A Genetic Screen Reveals an Unexpected Role for Yorkie Signaling in JAK/STAT-Dependent Hematopoietic Malignancies in Drosophila melanogaster

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ABSTRACT A gain-of-function mutation in the tyrosine kinase JAK2 (JAK2V617F) causes human myeloproliferative neoplasms (MPNs). These patients present with high numbers of myeloid lineage cells and have numerous complications. Since current MPN therapies are not curative, there is a need to find new regulators and targets of Janus kinase/Signal transducer and activator of transcription (JAK/STAT) signaling that may represent additional clinical interventions. Drosophila melanogaster offers a low complexity model to study MPNs as JAK/STAT signaling is simplified with only one JAK [Hopscotch (Hop)] and one STAT (Stat92E). hopTumorous-lethal (Tum-I) is a gain-of-function mutation that causes dramatic expansion of myeloid cells, which then form lethal melanotic tumors. Through an F1 deficiency (Df) screen, we identified 11 suppressors and 35 enhancers of melanotic tumors in hopTum-l animals. Dfs that uncover the Hippo (Hpo) pathway genes expanded (ex) and warts (wts) strongly enhanced the hopTum-I tumor burden, as did mutations in ex, wts, and other Hpo pathway genes. Target genes of the Hpo pathway effector Yorkie (Yki) were significantly upregulated in hopTum-l blood cells, indicating that Yki signaling was increased. Ectopic hematopoietic activation of Yki in otherwise wild-type animals increased hemocyte proliferation but did not induce melanotic tumors. However, hematopoietic depletion of Yki significantly reduced the hopTum-I tumor burden, demonstrating that Yki is required for melanotic tumors in this background. These results support a model in which elevated Yki signaling increases the number of hemocytes, which become melanotic tumors as a result of elevated JAK/STAT signaling.

KEYWORDS Drosophila melanogaster hopscotch Stat92E hippo expanded yorkie bantam warts Myc

The JAK/STAT pathway is evolutionarily conserved and plays critical roles in numerous developmental processes, including hematopoiesis (Levy 1999; Amoyel et al. 2014). JAKs are nonreceptor cytosolic tyrosine kinases that are normally activated by cytokines interacting with their cell surface receptors. The activated JAK–receptor complexes induce phosphorylation of STATs that subsequently bind specific DNA sequences and act as transcription factors (O’Shea et al. 2002). A dominant-active allele JAK2V617F leads to a constitutively-active, ligand-independent protein (James et al. 2005; Jones et al. 2005; Kralovics et al. 2005; Yan et al. 2005; Levine et al. 2005). This mutation is a causal event in the development of the human MPNs polycythemia vera, essential thrombocythemia, and primary myelofibrosis (Tefferi 2016). In mouse models of MPNs, JAK2V617F ectopically activates STAT5, and genetic removal of STAT5 impedes the development of these diseases (Walz et al. 2012; Yan et al. 2012; Sachs et al. 2016). MPN patients present with high numbers of myeloid lineage cells and a variety of symptoms, including splenomegaly, and they have complications such as heart attacks,

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stroke, and deep vein thrombosis (Wadleigh and Tefferi 2010). Multiple therapies including JAK2 inhibitors alleviate symptoms but are not curative (Tefferi 2016). Therefore, there is a pressing need to find new regulators and targets of JAK/STAT signaling that may provide druggable therapies.

In recent years, *Drosophila* has emerged as a powerful model organism for studying hematopoiesis [reviewed in Crozatier and Vincent (2011); Evans et al. (2014); Honti et al. (2014); and Gold and Bruckner (2015)]. There are two waves of hematopoiesis in *Drosophila*, one during embryogenesis and one during larval stages. The precursors of blood cells, called prohemocytes, form in the embryonic mesoderm, and most of them differentiate into plasmatocytes, which function as macrophages in removal of apoptotic corpses, wound healing, and immunity (Tepass et al. 1994; Wood and Jacinto 2007). Other prohemocytes develop into crystal cells, an insect-specific cell type that promotes melanization reactions (Lebestky et al. 2000). In larval stages, embryonically-derived plasmatocytes migrate to microenvironments called “hematopoietic pockets” located in the larval body wall (Makhijani et al. 2011; Makhijani and Bruckner 2012). These segmentally-repeated pockets, plasmatocytes form resident (or sessile) clusters and are supported by the peripheral nervous system (Markus et al. 2009; Makhijani et al. 2011). Pocket-associated plasmatocytes self-renew and proliferate, resulting in a nearly 30-fold expansion of the plasmatocyte pool during larval development (Leitao and Sucena 2015; Petraki et al. 2015). At the earliest larval stages, all plasmatocytes reside in hematopoietic pockets. From the second larval instar, some plasmatocytes in the pockets are progressively released into circulation (Makhijani et al. 2011). However, certain conditions, like injury or immune challenge, cause sessile hemocytes to prematurely enter circulation in large numbers (Markus et al. 2009; Makhijani et al. 2011; Gold and Bruckner 2015). These pockets are sites of bona fide hematopoiesis because, in addition to promoting plasmatocyte self-renewal, resident plasmatocytes can differentiate into crystal cells (Bretscher et al. 2015; Leitao and Sucena 2015). Under immune challenge, like parasitoid wasp ovidposition, these resident cells can also differentiate into lamellocytes, which are large, flat, adherent cells that are normally absent in wild-type larvae (Markus et al. 2009). Lamellocytes encapsulate foreign objects such as parasitoid wasp eggs that are too large to be phagocytosed.

