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JAK/STAT signalling in *Drosophila*: insights into conserved regulatory and cellular functions

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High levels of interspecies conservation characterise all signal transduction cascades and demonstrate the significance of these pathways over evolutionary time. Here, we review advances in the field of JAK/STAT signalling, focusing on recent developments in *Drosophila*. In particular, recent results from genetic and genome-wide RNAi screens, as well as studies into the developmental roles played by this pathway, highlight striking levels of physical and functional conservation in processes such as cellular proliferation, immune responses and stem cell maintenance. These insights underscore the value of model organisms for improving our understanding of this human disease-relevant pathway.

Introduction

The transduction of information from the outside of a cell to produce a specific response is an essential prerequisite for development, homeostasis and cellular survival, and is mediated by a small number of signal transduction cascades. As a result, the study of these pathways from a developmental viewpoint has been a focus of research for many years and have shown that signalling pathways are frequently reused in multiple tissues for diverse developmental processes. Here, we focus on one such cascade – the JAK/STAT pathway – the misregulation of which is associated with a wide range of human malignancies, including diverse haematopoietically derived cancers.

The human genome encodes multiple isoforms of all major JAK/STAT pathway components, many of which are co-expressed within the same cells and can form homo- and heterodimers to determine a variety of specific effects in response to a diverse range of ligands. By contrast, *Drosophila* contains a simpler 'streamlined' pathway that is nonetheless sufficient to mediate a multitude of different processes (reviewed by Bach and Perrimon, 2003; Castelli-Gair Hombría and Brown, 2002; Hou et al., 2002). However, despite the relative simplicity of the *Drosophila* pathway, molecular and functional data clearly indicate that a high level of conservation exists between the structural components of the insect and mammalian pathways. As a result, the low levels of redundancy in JAK/STAT pathway components, together with the availability of gain- and loss-of-function mutations, advanced genetic and molecular tools, and a multitude of markers, make *Drosophila* an excellent model for investigations into the JAK/STAT pathway.

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In this review, we focus on two aspects of JAK/STAT pathway biology. First, we examine recent advances in the identification and characterisation of pathway components and regulators. Second, we review results that highlight the evolutionary conservation of pathway function in vivo – with an emphasis on developmental aspects. In particular, we summarise information about conserved roles of the JAK/STAT pathway in the control of proliferation during larval development, and in immune responses and germ/stem cell development. Although the immune response is not central to the field of developmental biology, and has been reviewed in greater depth elsewhere (Agaisse and Perrimon, 2004; Tzou et al., 2002), these pathway functions serve to illustrate the functional conservation between flies and humans.

Given the levels of conservation in this pathway that are now becoming evident, it is hoped that the newly identified regulators and developmental roles being discovered in the *Drosophila* model system will advance our understanding of this important pathway and its roles in human disease.

The canonical JAK/STAT signalling pathway

Since the initial identification of a novel class of interferon-activated transcription factors over 14 years ago (Fu et al., 1992; Schindler et al., 1992), extensive studies have characterised the core components of the JAK/STAT signal transduction pathway, which include a wide and diverse range of extracellular ligands and transmembrane receptors, four Janus kinases (JAKs) and seven genes coding for signal transducers and activators of transcription (STATs) (Kisseleva et al., 2002). In addition to the identification of the pathway components themselves, a model of pathway activation has also been established (Fig. 1). In general, this entails the binding of an extracellular ligand to a transmembrane receptor, which results in the activation of the receptor-associated JAKs. These tyrosine kinases then phosphorylate themselves and their associated receptors to generate docking sites for the SH2 domains of STATs. According to the established models, STATs are normally present in the cytoplasm as inactive monomers before recruitment to the receptor/JAK complex. However, it has also been shown that STATs constitutively shuttle between the cytoplasm and nucleus before being retained in the nucleus following activation (reviewed by Vinkemeier, 2004). Once bound to the receptor/JAK complex, STAT molecules are themselves phosphorylated and dimerise. These dimers, stabilised by the interaction between the SH2 domain of one molecule and the phospho-Tyr of the other molecule, translocate to the nucleus where they bind to a palindromic DNA sequence in the promoters of pathway target genes to activate transcription (as shown in Fig. 1). Finally, although the dimerisation of STATs via an N-terminal domain interaction can occur prior to pathway stimulation, only complexes activated by Tyr-phosphorylation appear to induce target gene expression (Braunstein et al., 2003).

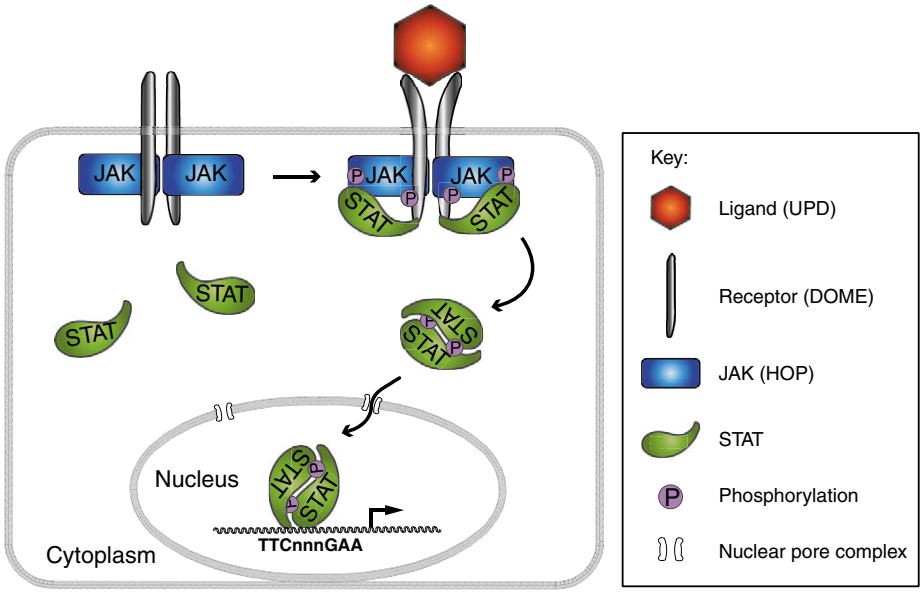

Fig. 1. The canonical model of JAK/STAT signalling. Pre-dimerised complexes of a pathway receptor (grey) and JAKs (blue) are activated following ligand (red) binding. Phosphorylation (purple circles) of the JAKs and the receptors generate docking sites for the normally cytosolic STATs that are recruited to the active complex. Following phosphorylation of the STATs, STAT dimers form, which translocate to the nucleus and bind to a palindromic DNA sequence in the promoters of target genes to activate their transcription. The names of the pathway components in *Drosophila* are provided in brackets in the key.

