Chondroitin sulfate proteoglycan Windpipe modulates Hedgehog signaling in *Drosophila*

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**ABSTRACT** Proteoglycans, a class of carbohydrate-modified proteins, often modulate growth factor signaling on the cell surface. However, the molecular mechanism by which proteoglycans regulate signal transduction is largely unknown. In this study, using a recently developed glycoproteomic method, we found that Windpipe (Wdp) is a novel chondroitin sulfate proteoglycan (CSPG) in *Drosophila*. Wdp is a single-pass transmembrane protein with leucin-rich repeat (LRR) motifs and bears three CS sugar chain attachment sites in the extracellular domain. Here we show that Wdp modulates the Hedgehog (Hh) pathway. In the wing disc, overexpression of *wdp* inhibits Hh signaling, which is dependent on its CS chains and the LRR motifs. The *wdp* null mutant flies show a specific defect (supernumerary scutellar bristles) known to be caused by Hh overexpression. RNA interference knockdown and mutant clone analyses showed that loss of *wdp* leads to the up-regulation of Hh signaling. Altogether, our study demonstrates a novel role of CSPGs in regulating Hh signaling.

**INTRODUCTION** Spatial and temporal regulation of growth factor signaling pathways is essential to proper development and disease prevention. Cell surface signaling events, such as ligand–receptor interactions, are often modulated by proteoglycans (Xu and Esko, 2014). Proteoglycans are carbohydrate-modified proteins that are found on the cell surface and in the extracellular matrix. They are composed of a core protein and one or more glycosaminoglycans (GAGs) covalently attached to specific serine residues on the core protein. GAGs are long, unbranched, and highly sulfated polysaccharide chains consisting of a repeating disaccharide unit. Based on the composition of the disaccharide units, proteoglycans are classified into several types, including heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs).

HSPGs function as coreceptors by interacting with a wide variety of ligands to modulate signaling activities (Lindahl and Li, 2009; Xu and Esko, 2014). *Drosophila* offers a powerful model system to study the functions of HSPGs in vivo because of its sophisticated molecular genetic tools and minimal genetic redundancy in genes encoding core proteins and HS synthesizing/modifying enzymes (Takemura and Nakato, 2015; Nakato and Li, 2016). In vivo studies using the *Drosophila* model have shown that HSPGs orchestrate information from multiple ligands in a complex extracellular milieu and sculpt the signal response landscape in a tissue (Nakato and Li, 2016). However, the molecular mechanisms of coreceptor activities of HSPGs still remain a fundamental question. Our previous studies predict that there are unidentified molecules involved in the molecular recognition events on the cell surface (Akiyama et al., 2008).

In addition to HS, *Drosophila* produces CS, another type of GAG (Toyoda et al., 2000). CSPGs are well known as major structural components of the extracellular matrix. CSPGs have also been shown to modulate signaling pathways, including Hedgehog (Hh), Wnt, and fibroblast growth factor signaling (Cortes et al., 2009; Townley and Bülow, 2018). Given the structural similarities between CS and HS, CSPGs may have modulatory, supportive, and/or complementary functions to HSPGs. However, the mechanisms by which CSPGs...
function as a coreceptor are unknown. In contrast to a large number of studies on HSPGs, very few CSPGs have been identified and analyzed in Drosophila (Momota et al., 2011). Unlike HSPGs, CSPG core proteins are not well conserved between species (Olson et al., 2006). Therefore, the identification of CSPGs cannot rely on the sequence homology to mammalian counterparts.

Recently, we have developed a glycoproteomic method to identify novel proteoglycans (Noborn et al., 2015, 2016, 2018). Briefly, this method includes trypsinization of protein samples, followed by enrichment of glycopeptides using strong anion exchange chromatography. After enzymatic digestion of HS/CS chains, the glycopeptides bearing a linkage glycan structure common to HS and CS chains are identified using nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS). This method has successfully identified novel CSPGs in humans (Noborn et al., 2015) and Caenorhabditis elegans (Noborn et al., 2018).

To study the function of CSPGs in signaling, we applied the glycoproteomic method to identify previously unrecognized CSPGs in Drosophila. We found that Windpipe (Wdp) is a novel CSPG and affects Hh signaling. Overexpression of wdp inhibits Hh signaling in the wing disc. This inhibitory effect of Wdp on Hh signaling is dependent on its CS chains and leucin-rich repeat (LRR) motifs. Consistent with the overexpression analysis, loss of wdp increases Hh signaling: wdp null mutant flies show a specific defect (supernumerary scutellar [SC] bristles) known to be caused by Hh overexpression. Our study highlights a novel function of CSPGs in cell signaling.