A second wave of hematopoiesis occurs in the larval lymph gland, a reservoir of prohemocytes that differentiate during second and third larval instars (Tepass et al. 1994; Lebestky et al. 2000; Mandal et al. 2004; Jung et al. 2005). Under normal, nonimmune challenged conditions, lymph gland prohemocytes give rise to ~2000–3000 blood cells, most of which are plasmatocytes with a smaller number of crystal cells. Under immune-challenged conditions, lymph gland prohemocytes can also differentiate into lamellocytes (Rizki 1978; Evans et al. 2003; Jung et al. 2005). All hemocyte differentiation in the lymph gland is completed by early pupal development, and differentiated blood cells are released into circulation when this organ disintegrates (Grigorian et al. 2011). Lineage-tracing studies have shown that the hemocyte pool in the adult is comprised of cells from both the embryonic and larval lineages (Holz et al. 2003). It is currently debated whether there is hematopoiesis in the adult with most groups finding no evidence of de novo generation of blood cells at this stage (Rizki 1978; Lanot et al. 2001; Horn et al. 2014; Van De Bor et al. 2015).

The *Drosophila* JAK/STAT pathway is simplified with only three ligands, one cytokine receptor, one JAK (Hop), and one STAT (Stat92E) (Amoyel et al. 2014). A gain-of-function, temperature-sensitive mutation in the *Drosophila* JAK, hop<sup>Tum-l</sup>, causes a dramatic increase in plasmatocytes and lamellocytes, leading to the formation of melanotic tumors that are visible in larval, pupal, and adult stages (Figure 1B, arrow; Corwin and Hanratty 1976; Hanratty and Dearolf 1993; Luo et al. 1995, 1997). Prior reports have characterized the phenotype caused by the hop<sup>Tum-l</sup> mutation. Melanotic tumors are not observed in this genotype until the middle of the third larval instar (Hanratty and Ryser 1981; Lanot et al. 2001). When reared at the restrictive temperature (29°C), lymph glands from late second and early third instar hop<sup>Tum-l</sup> larvae lack the anterior lobe, the primary site of larval hematopoiesis in wild-type animals, but contain an increased number of hypertrophied secondary lobes. By late third instar at 29°C, the lobes of the hop<sup>Tum-l</sup> lymph gland have dispersed, presumably releasing hemocytes into circulation (Hanratty and Ryser 1981; Jung et al. 2005; Sorrentino et al. 2007). When reared at 25°C, lamellocytes comprise 10–15% of circulating hemocytes in second instar hop<sup>Tum-l</sup>-labeled, and this percentage increases to nearly 50% by third instar (Lanot et al. 2001). By contrast, lamellocytes are not observed in healthy wild-type animals reared under the same conditions. Additionally, the premature differentiation of lamellocytes in lymph glands of hop<sup>Tum-l</sup> larvae can be observed as early as the second instar (Lanot et al. 2001). Cells from hop<sup>Tum-l</sup> lymph glands are neoplastic and, when serially transplanted into naive hosts, cause melanotic tumors for at least five generations (Hanratty and Ryser 1981). Reducing the genetic dose of Stat92E markedly reduces the number and size of melanotic tumors in hop<sup>Tum-l</sup>-labeled animals (Luo et al. 1997; Shi et al. 2006).

To identify genes that dominantly enhance or suppress the melanotic tumor burden in hop<sup>Tum-l</sup>-labeled animals, we conducted an F1 Df screen. Here, we report that genes encoding Hpo pathway components dominantly modify JAK/STAT-induced melanotic tumors. Hopo is a conserved tumor suppressor pathway that restrains the activity of Yki/Yes-associated protein (YAP) [reviewed in Irvine and Harvey (2015)]. Yki is a transcriptional coactivator that functions with several DNA-binding proteins, including a TEA/ATTS transcription factor Scalloped (Sd), to upregulate target genes that promote proliferation and inhibit apoptosis (Goulev et al. 2008; Wu et al. 2008). Through this F1 screen, we found that heterozygosity of Df(2L)al and of Df(3R)ED6346 strongly enhanced the tumor burden in hop<sup>Tum-l</sup>-labeled animals. Df(2L)al uncovers **ex**, which encodes a protein that acts upstream of the core Hpo kinase cascade (Boedigheimer and Laughon 1993; Hamaratoglu et al. 2006), and Df(3R)ED6346 uncovers **wts**, which encodes a serine–threonine kinase that inactivates Yki (Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003). Mutations in **ex** and **wts** and in genes encoding other Hpo components significantly modified the tumor burden in hop<sup>Tum-l</sup>-labeled animals. Expression of the Yki target genes **bantam** (bam), a micro RNA (miRNA), and **Myc** (Thompson and Cohen 2006; Neto-Silva et al. 2010) were significantly increased in hop<sup>Tum-l</sup>-labeled hemocytes compared to controls, indicating that Yki has increased activity in hop<sup>Tum-l</sup>-labeled blood cells. Finally, we showed that Yki is necessary for JAK/STAT-dependent melanotic tumors as hematopoietic depletion of Yki from hop<sup>Tum-l</sup>-labeled animals significantly reduced the tumor burden. While ectopic activity of Yki in a wild-type background increased plasmatocyte proliferation, it did not induce lamellocyte differentiation or melanotic tumors. These results support a model where increased hematopoietic activity of Yki in hop<sup>Tum-l</sup>-labeled animals promotes proliferation of circulating plasmatocytes, which can be induced to transdifferentiate into lamellocytes by the ectopic JAK/STAT signaling in these cells.

