Genome-wide RNAi analysis of JAK/STAT signaling components in *Drosophila*

Gyeong-Hun Baeg, Rui Zhou, and Norbert Perrimon

1Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA

The cytokine-activated Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway plays an important role in the control of a wide variety of biological processes. When misregulated, JAK/STAT signaling is associated with various human diseases, such as immune disorders and tumorigenesis. To gain insights into the mechanisms by which JAK/STAT signaling participates in these diverse biological responses, we carried out a genome-wide RNA interference (RNAi) screen in cultured *Drosophila* cells. We identified 121 genes whose double-stranded RNA (dsRNA)-mediated knockdowns affected STAT92E activity. Of the 29 positive regulators, 13 are required for the tyrosine phosphorylation of STAT92E. Furthermore, we found that the *Drosophila* homologs of RanBP3 and RanBP10 are negative regulators of JAK/STAT signaling through their control of nucleocytoplasmic transport of STAT92E. In addition, we identified a key negative regulator of *Drosophila* JAK/STAT signaling, protein tyrosine phosphatase PTP61F, and showed that it is a transcriptional target of JAK/STAT signaling, thus revealing a novel negative feedback loop. Our study has uncovered many uncharacterized genes required for different steps of the JAK/STAT signaling pathway.

*Keywords*: JAK/STAT signal transduction pathway; *Drosophila*; RNA interference

Supplemental material is available at http://genesdev.org.

Received April 1, 2005; revised version accepted June 20, 2005.

The evolutionarily conserved Janus kinase (JAK)/signal transducer and activator of transcription (STAT) cascade plays a key role in a wide variety of biological processes such as the immune response, tumorigenesis, and development. This pathway, regulated by a large number of cytokines and growth factors, has emerged as an essential facet of vertebrate signaling. Critical roles of the receptor-associated JAKs and their substrate transcription factors STATs have been demonstrated through the generation of gene knockout mice. JAK1-deficient mice die perinatally and are unable to nurse [Rodig et al. 1998], while JAK2 mutant mice display embryonic lethality due to anemia [Neubauer et al. 1998; Parganas et al. 1998]. Mice lacking JAK3 have profound reductions in thymocytes, B and T cells, as observed in the case of autosomal severe combined immune deficiency (SCID) mice [Nosaka et al. 1995; Park et al. 1995; Thomis et al. 1995]. Similarly, STAT-deficient mice are either immunocompromised or display hematopoietic defects [Durbin et al. 1996; Meraz et al. 1996]. On the other hand, constitutive activation of JAKs and/or STATs is correlated with tumorigenesis through their intimate connection to growth factor signaling, apoptosis, and angiogenesis [Yu and Jove 2004]. Furthermore, studies in model genetic systems, such as *Drosophila*, *Xenopus*, and zebrafish have shown that the JAK/STAT pathway participates in an unusually broad set of developmental decisions that include cell fate determination, cell migration, planar cell polarity, convergent extension, and stem cell maintenance [Hou et al. 2002]. Although much work has been done on this pathway, many questions remain to be addressed. In particular, the exact molecular mechanisms by which JAK/STAT signaling integrates and transduces cues from numerous extracellular signaling molecules to trigger specific genetic programs in vivo remain to be elucidated. In addition, STATs have been shown to be activated by at least four distinct mechanisms in mammals [Bromberg 2001], and many aspects of this regulation remain only partially understood.

