Developmental Cell
Short Article

Antagonistic Growth Regulation by Dpp and Fat Drives Uniform Cell Proliferation

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DOI 10.1016/j.devcel.2010.11.007

SUMMARY

We use the Dpp morphogen gradient in the *Drosophila* wing disc as a model to address the fundamental question of how a gradient of a growth factor can produce uniform growth. We first show that proper expression and subcellular localization of components in the Fat tumor-suppressor pathway, which have been argued to depend on Dpp activity differences, are not reliant on the Dpp gradient. We next analyzed cell proliferation in discs with uniformly high Dpp or uniformly low Fat signaling activity and found that these pathways regulate growth in a complementary manner. While the Dpp mediator Brinker inhibits growth in the primordium primarily in the lateral regions, Fat represses growth mostly in the medial region. Together, our results indicate that the activities of both signaling pathways are regulated in a parallel rather than sequential manner and that uniform proliferation is achieved by their complementary action on growth.

INTRODUCTION

During development of metazoans patterning and growth are tightly linked. This is partly due to the action of morphogens that serve not only to regulate patterning, but also growth. Gradients of morphogens precisely specify the position of cells within a tissue in a concentration dependent manner. If morphogens would also exert their mitotic function in a concentration dependent manner, one would expect cell proliferation rates to vary as a function of the distance to the source of the morphogen. Studies on organ growth, however, show that this is not the case (Milan et al., 1996; Ten Berge et al., 2008; Towers et al., 2008; Zhu et al., 2008). For example in the *Drosophila* wing imaginal disc, the morphogen Decapentaplegic (Dpp) is expressed in a narrow stripe of cells along the anteroposterior (A-P) compartment boundary, and spreads laterally to establish a gradient along the A-P axis of the primordium (a diagram of the wing primordium is shown in Figures 1A and 1B). In addition to regulating A-P patterning, Dpp also acts as a growth factor promoting cell proliferation. Despite being present in an A-P gradient though, the growth promoted by Dpp occurs uniformly. How this is achieved is a conundrum that has challenged the field for more than a decade (reviewed in Affolter and Basler, 2007; Day and Lawrence, 2000; Schwank and Basler, 2010).

Several models that attempt to explain this conundrum have been proposed and experimentally tested for Dpp in the wing disc. According to one of them—the memory model—the early Dpp gradient primes cells along the A-P axis to acquire different sensitivities toward the mitogenic activity of Dpp, so that to reach a certain proliferation rate cells closer to the source would require higher Dpp levels than cells further away from the source. The threshold model, on the other hand, posits that Dpp drives equal proliferation at any level exceeding a minimal degree of activity. However, both these hypotheses were refuted by experiments showing that uniform Dpp signaling, induced at the onset of wing disc development, leads to non-uniform growth with overproliferation in the lateral regions of the wing disc (Schwank et al., 2008).

The gradient models provided another potential explanation for uniform proliferation (Gelbart, 1989; Lawrence and Struhl, 1996). They predict that neighboring cells compare the levels of Dpp they are exposed to and proliferate if the differences are sufficiently high. Rogulja and Irvine (2005) proposed a refinement of the gradient idea, whereby the wing disc is divided into two distinct regions along the A-P axis, medial and lateral; proliferation of medial cells only depends on the *slope* of the Dpp gradient, whereas proliferation of lateral cells can also be triggered by *absolute* Dpp levels, even when these are uniform. In more recent work, Rogulja et al. (2008) provided evidence that the Dpp signaling gradient acts through the Fat tumor suppressor pathway. Based on these findings they proposed that the Dpp gradient regulates the expression and localization of Fat pathway components, and that Fat signaling through Dachs is necessary for the influence of the Dpp gradient on proliferation. Our own findings, however, challenged the validity of any gradient model to explain the mode of Dpp action on growth (Schwank et al., 2008). We found that juxtaposition of cells with different Dpp signaling levels is not necessary to drive proliferation, neither in the lateral nor medial area. Instead, our data suggested the existence of, at least, one additional, unidentified growth regulator, which acts mainly in parallel and complementary to Dpp, inhibiting growth predominantly in the medial region along the A-P axis of the wing primordium (the different regions of the wing primordium are illustrated in Figures 1A and 1B). The combined action of the two pathways then results in uniform proliferation.

