**Drosophila Rabex-5 restricts Notch activity in hematopoietic cells and maintains hematopoietic homeostasis**

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ABSTRACT

Hematopoietic homeostasis requires the maintenance of a reservoir of undifferentiated blood cell progenitors and the ability to replace or expand differentiated blood cell lineages when necessary. Multiple signaling pathways function in these processes, but how their spatiotemporal control is established and their activity is coordinated in the context of the entire hematopoietic network are still poorly understood. We report here that loss of the gene Rabex-5 in Drosophila causes several hematopoietic abnormalities including blood cell (hemocyte) overproliferation, increased size of the hematopoietic organ (the lymph gland), lamellocyte differentiation, and melanotic mass formation. Hemocyte-specific Rabex-5 knockdown was sufficient to increase hemocyte populations, increase lymph gland size, and induce melanotic masses. Rabex-5 negatively regulates Ras, and we show Ras activity is responsible for specific Rabex-5 hematopoietic phenotypes. Surprisingly, Ras-independent Notch protein accumulation and transcriptional activity in the lymph gland underlie multiple distinct hematopoietic phenotypes of Rabex-5 loss. Thus, Rabex-5 plays an important role in Drosophila hematopoiesis and may serve as an axis coordinating Ras and Notch signaling in the lymph gland.
INTRODUCTION

*Drosophila melanogaster* has served as a genetic model for studying signaling mechanisms controlling hematopoietic processes (Dearolf, 1998; Evans et al., 2003; Jung et al., 2005; Martinez-Agosto et al., 2007; Crozatier and Vincent, 2011). Regulation of hematopoiesis is similar in *Drosophila* and mammals; conserved pathways and transcription factors act in spatially and temporally distinct phases to ensure proper development and function of the hematopoietic system. While hematopoietic cell types differ between *Drosophila* and mammals, the regulation and activity of signaling pathways is highly conserved across species.

*Drosophila* blood cells, collectively known as hemocytes, arise from a common, multipotent progenitor population called prohemocytes in two waves of hematopoiesis: first during embryonic development and second during larval development. Prohemocytes differentiate into three distinct lineages: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes are present at all stages of *Drosophila* development and constitute 95% of hemocytes; they perform many functions of mammalian macrophages, as well as secrete cytokine-like molecules and antimicrobial peptides. Crystal cells are also present at all stages (Ghosh et al., 2015) and comprise 5% of hemocytes; they function in wound healing and the insect-specific immune process of melanization. Lamellocytes, a large and adherent cell type, only differentiate in the larval stage in response to large pathogens, wounding, and tissue overgrowth. They do not appear in unchallenged, wild-type larvae (Rizki and Rizki, 1992; Lanot et al., 2001; Sorrentino et al., 2002; Markus et al., 2005; Pastor-Pareja et al., 2008).

In the larval stages, hemocytes exist in three compartments: the hematopoietic organ known as the lymph gland, sessile islets under the cuticle, and the circulating hemolymph. The lymph gland is a series of bilateral lobes flanking the dorsal vessel. Hemocytes mature in the anterior-most pair of lobes, referred to as the primary lobes, while the subsequent secondary lobes of the lymph gland are primarily reservoirs of undifferentiated prohemocytes. Under normal conditions, hemocytes from the lymph gland are not released into the hemolymph until metamorphosis (Lanot et al., 2001; Holz et al., 2003; Grigorian et al., 2011a).
Ras signaling plays important roles in *Drosophila* hematopoiesis. *Heartless* (*htl*, an FGFR homolog) signaling is required for lymph gland progenitor development (Mandal et al., 2004; Grigorian et al., 2011b; Dragojlovic-Munther and Martinez-Agosto, 2013). Increased Ras activity causes hemocyte overproliferation and melanotic masses, but is insufficient for crystal cell specification (Asha et al., 2003; Zettervall et al., 2004).

Rabex-5 (also called RabGEF1) negatively regulates Ras by promoting Ras ubiquitination causing its relocalization to an endosomal compartment (Xu et al., 2010; Yan et al., 2010). We demonstrate here that loss of *Rabex-5* affects both hematopoietic waves and results in a number of hematopoietic abnormalities including increased hemocyte numbers, increased size of the larval lymph gland, lamellocyte differentiation, and formation of melanotic masses. Surprisingly, Ras dysregulation did not promote all of these abnormalities. We discovered increased Notch protein accumulation and transcriptional activity upon loss of *Rabex-5* in the lymph gland. Genetic interactions indicate that increased Notch activity is functionally relevant to *Rabex-5* crystal cell, larval lethality, melanotic mass, lamellocyte, and lymph gland size phenotypes. Thus, we identify *Rabex-5* as a negative regulator of Notch activity in the lymph gland with a role in blood cell progenitors to restrict Notch activity to ensure appropriate proliferation and differentiation of specific hemocyte lineages. Given that the interaction between Ras and Notch is synergistic or antagonistic depending on developmental context, a role for *Rabex-5* to regulate both Notch and Ras may elucidate how these complicated relationships are coordinated.
MATERIALS AND METHODS

Drosophila

Flies were raised at 25°C on standard media unless otherwise stated.

