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The *mir-7* and *bag of marbles* genes regulate Hedgehog pathway signaling in blood cell progenitors in *Drosophila* larval lymph glands

Tsuyoshi Tokusumi, **Yumiko Tokusumi**, and **Robert A. Schulz**

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556

Abstract

Hedgehog (Hh) pathway signaling is crucial for the maintenance of blood cell progenitors in the lymph gland hematopoietic organ present in *Drosophila* third instar larvae. Previous studies from our lab have likewise shown the importance of the *mir-7* and *bag of marbles* (*bam*) genes in maintaining the progenitor state. Thus we sought to investigate a possible interaction between the Hh pathway and *mir-7*/*bam* in the prohemocyte population within this hematopoietic tissue. Gain of function *mir-7* was able to rescue a blood cell progenitor depletion phenotype caused by Patched (Ptc) inhibition of Hh pathway signaling in these cells. Similarly, expression of a dominant/negative version of Ptc was able to rescue the severe reduction of prohemocytes due to *bam* loss of function. Furthermore, we demonstrated that *Suppressor of fused* [Su(fu)], another known inhibitor of Hh signaling, likely serves as a translational repression target of the *mir-7* miRNA. Our results suggest the *mir-7*/*bam* combination regulates the Hh signaling network through repression of Su(fu) to maintain hemocyte progenitors in the larval lymph gland.

Keywords

Bag of marbles; *Drosophila* blood cell progenitors; Hedgehog signaling pathway; *mir-7* miRNA; Patched; Suppressor of fused

1 / INTRODUCTION

Drosophila has emerged as an excellent model system for the study of hematopoiesis. Two distinct waves of blood cell formation exist during *Drosophila* development (Evans, Hartenstein, & Banerjee, 2003). The first wave occurs in the embryonic head mesoderm. Generated hemocytes are contributed to the larval form and persist in groups under the larval cuticle. The second wave occurs in the larval lymph glands, which are formed during embryogenesis and eventually degenerate during metamorphosis, releasing large numbers of mature blood cells that persist into the adult animal.

The lymph glands present in third instar larvae are composed of several pairs of lobes. The anterior, primary lobes consist of three distinct cellular domains: the medullary zone (MZ),

Correspondence: Robert A. Schulz, PhD, Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556. rschulz@nd.edu.

cortical zone (CZ) and posterior signaling center (PSC). The MZ is composed of blood cell progenitors, thought to function as hematopoietic stem-like cells. The CZ includes mature hemocytes such a plasmatocytes, crystal cells and lamellocytes. The PSC secretes multiple signaling molecules such as Unpaired-3, Serrate, Hedgehog (Hh) and Pvf to control progenitor cell maintenance versus blood cell differentiation onset (Jung et al., 2005; Lebestky, Jung, & Banerjee, 2003; Mandal et al., 2007; Mondal et al., 2011; Tokusumi et al., 2010).

The MZ cellular domain, initially marked with a *domeless* (*dome*) reporter, has now been characterized by many additional molecular markers expressed therein from multiple signaling pathway components and other genes (Benmimoun et al., 2012, 2015; Dragojilovic-Munther & Martinez-Agosto, 2012, 2013; Gao, Wu, & Fossett, 2011, 2013; Gao et al., 2016; Jung et al., 2005; Mandal et al., 2007; Mondal et al., 2014; Morin-Poulard et al., 2016; Oyallon et al., 2016; Shim, Mukherjee, & Banerjee, 2012; Sinenko et al., 2009; Tokusumi et al., 2011, 2017). The *hh* pathway is one important signaling pathway used to maintain blood cell progenitor quiescence (Mandal et al., 2007). The secreted Hh ligand binds to the receptor protein Patched (Ptc), preventing Ptc inhibition of Smoothened whose normal function is to activate the downstream transcriptional effector Cubitus interruptus (Ci) (Osterlund & Kogerman, 2006). The Ci protein is also regulated by other factors including the Suppressor of fused [Su(fu)] protein. Hh is expressed in PSC cells, which serves as the source for ligand communication with cells of the MZ. In the absence of *hh* function, or the inhibition of other positively-acting components of the Hh network, MZ cell quiescence is lost and the progenitor cells enter blood cell differentiation pathways (Giordani et al., 2016; Mandal et al., 2007).

