

# HSPG Modification by the Secreted Enzyme Notum Shapes the Wingless Morphogen Gradient

Antonio J. Giráldez, Richard R. Copley,  
and Stephen M. Cohen<sup>1</sup>  
European Molecular Biology Laboratory  
Meyerhofstrasse 1  
D-69117 Heidelberg  
Germany

## Summary

The secreted signaling protein Wingless acts as a morphogen to pattern the imaginal discs of *Drosophila*. Here we report identification of a secreted repressor of Wingless activity, which we call *Notum*. Loss of *Notum* function leads to increased Wingless activity by altering the shape of the Wingless protein gradient. When overexpressed, *Notum* blocks Wingless activity. *Notum* encodes a member of the  $\alpha/\beta$ -hydrolase superfamily, with similarity to pectin acetylesterases. We present evidence that Notum influences Wingless protein distribution by modifying the heparan sulfate proteoglycans Dally-like and Dally. High levels of Wingless signaling induce *Notum* expression. Thus, Wingless contributes to shaping its own gradient by regulating expression of a protein that modifies its interaction with cell surface proteoglycans.

## Introduction

Secreted signaling proteins of the Hedgehog, Wingless/Wnt, and Dpp/BMP/nodal families function as morphogens during animal development. In some cases, these proteins have been shown to form extracellular gradients that instruct cells in developing tissues about their prospective fate (Strigini and Cohen, 2000; Entchev et al., 2000; Teleman and Cohen, 2000; Lewis et al., 2001; Chen and Schier, 2001). Wingless (Wg), Dpp, and Hedgehog regulate the expression of genes that influence the shapes of their gradients (reviewed in Teleman et al., 2001). Each of these ligands regulates the level of expression of its receptor in ways that can influence its movement. Hedgehog induces Patched expression and thereby limits its ability to form a long-range gradient (Briscoe et al., 2001; Chen and Struhl, 1996). The Dpp receptor Thickveins is needed for Dpp movement, but at higher levels can sequester Dpp (Lecuit and Cohen, 1998; Entchev et al., 2000). Wg signaling also contributes to gradient formation by downregulating expression of its receptor Dfz2 (Cadigan et al., 1998).

The shape of ligand gradients can also be controlled by secreted and cell surface proteins distinct from the receptors. The Dpp/BMP binding proteins Chordin/Sog and Twisted Gastrulation act together with the protease Tolloid to shape the Dpp/BMP gradient in early embryos (Ashe and Levine, 1999; Oelgeschlager et al., 2000; Decotto and Ferguson, 2001; Ross et al., 2001). Other ligand binding proteins that contribute to spatial regulation of

signaling in vertebrate embryos include Wnt binding inhibitors of the sFRP and WIF families and Cerberus, which binds Wnt, BMP, and Nodal ligands (reviewed in Niehrs, 1999). Members of the Dkk family of Wnt inhibitors act by competing for binding to LRP6, an essential component of the Wnt receptor (Mao et al., 2001).

Heparan sulfate proteoglycans (HSPGs) have recently become the focus of considerable interest as modulators of intercellular signaling in development. Growth factors bind to the glycosaminoglycan side chains of HSPGs, which can mediate receptor-ligand interaction (Plotnikov et al., 1999; Reichsman et al., 1996). Genetic studies have implicated HSPGs in FGF, Hedgehog, Dpp/BMP, and Wg function in insect and vertebrate development (see Perrimon and Bernfield, 2000; Selleck, 2001 for review; Dhoot et al., 2001; Topczewski et al., 2001). Specific glypican HSPGs have been implicated in Wg/Wnt signaling in *Drosophila* and zebrafish (Lin and Perrimon, 1999; Tsuda et al., 1999; Topczewski et al., 2001). The *Drosophila* glypicans Dally and Dally-like (Dlp) have been shown to bind and stabilize Wg at the cell surface (Strigini and Cohen, 2000; Baeg et al., 2001). Dally is required for Wg activity, but has a limited capacity to increase the level of extracellular Wg binding to cells when overexpressed. In contrast, cells overexpressing Dlp accumulate Wg to considerably higher levels than surrounding cells. Dally and Dlp may help stabilize Wg and provide a pool of Wg protein that can become available for receptor binding on release from the HSPG. Thus, HSPGs may have a role in gradient formation.

In this report, we identify a novel secreted antagonist of Wg. On the basis of its mutant phenotypes in the adult, we name the gene *Notum*. *Notum* encodes an  $\alpha/\beta$ -hydrolase enzyme with similarity to pectin acetylesterases from plants. We present evidence that Notum acts as a secreted enzyme to shape the extracellular Wg gradient by modifying cell surface proteoglycans. Interestingly, high levels of Wg signaling induce expression of *Notum*, and thus, Wg acts via Notum to shape its own gradient.

## Results

### Isolation of a Novel Wingless Antagonist

The *Notum* gene was identified in a gain-of-function genetic screen (Mata et al., 2000) that caused loss of the wing and duplication of the dorsal thorax when expressed under *sd<sup>Gal4</sup>* control (Figures 1A and 1B). Replacement of the wing by a duplicated dorsal thorax resembles the defect caused by the *wg<sup>1</sup>* mutant (Morata and Lawrence, 1977; Sharma and Chopra, 1976), and can also be produced by Gal4-driven overexpression of the GSK3 homolog, *shaggy/ZW3*, an intracellular repressor of Wg signaling (Figure 1C). Expression of *Notum* throughout the embryo under *tubulin<sup>Gal4</sup>* control caused expansion of the denticle-producing zone at the expense of naked cuticle (Figures 1D and 1E). Similar phenotypes can also be obtained by overactivation of the

