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Growth regulation by Dpp: an essential role for Brinker and a non-essential role for graded signaling levels

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Morphogens can control organ development by regulating patterning as well as growth. Here we use the model system of the *Drosophila* wing imaginal disc to address how the patterning signal Decapentaplegic (Dpp) regulates cell proliferation. Contrary to previous models, which implicated the slope of the Dpp gradient as an essential driver of cell proliferation, we find that the juxtaposition of cells with differential pathway activity is not required for proliferation. Additionally, our results demonstrate that, as is the case for patterning, Dpp controls wing growth entirely via repression of the target gene *brinker* (*brk*). The Dpp-Brk system converts an inherently uneven growth program, with excessive cell proliferation in lateral regions and low proliferation in medial regions, into a spatially homogeneous profile of cell divisions throughout the disc.

KEY WORDS: Brinker, Dpp, Growth, Patterning, Morphogens

INTRODUCTION

How the growth and patterning of organs are regulated is one of the most fundamental questions in understanding animal development. Growth of organs is controlled by extrinsic and intrinsic factors. Extrinsic factors, such as temperature, stress and hormones, have an effect on the overall size of an animal and its organs; but the proportions of organs and tissues are determined by intrinsic growth programs. Evidence for organ-intrinsic mechanisms controlling growth derives from grafting experiments in invertebrates as well as vertebrates (Dittmer et al., 1974; French et al., 1976; Metcalf, 1964; Silber, 1976; Simpson et al., 1980; Twitty and Schwind, 1931). Studies in the chicken limb placed the intrinsic growth programs downstream of patterning (Wolpert, 1981). Also, experiments in the *Drosophila* wing imaginal disc support the hierarchical relationship between patterning and growth. The wing disc is first patterned into compartments, which then function as units of size (Day and Lawrence, 2000; Martin and Morata, 2006). Morphogens were shown to be essential for the correct patterning of organs. Produced at a local source, they spread into the tissue and form a concentration gradient to specify positional information. Besides their role in the patterning of the wing, morphogens also regulate wing growth. In summary, there is evidence that in invertebrates and vertebrates, patterning controls growth and not vice versa. However, the molecular details of how patterning and the intrinsic growth programs are linked remain poorly understood.

The *Drosophila* wing imaginal disc is a useful model system in which to study organ patterning and growth. It originates from an embryonic primordium containing ~40 cells that undergoes a sigmoidal growth period until it reaches its final size of 30,000-100,000 cells (Potter and Xu, 2001). Cells divide with a doubling time of ~10-12 hours (Garcia-Bellido and Merriam, 1971). Although cell divisions occur in clusters of cells and are therefore spatially and temporally dynamic, growth within the overall disc is even (Milan et al., 1996). The wing primordium is patterned by morphogen gradients, with Wingless (Wg) and Decapentaplegic (Dpp) expressed in stripes along the dorsal-ventral and anterior-posterior compartment boundaries, respectively. Movement of these secreted proteins establishes orthogonal concentration gradients along the two axes (Entchev and Gonzalez-Gaitan, 2002). The graded distribution of the Dpp ligand leads to changes in the Punt-Tkv receptor complex, which in turn control the ratio of phosphorylated to unphosphorylated Mad. Phosphorylated Mad (pMad) forms a complex with Medea, enters the nucleus and, together with its co-factor Schnurri, represses transcription at the *brinker* locus (*brk*), creating a gradient of *brk* expression that is reciprocal to the Dpp gradient. Brk is a transcriptional repressor that acts negatively in a dosage-dependent manner to establish the nested expression domains of other genes, such as *spalt* (*sal*; *spalt major* – FlyBase) and *optomotor-blind* (*omb*; *bifid* – FlyBase), which position longitudinal veins along the anteroposterior (AP) axis of the wing (Campbell and Tomlinson, 1999; Cook et al., 2004; Jazwinska et al., 1999; Marty et al., 2000; Minami et al., 1999; Sivasankaran et al., 2000; Sturtevant et al., 1997). Brk binds to these Dpp target genes via the sequence GGCGYY (Cordier et al., 2006) and uses different molecular mechanisms and co-repressors to regulate their expression, leading to the differential sensitivity of the target genes to Brk (Hasson et al., 2001; Winter and Campbell, 2004). Another difference between the regulation of *omb* and *sal* expression is that although the expression boundaries of both genes are solely set by Brk levels, the rate of *sal*, but not of *omb*, transcription requires a direct Dpp input (Marty et al., 2000). For a detailed review of the molecular mechanisms of Dpp morphogen readout see Affolter and Basler (Affolter and Basler, 2007).

Besides its well-described role in patterning, previous studies have indicated that Dpp also acts as a growth-promoting factor. Hypomorphic *dpp* alleles, which lead to a loss of Dpp expression in the wing imaginal discs, result in small wings (Spencer et al., 1982; Zecca et al., 1995), whereas ectopic expression of Dpp causes abnormally large discs (Burke and Basler, 1996; Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002). Furthermore, cell clones lacking Dpp signaling fail to survive, and clones in which the Dpp pathway is uniformly activated overgrow (Adachi-Yamada et al., 1999; Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002; Moreno et al., 2002). However, key aspects regarding the mechanism of growth control by Dpp remain to be resolved.

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One particularly intriguing issue is how a growth factor that is distributed in a gradient can lead to uniform growth in the wing disc. Several models have attempted to answer this conundrum. In one such model (the threshold model), the proliferation rate is constant for cells in which Dpp signaling activity exceeds a certain minimal level. Studies with the growth-promoting gene *vestigial* (*vg*) have shown that certain levels of Dpp and Wg together fuel a feed-forward autoregulation of *vg* expression (Zecca and Struhl, 2007a; Zecca and Struhl, 2007b), thus supporting the threshold model. However, the overproliferation behavior of clones with high experimental Dpp signaling levels calls for an additional mechanism of growth regulation by Dpp. In a second model (the cellular fate model), cells along the AP axis of the wing are programmed for differential sensitivity to Dpp. Such pre-patterning might occur in a Dpp-dependent or -independent manner. According to a third model (the inhibitor model), an additional growth-modulatory gradient exists, which parallels that of Dpp but acts in an antagonistic manner. Although no strong evidence exists against the cellular fate or inhibitor models, experimental support for the existence of pre-patterning or inhibitory factors is missing. Another concept suggests that growth depends on the slope of the Dpp gradient (Day and Lawrence, 2000; Gelbart, 1989; Lawrence and Struhl, 1996). A recent experimental study has led to a refinement of this model that incorporates aspects of the cellular fate model (Rogulja and Irvine, 2005): the proliferation of medial cells depends on the slope of the Dpp gradient, whereas proliferation in lateral regions can be triggered by both constant and graded signaling levels. However, this model fails to explain growth at the source of Dpp production, where a considerable area is exposed to equal, saturating levels of the Dpp signal. Moreover, medial clones in which Dpp signaling was uniformly activated showed no growth disadvantage (Martin-Castellanos and Edgar, 2002). A completely different explanation for uniform growth within the wing disc is provided by mechanical feedback models, in which growth rates differ initially owing to different Dpp levels. These differences cause mechanical stresses (compression and stretching) that in turn affect growth rates, resulting in uniform growth (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007; Shraiman, 2005).

Besides the problem of how the Dpp gradient directs uniform growth, the role of Brk in growth regulation remains elusive. The surprising discovery that the expression boundaries of the Dpp patterning targets *sal* and *omb* are set by Brk raised the question of whether Brk plays an equally important role in growth. Experiments conducted thus far indicate that Brk is at least partially involved in growth control. Overgrowth is not only observed in wing discs with constitutive Dpp signaling (Capdevila and Guerrero, 1994; Lecuit et al., 1996; Nellen et al., 1996), but also in *brk* mutant discs (Campbell and Tomlinson, 1999; Nellen et al., 1996), and cell clones that lack Dpp signaling, and therefore fail to survive, can be rescued by eliminating *brk* function (Marty et al., 2000). However, whether Dpp has an additional growth-promoting function in parallel to its repression of *brk* remained unresolved.

