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Dally is not essential for Dpp spreading or internalization but for Dpp stability by antagonizing Tkv-mediated Dpp internalization

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Abstract

Dpp/BMP acts as a morphogen to provide positional information in the *Drosophila* wing disc. Key cell-surface molecules to control Dpp morphogen gradient and signaling are heparan sulfate proteoglycans (HSPGs). In the wing discs, two HSPGs, the glypicans Division abnormally delayed (Dally) and Dally-like (Dlp) have been suggested to act redundantly to control these processes through direct interaction of their heparan sulfate (HS) chains with Dpp. Based on this assumption, a number of models on how glypicans control Dpp gradient formation and signaling have been proposed, including facilitating or hindering Dpp spreading, stabilizing Dpp on the cell surface, or recycling Dpp. However, how distinct HSPGs act remains largely unknown. Here we generate genome-engineering platforms for the two HSPGs and find that only Dally is critical for Dpp gradient formation and signaling through interaction of its core protein with Dpp. However, we find that this interaction is not sufficient and the HS chains of Dally are essential for Dpp gradient formation and signaling, but surprisingly, largely without interacting with Dpp. We provide evidence that the HS chains of Dally are not essential for spreading or recycling of Dpp but for stabilizing Dpp on the cell surface by antagonizing Dpp internalization through Tkv. These results provide new insights into how distinct HSPGs control morphogen gradient formation and signaling during development.

eLife assessment

This **important** study uses genomically-engineered glypican alleles to demonstrate **convincingly** that Dally (not Dally-like protein [Dlp]) is the key contributor to formation of the Dpp/BMP morphogen gradient in the wing disc of *Drosophila*. The authors provide **solid** genetic evidence that, surprisingly, the core domain of Dally appears to suffice to trap Dpp at the cell surface. They conclude with a model according to which Dally modulates the range of Dpp signaling by interfering with Dpp's internalization by the Dpp receptor Thickveins.

Introduction

Morphogens are signaling molecules secreted from localized source cells and control cell fates in a concentration dependent manner during development (Ashe and Briscoe, 2006; Rogers and Schier, 2011; Stapornwongkul and Vincent, 2021; Tabata, 2001). Amongst them, Decapentaplegic (Dpp), the vertebrate BMP2/4 homolog is the first validated secreted morphogen identified in *Drosophila*. Since its discovery, the role of Dpp signaling in the *Drosophila* wing imaginal disc (the larval precursor of the adult wing) has served as an excellent model to investigate how morphogen gradients form and act (Affolter and Basler, 2007; Kicheva and Gonzalez-Gaitan, 2008; Matsuda et al., 2016). In the wing disc, Dpp is expressed in an anterior stripe of cells along the anterior-posterior compartment boundary to control nested target gene expression and growth (Fig. 1A). Dpp binds to the type I receptor Tkv and the type II receptor Punt to phosphorylate the transcription factor Mad. Phosphorylated Mad (pMad) forms a complex with Co-Smad (Medea) to accumulate into the nucleus and activate or inhibit target gene transcription. Interestingly, the majority of Dpp target genes are repressed by a transcriptional repressor Brinker (Brk) while pMad directly represses *brk* expression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Thus, the pMad gradient regulates nested target gene expression mainly through generating an inverse gradient of Brk (Muller et al., 2003) (Fig. 1A). The nested target gene expression patterns are thought to position the future adult wing veins such as L2 and L5 (Cook et al., 2004; Sturtevant et al., 1997) (Fig. 1A). In addition to patterning, Dpp signaling controls growth of the wing through repressing Brk as the strong proliferation defects of *dpp* mutant wing discs can be reversed by removing *brk* (Campbell and Tomlinson, 1999; Schwank et al., 2008).

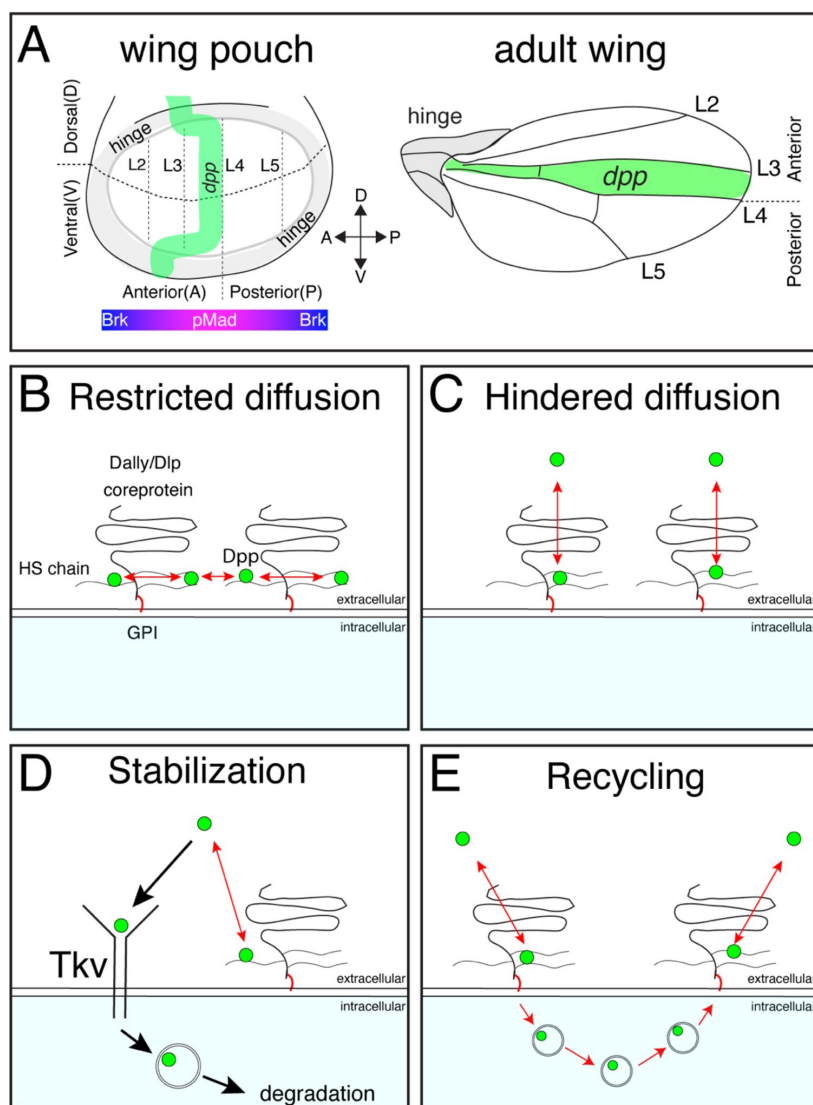


Fig.1

Distinct models on the roles of HSPGs on Dpp/BMP gradient formation

(A) Schematic view of the wing pouch (future wing tissue) and adult wing tissue. Dpp spreads from the anterior stripe of cells along the A-P compartment boundary to generate pMad gradient and an inverse Brk gradient to specify adult wing veins such as L2 and L5. (B) HSPGs are proposed to transport Dpp via repetitive interaction of HS chains with Dpp (Restricted diffusion model). (C) HSPGs are proposed to hinder Dpp spreading via reversible interaction of HS chains with Dpp (Hindered diffusion model). (D) HSPGs are proposed to stabilize Dpp on the cell surface via reversible interaction of HS chains with Dpp that antagonizes Tkv-mediated Dpp internalization. (E) HSPGs are proposed to internalize and recycle Dpp.

While endogenous Dpp is indeed distributed in a graded manner (Matsuda et al., 2021), how the gradient arises is highly controversial (Akiyama and Gibson, 2015; Kicheva and Gonzalez-Gaitan, 2008; Matsuda et al., 2016). A class of molecules that control gradient formation of various morphogens are glypicans, GPI-anchored heparan sulfate proteoglycans (HSPGs) consisting of a core protein and covalently attached heparan sulfate (HS) chains (Lin and Perrimon, 2002; Nybakken and Perrimon, 2002) (Yan and Lin, 2009). In *Drosophila*, two glypicans, Division abnormally delayed (Dally) (Nakato et al., 1995) and Dally-like (Dlp) (Khare and Baumgartner, 2000), are thought to redundantly act as co-receptors (Kuo et al., 2010) to regulate Dpp morphogen gradient formation (Belenkaya et al., 2004; Fujise et al., 2003) and control Dpp gradient scaling to tissue size (Romanova-Michaelides et al., 2015; Zhu et al., 2020).

The role of glypicans in Dpp signaling is dependent on HS chains. A variety of genes required for HS chain biosynthesis such as *sulfateless* (*sfl*) and a family of Ext genes (*sister of tout-velu* (*sotv*), *brother of tout-velu* (*botv*), and *tout-velu* (*ttv*)) (Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004) have been shown to be involved in Dpp distribution and signaling. Given that Dpp binds to heparin (Akiyama et al., 2008), the interaction of the HS chains of glypicans with Dpp is thought to be essential for their function.

Several, not necessarily mutually exclusive, models have been proposed on how glypicans control Dpp morphogen gradient formation. First, glypicans are suggested to be essential for Dpp spreading by handing it over from one cell to the next cell in a “bucket brigade” manner (so-called restricted diffusion model) (Kerszberg and Wolpert, 1998) (Fig. 1B). Consistently, FRAP assays using overexpression of GFP-Dpp revealed a low diffusion coefficient of Dpp ($D=0.1\mu\text{m}^2/\text{s}$) (Kicheva et al., 2007). Indeed, pMad signal and Dpp distribution have been shown to be reduced not only in glypican mutant clones but also in wild type cells located adjacent to the clone and distally to the Dpp source (so-called “shadow” effect), indicating that Dpp spreading requires glypicans (Belenkaya et al., 2004).

Second, and in contrast to the first model, glypicans have been proposed to interfere with Dpp spreading (Muller et al., 2013) (Fig. 1C). The “hindered diffusion” model postulates that Dpp spreads freely and transient interaction of Dpp with cell surface molecules such as glypicans hinders Dpp spreading (Muller et al., 2013).

Third, glypicans have been proposed to stabilize Dpp on the cell surface (Akiyama et al., 2008) (Fig. 1D). In a previous study, Dpp lacking the N-terminal HS binding site was less stable than wild type Dpp (Akiyama et al., 2008), indicating that Dally regulates Dpp stability through direct interactions of HS chains with Dpp. Genetic analyses indicated that Dally competes with Tkv for Dpp binding to antagonize Tkv-mediated Dpp internalization (Akiyama et al., 2008).

Fourth, glypicans are proposed to control recycling and re-secretion of Dpp, which is critical for extracellular Dpp gradient scaling (Romanova-Michaelides et al., 2022), a phenomenon in which Dpp gradient expands to match the growing wing disc size during development (Fig. 1E). Surprisingly, although receptor-mediated endocytosis is thought to be a mechanism to clear morphogens from the extracellular space (Fig. 1D), the same study showed that Dpp internalization was not affected in *tkv* mutant clones, indicating that Dally, but not Tkv, mediates Dpp internalization (Romanova-Michaelides et al., 2022).

Finally, a recent attempt to create a synthetic morphogen system using free diffusing GFP as a morphogen raises alternative possibilities (Stapornwongkul et al., 2020). When *dpp* was replaced with GFP, and anti-GFP nanobodies fused to Tkv and Punt that can activate pMad signal upon GFP binding were introduced, rescue of severe *dpp* mutant phenotypes was not perfect but improved by further introducing GPI-anchored non-signaling receptors (corresponding to glypicans). Supported by mathematical modeling, this study suggests that glypicans may contribute by blocking morphogen leakage and assisting morphogen spreading likely via their GPI-anchors (Stapornwongkul et al., 2020).

Previously, we generated a platform to introduce epitope tags and/or mutations in the *dpp* locus and protein binder tools to directly manipulate endogenous Dpp spreading (Matsuda et al., 2021). By visualizing and manipulating the endogenous Dpp morphogen gradient, we found that Dpp spreading is critical for posterior patterning and growth but largely dispensable for anterior patterning and growth (Matsuda et al., 2021). Although the requirements for Dpp spreading were thus less important than previously thought, endogenous Dpp gradient was easily visualized in the wing discs and Dpp spreading was still critical for posterior patterning and growth (Matsuda et al., 2021). Thus, wing imaginal discs serve as an excellent model to study Dpp morphogen gradient formation at physiological conditions.

In this study, to better understand how glypicans are involved in Dpp morphogen gradient formation and signaling, we generated genome-engineering platforms to manipulate both *Drosophila* glypicans, Dally and Dlp. Although the two glypicans are thought to act redundantly, we first find that only Dally is critical for Dpp gradient formation through interaction of its core protein with Dpp. Surprisingly, we found that, although the HS chains of Dally are essential for Dally’s function, *dpp* mutants that lack the interaction with HS

chains display minor phenotypes, indicating that the interaction of HS chains of Dally with Dpp is largely dispensable for Dpp gradient formation and signaling. We provide evidence that the HS chains of Dally are not essential for Dpp internalization or spreading but rather stabilize Dpp on the cell surface by antagonizing Tkv-mediated internalization of Dpp. These results provide new insights into how glypicans control dispersal and signaling of distinct morphogens during development.

Results

Dally but not Dlp interacts with Dpp

Previous genetic studies suggested that both Dally and Dlp are involved in extracellular Dpp distribution and signaling through the interaction of Dpp with HS chains (Belenkaya *et al.*, 2004). However, the relative contribution of each glypican remained unclear. To address this, we first expressed *dally* or *dlp* in the dorsal compartment of the wing disc using *ap*-Gal4 to compare their ability to interact with endogenous Dpp and activate pMad signal *in vivo*. To visualize endogenous extracellular Dpp distribution, we utilized a functional *Ollas-dpp* allele (Bauer Milena, 2022). Since Dpp distribution and pMad signal are similar between the dorsal and ventral compartments in discs expressing the *Ollas-dpp* allele (Fig. S1), the ventral compartment serves as an internal control in this assay. We found that expression of *dally* in the dorsal compartment increased HS chain levels, and expanded the extracellular Dpp distribution and pMad gradient compared to the control ventral compartment (Fig. 2A, B, E, F). We found that, in contrast to the broad extracellular Ollas-Dpp accumulation, the pMad signal dropped to background levels at the peripheral region (Fig. 2E, F), indicating that Dpp signal activation by Dally is inefficient likely due to sequestration of Dpp by overexpressed Dally. In contrast, expression of *dlp* using *ap*-Gal4 increased HS chain levels but rather reduced extracellular Ollas-Dpp distribution and pMad gradient in the dorsal compartment compared to the control ventral compartment (Fig. 2C, D, G, H), likely because ectopic Dlp expression sequestered HS (Fig. 2C) and blocked HS modification of Dally. Consistent with a previous report (Han *et al.*, 2005), Dlp expression, in the dorsal compartment efficiently expanded extracellular Wg distribution compared to Dally expression (Fig. S2). These results suggest that, although both Dally and Dlp are modified by HS chains, only Dally interacts with Dpp.

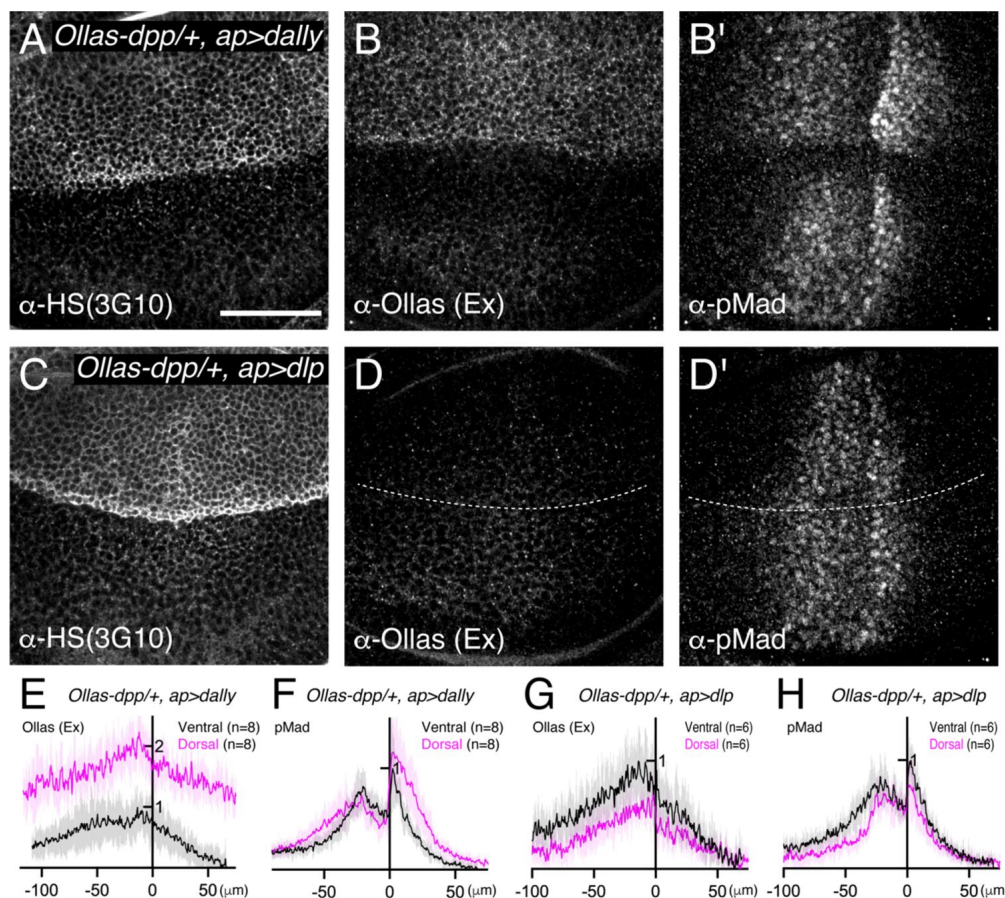


Fig. 2.

