

Drosophila Hox genes induce melanised pseudo-tumours when misexpressed in hemocytes

Titus Ponratnam

Centre for Cellular and Molecular Biology CSIR

Ravina Saini

Centre for Cellular and Molecular Biology CSIR

Rakesh Mishra (✉ mishra@ccmb.res.in)

Centre for Cellular and Molecular Biology CSIR <https://orcid.org/0000-0001-6636-7380>

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Abstract

Background: Hox genes are key early determinants of cell identity along the anterior-posterior body axis across bilaterians. Recently, several late non-homeotic functions of Hox genes have emerged in a variety of processes involved in organogenesis in several organisms, including mammals. Being crucial factors in determining cell identity and organogenesis, the misregulation of Hox genes is likely to be associated with defects in these processes. Several studies have reported the misexpression of Hox genes in a variety of malignancies including acute myeloid leukaemia.

Methods: The Hox genes *Dfd*, *Ubx*, *abd-A* and *Abd-B* were overexpressed via the UAS-Gal4 system using *Cg-Gal4*, *Lsp2-Gal4*, *He-Gal4* and *Hm1D3-Gal4* as specific drivers. Genetic interaction was tested by bringing overexpression lines in heterozygous mutant backgrounds of Polycomb and trithorax group factors. Larvae were visually scored for melanised bodies. Hemocytes were quantified by dissecting larvae for lymph in 4mm wells and staining nuclei with DAPI and tested for differentiation by staining them with anti-myospheroid and for proliferation with anti-PH3. Pupal lethality was carried out by letting pupae eclose and scoring those that failed after the time point.

Results: Expression of *Dfd*, *Ubx* and *abd-A*, but not *Abd-B* in the hematopoietic compartment of *Drosophila* led to the appearance of circulating melanised bodies, and increase in cell numbers, cell-autonomous proliferation and differentiation of hemocytes. Pupal lethality and the melanised pseudo-tumor phenotype were suppressed by the mutations in *Psc*¹ and *esc*² background while polycomb group member mutations *Pc*¹ and *Su(z)12*³ and trithorax group member mutation *TrlR*⁸⁵ increased the phenotype.

Conclusions: *Dfd*, *Ubx* and *abd-A* are leukemogenic. Mutations in Polycomb and trithorax group members, which are responsible for maintaining the expression state of the Hox genes, modulate the leukemogenic phenotype. *Drosophila*, widely used as a model for myeloid leukemias, can serve as a testbed for Hox expression induced leukemias.

Background

Highlights

1. Ectopic expression of Hox genes, *Dfd*, *Ubx*, *abd-A* and *Abd-B* in hematopoietic cells of *Drosophila* leads melanotic pseudo-tumors in larval stages.
2. The circulating pseudo-tumors in the viscera subsequently lead to extensive lethality at pupal stage.
3. The pseudo-tumor phenotype is also associated with an increase in blood cell number as well as their enhanced differentiation into lamellocytes.
4. Epigenetic factors, the polycomb and trithorax group of genes, modify the pseudo-tumor phenotype.

5. Our study reports the causal link of Hox genes to the process of cancer conditions.
6. These findings open a new possibility of addressing the function of Hox genes in leukemogenic hematopoiesis the fly as model system.

Life comes in a variety of body forms. Despite this variety, there is similarity at the genetic and molecular level in the developmental mechanisms that lead to this variety across species. For example, in spite of the evolutionary distance between vertebrates and *Drosophila*, many organ and tissue types show a degree of homology with each other and many key developmental pathways governing their development and function are conserved.

The hematopoietic system is no exception. Hemocytes of *Drosophila* resemble the myeloid lineage of blood cells¹. The most abundant cells, plasmatocytes, are the equivalent of macrophages and are involved in a variety of processes such as responses to pathogens, removal of apoptotic cells, deposition of the extracellular matrix during embryonic development, etc.². The next most abundant cells are Crystal cells, specialised to induce melanisation reactions in the presence of pathogens and wound healing³, resemble the granulocytes, and contribute about four per cent of the blood cells. Lamellocytes are the least abundant population of blood cells, usually only appearing in circulation upon the larva being challenged by any object too large to be cleared off by the macrophages, such as the eggs of a parasitoid wasp^{4,5}.

This conservation includes the molecular pathways involved in the development of these cell types. For example, *Drosophila serpent*⁶ is related to GATA 1, 2 and 3 of vertebrates. GATA-2 is responsible for blood progenitor proliferation and survival^{7,8} while GATA-1 is required for progenitor differentiation into erythrocytes, megakaryocytes and eosinophils⁹⁻¹¹. Similar to GATA-2, *srp* is required for progenitor maintenance and proliferation. Loss of function in *srp* leads to a reduced number of progenitors and a loss of all hemocytes. It is also required in plasmatocyte differentiation, similar to GATA-1¹². Additionally, *Drosophila u-shaped* is related to the Friend of GATA (FOG) family. FOG-1 and GATA-1 together are required for erythrocyte and megakaryocyte differentiation^{13,14}. FOG-1 interacts with GATA-1 to represses eosinophil differentiation and must be downregulated for eosinophil differentiation¹⁵. Similarly, *ush* is expressed in hemocyte precursors and plasmatocytes, and must be downregulated for crystal cell development¹⁶.

Signalling pathways involved in regulating hematopoiesis, as would be anticipated, are conserved between vertebrates and *Drosophila*. For example, Jagged-1, the vertebrate homolog of *Serrate* and a ligand of Notch, is produced by the stromal cells of the bone marrow, to regulate Hematopoietic Stem Cell (HSC) proliferation and survival¹⁷. *Ser* performs a similar role, being released by cells of the Posterior Signalling Centre (PSC)¹⁸ via cytonemes^{19,20}, a set of regulatory cells at the posterior end of the Lymph Gland (LG). Vertebrate JAK2 is required for erythropoiesis²¹, while STAT5 is required for proper progenitor and myeloid cell function²². The *Drosophila* JAK/STAT pathway is required within the LG for the maintenance of prohemocytes, among other things²³. Transformations in JAK2 can lead to

leukemogenesis in vertebrates²⁴, similar to how gain of function *hop* mutants behave²⁵. The Toll pathway is also conserved, playing a major role in innate immunity in both vertebrates and flies²⁶.

One aspect of vertebrate hematopoiesis that has not been mirrored in *Drosophila* is the role of Hox genes. Hox genes are well known for their conserved role in body axis formation across all bilaterians²⁷, but also play roles in vertebrate hematopoiesis²⁸, autophagy²⁹, as well as cell proliferation, differentiation, migration and apoptosis³⁰. Hox genes are transcribed in HSCs as well as lineage progenitors, and are suppressed in differentiated blood cells^{31–35}. Overexpression models show blockages in certain stages of development, expansion of HSCs, the circulation of blast cells, etc.^{36–41}. For example, *Hoxa7* and *Hoxa9* have been shown to have a role in the development of hematopoietic progenitors of different lineages in mice. On the other hand, in *Drosophila*, other than *Antp*, which is implicated in setting up the location of the LG⁴², as well as later marking the PSC⁴³, *Hox* genes have not been reported to play any role.

