GFP reporters detect the activation of the *Drosophila* JAK/STAT pathway *in vivo*

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Abstract

JAK/STAT signaling is essential for a wide range of developmental processes in *Drosophila melanogaster*. The mechanism by which the JAK/STAT pathway contributes to these processes has been the subject of recent investigation. However, a reporter that reflects activity of the JAK/STAT pathway in all *Drosophila* tissues has not yet been developed. By placing a fragment of the Stat92E target gene *Socs36E*, which contains at least two putative Stat92E binding sites, upstream of *GFP*, we generated three constructs that can be used to monitor JAK/STAT pathway activity *in vivo*. These constructs differ by the number of Stat92E binding sites and the stability of GFP. The *2XSTAT92E-GFP* and *10XSTAT92E-GFP* constructs contain 2 and 10 Stat92E binding sites, respectively, driving expression of enhanced *GFP*, while *10XSTAT92E-DGFP* drives expression of destabilized *GFP*. We show that these reporters are expressed in the embryo in an overlapping pattern with Stat92E protein and in tissues where JAK/STAT signaling is required. In addition, these reporters accurately reflect JAK/STAT pathway activity at larval stages, as their expression pattern overlaps that of the activating ligand *unpaired* in imaginal discs. Moreover, the *STAT92E-GFP* reporters are activated by ectopic JAK/STAT signaling. *STAT92E-GFP* fluorescence is increased in response to ectopic *upd* in the larval eye disc and mis-expression of the JAK kinase *hopscotch* in the adult fat body. Lastly, these reporters are specifically activated by Stat92E, as *STAT92E-GFP* reporter expression is lost cell-autonomously in *stat92E* homozygous mutant tissue. In sum, we have generated *in vivo* GFP reporters that accurately reflect JAK/STAT pathway activation in a variety of tissues. These reporters are valuable tools to further investigate and understand the role of JAK/STAT signaling in *Drosophila*.
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1. Results and discussion

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is an evolutionarily conserved signaling system that plays essential roles in numerous biological processes in vertebrates and inverte- brates, including immunity, hematopoiesis and prolifera- tion (reviewed in Levy and Darnell, 2002). Since *Drosophila* is highly amenable to genetic manipulations, it has served as an excellent model organism for studying this pathway (reviewed in Hombria and Brown, 2002; Hou et al., 2002). Genetic studies in *Drosophila* have identified several com- ponents of the JAK/STAT pathway, including three cyto- kine-like Unpaired (Upd) molecules (Upd, Upd2 and Upd 3) (Agaisse et al., 2003; Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005); the transmembrane receptor

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Domeless (Dome) (also called Master of Marelle), which is distantly related to the mammalian gp130 cytokine receptor (Brown et al., 2001; Chen et al., 2002); the JAK Hopscotch (Hop) (Binari and Perrimon, 1994), which is most similar to mammalian Jak2; the STAT Stat92E (Hou et al., 1996; Yan et al., 1996), which is homologous to mammalian Stat3 and Stat5; and *Socs36E*, a member of the SOCS/CIS/JAB family of JAK/STAT negative regulators (Alexander and Hilton, 2004; Callus and Mathey-Prevot, 2002; Karsten et al., 2002). The JAK/STAT signaling cascade is initiated when Upd binds to Dome, causing the receptor to undergo a conformational change. Hop molecules, which are constitutively associated with the Dome cytoplasmic domain, are then able to phosphorylate one another, as well as specific tyrosine sites on the receptor. Cytosolic Stat92E is recruited to these activated receptor sites and is subsequently phosphorylated on a specific tyrosine residue (Y711) by the associated Hop proteins (Yan et al., 1996). Activated Stat92E molecules dimerize and accumulate in the nucleus where they alter the transcription of target genes, such as *dome* and *Socs36E*, by binding to specific DNA sequences (consensus TTCNNNGAA) (Bach et al., 2003; Ghiglione et al., 2002; Karsten et al., 2002; Yan et al., 1996).

The *Drosophila* JAK/STAT pathway regulates many developmental processes, including sex determination, stem cell maintenance, oogenesis, border cell migration, embryonic segmentation, gut development, tracheal development, hematopoiesis, immunity, and eye development (Agaisse et al., 2003; Bach et al., 2003; Beccari et al., 2002; Binari and Perrimon, 1994; Brown et al., 2001; Johansen et al., 2003; Kiger et al., 2001; Sefton et al., 2000; Silver and Montell, 2001; Sorrentino et al., 2004; Tulina and Matunis, 2001; Xi et al., 2003). The contribution of JAK/STAT signaling to these processes has been the subject of recent investigations. However, an *in vivo* reporter to monitor the spatial and temporal activation of the *Drosophila* JAK/STAT pathway at multiple developmental stages is lacking.

