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

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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.

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-

Baeg et al.

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 tyrosine 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 frag-

ment 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 activ-
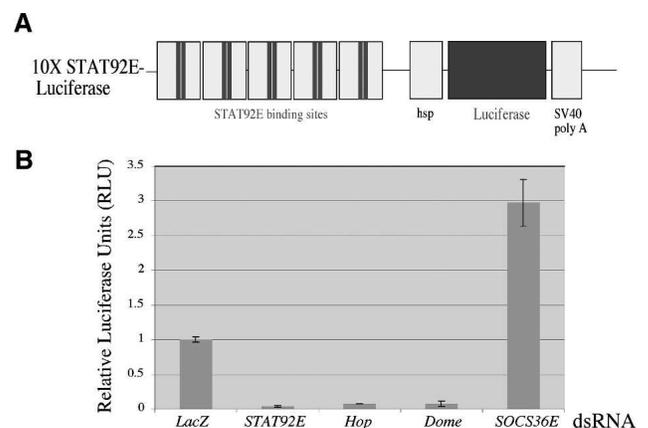
Figure 1. Generating a JAK/STAT reporter construct. (*A*) Schematic representation of the 10XSTAT92E–luciferase reporter construct. Five copies of a genomic fragment from the *SOCS36E* intronic region containing two STAT92E-binding sites were placed upstream of a *hsp* minimal promoter-driven firefly *luciferase* gene. (*B*) *Drosophila* S2-NP cells were transfected with 10XSTAT92E–luciferase and Act-*Renilla* together with dsRNAs against various canonical components of the JAK/STAT pathway. Luciferase assay was performed 4 d later, and the reporter activity was normalized as the ratio of firefly/*Renilla*. Note that the control value was set to 1. The results were from two independent experiments.

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-two 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.
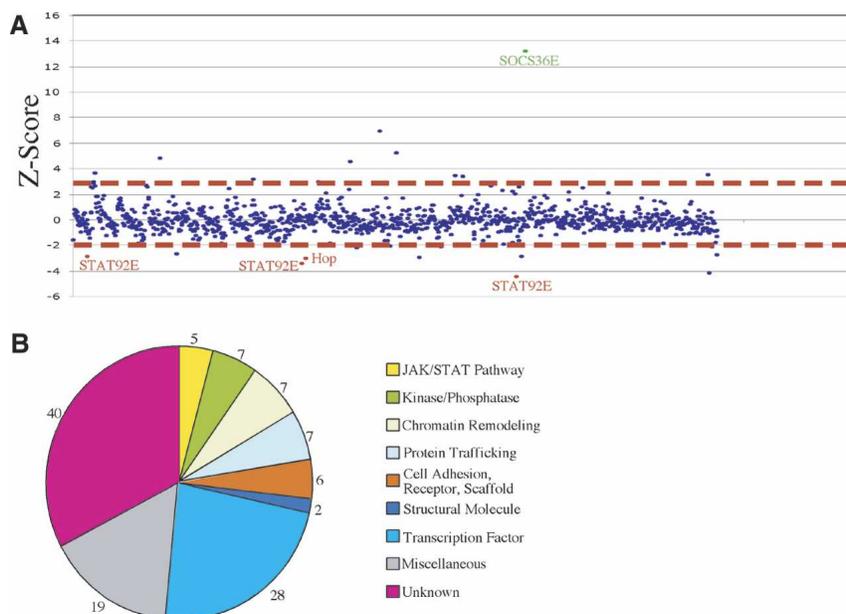
Figure 2. Data analysis for the JAK/STAT screen. (*A*) Scatter plot for three representative screen plates. Cutoffs were set as 2 SD below the mean or 3 SD above the mean RLU. Note that all three "spiked in" control dsRNAs against *STAT92E* were identified. (*B*) Pie chart depicting categories of genes identified in the JAK/STAT screen.

