Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors

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SUMMARY
The innate plasticity of hematopoietic progenitors is tightly regulated to supply blood cells during normal hematopoiesis and in response to stress or infection. We demonstrate that in the Drosophila lymph gland (LG) the tumor suppressors TSC and PTEN control blood progenitor proliferation through a common TOR- and 4EBP-dependent pathway. Tsc2 or Pten deficiency in progenitors increases TOR signaling and causes LG overgrowth by increasing the number of actively dividing cells that accumulate high levels of phosphorylated (p) 4EBP during a critical window of growth. These phenotypes are associated with increased reactive oxygen species (ROS) levels in the LG, and scavenging ROS in progenitors is sufficient to rescue overgrowth. Blood progenitor number is also sensitive to starvation and hypoxia in a TOR-dependent manner. Differences between Tsc1/2 and Pten function become apparent at later stages. Loss of Tsc1/2 autonomously increases p4EBP and decreases pAKT levels, expands the number of intermediate progenitors and limits terminal differentiation, except for a late induction of lamellocytes. By contrast, absence of PTEN increases p4EBP and pAKT levels and induces myeloproliferative expansion of plasmatocytes and crystal cells. This increased malignancy is associated with non-autonomous increases in p4EBP levels within peripheral differentiating hemocytes, culminating in their premature release into circulation and demonstrating potential non-autonomous effects of Pten dysfunction on malignancy. This study highlights mechanistic differences between TSC and PTEN on TOR function and demonstrates the multifaceted roles of a nutrient-sensing pathway in orchestrating proliferation and differentiation of myeloid-specific blood progenitors through regulation of ROS levels and the resulting myeloproliferative disorder when dysregulated.

KEY WORDS: Drosophila, Lymph gland, Myeloid, TSC, PTEN, TOR

INTRODUCTION
The innate plasticity of hematopoietic progenitors is tightly regulated to continuously replenish blood cells under steady-state conditions and to supply cells that are immediately available in response to stress. Similar to vertebrates, hematopoiesis in Drosophila requires a population of multipotent progenitor cells (Mandal et al., 2007). These progenitors give rise exclusively to myeloid-like lineages through mechanisms that are conserved at the cellular and molecular levels (Evans and Banerjee, 2000; Evans et al., 2003; Martinez-Agosto et al., 2007).

Mature myeloid cell types derived from Drosophila progenitors include: macrophage-like plasmatocytes (PLs); crystal cells (CCs); and a specialized cell type, the lamellocyte, which is not normally present in the larva but can be induced to differentiate in response to specific immune challenges (Lanot et al., 2001; Sorrentino et al., 2002; Owusu-Ansah and Banerjee, 2009; Krzemien et al., 2010). This plasticity of the LG to adapt to stress scenarios and regulate its growth is reminiscent of the Target of rapamycin (TOR) signaling network. TOR is a conserved serine/threonine kinase that forms part of a multiprotein complex (TORC1) that coordinates cell growth in response to environmental signals such as energy status, nutrient availability and cellular stressors (Yang and Guan, 2007; Wang and Proud, 2009; Russell et al., 2011). Its growth-promoting function is largely effected by regulating protein translation downstream of two major effectors: S6 kinase (S6K) and the translational initiation factor 4E-binding protein (4E-BP; Fig. 1B) (Fingar et al., 2002; Holz et al., 2005; Ma and Blenis, 2009).

Regulation of TORC1 signaling through upstream mediators occurs largely at the level of the pathway inhibitor Tuberous sclerosis complex 1 and 2 (TSC1/2; Fig. 1B). TSC1/2 blocks TORC1 activation by inhibiting RHEB, the direct activator of TORC1 (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Perhaps the best-characterized modulator of TORC1 signaling is AKT, a kinase that is activated downstream of growth factor signaling in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (Fig. 1B). Activated AKT phosphorylates TSC2 on multiple residues, which relieves RHEB inhibition and activates TORC1 (Inoki et al., 2002; Potter et al., 2002; Miron et al., 2003). PI3K-dependent activation of AKT is inhibited by the Phosphatase

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and tensin homolog (PTEN; Fig. 1B) as well as by a negative-feedback loop downstream of TORC1 signaling (Harrington et al., 2004; Kockel et al., 2010), adding additional layers of regulation to AKT-mediated TORC1 activation.

In this study, we demonstrate the common effects of Tsc2 and Pten loss on the regulation of early blood progenitor proliferation in a TORC1-, 4EBP- and ROS-dependent manner. We highlight differences in the expansion of intermediate progenitors and differentiated hemocytes that are unique to TSC and PTEN, as well as the roles of stress, feedback inhibition and AKT activity in TOR-dependent LG regulation.

**MATERIALS AND METHODS**

**Drosophila stocks**

All stocks were obtained from stock centers (VDRC, NIG, Bloomington) except: UAS-Tsc1/2 [A. Gould (Tapton et al., 2001)]; UAS-Pten and Pten

(A. Wodarz, Georg-August-Univ, Gottingen, Germany); Tsc2258 (T. Ip, University of Massachusetts, Medical Center, Worcester, MA, USA); UAS-GTPx1 (U. Banerjee, University of California, Los Angeles, CA, USA); and UAS-Akt254 (E. Hafen (Stocker et al., 2002)); w1118 was used for controls. Gal4 lines used: dom-gal4, UAS-2xEGFP, and dome-gal4; P[ubP-gal80]120 (U. Banerjee). dome-gal4 crosses were performed at 29°C, except dome=>Tsc2+/2, dome=>Pten+ and dome>=TorDN crosses which were grown at 18°C.

