Notum deacylates Wnt proteins to suppress signalling activity

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ARTICLE

Signalling by Wnt proteins is finely balanced to ensure normal development and tissue homeostasis while avoiding diseases such as cancer. This is achieved in part by Notum, a highly conserved secreted feedback antagonist. Notum has been thought to act as a phospholipase, shedding glypicans and associated Wnt proteins from the cell surface. However, this view fails to explain specificity, as glypicans bind many extracellular ligands. Here we provide genetic evidence in Drosophila that Notum requires glypicans to suppress Wnt signalling, but does not cleave their glycosphatidylinositol anchor. Structural analyses reveal glycosaminoglycan binding sites on Notum, which probably help Notum to co-localize with Wnt proteins. They also identify, at the active site of human and Drosophila Notum, a large hydrophobic pocket that accommodates palmitoleate. Kinetic and mass spectrometric analyses of human proteins show that Notum is a carboxylesterase that removes an essential palmitoleate moiety from Wnt proteins and thus constitutes the first known extracellular protein deacylase.

Negative feedback characterizes biological signalling1 and although often cell-intrinsic, is also mediated by secreted proteins. Cell- and non-cell-autonomous feedbacks modulate signal transduction by Wnt proteins, a class of secreted proteins characterized by the presence of palmitoleate acid appended on a conserved serine2,3. This palmitoleate acid moieties is essential for signalling24,25, contributing to interaction with Frizzled receptors3,6,7. Canonical Wnt signalling triggers expression of intracellular, extracellular and membrane-localized inhibitors of the pathway. Secreted inhibitors include Dickkopf (Dkk) family members, which bind to the extracellular domain of the Wnt co-receptor low-density-lipoprotein-receptor-related protein 5/6 (Lrp5/6), as well as Wnt inhibitory factor 1 (Wif1) and secreted Frizzled receptor proteins (Sfrp), which sequester Wnt proteins8. Tiki is a membrane-bound protease that cleaves the amino-terminal region of Wnt ligands9. Notum is also thought to act enzymatically9,10, but on glypicans, a class of heparan sulfate proteoglycans (HSPGs) implicated in the extracellular stabilization, movement, and/or surface retention of Wnt proteins, as well as of other signalling ligands12–14.

Notum orthologues are found in metazoans from planarians to humans and all bear the hallmark Ser-His-Asp catalytic triad of α/β-hydrolases10,31. The sequence similarity of Notum to plant pectin acetylases prompted the early suggestion that it could hydrolyse glycosaminoglycan (GAG) chains of glypicans30,31, thus affecting their ability to interact with Wnt ligands and somehow modulating signalling activity. It was subsequently reported that Notum triggers glypican shedding from cultured cells, perhaps by cleaving their glycosphatidylinositol (GPI) anchor15,16. Indeed, the currently accepted view is that Notum is a glycan-specific phospholipase27. However, glycan-based interactions also modulate Dpp (Drosophila TGF-β), Hedgehog, and fibroblast growth factor, as well as Wingless signalling12–14. One would expect therefore that these pathways would also be sensitive to Notum-induced glypican release. Yet, existing evidence suggests that Notum is primarily a feedback inhibitor of Wnt signalling. In planarian worms, Drosophila, zebrafish and hepatocarcinomas, notum expression is activated by Wnt signalling and, conversely, Notum seems to preferentially suppress Wnt signalling10,11,18–21. Because more pleiotropic effects would be expected from an enzyme that targets glypicans, we felt compelled to reassess Notum’s specificity and mode of action.

Notum specifically inhibits Wnt signalling

To investigate the specificity of Notum systematically, we analysed its effects on Drosophila wing imaginal discs, which require Wingless (the main Drosophila Wnt), Dpp and Hedgehog for patterning and growth22,23. As expected, overexpression of Drosophila Notum (dNotum) throughout the dorsal compartment prevented expression of senseless, a gene normally activated by high level Wingless signalling. By contrast, patched (ptc), a Hedgehog target gene24,25, was unaffected (Fig. 1a, b) and phospho-Mad immunoreactivity, a marker of Dpp signalling26, was only mildly reduced (Extended Data Fig. 1a, b). Loss-of-function assays, in homozygous notum knockout (notumKO) tissue, confirmed the specificity of dNotum to Wingless signalling (Fig. 1c and Extended Data Fig. 1c). Although complete loss of notum was lethal, strong hypomorphic animals (notum147/notum147) survived to adulthood. The wings of such animals had supernumerary margin bristles, consistent with excess Wingless signalling, but had no defects indicative of impaired Hedgehog or Dpp signalling (Extended Data Fig. 1d–g). Nevertheless, extensive evidence suggests that glypicans contribute to these two signalling pathways27–30. This is difficult to reconcile with the apparent specificity of Notum if it acts as a glycan-specific phospholipase.

Notum does not cleave the GPI anchor of glypicans

One previously reported observation, namely that dNotum inhibits signalling by membrane-tethered (that is, shedding-resistant) Wingless11 (Extended Data Fig. 2a, b), is incompatible with the view that Notum is a glycan-specific phospholipase. In addition, genetic removal of the two Drosophila glypicans Dally and Dally-like protein (Dlp) did not

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notum and dlp suggest a functional relationship\textsuperscript{31,32}. We therefore investigated the role of Dlp or Dally in the ability of dNotum to suppress Wingless signalling. dNotum overexpression, along the anterior–posterior (A–P) boundary, led to complete and long-range suppression of Senseless expression (Fig. 2a). In the absence of either Dlp or Dally, this activity was very much reduced, as indicated by the recovery of endogenous Senseless expression (Fig. 2b, c). Notably, Dally was also required for Notum to suppress signalling by membrane-tethered Wingless (Extended Data Fig. 3a). Because Dally is not essential for survival, its requirement for Notum’s ability to suppress Wingless signalling could be confirmed in adult wings (Extended Data Fig. 3b–d). To address the relevance of glypicans, we generated a transgene expressing Dlp–CD8 (34 carboxy-terminal amino acids of Dlp replaced by the CD8 transmembrane domain) under control of the tubulin promoter. This transgene restored the ability of overexpressed dNotum to repress Wingless signalling in dlp mutant homozygotes (Fig. 2d; compare to Fig. 2b), confirming the importance of glypicans but not their GPI anchor.

Glypicans bear sulfated glycans. In Drosophila, sulfation of the sugar chains requires Sulfatase, a GlnAc N-deacetylase/N-sulfotransferase (NDST)\textsuperscript{33}, which can be knocked down in vivo with an RNA interference (RNAi) transgene. Gal4 was used to express this transgene specifically in the posterior compartment, leaving the anterior compartment as a control. At the same time, a dpp-LexA driver was used to overexpress dNotum along the A–P boundary. Overexpressed dNotum inhibited Senseless expression in the control compartment but not in the territory deficient in sulfatase activity (Fig. 2e). Therefore, sulfation of HS PGs is needed for dNotum to act. Notably, overexpressed dNotum did not accumulate in the compartment expressing the sulfatase RNAi transgene (compare Fig. 2e to Fig. 2a, right panels). Likewise, dNotum was depleted from the surface of daily dlp double-mutant cells generated by mitotic recombination (Fig. 2f). These findings suggest that Dally and Dlp retain dNotum at the cell surface through interaction with their sulfated glycans. Indeed, dNotum bound specifically to sulfated glycans on a glycan array (Extended Data Fig. 4). In addition, surface plasmon resonance (SPR) showed that recombinant human (h) NOTUM\textsubscript{core} (Ser 81– Thr 451, Cys330Ser) bound to heparin and heparan sulfate with micromolar affinities. The dissociation constant of a complex comprising hNOTUM\textsubscript{core} and human glypican-3 (GPC3 Pro 31–Asn 538) was 104 µM (Fig. 3a). Consistent with the Drosophila genetic data, this binding relies largely on the two sulfated GAG chains in GPC3 as their removal led to a more than fivefold reduction in affinity (Fig. 3a). We conclude that sulfated GAG chains on glypicans probably mediate their interaction with Notum.

