Cell

The Hippo Pathway Regulates the *bantam* microRNA to Control Cell Proliferation and Apoptosis in *Drosophila*

Barry J. Thompson[1] and Stephen M. Cohen[1,*]
[1] European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg 69117, Germany
*Contact: cohen@embl.de
DOI 10.1016/j.cell.2006.07.013

SUMMARY

The Hippo signaling pathway acts upon the Yorkie transcriptional activator to control tissue growth in *Drosophila*. Activated Yorkie drives growth by stimulating cell proliferation and inhibiting apoptosis, but how it achieves this is not understood. Yorkie is known to activate Cyclin E (CycE) and the apoptosis inhibitor DIAP1. However, overexpression of these targets is not sufficient to cause tissue overgrowth. Here we show that Yorkie also activates expression of the *bantam* microRNA, a known regulator of both proliferation and apoptosis. *bantam* overexpression mimics Yorkie activation while loss of *bantam* function slows the rate of cell proliferation. *bantam* is necessary for Yorkie-induced overproliferation and *bantam* overexpression is sufficient to rescue survival and proliferation of *yorkie* mutant cells. Finally, we show that *bantam* levels are regulated during both developmentally programmed proliferation arrest and apoptosis. In summary, the results show that the Hippo pathway regulates expression of *bantam* to control tissue growth in *Drosophila.*

INTRODUCTION

How tissues grow to their correct sizes and shapes remains an unsolved problem in biology. In most animal species, tissue growth proceeds largely by an increase in cell number, which is determined by the rate of cell proliferation and the rate of cell death (Conlon and Raff, 1999). These processes are precisely controlled during normal development and their misregulation can produce tumors (Hanahan and Weinberg, 2000). Indeed, tumor formation appears to require both stimulation of cell proliferation and inhibition of apoptosis (Green and Evan, 2002).

Recently, a new tumor suppressor pathway that coordinately controls cell proliferation and apoptosis has been discovered in *Drosophila* (reviewed in Edgar, 2006). It consists of six negatively acting components (Merlin, Expanded, Hippo, Salvador, Warts, and Mats) that restrict tissue growth (Hamaratoglu et al., 2006; Harvey et al., 2003; Justice et al., 1995; Kango-Singh et al., 2002; Lai et al., 2005; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003) and one positively acting component (Yki; Huang et al., 2005) that promotes tissue growth. Merlin and Expanded are related FERM-domain proteins that localize to the cell cortex and are the most upstream components of the pathway identified to date. Hippo (Hpo), an MST-family kinase, acts with Salvador to control phosphorylation of Warts. Warts, an NDR-family kinase, then acts with Mats to control phosphorylation of Yki. Thus, the entire Hippo pathway acts to control the activity of Yki, which encodes a transcriptional coactivator.

All of the components of the Hippo pathway are conserved in humans and several have been implicated in human cancer, in particular Merlin, which in humans is encoded by the *Neurofibromatosis type-2* tumor suppressor gene. In *Drosophila*, mutation of the negatively acting pathway components or overexpression of Yki produces overgrown tissues due to increased cell proliferation and inhibition of apoptosis. However, it is not understood how this pathway causes these effects. Only two potential effector genes are known to be transcriptionally activated by Yki: Cyclin E (CycE), a cell cycle regulator, and DIAP1, an inhibitor of apoptosis (Huang et al., 2005). In addition to these effectors, Yki also activates transcription of two negatively acting Hippo components, Merlin and Expanded, as part of a negative feedback loop within the pathway (Hamaratoglu et al., 2006).

This current knowledge of genes regulated by the Hippo pathway is clearly incomplete because overexpression of any of the known Yki targets fails to mimic the effect of Yki itself in driving tissue growth. Here we show that Yki activates an additional target gene, *bantam*, encoding a microRNA that is known to control tissue growth in *Drosophila* (Brennecke et al., 2003; Hipfner et al., 2002). Furthermore, we show that *bantam* is essential for Yki-induced overproliferation and is sufficient to rescue survival and proliferation of *yki* mutant cells. Finally, we show that certain cells programmed to arrest their proliferation or undergo apoptosis during normal development downregulate levels of *bantam*.

RESULTS

Overexpression of *bantam* Produces *hippo*-Like Phenotypes

The Hippo pathway was discovered by genetic screens in the *Drosophila* eye, where mutations in *hippo* or genes encoding other negatively acting components cause eye overgrowth. The overgrown eyes consist of an abnormally large number of cells, which characteristically accumulate between the photoreceptor-containing ommatidial facets that compose the eye (for example, Udan et al., 2003). The same phenotype is observed when Yki is overexpressed (Huang et al., 2005). Previous work has shown that overexpression of the *bantam (ban)* microRNA also causes overgrowth in *Drosophila* tissues, including the eye. We therefore examined the cellular phenotype of eyes overexpressing *bantam* during the final stages of eye development when photoreceptor cells are differentiating and excess cells in between are normally killed off by apoptosis. We found that overexpression of *bantam*, like overexpression of Yki, causes accumulation of a large number of extra interommatidial cells (Figure 1). *bantam* causes this phenotype partly because it blocks apoptosis: apoptotic cells were readily detectable with an antibody recognizing the activated form of Caspase-3 in wild-type retinas (Figure 1A, Casp) but only infrequently in retinas expressing *bantam* (Figure 1B, Casp). However, it is known that blockade of apoptosis alone, for example by expression of the caspase inhibitor p35 (Kango-Singh et al., 2002) is not sufficient to produce the large number of extra interommatidial cells found in eyes expressing *bantam.* Thus, *bantam*, like Yki, must act both to promote cell proliferation and to limit apoptosis in a coordinated manner.

