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Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in *Drosophila*

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SUMMARY

The interconnected Insulin/IGF signaling (IIS) and Target of Rapamycin (TOR) signaling pathways constitute the main branches of the nutrient-sensing system that couples growth to nutritional conditions in *Drosophila*. Here, we addressed the influence of these pathways and of diet restriction on the balance between the maintenance of multipotent hematopoietic progenitors and their differentiation in the *Drosophila* lymph gland. In this larval hematopoietic organ, a pool of stem-like progenitor blood cells (prohemocytes) is kept undifferentiated in response to signaling from a specialized group of cells forming the posterior signaling center (PSC), which serves as a stem cell niche. We show that, reminiscent of the situation in human, loss of the negative regulator of IIS *Pten* results in lymph gland hyperplasia, aberrant blood cell differentiation and hematopoietic progenitor exhaustion. Using site-directed loss- and gain-of-function analysis, we demonstrate that components of the IIS/TOR pathways control lymph gland homeostasis at two levels. First, they cell-autonomously regulate the size and activity of the hematopoietic niche. Second, they are required within the prohemocytes to control their growth and maintenance. Moreover, we show that diet restriction or genetic alteration mimicking amino acid deprivation triggers progenitor cell differentiation. Hence, our study highlights the role of the IIS/TOR pathways in orchestrating hematopoietic progenitor fate and links blood cell fate to nutritional status.

KEY WORDS: *Drosophila*, Insulin, TOR, Hematopoiesis, Nutrition

INTRODUCTION

Thanks to the phylogenetic conservation of the genetic pathways and developmental strategies at stake during blood cell development, *Drosophila* has emerged as a valuable model organism to study the fundamental mechanisms underlying hematopoiesis (Hartenstein, 2006). Notably, the *Drosophila* larval hematopoietic organ, the lymph gland, provides a paradigm to reveal the relationships between the hematopoietic niche, the blood cell progenitors and their differentiated progenies (for a review, see Krzemien et al., 2010a). The lymph gland produces three mature blood cell types functionally related to mammalian myeloid cells: the plasmatocytes, the crystal cells and, upon some immune challenges, the lamellocytes (Lanot et al., 2001; Meister and Lagueux, 2003). During the two first instar larval stages, the lymph gland grows and is primarily composed of stem cell-like progenitor blood cells called prohemocytes (Crozatier et al., 2004; Jung et al., 2005; Krzemien et al., 2010b; Minakhina and Steward, 2010). In third instar larvae, the mature lymph gland consists of a pair of primary lobes and several smaller secondary lobes (Fig. 1A). The primary lobes are organized in three zones: the cortical zone (CZ), the medullary zone (MZ) and the posterior signaling center (PSC) (Jung et al., 2005). The MZ as well as the secondary lobes comprise quiescent prohemocytes whereas the CZ contains differentiated hemocytes that emerge from the MZ. Reminiscent of the mammalian hematopoietic system, the balance between stem cell-like and differentiated blood cells is controlled by the PSC, which acts as a niche and secretes diffusible molecules promoting prohemocyte maintenance (Krzemien et al., 2007; Mandal et al., 2007; Sinenko et al., 2009). However, only a handful of signaling pathways controlling niche development or hematopoietic progenitor maintenance are known.

An important challenge for every metazoan is to adjust its development to food availability, and growing evidence indicates that nutrition can influence stem/progenitor cell fate (Amcheslavsky et al., 2009; Chell and Brand, 2010; McLeod et al., 2010; Sousa-Nunes et al., 2011). The interconnected Insulin/IGF signaling (IIS) and Target of Rapamycin (TOR) pathways (Fig. 1B) are the integrators between tissue growth and dietary conditions (Tennessen and Thummel, 2011). Here, we show that these pathways play a crucial role during hematopoiesis in the *Drosophila* lymph gland. First, they regulate the size and activity of the hematopoietic niche. Second, they cell-autonomously control blood cell progenitor maintenance. Finally, they couple blood cell development to the larval nutritional status.