A number of tools have been developed previously to visualize the activity of the *Drosophila* JAK/STAT pathway. These include the βlue-βlau technique that detects homo-dimerization of the Dome receptor in *Drosophila* embryos (Brown et al., 2003), reagents to visualize Stat92E activation such as a Stat92E-GFP fusion protein that exhibits nuclear translocation in cultured cells upon activation (Karsten et al., 2006), and an antibody specific for the tyrosine phosphorylated form of activated Stat92E (Li et al., 2003). In addition, Gilbert and colleagues recently generated an *in vivo* reporter to monitor JAK/STAT pathway activity (Gilbert et al., 2005). In their reporter called (GAS)$_3$-*LacZ*, *LacZ* is driven by multimerized Gamma Activated Site (GAS) elements, to which mammalian Stat1 dimers bind with optimal affinity (Decker et al., 1997; Reich et al., 1989). The (GAS)$_3$-*LacZ* reporter accurately detects pathway activation in the embryo. However, no data was presented on the expression of this reporter at later developmental stages. Although useful for some studies, these reagents have some limitations. Here, we present a characterization of *in vivo* GFP reporters, generated by placing Stat92E binding sites from a Stat92E target gene (*Socs36E*) upstream of enhanced or destabilized GFP, that accurately reflect activity of the *Drosophila* JAK/STAT pathway. These reporters allow us to examine, for the first time, the spatial and temporal activity of the JAK/STAT pathway at all developmental stages in *Drosophila*.

1.1. Generating Drosophila JAK/STAT pathway in vivo reporters

One of the few characterized JAK/STAT target genes in *Drosophila* is *Socs36E*, which is transcriptionally activated by JAK/STAT signaling (Karsten et al., 2002). Socs36E acts as a negative regulator of this pathway, presumably by either blocking Hop activation, or by competing with Stat92E for activated receptor sites (Alexander and Hilton, 2004; Callus and Mathey-Prevot, 2002). The first intron of *Socs36E* contains a 441 bp fragment with at least two potential Stat92E binding sites (Karsten et al., 2002). We used tandem repeats of this fragment to drive expression of enhanced or destabilized GFP *in vivo*. We recently employed a similar strategy to generate a luciferase reporter to monitor JAK/STAT pathway activity *in vitro* (Baeg et al., 2005). Specifically, we generated a *2XSTAT92E-GFP* reporter and a *10XSTAT92E-GFP* reporter by placing one or five tandem repeats, respectively, of this 441 bp fragment upstream of a minimal heat-shock promoter (*hsp*) and a cDNA encoding enhanced *GFP* (Fig. 1A). We also generated a *10XSTAT92E* reporter driving expression of destabilized GFP (called *10XSTAT92E-DGFP*). While enhanced GFP is stable for more than 24 h, the destabilized form is only stable for ~8 h, and is therefore a better temporal marker of transcriptional activity than enhanced GFP (Li et al., 1998).