Structure-guided identification of GAG-binding sites
The above results indicate that glypicans contribute to Notum activity by localizing it at the cell surface, but are unlikely to be the target of Notum’s enzymatic activity. What could the target be? We started to address this question by solving the structures of hNOTUM\textsubscript{core} (in nine crystal forms at resolutions between 1.4 and 2.8 Å; see Supplementary Information) and of dNotum\textsubscript{Aloop} (in two crystal forms at resolutions of 2.4 and 1.9 Å) (Fig. 3b, Extended Data Fig. 5 and Supplementary Information). The structures exhibit a canonical α/β-hydrolase fold\textsuperscript{44}, as predicted\textsuperscript{10,11}. The conserved eight-stranded central β-sheet is extended on both sides by strands 4 and 14 and is flanked by the canonical six α-helices. This single domain topology is further extended by additional α-helices, two very short β-sheets, several long loops and seven stabilizing disulfides. The catalytic triad comprises Ser 232, Asp 340 and His 389 (hNOTUM residue numbering).

Seven sulfate binding sites were identified in hNOTUM\textsubscript{core} crystal form III (Fig. 3c and Extended Data Fig. 6). Among them, one (sulfate 1) was found by SPR to contribute substantially to heparin–Notum interactions (Fig. 3d). In addition, co-crystals with short heparin oligomers or sucrose octasulfate (SOS), a heparin mimic, were generated and analysed. These structural studies and additional biophysical analyses (described in Supplementary Information and illustrated in Extended Data...
Notum, the pectin acetylesterases of angiosperms (22% sequence identity to carboxylesterase. Furthermore, the closest non-animal homologues of homologues, including human esterase D36 and acyl-protein thioesterases, a grove between the top of the β-sheet and helix αK (Fig. 3c). Importantly, the GAG-binding surface on Notum is distant from the catalytic triad, consistent with our earlier evidence that Notum binds to glypicans, but does not act on them enzymatically.

Evidence for carboxylesterase activity

The α/β-hydrolase superfamily includes proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases. To identify which of these activities relate most closely to the activity of the Notum protein, we compared the structure of hNOTUM to those of all known α/β-hydrolases (PDBFold server). The search returned many weak homologues, including human esterase D and acyl-protein thioesterase 1 (APTI) (Extended Data Fig. 7a). A structure-based search for function using the ProFunc Server also suggested that Notum is a carboxylesterase. Furthermore, the closest non-animal homologues of Notum, the pectin acetylesterases of angiosperms (22% sequence identity to hNOTUM, Extended Data Fig. 7b) are carboxylesterases. We assessed the functional significance of these observations by measuring the activity of hNOTUMcore on p-nitrophenyl (pNP) acetate (pNP2), a chromogenic carboxylesterase substrate. Pronounced activity could be detected (Fig. 4a). This activity was strongly inhibited by Triton X-100, and by phenylmethanesulfonyl fluoride (PMSF), a compound known to covalently modify the catalytic serine of serine esterases and proteases (Extended Data Fig. 8a, b). By contrast, there was no measurable hNOTUM activity on representative sulfatase, phosphatase, phospholipase C or amidase/protease substrates (Fig. 4a). Addition of SOS or heparin resulted in a modest increase in Notum carboxylesterase activity (Extended Data Fig. 8a). The possibility that GAGs also contribute to Notum function by allosteric activation requires further investigation.

As a secreted carboxylesterase that inhibits Wnt signalling, Notum is likely to target a carboxy-oxoester or carboxy-thioester bond present on an extracellular component of the Wnt signal transduction machinery. The linkage between Wnt and palmitoleic acid is the only such
chemical bond described to date, suggesting that Notum could target Wnt proteins themselves. To evaluate this possibility, we treated mouse (m)Wnt3A with recombinant hNOTUMcore for specific durations, removed the hNOTUMcore, and used a cell-based luciferase assay to measure signalling activity of the remaining mWnt3A. This showed that hNOTUM inactivated mWnt3A directly, irreversibly and in a time-dependent manner (Fig. 4b), while no such effect could be detected on Norrin, a non-lipidated ligand that also acts via the Wnt receptors.

Remarkably, the Notum crystal structures revealed a large (\( \approx 380 \text{ Å}^3 \)), hydrophobic pocket adjacent to the catalytic triad (Fig. 3b, c). Computational docking showed that this pocket could accommodate long-chain fatty acids of up to 16 carbon atoms (C16). The size restriction imposed on saturated fatty acids was functionally assessed by measuring hNOTUM enzymatic activity on commercially available saturated linear carboxylic acids inhibited activity (Fig. 4d) while longer saturated fatty acids (C8-C12) were observed with the Wnt-associated carboxylic acids if they contain a C9–C10 cis double bond and therefore might hydrolyse the oxo-ester bond linking palmitoleate or myristoleate to Wnt proteins.

**Notum deacylates Wnt proteins**

To test directly Notum-mediated Wnt deacylation we turned to liquid chromatography–mass spectrometry (LC–MS) analysis. mWnt3A was purified from conditioned medium, treated with recombinant hNOTUMcore or a mock solution, differentially isotope labelled, and trypsinised. No notable identification could be obtained for the predicted palmitoleoylated tryptic peptide, indicating incompatibility with the LC–MS conditions. After treatment with hNOTUM, however, this peptide could be identified and quantified in non-acylated form (Fig. 5a, b and Extended Data Fig. 9a, b). The LC–MS measurements and label reversal consistently showed an increase in signal intensity for the hNOTUM-treated de-acylated peptide whereas control peptides were largely unaffected by hNOTUM treatment (Extended Data Fig. 9c, d). This suggests that treatment of mWnt3A with hNOTUM removes the palmitoleic acid moiety thus rendering the relevant peptide more hydrophilic and detectable by LC–MS. Encouraged by these results, we proceeded to assess the activity of hNOTUM on synthetic peptides. The predicted tryptic peptide from hWnt3A was synthesized in a disulphide-bonded form with a palmitoleate moiety on the relevant serine (Supplementary Information). These peptides were treated with recombinant hNOTUMcore, or with hNOTUMcore (S232A), which is predicted to be enzymatically inactive, and the reaction products were analysed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF). No significant deacylation was detected in hNOTUMcore(S232A)-treated samples, whereas hNOTUM-treated peptides were found to be extensively deacylated (Fig. 5c and Extended Data Fig. 9e). We conclude from these assays that Notum catalyses the removal of palmitoleic acid, which is normally O-linked to Ser 209 of hWnt3A. We also assayed the effect of hNOTUM on a synthetic peptide from human Sonic Hedgehog (SHH), which is N-palmitoylated at the amino terminus. No change in the level of acylation could be detected (Fig. 5d and Extended Data Fig. 9f), confirming that the activity of Notum on Wnt is specific, in agreement with our genetic evidence.