Dally, but not Dlp, interacts with Dpp

(A-D) α-HS (3G10) (A, C), extracellular α-Ollas (B, D), and α-pMad (B', D') staining of *Ollas-dpp/+*, *ap>dally* disc (A-B) and *Ollas-dpp/+*, *ap>dlp* disc (C-D). (E-H) Average fluorescence intensity profile of (B, B', D, D') respectively. Data are presented as mean $\pm$ SD. Scale bar: 50 μ m.

Dally, but not Dlp, is required for the Dpp activity gradient

To compare the requirement of Dally and Dlp for Dpp signaling gradient formation, we then generated null mutants for *dally* and *dlp* (*dally*^{KO} and *dlp*^{KO} respectively) by replacing the first exon of each gene by an attP cassette via genome engineering (Fig. S3). Although there is no good anti-Dally antibody, defects in pMad signal in *dally*^{KO} wing discs were similar to that in *dally*^{MH32} (a *dally* null allele (Franch-Marro et al., 2005)) wing discs (Fig. 3A-C, I) and *dally*^{KO} adult wings showed similar phenotypes to *dally*^{MH32} adult wings (Fig. 3E-G, J). Immunostaining for Dlp revealed that Dlp expression is completely lost in *dlp*^{KO} wing disc (Fig. S4). Thus, we confirmed that both *dally*^{KO} and *dlp*^{KO} are null alleles. These null alleles also serve as a platform to insert epitope tags or modified versions of *dally* and *dlp* in the endogenous genomic locus via the attP cassette.

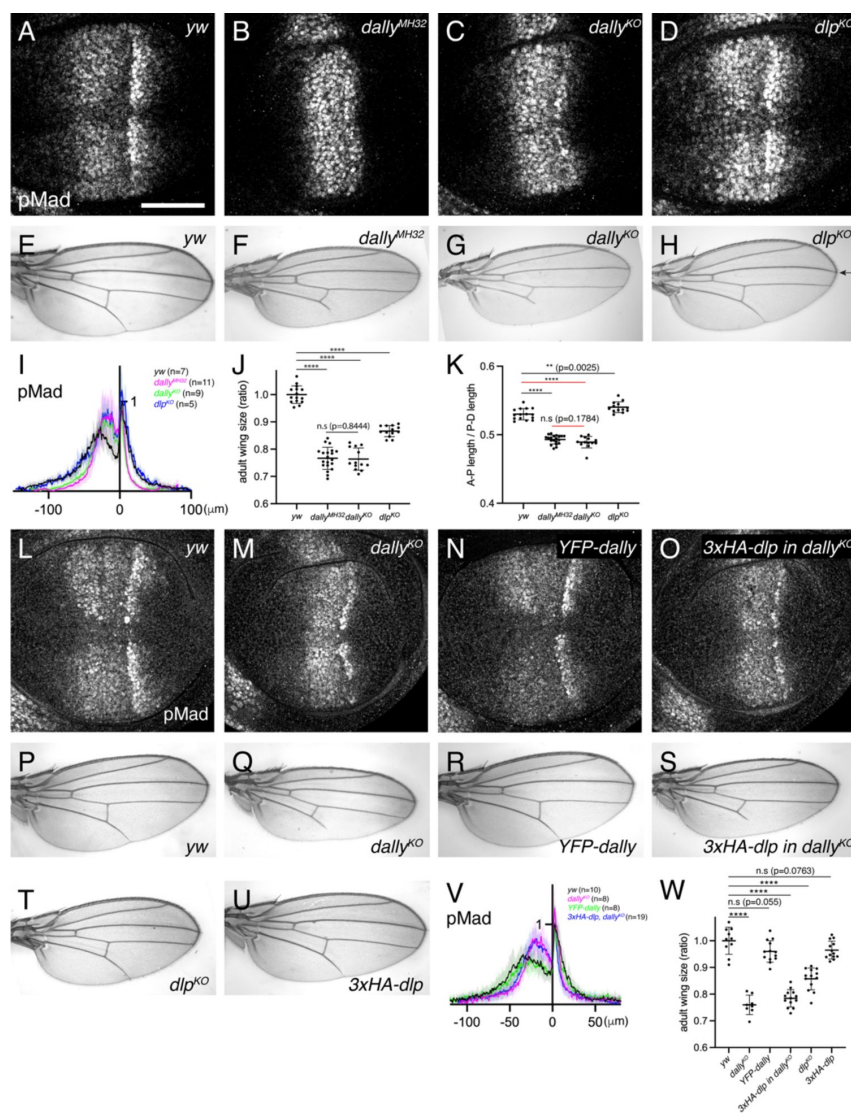


Fig. 3.

Dally, but not Dlp, is required for Dpp signaling gradient

(A–D) α -pMad staining of *yw* (A), *dally*^{MH32} (B), *dally*^{KO} (C), and *dlp*^{KO} (D) wing disc. (E–H) Adult wings of *yw* (E), *dally*^{MH32} (F), *dally*^{KO} (G), and *dlp*^{KO} (H). (I) Average fluorescence intensity profile of (A–D). Data are presented as mean $\pm$ SD. (J) Comparison of adult wing size of *yw* ($n=15$), *dally*^{MH32} ($n=20$), *dally*^{KO} ($n=13$), and *dlp*^{KO} ($n=13$). Data are presented as mean $\pm$ SD. Two-sided unpaired Student's *t* test with unequal variance was used for all the comparison. **** $p < 0.0001$. n.s.; not significant. (K) Comparison of normalized A-P length against D-V length of adult wings of *yw* ($n=15$), *dally*^{MH32} ($n=20$), *dally*^{KO} ($n=13$), and *dlp*^{KO} ($n=13$). Data are presented as mean $\pm$ SD. Two-sided unpaired Student's *t* test with unequal variance was used for comparison under black lines. Two-sided Mann-Whitney test was used for comparison under red lines. **** $p < 0.0001$, ** $p < 0.01$. (L–O) α -pMad staining of *yw* (L), *dally*^{KO} (M), YFP-*dally* (N), and 3xHA-*dlp* in *dally*^{KO} (O) wing disc. (P–U) Adult wings of *yw* (P), *dally*^{KO} (Q), YFP-*dally* (R), 3xHA-*dlp* in *dally*^{KO} (S), *dlp*^{KO} (T), and 3xHA-*dlp* (U). (V) Average fluorescence intensity profile of (L–O).

Data are presented as mean $\pm$ SD. (W) Comparison of adult wing size of *yw* ($n=10$), *dally*^{KO} ($n=8$), YFP-*dally* ($n=13$), 3xHA-*dlp* in *dally*^{KO} ($n=13$), *dlp*^{KO} ($n=13$), and 3xHA-*dlp* ($n=13$). Data are presented as mean $\pm$ SD. Two-sided unpaired Student's *t* test with unequal variance was used for all the comparison. **** $p < 0.0001$. Scale bar: 50 μ m.

We then compared the two null alleles and found that, while severely shrunk in *dally*^{KO} wing discs, the pMad signal was not affected in *dlp*^{KO} wing discs (Fig. 3C, D, I). In rare-occurring mutant adult wings, patterning defects linked to defects in Dpp signaling (truncated L5) were seen only in *dally*^{KO} mutants (Fig. 3G). In contrast, the distance of the distal tips of L3 and L4 veins was reduced in *dlp*^{KO} mutants, which is indicative of Hh signaling defects (see arrows in Fig. 3H). Although both *dally*^{KO} and *dlp*^{KO} adult wings are smaller than controls (Fig. 3J), only *dally*^{KO} adult wings are narrower along the A-P axis, consistent with the role of Dpp along this axis (Fig. 3K).

It has been shown that *dally* and *dlp* display distinct expression patterns in the wing disc since *dally* expression is controlled by Dpp signaling (Fujise *et al.*, 2003) and *dlp* expression is controlled by Wg signaling (Han *et al.*, 2005). Thus, the differential expression pattern of Dally and Dlp may underlie the differential requirements of glypicans in Dpp signaling. To address this, we inserted *dlp* in *dally* locus using the attP site in *dally*^{KO} allele to express *dlp*

in the *dally* expression pattern. As a control, *YFP-dally* inserted in *dally*^{KO} rescued pMad defects (Fig. 3L-N, V) and adult wing defects of *dally*^{KO} mutants (Fig. 3P-R, W). In contrast, *3xHA-dlp* insertion into *dally*^{KO} failed to rescue pMad defects (Fig. 3M, O, V) or adult wing defects in *dally*^{KO} mutants (Fig. 3Q, S, W), although *3xHA-dlp* insertion into *dlp*^{KO} restored adult wing growth in *dlp*^{KO} mutants (Fig. 3T, U, W). Taken together, these results indicate that *dally*, but not *dlp*, is required for Dpp signaling gradient formation.

Interaction of the core protein of Dally with Dpp

How can only Dally be involved in Dpp signaling although both HSPGs are modified with HS chains (Fig. 2A, C)? Dally has been shown to interact with Dpp *in vitro* not only through HS chains but also through the core protein (Kirkpatrick et al., 2006). Thus, we wondered whether the core protein of Dally can interact with Dpp in the wing disc to provide ligand specificity. To test this, we expressed *dally* or *dally*^{ΔHS}, a *dally* mutant lacking all HS chain attachment sites (Kirkpatrick et al., 2006) using *ap*-Gal4 to compare the ability of Dally and Dally^{ΔHS} to interact with Dpp and activate pMad signal. Upon *dally* expression in the dorsal compartment, HS chains, extracellular Dpp and pMad signal increased as compared to the control, ventral compartment (Fig. 4A-C, G, J). In contrast, upon *dally*^{ΔHS} expression, HS chains rather decreased (Fig. 4D), probably because the core protein of Dally sequesters the factors required for synthesis of HS chains. Under this condition, extracellular Dpp still increased but pMad signal rather decreased (Fig. 4E, F, H, K). These results indicate that the core protein of Dally can interact with Dpp; however, this interaction is not sufficient to activate pMad signal but requires the presence of the HS chains.

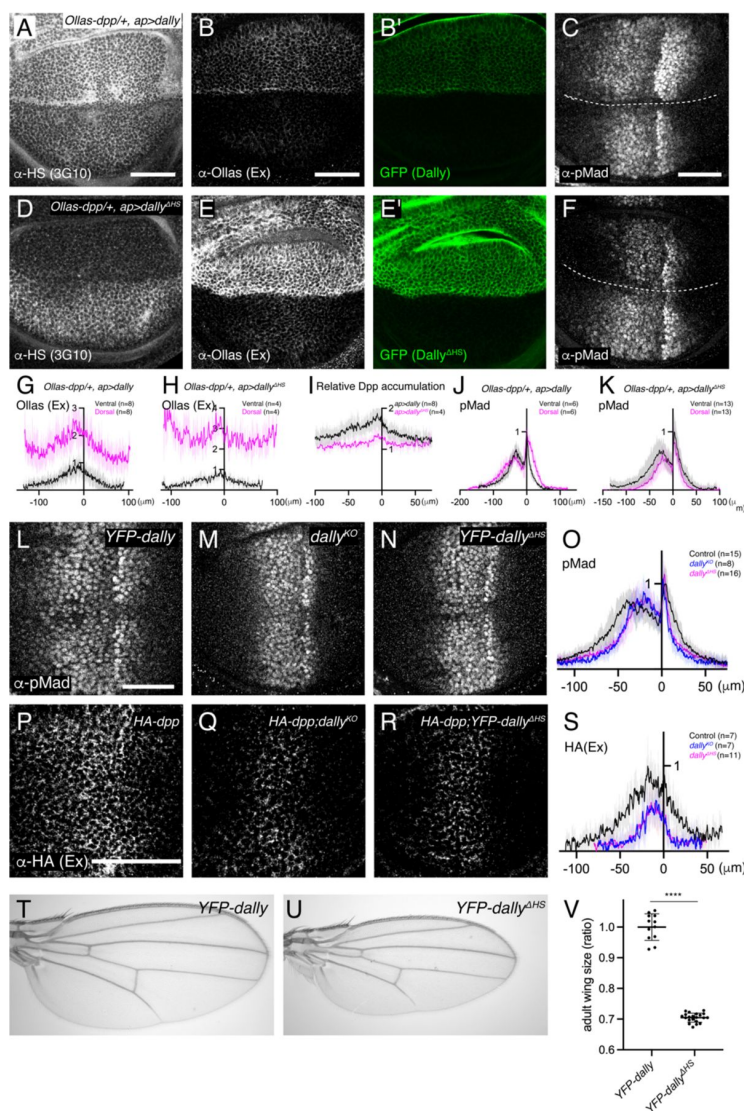


Fig. 4.

Interaction of core protein of Dally with Dpp

(A-F) α-HS (3G10) (A, D), extracellular α-Ollas (B, E), GFP (B', E'), α-pMad (C, F) staining of *Ollas-dpp/+*, *ap>dally* disc (A-C) and *Ollas-dpp/+*, *ap>dally^{ΔHS}* disc (D-F). (G) Average fluorescence intensity profile of (B). (H) Average fluorescence intensity profile of (E). (I) Average fluorescence intensity profile of (B, E) normalized against (B', E') respectively. (J) Average fluorescence intensity profile of (C). (K) Average fluorescence intensity profile of (F). (G-K) Data are presented as mean $\pm$ SD. (L-N) α-pMad staining of *YFP-dally* (L), *dally^{KO}* (M), and *YFP-dally^{ΔHS}* (N) wing disc. (O) Average fluorescence intensity profile of (L-N). Data are presented as mean $\pm$ SD. (P-R) Extracellular α-HA staining of *HA-dpp* (P), *HA-dpp;dally^{KO}* (Q), and *HA-dpp;YFP-dally^{ΔHS}* (R) wing disc. (S) Average fluorescence intensity profile of (P-R). Data are presented as mean $\pm$ SD. (T-U) Adult wings of *YFP-dally* (T) and *YFP-dally^{ΔHS}* (U). (V) Comparison of adult wing size of *YFP-dally* (n=12) and *YFP-dally^{ΔHS}* (n=20). Data are presented as mean $\pm$ SD. Two-sided unpaired Student's t test with unequal variance was used for the comparison. ****p < 0.0001. Scale bar: 50μm.

To address the relative contribution of the interaction of core protein and HS chains of Dally with Dpp, we measured the relative Dpp accumulation against Dally or Dally^{ΔHS} expression (Fig. 4I), since Dally^{ΔHS} expression was higher than Dally expression (Fig. 4B', E'). We found that the relative Dpp accumulation upon *dally^{ΔHS}* expression is only slightly reduced compared to that upon *dally* expression (Fig. 4I), indicating a major contribution of the core protein in the interaction between Dally and Dpp in the wing disc.

HS chains of Dally are critical for Dpp distribution and signaling

The relatively minor contribution of HS chains of Dally for Dpp interaction raises questions on how HS chains of Dally are involved in Dpp distribution and signaling. To address this, we inserted *YFP-dally^{ΔHS}*, a *dally* mutant allele lacking HS chain modification (Kirkpatrick *et al.*, 2006), in the attP site of the *dally^{KO}* allele. We found that both pMad signaling (Fig. 4L-O) and extracellular Dpp distribution (Fig. 4P-S) were severely affected in the *YFP-dally^{ΔHS}* wing discs resembling the phenotypes of *dally^{KO}* mutants. Consistently, the *YFP-dally^{ΔHS}* adult wings mimicked patterning and growth defects of *dally^{KO}* adult wings (Fig. 4T-V). These results show that the interaction of the core protein of Dally with Dpp is not sufficient and

that the HS chains of Dally are critical for Dpp distribution and signaling under physiological conditions.

