

Identification of JAK/STAT signalling components by genome-wide RNA interference

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Signalling pathways mediating the transduction of information between cells are essential for development, cellular differentiation and homeostasis¹. Their dysregulation is also frequently associated with human malignancies. The Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway represents one such signalling cascade whose evolutionarily conserved roles include cell proliferation and haematopoiesis². Here we describe a systematic genome wide survey for genes required for JAK/STAT pathway activity. Analysis of 20,026 RNA interference (RNAi) induced phenotypes in cultured *Drosophila melanogaster* haemocyte like cells identified interacting genes encoding 4 known and 86 previously uncharacterized proteins. Subsequently, cell based epistasis experiments were used to classify these proteins on the basis of their interaction with known components of the signalling cascade. In addition to multiple human disease gene homologues, we have found the tyrosine phosphatase Ptp61F and the *Drosophila* homologue of BRWD3, a bromo domain containing protein disrupted in leukaemia³. Moreover, *in vivo* analysis demonstrates that disrupted *dBRWD3* and overexpressed *Ptp61F* function as suppressors of leukaemia like blood cell tumours. This screen represents a comprehensive identification of novel loci required for JAK/STAT signalling and provides molecular insights into an important pathway relevant for human cancer. Human homologues of identified pathway modifiers may constitute targets for therapeutic interventions.

Developmental genetic screens in *Drosophila* have identified multiple JAK/STAT pathway components on the basis of their segmentation phenotype^{4,6}, and subsequent analysis of the pathway has characterized evolutionarily conserved roles during immune responses, haematopoiesis and cellular proliferation^{7,10}. The JAK/STAT signalling cascade in *Drosophila* comprises the extracellular ligand Unpaired (Upd)⁵, a transmembrane receptor with homology to the interleukin 6 (IL 6) receptor family termed Domeless (Dome)¹¹, a single Janus tyrosine kinase (JAK) called Hopscotch (Hop)⁴ and the Stat92E transcription factor^{6,12} (Fig. 1a). Known regulators of JAK/STAT signalling, including a family of SOCS like genes¹³, *dPIAS/Su(var)2 10* (ref. 14) and *STAM* (ref. 15), are functionally conserved and were identified based on their homology to components originally characterized in mammalian cell culture studies². Although successful in identifying the pathway members Upd, Dome, Hop and Stat92E, it is probable that forward genetic approaches have missed components, possibly due to non saturating mutagenesis, genetic redundancy or phenotypic pleiotropy.

In order to identify novel pathway components and circumvent limitations of classical genetic screens, we have undertaken a genome wide RNAi screen, a powerful technique for the identification of new components of diverse cellular pathways^{16,19}. To this end, we devised a quantitative assay for JAK/STAT signalling activity in

cultured *Drosophila* cells by multimerizing a STAT92E binding site from the *Draf* promoter²⁰ to generate the 6 × 2 × *DrafLuc* firefly luciferase reporter. Given the role for JAK/STAT signalling in haematopoiesis⁹, we used *Drosophila* haemocyte like Kc₁₆₇ cells because of their endogenous ability to respond to pathway activation (Fig. 1b). On transfection of the 6 × 2 × *DrafLuc* reporter and a plasmid to

