5.

(A) Anterior deficiency caused by the *Notum* MOs was rescued (over-rescued) by coexpression of *Dkk1* mRNA.

(B and C) Anterior deficiency caused by the *Notum* MOs was rescued (over-rescued) by co-injection of a *β-catenin* MO. The *Notum* MOs and/or the *β-catenin* MO were/was injected into the two A1 blastomeres at the 32-cell stage. Note that depletion of β-catenin at this stage caused enlarged head as reported (Heasman et al., 2000; Kim et al., 2013).

(D) The *Notum* MOs caused decreased expression of *Sox2* and *XAG* at stage 23, and expression of these markers was rescued by coexpression of *mNotum*, but not *Notum(S239A)*, mRNA. Anterior and ventro-anterior views were shown for *Sox2* and *XAG*, respectively. See also Table S2.

(E) RT-PCR revealed that expression of anterior neural and pan neural markers was decreased in Notum-depleted embryos, and was rescued by co-injection of *mNotum*, but not *Notum(S239A)*, mRNA. Expression of *Hbox6*, a posterior neural marker, was unaffected by Notum depletion. *XAG*, a cement gland marker; *Bf1* and *Pax6*, anterior neural markers; *Krox20*, a hindbrain marker; *Hbox6*, a posterior neural marker; *Sox2*, a pan-neural marker; *ODC*, a loading control.

(F) The *Notum* MOs did not affect expression of head organizer markers, *Chordin* and *Gsc,* or dorsal markers, *Xnot* and *Xnr3,* examined at the stage 10.5. See also Table S3.

Dorsal mesoderm is regulated by Wnt signaling, but why it is unaffected by Notum depletion? We think that any of following possibilities may account, in full or a part, for this observation: i) Notum action in dorsal mesoderm may be redundant with other Wnt antagonists expressed there, such as Tiki1 and Dkk1; ii) Notum produced in ectoderm may have limited diffusion ranges; iii) Notum may have Wnt-specificity that is yet to be understood, i.e., it may not inactivate some of the Wnts involved in dorsal mesoderm development; and iv) there may exist unknown negative regulators of Notum in dorsal mesoderm.

Figure S6. Notum is required for neural induction by Noggin, but is not required for Noggin or Chordin to antagonize BMP signaling. Related to Figure 6.

(A) Neural marker induction by the Noggin protein was suppressed by the *Notum* MOs in the animal pole explants, and was rescued by co-injection of *mNotum*, but not *Notum(S239A)*, mRNA. *XAG*, a cement gland marker; *Bf1* and *Pax6*, anterior neural markers; *Sox2*, a pan-neural marker; *N-tubulin*, a neuronal marker; *M-Actin*, a mesodermal marker; *ODC*, a loading control.

(B) The *Notum* MOs did not affect *Chordin* or Noggin activity in antagonizing *BMP4* induction of *Vent2* expression in animal pole explants.

Table S1. Statistics for Figure 5F.

Embryos displaying the described changes in marker gene expression																
Marker	**CoMO**				**Notum MOs**				**Notum MOs+mNotum**				**Notum MOs+S239A**			
	n	**Percentage (%)**			**n**	**Percentage (%)**			**n**	**Percentage (%)**			**n**	**Percentage (%)**		
		Absent	Reduced	NC*		Absent	Reduced	NC*		Absent	Reduced	NC*		Absent	Reduced	NC*
Bf1	81	0	0	100	54	5.6	64.8	29.6	31	0	32.3	67.7	33	3	78.8	18.2
Otx2	32	0	0	100	24	8.3	62.5	29.2	27	0	33.3	66.7	26	3.8	57.7	38.5
Krox20	107	0	0	100	78	1.3	61.5	37.2	58	0	31	69	59	3.4	66.1	30.5

* NC = No Change

Table S2. Statistics for Figure S5D. Related to Figure 5.