RESULTS
A glycoproteomic approach identified Wdp as a novel Drosophila CSPG

We investigated the potential presence of CSPGs in Drosophila using our recently developed glycoproteomic approach that identifies core proteins and their CS attachment sites. A general workflow for the sample preparation, CS-glycopeptide enrichment, LC-MS/MS analysis, and the subsequent data analysis is shown in Figure 1A. Briefly, Drosophila third-instar larvae were collected from two different genotypes (wild type [WT] [Oregon-R] and a loss-of-function mutant for tout-velu [ttv524]) and the material was homogenized in ice-cold acetone. The ttv encodes a Drosophila HS polymerase, and ttv mutants lack HS chains (The et al., 1999; Toyoda et al., 2000). The samples were incubated with trypsin and then passed over an anion exchange column equilibrated with a low-salt buffer. This procedure enriches for CS-attached glycopeptides as the matrix retains anionic polysaccharides and their attached peptides, whereas neutral or positively charged peptides flow through the column. The bound structures were eluted stepwise with three buffers of increasing sodium chloride concentrations. The resulting fractions were treated with chondroitinase ABC. This procedure
reduces the lengths of the CS chains and generates a residual hexa-
saccharide structure still attached to the core protein. The chondroit-
inase-treated fractions were analyzed with positive mode nLC-MS/ 
MS and an automated search strategy was used to identify CS-
modified peptides in the generated data set (Noborn et al., 2015).

The analysis revealed the Wdp protein as a novel CSPG, which 
was modified with three CS-polysaccharides on two unique peptides 
(Figure 1, B and C). We detected Wdp glycopeptides from both WT 
and tvt mutant samples, further supporting that Wdp bears CS 
chains, not HS. One of the identified precursor ions (m/z 983.38; 3+) 
equated to the mass of a peptide with a SDQVEGSSDLETSMELK 
sequence, derived from the middle part of the protein (amino 
acids 276–293) (Figure 1B). The peptide was modified with one 
hexasaccharide structure and one methionine oxidation. The mea-
sured mass (2947.1186 Da) deviated –3.27 ppm from the theoretical 
value. The other identified precursor ion (m/z 1276.76; 4+) equated 
to the mass of a peptide with a EEEHVKEDEDDDEGSSGGGLII 
DPSK sequence, located in proximity to the previous amino (amino 
acids 320–348) (Figure 1C). The peptide was found to be modified 
with two hexasaccharide structures and where one of the hexasac-
charides was modified with one phosphate modification. The mea-
sured mass (5102.9389 Da) deviated +3.05 ppm from the theoretical 
value. Detailed inspection of the spectra revealed several b- and y-
ions as well as the prominent diagnostic oxonium ion at m/z 362.1, 
corresponding to the disaccharide [GlcAGalNAc-H2O+]+ (Figure 
1, B and C). Furthermore, one of the glycans in Figure 1C was found 
modified with one phosphate group at a xylose residue (peptide + 
xylose + phosphate, m/z 1625.70; 2+).

Wdp is a single-pass transmembrane protein containing four LRR 
motifs in the extracellular domain (Huff et al., 2002). The three CS 
attachment sites (S282, S334, and S336) revealed by our glycopro-
tecmic analysis are located in the extracellular domain. Interestingly, 
a recent study reported that Wdp negatively regulates JAK–STAT 
signaling by promoting internalization and lysosomal degradation 
of the Domeless (Dome) receptor (Ren et al., 2015). We further in-
vestigated the role of Wdp, a novel CSPG, in signal transduction.

**Overexpression of wdp inhibits Hh signaling**

The growth and patterning of the Drosophila wing are controlled 
by multiple signaling pathways, including Decapentaplegic (Dpp; 
a Drosophila BMP), Wingless (Wg; a Drosophila Wnt), and 
Hh signaling. To determine the role of wdp in these developmental 
signaling pathways, we first asked whether overexpression of 
**wdp** affects adult wing morphology. **wdp** was overexpressed 
using BuMS1096-GAL4, which drives high levels of UAS transgene 
expression broadly in the wing pouch (Capdevila and Guerrero, 
1994; Mace and Tugores, 2004; Tripura et al., 2011). We found 
that wdp overexpression (BuMS1096-wdp) results in reduced 
wing size compared with control flies (BuMS1096+) (Figure 2, A–D). In 
addition, the distance between longitudinal wing veins 3 and 
4 (L3 and L4) was aberrantly narrower (Figure 2, A–C and E). This 
decreased distance between L3 and L4 is indicative of reduced 
Hh signaling during wing development (Mullor et al., 1997b; 
Strigini and Cohen, 1997).

