Development 130, 1515-1522

doi:10.1242/dev.00379

Dally regulates Dpp morphogen gradient formation in the *Drosophila* wing

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Accepted 8 January 2003

SUMMARY

Decapentaplegic (Dpp), a *Drosophila* TGFβ/bone morphogenetic protein homolog, functions as a morphogen to specify cell fate along the anteroposterior axis of the wing. Dpp is a heparin-binding protein and Dpp signal transduction is potentiated by Dally, a cell-surface heparan sulfate proteoglycan, during assembly of several adult tissues. However, the molecular mechanism by which the Dpp morphogen gradient is established and maintained is poorly understood. We show evidence that Dally regulates both cellular responses to Dpp and the distribution of Dpp morphogen in tissues. In the developing wing, *dally* expression in the wing disc is controlled by the same molecular pathways that regulate expression of *thickveins*, which encodes a Dpp type I receptor. Elevated levels of Dally increase the sensitivity of cells to Dpp in a cell autonomous fashion. In addition, *dally* affects the shape of the Dpp ligand gradient as well as its activity gradient. We propose that Dally serves as a co-receptor for Dpp and contributes to shaping the Dpp morphogen gradient.

Key words: *dally*, *dpp*, Morphogen gradient, Heparan sulfate proteoglycan, *Drosophila*

INTRODUCTION

Morphogens specify different cell fates in a concentration-dependent manner and their functions are fundamental to tissue patterning during development. In spite of the central role morphogens play in assembling tissues, the molecular mechanisms of morphogen gradient formation remain largely unknown. During development of the *Drosophila* wing, three molecules have been proposed to act as morphogens: Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp), a homolog of vertebrate bone morphogenetic proteins (BMPs). Dpp is expressed in a stripe of cells adjacent to the anterior (A)-posterior (P) compartment boundary (A/P border cells), and patterns the wing by inducing different target genes, such as *spalt* and *optomotor-blind*, at different extracellular concentrations (Lecuit et al., 1996; Nellen et al., 1996). Recently, two molecular tools have been developed to monitor the Dpp gradient formation. First, a biologically active GFP-tagged Dpp (Dpp-GFP) was used to directly visualize the Dpp gradient in the developing wing (Fig. 1A) (Entchev et al., 2000; Teleman and Cohen, 2000). Studies using Dpp-GFP successfully demonstrated that Dpp forms a long-range gradient throughout the wing pouch. Dpp-GFP moves quickly through the disc and is rapidly turned over. Second, an antibody that specifically recognizes the phosphorylated form of Mothers against Dpp (pMad) can be used to monitor the Dpp morphogen activity gradient by following the phosphorylation of Mad, a downstream transducer of the Dpp pathway (Fig. 1B) (Persson et al., 1998; Tanimoto et al., 2000). Generally, pMad levels are high at the central region of the wing disc and gradually decline toward the anterior and posterior distal cells. However, within the central region, pMad levels are lower at the A/P border cells owing to the reduced expression of *thickveins* (*tkv*), which encodes the type I receptor for Dpp. Although the ligand and activity gradients now can be visualized by using the tools mentioned above, the molecular basis for the Dpp gradient formation is not yet completely understood.

One of the determinants of the Dpp morphogen gradient is the Tkv receptor. As Dpp signaling negatively regulates *tkv* expression, the relative levels of *tkv* are high in cells at the peripheral region of the wing disc and are low within the central domain (Fig. 1C) (Lecuit and Cohen, 1998). In addition, *tkv* expression is strongly repressed by Hh signaling at the A/P border cells, which results in a reduction of pMad staining in this region. The basal level of *tkv* expression is higher in the P compartment than in the A compartment and is maintained by the activity of the P cell-specific selector gene, *engrailed* (*en*) (Funakoshi et al., 2001; Tanimoto et al., 2000). As higher levels of Tkv limit the movement of Dpp, Dpp does not spread as far in the P compartment, resulting in a steeper Dpp morphogen gradient. Overexpression of *tkv* was also shown to retard the movement of Dpp (Lecuit and Cohen, 1998; Tanimoto et al., 2000). Thus, Dpp gradient formation is in part controlled by the regulated expression of the *tkv* gene.

Several lines of evidence have suggested that heparan sulfate

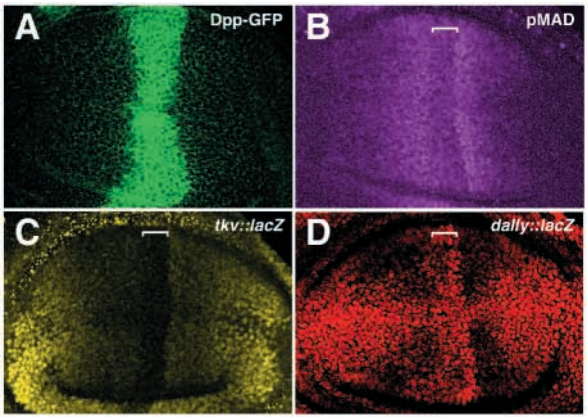


Fig. 1. Dpp morphogen gradients and expression of Dpp signaling components in the developing wing. (A,B) Patterns of Dpp-GFP expressed in A/P border cells by *dpp-GAL4* driver (A; *dpp^{d12}/+; dpp-GAL4/UAS-Dpp-GFP*) and Dpp activity gradient visualized by anti-pMad antibody staining (B). (C,D) Expression patterns of the Dpp type I receptor gene *tkv* (C) and a putative Dpp co-receptor gene *dally* (D), were followed by anti-β-galactosidase antibody staining of wing discs of the enhancer trap lines *tkv^{P906}* and *dally^{P2}*, respectively. Brackets show positions of the A/P border cells. Posterior is towards the right and ventral is towards the top of all wing discs and in all subsequent figures.

