Charting ESCRT function reveals distinct and non-compensatory roles in blood progenitor maintenance and lineage choice in *Drosophila*

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Abstract

Tissue heterogeneity permits diverse biological outputs in response to systemic signals but requires context-dependent spatiotemporal regulation of a limited number of signaling circuits. In addition to their stereotypical roles of transport and cargo sorting, endocytic networks provide rapid, adaptable, and often reversible means of signaling. Aberrant function of the Endosomal Sorting Complex Required for Transport (ESCRT) components results in ubiquitinated cargo accumulation, uncontrolled signaling and neoplastic transformation. However, context-specific effects of ESCRT on developmental decisions are not resolved. By a comprehensive spatiotemporal profiling of ESCRT in *Drosophila* hematopoiesis *in vivo*, here we show that pleiotropic ESCRT components have distinct effects on blood progenitor maintenance, lineage choice and response to immune challenge. Of all 13 core ESCRT components tested, only Vps28 and Vp36 were required in all progenitors, whereas others maintained spatiotemporally defined progenitor subsets. ESCRT depletion also sensitized posterior progenitors that normally resist differentiation, to respond to immunogenic cues. Depletion of the critical Notch signaling regulator Vps25 did not promote progenitor differentiation at steady state but made younger progenitors highly sensitive to wasp infestation, resulting in robust lamellocyte differentiation. We identify key heterotypic roles for ESCRT in controlling Notch activation and thereby progenitor proliferation and differentiation. Further, we show that ESCRT ability to regulate Notch activation depends on progenitor age and position along the anterior-posterior axis. The phenotypic range and disparity in signaling upon depletion of components provides insight into how ESCRT may tailor developmental diversity. These mechanisms for subtle control of cell phenotype may be applicable in multiple contexts.

Keywords

Blood cell homeostasis, Endosomal protein sorting, ESCRT, Progenitor homeostasis, Lineage choice, Ubiquitination, NICD, Notch signaling, Crystal Cell, Progenitor heterogeneity, Lamellocytes

Significance

The Endosomal Sorting Complex Required for Transport (ESCRT) machinery sorts ubiquitinated cargo for degradation or recycling. Aberrant ESCRT function is associated with many blood disorders. We did a comprehensive functional analysis of all 13 core ESCRT components in maintenance and differentiation of *Drosophila* larval blood progenitors. We show that ESCRT have diverse and non-compensatory functions in blood progenitors. ESCRT depletion from progenitors affects ubiquitination status cell autonomously and independent of progenitor maintenance. ESCRT function is more critical to maintain older progenitors and to prevent Notch-dependent crystal cell differentiation. Further, ESCRT depletion sensitizes refractile younger progenitors for lamellocyte differentiation. Our *in situ* developmental map of ESCRT function reveals critical checkpoints for cell fate choice and new paradigms for generating progenitor heterogeneity.

Introduction

Tissue patterning requires spatiotemporally controlled cell proliferation, progenitor specification and lineage differentiation. While a limited number of signaling circuits impact these complex cell properties, their context-dependent regulation elicits multiple diverse biological outputs. Endocytic trafficking can maintain, attenuate or amplify signaling to regulate inter or intracellular communication [1]. In addition to their stereotypical roles of transport and cargo sorting, endocytic networks can regulate the behaviour of individual cells as well as groups or collectives, thereby significantly impacting tissue homeostasis.

Protein trafficking and turnover through the endolysosomal route allows rapid post-translational adjustment of signal transduction efficiency. The conserved Endosomal Sorting Complex Required for Transport (ESCRT) actively controls the sorting of ubiquitinated cargoes for lysosomal degradation. This hetero-multimeric complex consists of four subunits (ESCRT-0, I, II and III) that are sequentially recruited onto endomembrane-bound ubiquitinated cargoes, allowing them to be sequestered in the intraluminal vesicles (ILV) of the multivesicular bodies (MVB). The initial steps of recognition and binding of ubiquitinated cargoes, converge into the subsequent crucial steps of membrane remodelling during ILV formation. ESCRT components act in a co-operative manner, following defined molecular stoichiometry, to successfully sequester cargoes for degradation. Hence, phenotypic diversity of ESCRT mutants is rare in unicellular organisms like budding yeast. However, dysfunction of metazoan ESCRT components can manifest as distinct and diverse cellular and histological phenotypes such as defective MVB biogenesis and incorrect cell fate choice, tissue hyperproliferation, apoptotic resistance, etc due to aberrant activation of signaling pathways such as Notch, EGFR and JAK/STAT that alter tissue homeostasis[2-5].

Blood progenitors are exposed to a plethora of signals and need to respond to a rapidly changing environment for stem- and progenitor cell maintenance and controlled differentiation. Previous genetic screens and knockout-based functional analyses in both *Drosophila* and mouse models showed a role of ESCRT in maturation of specific blood cell types in erythroid and lymphoid lineages and a possible functional link of ESCRT to blood cell homeostasis [6-8]. Several key endosomal proteins such as Rabex5, Atg6, Rab5 and Rab11 actively control endocytic trafficking and are implicated in developmental signaling and blood cell homeostasis [9-13]. The blood cell-enriched conserved endosomal regulator of hematopoiesis, Asrij interacts with ADP Ribosylation Factor 1 (ARF1-GTP), regulates the endocytic proteome and maintains stemness of blood progenitors in the *Drosophila* lymph gland [14]. Loss of Asrij promotes activation of Notch signaling in hematopoiesis, thereby leading to precocious differentiation to crystal cells [14]. Also, *asrij* mutant hemocytes accumulate

Notch intracellular domain (NICD) in the sorting endosomes, mimicking an endosomal sorting defect. Mouse Asrij also maintains hematopoietic stem cell quiescence [15]. This suggests conserved mechanisms of endosomal protein sorting in blood cell homeostasis that merit further investigation.

