FLY
2018, VOL. 12, NO. 2, 105–117
https://doi.org/10.1080/19336934.2018.1499370

Taylor & Francis
Taylor & Francis Group

bantam microRNA is a negative regulator of the Drosophila decapentaplegic pathway

Nanci S. Kane[a], Mehul Vora [a]*, Richard W. Padgett [a], and Ying Li[b]

[a]Waksman Institute, Department of Molecular Biology and Biochemistry, Cancer Institute of New Jersey, Rutgers University, Piscataway, NJ, USA; [b]Life Science Institute, Chongqing Medical University, Chongqing, China

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.

ARTICLE HISTORY
Received 23 April 2018
Revised 28 June 2018
Accepted 5 July 2018

KEYWORDS
Bantam; *decapentaplegic*; *Drosophila*; microRNAs; BMP

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 depression.

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, tkv^{Q253D}, 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.

CONTACT Ying Li liying@cqmu.edu.cn Life Science Institute, Chongqing Medical University, 1 Xueyuan Road, District Yuzhong, Chongqing 400016, China
*represents equal contribution

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 $ban^{\Delta 1}$ 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/* $ban^{\Delta 1}$ *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

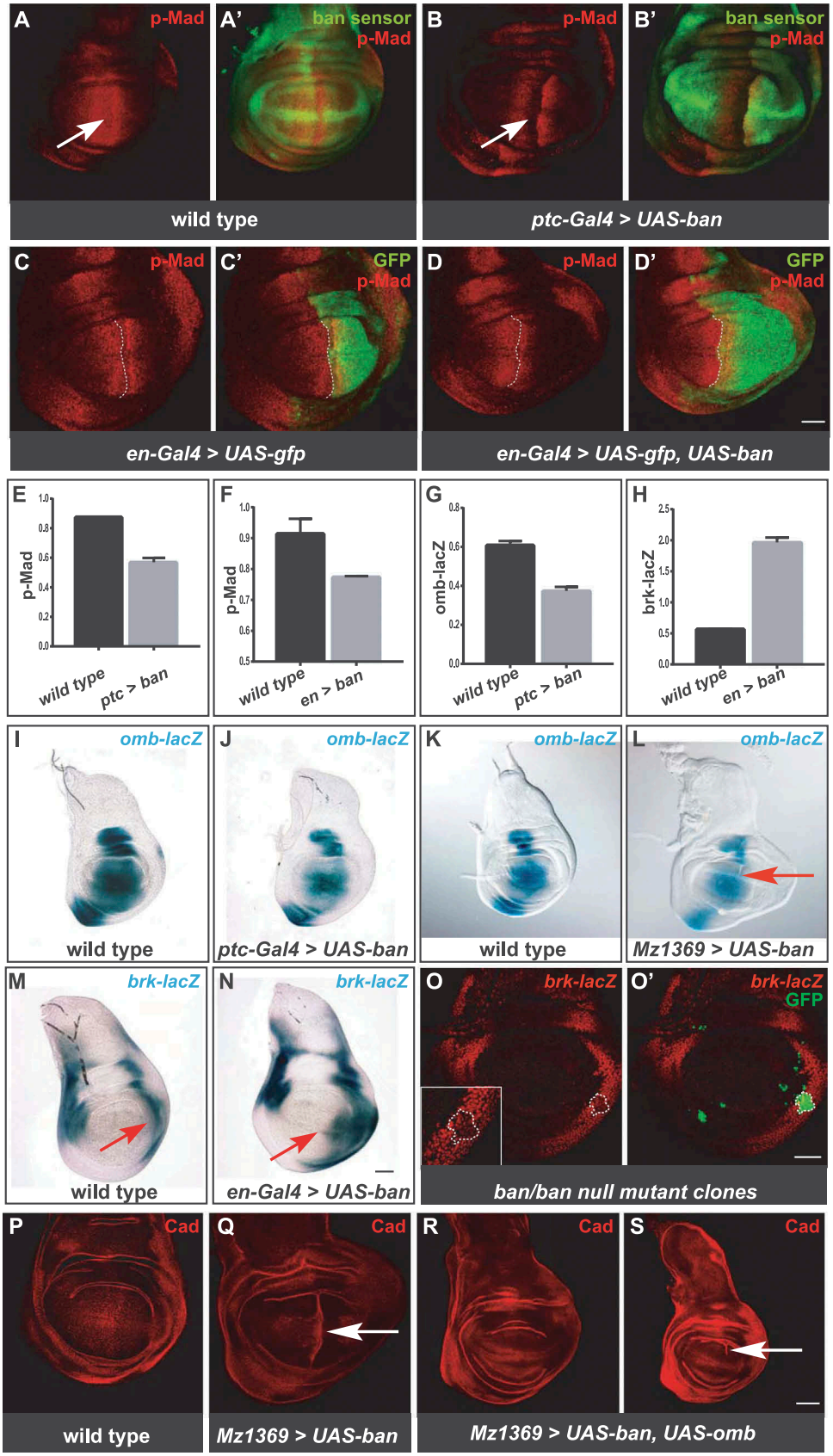
Third instar larvae were dissected and fixed as described above. Primary antibodies used for staining were rabbit anti-pMad (diluted as 1:4000) [52], rat anti-DE-Cadherin (diluted 1:20, Developmental Studies Hybridoma Bank, DCAD2), mouse anti-Wg (diluted 1:50, Developmental Studies Hybridoma Bank, 4D4), 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, $n = 6$, $p < 0.001$).


