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Activation of a *Drosophila* Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects

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In mammals, many cytokines and growth factors stimulate members of the Janus kinase (JAK) family to transduce signals for the proliferation and differentiation of various cell types, particularly in hematopoietic lineages. Mutations in the *Drosophila hopscotch* (*hop*) gene, which encodes a JAK, also cause proliferative defects. Loss-of-function alleles result in lethality and underproliferation of diploid tissues of the larva. A dominant gain-of-function allele, *Tumorous-lethal* ($hop^{Tum\text{-}l}$), leads to formation of melanotic tumors and hypertrophy of the larval lymph glands, the hematopoietic organs. We show that a single amino acid change in Hop is associated with the $hop^{Tum\text{-}l}$ mutation. Overexpression of either wild-type *hop* or $hop^{Tum\text{-}l}$ in the larval lymph glands causes melanotic tumors and lymph gland hypertrophy indistinguishable from the original $hop^{Tum\text{-}l}$ mutation. In addition, overexpression of Hop in other tissues of the larva leads to pattern defects in the adult or to lethality. Finally, overexpression of either *hop* or $hop^{Tum\text{-}l}$ in *Drosophila* cell culture results in tyrosine phosphorylation of Hop protein. However, overexpression of $hop^{Tum\text{-}l}$ results in greater phosphorylation than overexpression of the wild-type. We conclude that $hop^{Tum\text{-}l}$ encodes a hyperactive Hop kinase and that overactivity of Hop in lymph glands causes malignant neoplasia of *Drosophila* blood cells.

Key words: *Drosophila*/hematopoiesis/JAK tyrosine kinase/neoplasia/signal transduction

Introduction

The *hopscotch* (*hop*) gene of *Drosophila melanogaster* encodes a non-receptor tyrosine kinase (Binari and Perrimon, 1994), related to the Janus kinase (JAK) family identified in mammals (Firmbach-Kraft *et al.*, 1990; Wilks *et al.*, 1991). To date, the mammalian JAK family contains four members that share structural similarity (reviewed by Ihle *et al.*, 1994; Wilks and Harpur, 1994). This class of kinases is distinguished by the presence of a second, degenerate kinase-like domain for which no catalytic activity has been demonstrated (Wilks *et al.*, 1991). In addition, JAKs lack the SH2, SH3 and transmembrane domains found in many other tyrosine kinases.

The mammalian JAKs transduce signals through cytokine and growth factor receptors (see reviews, Kishimoto *et al.*, 1994; Wilks and Harpur, 1994; Ihle and Kerr, 1995). Many of these receptors lack intrinsic kinase activity, but recruit and activate JAKs when stimulated by ligand binding. Different receptor subunits interact preferentially with and activate specific JAKs (summarized by Briscoe *et al.*, 1994; Ihle and Kerr, 1995). Upon ligand binding, cytokine receptors dimerize and stimulate the tyrosine phosphorylation of bound JAKs, probably through auto- or *trans*-phosphorylation (see review, Wilks and Harpur, 1994). Activation of JAKs leads to tyrosine phosphorylation of downstream effectors, such as the STAT family of transcription factors (reviewed by Briscoe *et al.*, 1994; Darnell *et al.*, 1994; Ihle *et al.*, 1994). Phosphorylated STATs are translocated to the nucleus, where they induce transcription of other genes, thus potentiating cytokine signaling.

Drosophila hop has also been implicated in the regulation of cellular proliferation. Loss of *hop* function results in larval/pupal lethality and underproliferation of diploid tissues (Perrimon and Mahowald, 1986), indicating that *hop* is required for cell proliferation. However, unique among the JAKs is the role of a dominant gain-of-function *hop* mutation in neoplasia. Previous work has suggested that *Tumorous-lethal* (*Tum-l*), a mutation causing formation of melanotic tumors and proliferative defects in larval blood cells (Hanratty and Ryerse, 1981), is a lesion in the *hop* locus (Hanratty and Dearolf, 1993). No such oncogenic activity has been reported for a mammalian JAK. However, involvement of a JAK in neoplasia would not be surprising, as defects in many molecules involved in signal transduction within mitogenic or differentiative pathways have been associated with transforming activity in vertebrates (see reviews, Cantley *et al.*, 1991; Baserga, 1994).

Tum-l is an X-linked, dominant mutation that results in two phenotypes: formation of melanotic tumors and temperature-sensitive lethality. At restrictive temperatures (>25°C), *Tum-l* is a recessive lethal. Also at restrictive temperatures, *Tum-l* induces melanotic tumor formation in a dominant fashion, but is recessive at lower temperatures. Neoplastic transformation in the *Tum-l* mutation is limited to larval blood cells, which constitute the *Drosophila* immune system (reviewed by Gateff, 1978). The larval tissues that produce blood cells are the lymph glands, small organs arranged in pairs of lobes along the dorsal vessel, which are normally comprised of stem cells and undifferentiated blood cells. In circulation, these cells can divide or differentiate into either of the two classes of mature blood cells. The first class is the podocytes and lamellocytes, which are macrophage-like cells that are involved in encapsulation and phagocytosis of foreign objects. The second class is the crystal cells, which are involved in melanization. *Tum-l* causes hypertrophy of the

larval lymph glands (Hanratty and Ryerse, 1981) and premature differentiation of lamellocytes (Silvers and Hanratty, 1984). It is surmised that overproduction of lamellocytes leads to invasion of normal larval tissues, causing subsequent encapsulation and melanization. Moreover, the neoplastic lymph glands of *Tum-l* larvae retain the ability to overproliferate and cause lethality when transplanted to a wild-type host (Hanratty and Ryerse, 1981).

A number of other melanotic tumor mutations have been identified in *Drosophila* (Gateff, 1978; Watson *et al.*, 1991). One of the few melanotic tumor mutations that has been characterized molecularly is *aberrant immune response 8* (*air8*). Like *Tum-l*, *air8* causes overgrowth of the larval lymph glands, premature differentiation of lamellocytes with resultant melanized aggregates (Watson *et al.*, 1991), and blood cell-autonomous melanotic tumors (Bryant *et al.*, 1993). *air8* encodes the *Drosophila* homologue of the ribosomal S6 protein (Watson *et al.*, 1992; Bryant *et al.*, 1993). As with the JAKs, no mammalian neoplasm has been associated with mutations in S6, although phosphorylation of S6 has been correlated with the activity of mitogenic pathways (Traugh and Pendergast, 1986; Sturgill and Wu, 1991). The phenotypes of *hop* and *air8* mutations indicate that both cause regulatory defects in blood cell proliferation resulting in malignant melanotic neoplasia.

