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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, and 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.

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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 multi-lobed structure: the large primary lymph gland lobes contain differentiating cells in the cortical zone, whilst the medullary zone contains undifferentiated cells and the posterior signalling centre (PSC) acts as a hematopoietic niche which serves to maintain the medullary zone prohemocyte population [1, 2]. The secondary lobes, which vary in number but usually consist of between 2–4 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 co-activator protein Yki (Figures S1A–S1E). Some cells exhibited higher Yki expression (Yki^{high}), suggesting that Yki activity is non-uniform in lymph glands. 86% of Yki^{high} 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 analysed the number and location of the two predominant differentiated cells, plasmatocytes (P1+) and crystal cells (Hnt+), in lymph glands from *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 analyse 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 lymph glands, but both cell populations were prevalent in *wts* lymph glands (Figures 1I–1L′).

Differentiation into either a crystal cell or a plasmatocyte are mutually exclusive fate decisions [17, 18]. To determine whether this fate choice occurred normally in cells with aberrant Hippo pathway activity, we analysed lymph glands from 15 wild-type and 15 *wts* animals, using P1 antibodies and *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–1M″). 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 expressed P1, whereas12 *wts* lymph glands displayed more single *lz*+ cells than *lz*+ P1+ positive cells. Collectively, these results indicate two key roles for the Hippo pathway in hemocyte 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/plasmatoctye fate. The Hippo pathway regulates specific cell fate choices such as the decision between inner cell mass and trophectoderm in the early mouse embryo [19] and between R8 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-repressive function in other larval tissues such as the imaginal discs and brain (Figures 2A–2C and S2A–S2D) [22–24]. To analyse this more closely we assessed the proliferation profiles of age-matched *wts* mutant and wild-type lymph glands, throughout development. At each larval stage analysed (late second instar, early third instar and mid-third instar) *wts* lymph glands were larger and possessed significantly more cells in S phase than did control lymph glands (Figures 2D–2L). Similar results were observed when mitotic cells were analysed using phospho-Histone H3 antibodies (Figures 2M–2O), indicating that Wts normally limits lymph gland hemocyte proliferation. The fact that *wts* lymph glands were larger and more proliferative cannot account for the observed premature differentiation, as excess proliferation induced by loss of other growth suppressors in the lymph gland such as Tuberous Sclerosis Complex 2 does not cause premature differentiation [25].

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, yki^{B5}. *yki* mutant animals are lethal soon after embryogenesis [26]. Therefore, we measured the ratio of crystal cells per lymph gland cell (assessed by DAP1 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 RNA interference, or overexpressed hyperactive versions of Yki (Yki^{3SA} [27]) or Sd (Sd^{GA} [15]) under the control of the crystal cell-specific *lz-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–3I). Yki hyperactivation induced ectopic crystal cells in a cell autonomous manner, as *lz*-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 *lz-Gal4*. Unexpectedly, we observed a two-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 stage 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 *lz-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, L). Collectively, this data suggests that the Hippo pathway regulates proliferation and/or terminal differentiation of Lz+ progenitor cells, which differentiate into crystal cells.

The Hippo pathway regulates expression of the key crystal cell fate determinant, 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 homologues of Yki and Lz [YAP and Acute myeloid leukemia 3 (AML3), also known as Polyomavirus enhancer binding protein (PEBP2α)] 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 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 harbour 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″, S3B–S3B‴ and 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 larval 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 (Figure 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 (Figure S3F–S3G′ and not shown). To determine whether Yki regulates Lz at the level of transcription, we used a transgenic strain that harboured a 1.5kb fragment of the *lz* promoter fused to the *β-galactosidase* gene [31]. When *yki* was overexpressed with either *en-Gal4* or *ptc-Gal4*, we observed elevation of *lz*-

lacZ showing that Yki indeed can regulate *lz* at the level of transcription (Figures 4C–4C″ and Figure S3H–S3H″).

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, CATTCY and CATTYC, 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 co-transfected 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 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 directly activate *lz* gene expression via multiple cis-regulatory regions in the *lz* locus.

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 amplicons, 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 towards 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 analysing 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 (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 S4A–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-SdGA* lymph glands (Figure S4H–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)] (Figure S4J–S4L). Whilst 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].