<sup>1</sup>Correspondence: cohen@embl-heidelberg.de

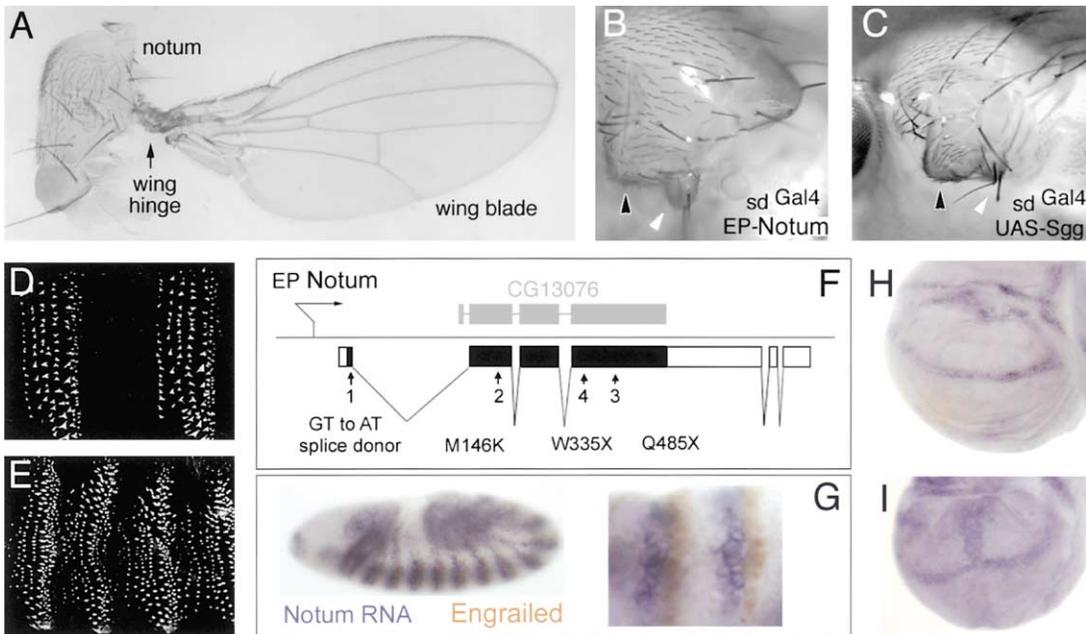


Figure 1. Identification and Characterization of the *Notum* Locus

- (A) Cuticle preparation of a wild-type wing and dorsal thorax (notum).  
 (B) *sd<sup>Gal4</sup>/+ EP-Notum/+* fly. Thoracic structures were duplicated at the expense of wing (arrowheads).  
 (C) *sd<sup>Gal4</sup>/+ UAS-sgg/+* fly. Expression of the Wg pathway antagonist Sgg/ZW3 produced the same phenotype.  
 (D and E) Ventral views of embryonic cuticle from the abdomen.  
 (D) Wild-type.  
 (E) *tubulin<sup>Gal4</sup>/UAS-Notum*. Note loss of naked cuticle and extra rows of denticles.  
 (F) Genomic organization of the *Notum* locus. Numbers under the exons indicate the positions of the molecularly characterized *Notum* mutant alleles. The associated defects are shown below. The predicted open reading frame (filled boxes) begins in exon 1.  
 (G) *Notum* mRNA (blue) and Engrailed protein (brown) visualized by in situ hybridization and antibody labeling in a germ band extended embryo. High-magnification view is shown on the right. *Notum* is expressed in cells anterior to the Engrailed-expressing cells.  
 (H) *Notum* mRNA visualized by in situ hybridization in a wild-type third instar wing imaginal disc. The expression pattern resembles Wg.  
 (I) Ectopic expression of *Notum* mRNA along the AP boundary in a *dpp<sup>Gal4</sup> UAS-Arm<sup>S10</sup>* wing disc.

EGF receptor. The balance between Wg and EGFR activity determines the domain in which naked cuticle forms by regulating expression of *shavenbaby* in the embryonic ectoderm (Payre et al., 1999). Likewise, subdivision of the wing disc into wing and thorax territories occurs during the second larval instar and depends on the balance between Wg and EGFR signaling (Ng et al., 1996; Wang et al., 2000). We also found that ventral expression of either *Notum* or an activated EGFR under *wg<sup>Gal4</sup>* control produced similar leg axis duplications (see Supplemental Figures S1A and S1B at <http://www.developmentalcell.com/cgi/content/full/2/5/667/DC1>). Thus, *Notum* might act as a repressor of the Wg pathway or as an activator of EGFR signaling when overexpressed. To distinguish between these possibilities, we examined the effects of *Notum* expression on wing vein formation, where the effects of Wg and EGFR signals differ. During third instar, ectopic EGFR signaling leads to formation of extra vein tissue in the wing blade. In contrast, *Notum* expression produced scalloping of the wing but had no effect on vein formation (Supplemental Figures S1C and S1D). This phenotype resembles late loss of Wg activity (Couso et al., 1994). Thus, we conclude that overexpression of *Notum* does not work by activation of the EGFR pathway, but rather interferes with Wg activity.

*EP-Notum* was inserted 5 kb from the predicted gene *CG13076*. The four predicted exons of *CG13076* were PCR amplified, assembled, and cloned into pUAST. Several independent transgenic lines were assayed. None had activity in vivo. Using RT-PCR, the predicted exon 2-3 splice product was amplified, but the exon 1-2 splice product was not, suggesting that the first exon predicted in *CG13076* is not used. DNA flanking *EP-Notum* was used to isolate a cDNA clone from embryo mRNA. This clone was capable of providing *Notum* function when expressed in vivo (Figure 1F and data not shown).

#### Wg Regulates *Notum* Expression

A number of genes that modulate Wg activity are spatially regulated by Wg signaling in the embryo, including *Dfz2*, *Dally*, and *naked* (Cadigan et al., 1998; Khare and Baumgartner, 2000; Zeng et al., 2000). We therefore asked whether Wg regulates *Notum* expression. In situ hybridization showed that *Notum* is expressed in a segmentally repeated pattern in two rows of cells anteriorly adjacent to the Engrailed-expressing cells (Figure 1G). This corresponds to the Wg stripe in the embryo. *Notum* expression also mirrors Wg expression in the wing disc (Figure 1H). Ectopic activation of the Wg pathway by expression of a constitutively active form of Armadillo induced ectopic *Notum* expression (Figure 1I). Thus,

high levels of Wg activity induce *Notum* expression, which in turn serves as a Wg antagonist.

#### Increased Wg Activity in *Notum* Mutants

To isolate loss-of-function mutations in the *Notum* gene, we performed a chemical mutagenesis screen for reversion of the thorax duplication caused by expression of EP-Notum. EP-Notum males were treated with EMS and crossed to *sd*<sup>Gal4</sup> females. Among 15,000 progeny, we recovered three flies that had normal wings despite overexpressing the endogenous *Notum* gene. These three alleles formed a single complementation group. *l(3)72Da* and *l(3)72CDf* were subsequently identified as *Notum* alleles and have been renamed *Notum*<sup>4</sup> and *Notum*<sup>5</sup>. Sequence analysis revealed an alteration of the splice donor site of exon 1 in *Notum*<sup>1</sup> and alterations in the coding sequence for the other alleles analyzed (Figure 1F).

