The Hippo Pathway Regulates Hematopoiesis in Drosophila melanogaster

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Summary

The Salvador-Warts-Hippo (Hippo) pathway is an evolutionarily conserved regulator of organ growth and cell fate. It performs these functions in epithelial and neural tissues of both insects and mammals, as well as in mammalian organs such as the liver and heart. Despite rapid advances in Hippo pathway research, a definitive role for this pathway in hematopoiesis has remained enigmatic. The hematopoietic compartments of Drosophila melanogaster and mammals possess several conserved features [1, 2]. D. melanogaster possess three types of hematopoietic cells that most closely resemble mammalian myeloid cells: plasmatocytes (macrophage-like cells), crystal cells (involved in wound healing), and lamellocytes (which encapsulate parasites). The proteins that control differentiation of these cells also control important blood lineage decisions in mammals [3–10]. Here, we define the Hippo pathway as a key mediator of hematopoiesis by showing that it controls differentiation and proliferation of the two major types of D. melanogaster blood cells, plasmatocytes and crystal cells. In animals lacking the downstream Hippo pathway kinase Warts, lymph gland cells overproliferated, differentiated prematurely, and often adopted a mixed lineage fate. The Hippo pathway regulated crystal cell numbers by both cell-autonomous and non-cell-autonomous mechanisms. Yorkie and its partner transcription factor Scalloped were found to regulate transcription of the Runx family transcription factor Lozenge, which is a key regulator of crystal cell fate. Further, Yorkie or Scalloped hyperactivation induced ectopic crystal cells in a non-cell-autonomous and Notch-pathway-dependent fashion.

Results and Discussion

Hippo Pathway Components Are Expressed in D. melanogaster Hematopoietic Cells

We adopted D. melanogaster as a system to investigate a potential role for the Hippo pathway in hematopoiesis since this pathway was first discovered and is best understood in this organism. The best-described hematopoietic organ in D. melanogaster is the larval lymph gland, which matures during larval development and ruptures during metamorphosis to give rise to circulating hemocytes in the pupa and adult [1, 2]. The lymph gland is a paired multilobed structure: the large primary lymph gland lobes contain differentiating cells in the cortical zone, whereas the medullary zone contains undifferentiated cells and the posterior signaling center (PSC) acts as a hematopoietic niche that serves to maintain the medullary zone precursors population [1, 2]. The secondary lobes, which vary in number but usually consist of between two and four paired lobes, contain undifferentiated hemocyte progenitor cells.

Initially, we studied expression of Hippo pathway components and found that the upstream pathway members Merlin (Mer), Fat (Ft), and four-jointed (fj) were expressed throughout the lymph gland, as was the key transcriptional coactivator protein Yorkie (Yki; Figures S1A–S1E available online). Some cells exhibited higher Yki expression (Ykihigh), suggesting that Yki activity is nonuniform in lymph glands. Eighty-six percent of Ykihigh cells were Hindsight positive (Hnt+), which marks terminally differentiated crystal cells [11] (Figures S1D’ and S1E’). Scalloped (Sd), the best-defined of Yki’s partner transcription factors [12–16], was observed throughout the lymph gland, was strongest in the primary lobe medullary zone, and was expressed at low levels in 89% of crystal cells (Figures S1F and S1G).

The Hippo Pathway Kinase Warts Regulates Blood Cell Differentiation

To determine whether the Hippo pathway regulates hematopoietic development, we analyzed the number and location of the two predominant differentiated cell types, plasmatocytes (P1+) and crystal cells (Hnt+), in lymph glands from wts (wts) hypomorphs. Both plasmatocytes and crystal cells were increased in number and were present throughout the medullary zone of the primary lobe and the secondary lobes, where normally only hemocyte progenitors reside, rather than being restricted to the cortical zone of the primary lobe (Figures 1A–1D). To analyze this more closely, we assessed hemocyte differentiation in wild-type and wts lymph glands throughout development in carefully staged animals. Plasmatocytes and crystal cells were absent from wild-type lymph glands at the late second larval instar; however, strikingly, the majority of wts mutant glands displayed strong expression of both Hnt and P1 (Figures 1E–1H). P1+, but not Hnt+, cells began to appear in wild-type lymph glands in early third larval instar development, but both cell populations were prevalent in wts lymph glands (Figures 1I–1L).