proteoglycans (HSPGs) are involved in distribution of morphogens. *tout velu* (*ttv*), a *Drosophila* homolog of the mammalian EXT tumor suppressor gene family, encodes a heparan sulfate co-polymerase (Bellaiche et al., 1998; The et al., 1999; Toyoda et al., 2000). Mutations in *ttv* disrupt the movement of Hh from its site of production. Thus, *ttv*-dependent synthesis of HSPG is required for normal distribution of Hh between cells. Similarly, Notum (Wingful) was recently reported to be required for gradient formation of the Wg morphogen (Gerlitz and Basler, 2002; Giraldez et al., 2002). This gene encodes a protein with homology to pectin acetyltransferases and is proposed to affect Wg distribution by modulating the structures of the heparan sulfate moiety of HSPGs. Recently, a novel membrane exovesicle structure, argosome, was identified and proposed to be involved in morphogen movements (Greco et al., 2001). The transportation of argosomes across cells and the localization of Wg protein in this structure suggests a possible role for argosomes in Wg distribution. Interestingly, when discs were treated with heparitinases, enzymes that digest heparan sulfate, Wg failed to accumulate properly in argosomes, although argosome distribution was not affected. Greco et al. proposed from this observation that interaction of Wg with HSPGs is required for the incorporation of Wg into argosomes (Greco et al., 2001). These findings collectively point to the possibility that HSPGs are involved in controlling the distributions and activities of morphogens during tissue patterning.

One of the *Drosophila* proteoglycan genes that has been shown to affect growth factor signaling in tissue patterning is *division abnormally delayed* (*dally*) (Nakato et al., 1995). *dally* encodes the core protein for glypican, a family of HSPGs that are linked to the cell membrane through a GPI anchor (Tsuda et al., 1999). *dally* affects signaling mediated by two known HS-binding growth factors in *Drosophila*: Dpp and Wg. Modulation of *dally* gene dosage dramatically alters the expression of Dpp target genes, as well as the patterning activity of Dpp in multiple imaginal tissues (Fujise et al., 2001; Jackson et al., 1997). In the embryonic epidermis, *dally* modulates patterning directed by Wg (Lin and Perrimon, 1999; Tsuda et al., 1999). These findings are consistent with a model in which HSPGs enhance the activity of growth factors on the cell surface by promoting the assembly and/or increasing the stability of signaling complexes. In this study, we focused on the function of *dally* in Dpp signaling during wing development. We found that elevated levels of *dally* increase the sensitivity of cells to Dpp, and that alterations in levels of *dally* affect formation of both Dpp ligand and activity gradients. In addition, we found that the same regulatory networks control expression of *dally* and *tkv*. These findings suggest that the regulated expression and function of Dally are essential components for Dpp morphogen gradient formation.

MATERIALS AND METHODS

Fly stocks

Enhancer trap lines, *dally^{P2}* (Fujise et al., 2001; Nakato et al., 1995) and *tkv^{P906}* (Tanimoto et al., 2000) were used to follow the expression patterns of *dally* and *tkv*, respectively. The mutations and transgenic animals used were as follows: *dally^{gem}* and *dally$^{\Delta P\text{-}527}$*, hypomorphic alleles of *dally* (Nakato et al., 1995); *Df en^{E}*, an embryonic lethal allele of *en* (Tabata et al., 1995); *tkv^{a12}* (Nellen et al., 1994; Nellen et al., 1996) and *tkv^{6}* (Singer et al., 1997), a null and hypomorphic allele of *tkv*, respectively; *UAS-dally* (Tsuda et al., 1999); *UAS-hhCD2*, a membrane-tethered form of Hh, the signaling fragment of Hh fused to the rat *CD2* gene (Strigini and Cohen, 1997); *UAS-tkv^{Q253D}*, a constitutively active form of *tkv* (Nellen et al., 1996; Wieser et al., 1995); *Act5C>y^{+}>GAL4*, a FLP-OUT cassette to misexpress the gene downstream of UAS (Ito et al., 1997); *UAS-Dpp-GFP*, a biologically active form of Dpp tagged with GFP (Teleman and Cohen, 2000); and *dpp-GAL4* (Morimura et al., 1996).

Immunostaining

Immunostaining was performed as previously described (Fujise et al., 2001) using rabbit anti-β-galactosidase (1:500, Cappel), rabbit anti-pMad [1:1000, a generous gift from T. Tabata and P. ten Dijke (Persson et al., 1998; Tanimoto et al., 2000)] and rat anti-Tkv [1:250, a generous gift from S. Cohen (Teleman and Cohen, 2000)]. The intensity profiles of pMad staining were generated by NIH Image using the plot function. The primary antibodies were detected with Alexa Fluor 568- or Alexa Fluor 488-conjugated secondary antibodies (Funakoshi).

Ectopic expression

Clones of cells that ectopically express *hhCD2*, *tkv^{Q253D}* and *dally* were induced by the GAL4/UAS system using a FLP-OUT cassette (Ito et al., 1997; Struhl and Basler, 1993). The genotypes of the flies used were:

y w hsp70-flp/+; Act5C>y^{+}>GAL4 UAS-GFP/+; UAS-hhCD2/ dally^{P2},

y w hsp70-flp/+; Act5C>y^{+}>GAL4 UAS-GFP/+; UAS-tkv^{Q253D}/ dally^{P2} and

y w hsp70-flp/+; Act5C>y^{+}>GAL4 UAS-GFP/+; UAS-dally/+.

