bantam microRNA is a negative regulator of the Drosophila decapentaplegic pathway

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ABSTRACT
Decapentaplegic (Dpp), the Drosophila homolog of the vertebrate bone morphogenetic protein (BMP2/4), is crucial for patterning and growth in many developmental contexts. The Dpp pathway is regulated at many different levels to exquisitely control its activity. We show that bantam (ban), a microRNA, modulates Dpp signaling activity. Over expression of ban decreases phosphorylated Mothers against decapentaplegic (Mad) levels and negatively affects Dpp pathway transcriptional target genes, while null mutant clones of ban upregulate the pathway. We provide evidence that dpp upregulates ban in the wing imaginal disc, and attenuation of Dpp signaling results in a reduction of ban expression, showing that they function in a feedback loop. Furthermore, we show that this feedback loop is important for maintaining anterior-posterior compartment boundary stability in the wing disc through regulation of optomotor blind (omb), a known target of the pathway. Our results support a model that ban functions with dpp in a negative feedback loop.

Introduction
A fundamental question in development is how growth, cell fate specification, and pattern formation are spatially and temporally regulated. Decapentaplegic (Dpp), an ortholog of vertebrate bone morphogenetic protein 2/4 (BMP2/4) [1], regulates both patterning and growth in Drosophila development [2]. Dpp acts through a well-characterized and conserved signal transduction pathway [3–5]. In the initial steps of activation, Dpp ligand binds the type I and type II receptors, Thickveins (Tkv) and Punt (Put). Tkv phosphorylates a receptor-regulated-SMAD (R-Smad), Mad. Phosphorylated Mad (pMad) then forms a complex with the common mediator SMAD (co-SMAD), Medea, which then translocates into the nucleus, forming a complex with other transcription co-factors, regulating target gene expression either by transcription activation or repression.

In larval wing imaginal discs, dpp expression in a narrow stripe of cells along the anterior-posterior (A/P) compartment boundary is essential for proper growth and patterning. Dpp functions as a gradient morphogen to divide the wing disc into different regions by directing the expression of different combinations of target genes. The graded distribution of Dpp ligands leads to the nested expression domains of target genes, such as spalt (sal) and optomotor blind (omb) (a synonym for bifid in FlyBase) and to the reciprocal gradient expression of brinker (brk). The characteristic expression patterns of these target genes play important roles in the positioning of wing veins along the anteroposterior axis [6,7].

Besides patterning, Dpp also functions as a growth-promoting factor. Ectopic expression of either dpp or an activated Dpp receptor, tkvQ253D, causes overgrowth in wing discs [8]. Loss or severe reduction of dpp expression in the wing primordium reduces the wing to a small stump [2]. Loss of the endogenous dpp stripe along the A/P boundary in the wing disc led to growth impairment, indicating Dpp is crucial for Drosophila wing disc growth [9,10]. Cell clones lacking Dpp signaling fail to survive, suggesting that Dpp also functions as a survival factor for wing cells [8,11]. However, the underlying mechanism of growth control by Dpp is only partially understood.
microRNAs (miRNAs) are an evolutionarily conserved, abundant class of small (20–22 nucleotides), non-coding RNAs, which affect translation and mRNA levels of target genes [12]. Each miRNA is thought to target multiple genes, perhaps hundreds [13]. In metazoans, miRNAs typically down regulate gene expression by binding to complementary sequences in the 3’ untranslated region (3’ UTR) of their target mRNAs, resulting in inhibition of protein translation and mRNA [14,15]. Although the overall complementation of miRNAs to their target mRNAs is imprecise, the region between nucleotides 2 through 8 or 9 at the 5’ end of the miRNA, known as the ‘seed’ region, maintains high complementarity with its target sequences [16–22]. miRNAs play widespread and critical roles in a variety of cellular processes including proliferation, differentiation, apoptosis, development, and tumor growth [12,23]. Identification of the mRNA targets of miRNAs is crucial for understanding miRNA functions.

