RESEARCH ARTICLE

Gα73B is a downstream effector of JAK/STAT signalling and a regulator of Rho1 in Drosophila haematopoiesis

Nina Bausek and Martin P. Zeidler*

ABSTRACT

JAK/STAT signalling regulates many essential developmental processes including cell proliferation and haematopoiesis, whereas its inappropriate activation is associated with the majority of myeloproliferative neoplasias and numerous cancers. Furthermore, high levels of JAK/STAT pathway signalling have also been associated with enhanced metastatic invasion by cancerous cells. Strikingly, gain-of-function mutations in the single Drosophila JAK homologue, Hopscotch, result in haemocyte neoplasia, inappropriate differentiation and the formation of melanised haemocyte-derived ‘tumour’ masses; phenotypes that are partly orthologous to human gain-of-function JAK2-associated pathologies. Here we show that Gα73B, a novel JAK/STAT homologue, partly orthologous to human gain-of-function JAK2-associated pathologies. Here we show that Gα73B, a novel JAK/STAT pathway target gene, is necessary for JAK/STAT-mediated tumour formation in flies. In addition, although Gα73B does not affect haemocyte differentiation, it does regulate haemocyte morphology and motility under non-pathological conditions. We show that Gα73B is required for constitutive, but not injury-induced, activation of Rho1 and for the localisation of Rho1 into filopodia upon haemocyte activation. Consistent with these results, we also show that Rho1 interacts genetically with JAK/STAT signalling, and that wild-type levels of Rho1 are necessary for tumour formation. Our findings link JAK/STAT transcriptional outputs, Gα73B activity and Rho1-dependent cytoskeletal rearrangements and cell motility, therefore connecting a pathway associated with cancer with a marker indicative of invasiveness. As such, we suggest a mechanism by which JAK/STAT pathway signalling may promote metastasis.

KEY WORDS: Drosophila melanogaster, Haematopoiesis, Tumorigenesis, JAK, Rho1

INTRODUCTION

The cellular co-ordination and exchange of information required during development is mediated by a relatively small group of evolutionarily conserved signal transduction pathways. One such pathway is the JAK/STAT signalling cascade named on the basis of its key components, the Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) families. Studied widely in both humans and model organisms such as Drosophila, JAK/STAT signalling is required for multiple developmental processes and its ectopic activation is also associated with numerous malignancies. For example, the JAK2 V617F allele is an important factor in the development of the majority of human myeloproliferative neoplasias (MPNs). This group of related diseases includes polycythaemia vera (>95% V617F positive), essential thrombocytthermia (50% V617F positive) and primary myelofibrosis (50% V617F positive), diseases that can progress to acute myeloid leukaemias and significantly affect quality of life (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005). In addition to these haematological malignancies, ectopic activation of JAK/STAT pathway signalling in human cancers has also been linked to the metastatic potential of solid tumours. For example, invasive prostate cancer cells have been shown to upregulate STAT upon epithelial to mesenchymal transition (Härmä et al., 2010). In addition, restoration of the protein inhibitor of activated STAT 1 (PIAS1) in gastric cancer tissues is sufficient to robustly decrease the expression of tumour migratory markers (Chen et al., 2012). Other reports suggest that JAK3 may directly activate phospholipase D to promote the invasive behaviour of breast adenocarcinoma cells (Henkels et al., 2011). Links between JAK/STAT signalling and the regulator of the F-actin cytoskeleton RhoA have also been associated with increased migratory and invasive potential of gastric cancer cells (Wei et al., 2013). However, although a consistent body of correlative evidence is building, the mechanistic causes of these links remain to be established.