Over the past decade, numerous studies have determined a wealth of information about the functions and activities of these core JAK/STAT signalling components (reviewed by Hou et al., 2002; Kisseleva et al., 2002). Studies in model systems, such as the fruitfly *Drosophila melanogaster*, the fish *Danio rerio* and the nematode *Caenorhabditis elegans*, have also identified conserved pathway components in these lower organisms (Hou et al., 2002). In particular, genetic analysis in *Drosophila* has identified all the components of the canonical JAK/STAT signalling cascade, and over recent years, this genetically tractable model organism has become an important focus of research in the field. In *Drosophila*, the core components of the JAK/STAT pathway consist of: three ligands [Unpaired (UPD; OS – FlyBase), UPD2 and UPD3 (Fig. 2A) (Agaisse et al., 2003; Castelli-Gair Hombría et al., 2005; Gilbert et al., 2005; Harrison et al., 1998)]; a transmembrane receptor called Domeless (DOME) (Fig. 2B) (Brown et al., 2001; Chen et al., 2002); a JAK kinase known as Hopscotch (HOP) (Fig. 2C) (Binari and Perrimon, 1994); and a transcription factor called STAT92E (Fig. 2D) (Hou et al., 1996; Yan et al., 1996). Although the core pathway components have been known for some time (reviewed by Bach and Perrimon, 2003; Castelli-Gair Hombría and Brown, 2002; Hou et al., 2002), these molecules still represent an area of active research that aims to characterise their biochemical and functional activity in vivo (Brown et al., 2003; Henriksen et al., 2002; Karsten et al., 2005). However, it is the identification and characterisation of additional components and regulators of the core pathway that will be discussed here.

JAK/STAT pathway regulators

Given the multitude of roles played by signal transduction pathways and the potential developmental consequences of their inappropriate activity, it is not surprising that multiple regulatory mechanisms exist to control them. These regulators can be broadly divided into two classes. Those whose activity is required for the transduction of pathway signalling, referred to as positive regulators, and those that act to reduce the strength of signalling – negative regulators.

Both positively and negatively acting factors that modulate JAK/STAT pathway signalling have been identified and include not only new ligands (Castelli-Gair Hombría et al., 2005), but also other interacting signal transduction cascades and other less well characterised molecules (Bach et al., 2003; Baeg et al., 2005; Mukherjee et al., 2006; Müller et al., 2005). These are discussed below.

Positive regulators

Pathway ligands

While the activation of JAK/STAT pathway signalling in mammalian systems is triggered by a wide range of interleukins, interferons and growth factors (Langer et al., 2004; Subramaniam et al., 2001), only a single closely related group of Unpaired-like pathway ligands have been identified in invertebrates (Agaisse et al., 2003; Castelli-Gair Hombría et al., 2005; Gilbert et al., 2005; Harrison et al., 1998). In addition, although retrospective alignments of Type II cytokines suggest that UPD may be related to the Leptin family of mammalian pathway ligands (Langer et al., 2004), no obvious UPD-like proteins can be detected beyond the *Drosophilidae* family of flies (Castelli-Gair Hombría and Brown, 2002; Castelli-Gair Hombría et al., 2005). In silico searches have, nonetheless, identified the three related *upd*-like genes (Fig. 2A) in the *Drosophilids*, for which genome sequence is available (Castelli-Gair Hombría et al., 2005), and characterisation of *upd* mutations in *Drosophila melanogaster* suggest that the canonical requirements for JAK/STAT pathway activity are likely to be mediated exclusively by the ligands UPD, UPD2 and UPD3 (Agaisse et al., 2003; Castelli-Gair Hombría et al., 2005; Harrison et al., 1998).

Mutations in the *upd* locus were first identified over 75 years ago (Müller, 1930), and the *upd* gene itself has since been shown to encode a secreted glycoprotein that activates the pathway via DOME (Brown et al., 2001; Harrison et al., 1998). When ectopically expressed, UPD can function over at least 20 cell diameters in vivo (Tsai and Sun, 2004) and non-autonomous signalling induced by endogenous levels of UPD expression has also been demonstrated (Bach et al., 2003; Karsten et al., 2002).

Although null mutations of *upd* show strong segmentation and posterior spiracle phenotypes (Brown et al., 2001; Harrison et al., 1998), these are generally less severe than those exhibited by loss of more downstream components, such as *hop* or *stat92E*, indicating that other ligands may partly compensate for the loss of *upd* (Castelli-Gair Hombría et al., 2005). The recent characterisation of

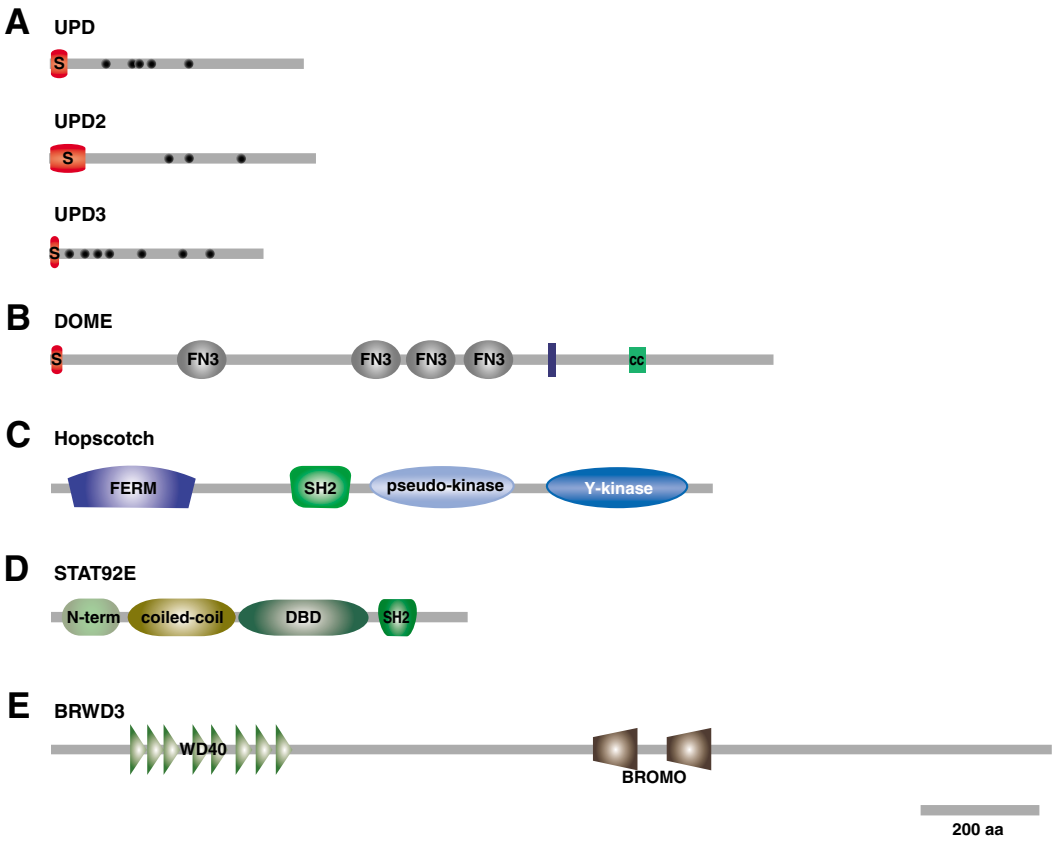

Fig. 2. Positive regulators of *Drosophila* JAK/STAT signalling. (**A**) Three UPD-related ligands, each of which contain N-terminal signal sequences (S) and have predicted N-linked glycosylation sites (dots). (**B**) DOME contains fibronectin type III-like repeats (FN3) (characteristic of cytokine receptors), a transmembrane region (blue box) and a coiled-coil domain (cc). (**C**) Hopscotch contains an N-terminal FERM (4.1, ezrin, radixin, moesin) domain found in all JAKs that is required for association with the receptor (Usacheva et al., 2002), an SH2 domain, a regulatory pseudo-kinase domain and a Tyr kinase (Y-kinase) domain. (**D**) STAT92E contains a N-terminal domain found in all STATs that mediates tetramerisation (Johnson et al., 1999; Vinkemeier et al., 1998), a coiled-coil region and a DNA-binding domain (DBD). An SH2 domain is also present, and is required for receptor binding and dimerisation. In mammalian STATs, the C terminus also contains a transcriptional activation domain. In ΔNSTAT92E, the absence of the N-terminal region produces a protein that acts as a dominant negative (Henriksen et al., 2002). (**E**) BRWD3 contains eight WD40 motifs and two BROMO domains, which are frequently present in chromatin-associated proteins. Proteins and domains are shown to scale.