The *Drosophila* larval lymph gland serves as a simple yet powerful model to study conserved mechanisms of hematopoiesis *in situ*. Using this *in vivo* model, we investigated the role of ESCRT components in spatiotemporal control of blood progenitor homeostasis and myelopoiesis. Blood progenitors reside in the multi-lobed lymph gland that flanks the cardiac tube in segments T3 and A1. The primary lobe of the lymph gland comprises of three distinct zones enriched in progenitors, differentiated blood cells (plasmacytes, crystal cells and lamellocytes) and the hematopoietic niche. Blood progenitors of *Drosophila* are linearly arranged in anterior (primary) and posterior lobes (secondary, tertiary and often quaternary) of the lymph gland and are characterized by the expression of several markers such as Domeless, TepIV and DE-Cadherin. The lymph gland develops in an anterior to posterior sequence, with younger progenitors in the posterior lobes [16]. Previous studies showed that the progenitor population is heterogeneous in gene expression, mitochondrial morphology and dynamics, signaling, differentiation potential and immune function [16, 17]. Posterior progenitors are refractile to immune challenge due to immature mitochondrial morphology and differential activation of JAK/STAT and Notch signaling [16, 17]. The lymph gland harbors the entire blood progenitor population of *Drosophila*, and hence allows complete sampling and a comprehensive study of progenitor homeostasis.

The *Drosophila* ESCRT is comprised of 13 core components [2, 18]. ESCRT-0 (Hrs, Stam) binds to the ubiquitinated cargoes through a ubiquitin-interacting motif. It then recruits ESCRT-I (Vps28, Tsg101, Vps37A, Vps37B) and ESCRT-II (Vps25, Vps22 and Vps36), which act as a bridging complex to assemble ESCRT-III (Vps32, Vps24, Vps20, Vps2). ESCRT-I-dependent membrane inward budding (negative curvature) and ESCRT-III-dependent membrane scission lie at the heart of endosomal protein sorting, resulting in the formation of intraluminal vesicles (ILV) containing the sequestered cargo [19]. Vps32 is the principal filament-forming component that undergoes activation and polymerization upon binding with various nucleating factors and integrates previous steps of endosomal sorting [5]. The final step involves disassembly of ESCRT subunits and scission of the membrane neck of the intraluminal vesicles, which is mediated by the Vps4-Vta1 mechanoenzyme complex.

Here, we provide a functional map of the role of all 13 *Drosophila* core ESCRT components in ubiquitinated cargo sorting and blood cell lineage choice across distinct progenitor subsets. We show that though ESCRT components are expressed in all cells of the lymph gland (LG), their roles in controlling lineage-specific differentiation and immune response of blood progenitors are distinct and

position-dependent. We also find that ESCRT dysfunction primarily affects Notch activation-dependent crystal cell differentiation. Our study supports heterogeneity of blood progenitors and highlights the role of ESCRT in spatiotemporal segregation of signaling.

Materials and methods

Fly stocks and genetics

Drosophila melanogaster stocks were maintained at 25°C as described previously [14]. The details of fly stocks, genetics and control genotypes used are in supplementary methods.

Immunostaining analysis

Drosophila third instar larval lymph glands were dissected in PBS as described before and immunostained for microscopic analysis [20]. The detailed protocol and reagents used are in supplementary methods.

Wasp parasitism assay

Wasp infestation was performed following standardised protocol as described in Rodrigues et al., 2021a [16]. Details are mentioned in supplementary methods.

Quantification and statistical analysis

Blood cell differentiation was quantified as described in Ray et al., 2021 [17]. The details of quantification of various parameters and statistical analysis are mentioned in supplementary methods.

Results

Divergent requirement for ESCRT components in ubiquitinated cargo sorting in the *Drosophila* lymph gland.

To explore whether ESCRT function may determine blood progenitor identity or potential, we depleted each of the 13 core ESCRT components individually in the lymph gland by RNAi mediated knockdown (KD) in domeless (dome) expressing blood progenitors (*domeGal4>UAS ESCRT RNAi*). As ESCRT plays an active role in ubiquitinated cargo sorting, the accumulation of ubiquitinated cargoes serves as a hallmark of dysfunctional ESCRT machinery and impaired endosomal protein sorting. A comprehensive analysis of conjugated ubiquitination (Ub) status (see methods) in the primary, secondary and tertiary lobes by immunostaining the complete lymph gland showed a range of effects with the phenotype varying among ESCRT components within a given ESCRT complex and between complexes.

Control LG showed low or no Ub in primary, secondary, and tertiary lobes (Fig 1A, B). A similar trend was seen on depletion of ESCRT-0 components Hrs or Stam, with an occasional increase in Ub in primary lobes, which was not significant (Figure 1A, B). In contrast ESCRT-I, -II and -III depletion had effects on all lobes, though not all components affected the Ub status. Among ESCRT-I components (Vps28, Tsg101, Vps37A, Vps37B), depletion of Vps28 or Tsg101 very significantly increased Ub in the primary lobe, Vps28 and Vps37A affected the secondary lobe and Vps37A showed an increase in Ub in the tertiary lobe. Vps37B depletion had a mild non-significant effect on the Ub status of the LG (Figure 1A, B). ESCRT-II components Vps25, Vps22 and Vps36 had no effect on the primary lobe. However, Vps22 depletion caused a dramatic increase in Ub in the secondary and tertiary lobes, where Vps25 and Vps36 depletion had no effect. Finally, depletion of ESCRT-III components (Vps32, Vps20 and Vps2) caused a significant increase in Ub in the primary lobes whereas secondary and tertiary lobes were sensitive only to Vps20 depletion.

In summary, our data indicates non-uniform response of progenitors to perturbation of the cargo sorting machinery, depending on the ESCRT component that is depleted as well as the target progenitor population (Figure 1B schematic). Of the 13 core ESCRT components, 7 caused increased Ub in the LG when depleted. Interestingly, the effects were not uniform amongst progenitor subsets - 5 affected Ub status in the primary lobes, 4 in the secondary lobes and 3 in the tertiary lobes. This is in agreement with the anterior-posterior developmental and functional heterogeneity of progenitors reported earlier [16] and suggests that younger progenitors have a reduced requirement for ESCRT function. Thus, our analysis provides a spatiotemporal correlation of Ub status to ESCRT depletion in

LG progenitor subsets. We next tested whether this correlation reflects the response of progenitors to maintenance and differentiation cues.