**Larval staging experiments**

Staging of dome> Tse2RNAI and dome>PtenRNAi was performed at 29°C, and the following time points after egg hatching (AEH) were used for larval stages: 30 hours AEH (early second instar, el2); 36 hours AEH (late second instar, lL2); 42 and 48 hours AEH (early third instar, el3); 54 hours AEH (mid third instar, ml3); 65 hours AEH (late third instar, lL3); and 72 hours AEH (wandering third instar, wL3).

**Clonal analysis**

FLP-out clones in the LG were generated using HHLT (hand-gal4, hml-gal4, UAS-2xEGFP, UAS-FLP, ASC-FRT-STOP-FRT-gal4) as described (Evans et al., 2009). For lineage tracing of circulating hemocytes, H-TRACE (hand-gal4, UAS-2xEGFP, UAS-FLP, ASC-FRT-STOP-FRT-gal4) was used. Genotypes for MARCM FRT clones are as follows: hs-flp FRT40A Pten 2L117 (E. Hafen (Stocker et al., 2002)); w1118 was used for controls.

**Immunohistochemistry, BrdU labeling and ROS detection**

Immunohistochemistry was performed as previously described (Jung et al., 2005). Antibodies used: mouse anti-PNX (1:400; L. Fessler, University of California, Los Angeles, CA, USA); rabbit anti-PPO (1:400; M. Kanost, Kansas State University, Manhattan, KS, USA); mouse anti-P1 and anti-L1 (both 1:20); I. Ando, Hungarian Academy of Sciences, Szeged, Hungary); rat anti-SHG (1:400; V. Hartenstein, University of California, Los Angeles, CA, USA); rabbit anti-p4EBP and anti-pAKT (1:300 and 1:40, respectively; Cell Signalling); mouse anti-LZ and anti-TSC2 (both 1:100; DSHB); rabbit anti-RHEB (1:200; Thermo Scientific); rabbit anti-Pten (1:400; A. Wodarz); mouse anti-Histone (pan) (1:100; Millipore); and anti-p-pH3 (1:300; Millipore and AbCam). BrdU labeling was performed as previously described with a 45-minute BrdU pulse (Mondal et al., 2011). ROS detection was performed as previously described (Owusu-Ansah and Banerjee, 2009) using dihydroethidium.

**Rapamycin experiments**

For early treatment, crosses were set up on 15-20 μM rapamycin food plates, and larvae were grown at 25°C until wL3. For later treatment, larvae were reared on normal food until el3 and transferred to rapamycin food plates until wL3.

**Circulating hemocyte analysis**

Circulating hemocytes from a single larva were isolated in 20 μl PBS and evenly dispersed on a 14 multi-well glass slide (Thermo Scientific, incubated for 30 minutes, and then fixed and immunostained as above. Relative quantification of circulating hemocytes is reported as the number of hemocytes per 0.25 mm² section of the glass well.

**Quantitative real-time PCR (Q-PCR)**

Isolation of total RNA and reverse transcription were performed according to standard methods. Q-PCR was performed using an ABI 7900HT Q-PCR machine and SYBR Green (Qiagen). Relative quantification of transcript levels was calculated using the comparative Ct method and normalized against rp49 (RpL32 – FlyBase). Tsc2 and rp49 primers were used as described (Amcheslavsky et al., 2011). Pten primers were: 5′-GCCAGAAAAATGCAAGCCTCA-3′; reverse, 5′-GCCGGAACATTGGTATGATTGGT-3′.

**Distribution of hemocyte and progenitor cell populations**

Methodology to determine ratios of dome+/Pxn- prohemocyte, dome+/Pxn+ intermediate progenitor and dome+/Pxn+ differentiated hemocyte populations was adapted from Shim et al. (Shim et al., 2012). Two-way ANOVA analysis was used to evaluate significance between hemocyte population distributions among genotypes.

**Mitotic index and CC ratios**

Mitotic index and ratio of CCs in the LG were measured as the total number of pH3+ or LZ+/PPO+ cells divided by the total number of cells. The method for estimating total cell numbers in the LG was adapted from Krzemien et al. (Krzemien et al., 2010). The distribution of mitoses at ml3 was assessed by quantifying the number of pH3+ cells in each population for all confocal sections as a ratio to total pH3+ hemocytes. Statistical significance was evaluated with Student’s t-test (mitotic index and CC ratios) and two-way ANOVA analysis (distribution of mitoses).

**Starvation and hypoxic stress**

Acute starvation was induced by transferring (fed) eL3 larvae (48 hours AEH at 29°C) to agar plates containing PBS/1% sucrose for 24 hours; larvae were dissected at 72 hours AEH. For hypoxia experiments, larvae were reared in normoxia until IL2 (36 hours AEH at 29°C) and then transferred to a 5% O2 hypoxia chamber until wL3.

