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SETDB1 modulates the differentiation of both the crystal cells and the lamellocytes in *Drosophila*

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ABSTRACT

Proper genetic and epigenetic regulation is necessary to maintain the identity and integrity of cells. Enzymes involved in post-transcriptional modifications of histones are key factors in epigenetic mechanisms. Such modifications are also gaining importance for their role in growth and development of cancer. SETDB1 catalyzes the epigenetic mark of lysine-9 methylation of histone-3. In this study, we explored the role of SETDB1 in *Drosophila* hematopoiesis. We show that SETDB1 controls the differentiation of matured blood cells in wandering third instar larvae. There are three matured blood cells in wild type *Drosophila melanogaster*: plasmatocytes, crystal cells and lamellocytes. We found that loss-of-function mutants of SETDB1 show hematopoietic defects; increased blood cell proliferation, decreased number of crystal cells, greater differentiation of blood cells into lamellocytes, dysplasia of the anterior lobes of lymph gland and presence of hematopoietic tumors. Cell type specific knockdown of SETDB1 provided similar phenotype i.e., decreased number of crystal cells and an increase in lamellocyte differentiation. In animals with loss of function of SETDB1, *Notch* pathway was downregulated. Further, overexpression of SETDB1 in blood cells resulted in an increase in the number of crystal cells. This increase is accompanied with an increase in the number of $Notch^{ICD}$ expressing cells. We therefore performed genetic rescue using UAS-GAL4 system to rescue loss of function *SETDB1* mutants. Our data show that the rescued larvae carrying a wild type copy of SETDB1 in mutant background are devoid of blood tumors. We have identified a novel dual function of SETDB1 methylatransferase as a critical regulator of two of the matured hemocytes, crystal cells and lamellocytes. We propose a novel role of SETDB1 in modulating the differentiation of crystal cells and lamellocytes from a common progenitor and underscore the importance of SETDB1 in *Drosophila* blood tumor suppression.

1. Introduction

Drosophila melanogaster has a simple hematopoietic system consisting of three known matured blood cells [plasmatocytes (>95%), crystal cells (<5%) and lamellocytes (<1%)] at larval stages (Crozatier and Meister, 2007; Lemaitre and Hoffmann, 2007; Meister and Lagueux, 2003). Blood cells are found in circulating hemolymph, hematopoietic organ, the lymph gland, and in the posterior hematopoietic compartment consisting of sessile cells residing under the cuticle (Crozatier and Meister, 2007; Jung, 2005; Krzemień et al., 2007; Lebestky et al., 2003; Lemaitre and Hoffmann, 2007; R. Markus et al., 2009; Meister and Lagueux, 2003). Some population of sessile cells are seen attached to the imaginal discs, but majority of the population of the sessile cells form “banded patterns” under the larval epidermis. Functional aspect of this posterior hematopietic compartment (PHC) is not yet understood. Previous studies suggest PHC replenishes the progenitor pool in the circulating hemolymph (R Markus et al., 2009) Lymph gland has a pair of anterior lobes and two to six pairs of posterior lobes along the dorsal vessel (Holz, 2003; Jung, 2005; Minakhina and Steward, 2010). Anterior lobes are compartmentalized into three different regions: medullary zone, cortical zone and posterior signaling center (PSC). Hematopoietic niche is considered as the PSC of lymph gland. Lymph gland harbors both mature (cortical zone) and precursor (medullary zone) blood cells, in the anterior lobes. While posterior lobes contain primarily the precursor and quiescent blood cells (Holz, 2003; Jung, 2005; Minakhina and Steward, 2010). Upon receiving signals for both, proliferation and differentiation,

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hematopoietic quiescence is lost and precursor cells differentiate forming matured hemocytes.

Prohemocytes are destined to differentiate into either progenitors or the matured blood cells (plasmatocytes, crystal cells or lamellocytes). Plasmatocyte differentiation occurs twice during *Drosophila* development: embryonic head development and larval development (Holz, 2003; Wood and Jacinto, 2007). Plasmatocytes are phagocytic macrophages required for elimination of bacteria, apoptotic cells and small foreign particles (Crozatier and Meister, 2007; Lemaitre and Hoffmann, 2007). Crystal cells contain paracrystalline inclusions within them. These cells have a role in wound healing and also during melanization reaction (Rizki and Rizki, 1959). Lamellocytes differentiate upon wasp infestation, to encapsulate parasitoid wasp egg (Lemaitre and Hoffmann, 2007; RIZKI and RIZKI, 1994; Small et al., 2012; Sorrentino et al., 2004). There are three prophenol oxidase enzymes know of which ProPO1 and ProPO2 are exclusively expressed in crystal cells and ProPO3 is expressed in blood cells of $hop^{Tum\text{-}l}$ (JAK-STAT mutant) mutants devoid of crystal cells containing increased lamellocytes (Irving et al., 2005; Nam et al., 2008). ProPO3 is shown to function in lamellocyte mediated melanization in *Drosophila* (Nam et al., 2008). Mutants with hyperactive hematopoietic signaling pathways such as JAK-STAT, RAS and NF-κB show increased number of lamellocytes in circulating hemolymph along with abnormal plasmatocytes (Asha et al., 2003; Kalamarz et al., 2012; Minakhina et al., 2007; Minakhina and Steward, 2010; Qiu et al., 1998).

Various existing models have speculated the formation of the three matured blood cells from a common prohemocyte progenitor (Lanot et al., 2001) (Krzemień et al., 2007).

In the past decades experimental evidences provided sufficient information for the existence of bipotent prohemocytes (Fossett, 2013; Gold and Bruckner, 2014; Honti et al., 2014; Kroeger et al., 2012; Owusu-Ansah and Banerjee, 2009; Stofanko et al., 2010; Wang et al., 2014). Previous literature also discuss the differentiation of lamellocytes from plasmatocytes due to over-expression of *Chn* (Stofanko et al., 2010). Gcm, a transcription factor is expressed in all prohemocytes in the early stages of larval development (Bataille et al., 2005). Glial cells missing (*Gcm*) and *Gcm2* are related transcription factors having a crucial role in the differentiation of plasmatocytes (Bernardoni et al., 1999) (Kammerer and Giangrande, 2001) (Alfonso and Jones, 2002). Lack of *Gcm* and *Gcm2* hinders plamatocyte differentiation although crystal cells are not affected. *Gcm* and *Gcm2* double mutants give rise to increased number of crystal cells with a reduction in the number of plasmatocyte population (Williams, 2007). These studies have shed light on the bipotent nature of the prohemocytes. Toll, JAK/STAT, Notch, Pvr, Wg, Hippo, hedgehog and Ras pathway are some of the signaling pathways crucial for the development of blood cells (Asha et al., 2003; Chiu et al., 2005; Duvic et al., 2002; Harrison et al., 1995; Makki et al., 2010; Mandal et al., 2004; Milton et al., 2014; Minakhina et al., 2011, 2007; Mondal et al., 2014; Morin-Poulard et al., 2013; Mukherjee et al., 2011; Qiu et al., 1998; Reimels and Pfleger, 2015; Tokusumi et al., 2010). Hematopoietic pathways are conserved between *Drosophila* and mammals including various genes crucial in deciding the fate of precursors to differentiate into matured blood cells. Some of these comprise Serpent (GATA transcription factor)(Fujiwara et al., 2004; Tokusumi et al., 2010), Lozenge (Acute Myeloid Leukemia-1 like protein)(Canon and Banerjee, 2000; Sinenko et al., 2010), Dorsal (Nuclear transcription Factor-kappa B)(Hayden et al., 2006; Qiu et al., 1998), Gcm (Glial cell missing)(Jacques et al., 2009), Pannier (related to GATA-1) (Minakhina et al., 2011), STAT (Minakhina et al., 2011; Morin-Poulard et al., 2013; Ward et al., 2000), U-Shaped (friend of GATA homologue)(Tokusumi et al., 2010) and Collier (*Drosophila* orthologue of EBF-1)(Krzemień et al., 2007). In the PSC we find expression of genes such as Antennapedia (homeotic gene), Serrate (Ligand for Notch pathway), collier (transcription factor), Hedgehog (segment polarity gene)(Krzemień et al., 2007; Lebestky et al., 2003; Mandal et al., 2007; Tokusumi et al., 2010).