The accuracy of the *2XSTAT92E-GFP* and *10XSTAT92E-GFP* reporters was confirmed by their embryonic expression patterns. Activation of the *Drosophila* JAK/STAT pathway results in increased levels and/or stability of the Stat92E protein (Chen et al., 2002; Johansen et al., 2003; Read et al., 2004). In wild type stage 10 embryos, Stat92E protein is detected in stripes (Fig. 1B), which is consistent with Upd and Upd2 expression domains (Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005). Both the *2X-* and *10XSTAT92E-GFP* reporters are expressed in a similar striped pattern in stage 10 embryos and specifically overlap with Stat92E protein (Fig. 1B′, B″ and data not shown). Previous work has demonstrated that JAK/STAT pathway activity is important for the development of polar cells and border cells in the ovary (Beccari et al., 2002; Silver and Montell, 2001), as well as that of posterior spiracles (Brown et al., 2001), hindgut (Johansen et al., 2003), and pharynx (Hombria et al., 2005) in the embryo. We therefore examined the expression of the *10XSTAT92E-GFP* reporter in these tissues. In the ovary, *upd* is expressed specifically in polar cells and in border cells (Beccari et al., 2002; Silver and Montell, 2001). The *10XSTAT92E-GFP* reporter is
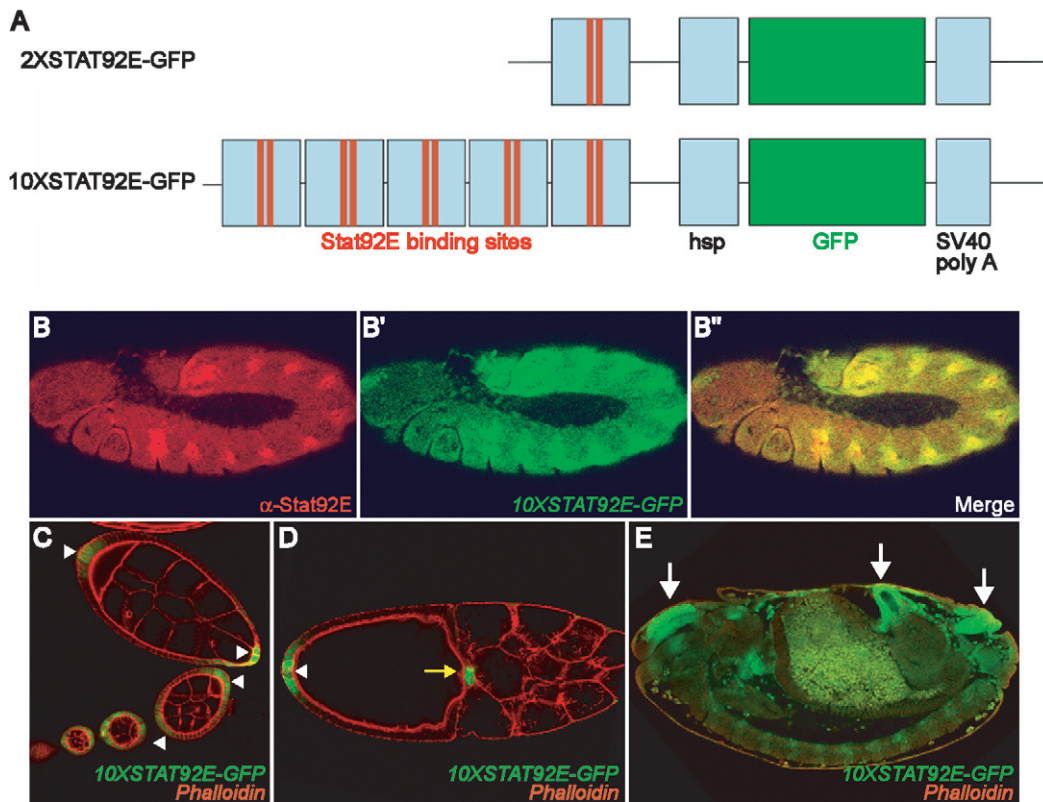
Fig. 1. *STAT92E-GFP in vivo* reporters detect JAK/STAT pathway activation in the *Drosophila* embryo. In this and all subsequent figures, anterior is to the left and dorsal is up. (A) Schematic representation of the *2XSTAT92E-GFP* and *10XSTAT92E-GFP* reporter constructs. One copy or five copies of a 441 bp genomic fragment from the *Socs36E* intron 1, which contains at least two Stat92E-binding sites, were placed upstream of an *hsp* minimal promoter-driven *Green Fluorescent Protein* (*GFP*) gene. (B–B″) The *10XSTAT92E-GFP* reporter detects JAK/STAT pathway activation in a stage 10 *Drosophila* embryo. Stat92E protein (red) is increased and/or stabilized in stripes as a result of JAK/STAT signaling (B). *10XSTAT92-GFP* reporter (green) overlaps with Stat92E protein (B′). Merge of red and green channels (yellow) (B″). Stat92E antibody (red), *10XSTAT92E-GFP* reporter (green). (C–E) The *10XSTAT92E-GFP* reporter detects JAK/STAT pathway activation in the ovary and in embryonic tissues that require JAK/STAT signaling. The *10XSTAT92E-GFP* reporter is expressed in the polar cells in stage 4–8 egg chambers (C, arrowheads), as well as in the neighboring cells, which is expected because Upd produced in the polar cells will diffuse locally. This reporter is also expressed in the polar cells (arrowhead) and in border cells (yellow arrow) in stage 10 egg chambers (D). The *10XSTAT92E-GFP* reporter is expressed in the posterior spiracle, hindgut and pharynx of a stage 16 *Drosophila* embryo (E, arrows). (C) stage 4–8 egg chambers, (D) stage 10 egg chamber, and (E) lateral view of a stage 16 embryo, all from *10XSTAT92E-GFP* transgenic flies. *10XSTAT92E-GFP* (green); Phalloidin (red).

expressed in polar cells in stage 4–10 egg chambers and in border cells in stage 10 chambers (Figs. 1C and D arrowheads and yellow arrow). In addition, cells neighboring the polar cells also express this reporter, which is expected due to local diffusion of Upd (Figs. 1C and D). Moreover, the posterior spiracles, hindgut and pharynx of a stage 16 *Drosophila* embryo all specifically express high levels of the *10XSTAT92E-GFP* reporter. These results indicate that our *STAT92E-GFP* reporters are specifically activated by JAK/STAT signaling in the embryo.

We next examined the ability of the *2X-* and *10XSTAT92E-GFP* reporters to respond to ectopic JAK/STAT signaling during later developmental stages. Wild type third instar larvae carrying a *2XSTAT92E-GFP* transgene exhibit minimal GFP fluorescence (Fig. 2A). However, increased GFP fluorescence is observed in *2XSTAT92E-GFP* larvae that also carry a *hop^{Tum-l}* allele, which encodes a hyper-activated Hop protein (Fig. 2B) (Harrison et al., 1995; Luo et al., 1995). Similar results are found in adult stages. Adults expressing the *2XSTAT92E-*

GFP transgene exhibit GFP fluorescence only in the eye (Fig. 2C). This is not due to auto-fluorescence, as it was not observed in a wild type adult in the absence of the transgene (data not shown). Using the UAS/GAL4 technique, when JAK/STAT signaling is ectopically induced by expression of *hop* in the fat body of female *2XSTAT92E-GFP* transgenic adults, there is a dramatic increase in GFP expression (Fig. 2D) (Brand and Perrimon, 1993). At both larval and adult stages, similar results were obtained with the *10XSTAT92E-GFP* reporter (data not shown). Therefore, both *2X-* and *10XSTAT92E-GFP* reporters are responsive to JAK/STAT pathway signaling in a range of tissues at various stages of development.

