Title page

Title:

Context-specific functions of Notch in *Drosophila* blood cell progenitors

Running title:

Notch roles in blood cell progenitors

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Summary statement

Notch signaling regulates differently distinct populations of blood cell progenitors of the *Drosophila* larval hematopoietic organ.

Abstract

Drosophila Larval hematopoiesis takes place at the lymph gland, where myeloid-like progenitors differentiate into Plasmatocytes and Crystal Cells, under regulation of conserved signaling pathways. It has been established that the Notch pathway plays a specific role in Crystal Cell differentiation and maintenance. In mammalian hematopoiesis, the Notch pathway has been proposed to fulfill broader functions, including HSCs maintenance and cell fate decision in downstream progenitors. In this work we describe different roles that Notch plays in the lymph gland. We show that Notch, activated by its ligand Serrate, expressed at the Posterior Signaling Center, is required for Core Progenitor maintenance. We define a novel population of blood cell progenitors that we name Distal Progenitors, where Notch, activated by Serrate expressed in cells at the Medullary Zone/Cortical Zone boundary, regulates a binary decision between Plasmatocyte and Crystal Cell fates. Thus, Notch plays context-specific functions in different blood cell progenitor populations of the *Drosophila* lymph gland.

Introduction

Hematopoiesis in *Drosophila* larvae takes place predominantly at the Lymph Gland (LG), which is composed of two primary lobes symmetrically localized at both sides of the dorsal vessel, and smaller posterior lobes that follow a similar array (1) (**Fig. 1a**). At the primary lobes, blood progenitors (prohemocytes) differentiate into myeloid-like lineages through evolutionary conserved mechanisms, making the LG an attractive model to explore the pathways involved in normal and oncogenic hematopoiesis (2-4). At the 3rd larval instar, prohemocytes are compactly arranged in an internal region of the primary lobe, called Medullary Zone (MZ), characterized by the expression of the JAK/STAT receptor *domeless* (*dome*) (**Fig. 1a, b**) (1, 5, 6). Maturing hemocytes are found in the peripheric region of the lobe, called Cortical Zone (CZ), which can be identified by the expression of the Von Willebrand-like factor *hemolectin* (*hml*) (**Fig. 1a, b**) (1). An Intermediate Progenitor (IP) population has been described at the MZ/CZ boundary, defined by the expression of both *dome* and *hml* (**Fig. 1b**), but its physiological relevance remains poorly defined (2, 7). Two types of differentiated populations of hemocytes occur at the CZ: Plasmatocytes (PLs) and Crystal Cells (CCs) (**Fig. 1a**), whereas a third cell type, the Lamellocytes, differentiate only following specific immune challenges. PLs, which constitute the bulk (95%) of mature hemocytes, are macrophages that retain *hml* expression, while they also express the receptors Nimrod (or P1-antigen) and Eater, both of them important for recognition of bacteria (8-10). The CCs, named after their cytoplasmic inclusions of Prophenoloxydase (ProPO), mediate melanization of pathogens and wounds, and constitute 5% of the total number of mature hemocytes. Mature CCs are thus characterized by the expression of ProPO, and no longer express the *hml* marker, characteristic of other CZ cells (5, 11). A population of CC Progenitors can also be identified at the CZ by the expression of the RUNX transcription factor Lozenge (Lz), homolog of the human AML1/Runx1 protein, frequently altered in Acute Myeloid Leukemias (1, 12). Another remarkable LG region, termed Posterior Signaling Center (PSC), occurs at the posterior tip of each primary lobe (**Fig. 1b**). It has been reported to function as the niche that maintains the progenitor population of the MZ in an undifferentiated state (13-15). PSC cells express the homeobox protein Antennapedia (Antp) (14), the signaling molecule Hedgehog (Hh) (14), the Notch ligand Serrate (Ser) (16), and the gene *collier* (*col*), ortholog of the mammalian Early B-cell Factor (EBF) (6, 17). Hh expressed at the PSC targets the Medullary Zone and is required to restrain progenitor differentiation (14). Recently the notion that the PSC functions as a hematopoietic niche has been challenged, as genetic ablation of this structure did not alter progenitor maintenance nor steady-state blood cell differentiation (18, 19).

Notch is a conserved signaling pathway utilized repeatedly during development of all metazoa. It is typically involved in cell differentiation, binary cell fate decisions, cell proliferation and cell survival (20). The Notch receptor, which gives the name to the pathway, can be activated by its transmembrane ligands Serrate (Ser) or Delta (Dl) expressed in adjacent cells (**Fig. 1c**). Once this interaction takes place, Notch undergoes two consecutive cleavage events, resulting in the

release of the Notch Intracellular Domain (NICD), which migrates into the nucleus and binds the transcription factor Suppressor of Hairless (Su(H)). The complex formed by NICD and Su(H) recruits transcriptional co-activators, thereby inducing transcription of Notch target genes. This canonical Notch pathway was reported to operate in *hml*-positive cells of the Cortical Zone to specify the CC fate (16, 21, 22). The source of Notch ligand in this context is Serrate expressed at cells localized at the MZ/CZ boundary (16, 23). Non-canonical ligand-independent Notch signaling is afterwards required for CC maturation and survival (22).

In mammalian hematopoiesis, Notch pathway functions have been explored quite extensively, although with contrasting results. Notch receptors (Notch 1-4) are expressed in Hematopoietic Stem Cells, hematopoietic progenitors and mature blood cells, suggesting that Notch is required at multiple stages of the differentiation cascade (24). Notch functions in mammalian hematopoietic stem cells are controversial (25). Results in mouse models suggest that it is dispensable for their maintenance (26, 27), however *in vitro* conflicting evidences favoring either a function of Notch in HSC differentiation (28, 29), or a requirement for HSC maintenance have been reported (30-32). It is however well established that in lymphoid progenitors Notch promotes differentiation and proliferation of T lymphocytes at the expense of B lymphocytes (33, 34). Given the involvement of Notch in mammalian hematopoiesis, it is not surprising that alterations of this pathway are associated with several types of leukemia (35).

Because of the diverse, yet unclear functions of Notch in mammalian hematopoiesis, and given the conservation that occurs between the mechanisms controlling fly and mammalian blood cell development, we sought to explore the functions that the Notch pathway plays in *Drosophila* blood cell progenitors. We show here that Notch has distinct functions in two different populations of hemocyte progenitors: 1) In the recently described “Core progenitors” (36) Notch is required for maintenance of an undifferentiated state, a function that depends on Ser expressed at the PSC; and 2) In Distal Progenitors, a cell population defined in this paper, Notch controls a binary decision towards a PL or a CC fate. This binary Notch-dependent choice depends on Ser expressed in cells at the MZ/CZ boundary. Thus, Notch plays context-specific functions in two different cell progenitor populations during *Drosophila* hematopoiesis.

Results

Redefining progenitor cell populations of the Medullary Zone

Before analyzing Notch functions in *Drosophila* blood cell progenitors, we sought to define precisely the different populations of progenitors that occur at the MZ. Cells of the MZ of wandering 3rd instar larvae lymph glands express *domeMESO* and include a subpopulation of internal cells, the Core Progenitors, characterized by the expression of *tepIV* (36). It is unclear in the literature whether all *domeMESO*-expressing cells that are negative for *tepIV* coexpress *hml,* and can be considered Intermediate Progenitors. We found that this is not the case: In

larvae that coexpress the Blue Fluorescent Protein (BFP) under a *tepIV*-Gal4 driver (*tepIV* > BFP), and GFP directly driven by a d*omeMESO* promoter (*domeMESO*-GFP), along with dsRed controlled directly by an *hml* promoter (*hml*-dsRed), three distinct cell populations can be recognized: 1) Core Progenitors positive for both *tepIV* > BFP and *domeMESO*-GFP (**Fig. 1d**, region 1); 2) Cells positive only for *domeMESO*-GFP (**Fig. 1d**, region 2); and 3) Intermediate Progenitors in which *domeMESO*-GFP and *hml*-dsRed signals overlap (**Fig. 1d**, region 3). Thus, an uncharacterized population of *domeMESO* progenitors which do not express *tepIV* or *hml* occurs in the lymph gland. We henceforth propose the name "Distal Progenitors" for this particular population, in reference to their distal location from the dorsal vessel and Core Progenitors. Interestingly, the membrane receptor Eater, often considered a PL-specific marker (10, 37), is also expressed in Distal Progenitors of wandering 3rd instar larvae (**Fig. 1e**, region 2), whereas it is barely detectable in Core Progenitors (**Fig. 1e**, region 1).

We conclude that three distinct populations of hemocyte progenitors occur at the Medullary Zone of 3rd instar larvae lymph glands: 1) Core Progenitors, which co-express *tepIV* and *domeMESO*; 2) Distal Progenitors that are positive for *domeMESO* but negative for *tepIV* and *hml*; and 3) Intermediate Progenitors, which co-express *domeMESO* and *hml* (**Fig. 1f**).

The Notch pathway is required for Core Progenitor maintenance

Notch expression is widespread throughout the lymph gland, suggesting that this pathway might operate in various cell types (**Fig. 2a**, upper). We initially analyzed Notch function in Core Progenitors by expressing a *notch* RNAi with *tepIV*-Gal4 (**Fig 2a**, middle), and observed a clear reduction of Core Progenitors (**Fig. 2a, b**), while both Plasmatocytes (PLs) and Crystal Cells (CCs), increased significantly (**Fig. 2b**). The number of cells of the PSC remained unaltered (**Fig. S1a**). As mentioned above, in wandering 3rd instar larvae the receptor Eater is expressed in Distal Progenitors, while it is almost undetectable in Core Progenitors, so we utilized Eater as another marker to assess the effect of Notch on progenitor populations. Notch silencing with *tepIV*-Gal4 resulted in a clear expansion of the *eater* expression domain (**Fig. 2c**), confirming that Notch is necessary for Core Progenitor maintenance. Consistent with this, silencing of another component of the canonical Notch pathway, the transcription factor Suppressor of Hairless (Su(H)), also provoked a reduction of Core Progenitors, while PLs and CCs increased significantly (**Fig. 2d**). Altogether, these results suggest that the Notch pathway is required cell-autonomously for maintenance of Core Progenitors in an undifferentiated state.

We next analyzed whether over-activation of the Notch pathway provokes the opposite phenotype, namely an increase of Core Progenitors and general differentiation impairment. This was not the case, as over-expression with *tepIV*-Gal4 of a full-length Notch construct (**Fig. S1b**) or Su(H) did not alter Core Progenitor, PL or CC populations (**Fig. S1c, d**). These results suggest that endogenous activity of the Notch pathway is already sufficient to prevent excessive differentiation of Core Progenitors. It was recently reported that Notch is expressed

transiently at the 1st larval instar (L1) in a small group of Hematopoietic Stem Cells (HSCs) (38), so we analyzed the possibility that the phenotype that we observed in Core Progenitors stems from an alteration of Notch function in L1 HSCs. To investigate this, we used a thermosensitive Gal80 construct to silence Notch expression only from mid-second larval instar onwards (**Fig. S1e**, upper). The results were identical to those in which Notch was constitutively silenced (**Fig. S1e** and **Fig. 2b**), ruling out the possibility that the loss of Core Progenitors observed after Notch silencing depends on an early role in L1 HSCs.

The findings shown in this section are consistent with a requirement of the Notch pathway for maintenance of Core Progenitors in an undifferentiated state.

Serrate expressed at the Posterior Signaling Center is required for Core progenitor Maintenance

As a next step, we studied the identity and source of the ligand for Notch activation in Core Progenitors. *Serrate* (*ser*) is expressed at high levels in PSC cells (1, 14, 16, 23), although no functions have been yet attributed to this expression. Since the PSC and Core Progenitors are in close proximity, and filopodia that emanate from PSC cells may play a role in transmitting signals to MZ progenitors (14), we asked whether Ser expressed at the PSC activates Notch for Core Progenitor maintenance. First, we silenced Ser at the PSC with an *antp*-Gal4 driver, and observed an increased number of PLs and CCs (**Fig. 3a**), indicating that Ser is required at the PSC to limit differentiation. In line with this, analysis of *eater*-dsRed expression following *antp*-Gal4 dependent silencing of Ser revealed a clear reduction of Core Progenitors (*eater*-negative) (**Fig. 3b**). These results indicate that Ser is required specifically at the PSC for Core Progenitors maintenance.

Recently, the notion that the PSC functions as a hematopoietic niche has been challenged, as *antp*-Gal4 driven Reaper expression (i.e. apoptotic ablation of the PSC) did not alter PL or CC differentiation (18). In that study, genetic ablation of the PSC was confirmed at L3 stage by the lack of expression of two classical PSC markers, Hh and Antp, whereas Ser expression was not assessed (18). We thus analyzed if *ser* expressing cells are still present in LGs in which the PSC was genetically ablated. Ablation was induced from L2 stage onwards by using a Gal80 thermosensitive construct to prevent Reaper expression and lethality at earlier stages. We monitored the expression of a *ser-LacZ* construct (1, 14, 16, 23, 39), and consistently observed the presence of *ser*-expressing cells in LGs where *antp*-positive cells were ablated (**Fig 3c**, lower). Importantly, in wild-type lymph glands we observed PSC cells that express *ser* but not *antp* (**Fig. 3c**, upper), suggesting that the PSC might encompass a mixed population of cells, with a large proportion of them co-expressing *antp* and *ser*, while some cells express *ser* but not *antp*. These observations are consistent with a model in which, after genetic ablation of the $antp^{+}$ cells of the PSC, the remaining ser^{+}, $antp^{-}$ PSC cells are sufficient to sustain normal Core Progenitor maintenance and hemocyte differentiation. Noteworthy, after *antp*-driven ablation,

a negative correlation between surviving *ser*$^{+}$, *antp*$^{-}$ PSC cells and hemocyte differentiation occurs: Lymph glands with a small *ser*-positive area at the PSC display greater PLs differentiation than those with a larger *ser*-positive area at the PSC (**Fig. 3d**). These results suggest that *ser* expressing cells of the PSC are important for Core Progenitor maintenance, and that the *ser* expressing cells that survive to *antp*-driven apoptotic cell ablation can support normal blood cell differentiation.

In Distal Progenitors the Notch pathway controls a binary cell fate decision

We have shown above that *notch* silencing with *tepIV*-Gal4 provokes enhanced differentiation of both PLs and CCs (**Fig. 2b**). In sharp contrast, *notch* silencing with *domeMESO*-Gal4 brought about almost complete loss of CCs accompanied by an increased proportion of PLs (**Figs. 4a and S2a**). Similar results were obtained when *su(H)* was silenced with the same driver (**Fig. 4b**), suggesting that the Notch pathway promotes CC differentiation in Distal Progenitors, while the PL fate is inhibited. To further explore this possibility, we over-expressed with *domeMESO*-Gal4 a full-length Notch construct (**Fig. S2b**) or the Notch Intracellular Domain (NICD), and observed in both cases that PL differentiation was virtually blocked, and CCs increased dramatically (**Fig. 4c**). Over-expression of Su(H) with *domeMESO*-Gal4 provoked the same effect (**Fig. 4d**). We thus conclude that an increase of Notch pathway activity in Distal Progenitors induces CC differentiation and inhibits PL differentiation.

To confirm that the activity of Notch is indeed required for normal differentiation in Distal Progenitors that are positive for *domeMESO* and negative for *hml* (**Fig. 1d, f**), *notch* silencing with *domeMESO*-Gal4 was repeated in a genetic background in which *notch* RNAi expression in cells that co-express *hml* was inhibited by QUAS-Gal80 (*domeMESO*-Gal4 > UAS-N^{RNAi}; *hml*-QF > QUAS-Gal80). Silencing of Notch in this genetic background provoked identical effects to those observed without expression of Gal80 in *hml*-positive cells (**Fig. S2c**). In a control experiment, *hml*-QF driven expression of QUAS-Gal80 effectively repressed Gal4 activity in the CZ (**Fig. S2d**). These results confirm that Notch operates in the Distal Progenitors, which express *domeMESO* but not *hml*.

Together, this set of experiments indicates that in Distal Progenitors the Notch pathway regulates a binary fate decision, promoting CC differentiation while inhibiting the PL fate.

Eater is an early Plasmatocyte fate marker in Distal Progenitors

Next, we explored further aspects of this Notch-dependent binary fate decision that takes place in Distal Progenitors. Noteworthy, a closer look to *eater*-dsRed reporter expression shown in **Fig. 1e** revealed that *eater-dsRed* can be detected in most Distal Progenitors but is virtually absent in a few cells within this region (**Fig. 5a**). A possible explanation is that *eater*-expressing Distal Progenitors at the 3rd instar might be already committed towards a PL fate, while those

Distal Progenitors with very low *eater* levels are likely committed to become CCs. Consistent with this notion, we observed at the CZ that *eater*-dsRed expression occurs in most *hml*-positive cells, while it is excluded from those cells that express the CC progenitor marker Lz (**Fig. 5b**). Lineage tracing experiments with an *eater*-Gal4 driver revealed that the lineage does not include CCs (**Fig. 5c**), supporting that *eater* expression in Distal Progenitors marks a Distal Progenitor subpopulation committed for a PL cell fate. *Eater*-Gal4 driven *notch* silencing or Notch over-expression did not recapitulate the effects observed when *domeMESO*-Gal4 was utilized to perform the same manipulations (compare **Figs. 4a, c** with **Fig. S3a**), suggesting that Notch operates in Distal Progenitors that have not yet begun to express *eater*. The above observations are consistent with a model in which, at an earlier developmental stage, *eater*-negative uncommitted Distal Progenitors make the binary cell fate decision regulated by Notch, while later, *eater* is expressed in cells committed to a PL fate.

If *eater* is indeed an early marker for Distal Progenitors committed to a PL fate, its expression should be controlled by the Notch pathway. We assessed this possibility by over-expressing Notch with *domeMESO*-Gal4, a treatment that induces massive differentiation to CCs (**Fig. 4c**), and observed almost complete absence of *dome*$^{+}$, *eater*$^{+}$ double-positive Distal Progenitors (**Fig. 5d**). Conversely, Notch silencing provoked a complete conversion of *dome*$^{+}$ *eater* $^{-}$ progenitors into *dome*$^{+}$, *eater*$^{+}$ double-positive Distal Progenitors (**Fig. 5d**). These observations suggest that *eater* expressed in Distal Progenitors is an early marker for a Notch-dependent binary fate decision, labeling cells committed to a PL fate.

Serrate expressed at the MZ/CZ boundary regulates the cell fate decision in Distal Progenitors

Groups of Serrate-expressing cells lying next to the MZ/CZ boundary, which instruct progenitors to acquire a CC fate have been previously reported (16, 23). We therefore investigated whether these cells provide the ligand for the Notch-dependent binary cell fate decision in Distal Progenitors. We noticed that a major proportion of these Ser-expressing cells, but not the Ser-expressing cells of the PSC, co-express *domeMESO* (**Fig. 6a**), so we utilized *domeMESO*-Gal4 to manipulate Ser expression. Silencing of *ser* with *domeMESO*-Gal4 virtually blocked CC differentiation, while it increased PL proportion (**Fig. 6b**), mimicking the effect of *notch* silencing with the same Gal4 driver (**Fig. 4a**). In agreement, overexpression of Ser rendered the opposite results, increasing CCs and reducing PLs (**Fig. 6c**). Thus Ser expressed in cells of the MZ/CZ boundary is required for Notch activation in Distal Progenitors, controlling the binary cell fate choice. The E3 ubiquitin ligase *neutralized* (*neu*) is necessary for Ser endocytosis and Notch activation in neighboring cells (40). Mimicking the results obtained after *ser* silencing with *domeMESO*-Gal4, expression of a *neu* RNAi with the same driver reduced CC number and increased PLs (**Fig. 6d**), further supporting the notion that Ser expressed at the MZ/CZ boundary activates Notch signaling for the binary cell fate decision in Distal Progenitors.

Discussion

In mammals, Notch receptors (Notch 1-4) are expressed in HSCs, hematopoietic progenitors and mature blood cells, suggesting that Notch is required at multiple levels of blood cell differentiation. Particularly, the role of the Notch pathway in HSC maintenance has been explored in depth, although with contrasting results. Some experiments in cell culture suggest that Notch is required for HSC maintenance (30-32), while others suggest that it promotes HSC differentiation towards the myeloid lineage (28, 29). Works *in vivo* in which interactions with the hematopoietic niche is still present, and therefore recapitulate better a physiologic situation, also yielded conflicting results. Studies utilizing conditional knock-out mice affecting different elements of the canonical Notch pathway suggested that Notch is dispensable for HSC maintenance (26, 27), while others indicated that alterations of Notch signaling are associated with development of myeloproliferative disease (MPD), characterized by accumulation of mature cells of the myeloid lineage (41). Thus, the role of Notch in adult HSC homeostasis is at least controversial and requires further examination. Many aspects of mammalian and *Drosophila* hematopoiesis are conserved, particularly at the level of the transcription factors and signaling pathways involved (2-4). One striking parallelism between *Drosophila* and mammalian models is the expression in the hematopoietic niche of the Notch ligand Serrate (Ser)/Jagged (JAG). While Ser is expressed in cells of the *Drosophila* PSC (16), mammalian JAG1 and JAG2 expression has been detected in different bone marrow cell types that fulfill niche functions, including endothelial cells and cells of the hematopoietic stroma (42-44). The wide array of genetic tools available in *Drosophila* allow for manipulations of gene expression with an exquisite temporal and spatial specificity that is currently not possible to the same extent in mammalian systems. Thus, the utilization of the fly model may provide clues for addressing unresolved issues related to Notch functions in mammalian hematopoiesis.