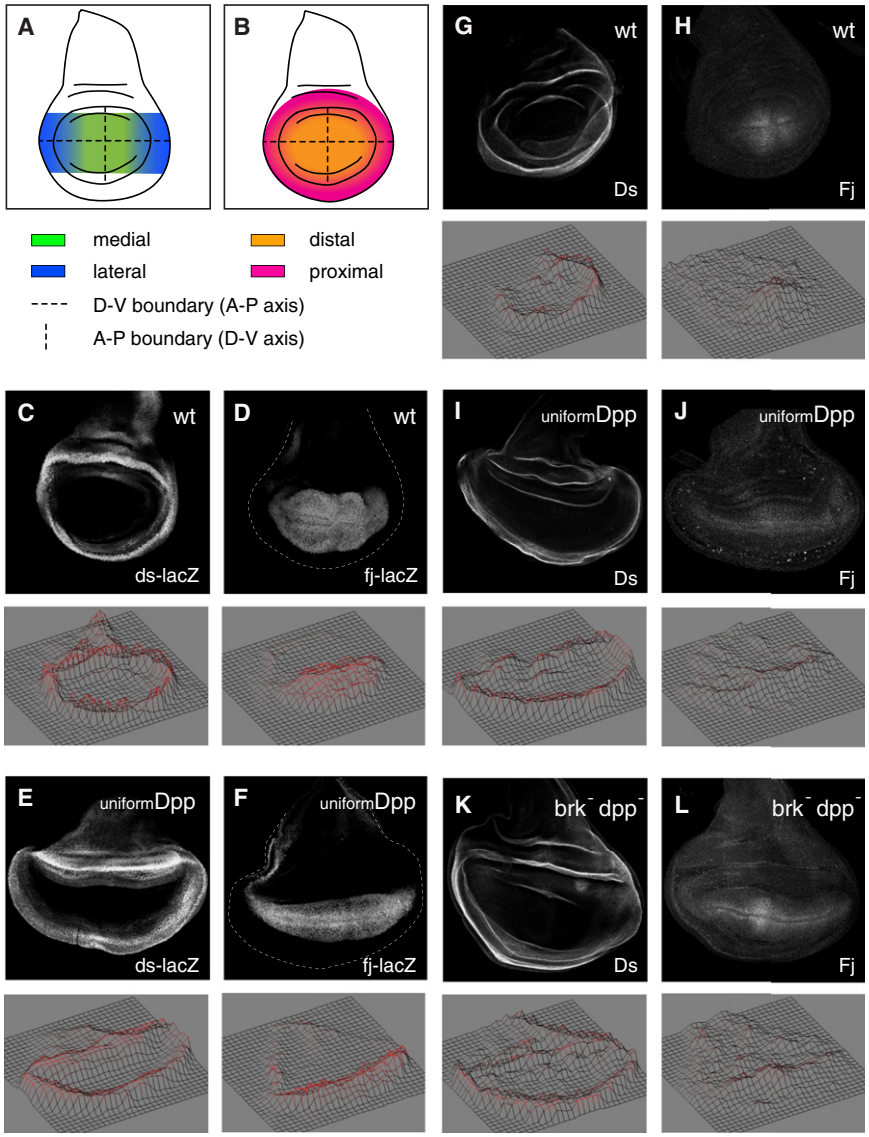

Figure 1. P-D *fj* and *ds* Gradients under Conditions of Uniform Dpp Signaling

(A and B) Diagrams of wild-type wing imaginal discs. We defined the A-P axis as a line approximated to the D-V compartment boundary (dashed horizontal line), and the D-V axis as a line approximated to the A-P boundary (dashed vertical line). In (A) we define the medial (green) and lateral (blue) regions along the A-P axis of the wing primordium, and in (B) we define the distal (orange) and proximal (magenta) regions along the P-D axis of the wing primordium. The borders between the medial-lateral and proximal-distal regions are overlapping and were defined according to high (lateral) versus low (medial) levels of *brk* expression along the A-P axis, and high *ds* (proximal) versus high *fj* (distal) expression levels along the P-D axis.

(C–F) Immunostainings for *ds-lacZ* and *fj-lacZ* reporters in wild-type (C and D), *C765 > dpp* (E) and *C765 > dpp;dpp^{8}/dpp^{12}* (F) discs. For verification of uniform Dpp signaling in discs expressing *dpp* under the *C765-Gal4* driver (see Schwank et al. [2008] and Figure S1).

(G–L) Immunostainings for Ds and Fj in wild-type (G and H), *C765 > dpp;dpp^{8}/dpp^{12}* (I and J) and *brk^{XA};dpp^{8}/dpp^{12}* (K and L) mutant wing discs. The intensities are projected as reliefs and are shown below the corresponding confocal images. Note that the *brk^{XA}* mutation is a *lac-Z* insertion in the *brk* locus, thus *ds-lacZ* and *fj-lacZ* reporters could not be used in this genetic background.

Here, we propose that the Fat signaling pathway is this second pathway. Taking advantage of two independently regulated binary expression systems, we are able to show that the Fat pathway activity is not dependent on graded Dpp levels, and that both pathways act in a complementary manner on growth, directing uniform proliferation along the A-P axis of the wing primordium.

RESULTS

The Proximodistal Expression Gradients of the Fat Regulators *four-jointed* and *dachsous* Are Established Independently of Graded Dpp Activity

To evaluate the role of the Fat pathway in mediating the growth-regulatory capacity of the Dpp gradient we first analyzed the expression of Fat pathway components in discs with uniform Dpp signaling activity. Two genes, *four-jointed* (*fj*), encoding a Golgi-associated kinase, and *dachsous* (*ds*), encoding an atypical cadherin, act genetically upstream of *fat* (Yang et al., 2002). *fat* encodes an atypical cadherin that functions as a transmembrane receptor for the Fat signaling cascade (Brittle et al., 2010; Cho and Irvine, 2004; Fanto et al., 2003; Willecke et al., 2006). *fj* and *ds* are expressed in inverse proximodistal (P-D) gradients in the wing primordium, with *fj* levels higher distally and *ds* levels higher proximally (for area definition, see Figures 1A and 1B); their graded expression is essential for the influence of Fat signaling on growth (Rogulja et al., 2008; Simon et al., 2010; Willecke et al., 2008).