Hh is produced in the posterior compartment of the wing disc 
and spreads toward the anterior compartment where it induces 
target gene expression in a concentration-dependent manner 
(Briscoe and Thérond, 2013; Gradilla and Guerrero, 2013; Hartl 
and Scott, 2014). Expression of high-threshold target genes, such 
as Patched (Ptc; the Hh receptor) (Capdevila et al., 1994) and En-
grailed (En) (Patel et al., 1989), are induced in anterior cells near 
the anteroposterior compartment boundary by high levels of Hh 
signaling (Figure 2, F and J; Jia et al., 2004). Lower levels of Hh 
signaling induce the expression of dpp and the accumulation of 
full-length cubitus interruptus (Ci; the transcriptional factor of Hh 
signaling) in a broader region (more distant away from the antero-
posterior boundary) (Figure 2, F and H). To determine whether Hh 
signaling is indeed affected by wdp, we overexpressed wdp spe-
cifically in the dorsal compartment of the wing disc using apterous 
**(ap)**-GAL4. We found that wdp overexpression in the dorsal com-
partment reduced the expression domains of both “high-thresh-
od” targets (Ptc and En) and “low-threshold” targets (dpp-
ac210638; a reporter for **dpp** expression, and full-length Ci) 
compared with those in the ventral compartment (Figure 2, G and I). To quantify the effect of wdp overexpression on Hh signaling, 
we generated signal intensity plots of the Ptc expression in the 
dorsal (red) and ventral (blue) compartments. In control wing discs 
**(ap>GFP)**, the Ptc signal is slightly higher in the dorsal compart-
ment (red) compared with the ventral compartment (blue), but the 
shape of the Ptc peak is similar in both compartments (Figure 2L).

On the other hand, in discs overexpressing **wdp** in the dorsal com-
partment **(ap>GFP-wdp)**, the width of Ptc-positive cells became 
significantly narrower specifically in the dorsal compartment 
(Figure 2M). Notably, overexpression of **wdp** did not affect the 
pattern of a **h**h transcriptional reporter **hh-lacZ**P30 (Figure 2K). 
Together, these results suggest that wdp acts as a negative 
modulator of Hh signaling without affecting **h**h transcription.

We next asked whether Wdp affects other developmental 
pathways: Dpp and Wg signaling. When wdp is overexpressed 
using ap-GAL4 or hh-GAL4 (a posterior compartment-specific GAL4 
driver), we did not observe apparent defects in Dpp signaling 
activity, which was monitored by the expression of phosphorylated 
Mad (pMad) and Spalt major (Salm) (readouts of Dpp signaling) 
(Supplemental Figure S1). Similarly, no changes in expression of 
Seneless (Sens) and Distal-less (Dll) (readouts of Wg signaling) were 
detected (Supplemental Figure S1). Thus, we did not detect any 
effect of Wdp on the Dpp and Wg downstream responses. This is 
consistent with a previous report (Ren et al., 2015).

We also noticed that overexpression of **wdp** induces massive 
apoptosis, as detected with anti-cleaved Caspase-3 antibody 
(Supplemental Figure S2B). This likely contributed to the smaller 
adult wing phenotype observed in BuMS1096-wdp flies. It was recently 
reported that Hh signaling is required for cell survival in wing disc 
cells (Lu et al., 2017). To determine whether reduced Hh signaling 
is responsible for the observed apoptosis, we first asked if reduced 
Hh signaling results in apoptosis. We inhibited Hh signaling either 
by expressing an RNA interference (RNAi) construct (TRiPHMC03577) 
targeting smoothened (smo) (Supplemental Figure S2E) or by 
overexpressing ptc in the dorsal compartment using ap-GAL4. We 
found that neither treatment increased signals for cleaved Caspase-3 
(Supplemental Figure S2F, G and H), indicating that reduced Hh 
signaling is not sufficient to induce massive apoptosis in the wing 
disc. Furthermore, coexpression of a constitutively active form of 
Smo with Wdp did not suppress apoptosis in the wing disc (Supple-
mental Figure S2H). These results suggest that overexpression of 
**wdp** induces apoptosis, independent of reduced Hh signaling.

