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bantam miRNA is important for *Drosophila* blood cell homeostasis and a regulator of proliferation in the hematopoietic progenitor niche

Victoria Lam, Tsuyoshi Tokusumi, Yumiko Tokusumi, Robert A. Schulz *

Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA

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ABSTRACT

The *Drosophila* hematopoietic system is utilized in this study to gain novel insights into the process of growth control of the hematopoietic progenitor niche in blood development. The niche microenvironment is an essential component controlling the balance between progenitor populations and differentiated, mature blood cells and has been shown to lead to hematopoietic malignancies in humans when misregulated. MicroRNAs are one class of regulators associated with blood malignancies; however, there remains a relative paucity of information about the role of miRNAs in the niche. Here we demonstrate that *bantam* miRNA is endogenously active in the *Drosophila* hematopoietic progenitor niche, the posterior signaling center (PSC), and functions in the primary hematopoietic organ, the lymph gland, as a positive regulator of growth. Loss of *bantam* leads to a significant reduction in the PSC and overall lymph gland size, as well as a loss of the progenitor population and correlative premature differentiation of mature hemocytes. Interestingly, in addition to being essential for proper lymph gland development, we have determined *bantam* to be a novel upstream component of the insulin signaling cascade in the PSC and have unveiled dMyc as one factor central to *bantam* activity. These important findings identify *bantam* as a new hematopoietic regulator, place it in an evolutionarily conserved signaling pathway, present one way in which it is regulated, and provide a mechanism through which it facilitates cellular proliferation in the hematopoietic niche.

1. Introduction

The cellular organization and molecular signaling that occurs in *Drosophila* hematopoiesis strikingly parallels that which is seen in the hematopoietic stem cell niches of vertebrate animals, including several mammals [1–3]. An elegant example of such similarity is the recent discovery of the *Drosophila* hematopoietic progenitor niche which like its vertebrate counterpart, uses homologous signaling pathways for hematopoietic progenitor maintenance [4–8]. The primary hematopoietic organ, the lymph gland, is comprised of three morphologically and molecularly distinct compartments: (1) the posterior signaling center (PSC), which is the stem cell-like niche, (2) the medullary zone comprised of self-renewing hematopoietic progenitors, that eventually undergo progressive differentiation into the increasingly lineage restricted mature blood cells of (3) the cortical zone which directly surrounds the medullary zone. Cells of the PSC are specified by the homeotic gene *Antennapedia* (*Antp*) and maintained by the *Drosophila* ortholog of vertebrate Early B-cell Factor: Collier (Col) [6,9]. Regulatory signals, such as Hedgehog (Hh), emanate from the PSC in a non-cell-autonomous manner to preserve the progenitor population of the medullary zone [5,6,10]. In lymph glands lacking a PSC or that do not express hh, there is depletion of the progenitor population and concomitant, premature differentiation of mature hemocytes.

This emphasizes the essential role of this cellular domain in controlling the vital balance between proliferation of progenitors and lineage commitment to mature blood cells. It is thus of fundamental importance to elucidate the signals that govern maintenance of the PSC [4–6,11–14].

Several reports have detailed key contributions by miRNAs in regulating the development of specific hematopoietic lineages [15–20]. Furthermore there is evidence of their causative role in mediating malignant diseases in the hematopoietic system [21–24]. Owing to their important developmental roles and ability to target known oncogenes and tumor suppressors, miRNAs are a

Abbreviations: PSC, posterior signaling center; Antp, Antennapedia; Col, Collier; Hh, hedgehog; HSC, hematopoietic stem cell; InR, insulin-like receptor; col-Gal4, P85col-Gal4; Foxo, forkhead-related transcription factor; Bam, bag of marbles; Rbf, retinoblastoma-family protein.

* Corresponding author at: Department of Biological Sciences, University of Notre Dame, 147 Galvin Life Science Building, Notre Dame, IN 46556, USA.
E-mail address: rschulz@nd.edu (R.A. Schulz).

