Gcn5 – mTORC1 – TFEB signalling axis mediated control of autophagy regulates *Drosophila* blood cell homeostasis

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Abstract:

Blood progenitors are regulated by a variety of signals from their environment. In the *Drosophila* lymph gland (LG), the Posterior Signalling Center (PSC) acts as a stem cell niche striking a balance between progenitors and differentiated blood cells. While the response of blood progenitors to extrinsic signals is well characterized, their ability to respond to cell intrinsic cues is unexplored. Autophagy is one such intrinsic cellular process that maintains cellular homeostasis by removing unnecessary or dysfunctional cell components through autophagic degradation and recycling. Here, we show that autophagy plays a critical role in regulating blood cell homeostasis in the lymph gland. General control non-derepressible 5 (Gcn5), a histone acetyltransferase is expressed in all the cellular subsets of the LG and modulation of Gcn5 levels in various cellular subsets of the LG perturbs LG homeostasis. Gcn5 through its known non-histone acetylation target, TFEB controls autophagic flux thereby regulating hematopoiesis. Additionally, we demonstrate that modulation of mTORC1 activity can perturb hematopoiesis. We show that Gcn5 acts as a nutrient sensor and mTORC1 activity regulates Gcn5. mTORC1 over-rides the effect exerted by Gcn5 in regulating LG hematopoiesis. Together, our findings indicate that Gcn5 – mTORC1 – TFEB signaling axis mediated control of autophagy is required for maintaining blood cell homeostasis in *Drosophila*.

Introduction:

Drosophila hematopoiesis occurs in two different waves: the first wave occurs in the embryonic stages and the second wave of definitive hematopoiesis occurs in the lymph gland (LG), the larval hematopoietic organ (1). The larval lymph gland can be divided into functionally distinct regions: the Posterior Signaling Center (PSC), which acts as the stem cell niche producing signals that are not only required for maintaining the prohemocytes but also for priming them towards differentiation. The Medullary Zone (MZ) is made up of prohemocytes (hematopoietic progenitors) that are capable of differentiating into three differentiated blood cell types namely plasmatocytes, crystal cells and lamellocytes. The Cortical Zone (CZ) houses the differentiated blood cell population (2). Single cell sequencing efforts have identified heterogeneity amongst the developing hemocytes in the lymph gland and have discovered previously unreported hemocyte types like adipohemocytes, stem-like prohemocytes, intermediate prohemocytes, early lamellocytes etc. (3). These recent single cell sequencing based studies show that the lymph gland is a complex organ with multiple hemocyte types and consists of an intricate array of signals that regulate them (3,4).

The cellular fate of prohemocytes in the medullary zone is intricately controlled by a set of extrinsic and intrinsic signals. Multiple signaling pathways like the JAK/STAT, Hedgehog, Wingless, Dpp have been reported to play an important role in prohemocyte maintenance (5-13). Stem cell and tissue homeostasis is closely linked to the nutritional status of the organism. There is increasing evidence indicating the involvement of nutrient signals in stem/progenitor cell fate (14-17). However, how these systemic signals are sensed by the prohemocytes to regulate cell fate determination is not clearly understood. Extrinsic signals often coordinate with cell intrinsic pathways to exert their effects. One such cell intrinsic cellular process that is responsive to extrinsic signals like nutritional status and stress is autophagy (18). Autophagy is a catalytic process that ensures cellular homeostasis by removing intracellular waste material, replaces old and damaged organelles and sustains cell survival during nutrient deprivation (19). One of the key nutrient sensing molecules, mTOR (molecular target of rapamycin) is an important regulator of autophagy. mTORC1 inhibits autophagy in nutrient replete conditions by targeting either initiation, expansion or termination of autophagy (20). Several studies indicate that autophagy is essential for the maintenance of hematopoietic stem cells (21-23). There have been several reports that investigate the role of key autophagy genes in hematopoiesis and show that mice lacking functional autophagy genes like Atg7, Atg12 or FIP200 lead to an exhaustion of the HSC pool especially long term HSCs thus showing that autophagy is critical for HSC maintenance (22, 24-25). Furthermore, HSCs lacking Atg12 displayed phenotypes similar to aged HSCs viz. increased mitochondrial content, heightened metabolic activity, myeloid differentiation bias (23). Mis-regulation of the autophagic process can lead to leukemogenesis (26). Though the role of autophagy has been explored in mammalian hematopoiesis, its role during *Drosophila* hematopoiesis is unexplored. There are various nutrient sensors that can sense systemic nutrient signals and regulate cellular processes like autophagy. In this study, we report that General control non-depressible 5 (GCN5),

a histone acetyltransferase, regulates the process of autophagy during hematopoiesis through its non-histone acetylation target, TFEB.

General control non-depressible 5 (GCN5) is the first nuclear histone acetyltransferase (HAT) to be identified with function in transcriptional regulation by serving as a key subunit in transcriptional coactivator complexes like SAGA (Spt-Ada-Gcn5-acetyltransferase) and ATAC (Ada-2A-containing) (27-29). The transfer of acetyl group to the lysine residues of histones mediates HAT activity (30). The primary acetylation site of Gcn5 is lysine 14 of Histone 3 (H3K14) whereas other lysine residues of Histone 3 like H3K9, H3K18, H3K23, H3K27 are also found to be acetylated by Gcn5 (31). Recent studies reported the additional role of Gcn5 in histone succinylation as well as crotonylation (32-34). Gcn5 has important functions in the extension of lifespan (35), metabolism (36), cellular differentiation (37), cell proliferation and growth (38), neuronal apoptosis (39) and DNA damage response (40). GCN5 acts as an oncoprotein in cancer and its inhibition can rescue cancer phenotypes in various cancers namely hepatocellular carcinoma, glioma, non-small cell lung carcinoma (41-43). Gcn5 is over-expressed in Acute Myeloid Leukemia (AML) and is needed for the survival of AML cell lines (44). Aberrant acetylation of H3K9 by GCN5 leads to all-trans Retinoic acid (ATRA) resistance in non-APL AML, thereby preventing the maturation of myeloid blasts and maintaining them in a leukemic state (45). GCN5 plays a role in drug resistance in the leukemic early drug resistant population (EDRP) where it interacts with ATM thereby recruiting ATM to the DNA damage site (46). With an evolutionary well conserved structure and function, the significance of Gcn5 in different cellular processes can be studied using a variety of model organisms (33). *Drosophila* has only one homolog of Gcn5 (dGCN5), hence making it an efficient model to study the functions of Gcn5 (47). In *Drosophila*, loss of Gcn5 function affects developmental processes like oogenesis, metamorphosis (48). Gcn5 plays an important role in germline stem cell maintenance (49). Other than the developmental processes, Gcn5 was shown to regulate a cellular process like autophagy by targeting TFEB, a non-histone acetylation target of Gcn5 that activates genes responsible for autophagosomal and lysosome biogenesis (50). mTORC1 regulates autophagy not only through the initiator kinase, Ulk1 arm but also through inhibitory phosphorylation of TFEB (51).

In the context of hematopoiesis, Gcn5 is necessary for T-cell development and function, and deletion of Gcn5 could affect the development of immune cells in vertebrates (37). Gcn5 expression increases during the differentiation phase of human CD34 cells indicating an important role of Gcn5 in erythroid differentiation (52). Although there are studies demonstrating the role of Gcn5 in leukemogenesis there is paucity of information on its role in physiological hematopoiesis. In *Drosophila*, a study reported that loss of a HAT like Gcn5 or Chameau phenocopies the differentiation defect seen upon loss of function of molecules regulating fatty acid oxidation (53). There are no other studies that have explored the role of Gcn5 during normal hematopoiesis in *Drosophila*.

Here, we show that Gcn5 plays an essential role in regulating blood cell homeostasis in the *Drosophila* lymph gland. Analysis of *gcn5* mutants, cellular subset specific modulation of Gcn5 levels in the LG and by utilizing structure function approaches in the LG, we show that Gcn5 function is important for developmental hematopoiesis. Our results demonstrate that modulation of Gcn5 levels alter blood cell homeostasis via its regulation of autophagy. We show that autophagy plays a critical role in the lymph gland by controlling blood cell differentiation. Gcn5 acts as a nutrient sensor and negatively regulates autophagy through its non-histone acetylation target, TFEB. Additionally, our findings indicate that modulation of mTORC1 activity can regulate Gcn5 levels and perturb LG hematopoiesis. mTORC1 over-rides the effect exerted by Gcn5 in regulating LG hematopoiesis. Taken together, we suggest that the Gcn5 – mTORC1 – TFEB signaling axis mediated control of autophagy is required for maintaining blood cell homeostasis in *Drosophila.*

Results:

Gcn5 is expressed in all the cellular subsets of the primary lymph gland lobe

General control non-depressible 5 (GCN5) is a histone acetyltransferase involved in transcriptional regulation. Gcn5 has been previously reported to be overexpressed in acute myeloid leukemias (AML) and is required for the survival of AML cells (44-46). In *Drosophila*, Gcn5 has been shown to play an important role during metamorphosis and in the maintenance of germline stem cells (48, 49). However, its role in *Drosophila* hematopoiesis is unknown. We first set out to understand the expression of Gcn5 in the *Drosophila* lymph gland where the primary lobe is the main site of active hematopoiesis. The primary lobe consists of various developmental zones housing different cell populations with identifiable markers (Fig. 1A, B). Gcn5 expression analysis shows that Gcn5 is expressed in all cellular subpopulations of the lymph gland including the Posterior Signalling Center (PSC, Fig 1C-E'), Medullary Zone (MZ, Fig 1D-F'), Cortical Zone (CZ, Fig 1G-I') and with HHLT-GFP positive pan hemocyte marker (Fig 1H-J').

Whole animal *gcn5* mutants show defective blood cell homeostasis

Since Gcn5 is expressed in all the cellular subpopulations of the lymph gland, we were interested to know if hematopoiesis gets affected in the whole animal *gcn5* mutants. We first characterized the hematopoietic phenotypes of the *gcn5* null allele, *gcn5[E333st]* and the hypomorphic allele, *gcn5[C137Y]* in heterozygous conditions. We observed that the Antennapedia positive PSC cell numbers were not affected in the *gcn5[C137Y/+]* or *gcn5[E333st/+]* heterozygous mutants compared to wildtype (Fig S1A-D). Complete blockage of P1 (NimRodC1) positive plasmatocyte differentiation was observed in the *gcn5[E333st/+]* mutant whereas no significant change was seen in the *gcn5[C137Y/+]* mutant compared to wildtype (Fig S1E-H). In contrast, a significant increase in Hindsight positive Crystal cell differentiation and gamma-H2Ax positive DNA damage

foci were observed in both *gcn5[C137Y/+]* and *gcn5[E333st/+]* mutants, compared to wildtype (Fig S1I-P).