The expression of genes of the *Hox* cluster during, and after development is regulated by two chromatin remodelers, Polycomb and trithorax group (PcG and trxG) of proteins, which were discovered as transcriptional repressors (PcG) and activators (trxG) of *Hox* genes in *Drosophila*⁴⁴. Later, these proteins were shown to regulate many biological processes such as cell fate and lineage, cellular memory, stem cell function, and tissue homeostasis in cell lines and mouse models^{45–48}. The deregulation of Hox genes via Polycomb or trithorax proteins can lead to leukemogenesis by misregulation of hematopoiesis. Furthermore, PcG members EZH2, a human homolog of *Drosophila* E(z) protein, EED (Esc in *Drosophila*), SUZ(12) (*Drosophila* Su(z)12) and BMI-1 (homolog of *Drosophila* Psc) have been shown to have a role in different cancers in knock out studies carried out in cell lines as well as mouse model^{49–52}. Mixed Lineage Leukemia (MLL), a human homolog of *Drosophila* Trithorax (Trx) protein, regulates *Hoxa* expression in HSCs. MLL is a frequent fusion protein partner in acute leukemia⁵³. Evidence for the role of PcG and trxG genes in regulating HSC development in *Drosophila* remains largely to be explored^{54,55}.

In this study, we show that overexpression of the Hox genes, *Dfd*, *Ubx* and *abd-A* in blood cells not only leads to melanised pseudo-tumors, but also to a significant increase in blood cell number and the induction of lamellocyte differentiation. Further, we present genetic evidence to show the role of PcG members, *Psc* and *Esc*, in the melanised pseudo-tumor formation induced by Hox genes. These findings will be helpful in understating the biological events associated with leukaemia in humans, which may open new possibilities of markers and therapy.

Methods

Fly strains and culture: Flies were cultured in standard cornmeal and sucrose agar. The wild-type flies used in this study were Canton-S. Flies were maintained at 25°C. For all experiments, flies were allowed to lay eggs for 6 hours before being transferred to a fresh vial. Larvae were screened and used for immunohistochemistry at 96-102 hours post egg laying, before the onset of metamorphosis. Supplementary Table S1 and S2 list the fly stocks used in this study.

Larval screening for percent penetrance and severity of the phenotype: For the over-expression of different Hox genes, the UAS-Gal4 binary system was used. To assess the effect of PcG and trxG members had in modifying the phenotype, heterozygous mutant lines were recombined with the *Cg-Gal4* driver (Supplementary Table S2 for all recombined stocks made in the lab). Confirmation of recombination was based on expression of *w+* linked with the *Cg-Gal4* transgene and lethality when backcrossed with the mutant line. Recombined mutants with *Cg-Gal4* were maintained over the *CyO-GFP* balancer for GFP screening. Third chromosome mutants were crossed with homozygous *Cg-Gal4* lines and maintained over TM6B for screening via the *Tubby* phenotype. Experimental crosses were set between recombined strains (*Cg-Gal4* with mutant) and *UAS-abd-A* at a density of 12 females and 6 males for each cross. Egg lay was allowed for 6 hours and progeny were collected after 96 hours post egg lay, at the L3F stage. Screening was done using a stereomicroscope. Penetrance was calculated by calculating the percentage of melanotic pseudo-tumor manifesting larvae. Severity of the phenotype was assessed visually. One-way ANOVA (Dunnett's multiple comparisons) was performed to test the significance.

Pupal lethality count: To assess the pupal lethality, larvae were allowed to develop into pupae and were observed beyond 10 days post egg-lay. Eclosed progenies were considered as survivors. Dead pupae were counted manually. For heterozygous mutant experimental pupae, larvae were first screened to confirm the presence of the *Cg-Gal4* driven expression of *UAS-abd-A* and the presence of the mutation before being transferred to fresh vials. Second chromosome mutants were confirmed by selecting non-GFP larvae while third chromosome mutations were *Tb*⁺

Immunostaining and cell quantification: For staining proliferative cells we made use the M-phase marker, Anti-PhosphoHistone 3 at serine 10, from Upstate (cat# 07-212, 1ng/μL). For confirming the presence of lamellocytes, we used anti-myospheroid (DSHB #CF.6G11, 27pg/μL). Blood cells were prepared using an established protocol⁵⁶. Blood cells numbers were quantified using a modified version of the protocol by Petraki, Alexander, & Bruckner, 2015⁵⁷. Larvae were dissected in 4mm wells, their hemolymph allowed to settle down, before being fixed with 1% formaldehyde and stained with DAPI. Each well was scanned using an Olympus IX83 at 20X, with 32 images stitched. Cells were quantified using CellProfiler by counting individual nuclei. Significance was tested using an unpaired t-test with Welch's correction between control and overexpression genotypes.

Visualisation of lymph glands in larvae over expressing Hox genes and Quantification of relative GFP levels: Larvae were grown as described above. Virgin *Hml-Gal4*, *UAS-GFP* flies were used to drive the expression of the individual Hox genes. *Cg-Gal4*, *Hml-Gal4*, *He-Gal4* and *Lsp2-Gal4* lines were crossed with *mcd8-GFP* lines. Larvae were harvested and visualised under a Zeiss Axiozoom.V16 for GFP. For comparison between *He-Gal4*, *Hml-Gal4* and *cg-Gal4*, whole larval maximum intensities of *He-Gal4* and *Hml-Gal4* were compared with regions devoid of the fatbody in *cg-Gal4*. For comparison between *cg-Gal4* and *Lsp2-Gal4*, whole larval maximum intensities were compared.

Results

Tumor phenotype correlates with the tissue specificity and strength of the driver:

In *Drosophila*, the *collagen-Gal4* (*Cg-Gal4*) driver induces the strong expression UAS tagged genes in the fatbody as well as in the hematopoietic system⁵⁸. The different UAS Hox genes lines, *Dfd*, *Ubx*, *abd-A* and *Abd-B*, when brought under the *Cg-Gal4* driver, induced melanised pseudo-tumors in larvae. This phenotype manifested in 26% of *Cg-Gal4>UAS Dfd* larvae, 60% of *Cg>Ubx* larvae, 82% of *Cg-Gal4>UAS abd-A* larvae and 4% of *Cg-Gal4>UAS-Abd-B* larvae (Figure 1, 2A, 2B and Supplementary Table S3).