The evolutionarily conserved, low complexity JAK/STAT pathway present in Drosophila is required for a diverse range of developmental processes during embryonic, larval and adult life. These functions include the regulation of stem cells, cell proliferation, haematopoiesis and the response to infection; conserved roles that also require JAK/STAT pathway activity in vertebrates (Arbouzova and Zeidler, 2006). In particular, JAK/STAT signalling has been shown to play an important role in normal Drosophila haematopoiesis (Minakhina et al., 2011). Under unchallenged conditions phagocytic plasmatocytes (>90% of circulating haemocytes) and crystal cells, which are involved in melanisation during immune response and healing, represent the principal cell types generated during the two rounds of Drosophila haematopoiesis. During the second, postembryonic haematopoiesis in the lymph gland, JAK/STAT signalling controls plasmatocyte differentiation through its target pannier, the Drosophila GATA factor orthologue (Minakhina et al., 2011). However, following infestation by the parasitic wasp Leptopilina boulardi, both circulating haemocytes and haemocyte precursors present within the lymph gland are triggered to differentiate into lamellocytes by a rapid downregulation of pathway signalling. These large lamellocyte cells then engulf and tightly encapsulate wasp eggs laid within the developing larva (Krzemień et al., 2007; Markus et al., 2009).
Although evolutionary divergence prevents a direct comparison with human MPN diseases, gain-of-function (GOF) mutations in the Drosophila JAK (hop\textsuperscript{Tuml} and hop\textsuperscript{T22}) also result in the neoplasia of fly blood cells and their inappropriate differentiation (metaplasia) into lamellocytes (Hanratty and Ryerse, 1981; Luo et al., 1997). This combination of neoplastic and metaplastic effects results in the generation of large numbers of ectopic lamellocytes and the spontaneous formation of melanised tumours composed of tightly associated plasmatocytes and lamellocytes.

Given the importance of JAK/STAT signalling for human health, the identification and characterization of components and regulators of the pathway has been extensively pursued (Bach et al., 2003; Baeg et al., 2005; Müller et al., 2005; Valentino and Pierre, 2006). However, identification of pathway target genes that act as in vivo phenotypic effectors of GOF JAK alleles has proved more elusive. We previously performed one such study to identify effectors required for the tumorous phenotype associated with the Drosophila JAK GOF Hop\textsuperscript{Tuml} allele (Bina et al., 2010).

Here we describe our analysis of a G-protein alpha subunit and putative effector of GOF JAK-induced tumour development, G₇₃B. Although G-alphas were originally described as the GTP-binding subunits that relay extracellular signals received by G-protein-coupled receptors (GPCR), we show here that Drosophila G₇₃B acts as an in vivo regulator of Rho1 activity and distribution.

**RESULTS**

**Generation of G₇₃B mutants**

We have previously used transcript profiling approaches to identify targets of Drosophila JAK/STAT pathway signalling and identified potential effectors of tumour development by in vivo knockdown in haematopoietic progenitor cells (Bina et al., 2010). One candidate selected for further study is the gene encoding G₇₃B, a 399 amino acid, GTP-binding protein sharing 41% identity with the GNAS family of human G alpha (G\alpha) proteins (Weinstein et al., 2006). However, although G₇₃B (also termed Gf alpha) has a diverse and dynamic expression pattern during embryonic, and especially mesodermal development (Furlong et al., 2001; Quan et al., 1993), very little functional data are available.

In order to better understand the GOF JAK–G₇₃B interaction phenotypes we confirmed expression of G₇₃B downstream of the JAK/STAT pathway. G₇₃B mRNA was upregulated in haemocyte-derived Kc\textsubscript{167} cells following addition of exogenous media conditioned with either Upd2 or Upd3, the JAK/STAT pathway ligands previously shown to be expressed in the lymph gland where haemocyte progenitors reside (Fig. 1A) (Bina et al., 2010; Makki et al., 2010). In addition, haemocytes bled from larvae misexpressing Hop\textsuperscript{Tuml} also showed significantly (\(P<0.001\)) increased G₇₃B expression levels (Fig. 1B), which is consistent with increased expression in haemocytes derived from GOF hop mutant backgrounds (Fig. 1E). Consistent with the JAK/STAT-induced increases in G₇₃B expression, we found

![Fig. 1. G₇₃B is a JAK/STAT target gene.](Image)

