

Dpp signaling inhibits proliferation in the *Drosophila* wing by Omb-dependent regional control of *bantam*

Xubo Zhang¹, Dan Luo¹, Gert O. Pflugfelder^{2,*} and Jie Shen^{1,*}

SUMMARY

The control of organ growth is a fundamental aspect of animal development but remains poorly understood. The morphogen Dpp has long been considered as a general promoter of cell proliferation during *Drosophila* wing development. It is an ongoing debate whether the Dpp gradient is required for the uniform cell proliferation observed in the wing imaginal disc. Here, we investigated how the Dpp signaling pathway regulates proliferation during wing development. By systematic manipulation of Dpp signaling we observed that it controls proliferation in a region-specific manner: Dpp, via *omb*, promoted proliferation in the lateral and repressed proliferation in the medial wing disc. Omb controlled the regional proliferation rate by oppositely regulating transcription of the microRNA gene *bantam* in medial versus lateral wing disc. However, neither the Dpp nor Omb gradient was essential for uniform proliferation along the anteroposterior axis.

KEY WORDS: Dpp signaling, *optomotor-blind*, *bifid*, *bantam*, Proliferation, Gradient, *Drosophila* wing disc

INTRODUCTION

The control of organ growth by extrinsic and intrinsic factors is a fundamental aspect of animal development that remains poorly understood (Baena-Lopez et al., 2012; Day and Lawrence, 2000; Johnston and Gallant, 2002; Schwank and Basler, 2010; Wartlick et al., 2011a). The *Drosophila* wing imaginal disc is a popular model for testing hypotheses on cell proliferation control. The wing disc grows rapidly (Bryant and Levinson, 1985; Bryant and Simpson, 1984; Garcia-Bellido and Merriam, 1971; Lawrence and Morata, 1977) and cell proliferation is largely uniform within the disc (Milán et al., 1996; Potter and Xu, 2001). The morphogen Decapentaplegic (Dpp) is expressed along the anterior/posterior (A/P) compartment boundary to form a precise concentration gradient along the A/P axis of the wing disc (Akiyama et al., 2008; Entchev et al., 2000; Fujise et al., 2003; Teleman and Cohen, 2000). This is essential for patterning the wing (Affolter and Basler, 2007; Ashe and Briscoe, 2006; Bollenbach et al., 2008; Cook et al., 2004; Lawrence and Struhl, 1996; Lecuit et al., 1996; Singer et al., 1997; Zecca et al., 1995). In contrast to the general accordance in views on wing patterning, how the Dpp gradient controls uniform proliferation across the wing disc remains controversial (Schwank et al., 2012; Wartlick et al., 2011b).

A number of models have been proposed to interpret how Dpp controls proliferation in the wing disc (Aegerter-Wilmsen et al., 2007; Day and Lawrence, 2000; Garcia-Bellido and Merriam, 1971; González-Gaitán et al., 1994; Hufnagel et al., 2007; Shraiman, 2005; Wartlick and González-Gaitán, 2011; Wartlick et al., 2011a). Recent observations argue that at least the spatial gradient of Dpp (and Wingless) is not essential for proliferation regulation in the wing disc (Baena-Lopez et al., 2009; Schwank et al., 2008). Dpp

has long been considered to exclusively promote growth and proliferation. This view was based on observations that overexpression of Dpp, or enhanced Dpp signaling, in the entire or lateral wing disc lead to overgrowth (Affolter and Basler, 2007; Burke and Basler, 1996; Capdevila and Guerrero, 1994; Lecuit et al., 1996; Martín-Castellanos and Edgar, 2002; Nellen et al., 1996; Zecca et al., 1995). However, clones lacking Dpp signaling activity can still proliferate in the medial wing disc even though they tend to be extruded from the epithelium (Gibson and Perrimon, 2005; Shen and Dahmann, 2005a). *Mad* and *brk* double-mutant clones grow normally in the central wing disc, which indicates that under normal circumstances (lack of central *brk* expression) Dpp is not directly required for proliferation in the medial wing disc (Schwank et al., 2012).

To explain why there is no correlation between local proliferation and the graded signaling activity, intricate cross-regulations between Wingless, Dpp and Hippo signaling (Baena-Lopez et al., 2012) or antagonistic effects of the Dpp and Fat pathways have been proposed (Schwank et al., 2011).

optomotor-blind (*omb*, *bifid* – FlyBase) is downstream of Dpp (Grimm and Pflugfelder, 1996), being repressed by Brinker (Brk) (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). Omb forms a gradient, similar to Dpp, that is required for wing patterning (Cook et al., 2004; Shen and Dahmann, 2005b; Shen et al., 2010). An effect on growth or tissue maintenance can be deduced from the reduced adult wing size of animals null or hypomorphic for *omb* (Grimm and Pflugfelder, 1996; Shen et al., 2008). A growth-repressive role of Omb has been identified in the ventral wing disc (del Álamo Rodríguez et al., 2004; Umemori et al., 2007).

Here, we investigated the role of the Dpp-Omb pathway in proliferation control in the *Drosophila* wing disc. By regionally and temporally manipulating the activity of the Dpp pathway, we demonstrate that Dpp-Omb medially inhibits and laterally promotes proliferation in the wing disc. Graded expression of neither protein is required for specifying an even proliferation rate. Mechanistically, we show that this differential function of Omb is reflected in a differential regulation of the proliferation-promoting microRNA *bantam* (*ban*).

¹Department of Entomology, China Agricultural University, 100193 Beijing, China.

²Institute of Genetics, Johannes Gutenberg University, 55128 Mainz, Germany.