ESCRT components play distinct roles in lymph gland progenitor maintenance.

As ESCRT components regulate ubiquitinated cargo sorting in the blood progenitors, they might potentially regulate progenitor homeostasis. Hence, we checked whether depleting the 13 ESCRT components individually from dome⁺ progenitor subsets (marked by GFP expression) (*domeGal4>2XEGFP/+; UAS ESCRT-RNAi/+; +/+*) may affect progenitor status similar to the effect on Ub accumulation. As in the case of Ub status, a comprehensive analysis of progenitor fraction (see methods) in the primary, secondary, and tertiary lobes of the lymph gland showed a range of effects with the phenotype varying among ESCRT components within a complex and between complexes (Figure 2A, B). Assessment of mitotically active nuclei and cell counts showed that ESCRT affects proliferation of blood progenitors (Fig S1A-C; see supplementary results).

In controls, anterior lymph gland lobe progenitors are restricted to the inner medullary zone (MZ) while the posterior lobes are composed almost entirely of progenitors [16]. ESCRT-0 (Hrs, Stam) knockdown did not show any significant change in progenitor status indicating non-essential roles for these in progenitor maintenance (Fig 2A, B). Depletion of ESCRT-I components Vps28 and Tsg101 caused reduction in progenitor fraction in the primary lobes whereas secondary lobe progenitors were reduced by depletion of Vps28, Vps37A or Vps37B but not of Tsg101. Interestingly, ESCRT-I components had no effect on tertiary lobe progenitors. Among ESCRT-II components, Vps25 had an effect on proliferation causing an absolute increase in primary lobe cell numbers with a concomitant decrease in progenitor fraction (Fig 2A, B; S1A, C; S6; Supplementary results). Vps22 also did not affect LG progenitor fraction. In contrast, Vps36 drastically reduced progenitor fraction in all LG lobes, with phenotype severity increasing from anterior to posterior. ESCRT-III had very restricted effects on progenitors with Vps32 KD causing a reduction only in anterior progenitors, Vps2 KD reduced both anterior and posterior progenitors and Vps24 and Vps20 had no effect (Fig. 2A-C).

Since increased Ub accumulation indicates dysfunctional cargo sorting and this is likely a cause of progenitor loss, we compared Ub status on ESCRT KD with progenitor maintenance. Superimposition of the phenotype chart for each of these (Fig 6B, C) showed that there was no absolute correlation between increased Ub and reduced progenitor numbers in older (primary lobe) or younger (posterior lobes) progenitors. While knockdown of some ESCRT components [(Vps28, Tsg101 (ESCRT-I), Vps32 and Vps2 (ESCRT-III)] caused increased Ub and reduced progenitors in the primary lobe, others

[(Vps25, Vps36 (ESCRT-II)) showed no change in Ub but progenitor numbers were reduced. Similarly, knockdown of Vps20 (ESCRT-III) had increased Ub but no effect on progenitors. Hence in addition to Ub cargo sorting, ESCRT components Vps25 and Vps36 have a critical independent role in progenitor maintenance. Notably, there was no correlation at all between Ub increase and progenitor reduction in the youngest progenitors (tertiary lobe). This suggests that notwithstanding defects in cargo sorting, posterior progenitors remain less sensitive to perturbation, indicating that they are maintained by other robust mechanisms.

Older progenitors are more prone to plasmatocyte differentiation upon ESCRT depletion

Plasmatocytes, marked by P1 expression, make up about 95% of the differentiated hemocyte population. In the LG, they are restricted to the cortical zone of the primary lobe, with occasional P1 positive cells seen in posterior lobes. Reduced progenitor numbers are expected to be accompanied by an increase in the plasmatocyte population due to differentiation. Enumeration of P1 positive cells in the ESCRT KD LG (*domeGal4 UAS 2XEGFP > UAS ESCRT RNAi*) showed an expected increase in the plasmatocyte fraction of the primary lobe for Vps28, Tsg101, Vps36 and Vps32, where the progenitor fraction was mostly reduced (Fig 2A, C) However, Vps25 depletion had no apparent effect on differentiation. This could be due to the failure of progenitors to terminally differentiate into plasmatocytes or due to non-autonomous overproliferation of the intermediate population. Additionally, Vps22 KD also showed increased plasmatocyte numbers though there was no significant effect on the progenitor fraction, suggesting possible non-autonomous overproliferation or exhaustion of the intermediate population. The remaining ESCRT components had no effect on primary lobe plasmatocytes. Interestingly, KD of ESCRT-0 component Hrs and ESCRT-III component Vps32, that had no effect on progenitors, caused an increase in plasmatocyte numbers only in the secondary lobes. This indicates that though there is no effect as assessed by progenitor marker analysis, Hrs or Vps32 depletion has sensitized the tissue to respond to proliferation and differentiation cues. Along similar lines, Vps36 and Vps2 depletion drastically reduced the secondary progenitor fraction and increased plasmatocyte differentiation. Except for Vps28, ESCRT KD did not induce plasmatocytes in the tertiary lobes, even when progenitors were lost (e.g. Vps36 KD and Vps2 KD).

ESCRT components are most effective in suppressing the crystal cell lineage.

Crystal cells make up only about 5% of the differentiated hemocyte pool. Under steady state conditions each primary lobe harbors approximately 0-10 crystal cells, while they are generally absent from posterior lobes, even upon immune challenge [16]. As seen earlier, 8 of the 13 core ESCRT components affect progenitor numbers in one or more lobes (Fig 3A, B). Further these effects are position-dependent. Hence, we next analysed crystal cell status by checking expression of ProPO in the ESCRT depleted LGs.