1.2. Spatial and temporal characterization of STAT92E-GFP reporters in imaginal discs

We next wanted to examine whether these reporters reflect JAK/STAT pathway activity in imaginal discs. We looked first at the eye disc, since the JAK/STAT pathway has been
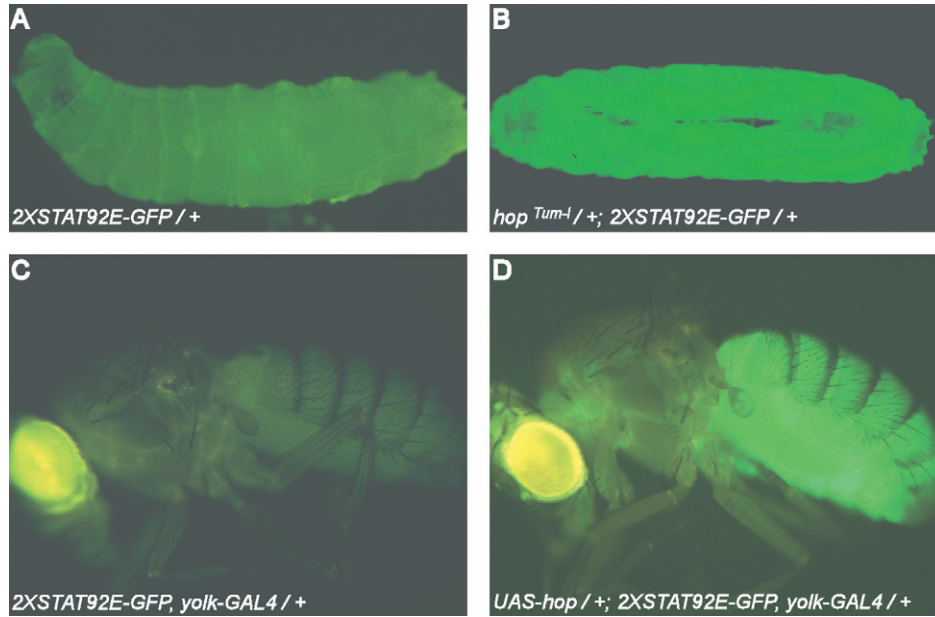
Fig. 2. *STAT92E-GFP in vivo* reporters detect ectopic activation of the JAK/STAT pathway in larval and adult stages. (A) *2XSTAT92E-GFP* reporter (green) has minimal expression in a wild type larva. (B) In a larva carrying a hyper-active allele of *hop* (hop^{Tum-l}), the *2XSTAT92E-GFP* reporter is strongly induced. (C) *2XSTAT92E-GFP* reporter has minimal expression in a wild type adult that carries a copy of the *yolk-Gal4* driver, which directs transcription of *UAS*-dependent genes in the fat body of adult females starting 3–5 days after hatching. In *2XSTAT92E-GFP, yolk Gal4/+* flies, the eye is the only tissue with detectable GFP expression, which is also observed in flies that carry the *2XSTAT92E-GFP* transgene alone. We presume that this is due to auto-fluorescence. (D) In adults carrying a *yolk-Gal4* driver, the *2XSTAT92E-GFP* reporter and a *UAS-hop* transgene, the *2XSTAT92E-GFP* reporter is strongly induced.

best studied in this tissue (Bach et al., 2003; Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005; Tsai and Sun, 2004; Zeidler et al., 1999). *In situ* hybridization reveals that *upd* is expressed at the posterior midline of the eye imaginal disc throughout the first and second instars, but its expression is not detected after early third instar, suggesting that Upd is active in early eye development (Figs. 3A–D, data not shown). Upd is a secreted molecule that acts cell non-autonomously (Bach et al., 2003; Tsai and Sun, 2004). However, the cells that respond to Upd and activate Stat92E have not yet been identified. We therefore examined the expression of both *upd*, using an *upd-Gal4, UAS-LacZ* (*upd > LacZ*) reporter, and the *STAT92E-GFP* reporters in the developing eye disc (Tsai and Sun, 2004). Like *upd* mRNA, the *upd > LacZ* reporter is expressed at the posterior margin of second and early third instar eye discs (Figs. 3F–H). However, β-galactosidase (β-Gal) protein is also detected in late third instar eye discs (Fig. 3I). Because *in situ* hybridization shows that *upd* mRNA is not present in middle and late third instar eye discs (Fig. 3I), the expression of *upd > LacZ* during this stage is due to the perdurance of β-GAL. Consistent with the expression pattern of *upd*, *10XSTAT92E-GFP* is highly activated in throughout the posterior domain of second instar eye discs (Figs. 3F and G). The *10XSTAT92E-GFP* reporter is expressed many cell diameters away from the *upd*-producing cells at the posterior midline, indicative of Upd's long-range effects. The expression of the *10XSTAT92E-GFP* reporter fades with time, as evidenced by reduced GFP fluorescence in the early and late third instar eye disc (Figs. 3H and I). Since perdurance is a common problem with *in vivo* reporters, we compared expres-

sion of the enhanced and destabilized *10XSTAT92E* reporters. In early and late second instar eye discs, both the enhanced and destabilized *10XSTAT92E* reporters have similar expression patterns (compare Figs. 3F and G, with Figs. 3Q and R, respectively). This indicates that during early larval stages, cells in the posterior half of the eye disc are continuously responding to Upd. However, the destabilized GFP reporter is not expressed in the third instar eye disc, demonstrating that expression of the enhanced GFP reporter in third instar is due to the perdurance of GFP protein (compare Fig. 3S with Figs. 3H and I).