the *upd2* locus has confirmed that UPD2 represents a second pathway activator that functions redundantly to UPD (Castelli-Gair Hombría et al., 2005; Gilbert et al., 2005). Like UPD, UPD2 can activate JAK/STAT pathway reporters both in tissue culture and in vivo. However, in contrast to UPD, UPD2 does not appear to attach to the extracellular matrix, but rather is secreted as a freely diffusible ligand (Castelli-Gair Hombría et al., 2005). The third UPD-like protein, UPD3, has been less extensively characterised. However, it is expressed in the developing gonads (Castelli-Gair Hombría et al., 2005), the larval lymph gland (Jung et al., 2005) and circulating haemocytes following septic injury (Agaisse et al., 2003).

BRWD3

The *Drosophila* homologue of human BRWD3 is a large WD40- and bromo-domain-containing protein (Fig. 2E) that was originally identified when two independent double-stranded (ds) RNAs targeting *BRWD3* mRNA were found to strongly suppress the transcription of STAT92E-dependent reporters in cell culture (Müller et al., 2005). Mutations in *BRWD3* also genetically interact with the JAK/STAT pathway to decrease the frequency of the melanotic tumour phenotype associated with the gain-of-function mutation $hop^{Tum\text{-}l}$ (Müller et al., 2005).

Of particular significance for the analysis of *BRWD3* is the recent discovery that the human homologue of *BRWD3* is disrupted in a large proportion of individuals with B-cell chronic lymphocytic leukaemia (B-CLL) (Kalla et al., 2005). Although a link between JAK/STAT signalling and the development of other haematopoietic malignancies has been demonstrated (Calò et al., 2003), the precise role of BRWD3 with regard to JAK/STAT signalling and the development of B-CLL has yet to be investigated.

Other signalling pathways

Recent investigations have identified that interactions occur between the JAK/STAT pathway and several other signal transduction cascades. For example, the Notch signalling cascade activates the JAK/STAT pathway in developing eye imaginal discs, where it induces expression of *upd* (Chao et al., 2004; Moberg et al., 2005; Reynolds-Kenneally and Mlodzik, 2005), and both pathways co-operate during foregut morphogenesis (Josten et al., 2004). In addition, the Notch cascade is known to affect JAK/STAT signalling via activation of JAK2 in the mouse (Kamakura et al., 2004), and strong genetic interactions between JAK/STAT pathway signalling and mutations in Notch pathway components, such as *Notch*, *Delta* and *bearded*, have been demonstrated in *Drosophila* (Mukherjee et

al., 2006). In addition, a requirement for STAT92E in mediating the eye overgrowth phenotype caused by ectopic activation of SRC has been reported (Read et al., 2004), and genetic screens have also identified interactions with components of the DPP/BMP pathway (such as *dpp*, *mothers against dpp*, *thickveins*, *bunched*) and the Hedgehog (*hh*) signalling pathway (Bach et al., 2003; Mukherjee et al., 2006).

Overall, it is becoming clear that no single pathway functions in isolation in a developmental context and although care must be taken in interpreting genetic interaction data, it is likely that an analysis of inter-pathway crosstalk will represent a key step towards our understanding of JAK/STAT signalling in vivo.

Other factors

Recent forward and reverse genetic screens (Box 1) (Bach et al., 2003; Baeg et al., 2005; Mukherjee et al., 2005; Müller et al., 2005) have identified multiple novel loci, the knockdown of which is sufficient to modulate the strength of JAK/STAT pathway activity. Strikingly, a comparison of the genes identified in these different screens reveals a relatively small overlap in the candidates identified. In the case of the genetic interaction screens (Bach et al., 2003; Mukherjee et al., 2005), genetic interaction can occur at many levels and may result from effects downstream of the pathway itself. In addition, as the genetic screens undertaken are not saturating, a limited overlap between the loci identified by genetic and RNAi screens is not necessarily surprising. By contrast, the results of two published RNAi screens (Baeg et al., 2005; Müller et al., 2005) are more striking and show a maximum overlap of only 20%. As both RNAi screens identified known pathway components, it is unlikely that false negatives have lead to the exclusion of many true interactors. Rather, it seems more likely that differences in the transcriptomes of the two cell lines used, in conjunction with alternative strategies of pathway stimulation and post-screen in silico analysis, are responsible for most of the differences in the results of these two screens. Given that false positive hits are also likely to be present, the in vivo validation of candidate pathway-regulating loci is still required.

Negative regulators

SOCS

The SOCS (suppressors of cytokine signalling) genes, originally characterised in vertebrates, are the best characterised of the JAK/STAT pathway negative regulators. In vertebrates, SOCS proteins have been shown to suppress JAK/STAT pathway signalling via several distinct mechanisms (Kile and Alexander, 2001; Krebs and Hilton, 2001). They are themselves target genes of this pathway, which are expressed in response to STAT activation, so forming negative-feedback loops to downregulate pathway activity (Box 2). On the basis of sequence homology, three SOCS-like genes have been identified in the *Drosophila* genome (Fig. 3A). Of these, *socs36E* functions as a potent pathway repressor, which, when ectopically expressed, mimics the outstretched wing phenotype of *upd* mutants and the venation defects associated with the $stat92E^{HJ}$ allele (Baeg et al., 2005; Rawlings et al., 2004). *socs36E* is also a transcriptional target of STAT92E (Baeg et al., 2005; Callus and Mathey-Prevot, 2002; Karsten et al., 2002; Rawlings et al., 2004). Although *socs44A*

Box 1. Screens for JAK/STAT pathway regulators

Whole-genome RNAi screens can now be routinely performed in *Drosophila* using libraries of double stranded (ds) RNA molecules, each ~500 bp long, and derived from every potential gene in the fly genome. The addition of dsRNA to cultured *Drosophila* cells leads to a potent and specific knockdown of the targeted transcript (Clemens et al., 2000). By using this RNAi effect in conjunction with STAT92E-responsive luciferase reporter constructs, two independent groups have recently published comprehensive lists of loci, the knockdown of which modulates reporter expression levels (Baeg et al., 2005; Müller et al., 2005). Such genome-wide RNAi screening approaches in *Drosophila* cells offer several major advantages. First, the low complexity of the *Drosophila* genome acts to minimise the number of false negatives that might otherwise occur because of redundancy within gene families. Second, unlike mammalian cells, dsRNA is taken up by *Drosophila* cells without the need to transfect them and is then efficiently spliced in vivo to generate diverse pools of short interfering RNA that have minimal non-specific effects.