CONCLUDING REMARKS

The Hippo pathway is an important regulator of tissue growth and cell-fate decisions in epithelial and neural tissues from 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. Whilst the anti-proliferative 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, *wts* loss in the larval eye delays or impairs differentiation of pro-neural 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 tumour suppressor pathway in human cancers [41, 44], this study provides rationale for an investigation of the Hippo pathway in the aetiology of hematopoietic malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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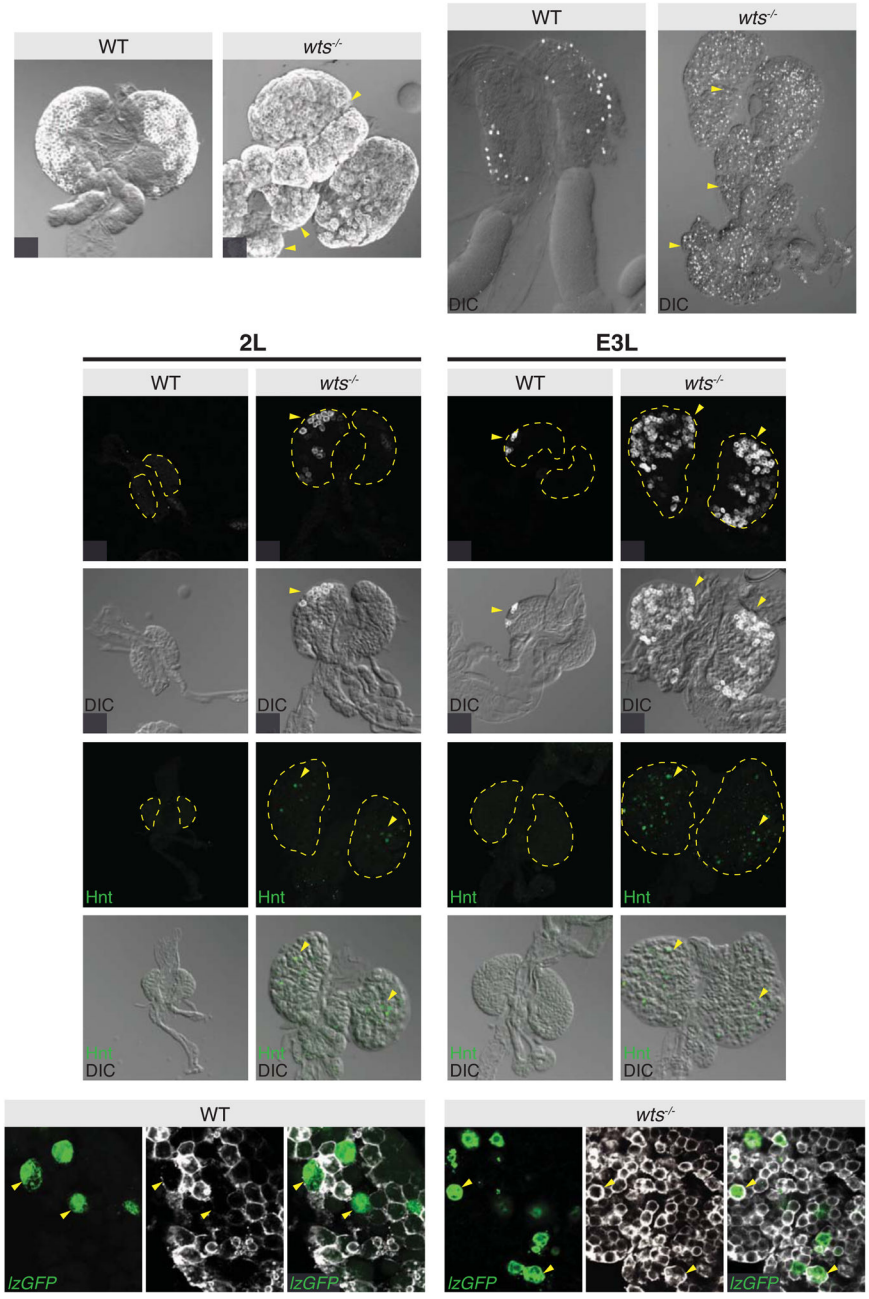