(A) Quantification of G₇₃B mRNA expression in haemocyte-derived Kc\textsubscript{167} cells after treatment with mock- or ligand-conditioned medium containing Upd2 or Upd3. (B) Quantification of G₇₃B gene expression in haemocytes from third instar larval haemolymph. Haemocytes of srp > Hop\textsuperscript{Tuml} larvae show significantly higher G₇₃B mRNA expression than control (srp > EYFP) haemocytes. (C) The G₇₃B genomic region on the left arm of chromosome 3. Untranslated 5’ and 3’ regions are shown in light grey, exons in dark grey, intronic regions by black lines. Positions of the indicated P-elements are depicted as triangles, and putative STAT92E binding sites as asterisks. The region deleted to produce the G₇₃B\textsuperscript{4B7} mutation is indicated by the dotted line. (D) Quantification of G₇₃B mRNA expression in whole flies of the indicated genotype. Expression is significantly reduced in flies homozygous for both the P (BAC) G₇₃B\textsuperscript{4B7} insertion (G₇₃B\textsuperscript{4B7}) and G₇₃B\textsuperscript{4B77} (E) Quantification of G₇₃B mRNA expression in haemocytes shows that levels of G₇₃B are significantly decreased in G₇₃B\textsuperscript{4B7} mutant larvae and upregulated in hop\textsuperscript{Tuml} larvae (\(P<0.001\)). Significance was calculated using t-tests and error bars represent standard error of the mean (s.e.m.). ***\(P<0.001\)
four putative STAT92E binding sites (with 4n spacers, Rivas et al., 2008) within the G73B locus (asterisks in Fig. 1C).

On the basis of these results we generated amorphic mutations in the G73B locus made up of a small, defined deficiency (shown by the dashed line in Fig. 1C and named G73B<sup>487</sup> hereafter) and also characterized P(BAC)G73B<sup>CG5874</sup> (Bellen et al., 2004), an insertion in the first G73B intron (Fig. 1C), subsequently termed G73B<sup>PH</sup>. When homozygous, both mutants expressed greatly reduced levels of G73B mRNA (Fig. 1D,E) with G73B<sup>PH</sup> likely to represent a strong hypomorphic allele. Both alleles are semi-lethal with homozygous and trans-heterozygous combinations recovered at ~20% of the expected Mendelian frequency.

**G73B promotes tumorigenesis**

In order to confirm the initial RNAi-derived identification of G73B (Bina et al., 2010), we undertook Hop<sup>Tuml</sup> tumour assays in genetic backgrounds that modulated G73B levels. Consistent with previous RNAi-derived results, reduction of G73B levels using the hypomorphic G73B<sup>PH</sup> allele was sufficient to significantly (P<0.001) reduce the average adult tumour burden in Hop<sup>Tuml</sup> individuals (Fig. 2A,B). Furthermore, overexpression of wild-type G73B within lymph gland haemocyte precursors is sufficient to increase tumour frequency and size distribution. RNAi-mediated knockdown of controls including stat92E or u-shaped (<i>ush</i>) act as expected (Fig. 2A,B) with decreased stat92E correlating with a decrease in tumour index (Bina et al., 2010), and loss of the haemocyte differentiation inhibitor <i>ush</i> leading to increased lamellocyte formation (Gao et al., 2009) and thus increased tumour frequency.

Although the GOF JAK allele hop<sup>Tuml</sup> has been widely studied (Bina et al., 2010; Harrison et al., 1995; Kwon et al., 2008; Stofanko et al., 2010), a second GOF HOP allele mutated in its JH2 pseudokinase domain and called hop<sup>T42</sup>, has also been identified (Luo et al., 1997). Given the key significance of the human JAK2 V617F pseudokinase domain mutation in MPNs, we also assessed the tumour burden in hop<sup>T42</sup> heterozygous adults with genetic backgrounds reducing stat92E or G73B levels (Fig. 2C). Consistent with the GOF hop<sup>Tuml</sup> allele, removal of one copy of either stat92E or G73B was sufficient to strongly and significantly (P<0.001) reduce tumour formation.

In order to better understand the cellular basis of the G73B-induced tumour phenotype we examined haemolymph from hop<sup>T42</sup> heterozygous larvae, either wild type or lacking one copy of the G73B locus. Although large tumours cannot be easily identified because of their dark melanisation, small ‘pre-tumorous’ masses of clumped haemocytes were recovered (Fig. 2D,E). By comparison with otherwise wild-type pre-tumours, a reduction in G73B gene dose frequently prevented formation of the tightly bound cell mass normally found in Hop<sup>T42</sup> backgrounds (compare Fig. 2D,E), and resulted in the formation of more loosely associated pre-tumorous masses (Fig. 2E). Taken together, these results suggest that the more loosely associated cell masses are not able to develop and generate the melanised tumours visible in adults and scored in Fig. 2A–C.

