

Transcriptional targets of *Drosophila* JAK/STAT pathway signalling as effectors of haematopoietic tumour formation

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Although many signal transduction pathways have been implicated in the development of human disease, the identification of pathway targets and the biological processes that mediate disease progression remains challenging. One such disease-related pathway is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) cascade whose constitutive misactivation by the JAK2 V617F mutation underlies most human myeloproliferative disorders. Here, we use transcript profiling of *Drosophila* haemocyte-like cells to identify JAK/STAT target genes, combined with an *in vivo* model for JAK-induced blood cell overproliferation, to identify the main effectors required for haematopoietic tumour development. The identified human homologues of the *Drosophila* effectors were tested for potential V617F-mediated transcriptional regulation in human HeLa cells and compared with small interfering RNA-derived data, quantify their role in regulating the proliferation of cancer-derived cell lines. Such an inter-species approach is an effective way to identify factors with conserved functions that might be central to human disease.

Keywords: Hop^{TumL}; Kc₁₆₇; transcript profiling; V617F; cancer
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INTRODUCTION

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signalling pathway has been conserved throughout evolution and consists of a large family of extracellular

ligands that bind to transmembrane receptors, which are themselves associated with JAK kinases. Following stimulation, ligand/receptor/JAK complexes are activated by tyrosine phosphorylation to recruit STATs, which are then tyrosine-phosphorylated and translocated to the nucleus. In the canonical model of pathway signalling, activated STAT dimers then bind to palindromic DNA-binding sites in the promoters of pathway target genes to activate transcription. In *Drosophila*, the DNA-binding sites recognized by the STAT92E transcription factor are separated either by three nucleotides (3n sites), generating a high-affinity site, or by four nucleotides (4n sites), which are bound with lower affinity (Rivas *et al*, 2008). In addition, recent reports suggest a non-canonical association of STAT92E/HP1 complexes to heterochromatic DNA in the absence of JAK signalling, thus representing an alternative mechanism by which STAT92E might indirectly regulate transcription *in vivo* (Shi *et al*, 2008).

Although JAK/STAT pathway signalling is required during development, its inappropriate activation is also responsible for a range of human malignancies. In particular, the dominant gain-of-function mutation JAK2 V617F is central to most myeloproliferative diseases (Kota *et al*, 2008). Interestingly, V617F is located in the regulatory JH2 domain of JAK2, the same domain that is affected by gain-of-function mutations in the *Drosophila* JAK homologue Hopscotch (Hop). The principal phenotype associated with these *Drosophila* mutations is an overproliferation of prohaemocytes and their inappropriate differentiation into lamellocytes, ultimately producing large black melanotic tumours (Luo *et al*, 1995).

The low redundancy of the evolutionarily conserved JAK/STAT pathway components, combined with the conservation of developmental roles of the pathway (Arbouzova & Zeidler, 2006), makes *Drosophila* an effective animal model for human disease development. Although JAK/STAT pathway components and regulators have been studied in detail and the tumour phenotypes are well characterized, the biological processes triggered by JAK/STAT signalling that produce the terminal phenotype are less well understood. Here, we use the strengths of the *Drosophila* system to identify candidate effectors that have a part in this process.