Here, we experimentally address whether Dpp regulates growth via Brk, and how the graded activity of Dpp can cause uniform growth. Our experiments demonstrate that Dpp regulates growth exclusively by repressing *brk*, and that the main function of the Dpp-Brk system is to limit proliferation in lateral areas of the wing disc. Second, we confirm that medial and lateral cells in the wing disc react differently to Dpp, and provide evidence that this distinct behavior depends on a mechanism independent of the Dpp-Brk system, effectively ruling out the possibility that the regionally different responses to Dpp are due to an earlier Dpp-dependent fate specification. Finally, we reveal that neither lateral nor medial cells need a Dpp gradient to proliferate. Repression of *brk* in the medial area and derepression of *brk* in the lateral area in a non-graded manner are sufficient for uniform growth in the entire disc.

Thus, our results challenge the gradient models, which imply that the juxtaposition of cells with different Dpp signaling levels induces proliferation. Indeed, the data suggest an alternative model in which the Dpp levels along the AP axis set different *brk* expression levels: high laterally and low medially. The high lateral Brk levels are needed to curb the overproliferation that would otherwise occur in this region.

MATERIALS AND METHODS

Clone generation, transgene induction and mutant genotypes

For the twin-spot analysis, the following larvae were generated.

yw hsp70-flp hsp70-GFP FRT19A/yw FRT19A.

w tubP-Gal80 hsp70-flp FRT19A/yw hsp70-flp hsp70-GFP FRT19A; tubP-Gal4/UAS-tkv^{Q235D}.

yw brk^{M68} hsp70-flp FRT19A/yw hsp70-flp hsp70-GFP FRT19A.

yw tubP-Gal80 brk^{M68} hsp70-flp FRT19A/yw hsp70-flp hsp70-GFP FRT19A; tubP-Gal4/UAS-tkv^{Q235D}.

To obtain these transgenic animals, the following stocks were used: *w tubP-Gal80 hs-flp FRT19A* (stock 5132, Bloomington), *tubP-Gal4* (stock 5138, Bloomington), *hsp70-GFP* (Vegh and Basler, 2003), *UAS-tkv^{Q235D}* (Nellen et al., 1996) and *brk^{M68}* (Jazwinska et al., 1999).

To induce clones, a heat shock of 15 minutes at 37°C was performed 48 hours AEL; 72 hours later the larvae were dissected. Discs were fixed in 4% paraformaldehyde (PFA) and stained with DAPI or propidium iodide. Standard protocols were followed. Twin-spot volumes were calculated as follows: the surface area of a clone was drawn in each *z*-stack of confocal images using Imaris (Bitplane, Scientific Solutions), and the volume was then calculated by the software. Volume ratios were calculated for each twin-spot individually.

Genotypes for the clonal analysis experiment in wing discs with uniform Dpp: *yw hsp70-flp; UAS-dpp; C765-Gal4/actin5>draf>nuc-lacZ*. Control experiment: *yw hsp70-flp; actin5>draf>nuc-lacZ*. The *actin5>draf>nuc-lacZ* cassette (Struhl and Basler, 1993), *C765-Gal4* driver (Nellen et al., 1996) and *UAS-dpp* construct (Ruberte et al., 1995) were described previously. Clones were induced by a heat shock (18 minutes at 34°C) 48 or 72 hours AEL, and larvae were dissected 120 hours AEL. Nuclei in wing disc cells were stained by DAPI. The volume of clones was calculated using Imaris.

Driver lines to induce the expression of *UAS-brk* (Moreno et al., 2002), *UAS-tkv^{Q235D}* (Nellen et al., 1996), *UAS-dpp* (Ruberte et al., 1995) or *UAS-GFP* transgenes: *C765-Gal4* (Nellen et al., 1996), *esg-Gal4* (Goto and Hayashi, 1999), *salE-Gal4* (Mosimann et al., 2006), *omb-Gal4* (Calleja et al., 1996), *en-Gal4* (obtained from the *Drosophila* Genetic Resource Center) and *act5cP(FRT.y^{+})-Gal4:PR* (Progesterone receptor) (Rogulja and Irvine, 2005). *tubP-Gal80^{ts}* stocks: stock 7108 and 7017 (Bloomington). *dpp* and *brk* mutants: *brk^{M68}* (Jazwinska et al., 1999), *brk^{XA}* (Campbell and Tomlinson, 1999), *dpp^{12}* and *dpp^{14}* (Spencer et al., 1982). *brk^{x47}-lacZ* line (Campbell and Tomlinson, 1999). The permissive temperature used for Gal80^{ts} was 18°C and the restrictive temperature was 29°C. *act5cp(FRT.y^{+})-Gal4:PR* was activated by adding progesterone to the food to a final concentration of 20 μg/ml.

Immunohistochemistry

Immunostaining was performed using standard protocols. Images were collected with Leica TCS SP1 and TCS SP5 confocal microscopes. Imaris was used to analyze the images. *z*-stacks were projected in the three-dimensional view.

For BrdU labeling, prior to fixation (4% PFA for 20 minutes), BrdU (0.1 mg/ml) was added to the Ringer's solution and discs were incubated for 30 minutes at room temperature. Before addition of the anti-BrdU antibody, discs were treated for 30 minutes with 2 M HCl. Primary and secondary antibody staining was then performed using standard protocols. For BrdU

co-stainings, prior to fixation, BrdU (0.1 mg/ml) was added to the Ringer's solution and the discs were incubated for 30 minutes at room temperature. Anti-pMad and anti-β-galactosidase (β-gal) stainings were performed using standard protocols. After addition of the secondary antibodies, discs were fixed with 4% PFA for 20 minutes, and then treated for 30 minutes with 2 M HCl before the anti-BrdU antibody was added.

Antibodies

The following antibodies were used: mouse anti-β-gal (1:2000; Promega); chicken anti-β-gal (1:2000; Promega); rabbit anti-pMad and guinea pig anti-pMad (1:1000; gift from Ed Laufer, Columbia University, New York); mouse anti-Wg (4D4; DSHB); guinea pig anti-Vg (gift from G. Struhl, Columbia University, New York); mouse anti-BrdU (1:50; BD-Pharmingen); rabbit anti-pH-H3 (1:400; Upstate); Alexa Fluor 488 goat anti-mouse, Alexa Fluor 594 goat anti-rabbit, Alexa Fluor 594 goat anti-chicken, Alexa Fluor 568 goat anti-guinea pig and Alexa Fluor 594 goat anti-mouse (all 1:500; Molecular Probes); goat anti-mouse Cy5 and goat anti-rabbit Cy5 (both 1:500; Jackson ImmunoResearch).

RESULTS

Equivalent growth behavior of *tkv*^{Q235D} and *brk*$^{-}$ clones

The Dpp gradient along the AP axis in the wing disc is converted into an inverse *brk* transcription gradient, which in turn defines the expression boundaries of the Dpp patterning targets (Affolter and Basler, 2007). To explore whether the effects of Dpp on growth are mediated via *brk* or whether Dpp regulates growth in a Brk-independent manner, we first performed a quantitative comparison of the growth behavior of cell clones with an activated Dpp signaling pathway versus clones lacking *brk* function. Since the growth response to altered Dpp signaling levels varies regionally along the AP axis (Martin-Castellanos and Edgar, 2002), phenotypes were analyzed separately in the medial and lateral areas (see Fig. 1A′-D′ for the location of the medial and lateral twin-spots). For the same reason, the twin-spot technique was used to monitor the clonal growth behavior (Lee and Luo, 2001). Clones that either exhibit an activated Dpp signaling pathway (by expressing a constitutively active form of the Dpp receptor Tkv, Tkv^{Q235D}) (Lecuit et al., 1996; Nellen et al., 1996), or that lacked *brk* function (by being homozygous mutant for the *brk*-null allele *brk*^{M68}) (Jazwinska et al., 1999), were induced at early second instar [48 hours after egg laying (AEL)] and compared with sibling control clones at the end of larval development (120 hours AEL). Owing to the uneven and folded nature of the wing disc epithelium at the edges, we assessed the volumes of the clones rather than their surface. Examples of such twin-spot clones are shown for each genotype in Fig. 1A-D.