In the current work, we have analyzed biological properties of *Drosophila* blood cell progenitors. This analysis led us to redefine the progenitor subpopulations of the larval lymph gland. A novel progenitor population, the “Distal Progenitors” that are positive for *domeMESO* and negative for both *tepIV* and *hml,* occurs at the medullary zone. Our results strongly suggest that the binary fate decision between PLs and CCs is made in naïve Distal Progenitors at early larval stages, while later in development, at wandering 3rd instar larvae, the PL-specific marker *eater* is expressed in most but not all Distal Progenitors, marking the cells that are already committed to a PL fate. The remaining Distal Progenitors with low *eater* levels are committed to become CCs, as suggested by *eater* lineage tracing, as well as by *eater* and Lz mutually exclusive expression in *hml*-positive cells. Thus, the Medullary Zone, in wandering 3rd instar larvae, encompasses an undifferentiated population of cells, the Core Progenitors, and a second population, the Distal Progenitors, which have already been instructed to become either PLs or CCs.

We have found that Notch, which is expressed throughout the lymph gland, plays distinct functions in Core Progenitors or Distal Progenitors (**Fig. 7**). In Core Progenitors Notch is required

for maintenance of the undifferentiated state; our results thus contribute to the incipient characterization of the Core Progenitor subpopulation, in which *collier* (*col*) is expressed at low but physiologically relevant levels (36). We found that Notch function in Core Progenitors depends on Ser that is expressed at the PSC. This Ser expression has been reported before (1, 14, 16, 23), but its function remained elusive. Filopodia emanating from PSC cells and intermingling between cells of the MZ have been described, and were proposed to participate in Hedgehog signaling (14). Given our observation that Ser expressed at the PSC is necessary for Notch activation in Core Progenitors, and considering that direct cell-cell interactions are required for Notch stimulation by its ligands, it seems reasonable to hypothesize that the filopodia that emanate from the PSC may play a role in Notch signaling as well (6).

It has been shown that genetic ablation of the PSC by expression of the proapoptotic protein Reaper (Rpr) does not alter Core Progenitor maintenance nor steady-state differentiation, challenging the notion that the PSC functions as a hematopoietic niche (18). Using the same PSC-ablation protocol with *antp*-Gal4 driven expression of Rpr from L2 stage onwards, we detected the presence of *ser*-positive cells in the PSC that escaped genetic ablation. Moreover, in wild-type lymph glands the PSC includes a subpopulation of cells that are *ser*-positive and *antp*-negative (ser^{+}, $antp^{-}$). It is therefore likely that those *ser*-positive cells that survived *antp*-Gal4 induced genetic ablation are responsible for maintaining Core Progenitors and for sustaining normal progenitor differentiation.

We have found that in Distal Progenitors Notch fulfills a totally different function; it regulates a binary cell fate decision, promoting CC differentiation, and inhibiting differentiation of PLs (**Fig. 7**). We propose that the activation of the Notch pathway in Distal Progenitors is achieved through direct interaction of early naive Distal Progenitors with Ser-expressing cells localized at the MZ/CZ boundary, thereby inducing CC differentiation and repressing the PL fate. On the other hand, naive Distal Progenitors that do not contact these Ser-expressing cells undergo a default differentiation program towards a PL fate (**Fig. 7**). Our finding that Notch regulates in Distal Progenitors a binary cell fate decision between a PL and a CC fate is in line with previous results by Tokusumi et al. (45): These authors utilized the allelic combination $su(H)^{1}/su(H)^{115B}$, and observed a total block of CC differentiation accompanied by massive differentiation of PLs that invaded the MZ. According to the model proposed in the current study (**Fig. 7**), this is indeed the expected outcome of Tukusumi et al. experiments (45), where a combined effect of Core Progenitor loss, along with inhibition of the CC fate and default differentiation towards the PL fate in Distal Progenitors is expected. It is still an open issue whether early fate specification in Distal Progenitors establishes the final (95:5) proportion of PLs versus CCs observed at the Cortical Zone, or if alternatively, the fate specification in Distal Progenitors brings about an initial 50:50 proportion of cells committed to one versus the other fate, followed by increased proliferative capacity of PL-committed Distal Progenitors.

The Notch pathway has been previously demonstrated to be necessary and sufficient for CC specification at the CZ (16, 21, 22). Our results suggest that Notch-dependent CC specification

occurs earlier in Distal Progenitors, and that Notch is probably required at later stages to sustain the original specification. Two works support this notion: Firsty, Krzemien et al. (46) utilized a clone labelling strategy at different larval stages, reaching the conclusion that blood cell progenitors of early L2 larvae, which have not yet developed a CZ, have already restrained their differentiating potential, either towards a PL or a CC fate. Secondly, at later stages of CC development, namely when Lz expression begins in unipotent CC progenitors at the CZ, it has been reported that Notch inhibits the expression of PL-specific genes (47). In this study, gene expression manipulations performed with *lz*-Gal4 demonstrated that the Notch target gene *klumpfuss* (*klu*) mediates repression of P1 expression in CC progenitors, thereby stabilizing CC commitment (47). We induced *klu* silencing with *domeMESO*-Gal4 in multipotent Distal Progenitors, and did not observe alterations of PL or CC differentiation (data not shown). This observation suggests that Notch target genes that regulate the early fate decision in Distal Progenitors, and those target genes that mediate CC-fate stabilization later at the CZ may be different. The identity of the Notch target genes that regulate initial cell fate determination in Distal Progenitors are not known and should be addressed in future investigations.

Notch function as a regulator of the cell fate choice in Distal Progenitors is remarkably similar to the role of Notch reported in the control of a binary cell fate decision in mammalian lymphoid progenitors (24, 33, 34, 48, 49). In this case, inhibition of the Notch pathway induces a default differentiation program towards a B lymphocyte fate, while the T lymphocyte fate is abolished (33, 34, 48). Conversely, over-activation of the Notch pathway induces a T cell fate, while B cell differentiation is inhibited (49). Even though *Drosophila* progenitors are considered myeloid-like lineage cells, this similarity in Notch-dependent blood progenitor cell fate decision with the mammalian lymphoid lineage suggests a primitive mechanism of cell communication that determines a balance of blood cell types in hematopoiesis.

The Notch pathway is therefore employed several times during hematopoiesis at the lymph gland. First: A possible role in HSC at the 1st larval instar (38); second: It is required for maintenance of Core Progenitors (this study); third: In Distal Progenitors it regulates the binary cell fate choice between PLs and CCs (this study); fourth: At the CZ, it is involved in fate stabilization in CC progenitors (47), and later it is required for CC maturation and survival (22). With the development of increasingly sophisticated genetic tools in mammalian systems, future studies may determine if Notch fulfills comparable context-specific functions throughout the mammalian hematopoietic hierarchy.

Materials and Methods

Fly Strains and crosses

The following *Drosophila* strains were used: *domeMESO*-GFP; *hml*-dsRed; *domeMESO*-Gal4; *antp*-Gal4 (U. Banerjee), *ser*-LacZ (A. Bachmann); *eater*-dsRed; *eater*-Gal4 (RA. Schulz). The

following stocks were obtained from the Bloomington Stock Center: UAS-GFP; UAS-BFP; *GFP* RNAi; QUAS-GFP; QUAS-Gal80; *hml*-Gal4; *hml*-QF; *notch* RNAi; *notch* RNAi (2); *su(H)* RNAi; *tub*-Gal80^{ts}; UAS-Su(H); UAS-Notch; UAS-NICD; *ser* RNAi; UAS-Ser; *neu* RNAi; UAS-RedStinger, UAS-Flp, Ubi-p63E(FRT.STOP)Stinger (G-TRACE, (50)). *TepIV*-Gal4 was obtained from the Vienna *Drosophila* Resource Center. All experimental crosses were performed at 25 ^{o}C and F1 larvae were incubated at 29 ^{o}C to maximize Gal4 activity, with the exception of experiments involving *tub*-Gal80^{ts}, in which larvae were kept at 18 ^{o}C and then transferred to 29 ^{o}C to induce Gal4 activity. Experiments involving *notch* RNAi (2) and *su(H)* RNAi were performed in a genetic background that included UAS-Dicer2 to enhance silencing.

Immunohistochemistry

Lymph glands were processed and stained as previously described (5): lymph glands were dissected from third-instar larvae (unless otherwise stated) in 1× PBS, fixed in 4% formaldehyde/1× PBS for 30 min, washed three times in 1×PBS with 0.4% Triton-X (1× PBST) for 15 min each, blocked in 10% normal goat serum/1× PBST for 30 min, followed by incubation with primary antibodies overnight at 4°C in blocking solution. Primary antibodies were washed three times in 1× PBST for 15 min each, re-blocked for 30 min, followed by incubation with secondary antibodies for 2 hr at room temperature. Samples were then washed three times in 1× PBST prior to mounting on glass slides in glycerol with Mowiol 4-88 anti-fade agent (EMD Millipore Corp., Billerica, MA). The following primary antibodies were used: rabbit α-βgal (Cappel), rabbit α-GFP (ThermoFisher, Waltham, MA), mouse α-Lz, mouse α-βgal, mouse α-Notch, mouse α-Antp (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse α-P1 (gift from I. Ando), rabbit α-ProPO (gift from G. Christophides). Alexa Fluor 488-, Alexa Fluor 647-, DyLight 405- and Cy3- conjugated secondary antibodies were used (Jackson Inmunoresearch, West Grove, PA).

Image acquisition and processing

Lymph glands were registered in a Zeiss LSM 510 confocal microscope, either as Z-stacks or single confocal planes as indicated in each case. Images were processed using ImageJ software. Quantification of the area occupied by the indicated markers was performed using whole Z-projections of confocal stacks or single confocal planes as mentioned in each case, and is expressed as the area occupied by the marker relative to the total lobe area. In the case of CCs, the total number of CCs relative to the total lobe area was quantified.

Statistical analysis

Two-tailed unpaired Student's t-tests or one-way ANOVAs (GraphPad Prism software) were used. The threshold for statistical significance was established as *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$.

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Competing interests

The authors declare no competing interests.

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Figure 1

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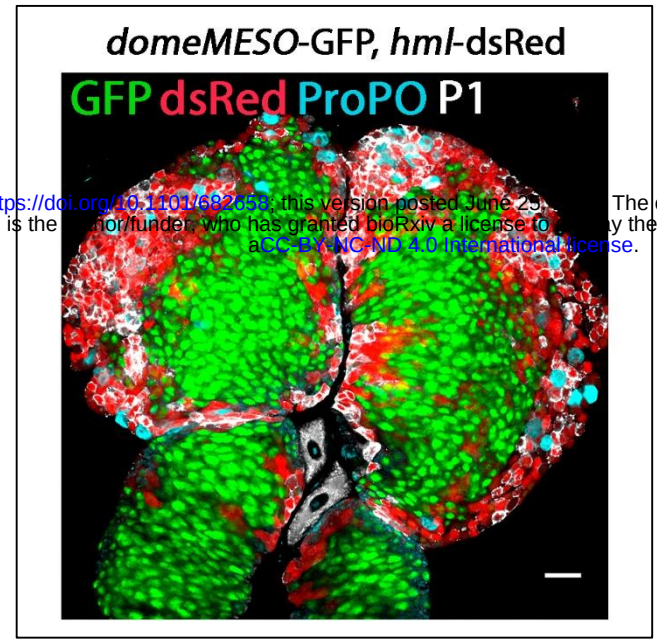

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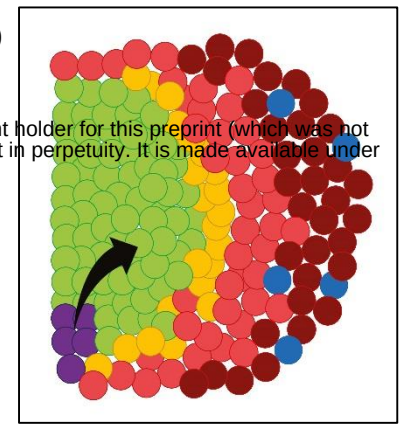

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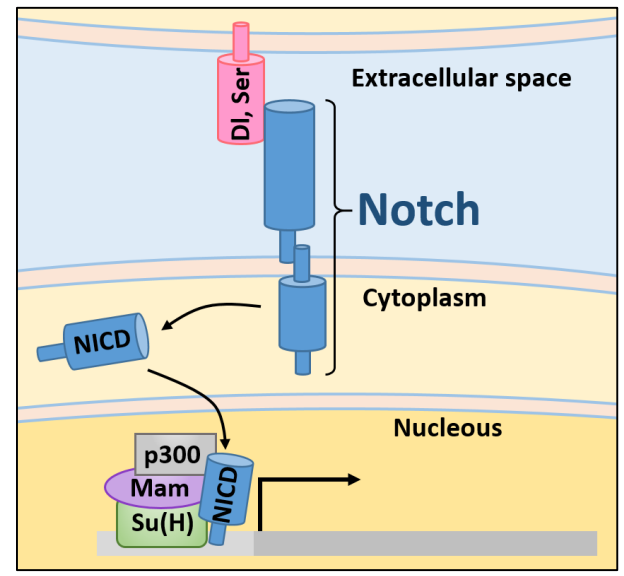

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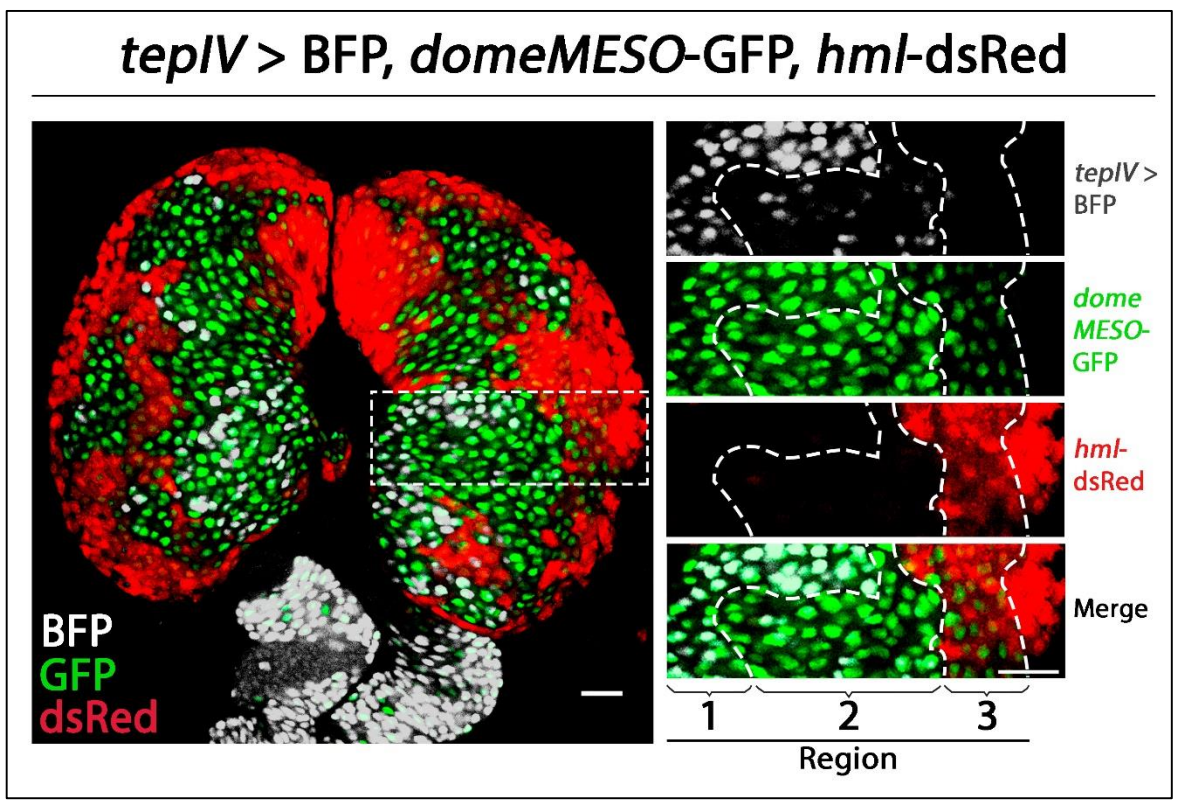

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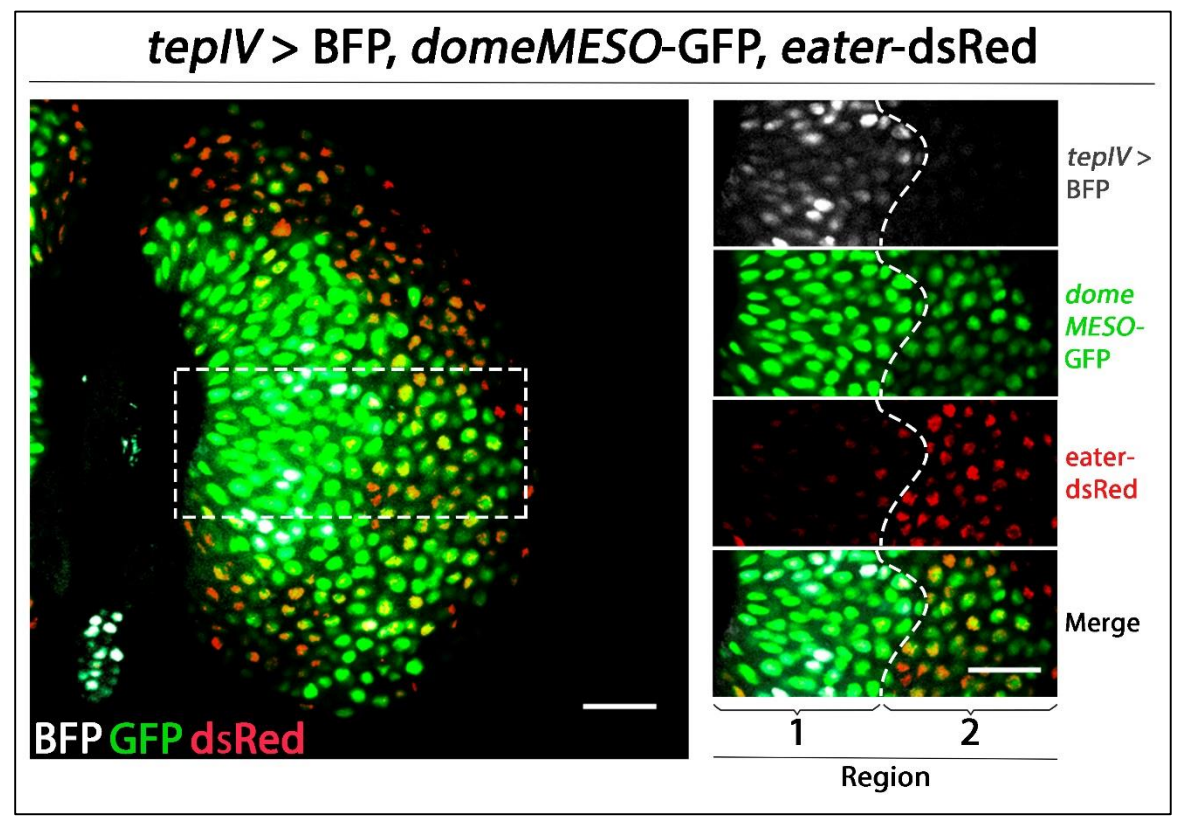

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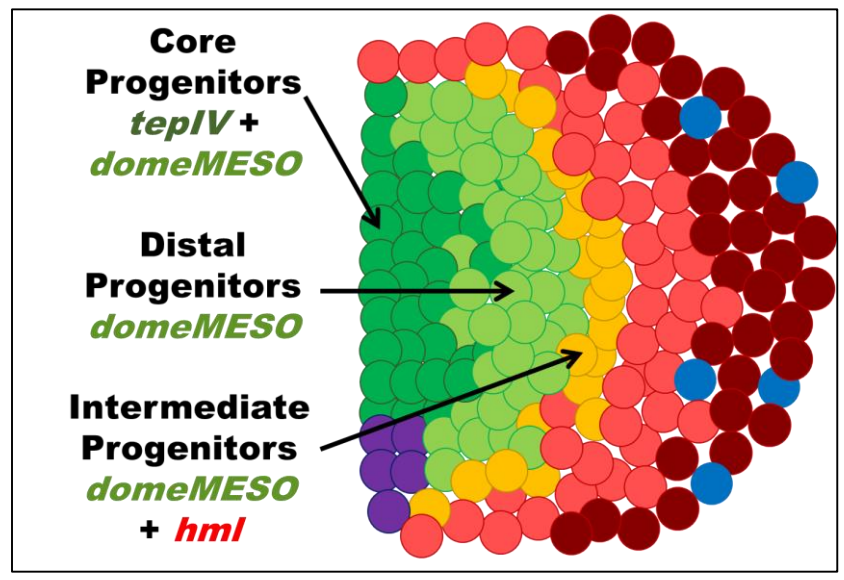