In mammals, genetic approaches to identify and characterize components of the JAK/STAT pathway have been predominantly dependent on cell-line-based genetics or gene targeting, which is labor-intensive and often time-consuming [Velazquez et al. 1992]. Moreover, interpretation of mammalian genetic studies is further complicated by the redundancy within individual components of the JAK/STAT pathway. In contrast, *Drosophila melanogaster* is highly amenable to genetic manipulations and has served as an excellent model organ-
ism to study the JAK/STAT pathway. Genetic studies in Drosophila have identified several canonical components of the JAK/STAT pathway, including cytokine-like molecules Unpaired (Upd), Domeless/Master of Marelle (Dome/Mom), the Upd receptor distantly related to the mammalian gp130 subfamily, Hopscotch (Hop), the Drosophila homolog of vertebrate JAK, STAT92E, the STAT protein, and SOCS36E, a negative regulator of the JAK/STAT pathway (Hou et al. 2002). However, the inherent limitations of forward genetic approaches make it likely that many genes remain unidentified. Recently, the development of high-throughput genome-wide RNAi-based technology in cultured Drosophila cells offers a rapid, systematic, and complementary methodology for dissecting gene functions (Boutros et al. 2004; Dasgupta et al. 2005). This quantitative cell-based RNAi analysis also offers the advantage of uncovering gene function associated with subtle phenotypes and/or redundancy that might not be readily identifiable through genetic studies, including those in sensitized genetic backgrounds (Bach et al. 2003). Furthermore, with abundant genetic tools readily available, Drosophila is a superior genetically tractable system for the in vivo validation of candidate genes.

There are a number of steps involved in signaling through the JAK/STAT pathway, including phosphorylation and nucleocytoplasmic shuttling of STAT92E. We hoped to identify new members of this canonical pathway as well as proteins that might function as modulators by regulating different steps of this pathway. To this end, we performed a genome-wide RNAi screen in cultured Drosophila cells and identified 121 genes that represent 29 potential positive and 92 negative regulators of the JAK/STAT pathway. Importantly, among these were five canonical components of the JAK/STAT pathway, including Upd2, Dome, Hop, STAT92E, and SOCS36E, indicating the robustness and validity of this approach. The 29 positive regulators were further analyzed by examining the effect of their double-stranded RNA (dsRNA)-mediated knockdown on STAT92E phosphorylation. We also demonstrate that Drosophila homologs of RanBP3 and RanBP10 are involved in STAT92E nucleocytoplasmic transport. Finally, we characterized the first protein tyrosine phosphatase, PTP61F, that negatively regulates the Drosophila JAK/STAT pathway. Together, these findings underscore the robustness of genome-wide RNAi screening approaches to uncover novel regulators involved in different steps in signaling pathways.

Results
Generating a JAK/STAT reporter gene in Drosophila

SOCS36E (Drosophila homolog of suppressor of cytokine signaling gene family) encodes a negative regulator of the JAK/STAT signaling pathway in Drosophila, and has been shown to be transcriptionally activated by JAK/STAT signaling (Callus and Mathey-Prevot 2002; Karsten et al. 2002). Upon close examination of the SOCS36E genomic region, we identified a 441-bp fragment in the enhancer of the SOCS36E gene that contains two potential STAT92E-binding sites. To generate a JAK/STAT reporter, we placed five tandem repeats of this genomic fragment upstream of a minimal heat-shock promoter-driven cDNA encoding the firefly luciferase gene (Fig. 1A), herein referred to as 10XSTAT92E-luciferase. To confirm that this reporter gene was responsive to JAK/STAT signaling and to select a Drosophila cell line that would allow for the identification of both positive and negative regulators of STAT92E activity, we first transfected various Drosophila cell lines with 10XSTAT92E-luciferase and an Actin promoter-driven Renilla luciferase expression vector (Act-Renilla) together with various dsRNAs. We tested dsRNAs against known JAK/STAT components and quantified the activity of JAK/STAT signaling by measuring relative luciferase units (RLU), which equaled the ratio of the absolute activity of firefly luciferase to Renilla luciferase. A Drosophila Schneider cell line derivative (S2-NP) exhibited robust endogenous JAK/STAT activity, and this activity was sensitive to RNAi manipulations. The addition of dsRNAs against positive regulators, such as STAT92E, Hop, and Dome, led to a 12- to 24-fold decrease in the reporter activity, whereas dsRNA against a negative regulator, SOCS36E, increased its activity by threefold (Fig. 1B). Thus the reporter gene faithfully reflected JAK/STAT signaling in S2-NP cells.