Figure 1. Warts regulates blood cell differentiation

(A–D) Control w^{1118} (A and C) and *wts* mutant primary lymph glands (B and D). Plasmatocytes were visualized by anti-P1 staining (greyscale in A and B) and merged with direct interference contrast images. *wts* mutant lymph glands exhibit P1 expression outside of its normal domain (yellow arrowheads). Crystal cells were visualized by anti-Hnt staining (greyscale in C and D). Primary and secondary lobes are labelled 1° and 2° respectively. *wts* mutant lymph glands possessed significantly more crystal cells as well as precocious differentiation of secondary gland hemocytes into crystal cells (yellow arrowheads in D).

(E–L) Late second instar control w^{1118} (E and G) and *wts* mutant (F and H) lymph glands, and early third instar control w^{1118} (I and K) and *wts* mutant (J and L) lymph glands. Plasmatocytes were visualized by anti-P1 staining (greyscale in E, F, I and J) and crystal cells were visualized by anti-Hnt staining (green in G, H, K and L) and merged with direct interference contrast images in (E′–L′). *wts* mutant lymph glands exhibited differentiation of both plasmatocytes and crystal cells at late second instar larval stages, while control lymph glands did not.

(M–N) Control w^{1118} (M–M″) and *wts* mutant primary lymph glands (N–N″). Crystal cells were marked with *lz-Gal4* driven expression of *UAS-GFP* (green in M and N) and plasmatocytes were visualized by anti-P1 staining (greyscale in M′ and N′). Crystal cells did not express P1 in control lymph glands (yellow arrowheads in M–M″), while there was a significant number of crystal cells that also expressed P1 in *wts* mutant lymph glands (yellow arrowheads in N–N″). GFP and protein expression images are merged in (M″ and N ″).

Scale bars = 20μm. See also Figure S1.