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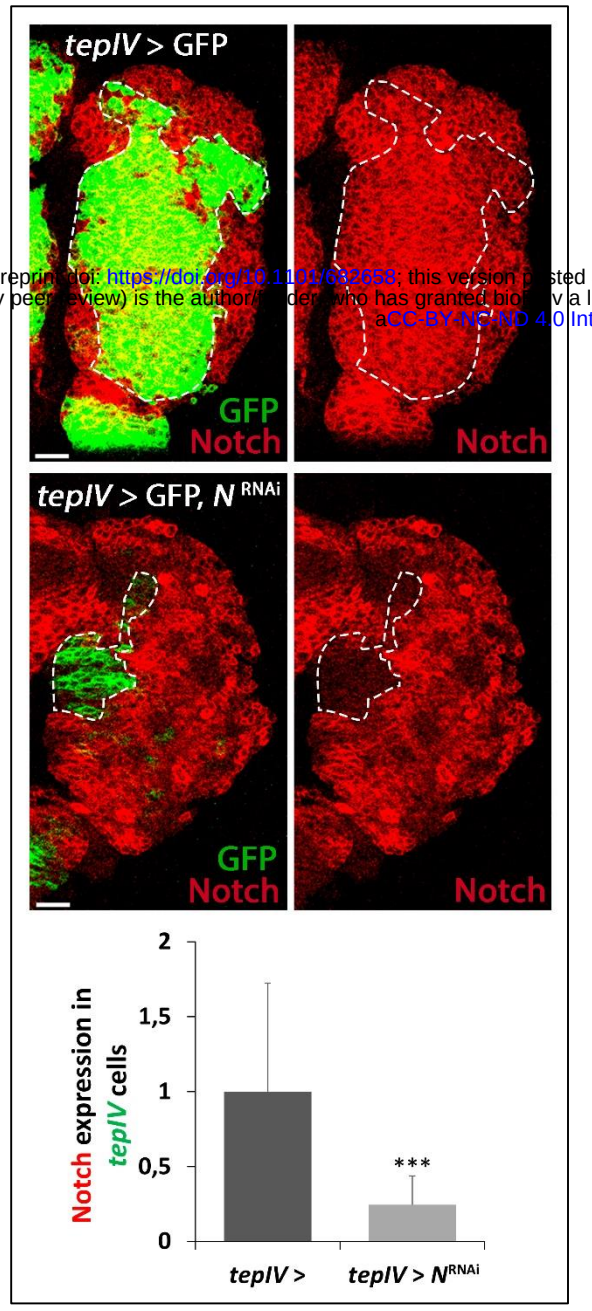

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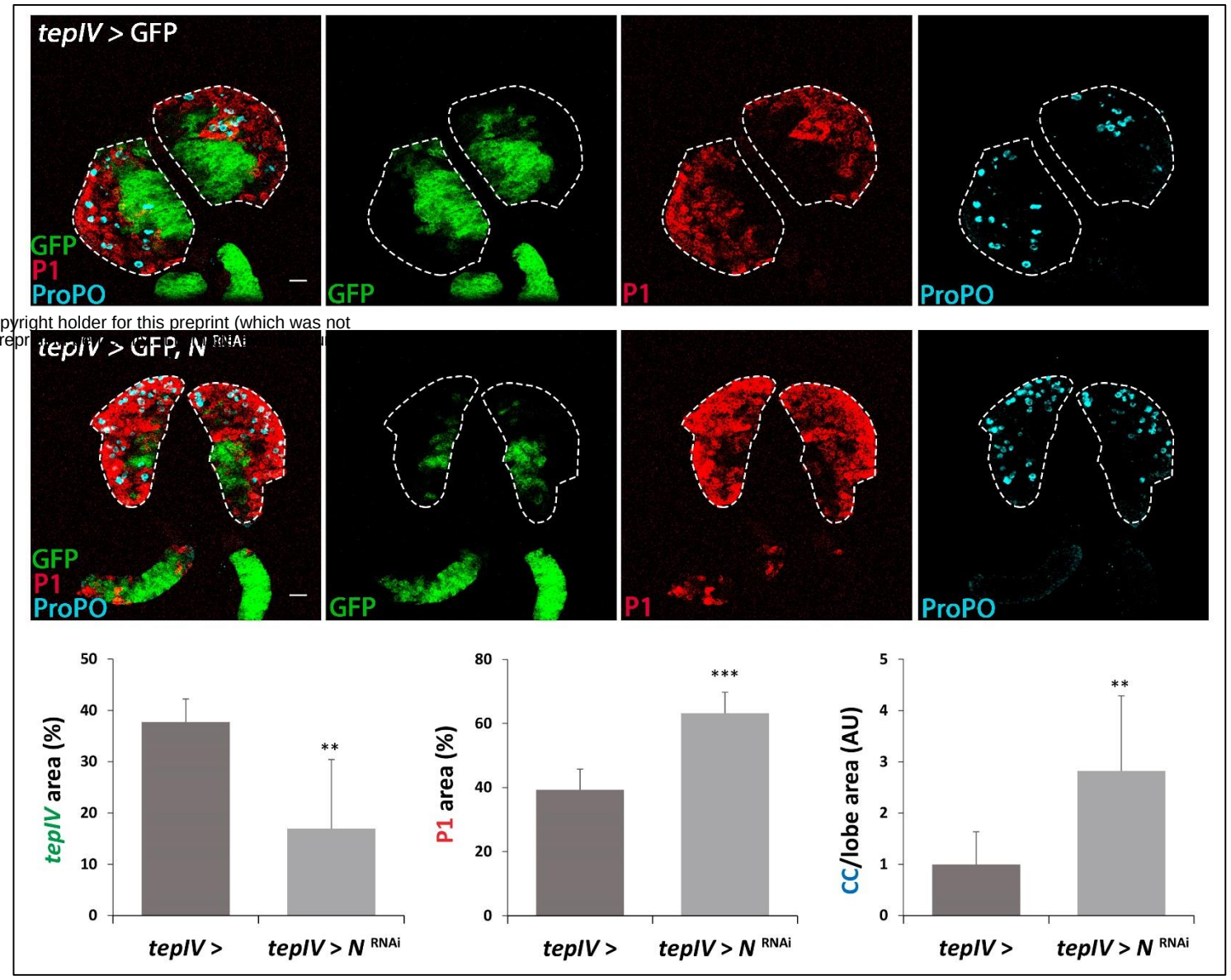

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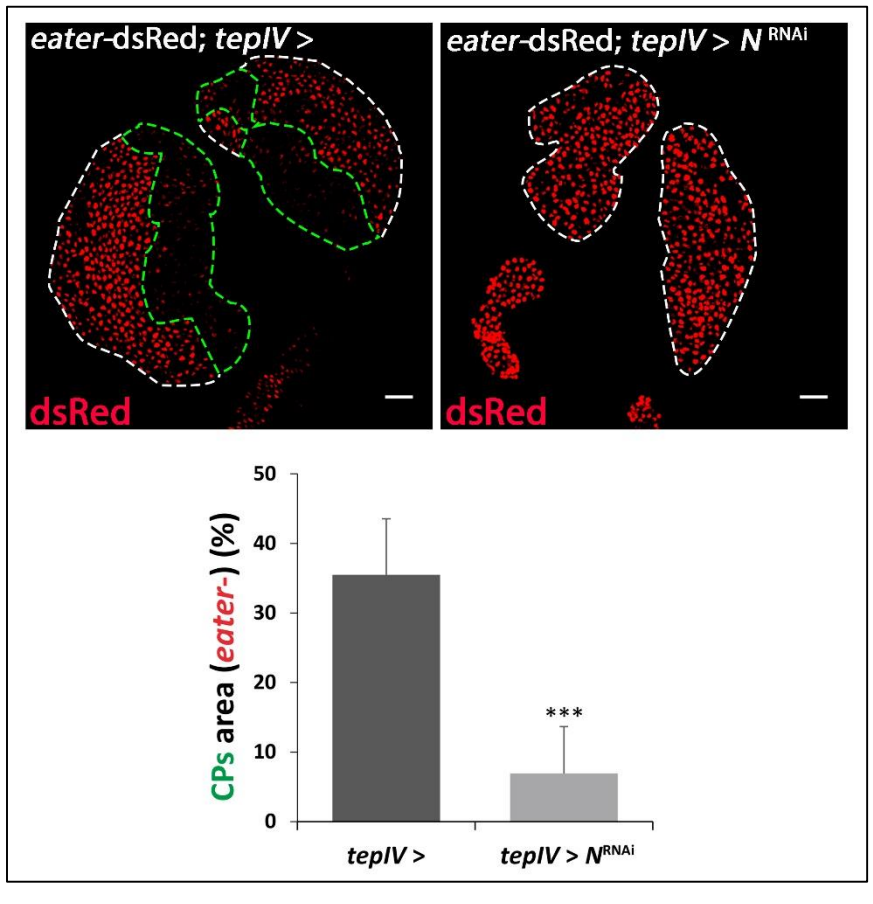

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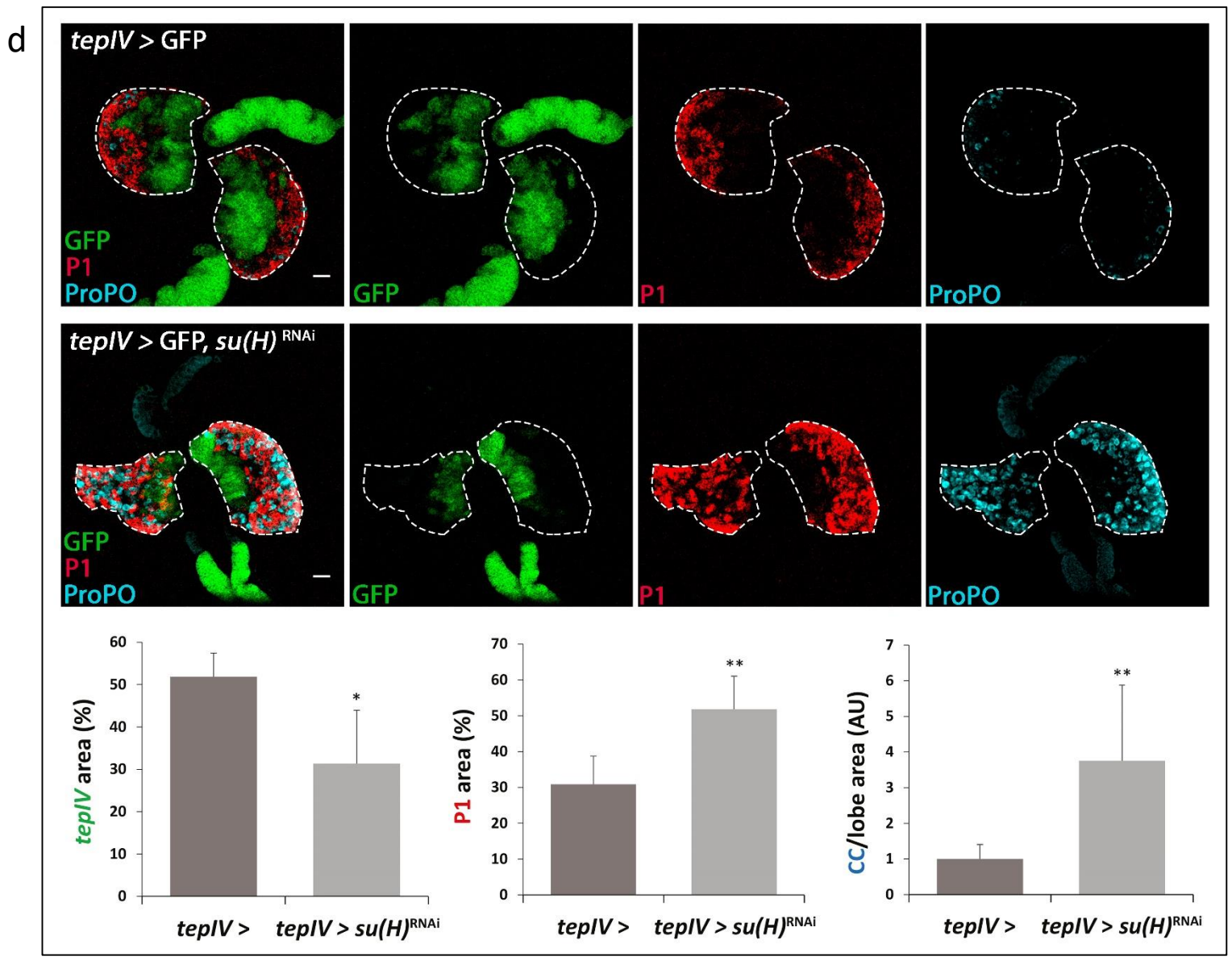

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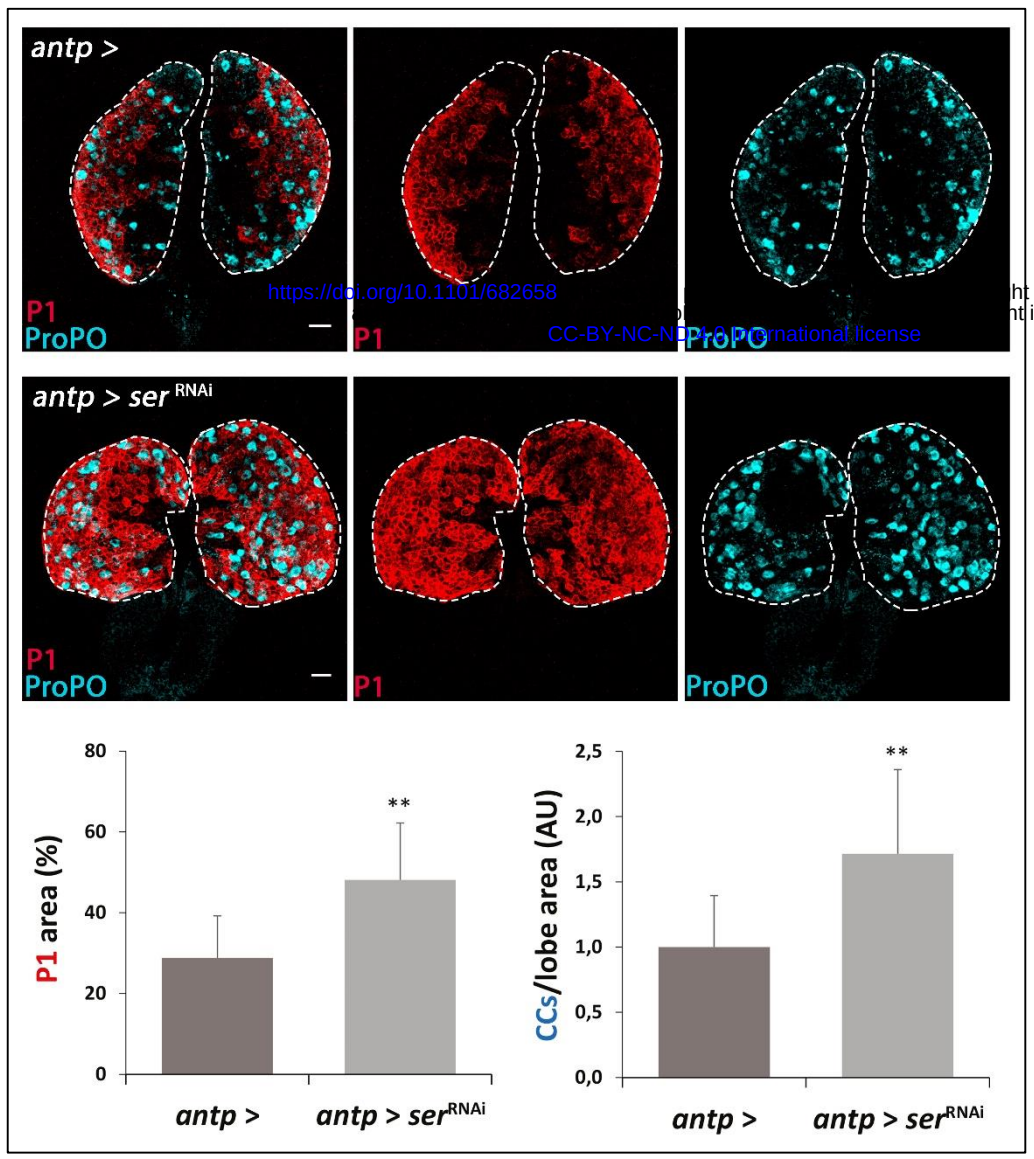

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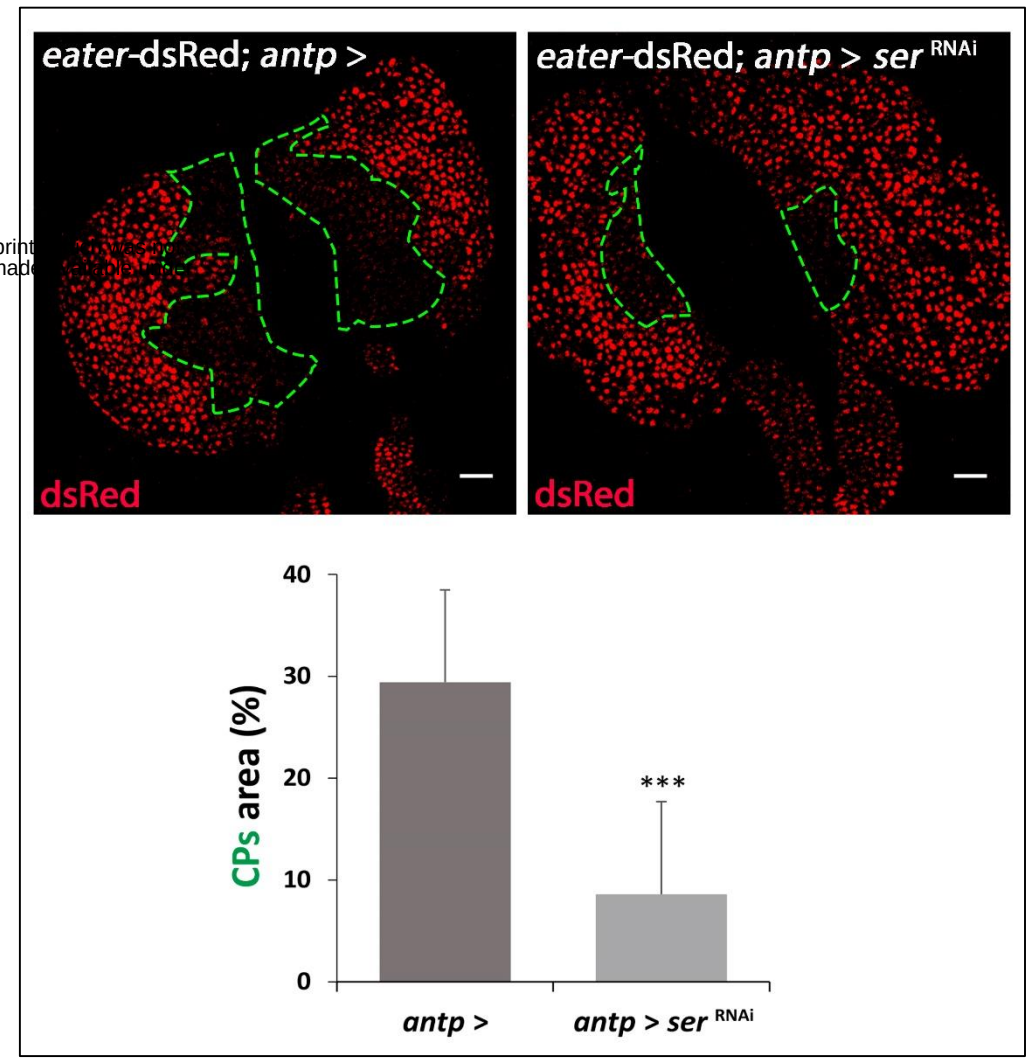

