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Review

Identification of JAK/STAT pathway regulators—Insights from RNAi screens

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ABSTRACT

While many core JAK/STAT pathway components have been discovered in *Drosophila* via classical genetic approaches, the identification of pathway regulators has been more challenging. Recently two cell-based RNAi screens for JAK/STAT pathway regulators have been undertaken using libraries of double-stranded RNAs targeting a large proportion of the predicted *Drosophila* transcriptome. While both screens identified multiple regulators, only relatively few loci are common to both data sets. Here we compare the two screens and discuss these differences. Although many factors are likely to be contributory, differences in the assay design are of key importance. Low levels of stimulation favouring the identification of negative pathway regulators and high levels of stimulation favouring the identification of positively acting factors. Ultimately, the results from both screens are likely to be largely complementary and have identified a range of novel candidate regulators of JAK/STAT pathway activity as a starting point for new research directions in the future.

Contents

1. Background

1.1. The JAK/STAT pathway

The JAK/STAT signal transduction pathway has been conserved through evolutionary time and, while most intensively studied in vertebrate models, a 'core' signalling pathway with homologues of the main pathway components is also present in the fruit fly

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Drosophila melanogaster. These include a trans-membrane receptor termed Domeless (Dome) with structural homology to members of the vertebrate IL-6R family including LIFR and gp130 [1,2], a receptor-associated Janus Kinase (JAK) termed Hopscotch (Hop; [3]) and a Signal Transducer and Activator of Transcription (STAT) termed STAT92E [4,5] (Fig. 1). Three secreted ligands termed Unpaired (Upd), Unpaired 2 (Upd2) and Unpaired 3 (Upd3) have also been identified and shown to have both distinct and overlapping functions in a range of developmental processes [6–8], although they have only limited homologies to vertebrate STAT pathway ligands.

In addition to these core components, a number of putative JAK/STAT pathway regulators have also been identified in *Drosophila* on the basis of their homology to genes originally characterised in vertebrates. These include members of the suppressors of cytokine signalling (SOCS) family and a protein inhibitor of activated STAT (PIAS) gene. In *Drosophila* the three *SOCS*-like genes *socs36E*, *socs16D* and *socs44A* are found. Of these, the homologue to human SOCS5, SOCS36E, is best characterised and is both a pathway target gene and negative regulator of pathway activity [9,10]. A *Drosophila* PIAS homologue has also been identified and shown to negatively regulate JAK/STAT signalling [11]. More recently, PIAS proteins have been shown to function as E3 SUMO ligases [12]. Alleles of *Su(var)2–10*, a class of mutations originally identified as suppressors of eye colour variegation in *Drosophila*, and proposed to be regulators of chromatin structure, have also been identified as allelic to *dPIAS* [13]. Although detailed analysis is still needed, PIAS-group proteins may therefore represent more general regulators of transcription [14,15].

The conservation of the core JAK/STAT pathway components, through the ~500 million years that have elapsed since the last common insect/vertebrate ancestor, probably reflects essential roles played by the pathway during development and homeostasis. For example, physiological roles for the pathway in innate immune responses [16,17], haematopoiesis [18–20], stem cell maintenance [21–23] and cellular proliferation [24,25] have all been conserved in both *Drosophila* and mammalian systems (reviewed in [26]).

Given the fundamental conservation of JAK/STAT pathway signalling at both the component and functional levels, *Drosophila* represents a promising model system to identify novel pathway components and regulators. The fruit fly *Drosophila melanogaster* is a widely used, developmentally well-characterised and genetically tractable organism. Extensive collections of both transposon-mediated and 'classical' mutations are available from stock centres [27,28] which, in combination with the detailed annotation of the sequenced genome [29,30], allows the rapid analysis of gene products and the elucidation of their roles during development. In particular, the relatively low complexity of the *Drosophila* genome results in low levels of redundancy so allowing the terminal phenotypes associated with the removal of a gene of interest to be determined and analysed.

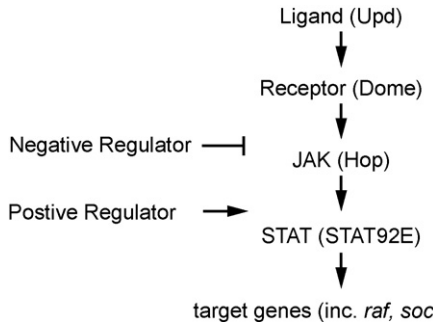

Fig. 1. Outline of the JAK/STAT signalling pathway indicating the principal core pathway components in *Drosophila* as well as the two pathway target genes used to generate pathway responsive reporter constructs (see text for details).

1.2. Screening approaches to identify novel JAK/STAT regulators

The use of *Drosophila* as a tool in the analysis of JAK/STAT signalling was initially facilitated by a number of genetic screens undertaken to identify genes required for segmentation of the larval cuticle (Fig. 2A). One particular phenotypic class, initially characterised as an 'atypical gap gene' [31,32], was subsequently shown to result from the disruption of JAK/STAT pathway components. Mutations in the genes of the pathway ligand Unpaired [31], the Domeless receptor [1], the Janus Kinase Hopscotch [33] and STAT92E [4] were all originally identified on the basis of this distinctive larval cuticle phenotype (Fig. 2B). This phenotype includes the deletion or fusion of the denticle belts secreted by abdominal segments *a4* and *a5*, reduction in the *a8* denticle belt, defects in the structures that make up the posterior spiracles as well as aspects of the head skeleton (labelled in Fig. 2B). Indeed, traditional genetic screening approaches have succeeded in identifying the core components of the canonical *Drosophila* JAK/STAT pathway largely on the basis of these phenotypes. While powerful, this approach is however limited as genes with multiple roles in different processes, regulatory functions in specific tissues or functions required for oocyte and early embryonic development are unlikely to be recovered in this way.