We then combined these alleles in homozygous and trans-heterozygous combinations. While *gcn5 [C137Y]* hypomorphic allele yielded viable homozygous larvae, the null allele *gcn5 [E333st]* was embryonic lethal in homozygous conditions. We also generated *gcn5 [C137Y]/gcn5 [E333st]* transheterozygotes in order to study the hematopoietic phenotypes of the *gcn5* mutants. Our analysis reveals that the PSC cell numbers were reduced in the *gcn5[E333st/C137Y]* transheterozygous mutant whereas no change was observed in the *gcn5[C137Y/C137Y]* homozygous mutant, compared to wildtype (Fig 2A-D). Plasmatocyte differentiation was significantly increased in the *gcn5[C137Y/C137Y]* homozygous mutant, whereas no significant change was observed in *gcn5[E333st/C137Y]* transheterozygous mutant compared to wildtype (Fig 2E-H). A significant increase in crystal cell differentiation and DNA damage was observed in both *gcn5[C137Y/C137Y]* and *gcn5[E333st/C137Y]* mutants, compared to wildtype (Fig 2I-P).

Lymph gland cellular subset-specific modulation of Gcn5 levels results in aberrant hematopoiesis

The observations from *gcn5* mutants led us to question if the modulation of Gcn5 levels in different cellular populations of the lymph gland affects hematopoiesis. In order to modulate Gcn5 levels, we have depleted or over-expressed Gcn5 in different cellular subsets of the lymph gland. Validation of *Hml-Gal4* mediated knockdown or overexpression of Gcn5 has been done using immunostaining with Gcn5 antibody (Fig S2A-C'). The FLAG-tagged Gcn5 overexpression construct has been validated using the anti-FLAG tag antibody by immunostaining (Fig S2D-E'). Validation of Gcn5 knockdown was also validated using protein expression analysis by western blotting (Fig S2F).

Since the balance between the prohemocyte population and the differentiated blood cells is a determinant of the homeostasis conditions in the lymph gland, we first depleted or overexpressed Gcn5 in the prohemocytes using *Dome-Gal4*. We observed that the PSC population remained unperturbed upon Gcn5 modulation in the prohemocytes as compared to wildtype (Fig 3A-C' and M). Over-expression of Gcn5 using *Dome-Gal4* led to a significant increase in both plasmatocyte and crystal cell differentiation whereas knockdown of Gcn5 did not affect blood cell differentiation compared to wildtype (Fig 3D-I', N and O). An increase in DNA damage marked by Gamma H2Ax was observed upon knockdown of Gcn5 in the Dome population, whereas overexpression of Gcn5 did not lead to any change in DNA damage, as compared to wildtype (Fig 3J-L' and P). Since PSC is known to produce signals that are required not only for maintaining the prohemocytes but also for priming them towards differentiation we modulated Gcn5 levels in the PSC using *Collier-Gal4*. Over-expression of Gcn5 in the PSC led to a cell-autonomous increase in PSC cell numbers whereas knockdown had no change in the PSC population as compared to the wildtype (Fig S3A-C' and M). Plasmatocyte differentiation was reduced upon PSC- specific knockdown of

Gcn5 whereas over-expression of Gcn5 did not affect plasmatocyte differentiation as compared to the wildtype (Fig S3D-F' and N). Crystal cell differentiation remained unaffected upon modulation of Gcn5 in the PSC as compared to wildtype (Fig S3G-I' and O). An increase in DNA damage was observed upon knockdown of Gcn5 in the PSC whereas over-expression did not lead to any change in DNA damage as compared to the wildtype (Fig S3J-L' and P).

Since over-expression of Gcn5 in the prohemocyte population resulted in an increase in blood cell differentiation we wanted to understand if perturbing Gcn5 levels in the differentiated blood cell population using *Hmldelta-Gal4* alters blood cell differentiation cell-autonomously. A non-cell-autonomous increase in the PSC population was observed upon knockdown or over-expression of Gcn5 using *Hmldelta-Gal4* as compared to wildtype (Fig S4A-C' and M). Plasmatocyte differentiation remained unaffected upon modulation of Gcn5 levels in the Hmldelta positive population compared to wildtype (Fig S4D-F' and N), whereas a cell-autonomous increase in the crystal cell differentiation was observed upon overexpression of Gcn5 in the Hmldelta positive population whereas knockdown of Gcn5 did not affect the crystal cell differentiation compared to wildtype (Fig S4G-I' and O). An increase in DNA damage was observed upon knockdown or over-expression of Gcn5 levels in the Hmldelta population, compared to wildtype (Fig S4J-L' and P). Overall, our analysis shows that modulation of Gcn5 levels in various sub-populations of the LG affects blood cell homeostasis.

Prohemocyte-specific expression of Gcn5 domain deletion constructs alters the hematopoietic program and results in increased DNA damage:

Gcn5 is an important component in the SAGA complex containing four different domains – the HAT, Pcaf, Ada, and Bromodomain (Fig.4A). We were further interested in investigating the functional role of each of the Gcn5 domains. In order to understand if any of the domain deletion constructs of Gcn5 perform a dominant negative function during hematopoiesis, we expressed Gcn5 lacking either the HAT, Pcaf, Ada or Bromodomain in the *Drosophila* prohemocytes using *tep4-Gal4*. A significant cell non-autonomous reduction in the PSC cell numbers was observed upon expression of Gcn5 domain deletion constructs in the prohemocyte population (Fig 4B-G). The tep4-GFP positive prohemocyte population was reduced upon expression of Bromo or Ada deletion constructs of Gcn5 using *tep4-Gal4*, whereas no change was observed upon expression of HAT or Pcaf domain deletion, compared to wildtype (Fig 4B'-F' and H). A significant decrease in plasmatocyte differentiation was seen upon expression of Pcaf domain deletion construct of Gcn5, whereas, expression of Bromo or Ada domain deletion constructs of Gcn5 led to a significant increase in plasmatocyte differentiation. On the other hand, expression of Gcn5 lacking HAT domain using *tep4-Gal4* did not have any significant change in plasmatocyte differentiation (Fig 4B"-F" and I). An increase in crystal cell differentiation was observed upon expression of HAT, Bromo, or Ada domain deletion of constructs of Gcn5, whereas expression of Gcn5 lacking Pcaf domain using *tep4-Gal4* did not affect crystal cell differentiation, compared to wildtype (Fig

4B"'-F"' and J). A significant increase in DNA damage was also observed upon expression of all domain deletion constructs of Gcn5 using *tep4-Gal4*, compared to wildtype (Fig S5A-F).

Autophagic flux in the *Drosophila* blood cells is negatively regulated by Gcn5

Increasing evidence suggests that Gcn5 can regulate gene expression programs linked to cellular metabolism (54). Autophagy is a cellular process that is closely associated with cell metabolism regulation and intracellular quality control as it is involved in the turnover of undesired cellular components, damaged or dysfunctional organelles (18-19, 55). Autophagic flux is regulated by a number of molecules, one of them is Transcription Factor EB (TFEB) which is a known non-histone acetylation target of Gcn5. TFEB regulates expression of genes related to autophagosome formation and lysosome production which are critical for autophagy. Mammalian cell culture based studies show that acetylation of TFEB by Gcn5 decreases TFEB transcriptional activity by disrupting its dimerization thereby affecting autophagy. Studies done in the *Drosophila* fat body indicate that Gcn5 negatively regulates formation of Atg8a puncta in starved conditions (50).

Hence we sought to determine if Gcn5 regulates autophagic flux in *Drosophila* blood cells. We genetically modulated Gcn5 levels using a pan hemocyte driver, *Hml-Gal4* and probed the lymph gland hemocytes for Ref(2)P/p62, an adaptor protein that is itself cleared during autophagy and Atg8 (homologue of human LC3). Our results indicate that there are no p62 puncta upon knockdown of Gcn5, whereas over-expression of Gcn5 led to an increase in p62 puncta, compared to wildtype (Fig 5A-C). Analysis of p62 levels using Western blotting also shows that Gcn5 knockdown leads to a reduction in p62 levels, whereas over-expression of Gcn5 results in an increase in p62 levels, compared to wildtype (Fig 5D). On the contrary, immunostaining with Atg8 showed an increase in Atg8 puncta upon knockdown of Gcn5, whereas over-expression of Gcn5 leads to a significant reduction in the Atg8 puncta compared to wildtype (Fig 5A'-C'). In corroboration with these results, we observed an increase in Atg8 levels upon Gcn5 knockdown, whereas over-expression of Gcn5 led to a reduction in Atg8 levels as compared to the wildtype (Fig 5E). We also looked at the transcript levels of a panel of Atg genes involved during autophagy upon over-expression of Gcn5 and our results indicate that Gcn5 over-expression using pan hemocyte driver, *Hml-Gal4* leads to a reduction in transcript levels of a panel of Atg genes (Fig S6)

Genetic and chemical ablation of autophagy boosts blood cell differentiation in the primary lymph gland lobe

Autophagy has been shown to regulate cellular homeostasis of all hematopoietic lineages in mammals (56). Mis-regulation of autophagy in hematopoietic stem cells (HSC) leads to dysfunction and loss of HSCs with age (23). Transcription factors that are considered to be master regulators of hematopoiesis have been shown to transcriptionally control autophagy related genes (21, 57). In *Drosophila*, Atg6 has been shown to play a role in multiple vesicle trafficking pathways and hematopoiesis (58).

Since we found that Gcn5 negatively regulates autophagy in *Drosophila* hemocytes, we asked the question whether key autophagy related genes regulate lymph gland hematopoiesis. Since Gcn5 regulates autophagy via TFEB, a key regulator in autophagosome and lysosome biogenesis, we perturbed TFEB and several autophagy effector genes like Atg8a, Atg5, or Atg18a specifically in the *Drosophila* prohemocytes using *tep4-Gal4*. We observed that depleting TFEB or the autophagy effector genes in the tep population led to a drastic increase in both plasmatocyte and crystal cell differentiation in the lymph gland (Fig 6A-L). In addition to the genetic perturbation based analysis, Chemical inhibition of autophagy by treating the larvae with autophagy inhibitor, chloroquine (CQ) also leads to a significant increase in blood cell differentiation in the lymph gland (Fig 6M-R).

Chemical modulation of mTORC1 activity controls blood cell differentiation

mTOR signaling, specifically mTORC1 has been shown to play a significant role in hematopoietic lineage commitment in mice (59). mTORC1 acts as a master regulator of autophagy and controls various steps of the autophagic process (20). In nutrient replete conditions, mTORC1 phosphorylates ULK1, a key initiator kinase in autophagy thereby disrupting its interaction with AMPK and ULK1 activation by AMPK (60-63). Similarly, mTORC1 phosphorylates TFEB in nutrient availability conditions at residues - S142 and S211 subjecting TFEB to localize in the cytoplasm rendering it inactive (64). Since mTORC1 regulates critical nodal points that control autophagy we were curious to know if modulating mTORC1 activity alters blood cell homeostasis in the LG.

We modulated mTORC1 activity by chemical modulation. We used 3BDO for mTOR activation and Rapamycin for mTOR inhibition. Upon treatment with the mTOR activator – 3BDO, a significant increase in both plasmatocyte and crystal cell differentiation was observed as compared to the control treatment (Fig 7A-F), whereas treatment with mTOR inhibitor – Rapamycin led to a decrease in plasmatocyte and crystal cell differentiation, compared to the control treatment (Fig 7G-L).

