Inositol phosphate kinase 2 is required for imaginal disc development in *Drosophila*

Andrew M. Seeds[a,1,2], Marco M. Tsui[a,1], Christine Sunu[a], Eric P. Spana[b], and John D. York[a,c,3]

[a]Department of Pharmacology and Cancer Biology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710; [b]Department of Biology, Duke University, Durham, NC 27708; and [c]Department of Biochemistry, Vanderbilt University Medical Center, Nashville, TN 37232

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved November 16, 2015 (received for review July 27, 2015)

Inositol phosphate kinase 2 (Ipk2), also known as IP multikinase IPMK, is an evolutionarily conserved protein that initiates production of inositol phosphate intracellular messengers (IPs), which are critical for regulating nuclear and cytoplasmic processes. Here we report that Ipk2 kinase activity is required for the development of the adult fruit fly epidermis. Ipk2 mutants show impaired development of their imaginal discs, the primordial tissues that form the adult epidermis. Although disk tissue seems to specify normally during early embryogenesis, loss of Ipk2 activity results in increased apoptosis and impairment of proliferation during larval and pupal development. The proliferation defect is in part attributed to a reduction in JAK/STAT signaling, possibly by controlling production or secretion of the pathway's activating ligand, Unpaired. Constitutive activation of the JAK/STAT pathway downstream of Unpaired partially rescues the disk growth defects in Ipk2 mutants. Thus, IP production is essential for proliferation of the imaginal discs, in part, by regulating JAK/STAT signaling. Our work demonstrates an essential role for Ipk2 in producing inositide messengers required for imaginal disk tissue maturation and subsequent formation of adult body structures and provides molecular insights to signaling pathways involved in tissue growth and stability during development.

inositol phosphates | inositol phosphate multikinase | Ipk2 | IPMK | Upd

At the confluence of numerous signaling pathways is phospholipase C (PLC)-mediated production of the second messenger inositol 1,4,5-trisphosphate (IP_3), a key regulator of intracellular calcium release (1). IP_3 also serves as an essential substrate for the synthesis of inositol phosphates (IPs) and pyrophosphates (PP-IPs) that are critical for eukaryotic cellular function in their own right (2–6). Phosphorylation of IP_3 by conserved inositol phosphate kinases (IPKs) leads to the synthesis of complex pools of different IP species (6). Insights into the cellular functions of IPs have been gleaned by perturbing their synthesis through genetic manipulations of the IPKs. Consequently, defects in specific cellular processes were attributed to losses of particular IPs. This revealed that IPs are specific regulators of diverse cellular processes, such as transcription, chromatin remodeling, DNA repair, RNA editing, and RNA export (3–7).

An essential enzyme for the conversion of IP_3 to the array of IP and PP-IP species found in cells is the evolutionarily conserved inositol phosphate kinase 2 (Ipk2, Fig. 1*A*), which is also known as IP multikinase (IPMK) (4). Ipk2 was first identified in yeast as a nuclear enriched protein whose activities toward the 6- and 3-positions on the inositol ring sequentially convert IP_3 to IP_4, and then IP_5 (Fig. 1*A*) (8–10). Yeast Ipk2 mutants that fail to produce IP_4 and IP_5 exhibit defects in transcriptional responses and chromatin remodeling (3). Additional "multikinase" activities described for certain Ipk2 orthologs indicate an array of IP substrates, as well as the inositol lipid PIP_2 (5, 11). Loss-of-function analysis of Ipk2 in a variety of species, cell types, and organisms demonstrates its requirement for proper metabolism and cellular functions (3, 12–14).

As we determine how IPs are synthesized in cells and identify the processes that they regulate, it is also critical to understand how these messengers function in metazoans. The generation of mutant animals has revealed that IPKs are required for development and viability; however, the loss of a gene product alone, without complementation analysis, limits interpretation of the role of the inositide products in organismal physiology. To further our understanding of the roles of IP messengers, we report here our examination of the role of Ipk2 and its kinase activity in the development of a genetically tractable metazoan, *Drosophila melanogaster*. Building on previous studies establishing a molecular basis for IP metabolism in flies (15), we report that Ipk2-dependent production of IP messengers is critical for adult epidermal development, specifically through regulation of cellular proliferation and apoptosis. Our data link Ipk2 activity to the control of proliferation, in part through interactions with JAK/STAT signaling. This demonstrates a specific role for Ipk2-produced IPs in regulating developmental pathways involved in tissue growth and stability.

Results

IPs Are Required for Development and Viability. To identify roles for IPs in *Drosophila*, we generated Ipk2 deletion mutants. The $ipk2^{G3545}$ line contains a P-element insertion within the 5′ untranslated region of the Ipk2 gene (Fig. S1*A*). Although these flies have near-complete losses of Ipk2 and its IP products, they are developmentally normal. We therefore generated Ipk2 null animals by mobilizing the $ipk2^{G3545}$ P-element and obtained two independent imprecise excision alleles: $ipk2^{20B}$ and $ipk2^{40B}$. The entire opening reading frame of Ipk2 is deleted in $ipk2^{20B}$, whereas ∼90% is excised in $ipk2^{40B}$ (Fig. S1 *A* and *B*). Ipk2 protein

Significance

Inositol phosphate kinase 2 (Ipk2) is a conserved protein that initiates the production of inositol phosphate intracellular messengers that are critical for regulating a variety of cellular processes. Here we explore the developmental roles Ipk2 and its products in *Drosophila*. We report that Ipk2 kinase activity is required to develop adult body structures including eyes, legs, and wings, which are formed by tissue known as imaginal discs. Although Ipk2 mutant discs seem to pattern normally in embryogenesis, during larval development they fail to undergo normal expansion. We find defects in signaling pathways that control both cell death and proliferation. Our work demonstrates a specific role for Ipk2-produced intracellular messengers in regulating developmental pathways involved in tissue growth and stability.

Author contributions: A.M.S., M.M.T., E.P.S., and J.D.Y. designed research; A.M.S., M.M.T., and C.S. performed research; A.M.S. and M.M.T. contributed new reagents/analytic tools; A.M.S., M.M.T., and J.D.Y. analyzed data; and A.M.S., M.M.T., and J.D.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]A.M.S. and M.M.T. contributed equally to this work.

[2]Present address: Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147.

