Bag of Marbles controls the size and organization of the Drosophila hematopoietic niche through interactions with the Insulin-like growth factor pathway and Retinoblastoma-family protein

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ABSTRACT

Bag of Marbles (Bam) is known to function as a positive regulator of hematopoietic progenitor maintenance in the lymph gland blood cell-forming organ during Drosophila hematopoiesis. Here, we demonstrate a key function for Bam in cells of the lymph gland posterior signaling center (PSC), a cellular domain proven to function as a hematopoietic niche. Bam is expressed in PSC cells, and gene loss-of-function results in PSC overgrowth and disorganization, indicating that Bam plays a crucial role in controlling the proper development of the niche. It was previously shown that Insulin receptor (InR) pathway signaling is essential for proper PSC cell proliferation. We analyzed PSC cell number in lymph glands double-mutant for bam and InR pathway genes, and observed that bam genetically interacts with PSC cell number in the formation of a normal PSC. The eIF4A protein is a translation factor downstream of InR pathway signaling, and functional knockdown of this crucial regulator rescued the bam PSC overgrowth phenotype, further supporting the cooperative function of Bam with InR pathway members. Additionally, we documented that the Retinoblastoma-family protein (Rbf), a proven regulator of cell proliferation, was present in cells of the PSC, with a bam function-dependent expression. By contrast, perturbation of Decapentaplegic or Wingless signaling failed to affect Rbf niche cell expression. Together, these findings indicate that InR pathway-Bam-Rbf functional interactions represent a newly identified means to regulate the correct size and organization of the PSC hematopoietic niche.

KEY WORDS: Bam, Drosophila, Hematopoietic niche, InR pathway, Rbf

INTRODUCTION

Drosophila melanogaster has emerged as a valuable model for the study of hematopoiesis. Blood cell production occurs in separate waves during two developmental stages (Evans et al., 2003; Fossett, 2013). The first wave occurs during embryogenesis, with hematopoietic progenitors initially derived from the cephalic mesoderm. The second wave takes place in a defined hematopoietic organ, termed the lymph gland, which is formed late in embryogenesis and grows substantially in size and progenitor cell number during the larval periods of development. This tissue is eventually composed of multiple pairs of lobes, with the primary lobes of the third-instar larval lymph gland containing three regionalized cell populations of distinct hematopoietic function. These populations include cells of the medullary zone, cortical zone and the posterior signaling center (PSC) (Jung et al., 2005; Krzemienie, 2005; Mandal et al., 2001). The medullary zone harbors progenitors called prohemocytes, whereas the outer cortical zone is occupied by differentiated hemocytes, such as plasmatocytes and crystal cells.

The PSC is a specialized cellular domain, as it functions as a hematopoietic progenitor niche. The PSC functions in the maintenance of blood cell progenitors, expressing several signaling molecules, such as Hedgehog, Serrate, Unpaired 3 and PDGF- and VEGF-related factor 1, and also plays a role in the induction of lamellocytes in response to wasp infestation (Krzemienie, 2007; Mandal et al., 2001, Sinenko et al., 2012; Mondal et al., 2011). The niche is composed of ∼2000 cells present in the lymph gland, and, given its important functions, PSC cell proliferation is tightly controlled by several signaling networks, including the Decapentaplegic (Dpp), Wingless (Wg) and Insulin-like growth factor pathways (Sinenko et al., 2009; Benanmoun et al., 2012; Penniet et al., 2012; Tokusumi et al., 2012).

The Bag of Marbles (Bam) protein is expressed in prohemocytes of the lymph gland, where it functions as a key regulator of hematopoietic progenitor maintenance (Tokusumi et al., 2011). Here, we demonstrate that Bam is also expressed in cells of the PSC, where it serves a seminal role in the control of niche cell number. Bam interacts genetically with members of the Insulin-like receptor (InR) pathway and positively regulates the expression of the Retinoblastoma-family protein Rbf, another crucial regulator of PSC cell proliferation. These results provide additional insights into the complexity of the genetic and molecular control of PSC niche size and organization.