One alternative to the screens already undertaken are F1 genetic interaction screens (reviewed in [34]). In this approach, traditional 'forward' genetic techniques are used to generate mutations in which a reduction in gene dosage leads to a visible interaction with a dominant phenotype. One such experimental design, termed *GMR-upd*, has been established to screen for modulators of JAK/STAT signalling and is based on the dominant eye overgrowth phenotype caused by constitutive misexpression of the Unpaired ligand in the developing eye. While JAK/STAT pathway activity is required for normal *Drosophila* compound eye development (Fig. 2C) [25,35,36], constitutive high-level Upd expression leads to over-proliferation of undetermined cells within the future eye which subsequently differentiate to produce an overgrown adult eye (compare Fig. 2C and D) [25].

A key characteristic of the F1 genetic screen is the fact that reducing the gene dose of known JAK/STAT pathway components is sufficient to reduce the eye overgrowth induced by *GMR-upd*. This results in adults heterozygous for genes of interest with eyes that are less severely overgrown than controls (compare Fig. 2D and E). By screening for this genetic interaction, mutations in previously unknown pathway regulators can be identified and ultimately identified. Two large-scale screens have been undertaken using this approach, and have both identified genes and genomic regions that modulate the activity of the pathway *in vivo* [25,37]. One validation of this approach is the subsequent characterisation of the *ken and barbie* (*ken*) locus [38]. *ken* mutations were originally identified as moderate enhancers of eye overgrowth [25] and shown to encode a transcriptional repressor. *In vivo*, Ken appears to competitively bind to a DNA sequence that largely overlaps that recognised by the STAT92E transcription factor to selectively modulate the transcriptional response to pathway stimulation [38]. As such Ken, and possibly also its human homologue BCL6, represents a novel class of JAK/STAT pathway regulators acting at the DNA level to specifically modulate a subset of the pathway-induced transcriptome.

1.3. Reverse genetic screening approaches

While traditional 'forward' genetic approaches have successfully identified both core JAK/STAT pathway components and pathway