**CS chains and LRR motifs are necessary for Wdp to inhibit 
Hh signaling**

Next, we asked whether the CS chains of Wdp are required for its 
function. In a CSPG core-protein, CS is attached to specific serine 
residues in the consensus serine-glycine dipeptide surrounded by 
acidic amino acids (Esko and Zhang, 1996). We generated a UAS-
wdpAGAG construct in which all three GAG-attachment serine
residues (S282, S334, and S336) were substituted with alanine residues so that CS cannot be attached to the core protein (Figure 3A).

The UAS-wdp and UAS-wdpΔGAG constructs were inserted in the same genomic location (ZH-86Fb) using the phiC31 site-specific integration system in order to ensure the same expression level of the UAS transgenes (Groth et al., 2004; Bischof et al., 2007).

Overexpression of wdpΔGAG by Bx MST106-GAL4 did not decrease the whole wing size (Figures 3, B and C, and 2D). No reduction in the distance between L3 and L4 was observed in Bx MST106-GAL4 > wdpΔGAG adult wings either (Figures 3, B and C, and 2E). Consistent with this, the expression of Ptc, En, Ci, and dpp-lacZ in the wing disc was not affected by wdpΔGAG overexpression in the dorsal compartment of the wing disc (Figure 3, D, E, and I). These results indicate that CS chains are required for Wdp’s activity to down-regulate Hh signaling.

To determine whether the LRR motifs and/or the intracellular domain of Wdp are necessary for inhibiting Hh signaling, we generated several Myc-tagged mutant constructs (Supplemental Figure S3) and examined their activities. Consistent with the earlier result (Figure 2, G and M), expression of a Myc-tagged WT Wdp (Myc:Wdp) led to a narrower Ptc expression domain (Figure 3, F and J). We found that a mutant wdp construct lacking LRR motifs (Myc:WdpΔLRRs) failed to inhibit Hh signaling (Figure 3, G and K). Thus, in addition to CS chains, the LRR motifs of Wdp are required for inhibiting Hh signaling. On the other hand, a truncated...
construct lacking the intracellular domain (Myc:WdpΔICD) retained the ability to inhibit Hh signaling (Figure 3, H and L). This result suggests that the Wdp intracellular domain may be dispensable for its function as a Hh-signaling regulator.

Wdp expression in the wing disc
To monitor Wdp expression, we generated transgenic flies (wdpKI.HA and wdpKI.OLLAS) expressing epitope-tagged Wdp protein from its endogenous locus. We inserted a spaghetti monster GFP with 10 copies of HA or OLLAS tags near the C-terminus of Wdp (after Q652; Figure 4A) using CRISPR–Cas9-mediated homology-directed repair (Gratz et al., 2014; Ren et al., 2014). Expression of a wdpRNAi construct (TRiP .HMC06302) using ap-GAL4 in wdpKI.HA/+ flies led to the loss of Wdp:HA staining specifically in the dorsal compartment (Figure 4B). This result validated the specificity of HA staining reflecting Wdp localization as well as the efficacy of RNAi-mediated knockdown of wdp. Expression of Wdp:HA and Wdp:OLLAS was detected in the eye disc, adult midgut, and tracheal system (Supplemental Figure S4), consistent with previous reports (Huff et al., 2002; Ren et al., 2015).

In the wing disc, Wdp:HA is expressed in most of the wing cells with enrichment in the basal side, as detected by anti-HA antibody (Figure 4, C and D). This result was confirmed by anti-OLLAS antibody staining of the wdpKI.OLLAS wing discs (Supplemental Figure S4, B and C). During mitosis, the nuclei of wing disc cells translocate to the apical surface to execute cell division, a phenomenon known as interkinetic nuclear migration (Ragkousi and Gibson, 2014). While both the nucleus and the bulk cytoplasm move toward the apical surface, these cells maintain connectivity with the basal side of the epithelium via long, thin, and F–actin-rich basal processes throughout mitosis (Meyer et al., 2011). Interestingly, Wdp:HA staining was particularly strong in the basolateral membrane of mitotic cells (Figure 4, E and F). The Wdp:HA signal is colocalized with F-actin, detected by Phalloidin staining (Figure 4G). Thus, Wdp is enriched in these basal extensions of mitotic cells during mitosis. At this point, however, biological significance of this localization of Wdp in the basal extensions of mitotic cells is unknown.