Previously, we demonstrated that the small regulatory RNA *mir-7* genetically interacts with the *bag of marbles* (*bam*) gene to maintain the lymph gland MZ prohemocyte population through the inhibition of the Yan pro-blood cell differentiation transcription factor (Tokusumi et al., 2011). In this study, we show that *mir-7* and *bam* genetically interact with components of the *hh* pathway in the maintenance of hematopoietic stem-like progenitors. Mechanistically, *mir-7* appears to function in the repression of Su(fu) protein expression, allowing for active *hh* pathway signaling in MZ cells and the maintenance of the blood cell progenitor state.

2 / RESULTS AND DISCUSSION

2.1 / The *mir-7* and *bam* genes regulate *hh* signaling to maintain prohemocytes in *Drosophila* larval lymph glands

The *hh* pathway plays a crucial role in maintaining blood cell precursors populating the MZ domain (Mandal et al., 2007). An MZ-specific Gal4 driver, *TepIV-Gal4*, was used to express the wild type *ptc* gene in lymph glands, with the result being a strong decrease in *TepIV-GFP* positive cells (Figure 1B, F). In contrast, expression of a dominant negative version of Ptc (ptc^{D584N}) induced a copious number of blood cell progenitors in the lymph glands (Figure 1C, F). These results agreed with previous MZ cell phenotypes elicited in *hh* mutant and Ci gain of function analyses (Mandal et al., 2007). Recently, two groups demonstrated that the PSC is not required for MZ cell maintenance and Hh signaling may not be necessary

for this process (Benmimoun et al., 2015; Oyallon et al., 2016). However, our results suggested the Hh signaling pathway clearly plays a role in maintaining the MZ prohemocyte population.

We previously demonstrated that the *mir*-7 miRNA is expressed in MZ cells and is required therein to maintain this population in a pluripotent progenitor state through its repression of Yan (Tokusumi et al., 2011). Intriguingly, *mir-7* gain of function led to an expanded MZ cell population, similar to the *TepIV-Gal4>ptc*^{D584N} result (Figure 1D, F). It is known that miRNAs can have multiple targets to regulate gene expression for tissue and organ homeostasis. We hypothesized that *mir-7* could control not only Yan expression, but also *hh* pathway signaling in the maintenance of the progenitor population. To address this possibility, we co-expressed *mir-7* and *ptc*^{wt} in MZ cells. This co-expression led to a near normal number of MZ cells, indicating *mir-7* expression can rescue the blood cell progenitor loss due to Ptc expression and Ptc inhibition of Hh signaling (Figure 1E, F). These results suggested the *mir-7* miRNA may also function through its regulation of the *hh* genetic network in the maintenance of the prohemocyte population.

We previously reported that Bam, initially identified as a germ line differentiating factor, functionally interacts with *mir-7* to control hematopoiesis in the larval lymph gland. Specifically, Bam and mir-7 act as positive regulators of hematopoietic progenitor maintenance in this developmental process (Tokusumi et al., 2011). Thus we sought to determine if *bam* gene function might also be involved in the regulation of progenitor cell number through an interaction with the *hh* pathway. As shown in a previous report, *bam*$^{\Delta 86}$ null mutant lymph glands present with a greatly diminished MZ cell population (Figure 2B, D and Tokusumi et al., 2011). This MZ reduction in the *bam* mutant lymph glands was rescued by *ptc*^{D584N} expression in MZ cells (Figure 2C, D). This finding is consistent with the *mir-7* analysis, suggesting the *bam* and *mir-7* genes functionally interact with components of the *hh* pathway in the maintenance of blood cell progenitors in the MZ domain of the larval lymph glands.

2.2 / mir-7 can negatively regulate a member of the *hh* pathway, Suppressor of fused

Several targets of the *mir-7* regulatory RNA have been identified in previous studies (Brennecke et al., 2005; Da Ros et al., 2013; Li & Carthew, 2005; Stark et al., 2003). We hypothesized one or more members of the *hh* pathway might be a target of *mir-7*. Therefore, we ran the search program Target Scan Fly (http://www.targetscan.org/fly_12/) to identify potential *mir-7* targets amongst *hh* pathway genes (Kheradpour et al., 2007). The program found two candidates: *interference hedgehog* (*ihog*) and *Su(fu). ihog,* which encodes a Hh receptor and is an inhibitor of Hh signal transduction, has already been shown to be a target of *mir-7* in imaginal discs (Da Ros et al., 2013). We examined if *TepIV>ihog RNAi* expression affected the MZ population, but could not detect a significant difference in MZ cell number as compared to wild type lymph glands (data not shown).