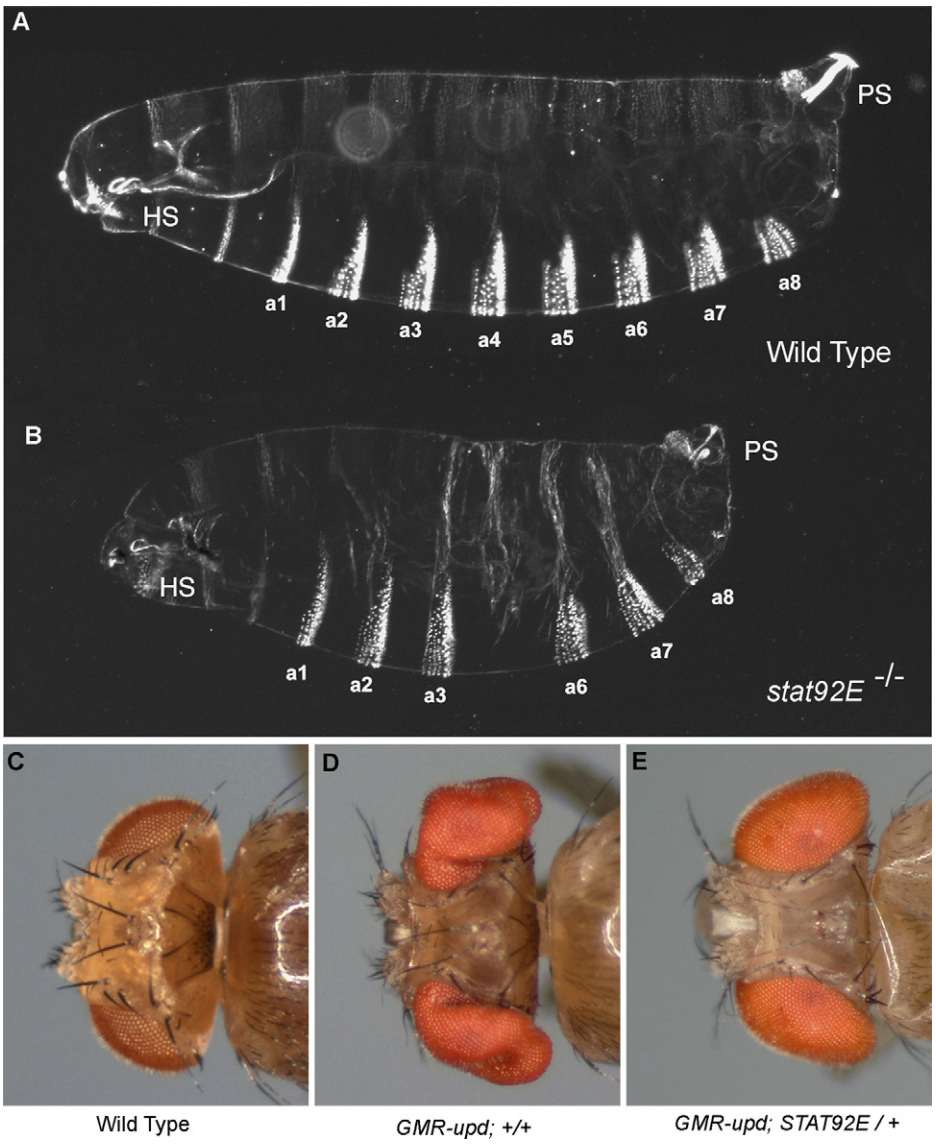

Fig. 2. Genetic screens for JAK/STAT pathway components. Wild type (A) and *stat92E* mutant (B) larval cuticles showing the distinctive defects associated with mutations in all core JAK/STAT pathway components. These include loss of the a4 and a5 denticle belts, disruption of a8 denticle belt and the head skeleton (HS) as well as a reduction in the posterior spiracles (PS). The *stat92E*^{06346} mutant shown has a strong segmentation phenotype but a PS reduction less severe than a full pathway null, possibly due to paternal rescue. The wild type eye of adult *Drosophila* (C) is significantly overgrown in a *GMR-upd* background in which the Unpaired ligand is expressed during eye development (D). However, removal of one copy of STAT92E is sufficient to reduce the degree of overgrowth (E) and indicates its role as a regulator of JAK/STAT pathway signalling.

regulators, these approaches have limitations. A powerful alternative is the use of 'reverse' genetics. In reverse genetic approaches, the activity of defined genes are reduced and the resulting phenotypes analysed. In such an approach, widely used in mouse knock out techniques, a gene of interest is targeted for mutation and the resultant mutant individuals examined for defects. Although mutation of endogenous loci via homologous recombination mechanisms has been demonstrated in *Drosophila* [39], it is still relatively cumbersome to undertake large scale gene knock-out in *Drosophila*, especially by comparison to other forward genetics approaches.

An alternative reverse genetic screening approach uses RNA interference (RNAi), a widely used tool to silence gene expression both in cultured cells and *in vivo*. First discovered in *C. elegans* [40], it was shown that silencing by RNAi relies on an ancient cellular response to infection by double-stranded RNA viruses and can be used to down-regulate gene expression in all metazoa (reviewed in [41]). Indeed, injection of double-stranded (ds) RNAs into *Drosophila* embryos or expression of hairpin dsRNAs from transgenes have both been shown to be effective *in vivo* [42–44]. Intriguingly, it was subsequently demonstrated that the addition of long dsRNA to cultured *Drosophila* cells is sufficient to trigger a potent down-regulation of target messages [45]. As invertebrates do not mount an innate cellular response against double stranded RNA viruses, cells do not respond to the dsRNA itself, but rather dice such molecules intracellularly to generate a diverse pool of small interfering RNAs (siRNAs).

Since the discovery of the long dsRNA effect in *Drosophila* cells, whole genome RNAi-based screens in cultured cells have been undertaken to study a broad range of cellular phenotypes, from simple life–death decisions to the study of signalling pathways and changes in cell shape [46–48]. Such experiments were enabled by large-scale libraries of dsRNAs that can be easily synthesised using gene-specific primer pairs to amplify templates for *in vitro* transcription. These libraries often consist of more than 20,000 elements covering a large proportion of all predicted protein coding genes. More recently, transgenic libraries of hairpin RNAi constructs

stably integrated into individual *Drosophila* stocks have become available [49]. These can be expressed in any tissue using the heterologous GAL4/UAS system [50], potentially enabling large-scale *in vivo* RNAi screening studies.

Since the transcript targeted is known, RNAi approaches can be used to rapidly generate lists of candidate genes involved in a particular process. However, this versatility comes at the cost of a certain 'fuzziness' of RNAi phenotypes. Given the high-throughput nature of a genome-wide RNAi screen, it is not possible to confirm the efficiency or degree of target transcript knock down in every case. Similarly, proteins that are very stable and which cannot be depleted within the time-frame of a given experiment may not be reduced to levels where their knock-down becomes phenotypic. In addition, gene families that have very homologous sequences, such as Histones, are frequently inhibited by the same dsRNAs and specific knock down of different isoforms of a single gene is usually not possible. More significantly, since the RNAi mechanism relies on 19–21mer siRNAs, even a diluted but highly efficient matching siRNA has the potential to cause 'off-target' silencing of unintended genes. Other elements, such as low-complexity regions encoding repeated amino acid motifs common to multiple transcripts have also been shown to induce silencing of unrelated genes [51,52].

Given these considerations, it is clear that both false negatives and false positives can arise in any genome-wide RNAi screening strategy. It is therefore important to keep in mind that hit lists from primary RNAi screens are only candidate modifiers that have to be confirmed by independent approaches.

2. RNAi screens for JAK/STAT regulators

In recent years, the technology of large-scale RNAi screening has been applied to the analysis of the JAK/STAT signalling pathway, and two genome-wide RNAi screens have been undertaken in *Drosophila* tissue culture systems. The first, undertaken jointly in the Boutros and Zeidler laboratories in Germany [53] and the second by the Perrimon laboratory in the US [54]. These screens will subsequently be referred to as the Müller and Baeg screens, respectively. In both screens, *Drosophila* tissue culture cells were transfected with reporter constructs engineered to drive expression of a firefly luciferase gene under the control of a STAT-dependent promoter (Fig. 3A). Although both groups used essentially the same library of dsRNA molecules, they used different pathway stimulation conditions, different cell lines and different reporters (see Table 1 for a description of the principal differences between the two screens). However, both groups set out to use genome-wide screening technology to identify the key factors regulating *Drosophila* JAK/STAT signalling in a relatively simple tissue culture system. Indeed, both groups were successful in identifying a considerable number of genes whose knock-down produced robust and reproducible interactions not only in cultured cells, but also *in vivo* [53,54]. In particular, the core pathway components *dome*, *hop*, *stat92E* and *socs36E* were identified in both screens, as was the protein tyrosine phosphatase *ptp61F*, a gene that appears to play an important role in the negative regulation of pathway activity *in vivo* [53,54].

