RESEARCH ARTICLE

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 that 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 might serve as an axis coordinating Ras and Notch signaling in the lymph gland.

KEY WORDS: Rabex-5, RabGEF1, Ras, Notch, Drosophila hematopoiesis, Leukemia, Hemocyte, Crystal cell, Lamellocyte, Lymph gland, Melanotic mass

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) for several decades. Regulation of hematopoiesis in Drosophila and mammals is similar; conserved pathways and transcription factors act in spatially and temporally distinct phases to ensure correct development and function of the hematopoietic system. Whereas 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, whereas 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 ubiquitylation 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 an increase in the accumulation of Notch protein and Notch 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 differentiation 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 in order to restrict Notch activity to ensure appropriate proliferation and differentiation of specific hematopoietic lineages. Given that the interaction between Ras and Notch is synergistic.

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or antagonistic depending on the developmental context, a role for Rabex-5 in the regulation of both Notch and Ras might elucidate how these complicated relationships are coordinated.

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 that lack 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-5ex42 (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 whether there is a requirement for Rabex-5 to prevent melanotic mass formation, we expressed wild-type Rabex-5 (Rabex-5WT) using Hemese-gal4 (He-gal4) or Serpent-gal4 (srp-gal4) (Fig. 1C, Table S1). 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 ~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 Rabex-5WT by 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-5WT by 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 whether hemocyte overproliferation contributes 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 to be pleiotropic; however, He-gal4

Fig. 1. Rabex-5 is required in blood cells to prevent melanotic mass formation. (A) Melanotic masses in larvae homozygous for the deletion allele Rabex-5ex42 (arrows; anterior, top). (B) At least one melanotic mass was seen in 3.8% of Rabex-5ex42 larvae compared to 0% in control larvae 6 days AEL. Incidence of melanotic masses in Rabex-5ex42 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-5ex42 background (Rabex-5ex42; srp-GFP, Rabex-5WT and Rabex-5ex42; He>GFP, Rabex-5WT) decreased the incidence of melanotic masses compared to those in controls (Rabex-5ex42; srp-GFP and Rabex-5ex42; He>GFP). (E) Expressing dap using either He-gal4 or srp-gal4 in a Rabex-5ex42 background (Rabex-5ex42; He>GFP, dap and Rabex-5ex42; srp-GFP, dap) decreased the incidence of melanotic masses compared to those in controls (Rabex-5ex42; He>GFP and Rabex-5ex42; srp-GFP) 14 days AEL. *P<0.05, *P<0.01.
directed Rabex-5 expression and He-gal4 or srp-gal4 directed dap expression decreased larval lethality (Fig. S1A,B). This suggests hemocyte overproliferation also contributes to Rabex-5-null larval lethality.

**Homzygous 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. 2Aiii, iv) compared to that in 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 the lymph gland area in control larvae (srp>dap) but restored the lymph glands of Rabex-5-null larvae to wild-type size (Rabex-5<sup>ex42/ex42</sup>; 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 120 h 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 might 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 does not result from increased hemocyte proliferation.