MATERIALS AND METHODS

Drosophila strains and larvae collections

The following fly stocks were used: *Pcol85-Gal4, UAS::mCD8-GFP* (Krzemien et al., 2007) referred here as *col-Gal4*, *ppl-Gal4/CyOGal80; slif^{Anti}*, *ppl-Gal4/CyO* (Colombani et al., 2003), *PTEN^{100}/CyO Act-GFP, PTEN^{117}/CyO Act-GFP* (Oldham et al., 2002), *UAS::TSC1*, *UAS::TSC2* (Tapon et al., 2001), *UAS::PTEN* (Huang et al., 1999), *UAS::PI3K^{caax}*/Cyo (Leevers et al., 1996), *UAS::dFoxo* (Puig et al., 2003), *tepIV-Gal4* (Avet-Rochex et al., 2010), *Cg25C-GFP* and *Bc-GFP* (Sorrentino et al., 2007), *Hh-GFP* (Tokusumi et al., 2011). The RNAi stocks were obtained from Vienna *Drosophila* RNAi Center (VDRC).

Embryos were collected for 6 hours and equivalent numbers of hatching first instar larvae were transferred to vials with regular food medium at 25°C until wandering third instar larval stage. Starvation experiments were performed as established by Ikeya et al. (Ikeya et al., 2002). Larvae from crosses of *UAS* or *Gal4* lines to *w^{1118}* were used as controls.

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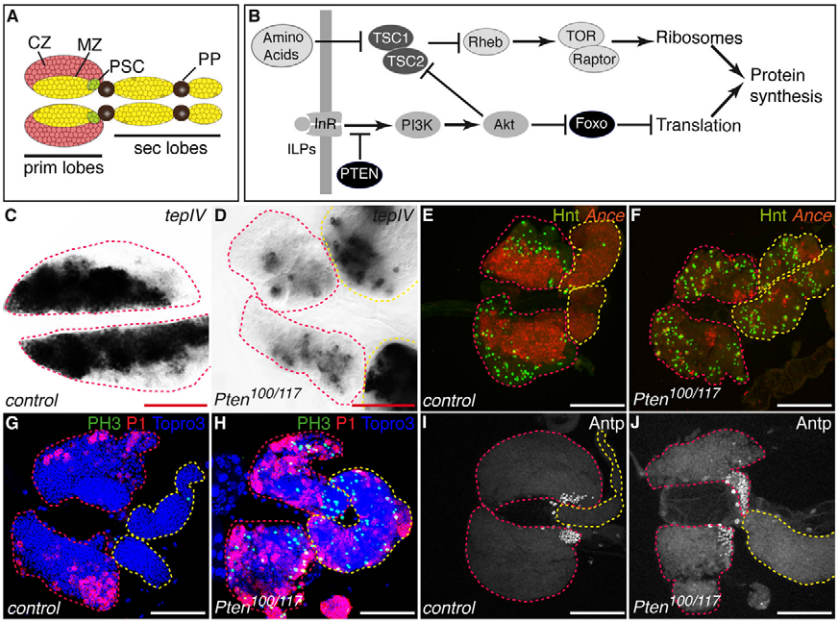

Fig. 1. IIS signaling controls lymph gland hematopoiesis. (**A**) Schematic of the *Drosophila* third instar lymph gland. The hematopoietic niche (green), blood cell progenitors (yellow) and mature blood cells (red) are depicted. Primary (prim) and secondary (sec) lobes are separated by pericardiac cells (black, PP). (**B**) Schematic of the IIS/TOR pathways. (**C-J**) Third instar *Drosophila* lymph glands. (C,D) Expression of the prohemocyte marker *TepIV*. (E,F) Expression of the crystal cell marker Hnt (green) and of the prohemocyte marker *Ance* (red). (G,H) Immunostaining against phosphorylated Histone H3 (PH3, green) and the plasmatocyte marker P1 (red). Nuclei are labeled with Topro3 (blue). (I,J) Expression of the PSC marker Antp. The PSC is composed of 42 (±14) or 55 (±11) cells in control or *Pten*$^{100/117}$ lymph glands, respectively. Dashed red and yellow lines outline primary and secondary lobes, respectively. Scale bars: 100 μm.