A cell-based genome-wide RNAi screen and data analysis

To identify additional modulators of the JAK/STAT pathway whose loss-of-function affects STAT92E activity.
ity, we performed a genome-wide RNAi screen using cultured Drosophila S2-NP cells in 384-well plates. We used a library of ~21,300 dsRNAs (Boutros et al. 2004) that target >95% of the annotated genes in the Drosophila genome [Hild et al. 2003]. Luciferase values were analyzed and potential candidate genes were assigned on the basis of their deviation from the plate average for each given plate [see Materials and methods]. In the primary screen, we identified 474 candidate genes that include 259 genes that reduced JAK/STAT signaling by more than two standard deviations (SD) and 215 genes that increased signaling by more than three SD when knocked down by RNAi [Fig. 2A]. Importantly, among these genes we independently identified five canonical components of the JAK/STAT pathway: Upd2, Dome, Hop, STAT92E, and SOCS36E, confirming the robustness of the screen (Fig. 2B).

Of these 474 candidate genes, 188 represent sequences not annotated by the Berkeley Drosophila Genome Project (which were based on an inclusive algorithm for determining ORFs in the fly genome) [Hild et al. 2003], ribosomal proteins, and proteins involved in RNA processing and translation [data not shown]. These genes were not pursued further. We next repeated the same assay with the remaining 286 genes in duplicate. Two-hundred-twenty candidate genes (71%) identified in the primary screen were verified. In the primary screen, we used Act–Renilla for normalizing the transfection efficiency. Because the candidate genes from the primary screen were determined by calculating the Firefly/Renilla ratio, it is conceivable that some of them might arise from the effect of certain dsRNAs on the Actin promoter. Thus, to remove candidate genes that might affect the Actin promoter and not the JAK/STAT responsive element, we repeated our reporter assay using a Pol III promoter-driven Renilla luciferase expression vector [pol III–Renilla]. We found that 81 candidates (40%) fell into this category and were not pursued further [data not shown]. Thus this screen identified 121 candidate genes that specifically modulate JAK/STAT signaling in S2-NP cells [Supplementary Table 1]. Importantly, Upd2, a cytokine-like molecule, is among the positive regulators identified in the screen. Thus, the endogenously expressed Upd2 is responsible for basal levels of JAK/STAT signaling in S2-NP cells.

These 121 genes were assigned to categories based on their predicted molecular functions, protein domains, and reports from the literature to help us to predict functions and generate testable hypotheses for further characterization [Fig. 2B]. These categories include (1) canonical JAK/STAT signaling pathway component, (2) kinase/phosphatase, (3) chromatin remodeling, (4) protein trafficking, (5) cell adhesion, (6) structural molecule, (7) transcription factor, and (8) miscellaneous.

Next, we assayed the 29 positive regulators in cells stimulated with exogenous Upd, a well-characterized ligand for JAK/STAT signaling. We found that 27 genes were validated in this assay, strongly suggesting that the screen has identified bona fide components of the JAK/STAT signaling pathway [Supplementary Table 1]. This assay revealed that Upd2 and CG17836 are not required for Upd-induced JAK/STAT signaling. Since Upd2 is an endogenously expressed cytokine responsible for basal levels of JAK/STAT signaling, it is not expected to be required for the JAK/STAT signal elicited by exogenous Upd.

Identification of genes that affect tyrosine phosphorylation of STAT92E

To more clearly elucidate the roles of positive regulators, we assayed their requirement for the phosphorylation of STAT92E. Tyrosine phosphorylation is a key step in STAT activation upon cytokine/receptor stimulation.