A
p-Mad
A'
ban sensor
p-Mad
B
p-Mad
B'
ban sensor
p-Mad
wild type
ptc-Gal4 > UAS-ban
C
p-Mad
C'
GFP
p-Mad
D
p-Mad
D'
GFP
p-Mad
en-Gal4 > UAS-gfp
en-Gal4 > UAS-gfp, UAS-ban
E
p-Mad
wild type
ptc > ban
F
p-Mad
wild type
en > ban
G
omb-lacZ
wild type
ptc > ban
H
brk-lacZ
wild type
en > ban
I
omb-lacZ
wild type
J
omb-lacZ
ptc-Gal4 > UAS-ban
K
omb-lacZ
wild type
L
omb-lacZ
Mz1369 > UAS-ban
M
brk-lacZ
wild type
N
brk-lacZ
en-Gal4 > UAS-ban
O
brk-lacZ
O'
brk-lacZ
GFP
ban/ban null mutant clones
P
Cad
wild type
Q
Cad
Mz1369 > UAS-ban
R
Cad
S
Cad
Mz1369 > UAS-ban, UAS-omb

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 (Figure1C 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, 1I 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.

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 sigficant 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 discs 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 discs 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 (I). (**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 discs 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 (I 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 $ban^{\Delta 1}/ban^{\Delta 1}$ mutant clone (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