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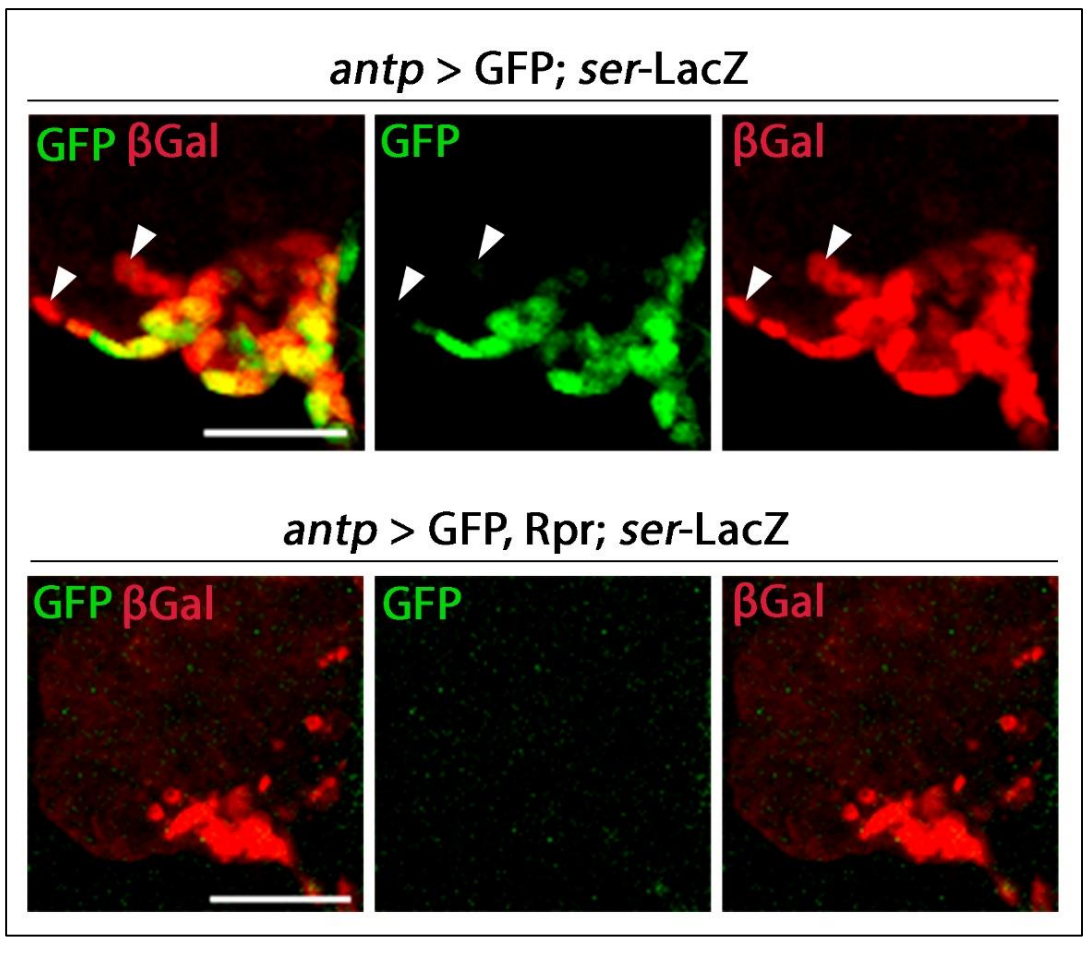

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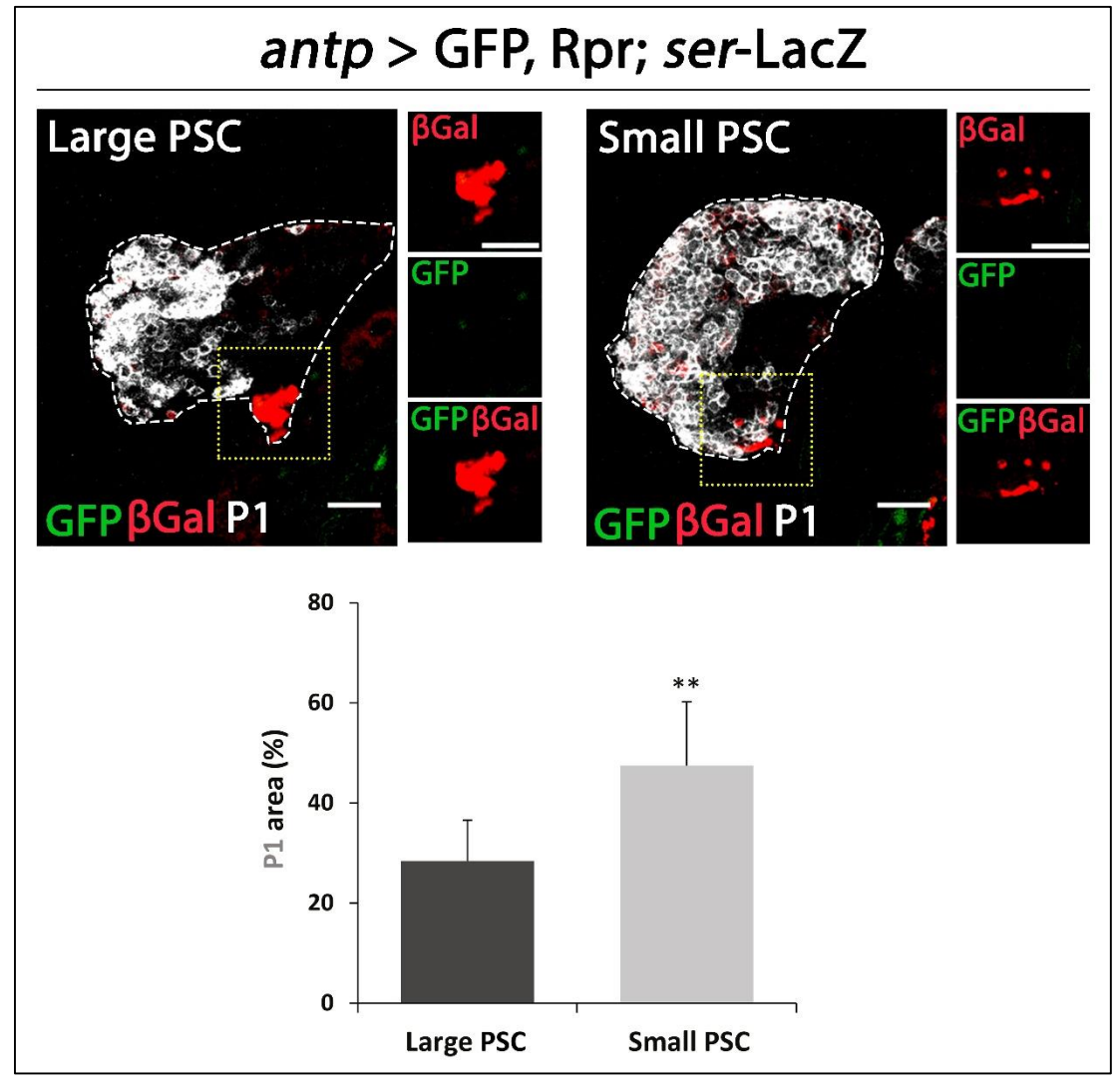

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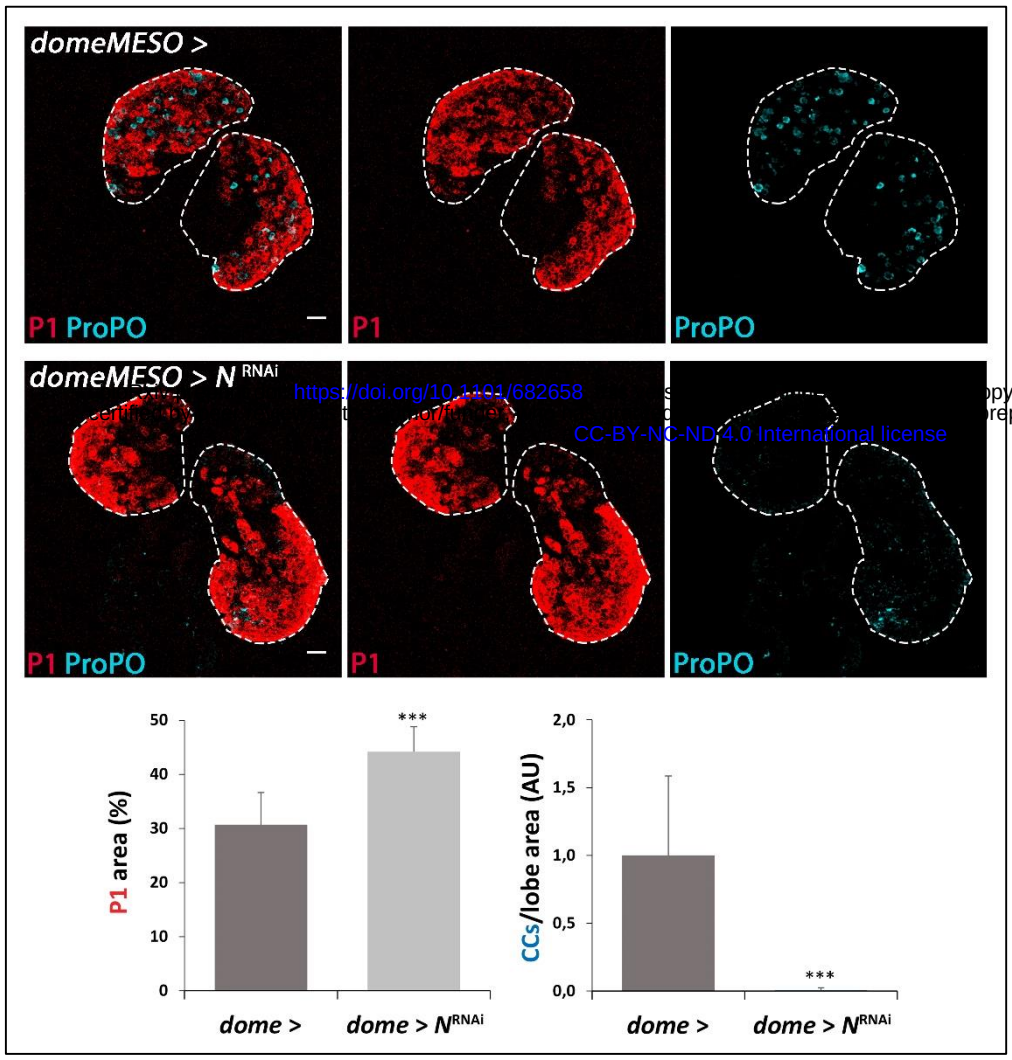

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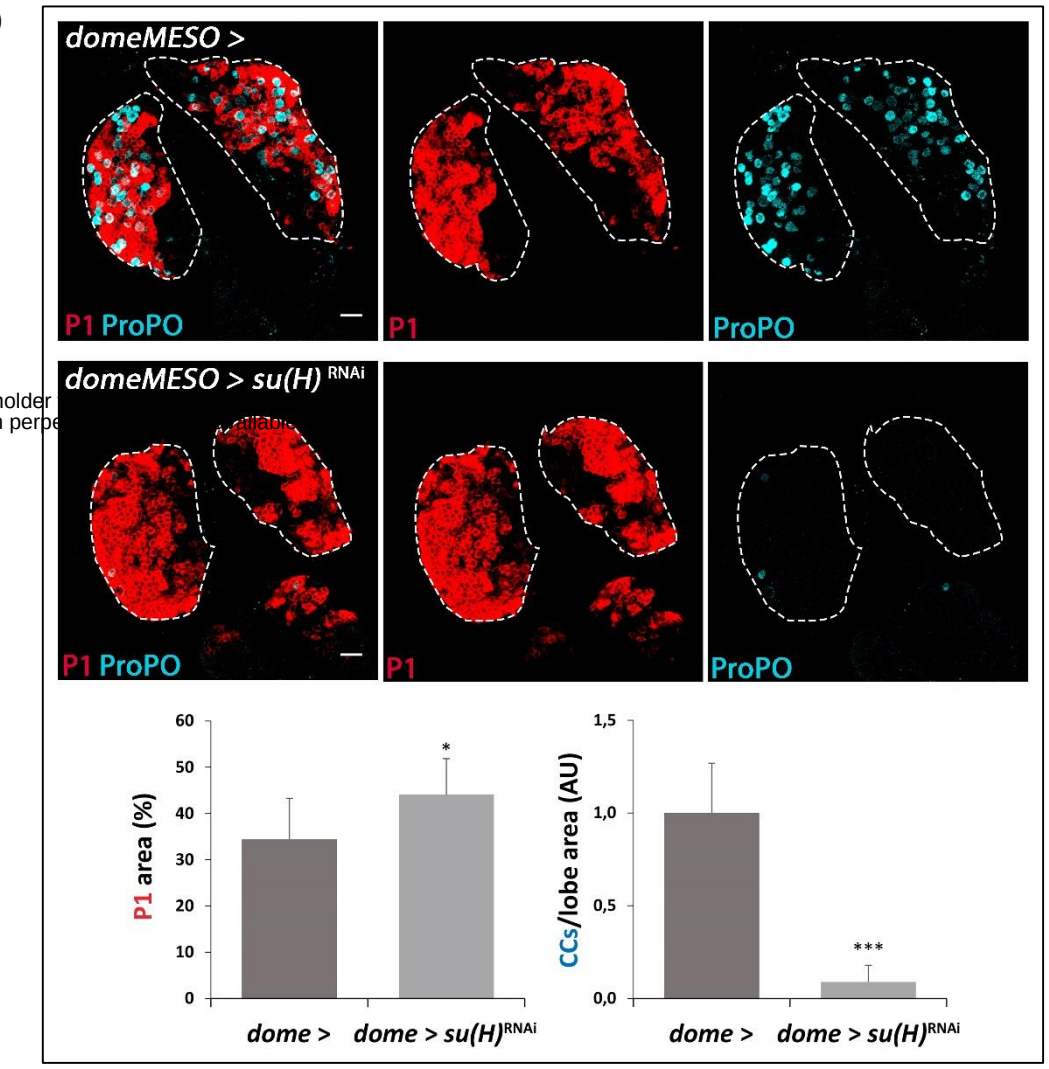

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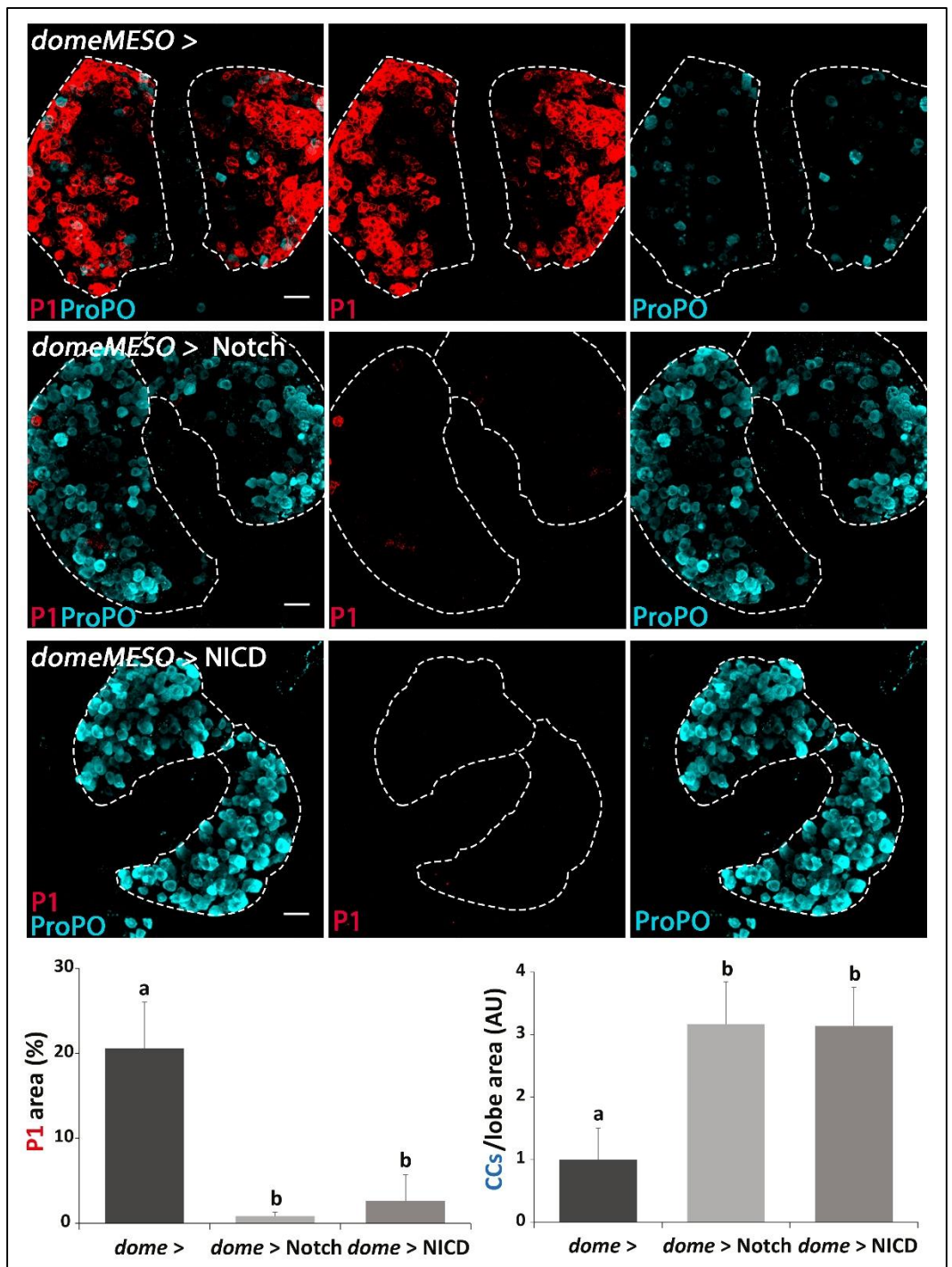

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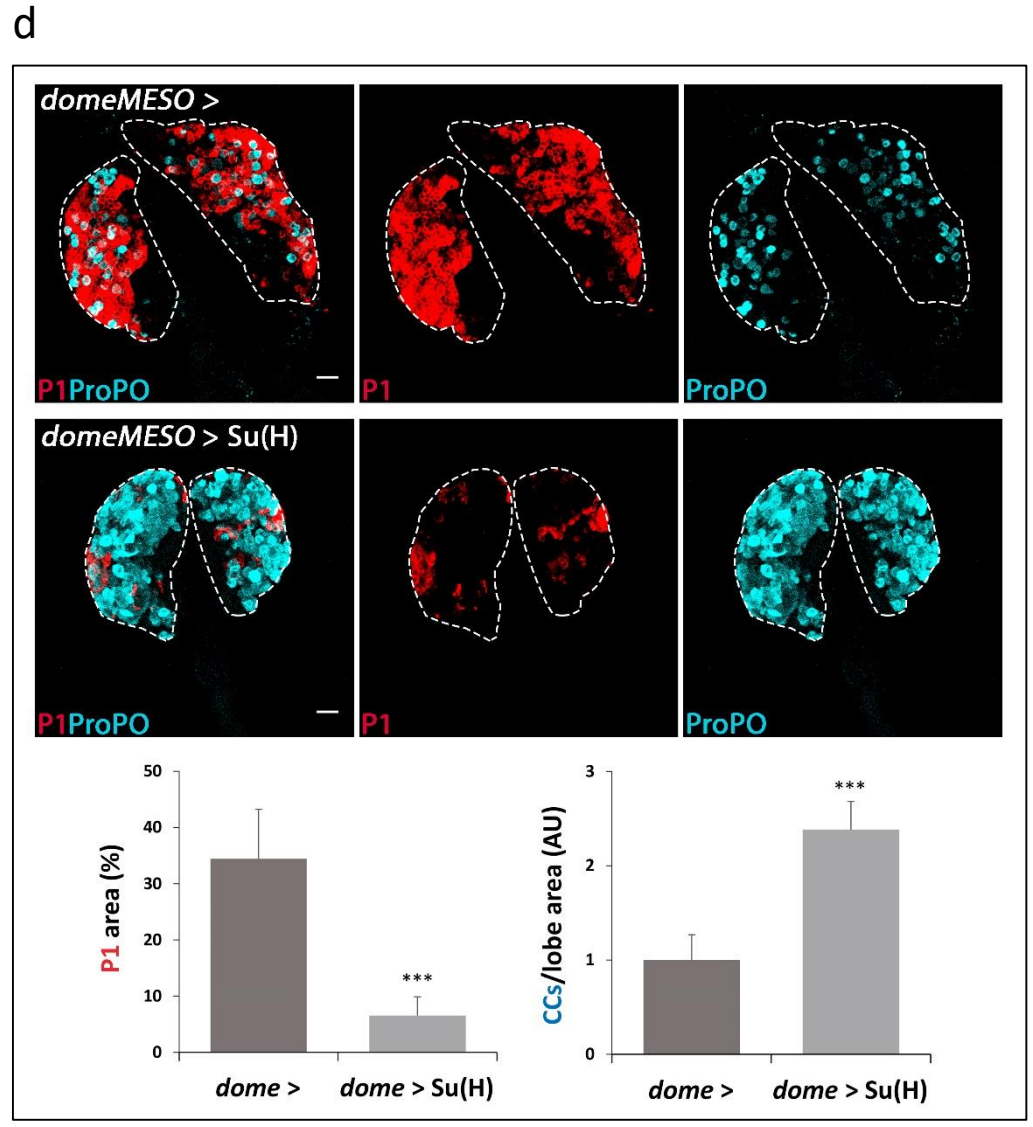