![Figure 2. Data analysis for the JAK/STAT screen. (A) Scatter plot for three representative screen plates. Cutoffs were set as 2 SD below the mean or 3 SD above the mean RLU. Note that all three “spiked in” control dsRNAs against STAT92E were identified. (B) Pie chart depicting categories of genes identified in the JAK/STAT screen.](https://example.com/figure2)
Thus, monitoring steady-state levels of phosphorylated STAT in dsRNA-treated cells would provide insight into the molecular functions of our candidate genes. As expected, we found that Upd stimulation of S2-NP cells leads to a dramatic increase in tyrosine-phosphorylated STAT92E, as shown by Western blot analysis (Fig. 3A). Next, we tested the effect of dsRNAs against the 29 positive regulators on Upd-induced STAT92E phosphorylation. Thirteen genes (besides STAT92E) were found to be required for Upd-induced STAT92E phosphorylation [Fig. 3B,C, Supplementary Table 1]. As expected, these genes included the canonical components Dome and hop. In contrast to the initial assay in the primary screen, here we used exogenous Upd to activate STAT92E phosphorylation, and thus we were unable to identify genes that act upstream of the receptor, such as Upd2. Notably, two of the 13 genes [CG16790 and CG4329] that regulate STAT92E phosphorylation have no predicted function, yet clearly have human orthologs; further investigation of their molecular functions in Jak/Stat signaling in Drosophila may advance our understanding of the mammalian pathway.

Interestingly, this assay revealed that RNAi knock-down of the cyclin-dependent kinase 2 gene (cdc2) resulted in a decrease in STAT92E tyrosine phosphorylation [Fig. 3B], suggesting that cdc2 modulates Jak/Stat signaling by affecting tyrosine phosphorylation of STAT92E. Consistent with this observation, Warts/Lats, which has been shown both biochemically and genetically to interact withcdc2 and to negatively regulate its kinase activity [Tao et al. 1999], was identified in our screen as a potential negative regulator of Jak/Stat signaling. These results suggest that STAT92E plays an important role in Warts/Lats-mediated inhibition of cell proliferation.

We also identified echinoid [ed] as a positive regulator required for Upd-dependent STAT92E tyrosine phosphorylation. ed encodes a cell adhesion molecule and has been shown to be a negative regulator of the EGFR signaling pathway during Drosophila eye development [Bai et al. 2001; Islam et al. 2003]. Previous experiments have shown both positive and negative interactions between the Jak/Stat pathway and the EGFR pathway. For example, STAT92E mutants phenocopy mutants in the EGFR pathway [Yan et al. 1996]. Furthermore, studies using mammalian tissue culture systems have demonstrated that EGFR signaling activates both Jak1 and Stat1 [Quelle et al. 1995; Leaman et al. 1996]. In addition, EGFR-induced cell migration is mediated predominantly by the Jak/Stat pathway in primary esophageal keratinocytes [Andl et al. 2004]. Similarly, ed has been shown to be responsible for defective cell migration in Caenorhabditis elegans [Vogel and Hedgecock 2001]. Therefore studying the role of ed in Jak/Stat signaling in different contexts may facilitate our understanding of the genetic and biochemical mode of Stat activation by EGFR signaling, and provide insights into the mechanisms governing cancer cell metastasis in humans.