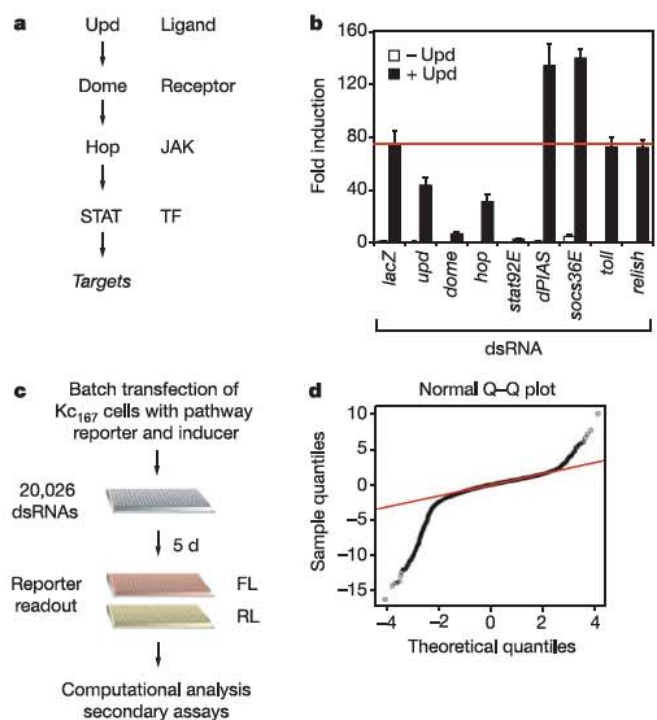


Figure 1 | Genome-wide RNAi screen for JAK/STAT signalling factors. **a**, Schematic representation of the *Drosophila* JAK/STAT signalling pathway. TF indicates transcription factor. **b**, Knock down of known JAK/STAT components leads to loss of pathway induction by Upd, whereas knock down of *lacZ*, *toll* and *relish* show no effect. The red line indicates an approximately 70 fold reporter induction relative to negative control dsRNA. Error bars represent standard deviations of six experiments. **c**, Schematic diagram of screening approach. A total of 20,026 dsRNAs were screened in duplicate in 384 well plates before computational analysis and re testing. FL indicates firefly luciferase; RL indicates *Renilla* luciferase. **d**, Q Q plot of normally distributed quantiles against actual pathway screening result quantiles. A fit to a normal distribution is represented by the red line. Tails of positively and negatively interacting dsRNAs at each extreme with a z score threshold of >2 and <-2 represent RNAi experiments with significant phenotypes.

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constitutively express the ligand Upd, a robust induction of reporter gene activity was observed (Fig. 1b). We examined whether depletion of known pathway components by RNAi²¹ modifies JAK/STAT signalling activity in *Kc₁₆₇* cells. We assessed the effect of double stranded (ds)RNAs targeting the mRNA of the genes *dome*, *stat92E* and *hop*, as well as dsRNAs directed against the negative regulators *socs36E* and *dPIAS*. As shown in Fig. 1b, knock down of JAK/STAT components results in significant changes in reporter activity, whereas reporter activity in uninduced cells remains at low levels (Fig. 1b).