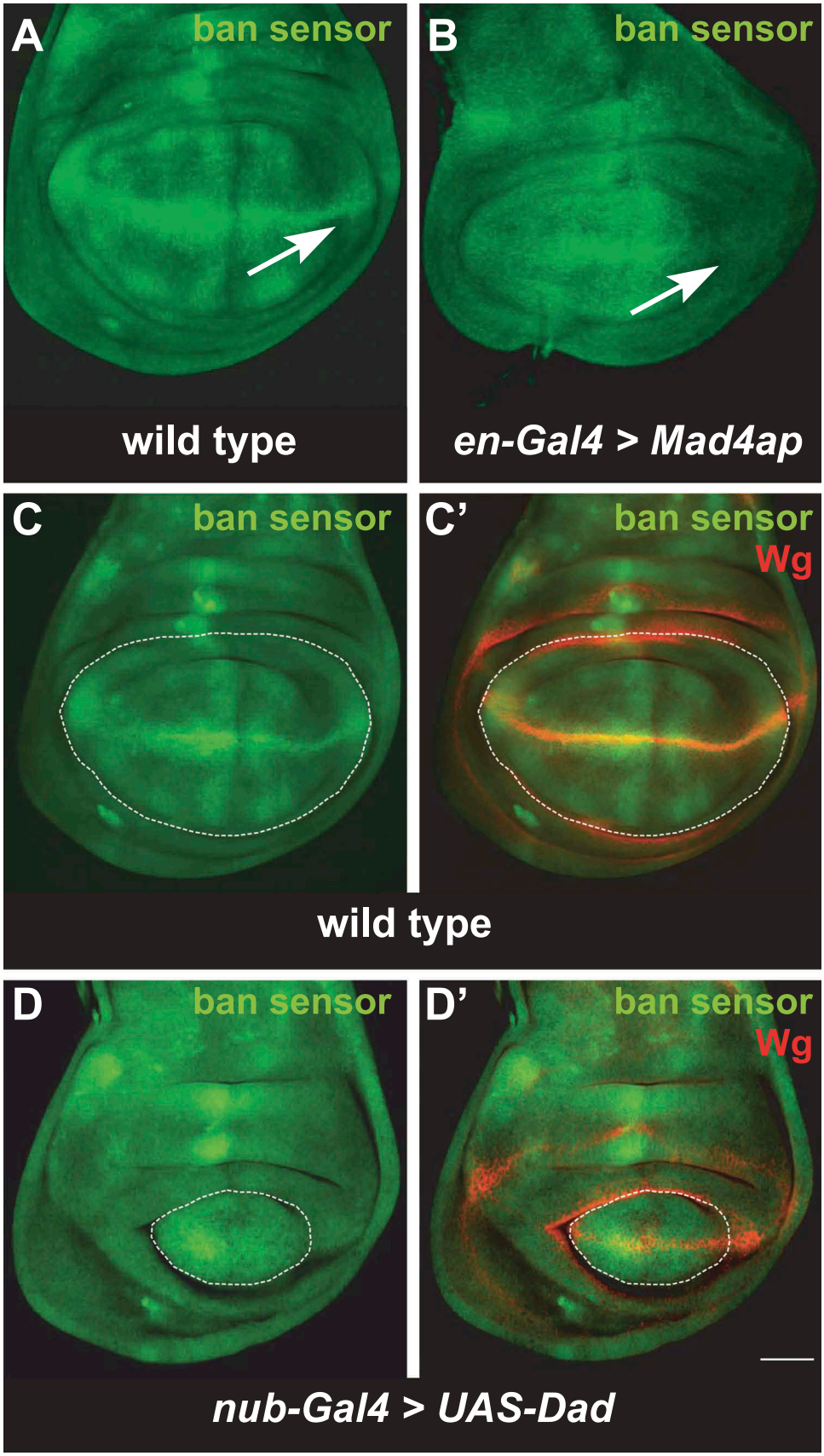


Figure 2. *ban* expression is regulated by Dpp signaling.

(**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 over expression of *ban* down regulated *omb* in the imaginal discs and caused a similar ectopic folding in the wing imaginal discs. Furthermore, over expression 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 *mz1369 > 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