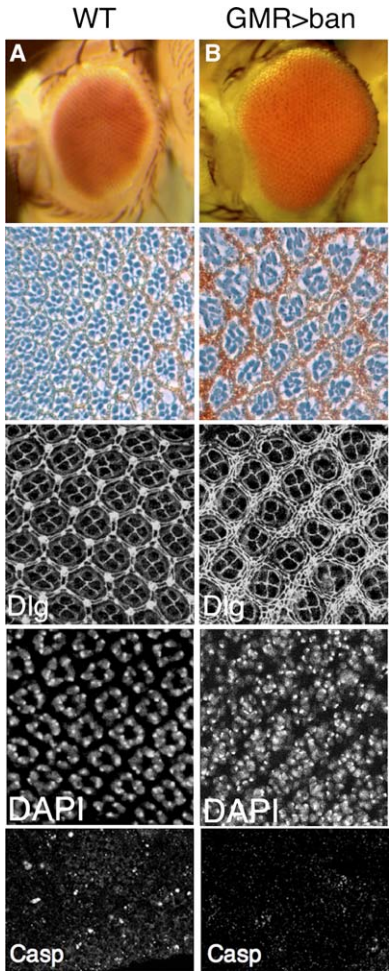

Figure 1. Overexpression of *bantam* Generates *hippo*-Like Phenotypes

(A) Wild-type *Drosophila* eye (top row), a histological section thereof (second row) and confocal sections through a wild-type pupal retina stained for Discs-Large (Dlg, third row), DAPI (marking nuclei, fourth row) and activated Caspase-3 (marking apoptotic cells, Casp, fifth row).

(B) Overexpression of *bantam* with the GMR.Gal4 driver causes eye overgrowth (top row), due mainly to excess interommatidial cells. These are the cells containing red pigment in a histological section (second row) and visible as extra small cells in a pupal retina stained for Dlg (third row) or extra nuclei staining for DAPI (fourth row). Apoptotic cells, marked by positive staining for activated Caspase-3, are not detected when *bantam* is overexpressed (Casp, fifth row). Excess interommatidial cells indicate that overexpression of *bantam* mimics loss of *hippo* in driving cell proliferation and inhibiting apoptosis.

bantam Mutant Cells Proliferate Slowly

Null mutants in *bantam* are lethal in *Drosophila* because the imaginal discs (which develop to form the adult structures of the fly, such as eyes and wings) of mutant animals fail to grow (Hipfner et al., 2002). To examine the role of *bantam* more closely, we generated clones of *bantam* mutant cells during imaginal disc growth using the FLP/FRT system. In this system, a mutant clone, marked by the absence of GFP, is generated along with a wild-type sister "twin-spot" clone, marked by two copies of GFP. The relative growth rates of the two clones can be directly compared. For example, in a control experiment in the wing imaginal disc, a wild-type clone and its wild-type sister twin-spot grow to similar sizes after three days (Figure 2A). In contrast, we found that growth of *bantam* mutant clones was dramatically reduced compared with sister twin-spot clones (Figure 2B). In addition, fewer *bantam* clones were recovered than twin-spot clones (Figure 2B), indicating that some of these clones are eliminated.

The reduced growth of *bantam* mutant clones could be due to either reduced rates of cell proliferation or increased apoptosis, or both. To distinguish between these possibilities, we induced large clones of *bantam* mutant cells by giving the mutant clone a competitive advantage over neighboring cells using the *Minute* technique (Morata and Ripoll, 1975). This method slows the growth of cells neighboring the clone so that the clone grows very large. For example, in a control experiment in the adult eye, large wild-type clones (marked by the absence of red pigment caused by the *white* mutation) grow to occupy most of the eye (Figure 2C). Under the same conditions, *bantam* mutant clones grow slowly to occupy a smaller proportion

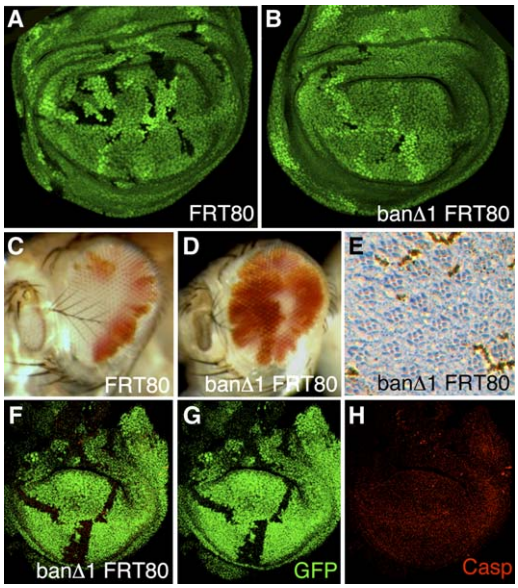

Figure 2. *bantam* Mutant Cells Proliferate Slowly

(A) The size of wild-type clones (marked by the absence of GFP) and their sister twin-spots (bright GFP) is roughly equal when induced in wing imaginal discs at 60 hr of development and examined at late third instar.