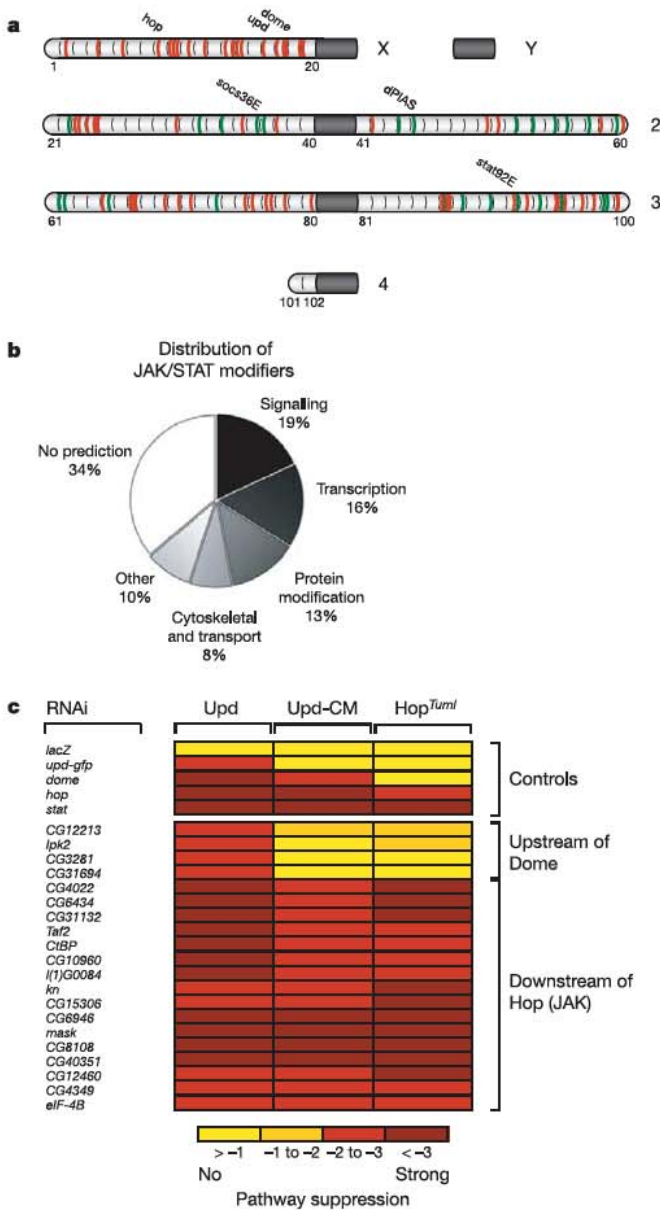


Figure 2 | Analysis of JAK/STAT activity modulators. a, Schematic representation of positive (red) and negative (green) regulator loci distributed within the *Drosophila* genome. An interactive version of this panel is available at <http://www.dkfz.de/signalling/jak> pathway/. b, Distribution of predicted gene functions. c, Epistasis analysis of the indicated positive pathway regulators showing interactions graded from none (yellow squares) to strong (red squares). Results shown have been obtained in two independent replicate experiments. Abbreviations used are: ectopic expression of *upd* (Upd); Upd conditioned medium (Upd CM); and expression of a constitutively active JAK allele (*Hop^{TumI}*) (ref. 26). Colour coding of z scores is shown.

We then set out to systematically identify genes required for JAK/STAT signalling by generating a library of 20,026 dsRNAs targeting ~91% of the predicted transcripts in the *Drosophila* genome (see Supplementary Information). Using this library we performed duplicate genome wide screens as outlined in Fig. 1c and Supplementary Fig. S1. After computational analysis to identify candidate pathway modifiers (Fig. 1d; see also Supplementary Information), dsRNAs were resynthesized and assayed with an independent reporter, derived from the promoter of the pathway target gene *socs36E*¹³, to exclude reporter specific artefacts. These approaches confirmed the identification of 67 dsRNAs that decrease pathway activity (putative positive regulators) and 24 dsRNAs that