To determine whether loss of Rabex-5 affects other hemocyte lineages, we examined crystal cell and plasmatocyte populations. We utilized Bc<sup>1</sup>, an allele of Black cells (Bc) that causes spontaneous melanization of crystal cells, to visualize crystal cells in vivo. Rabex-5-null larvae (Bc<sup>1</sup>; Rabex-5<sup>ex42/ex42</sup>) showed a marked increase in the number of melanized crystal cells compared to control larvae (Bc<sup>1</sup>; Fig. 2G). The percentage of melanized crystal cells in the hemolymph increased with decreasing levels of Rabex-5 (Fig. 2H). A similar ~1.5-fold increase in the percentage of crystal cells upon loss of Rabex-5 was confirmed by using an antibody against lozenge, a transcription factor required for crystal cell specification (Lebestky et al., 2000), and by using heat to induce melanization of crystal cells in vivo (Fig. S2E,F). Excess crystal cells might reflect overproliferation and release from the sessile compartment or transdifferentiation from plasmatocytes (Leitao and Sucena, 2015). The percentage of plasmatocytes present in the hemolymph, which were detected by using a mixture of P1a and P1b antibodies (Kurucz et al., 2007), decreased in Rabex-5-null larvae (Rabex-5<sup>ex42/ex42</sup>; srp>GFP and Rabex-5<sup>ex42/ex42</sup>; He>GFP) compared to controls (srp>GFP and He>GFP, Fig. 2I and Fig. 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 might explain the decrease in circulating plasmatocytes (Honti et al., 2010; Krzemien et al., 2010; Stofanko et al., 2010). Alternatively, loss of Rabex-5 might 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 mRNA 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 performed RNA interference (RNAi) of Rabex-5 by using srp-gal4 and an inducible inverted repeat allele, Rabex-5<sub>IR</sub>, we characterized previously (Yan et al., 2010). Surprisingly, reducing Rabex-5 levels by 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 insufficient to increase hemocyte concentration (Fig. 3C), it was sufficient to alter circulating hemocyte proportions. Compared to that of controls, RNAi of Rabex-5 in hemocytes increased the percentage of GFP-positive hemocytes in circulation (Fig. 3D) to an extent similar to that observed in Rabex-5-null larvae (Fig. S2C). RNAi of Rabex-5 increased the percentage of circulating crystal cells (melanized cells, Fig. 3E) to an extent similar to that seen in Rabex-5 heterozygous larvae (Fig. 2H). The basement membrane of lymph glands, marked by Trol expression, remained intact upon loss of Rabex-5 (Fig. S3); the increased percentage of circulating hemocytes did not result from rupture or emptying of the lymph gland. In contrast, RNAi of Rabex-5 did not significantly increase the percentage of plasmatocytes in circulation compared to that of controls (P1a/ P1b-positive cells, Fig. 3F). These data indicate an intrinsic requirement for Rabex-5 in the hematopoietic system in order to prevent melanotic masses, restrict proliferation in the primary lymph gland and maintain appropriate proportions of hemocytes in the hemolymph.

Hematopoiesis in Drosophila occurs in two waves. To determine whether Rabex-5 is required to maintain hematopoietic balance in the embryonic wave, the larval wave or both, we used croquemort-gal4 (crg-gal4) to perform RNAi of Rabex-5 specifically in hemocytes of embryonic origin (Fig. 1C, Table S1). Rabex-5 knockdown by using crq-gal4 (crg>Rabex-5<sup>IR</sup>) 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 those in controls (crg>GFP).
Similarly, we used domeless-gal4 (dome-gal4) to reduce Rabex-5 specifically in hemocytes of larval origin. Rabex-5 knockdown by using dome-gal4 (dome-Rabex-5IR) increased the area of the primary lobes (Fig. 3I) and increased crystal cell numbers (Fig. 3J) compared to those in controls (dome>GFP). These results suggest that Rabex-5 is required during each wave of hematopoiesis but may have developmental stage-specific functions.

Fig. 2. See next page for legend.
Fig. 2. Loss of Rabex-5 causes a range of hematopoietic abnormalities. (A) Wild-type (He>GFP, i; i) and Rabex-5ex42/ex42 (Rabex-5ex42/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. The strong anterior signal is GFP fluorescence in Rabex-5 of mass formation (Fig. 4D), lamellocyte differentiation (Fig. 4E) and wing area to those of controls, and largely suppressed the hematopoietic phenotypes, we reduced gene dosage or full-length Rabex-5 (Melanized cells, Fig. 4G), suggesting that melanotic mass formation, lamellocyte differentiation and increased numbers of crystal cells are not the result of increased Ras activity.

Rabex-5 knockdown, however, was sufficient to increase the percentage of circulating crystal cells (Fig. 3E). Given the instructive role of Notch signaling in crystal cell specification (Duvic et al., 2002; Lebestky et al., 2003; Small et al., 2007), we further investigated the involvement of Notch. Encouragingly, genetic modification of certain Notch signaling components changed 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 (Ser), Serdβ, 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, and enhanced by Notch duplication (DpN).