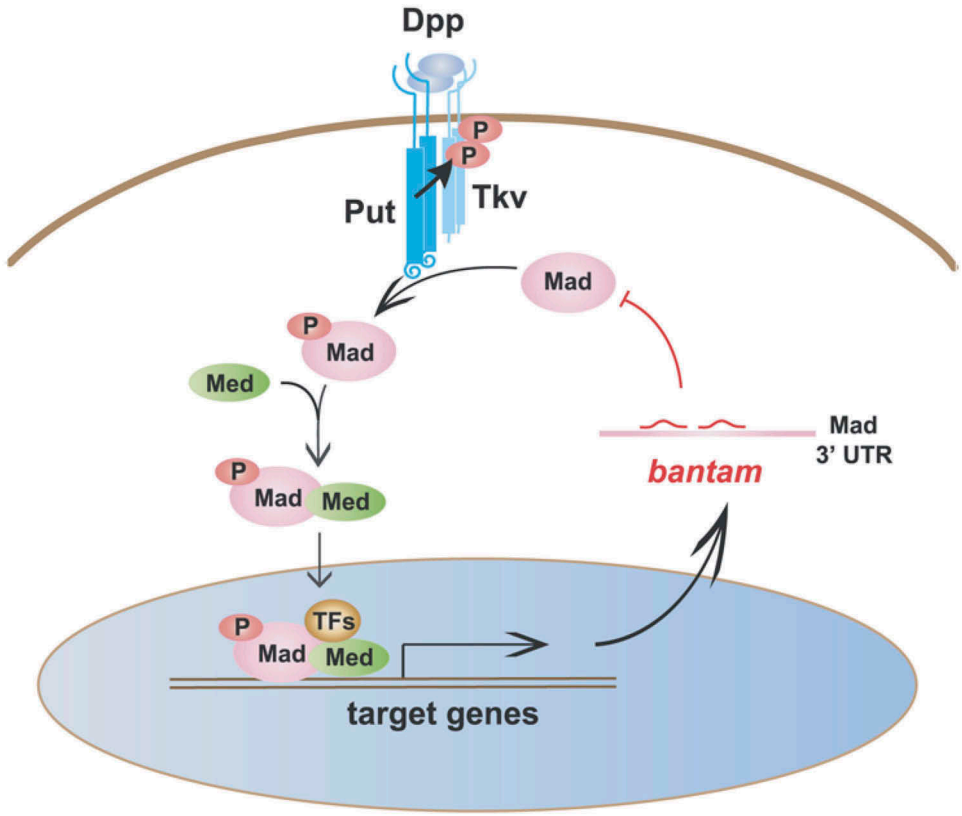


Figure 3. Model of *ban* regulation of *Mad* and Dpp signaling in *Drosophila* wing imaginal disc cells.

In our model, we propose a feedback loop regulation between *ban* and Dpp signaling in the wing imaginal disc. First, extracellular Dpp ligands bind to the cell surface type I and type II receptors, Tkv and Put, respectively. Constitutively active Put phosphorylates Tkv, which in turn phosphorylates the R-Smad, Mad. pMad forms a complex with the Co-Smad, Medea (Med), and translocates into the nucleus, where tissue specific transcription is activated or repressed with the cooperation of other transcription factors (TFs). In cells expressing *ban*, Dpp signaling can be fine-tuned through the inhibitory effect of *ban* on *Mad*. *ban* is up-regulated by Dpp to further ensure the appropriate Dpp activity for developmental requirements. Thus, we propose a model by which *ban* fine-tunes Dpp signaling possibly through its regulation of *Mad*.

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 and to clarify how components in these other pathways affect *ban* expression are warranted.

Acknowledgments

We would like to thank Drs. K. Hofmeyer, G. Pflugfelder, S. Cohen and N. Yakoby, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank at the University of Iowa for generously supplying the fly stocks and reagents. We also want to thank Harlan Robins for his observation that *Mad* contains two *ban* binding sites. This work was partially supported by grants from the NSF (IOS-0641173) and NJ Commission on Spinal Cord Research (CSCR11ERG017) to RWP and grants from the National Natural Science Foundation of China (31501039), Chongqing Yuzhong District Science and technology project (20140104), Chongqing Basic and Frontier Research Project (cstc2016jcyjA0305), Open Project of Key Laboratory of Oncology and Immunology of Ministry of Education (2012jszl10) to YL. YL and MV were recipients of a Charles and Johanna Busch Fellowship.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [31501039];National Science Foundation [IOS-0641173];Chongqing Basic and Frontier Research Project [cstc2016jcyjA0305];Open Project of Key Laboratory of Oncology and Immunology of Ministry of Education [2012jszl10];Charles and Johanna Busch Fellowship;NJ Commission on Spinal Cord Research [CSCR11ERG017];Chongqing Yuzhong District Science and technology project [20140104];

ORCID

Mehul Vora http://orcid.org/0000-0002-1239-6458
Richard W. Padgett http://orcid.org/0000-0002-5740-3324