**RESULTS**

**TSC and PTEN regulate TORC1 signaling in Drosophila blood progenitors**

A search for cell type-specific markers in the LG identified higher expression of RHEB and TSC2 in MZ progenitors (prohemocytes) than in CZ hemocytes at lL3 (Fig. 1C–D′). Similar expression was observed for the upstream regulator of TORC1 activity, PTEN (Fig. 1E, F′), as well as for phosphorylated (p) AKT (AKT1 – FlyBase) (Fig. 1F, F′), indicating that active PI3K-AKT signaling occurs in prohemocytes. Expression of p4EBP (THOR – FlyBase), a marker of active TOR kinase activity (Miron et al., 2003), was also higher in MZ progenitors (prohemocytes) than in CZ hemocytes at wL3 (Fig. 1C–D′). We confirmed the central role for TSC in regulating TORC1 signaling during normal Drosophila hematopoiesis by examining p4EBP expression upon Tsc1/2 loss-of-function (LOF). Tsc1 and Tsc2 (gigas – FlyBase) homozygous mutants die at early larval stages (Ito and Rubin, 1999). Interestingly, heterozygosity for mutations in Tsc1 or Tsc2 specifically increases p4EBP levels in dome+ progenitors at wL3 (supplementary material Fig. S1A–C), whereas heterozygosity for a Pten mutation increases p4EBP throughout the LG (supplementary material Fig. S1D, D′). A similar effect occurs upon progenitor-specific downregulation of Tsc2 or Pten in the LG (supplementary material Fig. S1E–F′).

We next examined the phenotypic consequence of TORC1 hyperactivation, induced by downregulation of Tsc1/2 or Pten function, on hemocyte progenitors. Growth of the LG occurs via extensive cell proliferation throughout larval development, from an initial group of ~30 progenitors per lobe in the late embryo to a
mature LG consisting of ~5000 hemocytes (Krzemien et al., 2010). Onset of differentiation occurs during the second larval instar stage (L2), invariably at the periphery of the tissue (Fig. 1H). Early differentiating hemocytes express both the prohemocyte marker dome and the early differentiation marker Peroxidasin (PXN) at low levels (dome+/PXN−), and represent a population of intermediate progenitors. By contrast, fully differentiated hemocytes lose dome, upregulate PXN expression (dome+/PXN+), and proceed to express a marker of terminal differentiation: P1 (PLs), Prophenoloxidase (PPO; CCs) or L1 (lamellocytes). Tsc2 or Pten downregulation in prohemocytes increases the rate of tissue growth during L2, eL3 and mL3 instar stages (Fig. 1I,J). In Tsc2-deficient LGs, differentiation onset and progression remain intact (compare Fig. 1H with 1I), and, accordingly, the proportion of dome+/PXN+ prohemocytes by mL3 (64%) is the same as in wild type (WT; 65%) (supplementary material Fig. S1G). By contrast, Pten deficiency expands differentiating hemocytes from IL2 to mL3 (Fig. 1J and supplementary material Fig. S1G), suggesting a specific role for Pten in prohemocytes in regulating their transition to differentiation during development.

**TORC1 activity correlates with early proliferating progenitors**

The accelerated rate of tissue growth that we observe upon Tsc2 and Pten LOF suggested that they might regulate LG proliferation. We tested this hypothesis by comparing the populations of phospho-histone H3 (pH3+) cells in WT versus Tsc2- or Pten-deficient LGs, and observed an increased number of mitotically dividing cells upon Tsc2 or Pten disruption throughout all stages (compare Fig. 2A,D,G with 2B,E,H and 2C,F,I). The percentage of pH3+ cells in the developing LG (mitotic index) is highest during L2 and eL3 (Fig. 2J) (Krzemien et al., 2010). Importantly, we found that the mitotic index of Tsc2- or Pten-deficient LGs is significantly increased during this critical window of LG growth (Fig. 2J), accounting for the increased rate of tissue growth observed (Fig. 1H-J). Interestingly, high levels of cytoplasmic p4EBP (p4EBPhigh) are expressed in a small population of scattered cells (Fig. 2A,D,G) in WT LGs during L2 and eL3, the majority of which colocalize with pH3 and represent mitotically dividing hemocytes (Fig. 2A,D,G). Downregulation of either Tsc2 or Pten in progenitors increases the population of pH3+/p4EBPhigh cells at these early stages (Fig. 2B,E,H and 2C,F,I), suggesting that TORC1 activity promotes the early proliferation and expansion of progenitor pools. Expression of high p4EBP levels in pH3+ cells during early larval development is specific for M phase, as overlap of p4EBP with the S-phase marker BrdU is not observed in any background (supplementary material Fig. S1H-M).

By mL3 the mitotic index drops significantly in WT, but more dramatically in Tsc2- or Pten-deficient backgrounds (Fig. 2J), emphasizing their role during the phases of highest proliferation in early LG development. Moreover, a higher proportion of mitoses in Pten-deficient LGs at mL3 occur within PXN+ hemocytes as compared with WT or Tsc2-deficient LGs (supplementary material Fig. S1N), consistent with the increased differentiation observed at mL3 (Fig. 1J and supplementary material Fig. S1G). In addition, the population of p4EBPhigh cells at mL3 in Tsc2- and Pten-deficient LGs is much larger than that of pH3+ cells, although some colocalization still occurs (Fig. 2H,I).