Differentiation into either a crystal cell or a plasmocyte are mutually exclusive fate decisions [17, 18]. To determine whether this fate choice occurred normally in cells with aberrant Hippo pathway activity, we analyzed lymph glands from 15 wild-type and 15 wts animals, using P1 antibodies and lozenge (lz)–Gal4-driven expression of UAS-GFP, which marks crystal cells. All wild-type lymph glands displayed either lz+ or P1+ cells but never lz+ P1+ (double-positive) cells (Figures 1M–1N”). By contrast, all 15 wts lymph glands displayed multiple cells that were positive for both lz-Gal4 and P1 (Figures 1N–1N”). In three wts lymph glands, the majority of Lz+ cells also
Figure 1. Warts Regulates Blood Cell Differentiation

(A–D) Control w^{1178} (A and C) and wts mutant lymph glands (B and D). Plasmatocytes were visualized by anti-P1 staining (grayscale in A and B) and merged with direct interference contrast (DIC) images. wts mutant lymph glands exhibit P1 expression outside of its normal domain (yellow arrowheads). Crystal
expressed P1, whereas 12 wts lymph glands displayed more single Iz" cells than Iz" P1" cells. Collectively, these results indicate two key roles for the Hippo pathway in hematopoietic differentiation: (1) It maintains the progenitor state of hemocyte progenitors during lymph gland development and (2) it prevents hemocyte progenitors from differentiating inappropriately to adopt a mixed crystal cell/plasmatocyte fate. The Hippo pathway regulates specific cell-fate choices, such as the decision between inner cell mass and trophoderm in the early mouse embryo [19] and between R6 photoreceptor subtypes in the D. melanogaster eye [20, 21]. In these scenarios, the Hippo pathway regulates a binary fate choice, i.e., a decision between one cell type or another. Our data show that the Hippo pathway does not regulate blood cell fate by stimulating binary fate decisions, but rather prevents premature differentiation of both major D. melanogaster blood lineages.

The Hippo Pathway Kinase Warts Regulates Blood Cell Proliferation and Lymph Gland Size

We also noted that wts lymph glands were larger than in control animals. When quantified, wts lymph glands were 58% larger in size than controls, showing that the Hippo pathway also limits lymph gland growth, comparable to its growth-larger in size than controls, showing that the Hippo pathway complex 2 does not cause premature differentiation [25].

Differentiation, as excess proliferation induced by loss of other control animals. When quantified, wts

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expression images are merged in (M–O), indicating that Wts normally limits lymph gland hemocyte proliferation. We also noted that wts lymph glands were larger and possessed significantly more cells in S phase than did control lymph glands (Figures 2D–2L).

To analyze this more closely, we assessed the proliferation profiles of age-matched wts mutant and wild-type lymph glands throughout development. At each larval stage analyzed (late second instar, early third instar, and mid third instar), wts lymph glands were larger and possessed significantly more cells. Collectively, these results suggest that it is not an important regulator of crystal cell number or fate (Figures S2K and S2L). These data suggest that the Hippo pathway regulates proliferation and/or terminal differentiation of Iz" progenitor cells, which differentiate into crystal cells.

The Key Hippo Pathway Transcriptional Regulator Yorkie Influences Crystal Cell Numbers

Yki is the key effector of the Hippo pathway and is a direct substrate of the Wts kinase [26]. To explore a role for Yki in hematopoiesis, we employed a null allele, yki06. yki mutant animals are lethal soon after embryogenesis [26]. Therefore, we measured the ratio of crystal cells per lymph gland cell (assessed by DAPI intensity) in third-instar larval control animals or yki hemizygotes. As shown in Figures 3A–3C, yki hemizygotes displayed a significant reduction in crystal cells compared to wild-type animals, suggesting that full Yki activity is required for development of the complete complement of crystal cells.

To determine whether the Hippo pathway regulates crystal cells in a cell-autonomous fashion, we depleted Wts by RNAi or overexpressed hyperactive versions of Yki (Yki3SA [27]) or Sd (Sd3SA [18]) under the control of the crystal-cell-specific Iz-Gal4 driver. In each case, we observed a significant increase in crystal cells compared to controls (Figures 3D–3E and Figures S2E–S2H). The increase in crystal cells upon Wts depletion was dependent on Yki, as when both proteins were knocked down, the increase in crystal cells was significantly suppressed (Figures 3F–3J). Yki hyperactivation induced ectopic crystal cells in a cell-autonomous manner, as Iz-driven GFP completely overlapped with Hnt (Figures 3D", 3E", and S2E").

