The Role of Variant Histone H2AV in *D. melanogaster* Larval Hematopoiesis

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Summary Statement: The variant histone H2AV is required for the normal differentiation of blood cells in Drosophila larval lymph glands.

ABSTRACT

Replication-independent histone variants can replace the canonical replication-dependent histones. Vertebrates have multiple H2A variant histones, including H2AZ and H2AX that are present in most eukaryotes. H2AZ regulates transcriptional activation as well as maintenance of gene silencing, while H2AX is important in DNA damage repair. The fruit fly *Drosophila melanogaster* has only one histone H2A variant (H2AV), which is a chimera of H2AZ and H2AX. In this study we found that lack of H2AV led to the formation of black melanotic masses in the third instar larvae of Drosophila. The formation of these masses was found in conjunction with a loss of a majority of the primary lymph gland lobes. Interestingly, the cells of the posterior signaling center were preserved in these mutants. Reduction of H2AV levels by RNAi knockdown caused a milder phenotype that preserved the lymph gland structure, but that included precocious differentiation of the prohemocytes located within the medullary zone and secondary lobes of the lymph gland. Mutant rescue experiments suggest that the H2AZ-like rather than the H2AX-like function of H2AV is primarily required for normal hematopoiesis.

INTRODUCTION

Within the nucleus the compaction of chromatin is important in not only protecting DNA from damage but also in controlling access of the transcriptional machinery to the genome (Lorch and Kornberg, 2015). Chromatin compaction is carried out with the aid of highly conserved histone proteins, which are assembled into the nucleosome structure. Each nucleosome is composed of 146 bp of DNA wrapped around an octamer of histones. This octamer includes two molecules each of the histones H2A, H2B, H3 and H4 (Luger et al., 1997). In order for gene expression to occur, the compaction of chromatin must be relieved, allowing transcriptional machinery access to the DNA. One method whereby this is thought to be regulated is with the replacement of the replication-dependent canonical histones with replication-independent histone variants that can alter nucleosome structure and function (Luger et al., 1997).

Two variants of H2A that have been highly conserved in eukaryotes are H2AZ and H2AX (Malik and Henikoff, 2003). H2AZ can be found at gene regulatory regions and has been implicated in both the transcriptional activation of genes and in the maintenance of gene silencing (Billon and Cote, 2013). In contrast, H2AX is important in the DNA damage repair response (Scully and Xie, 2013). In particular, the phosphorylation of the carboxy-terminal tail of H2AX is critical for mediating the assembly of the repair machinery in response to double-stranded breaks in chromosomal DNA. Unlike many other eukaryotes, the model organism *Drosophila melanogaster* has only one H2A variant, H2AV, which is a structural and functional chimera of H2AZ and H2AX (Baldi and Becker, 2013).

Previous studies of ATP-dependent chromatin remodeling enzymes have shown a role for these complexes in Drosophila hematopoiesis (Badenhorst, 2014).

NURF (nucleosome remodeling factor), a chromatin remodeling complex that catalyzes nucleosome sliding, was found to play a role in hematopoiesis. Mutants of NURF display hemocyte-containing black melanotic masses, as well as increases in hemocyte number (Badenhorst, 2014; Kwon et al., 2008). Domino (Dom), a catalytic subunit of the Drosophila TIP60 complex (dTIP60), has also been implicated in the regulation of hematopoiesis. The dTIP60 complex facilitates the acetylation and exchange of the phosphorylated histone 2A variant (H2AVph) with non-phosphorylated H2AV (Kusch et al., 2004). *domino* (*dom)* loss-of-function mutants in Drosophila larvae form black melanotic masses that are composed of necrotic prohemocytes (Braun et al., 1997). These results suggested a role for Dom in prohemocyte survival. This function appears to have been conserved in mice, where a Dom homolog was shown to be important both in primitive hematopoiesis in the embryo and in the maintenance of hematopoietic progenitor cells in the adult bone marrow (Fujii et al., 2010; Ueda et al., 2007).

Given the role that the dTIP60 complex plays in both H2AV exchange and hematopoiesis, we thought it would be informative to study a potential role for H2AV itself in hematopoiesis. Drosophila has three types of hemocytes that are most similar to members of the myeloid lineage of blood cells found in vertebrates (Evans et al., 2003). The most prevalent Drosophila blood cells are the plasmatocytes, which make up 90-95% of the mature hemocytes and are phagocytic in function. The second most prevalent blood cells are the crystal cells, which comprise approximately 5% of the mature hemocytes and contain prominent cytoplasmic granules. Crystal cells aid in melanization, which is important for wound healing and the innate immune response. Finally, the rarest of blood cells are the lamellocytes, which are seldom seen under normal conditions. If an immune challenge is detected, the differentiation of these

large cells can be upregulated drastically. The role of the lamellocytes is to encapsulate and neutralize any objects too large to be phagocytized by plasmatocytes (Evans et al., 2003).

The larval hematopoietic organ, the lymph gland, is a bilaterally symmetric structure that flanks either side of the dorsal vessel or heart tube (Fig. 1). Multiple lobes are located on either side of the heart and are termed primary, secondary and tertiary lobes. The primary lobes contain not only undifferentiated prohemocytes, but also differentiated hemocytes. The secondary and tertiary lobes commonly contain undifferentiated prohemocytes during larval stages. The primary lobes of the lymph gland, which are located at the anterior-most region of the hematopoietic organ, can each be sub-divided into three distinct zones. The differentiated hemocytes are located in the outermost region called the cortical zone. The undifferentiated prohemocytes are located in the central region called the medullary zone. Finally, there is a small subset of cells located in the posterior medial portion of each primary lobe called the posterior signaling center (PSC) (Jung et al., 2005) . It is the role of the PSC to send signals to the other cells of the lymph gland, thereby orchestrating their differentiation. The PSC sends out multiple signals that not only keep the prohemocytes of the medullary zone in an undifferentiated state, but that also lead to the differentiation of hemocytes within the cortical zone (Krzemien et al., 2007; Mandal et al., 2007). We now show that the absence of H2AV causes a variety of hematopoietic abnormalities in Drosophila larvae including the formation of melanotic masses.

RESULTS

Loss of H2AV causes melanotic masses in Drosophila larvae.

Unlike most other eukaryotes studied thus far, *D. melanogaster* has only one histone H2A variant, H2AV. This protein is thought to be a functional chimera of the H2AX and H2AZ proteins present in most other eukaryotes including humans (Baldi and Becker, 2013). Comparison of the Drosophila H2AV protein to the two most common H2A variants in humans reveals that H2AV is much more closely related to human H2AZ than to human H2AX (Fig. 2A-B). However, the carboxy-terminal tail of Drosophila H2AV does contain an H2AX-like SQXY peptide that can be phosphorylated in response to DNA damage (Madigan et al., 2002). The rest of the human H2AX protein is far more closely related to the canonical H2A proteins of human and Drosophila, than to human H2AZ and Drosophila H2AV. These observations are consistent with a model in which the *H2AZ* gene family arose via duplication of and divergence from an ancestral *H2A*-like gene early during eukaryotic evolution (Malik and Henikoff , 2003). On the other hand, genes encoding H2AX appear to have arisen multiple times during eukaryotic evolution. This most frequently occurred by a more recent duplication of an H2A-encoding gene followed by the addition of sequences encoding an SQXY-containing carboxy-terminal tail to one duplicated copy. In *D. melanogaster*, sequences encoding an SQXY- containing tail appear instead to have been added to an unduplicated H2AZ-encoding gene, thereby giving rise to the gene encoding H2AV.

In order to study the role of the *His2Av* gene that encodes H2AV in *D. melanogaster*, we examined $His2Av^{810}$ / $His2Av^{810}$ homozygous null mutants and observed a previously unreported formation of black melanotic masses during the larval stages of the organism (Fig. 2C). $His2Av^{810}$ is a null allele that contains a 311

base pair deletion that removes the region surrounding and including the second exon of the gene (van Daal and Elgin, 1992). Homozygous *His2Av*^{810} / *His2Av*^{810} mutants arrest in development during late third instar or prepupal stages (Kotova et al., 2011). In order to test whether the formation of the melanotic masses was due to the loss of H2AV, rather than some other unknown mutation in the genetic background of the homozygous animals, we crossed the *His2Av*^{810} / + heterozygotes with flies heterozygous for *Df(3R)BSC524*, a large deletion mutant with molecularly defined endpoints in the same region of the genome (Cook et al., 2012). This chromosomal deficiency (Df) lacks 312,855 contiguous basepairs on the right arm (R) of chromosome 3, including the entire *HisAv* gene. In heterozygous *His2Av*^{810} / *Df(3R)BSC524* larvae we observed the formation of similar melanotic masses, implying that the absence of H2AV is indeed the cause of this phenotype (Fig. 2D).

To further test whether the absence of H2AV alone is responsible for the observed melanotic masses, we introduced a transgene expressing an H2AV-RFP fusion protein under the control of the *His2Av* promoter into homozygous *His2Av*^{810} / *His2Av*^{810} animals. No melanotic masses were observed (data not shown). These results again imply that the absence of H2AV is indeed the cause of this phenotype.

The formation of melanotic masses in Drosophila has been described in the past, and has been commonly linked to the immune response (Minakhina and Steward, 2006; Rodriguez et al., 1996). Generally hemocytes undergo melanization as an immune response to a present threat. However, in certain mutants, this response occurs without the presence of any discernable immune challenge (Harrison et al., 1995; Lemaitre et al., 1995; Minakhina and Steward, 2006; Zettervall et al., 2004). In order to determine whether the melanotic masses we observed included hemocytes, and if so, which types of hemocytes, we immunostained the melanotic masses using

antibodies against three different hemocyte cell types (Evans et al., 2014). A Nimrod (P1) antibody was used to detect plasmatocytes, a Prophenyloxidase (Propo) antibody to detect crystal cells, and an Atilla (L1) antibody to detect lamellocytes. We identified plasmatocytes and crystal cells within the melanotic masses (Fig. 2E), but did not find any lamellocytes (Fig. 2F). These results imply that the melanotic tumors observed in the absence of H2AV are composed of hemocytes.

Loss of H2AV results in premature dispersal of the prohemocytes and hemocytes found within the larval lymph gland.