**MATERIALS AND METHODS**

**Genetics and fly stocks**

We obtained the following stocks from the Bloomington *Drosophila* Stock Center (BDSC): Df stocks covering chromosomes 2 and 3 from
Figure 1 Characterization of the hematopoietic system in hopTum-l animals. (A and B) Light micrographs of third instar wild-type (A) and hopTum-l (B) larvae. The arrow in (B) highlights a large melanotic tumor in the hopTum-l larva, which is not seen in wild-type (A). (C–H) Hemolymph bleeds from control HaHmlLT > GFP larvae reveal P1-positive (red) plasmatocytes (C) but not L1-positive (red) lamellocytes (D) in circulation. Hemolymph bleeds from hopTum-l; HaHmlLT > GFP larvae reveal the presence of both P1- and L1-positive cells in circulation (E–F). Bleeds from HaHmlLT > hopTum-l, where UAS-hopTum-l is mis-expressed in the entire hematopoietic compartment, show P1- and L1-positive cells in circulation (G–H) as well as microtumors (H’, arrow). P1 is red in (C), (E), and (G); L1 is red in (D), (F), and (H). GFP labels hemocytes and F-actin is blue. (I–L) In control larvae, X-gal staining shows that srpHemo-Gal4 lineage hemocytes (I, arrow) are present in larval circulation. In hopTum-l larvae, X-gal staining reveals that the srpHemo-Gal4 lineage gives rise to plasmatocytes and lamellocytes (J, arrow and arrowhead, respectively). In control larvae, no circulating cells are positive for the Dot-Gal4 lineage (K). However, in hopTum-l larvae, both plasmatocytes and lamellocytes arise from the Dot-Gal4 lineage (L, arrow and arrowhead, respectively). (M–O) Hematopoietic pockets (M, arrows) from a control (HaHmlLT > GFP) larva where the entire hematopoietic compartment is labeled with GFP. Pockets are not observed in a hopTum-l; HaHmlLT > GFP larva (N). The lack of hematopoietic pockets is autonomous to the hematopoietic lineage because hemocyte residence in these structures is not observed when UAS-hopTum-l is mis-expressed in a HaHmlLT > GFP larva (O). The arrowheads in (N) and (O) mark GFP-positive microtumors within the abdominal cavity. Anterior is to the left and dorsal is up in (M–O). Bar, 50 μM. GFP, green fluorescent protein.
that covers one complete abdominal segment was classified as a 1.0, and so forth. Each individual progeny was given a TI, which is the sum of all tumor sizes per animal [adapted from Shi et al. (2006)]. For statistical purposes, the TI for each individual was normalized to the average TI of the control hopTum-l/+ outcross set up in parallel and divided by the SD of that control using the following formula: 

\[ \text{Normalized TI} = \frac{\text{[TI]}}{\text{[TI]control} - \text{[SD]control}} \]

We then computed the average normalized TI (NTI) of all individuals in each genotype. NTIs were graphed with SE bars by GraphPad Prism 7. Genotypes that significantly modified the NTI by 1 SD above or below that of the control were classified as enhancers or suppressors, respectively. Except for rare cases of unhealthy crosses, the minimum sample size of each Df screened (hopTum-l/+; Df/+)) was 15. Modifying Dfs were independently validated.

**Immunohistochemistry**

Hemocytes were bled from wandering third instar larvae into 30 μl phosphate buffered saline (1X PBS) and allowed to settle for 30–45 min on Superfrost Plus microscope slides (Fisher Scientific Catalog# 1255034). Hemocytes were fixed with 4% formaldehyde or 4% paraformaldehyde in PBS for 10 min, washed twice for 10 min in 0.1% Triton, 1X PBS (PBS-T), and then blocked for 1 hr at room temperature or overnight at 4°C in 10% normal goat serum (NGS, Vector, Catalog# S-1000) in PBS-T. Hemocytes were incubated in primary antibody diluted in blocking solution overnight at 4°C, with the exception of anti-Stat92E, which was incubated overnight at room temperature. The following antibodies were used: rabbit anti-Stat92E [1:250, Flaherty et al. (2010)]; rabbit anti-Myc (1:1000, a gift of R. Eisenman); mouse anti-Pi (Nimrod C) and mouse anti-L1 (Atilla) [both at 1:10, a gift of I. Andó (Kurucz et al. 2007)]; mouse anti-BGal (1:250, Developmental Studies Hybridoma Bank); chicken anti-BGal (1:250, Immunology Consultants Lab, Catalog# CGAL-45A-Z); chicken anti-GFP (1:500; Aves labs, Catalog# A6455); rabbit anti-RFP (1:500, Medical & Biological Laboratories Co., Ltd., Catalog # PM005); guinea pig anti-Scallop (1:500, gift from J. Zeitlinger [Ikmi et al. 2014]); mouse anti-LaminD (1:100); and Phalloidin, which stains F-actin (1:25, Molecular Probes). All secondary antibodies (Jackson Immunologicals) were used at 1:200. Samples were mounted in Vectashield (Vector, Catalog# H-1000). Images were acquired using a Zeiss LSM510 confocal microscope. Images were processed using Adobe Photoshop and figures were made with Adobe Illustrator.