**G73B does not regulate haematopoiesis**

Given that G73B is expressed in haemocytes throughout development, we next tested for a potential requirement during normal haematopoiesis. We first examined molecular markers of lymph gland development, including the posterior signalling centre (PSC) marked by Collier expression (Fig. 3A,B), and
plasmatocyte differentiation within the cortical zone of the lymph gland marked by the P1 antibody (Fig. 3C,D). Although inherently difficult to visualise and photograph in two dimensions, careful examination of multiple lymph glands suggested that the size and morphology of the PSC are indistinguishable in wild-type and \( \alpha 73B \) mutant primary lymph gland lobes (Fig. 3A,B). In addition, mature, P1-positive plasmatocytes were present within cortical zones of wild type and \( \alpha 73B \) mutants in comparable amounts (Fig. 3C,D). These results suggest that \( \alpha 73B \) is not required for normal haemocyte development.

As changes in the expression levels of \( \alpha 73B \) alter Hop\(^{\text{TumI}}\)-mediated tumour formation, which is initiated by aberrant lamellocyte production, we also examined lamellocyte differentiation triggered by parasitic wasp infestation. Differentiation of haemocyte precursors within the third instar lymph gland towards the lamellocyte lineage following infestation (as shown by the lamellocyte marker PS4) was rapid and occurred in both wild-type and \( \alpha 73B \) individuals (Fig. 3E,F), and both wild-type and \( \alpha 73B^{4B7} \) larvae contained circulating plasmatocytes and lamellocytes after infestation (arrowheads and arrows in Fig. 3G,H).

Finally, we tested whether the effect of reduced levels of \( \alpha 73B \) on Hop\(^{\text{TumI}}\)-mediated tumour formation could be the result of a decrease in haemocyte number. In a wild-type background, average absolute numbers of circulating haemocytes were somewhat reduced in \( \alpha 73B \) homozygotes, however, this reduction was not statistically significant (Fig. 4A). Furthermore, we confirmed the presence of sessile and circulating larval crystal cells in both wild-type and \( \alpha 73B^{4B7} \) homozygous larvae (supplementary material Fig. S1). We also examined the haemocytes present in hop\(^{\text{TumI}}\) genetic backgrounds and found that lamellocyte differentiation induced by Hop\(^{\text{TumI}}\) was not significantly affected by the loss of \( \alpha 73B \) with circulating lamellocytes present in both genetic backgrounds (Fig. 4B–D).

---

**Fig. 3.** Parasite infestation can trigger an immune response in \( \alpha 73B \) mutant larvae. (A,B) Wild-type \( w^{1118} \) (A) and homozygous \( \alpha 73B^{4B7} \) (B) third instar larval lymph glands both contain normal posterior signalling centre (PSC) and medullary zones as shown by anti-Collier immunostaining (red). DNA in nuclei were visualised by ToPro3 (blue). (C,D) The cortical zone of the primary lobes of wild-type \( w^{1118} \) (C) and homozygous \( \alpha 73B^{4B7} \) (D) third instar larvae showing mature plasmatocytes (as identified by the P1 antibody, red) and DNA in nuclei (ToPro3, blue). (E,F) Lymph glands from third instar larvae of \( w^{1118} \) (E) and homozygous \( \alpha 73B^{4B7} \) (F) individuals dissected 30 hours post infestation with *Leptopilina boulardi*. Lamellocytes are identified by the PS4 antibody (green) and the PSC (anti-Collier) and crystal cells (anti-ProPo) are shown in red; DNA in nuclei is shown in blue (ToPro3). (G,H) Circulating haemocytes from \( w^{1118} \) (G) and homozygous \( \alpha 73B^{4B7} \) (H) individuals dissected 90 hours (at 18°C) post infestation with *Leptopilina boulardi* and stained to visualise F-actin and cellular morphology. Based on morphological criteria, both plasmatocytes (arrowheads) and presumptive lamellocytes (arrows) are present in both backgrounds.

---

**Fig. 4.** \( \alpha 73B \) is not necessary for haemocyte differentiation. (A) The number of haemocytes present in the haemolymph of third instar larvae of the indicated genotypes (\( n=9, P=0.63 \)). (B) The percentage of lamellocytes within the haemocyte population of third instar larvae of the indicated genotypes as determined by immunostainings of third instar larval haemocytes heterozygous for hop\(^{\text{TumI}}\) and the indicated genotype (\( n=8, P=0.59 \)). (C,D) Haemocytes from third instar larvae of the indicated genotypes stained to visualise \( \alpha \)-tubulin (green) and lamellocytes as identified by the L1 antibody marker (magenta).
Conversely, we did not observe the formation of melanised tumours or inappropriate lamellocyte differentiation following overexpression of Gz73B in a wild-type genetic background (supplementary material Fig. S2).