Fly Genotypes from Data Appearing in Images and Graphs

- w; FRT80B Rabex-5ext42 (Fig. 1A, 1B, 2F, 4A, 4B, 4D, 4E, 4H, 8A, 8B, 8C)
- w; FRT80B (Fig. 1B, 2F)
- w; He-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1D, 1E, 2A, 2E, 4C)
- w; UAS Rabex-5WT; He-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1D, 4C)
- w; srp-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1D, 1E, 2C, 2D, 2I)
- w; UAS Rabex-5WT; srp-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1D)
- w; UAS dap; He-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1D)
- w; UAS dap; srp-gal4, UAS GFP, FRT80B Rabex-5ext42 (Fig. 1E, 2C)
- w; He-gal4, UAS GFP (Fig. 2A)
- w, Trol-GFP/w (Fig. 2B)
- w, Trol-GFP/w; FRT80B Rabex-5ext42 (Fig. 2B)
- w; srp-gal4, UAS GFP (Fig. 2C, 2D, 2I, 3A, 3C, 3D, 3F)
- w; UAS dap; srp-gal4, UAS GFP (Fig. 2C)
- w; Be1/+ (Fig. 2G, 4F)
- w; Be1+/+; FRT80B Rabex-5ext42 (Fig. 2G, 4F)
- w; Be1+/+; srp-gal4, UAS GFP/+ (Fig. 2H, 3E, 4G)
- w; Be1+/+; srp-gal4, UAS GFP, FRT80B Rabex-5ext42/+ (Fig. 2H)
- w; Be1/UAS Rabex-5IR; srp-gal4, UAS GFP, FRT80B Rabex-5ext42/+ (Fig. 2H)
- w; UAS Rabex-5IR; srp-gal4, UAS GFP (Fig. 3A, 3C, 3D, 3F)
- w; srp-gal4, UAS GFP/+ (Fig. 3B, 8E)
- w; UAS Rabex-5IR/+; srp-gal4, UAS GFP/+ (Fig. 3B, 8E)
- w; Be1/UAS Rabex-5IR; srp-gal4, UAS GFP/+ (Fig. 3E)
- w; crq-gal4, UAS GFP/+ (Fig. 3G)
- w; UAS Rabex-5IR; crq-gal4, UAS GFP/+ (Fig. 3G)
- w; Be1/+; crq-gal4, UAS GFP/+ (Fig. 3H)
- w; Be1/UAS Rabex-5IR; crq-gal4, UAS GFP/+ (Fig. 3H)
- w; dome-gal4, UAS GFP/w (Fig. 3I, 3J, 6B, 6C)
w; dome-gal4, UAS GFP/w; UAS Rabex-5IR/+ (Fig. 3I, 3J, 6B, 6C)
w; Raselb, FRT80B Rabex-5ex42/FRT80B Rabex-5ex42 (Fig. 4A, 4B, 4D, 4E)
w; + (Fig. 4B, 4H)
w; Raselb/+ (Fig. 4B)
w; UAS Rabex-5DPfT, srp-gal4, UAS GFP, FRT80B Rabex-5ex42 (Fig. 4C)
w; BeI/+; Raselb/+ (Fig. 4F)
w; BeI/+; Raselb, FRT80B Rabex-5ex42/FRT80B Rabex-5ex42 (Fig. 4F)
w; BeI/UAS RasV12; srp-gal4, UAS GFP/+ (Fig. 4G)
w; SerBD3/+ (Fig. 4H)
w; DI7/+ (Fig. 4H)
w; DpN/+ (Fig. 4H)
w; SerBD3, FRT80B Rabex-5ex42/FRT80B Rabex-5ex42 (Fig. 4H, 8A, 8B, 8C)
w; DI7, FRT80B Rabex-5ex42/FRT80B Rabex-5ex42 (Fig. 4H, 8A, 8B, 8C)
w; DpN/+; FRT80B Rabex-5ex42 (Fig. 4H, 8A, 8B, 8C)
w; Dome-meso-EBFP2/w; srp-gal4, UAS GFP/+ (Fig. 5A-A‴, 5D, 5E, 5F)
w; Dome-meso-EBFP2/w; UAS Rabex-5IR/+; srp-gal4, UAS GFP/+ (Fig. 5B-B‴, 5D, 5E, 5F)
w; Dome-meso-EBFP2/w; UAS RasV12/+; srp-gal4, UAS GFP/+ (Fig. 5C-C‴, 5D, 5E, 5F)
w; dome-gal4, UAS GFP/w; UAS RasV12/+ (Fig. 6C)
w; Dome-meso-EBFP/w; antp-gal4, UAS GFP/+ (Fig. 6D, 6E, 6F)
w; Dome-meso-EBFP/w; UAS Rabex-5IR/+; antp-gal4, UAS GFP/+ (Fig. 6D, 6E, 6F)
w; crq-gal4, UAS GFP/+ (Fig. 6G)
w; UAS Rabex-5IR/+; crq-gal4, UAS GFP/+ (Fig. 6G)
w; UAS RasV12; crq-gal4, UAS GFP/+ (Fig. 6G)
w; srp-gal4, UAS GFP/12xSu(H)bs-lacZ (Fig. 7A, 7C, 7D)
w; UAS Rabex-5IR/+; srp-gal4, UAS GFP/12xSu(H)bs-lacZ (Fig. 7A, 7B, 7C, 7D)
w; UAS RasV12/+; srp-gal4, UAS GFP/12xSu(H)bs-lacZ (Fig. 7A, 7C, 7D)
w; srp-gal4, UAS GFP/UAS NIR (Fig. 8E)
w; UAS Rabex-5IR/+; srp-gal4, UAS GFP/UAS NIR (Fig. 8E)
Larval staging and Quantification

For lethality, melanotic mass, and lamellocyte quantification, flies were permitted to lay eggs for 1 day. Control larvae were evaluated 6 days after egg laying (AEL), and experimental larvae were evaluated 6, 9, or 14 days AEL. For circulating hemocyte quantification, flies were permitted to lay eggs for 2 hours. Control larvae were evaluated 120 hours AEL, and experimental larvae were evaluated 120 hours or 9 days AEL. Hemolymph from individual larva was collected by tearing open and inverting the cuticle in 5μl drops of phosphate-buffered saline (PBS) on coverslips pretreated with poly-D-lysine. Hemolymph was fixed with 5μl of 7.5% paraformaldehyde in PBS for 15 minutes at room temperature (RT) and washed 3 times with PBS. Cells were permeabilized with 0.1% Tween-20 in PBS with 5% either natural goat serum, natural donkey serum, or bovine serum albumin (BSA) for 20 minutes at RT. Hemocytes were counted from at least 5 random frames per individual larva. The percent of hemocytes, plasmatocytes, and crystal cells were calculated by dividing the number of GFP-positive, P1a/P1b-positive, lozenge-positive, or melanized cells by the number of DAPI-positive cells.

Circulating hemocyte concentrations

Flies were permitted to lay eggs for 2 hours. Control larvae were evaluated 120 hours AEL. Experimental larvae were evaluated 120 hours, 9 days, and 14 days AEL. Hemolymph from individual larva was collected in 20μl of PBS and kept on ice. Hemocyte concentration was measured in cells/mL using a Countess Automated Cell Counter from Invitrogen and graphed as relative concentration. Minimum and maximum cell sizes were set to 2μm and 22μm, respectively, and circularity was restricted to 75-80% roundness.

Crystal cell melanization

Flies were permitted to lay eggs for 1 day. Third instar larvae were collected and washed in PBS, dried, and individually placed in PCR tubes. Larvae were heated at
60°C for 10 minutes in an Eppendorf Mastercycler EP Gradient S thermal cycler to induce melanization of crystal cells. Two lab members blindly scored larvae.

**Lymph gland preparations**

Flies were permitted to lay eggs for 6 hours. Lymph glands were dissected as described in standard protocols (Evans et al., 2014) 4 or 5 days AEL, fixed with 3.7% paraformaldehyde in PBS for 30 minutes on ice, washed 3 times with PBS, and permeabilized in PBS with 0.1% Tween-20 and 5% BSA for 20 minutes at RT. Lymph glands were incubated in antibodies (below) and mounted according to standard protocols (Evans et al., 2014).

**Immunohistochemistry**

Antibodies were diluted in 0.1% Tween-20 in PBS with 5% either natural goat serum, natural donkey serum, or BSA. P1a/1b and L1a/L1b/L1c antibody mixtures were diluted 1:750 (I. Andó). Antibodies used were: Lozenge antibody at 1:20 (anti-lozenge, DSHB), Notch intracellular domain antibody at 1:500 (C17.9C6, DSHB), β-galactosidase antibody at 1:1000 (G4644, Sigma), Alexa-Fluor 555 goat anti-mouse HCA secondary antibody at 1:2000 (Invitrogen). Incubations were overnight at 4°C for primary antibodies and at least 2 hours at RT for secondary antibodies.

**Microscopy**

Larvae with melanotic masses (Fig. 1A) and melanized crystal cells (Fig. 2G, 3H, 3J, 4H, S2F) were imaged with a Nikon SMZ1000 stereomicroscope. Still frames from movies of live larvae with GFP-labeled hemocytes (Fig. 2A) were taken with a Zeiss Axio Observer.Z1. Fixed hemocytes (Fig. 2E) were imaged with a Zeiss Axio Imager.Z1. Z-stacks of lymph glands were taken with a Zeiss Axio Imager.Z1 and analyzed using Zen software. Regions of interest (ROIs) surrounding the primary lymph gland lobes and 21 Z-positions (9.8 μm) surrounding the center Z-position were selected. With the exception of lymph glands marked with Trol (Fig. 2B, S3), constrained iterative deconvolution was applied to all lymph gland images prior to
analysis of primary lobes (Fig. 3B, 5A-F, 6B-G, 7A-D, 8E, S2A). All lymph gland images are presented as a single maximum intensity projection.