Reduction of 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 to cause specification and differentiation defects, such as ectopic wing veins and eye/antennal transformations (Yan et al., 2010). These phenotypes are sensitive to Ras activity; reducing the gene dosage of Ras restored body size and wing area to those 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 whether 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 by using the loss-of-function allele Ras1 proficient suppressed larval lethality in Rabex-5-null larvae (Rabex-5ex42/ex42; Ras1+/–; Fig. 4A) and restored the size of the lymph gland (Fig. 4B). To restore Rabex-5 E3 ligase function, we used He-gal4 in order to express either Rabex-5CR or full-length Rabex-5 with an intact E3 ligase domain and an inactive Rab5 GEF domain (Rabex-5ΔT) that had been characterized previously (Yan et al., 2010). Expressing Rabex-5ΔT suppressed larval lethality in a Rabex-5-null background (Fig. 4C, Fig. S1A).

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

Reducing the Ras gene dosage did not suppress melanotic mass formation (Fig. 4D), lamellocyte differentiation (Fig. 4E) or the phenotype of increased crystal cell numbers (Fig. 4F) in Rabex-5-null larvae. Furthermore, expressing constitutively active Ras12T did not increase the percentage of circulating crystal cells (melanized cells, Fig. 4G), suggesting that melanotic mass formation, lamellocyte differentiation and increased numbers of crystal cells are not the result of increased Ras activity.

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; Small et al., 2014). The primary lymph gland lobes contain distinct zones: the medullary zone (MZ) comprising slowly proliferating prohemocytes, the cortical zone (CZ) containing differentiating hemocytes and a small cluster of cells called the posterior signaling center (PSC), which controls the balance of prohemocytes and differentiating hemocytes. We investigated Notch dysregulation upon Rabex-5 knockdown within the primary lobes by 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,D, Table S1). In control larvae, Notch antibody staining within the MZ was moderate and uniform. This was easily discernable from intense and heterogeneous Notch antibody staining within 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 by using srp-gal4 (srp>Rabex-5ΔT) 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 percentage 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>Ras12T). Ras12T did not significantly alter the percentage of lymph glands displaying differential Notch staining (Fig. 5C,D) but significantly decreased the lymph gland area (Fig. 5E). Surprisingly, Ras12T expression decreased Notch fluorescence intensity (Fig. 5F) of the primary lobe. This suggests that increased Ras activity is not sufficient to promote Notch signaling in the lymph gland.
Fig. 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-5*iso) 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-5*iso) increased the area of the primary lymph gland lobes compared to those in controls (srp>GFP) 4 days AEL. DAPI staining is shown in blue. Scale bars: 50 μm. (C) *Rabex-5* RNAi (srp>GFP, *Rabex-5*iso) did not change circulating hemocyte concentrations compared to those in controls (srp>GFP) 120 h AEL. (D) *Rabex-5* RNAi in hemocytes (srp>GFP, *Rabex-5*iso) increased the percentage of circulating GFP-positive hemocytes compared to that of controls (srp>GFP) 120 h AEL. (E) *Rabex-5* RNAi in hemocytes in a *Bc*iso heterozygous background (Bciso; srp>GFP, *Rabex-5*iso) increased the percentage of melanized crystal cells in the hemolymph compared to that of controls (Bciso; srp>GFP) 120 h AEL. (F) *Rabex-5* knockdown by using srp-gal4 (srp>GFP, *Rabex-5*iso) did not change the percentage of circulating plasmatocytes compared to that of controls (srp>GFP) 120 h AEL. (G) *Rabex-5* RNAi in embryonic hemocytes (crq>GFP, *Rabex-5*iso) increased the area of the primary lymph gland lobes compared to that in controls (crq>GFP) 5 days AEL. (H) *Rabex-5* RNAi in embryonic hemocytes in a *Bc*iso heterozygous background (Bciso; crq>GFP, *Rabex-5*iso) did not alter the percentage of melanized crystal cells in the hemolymph compared to that in controls (Bciso; crq>GFP) 120 h 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*iso) increased the area of the primary lobes 4 days AEL and increased crystal cell numbers visualized by heating larvae (J) compared to those of controls (dome>GFP). *P < 0.01.
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 dome-gal4 or exclusively in the PSC using antennapedia-gal4 (antp-gal4) (Fig. 6A, Table S1). A significant increase in Notch intensity across the entire lymph gland was seen upon RNAi of Rabex-5 in the MZ (dome>Rabex-5IR, Fig. 6B). Per lobe, the average number of cells with strong Notch expression increased, even when larvae were raised at 18°C to minimize the effect of RNAi (Fig. 6C). Similar to Rabex-5 reduction in the entire primary lobe, Rabex-5 reduction exclusively in the MZ promoted Notch accumulation, 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 by using dome-gal4 did not significantly alter the average number of cells per lobe that strongly express Notch (dome>RasV12, Fig. 6C).