(B) *bantam* homozygous mutant clones (absence of GFP) are very small compared to their sister twin-spots (bright GFP) when induced in wing imaginal discs at 60 hr of development and examined at late third instar.

(C) Very large wild-type clones are produced in the adult eye (white cells) when they are given a competitive advantage over their neighboring *Minute* heterozygous cells (marked with red pigment).

(D) *bantam* homozygous mutant clones (white cells) remain relatively small even with a competitive advantage over their *Minute* heterozygous neighbors. However, these clones are larger than those in (B).

(E) Histological section through an adult eye containing a large *bantam* mutant clone induced using the *Minute* technique (as in [D]). The clone is marked by the absence of red pigment from interommatidial cells. The orderly pattern of photoreceptors remains normal despite the loss of *bantam*.

(F–H) Apoptosis, detected with an anti-activated Caspase-3 antibody (H), is not induced in *bantam* mutant clones (induced with the *Minute* technique and marked by the absence of GFP, [G]) in third instar wing discs.

of the tissue (Figure 2D). However, the resulting *bantam* mutant clone is large enough to examine whether excess apoptosis is occurring. Excess apoptosis in the eye causes a "rough" eye phenotype featuring a disorganized pattern of ommatidia. Interestingly, the *bantam* mutant clones showed no evidence of increased apoptosis, and gave rise to a normal pattern of ommatidial facets (Figure 2E). Furthermore, *bantam* mutant clones in the wing imaginal disc similarly show no evidence of increased apoptosis, as is normally detectable with an antibody against activated Caspase-3 (Figures 2F–2H). The results show that *bantam* mutant clones grow poorly due to a reduced rate of cell proliferation. Thus, the *bantam* loss-of-function phenotype resembles the *yki* loss-of-function phenotype with respect to cell proliferation, but differs with respect to apoptosis, which is increased in *yki* mutant clones.

The Hippo Pathway Regulates Expression of *bantam*

The activity of the *bantam* microRNA can be detected using a "sensor" transgene that encodes GFP under the control of a ubiquitous promoter and contains two perfectly matching target sites for *bantam* in its 3′ UTR (Brennecke et al., 2003). The level of GFP expression from the sensor is very sensitive to *bantam* microRNA levels and shows a dynamic pattern during imaginal disc development (Figure 3A). When cells begin to arrest proliferation, such as along the dorsal-ventral boundary of the wing imaginal disc at the end of larval development, *bantam* is downregulated and a stronger stripe of sensor expression is detected. The observed modulations have been shown to depend on the presence of the *bantam* microRNA as, in the absence of *bantam*, sensor levels are uniformly high (Brennecke et al., 2003).

We have found that manipulation of Hippo pathway activity has strong effects on *bantam* sensor levels. Overexpression of Yki in a stripe adjacent to the anterior-posterior boundary of the wing imaginal disc causes a clear downregulation of sensor levels (vertical stripe in Figure 3B), suggesting that *bantam* levels have increased. Conversely, inhibition of Yki activity by overexpression of Hippo with the same driver causes a clear upregulation of sensor levels (Figures 3C and 3D), suggesting reduced *bantam* levels. In this experiment, we coexpressed DIAP1 with Hippo in order to prevent apoptosis of the cells. Interestingly, these cells showed abnormalities in their epithelial architecture, with cells moving basally to form a fold in the epithelium (Figures 3C, 3D, and 3F; compare Figure 3F with wild-type, Figure 3E).

To confirm that the ability of Yki to repress the sensor is due to upregulation of *bantam* microRNA, we analyzed *bantam* levels by using Northern blots and quantitative-PCR. Total RNA was extracted from both wild-type larvae and larvae expressing Yki in the wing imaginal disc with nubbin-Gal4. Yki expression increased *bantam* RNA levels (Figure 3G). To estimate of the degree of induction, we performed quantitative-PCR on dissected wing discs and found that Yki overexpression induced *bantam* levels by 6-fold (Figure 3H). Together, the sensor, RNA blot, and quantitative-PCR results show that the Hippo pathway regulates *bantam* expression. In a control experiment, we found that another growth regulatory pathway, the Insulin/PI3K pathway, did not regulate *bantam* in the same manner, suggesting that its regulation is not a consequence of proliferation control in general, but rather a specific output of the Hippo pathway (see Figure S1 in the Supplemental Data).

bantam Is Required for Yki-Induced Overproliferation

Overexpression of Yki in clones of cells has been shown to cause a dramatic increase in clonal growth rates (Huang et al., 2005). We have used the MARCM system to test whether this Yki-induced overproliferation is affected by loss of *bantam*. We induced clones of wild-type cells (Figure 4A), Yki-overexpressing cells (Figure 4B), *bantam*