**Statistical analysis**

Student unpaired t-tests compared lymph gland areas, hemocyte concentrations, and fluorescence intensities. Error bars represent s.e.m. Chi-square tests compared percentages of larvae with melanotic masses, larval lethality, hemocyte percentages, lamellocyte percentages, and lymph gland percentages. Statistically significant P values are indicated in the figure panels, where ^ indicates p≤0.05 and * indicates p≤0.01. For lamellocyte experiments, n≥6 larvae of each genotype. For quantification of hemocyte numbers, n≥6 larvae of each genotype. For lymph gland area measurements, n≥6 larvae of each genotype. For fluorescence intensity measurements, n≥8 larvae of each genotype. For heat-induced melanization experiments, n≥11 larvae of each genotype. For larval lethality experiments, n≥20 larvae of each genotype. For melanotic mass experiments n≥20 with the exception of flies carrying DpN where n=9. Data shown are representative results from reproducible experiments.

**RESULTS**

*Rabex-5 is required in Drosophila blood cells to prevent melanotic masses*

We previously reported melanotic mass formation (Fig. 1A) and larval and pupal lethality in *Drosophila* lacking the neoplastic tumor suppressor Rabex-5 (Yan et al., 2010). At least one melanotic mass was found in 3.8% of larvae homozygous for the deletion allele *Rabex-5*Δ42 (referred to as *Rabex-5* null larvae) 6 days after egg laying (AEL). The incidence of melanotic masses increased over time to 45% 14 days AEL (Fig. 1B). Melanotic masses were of variable size, number, and location within the body cavity. In the absence of parasitization, melanotic masses are often associated with abnormalities in the hematopoietic system, including autoimmune-like responses to self-tissue and dysregulation of proliferation leading to excess hemocyte numbers (Watson et al., 1994; Asha et al., 2003; Zettervall et al., 2004; Minakhina and
Steward, 2006). To establish if there was a requirement for Rabex-5 to prevent melanotic mass formation, we expressed wild-type Rabex-5 using Hemese-gal4 (He-gal4) or Serpent-gal4 (srp-gal4) (Fig. 1C, Supplemental Table 1). Hemese is a transmembrane protein expressed in all hemocyte lineages beginning in the second larval instar (Kurucz et al., 2003; Jung et al., 2005). He-gal4 expresses in approximately 70% of circulating hemocytes, in sessile hemocytes, and at low levels in the larval lymph gland (Zettervall et al., 2004) but does not express in the embryo. Serpent is a GATA family member and the earliest known transcription factor required for embryonic and larval hemocyte development (Rehorn et al., 1996; Lebestky et al., 2000). Srp-gal4 expresses in embryonic hemocytes (Narbonne-Reveau et al., 2011) as well as in prohemocytes and all lymph gland cells of the larval stages (Jung et al., 2005). In Rabex-5 null larvae, expressing wild-type Rabex-5 using He-gal4 (Rabex-5ex42/ex42; He>Rabex-5WT) did not reduce the incidence of melanotic masses observed at 14 days AEL; however, expressing Rabex-5 using srp-gal4 (Rabex-5ex42/ex42; srp>Rabex-5WT) reduced melanotic mass formation more than twofold (Fig. 1D). This indicates a specific requirement for Rabex-5 during hematopoiesis to prevent melanotic masses. To determine if hemocyte overproliferation contributed to the melanotic mass phenotype, we utilized Drosophila cyclin dependent kinase inhibitor dacapo (dap). In Rabex-5 null larvae 14 days AEL, expressing dap in the hematopoietic system reduced melanotic mass formation (Rabex-5ex42/ex42; He>dap and Rabex-5ex42/ex42; srp>dap, Fig. 1E). Taken together, these findings suggest a role for Rabex-5 to restrict hemocyte proliferation and prevent melanotic mass formation. Rabex-5 null lethality is likely pleiotropic; however, He-gal4 directed Rabex-5 expression and He-gal4 or srp-gal4 directed dap expression decreased larval lethality (Fig. S1A, S1B). This suggests hemocyte overproliferation also contributes to Rabex-5 null larval lethality.

**Homozygous loss of Rabex-5 in Drosophila larvae causes hematopoietic abnormalities**

Because the Rabex-5 null melanotic mass phenotype depends on proliferation of hemocytes, we further investigated the role of Rabex-5 within the hematopoietic system. Visualizing hemocytes in vivo using He>GFP, we observed a dramatic disruption of the hematopoietic system in Rabex-5 null larvae (Fig. 2Ai, ii)
compared to controls (Fig. 2Ai, ii). The hematopoietic organ, the lymph gland, became clearly visible through the cuticle of Rabex-5 null larvae (arrow in Fig. 2Aiii) but not control larvae (Fig. 2Ai). The size of Rabex-5 null lymph glands increased drastically (Fig. 2B, C and Fig. S2A); lymph glands became so overgrown that they dissociated from the dorsal vessel upon dissection, were morphologically unrecognizable, and/or physically indistinguishable from other overgrown tissues. This is consistent with overgrowth seen previously for Rabex-5 mutant epithelial tissues (Yan et al., 2010; Thomas and Strutt, 2014). Srp-gal4 directed dap expression did not affect lymph gland area in control larvae (srp>dap) but restored the lymph glands of Rabex-5 null larvae to wild-type size (Rabex-5ex42/ex42; srp>dap, Fig. 2C).

Rabex-5 null larvae also showed a dramatic increase in hemocyte numbers throughout the body cavity (Fig. 2Aiii, iv compared to 2Ai, ii). At 120h AEL, hemocyte concentrations in Rabex-5 null larvae are similar to the control; hemocyte concentrations increased in Rabex-5 null larvae by 9 days AEL (Fig. 2D, Fig. S2B). Changes in hemocyte proportions as monitored by srp>GFP and He>GFP were also seen by 9 days AEL (Fig. S2C). These increases did not result from a ruptured or empty lymph gland because the lymph gland remained populated and the basement membrane, marked by Trol expression (Grigorian et al., 2011a), remained intact in Rabex-5 null larvae (Fig. 2B). Given increased hemocyte concentrations, the increase in GFP-positive hemocytes in Rabex-5 null larvae may result from hemocyte overproliferation or from dysregulation of hemocyte lineages and markers.

Unexpectedly, we observed lamellocytes in the hemolymph of Rabex-5 null larvae, detected by a mixture of L1a, L1b, and L1c antibodies (Kurucz et al., 2007). Lamellocytes in wild-type larvae only differentiate in response to specific immune challenges. Despite the lack of external immune challenges sufficient to induce lamellocyte differentiation in our system, lamellocytes were observed in 95% of Rabex-5 null larvae, compared to 0% of control larvae 6 days AEL (Fig. 2E, F). Expression of dap in hemocytes did not suppress lamellocyte differentiation (Fig. S2D), suggesting that lamellocyte differentiation did not result from increased hemocyte proliferation.
To determine if Rabex-5 loss affected other hemocyte lineages, we examined crystal cell and plasmatocyte populations. We utilized Bc\(^1\), an allele of Black cells (Bc) that causes spontaneous melanization of crystal cells, to visualize crystal cells in vivo. Rabex-5 null larvae (Bc\(^{1+}\); Rabex-5\(^{ex42/ex42}\)) showed a marked increase in the number of melanized crystal cells compared to control larvae (Bc\(^{1+}\), Fig. 2G). The percentage of melanized crystal cells in the hemolymph increased with decreasing levels of Rabex-5 (Fig. 2H). A similar fold increase in the percentage of crystal cells upon Rabex-5 loss was confirmed using an antibody against lozenge, a transcription factor required for crystal cell specification (Lebestky et al., 2000), and using heat to induce melanization of crystal cells in vivo (Fig. S2E, F). Excess crystal cells may reflect overproliferation and release from the sessile compartment or transdifferentiation from plasmatocytes (Leitao and Sucena, 2015). The percentage of plasmatocytes present in the hemolymph detected by a mixture of P1a and P1b antibodies (Kurucz et al., 2007), decreased in Rabex-5 null larvae (Rabex-5\(^{ex42/ex42}\); srp>GFP and Rabex-5\(^{ex42/ex42}\); He>GFP) compared to controls (srp>GFP and He>GFP, Fig. 2I and S2G). Given that plasmatocytes have been reported to transdifferentiate to lamellocytes as well, the appearance of large numbers of lamellocytes and the increase in crystal cells may explain the decrease in circulating plasmatocytes (Honti et al., 2010; Krzemien et al., 2010; Stofanko et al., 2010). Alternatively, loss of Rabex-5 may promote a progenitor-like state or alter gene expression patterns such that plasmatocyte-specific epitopes are no longer present. Hemocytes overexpressing activated Ras have been reported to alter expression compared to wild-type hemocytes (Asha et al., 2003).