Loss of wdp leads to higher levels of Hh signaling
To determine whether loss of wdp affects Hh signaling activity, we examined the effect of wdp RNAi knockdown in the wing disc. The effect of wdp knockdown on Hh signaling was assessed using the Ptc expression level as a readout of the Hh signaling activity. The wdpRNAi expression using ap-GAL4 significantly increased the signal intensity of Ptc staining only in the dorsal compartment (Figure 5A). As stated earlier, the patterns of the Ptc signal in the dorsal and ventral compartments are similar in control wing discs (Figure 5, B and C). During mitosis, the nuclei of wing disc cells translocate to the apical surface to execute cell division, a phenomenon known as interkinetic nuclear migration (Ragkousi and Gibson, 2014). While both the nucleus and the bulk cytoplasm move toward the apical surface, these cells maintain connectivity with the basal side of the epithelium via long, thin, and F–actin-rich basal processes throughout mitosis (Meyer et al., 2011). Interestingly, Wdp:HA staining was particularly strong in the basolateral membrane of mitotic cells (Figure 4, E and F). The Wdp:HA signal is colocalized with F-actin, detected by Phalloidin staining (Figure 4G). Thus, Wdp is enriched in these basal processes during mitosis. At this point, however, biological significance of this localization of Wdp in the basal extensions of mitotic cells is unknown.

To determine whether loss of wdp affects Hh signaling activity, we examined the effect of wdp RNAi knockdown in the wing disc. The effect of wdp knockdown on Hh signaling was assessed using the Ptc expression level as a readout of the Hh signaling activity. The wdpRNAi expression using ap-GAL4 significantly increased the signal intensity of Ptc staining only in the dorsal compartment (Figure 5A). As stated earlier, the patterns of the Ptc signal in the dorsal and ventral compartments are similar in control wing discs.
As an additional control, overexpression of flp (ap>flp) did not change the Ptc levels (Figure 5B). On the other hand, Ptc signal intensity plots showed that in discs expressing wdp RNAi with ap-Gal4 (ap>GFP + wdp RNAi), the Ptc peak in the dorsal compartment is significantly higher and broader than that in the ventral compartment (Figure 5C). In addition, we observed that the dpp-lacZ expression domain was expanded anteriorly by wdp knockdown (Figure 5, D and E). The number and position of these notal mechanosensory bristles, called macrochaetes, are controlled by prepattern genes (Jan and Jan, 1990). For example, Wg and Dpp act as prepattern genes to regulate the specification of presutural (PS) and dorsocentral (DC) bristles, respectively (Whittle and Phillips, 1993; Couso et al., 1994; Tomoyasu et al., 1998). The formation of SC bristles is controlled by Hh signaling (Mullor et al., 1997a). Hh overexpression results in supernumerary SC bristles (Porter et al., 1996). Thus, loss of wdp function led to a known specific phenotype caused by hh overexpression.

We found that deleting one copy of hh in wdp homozygotes (wdp/wdp; hh+/−) almost completely suppressed the SC phenotype.
The authors showed that Wdp inhibits Hh signaling least one clone in all wing discs we examined (Figure 6K). Taken together, our results consistently show that wdp negatively regulates Hh signaling in the Drosophila wing.

DISCUSSION

Our glycoproteomic analysis identified Wdp as a novel CSPG. Apart from Wdp, we did not find any additional novel core proteins in this study. However, some previously established core proteins were also identified, which were found with both CS and/or HS modifications (unpublished data). In a recent glycoproteomic study of C. elegans, we identified 15 novel chondroitin core proteins, in addition to the nine previously established (Noborn et al., 2018). The reason for this discrepancy with regard to the number of identified core proteins in the two model organisms is unclear, but it may suggest that optimization of sample preparation is necessary for identifying additional CSPGs in Drosophila.

Although Wdp was found modified with CS in both WT and ttv backgrounds, general assessment of spectral intensities suggests that Wdp was present in higher abundance in the ttv samples. Earlier studies in zebrafish, mammalian cells, and C. elegans indicated that reduced HS sulfation results in increased CS sulfation (Dierker et al., 2016). Thus, it is not surprising to see a compensatory increase of CS synthesis in a strain lacking HS polymerase (ttv). It should be noted that we did not detect Wdp modified with HS in WT flies, although we explicitly looked for this variant.