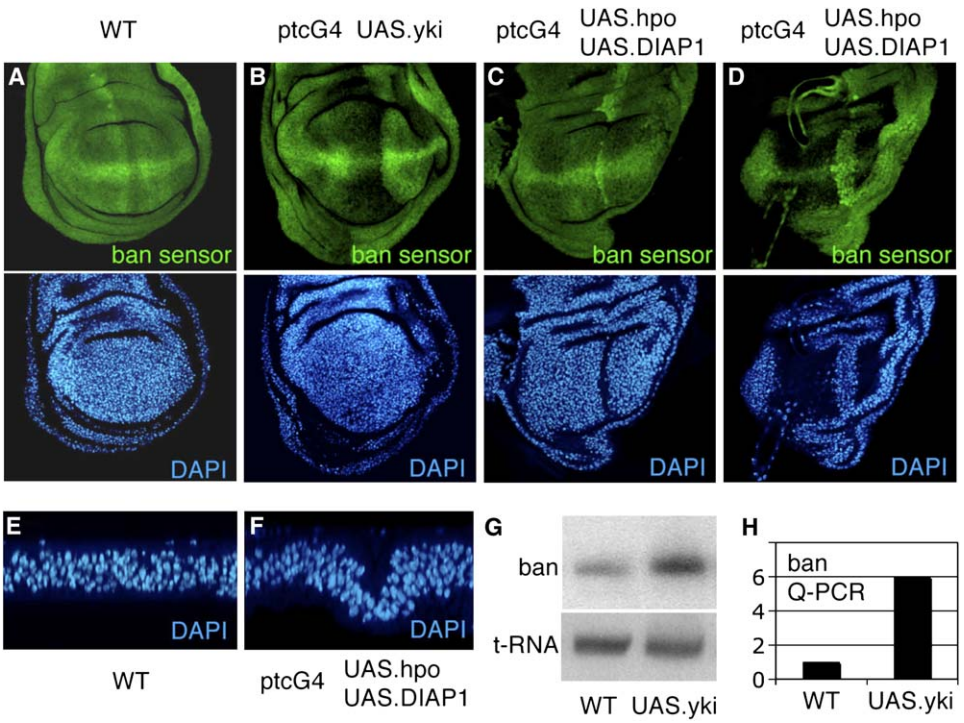

Figure 3. The Hippo Pathway Regulates Expression of *bantam*

(A–F) DAPI marks nuclei and shows the morphology of the epithelium. (A) The pattern of expression of *bantam*, visualized with the *bantam* sensor in wild-type third instar wing disc. (B) Overexpression of Yki in a vertical stripe adjacent to the anterior-posterior compartment boundary with the ptc.Gal4 driver induces expression of *bantam*, which consequently downregulates the *bantam* sensor. (C and D) Inhibition of Yki by overexpression of Hippo (and DIAP1) reduces *bantam* expression and allows upregulation of the *bantam* sensor. (C) Apical view and (D) basal view, showing folding of the epithelium where ptc.Gal4 is expressed. (E) Z-section through a wild-type wing pouch epithelium, showing the normal pseudo-stratified organization. (F) Z-section through a ptc.Gal4 UAS.hpo UAS.DIAP1 wing pouch (as in C and D) showing basal outfolding of the epithelium.

(G) Yki overexpression increases *bantam* levels. (Upper panel) Northern blot probed with radiolabeled anti-*ban* oligonucleotide. Left lane, 30 μg of total RNA from wild-type larvae. Right lane, 30 μg of total RNA from nubbin.Gal4 UAS.yki animals. Samples were anterior halves of larvae containing brain and imaginal discs, as well as body wall, but from which other internal tissues were dissected away. The wing discs in which nubbin.Gal4 is expressed comprise a small proportion of the total tissue. (Lower panel) The same blot probed with an anti-tRNA radiolabeled oligonucleotide as a loading control.

(H) Yki overexpression increases *bantam* levels. Quantitative-PCR analysis of *bantam* microRNA levels, normalized to two reference microRNAs (*miR-14* and *miR-125*). RNA was isolated from wing discs, and dissected from wild-type larvae (WT) and from nubbin.Gal4 UAS.yki (UAS.yki) larvae. *bantam* levels were induced 6-fold in the UAS.yki sample compared with the wild-type control.

mutant cells (Figure 4C), and Yki-overexpressing cells that are also mutant for *bantam* (Figure 4D). In this experiment all clones were induced simultaneously and allowed to grow for the same amount of time. We found that Yki-overexpressing clones grew much larger than wild-type clones and that loss of *bantam* strongly reduced the growth of both wild-type and Yki-overexpressing clones (Figure 4E). This result indicates that Yki requires *bantam* to fully stimulate cell proliferation. However, we note that Yki still retains some activity even in the absence of *bantam*, as *bantam* mutant clones grow slightly larger when Yki is overexpressed.