To gain structural insight into Wnt–Notum recognition, we co-crystallized inactive hNOTUMcore(S232A) with a palmitoleoylated disulphide-bonded peptide corresponding to hWNT7A(Cys 202–Cys 209). The crystal structure revealed the palmitoleoyl group occupying the active site pocket (Fig. 5e and Extended Data Fig. 9g). Electron density was also evident for the ester bond. No interpretable density was found for the peptide, probably owing to disorder. This apparent lack of interaction with the peptide concurs with the general observation that esterases/lipases of the \( \beta / \beta \)-hydrolase family bind only to the acid part of the ester substrate. The carbohydrate acid carbon is 3.3 Å from the CB of the mutated serine nucleophile, a distance consistent with ideal positioning of the hydroxyl for nucleophilic attack. Classically esterase-catalysed hydrolysis proceeds through a tetrahedral transition state characterized by a negatively charged carbonyl oxygen stabilized by two canonical backbone amides, the oxyanion hole. In hNOTUM, the Gly 127–Trp 128 amide participates in formation of the oxyanion hole in addition to the canonical Ser 232–Ala 233 and Gly 126–Gly 127 amides, thereby providing optimal stabilization during the transition state (Extended Data Fig. 9g). The kinked cis double bond (C9–C10) of the acyl tail is positioned at the base of the pocket between Ile 291, Phe 319 and Phe 320. We found a similar binding mode for a hNOTUM–myristoleate crystal structure (Extended Data Fig. 9h). Thus, the binding pocket can accommodate extended carbon tails up to C8/C10 but longer fatty acid chains must be kinked at this point in order to fit in. Saturated fatty acids generally adopt an extended conformation, explaining the preference of Notum for palmitoleate and myristoleate (both cis-unsaturated acids kinked at C9–C10) over palmitate and myristate. The pocket entrance (lined by Ser 232 and His 389) is relatively narrow, but...
membrane-bound O-acyl transferases (MBOATs). Porcupine, the Wnt MBOAT, appendes palmitoleate and shorter cis-unsaturated fatty acids onto Wnt. We have shown here that Notum specifically deacylates Wnt (Fig. 5f) and is thus the first enzyme known to deacylate an extracellular protein. The specificity of Notum can be traced to the shape of its hydrophobic pocket, which can accommodate cis-unsaturated fatty acids such as myristoleate and palmitoleate, and the nature of its enzymatic activity, a carboxyl oxoesterase. These characteristics ensure that Notum preferentially acts on Wnt proteins, the only secreted proteins known to be O-palmitylated on a serine residue. Notum enzymatically inhibits signaling activity by removing the palmitoleate moiety of Wnt proteins, which contributes directly to receptor binding. Notum could also interfere non-catalytically with the formation of the Wnt–Frizzled complex by sequestering the palmitoleate moiety as overexpressed dNotum(S237A) mildly suppressed Wingless signalling in vivo (data not shown). We have found that glypicans are required for Notum function and that Notum binds to the sulfated GAGs of HSPGs. Glypicans can have stimulatory roles in Wnt signalling. However, in the presence of Notum, we suggest that glypicans are also inhibitory by acting as a scaffold that co-localizes Notum and its substrate (Wnts) at the cell surface (Fig. 5f).

Our results point to Notum’s physiological targets being exclusively Wnt family members. Notum is the only secreted Wnt feedback inhibitor found across the metazoan kingdom, from planarians to humans, although it is seemingly absent from Caenorhabditis elegans. Notum’s Wnt-deacylation activity, along with other means of feedback inhibition such as ligand sequestration, receptor blocking, receptor downregulation and proteolytic degradation, undoubtedly contributes to the fine balancing of Wnt signalling both during development, for cell fate specification, and in adults, for stem cell maintenance. Indeed, insufficient or excessive Wnt signalling has been associated with diseases such as neurodegeneration or cancer, respectively. Our binding data suggest that Notum could possibly be modulated by dietary cis-unsaturated fatty acids. Moreover, because Notum is an extracellular enzyme with a well-defined and large active site pocket, it is probably amenable to chemical inhibition to alleviate conditions associated with insufficient Wnt signalling. Conversely, recombinant Notum could be considered as a therapeutic agent to prevent excess Wnt signalling such as in Wnt-driven cancers.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper, references unique to these sections appear only in the online paper.

Received 21 October 2014; accepted 26 January 2015.
Published online 25 February 2015.

1. Freeman, M. Feedback control of intercellular signalling in development. Nature 408, 313–319 (2000).
2. Takada, R. et al. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev. Cell 11, 791–801 (2006).
3. Janda, C. Y. Waghray, D., Levin, A. M., Thomas, C. & Garcia, K. C. Structural basis of Wnt recognition by Frizzled. Science 337, 59–64 (2012).
4. Willett, K. et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452 (2003).
5. Tang, X. et al. Roles of N-glycosylation and lipidation in Wg secretion and signalling. Dev. Biol. 364, 32–41 (2012).
6. Clevers, H. & Nusse, R. Wnt-β-catenin signaling and disease. Cell 149, 1192–1205 (2012).
7. Kim, S. E. et al. Wnt Stabilization of β-Catenin Reveals Principles for Morphogen Receptor-Scaffold Assemblies. Science 340, 867–870 (2013).
8. Niehrs, C. The complex world of WNT receptor signalling. Nature Rev. Mol. Cell Biol. 13, 767–779 (2012).
9. Zhang, X. et al. Tik1 is required for head formation via Wnt clearance-oxidation and inactivation. Cell 149, 1565–1577 (2012).
10. Giráldez, A. J., Copley, R. R. & Cohen, S. M. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. Dev. Cell 22, 667–676 (2007).
11. Gerlitz, O. & Basler, K. Wingful, an extracellular feedback inhibitor of Wingless. Genes Dev. 16, 1055–1059 (2002).
12. Filus, J., Capurro, M. & Rast, J. Glypicans. Genome Biol. 9, 224 (2008).
13. Yan, L. & Lin, X. Shaping morphogen gradients by proteoglycans. Cold Spring Harb. Perspect. Biol. 1, a002493 (2009).

Figure 5 | Wnt-deacylation by Notum. a, LC–MS analysis of mWnt3A protein treated with hNOTUM or a mock solution. By comparison to mock treatment (light label), addition of hNOTUM (heavy label) caused a significant increase in the signal intensity of unlipidated CHGLSGSCEVK. b, LC–MS peak areas from a, shown as mean ± s.e.m. (n = 2). c, d, Quantification from MALDI analysis of synthetic lipid-bearing peptides treated with hNOTUM or its Ser232Ala variant. Bars (grey denotes lipidated; white denotes delipidated) show mean ± s.e.m. (n = 3). c, Palmitoleoylated hWNT3A peptide, but not palmitoylated hSHH peptide, was specifically deacylated by the wild-type enzyme. d, Close-up view on the seryl-palmitoleate active site complex of hNOTUM. The experimental omit electron density is contoured at 2σ. e, f, Feedback control by Notum. Notum deacylates Wnt in a glycan-assisted fashion.

comparisons of all hNOTUM structures suggest substantial flexibility, compatible with palmitoleate entry and release (Extended Data Fig. 5b). Therefore, crystallographic evidence strengthens our observation that Notum is a Wnt-specific deacylase with preference for cis-unsaturated long chain lipids.