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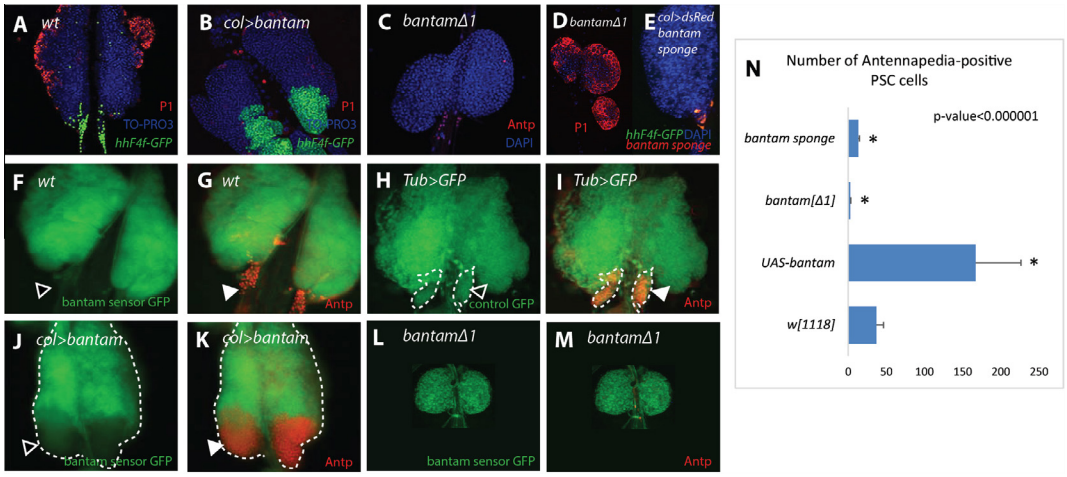

Fig. 1. Characterization of *bantam* miRNA expression and function in the PSC of the lymph gland. Anti-Antp staining and *hhF4f-GFP* transgene expression mark the PSC. Mature plasmatocytes are labeled with P1 staining and cell nuclei are stained with DAPI. (A) Wild type (wt) control lymph gland w^{1118} showing normal PSC size. (B) PSC specific overexpression of *bantam*, through *col-GAL4*. (C and D) lymph glands from $bantam^{\Delta 1}$ homozygotes. (E) *col*-driven expression of a *bantam sponge-dsRed* transgene. (F) Region of endogenous *bantam* activity indicated by loss of GFP expression in *bantam* sensor GFP background (empty arrowhead). (G) Anti-Antp staining indicates that the observed region of endogenous *bantam* activity occurs in the cells of the PSC (arrowhead). (H) Lymph gland expressing control sensor transgene express GFP due to insensitivity to *bantam* activity (empty arrowhead) (I) Region verified as PSC with anti-Antp staining (arrowhead) (J and K) *col*-driven *bantam* in *bantam sensor* GFP background. (J) Region of *bantam* activity (empty arrowhead) verified as (K) expanded PSC by anti-Antp staining (arrowhead). (L and M) Loss of *bantam*, in $bantam^{\Delta 1}$ homozygous null mutants. (N) Statistical analysis of PSC cells in loss of function and gain of function of *bantam*.

key part of the intricate molecular networks that drive or suppress cancer development and progression [16]. However, despite their clear relationship with hematopoietic development and cancer, there remains a paucity of knowledge regarding the expression patterns, the identity of their target genes, and in vivo function of miRNAs in hematopoietic stem cell (HSC) populations and their niches [25–27]. Since both uncontrolled HSC expansion as well as loss of HSC is deleterious for humans, an understanding of the molecular mechanisms of HSC fate decision is thus of considerable importance.

In this study, we investigate the role of *bantam* miRNA, in hematopoiesis and elucidate its relationship with the Insulin-like receptor (InR) pathway and dMyc in positively regulating growth [28–30]. The InR pathway is a critical, conserved pathway responsible for regulating cell size and number in *Drosophila* [31–33]. Previous studies have shown that InR pathway signaling impacts PSC size and maintenance of the blood progenitor population [34–38]. Similarly dMyc, the *Drosophila* transcriptional activator orthologous to the mammalian proto-oncoprotein c-Myc, has also been shown to be an important positive regulator of growth in the PSC [35,39–41]. Our studies present a novel role for this crucial miRNA specifically in regulating the hematopoietic niche through interacting with these signaling factors established to be essential in controlling growth and proliferation.