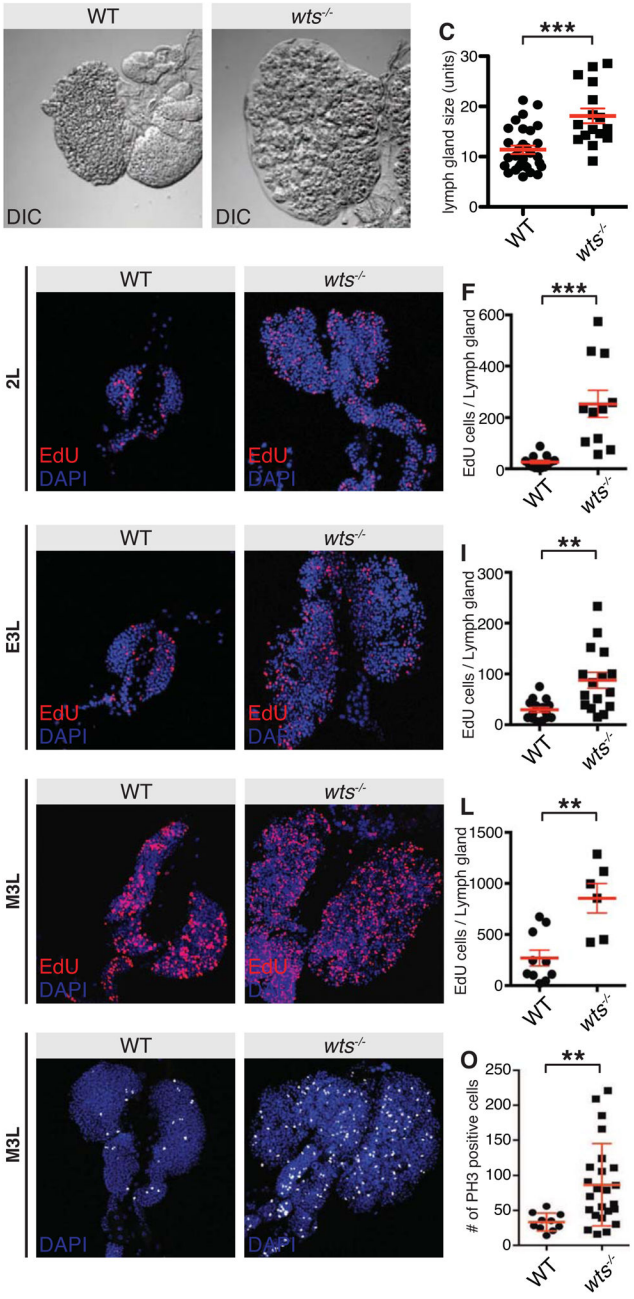

Figure 2. Warts regulates lymph gland size and hemocyte proliferation

(A–C) Direct interference contrast images of control w^{1118} (A) and *wts* mutant late third instar lymph glands (B). Lymph gland size was quantified in (C). *wts* mutant lymph glands were significantly larger than controls ($p<0.0001$; n=30 for w^{1118}, n=16 for *wts-Z*; error bars represent SEM).

(D–L) Control w^{1118} (D, G and J) and *wts* mutant lymph glands (E, H and K) at late second instar (D–E), early third instar (G–H) and mid third instar (J–K). Cells undergoing DNA replication were visualised using EdU (red in D, E, G, H, J and K) and nuclei were marked

with DAPI (blue in D, E, G, H, J and K). The number of EdU positive cells per lymph gland was quantified in (F, I and L).

(M–O) Control w^{1118} (M) and *wts* (N) mutant mid third instar larval lymph glands. Mitotic cells were visualised using anti phospho-Histone H3 (white) and nuclei were marked with DAPI (blue). The number of phospho-Histone H3 positive cells per lymph gland was quantified in (O).

Scale bars = 20 μm. See also Figure S2.