**Rabex-5 is required in hemocytes to maintain hematopoietic balance**

Both srp-gal4 directed expression of Rabex-5 and of dap in Rabex-5 null larvae suppressed melanotic mass formation (Fig. 1D, E), indicating a requirement for Rabex-5 specifically in the hematopoietic system and suggesting a role for Rabex-5 to restrict hemocyte proliferation. To investigate a specific requirement for Rabex-5 within the hematopoietic system, we utilized Rabex-5 RNAi using srp-gal4 and an inducible inverted repeat allele, Rabex-5\(^{IR}\), we characterized previously (Yan et al., 2010). Surprisingly, reducing Rabex-5 levels using srp-gal4 was sufficient to cause melanotic masses in 6.7% of larvae (Fig. 3A). Rabex-5 knockdown increased the area and the GFP intensity of the primary lymph gland lobes (Fig. 3B). Although Rabex-5
knockdown was not sufficient to increase hemocyte concentration (Fig. 3C), it was sufficient to alter circulating hemocyte proportions. Compared to controls, Rabex-5 RNAi in hemocytes increased the percentage of GFP-positive hemocytes in circulation (Fig. 3D) to a similar extent observed in Rabex-5 null larvae (Fig. S2C). Rabex-5 RNAi increased the percentage of circulating crystal cells (melanized cells, Fig. 3E) to a similar extent seen in Rabex-5 heterozygous larvae (Fig. 2H). The basement membrane of lymph glands, marked by Trol expression, remained intact upon Rabex-5 loss (Fig. S3); the increased circulating hemocyte percentages did not result from rupture or emptying of the lymph gland. In contrast, Rabex-5 RNAi did not significantly increase the percentage of plasmatocytes in circulation compared to control (P1a/P1b-positive cells, Fig. 3F). These data indicate an intrinsic requirement for Rabex-5 in the hematopoietic system to prevent melanotic masses, to restrict proliferation in the primary lymph gland, and to maintain appropriate proportions of hemocytes in the hemolymph.

Hematopoiesis in Drosophila occurs in two waves. To determine if Rabex-5 was required to maintain hematopoietic balance in the embryonic wave, the larval wave, or both, we used croquemort-gal4 (crq-gal4) to express RNAi to Rabex-5 specifically in hemocytes of embryonic origin (Fig. 1C, Supplemental Table 1). Rabex-5 knockdown using crq-gal4 (crq>Rabex-5IR) increased the area of the primary lymph gland lobes (Fig. 3G) but did not affect crystal cell populations (Fig. 3H) or induce melanotic masses (not shown) compared to controls (crq>GFP). Similarly, we used domeless-gal4 (dome-gal4) to reduce Rabex-5 specifically in hemocytes of larval origin. Rabex-5 knockdown using domeless-gal4 (dome>Rabex-5IR) increased the area of the primary lobes (Fig. 3I) and increased crystal cell numbers (Fig. 3J) compared to controls (dome>GFP). These results suggest Rabex-5 is required during each wave of hematopoiesis but may have developmental stage-specific functions.

Reducing Ras gene dosage suppresses larval lethality and lymph gland size but not other hematopoietic abnormalities

Rabex-5 loss in Drosophila was originally reported to increase both organismal and organ size as well as cause specification and differentiation defects such as ectopic wing veins and eye/antennal transformations. These phenotypes were sensitive to Ras
activity; reducing the gene dosage of Ras restored body size and wing area to that of controls, and largely suppressed the specification and differentiation defects (Yan et al., 2010). Rabex-5 restricted ERK activation through its E3 ubiquitin ligase activity (Xu et al., 2010; Yan et al., 2010).

To determine if Ras inhibition underlies Rabex-5 null hematopoietic phenotypes, we reduced Ras gene dosage or restored the Rabex-5 E3 ligase domain in the hematopoietic system. Reducing Ras gene dosage using loss-of-function allele Ras<sup>e1b</sup> suppressed larval lethality in Rabex-5 null larvae (Rabex-5<sup>ex42/ex42</sup>; Ras<sup>e1b/+</sup>, Fig. 4A) and restored the size of the lymph gland (Fig. 4B). To restore Rabex-5 E3 ligase function, we used He-gal4 to express either wild-type Rabex-5 or full-length Rabex-5 with an intact E3 ligase domain and an inactive Rab5 GEF domain (Rabex-5<sup>DPYT</sup>) characterized previously (Yan et al., 2010). Expressing Rabex-5<sup>DPYT</sup> suppressed and expressing wild-type Rabex-5 showed a trend to suppress larval lethality in a Rabex-5 null background (Fig. 4C, Fig. S1A).

The ability of Ras mutation and the Ras inhibitory domain of Rabex-5 to suppress larval lethality and increased lymph gland size are consistent with these phenotypes resulting in part from increased Ras activity in the hematopoietic system. Taken together with dap-dependent suppression of these phenotypes (Fig. 2C and S1B), this may indicate that excess proliferation due to elevated Ras activity in the hematopoietic system contributes to larval lethality and increased lymph gland size.

Reducing Ras gene dosage did not affect melanotic mass formation (Fig. 4D), lamellocyte differentiation (Fig. 4E), or increased crystal cell numbers (Fig. 4F) in Rabex-5 null larvae. Furthermore, expressing constitutively active Ras<sup>V12</sup> did not increase circulating crystal cells (melanized cells, Fig. 4G), suggesting that melanotic mass formation, lamellocyte differentiation, and increased crystal cells do not result from increased Ras activity.

Rabex-5 knockdown, however, was sufficient to increase the percentage of circulating crystal cells (Fig. 3E). Given the instructive role for Notch signaling in crystal cell specification (Duvic et al., 2002; Lebestky et al., 2003; Mandal et al., 2007; Krzemien et al., 2010) and reported roles in lamellocyte differentiation (Duvic et al., 2002;
Small et al., 2014), we investigated Notch involvement. Encouragingly, components of Notch signaling genetically modified the Rabex-5 null crystal cell phenotype (Fig. 4H, summarized in Fig. 8D). The Rabex-5 null crystal cell phenotype was strongly suppressed by a dominant-negative allele of Notch ligand Serrate (Serr), Ser\textsuperscript{Bd-3}, consistent with reported effects of this allele on crystal cells (Lebestky et al., 2003). The crystal cell phenotype was subtly suppressed by a loss-of-function allele of Notch ligand Delta (Dl), Dl\textsuperscript{7}, and enhanced by Notch duplication (DpN).