The Growth of *yki* Mutant Clones Is Fully Rescued by *bantam* Overexpression

Our results indicate that cells with low *yki* activity will have low levels of *bantam* activity. *yki* mutant clones have been shown to fail to grow and to undergo apoptosis (Huang et al., 2005). This phenotype cannot be rescued by mutations in *hippo* or other tumor suppressor components of the pathway, indicating that these genes act upstream of *yki*. We therefore tested whether restoring *bantam* expression could rescue *yki* mutant clones. We induced four types of clones simultaneously: wild-type clones; *yki* mutant clones; *yki* mutant clones expressing DIAP1 to inhibit apoptosis; and *yki* mutant clones expressing *bantam*. In comparison to wild-type clones (Figure 5A), *yki* mutant clones were almost completely eliminated from the tissue (Figure 5B). The survival of these clones could be rescued by expression of DIAP1 (Figure 5C), producing clones smaller than wild-type. Strikingly, we found that both survival and proliferation of *yki* mutant clones could be completely rescued by expression of *bantam*, producing clones that were even larger than wild-type (Figures 5D

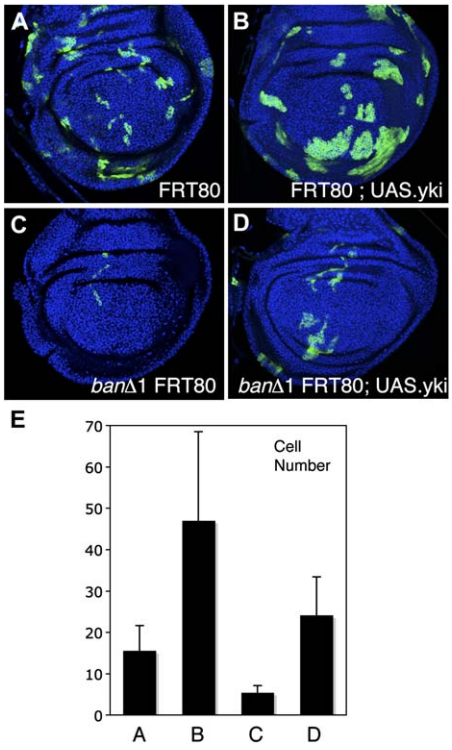

Figure 4. *bantam* Is Required for Yki-Induced Overproliferation

Four types of clones were induced simultaneously with the MARCM system and allowed to grow for an identical period of time (clones marked by expression of GFP). (A) Wild-type control clones (induced with a wild-type FRT80 chromosome). (B) Wild-type clones overexpressing Yki grow much larger than controls. (C) *bantam* mutant clones grow smaller than wild-type clones. (D) *bantam* mutant clones overexpressing Yki grow smaller than wild-type clones overexpressing Yki.

(E) Quantification of clone size (measured as average number of cells within clones of a certain genotype) for each of the genotypes in (A)–(D). At least 20 clones were measured in each case. Error bars indicate one standard deviation from the mean.

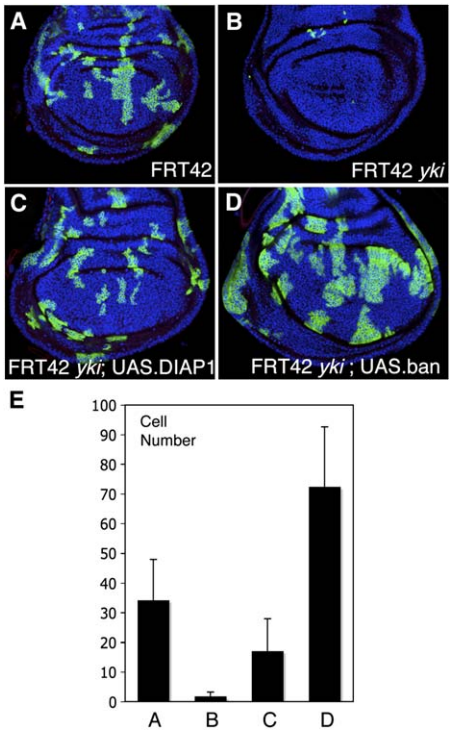

Figure 5. Survival and Proliferation of *yki* Mutant Clones Is Fully Rescued by Overexpression of *bantam*

Four types of clones were induced simultaneously with the MARCM system and allowed to grow for an identical period of time (clones marked by expression of GFP). (A) Wild-type control clones (induced with a wild-type FRT42 chromosome). (B) *yki* mutant clones are almost completely eliminated from the tissue. (C) Expression of DIAP1 rescues survival of *yki* mutant clones. (D) Expression of *bantam* rescues survival and proliferation of *yki* mutant clones, which are able to grow even larger than wild-type clones.

(E) Quantification of clone size (measured as average number of cells within clones of a certain genotype) for each of the genotypes in (A)–(D). At least 20 clones were measured in each case. Error bars indicate one standard deviation from the mean.

and 5E). These results show that *bantam* acts downstream of *yki* and that *bantam* overexpression is sufficient to rescue *yki* loss of function.

bantam Does Not Regulate the Hippo Pathway

Since our results show that *bantam* acts downstream of *yki*, a clear prediction is that *bantam* should not affect the activity of the Hippo pathway upstream of Yki. The activity of the Hippo pathway can be measured independently of the *bantam* sensor using expression of Expanded (which is activated in a negative feedback loop) as a readout. We tested whether *bantam* regulates Expanded levels by generating clones of cells that overexpress *bantam*. We found that *bantam*-expressing clones had no effect on Expanded levels (Figure 6A). In a control experiment, Yki-expressing clones were found to have increased Expanded levels (Figure 6B) and this phenotype was unaffected when such clones were also mutant for *bantam* (Figure 6C). These results show that *bantam* does not regulate the activity of the Hippo pathway upstream of Yki.

bantam Is Downregulated in Apoptotic Cells at the Eye Margin

A previous analysis of *bantam* expression found that *bantam* is downregulated as cells arrest proliferation in the wing imaginal disc (Brennecke et al., 2003). Given the additional role of the Hippo pathway in controlling apoptosis, we examined *bantam* expression in cells destined for apoptosis during normal development. We have used the programmed cell death of the cells at the edge of the pupal retina (rim cells) as a model. These cells are trimmed by apoptosis to generate a smooth eye margin (Brachmann and Cagan, 2003; Lin et al., 2004). Large numbers of dying rim cells are detectable with an antibody to the activated form of Caspase-3 (Figure 7A).