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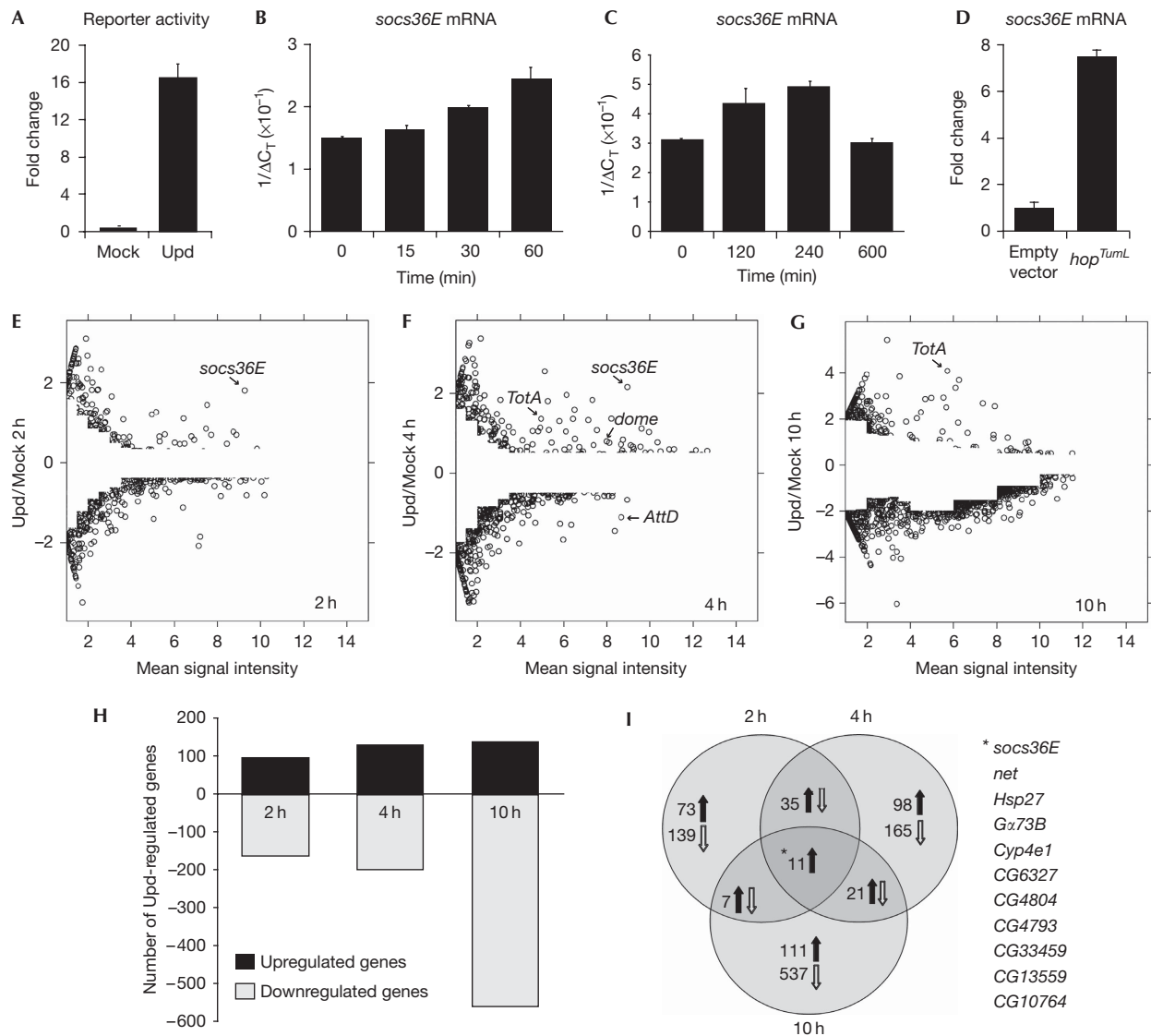


Fig 1 | Transcript profile analysis of Upd-induced JAK/STAT activation. (A) Upd is able to stimulate a luciferase JAK/STAT pathway reporter construct (Müller *et al*, 2005). (B–D) Quantitative PCR showing *socs36E* mRNA levels in *Kc167* cells after either Upd stimulation (B,C) or 72 h after Hop^{TumL} transfection (D). (E–G) Plots of genes expressed at the indicated time points showing the log₂ ratio for each gene as a function of the mean signal intensity. Differentially expressed genes were identified by calculating intensity-dependent z-scores and filtered to remove those differing by less than two standard deviations from the mean. (H) The number of genes either upregulated or downregulated at the 2 h, 4 h and 10 h time points. (I) Venn diagram showing the distribution of upregulated and downregulated genes at the 2 h, 4 h and 10 h time points. The 11 loci upregulated at all time points are listed. Hop, Hopscotch; JAK, Janus kinase; STAT, signal transducer and activator of transcription; Upd, Unpaired.

RESULTS AND DISCUSSION
Transcript profiling

Drosophila haemocyte-like *Kc167* cells endogenously express JAK/STAT pathway components, respond to the Unpaired (Upd) ligand (Harrison *et al*, 1998) and have been used to undertake genome-wide RNA interference (RNAi) screens for regulators of pathway signalling (Müller *et al*, 2005). We therefore used this *ex vivo* system as a starting point to identify pathway targets and mediators of JAK/STAT signalling in *Drosophila* haematopoiesis. Treating *Kc167* cells with Upd-conditioned media is sufficient to induce luciferase-based reporters (Fig 1A). To test the temporal expression profile mediated

by Upd stimulation, the immediate-early pathway target gene *socs36E* was assessed by quantitative PCR (Q-PCR) and found to be expressed rapidly and strongly over several hours (Fig 1B,C), an upregulation recapitulated by Hop^{TumL} stimulation (Fig 1D). To reflect the *socs36E* profile and the time course of haematopoietic organ development *in vivo*, 2 h, 4 h and 10 h time points were selected for analysis. The aim was to capture the response of genes stimulated directly by the pathway (2 h), later genes representing both direct targets and early secondary effects (4 h), and longer term responses probably representing the secondary and possibly tertiary effects of JAK/STAT signalling (10 h).