For mixed cell experiments, hemolymph from DsRed-positive control larvae was bled into the same well as that from unlabeled experimental larvae. Fixation and immunostaining were performed as described above.

**5-ethyl-2’-deoxyuridine (EdU) labeling**

EdU-labeling of hemocytes was performed using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen, Molecular Probes #C10356). Hemocytes from third instar larvae were dissected into 15 μl of 10 μM EdU and allowed to settle for 30 min into 5 mm wells on Superfrost Plus microscope slides made with a pip pen (Sigma-Aldrich #Z377821). After 30 min of EdU incorporation, the solution was removed and replaced with 4% paraformaldehyde for 10 min. The samples were washed twice with PBS-T. Samples were incubated with primary mouse anti-Pi and fluorescent secondary antibodies as described above. The cycloaddition reaction was performed per the manufacturer’s instructions. Samples were mounted in Vectashield. We imaged 10–15 areas of the slide at random at 25x using a Zeiss.
LSM510 confocal microscope. To calculate the percentage of proliferating plasmatocytes, we used ImageJ to quantify the number of P1-positive and EdU-positive cells.

**Statistics**

For quantification of intensity of GFP, Myc, or Stat92E, we used ImageJ. First, we defined single cells (either DsRed-positive control hemocytes or DsRed-negative hopTum-l allele Hemocytes) in a single 1 μm confocal slice or maximum intensity projection. We then determined the average gray value of the green channel for each genotype. The gray values of the controls were normalized to 1, and then the hopTum-l samples were normalized to the control.

All numerical data were plotted and analyzed using GraphPad Prism7, and Student’s t-tests were used determine statistical significance.

**Lineage-tracing experiments**

y w UAS-flp/Y, tub-Gal80°; act5c FRT stop FRT nuc lacZ/Tb males were crossed separately to virgins of these four genotypes: (1) srpHemoGal4, UAS-srceGFP, (2) hopTum-l/++; srpHemoGal4, UAS-srceGFP; (3) Dot-Gal4, and (4) hopTum-l/++; Dot-Gal4. Crosses were reared at 29°C to inactivate Gal80. Hemocytes were bled from wandering third instar larvae into 30 μl 1× PBS and allowed to settle for 30 min on Superfrost Plus microscope slides. They were then fixed in 1% glutaraldehyde for 15 min, followed by X-gal staining at 37°C for 30 min. DIC images of hemocytes (at 40x) were obtained using a Zeiss Axioplan microscope with a Retiga Evi (QImaging) digital camera and QCapture Pro 6.0 software.

**Hematopoietic pockets**

HaHmlLT > GFP flies were crossed with Ore°, UAS-hopTum-l, hopTum-l/FM7, or UAS-YkT(Δ166A)V3 flies and reared at 29°C. Third instar larvae of the correct genotype were collected and washed in 1× PBS. Handling of the larvae, which can disrupt hematopoietic pockets (Makhijani et al. 2011), was kept to a minimum. Larvae were immobilized on chilled microscope slides and images were obtained using a Nikon D5100 camera mounted on a Nikon SMZ 1500 dissecting microscope with UV X-Cite 120 at 5× magnification.

**Data availability**

Sticks are available upon request or from the BDSC. Table S1 contains the full data set for the hopTum-l DF screen. Figure S1 contains expression patterns of Dia1p-lacZ and Scalloped in control and hopTum-l hemocytes. Figure S2 contains expression pattern of Stat92E in in control and hopTum-l hemocytes.

