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

Xubo Zhang¹, Dan Luo¹, Gert O. Pflugfelder²,* and Jie Shen¹,*

**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; Law 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; Telemán 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 Stuhl, 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, 2007; Day and Lawrence, 2000; 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; Telemán 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 Stuhl, 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).

**Accepted 13 May 2013**
MATERIALS AND METHODS

Drosophila stocks

Mutant alleles were l(1)ombD4 (Poeck et al., 1993), dpp612, dpp614 (Spencer et al., 1982). Transgenes were: tubP-Gal80ts (McGuire et al., 2003), dpp-GAL4 (Shen and Mardon, 1997), 30A-GAL4 (Brand and Perrimon, 1993), en-Gal4, nud-Gal4, omb-Gal4MD653 (Calleja et al., 1996), C765-Gal4 (Nellen et al., 1996), UAS-GFP, UAS-bskD (Bloomington Stock Center), UAS-CD8-GFP (Lee and Luo, 1999), UAS-omb (Grinn and Pflugfelder, 1996), UAS-TkvQD (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 brk47-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 Gal80ts-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 TkvQD enhances lateral proliferation. 

(R,R’) Lateral omb expression enhances lateral proliferation. Omb expression was temporally controlled by tub-Gal80ts. 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 Martin et al. (Martin 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>TkvQD+omb-RNAi or nub>TkvQD+omb-RNAi causes an inverted Omb gradient and induces an almost even rate of proliferation across the width of the wing disc.
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 Gal80ts 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 TkvQD-induced omb expression promoted lateral growth and proliferation (Fig. 1Q-1R). 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>TkvQD discs. Since omb can be activated by TkvQD, this should result in medially reduced proliferation. However, we and others have shown that in the medial domain TkvQD does not activate omb beyond its normal central level. Direct overexpression (i.e. not via TkvQD) 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.

**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 TkvOD 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 part complementary to the pouch expression of omb (Fig. 3A,B). Consistent with the effect of lateral TkvQD clones (Oh and Irvine, 2011), elevated Dpp signaling in the lateral region of the wing disc, induced by 30A>TkvQD, 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 TkvQD 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. 5B,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.

**Funding**

This research was supported by the 973 Program [2013CB127603]; National Natural Science Foundation of China [NSFC31071698]; New Century Excellent Talent Award Program from the Ministry of Education of China [NCET090734]; the Doctoral Program of Higher Education Research Fund [20100008120022, 20120008110005]; and Deutsche Forschungsgemeinschaft [PJ 163/15-1].

**Competition 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

**References**


