



The roles of the *Drosophila* JAK/STAT pathway

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The JAK/STAT signal transduction pathway has been conserved throughout evolution such that true structural and functional homologues of components originally identified in vertebrate systems are also present in the model genetic system *Drosophila melanogaster*. In addition to roles during larval hematopoiesis reminiscent of the requirement for this pathway in mammalian systems, the JAK/STAT pathway in *Drosophila* is also involved in a number of other developmental events. Recent data has demonstrated further roles for the JAK/STAT pathway in the establishment of sexual identity via the early embryonic expression of *Sex lethal*, the segmentation of the embryo via the control of pair rule genes including *even skipped* and the establishment of polarity within the adult compound eye via a mechanism that includes the *four jointed* gene. Use of the powerful genetics in the model organism *Drosophila* may identify new components of the JAK/STAT pathway, define new roles for this pathway, and provide insights into the function of this signal transduction system. Here we review the roles of STAT and its associated signaling pathway during both embryonic and adult stages of *Drosophila* development and discuss future prospects for the identification and characterization of novel pathway components and targets. *Oncogene* (2000) 19, 2598–2606.

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Introduction

The JAK/STAT signal transduction cascade was first identified in mammalian systems because of its role in the transduction of a variety of cytokines and growth factor signals (reviewed in Darnell, 1997). Extensive studies in mammalian systems have led to the development of a canonical model in which the non-receptor tyrosine kinase JAK is associated with the intracellular portion of transmembrane cytokine receptors (Figure 1a). Following ligand binding, receptors dimerize bringing two JAK molecules into juxtaposition where they trans-phosphorylate one another. JAKs activated in this manner then tyrosine phosphorylate their associated receptors causing normally cytosolic STAT molecules to bind to the receptor complex via their SRC homology 2 (SH2) domains. The STAT molecules recruited in this manner are themselves activated by JAK-mediated phosphorylation of an invariant tyrosine residue in their C-terminal region and then either homo- or hetero-dimerize prior

to nuclear translocation. Once in the nucleus activated STAT dimers bind to consensus DNA target sites where they act as activators of transcription (Figure 1a and other reviews in this issues).

Interestingly the JAK/STAT pathway and STAT-like molecules in particular are present throughout evolution. To date one (or possibly two) STATs have been found in the nematode *C. elegans* (Lui *et al.*, 1999), and three STAT-like molecules have been identified in the slime mould *Dictyostelium* (M Fukazawa and JG Williams, unpublished data reported in Williams, 1999). However, neither organism appears to have JAK-like protein kinases.

In the *Drosophila* system a ‘complete’ JAK/STAT pathway that consists of one JAK (called *hopscotch*) and one STAT (called *stat92E*) has been identified, in stark contrast to the multitude of JAK and STAT homologues present in mammals. The presence of a relatively simple JAK/STAT pathway, together with the wealth of sequence data and genetic techniques available, make *Drosophila* a very powerful system with which to study this pathway. These advantages, especially with respect to developmental requirements for JAK/STAT signaling, are particularly relevant for the fly model system and hold much promise for the future elucidation of interactions between the JAK/STAT pathway and other signal transduction cascades involved in developmental decisions.

In this review we discuss recent developments into our understanding of the components and roles of the JAK/STAT pathway during *Drosophila* development and address potential future directions in the field.

Characterization of *Drosophila* JAK/STAT pathway components

With the recent cloning of *unpaired* (*upd*) and its identification as a potential ligand for the *Drosophila* JAK/STAT pathway (Harrison *et al.*, 1998), three pathway components have been identified on the basis of their biochemical, genetic and mutant phenotypes.

The *hopscotch* (*hop*) locus encodes a 1177 amino acid protein that shares all the characteristics of mammalian JAK family non-receptor tyrosine kinases. Hop is most similar to human JAK2 (27% identity), with higher levels of homology in kinase and kinase-like domains (Figure 1b, Binari and Perrimon, 1994). The *Drosophila* STAT homologue Stat92E is also highly homologous to human STAT5 (37% identity). Stat92E includes a SH2 domain, DNA binding domain and the single tyrosine residue around position 700 found in all STAT-like genes (Figure 1b, Hou *et al.*, 1996; Yan *et al.*, 1996a). Indeed this residue in Stat92E (Y711) has been shown to be phosphorylated in response to

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pathway activation in tissue culture systems (Yan *et al.*, 1996a).

It has previously been reported that a second, non-Stat92E derived STAT-like activity is detectable in *Drosophila* cell extracts (Yan *et al.*, 1996a; Sweitzer *et al.*, 1995). A series of searches of the *Drosophila* genome sequencing project data were therefore conducted on a complete 10× coverage database to address this question. Using Stat92E protein sequences as 'probes' for tblastn search programs no candidate STAT-like molecules, other than *stat92E* itself, were identified (E Spana, personal communication). While, it remains possible that a STAT-like protein, which has diverged sufficiently far from Stat92E to be undetectable with sequence based searches, is in fact present, it seems that the complex situation in vertebrate model systems is not mirrored in *Drosophila*. Therefore both Hopscotch and Stat92E probably represent the sole

examples of canonical JAK- and STAT-like proteins present in flies.