ban was one of the first miRNAs studied in Drosophila and has been shown to affect cell death and growth [24,25]. Originally thought to be unique to Drosophila and related species (www.mirbase.org), it is now known that ban has conserved orthologs across phyla[26]. First identified in a gain-of-function screen for genes that affect tissue growth [25], the ban gene is expressed in a spatio-temporally restricted manner throughout development. ban miRNA stimulates cell proliferation through unknown downstream targets and inhibits apoptosis through its regulation of the pro-apoptotic gene head involution defective (hid) [24]. Studies of elevated ban expression in hippo mutant cells provided evidence that ban is a downstream target of the Hippo tumor-suppressor pathway [27–29]. Furthermore, Yorkie (Yki), a transcriptional effector of the Hippo pathway, induces ban, and ban overexpression is sufficient to rescue the growth defects of yki mutant cells. However, there is no evidence that Yki and ban function in a feedback loop [27,29]. In eye imaginal discs, Yki acts together with Homothorax (Hth) and Teashirt (Tsh), two DNA-binding transcription factors, upregulating ban to promote cell proliferation and survival in the progenitor domain [30]. Hth and Yki are bound to a DNA sequence ~ 14 kb upstream of the ban hairpin in eye imaginal disc cells by chromatin immunoprecipitation, suggesting that this regulation might be direct. ban is also cooperatively regulated by Yki and Mad with both transcription factors binding to a 410 bp enhancer in the ban promoter [28]. This suggests that ban is an important modulator of growth and may be involved in feedback loops [31] to canalize development and growth – a key function of most miRNAs [32].

Other roles for ban in cellular regulation, especially growth and proliferation, have been uncovered. ban expression in interommatidial cells in the larval eye imaginal discs modulates the survival of cells mutant for Retinoblastoma-family proteins [33]. In addition, germline stem cell (GSC) maintenance in adult Drosophila testes and ovaries requires ban [34,35]. In the Drosophila nervous system, ban inhibits polyQ – and tau-induced neurodegeneration [36,37] as well as the control of proliferation of neuroblasts in the brain [38] and glial cells [39]. Furthermore, a core circadian clock gene, clock, is regulated by ban in circadian cells [40]. Finally, growth of dendrite arbors in the Drosophila peripheral nervous system is also regulated by ban [41].

Based on our previous findings in Drosophila S2 cells which showed that Mad is a target of ban [22], we investigated whether ban affects Dpp signaling in vivo. We provided the evidence that ban regulates Dpp signaling in a negative feedback loop, which is important for maintaining the anterior-posterior (A/P) compartment boundary stability in the wing disc through regulation of omb..

Methods

Drosophila strains and genetics

The Gal4/UAS system was used to over express transgenes [42,43]. The following strains were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN): patched-Gal4 (ptc-Gal4, FBti0002124) which expresses in the wing disc A/P boundary, nubbin-Gal4 (nub-Gal4, FBst0042699) which expresses in the wing pouch, engrailed-Gal4 (en-Gal4, FBal0246629) which expresses in the wing disc posterior compartment, and Mz1369-Gal4 (FBal0052386) which expresses uniformly in the wing discs and in the optic lobe of the brain [44]. The following UAS strains were used: (1) GS-ban (FBal0268610), which contains an insertion of the gene search UAS element upstream near the ban
gene, allowing ban to be over expressed by Gal4 (also referred to as UAS-ban) [45], (2) UAS-omb (FBal0049358) [46], (3) UAS-EGFP (FBti0013986), a green fluorescent protein reporter, (4) UAS-Mad4ap, an activated Mad, which contains a mutation of serines into alanines at the four possible mitogen-activated protein kinase (MAPK) sites in the Mad linker region (S-H. Cho and R.W.P., unpublished results), (5) UAS-Daughters against dpp (UAS-Dad, FBal0066214) [47]. Other fly strains used in this study include: a ban sensor (FBtp0017239), a line which contains tub-EGFP and two copies of the ban target sequence in the 3’UTR, and banΔ1 FRT80B/TM6B (FBab0029992) [24], omb-lacZ (FBal0040912) [48], and brk-lacZ (FBal0097347) [49].