To investigate the proliferative defects associated with mutations in *hop*, we have analyzed *Tum-l* molecularly and have overexpressed both *Tum-l* and wild-type *hop* *in vitro* and *in vivo*. A specific amino acid alteration is identified in the *Tum-l* allele. Furthermore, it is demonstrated that the defects induced by *Tum-l* can be recapitulated by the overexpression of wild-type *hop*. Biochemical analysis suggests that this gain-of-function phenotype reflects Hop activation and is caused by increased tyrosine kinase activity of Hop.

Results

Characterization of the Tum-l DNA lesion

Genetic mapping of *Tum-l* placed it near *hop* on the X chromosome (Hanratty and Ryerse, 1981). All revertants of *Tum-l* also failed to complement recessive alleles of *hop*, indicating that *Tum-l* is either close or allelic to *hop* (Hanratty and Dearolf, 1993). Consistent with this hypothesis, Southern blot analysis of DNA from *Tum-l* and independent revertants of *Tum-l* showed that four of the five revertants contain detectable lesions within the DNA that encodes *hop* (Figure 1A). To determine whether *Tum-l* is a subtle alteration in *hop*, the *hop* coding region was sequenced from *Tum-l* DNA. When compared with wild-type strains, *Tum-l* was found to contain one nucleotide alteration resulting in an amino acid change. *Tum-l* encodes the substitution of glutamic acid for glycine at residue 341, in a region of *hop* for which a function has yet to be described (Figure 1B). Based on the failure of *Tum-l* revertants to complement *hop*, the alteration of *hop* DNA associated with those revertants, and the single amino acid substitution in *hop* found in *Tum-l*, we conclude that *Tum-l* is allelic to *hop*. Consistent with standard *Drosophila* nomenclature, we will refer to *Tum-l* as $hop^{Tum\text{-}l}$.

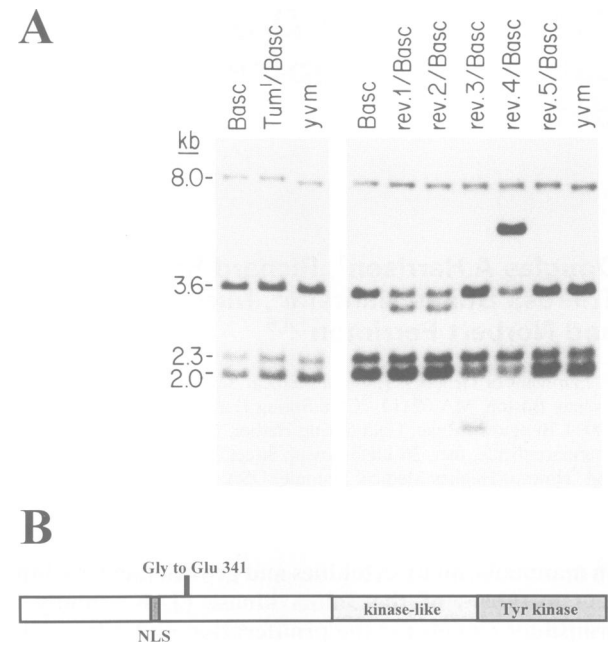

Fig. 1. Molecular analysis of $hop^{Tum\text{-}l}$ and $hop^{Tum\text{-}l}$ revertants. (**A**) To detect the positions of the lesions associated with mutations, equal amounts of DNA digested with *Sal*I were probed with the *hop* 5-1 cDNA (Binari and Perrimon, 1994). The left panel shows the absence of any alteration in $hop^{Tum\text{-}l}$ DNA as compared with either the *Basc* balancer chromosome or the *y v m* background chromosome. The right panel shows alterations found in DNA from four of five γ-ray-induced revertants of $hop^{Tum\text{-}l}$ ($hop^{Tum\text{-}l}$ revertants 1–4) (Hanratty and Dearolf, 1993) as compared with the *Basc* or *y v m* chromosomes. In each case, an alteration is detected in one or more of the *Sal*I genomic fragments to which the *hop* cDNA has been mapped (Binari and Perrimon, 1994). (**B**) DNA from $hop^{Tum\text{-}l}$ males was sequenced and compared with the sequence of nine different wild-type strains. A single alteration was found in $hop^{Tum\text{-}l}$ that was not present in any of the wild-type DNAs. This mutation is a G to A transversion at nucleotide 1641 resulting in the substitution of a Glu for a Gly at amino acid 341. The position of the amino acid change is indicated on a schematic of the Hop protein. The positions of the tyrosine kinase domain (Tyr kinase), the kinase-like domain (kinase-like), and a putative nuclear localization signal (NLS) are indicated.

Overexpression of wild-type hop causes tumors

To understand how $hop^{Tum\text{-}l}$ causes dominant defects, wild-type *hop* was overexpressed to try to reproduce the $hop^{Tum\text{-}l}$ phenotype. Flies were generated which carry a transposon bearing the *hop* cDNA under direct or indirect control of a heat shock promoter; both will be referred to as *hshop* animals. Direct control was achieved by fusion with the *Drosophila hsp70* promoter, while indirect heat shock expression was generated using a fusion of the yeast GAL4 UAS with *hop* and a separate insertion of the *hsp70*-GAL4 construct (Brand *et al.*, 1994). Animals were subjected to various heat shock regimens during development. Using indirect expression, a single heat shock of as little as 10 min during second or third instar larval development was sufficient to produce melanotic masses in larvae and pupae within 1–2 days (Figure 2). Tumor formation correlated closely with death and occurred only in animals containing both the hs-GAL4 and UAS-hop constructs, while animals carrying either construct alone survived and did not develop tumors. Direct heat shock expression gave similar results, but required longer heat shocks to obtain the same level of