RNAi depletion of Rabex-5 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). RNAi of Rabex-5 in the PSC did not increase the average number cells per lobe, Rabex-5 reduction exclusively in the MZ promoted Notch accumulation, 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 by using dome-gal4 did not significantly alter the average number of cells per lobe that strongly express Notch (dome>RasV12, Fig. 6C).

\[ P \leq 0.05, \ast P \leq 0.01. \]
lobe that strongly express Notch (Fig. 6F). These results indicate that \textit{Rabex-5} functions in the prohemocytes of the MZ, but not in the PSC, to prevent Notch accumulation. Given that \textit{Rabex-5} knockdown in embryonic hemocytes did not affect crystal cell numbers (Fig. 3H), we assessed the requirement for \textit{Rabex-5} to regulate Notch specifically in hemocytes of embryonic origin. \textit{Rabex-5} knockdown in embryonic hemocytes (\textit{crq}>\textit{Rabex-5}RNAi) increased Notch expression in the MZ compared to that in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). \textit{RasV12} expression (\textit{crq}>\textit{RasV12}) did not increase Notch expression in the MZ. Scale bars: 50 μm. (D) \textit{Rabex-5} RNAi, but not \textit{RasV12}, in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{Rabex-5}RNAi and \textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{RasV12}) decreased the percentage of lymph glands in which the MZ and CZ are discernible by Notch expression compared to that in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). \textit{Rabex-5} RNAi in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{Rabex-5}RNAi) increased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to those in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). \textit{RasV12} in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{RasV12}) decreased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to those in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). Lymph glands were dissected 4 days AEL. *P≤0.05, **P≤0.01.

Fig. 5. Loss of \textit{Rabex-5} leads to Ras-independent dysregulation of Notch protein across the primary lymph gland lobes. (A–C) (A–C,A″–C″) Expression of EBFP2 (traced in white) marked the medullary zone (MZ) of the primary lymph gland. (A) In control larvae (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}) Notch expression was low in the MZ and distinct from the high expression in the outer, cortical zone (CZ). (B) \textit{Rabex-5} RNAi in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{Rabex-5}RNAi) increased Notch expression in the MZ compared to that in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). (C) Expressing \textit{RasV12} in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{RasV12}) did not increase Notch expression in the MZ. (D) \textit{Rabex-5} RNAi, but not \textit{RasV12}, in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{Rabex-5}RNAi and \textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{RasV12}) decreased the percentage of lymph glands in which the MZ and CZ are discernible by Notch expression compared to that in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). \textit{Rabex-5} RNAi in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{Rabex-5}RNAi) increased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to those in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). \textit{RasV12} in hemocytes (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}, \textit{RasV12}) decreased the area (E) and the Notch fluorescence intensity (F) of the primary lobes compared to those in controls (\textit{Dome-meso-EBFP2}/+; \textit{srp}>\textit{GFP}). Lymph glands were dissected 4 days AEL. *P≤0.05, **P≤0.01.