Based on data resulting from clonal activation of the Dpp pathway, it was postulated that graded Dpp levels drive growth via the Fat pathway by influencing the expression of *fj* and *ds* (Rogulja et al., 2008). To test this model in a more direct way, we examined whether the P-D expression gradients of *fj* and *ds* are altered in discs where Dpp signaling is not graded. Uniform Dpp signaling was achieved by expressing *dpp* under control of a uniformly active *Gal4* driver in a wild-type (*C765 > dpp*) and in a *dpp* mutant background (*C765 > dpp; dpp^{8}/dpp^{12}*). We verified that Dpp signaling was uniform by monitoring the phosphorylation of Mad (pMad) and the expression of the Dpp target gene *brinker* (*brk*) (Schwank et al., 2008) (see Figure S1 available online). Although uniform Dpp signaling leads to a change in the shape of the wing pouch and thus to

a change in the geometry of *fj* and *ds* expression patterns, the loss of the Dpp gradient did not diminish the P-D expression gradients of either gene (Figures 1C to 1J). The highest expression levels of *fj* and *ds* were in distal and proximal cells, respectively. In an alternative approach we eliminated the Dpp gradient by genetically removing *dpp* together with *brk* ($brk^{XA};dpp^{8}/dpp^{12}$). The *brk* gene, which encodes a growth-inhibitory transcription factor, is a negative target of Dpp, and discs mutant for *dpp* and *brk* phenotypically mimic discs with ectopic uniform Dpp signaling (Campbell and Tomlinson, 1999; Schwank et al., 2008). Also in this genetic setup the loss of the Dpp gradient did not disrupt the *ds* and *fj* gradients (Figure 1K and 1L). Our experiments thus indicate that the P-D expression gradients of *fj* and *ds* are not directly dependent on the slope of the Dpp gradient.

We next examined what other factors could account for the graded expression of *fj* and *ds*. One factor that has previously been implicated in influencing *fj* and *ds* expression is Vestigial (Vg) (Baena-Lopez and Garcia-Bellido, 2006; Cho and Irvine, 2004). In wild-type discs *vg* is expressed at peak levels in the distal region of the wing primordium. Whereas *vg* expression overlaps in large parts with *fj* expression, *vg* and *ds* expression mainly exclude each other. We found that driving *vg* expression uniformly within the entire wing disc under the *escargot-Gal4* driver leads to ectopic downregulation of *ds* and upregulation of *fj* expression (data not shown). Additionally, uniform Dpp signaling activity does not abolish the P-D component of the *vg* expression pattern (Schwank and Basler, 2010), and as for *fj* and *ds*, the graded expression of *vg* has been shown to be essential for promoting growth (Baena-Lopez and Garcia-Bellido, 2006). Taken together, these results argue that *vg* plays an important role in establishing the expression gradients of *ds* and *fj*, and thus in regulating growth via Fat signaling. Although it is not fully understood how the *vg* expression gradient is established, one elegant model implicates an autoregulated feed-forward loop as the primary means by which *vg* propagates and establishes its own expression (Zecca and Struhl, 2007a, 2007b, 2010).

Fat Pathway Activity Is Not Dependent on the Dpp Gradient

Although Dpp did not affect the expression of the upstream components *fj* and *ds* of the Fat pathway, the Dpp gradient might still have an effect on the activity of the Fat pathway. We therefore analyzed the relationship between the Dpp gradient and the activity of the Fat signaling pathway downstream of *fat* by directly monitoring the subcellular localization of Dachs in discs with uniform Dpp signaling. Mutations in *dachs* completely suppress the overgrowth phenotype of *fat* mutant clones, and *dachs* encodes an unconventional myosin whose subcellular localization is regulated by Fat, indicating that it acts downstream of Fat in the Fat signaling network (Mao et al., 2006). Dachs localization can be visualized in clones expressing a V5 epitope-tagged fusion protein when expressed at high levels by the Gal4 system. Such Dachs:V5 protein preferentially accumulates at membranes on the distal side of cells within the clones (Mao et al., 2006; Rogulja et al., 2008). This P-D polarity of Dachs has been proposed to result from a combination of cues emanating from the two compartment boundaries, with the A-P component being defined by the Dpp gradient (Rogulja et al., 2008).

To test whether the P-D polarity of Dachs localization depends on the Dpp gradient, we wanted to monitor clonally expressed Dachs:V5 in discs uniformly expressing Dpp in a *dpp* mutant background. To achieve ubiquitous expression of Dpp as well as clonal expression of Dachs:V5 at high levels, we had to employ a second binary expression system in addition to Gal4. We equipped the DNA binding domain of bacterial LexA (L) with the hinge region (H) and the activation domain of Gal4 (G) and generated an *actin5c* > y^{+} > *LHG* transgene that potently activates a *lexO-Dachs:V5* construct on Flp-mediated recombination (see Experimental Procedures and Yagi et al., 2010).