However, despite the similar goals and apparently similar approaches of both screens, the total overlap in the published lists generated by both groups is surprisingly low—in the range of 5–6% (6 of 121/90, respectively). An initial comparison of the two data sets suggests that this relatively low level of overlap may result from a fundamental difference in the nature of the regulators identified in each screens. While 73% of the genes identified in the Müller screen are positive regulators of pathway activity, over three quarters of the genes identified by the Baeg screen represent putative negative regulators. Although some of these differences may be the result

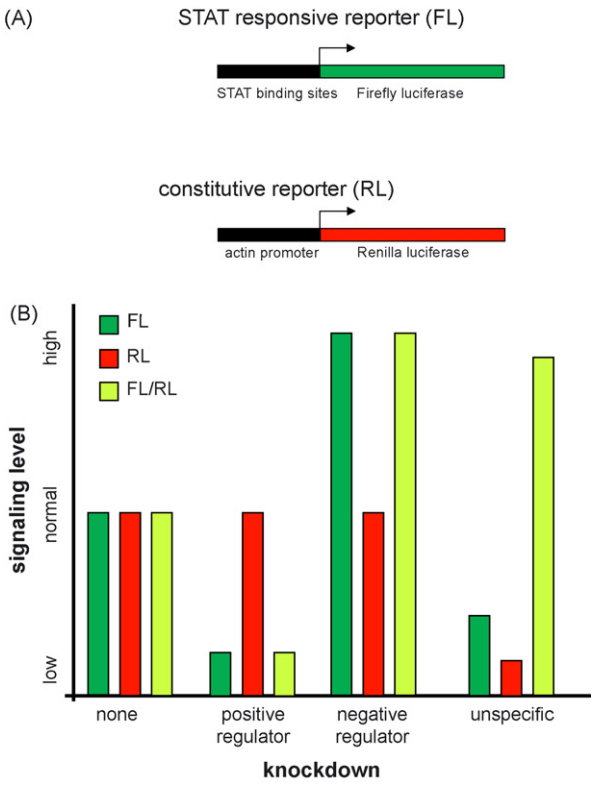

Fig. 3. Outline of the RNAi assay approaches used by both Baeg and Müller whole genome screens. (A) STAT responsive (FL) and constitutive (RL) reporter constructs used. (B) Hypothetical responses of both reporters to treatment by dsRNAs that target a non-interacting gene (non), a positive regulator, a negative regulator and a non-specific dsRNA that reduces cell viability.

of normalisation artefacts (see below and Table 2) it is likely that alternative assay designs and different levels of pathway stimulation may be responsible for much of this bias. These possibilities are discussed in greater detail below.

2.1. The Müller et al. JAK/STAT RNAi screen

The Müller screen used the haemocyte-like *Drosophila* Kc_{167} cell line, which is derived from cells in which the JAK/STAT pathway is active *in vivo* [19]. In order to sensitise the system, we co-transfected the cells with the dsRNA transporter SID-1, which had been shown to enhance the uptake of RNAi [55]. The JAK/STAT pathway reporter was based on the promoter of a previously published JAK/STAT target gene, *raf* [56], driving the transcription of the firefly luciferase gene (FL). To increase sensitivity, we multimerised the STAT binding sites in this promoter element to generate a reporter with 12 STAT binding sites, which was termed *p6x2xDrafLuc*. To induce the pathway, cells were transfected with a plasmid coding for the Upd ligand, generating reporter activity ~70-fold over basal levels. In order to monitor effects of dsRNAs on cell viability, we also transfected a reporter, in which the *Renilla* luciferase (RL) was constitutively expressed under the control of an *actin* promoter (Fig. 3A).

Having generated a reporter system, we showed that reporter activity can be specifically modulated by RNAi mediated knockdown of JAK/STAT pathway components, while remaining unaffected by knockdown of other signalling pathways. Using this system, two parallel genome-wide screens were undertaken using a library of 20,026 dsRNAs targeting approximately 91% of all annotated *Drosophila* genes [57,58]. Five days after treatment with dsRNAs, cells were lysed and the activities of the STAT92E reporter