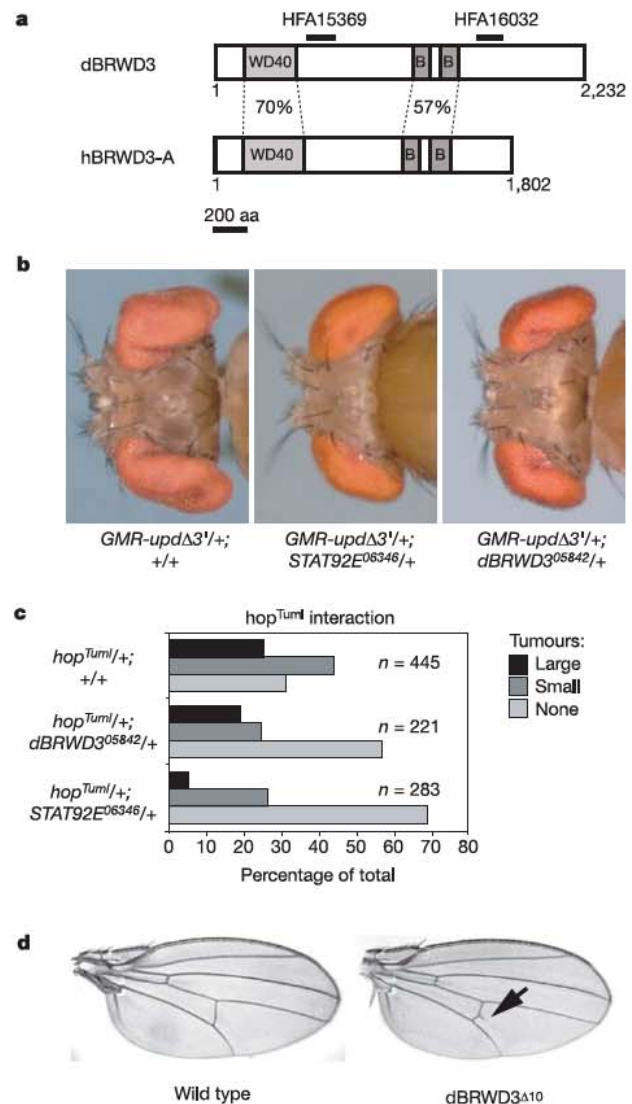


Figure 3 | dBRWD3 functions as a JAK/STAT pathway component. a, Domain structure and sequence similarity of *Drosophila* and human BRWD3 proteins. Percentages show the similarity in the amino acid sequence, and regions targeted by two independent dsRNAs independently recovered in the screen are shown. b, Adult *Drosophila* heads heterozygous for the *GMR updΔ3'* transgene crossed to wild type (left panel), *stat92E* (middle panel) and *dBRWD3* (right panel) mutants. Note the reduction in eye overgrowth after removal of pathway components. c, The size and frequency of *hop^{TumI}* induced tumour formation is significantly decreased in *stat92E* and *dBRWD3* heterozygous backgrounds. d, Compared to adult wild type wings (left), ectopic wing vein material (arrow) is present in homozygous *dBRWD3^{Δ10}* mutant flies (a putative hypomorphic allele, right), a phenotype reminiscent of the *stat92E^{HJ}* mutant¹².

increase pathway activity (putative negative regulators) (see also Supplementary Materials and Table 1), targeting a total of 90 predicted genes. Although most modifiers are distributed throughout the genome (Fig. 2a; see also Supplementary Table 7), the X chromosome appears to be devoid of negative regulators, a finding that may be linked to the role of the pathway in X:autosome ratio detection during *Drosophila* sex determination²².

Based on Interpro and gene ontology (GO) annotations, pathway modifiers were classified according to their predicted functions (see Supplementary Information). Signalling factors, enzymes mediating post translational protein modifications and transcription factors cumulatively represent 48% of the genes identified (Fig. 2b). Examples include *CG11501* encoding a putatively secreted, negative regulator of JAK/STAT signalling previously demonstrated to be a pathway target gene⁸, *enok/CG11290* encoding an acetyl transferase, and the *tumour suppressor protein 101/CG9712* gene, which encodes a ubiquitin conjugating enzyme. The molecular role of these genes in the regulation of JAK/STAT signalling remains to be determined. Seventy four per cent of the identified loci possess human homologues (*E* values <10⁻¹⁰, compared to 62% for the whole proteome), of which 39% have been implicated in human disease (see Supplementary Tables 5 and 6).

A genetic technique to characterize signalling molecules is the determination of their epistatic relationship with respect to defined pathway components. We therefore performed cell based epistatic assays to determine the pathway response to expression of *upd*, Upd conditioned medium or expression of the constitutively active JAK allele *hop^{TumI}* (refs 5, 23) while simultaneously targeting a subset of positive regulators. In this way, dsRNA inactivated genes required upstream in the pathway can be characterized on the basis of their rescue by pathway activation further downstream (Fig. 2c). For example, although depletion of the γ interferon related protein *CG31694* results in downregulation of signalling stimulated by expression of *upd*, activation by Upd conditioned medium or

hop^{TumI} is unaffected (Fig. 2c). This suggests that *CG31694* is required for the production and/or activity of the Upd ligand. Conversely, loss of pathway activity resulting from the knock down of *CG31132* cannot be rescued by any form of pathway stimulus, implying a function downstream of JAK (Fig. 2c). Although this analysis suggests a role for multiple genes upstream of Dome, this classification is based on the lack of interaction observed under differing experimental conditions and the molecular basis of these results remains to be established.

In order to confirm the function of candidate genes *in vivo*, we tested examples of both positive and negative regulators of the JAK/STAT signalling pathway. One positive regulator mentioned above is *CG31132*, which encodes a 2,232 amino acid WD40 and bromo domain containing protein homologous to human BRWD3 (Fig. 3a; see also Supplementary Information). In the screen, a strong reduction of pathway activity was observed for two independent dsRNAs that target different regions of the transcript (Fig. 3a). BRWD3 is a functionally uncharacterized locus recently identified at the break point of t(X;11)(q13;q23) translocations derived from multiple B cell chronic lymphocytic leukaemia (B CLL) patients³.