In a control experiment, we confirmed that wild-type twin-spot clones that differed only in their GFP expression levels grew to equal sizes, irrespective of their position along the AP axis (Fig. 1A,E), indicating that neither the genetic set-up nor the volumetric analysis introduces any experimental distortions.

We then compared twin pairs, of which one clone was wild-type and the other expressed Tkv^{Q235D}. Confirming previous studies, Tkv^{Q235D}-expressing clones grew faster than their wild-type twins in the lateral area (Martin-Castellanos and Edgar, 2002). The volume of lateral *tkv*^{Q235D} clones was 3.9±1.9-fold larger [values are shown ± 95% confidence interval (CI)] than the volume of their corresponding wild-type siblings, whereas medial *tkv*^{Q235D} clones exhibited the same volume as their siblings (Fig. 1B,F). We then analyzed the growth behavior of *brk*^{M68} clones and observed that they closely reflected the phenotype of *tkv*^{Q235D} clones. When located laterally, their volume was 3.8±1.7-fold larger than the volume of their corresponding wild-type twins; when located medially, they did not show any extra growth (Fig. 1C,G). The observation that the relative growth differences between *tkv*^{Q235D} and wild-type control clones parallel those of *brk* mutant versus wild-type clones indicates that the activation of the Dpp pathway has the same effect on growth as the removal of Brk function. To more directly corroborate the equivalency of the two conditions, we set up mitotic recombination events in which the gain of Tkv^{Q235D} and the loss of *brk* occurred concomitantly in complementary daughter cells. This led to clone pairs of

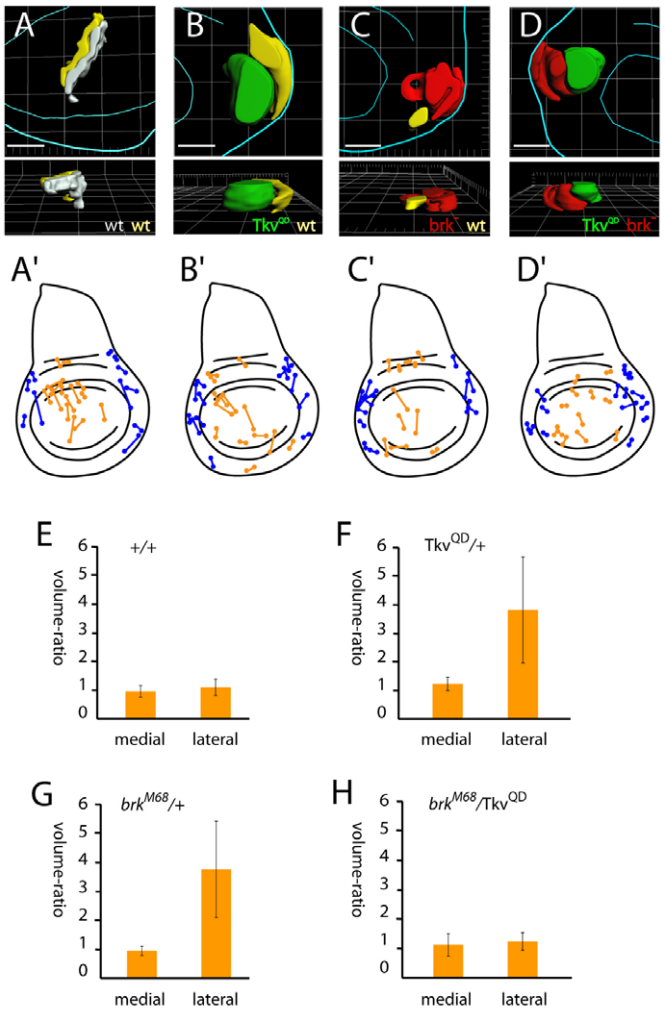
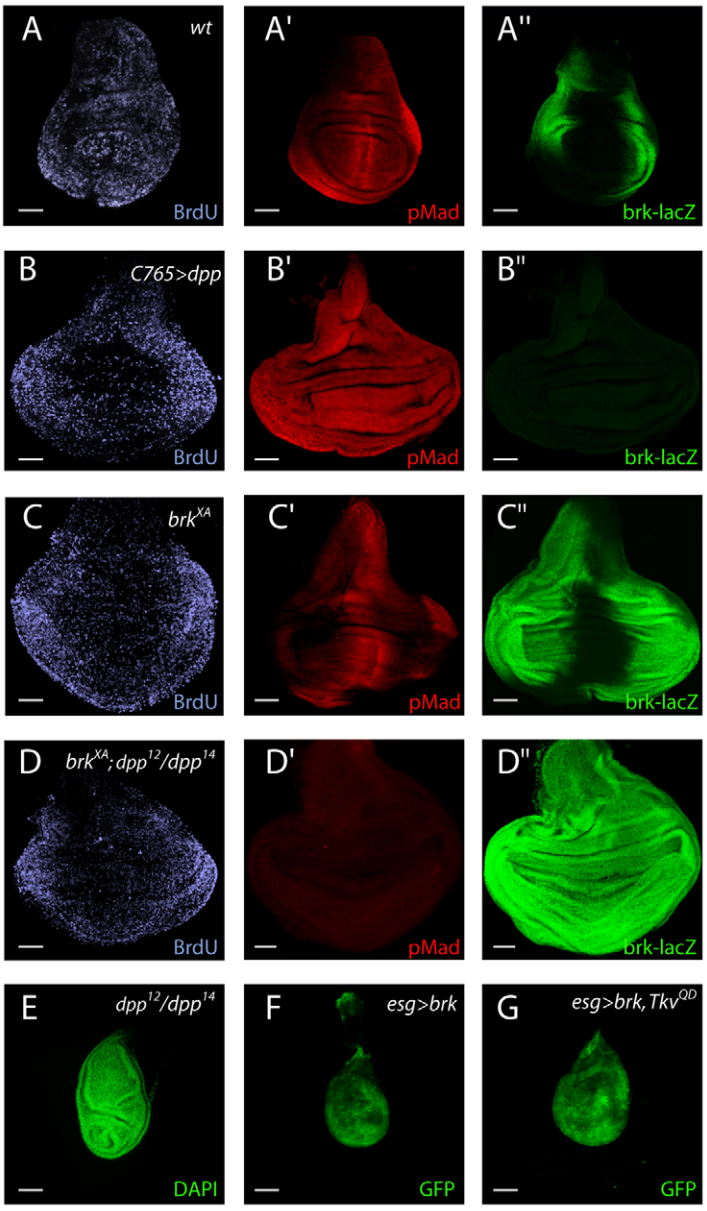


Fig. 1. Quantitative comparison of the growth behavior of wild-type, *tkv*^{Q253D} and *brk*$^{-}$ clones. (A,A′,E) Wild-type (wt) *Drosophila* twin-spots. (B,B′,F) *tkv*^{Q235D}/wt twin-spots. (C,C′,G) *brk*^{M68}/wt twin-spots. (D,D′,H) *brk*^{M68}/*tkv*^{Q235D} twin-spots. (**A-D**) Illustrations of the volumes of twin-spots. Disc margin and folds are indicated by cyan lines. Representative twin-spots for each genotype were chosen. Volumes were defined and measured using Imaris software. The image in B highlights the importance of using the volume to measure clone size. Although the apical area of the *tkv*^{Q235D} clone is similar to that of the wild-type clone, its volume is 2.9-fold larger. Scale bars: 50 μm. (**A′-D′**) The locations of the measured twin-spots are indicated; lateral (blue), medial (orange). (**E-H**) Volume ratio of medial and lateral twin-spots of each genotype. Error bars represent 95% confidence intervals (CIs). Number of twin-spots measured (*n*): (E) medial, *n*=14; lateral, *n*=11; (F) medial, *n*=14; lateral, *n*=13; (G) medial, *n*=11; lateral, *n*=14; (H) medial, *n*=13; lateral, *n*=13.