Table 1
Comparison of JAK/STAT RNAi screens

Experimental condition	Baeg	Müller
Biological differences		
Pathway stimulation	Endogenous Upd2	Ectopically expressed Upd-GFP
Cell line	S2-NP	Kc_{167}
Screening procedure		
Coverage of library	21,300 dsRNAs	20,026 dsRNAs
Screening reporter	*10xStat92E-luciferase* (FL) containing five tandem repeats of a 441bp fragment from the *socs36E* enhancer (each with four potential Stat92E binding sites)	*p6x2xDrafLuc* containing six repeats of a 165 bp fragment from the *raf* promoter (each with two Stat92E binding sites)
Co-reporter	*pAct-RL*	*pAct-RL*
RNA concentration per well	80 ng/well	500 ng/well
Cells seeded per well of a 384-well plate	40,000	15,000
Transfection of reporter	Per well	In batch
dsRNA uptake	Transfection	Bathing + SID-1 dsRNA trans-porter
Time for RNAi	4 days	5 days
Replicate datasets	Two	Two
Data processing		
Data normalisation	Fold SD from the plate mean of FL/RL ratio for each plate	Fold MAD from the plate median of FL channel for each plate
Selection of positive regulators	<2 SD below plate mean	<2 MAD below plate median
Selection of negative regulators	>3 SD above plate mean	>2 MAD above plate median
Exclusion of genes	Genes not annotated by BDGP, ribosomal proteins, proteins involved in RNA processing and translation	Previously published cell viability modifiers, treatments with high variability between replicates, treatments with *z*-scores >2 or <2 in the RL channel, genes with phenotypes in other screens
False-positive rate (determined by re-screens of primary hits)	29%	15%
Human homologs of hits	73%	74%

Table 2
Comparison of common results identified in different JAK/STAT modulator screens

Gene		Baeg (FL/RL)	Müller (FL)	Müller (FL/RL)	Human homologue
dome	*	–	–	–	gp130[a]
hop	*	–	–	–	JAK2[b,d,g]
stat92E	*	–	–	–	STAT5[c,d,g]
cdc2	*	–	–	–	CDC2[d,g]
CG11700	*	–	–	NC	UBC[h]
CG17836	*	–	–	–	None
apt		+	+	+	None
brm	*	+	–	–	SMARCA2[g]
CG15563		+	–	NC	None
CG30460		+	–	NC	C8ORF70[g]
enok	*	+	+	+	MYST3[d,g]
hsc70-4		+	–	NC	HSPA8[g]
ken	*	+	–	NC	BCL6[e]
mbl		+	–	NC	MBNL1[d,g]
mor	*	+	–	–	SMARCC2[d,g]
proct		+	–	NC	None
ptp61F	*	+	+	+	PTPN1[d,g]
samuel		+	–	+	SAMD11[h]
socs36E	*	+	+	+	SOCS5[f]
ssdp	*	+	+	NC	SSBP3[d,g]

Data from Baeg et al. ('Baeg') [54] and from Müller et al 2005 ('Müller') [53].
Minus symbols (–) indicate down-regulation, plus symbols (+) indicate up-regulation and NC indicates no change of the pathway reporter after RNAi according to the normalisation approach indicated in the respective columns. FL indicates changes for the pathway reporter, while FL/RL indicate changes of the pathway reporter after normalization to a co-reporter.
* indicates that the interaction was confirmed by an independent dsRNA [61].
[a] Domains in the Dome protein have most homology with the IL-6R family of receptors, including gp130 and LIFR [1].
[b] Binari and Perrimon [3]
[c] Hou et al. [4].
[d] Human homologene.
[e] Arbouzova et al. [38].
[f] Karsten et al. [10].
[g] Inparanoid.
[h] Best reciprocal BLAST search.

(FL) and control plasmids (RL) determined. To calculate the effect of each dsRNA treatment, the median of FL and RL activities were calculated independently for each plate and *z*-scores determined by calculating how many median absolute deviations (MAD) each dsRNA differed from the plate median [53]. To identify those dsRNAs that specifically modulated the reporter system, we selected dsRNAs with a significant *z*-score in the FL channel which did not have a significant *z*-score in the co-reporter RL channel (schematically shown in Fig. 3B). To further increase the specificity of the screen, hits were also filtered against genes previously identified in other available genome-wide RNAi data sets.

Having identified a preliminary list of pathway modulating dsRNAs, we then sought to exclude potential artefacts arising from the use of the multimerised reporter. The putative interactors were therefore re-tested using a reporter in which FL activity was driven by a non-multimerised promoter from the JAK/STAT target gene *socs36E*. Ultimately, re-screening and secondary screens confirmed the effect of 91 dsRNAs targeting 90 genes including the previously identified pathway components *dome*, *hop stat92E* and *socs36E*. Of these, approximately one third were negative regulators and two thirds positive regulators of JAK/STAT pathway activity.

Finally, the cell culture model was used to undertake epistasis experiments to further categorise positively acting factors. To identify dsRNAs that putatively act in the processing of the ligand or in the binding to the receptor, we compared reporter activities of cells expressing the Upd ligand to reporter activities of cells stimulated with Upd conditioned medium. To identify dsRNAs that may act upstream or downstream of JAK, we further induced pathway activity in dsRNA-treated cells using expression of the constitutively active JAK allele $hop^{Tum\text{-}L}$ and compared the results to the treatments with Upd ligand. These experiments indicated that most of the positive regulators identified act downstream of JAK [53].

2.2. The Baeg et al. JAK/STAT RNAi screen

In a second RNAi-based screen for JAK/STAT signalling modulators Baeg and colleagues generated a luciferase-based reporter containing multimerised STAT92E binding sites present within the *socs36E* enhancer [54,59]. As expected, this reporter, termed *10xStat92E-luciferase* (which actually contains 20 potential STAT92E binding sites), reflects JAK/STAT signalling activity and was used in conjunction with a Schneider cell line [60] derivative, S2-NP [54]. Intriguingly, S2-NP cells appear to express sufficient levels of endogenous pathway ligand to activate signalling. However, while Upd is the major JAK/STAT pathway ligand *in vivo* [7], RNAi knockdown suggests that it is the closely related Upd2 ligand that activates the pathway in S2-NP cells [54]. To compensate for potential differences in transfection efficiency, the *10xSTAT92E-luciferase* reporter activity was normalised to the activity of the co-transfected co-reporter *Act-RL*. Using this assay system, S2-NP cells were transfected with *10xSTAT92E-luciferase* reporter (FL) and *Act-RL* co-reporter (RL) along with individual dsRNAs on a well-by-well basis with reporter activities determined 4 days later. For candidate selection, the plate averages for the ratios of reporter to co-reporter (FL/RL) were calculated and phenotypes expressed as the fold standard deviation (SD) from the mean of each given plate. Genes not annotated by BDGP, genes encoding ribosomal proteins and genes coding for proteins involved in RNA processing and translation were excluded from retesting as were genes affecting the *actin* promoter of the co-reporter.