*Authors for correspondence (pflugfel@uni-mainz.de; shenjie@cau.edu.cn)

MATERIALS AND METHODS

Drosophila stocks

Mutant alleles were *l(1)omb^{D4}* (Poeck et al., 1993), *dpp^{d6}*, *dpp^{d12}*, *dpp^{d14}* (Spencer et al., 1982). Transgenes were: *tubP-Gal80^{ts}* (McGuire et al., 2003), *dpp-GAL4* (Shen and Mardon, 1997), *30A-GAL4* (Brand and Perrimon, 1993), *en-Gal4*, *nub-Gal4*, *omb-Gal4^{MD653}* (Calleja et al., 1996), *C765-Gal4* (Nellen et al., 1996), *UAS-GFP*, *UAS-bsk^{DN}* (Bloomington Stock Center), *UAS-CD8-GFP* (Lee and Luo, 1999), *UAS-omb* (Grimm and Pflugfelder, 1996), *UAS-Tkv^{OD}* (Nellen et al., 1996), *UAS-brk* (Moreno et al., 2002), *UAS-omb-RNAi* (Shen et al., 2008), *UAS-Dad* (Tsuneizumi et al., 1997), *UAS-P35* (Bloomington Stock Center), *UAS-ban* (Li and Padgett, 2012). The *brk* enhancer trap line was *brk^{X47}-lacZ* (Campbell and Tomlinson, 1999). The *ban* enhancer reporter line was *br-C12-lacZ* (Oh and Irvine, 2011).

Larvae were raised at 25°C unless stated otherwise. For efficient expression of RNAi transgenes, larvae were raised at 29°C. Larvae containing *Gal80^{ts}-Gal4* combinations were raised at 18°C and then were shifted to 29°C for strong UAS transgene expression or to 27°C for weaker expression for the indicated duration before dissection.

Immunohistochemistry

Dissected wing imaginal discs were fixed and stained with antibodies according to standard procedures. The primary antibodies used were: rabbit anti-Omb, 1:1000; mouse anti- β -galactosidase, 1:2000 (Promega); mouse anti-BrdU, 1:100 (MBL); rabbit anti-PH3, 1:700 (Sigma). Secondary antibodies were goat anti-mouse DyLight 549 and goat anti-rabbit DyLight 488, 1:200 (Agrisera). Images were collected using a Leica TCS-SP2-AOBS confocal microscope and assessed using ImageJ (NIH).

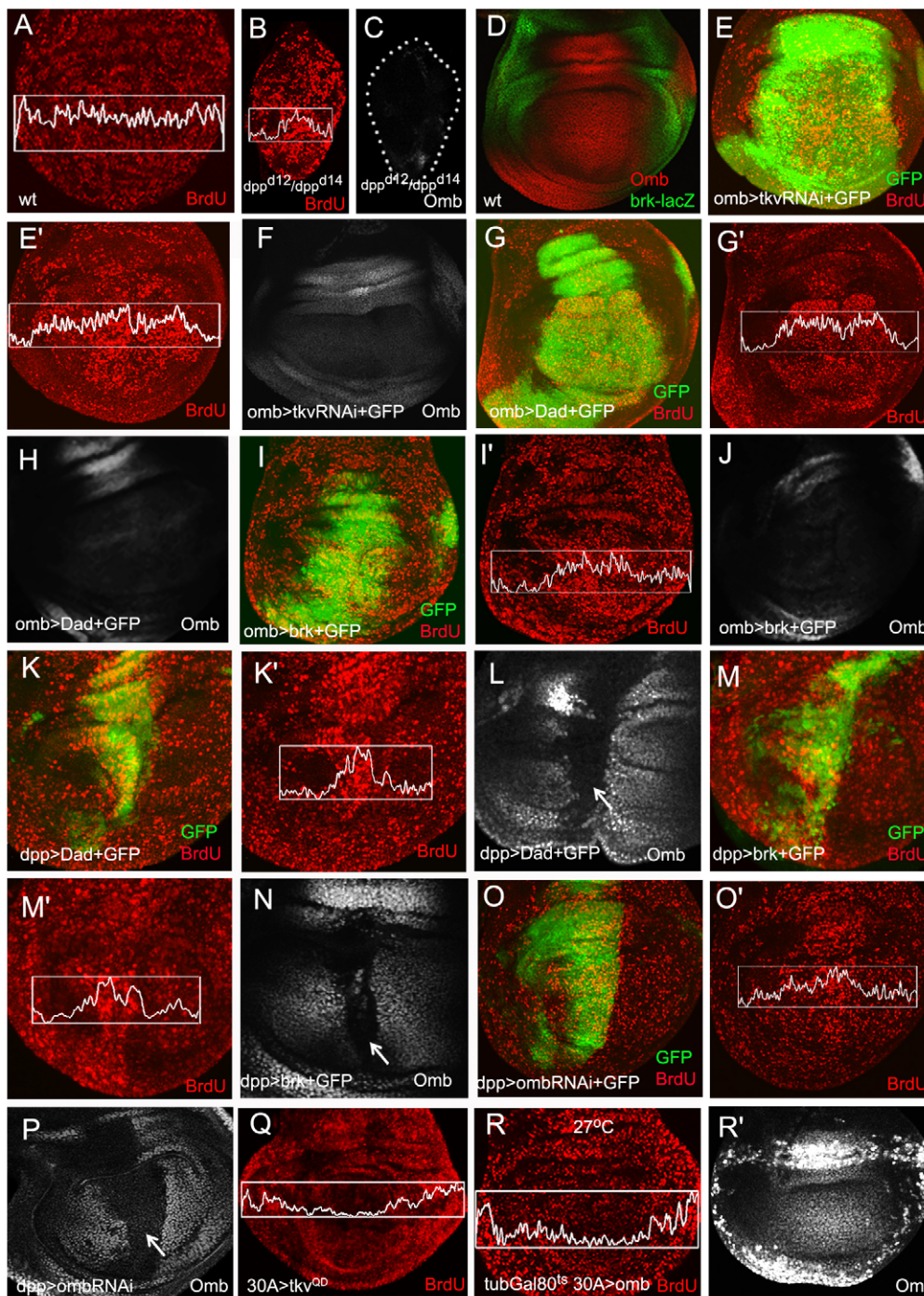


Fig. 1. Reduced Dpp signaling promotes proliferation in the *Drosophila* medial wing disc.