Notch accumulation upon \textit{Rabex-5} loss leads to increased transcriptional outputs
To establish whether Notch protein accumulation results in increased transcriptional activity, we examined the effect of \textit{Rabex-5} knockdown on a Notch transcriptional reporter, 12x\textit{Su(H)bs-lacZ} (Go et al., 1998). Reducing \textit{Rabex-5} across the primary lymph gland lobes (\textit{srp}>\textit{Rabex-5}RNAi) increased β-
galactosidase (β-gal) fluorescence intensity compared to that in 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 leads to functionally increased Notch transcriptional activity. RasV12 expression (srp>RasV12) 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 whether the increased Notch activity is functionally relevant to Rabex-5 hematopoietic phenotypes, we performed genetic interactions by using the Notch pathway components Notch, Delta and Serrate (Fig. 8A-C, summarized in 8D), or performing RNAi of Notch (Fig. 8E). Reducing Delta gene dosage using the Df3 allele suppressed larval lethality in a Rabex-5-null background. Notch duplication (DpN) enhanced larval lethality. Surprisingly, larval lethality was also enhanced by the SerAd3 allele (Fig. 8A,D), which produces a protein that lacks the intracellular and transmembrane domains but retains the Notch binding domain (Hukriede and Fleming, 1997). If this truncated Serrate is able to activate Notch in some contexts, it might modify Rabex-5-null phenotypes similar to Notch duplication. Similarly, Df3 suppressed the Rabex-5-null melanotic mass phenotype, whereas DpN and SerAd3 enhanced the phenotype (Fig. 8B,D). Df3 suppressed lamellocyte differentiation in Rabex-5-null larvae, DpN dramatically enhanced lamellocyte differentiation, and SerAd3 had no effect (Fig. 8C,D). RNAi of Notch, by using the 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 differentiation 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, to direct or prevent differentiation into specific lineages, and to maintain hematopoietic homeostasis.

Fig. 8. Rabex-5 negative regulation of Notch is required for proper regulation of hematopoiesis during development. (A) In a Rabex-5ex42ex42 background, DpN and SerBd-3 (Rabex-5ex42ex42; DpN/+ and Rabex-5ex42ex42; SerBd-3/) increased larval lethality compared to that in controls (Rabex-5ex42ex42). Dl7 (Rabex-5ex42ex42; Dl7/) suppressed larval lethality. (B) In a Rabex-5ex42ex42 background 14 days AEL, DpN and SerBd-3 (Rabex-5ex42ex42; DpN/+ and Rabex-5ex42ex42; SerBd-3/) increased the incidence of melanotic masses and Dl7 (Rabex-5ex42ex42; Dl7/) decreased the incidence of melanotic masses compared to those in controls (Rabex-5ex42ex42). (C) In a Rabex-5ex42ex42 background 6 days AEL, DpN (Rabex-5ex42ex42; DpN/) increased the percentage of larvae with lamellocytes compared to that in controls (Rabex-5ex42ex42). Dl7 (Rabex-5ex42ex42; Dl7/) decreased the percentage of larvae with lamellocytes. SerBd-3 (Rabex-5ex42ex42; SerBd-3/) did not alter the percentage of larvae with lamellocytes compared to that in controls (Rabex-5ex42ex42). (D) Summary of Rabex-5ex42ex42 genetic interactions with Notch, Delta and Serrate. (E) Notch RNAi in hemocytes (srp>GFP, N^R) did not affect the area of the primary lymph gland lobes at 21°C but reduced the enlarged lymph glands (srp>GFP, N^R, Rabex-5^R) resulting from Rabex-5 RNAi (srp>GFP, Rabex-5^R) to control area (srp>GFP) 7 days AEL. DAPI staining is shown in blue. Scale bars: 50 μm; *P<0.05, *P<0.01.
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 almost all *Rabex-5*-null phenotypes. This indicates 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 that of wild-type control BMCMCs (Tam et al., 2004). These similarities suggest that the function of *Rabex-5* within the hematopoietic system is conserved in mammals.