HS chains of Dally control Dpp distribution and signaling largely independent of interaction with Dpp

How do HS chains of Dally control Dpp distribution and signaling gradient? We first asked whether a direct interaction of the HS chains of Dally and Dpp is critical. A previous study showed that 7 basic amino acids at the N-terminus of the Dpp mature domain are crucial for the interaction with heparin but dispensable for interaction with receptors to activate downstream signaling (Akiyama *et al.*, 2008). To test the importance of this interaction *in vivo*, we generated *dpp^{ΔN}*, a *dpp* mutant allele lacking the basic amino acid stretch. We found that the *dpp^{ΔN}* allele is embryonic lethal, likely because the basic amino acids overlap with a collagen binding site and the Dpp-Collagen IV interaction is important for Dpp gradient formation in the early embryo (Wang *et al.*, 2008). To bypass this embryonic lethality without affecting wing disc development, we utilized a previously reported transgene called JAX, which contains the genomic region of *dpp* (JAX) critical for the early embryogenesis (Hoffmann and Goodman, 1987) but does not rescue the wing phenotypes of *dpp* mutants (Fig. S5). Indeed, the early lethality of homozygous *HA-dpp^{ΔN}* was greatly rescued in the presence of this transgene (*JAX; HA-dpp^{ΔN}*), thus allowing to investigate the requirement of the interaction of Dpp with the HS chains of Dally in later stages. Surprisingly, and in contrast to *dally^{KO}* or *dally^{ΔHS}* flies (Fig. 3, 4), we found that *JAX; HA-dpp^{ΔN}* allele showed only minor defects in Dpp distribution and signaling in wing discs (Fig. 5A-D) and in patterning and growth in adult wings (Fig. 5E-G). Indeed, while *dally^{KO}* and *dally^{ΔHS}* adult wings are 25-30% smaller than control wings (Fig. 3J, W, 4V), *JAX; HA-dpp^{ΔN}* adult wings are only 6.4% smaller than control wings (Fig. 5G).

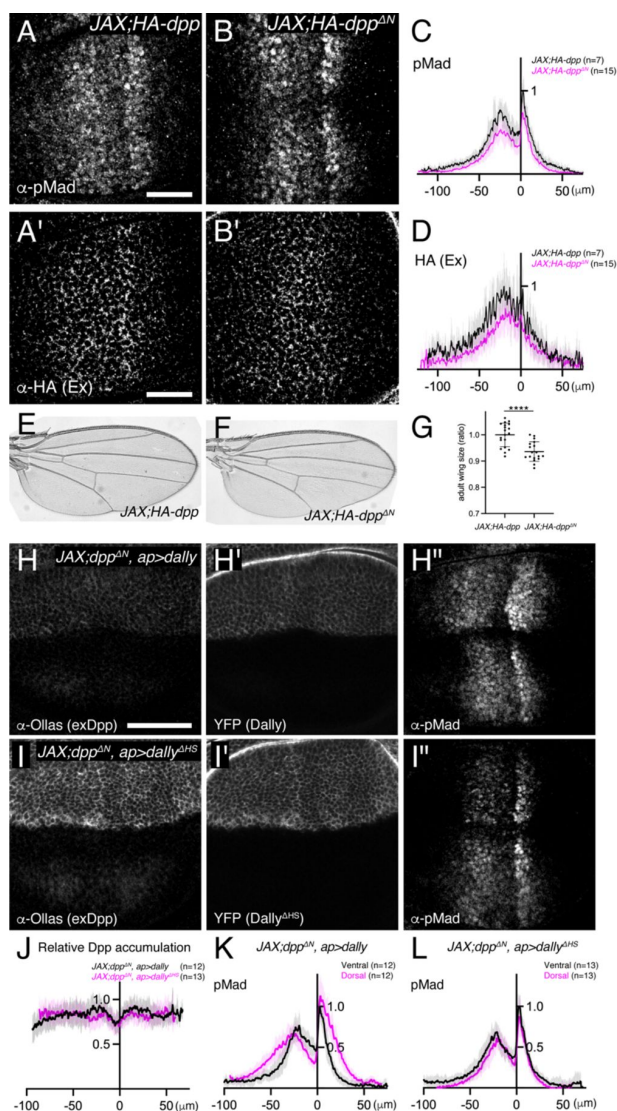


Fig. 5.

HS chains of Dally act largely independent of interaction with Dpp

(A–B) α -pMad (A, B) and extracellular α -HA (A', B') staining of *JAX;HA-dpp* disc (A, A') and *JAX;HA-dpp Δ N* disc (B, B'). (C) Average fluorescence intensity profile of (A, B). Data are presented as mean $\pm$ SD. (D) Average fluorescence intensity profile of (A', B'). Data are presented as mean $\pm$ SD. (E–F) Adult wings of *JAX;HA-dpp* (E) and *JAX;HA-dpp Δ N* (F). (G) Comparison of adult wing size of *JAX;HA-dpp* ($n=19$) and *JAX;HA-dpp Δ N* ($n=18$). Data are presented as mean $\pm$ SD. Two-sided unpaired Student's *t* test with unequal variance was used for the comparison. **** $p < 0.0001$. (H–I) extracellular -Ollas (H, I), YFP (H', I'), and α -pMad (H'', I'') staining of *JAX;HA-dpp Δ N*, *ap>dally* disc (H–H'') and *JAX;HA-dpp Δ N*, *ap>dally Δ HS* disc (I–I''). (J) Average fluorescence intensity profile of (H, I) normalized against (H', I') respectively. (K) Average fluorescence intensity profile of (H''). Data are presented as mean $\pm$ SD. (L) Average fluorescence intensity profile of (I''). Data are presented as mean $\pm$ SD.

The slight but consistent reduction of relative Dpp accumulation upon Dally Δ HS expression compared to Dally expression indicates minor interaction of HS chains of Dally with Dpp (Fig. 4I). In contrast, the relative Dpp Δ N accumulation upon Dally or Dally Δ HS expression in *JAX;dpp Δ N* wing discs was comparable (Fig. 5H–J). This indicates that, consistent with a previous report (Akiyama *et al.*, 2008), Dpp Δ N lacks the ability to interact with HS chains of Dally.

Using the same experimental setup, we then compared the ability of Dally and Dally Δ HS to activate Dpp signal in *JAX;dpp Δ N*, in which HS chains-Dpp interaction is lost (Fig. 5J). We found that Dally expression expanded Dpp signaling in *JAX;dpp Δ N* (Fig. 5H'', K), indicating that Dally can activate Dpp signaling independent of interaction of HS chains of Dally with Dpp. In contrast, Dally Δ HS expression failed to activate Dpp signaling in *JAX;dpp Δ N* (Fig. 5I'', L), indicating that co-receptor function of HS chains of Dally does not require direct interaction with Dpp. Taken together, these results suggest that, although HS chains of Dally are essential for Dpp distribution and signaling, a direct interaction of HS chains of Dally with Dpp is largely dispensable.

HS chains of Dally stabilize Dpp by antagonizing Tkv-mediated Dpp internalization

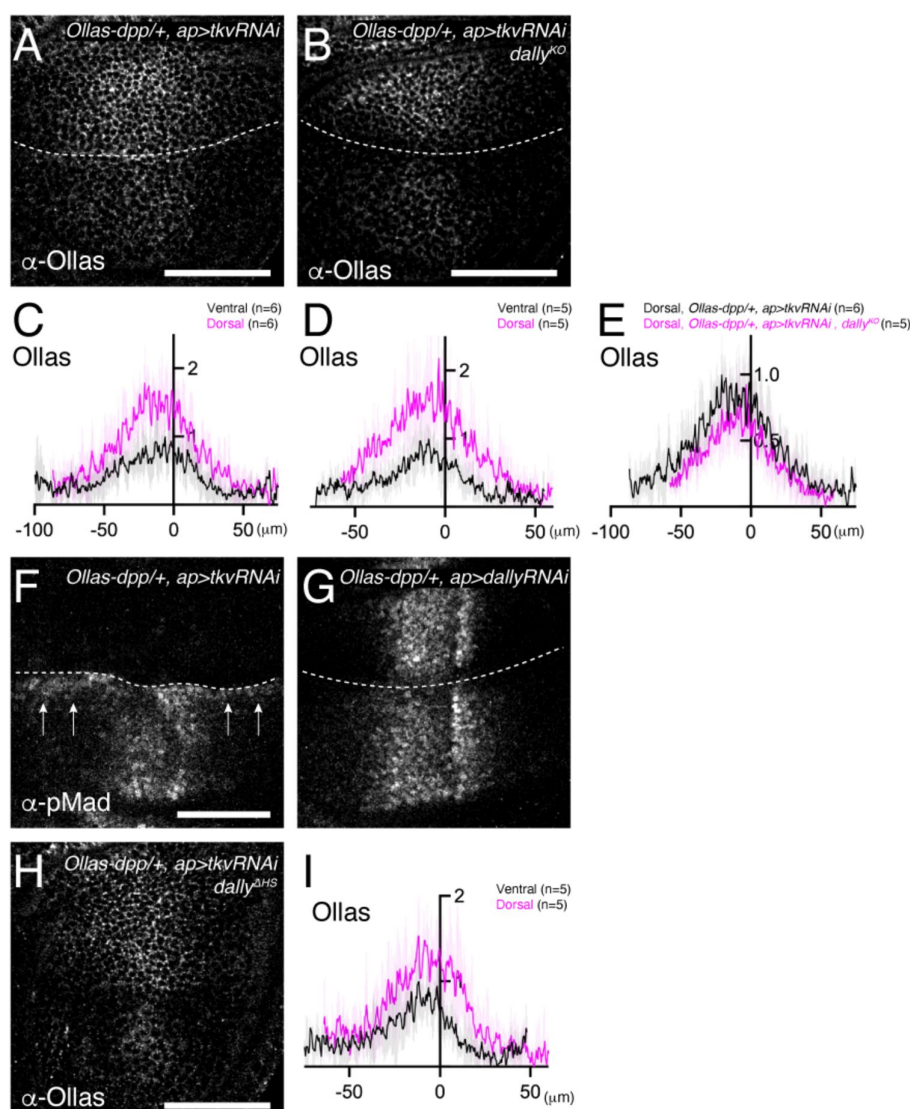
So far, all the models on how glypicans control Dpp distribution and signaling are based on direct interaction of the HS chains with Dpp (Fig. 1). How can the HS chains of Dally control Dpp distribution and signaling without direct interaction with Dpp? Among the models, we wondered whether Tkv is a factor through which HS chains of Dally can act (Fig 1D), although HS chains are thought to act through Dpp in the original model (Akiyama *et al.*, 2008).

We first re-investigated the role of Tkv. According to the model (Akiyama *et al.*, 2008) (Fig. 1D), Tkv is the Dpp receptor on the cell surface that internalizes and degrades Dpp in addition to activating pMad signal. In contrast, a recent study proposed that it is Dally, but not Tkv, that internalizes Dpp (Romanova-Michaelides *et al.*, 2022). To address this discrepancy, we first depleted *tkv* by RNAi using *ap*-Gal4 and visualized endogenous extracellular Dpp distribution. If Tkv is required for Dpp internalization, extracellular Dpp distribution should increase by removing Tkv. Indeed, we found that extracellular Dpp increased and expanded in the dorsal compartment (Fig. 6A, C), indicating that Tkv is required to internalize Dpp.

Fig. 6.

HS chains of Dally stabilize Dpp by antagonizing Tkv-mediated Dpp internalization

(A, B) Extracellular α -Ollas staining of *Ollas-dpp/+*, *ap>tkv RNAi* wing disc (A) and *dally^{KO}*, *Ollas-dpp/+*, *ap>tkv RNAi* wing disc (B). (C, D) Average fluorescence intensity profile of (A, B) respectively. Data are presented as mean $\pm$ SD. (E) Average fluorescence intensity profile of the dorsal compartment of (A, B). Data are presented as mean $\pm$ SD. (F, G) α -pMad staining of *Ollas-dpp/+*, *ap>tkv RNAi* wing disc (F), and *Ollas-dpp/+*, *ap>dally RNAi* wing disc (G). (H) Extracellular α -Ollas staining of *dally^{AHS}*, *Ollas-dpp/+*, *ap>tkv RNAi* wing disc. (I) Average fluorescence intensity profile of (H). Data are presented as mean $\pm$ SD. Scale bar: 50 μ m.



To address whether Dally antagonizes Tkv-mediated Dpp internalization, we asked if the defects in Dpp distribution in *dally* mutants can be reduced by RNAi-mediated depletion of *tkv*. We found that the extracellular Dpp gradient was expanded in the dorsal compartment (Fig. 6B, D), indicating that Dally antagonizes Tkv-mediated Dpp internalization. Expanded Dpp distribution in *dally* mutants upon blocking Dpp internalization also suggest that Dally is largely dispensable for Dpp spreading, and that there are factors other than Tkv and Dally that bind to Dpp on the cell surface. We then asked if Dally contributes to internalization of Dpp by comparing extracellular Dpp levels in the dorsal compartment of *ap>tkv RNAi* wing discs (Fig. 6A) and that of *ap>tkv RNAi, dally^{KO}* wing discs (Fig. 6B), since extracellular Dpp is removed by Tkv in *dally* mutants. We found that extracellular Dpp levels do not increase (Fig. 6E), indicating that Dally is not essential to internalize Dpp.

Interestingly, when *tkv* was depleted in the dorsal compartment, pMad was not only lost from the dorsal compartment but was also upregulated in ventral cells along the dorsal-ventral compartment boundary (arrow, Fig. 6F). This observation is consistent with the idea that, upon blocking internalization, Dpp from the dorsal compartment can reach to the ventral compartment to activate pMad through Tkv binding. In contrast, this phenotype was not seen upon depletion of *dally* by RNAi, although depletion of *dally* in the dorsal compartment mimicked the pMad defects of *dally* mutants (Fig 6G).

Cumulatively, these results suggest that Tkv, but not Dally, is essential to internalize Dpp, and support a model, in which Dally stabilizes Dpp on the cell surface by blocking Tkv-mediated Dpp internalization (Akiyama *et al.*, 2008). While HS chains of Dally are thought to compete with Tkv for binding to Dpp in the original model, the minor phenotypes of *dpp^{ΔN}* (Fig. 4, 5) suggest that HS chains act through a different mechanism. To test if the HS chains of Dally act through Tkv, we depleted *tkv* by RNAi using *ap-Gal4* in *dally^{ΔHS}* mutants. We found that extracellular Dpp gradient expanded in the dorsal compartment (Fig. 6H, I), indicating that the HS chains of Dally antagonize Tkv-mediated Dpp internalization through Tkv.

Discussion

HSPGs are essential for Dpp/BMP morphogen gradient formation and signaling in the *Drosophila* wing disc. Although a variety of models have been proposed, it remains largely unknown how distinct HSPGs control Dpp gradient formation and signaling. In this study, we generated and utilized genome engineering platforms for two HSPGs, *dally* and *dlp*, and *dpp* (Matsuda *et al.*, 2021) to address this question.

Mechanism of Dally function

It has been thought that Dally and Dlp act redundantly to control Dpp signaling. However, we demonstrate here that Dally, but not Dlp, is critical for Dpp distribution and signaling through interaction of the core protein of Dally with Dpp (Fig. 2-4). We do not know if the interaction is direct but previous surface plasmon resonance (SPR) and co-immunoprecipitation experiments suggest that this is the case (Kirkpatrick *et al.*, 2006). It has recently been shown that a specific structure, present in the core protein of Dlp but absent in Dally, shields the lipid moiety of Wg and is important for its spreading (McGough *et al.*, 2020). Thus, each glypican appears to acquire ligand specificity through its core protein. In contrast to Wg, Dpp has no lipid modification and is diffusible by nature. Indeed, the expanded Dpp distribution in *dally* mutants upon blocking Dpp internalization (Fig. 6) does not support the idea that Dally is required for Dpp spreading itself (Belenkaya *et al.*, 2004; Schwank *et al.*, 2011; Stapornwongkul *et al.*, 2020) (Fig. 1B) or that Dally and Tkv are essential for blocking Dpp leakage from the wing epithelia (Stapornwongkul *et al.*, 2020). These results

also suggest that there are other cell surface molecules that bind to Dpp on the cell surface. Furthermore, the expanded extracellular Dpp distribution upon *dally* expression (Fig. 2) suggests that the ability of Dally to hinder Dpp spreading is, if any, mild (Fig. 1C).