After retesting of the remaining candidates, Baeg and colleagues identified 121 novel genes that regulate JAK/STAT signalling in *Drosophila* including the previously identified JAK/STAT pathway components *upd2*, *dome*, *hop*, *stat92E* and *socs36E*. By stimulating the cells with Upd, Baeg and colleagues further found that 27 of 29 positive regulators had similar phenotypes following activation by both Upd and Upd2, indicating that these are regulators of a core pathway for which stimulation with different ligands has no influence. Of the 29 positive regulators tested, 13 (including *dome* and *hop*) affected phosphorylation levels of STAT92E. Furthermore, using a microscopic assay for nuclear translocation of STAT92E, they found homologs of *ranBP3* and *ranBP10* to be involved in nucleocytoplasmic shuttling of STAT92E.

In 2007, Baeg and colleagues further re-assessed their 121 hits using independent dsRNAs to exclude potential off-target effect induced artefacts [61]. Using the initial dsRNAs present in the genome-wide screen, 111 of 121 (92%) of the hits could be confirmed with 50 of these 111 (i.e. 41% of the initial hits) re-confirmed using multiple independent dsRNAs.

3. Differences between the Baeg and Müller screens

One fundamental question arising from a comparison of the Baeg and Müller RNAi screens is why only relatively few genes are common to both lists of validated pathway regulators. Genes central to JAK/STAT signalling were identified in both screens suggesting that both approaches are robust enough to identify primary pathway components and regulators such as *socs36E* and *ptp61F*. However, a majority of the genes identified by each screen was not found in the other representing a population of 'orphan' regulators identified only under one set of screening conditions. Ultimately, differences in the results obtained in both screens are likely to arise from either biological differences in the screening systems, or experimental differences inherent in the RNAi approach itself.

3.1. Biological differences

The independent design and execution of the Baeg and Müller JAK/STAT RNAi screens has resulted in a number of biological differences between the two strategies including differences in the cells used, the reporter and the method of pathway stimulation.

One obvious biological factor are differences in the transcriptomes of the two cell lines independently derived from the same organism. Indeed, comparison of the transcriptome of Kc_{167} and S2 cells (the parental cells from which the S2-NP cells used in the Baeg screen were derived) suggests that 5567 of the probe sets present on the Affymetrix *Drosophila* Genome 1 chip were called present in S2 cells (40%) while 5773 are expressed in Kc_{167} cells (41%). In total, 5214 genes were expressed in both Kc_{167} and S2 (6126 expressed in either) giving an overlap in the present call between Kc_{167} and S2 cells of 85% (D. Sims, B. Baum, personal communication). Differences in the absolute levels of gene expression between cell lines as well as low level gene expression insufficient to register as 'present' using Affimetrix technology would further increase the effective differences in transcription between the cell lines used.

Although transcriptome data for the S2-NP cell line itself is not available, its parental S2 strain, as well as Kc_{167} cells, show relatively low levels of *upd* and higher levels of *stat92E* expression (data retrieved from the FLIGHT database [62]). Consistent with the low levels of *upd* in Kc_{167} cells, basal levels of pathway induction in the Müller screen were very low in the absence of ectopic ligand [53]. By contrast, S2-NP cells appear to express *upd2* at a level sufficient for at least moderate levels of pathway induction [54]. Although the functional consequences of the different expression levels of both pathway components and regulators are unclear, it seems likely that differing transcription profiles may at least partly account for the orphan regulators identified by the two screens.

A further source for potential differences between the two screens lies in the reporters used. While the Baeg screen used a reporter based on the *socs36E* target gene, the Müller screen used a variant of the STAT92E dependent site previously identified within the *raf* promoter [56]. Although the regulation of *Drosophila raf* expression includes a role for STAT92E, *raf* is expressed in many tissues throughout development and is likely to be under the control of a number of transcriptional regulators. By contrast, *socs36E* is one of the strongest JAK/STAT pathway target genes *in vivo* and in cultured cells (MPZ, unpublished). In addition, its expression appears to be almost exclusively dependent on JAK/STAT pathway activity with an embryonic expression pattern closely matching that of Upd itself [10]. As such, it is possible that a reporter derived from the *raf* promoter may exhibit requirements for co-factors or other signalling cascades that would not influence a reporter based on the *socs36E* promoter. Ultimately, both groups used reporters containing multimerised versions of their respective STAT92E response elements, taken out of chromosomal context and engineered into a transiently transfected plasmid for their primary screens. However, it should be noted that the Müller screen retested all potential interacting dsRNAs using a second, non-multimerised *socs36E* derived reporter [53] and should have therefore excluded multimerisation artefacts on this basis (see Table 1).