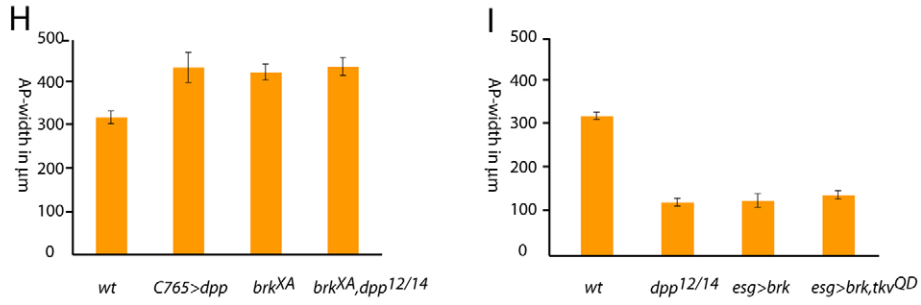


Fig. 2. Comparison of cell proliferation, disc size and Dpp signaling activity between wild-type discs and discs with altered *brk* levels or Dpp pathway activity. (**A-D″**) *Drosophila* third instar discs stained for BrdU (A-D). Dpp signaling activity assayed by (A′-D′) pMad staining (Tanimoto et al., 2000) and (A″-D″) *brk-lacZ* staining. Note that in A″ and B″, the brk^{x47}*-lacZ* reporter (Campbell and Tomlinson, 1999) was used, which recapitulates *brk* expression but does affect *brk* functionally. In C″ and D″, the brk^{XA}*-lacZ* allele was used, which recapitulates *brk* expression and disrupts *brk* function. (**A‴-D‴**) Illustrations of Dpp levels, Brk levels and cell proliferation in discs from wt (A), *C765>dpp* (B), brk^{XA} (C) and $dpp^{12/14}$;brk^{XA} double-mutant (D) animals. (**E-G**) $dpp^{12/14}$ (E, DAPI), *esg>brk* (F, GFP) and *esg>brk,dpp* (G, GFP) discs illustrating the similar size of discs of these three genotypes. Note that activation of Brk with *esg-Gal4* in the wing disc precursors blocks wing disc specification and therefore we blocked the system until the first instar stage (80 hours AEL at 18°C) using the $Gal80^{ts}$ system. (**H**) Comparison of the sizes of wild-type (n=37), *C765>dpp* (n=15), brk^{XA} (n=27) and $dpp^{12/14}$;brk^{XA} (n=27) discs along the AP axis. (**I**) Comparison of the sizes of wild-type (n=13), $dpp^{12/14}$ (n=27), *esg>brk* (n=13) and *esg>brk,dpp* (n=25) discs along the AP axis. Error bars in H and I indicate 95% CIs. Scale bars: 50 μm.

opposite genotypes, which could be directly compared in a quantitative manner. As shown in Fig. 1D,H, no size differences were observed between tkv^{Q235D} and brk^{M68} twins in either lateral or medial regions. Taken together, these results indicate that, with respect to clonal growth, the activation of the Dpp pathway is equivalent to the loss of *brk*. This is consistent with the notion that Dpp regulates growth in the wing disc solely by repressing *brk* and that is does not use a parallel, *brk*-independent output.

The Dpp-Brk system regulates the size of wing discs by defining Brk levels

The discovery that brk^{M68} clones behave like tkv^{Q235D} clones raised the possibility that all aspects of imaginal disc growth attributed to the Dpp gradient, such as disc size and cell proliferation pattern, are also controlled by Brk. To investigate this hypothesis, we compared the size and proliferation pattern of third instar wing discs exhibiting either uniform Dpp signaling

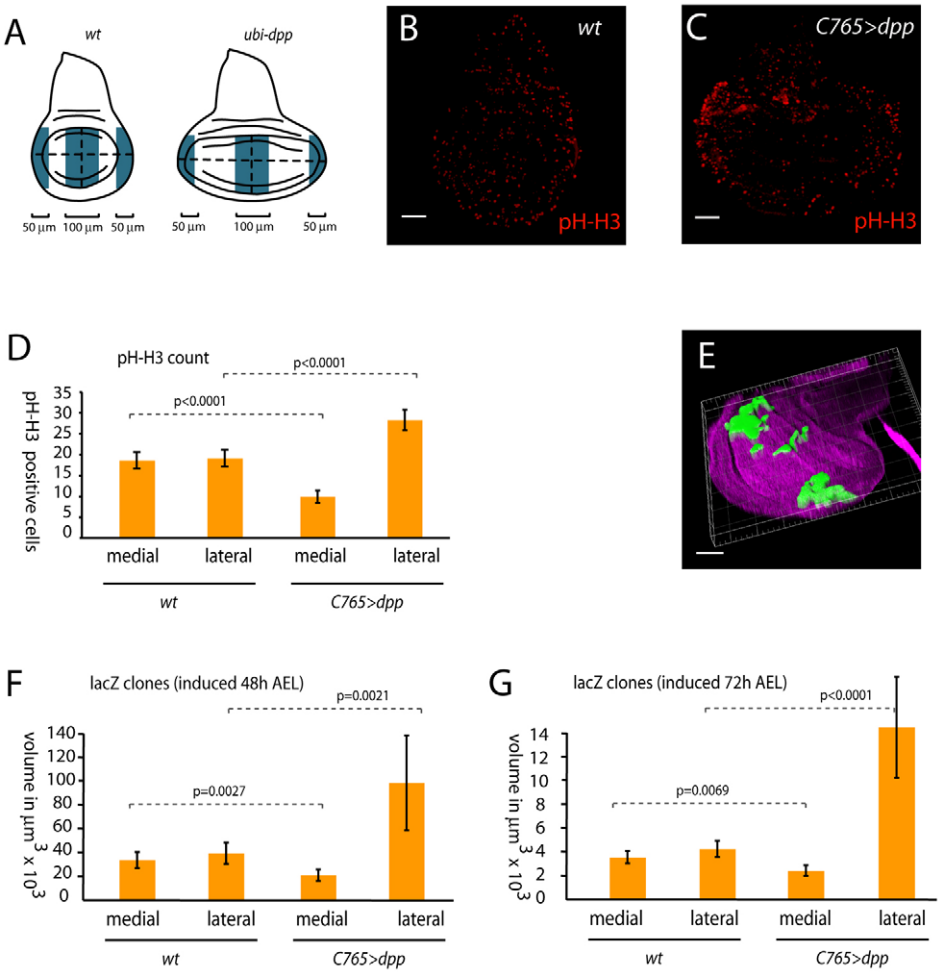


Fig. 3. Uniform activation of the Dpp pathway leads to enhanced proliferation in the lateral areas and reduced proliferation in the medial area. (**A**) Definition of the medial and lateral areas. Note that the lateral region overlaps with the outer region of the pouch and the medial region with the *patched* (*ptc*) expression domain (see Fig. S1A,B in the supplementary material). (**B**,**C**) Wild-type (B) and *C765>dpp* (C) *Drosophila* larva wing discs stained for phosphorylated histone H3 (pH-H3). (**D**) Quantification of pH-H3-positive cells in wild-type and *C765>dpp* discs. The numbers of pH-H3-positive cells in rectangles of 50×75 µm in the medial or lateral areas were counted. Wild-type discs, *n*=22; *C765>dpp* discs, *n*=27. (**E-G**) Comparative analysis of the behavior of wild-type *lacZ*-expressing clones in the medial and lateral areas of *C765>dpp* discs and wild-type discs. In some cases, owing to their size, lateral clones extended the defined border. (E) A *C765>dpp* disc containing medial and lateral clones (green). Volumes were calculated and are illustrated using Imaris software. (F) Comparison of the volume of medial and lateral clones induced 48 hours AEL in wild-type and *C765>dpp* discs. Wt: medial, *n*=25; lateral, *n*=24. *C765>dpp*: medial, *n*=45; lateral, *n*=17. (G) Comparison of the volume of clones induced 72 hours AEL. Wt: medial, *n*=61; lateral, *n*=29. *C765>dpp*: medial, *n*=139; lateral, *n*=34. Error bars (D,F,G) represent 95% CIs. Scale bars: 50 µm.