Genetic aberrations affecting NF-κB, JAK/STAT, and Ras pathways show inflammation and dysplasia of blood cells leading to development of blood tumor formation (Asha et al., 2003; Chiu et al., 2005; Harrison et al., 1995; Paddibhatla et al., 2010). Hematopoietic mutants with increased lamellocytes such as $hop^{Tum\text{-}l}$, $Ubc9^{-/-}$show aggregates and melanotic tumors in circulating hemolymph. Aggregates and microtumors are differentiated based on their sizes or number of blood cells found in each of these structures. Tumors show projection area >10,000 μm^2 while aggregates are <10,000 μm^2 (Kalamarz et al., 2012). Hallmarks of cancer arise due to alterations in the genome. Sometimes, epigenetic regulation is also disrupted in cancerous cells. Epigenetic modification such as DNA methylation, histone methylation and acetylation need proper regulation for the functioning of normal cells (Costa, 2010; Grønbæk et al., 2007; Hanahan and Weinberg, 2000) Hanahan and Weinberg, 2011. DNA hypermethylation is a mechanism known to inhibit the expression of tumor suppressor genes (Gou et al., 2010; Vincent et al., 2007; Wajed et al., 2001). Even though research on the signaling pathways affected during cancer growth and development has been ongoing, there exists little evidence for the role of epigenetic regulators, specifically epigenetic mechanisms in melanotic tumor growth and development in *Drosophila*. Inspite of researchers being cognizant about the role of epigenome in hematopoiesis it remains an arena unexplored from the perspective of single gene mutations causing the hematopoietic defects. Various scientific groups shed light on hematopoietic tumors in *Drosophila* (Crozatier and Vincent, 2011; Kalamarz et al., 2012; Minakhina et al., 2007; Minakhina and Steward, 2006; Morán et al., 2015; Paddibhatla et al., 2010; Reimels and Pfleger, 2015). Knowledge gained on epigenetic mechanisms playing a role for hematopoiesis and understanding the perturbations in epigenome of blood cells in *Drosophila* can help to further decipher the steps involved in growth and development of blood tumors.

We explore through this study the role of a histone modifying enzyme, SETDB1 in *Drosophila* hematopoiesis. SETDB1 belongs to the KMT1 family of methyltransferases, methylating histone-3-lysine-9 for which SET domain and the two neighboring pre-SET and post-SET domains are required (Schultz et al., 2002; Stabell et al., 2006). Wild type *Drosophila* express SETDB1 in all the developmental stages of the life cycle (Stabell et al., 2006). Referred also as *eggless*, this gene is expressed during oogenesis in both, germ cells and somatic cells. Preceding studies established the role of SETDB1 for (1) egg chamber formation during oogenesis, (2) wing development and (3) eye development (Clough et al., 2014, 2007; Gou et al., 2010; Li et al., 2006; Lundberg et al., 2013; Seum et al., 2007; Tzeng et al., 2007; Yoon et al., 2008). Apart from these results we also know from earlier literature that SETDB1 is required for silencing *retinoblastoma* gene (Gou et al., 2010). Furthermore it has a significant contribution in silencing chromosome four in *Drosophila* (Seum et al., 2007). We present data through this study on *Drosophila* SETDB1's novel function as a tumor suppressor in larval hematopoiesis.

Based on our experimental evidences we bring to focus a novel role of SETDB1 in (1) differentiation of both crystal cells and lamellocytes (2) in suppressing blood tumor formation.

2. Results

2.1. SETDB1 mutants exhibit hematopoietic defects

To elucidate the importance of SETDB1 in *Drosophila* hematopoiesis we studied loss-of- function mutants of *SETDB1*. Wandering third instar mutant larvae showed melanotic microtumors through the cuticle as depicted by tumor penetrance also in transheterozygotic mutant larvae i.e. $SETDB1^{235/1473}$, $SETDB1^{235/235}$, $SETDB1^{1473/1473}$ (Fig. 1A–D). To determine if the blood cells involved in and contributing to the formation of these melanotic microtumors we counterstained the dissected circulating hemolymph from wandering third instar larvae with nuclear dye (DAPI-blue) and cytoskeletal specific dye (polymerized F-actin-red). Hemolymph in heterozygote larva ($SETDB1^{+/-}$) show typical wild type larval blood cells (Fig. 1E). Compared to heterozygote siblings hemolymph from transheterozygotic mutants ($SETDB1^{235/1473}$) showed

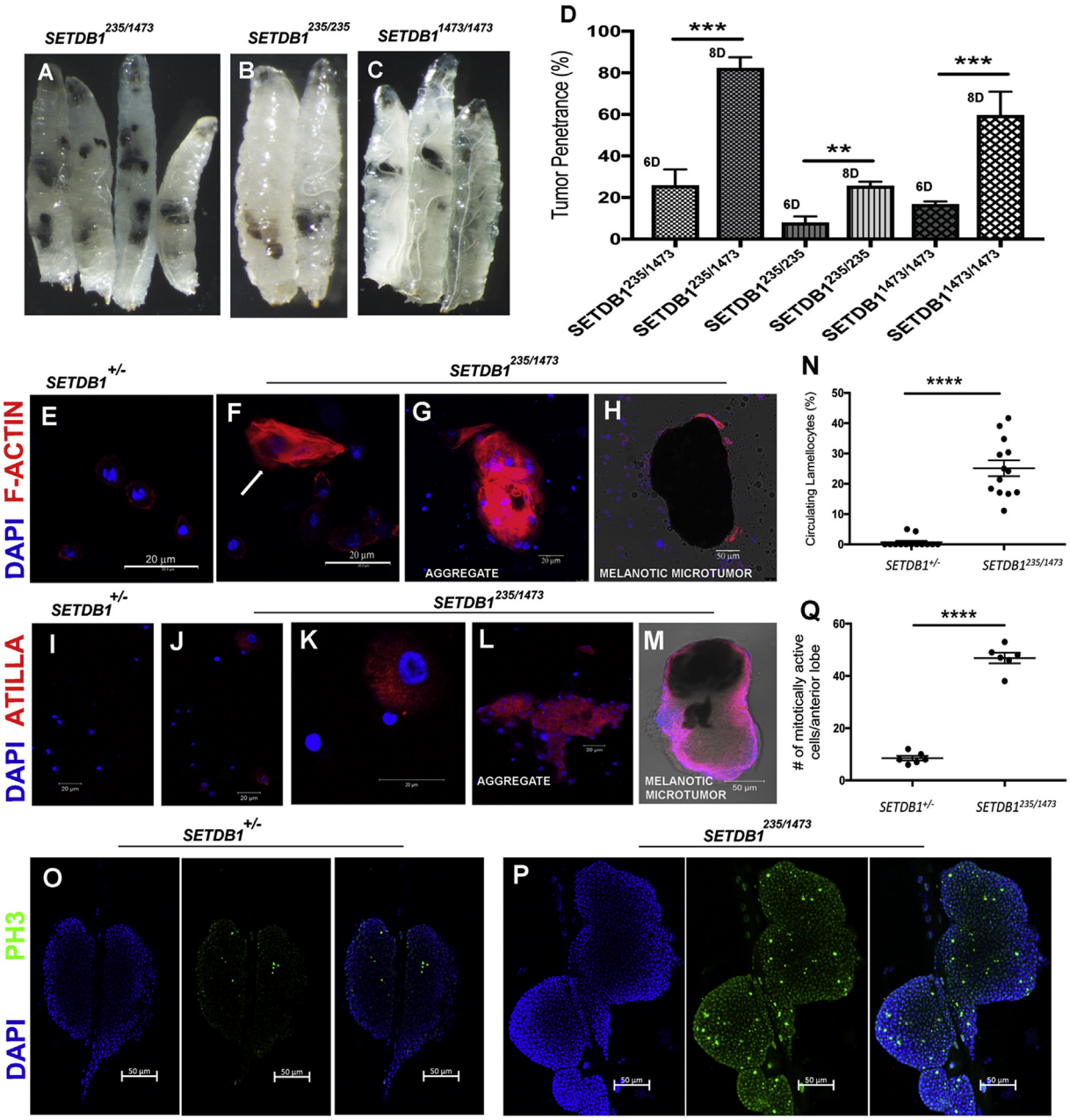

Fig. 1. Loss-of-function mutants of *SETDB1* show melanotic microtumor formation. Melanized microtumors visible through cuticle of wandering third instar whole larva from $SETDB1^{235/1473}$ (A), $SETDB1^{235/235}$ (B), *and* $SETDB1^{1473/1473}$ (C) mutant background. Graphical representation of the tumor penetrance observed in circulating hemolymph in six day old larvae and eight day old mutant larvae [$SETDB1^{235/1473}$ 6D (N=3, n=20) and 8D (N=3, n=22), $SETDB1^{235/235}$ 6D and 8D (N=3, n=19), *and* $SETDB1^{1473/1473}$ 6D (N=3, n=17) and 8D (N=3, n=12). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**), $p \leq 0.001$ (***). Circulating blood cells stained for polymerized F-actin (red) and nuclei (blue) from hemolymph of $SETDB1^{+/-}$ heterozygote larva (E) and hemolymph of $SETDB1^{235/1473}$ mutant larva (F). White arrow pointing to lamellocyte. Aggregate (G) and melanotic microtumor (H) from circulating hemolymph of $SETDB1^{235/1473}$ mutant larva stained for polymerized F-actin (red) and nuclei (blue). Circulating blood cells immunostained for lamellocytes using anti-Atilla antibody (red) and nuclei (blue) from hemolymph of $SETDB1^{+/-}$ heterozygote larva (I) and hemolymph of $SETDB1^{235/1473}$ mutant larva (J-K). Aggregate (L) and melanotic microtumor (M) from circulating hemolymph of $SETDB1^{235/1473}$ mutant larva immunostained for lamellocytes using anti-Atilla (red) and nuclei (blue). Graphical representation of the number of lamellocytes (N=3, n1=4, n2=4, n3=5, total n=13 individually plotted data points) in $SETDB1^{+/-}$ and $SETDB1^{235/1473}$ wandering third instar larvae (N). Student t-test to confirm significance shows $p \leq 0.0001$ (****). Anterior lobes of lymph glands stained for mitotically active cells using anti-Phospho-Histone-3 antibody (0-P, green). Graphical representation of mitotically active blood cells per anterior lobe (N=3, n1=2, n2=2, n3=3, total n=6 individually plotted data points) in $SETDB1^{+/-}$ and $SETDB1^{235/1473}$ in early third instar larvae (Q). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****).