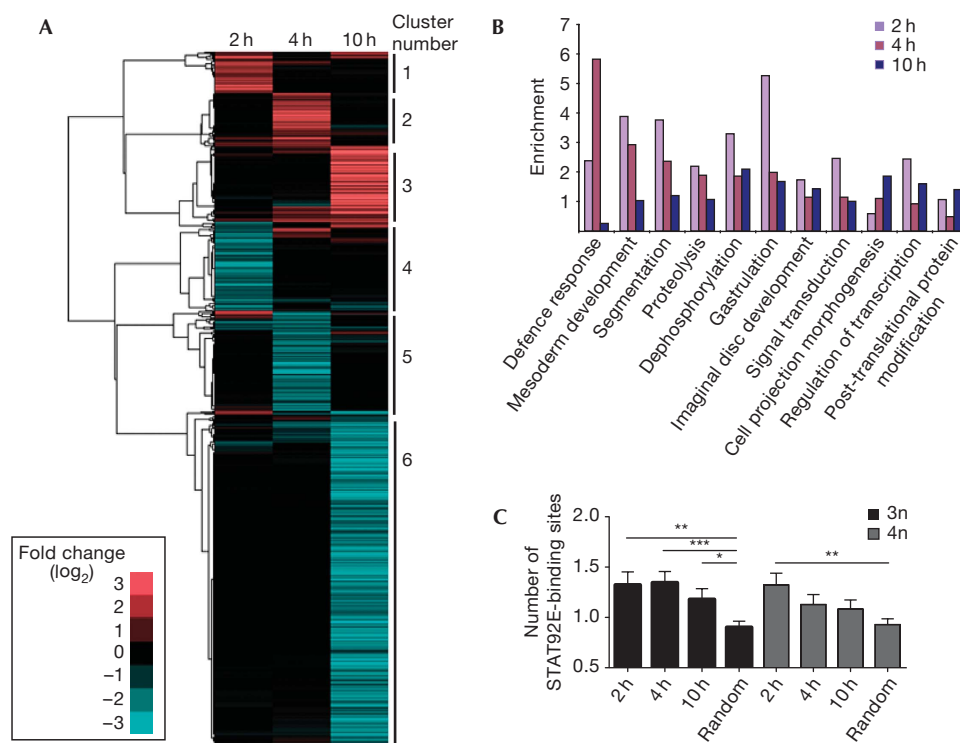


Fig 2 | Clustering, gene ontology and binding-site analysis of putative JAK/STAT target genes. (A) Hierarchical cluster analysis of 1,168 Upd-regulated genes using log₂ ratios. The blue tiles represent downregulated genes and red tiles indicate upregulated genes. Six main clusters were identified. (B) The functional enrichment of the 1,168 Upd-regulated genes according to gene ontology terms. (C) The average number of potential 3n and 4n STAT92E-binding sites present in the upstream region of positively regulated genes at each time point, compared with the number of sites in a randomly selected 'control' group of genes. **P*<0.05, ***P*<0.005, ****P*<0.0005. JAK, Janus kinase; STAT, signal transducer and activator of transcription; Upd, Unpaired.

The transcription profiles generated from microarrays were analysed by using the propagating uncertainty in microarray analysis (PUMA) algorithm to assign a confidence score to each gene (Pearson *et al*, 2009). A differential fold change cutoff was applied to define genes whose expression was significantly different (*P*<0.05) from that of the mock control at various expression levels (Fig 1E–G; Yang *et al*, 2002). This approach increases the stringency applied to genes expressed at lower levels where the effect of experimental noise and non-specific hybridization is greater (Fig 1E–G). In total, 1,168 genes were identified as being expressed differentially at one or more time point (Fig 1H–I; supplementary Fig S1 and Table S1 online).