While mutations in the *unpaired* locus have been known for over 75 years, only recently has the associated gene been identified as a ligand for the *Drosophila* JAK/STAT pathway (Venderosa and Muller, 1954; Harrison *et al.*, 1998). The *unpaired* locus encodes a predicted 47 kDa secreted protein with an N-terminal signal sequence and several potential N-linked glycosylation sites (Figure 1b). While a protein similar to Upd called Om1E has been identified in the closely related fruit fly *Drosophila ananassae*, no known vertebrate homologues have been found in publicly available databases to date (Harrison *et al.*, 1998; Juni *et al.*, 1996). Biochemical analysis of Upd revealed that the protein has an apparent molecular weight of 65 kDa, is indeed glycosylated and is localized in the extracellular matrix. Furthermore, tissue culture experi-

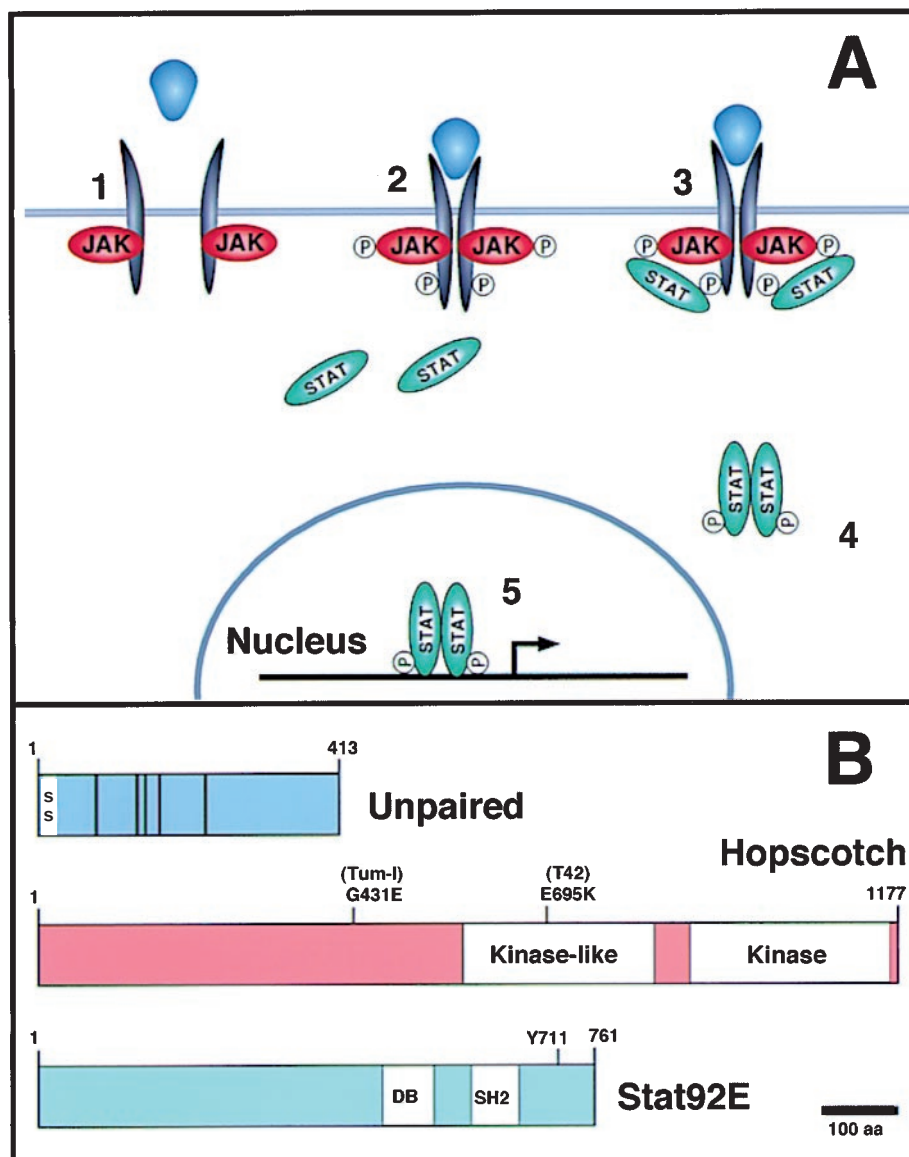


Figure 1 (a) The JAK/STAT pathway in which receptor/JAK (red) complexes dimerize on binding of ligand (blue) (1). Following receptor and JAK phosphorylation (2) cytosolic STATs (green) are recruited to the complex (3). Phosphorylated STAT molecules dimerize (4) and translocate to the nucleus where they bind DNA and activate transcription (5). (b) Schematic representation of the *Drosophila* Unpaired (blue), Hopscotch (red) and Stat92E (green) proteins. Gray bars in Unpaired represent potential amino-linked glycosylation sites. The activating mutations in Hopscotch are indicated (see text for details) as is the invariant tyrosine residue in Stat92E. SS = signal sequence, DB = DNA binding domain and SH2 = Src Homology 2 domain. Each protein is drawn to scale (scale bar is 100 amino acids) and numbers represent amino acid position

ments have shown that Upd is capable of inducing Hop phosphorylation and activation in Clone 8 tissue culture cells. Furthermore, activation of the JAK/STAT pathway by Upd does not require co-expression of Upd in the signaling cell as Upd supplied by either a physically separate, but co-cultured, (S2) cell type, or even from previously conditioned media, is sufficient to activate Hop (Harrison *et al.*, 1998). Taken together with *in vivo* evidence showing that Upd functions as an activator of JAK/STAT signaling in the eye (Zeidler *et al.*, 1999b), it is clear that Unpaired represents a *bona fide* JAK/STAT pathway ligand.

Although the identity of *Drosophila* JAK and STAT homologues and a pathway ligand are now known, the identity of a pathway receptor(s) remains unclear. Vertebrate studies have shown that the JAK/STAT pathway is downstream of cytokine receptors and receptor tyrosine kinases such as EGFR and PDGFR (Darnell, 1997). While a similar situation may also be the case in *Drosophila* no evidence had emerged to date regarding the nature of a potential pathway receptor.