In order to understand whether the absence of H2AV was having an effect on the cells of the lymph gland and whether the lymph gland was the possible source of the hemocytes that resulted in the formation of the melanotic masses, we first looked for the expression of H2AV in normal non-mutant lymph gland lobes. We wished to know whether H2AV would be found at varying expression levels within specific zones and cell types of the lymph gland. However, immunostaining revealed ubiquitous and uniform expression of H2AV in all of the different zones within the primary lymph gland lobes (Fig. 3A). We also looked at H2AV phosphorylation in the lymph gland in order to discern whether significant levels of DNA damage were present. We saw only small numbers of cells within the lymph gland that were positive for H2AVph under normal conditions (Fig. 3B-B'). In order to ascertain whether the cells of the lymph gland might be contributing to the hemocytes found within the melanotic masses that formed in the absence of H2AV, we looked at the primary lymph gland lobes of homozygous *His2Av*^{810} / *His2Av*^{810} and heterozygous *His2Av*^{810} / + mutants.

We found that the heterozygous mutants had larval lymph glands that appeared to be very similar to those of wild type (*WT*) animals. Further investigation using antibodies against specific zones and cell types within the lymph glands of heterozygotes showed a *WT* pattern of expression of these markers (Fig. 3C,E,G). P1 was used to label plasmatocytes within the cortical zone (Fig. 3C), while Antennapedia (Antp) and Prophenyloxidase (Propo) were used to label the PSC and the crystal cells, respectively (Fig. 3E,G).

In contrast to the *WT*-like phenotype seen in heterozygous *His2Av*^{810} / + larvae, examination of the homozygous *His2Av*^{810} / *His2Av*^{810} larvae revealed a structural loss of most of the primary lymph gland lobes (Fig. 3D,F,H). A majority of the differentiated plasmatocytes and crystal cells had dispersed from the primary lobes in homozygous mutant larvae (Fig. 3D,H), along with a majority of the undifferentiated prohemocytes normally found within the medullary zone of the primary lobes (Fig. 3D,F,H). Though some crystal cell staining could still be seen within the primary and secondary lobes of the lymph gland (Fig. 3H), the staining for Propo appeared hazy, suggesting that the crystal cells may have burst and released the Propo protein into the surrounding area.

Interestingly, the one portion of the lymph gland that appeared to remain intact in homozygous *His2Av*^{810} / *His2Av*^{810} mutant larvae was the Antp-positive PSC (Fig. 3F,H). Some of the homozygous mutant larvae did contain ectopic clusters of Antp-positive cells distinct from the PSC within the primary lobes (Fig. 3H’), as well as the abnormal presence of Antp-positive cells within the secondary or tertiary lobes of the lymph gland (Fig. 3H). Normally Antp is restricted to a discrete region within the primary lobes of the lymph gland. Although the area occupied by the PSC appeared to be expanded within some of the homozygous mutant larvae relative to

heterozygous larvae, the number of Antp-positive cells was similar in the primary lobes of the lymph glands in both genotypes (Fig. S1 in the supplementary material). Rather, the proportion of the primary lobe occupied by Antp-positive PSC cells increased dramatically from a mean of 4% in heterozygotes to 79% in homozygotes (Fig. S1 in the supplementary material).

Together, these results suggest that H2AV is required within prohemocytes and its absence may alter the response of these cells to signals originating from the PSC. Consistent with this hypothesis, the Hedgehog (Hh) ligand that prevents differentiation of prohemocytes was detected by immunostaining in the PSC cells of both heterozygous and homozygous *His2Av*^{810} mutant lymph glands (Fig. S2A-B in the supplementary material) (Mandal et al., 2007). Likewise, levels of Wingless (Wg) protein expression appeared similar in heterozygous and homozygous *His2Av*^{810} mutant lymph gland lobes (Fig. S2C-D in the supplementary material). Past studies have shown that Wg plays a role in both maintaining undifferentiated prohemocytes and in dictating PSC cell number (Sinenko et al., 2009).

An H2AV-RFP fusion protein is able to partially rescue the homozygous mutant lymph gland phenotype.

In order to determine whether we could rescue the homozygous *His2Av*^{810} / *His2Av*^{810} mutant lymph gland phenotype, we introduced a transgene expressing an H2AV-RFP fusion protein into the *His2Av*^{810} / *His2Av*^{810} background. We found that we were able to rescue most of the dispersal phenotype of the lymph glands of homozygous mutant larvae (Fig. S3A-F in the supplementary material). However, we did observe an increase in plasmatocyte differentiation in both the primary and secondary lobes (Fig. S3C-D). Crystal cells, present in both primary and secondary

lobes, appeared to have burst (arrows; Fig S3E-F). It is possible that the incomplete nature of the rescue we observed was due to the RFP domain that is fused to the H2AV protein we tested for rescue. In this regard, others have previously reported that a similar fusion of GFP can disrupt some of the normal functions of H2AV *in vivo* (Kotova et al., 2011).

Reducing levels of H2AV in prohemocytes results in their premature differentiation within the medullary zone, but not in the early dispersal of hemocytes from the primary lymph gland lobes.

In order to determine whether the premature differentiation of prohemocytes in the absence of H2AV was a cell-autonomous property, we drove expression of the *UAS-His2Av*^{HMO5177} *RNAi* with a *domeless-GAL4* driver. A *UAS-GFP* transgene acted as a reporter for the location of *domeless-GAL4* and *UAS-His2Av RNAi* expression. Importantly, *domeless-GAL4* is expressed in the prohemocytes but not in the PSC of the primary lobes of the lymph gland (Jung et al., 2005). *domeless-GAL4* expression initially occurs in a majority of the prohemocytes of the primary lobe, but continued expression then becomes restricted to the undifferentiated prohemocytes within the medullary zone as the cells within the cortical zone start to differentiate. Expression of *domeless* also occurs in the prohemocytes of the secondary lobes. In order to test whether we were getting a significant reduction of H2AV protein levels, we carried out immunostaining with an anti-H2AV antibody (Fig. 4A-B'). We observed a dramatic reduction of H2AV protein levels in a majority of the prohemocytes found within the primary lobes of the lymph gland, whereas H2AV protein was still expressed in the cells of the PSC as expected (Fig. 4A-A').

Lymph glands in which levels of H2AV protein were greatly reduced in the prohemocytes, via *domeless-GAL4* driven RNAi, displayed a marked increase in the differentiation of plasmatocytes and crystal cells in comparison to controls (Fig. 5A-C, E-G). This increased differentiation of plasmatocytes and crystal cells was seen not only in the medullary zone of the primary lobes, but was also present extensively within the secondary lobes of the lymph gland (Fig. 5C,G). In contrast, control lymph glands did not display differentiation within the secondary lobes at similar stages (Fig. 5A,E). We also looked for differentiation of lamellocytes, but did not see a large increase in the number of differentiating lamellocytes in comparison to controls (data not shown). Since differentiation can be effected by the presence of the PSC, we also looked at the expression of Antp within these lymph glands using an Antp-specific antibody (Fig 5I-K). However, we did not see any discernable differences in the number or location of Antp-positive cells in the PSC of *domeless-GAL4* driven *UAS-His2Av RNAi* lymph glands. These results are consistent with a cell-autonomous function of H2AV within the prohemocytes of the lymph gland.

In order to test whether H2AV was also required within cells of the PSC for normal prohemocyte differentiation, we drove *UAS-His2Av RNAi* and *UAS-GFP* expression with an *Antp-GAL4* driver that is expressed in the PSC cells, but not in the other cells of the lymph gland (Mandal et al., 2007). We used anti-H2AV immunostaining to verify that the expected reduction in H2AV protein levels occurred only within the cells of the PSC, but not in other cells found within the lymph gland (Fig. 4B-B'). In lymph glands with greatly reduced levels of H2AV in the PSC, plasmatocyte differentiation appeared normal and the medullary zone was maintained (Fig. 5D). However, a decrease in crystal cells was observed in comparison to controls (Fig. 5H). Given the possible bursting of the crystal cells observed in the

homozygous *His2Av*^{810} / *His2Av*^{810} mutants and the reduction in crystal cell number when an *Antp-GAL4* driver was used to greatly reduce levels of H2AV in the PSC, it is possible that proper H2AV expression within the PSC is required for crystal cell differentiation and maintenance.

A mutant H2AV protein lacking the C-terminal H2AX-like SQAY motif can partially rescue larval hematopoiesis.

In Drosophila, the H2AX-like function of H2AV requires a carboxy-terminal SQAY motif that is phosphorylated on the serine when double stranded breaks in DNA are present nearby (Madigan et al., 2002). In order to determine whether loss of the H2AX-like function of H2AV led to the hematopoietic phenotypes seen in homozygous *His2Av*^{810} / *His2Av*^{810} mutant larvae, we tested for rescue with a previously described H2AV mutant protein that lacks the carboxy-terminal 14 amino acids including the SQAY motif (H2AV^{CT}) (Clarkson et al., 1999). This mutant protein was also able to rescue most of the lymph gland dispersal phenotype (Fig. 6B,E). The number of Antp-positive PSC cells in the CT-rescued *His2Av*^{810} / *His2Av*^{810} mutant larvae primary lobes was similar to that in heterozygous larvae with or without the CT transgene (Fig. S1 in the supplementary material). However, some abnormalities were present in the CT-rescued homozygous mutant larvae, including the presence of Antp-positive cells in the secondary lobes (Fig 6B). Crystal cell and plasmatocyte differentiation appeared to be increased, and some shedding of hemocytes was still observed (Fig. 6E), though not to the extent seen in unrescued homozygous *His2Av*^{810}/ *His2Av*^{810} mutants.

Because the rescue of normal larval hematopoiesis by the H2AV^{CT} mutant was incomplete, we wondered whether this mutant protein might itself have a dominant phenotype. When the H2AV^{CT} protein was expressed in heterozygous *His2Av*^{810} / +

larvae, the lymph gland phenotype was different than that seen in heterozygous *His2Av*^{810} / + larvae, which themselves had a normal *WT* phenotype. H2AV^{CT} caused crystal cell and plasmatocyte differentiation to no longer be restricted to the cortical zone, and some early shedding of differentiated hemocytes was seen (Fig. 6D).