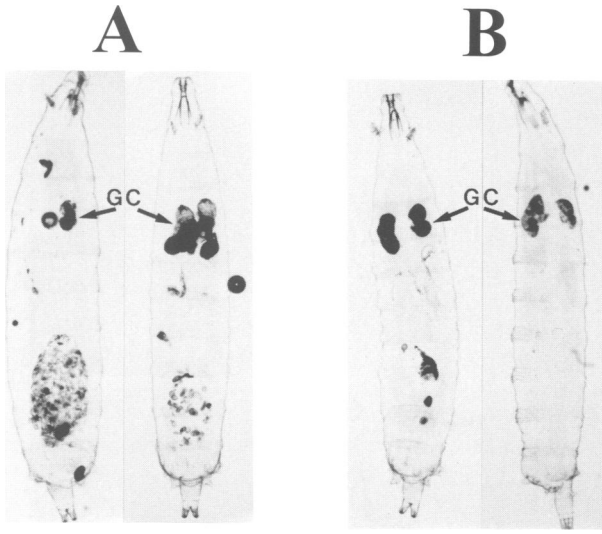

Fig. 2. Production of melanotic masses. (**A**) Melanotic masses of various sizes can be seen in several tissues of $hop^{Tum\text{-}l}$ larvae reared at the restrictive temperature of 29°C. The gastric cecae (GC), part of the larval anterior midgut, are melanized in most $hop^{Tum\text{-}l}$ animals. (**B**) Similarly prepared larvae are shown in which wild-type *hop* is overexpressed by heat shock. These animals also contain melanized tissues, including gastric cecae. The larvae shown carry a single copy of the two transposons, pUAS-hop and hs-GAL4. Similar results were obtained with flies in which *hop* was directly overexpressed under control of the *hsp70* promoter using the pCaShs-hop construct (data not shown).

melanotic tumor formation (not shown). Similar results were also obtained using a UAS-Tum construct (not shown). The rapid growth, invasiveness and lethality associated with the induced tumors indicate that the overexpression of *hop* leads to malignant neoplasia, indistinguishable from that seen in the $hop^{Tum\text{-}l}$ mutation.

As with $hop^{Tum\text{-}l}$, *hshop* larvae with tumors contained hypertrophied lymph glands (Figure 3). Although strictly organ-specific melanotic tumors have not been found in *Drosophila*, the tissues that become encapsulated can be diagnostic for particular melanotic tumor mutations (Sparrow, 1978). While many other tissues may be affected, the gastric cecae of the anterior midgut are the most commonly melanized structures of $hop^{Tum\text{-}l}$ larvae (Hanratty and Ryerse, 1981). In *hshop* animals, the primary target of blood cell encapsulation is also the gastric cecae (Figures 2 and 3). These similarities between the phenotypes of *hshop* and $hop^{Tum\text{-}l}$ suggest that they are mechanistically related and imply that the $hop^{Tum\text{-}l}$ lesion causes overactivity of Hop.

Tumor formation correlates with overexpression of hop in lymph glands

Tissue transplantation from $hop^{Tum\text{-}l}$ donor animals has indicated that the lymph glands can autonomously generate the melanotic masses associated with this mutation. The ability to overexpress *hop* under the direction of GAL4 has allowed us to further explore the spatial and temporal requirements for generation of melanotic tumors. Approximately 200 characterized and uncharacterized enhancer trap insertions of GAL4 expressed in a variety of patterns were crossed with UAS-hop or UAS-Tum insertion strains to examine the effects of restricted overexpression. Twelve of these GAL4 lines reproducibly caused the formation of melanotic masses in UAS-hop or UAS-Tum animals. The patterns of expression of GAL4 in third instar larvae were examined for these 12 lines and compared with the expression patterns for several lines which did not induce tumor formation (Table I). We found that the lymph glands of *hop*-overexpressing animals are responsible for the formation of melanotic tumors, consistent with $hop^{Tum\text{-}l}$ transplantations performed by Hanratty and Ryerse (1981). All 12 of the GAL4 lines which direct tumor formation had detectable expression of GAL4 in the third instar larval lymph glands (Table I). In nine of these lines, GAL4 was expressed uniformly throughout all the lobes of the lymph glands, although levels varied from line to line. In the remaining three lines, there was strong but patchy expression in multiple lobes of the lymph glands. While none of these lines expressed solely in the lymph glands, the lymph glands are the only tissue in which all 12 of these lines expressed GAL4.

By contrast, expression was analyzed from nine GAL4 lines which do not direct tumors, but do direct other defects in UAS-hop or -Tum animals (Table I). Five of these lines did not express at all in the lymph glands, while three others had weak, patchy expression in small regions of some of the smaller posterior lymph gland lobes. The ninth line showed strong expression of GAL4, but only in very few cells of the lymph glands. Furthermore, as a group, these GAL4 lines showed extensive, strong expression in essentially all other third instar larval tissues. The lack of melanotic tumors in UAS-hop and -Tum animals containing these GAL4 lines strongly suggests that overexpression of *hop* in tissues other than lymph glands cannot induce melanotic tumors.

Overexpression of hop in imaginal tissues correlates with adult defects

The effects of overexpression of *hop* are not restricted to larval blood cells. Overexpression of *hop* or *Tum* directed by GAL4 lines which express in imaginal tissues, the cells which give rise to adult structures, can cause defects in the adult fly. Imaginal tissues are derived from several small clusters of cells that are set aside during embryogenesis and ultimately give rise to adult structures. These imaginal cells proliferate and differentiate during larval development, then undergo morphological changes during pupariation to form the appropriate adult structures (see review, Bryant, 1978).

Directed overexpression of *hop* in larval imaginal discs caused adult defects that correlated closely with the larval expression patterns of the GAL4 lines. The wing imaginal disc gives rise to both wing blade and notum, the thoracic cuticle to which the wing is attached. Expression of *hop* in the wing disc can cause defects of the wing blade itself, such as ectopic vein in the proximal wing, loss of vein in distal wing, ectopic sensory organs and duplications of wing structures (Figure 4B and D). Expression in presumptive notal regions can result in notal outgrowths, ectopic notal bristles, loss of the scutellum and outstretched wings (Figure 4D and F). The eye–antennal imaginal discs give rise to most cuticular structures of the head. *hop* overexpression in the eye–antennal imaginal discs can cause large, misshapen or ectopic eye structures (Figure 4H). Expression during development of other imaginal