Mosaic analyses

Homozygous mutant clones were induced by FLP-mediated mitotic recombination (Golic, 1991; Xu and Rubin, 1993). Larvae were heat shocked at 24-48 hours after egg-laying at 37°C for 10-60 minutes to induce recombination. Discs were dissected and analyzed 48-96 hours

after the induction. The genotypes of larvae we used to examine the effect of *tkv* mutations on *dally::lacZ* expression were: *Ubi-nlsGFP FRT40/tkv^{a12} FRT40*; *dally^{P2}/FLP3 Sb MKRS* and *Ubi-nlsGFP FRT40/tkv^{6} FRT40*; *dally^{P2}/FLP3 Sb MKRS*. To induce *en* mutant clones, we used discs from *y w hsp70-flp/+*; *FRT42D y^{+} Df en^{E}/FRT42D y^{+} Ubi-GFP*; *dally^{P2}/+* larvae.

Expression and detection of Dpp-GFP

To monitor the Dpp-GFP distribution, we dissected wing discs of *dpp^{d12}/+; UAS-dpp-GFP/dpp-GAL4* larvae. Distribution of Dpp-GFP in *dally* mutants was observed using discs from *dpp^{d12}/+; dpp-GAL4 dally^{gem}/UAS-dpp-GFP dally^{gem}* larvae. The effect of *dally* overexpression in A/P border cells on Dpp-GFP distribution was analyzed using discs from *dpp^{d12}/+; UAS-dpp-GFP UAS-dally/dpp-GAL4* larvae. In previous studies (Entchev et al., 2000; Teleman and Cohen, 2000), Dpp-GFP was expressed in *dpp* homozygous animals (*dpp^{d8}/dpp^{d12}* or *dpp^{d12}/dpp^{d16}*). However, because animals homozygous for both *dpp* and *dally* rarely survive to the third instar, we expressed Dpp-GFP in a *dpp* heterozygous background (*dpp^{d12}/+*). After a brief fixation (10 minutes) of discs with 4% formaldehyde, signals for Dpp-GFP were imaged using confocal microscopy (LSM410, Carl Zeiss). Average intensity profiles for different genotypes were generated using NIH Image.

RESULTS

Expression pattern of *dally* along the AP axis of the wing disc

The expression pattern of *dally*, monitored by *dally::lacZ* enhancer-trap expression, in the developing wing along the AP axis shows a peak of expression at the A/P border cells, and *dally* levels are lowest in cells adjacent to this region (Fig. 1D). Furthermore, *dally* levels gradually increase toward the anterior and posterior distal cells. This pattern correlates with the expression patterns of several genes involved in pattern formation along the AP axis, such as *tkv* and *master of thick veins* (*mtv*; *Sbb* – FlyBase), which suggests that *dally* also participates in this process. Expression of the *tkv* gene is controlled by two distinct pathways. First, Hh represses *tkv* expression at the A/P border cells, and En regulates a high basal level of *tkv* in the P compartment (Funakoshi et al., 2001; Tanimoto et al., 2000). The activities of both *hh* and *en* genes are mediated by a putative transcription factor, Mtv. Second, *tkv* levels are downregulated by Dpp signaling (Lecuit and Cohen, 1998). By this mechanism, *tkv* expression is maintained at low levels in the center of the disc and at higher levels toward the anterior and posterior edges. The correlation between expression patterns of *dally* and *tkv* prompted us to analyze the *dally* function in Dpp signaling in this tissue. As a first step toward this, we analyzed the regulatory pathways controlling *dally* expression and compared them with those controlling *tkv* expression.

dally expression is regulated by Hh and En

In a previous study, we showed that Hh signaling induces *dally* expression at the A/P border cells (Fujise et al., 2001). *dally* expression was absent in the *smoothened* (*smo*) mutant clones generated in the A compartment, where the Hh signaling is blocked (Alcedo et al., 1996), indicating that Hh signaling is required for activation of *dally* at the A/P border cells. To further determine whether Hh signaling is sufficient for the induction of *dally*, we examined clones that ectopically express *hhCD2*, which encodes a membrane-tethered form of Hh (Strigini and Cohen, 1997), using the FLP-OUT system. In the A compartment, *dally* expression levels were increased in *hhCD2*-expressing cells and in cells immediately adjacent to them (Fig. 2A-C). This result shows that Hh expression is sufficient to induce *dally* expression in the A compartment. To determine if *dally* expression is controlled by *en*, which upregulates *tkv* expression, we induced clones of *en*-mutant cells using the FLP-FRT mosaic analysis system. Within *en*-mutant clones in the P compartment, *dally* levels were dramatically increased (Fig. 2D-F), which indicates that *dally* expression is negatively regulated by *en*.

Expression of *dally* enhancer trap is repressed by Dpp signaling

To determine whether modulation of Dpp signaling affects *dally* expression, we first compared the *dally::lacZ* expression between wild-type and *tkv* heterozygous cells. Clones mutant for *tkv* were generated in a heterozygous background (*tkv^{a12}/+*)