We then used the *Hemese-Gal4* (*He-Gal4*) driver, which expresses throughout the lymph gland as well as in circulating hemocytes, and the *HemolectinD3-Gal4* (*HmID3-Gal4*) driver, which expresses in the cortical region of the lymph gland as well as in mature circulating hemocytes. While melanised pseudo tumours were observed in these genotypes, they appeared smaller and the penetrance of the phenotype was very low, manifesting in 3% of *He-Gal4>UAS Dfd*, 6% in *HmID3-Gal4>UAS Dfd*, 9% in *He-Gal4>UAS Ubx*, 2% in *HmID3-Gal4>UAS Ubx*, 8% in *He-Gal4>UAS abd-A*, 4% in *HmID3-Gal4>UAS abd-A*, 3% in *He-Gal4>UAS Abd-B* and 2% in *HmID3-Gal4>UAS Abd-B* (Figure 1, 2A, 2B and Supplementary Table S3). *He-Gal4* induces expression throughout the lymph gland and in sessile cell pockets which reside underneath the larval cuticle. Thus, it expresses in all areas involved in hematopoiesis⁵⁹. Over-expression of *Hox* genes with the *He-Gal4* driver always showed a higher penetrance of the phenotype when compared to *HmID3-Gal4*. Lamellocytes are responsible for the encapsulation mechanism in combating an immune challenge, and they do not express *Hemolectin*. The low penetrance of the phenotype in *HmID3-Gal4* could be due to a lack of expression in lamellocytes⁶⁰. Also, *Hemolectin* does not express in the medullary zone of the lymph gland, where cell proliferation and differentiation takes place⁶¹. It shows the phenotype is associated with active proliferation and differentiation of hemocytes of developing larvae. To test that the phenotype was not due to expression of the Hox genes in the fatbody (as *Cg-Gal4* expresses in both blood cells as well as the fatbody) we over-expressed these genes using the fatbody specific driver *Lsp2-Gal4*. *Lsp2-Gal4* functions in L3 larval fat bodies⁶². No melanised spots were observed in such larvae, indicating that the pseudo-tumor phenotype is not induced by the misexpression of of Hox gene in the fatbody. To test whether the relative strength of the Gal4s, we overexpressed mcd8-GFP under *Cg-Gal4*, *Hml-Gal4*, *He-Gal4* and *Lsp2-Gal4*. *Hml-Gal4* was significantly weaker than *He-Gal4* and *cg-Gal4*. *He-Gal4* and *cg-Gal4* appear to drive expression at similar levels. However, as we compared whole larvae of *He-GFP* expressing larvae to regions devoid of the fatbody in *cg-Gal4* larvae, this similarity may be artefactual (Figure 7A, Supplementary table 11A). *Lsp2-Gal4* and *cg-Gal4* drove GFP at similar levels in the fatbody (Figure 7B, Supplementary table 11B). of the respective drivers in hemocytes (Figure 1, 2A, 2B and Supplementary Table S3). Taken together, this implied that the melanised pseudo-tumour phenotype we observe is of hemocyte origin.

Tumor phenotype is co-related with lethality at the pupal stage:

We also noticed a significant level of pupal lethality when *Hox* genes were misexpressed in these conditions. Pupal lethality with the *Cg-Gal4* driver was highest when it drives *UAS-abd-A* (99 %). *Cg-Gal4>UAS Dfd* (53%) and *Cg>Ubx* (24%) also show an increased lethality at pupal stage. It was negligible

in *Cg-Gal4>UAS-Abd-B* (2%). We observed lethality when the same genes were over expressed in the fatbody with *Lsp2-Gal4*. However, *Lsp2-Gal4* driven Hox expression induced lethality was lower compared to *Cg-Gal4* driven Hox expression induced lethality. But it must be noted that it was greater than that induced by the blood specific drivers used by us. Pupal lethality with *Lsp2-Gal4* driver was observed 9% in *Lsp2-Gal4>UAS Dfd*, 26% in *Lsp2-Gal4>UAS Ubx* and 31% in *Lsp2-Gal4>UAS abd-A*. It has previously been shown that aberrant blood cells can induce pupal lethality⁶³. However, while we did observe some pupal lethality when the Hox genes were expressed under *He* and *Hml*, the lethality was most prominent in when the *Cg-Gal4* or *Lsp2-Gal4* driver was used (Figure 2B, Supplementary Table S4) which supports the earlier report suggesting that Hox genes are repressors of autophagy in the fatbody²⁹. Thus, while we do observe insignificant lethality with blood specific drivers since the expression of Hox genes in the fatbody does indeed induce lethality, the greater lethality when *Cg-Gal4* is used may be due to the concomitant expression induced in the fatbody as well as blood cells.

Hox genes over-expression induces hemocyte proliferation and differentiation:

Change in number cells and types of cells become important considering the phenotype observed upon misexpression of Hox genes. We quantified the number of blood cells in our overexpression lines using a modified version of established methods^{56,57}. When expressed by blood specific driver, *Dfd*, *Ubx* and *abd-A* led to a significant increase in the number of circulating hemocytes (Figure 3A and 3B, Supplementary Table S5-8). Interestingly, while the penetrance of melanised spots was lower, blood specific drivers showed a larger number of blood cells (Figure 3B). Under the control of, *Lsp2*, the fatbody exclusive driver, however, *Ubx* and *abd-A* gave a significant increase in hemocyte number, despite them not manifesting melanised spots. Our results show that melanised spots (or pseudo-tumors), which have been reported as the hallmarks of a “leukemia-like” phenotype in *Drosophila*, may not reflect an actual increase in hemocytes. Additionally, many studies have used the strong driver *Cg-Gal4*, which drives expression in fatbody as well as the blood cells. As our results show that perturbations in the fatbody may indeed lead to an increase in circulating hemocytes. It may also be that the number of circulating cells when we expressed the hox genes under *cg-Gal4* may be due to circulating cells being trapped in the melanised pseudo tumors.

Previous studies have shown that cells of the LG do not enter into circulation until the onset of metamorphosis. However, *Hml* and *Cg* express in the cortical region of the LG, and *He* expresses throughout. Thus, the question arose as to whether the increase in cell number was due to an increase in cell proliferation at the LG or were circulating cells proliferating in a cell-autonomous manner. Hence, we checked for the presence of the mitotic cell marker PH3. We observed cells positive for PH3, when Hox genes were expressed in the blood cells, and not when expressed exclusively in the fatbody (Figure 3A, Supplementary Figure 1A-D). Unlike previous reports, we did not find proliferative cells in our control experiments⁶⁴. This may be due to a loss of cells in our preparations or more robust immunostaining on our part. Thus, while we cannot rule out the possibility that LG cells contribute to this increase, at least a fraction of the increase takes place due to the cell autonomous division of Hox overexpressing cells. As cells of the LG could potentially prematurely be released into circulation on account of the Hox gene over

expression, we checked for the integrity of the LG by overexpressing *UAS-Dfd*, *UAS Ubx* and *UAS-abd-A* in an *Hml-Gal4*, *UAS-GFP* background. LGs remained intact 96hrs post egglay (Figure 6)

While imaging the blood cells, we noticed that there were larger, flattened cells in circulation, reminiscent of lamellocytes. To test whether they were bonafide lamellocytes, we stained the hemocytes for the lamellocyte marker myospheroid (Figure 4A, Supplementary Figure 1A-D). Control larvae infrequently showed the presence of lamellocytes. In our overexpression lines, however, we noticed that a significant number of cells were lamellocytes *mys*⁺. Some plasmatocytes also stained positive for *mys*. None of the plasmatocytes in the control flies or those overexpressing *Abd-B* were positive for *mys*. Previous reports have suggested that circulating plasmatocytes may differentiate into lamellocytes^{65,66}. Thus, it may be that these circulating *mys*⁺ plasmatocyte like cells are differentiating into lamellocytes. However, *Lsp2-Gal4>UAS Ubx* also had a significant number of lamellocytes. This is in keeping with reports that signals from the fat body can drive lamellocyte differentiation^{67,68}. Thus, we speculate that these cells, upon Hox overexpression, are pushed toward the lamellocyte fate (Figure 4A, 4B).