Gcn5 acts as a nutrient sensor and is regulated by mTORC1

Enzymatic activity of Gcn5 is regulated by the cellular and metabolic energy state which in turn controls gene expression programs (54). Gcn5 is capable of sensing Acetyl Co-A which is a central metabolic intermediate (65). Since Gcn5 levels are critical in controlling gene expression programs and transcriptional noise (66), we tested if Gcn5 levels are responsive to altered nutrient conditions. We subjected *Drosophila* larvae to different dietary conditions viz. Fed, starved and high fat diet (HFD) and probed for Gcn5 levels in these conditions. Our results indicate that there is a decrease in Gcn5 levels upon starvation whereas an increase in high fat diet (HFD) conditions (Fig 7M). mTOR is known to control protein translation in response to nutrient stress signals; under nutrient deprivation, mTOR is inhibited thereby inducing autophagy. There are few studies that have linked mTOR to histone acetyltransferase Gcn5 and therefore nutrient response (50, 67).

To further determine if Gcn5 levels are regulated by mTORC1 activity, we treated larvae with mTOR inhibitor Rapamycin and probed for Gcn5 levels. We found that there was a decrease in Gcn5 levels upon mTOR inhibition as compared to control (Fig 7N).

mTORC1 overrides the effect of Gcn5 in regulating blood cell homeostasis in the lymph gland

mTORC1 regulates autophagy at multiple nodal points for example by phosphorylation of initiator kinase ULK1 or by phosphorylating TFEB preventing its nuclear translocation. On the other hand, Gcn5 regulates autophagy by acetylation of TFEB preventing its dimerization and nuclear translocation thereby inhibiting autophagy (50). Since both mTORC1 and Gcn5 converge on and exert their effect over TFEB by phosphorylation or acetylation respectively, we wanted to investigate if one can override the effector function of the other. Since our results indicate that mTOR activity can regulate Gcn5 levels, we wanted to test if mTORC1 can override the effect of Gcn5 modulation in controlling LG hematopoiesis. Activation of mTOR by treatment with 3BDO in *tep4-Gal4* mediated prohemocyte specific Gcn5 knockdown genetic background led to increased plasmatocyte and crystal cell differentiation as compared to the *tep4-Gal4* mediated prohemocyte specific Gcn5 knockdown control (Fig 8A-D', I and J). Similarly, inhibition of mTOR by Rapamycin treatment in the *dome-Gal4* mediated prohemocyte specific Gcn5 over-expression genetic background resulted in a significant reduction in plasmatocyte and crystal cell differentiation as compared to the *dome-Gal4* mediated prohemocyte specific Gcn5 over-expression control (Fig 8E-H', K and L). Taken together, our results demonstrate that a fine balance between mTORC1 mediated phosphorylation of TFEB and Gcn5 mediated acetylation of TFEB maintains the autophagic flux. Nutrient conditions, mTORC1 activity and Gcn5 levels are key determinants of autophagic flux in the LG. Maintenance of the autophagic flux and turnover is critical for regulating blood cell homeostasis in the LG. Thus, Gcn5-mTOR-TFEB signaling axis controls autophagy thereby regulating *Drosophila* blood cell homeostasis in the LG (Fig 9).

Discussion

Gcn5 is highly expressed in acute myeloid leukemia (AML). It helps in the maintenance of immature leukemic blasts and contributes to All-Trans Retinoic Acid (ATRA) resistance in non-APL AML (non-acute promyelocytic leukemia AML) (45-46). While its function in the context of leukemia is well studied, the molecular function of Gcn5 during physiological hematopoiesis is unexplored. Here, we employ *Drosophila* as a model to understand Gcn5 role during normal physiological hematopoiesis. Our work shows that Gcn5, a histone acetyltransferase plays an important regulatory role during *Drosophila* lymph gland hematopoiesis. We find that Gcn5 is expressed in all the cellular subsets in the LG and its levels are critical for controlling hematopoiesis. We investigated the role of Gcn5 in the LG prohemocytes primarily as the medullary zone prohemocyte population is critical for maintaining blood cell homeostasis in

Drosophila. Structure-function analysis of Gcn5 and its constituent domains shows that expression of Gcn5 lacking any of its constituent domains in wild type genetic background results in a dominant negative phenotype in the LG. Our results indicate that optimum levels of Gcn5 have to be maintained in the prohemocytes as excess Gcn5 levels lead to increased blood cell differentiation and loss of blood cell homeostasis. Further, we find that Gcn5 acts as a nutrient sensor and its levels are critical for controlling the autophagic flux in the LG. In order to investigate whether autophagy players regulate LG hematopoiesis, we carried out a systematic genetic perturbation analysis of key autophagy regulatory genes and our results indicate that disrupting autophagy abrogates LG hematopoiesis. Increased Gcn5 levels in the hemocytes led to a decrease in the autophagic flux whereas depleting Gcn5 resulted in increased autophagy which also correlates with the corresponding hematopoietic phenotypes in the LG.

Since mTORC1 is a known molecular player regulating autophagy in response to nutrient conditions, we investigated whether modulating mTORC1 activity affects hematopoiesis. Our data elucidates that modulating mTORC1 activity can regulate hematopoiesis and these effects on hematopoiesis are consistent with our observations obtained upon genetically modulating the regulators of autophagy. Modulating mTORC1 activity by inhibition using Rapamycin results in the down-regulation of Gcn5 levels establishing a regulatory link between mTORC1 and Gcn5. One of the common molecular nodal points through which both mTORC1 and Gcn5 could potentially regulate autophagy is through Transcription factor EB (TFEB), a member of the microphthalmia-associated transcription factor/transcription factor E (MiTF/TFE) family (68). Our results demonstrate that mTORC1 can override the effect exerted by Gcn5 in regulating LG hematopoiesis. Overall, in this study we demonstrate that the Gcn5-TFEB-mTORC1 signaling axis plays an important role in regulating *Drosophila* LG hematopoiesis via its control over autophagy.

Expression analysis of Gcn5 in the LG indicates that Gcn5 is expressed in all the cellular sub-populations of the LG. Since Gcn5 is ubiquitously found, it prompted us to look at the whole animal mutants of Gcn5. *gcn5* mutants display loss of blood cell homeostasis resulting in increased blood cell differentiation in the LG. Increase in plasmatocyte or crystal cell differentiation could be due to a contribution of systemic signals that are altered upon loss of Gcn5 in the entire organism as the lymph gland is capable of responding to external stimuli (69). *gcn5* mutant alleles either in homozygous or trans-heterozygous conditions show an increased accumulation of DNA damage indicative of its role in DNA damage repair pathways (40, 70). Modulation of Gcn5 levels in specific cellular subsets of the LG results in a number of hematopoietic phenotypes. Prohemocyte specific genetic perturbation of Gcn5 indicates that over-expression of Gcn5 leads to increased plasmatocyte as well as crystal cell differentiation whereas depletion of Gcn5 levels has no significant effect on differentiation. *Hmldelta-Gal4* mediated Cortical Zone (CZ) specific over-expression of Gcn5 resulted in a cell autonomous increase in crystal cell differentiation whereas Gcn5 knockdown had no effect. These results show that optimum levels of Gcn5 are important for maintaining blood cell homeostasis in the LG. Higher levels of Gcn5 skews the balance towards increased blood cell differentiation. Higher Gcn5 expression has been observed previously in many

carcinomas and leukemia promoting cell proliferation, growth and cancer progression (41-46). We also performed structure function analysis of Gcn5 by expressing domain deletion mutants of Gcn5 in wild type genetic background in the LG prohemocyte population. Our analysis shows that expression of Gcn5 lacking the Bromo or Ada domain in the wild type genetic background results in a dominant negative phenotype in terms of the maintenance of prohemocyte population itself and plasmatocyte differentiation whereas expression of Gcn5 lacking HAT or Bromo or Ada results in a dominant negative effect on crystal cell differentiation indicating that the constituent domains of Gcn5 might be playing specific functions in regulating various aspects of LG hematopoiesis by aiding in interactions with specific molecules that are involved in hematopoiesis.

Gcn5 has been shown to be responsive to changes in cellular energetic and metabolic states (54). Gcn5 via its non-histone acetylation target, Transcription factor EB (TFEB) is capable of regulating an intracellular process called autophagy which is directly linked to the nutritional status of the cell. TFEB acetylation by Gcn5 is inhibitory for TFEB as it prevents its dimerization and hence nuclear translocation. Upon nuclear translocation, TFEB activates genes responsible for autophagosome and lysosome biogenesis (50). In order to probe whether Gcn5 mediated regulation of hematopoiesis is due to its control over autophagy, we probed whether perturbing Gcn5 expression has any effect on the autophagic flux in the hemocytes. Our results suggest that depletion of Gcn5 leads to an upregulation of autophagy increasing the flux whereas over-expression of Gcn5 results in downregulation of autophagy lowering the autophagic flux. These observations were intriguing as this establishes an important role of autophagy in maintaining the homeostasis paradigm in the LG. We investigated this further to find out if there is a direct link between autophagy regulators (Atg genes) including TFEB in controlling LG hematopoiesis. Genetic perturbation of the autophagy pathway regulators or TFEB in the prohemocytes leads to an increase in blood cell differentiation. Chemical inhibition of autophagy induced by Chloroquine also recapitulates this phenotype. There is literature indicating a critical role for autophagy in development of the blood system, self renewal of HSCs and their mobilization (23-26). Our observations on the role of autophagy in *Drosophila* LG hematopoiesis support these findings. We then moved on to test if mTORC1 could regulate hematopoiesis in order to investigate other possible molecules that could control autophagy in this context. Chemical modulation of mTORC1 activity which is a master molecule that regulates autophagy alters LG hematopoiesis. mTORC1 activity regulates Gcn5 levels as inhibition of mTORC1 activity resulted in lower levels of Gcn5.

mTORC1 regulates autophagy via two arms - One arm is where it regulates the initiator kinase, Ulk1 in response to either nutrient depletion or replete conditions via inhibitory phosphorylation of TFEB (61-63). TFEB phosphorylation by mTORC1 subjects it to degradation thereby inhibiting autophagy (71). Since TFEB is the common molecular nodal point that is regulated by both mTORC1 and Gcn5, we were curious to understand whether one is epistatic to the other in controlling hematopoiesis. We performed experiments to delineate the mechanism and our analysis shows that mTORC1 can override the effect exerted by Gcn5 in controlling hematopoiesis. We speculate that during normal physiological hematopoiesis a balance is struck

between phosphorylation and acetylation of TFEB mediated by mTORC1 and Gcn5 respectively in order to regulate and fine tune levels of autophagy. Perturbation of Gcn5 levels or mTORC1 activity could disturb this balance thereby deregulating hematopoiesis. Our findings shed light on the importance of non histone acetylation function of Gcn5 in a vital process like hematopoiesis. This could especially be important as the LG prohemocytes are known to respond to cell intrinsic and extrinsic nutrient signals (72). The nutrient state can in turn determine the activation or inhibition of a cellular process like autophagy thereby dictating the differentiation trajectory of the precursor cells. Since Gcn5 is highly expressed in acute myeloid leukemia and is responsible for providing chemoresistance to leukemic cells (45, 46) it becomes important to understand how Gcn5 level is regulated and what cellular processes Gcn5 is capable of regulating in order to control tissue homeostasis. Our work sheds novel insights on the role of Gcn5 during normal hematopoiesis and the cellular processes it regulates in order to control hematopoiesis. Our results illustrate that Gcn5 in the LG is capable of sensing and responding to external stimuli by calibrating the levels of autophagy in blood cells. We show that Gcn5 fine tunes autophagy in conjunction with mTORC1 where mTORC1 activity is capable of regulating Gcn5 and can override its function in modulation autophagy. Our analysis indicates that the process of autophagy is critical for controlling the hematopoietic program. It would be interesting to further understand how an optimal balance of phosphorylation or acetylation of TFEB by mTORC1 or Gcn5 respectively modulates the resultant autophagic flux in order to control various cellular functions like stem cell homeostasis. Overall, our work uncovers a novel Gcn5-mTORC1-TFEB signaling axis that regulates *Drosophila* hematopoiesis via its control over autophagy (Figure 9).