A previously uncharacterized mutagenic P element inserted in the fourth intron of *CG31132* (hereafter termed *dBRWD3⁰⁵⁸⁴²*) has been generated by the *Drosophila* genome project²⁴ and its remobilization indicates that the transposon insertion is responsible for late embryonic lethality (see Supplementary Information). We therefore tested for genetic interactions between *dBRWD3* and JAK/STAT signalling by crossing the *dBRWD3⁰⁵⁸⁴²* allele to *GMR updΔ3'* (ref. 25). The *GMR updΔ3'* transgene ectopically misexpresses *upd* during eye development, resulting in cellular overproliferation and an enlarged adult eye (Fig. 3b, left panel). Furthermore, removal of one copy of *stat92E* significantly suppresses eye overgrowth (Fig. 3b, middle panel) due to a reduction in the potency of JAK/STAT signalling²⁵. Removal of a single copy of *dBRWD3* was also able to suppress the *GMR updΔ3'* phenotype (Fig. 3b, right panel) as expected for a

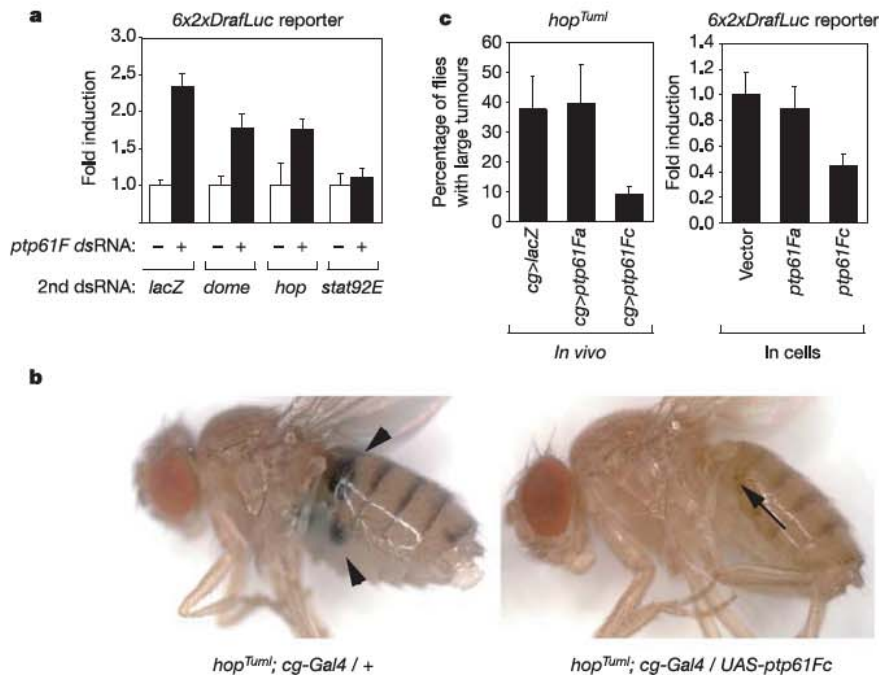


Figure 4 | Ptp61F is a tumour suppressor *in vivo*. **a**, Epistasis analysis of *ptp61F* dsRNA in cell culture indicates that it acts downstream of Hop and upstream or parallel to STAT92E. **b**, Haemocyte specific misexpression of *ptp61F* can protect *hop^{TumI}* mutants from melanotic tumour formation. Compare large black tumours in controls (arrowheads, left) with small tumours present in a *ptp61F* expressing individual (right). **c**, Quantitative

analysis of large tumour formation in *hop^{TumI}* mutants expressing cytoplasmic Ptp61Fa and nuclear Ptp61Fc, showing specificity of rescue for the nuclear isoform (left), an effect that is mirrored by overexpression of the same isoforms in tissue culture based reporter assays (right). Error bars represent standard deviations of three or four independently tested transgenic lines or eight parallel cell culture experiments.

positive regulator of JAK/STAT signalling. A chromosomal deficiency removing the region has previously been independently identified as a suppressor of *GMR updΔ3'* (ref. 25).

One phenotypic consequence of constitutive JAK/STAT activation caused by the gain of function JAK allele *hop^{TumI}* is the overproliferation of haemocytes and the frequent formation of melanotic tumours, a phenotype described as a *Drosophila* model for leukaemia^{23,26}. We found that removal of one copy of *dBRWD3* is sufficient to reduce the size and the frequency of *hop^{TumI}* induced melanotic tumours (Fig. 3c; and see also Supplementary Table 3). Moreover, homozygous escapers of a putative hypomorphic allele of *dBRWD3*, generated by excision of the original P element, frequently develop ectopic wing vein material (Fig. 3d), a phenotype reminiscent of the weak loss of function *stat92E^{HJ}* allele²⁷. Taken together, these experiments suggest a role for *dBRWD3* in JAK/STAT signalling.