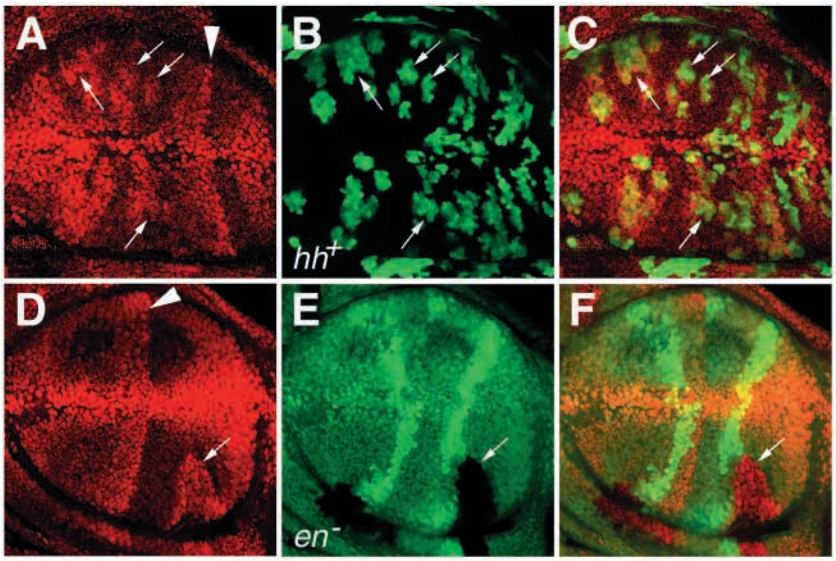
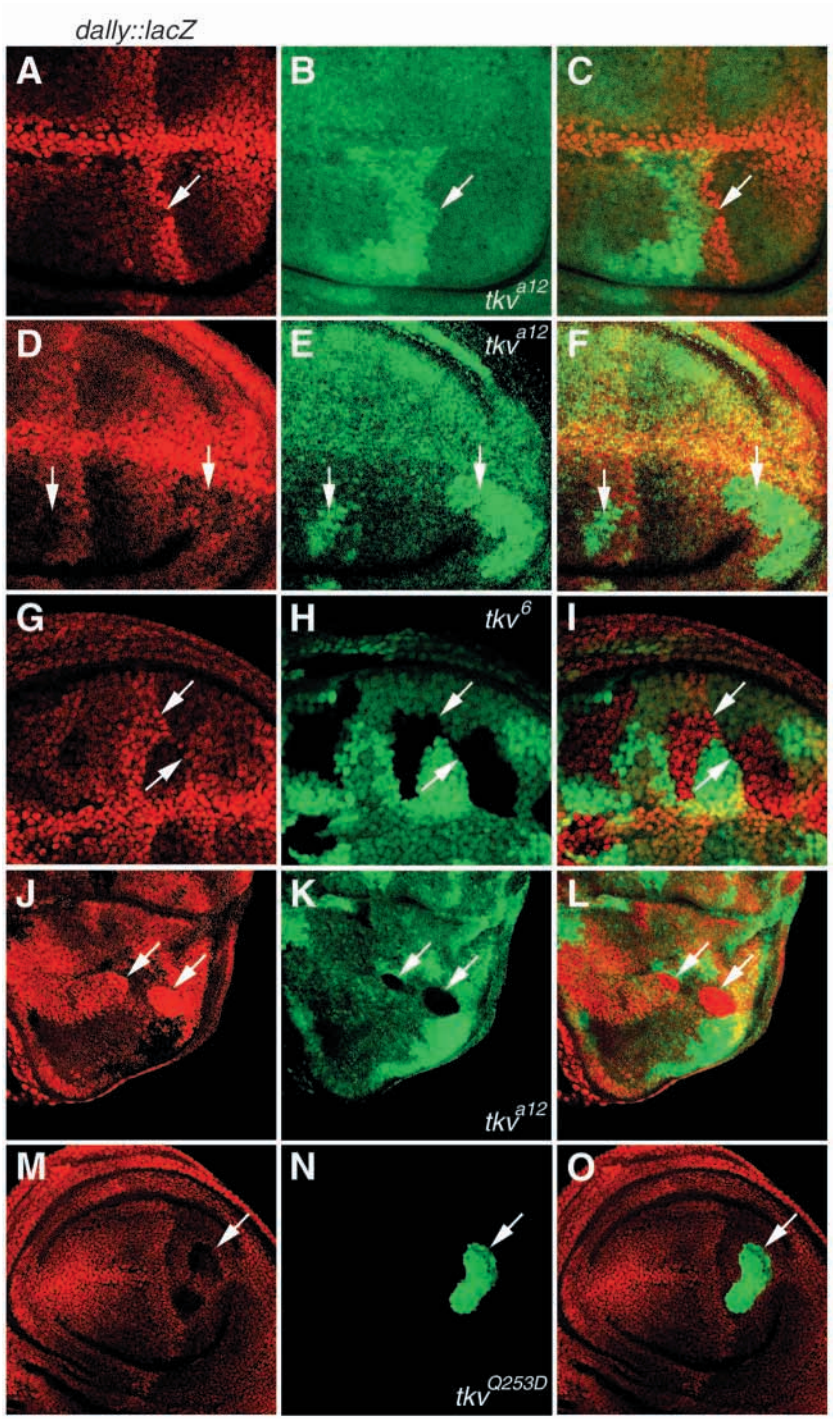


Fig. 2. Expression of *dally* is regulated by Hh and En. (A-C) Misexpression of the membrane-tethered form of Hh, HhCD2, induces ectopic expression of *dally*. *dally::lacZ* expression was followed using anti-β-galactosidase antibody staining (A). Clones expressing *hhCD2* are marked by GFP in (B). (C) A merged image of A and B. Levels of *dally*::*lacZ* are increased both in cells inside and adjacent to the clones located in the A compartment (arrows), while clones in the P compartment do not alter *dally* expression. (D-F) *dally* is repressed by *en* in the P compartment. (D) Expression pattern of *dally::lacZ*. (E) Positions of *en*-mutant clones are shown by loss of GFP signal. (F) A merged image of D and E. *dally* expression is significantly elevated in *en*-mutant clones induced in the P compartment (arrow), indicating that *dally* is normally downregulated by *en*. Arrowheads in A and D indicate the AP boundary.