References

1. Padgett RW, Wozney JM, Gelbart WM. Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. Proc Natl Acad Sci U S A. 1993 Apr 1;90(7):2905–2909. PubMed PMID: 8464906; eng
2. Spencer FA, Hoffmann FM, Gelbart WM. *Decapentaplegic*: a gene complex affecting morphogenesis in Drosophila melanogaster. Cell. 1982 Mar;28 (3):451–461. 0092–8674(82)90199–4 [pii]. PubMed PMID: 6804094; eng
3. Parker L, Stathakis DG, Arora K. Regulation of BMP and activin signaling in Drosophila. Prog Mol Subcell Biol. 2004;34:73–101. PubMed PMID: 14979665; eng
4. Raftery LA, Sutherland DJ. TGF-?2 family signal transduction in *Drosophila* development: from Mad to Smads. Dev Biol. 1999 Jun 15;210(2):251–268. . PubMed PMID: 10357889; eng.
5. Upadhyay A, Moss-Taylor L, Kim MJ, et al. TGF-?2 Family Signaling in Drosophila. Cold Spring Harb Perspect Biol. 2017 Feb 13. DOI:10.1101/cshperspect.a022152. [PubMed PMID: 28130362].
6. Affolter M, Basler K. The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat Rev Genet. 2007 Sep;8(9):663–674. .PubMed PMID: 17703237; eng
7. De Celis JF, Barrio R, Kafatos FC. A gene complex acting downstream of *dpp* in Drosophila wing morphogenesis. Nature. 1996 May 30;381(6581):421–424. . PubMed PMID: 8632798; eng.
8. Martin-Castellanos C, Edgar BA. A characterization of the effects of Dpp signaling on cell growth and proliferation in the Drosophila wing. Development. 2002 Feb;129(4):1003–1013. PubMed PMID: 11861483; eng
9. Bosch PS, Ziukaite R, Alexandre C, et al. Dpp controls growth and patterning in Drosophila wing precursors through distinct modes of action. eLife. 2017 Jul;4:6. PubMed PMID: 28675374; PubMed Central PMCID: PMC5560859. eng.
10. Matsuda S, Affolter M. Dpp from the anterior stripe of cells is crucial for the growth of the Drosophila wing disc. eLife. 2017 Jul;4:6. PubMed PMID: 28675373; PubMed Central PMCID: PMC5560856. eng.
11. Burke R, Basler K. Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. Development. 1996 Jul;122(7):2261–2269. PubMed PMID: 8681806; eng
12. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell. 2003 Jun 13;113(6):673–676. S0092867403004288 [pii]. PubMed PMID: 12809598; eng
13. Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005 Feb 17;433(7027):769–773. PubMed PMID: 15685193.
14. Baek D, Villen J, Shin C, et al. The impact of microRNAs on protein output. Nature. 2008 Sep 04;455(7209):64–71. PubMed PMID: 18668037; PubMed Central PMCID: PMCPMC2745094.
15. Guo H, Ingolia NT, Weissman JS, et al. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010 Aug 12;466(7308):835–840. PubMed PMID: 20703300; PubMed Central PMCID: PMCPMC2990499.
16. Brennecke J, Stark A, Russell RB, et al. Principles of microRNA-target recognition. PLoS Biol. 2005 Mar;3 (3):e85. PubMed PMID: 15723116; eng.