abnormal cells that are larger in size like lamellocytes (Fig. 1F-white arrow pointing the differentiated lamellocyte like cell), aggregates (Fig. 1G) and melanotic microtumors (Fig. 1H) composed of blood cells. To identify the blood cell type in the hemolymph of mutants ($SETDB1^{235/1473}$) we stained the hemolymph with a plasmatocyte specific marker (Supplementary Fig. 1, P1/Nimrod C-green). Unlike the plasmatocytes found in wild type larvae, mutant blood cells from circulation showed few abnormal plasmatocytes that were positive for anti-P1 antibody staining. Aggregates found in mutants also show presence of these abnormal plasmatocytes along with region of melanization (Supplementary Fig. 1C, * indicating the region of melanization). While there is increased differentiation of blood cells which appear like lamellocytes in

circulating hemolymph of mutants (Fig. 1F–H) we examined with anti-atilla antibody, the morphology of these blood cells to determine presence of lamellocytes. Our results indicate presence of anti-atilla positive lamellocytes (Fig. 1J–K and N), aggregates (Fig. 1L), and melanotic microtumors (Fig. 1M) in circulating blood cells of mutants, *SETDB1*$^{235/1473}$ compared to heterozygote *SETDB1*$^{+/-}$ controls (Fig. 1I). To ascertain weather loss of function mutants, *SETDB1*$^{235/1473}$ compared to heterozygote *SETDB1*$^{+/-}$ controls have an increased proliferation of blood cells in the anterior lobes of the lymph glands we analyzed the expression of phospho-Histone H3 (H3P) using anti-H3P antibody (M-phase marker) in the anterior lobes. We noticed an increase in the number of mitotically active cells in the anterior lobes of lymph glands with significant differences between the heterozygotes and mutants (Fig. 1O–Q).

To examine the blood cells in the anterior lobes we first dissected out the anterior lobes from day five and day six old larvae. We documented the differences in feeding third instar five day old larva (5D) and

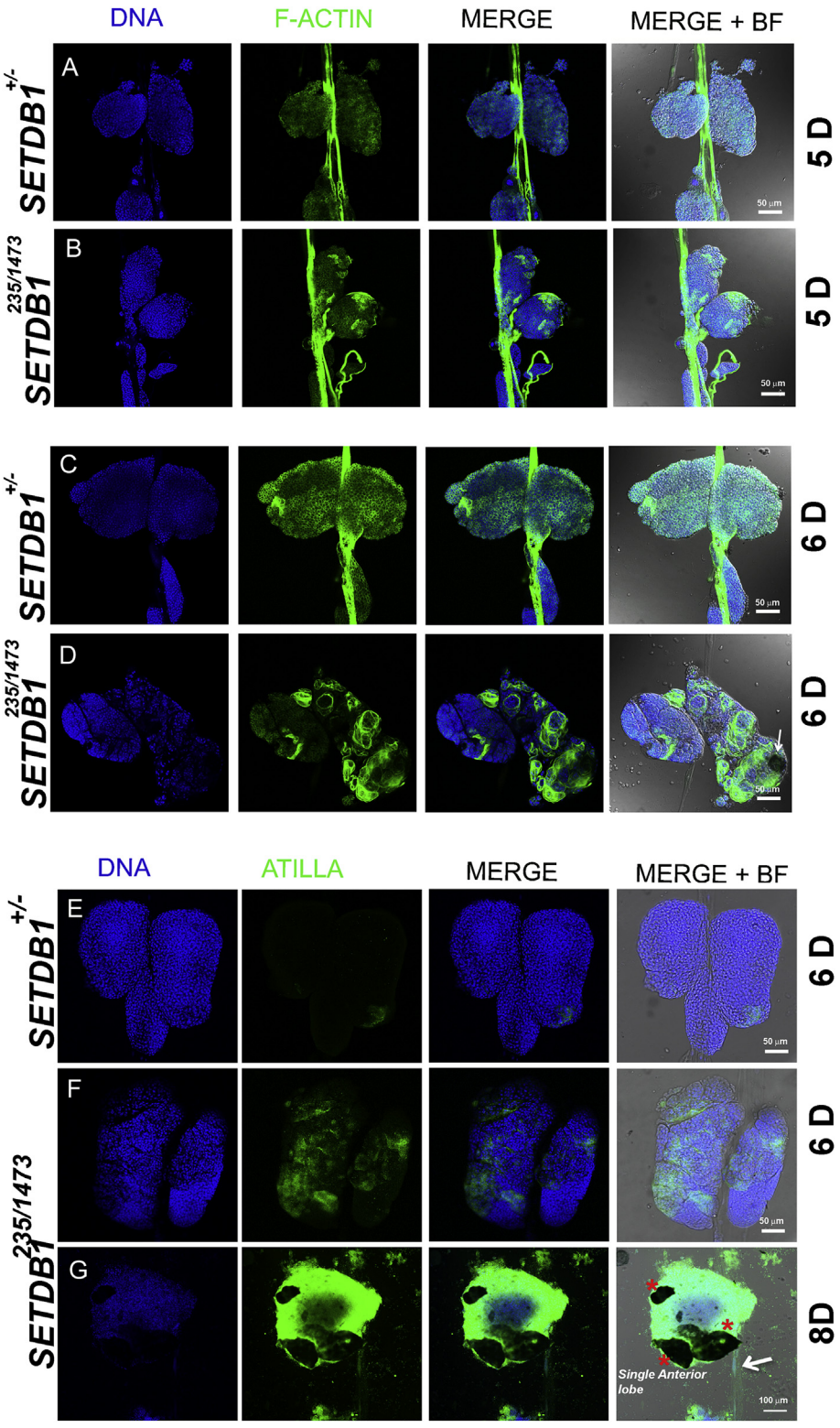

Fig. 2. *SETDB1*$^{235/1473}$ mutants show lymph gland hypertrophy with increased lamellocytes. Anterior lobes (AL) of the lymph glands from both heterozygote (*SETDB1*$^{+/-}$) and mutant (*SETDB1*$^{235/1473}$) siblings dissected from feeding third instar larva (FL3) five day old (5D), wandering third instar larva (WL3) six day old (6D), and mutants from delayed third instar larval stage eight day old (8D). A to D anterior lobes stained for polymerized F-actin (green) and nuclei (blue). White arrow in panels D and G are indicating the regions of melanizationin the anterior lobe. A. *SETDB1*$^{+/-}$ FL3 larva (5D) B. *SETDB1*$^{235/1473}$ FL3 larva (5D) C. *SETDB1*$^{+/-}$ WL3 larva (6D). D. *SETDB1*$^{235/1473}$ WL3 larva (6D). E to G anterior lobes immunostained for lamellocyte marker Atilla (green) and nuclei (blue) E. *SETDB1*$^{+/-}$ WL3 larva (6D) F. *SETDB1*$^{235/1473}$ WL3 larva (6D) G. *SETDB1*$^{235/1473}$ WL3 larva (8D).

wandering third instar six day old larva (6D) of heterozygote (*SETDB1*$^{+/-}$) and *SETDB1*$^{-/-}$ mutants (Fig. 2A–D). The lymph gland anterior lobes of *SETDB1*$^{+/-}$ heterozygote larvae in both the feeding stage and the wandering stage show normal wild type morphology (Fig. 2A, C) while the anterior lobes with regions of intense polymerized F-actin (green) staining is very enhanced in *SETDB1*$^{-/-}$mutants (Fig. 2B, D) indicating presence of differentiated cells. Furthermore, anterior lobe of the lymph gland in wandering third instar (6D) larva of (*SETDB1*$^{-/-}$) mutant showed melanized region (Fig. 2D, star indicating to melanized region). Using lamellocyte specific anti-L1/Atilla antibody we performed immunostaining to identify the lamellocyte population in these mutant' anterior lobes. Indeed as expected we observed increased number of lamellocytes that were Atilla positive in the six-day old mutant *SETDB1*$^{-/-}$ anterior lobes compared to heterozygotes (Fig. 2E and F). We extended the study to document the phenotype of the anterior lobes of eight-day old *SETDB1*$^{-/-}$ mutants to determine if the phenotype deteriorated with time. We noticed lamellocytes were extensively stained for Atilla in the anterior lobes of eight-day old larva and also observed increased regions of melanization (Fig. 2G, * in red indicating regions of melanization). These results clearly establish that the *SETDB1*$^{-/-}$ mutants show dysplasia with enlarged anterior lobes with greater number of lamellocytes and the anterior lobes forming melanotic microtumors within itself.

With the presence of abnormal plasmatocytes and lamellocytes it is indicative that there is hypertrophy of anterior lobes in lymph glands and in the circulating hemolymph of *SETDB1* mutants. This is the first study where anterior lobes of a lymph gland are shown with regions of melanization. Our results suggest a role for SETDB1 in hematopoietic blood cell maintainance.