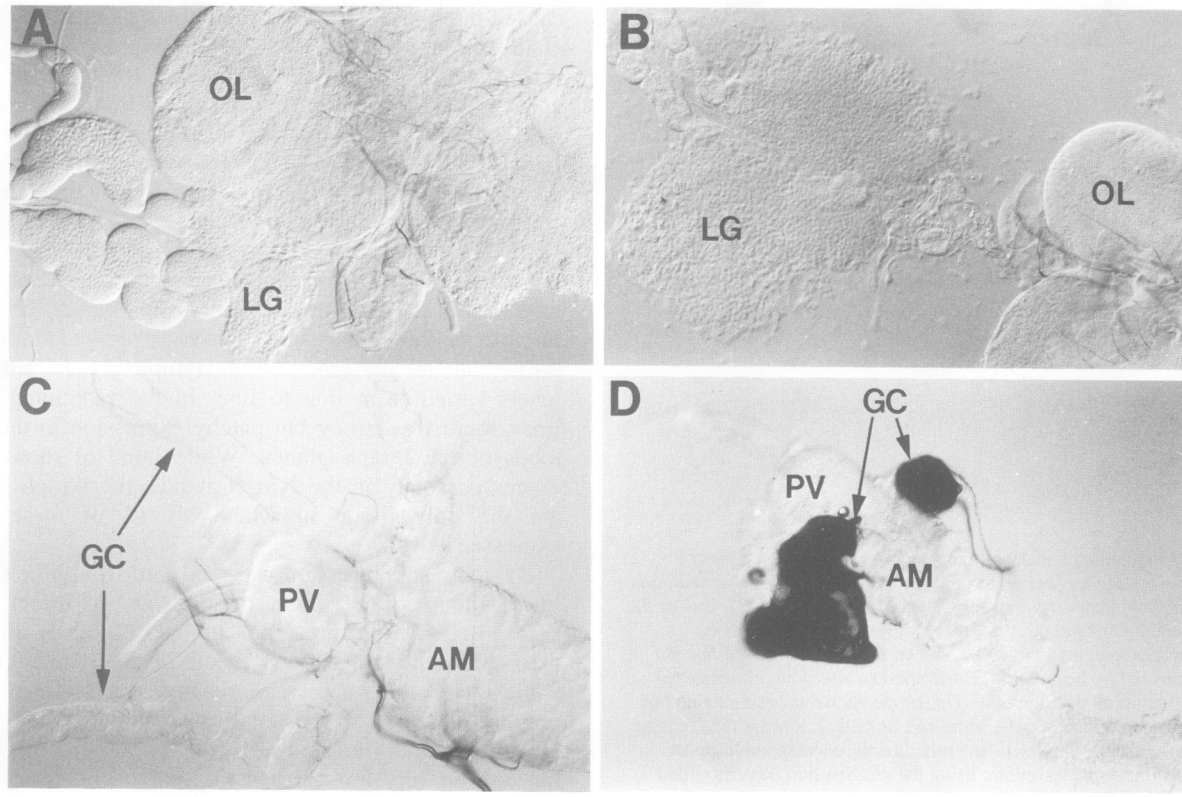

Fig. 3. Abnormal lymph glands and gastric cecae of *hshop* animals. Lymph glands from (**A**) wild-type, or (**B**) animals that carry both hs-GAL4 and pUAS-hop, are shown. The most anterior lymph gland lobes (LG) are grossly hypertrophied in the *hshop* animal. The overgrown lymph glands become very fragile and adherent and typically cannot be dissected without rupturing. The optic lobes (OL), dorsal vessel and pericardial cells are also visible. Gastric cecae are also shown from similarly prepared animals. The anterior portion of the gut of a wild-type larva is shown in (**C**), while melanized gastric cecae (GC) of a *hs-GAL4, UAS-hop* animal can be seen in (**D**). Other structures are the proventriculus (PV) and the anterior midgut (AM).

Table I. Comparison of tissue-specific expression with the formation of melanotic tumors

GAL4 line	Tumor formation	Lymph gland expression	Other expression
76B	Yes	Strong, patchy	SG, tr, OL, discs, brain
c135	Yes	Uniform	SG, FB, tr, Malp, OL, gut, brain
c273	Yes	Strong, patchy	SG, FB, tr, Malp, OL, cut, discs, gut, brain
c355	Yes	Uniform	SG, FB, tr, Malp, OL, cut, discs, gut, brain
c564	Yes	Uniform	SG, FB, discs, gut, brain
c729	Yes	Uniform	SG, FB, tr, Malp, discs, gut
c736	Yes	Uniform	SG, FB, tr, discs, gut
c754	Yes	Uniform	SG, FB, Malp, OL, discs, gut
e33C	Yes	Uniform	tr, Malp, OL, gut, brain
T32	Yes	Patchy	SG, FB, tr, discs
T59	Yes	Uniform	SG, FB, tr, OL, discs, gut, brain
T155	Yes	Uniform	SG, FB, tr, OL, cut, discs, gut, brain
c591	No	None	SG, FB, tr, Malp, discs, gut
e8A	No	None	cut, gut, brain
e13C	No	None	SG, FB, Malp, discs, gut
e16E	No	None	FB, discs, cut, gut
T13	No	Very weak, patchy	SG, FB, tr, Malp, OL, eye disc, brain
T76	No	Strong, patchy	SG, FB, tr, OL, discs, gut, brain
T98	No	Very weak, patchy	SG, tr, OL, discs, gut
T100	No	Weak, patchy	SG, tr, discs, gut
T110	No	None	SG, FB, Malp, discs, gut

Approximately 200 enhancer trap GAL4 lines (obtained from various sources listed in Materials and methods) were tested for ability to direct UAS-hop or UAS-Tum to generate melanotic tumors. Patterns of expression of all tumorigenic and some non-tumorigenic GAL4 lines were determined by mating to flies carrying an insertion of UAS-*lacZ* with flies from each GAL4 line. Third instar larval progeny were dissected and stained for β-galactosidase activity. SG, salivary glands; FB, fat bodies; tr., trachea, Malp, Malpighian tubules; OL, optic lobes; cut, cuticle; discs, imaginal discs.