Effect of PcG and trxG genes

PcG members are known to function primarily through two distinct complexes, PRC1 (consisting of Pc, Psc, Su(z)2 and Sce) and PRC2 (consisting of E(z), Su(z)12, Esc and Caf 1-55)⁶⁹. Members of the PcG and trxG have been shown to have a role in hematological malignancies in different clinicopathological data in leukemic patients and mice models^{70,71}. To determine their role in melanized pseudo-tumor formation in flies, we over-expressed *abd-A* using *Cg-Gal4* in the background of different PcG and trxG mutants. We selected *Psc*¹, *Pc*¹, *Su(z)2*, *Su(z)12*, *E(z)* and *esc2* from the PcG and *brm*², *Trl* from the trxG. Melanotic pseudo-tumor phenotype was used in our study to assay the effect of the mutants as it is convenient and robust. All experiments were performed in biological triplicates. The PcG mutants *Pc*¹, *Su(z)12*³, and trxG member *brm*² showed an increase in melanotic body formation (Figure 5A, 5B and Supplementary Table S9), and enhanced the phenotype upto 100 per cent. *Pc*¹ and *Su(z)12*³ not only enhanced the penetrance (percentage phenotype showing larvae) but showed an increase in severity (scored as number and size of the black spots) compared to *abd-A* over-expressed in absence of mutants (Figure 5A). Pc and Su(z)12, both are the core proteins of PRC1 complex and play a role in negative regulation of their target genes. Our results indicate these proteins might regulate melanotic body formation. Surprisingly, *E(z)* does not show any significant effect on penetrance. On the other hand, *esc*² (PRC2 member) and *Psc*¹ (PRC1 member) showed a significant decrease in penetrance 15% and 17% respectively (Figure 5B, Supplementary Table S9). The severity of the phenotype is also reduced in both the mutant background. These results indicate that genes involved in melanotic pseudo-tumor causing phenotype might be the target of the Esc and Psc proteins. Although it has been shown that Esc-E(z) complex is a thousand times effective to *E(z)* alone^{72,73}, our results suggest that Esc regulates its targets independent of *E(z)* activity or, for that matter, any other member of the PRC2 complex in the observed phenotype. Similarly, *Psc* mutation rescued the phenotype. We tested whether bringing our overexpression in the PcG and trxG backgrounds affected the number of PH3 positive nuclei. We did not

observe a significant change (Figure 5D, Supplementary table 12). As the average number of PH3+ nuclei in *cg-Gal4>UAS abd-A* larvae was 0.08% of the average of total hemocytes, it may be that the total number of dividing nuclei are too few to significantly differ.

Effect of PcG mutants on the melanized pseudo-tumor related pupal lethality:

To test the effect of mutations on pupal lethality, L3F larvae from each combination, which manifested melanised pseudo-tumours, were transferred to fresh vials and allowed to pupate and eclose. Larvae from overexpressed *abd-A* (driven by *Cg-Gal4*) with melanotic body showed up to 99% lethality at the pupal stage. Further, we checked pupal lethality in mutant background. Since all mutants are maintained over balancers (Table S2), we selected overexpressed progenies without balancer to confirm mutant in the same progeny and transferred them in new food vials. Pupal lethality in *Su(z)12³*, *Pc¹* and *Su(z)2^{1.a1}* was always 100% while we could get a few survivors from *Cg-Gal4>UAS abd-A* (Figure 3C, Table S10). A decline in lethality was seen in *Psc¹*, *esc²*, *brm²* and *Trl^{R85}*. The survivors from *Psc¹* and *esc²* were quite healthy as compared to the survivors of *Cg-Gal4>UAS abd-A*. This reduction in lethality indicates that Esc and Psc proteins are strongly suppressing the melanotic pseudo-tumour phenotype and its consequences on development. Although *brm²* showed an increase in penetrance it decreases pupal lethality 89% compare to *abd-A* alone. *Trl^{R85}* showed a decrease in pupal lethality (79%).

Conclusion

Homeotic genes or Hox genes determine the cell identity across the anterior-posterior body axis early during development, a function that is conserved across all bilaterians. The later functions played by these genes, however, are less well studied. A number of reports indicate that they play a variety of non-homeotic functions later in development. Our lab has shown that *abd-A*, one of the three Hox genes of the bithorax complex of *Drosophila*, acts as a growth promoter in Histoblast Nest Cells⁷⁴. We show this to be a normal additional function of *abd-A* which involved adult cuticle formation during pupation. In the present study, we tested four Hox genes of *Drosophila* by ectopically expressing them in the blood cells and show that they are capable of inducing melanised bodies in circulation. These melanotic spots appear only when expressed in the lymph gland and circulating blood cells. The ectopic expression of the Hox genes also triggers cell proliferation. The cells appear to divide in a cell-autonomous manner, which is reflected in the detection of PH3+ cells in circulation. The presence of *myospheroid* positive elongated cells, seen in circulation, also suggests that Hox overexpression leads to the differentiation of the circulating blood cells into lamellocytes. Overexpression of *abd-A*, shows a relatively stronger phenotype while *Abd-B* overexpression does not. It supports an earlier finding in which we observed a non-homeotic growth promoter role of *abd-A* but not of *Abd-B* during the formation of the adult cuticle during pupation⁷⁴.

Our results indicate that Hox genes are causal in leukaemia, reinforcing previous studies in vertebrate model systems, and extending these findings to *Drosophila*. This also opens the possibility that Hox gene induced leukaemias, especially those of the myeloid lineage, can be studied and modelled in *Drosophila*.

Till date, the only known Hox gene to participate in *Drosophila* hematopoiesis is *Antp*, which marks in the PSC, and provides spatial signals for the development of the LG. In vertebrates, Hox genes have been shown to express within progenitor cells and are rapidly switched off during cell maturation. As our overexpression lines perturb both cell number and differentiation, it is possible that multiple *Drosophila* Hox genes are involved in finetuning the precise programme of *Drosophila* blood cell development as well.

The fact that the cells appear to be phenotypically confined to plasmatocytes and lamellocytes implies that expression of these genes works in tandem with, and above the specific programme of the cell types. It would be interesting to know which genes are being modulated in our overexpression lines, by profiling their transcription states as well as the binding sites of the individual Hox proteins. In the absence of this information, we speculate that Hox gene overexpression leads to the aberrant transcription of genes. It is known from previous studies that Hox dysregulation in leukaemia is usually concomitant with gain or loss of function mutations in upstream regulators, most commonly in Mixed Lineage Leukemia-1 (MLL-1) fusion proteins^{75,76}, or loss of function Enhancer of Zeste Homolog 2 (EZH2) mutations⁷⁷. It has been reported that EZH2 mutations have the lowest number of co-operating mutations to induce leukemogenesis⁷⁸.

Interestingly, we observe polycomb members *Psc* and *Esc* have a role in suppressing melanised pseudo-tumour formation. Both *Psc* and *Esc* mutants rescued the phenotype significantly which suggests that tumour suppressor genes may be their targets for repression. However, while there is evidence that Hox overexpression in vertebrate blood cells do induce leukemia, the modulation of the phenotype by PcG and trxG mutant backgrounds may be due to the differential regulation of immune genes in the overexpression background. As many Hox induced leukemia occur in the background of PcG loss of function and trxG gain of function backgrounds, this may lead to the differential accessibility of the overexpressed transcription factor to immune genes, thus either enhancing or suppressing the phenotype. BMI1, a mammalian counterpart of *Psc*, was discovered as a proto-oncogene. Overexpression of BMI1 in mouse models has been shown to induce both types of leukaemia (lymphoid and myeloid origin)^{53,79,80}. BMI1 cooperates with *c-myc* in the generation of lymphomas. MYC is a transcription factor that regulates many cellular processes, including proliferation and apoptosis. c-myc is an attractive target for chemotherapy as it has a role in multiple converging signalling cascades. In many cancers, MYC protein is overexpressed due to various processes like translocation, duplication or epigenetic misregulation⁸¹⁻⁸³. BMI1 regulates MYC protein expression by inhibiting its downstream target ink4a-ARF, a tumour suppressor gene⁸⁴. The role of EED, a mammalian homolog of *Drosophila Esc*, in leukaemia is not very well understood. Mutations in EED have been indicated to impair polycomb complex (PRC2) functionality and it is associated with myelodysplastic neoplasm^{85,86}. While PcG and trxG genes are known to function in a complex, results in our lab indicate that they may have functions outside their canonical pathways, which would explain why genes within similar complexes elicit different effects on our overexpression backgrounds^{87,88}.