2.2. Knockdown of SETDB1 contributed to lamellocyte differentiation

To further explore the role of SETDB1 for differentiation of blood cells into lamellocytes we performed RNAi knockdown of SETDB1 using classical UAS-GAL4 system (Brand and Perrimon, 1993). We used TRiP RNAi stock for knockdown of SETDB1 protein (*UAS-SETDB1*^{RNAi}). We utilized two different GAL4 drivers i.e. *Tubulin-Gal4* (ubiquitious) and *MSNF9-Gal4* (lamellocytes) to knockdown SETDB1. A single anterior lobe of control class (*Tubulin-Gal4*) and experimental class (*Tubulin* > *SETDB1*^{RNAi}) were compared through immunostaining experiment using anti-Atilla antibody specific for lamellocytes; those larvae carrying only one component of the expression system, *Tubulin-Gal4* (control class) were compared to the larvae carrying both components of the expression system, *Tubulin* > *SETDB1*^{RNAi} (experimental class). We found differentiated lamellocytes that were Atilla positive in *Tubulin* > *SETDB1*^{RNAi} (Supplementary Fig. 2B-B1, green) compared to control larvae carrying only *Tubulin-Gal4* (Supplementary Fig. 2A). To identify if there were other effects due to ubiquitous loss of expression of SETDB1 (*Tubulin* > *SETDB1*^{RNAi}), we analyzed developmental stages of the growing experimental animals. Larvae underwent through all developmental stages but we found wing defects in adult flies (with 100% penetrance, DNS). We next used lamellocyte specific driver, *MSNF9-GAL4* to study the loss of SETDB1 expression. Anterior lobes of *MSNF9*>*SETDB1*^{RNAi} experimental larva show significant increase in the differentiation of lamellocytes (Fig. 3B-B1 shown via graphical representation (Fig. 3G) compared to anterior lobes of control larva (Fig. 3A).

To elucidate the requirement of SETDB1 for lamellocyte differentiation we subsequently generated heat shock FLP-OUT clones of *SETDB1 [RNAi]* LOF. Experimental larvae carrying heat shock flippase enzyme, *Actin* > *FRT, CD2*>*GAL4, UAS-GFP* and *UAS-SETDB1*^{RNAi} were exposed to heat shock and compared to non-heat shock larva of same genotype. We observed upon heat shock experimental larvae showed differentiated lamellocytes that were atilla positive GFP clones (Fig. 3F-F1, Green and Red positive cells) compared to non-heat shock experimental larva (Fig. 3E) and control larva not carrying *UAS-SETDB1*^{RNAi} (with or without heat shock, Fig. 3C and D) clearly establishing that loss of SETDB1 expression leads to lamellocyte differentiation. We found

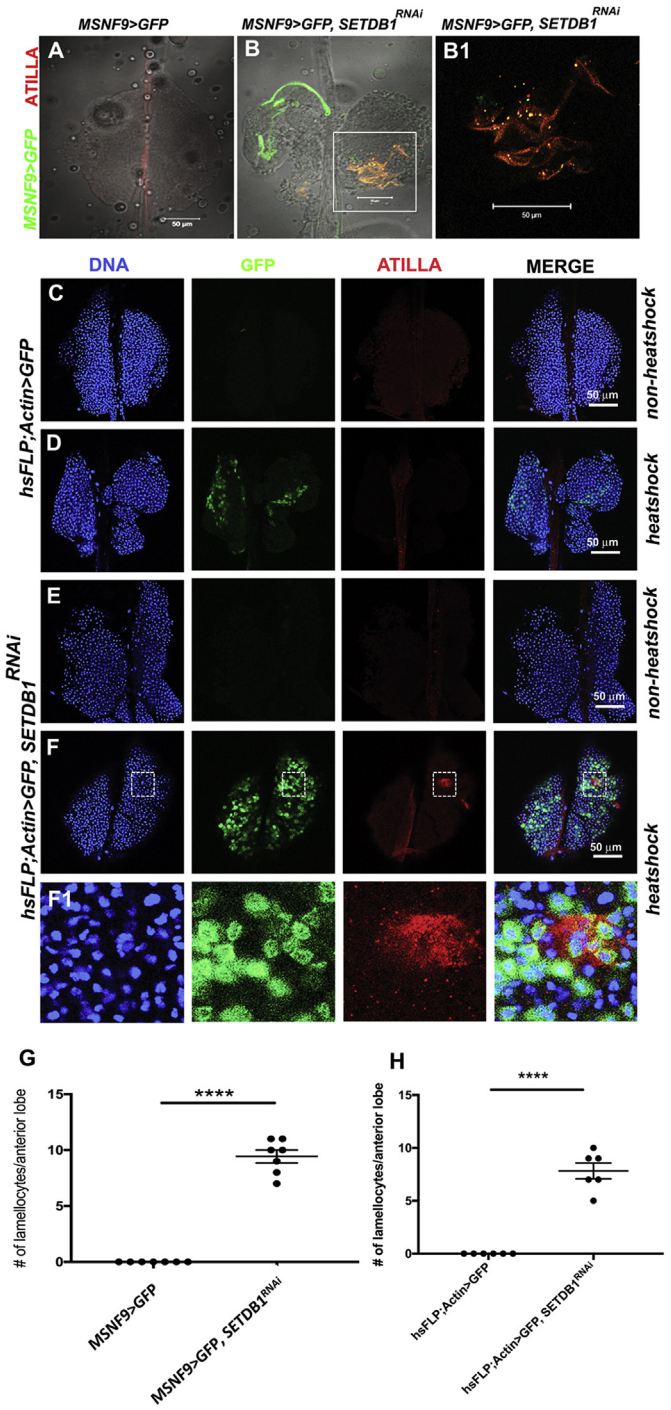

Fig. 3. Knockdown of *SETDB1* leads to lamellocyte production. Anterior lobes of the lymph glands from wandering third instar wild type larva, *MSNF9>GFP* (A), and experimental larva, *MSNF9>GFP, SETDB1*^{RNAi} (B-B1) stained for lamellocytes (anti-Atilla, red). Anterior lobes of the lymph gland stained for lamellocytes (anti-Atilla-red) in control larva *hsFLP;Actin > GFP* without heat shock (C), in experimental larva *hsFLP;Actin > GFP, SETDB1*^{RNAi} without heat shock (E), in control larva *hsFLP;Actin > GFP* with heat shock (D), and in experimental larva *hsFLP;Actin > GFP, SETDB1*^{RNAi} with heat shock (F). High magnification image of a region from F (F1). Graphical representation of number of lamellocytes in *MSNF9>GFP* and *MSNF9>GFP, SETDB1*^{RNAi} (G, n=7) larvae and in *hsFLP;Actin > GFP* and *hsFLP;Actin > GFP, SETDB1*^{RNAi} with heat shock (H, n=6). Student *t-test* (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****).

statistically significant differences after heat shock in the number of lamellocytes as represented graphically (Fig. 3H). These results suggest an autonomous function of SETDB1 in the differentiation of blood cells into lamellocytes (due to presence of Atilla positive cells that were also GFP positive cells). However, it is clear that not all the cells with lack of SETDB1 differentiate into lamellocytes since there exist some GFP cells that are not lamellocytes. Therefore, the requirement of SETDB1 is yet to be confirmed in maintaining few blood cells from differentiating into lamellocytes.