Fly homologues of other components of the mammalian JAK/STAT pathway, specifically PIAS, SOCS, and STAM, have also been identified. Two of these are homologues of previously identified negative regulators of the mammalian JAK/STAT pathway; Protein Inhibitor of Activated STAT (PIAS) and Suppressor Of Cytokine Signaling (SOCS). PIAS proteins contain a putative zinc-finger domain, and specific PIAS proteins bind to and inhibit the activity of specific STATs (Chung *et al.*, 1997). In *Drosophila*, a PIAS homologue named *zimp*, has been identified by sequence homology and although mutations are lethal, no further functional data has been published. It is therefore not yet clear whether this protein inhibits the activity of Stat92E (Mohr and Boswell, 1999). The SOCS family of proteins are characterized by the presence of a highly conserved 40-amino acid C-terminal SOCS domain preceded by an SH2 domain (Nicholson *et al.*, 1999). Studies indicate that SOCS proteins can inhibit cytokine signaling either directly by inhibition of JAKs or indirectly by competition with STAT for a phospho-tyrosine binding site on the receptor. Interestingly a fly homologue of SOCS-5 has recently been identified, that retains sequence identity in both the SH2 domain and regions previously identified as being required for SOCS activity (Nicholson *et al.*, 1999). Currently there are no mutations in the *Drosophila* SOCS gene, and thus the physiological function of this gene is presently not known. The final *Drosophila* homologue to human pathway components is the Signal Transducing Adaptor Molecule (STAM). STAMs were originally identified as positive regulators of cytokine-dependent signal transduction, contain a SRC homology 3 (SH3) domain and associate with Jak2 and Jak3 (Takeshita *et al.*, 1997). The fly homologue of STAM, also called *stam*, was cloned by homology, and again no functional data is available for this gene (Mesilaty-Gross *et al.*, 1999). Future genetic and biochemical characterization of these *Drosophila* homologues may establish that *Drosophila* PIAS, SOCS and STAM are true orthologues and represent additional levels of JAK/STAT pathway complexity in the fly model system.

Sex determination

The *sisterless C* (*sisC*) gene has been known for some time as a component of the *Drosophila* sex determination system (Cline, 1993) and the recent identification of *sisC* as an allele of Upd has implicated the JAK/STAT pathway in one of the earliest processes of embryonic development.

The process of sexual identity determination in *Drosophila* is based on very early mechanisms which detect the X chromosome to autosome ratio of the newly fertilized embryo (with XY individuals becoming males and XX developing into females). The cellular mechanism which determines the number of X chromosomes establishes the expression state of the *Sex lethal* gene which acts as a 'master switch' to determine sexual identity and X chromosome dosage compensation. The dose of X chromosomes present in a newly fertilized embryo is relayed into this sexual determination machinery via the activity of a number of X-linked signal element (XSE) genes which are encoded on the X chromosome and include *sisA*, *sisB* and *sisC* (see Cline, 1993 for review). The two copies of these XSE genes produced in future females is sufficient to activate the '*Sex lethal* establishment' promoter and cause *Sex lethal* expression which, via a positive autoregulatory feedback loop, is then maintained throughout the life of the fly. In future males the XSE genes present on the single X chromosome are expressed at insufficient levels to activate the *Sex lethal* promoter.

A recent report has identified the XSE gene *sisC* as being allelic to *upd*. While previously identified XSE genes, *sisA* and *sisB* represent transcription factors that can bind directly to the *Sex lethal* promoter, *sisC/upd* obviously represents an extracellular JAK/STAT pathway ligand. This finding suggests that the JAK/STAT pathway is required for the transduction of the *SisC/Upd* signal and, either directly or indirectly, controls *Sex lethal* expression (Thomas Cline, personal communication).

Although a similar role for the JAK/STAT pathway in sexual identity determination has not been previously described in other systems, it is clear that the mechanisms by which sexual identity is determined in other animals are very diverse. As a result it is possible that research into a potential role for the JAK/STAT pathway in sex determination will provide insights not only into the rapid evolutionary development of sexual identity, but also the mechanisms whereby established signal transduction pathways are co-opted into new developmental roles.

Embryonic segmentation

Both *hopscotch* and *stat92E* are deposited maternally in the developing oocyte during oogenesis. Hop is expressed essentially uniformly throughout the various stages of embryonic development, while Stat92E is expressed uniformly during early stages but subsequently resolves initially into seven and then 14 segmental stripes (Binari and Perrimon, 1994; Hou *et al.*, 1996; Yan *et al.*, 1996a). Upd, in contrast, is not maternally supplied but is expressed from the zygotic genome in a highly dynamic pattern: broadly in the trunk and in a stripe in the head before cellularization,

seven stripes during cellularization, 14 stripes during gastrulation and later in the tracheal pits (Harrison *et al.*, 1998). Despite these very different patterns of expression the three known components of the JAK/STAT pathway exhibit very similar, loss-of-function phenotypes in the embryo. However because of the maternal contributions, the roles of *hop* and *stat92E* in the embryo can be only assessed in embryos that lack this contribution and that are derived from females with homozygous mutant germlines. Using a genetic method, the 'dominant female sterile technique', which allows the generation of females that have a homozygous mutant germ-line in an otherwise wild type fly (Chou and Perrimon, 1996), embryos that lack maternally contributed *hop* or *stat92E* can be generated (hereafter referred to as *hop* and *stat92E* mutant embryos). It should however be noted that maternal deposition of *hop* and *stat92E* does not appear to be absolutely essential. The embryonic phenotypes associated with removal of maternally supplied *hop* or *stat92E* can be partially rescued by a wild type copy of the gene supplied paternally and also by the injection of wild type *stat92E* mRNA into pre-blastoderm *stat92E* mutant embryos (Binari and Perrimon, 1994; Hou *et al.*, 1996).