Knockdown of 12 out of 13 ESCRTs increased crystal cell differentiation in the primary lobes. Vps25 depletion in the progenitors had no effect on crystal cell differentiation. Secondary lobes were sensitive to depletion of Hrs, Vps28, Vps36 and Vps2 showing increased crystal cell numbers in all cases. Tertiary lobes showed increase in crystal cell numbers only on Vps28 or Vps36 depletion. This suggested that cargo sorting is critical to regulate crystal cell differentiation. Perturbation in the ESCRT machinery results in activation of signaling pathways that promote crystal cells. Since Notch pathway activation is a key requirement of crystal cell differentiation and Notch is a well-known target of ESCRT-mediated cargo sorting [3, 21-23], we next checked for the status of Notch pathway activation upon ESCRT depletion.

ESCRT depletion in blood progenitors ectopically activates Notch signaling.

Increased crystal cell differentiation upon depletion of some ESCRT components suggests that they normally suppress Notch pathway activation. We focused our study on analysis of 8 ESCRT components [Hrs, Stam (ESCRT-0); Vps28, Tsg101 (ESCRT-I); Vps25, Vps22 (ESCRT-II); Vps32, Vps24 (ESCRT-III)] as these are well known for their role in NICD trafficking and Notch pathway activation [2, 4]. These components showed uniform expression across different lobes and developmental zones of the lymph gland, as assessed by immunofluorescence (IF) or RNA *in situ* hybridisation in wild type lymph gland (Fig S2). *DomeGal4* driven knockdown of the ESCRT components was validated using immunofluorescence analysis where antibodies were available or by *in situ* hybridisation and RT-qPCR for analysing transcript levels (Fig S3). The Notch response element driving GFP (NRE-GFP) is a useful reporter to assess activation of Notch signaling. Upon KD in the lymph gland progenitors, except Vps25, all components tested [Hrs, Stam (ESCRT-0); Vps28, Tsg101 (ESCRT-I); Vps22 (ESCRT-II); Vps32, Vps24 (ESCRT-III)] caused upregulation of Notch signaling as interpreted by an increase in NRE-GFP positive cells in the primary lobe (Fig 4A). This is in concordance with the crystal cell differentiation phenotype as all of the ESCRT components except Vps25 cause increased crystal cell differentiation in

the primary lobe upon knockdown. Our result also indicates that these 7 ESCRT components are indispensable for regulation of Notch signaling in the blood cell progenitors possibly with non-compensatory roles.

Knockdown of 3 out of the 8 ESCRT components [Hrs, Stam (ESCRT-0) and Vps28 (ESCRT-I)] resulted in increased Notch signaling activation in the secondary lobe (Fig 4A, S4A). Of these only Hrs and Vps28 knockdown resulted in increased crystal cell differentiation in the secondary lobe (Fig 3), suggesting additional mechanisms downstream of Notch activation prevent crystal cell differentiation in the absence of Stam. Interestingly, in the tertiary lobe, both Hrs or Stam knockdown resulted in Notch activation (Fig4A, S4A) though neither resulted in crystal cell differentiation (Fig 3). In either case, tertiary lobe progenitors fail to differentiate, indicating their immature nature (Fig4A, S4A). Also, though Vps28 depletion does not significantly activate Notch signaling in the tertiary lobe it can promote crystal cell differentiation, suggesting a possible mechanism to downregulate Notch signaling likely after progenitors differentiate. However, we do see occasional increase in Notch activation in Vps28 KD tertiary lobes. Hence, crystal cell differentiation in the tertiary lobe could be under complex temporal regulation. Our analysis demonstrates the active role of ESCRT components in regulating Notch signaling, which may contribute to crystal cell differentiation and also differential response of the progenitor subsets.

NICD cleavage and transport to the nucleus to activate target gene transcription is key to effecting canonical and non-canonical modes of Notch signaling. Accumulation of NICD may lead to aberrant activation of Notch signaling. Progenitor-specific knockdown of all tested ESCRT components, except Vps25, resulted in increase in the number of cells accumulating NICD in the primary lobe (Fig 4B). This suggests a role for a majority of the ESCRT components in NICD trafficking, which may affect Notch signaling. The absence of any phenotype due to Vps25 knockdown suggests compensatory mechanisms may regulate cargo trafficking and lineage-specific signaling activation, which is sufficient to maintain progenitors at steady state.

Knockdown of 4 components [Hrs, Stam (ESCRT-0), Vps28 (ESCRT-I) and Vps24 (ESCRT-III)] led to an increase in the number of NICD accumulating cells in the secondary lobe (Fig 4B, S4A). However, only Hrs and Stam knockdown resulted in NICD accumulation in the tertiary lobe. This is in concordance with the phenotype of Notch activation upon Hrs, Stam and Vps28 knockdown in the posterior lobes. Our results show that NICD accumulation and Notch pathway activation correlate perfectly with crystal cell differentiation upon ESCRT knockdown. Notch signaling is known to be sensitive to endocytic sorting defects due to ESCRT in other contexts [24]. Similar effects may result in ectopic activation and promoting crystal cell differentiation. Despite a differential effect on NICD trafficking

and Notch activation across progenitor subsets, immunolocalization and proximity ligation assay indicated uniform interaction of ESCRT with NICD (Fig S4B, C; see supplementary results). Also, Notch activation triggered by ESCRT depletion in blood progenitors may be independent of the status of Notch ubiquitination (Fig S4D, E; see supplementary results). Hence the role of ESCRT in Notch signaling may lie downstream of subtle post-translational regulatory mechanisms.

ESCRT depletion sensitizes progenitors for lamellocyte differentiation.

Lamellocytes are rarely present in the larva without any wasp infestation. However, progenitor-specific knockdown of 6 ESCRT components [Tsg101 and Vps37A (ESCRT-I); Vps36 (ESCRT-II); Vps32, Vps20 and Vps2 (ESCRT-III)] induced lamellocyte differentiation in the primary lobe, as visualized by Phalloidin (F-actin) staining, even without any immune challenge (Fig 5A-C). Only 2 components (Vps36 and Vps2) caused lamellocyte differentiation in the secondary lobe when knocked down and only Vps36 knockdown triggered lamellocyte differentiation in the tertiary lobe (Fig 5A, B). This indicates that the majority of the ESCRT components are not involved in suppressing lamellocyte differentiation in the refractile posterior progenitors at steady state. However, it is likely that KD progenitors may be more sensitive to immunogenic cues as compared to normal, unperturbed progenitors.