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 knockdown 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 with cdc2 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.
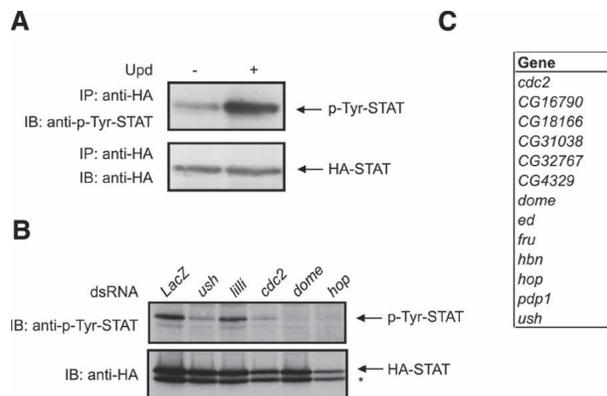
Figure 3. Identification of genes required for Upd-induced tyrosine phosphorylation of STAT92E. (*A*) Act-STAT92E-HA was transfected into S2-NP cells together with dsRNA against *lacZ*. Cells were split into two dishes 3.5 d after transfection. Half of the cells were cocultured with S2-NP cells transfected with Act-Upd ~12 h prior to harvest and the other half remained untreated. Cell extracts were subjected to immunoprecipitation using anti-HA antibodies and the immunoprecipitates were analyzed by immunoblotting using anti-phospho-Tyr-STAT92E and HA antibodies. Note that Upd-induction leads to a dramatic increase in STAT92E phosphorylation. (*B*) Act-STAT92E-HA was transfected into S2-NP cells together with various dsRNAs targeting positive regulators identified in the screen. Cells were stimulated with Upd ~12 h prior to harvest. Cell extracts were subjected to Western blot analysis using anti-phospho-Tyr-STAT92E and HA antibodies. dsRNAs against *LacZ* and *lilli* serve as control. (*C*) List of genes required for Upd-induced STAT92E phosphorylation.

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 nucleocytoplasmic 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

Figure 4. *Drosophila* homologs of RanBP3 (CG11763) and RanBP10 (CG10225) are involved in phosphorylated STAT92E nucleocytoplasmic shuttling. Cells were treated with various dsRNAs and then transfected with Act-Upd 4 d later (*G–O*) or remained untransfected (*A–F*). Immunostaining was performed using anti-phospho-Tyr-STAT92E antibody (green). DAPI staining was employed to visualize the nuclei (red). Note that a significant accumulation of phosphorylated STAT92E in the nuclei of cells treated with dsRNA against either *CG11763* or *CG10225* was detected upon Upd induction, compared with cells treated with dsRNA for *lacZ*.

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* (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

Baeg et al.

Figure 5. PTP61F negatively regulates the JAK/STAT pathway in *Drosophila*. (*A*) Knockdown of *PTP61F* by RNAi activates the JAK/STAT reporter activity. *Drosophila* S2-NP cells were transfected with 10XSTAT92E–luciferase and Act-*Renilla* together with dsRNAs against *lacZ* or *PTP61F*. Luciferase assay was performed 4 d later and the reporter activity was normalized as the ratio of firefly to *Renilla*. The control value was set as 1. The results were from two independent experiments. (*B*) Act-Myc-Hop was transfected into S2-NP cells together with dsRNAs against *lacZ* or *PTP61F*. Cells were harvested and cell lysates were immunoprecipitated with anti-Myc antibody. Immunoprecipitates were analyzed by immunoblotting using anti-phospho-Tyr or anti-Myc antibodies. Note that an increase in phospho-Hop levels was detected upon RNAi knockdown of *PTP61F*. (*C*) Act-STAT92E-HA was transfected into S2-NP cells together with dsRNAs against *lacZ* or *PTP61F*. Cells were harvested and cell lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates were analyzed by immunoblotting using anti-phospho-Tyr-STAT92E or anti-HA antibodies. An increase in phospho-STAT92E levels was detected upon RNAi knockdown of *PTP61F*. (*D*) RNA in situ hybridization using *STAT92E* or *PTP61F* probes was performed on wild-type stage 9–10 embryos (panels *a,b*), embryos overexpressing Upd under the control of *paired-Gal4* (panel *c*), and *hop* GLC embryos (panel *d*). Note that *PTP61F* transcript levels are dramatically increased in the *paired* domain (panel *c*) and decreased in embryo lacking *hop* activity (panel *d*). (*E*) Genetic interactions between *Upd* and *PTP61F*. Overexpression of *Upd* in the eye under the control of *GMR-Gal4* results in a dramatic overgrowth and deformation in the adult eye (cf. panels *a* and *b*). Removing one copy of *PTP61F* further enhances this phenotype (panel *c*), whereas introduction of a *PTP61F* transgene rescues this phenotype (panel *d*).