JAK/STAT pathway stimulation has long been considered a positive regulator of transcription. However, treatment of Kc₁₆₇ cells with Upd clearly induces both upregulation and downregulation of a wide range of transcripts, with the proportion of downregulated loci increasing at later time points (Fig 1E–I). Q-PCR analysis of selected loci validated the chip-derived data and confirmed the existence of downregulated genes (supplementary Fig S1A,B online). While many of the loci regulated negatively at 10h might represent secondary or tertiary consequences of pathway activation, more than 150 loci are also regulated negatively at 2h. Although mechanisms responsible for these early effects remain to be identified, this negative transcriptional regulation is not unprecedented, with examples of downregulation in *Dictyostelium* (Mohanty *et al*, 1999), the

Drosophila eye (Mukherjee *et al*, 2006) and vertebrate cancers (Dauer *et al*, 2005; Qu *et al*, 2009).

Clustering of genes and functional enrichment

Hierarchical clustering of the data set (Eisen *et al*, 1998) identified six distinct clusters of co-regulated loci (Fig 2A), with subsequent analysis of associated gene ontology (GO) terms showing enrichment of functionalities such as endopeptidase activity (cluster 3), cell differentiation and morphogenesis (cluster 6), and humoral immune responses (cluster 5). Interestingly, when similar GO analysis is undertaken within each time point (Fig 2B), processes such as mesoderm development, segmentation and gastrulation are represented strongly at 2h, defence responses are enriched at 4h and cell morphogenesis and post-translational modifications are more prevalent at 10h. These roles are consistent with the known developmental roles of JAK/STAT pathway signalling, including haematopoiesis and immune responses (Arbouzova & Zeidler, 2006). As such, GO-based analysis suggests that the genes identified reflect a bona fide response to pathway stimulation.

STAT92E-binding sites

The single *Drosophila* STAT92E protein has recently been shown to bind to both 3n and 4n DNA-binding sites, with higher affinity for 3n sites (Rivas *et al*, 2008). Given that co-expression (or repression) of genes is probably a consequence of common

Table 1 | Upd-responsive genes selected for *in vivo* RNAi assays

Gene name <i>Drosophila melanogaster</i>	Fold change log ₍₂₎			STAT92E-binding sites		Gene ontology	Gene name <i>Homo sapiens</i>
	2 h	4 h	10 h	3n	4n		
<i>baz</i>	-0.2	-0.6	-1.9	0	1	Asymmetric protein localization	<i>PARD3, PARD3B, MPDZ</i>
<i>CG10764</i>	0.5	1.3	2.5	6	4	Serine endopeptidase	—
<i>CG13559</i>	1.5	1.8	2.6	2	1	—	<i>LITAF</i>
<i>CG15211</i>	0.0	0.6	0.0	1	0	—	—
<i>CG15678</i>	0.8	0.1	0.7	1	0	Negative regulation of innate immune response	—
<i>CG1572</i>	0.0	0.6	0.6	2	1	—	—
<i>CG31158</i>	0.7	0.7	0.0	1	0	Regulation of ARF protein signal transduction	<i>PSD3, PSD, PSD2</i>
<i>CG3829</i>	0.7	1.4	0.1	2	0	Defence response	<i>SCARB1, SCARB2, CD36</i>
<i>CG4080</i>	0.7	1.1	0.0	1	1	Protein binding	<i>MARCH8, MARCH2, MARCH1</i>
<i>CG4793</i>	0.7	1.6	3.7	6	2	Serine endopeptidase	<i>TMPRSS13, KLKB1, CTRB2</i>
<i>CG4804</i>	1.1	2.0	1.8	4	2	Serine endopeptidase	<i>PAI-2, SERPIN11, SERPINB8</i>
<i>CG6014</i>	0.0	1.0	0.0	2	0	—	<i>REG1A</i>
<i>Gα73B</i>	1.2	2.6	2.9	2	0	GTPase activity	<i>GNAS, GNAL, GSA</i>
<i>idgf1</i>	0.0	0.9	2.9	0	0	Imaginal-disc development	<i>CHIT1, CHI3L2, CHIA</i>
<i>l(2)gl</i>	0.0	1.3	2.4	1	0	Protein localization	<i>LLGL1, LLGL2, STXBPS</i>
<i>mfas</i>	0.0	0.8	1.0	2	0	Axonegenesis	<i>TGFBI, POSTN</i>
<i>mthl3</i>	0.1	0.8	0.2	6	4	G-protein-coupled receptor activity	<i>GPR112</i>
<i>net</i>	2.3	1.2	2.2	2	2	Imaginal-disc-derived wing vein morphogenesis	<i>ATOH8</i>
<i>pxb</i>	-0.7	-0.8	0.0	0	0	Smoothened signalling pathway	—
<i>socs36E</i>	1.8	2.2	1.2	5	3	Negative regulation of JAK/STAT cascade	<i>SOCS5, SOCS4, SOCS6</i>
<i>TotA</i>	0.0	1.1	4.1	2	2	Response to stress	—
<i>Wnt4</i>	2.2	-1.5	0.0	0	2	Signal transducer activity	<i>WNT9A, WNT9B, WNT16</i>