The phenotype seen when H2AV^{CT} is expressed in a heterozygous *H2AV*^{810} / + background suggested a possible dominant effect, as the overall phenotype with H2AV^{CT} present was more severe than what is seen in heterozygous *His2Av*^{810} / + mutants alone. To further test this hypothesis, we examined larvae containing the H2AV^{CT} protein in a *His2Av* wild-type background. We found that H2AV^{CT} again caused a phenotype that included differentiation of crystal cells and plasmatocytes within the secondary lobes (Fig. 6F). Shedding of hemocytes from the primary lobes was also observed.

We wished to distinguish among different hypotheses that might explain the dominant lymph gland phenotypes caused by the presence of the mutant H2AV^{CT} protein described above. The observed dominant H2AV^{CT} phenotype could be due to the absence of a short peptide upstream of the SQAY motif. Alternately, the phenotype might also be caused by the dilution of wild type H2AV proteins capable of responding to DNA damage by mutant H2AV^{CT} proteins, thereby causing increased levels of cell death within the organism. This in turn might result in increased mobilization of hemocytes from the lymph gland in response to cell damage and/or cell death. Finally, it is possible that an increased dosage of mutant or wild type H2AV might cause a dominant phenotype in larval hematopoiesis.

Overexpression of wild type H2AV protein in the lymph gland via either a *domeless-GAL4* or an *Antp-GAL4* resulted in less cohesion of the Antp-positive cells of the PSC (Fig. S4C-E in supplemental materials). Overexpression of wild type

H2AV in the PSC via *Antp-GAL4* also resulted in lower numbers of differentiated crystal cells and plasmatocytes (Fig. S4D-E). However, a similar alteration in differentiated hemocyte numbers was not observed when the overexpression of wild type H2AV was driven via a *domeless-GAL4* driver (Fig. S4A-B). Similar alterations in hemocyte differentiation or PSC cell cohesion were also observed when we drove expression of either a mutant H2AV protein that mimicked constitutive phosphorylation of the serine 137 residue within the SQAY motif ($H2AV^{SE}$; Fig. S4F-J), or a mutant H2AV protein that was non-phosphorylatable due to a serine-to-alanine substitution ($H2AV^{SA}$; Fig. S4K-O) with *domeless-GAL4* or an *Antp-GAL4* (Kotova et al., 2011). Taken together these results show that increasing the dosage of H2AV proteins, whether wild type or mutant, can also cause alterations in larval hematopoiesis.

DISCUSSION

We discovered that absence of the variant histone protein H2AV results in the formation of larval melanotic masses containing plasmatocytes and crystal cells. Previous studies have proposed that the formation of melanotic masses can be due either to the response of a normal immune system to abnormal tissue formed during development, or to a developmental defect in the hemocytes of the lymph gland (Rizki and Rizki, 1980; Rodriguez et al., 1996; Watson et al., 1991). Our data showing the loss of a majority of the primary lymph gland lobes in the $His2Av^{810}$ null mutant, as well as the early differentiation of the medullary zone and secondary lobe prohemocytes when H2AV levels were reduced via RNAi are consistent with the latter model. Our results demonstrate an important role for H2AV during normal hemocyte differentiation and dispersal. Interestingly, studies using a human

histiocytic lymphoma cell line or normal macrophages differentiated with macrophage colony stimulating factor (M-CSF) have shown an upregulation of the *His2Av*-related human *H2A.Z* gene during macrophage differentiation (Baek et al., 2009). These results imply an evolutionarily conserved role for the closely related H2AV and H2AZ histone variants in blood cell differentiation.

The presence of black melanotic masses in Drosophila larvae is not restricted to *His2Av* mutants. This phenotype has previously been observed in mutants of two different ATP-dependent chromatin-remodeling complexes. Dom, which is a catalytic subunit of the Tip60 complex, plays a role in H2A variant exchange in nucleosomes, as well as in DNA-damage repair (Kusch et al., 2004). *dom* loss-of-function mutants display black melanotic masses that are comprised of melanized lymph glands (Braun et al., 1997). Mutants of the vertebrate homolog of *dom* have also been shown to be required for both embryonic and adult hematopoiesis in the laboratory mouse (Fujii et al., 2010; Ueda et al., 2007). Loss of another ATP-dependent chromatin remodeling enzyme, NURF, also causes melanotic masses (Badenhorst, 2014). In addition, melanotic masses have been observed in mutations that affect various signaling pathways. For example, constitutive activation of the JAK-STAT pathway via the dominant gain-of-function Hop^{TUM} mutation results in the formation of melanotic masses (Hanratty and Dearolf, 1993). Constitutive activation of the Toll pathway via the dominant gain-of-function Tl^{10b} mutation also causes melanotic masses (Lemaitre et al., 1995). These observations raise the question of whether the closely related variant histones H2AV and H2AZ might be required to repress these evolutionarily conserved signaling pathways in hematopoietic cells.

Although the majority of the cells in the primary lymph gland lobes in *His2Av* mutants are lost, the Antp-positive cells comprising the PSC are spared, and can be

seen adjacent to the cardioblasts of the dorsal vessel. In addition, these cells express the Hh ligand that normally prevents premature differentiation of hemocyte precursors. The presence of Antp-positive cells can also be observed in posterior lymph gland lobes, where *Antp* is not normally expressed. In this regard, previous studies have shown that *His2Av* can function as a Polycomb Group (PcG) gene, and PcG genes are known to be important for repressing the transcription of homeotic genes such as *Antp* (Swaminathan et al., 2005). In particular, it has been reported that *Antp* expression is expanded in the central nervous system of larvae that are mutant for *His2Av*. Our results show that the Antp-positive PSC is also expanded in the lymph gland when the H2AV protein is absent or reduced in level. Reduction of H2AV levels via RNAi in the prohemocytes of the primary lobes, as well as in the secondary lobes, led to increased differentiation of plasmatocytes and crystal cells. This result suggests that H2AV also acts downstream of the signals that originate from the PSC and that maintain the prohemocytes of the medullary zone in an undifferentiated state.

Reduction of H2AV levels via RNAi cause a less severe phenotype than that of *His2Av* null mutants, in that the primary lobes of the lymph gland are preserved. However, there is a loss of the undifferentiated prohemocytes found within the medullary zone, as these cells differentiate into mature hemocytes. Previous studies in the testis of *Drosophila* have shown an important role for H2AV in the maintenance of both the germline and cyst stem cells (Morillo Prado et al., 2013). Together these results suggest a possible role for H2AV in the transcriptional control of genes important for stem cell maintenance in general. In this regard, the closely related H2AZ protein of mammals has been reported to be important for the differentiation of embryonic stem cells in culture (Creyghton et al., 2008).

H2AV may be exerting its effects on the lymph gland through various signaling pathways that have been shown to orchestrate prohemocyte differentiation. Two pathways that may be affected include the Hh and Wg signaling pathways. Hh has been implicated in maintaining the prohemocytes in an undifferentiated state (Mandal et al., 2007). However, we observed Hh expression in the PSC of both heterozygous and homozygous mutant *His2Av*^{810} larval lymph glands . Meanwhile, Wg has been reported to not only maintain the prohemocyte population in an undifferentiated state, but to also dictate the PSC cell number (Sinenko et al., 2009). We did not detect any significant differences in the staining of prohemocytes and PSC cells with anti-Wg antibodies in homozygous versus heterozygous mutant *His2Av*^{810} larval lymph glands. These results suggest that loss of H2AV may alter the intracellular responses to these ligands rather than their expression.

Drosophila H2AV is a chimeric protein that plays the roles of two widely conserved variant histones, H2AX and H2AZ (Baldi and Becker, 2013). H2AX is important for the DNA-damage repair response (Scully and Xie, 2013), while H2AZ is important for both transcriptional activation and gene silencing (Billon and Cote, 2013). Previous studies have shown that H2AV^{CT}, which lacks H2AX function, is able to rescue the lethal phenotype that is seen in *His2Av*^{810} null mutants, allowing the organisms to progress to pupation and adulthood (Clarkson et al., 1999). We found that H2AV^{CT} was able to partially rescue the *His2Av*^{810} null larval hematopoietic phenotype, arguing that an H2AZ-like function rather than an H2AX-like function of H2AV is required for hematopoiesis. Nevertheless, differentiation within the lymph gland still appeared disrupted and partial loss of the primary lymph gland lobes could be seen. In addition, the expression of Antp still appeared to be expanded. This lack of full rescue could be due to a decreased stability of the CT mutant protein.

However, the presence of $H2AV^{CT}$ in an otherwise *His2Av* wild type background was sufficient to cause abnormalities of the lymph gland lobes. Furthermore, the overexpression of wild type or of phosphorylation mutants of H2AV also caused hematopoietic abnormalities. Together these results imply that the precise dosage of the H2AV protein is essential for normal hematopoiesis in Drosophila. Similar alterations in differentiation may also occur in other organs and tissues. In this regard, care should be taken when using *His2Av-GFP* and *His2Av-RFP* transgenes, which are popular markers in live imaging.

The formation of black melanotic masses in the $His2Av^{810}$ null mutant establishes larval hemocytes as a useful tool for further studies of H2AV function. Futhermore, given the role that H2AV plays in not only undifferentiated prohemocytes, but also in the germline and cyst stem cells found in the testis (Morillo Prado et al., 2013), it will be interesting to test whether H2AV also regulates stem cells found in other tissues.

MATERIALS AND METHODS

Fly Lines and Crosses

Mutant or transgenic fly stocks obtained for use in these studies included: *domeless-GAL4, UAS-GFP* (Grigorian et al., 2013; Mandal et al., 2007); *Antennapedia-GAL4, UAS-GFP* (Grigorian et al., 2013; Mandal et al., 2007); $His2Av^{810}/TM3,Sb^{1}$ (Bloomington Drosophila Stock Center); $His2Av^{CT}$ (Clarkson et al., 1999); $UAS\text{-}His2Av^{WT}$ (Kotova et al., 2011); $UAS\text{-}His2Av^{SE}$ (Kotova et al., 2011); $UAS\text{-}His2Av^{SA}$ (Kotova et al., 2011); $UAS\text{-}His2Av^{HMO5177}$ *RNAi* (Bloomington Drosophila Stock Center) ; *His2Av-RFP* (Schuh et al., 2007). Additional stocks with balancer chromosomes expressing dominant larval markers were obtained from the

Bloomington Stock Center. All *GAL4 x UAS-RNAi* crosses were carried out at 29°C. All other stocks and crosses were grown out at 25°C in standard laboratory conditions.