(Fig. 5D, panels a,b). 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). The severity of this phenotype is proportional to the strength of the JAK/STAT-mediated signal, as removing one copy of *STAT92E* significantly suppresses the *GMR-Upd* eye phenotype (Bach et al. 2003; data not shown). 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 heterozygous for *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 (*Hop^Tum-1*) 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 *Hop^Tum-1* and compared these results to females heterozygous for both *Hop^Tum-1* and *Df(3)ED4238*. Removing one copy of *PTP61F* in *Hop^Tum-1* 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) CTGCAGGAAC CACTCAGAGTGCCTGCGTGT (PstI), GAATTCATACAAAA CTGTCTTAGGTGTTTA (EcoRI); (2) CTGCAGGAACCACT CAGAGTGCCTGCGTGT (PstI), CTGCAGATACAAAACT GTCTTAGGTGTTTA (PstI); (3) GAATTCGAACCACTCA GAGTGCCTGCGTGT (EcoRI), GAATTCATACAAAACTGT CTTAGGTGTTTA (EcoRI); (4) AGATCTGAACCACTCA GAGTGCCTGCGTGT (BglII), AGATCTATACAAAACTGT CTTAGGTGTTTA (BglII); (5) GCGGCCGCGAACCACTCAG AGTGCCTGCGTGT (NotI), GCGGCCGCATACAAAACTGT CTTAGGTGTTTA (NotI). Each amplified genomic fragment containing different restriction enzyme sites was sequentially subcloned into pP[UAST] (Phelps and Brand 1998). The genomic fragment amplified using the first set of oligos was subcloned into the PstI/EcoRI sites of pP[UAST] to generate 2XSTAT92E. The genomic fragment amplified using the second set of oligos was subcloned into the PstI site of 2XSTAT92E to generate 4XSTAT92E. The genomic fragment amplified using the third set of oligos was subcloned into the EcoRI site of 4XSTAT92E to generate 6XSTAT92E. The genomic fragment amplified using the fourth set of oligos was subcloned into the BglII site of 6XSTAT92E to generate 8XSTAT92E. Next, the *hsp70* minimal promoter element was amplified from pP[UAST] by PCR using oligos GCGGCCGCAGCGGAGACTCTAGCGAGCG (NotI) and CTCGAGAATTCCCTATTCAGAGTTCT (XhoI). This *hsp70* minimal promoter was subcloned into the NotI/XhoI sites of 8XSTAT92E to generate 8XSTAT92E–hsp70. Again, the genomic fragment amplified using the fifth set of oligos was subcloned into the NotI site of 8XSTAT92E–hsp70 vector to generate 10XSTAT92E–hsp70. Finally, an XhoI/XbaI fragment containing the firefly *luciferase* gene from the pGL3–luciferase vector (Promega) was subcloned into the XhoI/XbaI sites of 10XSTAT92E–hsp70 to generate 10XSTAT92E–luciferase.

To generate a reporter construct containing six STAT consensus sites, two pairs of oligos—(1) TTCTGGGAAACCGTTTA TACGCTGCGTTCGCGGAAACCGTTT ATACGCTGCGTT C TGGGAAACCGTTTATAC, AACGGTTTCCCAGAACGCAG

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

Genotype	Viable	% with tumors
Tum-l/+; *TM3, Sb/+*	350	19.43
Tum-l/+; *Df(3)ED4238/+*	177	89.83

Results from three independent experiments.
Female flies heterozygous for both *Df(3)ED4238* and *hop^Tum-1* displayed a significant decrease in viability and a dramatic increase in melanotic tumor formation compared to *hop^Tum-1* female flies heterozygous for only *hop^Tum-1*.