Immunohistochemistry, in situ hybridization and quantification

Lymph glands were processed and stained as described (Avet-Rochex et al., 2010). The following primary antibodies were used: anti-Antp, anti-Hnt (Developmental Studies Hybridoma Bank), anti-Col (Crozatier et al., 2004), anti-P1/NimrodC1 (Vilmos et al., 2004), anti-H3P (Upstate), anti-β-Gal (Cappel), anti-GFP (Torrey Pines). For in situ hybridization, DIG-labeled antisense RNA probes against *TepIV*, *Ance* and *α-PS4* were used. Samples were visualized with a laser scanning confocal microscope (Leica). Confocal images are displayed as maximum intensity projections of the lymph glands.

For quantification, lymph glands were scanned at an optimized number of slices using a Leica SP5 microscope. Crystal cell and plasmatocyte differentiation indexes were defined as the number of Hnt^{+} cells (quantified using Volocity software) or the level of Cg25C-GFP expression (measured with Image J software), respectively, in each primary lobe reported to the estimated lobe's volume. PSC cell number and MZ or lobe size were quantified using Image J software. A minimum of 12 lobes was scored per genotype.

RESULTS AND DISCUSSION

IIS controls lymph gland homeostasis

To test whether the IIS pathway controls lymph gland homeostasis, we used a combination of *Pten* mutant alleles for which third instar larvae can be obtained (Oldham et al., 2002). *Pten* codes for a protein phosphatase that antagonizes IIS-induced activation of the Phosphoinositol-3-Kinase (PI3K) (Fig. 1B). Hence, *Pten* larvae exhibit increased IIS activity. In contrast to wild-type lymph glands, which exhibited strong *TepIV* and *Ance* expression in the MZ of their primary lobes and in their secondary lobes, *Pten* lymph glands displayed markedly reduced expression of these two prohemocyte markers (Fig. 1C-F; supplementary material Fig. S1). Conversely, immunostaining against the crystal cell marker Hindsight (Hnt; Peb – FlyBase) (supplementary material Fig. S1) or the plasmatocyte marker P1, showed that whereas differentiated hemocytes are normally restricted to the CZ, they filled *Pten* lymph glands primary lobes and were also present in the secondary lobes (Fig. 1E-H), which is a characteristic sign of precocious differentiation (Owusu-Ansah and Banerjee, 2009). In addition, in situ hybridization against *α-PS4* revealed the presence of lamellocytes, which are seldom observed in the normal situation (supplementary material Fig. S2). Furthermore, *Pten* lymph glands frequently exhibited premature primary lobe dispersal and displayed overgrown primary and secondary lobes (supplementary material Fig. S5). Consistent with this observation, anti-phospho-H3 labeling showed that *Pten* lymph glands contained numerous proliferating cells (Fig. 1G,H). Thus, as in mammals, in which *Pten* loss induces hematopoietic stem cell exhaustion and myeloproliferative disease (Yilmaz et al., 2006; Zhang et al., 2006), *Pten* is required to prevent the aberrant differentiation of the blood cell progenitors into the three mature blood cell types found in *Drosophila*.

To ensure that the massive differentiation observed in *Pten* mutants was not due to a defect in PSC cell specification, we monitored the expression of the two key regulators of PSC fate: Antennapedia (Antp) and Collier (Col; Kn – FlyBase) (Krzemien et al., 2007; Mandal et al., 2007). Although immunostaining against these two proteins demonstrated that PSC cells were correctly specified, we found that the size of the niche was strongly increased (Fig. 1I,J; data not shown), suggesting that IIS normally controls PSC cell number. As the presence of a larger niche was nonetheless associated with increased prohemocyte differentiation, we surmised that these contradictory phenotypes might arise from a dual activity of the IIS in the PSC versus the MZ. We thus assessed its role specifically in these two domains.