Taken together, these results suggest that Gz73B is not required for the processes that differentiate plasmatocytes and crystal cells under normal conditions or for the differentiation of lamellocytes in either Hop\textsuperscript{Tuml} or wasp-induced conditions. Furthermore, Gz73B does not appear to play a major role in the haemocyte neoplasia associated with Hop\textsuperscript{Tuml}.

G73B regulates haemocyte morphology and F-actin dynamics

Given that G73B does not appear to be required for the haemocyte neoplasia or lamellocyte metaplasia phenotypes associated with Hop\textsuperscript{Tuml}, we examined whether normal plasmatocyte morphology was disrupted in Gz73B mutants. It has previously been described that circulating plasmatocytes bled from third instar larvae can adhere to glass coverslips in a Rho1-dependent process, where they spread by forming lamellipodia and F-actin-rich filopodia (Kadandale et al., 2010). Examination of plasmatocyte morphology in wild-type and Gz73B mutants revealed a striking difference in this behaviour. By contrast to wild-type haemocytes (Fig. 5A), filopodia in cells lacking Gz73B (Fig. 5B) were often composed of thicker bundles of F-actin than in wild-type cells, and protruded from both distal and more medial regions of their lamellipodia with ‘three dimensional’ spikes protruding upwards from the focal plain (Fig. 5B). In order to better describe and quantify these changes we measured the ratio of cell perimeter to total surface area and found that haemocytes lacking Gz73B had a significantly (P<0.001) higher perimeter:area ratio (Fig. 5C) and consistently projected more filopodial spikes (Fig. 5D). Gz73B mutant cells also appeared to contain increased levels of F-actin, alterations reminiscent of the changes that occur during haemocyte activation following injury or misexpression of Rho1 (Williams et al., 2007).

We next set out to examine filopodial dynamics in living cells. Using time-lapse microscopy of live wild-type and Gz73B mutant plasmatocytes (supplementary material Movies 1 and 2), we generated kymographs of filopodial movements and counted protrusion events and lengths over a given time course (see supplementary material Fig. S3). Analysing these, we observed that Gz73B mutant filopodia were significantly (P<0.001) more dynamic, extending and retracting more frequently than controls (Fig. 5E), whereas they were slightly (but not statistically significantly) shorter (Fig. 5F).

Live imaging suggested that Gz73B mutant plasmatocytes not only have increased numbers of filopodia, but that these are also more active. Given the ex vovo nature of this assay, we are unable to examine the migration effects these changes may elicit in vivo. However, these data do indicate that Gz73B is required for the normal regulation of the F-actin cytoskeleton in circulating Drosophila haemocytes.

G73B is a regulator of Rho1

Mutations in Drosophila Gz73B have previously been identified in a screen for modulators of a Rho1-dependent rough eye phenotype (Gregory et al., 2007). Consistent with this, Rho1 is a principal regulator of the F-actin cytoskeleton and one of a small group of related Rho GTPases required during Drosophila development. Furthermore, Rho1 has been shown to be involved in lamellipodium and filopodium formation in normal and activated Drosophila haemocytes (Williams et al., 2007), but is not required for embryonic haemocyte migration (Paladi and Tepass, 2004; Stramer et al., 2005).

In the light of these potential links, we examined the effect of expressing Rho1 dominant GOF (Rho1\textsuperscript{V14}) and dominant-negative mutant constructs (Rho1\textsuperscript{N19}) (Fanto et al., 2000; Strutt et al., 1997) on filopodial outgrowth in ex vovo haemocytes (Fig. 6A–D). Expression of either form of Rho1 resulted in a marked increase in the number of filopodia comparable with that produced by loss of Gz73B. Furthermore, we found that both constitutively active and dominant-negative forms of Rho1 alter the dynamics of filopodial outgrowth and actin dynamics in plasmatocytes, suggesting that normal cellular morphology requires a balance of Rho1 activity that is neither too strong nor too weak. This finding is consistent with the finding that both

![Fig. 5. Loss of Gz73B causes changes in filopodium dynamics in haemocytes.](image-url)
RhoV14 and RhoN19 constructs have the same effect on cell division (Williams et al., 2007). We therefore suggest that filopodium formation in haemocytes is sensitive to the level of Rho1 activity in vivo.