[3]To whom correspondence should be addressed. Email: john.york@vanderbilt.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1514684112/-/DCSupplemental.

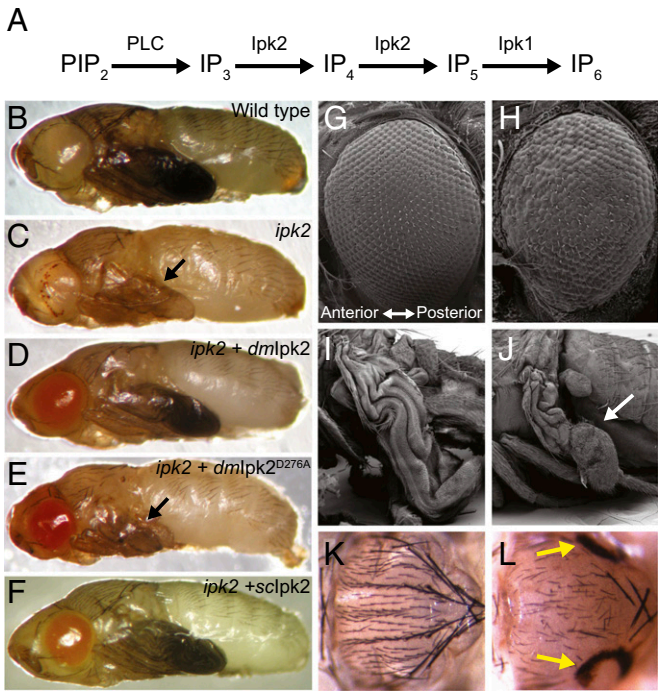

Fig. 1. Ipk2 and its products are required for pupal development. (*A*) Synthesis pathway for producing the bulk of cellular IPs. PIP_2 is hydrolyzed by PLC to produce soluble IP_3. Ipk2 phosphorylates IP_3 to IP_5 that serves as a substrate for the production of IP_6 by Ipk1. (*B* and *C*) Pupae were compared at the latest stage to which the *ipk2* mutants could develop. Shown are (*B*) wild type and (*C*) *ipk2*. (*D*–*F*) *ipk2* mutants with the following UAS transgenes expressed under control of Actin-GAL4: (*D*) *UAS-dmIpk2*, (*E*) *UAS-dmipk2*^{D276A}, and (*F*) *UAS-scIpk2*. Black arrows point to defective wings. (*G*–*J*) Scanning electron microscope images of (*G*) wild-type and (*H*) *ipk2* mutant eyes and (*I*) wild-type and (*J*) *ipk2* mutant wings. The mutant wing is indicated with the white arrow. (*K* and *L*) Images of (*K*) wild-type (*L*) and *ipk2* mutant notums. Degenerative holes are shown with yellow arrows.

was not detectible in homozygous *ipk2*^{20B} third-instar larvae, further confirming that the gene had been removed (Fig. S1*D*). Eighty-five percent of homozygous mutants for either *ipk2* allele survived through the larval stages, but their pupation was delayed by about 12 h relative to wild type (Fig. S1*C*). Homozygotes and transheterozygotes (*ipk2*^{20B}/*ipk2*^{40B}) died during pupation, with no flies reaching eclosion. We focused on the *ipk2*^{20B} allele in subsequent experiments (hereafter referred as *ipk2*). This preliminary analysis of *ipk2* mutants indicates that the gene is critical for progression through development, and for viability.

Examination of the mutant pupae revealed defective eyes, wings, and notum (Fig. 1 *B*, *C*, and *G*–*L*). The "rough" eyes had missing or disordered omatidia and bristles that became progressively less defective from anterior to posterior (Fig. 1 *G* and *H*). *ipk2* wings were small (Fig. 1 *B*, *C*, *I*, and *J*) and bristles on the notum were disordered and missing (Fig. 1 *K* and *L*). Fifty-six percent of *ipk2* mutants formed symmetrical degenerative holes on their posterior notums (Fig. 1 *K* and *L*), with a similar percentage developing a single hole in the thoracic region of their ventral midline. These defects suggest an Ipk2 requirement in forming and/or maintaining external body structures.

We tested the requirement of Ipk2 and its IP products for development and viability. The morphological phenotypes and lethality of the *ipk2* mutants were fully rescued when an Ipk2 transgene (*UAS-Ipk2*) (15) was ubiquitously expressed in homozygous *ipk2* mutants, demonstrating that the phenotypes were due to loss of the protein (Fig. 1*D* and Fig. S2). In contrast, a catalytically inactive point mutant (*UAS-ipk2*^{D276A}) could not rescue any mutant phenotypes, indicating that kinase activity was critical for Ipk2 function (Fig. 1*E* and Fig. S2). Expression of catalytically conserved orthologs from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (*sc*Ipk2 and *at*Ipk2, respectively) (10, 16) also rescued *ipk2* lethality and developmental defects, despite their low sequence identities with *Drosophila* Ipk2 (22 and 31% identity, respectively; in Fig. 1*F* *sc*Ipk2 is shown). Thus, the kinase activity of Ipk2, and hence its IP products, are required for normal development and viability.

An Essential Role for Ipk2 in Body Morphogenesis. Analysis of embryonic (17) and larval (Fig. S1*E*) gene expression revealed that maternally derived mRNA caused Ipk2 to persist into the second instar larval stages in *ipk2* mutants. We hypothesized that this maternal contribution had partially rescued the *ipk2* zygotic (*ipk2* ZYG) mutants described above. Therefore, *ipk2* germ-line clones (*ipk2* GLCs) were generated using the flippase-dominant female sterile technique (18) to abolish the maternal mRNA and test the consequence of removing the gene product throughout development (Fig. S1*D*). Like the ZYG mutants, the *ipk2* GLCs survived through larval development and arrested as pupae (Fig. 2 *A*–*C*). This suggested that larval tissues were largely unaffected in the GLC mutants, despite removal of maternal and zygotic Ipk2.