**Rabex-5 knockdown increases Notch accumulation in the larval lymph gland**

The larval lymph gland is a site of hemocyte proliferation and differentiation with known roles for Notch signaling (Duvic et al., 2002; Lebestky et al., 2003; Krzemien et al., 2007; Small et al., 2014). The primary lymph gland lobes contain three distinct zones: the medullary zone (MZ) comprised of slowly proliferating prohemocytes, the cortical zone (CZ) containing differentiating hemocytes, and a small cluster of cells called the Posterior Signaling Center (PSC) which control the balance of prohemocytes and differentiating hemocytes. We investigated Notch dysregulation upon Rabex-5 knockdown within the primary lobes using an antibody that recognizes the intracellular domain of Notch (C17.9C6, DSHB). The MZ of the primary lymph gland lobes was marked using domeless-meso-EBFP2 (Fig. 5A-C, A’’-C’’), Supplemental Table 1). In control larvae, Notch antibody staining in the MZ was moderate and uniform. This was easily discernable from high and heterogeneous Notch antibody staining in the CZ. Thus, in 80% of control larvae, Notch expression also delineated the boundary between the MZ and CZ (Fig. 5A’, D). Reducing Rabex-5 levels across the entire primary lobe using srp-gal4 (srp>Rabex-5\textsuperscript{IR}) dramatically increased Notch antibody staining in the MZ (Fig. 5B’), making the MZ/CZ boundary no longer discernable by Notch expression patterns. Consequently, Rabex-5 reduction decreased the percent of lymph glands that display differential Notch staining between the MZ and CZ from 80% to 25% (Fig. 5D). The area (Fig. 5E) and Notch fluorescence intensity (Fig. 5F) of the entire primary lobe also increased upon Rabex-5 reduction.

Notch and Ras demonstrate context-dependent interactions (Sundaram, 2005). To rule out a role for increased Ras activity in Notch dysregulation in the larval lymph gland,
we expressed constitutively active Ras across the entire primary lobe (srp>RasV12). RasV12 did not significantly alter the percent of lymph glands with differential Notch staining (Fig. 5C’, D) but significantly decreased lymph gland area (Fig. 5E). Surprisingly, RasV12 expression decreased Notch fluorescence intensity (Fig. 5F) of the primary lobe. This suggests that increased Ras is not sufficient to promote Notch signaling in the lymph gland.

**Rabex-5 is required in embryonic and medullary zone hemocytes**

To determine which zone of the primary lobe required Rabex-5 to regulate Notch, we knocked down Rabex-5 exclusively in the MZ using domeless-gal4 (dome-gal4) or exclusively in the PSC using antennapedia-gal4 (antp-gal4) (Fig. 6A, Supplemental Table 1). A significant increase in Notch intensity across the entire lymph gland was seen upon Rabex-5 RNAi in the MZ (dome>Rabex-5IR, Fig. 6B). The average number of cells per lobe with strong Notch expression increased, even when larvae were raised at 18°C to minimize the level of RNAi (Fig. 6C). Rabex-5 reduction exclusively in the MZ promoted Notch accumulation similar to Rabex-5 reduction in the entire primary lobe, supporting a model that Rabex-5 is required to restrict Notch accumulation in the prohemocytes of the MZ. Constitutive Ras activation in the MZ had no effect on Notch expression; at 18°C, RasV12 expression using dome-gal4 did not significantly alter the average number of cells per lobe that strongly express Notch (dome>RasV12, Fig. 6C).

Rabex-5 RNAi specifically in the PSC (antp>Rabex-5IR) did not change Notch fluorescence intensity over the entire primary lobe (Fig. 6D) or in the PSC itself (Fig. 6E). Rabex-5 RNAi in the PSC did not increase the average number cells per lobe that strongly express Notch (Fig. 6F). These results indicate that Rabex-5 functions in the prohemocytes of the MZ, but not in the PSC, to prevent Notch accumulation.

Given that Rabex-5 knockdown in embryonic hemocytes did not affect crystal cell numbers (Fig. 3H), we assessed the requirement for Rabex-5 to regulate Notch specifically in hemocytes of embryonic origin. Rabex-5 knockdown in embryonic hemocytes (crq>Rabex-5IR) was sufficient to increase Notch fluorescence intensity across the primary lymph gland lobe. Consistent with our previous results, RasV12
expression (crq>Ras\textsuperscript{V12}) did not promote Notch accumulation in the lymph gland (Fig. 6G). These data demonstrate that Rabex-5 functions both in embryonic hemocytes and in the larval lymph gland to restrict Notch accumulation.

Notch accumulation upon Rabex-5 loss leads to increased transcriptional outputs

To establish if Notch protein accumulation resulted in increased Notch transcriptional activity, we examined the effect of Rabex-5 knockdown on a Notch transcriptional reporter, 12xSu(H)bs-lacZ (Go et al., 1998). Reducing Rabex-5 across the primary lymph gland lobes (srp>Rabex-5\textsuperscript{IR}) increased β-galactosidase (β-gal) fluorescence intensity compared to controls (Fig. 7A). In 81% of control lymph glands, β-gal staining was uniform and low. The remaining 19% of lymph glands showed individual cells with elevated reporter activity (arrows in Fig. 7B, quantification in 7C). Rabex-5 reduction increased the percent of lymph glands showing individual cells with elevated reporter activity from 19% to 75%. Compared to controls, Rabex-5 reduction also increased the average number of individual cells with elevated activity per primary lobe (Fig. 7D). These findings indicate that Notch protein accumulation upon Rabex-5 knockdown in the lymph gland led to functionally increased Notch transcriptional activity.

Ras\textsuperscript{V12} expression (srp>Ras\textsuperscript{V12}) had no effect on β-gal fluorescence intensity (Fig. 7A), did not significantly alter the percentage of lymph glands showing individual cells with elevated reporter activity (Fig. 7C), and did not significantly alter the average number of cells displaying elevated reporter activity per lobe (Fig. 7D). These results indicate that increased Ras activity is not sufficient to increase Notch transcriptional activity in the lymph gland.

Increased Notch activity mechanistically underlies specific Rabex-5 hematopoietic phenotypes

To establish if the increased Notch activity was functionally relevant to Rabex-5 hematopoietic phenotypes, we performed genetic interactions using Notch pathway components Notch, Delta, and Serrate (Fig. 8A-C, summarized in 8D) or Notch RNAi (Fig. 8E). Reducing Delta gene dosage using the Dl\textsuperscript{7} allele suppressed larval lethality
in a Rabex-5 null background. Notch duplication (DpN) enhanced larval lethality. Surprisingly, larval lethality was also enhanced by the SerBd-3 allele (Fig. 8A, D), which produces a protein lacking the intracellular and transmembrane domains but retaining the Notch binding domain (Hukriede and Fleming, 1997). If this truncated Serrate is able to activate Notch in some contexts, it may modify Rabex-5 null phenotypes similar to Notch duplication. Similarly, Dl7 suppressed the Rabex-5 null melanotic mass phenotype, while DpN and SerBd-3 enhanced the phenotype (Fig. 8B, D). Dl7 suppressed lamellocyte differentiation in Rabex-5 null larvae, DpN dramatically enhanced lamellocyte differentiation, and SerBd-3 had no effect (Fig. 8C, D). RNAi to Notch using an inducible inverted repeat allele, NIR, in hemocytes (srp>NIR) did not affect the size of the lymph gland at 21°C but suppressed the increased lymph gland area resulting from Rabex-5 knockdown (srp>GFP, Rabex-5IR and srp>GFP, NIR, Rabex-5IR, Fig. 8E). These results indicate that increased Notch activity contributes to larval lethality and is functionally relevant to the melanotic mass, lamellocyte, and lymph gland size phenotypes. These data and our earlier findings are consistent with a model that Rabex-5 regulates not only Ras activity (Yan et al., 2010) but also Notch activity in a Ras-independent manner during hematopoiesis to ensure proper restriction of hemocyte proliferation, direct or prevent differentiation into specific lineages, and maintain hematopoietic homeostasis.