17. Didiano D, Hobert O. Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat Struct Mol Biol. 2006 Sep;13(9):849–851. . PubMed PMID: 16921378; eng
18. Jackson AL, Burchard J, Schelter J, et al. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA. 2006 Jul;12(7):1179–1187. PubMed PMID: 16682560; eng.
19. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet. 2005 May;37(5):495–500. PubMed PMID: 15806104; eng.
20. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005 Jan 14;120(1):15–20. S0092867404012607 [pii]10.1016/j.cell.2004.12.035. PubMed PMID: 15652477; eng
21. Nahvi A, Shoemaker CJ, Green R. An expanded seed sequence definition accounts for full regulation of the hid 3′ UTR by *bantam* miRNA. RNA. 2009 May;15(5):814–822. .PubMed PMID: 19286629; eng
22. Robins H, Li Y, Padgett RW. Incorporating structure to predict microRNA targets. Proc Natl Acad Sci U S A. 2005 Mar 15;102(11):4006–4009. . PubMed PMID: 15738385; eng.
23. Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007;23:175–205. .PubMed PMID: 17506695; eng
24. Brennecke J, Hipfner DR, Stark A, et al. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell. 2003 Apr 4;113(1):25–36. PubMed PMID: 12679032; eng
25. Hipfner DR, Weigmann K, Cohen SM. The bantam gene regulates Drosophila growth [research support, Non-U.S. Gov't]. Genetics. 2002 Aug;161(4):1527–1537. PubMed PMID: 12196398; PubMed Central PMCID: PMC1462212. eng
26. Ibanez-Ventoso C, Vora M, Driscoll M. Sequence relationships among C. elegans, D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology [research support, N.I.H., extramural]. PloS One. 2008 Jul 30;3(7):e2818. . PubMed PMID: 18665242; PubMed Central PMCID: PMC2486268. eng.
27. Nolo R, Morrison CM, Tao C, et al. The *bantam* microRNA is a target of the *hippo* tumor-suppressor pathway. Curr Biol. 2006 Oct 10;16(19):1895–1904. PubMed PMID: 16949821; eng.
28. Oh H, Irvine KD. Cooperative regulation of growth by Yorkie and Mad through bantam [research support, N.I.H., extramural research support, Non-U.S. Gov't]. Dev Cell. 2011 Jan 18;20(1):109–122. . PubMed PMID: 21238929; PubMed Central PMCID: PMC3033745. eng.
29. Thompson BJ, Cohen SM. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. Cell. 2006 Aug 25;126(4):767–774. . PubMed PMID: 16923395; eng.
30. Peng HW, Slattery M, Mann RS. Transcription factor choice in the Hippo signaling pathway: *homothorax* and *yorkie* regulation of the microRNA *bantam* in the progenitor domain of the Drosophila eye imaginal disc. Genes Dev. 2009 Oct 1;23(19):2307–2319. . PubMed PMID: 19762509; eng.
31. Herranz H, Hong X, Cohen SM. Mutual repression by bantam miRNA and Capicua links the EGFR/MAPK and Hippo pathways in growth control. Current Biology: CB. 2012 Apr 24;22(8):651–657. . PubMed PMID: 22445297; eng.
32. Cassidy JJ, Straughan AJ, Carthew RW. Differential masking of natural genetic variation by miR-9a in Drosophila. Genetics. 2016 Feb;202(2):675–687. . PubMed PMID: 26614743; PubMed Central PMCID: PMCPMC4788242
33. Tanaka-Matakatsu M, Xu J, Cheng L, et al. Regulation of apoptosis of *rbf* mutant cells during Drosophila development. Dev Biol. 2009 Feb 15;326(2):347–356. PubMed PMID: 19100727; eng.
34. Shcherbata HR, Ward EJ, Fischer KA, et al. Stage-specific differences in the requirements for germline stem cell maintenance in the Drosophila ovary. Cell Stem Cell. 2007 Dec 13;1(6):698–709. PubMed PMID: 18213359; eng.
35. Yang Y, Xu S, Xia L, et al. The *bantam* microRNA is associated with drosophila fragile X mental retardation protein and regulates the fate of germline stem cells. PLoS Genet. 2009 Apr;5(4):e1000444. PubMed PMID: 19343200; eng.
36. Bilen J, Liu N, Bonini NM. A new role for microRNA pathways: modulation of degeneration induced by pathogenic human disease proteins. Cell Cycle. 2006 Dec;5(24):2835–2838. 3579 [pii]. PubMed PMID: 17172864; eng
37. Bilen J, Liu N, Burnett BG, et al. MicroRNA pathways modulate polyglutamine-induced neurodegeneration. Mol Cell. 2006 Oct 6;24(1):157–163. PubMed PMID: 17018300; eng.
38. Weng R, Cohen SM. Control of Drosophila type I and type II central brain neuroblast proliferation by bantam microRNA [research support, Non-U.S. Gov't]. Development. 2015 Nov 1;142(21):3713–3720. . PubMed PMID: 26395494; PubMed Central PMCID: PMC4647215. eng.
39. Li Y, Padgett RW. *bantam* is required for optic lobe development and glial cell proliferation. PLoS One. 2012;7(3):e32910. .PubMed PMID: 22412948; PubMed Central PMCID: PMCPMC3297604
40.. Kadener S, Menet JS, Sugino K, et al. A role for microRNAs in the Drosophila circadian clock. Genes Dev. 2009 Sep 15;23(18):2179–2191. PubMed PMID: 19696147; eng.
41. Parrish JZ, Xu P, Kim CC, et al. The microRNA *bantam* functions in epithelial cells to regulate scaling growth of dendrite arbors in Drosophila sensory neurons. Neuron. 2009 Sep 24;63(6):788–802. PubMed PMID: 19778508; eng.