Discussion

Only a small number of secreted proteins, Wnts, Hedgehogs and Ghrelin, are known to be acylated. In all cases, this post-translational modification is essential for activity and is carried out by dedicated
14. Bornemann, D. J., Duncan, J. E., Staatz, W., Selleck, S. & Warrior, R. Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. Development 131, 1927–1938 (2004).
15. Kreuger, J., Perez, L., Giraldes, A. J. & Cohen, S. M. Opposing activities of Dally-like glypicans at high and low levels of Wingless morphogen activity. Dev. Cell 7, 503–512 (2008).
16. Trastler, A., Shi, W. & Filmar, J. Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cell surface. Biochem. J. 410, 503–511 (2008).
17. Hacker, U., Nybakken, K. & Perrimon, N. Heparan sulphate proteoglycans: the sweet side of development. Nature Rev. Mol. Cell Biol. 6, 530–541 (2005).
18. Petersen, C. P. & Redden, P. W. Polarized notum activation at wounds inhibits Wnt function to promote planar head regeneration. Science 332, 852–855 (2011).
19. Chang, M. V., Chang, J. L., Gangopadhyay, A., Shearer, A. & Cadigan, K. M. Activation of wingless targets requires bipartite recognition of DNA by TCF. Curr. Biol. 18, 1877–1881 (2008).
20. Flowers, G. P., Topczewska, J. M. & Topczewski, J. A. zebrafish Notum homolog specifically blocks the Wnt/b-catenin signaling pathway. Development 139, 2416–2429 (2012).
21. Torisu, Y. et al. Human homolog of NOTUM, overexpressed in hepatocellular carcinoma, is regulated transcriptionally by b-catenin/TCF. Cancer Sci. 99, 1139–1146 (2008).
22. Baena-Lopez, L. A., Nojima, H. & Vincent, J.-P. Integration of morphogen signaling within the growth regulatory network. Curr. Opin. Cell Biol. 24, 166–172 (2012).
23. Alberts, L. J. et al. Molecular Biology of the Cell, 2nd edn (Garland Science, 2008).
24. Basler, K. & Struhl, G. Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature 368, 208–214 (1994).
25. Alexandre, C., Jacinto, A. & Ingham, P. W. Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus transcript, a member of the Gli family of zinc finger DNA-binding proteins. Genes Dev. 10, 2003–2013 (1996).
26. Telemann, A. A. & Cohen, S. M. Dpp gradient formation in the Drosophila wing imaginal disc. Cell 103, 971–980 (2000).
27. Whalen, D. M., Malinauskas, T., Gilbert, R. J. C. & Siebold, C. Structural insights into proteoglycan-shaped Hedgehog signaling. Proc. Natl Acad. Sci. USA 110, 16420–16425 (2013).
28. Bellenkaya, T. Y. et al. Drosophila Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypicans members of heparan sulfate proteoglycans, Cell 119, 231–244 (2004).
29. Lum, L. et al. Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. Science 299, 2039–2043 (2003).
30. Akiyama, T. et al. Daily regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. Dev. Biol. 313, 408–419 (2008).
31. You, J., Bellenkaya, T. & Lin, X. Sulfated is a negative feedback regulator of wingless in Drosophila. Dev. Dyn. 240, 640–648 (2011).
32. Kirkpatrick, C. A., Dimitroff, B. D., Rawson, J. M. & Selleck, S. B. Spatial regulation of Sonic hedgehog distribution and signaling by Dally-like protein. Dev. Cell 18, 1877–1881 (2008).
33. Xu, Q. et al. Vascular development in the retina and inner ear: control by Norrin and Fzd4, a high-affinity ligand-receptor pair. Cell 116, 883–899 (2004).
34. Pepinsky, R. B. et al. Identification of a palmitic acid-modified form of human Sonic hedgehog. J. Biol. Chem. 273, 14037–14045 (1998).
35. Rios-Esteses, J. & Resh, M. D. Stearoyl CoA desaturase is required to produce active, lipid-modified Wnt proteins. Cell Reports 4, 1072–1081 (2013).
36. Reichman, F., Smith, L. & Cumming, S. Glycosamionglycans can modulate extracellular localization of the wingless protein and promote signal transduction. J. Cell Biol. 155, 819–827 (1996).
37. Fuerer, C., Habib, S. J. & Nusse, R. A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. Dev. Dyn. 239, 184–190 (2010).
38. Zeibisch, M. et al. Structural and molecular basis of ZNRF3/RNF43 transmembrane ubiquitin ligase inhibition by the Wnt antagonist R-spondin. Nature Commun. 4, 2787 (2013).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank K. Dingwell for supplying purified mWnt3A, H. Bellen for anti-Sanseelcs, C. Alexandre for plasmids and advice, W. Chai for glycosaminoglycan probes, T. Holder for suggestions, T. Malinauskas and C. Lorenz for advice and technical support, T. Walter for technical support with crystallization, W. Lu and Y. Zhao for help with tissue culture, and the organisers of the EMBO Wnt meeting 2012 where our collaboration began. We thank staff at Diamond Light Source beamlines (I02, I03, I04, I04-1, I24) for assistance with data collection (proposal mx4823). This work was supported by the MRC (U11T584268 to J.-P.V., P0900084 to E.Y.J.), the UK Research Council Basic Technology Initiative (Glycomarries Grant GRS/79268 and EPSRC Translational Grant EP/G037604/1), the Wellcome Trust (Biomedical Resource Grants WT093378MA and WT099197MA to T.F.), the European Union (ERC grant WNTEXPORT; 294523 to J.-P.V., a Marie Curie IEF grant to M.Z.), Cancer Research UK (C375/A10976 to E.Y.J.), and the Japan Society for the Promotion of Science (to S.K.), T.-H.C. was funded by a Nuffield Department of Medicine Prize Studentship in conjunction with Clarendon and Somerville College Scholarships. The Wellcome Trust Centre for Human Genetics is supported by Wellcome Trust Centre grant 090532/Z/09/Z.

Author Contributions Experimental contributions were as follows: Drosophila developmental genetics (P.F.L. and S.K.); Drosophila cell-based assays (S.K.); human cell-based assays (M.Z. and T.-H.C.); mass spectrometry (S.H., S.K. and A.P.S.); glycan synthesis (G.B. and N.O’R.). The project was conceived by S.K., P.F.L., M.Z., E.Y.J. and J.-P.V. The first draft of the paper was written by M.Z., E.Y.J. and J.-P.V. with substantial contributions from P.F.L., S.K. and A.P.S. and experimental contributions were as follows: P.F.L. and S.K.; human cell-based assays (M.Z. and T.-H.C.); mass spectrometry (S.H., S.K. and A.P.S.); glycan synthesis (G.B. and N.O’R.). The project was conceived by S.K., P.F.L., M.Z., E.Y.J. and J.-P.V. The first draft of the paper was written by M.Z., E.Y.J. and J.-P.V. with substantial contributions from P.F.L., S.K. and A.P.S. All authors contributed to the design and interpretation of experiments. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.Z. (matthias.zeibisch@evotec.com), E.Y.J. (yvonne@strub.ox.ac.uk) or J.-P.V. (jvrcen@nimr.mrc.ac.uk).
**METHODS**

Immunostaining and microscopy. The following primary antibodies were used: guinea-pig anti-Senseless (1:1000, gift from H. Bellen), mouse anti-Patched (1:50, Hybridoma bank), rabbit anti-V5 (1:500, Abcam), mouse anti-V5 (1:500, Invitrogen), rabbit anti-p-Smad3 (1:500, Epitomics), mouse anti-Dlp (1:50, Hybridoma bank), rabbit anti-GFP (1:500, Abcam), mouse anti-Wingless (1:200, Hybridoma bank). Secondary antibodies were used Alexa 488, Alexa 555 and Alexa 647 (1:500, Molecular Probes). Total and extracellular immunostaining of imaginal discs was performed as previously described. Imaginal discs were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and imaged using a Leica SP5 confocal microscope. Confocal images were processed with ImageJ (NIH) and Photoshop CS1 (Adobe). All confocal images show a single confocal section. Adult wings were mounted in Euparal (Fisher Scientific) and imaged with a Zeiss Axiophot2 microscope with an AxioCam HRC camera. Adult wing size and L3–L4 intervein distance was measured with ImageJ.