More-traditional 'forward' genetic screens have also been employed to identify mutations that interact with JAK/STAT signalling, using the principal of a 'sensitised system'. In this approach, candidate pathway modulators are identified on the basis of a genetic interaction with a dominant phenotype. In the case of the recently completed JAK/STAT pathway screens, ectopic pathway activation in the developing fly eye leads to a massive increase in cellular proliferation and an overgrown adult eye. Importantly, this large eye phenotype is sensitive to the strength of the endogenous pathway and loss of one copy of a pathway component is sufficient to produce a readily recognisable decrease in eye size (Bach et al., 2003). Screens using this technique have been used to identify genetic regions, candidate mutations and randomly induced transposon insertions that interact with the JAK/STAT pathway in vivo (Bach et al., 2003; Mukherjee et al., 2006).

Box 2. Feedback loops and JAK/STAT pathway signalling

Several feedback loops consisting of putative pathway target genes function in the JAK/STAT pathway to modulate its signalling. In such feedback loops, pathway activation leads to target gene expression, so increasing the influence of that gene product. Where a target gene normally negatively regulates JAK/STAT signalling a negative-feedback loop is formed that acts to decrease the strength and duration of pathway activity. Where the target gene is a positively acting pathway component, a positive feedback loop acts to increase the strength of signalling.

One of the first feedback loops identified in JAK/STAT signalling is that formed by the SOCS protein family, the pathway-dependent expression and negative regulation of the JAK/receptor complex of which acts as a potent negative feedback loop in both mammals and *Drosophila* (Kile and Alexander, 2001). More recently, another negative feedback loop defined by the putative target gene *PTP61F*, a fruitfly homologue of the human PTPB1 phosphatase (see above), has been identified in *Drosophila* and may represent a mechanism of pathway regulation also utilised in vertebrates (Baeg et al., 2005).

By contrast, positive feedback loops appear to be involved in regulating JAK/STAT pathway activity. For example, upregulation of STAT3 is often observed in mammalian cells upon interleukin 6 (IL6) stimulation, a response elicited by IL6-response elements found in the STAT3 promoter (Ichiba et al., 1998). Similarly, the stability of *Drosophila* STAT92E also appears to be enhanced in cells with high JAK/STAT pathway activity (Xi et al., 2003), while the expression of the Domeless receptor is also strongly upregulated by pathway activity (Brown et al., 2001).

Although yet to be demonstrated molecularly, the interplay of positive feedback loops generated by the upregulation of pathway components, and of negative feedback loops generated by SOCS and PTP61F, is likely to represent an important aspect of the differing levels of pathway sensitivity and perdurance in vivo.

does not show JAK/STAT-dependent expression, it can inhibit pathway activity (Rawlings et al., 2004). *socs16D* is as yet uncharacterised.

ZIMP/PIAS

PIAS proteins (protein inhibitors of activated STAT) represent another well-characterised group of pathway suppressors that bind STATs and target them for degradation via SUMOlation (Kotaja et al., 2002; Ungureanu et al., 2003; Wormald and Hilton, 2004). A single *Drosophila* PIAS-like protein, ZIMP (Fig. 3B), also physically interacts with STAT92E and can suppress the formation of haematopoietic tumours caused by ectopic overactivation of the pathway (Betz et al., 2001).

ΔNSTAT92E

Although the *Drosophila* genome contains a single STAT-encoding gene (Yan et al., 1996), a negatively acting truncated form of STAT92E has recently been characterised (Fig. 3C) (Henriksen et al., 2002). In contrast to the C-terminal truncated β-forms of mammalian STATs, which lack a transcriptional activation domain, *Drosophila* ΔNSTAT92E lacks an N-terminal region, which has been shown in mammalian STATs to promote tetramerisation and nuclear re-export to the cytoplasm (Johnson et al., 1999; Levy and Darnell, 2002; Meyer and Vinkemeier, 2004; Vinkemeier et al., 1998). When ectopically expressed, ΔNSTAT92E exerts a dominant-negative effect on the expression of the JAK/STAT pathway target genes *even-skipped* and *trachealess* (Henriksen et al., 2002; Karsten et al., 2005). The ratio of full-length to ΔNSTAT92E expression also varies throughout development (Henriksen et al., 2002; Mukherjee et al., 2005) and it will be intriguing to determine the mechanisms by which the truncated:full-length STAT92E ratio is regulated in vivo.

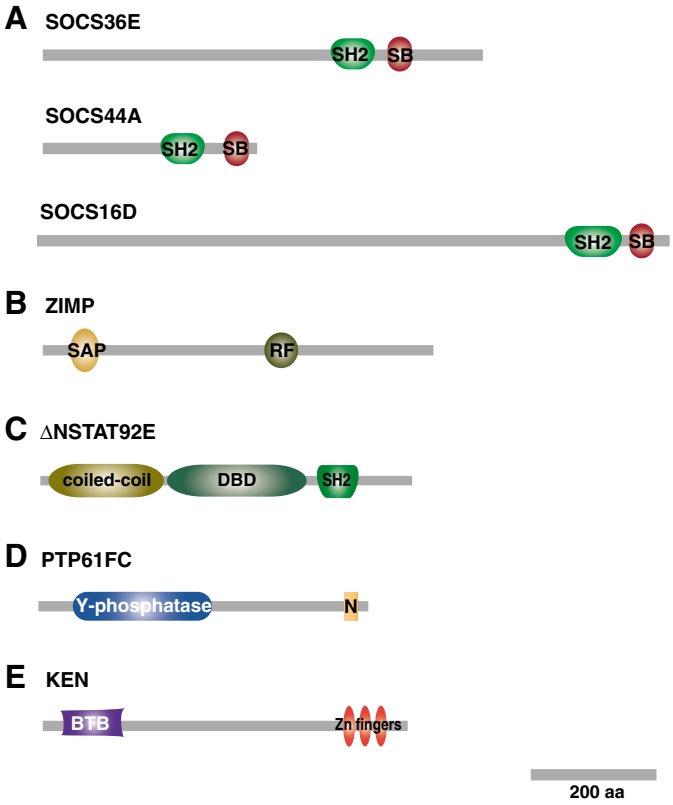

Fig. 3. Negative regulators of *Drosophila* JAK/STAT signalling. (**A**) The SOCS genes are named for their chromosomal locations, and each contains an SH2 domain and a SOCS box (SB). (**B**) ZIMP [also called PIAS or Su(var)2-10] contains a putative chromatin binding SAP (SAF, Acinus and PIAS) domain and a zinc-containing ring finger (RF) implicated in SUMOlation. (**C**) A truncated STAT92E splice variant lacking an N-terminal protein:protein interaction domain results in a dominant-negative protein (Henriksen et al., 2002). Compare to Fig. 2D. (**D**) PTP61FC is a spliceform of *ptp61F* that contains a C-terminal nuclear localisation signal (N) and an Y-phosphatase domain. (**E**) KEN contains an N-terminal BTB/POZ (BTB) domain and three C-terminal C2H2 zinc-finger motifs. Proteins and domains shown to scale.