using the FLP-FRT system, which should, as a consequence, produce both mutant (tkv^{a12}/tkv^{a12}) and wild-type sister clones (+/+). However, tkv^{-} cells do not survive in the wing pouch as Tkv activity is indispensable for growth and, thus, only wild-type sister clones survived (Burke and Basler, 1996). In resultant mosaic discs with wild-type and *tkv*-heterozygous cells, *dally* expression was decreased cell autonomously in wild-type (+/+) clones at the AP border (Fig. 3A-C) and peripheral to the border (Fig. 3D-F). To further confirm this result, we also examined the effect of *tkv*-hypomorphic clones (tkv^{6}/tkv^{6}) on *dally* expression. In such clones, where *tkv* activity is partially compromised, the levels of *dally* expression were elevated (Fig. 3G-I). In the notum region of the wing disc, we could generate *tkv*-null clones (tkv^{a12}/tkv^{a12}) in which a substantial increase of *dally* expression was observed (Fig. 3J-L). Finally, we tested the effect of increased Dpp signaling on *dally* expression by using the FLP-OUT method to induce clones of cells that express tkv^{Q253D}, a constitutively active form of *tkv*, in the wing pouch. We found that the level of *dally::lacZ* expression was autonomously reduced in the tkv^{Q253D}-expressing clones (Fig. 3M-O). All of these results consistently indicate that *dally* expression in the wing disc is negatively regulated by Dpp signaling, as has been shown for *tkv* (Lecuit and Cohen, 1998). Thus, *dally* and *tkv* are regulated by the same set of molecular pathways: Hh, En and Dpp signaling.



dally affects the sensitivity of cells to Dpp

Although Dally was shown to be involved in Dpp signaling in several imaginal tissues (Jackson et al., 1997), the function of Dally in this signaling pathway during wing development remains unclear. To investigate whether *dally* indeed affects Dpp signaling activity in the developing wing, we examined the effect of *dally* misexpression on pMad levels. We found that the pMad signal was significantly elevated in *dally*-overexpressing clones induced by the FLP-OUT system (Fig. 4). From the observation of 38 clones, this effect of $dally^{+}$ expression on pMad levels was found to be strictly cell autonomous. This is consistent with the idea that Dally serves as a co-receptor for Dpp, which facilitates the assembly of a signaling complex on the cell surface. Given that Dpp is an unstable molecule with a short half-

Fig. 3. Expression of *dally* is repressed by Dpp signaling. (A-F) Clonal analysis for a severe *tkv* allele, tkv^{a12}, resulted in loss of the clones homozygous for *tkv*, because of growth defects, and preferential growth of their sister clones carrying two copies of the wild-type *tkv* in a genetic background heterozygous for *tkv* (one copy of wild-type *tkv*). The sister clones are marked by two copies of GFP (+/+), giving brighter signals than the single copy of GFP (+/–) (B,E). Wild-type (+/+) clones at the A/P border (A-C) and peripheral to the border (D-F) autonomously decrease the expression of *dally*::*lacZ* (arrows). (G-I) Clonal analysis for a mild *tkv* allele. *dally::lacZ* expression (G) is elevated in clones of cells homozygous for a partial loss-of-function *tkv* allele, tkv^{6} (arrows). (J-L) Effects of tkv^{a12} mutation on *dally::lacZ* expression in the notum. Levels of *dally* expression (J) were significantly increased in tkv^{a12} clones (arrows). Positions of clones are shown by loss of the GFP signal (H,K). (M-O) Effects of ectopically activated Dpp signaling on *dally::lacZ* expression. Expression of *dally* was repressed in the FLP-OUT clones that express a constitutively active form of *tkv*, tkv^{Q253D} (M). Positions of the FLP-OUT clones are marked by GFP (N). (C,F,I,L,O) Merged images of the two left-hand images on each row.

life (Teleman and Cohen, 2000), it is also likely that Dally stabilizes Dpp or the Dpp/receptor complex by protecting them from extracellular degrading enzymes, or by reducing the rate at which the internalized signaling complexes are delivered to lysosomes. Increased pMad staining was observed also in several clones induced at the hinge region (Fig. 4D-F). However, in some clones located in the peripheral wing pouch, no increase of pMad staining was seen (Fig. 4G-I). As Dpp levels are low and the levels of endogenous *dally* expression are already high in these regions, the elevated levels of Dally in this experiment might not be sufficient to induce pMad accumulation. Thus, *dally* positively controls Dpp signaling in a cell autonomous fashion, and the sensitivity of cells to *dally* misexpression is different depending on the position of the misexpressing cells in the wing pouch.

dally affects the Dpp morphogen activity gradient formation

We next tested whether *dally* mutations affect the spatial patterning of Dpp activity by using an anti-pMad antibody. As is evident from Fig. 5, pMad patterns in wing discs from *dally*-hypomorphic mutants ($dally^{gem}/dally^{gem}$ and $dally^{gem}/dally^{\Delta P\text{-}527}$) show two abnormal features. First, the mutant discs lose the ability to downregulate Dpp signaling in A/P border cells. Second, in the receiving cells, the smooth gradient of the pMad signal was not seen in the mutant discs. This was particularly evident in the P compartment; the pMad levels are high at the central domain (two-cell widths from the compartment boundary) but suddenly fall in sites where normally they would decline gradually. As a result, *dally* mutant discs show abnormally high levels of pMad in the center of the disc and low pMad levels in the peripheral regions (Fig. 5C,G). We also observed a similar but less severe phenotype in *dally* heterozygotes (Fig. 5B,F), which suggests that gradient formation seems to be sensitive to *dally* gene dosage.

We found that overexpression of *dally* at the A/P border cells also results in abnormal pMad distribution (Fig. 5D,H). The pMad levels in these discs were high at the central region but abnormally low in the receiving cells. Unlike the pMad patterns observed in *dally* mutants, the high pMad signals were restricted to the *dally*-overexpressing domain and were not seen in the receiving cells. This pattern suggests that excess Dally sequesters Dpp proteins at the site of expression.