Materials and Methods

List of *Drosophila* stocks, antibodies used for immunofluorescence-based experiments have been listed in a detailed manner in the Supplemental Information (SI). The experimental protocol for lymph gland dissection, immunohistochemistry and image analysis have been mentioned in the SI. The procedure for protein extraction, western blotting and quantitative real time PCR have been mentioned in the SI along with the list of primers used for qRT experiments. The chemical treatment procedure has been described in a detailed manner in the SI. Statistical analysis performed for each of the experiments has been described in the statistical analysis section in the SI.

Data, Materials, and Software availability:

All the data related to this study are included in the article and/or in the SI.

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Author contributions:

Conceptualization: AAR and RJK; Methodology: AAR, SM and LK; Validation: AAR; Formal analysis: AAR, SM and LK; Investigation: AAR, SM and LK; Resources: RJK ; Data curation: AAR; Writing - original draft: AAR and RJK; Writing - review & editing: AAR and RJK; Visualization: AAR and RJK; Supervision: RJK; Project administration: RJK; Funding acquisition: RJK

Competing interests:

The authors declare no competing interest

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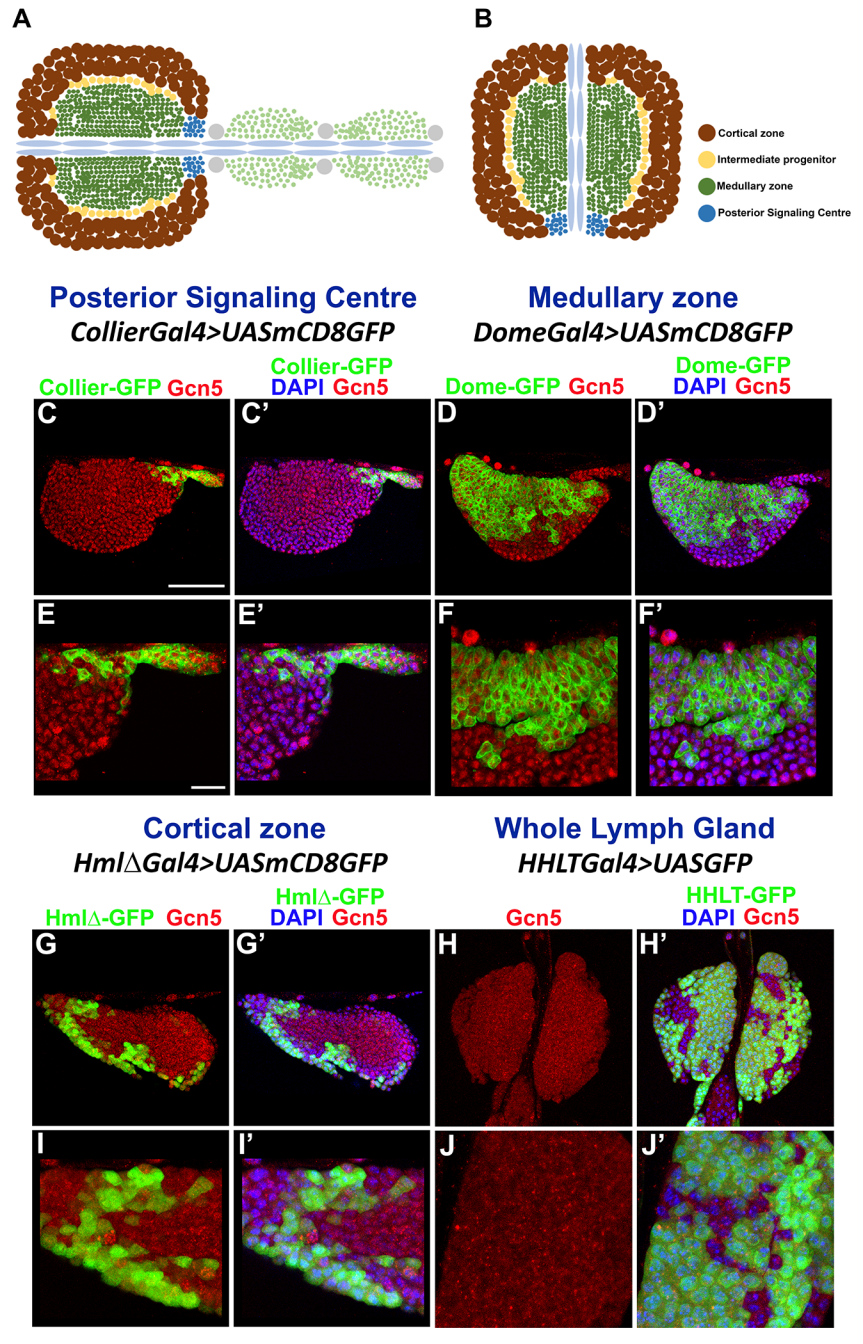
A
B
Cortical zone
Intermediate progenitor
Medullary zone
Posterior Signaling Centre
Posterior Signaling Centre
CollierGal4>UASmCD8GFP
Medullary zone
DomeGal4>UASmCD8GFP
Collier-GFP Gcn5
Collier-GFP
DAPI Gcn5
Dome-GFP Gcn5
Dome-GFP
DAPI Gcn5
C
C'
D
D'
E
E'
F
F'
Cortical zone
HmlΔGal4>UASmCD8GFP
Whole Lymph Gland
HHLTGal4>UASGFP
HmlΔ-GFP Gcn5
HmlΔ-GFP
DAPI Gcn5
Gcn5
HHLT-GFP
DAPI Gcn5
G
G'
H
H'
I
I'
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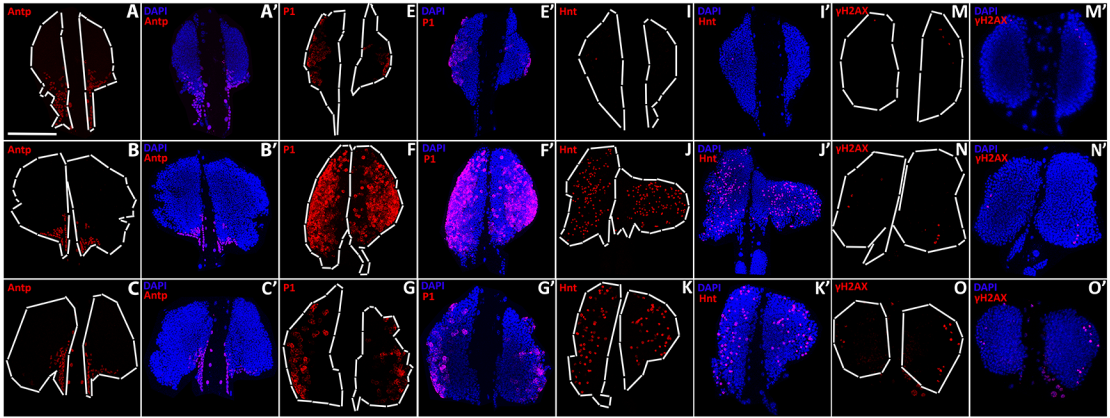
J'

Figure 1. Gcn5 is expressed in all compartments of the *Drosophila* larval lymph gland.

Cartoon of the third instar larval lymph gland, indicating the anterior primary lobes and the posterior lobes (A). The primary lobe of the lymph gland depicting different subpopulations: the Posterior Signaling Centre (PSC: blue) functioning as the niche, Medullary zone (MZ: green) houses the progenitors, Intermediate progenitor zone (IZ: yellow) which contains the cells transitioning from progenitors to specific lineages, and the Cortical zone (CZ: brown) containing the differentiated hemocytes population – plasmatocytes and crystal cells (B). Gcn5 expression in different compartments of lymph gland (C-J) Gcn5 (red) expression in the - Posterior Signaling Centre (PSC) using Collier-GFP (green) (C-C', E-E'), Medullary zone prohemocytes using Dome-GFP (green) (D-D', F-F'), Differentiated hemocytes in the Cortical zone using HmlΔ-GFP (green) (G-G', I-I'), and the Whole Lymph Gland using HHLT-GFP (green) (H-H', J-J'). Nuclei are stained with DAPI (blue). GFP (green) is driven by respective Gal4 drivers. Scale Bar: 50µm (C-D', G-H') and 30µm (E-F', I-J')


wt
gcn5[C137Y/C137Y]
gcn5[E333st/C137Y]
Antp
DAPI
P1
Hnt
γH2AX
A
A'
B
B'
C
C'
E
E'
F
F'
G
G'
I
I'
J
J'
K
K'
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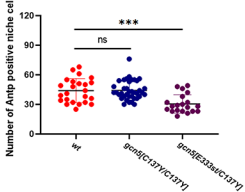
N
N'
O
O'


D
Number of Antp positive niche cells
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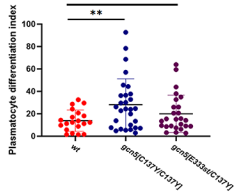
90
60
30
0
***
ns
wt
gcn5[C137Y/C137Y]
gcn5[E333st/C137Y]


H
Plasmatocyte differentiation index
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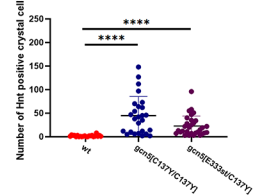
60
40
20
0
ns
**
wt
gcn5[C137Y/C137Y]
gcn5[E333st/C137Y]


L
Number of Hnt positive crystal cells
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200
150
100
50
0
****
****
wt
gcn5[C137Y/C137Y]
gcn5[E333st/C137Y]