Phosphatases

Given the central role of the JAK tyrosine kinase in pathway signalling, phosphatase activity is likely to represent an important regulatory mechanism. Recently, two independent RNAi screens have identified *Drosophila* PTP61F (Fig. 3D), a homologue of human PTPB1 (phospho-Tyr phosphatase B1), as a suppressor of STAT92E-dependent transcription (Baeg et al., 2005; Müller et al., 2005). *Ptp61f* is expressed in a pattern that mirrors that of *upd* and that appears to be JAK/STAT pathway-dependent (see also Box 2). As expected, reducing PTP61F activity in cultured Kc_{167} and S2-NP cells by RNAi leads to dramatic increases in both the activity of STAT-responsive reporters and the levels of Tyr-phosphorylated HOP and STAT92E protein (Baeg et al., 2005). However, the exact mechanism by which PTP61F can downregulate pathway signalling is not entirely clear. Only a nuclear localised splice form of PTP61F, termed PTP61FC, can affect pathway activity in vivo but RNAi knockdown of both splice forms affects the pathway epistatically downstream of HOP (Müller et al., 2005). PTP61FC therefore probably acts at the level of STAT92E, a conclusion that is also supported by in silico modelling, which shows that pathway activity is most strongly influenced by altering the level of nuclear STAT-interacting phosphatases (Zi et al., 2005).

Ken & Barbie/BCL6

A role for Ken & Barbie (KEN) in the selective regulation of STAT92E activity has also recently been demonstrated, a finding that could advance our understanding of how a single pathway can mediate diverse developmental processes (Arbouzova et al., 2006). KEN is the *Drosophila* homologue of human BCL6 (B-cell lymphoma 6) and belongs to the family of BTB/POZ domain-containing transcriptional repressors (Fig. 3E). In vitro selection experiments show that KEN recognises a DNA sequence that partially overlaps that of STAT92E in vitro (Fig. 4A), while tissue culture assays indicate that it can specifically downregulate JAK/STAT pathway reporters only when they contain the consensus KEN DNA-binding site (Fig. 4B). In vivo, both ectopically expressed and endogenous KEN downregulate only a subset of likely STAT92E target genes.

The human BCL6 locus is itself the focus of considerable interest because of its mutation in a large proportion of diffuse large B-cell non-Hodgkins lymphomas (Pasqualucci et al., 2003). Although no direct link has been made between the requirement for BCL6 during B-cell development and the JAK/STAT pathway in vivo, BCL6 has also been shown to repress STAT6-dependent transcription in cell culture (Harris et al., 1999; Harris et al., 2005; Hartatik et al., 2001). Further analysis of the interplay between KEN/BCL6 and JAK/STAT signalling promises to be an interesting area for future research.

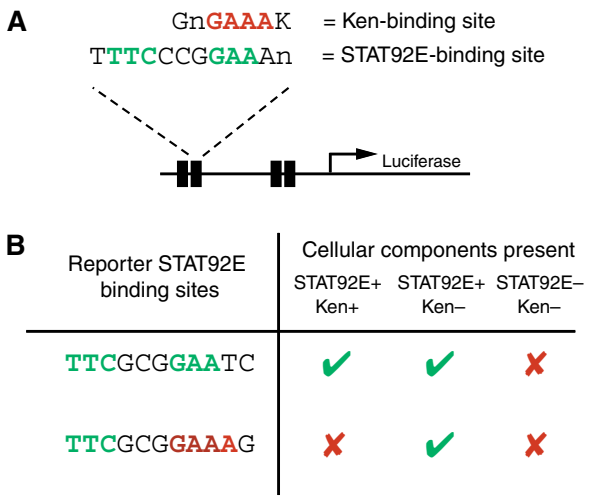

Fig. 4. KEN selectively regulates STAT92E targets depending on the binding sites present. (**A**) In vitro selection experiments have defined the optimal DNA-binding sites of KEN and STAT92E. The 'core' positions essential for binding are shown in red (for KEN) or green (for STAT92E). There is overlap in the sequences recognised. (**B**) Reporters differing only in their STAT92E-binding sites respond differently to the activity of STAT92E and KEN. In reporters to which only STAT92E can bind (green; top row), activation by STAT92E cannot be modulated by KEN. When both STAT92E and KEN are able to bind to a reporter (green and red; lower row), activation by STAT92E can be countered by the co-expression of KEN. Neither reporter is active in the absence of STAT92E. Green tick represents activation of reporter transcription, red cross implies repression.

Conserved roles for JAK/STAT signalling during development

In addition to the identification of new factors that regulate JAK/STAT signalling, significant progress has also been made in our understanding of the roles played by the pathway during development (reviewed by Bach and Perrimon, 2003; Castelli-Gair Hombría and Brown, 2002; Hou et al., 2002). Several recent studies of the role of the *Drosophila* JAK/STAT pathway have identified examples in which both the mechanisms and functions of the pathway are conserved between vertebrates and *Drosophila*. Here, we review these findings with a particular focus on cellular proliferation, the innate immune response and stem/germ cell development.

JAK/STAT signalling and the control of cellular proliferation

Several lines of evidence indicate that the JAK/STAT pathway functions in the regulation of cellular proliferation – a process that is essential to many aspects of development. First, the constitutive activation of several STATs has been observed in multiple human cancers, including blood malignancies (such as leukaemias, lymphomas and myelomas) and solid tumours [including brain, breast, lung, pancreatic and prostate cancers (reviewed by Calò et al., 2003)]. Second, chronic myeloproliferative diseases (MPDs; a term for diseases in which myeloid cell types undergo abnormal proliferation and/or survival) are often associated with the activation of tyrosine kinases, many of which have the potential to activate STATs (De Keersmaecker and Cools, 2005). In particular, a recent analysis of polycythaemia vera, a MPD characterised by the overproliferation of erythrocytes, has revealed that over 80% of affected individuals carry a V617F mutation in the regulatory pseudokinase domain of JAK2. This mutation results in constitutive kinase activity and, presumably, in ectopic pathway activation (James et al., 2005; Staerk et al., 2005).

Similar gain-of-function mutations in the *Drosophila* JAK homologue HOP have also been identified. The hop^{T42} allele, for example, also contains a pseudo-kinase domain mutation, which leads to the constitutive activity of *Drosophila* HOP, while the corresponding mutation generated in mouse JAK2 also results in pathway overactivation (Luo et al., 1997). In vivo, hop^{T42} induces the overproliferation and premature differentiation of larval blood cells, which then form melanotic tumours (Fig. 5B) (Luo et al., 1997), a phenotype essentially identical to that observed in the $hop^{Tum\text{-}l}$ gain-of-function mutant (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1995). Thus, it appears that in addition to the conservation of the JAKs themselves, both the potential to generate activating mutations and their in vivo haematopoietic overproliferation phenotypes have been conserved through evolution. A role for the pathway during normal haematopoietic development is also implied by the observation that *upd3* and *dome* are expressed in the main *Drosophila* larval haematopoietic organ – the lymph gland (Jung et al., 2005). Although parallels between *Drosophila* and vertebrate haematopoiesis have been drawn in the past (Evans et al., 2003), it will be intriguing to determine exactly how far the functional conservation at the level of individual signalling pathways, such as the JAK/STAT cascade, has been maintained.