IIS and TOR pathways cell-autonomously control the size of the hematopoietic niche

To test whether the IIS pathway is cell-autonomously required in the PSC, we used the *col-Gal4* driver (Krzemien et al., 2007), expression of which is strictly confined to the PSC during lymph gland ontogeny, as demonstrated by a lineage tracing experiment (supplementary material Fig. S3). In addition, we took advantage of *col-Gal4*-driven expression in the wing disc to confirm the specificity of the *UAS* transgenes used in this study (supplementary material Fig. S4). As observed in *Pten* larvae, over-activation of IIS in the PSC, induced by expressing either *Pten* RNAi or an active form of PI3K (PI3K^{caax}) (Leevers et al., 1996), led to a strong increase in PSC size (Fig. 2B,C). This phenotype correlated with a rise in PSC cell number (Fig. 2G). Conversely, knocking down *InR* by RNAi or overexpressing *Pten* caused a reduction in PSC cell

number (Fig. 2D,G). As IIS impinges on TOR activity (Fig. 1B), we tested whether this pathway also regulates PSC development. PSC cell number diminished when the TOR pathway was inactivated either by overexpressing both *TSC1* and *TSC2* (*gig* – FlyBase) or by downregulating *raptor* by RNAi (Fig. 2E,G; data not shown). Of note, *TSC1/TSC2* overexpression seemed to reduce PSC cell size (Fig. 2E′). Conversely, *TSC1* RNAi expression, which resulted in a larger PSC (Fig. 2F), did not significantly affect cell number but increased cell size (Fig. 2G,H). This suggests that TOR signaling not only supports PSC cell proliferation but also their growth. Finally we observed a strong drop in PSC cell number when we overexpressed Foxo (Fig. 2G), which is the main effector of IIS and whose targets are concomitantly regulated by the TOR kinase (Edgar, 2006). Together, these data indicate that IIS and TOR pathways are required in the PSC to promote niche cell proliferation/maintenance and growth.

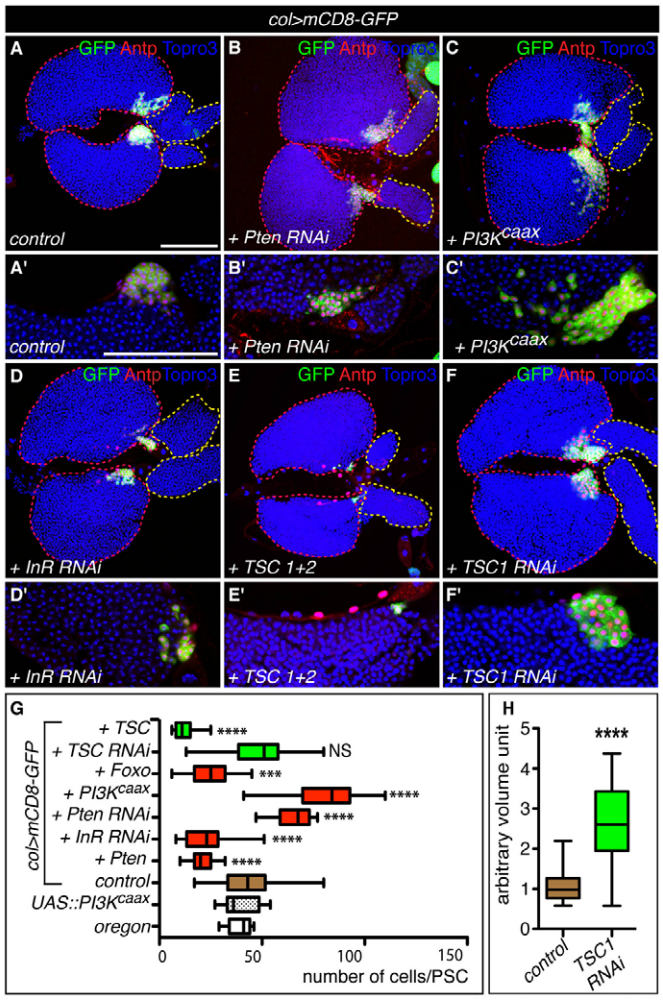

Fig. 2. IIS and TOR signaling cell-autonomously control the size of the PSC. (**A-F′**) Antp immunostaining (red) on lymph glands expressing the indicated transgene and a membrane-linked GFP (mCD8-GFP, green) under the control of *col-Gal4*. A′-F′ show high magnification views of the PSC region. Nuclei are stained with Topro3 (blue). Dashed red and yellow lines outline primary and secondary lobes, respectively. Scale bars: 100 μm. (**G**,**H**) Quantification of the number of cells per PSC (G) or the volume of the PSC (H) in control larvae and in larvae expressing the indicated transgenes in the PSC. Data are presented as box-and-whisker plots. Student's *t*-test: ***P<0.0005; ****P<0.0001; NS, not significant.