RESULTS AND DISCUSSION

Bam expression and function in lymph gland PSC cells

Previous studies demonstrated that Bam is expressed in prohemocytes of the lymph gland medullary zone, where it functions as a key regulatory protein that promotes blood cell precursor maintenance and prevents hemocyte differentiation (Tokusumi et al., 2011). To investigate the possibility of Bam expression in the PSC domain of the lymph gland, we immunostained lymph glands for Bam protein and hhF4f-GFP expression, the latter being a definitive marker of PSC cells (Tokusumi et al., 2010). Co-localization of Bam with the GFP marker demonstrated that Bam is expressed in cells of the hematopoietic progenitor niche (Fig. 1A). Bam expression was completely abolished in PSC cells of bam null lymph glands or decreased in col>bam RNAi PSC cells (supplementary material Fig. S1A,C). To investigate Bam function in these cells, we assessed PSC cell number in lymph glands from bam homozygous and col>bam RNAi mutant larvae, the latter being a genetic condition in...
Fig. 1. Bam PSC expression and loss-of-function phenotypes. (A) Bam protein (red) co-localization with the PSC-specific marker hhF4f-GFP (green). (A'-A'') Higher magnification views of box in A. Dashed area indicates PSC. (B) PSC cell numbers in lymph glands from wild-type and bam mutant larvae. hhF4f-GFP is used as control strain. (C-E) Antp protein (red) and col>gapGFP (green) expression serve as markers for PSC cells in lymph glands from (C) wild-type, (D) bamΔ86 homozygous mutant and (E) col>bam RNAi mutant larvae. (F-H) Monitoring of PSC cell cycle by FlyFUCCI in (F) wild-type and (G) bamΔ86 mutant lymph glands. (H) Quantification of these results. (I) col>gapGFP (green) and dome-MESO (red) serve as marker for PSC cells and for hematopoietic progenitors in wild-type lymph glands, respectively. (J) PSC cells and hematopoietic progenitors in lymph glands from col>bam RNAi mutant larvae. (K) Densitometric means of anti-β-Gal (dome-MESO) staining in col>wt and col>bam RNAi lymph glands. (L,M) hhF4f-GFP (green) serves as marker for PSC cells, and Eater protein (red) serves as marker for differentiated plasmatocytes in lymph glands from (L) wild-type and (M) col>bam RNAi mutant larvae. P-values (Student’s t-test) indicate significant differences. Scale bar: 20 μm in all images except higher magnification views.
which bam function is selectively knocked down in PSC cells. In both cases, we observed the production of supernumerary PSC cells located throughout the lymph gland (Fig. 1B,D,E). To monitor the status of the cell cycle in PSC cells with a bam mutant background, we introduced the FUCCI system into this analysis (Sakaue-Sawano et al., 2008; Zielke et al., 2014). The system revealed that bam loss-of-function enhanced the proliferation status of PSC cells compared with wild-type PSC cells (Fig. 1F-H). These studies demonstrated a crucial role for Bam in the control of niche cell proliferation and PSC organization. To investigate whether the expanded PSC in lymph glands of the genotype col>bam RNAi maintained its ability to positively regulate progenitor cell maintenance while preventing premature hemocyte differentiation, we assayed these lymph glands for the expression of the prohemocyte marker dome-MESO and plasmatocyte marker Eater (Hombria et al., 2005; Kocks et al., 2005; Gao et al., 2009; Chung and Kocks, 2011). In contrast to lymph glands from control animals (Fig. 1I,K), col>bam RNAi lymph glands contained a substantially increased population of hematopoietic progenitors (Fig. 1J,K) and a greatly diminished population of differentiated plasmatocytes (Fig. 1L,M). Both findings support the notion of the expanded PSC niche in bam mutant lymph glands being functional in its communication with neighboring progenitor cells.