In contrast to *ipk2* ZYG animals, the *ipk2* GLCs failed to form an adult outer body (epidermis) during pupation, including the head, thorax, wings, abdomen, and legs (compare Fig. 2 *B* and *C*). *ipk2* GLC larvae contained a central nervous system but lacked the larval precursor tissues that form the adult epidermis, the imaginal discs (Fig. 2 *D* and *E*). This revealed a critical role for Ipk2 in forming imaginal discs. One possible explanation for their absence in the *ipk2* GLCs was their lack of specification as imaginal disk precursor cells in embryogenesis. To test this, we probed for the presence of *Distal-less* transcripts, which mark leg disk precursor cells (19). *ipk2* GLCs and wild-type embryos had similar *Distal-less* expression patterns, indicating that the mutant embryos specified leg discs normally (Fig. 2 *F* and *G*). This indicates that the disk precursors formed in *ipk2* GLCs and were either lost through cell death or failed to proliferate during early larval stages.

***ipk2* Mutants Undergo Apoptosis.** We hypothesized that increased cell death could explain *ipk2* defects, such as the "rough" eyes

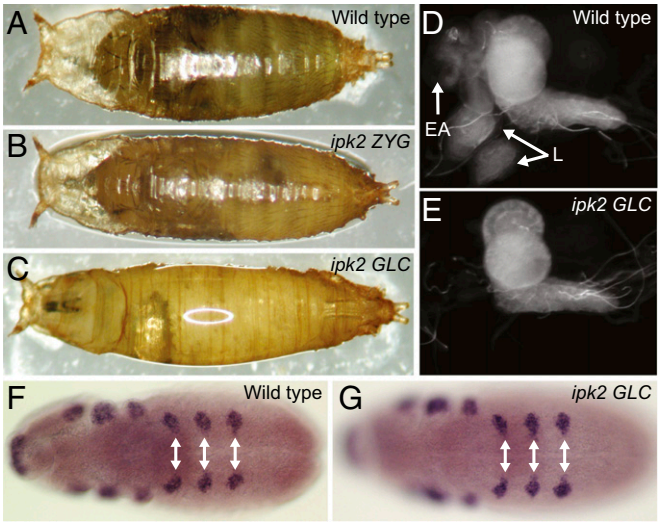

Fig. 2. *ipk2* germ-line clones fail to develop adult body structures. (*A*–*C*) Pupal images of (*A*) wild-type, (*B*) *ipk2* ZYG, (*C*) and *ipk2* germ-line GLC pupae. (*D* and *E*) Imaginal tissues are absent in the *ipk2* GLC. Central nervous system and associated imaginal discs from (*D*) wild-type (*E*) and *ipk2* GLC third-instar larvae. White arrows point to eye/antennal (EA) and leg discs (L). (*F* and *G*) Leg discs are specified normally in *ipk2* GLCs. In situ hybridization using *Distal-less* probes on (*F*) wild-type and (*G*) *ipk2* GLC germband-extended embryos. White arrows show leg disk primordial cells.

CELL BIOLOGY

and small wings (Fig. 1 *H* and *J*), the degenerative holes on the thorax (Fig. 1*L*), and the lack of imaginal discs despite their specification in the GLCs (Fig. 2 *C*, *E*, and *G*). Therefore, we tested whether loss of Ipk2 leads to increased cell death. Indeed, *ipk2* ZYG imaginal discs were prone to apoptosis as they showed increased DNA fragmentation (Fig. 3 *A* and *B*), a hallmark of programmed cell death (20). Increased cell death was found in all mutant imaginal disk tissues but was similar to wild type in other tissues such as salivary glands, gut, and fat bodies.

We examined the requirement of the canonical apoptotic machinery in mediating cell death in *ipk2* imaginal discs. Apoptosis requires the activation of caspases that cleave different substrates to induce an orderly dismantling of the cell (21). The baculovirus caspase inhibitor protein p35 is a specific genetically encoded tool capable of blocking apoptosis (22). Expression of p35 in *ipk2* wing discs suppressed the abnormal cell death, indicating that it was caspase-mediated apoptosis (Fig. 3 *C* and *D*). Caspases are classified as either initiators or effectors, with initiators inducing apoptosis by cleaving and activating the effectors that bring about cell death (21). The major initiator caspase in *Drosophila* is Dronc, and therefore we examined the consequence of its removal in *ipk2* mutants. *ipk2 dronc* double-null flies lacked increased cell death, indicating that the caspase is necessary for apoptosis in the *ipk2* discs (Fig. 3*E*). These data are consistent with a role for IPs in regulating apoptosis via a caspase-dependent pathway.

We examined possible upstream signaling pathways that could initiate apoptosis in the *ipk2* mutants. p53 initiates apoptosis during stress responses and development (23). Apoptosis was not suppressed in an *ipk2 p53* double mutant (24), where the entire p53 gene was excised (Fig. 3*F*). Additionally, a transgene expressing a dominant-negative form of p53 (25) was unable to suppress apoptosis in the *ipk2* mutant. Thus, p53 does not contribute to IP-mediated apoptosis.

We previously examined altered gene expression in *ipk2* mutant imaginal discs and found that two transcripts linked to apoptosis, Puckered and Reaper, had increased expression relative to wild type, and could possibly be the cause of apoptosis. Puckered is a phosphatase whose expression increases to antagonize activated JNK signaling that can induce Reaper-mediated apoptosis (26). This prompted us to investigate the possibility that the JNK pathway induces apoptosis in *ipk2* mutants. We generated *ipk2* flies containing reporters of either Puckered or Reaper expression. *ipk2* mutants showed increased expression of the Puckered reporter (Fig. 3 *G* and *H*), which indicated that JNK was activated (26). Additionally, Reaper expression was detected throughout the wing disk compared with its restricted pattern in a wild-type disk (Fig. 3 *I* and *J*). This suggested that IPs negatively regulate Reaper-induced apoptosis in imaginal discs through JNK signaling.

To further test whether JNK mediates apoptosis in the *ipk2* mutant, we expressed a dominant negative form of JNK (JNK.DN) (27) in the posterior half of the *ipk2* mutant wing disk. The JNK.DN transgene effectively blocked Puckered expression as judged by the loss of puc-lacZ staining (Fig. 3*K*), indicating that the increased JNK signaling in the *ipk2* mutant discs was successfully suppressed. Although JNK.DN effectively suppressed JNK signaling as measured by decreased puc-LacZ expression, the transgene was unable to decrease the levels of apoptotic cells (Fig. 3*L*), suggesting that JNK is not required for initiation of apoptosis in *ipk2* mutants. Therefore, apoptosis due to the loss of *ipk2* occurs through a different pathway.