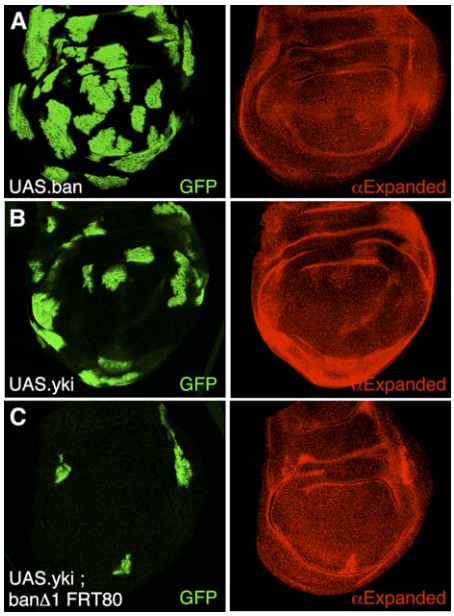

Figure 6. *bantam* Does Not Regulate the Hippo Pathway
The activity of the Hippo pathway was measured by the level of Expanded expression. Clones were induced with the MARCM system and are marked by expression of GFP. (A) Clones expressing *bantam* do not affect the level of Expanded. (B) Clones expressing Yki upregulate Expanded levels. (C) *bantam* mutant clones expressing Yki also upregulate Expanded levels.

We examined the pattern of expression of *bantam* under these circumstances using the *bantam* sensor. We find that sensor levels are always high in the arrested, differentiating photoreceptor cells of each ommatidium (Figure 7A), indicating low *bantam* levels. In contrast, sensor levels are very low in most interommatidal cells (positive for DLG, Figure 7A), indicating that these cells express *bantam*. At the retinal margin, where many cells are undergoing apoptosis, sensor levels are again high—suggesting that *bantam* is downregulated in these dying cells. This downregulation of *bantam* appears to be essential for apoptosis because restoration of *bantam* to high levels prevents apoptosis of these cells (Figure 7B). The results show that *bantam* expression is downregulated during developmentally programmed apoptosis in the eye and that low levels of *bantam* are essential for apoptosis to proceed.

The Hippo Pathway Controls Epithelial Morphogenesis Independently of *bantam*

During the foregoing analysis we noticed that manipulation of the Hippo pathway caused changes in the epithelial architecture of imaginal discs (Figure 3F). We examined these effects more closely, in confocal Z-sections of the wing disc, and found that compared with wild-type cells (Figure S2A), Yki-expressing cells caused the epithelium to bulge apically, due to a change in cell morphology in which nuclei moved to a more apical position (Figure S2B).

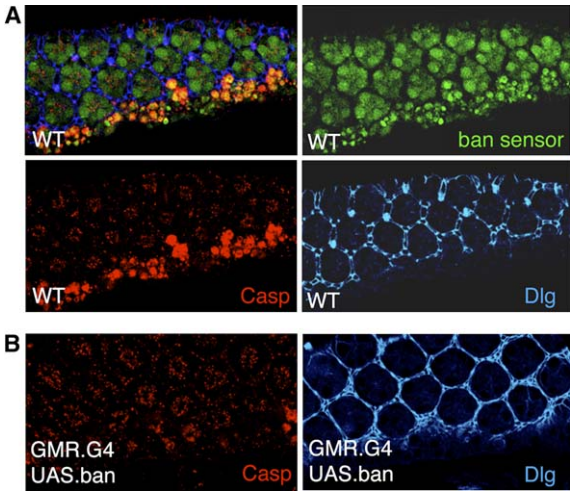

Figure 7. Expression of *bantam* Is Downregulated in Rim Cells Destined for Apoptosis
(A) In the pupal retina, *bantam* is expressed in interommatidial cells (staining positive for Dlg), indicated by low levels of *bantam* sensor expression. Sensor expression is high in arrested photoreceptor cells and cells undergoing apoptosis at the margin of the retina (the rim cells stain positively for cleaved Caspase-3).
(B) When downregulation of *bantam* is prevented by placing *bantam* expression under control of GMR.Gal4, apoptosis of both interommatidial and margin cells is prevented.

In contrast, Hippo-expressing cells (rescued for apoptosis by expression of DIAP1) behaved oppositely, with nuclei moving basally and cells changing shape to cause a basally-directed folding of the epithelium (Figure S2C).

These effects appear to be independent of *bantam* because neither loss of function nor gain of function of *bantam* affects epithelial morphogenesis. For example, the effects of overexpressing *bantam* and Yki in the posterior compartment of the wing disc are compared in Figures S2D and S2E, respectively. Both *bantam* and Yki drive overgrowth, but only Yki additionally causes a striking folding of the epithelium (Figure S3). Thus, the Hippo pathway controls epithelial morphogenesis independently of *bantam*.