**RESULTS AND DISCUSSION**

**Characterizing the hopTum-l allele**

After cleaning up the hopTum-l chromosome (see Materials and Methods), we wanted to characterize this allele. We crossed virgin hopTum-l females balanced over an FM7 chromosome to Ore° males. At 25°C, 68% of heterozygous hopTum-l/+ adult animals developed melanotic tumors. At 29°C, the penetrance increased to 99%, consistent with an earlier report (Corwin and Hanratty 1976). We labeled the entire hematopoietic lineage using the HaHmlLT-G4b driver and UAS-GFP crossed to Ore° (for the control) or hopTum-l. As expected, hemolymph bleeds from control HaHmlLT > GFP larvae contained P1-positive plasmatocytes but no lamellocytes (Figure 1, C–D’). By contrast, hemolymph bleeds from hopTum-l, HaHmlLT > GFP third instar larvae revealed the presence of both plasmatocytes and lamellocytes [Figure 1, E–F and Lanot et al. (2001)]. The precocious appearance of lamellocytes in hopTum-l animals is due to autonomous activation of the JAK/STAT pathway within hemocytes because these observations can be recapitulated by mis-expression of UAS-hopTum-l within the hematopoietic lineage (Figure 1, G–H’). Because the lymph gland in hopTum-l animals disperses prior to pupariation, we wanted to determine whether lymph gland-derived hemocytes were present in the circulating pool in hopTum-l larvae (Hanratty and Ryerse 1981; Jung et al. 2005; Sorrentino et al. 2007). We used a lineage-tracing technique and Gal4 lines that are restricted to the embryonic lineage [srpHemo-Gal4 (Bruckner et al. 2004)] or larval lymph gland [Dot-Gal4 (Honti et al. 2010)]. As expected, we found embryonic-lineage hemocytes in larval circulation in controls [Figure 11, arrow and (Bruckner et al. 2004)]. In hopTum-l larvae, embryonic-lineage hemocytes gave rise to labeled plasmatocytes as well as lamellocytes (Figure 1J, arrow and arrowhead, respectively). In control larvae, lymph gland-lineage cells were not found in circulation (Figure 1K). In contrast, lymph gland-lineage hemocytes, including cells with lamellocyte characteristics, were present in circulation in hopTum-l larvae (Figure 1L, arrow and arrowhead). At larval stages, sessile hemocytes reside in hematopoietic pockets in the body wall (Markus et al. 2009; Makhijani et al. 2011). Whereas hematopoietic pockets were apparent in control HaHmlLT > GFP larvae (Figure 1M, arrows), they were not observed in hopTum-l, HaHmlLT > GFP larvae (Figure 1N). The cell-autonomy and specificity of this result were confirmed by mis-expressing UAS-hopTum-l using the HaHmlLT-G4b driver. No pockets were observed in HaHmlLT > hopTum-l larvae (Figure 1O). Therefore, in hopTum-l larvae the circulating pool of hemocytes is comprised of both embryonic- and lymph gland-derived cells, and there are alterations in hemocyte residence in larval hematopoietic pockets.

**A screen for modifiers of hopTum-l melanotic tumors**

We wanted to establish an F1 genetic screen with the goal of identifying novel modifiers of JAK/STAT-induced melanotic tumors. We first screened the left arm of the second chromosome (2L). hopTum-l virgin females were crossed to DF males, and the crosses were reared at 29°C. F1 adult females that were heterozygous for hopTum-l and for the DF were scored for the presence and size of abdominal melanotic tumors. As described in the Materials and Methods, we used the abdominal segments as a ruler to measure how much of each segment a melanotic tumor covered. The sum of all tumor sizes within an adult was that individual’s T. A normalized T (NTI) for each individual adult was obtained by comparing its T to that of an outcross control (hopTum-l/FM7 virgins × Ore° males) set up at the same time and reared under identical conditions. The NTI for each genotype was calculated by averaging all of the T scores for each individual in that genotype. An interaction was considered significant if the NTI was > 1 SD above or below the relevant control (see Materials and Methods). Although we observed increased lethality accompanied by lower recovery of progeny at 29°C, we identified multiple DNs on 2L that significantly modified the hopTum-l melanotic tumor phenotype (Figure 2 and Table S1). However, we chose to perform the rest of screen at 25°C to mitigate the enhanced lethality at 29°C.

Overall, we tested 363 fly stocks, covering chromosomes 2 and 3, from the Bloomington DF Kit. The majority of DNfs tested fell within 1 SD of the control and were considered to have no effect (see Materials and Methods, Table S1). Two DNfs resulted in synthetic lethality as no adult offspring were recovered (Table S1). However, 46 DNfs significantly altered the hopTum-l melanotic tumor phenotype (Figure 2 and Table S1). Specifically, 35 DNfs enhanced the NTI, while 11 DNfs suppressed it. We compared our list of modifier regions to genes previously reported
to be involved in inducing blood cell masses when mutated or overexpressed (Betz et al. 2001; Hwang et al. 2002; Baeg et al. 2005; Muller et al. 2005; Minakhina and Steward 2006; Shi et al. 2006; Shravage et al. 2013). Included in this list are Dfs uncovering plume, HEM-protein, hairy, polo, and Autophagy-related 6 (Atg6) (Table S2; Shi et al. 2006; Shravage et al. 2013). The limited overlap of our screen with other known factors suggests that we may discover new modifiers of JAK/STAT signaling or of blood cell development.