Wild type larvae are generally able to mount a sufficiently robust immune response against wasp infestation, that aids their survival and eclosion. Systemic signals are generated upon wasp infestation and are received by the lymph gland progenitors [25], possibly through a complex extracellular matrix [16]. This results in lamellocyte differentiation in the primary lobe followed by disintegration and release of lamellocytes into circulation. Secondary and tertiary lobes are refractile to wasp infestation and do not form lamellocytes even upon immune challenge. Hence, we chose to test the response to wasp infestation in- a] ESCRT KD that had no effect on lamellocyte formation (Vps25 KD) and b] ESCRT KD that caused lamellocyte differentiation only in the primary lobe (Vps32 KD). Knockdown of both Vps25 and Vps32 triggered lamellocyte differentiation across all progenitor subsets upon immune challenge with wasp (Fig 5C). This shows that Vps25 and Vps32 play essential roles in preventing all posterior progenitors from lamellocyte differentiation in response to a natural immune challenge. Further, loss of ESCRT sensitizes progenitors to systemic cues by unlocking differentiation programs.

Our detailed analyses of blood progenitor differentiation upon knockdown of the 13 core ESCRT components yielded a functional map that reflects distinct lineage-specific roles of ESCRT in blood cell homeostasis and reduced sensitivity of younger progenitors to endocytic perturbation (Fig 6 A, B).

ESCRT regulates cargo sorting in a cell-autonomous manner in blood progenitors.

Progenitor-specific downregulation of ESCRT expression leads to accumulation of ubiquitinated cargoes. However, a majority of the ubiquitin aggregates were found to accumulate in non-progenitor (domeless⁻) cells, suggesting a possible cell non-autonomous effect. To test whether this could be due to ubiquitin accumulation in progenitors prior to their differentiation (cells lose domeless marker expression), we generated homozygous mutant mitotic recombinant clones for a representative ESCRT gene Vps32 (shrub), in progenitors. Vps32 is a terminally acting ESCRT that affects ubiquitinated cargo sorting and its depletion affects all blood cell lineages (Fig. 6). Hence it serves as a good model to assess cell autonomous function of ESCRT. Staining for conjugated ubiquitin revealed accumulation of ubiquitin aggregates in the homozygous mutant patch of the tissue (GFP⁻) indicating Vps32 has a cell autonomous role in cargo sorting in blood progenitors (Fig 7A). Hence, it is likely that ubiquitin seen in dome⁻ cells (Fig. 1) accumulated when the cells were still expressing domeless. This suggests that despite a decrease in dome expression in the knockdown cells, ubiquitin aggregates may persist during differentiation, likely due to dysfunctional cargo sorting in a cell-autonomous manner.

ESCRT affects progenitor differentiation in a cell non-autonomous manner.

We analysed differentiation in progenitor-specific mitotic clones of ESCRT. Vps32 knockdown results in increased crystal cell differentiation and triggers lamellocyte differentiation, as described earlier (Fig 3, 5-6). ProPO staining showed both wild type and mutant origin of crystal cells as revealed by overlap with GFP expression in the mutant tissue (Fig 7B). As crystal cells are usually present in the lymph gland in low numbers, it is difficult to interpret cell-autonomous origin of crystal cells from mutant progenitors. However, lamellocytes are completely absent in the control lymph gland at steady state (Fig 7C). Phalloidin staining in the Vps32 mutant clone showed GFP⁺ elongated or coalescing cells, indicating the presence of lamellocytes and possibly their precursors (Fig 7C). This suggests non-autonomous regulation of lamellocyte differentiation by ESCRT. Hence, ESCRT may regulate progenitor differentiation in both a cell-autonomous as well as non-cell-autonomous manner.

Vps25 knockdown did not affect the status of ubiquitination, progenitor maintenance or differentiation to any particular blood cell lineage despite its expression in the lymph gland. To further verify this, we generated lymph gland progenitor-specific homozygous mitotic clones of Vps25 loss of function mutation (Vps25^{A3}). There was no accumulation of ubiquitin aggregates (Fig S5A) or any change in the status of the progenitor (Fig S5B), plasmatocyte (Fig S5C), crystal cell (Fig S5D) and

lamellocyte differentiation (Fig S5E). However, the mutant lymph glands showed enlargement of the primary lobe, suggesting possible increase in blood cell proliferation upon loss of Vps25, due to non-autonomous effects. Also, phalloidin staining revealed appearance of binucleate, large cells and also very small cells occasionally, along with increase in F-actin content in some patches of the tissue, mostly in a cell autonomous manner (visible in GFP negative area of the tissue) (Fig S5E). Hence, Vps25 possibly inhibits uncontrolled cell proliferation and may contribute to critical steps of cell division that may dictate cell shape, number and polarity.

Discussion

Cargo sorting by the ESCRT machinery is a ubiquitous requirement. We asked whether ESCRTs play a decisive role in progenitor maintenance and lineage choice during development. For this we chose the well-conserved *Drosophila* hematopoietic system as a model. It is a simple, accessible and genetically tractable developmental model with limited cell types, whose development is regulated by conserved signaling networks.

Our study shows the active role of endosomal protein sorting in hematopoietic homeostasis *in vivo*. The detailed functional map of all 13 ESCRT core components in lymph gland hematopoiesis highlights distinct regulatory roles of individual components in lineage-specific progenitor differentiation. The functional chart reveals the most crucial steps of endosomal protein sorting in blood progenitor fate choice. ESCRT-I remodels the endosomal membrane through budding and ESCRT-III carries out scission, to allow cargo sorting. Loss of ESCRT-I or ESCRT-III components result in progenitor differentiation to all blood cell lineages. Moreover, Vps28 knockdown significantly affects crystal cell differentiation in all lymph gland lobes and Vps2 depletion induces posterior progenitor differentiation to lamellocytes. Hence, membrane budding and scission during endosomal protein sorting appear to affect a wide range of signaling pathways across distinct progenitor subsets. Recent reports highlight the universal role of ESCRT-III, often in concert with ESCRT-I, in various ESCRT-dependent membrane remodelling processes such as nuclear envelope reformation, lysosomal membrane repair, cytokinetic abscission, macroautophagy and exocytosis [5]. Whether such moonlighting functions of ESCRT-I and ESCRT-III impact signaling that determines lineage specification merits further investigation.