All embryos mutant for *hop*, *stat92E* or zygotic *upd* exhibit a characteristic segmentation defect. These defects are readily identifiable as a disruption in the normal pattern of hairs or denticles that make up part of the external cuticle secreted by the embryo shortly before hatching. While wild type embryos have a stereotyped arrangement of denticle belts that correspond to each thoracic and abdominal segment (Figure 2a), JAK/STAT mutant embryos typically exhibit a loss of the fifth segment with variable deletion of the fourth and eighth segments, as well as occasional fusion of the sixth and seventh segments (Figure 2b;

Binari and Perrimon, 1994; Harrison *et al.*, 1998; Hou *et al.*, 1996; Yan *et al.*, 1996a).

Proper segmentation of the embryo is established by several sets of temporally expressed genes. Maternal genes establish the anteroposterior and dorsoventral axes of the embryo, and along the anteroposterior axis the sequential expression and activity of zygotic segmentation genes (gap, pair-rule, and segment-polarity) progressively refine the number and orientation of the segments. Mutations in the gap genes result in deletion of multiple adjacent segments, those in the pair-rule genes in deletion of alternative segments, and those in the segment polarity genes result in loss of part of each segment. However the distinctive cuticle phenotypes of *upd*, *hop* and *stat92E* mutant embryos are shared only by mutations now identified as being components of the JAK/STAT pathway, and were therefore classified as a separate class of segmentation mutants. In *upd*, *hop* and *stat92E* mutant embryos, the gap-genes are expressed normally. However, there are defects in the expression of the pair-rule genes *even-skipped* (*eve*), *fushi tarazu* and *runt* (Binari and Perrimon, 1994; Hou *et al.*, 1996).

The effect of mutations in the JAK/STAT pathway on pair rule gene expression is quite variable and depends on the stripe in question. Levels of endogenous *eve* stripes 3 and 5 are significantly reduced in *upd*, *hop* and *stat92E* mutant embryos (Binari and Perrimon, 1994; Harrison *et al.*, 1998; Hou *et al.*, 1996; Yan *et al.*, 1996a). Although the regulatory domains controlling stripe 5 have not been identified, a 500 bp regulatory sequence in the *eve* promoter has been defined that controls expression of *eve* stripe 2, 3 and 7 (Binari and Perrimon, 1994; Small *et al.*, 1996). When this enhancer is fused to a lacZ reporter and introduced into wild type embryos β -galactosidase activity is detected in three stripes corresponding to

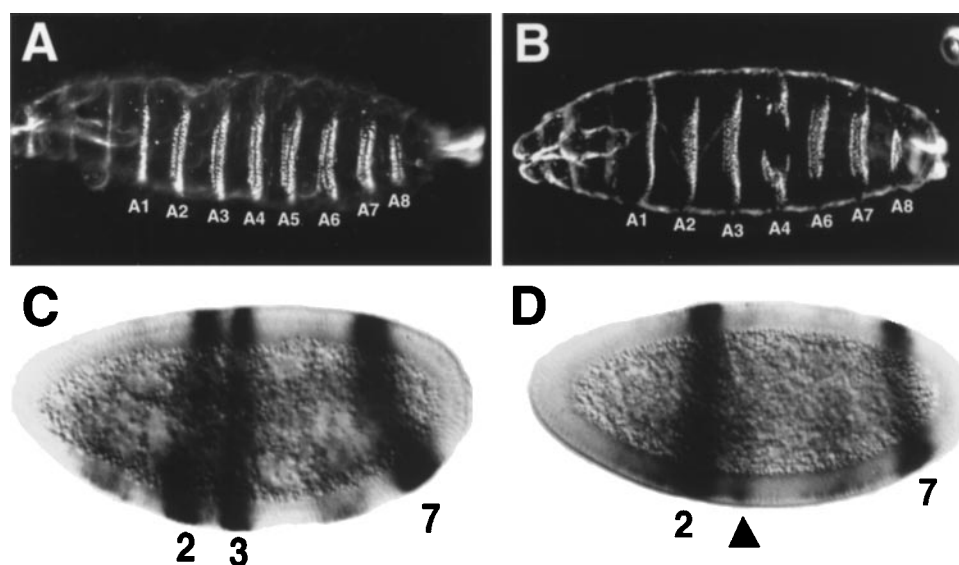


Figure 2 (a) The characteristic pattern of denticle belts visible in a wild type embryonic cuticle showing abdominal (A1-A8) denticle bands. (b) The characteristic pattern of a *Stat92E* mutant embryo lacking any maternal contribution. As is characteristic for all JAK/STAT mutant embryos, bands A1-3 are normal. However A4 is partially deleted and A5 is absent. The occasional reduction or loss of A6-A8 in *stat92E* mutant embryos is not shown in this example. (c) The pattern of lacZ driven by the 500 bp *eve* promoter fragment in a wild type embryo. Bands that correspond to the positions of *eve* stripes 2,3 and 7 are visible. (d) The pattern of lacZ driven by the 500 bp *eve* promoter fragment in a *stat92E* mutant embryo. The band corresponding to stripe 3 is greatly reduced in intensity (arrow head). A similar reduction in stripe 3 expression is also produced in *upd* and *hop* mutant embryos and if the *Stat92E* binding sites in the 500 bp *eve* promoter are mutated

the endogenous *eve* stripes 2, 3 and 7 (Figure 2c). When this enhancer, is introduced into *upd*, *hop* or *stat92E* mutant embryos, expression of stripe 3 is almost completely abolished (Figure 2d; Binari and Perrimon, 1994; Hou *et al.*, 1996; Harrison *et al.*, 1998). This enhancer contains two sequences that closely resemble the mammalian consensus STAT binding site, and *in vitro* assays indicate that, tyrosine-phosphorylated, activated *stat92E* can bind to these sites. Moreover, when these STAT-binding sites are mutated, the 500 bp enhancer no longer drives *eve* stripe 3 expression. Taken together these results indicate that the JAK/STAT pathway is directly required for expression of *eve* stripe 3 (Hou *et al.*, 1996; Yan *et al.*, 1996a).