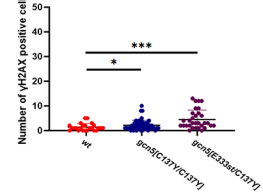
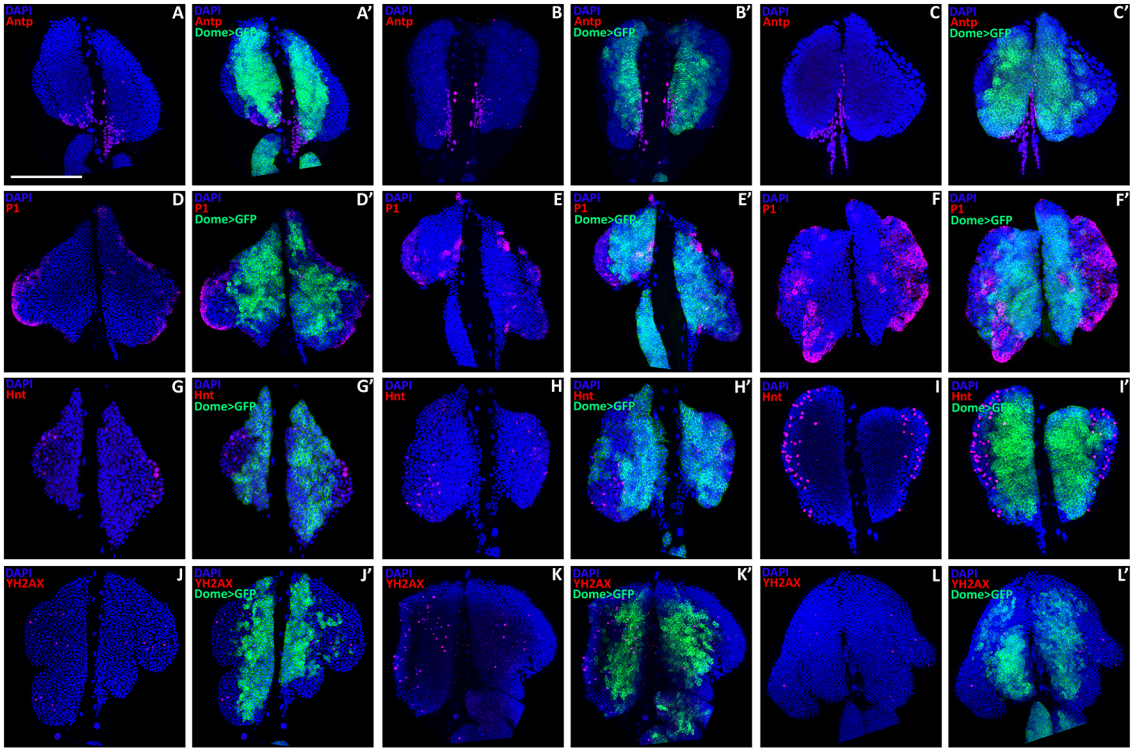

P
Number of γH2AX positive cells
50
40
30
20
10
0
***
*
wt
gcn5[C137Y/C137Y]
gcn5[E333st/C137Y]

Figure 2. Whole animal *gcn5* mutants show defective lymph gland homeostasis.

Posterior Signaling Center (PSC) cell numbers marked by Antennapedia (red) in *gcn5[C137Y/C137Y]* (B-B') and *gcn5[E333st/C137Y]* (C-C') as compared to *wildtype* (A-A'). Graphical representation of PSC cell numbers of the *gcn5* mutants as compared to the wildtype (D), n=24 for *wildtype*, n=38 for *gcn5[E333st/C137Y]*, and n=20 for *gcn5[E333st/C137Y]* for PSC cell number quantification. Plasmatocyte differentiation was marked by P1 (red) in *gcn5[C137Y/C137Y]* (F-F') and *gcn5[E333st/C137Y]* (G-G') as compared to the *wildtype* (E-E'). Graphical representation of plasmatocyte differentiation index of the *gcn5* mutants compared to *wildtype* (H), n=20 for *wildtype*, n=29 for *gcn5[C137Y/C137Y]*, and n=26 for *gcn5[E333st/C137Y]* for quantification. Crystal cell differentiation marked by Hnt (red) in *gcn5[C137Y/C137Y]* (J-J') and *gcn5[E333st/C137Y]* (K-K') as compared to *wildtype* (I-I'). Graphical representation of the number of crystal cells for *gcn5* mutants compared to *wildtype* (L), n=22 for *wildtype*, n=26 for *gcn5[C137Y/C137Y]*, and n=31 for *gcn5[E333st/C137Y]* for quantification. Cells undergoing DNA damage marked by γH2AX (red) in *gcn5[C137Y/C137Y]* (N-N') and *gcn5[E333st/C137Y]* (O-O') as compared to *wildtype* (M-M'). Graphical representation of γH2AX positive cells undergoing DNA damage in the *gcn5* mutants compared to the *wildtype* (P), n=24 for *wildtype*, n=54 for *gcn5[C137YC137Y]*, and n=31 for *gcn5[E333st/C137Y]* for quantification. Nuclei are stained with DAPI (Blue). n represents the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (*$p<0.05$; **$p<0.01$; ***$p<0.001$, ****$p<0.0001$, Student's t-test with Welch's correction). Scale Bar: 50µm (A-O')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DomeGal4UASmCD8GFP x UAS-Gcn5RNAi
DomeGal4UASmCD8GFP x UAS-Gcn5
DAPI
Antp
Dome>GFP
P1
Hnt
YH2AX
A
A'
B
B'
C
C'
D
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L
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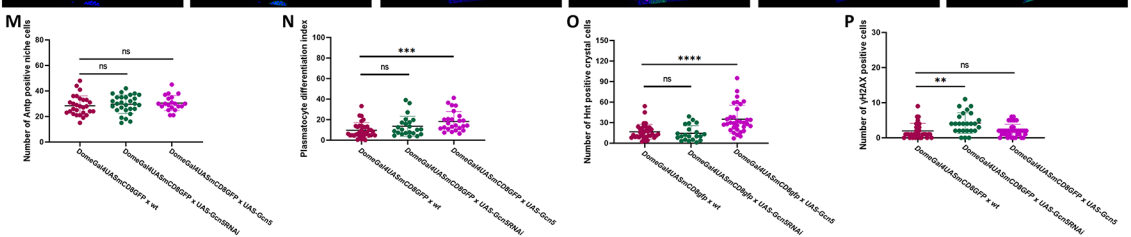

M
Number of Antp positive niche cells
ns
ns
N
Plasmatocyte differentiation index
***
ns
O
Number of Hnt positive crystal cells
****
ns
P
Number of γH2AX positive cells
ns
**
DomeGal4UASmCD8GFP x wt
DomeGal4UASmCD8GFP x UAS-Gcn5RNAi
DomeGal4UASmCD8GFP x UAS-Gcn5

Figure 3. Modulation of Gcn5 levels in the prohemocytes alters blood cell homeostasis in the lymph gland

Posterior Signalling Center (PSC) population marked by Antennapedia (red) upon *Dome-Gal4* mediated knockdown (B-B') or over-expression (C-C') of Gcn5 as compared to wildtype (A-A'). Plasmatocyte differentiation marked by P1 (red) upon *Dome-Gal4* mediated knockdown (E-E') or over-expression (F-F') of Gcn5 as compared to wildtype (D-D'). Crystal cell differentiation marked by Hnt (red) upon *Dome-Gal4* mediated knockdown (H-H') or over-expression (I-I') of Gcn5 as compared to wild type (G-G'). Cells undergoing DNA damage marked by γH2AX (red) upon *Dome-Gal4* mediated knockdown (K-K') or over-expression (L-L') of Gcn5 as compared to wild type (J-J'). Graphical representation of PSC numbers upon modulation of Gcn5 levels in the Dome positive population compared to wildtype (M), n=28 for wildtype, n=28 for Gcn5 knockdown, and n=20 for Gcn5 over-expression. Graphical representation of plasmatocyte differentiation index upon modulation of Gcn5 levels in dome population compared to wildtype (N), n=35 for wildtype, n=23 for Gcn5 knockdown, and n=25 for Gcn5 over-expression. Graphical representation of numbers of Crystal cells upon modulation of Gcn5 levels in the dome population compared to wildtype (O), n=37 for wildtype, n=21 for Gcn5 knockdown, and n=33 for Gcn5 over-expression. Graphical representation of γH2AX positive cells upon modulation of Gcn5 levels in the dome population compared to wildtype (P), n=33 for wildtype, n=27 for Gcn5 knockdown, and n=38 for Gcn5 over-expression. Nuclei are stained with DAPI (blue). Dome>GFP positive population (Green) indicates the expression domain of *Dome-Gal4* activity in the LG. n represent the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (**$p<0.01$; ***$p<0.001$; ****$p<0.0001$, Student's t-test with Welch's correction). Scale Bar: 50µm (A-L')