DISCUSSION

Efforts in the field of developmental biology have revealed much about how patterns of different cell types arise in developing tissues (Lawrence and Struhl, 1996; Neumann and Cohen, 1997). What remains unclear is how developing tissues grow to their correct sizes and shapes (Conlon and Raff, 1999). The discovery of the Hippo pathway represents a major milestone in understanding the control of tissue growth. The Hippo pathway is unlike most developmental signaling pathways (such as Wnt, Hh, Notch, EGFR, and TGFβ), which provide positional information to cells and therefore affect all aspects of tissue development, including both tissue growth and patterning. The Hippo pathway is also distinct from the Insulin/PI3K pathway,

which mediates the hormonal modulation of growth across the whole organism in response to nutrition. Thus, the Hippo pathway is unique in its direct and dedicated role in the intrinsic program of growth in proliferating tissues.

The potency of the Hippo pathway in driving tissue growth appears to reside in its ability to coordinately stimulate cell proliferation and suppress apoptosis. A key goal is to understand how this coordinate control is achieved. Our results show that the *bantam* microRNA, a known regulator of both cell proliferation and apoptosis (Brennecke et al., 2003), is a critical target of the Hippo pathway. Activated Yki is necessary and sufficient to induce *bantam* expression and to stimulate cell survival and proliferation. *bantam* appears to be a key target of Yki because loss of Yki can be rescued by overexpression of *bantam*. Finally, *bantam* clearly has an important role in both normal growth and Yki-driven overgrowth because loss of *bantam* strongly reduces the rate of cell proliferation in either case. Although the *bantam* microRNA appears not to be conserved in vertebrates, it is possible that other microRNAs play a functionally equivalent role as effectors of the Hippo pathway. Recent work has identified human microRNAs involved in this pathway (Voorhoeve et al., 2006).

Two lines of evidence indicate that *bantam* is not the only relevant target of the Hippo pathway. Firstly, loss of *bantam* does not completely mimic loss of Yki in every respect, because *bantam* mutant cells do not undergo apoptosis. This difference is likely to reflect the contribution of the Yki target DIAP1, whose absence is known to trigger apoptosis. Secondly, Yki retains some ability to stimulate cell proliferation even in the absence of *bantam*. Again, this activity may reflect the role of other Yki targets, including CycE, in driving cell proliferation. Thus, the results favor the view that *bantam* acts in a highly cooperative way with other Yki target genes to mediate the effects of the Hippo pathway on cell proliferation and apoptosis.

The expression of *bantam* during normal development shows a striking pattern of regulation; it is expressed in proliferating cells but not in quiescent cells or, as we have shown in this work, in certain cells destined for apoptosis. These findings indicate that regulation of *bantam* is a key feature of the normal program of tissue growth. Previous work has shown that high levels of the Wingless (Wnt) morphogen represses *bantam* as cells arrest proliferation at the presumptive wing margin (Brennecke et al., 2003). Since our results show that the Hippo pathway regulates *bantam*, the pattern of *bantam* expression may reflect regulation of Hippo pathway activity by positional signals. Thus, positional signals could determine the behavior of cells along the spectrum from rapid proliferation to apoptosis simply by controlling the Hippo pathway. Alternatively, positional signals and the Hippo pathway may act independently, with the *bantam* locus being a regulatory nexus that integrates information from a number of different signaling pathways.

Our final finding is that the Hippo pathway also influences epithelial morphogenesis. This function appears to be independent of its role in controlling cell survival and proliferation and does not involve *bantam*. Interestingly, expression of Yki and Hippo have reciprocal effects on the epithelium, with overexpressed Yki driving apical bulging and overexpressed Hippo causing basal outfolding. In both cases, the cells remain epithelial, indicating that the Hippo pathway controls cell shape without affecting epithelial polarity or integrity. These observations are consistent with previous reports that clones of cells with elevated pathway activity (i.e. mutant for Hippo or other negatively acting components) have a rounded appearance, indicating altered cell affinities, and that mutation of *warts* also causes apical bulging of epithelia that is attributed to an expanded apical membrane domain. Why cells use the same pathway to control survival, proliferation, and shape remains an intriguing question.

A fuller understanding of the network connecting the Hippo pathway with the basic machinery controlling survival, proliferation, and morphology will be needed to understand how size regulation is connected to pattern formation during normal development. This work allows us to sketch the outline of one facet of this network, with the Yki targets *bantam*, CycE, and DIAP1 cooperating to control survival and proliferation.

EXPERIMENTAL PROCEDURES

Transgenic Overexpression with the Gal4/UAS System

This system is reviewed in Duffy (2002). The GMR.Gal4, ptc.Gal4 and en.Gal4 drivers are available from the Bloomington *Drosophila* Stock Centre. Other stocks are described in the following references: UAS.ban, UAS.D; Brennecke et al. (2003); UAS.DIAP1 in Wang et al. (1999); UAS.hpo in Udan et al. (2003); and UAS.yki in Huang et al. (2005). The bantam sensor transgene is described in Brennecke et al. (2003).