Identification of genes that affect nuclear translocation of STAT92E

Another step in the activation of the Jak/Stat signaling pathway is the translocation of Stats into the nucleus. In resting cells, Stats reside mainly in the cytoplasm. Upon cytokine stimulation, they are phosphorylated on key tyrosine residues and rapidly translocate to the nucleus, where they trans-activate target genes. Previous studies have shown that Importin α5 and Ran are required for the nuclear import of phosphorylated [activated] Stats [Sekimoto et al. 1997]. To reset the cells after stimulation, Stats are exported out of the nucleus into the cytoplasm in preparation for the next round of signaling using an exportin-1/Crm-1-dependent mechanism [McBride et al. 2000]. These observations suggest that defective nuclearcytoplasmic shuttling of Stats can disrupt steady-state distribution of Stats and induce aberrant biological responses. Among all 121 candidates, we identified seven genes that are potentially involved in protein trafficking based on their predicted molecular functions and protein domains [Fig. 2B, Supplementary Table 1]. These include Rab26, Ran, CG10225, which encodes the Drosophila homolog of Ran-binding protein 3 [RanBP3], CG11763, which encodes the Drosophila homolog of Ran-binding protein 10 [RanBP10], and the Drosophila homolog of Cellular Apoptosis Susceptibility gene product [CAS] that was initially identified as a Ran-binding protein. In addition, we
also identified *Drosophila* homologs of Transportin 1 and Nucleoporin 196, which have been implicated in protein import and/or export in mammals. We therefore examined the subcellular localization of phosphorylated STAT92E under conditions where each of the seven candidates was depleted by RNAi except Rad26. As a control we found that under resting conditions tyrosine phosphorylated STAT92E was detected predominantly in the cytoplasm [Fig. 4A–C]. Moreover, we observed a significant reduction in phosphorylated STAT92E levels in the cytoplasm when cells were treated with dsRNA against the receptor *dome* [Fig. 4D–F]. Upon stimulation with Upd, STAT92E accumulates in the nuclei of 27.2% (n = 162) of cells [Fig. 4, cf. G–I and A–C]. These results illustrate the specificity and sensitivity of our assay. Interestingly, we found that cells treated with dsRNAs against *CG11763* or *CG10225* displayed a significant increase in phospho-STAT92E nuclear accumulation upon Upd stimulation [Fig. 4, cf. J–L,M–O and G–I, 80%, n = 200 and 59%, n = 200, respectively]. This was not due to changes in the total phosphorylation levels of STAT92E [data not shown]. We could not detect significant effects of dsRNA-mediated knockdown of *Cas* or *Trn* on STAT92E translocation [data not shown]. On the other hand, the role of Ran and Nup98 in STAT92E translocation could not be assessed in this assay due to difficulties in introducing the Upd expression vector into cells upon RNAi knockdown of these two genes [data not shown]. Taken together, these results strongly suggest that the *Drosophila* homologs of RanBP3 and RanBP10 are novel regulators of JAK/STAT signaling that affect signal-dependent STAT92E nuclear transport.

The role of protein tyrosine phosphatase 61F (PTP61F) in the JAK/STAT pathway

Another important step in the JAK/STAT signal transduction pathway is the dephosphorylation of the signaling molecules JAKs and STATs. In mammals, several PTPs have been implicated in the dephosphorylation of JAK and/or STAT proteins both in the cytoplasm and in the nucleus [Shuai and Liu 2003]. In contrast, no PTPs have been identified that regulate JAK/STAT signaling in *Drosophila*. PTP61F was identified as a strong negative regulator in our screen. Knockdown of PTP61F by RNAi resulted in a more than fourfold increase in STAT92E-dependent reporter activity (Fig. 5A). PTP61F encodes the *Drosophila* homolog of mammalian PTP-1B, which has been shown to attenuate insulin, PDGF, EGF, and IGF-I signaling by dephosphorylating tyrosine residues of JAKs and/or STATs in mammalian tissue culture [Aoki and Matsuda 2000; Myers et al. 2001]. We therefore tested the hypothesis that PTP61F might serve as the tyrosine phosphatase for Hop. We observed a dramatic increase in tyrosine phosphorylation of Hop upon RNAi knockdown of PTP61F by RNAi [Fig. 5B], suggesting that Hop may be a substrate of PTP61F. We also detected a significant increase in STAT92E phosphorylation in cells treated with dsRNA against PTP61F [Fig. 5C]. This is consistent with the notion that STAT92E is a downstream target of Hop, although we cannot rule out the possibility that both Hop and STAT92E may be targets of PTP61F.