Positively marked clones were generated with the Mosaic Analysis with a Repressible Cell Marker (MARCM) system [50]. ban null mutant clones were generated with the aid of UAS-p35 (FBti0012594), which reduces cell death in the clones. Animals were heat shocked at 37°C for one hour after 72 hours of development. Third instar larval wing discs were dissected for staining. The genotype analyzed was brk-lacZ/yw hsFlp tub-Gal4 UAS-GFP; UAS-p35/+; tub-Gal80 FRT 80B/banΔ1 FRT80B.

X-Gal staining

X-Gal staining was performed in order to visualize enhancer trap lines omb-lacZ and brk-lacZ. Third instar larvae were rinsed and dissected in chilled 1x Ringers solution [51]. Larval heads with discs attached were fixed in formalin (Sigma-Aldrich) for 10 minutes and then rinsed for 10 minutes in assay buffer (5 mM KH2PO4, 5 mM K2HPO4, 2 mM MgCl2, 100 mM KCl, 4 mM K3[Fe(III) (CN)6], 4 mM K4[Fe(II)(CN)6])). Next, they were incubated in pre-warmed reaction buffer (1.5 mg/ml X-Gal in assay buffer) for four hours at room temperature or overnight at 4°C. Samples were rinsed in assay buffer to stop the reaction.

Antibody staining

Third instar larvae were dissected and fixed as described above. Primary antibodies used for staining were rabbit anti-pMad (diluted as 1:4000) [52], rat anti-DE-Cadherin (diluted 1:20, Developmental Studies Hybridoma Bank, DCAD2), mouse anti-Wg (diluted 1:50, Developmental Studies Hybridoma Bank, 4D4), rabbit anti-beta-GAL (diluted 1:8000, Cappel). Secondary antibodies, conjugated to Cy3, were used for detection (diluted 1:200, Jackson ImmunoResearch Lab). Wing imaginal discs were mounted in Vectashield mounting medium (Vector Laboratories) and analyzed using confocal microscopy (Leica TCS SP2 and SP5).

Results

ban negatively affects dpp signaling

Based upon our previous findings in Drosophila S2 cells which showed that Mad is a target of ban [22], we investigated whether ban affects Dpp signaling in vivo. We first examined how the output of Dpp signaling is affected when the expression level of ban is modulated. ban expression was monitored using the ban sensor (Fig. S1B2). This ban sensor identifies endogenous levels of ban as well as cells where ban is over expressed. The sensor consists of the green fluorescent protein (GFP) under the transcriptional control of a tubulin promoter containing an SV40 3’UTR that has two perfectly complementary ban binding sites.

The level of Dpp signaling can be monitored by changes in the level of the phosphorylated form of Mad (pMad) using an antibody specific for the phosphorylated form [53]. In the wild-type wing discs, pMad levels are high in the center, and then graded towards the peripheral region of the wing pouch as observed by immunostaining (Figure 1A), consistent with other’s reports [54]. Over expression of ban along the A/P boundary in the wing imaginal disc by ptc-Gal4 resulted in greatly decreased pMad levels along the A/P boundary in these animals (Figure 1B). Fluorescent intensity measurements of pMad in the AP boundary vs nearby posterior region in discs, indicate that there is a 34.5% decrease in pMad levels when ban is overexpressed (Figure 1E, pMad ratios of A/P to P were 0.57 and 0.87 respectively in ban overexpressing discs and in wild type discs, an independent-samples two-tailed t-test, n = 6, p < 0.001).
To further confirm the effects of ban on Dpp signaling, we used engrailed-Gal4 to overexpress ban in the posterior compartment and then assayed pMad levels in the posterior portion of the A/P boundary and compared it to the anterior region of the A/P boundary (Figure 1C and 1D). Similar to ptc-Gal4 driven ban expression, compared to the wt, we observe a significant decrease in pMad staining in the posterior compartment when ban is overexpressed (0.91 vs 0.77, wt vs ban+, p < 0.05 using an independent-samples two-tailed t-test, n = 6.) (Figure 1F). Taken together, these results suggest that ban is able to negatively regulate dpp signaling.