**DISCUSSION**

We report a requirement for Rabex-5 to ensure proper hematopoiesis in Drosophila. Rabex-5 null mutants exhibited a range of hematopoietic abnormalities including hemocyte overproliferation, increased lymph gland size, increased crystal cell populations, lamellocyte differentiation, and melanotic mass formation. Rabex-5 is a known Drosophila neoplastic tumor suppressor (Yan et al., 2010; Thomas and Strutt, 2014); inactivating mutations in Rabex-5 cause tissue overgrowth and extend larval development. Immune responses to overgrown tissue have been demonstrated in both Drosophila (Pastor-Pareja et al., 2008; Hauling et al., 2014) and mammalian systems (Hanahan and Weinberg, 2011). We cannot exclude that an immune response to overgrowing tissue or a prolonged larval period partly contributes to Rabex-5 null phenotypes. However, restoring wild-type Rabex-5 activity in hemocytes suppressed
melanotic mass formation and reducing Rabex-5 in the hematopoietic system, which
does not delay development, was sufficient to reproduce nearly all Rabex-5 null
phenotypes, indicating a role for Rabex-5 specifically in the hematopoietic system.
Interestingly, a Rabex-5 knockout mouse model shows skin inflammation, increased
mast cell numbers, and perinatal lethality. Bone marrow cultured mast cells
(BMCMCs) derived from Rabex-5 knockout mice show enhanced and prolonged
activation upon stimulation compared to wild-type control BMCMCs (Tam et al.,
2004). These similarities suggest Rabex-5 function within the hematopoietic system
may be conserved in mammals.

We provide evidence that Rabex-5 restricts both Ras and Notch signaling to establish
proper lymph gland size and to promote organismal survival. Reducing Ras or Notch
activity, as well as restricting hemocyte proliferation, suppressed the increased lymph
gland size and larval lethality resulting from Rabex-5 loss. This suggests that Rabex-5
restricts proliferation of hemocytes by downregulating Ras, consistent with reports
that excess Ras signaling causes overproliferation of hemocytes (Asha et al., 2003;
Zettervall et al., 2004), and also by downregulating Notch. Surprisingly, melanotic
mass formation was dependent upon hemocyte proliferation and increased Notch
activity, but not increased Ras activity. In this context, Rabex-5 may restrict hemocyte
proliferation through a distinctively Notch-mediated mechanism. Consistent with the
requirement for Notch, but not Ras, activity in crystal cell specification, the increase
in crystal cells observed upon Rabex-5 loss was dependent upon increased Notch, but
not Ras, activity. Additionally, increased Notch, but not increased Ras, activity was
relevant to the Rabex-5 null lamellocyte phenotype, indicating a function for Rabex-5
to regulate Notch during hemocyte specification. All together, these results are
consistent with a role for Rabex-5 to restrict both Ras and Notch signaling in
hemocytes.

Convergence of the Ras and Notch pathways is required for specification of blood
progenitors in the Drosophila embryo (Grigorian et al., 2011b). Importantly, we
provide evidence that Rabex-5 does not regulate Notch via its regulation of Ras in the
hematopoietic system. Constitutively active Ras expression did not phenocopy Rabex-
5 loss in the lymph gland and was not sufficient to induce phenotypes associated with
Notch pathway dysregulation, such as increased crystal cell populations. Few specific
regulators have been identified in any system as links between these two pathways. We identify Rabex-5 as a modulator of both Ras and Notch activity that ensures hematopoietic homeostasis; this dual role may implicate Rabex-5 as a nexus coordinating activity of these pathways and raises interesting questions regarding the spatiotemporal regulation of Ras and Notch by Rabex-5 specifically in the hematopoietic system, and more generally in developmental contexts requiring Ras and Notch interplay.

To this point, we reveal a spatiotemporal requirement for Rabex-5 during the two waves of Drosophila hematopoiesis. Reducing Rabex-5 in hemocytes specifically of embryonic or of larval origin was sufficient to increase lymph gland size and Notch accumulation. Rabex-5 reduction in larval, but not embryonic, hemocytes increased crystal cell numbers. Rabex-5 reduction in both embryonic and larval hemocytes, but not in embryonic hemocytes alone, was sufficient to induce melanotic masses. These findings demonstrate an intrinsic requirement for Rabex-5 in the hematopoietic system with overlapping and distinct roles during embryonic and larval hematopoiesis.

Excitingly, our data may implicate Delta in Drosophila hematopoiesis. With the exception of controlling blood progenitor specification and proliferation in the embryo (Mandal et al., 2004; Grigorian et al., 2011b), Delta has not been demonstrated to function in Drosophila hematopoietic processes. Rather, Serrate is the primary ligand that activates Notch during hematopoiesis. Notch activation via Serrate is required for crystal cell formation (Duvic et al., 2002; Lebestky et al., 2003; Mandal et al., 2007; Krzemien et al., 2010), maintains PSC identity (Lebestky et al., 2003; Krzemien et al., 2007), and prevents lamellocyte differentiation (Small et al., 2014). We show reducing Delta gene dosage in Rabex-5 null larvae suppressed lethality and melanotic masses, phenotypes dependent on hemocyte proliferation. A dominant-negative Serrate allele suppressed increased crystal cell numbers in a Rabex-5 null background, whereas suppression by reducing Delta was subtle. One interpretation of these findings is that Notch activation by Delta affects hemocyte proliferation, while Notch activation by Serrate affects hemocyte differentiation.
Our findings may have implications for human disease. In mammals, Notch controls decisions of multipotent hematopoietic cells to self-renew, proliferate, commit, and differentiate to specific lineages. The importance of Notch in mammalian hematopoiesis is emphasized by the frequency of Notch alterations in human hematological malignancies, including leukemia. Excitingly, we identify Rabex-5 as an important regulator of Notch in the prohemocytes of the larval lymph gland. Prohemocytes most closely resemble the mammalian common myeloid progenitor, and evidence for Notch involvement in myeloid leukemias is emerging. Sequencing of acute myeloid leukemias (AML) revealed that two-thirds of AML cases with downregulated Rabex-5 mRNA show Notch, Delta, or Jagged2 (a mammalian Serrate ortholog) mRNA upregulation (Cerami et al., 2012; Cancer Genome Atlas Research, 2013; Gao et al., 2013). However, there are conflicting reports on the role of Notch signaling in AML, which may reflect unresolved heterogeneity within this cancer type (Tohda and Nara, 2001; Asha et al., 2003; Tohda et al., 2005; Kannan et al., 2013; Zhang et al., 2013). Rabex-5 status may help further define subsets of AML, and provide tremendous opportunities to elucidate the etiology and inform the treatment of human leukemia.
AUTHOR CONTRIBUTIONS

CMP and TAR contributed to study design, data analysis and interpretation, and preparation of the article. TAR performed all of the experiments shown in the figures.

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Figure 1: Rabex-5 is required in blood cells to prevent melanotic mass formation.

