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Bantam is essential for *Drosophila* intestinal stem cell proliferation in response to Hippo signaling

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ABSTRACT

The *Drosophila* midgut has emerged as an attractive model system to study stem cell biology. Extensive studies have been carried out to investigate the mechanisms of how the signaling pathways integrate to regulate intestinal stem cells (ISCs), yet, whether the microRNAs are involved in ISC self-renewal and maintenance is unknown. Here we demonstrate that the bantam microRNA is expressed specifically at high levels in *Drosophila* midgut precursor cells (including ISCs and enteroblasts) and secretory enteroendocrine cells while at extremely low levels in enterocytes. Furthermore, overexpression of bantam microRNA results in increase of the division of the midgut precursor cells, whereas loss of bantam microRNA decreases their proliferation. The mechanical studies show that bantam microRNA is essential for the Hpo pathway induced cell-autonomous ISC self-renewal, while it is disposable for EGFR and Notch pathways mediated ISC proliferation. More interestingly, we find that bantam microRNA is not required for the Hpo pathway mediated non-cell-autonomous ISC proliferation, revealing a novel mechanism by which the Hpo signaling pathway specifies its transcriptional targets in specific tissue to exhibit its biological functions.

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Introduction

Accumulating evidence has suggested that the *Drosophila* adult midgut is an attractive model for studying stem cell biology (Casali and Batlle, 2009). The *Drosophila* adult midgut contains intestinal stem cells (ISCs) that are located basally to the basement membrane of the midgut epithelium (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The division of ISCs gives rise to renewed ISCs or enteroblasts (EBs) that undergo further differentiation to become either larger enterocytes (ECs) or small secretory enteroendocrine cells (EEs), both of which are terminally differentiated cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs can be distinguished by their small nuclear sizes with the specific expression of the Notch ligand Delta (Dl) (Supplementary material Fig. 1). ISCs and EBs, the so-called midgut precursor cells, express the Snail/Slug family transcription factor escargot (esg) (Micchelli and Perrimon, 2006). Over 90% of EBs differentiate into ECs, which can be distinguished by their large endoreplicating nuclei and Pdm1 expression (Singh et al., 2012). The EEs are characterized by their small size and Prospero(Pros) expression (Supplementary material Fig. S1A) (Singh et al., 2012).

Several signaling pathways including Notch (Ohlstein and Spradling, 2007), Wingless (Wg)/Wnt (Lin et al., 2008), and JAK-STAT play curial roles in ISC maintenance, proliferation and differentiation in *Drosophila* midgut (Jiang et al., 2009; Liu et al., 2010). Hyperactivation of Notch signaling in ISCs leads to a loss of ISCs and the formation of ECs, whereas inactivation of Notch signaling results in tumor-like ISC accumulation. The JAK-STAT pathway is important for damage-induced ISC proliferation and subsequent midgut regeneration while the Wnt Pathway constitutes the ISC niche in regulating ISC self-renewal and differentiation. A significant role of the Hippo (Hpo) pathway in *Drosophila* midgut proliferation has been illustrated recently (Poernbacher et al., 2012; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010). Several lines of evidence pointed out that the Hpo pathway mediates ISC proliferation in the normal physiological environment as well as in the damage-induced regeneration. Despite the great efforts have been paid to investigate the functions of several signaling pathways in ISC self-renewal or differentiation, whether microRNA participates in the regulation of ISC proliferation and maintenance is unknown.

Here, we report that *bantam* microRNA exhibits cell lineage specific expression pattern in *Drosophila* midgut. We find that both overexpression and inactivation of bantam regulate ISC renewal without disrupting ISC differentiation in *Drosophila* midgut. Furthermore, bantam is required in precursor cells for injury-induced ISC proliferation. Despite that *bantam* microRNA is indispensible for

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Hippo signaling (Thompson and Cohen, 2006), EGFR signaling (Herranz et al., 2012a, 2012b) and Notch signaling (Becam et al., 2011) induced proliferation or differentiation in imaginal disc, it is only required for loss of Hpo signaling-induced cell-autonomous ISC proliferation. Combing our subsequent finding that bantam microRNA is not required for the Hpo pathway transcriptional co-activator Yorkie (Huang et al., 2005) induced non-cell-autonomous ISC proliferation, we propose an updated model that Hpo signaling regulates the ISC proliferation in *Drosophila* through specifying its transcriptional targets in specific tissue.

Results

Bantam exhibits cell linage specific expression pattern in Drosophila midgut

Although the functions of several signaling pathways have been identified in adult midgut, whether microRNAs play roles in ISC biology remains elusive. Occasionally, we found that *bantam-LacZ*, which reflects the expression level of bantam microRNA, was highly expressed in esgGFP$^+$ cells (ISCs/EBs) and Pros$^+$ cells (EEs) (Fig. 1A–A‴). *Esg-Gal4* enables transgenes to be expressed specifically in ISCs and EBs; therefore, esgGFP$^+$ marks the midgut precursor cells (Micchelli and Perrimon, 2006). To confirm this discovery, we combined *esg-LacZ* with *bantam Sensor GFP* (*BSGFP*) to check the expression pattern of bantam. Flies carrying *BSGFP* express GFP containing two copies of the *bantam* target sequence in the 3′-UTR under the control of the tubulin promoter (Brennecke et al., 2003; Thompson and Cohen, 2006). As predicted, BSGFP was expressed at a high level in ECs while is nearly undetectable in precursor cells and EEs (Fig. 1B-B‴). Furthermore, we found that the expression of BSGFP was exclusive of the expression of Dl (Supplementary material Fig. S1B–B‴), which serves as a highly specific ISC marker (Micchelli and Perrimon, 2006). To further strengthen our findings, we then generated flip-out clones of N-RNAi, which produced both ISC-like and EE-like tumors (Lin et al., 2008; Ohlstein and Spradling, 2007). As shown in Supplementary material Fig. S1C–C‴, BSGFP was expressed with a higher level in Dl$^-$ and Pros$^-$ ECs. Collectively, our study demonstrates that bantam microRNA is expressed at a high level in precursor cells and EEs, suggesting a possible role of bantam in ISC proliferation and/or differentiation.