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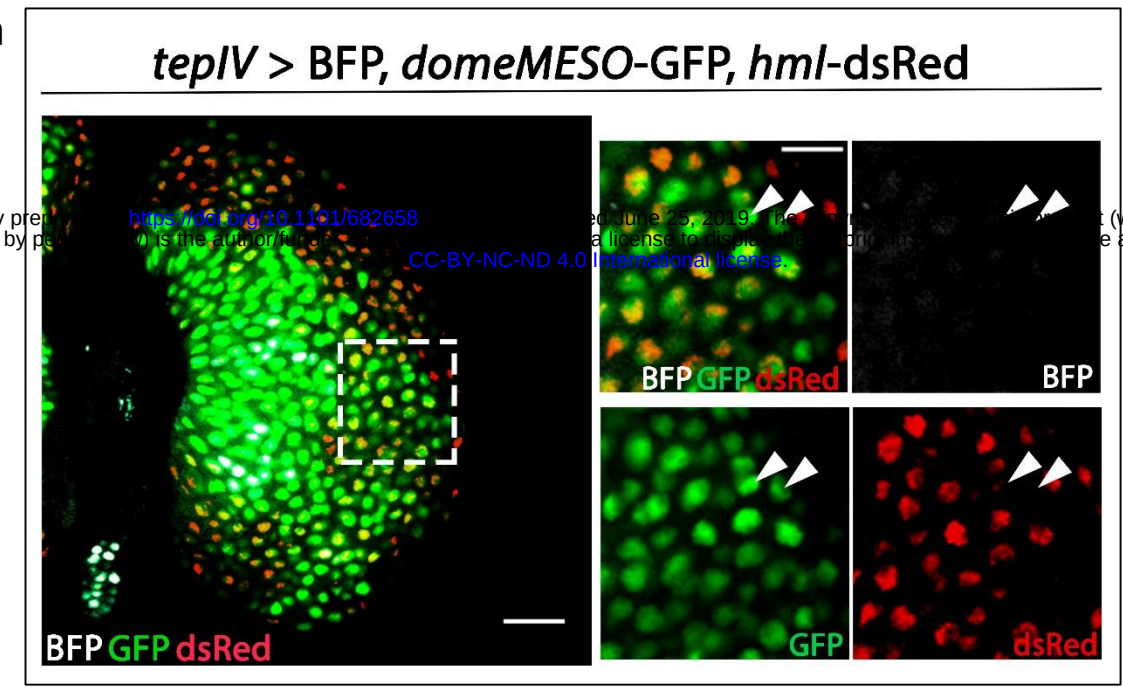

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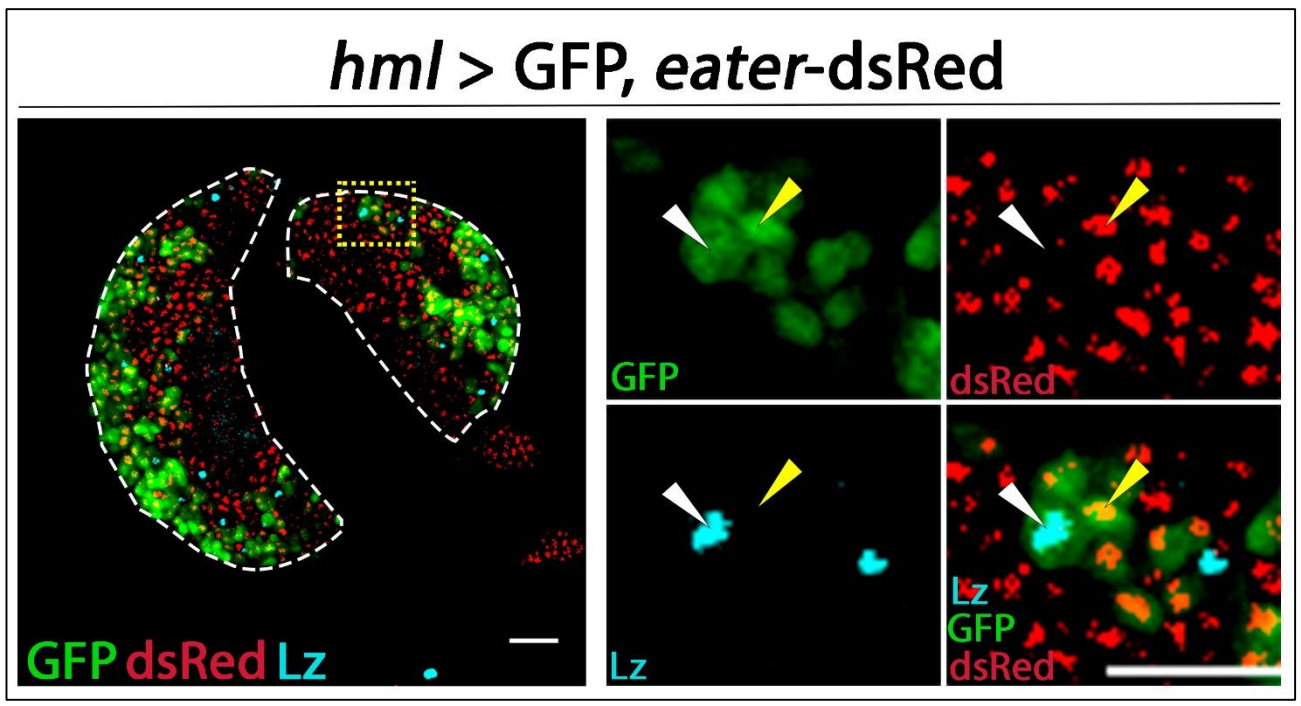

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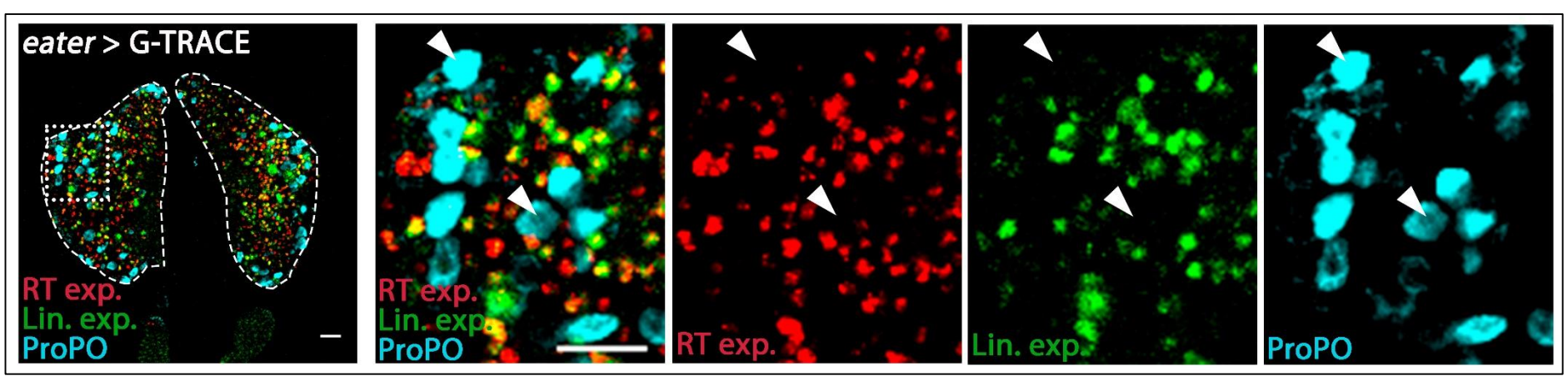

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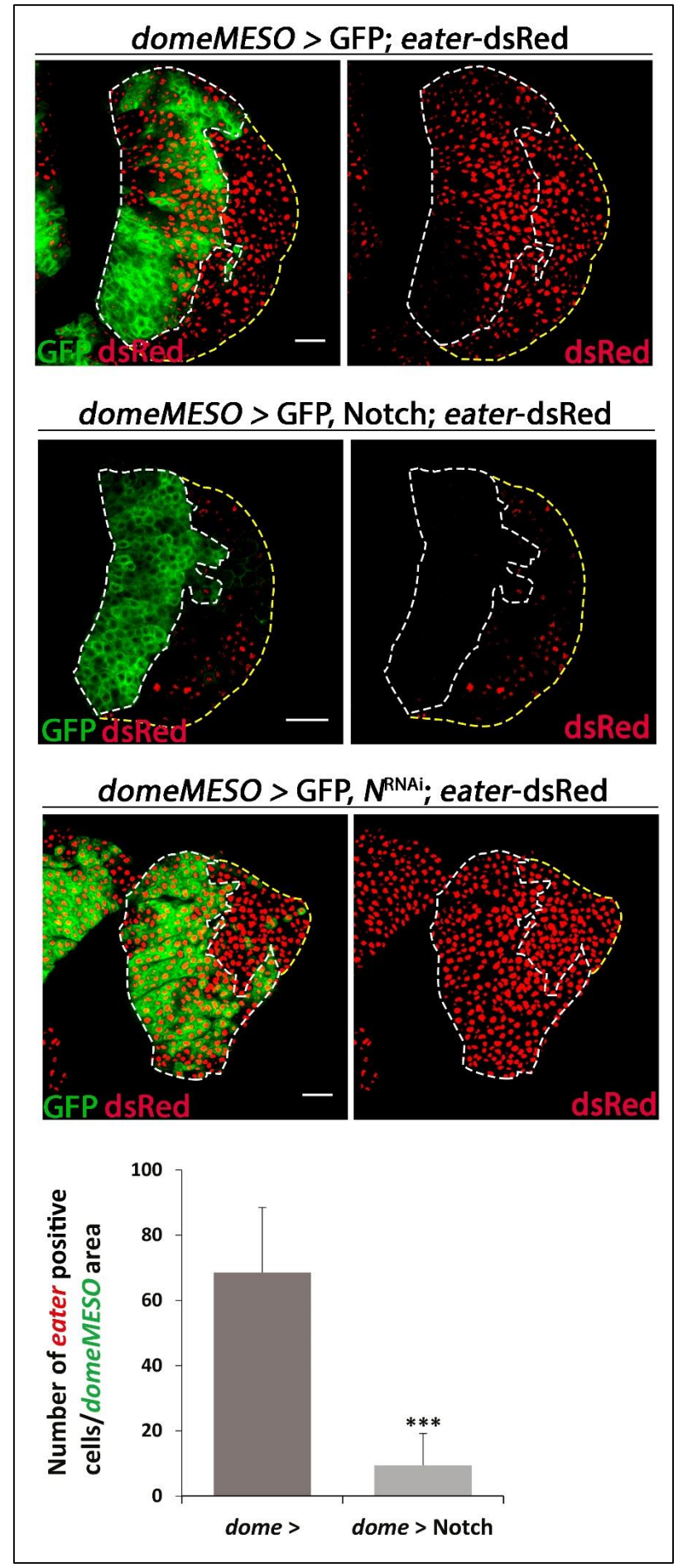

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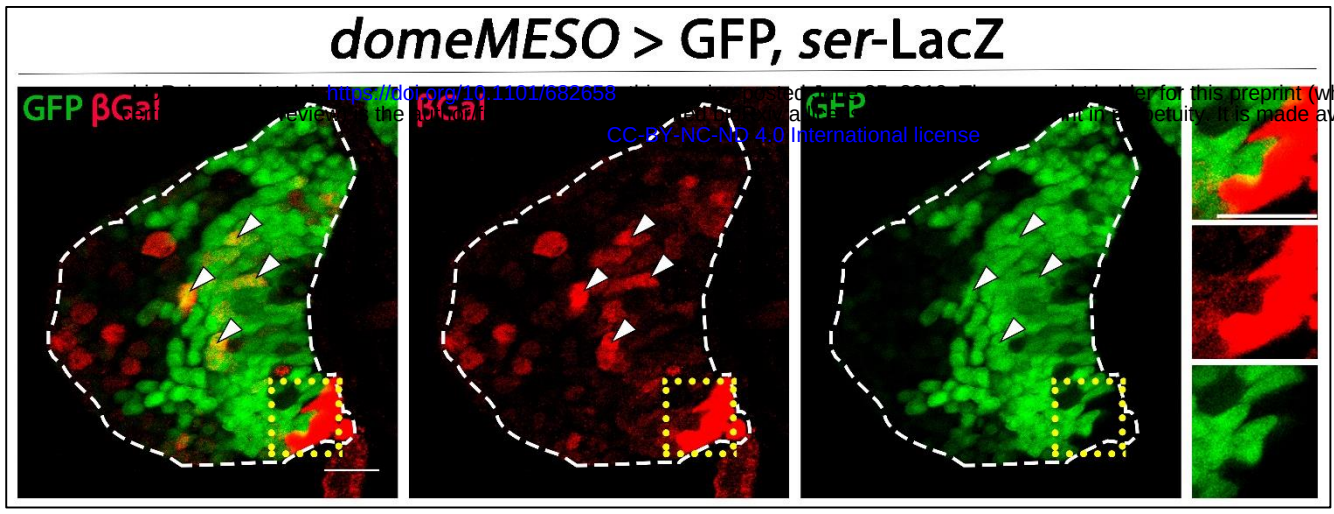

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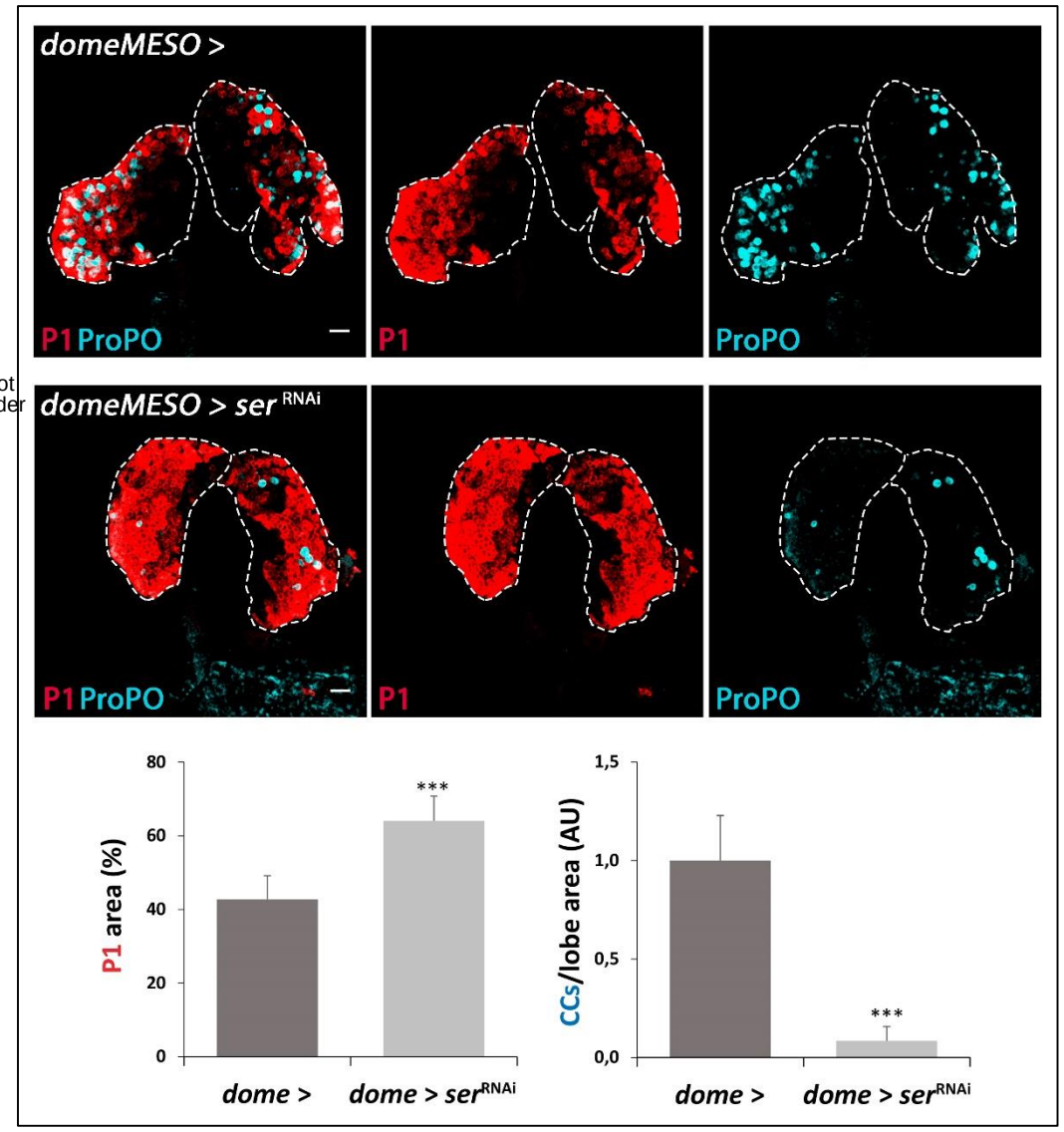

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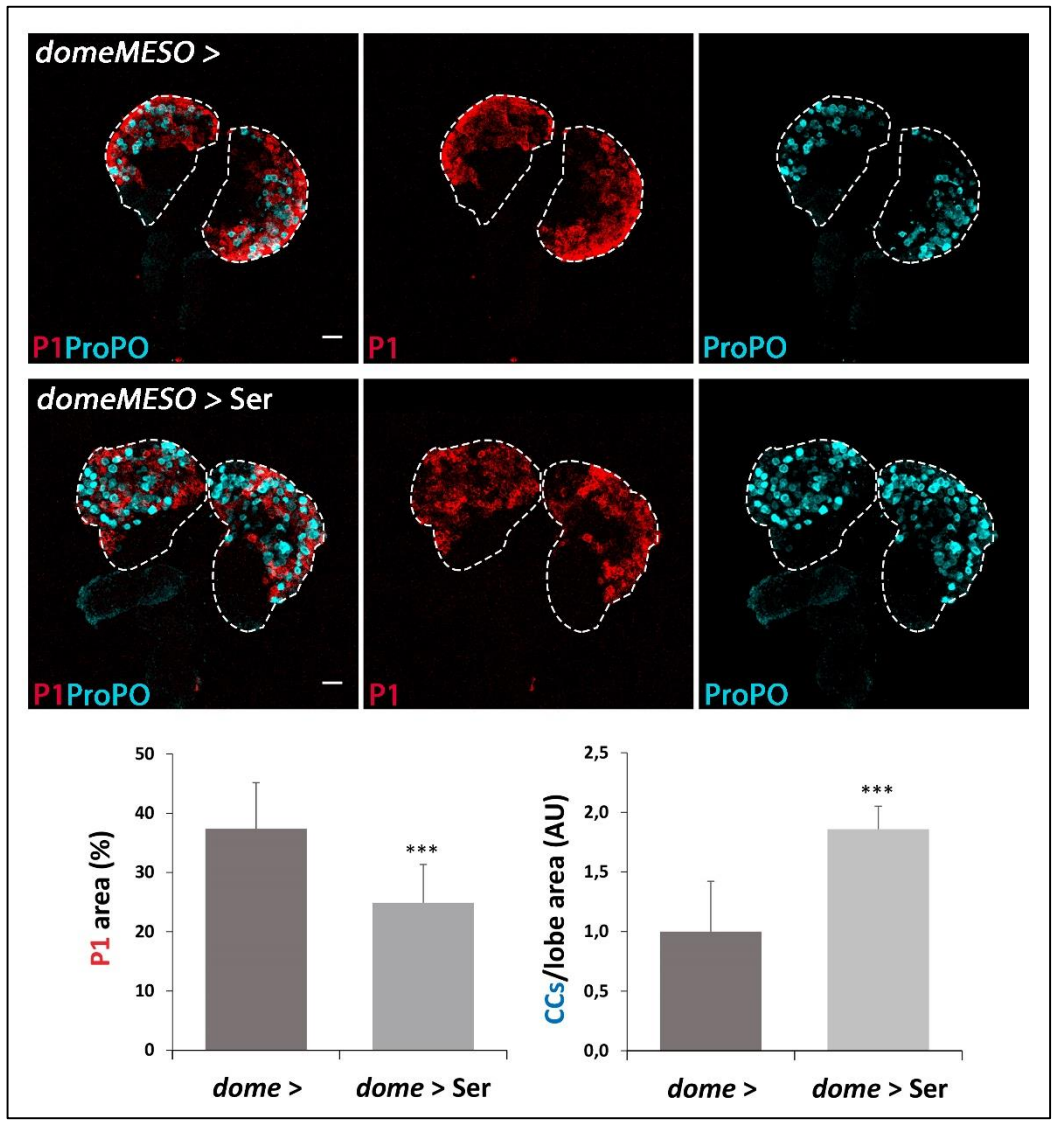