activity or uniform Brk expression with discs entirely lacking *brk* or *dpp* expression. If the only growth-regulating function of Dpp is to define *brk* levels, *brk* mutant discs should resemble discs with uniform and high Dpp signaling activity. It has been reported that uniform expression of the ligand Dpp or of the constitutively active receptor Tkv^{Q235D} leads to overgrowth as well as regional differences in proliferation, with higher levels laterally and lower levels medially (Martin et al., 2004; Rogulja and Irvine, 2005). We confirmed these observations by expressing Dpp with the *C765-Gal4* driver, which is uniformly active in the wing disc from the second instar onwards (data not shown). In *C765>dpp* discs, the Dpp signaling activity was uniformly activated at high levels (Fig. 2, compare B′,B″ with A′,A″; Fig. S1, compare D with C in the supplementary material). Such discs become larger than the wild-type control discs, measuring 436±35 µm along the AP axis at third instar, as compared with 321±15 µm (Fig. 2H). Moreover, the uniform proliferation pattern observed in wild-type wing discs was lost in *C765>dpp* third instar discs: BrdU incorporation was enhanced in the lateral area compared with the medial area (Fig. 2A,B) (for area definition see Fig. 3A,B). The ring of Wg staining indicated that the pouch in *C765>dpp* discs was expanded almost to the lateral edges of the wing disc (see Fig. S1B in the supplementary material). Thus, the overproliferating lateral area partly overlapped with the wing pouch, leading to its enlargement. Activation of the Dpp pathway by overexpressing Tkv^{Q235D} instead of Dpp yielded equivalent results (see Fig. S3A,C in the supplementary material).

Next, we analyzed wing discs mutant for *brk*. To circumvent embryonic lethality of the *brk*-null alleles we used the hypomorphic brk^{XA} allele, which exhibits expanded Dpp target gene expression in the enlarged pouch, indicating abrogated *brk* function (Campbell and Tomlinson, 1999) (see Fig. S1E-J in the supplementary material), but allows development until pupation. brk^{XA} mutant discs showed the same phenotype as *C765>dpp* discs (Fig. 2C,H); they measured 425±20 µm along the AP axis, and BrdU uptake was enhanced in the lateral region compared with the medial region. Thus, although Dpp signaling was limited to a narrow gradient along the AP axis (as assessed by the levels of pMad; see Fig. 2C′,C″), the growth of brk^{XA} discs was comparable to that of discs with ubiquitous Dpp signaling.

If, as suggested by the above results, Dpp solely affects growth by regulating *brk*, the levels and spatial distribution of Dpp signaling should be irrelevant in *brk* mutant discs or in discs expressing *brk* constitutively. To test the first of these predictions, we analyzed the growth phenotype of third instar discs lacking both *brk* and *dpp*. To circumvent embryonic lethality of *dpp* mutants, we used the *dpp* disc alleles dpp^{12} and dpp^{14} ($dpp^{12/14}$), which lack Dpp expression only in the wing imaginal discs and not in the embryo (St Johnston et al., 1990). Strikingly, although no Dpp signaling activity was detected in brk^{XA} $dpp^{12/14}$ discs (Fig. 2D′,D″), they overgrew (435±23 µm) and exhibited enhanced BrdU incorporation in lateral as compared with medial areas (Fig. 2D,H). In the reciprocal experiment, we tested whether discs constitutively expressing Brk mimic the reduced size of discs mutant for *dpp*, regardless of their Dpp signaling levels. Indeed, discs expressing Brk uniformly and at high

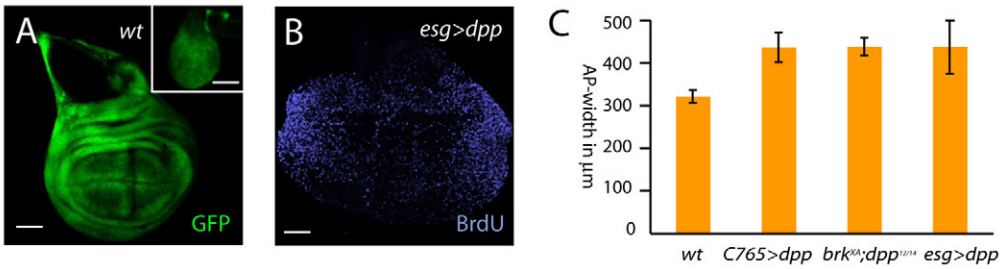


Fig. 4. In the wing disc, two areas with distinct growth properties are defined by a Dpp-independent mechanism. (**A**) *esg>GFP* discs at the second (inset) and third instar stage. The activity of the *esg-Gal4* driver is uniform, with the exception of the notum, which is taken into consideration in our studies. (**B**) Disc of an *esg>dpp Drosophila* larva. (**C**) Bar chart showing the size of discs exposed to either uniform Dpp signaling from late second instar onwards (*C765>dpp*), discs exposed to uniform Dpp signaling from the beginning of wing disc development (*esg>dpp*), or discs lacking the Dpp-Brk system ($dpp^{12/14};brk^{XA}$). Wild-type discs span 321±15 μm along the AP axis, *C765>dpp* discs 436±35 μm, *esg>dpp* discs 438±63 μm and $dpp^{12/14};brk^{XA}$ discs 438±21 μm (± 95% CIs). Wild-type discs, *n*=37; *C765>dpp* discs, *n*=15; $dpp^{12/14};brk^{XA}$ discs, *n*=27; *esg>dpp* discs, *n*=9. Scale bars: 50 μm.

levels under the *escargot* (*esg*)-*Gal4* driver, which is active uniformly during wing disc development, measured only 128±16 μm along the AP axis, resembling $dpp^{12/14}$ discs (124±9 μm) (Fig. 2E,F,I). Even massive and ubiquitous Dpp signaling (by means of $UAS\text{-}tkv^{Q235D}$ under *esg-Gal4* control) could not rescue the phenotype of such discs (Fig. 2G,I). Consistent with these results, expressing Brk and Tkv^{Q235D} under *C765-Gal4* led to the same phenotype as when expressing only Brk. However, owing to the later activation of the *C765-Gal4* driver, discs were larger than $dpp^{12/14}$ discs (see Fig. S4 in the supplementary material).

In conclusion, the growth phenotypes of discs with non-regulatable *brk* levels (no Brk or ubiquitous and high levels of Brk) no longer respond to Dpp signaling. We interpret this epistatic relationship between Brk and Dpp to indicate that Dpp normally regulates the growth and size of wing discs exclusively by repressing *brk*.

Uniform activation of the Dpp pathway enhances lateral and reduces medial growth

The patterns of BrdU incorporation described above confirmed previous observations (Rogulja and Irvine, 2005) and suggest that in wing discs with ubiquitous Dpp signaling, cell proliferation is enhanced in the lateral regions (compared with the medial area). The extended shape of the wing disc along the AP axis also suggests that the growth rate of lateral cells is higher than in wild-type discs. However, as BrdU is only a marker for cells in S phase, these results alone are not conclusive evidence for a decrease in the growth rate of medial cells, as compared with the situation in wild-type discs. We therefore examined the growth rates in discs with uniform Dpp signaling in more detail. First, we used the anti-phosphohistone H3 (pH-H3) antibody to identify cells in M phase, which represent proliferating cells. The lower percentage of cells found in M phase, as compared with cells in S phase, renders the pH-H3 assay more appropriate for quantification. Furthermore, the reduced variability in the pH-H3 (relative to the BrdU) assays enabled a more reliable comparison of the results from discs of different larvae. *C765>dpp* discs showed an increase in pH-H3-positive cells in the lateral area, and a decrease in the medial area, confirming the BrdU results (Fig. 3B,C) (for area definition see Fig. 3A). Quantification of the pH-H3-positive cells demonstrated that both the increase in proliferation levels in the lateral areas and the decrease in the medial areas, as compared with the wild type, were statistically significant (Fig. 3D). The earliest time point at which we observed differences between medial and lateral cells was at the mid-third instar stage, 96 hours AEL (see Fig. S2 in the supplementary material). As an additional confirmation of these observations, we used an independent quantitative assay to compare the proliferation behavior of medial and lateral cells. Randomly positioned, neutral *lacZ*-expressing clones were induced both in *C765>dpp* discs and in wild-type discs at 48 hours and 72 hours AEL and allowed to grow until 120 hours AEL. The volumes of these clones were recorded separately for medial and lateral regions (Fig. 3A,E-G). In agreement with the other assays, clone size, and hence growth, was enhanced in the lateral area and reduced in the medial area of *C765>dpp* discs, as compared with wild-type discs, in a manner that was statistically significant.