However, the control of endogenous *eve* stripe 3 expression is not solely a consequence of JAK/STAT activity. Additional levels of regulation must exist as endogenous *eve* stripe 3 is not completely abolished in JAK/STAT pathway mutant embryos. Therefore it has been suggested that at least one additional unidentified factor co-operates with Stat92E to activate stripe 3 expression in the embryo. Similarly, because the expression of stripe 7, as reported by the 500 bp enhancer, is not diminished in *upd*, *hop* or *stat92E* mutant embryos, another unidentified co-activator that acts within the enhancer element to control stripe 7 expression must also exist.

The analysis of *eve* stripe 3 regulation by JAK/STAT pathway has led to the model that this pathway plays a permissive, and not an instructive, role in embryonic patterning at this stage. In this model, the pathway acts to potentiate and amplify an unknown factor that drives *eve* stripe 3 expression rather than being responsible for the generation of the primary signal that positions *eve* stripe 3 directly. Genetic analysis supports this view and has shown that the anterior and posterior domains of *eve* stripe 3 expression are controlled by the Hunchback and Knirps proteins, both of which are transcriptional repressors. Removal of Hunchback expands the domain of stripe 3 anteriorly while removal of Knirps expands it posteriorly. When both repressors are removed, stripe 3 expands in both directions and is expressed throughout the embryo. Therefore, because the borders of *eve* stripe 3 expression are already tightly controlled by Hunchback and Knirps, the JAK/STAT pathway does not have to be activated in a spatially restricted domain. Consistent with this model, precise spatial activation of the JAK/STAT pathway by the ligand Upd does not appear to be essential for proper segmentation.

The JAK/STAT pathway and larval hematopoiesis

As is also the case in mammalian systems, a requirement for the JAK/STAT pathway during *Drosophila* hematopoiesis has also been established. Although there are many unresolved issues about the ontogeny of the cells in *Drosophila* hemolymph, it is thought that the larval hematopoietic organ, the lymph gland, gives rise to plasmacytes which make up approximately 90% of the circulating cells in the larva. Plasmacytes are phagocytic cells that play an important role in immune defense and cell-scavenging, and it is these cells which then terminally differentiate

into encapsulating lamellocytes, at the end of larval development (see Mathey-Prevot and Perrimon, 1998, for recent review).

One of the first indications that the JAK/STAT pathway is critical to the development of these circulating cells was the identification of dominant gain-of-function alleles of *hop*. Two temperature sensitive mutations in the *hop* locus have been identified that result in proteins with constitutively hyperactivated kinase activity. The *hop^{Tum-l}* allele is a point mutation (G431E, see Figure 1b) of a residue otherwise unconserved in other JAKs (Hanratty and Dearolf, 1993). The other, *hop^{T42}*, is slightly stronger than *hop^{Tum-l}* and also contains a single amino acid substitution (E695K, see Figure 1b) present in the kinase-like domain and represents the mutation of a residue conserved in all known JAK homologues (Luo *et al.*, 1997). These substitutions represent the only hyper-activating mutations currently identified in any JAK kinase. An equivalent substitution to the *hop^{Tum-l}* allele engineered into murine JAK2 results in similar overactive molecules (Luo *et al.*, 1997). *hop^{Tum-l}* individuals form 5–20-fold more plasmacytes when raised above the restrictive temperature. Many of these prematurely differentiate into lamellocytes, form large aggregates and are encapsulated to form melanotic tumors. When transplanted into a wild type host, *hop^{Tum-l}* hypertrophied larval lymph glands retain the ability to cause overproliferation of plasmacytes and melanotic tumors (Hanratty and Dearolf, 1993; Harrison *et al.*, 1995; Luo *et al.*, 1995).

Overexpression experiments have also shown that wild type *hop* misexpression can also produce a melanotic blood cell tumor phenotype similar to *hop^{Tum-l}*. Furthermore, both *hop* overexpression and *hop^{Tum-l}*-induced melanotic blood cell tumor phenotypes can be suppressed by the removal of a copy of the *stat92E* locus, and the resultant reduction in the level of Stat92E activity. However, while Stat92E is obviously important for the melanization of tumors, it has been reported that the blood cell overproliferation phenotype is not effected by changes in Stat92E. It was therefore suggested that the pathway may bifurcate downstream of *hop*, proliferation being a Stat92E-independent process (Luo *et al.*, 1995). While this may be the case, it is also possible that proliferation is simply not as easily suppressed as the melanization by removal of a single copy of *stat92E* in this context.

JAK/STAT functions at other developmental stages

While Hop misexpression of Hop^{Tum-l} activity in the lymph gland leads to blood cell overproliferation, the situation in imaginal discs, tissue destined to form the adult fly, is not as straightforward. Over- or mis-activation of the pathway in imaginal tissue by ectopic expression of Hop or Hop^{Tum-l} does not generally produce an overproliferation of target tissue. Rather such ectopic activation results in a range of as yet largely uncharacterized fate changes and developmental defects (Harrison *et al.*, 1995). While these effects are intriguing and indicative of uncharacterized roles for the JAK/STAT pathway in adult development, it does not at present appear that the JAK/STAT pathway is linked to tissue neoplasia in imaginal tissue.

Loss of Hop activity in hetero-allelic mutant combinations result in 'small' or 'no' disc phenotype (Perrimon and Mahowald, 1986). In addition the removal of the *hop* locus from imaginal cells results in a mutant clone that is often far smaller than expected given the size of the 'twin spot' WT clone produced during mitotic recombination. This indicates that these mutant cells are at a proliferative disadvantage (Luo *et al.*, 1999, D Strutt and MP Zeidler, unpublished observations). However it is not clear that *stat92E* mutant clones show a similar 'undergrowth' and both *hop* and *stat92E* mutant tissue can survive and proliferate to some degree. Therefore it would seem that the JAK/STAT pathway is not absolutely required for cellular proliferation in the fly.