We noticed that the pMad pattern seen at the *dally* mutants is similar to the abnormality observed in discs overexpressing *tkv*, in which the elevated levels of Tkv retard the distribution of Dpp protein (data not shown) (Lecuit and Cohen, 1998; Tanimoto et al., 2000). This similarity between *dally* mutants and *tkv*-overexpressing discs raises the possibility that *dally* normally downregulates *tkv* expression. In this scenario, *dally* mutations would induce high levels of *tkv* expression, resulting in abnormal distribution of pMad signals. To determine if this is the case, we analyzed *tkv* expression in the same *dally*-mutant backgrounds that had been used for the analysis of the pMad distribution ($dally^{gem}/dally^{gem}$ and $dally^{gem}/dally^{\Delta P\text{-}527}$). We found that levels and expression patterns of *tkv::lacZ* (Fig. 6A,B) and Tkv protein (Fig. 6C,D) in *dally* mutants were indistinguishable from those in wild-type discs. This result indicates that Dally regulates Dpp activity

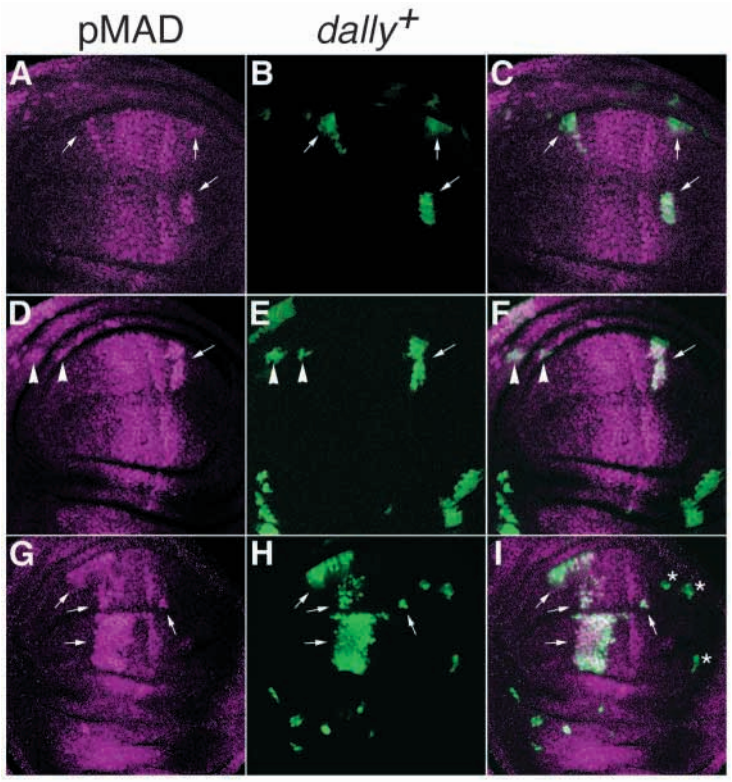


Fig. 4. Ectopic expression of *dally* increases the sensitivity of cells to Dpp. Three examples are shown for anti-pMad antibody staining of wing discs bearing clones of cells overexpressing *dally*. (A,D,G) Patterns of pMad. (B,E,H) Position of *dally*-expressing clones marked by GFP expression. (C,F,I) Overlay images. The pMad levels are increased in the *dally*-expressing clones in the wing pouch (arrows) and the hinge region (arrowheads). Clones induced in the peripheral domain did not show the elevated pMad signals (asterisks).

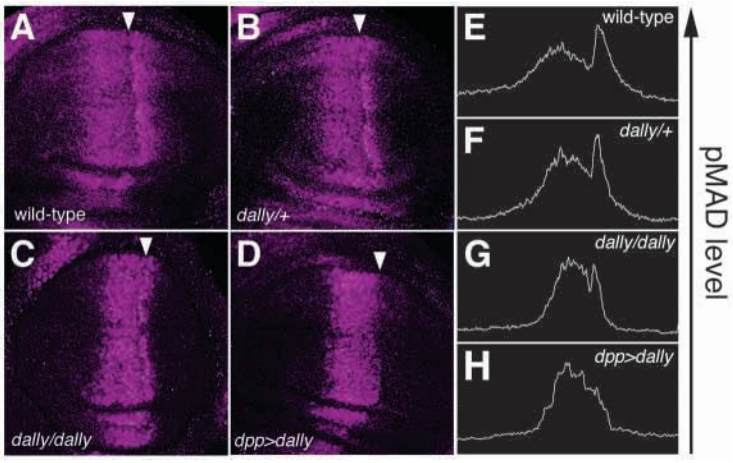


Fig. 5. *dally* affects the Dpp morphogen activity gradient. (A-D) Anti-pMad antibody staining of the wing discs from wild-type (A), $dally^{gem}/+$ (B), $dally^{gem}/dally^{gem}$ (C) and *dpp-GAL4/UAS-dally* (D) animals. Arrowheads indicate the AP boundary. (E-H) Graphs indicate intensity profiles for pMad levels shown in A-D.

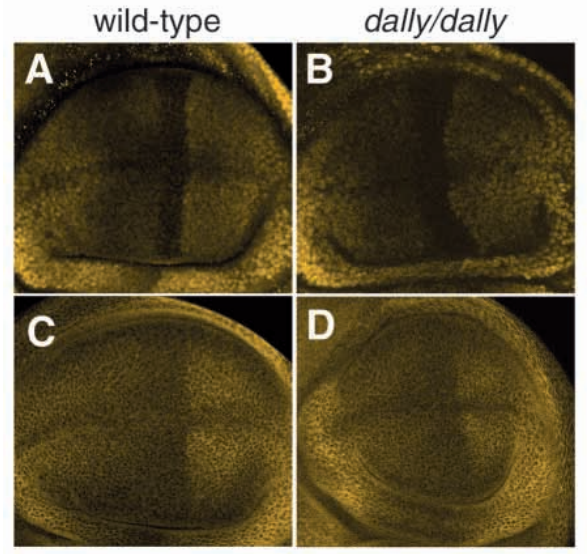


Fig. 6. Expression of *tkv* in *dally* mutant wing disc. (A,B) *tkv*-enhancer trap expression was monitored using anti-β-galactosidase antibody staining in wild-type (A) and $dally^{gem}/dally^{gem}$ (B) backgrounds. (C,D) Anti-Tkv antibody staining of wild-type (C) and $dally^{gem}/dally^{gem}$ (D) wing discs. *tkv* expression is not affected by the combinations of *dally* hypomorphic alleles, $dally^{gem}/dally^{gem}$ and $dally^{gem}/dally^{\Delta P\text{-}527}$ (data not shown), that alter the shape of the Dpp activity gradient (Fig. 5C).