In both mammals and *Drosophila*, SOCS, a negative regulator of the JAK/STAT pathway, has been shown to be transcriptionally activated by JAK/STAT signaling, thus generating a negative feedback loop. This prompted us to examine the expression pattern of PTP61F and whether its expression is responsive to JAK/STAT signaling in vivo. We found PTP61F is expressed in a striped pattern, reminiscent of the STAT92E expression pattern.
In addition, overexpression of Upd under the control of prd-Gal4 resulted in a dramatic increase in PTP61F transcript levels in the paired domain (Fig. 5D, panel c). Furthermore, levels of the PTP61F transcript were greatly reduced in embryos lacking Hop activity (Fig. 5D, panel d), suggesting that PTP61F transcription is dependent on active JAK/STAT signaling. Taken together, these results demonstrate that PTP61F expression responds to JAK/STAT signaling in vivo.

These data suggested that loss of PTP61F would result in an increase in JAK/STAT signaling. Thus, we next examined the genetic interaction between PTP61F and canonical components of the JAK/STAT pathway, using Df(3)ED4238, a deficiency uncovering the PTP61F gene. We tested the interaction in the Drosophila eye following overexpression of Upd using GMR-Gal4 driver, which causes a dramatic overgrowth and deformation of the adult eye [Fig. 5E, panel b] (Bach et al. 2003; Chen et al. 2003). Consistent with PTP61F being a negative regulator of the JAK/STAT signaling pathway, flies heterozygous for Df(3)ED4238 showed an enhanced deformed eye phenotype (Fig. 5E, panel c). A PTP61F transgene rescues this enhanced deformed eye phenotype in flies carrying UAS-Upd GMR-Gal4/+; Df(3)ED4238/+ (Fig. 5E, panel d). In addition, the PTP61F transgene also rescues lethality in flies carrying UAS-Upd GMR-Gal4/+; Df(3)ED4238/+; presumably caused by leaky expression of UAS-Upd in conjunction with PTP61F deficiency [Supplementary Table 2].

Next we examined the genetic interaction between
PTP61F and Hop. Flies carrying a dominant hyperactive Hop allele [HopTum-l] display decreased viability and the formation of melanotic tumors [Harrison et al. 1995; Dearolf 1998]. This tumor formation phenotype is sensitive to gene dosage. Previous studies have shown that reducing the levels of positive regulators, such as STAT92E, Cdk4, and CycE, increases the viability and/or decreases tumor formation [Hou et al. 1996; Chen et al. 2003]. We therefore monitored both viability and melanotic tumor formation in females heterozygous for HopTum-l and compared these results to females heterozygous for both HopTum-l and Df(3)ED4238. Removing one copy of PTP61F in HopTum-l heterozygous females leads to a significant decrease in survival rate and a dramatic enhancement in the formation of melanotic tumors (Table 1). Altogether, these results demonstrate that PTP61F is a bona fide negative regulator of the JAK/STAT pathway in Drosophila.

Discussion

Here, we report the first genome-wide RNAi screen for novel components of the JAK/STAT signal transduction pathway. This screen has uncovered 116 novel genes that regulate JAK/STAT signaling in Drosophila, in addition to five previously characterized canonical JAK/STAT components. This demonstrates that our screen was successful in identifying genes with specific roles in the JAK/STAT pathway. We further showed that 13 of the 29 positive regulators are required for Upd-induced STAT92E phosphorylation. In addition, we found two novel regulators of STAT92E nuclear translocation. Finally, we identified PTP61F as the first protein tyrosine phosphatase that negatively regulates JAK/STAT signaling in Drosophila both in vitro and in vivo, and demonstrated that PTP61F is a transcriptional target of JAK/STAT signaling.

Among the identified genes, 40 (32.8%) had no predicted molecular function and/or recognizable protein domain, suggesting that the screen identified many uncharacterized genes with essential roles in JAK/STAT signaling (Fig. 2B, Supplementary Table 1). Notably, “Reciprocal-Best-Blast” (RBB) analysis revealed that 88 genes (72.7%) identified in the screen have human orthologs, suggesting a conserved role in the mammalian JAK/STAT signaling pathway (Supplementary Table 1).