We next examined the expression of the *STAT92E-GFP* reporters in other imaginal discs, including wing, antenna, and leg. The pattern of *upd* mRNA expression in the wing disc has been previously reported and is consistent with the *upd > LacZ* expression pattern (compare Figs. 3O, P in this study with Figs. 3b, c in Mukherjee et al., 2005). *upd > LacZ* is expressed in a large domain in the medial dorsal compartment of second instar wing discs (Figs. 3O, P). In third instar, there are five clearly defined domains of *upd* expression, three in the medial dorsal compartment, one in the anterior margin of the dorsal/ventral boundary and one in the ventral posterior region (Fig. 3P). Activity of the *10XSTAT92E-GFP* reporter overlaps with *upd* expression in both second and third instar wing discs (Fig. 3O). Interestingly, this reporter is not expressed in the wing pouch proper, but rather entirely surrounds it. Similar to what we observe in the eye disc, the *10XSTAT92E-GFP* reporter is most strongly expressed in the wing disc during early larval stages (Fig. 3O).
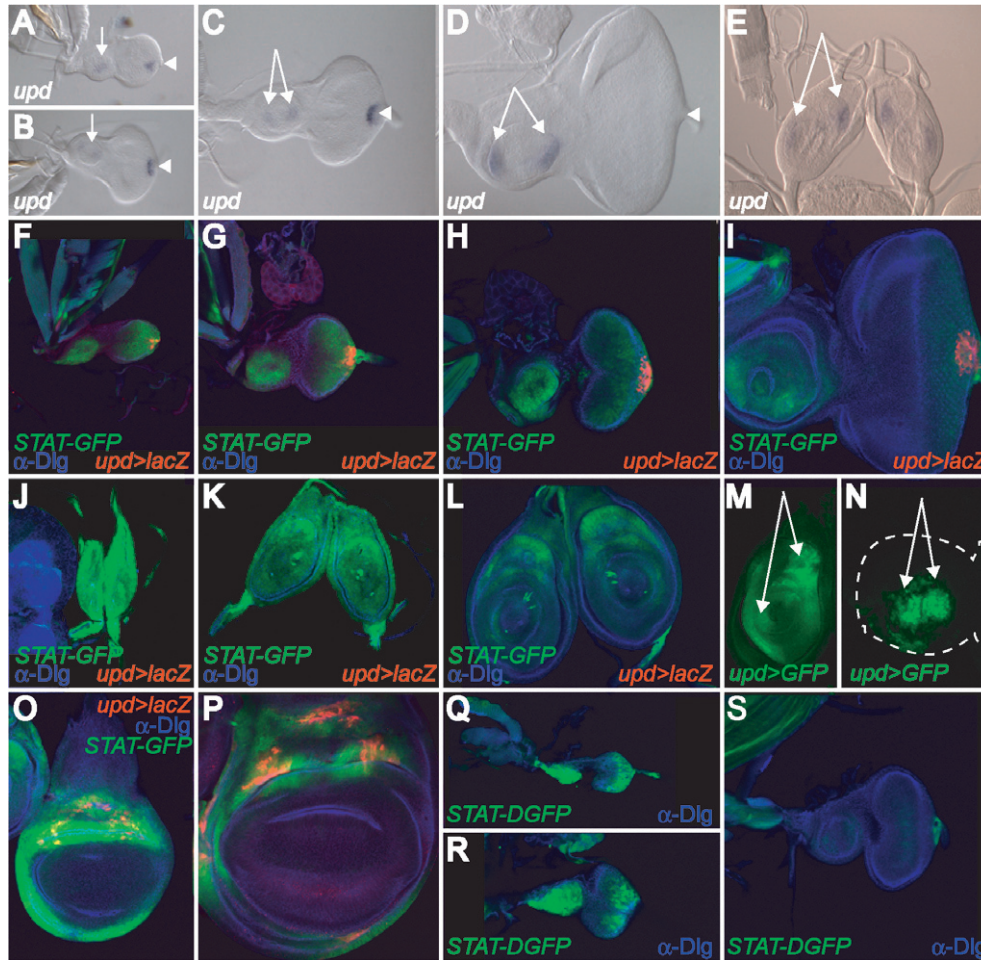
Fig. 3. The *STAT92E-GFP* reporters detect JAK/STAT pathway activation in imaginal discs. (A–E) *upd* mRNA expression in wild type imaginal discs. (A–D) *upd* is expressed by cells at the posterior margin next to the optic stalk in the eye disc in early second (A, arrowhead), late second (B, arrowhead) and early third (C, arrowhead) instar. However, *upd* is not expressed in the late third instar eye disc (D, arrowhead). In the antennal disc, *upd* first appears in the distal antenna in second instar (A, B arrows). In subsequent stages, it is expressed in two domains, one anterior and one posterior in the distal ventral antenna (C,D, arrows). In the leg disc, *upd* is expressed two domains, one ventral anterior and the other dorsal posterior in late second instar (E, arrows). (F–L, O, P) Imaginal discs from *upd > LacZ/+*; *10XSTAT92E-GFP/+* larvae. In early and late second instar eye discs, expression of the *10XSTAT92E-GFP* reporter in green (abbreviated *STAT-GFP*) overlaps with *upd* expression in red (β-Gal expressed from *upd > LacZ*) (F, G). In addition, the *10XSTAT92E-GFP* reporter is expressed in cells at a distance from the *upd* source, indicating Upd's long-range effects. In early and late third instar, the *10XSTAT92E-GFP* reporter has greatly reduced expression (H, I). In second and third instar antennal discs, this reporter's expression overlaps with that of *upd* (F–I). The *10XSTAT92E-GFP* reporter is highly expressed throughout second instar (J) and in the dorsal domain of third instar leg discs (K, L). Although *upd* is expressed in the ventral domain during these time points, we could not detect *10XSTAT92E-GFP* reporter activity ventrally (K, L). In second instar wing discs, *upd > LacZ* is expressed in the dorsal medial domain (O). *10XSTAT92E-GFP* overlaps with *upd* expression and surrounds the wing pouch proper (O). In third instar, *upd > LacZ* expression has resolved into five domains, three in the dorsal compartment, one at the anterior lateral of the dorsal-ventral boundary and one in the ventral posterior (P). *upd > LacZ* is not detected by β-gal staining in antennal and leg discs (F–L). However, when the same *upd-Gal4* insertion drives expression of *GFP* (from a *UAS-GFP* transgene), (*upd > GFP*), GFP is easily observed in leg (M) and antennal (N) discs. (Q–S) Eye – antennal discs expressing two copies of the destabilized *10XSTAT92E-DGFP* reporter. In early and late second instar, the *10XSTAT92E-DGFP* reporter (labeled *STAT-DGFP*) is expressed in the posterior half of the eye disc and in the distal antenna in a manner similar to the enhanced *10XSTAT92E-GFP* reporter (compare F and G with Q and R, respectively). However, in third instar eye–antennal discs, the destabilized *10XSTAT92E-DGFP* reporter is not expressed (S). Discs-large (Dlg), which marks cell outlines, is in blue (F–S).