Although *Drosophila* does not have *ink4a* homolog, it will be interesting to see the mechanism of regulation of melanotic tumor formation and over-proliferation of blood cells by Psc. Taken together, we speculate that Hox gene activation in hemocytes causes cell-autonomous proliferation and differentiation and induces leukaemia via aberrant transcription.

In summary, *Drosophila* the Hox genes *Dfd*, *Ubx* and particularly *abd-A*, when expressed in blood cells, are leukemogenic. This link of Hox genes to the pseudo-tumor phenotype supports the non-homeotic role of *abd-A* as a growth promoter later during development. The disease phenotype is modified by select PcG/trxG members. This reinforces previous studies in vertebrates that report the misregulation of Hox genes in several cancers and implicate the role of epigenetic factors in them. Hox induced leukaemia in *Drosophila* offers advantages of the fly model to explore the biology of the process and develop novel potential markers and therapeutic options.

Abbreviations

PH3	Phospho Histone 3 at Serine 10
LG	Lymph Gland
PcG	Polycomb Group of Proteins
TrxG	Trithorax Group of Proteins
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2

Declarations

-Ethics approval and consent to participate- Not applicable

-Consent for publication

All the authors have consented for publication of this work.

-Availability of data and material

Data and material are available on request.

-Competing interests

Authors declare no competing interests.

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-Authors' contributions

TP carried out experiments, contributed to the design, interpretation of the experiments and writing the manuscript, RS carried out experiments on epigenetic effects, and RKM conceived the project, wrote the manuscript and supervises the work.

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Figures

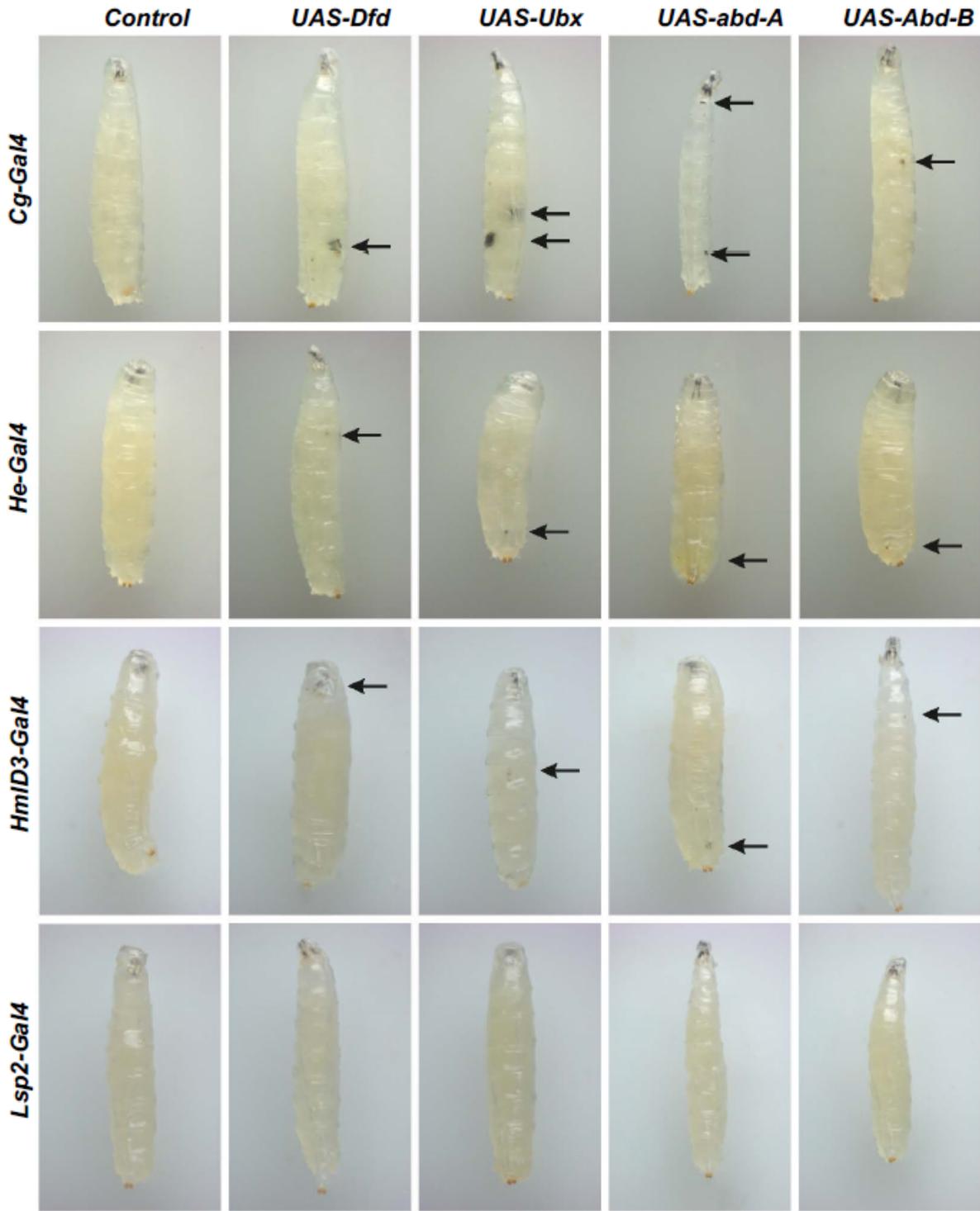


Figure 1

Larvae with subcutaneous tumors. *Dfd*, *Ubx*, *abd-A* and *Abd-B*, when expressed under the drivers *Cg*, *He* and *HmI D3* lead to melanised bodies in the viscera.