(A) Uniform BrdU incorporation in the wild-type wing disc. White boxes define the area of fluorescence quantification of BrdU staining. (B,C) *dpp* mutant wing discs (dotted outline in C) exhibit enhanced medial proliferation (B) and lack Omb (C). (D) *brk-lacZ* (immunodetection of β -galactosidase) and Omb expression in the wild-type wing disc are complementary. (E-N) Expressing *tkv-RNAi*, *Dad* or *brk* under *omb-Gal4* or *dpp-Gal4* control induces high medial proliferation and represses *omb*. Arrows (L,N) indicate repression of Omb. (O-P) *omb-RNAi* enhances medial proliferation (O,O') and efficiently represses *omb* (P, arrow). (Q) Lateral *Tkv^{OD}* enhances lateral proliferation. (R,R') Lateral *omb* expression enhances lateral proliferation. Omb expression was temporally controlled by *tub-Gal80^{ts}*. In this and subsequent figures, images of L3 wing discs are oriented with dorsal up and anterior left.

RESULTS AND DISCUSSION

Dpp signaling inhibits, rather than promotes, cell proliferation in the medial region of the wing pouch

It was noted early on that in larval-viable *dpp* mutants imaginal disc development is severely compromised (Spencer et al., 1982; Zecca et al., 1995). However, this cannot be attributed to a failure in cell proliferation because loss of Dpp signaling in medial clones does not block proliferation (Gibson and Perrimon, 2005; Kim et al., 1996; Schwank et al., 2012). We observed that in the tiny *dpp* mutant wing disc proliferation did occur but was not uniform (Fig. 1B; supplementary material Fig. S1H). No Omb could be detected (Fig. 1C). The ubiquitous lack of Dpp might cause changes in the pre-pattern or a respecification of the wing disc, thus preventing bulk growth of the tissue. In order to reduce interference with such Dpp functions, we manipulated Dpp signaling regionally. Along the A/P axis the *Drosophila* wing disc is subdivided into A and P compartments by a cell lineage restriction boundary

(Dahmann and Basler, 1999). Each compartment appears to control growth autonomously (Martín and Morata, 2006). Less distinctly, the wing disc can also be subdivided into lateral and medial regions. We used the largely complementary expression domains of *brk* and *omb* to define lateral and medial (Fig. 1D).

Attenuation of Dpp signaling by expressing *tkv*-RNAi, *Dad* or *brk* in the *omb*-Gal4 or *dpp*-Gal4 domain in all cases induced increased medial cell proliferation (Fig. 1E-M'), compared with the uniform proliferation seen in wild type (Fig. 1A). This was mimicked by direct repression of *omb* (Fig. 1O-P). Data from Martín et al. (Martín et al., 2004) appear to contradict our results: they reported that expressing *brk* or *Dad* by the strong and more widely expressed (Fig. 2H) *nub*-Gal4 line inhibits cell proliferation and reduces adult wing size. However, we observed the occurrence of severe apoptosis, cell extrusion and disruption of normal tissue morphology in *nub*>*brk* wing discs (supplementary material Fig. S1B,C). Repressing apoptosis could rescue, at least in part, the deficit in medial cell proliferation (supplementary material Fig.