As a second example we analysed Ptp61F, a protein tyrosine phosphatase whose depletion led to an increase in JAK/STAT signalling activity. To perform an epistasis analysis we removed known pathway components and tested for the effect of simultaneously targeting *ptp61F*. Double RNAi against *ptp61F* together with *lacZ*, *dome* or *hop* results in pathway stimulation (Fig. 4a). However, simultaneous removal of *ptp61F* and *stat92E* is sufficient to prevent signalling (Fig. 4a). Loss of this phosphatase therefore results in the stimulation of Stat92E activity even in the absence of upstream components, indicating that Ptp61F negatively regulates the pathway downstream of JAK. We next asked whether Ptp61F also interferes with JAK/STAT signalling *in vivo* by using the *cg Gal4* driver to misexpress *ptp61F* in blood cells of *hop^{TumI}* mutants. Misexpression of *ptp61F_c* in a *hop^{TumI}* mutant background resulted in suppression of melanotic tumour formation, with the average frequency of large tumours reduced by approximately fourfold, an effect also observed after the misexpression of *dPIAS¹⁴* (Fig. 4b; see also Supplementary Table 3). Alternative splicing of *ptp61F* gives nuclear and cytoplasmic protein forms that both contain the same phosphatase domain²⁸. However, the tumour suppressor phenotype is only observed with nuclear Ptp61F_c (Fig. 4c), an effect that is reproduced by overexpression in cell culture (Fig. 4c). These results are consistent with our identification of Ptp61F as a negative regulator of pathway activity and suggest that it may function by targeting phosphorylated, nuclear localized Stat92E for deactivation.

Aberrant JAK/STAT signalling has been implicated in multiple human malignancies and its components have been proposed as molecular targets for the development of therapeutic compounds^{29,30}. The genome wide screen presented here has identified known and previously unknown genes and we have characterized their probable level of interaction using cell based epistasis analysis. Of the 90 JAK/STAT modifiers identified, many have human homologues that remain to be characterized. We have performed an analysis of two examples *in vivo* and demonstrate their roles in regulating the pathway in *Drosophila*. One of these is a homologue of human *BRWD3*, a gene recently identified at the break point of a translocation isolated from multiple B CLL patients³. Given our functional analysis of *dBRWD3* and the known roles for JAK/STAT signalling during normal haematopoiesis, it is possible that a break down in *BRWD3* mediated STAT regulation may represent a molecular mechanism leading to the development of B CLL. Thus, comprehensive genetic surveys by RNAi using *Drosophila* as a model organism represent a powerful approach for identifying targets relevant to human diseases.

METHODS

Constructs. The JAK/STAT firefly luciferase reporter $6 \times 2 \times \text{DrafLuc}$ was constructed by multimerization of a molecularly characterized Stat92E binding site present in the promoter of the endogenous target gene *Draf⁰*, whereas the $4 \times \text{SocsLuc}$ reporter is based on a single 290 base pair (bp) region containing four potential Stat92E binding sites present within the first intron of *socs36E* (ref. 13) (see Supplementary Information for details). A *Renilla* luciferase reporter gene

under the control of the constitutively active *Actin5C* promoter was co transfected and used to monitor cell number (see Supplementary Information).

Genome wide RNAi screening. A genome wide RNAi library based on polymerase chain reaction (PCR) templates with an average length of 408 bp (flanked by T7 promoter binding sites) was generated by *in vitro* transcription¹⁷ (see Supplementary Information). Primer and amplicon sequence information can be found at <http://rnai.dkfz.de>. For screening experiments, *Drosophila* Kc₁₆₇ cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA) and 100 µg ml⁻¹ penicillin streptomycin (Invitrogen). A total of 57 384 well screening plates were loaded with approximately 75 nM dsRNA in 5 µl of 1 mM Tris at pH 7. Kc₁₆₇ cells were batch transfected with the appropriate reporters, *pAct Renilla* and *pAct UpdGFP*, and transferred to dsRNA containing screening plates in serum free medium after 7 h. For 384 well plates, 15,000 cells in 20 µl were dispensed per well using an automated liquid dispenser (MultiDrop, Thermo Labsystems) and incubated for 60 min before addition of serum containing medium. After 5 d, medium was removed, cells were lysed and both firefly and *Renilla* luciferase activities were determined (see also Supplementary Information for computational analysis).