*Notum* repressed Wg activity when overexpressed. *Notum* zygotic mutant embryos produced a variable naked cuticle phenotype, typical of excess Wg activity (not shown). The embryonic phenotype was weak, perhaps due to maternal contribution, so we turned to wing discs from *Notum* mutant larvae to examine the effects of removing Notum activity. The primordia of the adult wing and thoracic body wall can be visualized in the wing disc by expression of Nubbin and Teashirt proteins (Figure 2A). Nubbin is a POU homeodomain protein expressed in the presumptive wing blade and wing hinge. Tsh is a zinc finger protein expressed in the presumptive thorax. Ectopic Wg activity can lead to duplication of wing structures at the expense of thorax (Ng et al., 1996; Wang et al., 2000). *Notum* mutant discs showed this phenotype. The severity of these defects ranged from duplication of the wing pouch and hinge associated with a reduced thorax (Figure 2B) to almost complete loss of thorax associated with a severely abnormal wing duplication (Figure 2C, see legend). The same range of phenotypes was obtained by activation of the Wg pathway in the early wing disc using *Arm*<sup>S10</sup>. Figure 2D shows an example of the milder phenotype.

#### *Notum* Shapes the Wingless Gradient

Wg forms a long-range protein gradient and regulates several target genes in different spatial domains in the wing disc (Zecca et al., 1996; Neumann and Cohen, 1997). *Achaete-Scute* is a high-threshold Wg target, expressed in cells close to the DV boundary. *Achaete-Scute* expression specifies the proneural region in the anterior wing margin in which the single row of sense organ precursor (SOP) cells will form. In wild-type discs, a single row of SOPs forms on each side of the Wg stripe in the anterior compartment (Figure 2E). Large clones of cells mutant for *Notum*<sup>3</sup> formed additional rows of SOPs and produced extra mechanosensory bristles (Figure 2F). Thus, the region in which cells show a high-threshold response to Wg was broadened in the *Notum* mutant tissue. We noted that this defect occurred only when large clones affected both sides of the DV boundary. The defect was rescued when cells on one side of the boundary were able to produce Notum. This suggests that Notum can act nonautonomously. In addition, we noted that some of the ectopic bristles derived from wild-type cells. This indicates that loss of Notum activity

in a mutant clone can lead to increased Wg signaling in nearby wild-type cells. These observations suggest that Notum acts nonautonomously to affect the range of Wg action.

To examine the effect of large clones of *Notum* mutant cells on Wg, clones were produced using engrailed<sup>Gal4</sup> to drive Flp recombinase in posterior cells. The mutant posterior compartment was overgrown and the size of the Distal-less (Dll) expression domain increased, suggesting an increased range of Wg action (Figure 2G). Antibody labeling showed that the level of Wg protein was elevated in the posterior compartment. The number and brightness of Wg protein vesicles was used to visualize the Wg gradient. Both were increased in the *Notum* mutant tissue (Figures 2G and 2H). Thus, reducing *Notum* activity increased the level of Wg protein and broadened its distribution. We noted that the effects on Wg distribution did not make an abrupt transition at the AP compartment boundary. Instead, it increased with distance from the boundary, suggesting that the mutant phenotype may be partially rescued by Notum protein produced by the wild-type anterior cells.

#### Notum Protein Is Secreted and Acts Nonautonomously

The results of the clonal analyses suggested that Notum acts nonautonomously. The predicted Notum protein contains a hydrophobic sequence near its amino terminus that might function as a signal peptide. To determine whether Notum is secreted, we transfected S2 cells with expression constructs for wild-type Notum, the *Notum*<sup>2</sup> mutant, and a Golgi-tethered form of Notum, in which the signal peptide was replaced with the transmembrane domain from a Golgi-resident enzyme (Notum-GT). Secretion was assayed by immunoprecipitation from cell lysates and from the medium in which the cells were grown. Wild-type Notum protein was recovered from the conditioned medium (Figure 3A). Notum-GT was not recovered from conditioned medium. The *Notum*<sup>2</sup> mutant protein was recovered at low levels from the medium, suggesting impaired secretion.

The activities of wild-type and Golgi-tethered Notum were compared in vivo to verify that Notum can act nonautonomously. In wild-type discs, Dll and Hnt are expressed symmetrically in D and V compartments in response to Wg (Figure 3B). When overexpressed in the D compartment under apterous<sup>GAL4</sup> control, Notum protein was seen at elevated levels throughout the disc, indicating that it is secreted in vivo (Figure 3D). Hnt expression was lost and Dll expression reduced symmetrically in both D and V compartments. This led to scalloping of the wing (Figures 3C and 3E). In contrast, expression of Notum-GT only affected the D compartment (Figure 3F). The reduced size of the Dll domain can be attributed to a reduced level and range of Wg in the D compartment (Figure 3G). This led to a small D compartment and loss of dorsal wing margin, without any effect on the ventral margin (Figure 3H). Thus, overexpression of Notum-GT in the D compartment produced an asymmetric Wg gradient, whereas overexpression of the wild-type protein in the D compartment reduced the Wg gradient symmetrically in both compartments. These observations indicate that wild-type Notum can act as a secreted protein to reduce the effective