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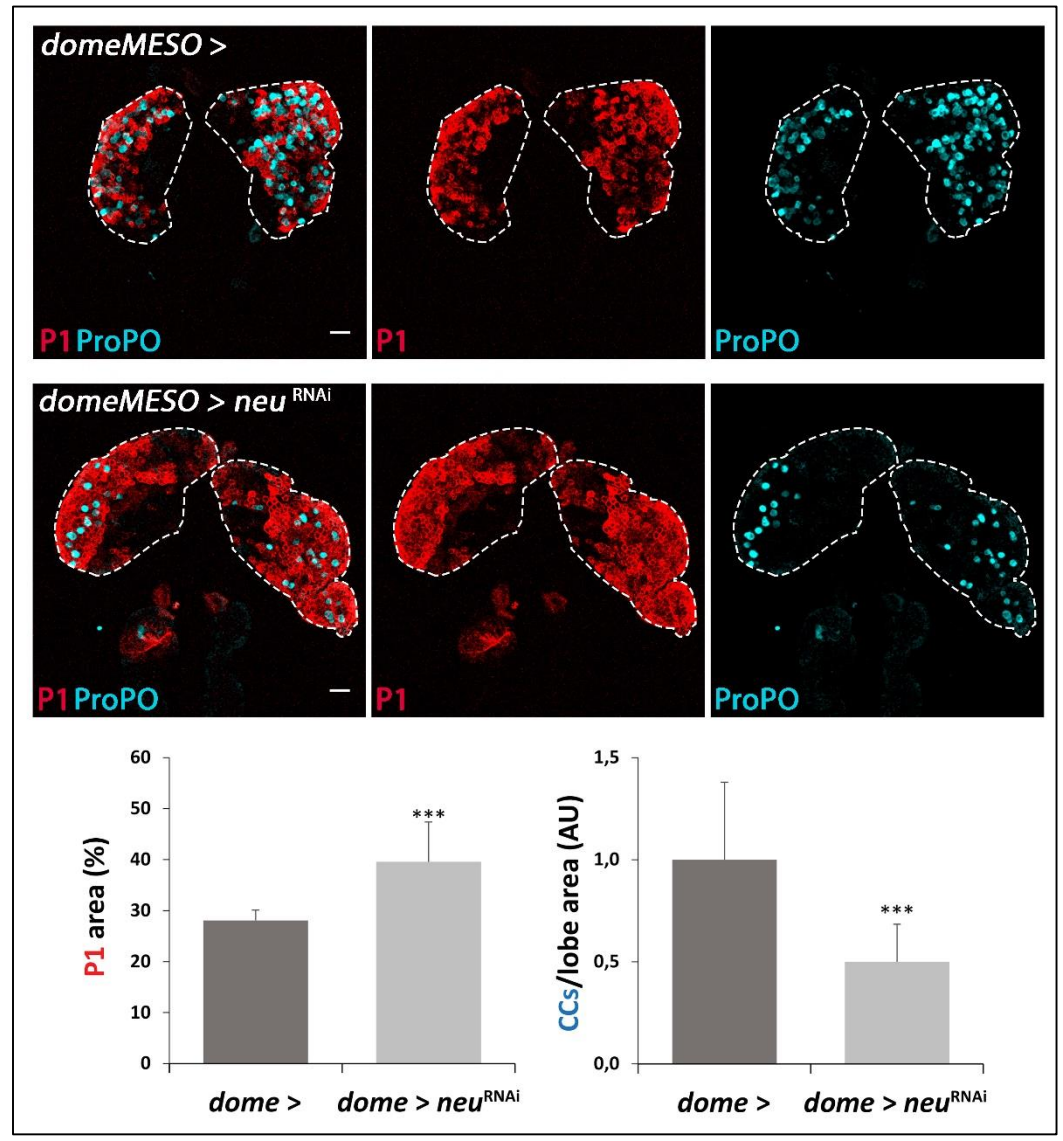

Figure 7

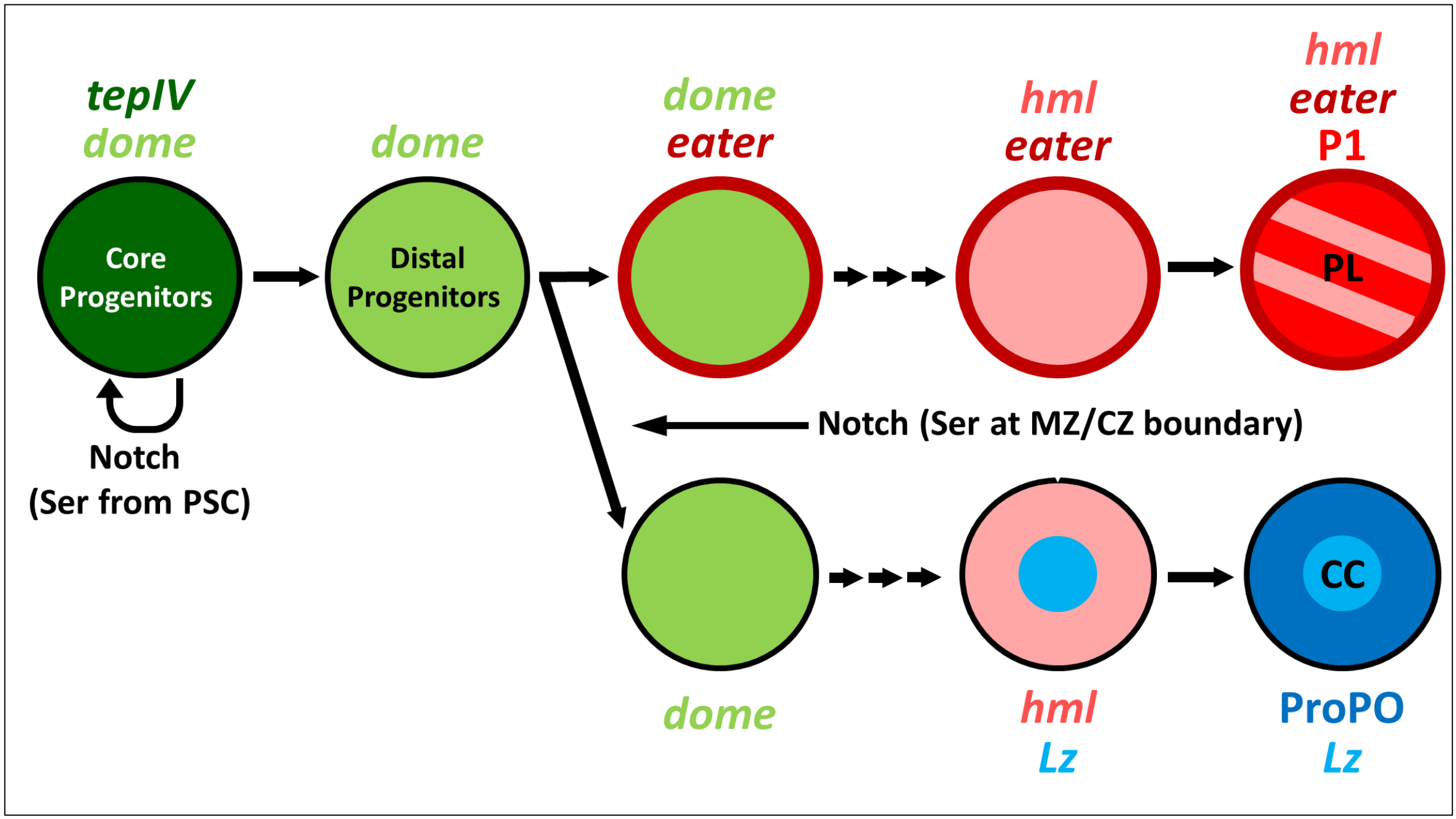

Figure legends

Figure 1. Progenitor populations of the lymph gland

(a) Cell populations of the lymph gland. Blood cell progenitors (green; *domeMESO*-GFP); differentiating cells (red; *hml*-dsRed); Crystal Cells (cyan, anti-ProPO staining) and Plasmatocytes (white, anti-P1 staining). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm.

(b) Schematic representation of a wandering 3rd instar lymph gland lobe. Purple: Posterior Signaling Center; green: Blood cell progenitors; yellow: Intermediate Progenitors; pink: Differentiating cells; dark red: Plasmatocytes; blue: Crystal Cells. The black arrow indicates progenitor maintenance signals that emanate from the PSC.

(c) Simplified scheme of the Notch pathway. Dl: Delta, Ser: Serrate. NICD: Notch Intracellular Domain, Su(H): Supressor of Hairless, Mam: Mastermind.

(d) Three populations of blood cell progenitors occur in the lymph gland. Region 1: Cells that express both *tepIV* > BFP (white) and *domeMESO*-GFP (green). Region 2: Cells that express *domeMESO*-GFP but not *tepIV* > BFP or *hml*-dsRed (red). Region 3: Cells in which *domeMESO*-GFP and *hml*-dsRed signals coexist. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm.

(e) The Plasmatocyte marker *eater* is expressed in a subpopulation of blood cell progenitors. Progenitors that express both *tepIV* > BFP (white) and *domeMESO*-GFP (green) co-express almost undetectable levels of the plasmatocyte marker *eater*-dsRed (red) (region 1). In contrast, *eater*-dsRed expression can be readily detected in Distal Progenitors that are *domeMESO*-GFP positive and negative for *tepIV* > BFP (region 2). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm.

(f) Schematic representation of a wandering 3rd instar wild-type lymph gland lobe with redefined progenitor populations. Progenitors encompass three subpopulations: 1) Core Progenitors, positive for *domeMESO* and *tepIV* (dark green); 2) Distal Progenitors, negative for *tepIV* and *hml* and positive for *domeMESO* (light green); and 3) Intermediate Progenitors, which are positive for both *domeMESO* and *hml* (yellow). The color code for the remaining cell populations are as in Fig. 1b: Purple: Posterior Signaling Center; pink: *hml*-positive differentiating cells; dark red: Plasmatocytes; blue: Crystal Cells.

Figure 2. The Notch pathway is required for Core Progenitor maintenance.

(a) Notch is expressed throughout the lymph gland. Staining with an anti-Notch antibody (red) in control lymph glands (upper panels), or in lymph glands expressing a *tepIV*-Gal4 driven *notch* RNAi (N^{RNAi}) (middle panels). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm. *TepIV* > GFP marks the area of Core Progenitors, which is also delimited with a white dashed line. Note that following N^{RNAi} expression, Notch staining was reduced in CPs (middle panels). Lower panel: Quantification of mean anti-Notch signal levels in Core Progenitors (***p < 0.001). Error bars represent SD. *tepIV* >, n = 16; *tepIV* > N^{RNAi}, n = 18.

(b) Notch is required for Core Progenitor maintenance. *Notch* RNAi (N^{RNAi}) expression in Core Progenitors, driven by *tepIV*-Gal4, provoked loss of Core Progenitors (green: *tepIV* > GFP), along with increased Plasmatocytes (red: P1 staining) and Crystal Cells (cyan: ProPO staining). Compare control lymph glands in upper panels with lymph glands expressing N^{RNAi} in middle panels. Whole Z-projection confocal images of primary lobes from wandering third instar

larvae are depicted. Graphs in lower panels show quantification of the indicated markers (**p < 0.01, ***p < 0.001). Error bars represent SD. *tepIV* >, n = 8; *tepIV* > N^{RNAi}, n = 8.

(c) *eater*-dsRed reporter expression domain is expanded upon Core Progenitor loss. Expression of the *eater*-dsRed reporter (red) in control lymph glands (left panel) and in lymph glands expressing *notch* RNAi (N^{RNAi}) in Core Progenitors under the *tepIV*-Gal4 driver (right panel). The green dashed line marks *eater*-negative Core Progenitors area. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. The graph shows quantification of *eater*-negative area corresponding to Core Progenitors (***p < 0.001). Error bars represent SD. *tepIV* > , n = 8; *tepIV* > N^{RNAi}, n = 15.

(d) Supressor of Hairless (Su(H)) is required for Core Progenitor maintenance. $Su(H)^{RNAi}$ expression with *tepIV*-Gal4 caused a reduction of Core Progenitors (green: *tepIV* > GFP), and a simultaneous increase of Plasmatocytes (red: P1 staining) and Crystal Cells (cyan: ProPO staining). Upper panels: Controls without RNAi; Middle panels: Expression of $su(H)^{RNAi}$. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are depicted. Graphs in lower panels show quantification of the indicated markers (*p < 0.05, **p < 0.01). Error bars represent SD. *tepIV* >, n = 8; *tepIV* > $su(H)^{RNAi}$, n = 5.

Figure 3. Serrate is required at the Posterior Signaling Center for Core Progenitor maintenance.

(a) *Serrate (Ser)* silencing at the Posterior Signaling Center provokes increased differentiation of Plasmatocytes and Crystal Cells. Following *serrate* RNAi (ser^{RNAi}) expression with an *antp*-Gal4 driver, a general increase of cell differentiation was observed. Plasmatocytes are in red (P1 staining) and Crystal Cells in cyan (ProPO staining). Upper panels: Control lymph glands; middle panels: Lymph glands with expression of ser^{RNAi} driven by *antp*-Gal4. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm. Lower panels: Quantification of the results (**p < 0.01). Error bars represent SD. *Antp* >, n = 10; *antp* > ser^{RNAi}, n = 14.

(b) *Serrate* (*ser*) silencing at the Posterior Signaling Center provokes a reduction of Core Progenitors. Expression of the *eater*-dsRed reporter is shown, and the area that lacks its expression corresponds to Core Progenitors (marked with green dashed lines). Left panel: Control Lymph gland; right panel: Lymph gland with *ser* silencing at the Posterior Signaling Center. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm. Lower panel: quantification of the area occupied by *eater*-negative Core Progenitors. Error bars represent SD. *Antp* >, n = 14; *antp* > ser^{RNAi}, n =19.

(c) *Serrate*-expressing cells of the Posterior Signaling Center survive to genetic ablation of *antennapedia*-expressing cells. A*ntennapedia (antp)*-positive cells of the Posterior Signaling Center (PSC) are visualized with *antp*-Gal4 driven expression of GFP (*antp* > GFP, green), while *serrate* (*ser*) expressing cells are shown with anti-βGal staining (red) of *ser*-LacZ reporter. Expression of the pro-apoptotic protein Reaper (Rpr) with an *antp*-Gal4 driver from L2 stage onwards (see text) eliminates all *antp*-positive cells, but many *ser* expressing cells survive. Upper panels: control lymph glands without Rpr expression. The wild-type PSC comprises a mixed population of *antp* and *ser* double-positive cells (ser^+, $antp^+$) and *ser* positive-*antp* negative cells (ser^+, $antp^-$) (examples of the latter are shown with arrowheads); lower panels: lymph glands expressing Rpr under *antp*-Gal4. After genetic ablation, all of the observed PSC cells express *ser* and lack *antp* expression. Single-plane confocal images of the PSC region of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm.

(d) After *antennapedia*-Gal4 driven genetic ablation, the abundance of the surviving *serrate*-expressing cells of the Posterior Signaling Center correlates negatively with the amount of Plasmatocytes. In lymph glands in which *antp*-expressing cells of the Posterior Signaling Center (PSC) have been ablated by *antp*-Gal4 driven expression of Reaper (Rpr), Plasmatocytes differentiation (white, P1 staining) correlates negatively with the area occupied by *serrate*-LacZ expressing cells that survived genetic ablation (red, βGal staining). Examples of large and small *ser*-positive PSC areas are shown ("Large PSC" on the left panel and "Small PSC" on the right panel, respectively). Inset shows a magnification of the PSC region, with all cells of the PSC expressing *ser* but not *antp*. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are depicted. Scale bars, 20 µm. The graph shows quantification of P1-positive area in *antp*-ablated lymph glands that exhibit large or small PSC area. PSC area was considered small if it was less than %1.5 of the total lobe area). Large PSC, n = 7, Small PSC, n = 8.

Figure 4. Notch regulates the Plasmatocyte/Crystal Cell fate decision in Distal Progenitors.

(a) *Notch* (*N*) silencing in *domeMESO*-positive progenitors abolishes Crystal Cell specification and promotes the Plasmatocyte cell fate. *Notch* RNAi (N^{RNAi}) expression with the *domeMESO*-Gal4 driver provoked loss of Crystal Cells (cyan: ProPO staining), along with increased Plasmatocytes (red: P1 staining). Compare control lymph glands in upper panels with lymph glands expressing N^{RNAi} in middle panels. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are displayed. Charts in lower panels show quantification of the indicated markers (***p < 0.001). Error bars represent SD. *dome* >, n = 8; *dome* > N^{RNAi}, n = 8.

(b) *Suppressor of hairless* (*su(H)*) silencing in *domeMESO*-positive progenitors is similar to *notch* silencing. *Su(H)* RNAi ($su(H)^{RNAi}$) expression with the *domeMESO*-Gal4 driver provoked loss of Crystal Cells (cyan: ProPO staining) and increased Plasmatocytes (red: P1 staining). Upper panels: control lymph glands; middle panels*:* lymph glands expressing $su(H)^{RNAi}$. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are shown. Lower panels: quantification of the indicated markers (*p < 0.05, ***p < 0.001). Error bars represent SD. *dome* >, n = 10; *dome* > $su(H)^{RNAi}$, n = 14.

(c) Increased Notch activity in *domeMESO*-positive progenitors favors the Crystal Cell fate and inhibits the Plasmatocyte fate. Full-length Notch (Notch) or the Notch Intracellular Domain (NICD) were over-expressed with a *domeMESO*-Gal4 driver. In the upper panels, a control lymph gland is shown. In the rows below lymph glands overexpressing Notch (2^{nd} row) or NICD (3^{rd} row) are shown. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are depicted. Bottom row: Quantification of the results. Different letters indicate statistical difference (Tukey´s multiple comparison test). Error bars represent SD. *dome* >, n = 6; *dome* > Notch, n = 6; *dome* > NICD, n = 4.

(d) Over-expression of Suppressor of Hairless (Su(H)) in *domeMESO*-positive progenitors provokes reduction of Plasmatocytes and increase of Crystal Cells, similarly to Notch over-expression. Plasmatocytes are visualized in red (P1 staining) and Crystal Cells in cyan (ProPO staining). Upper panels: Control lymph glands; middle panels: Lymph glands with *domeMESO*-Gal4 driven over-expression of Su(H). Whole Z-projection confocal images of primary lobes from wandering third instar larvae are displayed. Scale bar, 20 µm. Lower panels: Quantification of the results (***p < 0.001). Error bars represent SD. *dome* >, n = 7; *dome* > Su(H), n = 8.

Figure 5. Eater is an early Plasmatocyte fate marker in Distal Progenitors.

(a) Eater is expressed in all but a few Distal Progenitors of wandering 3rd instar lymph glands. Pictures show a magnification of the Region 2 of Fig. 1e, which corresponds to the Distal Progenitor region that expresses the *domeMESO*-GFP reporter (green), but lacks *tepIV*-Gal4 driven BFP expression (*tepIV* > BFP, white). *Eater*-dsRed reporter (red) is expressed at high levels in most of Distal Progenitors, with exception of a few cells that show reduced or null levels (examples depicted with arrowheads). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 10 µm.

(b) In the Cortical Zone, the few differentiating cells that do not express *eater* are Crystal Cell Progenitors. Lozenge (Lz) staining (cyan) and *eater*-dsRed expression (red) is shown in lymph glands that also express GFP under *hml*-Gal4 control (*hml* > GFP, green). Inset shows an example of an *hml* > GFP positive cell that expresses the CC Progenitor marker Lz (white arrowhead), and another example of a *hml* > GFP positive cell that expresses *eater*-dsRed (yellow arrowhead). The Lz-expressing CC Progenitor lacks *eater*-dsRed expression (white arrowhead). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm.

(c) *Eater* is never expressed during Crystal Cell development. *Eater*-Gal4 driver was used to express the G-TRACE system, which allows real time and lineage detection of the expression of the Gal4 driver of interest. Crystal Cells are visualized with ProPO staining (cyan), and display neither real time (RT exp., red) nor lineage expression (Lin exp., green) of the *eater*-Gal4 driver (two examples are indicated by arrowheads). Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm.