Curiously, we observed drastic functional diversity of ESCRT-II components in progenitor fate specification. While Vps36 depletion affected all lineages across progenitor subsets, Vps25 depletion

did not affect differentiation. Loss of Vps25 caused hyperproliferation in blood cells and failed to activate signaling pathways such as Notch, which are necessary for progenitor differentiation. Though Vps25 is a critical player in endosomal protein sorting in epithelial tissues [23, 26], its redundancy in lineage-specific differentiation suggests alternate routes for endosomal protein sorting in blood progenitors or a temporally regulated, developmental stage-specific role that has not yet been identified. Notably, though Vps25 is dispensable for steady state hematopoiesis, its depletion sensitizes all progenitors to differentiate upon immune challenge. This supports the possibility that the diverse roles of ESCRT may contribute to differential regulation of steady state and stress hematopoiesis.

Our study shows the distinct role of individual components of ESCRT in Notch signaling in blood progenitors. Expectedly, regulation of Notch signaling in lymph gland progenitors relies heavily on the ESCRT machinery. Our previous reports highlight the potential functional link of endosomal protein sorting with blood progenitor homeostasis [14, 27]. Though Hrs and Stam depletion promoted crystal cell differentiation in the lymph gland, it hardly affected plasmacyte and lamellocyte differentiation. In epithelial tissue, though Hrs and Stam regulate Notch trafficking, they do not regulate Notch pathway activation and downstream phenotypes such as cell polarity and proliferation [4]. Similar mechanisms specific to hematopoietic tissue are not yet explored. It is notable that ALIX and its yeast homolog Bro1 can recognize non-ubiquitinated cargoes and sort them independent of ESCRT-0 [28]. Also, Bro1, ALIX and HD-PTP act as alternate bridging factors to ESCRT-II to mediate endosomal protein sorting in yeast and mammalian cells [29, 30]. Even post-translational regulatory mechanisms may render ESCRT components inactive [31]. The non-compensatory roles of only a limited number of ESCRT components, in spite of uniform expression across the lymph gland, supports the idea that endosomal protein sorting possibly acts through multiple analogous components and parallel routes, with only a few indispensable, critical regulatory nodes.

ESCRT may potentially regulate EGFR cargo transport, sorting and signaling activation that governs plasmacyte proliferation and lamellocyte differentiation. Also, JAK/STAT and Hedgehog signaling can be regulated at the level of endosomal sorting in the blood progenitor. We speculate that lineage-specific signaling activation could be achieved through modulation of individual ESCRT component expression and function at the post-transcriptional or post-translational level. Though Deltex and eIF3f1 positively regulate Notch signaling in epithelial tissues [32-34], they appear dispensable for controlling Notch activation in blood progenitors upon ESCRT knockdown. While other E3 ubiquitin ligases and deubiquitinases may possibly complement for Deltex or eIF3f1 depletion, signaling activation may not always depend on the status of ubiquitination. For example, Vps36 depletion elicits a strong phenotype of differentiation without causing ubiquitinated cargo accumulation. Further

genetic interaction-based studies with other regulators of Notch signaling may reveal ESCRT-dependent mechanisms of Notch activation.

Lymph gland progenitor subsets are functionally heterogeneous and show reduced sensitivity to differentiation cues from anterior to posterior [16, 17]. Posterior progenitors resist differentiation upon immune challenge suggesting that they have additional signal regulatory checkpoints. What remains largely underexplored is the mechanism through which progenitor subsets differentially respond to systemic cues. One possibility is that younger progenitors have inherently low levels of ubiquitination and protein turnover and hence show mild effects on ESCRT depletion. Depletion of Vps36 and Vps2 can trigger lamellocyte differentiation in refractile progenitors even without any immune challenge, indicating that they actively prevent differentiation. Also, while Hrs knockdown activates Notch signaling and crystal cell differentiation in posterior progenitors, Stam knockdown fails to trigger terminal differentiation to crystal cells despite Notch activation. This indicates existence of multiple checkpoints and highlights the complexity of mechanisms that progenitors may employ to maintain their identity. Elucidating expression and function at the single cell level may aid in an improved understanding of ESCRT-dependent lineage specification across these distinct progenitor subsets. Such candidates can be screened further for efficient modulation of vertebrate blood regeneration *in vitro* as well as *in vivo*.

Loss of function mutation in ESCRT genes result in cell-autonomous cargo accumulation in *Drosophila* epithelial tissues [2, 3]. However, the cell non-autonomous role of ESCRT in cell proliferation as well as neoplastic transformation, suggests altered intercellular communication and aberrant signaling activation in the neighboring cell population [2, 3, 23]. In concordance with the previous reports, we observed a cell-autonomous role of ESCRT in regulating ubiquitinated cargo sorting in the blood progenitors. However, analysis of lamellocyte differentiation suggests that ESCRT may regulate lineage-specification non-autonomously. Both progenitor differentiation and proliferation can influence blood cell homeostasis in the lymph gland. ESCRT depletion not only activates lineage-specific signaling pathways but also promotes blood cell proliferation. Cell type-specific increase in proliferation and enlargement of lymph gland lobes can affect the proportion of different types of hemocytes. Elucidating the interplay between ESCRT components and the mitogenic signaling machinery could reveal whether downregulation of mitotic potential may restore steady state hematopoiesis.