**Drosophila husbandry and clone induction.** All crosses were performed at 25 °C except those to generate discs shown in Figs 1a, b, 2 and Extended Data Figs 1a, b and 2g, h, k, l in which larvae were reared at 18 °C, the Gal80° permissive temperature, and then shifted to 29 °C, the restrictive temperature, 16 h before dissection to induce UAS-notum-V5 expression. To generate mutant clones, larvae were heat-shocked for 1 h at 37 °C at 60 h (±12 h) after egg-laying, except for the crosses to generate the disc shown in Fig. 2f, which was heat-shocked for 1 h at 37 °C at 84 h (±12 h) after egg-laying. Large mutant clones were generated by including a Minute mutation on the homologous chromosome.

**Drosophila genotypes.** The following Drosophila genotypes were used: CyO / UAS-notum-V5 ; tub::Gal80ts/+ (Fig. 1a); ap-Gal4 / UAS-notum-V5 ; tub::Gal80ts/+ (Fig. 1b); yw hs-FLP; notumKO FRT2A / Ubi::GFP M FRT2A (Fig. 1c); yw hs-FLP; dlp-dpl-MHA dlp-dl-2A (Fig. 2a); UAS-notum-V5 / + ; dpp-Gal4 dlp-MHA dlp-dl-2A (Fig. 2b); UAS-notum-V5 / + ; dpp-Gal4 dlp-MHA dlp-dl-2A / FRT2A (Fig. 2c); UAS-notum-V5; tub:GFP-C8; dlp-dlp-MHA dlp-dl-2A / FRT2A (Fig. 2d); sex-O-potum-V5 / en-Gal4 UAS::GFP; dlp-dlp-MHA dlp-dl-2A / FRT2A (Extended Data Fig. 1f); Cyo / UAS-notum-V5 ; tub::Gal80ts/+ (Extended Data Fig. 1a); ap-Gal4 / UAS-notum-V5 ; tub::Gal80ts/+ (Extended Data Fig. 1b); yw hs-FLP; notumKO FRT2A / Ubi::GFP M FRT2A (Extended Data Fig. 1c); notum141 FRT2A / FRT2A (Extended Data Fig. 1d); Notum-V5 or pLotattB-Notum-V5, respectively. A stable S2 line expressing V5-tagged dNotum was overexpressed in Schneider's 2/3 medium, 10 h after egg-laying, except where noted otherwise. Large mutant clones were generated by including a Minute mutation on the homologous chromosome.

**Generation of Notum knock-out by homologous recombination.** notumKO was generated by homologous recombination using NRT-wg and crossing schemes described previously. The homolog arms were amplified from w1118 genomic DNA. The primers 5′-GATGCTGATCGGCGGAGAAGACCAAACAGGATAC 3′ and 5′-GATGCTGATCGGCGGAGAAGACCAAACAGGATAC 3′ were used to amplify the upstream 5-kilobase (kb) homology arm, which was cloned into pTV as a SpeI-BglII fragment. GGAATTGATTTGATTCGATTGCGGTG-3′ was used to amplify the downstream 3-kb homology arm, which was cloned into pTUB as a SpeI-BglII fragment.

**Expression vectors for cultured Drosophila cells.** Drosophila S2 or 28R + (Drosophila Genomics Resource Centre, DGRC), were cultured at 25 °C in Schneider's medium plus 1-glutamine (Sigma) containing 10% (v/v) fetal FBS (Life Technologies) and 0.1 mg ml⁻¹ pen/strep (Life Technologies). To generate plasmids expressing V5-tagged dNotum, the dNotum cDNA (from S. Cohen) was amplified, adding a V5 tag (GKPPIPNLLGLDST) at the C terminus. This fragment was then inserted into pActin, pUASt or pLotattB to generate pAct-notum-V5, pUASt-Notum-V5 or pLotattB-Notum-V5, respectively. A stable S2 line expressing V5-tagged dNotum (52 act-Notum-V5) was generated by transfection of S2 cells with act-Notum-V5 and pCoHygro (Invitrogen) followed by drug selection. Wingless was expressed from pTub-Wg, which was prepared by inserting the Wg cDNA from pKS-Wg into pTubulin. HA-tagged Dally was expressed from pAct-Dally-HA, prepared by inserting Dally-HA excised from pMT-Dally-HA (from S. Cohen) into pActin. To conveniently manipulate the coding sequence of Dlp, three nucleotides (GTC) were inserted at positions 2100–2102 (nucleotide numbering with the A of first codon at position 1) to introduce a Sall site in KS-Dlp. This was used to insert DNA encoding an HA tag flanked by Glycine (GTPYDVPDYAG) and thus generate KS-P5-Dlp-HA. The KS-Dlp-HA was then inserted into pTubulin to make pTub-Dlp-HA.

**PIPIL treatment of imaginal discs and cultured cells.** Wing imaginal discs were treated with PIPIL as previously described with some modifications. In brief, discs were dissected from third instar larvae and incubated in Schneider's medium with 10% FBS containing 1 U ml⁻¹ of PIPIL (Molecular Probes) at room temperature for 30 min. After treatment, the discs were washed three times with Schneider's medium before extracellular staining (no detergent). S2 cells transfected with pTub-Dlp-HA and pActin (mock), or pTub-Dlp-HA and pAct-Notum-V5 as well as the corresponding conditioned medium were collected (total volume 300 μl) and treated with PIPIL (final concentration 1 U ml⁻¹) for 1.5 h at 25 °C before phase separation.

**Phase separation assay.** The phase separation assay was performed as previously described with some modifications. After PIPIL treatment, 200 μl of precondensed Triton X-114 (Sigma) was added to the reaction mixtures (Triton X-114 final concentration ~2%). The extracts were incubated for 15 min on ice and then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant were transferred to new tubes and warmed at 37 °C in a water bath for 10 min. After a second centrifugation (10,000 g for 10 min at room temperature), the upper phases (aqueous) and lower phases (detergent) were collected separately and mixed with 4× sample buffer (Life Technologies) for analysis by immunoblotting.

**Immunoblotting.** Samples were run on 4–12% Bis-Tris NuPAGE gels (Invitrogen) with MOPS buffer. Proteins on gel were transferred onto nitrocellulose membrane using iBlot gel transfer System (Invitrogen). The membranes were washed with dH₂O and blocked with 5% skimmed milk in 0.1% Tween-20 PBS (PBS-T) for 30 min at room temperature. Membranes were incubated with primary antibodies (mouse monoclonal anti-V5; Life Technologies, 1:5,000 and rat anti-HA; Roche, 1:2,500) diluted in 5% milk PBS-T overnight at 4 °C before phase separation.