Probably one of the most significant biological differences between the Baeg and Müller screens is the mechanism of pathway activation employed. In the Müller screen, a plasmid constitutively expressing the primary *in vivo* pathway ligand Upd was co-transfected with the reporter before treatment with RNAi. This transfection occurred 5 days before the ultimate readout and resulted in prolonged high-levels of pathway stimulation. By contrast the Baeg screen relied on the endogenous expression of Upd2 in S2-NP cells as a pathway stimulus, resulting in an equally prolonged, but lower level, of pathway stimulus. Because of these differences in stimulation, high levels of firefly luciferase in the Müller screen resulted in knock down of positive pathway regulators being readily identified while the available 'headroom' of increased signalling caused by the knock down of negative regulators was more constrained. By contrast, the lower levels of reporter activity generated by endogenous Upd2 resulted in positive regulator knock down that reduced reporter activity to levels difficult to statistically distinguish from the inherent noise of the system. By contrast, removal of negative regulators which increase pathway activity are easily identified. This fundamental difference in experimental design is probably a very significant factor in the identification of 'orphan' regulators a hypothesis supported by the fact that 76% (92/121) of the Baeg screen hits represent negative regulators while 73% (66 of 90) of the Müller screen hits are positively acting factors.

Finally, the pathway signalling response to Upd (as used in the Müller screen) and Upd2 (as secreted by the S2-NP cells used in the Baeg screen) may also differ for these two related, but clearly different ligands [7]. Indeed, re-screening of 29 positive regulators from the Baeg screen using Upd as a stimulus re-identified 27 as common to both ligands [54], suggesting that at least some loci are ligand specific.

3.2. Experimental differences

A further potential explanation for the differences between the Baeg and Müller screens are technical differences in the design, execution and post-screen statistical analysis of the data generated. As with any complex experimental design, technical aspects are likely to influence the results ultimately obtained. One of the most significant of these is likely to be the post-screen statistical analysis of the data sets generated and the filtering undertaken.

High-throughput experiments and the resulting data sets have previously been shown to be prone to artefacts. Discrepancies between microarray datasets analysing the same biological process in yeast, but generated in different laboratories, represent a particularly striking example [63,64]. Furthermore, the overlap in other genome-wide proteomic and protein–protein interaction datasets can be very low (reviewed in [65]). As such, accounting for false positives and false negatives is of crucial importance in any high-throughput experiment with one particularly significant factor being the different normalisation approaches used [66]. Most strikingly, different normalisation approaches can lead to the assignment of different regulatory roles for a candidate modulator. For example, failure of reporter and control luciferase activities to follow fluctuations in cell numbers or viability may lead to artefacts if activities are normalised to each other (Fig. 3B). Ideally, a control reporter will not respond to changes in signalling pathway activity and has a robust basal activity [67], criteria met by both screens which each used a similar co-reporter (Act-RL). However, in contrast to the Baeg screen, co-reporter activity was not used to normalise for transfection efficiency in the Müller screen. Instead, transfection of cells 'in batch' before bathing with individual dsRNAs allowed the use of the co-reporter as a separate filter criterion to identify screening and viability artefacts. Table 2 shows the phenotypes of overlapping hits in both screens and demonstrates how some dsRNAs that were altered in both the reporter and co-reporter channels, do not show a change in signalling activity following FL/RL normalisation. Most of the identical phenotypes between Baeg and Müller screens, however, are robust to this kind of normalisation (i.e. *stat92E, dome, hop, CG17836, cdc2, apt, enok socs36E, ptp61F*). Furthermore, different sensitivity and signal-to-noise ratios in both screens could lead to different outcomes, and as a consequence, the selected cut-off threshold can significantly affect the final candidate lists.

While the design and *in silico* optimisation of second and third generation collections of dsRNA molecules will undoubtedly improve library specificity, technical differences will probably always represent a potential source of false negatives.

Given the essentially unavoidable presence of false negative calls, it is tempting to speculate that many of the JAK/STAT pathway regulators identified by only one screen may have been 'orphaned' by such a false negative artefact in the other. Although this is certainly a potential explanation for some orphan interactors, individual validation and analysis of these loci will ultimately be required to determine if this is indeed the case. It is curious however that the strongest (mostly core components) have been found in both screens, indicating that the false negative/positive rate might differ for functionally different subsets of the genes identified.

4. Potential optimisation of future screens

Although the results obtained from the two RNAi screens described above have identified a range of genes for future analysis, it is not clear what proportion of potential JAK/STAT pathway regulators have been determined. Indeed, it would seem unlikely that 100+ additional 'core' components of a genetically well-studied signalling pathway remain to be identified. As such, many components identified in a quantitative assay are likely to represent regulatory factors whose roles are highly context sensitive, although it is unlikely that any cell-based assay could reproduce the complexities of pathway regulation and inter-pathway crosstalk that occurs during the course of embryonic and adult life.

Recent developments in the technology of genome-wide RNAi screening in *Drosophila* cells suggest improvements that could be achieved. These include the optimisation of assays to address

specific aspects of pathway signalling, the use of improved RNAi libraries and the use of more sophisticated *in silico* analytical tools. Such developments promise to not only extend, but also refine and validate the existing inventory of putative JAK/STAT pathway regulating genes.