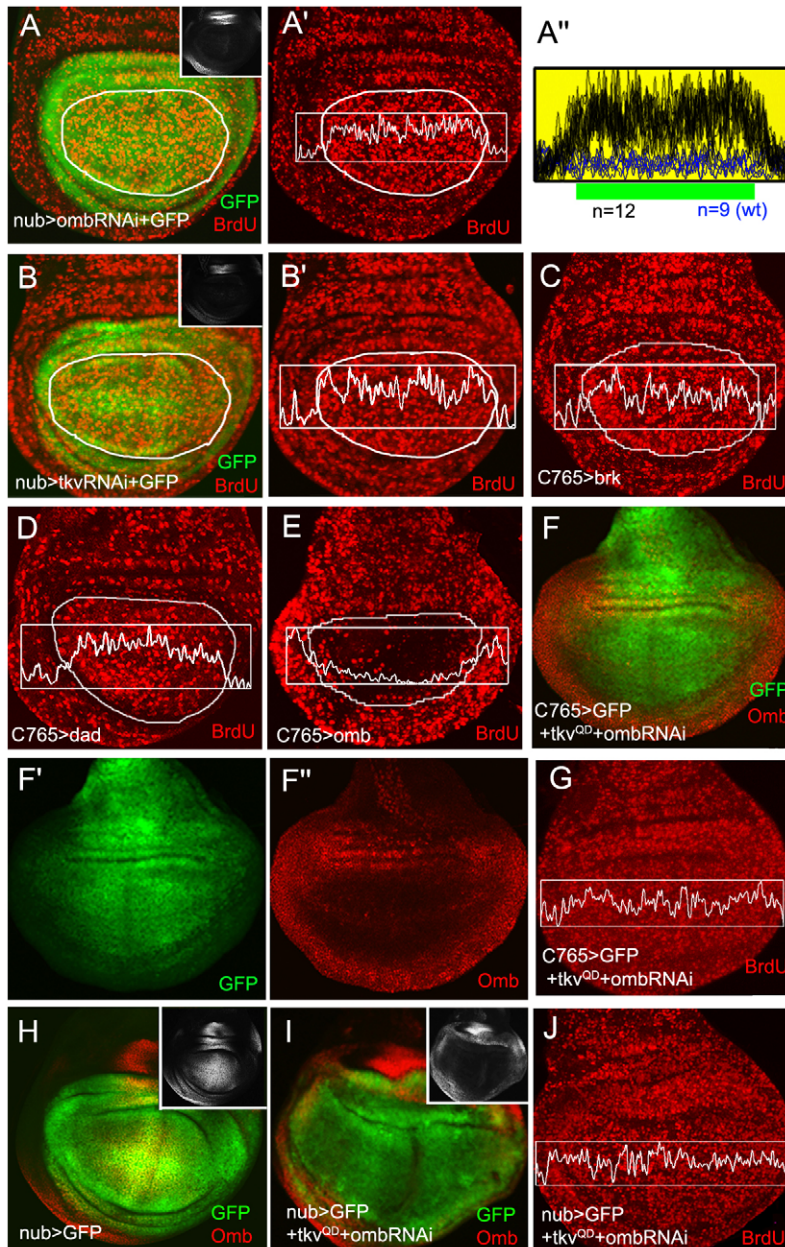


Fig. 2. Dpp and Omb gradients are not required for an even rate of proliferation along the A/P axis of the wing disc. Insets show anti-Omb staining. White circumferential outlines demarcate the wing pouch. White boxes define the area of fluorescence quantification of BrdU staining. (A,A') *nub*>*omb*-RNAi causes uniform and increased proliferation in the wing pouch. (A'') Merged traces of BrdU incorporation collected from 12 *nub*>*omb*+GFP (black profiles) and nine wild-type wing discs (blue profiles). (B,B') *nub*>*tkv*-RNAi enhances proliferation in the pouch. (C,D) *nub*C765>*brk* or *Dad* enhances proliferation in the wing pouch. (E) C765>*omb* promotes proliferation in the lateral wing disc and reduces proliferation in the pouch. (F-J) C765>*Tkv*^{QD}+*omb*-RNAi or *nub*>*Tkv*^{QD}+*omb*-RNAi causes an inverted Omb gradient and induces an almost even rate of proliferation across the width of the wing disc.

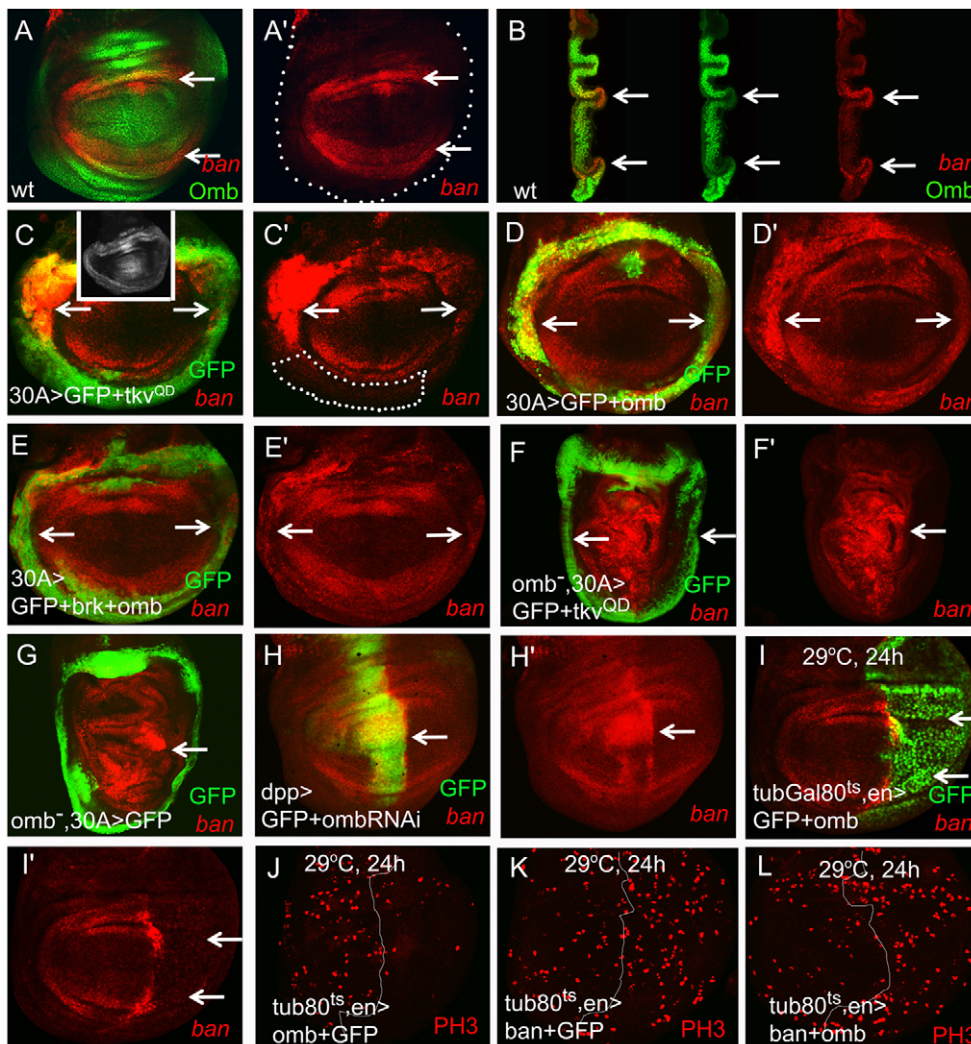


Fig. 3. *omb* regulates *ban* br-C12-lacZ enhancer reporter expression. br-C12-lacZ is abbreviated to *ban* in A-I'. (A-B) *ban* expression in the wing disc (dotted outline in A') is maximal in cells along the circumferential hinge/blade fold. Peak expression of *ban* (arrows) and of Omb are complementary in the wing pouch (x-z section in B). (C-D') 30A>TkV^{QD} or Omb induces lateral *ban* expression (arrows). Immunostaining of Omb (inset in C) shows circumferential upregulation of Omb in the 30A-Gal4 domain. *ban* was not induced in the ventral pleura (dotted outline in C'), a region with strong endogenous Omb expression (A). (E-E') 30A>brk+omb does not prevent *ban* expression in the lateral region (arrows). (F,F') 30A>TkV^{QD} in an *omb* mutant background does not induce lateral *ban* expression (arrows). (G) Lack of *omb* induces *ban* expression in the medial region (arrow). (H,H') *dpp*>*omb*-RNAi induces *ban* expression in the central region (arrows). (I,I') *en*>*omb* represses *ban* expression in the P compartment (arrows). (J) *en*>*omb* represses mitotic activity in the posterior pouch. (K) *en*>*ban* increases posterior proliferation. (L) This enhanced posterior proliferation is not repressed by co-expression of Ban and Omb. PH3, phosphohistone H3 (mitotic marker).