Antibodies

α-Antennapedia (mouse; 1:4; Developmental Studies Hybridoma Bank), α-P1 (mouse; 1:10)(Kurucz et al., 2007), α-ProPo (rabbit; 1:1000) (Muller et al., 1999), α-L1 (mouse; 1:10) (Kurucz et al., 2003), α-H2AV (rabbit; 1:1000) (Leach et al., 2000), α- H2AVph (mouse; 1:25; Developmental Studies Hybridoma Bank) (Lake et al., 2013), α-Hedgehog (rabbit; 1:200) (Taylor et al., 1993), α- Wingless (mouse; 1:50; Developmental Studies Hybridoma Bank), α-GFP (rabbit; 1:1000; Abcam ab6556), α-GFP (mouse; 1:250; Abcam ab1218). All fluorescent secondary antibodies were conjugated to Alexa Fluor® dyes (Thermo Fisher Scientific) and were used at a concentration of 1:500. Samples were counterstained with the DNA dye TOTO-3 iodide (Thermo Fisher Scientific) added at a concentration of 1:250-1:500 to the Vectashield (Vector Labs) in which the samples were mounted.

Larval Dissection, Fixation and Immunostaining

Lymph glands were dissected from wandering third instar larvae. Lymph glands were dissected by pulling out mouth hooks with lymph glands attached. Tissue was fixed for 10 minutes in a 1:3 solution of 37% formaldehyde: 1X PBS. Immunostaining was carried out as previously described (Grigorian et al., 2011).

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COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

All experiments were performed by M.G. and H.D. Analysis of data, conceptual planning, and preparation of the manuscript were done by M.G. and J.S.L.

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REFERENCES

Badenhorst, P. (2014). What Can We Learn from Flies: Epigenetic Mechanisms Regulating Blood Cell Development in *Drosophila*. In *Transcriptional and Epigenetic Mechanisms Regulating Normal and Aberrant Blood Cell Development* (ed. C. Bonifer & P. Cockerill), pp. 15-47. Verlag Berlin Heidelberg Springer Science and Business Media.

Baek, Y. S., Haas, S., Hackstein, H., Bein, G., Hernandez-Santana, M., Lehrach, H., Sauer, S. and Seitz, H. (2009). Identification of novel transcriptional regulators involved in macrophage differentiation and activation in U937 cells. *BMC Immunol* **10**, 18.

Baldi, S. and Becker, P. B. (2013). The variant histone H2A.V of Drosophila--three roles, two guises. *Chromosoma* **122**, 245-258.

Billon, P. and Cote, J. (2013). Precise deposition of histone H2A.Z in chromatin for genome expression and maintenance. *Biochim Biophys Acta* **1819**, 290-302.

Braun, A., Lemaitre, B., Lanot, R., Zachary, D. and Meister, M. (1997). Drosophila immunity: analysis of larval hemocytes by P-element-mediated enhancer trap. *Genetics* **147**, 623-634.

Clarkson, M. J., Wells, J. R., Gibson, F., Saint, R. and Tremethick, D. J. (1999). Regions of variant histone His2AvD required for Drosophila development. *Nature* **399**, 694-697.

Cook, R. K., Christensen, S. J., Deal, J. A., Coburn, R. A., Deal, M. E., Gresens, J. M., Kaufman, T. C. and Cook, K. R. (2012). The generation of chromosomal deletions to provide extensive coverage and subdivision of the Drosophila melanogaster genome. *Genome Biol* **13**, R21.

Creyghton, M. P., Markoulaki, S., Levine, S. S., Hanna, J., Lodato, M. A., Sha, K., Young, R. A., Jaenisch, R. and Boyer, L. A. (2008). H2AZ is enriched at polycomb complex target genes in ES cells and is necessary for lineage commitment. *Cell* **135**, 649-661.

Evans, C. J., Hartenstein, V. and Banerjee, U. (2003). Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. *Dev Cell* **5**, 673-690.

Evans, C. J., Liu, T. and Banerjee, U. (2014). Drosophila hematopoiesis: Markers and methods for molecular genetic analysis. *Methods* **68**, 242-251.

Fujii, T., Ueda, T., Nagata, S. and Fukunaga, R. (2010). Essential role of p400/mDomino chromatin-remodeling ATPase in bone marrow hematopoiesis and cell-cycle progression. *J Biol Chem* **285**, 30214-30223.

Grigorian, M., Liu, T., Banerjee, U. and Hartenstein, V. (2013). The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the Drosophila lymph gland. *Dev Biol* **384**, 301-312.

Grigorian, M., Mandal, L. and Hartenstein, V. (2011). Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymph gland. *Dev Genes Evol* **221**, 121-131.

Hanratty, W. P. and Dearolf, C. R. (1993). The Drosophila Tumorous-lethal hematopoietic oncogene is a dominant mutation in the hopscotch locus. *Mol Gen Genet* **238**, 33-37.

Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M. and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *Embo J* **14**, 2857-2865.

Jung, S. H., Evans, C. J., Uemura, C. and Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. *Development* **132**, 2521-2533.

Kotova, E., Lodhi, N., Jarnik, M., Pinnola, A. D., Ji, Y. and Tulin, A. V. (2011). Drosophila histone H2A variant (H2Av) controls poly(ADP-ribose) polymerase 1 (PARP1) activation in chromatin. *Proc Natl Acad Sci U S A* **108**, 6205-6210.

Krzemien, J., Dubois, L., Makki, R., Meister, M., Vincent, A. and Crozatier, M. (2007). Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. *Nature* **446**, 325-328.

Kurucz, E., Vaczi, B., Markus, R., Laurinyecz, B., Vilmos, P., Zsamboki, J., Csorba, K., Gateff, E., Hultmark, D. and Ando, I. (2007). Definition of Drosophila hemocyte subsets by cell-type specific antigens. *Acta Biol Hung* **58 Suppl**, 95-111.

Kurucz, E., Zettervall, C. J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedus, Z., Ando, I. and Hultmark, D. (2003). Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila. *Proc Natl Acad Sci U S A* **100**, 2622-2627.

Kusch, T., Florens, L., Macdonald, W. H., Swanson, S. K., Glaser, R. L., Yates, J. R., 3rd, Abmayr, S. M., Washburn, M. P. and Workman, J. L. (2004). Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. *Science* **306**, 2084-2087.

Kwon, S. Y., Xiao, H., Glover, B. P., Tjian, R., Wu, C. and Badenhorst, P. (2008). The nucleosome remodeling factor (NURF) regulates genes involved in Drosophila innate immunity. *Dev Biol* **316**, 538-547.

Lake, C. M., Holsclaw, J. K., Bellendir, S. P., Sekelsky, J. and Hawley, R. S. (2013). The development of a monoclonal antibody recognizing the Drosophila melanogaster phosphorylated histone H2A variant (gamma-H2AV). *G3 (Bethesda)* **3**, 1539-1543.

Leach, T. J., Mazzeo, M., Chotkowski, H. L., Madigan, J. P., Wotring, M. G. and Glaser, R. L. (2000). Histone H2A.Z is widely but nonrandomly distributed in chromosomes of Drosophila melanogaster. *J Biol Chem* **275**, 23267-23272.

Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J. M. and Hoffmann, J. A. (1995). Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. *Embo J* **14**, 536-545.

Lorch, Y. and Kornberg, R. D. (2015). Chromatin-remodeling and the initiation of transcription. *Q Rev Biophys* **48**, 465-470.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251-260.

Madigan, J. P., Chotkowski, H. L. and Glaser, R. L. (2002). DNA double-strand break-induced phosphorylation of Drosophila histone variant H2Av helps prevent radiation-induced apoptosis. *Nucleic Acids Res* **30**, 3698-3705.

Malik, H. S. and Henikoff, S. (2003). Phylogenomics of the nucleosome. *Nat Struct Biol* **10**, 882-891.

Mandal, L., Martinez-Agosto, J. A., Evans, C. J., Hartenstein, V. and Banerjee, U. (2007). A Hedgehog- and Antennapedia-dependent niche maintains Drosophila haematopoietic precursors. *Nature* **446**, 320-324.

Minakhina, S. and Steward, R. (2006). Melanotic mutants in Drosophila: pathways and phenotypes. *Genetics* **174**, 253-263.

Morillo Prado, J. R., Srinivasan, S. and Fuller, M. T. (2013). The histone variant His2Av is required for adult stem cell maintenance in the Drosophila testis. *PLoS Genet* **9**, e1003903.

Muller, H. M., Dimopoulos, G., Blass, C. and Kafatos, F. C. (1999). A hemocyte-like cell line established from the malaria vector Anopheles gambiae expresses six prophenoloxidase genes. *J Biol Chem* **274**, 11727-11735.

Rizki, T. M. and Rizki, R. M. (1980). Developmental analysis of a temperature-sensitive melanotic tumor mutant in Drosophila melanogaster *Wilhelm Roux's Arch. Dev. Biol.* **189**, 197-206.

Rodriguez, A., Zhou, Z., Tang, M. L., Meller, S., Chen, J., Bellen, H. and Kimbrell, D. A. (1996). Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in Drosophila melanogaster. *Genetics* **143**, 929-940.

Schuh, M., Lehner, C. F. and Heidmann, S. (2007). Incorporation of Drosophila CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr Biol* **17**, 237-243.

Scully, R. and Xie, A. (2013). Double strand break repair functions of histone H2AX. *Mutat Res* **750**, 5-14.

Sinenko, S. A., Mandal, L., Martinez-Agosto, J. A. and Banerjee, U. (2009). Dual role of wingless signaling in stem-like hematopoietic precursor maintenance in Drosophila. *Dev Cell* **16**, 756-763.

Swaminathan, J., Baxter, E. M. and Corces, V. G. (2005). The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of Drosophila heterochromatin. *Genes Dev* **19**, 65-76.