4.1. The next generation of cell culture-based RNAi screens

In the case of optimised screening approaches, a very wide range of potential screens could be envisaged. These include modified transcriptional, luciferase-based reporters using promoters derived from various pathway target genes as well as different modes of autocrine and paracrine stimulation with one or more of the three *Drosophila* Unpaired-like ligands using cell lines derived from multiple *Drosophila* tissues. Alternatively, more specialised high-content assays of specific aspects of pathway activity may also be undertaken—these could include microscopic screens to automatically quantify the nuclear translocation of activated STAT92E [68] or assays to visualise the pre-dimerisation of the Domeless receptor [69]. In addition, biochemical assays of STAT92E phosphorylation, DNA binding or protein stability may also be viable when combined with screens of smaller subsets of the available RNAi library. A combination of several of these readouts at the same time in a multi-parametric screen may allow the direct role of a targeted gene to be quickly assessed.

In addition to new screens, the use of new libraries will also lead to a significant improvement in the quality of data generated from renewed screening approaches. In particular, second generation libraries have been generated and optimised to avoid sequences homologous to other genes and sequence motifs that might lead to off-target effects [70]. In such libraries, computer based approaches have been used to select dsRNAs which are diced *in silico* with the resulting population of 21 bp siRNAs individually compared to the predicted transcriptome. The resulting data, when analysed in the light of advances in our understanding of the mechanistic basis of RNAi (reviewed in [71]), can be used to generate a prediction of the strength of potential target gene knock down as well as the identification of transcripts potentially affected by off-target siRNAs [70].

Ultimately, third generation libraries will also be developed to include multiple independent dsRNAs per gene, particularly where *in silico* predictions suggest dsRNAs with potential off-target effects.

4.2. Data analysis

Finally, experience gained during the analysis of the Müller screen has underlined the significant influence played by the post-screen *in silico* processing used to analyse the large datasets generated. Compared to other data sets in functional genomics, common standards for RNAi experiments have not yet been established. One such standard exists for microarray experiments, which allows for reproducible and independent data analysis. The MIAME standard represents the minimum information about a microarray experiment, which includes annotations about the experimental design and the microarray probes, the primary raw data of the experiments, as well as the normalised data and information about normalisation methods [72]. The RNAi Global Initiative is currently developing such a standardized protocol for RNAi screenings, termed MIARE for minimal information about RNAi experiments (http://www.miare.org), which will likely facilitate the establishment of a public raw data repository as well as comparisons between different screens and the integration of other datasets in meta-analyses.

Furthermore, reliable and robust statistical procedures are important to identify interactors for downstream analysis. Although statistical tools for the analysis of RNAi screens have been developed [73,74], analysis approaches and software for multi-parametric readouts are still needed. In particular, it is currently unclear which normalisation method will identify *bona fide* candidates if several parameters, such as the activity of two luciferase reporters, are measured at the same time. As such, it would be very interesting to ascertain the overlap between the two datasets of the Baeg and Müller screens using the same normalisation methods.

4.3. Data validation

The potentially high rate of false-positives in RNAi screening experiments has been pointed out and emphasises the need to carefully validate the initial screening hits using independent reagents in independent experiments [75]—for example by using more than one independent dsRNA targeting different regions of the mRNA.

A more rigorous validation of screening hits utilizes the potential evolutionary conservation of gene function, assuming that the knockdown of gene activity in a conserved pathway would have the same functional consequences in different animal species [76,77]. The JAK/STAT pathway has been initially discovered in mammals and commercial tools for pathway analysis are available. These include highly specific antibodies as well as a range of siRNAs. More importantly, the JAK/STAT pathway is more complex with multiple extracellular ligands, JAKs and STATs, all of which contribute to distinct biological responses. Using a vertebrate system with multiple JAK/STAT pathways to validate interactors identified in an organism with only one JAK/STAT pathway may reveal differential roles for pathway modulators and would represent a powerful vindication of the RNAi screening approaches described here.

Ultimately, the results of any screen only represent the identification of loci which potentially interact with the biological process of interest. As such, lists generated by even the most carefully designed and executed cell culture-based screen merely represent candidates awaiting *in vivo* validation. In this context, the recent development of libraries of transgenic *Drosophila* stocks expressing inducible dsRNA constructs represents a potentially very valuable tool [49]. Using such reagents, it will be possible to move on from the cell-based screening described here to the detailed, gene specific developmental genetic analysis that will ultimately be required to fully investigate the loci identified. One potential example would be to combine the recently developed *10xSTAT-GFP in vivo* JAK/STAT pathway reporter [59] with the Gal4/UAS system to express RNAi targeting putative pathway regulator genes.

5. Conclusions

The two whole-genome RNAi screens discussed here have identified a significant number of positive and negatively acting loci with potential roles as JAK/STAT pathway regulators. Intriguingly, each screen appears to have enriched for a specific category of pathway regulator with negatively acting factors predominant in the Baeg data set and positively acting factors making up a large proportion of the Müller gene list. As such, it is likely that the results generated by the two screens are largely complementary to one another. It is important to stress however, that the results of any large-scale genetic screen are just a first step in the characterisation of potential pathway components. The results generated by both genome-wide RNAi screens and more traditional forward genetic approaches all ultimately need to be validated by a detailed *in vivo* analysis and only then will the accuracy of the results generated by RNAi screens become apparent.

While the precise functions of many of the loci identified remains to be determined, a number, including BRDW3 and the his-

tone acetyl transferase enok, represent the homologues of human disease genes already implicated in a range of leukaemias and lymphomas [53]. By linking such disease genes with a signal transduction pathway the possibility to treat these diseases via intervention at other points in the pathway is made available. While such approaches remain to be validated, it is hoped that comparative, cross-species approaches will help to accelerate the rate at which understanding and insight into human disease can be gained.

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