S1D',G'). Similarly, in the tiny *dpp* mutant wing discs, extrusion of cells occurred leading to disruption of normal tissue morphology (supplementary material Fig. S1I,J). The medial proliferation was still enhanced to some extent in such wing discs (Fig. 1B; supplementary material Fig. S1H).

To reduce secondary effects, such as apoptosis (Adachi-Yamada and O'Connor, 2002) and cell elimination (Shen et al., 2010) caused by sharp and lasting discontinuities in Dpp signaling activity, we induced transgene expression for a relatively short time during the third larval instar under Gal80^{ts} control (McGuire et al., 2003). Adults emerging from this regime had well developed and patterned wings, with typical wing blade differentiation in the area of experimental manipulation (supplementary material Fig. S2), indicating that tissue respecification did not occur. Compared with the control genotype, medial downregulation of Dpp signaling by expressing *Dad* or *omb*-RNAi increased the distance between veins L3 and L4, whereas overexpressed *omb* decreased the distance between L3 and L4 (supplementary material Fig. S2). Trichome density in the interval between L3 and L4 was not significantly different between these genotypes, indicating that the observed effects were caused by changes in proliferation rather than cell size. In the lateral region, direct or TkV^{QD}-induced *omb* expression promoted lateral growth and proliferation (Fig. 1Q-R'). Therefore,

Dpp-Omb signaling regulates proliferation in a region-specific manner: proliferation is inhibited medially and promoted laterally.

Schwank et al. (Schwank et al., 2008) reported that proliferation is uniform in *sal*>TkV^{QD} discs. Since *omb* can be activated by TkV^{QD}, this should result in medially reduced proliferation. However, we and others have shown that in the medial domain TkV^{QD} does not activate *omb* beyond its normal central level. Direct overexpression (i.e. not via TkV^{QD}) is necessary to increase Omb beyond the endogenous peak level (Nellen et al., 1996; Shen et al., 2010).

The Omb vertebrate orthologs Tbx2 and Tbx3 also control proliferation in a tissue-specific manner in some tissues downstream of Bmp2 (Manning et al., 2006; Redmond et al., 2010; Ribeiro et al., 2007).

The region of expression but not the gradient is important for proliferation control by Dpp-Omb

The Dpp gradient has been experimentally demonstrated not to be important for proliferation regulation in the medial wing disc (Schwank et al., 2008). Omb, too, shows a graded distribution along the A/P axis of the wing disc (Shen et al., 2010). To test the relevance of graded Omb expression for uniform growth in the wing pouch, we measured the profiles of anti-BrdU fluorescence intensity

in wing discs in which *omb* expression was variously manipulated. This was achieved either by indirect downregulation of *omb* (expression of *tkv*-RNAi, *brk* or *Dad*; Fig. 2B-D) or by directly affecting *omb* (expression of *omb*-RNAi or *omb*; Fig. 2A,E). When nub-Gal4 (Fig. 2H) was used to drive *omb*-RNAi, upregulation of proliferation was restricted to the *omb* domain (Fig. 2A-A''). Within the pouch, the level of BrdU incorporation was roughly even (Fig. 2A''). This suggests that the normal Omb gradient is not required for specifying an even growth rate in the wing pouch. To further demonstrate the irrelevance of the normal Omb gradient for an even rate of proliferation, we generated an inverted Omb gradient (Fig. 2F'') by co-expressing *Tkv*^{QD} and *omb*-RNAi using nub-Gal4 or C765-Gal4, which in this background has a relatively weak activity laterally (Fig. 2F'). Even under these conditions, uniform growth along the A/P axis was maintained (Fig. 2G,J). Therefore, the medial-to-lateral Dpp and Omb gradients are not required to maintain an even rate of proliferation in the wing disc.

***ban* mediates Dpp-Omb signaling in proliferation regulation**

Previous reports showed that the Dpp and Fat-Hippo signaling pathways regulate expression of the microRNA gene *ban*, which promotes proliferation (Brennecke et al., 2003; Nolo et al., 2006; Thompson and Cohen, 2006). In the wing disc, *ban* is repressed laterally by Brk and induced medially by Yorkie-Mad (Martin et al., 2004; Doumpas et al., 2013; Oh and Irvine, 2011).

To determine the relationship between Omb and *ban*, we monitored *ban* transcription using the enhancer reporter br-C12-lacZ (Oh and Irvine, 2011). *ban* was mainly transcribed in the hinge/blade folds surrounding the wing pouch and thus was partly complementary to the pouch expression of *omb* (Fig. 3A,B). Consistent with the effect of lateral *Tkv*^{QD} clones (Oh and Irvine, 2011), elevated Dpp signaling in the lateral region of the wing disc, induced by 30A>*Tkv*^{QD}, upregulated *ban*. Upregulation only occurred where *omb* was also induced. No upregulation was seen in pleura and medial hinge (Fig. 3C', dotted outline) where endogenous Omb expression is strong. Direct overexpression of *omb* by 30A>*omb* induced *ban* expression in the entire 30A-Gal4 domain (Fig. 3D). When *brk* and *omb* were co-expressed, *ban* was still upregulated laterally (Fig. 3E). The lateral upregulation of *ban* by *Tkv*^{QD} was suppressed by lack of *omb* (Fig. 3F). Quantification of *ban* expression along the A/P axis is shown in supplementary material Fig. S3. Stronger *ban* induction at the anterior compared with the posterior disc periphery probably reflects local differences in the strength of the 30A-Gal4 driver. The data indicate that *omb* is downstream of *brk* and that *omb* is necessary and sufficient for lateral *ban* induction.