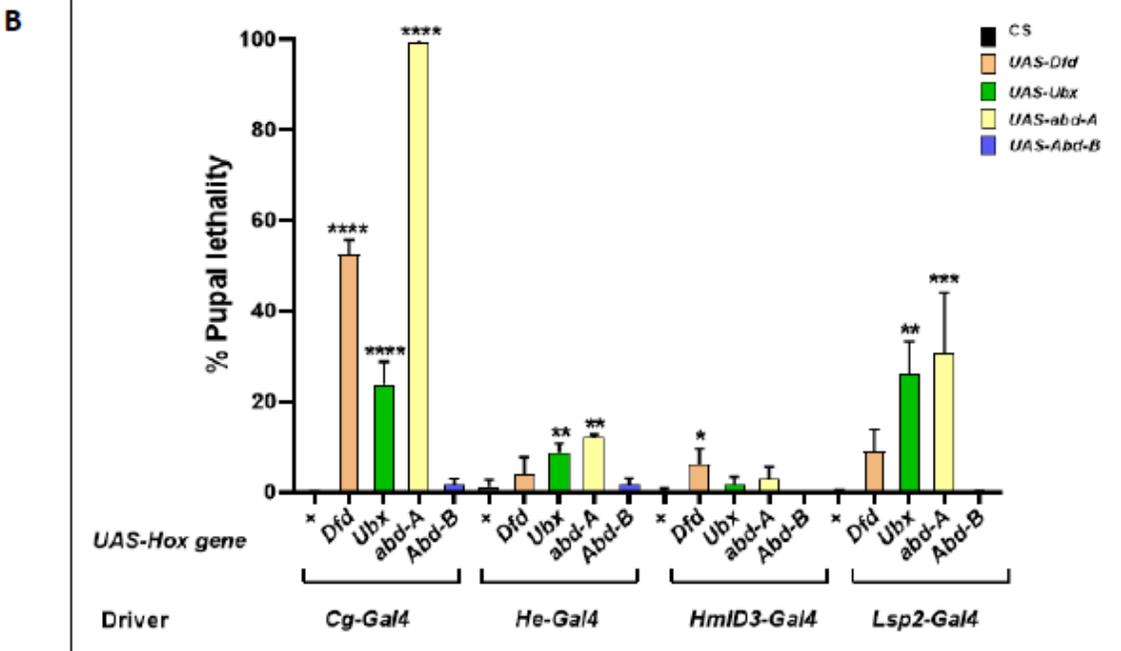
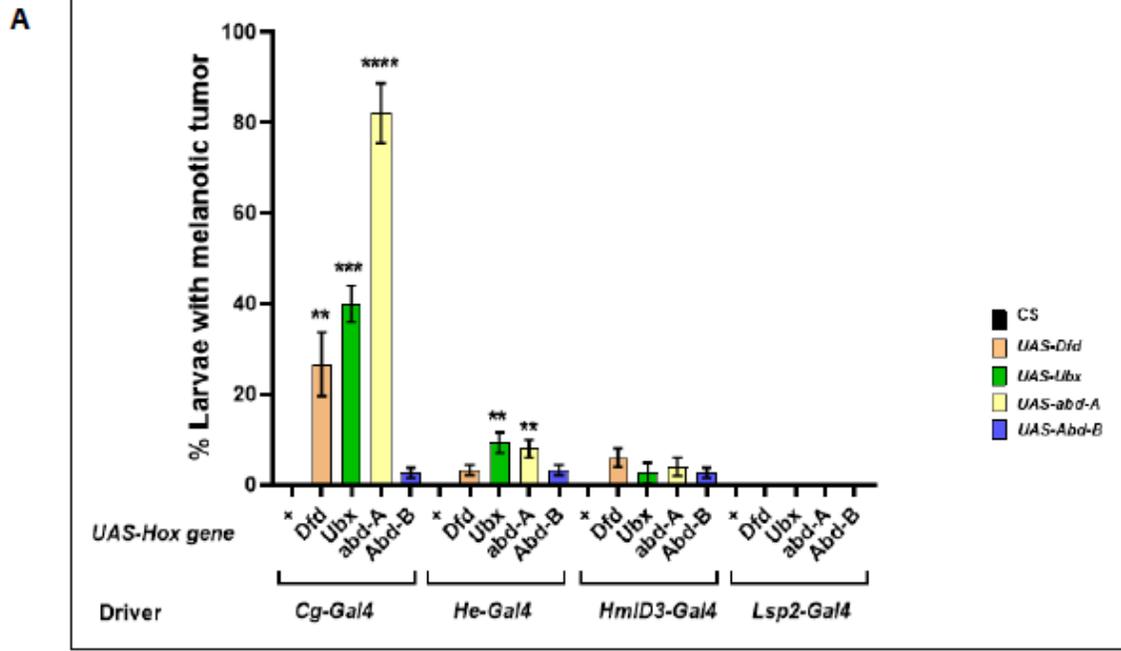


Figure 2

Tumor phenotype in larvae and pupal lethality. A) The size and penetrance of melanised bodies was maximum when expressed under Cg, while tumors do manifest when He and Hml are used, they are much rarer and smaller. Expression under Lsp2-Gal4 does not lead to the formation of such bodies. B) Percentage of pupal lethality, indicated by larvae that fail to eclose. When Cg-Gal4 drives the genes Dfd, Ubx, abd-A and Abd-B do cause lethality, so does expressing them in the fatbody under Lsp2-Gal4. Driving

increases significantly when Dfd is over expressed using Cg-Gal4 ($p=0.0003$), He-Gal4 ($p<0.0001$) and HmID3-Gal4 ($p<0.0001$), Ubx when over expressed with Lsp2-Gal4 ($p<0.0001$) and HmID3-Gal4 ($p=0.0011$), abd-A when over expressed with Cg-Gal4 ($p<0.0001$), Lsp2-Gal4 ($p=0.0028$), He-Gal4 ($p=0.0018$) and HmID3-Gal4 ($p=0.0002$), Abd-B when over expressed with Cg-Gal4 ($p<0.0001$). B) PhosphoHistone3+ nuclei appear when these genes are expressed in the blood cells, indicating that some of the increase in cell number maybe due to cell autonomous proliferation.