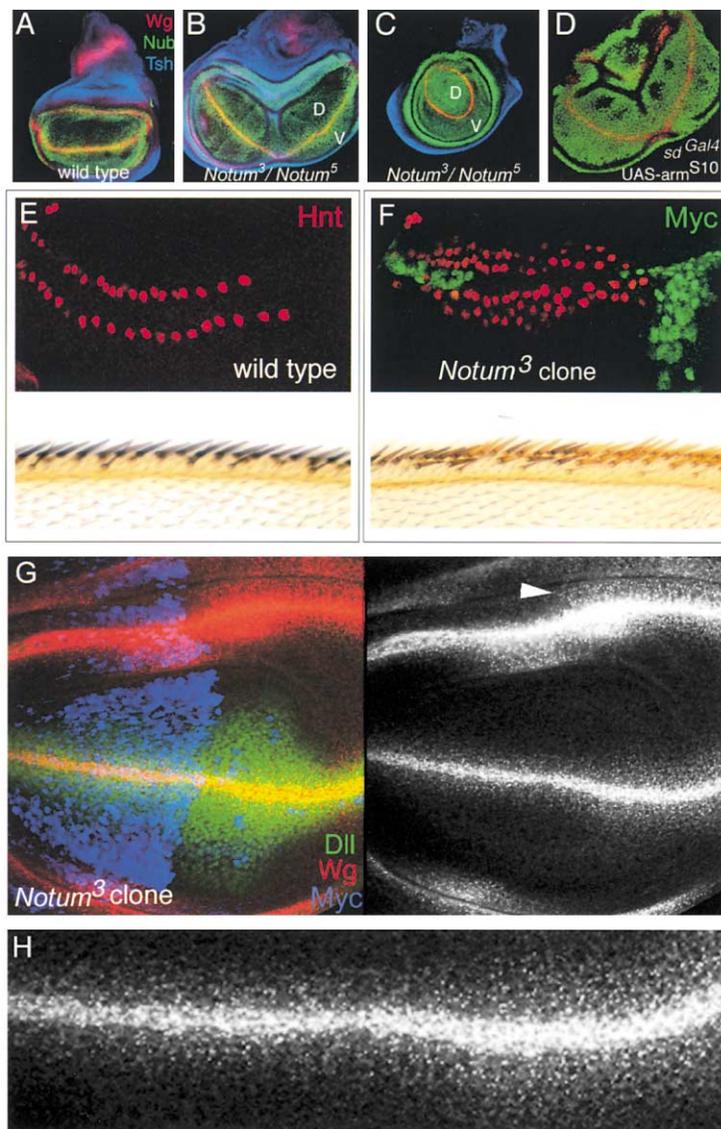


Figure 2. *Notum* Mutants Cause Wg Gain-of-Function Phenotypes

(A–D) Wing imaginal discs labeled with antibodies to Wg (red), Nubbin (green), and Teashirt (blue).

(A) Wild-type. Nubbin labels the wing pouch and hinge. Teashirt labels the presumptive body wall. Wg is expressed along the DV boundary, in rings around the wing pouch, and in a stripe in the notum. The two rings of Wg expression in the hinge region are only partially in focus.

(B and C) *Notum<sup>3</sup>/Notum<sup>5</sup>* discs.

(B) A duplicated wing pouch is shown. D and V indicate dorsal and ventral compartments. (C) A more extreme phenotype in which the dorsal thorax is lacking is shown.

(D) *sd<sup>Gal4</sup>/+ UAS-arm<sup>S10</sup>/+* disc shows that wing duplications comparable to (B) can result from activation of the Wg pathway.

(E) Wild-type anterior wing margin. Upper panel: sense organ precursor cells labeled with anti-Hindsight (red). Lower panel: dorsal view of the anterior wing margin. Note the single row of stout mechanosensory bristles and the second row of thinner, curved chemosensory bristles along the edge of the wing.

(F) Anterior wing margin in a wing disc with several large *Notum<sup>3</sup>* mutant clones. Mutant cells are marked by the absence of the Myc marker (green). Additional rows of Hnt-expressing sense organ precursors form. Lower panel: mutant sensory bristles were marked with yellow. Some ectopic bristles were wild-type.

(G) Wing disc with a large *Notum<sup>3</sup>* mutant clone filling the P compartment. Mutant cells are marked by the absence of Myc (blue). Dll protein (green) is expressed in cells further from the source of Wg in the mutant tissue of the P compartment. Wg protein (red) is shown separately in the right hand panel.

(H) Higher magnification view of the wing margin showing Wg expression. Note the increased level of Wg and the increased range of Wg movement in the clone.

range of the Wg gradient. However, Notum can also act when retained in the Golgi.

#### ***Notum* Encodes an $\alpha/\beta$ -Hydrolase Related to Plant Pectin Acetylsterases**

Using the globular region of the Notum protein, we searched the nonredundant protein sequence database from the NCBI, using PSI-Blast with default parameters. The first search revealed significant sequence similarity to hypothetical plant proteins from *Arabidopsis*, rice, and a single protein from mung bean. The latter protein has been experimentally characterized as a pectin acetylsterase (Breton et al., 1996). Additional searches revealed more distantly related homologs in the prokaryotes *Archaeoglobus fulgidus* and *Thermus thermophilus*. Searches of other databases revealed statistically significant matches to a human protein predicted by the Ensembl system (<http://www.ensembl.org>) and a mouse cDNA.

Phylogenetic analysis using the neighbor-joining algorithm implemented in ClustalW (Thompson et al., 1994)

suggests that the mouse, human, and fly genes are orthologs (i.e., related by speciation events, and therefore likely to have similar functions). However, no closely related sequences were found from *C. elegans*, other metazoa, or yeasts. The length of the region conserved in all proteins identified is  $\sim 325$  amino acids (for alignment, see Supplemental Figure S2). The most distinctive sequence feature is a conserved block of small residues flanking a central serine (Figure 4A). Database searches revealed a number of hits of marginal statistical significance that shared this G-X-S-X-G motif with Notum. To investigate the biological relevance of these matches, we performed additional PSI-Blast searches, initiated with the Notum homolog from *Archaeoglobus fulgidus*. This revealed similarity to the rat carboxylesterase precursor (E value  $< 0.004$ ). Representative protein structures show that members of this family of esterases belong to the wider  $\alpha/\beta$ -hydrolase superfamily, which includes peptidases, lipases, esterases and a variety of other hydrolytic enzymes (Nardini and Dijkstra, 1999).