In addition to pMad levels, we also examined two known Dpp transcriptional target gene levels, omb and brk. omb is a Drosophila T-box gene positively regulated by Dpp [48,55] and is expressed in a broad region in the middle of the wing disc (Figure 1I). X-Gal staining was used to monitor the expression levels of omb-lacZ in wing discs, the enhancer trap lines for omb. ban overexpression by ptc-Gal4 decreased omb-lacZ 39.3% around the A/P boundary in the wing imaginal disc (Figure 1G, II and 1J, 0.61 vs 0.37, wt vs ban+, p < 0.001 using an independent-samples two-tailed t-test, n = 15.).

brk encodes a transcriptional repressor and is a key target of the Dpp pathway that is negatively regulated by Dpp signaling throughout embryonic and larval development [56,57]. brk is highly expressed in the lateral regions of the wing disc, forming a gradient reciprocal to the Dpp gradient (Figure 1M). en-Gal4 was used to overexpress ban in the posterior region of the wing discs, while the anterior compartment was not changed. Expression regions of brk-lacZ in the anterior and posterior compartments were measured. P/A ratios of brk-lacZ expression regions in wing discs were compared between ban overexpression and wild type conditions. There was a significant expansion of brk-lacZ expression in posterior compartment toward the A/P border in wing discs of en-Gal4 > UAS-ban (P/A = 1.97) compared to wild type (P/A = 0.57) conditions (Figure 1H, 1M and 1N, an independent-samples t-test, n = 7, p < 0.001). Furthermore,

![Figure 1. ban down regulates Dpp signaling.](image-url)