Genetic interactions of bam with InR pathway and elf4A genes

Previous studies documented a central role for InR pathway signaling in the control of PSC cell proliferation in response to altered animal nutrition status (Benmimoun et al., 2012; Tokusumi et al., 2012). Noteworthy is the observation that lymph glands mutant for the Pten or dFOXO genes, two negative regulators of InR pathway signaling, presented with the same phenotype of PSC overgrowth and dispersion as bam mutant lymph glands (Tokusumi et al., 2012). These comparable phenotypes led us to hypothesize that bam functions with InR pathway genes in the control of niche size. To address this possibility, we generated animals that were double-heterozygous for bam and Pten (Fig. 2A) or for bam and dFOXO (Fig. 2B). In both genetic backgrounds, we observed an expanded PSC domain with increased cell number (Fig. 2G). In further support of bam and dFOXO functional interactions, forced expression of dFOXO in niche cells suppressed the increased PSC cell phenotype in lymph glands from bam^{86}/bam^{X6};col>dFOXO mutant larvae (Fig. 2E,G). Additionally, RNAi-mediated knockdown of dAkt1 function, a proven positive regulator of InR pathway signaling, likewise suppressed the increased PSC cell phenotype due to bam loss-of-function (Fig. 2D,G). Together, these findings indicate that Bam has an important functional interaction with the InR pathway in the control of hematopoietic niche cell number.

Thor/4EBP is a component of the elf4F translational control complex and is a known target of FOXO transcriptional regulation. Thor mutants show a comparable expanded PSC phenotype, as observed in dFOXO mutant lymph glands, suggesting that the elf4F complex functions downstream of dFOXO and InR pathway signaling (Tokusumi et al., 2012). It has been shown that, in germline cells, Bam negatively interacts with the elf4A protein, a functional component of the elf4F complex, to prevent the translation of the DE-cadherin protein (Shen et al., 2009). As we had previously demonstrated that selective RNAi-mediated knockdown of elf4A function in PSC cells resulted in a decreased population of niche cells (Tokusumi et al., 2012), we tested the effect of elf4A loss-of-function in a bam mutant background. Intriguingly, knockdown of elf4A function suppressed the increased PSC cell phenotype normally seen in bam mutant lymph glands (Fig. 2F,G), suggesting that the Bam and elf4A proteins function antagonistically to each other in the tight control of PSC cell number during lymph gland development.

Rbf expression and function in the hematopoietic progenitor niche

Rbf is a proven controller of cell proliferation and tissue growth (Gordon and Du, 2011). Owing to these vital functions, we assayed lymph glands for the potential expression of this regulatory protein. We observed that Rbf is expressed in cells populating the medullary zone and also in PSC cells (Fig. 3E). Rbf proteins have been characterized as tumor suppressor proteins, as their mutation results in cell overproliferation and tumor formation in various tissue types (Gordon and Du, 2011). Owing to its expression in cells of the PSC, we sought to identify Rbf/PSC phenotypes due to gene mutation or overexpression. To assess whether Rbf loss-of-function culminated in a hyperproliferation of PSC cells, we assayed lymph glands of the genotypte Rbf^{20-3}/Rbf^{56/4} for Antp protein expression and observed a doubling of PSC cell number (Fig. 3B,D). To test for a cell-autonomous function for Rbf in the control of niche cell proliferation, we knocked down Rbf function selectively in the PSC and again quantified the number of Antp^{+} cells. col>Rbf RNAi lymph glands likewise contained a PSC of increased cell number, demonstrating the cell-autonomous requirement of Rbf in niche cells for their correctly controlled proliferation (Fig. 3D,F). This study also demonstrated the absence of Rbf protein from hhF4f-GFP^{+} PSC cells due to the targeted functional knockdown (Fig. 3F). Finally, we forced the expression of Rbf in PSC cells, which resulted in a significant reduction of this cell population in the gain-of-function condition (Fig. 3C,D).

It is known that Rbf negatively regulates the G1/S transition of the mitotic cell cycle (Du and Dyson, 1999). To monitor the status of the PSC cell cycle in the various Rbf genetic backgrounds, we employed the Fly-FUCCI assay system. We observed that, owing to Rbf gain-of-function, PSC cells were arrested at the G1 phase (Fig. 3G,I), whereas Rbf loss-of-function increased the number of S phase cells (Fig. 3G,J). Together, these findings identified Rbf as an essential regulator of niche cell number through regulation of cell G1/S phase transition.