Bold gene names indicate homologues tested by quantitative PCR.
baz, bazooka; RNAi, RNA interference; STAT, signal transducer and activator of transcription; Upd, Unpaired.

cis-regulatory elements within promoters, we assessed the frequency with which either 3n or 4n sites are found in the genomic interval 3 kb upstream of the transcriptional start site of the 1,168 genes identified and a randomly selected group of unrelated *Drosophila* genes. The negatively regulated genes did not show enrichment of STAT92E-binding sites and are therefore unlikely to be regulated by the canonical STAT92E-binding sites (data not shown). Interestingly, for upregulated loci, 3n sites are present at significantly higher frequencies at each time point (Fig 2C), whereas 4n sites are only enriched at 2 h. Given that Upd-induced STAT92E activity is probably strongest at 2 h (Fig 1B,C), it seems plausible that transcription driven by lower affinity 4n STAT92E-binding sites reflects a requirement for higher levels of STAT92E activity.

Roles in *Drosophila* haematopoietic tumour development

Given the number of loci differentially regulated by Upd, other criteria were used to select potential JAK/STAT pathway effectors

for testing *in vivo*. The 1,168 genes were therefore compared with genes identified in published microarray experiments and RNAi screens focused on *Drosophila* immunity. Comparison of these data sets identified 22 differentially regulated genes (including *socs36E*) that may represent potential mediators of JAK/STAT pathway signalling *in vivo* (Table 1). These include components of other signalling pathways such as the Wnt4 ligand, the Gα73B subunit and the negative regulator of Hedgehog signalling *pxb*, as well as *l(2)gl* and the negatively regulated *bazooka* (*baz*), two loci previously implicated in tumorigenesis and cell polarity.

JAK/STAT pathway signalling has an important role in the larval haematopoietic organ (Krzemien *et al*, 2007), where haematopoietic stem cells are maintained in the medullary zone (MZ) of the lymph gland by pathway activity. The MZ is marked by expression of the pathway receptor *dome* (Fig 3A, green). However, in the *hop^{Tuml}* gain-of-function mutant, increased levels of JAK/STAT pathway signalling result in additional cellular proliferation in the lymph gland, a massive increase in circulating