**Dynamic p4EBP expression downstream of Pten and Tsc2 deficiencies at later third instar stages**

The significant increase in p4EBP levels in Tsc2- or Pten-deficient LGs by mL3 (Fig. 2H,I) occurs during a stage when increased differentiation in the LG expands the early CZ. In both Tsc2- and Pten-deficient backgrounds at mL3, a pronounced accumulation of p4EBP occurs in differentiating cells that express low levels of...
PXN along the early MZ/CZ boundary (Fig. 3A-F/H11630), marking a transition in high TORC1 activation from proliferating hemocytes to differentiating hemocytes at mL3. A subset of these p4EBP high cells express low levels of P1 (supplementary material Fig. S2A-C/H11630/H11032, arrows) but not the CC progenitor marker Lozenge + (LZ+; supplementary material Fig. S2D-F/H11630/H11032), demonstrating that high p4EBP expression is specific for differentiating dome+ or PXN+ PLs, and not for fully differentiated (dome–) PLs at the LG periphery or CC progenitors. Lamellocytes are not observed at this stage.

At lL3 (Fig. 3G-I/H11630), the p4EPB patterns become markedly distinct between Tsc2- and Pten-deficient LGs (compare Fig. 3H/H11033 with 3I/H11033). High p4EBP expression extends medially in Tsc2-deficient LGs, overlapping largely with dome+/PXN+ cells, and is absent in the LG periphery (Fig. 3H-H11630). By contrast, high p4EBP expression extends peripherally in differentiating hemocytes in Pten-deficient LGs, including within buds of tissue that pinch off from the CZ, and is expressed at lower levels in MZ prohemocytes (Fig. 3I-I/H11630). By wL3, p4EBPhigh cells are observed in dome+ hemocytes in both backgrounds, but continue to extend peripherally upon progenitor-specific Pten downregulation (supplementary material Fig. S1E-F). Collectively, these data demonstrate non-autonomous activation of TORC1 activity upon Pten, but not Tsc2, downregulation in progenitors at late stages.

We further confirmed our findings from Tsc1/2 and Pten deficiencies by performing mutant clonal analysis. Consistent with our findings from when Tsc2 is downregulated in progenitors, high levels of p4EBP are autonomously upregulated in Tsc1–/– clones (Fig. 4B-B/H11033). Although low levels of PXN are expressed in Tsc1–/– cells (Fig. 4B/H11630, gray), fully differentiated PXNhigh cells are found only at the very periphery of the LG (Fig. 4B/H11630, white), which are p4EBP negative. By contrast, accumulation of p4EBP in Pten–/– cells depends on clone location. Medially localized Pten–/– cells are p4EBPlow and undifferentiated, similar to Pten downregulation in prohemocytes (Fig. 4D-D/H11630, yellow arrows; compare with Fig. 3I-I/H11630). Scattered p4EBPhigh expression is observed within a subset of more peripheral Pten–/– cells, as well as non-autonomously within WT PXNhigh cells (Fig. 4D-D/H11630, white arrows). Interestingly, the localization of large Pten–/– clones in the most peripheral LG regions was more likely associated with autonomous p4EBP upregulation (Fig. 4E,E/H11032), differentiation, and ‘pinching off’ of LG tissue (Fig. 4F,F/H11032), suggesting that the absence of Pten function might selectively affect differentiating hemocyte progenitors as opposed to the undifferentiated prohemocyte population. Furthermore, these findings substantiate the differences observed upon progenitor-specific downregulation of Pten and Tsc.

Tsc2 and Pten deficiencies expand distinct cell populations at wandering third instar

In addition to their divergent p4EBP patterns at late stages, striking differences are observed between Tsc2- and Pten-deficient LGs in the expansion of distinct cell lineages by wL3. Whereas WT LGs...
have a small population of intermediate progenitors at the MZ/CZ boundary (5±2% of the LG; Fig. 5A-A
/H11033, P), as identified by co-expression of dome and PXN (Fig. 5A-A
/H11033, insets), Tsc2 downregulation in the MZ induces severe LG overgrowth and dramatically expands the proportion of dome+/PXN+ intermediate progenitors (51±10%; P<0.0001; Fig. 5E-E
/H11033, P). This proportion of dome+/PXN+ cells at wL3 is much higher than that observed at mL3 (supplementary material Fig. S1G), demonstrating a late accumulation of intermediate progenitors upon Tsc2 downregulation. By contrast, the population of fully differentiated hemocytes (dome–/PXN+) decreases relative to WT (23±4% versus 40±3%, respectively; P<0.0001; Fig. 5P). Terminally differentiated PLs and CCs are found only in a thin cortical layer around the LG.
periphery (Fig. 5F,G), and the ratio of CCs to total LG cells is significantly reduced compared with WT (Fig. 5Q). By contrast, lamellocytes, which are not normally present in a healthy larva (Fig. 5D), are dispersed throughout the LG (Fig. 5H). LG-specific clonal downregulation of $Tsc2$ also induces overgrowth (supplementary material Fig. S3D-F), expands the population of PXN+ hemocytes (supplementary material Fig. S3D/H11032, D/H11033) and induces lamellocytes (supplementary material Fig. S3F), demonstrating the LG-specific effects of $Tsc2$ deficiency.

By contrast, $Pten$ deficiency expands the populations of dome$^+$/PXN$^-$ intermediate progenitors (19±9%, compared with 5±2% in WT; $P<0.0005$; Fig. 5P), dome$^+$/PXN$^+$ cells (54±8%, compared with 40±3% in WT; $P<0.0005$; Fig. 5I-I/H11033), and fully differentiated PLs (Fig. 5J) at the cost of the MZ. Unlike $Tsc2$ downregulation, the ratio of CCs to total LG cells is significantly increased in the LG upon progenitor-specific $Pten$ downregulation (Fig. 5K,Q), and lamellocytes are rarely observed (Fig. 5L).