BloodSpot Leukemia MILE and COSMIC databases show aberrant expression of ESCRT genes in hematopoietic and lymphoid tissues in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), myelodysplastic syndromes, etc (<https://servers.binf.ku.dk/bloodspot/>;

<https://cancer.sanger.ac.uk/cosmic>). Our study has application in understanding endosomal regulation of proliferative hematological pathologies. Further, as the role of endosomal protein sorting and the ESCRT machinery in vertebrate hematopoiesis is largely unexplored, it could help identify new regulators of hematopoietic homeostasis and provide novel targets for therapies, especially for improved blood regeneration during autologous transplantation. Further understanding of context-specific functions of ESCRT may improve our understanding of lineage-specific regeneration of tissue, including blood.

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Author Contributions

A.R. and M.S.I. designed research; A.R. and Y.R. performed experiments; M.S.I. contributed reagents and materials; A.R., Y.R. and M.S.I. analysed the data; AR and MSI wrote the manuscript.

Competing interest

The authors declare no competing interest.

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Figures

Figure 1. ESCRT components regulate ubiquitinated cargo sorting in the lymph gland.

(A) Whole-mount larval lymph gland showing accumulation of conjugated ubiquitin (FK2) in the lymph gland upon progenitor-specific (*domeGal4 UAS 2xEGFP* driven) knockdown of 7 *Drosophila* ESCRT components indicated (Vps28, Tsg101, Vps37A, Vps22, Vps32, Vps20, Vps2). Ubiquitin staining is shown in gray scale. Accumulation of ubiquitin aggregates is marked by arrowhead and magnified in insets. Scale bar: 100 μ m. (B) Bar diagrams show quantification of the fraction of cells accumulating ubiquitin aggregates in primary, secondary and tertiary lobes upon knockdown of all 13 core ESCRT components. n indicates the number of individual lobes analysed and N indicates the number of larvae analysed. Error bars represent SEM. Kruskal Wallis test was performed to determine the statistical significance. *P<0.05, **P<0.01, ***P<0.001. Summary chart indicating presence (colored box) or absence (white box) of ubiquitin accumulation in the primary, secondary and tertiary lobes upon depletion of the respective ESCRT component (left).

Figure 2. ESCRT components regulate progenitor maintenance and plasmatocyte differentiation in the lymph gland.

(A) Whole-mount larval lymph gland showing change in the fraction of dome>2xEGFP+ve progenitors (green) or P1+ve plasmatocytes (red) in the lymph gland upon progenitor-specific knockdown of 6 ESCRT components (Hrs, Vps28, Vps37A, Vps36, Vps32, Vps2). Scale bar: 100 μ m. (B-C) Bar diagrams shows quantification of the fraction of progenitors (B) and plasmatocytes (C) in primary, secondary and tertiary lobes upon knockdown of all 13 core ESCRT components. n indicates the number of individual lobes analysed and N indicates the number of larvae analysed. Error bars represent SEM. Kruskal Wallis test was performed to determine the statistical significance. *P<0.05, **P<0.01, ***P<0.001. Summary chart indicating presence (colored box) or absence (white box) of phenotypes of progenitor loss (B) or increased plasmatocytes differentiation (C) in the primary, secondary and tertiary lobes upon depletion of the respective ESCRT component (left). Also see Fig. 6, Fig.S1.

Figure 3. ESCRT components differentially regulate crystal cell differentiation of lymph gland progenitors.

(A) Whole-mount larval lymph gland showing increase in differentiation of ProPO+ve crystal cells (red) in the lymph gland upon progenitor-specific knockdown of 12 core ESCRT components (All except Vps25). Dome>2xEGFP (green) marks the progenitors across different lobes. Arrowheads mark

presence of crystal cells in posterior lobes. Scale bar: 100 μ m. **(B)** Bar diagram shows quantification of the fraction of crystal cells in primary, secondary and tertiary lobes upon knockdown of all 13 core ESCRT components. n indicates the number of individual lobes analysed and N indicates the number of larvae analysed. Error bars represent SEM. Kruskal Wallis test was performed to determine the statistical significance. *P<0.05, **P<0.01, ***P<0.001. Summary chart indicating presence (colored box) or absence (white box) of phenotypes of increased crystal cell differentiation in the primary, secondary and tertiary lobes upon depletion of the respective ESCRT component (left). Also see Fig. 6.

Figure 4. ESCRT components regulate Notch activation and NICD trafficking in the lymph gland.

(A) Whole-mount larval lymph gland showing NRE-GFP+ve (Notch reporter) cells (green) and dome+ve progenitors (red) in primary lobes upon progenitor-specific knockdown of 8 representative ESCRT components indicated [Hrs, Stam (ESCRT-0); Vps28, Tsg101 (ESCRT-I); Vps25, Vps22 (ESCRT-II); Vps32, Vps24 (ESCRT-III)]. Scale bar: 100 μ m. Bar diagrams show quantification of the number of NRE-GFP positive cells in primary, secondary and tertiary lobes upon knockdown of the same 8 ESCRT components. **(B)** Whole-mount larval lymph gland showing NICD expression (shown in red in the upper panel and in gray scale in the lower panel) in primary lobes upon progenitor-specific knockdown of the same 8 aforementioned ESCRT components. Progenitors are marked by dome>2xEGFP (green). Scale bar: 100 μ m. **(B')** Magnified view showing lymph gland hemocytes with (arrow) or without (arrowhead) NICD accumulation. Scale bar: 10 μ m. Bar diagrams show quantification of the number of NICD accumulating cells in primary, secondary and tertiary lobes. n indicates the number of individual lobes analysed and N indicates the number of larvae analysed. Error bars represent SEM. One-way ANOVA was performed to determine the statistical significance. *P<0.05, **P<0.01, ***P<0.001. See also Fig S4.

Figure 5. ESCRT components regulate lamellocyte differentiation in the lymph gland.