The expression pattern of *upd* in the antennal disc has not been previously reported. In early second instar, *upd* mRNA is expressed in the ventral distal antenna, and becomes restricted to two distinct regions (one anterior and one posterior) in the third instar distal antenna (Figs. 3A–D). The *upd > LacZ* reporter cannot be detected by β-Gal staining in either the antennal or leg disc (Figs. 3J–L). However, the same *upd-Gal4* insertion driving expression of *UAS-GFP* (*upd > GFP*) is detected in both anten-

nal and leg discs in a pattern that completely overlaps with *upd* mRNA as detected by *in situ* hybridization (compare Figs. 3M and N to Figs. 3E and C, respectively). Both the *10XSTAT92E-GFP* and *10XSTAT92E-DGFP* reporters are expressed in the distal antenna in second instar in a broad pattern that partially overlaps with *upd* (Figs. 3F, G, Q and R). In third instar, the enhanced GFP reporter becomes concentrated in two domains in the distal ventral antenna (Figs. 3H and I). However, the destabilized GFP
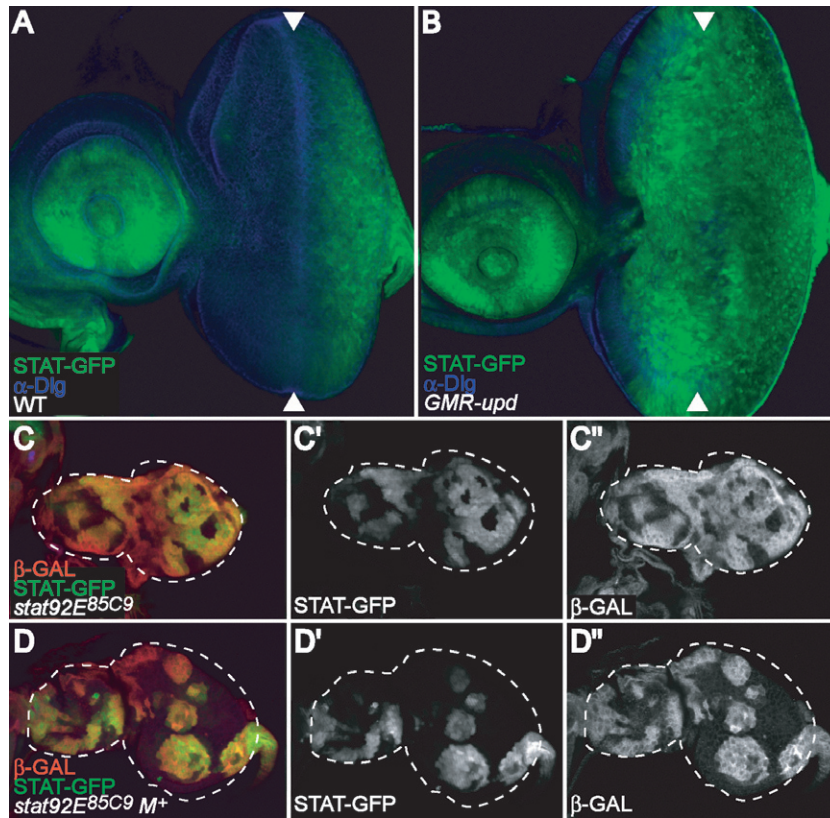
Fig. 4. Activity of the *10XSTAT92E-GFP* reporter requires a functionally active Stat92E protein. (A, B) Ectopic activation of the JAK/STAT pathway in the third instar eye disc induces expression of the *10XSTAT92E-GFP* reporter. Wild type (A) or *GMR-upd* (B) third instar eye discs expressing one copy of the *10XSTAT92E-GFP* reporter in green (abbreviated *STAT-GFP*) and stained with anti-Dlg in blue. In wild type third instar eye discs, the *10XSTAT92E-GFP* reporter is not expressed anterior to the morphogenetic furrow (A). However, ectopic expression of *upd* using the *GMR* promoter causes cells anterior to the furrow to express the *10XSTAT92E-GFP* reporter (B). In A and B, the morphogenetic furrow is marked by white arrowheads. (C, D) Expression of the *10XSTAT92E-GFP* reporter requires a functionally active JAK/STAT pathway. (C–C ′) *stat92E*[85C9] clones in the eye-antennal disc were induced using *ey-flp* and are marked by the absence of β-Gal (red). In *stat92E*[85C9] clones, *10XSTAT92E-GFP* expression (green) directly overlaps with wild type tissue (red) and is not expressed in *stat92E*[85C9] clones. Merge of red and green channels (C); green (*10XSTAT92E-GFP*) channel (C′); red (β-Gal) channel (C″). (D) *stat92E*[85C9] clones in the eye-antennal disc were induced using *ey-flp* in a *Minute* background and are marked by the absence of β-Gal (red). In *stat92E*[85C9] clones in a *Minute* background, *10XSTAT92E-GFP* expression (green) directly overlaps with heterozygous (*Minute/+*) tissue (red), but is not expressed in *stat92E*[85C9] clones. Merge of red and green channels (D); green (*10XSTAT92E-GFP*) channel (D′); red (β-Gal) channel (D″).