The second potential target, *Su(fu)*, encodes a protein found in the cytoplasm wherein it binds to the Ci factor and inhibits Ci transcriptional activity. One potential binding site for *mir-7* was found in the 3′ UTR of the *Su(fu)* gene, and this site is perfectly conserved in the *Su(fu)* genes of 12 sequenced *Drosophila* species (Figure 3). We confirmed that the Su(fu)

protein was expressed in the lymph glands, including cells of the MZ domain (Figure 4A, A′). Based on this observation, we tested if *mir-7* expression in hemocyte progenitors could negatively affect Su(fu) protein accumulation in these cells. *TepIV-Gal4* driven expression of *UAS-mir-7* significantly diminished the level of Su(fu) in MZ cells (Figure 4B, B′, C). To determine whether *Su(fu)* loss-of-function could affect the MZ prohemocyte population, we examined the $Su(fu)^{LP}$ homozygous phenotype in lymph glands. These lymph glands showed a strong increase of blood progenitor cells in parallel to a reduction of mature hemocytes (Figure 4D, 4E, 4F). To further support the hypothesis, we examined if Su(Fu) expression is affected in *mir-7* mutants. At the second or the early third instar stage, there are detectable MZ cells in mir-7 mutants, although they are rapidly reduced after the mid third instar stage. We confirmed Su(Fu) expression in mir-7 mutant lymph glands was higher than the protein level detected in wild type lymph glands. These results indicated the *mir-7* miRNA could negatively attenuate *Su(fu)* gene expression, allowing for Hh signaling and the maintenance of the MZ progenitor cells.

2.3 / Summary

In this study, we demonstrated *mir-7* can regulate the *hh* pathway via repression of Su(fu) expression, with the reduction of the level of Su(fu) binding to the Ci transcriptional factor allowing for enhanced Hh signaling (Figure 5). The *hh* pathway plays important roles in lymph gland hematopoiesis, especially prohemocyte maintenance. Without the Hh ligand, Ptc inhibits the activation of Ci, resulting in the promotion of blood cell differentiation. In previous studies, *hh* gene expression in PSC niche cells was shown to be stable during larval stages under various stress conditions such as animal starvation and injury. Under these conditions, blood cell progenitors are maintained in the lymph gland MZ domain, with the exception of wasp infestation (Krzemien et al., 2007; Tokusumi et al., 2012; unpublished data). A question can be raised as to how Hh signaling is fine-tuned under various stress conditions, even if the *hh* expression level is the same during larval stages. Previously, we suggested that *mir-7* interacts with *bam* to maintain hematopoietic progenitor cells through their negative regulation of Yan which normally functions as a factor that promotes blood cell differentiation (Tokusumi et al., 2011). One major function of miRNAs is to attenuate gene expression to promote tissue and organ homeostasis in response to changing physiological environments (Carthew, Agbu, & Giri, 2016). Our findings implicate that the *mir-7* and *bam* genes may be able to positively regulate Hh signaling, towards the goal of blood cell progenitor maintenance and lymph gland homeostasis in response to various stress conditions.

3 / MATERIALS AND METHODS

3.1 / *Drosophila* strains

The following strains were used in this study: *UAS-mCD8GFP*, *UAS-ptc*, $UAS\text{-}ptc^{D584N}$, $bam^{\Delta 86}$, $Su(fu)^{LP}$ (Bloomington *Drosophila* Stock Center); *TepIV-Gal4* (DGRC, Kyoto); *UAS-mir-7* (Li & Carthew, 2005).

3.2 / Tissue staining

Tissue staining methods were described previously (Tokusumi et al., 2015). We dissected lymph glands at the mid 3rd instar larval stage and fixed them with 4% paraformaldehyde in PBS for 30 min. For antibody staining, the fixed samples were blocked with a PBSTB solution consisting of 5% goat serum and 0.05% Triton X-100 in PBS, for 1 hr and then incubated with an antibody solution diluted with PBSTB overnight at 4°C. The following primary antibodies were used: mouse anti-Su(Fu) antibody (1:100, Developmental Studies Hybridoma Bank); anti-β-galactosidase (1:100, Promega), anti- Eater (1:1000, Chung & Kocks, 2011). After washing 3 times with 0.05% Triton X-100 in PBS, we incubated the samples with Alexa-555 conjugated anti-mouse antibody for 1 hr at room temperature (1:500, Thermo Fisher Scientific). After washing 3 times with 0.05% Triton X-100 in PBS, tissues were mounted in 50% glycerol in PBS. Images were captured with a Nikon A1R laser-scanning confocal microscope.