Loss of *omb* increased *ban* in the medial region (Fig. 3F-G), suggesting a repressive role of Omb on *ban* expression in this region. To test this assumption, we downregulated *omb* in the dpp-Gal4 domain. This caused upregulation of *ban* expression (Fig. 3H). When *omb* was expressed in the en-Gal4 domain for 24 hours, *ban* expression and cell proliferation in the P compartment were efficiently reduced (Fig. 3I-J). Co-expression of *ban* and *omb* rescued the medial growth repression caused by *omb* expression (Fig. 3K,L). When *omb* was expressed in the en-Gal4 domain for 40 hours, *ban* expression in the lateral region was elevated and the posterior pouch was reduced as a consequence of low proliferation (supplementary material Fig. S4B,B'). Taken together, our results demonstrate that Omb oppositely regulates *ban* in lateral versus medial regions of the wing imaginal disc to control proliferation.

ban can be activated by a complex of Yorkie (Yki) and Mad. However, Brk represses *ban* even in the presence of a constitutively

active form of Yki (Oh and Irvine, 2011). Omb appears to be more potent than Yki in antagonizing Brk. Expression of *omb* in the lateral wing disc overcame the repressive effect of even high Brk concentrations (Fig. 3E). The *ban* enhancer reporter carries a weak potential binding site for the T-box transcription factor Omb, which, however, is poorly conserved among *Drosophila* species suggesting that regulation by Omb is indirect.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

J.S. and G.O.P. conceived and designed the experiments, analyzed data and wrote the manuscript. Experiments were performed by X.Z. and D.L.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094300/-/DC1>

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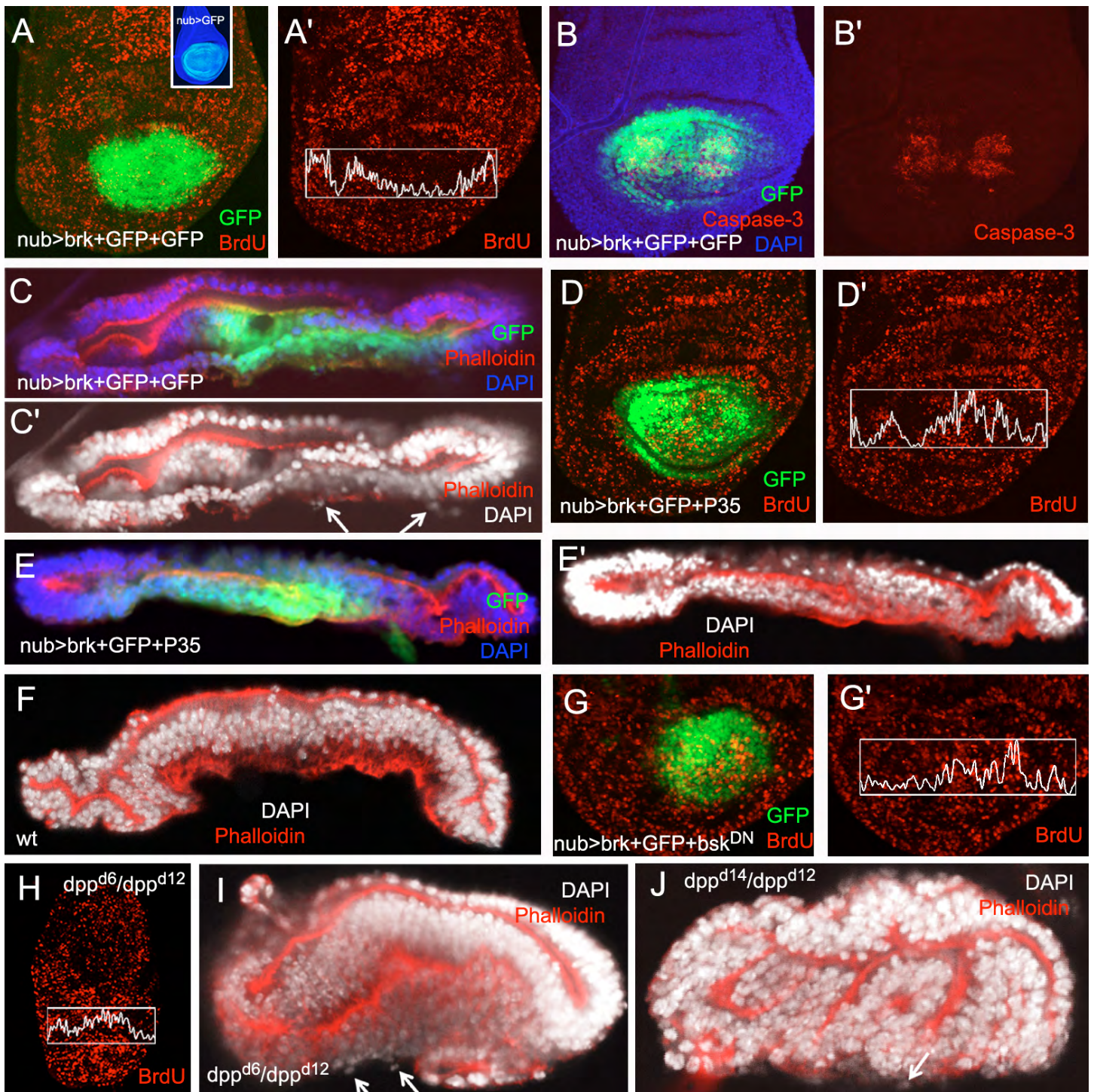


Fig. S1. *nub>brk* induces apoptosis and defective cell/tissue morphogenesis. (C,E-G,I,J) Transverse sections (*x-z* images). Arrows indicate extruded cells. (A-C') Expressing *brk* by *nub*-Gal4 induced decreased proliferation (A), cell death (B) and changes in tissue morphogenesis as well as cell extrusion (C). Inset in A shows the *nub*-Gal4 expression pattern. (D-G') Repressing cell death rescued the defect in proliferation (D,G) but not in tissue morphogenesis (E). Wild-type control (F). (H-J) *dpp* mutant wing discs showed medially enhanced proliferation (H) along with extrusion of cells and disruption of normal tissue morphology (I,J).