bam function is required for Rbf protein expression in PSC niche cells

As lymph glands mutant for the bam or Rbf genes displayed the same phenotypes of PSC cell overproliferation, we hypothesized that a regulatory relationship between the two genes might exist. To investigate this possibility, we forced the expression of Rbf in PSC cells in bam loss-of-function lymph glands and observed that Rbf expression in niche cells suppressed the increased PSC phenotype normally found due to bam mutation (Fig. 4A,B). Additionally, we observed a lack of Rbf protein in PSC cells in lymph glands from bam^{X6}/bam^{X6} larvae (Fig. 4C). These findings allowed the conclusion that Bam function is required for Rbf expression in PSC cells, and overproliferation of niche cells due to bam loss-of-function is probably due to the absence of downstream Rbf protein function. Similarly, Rbf protein failed to be expressed in dFOXO mutant lymph glands (Fig. 4D), and PSC cell overproliferation induced by InR expression was suppressed due to Rbf expression (supplementary material Fig. S2). Together, these results support the hypothesis of a functional interaction of Bam, Rbf and InR pathway components.

It has been reported that Dpp and Wg signaling controls PSC cell number through regulation of the dMyc cell proliferation factor (Sinенко et al., 2009; Pennettier et al., 2012). To investigate a potential relationship between the Dpp/Wg/dMyc effectors and Rbf...
function, we assayed for Rbf protein expression in genetic backgrounds that perturbed Dpp and Wg signaling or enhanced dMyc function. Preventing Dpp signaling in PSC cells had no effect on Rbf expression, as expressing a dominant-negative version of Tkv resulted in an increased niche size but normal Rbf expression (Fig. 4E). Additionally, forced expression of Wg in the niche domain resulted in supernumerary PSC cells, but did not affect Rbf expression (supplementary material Fig. S3). Comparably, forced expression of dMyc selectively in the PSC resulted in an increased number of niche cells, but again, PSC cells showed normal Rbf expression (Fig. 4F). These findings suggested that the Bam/Rbf regulators might regulate PSC cell number and organization via a mechanism that functioned in parallel to Dpp/Wg signaling and dMyc-negative regulation. However, co-expression of both dMyc and Rbf suppressed the PSC cell overproliferation phenotype normally observed in col>dMyc lymph glands (Fig. 4I), and, strikingly, there was an absence of dMyc protein in PSC cells due to forced Rbf expression in niche cells (Fig. 4I). Finally, there was also an absence of dMyc protein in PSC cells due to E2F gene function knockdown (Fig. 4J). These results indicate that Rbf can directly or indirectly repress dMyc expression, and the co-expression of the two cell-proliferation regulators leads to a near-normal PSC cell number, due to Rbf suppression of dMyc and resulting rescue of the PSC overproliferation phenotype. Thus, it appears that the dMyc cell proliferation controller is a common target for Dpp pathway signaling and InR/Bam/Rbf functional interactions. Such an integration of the Dpp, Wg and InR/Bam/Rbf genetic regulators in the control of PSC cell number is shown in Fig. 4K.

Taken together, this work has demonstrated a vital function for Bam in the regulation of cell number and organization of the hematopoietic progenitor niche. bam works in conjunction with members of the InR signaling pathway and has an essential role in promoting the expression of the Rbf protein, which in turn is a direct or indirect regulator of dMyc expression. The question remains as to how...
Bam positively regulates Rbf expression. As Bam has been shown to function in germline cells as a translational regulator through binding to target mRNA 3′-UTR sequences (Shen et al., 2009; Li et al., 2009; Inesco et al., 2012), Bam might function to prevent the expression or activity of a repressor that would normally inhibit Rbf expression (Fig. 4K). Also possible is a mechanism in which Bam directly or indirectly facilitates Rbf gene expression and/or subsequent mRNA translation and protein synthesis. In either case, Bam has been proven to be an indispensable regulator of the final phase of Drosophila hematopoiesis through its documented functions in both hematopoietic progenitors and their instructive niche cells.

MATERIALS AND METHODS

Drosophila strains

Drosophila strains used in this study are described in the supplementary material.