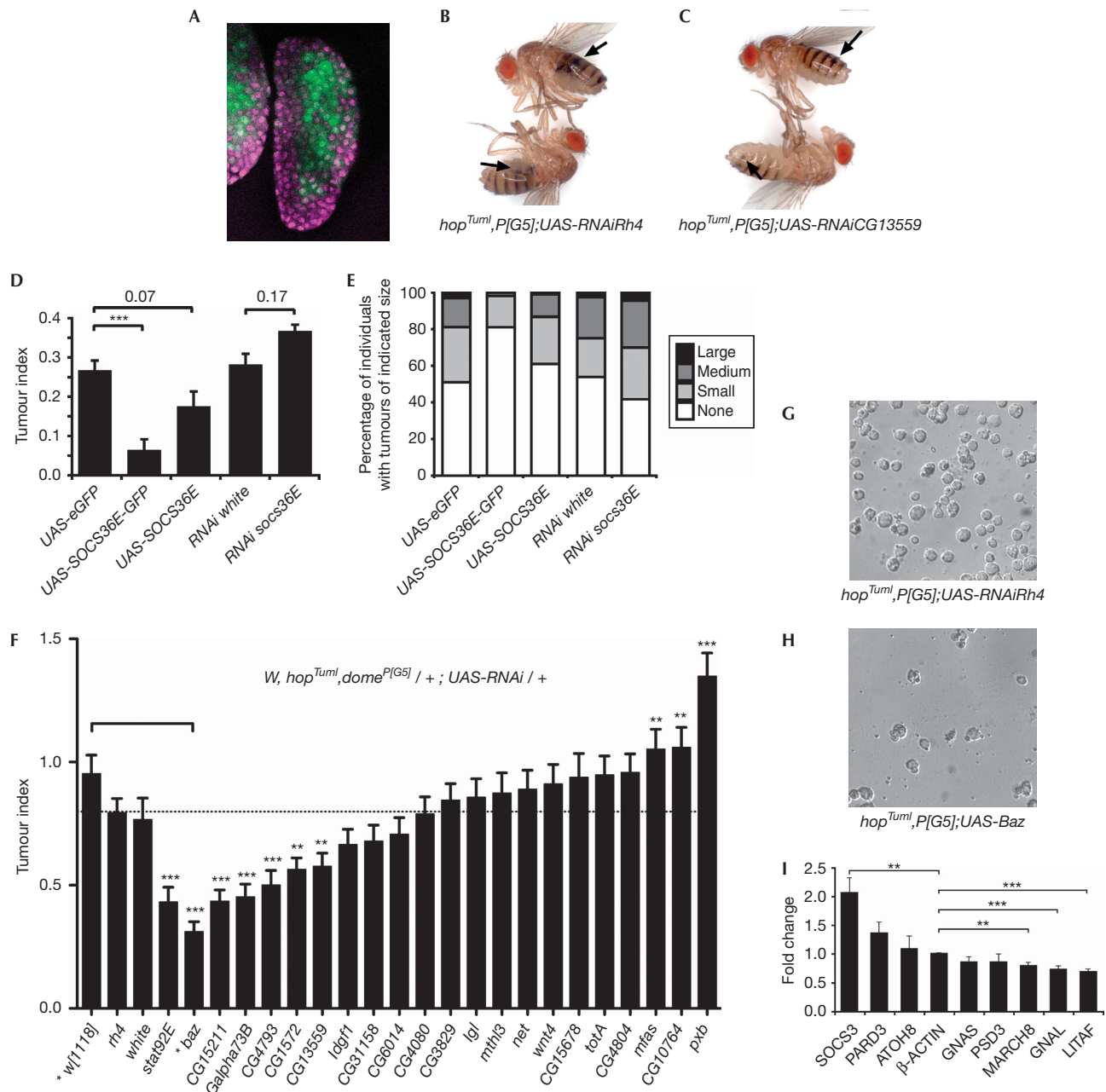


Fig 3 | *In vivo* characterization of JAK/STAT targets. (A) The third-instar lymph gland showing the domain of p[G5] expression (green) in the central MZ. DNA is shown in magenta. (B,C) Adult flies of the specified genotypes carrying the gain-of-function *hop^{TumL}* allele contain multiple, large black tumours (indicated by arrows) when a control shRNA targeting *rhodopsin 4* (*rh4*) is expressed in the MZ by the p[G5] driver (B). Flies in which the pathway effector *CG13559* is knocked down contain fewer, smaller tumours (C). The TI (D) and percentage of adults with tumours of the indicated size (E) are shown for the specified genotypes, which either overexpress or knock down *socs36E*. Overexpression using *UAS-SOCS36E-GFP* flies showed significant reduction in TI as compared with that in the control (D). (F) The TI of flies of the indicated genotype expressing shRNAs targeting 21 potential effectors as well as *rh4* and *white* as controls. The bar indicates the link between *baz* misexpression and *w[1118]* used as the control for this cross. (G,H) Representative differential interference contrast image of haemocytes from *hop^{TumL}* larvae of the indicated genotypes showing reduction in circulating cells present after *UAS-baz* overexpression. (I) Fold change in the expression of selected homologues of *Drosophila* genes in HeLa cells after stimulation by the gain-of-function JAK2 V617F mutation. For all graphs, error bars represent standard error. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *baz*, *bazooka*; GFP, green fluorescent protein; *hop*, Hopscotch; JAK, Janus kinase; MZ, medullary zone; shRNA, short-hairpin RNA; STAT, signal transducer and activator of transcription; TI, tumour index.

haemocytes, engulfment of 'self' tissue and ultimately formation of black melanotic tumours (Fig 3B,C; Luo *et al*, 1995). Altered expression of JAK/STAT pathway targets and effectors in the lymph gland, elicited by the activating Hop^{TumL} mutation (Fig 1D), might therefore be central to melanotic tumour formation.