**Mutation of the Hpo component Ex enhances hopTum-1 melanotic tumors**

One of the strongest enhancers identified from our screen was Df(2L)al, which deletes the cytological region 21C1–21C7 (Figure 2). Among the genes uncovered by this deficiency, we thought that ex was the strongest candidate for being the underlying modifier. ex encodes a FERM domain-containing protein that promotes Hpo activity. Loss of ex leads to decreased Hpo activity and increased Yki activity (Hamaratoglu et al. 2006). Other laboratories have described a positive role for Yki in promoting plasmatocyte proliferation and crystal cell differentiation in the larval lymph gland (Ferguson and Martinez-Agosto 2014; Milton et al. 2014), supporting our hypothesis that Yki activation will promote JAK/STAT-induced tumorigenesis. We tested three different alleles of ex and found that two (ex1 and exNTI) significantly enhanced the hopTum-1 tumor phenotype to similar levels seen with Df(2L)al (Figure 3, A–C). A third allele (ex697, an enhancer trap) mildly enhanced the hopTum-1 tumor burden (Figure 3C).

**Mutations in several Hpo pathway genes modify the tumor index in hopTum-1 animals**

As Ex primarily functions through the Hpo signaling pathway to regulate cell proliferation and apoptosis (Hamaratoglu et al. 2006), we examined our Df screen data to determine if other Dfs uncovering Hpo components were putative modifiers of hopTum-1. We found that a Df uncovering wts was the strongest enhancer in the screen: Df(3R)ED6346 (Figure 2). While this does not prove that the Hpo pathway is a general modifier of hopTum-1, it suggests that Ex is not acting independently in this role. We sought to clarify this by testing if single hypomorphic mutations of other Hpo components were able to alter JAK/STAT-induced tumorigenesis. To accomplish this, we crossed hopTum-1 females to males carrying known Hpo pathway mutant alleles and scored the NTI in the F1 double heterozygote progeny (i.e., hopTum-1/+; mutation/+). We found that heterozygosity for alleles in multiple negative regulators of Yki activity (wts, ex, mats, hpo, deo, kibra, sav, and ft) significantly enhanced the NTI in hopTum-1 animals (Figure 3C, green bars). Moreover, heterozygosity for yki itself significantly suppressed the NTI in hopTum-1 animals, similar to that observed with Stat92E heterozygosity (Figure 3C, purple bar for yki and red bar for Stat92E). These data suggest that enhancing Yki activity in hopTum-1 animals increases the melanotic tumor burden.

**The Yki targets ban and Myc are upregulated in hopTum-1 hemocytes**

Yki has been shown to upregulate multiple transcriptional targets, including ban miRNA, Diap1, ex, and Myc (Huang et al. 2005; Hamaratoglu et al. 2006; Thompson and Cohen 2006; Neto-Silva et al. 2010). In order to determine if hopTum-1 might function through any of these well-characterized Yki targets, we compared their expression on the same slide using mixed-cell experiments where we bledd onto the same slide DsRed-positive control hemocytes and DsRed-negative hopTum-1 hemocytes. To monitor expression of the ban miRNA, we used a ban sensor where two copies of a 31 bp ban target sequence have been inserted into the 3’UTR of Tub-eGFP.
et al. 2003). When ban is present, GFP expression is lower. When ban is reduced or absent, GFP expression is higher. We crossed the ban sensor into both HmlΔDsRed controls and DsRed-negative hopTum-l animals. In mixed-cell experiments, we found that while there was variable but detectable expression of the ban sensor in DsRed-positive control hemocytes (Figure 4, A–A”, open arrowheads), the sensor was expressed at significantly lower levels in DsRed-negative hopTum-l blood cells (Figure 4, A–A”, solid arrowheads, quantified in Figure 4A”).

Figure 3 Mutations in Hpo pathway components significantly modify hopTum-l melanotic tumors. (A and B) Light micrographs of a representative hopTum-l/+;+/+ adult female (A) showing a typical abdominal melanotic tumor and of a representative hopTum-l/+;exWTY/+ adult male showing enhanced tumor size. Red bracketed lines indicate the extent of the melanotic tumors in (A and B). (C) Graph of NTI ± SE for each genotype. Heterozygosity for mats, ex, wts, hpo, kibra, ft, and dco (green bars) enhanced tumors in hopTum-l/+ flies, while heterozygosity for yki (purple bar) strongly suppresses them. Heterozygosity for crb and ed (gray bars) had no appreciable effect on tumor formation in hopTum-l/+ flies. The red bar indicates the positive control for suppression, heterozygosity for strong hypomorphic allele Star92E797. Hpo, Hippo; LFO, loss-of-function; NTI, normalized tumor index.