Taylor, A. M., Nakano, Y., Mohler, J. and Ingham, P. W. (1993). Contrasting distributions of patched and hedgehog proteins in the Drosophila embryo. *Mech Dev* **42**, 89-96.

Ueda, T., Watanabe-Fukunaga, R., Ogawa, H., Fukuyama, H., Higashi, Y., Nagata, S. and Fukunaga, R. (2007). Critical role of the p400/mDomino chromatin-remodeling ATPase in embryonic hematopoiesis. *Genes Cells* **12**, 581-592.

van Daal, A. and Elgin, S. C. (1992). A histone variant, H2AvD, is essential in Drosophila melanogaster. *Mol Biol Cell* **3**, 593-602.

Watson, K. L., Johnson, T. K. and Denell, R. E. (1991). Lethal(1) aberrant immune response mutations leading to melanotic tumor formation in Drosophila melanogaster. *Dev Genet* **12**, 173-187.

Zettervall, C. J., Anderl, I., Williams, M. J., Palmer, R., Kurucz, E., Ando, I. and Hultmark, D. (2004). A directed screen for genes involved in Drosophila blood cell activation. *Proc Natl Acad Sci U S A* **101**, 14192-14197.

Figures

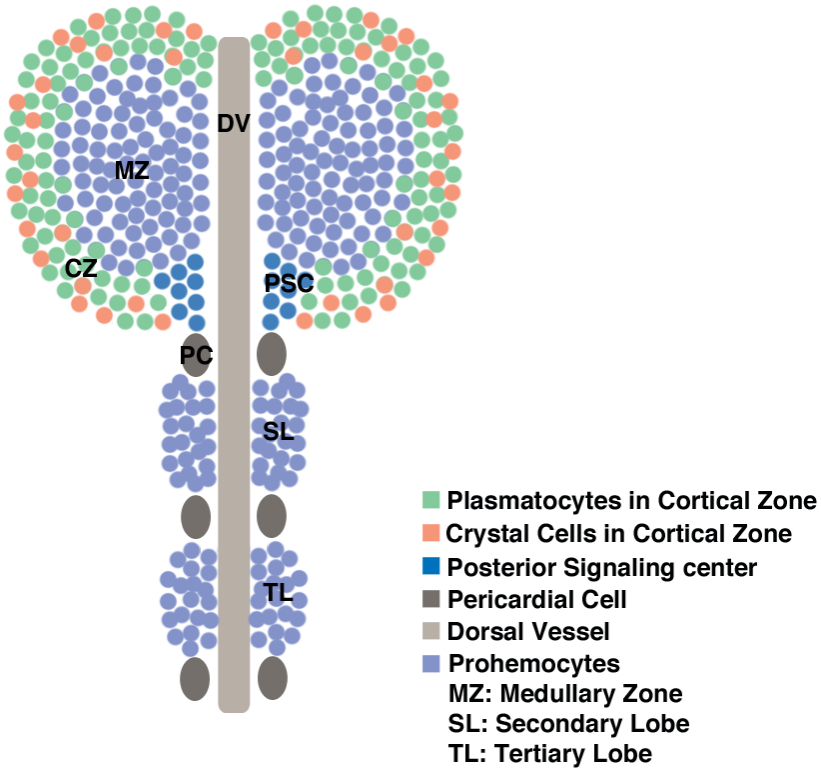


Figure 1

Architecture of a third instar larval lymph gland. Abbreviations: MZ: medullary zone; CZ: cortical zone; DV: dorsal vessel; PSC: posterior signaling center; PC: pericardial cell; SL: secondary lobe; TL: tertiary lobe. Differentiated plasmatocytes and crystal cells are shown in the cortical zone. Due to their rarity, lamellocytes have not been depicted.

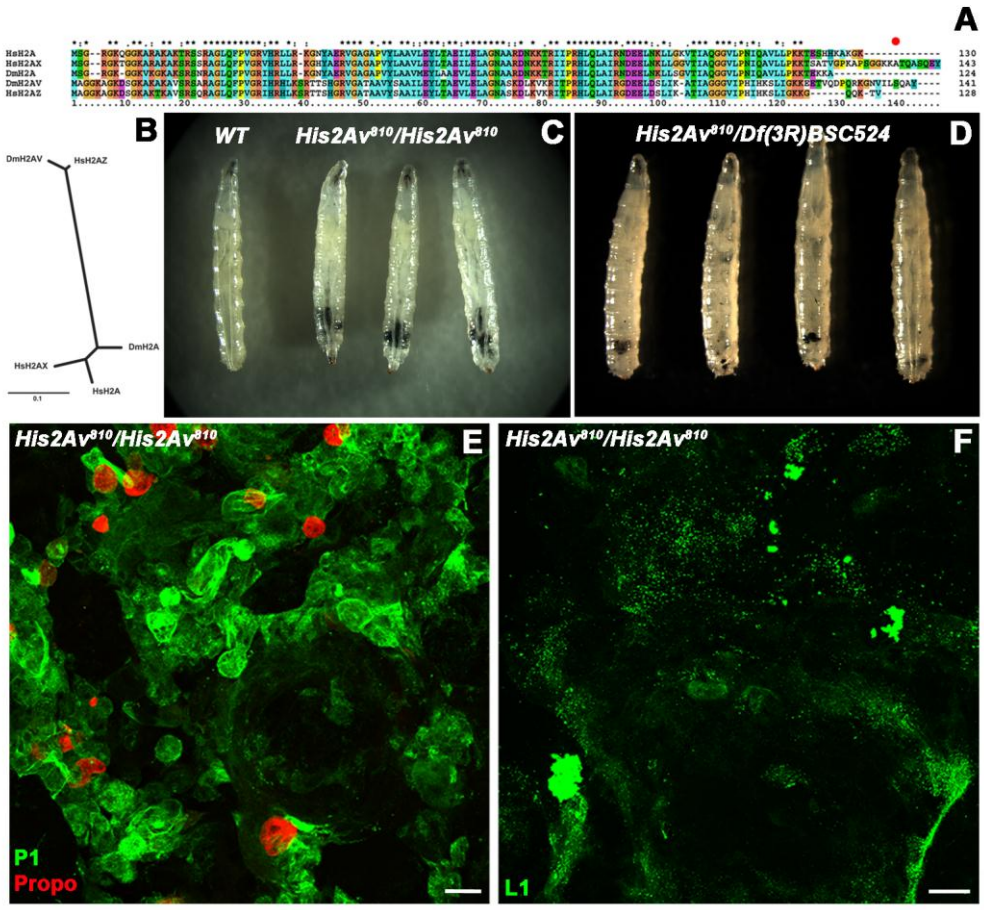


Figure 2

Lack of H2AV causes melanotic masses in mutant larvae. (A) Sequence alignment of *H. sapiens* H2A, H2AX and H2AZ proteins and *D. melanogaster* H2A and H2AV proteins. The red dot indicates the site of serine phosphorylation in H2AV. (B) Phylogenetic tree showing relatedness of *H. sapiens* H2A, H2AX and H2AZ proteins and *D. melanogaster* H2A and H2AV proteins. (C) Comparison of wild type (WT) third instar larva and homozygous *His2Av*^{810} mutant larvae. Black melanotic masses can be seen in homozygous *His2Av*^{810}mutant larvae. (D) Larvae hemizygous for the *His2Av*^{810} mutation over the chromosomal deletion *Df(3R)BSC524* also show

black melanotic masses. (E) Dissected black melanotic masses from homozygous *His2Av*^{810} mutant larvae immunostained for plasmatocytes (P1, green) and crystal cells (Propo, red). (F) Dissected black melanotic masses from homozygous H2AV^{810} mutant larvae immunostained for lamellocytes (L1, green). Scale Bars: E, 23μm ; F, 32μm.