2.3. Loss-of-function of SETDB1 affects crystal cell differentiation

We next wanted to determine the effect of loss-of-function of SETDB1 on crystal cells. Anterior lobes of *SETDB1*$^{235/1473}$ mutants and *SETDB1*$^{+/-}$ controls were stained for crystal cells (anti-ProPO antibody) and cells in the niche (anti-Antp antibody). We observed crystal cells in the anterior lobes of control larva (Fig. 4A, red) that were absent in the anterior lobes of the mutant larva (Fig. 4B). Antennapedia positive niche cells were reduced in mutants (Fig. 4B, green) compared to the heterozygote controls (Fig. 4A, green). These results show the loss of crystal cells in the *SETDB1* mutants with the loss of Antennapedia positive niche cells. We know from published literature that crystal cell differentiation requires Notch signaling pathway in larvae. Apart from Lozenge, Notch also has a role in facilitating the differentiation of crystal cells. Notch undergoes proteolytic cleavage upon receptor activation there by Notch intracellular domain is formed (Bataille et al., 2005; Duvic et al., 2002; Lebestky et al., 2003; Mukherjee et al., 2011; Small et al., 2014). Therefore, we asked if there is a difference in Notch-ICD expression and further determined Notch-ICD protein expression (Notch intracellular domain) along with *Notch* and *Su(H)* gene expression. Su(H) is a transcription factor required for crystal cell development (Lebestky et al., 2003). Our results indicate lower levels of N-ICD expression in anterior lobes of mutants (Fig. 4D-D1, Green) compared to heterozygote controls (Fig. 4C-C1, Green). Consistent with the protein levels we found reduced levels of *Notch* and *Su(H)* gene expression in mutants compared to wild type control larvae (Fig. 4K). We next investigated the requirement of SETDB1 for crystal cell differentiation using *Lozenge*-GAL4 driver carrying *UAS-GFP* (*Lz-Gal4* is specific for crystal cell progenitor and mature crystal cells). To observe N-ICD protein expression we performed immunostaining. Results indicate an increase in the number of crystal cells (Green) with an increased N-ICD expression (red) in anterior lobes of *Lz > SETDB1, GFP* (Fig. 4F) compared to *Lz > GFP* controls (Fig. 4E). There are blood cells exclusively *Lozenge > GFP* (only green), positive for both, *Lozenge > GFP* and Notch^{ICD} (yellow) and positive only for Notch^{ICD} (red) in anterior lobes of both *Lz > GFP* and *Lz > GFP, SETDB1*. Blood cells that were positive for only Notch^{ICD} (red) and for both *GFP* and Notch^{ICD} (yellow) were higher in experimental class when compared to the control anterior lobes, suggestive of SETDB1 playing a role in increased expression of Notch. Upon RNAi knockdown we not only observed a decrease in the number of crystal cells (green) in the anterior lobes of the lymph gland of *Lz > SETDB1*^{RNAi} (Fig. 4G–L) compared to *Lz > GFP* controls (Fig. 4E, L), but also noticed reduced N-ICD expression in experimental *Lz > SETDB1*^{RNAi} compared to *Lz > GFP* controls (Fig. 4E and G, Red). These results clearly signify the role of SETDB1 in the Notch expression. We also performed immunostaining experiments (using anti-Atilla antibody) to determine if the loss of SETDB1 in *Lozenge > SETDB1[RNAi]* larvae can affect blood cells originating from the lymph glands to differentiate into lamellocytes. Our results clearly showed no such effect (data not shown). Apart from SETDB1, in *Drosophila* there exist another histone methyltransferase Su(var)$^{3-9}$ that methylate Histone-3 at lysine-9. Su(var)$^{3-9}$ also plays a role in oogenesis and its function comes into play after SETDB1 (Clough et al., 2014). Therefore, we wanted to explore the possible role of *Su(var)*$^{3-9\ GOF}$ [constitutively expressed gain of function allele (Schotta et al., 2003)] in *Drosophila* larval hematopoiesis. Thus, we determined if Su(var)$^{3-9}$, is essential for blood cell differentiation. We found that unlike SETDB1's function Su(var)$^{3-9}$ does not affect either crystal cells or lamellocytes (Fig. 4H–J, 4M and Supplementary Fig. 3) as shown through graphical representation (Fig. 4M). These results indicate that *Su(var)*$^{3-9}$ is not functionally similar to SETDB1 in hematopoietic tissues of *Drosophila*. To determine the possibility of decreased number of crystal cells in circulating hemolymph we performed cooking assay on third instar larvae with knockdown of SETDB1 expression using the three drivers *Tubulin-Gal4*, *Hemolectin-Gal4* and *Lozenge-Gal4*. Simultaneously, similar experiment was also performed using the heterozygote, *SETDB1*$^{+/-}$ and loss-of-function mutants of SETDB1, *SETDB1*$^{235/1473}$. Our results from cooking assay elucidated the reduced crystal cell numbers in the hemolymph of wandering third instar larvae due to loss of *SETDB1* (Fig. 4N).

The UAS TRiP RNAi stock that was used in our experiments for knockdown of SETDB1 protein has not been used earlier hence we wanted to see if we could reproduce and validate earlier published eye phenotype results with a different *UAS-SETDB1*^{RNAi} stock using the TRiP RNAi line (Gou et al., 2010). Our results confirm previously recognized eye phenotype of *Lozenge > SETDB1*^{RNAi} as we also observed same phenotypes of the eye. Adults show fused ommatidia as documented using scanning electron microscopy (Supplementary Figs. 4A–B).

2.4. Over-expression of SETDB1 affects crystal cell number

We first performed experiments using *Hemolectin-Gal4* driver (*Hml-Gal4,UAS- GFP* represents approximately 66% of the circulating GFP positive cells) to determine the effect of SETDB1 expression on plasmatocytes (Shia et al., 2009). There was no significant difference in the plasmatocyte population (GFP positive cells) stained with anti-P1/Nimrod-C antibody in the anterior lobes of lymph gland in *Hml > GFP, SETDB1* compared to the *Hml > GFP* (Fig. 5A and B). However, an increased crystal cell numbers marked by the crystal cell specific antibody (anti-C4 (Kurucz et al., 2007),) in the anterior lobes was observed in larvae from similar experiment (Fig. 5C and D). In both the experimental and control anterior lobes some of the crystal cells that were C4 positive (red) were also *Hml > GFP* positive. These doubly marked cells were more in *Hml > GFP, SETDB1* anterior lobes (Fig. C1-D2). To find out if similar effect is observed on crystal cells in the circulating hemolymph we performed the cooking assay and we utilized three different drivers, viz., *Tubulin-Gal4, Hml-Gal4* and *Lozenge-Gal4*. Our results from these experiments indicate an increase in the crystal cell population in the last two segments of the whole larva compared to the wild type larvae (Fig. 1E and F). While there is a significant difference between the wild type and experimental class we observed that it is the *Hml > GFP, SETDB1* larvae that showed greater number of crystal cells than the other two experimental classes, *Tubulin > SETDB1* and *Lozenge > GFP, SETDB1*. In *Hml > GFP, SETDB1* we also found crystal cells in the region of anterior lobes of the lymph gland (indicated by *) unlike other experimental larvae.

2.5. Rescue of SETDB1 mutants

To ascertain if the hematopoietic phenotypes observed in mutants of SETDB1 are due to its loss-of-function we performed a genetic rescue using *Tubulin–Gal4*, ubiquitous driver and exogenously expressed SETDB1 in mutants. Heterozygote siblings with only one copy of the mutant allele of *SETDB1* gene (*SETDB1*$^{+/-}$) and heterozygotes along with an exogenously expressed wild type copy (*SETDB1*$^{+/-}$*, Tubulin > SETDB1*) appeared to have normal circulating hemolymph (Fig. 6A and B). As shown earlier, mutants (*SETDB1*$^{235/1473}$) have increased number of lamellocytes, aggregates and melanotic microtumors (Figs. 1 and 6C and Supplementary Fig. 1) but mutants carrying an exogenously expressed wild type copy of SETDB1 (*SETDB1*$^{235/1473}$*, Tubulin > SETDB1*) show loss of lamellocytes and they are devoid of aggregates, and microtumors (Fig. 6D).

These results confirm the essentiality of the gene *SETDB1* in *Drosophila* for two of the matured hematopoietic cell formation i.e.