Control of hematopoietic niche size by IIS and TOR signaling affects blood cell differentiation

Previous work showed that the PSC controls prohemocyte maintenance (Krzemien et al., 2007; Mandal et al., 2007). Notably, in contrast to what we observed in *Pten* larvae, increased PSC size has been associated with decreased differentiation (Mandal et al., 2007; Sinenko et al., 2009; Tokusumi et al., 2011). We thus assessed the effect of increased IIS/TOR signaling in the PSC on hemocyte differentiation. Although *PI3K^{caax}* overexpression or *TSC1* RNAi increased PSC size by more than twofold, P1 and Hnt immunostaining did not reveal an obvious reduction in either plasmatocyte or crystal cell differentiation (Fig. 3A-F). To ensure that we did not overlook a subtle phenotype, we measured crystal cell or plasmatocyte differentiation index on a minimum of 20 samples (see Materials and methods). We found that over-activation of the IIS or TOR pathway in the PSC significantly inhibited the differentiation of both type of hemocytes (Fig. 3G,H). Moreover, expression of an *Hh-GFP* reporter line (Tokusumi et al., 2011) was observed in all the PSC cells expressing either *PI3K^{caax}* or *TSC1* RNAi, suggesting that increased PSC size is associated with increased production of this inhibitor of prohemocyte differentiation

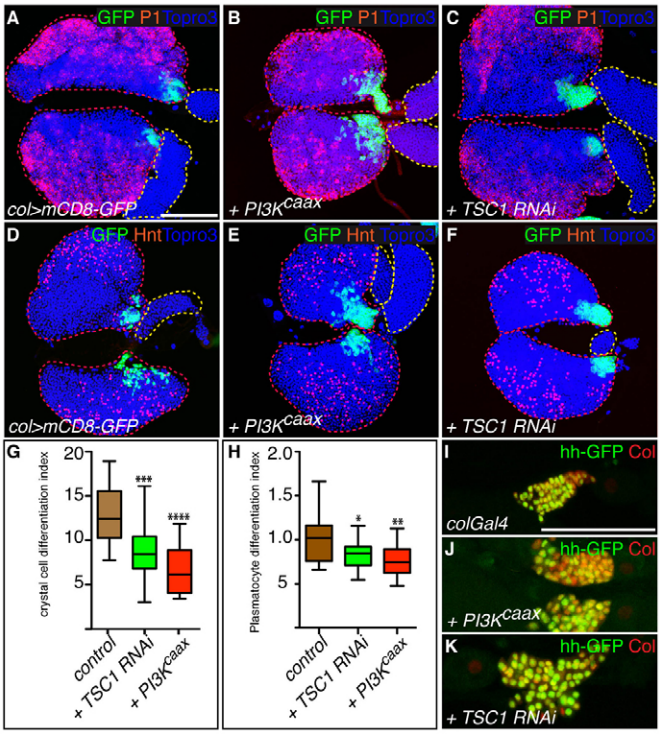

Fig. 3. Increasing IIS or TOR signaling in the PSC impairs blood cell differentiation. (**A-F**) Immunostaining against the plasmatocyte marker P1 (A-C) or the crystal cell marker Hnt (D-F) on lymph glands of third instar larvae expressing mCD8-GFP and the indicated transgene in the PSC (*col-Gal4*). Nuclei are stained with Topro3 (blue). Dashed red and yellow lines outline primary and secondary lobes, respectively. Scale bar: 100 μm. (**G**,**H**) Quantification of the crystal cell differentiation index (number of crystal cells per primary lobe/lobe volume) (G) and plasmatocyte differentiation index (Cg25C-GFP expression level per primary lobe/lobe volume) (H) in control larvae and in larvae expressing the indicated transgenes in the PSC. Arbitrary units. Student's *t*-test: *P<0.05; **P<0.005; ***P<0.0005. (**I-K**) High magnification views of the PSC region showing Hh-GFP and Col expression.

(Mandal et al., 2007). Together, our results indicate that increased IIS/TOR signaling not only supports *Drosophila* hematopoietic niche growth but also promotes its activity.