reporter is not expressed in the antenna after early third instar (Fig. 3S).

In the leg disc, *upd* mRNA and the *upd > GFP* reporter are expressed in two distinct domains, one ventral anterior and the other dorsal posterior (Figs. 3E and M). The leg disc exhibits a dynamic pattern of *10XSTAT92E-GFP* expression (Figs. 3J–L). In early second instar, this reporter is expressed throughout the leg disc and becomes restricted to the dorsal domain in second and third instar (Figs. 3J–L). Interestingly, the ventral anterior domain of *upd* expression (Figs. 3E and M) does not have a corresponding region of *10XSTAT92E-GFP* reporter activity in either second and third instar leg discs (Figs. 3K and L). Similar to what is observed in eye and wing discs, the antennal and leg discs have the highest level of JAK/STAT signaling during early larval stages.

1.3. Expression of the STAT92E-GFP reporters requires Stat92E

To confirm that the *STAT92E-GFP* reporters are responsive to JAK/STAT signaling in imaginal discs, we

assessed at the ability of ectopic *upd* to activate the *10XSTAT92E-GFP* reporter. We and others have previously shown that ectopic expression of *upd* using the *GMR* promoter causes cells anterior to the furrow to undergo additional rounds of mitosis and to upregulate expression of target genes, such as *dome* and *Socs36E* (Bach et al., 2003; Karsten et al., 2002; Tsai and Sun, 2004). Consistent with this finding, in the presence of ectopic *upd* using a *GMR-upd* transgene, the *10XSTAT92E-GFP* reporter is ectopically activated in the region anterior to the furrow (Fig. 4B). In contrast, the *10XSTAT92E-GFP* reporter is not activated anterior to the furrow in wild type eye discs (Fig. 4A).

To ensure that the *10XSTAT92E-GFP* reporter is activated only by JAK/STAT signaling, we examined its expression in JAK/STAT loss-of-function clones. We used the *stat92E*[85C9] allele, which is a strong hypomorphic mutation resulting from an R442P substitution (Silver and Montell, 2001). While the *stat92E*[85C9] mutation is not a protein null, it generates stronger phenotypes than the putative null allele *stat92E*[06346] (L.A.E., A.A.C. and E.A.B.,

unpublished observations). In *stat92E*[85C9] mosaic clones, *10XSTAT92E-GFP* expression directly overlaps with wild type tissue within its normal range of expression (Figs. 4C–C″). As expected, GFP is lost from *stat92E*[85C9] clones in a cell autonomous manner (Figs. 4C–C″). Reporter expression is also lost in *stat92E*[85C9] clones in a *Minute* background, in which *stat92E* mutant tissue has a growth advantage over *Minute*/+ tissue (Figs. 4D–D″) (Morata and Ripoll, 1975). In these discs, *10XSTAT92E-GFP* is expressed only in heterozygous (*Minute*/+) tissue, which contains one wild type copy of *stat92E* (Figs. 4D–D″). We obtained similar results for the requirement of Stat92E in activation of the destabilized *10XSTAT92E-DGFP* reporter (data not shown). The *10XSTAT92E-GFP* reporters are therefore activated by JAK/STAT signaling through Stat92E. In the absence of a functional *Drosophila* STAT protein, these reporters cannot be activated.