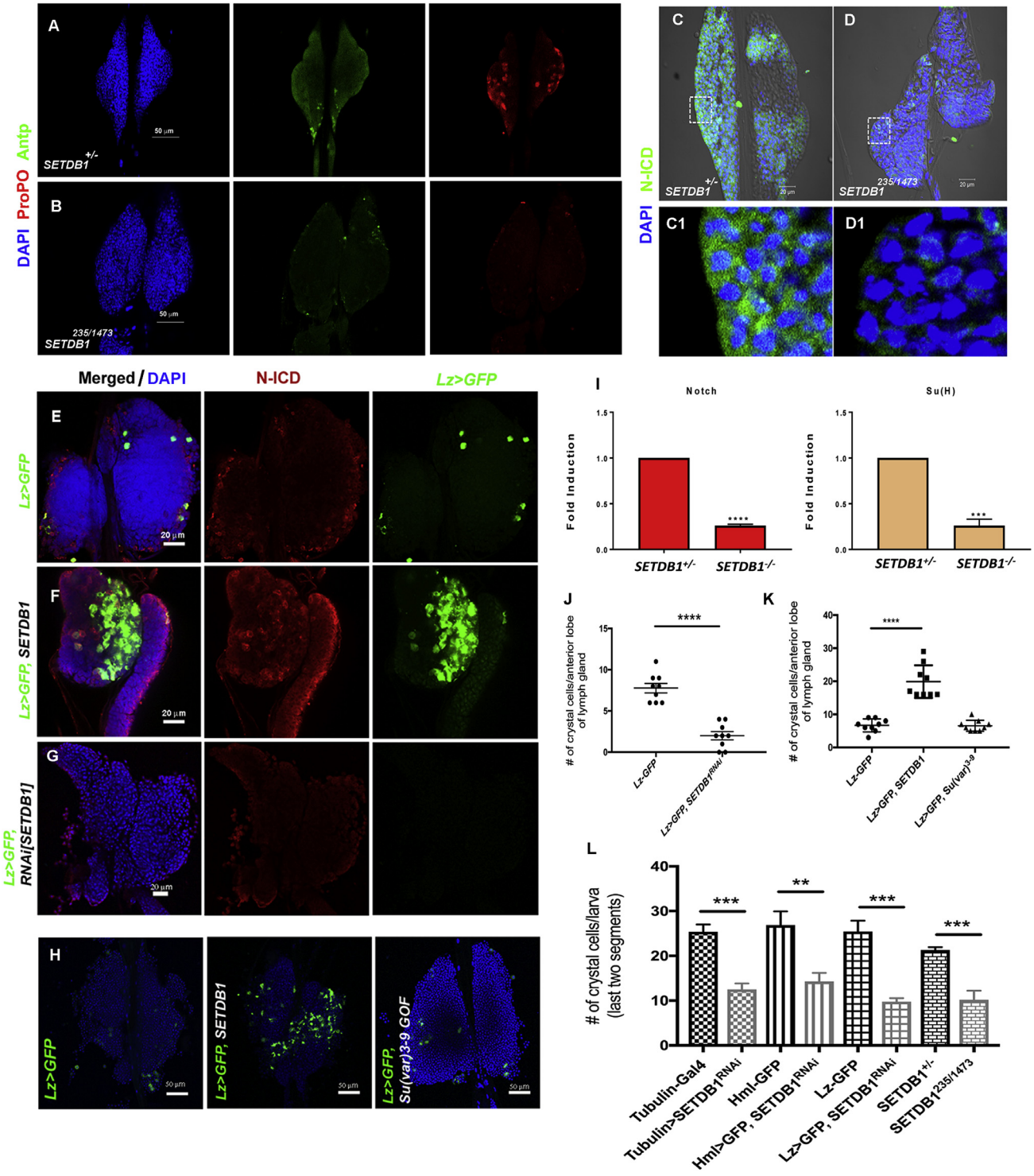

Fig. 4. SETDB1 affects crystal cell differentiation and Notch pathway. Anterior lobes showing expression of both ProPO (red) and Antp (Green) in *SETDB1*$^{+/-}$ (A) and *SETDB1*$^{235/1473}$ (B). Anterior lobes showing expression of N-ICD (Notch-Intra Cellular Domain, Green) in *SETDB1*$^{+/-}$ (C-C1) and *SETDB1*$^{235/1473}$ (D-D1). GFP positive crystal cells in the anterior lobes stained for expression of N-ICD (red) and DNA (blue) dissected from *Lz > GFP* larva (E), from *Lz > GFP, SETDB1* larva (F) and *Lz > GFP, SETDB1*^{RNAi} larva (G). Anterior lobes showing GFP positive crystal cells of *Lz > GFP* larva (H), *Lz > GFP, SETDB1* larva (I) and *Su(var)3–9* GOF, *Lz > GFP* larva (J) stained for nuclei (blue). Graphical representation of the fold induction of *Notch* and *Su(H)* in *SETDB1*$^{+/-}$ and *SETDB1*$^{235/1473}$ larvae (K), (N=3, n=50). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****), $p \leq 0.001$ (***). Graphical representation of the number of crystal cells from single lobe of anterior lobes (n=9) from both *Lz > GFP* and *Lozenge > SETDB1*^{RNAi} (L). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****). Graphical representation of the number (N=3, n=3) of crystal cells per anterior lobe of *Lz > GFP, Lozenge > SETDB1* and *Su(var)3–9* GOF, *Lz > GFP* larva (M). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****). Graphical representation of the number of crystal cells [*Tubulin-Gal4, Tubulin > SETDB1*^{RNAi} (N=3, n=9), *Hml > GFP, Hml > GFP, SETDB1*^{RNAi} (N=3, n=8), *Lz > GFP, Lz > GFP, SETDB1*^{RNAi} (N=3, n=9), *SETDB1*$^{+/-}$ and *SETDB1*$^{235/1473}$ (N=3, n=9)] in the last two segments of the wandering third instar larvae (E). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

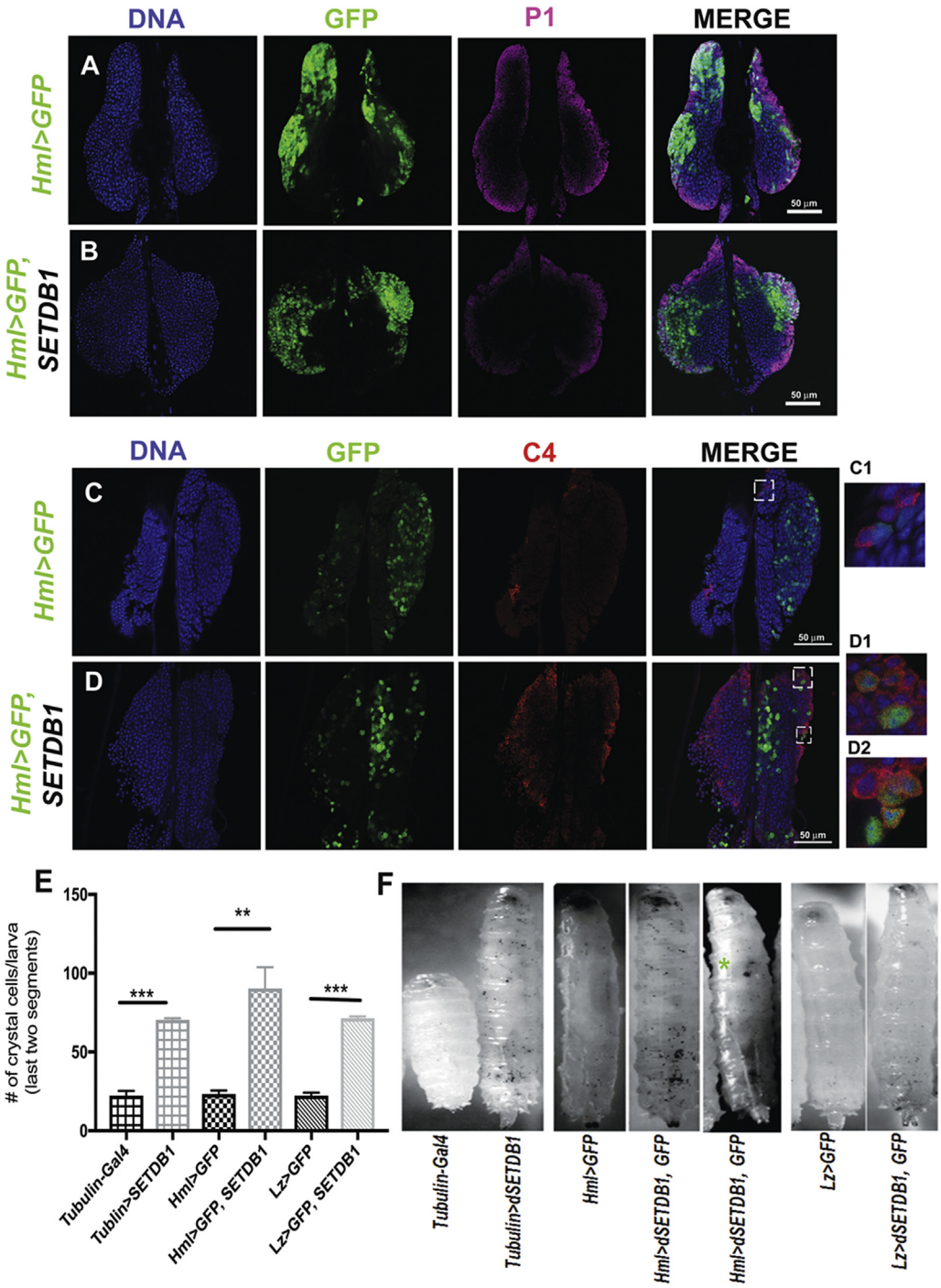

Fig. 5. Overexpression of SETDB1 in blood cells of both hemolymph and the lymph gland results in increased crystal cells. Anterior lobes immunostained for plasmatocyte specific protein expression [P1/Nimrod-C, magenta from *Hemolectin > GFP* (*Hml > GFP*)] (A), and from *Hml > GFP, SETDB1* (B). Anterior lobes immunostained for crystal cell specific protein expression (anti-C4, red) from *Hml > GFP* (C-C1) and from *Hml > GFP, SETDB1* (D-D2). Graphical representation of the number of crystal cells in the last two segments of the [*Tubulin-Gal4*, *Tubulin > SETDB1* (N=3, n=8), *Hml > GFP, Hml > GFP, SETDB1*, *Lozenge > GFP* (*Lz > GFP*) and *Lz > GFP*, *SETDB1* (N=3, n=9)] third instar larvae (E). Student *t-test*, (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Whole larvae from various genotypes showing crystal cells (blackened) after cooking assay (F, green star indicates region of crystal cells found near the anterior lobes of the lymph gland when *SETDB1* is over expressed with *Hml > GFP*).

crystal cells and lamellocytes and further substantiate the role of SETDB1 in blood tumor suppression.

3. Discussion

Drosophila SETDB1 is identical to its human and mouse homologue in its enzymatic activity as a methyltransferase. Studies using vertebrate and mammalian models brought out the function of SETDB1 in accelerating melanoma (human melanoma cells, ovarian cancer, non-small-cell lung cancer, small-cell lung cancer, hepatocellular carcinoma, and breast cancer)(Ceol et al., 2011). Mice, that fail to perform proper histone modifications exhibit hematopoietic defects (Thomas et al., 2006). Recent literature identified the role of SETDB1 in mice for hematopoietic stem cell development (Koide et al., 2016). Various publications contributed to the existing knowledge about *SETDB1*'s role in different developmental stages of *Drosophila*. However, little is known about SETDB1's requirement at tissue level (but for oogenesis) inspite of it being expressed in all developmental stages. Loss of SETDB1 leads to early stage arrest of oogenesis (Clough et al., 2014, 2007). We provide data in the current study taking advantage of fruit flies' genetic system to unravel the function of SETDB1 in hematopoiesis.