We provide evidence that *Rabex-5* restricts both Ras and Notch signaling in order 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 that results 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* might 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 activity of Notch but not Ras. 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 through its regulation of Ras in the hematopoietic system. Constitutively active Ras expression did not phenocopy the effect of *Rabex-5* loss in the lymph gland and was insufficient 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 to ensure hematopoietic homeostasis; this dual role might 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 that require 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 might implicate Delta in *Drosophila* hematopoiesis. Except in the control of 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 through 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 that the reduction of Delta gene dosage in *Rabex-5*-null larvae suppressed lethality and melanotic masses, both of which are phenotypes that depend on hemocyte proliferation. A dominant-negative Serrate allele suppressed the increase in crystal cell numbers in a *Rabex-5*-null background, whereas any suppression mediated by the reduction of Delta was subtle. One interpretation of these findings is that Notch activation via Delta affects hemocyte proliferation, whereas Notch activation through Serrate affects hemocyte differentiation.

Our findings 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 (AMLs) revealed that two-thirds of AML cases in which *Rabex-5* mRNA is downregulated show upregulation of Notch, Delta or Jagged2 (a mammalian Serrate ortholog) mRNA (Cerami et al., 2012; The Cancer Genome Atlas Research Network, 2013; Gao et al., 2013). However, there are conflicting reports on the role of Notch signaling in AMLs, which might 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). The status of *Rabex-5* might help to further define subsets of AML, and might provide tremendous opportunities to elucidate the etiology – and inform on the treatment – of human leukemia.

MATERIALS AND METHODS

*Drosophila*
Flies were raised at 25°C on standard medium unless otherwise stated. Fly genotypes are listed in Table S2.

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 hatching.
Circulating hemocyte concentrations

Flies were permitted to lay eggs for 2 h. Control larvae were evaluated 120 h AEL. Experimental larvae were evaluated 120 h, 9 days and 14 days AEL. Hemolymph from each individual larva was collected in 20 μl of PBS and kept on ice. Hemocyte concentration was measured in cells/ml using a Countess Automated Cell Counter from Invitrogen and plotted as relative concentration. Minimum and maximum cell sizes were set to 2 μm and 22 μm, 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 min 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 h. 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 min on ice, washed three times with PBS, and permeabilized in PBS with 0.1% Tween-20 and 5% BSA for 20 min at RT. Immunohistochemistry

Antibodies were diluted in 0.1% Tween-20 in PBS with 5% natural goat serum, natural donkey serum or BSA. P1a/P1b-positive, lozenge-positive or melanized cells by the number of DAPI-positive cells.

Microscopy

Larvae with melanotic masses (Fig. 1A) and melanized crystal cells (Figs 2G, 3H,3,J) and 4H, Fig. 2F) 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 Axios 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, Fig. S3), constrained iterative deconvolution was applied to all lymph gland images prior to analysis of primary lobes (Figs 3B, 5A-F, 6B-G, 7A-D and 8E, Fig. S2A). All lymph gland images are presented as a single maximum-intensity projection.

Statistical analysis

Student’s unpaired t-tests compared lymph gland areas, hemocyte concentrations and fluorescence intensities. Error bars represent ±s.e.m. P values are indicated in the figure panels; *P<0.05 and **P<0.01. For melanized cell experiments, n≥26 larvae of each genotype were scored. For quantification of hemocyte numbers, n≥6 larvae of each genotype were scored. For fluorescence intensity measurements, n≥8 larvae of each genotype were scored. For heat-induced melanization experiments, n≥11 larvae of each genotype were scored. For larval lethality experiments, n≥20 larvae of each genotype were scored. For melanotic mass experiments: n≥20 with the exception of flies carrying DopN, n=9. Data shown are representative results from reproducible experiments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.M.P. and T.A.R. contributed to study design, data analysis and interpretation, and preparation of the article. T.A.R. performed all of the experiments shown in the figures.

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Supplementary information

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