***ipk2* Mutants Have Cellular Proliferation Defects.** The finding that *ipk2* GLC larvae lack imaginal discs may suggest that they fail to proliferate after being specified in embryogenesis. We examined whether *ipk2* ZYG wing discs displayed cellular proliferation defects; 5′-bromo-2′-deoxyuridine (BrdU) incorporation analysis revealed that *ipk2* ZYG discs from third-instar larvae had reduced DNA synthesis relative to wild type, suggesting that loss of Ipk2 leads to proliferation defects (Fig. S3*A*). We also used clonal analysis to compare the proliferation rates between wild-type and *ipk2* wing discs (Fig. S3*B*). The median cell doubling

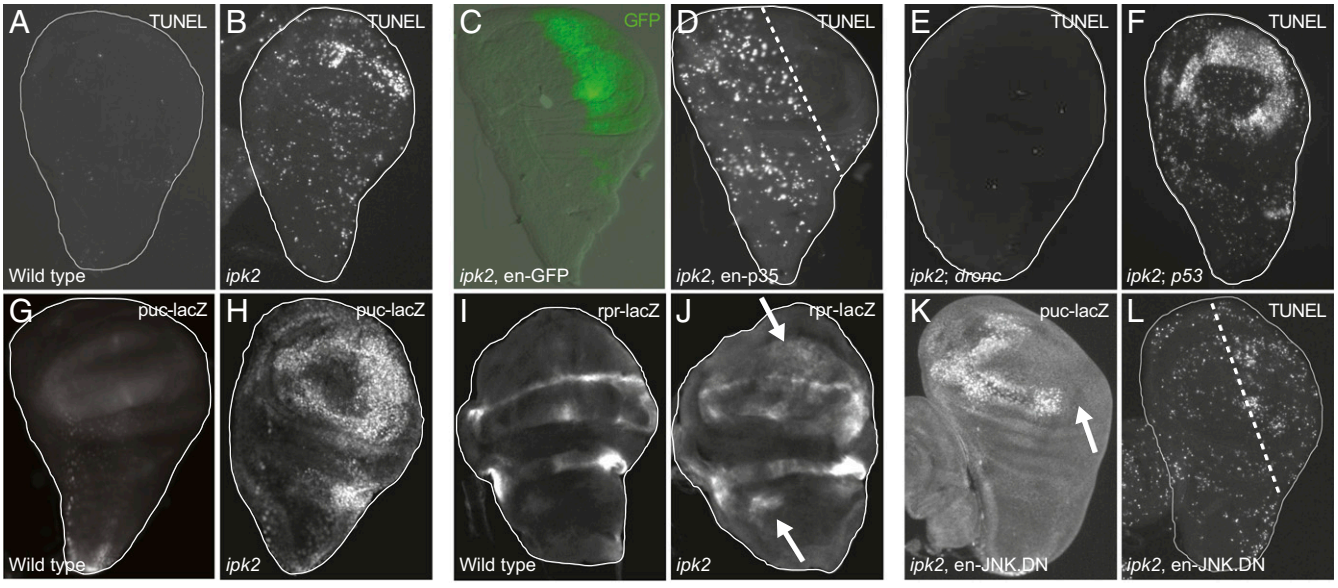

Fig. 3. *ipk2* imaginal discs undergo apoptosis. (*A* and *B*) TUNEL stain of wing imaginal discs from (*A*) wild-type and (*B*) *ipk2* ZYG. (*C* and *D*) Suppression of apoptosis by expression of caspase inhibitor p35 in the posterior compartment of *ipk2* ZYG wing discs. (*C*) Engrailed-GAL4 expression pattern (green) in the posterior compartment (*ipk2*, en-GAL4, *UAS-GFP*). (*D*) TUNEL stain of an *ipk2* ZYG wing disk expressing p35 in the same cells (en-GAL4, *UAS-p35*). Area right of the dashed line expresses p35. (*E*) Suppression of apoptosis in *ipk2*, $dronc^{I29}$ double mutants (TUNEL stain). (*F*) No suppression of apoptosis with loss of p53. TUNEL stain of an *ipk2* ZYG; $p53^{5A\text{-}1\text{-}4}$ double-mutant wing disk. (*G* and *H*) Expression of a Puckered reporter (puc-lacZ) in (*G*) wild-type or (*H*) *ipk2* wing discs. Discs were stained using anti–β-galactosidase antibodies (*G*–*K*). (*I* and *J*) Expression of a Reaper reporter (rpr-lacZ) in (*I*) wild-type or (*J*) *ipk2* wing discs. Arrows point to example areas of abnormal Reaper expression. (*K*) Suppression of puc-lacZ reporter expression in an *ipk2* wing disk expressing JNK.DN in the posterior compartment. Arrow points to area of suppression. (*L*) No suppression of apoptosis in *ipk2* wing discs by expressing dominant-negative JNK in the posterior compartment (*ipk2*, en-GAL4, *UAS-JNK.DN*). The area to the right of the dashed line would lack TUNEL staining had JNK.DN suppressed apoptosis.

time for wild-type and *ipk2* mutants was 14.3 and 18 h, respectively. Relative to wild type, *ipk2* clones contained fewer cells (on average six compared with nine cells per clone). About 30% of the wild-type clones consisted of greater than 13 cells, compared with less than 10% of *ipk2* cells. These data demonstrate that IPs positively regulate cellular proliferation in imaginal discs.

Ipk2 Mediates Proliferation Through JAK/STAT Signaling. We next considered how IPs might mediate proliferation. Müller et al. (28) previously identified Ipk2 in an RNAi screen for regulators of JAK/STAT-mediated transcription. This signaling pathway consists of a secreted extracellular ligand Unpaired (Upd), its transmembrane receptor Domeless (Dome), a Janus tyrosine kinase Hopscotch (Hop), and the Stat92E transcription factor. In their assay, cultured cells were cotransfected with a luciferase transcriptional reporter containing Stat92E-binding sites and a plasmid that induces JAK/STAT signaling by constitutively expressing Upd. RNAi-mediated knockdown of Ipk2 in these cells caused reduced STAT reporter expression (28). This led us to examine the role of Ipk2 in JAK/STAT signaling, given that the pathway regulates cellular proliferation (29).