Figure 4 Expression of Yki targets ban and Myc are significantly increased in hopTum-l hemocytes. (A and B) Mixed-cell experiments of hemo-lymph bleeds where control hemocytes are DsRed-positive and hopTum-l hemocytes are DsRed-negative. Both genotypes express the ban sensor. Open arrowheads (A–A”) indicate DsRed-positive control hemocytes that have appreciable levels of GFP, indicating that ban miRNA is low in these cells. By contrast, solid arrowheads (A–A”) indicate DsRed-negative hopTum-l hemocytes that have low levels of GFP, indicating that ban miRNA is higher in these cells. (A”) Quantification of fluorescence intensity of the ban sensor. hopTum-l hemocytes (green bar) have significantly lower ban sensor levels than the control (red bar) (P < 0.0001). (B) Mixed-cell experiment with anti-Myc (green). Open arrowheads (B–B”) indicate DsRed-positive control hemocytes that have appreciable levels of Myc. Solid arrowheads (B–B”) indicate DsRed-negative hopTum-l hemocytes that have higher levels of Myc than controls. (B”) Quantification of fluorescence intensity of Myc. hopTum-l hemocytes (green bar) have significantly higher levels of Myc than the control (red bar) (P < 0.0001). DsRed is red and Lamin D, which marks the nucleus, is blue in (A and B). Bar, 10 μM. GFP, green fluorescent protein; miRNA, micro RNA; Yki, Yorkie.
These data indicate that ban miRNA is expressed at higher levels in hopTum-l hemocytes than controls, suggesting that Yki activity is higher in the former. We also observed significantly higher Myc protein expression in hopTum-l hemocytes compared to controls [compare solid (hopTum-l) arrowheads to open (control) arrowheads in Figure 4, B–B’], quantified in Figure 4B” (P, 0.0001). These data also suggest that Yki activity is higher in hemocytes with sustained JAK/STAT activity. However, we observed no significant change in Diap1-lacZ expression [Figure S1A”, compare solid (hopTum-l) arrowheads to open (control)] in these cells. Finally, we sought to determine whether Sd, the Yki binding partner involved in crystal cell specification in the lymph gland (Ferguson and Martinez-Agosto 2014; Milton et al. 2014), was expressed in peripheral blood cells and whether it had altered expression in hopTum-l hemocytes. Using the mixed-cell experimental approach, we observed no alteration in Sd expression in DsRed-negative hopTum-l vs. DsRed-positive control hemocytes [Figure S1B”, compare solid (hopTum-l) arrowheads to open (control)]. Altogether, these data reveal that a subset of Yki target genes are upregulated in hopTum-l animals heterozygous for Hpo pathway components.

Ectopic Yki signaling does not upregulate JAK/STAT activity

We reasoned that increased Yki activity may enhance hopTum-l melanotic tumors by elevating JAK/STAT signaling in hemocytes. To test this hypothesis, we monitored Stat92E activation in DsRed-positive control hemocytes and in DsRed-negative hemocytes mis-expressing an activated form of Yki (UAS-YkiS168A.V5) (Oh and Irvine 2008). We stained this mixed-cell experiment with an antibody to Stat92E, a well-established read-out for JAK/STAT pathway activation (Flaherty et al. 2010). We observed no alteration in Stat92E protein staining in Hml > YkiS168A.V5 hemocytes compared to controls [compare solid (hopTum-l) arrowheads to open (control) in A–F], indicating that not all Yki targets are upregulated in these cells. Finally, we sought to determine whether Sd, the Yki binding partner involved in crystal cell specification in the lymph gland (Ferguson and Martinez-Agosto 2014; Milton et al. 2014), was expressed in peripheral blood cells and whether it had altered expression in hopTum-l hemocytes. Using the mixed-cell experimental approach, we observed no alteration in Sd expression in DsRed-negative hopTum-l vs. DsRed-positive control hemocytes [Figure S1B”, compare solid (hopTum-l) arrowheads to open (control)]. Altogether, these data reveal that a subset of Yki target genes are upregulated in hopTum-l animals heterozygous for Hpo pathway components.

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arrowheads to open (control) arrowheads in Figure S2A–A”, quantified in Figure S2A”. These data suggest that ectopic Yki activation does not upregulate Stat92E function in hemocytes and that enhanced melanotic tumor formation upon reduced Hpo activity is not a result of augmented JAK/STAT signaling by Yki.

**Ectopic Yki activation in hemocytes increases plasmatocyte proliferation but does not induce lamellocyte differentiation**

One possibility is that sustained Yki activation in peripheral blood cells induces plasmatocyte proliferation and lamellocyte differentiation. This hypothesis is consistent with the known role of Yki in promoting proliferation of plasmatocytes in the lymph gland (Ferguson and Martinez-Agosto 2014; Milton et al. 2014). However, Yki is not required for lamellocyte differentiation in response to waps infestation, suggesting that Yki activation is not sufficient to induce the lamellocyte fate (Ferguson and Martinez-Agosto 2014). To test our hypothesis directly, we mis-expressed activated Yki (UAS-YkiS168A.V5) using the HaHmlLT-Gal4 driver. Hemolymph from larvae with hematopoietic Yki activation contained numerous plasmatocytes compared to controls, but we observed no lamellocytes in these larvae (Figure 5, C–D). Additionally, these animals (HaHmlLT > YkiS168A.V5, hopTum-L; HaHmlLT-Gal4) induced robust lamellocyte differentiation and the formation of microtumors (Figure 5, E and F, arrow). These results demonstrate that Yki activation in hematopoietic tissue is not sufficient to induce lamellocyte differentiation. Furthermore, they suggest that Yki activity expands the circulating plasmatocyte pool by increasing proliferation. To test this hypothesis, we ex vivo labeled circulating plasmatocytes with the S phase marker EdU in control, HaHmlLT > YkiS168A.V5, hopTum-L, and HaHmlLT > hopTum-L larvae. There were significantly more EdU-positive, P1-positive cells in HaHmlLT > YkiS168A.V5 larvae compared to control [Figure 5G, blue bar (Control), yellow bar (UAS-YkiS168A.V5), P < 0.01]. We also observed significantly more cycling plasmatocytes in hopTum-L and HaHmlLT > hopTum-L larvae compared to control [Figure 5G, green bar (hopTum-L), pink red (UAS-hopTum-L) P < 0.001 for hopTum-L and P < 0.01 for UAS-hopTum-L]. Furthermore, unlike hopTum-L and HaHmlLT > hopTum-L larvae, which had altered hemocyte residence in hematopoietic pockets (Figure 1, N and O), HaHmlLT > YkiS168A.V5 larvae had hematopoietic pockets that appeared similar to control larvae (compare Figure 5H to Figure 1M). These results indicate that ectopic Yki activation was not sufficient to perturb hemocyte residency in pockets. Taken together, these results indicate that Yki activation in hemocytes increases the rate of plasmatocyte proliferation but does not directly promote lamellocyte differentiation.