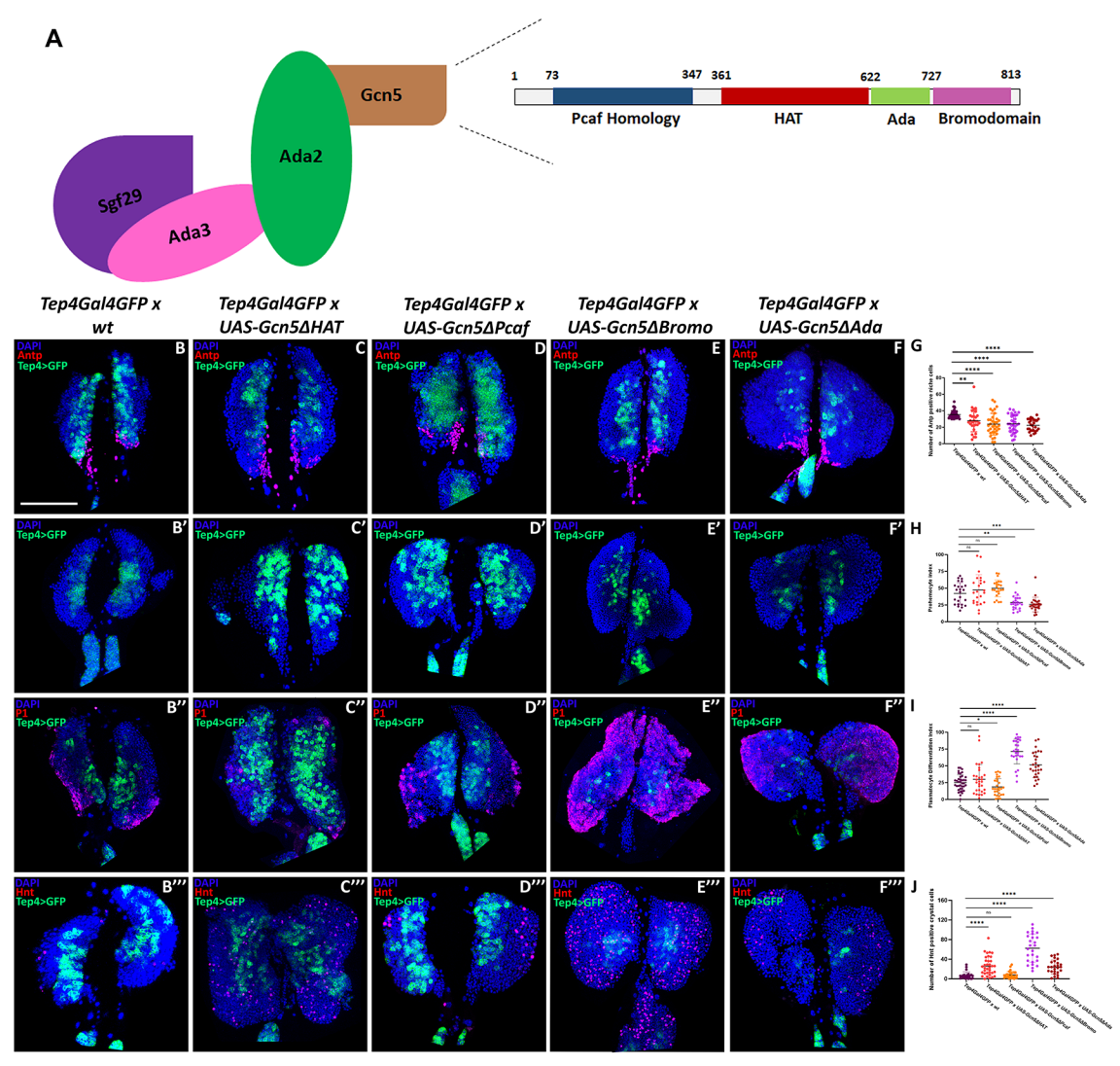
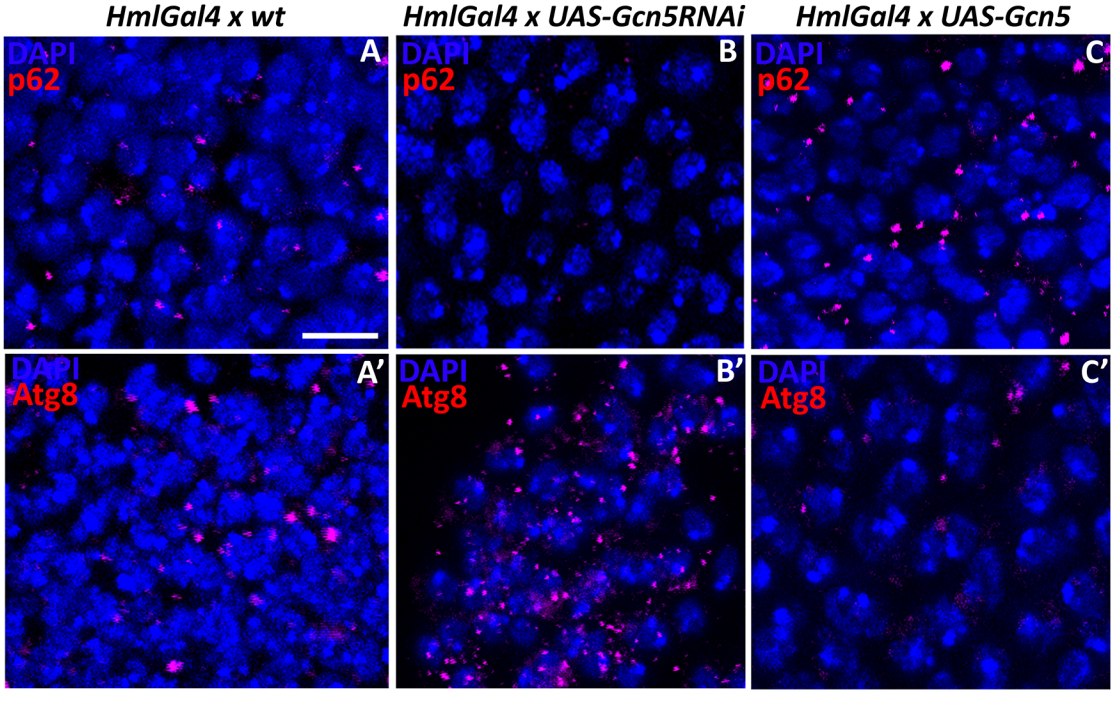
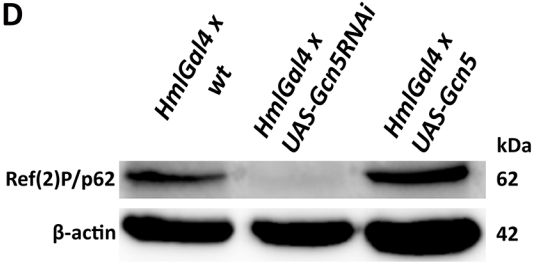

A
Gcn5
Ada2
Sgf29
Ada3
1
73
347
361
622
727
813
Pcaf Homology
HAT
Ada
Bromodomain
Tep4Gal4GFP x wt
Tep4Gal4GFP x UAS-Gcn5ΔHAT
Tep4Gal4GFP x UAS-Gcn5ΔPcaf
Tep4Gal4GFP x UAS-Gcn5ΔBromo
Tep4Gal4GFP x UAS-Gcn5ΔAda
DAPI
Antp
Tep4>GFP
P1
Hnt
B
C
D
E
F
B′
C′
D′
E′
F′
B″
C″
D″
E″
F″
B‴
C‴
D‴
E‴
F‴
G
H
I
J
Number of Antp positive niche cells
Prohemocyte index
Plasmocyte Differentiation Index
Number of Hnt positive crystal cells
Tep4Gal4GFP x wt
Tep4Gal4GFP x UAS-Gcn5ΔHAT
Tep4Gal4GFP x UAS-Gcn5ΔPcaf
Tep4Gal4GFP x UAS-Gcn5ΔBromo
Tep4Gal4GFP x UAS-Gcn5ΔAda

Figure 4. Prohemocyte-specific expression of Gcn5 domain deletion constructs affects LG homeostasis.

Schematic of the SAGA HAT complex containing different modules – Sgf29, Ada2, Gcn5, and Ada3. Gcn5 is an 813 aa protein in *Drosophila* containing the Pcaf homology domain, HAT domain, Ada domain, and Bromodomain (A). Posterior Signalling Center (PSC) numbers marked by Antennapedia (red) upon expression of different Gcn5 domain deletion constructs in the tep-GFP (green) population using *tep4-Gal4* (C-F), compared to wildtype (B). Progenitor index marked by tep (green) upon expression of different Gcn5 domain deletion constructs (C'-F'), compared to wildtype (B'). Plasmatocyte differentiation marked by P1 (red) upon expression of different Gcn5 domain deletion constructs in the tep-GFP population using *tep4-Gal4* (green) (C"-F"), compared to wildtype (B"). Crystal cell differentiation marked by Hnt (red) upon expression of different Gcn5 domain deleted constructs in the tep-GFP population using *tep4-Gal4* (green) (C"'-F"'), compared to wildtype (B"'). Graphical representation of PSC numbers upon expression of Gcn5 domain deletion constructs, compared to wildtype (G), n=40 for wildtype, n=29 for Gcn5ΔHAT expression, n=35 for Gcn5ΔPcaf expression, n=33 for Gcn5ΔBromo expression, and n=26 for Gcn5ΔAda expression in the tep population. Graphical representation of Prohemocyte index upon expression of Gcn5 domain deletion constructs, compared to wildtype (H), n=24 for wildtype, n=24 for Gcn5ΔHAT expression, n=34 for Gcn5ΔPcaf expression, n=24 for Gcn5ΔBromo expression, and n=24 for Gcn5ΔAda expression in the tep population. Graphical representation of Plasmatocyte differentiation index upon expression of Gcn5 domain deleted constructs, compared to wildtype (I), n=38 for wildtype, n=30 for Gcn5ΔHAT expression, n=29 for Gcn5ΔPcaf expression, n=27 for Gcn5ΔBromo expression, and n=27 for Gcn5ΔAda expression in the tep population. Graphical representation of Crystal cell numbers upon expression of Gcn5 domain deleted constructs, compared to wildtype (J), n=23 for wildtype, n=35 for Gcn5ΔHAT expression, n=26 for Gcn5ΔPcaf expression, n=26 for Gcn5ΔBromo expression, and n=26 for Gcn5ΔAda expression in the tep population. Nuclei are stained with DAPI (blue). n represent the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, Student's t-test with Welch's correction). Scale Bar: 50µm (B-F''')


HmlGal4 x wt
HmlGal4 x UAS-Gcn5RNAi
HmlGal4 x UAS-Gcn5
DAPI
p62
A
B
C
Atg8
A'
B'
C'


D
HmlGal4 x wt
HmlGal4 x UAS-Gcn5RNAi
HmlGal4 x UAS-Gcn5
kDa
Ref(2)P/p62
62
β-actin
42

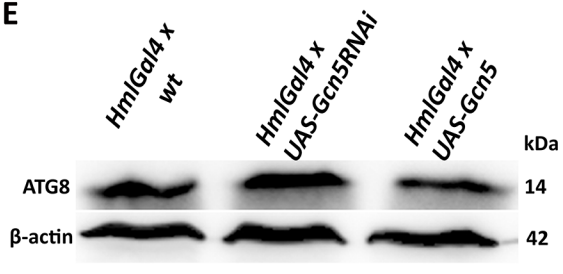

E
HmlGal4 x wt
HmlGal4 x UAS-Gcn5RNAi
HmlGal4 x UAS-Gcn5
kDa
ATG8
14
β-actin
42

Figure 5. Autophagic flux in *Drosophila* blood cells is negatively regulated by Gcn5

p62 (red) labeling the autophagy adaptor protein upon *Hml-Gal4* mediated Gcn5 knockdown (B), or overexpression (C) as compared to wildtype (A). Atg8 (red), the autophagosomal marker upon *Hml-Gal4* mediated Gcn5 knockdown (B') or over-expression (C') as compared to wildtype (A'). Nuclei were stained with DAPI (blue). Immunoblot showing p62 (62kDa) protein levels upon knockdown or over-expression of Gcn5 in the Hml population, compared to wildtype (D). Immunoblot showing ATG8 (14kDa) protein levels upon knockdown or over-expression of Gcn5 in the Hml population, compared to wildtype (E). β-actin (42kDa) was used as the loading control. Scale Bar: 20µm (A-E')