First, we verified that our setup is able to reveal the P-D asymmetry of Dachs localization in wild-type discs. In agreement with previous results, we found that Dachs accumulates at the membranes on the distal side of the clones (Figure 2A). Previous studies indicated that not all Dachs clones show polarization (Mao et al., 2006). Consistent with this observation, in our setup 148 of 283 clones were polarized. The direction of Dachs localization in each polarized clone was determined by an unbiased blind test (described in Experimental Procedures) and plotted as a vector in a position map based on the relative position of the clone within the wing disc (Figure 2B). This quantitative data was then used to generate a P-D Dachs polarization diagram (Figure 2C and Experimental Procedures). Clones in the very distal region of the wing primordium showed only weak polarization, and therefore were dismissed in the blind test. Next we examined the direction of Dachs polarization in discs with uniform Dpp signaling (Figures 2D–2F). A total of 189 of 384 clones showed polarization, and we again plotted the direction vectors of the polarized clones together with their relative position into a position map (Figure 2E) to generate the polarization diagram (Figure 2F). Our results unambiguously show that Dachs is still polarized on the distal clone edges in discs with uniform Dpp and that the P-D polarization and its A-P component are not lost. These results combined with our analysis of *fj* and *ds* expression patterns indicate that the Fat pathway activity is regulated independently of the slope of the Dpp gradient.

Dpp/Brk and Fat regulate growth in a Complementary Manner along the A-P Axis of the Wing Primordium

In our recent analysis of cell proliferation in response to the Dpp gradient we observed that the Dpp/Brk system curbs proliferation mainly in the lateral regions along the A-P axis of the wing primordium, where *brk* is normally expressed (Schwank et al., 2008). Based on this we postulated the existence of a Dpp-independent signaling system that takes over this inhibitory role in the medial region to ensure even growth along the entire A-P axis of the primordium in wild-type (Schwank et al., 2008). The results presented above provide evidence that the profile of Fat pathway activity is not dependent on the Dpp gradient. Conversely, the profile of Dpp pathway activity does not depend on Fat signaling because neither the Dpp gradient nor the A-P expression pattern of the Dpp target genes *brk*, *spalt* (*sal*) and *optomotor-blind* (*omb*) are lost in *fat* mutant discs (Figure S2). The independent regulation of both pathways, and the fact that Dachs:V5 is polarized stronger in the proximal

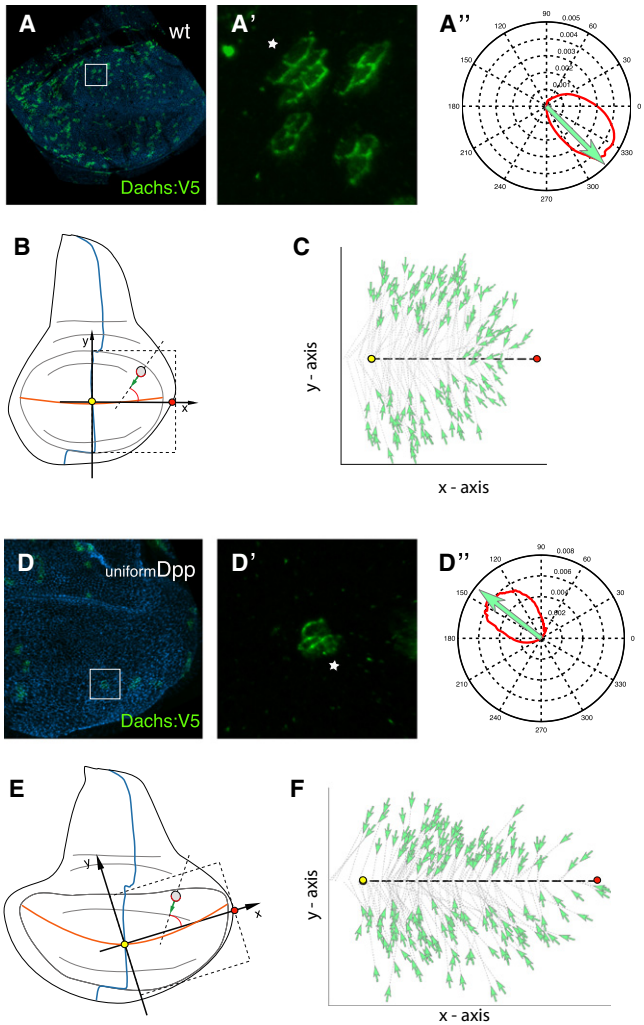

Figure 2. Polarization of Dachs:V5 Localization under Conditions of Uniform Dpp Signaling

Examples of wild-type (A) and *C765 > dpp:GFP; dpp^{8}/dpp^{12}* (D) wing imaginal discs expressing *dachs:V5* (green) in clones via the LexA system. The apical surface of the discs is marked by E-cadherin staining (blue).

(A′) and (D′) show magnifications of the marked regions. (A″) and (D″) illustrate the polarization vectors of the Dachs:V5 localization for the clones marked with a star in (A′) and (D′), respectively. The direction was calculated from signal intensities at the clone borders (described in Experimental Procedures).