While the *hop^{Tum-I}* gain-of-function mutation discussed above indicates an important role for the JAK/STAT pathway during hematopoiesis, further functions for the pathway during adult development have been indicated by a range of putative regulatory and partial loss of function alleles, of pathway components, recovered by virtue of their adult phenotypes (Perrimon and Mahowald, 1986; Venderosa and Muller, 1954). Of these, alleles of *upd* were originally identified (and named) on the basis of their distinctive 'outstretched' wing phenotype in which wings are held out from the body of the adult fly and a 'small eye' phenotype characterized by a small roughened adult eye or both 'outstretched-small eye' defects (Venderosa and Muller, 1954). All of these appear to represent regulatory alleles of the *unpaired* locus in which protein expression domains or levels are altered (Harrison *et al.*, 1998). In addition to these *upd* alleles a number of weak loss-of-function alleles of *hop* have also been identified (Perrimon and Mahowald, 1986; Luo *et al.*, 1999). In different hetero-allelic combinations *hop* alleles can give rise to both small or no disc phenotypes as well as adult animals with held out wings and rough or disrupted eye phenotypes (Luo *et al.*, 1999). Finally a weak *stat92E* allele, called *stat92E^{hi-jak}*, has also been described and shows a range of adult phenotypes, including a subtle but clearly reproducible ectopic wing vein formation (Yan *et al.*, 1996b).

Taken together it is clear that the components of the *Drosophila* JAK/STAT signaling pathway are involved in multiple aspects of adult development, including wing vein, wing hinge and eye development. However, the precise roles of the pathway in the development of these tissues remain to be characterized.

Ommatidial polarity

One adult tissue in which the role of the JAK/STAT pathway has been studied in detail is the eye. The *Drosophila* compound eye consists of approximately eight hundred 20-cell subunits called ommatidia that form the individual facets of the adult eye. These ommatidia are formed in the eye imaginal disc and rotate during development to assume dorsal and ventral rotational fates (shown as green and red clusters in Figure 3a) in each hemisphere of the future eye separated by a central line of mirror image symmetry known as the equator (Figure 3a and reviewed in Wolff and Ready, 1993). It has recently been shown that there is a requirement for the JAK/STAT pathway for the process of ommatidial rotation (Zeidler *et al.*, 1999b). Moreover the process of

ommatidial rotation requires not only JAK/STAT activity, but also both Wingless (Heberlein *et al.*, 1998; Wehrli and Tomlinson, 1998), and Notch function (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos *et al.*, 1998).

While clones lacking either *hop* or *stat92E* generated in the developing eye primordia are generally smaller than expected, they can proliferate and give rise to normal ommatidial clusters. Ommatidia within JAK/STAT mutant clones contain all cell types and appear to differentiate correctly and most rotate as anticipated. However, ommatidia situated close to the margin of large or broad mutant regions furthest from the equator often assume a 180° inverted orientation (note red clusters in Figure 3b). Consequently dorsally oriented ommatidia can be associated with *hop* clones in the ventral hemisphere of the eye and ventrally rotated ommatidia can be associated with dorsal clones (as shown in Figure 3b). Interestingly, inverted ommatidia present near the margins of *hop* mutant clones do not always lack JAK/STAT signaling but are sometimes comprised exclusively of wild type cells (arrows in Figure 3b). The fact that ommatidial clusters at this boundary composed entirely of wild type cells can still be inverted suggests that the JAK/STAT pathway, which molecular evidence suggests should be acting autonomously (within a single cell), is actually acting in a non-autonomous manner (on neighboring cells). This unexpected non-autonomous effect of *hop* and *stat92E* mutant clones is thought to result from a second, genetically downstream diffusible molecule that is controlled by the JAK/STAT pathway and is able to act non-autonomously on the juxtaposed wild type tissue.

A second signal and ommatidial polarity determination

The finding that clones of autonomously acting JAK/STAT pathway components can give rise to non-autonomous ommatidial inversion phenotypes, while unexpected, is not entirely without precedent. Similar results have also been reported for the Wingless signaling pathway (Wehrli and Tomlinson, 1998). As is the case for *hop* and *stat92E*, autonomously acting mutants of Wingless pathway components can also result in the inversion of entirely wild type ommatidia adjacent to mutant clonal areas. The existence of non-autonomous ommatidial inversion phenotypes has been interpreted to suggest that both the JAK/STAT and Wingless pathways, cannot be acting directly, but rather must be exerting their effects via the production of one or more intermediate molecules of signaling pathways capable of acting at a distance. These factors have been given various names including, the 'second signal' (the term we shall use in this review), factor X and Wnt X (Papayannopoulos *et al.*, 1998; Wehrli and Tomlinson, 1998; Zeidler *et al.*, 1999b). The identity of a putative second signal has remained elusive despite the fact that a number of predictions regarding the molecular nature and expression pattern of the second signal can be made (Zeidler *et al.*, 1999b). However, a recent report has described the identification of such a molecule (Zeidler *et al.*, 1999a). Four jointed (Fj) is a putative secreted type II trans-membrane molecule that is expressed in a broad gradient across the developing eye (Figure 3c; Brodsky and Steller, 1996; Villano and