All discs are oriented with anterior to the left and ventral down. **(A)** Wild-type wing discs were stained for pMad (red), an indicator of Dpp activity level, showing highest levels along the A/P boundary. **(B)** Over expression of ban by ptc-Gal4 along the A/P boundary of the wing disc, shows that pMad staining decreases along the A/P boundary. White arrows in (A) and (B) indicate altered levels of pMad at the A/P boundary. **(C, C')** Wild type wing discs were stained for pMad (red), posterior compartment was labeled by GFP driven by en-Gal4. **(D, D')** Over expression of ban by en-Gal4, shows that pMad (red) is greatly decreased in the posterior compartment (green region) of the wing disc. Scale bar indicates 50μm (A-D'). **(E, F)** Quantification of fluorescent intensity of pMad in wing discs. Overexpression of ban led the significant decrease of pMad levels in the wing discs. **(E)** pMad ratios of A/P to P were 0.87 and 0.57 respectively in wild type disc and in ptc-Gal4 > UAS-ban discs (an independent-samples two-tailed t-test, n = 6, p < 0.001). **(F)** pMad ratios of P to A were 0.91 and 0.77 respectively in wild type disc and in en-Gal4 > UAS-ban discs (an independent-samples two-tailed t-test, n = 6, p < 0.05). **(G)** Quantification of omb-lacZ levels in wing discs. ban overexpression by ptc-Gal4 decreased omb-lacZ around the A/P boundary in the wing discs (0.61 vs 0.37, wild type vs ptc-Gal4 > UAS-ban, an independent-samples two-tailed t-test, n = 15, p < 0.001). **(H)** Quantification of brk-lacZ expression regions in wing discs. ban overexpression by en-Gal4 led a significant expansion of brk-lacZ expression in posterior compartment toward the A/P border in wing discs (P/A ratios of brk-lacZ expression regions were 0.57 vs 1.97, wild type vs en-Gal4 > UAS-ban, an independent-samples two-tailed t-test, n = 7, p < 0.001). **(I-L)** X-Gal staining was used to monitor the expression levels in wing discs of the enhancer trap lines for omb, a downstream target gene of Dpp signaling. Discs from wild-type (I, K) or over expressed ban (J, L) were incubated with X-Gal for equal periods of time. **(J)** When ban was over expressed along the A/P boundary by ptc-Gal4, omb expression was decreased compared to wild type (L). **(L)** When ban was over expressed throughout the wing disc by Mz1369-Gal4, omb expression was decreased compared to wild type (K). Red arrow indicates an apical fold at the A/P boundary. **(M, N)** X-Gal staining was used to monitor the expression levels in wing disc of the enhancer trap lines for brk, a downstream target gene of Dpp signaling. Discs were incubated with X-Gal for equal periods of time. brk expression was expanded anti-parallel to Dpp gradient as indicated by the red arrows when ban over expressed by en-Gal4. Scale bar indicates 50μm (l to N). **(O, O')** ban null mutant clones are marked by expression of GFP. Apoptosis was prevented by the use of UAS-p35. Anti-β-GAL antibodies were used to monitor brk-lacZ levels. brk-lacZ decreases inside of banΔ/Δ mutation (white dashed line) compared to the upper and lower wild type cells (inset in O is a magnification of the clone). Scale bar indicates 50μm. **(P-S)** discs stained with anti-DE-Cadherin (red) to view the morphology of wing discs, **(P)** wild-type wing disc, **(Q)** over expression of ban by Mz1369-Gal4. ban causes an apical fold morphology defect along the A/P boundary (white arrow). **(R)** Coexpression of ban with omb can fully rescue ban (notice no ectopic fold along A/P boundary), or **(S)** partially rescue ban defects (notice that only a short ectopic fold was seen along the A/P boundary). Scale bar indicates 50μm (P to S).
when ban null mutant clones were located in brk-expressing cells in the lateral region of the wing disc, the level of brk-lacZ was strongly reduced (35%, n = 6) compared to the adjacent wild-type cells (Fig. 1O, 1O'). Since ban mutant clones are almost the same distance from the source of Dpp as the adjacent wild-type cells, the decrease of brk-lacZ in ban clones is not due to its position relative to the Dpp source, but to the loss of ban. All of these results demonstrated that ban down regulates Dpp signaling.

Over expression of ban caused an apical fold defect along the wing disc A/P boundary

In wild type wing disc of Drosophila, omb expression is required in posterior cells to suppress fold formation at the anterior/posterior (A/P) compartment boundary, in order to develop the flat wing surface. Reduction of omb by omb hypomorphic alleles have an apical fold morphogenetic defect in the middle of the wing disc [58–61].

When ban was over expressed by Mz1369-Gal4 throughout the entire wing imaginal disc, omb-lacZ expression decreased and ectopic folding increased in the middle of the wing disc (Figure 1L, 1Q) compared to wild type (Figure 1K, 1P), similar to the folding caused by hypomorphic omb [59]. When omb was over expressed with the Mz1369-Gal4 driver, most animals died as embryos [62]. However, when both omb and ban were over expressed together using the Mz1369-Gal4 driver, approximately 40% of the discs (n = 35) appeared wild type (Figure 1R), and the remaining discs had a less severe phenotype (Figure 1S) than when ban was over expressed alone (Figure 1Q).

These results implied that the ectopic folding caused by ban was at least in part due to the decrease in omb by downregulation of Dpp signaling by ban.