(B and E) Schematics of wild-type (B) and *C765 > dpp:GFP; dpp^{8}/dpp^{12}* (E) wing discs. The dashed box and the x- and the y-axes illustrate the area of the corresponding vector plots. The same area in the anterior compartment was analyzed. No difference in P-D polarization of Dachs:V5 was observed between clones of both regions, therefore the data was plotted in the same graph. (C and F) Vector plots showing the polarization vectors of Dachs:V5 expressing clones, obtained in the unbiased blind test. The P-D asymmetry of Dachs polarization was not lost in discs with uniform Dpp signaling, thus the data indicates that Fat signaling activity is not reliant on the Dpp gradient. Likewise, also the Dpp pathway activity does not depend on Fat signaling (see Figure S2).

compared to the distal region of the wing disc, raises the possibility that the Fat pathway represents the complementary growth system that we proposed to curb growth in the medial region along the A-P axis of the wing primordium.

To test this possibility we compared the cell proliferation pattern of discs with uniform Dpp signaling (lacking *brk* activity), with that of *fat* discs (lacking Fat pathway activity). Uniform Dpp signaling was again achieved by means of the *C765-Gal4* driver (*C765 > dpp*) and genetically by removing *dpp* together with *brk* (*brk^{XA};dpp^{8}/dpp^{12}*). The Fat pathway was blocked by combining the two amorphic *fat* alleles *ft^{fd}* and *ft^{GR-V}* (Bryant et al., 1988; Willecke et al., 2006). Cell proliferation was monitored by incorporation of 5-ethynyl-2′-deoxyuridine (EdU), a marker for cells in S-phase. To better control for differences in the proliferation pattern among individual discs, EdU positive nuclei were recorded from 10 discs with a nuclei detector, integrated and displayed as a cell proliferation map (Figure 3) (for a detailed description and EdU sample images, see Figure S3 and Experimental Procedures). As was previously observed, the proliferation pattern in discs with ubiquitous Dpp signaling is no longer uniform, but is higher in the lateral regions than in the medial region of the primordium (Figures 3B and 3E; results for *brk^{XA};dpp^{8}/dpp^{12}* discs see Figure 3J). Interestingly, *ft$^{fd/GR-V}$* mutant discs exhibit the opposite phenotype, and proliferation along the A-P axis of the primordium is increased in the medial region compared to lateral regions (Figures 3C and 3F). Nonuniformity in proliferation can be observed already at 96 hr after egg laying (AEL) and becomes stronger later in development (120 hr AEL). Increase in nonuniformity becomes enforced because overproliferating cells can curb growth in proximate regions of the wing disc (Schwank et al., 2008). This effect is seen in discs with uniform Dpp signaling as well as *fat* mutant discs (Figures 3E and 3F). Staining for cleaved caspase-3 indicates that apoptosis is neither increased in discs with ubiquitous *dpp* signaling nor in *fat* mutant discs (data not shown). These results are consistent with the notion that along the A-P axis of the wing primordium the Dpp/Brk and the Fat systems inhibit growth in a complementary manner to achieve uniform proliferation (Figure 4).

Finally, we also tested this model genetically. If the two systems act in a complementary manner to control growth, one would expect that in discs mutant for *fat* and uniformly expressing *dpp* the proliferation levels will be high both in medial *and* lateral regions of the wing primordium. This was in fact what we observed when the ubiquitous Dpp expression system was introduced into a *fat* mutant background: overgrowth is enhanced and more homogenous compared to the effects resulting from the individual manipulations alone (Figure 3G and 3K).

A further prediction of the model is that along the A-P axis overproliferation in the medial region of *fat* mutant discs should be counteracted by inhibition of the Dpp pathway, whereas overproliferation in the lateral regions caused by uniform Dpp signaling should be counteracted by ectopic activation of the Fat pathway. To attain the first situation we ubiquitously expressed *daughter-against-dpp* (*dad*, encoding a negative feedback regulator of the Dpp pathway) in a *fat* mutant background. Inhibition of the Dpp pathway by *dad* overexpression fully counteracted the increased proliferation occurring in the medial region of *fat^{-}* discs (Figures 3H and 3K). Conversely, overproliferation in the lateral regions by uniform *dpp* expression was blocked by the ectopic Fat pathway activity occurring in a *dachs* mutant background (Figures 3I and 3K). In both cases the proliferation pattern became more uniform. Taken together,