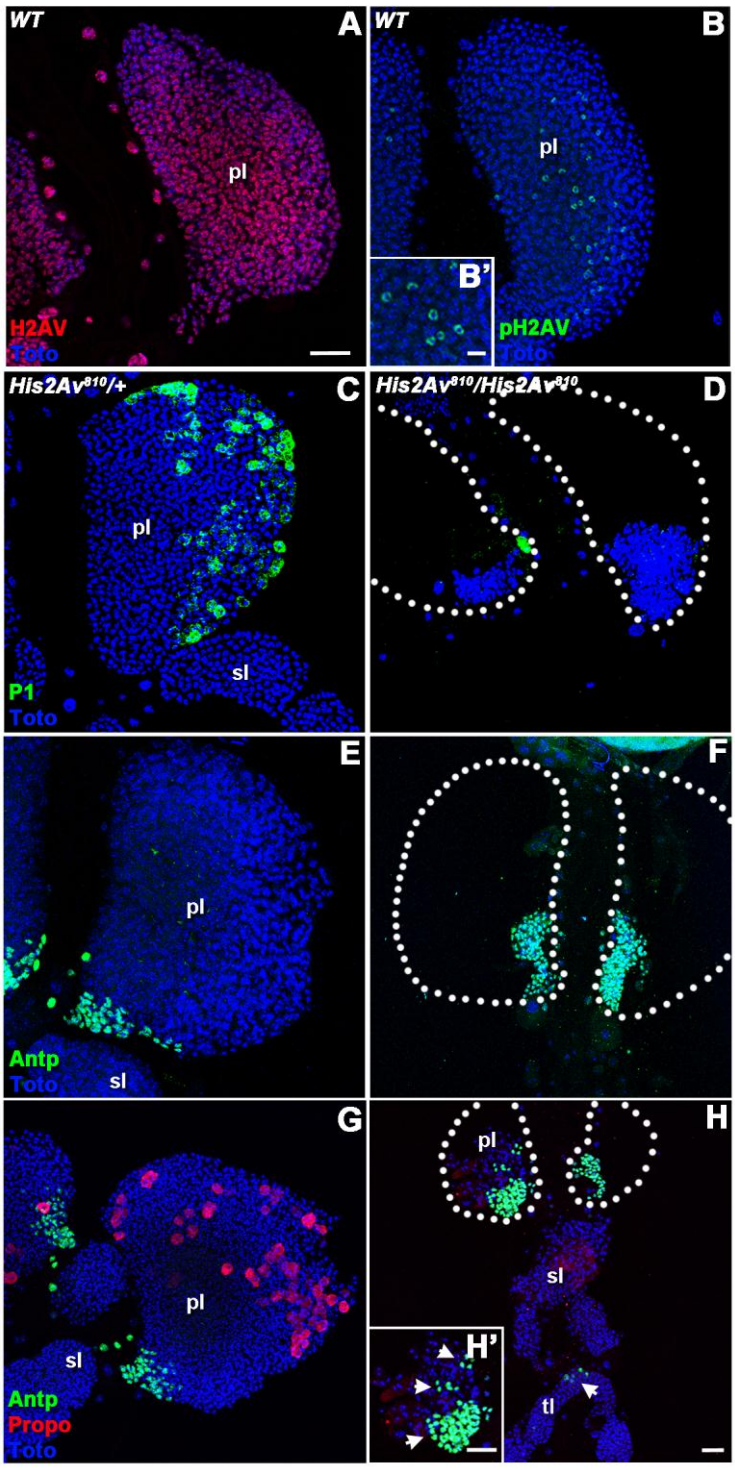


Figure 3

H2AV is required for normal larval hematopoiesis. (A and B) WT larval lymph gland immunostained for H2AV (red) and phospho-H2AV (pH2AV, green). Inset (3B') shows a magnified area of pH2AV staining in a central region of a primary lobe. (C-H) Heterozygous *His2Av*^{810}/ + (C,E,G) and homozygous *His2Av*^{810}/ *His2Av*^{810} (D,F,H) larval lymph gland lobes were immunostained for plasmatocytes (P1, green; C,D), the posterior signaling center (Antennapedia [Antp], green; E-H'), and crystal cells (Propo, red; G,H). Nuclei were labeled with a DNA dye (Toto, blue; A-H). Prospective "missing" areas of homozygous *His2Av*^{810}/ *His2Av*^{810} primary lymph gland lobes are outlined in white dots (D,F,H). Inset H' depicts a magnified area of the primary lymph gland lobe shown in H. Discontinous areas of the PSC are marked with arrowheads (H and H'). Note that panel H is shown at lower magnification than other panels in order to display the secondary and tertiary lobes, which normally contain no Antp positive cells. Arrow in H shows aberrant Antp expression in the tertiary lobe. Abbreviations: pl: primary lobe; sl: secondary lobe; tl: tertiary lobe. Scale Bars: A-G, 20μm ; B', 10μm ; H', 20μm ; H, 20μm.

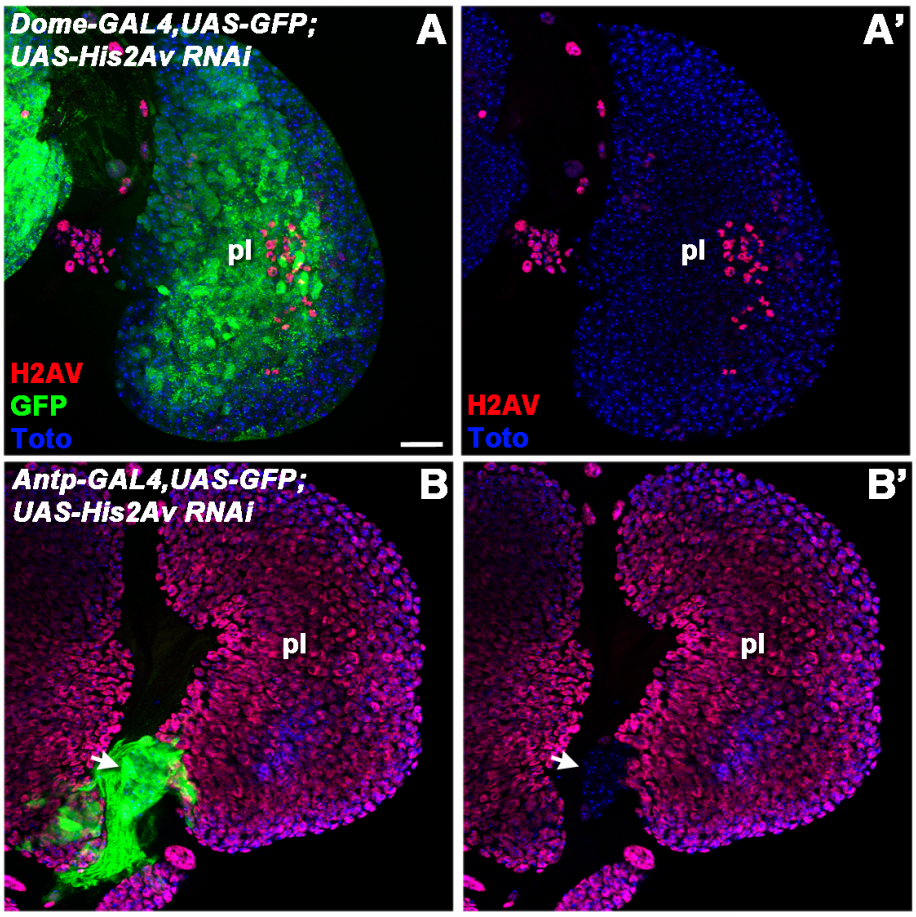


Figure 4

Cell-Type Specific Knockdown of H2AV with GAL4-driven RNAi. *domeless-GAL4* (A,A') or *Antp-GAL4* (B,B') were used to drive *UAS-GFP* and *UAS-His2Av RNAi*^{HM05177} in lymph glands. GFP expression indicates areas in which the drivers are active (green; A,B). H2AV protein was detected by immunolabeling (red; A,A'-B,B'). Nuclei were labeled with a DNA dye (Toto, blue; A,A',B,B'). Arrow indicates an area in which H2AV protein is absent and the *Antp-GAL4* driver is active (B,B'). Abbreviations: pl: primary lobe. Scale Bars: A-B', 20μm.

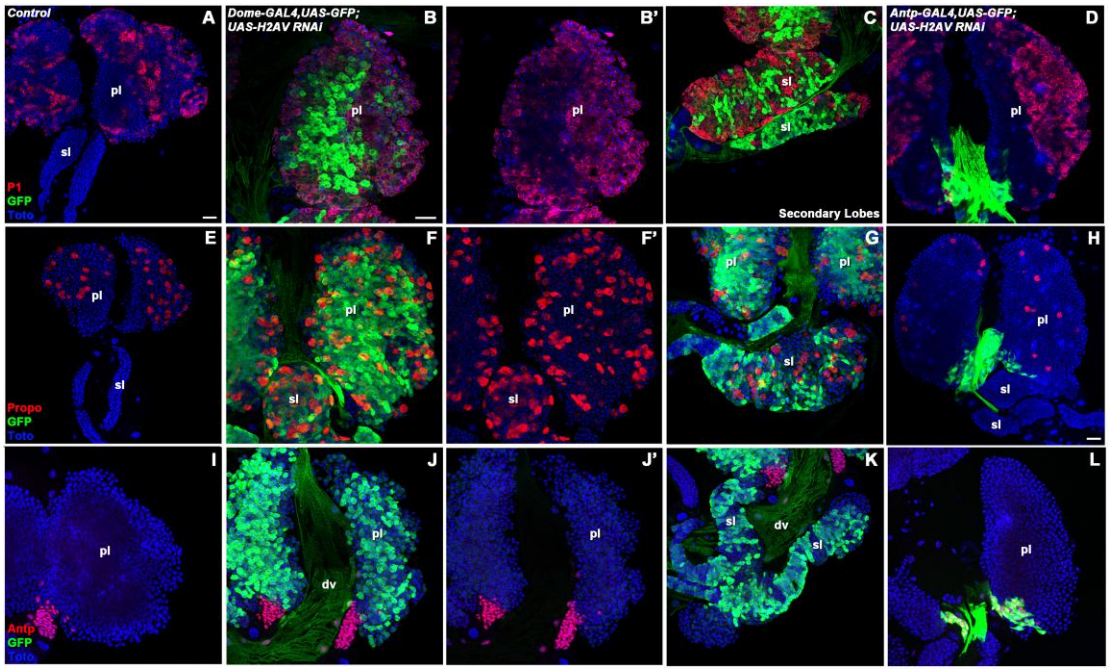


Figure 5

RNAi knockdown of H2AV in the lymph gland causes abnormal hemocyte differentiation. *domeless-GAL4* (*dome-GAL4*) driven *UAS-GFP* (and by inference, *UAS-His2Av RNAi*) expression is shown in green in the primary (B,F,J) and secondary (C,G,K) lobes of the lymph gland. Expanded differentiation of plasmatocytes (P1; red) can be seen via immunostaining in the primary (B-B') and secondary (C) lobes in comparison to the control (A). Crystal cell (Propo; red) differentiation, shown via immunostaining, is also expanded in the primary (F-F') and secondary (G) lobes in comparison to the control (E). The posterior signaling center (PSC) visualized with immunostaining for Antennapedia (Antp; red) appeared comparable in control lymph glands (I) and those with *dome-GAL4* driven *UAS-His2Av RNAi* (J-K). *Antennapedia-GAL4* was used to drive *UAS-His2Av RNAi*, as well as a *UAS-GFP* reporter (green) in the PSC (D,H,L). Plasmatocyte differentiation in an *Antp-GAL4* driven knockdown

(D) was comparable to controls (A). However crystal cell numbers (H) appeared reduced in these knockdown lymph glands in comparison to the control (E). PSC size itself (L) was comparable to the control (I). Abbreviations: dv: dorsal vessel; pl: primary lobe; sl: secondary lobe. Scale Bars: A-B, A'-B', 20μm.