Baeg et al.

CGTATAAACGGTTTCCGCGAACGCAGCGTATAAACGGTT
TCCCAGAATGCA and (2) GCTGCGTTCGCGGAAACCGTT
TATACGCTGCGTTCTGGGAAACCGTTTATACGCTGCGT
TCGCGGAA, AATTTTCCGCGAACGCAGCGTATAAACG
GTTTCCCAGAACGCAGCGTATAAACGGTTTCCGCGAA
CGCAGCGTATA—were annealed, respectively, and cloned to-
gether into pP[UAST] using PstI and EcoRI sites. Subsequently,
an XhoI/XbaI fragment containing the firefly *luciferase* gene
from the pGL3–luciferase vector (Promega) and the *hsp70* mini-
mal promoter were subcloned into the resulting vector using
XhoI/XbaI and NotI/XhoI sites, respectively, to generate the
6XSTAT–synthetic-luc construct.

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* ge-
nome was aliquotted into 384-well plates (~80 ng dsRNA/well).
For each well, 0.5 ng 10XSTAT92E–luciferase, 10 ng Act-*Re-
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
were added and the mixture was immediately dispensed into
each well containing dsRNA. After incubation at room tem-
perature for 10 min, 40 μL S2-NP cells (10^6 cells/mL) were dis-
pensed 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 calcu-
lated. 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 dupli-
cate 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 aliquotted into 384-well plates (80 ng dsRNA/
well). Transfection and luciferase assay were performed as de-
scribed 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 experi-
ments, luciferase assays were performed 96 h after transfection.

Immunoprecipitation and Western blot analysis

For analyzing Upd-induced STAT92E phosphorylation, an ex-
pression 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 cocultured 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 anti-
bodies. The effect of RNAi knockdown of 29 potential positive
regulators on STAT92E tyrosine phosphorylation was investi-
gated 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 immunoblot-
ting 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 *PTP61F*. 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-Tyr-STAT92E antibodies, respectively. The mem-
branes were stripped and reprobed with anti-Myc or anti-HA
antibodies, respectively.

Immunohistochemistry

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 stan-
dard 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.

Fly stocks and genetic interaction

Fly lines were maintained according to standard procedure. The
following fly lines were used: *hop^{C111}*/FM7 (Binari and Perrimon
1994), *hop^{Tum-1}*/FM7 (a dominant temperature-sensitive allele)
(Hanratty and Dearolf 1993), *paired-Gal4* (Brand and Perrimon
1993), *UAS-Upd* (Harrison et al. 1998), *UAS-Upd GMR-Gal4/
CyO* (this study), *UAS-PTP61F* (this study), and *UAS-PTP61F
Df(3)ED4238/TM3* (this study). Females carrying germline
clones of *hop^{C111}* were generated using the FLP-DFS technique
(Chou and Perrimon 1996). Virgin females of the genotype
hop^{C111} FRT^{101}/FM7 were mated with males of the genotype
ovo^{D1} FRT^{101}/Y; *FLP^{38}*. The resulting larvae were heat-shocked
for 2 h at 37°C. *hop^{C111} FRT^{101}*/ovo^{D1} FRT^{101} females were
crossed with *FM7/Y* males. For *PTP61F* genetic interaction stud-
ies, the eye phenotype of *UAS-Upd GMR-Gal4/+* adult flies was
compared to that of *UAS-Upd GMR-Gal4/+; Df(3)ED4238/+*
adult flies. *hop^{Tum-1}*/+;*TM3, Sb*/+ and *hop^{Tum-1}*/+;*Df(3)ED4238/+*
females were generated by crossing *hop^{Tum-1}*/Y males with
Df(3)ED4238/TM3, Sb females, were maintained at 29°C, and
were scored for viability and the presence of melanotic tumors.