**Glycan array.** CM obtained from S2 cells expressing V5-tagged dNotum was overlaid on a focused neoglycolipid-based glycan array containing lipid-linked GAG oligosaccharide probes (see http://www1.imperial.ac.uk/glycosciences/ and refs 56, 57) and allowed to bind for 90 min. The array was then washed and stained with anti-V5 mouse monoclonal antibody (Invitrogen) followed by biotinylated anti-mouse IgG (Sigma). Binding was detected with Alexa Fluor 647-labelled streptavidin. Fluorescence intensity was quantified and data analysis was performed with dedicated microarray software. No binding was observed when control medium was used instead of the conditioned medium or when the anti-V5 was used in the absence of the conditioned medium (data not shown).

**Large-scale expression of Notum constructs.** The CDNA coding for mature hNOTUM (residues Arg38–Ser 496) was cloned into the PHLsec vector that adds a C-terminal His6- or His10-tag. After the crystal structure was solved in crystal forms I and II (see below) and the folded region identified, a shorter construct hNOTUMcore comprising Ser 81–Thr 451, Cys330Ser, was found to provide higher expression levels, thanks in part to the removal of the non-conserved Cys330, which provides a free, surface-exposed sulfhydryl. Expression of wild-type protein resulted in non-quantitative spontaneous crosslinking of the protein, a problem that was not observed with the Cys330Ser variant.

For dNotum, we initially attempted to express Asp83–Thr 617. However, a large unstructured and non-conserved domain of 22 kDalanons (kDa) (Arg416–Lys 597) was found to interfere with crystallization. This domain, which was not present in hNOTUM, was deleted and replaced by GNNNG to generate dNotumDalloop. Note that this domain could provide an additional glycan-binding surface since it is...
highly basic (pI = 12.4). Proteins were transiently expressed in HEK293T cells and purified as described17. Proteins for crystallization were expressed either in GntI-deficient HEK293 cells or in HEK293 cells treated with kifunensine (1 mg ml\(^{-1}\)). Before crystallization the proteins were treated with endoglycosidase F1 at a ratio of 1:100. Proteins intended for kinetic studies were stored in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 50% (v/v) glycerol at −20 °C.

**SPR equilibrium binding studies.** Affinity between variants of hNOTUM and GPC3 or sulfated GAG was measured at 25 °C in 10 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, and 0.005% (v/v) Tween20 using a Biacore T200 machine (GE Healthcare). GPC3 constructs (see below) or sulfated GAGs were coupled to a streptavidin-coated sensor chip via a biotin label and purified Notum proteins were used as analyte. Biotinylated GAGs were produced as described17. To produce biotinylated GPC3 we proceeded as follows. The cDNA encoding human GPC3 (full-length except for the lack of endogenous signal sequence, Pro31–Asn538) or GPC3\(_{\text{CAG}}\) (lacking a C-terminal stretch that normally contains the GAG attachment sites, Pro31–Phe493) was cloned into a variant of the PhLSec vector, which introduces a recognition sequence for the Escherichia coli BirA enzyme at the C-terminus. Biotinylation at this sequence tag was performed by co-transfection of HEK293T cells with the GPC3 construct and an E. coli BirA expression construct. The synthetic BirA gene was codon-optimized and carried a C-terminal KDEL-tag for retention in the endoplasmic reticulum. The BirA plasmid was used at 20% of total DNA. The expression medium was supplemented with 100 μM of sterile biotin prepared as a 2 mM stock in PBS. After 3 days, the conditioned medium was cleared from cell debris and repeatedly buffer-exchanged to remove free biotin. The chip surface was precoupled with approximately 10,000 resonance units (RU) of streptavidin. Approximately 500 RU of GPC3 was immobilized. The amount of immobilized GAGs could not be measured. After each injection of analyte the chip surface was regenerated with 1 M NaCl, 10 mM HEPES/NaOH, pH 7.5, to return to baseline levels. Data were fit to a Langmuir adsorption model (\(B = \frac{B_{\text{m}}K_{d}}{C + K_{d}}\), where \(B_{\text{m}}\) is the amount of bound analyte and \(C\) is the concentration of analyte in the sample. Data were then normalized to a maximum analyte binding value of 100. For the design of heparin binding site mutants, the following considerations were taken into account. If, based on the crystal structure, the hydrophobic part of the side chain (for example, Arg, Lys, His) was estimated to be of no structural importance, then the residue was mutated to serine. In all other cases it was mutated to glutamine (Arg, Lys) or asparagine (His) to keep the overall structure as native as possible.

**Heparin affinity chromatography.** We compared the affinity of hNOTUM variants for heparin using a 1 ml HiTrap Heparin HP column (GE Healthcare). The flow rate was 2 ml min\(^{-1}\). 

**Cell-based Notum activity assays.** To assay Wingless signalling in Drosophila cells, a modified TOPFlash vector called WISIR, comprising a TCF-responsive promoter and luciferase reporter was used. The construct was transiently co-transfected with 20% of total DNA with Renilla luciferase using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions of the manufacturer. Data represent average of quadruplicate measurements ± s.d. The incubation time with cells was kept constant for all compared samples.

**Mass spectrometric analysis of the effect of Notum on Wnt3A protein.** As a general method to quantify the levels of delipidated Wnt3A protein by LC–MS–MS, we used an isotope coded alkylation reaction targeting cysteines to multiplex mass spectrometry signal. One sample was reacted with heavy iodoacetamide (IAA, \(^{13}\text{C}_{2}\text{D}_{2}\text{HNO}) and the other with the light version (\(^{12}\text{C}_{2}\text{HNO}) and consequently, peptide signals were separated at a 1:1 ratio at per well into 96-well plates.

**Supplementary Information** with 1 ml of purified hNOTUM core at 25 ng μl\(^{-1}\) in the following buffer: 20 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl, 0.5 mM EDTA, 0.5% CHAPS and 5% glycerol and left together for 16 h at 25 °C. The reaction was quenched by addition of 4% LIDS sample buffer (Life Technologies). Coomassie blue stained bands from SDS–PAGE were excised from the gel and cut in half and destained by incubating for 45 mins with 200 mM ammonium bicarbonate (ABC) 60% acetonitrile (ACN). To reduce cysteines, buffer was refreshed with the inclusion of 10 mM dithiothreitol (DTT) for 15 min. After washing, half of the gel pieces were incubated in 20 mM heavy or light IAA in 100 mM ABC/60% ACN buffer in the dark for 30 min. Proteins were digested using 4 h in-gel trypsin digestion step in 100 mM ABC and then quenched with 0.1% TFA. Equal aliquots of heavy and light reaction were mixed to generate forward and reverse labelled samples. Duplicate LC–MS analysis was performed using an Ultimate3000 RSLC system coupled to a LTQ-Orbitrap Velos-Pro mass spectrometer (Thermo Scientific). The instrument was operated in an alternating targeted MS/MS and data dependent acquisition mode. CHGlSGCSEVK and the control peptide AGIQECQHQFR were targeted for MS/MS. MS/MS spectra were searched using Mascot v2.3 and identified in Skyline software v2.6.0.6709. Skyline was used for peaks extraction and areas determination.