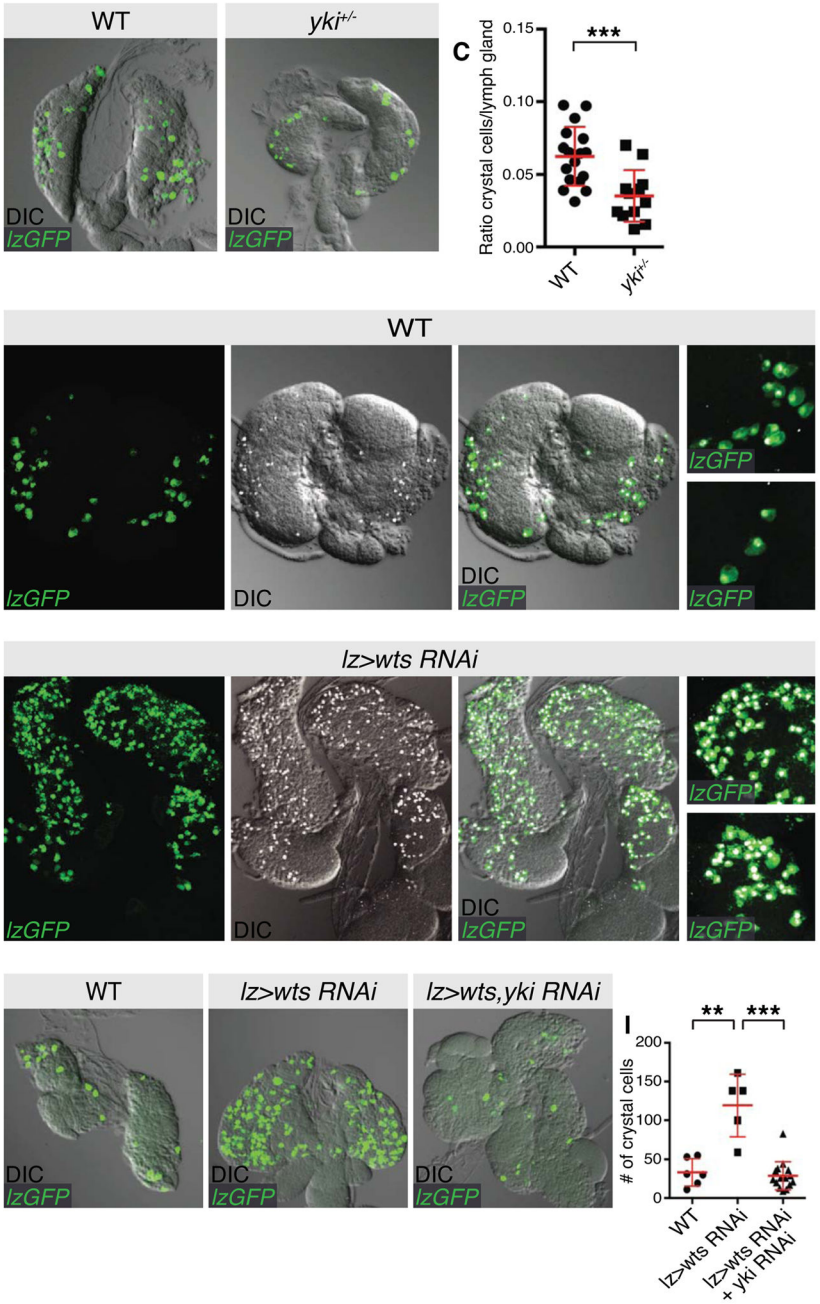

Figure 3. The Hippo pathway transcriptional regulator Yorkie influences crystal cell numbers

(A–C) A control w^{1118} lymph gland (A) and a lymph gland that was hemizygous for yki^{B5} (B). Crystal cells were visualized by expression of *UAS-GFP* (green) with the *lz-Gal4* driver. The ratio of GFP-positive crystal cells relative to DAPI-positive lymph gland cells from multiple animals were quantified in (C). Lymph glands from yki^{B5} hemizygous animals possessed significantly fewer crystal cells (p=0.0008; n=17 for A, n=12 for B; error bars represent SEM).

(D–E) Control lymph glands (D–D‴) or lymph glands expressing the *wts* RNAi transgene (E–E‴) under the control of the *lz-Gal4* driver. Crystal cells were visualized by co-

expression of *UAS-GFP* (green in D–E) and staining for Hnt (greyscale in A′–C′ and merged with direct interference contrast images). GFP and protein expression images are merged with direct interference contrast images in (D″–E″). Magnified images are shown in (D‴–E‴) showing that *lz-Gal4 GFP* and Hnt expression overlap in both genotypes.
(F–I) Control lymph glands (F), lymph glands expressing the *wts* RNAi transgene (G) or lymph glands expressing RNAi transgenes for *wts* and *yki* (H) under the control of the *lz-Gal4* driver. Crystal cells were visualized by expression of *UAS-GFP* (green in F–H) and merged with direct interference contrast images. GFP-positive crystal cells were quantified in (I). *wts RNAi*-expressing lymph glands possessed significantly more crystal cells than controls ($p=0.001$; n=6 for F, n=5 for G; error bars represent SEM), while *wts* RNAi, *yki* RNAi-expressing lymph glands significantly suppressed this increase ($p<0.0001$; n=5 for G, n=15 for H; error bars represent SEM).
Scale bars = 20 μm. See also Figure S2.