Our genetic analyses of Wdp showed that it acts as a negative modulator of Hh signaling in a CS- and LRR motif-dependent manner. It has also been reported that Wdp negatively regulates JAK–STAT signaling and controls adult midgut homeostasis and regeneration (Ren et al., 2015). The authors showed that Wdp interacts with the Dome receptor and promotes its endocytosis and lysosomal degradation. Although we do not know the mechanism by which Wdp regulates Hh signaling, it is possible that Wdp modulates these two pathways via a similar mechanism: by controlling the stability of cell surface components of the pathways. Hh signaling is controlled by two key membrane proteins—Ptc and Smo. In the absence of Hh, Ptc inhibits the phosphorylation of Smo, which is internalized and degraded (Zhu et al., 2003). In the presence of Hh, restriction of Ptc on Smo is relieved, allowing Smo to accumulate on the cell surface and activate Hh signaling. Our preliminary observation showed that knockdown of wdp increases Smo protein levels (data not shown). Thus, Wdp may down-regulate Hh signaling by affecting Smo levels (e.g., disrupting Smo translocation to the cell membrane or the stability of Smo on the cell surface; Figure 7). However, this does not exclude other possibilities for Wdp action, such as sequestering the ligand, inhibiting Ptc in its Smo phosphorylation/activation, and competing with a HSPG coreceptor. In mice, sulfated CS is necessary for Indian hedgehog (Ihh) signaling in the developing growth plate (Cortes et al., 2009). Although Ihh and Sonic hedgehog (Shh) have been shown to bind to CS (Zhang et al., 2007; Cortes et al., 2009; Whalen et al., 2013), the molecular mechanisms of CSPG function in Hh signaling remain to be elucidated.

It is worth noting that both JAK–STAT and Hh signaling, the two pathways negatively controlled by Wdp, are also regulated by HSPGs. Daily-like, a glypican family of HSPGs, positively regulates Hh signaling (Desbordes and Sanson, 2003; Lum et al., 2003; Williams et al., 2010; Yan et al., 2010; Kim et al., 2011). In the
**FIGURE 6:** The *wdp* mutant phenotypes. (A) Schematic of the generation of a *wdp* loss-of-function allele (*wdp*ΔCDS) lacking most of the *wdp* CDS using the CRISPR–Cas9 system (top left). A primer set shown as cyan arrows was used for PCR-based genotyping (top right). The results are shown for the WT and *wdp*ΔCDS allele (KO). Genomic sequencing of the *wdp* endogenous locus targeted by CRISPR–Cas9 showed a deletion of most of the *wdp* CDS (bottom). A small insertion is shown in green. (B, C) Control (WT; B) and *wdp/wdp* (C) SC bristles. Anterior and posterior SC bristles are marked (Asc and pSc, respectively). The *wdp* homozygous mutants show duplication or triplication of SC bristles (arrowheads). (D) Genetic interactions between *wdp* and hh or Pka-C1 on the notum phenotype. Penetration of SC phenotypes is shown. Deleting one copy of hh in *wdp* homozygotes almost completely suppressed the SC phenotype (*wdp/wdp; hhAC/I*), while Pka-C1ΔN significantly enhanced it (*Pka-C1ΔN, wdp/wdp*). The bristles were scored for at least 200 specimens per genotype. (E–J) Somatic mosaic clones of *wdpKO* were induced in the wing pouch using Act5C-GAL4 UAS-FLP. Homozygous *wdp*ΔCDS mutant cells are marked by loss of GFP (green) and discs were stained with anti-Ptc antibody (magenta). The clone borders are marked with yellow lines. Increased Ptc expression was observed in *wdp* mutant clones. Zoom-out images of two wing discs bearing three *wdp* mutant clones for 6F, G, and H are shown in Supplemental Figure S5 to indicate the relative positions of these clones in the wing disc. (K) Boxplots showing the effect of *wdp* mutant clones on Ptc staining signal intensity. Ptc staining signal intensity in randomly selected *wdp* mutant clones was compared with that in immediate neighboring WT cells (*n = 49* pairs). ***p < 0.001. The Wilcoxon rank sum test was used to compare two paired samples.
Materials and Methods

Preparation of GAG-glycopeptides and LC-MS/MS analysis
GAG-glycopeptide samples were prepared from WT (Oregon-R) and tvt mutant (ttv237) third-instar larvae as previously described (Noborn et al., 2015, 2018). Briefly, 200–400 third-instar larvae (wet weight, 200–400 mg) were lysylphosphatidyl and homogenized using a motor pestle in 1 ml of ice-cold acetone. After extensive washes with acetone, the insoluble fraction was recovered by centrifugation. After overnight desiccation, the pellet was dissolved in 1.5 ml of 0.2 M MgCl2 and incubated with 3 μl Benzonase (MilliporeSigma, Burlington, MA) at 37°C for 3 h. After heat-inactivation of Benzonase, the sample was centrifuged and the supernatant was collected in a new tube.