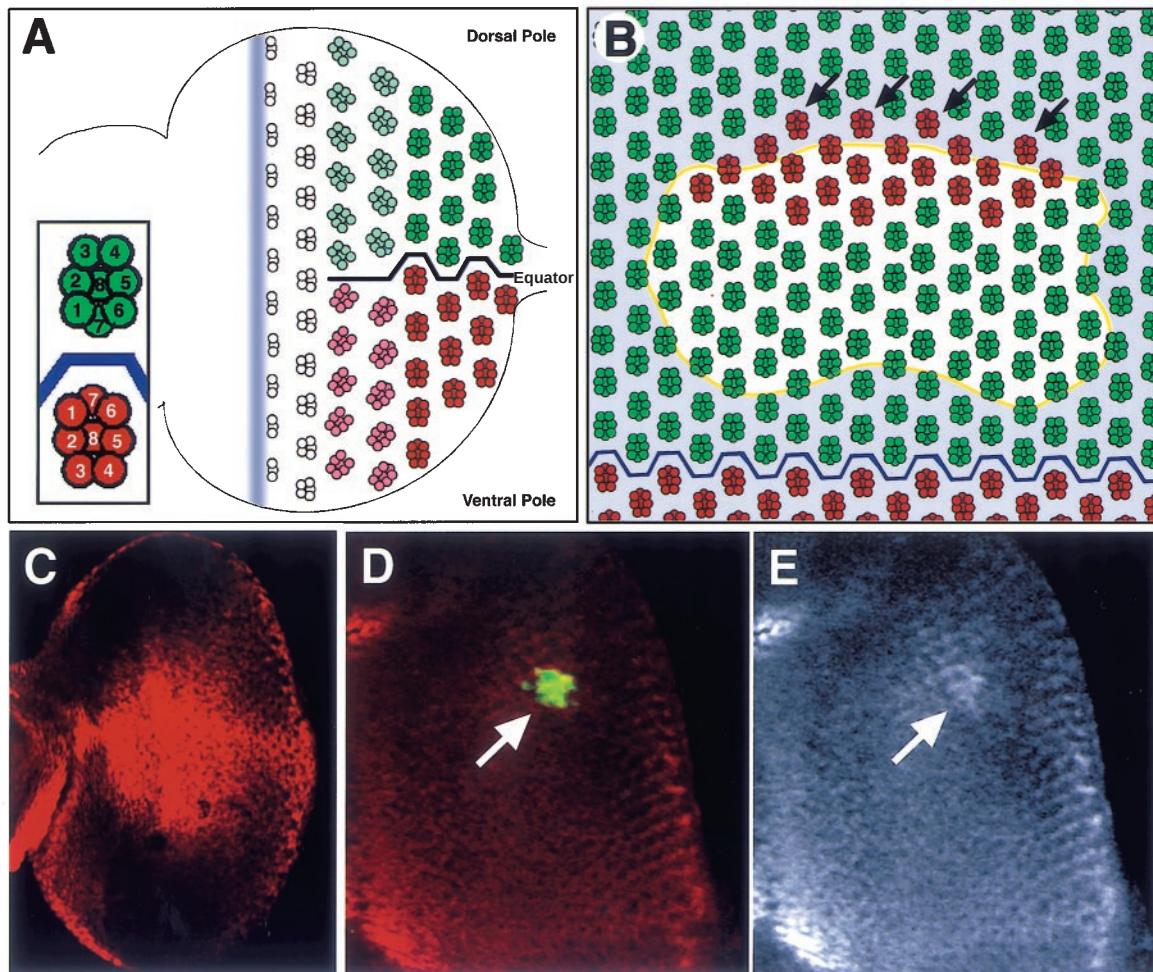


Figure 3 (a) A schematic cartoon illustrating the stages of ommatidial development and rotation in the eye imaginal disc. After beginning their development in the morphogenetic furrow (vertical gray line) the future ommatidia grow by recruiting surrounding cells to the cluster. Dorsal (green) and ventral (red) clusters then begin to rotate and form a line of mirror image symmetry along the midline of the disc known as the equator (blue line). An example of dorsal and ventral clusters that contain all eight photoreceptor cells is shown (insert). (b) A schematic representation of an eye imaginal disc that contains a mutant clone lacking all *hopscotch* activity (white background outlined in yellow) in an otherwise wild type background (gray background). While the clone is in the dorsal hemisphere of the eye and most mutant ommatidia have adopted the dorsal fate (green), ventrally oriented ommatidia (red) are present at the margin of the clone. Furthermore ommatidia entirely within the wild type (gray) region have also adopted a ventral (red) fate (indicated by arrows). (c) The expression pattern of the second signal molecule *four jointed* in the developing eye imaginal disc. Highest levels of expression are present at the midline of the disc. (d, e) Ectopic expression of Unpaired in a small region within the developing eye disc (green spot in d) causes the up-regulation of *four jointed* expression both in and around the Unpaired expressing clone (red in d and gray in e). (e) shows only the red channel of (d) for clarity. Arrow indicates the position of the Unpaired expressing clone

Katz, 1995). Both *ff* gain- and loss-of-function clones produce ommatidial inversion phenotypes as predicted for a diffusible second signal proposed to be downstream of the Wingless and JAK/STAT pathways (Zeidler et al., 1999a). Interestingly, *ff* expression is regulated by both Upd and the JAK/STAT pathway as would be expected of a *bona fide* second signal. *ff* is down regulated in loss-of-function clones lacking *hop* and is upregulated in and around clones of cells that misexpress Upd (Figure 3d,e). Further research is required to determine whether this effect is a direct consequence of Stat92E mediated expression as the promoter region of *four jointed* has not been analysed, and details regarding the mechanism of activation by the Upd and JAK/STAT pathway are as yet unknown. However, *ff* appears to fulfil the requirements for a second signal and may well represent another developmentally relevant, *in vivo* target of the JAK/STAT pathway.