The list of candidate genes identified in this screen only minimally overlaps with that generated from similar genome-wide RNAi studies on the Wnt and Hedgehog signaling pathways [Dasgupta et al. 2005; K. Nybakken, pers. comm.], indicating that we have identified many genes that have a specific role in the JAK/STAT signaling pathway. Moreover, ~73% of our candidate genes have well-conserved human orthologs, suggesting that cell-based assays in Drosophila can serve as a simple assay system to rapidly identify and characterize genes that may play similar roles in the mammalian JAK/STAT pathway.

Clearly the results from the screen presented here will provide the foundation for many follow-up investigations, as each of the newly identified genes will need to be carefully validated in vivo for their roles in JAK/STAT signaling. Validation in the fly system, as well as in other model systems for those evolutionarily conserved factors, will provide further insights into our global understanding of JAK/STAT signaling.

Materials and methods

JAK/STAT reporter gene

A 441-bp genomic fragment in the enhancer of SOCS36E containing two potential STAT92E-binding sites was amplified by PCR, using five different sets of oligos: (1) CTGCAGGAACACCTCATAGATGCTGAGTCCTGTGCTGTTAAC, AACGGTTTCCCAGAACGCAG

Table 1. Loss of function of PTP61F enhances Tum-l phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viable</th>
<th>% with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tum-l+/+; TM3, Sh/+</td>
<td>350</td>
<td>19.43</td>
</tr>
<tr>
<td>Tum-l+/+; Df(3)ED4238/+</td>
<td>177</td>
<td>89.83</td>
</tr>
</tbody>
</table>

Results from three independent experiments. Female flies heterozygous for both Df(3)ED4238 and hopTum-l displayed a significant decrease in viability and a dramatic increase in melanotic tumor formation compared to hopTum-l female flies heterozygous for only hopTum-l.
were annealed, respectively, and cloned to-

hsp70

UAS-Upd GMR-Gal4/+; Df(3)ED4238

hop

PTP61F

paired-Gal4 (Harrison et al. 1998), °

Drosophila

Renilla

PTP61F (a dominant temperature-sensitive allele) (Brand and Perrimon ∼

UAS-PTP61F

hop

were generated using the FLP-DFS technique

hop

/+

Renilla

RNAi Screening Center for reagents and technical

Renilla

FRT

hop

∼

males with

FRT

or

UAS-Upd

(this study),

luciferase

UAS-PTP61F

FM7/Y

°

adult flies was

females, were maintained at 29 °C

Fly stocks and genetic interaction

For image analysis, cells were bathed with various dsRNAs for 4 d and then transfected with Act-Upd or left untreated. Twenty-four hours after transfection, cells were fixed and standard immunohistochemistry was performed using an antibody against phospho-Tyr-STAT92E. DAPI staining was employed to visualize the nuclei. Accumulation of phosphorylated STAT92E in the nuclei was analyzed and images acquired under the confocal microscope.

Cell lines

The cell line that we used is a derivative of S2 cells and was originally a Peptidoglycan-responsive cell line. During the course of maintenance in our lab, however, we have noticed subtle morphological changes in these cells. Most importantly, they are no longer responsive to Peptidoglycan treatment. Thus, these cells have evolved to a new S2 cell derivative, and were thus referred to as “S2-NP.”

A cell-based RNAi screen

A library of ~21,300 dsRNAs representing the Drosophila genome was aliquoted into 384-well plates (~80 ng dsRNA/well). For each well, 0.5 ng 10XSTAT92E-luciferase, 10 ng Act-Remi