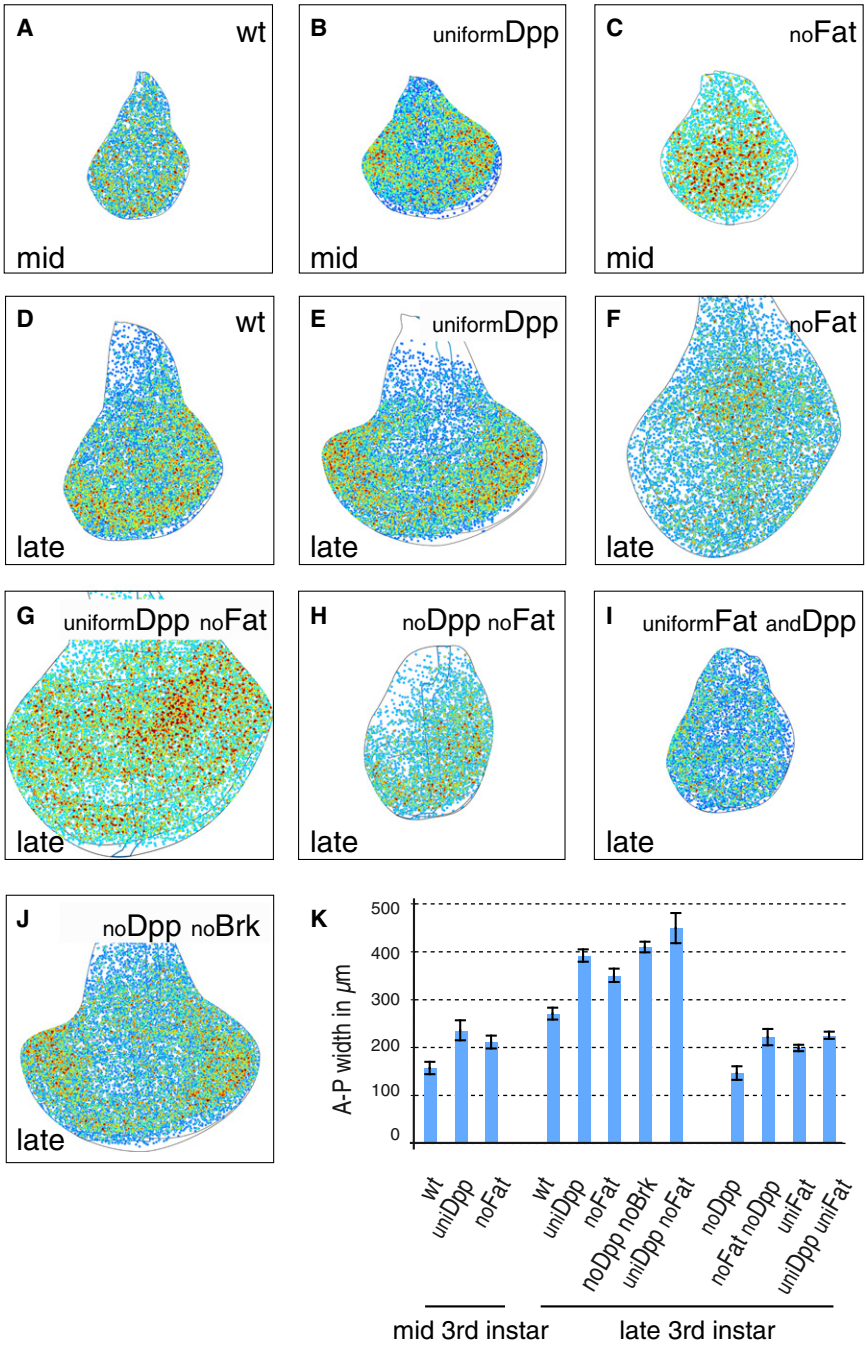

Figure 3. Dpp and Fat Signaling Regulate Growth in the Wing Disc in a Complementary Fashion

(A–J) Proliferation pattern displayed by cell proliferation maps in wing discs with modulated Dpp and/or Fat signaling activities. The maps illustrate the growth constant *k* (described in Experimental Procedures). The scale bar is standardized, making the rates comparable between the different genotypes. The highest values are shown in red, and the lowest values in blue/transparent. The rates were calculated from EdU incorporation data of 10 discs per genotype. (A–C) Cell proliferation maps of wing discs at mid third instar stage, 96 hr after egg laying (AEL).

(D–J) Cell proliferation maps of wing discs at late third instar stage, 120 hr AEL. Discs are from wild-type (A and D), *C765 > dpp* (B and E), $fat^{GR\text{-}V}/fat^{fd}$ (C and F), *C765 > dpp;* $fat^{GR\text{-}V}/fat^{fd}$ (G), *esg > dad;* $fat^{GR\text{-}V}/fat^{fd}$ (H), $dachs^{GC13}$; *C765 > dpp* (I), and brk^{XA};dpp^{8}/dpp^{12} (J) larvae. Note that $dachs^{GC13}$ and brk^{XA};dpp^{8}/dpp^{12} mutant larvae are delayed in development for 24 hr; to adjust for this delay $dachs^{GC13}$, *C765 > dpp;*$dachs^{GC13}$, and brk^{XA};dpp^{8}/dpp^{12} larvae were dissected 144 hr AEL. (K) Comparison of the sizes of discs with the different genotypes. Error bars indicate 95% confidence intervals. Example images for EdU stainings for each genotype are shown in Figure S3.

our results indicate that the Fat and the Dpp/Brk systems act together to regulate growth, and that normally their combined activities produce uniform proliferation along the A-P axis of the wing primordium.

DISCUSSION

One key question in development concerns the effect of morphogens on growth: how can a gradient of a proliferation-inducing factor ultimately result in uniform cell proliferation?

The Dpp gradient in the *Drosophila* wing imaginal disc has emerged as an important model to study how morphogen gradients are established and how they operate to orchestrate patterning and growth.