1.4. Discussion

While a number of developmental processes that require JAK/STAT signaling have already been reported, there are likely additional requirements for this pathway that have yet to be identified. We have developed a tool to examine the *in vivo* activity of the JAK/STAT pathway in a variety of tissues and developmental stages in *Drosophila*. Both the *2X-* and *10XSTAT92E-GFP* reporters are expressed in the embryo in an overlapping pattern with Stat92E, and, as expected, in a domain slightly broader than *upd* in a variety of imaginal discs. In nearly every disc examined, *10XSTAT92E-GFP* reporter activity overlaps with *upd* expression. The one exception is the ventral anterior domain of the leg disc, in which we observe *upd* expression but no corresponding activity of the *10XSTAT92E-GFP* reporter. The reason for this discrepancy is unclear as the functional role of the JAK/STAT pathway in leg development is currently not known. However, potential explanations include the lack of *dome* expression, or the lack of another positive regulator of this pathway, in this region. Nevertheless, we demonstrate that when Stat92E is removed, expression of the *10XSTAT92E-GFP* reporter is extinguished in an autonomous manner. Conversely, ectopic activation of JAK/STAT signaling leads to the expression of this reporter.

Our reporter is a more sensitive assay of JAK/STAT pathway activation than monitoring *Socs36E* mRNA. *Socs36E* expression patterns have been reported for the embryo, leg, wing and eye imaginal discs, and in the ovary (Callus and Mathey-Prevot, 2002; Karsten et al., 2002; Rawlings et al., 2004). In the embryo and ovary, our GFP reporters and published *Socs36E* mRNA share a very similar expression domain (compare Fig. 1 in this study to figures in (Callus and Mathey-Prevot, 2002; Karsten et al., 2002; Rawlings et al., 2004)). However, in imaginal discs, our reporters appear to be more sensitive than *Socs36E* mRNA as detected by *in situ* hybridization (compare Fig. 3 in our study to Fig. 3 in (Karsten et al., 2002)).

Thus, the *STAT92E-GFP* reporters we have developed provide *in vivo* tools to further investigate the JAK/STAT pathway and offer several advantages over other previously published *in vivo* JAK/STAT reporters. First, using our reporters, the *bona fide* activity of Stat92E in a living organism can be monitored by GFP. Second, we developed a destabilized GFP reporter, which is a more accurate temporal marker than enhanced GFP. Third, we document the expression of the *10XSTAT92E-GFP* reporter in a wide variety of tissues and developmental stages. In contrast, the expression pattern of other reporters, such as βlue-βlau, (Brown et al., 2003), Stat92E-GFP (Karsten et al., 2006), and (GAS)₃-*LacZ* (Gilbert et al., 2005), have only been reported in the embryo or in cultured cells. Lastly, our reporters can be used to conduct modifier screens in which mutations can be isolated based on their ability to change the activation of Stat92E rather than on their loss of function phenotype.

2. Experimental procedures

2.1. Drosophila stocks

hop[Tum-l] (Harrison et al., 1995); *yolk-GAL4* (Georgel et al., 2001); *UAS-hop* (Harrison et al., 1995); *stat92E*[85C9] (Silver and Montell, 2001); *upd-GAL4* (Halder et al., 1995; Tsai and Sun, 2004); *GMR-upd* (Bach et al., 2003); *ey-flp* (Newsome et al., 2000).

2.2. Generation of GFP reporter constructs and transgenic lines

The *STAT92E* reporters were made as described in (Baeg et al., 2005), the only difference being that an *XhoI/XbaI* fragment containing a *luciferase* gene in (Baeg et al., 2005) was replaced with an *XhoI/XbaI* fragment containing either *enhanced GFP* (pEGFP-N1, Clontech) or *destabilized GFP* (pd2EGFP, Clontech). Transgenic animals were generated by standard procedures (Bach et al., 2003). The #6-1 *2XSTAT92E-GFP* line is a homozygous viable insertion on the 3rd chromosome. The #1 and #2 *10XSTAT92E-GFP* lines are homozygous viable insertions on the 2nd and 3rd chromosome, respectively. The *10XSTAT92E-DGFP* line is a homozygous viable insertion on the 2nd chromosome.

2.3. Mosaic clones

stat92E mosaic clones were made by the FLP/FRT technique using *ey-flp; P{neoFRT}*[82B]*P{arm-lacZ.V}83B/TM6C, Sb*[1] *Tb*[1] (Xu and Rubin, 1993). *Minute* clones were generated in the developing eye using *ey-flp; P{neoFRT}*[82B]*M(3)96C, arm-lacZ/TM6B. ey-flp* is active in the eye disc from early larval stages until late third instar (Newsome et al., 2000).

2.4. In situ hybridization, antibody staining, and microscopy

In situ hybridization was carried out according to the protocol in (Bach et al., 2003).

We performed antibody staining as previously described (Bach et al., 2003). We used the following primary antibodies: rabbit anti-Stat92E (1:1000) (Chen et al., 2002); 40-1A (mouse anti-β-Galactosidase) (1:50) and 4F3 (mouse anti-Discs large) (1:50) (both from Developmental Studies Hybridoma Bank); rabbit anti-β-Galactosidase (1:100) (Cappel); Alexa-Fluor[546] Phalloidin (1:400) (Invitrogen). We used fluorescent secondary antibodies at 1:300 (Jackson Laboratories). Samples were mounted on microscopes slides using Slow-fade Gold (Invitrogen). We collected fluorescent images using a Zeiss LSM 510 confocal microscope, and brightfield pictures using a Zeiss Axioplan microscope with a Nikon

Digital Sight DL-UL camera, a Leica MZ8 microscope with an optronics camera, or a Zeiss Axioskop with a Spot Insight QE camera.

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