To test this hypothesis, we first overexpressed the pathway target gene and negative regulator *socs36E* in the MZ of Hop^{TumL} individuals. Counts of the size and frequency of melanotic masses present in adult flies were used to generate a quantitative 'tumour index' (TI; Shi *et al*, 2006). Notably, overexpression of SOCS36E by two independently generated transgenes reduced the TI of Hop^{TumL} individuals (Fig 3D), with a corresponding decrease in the distribution of tumour sizes (Fig 3E). Conversely, *in vivo* RNAi constructs targeting *socs36E* mRNA marginally increased the TI and the proportion of larger tumours (Fig 3D,E). These results indicate that modulation of JAK/STAT pathway activity by overexpression, or knockdown of a JAK/STAT pathway regulator in the MZ, is sufficient to alter the Hop^{TumL}-induced tumour phenotype.

To test the functional roles of the potential JAK/STAT effectors *in vivo*, RNAi was used to knock down the expression of the remaining 21 loci in the MZ of *hop^{TumL}* mutants with the TI of the resulting adults compared to a range of controls (Fig 3F). In addition to *stat92E* itself, five novel effector loci—*CG15221*, *Gα73B*, *CG4793*, *CG1572* and *CG13559*—were identified whose knockdown significantly reduces the development of tumours in the adult fly, three of which (*Gα73B*, *CG4793* and *CG13559*) are also upregulated at each of the three time points assayed originally (Fig 1I). This suggests that these five loci represent true *in vivo* effectors of JAK/STAT-mediated tumorigenesis. In addition, knockdown of three loci—*mfas*, *CG10764* and *pxb*—results in significant increases in TI (Fig 3F), suggesting that they function as 'anti-oncogenes' *in vivo*. This result is consistent with the Upd-mediated decrease in *pxb* expression observed at both 2 h and 4 h time points (Table 1).

In addition to RNAi-mediated knockdown, overexpression of Baz was also tested, given the decrease in observed *baz* expression after pathway stimulation. Strikingly, ectopic *baz* expression is one of the strongest suppressors of tumour development, significantly reducing both the size and frequency of tumours (Fig 3F; supplementary Fig S2 online), as well as the number of circulating haemocytes present in late third-instar larvae (Fig 3G,H). However, knockdown of *baz* mRNA also seems to produce a reduction in TI and size (supplementary Fig S2 online), suggesting that any alteration of Baz is sufficient to reduce Hop^{TumL}-induced tumorigenesis. Interestingly, Baz has recently been shown to drive the apical localization of STAT92E in ectodermally derived tissues *in vivo* (Sotillos *et al*, 2008), suggesting a model in which increased Baz binds to and sequesters STAT92E, thereby attenuating its tumorigenic function. However, the validity of this model in mesodermally derived haematopoietic precursors, and the relative expression of endogenous Baz in wild-type and *hop^{TumL}* lymph glands, remains to be determined.

In addition to Baz, other JAK/STAT effectors identified have close human homologues, including the *Gα73B* putative homologues GNAL and GNAS, which are key elements of G-protein pathways. In a striking parallel to our findings from the *Drosophila* system, mutations in the GNAS locus, which is heavily maternally imprinted and associated with a range of human diseases, have been reported to be a marker of chronic lymphocytic leukaemia disease progression (Frey *et al*, 2006).

JAK/STAT effectors in humans

To extend our studies and begin to test the relevance of our findings to humans, we used one-way and reciprocal BLAST searches to identify the putative human homologues of the 1,168 differentially regulated *Drosophila* genes. These searches identified 1,818 human genes (292 from reciprocal alignments) representing putative homologues of 717 (61%) of the *Drosophila* genes identified (supplementary Table S1 online). Direct comparison of these human loci to published lists of genes differentially regulated by STAT3 seems to identify relatively few common targets, with an overlap of eight out of 100 genes identified in human lung-carcinoma cells (Dauer *et al*, 2005), and 12 out of 159 genes (7.5%) in murine lung tumours (Li *et al*, 2007; supplementary Table S4 online). However, both overlaps represent significant scores when compared to a randomly selected set of control genes (*z*-scores of 2.34 and 3.25, respectively), suggesting that downstream targets of STAT signalling have been partly conserved through evolution.

To obtain data relating directly to the *Drosophila* effectors tested *in vivo*, we examined the expression of human homologues in cancer-derived HeLa cells stimulated by transfection with the dominant gain-of-function JAK2 V617F mutation (Kota *et al*, 2008). Of 15 homologues tested (Table 1, bold), eight are expressed in HeLa cells. Of these, GNAL, LITAF, MARCH8 and SOCS3, used as a positive control, were all significantly differentially regulated (Fig 3I; supplementary Table S3 online). This suggests that, although direction is not necessarily maintained, changes in the expression of these loci after JAK/STAT signalling are common to both *Drosophila* and human systems.