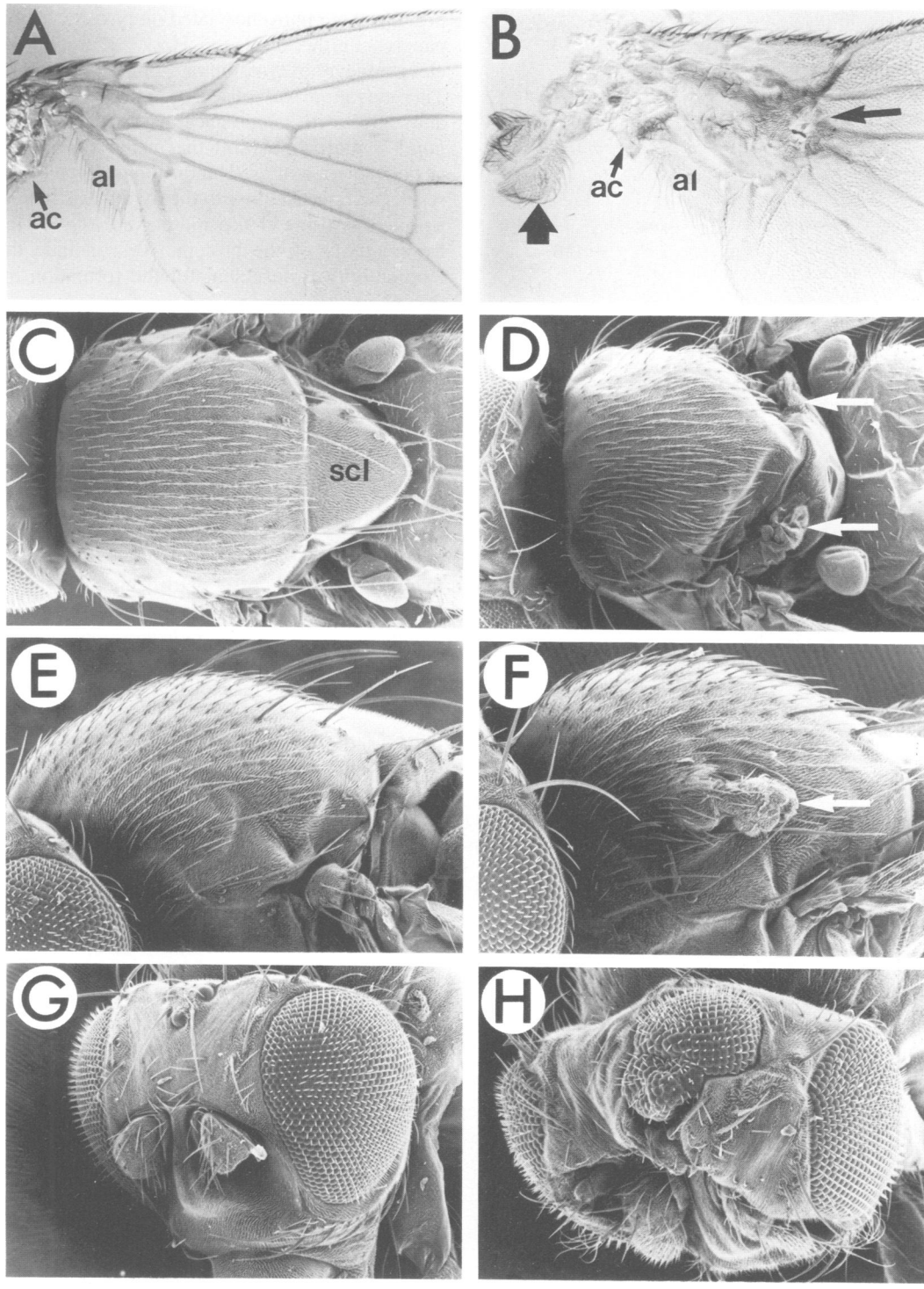

Fig. 4. Adult pattern defects caused by *hop* overexpression. *hop* was overexpressed in restricted imaginal tissues using enhancer trap GAL4 lines crossed with UAS-hop lines. Wings from (**A**) wild-type and (**B**) an adult carrying UAS-hop plus GAL-T59 are shown. GAL-T59 expresses GAL4 in both the blade and notal regions of the third larval instar wing imaginal disc. Adults from crosses with UAS-hop have excessive proximal wing vein (thin arrow) with ectopic sensory organs, loss of distal wing vein (not shown) and variable wing structure duplications (thick arrow). These duplications are usually located at the base of the axillary cord (ac) and resemble the allula (al) in general structure and bristle type. Scanning electron micrographs (SEMs) show (**C**) the wild-type notum as compared with (**D**) the notum from a *UAS-hop, dpp-GAL4* fly. dpp-GAL4 (Staehling-Hampton *et al.*, 1994) directs overexpression of *hop* to the anterior–posterior border in both the blade and notal regions of the wing imaginal disc. This results in blade disruptions similar to those seen with GAL-T59, as well as wing structure duplications (arrows) and almost complete loss of the scutellum (scl), also seen with *UAS-hop, GAL-T59* flies. SEMs of the lateral notum of (**E**) wild-type and (**F**) a *UAS-hop, GAL-c754* fly show an outgrowth of notal tissue (arrow) that is representative of abnormalities seen when *hop* overexpression is driven by some GAL4 lines. This defect is more variable than those described above and results in formation of indeterminate structures. GAL-c754 expresses GAL4 weakly in the notal region of the wing imaginal disc. SEMs show in panel (**G**) a wild-type adult head compared with (**H**) the head of a *UAS-hop, GAL-T13* adult. Overexpression of hop in the eye imaginal disc, as directed by GAL-T13, causes the formation of ectopic head structures, including extra eye material and the large orbital bristles that surround it.

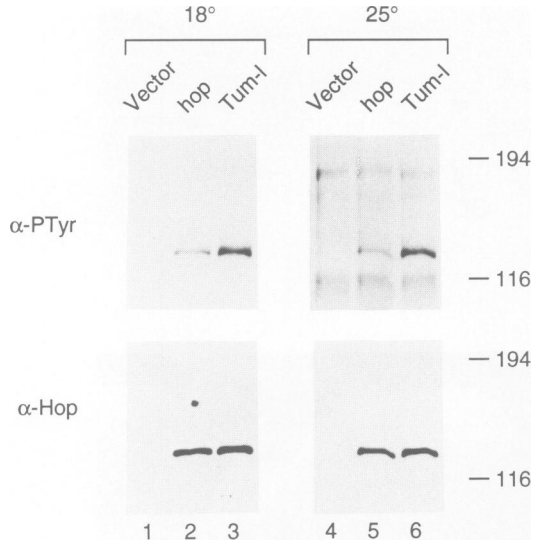

Fig. 5. Tyrosine phosphorylation of Hop proteins overexpressed in *Drosophila* S2 cells. S2 cells were transfected with empty vector (lanes 1 and 4), vector expressing Hop (lanes 2 and 5), or vector expressing Hop^{Tum-l} (lanes 3 and 6), as described in Materials and methods. Transfected cells were incubated for 18 h at 25°C and harvested directly (lanes 4–6) or shifted to 18°C for an additional 27 h before harvest (lanes 1–3). Whole-cell lysates were fractionated by SDS–PAGE, transferred to filters, probed with antibody to Hop (lower panels), stripped and re-probed with antibody to phosphotyrosine (upper panels). Antibody bands were visualized by enhanced chemiluminescence. Migration of marker proteins is indicated on the right.

structures can result in defects of the adult legs, genitalia or abdominal cuticle (not shown). The mechanisms by which these defects occur has yet to be determined, but *hop* overactivity in imaginal discs is clearly not associated with neoplasia or melanotic masses.