The two distinct areas of growth in the wing disc are defined by a Dpp-independent mechanism

The experiments above show that the wing disc consists of at least two distinct cell populations, the medial and lateral, which exhibit different proliferation rates under conditions of uniform Dpp signaling. In principle, two different explanations could account for the existence and divergent behavior of medial and lateral cells. The Dpp gradient itself could provide early positional information along the AP axis, causing the subdivision of cells into a mirror-symmetric arrangement of lateral, medial and again lateral cells. Later in development, these distinct cell populations react differently to experimental, uniformly high Dpp signaling levels. Alternatively, a Dpp-independent system might determine medial and lateral regions. To distinguish between these two scenarios, we sought to analyze discs that were never exposed to graded Dpp signaling during their entire developmental history. This criterion is fulfilled by discs of $dpp^{12/14};brk^{XA}$ genotype. As mentioned above and shown in Fig. 2D, the proliferation of lateral cells in such discs is enhanced, indicating that the two populations can form in the absence of a detectable Dpp gradient (Fig. 2D′,D″). Since it was theoretically possible that the hypomorphic *dpp* disc alleles might still provide some early graded distribution of Dpp, we also examined discs from *esg>dpp* larvae. *esg* is expressed uniformly at high levels during embryogenesis in the wing disc precursors (Fuse et al., 1994), as well as during all larval stages of wing development in the regions of interest (Fig. 4A). Hence, the *esg>dpp* transgene combination leads to saturating Dpp signaling activity in all cells from the very beginning of disc development. Strikingly, third instar wing discs still showed a differential proliferation pattern in medial versus lateral regions (Fig. 2D, Fig. 4B). Moreover, no significant size

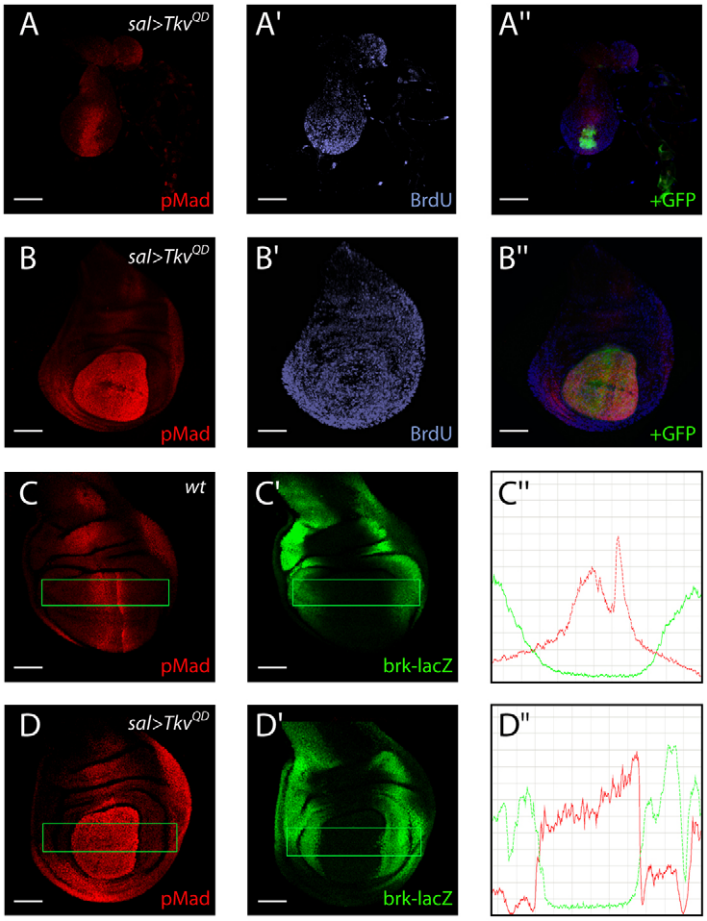


Fig. 5. Graded Dpp pathway activity levels in the medial area of the wing disc are not required to drive cell proliferation. (**A-A″**) A second instar wing disc from a *Drosophila* larva expressing tkv^{Q235D} in the medial area (*salE-Gal4* driver). Gal4 function was inhibited during early embryogenesis by the $Gal80^{ts}$ system. Twenty-four hours AEL, embryos were shifted for 48 hours from 18°C to the permissive temperature of 29°C. (**B-B″**) Third instar wing discs of larvae expressing tkv^{Q235D} under the *salE-Gal4* driver. $Gal80^{ts}$ inhibited tkv^{Q235D} expression during embryogenesis; 24 hours AEL, larvae were shifted for 96 hours to 29°C. (**C-C″**) The pMad and *brk-lacZ* gradient in wild-type wing discs. Quantification of antibody staining within the area of the green rectangles, from anterior to posterior, is shown in C″. (**D-D″**) pMad and *brk-lacZ* staining of *salE>*tkv^{Q235D} discs (processed as in B); quantification is shown in D″. Scale bars: 50 μm.

difference could be detected between discs with early uniform Dpp signaling ($dpp^{12/14}$;brk^{XA} double-mutant discs and *esg>dpp* discs) and discs with uniform Dpp signaling from second instar onwards (*C765>dpp*) (Fig. 4C). Expressing Tkv^{Q235D} instead of Dpp resulted in the same phenotype (see Fig. S3B,C in the supplementary material). Together, these experiments argue for the existence of a second AP patterning system that acts independently of Dpp to specify the distinct growth behaviors of medial and lateral cells.

Graded Dpp pathway activity is not required in the medial area of the wing disc to permit cell proliferation

In response to uniform activation of the Dpp pathway in the wing disc, lateral cells seem to proliferate at elevated rates, and medial cells at reduced rates. These distinct behaviors were previously explained by the ability of lateral cells to proliferate in response to absolute Dpp levels and the necessity of medial cells to sense a difference in Dpp pathway activity (relative to that in neighboring cells) for proliferation (Rogulja and Irvine, 2005). However, during wing disc development, proliferation occurs in a normal manner at the source of Dpp expression, where cells are exposed to saturating and equal Dpp signaling levels. This prompted us to reassess the gradient model by analyzing the proliferation pattern in a situation in which the Dpp pathway is uniformly activated in the medial area of the wing primordium. Tkv^{Q235D} was expressed under the control of the *sal-Gal4* driver, which is active in the medial area from early second instar (Fig. 5A″) until third instar (Fig. 5B″). Intensity measurements for pMad and *brk-lacZ* confirmed uniform and full activation of the Dpp pathway within the *sal* domain (Fig. 5D): *brk-lacZ* expression was fully repressed within this domain, and the *brk-lacZ* gradient observed in wild-type discs was lost (Fig. 5, compare C with D). The uniform and high *brk* expression observed lateral to the *sal* domain can be attributed to the ability of Tkv to sequester Dpp and thus hinder its movement (Belenkaya et al., 2004; Crickmore and Mann, 2006; Lecuit and Cohen, 1998). As shown in Fig. 5B, despite uniform activation of the Dpp pathway – and hence the absence of a Dpp signaling gradient – in the *sal* domain, close to normal growth was observed. The *sal* expression domain expanded over time (Fig. 5, compare A with B, second instar discs compared with third instar discs), and *sal>*tkv^{Q235D} third instar discs were approximately the size of control discs (305±11 μm along the AP axis compared with 321±15 μm). Importantly, BrdU staining was uniform in *sal>*tkv^{Q235D} discs, indicating even growth throughout the entire wing disc (Fig. 5B′). Expression of tkv^{Q235D} under the *omb-Gal4* driver, which is also downstream of Dpp signaling but expressed in a broader domain, led to similar results (see Fig. S5 in the supplementary material). Together, these results demonstrate that uniform Dpp signaling levels do not prevent cell proliferation in the medial area and thus strongly argue against the gradient slope model.