As previously reported, RNAi-mediated knockdown of Dome or Ipk2 in cell culture caused a loss of transcription from the STAT reporter (Fig. 4*A*). We addressed whether this defect was due to loss of Ipk2 products or downstream IPs. Ipk2 initiates the synthesis of the majority of IPs in flies by sequentially phosphorylating IP_3 to IP_4 and IP_5, which then is phosphorylated by Ipk1 to produce IP_6 (Fig. 1*A*). Consequently, RNAi knockdown of Ipk2 causes a reduction in cellular levels of IP_6 (15) (Fig. S4*A*), which raises the possibility that it is the IP mediator of JAK/STAT signaling. However, RNAi knockdown of Ipk1 did not decrease transcription, but instead caused increased reporter activity (Fig. 4*A*). This increase might be explained by the observation that knockdown of Ipk1 causes a corresponding buildup of its IP_5 substrate (15) (Fig. S4*A*), and therefore the increased IP_5 levels might boost JAK/STAT-mediated transcription. Our data are most consistent with the conclusion that production of IP_4 and/or IP_5 is sufficient for JAK/STAT-mediated transcription, whereas IP_6 seems dispensable. However, we cannot exclude that possibility that other potential downstream metabolites (e.g., $PP\text{-}IP_4$) may also be involved as mediators of this transcription.

Müller et al. (28) further demonstrated that the defect in STAT-mediated transcription caused by loss of Ipk2 could be suppressed by treating cells with Upd-conditioned culture medium. This suggests that Ipk2 functions upstream of the Upd receptor Dome, possibly by regulating the production or secretion of the Upd ligand. To investigate this further, we examined the levels of secreted Upd in cultured cells (30). Indeed, we found decreased secretion of Upd in cells treated with Ipk2 RNAi (Fig. 4*B* and Fig. S4*B*). This reduction could be rescued by cotransfection with *at*Ipk2, indicating that the decreased Upd was due to the loss of Ipk2 activity (Fig. 4*B* and Fig. S4*B*). Thus, Ipk2 products positively regulate Upd production or secretion.

We next examined the role of Ipk2 in JAK/STAT-mediated proliferation. We used a sensitized transgenic assay to test whether loss of Ipk2 modulates JAK/STAT signaling in the eye (31). In this assay, Upd is ectopically expressed in developing eye discs under control of GMR-GAL4. This leads to hyperactivation of JAK/STAT-mediated proliferation, causing enlarged eyes in the pharate adults, compared with wild-type eyes (~40% increase in eye surface area; Fig. 4 *C* and *D*, compare *3* and *1*). Using eye size as a readout of JAK/STAT activation, we examined whether the Upd-mediated enlarged eyes could be suppressed in *ipk2* mutants. We observed an average ~50% decrease in eye size in the *ipk2* mutants, compared with those expressing the Upd transgene in a wild-type background (Fig. 4 *C* and *D*, compare *3* and *4*). This size decrease was unlikely due to the cell death that occurs in *ipk2* mutants, because expression of p35 did not relieve the suppression (Fig. 4 *C* and *D*, compare *5* and *6*). These results indicate that Ipk2 is required for efficient JAK/STAT-mediated proliferation.

We next considered whether JAK/STAT activation could suppress *ipk2* developmental phenotypes. The loss of Ipk2 by RNAi causes a reduction in the levels of secreted Upd in cell culture (Fig. 4*B*) that can be bypassed by activating downstream components through addition of Upd-conditioned medium (28). Therefore, if the developmental defects in *ipk2* mutants are due to a loss of JAK/STAT-mediated proliferation, then it may be possible to rescue these phenotypes by activating the JAK/STAT pathway downstream of Upd. To test this, we used a dominant gain-of-function mutant of the JAK kinase Hop ($hop^{Tum\text{-}l}$), which contains a mutation that causes hyperphosphorylation and hyperactivation of Stat92E (32, 33). $hop^{Tum\text{-}l}$ partially rescued the small wing phenotype of the *ipk2* mutant (Fig. 4 *E* and *F*), further suggesting that Ipk2 acts upstream of Hop. However, JAK/STAT activation could not completely rescue this wing phenotype, nor could it rescue *ipk2* lethality. Taken together, these results demonstrate that Ipk2 products regulate JAK/STAT signaling to control proliferation in development, yet other processes are also likely involved.

Discussion

Inositol phosphate kinases are highly conserved, underscoring the importance of IP production for both single and multicelled eukaryotes. In particular, Ipk2 and its products serve an essential function for yeast adaptive responses and proper organismal development. Our study of fly Ipk2 provides molecular insights into how production of IP products contributes to developmental programs and cellular homeostasis. Our initial characterization of IP synthesis in *Drosophila* established that loss or gain of Ipk2 kinase activity had profound effects on cellular levels of IP_4, IP_5, and IP_6 in flies (15). Here, we find that loss of these IP products through deletion of *ipk2* results in inviability, and more specifically a "disc-less" phenotype, which causes major defects in the development and maintenance of adult epidermal tissues. Our data are most consistent with a role for proper spatiotemporal IP production during organismal development as a key regulator of proliferative and degenerative signaling pathways. The failure of imaginal disk primordial cells to proliferate and form an epidermis can be partially explained by defective JAK/STAT signaling. Although multiple different processes are likely affected by the loss of IPs to contribute to their essential functions, this work provides a foundation for determining the mechanisms of IP regulation in a metazoan.

Ipk2 Kinase Activity Plays a Key Role in Organismal Development. An important goal of the complementation analysis and genetic studies reported here is to narrow down which of the "multikinase" activities of Ipk2, if any, play a role in development. Our data clearly demonstrate that kinase activity, and therefore the products of Ipk2, are required for imaginal disk development and regulation of signaling pathways. To distinguish which of the products of Ipk2 are responsible, for example water-soluble IP messengers versus lipophilic PIPs, we complemented mutant flies with *Arabidopsis* Ipk2 because it has been reported to only harbor water-soluble but not lipid kinase activity (11). From these analyses, we define a kinase-dependent role for water-soluble IP production, but not inositol lipids, in regulating *Drosophila* development. The ability of heterologous expression of either yeast or plant Ipk2 orthologs, despite their low sequence identity to *Drosophila* Ipk2, to completely rescue viability and imaginal disk development further supports our hypothesis that it is the conversion of IP_3 to IP_4 and IP_5 that plays an important role in signaling. With respect to proliferation and regulation of JAK/STAT pathways, our data demonstrate that loss of IP_6 production does not account for the phenotypes observed. Our work further pinpoints a role for Ipk2 products IP_4 and IP_5 as regulators of the JAK/STAT pathway; however, given that other inositide metabolites are produced downstream of Ipk2, such as PP-IPs, we leave open the possibility that such messengers may account for some of the phenotypes reported.