42. Elliott DA, Brand AH. The GAL4 system: a versatile system for the expression of genes. Methods Mol Biol. 2008;420:79–95. .PubMed PMID: 18641942; eng
43. Phelps CB, Brand AH. Ectopic gene expression in Drosophila using GAL4 system. Methods. 1998 Apr;14(4):367–379. .PubMed PMID: 9608508; eng
44. Hiesinger PR, Reiter C, Schau H, et al. Neuropil pattern formation and regulation of cell adhesion molecules in Drosophila optic lobe development depend on synaptobrevin. J Neurosci. 1999 Sep 1;19(17):7548–7556. PubMed PMID: 10460261; eng
45. Cho E, Feng Y, Rauskolb C, et al. Delineation of a Fat tumor suppressor pathway. Nat Genet. 2006 Oct;38 (10):1142–1150. PubMed PMID: 16980976; eng.
46. Hofmeyer K, Kretzschmar D, Pflugfelder GO. Optomotor-blind expression in glial cells is required for correct axonal projection across the Drosophila inner optic chiasm [research support, Non-U.S. Gov't]. Dev Biol. 2008 Mar 1;315(1):28–41. . PubMed PMID: 18234176; eng.
47. Tsuneizumi K, Nakayama T, Kamoshida Y, et al. Daughters against dpp modulates *dpp* organizing activity in Drosophila wing development. Nature. 1997 Oct 9;389(6651):627–631. PubMed PMID: 9335506; eng.
48. Nellen D, Burke R, Struhl G, et al. Direct and long-range action of a DPP morphogen gradient [research support, Non-U.S. Gov't]. Cell. 1996 May 3;85(3):357–368. PubMed PMID: 8616891; eng
49. Minami M, Kinoshita N, Kamoshida Y, et al. *brinker* is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. Nature. 1999 Mar 18;398 (6724):242–246. PubMed PMID: 10094047; eng.
50. Wu JS, Luo L. A protocol for mosaic analysis with a repressible cell marker (MARCM) in Drosophila. Nat Protoc. 2006;1(6):2583–2589. .PubMed PMID: 17406512; eng
51. Van De Bor V, Delanoue R, Cossard R, et al. Truncated products of the *vestigial* proliferation gene induce apoptosis. Cell Death Differ. 1999 Jun;6(6):557–564. PubMed PMID: 10381644; eng.
52. Yakoby N, Lembong J, Schupbach T, et al. Drosophila eggshell is patterned by sequential action of feedforward and feedback loops. Development. 2008 Jan;135 (2):343–351. PubMed PMID: 18077592; eng.
53. Persson U, Izumi H, Souchelnytskyi S, et al. The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. FEBS Lett. 1998 Aug 28;434(1–2):83–87. S0014–5793(98)00954–5 [pii]. PubMed PMID: 9738456; eng
54.. Tanimoto H, Itoh S, Ten Dijke P, et al. Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs [research support, Non-U.S. Gov't]. Mol Cell. 2000 Jan;5 (1):59–71. PubMed PMID: 10678169; eng
55. Sivasankaran R, Vigano MA, Muller B, et al. Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker [research support, Non-U.S. Gov't]. EMBO J. 2000 Nov 15;19(22):6162–6172. PubMed PMID: 11080162; PubMed Central PMCID: PMC305821. eng.
56. Campbell G, Tomlinson A. Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by *brinker*. Cell. 1999 Feb 19;96(4):553–562. S0092–8674(00)80659–5 [pii]. PubMed PMID: 10052457; eng
57. Jazwinska A, Rushlow C, Roth S. The role of *brinker* in mediating the graded response to Dpp in early Drosophila embryos. Development. 1999 Aug;126 (15):3323–3334. PubMed PMID: 10393112; eng
58. Shen J, Dahmann C, Pflugfelder GO. Spatial discontinuity of optomotor-blind expression in the Drosophila wing imaginal disc disrupts epithelial architecture and promotes cell sorting. BMC Developmental Biology. 2010 Feb 23;10:23. . PubMed PMID: 20178599; PubMed Central PMCID: PMC2838827. eng.
59. Shen J, Dorner C, Bahlo A, et al. optomotor-blind suppresses instability at the A/P compartment boundary of the Drosophila wing [research support, Non-U.S. Gov't]. Mech Dev. 2008 Mar-Apr;125(3–4):233–246. PubMed PMID: 18171611; eng.
60. Umemori M, Takemura M, Maeda K, et al. Drosophila T-box transcription factor Optomotor-blind prevents pathological folding and local overgrowth in wing epithelium through confining Hh signal [research support, Non-U.S. Gov't]. Dev Biol. 2007 Aug 1;308(1):68–81. PubMed PMID: 17573067; eng.
61. Pflugfelder GO, Eichinger F, Shen J. T-Box genes in Drosophila limb development [review]. Curr Top Dev Biol. 2017;122:313–354. .PubMed PMID: 28057269; eng
62. Del Alamo Rodriguez D, Terriente Felix J, Diaz-Benjumea FJ. The role of the T-box gene optomotor-blind in patterning the Drosophila wing. Dev Biol. 2004 Apr 15;268(2):481–492. . PubMed PMID: 15063183; eng.
63. Eivers E, Demagny H, De Robertis EM. Integration of BMP and Wnt signaling via vertebrate Smad1/5/8 and Drosophila Mad. Cytokine Growth Factor Rev. 2009 Oct-Dec;20(5–6):357–365. .PubMed PMID: 19896409; eng
64. Eivers E, Fuentealba LC, Sander V, et al. *Mad* is required for *wingless* signaling in wing development and segment patterning in Drosophila. PLoS One. 2009;4(8):e6543. .PubMed PMID: 19657393; eng
65. Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-?2 family mediator Smad1. Nature. 1997 Oct 9;389 (6651):618–622. . PubMed PMID: 9335504; eng.
66.. Martin FA, Perez-Garijo A, Moreno E, et al. The brinker gradient controls wing growth in Drosophila [research support, Non-U.S. Gov't]. Development. 2004 Oct;131(20):4921–4930. PubMed PMID: 15371310; eng.
67. Li Y, Wang F, Lee JA, et al. MicroRNA-9a ensures the precise specification of sensory organ precursors in