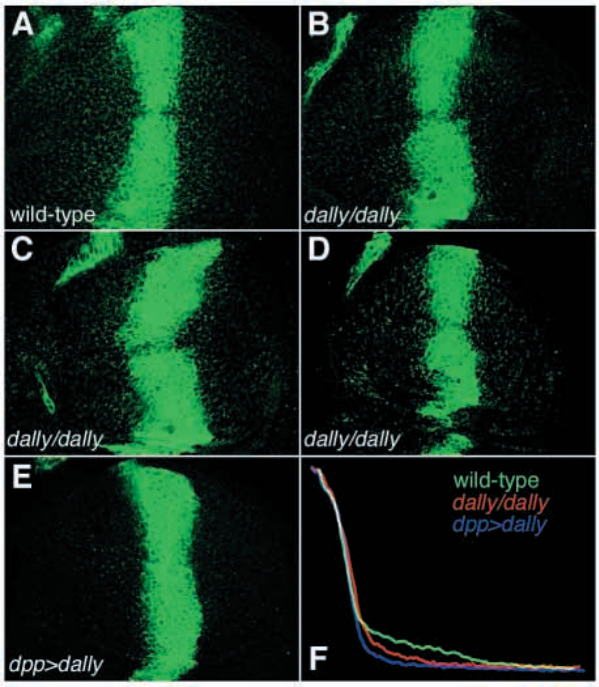


Fig. 7. Distribution of Dpp-GFP in *dally* mutant wing disc. Dpp-GFP was expressed in A/P border cells by *dpp-GAL4* and its distribution was monitored. Wild-type for *dally* gene (A), $dally^{gem}/dally^{gem}$ (B-D), and *dpp-GAL4/UAS-dally* (E) larvae. All discs were heterozygous for dpp^{d12} (see Materials and Methods). (F) Intensity profiles for Dpp-GFP in the posterior half of discs with three different genotypes are shown. 10, 8 and 12 samples were used for wild-type (green), *dally* homozygote (red) and *dpp-GAL4/UAS-dally* (blue), respectively, to obtain the averaged profiles.

gradient formation via a mechanism that is independent of *tkv* expression.

dally affects the Dpp ligand gradient formation

To examine the effect of *dally* mutations on the distribution of Dpp morphogen, we expressed Dpp-GFP in the region where it is endogenously expressed using *dpp-GAL4*. In wild-type discs, Dpp-GFP was detectable as intracellular punctate spots and on the surface of the receiving cells (Fig. 7A), as previously reported (Entchev et al., 2000; Teleman and Cohen, 2000). Dpp-GFP migrates throughout the wing pouch region, forming a shallow but evident gradient. However, in *dally*-mutant discs, we could not detect an evident gradient of Dpp distribution in the receiving cells (Fig. 7B-D). In general, mutant discs showed a lower level of cell surface signals, suggesting reduced stability of Dpp.

To determine whether *dally* overexpression at the A/P border cells, which causes abnormal patterns of pMad (Fig. 5D,H), also affects Dpp ligand gradient formation, we observed Dpp-GFP distribution in discs where *dally* was co-expressed with Dpp-GFP using *dpp-GAL4*. Consistent with the pMad patterns (Fig. 5D), Dpp was restricted to the *dally*-overexpressing region and failed to migrate properly (Fig. 7E). This suggests that Dally binds to Dpp protein and limits its distribution. Intensity profiles of these discs (compared in Fig. 7F) show that both reduction of *dally* and overexpression of *dally* at the A/P border cells result in a shallower gradient and lower levels of Dpp in the receiving cells. Taken together, Dally regulates formation of both Dpp ligand and activity gradients. In addition, our results strongly suggest that Dally plays at least two roles in the formation of the Dpp signaling gradient: (1) it regulates the sensitivity of cells to Dpp in a cell autonomous fashion; and (2) it affects Dpp protein distribution, which is a non-autonomous effect.

DISCUSSION

Mechanisms of Dally function in Dpp signaling

Although Dpp is one of the most extensively studied morphogens, the molecular mechanisms by which the Dpp morphogen gradient is generated and maintained are poorly understood. Previously, it has been suggested that HSPGs affect signaling and distribution of BMPs (Grisaru et al., 2001; Jackson et al., 1997; Paine-Saunders et al., 2002; Paine-Saunders et al., 2000). The present study demonstrates that *dally* controls shape of both the ligand and the activity gradients of Dpp in the developing wing. How does *dally* contribute to the Dpp gradient formation? In vitro analyses using mammalian tissue culture cells have established that HSPGs can increase FGF signaling by stabilizing FGF/FGF receptor complexes (Sperinde and Nugent, 1998; Sperinde and Nugent, 2000). Several lines of evidence indicated that the dosage of HSPGs is an important factor for FGF signaling. For example, sodium chlorate treatment, which inhibits the sulfation of heparan sulfate, reduces the biological response of cells to FGF; the response can be restored by an exogenous supplement of heparin. However, restoration is seen only at an optimal concentration of heparin; excess heparin competes for FGF with signaling complex, resulting in a reduction of signaling (Krufka et al., 1996). In the *Drosophila* wing, ectopic