The production of IP_4/IP_5 in many cells requires the hydrolysis of PIP_2 to produce IP_3 and thus raises the question as to whether

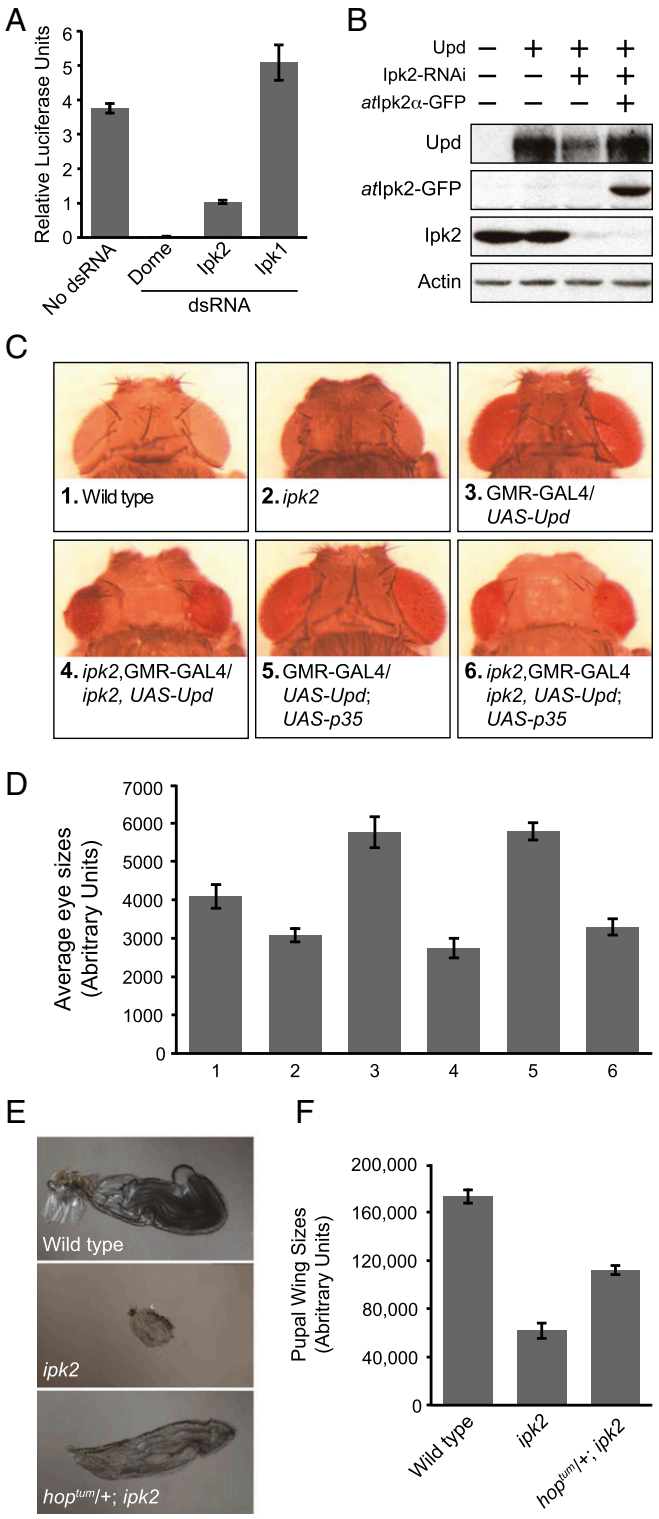

Fig. 4. Interaction between Ipk2 and JAK/STAT. (*A*) Ipk2, but not Ipk1, is required for JAK/STAT-mediated transcription. Kc_{167} cells were treated with dsRNA targeted against Dome, Ipk2, and Ipk1 and assayed using a STAT transcriptional reporter. Relative luciferase units are plotted (mean ± SEM). (*B*) Ipk2 knockdown causes a reduction in secreted Upd. Cells were transfected with combinations of Upd plasmid, *at*Ipk2-GFP plasmid, and treated with Ipk2 dsRNA, as indicated by the + and − signs. Protein extracts and conditioned medium were immunoblotted with antibodies against Upd, Ipk2, GFP, or actin. (*C* and *D*) *ipk2* suppresses the eye overproliferation phenotype caused by ectopic expression of Upd. (*C*) Shown are representative images of heads from pharate adults of the genotypes indicated below the images. (*D*) Mean eye sizes of the genotypes indicated in *C* (mean ± SEM, $n \geq 6$). (*E* and *F*) $hop^{Tum\text{-}l}$ partially rescues the small wing phenotype of *ipk2*. (*E*) Representative images of pupal wings of the indicated genotypes. (*F*) Mean pupal wing sizes of these flies (mean ± SEM, $n \geq 40$).

Ipk2-mediated development is dependent on phospholipase C activity. In budding yeast, it has been possible to delineate this through genetic and biochemical approaches because there is a single phospholipase C gene product Plc1. *Drosophila* possesses three different isoforms of PLC [NorpA, Plc21C, and Small wing (Sl)] (34), and although triple-deletion mutants have not been reported in the literature, individual PLC mutants show interesting phenotypes, including small wings and rough eyes (35), similar to the zygotic *ipk2* mutant defects we report. Evidence in this study hints at a different mechanism but it is also quite likely that the different PLC orthologs can compensate for each other, confounding interpretation.

JAK/STAT-Mediated Signaling and Cell Proliferation. Previous work implicated Ipk2 as necessary for JAK/STAT-mediated transcription in cultured cells (28), which prompted us to explore the relationship between this signaling pathway and IP production. We extended this finding by showing that JAK/STAT-mediated transcription requires the production of Ipk2 products, IP_4 and IP_5, whereas IP_6 production by Ipk1 is dispensable. Further, IP regulation of JAK/STAT occurs through modulation of the production or secretion of the Upd ligand. This is in agreement with the finding that decreased JAK/STAT-mediated transcription caused by knockdown of Ipk2 can be relieved by addition of Upd-conditioned medium (28). An important next step will be to determine the mechanism by which IPs control the process that leads to Upd secretion.