Overexpression of hop^{Tum-l} in Schneider cells results in increased tyrosine phosphorylation

The phenotypic similarities between *hop^{Tum-l}* and overexpressed *hop* suggest that *hop^{Tum-l}* encodes an activated form of Hop. Evidence from mammalian JAKs indicates that tyrosine phosphorylation of JAKs correlates with enzyme activation (Argetsinger *et al.*, 1993; Shuai *et al.*, 1993; Silvennoinen *et al.*, 1993; Witthuhn *et al.*, 1993). To investigate whether similar phosphorylation occurs in Hop activation, we examined the level of phosphotyrosine in Hop and Hop^{Tum-l} proteins overexpressed in *Drosophila* Schneider cells. The *hop* and *hop^{Tum-l}* cDNAs were placed under the control of the cytoplasmic actin promoter, and the resulting plasmids, pDAC5hop and pDAC5Tum, were transiently transfected into Schneider cells and grown at either 18° or 25°C. Whole-cell lysates were fractionated on gels, transferred to filters, and probed sequentially with Hop antiserum and with phosphotyrosine antiserum.

Schneider cells transfected with plasmid vector alone contained little protein reactive with the anti-Hop serum, while cells transfected with pDAC5hop and pDAC5Tum expressed similar significant amounts of an immunoreactive protein with molecular weight close to that predicted for Hop (Figure 5, lower panels). Consistent with studies of the mammalian JAKs (Silvennoinen *et al.*, 1993), both pDAC5hop and pDAC5Tum overexpressed proteins were phosphorylated on tyrosine (Figure 5, upper panels). Furthermore, the Hop^{Tum-l} protein exhibited an elevated level of tyrosine phosphorylation relative to Hop. This elevation was observed in cells grown at both 18° and 25°C, in accordance with the observation that *hop^{Tum-l}* mutants exhibit the melanotic tumor phenotype at both temperatures (Hanratty and Ryerse, 1981). These results are consistent with the hypothesis that *hop* encodes a protein tyrosine kinase and that the *hop^{Tum-l}* lesion causes hyperactivity of the protein. We conclude that elevated Hop activity is sufficient for the formation of melanotic tumors in *Drosophila*.

Discussion

The dominant, tumorigenic mutation *hop^{Tum-l}* is associated with a single amino acid substitution in the *hop* gene product. Hematopoietic neoplasia indistinguishable from the *hop^{Tum-l}* phenotype can be induced by the overexpression of either wild-type *hop* or *hop^{Tum-l}*. Formation of these tumors is closely correlated with the overexpression of *hop* in the larval lymph glands. Overexpression of *hop* in other tissues is also detrimental, but does not cause neoplasia. Analysis of Hop from *Drosophila* cells that overexpress the protein indicates that activated Hop is phosphorylated on tyrosine and that more Hop phosphorylation occurs on Hop^{Tum-l} than on wild-type Hop.

Blood cell involvement in the melanotic tumor phenotype

The aberrations associated with Hop overactivity represent the first dominant defects described for a JAK. Overexpression throughout the larva results in melanotic tumors and lethality, identical to the *hop^{Tum-l}* phenotype. Restricted expression of *hop* has implicated the lymph glands as the tissue responsible for melanotic tumor formation, consistent with results of *hop^{Tum-l}* lymph gland transplantations (Hanratty and Ryerse, 1981). Furthermore, essentially all larval tissues other than blood cells can be discounted as tumorigenic because overexpression of Hop in other tissues did not cause tumors. Similarly, mammalian JAKs have been implicated as components of signaling for proliferation principally in hematopoetic lineages (Briscoe *et al.*, 1994; Wilks and Harpur, 1994). Perhaps this similarity reflects a common origin of JAKs as mitogenic signaling molecules involved in blood cell development.

Activation of Hop by two distinct mechanisms

Dominant oncogenes can exert their transforming activity by one of two mechanisms: alteration of the normal protein product, such as with N-terminal truncations of *raf* or missense mutations in *ras*, or inappropriate expression of the normal protein product, such as with overexpression of *myc* (see reviews by Marshall, 1989; Baserga, 1994). Hop can induce neoplasia in *Drosophila* by either mechanism. The *hop^{Tum-l}* alteration results in an activated protein, as does the overexpression of wild-type Hop. The phenotypic similarities between these two activation mechanisms and the hyperphosphorylation of Hop^{Tum-l} relative to Hop in Schneider cells suggest that the *hop^{Tum-l}* lesion causes overactivity of Hop^{Tum-l}. This hyperactivity may occur because the *hop^{Tum-l}* mutation compromises a regulatory domain or may result in altered

affinity or specificity for substrate molecules. Overexpression of wild-type Hop may mimic the $hop^{Tum\text{-}l}$ phenotype by overwhelming regulatory molecules or by altering the kinetics of interaction with normal or inappropriate substrates. Alternatively, the lesion may result in increased stability of the $Hop^{Tum\text{-}l}$ protein. However, this seems unlikely, because equal amounts of pDAC5hop and pDAC5Tum plasmid used in transient transfections consistently resulted in less accumulation of $Hop^{Tum\text{-}l}$ than wild-type Hop (data not shown), suggesting that $Hop^{Tum\text{-}l}$ is actually less stable than wild-type.

Tyrosine phosphorylation associated with Hop activation

The defects resulting from Hop overactivity are apparently caused by ectopic activation of Hop associated with tyrosine phosphorylation. Tyrosine phosphorylation of other JAKs has been associated with activation by cytokines and growth factors (Argetsinger *et al.*, 1993; Shuai *et al.*, 1993; Silvennoinen *et al.*, 1993; Witthuhn *et al.*, 1993). Similarly, overexpression of Hop in *Drosophila* cells results in significant tyrosine phosphorylation of Hop, suggesting that the dominant phenotypes from Hop overexpression are due to activation of Hop signaling rather than dominant interference in other signaling pathways. Evidence from mammalian cells suggests that phosphorylation of JAKs occurs by an auto- or *trans*-phosphorylation mechanism (Argetsinger *et al.*, 1993; Muller *et al.*, 1993; Silvennoinen *et al.*, 1993). Consistent with these observations, overexpression of Hop leads to its own tyrosine phosphorylation. The simplest mechanism to explain this result is that Hop molecules can dimerize, then activate each other by tyrosine phosphorylation. Overexpression may simply increase Hop concentration, thus driving dimerization. Such a mechanism is consistent with current models for activation of other JAKs (see reviews, Ihle *et al.*, 1994; Wilks and Harpur, 1994).