A gradient of JAK/STAT signaling activity across the eye

Analysis of an enhancer detector P-element insertion in the *stat92E* locus has shown this insertion (known as STAT-lacZ) to act as an *in vivo* reporter of JAK/STAT pathway activity. The activity of the reporter is inversely proportional to the actual level of JAK/STAT pathway activity. Clones of cells mutant for *hop* result in up-regulation of STAT-lacZ while clones of cells ectopically expressing *upd* strongly down-regulate STAT-lacZ, not only within the region of Upd mis-expression, but also in surrounding tissues (Zeidler et al., 1999b). Based on these findings, it is clear that the pattern of STAT-lacZ in the wild type situation can be used to indicate the level of JAK/STAT pathway signaling. Such an analysis shows that a clear gradient of JAK/STAT pathway activity exists across the developing eye primordia with highest activity at the midline of the eye imaginal disc and

lowest at the dorsal and ventral poles. The pattern of JAK/STAT pathway activity implied by STAT-lacZ is consistent with the pattern of Unpaired expression. Both immunological and enhancer detector based assays show expression of Upd from an early stage at the posterior midline of the developing eye (Zeidler *et al.*, 1999b; H Sun, personal communication). This finding represents one of the clearest correlations between the pattern of Upd and the resultant activity of the JAK/STAT pathway *in vivo*, further strengthening the link between Upd and its role in the activation of the JAK/STAT pathway. The fact that the gradient of Upd induced JAK/STAT pathway activity (as demonstrated by STAT-lacZ) is central to the correct orientation of ommatidia is supported by experiments in which Upd is mis-expressed. Experiments in which Upd is mis-expressed in domains other than the midline of the disc (where endogenous Upd is present) change the local gradient of JAK/STAT activity and have been shown to result in ommatidial inversion phenotypes (Zeidler *et al.*, 1999b), presumably via the resultant ectopic expression of the second signal *four jointed* (Figure 3d,e). This is the first case in which a gradient of JAK/STAT pathway signaling activity has been shown to exist, to be required for normal development, and to be the result of localized expression of a pathway ligand.

Redundancy in JAK/STAT signaling

Although both *hop* and *stat92E* loss of function clones can generate ommatidial inversion phenotypes, the relative strength (or expressivity) of these phenotypes are very different. Alleles of *stat92E* thought to be amorphic (total loss of function) produce effects at a much lower frequency than amorphic alleles of *hop* (Zeidler *et al.*, 1999b). It was originally suggested that this difference in phenotypic strength could be due to partial redundancy between *stat92E* and other as yet undiscovered *Drosophila* STAT molecules. However, as discussed above, that explanation now seems increasingly unlikely. As *stat92E* probably represents the only *Drosophila* STAT, it is possible that another as yet unidentified mechanism exists downstream of Hop, and parallel to Stat92E. While a potential explanation is simply that an alternative STAT like molecule is too divergent to be identified by sequence searches, the precise nature of the mechanisms that would partially mediate the transduction of Upd initiated Hop signaling remain unidentified.

Future directions

Our understanding of the roles of the JAK/STAT pathway during *Drosophila* development has advanced considerably in recent years and the study of the pathway is sure to be a source of new and unexpected findings in the years to come. However, a number of outstanding questions remain in our understanding of both the pathway itself, its functions during development and its interactions with other signal transduction cascades. The answers to these questions are likely to lie in a number of directions. While a number of putative additional JAK/STAT pathway components have been identified by homology, many components

of this pathway have yet to be identified genetically in flies. Given the genetic nature of the *Drosophila* system, the generation and identification of mutations in these components will be essential. It is conceivable that screens to identify mutations in these components could be based on a number of different aspects of the JAK/STAT pathway. These include further screens designed to identify the characteristic JAK/STAT-like embryonic cuticle phenotype in germline clones, suppression of the dominant phenotypes associated with *hop^{Tum-I}* or *hop^{T42}* mutations or sensitized systems that select candidate mutations by their interaction with an engineered over-activation phenotype. Indeed each of these possibilities either has been, or is being, pursued in a number of laboratories and the next few years should lead to a significant increase in the number of identified, genetically defined, JAK/STAT pathway components.

A further major advance in our understanding of JAK/STAT signaling will undoubtedly come from the analysis of other aspects of both embryonic and adult development that require the pathway. These may include other stages and tissues of embryonic development such as the tracheal pits in which Upd is strongly expressed (Harrison *et al.*, 1998), larval blood cell development and the insect immune response (Barillas *et al.*, 1999). Moreover, the analysis of other stages of adult development such as wing vein and wing hinge defects observed to be part of existing partial loss-of-function pathway mutant phenotypes may also prove fruitful.

In addition to these developmental roles, it is becoming increasingly clear that the JAK/STAT pathway cannot function in isolation. Indeed analysis of *eve* expression in mutant embryos indicates that while the JAK/STAT pathway is undoubtedly important in the regulation of *eve* stripe 3 expression, additional signals feed in to maintain low levels of *eve* expression in this third stripe even in the total absence of JAK/STAT activity (Hou *et al.*, 1996; Yan *et al.*, 1996a). In addition the proper expression of *ff* in the developing eye is not simply a consequence of Upd/JAK/STAT signaling but rather appears to require the additional involvement of Wingless signaling, Notch activity and an as yet uncharacterized component of *ff* autocrine feedback (Zeidler *et al.*, 1999a). No doubt similar complications will arise with regard not only to JAK/STAT signaling, but a wide range of signal transduction responses as the tools available and our resultant understanding of the systems being analysed continue to improve.

One such set of tools which would facilitate our understanding of JAK/STAT signaling would be reagents with which to visualize the activity of the pathway *in vivo*. While STAT-lacZ is able to report JAK/STAT activity in imaginal discs this does not represent an assay suitable for individual cells or tissue culture based systems and additional techniques would also be useful. Given the recent development and use of antibodies specific for activated forms of *Drosophila* ERK (Gabay *et al.*, 1997) and the commercial availability of phospho-specific forms of mammalian STATs, it seems plausible that similar antibodies that recognize activated JAK or STAT could be developed. Alternatively the subcellular translocation of STAT into the nucleus following activation may also provide the basis of such techniques.

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