2. Materials and methods

2.1. Fly stocks

We used the following lines in this study: *P85col-Gal4* (*col-Gal4*) (M. Crozatier) [5,9]; *hhF4f-GFP* [14]; *bantam sensor GFP*, *control sensor*, *UAS-bantam-A*, $bantam^{\Delta 1}$ and *UAS-dsRed bantam sponge* (S. Cohen) [28,42]; UAS-myr::tdTomato, UAS-InR, UAS-dMyc, UAS-dMyc RNAi (TRiP JF1761), UAS-foxo, and w^{1118} obtained from the Bloomington Stock Center and UAS-Akt1 RNAi (GD1361) obtained from the Vienna Drosophila RNAi Center. Flies were reared on a conventional cornmeal-based medium. Egg-lays were done at 25 °C and the lymph glands of 5-days old (unless otherwise indicated) 3rd instar larvae were dissected. As a consequence of the delayed developmental timeframe of $bantam^{\Delta 1}$ homozygous null mutants, due in part to reduced growth rates, all larvae analyzed were 7-days old and kept at 29 °C for purposes of keeping comparative analysis consistent [29].

2.2. Immunostaining

Immunostaining was performed as described previously [14]. The following primary antibodies were used to determine protein expression in lymph glands dissected from control or mutant animals: mouse anti-plasmatocyte (P1) antibody (1:100) [43,44] and mouse anti-Antp antibody (1:100; 4C3, Developmental Studies Hybridoma Bank) [6]. As secondary antibodies, we used Alexa 488 or 555-conjugated mouse IgG antibodies (Invitrogen). Cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Samples were analyzed and imaged with a Zeiss Axioplan 2 fluorescence microscope or a Nikon A1R-NP laser-scanning confocal microscope.

2.3. Counting PSC cell number

A minimum of twenty, individual lymph glands were analyzed for each genotype. Statistical analyses of Antp-positive cell counts were performed as previously described [45] and analyzed with a *t*-test.

3. Results and discussion

3.1. bantam miRNA is an essential regulator of growth in the PSC and blood cell homeostasis in the lymph gland

Extensive studies have been conducted to determine the functions and interactions of signaling pathways in regulating the larval hematopoietic organ. However, it is not currently known whether miRNAs are involved in PSC development. We were specifically interested in investigating the impact of *bantam* miRNA, a known positive regulator of growth in *Drosophila*, on the hematopoietic system [28,30,46–48].

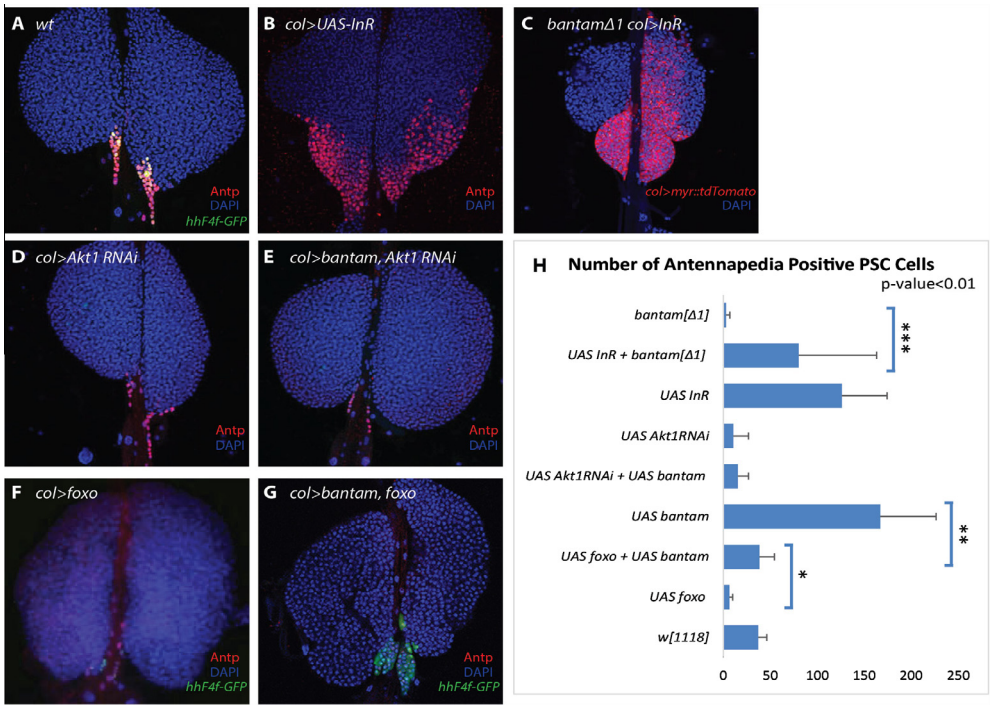

Fig. 2. Relationship between *bantam* and InR pathway components. The PSC is marked by *hhF4F-GFP, UAS-myr::tdTomato* or anti-Antp staining. (A) wt lymph gland. The following assays were conducted using the PSC-specific *col*-GAL4 driver: (B) *UAS-InR*, (C) *bantam mutant* and *UAS-InR*, (D) *UAS-Akt1 RNAi*, (E) *UAS-Akt1 RNAi* and *UAS-bantam*, (F) *UAS-foxo*, (G) *UAS-foxo* and *UAS-bantam*. (H) Quantification of the number of Antp-positive PSC cells in 5-days old larvae. (∗, ∗∗, ∗∗∗) indicate significant difference in respective pairwise comparisons.