Mechanism of hop defects in imaginal tissues

Overexpression of Hop in tissues other than the lymph glands can cause defects. GAL4-driven expression of Hop in imaginal tissues results in patterning defects in corresponding adult structures. These defects primarily involve gain of ectopic structure or apparent changes in cell fate, suggesting that *hop* overexpression can interfere with proper differentiation of imaginal tissues. In contrast, loss of *hop* results in proliferative defects in imaginal discs (Perrimon and Mahowald, 1986). Interestingly, overexpression of *hop* in the wing disc results in vein phenotypes similar to those seen in animals which express activated forms of *D-raf* (E.Noll, J.B.Duffy, X.Lu and N.Perrimon, in preparation). Loss-of-function *D-raf* mutations result in larval/pupal lethality with underproliferation of diploid tissues (Perrimon *et al.*, 1985), similar to *hop* mutations. Given the similarities in phenotypes, *hop* and *D-raf* may be components of overlapping signaling pathways. A possible common effector is the *Drosophila* epidermal growth factor receptor homologue (*DER*), which is also involved in wing vein formation (Clifford and Schupbach, 1989) and is genetically upstream of *D-raf* in other developmental processes (Brand and Perrimon, 1994; Diaz-Benjumea and Hafen, 1994). As it has also been demonstrated that vertebrate EGF signaling induces the activation of JAK1 (Shuai *et al.*, 1993), it is tempting to speculate that *DER* signaling may activate Hop as well.

Implications of Hop activation for neoplasia in Drosophila

As described, one class of tumorigenic mutations results in blood cell neoplasia and formation of melanotic masses. A second class of larval tumor mutants causes unregulated neural or epithelial proliferation and includes mutations in *discs-large (dlg)*, *lethal(2)giant larvae* and *fat*. These mutations cause imaginal discs to become disorganized, overgrow and lose the ability to differentiate (Gateff, 1978; Woods and Bryant, 1989; Mahoney *et al.*, 1991). In contrast to recessive mutations in genes such as *dlg* and *fat* (Bryant and Schmidt, 1990), Hop activation in imaginal tissues does not induce neoplasia or hyperplasia. Lack of a disc overgrowth phenotype associated with Hop activation or any other melanotic tumor mutation (Gateff, 1978; Watson *et al.*, 1991) suggests that neoplasia of the blood cells arises quite differently from that of imaginal tissues. The distinction between these two classes of tumorigenic mutations is supported by the finding that the molecules involved in imaginal disc overgrowth seem to have roles in cell adhesion and/or cell–cell interaction (reviewed by Bryant and Schmidt, 1990; Bryant *et al.*, 1993), while the characterized melanotic tumor mutations *air8* and *hop* are presumed to potentiate mitogenic signals (Watson *et al.*, 1992; Binari and Perrimon, 1994). This may reflect a basic difference between regulation of proliferation in imaginal discs and blood cells of the larva. The nature of the defects associated with overactivity of Hop in the lymph glands is apparently proliferative, while that of the imaginal tissues seems to be perturbation of cell fate. Perhaps this dichotomy results because the blood cells retain the ability to respond to exogenous proliferative signals throughout development (Gateff, 1978), while larval imaginal discs are already fated to become adult structures. Spurious signal transduction within the imaginal discs may only be able to alter the refinement of the adult pattern, whereas signaling in the blood cells triggers normal proliferation. Perhaps induction of neoplasia in imaginal tissues requires a more severe perturbation, such as complete disruption of the intimate cell associations of the disc by gross alteration of cell adhesion.

Materials and methods

Southern blot and DNA sequence analysis

Equal amounts of genomic fly DNA (Junakovic and Angelucci, 1986) were digested with *Sal*I and subjected to Southern blot analysis (Sambrook *et al.*, 1989), using ^{32}P-labeled *hop* cDNA (Binari and Perrimon, 1994) as probe. The sequence of the $hop^{Tum\text{-}l}$ allele was determined by sequencing cloned PCR fragments covering the entire *hop* coding region. Oligonucleotides used for PCR were as follows:

Pair A: ol24-GCTCTAGACCGATAGCGCTTTAAGCC
ol25-GCTCTAGAGTGGCCAAAAAACTGCCC
Pair B: ol26-GCTCTAGACTCGGTTGGGTCATACTC
ol27-GCTCTAGACCGGAAAACTTGTTCCG
Pair C: ol30-GCTCTAGAGAGTATGACCCAACCGAG
ol31-GCTCTAGACGCTTACTGATGATTGCC
Pair D: ol32-GCTCTAGAGGCCAGCATTAAAATGCGC
ol33-GCTCTAGAGCCAAGGAACTGGGCTTGC
Pair E: ol34-GCTCTAGAGCCTGATAACCAGCGAGG
ol35-GCTCTAGAGGACAGCAACAAATGTGCGC

Pair F: ol36-GCTCTAGAGGAAGCGAACTCTTTGGG
ol37-GCTCTAGACGCTGGCAAAGATTCTTG.

*Xba*I-cut PCR fragments were cloned into pBSK$^+$ (Stratagene), sequenced (Sanger *et al.*, 1977), and compared with wild-type genomic and cDNA sequences (Binari and Perrimon, 1994; R.Binari, unpublished). Three independent clones were sequenced from $hop^{Tum\text{-}l}$ to confirm any difference from wild-type. Two consistent changes leading to amino acid substitutions were found between $hop^{Tum\text{-}l}$ and the original wild-type genomic and cDNA clones. Genomic DNA from seven additional strains wild-type for *hop* was amplified by PCR and sequenced directly with the Stratagene Cyclist Pfu Exo$^-$ sequencing kit. One alteration was found in five of the additional wild-type strains and was discounted as a polymorphism, while the second change, a G to A at nucleotide 1641, was found in none of the wild-type strains. This mutation replaces Gly with Glu at amino acid 341 and is attributed to the $hop^{Tum\text{-}l}$ lesion (see text).

Overexpression of hop and Hop$^{Tum\text{-}l}$

The pUAS-hop construct was generated by insertion of a *Not*I–*Xba*I fragment containing the entire *hop* coding region from the hop 5-1 cDNA (Binari and Perrimon, 1994) into pUAST (Brand and Perrimon, 1993). The pCaShs-hop construct was generated by insertion of the same *Not*I–*Xba*I fragment of the *hop* cDNA into the CaSpeR-hs vector (Thummel and Pirrotta, 1992). The pCaShs-Tum construct was generated by the replacement of a 220 bp *Sac*II–*Bst*EII fragment from pCaShs-hop with the same fragment of PCR-generated DNA from $hop^{Tum\text{-}l}$ hemizygous flies. The pUAS-Tum was made by the insertion of the *Not*I–*Xba*I fragment from pCaShs-Tum into pUAST. These constructs were introduced into *y w; Δ2–3, Sb/TM6* flies by P element-mediated transformation (Spradling, 1986). Three independent transformant lines were recovered containing the pUAS-hop transposon, one line bearing the pUAS-Tum construct, and two lines with the pCaShs-hop transposon. Genomic DNA from each line was sequenced using a transposon-specific primer to verify the sequence of the *hop*/$hop^{Tum\text{-}l}$ divergent region.