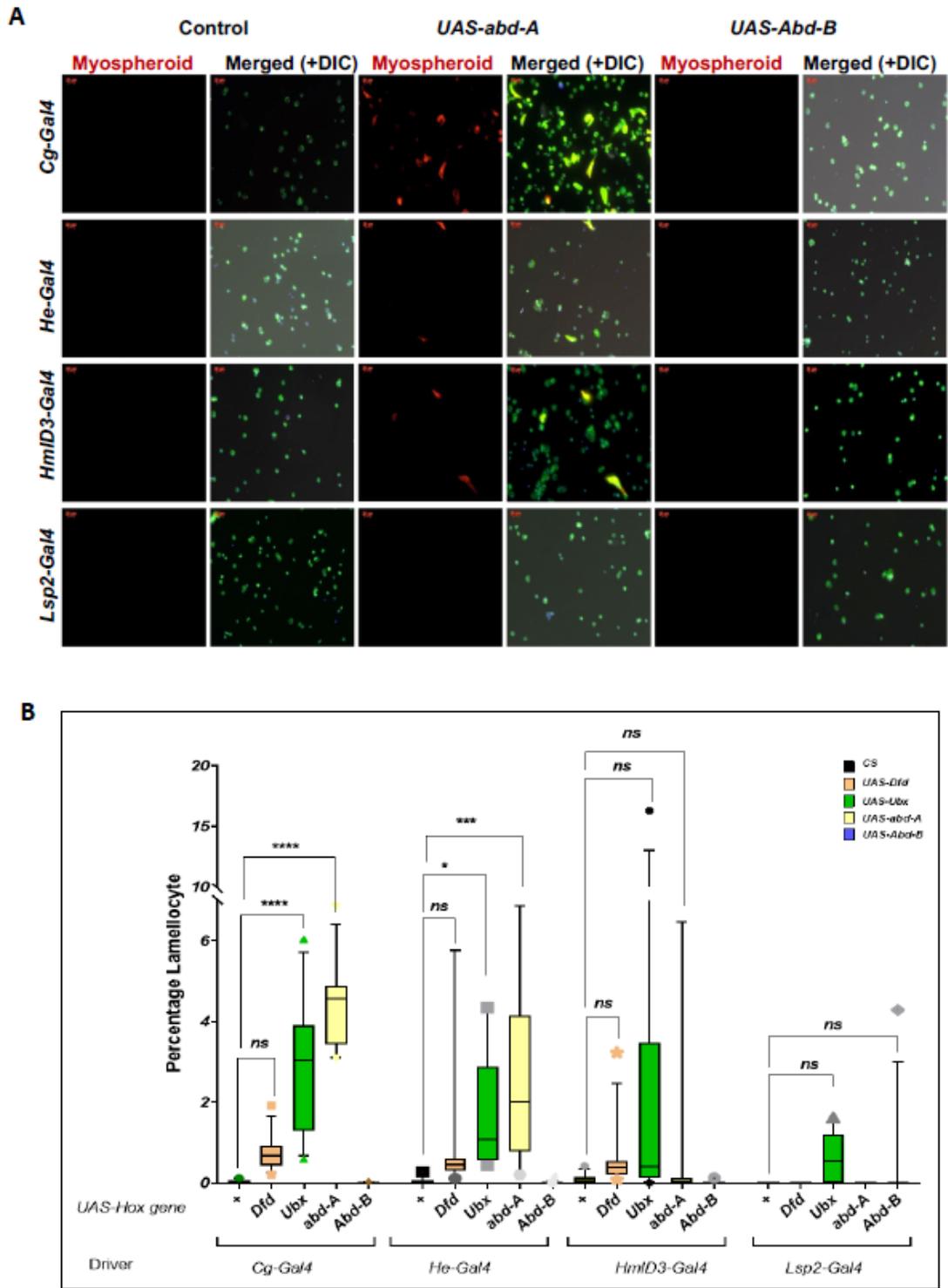


Figure 4

Myospheroid staining and quantification of lamellocytes. A) When Dfd, Ubx, abd-A, but not Abd-B are driven in blood cells (under Cg-Gal4, He-Gal4 or Hm1D3-Gal4), but not in the fatbody (Lsp2-Gal4), large, dorsoventrally flattened cells begin to appear in circulation. These stain positive for mys. Some circulating plasmatocytes also appear to mys+. This indicates that they might be in the process of differentiating. B) Percentage of lamellocyte is plotted on Y-axis. Ubx over-expression with Cg-Gal4 ($p < 0.0001$) and He-Gal4 ($p = 0.0455$) has a significant increase while Lsp2-Gal4 and Hm1D3-Gal4 do not show any significant increase. abd-A with Cg-Gal4 and He-Gal4 has comparatively high percent of increase to control as well as Ubx over-expressed.

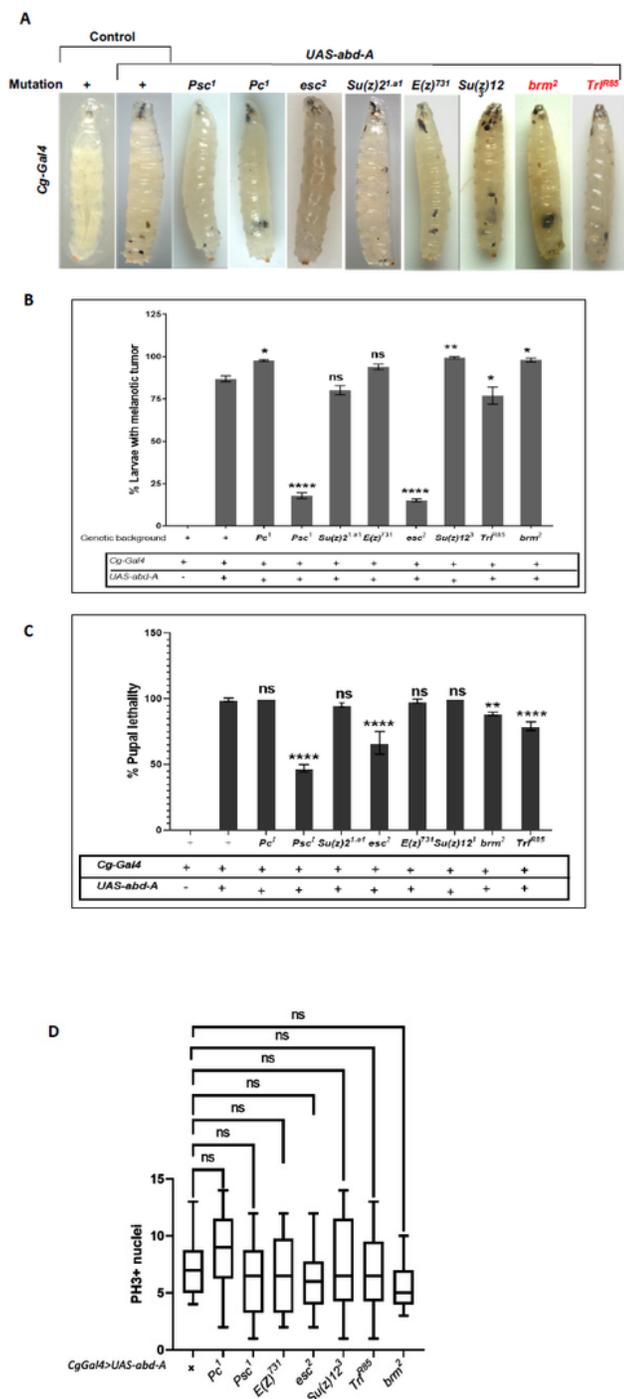


Figure 5

Effect of PcG and trxG mutations on the *abd-A* induced tumor phenotype. A) Effect of PcG and trxG on subcutaneous melanotic tumor formation. (trxG members have been highlighted in red in 5A) Over-expression of *abd-A* gene is driven by *Cg-Gal4*. Most of PcG and trxG mutants have effect on the phenotype. Polycomb mutants, *esc2* (PRC2 member) and *Psc1* (PRC1 member) show a decrease in severity of phenotype (size and numbers of melanotic body). *Su(z)123* and *Pc1* have increased severity.

Brm2, TrlR85, Su(z)21.a1 and E(z)731 do not show any change in severity compare to Cg-Gal4 driven *abd-A* over-expressed individual. B) Comparative quantification of melanotic tumor formation phenotype in *abd-A* over-expressed (driven by Cg-Gal4) individual in different PcG and *trxG* background. Percentage of tumor showing individuals is plotted on Y-axis (no. of animal screened is >80 in each case, error bars represent the standard error). Phenotype is rescued in *esc2* ($p < 0.0001$), *Psc1* ($p < 0.0001$), and *TrlR85* ($p = 0.0223$) mutants while *Pc1* ($p = 0.0109$), *Su(z)123* ($p = 0.0028$) and *Brm2* ($p = 0.0088$) have a significant increase in melanotic tumor formation. C) Percent pupal lethality in PcG and *trxG* mutant background. *Psc1* ($p < 0.0001$), *esc2* ($p < 0.0001$), *brm2* ($p = 0.0042$) and *TrlR85* ($p < 0.0001$) show decrease in pupal lethality. D) Number of PH3 positive larvae in mutant backgrounds, Compared to Cg-Gal4 overexpression of *abd-A* alone, the overexpression did not significantly alter the number of PH3+ hemocytes in *Pc1* ($p = 0.2554$), *Psc1* ($p = 0.1275$), *E(Z)731* ($p = 0.7907$), *esc2* ($p = 0.3282$), *Su(z)123* ($p = 0.8642$), and *TrlR85* ($p = 0.8975$) backgrounds.