As described above, analyses of gain-of-function alleles have shown that ectopic JAK/STAT pathway activation is sufficient to induce overproliferation of at least some cell types. Another tissue in which this occurs is the developing eye imaginal disc. During normal eye development, a physical indentation, termed the morphogenetic furrow (MF), moves from the posterior to the anterior of a field of undetermined retinal cells. The MF marks the beginning of cellular determination and is associated with the first and second mitotic 'waves' of cell division that occur ahead of and behind the MF, respectively (Fig. 5C) (reviewed by Voas and Rebay, 2004). This pattern of proliferation is enhanced by the ectopic stimulation of the JAK/STAT pathway, which results in a large increase in the number of mitotic cells within the first mitotic wave (Fig. 5D) (Bach et al., 2003; Tsai and Sun, 2004). However, despite the overproliferation of pluripotent cells ahead of the MF, cells already determined to generate the adult eye posterior to the furrow do not respond proliferatively and differentiate essentially normally (Fig. 5D) (Bach et al., 2003). As a result of the increase in proliferation, more cells are available to be recruited into ommatidia, giving rise to an enlarged and overgrown adult eye (compare Fig. 5E with 5F). This phenotype is sensitive to the dose of downstream pathway genes and has been used as the basis of genetic interaction screens (see Box1) (Bach et al., 2003; Mukherjee et al., 2006).

The analyses of loss-of-function mutations also indicate that the JAK/STAT pathway is required for normal cellular proliferation. The epithelial cells that make up the imaginal discs destined to form the adult fly are diploid cells, have a normal G1-S-G2-M cell cycle, and so represent a good model for analysing cellular proliferation in vivo (reviewed by Baker, 2001). Loss of *hop* leads to the under-proliferation of these imaginal disc cells, resulting in small discs (Mukherjee et al., 2005; Perrimon and Mahowald, 1986). In addition, several hypomorphic *upd* alleles display small eye phenotypes (Müller, 1930) – an effect that is also sensitive to the dose of downstream pathway genes (Mukherjee et al., 2006; Tsai and Sun, 2004).

More recently, a detailed analysis of the requirement for *stat92E* in the cellular proliferation of the wing imaginal disc has revealed an unexpected and more complex situation. By inducing mitotic recombination, heterozygous *stat92E* mutant cells can be forced to divide to generate a pair of daughter cells: one of which is homozygous mutant for *stat92E,* while the other is wild type. Given that the two daughter cells are born at the same time and in the same local environment, differences in the subsequent proliferation of these two cells can be directly compared. By analysing the growth of such clonally related cell populations at different time points during larval development, a major (although not essential) role for *stat92E* in the promotion of cell proliferation has been demonstrated during early larval development. Surprisingly, the converse occurs at later larval stages with *stat92E* mutant cells proliferating significantly more rapidly than wild type (Mukherjee et al., 2005). This changing proliferative phenotype implies that endogenous STAT92E present in wing disc cells at later stages normally functions to reduce the rate of proliferation. Even more surprising is that this endogenous anti-proliferative activity is a result of non-canonical (UPD- and HOP-independent) STAT92E activation – although ectopically induced canonical pathway stimulation is also sufficient to produce the same effect (Mukherjee et al., 2005).

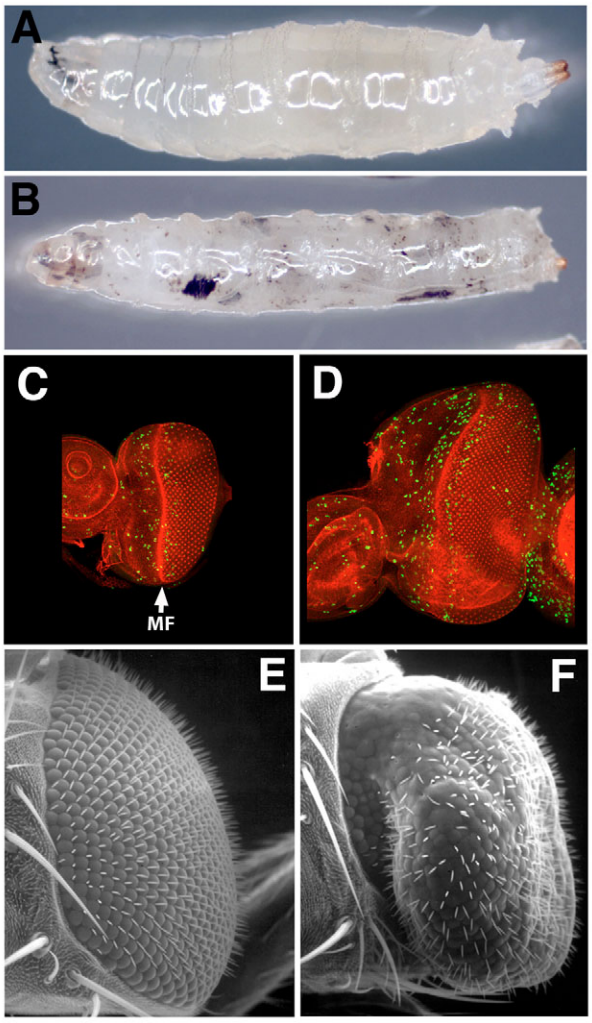

Fig. 5. JAK/STAT pathway activation leads to cellular overproliferation. (**A**,**B**) Late third instar *Drosophila* larvae: (A) wild type or (B) carrying a gain-of-function *hop* allele. Constitutively activated JAK/STAT signalling leads to the proliferation of lamellocytes, which aggregate to form prominent and, ultimately lethal, melanised tumours (dark cells). (**C**) Wild-type and (**D**) *upd*-overexpressing *Drosophila* eye imaginal discs, at the same magnification, are stained to show the structure of the disc (red) and mitotic cells (green). The morphogenetic furrow (MF) is marked, with the first and second mitotic waves visible ahead and behind it. The increased density of mitotic cells in the first mitotic wave is visible in the dorsal region of the *upd*-overexpressing disc. (**E**,**F**) Scanning electron micrographs of the adult eyes derived from (E) wild-type and (F) *upd*-overexpressing *Drosophila* showing the dorsal overgrowth of the adult eye that results from pathway mis-activation. (E) Reproduced, with permission, from Bach et al. (Bach et al., 2003).

The two findings, that STAT92E activity is both non-canonically stimulated and exhibits changing proliferative functions, are not entirely without precedent. First, mammalian STATs can also be activated non-canonically, for example by Src family kinases (Silva, 2004; Silva et al., 2003). Similarly in *Drosophila*, cellular overproliferation caused by ectopic SRC activation in the adult eye is a consequence of STAT92E stimulation (Read et al., 2004), while STAT92E activity in embryonic primordial germ cells (PGCs), also termed pole cells, is a consequence of stimulation by the receptor tyrosine kinase TORSO (Li et al., 2003). Second, although it is as yet unclear how STAT92E elicits both proliferative and anti-proliferative effects, this finding also has parallels in mammalian systems. STAT1 is known to promote cell cycle arrest and apoptosis (Stephanou and Latchman, 2005), while the activities of STAT3 and STAT5 promote pro-proliferative cellular responses (Bowman et al., 2000; Stephanou and Latchman, 2005). By contrast, the single *Drosophila* STAT-like gene can fulfil both of these functions. Whether this is a consequence of an ancestral activity subsequently assigned to distinct vertebrate STAT proteins or an example of genome ‘simplification’ in *Drosophilids* (Raible et al., 2005) is as yet unknown.