IIS and TOR signaling cell-autonomously controls blood cell progenitor maintenance

Next, we modulated the activity of the IIS/TOR pathways specifically in the prohemocytes using the *TepIV-Gal4* driver (Avet-Rochex et al., 2010). Reminiscent of the phenotypes observed in *Pten* larvae, over-activation of IIS by PI3K^{caax} expression induced a massive differentiation of plasmatocytes (labeled with either Cg25C-GFP or P1) and crystal cells (labeled with either Hnt or Bc-GFP) in the primary and secondary lobes, paralleled by a strong reduction in the pool of prohemocytes (labeled with either *Ance* or a UAS-mCherry) (Fig. 4B,H; supplementary material Fig. S6B,G), and an overgrowth of the secondary lobes (supplementary material Fig. S5). Unexpectedly, knocking down IIS by expressing *InR* RNAi also favored prohemocyte differentiation (Fig. 4C,H; supplementary material Fig. S6C,G). However, in contrast to IIS activation, IIS knockdown reduced lymph gland growth (supplementary material Fig. S5) and did not cause lamellocyte differentiation (supplementary material Fig. S2). Finally, lowering TOR activity using *raptor* RNAi promoted hemocyte differentiation and significantly reduced the proportion of prohemocytes present in the primary lobes (Fig. 4D,H; supplementary material Fig. S6D,G). Similar phenotypes were observed when IIS and TOR signaling were reduced in the MZ using the *dome-Gal4* driver (data not shown). Thus, a fine-tuning of IIS/TOR pathways in the blood cell progenitors seems to be crucial to prevent their differentiation and to control lymph gland growth. Furthermore, as increased PSC size in *Pten* zygotic mutants does not prevent the massive differentiation of the prohemocytes, we propose that over-activation of the IIS/TOR pathways in the MZ bypasses the progenitor-promoting activity of the PSC.

Starvation causes prohemocyte differentiation

One main function of the IIS pathway is to link tissue growth to nutrient sensing. Indeed, food shortage induces a reduction of insulin-like peptide (ILP) secretion by the insulin-producing cells (IPC) located in the brain (Edgar, 2006). The consecutive reduction in IIS signaling diminishes larval growth. The fat body acts as a nutrient sensor that restricts global growth through a humoral mechanism: upon diet restriction, ILP production by the IPC is reduced in response to the inactivation of the TOR pathway in the fat body. Accordingly, depletion of the amino acid transporter Slimfast (Slif) in the fat body disrupts TOR signaling and is sufficient to mimic diet restriction (Colombani et al., 2003). As IIS/TOR signaling is active in the lymph gland and controls prohemocyte maintenance, we investigated whether the larval nutritional status alters lymph gland homeostasis. When the larvae were starved for 24 hours, we observed a strong increase in plasmatocyte differentiation as revealed by Cg25C-GFP expression, and a concomitant decrease in *Ance* expression, indicative of a switch from prohemocyte maintenance towards differentiation (Fig. 4F,J). Unexpectedly, only a few crystal cells were present in the lymph glands of starved third instar larvae and we observed that they burst (Fig. 4J; data not shown). However, the examination of starved second instar larvae revealed the precocious differentiation of crystal cells associated with a decreased in the MZ (Fig. 4J′). Hence, starvation induces precocious differentiation of prohemocytes into both crystal cells and plasmatocytes. Of note, it is possible that starvation also directly affects differentiated hemocytes, notably crystal cells. We then tested whether food shortage was sensed directly by the prohemocytes or mediated by a systemic humoral response. To do so, we mimicked amino-acid deprivation by inhibiting Slif expression either in the prohemocytes or in the fat body, where its downregulation causes a systemic reduction in ILP levels (Colombani et al., 2003). Whereas