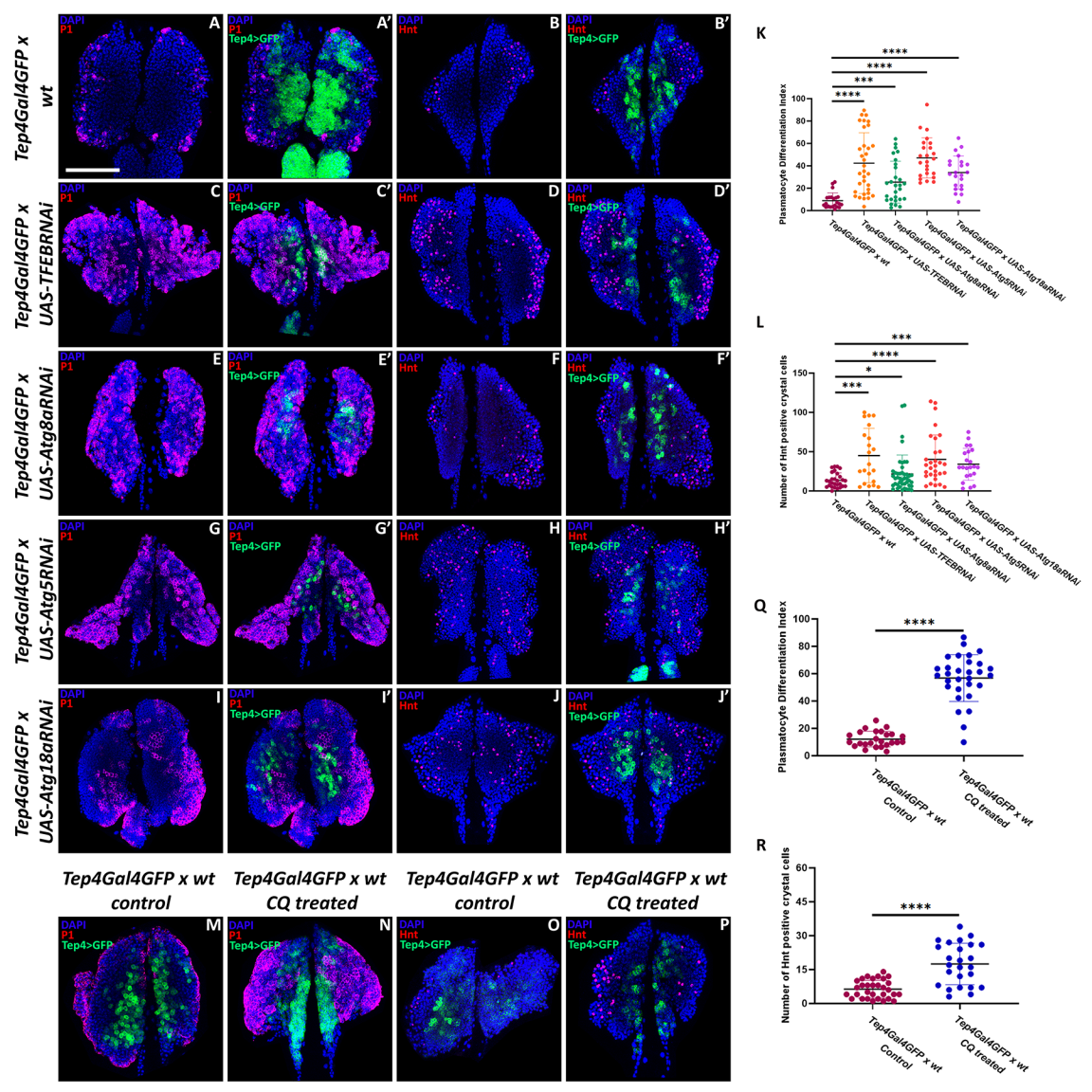

Tep4Gal4GFP x wt
Tep4Gal4GFP x UAS-TFEBRNAi
Tep4Gal4GFP x UAS-Atg8aRNAi
Tep4Gal4GFP x UAS-Atg5RNAi
Tep4Gal4GFP x UAS-Atg18aRNAi
DAPI
P1
Hnt
Tep4>GFP
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'
I
I'
J
J'
Tep4Gal4GFP x wt control
Tep4Gal4GFP x wt CQ treated
Tep4Gal4GFP x wt control
Tep4Gal4GFP x wt CQ treated
M
N
O
P
K
Plasmatocyte Differentiation Index
Tep4Gal4GFP x wt
Tep4Gal4GFP x UAS-TFEBRNAi
Tep4Gal4GFP x UAS-Atg8aRNAi
Tep4Gal4GFP x UAS-Atg5RNAi
Tep4Gal4GFP x UAS-Atg18aRNAi
L
Number of Hnt positive crystal cells
Q
Control
CQ treated
R

Figure 6. Genetic and chemical ablation of autophagy leads to aberrant blood cell differentiation.

Plasmatocyte differentiation marked by P1 (red) upon *tep4-Gal4* mediated knockdown of TFEB (C-C'), Atg8a (E-E'), Atg5 (G-G'), or Atg18 (I-I') in the tep population (green) as compared to wildtype (A-A'). Crystal cell differentiation marked by Hnt (red) upon *tep4-Gal4* mediated knockdown of TFEB (D-D'), Atg8a (F-F'), Atg5 (H-H'), or Atg18 (J-J') in the tep population (green) as compared to wildtype (B-B'). Graphical representation of Plasmatocyte differentiation index upon knockdown of TFEB (n=33), Atg8a (n=28), Atg5 (n=24), or Atg18 (n=22) in the tep population, compared to the wildtype (n=22) (K). Graphical representation of Crystal cell numbers upon knockdown of TFEB (n=21), Atg8a (n=44), Atg5 (n=30), or Atg18 (n=58) in the tep population, compared to the wildtype (n=28) (L). Plasmatocyte differentiation (red) upon Chloroquine treatment (N) as compared to control (M). Crystal cell differentiation upon Chloroquine treatment (P) as compared to control (O). Graphical representation of Plasmatocyte Differentiation Index upon Chloroquine treatment (n=30), compared to control (n=25) (Q). Graphical representation of Crystal Cell numbers upon Chloroquine treatment (n=25), compared to control (n=31) (R). Nuclei are stained with DAPI (blue). n represents the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (*$p<0.05$; ***$p<0.001$; ****$p<0.0001$, Student's t-test with Welch's correction). Scale Bar: 50µm (A-P)

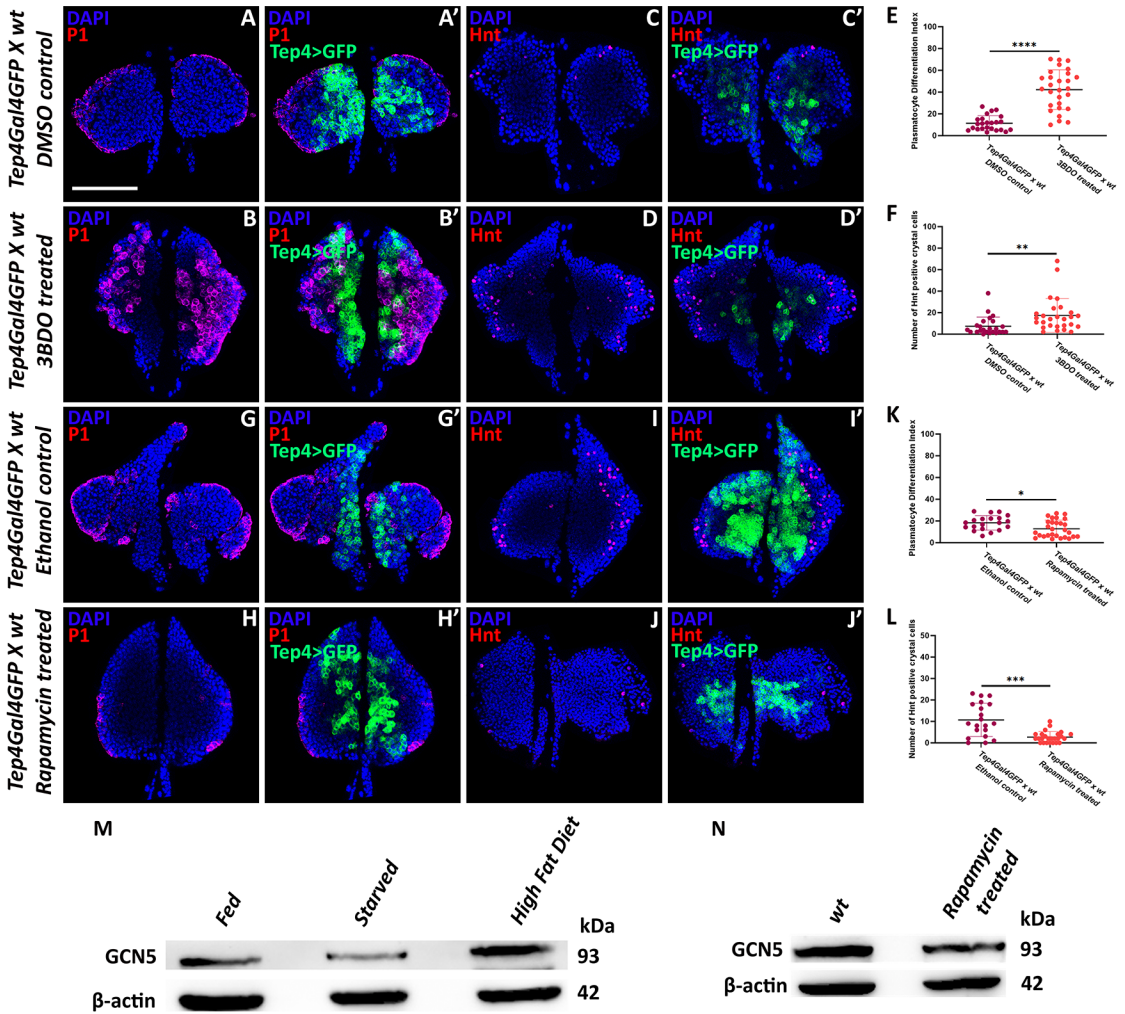
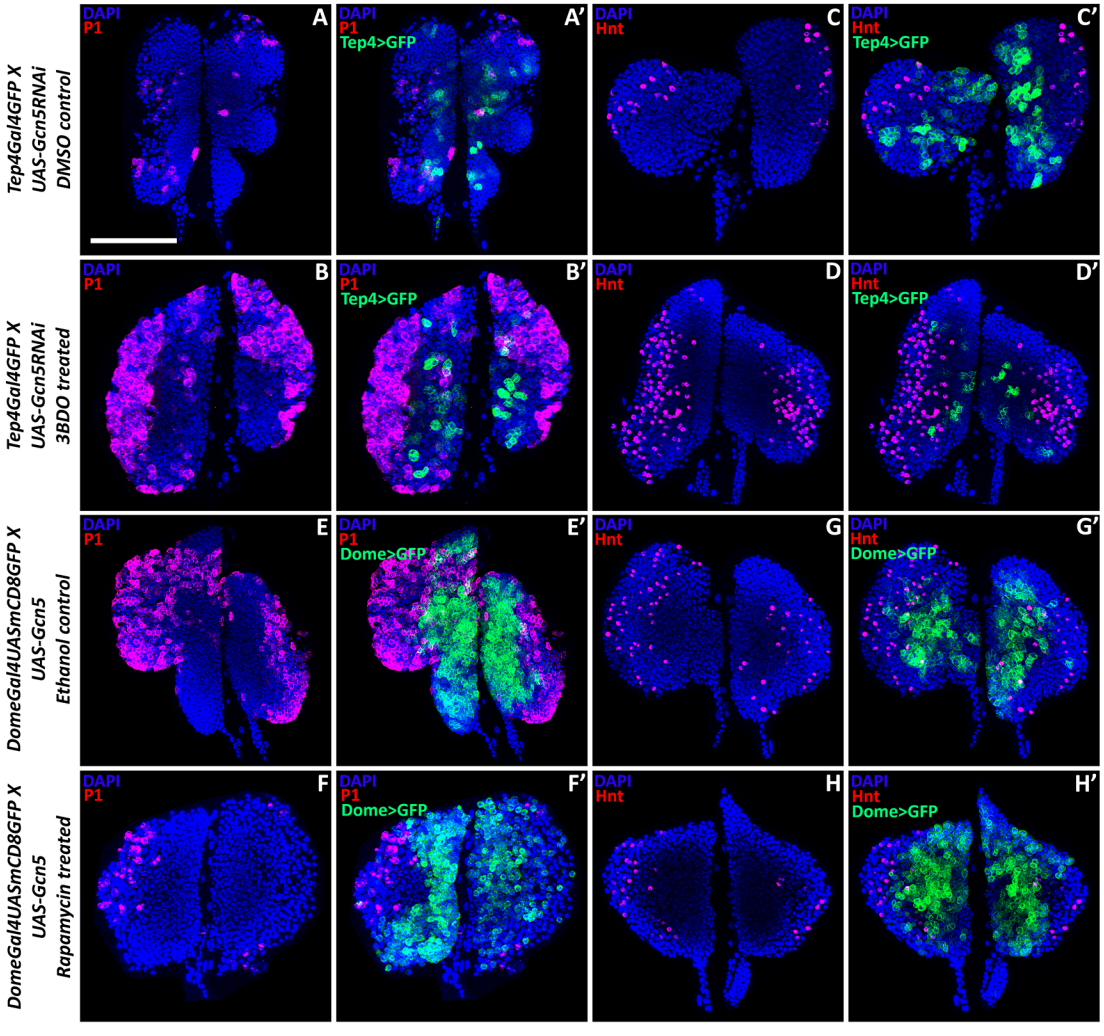