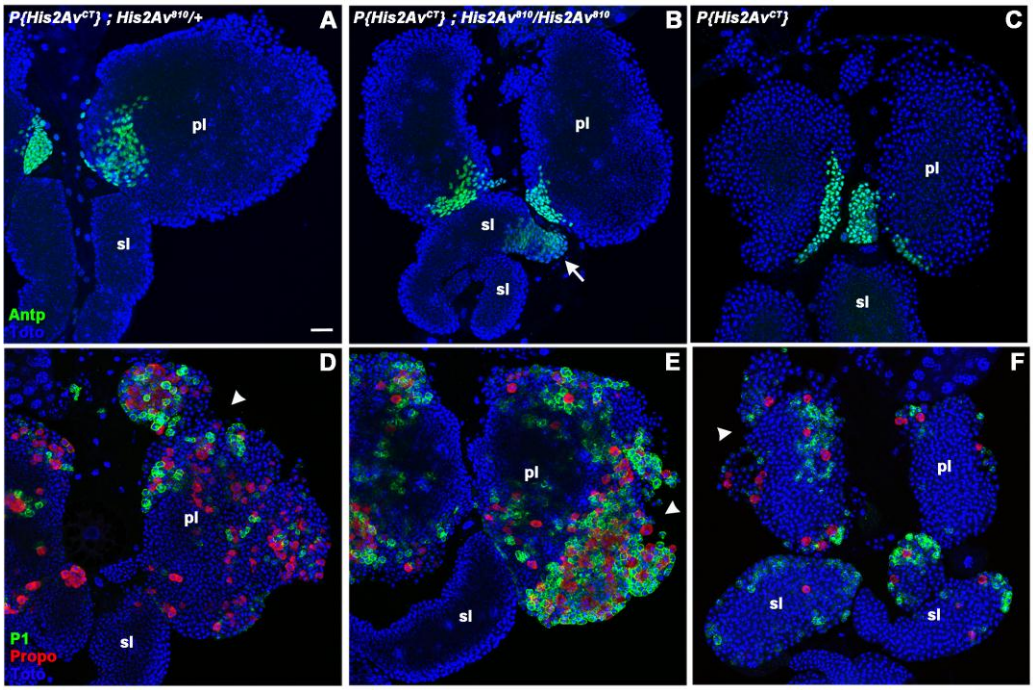
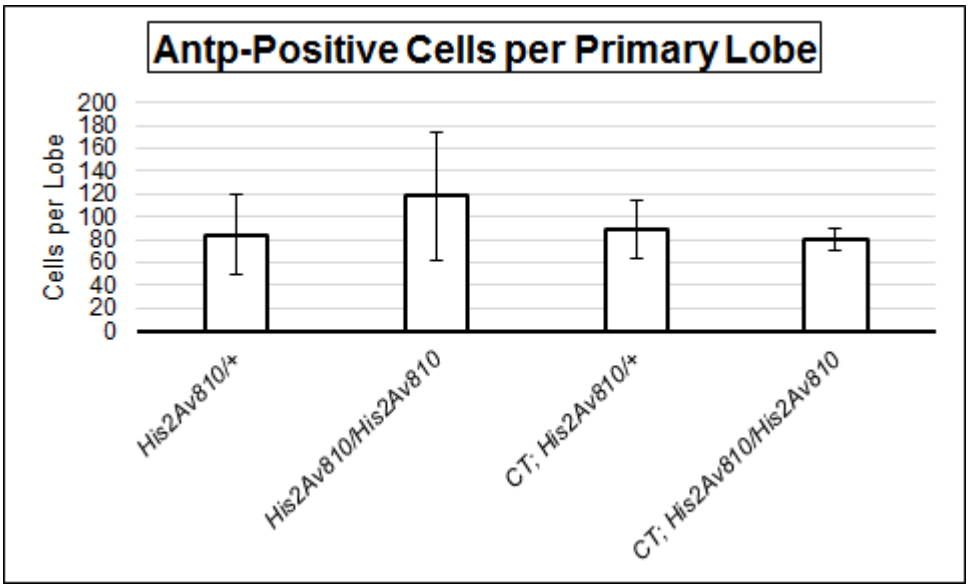
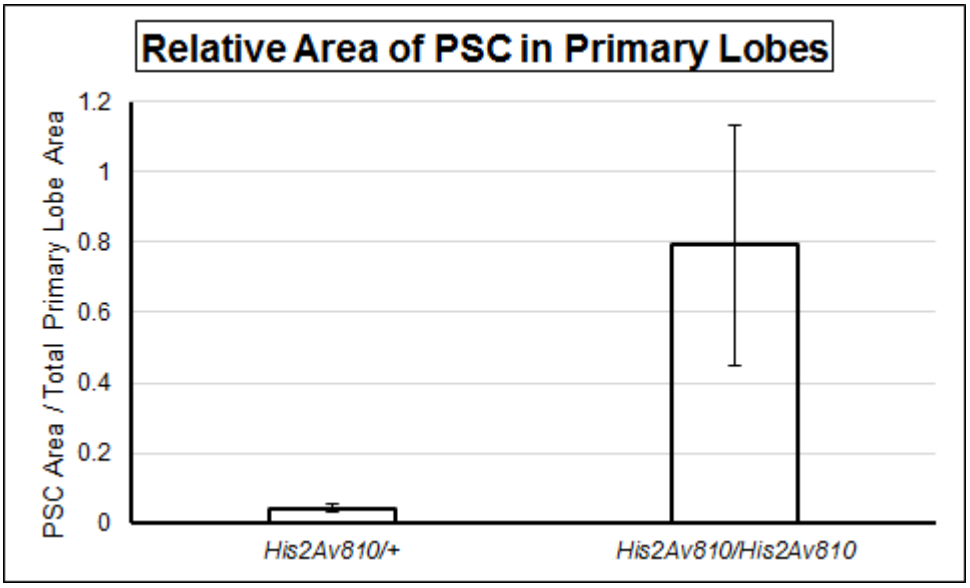


Figure 6

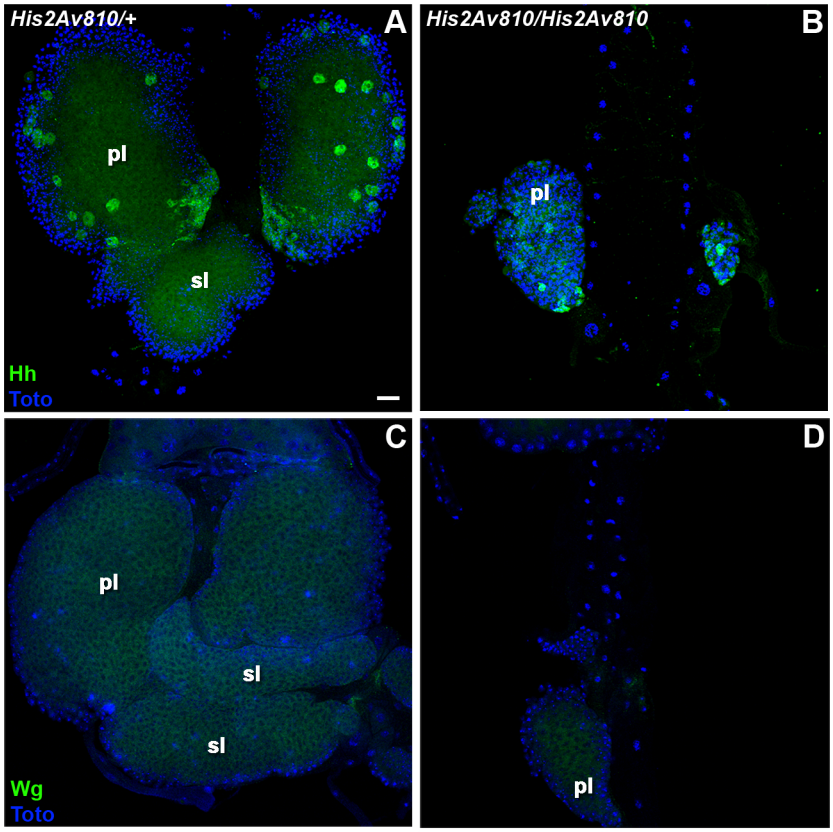
A mutant H2AV protein lacking the C-terminal H2AX-like phosphorylation site rescues larval lymph gland structure and function in a *His2Av* null mutant. A transgene that expresses an H2AV protein lacking fourteen C-terminal amino acids including the SQAY motif, *P{His2Av^{CT}}*, was tested in an otherwise wild-type *His2Av* background (C,F), in a *His2Av^{810}* / *His2Av^{810}* homozygous mutant background (B,E), and in a *His2Av^{810}*/ + heterozygous mutant background (A,D). The PSC was labeled via immunostaining (Antp, green, A-C). Arrow marks area of Antp expansion into the secondary lobe (B). Plasmatocytes (P1, green) and crystal cells (Propo, red) were also labeled via immunostaining (D-F). Arrowheads mark areas of hemocyte dispersal from the primary lymph gland lobes (D-F). Nuclei were labeled with a DNA dye (Toto, blue; (A-F). Abbreviations: pl: primary lobe; sl: secondary lobe. Scale Bars: A-F, 20μm.





Supplementary Figure S1

PSC cell number is unaffected, but non-PSC cells are greatly diminished in a *His2Av* null mutant. Top panel: Antp-positive cells were counted in primary lobes of lymph glands from larvae of the indicated genotypes. Bars indicate the mean values of four to six samples for each genotype with error bars indicating standard deviations. No statistically significant differences ($P < 0.05$) between pairs of genotypes were detected using a two-tailed Student t-test. Bottom panel: Image J was used to measure the areas of entire primary lymph gland lobes and the areas occupied by Antp-positive cells of the PSC. Bars indicate the mean values of the ratio of PSC area to total area for each genotype with error bars indicating standard deviations. A statistically significant difference ($P = 0.0005$) between the two genotypes was detected using a two-tailed Student t-test.