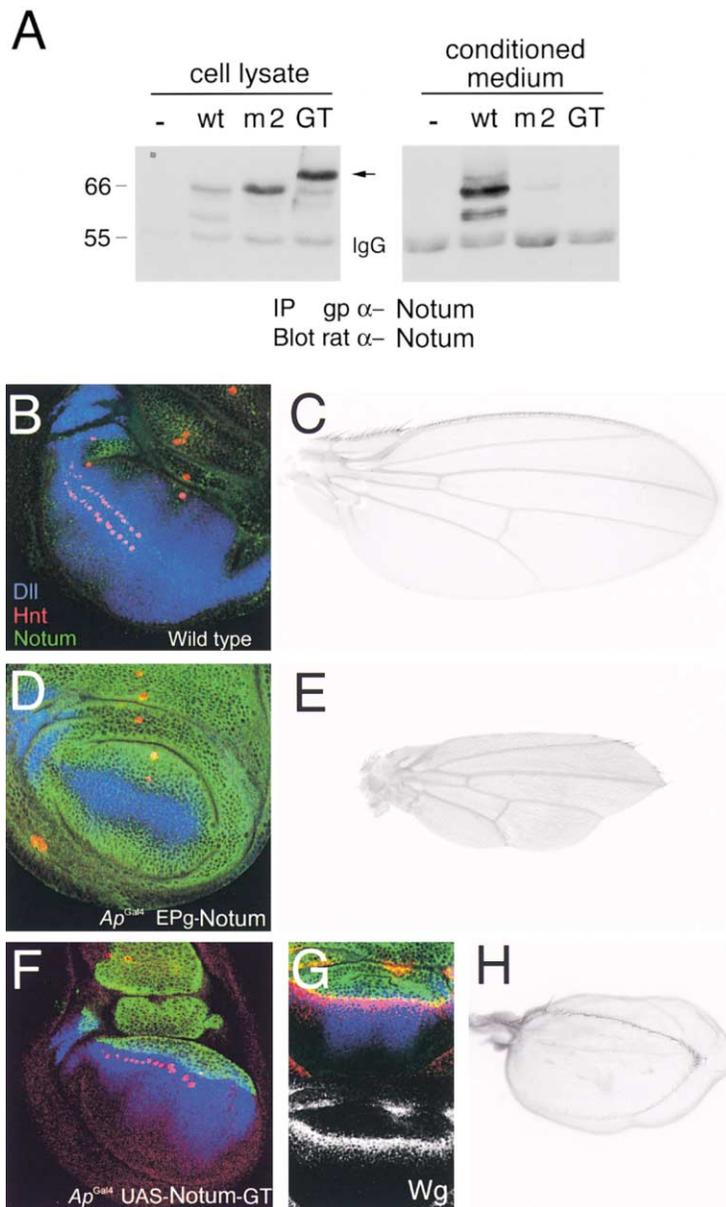


Figure 3. Notum Acts as a Secreted Protein

(A) Immunoblots of S2 cells transfected to express wild-type Notum (wt), the Notum<sup>2</sup> mutant (m2), Golgi-tethered Notum (GT), or with empty vector (-). Cell lysates and conditioned medium were immunoprecipitated with guinea pig anti-Notum and blots were probed with rat anti-Notum. IgG indicates crossreaction with the heavy chain band. Two forms of Notum protein were seen in cell lysates. Both forms were recovered by immunoprecipitation from conditioned medium from cells expressing wild-type Notum. The slower migrating form at ~66 kDa was the major form seen in the m2 cells. Very little m2 protein was recovered in the conditioned medium. Notum GT runs at a higher molecular weight due to addition of the 121 amino acid transmembrane domain from Gal-T3. Notum-GT was not recovered from the conditioned medium.

(B) Wild-type wing disc labeled with anti-Notum (green), anti-Hnt (red), and anti-Dll (blue).

(C) Cuticle preparation of a wild-type wing.

(D) *ap<sup>GAL4</sup>* + EPg-Notum wing disc.

(E) *ap<sup>GAL4</sup>* + EPg-Notum wing. Note the scalloping of the margin.

(F) *ap<sup>GAL4</sup>* + UAS-Notum-GT wing disc. Note the intact ventral row of Hnt expression. Dll expression was reduced in the dorsal compartment.

(G) *ap<sup>GAL4</sup>* + UAS-Notum-GT wing disc labeled with anti-Wg (red), anti-Dll (blue), and anti-Notum (green). Wg is shown separately below.

(H) *ap<sup>GAL4</sup>* + UAS-Notum-GT wing. Note the reduced D compartment and loss of dorsal margin bristles.

In these structures, G-X-S-X-G is a conserved active site motif, termed a “nucleophile-elbow,” which forms part of a Ser, Asp, His catalytic triad. The hydroxyl group of the serine residue is essential for the nucleophilic attack in the first step of the hydrolysis of ester or amide bonds by these enzymes. On this basis, Ser 237, Asp 338, and His 384 were predicted to form the catalytic triad of Notum.

#### Modification of the Glypicans Dally and Dlp by Notum

Pectin acetyltransferases from plant pathogens act as secreted enzymes to deacetylate pectins in plant cell walls. Pectins are composed mainly of galacturonic acid residues, some of which are methylated or acetylated. Pectin acetyltransferases hydrolyze the ester bond linking acetyl groups to galacturonic acid. Glycosaminoglycans

consist of repeated glucuronic acid and GlcNAc disaccharide units. Although GAGs are different in structure from pectins (Figure 6C), the similarity to pectin acetyltransferases raised the possibility that Notum might act on the GAG side chains of HSPGs. As a first step toward addressing this possibility, we asked whether Notum could modify Dally and Dlp when coexpressed in S2 cells. Coexpression of Dally-HA with Notum reduced the amount of Dally-HA recovered in S2 cell lysates (Figure 4B, lanes 4 and 5). In *sulfateless* mutants, which lack N-deacetylase/N-sulfotransferase (NDST) activity, the level of Dally and Dlp proteins were also strongly reduced, perhaps indicating altered stability of the immature protein (as noted by Selleck 2001; data in Lin and Perrimon 1999; Baeg et al., 2001). Coexpression of HA-tagged Dlp with Notum altered the electrophoretic mobility of Dlp-HA, without causing substantial loss of the