3.3 / Quantification of labeled cells in lymph glands

Densitometric means of GFP-labeled or immunostained samples were quantified with a previously described method and the values were analyzed with the Mann-Whitney's U test for statistical analyses (Gao, Wu, & Fossett, 2009; Tokusumi et al., 2015). In all bar graphs, error bars are indicated with standard error.

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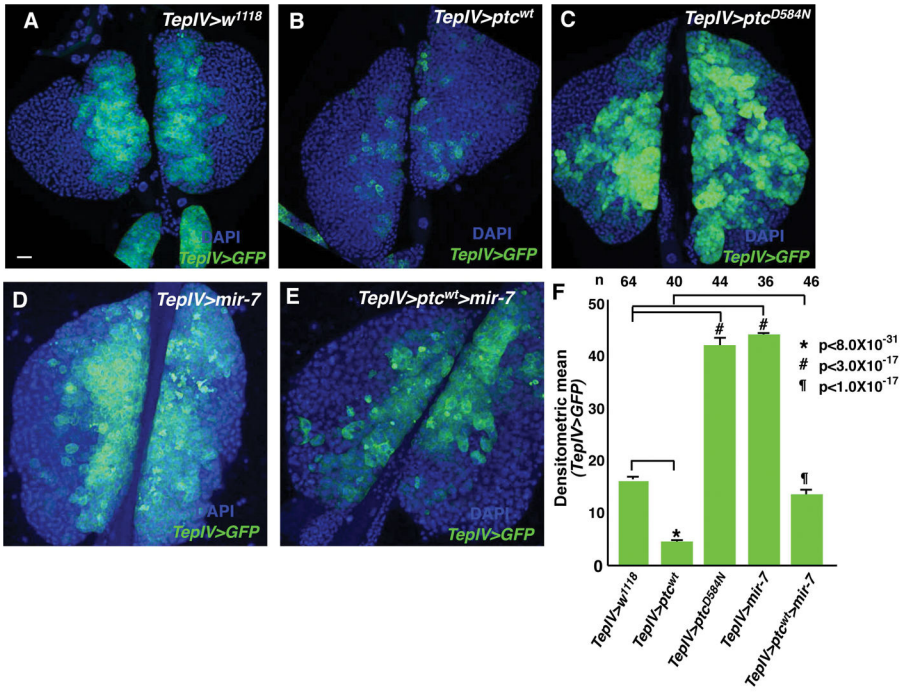

Figure 1.

Medullary zone cell reduction due to *ptc* expression and inhibition of *hh* pathway signaling is rescued by *mir-7* expression. (A) *TepIV>w*^{1118} serves as a wild type control. (B) *TepIV>ptc*^{wt} lymph glands. (C) *TepIV*-driven dominant negative form *ptc*^{D584N} lymph glands. (C) *TepIV>mir-7* lymph glands. (D) *TepIV*-driven co-expression of *mir-7* and *ptc*^{wt} lymph glands. In all panels, blue corresponds to DAPI staining and green indicates *TepIV>UAS-GFP* expression marking MZ cells. Bar in (A) indicates 20μm. All lymph gland images are at the same magnification. (F) Relative values of densitometric scans of *TepIV>GFP* expression in the various genetic backgrounds. P-values indicate significance differences.