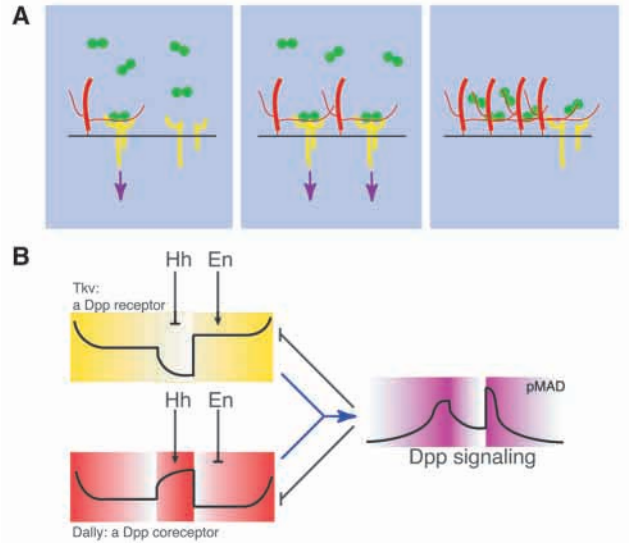


Fig. 8. Expression patterns and function of *dally* contribute to shaping the Dpp morphogen gradient in wing disc. (A) Model for Dally function in Dpp signaling. Dally (red) forms a signaling complex with Dpp (green) and receptor molecules (yellow) on the cell surface (left). Purple arrows represent signaling activity. Increased levels of Dally can enhance Dpp signaling by stabilizing the signaling complex (middle). However, excess levels of Dally sequester Dpp protein and show an inhibitory effect on signaling (right). (B) Levels of *tkv* expression (yellow), *dally* expression (red) and Dpp signaling (purple). Expression of both Tkv (a Dpp receptor) and Dally (a Dpp co-receptor) is regulated by several common molecular pathways in the wing. (1) Hh signaling suppresses *tkv* and activates *dally*. (2) En induces *tkv* and represses *dally*. (3) Dpp signaling downregulates both genes. As the Tkv receptor and Dally co-receptor mediate Dpp signaling, this regulatory pathway forms a negative-feedback loop. At anterior and posterior edges of the wing pouch, lower levels of Dpp signaling result in high levels of Tkv and Dally, which sensitize cells to Dpp.

expression of Dally-like, another glypican related to Dally, leads to a massive accumulation of extracellular Wg protein and compromises Wg signal transduction, suggesting that the glypicans can affect ligand stability and distribution (Baeg et al., 2001).

On the basis of these previous studies as well as our data, Dally would appear to have both positive and negative roles on Dpp signaling (Fig. 8A). In its positive role, Dally serves as a co-receptor for Dpp, stabilizing Dpp protein and enhancing signaling. Conversely, given that Dpp is a heparin-binding protein (Groppe et al., 1998), Dally may bind Dpp through its heparan sulfate chains and reduce the amount of free Dpp ligands. Thus, Dally affects the Dpp gradient at two distinct steps: signal transduction (autonomous effect) and ligand distribution (non-autonomous effect). We propose a model in which alterations in the shapes of the Dpp ligand and the activity gradients caused by *dally* mutations and *dally* overexpression are interpreted as sum of these plus and minus effects of Dally function. In this model, Dally normally sequesters Dpp protein to some extent in A/P border cells, where *dally* levels are very high. Therefore, reduced levels of Dally in mutant discs may result in the release of Dpp ligand and, consequently, higher levels of signaling activity in the central region. Therefore, *dally* mutations may severely reduce the stability of Dpp protein as well as its signaling activity in the receiving cells. When *dally* is overexpressed in A/P border cells, Dpp is trapped by binding to excess Dally and fails to distribute properly.

The model described above is based on the idea that the Dpp gradient is established by diffusion. The diffusive mechanisms of morphogen gradient formation are supported by a recent theoretical analysis (Lander et al., 2002). However, our results do not rule out the possibility that Dally plays a more active role in facilitating Dpp diffusion or 'carries' Dpp protein. For example, it is possible that Dally is required for the Dpp movement through the transcytosis pathway (Entchev et al., 2000; Gonzalez-Gaitan and Jackle, 1999) or other transport systems, such as cytonemes (Ramirez-Weber and Kornberg, 1999) and argosomes (Greco et al., 2001).

Regulated expression of receptor and co-receptor for stable morphogen gradient

We also showed that *dally* expression is regulated by the same set of signaling pathways that control expression of *tkv*. Both genes are regulated by Hh in A/P border cells and by En in the P compartment (Fig. 1, Fig. 8B), but the effects of Hh and En on *dally* are opposite to those on *tkv*. In addition, *dally* expression is negatively controlled by Dpp signaling. Through this mechanism, relative levels of *dally* expression are higher at the anterior and posterior distal edges. Therefore, *dally* and *tkv* show similar patterns of expression with one exception: the level of *dally* expression is high in A/P border cells, where Dpp is synthesized and secreted, but by contrast, *tkv* expression levels are low in this region (Fig. 1, Fig. 8B). The high levels of *dally* in the peripheral regions could sensitize cells to low levels of Dpp, as has been shown for *tkv* (Lecuit and Cohen, 1998). These regulatory pathways appear to form negative feedback loops, which may stabilize the shape of the Dpp morphogen gradient. Thus, the regulated expression and function of Dally are crucial factors in the generation and maintenance of the Dpp morphogen gradient.

We are grateful to S. Cohen, M. Nakamura, T. Tabata, P. ten Dijke and the Bloomington Stock Center for antibodies and fly stocks. We thank S. Selleck, S. Stringer and W. Waldrip for helpful discussions. This work was supported in part by the Human Frontier Science Program.

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