Embryos displaying the described changes in marker gene expression																
Marker	**CoMO**				**Notum MOs**				**Notum MOs+mNotum**				**Notum MOs+S239A**			
	n	**Percentage (%)**			**n**	**Percentage (%)**			**n**	**Percentage (%)**			**n**	**Percentage (%)**		
		Absent	Reduced	NC*		Absent	Reduced	NC*		Absent	Reduced	NC*		Absent	Reduced	NC*
Sox2	12	0	0	100	17	52.9	35.3	11.8	14	7.1	28.6	64.3	14	57.1	35.8	7.1
XAG	14	0	0	100	14	28.6	57.1	14.3	10	0	30	70	7	28.6	42.8	28.6

* NC = No Change

Table S3. Statistics for Figure S5F. Related to Figure 5.

Embryos displaying the described changes in marker gene expression									
Marker	**CoMO**			**Notum MOs**			**Notum MOs+mNotum**		
	n	**Percentage (%)**		**n**	**Percentage (%)**		**n**	**Percentage (%)**	
		NC*	Reduced		NC*	Reduced		NC*	Reduced
Chordin	29	97	3	30	84	16	29	87	13
Gsc	31	91	9	29	87	13	32	88	12
Xnot	26	100	0	25	100	0	25	100	0
Xnr3	15	93	7	14	86	14	16	87	13

* NC = No Change

Table S4. Statistics for Figure 6A.

Embryos displaying the described changes in marker gene expression												
Marker	CoMO			Notum MOs			Notum MOs+mNotum			Notum MOs+S239A		
	n	Percentage (%)		n	Percentage (%)		n	Percentage (%)		n	Percentage (%)	
		NC*	Affected		NC*	Affected		NC*	Affected		NC*	Affected
Sox2	32	96.9	3.1 (down)	30	30	70 (down)	28	67.9	32.1 (down)	33	30.3	69.7 (down)
Keratin	37	100	0	36	30.6	69.4 (up)	37	75.7	24.3 (up)	28	28.6	71.4 (up)

* NC = No Change

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture, transfection and reporter assay

HEK293T (from ATCC) and mouse embryonic fibroblasts (MEFs) (isolated from E13-14 mouse embryos and immortalized by the SV40 T antigen) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for transfections. TOPFLASH reporter assay was performed as previously described (Zhang et al., 2012).

Antibodies and immunoblotting

Antibodies were used according to manufacturers' instructions. Immunoblotting was performed as previously described (Zhang et al., 2012). The rabbit monoclonal anti-Wnt3a (#2721, 1:1000), anti-HA (#3724, 1:1000), anti-Wnt5a (#2530, 1:1000), anti-FLAG (#8146, 1:2000), anti-Dvl2 (#3224, 1:1000), and anti-pLRP6 (S1490, #2568, 1:1000) were from Cell Signaling. Rabbit polyclonal anti-Notum (HPA023041, 1:1000) was from ATLAS Antibodies. Mouse monoclonal anti-HA (12CA5) was from Roche. Anti-FLAG M2 (A2220) and anti-HA (A2095) Affinity gels were from Sigma.

RT-PCR primers and Morpholinos

RT-PCR primers for *Xenopus laevis* Notum: Forward, 5'-AAGAGAATCCGCACTGGTGG-3' (Notum), 5'-AAGAGAATCCTCACTGGTGG-3' (Notum'); Reverse, 5'-TCTAGGGAAGGGACTGGACG-3' (Notum and Notum'). The MO sequences: xNotum MO, 5'-GTGCTCAGTGCCTGGCATGGCTGGG-3'; xNotum', 5'-TGAAGGGTGGTGCTTGGCATGGCTG-3'; standard control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3' (Gene Tools, LLC).

SUPPLEMENTAL REFERENCES

De Cat, B., Muyldermans, S.Y., Coomans, C., Degeest, G., Vanderschueren, B., Creemers, J., Biemar, F., Peers, B., and David, G. (2003). Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. The Journal of cell biology *163*, 625-635.

Gonzalez, A.D., Kaya, M., Shi, W., Song, H., Testa, J.R., Penn, L.Z., and Filmus, J. (1998). OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. The Journal of cell biology *141*, 1407-1414.

Kim, S.E., Huang, H., Zhao, M., Zhang, X., Zhang, A., Semonov, M.V., MacDonald, B.T., Garcia Abreu, J., Peng, L., and He, X. (2013). Wnt stabilization of beta-catenin reveals principles for morphogen receptor-scaffold assemblies. Science *340*, 867-870.