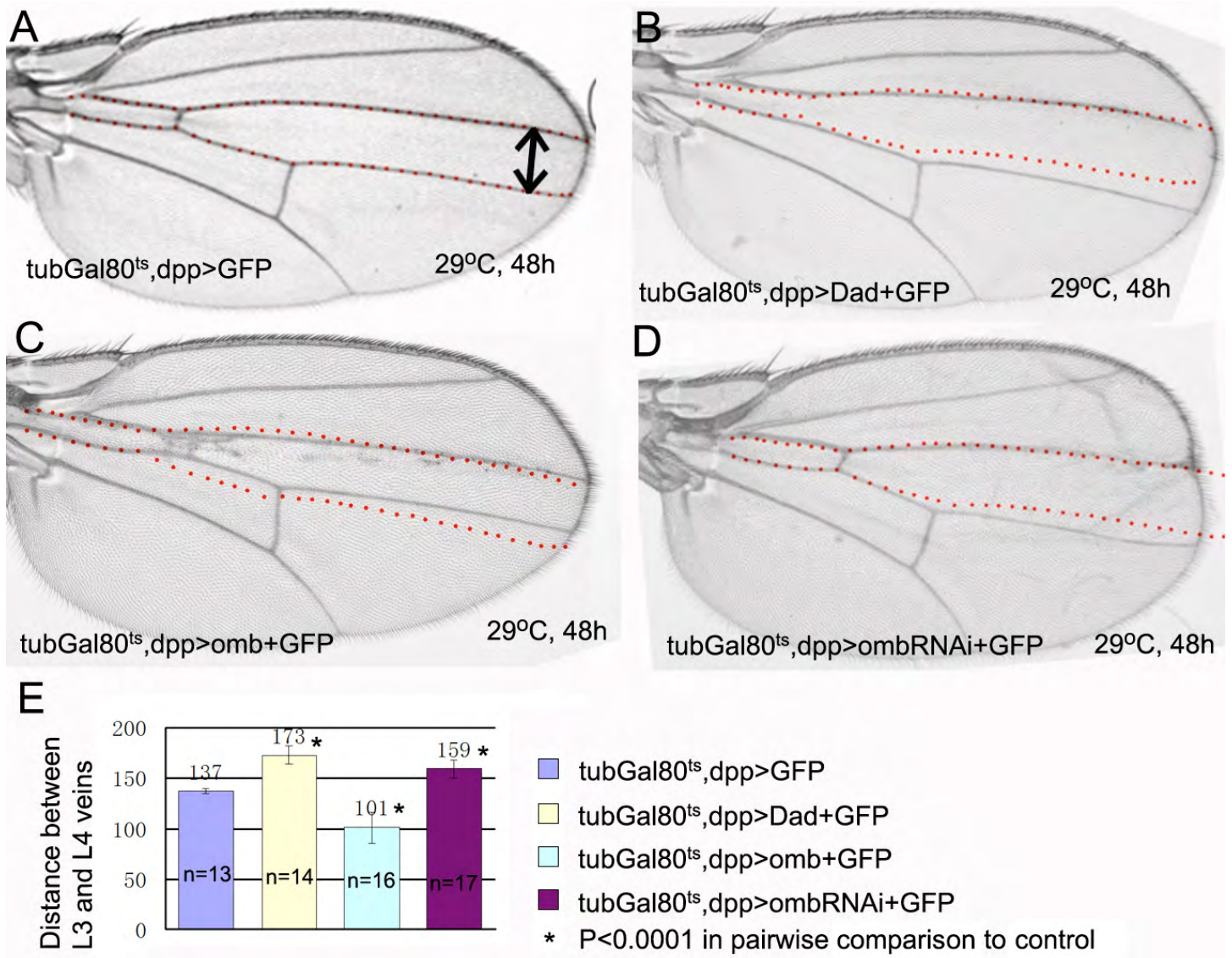


Fig. S2. Attenuated Dpp-Omb signaling increases the distance between L3 and L4 in the adult wing. Red dotted lines outline the normal position of L3 and L4 as observed in the control wing. (A) Control wing. (B) Repression of Dpp signaling by expressing *Dad* in the *dpp*-Gal4 domain increased the distance between L3 and L4. (C) Expressing *omb* in the *dpp*-Gal4 domain reduced the distance between L3 and L4. (D) Repression of *omb* in the *dpp*-Gal4 domain increased the distance between L3 and L4. (E) Statistics of the L3-L4 distance measurements (in arbitrary units). *t*-tests of pairwise comparisons showed significant differences between control and the three experimental genotypes ($P < 0.0001$). Error bars indicate s.d.