(A) Melanotic masses in larvae homozygous for the deletion allele Rabex-5<sup>ex42</sup> (arrows; anterior, top). (B) At least one melanotic mass was seen in 3.8% of Rabex-5<sup>ex42/ex42</sup> larvae compared to 0% in control larvae 6 days AEL. Incidence of melanotic masses in Rabex-5<sup>ex42/ex42</sup> larvae increased to 15% 9 days AEL and 45% 14 days AEL. (C) Serpent- (srp) and croquemort- (crq) gal4 express in embryonic hemocytes. Srp-gal4 also expresses in all hemocytes of the larval lymph gland and in circulating prohemocytes. Hemese- (He) gal4 expresses in larval hemocytes. (D) Expressing wild-type Rabex-5 using srp-gal4, but not He-gal4, in a Rabex-5<sup>ex42/ex42</sup> background (Rabex-5<sup>ex42/ex42</sup>; srp>GFP, Rabex-5<sup>WT</sup> and Rabex-5<sup>ex42/ex42</sup>; He>GFP, Rabex-5<sup>WT</sup>) decreased the incidence of melanotic masses compared to controls (Rabex-5<sup>ex42/ex42</sup>; srp>GFP and Rabex-5<sup>ex42/ex42</sup>; He>GFP) 14 days AEL. (E) Expressing dap using either He-gal4 or srp-gal4 in a Rabex-5<sup>ex42/ex42</sup> background (Rabex-5<sup>ex42/ex42</sup>; He>GFP, dap and Rabex-5<sup>ex42/ex42</sup>; srp>GFP, dap) decreased the incidence of melanotic masses compared to controls (Rabex-5<sup>ex42/ex42</sup>; He>GFP and Rabex-5<sup>ex42/ex42</sup>; srp>GFP) 14 days AEL. For this and subsequent figures, ^ indicates p≤0.05 and * indicates p≤0.01.
Figure 2: Loss of Rabex-5 causes a range of hematopoietic abnormalities.

(A) Wild-type (He>GFP, i,ii) and Rabex-5\textsuperscript{ex42/ex42} (Rabex-5\textsuperscript{ex42/ex42}; He>GFP, iii,iv) larvae expressing GFP in hemocytes. Arrowheads (ii) indicate sessile hemocytes, arrow (iii) indicates lymph gland, and asterisks (i, iii) mark mouth hooks for a reference point. Strong anterior signal is GFP fluorescence in the salivary glands seen with many Gal4 drivers. (B) Lymph glands dissected from control and Rabex-5\textsuperscript{ex42/ex42} larvae 5 days AEL. The basement membrane was marked by Trol expression. Scale bars represent 50 μm. (C) Compared to controls (srp>GFP), the area of the primary
lymph gland lobes increased in Rabex-5<sup>ex42/ex42</sup> larvae (Rabex-5<sup>ex42/ex42</sup>; srp>GFP) but was restored with hemocyte-specific expression of dap (Rabex-5<sup>ex42/ex42</sup>; srp>GFP, dap). (D) At 120h AEL, circulating hemocyte concentrations of Rabex-5<sup>ex42/ex42</sup> larvae (Rabex-5<sup>ex42/ex42</sup>; srp>GFP) were similar to controls (srp>GFP). Hemocyte concentrations of Rabex-5<sup>ex42/ex42</sup> larvae increased 9 days and 14 days AEL. (E) Lamellocytes were detected by L1a, L1b, and L1c antibodies in the hemolymph of Rabex-5<sup>ex42/ex42</sup> larvae (arrow, Rabex-5<sup>ex42/ex42</sup>; He>GFP). (F) Lamellocytes were detected in 95% of Rabex-5<sup>ex42/ex42</sup> larvae and 0% of controls larvae 6 days AEL. (G) Melanized crystal cells were visible in a heterozygous Bc<sup>1</sup> background (left, Bc<sup>1/+</sup>). Rabex-5<sup>ex42/ex42</sup> larvae (right, Bc<sup>1/+</sup>; Rabex-5<sup>ex42/ex42</sup>) showed a marked increase in the number of melanized crystal cells. (H) The percentage of melanized crystal cells in the hemolymph of larvae 120h AEL increased with decreasing Rabex-5 levels. (I) The percentage of circulating plasmatocytes detected by P1a and P1b antibodies decreased in Rabex-5<sup>ex42/ex42</sup> larvae (Rabex-5<sup>ex42/ex42</sup>; srp>GFP) 9 days AEL compared to controls (srp>GFP) at 120h AEL.
Figure 3: Rabex-5 is required in blood cells to restrict proliferation, differentiation, and the size of the lymph gland. (A) Rabex-5 RNAi (srp>GFP, Rabex-5IR) caused melanotic masses in 6.7% of larvae compared to 0% in control larvae (srp>GFP) 6 days AEL. (B) Rabex-5 RNAi (srp>GFP, Rabex-5IR) increased the area of the primary lymph gland lobes compared to controls (srp>GFP) 4 days AEL. DAPI staining is shown in blue. Scale bars represent 50 μm. (C) Rabex-5 RNAi (srp>GFP, Rabex-5IR) did not change circulating hemocyte concentrations compared to controls (srp>GFP) 120h AEL. (D) Rabex-5 RNAi in hemocytes (srp>GFP, Rabex-5IR) increased the percentage of circulating GFP-positive hemocytes compared to controls (srp>GFP) 120h AEL. (E) Rabex-5 RNAi in hemocytes in a Bc1 strain.
heterozygous background ($B^{c1/+}; srp>GFP, Rabex-5^{IR}$) increased the percentage of melanized crystal cells in the hemolymph compared to controls ($B^{c1/+}; srp>GFP$) 120h AEL. (F) Rabex-5 knockdown using $srp$-gal4 ($srp>GFP, Rabex-5^{IR}$) did not change the percentage of circulating plasmatocytes compared to controls ($srp>GFP$) 120h AEL. (G) Rabex-5 RNAi in embryonic hemocytes ($crq>GFP, Rabex-5^{IR}$) increased the area of the primary lymph gland lobes compared to controls ($crq>GFP$) 5 days AEL. (H) Rabex-5 RNAi in embryonic hemocytes in a $B^{c1}$ heterozygous background ($B^{c1/+}; crq>GFP, Rabex-5^{IR}$) did not alter the percentage of melanized crystal cells in the hemolymph compared to controls ($B^{c1/+}; crq>GFP$) 120h AEL. Heat-induced melanization of crystal cells in vivo also showed no difference. (I) Rabex-5 RNAi in the medullary zone of the larval lymph gland ($dome>GFP, Rabex-5^{IR}$) increased the area of the primary lobes 4 days AEL and increased crystal cell numbers visualized by heating larvae (J) compared to controls ($dome>GFP$).
Figure 4: Melanotic mass formation and lamellocyte differentiation are not sensitive to Ras gene dosage. (A) Reducing Ras gene dosage with loss-of-function allele *Ras*<sup>e1b</sup> in a *Rabex-5<sup>ex42/ex42</sup>* background (*Rabex-5<sup>ex42/ex42</sup>; *Ras*<sup>e1b/+</sup>) decreased larval lethality compared to controls (*Rabex-5<sup>ex42/ex42</sup>*). (B) Reducing Ras gene dosage restored lymph gland area in a *Rabex-5<sup>ex42/ex42</sup>* background compared to controls (*Rabex-5<sup>ex42/ex42</sup>; *Ras*<sup>e1b/+</sup> and *Rabex-5<sup>ex42/ex42</sup>* but had no effect on lymph gland area alone (*Ras*<sup>e1b/+</sup>) 5 days AEL. (C) In a *Rabex-5<sup>ex42/ex42</sup>* background, expressing GEF-inactive *Rabex-5* (*Rabex-5<sup>ex42/ex42</sup>; *He>GFP, Rabex-5<sup>DPYT</sup>* suppressed wild-type *Rabex-5* (*Rabex-5<sup>ex42/ex42</sup>; *He>GFP, Rabex-5<sup>WT</sup>* showed a trend to suppress larval lethality compared to controls (*Rabex-5<sup>ex42/ex42</sup>; *He>GFP*). Reducing Ras gene dosage in a *Rabex-5<sup>ex42/ex42</sup>* background (*Rabex-5<sup>ex42/ex42</sup>; *Ras*<sup>e1b/+</sup>) did not affect the incidence of melanotic masses (D), the percentage of larvae containing lamellocytes (E) 14 days AEL, or the increased crystal cells (F) 9 days AEL. (G) Expressing constitutively active *Ras*<sup>V12</sup> in a *Bc<sup>1</sup>* heterozygous background (*Bc<sup>1/+</sup>; *srp>GFP, Ras<sup>V12</sup>* did not increase the percentage of melanized crystal cells in the hemolymph compared to controls (*Bc<sup>1/+</sup>; *srp>GFP*) 120h AEL. (H) Crystal cells were visualized by heating...
larvae 5 days AEL. Dominant-negative Serrate, SerBd-3, strongly suppressed the increased crystal cell numbers in a Rabex-5ex42/ex42 background. The loss-of-function Delta allele, Dl7, subtly suppressed the increased crystal cell numbers. Duplication of Notch (DpN) further increased crystal cell numbers in a Rabex-5ex42/ex42 background.
Figure 5: Loss of Rabex-5 leads to Ras-independent dysregulation of Notch protein across the primary lymph gland lobes. (A-C) Scale bars represent 50 μm. (A-C, A’’-C’’’) Expression of EBFP2 (traced in white) marked the medullary zone (MZ) of the primary lymph gland. (A’) In control larvae (Dome-meso-EBFP2/+; srp>GFP) Notch expression was low in the MZ and distinct from the high expression in the outer, cortical zone (CZ). (B’) Rabex-5 RNAi in hemocytes (Dome-meso-EBFP2/+; srp>GFP, Rabex-5IR) increased Notch expression in the MZ compared to controls (Dome-meso-EBFP2/+; srp>GFP). (C’) Expressing RasV12 in hemocytes (Dome-meso-EBFP2/+; srp>GFP, RasV12) did not increase Notch expression in the MZ. (D) Rabex-5 RNAi, but not RasV12, in hemocytes (Dome-meso-EBFP2/+; srp>GFP, Rabex-5IR and Dome-meso-EBFP2/+; srp>GFP, RasV12) decreased the percentage of lymph glands in which the MZ and CZ are discernable by Notch expression compared to controls (Dome-meso-EBFP2/+; srp>GFP). Rabex-5 RNAi in hemocytes (Dome-meso-EBFP2/+; srp>GFP, Rabex-5IR) increased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to controls.