Increased lamellocyte differentiation upon $Tsc1/2$ versus $Pten$ LOF was also demonstrated by LG-specific clonal downregulation (supplementary material Fig. S3F,I) and mutant clonal analysis (supplementary material Fig. S3M-P). $Pten$ downregulation also increases the number of circulating hemocytes compared with WT or $Tsc2$ deficiency (compare Fig. 5O with 5M,N), suggesting premature dissemination of LG hemocytes upon $Pten$ disruption, which do not normally enter circulation until the onset of metamorphosis when the LG disintegrates (Grigorian et al., 2011). Lineage-tracing analysis demonstrated the LG origin of the increased circulating hemocytes upon LG-specific $Pten$ downregulation (supplementary material Fig. S3J-L). These phenotypic differences in $Tsc2$- and $Pten$-deficient LGs are not the...
Inhibition of TORC1 signaling reduces progenitor pools and impairs early, but not late, LG growth

We next examined the phenotypic consequence of Tsc1/2 and Pten overexpression on prohemocytes. Inhibition of TORC1 signaling by overexpression of Tsc1\textsuperscript{1-2} and Pten\textsuperscript{wt} in progenitors reduces overall LG size at wL3 and significantly increases the proportion of PXN\textsuperscript{high} hemocytes (Fig. 6A,E,I and Fig. 5P). Temporally differentiated hemocyte lineages are also expanded (Fig. 6F-H,J-L) and the ratio of CCs to total LG cells is significantly increased (Fig. 5Q). LG-specific clonal expression of Tsc1\textsuperscript{1-2} and Pten\textsuperscript{wt} also decreases total LG size and increases differentiation throughout the tissue (supplementary material Fig. S5A-C). At L2, LGs in which prohemocytes overexpress Tsc1\textsuperscript{1-2} or Pten\textsuperscript{wt} are already markedly smaller than controls (supplementary material Fig. S5D-F), which can be attributed to reduced progenitor proliferation at this stage (supplementary material Fig. S5G).

The decreased LG size and increased differentiation that we observe upon Tsc1\textsuperscript{1-2} or Pten\textsuperscript{wt} overexpression is phenocopied by overexpression of a dominant-negative Tor allele in progenitors and through systemic treatment with the TORC1 inhibitor rapamycin (Fig. 6M-T). Both conditions increase the proportion of dome-'PXN\textsuperscript{high}' hemocytes in the LG as well as the ratio of CCs to total LG cells (Fig. 5P,Q). Delaying treatment with rapamycin until later stages is sufficient to increase hematocyte differentiation as well (Fig. 6U-X and Fig. 5P,Q), but does not significantly affect further LG growth. A similar effect occurs upon conditionally overexpressing Tsc1\textsuperscript{1-2} in prohemocytes only after larvae reach later stages (supplementary material Fig. S5H-I). These results highlight the early role of TORC1 signaling in regulating prohemocyte proliferation and a later role in regulating differentiation.

**Tsc2 and Pten phenotypes are sensitive to stress and depend on TORC1, 4EBP and pAKT**

That a nutrient-sensing signaling pathway regulates progenitor number during normal *Drosophila* hematopoiesis raises the intriguing possibility that nutritional/environmental stress signals might utilize the same developmental hematopoietic signaling pathway to regulate progenitor adaptations to stress. The response of blood progenitors to stress conditions is evident upon rearing WT larvae under acute starvation and hypoxia, both of which increase the size of the CZ at the expense of MZ progenitors (Fig. 7A-A', Fig. 5P) and phenocopy conditions of reduced TORC1 signaling induced by overexpression of Tsc1\textsuperscript{1-2} and Pten\textsuperscript{wt} (Fig. 5P). Furthermore, the LG overgrowth characteristic of Tsc2- and Pten-deficient LGs is attenuated by these stressors (Fig. 7B-B',C-C'), suggesting that the effects of starvation and hypoxia on hematocyte progenitors might be mediated by reducing TORC1 signaling.