Our results indicate for the first time an unidentified function of SETDB1 for the differentiation of crystal cells and lamellocytes from a common progenitor in *Drosophila* larva. Experimental evidences to decipher the regulatory function of SETDB1 for the mature blood cells' differentiation were obtained by performing experiments using classical

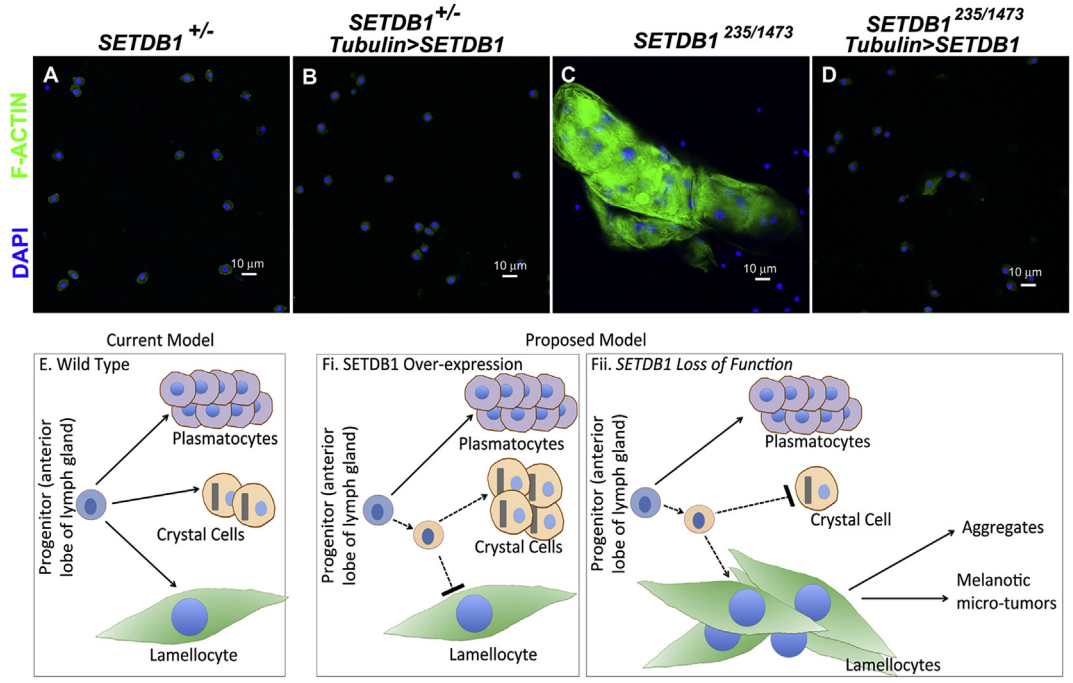

Fig. 6. A-D. Genetic rescue of *SETDB1* mutants with exogenous expression of SETDB1 ubiquitously: Circulating blood cells from hemolymph stained for polymerized F-actin (green) and nuclei (blue) dissected from *SETDB1*$^{+/-}$ heterozygote larva (A), *SETDB1*$^{+/-}$heterozygote larva carrying *Tubulin* > *SETDB1* (B), *SETDB1*$^{235/1473}$ mutant larva with an aggregate (C) and in genetically rescued mutants, *SETDB1*$^{235/1473}$ carrying *Tubulin* > *SETDB1* (D). **E-F. Schematic representing the function of SETDB1 in maintaining the blood cell homeostasis**: Current lineage showing progenitor from the anterior lobe of the lymph gland, differentiating into matured blood cells; plasmatocytes, crystal cells and lamellocytes (E). Proposed lineage shows a progenitor differentiation in two different genetic conditions (Fi-Fii). Upon over-expression of SETDB1, a common progenitor differentiates into increased number of crystal cells inhibiting lamellocytes formation (Fi). While due to loss of function of *SETDB1* a common progenitor differentiates into increased lamellocytes leading to aggregate and melanotic micro-tumor formation with a simultaneous hold on crystal cell differentiation (Fii).

UAS-GAL4 system for both over-expression and knockdown of *SETDB1*. Results obtained from loss-of-function mutants of SETDB1 (*SETDB1*$^{235/1473}$) along with knockdown of SETDB1 (RNAi) using *Tubulin-Gal4*, *Msnf9-Gal4* and *Lozenge-Gal4* clearly establish the requirement of SETDB1 in regulating lamellocyte and crystal cell numbers in wild type. Greater number of lamellocytes, aggregates and microtumors, are found in circulating hemolymph of LOF *SETDB1* mutants. In accordance with the results obtained from circulating hemolymph of LOF mutants we found that morphology of hematopoietic organ in these mutants is also being affected with an abundance of lamellocytes and an enlarged anterior lobe implicating dysplasia of the hematopoietic organ. The morphological changes in the mutant's blood cells eventually led to the formation of neoplastic hematopoietic tumors within the circulating hemolymph and lymph gland anterior lobes which are either non-melanized or melanized. These hematopoietic defects coincide with the phenotypes observed where there is hyperactivation of hematopoietic pathways (for e.g. NF-κB, JAK/STAT and Ras) that show similar blood cell defects in *Drosophila*. To corroborate our findings we further verified by clonal analysis that loss of SETDB1 leads to increased lamellocyte differentiation in wild type. Clonal analysis clearly demonstrates the dependency of few of the progenitor cells on SETDB1 for restricting their differentiation into lamellocytes. Over-expression studies are suggesting a requirement for SETDB1 for the differentiation into crystal cells. Increased number in the crystal cell population was detected in both circulating blood cells and anterior lobes of lymph glands compared to wild type wandering third instar larva due to over-expression of SETDB1 using *Hml-Gal4*, *Lozenge-Gal4* and *Tubulin-Gal4*. Our results indicate a function for SETDB1 in regulating the bipotent progenitor.

Furthermore, we noticed a decrease in the antennapedia expressing cells in mutants with a loss of crystal cells compared to anterior lobes of heterozygotes. Antennapedia is expressed in the niche cells of the lymph gland in wild type maintaining the progenitor population (Mandal et al., 2007). On the basis of these results we understand a requirement for Antp expression in the niche for maintaining progenitor population in *SETDB1* mutants. Consistent with the reduced crystal cell numbers we also noticed a decrease in Notch^{ICD} expression and *Notch*, *Su(H)* gene expression in both experimental conditions i.e. *SETDB1* mutants and also knockdown of *SETDB1* (RNAi). Notch^{ICD} protein expression was reversed upon over-expression of SETDB1 iterating the involvement of SETDB1 in regulating Notch pathway. It is therefore possible that the upsurge in number of crystal cells is a consequence of increased Notch protein in SETDB1 over-expression experiments. We therefore propose the existence of a bipotent progenitor that has the potential to differentiate into either a crystal cell or a lamellocyte without affecting the plasmatocyte population (Fig. 6E-Fii).

Further investigation can shed light on the mechanism involving Notch pathway. It is an oncogenic pathway used as a very strong therapeutic target while designing cancer drugs. Our experimental observations also direct a need to explore the mechanisms, involving SETDB1 in progenitors to decipher its requisite in not only controlling the bipotent progenitor cells' fate from differentiating into lamellocytes, but also in limiting the differentiation of precursors into crystal cells in wild type larva. While SETDB1 has a role in the development of crystal cells and lamellocytes it is crucial to have a better understanding of how SETDB1 is playing such role. It would be interesting and valuable to elucidate the role of SETDB1 in regulating hematopoietic pathways crucial for proliferation and differentiation of precurssor and progenitor cells. Yet to be answered is the connecting link between the genes or signaling pathways that are silenced by SETDB1 implicated in hematopoietic development. Further research needs to be carried out to elucidate the expression of SETDB1 protein in different blood cells and to determine the specific blood cell type in which SETDB1 plays an important role.

Genetic rescue experiments authenticate the requirement of SETDB1 in larval stages in blood tumor suppression. Upon expression of a wild type SETDB1 protein in the SETDB1 mutants' background not only there

is a reduction in the circulating lamellocytes but also melanotic micro-tumors are not formed. This is the first study highlighting the importance of a gene affecting epigenetics (modifying chromatin) involved in hematopoiesis and hematopoietic tumor regulation in *Drosophila*. Whether the effects of SETDB1 in *Drosophila* blood cells are dependent on H3K9me3 is however to be elucidated in future studies for mechanistic purposes. Since the domains involved in methyltransferase enzymatic function are similar in both, humans and fruit flies, further investigations can be carried out to perceive if human SETDB1 can liberate hematopoietic blood tumors in *SETDB1* larval mutants. Previous studies described the requirement of SETDB1 along with HP1 and Dnmt2 in *Drosophila* for DNA methylation for the silencing of target genes like retinoblastoma, a tumor suppressor gene (Gou et al., 2010). Extensive research is required for establishing how different levels of SETDB1 silence oncogenic and tumor suppressor pathways that affect the blood cell progenitor differentiation.