(d) Notch regulates *Eater* expression in *domeMESO*-positive cells. *Eater*-dsRed reporter expression (red) is shown for control lymph glands (upper panels), and for lymph glands with *domeMESO*-Gal4 driven over-expression of Notch (2nd row panels) or *domeMESO*-Gal4 driven silencing of *notch* (3rd row panels). *DomeMESO*-positive cells are shown by *domeMESO*-Gal4 driven expression of GFP (*domeMESO* > GFP, green). Notch over-expression with *domeMESO*-Gal4 dramatically reduces *eater*-dsRed expression in all *domeMESO*-positive cells, while *notch* RNAi (N^{RNAi}) expression with *domeMESO*-Gal4 provokes that all *domeMESO*-positive cells express *eater*-dsRed. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panel: Quantification of double positive $dome^{+}$, $eater^{+}$ cells in control and Notch over-expressing lymph glands (***$p < 0.001$). Error bars represent SD. *Dome* >, n = 9; *dome* > Notch, n = 9.

Figure 6. Serrate expressed at the MZ/CZ boundary regulates the Plasmatocyte/Crystal Cell fate in Decision Progenitors.

(a) A fraction of s*er*-expressing cells at the MZ/CZ boundary expresses *domeMESO*. *Ser*-positive cells are visualized with βGal staining (red) of the *ser*-LacZ reporter. *DomeMESO-Gal4* driver expression of GFP (*domeMESO* > GFP, green) is observed in a proportion of *ser*-expressing cells at the MZ/CZ boundary (examples are shown with arrowheads). The yellow dashed line marks the Posterior Signaling Center area. On the right, a magnification of this area shows that *ser* is also expressed in these cells, but in this case *domeMESO* is not co-expressed. Single-plane confocal images of a primary lymph gland lobe from mid-3rd instar larvae are shown. Scale bar, 20 µm.

(b) Serrate (Ser) at the Medullary Zone is required for Crystal Cell differentiation and Plasmatocyte fate inhibition. Plasmatocytes are visualized in red (P1 staining) and Crystal Cells in cyan (ProPO staining), in control lymph glands (upper panels) and in lymph glands that

express a *ser* RNAi (*ser*^{RNAi}) under *domeMESO*-Gal4 driver control (middle panels). Whole Z-project confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the results (***p < 0.001). Error bars represent SD. *dome* >, n = 10; *dome* > *ser*^{RNAi}, n = 12.

(c) Over-expression of Serrate (Ser) at the Medullary Zone provokes an increase in Crystal Cell numbers and a reduction in Plasmatocyte differentiation. Pictures show Plasmatocytes (red, P1 staining) and Crystal Cells (cyan, ProPO staining) in control lymph glands (upper panels) and in lymph glands with *domeMESO*-Gal4 driven Ser over-expression (middle panels). Whole Z-project confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the results (***p < 0.001). Error bars represent SD. *Dome* >, n = 12; *dome* > Ser, n = 12.

(d) Neuralized (Neu) at the Medullary Zone is required for Crystal Cell differentiation and Plasmatocyte fate inhibition. Plasmatocytes (red, P1 staining) and Crystal Cells (cyan, ProPO staining) is shown in control lymph glands (upper panels) and in lymph glands with *neu* RNAi (*neu*^{RNAi}) expression under *domeMESO*-Gal4 driver control (middle panels). Whole Z-project confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the indicated markers (***p < 0.001). Error bars represent SD. *Dome* >, n = 10; *dome* > *neu*^{RNAi}, n = 10.

Figure 7. Model showing the redefinition of progenitor populations and the functions of the Notch pathway. In *tepIV*$^{+}$, *dome*$^{+}$ Core Progenitors (dark green) Notch is activated by its ligand Ser (Ser), expressed at Posterior Signaling Center (PSC) cells. This Notch activation is required to avoid premature Core Progenitor differentiation towards both the Plasmatocyte (PL) and Crystal Cell fate (CC) (upper and lower rows of cells, respectively). Thereafter *tepIV* expression ceases, while *dome* expression continues, defining a second population of progenitors, the Distal Progenitors (light green). Early Distal Progenitors do not express any differentiation marker, and constitute the stage where a cell fate decision between the Plasmatocyte and the Crystal Cell fate is made. This cell fate decision also depends on Notch, which is activated by Ser expressed at the MZ/CZ boundary. Notch activation in Distal Progenitors induces CC differentiation and inhibits the PL fate. Without contact from Ser-expressing cells, Distal Progenitors follow a default PL differentiation program. This early fate decision is evidenced later in development by the expression of *dome* and *eater* in Distal Progenitors destined to a PL fate (*eater* expression is represented with a dark red outline), while Distal Progenitors destined to CCs lack *eater* expression. Once blood cells start expressing *hemolectin* at the Cortical Zone (*hml,* pink), they are already committed either to a PL or to a CC fate. Cells committed to a PL fate continue expressing *eater,* while cells committed to become CCs now express Lozenge (Lz, light blue). Finally, mature PLs co-express *eater*, *hml* and the P1-antigen (red); while mature CCs co-express Lz and ProPO (dark blue) but have ended *hml* expression.

Supplementary Figure 1

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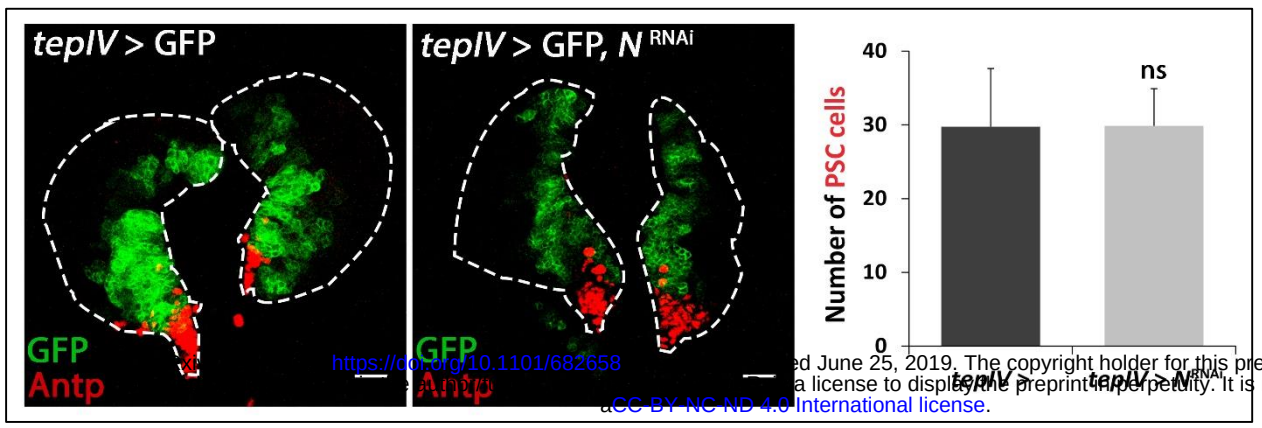

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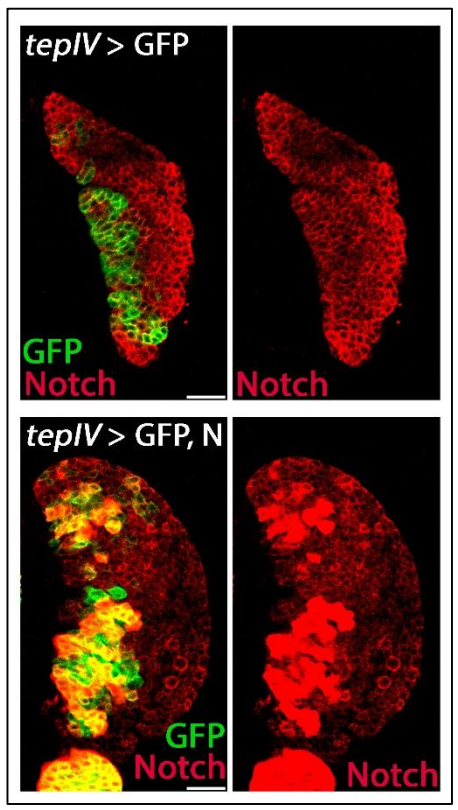

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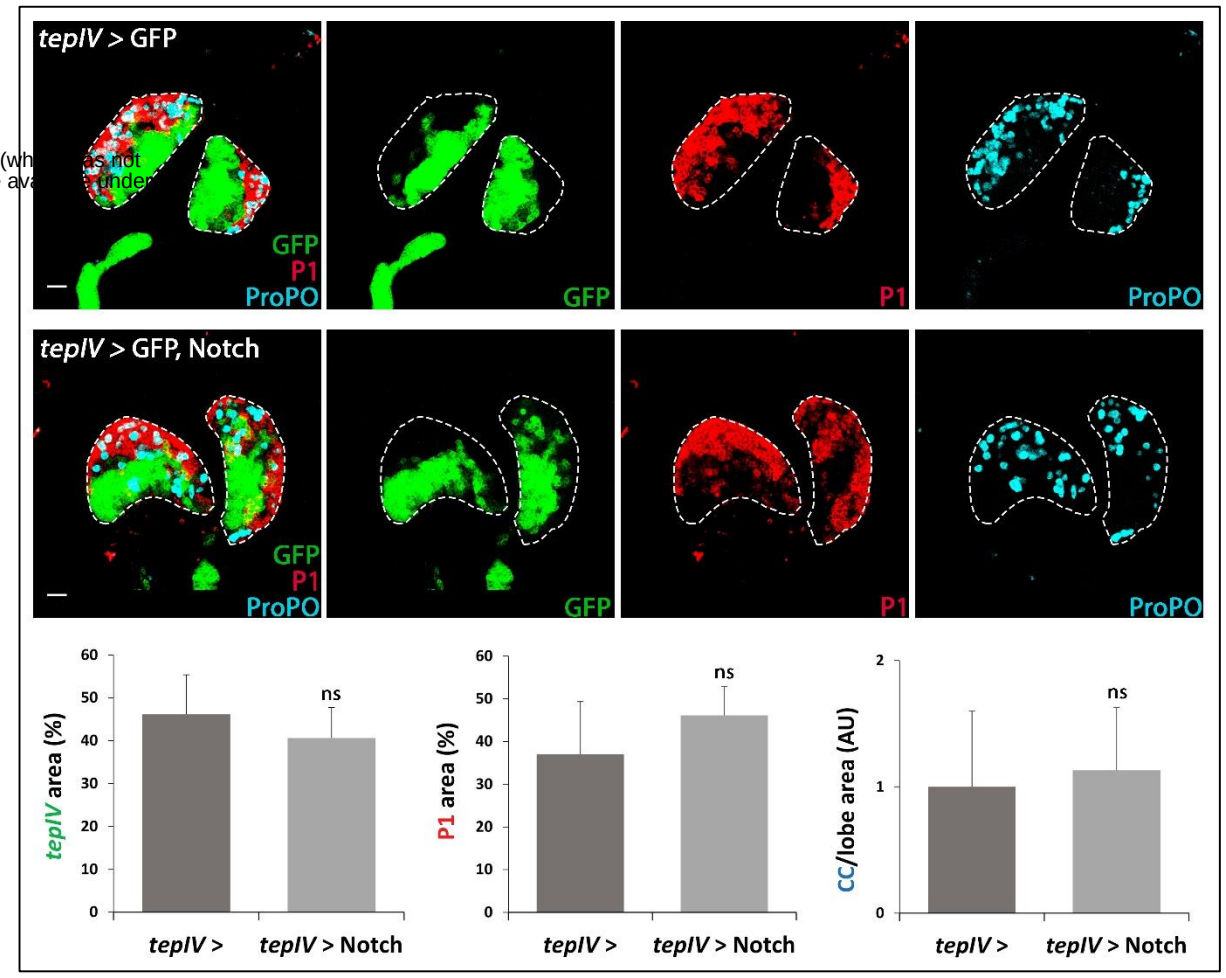

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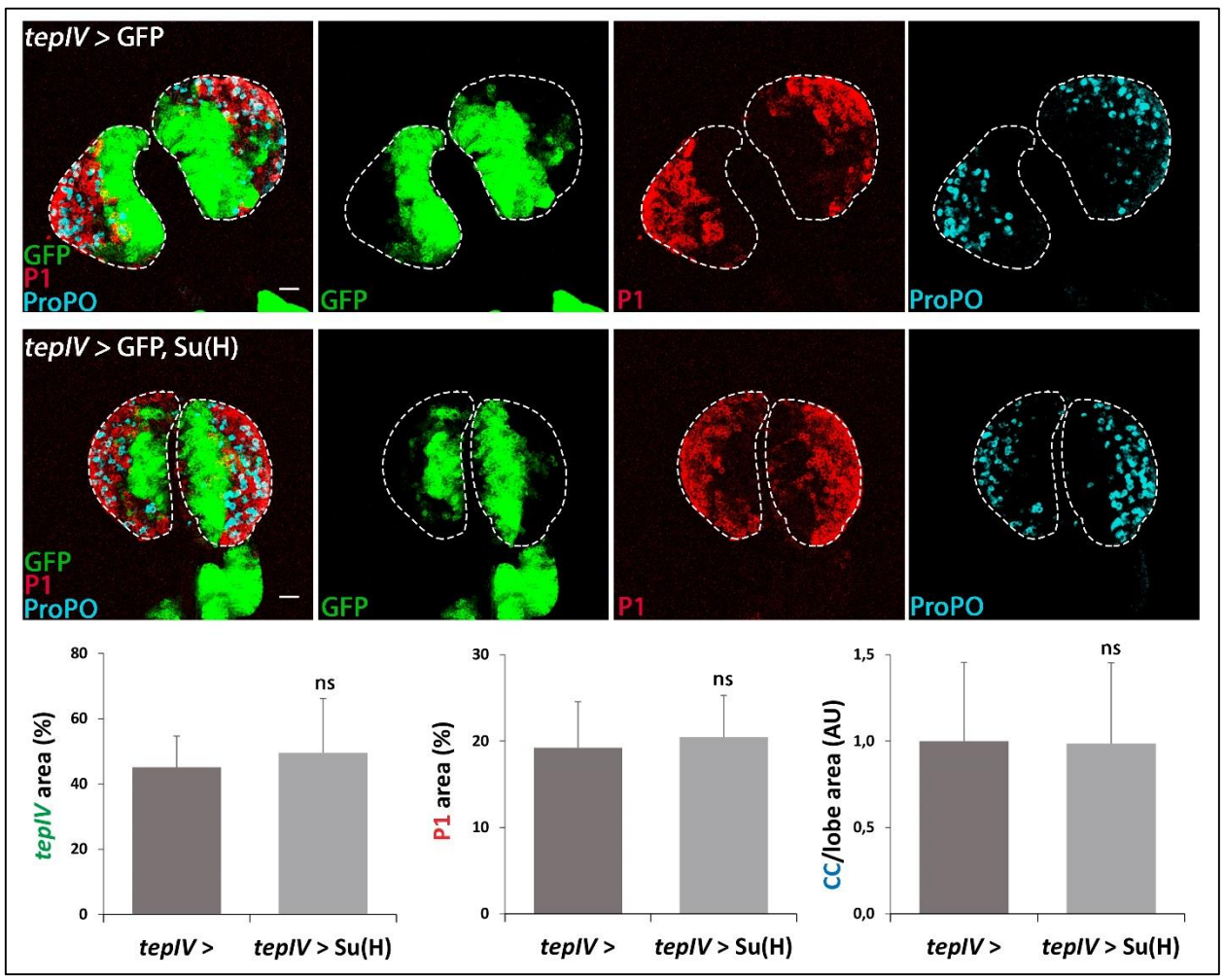

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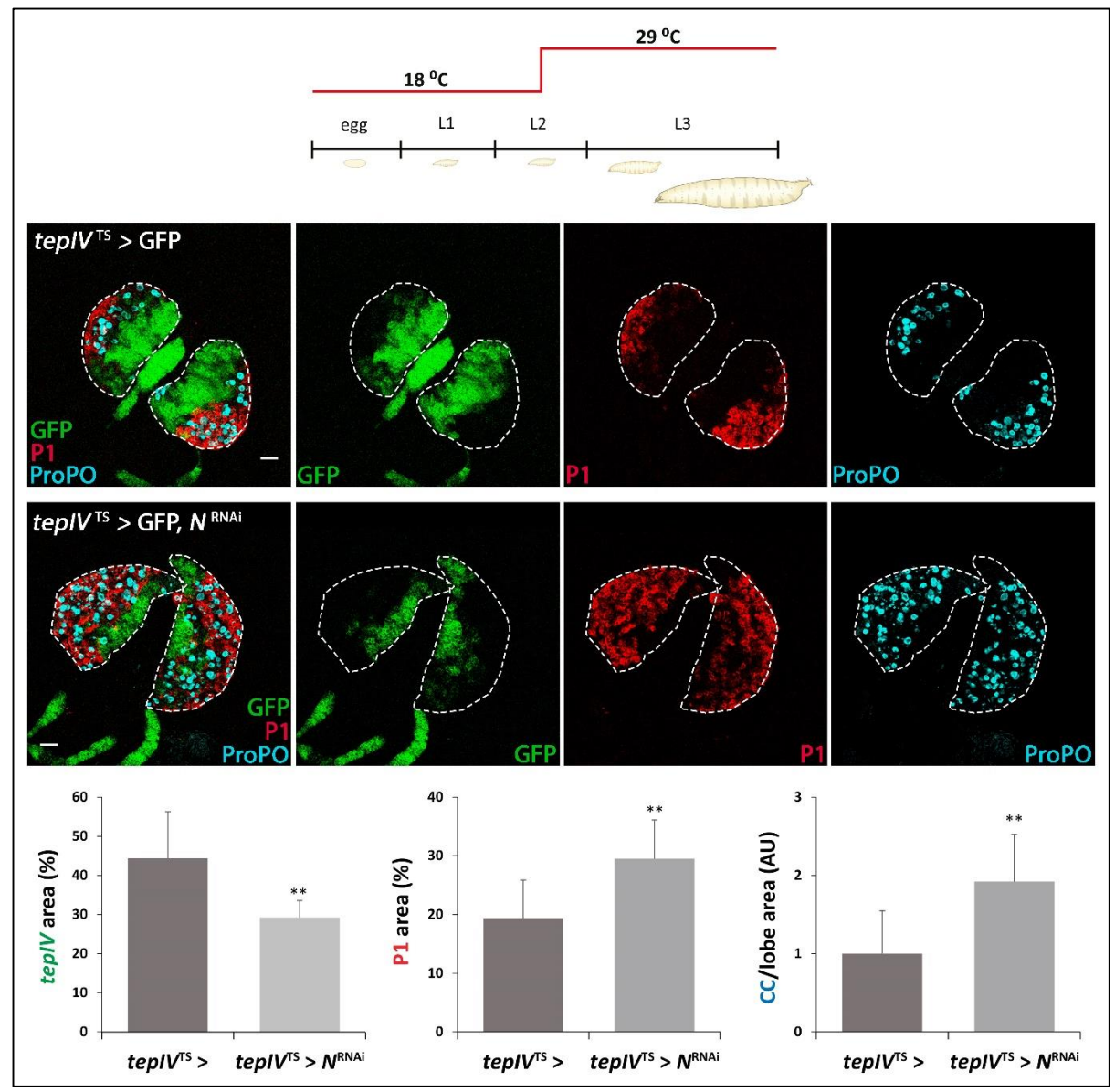