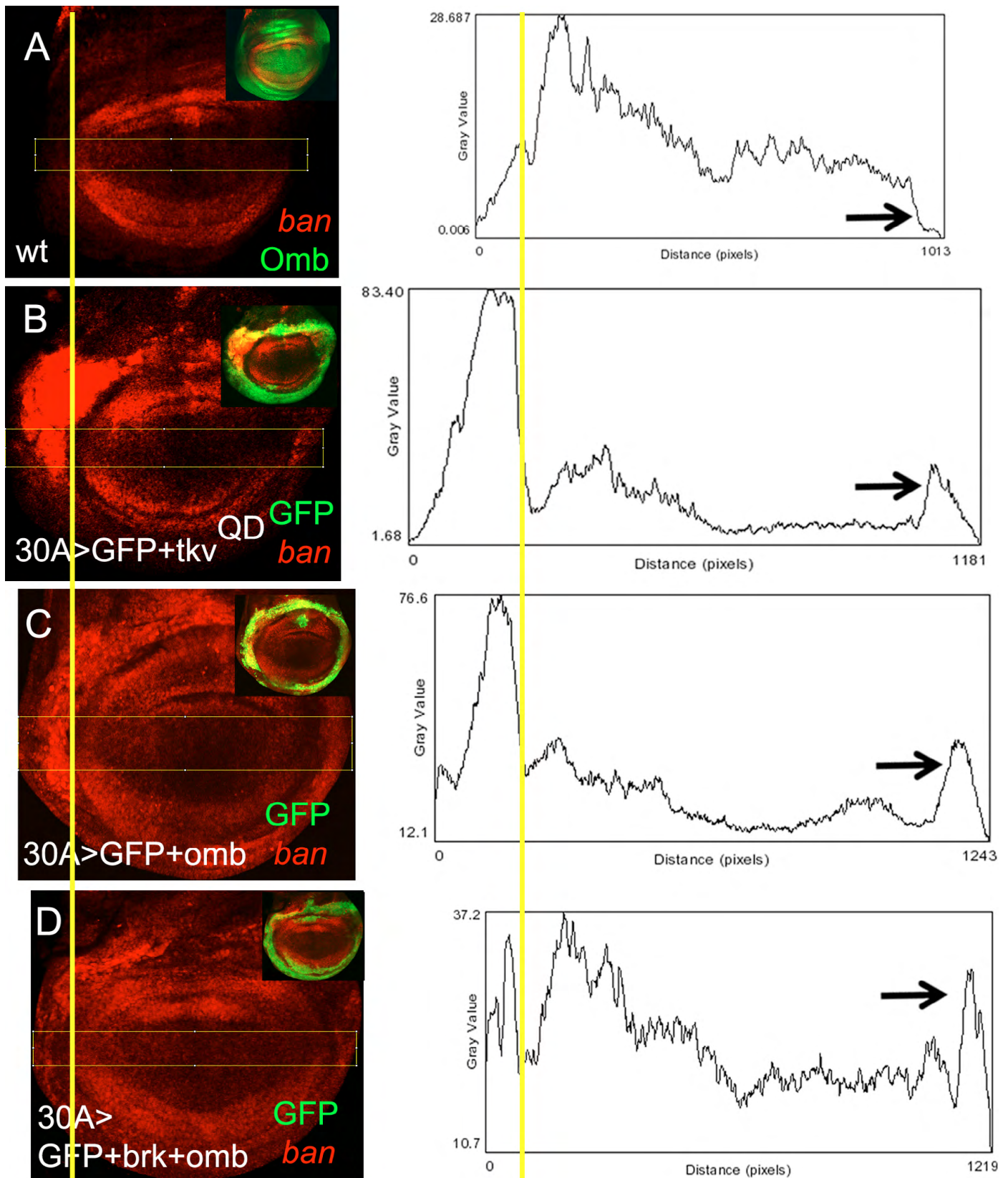


Fig. S3. Quantification of *ban* expression. Omb or Dpp signaling was manipulated in the 30A-Gal4 domain as indicated. A to D correspond to A, C, D and E of Fig. 3. Fluorescence intensities were measured (by ImageJ) in the boxed regions. Panels are oriented such that the anterior hinge-pouch fold is in vertical register (yellow line). (A) Low *ban* expression in lateral wing disc. (B) Lateral Tk v^{QD} induced *ban* expression in the lateral wing disc. Upregulated *ban* only occurred in the lateral regions where *omb* was elevated, but not in pleura and medial hinge where *omb* was not induced. (C) 30A>*omb* induced *ban* expression in the entire 30A-Gal4 domain. (D) When *brk* and *omb* were co-expressed, *ban* was still upregulated laterally. Note that the 30A-Gal4 activity in D was weaker than in B and C. *brk* overexpression apparently attenuated 30A-Gal4 activity. In all three 30A-Gal4-driven experiments, lateral upregulation of *ban* by Tk v^{QD} or Omb was stronger anteriorly than posteriorly, which reflects lateral differences in Gal4 activity (see insets in B-D). This also implies that *ban* is sensitive to the level of Omb in the lateral wing.

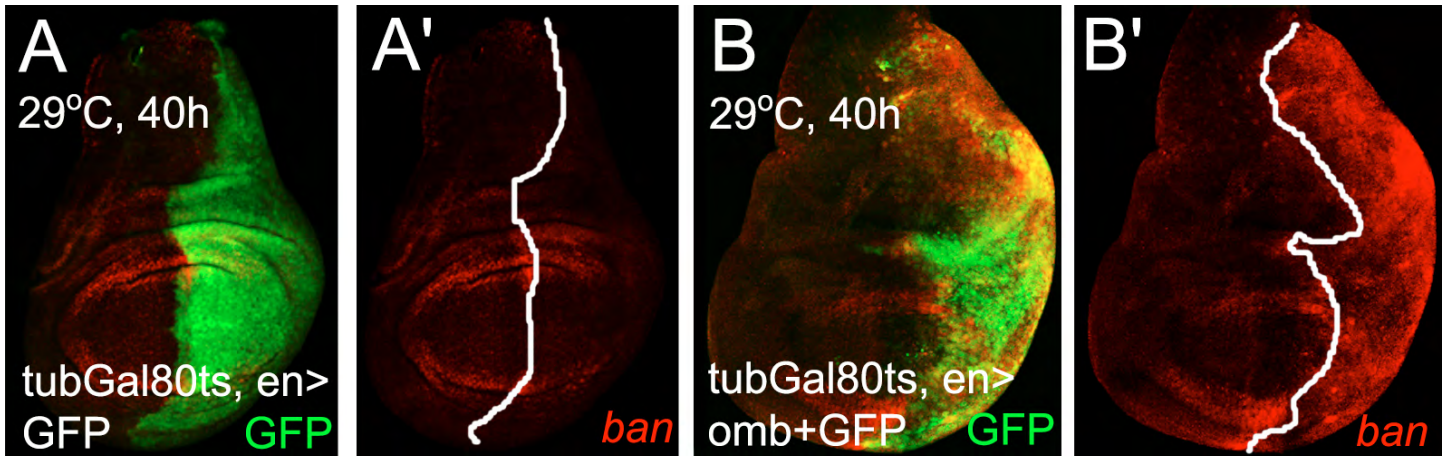


Fig. S4. *Omb* induces *ban* expression in the lateral wing. (A,A') Control experiment. (B,B') Expressing *omb* by en-Gal4 under temporal Gal80^{ts} control for 40 hours induced the elimination of posterior pouch and enhanced lateral *ban* expression.