Ectopic Dpp pathway activity in lateral cells causes reduced proliferation in the residual disc

The experiments above indicate that the slope of the Dpp gradient does not play a major role in determining the growth rates of the wing disc. However, the need for differences in Dpp signaling levels between adjacent cells was proposed to explain the reduced medial proliferation rates in discs with uniform Dpp signaling (Rogulja and Irvine, 2005). An alternative explanation for this phenomenon is that

ectopic Dpp signaling in the lateral area causes reduced proliferation in the medial area. To test this idea, we analyzed proliferation in discs that exhibit the endogenous Dpp gradient in the medial area, but ectopic Dpp signaling in the lateral area. One way to obtain such a situation is the generation of Tkv^{Q235D}-expressing clones. Fig. 6A shows a lateral clone in which the hormone-inducible Gal4, Gal4:PR, drives Tkv^{Q235D} expression. Upon induction, Dpp signaling activity within the clone is uniformly high, but the Dpp activity gradient outside of the clone is unaffected (Fig. 6A′). In contrast to the autonomous increase in proliferation within the clone, proliferation was reduced in the rest of the disc (Fig. 6A). Next, we directly compared the non-autonomous influence of lateral Dpp signaling on medial cells in the presence and absence of a Dpp signaling gradient. We used an *engrailed* (*en*)-*Gal4* transgene to drive Tkv^{Q235D} expression in the P compartment, and the $Gal80^{ts}$ system for temporal control. In such *en>tkv*^{Q235D} discs we assayed proliferation 24 and 48 hours after induction, and observed an overproliferation of posterior lateral cells (Fig. 6B-D) (for area definition see Fig. 3A,B). Importantly, in these discs only cells in the anterior half of the medial area are exposed to a Dpp gradient, whereas cells in the posterior medial half exhibit saturating and uniform Dpp pathway activity. Despite this difference, proliferation was significantly reduced in the medial parts of both the anterior and posterior halves of the discs as assessed by BrdU (Fig. 6B,C). For the 48-hour induction time point, proliferation was also assayed by pH-H3 staining and quantified (Fig. 6D,E) (CIs of pH3-positive cells in wild-type discs do not overlap with the CIs of pH3-positive cells in the medial areas and the posterior lateral area of *en>tkv*^{QD} discs; $P<0.0001$). Besides the reduction of proliferation in the medial areas, proliferation was also reduced in the anterior lateral region ($P=0.0004$), indicating that the growth-curbing signal acts on all cells within the disc except those with an additional growth advantage. Taken together, our results provide an alternative explanation for the reduced growth rates in the medial area of discs with uniform Dpp pathway activity. It seems that this effect is not caused by the loss of the gradient, but rather by a non-autonomous effect of overproliferating lateral cells on medial cells. This model is now also in agreement with our finding that only in the medial area are uniform and high Dpp signaling levels sufficient for normal growth.

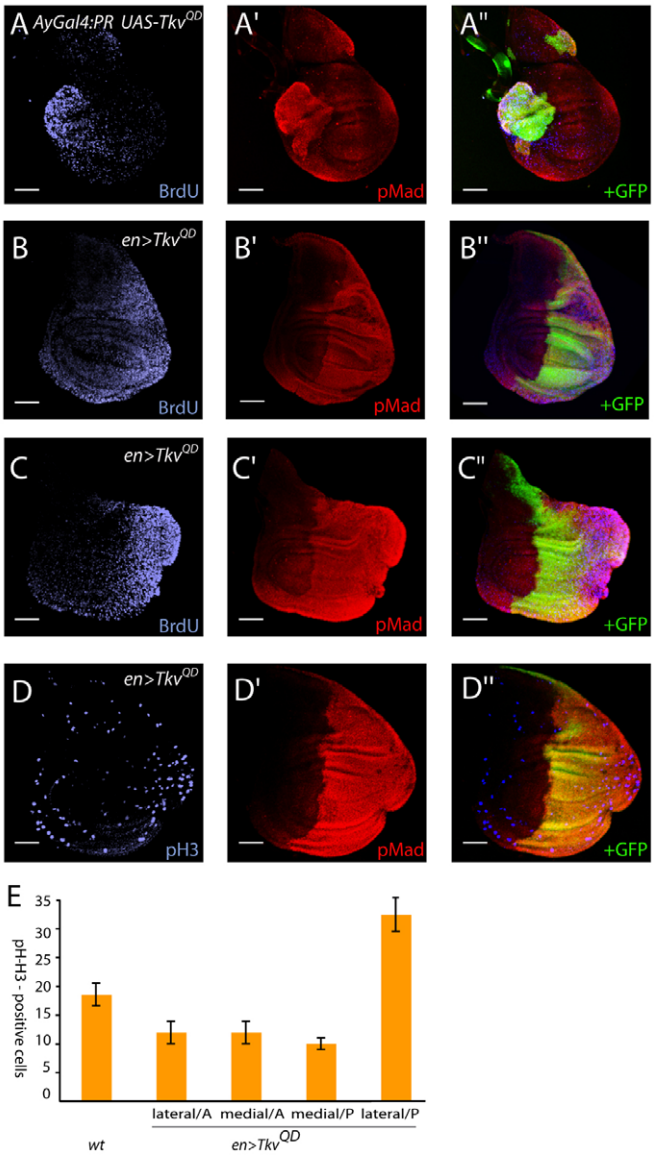


Fig. 6. Ectopic Dpp signaling in the lateral area causes reduced proliferation in the medial area. (**A-A″**) *tkv*^{Q235D} expression in a lateral *AyGal4:PR* clone. *Gal4:PR* activity was induced by the addition of progesterone from 72 hours AEL for 48 hours. (**B-B″**) *tkv*^{Q235D} was expressed under the *en-Gal4* driver for 24 hours from 120 hours AEL (at 18°C); the $Gal80^{ts}$ system was used to temporally control Gal4 activity. (**C-D″**) *tkv*^{Q235D} was expressed under the *en-Gal4* driver for 48 hours from 120 hours AEL (at 18°C). (**E**) Quantification of the number of pH-H3-positive cells in a rectangle of 75×50 μm in the lateral-anterior, medial-anterior, medial-posterior and lateral-posterior areas of *en>tkv*^{Q235D} discs as compared with the corresponding regions in wild-type *Drosophila* discs. Note that in wild-type discs, proliferation is uniform across the disc (see Fig. 3A) and therefore, for simplification, only the mean proliferation levels are shown here. Wild-type discs, $n=22$; *en>tkv*^{Q235D} discs, $n=32$. Error bars display 95% CIs. A, anterior; P, posterior. Scale bars: 50 μm.

DISCUSSION

Morphogen gradients play essential roles in pattern formation during animal development. They direct the transcriptional on and off states of genes in a concentration-dependent manner (Gurdon and Bourillot, 2001; Lawrence, 2001; Vincent and Briscoe, 2001) in various embryonic organ systems. The tight link between organ patterning and organ growth raised the notion that morphogens also determine cell proliferation rates and final tissue size. This latter aspect of the morphogen concept, however, is not well understood. Indeed, it is not clear whether the nuclear response to morphogen signals that directs the transcription of patterning genes also regulates growth. And what property of a morphogen signaling system explains how uniform growth rates can ensue in response to a graded input? Here we address these questions in the experimental system of the Dpp gradient, a key determinant in pattern formation and growth of the *Drosophila* wing.