Given that RhoV14 and RhoN19 expression result in defects in cytokinesis and hence multinucleate haemocytes (Williams et al., 2007), we used a GFP-tagged Rho1 expressed at physiological levels (Rosales-Nieves et al., 2006) as an alternative tool to examine the distribution of Rho1 in haemocytes. Subcellular localization of Rho1 has previously been shown to be diagnostic for its localized activity within the cell (Simões et al., 2006), and in wild-type haemocytes Rho1–GFP was localized predominantly within the cytoplasm of the cell (Fig. 6E). In general, lower levels of Rho1–GFP were detected in lamellipodia surrounding the cell, but were frequently enriched within filopodia themselves (Fig. 6F,G). By contrast, in Gx73B mutant haemocytes overall levels of Rho1–GFP were lower (compare Fig. 6E–G with H–J for which microscope settings were identical) and almost undetectable in filopodia (Fig. 6H–J).

In the converse experiment, we also examined the levels of Rho1 expressed by cultured Drosophila S2R+ cells transfected with a plasmid expressing exogenous Gx73B (Fig. 6K). Strikingly, levels of endogenous Rho1 were strongly upregulated (arrows in Fig. 6K’) with staining also visible in cellular filopodia.

Overall, our results show that levels of Rho1 are regulated by Gx73B and also suggest that Gx73B may also be required for the translocation of Rho1 to filopodia – although it is possible that this finding is influenced by changes in overall expression level.

Although the levels and distribution of Rho1 within both haemocytes and cultured cells is clearly altered in a Gx73B-dependent manner, Rho1 activity is also dependent on its association with GTP or GDP. We therefore use a reporter based on a modified, GFP-tagged, Rho1–GTP-interacting protein PKN (Lu and Settleman, 1999; Simões et al., 2006). Using this PKNG58AeGFP reporter we found that haemocytes bled from otherwise wild-type larvae had a wide distribution of reporter activity, with most cells having medium or high levels of Rho1–GTP (Fig. 7A,G); a result that reflects the functional diversity of the haemocyte population. Consistent with other approaches (Williams et al., 2007), we found that prior injury of larvae substantially increases the absolute levels of reporter activity to levels approaching those in injured wild-type larvae was still sufficient to trigger a substantial increase in reporter activity to levels approaching those in injured wild-type larvae (Fig. 7H). This suggests that the lower levels of Rho1 present in cells lacking Gx73B was still able to respond to injury.

However, whereas reporter activity increased to similar levels, wasp infestation (Howell et al., 2012). In addition, the fidelity of the reporter was demonstrated by RNAi-mediated knockdown of Rho1, which resulted in a dramatic decrease in PKNG58AeGFP levels (Fig. 7G).
Finally, given the changes in Rho1 subcellular distribution following loss of Gz73B, we tested for a potential genetic interaction between loss of Rho1 and GOF Hop alleles. Strikingly, we found that expression of both Rho1V14 and Rho1N19 in vivo using the P[G5] dome-Gal4 line was 100% lethal in a genetic background simultaneously heterozygous for the hopTuml GOF allele – a synthetic lethality that was not observed following the same misexpression in a wild-type genetic background. In addition, the tumour phenotype associated with the GOF hopT42 allele in vivo was also significantly (P<0.001) reduced by the removal of a single copy of the Rho1 gene, a genetic interaction that was as strong as the removal of a single copy of Gz73B or stat92E (Fig. 7I). These interactions are consistent with a model in which the interaction between GOF hop alleles and Gz73B mutants is being mediated by a reduction in Rho1 activity.

**DISCUSSION**

Here we show that Gz73B is a transcriptional target of JAK/STAT pathway signalling that plays a key role in the formation of the haemocyte-containing tumours which characterise GOF JAK genetic backgrounds. We show that Gz73B modulates the levels and activity of the small GTPase Rho1 to affect the F-actin cytoskeleton, and thus filopodium formation – a link that is supported by genetic interactions between loss of Rho1 and GOF JAK-induced phenotypes.