Tissue immunostaining

Dissected lymph glands were fixed with 4% paraformaldehyde for at least 30 min. After fixation, samples were incubated with blocking solution containing 5% goat serum in 0.05% Triton X-100 in PBS (PBST) for 1 h at room temperature and then incubated with primary antibody dilution with blocking solution at room temperature for 1 h (anti-Antp) or at 4°C overnight (other antibodies). See supplementary material Table 1 for antibodies used. After washing with PBST four times, Alexa 555-conjugated anti-mouse or anti-rabbit antibody (1:500; Invitrogen, A-21422, A-21428, respectively) was added to the samples for at least 1 h. After washing again, samples were mounted with 50% glycerol in PBS. Images of immunostained samples were captured using a Nikon A1R laser-scanning confocal microscope.

PSC cell counting

Quantification of PSC cell numbers was performed as previously described (Tokusumi et al., 2012). Antp+ PSC cells in lymph glands from mid-third-instar larvae were counted. Error bars in each graph show s.d. Natural
Fig. 4. Positioning bam within the genetic hierarchy controlling PSC cell number. (A) PSC cells in bam\textsuperscript{Δ86}/bam\textsuperscript{Δ86} mutant larvae. Antp protein (red) serves as niche cell marker. (B) PSC cell numbers in lymph glands from wild-type and mutant larvae. (C) Lack of Rbf protein in PSC cells in lymph glands from bam\textsuperscript{Δ86}/bam\textsuperscript{Δ86} mutant larvae. (C', C'') Higher magnification views of box in C. (D) dFOXO mutant diminishes Rbf expression in PSC cells. (D', D'') Higher magnification views of box in D. (E) Expressing a dominant-negative version of Tkv in PSC cells results in an increased niche size but normal Rbf expression. (E', E'') Higher magnification views of box in E. (F) Forced expression of dMyc selectively in the PSC results in an increased number of niche cells. (F', F'') Higher magnification views of box in F. (G) Forced expression of Rbf suppresses the increased PSC cell phenotype in lymph glands from col\textsuperscript{dMyc}>Rbf larvae. Antp protein (red) and hhF4f-GFP expression (green) serve as PSC cell markers. (H) Control lymph gland stained for the PSC cell marker hhF4f-GFP (green) and dMyc protein (red). (H', H'') Higher magnification views of box in H. (I) Reduced number of PSC cells in lymph glands from Rbf gain-of-function larvae. (I', I'') Higher magnification views of box in I. (J) Knockdown of E2F function results in a decreased PSC cell number in lymph glands from col\textsuperscript{E2F RNAi} larvae. (J', J'') Higher magnification views of box in J. Filled arrowheads highlight hhF4f-GFP\textsuperscript{+}, Rbf\textsuperscript{+} or dMyc\textsuperscript{+} PSC cells, whereas open arrowheads highlight Rbf\textsuperscript{-} or dMyc\textsuperscript{-} PSC cells. (K) Placing Bam within a genetic hierarchy controlling PSC cell number and niche size. P-values (Student’s t-test) indicate significant differences. Scale bar: 20 μm in all images except higher magnification views.
logarithms of cell numbers for each PSC were determined and analyzed by Student’s t-test (Sorrentino et al., 2007). All calculations were obtained with Microsoft Excel or Apple Numbers.

Quantification of progenitor cells in lymph glands

Densitometric means of dome-MESO+ cells, which are blood progenitor cells in lymph glands, were quantified with a modified method, as previously described (Gao et al., 2009). After anti-β-galactosidase staining, lymph gland images were captured with a Zeiss Axioplan, and their size and densitometric mean values were determined with ImageJ (NIH).

Cell cycle monitoring

To monitor cell cycle status of PSC cells in various genetic backgrounds, the fluorescent ubiquitylation-based cell cycle indicator (Fucci) for Drosophila (FlyFucci) system was used (Sakaue-Sawano et al., 2008; Zielke et al., 2014). See supplementary material for fly stocks used. We assessed mid-third instar col-Fucci lymph glands in various genetic backgrounds with a Nikon A1-R or C2 confocal microscope and counted each color of the PSC cells (GFP+ or mRFP, only GFP and only mRFP).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.T., Y.T., D.W.H. and R.A.S. conceived/designed experiments; T.T., Y.T. and D.W.H. performed experiments; T.T., Y.T., D.W.H. and R.A.S. analyzed the data; and T.T and R.A.S. wrote the paper.

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