Brk plays an equally important, and essential, role in patterning and growth

Studies from the past decade have shown that the Dpp gradient in the wing disc does not define the expression boundaries of subordinate patterning genes directly via its nuclear mediators, but does so indirectly by setting up an inverse gradient of the transcriptional repressor Brk (Affolter and Basler, 2007). Here we have investigated the potential role of this indirect mechanism in growth regulation and found that it is equally important, and essential, for the ability of Dpp to promote growth. Clones of cells

with a constitutively active Dpp signaling pathway exhibited qualitatively and quantitatively the same growth behavior as *brk*⁻ clones, overgrowing when located in the lateral area. Moreover, the phenotype of discs in which Brk levels can no longer be regulated by Dpp (because *brk* is either lacking genetically, or controlled by a heterologous promoter) are insensitive to experimentally varying Dpp signaling levels. Thus, our experiments demonstrate that the growth output of the Dpp pathway is entirely funneled through the regulation of the *brk* gene.

The paradigm of Dpp directing pattern formation via *brk* repression thus also explains how Dpp controls growth. This observation serves to validate the connection between morphogen-mediated patterning and the control of organ size. Our results indicate that for the Dpp system, any mechanistic bifurcation of the two outputs occurs downstream of the first tier of transcriptional regulation.

The Dpp-Brk system evens out regional differences in wing disc growth

Discs lacking both *dpp* and *brk* functions grow to a larger size than wild-type discs. Importantly, in this state, in contrast to the normally uniform profile, cell proliferation also occurs unevenly across the disc, with higher rates in the lateral areas and lower rates in the medial area. Based on this difference, we conclude that the Dpp-Brk system is not a growth promoter but is rather a growth-modulatory system, ironing out inherent regional differences in proliferation rates.

The origin of the regional proliferative differences in discs devoid of the Dpp-Brk system is unknown. Since such discs lack Dpp, as the only agent known to impose mirror-symmetric differences along the AP axis, no pre-patterning mechanism that depends on it can be postulated. The smooth transitions to higher proliferation rates between medial and lateral areas would be consistent with a diffusible factor that acts in a concentration-dependent manner. This hypothetical factor could originate, for example, at the border between the disc proper and the adjacent peripodial membrane and promote growth laterally. Alternatively, the factor could be a growth inhibitor with high activity in the center of the disc and low activity peripherally. Expression of the factor could be controlled by Hedgehog in a Dpp-independent manner. But this is pure speculation because to date there is no evidence for the existence of such a factor(s) in the developing wing discs.

An entirely different explanation for our experimental observations could be a growth-regulatory mechanism that depends on mechanical forces. It has been proposed that during growth, mechanical compression of cells increases in the center, while cells in the peripheral regions become stretched (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007). Assuming a growth-stimulatory role for stretching and a growth-inhibitory role for compression, growth would be facilitated in the peripheral regions during normal development, and Brk would counter this advantage and thus ensure uniform growth (Aegerter-Wilmsen et al., 2007; Martin et al., 2004). In the absence of the Dpp-Brk system, the amount and distribution of mechanical stresses are likely to differ significantly, which in turn could feed back on growth and lead to the observed differences between the lateral and medial regions of the disc.

Cell proliferation under conditions of uniform Dpp signaling

Here we have confirmed and extended previous findings that in wing discs with uniform Dpp signaling, lateral cells proliferate faster, and medial cells slower, than cells of wild-type discs (Martin et al., 2004; Rogulja and Irvine, 2005). The inhibition of cell proliferation in the medial region is an important pillar for the model which proposes that it is the slope of the Dpp morphogen gradient that serves as the driving force behind medial wing cell proliferation during normal development (Rogulja and Irvine, 2005). Contradicting the proposed requirement for disparate Dpp signaling activities among adjacent cells, we found that when uniform pathway activity is established in, and limited to, the medial area, no deficit in cell proliferation rates occurs. Indeed, the medial domain of discs with such even Dpp signaling levels expands, and proliferation is uniform (Fig. 5). This finding is consistent with results from our twin-spot analysis, which showed that the growth rates of medial tkv^{Q235D} and brk^{M68} clones are identical to those of wild-type clones (Fig. 1). Thus, the transient effect of additional proliferation at clonal boundaries observed by Rogulja and Irvine (Rogulja and Irvine, 2005) seems to be more important for situations such as wound healing, in which cells of different Dpp signaling levels become juxtaposed, than for the normal growth of a wild-type wing disc. We found that a reduction in proliferation rates in the medial area only occurs when Dpp activity is driven in the lateral area, independent of the presence or absence of a Dpp signaling gradient. Ectopic Dpp pathway activation in lateral cells is not only necessary, but also sufficient, to impede proliferation of medial cells. Thus, overproliferating lateral cells appear to exert a proliferation-

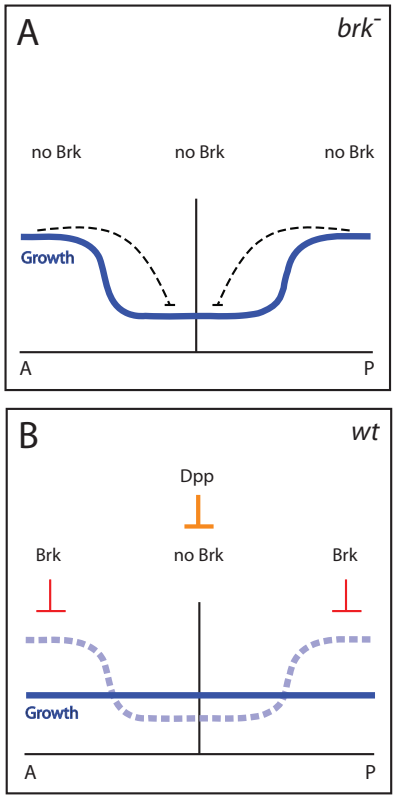


Fig. 7. Model of growth regulation in *Drosophila* wing discs by the Dpp-Brk system. (**A**) In wing discs without the Dpp-Brk system, growth is uneven. Lateral cells have a growth advantage over medial cells and overproliferate. By an unknown mechanism, lateral overproliferation inhibits growth in the medial area. (**B**) In wild-type wing discs, Dpp smooths out these local growth differences. High-level Dpp signaling prevents the expression of the growth inhibitor Brk in the medial area but not in the lateral area. High levels of Brk in the lateral area curb proliferation to levels similar to those in the medial area. A, anterior; P, posterior.

retardant effect on other cells. Whether this effect underlies a mechanism also used to control proliferation rates during wild-type development, or whether it is 'only' a back-up mechanism used if something goes wrong during development (e.g. wound healing and regeneration), is not known. Moreover, as noted earlier, the mechanistic nature of the communication between lateral and medial cell populations remains speculative. It is possible that high Dpp signaling in lateral cells not only provides them with a growth advantage, but also causes the expression of a factor that spreads within the entire disc to reduce proliferation of cells without an additional growth advantage. Other possible explanations include the competition among wing cells for a limiting proliferation factor (whereby ectopic Dpp-transducing cells prevail), or the negative impact that overproliferating cells might exert on remaining cells via metabolic side-products or increased mechanical compression. These models would also be consistent with the observation that proliferation is reduced in all cells of the wing disc except those with an additional growth advantage.

Growth regulation of the wing disc by the Dpp-Brk system

Based on our observations and the above conclusions we summarize Dpp-mediated growth control in the wing disc as follows (Fig. 7). The disc consists of at least two different cell populations, medial and lateral, which have distinct abilities to proliferate. The Dpp signal is required to even out these growth differences and establish a uniform pattern of cell proliferation within the wing primordium. Medial cells must sense high levels of Dpp to shut down *brk* expression, which consequently promotes medial proliferation. Lateral cells have a growth advantage and must receive little or no Dpp input to allow *brk* expression. The action of Brk curbs lateral proliferation. We do not know how intermediate Brk levels affect the proliferative behavior of cells situated between lateral and medial cells. However, we can conclude from our results that differential pathway activity between neighboring cells is not necessary to direct proliferation, as constitutively high Dpp levels in the medial area and nil or low levels in the lateral areas are sufficient for uniform and normal cell proliferation rates throughout the disc.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4003/DC1

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