Our results thus suggest that Dally is not critical for Dpp spreading and that interaction of Dally with Dpp occurs downstream of Dpp spreading. Despite the interaction of the core protein of Dally with Dpp upon Dally overexpression, this interaction is not sufficient to control Dpp distribution and Dpp signaling at its physiological level, while HS chains of Dally are absolutely essential for both processes (Fig. 4). Since increase of HS chains was not sufficient for interaction with Dpp (Fig. 2C, D), we speculate that HS chains of Dally act downstream of the interaction of the core protein of Dally with Dpp.

Among the proposed models (Fig. 1), our results support a model, in which HS chains of Dally stabilize Dpp on the cell surface by antagonizing Tkv-mediated internalization of Dpp (Akiyama *et al.*, 2008) (Fig. 1D, 7A). Consistently, pulse-chase experiments showed that Dpp is less stable in *dally* mutants (Akiyama *et al.*, 2008). However, while the original model suggests that HS chains compete with Tkv for Dpp binding (Akiyama *et al.*, 2008) (Fig. 1D, 7A), our results suggest that HS chains act largely through Tkv based on two observations (Fig. 7B). First, although the *dally*^{ΔHS} allele showed severe defects similar to the *dally*^{KO} allele, the defects of the *dpp*^{ΔN} allele were surprisingly mild despite the lack of interaction with HS chains (Fig. 4, 5), indicating that HS chains act largely through other molecules. Second, defects in Dpp distribution in *dally*^{ΔHS} were rescued by removing *tkv* (Fig. 6). These results indicate that HS chains act through Tkv rather than through Dpp to stabilize Dpp on the cell surface (Fig. 7C-E).

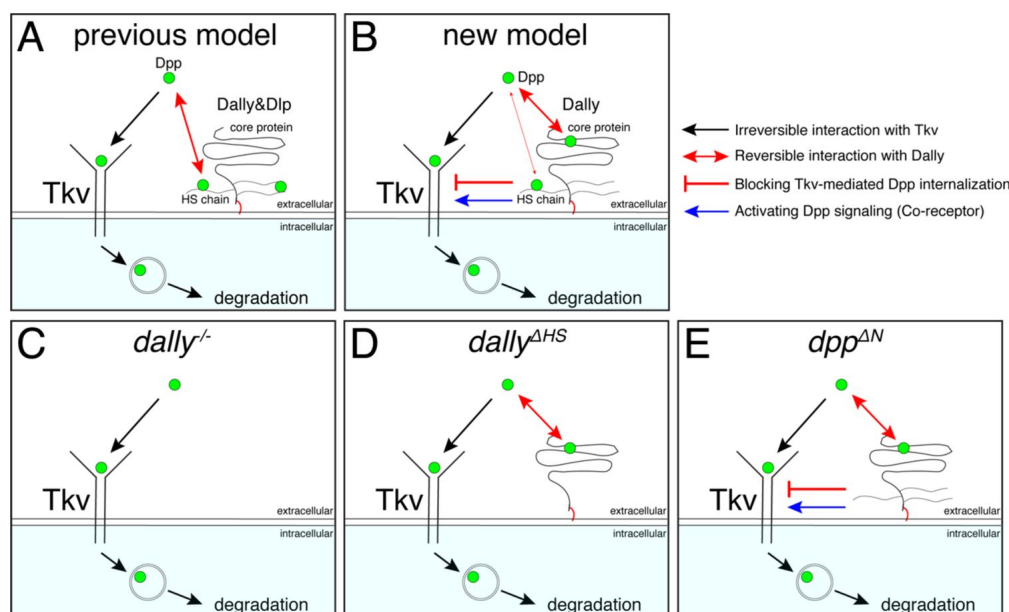


Fig. 7.

A revised model on the roles of Dally on Dpp/BMP gradient formation and signaling

(A) A previous model. Dally and Dlp competes with Tkv for Dpp binding to antagonize Tkv-mediated Dpp internalization. (B) A revised model. Dally, but not Dlp, reversibly interacts with Dpp partially through its HS chains and mainly through its core protein. The former interaction is not essential and the latter

interaction is not sufficient to antagonize Tkv-mediated Dpp internalization under physiological conditions. Upon interaction of Dpp with the core protein of Dally, HS chains of Dally antagonize Tkv-mediated Dpp internalization through Tkv to stabilize Dpp on the cell surface. HS chains of Dally control Dpp signaling also independent of interaction with Dpp likely through Tkv. It remains unknown whether and how Dpp signal activation by core protein bound Dpp and stability by HS chains are coordinated. (C) In *dally*^{-/-} mutants, Tkv-mediated Dpp internalization by Tkv is not antagonized. Dpp is irreversibly bound to Tkv and then removed by Tkv from the extracellular space and degraded. (D) In *dally*^{AHS} mutants, the core protein of Dally can interact with Dpp but this interaction is not sufficient to antagonize Tkv-mediated Dpp internalization. Without HS chains, Dpp is irreversibly bound to Tkv and then removed by Tkv from the extracellular space and degraded. (E) In *dpp*^{ΔN} mutants, Dpp can interact with core protein but not with HS chains of Dally. Without interaction of HS chains with Dpp, HS chains can antagonize Tkv-mediated Dpp internalization through Tkv to stabilize Dpp on the cell surface.

It remains unknown how HS chains of Dally stabilize Dpp through Tkv. Since heparin has been shown to bind to BMP receptors (Kanzaki et al., 2008), the HS chains of Dally may directly interact with Tkv to stabilize Dpp on the cell surface. Interestingly, we found that the HS chains of Dally control Dpp signaling also largely independent of interaction with Dpp (Fig. 5). The simplest possibility is that the HS chains of Dally control both Dpp stability and signaling through Tkv (Fig. 7), but it is equally possible that the HS chains of Dally control Dpp signaling through other factors. In the future, it will be also important to address how the interaction of the core protein of Dally with Dpp and functions of HS chains through Tkv are coordinated.

Mechanisms of Dpp internalization

Our proposed model is based on the result that Dpp is internalized by Tkv (Fig. 6), consistent with a widely accepted idea that extracellular morphogens are removed by their receptors. However, this result contradicts a recent report proposing that Dally, but not Tkv, internalizes and recycles Dpp (Romanova-Michaelides et al., 2015). How can we explain the discrepancy?

If Tkv is involved in internalization of Dpp, internalized Dpp should decrease upon loss of *tkv*. Romanova-Michaelides et al. applied a nanobody internalization assay to visualize

internalized Dpp (Romanova-Michaelides *et al.*, 2015). In this assay, extracellular GFP-Dpp was first labeled using fluorophore conjugated anti-GFP nanobodies under endocytosis-restricted condition. After allowing internalization of the labelled GFP-Dpp, extracellular anti-GFP nanobodies were then stripped away by acid wash to follow only the internalized Dpp. Using this assay, Romanova-Michaelides *et al.* surprisingly found that internalization of GFP-Dpp was not affected in *tkv* mutant clones. However, the experiment was performed under overexpression of GFP-Dpp from the anterior stripe of cells, in which the authors estimate 400 times higher expression than the physiological level. Under such unphysiological conditions, Dpp internalization through other Dpp binding factors can easily mask the impact of Tkv on Dpp internalization. Consistently, we found that Dpp binds to the cell surface without Tkv and Dally (Fig. 6).

If Tkv is involved in Dpp internalization, extracellular Dpp should increase. Romanova-Michaelides *et al.* used the observation that extracellular GFP-Dpp did not increase in *tkv* mutant clones upon GFP-Dpp expression from the anterior stripe of cells (Schwank *et al.*, 2011) to further support Tkv-independent Dpp internalization (Romanova-Michaelides *et al.*, 2015). However, under 400 times higher expression levels of GFP-Dpp than the physiological level, it is not surprising that an increase of extracellular Dpp can be missed. Indeed, we find that endogenous extracellular Dpp is increased upon *tkv* depletion, indicating that Dpp is internalized through Tkv (Fig. 6). Notably, even when GFP-Dpp was overexpressed, accumulation of GFP-Dpp on the surfaces of cells lacking Tkv have been previously reported (Entchev *et al.*, 2000).

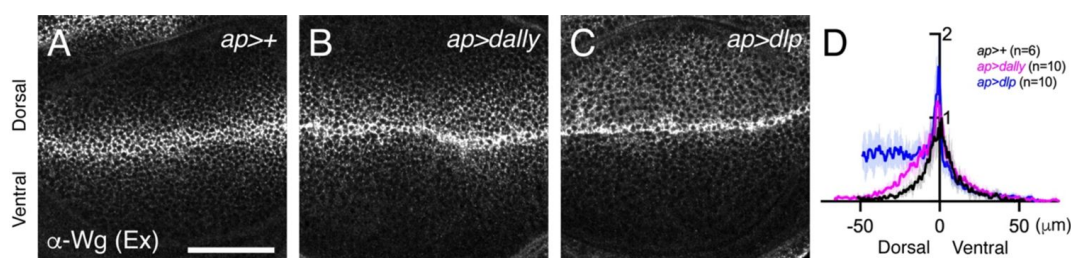
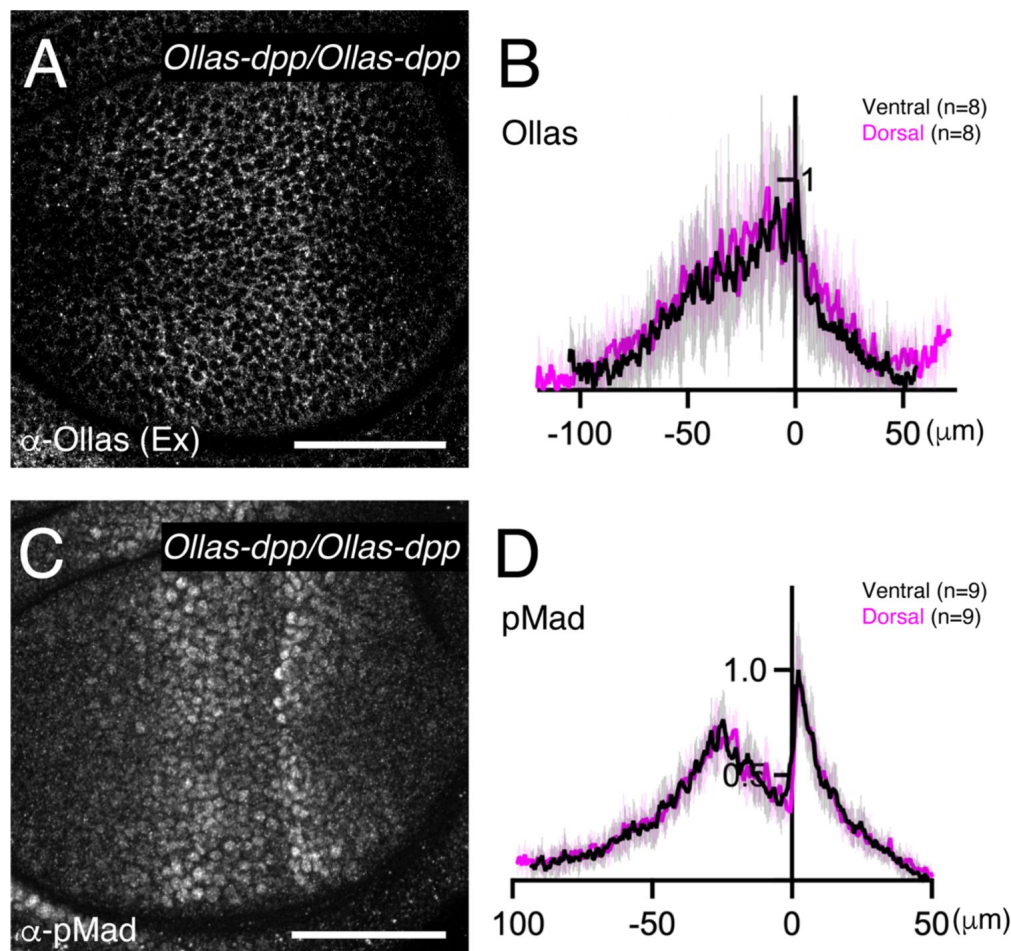
Romanova-Michaelides *et al.* proposed that, instead of Tkv, Dally is required for internalization of Dpp based on reduced internalization of endogenous GFP-Dpp upon blocking HS chain synthesis or upon cleaving HSPGs using the nanobody internalization assay (Romanova-Michaelides *et al.*, 2015). In contrast, our results suggest that Dally rather antagonizes internalization of Dpp by blocking Tkv-mediated internalization of Dpp (Fig. 6). How can Dpp internalization be reduced in *dally* mutants if Dally antagonizes internalization of Dpp? We found that extracellular Dpp distribution is severely reduced in *dally* mutants due to Tkv-mediated internalization of Dpp (Fig. 6), indicating that the amount of extracellular Dpp available for labeling with nanobody is reduced in *dally* mutants. Thus, we speculate that the reduced internalization of Dpp in *dally* mutants revealed by the nanobody internalization assay reflects the reduced extracellular Dpp rather than reduced ability to internalize Dpp.

Our results do not thus support Dally-mediated internalization and recycling of Dpp as a mechanism of Dpp gradient scaling, (Romanova-Michaelides *et al.*, 2015), although recycling of Dpp through other factors may contribute to Dpp gradient scaling. It would be interesting to test if the Dally-mediated Dpp stabilization on the cell surface is involved in the Dpp gradient scaling. Consistent with this, decrease of Dpp degradation rates has previously been proposed as a mechanism for Dpp gradient scaling (Wartlick *et al.*, 2011). Furthermore, previous pulse-chase experiments showed that, similar to Dally, Pentagone (Pent), a secreted feedback factor required for Dpp gradient scaling, is required for Dpp stability (Vuilleumier *et al.*, 2010). Interestingly, Pent has been shown to interact with HS chains of Dally (Vuilleumier *et al.*, 2010) (Norman *et al.*, 2016), raising a possibility that interaction of HS chains of Dally with Pent is critical for Dpp stability, thereby Dpp gradient scaling.

Summary

To recognize and activate the specific signal, the affinity of a morphogen with its receptors is high. However, the high affinity of the morphogen-receptor interaction could at the same time compromise the range of morphogens through receptor-mediated removal of morphogens from the extracellular space. Thus, there must exist mechanisms to ensure a long-range action of the morphogen despite the high affinity of the morphogen with its

receptors. Our results suggest that Dally counteracts the internalization of Dpp through Tkv to increase the chance that Dpp can reach further despite the high affinity of Dpp with Tkv.



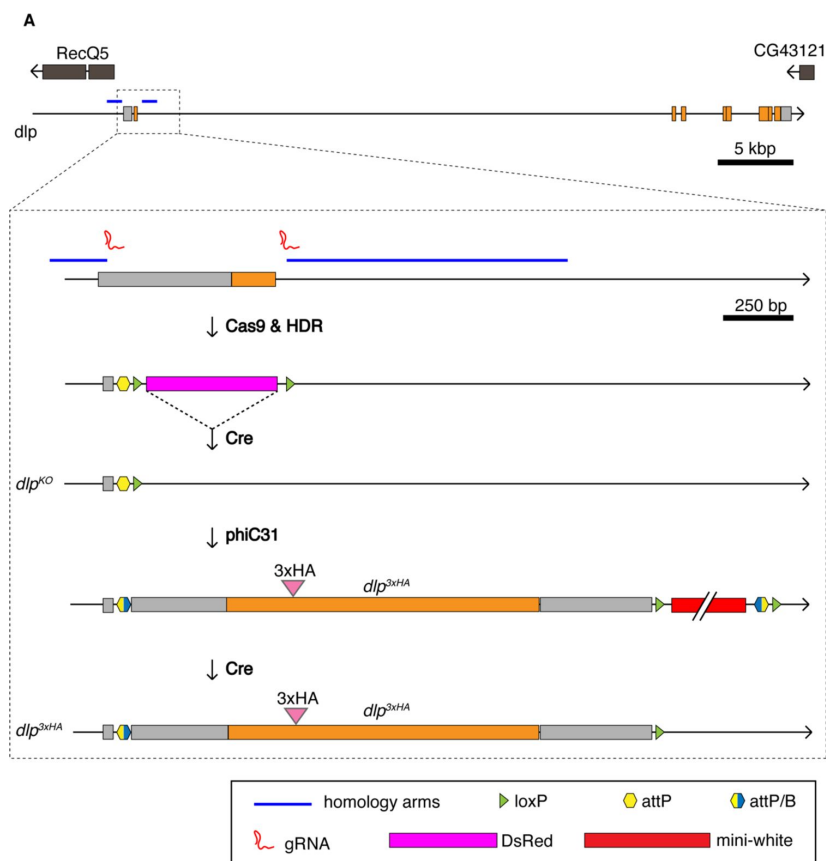


Fig. S3

Generation of *dally*^{KO} and *dlp*^{KO}

(A) Schematic view of manipulation of the glypican genomic loci exemplified with *dlp*. (B) the exact sequences at the lesion of *dally*^{KO} and *dlp*^{KO}: color code as in (A) (blue: genomic sequences at the inner boundaries of homology arms for HDR, yellow: attP, green: loxP, black: “scars” (remnants of vector sequences including cloning sites)).