Although the activation of JAK/STAT signalling appears to be both necessary and sufficient to modulate cellular proliferation in multiple tissues, the exact mechanism by which the pathway regulates cell division is not clear. In humans, the STAT-activated expression of *cyclin D1*, which encodes a regulatory subunit of CYCD/CDK4 complex that promotes G1/S transition, and of *c-myc* (*dm* – FlyBase), which encodes a transcriptional regulator of cell cycle progression, can account for the proliferative effect of the JAK/STAT pathway (Bowman et al., 2000; Calò et al., 2003). Similarly, *Drosophila* CYCD/CDK4 and CYCE/CDK2 complexes have been reported to interact with STAT92E (Chen et al., 2003). However, these and recent observations describing JAK/STAT induced upregulation of *cycD* in the eye imaginal disc (Tsai and Sun, 2004) do not represent a complete explanation of the overproliferation phenotype. Rather, the *Drosophila* homologues of the CYCD/CDK4 complex is known to promote cellular growth with only an indirect effect on proliferation (Datar et al., 2000; Meyer et al., 2000; Tsai and Sun, 2004). Furthermore, loss of a single copy of *cycD* does not reduce the eye overgrowth phenotype caused by ectopic activation of the JAK/STAT pathway (Mukherjee et al., 2006). Thus, although links have been demonstrated, the exact mechanisms that connect the *Drosophila* JAK/STAT pathway to the cell cycle remain to be elucidated.

Immune responses

The JAK/STAT cascade was originally discovered as a cytokine-induced signalling pathway required by myeloid and lymphoid cell lineages (reviewed by Ihle, 1995). It has subsequently been shown to play a central role in orchestrating mammalian immune responses

(reviewed by Boehm et al., 1997; Hanlon et al., 2002; Trinchieri, 2003; Watford et al., 2003). Consequently, lack of pathway activity leads to defects in B- and T-cell functions, and results in severe immunodeficiencies, such as SCID (severe combined immunodeficiency) (reviewed by Schindler, 2002). An increasing amount of evidence also indicates that the pathway is required to control the innate immune response and the haematopoietic development of the fruitfly. One of the first demonstrations of this was the melanised haematopoietic tumour phenotype that results from the overactivation of the pathway (Fig. 5A,B) (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1997). In this case, tumourigenesis is caused by gain-of-function *hop* alleles, the constitutive activation of which results in the overproliferation of lamellocytes, one of the three lineages of the *Drosophila* haemocytes (Sorrentino et al., 2004).

Lamellocyte differentiation and proliferation, similar to that observed in *hop* gain-of-function mutants, is also part of the normal immune response to wounding and infection (Tzou et al., 2002). Given this similarity, a role for the endogenous JAK/STAT pathway in immune responses has also been suggested (Lanot et al., 2001; Markus et al., 2005). Indeed, while the number of circulating haemocytes is not affected, the ability to activate immune responses upon infection is largely suppressed in *hop* and *stat92E* partial loss-of-function mutants (Sorrentino et al., 2004). Consistent with this are recent studies that show STAT92E is activated in fat body cells upon immune challenge. The *Drosophila* fat body is an essential organ of the fly immune response that is the functional analogue of the mammalian liver. STAT92E activation in the fat body results in the expression of the antimicrobal peptides TotA and TotM, as well as of the *D-raf* (*phl* – FlyBase) proto-oncogene. In the case of TotA, this response can also be blocked by a dominant-negative form of DOME (Agaisse et al., 2003; Boutros et al., 2002; Dostert et al., 2005; Kwon et al., 2000). Interestingly, this fat body specific activation of JAK/STAT signalling requires *upd3*, but not *upd* or *upd2*. *upd3* is expressed by circulating haemocytes at the site of septic injury, with the cytokine functioning non-autonomously to activate the pathway in fat body cells throughout the larva (Agaisse et al., 2003). Further evidence also suggests a role of the *Drosophila* JAK/STAT pathway in mediating an innate antiviral response. Transcription of *vir-1* (*virus-induced RNA 1*), an as yet uncharacterised protein that is expressed upon viral challenge, may be STAT92E dependent, as STAT92E binds to the *vir-1* promoter in vitro. Moreover, *hop* mutants are hyper-sensitive to viral infection (Dostert et al., 2005). Consistent with this, studies in the mosquito *Anopheles gambiae* (*Ag*) also implicate the JAK/STAT pathway in the immune response, with the activation of *Ag*-STAT occurring in cell culture and in cell lysates obtained from immune-challenged adult individuals (Barillas-Mury et al., 1999).

Taken together, it appears that key roles for the JAK/STAT signal transduction cascade in the development of the immune system, and the signalling required to activate it, have been conserved during evolution. Although the details and cell types involved in *Drosophila* and mammals differ, analysis of the promoters of the putative STAT92E target genes represents a future direction for studies into the immune functions of JAK/STAT signalling.

Primordial germ cells, stem cells and JAK/STAT signalling

The identification, maintenance and potential use of pluripotent stem cells is a field of considerable potential significance for human health. As such, research into stem cell biology has attracted considerable interest over recent years. In particular, a thorough understanding of the cellular environment (the so-called stem cell niche) in which stem cells are maintained in vivo represents an essential prerequisite for such efforts and, as a result, the niches required by stem cells in vivo are increasingly being studied in *Drosophila*. Although several signalling pathways, including the Wnt and BMP pathways, have been shown to be repeatedly required to define stem cell niches in both mammals and flies (reviewed by Li and Xie, 2005), the role of JAK/STAT pathway signalling in this process is especially pertinent for this review (Rao, 2004; Varga and Wrana, 2005). One of the first indications for such a requirement is derived from experience in cell culture, which has shown that stimulation of mouse STAT3 by LIF (leukaemia inhibitory factor) and other interleukin 6-family cytokines is required to maintain the undifferentiated status of murine embryonic stem (ES) cells ex vivo (Boeuf et al., 1997; Hao et al., 2006; Humphrey et al., 2004; Niwa et al., 1998). Although stimulation by STAT3 does not appear to be sufficient to support the self-renewal of human ES cells (Daheron et al., 2004; Sato et al., 2004), it is possible that the activity of other STATs may be required by human ES cells.

The JAK/STAT pathway is also essential for stem cell maintenance in *Drosophila*. At early stages of *Drosophila* embryogenesis, the zygotic nuclei that migrate to the posterior pole of the embryo give rise to PGCs, termed pole cells, which require JAK/STAT signalling at multiple stages. During the early blastoderm stage, pole cell proliferation requires the activation of STAT92E, which is mediated by the receptor tyrosine kinase TORSO (Li et al., 2003). Later, 6-7 hours after egg laying, STAT92E activity is necessary for the pole cells to migrate through the wall of the hindgut and towards the embryonic gonads (Li et al., 2003). After ~12 hours, the pole cells use guidance cues, probably supplied by the localised expression of JAK/STAT pathway ligands, to coalesce and form the embryonic gonads (Brown et al., 2006; Li et al., 2003).