We confirmed the TORC1 dependence of Tsc2 or Pten deficiency-induced LG overgrowth by treating larvae with rapamycin throughout development, which was sufficient to rescue the phenotype in both backgrounds (Fig. 7B'B'',C'C'). The tight correlation of p4EBP expression with mitotically dividing cells during early LG development suggested that 4EBP might...
Fig. 7. Dependence of Tsc2 and Pten phenotypes on TORC1 and 4EBP but not S6K. All panels show wL3. In A-U, dome-gal4, UAS-2xEGFP is used to drive expression of the specified genetic constructs (green channel is omitted in M-U, but dome+ progenitors are outlined by dotted lines). PXN is in red in A-L and pAKT is in red in M-Y. (A-A') WT LGs (dome>). Starvation (A'), hypoxia (A') or rapamycin treatment (A') all increase PXN+ hemocytes (red) at the expense of MZ prohemocytes (green) compared with controls (fed/normoxic, A). (B-C') Overgrowth associated with Tsc2 and Pten downregulation (B,C) is attenuated in conditions of starvation (B,C), hypoxia (B,C) and rapamycin treatment (B,C). (D-F) Overexpression of d4EBP(LL) decreases LG size (D) and is sufficient to attenuate LG overgrowth in Tsc2-deficient (E) or Pten-deficient (F) LGs (compare with B,C). (G-I) Reducing S6K function (dome>S6kDN) does not affect LG size and differentiation (G), and does not rescue LG overgrowth in Tsc2-deficient (H) or Pten-deficient (I) LGs. (J,K) Downregulation of 4EBP (J) increases LG size to a greater extent than activation of S6K (K). (L) Overexpression of Tsc12+ upon Pten downregulation rescues LG overgrowth but does not rescue the increased differentiation observed upon Pten downregulation alone (compare with C). (M) WT LG. Expression of pAKT is highest medially, within dome+ progenitors. (N) Pten downregulation increases pAKT levels in progenitors (N), whereas Tsc2 deficiency reduces pAKT expression (O). (P,Q) Activating (dome>S6kActivated, P) or reducing (dome>S6kDN, Q) S6K function does not affect pAKT expression. (R) Downregulation of S6K activity upon Tsc2 downregulation does not increase pAKT levels compared with conditions of Tsc2 downregulation alone (compare with O). (S-U) Reducing TORC1 signaling via systemic rapamycin treatment (S) or overexpression of Tsc12+ (T) or Tsc12+ (U) in prohemocytes increases pAKT expression. (V-X') MARCM clones for WT [hs-flp FRT82B Tub-mCD8-GFP (V-X'), Tsc12+ (hs-flp FRT82B; FRT82B Tub-mCD8-GFP; W-W') and Pten2177 (hs-flp FRT40A Pten2177; FRT40A Tub-nGFP; Y-X')]. In WT, pAKT expression is highest in PXN-negative cells (arrows, V-V' and X-X') and gradually decreases in PXN+ cells (asterisk, V-V' and X-X'). Tsc12+ clones demonstrate reduced pAKT expression in progenitors (PXN-negative cells; arrows, W-W'). Pten2177 clones in medial LG regions demonstrate autonomous increases in pAKT expression (PXN-negative cells; arrows, Y-Y'). Scale bars: 20 μm; A for A-L; in V for V-Y'.
itself be regulating the proliferation of blood progenitors. Phosphorylation of 4EBP by active TORC1 relieves translation initiation factor 4E (eIF-4E) from inhibitory 4EBP binding and promotes growth (Gingras et al., 2001). Expression of a mutant form of Drosophila 4EBP (d4EBP(LL)) that binds more tightly to eIF-4E decreases LG size and increases differentiation, similar to rapamycin treatment (compare Fig. 7D with 7A,A’). Overexpression of d4EBP(LL) upon Tsc2 or Pten downregulation in progenitors is sufficient to block LG overgrowth (Fig. 7E,F), confirming a role for 4EBP downstream of TSC and PTEN in hemocyte progenitor proliferation. Unexpectedly, overexpression of S6kDN in prohemocytes is not sufficient to significantly alter LG size and differentiation (Fig. 7G), or to rescue overgrowth of Tsc2- and Pten-deficient LGs (Fig. 7H,I). The more prominent role for 4EBP as compared with S6k in the LG is also demonstrated in that downregulation of 4EBP in prohemocytes increases LG size compared with controls (compare Fig. 7J with 7A), whereas overexpression of

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**Fig. 8. ROS-dependent effects of TSC and PTEN in prohemocytes.** All panels show wL3. dome-gal4, UAS-2xEGFP (green) drives expression of each genetic construct in prohemocytes. ROS levels are in white (A’,F’). PXN (G-O) or P1 (P-R) are in red. (A,A’). WT. ROS levels are highest in dome+ prohemocytes. (B-C’) Downregulation of Tsc2 (B,B’) or Pten (C,C’) increases ROS levels. (D,D’) Overexpression of the ROS scavenger GTPx1 reduces ROS levels. (E-F’) GTPx1 expression reduces ROS levels in Tsc2-deficient (E,E’) and Pten-deficient (F,F’) LGs. (G-O) Overexpression of the ROS scavengers GTPx1 (J) or catalase (M) upon Tsc2 or Pten downregulation rescues their overgrowth (compare K,L and N,O with H,I). (P) WT LG. (Q) Downregulation of ND42 increases PL differentiation and reduces the progenitor population (green) while LG size is unaffected. (R) Systemic rapamycin treatment of ND42-deficient LGs does not reverse the increase in differentiation. (S) TORC1 signaling to 4EBP and S6K is inhibited by TSC and PTEN. The presence of a TORC1-dependent negative-feedback loop in the LG regulates AKT activity upon TORC1 activation (crosshatched line). Pten disruption hyperactivates AKT, which can activate TORC1 signaling as well as TOR- and FOXO-independent effectors. Conditions of hypoxia and starvation are able to reduce TORC1 signaling downstream of PTEN and TSC. (T) Disruption of Tsc2 and Pten in hemocyte progenitors (green) increase proliferation in a TOR-, 4EBP- and ROS-dependent manner at early stages. (U) At late LG stages, Tsc1/2 and Pten deficiencies affect distinct hemocyte lineages. Tsc1/2 disruption autonomously increases p4EBP and ROS levels and expands intermediate progenitors (yellow) and lamellocytes (gray). Pten disruption autonomously increases AKT activation and ROS levels, non-autonomously induces p4EBP expression and expands fully differentiated PLs (red) and CCs (blue). White arrows with gray outlines represent increases in specified marker expression; white arrows with black outlines represent increases in specified cell lineages. DV, dorsal vessel. Scale bars: 20 μm; in G for G-R.
S6k^{Activated} does not (Fig. 7K). Epistasis of Pten and Tsc1/2 was also examined, and, similar to rapamycin treatment, Tsc^{1-2} overexpression is sufficient to block overgrowth of Pten-deficient LGs, although substantial differentiation still occurs (Fig. 7L). These data suggest that, although a common TOR-mediated signaling network regulates progenitor proliferation downstream of TSC and Pten, TSC/TOR-independent effectors might contribute to the increased differentiation and myeloproliferative effects observed upon Pten deficiency.