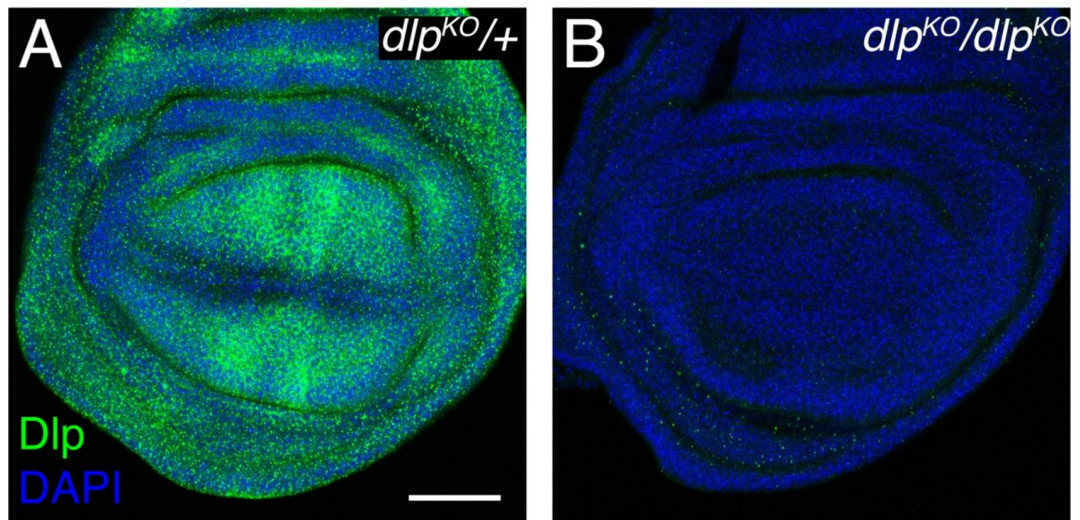


Fig. S4

Validation of *dlp*^{KO} allele

(A, B) α -Dlp and DAPI staining of *dlp*^{KO/+} (A) and *dlp*^{KO/dlp}^{KO} (B).

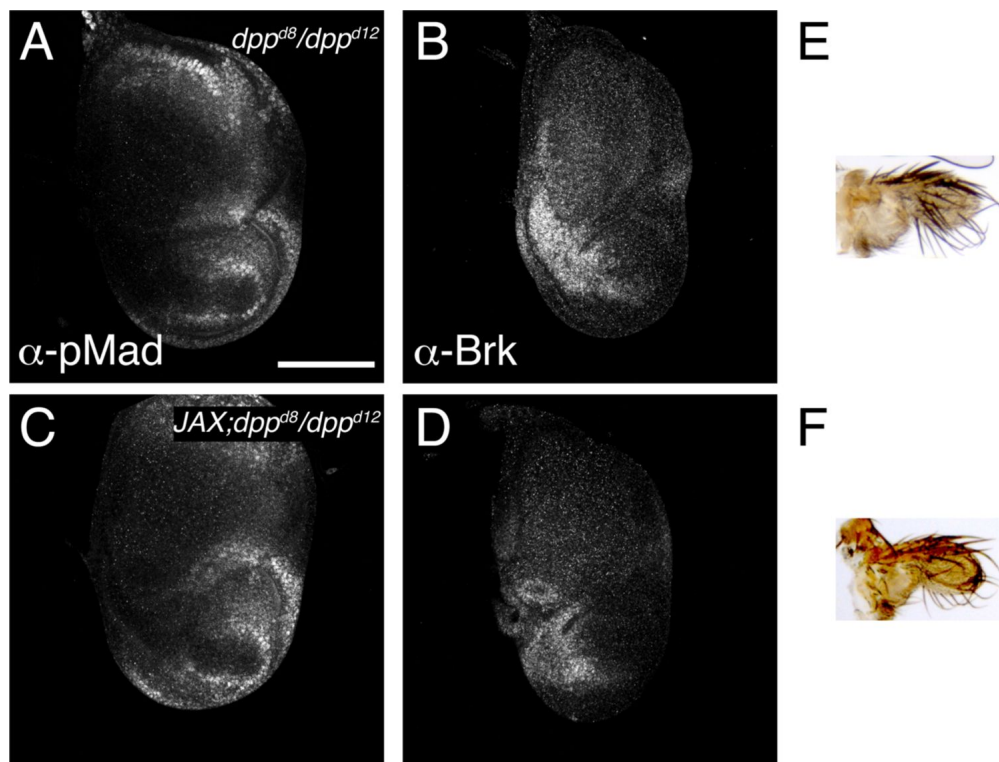


Fig. S5

JAX does not rescue *dpp* disc allele

(A-D) α -pMad staining (A, C) and α -Brk staining (B, D) of *dpp*^{d8/dpp}^{d12} (A, B) and *JAX;dpp*^{d8/dpp}^{d12} (C, D). (E, F) Adult wings of *dpp*^{d8/dpp}^{d12} (E) and *JAX;dpp*^{d8/dpp}^{d12} (F).

Materials and Methods

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Fly stocks

Flies for experiments were kept at 25°C in standard fly vials containing polenta and yeast. The following fly lines were used: *UAS-tkv-RNAi* (Bloomington 40937), *UAS-dally-RNAi* (VDRC 14136), *Ollas-dpp* (Bauer Milena, 2022), *UAS-GFP-HA-dally* (Suzanne Eaton), *UAS-dlp* (Baeg et al., 2001), *HA-dpp* (Matsuda et al., 2021), *HA-dpp^{ΔN}* (this study), *dlp [ko;attB]* (this study), *dally[ko;attB]* (this study), *dally[YFP-dally;attP]* (this study), *dlp[3xHA-dlp;attP]* (this study) *dally[YFP-dally^{ΔHS};attP]* (this study), *dally[3xHA-dlp;attP]* (this study), *ap-Gal4* (Markus Affolter), *JAX* (Hoffmann and Goodman, 1987), *Ollas-HA-dpp^{ΔN}* (this study), *hh-Gal4* (Gift from Dr Manolo Calleja), *dally^{MH32}* (Franch-Marro et al., 2005). *yw*, *dpp^{d8}* and *dpp^{d12}* are described from Flybase.

Genotypes by figures

Fig. 2A, B; <i>ap-Gal4, Ollas-dpp/+ ; UAS-GFP-HA-dally/+</i>
Fig. 2C, D; <i>ap-Gal4, Ollas-dpp/+ ; UAS-dlp/+</i>
Fig. 3A, E; <i>yw</i> (control)
Fig. 3B, F; <i>dally^{MH32}/dally^{MH32}</i>
Fig. 3C, G; <i>dally[KO;attB]/dally[KO;attB]</i>
Fig. 3D, H; <i>dlp[KO;attB]/dlp[KO;attB]</i>
Fig. 3L, P; <i>yw</i> (control)
Fig. 3M, Q; <i>dally[KO;attB]/dally[KO;attB]</i>
Fig. 3N, R; <i>dally[YFP-dally;attP]/dally[YFP-dally;attP]</i>
Fig. 3O, S; <i>dally[3xHA-dlp;attP]/dally[3xHA-dlp;attP]</i>
Fig. 3T; <i>dlp[KO;attB]/dlp[KO;attB]</i>
Fig. 3U; <i>dlp[3xHA-dlp;attP]/dlp[3xHA-dlp;attP]</i>
Fig. 4A-C; <i>ap-Gal4, Ollas-dpp/+ ; UAS-GFP-HA-dally/+</i>
Fig. 4D-F; <i>ap-Gal4, Ollas-dpp/+ ; UAS-GFP-dally^{ΔHS}</i>
Fig. 4L; <i>dally[YFP-dally;attP]/dally[YFP-dally;attP]</i>
Fig. 4M; <i>dally[KO;attB]/dally[KO;attB]</i>
Fig. 4N; <i>dally[YFP-dally^{ΔHS};attP]/dally[YFP-dally^{ΔHS};attP]</i>
Fig. 4P; <i>HA-dpp/HA-dpp</i>
Fig. 4Q; <i>HA-dpp/HA-dpp;dally[KO;attB]/dally[KO;attB]</i>
Fig. 4R; <i>HA-dpp/HA-dpp;dally[YFP-dally^{ΔHS};attP]w+/dally[YFP-dally^{ΔHS};attP]w+</i>
Fig. 4T; <i>dally[YFP-dally;attP]/dally[YFP-dally;attP]</i>
Fig. 4U; <i>dally[YFP-dally^{ΔHS};attP]/dally[YFP-dally^{ΔHS};attP]</i>
Fig. 5A; <i>JAX;HA-dpp/HA-dpp</i>
Fig. 5B; <i>JAX;HA-dpp^{ΔN}/HA-dpp^{ΔN}</i>
Fig. 5H; <i>JAX; ap-Gal4,Ollas-HA-dpp^{ΔN}/HA-dpp^{ΔN};UAS-GFP-HA-dally/+</i>
Fig. 5I; <i>JAX; ap-Gal4,Ollas-HA-dpp^{ΔN}/HA-dpp^{ΔN};UAS-GFP-dally^{ΔHS}/+</i>
Fig. 6A; <i>ap-Gal4, Ollas-dpp/UAS-tkvRNAi</i>
Fig. 6B; <i>ap-Gal4, Ollas-dpp/UAS-tkvRNAi;dally[KO;attB]/dally[KO;attB]</i>
Fig. 6F; <i>ap-Gal4, Ollas-dpp/UAS-tkvRNAi</i>
Fig. 6G; <i>ap-Gal4, Ollas-dpp/+; UAS-dallyRNAi/+</i>
Fig. 6H; <i>ap-Gal4, Ollas-dpp/UAS-tkvRNAi;dally[YFP-dally^{ΔHS};attP]w+/dally[YFP-dally^{ΔHS};attP]w+</i>
Fig. S1A, C; <i>Ollas-dpp/Ollas-dpp</i>
Fig. S2A; <i>ap-Gal4, Ollas-dpp/+</i>
Fig. S2B; <i>ap-Gal4, Ollas-dpp/+; UAS-GFP-HA-dally/+</i>
Fig. S2C; <i>ap-Gal4, Ollas-dpp/+; UAS-dlp/+</i>
Fig. S4A; <i>dlp[KO;attB]/+</i>
Fig. S4B; <i>dlp[KO;attB]/dlp[KO;attB]</i>
Fig. S5A, B, E; <i>dpp^{d8}/dpp^{d12}</i>
Fig. S5C, D, F; <i>JAX; dpp^{d8}/dpp^{d12}</i>

Generation of transgenic flies and alleles

HA-dpp^{ΔN} plasmid was constructed by GENEWIZ by removing 21bp encoding the 7 basic amino acids (RRPTRRK) from *HA-dpp* plasmid (Matsuda *et al.*, 2021).

Ollas-HA-dpp^{ΔN} plasmid was constructed by using the *HA-dpp^{ΔN}* plasmid and the *Ollas-HA-dpp* plasmid as templates. Both plasmids were digested using XhoI and BspEI as restriction enzymes. The small fragment from the *HA-dpp^{ΔN}* plasmid and the large fragment from the *Ollas-HA-dpp* plasmid were used for ligation. Generation of endogenous *dpp* alleles using these plasmids were previously described (Matsuda *et al.*, 2021).

Genomic manipulation of glypicans

The *dally^{KO}* and *dlp^{KO}* alleles were generated by replacing parts of the first exon of the genes with attP cassettes. In both cases, the deleted fragment comprise part of the 5' UTR, and the complete coding sequence, including the start codon and the signal peptide encoding sequence. For the generation of *dally^{KO}* homology arms flanking the target region were amplified by genomic PCR using primer pairs *dally_5'_for* (ATAGCTAGCCTCTAAAGACTCAAATTAATTAATTCATTTCAGATTGCGC) and *dally_5'_rev* (ATACATATGTTTTGATGGGTGATTTCTGTGTGCAGACACAGTG), or *dally_3'_for* (TATACTAGTGTAAGTTCACGCCATCCATCCGTAGAGTTATAATATCG) and *dally_3'_rev* (ATAGGCGCGCCTTTCACCTCAATTACACGAAACAGATATATATTGGGTACATTCCG) and cloned into the 5' MCS and 3' MCS of vector pTV(Cherry) using restriction enzymes NheI and NdeI or SpeI and AscI, respectively (restriction sites underlined). The construct was subjected to P-element transgenesis and was subsequently mobilized and linearized by crossing transgenic flies to flies carrying hsFLP and hsSceI and applying heat-shock as described (Baena-Lopez *et al.*, 2013).

The *dlp^{KO}* allele was generated by Cas9/CRISPR mediated homologous repair. Guide RNA sequences flanking the target region were GAAGCAATTGAAGTGCAACA and AATATCGGACATACCGTTAC and were cloned into plasmid pCFD3:U6:3. The two homology arms for homologous-directed repair were amplified by genomic PCR using primer pairs *dlp_5'_for* (TTTTCTCGAGCATGCTGGGCATCGACACATACACATCC) and *dlp_5'_rev* (TTTTGCGGCCGCATGGTTGTCGGGTGTTATTAAATCGG), or *dlp_3'_for* (TTTTTCTAGATGTCCGATATTATATACCAATGGC) and *dlp_3'_rev* (TTTTCTCGAGCCAACGATGCTCTACTGTATGC) and cloned into vector pHD-DsRed-attP using restriction enzymes SphI and NotI or SpeI and XhoI, respectively. Homologous directed repair was achieved by co-injecting gRNA plasmids and the repair donor as described (Gratz *et al.*, 2014).

For both *dally^{KO}* and *dlp^{KO}*, successful genomic integrations were identified by the presence of eye markers (mini-white and dsRed, respectively) followed by Cre-mediated excision of marker cassettes. The integrity of the deletion and attP integration was verified by genomic PCR and subsequent sequencing. The exact sequences at the manipulated loci are depicted in Fig. S3. Dally and Dlp genomic constructs were generated in the reintegration vector pRIVwhite and incorporated into the attP site of the *dally^{KO}* and *dlp^{KO}* alleles by phiC31/attB integration (Baena-Lopez *et al.*, 2013). The YFP sequence in Dally constructs was inserted after lysine 90. Dally^{ΔHS} contains serine to alanine amino acid substitutions at positions 549, 569, 573 and 597. Three copies of the influenza hemagglutinin derived HA epitope were inserted after glycine 69 of Dlp.

Immunohistochemistry

Total staining

Wing discs from third instar larvae were dissected and stored temporarily in Phosphate Buffered Saline (PBS) (Gibco) on ice until enough samples were collected. The discs were then fixed in 4% Paraformaldehyde (PFA) in PBS for 30min on the shaker at room temperature (25°C). After fixation, the discs were rinsed three times quickly with PBS and three times for 15 min with PBS at 4°C. Wing discs were permeabilized in PBST (0.3% Triton-X in PBS) and then blocked in 5% normal goat serum (NGS) in PBST for at least 30min. Primary antibodies were added in 5% normal goat serum (NGS) in PBST for incubation over night at 4°C. The next day, the primary antibodies were carefully removed and the samples were rinsed three times quickly in PBST and three times 15 min at room temperature in PBST. Discs were incubated in secondary antibody for 2h at room temperature. Afterwards the samples were washed again three times quickly and three times 15 min in PBST at room temperature. After the final washing the PBST was rinsed with PBS, then the PBS was removed completely and the samples were mounted in VECTORSHIELD on glass slides. For HS stainings, wing discs were dissected as described above. After fixation, discs were washed in PBS and blocked in 5% NGS in PBST, then treated with Heparinase III (Sigma-Aldrich) for 1.5h at 37°C. Afterwards the discs were blocked again in 5% NGS in PBST and stained as described above.