(Dome-meso-EBFP2/+; srp>GFP). RasV12 in hemocytes (Dome-meso-EBFP2/+; srp>GFP, RasV12) decreased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to controls (Dome-meso-EBFP2/+; srp>GFP). Lymph glands were dissected 4 days AEL.
Figure 6: Rabex-5 is required in the medullary zone of the larval lymph gland to restrict Notch accumulation (A) Schematic depicting primary lobe of the larval lymph gland. Srp-gal4 is expressed across the entire lobe. Dome-gal4 is expressed exclusively in the MZ. Antp-gal4 is expressed exclusively in the PSC. (B) Rabex-5 RNAi in the MZ (dome>GFP, Rabex-5IR) increased Notch fluorescence intensity over the entire lobe compared to controls (dome>GFP) 4 days AEL. (C) At 18°C, Rabex-5 RNAi in the MZ (dome>GFP, Rabex-5IR) increased the average number of cells per lobe that strongly express Notch compared to controls (dome>GFP). RasV12 expression in the MZ (dome>GFP, RasV12) did not alter the average number of cells per lobe that strongly express Notch. Rabex-5 RNAi in the PSC (antp>GFP, Rabex-5IR) did not change Notch fluorescence intensity over the entire primary lobe (D) or in the PSC (E) compared to controls (antp>GFP). (F) Rabex-5 RNAi in the PSC
(antp>GFP, Rabex-5IR) had no effect on the average number of cells per lobe that strongly express Notch compared to controls (antp>GFP). (G) Rabex-5 RNAi, but not RasV12, in embryonic hemocytes (crq>GFP, Rabex-5IR and crq>GFP, RasV12) increased Notch fluorescence intensity of the primary lobes compared to controls (crq>GFP) 5 days AEL.
Figure 7: Notch accumulation upon Rabex-5 loss leads to increased transcriptional outputs. (A) Rabex-5 RNAi, but not Ras<sup>V12</sup>, in hemocytes (srp>GFP, Rabex-5<sup>IR</sup> and srp>GFP, Ras<sup>V12</sup>) increased β-gal fluorescence intensity across the primary lymph gland lobes compared to controls (srp>GFP). (B) Representative image showing individual cells with elevated reporter activity (arrows) in lymph glands expressing GFP in hemocytes. DAPI staining is shown in blue. Scale bars represent 10 μm. (C) Rabex-5 RNAi, but not Ras<sup>V12</sup>, in hemocytes (srp>GFP, Rabex-5<sup>IR</sup> and srp>GFP, Ras<sup>V12</sup>) increased the percentage of lymph glands with elevated reporter activity in individual cells compared to controls (srp>GFP). (D) Rabex-5 RNAi, but not Ras<sup>V12</sup>, in hemocytes (srp>GFP, Rabex-5<sup>IR</sup> and srp>GFP, Ras<sup>V12</sup>) increased the average number of cells per lobe with elevated reporter activity compared to controls (srp>GFP).
Figure 8: Rabex-5 negative regulation of Notch is required for proper regulation of hematopoiesis during development. (A) In a Rabex-5^{ex42/ex42} background, DpN and Ser^{Bd-3} (Rabex-5^{ex42/ex42}; DpN/+ and Rabex-5^{ex42/ex42}; Ser^{Bd-3/+}) increased larval lethality compared to controls (Rabex-5^{ex42/ex42}). Dl^{7} (Rabex-5^{ex42/ex42}; Dl^{7/+}) suppressed larval lethality. (B) In a Rabex-5^{ex42/ex42} background 14 days AEL, DpN and Ser^{Bd-3} (Rabex-5^{ex42/ex42}; DpN/+ and Rabex-5^{ex42/ex42}; Ser^{Bd-3/+}) increased the incidence of melanotic masses and Dl^{7} (Rabex-5^{ex42/ex42}; Dl^{7/+}) decreased the incidence of melanotic masses compared to controls (Rabex-5^{ex42/ex42}). (C) In a Rabex-5^{ex42/ex42} background 6 days AEL, DpN (Rabex-5^{ex42/ex42}; DpN/+).
percentage of larvae with lamellocytes compared to controls (Rabex-5\textsuperscript{ex42/ex42}). Dl\textsuperscript{7} (Rabex-5\textsuperscript{ex42/ex42}; Dl\textsuperscript{7/+}) decreased the percentage of larvae with lamellocytes. Ser\textsuperscript{Bd-3} (Rabex-5\textsuperscript{ex42/ex42}; Ser\textsuperscript{Bd-3/+}) did not alter the percentage of larvae with lamellocytes compared to controls (Rabex-5\textsuperscript{ex42/ex42}). (D) Summary of Rabex-5\textsuperscript{ex42/ex42} genetic interactions with Notch, Delta, and Serrate. (E) Notch RNAi in hemocytes (srp＞GFP, N\textsuperscript{IR}) did not affect the area of the primary lymph gland lobes at 21°C but reduced the enlarged lymph glands (srp＞GFP, N\textsuperscript{IR}, Rabex-5\textsuperscript{IR}) resulting from Rabex-5 RNAi (srp＞GFP, Rabex-5\textsuperscript{IR}) to control area (srp＞GFP) 7 days AEL. DAPI staining is shown in blue. Scale bars represent 50 μm.