An answer to this problem was provided by Rogulja and Irvine (2005) who demonstrated that the gradient of Dpp pathway activity can influence cell proliferation. Their arguments were mainly based on the observation that sharp discrepancies in Dpp transduction levels can lead to local, transient overproliferation (Rogulja and Irvine, 2005). A follow-up study provided evidence that this effect is exerted via the Fat signaling network (Rogulja et al., 2008). Based on these findings the authors proposed a model, in which graded Dpp levels drive growth by regulating the expression and localization of Fat pathway components. However, the proposed necessity of a Dpp gradient to induce growth was challenged by our finding that if lateral overproliferation is avoided, cell proliferation occurs normally in areas with uniform Dpp signaling (Schwank et al., 2008). Here we further confront the model of Rogulja et al. (2008) by demonstrating that the profile of Fat pathway activity does not depend on the Dpp gradient. Conditions of uniform Dpp signaling neither affected the graded expression of *fj* and *ds*, which encode upstream regulators of the Fat pathway, nor

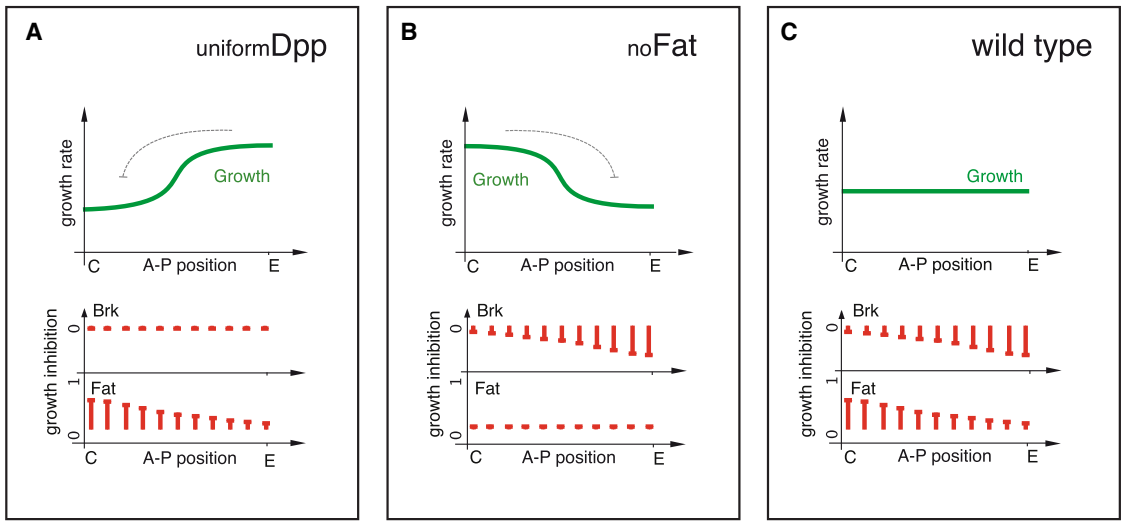

Figure 4. The "Opposing Growth Pathways" Model Explains Uniform Growth along the A-P Axis of the Wing Primordium

Illustrated are growth rates (upper panels) and Brk and Fat levels (lower panels) along the A-P axis (D-V boundary) of the wing primordium. C stands for center and E for edge of the primordium.

(A) Under conditions of uniform Dpp signaling Brk is repressed everywhere and growth is uneven, with higher proliferation levels in the lateral regions. This non-uniformity derives from remaining differences in Fat activity along the A-P axis.

(B) Removal of Fat leads to the converse proliferation pattern, with higher proliferation levels in the medial region due to remaining differences in Brk levels. Overproliferating cells in (A) and (B) curb proliferation in proximate areas of the wing primordium by an unknown mechanism.

(C) In a wild-type situation the distinct activity outputs of Dpp/Brk and Fat complement each other, and together lead to uniform proliferation along the A-P axis.

the intracellular polarization of Dachs, which serves as a mediator and read-out for Fat pathway activity. We suggest that the transient proliferation induced by sharp appositions in Dpp signaling levels probably does not reflect the mechanism controlling normal growth in the context of an entire disc. Instead this effect might mimic a situation related to wound healing, where considerable differences of morphogen pathway activity become juxtaposed.

If the gradient model does not account for the growth-stimulatory effect of Dpp, how can the uniform growth in a tissue exposed to graded Dpp be explained? Based on our previous observation that in the complete absence of the Dpp/Brk system growth along the A-P axis of the wing primordium occurs nonhomogenously with overproliferation in lateral regions, we proposed the idea that multiple systems modulate and "even-out" growth rates (Schwank and Basler, 2010). In this model, the Dpp/Brk system inhibits growth along the A-P axis of the primordium primarily in the lateral regions, whereas one or more complementary system(s) would inhibit growth mostly in the medial region. The asymmetric proliferation pattern in discs lacking the Dpp/Brk system indicates that the activity profile of the second system must not strictly depend on, and thus act downstream of, Dpp signaling. It is important to note, however, that both systems might still partly influence the activity of each other and could also share target genes via which they modulate growth.