**Hematopoietic depletion of Yki reduces the tumor burden in hopTum-L animals**

Finally, we wanted to determine the hematopoietic importance of Yki in JAK/STAT-dependent melanotic tumors. To accomplish this, we depleted Yki from hopTum-L animals by RNAi using HaHml-Gal4 that targets the entire hematopoietic lineage. Two independent UAS-RNAi lines targeting Yki (HMS00041 and JF03119) significantly reduced the NTI of hopTum-L adult animals (Figure 6, light and dark purple bars; P < 0.0283 for HMS00041 and P < 0.0023 for JF03119). However, neither suppressed the NTI to the extent observed with heterozygosity for a hypomorphic Stat92E mutation (Figure 6, red bar; P < 0.0001). While this could be due to inefficiency of RNAi, we favor the interpretation that knockdown of Yki suppresses the enhanced proliferation of hemocytes in hopTum-L animals, while reduction of Stat92E in this background suppresses plasmatocyte proliferation and lamellocyte differentiation.

**DISCUSSION**

Using an F1 genetic modifier screen, we have identified Dfs uncovering ex and wts as the strongest enhancers of JAK/STAT-induced melanotic tumors. Furthermore, we determined that reducing the genetic dose of ex, wts, or other negative regulators of Yki significantly enhanced the tumor burden in hopTum-L animals, while reducing yki significantly suppressed it. We showed that the Yki targets ban and Myc are upregulated in hopTum-L hemocytes compared to controls and that knockdown of Yki in the hemocyte lineage suppresses the hopTum-L phenotype. Future work will need to determine how Yki activity is upregulated in hopTum-L hemocytes. One possibility is that ectopic JAK/STAT signaling inhibits the expression or function of a negative regulator of Yki (e.g., Ex, Hpo, or Wts). Another potential mechanism is that JAK/STAT signaling promotes the expression or function of a positive regulator of Yki, possibly even Yki itself. One issue that arises from our study is how Hpo signaling might act in circulating hemocytes, as the pathway has generally been studied in the context of cell-cell contact in epithelial tissue (Irvine and Harvey 2015). Notably, Yki has been...
reported to regulate Myc through ban miRNA in glial cell proliferation (Reddy and Irvine 2011), suggesting that Hpo signaling plays a role in nonhemipithelial cells. Moreover, this regulation appears to be through Merlin–Hpo, not through other upstream components like Ex, suggesting that Hpo–Yki signaling may be regulated differently in certain cell types or tissues.

We plan to map, at the level of the gene, other modifiers identified through this screen in the future. While we identified interactors common to other JAK/STAT gain-of-function screens, numerous hits were unique to ours. The differences between this report and a previous screen for JAK/STAT-mediated tumorigenesis might be due to (1) the parameters we used for scoring and defining significance, resulting in differences in classification, and (2) the use of earlier Df kits by Shi et al. (2006) that contain Dfs with different coverage, thus similar regions may not be identified. Going forward, we will also need to consider interacting Dfs/underlying modifiers in light of our new finding that residence in hematopoietic pockets is perturbed in hopTum-l larvae and results from autonomous JAK/STAT activation in the hematopoietic compartment. Subsequent work will be needed to determine whether homing to pockets, length of residence in the pockets, cell-cell contact in pockets, and/or transit between pockets and circulation are altered in hopTum-l animals, and whether modifiers identified by the screen impact any of these parameters.

Reports from other laboratories have shown that dysregulation of Jun N-terminal Kinase (JNK), Toll (Tl), and Ras causes lamellocyte differentiation and melanotic tumors (Lemaître et al. 1995; Luo et al. 1995; Qiu et al. 1998; Asha et al. 2003; Zettervall et al. 2004; Minakchina and Steward 2006). In the future, it will be of interest to determine if Hpo signaling specifically plays a role in hopTum-l-induced melanotic tumors, or if Hpo has a broader role in melanotic tumors induced by other pathways. Finally, it would be important to determine if increased YAP activity is observed in samples from MPN patients or from mice harboring the JAK2V617F mutation.

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