nilla, and 110 ng pAc-PL (serving as carrier DNA) were mixed with 0.96 µL Enhancer in 15 µL EC (Qiagen) and incubated at room temperature for 5 min. Then 0.42 µL of Effectene reagent was added and the mixture was immediately dispensed into each well containing dsRNA. After incubation at room temperature for 10 min, 40 µL S2-NP cells (10⁶ cells/mL) were dispensed into the well. Luciferase assays were performed 96 h after transfection using DualGlo reagents (Promega). For each well, the reporter activity, referred to as relative luciferase units (RLU), was calculated as the ratio of firefly luciferase to Renilla luciferase. For each plate, the mean and SD of RLU were calculated. dsRNAs that caused a RLU value to be either two SD or more below the mean or three SD or more above the mean were selected as candidate genes. The assay was conducted in duplicate to reduce the rate of false positives and to increase the reproducibility of individual candidates. For the secondary screen, 286 dsRNAs were resynthesized using the MegaScript kit (Ambion) and aliquoted into 384-well plates (80 ng dsRNA/well). Transfection and luciferase assays were performed as described above. In experiments involving pol III-Renilla, the same amount of pol III-Renilla was used as with Act-Renilla. To assay candidate genes in cells stimulated with Upd, 20 pg 10XSTAT92E-luciferase, 5 ng Act-Renilla, 105 ng pAc-PL, and 1 ng Act-Upd were transfected to S2-NP cells together with 80 ng dsRNA. In experiments involving 6XSTAT–synthetic-luc, 2 ng 6XSTAT–synthetic-luc, 20 ng pol III-Renilla, 80 ng pAc-PL, and 20 ng Act-Upd were transfected to S2-NP cells together with 80 ng dsRNA per well. In all the above-mentioned experiments, luciferase assays were performed 96 h after transfection.

Immunoprecipitation and Western blot analysis

For analyzing Upd-induced STAT92E phosphorylation, an expression plasmid for HA-tagged STAT92E (Act-STAT92E-HA) was transfected into S2-NP cells together with dsRNA against LacZ. Cells were split into two dishes 3.5 d after transfection. Half of the cells were co-cultured with S2-NP cells transfected with Act-Upd ~12 h prior to harvest (Harrison et al. 1998) and the other half remained untreated as control. Cell extracts were subjected to immunoprecipitation using anti-HA antibodies, and the immunoprecipitates were analyzed by immunoblotting analysis using anti-phospho-Tyr-STAT92E and anti-HA antibodies. The effect of RNAi knockdown of 29 potential positive regulators on STAT92E tyrosine phosphorylation was investigated by Western blot analysis. S2-NP cells were transfected with Act-STAT92E-HA together with dsRNA targeting each of the positive regulators. Four days after transfection, cells were cocultured with S2-NP cells transfected with Act-Upd for ~12 h prior to harvest. The cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane, and subjected to immunoblotting analysis using anti-phospho-Tyr-STAT92E antibody (Cell Signaling). The membrane was then stripped and reprobed with anti-HA antibody (Upstate) to detect STAT92E-HA as a loading control. To examine the effect of dsRNA-mediated knockdown of PTP61F on the phosphorylation status of Hop and STAT92E, Act-Myc-Hop or Act-STAT92E-HA was transfected into S2-NP cells together with dsRNAs against lacZ or PTTP61F. Cells were harvested and cell lysates were immunoprecipitated with anti-Myc or anti-HA antibodies, respectively. Immunoprecipitates were analyzed by immunoblotting using anti-phospho-Tyr or anti-phospho-Hop-STAT92E antibodies, respectively. The membranes were stripped and reprobed with anti-Myc or anti-HA antibodies, respectively.

Acknowledgments

We thank Dr. Kent Nybakken for kindly providing the Act-Renilla and pol III-Renilla plasmids. We thank members of the Drosophila RNAi Screening Center for reagents and technical assistance. We thank Ramanuj Dasgupta and Kent Nybakken.
for communicating data prior to publication. Special thanks go to Bernard Mathey-Provot, Sara Cherry, Ramanuj Dasgupta, Pamela Bradley, and Richard Binari for critically reading the manuscript. N.P. is a Howard Hughes Medical Institute investigator. G.-H.B. was supported by The Medical Foundation/Charles A. King Trust post-doctoral fellowship. R.Z. is a Leukemia and Lymphoma Society fellow.

Note added in proof

Supplementary Table 1 lists the information on the dsRNAs. Please note that the results obtained with dsRNAs with potential off-target effects will need further validation with newly synthesized independent dsRNAs.

References