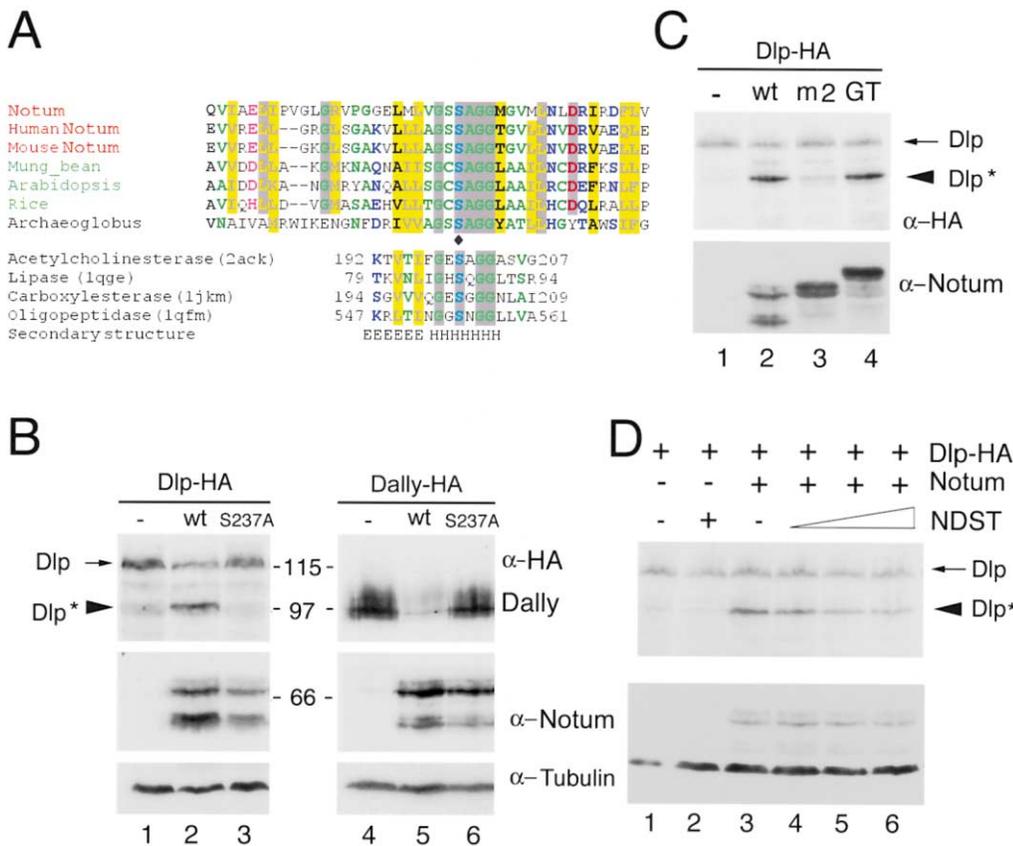


Figure 4. Notum Modifies Dally-like

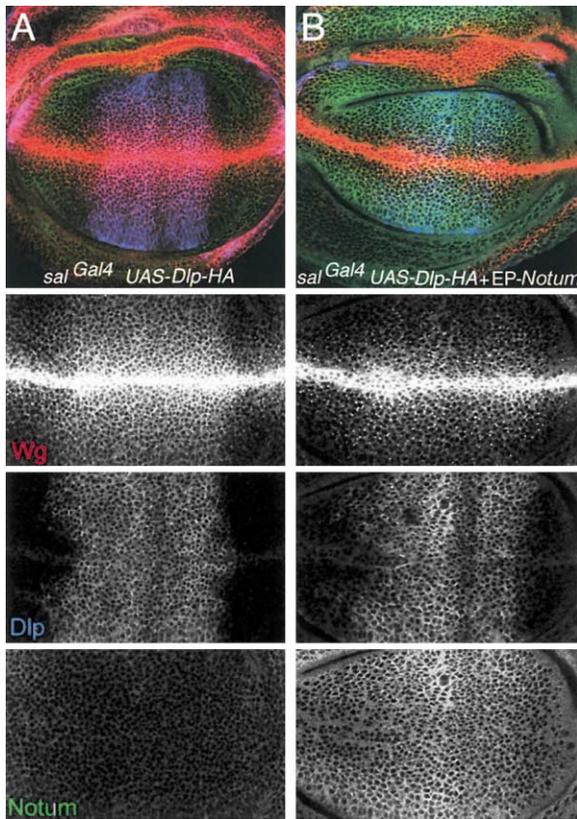
(A) Alignment of representative sequences highlighting the GXSXG motif. The alignment also shows sequences from solved structures in the region of the “nucleophile-elbow” motif (Protein Data Bank accession numbers are in parentheses). The putative active site serine is marked (◆). The alignment is colored using the CHROMA program with default parameters, and an 80% conservation threshold (PMID: 11590103). (B) Immunoblots of S2 cells transfected to express Dlp-HA (lanes 1–3) or Dally-HA (lanes 4–6). Lanes 1 and 4: cotransfected with empty vector. Lanes 2 and 5: cotransfected to express Notum. Lanes 3 and 6: cotransfected to express the S237A mutant form of Notum. Upper panels: probed with anti-HA. Middle panels: reprobed with anti-Notum. Lower panels: reprobed to show tubulin as a loading control. (C) Immunoblots of S2 cells transfected to express Dlp-HA alone or with wild-type (wt), M146K mutant (m2), or Golgi-tethered (GT) Notum. Upper panel: probed with anti-HA. Lower panel: same blot reprobed with anti-Notum. (D) Immunoblot of S2 cells transfected to express Dlp-HA, Notum, and varying amounts of NDST. Lane 1: Dlp-HA alone. Lane 2: Dlp-HA + NDST (0.5  $\mu$ g DNA). Lane 3: Dlp-HA + Notum. Note the increase in Dlp\*. Lanes 4–6: Dlp-HA + Notum with increasing amounts of NDST (0.06, 0.12, and 0.24  $\mu$ g DNA).

protein. Dlp-HA migrated as a broad band at  $\sim$ 115 kDa, with minor bands at 97 and 105 kDa (Figure 4B, lane 1). Coexpression with wild-type Notum increased the amount of the 97 kDa form (Dlp\*), apparently at the expense of the 115 kDa form. The small amount of this band present in S2 cells expressing Dlp-HA may reflect activity of endogenous Notum protein, which was detected on longer exposures.

To assess the prediction that Notum is structurally and functionally related to  $\alpha/\beta$ -hydrolases, we mutated the predicted active site Ser 237 to Ala. This replaces the essential hydroxyl group of the serine with hydrogen and should render the protein catalytically inactive. As expected, Notum<sup>S237A</sup> was ineffective in reducing the level of Dally-HA and in producing the 97 kDa form of Dlp\* (Figure 4B). These results support the proposal that Ser 237 plays an important role in the catalytic activity of Notum. The EMS-induced M146K Notum mutant also had reduced activity in this assay, indicating that *Notum*<sup>2</sup>

is likely to be a hypomorphic allele (Figure 4C). Notum-GT had the same activity as the wild-type protein.