Finally, we cross-referenced the human loci homologous to 16 of the 22 selected *Drosophila* genes with a database of three genome-wide, small interfering RNA-mediated cell viability screens undertaken with HeLa, HEK293T and HepG2 cells (T. Horn & M. Boutros, www.genomeRNAi.org). Although these viability data remain to be validated, the human homologues MARCH2, GPR112 and LLGL1 represent strong suppressors of proliferation in one or more human cancer cell lines (supplementary Table S2 online). These results indicate a potential role for these loci in the development or progression of cancer, and represent a potentially important insight into the role of these putative effectors.

The GNAL/*Gα73B* locus is a particularly intriguing example of a gene identified repeatedly in this study. We have shown that *Drosophila* *Gα73B* is upregulated at all three time points in response to Upd (Fig 1I and Table 1) and its knockdown in *hop^{TumL}* flies significantly reduces tumorigenesis (Fig 3F). By contrast, its human homologue GNAL is downregulated significantly by V617F in HeLa cells (Fig 3I), while its knockdown in the HepG2 cancer cell line significantly increases cellular proliferation. Therefore, in both systems, disease-related JAK mutations result in differential regulation of this *Gα* subunit to promote cellular proliferation and tumorigenesis, respectively. Encouragingly, previous reports have identified downregulation of GNAL in leukaemic cells (Koldehoff *et al*, 2008), supporting the possibility that this locus has an important effector function downstream from JAK/STAT signalling.

Although encouraging, cell culture-based proliferation data must be interpreted with caution and ultimate proof that the genes identified represent the bona fide effectors of tumorigenesis *in vivo* remains to be determined. However, we have identified JAK/STAT pathway effectors in *Drosophila* and have identified conserved

homologues of these in humans. In addition, we have shown examples of common transcriptional regulation as well as potential links with haematopoietic malignancies and proliferation of cancer-derived cell lines. Although detailed mechanistic analysis of individual effectors and their roles in disease progression remains the ultimate goal, our genetic approach has effectively identified pathway effectors likely to mediate human disease.

METHODS

Upd-conditioned media and transcript profiling. Conditioned media was prepared as described by Harrison *et al* (1998) and assayed for activity by using the *6x2xDrafluc* reporter (Müller *et al*, 2005). Kc₁₆₇ cells were treated with Upd or mock-conditioned media for 30 min, washed and total RNA was extracted after 2 h, 4 h or 10 h according to the Affymetrix protocols. Twelve samples were hybridized to the GeneChip® *Drosophila* Genome 2.0 arrays. The raw probe set data were analysed by using the PUMA package (Pearson *et al*, 2009) to generate intensity-dependent z-scores for each log₂ ratio (Yang *et al*, 2002). Genes ≥ 2 standard deviations from the mean were flagged as differentially expressed. Raw data have been deposited in the Gene Expression Omnibus database under accession number GSE15584.

Q-PCR of Kc₁₆₇ cells. Q-PCR used the total RNA and primers shown in supplementary Table S4 online, the SYBR® Green JumpStart™ TaqReadyMix™ (Sigma-Aldrich, St Louis, MO, USA) and followed a standard Verso™ reverse transcription-PCR (Thermo Scientific, Waltham, MA, USA) protocol. PCR was performed in triplicate in a Bio-Rad MyIQ™, analysed by $\Delta\Delta C_T$ and normalized to *Rpl32*.

Cell culture. HeLa cells grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum were transfected with pMX-IRES-JAK2 V617F or empty vector using Lipofectamine (Invitrogen, Paisley, UK). The cells were harvested after 24 h and total RNA was extracted. Q-PCR was performed in triplicate as described above and normalized to β -actin.

In vivo tumour assays. *In vivo* tumour assays used a *hop^{TumI}, dome^{P[G5]}* recombinant crossed to the individual inducible *UAS-RNAi* and misexpression stocks as described by Dietzl *et al* (2007; supplementary Table S5 online). The TI scores of the resulting adults were calculated as described by Shi *et al* (2006). For blood-cell pictures, wandering third-instar larvae were bled by tearing the larva in a 15 μ l drop of serum-free Schneider's medium placed on a haemocytometer.

Further details, including bioinformatic techniques, are available in the supplementary information online or from the authors on request.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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