Cell based epistasis experiments. For epistasis experiments, cells were transfected with expression vectors to stimulate pathway activity (see below). After 7 h, 30,000 cells in 50 µl of serum free medium were seeded into 96 well plates (Greiner) containing the dsRNAs to be tested (listed in Fig. 2c). One hour later, 75 µl medium supplemented with 10% fetal bovine serum was added to the cells. Cells were lysed after 5 d and luciferase activity measured.

Each dsRNA was tested for its ability to suppress pathway activity under three conditions: (1) in Upd expressing cells (screening conditions); (2) in cells treated with Upd conditioned medium (Upd CM); and (3) in cells expressing *hop^{TumI}*. Specifically, for Upd overexpression 5×10^6 Kc₁₆₇ cells were transfected with 600 ng *pAct UpdGFP*, 500 ng $6 \times 2 \times \text{DrafLuc}$ reporter, 250 ng *pAc5.1 Sid 1*, 25 ng *pAct RL* and *pAc5.1* to a total of 2 µg DNA. For *hop^{TumI}* overexpression, 5×10^6 Kc₁₆₇ cells were transfected with 200 ng *pAct hop^{TumI}*, 500 ng $6 \times 2 \times \text{DrafLuc}$ reporter, 250 ng *pAc5.1 Sid 1*, 25 ng *pAct RL* and *pAc5.1* to a total of 2 µg DNA. To analyse processes upstream of Upd, two batches of cells were transfected separately to generate 'responder' and 'Upd producer' cells. The responder cells were batch transfected with 500 ng $6 \times 2 \times \text{DrafLuc}$ reporter, 250 ng *pAc5.1 Sid 1*, 25 ng *pAct RL* and *pAc5.1* to a total of 2 µg plasmid DNA and subsequently seeded into 96 well plates containing the respective dsRNAs as described above. The Upd producing cells were transfected with 2 µg *pAct UpdGFP* and cultured in 10 cm dishes (Falcon). After 4 d, the cells were treated with 50 µg ml⁻¹ heparin (Sigma) for 24 h and then the supernatant was collected, cleared by centrifugation and passed through a 0.2 µm filter (Millipore). 50 µl of this Upd conditioned medium was then used to stimulate pathway activity in the responder cells for 24 h. Control medium from untransfected heparin treated cells did not elicit pathway activity (data not shown). Epistasis analysis of *ptp61F* by double RNAi is described in Supplementary Information.

Genetics. For genetic interaction assays, females of the stock *y, w, hop^{TumI}/FM7; P[w⁺, cg Gal4.A]2* (ref. 26) were crossed to wild type controls (*OreR* and *w¹¹¹⁸*) and mutations in *stat92E* and *dBRWD3*. The haemocyte specific Gal4 driver line *P[w⁺, cg Gal4.A]2* allowed specific misexpression constructs with upstream activating sequences (UAS) to be tested for their potential influence on tumour formation. Transgenic animals carrying *UAS EGFP* or *UAS β galactosidase* were used as negative controls whereas *UAS dPIASGFP* served as a positive control¹⁴ (see Supplementary Table 3).

Crosses were incubated at 25 °C and adult females heterozygous for the *hop^{TumI}* chromosome were scored within 24 h of eclosion for the presence of tumours, classified as small (one or two small melanotic spots as shown in Fig. 4b, right panel) or large (one or more large melanized growths or more than three small spots; Fig. 4b, left panel). Survival rates for *hop^{TumI}* females seem to be independent of tumour frequency at the time point counted (data not shown). Assays were repeated at least twice for each genotype and a representative example from one experiment is shown (Fig. 4b).

Genetic interaction with *P[w⁺, GMR updΔ3']¹⁹* (termed *GMR updΔ3'* in the text) was undertaken as described²⁵ using *OreR* and *stat92E⁰⁶³⁴⁶* as negative and positive controls, respectively. Suppression of *GMR updΔ3'* induced eye overgrowth by *dBRWD3⁰⁵⁸⁴²* was observed in multiple independent experiments in a majority of individuals of the appropriate genotype. *Drosophila* heads were photographed using a Zeiss STEMI 2000 C binocular microscope and AxioCam camera.

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