**Mass spectrometric analysis of Wnt3A and Shh peptides.** Delipidation assays were performed by reacting 3 μg of synthetic peptides (synthesis described in Supplementary Information) with 1 μl of enzyme (hNOTUM\(_{\text{core}}\) or hNOTUM\(_{\text{core}}\) with WISIR alone or with WISIR and pTub-Wg were mixed with mock-treated cells. Firefly luciferase activity was normalized to Renilla luciferase activity and the average of triplicate samples was calculated.

**hNOTUM inhibition of Wnt signalling in mammalian cells was assessed in stably transfected SuperTopFlash (STF) HEK293 cells48. These were treated with conditioned medium from Wnt3A-producing L cells with or without recombinant purified hNOTUM. To reveal the direct action of hNOTUM on Wnt we proceeded as follows. Wnt3A CM was dialysed for 24 h against ten volumes of tissue culture-grade PBS and then sterile-filtered with the aim to remove chemicals that might interfere with TALON-binding (see below). To 500 μl of such dialysed Wnt3A, 5 μl hNOTUM protein or an unrelated control protein (mock) at a concentration of 1 mg ml\(^{-1}\) was added and incubated for the indicated time at room temperature (23 °C). To stop the enzymatic reaction we added 50 μl of fresh 50% slurry of cobalt affinity beads (TALON resin) equilibrated against tissue culture grade PBS and 5 μl of 500 mM imidazole in PBS. After vigorous shaking the solution was incubated for 1 h at room temperature on a vertical rotator. Beads containing the His\(_{10}\)-tagged hNOTUM protein were removed by centrifugation (3,000 g, 5 min) and discarded. The supernatant was cleared again by centrifugation at maximum speed (16,000 g, 5 min). 100 μl of the reaction solution was then added to STF cells seeded the previous day at 50,000 cells per 100 μl and per well into 96-well plates. The Wnt-induced luciferase activity was measured after 16–20 h using the Glo kit (Promega) and an Ascent Luminoskan luminometer (Labystems) following the instructions of the manufacturer. Data represent average of quadruplicate measurements ± s.d. The incubation time with cells was kept constant for all compared samples.

**To assess hNOTUM inhibition of Norrin signalling, STF cells seeded in 96-well plates were transfected after 24 h with 200 ng DNA: 60 ng each of hFZ4 and hLRP6 plasmids, 30 ng of Tspan-12 plasmid, and 50 ng constitutive Renilla luciferase plasmid. Wnt3A protein was stimulatued 24 h after transfection with 10 μg ml\(^{-1}\) recombinant Norrin (T.-H. Chang et al. manuscript in preparation), which had been preincubated for 24 h with 10 μg ml\(^{-1}\) hNOTUM variants or FCS as a control. Firefly and Renilla luciferase activities were measured 48 h later with Dual-Glo luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and the average of triplicate samples was calculated.

**Crytalization, data collection and structure determination.** Concentrated proteins were subjected to sitting drop vapour diffusion crystallization trials employing a Cartesian Technologies pipetting robot and typically consisted of 100–300 nl of protein solution and 100 nl of reservoir solution. A detailed discussion of the multiple conditions in which crystal growth occurred is provided in Supplementary Information. Standard methods were used for X-ray diffraction data collection and structure determination, distinctive details for the series of crystal structures are discussed in Supplementary Information.
(S232A); 25 ng µl\(^{-1}\)) in 20 mM ammonium bicarbonate buffer (total volume 5 µl) for 16 h at 25 °C. The reaction was quenched with 0.1% TFA and samples were desalted using c18 zip tips. Samples were prepared in α-cyano-4-hydroxycinnamic acid in 50:50 water/acetonitrile with 0.1% TFA. MALDI-TOF spectra were acquired using an ABSCIEX 5800 TOF/TOF systems and analysed using data explorer v4.11.

**Statistics.** No statistical methods were used to predetermine sample size.

49. Beckett, K. *et al.* *Drosophila* S2 cells secrete wingless on exosome-like vesicles but the wingless gradient forms independently of exosomes. *Traffic* **14**, 82–96 (2013).

50. Vincent, J.-P., Kolahgar, G., Gagliardi, M. & Piddini, E. Steep differences in wingless signaling trigger myc-independent competitive cell interactions. *Dev. Cell* **21**, 366–374 (2011).

51. Baena-Lopez, L. A., Alexandre, C., Mitchell, A., Pasakarnis, L. & Vincent, J. P. Accelerated homologous recombination and subsequent genome modification in *Drosophila*. *Development* **140**, 4818–4825 (2013).

52. Yagi, R., Mayer, F. & Basler, K. Refined LexA transactivators and their use in combination with the *Drosophila* Gal4 system. *Proc. Natl Acad. Sci. USA* **107**, 16166–16171 (2010).

53. Alexandre, C., Baena-Lopez, A. & Vincent, J.-P. Patterning and growth control by membrane-tethered Wingless. *Nature* **505**, 180–185 (2014).

54. Marois, E., Mahmoud, A. & Eaton, S. The endocytic pathway and formation of the Wingless morphogen gradient. *Development* **133**, 307–317 (2006).

55. Doering, T. L., Englund, P. T. & Hart, G. W. Detection of glycosphingolipid anchors on proteins. *Curr. Prot. Prot. Sci. Chapter 12*, Unit 12.15 (2001).

56. Fukui, S., Feizi, T., Galustian, C., Lawson, A. M. & Chai, W. Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. *Nature Biotechnol.* **20**, 1011–1017 (2002).

57. Palma, A. S., Feizi, T., Childs, R. A., Chai, W. & Liu, Y. The neoglycolipid (NGL)-based oligosaccharide microarray system poised to decipher the meta-glycome. *Curr. Opin. Chem. Biol.* **18**, 87–94 (2014).

58. Aricescu, A. R., Lu, W. & Jones, E. Y. A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr. D* **62**, 1243–1250 (2006).

59. Malinauskas, T., Aricescu, A. R., Lu, W., Siebold, C. & Jones, E. Y. Modular mechanism of Wnt signaling inhibition by Wnt inhibitory factor 1. *Nature Struct. Mol. Biol.* **18**, 886–893 (2011).

60. Gagliardi, M., Hernandez, A., McGough, I. J. & Vincent, J. P. Inhibitors of endocytosis prevent Wnt/Wingless signalling by reducing the level of basal β-Catenin/Armadillo. *J. Cell Sci.* **127**, 4918–4926 (2014).

61. Glise, B. *et al.* Shifted, the *Drosophila* ortholog of Wnt inhibitory factor-1, controls the distribution and movement of Hedgehog. *Dev. Cell* **8**, 255–266 (2005).
Extended Data Figure 1 | Notum modulates Wingless, but not Dpp or Hedgehog signalling. a, b, Overexpression of dNotum-V5 with the apterous-Gal4 driver, which is expressed in the dorsal compartment, prevents expression of Senseless (Sens) (b, middle), a Wingless target gene, but has little effect on phospho-Mad (pMad) immunoreactivity (b), an indicator of Dpp signalling. c, Loss of notum activity, achieved by generating large patches of notum\( ^{\text{KO}} \) tissue (see Methods), marked by the loss of GFP, leads to broadening of Senseless expression but does not affect pMad immunoreactivity.