Taken together, current findings support the idea that SETDB1, a histone methyltransferase, has a novel role in regulating differentiation of progenitor cell's fate into either a crystal cell or a lamellocyte. The results obtained from our research lead to further questions in exploring SETDB1s′ contribution in the mechanisms involved for maintaining progenitors thereby limiting the formation of blood tumors.

4. Materials and methods

4.1. Fly stocks

All the stocks and transgenic lines of *Drosophila* were raised at 25 °C on standard media. *Tubulin-Gal4* [*Bloomington Drosophila Stock Center*, BDSC (Mueller et al., 2007)], *Hemolectin-Gal4, UAS-GFP* [Gift from Dr. Utpal Banerjee's Laboratory-UCLA (Sinenko and Mathey-Prevot, 2004)], *Lozenge-Gal4; UAS-GFP* [Gift from Dr. Utpal Banerjee's Laboratory-UCLA (Jung, 2005)], *MSNF9-GAL4* and *y w hs FLP; + / + ; actin > CD2>GAL4, UAS-GFP/TM6 Tb* [Gift from Dr. Shubha Govind, City College, CUNY (Small et al., 2014)], *UAS-SETDB1* [gift from Dr. Andreas Wodarz (Koch et al., 2009)], *UAS-SETDB1^{RNAi}* [Stock # 34803, Expresses dsRNA for RNAi of egg (FBgn0086908) under UAS control. Yale TRiP RNAi at Harvard Medical School (Ni et al., 2011)], *egg^{235}/SM1* [BDSC (Clough et al., 2007)], *egg^{1473}/SM1* [BDSC (Clough et al., 2007)], *Su(var) 3-9^{06}/TM6 Tb* [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)], *Su(var)3-9^{02}/TM6 Tb* [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)], *Su(var)3–9* gain-of-function [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)]

4.2. Mutants

Egg mutants: *egg^{235}/SM1* (null mutant) and *egg^{1473}/SM1* (mutant with loss of SET domain). We replaced SM1 balancer with *CyO GFP* balancers (Prasad, 2003) on chromosome two in both the mutant stocks. To generate heteroallelic combination in trans (transheterozgote mutants) we crossed *egg^{1473}/Cy O GFP* flies to *egg^{235}/Cy O GFP* flies.

4.3. Circulating hemolymph and lymph gland preparations

Crosses were set up and allowed to lay eggs for 6 h. Third instar larvae (5D-feeding third instar [FTI] or 6D and 8D-wandering third instar [WTI]) were collected for washes with 1X phosphate buffer saline, ddH2O, 75% alcohol, ddH2O, and finally in 1xPBS. Blood smears (circulating hemolymph) and hematopoietic organs (lymph glands) were dissected using fine forceps (FST- M5S 11200–14/Inox-Electronic by DUMONT Switzerland) as described in Small et al. (2012) (Small et al., 2012).

4.4. Immunohistochemistry

Antibody staining: Third instarlarval lymph glands or hemolymph were air dried on slides, samples were fixed in 4% paraformaldehyde and incubated with 1% Bovine Serum Albumin in 1x Phosphate Buffer Saline (1x PBS) for 30 min at room temperature, followed by incubation overnight with the primary antibody. After three washes with 1x PBST (15 min each), samples were incubated for 3 h atroom temperature with the respective fluorescently-labeled secondary antibodies (Invitrogen and Jackson Immunochemicals). After three washes in 1X PBST and one final wash with 1x PBS, the samples were counterstained with nuclear dye Hoechst 33258 (1:500) and/or F-actin (phalloidin)(1:200) where specified, then mounted in glycerol containing antifade (N-propylgallate). Primary antibody concentrations used were anti-Nimrod-C (gift from Dr. Istvan Ando) 1:20, anti-Atilla (gift from Dr. Istvan Ando) 1:20, anti-C4 (gift from Dr. Istvan Ando) 1:20 for staining matured blood cells (Kurucz et al., 2007), anti-PH3 (gift from Dr. Krishnaveni Mishra, University of Hyderabad, School of Life Sciences, rabbit, abcam) 1:100 and anti-Notch-ICD (Developmental Studies Hybridoma Bank (DSHB)) 1:10. Flourescently labeled secondary antibodies [TRITC/FITC/Cy5-conjugated goat anti-mouse (1:50) and goat anti-rabbit (1:50)] were commercially obtained from Jackson Immuno Research Laboratories. Anti-ProPO antibody was (gift from Dr. Tina Mukherjee) was diluted 1:100, Anti-Antp (8C11) was commercially obtained from DSHB (1:100).

4.5. Microscopy

Whole larvae: Larvae of interest were washed as mentioned above in "hemolymph sample preparations". Using Zeiss Axio CS microscope larvae were imaged. Immunohistochemistry samples: Circulating hemolymph and lymph gland samples stained and mounted were imaged with LAS AF SP8confocal microscope and images were processed using the LAS AF SP8 software. Final images were processed using Adobe Photoshop CS3 software.

4.6. Flp-out technique

Flp-out technology is used for the spatial and temporal restriction of transgene expression. In this technique there is use of the hybrid flipout (Flp) and GAL4 activation [*hsp70-flp*; *Actin > CD2>GAL4*] and *UAS-NLS-GFP* transgenes or those larvae with an additional *UAS-SETDB1^{RNAi}* transgenes, were heat shocked at 37° in a water bath for 15 min. After removing them from water bath they were placed at 25°. Lymph glands were dissected on day 6 from mid-third instar larvae, 20 h post heat shock. Developmentally synchronized larvae from controls class (without *UAS-SETDB1^{RNAi}* transgenes) and experimental class (with *UAS-SETDB1^{RNAi}* transgenes) both carried *UAS-NLS-GFP* transgenes. These larvae were compared without and with heat shock.

4.7. Crystal cell melanization assay (cooking assay)

Wandering third instar larvae were washed with 1X phosphate buffer saline, ddH2O, 75% alcohol, ddH2O, and finally in 1xPBS. Larvae were then transferred into 1X PBS containing centrifuge tubes. Tubes were placed inside the water bath at 60 °C for 10 min. Both wild type larvaeand experimental larvae were then removed from water bath, and left at roomtemperature for some time. Number of crystal cells in the last two segments of wandering third instar larvae were counted. N=3, n > 8.

4.8. Scanning electron microscopy

Samples were prepared as per the standardprotocols as mentioned. Imaging was done using the Hitachi SEM.

Measurement of crystal cells, lamellocytes, mitotic index and tumor penetrance.

Unless mentioned third instar larvae were used for experiments. All samples under observation for data collection were randomly picked for the experiments (Sample size chosen as noted from earlier published

research studies). Statistical analysiswas performed using GraphPad Prism 7 software. All the samples were included for statistical analysis. For comparisons between two groups, we utilized Student's *t*-test (unpaired, two-tailed). All graphs show mean ± SEM. In all cases (not significant) $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p < 0.001$ and $^{****}p \leq 0.0001$. Biological repeats (N), sample size (n) and *t-test* results are mentioned in the legends section. Crystal cell indexes displayed were *Lozenge > GFP* positive crystal cells counted per anterior lobes. Lamellocyte counts in circulating hemolymph is displayed as a number of Atilla positive cells after staining with anti-Atilla antibody used for staining circulating lamellocytes. Using LAS AF SP8 confocal microscopy lymph glands were laser scanned and Z stack images were collected. Mitotically active cells per anterior lobe were counted from the images obtained. Wandering third instar six day old larvae and eight day old larvae were dissected and observed formicrotumors in circulating hemolymph for tumor penetrance.

4.9. RNA collection and real time-PCR

Fifty synchronized 3rd instar larva of the appropriate genotypes (six days after egg lay) were collected for RNA extraction (Trizol method, Invitrogen, Life technologies, Carlsbad, CA). RNA was quantified by a StepOnePlus ™ System (Applied Biosystem, Thermo Fischer Scientific). 1.5 μg of total RNA was used as template for cDNA synthesis (iscript ™; Bio-rad Laboratories, Hercules, CA). Real time PCR was performed running the standard two-step PCR program: 1 μl of the cDNA sample was mixed with KAPA SYBR^{R} FAST Universal (KAPA Biosystems, Lot # 006255-8-1) and primers to set up a 25-μl reaction mix. Transcript levels detected were normalized to *rp49* mRNA values. Primers used:

Notch: Forward primer (5′) *AGC GAA ATG GAG TCG GTC CCG* (3′); Reverse primer (5′) *GAT GGC GAG CCC AAG TAG GCA* (3′)

SU(H): Forward primer (5′)*AAT GGT CCT TGC AGG TAC GTC* (3′); Reverse primer (5′) *ATC CTC GGC CTG TGT ATT GC* (3′)

RP 49 Forward primer (5') GAC GCT TCA AGG GAC AGT ATC TG (3′); Reverse primer (5′) AAA CGC GGT TCT GCA TGA G (3′)

Author contributions

I. P. conceived and designed the study; I. P. performed the experiments; D. K. G. performed experiments for Fig. 4K. I. P., D. K. G. and R. K. M. analyzed the data and wrote the paper.

Conflict-of-interest disclosure

Authors declare they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.008.

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