Drosophila. Genes Dev. 2006 Oct 15;20(20):2793–2805. PubMed PMID: 17015424; eng.

68. Chang S, Johnston RJ Jr., Frkjr-Jensen C, et al. MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. Nature. 2004 Aug 12;430(7001):785–789. 10.1038/nature02752 nature02752. PubMed PMID: 15306811; eng.
69. Johnston RJ, Hobert O. A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature. 2003 Dec 18;426(6968):845–849. 10.1038/nature02255 nature02255. PubMed PMID: 14685240; eng
70. Grimm S, Pflugfelder GO. Control of the gene optomotor-blind in Drosophila wing development by decapentaplegic and wingless [research support, Non-U.S. Gov't]. Science. 1996 Mar 15;271(5255):1601–1604. PubMed PMID: 8599120; eng
71. John B, Enright AJ, Aravin A, et al. Human MicroRNA targets. PLoS Biol. 2004 Nov;2(11):e363. PubMed PMID: 15502875; eng.
72. Carthew RW. Gene regulation by microRNAs. Curr Opin Genet Dev. 2006 Apr;16(2):203–208. .PubMed PMID: 16503132; eng
73. Li X, Carthew RW. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell. 2005 Dec 29;123(7):1267–1277. . PubMed PMID: 16377567; eng.
74. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis [research support, Non-U.S. Gov't]. Cell. 2005 Dec 2;123(5):819–831. PubMed PMID: 16325577; eng.
75. Yoo AS, Greenwald I. LIN-12/Notch activation leads to microRNA-mediated down-regulation of Vav in C. elegans. Science. 2005 Nov 25;310(5752):1330–1333. . PubMed PMID: 16239437; eng.
76. Martin DN, Baehrecke EH. Caspases function in autophagic programmed cell death in Drosophila. Development. 2004 Jan;131(2):275–284. .PubMed PMID: 14668412; eng
77. De Lucas MP, Saez AG, Lozano E. miR-58 family and TGF-?2 pathways regulate each other in Caenorhabditis elegans. Nucleic Acids Res. 2015 Nov 16;43(20):9978–9993. . PubMed PMID: 26400166; PubMed Central PMCID: PMCPMC4783514.
78. Herranz H, Perez L, Martin FA, et al. A Wingless and Notch double-repression mechanism regulates G1-S transition in the Drosophila wing. EMBO J. 2008 Jun 4;27(11):1633–1645. PubMed PMID: 18451803; eng.