Supplementary Figure 2

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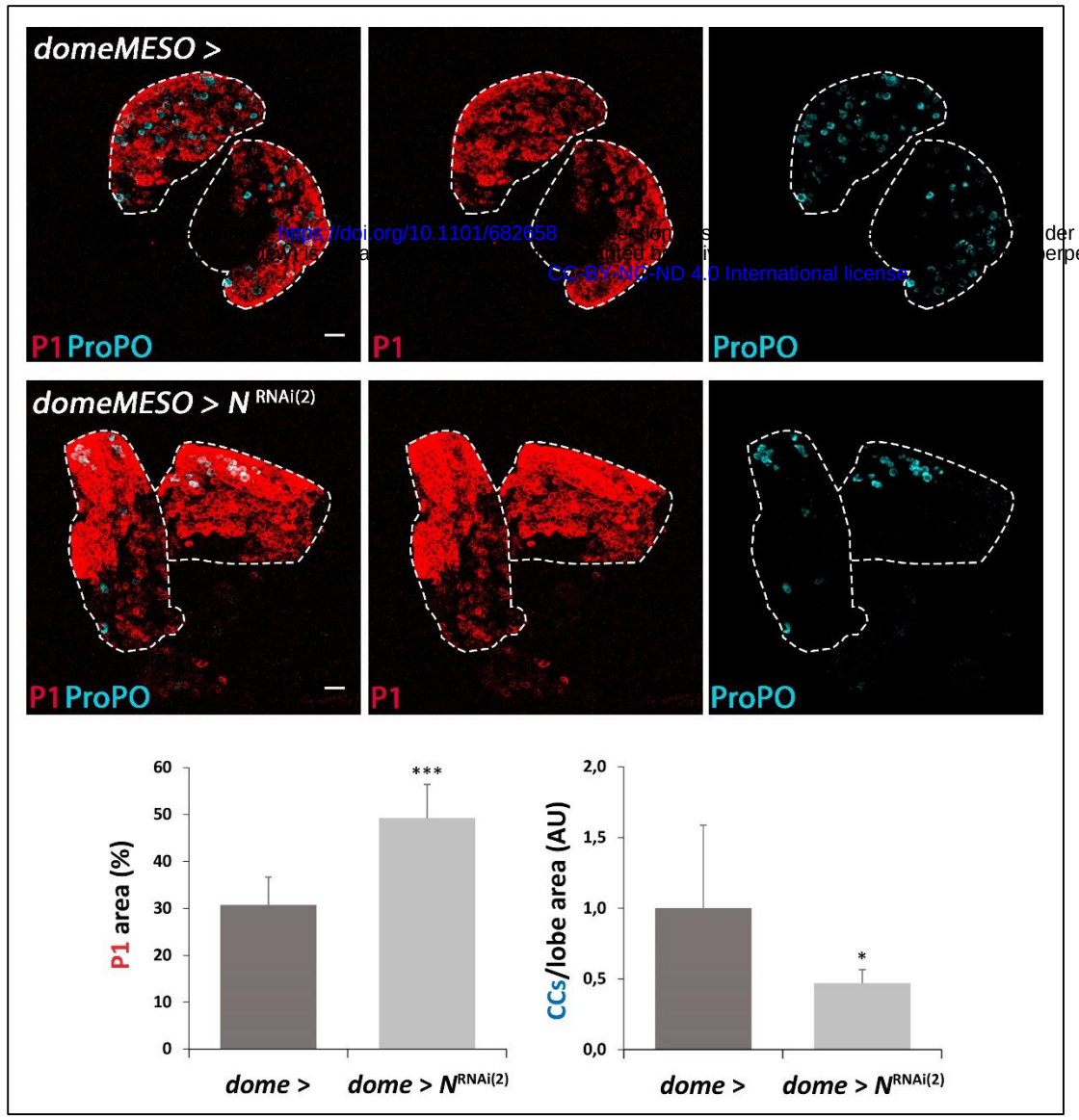

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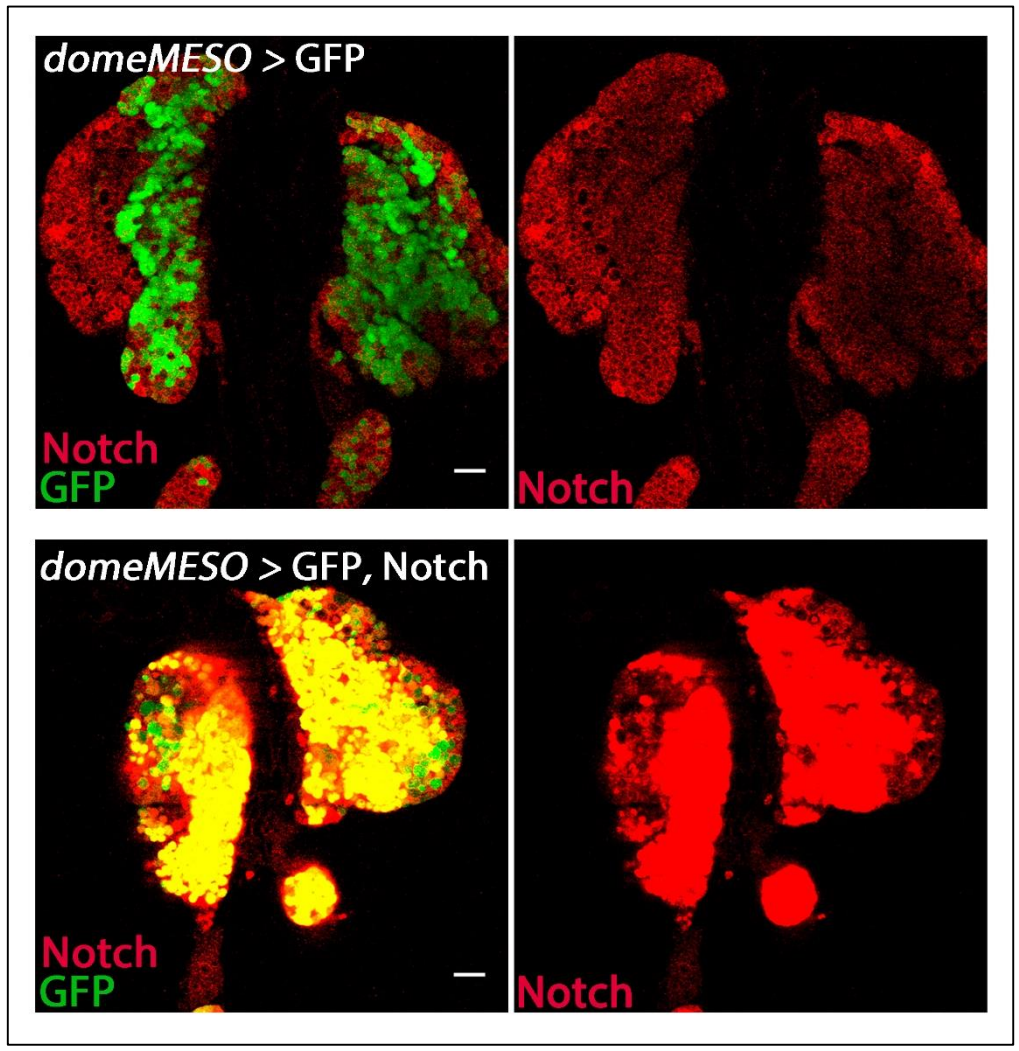

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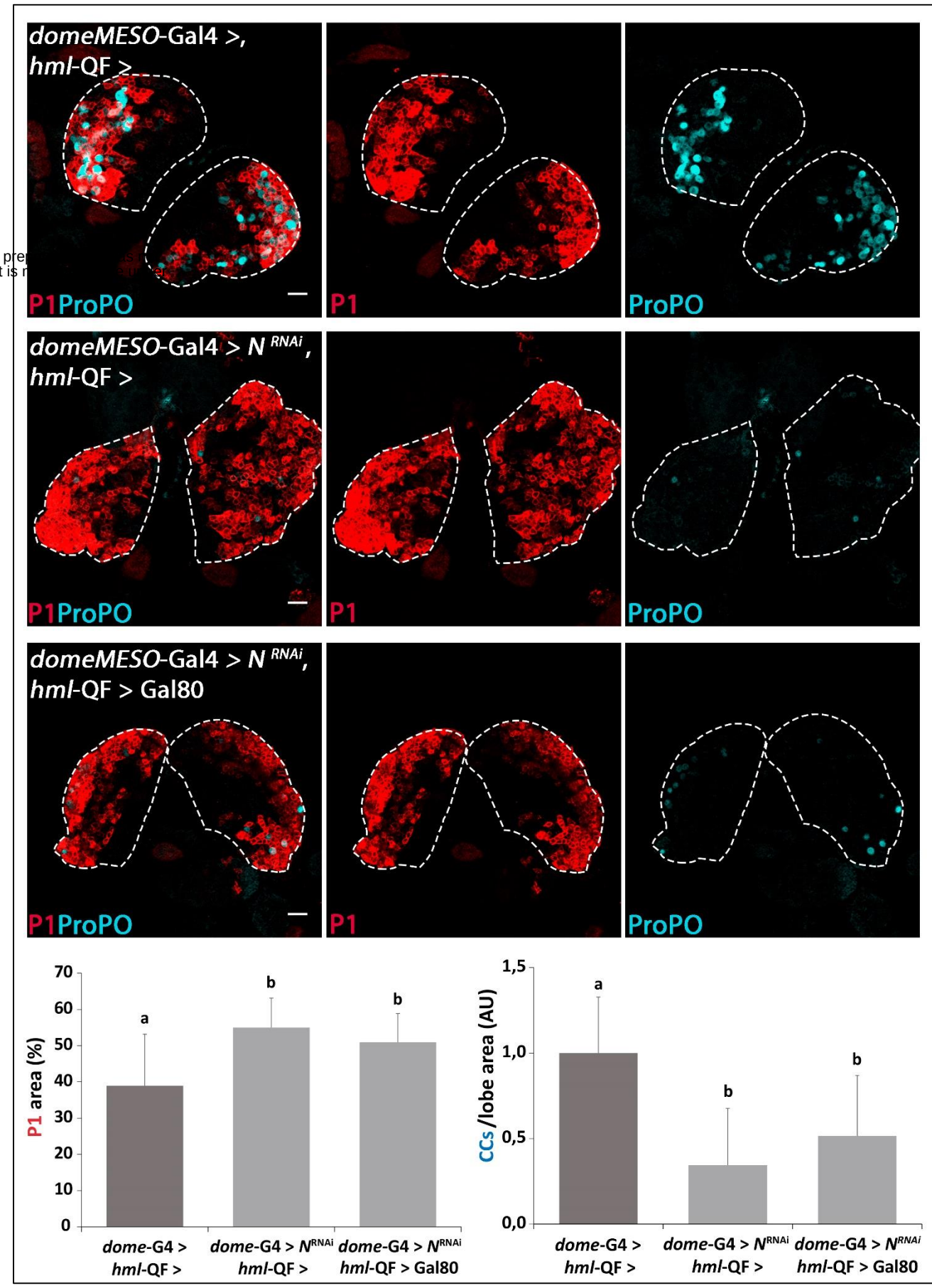

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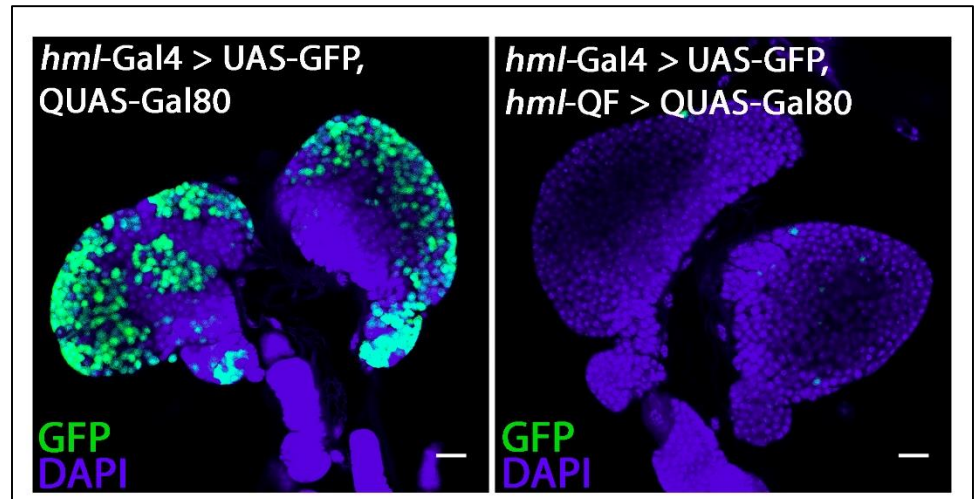

Supplementary Figure 3

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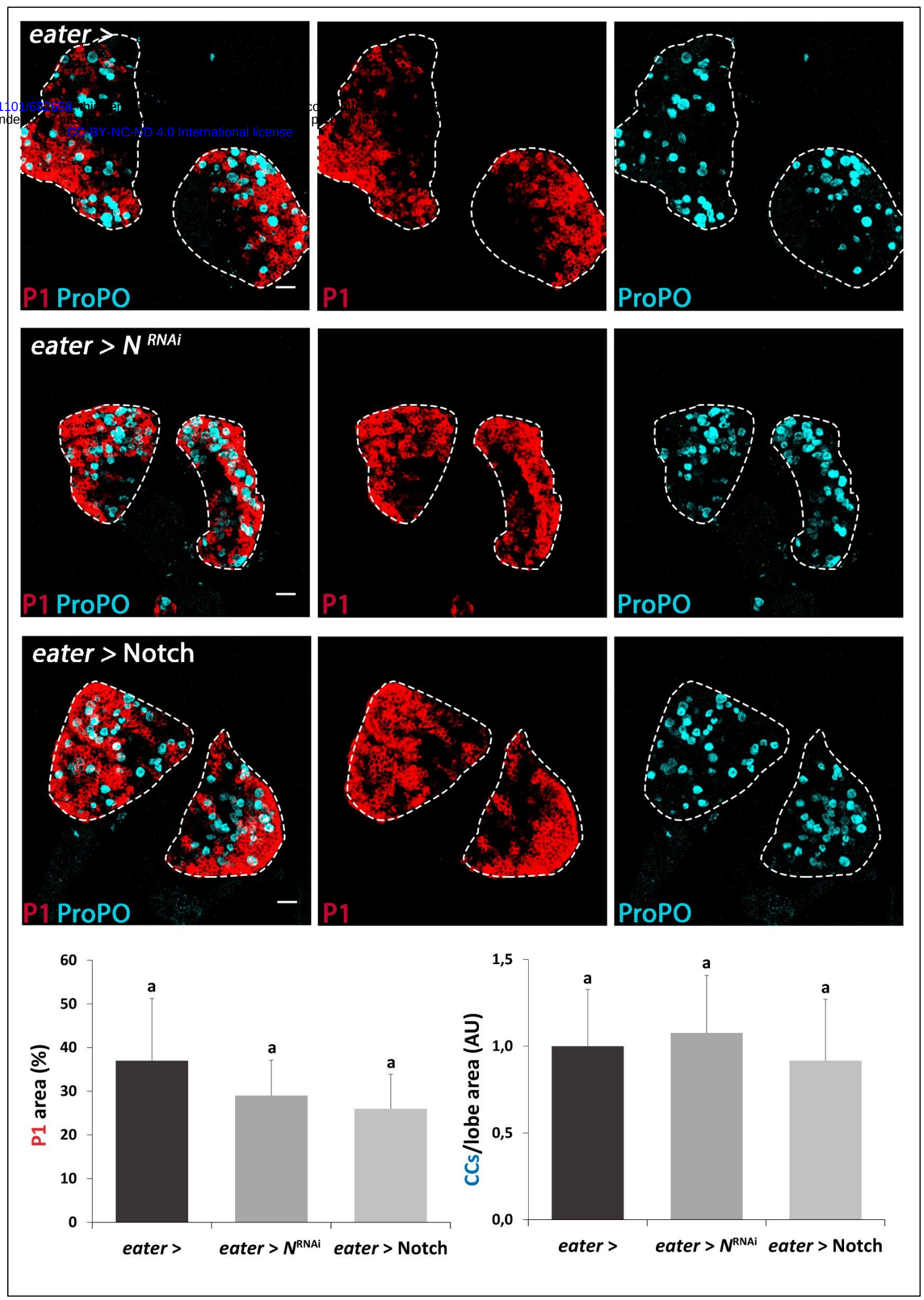

Supplementary Figure legends

Figure S1.

(a) *Notch* silencing in Core Progenitors does not affect the size of the Posterior Signaling Center. *TepIV*-Gal4 driven expression of a *notch* RNAi (N^{RNAi}), does not affect the number of Posterior Signaling Center cells, as shown by an anti-Antennapedia (Antp) staining (red). *TepIV* > GFP expression (green) labels Core Progenitors. Left panel: Control lymph gland without RNAi expression; central panel: Lymph gland that expresses N^{RNAi} driven by *tepIV*-Gal4. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm. Right panel: Quantification of the number of Posterior Signaling Center cells per lobe (ns: Non-significant). Error bars represent SD. *tepIV* >, n = 8; *tepIV* > N^{RNAi}, n = 8.

(b) Notch over-expression in Core Progenitors. Anti-Notch (N) staining (red) was utilized to demonstrate an increase of Notch levels in Core Progenitors following *tepIV*-Gal4 driven over-expression of full-length Notch. Core progenitors are visualized in green (*tepIV* > GFP). Upper panel: Control lymph glands; lower panel: lymph glands that over-express Notch. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm.

(c-d) Over-expression of Notch (d) or Supressor of Hairless (e) in Core Progenitors has no effect on cell differentiation. Core Progenitors are visualized in green (*tepIV* > GFP); Plasmatocytes in red (P1 staining); and Crystal Cells in cyan (ProPO staining). Upper panels: Control lymph glands; middle panels: Lymph glands with *tepIV*-Gal4 driven over-expression of full-length Notch (d) or Su(H) (e). Whole Z-projection confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the results (ns: Non-significant). Error bars represent SD. *tepIV* >, n = 8; *tepIV* > Notch, n = 8; *tepIV* > Su(H), n = 8.

(e) Notch is not required for Core Progenitor maintenance at L1 larval stage. *Notch* (*N*) silencing was induced in Core Progenitors from mid L2 stage onwards using the *tub*-Gal80^{ts}, *tepIV*-Gal4 genetic combination ($tepIV^{TS}$), leading to Core Progenitor loss (green: *tepIV* > GFP), and enhanced differentiation of Plasmatocytes (red: P1 staining) and Crystal Cells (cyan: ProPO staining). Top: temperature protocol utilized in the experiment. Upper panels: control lymph glands without RNAi expression; middle panels: Lymph glands expressing N^{RNAi}. Whole Z-projection confocal images of wandering third instar larvae are shown. Scale bars, 20 µm. Lower panels: Quantification of the results (**p < 0.01). Error bars represent SD. $tepIV^{TS}$>, n = 9; $tepIV^{TS}$>N^{RNAi}, n = 10.

Figure S2.

(a) *Notch* silencing with the *domeMESO*-Gal4 driver, utilizing an alternative *notch* RNAi construct ($N^{RNAi(2)}$), also provoked increase of Plasmatocytes and reduction of Crystal Cells. Plasmatocytes are visualized in red (P1 staining) and Crystal Cells in cyan (ProPO staining). Upper panels: Control lymph glands; middle panels: Lymph glands with expression of a $N^{RNAi(2)}$ construct with a different target sequence than the N^{RNAi} used in Fig. 4a. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the results (*p < 0.05, ***p < 0.001). Error bars represent SD. *dome* >, n = 8; *dome* > $N^{RNAi(2)}$, n = 5.

(b) Notch over-expression in *domeMESO*-positive progenitors. Anti-Notch (N) staining (red) was utilized to demonstrate an increase of Notch levels in progenitors (visualized in green; *domeMESO* > GFP) following *domeMESO*-Gal4 driven over-expression of full-length Notch.

Upper panel: Control lymph glands, lower panel: lymph glands that over-express Notch. Single-plane confocal images of primary lobes from wandering third instar larvae are shown. Scale bars, 20 µm.

(c) *Notch* silencing in *domeMESO*-positive progenitors which do not express *hemolectin* (*hml*) recapitulates increase of Plasmatocytes and reduction of Crystal Cells. *Notch* RNAi (N^{RNAi}) was expressed with the *domeMESO*-Gal4 driver, together or not with the Gal4 inhibitor Gal80 in *hml*-positive cells (achieved through *hml*-QF driven expression of QUAS-Gal80). Plasmatocytes are visualized in red (P1 staining) and Crystal Cells in cyan (ProPO staining). Upper panels: control lymph glands not expressing N^{RNAi}; 2nd row panels: lymph glands expressing N^{RNAi}, without Gal80 expression; 3rd row panels: lymph glands expressing N^{RNAi}, with Gal80 expression in *hml*-positive cells. Whole Z-projection confocal images of primary lobes from wandering third instar larvae are shown. Scale bar, 20 µm. Lower panels: Quantification of the indicated markers. The letters indicate statistical difference (Tukey´s multiple comparison test). Error bars represent SD. *Dome*-G4 >, *hml*-QF >, n = 10; *dome*-G4 > N^{RNAi} , *hml*-QF >, n = 10; *dome*-G4 > N^{RNAi} , *hml*-QF > Gal80, n = 14.

(d) *Hml*-QF driven expression of QUAS-Gal80 is effective in repressing Gal4 activity at the Cortical Zone. Gal80 is effective in inhibiting Gal4 activity, as assessed by a virtual block of *hml*-Gal4 driven UAS-GFP expression (green) when Gal80 is expressed in the same cells using *hml*-QF > QUAS-Gal80 (right panel). Left panel: control lymph glands without expression of Gal80. DAPI staining shows nuclei. Single-plane confocal images of wandering third instar primary lymph gland lobes are shown. Scale bars, 20 µm.

Figure S3.

(a) Notch does not affect Plasmatocyte or Crystal Cell differentiation after *eater* expression is initiated. Pictures show expression of the Plasmatocyte marker P1 (red) and the Crystal Cell marker ProPO (cyan) in control lymph glands (upper panels) and in lymph glands with *eater*-Gal4 driven expression of *notch* RNAi (2nd row panels) or full-length Notch (3rd row panels). Whole Z-projection confocal images of primary lobes from wandering third instar larvae are depicted. Scale bar, 20 µm. Lower panels: Quantification of the indicated markers. Same letter indicates no statistical difference between means (Tukey´s multiple comparison test). *Eater* >, n = 8; *eater* > N^{RNAi}, n = 10; *eater* > Notch, n = 10.