Generation of Clones

Clones were generated using the FLP/FRT system (reviewed in Duffy [2002]). When the heat shock (hs) promoter was used to drive expression of the Flp recombinase, clones were induced by heat shocking larvae at 60 hr (±12 hr) of development and larvae were dissected at the third instar stage. The following genotypes were employed: (1) hs.Flp; ubi.GFP FRT80 / FRT80; (2) hs.Flp; ubi.GFP FRT80 / banΔ1 FRT80; (3) hs.Flp; ubi.GFP Minute FRT80 / banΔ1 FRT80; (4) ey.Flp; w+ Minute FRT80 / banΔ1 FRT80; and (5) ey.Flp; w+ Minute FRT80 / FRT80.

MARCM clones (Lee and Luo, 2001) were generated using the following genotypes: (1) hs.Flp UAS.GFP; tub.Gal80 FRT80 tub.Gal4 / FRT80; (2) hs.Flp UAS.GFP; tub.Gal80 FRT80 tub.Gal4 / UAS.yki; FRT80; (3) hs.Flp UAS.GFP; tub.Gal80 FRT80 tub.Gal4 / UAS.yki; banΔ1 FRT80; (4) hs.Flp UAS.GFP; tub.Gal80 FRT80 tub.Gal4 / banΔ1 FRT80; (5) hs.Flp UAS.CD8GFP; FRT42 tub.Gal80 / FRT42; tub.Gal4; (6) hs.Flp UAS.CD8GFP; FRT42 tub.Gal80 / FRT42 yki; tub.Gal4; (7) hs.Flp UAS.CD8GFP; FRT42 tub.Gal80 / FRT42 yki; tub.Gal4 / UAS.DIAP1; and (8) hs.Flp UAS.CD8GFP; FRT42 tub.Gal80 / FRT42 yki; tub.Gal4 / UAS.ban.

Immunofluorescent Staining

Imaginal discs or pupal retinas were fixed and stained by standard procedures using the following antibodies: rabbit anti-GFP (1:400, Torrey Pines), guinea-pig anti-Expanded (1:1000, gift of R. Fehon); mouse anti-DLG (Developmental Studies Hybridoma Bank), rabbit anti-cleaved Caspase-3 (1:200, Cell Signaling Technologies). Images were taken with a Leica TCS SP2 Confocal Microscope.

Histology

Adult eye sections were prepared as described in Freeman et al. (1992).

microRNA Northern Blotting

Northern blotting was performed as described in Brennecke et al. (2003). A detailed protocol is available upon request.

Quantitative-PCR Analysis of microRNAs

Primer sets designed to amplify mature microRNAs (*ban, miR-14, mir-125*) were obtained from Applied Biosystems. Products were amplified from 10 ng of total RNA with the "TaqMan MicroRNA Assay," Quantitative-PCR machine, and software from Applied Biosystems. The fold induction of *bantam* was calculated relative to either of the two references, *miR-14* and *miR-125.*

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/126/4/767/DC1/.

ACKNOWLEDGMENTS

We thank Natascha Bushati for assistance with microRNA Northern analysis and Vladimir Benes and Jens Stolte for assistance with the TaqMan MicroRNA Assay, which was supplied by TL Ceccardi at Applied Biosystems. We also thank David Hipfner for many useful discussions and Richard Fehon for providing the guinea-pig anti-Expanded antibody.

Received: February 28, 2006
Revised: May 12, 2006
Accepted: July 5, 2006
Published: August 24, 2006

REFERENCES

Brachmann, C.B., and Cagan, R.L. (2003). Patterning the fly eye: the role of apoptosis. Trends Genet. *19*, 91–96.

Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., and Cohen, S.M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell *113*, 25–36.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 235–244.

Duffy, J.B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis *34*, 1–15.

Edgar, B.A. (2006). From cell structure to transcription: Hippo forges a new path. Cell *124*, 267–273.

Freeman, M., Klambt, C., Goodman, C.S., and Rubin, G.M. (1992). The argos gene encodes a diffusible factor that regulates cell fate decisions in the Drosophila eye. Cell *69*, 963–975.

Green, D.R., and Evan, G.I. (2002). A matter of life and death. Cancer Cell *1*, 19–30.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. (2006). The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat. Cell Biol. *8*, 27–36.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457–467.

Hipfner, D.R., Weigmann, K., and Cohen, S.M. (2002). The bantam gene regulates Drosophila growth. Genetics *161*, 1527–1537.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell *122*, 421–434.

Justice, R.W., Zilian, O., Woods, D.F., Noll, M., and Bryant, P.J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. *9*, 534–546.

Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P.R., Bellen, H.J., and Halder, G. (2002). Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. Development *129*, 5719–5730.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. (2005). Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell *120*, 675–685.

Lawrence, P.A., and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? Cell *85*, 951–961.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci. *24*, 251–254.

Lin, H.V., Rogulja, A., and Cadigan, K.M. (2004). Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis. Development *131*, 2409–2418.

Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. Dev. Biol. *42*, 211–221.

Neumann, C., and Cohen, S. (1997). Morphogens and pattern formation. Bioessays *19*, 721–729.

Pantalacci, S., Tapon, N., and Leopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat. Cell Biol. *5*, 921–927.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. (2002). salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell *110*, 467–478.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. *5*, 914–920.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell *124*, 1169–1181.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A., and Hay, B.A. (1999). The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell *98*, 453–463.

Wu, S., Huang, J., Dong, J., and Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell *114*, 445–456.