Directed overexpression of *bantam*, through utilizing the PSC-specific *collier-Gal4* driver (*col-Gal4*), accelerated cell proliferation and was sufficient to cause significant overgrowth of the niche. This quadrupling of niche cells lead to a corresponding increase in the size of the hemocyte progenitor population of the medullary zone and an observed reduction in mature, P1-positive plasmatocytes in the cortical zone (Fig. 1B).

In contrast, in homozygous null mutants ($bantam^{\Delta 1}$) (Fig. 1C) and reduction of endogenous *bantam* through targeted expression of the *bantam sponge*-transgene (Fig. 1E), there is a significantly diminished to absent PSC [42,49,50]. Accordingly due to this decrease in number of PSC cells, there was also a substantial decrease of the instructive *hh* signal, as measured by the expression of *hhF4f-GFP* transgene, necessary for maintenance of the medullary zone (Fig. 1E). PSCs of *bantam* loss of function mutants had an average of just 2.75 PSC cells compared with 37 in control lymph glands (Fig. 1N). Reduction of the PSC culminated in a concomitant depletion of hematopoietic progenitors in the medullary zone and the swelling of the lymph gland with differentiated blood cells (Fig. 1D). This suggests that basal activity of *bantam* is specifically necessary for proper development of the PSC.

To verify both the location and expression levels of endogenous *bantam* miRNA in the lymph gland, we utilized a *bantam sensor GFP* transgene, comprised of a ubiquitously-active *tubulin* promoter driving the expression of a GFP gene with two perfect *bantam* target sequences located in the 3′UTR [28]. Cells without *bantam* activity remain fluorescent; however, in cells in which *bantam* miRNA is present, *GFP* mRNA is bound by *bantam* miRNA and degraded. Efficacy of this tool is demonstrated with a control transgene (*control GFP*) lacking any *bantam* target sequences in the 3′UTR, and thus is insensitive to *bantam* miRNA activity and shows no reduction in GFP (Fig. 1H and I). We used this as a means to demonstrate that *bantam* is indeed endogenously expressed in the lymph gland (empty arrowhead Fig. 1F) and confirmed, through labeling with anti-Antp antibody, that *bantam* miRNA expression is specific to the hematopoietic progenitor niche (arrowhead Fig. 1G). As a comparison, *col-Gal4* driven *bantam* overexpression leads to substantial GFP reduction in the expanded cell population (Fig. 1J), which is indicative of increased *bantam* activity in the PSC (Fig. 1K). It was postulated that with the loss of *bantam*, GFP levels would not be reduced. However, due to the substantial reduction in PSC cell number in *bantam* null lymph glands, it was difficult to ascertain whether GFP levels changed in the remaining niche cells (Fig. 1L and M).

Together, these pieces of evidence indicate that *bantam* is an essential component in regulating hematopoietic homeostasis and a critical factor for proper PSC maintenance at endogenous levels. Furthermore, this implies that unlike many miRNAs that operate in slightly modulating or fine-tuning expression of target genes, and whose loss or overexpression only subtly impact cellular phenotypes, *bantam* function in the primary hematopoietic organ is both necessary for proper lymph gland size, development of the PSC, and general homeostasis during blood development and is sufficient for driving substantial proliferation in the niche.

3.2. bantam is dependent on Akt activity to positively regulate proliferation in the PSC

The InR pathway is a critical pathway responsible for regulating cell size and number in *Drosophila* and is evolutionarily conserved as a global regulator of growth in mammals [31–33,51,52]. Previous studies have shown that InR pathway signaling impacts PSC size and maintenance of the blood progenitor population [34–38]. The dominant-negative and RNAi analysis of positive regulators of growth within the InR pathway resulted in greatly diminished niches, a phenotype strikingly similar to that of PSCs in a *bantam* null background [35]. This led us to investigate a potential interaction between *bantam* and the InR pathway.