(A) Whole-mount larval lymph gland showing Phalloidin staining (red) to visualise elongated morphology of lamellocytes upon progenitor-specific knockdown of 5 ESCRT components (Tsg101, Vps37A, Vps36, Vps20, Vps2). Blue arrowheads mark the region from primary, secondary or tertiary lobes, magnified in the insets. The inset panel shows enlarged view of Phalloidin staining with lamellocytes marked by orange arrowhead. Scale bar: 100 μ m. **(B)** Bar diagram shows quantification of the percentage of lymph glands showing lamellocyte differentiation in primary, secondary and

tertiary lobes upon knockdown of all 13 core ESCRT components, without any immune challenge. Values in the columns indicate the number of larvae analysed for presence or absence of lamellocytes. Summary chart indicating presence (colored box) or absence (white box) of lamellocytes differentiation in the primary, secondary and tertiary lobes upon depletion of the respective ESCRT component (left). **(C)** Whole-mount lymph glands of Vps25 KD (*domeGal4 UAS 2xEGFP; UAS Vps25 RNAi;+/+*) and Vps32 KD (*domeGal4 UAS 2xEGFP; UAS Vps32 RNAi;+/+*) larvae uninfested or 3 days after wasp infestation. Phalloidin staining shows presence of lamellocytes (marked by orange arrowhead in the inset). Schematic representing the phenotype of Vps25 KD and Vps32 KD lymph glands with and without wasp infestation. Box marks lamellocyte differentiation in posterior lobes. See also, Fig 6.

Figure 6. A spatiotemporal map indicating ESCRT function in cargo sorting, progenitor maintenance and lineage-specific differentiation in *Drosophila* larval hematopoiesis.

(A) Schematic representation of lymph gland lobes from anterior to posterior (left to right). Green circle (medullary zone) in primary lobe and green dotted lines in posterior lobe indicates the progenitor pool depleted of ESCRT components (13 genes from ESCRT-0, I, II and III). Blood cell types are represented as violet (plasmacytes), pink (crystal cells) and blue (lamellocytes). Red font denotes ESCRT components whose depletion affected a given lineage whereas grey font indicates that there was no discernible change. Red monochrome heatmap indicates position-dependent progenitor sensitivity to depletion of ESCRT. See also Fig S2. **(B)** Comprehensive summary chart of the effects of individual ESCRT depletion on the various aspects of hematopoiesis as indicated. Presence or absence of a phenotype is depicted by colored or white boxes respectively. Red asterisk indicates Notch pathway activation and grey asterisk indicates no change, for components that were tested. **(C)** Venn diagram showing superimposition of phenotypes caused by the knockdown of different ESCRT components from progenitors. The underlined components in the lower panel affect both ubiquitin accumulation and progenitor status as described in the upper panel and (B).

Figure 7. ESCRT cell-autonomously regulates ubiquitinated cargo sorting in the lymph gland progenitors but may regulate differentiation in non-autonomous manner as well.

(A) Whole-mount lymph glands showing immunostaining for conjugated ubiquitin (red) in control (*domeGal4/+; neoFRT42D/+; UAS mCD8 RFP/+*) and progenitor-specific mutant clone of representative ESCRT component Vps32/shrub (*domeGal4/+; shrub^{GS} neoFRT42D/neoFRT42D; UAS*

677 *mCD8 RFP/UAS FLP*). Area marked by blue arrowheads are magnified in the insets to show
678 homozygous mitotic clones (GFP-ve patch, demarcated by dotted white line). Orange arrowheads
679 indicate ubiquitin accumulation in the mutant cells. DAPI marks the nuclei. **(B)** Primary lobe of control
680 and Vps32 mutant clone showing ProPO staining (red) to mark crystal cells. Arrowheads mark the
681 crystal cells which are GFP-ve (homozygous mutant). **(C)** Phalloidin staining in the same genotypes
682 shows GFP+ elongated and coalescing cells marked by arrowheads (insets) in the mutant clone. Scale
683 bar in all image panels: 100 μ m.

Conjugated Ub

	Pri.	Sec.	Tert.
Hrs			
Stam			
Vps28			
Tsg101			
Vps37A			
Vps37B			
Vps25			
Vps22			
Vps36			
Vps32			
Vps24			
Vps20			
Vps2			

Figure 1

A.

B.

Progenitors

	Pri.	Sec.	Tert.
Hrs			
Stam			

Vps28			
-------	--	--	--

Tsg101			
--------	--	--	--

Vps37A			
--------	--	--	--

Vps37B			
--------	--	--	--

Vps25			
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Vps22			
-------	--	--	--

Vps36			
-------	--	--	--

Vps32			
-------	--	--	--

Vps24			
-------	--	--	--

Vps20			
-------	--	--	--

Vps2			
------	--	--	--

C.

Plasmatocytes

	Pri.	Sec.	Tert.
Hrs			
Stam			

Vps28			
-------	--	--	--

Tsg101			
--------	--	--	--

Vps37A			
--------	--	--	--

Vps37B			
--------	--	--	--

Vps25			
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Vps22			
-------	--	--	--

Vps36			
-------	--	--	--

Vps32			
-------	--	--	--

Vps24			
-------	--	--	--

Vps20			
-------	--	--	--

Vps2			
------	--	--	--

Figure 2

A.

B.

Crystal cells

Pri. Sec. Tert.

Hrs			
Stam			

Vps28			
Tsg101			
Vps37A			
Vps37B			

Vps25			
Vps22			
Vps36			

Vps32			
Vps24			
Vps20			
Vps2			

Figure 3

A.

ESCRT-0

ESCRT-I

ESCRT-II

ESCRT-III

NRE-GFP dome>RFP

Notch activation

B.

dome>2xEGFP NICD

NICD accumulation

Figure 4

A.

ESCRT-II

ESCRT-I

ESCRT-III

B.

Lamellocyte

Lamellocytes			
	Pri.	Sec.	Tert.
Hrs			
Stam			
Vps28			
Tsg101			
Vps37A			
Vps37B			
Vps25			
Vps22			
Vps36			
Vps32			
Vps24			
Vps20			
Vps2			

C.

dome>Vps25 RNAi

dome>Vps32 RNAi

Figure 5

- Plasmatocytes
- Crystal cells
- Lamellocytes
- Domeless +veProhemocytes

Primary lobe

C.

A.

B.

Crystal cells

C.

Lamellocytes

Figure 7