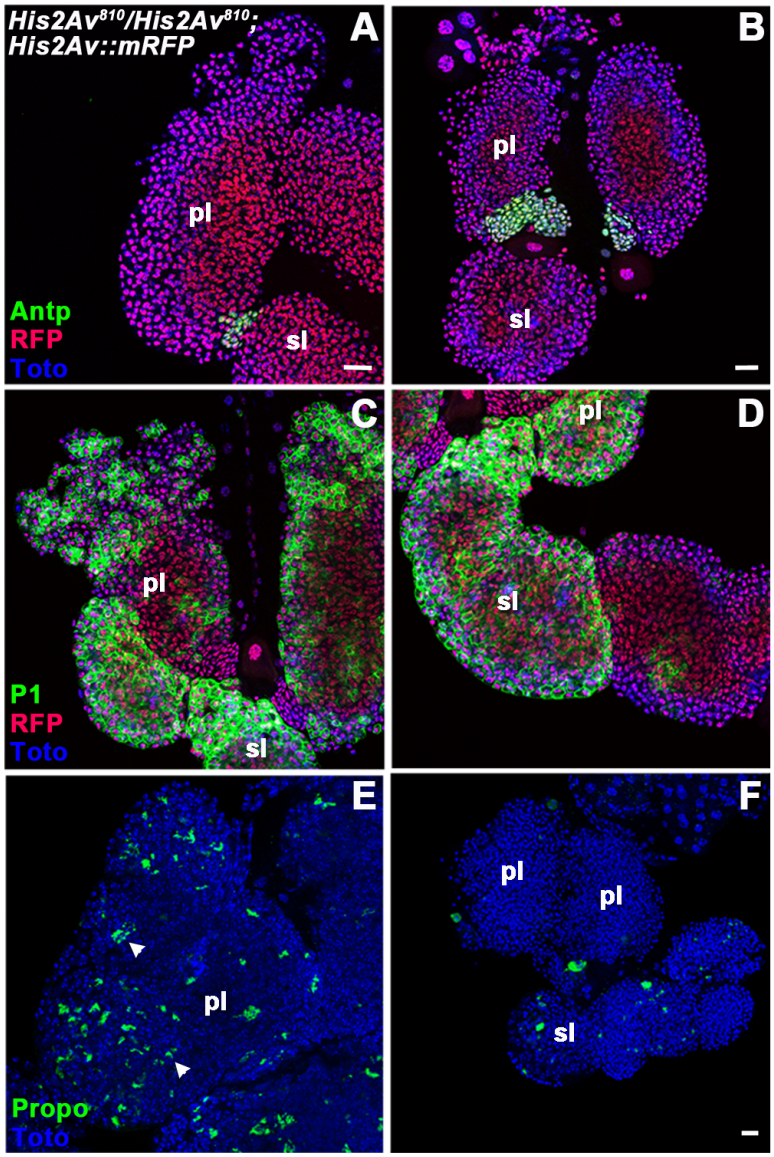


Grigorian, et al, Supplementary Figure S2

Supplementary Figure S2

Hedgehog and Wingless proteins are present in the cells of *His2Av* null mutant lymph glands. Larval lymph glands of the indicated genotypes were dissected, fixed, and stained with either anti-Hedgehog antibody (Hh, green, A-B) or with anti-Wingless antibody (Wg, green, C-D), as well as with the DNA dye TOTO-3 (Toto, blue, A-D). A tight cluster of Hh-positive cells is present in the PSC regions of each primary lymph gland lobe in *His2Av*^{810} / + heterozygous larvae as well as scattered Hh-positive hemocytes in the cortical zone (A). A tight cluster of Hh-positive cells is also present in the PSC regions of each primary lymph gland lobe in *His2Av*^{810} / *His2Av*^{810} homozygous larvae (B). Few if any prohemocytes are present in the homozygous mutant lymph glands. The differences in relative areas of the primary lobe occupied by Hh-positive cells were similar to those observed for Antp-positive cells in Figure S1 (0.25 for

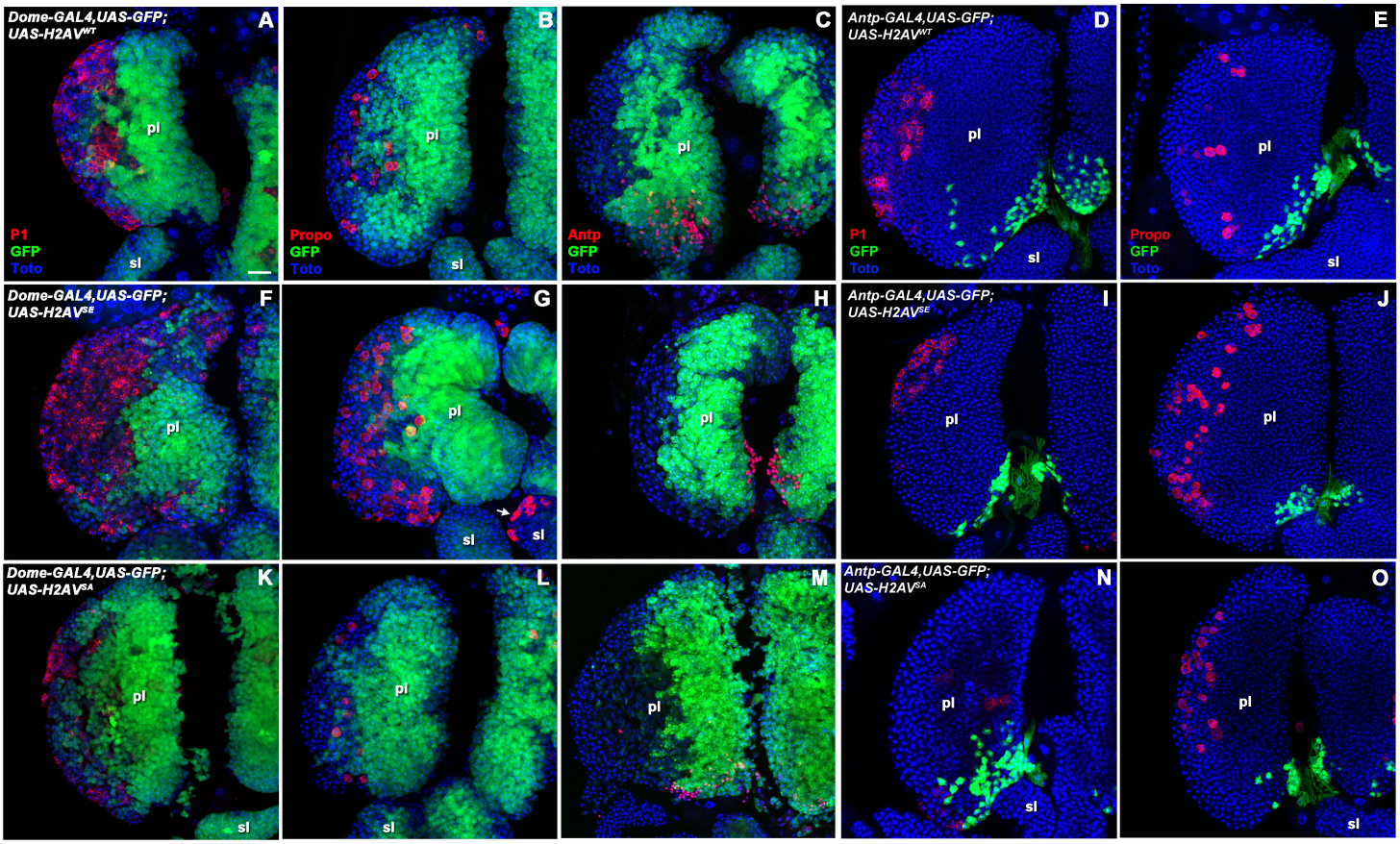
homozygotes; 0.08 for heterozygotes). Wg expression can be seen in the primary and secondary lobes of the *His2Av*^{810} / + heterozygous larval lymph gland (C). Wg expression is also seen in what remains of the primary lymph gland lobe of *His2Av*^{810} / *His2Av*^{810} homozygous larvae (D). Abbreviations: pl: primary lobe; sl: secondary lobe. Scale Bars: A-D, 20μm.



Grigorian, et al, Supplementary Figure S3

Supplementary Figure S3

An H2AV-mRFP fusion protein rescues larval lymph gland structure and function in a *His2Av* null mutant. A transgene expressing an H2AV protein fused to mRFP *P{His2Av::mRFP}* was tested for lymph gland rescue in a $His2Av^{810}$ / $His2Av^{810}$ homozygous mutant background. Immunostaining was used to detect H2AV-mRFP (RFP, red; A-D), the PSC (Antp, green;A-B), plasmatocytes (P1,green; C-D), and crystal cells (Propo, green; E-F). Nuclei were stained with a DNA dye (Toto, blue; A-F). Arrowheads mark areas where crystal cells appear to have burst and left debris (E). Panels B and F are shown at lower magnification to facilitate visualization of the secondary lobes. Abbreviations: pl: primary lobe; sl: secondary lobe. Scale Bars: A,C,D,E, 20μm ; B, 20μm ; F, 20μm.



Grigorian, et al, Supplementary Figure S4

Supplementary Figure S4

Ectopic expression of H2AV proteins leads to changes in the lymph gland. *Domeless-GAL4* (*Dome-GAL4*) was used to drive the expression of *UAS-GFP* along with *UAS-H2AV^{WT}* (A-C), *UAS-H2AV^{SE}* phospho-mimic (F-H), or *UAS-H2AV^{SA}* phospho-mutant (K-M) in an otherwise wild-type *His2Av* background. Lymph glands were immunostained for the presence of plasmatocytes (P1, red; A,F,K), crystal cells (Propo,red; B,G,L), or the PSC (Antp, red; C,H,M). The arrow points to an area of abnormal crystal cell differentiation within the secondary lobe (G). *Antennapedia-GAL4* (*Antp-GAL4*) was used to drive *UAS-GFP* along with *UAS-H2AV^{WT}* (D,E), *UAS-H2AV^{SE}* phospho-mimic (I,J), or *UAS-H2AV^{SA}* phospho-mutant (N,O). Plasmatocytes (P1, red; D,I,N) and crystal cells (Propo, red; E,J,O) were detected by immunostaining. GFP indicates the areas in which the GAL4 drivers are active (green; A-O). Nuclei were labeled with a DNA dye (Toto, blue; A-O). Abbreviations: pl: primary lobe; sl: secondary lobe. Scale Bars: A-O, 20μm.

The arrow points to an area of abnormal crystal cell differentiation within the secondary lobe (G). *Antennapedia-GAL4* (*Antp-GAL4*) was used to drive *UAS-GFP* along with *UAS-H2AV*^{WT} (D,E), *UAS-H2AV*^{SE} phospho-mimic (I,J), or *UAS-H2AV*^{SA} phospho-mutant (N,O). Plasmatocytes (P1, red; D,I,N) and crystal cells (Propo, red; E,J,O) were detected by immunostaining. GFP indicates the areas in which the GAL4 drivers are active (green; A-O). Nuclei were labeled with a DNA dye (Toto, blue; A-O). Abbreviations: pl: primary lobe; sl: secondary lobe. Scale Bars: A-O, 20μm.