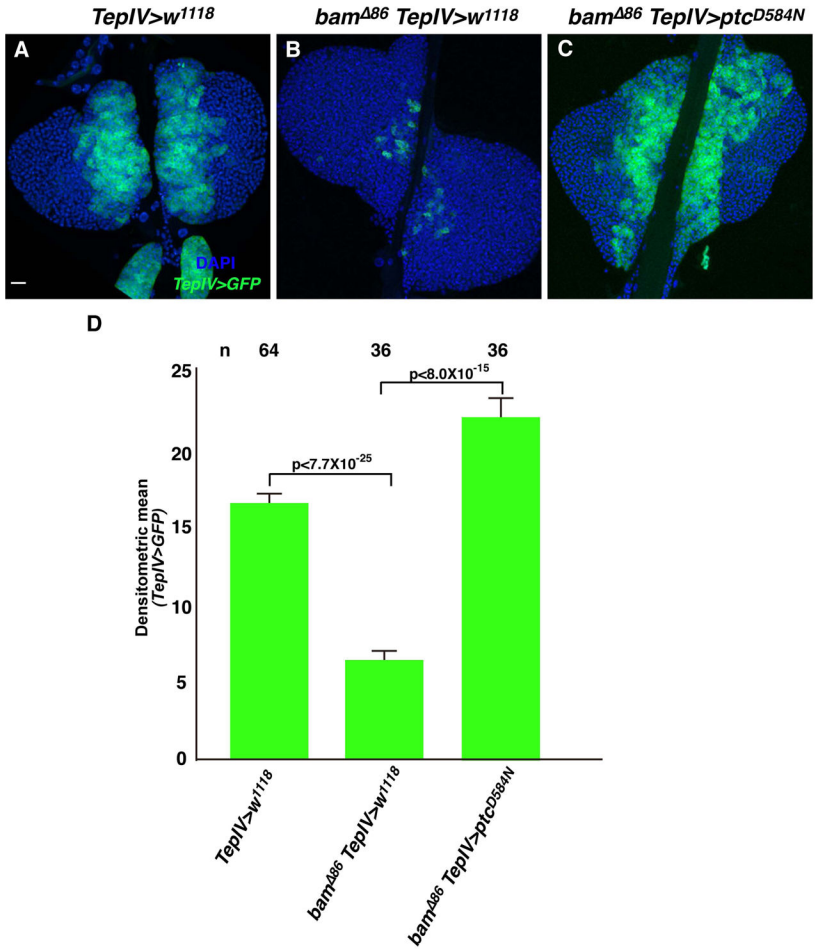

Figure 2.

Medullary zone cell reduction due to *bam* loss of function is rescued by enhanced *hh* pathway signaling due to dominant-negative Ptc expression. (A) w^{1118} lymph glands serve as a wild type control. (B) $bam^{\Delta 86}$ null mutant lymph glands. (C) $TepIV>ptc^{D584N}$ expression rescues the *bam* loss of function phenotype of reduced MZ cells. In all panels, blue corresponds to DAPI staining and green indicates *TepIV>UAS-GFP* expression marking MZ cells. Bar in (A) indicates 20μm. All lymph gland images are at the same magnification. (D) Relative values of densitometric scans of *TepIV>GFP* expression in the various genetic backgrounds. P-values indicate significant differences.

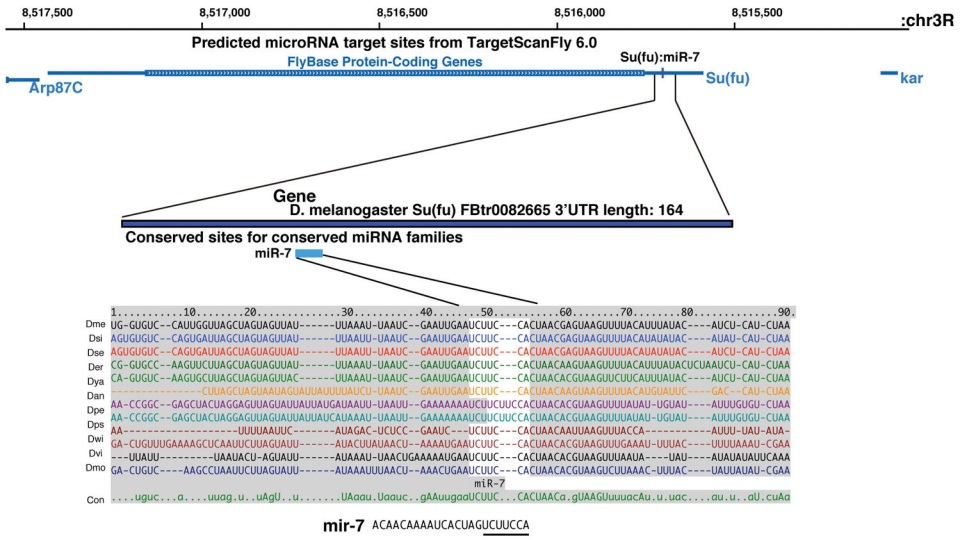

Figure 3.

Conservation of a consensus *mir-7* binding site in the 3′UTR of the *Su(fu)* gene in the genomes of 12 *Drosophila* species.