The GAG side chains of HSPGs consist mainly of repeated dimers of N-acetylglucosamine (GlcNAc) and glucuronic acid. The first step in modification of the side chains involves replacement of the acetyl moiety on GlcNAc with a sulfate moiety by N-deacetylase/N-sulfotransferase. NDST modifies  $\sim$ 50% of the GlcNAc residues, in blocks along the GAG side chain. The structural similarity between pectins and GAGs raised the possibility that Notum might act by removing acetyl groups from GlcNAc residues of GAGs. If this is the case, GlcNAc residues modified by NDST should not be a good substrate for Notum in cells. To address this possibility, we transfected S2 cells to express a constant amount of Notum and increasing amounts of NDST and assayed the ability of Notum to modify Dlp. Increasing the ratio of NDST to Notum reduced the amount of Dlp\* (Figure 4D). These observations support the proposal



**Figure 5. Notum Acts on Dally-like to Regulate Wg Accumulation**  
Wing discs labeled with anti-Wg (red), anti-HA to visualize Dlp-HA (blue), and anti-Notum (green). Upper panels show overviews of the wing pouch. Higher magnification views of the wing margin are shown below (Wg, HA, and Notum channels, shown separately).  
(A) Ectopic expression of Dlp-HA in the *spalt<sup>Gal4</sup>* domain caused accumulation of Wg protein at the cell surface. Few spots of internalized Wg were seen. Although Wg protein was bound to the cell surface, this genotype caused scalloping of the wing, presumably due to reduced availability of Wg for binding to its receptor (see also Baeg et al., 2001).  
(B) Coexpression of Notum with Dlp-HA reduced accumulation of Wg protein. Note the punctate appearance of Wg, suggesting that it has not been sequestered at the cell surface by binding to Dlp and can be internalized into cells.

that NDST and Notum could act on the same substrate. NDST is a Golgi-resident enzyme. In cells where the two proteins are coexpressed, NDST and Notum might compete for modification of GAG side chains, as illustrated by the activity of Notum-GT when expressed in the wing disc. As there are few cells in the disc where Notum is expressed, we suggest that secreted Notum may act on HSPGs at the cell surface to deacetylate the blocks of GlcNAc residues that were not modified by NDST during GAG biosynthesis.

#### Notum Acts on Dlp to Shape the Wg Gradient

The glypicans Dally and Dlp have been shown to bind and stabilize extracellular Wg, although Dlp is considerably more effective (Strigini and Cohen 2000; Baeg et al., 2001). To ask whether Notum modifies the ability of Dlp to bind Wg, we examined Wg protein in discs expressing Dlp-HA and Notum. Expression of Dlp-HA

in a broad band of cells in the center of the wing disc under *spalt<sup>Gal4</sup>* control caused accumulation of Wg protein, mainly outlining the cell surface (Figure 5A). Under these conditions, we observed scalloping of the wing margin (not shown), suggesting that Wg is partially sequestered to Dlp and is less available for binding to its receptor. The distribution of Wg differed in discs expressing Dlp-HA and Notum (Figure 5B). The total level of Wg accumulation was considerably lower, although Dlp was expressed at a comparable level. Second, much of the Wg protein appeared in intracellular vesicles, instead of outlining the cell surface. These findings support the proposal that Notum modifies Dlp to render it less able to bind and stabilize Wg. Thus, Notum contributes to shaping the Wg gradient by altering the ability of the cell surface glypican Dlp to stabilize extracellular Wg.

#### Discussion

##### HSPGs and Wg/Wnt Signaling

Heparan sulfate proteoglycans are present in very large numbers on the cell surface and are thought to provide low-affinity binding sites for secreted signaling proteins, including Wg/Wnt. Wg has been shown to bind to GAGs in cell culture (Reichsman et al., 1996). The cell surface proteoglycans Dally and Dlp have been implicated in Wg signaling (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Dally and Dlp expression levels can each influence the amount of Wg bound to the cell surface, though Dlp is considerably more effective (Baeg et al., 2001; Strigini and Cohen, 2000). Our findings suggest that Notum acts to modify Dlp and Dally and thereby alters the extracellular gradient of Wg protein.

Modification of HSPGs by sulfation is important for their function. Deacetylation and sulfation of a subset of the GlcNAc residues in the GAG side chains of HSPGs by NDST enzymes is required for subsequent epimerization and sulfation reactions that enrich the structural complexity of the GAG side chains (reviewed in Perrimon and Bernfield, 2000). The *Drosophila* NDST enzyme is encoded by the *sulfateless* gene. Cells lacking NDST activity show reduced Dally and Dlp levels, reduced Wg binding, and reduced sensitivity to Wg in vivo (Baeg et al., 2001; Lin and Perrimon, 1999). Overexpression of HSPGs can also reduce Wg activity, apparently by sequestering Wg at the cell surface and reducing its availability for binding to its receptor. This indicates that the level of Wg binding activity conferred by HSPGs is critically important.

Our findings provide evidence that Notum acts to modify the ability of glypicans to bind Wg at the cell surface. In view of the finding that Notum acts nonautonomously in vivo, we propose that Notum can act extracellularly on the GAG side chains of mature HSPG. NDST-dependent sulfation and subsequent modification occurs on only about half of the GlcNAc residues in the GAG side chains. An intriguing possibility is that Notum might act by deacetylating the unmodified GlcNAc residues to produce amino groups and reduce the ability of the GAGs to bind Wg (illustrated in Figure

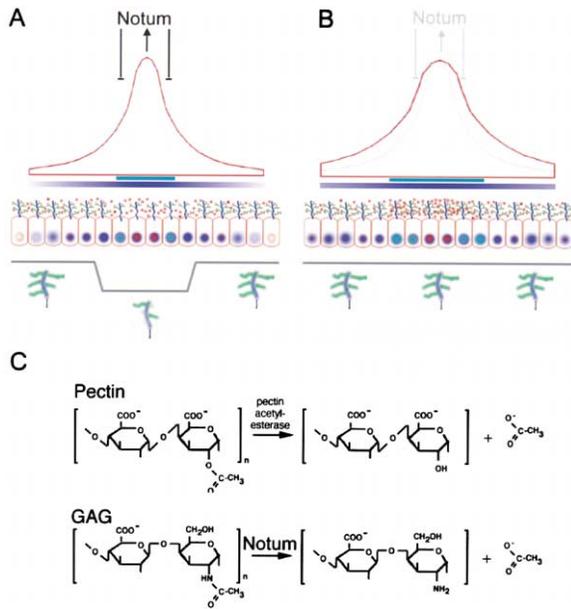


Figure 6. Model of Action of Notum

(A and B) Schematic representation of the Wingless morphogen gradient (red line) in the presence or absence of Notum activity. Wingless induces the expression of Notum, which modifies the ability of Dally and Dally-like to bind Wg. The gray line represents the affinity of HSPG for Wingless. Under these lines the proteoglycans with different affinities to Wg are represented. Reduced Notum activity allows increased binding and stabilization of Wg. The level and effective range of Wg increase so that Wg target genes are activated in broader domains.