To determine whether Sd reduction displayed a similar phenotype to yki hemizygosity, we depleted Sd by RNAi in developing crystal cells using Iz-Gal4. Unexpectedly, we observed a 2-fold increase in crystal cells (Figures S2I, S2J, and S2L). This observation was reminiscent of Yki and Sd’s role in posterior follicle cell fate in the D. melanogaster ovary. At stages 7–9, both loss-of-function or gain-of-function Sd induces the same phenotype, i.e., increased Cut" cells [28], because Sd functions with Yki to induce Cut expression in these cells but also acts as a default repressor of Cut when Yki activity is low. However, depletion of the Sd co-repressor Tgi using Iz-Gal4 by RNAi did not affect crystal cell number, suggesting that it is not an important regulator of crystal cell number or fate (Figures S2K and S2L). Collectively, these data suggest that the Hippo pathway regulates proliferation and/or terminal differentiation of Iz" progenitor cells, which differentiate into crystal cells.

The Hippo Pathway Regulates Expression of the Key Crystal Cell FateDeterminant, Lozenge

The above data show that total crystal cell numbers in larval lymph glands are sensitive to modulation of Hippo pathway activity during crystal cell maturation. Therefore, we investigated functional links between Lz and the Hippo pathway further. The human homologs of Yki and Lz (YAP and acute myeloid leukemia 3 [AML3], also known as polymavirus enhancer binding protein [PEBP2]) form a physical complex, and YAP activates AML3 in transcription assays [29]. Therefore, we tested whether Yki and Lz formed a physical interaction in S2 cells, but we failed to detect such an association (Figure S3A).

Next, given that wts loss led to premature induction of crystal cells, as well as ectopic crystal cells in lymph gland regions that normally only harbor progenitor cells, we considered the possibility that Yki and Sd regulate Lz expression. To test this, we induced small Yki clones in lymph glands using the Actin-Gal4 flip-out technique. Normally, Lz is expressed in crystal cells of the cortical zone of the primary lymph gland, as well as in the differentiating photoreceptor cells of the third-instar larval eye disc [4, 30]. Strikingly, in yki-overexpressing clones,
we observed ectopic induction of Lz in both the primary and secondary lymph gland lobes (Figures 4A–4A’ and S3B–S3B’ and data not shown). The ability of Yki to induce Lz expression in normally Lz- cells was independent of proliferation, as Lz was induced in single cell yki-expressing clones (Figures S3B–S3B’). We also observed ectopic expression of Lz in yki-overexpressing clones anterior to the normal Lz expression domain in third-instar larvae eye-antennal discs (Figures 4B–4B’). This effect was independent of the UAS-yki transgene utilized, as we observed the same result with three independent transgenes (Figures S3C–S3E’). Furthermore, we observed ectopic Lz expression in yki-overexpressing clones in larval tissues that do not normally express Lz expression, such as the brain, wing, and leg discs (Figures S3F and S3G’ and data not shown).

Scalloped and Yorkie Directly Activate lozenge Transcription in Kc167 Cells

Our finding that ectopic Yki cell-autonomously induced Lz expression in vivo prompted us to search for direct evidence for regulation of Lz transcription by Yki and one of its partner transcription factors, Sd. Sd was found to bind the TEAD/TEF family DNA binding motif, CATTCCA, in a Sd/Yki-responsive enhancer of diap1 [14]. A similar but more divergent consensus emerged from genome-wide analysis of Sd binding [32]. We identified 19 candidate Sd motifs, CATTCCY and CATTCY, in the entire Lz gene that exhibited some evidence for conservation (Figure 4D). Of these, 15 sites were well conserved based on their preservation to D. pseudoobscura or beyond (Figure 4D), suggesting that Lz is under strong and extensive evolutionary constraint for regulation by Sd.

We assayed four genomic regions (lz-R1, lz-R2, lz-R3, and lz-R4) containing multiple conserved Sd binding sites for responsiveness to ectopic Sd/Yki in reporter assays in Kc167 cells, which display hemocyte properties [33, 34] and express Lz [18]. When Kc167 cells were cotransfected with Sd and Yki, we observed upregulation of all four lz reporters but no effect on reporters bearing regulatory regions from the vestigial and fringe loci (Figure 4E) [35], neither of which contain Sd binding sites. All four lz reporters required Sd binding for activation by Yki, as mutation of the Sd sites suppressed or ablated luciferase activity (Figure 4E). These data provide evidence that Sd and Yki can indeed regulate Lz at the level of transcription (Figures 4C–4C’ and S3H–S3H’).