\( d-g \), Strong, but not complete, reduction of notum activity led to ectopic wing margin bristles (compare insets in \( d \) and \( e \)) but had no significant effect on wing area, which is sensitive to Dpp signalling (\( f \) (\( P = 0.26 \), Student’s t-test), or on the distance between L3 and L4 veins, which is affected by changes in Hedgehog signalling (\( g \) (\( P = 0.41 \), Student’s t-test). In total, 19 control (notum\( ^{+/+} \)) and 17 mutant (notum\( ^{141/KO} \)) wings were analysed. Error bars in \( f \) and \( g \) are s.d.
Extended Data Figure 2 | dNotum does not cleave the GPI anchor of glypican s. a, b, Ectopic expression of Senseless caused by NRT-wingless, as well as endogenous Senseless, is suppressed by co-expression of dNotum. NRT-wingless and notum are expressed in a vertical band under the control of dpp-Gal4. c, Western blot analysis of phase-separated extracts of S2 cells transfected with a plasmid expressing HA-tagged Dally. In control extracts, Dally is found largely in the detergent (D) phase. Coexpression of dNotum–V5 from a plasmid had no effect, while treatment with PIPLC shifted all detectable Dally to the aqueous (A) phase. d, dNotum-V5 expression as in c was sufficient to suppress Wingless-induced TOPFlash activity. Cells were transfected with a dual luciferase TOPFlash reporter along with a mock plasmid (−), tubulin::wingless (Wg), or tubulin::wingless + actin::notum-V5 (Wg + Notum). e–h, Extracellular Dlp in control (e, g), PIPLC-treated (f) or apterous-Gal4 UAS-notum-V5 (h) imaginal discs. i–l, Extracellular anti-GFP staining of imaginal discs from gene trap line expressing Dally–GFP fusion protein. Discs were treated with a mock solution (i) or PIPLC (j) (same discs as in e or f, respectively, but here showing Dally protein). In a separate experiment, dNotum was overexpressed with apterous-Gal4 in the Dally–GFP background (l). No change in the distribution of extracellular GFP could be seen compared to that in control discs (k, no apterous-Gal4).
Extended Data Figure 3 | dNotum requires Dally to inhibit Wingless signalling. a, Wingless and Senseless expression in a dally heterozygous wing imaginal disc expressing NRT-wingless and notum under the control of dpp-Gal4. Some senseless expression remains, indicating that, in the absence of Dally, dNotum is a poor inhibitor of NRT-Wingless-induced (as well as endogenous) signalling. b–d, Anterior margin of wings from control, spalt (sal)-Gal4 UAS-notum-V5, and sal-Gal4 UAS-notum-V5 dally null animals. Removal of dally rescues the loss of margin bristles caused by dNotum overexpression.
Extended Data Figure 4 | dNotum binds to sulfated glycans. Binding of dNotum-V5 to a GAG oligosaccharide array, detected by immunofluorescence. CSA/B/C, chondroitin sulfate A/B/C; HA, hyaluronic acid; hep, heparin; HS, heparan sulfate. Details on the array are provided in the Methods.
Extended Data Figure 5 | Additional structural information on Notum.

a, Topology plot of hNOTUM. β-strands are shown as numbered triangles and α-helices as circles labelled in alphabetical order from the N to C terminus (NT to CT). Structural elements conserved among most α/β-hydrolases are outlined in grey.

b, Comparison of the two most conformationally distinct hNOTUM structures (from crystal forms III and V). Crystal form III is the most structurally different. All other structures superimpose with root mean squared deviation (r.m.s.d.) of ~0.7 Å. Circles highlight the most flexible regions.

c, Comparison between the structures of hNOTUM (form V) and dNotum (form I). The circle highlights the lack of a cysteine bridge in dNotum.
Extended Data Figure 6 | Structural and biophysical analysis of heparin binding. a, Heparin affinity chromatography of wild-type hNOTUM and selected surface variants. b–e, Close-up views of additional sulfate binding sites on hNOTUM, crystal form III. Each view is accompanied with SPR heparin affinity data corresponding to each hNOTUM variant.
Extended Data Figure 7 | Relation of Notum to other esterases of the α/β hydrolase family. a, Comparison between hNOTUM and human esterase D (hESTD), showing structural relatedness. hNOTUM is also related to hAPT1, a cytosolic esterase used in this study as a positive control for fatty acid esterase activity. In the views shown here, the hNOTUM structure has been rotated by 90° around the x axis relative to the structure shown in Fig. 3b. b, Rootless phylogenetic tree of animal Notum proteins (red) and plant pectin acetyl esterases (PAE, green). Extent of sequence identity to hNOTUM is shown next to species name. Percentages between branches indicate sequence identity between neighbours.
Extended Data Figure 8 | Substrates and inhibitors of hNOTUM.

a, Inhibition of hNOTUM activity on pNP-butyrate (pNP4) by PMSF (30 min pre-incubation with 2 mM PMSF) as well as by Triton X-100 and CHAPS (0.5%). Presence of 20 mM SOS and 50 mg l−1 heparin results in a minor increase of esterase activity. The height of each bar represents activity relative to the mean of four control samples lacking the additives.

b, Saturable inhibition of hNOTUM by Triton X-100. Triton X-100 inhibits many esterases owing to binding to the acyl binding pocket through its hydrophobic group.

c, Lack of inhibition of Norrin-mediated β-catenin stabilization by Notum. Recombinant Norrin was pretreated with hNOTUMcore at a concentration sufficient to suppress Wnt3A-mediated signalling.

d, e, Saturation kinetics of the action of hNOTUM on pNP-octanoate (pNP8) and pNP-butyrate (pNP4, e).

The activity was normalized to the Amax calculated for hNOTUMcore. The activity values for the larger, full length protein were adjusted to compensate for the increased mass. Apparent Km values in d were corrected for the inhibition caused by Triton X-100.

f, Saturation inhibition kinetics with myristoleic and palmitoleic acid. pNP8 was used at a concentration of 1 mM and 250 μM, respectively.
Extended Data Figure 9 | Additional mass spectrometric analysis of the hNOTUM deacylase activity. a, Mass spectra of CHGLSGSCVEVK from trypsinized Wnt3A protein mock-treated or treated with hNOTUMcore. Left-hand graph is the same as that shown in Fig. 5a, while the right-hand side shows the results of a separate experiment performed with the labels reversed. b, Duplicate LC–MS peak areas with label reversal. Irrespective of the nature of the label (grey indicates light label, black indicates heavy label), hNOTUMcore triggered an increase in peak area of the delipidated Wnt3A tryptic peptide. c, d, Two control Wnt3A cysteine-containing peptides from the same data set were not affected by hNOTUMcore. e, Activity of hNOTUMcore and its Ser232Ala variant on a synthetic disulphide-bonded Wnt3A peptide (CHGLSGSCVEVK) palmitoleoylated on the first serine. Both lipidated and unlipidated peptide could be detected by MALDI-TOF. Incubation with hNOTUMcore, but not its Ser232Ala variant, caused significant delipidation (peak corresponding to delipidated peptide is marked by asterisk). Quantification of triplicate experiments is shown in Fig. 5c. f, MALDI-TOF analysis shows that neither hNOTUMcore nor its Ser232Ala variant delipidated a synthetic SHH peptide (CGPGRGFGKRR) palmitoylated on its N-terminal cysteine. Quantification of triplicate experiments is shown in Fig. 5d (peak corresponding to lipidated peptide is marked by black triangle). g, Two-dimensional active site schematic relating to Fig. 5e. Additional hydrogen bonds and electron pair movements thought to occur during hydrolysis by the wild type protein are shown in grey. h, Close-up view on the myristoleate active site complex of hNOTUMcore (crystal form I). The experimental omit electron density is contoured at $2\sigma$. 

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