Extracellular staining

Wing discs from third instar larvae were dissected and stored temporarily in Schneider's Drosophila medium (S2) on ice until enough samples were collected. The discs were then blocked in cold 5% NGS in S2 medium on ice for 10min. The blocking solution was removed carefully and the primary antibody was added for 1h on ice. During the 1h incubation period, the tubes were tapped carefully every 15min, to make sure the antibody is distributed evenly. After 1h incubation on ice, the antibody was removed and the samples were washed at least 6 times with cold S2 medium and another two times with cold PBS to remove excess primary antibody. Wing discs were then fixed with 4% PFA in PBS for 30min on the shaker at room temperature (25°C). After fixation the protocol continued as described in total stainings.

Antibodies

Primary antibodies: rabbit-anti-phospho-Smad1/5 (41D10, Cell Signaling, #9516; 1:200), mouse-anti-Wg (4D4, DSHB, University of Iowa; total staining: 1:120, extracellular staining: 1:120), mouse-anti-Ptc (DSHB, University of Iowa; total staining: 1:40), rat-anti-HA (3F10, Roche, 11867423001; total staining: 1:300, extracellular staining: 1:20), rat-anti-Ollas (L2, Novus Biologicals, NBP1-06713; total staining: 1:300, extracellular staining: 1:20), mouse-anti-HS (F69-3G10, amshio; 1:100)

The following secondary antibodies were used at 1:500 dilutions in this study. Goat anti-rabbit IgG (H+L) Alexa Fluor™ 488 (A11008 Thermo Fischer), goat anti-rabbit IgG (H+L) Alexa Fluor™ 568 (A11011 Thermo Fischer), goat anti-rabbit IgG (H+L) Alexa Fluor™ 680 (A21109 Thermo Fischer), goat anti-rat IgG (H+L) Alexa Fluor™ 568 (A11077 Thermo Fischer), goat anti-mouse IgG (H+L) Alexa Fluor™ 568 (A11004 Thermo Fischer), goat anti-rat IgG Fc 488 (ab97089 abcam), goat anti-mouse IgG Fc Alexa Fluor™ 680 (115625071 Jackson Immuno Research).

Imaging

Samples were imaged using a Leica SP5-II-MATRIX confocal microscope and Leica LAS AF and Images were analyzed using ImageJ. Figures were obtained using Omero and Adobe Illustrator.

Quantification of pMad and extracellular staining

From each z-stack image, signal intensity profile along A/P axis was extracted from average projection of three sequential images using ImageJ (v.2.0.0-rc69/1.52p). Each signal intensity profile collected in Excel (Ver. 16.51) was aligned along A/P compartment boundary (based on anti-Ptc staining or pMad staining) and average signal intensity profile from different samples was generated and plotted by the script (wing_disc-alignment.py). The average intensity profile from control and experimental samples was then compared by the script (wingdisc_comparison.py). Both scripts can be obtained from https://etiennes.github.io/Wing_disc-alignment/. The resulting signal intensity profiles (mean with SD) were generated by Prism (v.8.4.3(471)). Figures were prepared using Omero (ver5.9.1) and Illustrator (24.1.3).

Quantification of adult wing size

The size of each compartment was measured using ImageJ (v.2.0.0-rc69/1.52p) and collected in Excel (Ver. 16.51). Scatter dot plots (mean with SD) were generated by Prism (v.8.4.3(471)). Figures were prepared using Omero (ver5.9.1) and Illustrator (24.1.3).

Statistics

All images were obtained from multiple animals ($n > 3$). The experiments were repeated at least two times independently with similar results. Statistical significance was assessed by Prism (v.8.4.3(471)) based on the normality tests. The observed phenotypes were highly reproducible as indicated by the significance of p values obtained by statistical tests.

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References

Affolter M., Basler K. (2007) **The Decapentaplegic morphogen gradient: from pattern formation to growth regulation** *Nature reviews.Genetics* **8**:663–674
<https://doi.org/10.1038/nrg2166>

Akiyama T. , Gibson M.C. (2015) **Morphogen transport: theoretical and experimental controversies** *Wiley interdisciplinary reviews. Developmental biology* **4**:99–112
<https://doi.org/10.1002/wdev.167>

Akiyama T. , Kamimura K. , Firkus C. , Takeo S. , Shimmi O. , Nakato H. (2008) **Dally regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface** *Dev Biol* **313**:408–419
<https://doi.org/10.1016/j.ydbio.2007.10.035>

Ashe H.L. , Briscoe J. (2006) **The interpretation of morphogen gradients** *Development (Cambridge, England)* **133**:385–394

Baena-Lopez L.A. , Alexandre C. , Mitchell A. , Pasakarnis L. , Vincent J.P. (2013) **Accelerated homologous recombination and subsequent genome modification in Drosophila** *Development* **140**:4818–4825
<https://doi.org/10.1242/dev.100933>

Bauer Milena A.G. , Kristi Wharton , Shinya Matsuda , Markus Affolter (2022) **Heterodimerization-dependent secretion of BMPs in Drosophila**
<https://doi.org/10.1101/2022.08.04.502599>

Belenkaya T.Y. , Han C. , Yan D. , Opoka R.J. , Khodoun M. , Liu H. , Lin X. (2004) **Drosophila Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans** *Cell* **119**:231–244
<https://doi.org/10.1016/j.cell.2004.09.031>

Bornemann D.J. , Duncan J.E. , Staatz W. , Selleck S. , Warrior R. (2004) **Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways** *Development* **131**:1927–1938
<https://doi.org/10.1242/dev.01061>

Campbell G. , Tomlinson A. (1999) **Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker** *Cell* **96**:553–562
[https://doi.org/10.1016/S0092-8674\(00\)80659-5](https://doi.org/10.1016/S0092-8674(00)80659-5)

Cook O. , Biehs B. , Bier E. (2004) **brinker and optomotor-blind act coordinately to initiate development of the L5 wing vein primordium in Drosophila** *Development* **131**:2113–2124
<https://doi.org/10.1242/dev.01100>

Entchev E.V. , Schwabedissen A. , Gonzalez-Gaitan M. (2000) **Gradient formation of the TGF-beta homolog Dpp** *Cell* **103**:981–991
[https://doi.org/10.1016/S0092-8674\(00\)00200-2](https://doi.org/10.1016/S0092-8674(00)00200-2)

Franch-Marro X. , Marchand O. , Piddini E. , Ricardo S. , Alexandre C. , Vincent J.P. (2005) **Glypicans shunt the Wingless signal between local signalling and further transport** *Development* **132**:659–666
<https://doi.org/10.1242/dev.01639>

Fujise M. , Takeo S. , Kamimura K. , Matsuo T. , Aigaki T. , Izumi S. , Nakato H. (2003) **Dally regulates Dpp morphogen gradient formation in the Drosophila wing** *Development* **130**:1515–1522
<https://doi.org/10.1242/dev.00379>

Gratz S.J. , Ukken F.P. , Rubinstein C.D. , Thiede G. , Donohue L.K. , Cummings A.M. , O'Connor-Giles K.M. (2014) **Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in Drosophila** *Genetics* **196**:961–971
<https://doi.org/10.1534/genetics.113.160713>

Han C. , Belenkaya T.Y. , Khodoun M. , Tauchi M. , Lin X. , Lin X. (2004) **Distinct and collaborative roles of Drosophila EXT family proteins in morphogen signalling and gradient formation** *Development* **131**:1563–1575
<https://doi.org/10.1242/dev.01051>

Han C. , Yan D. , Belenkaya T.Y. , Lin X. (2005) **Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc** *Development* **132**:667–679
<https://doi.org/10.1242/dev.01636>

Hoffmann F.M. , Goodman W. (1987) **Identification in transgenic animals of the Drosophila decapentaplegic sequences required for embryonic dorsal pattern formation** *Genes Dev* **1**:615–625
<https://doi.org/10.1101/gad.1.6.615>

Jazwinska A. , Rushlow C. , Roth S. (1999) **The role of brinker in mediating the graded response to Dpp in early Drosophila embryos** *Development* **126**:3323–3334
<https://doi.org/10.1242/dev.126.15.3323>

Kanzaki S. , Takahashi T. , Kanno T. , Ariyoshi W. , Shinmyouzu K. , Tujisawa T. , Nishihara T. (2008) **Heparin inhibits BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMP receptor** *J Cell Physiol* **216**:844–850
<https://doi.org/10.1002/jcp.21468>

Kerszberg M. , Wolpert L. (1998) **Mechanisms for positional signalling by morphogen transport: a theoretical study** *J Theor Biol* **191**:103–114
<https://doi.org/10.1006/jtbi.1997.0575>

Khare N. , Baumgartner S. (2000) **Dally-like protein, a new Drosophila glypican with expression overlapping with wingless** *Mech Dev* **99**:199–202
[https://doi.org/10.1016/s0925-4773\(00\)00502-5](https://doi.org/10.1016/s0925-4773(00)00502-5)

Kicheva A. , Gonzalez-Gaitan M. (2008) **The Decapentaplegic morphogen gradient: a precise definition** *Current opinion in cell biology* **20**:137–143
<https://doi.org/10.1016/j.ceb.2008.01.008>

Kicheva A. , Pantazis P. , Bollenbach T. , Kalaidzidis Y. , Bittig T. , Julicher F. , Gonzalez-Gaitan M. (2007) **Kinetics of morphogen gradient formation** *Science* **315**:521–525
<https://doi.org/10.1126/science.1135774>

Kirkpatrick C.A. , Knox S.M. , Staatz W.D. , Fox B. , Lercher D.M. , Selleck S.B. (2006) **The function of a *Drosophila* glypican does not depend entirely on heparan sulfate modification** *Dev Biol* **300**:570–582
<https://doi.org/10.1016/j.ydbio.2006.09.011>

Kuo W.J. , Digman M.A. , Lander A.D. (2010) **Heparan sulfate acts as a bone morphogenetic protein coreceptor by facilitating ligand-induced receptor hetero-oligomerization** *Mol Biol Cell* **21**:4028–4041
<https://doi.org/10.1091/mbc.E10-04-0348>

Lin X. , Perrimon N. (2002) **Developmental roles of heparan sulfate proteoglycans in *Drosophila*** *Glycoconj J* **19**:363–368
<https://doi.org/10.1023/A:1025329323438>

Matsuda S. , Harmansa S. , Affolter M. (2016) **BMP morphogen gradients in flies** *Cytokine & growth factor reviews* **27**:119–127
<https://doi.org/10.1016/j.cytogfr.2015.11.003>

Matsuda S. , Schaefer J.V. , Mii Y. , Hori Y. , Bieli D. , Taira M. , Pluckthun A. , Affolter M. (2021) **Asymmetric requirement of Dpp/BMP morphogen dispersal in the *Drosophila* wing disc** *Nat Commun* **12**:6435
<https://doi.org/10.1038/s41467-021-26726-6>

McGough I.J. , Vecchia L. , Bishop B. , Malinauskas T. , Beckett K. , Joshi D. , O'Reilly N. , Siebold C. , Jones E.Y. , Vincent J.P. (2020) **Glypicans shield the Wnt lipid moiety to enable signalling at a distance** *Nature* **585**:85–90
<https://doi.org/10.1038/s41586-020-2498-z>

Minami M. , Kinoshita N. , Kamoshida Y. , Tanimoto H. , Tabata T. (1999) **brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes** *Nature* **398**:242–246
<https://doi.org/10.1038/18451>

Muller B. , Hartmann B. , Pyrowolakis G. , Affolter M. , Basler K. (2003) **Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient** *Cell* **113**:221–233
[https://doi.org/10.1016/s0092-8674\(03\)00241-1](https://doi.org/10.1016/s0092-8674(03)00241-1)

Muller P. , Rogers K.W. , Yu S.R. , Brand M. , Schier A.F. (2013) **Morphogen transport** *Development* **140**:1621–1638
<https://doi.org/10.1242/dev.083519>

Nakato H. , Futch T.A. , Selleck S.B. (1995) **The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*** *Development* **121**:3687–3702
<https://doi.org/10.1242/dev.121.11.3687>

Norman M. , Vuilleumier R. , Springhorn A. , Gawlik J. , Pyrowolakis G. (2016) **Pentagone internalises glypicans to fine-tune multiple signalling pathways** *eLife* **5**:
<https://doi.org/10.7554/eLife.13301>

- Nybakken K. , Perrimon N. (2002) **Heparan sulfate proteoglycan modulation of developmental signaling in Drosophila** *Biochim Biophys Acta* **1573**:280–291
[https://doi.org/10.1016/s0304-4165\(02\)00395-1](https://doi.org/10.1016/s0304-4165(02)00395-1)
- Rogers K.W. , Schier A.F. (2011) **Morphogen gradients: from generation to interpretation** *Annual Review of Cell and Developmental Biology* **27**:377–407
<https://doi.org/10.1146/annurev-cellbio-092910-154148>
- Romanova-Michaelides M. , Aguilar-Hidalgo D. , Julicher F. , Gonzalez-Gaitan M. (2015) **The wing and the eye: a parsimonious theory for scaling and growth control** *Wiley interdisciplinary reviews. Developmental biology* **4**:591–608
<https://doi.org/10.1002/wdev.195>
- Romanova-Michaelides M. , Hadjivasiliou Z. , Aguilar-Hidalgo D. , Basagiannis D. , Seum C. , Dubois M. , Julicher F. , Gonzalez-Gaitan M. (2022) **Morphogen gradient scaling by recycling of intracellular Dpp** *Nature* **602**:287–293
<https://doi.org/10.1038/s41586-021-04346-w>
- Schwank G. , Dalessi S. , Yang S.F. , Yagi R. , de Lachapelle A.M. , Affolter M. , Bergmann S. , Basler K. (2011) **Formation of the long range Dpp morphogen gradient** *PLoS Biol* **9**:
<https://doi.org/10.1371/journal.pbio.1001110><https://doi.org/10.1371/journal.pbio.1001111>
- Schwank G. , Restrepo S. , Basler K. (2008) **Growth regulation by Dpp: an essential role for Brinker and a non-essential role for graded signaling levels** *Development (Cambridge, England)* **135**:4003–4013
<https://doi.org/10.1242/dev.025635><https://doi.org/10.1242/dev.025635>
- Stapornwongkul K.S. , de Gennes M. , Cocconi L. , Salbreux G. , Vincent J.P. (2020) **Patterning and growth control in vivo by an engineered GFP gradient** *Science* **370**:321–327
<https://doi.org/10.1126/science.abb8205>
- Stapornwongkul K.S. , Vincent J.P. (2021) **Generation of extracellular morphogen gradients: the case for diffusion** *Nat Rev Genet* **22**:393–411
<https://doi.org/10.1038/s41576-021-00342-y>
- Sturtevant M.A. , Biehs B. , Marin E. , Bier E. (1997) **The spalt gene links the A/P compartment boundary to a linear adult structure in the Drosophila wing** *Development* **124**:21–32
<https://doi.org/10.1242/dev.124.1.21>
- Tabata T. (2001) **Genetics of morphogen gradients** *Nature reviews. Genetics* **2**:620–630
<https://doi.org/10.1038/35084577>
- Takei Y. , Ozawa Y. , Sato M. , Watanabe A. , Tabata T. (2004) **Three Drosophila EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans** *Development* **131**:73–82
<https://doi.org/10.1242/dev.00913>

Vuilleumier R. , Springhorn A. , Patterson L. , Koidl S. , Hammerschmidt M. , Affolter M. , Pyrowolakis G. (2010) **Control of Dpp morphogen signalling by a secreted feedback regulator** *Nat Cell Biol* **12**:611–617
<https://doi.org/10.1038/ncb2064>

Wang X. , Harris R.E. , Bayston L.J. , Ashe H.L. (2008) **Type IV collagens regulate BMP signalling in Drosophila** *Nature* **455**:72–77
<https://doi.org/10.1038/nature07214>

Wartlick O. , Mumcu P. , Kicheva A. , Bittig T. , Seum C. , Julicher F. , Gonzalez-Gaitan M. (2011) **Dynamics of Dpp signaling and proliferation control** *Science* 1154–1159
<https://doi.org/10.1126/science.1200037>

Yan D. , Lin X. (2009) **Shaping morphogen gradients by proteoglycans** *Cold Spring Harb Perspect Biol* **1**:
<https://doi.org/10.1101/cshperspect.a002493>

Zhu Y. , Qiu Y. , Chen W. , Nie Q. , Lander A.D. (2020) **Scaling a Dpp Morphogen Gradient through Feedback Control of Receptors and Co-receptors** *Developmental cell* **53**:724–739

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Reviewer #1 (Public Review):

How morphogens spread within tissues remains an important question in developmental biology. Here the authors revisit the role of glypicans in the formation of the Dpp gradient in wing imaginal discs of *Drosophila*. They first use sophisticated genome engineering to demonstrate that the two glypicans of *Drosophila* are not equivalent despite being redundant for viability. They show that Dally is the relevant glypican for Dpp gradient formation. They then provide genetic evidence that, surprisingly, the core domain of Dally suffices to trap Dpp at the cell surface (suggesting a minor role for GAGs). They conclude with a model that Dally modulates the range of Dpp signaling by interfering with Dpp's degradation by Tkv. These are important conclusions, but more independent (biochemical/cell biological) evidence is needed.