Dpp signaling modulates ban expression

Consistent with previous reports, we find that ban is spatially restricted in the wing disc [24] and showed low expression of the sensor in the wing pouch but high expression along the A/P and D/V boundaries (Figure 2A and Fig. S1B2). This expression pattern indicates ban levels are high in the wing pouch but not at the axis boundaries. To determine if Dpp signaling and ban function in a feedback loop, we modulated Dpp signaling activity to examine the effect on ban. An activated Mad (generated by removing putative MAP kinase sites in the linker region) [63–65] was over expressed using the en-Gal4 driver in the posterior compartment of the wing imaginal disc (Figure 2B). We found that the ban sensor was decreased in the posterior compartment of the wing disc and more obviously in the posterior lateral region. No comparable changes were seen in the anterior compartment where Mad levels were not changed (Figure 2B compared to Figure 2A). This decline in the ban sensor indicated that increased Dpp signaling increased ban expression in the wing disc, as was seen previously using brk overexpression [66]. Further support of this was seen when Dad, which negatively regulates MAD phosphorylation, was over expressed by nub-Gal4 in the wing pouch (circular region marked by Wg) to inhibit Dpp. The ban sensor expression was greatly increased (Figure 2D compared to Figure 2C), which shows that ban levels were decreased upon inhibition of Dpp signaling. Taken together, our results suggest that ban is regulated by Dpp signaling in the wing disc and exists in a negative feedback loop.

Discussion

In this report, we provide evidence that ban and Dpp signaling exist in a negative feedback loop (Figure 3). Over expression of ban changes the levels of pMad and Dpp transcriptional target genes, omb and brk. When ban is over expressed, brk levels increase and pMad and omb levels decrease, as expected if ban affects Dpp signaling.

ban regulates aspects of dpp functions

The mechanisms of action of miRNAs on biological events vary. Most miRNAs are thought to function subtly to fine-tune the biological processes they are regulating by ensuring the appropriate level of gene expression during different developmental processes. For example, Drosophila mir-9a regulates the level of expression of its target...
Figure 2. ban expression is regulated by Dpp signaling.

(A) The ban sensor levels in a wild-type disc and, (B) ban sensor expression of wing disc in which activated Mad (Mad4ap) was expressed by en-Gal4 in the posterior compartment. (C, D) Dad decreases ban levels. Wg staining (red) was used to outline the wing pouch (samples were imaged with the same settings). (C, C') the ban sensor in a wild-type disc, (D, D') ban sensor of the wing disc in which Dad was over expressed by nub-Gal4 in the wing pouch. Note that the ban sensor was increased inside of wing pouch in this case (compare GFP levels inside area denoted by white dashed lines in C and D). Scale bar indicates 50μm.
gene senseless to ensure the generation of precise numbers of sensory organs in Drosophila embryos and adults [67]. Rarely, miRNAs act as a switch, such as C. elegans lsy-6 and mir-273, which are thought to operate in a double negative-feedback loop to specify left-right asymmetry of chemosensory neurons [68,69]. Our results demonstrated that ban is a negative regulator of the Dpp pathway, but we do not believe that ban is acting as a switch. Instead, we propose that ban functions by fine-tuning Dpp signaling to regulate the signaling strength or the gradient of Dpp signaling.

omb is required in the posterior cells of the wing disc to prevent aberrant apical fold formation at the A/P boundary, and hypomorphic omb alleles exhibit ectopic folding [58–60]. We found that overexpression of ban down regulated omb in the imaginal discs and caused a similar ectopic folding in the wing imaginal discs. Furthermore, overexpression of omb partially rescued the folding defects caused by ban. The partial rescue of omb folding defects rather than total rescue could be explained by regulation of omb by other genes, such as Wg, which has been shown to regulate omb in conjunction with Dpp [70]. Besides the function of maintaining normal cell morphology in the wing disc, omb has roles in growth control of the wing disc. A growth-repressive role of Omb has been found in the wing disc [60], which might be the reason that the size of wing discs of mzl369 > UAS-ban + UAS-omb is smaller than wild type ones. Future experiments to explore the underlying mechanism will be of interest.

ban has putative binding sites on mad 3’UTR mRNA and could possibly regulate mad

Computational algorithms, including our own, to predict target genes for miRNAs, identified Mad as a potential target of ban (TargetScan, miRanda) [20,71] which was subsequently validated by our group in vitro in Drosophila S2 cells [22]. We find two putative ban binding sites in the Mad 3’UTR (Fig. S1A), which are physically close to each other and are evolutionarily conserved in other fly species (data not shown).