Animals with tumors were generated by heat shock either of heterozygous pCaShs-hop-transformed animals or progeny from mating balanced lines of pUAS-hop or pUAS-Tum with a homozygous hsp70-GAL4 line (Brand *et al.*, 1994). Animals in plastic vials were heat shocked at 37°C for 0–30 min in a circulating water bath during second or third larval instar. Larvae were recovered 2 days later and mounted in Hoyer's mountant with lactic acid. All overexpression lines resulted in production of melanotic masses; however, there were quantitative variations among the different insertions.

To examine lymph gland size and morphology, animals were heat shocked as described above and lymph glands were dissected 2 days later, fixed 5 min in 3.7% formaldehyde in PBS, and mounted in 70% glycerol. Only the strongest-expressing insertion lines showed dramatic lymph gland hypertrophy.

GAL4 enhancer trap insertion lines

Approximately 200 enhancer trap insertions of GAL4 were kindly provided by several laboratories. Those described specifically in this work were obtained as follows: GAL-76B, -e8A, -e13C, -e16E, and -e33C were provided by A.Brand and K.Yoffe. GAL-c135, -c273, -c355, -c564, -c591, -c729, -c736, and -c754 were generated by K.Kaiser. GAL-T13, -T32, -T59, -T76, -T98, -T100, -T110, -T155 were generated by J.Urban and G.Technau. dpp-GAL4 was provided by K.Staehling-Hampton and F.M.Hoffman (Staehling-Hampton *et al.*, 1994).

Analysis of GAL4 expression by X-gal staining

The tissues in which GAL4 is active were determined for a number of enhancer trap lines (described in Table I) by the ability to drive expression of a UAS-*lacZ* reporter construct (Fischer *et al.*, 1988; Brand and Perrimon, 1993). Females homozygous for the UAS-*lacZ* insertion (Bg4-1-2) were mated with males from the GAL4 enhancer trap lines of interest. The progeny were dissected at third larval instar and stained for β-galactosidase activity using standard histochemical techniques. Dissected larvae were fixed for 5 min in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBT), washed in PBT and stained in X-gal staining solution (Klambt *et al.*, 1991) until color developed (~1 h).

Overexpression of hop and hop$^{Tum\text{-}l}$ in imaginal tissues

The consequences of *hop* and $hop^{Tum\text{-}l}$ overexpression in imaginal discs were determined by examining the progeny resulting from matings of UAS-hop and UAS-Tum lines with enhancer trap lines that express GAL4 in imaginal tissues. Crosses were performed at 25°C and viable adult or pharate adult progeny were collected. Wings from some adults were dissected and mounted in Hoyer's mountant with lactic acid in preparation for light microscopy. Other adults were preserved in 95% ethanol for analysis by scanning electron microscopy (SEM). Flies were prepared for SEM by critical point drying in CO_2 and coating with gold–palladium. Images were obtained on an AMRAY1000A scanning electron microscope.

Generation of Hop antisera

A 740 bp *Bam*HI fragment from the 5′ translated region of hop (Binari and Perrimon, 1994) was fused to a T7 RNA polymerase-inducible promoter at the *Bam*HI site of the pAR3040 vector (Rosenberg *et al.*, 1987). The ~34 kDa peptide from *hop* was overexpressed in *Escherichia coli* strain BL21(DE3) after induction with IPTG (Studier and Moffatt, 1986). Using standard methods (Harlow and Lane, 1988), cell extracts were electrophoresed in SDS–polyacrylamide gels and the band corresponding to the induced hop fusion protein was cut from the gel, washed, macerated and suspended in Freund's adjuvant. The emulsion was injected into rats to induce an immunological response. After two boosts, serum was recovered from the rats and used for Western analysis.

Overexpression of Hop and Hop$^{Tum\text{-}l}$ in S2 cells

Fragments carrying the entire coding region of the *hop* and $hop^{Tum\text{-}l}$ cDNAs were generated by PCR amplification using recombinant Pfu DNA Polymerase (Stratagene) and inserted into the *Drosophila* expression vector pDAC5, which expresses inserted genes from the actin-5C promoter (Natesan and Gilman, 1993). Constructs were verified by DNA sequencing.

Schneider line 2 cells were grown in Schneider's Insect Medium (Sigma) supplemented with 10% heat inactivated FBS (Hyclone), penicillin and streptomycin. Cells were grown at 25°C in the absence of CO_2 and subcultured 1:6 twice weekly, allowing the cells to reach a density of ~4×10^6 cells/ml. Cells were split 5–6 h before transfection to obtain a final density of 5×10^5 cells/ml in a total volume of 7 ml for each transfection. Cells were transfected by calcium phosphate co-precipitation (Natesan and Gilman, 1993). Transfection precipitates contained one of the following: 6 μg pDAC5, 3 μg pDAC5hop, or 6 μg pDAC5Tum-l. All transfection precipitates contained 1 μg of pACLacZ, a β-galactosidase reporter plasmid, which was used to monitor transfection efficiency, pUC119 DNA to a total of 20 μg in the final precipitate. Cells were incubated in the presence of transfection precipitate for 18 h at 25°C, and then harvested directly or shifted to 18°C for 27 h before harvest.

Analysis of Hop phosphorylation

Transfected cells were collected by centrifugation, washed in PBS containing 2 mM Na_3VO_4, and resuspended in an equal volume of buffer containing 120 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 300 mM 2-mercaptoethanol, 0.002% bromophenol blue, 2 mM EDTA, and 4 mM Na_3VO_4. A portion of the original cell suspension was reserved for determination of protein concentration by the Bradford assay using bovine gamma globulin as a standard.

Cell lysates containing 45 μg protein were fractionated on 7.5% polyacrylamide–SDS gels under standard conditions. Protein was electrophoretically transferred to Immobilon-P filters (Millipore) using a transfer buffer consisting of 50 mM Tris, 40 mM glycine, 20% MeOH, 0.02% SDS. Filters were probed first with rat anti-Hop serum (1:500 dilution) followed by HRP-conjugated sheep anti-rat antibody (Amersham, 1:3000 dilution). The filters were stripped in buffer containing 62.5 mM Tris, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS. They were re-probed with monoclonal anti-phosphotyrosine antibody 4G10 (UBI, 1:1000 dilution), followed by HRP-conjugated sheep anti-mouse antibody (Amersham, 1:3000). Bound antibody was visualized using the enhanced chemiluminescence system (ECL, Amersham) and exposure to Kodak XAR-5 film.

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