Since these were ectopic tests, we wished to assess whether endogenous Yki could be detected at the Lz locus. We used chromatin immunoprecipitation (ChIP) to query Yki occupancy at the Lz enhancers in Kc167 cells. We observed ~2-fold enrichment of Yki to the lz-R1b and lz-R2 ChIP amplimers, but not to a control region upstream of Lz that lacked a Sd consensus site (Figure 4F). The conserved sites within the lz-R1 enhancer are toward the 5’ end of this region, but this region lacks desirable amplicon properties. However, we assayed another amplicon (R1a) and found strong (~10-fold) enrichment to Yki at this genomic site (Figure 4F). Taken together, these data support the notion that Yki is endogenously recruited to these Sd sites in hemocytes and that the Yki/Sd complex activates Lz transcription.
The Hippo Pathway Non-Cell-Autonomously Influences Crystal Cell Numbers in a Notch-Pathway-Dependent Fashion

When analyzing the effects of Yki overexpression on Lz in lymph glands, we noted that expression of hyperactive Yki (Yki^{3SA}) sometimes led to non-cell-autonomous induction of Lz (data not shown). To investigate this further, we expressed either Yki^{3SA} or Sd^{GA} with hml-Gal4 [1]. Expression of either transgene induced a significant increase in crystal cell numbers (Figures S4 A–S4D). Upon close examination, crystal cells almost never overlapped with hml-expressing cells, indicating that induction of ectopic crystal cells by hyperactive Yki and Sd occurred in a non-cell-autonomous fashion (Figures S4E–S4G). A major determinant of crystal cells is the Notch pathway [5, 6, 18], which acts during the third-instar larval period of development to promote differentiation of crystal cell progenitors to become mature crystal cells [17]. Consistent with this, a substantial increase in cells displaying Notch activity, observed using a Notch response element GFP reporter [18], was observed in hml-Sd^{GA} lymph glands (Figures S4H and S4I). Furthermore, the ability of hml-driven Yki^{3SA} to induce ectopic crystal cells was reliant on full Notch pathway activity, as this phenotype was suppressed in animals that were heterozygous for the Notch pathway transcription factor Suppressor of Hairless [Su(H)] (Figures S4J–S4L). While a mechanistic understanding of Notch-Hippo collaboration in the lymph gland requires further study, it is interesting to note that these pathways collaborate to effect numerous development processes in tissues such as the D. melanogaster ovary and brain [36, 37] and the murine embryo and liver [38–40].

Conclusions

The Hippo pathway is an important regulator of tissue growth and cell-fate decisions in epithelial and neural tissues from...
Figure 4. The Hippo Pathway Regulates Expression of the Key Crystal Cell-Fate Determinant, Lozenge

(A–B) A primary third-instar larval lymph gland (A–A') and eye-antennal disc (B–B') harboring clones of tissue expressing UAS-yki transgenes (marked by GFP in green). Expression of Lz (grayscale in A and B, red in the merged image in B') and Yki (red in A') are shown. Ectopic expression of Lz was induced by yki overexpression in both lymph glands (indicated by arrowheads in A–A') and eye-antennal discs (indicated by arrowheads in B–B'). Asterisks mark endogenous Lz expression in the lymph gland (A–A') and the posterior region of the eye-antennal disc (B).

(C–C') Lz expression, as reported by a 1.5 kb region of the lz promoter fused to the lacZ gene in a wing imaginal disc expressing UAS-yki in the posterior domain under the control of en-gal4. Lz expression is grayscale in the single channel (C) and red in the merged image (C'), while UAS-yki expression is marked by coexpression of UAS-GFP (green in C' and C').

(legend continued on next page)
both insects and mammals [41]. Here we show that the Hippo pathway controls the overall size of the major hematopoietic organ of D. melanogaster, the larval lymph gland, and promotes the progenitor cell state by repressing Yki activity. While the antiproliferative activity of the Hippo pathway in the lymph gland mirrors its growth-suppressive role in many D. melanogaster and mammalian tissues, its effects on differentiation do not. For example, wt loss in the larval eye delays or impairs differentiation of proneural cells [42, 43] and blocks the transition from neuroepithelium to neuroblasts in the larval brain [36].

In mammals, Runx1, GATA2, and the Notch pathway cooperate to regulate differentiation of megakaryocytes [9]. These same factors also regulate crystal cell fate in D. melanogaster [1-3]. Given that the Notch and Hippo pathways appear to collaborate to regulate crystal cell fate and that Yki can induce expression of the key Runx family transcription factor, Lz, it is possible that the Hippo pathway also controls mammalian megakaryocyte differentiation. Further, given the well-documented role of the Hippo pathway as a tumor-suppressor pathway in human cancers [41, 44], this study provides rationale for an investigation of the Hippo pathway in the etiology of hematopoietic malignancies.

**Supplemental Information**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.10.031.

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