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.

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Addendum

Genes & Development 19: 1861–1870 (2005)

Genome-wide RNAi analysis of JAK/STAT signaling components in *Drosophila*
Gyeong-Hun Baeg, Rui Zhou, and Norbert Perrimon

Recently it was shown that long double-stranded RNAs (dsRNAs) can lead to "off-target effects" (OTE) in *Drosophila* cells (Kulkarni et al. 2006; Ma et al. 2006). We therefore generated one or two additional independent dsRNAs for each of the 121 candidate genes of the JAK/STAT signaling pathway that we initially reported (Baeg et al. 2005). Each of the newly generated dsRNAs was designed to be free of 19 base pairs (bp) or longer overlap with other genes. We retested these new dsRNAs in parallel with the original dsRNAs identified from the screen and found that 111 original dsRNAs scored, and among them, 50 could be further confirmed by one or two independent dsRNAs (Table 1). Of interest, we note that 17 of the original dsRNAs that were devoid of any 19-bp homology with other genes failed to be confirmed by additional dsRNAs. This finding suggests that other OTE rules that we have not been able to identify (such as interference with miRNA function through potential seed regions found in small interfering RNAs [siRNAs] [Lewis et al. 2003]) may also lead to false positives in large-scale screens in *Drosophila* cells. Alternatively, it is possible that knockdown efficiency varies among different long dsRNAs. In addition, nine of the dsRNAs in the initial 121 positives that, based on our in silico analysis, were predicted to have off-target sequences targeting 15 or more other genes could be confirmed with a second or third dsRNA. Taken together, our data strongly support the recommendation made by Echeverri et al. (2006) that testing of two or more independent dsRNAs should be performed, and will help minimize the risk of reporting false positives in RNA interference (RNAi)-based assays. In conclusion, cell-based assays and RNAi, when well controlled, constitute a valid approach for identification of genes potentially involved in a given biological process, but more detailed biochemical and genetic analyses will be necessary to validate these candidate genes.

Table 1. *List of genes that were confirmed by two or three independent dsRNAs in the JAK/STAT assay*

Negative regulators			Positive regulators		
Amplicon	Gene	Fold change	Amplicon	Gene	Fold change
DRSC11325	ash1	3.43	DRSC03504	cdc2	0.40
DRSC32654	ash1	3.81	DRSC30705	cdc2	0.39
DRSC32655	ash1	2.64	DRSC30706	cdc2	0.38
DRSC19337	Bap60	1.47	DRSC17794	CG11700	0.27
DRSC32656	Bap60	1.70	DRSC31545	CG11700	0.22
DRSC32657	Bap60	1.53	DRSC08254	CG12104	0.65
DRSC11330	brm	1.89	DRSC32318	CG12104	0.67
DRSC30901	brm	1.32	DRSC32317	CG12104	0.55
DRSC03287	Cas	1.43	DRSC15283	CG17836	0.24
DRSC32658	Cas	1.48	DRSC32680	CG17836	0.29
DRSC32659	Cas	1.76	DRSC32681	CG17836	0.29
DRSC04085	CG10955	1.43	DRSC18386	CG32767	0.30
DRSC30727	CG10955	1.55	DRSC32396	CG32767	0.54
DRSC30728	CG10955	1.81	DRSC13053	CG3563	0.54
DRSC09878	CG12310	2.22	DRSC30936	CG3563	0.73
DRSC25358	CG12310	1.48	DRSC10516	CG5546	0.44
DRSC04191	CG13550	2.44	DRSC30863	CG5546	0.34
DRSC31776	CG13550	1.37	DRSC30862	CG5546	0.49
DRSC31777	CG13550	1.50	DRSC10563	CG5971	0.78
DRSC00447	CG15432	1.48	DRSC32714	CG5971	0.63
DRSC32675	CG15432	1.25	DRSC19969	CG5988	0.26
DRSC06127	CG30089	3.36	DRSC32715	CG5988	0.23
DRSC32689	CG30089	1.27	DRSC32716	CG5988	0.35
DRSC10977	CG32365	1.74	DRSC18427	CG8636	0.35
DRSC32700	CG32365	1.29	DRSC32087	CG8636	0.41
DRSC11697	CG32428	2.80	DRSC32088	CG8636	0.55
DRSC32701	CG32428	1.24	DRSC19583	dome	0.17
DRSC06562	CG33455	2.01	DRSC32731	dome	0.16
DRSC32709	CG33455	1.47	DRSC32732	dome	0.13