An aliquot of the preparation (1 mg of protein) was further used. The sample was reduced and alkylated in 1 ml of 50 mM NH4HCO3 and trypsinized at 37°C overnight with 20 μg trypsin (Promega, Madison, WI). The digested samples were applied onto DEAE (GE Healthcare, Chicago, IL) columns (600 μl) at 4°C. The columns were washed with three different low-salt washing solutions at 4°C: 50 mM Tris-HCl, 100 mM NaCl, pH 8.0; 50 mM NaAc, 100 mM NaCl, pH 4.0; and 100 mM NaCl. The glycopeptides that were bound to DEAE were eluted stepwise with four buffers with increasing sodium chloride concentrations at 4°C: 4 ml 250 mM NaCl, 400 mM NaCl, 800 mM NaCl, and 3 ml 1500 mM NaCl. Each fraction was desalted using PD10-columns (GE Healthcare).

All fractions were lyophilized and the salt-free samples were then individually treated with 1 μM of chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) for 3 h at 37°C. Prior to MS analysis, the samples were desalted using a C18 spin column (8 mg resin) according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA) and lyophilized. LC-MS/MS analysis was performed as previously described (Noborn et al., 2015, 2018). In brief, the samples were analyzed on a Q Exactive mass spectrometer coupled to an Easy-nLC 1000 system (Thermo Fisher Scientific). The glycopeptides (2-μl injection volume) were separated using an analytical column with Reprosil-Pur C18-AQ particles (Dr. Maisch GmbH, Ammerbuch, Germany). The following gradient was run at 300 nL/min; from 7 to 35% B-solvent (acetonitrile in 0.2% formic acid) over 75 min to 100% B-solvent over 5 min, with a final hold at 100% B-solvent for 10 min. The A-solvent was 0.2% formic acid. Spectra were recorded in positive ion mode and MS scans were performed at 70,000 resolution with a mass range of m/z 600–1800. The MS/MS analysis was performed in a data-dependent mode, with the top 10 most abundant precursor ions in each MS scan selected for fragmentation (MS2) by higher-energy collision dissociation (HCD) with a normalized collision energy value of 30%. The MS2 scans were performed at a resolution of 35,000 (at m/z 200). The data analyses were performed as previously described (Noborn et al., 2015) with some small adjustments. In brief, the HCD.raw spectra were converted to Mascot .mgf format using Mascot distiller (version 2.3.2.0, Matrix Science, London, UK). The ions were presented as singly protonated in the output Mascot file. Searches were performed using an in-house Mascot server (version 2.3.02) with the enzyme specificity set to Trypsin, and then to Semi-trypsin, allowing for one or two missed cleavages, in subsequent searches on Drosophila sequences of the UniprotKB (42, 507, sequences, 2018-06-18). The peptide tolerance was set to 10 parts per million (ppm) and fragment tolerance was set to 0.1 Da. The searches were allowed to include variable modifications at serine residues of the partial hexasaccharide structure [GlcAl-H2O]Gal-NAcGlcAGalGalXyl-O-] with 0, (C2H7NO3S, 993.2809 Da), 1 (C2H7H5NO3S2, 1063.1945 Da) sulfate groups attached.