We modified the ban sensor by replacing the SV40 3’UTR with a wild-type Mad 3’UTR, or a mutated Mad 3’UTR (Fig. S1B3,B4) to determine if loss of target sites would change transgene expression patterns. The wild-type Mad sensor (Fig. S1B3) showed similar patterns to the ban sensor (Fig. S1B2) in the wing pouch, indicating that in regions of high ban expression, the wild-type Mad sensor had been also down regulated. The mutated Mad sensor (Fig. S1B4) lacked this pattern, showing high expression levels in the entire disc similar to the control sensor that lacked ban target sites (Fig. S1B1). Since the mutated Mad sensor differed from the wild-type Mad sensor by only two nucleotide (AU to UA) changes in each of the two putative ban binding regions (Fig. S1A), the expression pattern difference between them suggests that these two ban binding sites are sensitive to the endogenous expression level of ban. Taken together, these data suggest that ban modulates Dpp signaling activity, possibly through downregulation of Mad (Figure 3). However, we state this cautiously as it is important to determine whether endogenous Mad protein levels are affected by ban – a study that is made difficult by small size of ban null clones, low sensitivity of pMad antibody and that pMad levels do not appreciably change under mild perturbation of Dpp signaling [9].

Feedback loop between ban and dpp

Reciprocal feedback loops between miRNAs and the pathways they regulate can play important roles in their functions [68,72]. In Drosophila, reciprocal negative feedback between mir-7 and its target Yan reinforces the photoreceptor differentiation induced by the EGF signal in developing eyes [73]. A similar negative feedback regulatory circuitry involving miR-223 and two transcriptional factors, NFI-A and C/EBPα, is important in human granulocytic differentiation [74]. In C. elegans, a positive feedback loop between lin-12, mir-61, and vav-1 was reported to maximize LIN12 activity and specify the secondary vulva cell fate [75]. In our work, we provided evidence that ban can negatively affect Dpp signaling, while Dpp activity in turn affects ban expression. Our results support a model in which ban and Dpp signaling regulate each other in a negative feedback loop (Figure 3). Consistent with our model, brk has been shown to inhibit ban in the wing disc cells [76], and Mad can bind the enhancer region.
of ban [28]. The regulation of ban by Dpp we observed might be the result of Mad-directed transcriptional regulation or indirect regulation by Brk, or by the cooperation of both. In cells expressing ban, Dpp signaling activity may be fine-tuned through ban’s negative regulatory effect on Mad, which would in turn ensure the precise transcription of Dpp target genes in specific temporal and spatial patterns during development. Upon the stimulation of the Dpp pathway, cells increase the level of ban, which can further down regulate the Dpp pathway to a level needed for development. Interestingly, a recent study has shown that the ban orthologs in C. elegans, the mir-58 family, also directly regulate the BMP pathway by directly binding to and repressing the Type I and Type II receptors (sma-6 and daf-4 respectively) and the ligand (dbl-1) [77]. This study has further shown that a negative feedback loop exists between the BMP pathway and the expression of mir-58 (and related mir-80) similar to what we have reported for Dpp signaling and ban in Drosophila. Taken together, these data suggest that fine tuning of these related pathways by miRNAs is evolutionary conserved.

It is possible that this feedback loop regulation between ban and Dpp could be regulated only in a specific developmental context as a way to fine-tune the regulation of the pathway. Likewise, ban is regulated by a growing number of genes. For example, Notch signaling inhibits ban expression in the wing disc [78]. ban is also a target of the Hippo pathway [27–29], making it an ideal candidate for mediating crosstalk between different signaling pathways. Future studies to understand how ban is integrated into other signaling pathways.
pathways and to clarify how components in these other pathways affect ban expression are warranted.

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Disclosure statement

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