(*continued*)

Table 1. (*continued*)

Amplicon	Gene	Fold change	Amplicon	Gene	Fold change
	Negative regulators			**Positive regulators**	
DRSC32710	CG33455	1.38	DRSC16704	Hmgcr	0.34
DRSC04360	CG3363	1.37	DRSC31628	Hmgcr	0.77
DRSC32064	CG3363	1.90	DRSC20340	hop	0.23
DRSC18349	CG4136	3.83	DRSC32739	hop	0.25
DRSC32406	CG4136	3.27	DRSC32740	hop	0.23
DRSC32407	CG4136	3.59	DRSC00708	lilli	0.56
DRSC10635	CG6434, CG5585	1.51	DRSC32745	lilli	0.49
DRSC32720	CG6434	1.24	DRSC32746	lilli	0.65
DRSC11848	CG7752	1.53	DRSC11251	Pdp1	0.58
DRSC31702	CG7752	1.29	DRSC32517	Pdp1	0.77
DRSC31701	CG7752	1.48	DRSC11285	Snap	0.54
DRSC20132	CG8949	1.89	DRSC31261	Snap	0.20
DRSC32727	CG8949	1.44	DRSC16870	Stat92E	0.17
DRSC32728	CG8949	1.43	DRSC32773	Stat92E	0.23
DRSC04096	enok	2.51	DRSC32774	Stat92E	0.27
DRSC32735	enok	1.81	DRSC00843	ush	0.14
DRSC32736	enok	2.38	DRSC32226	ush	0.42
DRSC16651	jumu	1.59	DRSC32227	ush	0.34
DRSC32741	jumu	2.02			
DRSC32742	jumu	1.81			
DRSC04696	ken	4.83			
DRSC31748	ken	1.69			
DRSC06948	lolal	1.76			
DRSC32751	lolal	1.64			
DRSC32752	lolal	1.78			
DRSC15378	mor	1.35			
DRSC32754	mor	1.30			
DRSC14209	Nup98	1.38			
DRSC31803	Nup98	1.29			
DRSC11874	Pitslre	1.55			
DRSC31971	Pitslre	1.56			
DRSC08683	Ptp61F	4.39			
DRSC32761	Ptp61F	4.52			
DRSC32762	Ptp61F	4.01			
DRSC17034	puc	1.84			
DRSC31024	puc	1.34			
DRSC02455	Socs36E	5.46			
DRSC30658	Socs36E	2.32			
DRSC30659	Socs36E	5.54			
DRSC16211	Ssdp	1.46			
DRSC31311	Ssdp	1.29			
DRSC31310	Ssdp	1.51			
DRSC11309	Trn	1.53			
DRSC32778	Trn	1.22			
DRSC17089	trx	4.90			
DRSC32779	trx	4.20			
DRSC32780	trx	5.87			
DRSC20381	unc-4	1.99			
DRSC32781	unc-4	1.61			
DRSC03641	zf30C	1.55			
DRSC32783	zf30C	1.83			
DRSC32784	zf30C	1.77			

The average reporter activity from multiple control samples treated with *lacZ* dsRNA was set as 1 and those from samples treated with test dsRNAs were calculated accordingly. Results shown were the average values from three independent experiments conducted in duplicate. The cut-off values are 1.2 and 0.8 for negative and positive regulators, respectively. The original amplicons are shaded.

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

Gyeong-Hun Baeg, Rui Zhou and Norbert Perrimon

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