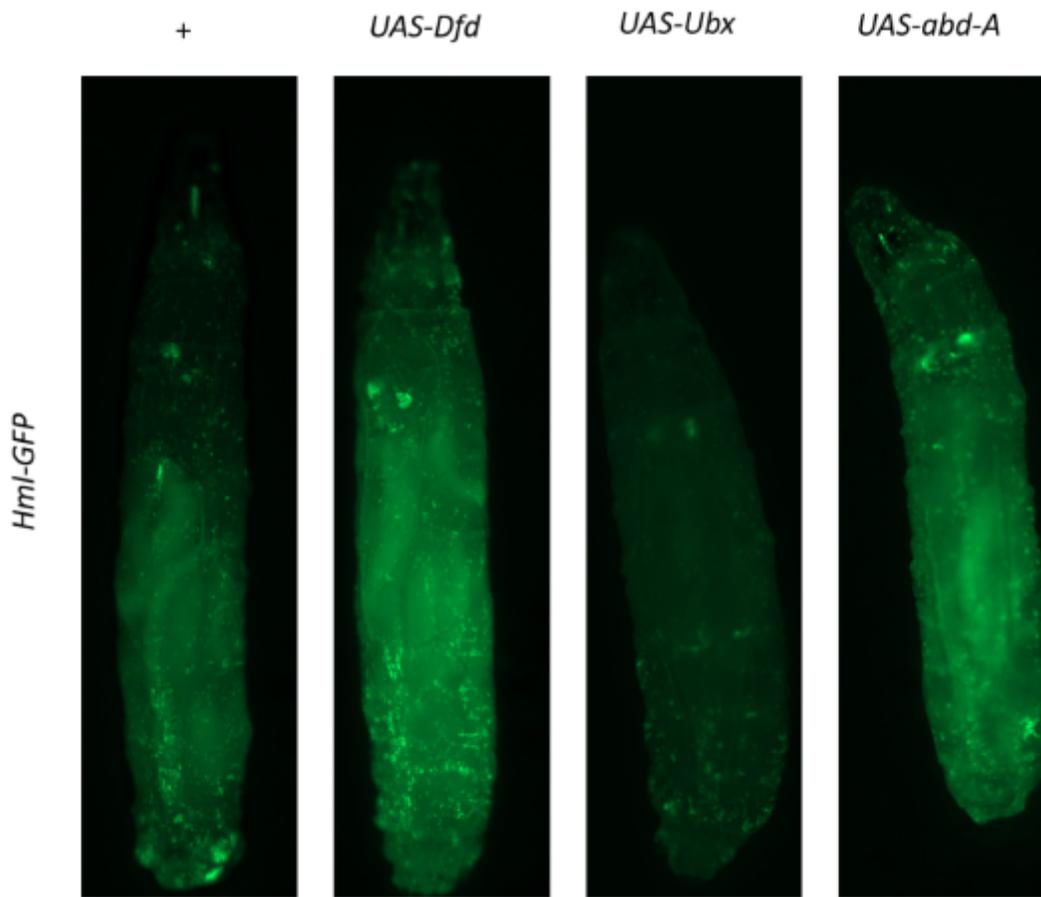


Figure 6

Visualisation of lymph glands in larvae over expressing Hox genes. *UAS-Dfd*, *USA-Ubx* and *UAS-abd-A* were over expressed in a *Hml-Gal4*, *UAS-GFP* background, to test whether overexpression of the genes led to the premature release of cells resident in the cortical zone of the lymph gland. The cortical zones of the over expression larvae appeared to be GFP positive.

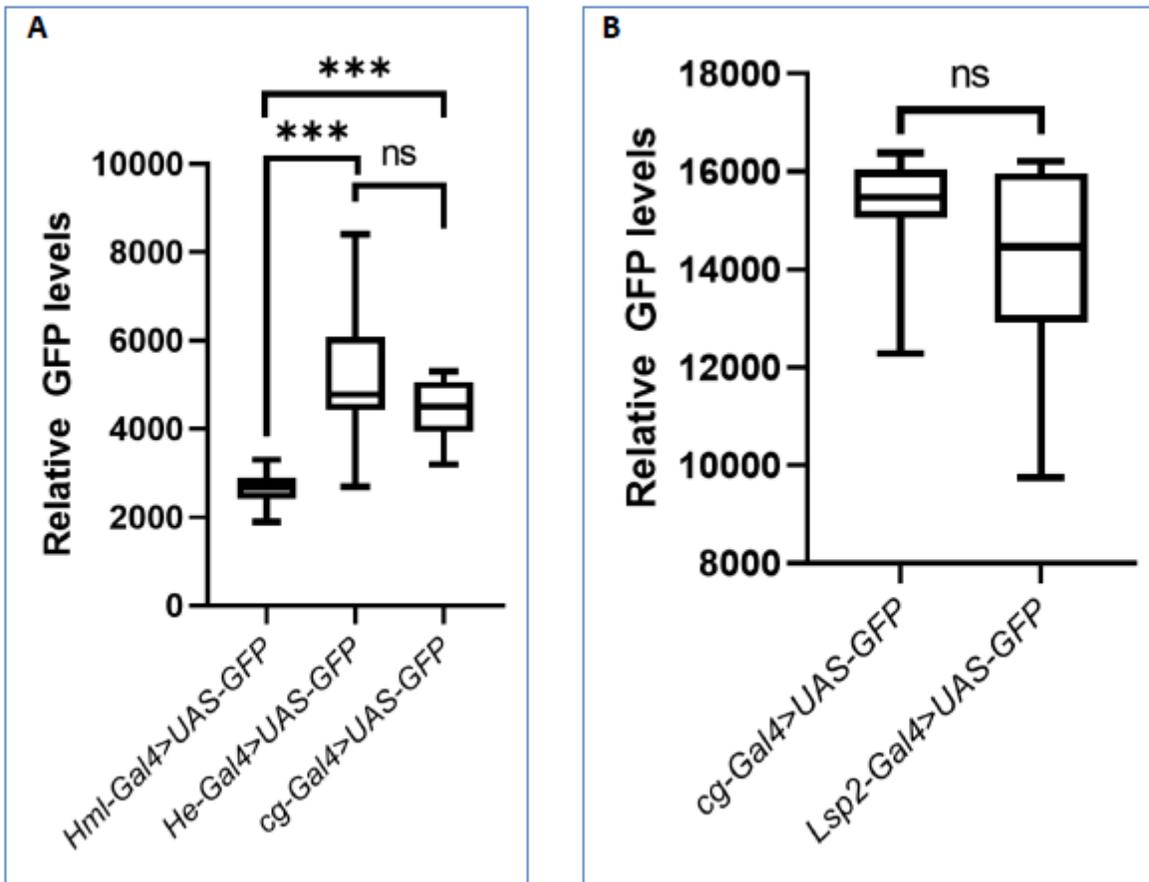


Figure 7

Quantification of relative Gal4 strength by relative GFP levels. Hml-Gal4, He Gal4, cg-Gal4 and Lsp2-Gal4 were used to drive the expression of UAS-mcd8-GFP. A) Average maximum GFP intensities of whole larvae of Hml-Gal4>UAS mcd8-GFP and He-Gal4>UAS mcd8-GFP were compared with regions excluding the fat body in cgGal4>UAS mcd8-GFP. The Hml-Gal4 driver was weaker than He-Gal4 ($p < 0.0001$) as well as cg-Gal4 ($p = 0.0005$). He-Gal4 was not significantly different from cg-Gal4 ($p = 0.1903$) B) Average maximum intensities of whole larvae of cg-Gal4>UAS mcd8-GFP were compared with Lsp2-Gal4>UAS-GFP. GFP levels were not significantly different ($p = .1261$)

Supplementary Files

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