Tep4Gal4GFP X wt DMSO control
Tep4Gal4GFP X wt 3BDO treated
Tep4Gal4GFP X wt Ethanol control
Tep4Gal4GFP X wt Rapamycin treated
DAPI
P1
Hnt
Tep4>GFP
A
A'
B
B'
C
C'
D
D'
G
G'
H
H'
I
I'
J
J'
E
F
K
L
Plasmatocyte Differentiation Index
Number of Hnt positive crystal cells
Tep4Gal4GFP x wt DMSO control
Tep4Gal4GFP x wt 3BDO treated
Tep4Gal4GFP x wt Ethanol control
Tep4Gal4GFP x wt Rapamycin treated
****
**
*
***
M
Fed
Starved
High Fat Diet
kDa
GCN5
β-actin
93
42
N
wt
Rapamycin treated
kDa
GCN5
β-actin
93
42

Figure 7. Modulation of mTORC1 activity regulates blood cell differentiation in the Lymph gland.

Plasmatocyte differentiation marked by P1 (red) upon 3BDO treatment (B-B') as compared to control (A-A'). Crystal cell differentiation marked by Hnt (red) upon 3BDO treatment (D-D') compared to control (C-C'). Graphical representation of Plasmatocyte Differentiation Index upon 3BDO treatment (n=28) compared to control (n=24) (E). Graphical representation of Crystal cell numbers upon 3BDO treatment (n=27) compared to control (n=24) (F). Plasmatocyte differentiation marked by P1 (red) upon Rapamycin treatment (H-H') compared to control (G-G'). Crystal cell differentiation marked by Hnt (red) upon Rapamycin treatment (J-J') compared to control (I-I'). Graphical representation of Plasmatocyte Differentiation Index upon Rapamycin treatment (n=28) compared to control (n=20) (K). Graphical representation of Crystal cell numbers upon Rapamycin treatment (n=25) compared to control (n=20) (L). Immunoblot showing GCN5 (93 kDa) protein levels in different diet conditions – fed, starved, and high-fat diet (M). Immunoblot showing GCN5 (93 kDa) protein levels upon Rapamycin treatment compared to wildtype (N). β-actin (42kDa) was used as the loading control. Nuclei are stained with DAPI (blue). n represent the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, Student's t-test with Welch's correction). Scale Bar: 50µm (A-J')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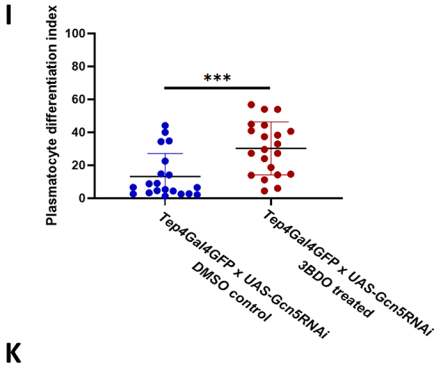
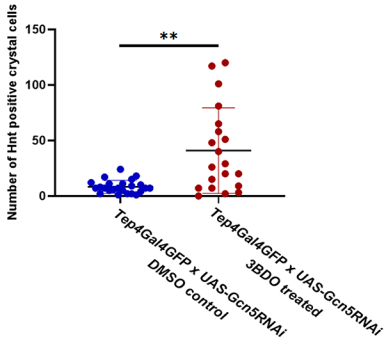
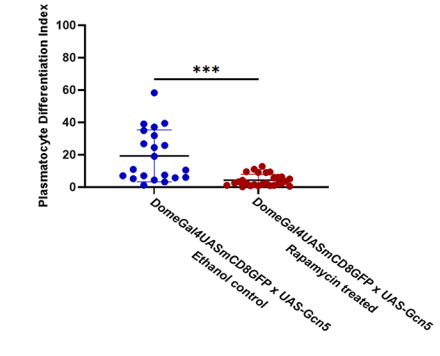
UAS-Gcn5RNAi
DMSO control
Tep4Gal4GFP X
UAS-Gcn5RNAi
3BDO treated
DomeGal4UASmCD8GFP X
UAS-Gcn5
Ethanol control
DomeGal4UASmCD8GFP X
UAS-Gcn5
Rapamycin treated
DAPI
P1
Hnt
Tep4>GFP
Dome>GFP
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'


I
Plasmatocyte differentiation index
100
80
60
40
20
0
***
Tep4Gal4GFP x UAS-Gcn5RNAi
DMSO control
Tep4Gal4GFP x UAS-Gcn5RNAi
3BDO treated


J
Number of Hnt positive crystal cells
150
100
50
0
**
Tep4Gal4GFP x UAS-Gcn5RNAi
DMSO control
Tep4Gal4GFP x UAS-Gcn5RNAi
3BDO treated


K
Plasmatocyte Differentiation Index
100
80
60
40
20
0
***
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Ethanol control
DomeGal4UASmCD8GFP x UAS-Gcn5
Rapamycin treated

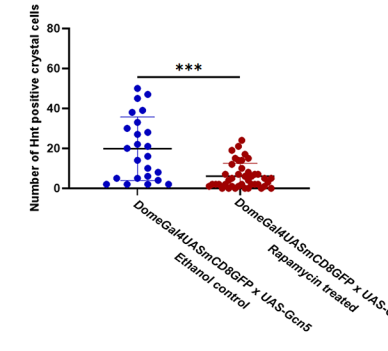
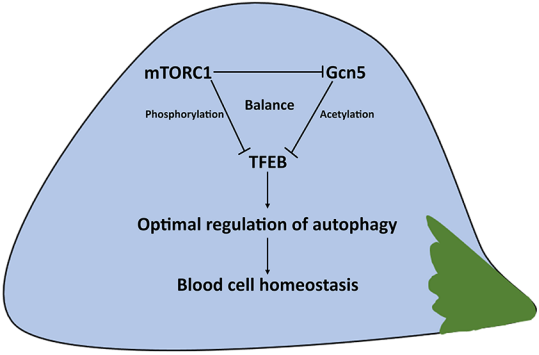
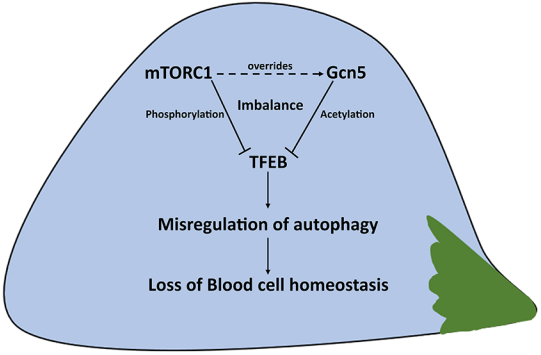

L
Number of Hnt positive crystal cells
80
60
40
20
0
***
DomeGal4UASmCD8GFP x UAS-Gcn5
Ethanol control
DomeGal4UASmCD8GFP x UAS-Gcn5
Rapamycin treated

Figure 8. mTORC1 can override the effect of Gcn5 level modulation over autophagy.

Plasmatocyte differentiation marked by P1 (red) upon 3BDO treatment in *tep4-Gal4* mediated (green) Gcn5 knockdown background (B-B') compared to control treatment in the same genetic background (A-A'). Crystal cell differentiation marked by Hnt (red) upon 3BDO treatment in the tep-mediated (green) Gcn5 knockdown background (D-D') compared to control treatment in the same genetic background (C-C'). Plasmatocyte differentiation marked by P1 (red) upon Rapamycin treatment in Dome-mediated (green) Gcn5 over-expression background (F-F') compared to control treatment in the same genetic background (E-E'). Crystal cell differentiation marked by Hnt (red) upon Rapamycin treatment in *Dome-Gal4* mediated (green) Gcn5 over-expression background (H-H') as compared to control treatment in the same genetic background (G-G'). Graphical representation of Plasmatocyte Differentiation Index upon 3BDO treatment (n=21) and control treatment (n=20) in the tep-mediated Gcn5 knockdown background (I). Graphical representation of Crystal cell numbers upon 3BDO treatment (n=20) and control treatment (n=24) in the tep-mediated Gcn5 knockdown background (J). Graphical representation of Plasmatocyte Differentiation Index upon Rapamycin treatment (n=27) and control treatment (n=21) in the Dome-mediated Gcn5 over-expression background (K). Graphical representation of Crystal Cell numbers upon Rapamycin treatment (n=42) and control treatment (n=24) in the Dome-mediated Gcn5 over-expression background (L). Nuclei are stained with DAPI (blue). n represent the number of individual primary lobes of the Lymph Gland. Individual data points in the graphs represent individual primary lobes of the Lymph Gland. Values are mean ± SD, and asterisk marks statistically significant differences (**$p<0.01$; ***$p<0.001$, Student's t-test with Welch's correction). Scale Bar: 50µm (A-H')



wild type



Perturbation of Gcn5-mTOR-TFEB signalling axis

Figure 9: Gcn5-mTORC1-TFEB signaling axis regulates autophagy to control blood cell homeostasis.

A cartoon summarizing how Gcn5-mTORC1-TFEB signaling axis regulates autophagy to control blood cell homeostasis in the *Drosophila* Lymph Gland.