A significant contribution of this work is the demonstration of a genetic interaction between Ipk2 and JAK/STAT in controlling cellular proliferation. *ipk2* mutants suppress JAK/STAT-mediated proliferation during eye development, possibly because of decreased Upd secretion. Conversely, the partial rescue of the *ipk2* mutant small-wing phenotype using $hop^{Tum\text{-}l}$ indicates that up-regulation of the JAK/STAT pathway can partially overcome an *ipk2* mutant phenotype. The gain-of-function $hop^{Tum\text{-}l}$ mutant likely rescues *ipk2* by bypassing the reduced levels of Upd. Thus, our results provide a partial explanation for why *ipk2* mutants have disk proliferation defects.

The interaction between Ipk2 and JAK/STAT raises the question of whether IP production is always required for the signaling pathway. *ipk2* phenotypes are reminiscent of the small or absent imaginal discs exhibited by some *hop* and *stat92E* mutants (36, 37). However, modulation of JAK/STAT activity by IPs may be restricted to the imaginal discs, because *ipk2* mutants do not seem to have the embryonic segmentation or sex determination defects exhibited by JAK/STAT pathway mutants (38). Given the multitude of products generated by Ipk2 and the growing number of processes regulated by these products and downstream metabolites, such as inositol pyrophosphates, it is not surprising that *ipk2* phenotypes cannot be fully rescued by up-regulation of the JAK/STAT pathway. Further genetic analyses in *Drosophila* of Ipk1, IP6K, and Vip1 mutants will greatly enhance our ability to further assign the roles of IP_6 and PP-IPs, such as IP_7 and IP_8.

Tissue Degeneration and Apoptosis. *ipk2* mutants have tissue stability defects, which may be caused by increased apoptosis. For example, *ipk2* ZYG eyes become increasingly disordered from posterior to anterior. This coincides with a gradual loss of maternally supplied Ipk2 to rescue the developing eye, because the anterior end of the eye differentiates later in development (39). Rough eye phenotypes have been previously linked to aberrant apoptosis (40). Additionally, holes form in the cuticle of *ipk2* mutants, providing evidence of tissue degeneration. The apparent defects in tissue stability in *ipk2* mutants led us to find that the imaginal discs undergo increased caspase-dependent apoptosis.

Previous work in mammalian cells demonstrated that IP_6 is protective from apoptosis (41), consistent with our results that loss of IP production leads to increased cell death.

Materials and Methods

Analysis of JAK/STAT-Mediated Signaling in Kc_{167} Cells. Primers, ESTs, and protocols for generating dsRNA and performing RNAi in cells were the same as described previously (15, 28). For JAK/STAT reporter assays (28), Kc_{167} cells that were treated with dsRNA for 4 d were transfected with *pAct-UpdGFP* (Upd 1), 6 × *2x DrafLuc* firefly luciferase reporter, and *pAct-Renilla* using Effectene (Qiagen) and treated with dsRNA for an additional 3 d. Assays were done using the Luciferase Assay System (Promega). Data are plotted (Fig. 4*A*) as relative luciferase units that were corrected for transfection efficiency by calculating the firefly versus *renilla* luciferase ratios.

For detection of Upd after Kc_{167} cells were treated with dsRNA for 4 d, the cells were transfected with Ubiquitin-GAL4, a combination of *pUAST-Upd* and *pUAST-GFP-atIpk2α*, as indicated in Fig. 4*B*, and pUAST (empty vector) to equalize the amount of total DNA input in each transfection. Cells were then treated with dsRNA for an additional 3 d. Twenty-four hours before harvesting, the cells were collected, washed, and plated with Sf-900 II Serum-Free Medium; 50 μg/mL heparin (Sigma H-9399) was added to the medium to release secreted Upd from the extracellular matrix (30). The conditioned medium for each sample was collected, centrifuged to remove cells, and concentrated to the same volume using a 10,000 molecular weight-cutoff concentrator (Sartorius). Cells were lysed in 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris (pH 7.5) buffer, 1 mM PMSF, and complete protease inhibitor (Roche). Laemmli loading buffer (5×) was added to the cell lysates and concentrated conditioned medium and boiled for 5 min. Twenty micrograms of protein was loaded for the cell protein extracts, and 2 μL of the concentrated conditioned medium (0.8% total medium protein extracted) was loaded per lane onto a 10% (wt/vol) SDS/PAGE gel. Western blots were performed using a 1:5,000 dilution of rabbit anti-Upd from Doug Harrison, University of Kentucky, Lexington, KY.

Genetic Interaction Between Ipk2 and JAK/STAT. Flies of the genotypes indicated in Fig. 4 *C* and *E* were raised on standard food at room temperature, under noncrowded conditions. Pharate adults were dissected from their pupal cases to expose the eyes (Fig. 4*C*). The pupae were placed on a glass slide in the same orientation. The wings were dissected and mounted on slides in 50% (vol/vol) glycerol. Heads and wings were imaged at 10× magnification. The eyes and wings in the images were segmented using manual tracing and show region statistics, and then region measurement tools were used to measure the areas (in arbitrary units) using Metamorph (Molecular Devices).

See *SI Materials and Methods* for additional materials and methods.

ACKNOWLEDGMENTS. We thank members of the J.D.Y. laboratory, especially Dr. Jessica Monserrate, for helpful discussions; Drs. Patrick Müller and Michael Boutros for sharing JAK/STAT pathway reagents; and Rick Fehon, Donald McEwen, Martin Zeidler, Andreas Bergmann, and Doug Harrison for fly stocks and reagents. This work is supported by funds from HHMI and NIH Grant HL-55672. J.D.Y. is an Alumni Investigator of HHMI.

1. Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 1793(6):933–940.
2. Irvine RF, Schell MJ (2001) Back in the water: The return of the inositol phosphates. *Nat Rev Mol Cell Biol* 2(5):327–338.
3. Seeds AM, York JD (2007) Inositol polyphosphate kinases: Regulators of nuclear function. *Biochem Soc Symp* 74:183–197.
4. Hatch AJ, York JD (2010) SnapShot: Inositol phosphates. *Cell* 143(6):1030–1030.e1.
5. Koldobskiy MA, Snyder SH (2011) Inositol pyrophosphates in cell death and life. *Cell Cycle* 10(4):568–570.
6. Wilson MSC, Livermore TM, Saiardi A (2013) Inositol pyrophosphates: Between signalling and metabolism. *Biochem J* 452(3):369–379.
7. Lee J-Y, Kim Y-R, Park J, Kim S (2012) Inositol polyphosphate multikinase signaling in the regulation of metabolism. *Ann N Y Acad Sci* 1271:68–74.
8. York JD, Odom AR, Murphy R, Ives EB, Wente SR (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science* 285(5424):96–100.
9. Saiardi A, Caffrey JJ, Snyder SH, Shears SB (2000) Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in *Saccharomyces cerevisiae*. *FEBS Lett* 468(1):28–32.
10. Odom AR, Stahlberg A, Wente SR, York JD (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. *Science* 287(5460):2026–2029.
11. Maag D, et al. (2011) Inositol polyphosphate multikinase is a physiologic PI3-kinase that activates Akt/PKB. *Proc Natl Acad Sci USA* 108(4):1391–1396.
12. Kim E, et al. (2013) Inositol polyphosphate multikinase is a coactivator for serum response factor-dependent induction of immediate early genes. *Proc Natl Acad Sci USA* 110(49):19938–19943.
13. Wickramasinghe VO, et al. (2013) Human inositol polyphosphate multikinase regulates transcript-selective nuclear mRNA export to preserve genome integrity. *Mol Cell* 51(6):737–750.
14. Xu R, et al. (2013) Inositol polyphosphate multikinase is a transcriptional coactivator required for immediate early gene induction. *Proc Natl Acad Sci USA* 110(40): 16181–16186.
15. Seeds AM, Sandquist JC, Spana EP, York JD (2004) A molecular basis for inositol polyphosphate synthesis in *Drosophila melanogaster*. *J Biol Chem* 279(45):47222–47232.
16. Stevenson-Paulik J, Odom AR, York JD (2002) Molecular and biochemical characterization of two plant inositol polyphosphate 6-/3-/5-kinases. *J Biol Chem* 277(45): 42711–42718.
17. Tomancak P, et al. (2002) Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol* 3(12)research0088.1–88.14.
18. Chou TB, Perrimon N (1996) The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* 144(4):1673–1679.
19. Cohen B, Simcox AA, Cohen SM (1993) Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. *Development* 117(2):597–608.
20. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119(3):493–501.
21. Parrish AB, Freel CD, Kornbluth S (2013) Cellular mechanisms controlling caspase activation and function. *Cold Spring Harb Perspect Biol* 5(6):a008672.
22. Hay BA, Wolff T, Rubin GM (1994) Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* 120(8):2121–2129.
23. Mollereau B, Ma D (2014) The p53 control of apoptosis and proliferation: Lessons from *Drosophila*. *Apoptosis* 19(10):1421–1429.
24. Rong YS, et al. (2002) Targeted mutagenesis by homologous recombination in *D. melanogaster*. *Genes Dev* 16(12):1568–1581.
25. Ollmann M, et al. (2000) *Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* 101(1):91–101.
26. McEwen DG, Peifer M (2005) Puckered, a *Drosophila* MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. *Development* 132(17):3935–3946.
27. Adachi-Yamada T, et al. (1999) p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in *Drosophila* wing morphogenesis. *Mol Cell Biol* 19(3):2322–2329.
28. Müller P, Kuttenkeuler D, Gesellchen V, Zeidler MPM, Boutros M (2005) Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature* 436(7052):871–875.
29. Mukherjee T, Hombría JC-G, Zeidler MP (2005) Opposing roles for *Drosophila* JAK/STAT signalling during cellular proliferation. *Oncogene* 24(15):2503–2511.
30. Harrison DA, McCoon PE, Binari R, Gilman M, Perrimon N (1998) *Drosophila* unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes Dev* 12(20):3252–3263.
31. Bach EA, Vincent S, Zeidler MP, Perrimon N (2003) A sensitized genetic screen to identify novel regulators and components of the *Drosophila* janus kinase/signal transducer and activator of transcription pathway. *Genetics* 165(3):1149–1166.
32. Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N (1995) Activation of a *Drosophila* Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *EMBO J* 14(12):2857–2865.
33. Luo H, et al. (1997) Mutation in the Jak kinase JH2 domain hyperactivates *Drosophila* and mammalian Jak-Stat pathways. *Mol Cell Biol* 17(3):1562–1571.
34. Singer WD, Brown HA, Sternweis PC (1997) Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu Rev Biochem* 66(1): 475–509.
35. Murillo-Maldonado JM, Zeineddine FB, Stock R, Thackeray J, Riesgo-Escovar JR (2011) Insulin receptor-mediated signaling via phospholipase C-γ regulates growth and differentiation in *Drosophila*. *PLoS One* 6(11):e28067.
36. Binari R, Perrimon N (1994) Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in *Drosophila*. *Genes Dev* 8(3):300–312.
37. Ekas LA, Baeg G-H, Flaherty MS, Ayala-Camargo A, Bach EA (2006) JAK/STAT signaling promotes regional specification by negatively regulating wingless expression in *Drosophila*. *Development* 133(23):4721–4729.
38. Chen Q, Giedt M, Tang L, Harrison DA (2014) Tools and methods for studying the *Drosophila* JAK/STAT pathway. *Methods* 68(1):160–172.
39. Baker NE (2001) Cell proliferation, survival, and death in the *Drosophila* eye. *Semin Cell Dev Biol* 12(6):499–507.
40. Meier P, Silke J, Leevers SJ, Evan GI (2000) The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J* 19(4):598–611.
41. Verbsky J, Majerus PW (2005) Increased levels of inositol hexakisphosphate (InsP6) protect HEK293 cells from tumor necrosis factor (alpha)- and Fas-induced apoptosis. *J Biol Chem* 280(32):29263–29268.
42. González B, et al. (2004) Structure of a human inositol 1,4,5-trisphosphate 3-kinase: Substrate binding reveals why it is not a phosphoinositide 3-kinase. *Mol Cell* 15(5): 689–701.
43. Miller GJ, Hurley JH (2004) Crystal structure of the catalytic core of inositol 1,4,5-trisphosphate 3-kinase. *Mol Cell* 15(5):703–711.
44. Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D (1997) The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* 124(4):761–771.