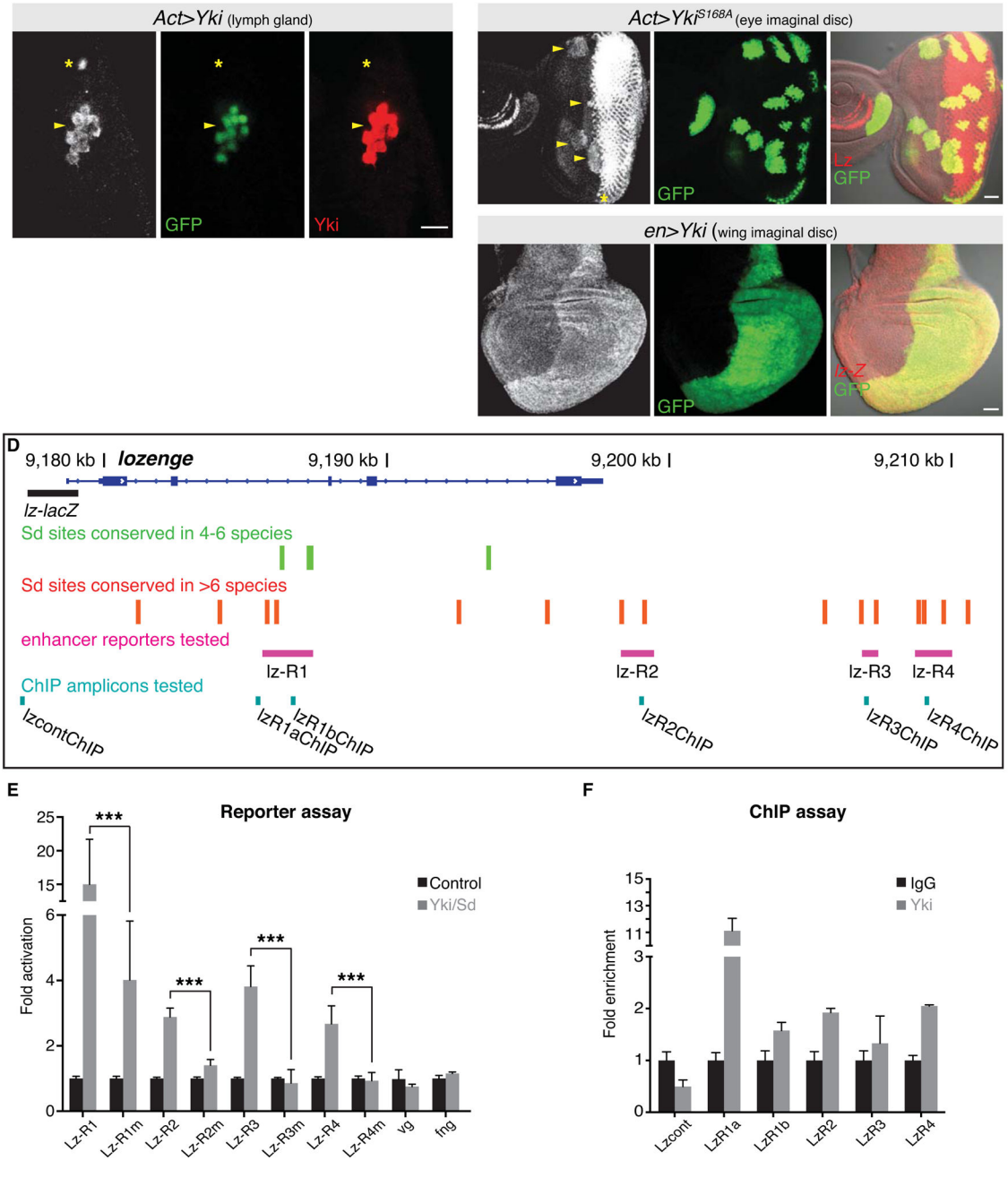

Figure 4. The Hippo pathway regulates expression of the key crystal cell fate determinant, Lozenge

A–B) A primary third instar larval lymph gland (A–A″) and eye-antennal disc (B–B″) harbouring clones of tissue expressing *UAS-yki* transgenes (marked by GFP in green). Expression of Lz (greyscale in A and B, red in merged image B″) and Yki (red in A″) are shown. Ectopic expression of Lz was induced by *yki* overexpression in both lymph glands (indicated by arrowhead 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) *lz* expression, as reported by a 1.5kb 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 greyscale in the single channel (C) and red in the merged image (C″), while *UAS-yki* expression is marked by co-expression of *UAS-GFP* (green in C′ and C″).
(D) Schematic of the *lz* gene locus showing conserved Sd sites, the *lz-lacZ* reporter, the *lz* reporters (pink) and the ChIP-qPCR amplicons (turquoise).
(E) Reporter assays demonstrate that several regions of the *lz* locus that bear conserved Sd sites can mediate transcriptional activation by Sd/Yki in Kc167 cells. When these conserved Sd sites were mutated ("m"), this activation was suppressed or abolished. The *vg* and *fng* reporters serve as negative controls.
(F) ChIP-qPCR validates binding of endogenous Yki to several of these reporter regions in Kc167 cells, particularly to the R1 region. Yki did not associate with the control region lacking Sd sites.
Scale bars = 20μm. See also Figures S3 and S4.