Drosophila strains
The following fly strains were used in this study: Oregon-R, w1118 (Bloomington Drosophila Stock Center [BDSC] #5905), tvt237, ap-GAL4, hh-GAL4, Bx551916-GAL4 (BDSC #8860), AB1-GAL4 (BDSC #1824), elavG588>GAL80 (BDSC #1416), UAS-GFP (BDSC #1521), UAS-ttdTomato (BDSC #36327 and #36328), UAS-FLP (BDSC #4539 and #4540), UAS-pect (BDSC #44614), nub-GAL4 (BDSC #25754), Act5C-GAL4 (BDSC #3954), FRT42D 2xUbI-GFP, UAS-smo-GFP (BDSC #44624), UAS-FLAG:smoAcT (BDSC #44621), UAS-wdpGAL4 (TRIP:HM05118, BDSC #28907), UAS-smoRNAi (TRIP:HM03577, BDSC #53348), hh-lacZ203 (a gift from Gary Struhl), dpp-lacZ1638 (BDSC #12379), vas-Cas9 (BDSC #55821), and esg-GAL4 (DGRC #113886). The UAS-wdp, UAS-wdpGAL4, UAS-3xMyc:wpd, UAS-3xMyc:wpdGAL4, UAS-3xMyc:wpdGAL4, UAS-3xMyc:wpdGAL4, UAS-3xMyc:wpdGAL4 files were generated in this study. A full list of genotypes used in this study can be found in Supplemental Table S1.
For constructing UAS-wdp, wdp CDS (corresponding to wdp-RA-E in FlyBase) was inserted into the Xhol- and XbaI-digested pJFRC7 vector (a gift from Gerald Rubin, Howard Hughes Medical Institute; Addgene #26220) using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs [NEB], Ipswich, MA, E2621S). Similarly, wdp<sup>ΔCDs</sup> (S282A, S334A, and S336A), Myc:wdp, Myc:wdp<sup>ΔRR</sup>, and Myc:wdp<sup>ΔCDs</sup> were inserted into the pJFRC7 vector. The UAS transgenic flies were generated using phiC31 integrase-mediated transgenesis at the ZH-86Fb attP (Fbit007525) integration site (Bischof et al., 2007). Because these UAS transgenes were integrated into an identical genomic location, the phenotypic differences reflect the activity of each construct rather than differential expression levels due to positional effects (Klein-schmit et al., 2010; Dejima et al., 2013; Kleinschmit et al., 2013). Embryonic injection was performed by BestGene (Chino Hills, CA). Primers used in this study will be available on request.

To generate the wdp<sup>KO,ΔCDs</sup> allele, two sgRNAs (pU6-sgRNA-wdp-1 and pU6-sgRNA-wdp-2) were introduced to delete the wdp CDS. To construct sgRNA plasmids, 5′-CTTCGACAGGGCAACACGCCCCGTCG-3′ and 5′-AAACGACGCCTGGTGGCCTGTCG-3′ were annealed (pU6-sgRNA-wdp-1), and 5′-CTTCGATGCGCCATTGATACCTGGG-3′ and 5′-CACCAAAAGGTATCAACAAGCCATTGCTC-3′ (pU6-sgRNA-wdp-2) were annealed and ligated in the BbsI-digested pU6-BbsI-chiRNA plasmid (a gift from Melissa Harrison, University of Wisconsin; Kate O’Connor-Giles, Brown University; and Jill Wildonger, University of Wisconsin; Addgene #54926). A mixture of 50 ng/μl of pU6-sgRNA-wdp-1 and pU6-sgRNA-wdp-2 was injected into the embryos of the vas-Cas9 flies, which express Cas9 under the control of the germline vasa regulatory elements, by BestGene. The wdp<sup>KO,ΔCDs</sup> allele was screened by PCR and verified by Sanger sequencing.

To generate the wdp<sup>X,LHA</sup> allele, we constructed a donor plasmid, which contained a Gly-Gly-Ser linker, smGFP-HA, and approximately 1-kb homology arms to wdp flanking the linker and smGFP-HA, for homology-directed repair. The smGFP-HA and the wdp homology sequences on either side of the targeted DSB were PCR-amplified from pJFRC201-10XUAS-FRT>STOP>FRRT-myrm:smGFP-HA (a gift from Gerald Rubin; Addgene plasmid #63166) and genomic DNA extracted from the vas-Cas9 flies, respectively. These fragments were cloned into the phD-DsRed-attP backbone (a gift from Melissa Harrison, Kate O’Connor-Giles, and Jill Wildonger; Addgene #51019) using NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621S). Similarly, we generated a donor plasmid with OLLAS tags amplified from pJFRC7 vector (a gift from Gerald Rubin, Howard Hughes Medical Institute; Addgene #51019) using NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621S).

Preparation of adult wings and notums
The right wings from female flies were dehydrated in ethanol and subsequently with xylene. Adult cuticles of the notum were boiled in 2.5 N sodium hydroxide, washed in distilled water, and dehydrated in 2-propanol (Fujise et al., 2001). The specimens were mounted in Canada balsam (Benz Microscope, BB0020).

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