(C) Schematic representation of the reaction carried out by pectin acetyltransferase and the proposed reaction carried out by Notum.

6). Recent studies suggest that secondary modification of residues sulfated by NDST is also important for Wnt function. Expression of Qsulf1, a GlcNAc sulfatase that is thought to remove 6-O-sulfate groups from GlcNAc residues in HSPG, increased Wnt responsiveness in cell culture (Dhoot et al., 2001). It is not clear at present whether this modification allows increased Wnt signaling by increasing or decreasing the affinity of GAGs for Wnts.

### Spatial Pattern and Gradient Formation

Wg, Dpp, and Hh each regulate the expression of their receptors in ways that can influence the shape of the gradient (reviewed in Teleman et al., 2001). Our findings indicate that Wg can also influence formation of its own gradient by modulating the activity of cell surface HSPGs (illustrated in Figure 6). Wg induces expression of Notum, which can act either during GAG biosynthesis or as a secreted protein to modify cell surface HSPGs. Reduced Notum activity allows excess accumulation of Wg protein, resulting in an increased range of Wg activity (Figure 6B). Conversely, overexpression of Notum limits the ability of cells to bind and stabilize Wg, thereby limiting the ability of Wg to spread in the disc epithelium and form a long-range gradient. In the embryo, Notum overexpression produced segment polarity defects comparable to those caused by reduced Wg activity. In the wing disc, elevating Notum levels limited Wg movement and caused phenotypes ranging from scalloping

of the wing to early failure of wing pouch specification. These changes in the shape of the Wg gradient can be attributed to the effect of Notum on Dally and Dlp. Coexpression of Notum with Dly limited the ability of overexpressed Dlp to accumulate Wg protein. We suggest that the role of Notum is to limit the ability of Dlp and Dally to bind Wg.

In conclusion, our findings suggest that a novel type of GAG modification contributes to Wg gradient formation during development. In view of the complexity of HSPG modification in vivo, we anticipate that there will be many opportunities for regulation of intercellular signaling by enzymes that modify GAGs to confer specific activities. The possibility also exists that enzymes might act on specific subsets of GAGs to confer specific functions, as may be the case for the heparan sulfate copolymerase enzyme Tout-velu (The et al., 1999). Morphogen gradient formation in tissues is a complex process about which much remains to be learned.

### Experimental Procedures

#### Notum cDNA

A cDNA library was prepared using mRNA from 0- to 24-hr-old embryos expressing *Arm<sup>S10</sup>* under tubulin<sup>Gal4</sup> control and screened with genomic DNA flanking the EPg insertion site. *Arm<sup>S10</sup>* was used to increase Notum mRNA levels. UAS-Notum was produced by cloning the full-length cDNA in pUAST. The Notum M146K and S237A mutants were produced by PCR amplification.

#### Expression Constructs and Immunoprecipitation

Notum-GT consists of amino acids 1–122 from GalNAc-T3 (GenBank accession number X92689) cloned in-frame with amino acids 58–673 of Notum in pUAST and pRmHa3. Dlp-HA was produced by inserting an oligonucleotide encoding the HA sequence in-frame at a unique NdeI site in the coding sequence of Dlp (GenBank accession number AF317090). Dally-HA was recloned into pRmHa3 by PCR from pUAST Dally-HA (Tsuda et al., 1999). S2 cells were transfected with 2–6  $\mu$ g of DNA using 4  $\mu$ l per well of CellFectin (Invitrogen). After 14–18 hr, the transfection mix was removed, cells were recovered in 3 ml of SMF medium (GIBCO) for 6–8 hr, and induced with 0.7 mM CuSO<sub>4</sub> for 3 days. Cells were lysed in 200  $\mu$ l of 5 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, and protease inhibitors supplemented with 1 mM PMSF. One-tenth of the cell lysate was diluted in 1 ml of lysis buffer and immunoprecipitated with 2  $\mu$ l of anti-Notum. Three milliliters of conditioned medium from the same wells were adjusted to 0.1% Triton X-100 and immunoprecipitated with 1  $\mu$ l/ml of anti-Notum.

#### Fly Strains

*wg<sup>Gal4</sup>* was produced by replacing the PZ element inserted in *wg<sup>R0727</sup>* with the P{GAWB} element in *sc<sup>Gal4</sup>* as described in Preston and Engels (1996). UAS-FLP is described in Campbell and Tomlinson (1998). UAS-dally is described in Jackson et al. (1997). UAS-Dlp is described in Baeg et al. (2001). UAS-*arm<sup>S10</sup>* is described in Pai et al. (1997). UAS-*sgg* is described in Steitz et al. (1998).

#### Genetic Mosaics

*yw* *hsFlp1/yw*; *Notum<sup>3</sup> FRT80B/{y<sup>+</sup>}* *M* *hs $\pi$ Myc FRT80B yw*; *en<sup>Gal4</sup>/UAS-FLP*; *Notum<sup>3</sup> FRT80B/{y<sup>+</sup>}* *M* *hs $\pi$ Myc FRT80B* Heat shock was performed for 60 min at 38°C at 48  $\pm$  12 hr to induce clones. Two hours before dissection, larvae were heat shocked for 30 min to induce *hs $\pi$ Myc* expression.

#### Antibodies

Notum antibodies were raised against amino acids 58–629 expressed in bacteria. Other antibodies used were rabbit anti-Tsh (Wu and Cohen, 2000), mouse anti-Wg (Brook and Cohen, 1996), rat anti-Nubbin (Averof and Cohen, 1997), mouse anti-Hindsight (1G9; Developmental Studies Hybridoma Bank at the University of Iowa),

rat monoclonal anti-HA (clone 3F10; Roche), and rabbit anti-c-Myc (A-14; Santa Cruz Biotechnology).

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#### Accession Numbers

The GenBank accession number for Notum cDNA is AJ457833.