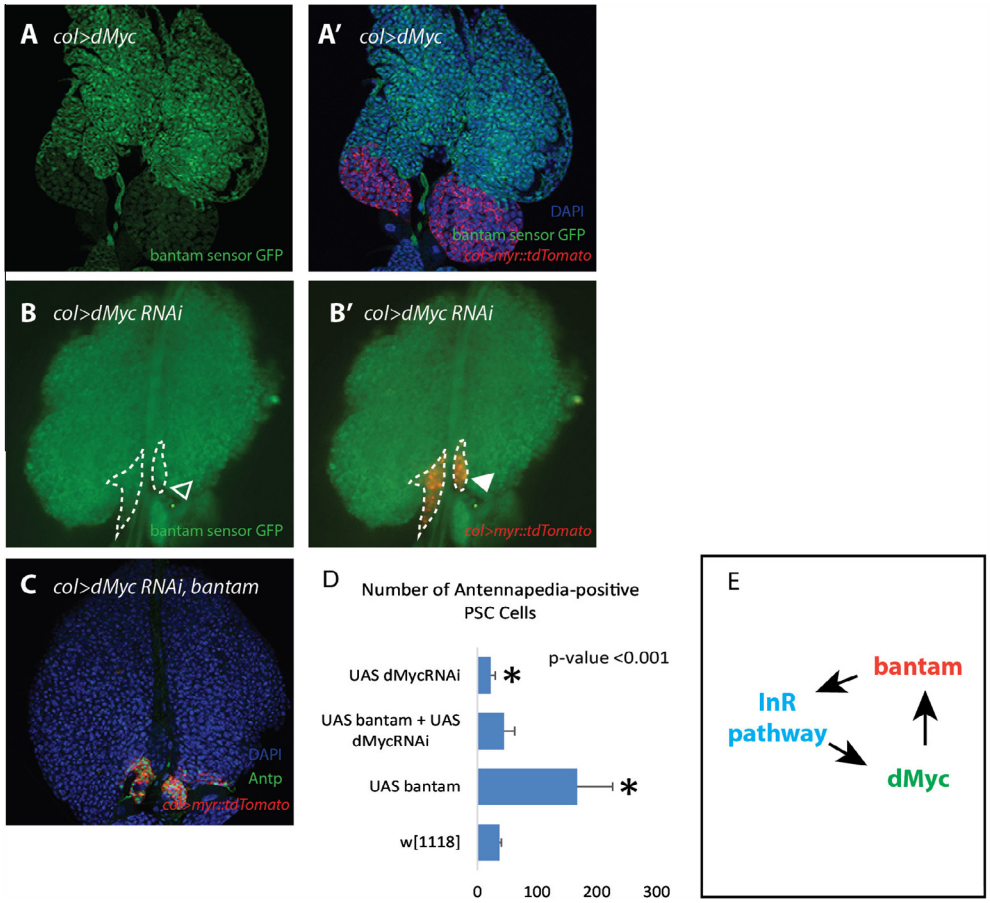

Fig. 3. *Bantam* is dependent on dMyc for positively regulating proliferation in the PSC. PSC cells are marked by anti-Antp staining or *UAS-myr::tdTomato* in the niche cells. (A and A′) *bantam sensor in col-Gal4>dMyc* background. (B and B′) *bantam sensor in col-GAL4>UAS-dMyc RNAi.* (B) Empty arrowhead marks lack of change in GFP in the PSC (B′) as indicated by *col>myr::tdTomato* (arrowhead). (C) *col-GAL4>UAS-bantam>UAS-dMycRNAi.* (D) Quantification of Antp-positive PSC cells in 5-days old larvae (E) Proposed model for placement of *bantam* in the InR pathway in regulation of the hematopoietic niche.

When InR is overexpressed in the PSC, it causes massive expansion (Fig. 2B) a phenotype that persists in the absence of *bantam* (Fig. 2C). This suggests that *bantam* may be an upstream regulator of the InR pathway specifically in the hematopoietic niche, thus providing a mechanism through which *bantam* promotes growth in the blood system.