Bantam promotes ISC renewal without disrupting ISC differentiation in Drosophila midgut

To explore the function of *bantam* microRNA in midgut homeostasis, we expressed *UAS-bantam* under the control of *esg-Gal4*. Overexpression of bantam increased the number of esgGFP$^+$ cells dramatically (compare Fig. 2A with B), suggesting a role of bantam in expanding the precursor cell population. The BrdU cell proliferation assay was then carried out to determinate whether bantam affects ISC proliferation. Indeed, overexpressing bantam in precursor cells resulted in increase of ISCs that are undergoing DNA replication (compare Fig. 2A′ with B′, and C).

Previous studies indicated that JAK/STAT pathway is critical for ISC homeostasis (Jiang et al., 2009). To strengthen our finding, we examined the mRNA levels of ISCs marker genes, such as Dl and Esg, and the mRNA levels of JAK/STAT pathway ligand and target genes using real-time PCR when expressed bantam using *esg-Gal4*. As shown in Fig. 2D, the activation of bantam was sufficient to promote expression of Dl, Esg, JAK-STAT pathway ligand Upd3 and JAK-STAT pathway target SOC36E, suggesting that bantam regulates the proliferation of ISCs.

Since *esg-Gal4* is supposed to drive transgene expression as early as larvae stage (Ren et al., 2010), whether the gain-of-function of bantam is sufficient to affect ISC proliferation at the adult stage requires further investigation. We took the advantage of a *tublin-Gal80^{ts}* to temporarily control *esg-Gal4*-mediated gene expression (*esg^{ts}*). To induce transgene expression, adult flies were shifted to 29 °C for 7 days. As shown in Supplementary material Fig. S1D–D″ and E–E″, the midguts overexpressing bantam generated more precursor cells compared with control midguts. Moreover, the pH3 staining results indicated that overexpression of bantam in *Drosophila* adult midgut was sufficient to promote the proliferation of ISCs (Supplementary material Fig. S1F).

To further explore the function of bantam in the growth of intestinal epithelial tissue and ISC maintenance and differentiation, we used mosaic analysis with a repressible cell marker (MARCM) to express bantam in GFP positive clones, which enable us to trace the lineage of ISCs. As revealed in Fig. 2F–F″, MARCM clones

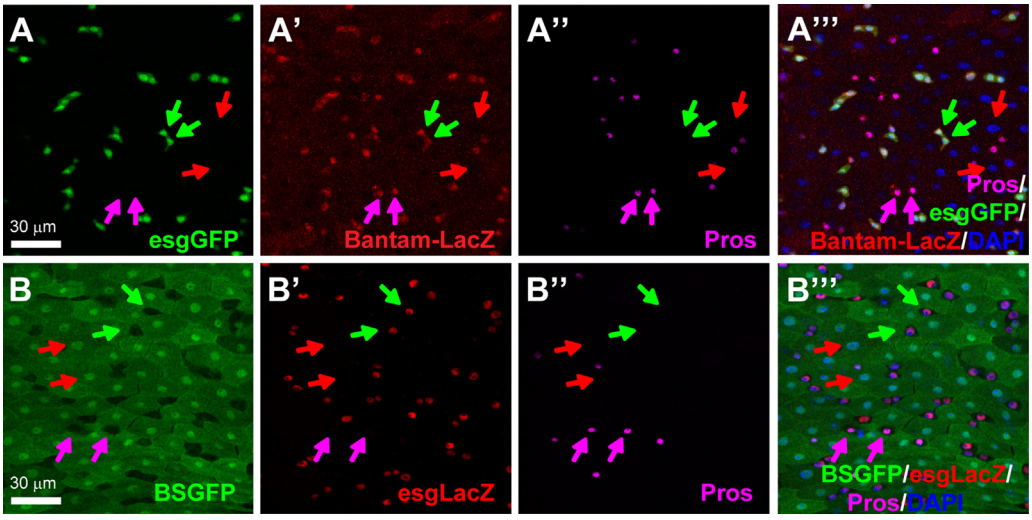

Fig. 1. *bantam* microRNA exhibits cell linage specific expression pattern in *Drosophila* midgut. (A–B‴) Bantam is expressed at a high level in *Drosophila* midgut precursor cells and EEs. *Drosophila* adult female midguts with BSGFP and esgLacZ expression (B–B‴) or esgGFP and *bantam*-Lacz expression (A–A‴) were dissected out and immunostained with indicated antibodies. *BSGFP* is the *bantam* sensor line, whose expression level is reversely correlated with that of bantam. esgGFP$^+$ and Pros$^+$ cells stand for precursor cells and EEs respectively. The representative precursor cells, EEs and large-nuclei ECs were pointed out by green, pink, and red arrows. Note that BSGFP exhibited higher expression only in ECs (B–B‴), while *bantam*-LacZ is expressed at a much higher level in both precursor cells and EEs (A–A‴).

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