Although it is not entirely clear whether the proliferation and migration of PGCs requires JAK/STAT pathway activity in both sexes, a specific role for JAK/STAT pathway signalling in embryonic male gonads has also been established. In particular, a recent report has shown that *upd* is expressed in somatic cells of the embryonic testis, where it induces the phosphorylation of STAT92E specifically in the male germ cells (Wawersik et al., 2005). This pathway activation results in the expression of the male-specific markers *mgm-1*, *dpa* and *mcm5*. Blocking the activity of the pathway by removing zygotic STAT92E in the male germ cells results in the loss of marker expression, while activation of the pathway in female germ cells induces STAT92E activity and masculinisation (Wawersik et al., 2005).

A key role of JAK/STAT signalling for the maintenance and proliferation of the stem cells within the gonads of both sexes has also been demonstrated during adult life. In adult *Drosophila* males, self-renewing germline stem cells (GSCs) are arranged around a small group of *upd*-expressing somatic cells at the apical tip of each testis, called the hub (Fig. 6A) (Fuller, 1998). The polarised division of stem cells produces one self-renewed stem cell proximal to the hub and one distal gonialblast committed to differentiation (Fig. 6A).

It has been proposed that JAK/STAT signalling in GSCs is required to maintain stem cell state and/or proliferation, a suggestion strongly supported by the expression of UPD in hub cells (Fig. 6B). In this model, the more distal daughter cell arising from the division of GSCs is further away from the source of pathway ligand (Fig. 6A) and so is no longer exposed to sufficiently high levels of stimulation.

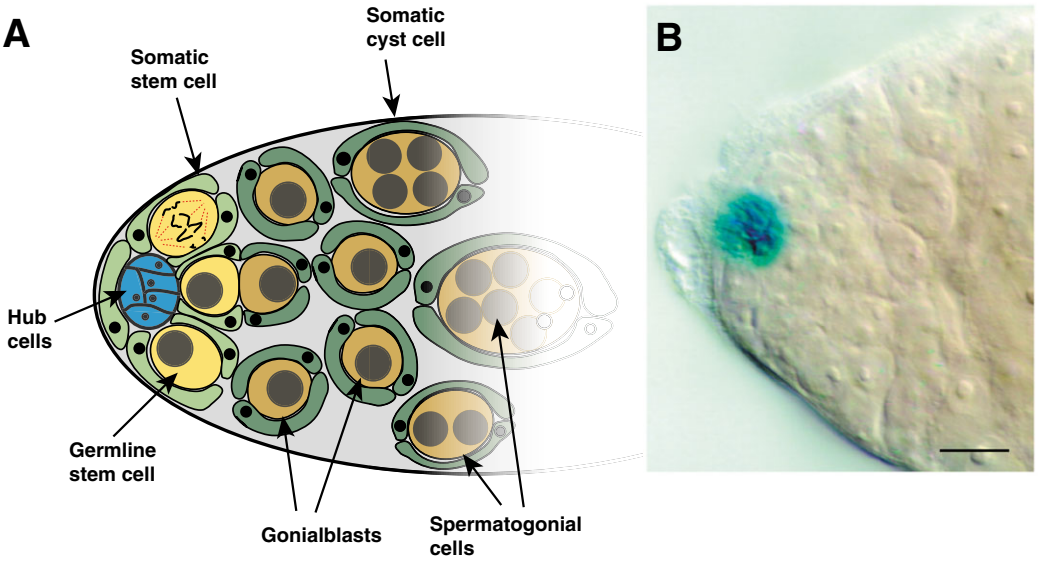

Fig. 6. The stem cell niche at the tip of the *Drosophila* testis. (**A**) A small group of somatic hub cells (blue) are located at the tip of the *Drosophila* testis and express the UPD ligand and so maintain the fate of adjacent stem cells (yellow). As the germline stem cells divide (top), daughter cells are born (middle), with one remaining adjacent to the hub and maintaining stem cell fate, and the other beginning to differentiate towards the gonialblast and spermatogonial fate (dark yellow). Somatic stem cells (light green) are also present that give rise to the cyst cells (dark green), which encase the differentiating spermatogonia. (**B**) An enhancer trap in the *upd* locus mirrors the hub cell-specific expression of *upd* at the testis tip. Scale bar: 10 μm. Reproduced, with permission, from Tulina and Matunis (Tulina and Matunis, 2001).

Consistent with this, analysis of *hop* and *stat92E* mutants reveals that mutant testes lose their GSCs during larval development, although mutant spermatogonia are able to differentiate normally into sperm (Brawley and Matunis, 2004; Kiger et al., 2001; Tulina and Matunis, 2001). Conversely, ectopic expression of *upd* results in the expansion of GSCs at the expense of differentiating spermatogonia (Kiger et al., 2001; Tulina and Matunis, 2001). Strikingly, one report has indicated that spermatogonia, which have initiated differentiation following a decrease in JAK/STAT signalling, can repopulate the niche after restoration of appropriate signalling, implying that pathway activity may also be sufficient to 'de-differentiate' cells in this particular environment (Brawley and Matunis, 2004).

Analysis of JAK/STAT signalling in the female *Drosophila* ovary have also identified several, apparently independent, requirements for pathway signalling. For example, border cell migration, maintenance of epithelial polarity in the follicle cells and the development of the polar/stalk cells are all disrupted by a loss of pathway signalling (Baksa et al., 2002; Ghiglione et al., 2002; McGregor et al., 2002; Silver and Montell, 2001). More specifically related to stem cell maintenance is a recent description of somatic 'escort stem cells' and germline stem cells present at the very earliest stages of oocyte development (Decotto and Spradling, 2005). The maintenance of the escort stem cells and the development of the oocyte have also been shown to depend on the activity of the JAK/STAT pathway, and although the precise interactions between escort and germline stem cells remains to be determined, it appears that the pathway is required for the maintenance of at least a subset of male and female stem cells in vivo (Decotto and Spradling, 2005).

Conclusions and perspectives

Comparative analysis of data published over recent years shows that the JAK/STAT pathway has been conserved during evolution, not only at the level of its structural components but also functionally. However, despite the relative simplicity of the *Drosophila* JAK/STAT pathway, it still fulfils many of the same functions observed in vertebrates.

Given this degree of inter-species conservation, the systematic exploitation of the genetic strengths inherent in the *Drosophila* system represents a particularly valuable approach for the future. Indeed, genetic and genome-wide RNAi screens that have already been undertaken (Box 1) have almost certainly identified pathway components, regulators and interacting genes whose human homologues fulfil similar roles in vivo. Indeed, an essential task for the near future will be to identify these human orthologues from amongst the genetic 'noise' inherent in any screen.

After the identification of the orthologous genes, one approach that will undoubtedly prove to be helpful for the further characterisation of both human and *Drosophila* pathway regulators is the analysis of common roles in the conserved pathway functions of stem cell maintenance, immunity and cellular proliferation. For example, the JAK2 mutations recently identified as being responsible for human polycythema vera have been known to produce leukaemia-like haemocyte overproliferation in *Drosophila* for almost 10 years (Luo et al., 1995; James et al., 2005).

In the longer term, it is to be hoped that both the *Drosophila* and mammalian JAK/STAT fields can recognise and exploit their inter-species synergies to use the strengths of both systems to determine the identify and functions of the key regulators of this medically important signal transduction pathway.

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