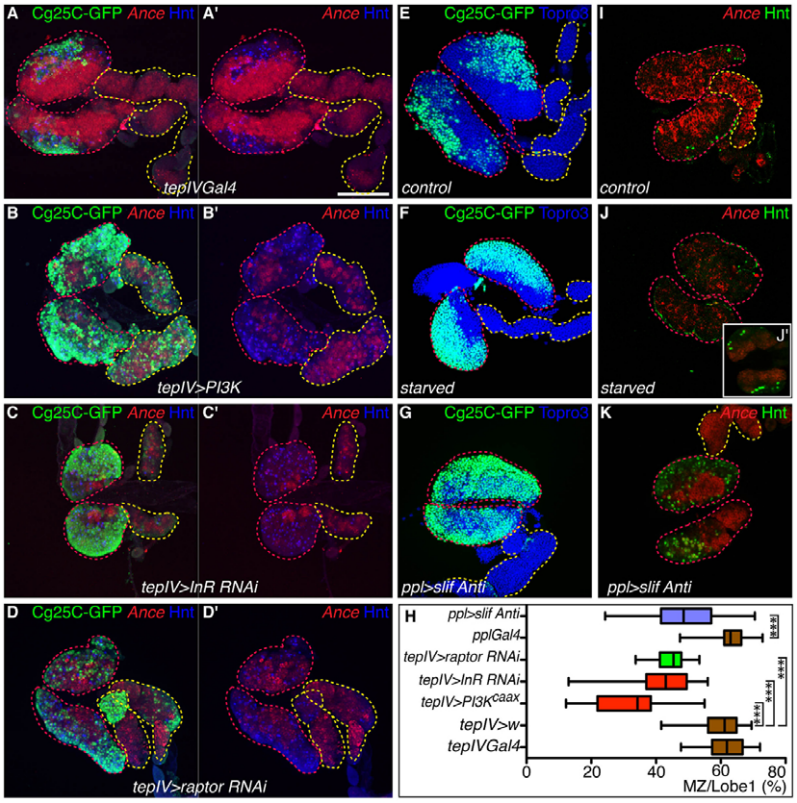

Fig. 4. IIS and TOR signaling functions cell-autonomously in the prohemocytes to sense systemic diet restriction. (**A-G**,**I-K**) Third instar larval lymph glands. Plasmatocytes, crystal cells and prohemocytes are labeled with Cg25C-GFP, Hnt and *Ance*, respectively. Scale bar: 100 μm. (A-D′) *TepIV-gal4* was used to drive the expression of the indicated transgene in the prohemocytes. (E-G,I-K) Diet restriction was induced either by starvation (F,J) or by depletion of *slif* activity in the fat body (*ppl>slif^{anti}*) (G,K). (J′) Effect of starvation on Hnt and *Ance* expression in second larval instar lymph gland. (E-G) Nuclei are stained with Topro3. Dashed red and yellow lines outline primary and secondary lobes, respectively. (**H**) Proportion of prohemocytes (MZ area/primary lobe area) in the primary lobes of control larvae and in larvae expressing the indicated transgenes. Box-and-whisker representation. Student's *t*-test: ***P<0.0005.

expression of the RNA antisense *slif*^{Anti} in the prohemocytes did not affect their maintenance (supplementary material Fig. S6E,G), its expression with the fat body-specific *pumpless-Gal4* (*ppl-Gal4*) driver caused massive plasmatocyte differentiation and decreased the prohemocyte pool (Fig. 4G,H,K; supplementary material Fig. S6F). Therefore, the systemic response to nutrient availability controls blood cell progenitor maintenance in the lymph gland.

Conclusions

Our results demonstrate that IIS/TOR signaling plays a dual role in the maintenance of the blood cell progenitors by acting both within the hematopoietic niche to control its size and its activity, and within the prohemocytes to control their fate. To gain a comprehensive view of IIS/TOR function in *Drosophila* hematopoiesis and in light of the recent report showing that differentiated hemocytes can feedback on prohemocyte maintenance (Mondal et al., 2011), it will be interesting to explore the role of these pathways in the differentiated blood cells. In addition, our data are consistent with a model whereby the IIS/TOR pathways link prohemocyte maintenance to the *Drosophila* larvae nutritional status. We speculate that food shortage, by sensitizing blood cell progenitors to differentiation, might affect the cellular immune response. Along this line, the rate of encapsulation of parasitoid wasp eggs, which relies primarily on the differentiation of lamellocytes, has been shown to diminish in larvae that were deprived of yeast before infestation (Vass and Nappi, 1998). We anticipate that future studies will allow further understanding of how developmental and environmental cues are integrated by IIS/TOR signaling to control blood cell homeostasis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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