For supporting evidence of this notion, we investigated two additional downstream components of the insulin signaling cascade: forkhead-related transcription factor (Foxo) and the antagonistic modulator of Foxo, the serine/threonine protein kinase Akt1 [53,54]. Foxo is the singular *Drosophila* ortholog of mammalian foxo, which functions in growth inhibition in response to reduced insulin signaling, through positively regulating cell death factors and growth-suppressors and inhibitors of cell cycle [53,54]. Expression of *foxo* in the niche resulted in significant reduction of both *hhF4f-GFP* and Antp-positive PSC cells (Fig. 2F). When both *bantam* and *foxo* are co-expressed in the PSC (Fig. 2G), *foxo* expression offsets the effects of proliferation-permissive *bantam* miRNA [55]. The overgrowth seen with *bantam* alone is lost with the niche exhibiting no significant difference in average Antp-positive cell number compared with wildtype (Fig. 2A and H).

Consistent with previous findings, loss of growth factor Akt1 in the PSC, through *Akt1 RNAi* expression, leads to a significant reduction in niche cell number (Fig. 2D) [35]. Additionally, despite concurrent expression of *bantam*, the loss of Akt1 also leads to a loss of PSC expansion (Fig. 2E), thus suggesting that *bantam*-induced overgrowth is reliant on Akt1 activity. Taken together we observed that *bantam*-induced overgrowth is abrogated by both the loss of positive regulator of growth, Akt1, and overexpression of negative regulator of growth, Foxo, both of which are key components of the InR pathway. Statistically identical outcomes of reduced Antp-positive PSCs, for both overexpression of *Akt1 RNAi* as well as co-expression of *Akt1 RNAi* and *bantam*, suggest that *bantam* is upstream of Akt1 and dependent on activation of the InR pathway to facilitate proliferation. Furthermore, consistent with *bantam* being upstream of this established growth-regulatory pathway, overexpression of InR is sufficient to rescue the effects of the loss of *bantam* (Fig. 2C).

3.3. *bantam* is dependent on dMyc to facilitate cell proliferation in the niche

dMyc has been extensively studied as a positive regulator of cell growth and the activator of genes responsible for DNA replication and progression through from G1 to S-phase [56–58]. It has been shown that dMyc is also an important growth regulator in the PSC and we have confirmed that the InR pathway positively regulates dMyc [39]. Building upon this model, we have recently determined that dMyc activity is promoted through InR pathway interaction with the Bag of marbles (bam)/Retinoblastoma-family protein (Rbf) pathway, which regulates genes important for mitotic G1-S phase progression. (Tokusumi et al., submitted).

Consistent with this, overexpression of *dMyc* in the PSC causes an expansion similar to that of *bantam* overexpression (Fig. 3A, A′, B). Additionally all of these PSC cells display *bantam* activity (Fig. 3A), which led us to investigate whether *bantam* and *dMyc*

interact in the niche to promote proliferation. Our *bantam* sensor results indicated that endogenous *bantam* activity is significantly reduced with *dMyc RNAi* (Fig. 3B and B′). Further evidence, consistent with *bantam* reliance on dMyc to facilitate proliferation, is that the size of the niche (normally expanded to several hundred cells with *bantam* overexpression) becomes statistically identical to wild type (Fig. 3D), due to the loss of dMyc regardless of persistent *bantam* expression. These cells also expressed *hhF4f-GFP* suggesting functional production of the hh signal (data not shown). *bantam* miRNA operates in a permissive capacity in alleviating the repression of cell proliferation by targeting inhibitors of growth, however, for growth to take place, positive regulators of growth must also be present. These results can thus be interpreted as limited PSC cell number ultimately due to missing dMyc despite active inhibition of negative regulators by *bantam*.

We draw from this evidence that *bantam*-induced PSC expansion is in part dependent on dMyc activity, as loss of dMyc restricts the extent of *bantam*-induced niche expansion (Fig. 3C) and negatively impacts endogenous *bantam* activity (Fig. 3B and B′). Taken together, these results suggest that dMyc is a crucial positive regulator of *bantam* in the lymph gland, a stipulation that is additionally supported by overexpression of *dMyc* causing a net increase in *bantam* activity (Fig. 3A and A′). Overall the result is a signal-enhancing, positive feedback loop, in which *bantam* indirectly promotes growth through the InR cascade, which leads to dMyc activation and in turn, upregulation of *bantam* activity (Fig. 3E).

In summary, our studies demonstrate the direct role of *bantam* miRNA in the normal maintenance of pluripotent hematopoietic progenitors and how deregulation of this vital miRNA causes either overproliferation of blood cells or loss of vital hematopoietic populations. These findings not only identify a novel role for *bantam* in the lymph gland and unveil a new level complexity to the well-characterized function of *bantam* in growth control, but also broaden our understanding of how miRNAs contribute to blood cell development and malignant blood cell disorders.

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