As indicated above, the genetic evidence for the predominant role of Dally in Dpp protein/signalling gradient formation is strong. In passing, the authors could discuss why overexpressed Dlp has a negative effect on signaling, especially in the anterior compartment. The authors then move on to determine the role of GAG (=HS) chains of Dally. They find that in an overexpression assay, Dally lacking GAGs traps Dpp at the cell surface and, counterintuitively, suppresses signaling (fig 4 C, F). Both findings are unexpected and therefore require further validation and clarification, as outlined in a and b below.

a) In loss of function experiments (dallyDeltaHS replacing endogenous dally), Dpp protein is markedly reduced (fig 4R), as much as in the KO (panel Q), suggesting that GAG chains do contribute to trapping Dpp at the cell surface. This is all the more significant that, according to the overexpression essays, DallyDeltaHS seems more stable than WT Dally (by the way, this difference should also be assessed in the knock-ins, which is possible since they are YFP-tagged). The authors acknowledge that HS chains of Dally are critical for Dpp distribution (and signaling) under physiological conditions. If this is true, one can wonder why overexpressed dally core 'binds' Dpp and whether this is a physiologically relevant activity.

b) Although the authors' inference that dallycore (at least if overexpressed) can bind Dpp. This assertion needs independent validation by a biochemical assay, ideally with surface plasmon resonance or similar so that an affinity can be estimated. I understand that this will

require a method that is outside the authors' core expertise but there is no reason why they could not approach a collaborator for such a common technique. In vitro binding data is, in my view, essential.

In a subsequent set of experiments, the authors assess the activity of a form of Dpp that is expected not to bind GAGs (DppDeltaN). Overexpression assays show that this protein is trapped by DallyWT but not dallyDeltaHS. This is a good first step validation of the deltaN mutation, although, as before, an invitro binding assay would be preferable. Nevertheless, the authors show that DppDeltaN is surprisingly active in a knock-in strain. At face value (assuming that DeltaN fully abrogates binding to GAGs), this suggests that interaction of Dpp with the GAG chains of Dally is not required for signaling activity. This leads to authors to suggest (as shown in their final model) that GAG chains could be involved in mediating the interactions of Dally with Tkv (and not with Dpp. This is an interesting idea, which would need to be reconciled with the observation that the distribution of Dpp is affected in dallyDeltaHS knock-ins (item a above). It would also be strengthened by biochemical data (although more technically challenging than the experiments suggested above).

In an attempt to determine the role of Dally (GAGs in particular) in the signaling gradient, the paper next addresses its relation to Tkv. They first show that reducing Tkv leads to Dpp accumulation at the cell surface, a clear indication that Tkv normally contributes to the degradation of Dpp. From this they suggest that Tkv could be required for Dpp internalisation although this is not shown directly. The authors then show that a Dpp gradient still forms upon double knockdown (Dally and Tkv). This intriguing observation shows that Dally is not strictly required for the spread of Dpp, an important conclusion that is compatible with early work by Lander suggesting that Dpp spreads by free diffusion. These result show that Dally is required for gradient formation only when Tkv is present. They suggest therefore that Dally prevents Tkv-mediated internalisation of Dpp. Although this is a reasonable inference, internalisation assays (e.g. with anti-Ollas or anti-HA Ab) would strengthen the authors' conclusions especially because they contradict a recent paper from the Gonzalez-Gaitan lab.

The paper ends with a model suggesting that HS chains have a dual function of suppressing Tkv internalisation and stimulating signaling. This constitutes a novel view of a glypican's mode of action and possibly an important contribution of this paper. As indicated above, further experiments could considerably strengthen the conclusion. Speculation on how the authors imagine that GAG chains have these activities would also be warranted.

Reviewer #2 (Public Review):

The authors are trying to distinguish between four models of the role of glypicans (HSPGs) on the Dpp/BMP gradient in the Drosophila wing, schematized in Fig. 1: (1) "Restricted diffusion" (HSPGs transport Dpp via repetitive interaction of HS chains with Dpp); (2) "Hindered diffusion" (HSPGs hinder Dpp spreading via reversible interaction of HS chains with Dpp); (3) "Stabilization" (HSPGs stabilize Dpp on the cell surface via reversible interaction of HS chains with Dpp that antagonizes Tkv-mediated Dpp internalization); and (4) "Recycling" (HSPGs internalize and recycle Dpp).

To distinguish between these models, the authors generate new alleles for the glypicans Dally and Dally-like protein (Dlp) and for Dpp: a Dally knock-out allele, a Dally YFP-tagged allele, a Dally knock-out allele with 3HA-Dlp, a Dlp knock-out allele, a Dlp allele containing 3-HA tags, and a Dpp lacking the HS-interacting domain. Additionally, they use an OLLAS-tag Dpp (OLLAS being an epitope tag against which extremely high affinity antibodies exist). They examine OLLAS-Dpp or HA-Dpp distribution, phospho-Mad staining, adult wing size.

They find that over-expressed Dally - but not Dlp - expands Dpp distribution in the larval wing disc. They find that the Dally[KO] allele behaves like a Dally strong hypomorph Dally[MH32]. The Dally[KO] - but not the Dlp[KO] - caused reduced pMad in both anterior and posterior domains and reduced adult wing size (particularly in the Anterior-Posterior axis). These defects can be substantially corrected by supplying an endogenously tagged YFP-tagged Dally. By contrast, they were not rescued when a 3xHA Dlp was inserted in the Dally locus. These results support their conclusion that Dpp interacts with Dally but not Dlp.

They next wanted to determine the relative contributions of the Dally core or the HS chains to the Dpp distribution. To test this, they over-expressed UAS-Dally or UAS-Dally[deltaHS] (lacking the HS chains) in the dorsal wing. Dally[deltaHS] over-expression increased the distribution of OLLAS-Dpp but caused a reduction in pMad. Then they write that after they normalize for expression levels, they find that Dally[deltaHS] only mildly reduces pMad and this result indicates a major contribution of the Dally core protein to Dpp stability. The "normalization" is a key part of this model and is not mentioned how the normalization was done. When they do the critical experiment, making the Dally[deltaHS] allele, they find that loss of the HS chains is nearly as severe as total loss of Dally (i.e., Dally[KO]). Additionally, experimental approaches are needed here to prove the role of the Dally core.

Prior work has shown that a stretch of 7 amino acids in the Dpp N-terminal domain is required to interact with heparin but not with Dpp receptors (Akiyama, 2008). The authors generated an HA-tagged Dpp allele lacking these residues (HA-dpp[deltaN]). It is an embryonic lethal allele, but they can get some animals to survive to larval stages if they also supply a transgene called "JAX" containing dpp regulatory sequences. In the JAX; HA-dpp[deltaN] mutant background, they find that the distribution and signaling of this Dpp molecule is largely normal. While over-expressed Dally can increase the distribution of HA-dpp[deltaN], over-expression of Dally[deltaHS] cannot. These latter results support the model that the HS chains in Dally are required for Dpp function but not because of a direct interaction with Dpp.

In the last part of the results, they attempt to determine if the Dpp receptor Thickveins (Tkv) is required for Dally-HS chains interaction. The 2008 (Akiyama) model posits that Tkv activates pMad downstream of Dpp and also internalizes and degrades Dpp. A 2022 (Romanova-Michaelides) model proposes that Dally (not Tkv) internalizes Dpp.

To distinguish between these models, the authors deplete Tkv from the dorsal compartment of the wing disc and found that extracellular Dpp increased and expanded in that domain. These results support the model that Tkv is required to internalize Dpp. They then tested the model that Dally antagonizes Tkv-mediated Dpp internalization by determining whether the defective extracellular Dpp distribution in Dally[KO] mutants could be rescued by depleting Tkv. Extracellular Dpp did increase in the D vs V compartment, potentially providing some support for their model. However, there are no statistics performed, which is needed for full confidence in the results. The lack of statistics is particularly problematic (1) when they state that extracellular Dpp does not rise in ap>tkv RNAi vs ap>tkv RNAi, dally[KO] wing discs (Fig. 6E) or (2) when they state that extracellular Dpp gradient expanded in the dorsal compartment when tkv was dorsally depleted in dally[deltaHS] mutants (Fig. 6I). These last two experiments are important for their model but the differences are assessed only visually. In fact, extracellular Dpp in ap>tkv RNAi, dally[KO] (Fig. 6B) appears to be lower than extracellular Dpp in ap>tkv RNAi (Fig. 6A) and the histogram of Dpp in ap>tkv RNAi, dally[KO] is actually a bit lower than Dpp in ap>tkv RNAi. But the author claim that there is no difference between the two. Their conclusion would be strengthened by statistical analyses of the two lines.

Strengths:

1. New genomically-engineered alleles

A considerable strength of the study is the generation and characterization of new Dally, Dlp and Dpp alleles. These reagents will be of great use to the field.

2. Surveying multiple phenotypes

The authors survey numerous parameters (Dpp distribution, Dpp signaling (pMad) and adult wing phenotypes) which provides many points of analysis.

Weaknesses:

1. Confusing discussion regarding the Dally core vs HS in Dpp stability. They don't provide any measurements or information on how they "normalize" for the level of Dally vs Dally[deltaHS]? This is important part of their model that currently is not supported by any measurements.

2. Lacking quantifications and statistical analyses:

a. Why are statistical significance for histograms (pMad and Dpp distribution) not supplied? These histograms provide the key results supporting the authors' conclusions but no statistical tests/results are presented. This is a pervasive shortcoming in the current study.

b. dpp[deltaN] with JAX transgene - it would strengthen the study to supply quantitative data on the percent survival/lethal stage of dpp[deltaN] mutants with or without the JAK transgene

c. The graphs on wing size etc should start at zero.

d. The sizes of histograms and graphs in each figure should be increased so that the reader can properly assess them. Currently, they are very small.

The authors' model is that Dally (not Dlp) is required for Dpp distribution and signaling but that this is not due to a direct interaction with Dpp. Rather, they posit that Dally-HS antagonize Tkv-mediated Dpp internalization. Currently the results of the experiments could be considered consistent with their model, but as noted above, the lack of statistical analyses of some parameters is a weakness. One problematic part of their result for me is the role of the Dally core protein (Fig. 7B). There is a mis-match between the over-expression results and Dally allele lacking HS (but containing the core). Finally, their results support the idea that one or more as-yet unidentified proteins interact with Dally-HS chains to control Dpp distribution and signaling in the wing disc.

There is much debate and controversy in the Dpp morphogen field. The generation of new, high quality alleles in this study will be useful to Drosophila community, and the results of this study support the concept that Tkv but not Dally regulate Dpp internalization. Thus the work could be impactful and fuel new debates among morphogen researchers.

The manuscript is currently written in a manner that really is only accessible to researchers who work on the Dpp gradient. It would be very helpful for the authors to re-write the manuscript and carefully explain in each section of the results (1) the exact question that will be asked, (2) the prior work on the topic, (3) the precise experiment that will be done, and (4) the predicted results. This would make the study more accessible to developmental biologists outside of the morphogen gradient and Drosophila communities.

Author Response

Reviewer #1 (Public Review):

How morphogens spread within tissues remains an important question in developmental biology. Here the authors revisit the role of glypicans in the formation of the Dpp gradient in wing imaginal discs of Drosophila. They first use sophisticated genome engineering to demonstrate that the two glypicans of Drosophila are not equivalent despite being redundant for viability. They show that Dally is the relevant glypican for Dpp gradient formation. They then provide genetic evidence that, surprisingly, the core domain of Dally suffices to trap Dpp at the cell surface (suggesting a minor role for GAGs). They conclude with a model that Dally modulates the range of Dpp signaling by interfering with Dpp's degradation by Tkv. These are important conclusions, but more independent (biochemical/cell biological) evidence is needed.

As indicated above, the genetic evidence for the predominant role of Dally in Dpp protein/signalling gradient formation is strong. In passing, the authors could discuss why overexpressed Dlp has a negative effect on signaling, especially in the anterior compartment. The authors then move on to determine the role of GAG (=HS) chains of Dally. They find that in an overexpression assay, Dally lacking GAGs traps Dpp at the cell surface and, counterintuitively, suppresses signaling (fig 4 C, F). Both findings are unexpected and therefore require further validation and clarification, as outlined in a and b below.

a) In loss of function experiments (dallyDeltaHS replacing endogenous dally), Dpp protein is markedly reduced (fig 4R), as much as in the KO (panel Q), suggesting that GAG chains do contribute to trapping Dpp at the cell surface. This is all the more significant that, according to the overexpression essays, DallyDeltaHS seems more stable than WT Dally (by the way, this difference should also be assessed in the knock-ins, which is possible since they are YFP-tagged). The authors acknowledge that HS chains of Dally are critical for Dpp distribution (and signaling) under physiological conditions. If this is true, one can wonder why overexpressed dally core 'binds' Dpp and whether this is a physiologically relevant activity.

According to the overexpression assay, DallyDeltaHS seems more stable than WT Dally (Fig. 4B', E', 5H, I). As the reviewer suggested, we addressed the difference using the two knock-in alleles and found that DallyDeltaHS is more stable than WT Dally (Fig. 4 L, M inset), further emphasizing the insufficient role of core protein of Dally for extracellular Dpp distribution.

(During the revising our figure, we found labeling mistake in Fig. 4M, N and Fig. 4Q, R and corrected the genotypes.)

In summary, we showed that, although Dally interacts with Dpp mainly through its core protein from the overexpression assay (Fig. 4E, I), HS chains are essential for extracellular Dpp distribution (Fig. 4R). Thus, the core protein of Dally alone is not sufficient for extracellular Dpp distribution under physiological conditions. These results raise a question about whether the interaction of core protein of Dally with Dpp is physiologically relevant. Since the increase of HS upon dally expression but not upon dlp expression resulted in the accumulation of extracellular Dpp (Fig. 2) and this accumulation was mainly through the core protein of Dally (Fig. 4E, I), we speculate that the interaction of the core protein of Dally with Dpp gives ligand specificity to Dally under physiological conditions.

To understand the importance of the interaction of core protein of Dally with Dpp under physiological conditions, it is important to identify a region responsible for the interaction. Our preliminary results overexpressing a dally mutant lacking the majority of core protein (but keeping the HS modified region intact) showed that HS chains modification was also lost. Although this is consistent with our results that enzymes adding HS chains also interact with the core protein of Dally (Fig. 4D), the dally mutant allele lacking the core protein would hamper us from distinguishing the role of core protein of Dally from HS chains.

Nevertheless, we can infer the importance of the interaction of core protein of Dally with Dpp using dally[3xHA-dlp, attP] allele, where dlp is expressed in dally expressing cells. Since Dally-like is modified by HS chains but does not interact with Dpp (Fig. 2, 4), dally[3xHA-dlp, attP] allele mimics a dally allele where HS chains are properly added but interaction of core protein with Dpp is lost. As we showed in Fig. 3O, S, the allele could not rescue dallyKO phenotypes, consistent with the idea that interaction of core protein of Dally with Dpp is essential for Dpp distribution and signaling and HS chain alone is not sufficient for Dpp distribution.

b) Although the authors' inference that dallycore (at least if overexpressed) can bind Dpp. This assertion needs independent validation by a biochemical assay, ideally with surface plasmon resonance or similar so that an affinity can be estimated. I understand that this will require a method that is outside the authors' core expertise but there is no reason why they could not approach a collaborator for such a common technique. In vitro binding data is, in my view, essential.

We agree with the reviewer that a biochemical assay such as SPR helps us characterize the interaction of core protein of Dally and Dpp (if the interaction is direct), although the biochemical assay also would not demonstrate the interaction under the physiological conditions.