Our observations presented here suggest that the Fat signaling pathway could serve as such an "independent," complementary growth regulation system. First, the activity profile of the Fat pathway is not lost under conditions of uniform Dpp signaling. Second, in contrast to the proliferation pattern in discs with uniform Dpp signaling, inhibition of the Fat pathway leads to overgrowth in the medial region along the A-P axis of the wing primordium. Moreover, overgrowth induced by ectopically modulating one system can be counteracted by ectopically inhibiting growth via the other pathway. Thus we suggest the "opposing growth-pathways" model, by which the distinct activities in Dpp and Fat signaling complement each other and provide the basis for a relatively even proliferation pattern along the A-P axis of the wing primordium (Figure 4).

Because the Dpp gradient is deployed along the A-P axis, and components of the Fat pathway are graded along the P-D axis, the effects of additional growth factors (e.g., deployed along the D-V axis) need to be integrated to achieve uniform proliferation not only along the A-P axis but within the entire wing disc. Further studies are needed to discover such additional growth signals and/or mechanical forces. Finally, it will also be interesting to test if similar regulatory strategies to those described here are used in vertebrates to derive uniform growth rates from the concentration gradients of morphogens.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

Immunostaining was performed using standard protocols. Images were collected with a Zeiss LSM710 confocal microscope. Imaris and ImageJ was used to analyze the images, Z stacks were projected in the three-dimensional view.

EdU Labeling

Before fixation discs were incubated with 5-ethynyl-2′-deoxyuridine (EdU, 10 mM) in clone8 medium for 30 min at room temperature. For EdU staining the Click-iT EdU Alexa Fluor 647 Imaging Kit was used (Invitrogen).

The following antibodies were used: mouse anti-β-Gal (Promega); rat anti-Ds (Yang et al., 2002), rabbit anti-pMad (gift from Ed Laufer, Columbia University, New York), rabbit anti E-cadherin (Santa Cruz Biotechnology), mouse anti-V5 (Invitrogen), mouse anti-Patched (Hybridoma bank), mouse anti-Nubbin (gift from Steven Cohen, National University of Singapore), guinea pig anti-Vg (gift from Gary Struhl, Columbia University), rabbit anti-Fj (gift from David Strutt, University of Sheffield), and rabbit anti-Sal (gift from Ronald Kühnlein, Max-Planck-Institute, Göttingen).

Quantitative Image Analysis

Reliefs of the Fj, Ds, and *lacZ* signal intensities were generated using the ImageJ imaging software.

Radial Plot for Dachs Polarization

The intensities of V5 along the outline of a clone (determined by E-cadherin staining) can be used to estimate the angle α of the polarization vector. For a discrete set of possible angles α_i, the V5 signal is projected onto a line in α_i direction. The projected signal is fitted to a line of slope a_i using least-squares. In Figures 2A and 2D, the slopes a_i are plotted against the angles α_i for one selected clone from each of the genotypes. The polarization vector (arrow) is chosen to be the angle with the largest slope, which corresponds to the strongest localization of Dachs.

Quantitative Vector Plot

The directions of the Dachs polarization vectors were determined in a blind test. All 334 clones with polarized Dachs:V5 were cut out from a print-out on paper, numbered on the backside, mixed, and analyzed without knowing their position, orientation, and genotype. The polarization vectors of the clones were then plotted according to their relative position along the x and y axis using MATLAB (x and y-axes are illustrated in Figures 2B and 2E).

Generation of Cell Proliferation Maps

EdU positive cells were recorded using a nuclei detector in Imaris. Each disc was also marked at 10 reference points (see Figure S3A′) to integrate the EdU results of 10 discs per genotype. The reference points were used to determine the best fitting (in the least-squares sense) affine transformation that maps locations of one disc to a reference disc. The average number of detected nuclei at each point on the disc was used to estimate the probability p for a cell at that position to be actively proliferating. By assuming exponential area growth $A(t) = A_0 e^{kt}$ for the tissue and an expected relative area increase by a factor of 1 + p within the duration Δt_{cc} of a cell cycle, the growth constant k can be computed as $k = \ln(1 + p)/\Delta t_{cc}$. The growth constant k as it varies in space is shown on the cell proliferation maps in Figure 3 and represents the relative rate of area growth ($dA/dt = kA$). The maps were plotted on sketches, which feature the disc shape of each particular genotype and include the *patched* and *nubbin* expression domains illustrated as blue and black lines, respectively. The scale is standardized to have the highest values (red) at p = 1 and the lowest values (blue, transparent) at p = 0.

All mutant genotypes used in this study, and plasmid construction for the LexA system are described in detail in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.devcel.2010.11.007.

ACKNOWLEDGMENTS

We thank R. Kühnlein, E. Laufer, M. Simon, G. Struhl, and D. Strutt for antibodies; K. Irvine for plasmids; and G. Hausmann for critical comments on the manuscript. This work was supported by the Swiss National Science Foundation, the Kanton of Zurich, the SystemsX.ch initiative within the framework of the WingX Project, and the European Research Council under the European Community's Seventh Framework Programme.

Received: June 9, 2010
Revised: September 22, 2010
Accepted: October 25, 2010
Published: January 18, 2011

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