One candidate effector molecule that may be differentially regulated upon Tsc2 and Pten downregulation is AKT. Whereas Pten loss leads to constitutive activation of AKT, hyperactive TORC1 signaling is known to trigger a negative-feedback loop that attenuates PI3K-AKT signaling (Harrington et al., 2004; Kockel et al., 2010). Pten downregulation in prohemocytes increases pAKT levels in the LG (Fig. 7N), whereas PAKT expression is nearly absent in Tsc2-deficient LGs (Fig. 7O). Although this feedback inhibition downstream of TORC1 signaling could be S6K mediated (Harrington et al., 2004), we found no evidence of S6K-dependent feedback in Drosophila prohemocytes (Fig. 7P-R). By contrast, reducing TORC1 signaling in prohemocytes via rapamycin treatment (Fig. 7S), Tor^{DN} overexpression (Fig. 7T), or Tsc^{1-2} overexpression (Fig. 7U) did increase PAKT levels. Our data thus describe a TORC1-dependent, S6K-independent negative feedback that attenuates signaling to AKT in the LG.

Disparate PAKT expression among Tsc2- and Pten-deficient LGs raises the possibility that AKT might be a functionally relevant target downstream of Pten but not TSC signaling, which might account for some of the observed phenotypic differences in these backgrounds. We confirmed differential regulation of pAKT upon Tsc1/2 and Pten LOF through MARCM clonal analysis (Fig. 7V-Y”), which demonstrates cell-autonomous increases in pAKT expression in medial Pten^{1-} cells, but absence of PAKT in Tsc1^{1-} cells. Although constitutive activation of AKT in prohemocytes does not induce hypertrophy, unlike in Pten-deficient LGs, we often observed an increase in PXN+ cells, with occasional fragments of differentiated tissue ‘pinching’ off from the LG, similar to the effects of Pten loss (compare supplementary material Fig. S6C with S6B). Additionally, the ratio of Ccs to total LG cells is significantly increased upon AKT activation (Fig. 5Q), whereas lamellocyte numbers are not altered (data not shown), further demonstrating the similarity of AKT-dependent phenotypes to Pten but not Tsc1/2 loss. Perhaps the best-described targets of AKT are the Forkhead box O (FoxO) family of transcription factors, which are inhibited by activated AKT (Castellino and Durden, 2007). Downregulation of foxo in prohemocytes does not phenocopy Pten silencing or AKT activation (compare supplementary material Fig. S6D with S6B,C), and overexpression of foxo is not sufficient to rescue the increased differentiation or overgrowth of Pten-deficient LGs (supplementary material Fig. S6E,F). Although a common TORC1- and 4EBP-dependent signaling pathway mediates the early proliferative effects downstream of Tsc2 and Pten deficiencies, the additive effects of activating both AKT and TORC1 signaling upon Pten disruption may account for the increased differentiation and myeloproliferation observed.

**DISCUSSION**

**TORC1 signaling regulates hemocyte progenitor proliferation**

Our data demonstrate a crucial role of TORC1 signaling in regulating the proliferation of Drosophila hemocyte progenitors at early developmental stages, when rapid growth of the LG occurs. The specific accumulation of p4EBP in pH3^{+} cells at early stages highlights the central function of TORC1 signaling in actively dividing hemocyte progenitors. These data complement recent findings in Drosophila ovarian germ line stem cells (GSCs) in which p4EBP levels were specifically elevated in M phase (LaFever et al., 2010). However, in contrast to Drosophila GSCs, in which 4EBP does not mediate Tor-associated phenotypes (LaFever et al., 2010), our data demonstrate a central role for 4EBP in regulating hemocyte progenitor proliferation downstream of Tsc and Pten disruption (Fig. 8S,T). The sufficiency of scavenging ROS in Pten- and Tsc2-deficient LGs to rescue associated LG overgrowth also highlights the role of ROS in mediating hemocyte proliferation upon TORC1 hyperactivation (Fig. 8T). A limited number of studies in vertebrate hematopoietic stem cells (HSCs) deficient for Tsc1 and Pten consistently demonstrate their initial expansion due to aberrant cell cycling, but eventual exhaustion caused by reduced self-renewal capacity (Yilmaz et al., 2006; Zhang et al., 2006; Chen et al., 2008). Our findings suggest that 4EBP-mediated TORC1 activity might be particularly relevant in hematopoietic progenitors, warranting its further investigation in vertebrates.