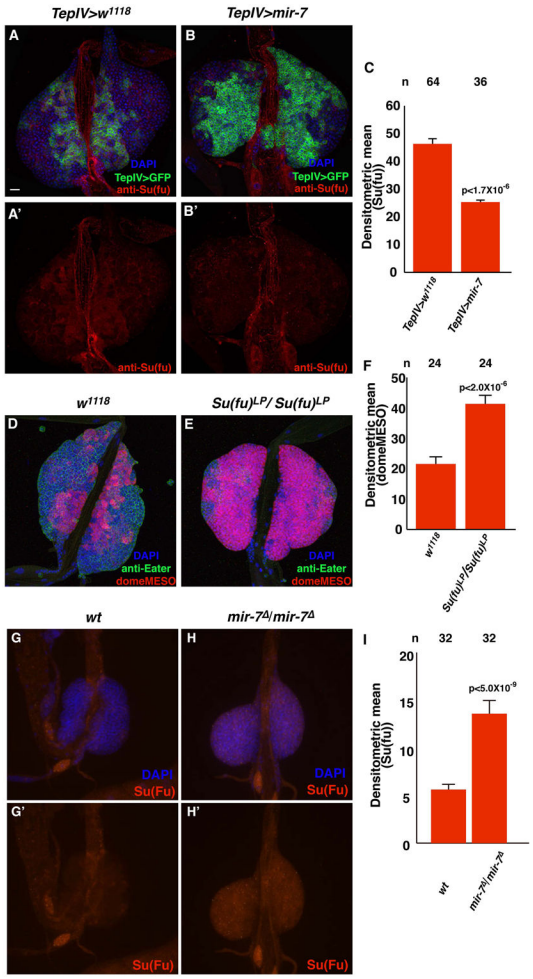

Figure 4.

Su(fu) is a likely target of translational repression by the *mir-7* miRNA. (A, A′) Localization of Su(fu) proteins in wild type lymph glands. (B, B′) *TepIV*-driven *mir-7* expression in MZ cells reduces the level of Su(fu) protein in lymph gland cells. Blue, green and red indicate DAPI nuclear staining, *TepIV-UAS-GFP* MZ cell expression and Su(fu) protein expression, respectively. Bar in (A) indicates 20μm. All lymph gland images are at the same magnification. (C) Relative values of densitometric scans of Su(fu) protein expression in the two different genetic backgrounds. MZ marker *domeMESO* and plasmatocyte marker anti-Eater antibody staining patterns in (D) wild type and (E) *Su(fu)* loss-of-function mutant $Su(fu)^{LP}$ lymph glands. Blue, green and red show respectively nucleus (DAPI), Eater protein and *domeMESO*. (F) Densitometric relative values of *domeMESO* expression in wild type and $Su(fu)^{LP}$ lymph glands. Su(fu) protein expression at the early third instar stage in (G, G′) wild type and (H, H′) *mir-7* mutants. Blues and red mean DAPI and Su(fu) protein, respectively. (I) Densitometric relative values of Su(fu) expression in wild type and $mir\text{-}7^{\Delta}$ lymph glands at the second or the early third instar stage. All p-values (C, F, and I) indicate significant differences.

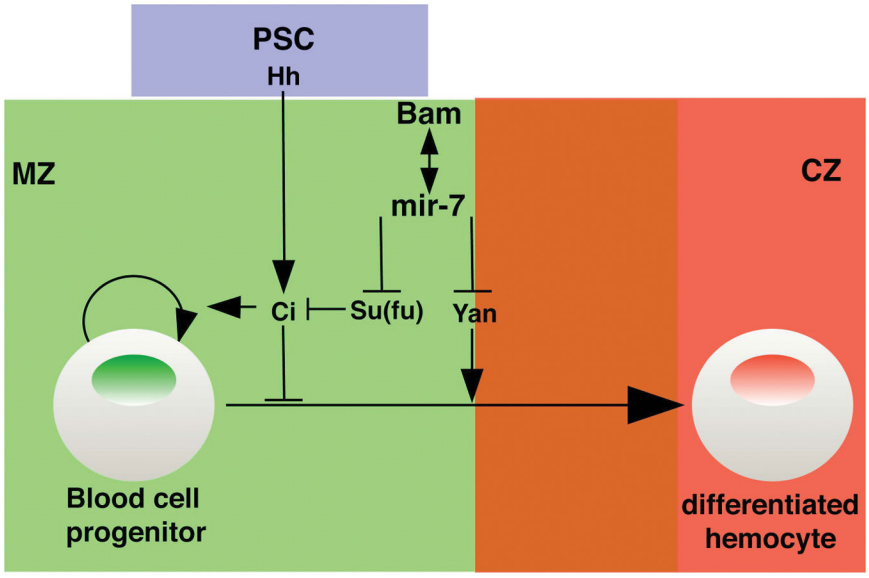

Figure 5.

Model of the interaction between the *hh* signaling pathway and *mir-7/bam* genes.

Abbreviations: CZ, cortical zone; MZ, medullary zone; PSC, posterior signaling center.