However, SPR has never been applied in the case of Dpp, probably because purifying functional refolded Dpp dimer from bacteria has previously been found to be stable only in low pH and be precipitated in normal pH buffer (Groppe J, et al., 1998)(Matsuda et al., 2021). As the reviewer suggests, collaborating with experts is an important step in the future.

Nevertheless, SPR was applied for the interaction between BMP4 and Dally (Kirkpatrick et al., 2006), probably because BMP4 is more stable in the normal buffer. Although the binding affinity was not calculated, SPR showed that BMP4 directly binds to Dally and this interaction was only partially inhibited by molar excess of exogenous HS, suggesting that BMP4 can interact with core protein of Dally as well as its HS chains. In addition, the same study applied Co-IP experiments using lysis of S2 cells and showed that Dpp and core protein of Dally are co-immunoprecipitated, although it does not demonstrate if the interaction is direct.

In a subsequent set of experiments, the authors assess the activity of a form of Dpp that is expected not to bind GAGs (DppDeltaN). Overexpression assays show that this protein is trapped by DallyWT but not dallyDeltaHS. This is a good first step validation of the deltaN mutation, although, as before, an invitro binding assay would be preferable.

Our overexpression assays actually showed that DppDeltaN is trapped by DallyWT and by dallyDeltaHS at similar levels (Fig. 5H-J), indicating that interaction of DppDeltaN and HS chains of Dally is largely lost but DppDeltaN can still interact with core protein of Dally.

(Related to this, we found typo in the sentence "In contrast, the relative DppΔN accumulation upon DallyΔHS expression in JAX;dppΔN was comparable to that upon DallyΔHS expression in JAX;dppΔN (Fig. 5H-J)." and corrected as follows, "In contrast, the relative DppΔN accumulation upon Dally expression in JAX;dppΔN was comparable to that upon DallyΔHS expression in JAX;dppΔN (Fig. 5H-J)."

We thank the reviewer for the suggesting the in vitro experiment. Although we decided not to develop biophysical experiments such as SPR for Dpp in this study due to the reasons discussed above, we would like to point out that our result is consistent with a previous Co-IP experiment using S2 cells showing that DppDeltaN loses interaction with heparin (Akiyama2008).

However, in contrast to our results, the same study also proposed by Co-IP experiments using S2 cells that DppDeltaN loses interaction with Dally (Akiyama2008). Although it is hard to conclude since western blotting was too saturated without loading controls and normalization (Fig. 1C in Akiyama 2008), and negative in vitro experiments do not necessarily demonstrate the lack of interaction in vivo. One explanation why the interaction was missed in the previous study is that some factors required for the interaction of DppDeltaN with core protein of Dally are missing in S2 cells. In this case, in vivo interaction assay we used in this study has an advantage to robustly detect the interaction.

Nevertheless, the authors show that DppDeltaN is surprisingly active in a knock-in strain. At face value (assuming that DeltaN fully abrogates binding to GAGs), this suggests that interaction of Dpp with the GAG chains of Dally is not required for signaling activity. This leads to authors to suggest (as shown in their final model) that GAG chains could be involved in mediating the interactions of Dally with Tkv (and not with Dpp. This is an interesting idea, which would need to be reconciled with the observation that the distribution of Dpp is affected in dallyDeltaHS knock-ins (item a above). It would also be strengthened by biochemical data (although more technically challenging than the experiments suggested above). In an attempt to determine the role of Dally (GAGs in particular) in the signaling gradient, the paper next addresses its relation to Tkv. They first show that reducing Tkv leads to Dpp accumulation at the cell surface, a clear indication that Tkv normally contributes to the degradation of Dpp. From this they suggest that Tkv could be required for Dpp internalisation although this is not shown directly. The authors then show that a Dpp gradient still forms upon double knockdown (Dally and Tkv). This intriguing observation shows that Dally is not strictly required for the spread of Dpp, an important conclusion that is compatible with early work by Lander suggesting that Dpp spreads by free diffusion. These result show that Dally is required for gradient formation only when Tkv is present. They suggest therefore that Dally prevents Tkv-mediated internalisation of Dpp. Although this is a reasonable inference, internalisation assays (e.g. with anti-Ollas or anti-HA Ab) would strengthen the authors' conclusions especially because they contradict a recent paper from the Gonzalez-Gaitan lab.

Thanks for suggesting the internalization assay. As we discussed in the discussion, our results suggest that extracellular Dpp distribution is severely reduced in dally mutants due to Tkv mediated internalization of Dpp (Fig. 6). Thus, extracellular Dpp available for labelling with nanobody is severely reduced in dally mutants, which can explain the reduced internalization of Dpp in dally mutants in the internalization assay. Therefore, we think that the nanobody internalization assay would not distinguish the two contradicting possibilities.

The paper ends with a model suggesting that HS chains have a dual function of suppressing Tkv internalisation and stimulating signaling. This constitutes a novel view of a glypican's mode of action and possibly an important contribution of this paper. As indicated above, further experiments could considerably strengthen the conclusion. Speculation on how the authors imagine that GAG chains have these activities would also be warranted.

Thank you very much!

Reviewer #2 (Public Review):

The authors are trying to distinguish between four models of the role of glypicans (HSPGs) on the Dpp/BMP gradient in the Drosophila wing, schematized in Fig. 1: (1) "Restricted diffusion" (HSPGs transport Dpp via repetitive interaction of HS chains with Dpp); (2) "Hindered diffusion" (HSPGs hinder Dpp spreading via reversible interaction of HS chains with Dpp); (3) "Stabilization" (HSPGs stabilize Dpp on the cell surface via reversible interaction of HS chains with Dpp that antagonizes Tkv-mediated Dpp internalization); and (4) "Recycling" (HSPGs internalize and recycle Dpp).

To distinguish between these models, the authors generate new alleles for the glypicans Dally and Dally-like protein (Dlp) and for Dpp: a Dally knock-out allele, a Dally YFP-tagged allele, a Dally knock-out allele with 3HA-Dlp, a Dlp knock-out allele, a Dlp allele containing 3-HA tags, and a Dpp lacking the HS-interacting domain. Additionally, they use an OLLAS-tag Dpp (OLLAS being an epitope tag against which extremely high affinity antibodies exist). They examine OLLAS-Dpp or HA-Dpp distribution, phospho-Mad staining, adult wing size.

They find that over-expressed Dally - but not Dlp - expands Dpp distribution in the larval wing disc. They find that the Dally[KO] allele behaves like a Dally strong hypomorph Dally[MH32]. The Dally[KO] - but not the Dlp[KO] - caused reduced pMad in both anterior and posterior domains and reduced adult wing size (particularly in the Anterior-Posterior axis). These defects can be substantially corrected by supplying an endogenously tagged YFP-tagged Dally. By contrast, they were not rescued when a 3xHA Dlp was inserted in the Dally locus. These results support their conclusion that Dpp interacts with Dally but not Dlp.

They next wanted to determine the relative contributions of the Dally core or the HS chains to the Dpp distribution. To test this, they over-expressed UAS-Dally or UAS-Dally[deltaHS] (lacking the HS chains) in the dorsal wing. Dally[deltaHS] over-expression increased the distribution of OLLAS-Dpp but caused a reduction in pMad. Then they write that after they normalize for expression levels, they find that Dally[deltaHS] only mildly reduces pMad and this result indicates a major contribution of the Dally core protein to Dpp stability.

Thanks for the comments. We actually showed that compared with Dally overexpression, Dally[deltaHS] overexpression only mildly reduces extracellular Dpp accumulation (Fig. 4I). This indicates a major contribution of the Dally core protein to interaction with Dpp, although the interaction is not sufficient to sustain extracellular Dpp distribution and signaling gradient.

The "normalization" is a key part of this model and is not mentioned how the normalization was done. When they do the critical experiment, making the Dally[deltaHS] allele, they find that loss of the HS chains is nearly as severe as total loss of Dally (i.e., Dally[KO]). Additionally, experimental approaches are needed here to prove the role of the Dally core.

Since the expression level of Dally[deltaHS] is higher than Dally when overexpressed, we normalized extracellular Dpp distribution (a-Ollas staining) against GFP fluorescent signal (Dally or Dally[deltaHS]). To do this, we first extracted both signal along the A-P axis from the same ROI. The ratio was calculated by dividing the intensity of a-Ollas staining with the intensity of GFP fluorescent signal at a given position x. The average profile from each normalized profile was generated and plotted using the script described in the method (wingdisc_comparison.py) as other pMad or extracellular staining profiles.

Although this analysis provides normalized extracellular Dpp accumulation at different positions along the A-P axis, we are more interested in the total amount of Dpp or DppDeltaN accumulation upon Dally or dallyDeltaHS expression. Therefore, we plan to analyze the normalized total amount of Dpp against GFP fluorescent signal (Dally or Dally[deltaHS]) in the revised ms. In this case, normalization will be performed by dividing total signal intensity of extracellular Dpp staining (ExOllas staining) divided by GFP fluorescent signal (Dally or Dally[deltaHS]) in ROI in each wing disc.

We agree with the reviewer that additional experimental approaches are needed to address the role of the core protein of Dally. As we discussed in the response to the reviewer¹, to understand the importance of the interaction of core protein of Dally with Dpp, it is important to identify a region responsible for the interaction. Our preliminary results overexpressing a dally mutant lacking the majority of core protein (but keeping the HS modified region intact) showed that HS chains modification was also lost. Although this is consistent with our results that enzymes adding HS chains also interact with the core protein of Dally (Fig. 4D), the dally mutant allele lacking the core protein would hamper us from distinguishing the role of the core protein of Dally from HS chains.

Nevertheless, we can infer the importance of the interaction of core protein of Dally with Dpp using dally[3xHA-dlp, attP] allele, where dlp is expressed in dally expressing cells. Since Dally-like is modified by HS chains but does not interact with Dpp (Fig. 2, 4), dally[3xHA-dlp, attP] allele mimics a dally allele where HS chains are properly added but interaction of core protein with Dpp is lost. As we showed in Fig. 3O, S, the allele could not rescue dallyKO phenotypes, consistent with the idea that interaction of core protein of Dally with Dpp is essential for Dpp distribution and signaling.

Prior work has shown that a stretch of 7 amino acids in the Dpp N-terminal domain is required to interact with heparin but not with Dpp receptors (Akiyama, 2008). The authors generated an HA-tagged Dpp allele lacking these residues (HA-dpp[deltaN]). It is an embryonic lethal allele, but they can get some animals to survive to larval stages if they also supply a transgene called "JAX" containing dpp regulatory sequences. In the JAX; HA-dpp[deltaN] mutant background, they find that the distribution and signaling of this Dpp molecule is largely normal. While over-expressed Dally can increase the distribution of HA-dpp[deltaN], over-expression of Dally[deltaHS] cannot. These latter results support the model that the HS chains in Dally are required for Dpp function but not because of a direct interaction with Dpp.

Our overexpression assays actually showed that both Dally and Dally[deltaHS] can accumulate Dpp upon overexpression and the accumulation of Dpp is comparable after normalization (Fig. 5H-J), consistent with the idea that interaction of DppdeltaN and HS chains are largely lost. As the reviewer pointed out, these results support the model that the HS chains in Dally are required for Dpp function but not because of a direct interaction with Dpp.

In the last part of the results, they attempt to determine if the Dpp receptor Thickveins (Tkv) is required for Dally-HS chains interaction. The 2008 (Akiyama) model posits that Tkv activates pMad downstream of Dpp and also internalizes and degrades Dpp. A 2022 (Romanova-Michaelides) model proposes that Dally (not Tkv) internalizes Dpp.

To distinguish between these models, the authors deplete Tkv from the dorsal compartment of the wing disc and found that extracellular Dpp increased and expanded in that domain. These results support the model that Tkv is required to internalize Dpp.

They then tested the model that Dally antagonizes Tkv-mediated Dpp internalization by determining whether the defective extracellular Dpp distribution in Dally[KO] mutants could be rescued by depleting Tkv. Extracellular Dpp did increase in the D vs V compartment, potentially providing some support for their model. However, there are no statistics performed, which is needed for full confidence in the results. The lack of statistics is particularly problematic (1) when they state that extracellular Dpp does not rise in ap>tkv RNAi vs ap>tkv RNAi, dally[KO] wing discs (Fig. 6E) or (2) when they state that extracellular Dpp gradient expanded in the dorsal compartment when tkv was dorsally depleted in dally[deltaHS] mutants (Fig. 6I). These last two experiments are important for their model but

histogram of Dpp in ap>tkv RNAi, dally[KO] is actually a bit lower than Dpp in ap>tkv RNAi, But the author claim that there is no difference between the two. Their conclusion would be strengthened by statistical analyses of the two lines.

We will provide the statistical analyses in the revised ms.

Strengths:

1. New genomically-engineered alleles

A considerable strength of the study is the generation and characterization of new Dally, Dlp and Dpp alleles. These reagents will be of great use to the field.

Thanks. We hope that these resources are indeed useful to the field.

2. Surveying multiple phenotypes

The authors survey numerous parameters (Dpp distribution, Dpp signaling (pMad) and adult wing phenotypes) which provides many points of analysis.

Thanks!

Weaknesses:

1. Confusing discussion regarding the Dally core vs HS in Dpp stability. They don't provide any measurements or information on how they "normalize" for the level of Dally vs Dally[deltaHS]? This is important part of their model that currently is not supported by any measurements.

We explained how we normalized in the above section. We will update the analysis in the revised ms.

2. Lacking quantifications and statistical analyses:

a) Why are statistical significance for histograms (pMad and Dpp distribution) not supplied? These histograms provide the key results supporting the authors' conclusions but no statistical tests/results are presented. This is a pervasive shortcoming in the current study.

Thanks. We will provide statistics in the revised ms.

b) dpp[deltaN] with JAX transgene - it would strengthen the study to supply quantitative data on the percent survival/lethal stage of dpp[deltaN] mutants with or without the JAX transgene

In this study, we are interested in the role of dpp[deltaN] during the wing disc development. Therefore, we decided not to perform the detailed analysis on the percent survival/lethal stage of dpp[deltaN] mutants with or without the JAX transgene in the current study. Nevertheless, the fact that dpp[deltaN] allele is maintained with a balanced stock and JAX;dpp[deltaN] allele can be maintained as homozygous stock indicates that the lethality of dpp[deltaN] allele comes from the early stages. Indeed, our preliminary results showed that pMad signal is severely lost in the dpp[deltaN] embryo without JAX (data not shown), indicating that the allele is lethal at early embryonic stages.

c) The graphs on wing size etc should start at zero.

Thanks. We corrected this in the current ms.

d) The sizes of histograms and graphs in each figure should be increased so that the reader can properly assess them. Currently, they are very small.

Thanks. We changed the sizes in the current ms.

The authors' model is that Dally (not Dlp) is required for Dpp distribution and signaling but that this is not due to a direct interaction with Dpp. Rather, they posit that Dally-HS antagonize Tkv-mediated Dpp internalization. Currently the results of the experiments could be considered consistent with their model, but as noted above, the lack of statistical analyses of some parameters is a weakness.

Thanks. We will perform the statistical analyses in the revised ms.

One problematic part of their result for me is the role of the Dally core protein (Fig. 7B). There is a mis-match between the over-expression results and Dally allele lacking HS (but containing the core). Finally, their results support the idea that one or more as-yet unidentified proteins interact with Dally-HS chains to control Dpp distribution and signaling in the wing disc.

Our results simply suggest that Dpp can interact with Dally mainly through core protein but this interaction is not sufficient to sustain extracellular Dpp gradient formation under physiological conditions (dallyDeltaHS) (Fig. 4Q). We find that the mis-match is not problematic if the role of Dally is not simply mediated through interaction with Dpp. We speculate that interaction of Dpp and core protein of Dally is transient and not sufficient to sustain the Dpp gradient without HS chains of Dally stabilizing extracellular Dpp distribution by blocking Tkv-mediated Dpp internalization.

There is much debate and controversy in the Dpp morphogen field. The generation of new, high quality alleles in this study will be useful to Drosophila community, and the results of this study support the concept that Tkv but not Dally regulate Dpp internalization. Thus the work could be impactful and fuel new debates among morphogen researchers.

Thanks.

The manuscript is currently written in a manner that really is only accessible to researchers who work on the Dpp gradient. It would be very helpful for the authors to re-write the manuscript and carefully explain in each section of the results (1) the exact question that will be asked, (2) the prior work on the topic, (3) the precise experiment that will be done, and (4) the predicted results. This would make the study more accessible to developmental biologists outside of the morphogen gradient and Drosophila communities.

Thanks. We will modify our texts to help non-experts understand our story in the revised ms.