**Differential effects of Tsc1/2 and Pten function on blood progenitors**

Our findings demonstrate phenotypic differences upon Tsc1/2 and Pten manipulation in blood progenitors. Mutant Tsc1 clones autonomously express high levels of p4EBP and are associated with low PXN expression. Progenitor-specific downregulation of Tsc2 does not alter differentiation onset and progression, but by late L3 stages the population of p4EBP^{high} dome” / PXN” intermediate progenitors dramatically expands, and retention of this pool of progenitors restricts the populations of fully differentiated PLs and CCs while still allowing large numbers of lamellocytes to differentiate (Fig. 8U). By contrast, Pten silencing promotes an
early increase in differentiation that proceeds to expand terminally differentiated PLs and CCs and also leads to premature release of hemocytes from the LG (Fig. 8U). Interestingly, the increased malignancy induced by Pten disruption in progenitors is associated with non-autonomous increases in p4EBP levels within peripheral differentiating hemocytes and ‘buds’ of CZ tissue (Fig. 8U, Fig. 3I, Fig. 4D and supplementary material Fig. S1F). Further studies are needed to clarify whether non-autonomous TORC1 activation induced by Pten disruption is common to other cell types and might contribute to the greater malignancy potential associated with Pten mutations as compared with Tsc mutations in human disease (Manning et al., 2005).

In contrast to the non-autonomous effects of Pten LOF on p4EBP accumulation, Pten loss autonomously increases AKT activation. Further, the phenotypic similarities between AKT activation and Pten loss in progenitors suggests that the additive effects of activating both AKT and TORC1 signaling in Pten-deficient hemocytes might contribute to the more dramatic effects on terminal differentiation and premature release of LG hemocytes into circulation. Previous studies in vertebrates have shown that Pten loss (Yilmaz et al., 2006; Zhang et al., 2006) and AKT activation (Kharas et al., 2010) in HSCs as well as in myeloid progenitors (Yu et al., 2010) induces myeloproliferative disorder (MPD) and leukemia. Although reduced expression of TSC has been reported in a subset of acute leukemia patients (Xu et al., 2009), Tsc1 loss in HSCs is not sufficient to induce leukemia in mice (Chen et al., 2008). Attenuation of AKT signaling upon Tsc1/2 disruption might account for the decreased malignant potential of Tsc1/2-deficient LGs compared with Pten loss, similar to previous reports (Harrington et al., 2004; Manning et al., 2005).

Unexpectedly, both activation and suppression of TORC1 signaling increases the proportion of PXN+ cells in the LG at the expense of dome+ progenitors. The increased AKT activation that we observe upon conditions of TORC1 pathway suppression, induced via relief of a TOR-dependent negative-feedback loop, may account for the expansion of differentiated hemocytes and CCs observed, although we cannot rule out the possibility that other pathways might contribute to this effect, such as changes in Wingless levels (Shim et al., 2012).

TORC1-dependent effects of stress and elevated ROS in progenitors

Consistent with the nutrient-sensing function of TOR signaling (Wang and Proud, 2009) and our findings concerning its central role in regulating Drosophila hemocyte progenitor proliferation and differentiation, we observed increased differentiation under starvation and low oxygen conditions, which phenocopied reduced TORC1 signaling in the LG. Furthermore, starvation specifically decreased WT LG size (Fig. 7A’), and rearing Tsc2-deficient LGs under conditions of starvation rescues their overgrowth, demonstrating the TOR-dependent effects of nutrient availability on proliferation, similar to findings for neural progenitors in Drosophila (Sousa-Nunes et al., 2011). An additional metabolic stressor that has been shown to affect LG progenitor differentiation is elevated ROS (Owusu-Ansah and Banerjee, 2009). Manipulation of mitochondrial electron transport chain proteins increases ROS levels in the LG and causes precocious progenitor differentiation, but does not affect tissue size. These effects are not TOR dependent, consistent with their distinct phenotype compared with Tsc2 and Pten disruption. The lack of change in LG size upon rapamycin treatment of ND42RNAi larvae is likely to be due to compensatory proliferation of differentiated hemocytes, independent of TOR, which may result from the early loss of progenitors in this background (Owusu-Ansah and Banerjee, 2009). The ROS-dependent LG overgrowth observed upon TORC1 hyperactivation demonstrates mechanistic differences in ROS signaling between conditions of mitochondrial dysfunction and of increased TOR signaling in blood progenitors. Conditional Tsc1 deletion in mouse HSCs increases ROS, which induces a loss of quiescence and increased cycling that is rescued by decreasing ROS levels (Chen et al., 2008). This and several other studies consistently demonstrated a link between HSC proliferation/quiescence and intracellular oxidation status (Ito et al., 2006; Arai and Suda, 2007; Jang and Sharkis, 2007; Tothova et al., 2007), similar to our findings.

Our results highlight differences in the expansion of intermediate progenitors, differentiated hemocytes and LG-derived circulating hemocytes that are unique to TSC and PTEN, resulting from differential effects on feedback inhibition, AKT activity and autonomous versus non-autonomous TOR activation. We also demonstrate the common effects of Tsc2 and Pten loss on regulating early blood progenitor proliferation in a TORC1-, 4EBP- and ROS-dependent manner and the effects of starvation and hypoxia on TOR-dependent LG growth. This study demonstrates the multifaceted roles of a nutrient-sensing pathway in orchestrating the growth and differentiation of myeloid-specific blood progenitors through regulation of ROS levels and the resulting MPD when dysregulated, highlighting potential non-autonomous effects of Pten dysfunction on malignancy.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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