**Gz73B is a JAK/STAT effector**

Ectopic activation of the JAK/STAT signalling pathway is associated with a wide range of solid and haematological malignancies (Buettner et al., 2002). We have previously
attempted to identify cancer-relevant effectors using the low complexity Drosophila system in which GOF mutations in JAK result in haemocyte neoplasia (Bina et al., 2010). Expression of one candidate effector, Gz73B, is strongly regulated by the JAK/STAT pathway in haemocytes (Fig. 1A,B,E). Although the genomic region encoding Gz73B does not contain putative STAT92E binding sites with conventional 3n spacing (TTCCnnnGGA), the region does contain three putative sites separated by four nucleotides (TTCCnnnGGA), an alternative binding site recognized by STAT92E both in vivo and in vitro and required to drive the mesodermal-specific expression of domeless (Rivas et al., 2008). Given the mesodermal origin of blood cells it seems likely that the 4n sites present in Gz73B are responsible for its JAK/STAT pathway responsiveness, although more detailed promoter analysis and occupancy studies would be required to prove this definitively.

**Regulation of the F-actin cytoskeleton by Gz proteins**

The alpha subunits of heterotrimeric G proteins are the effector units that relay extracellular signals received by G-protein-coupled receptors (GPCRs) to generate a cellular response (Louet et al., 2012). However, in addition to their role in canonical GPCR signalling, recent evidence suggests a more versatile role for Gz subunits. Studies from in vivo models have implicated Gz in mitotic spindle organisation (Wilkie and Kinch, 2005) and microtubule and cytoskeleton dynamics (Giretti et al., 2008). Gz proteins are also involved in the regulation of Hippo–YAP (Yu et al., 2012) signalling. Moreover, some of these novel regulatory roles of Gz subunits occur in the absence of GPCR signalling (Wilkie and Kinch, 2005). Consistent with these alternative functions, another Drosophila Gz subunit, Concertina, has been shown to activate the Rho1 GEF, RhoGEF2, and is involved in cytoskeletal rearrangements during early morphogenetic events in the embryo (Fox and Peifer, 2007). Furthermore, the human Gz subunit Gz(olf) has also been shown to promote cellular invasion and survival, in a RhoA-dependent manner (Régnauld et al., 2002). Taken together with the results shown here, we suggest that Gz proteins have conserved roles in regulating the F-actin cytoskeleton through interactions with Rho1/RhoA GTPases.

**Rho1, JAK/STAT and cancer**

Tumour metastasis and invasiveness are tightly linked to changes in migratory behaviour and cell motility, which are themselves associated with changes in the cytoskeleton. Rho1 is one of the main factors that regulate cytoskeletal rearrangements, and has been implicated in the metastatic potential of cancer cells (Fagan-Solis et al., 2013). Although the molecular basis of Rho1 activation has been studied in detail, and its downstream modulation of the F-actin cytoskeleton has been extensively characterised, the mechanisms that function upstream to regulate its activity in a signalling context are less well understood. Here, we have identified a previously unknown regulator of one aspect of Rho1 activation and show that Gz73B is itself downstream of a cancer-related signalling pathway. Furthermore, given the conservation of Gz proteins through evolution and the high levels of functional conservation of JAK/STAT pathway regulators (Müller et al., 2012), it is possible that JAK/STAT pathway signalling in human cancers has also been linked to metastatic potential. For example, invasive cancer cells have high levels of STAT activity (Harmá et al., 2010) and this invasive potential is dependent on JAK3 (Henkels et al., 2011). As Gz73B is a direct target gene of JAK/STAT signalling, it could provide the link between pathway activity and Rho1-mediated cytoskeletal rearrangements. These links could also be of wider clinical importance, as it has recently been shown that JAK/STAT signals through Rho-kinase to enable carcinoma and melanoma cells to initiate migration, a key step towards metastasis (Sanz-Moreno et al., 2011). Furthermore, links between JAK/STAT signalling and RhoA have also been associated with increased migratory and invasive behaviour of gastric cancer cells (Wei et al., 2013), although the mechanistic link between JAK/STAT pathway stimulation and RhoA activity here is not clear. As such, our analysis, using Drosophila haemocytes and tumour formation as a genetically tractable low complexity in vivo system has identified a potentially crucial link. It will be intriguing to discover whether the Gz-mediated link between cytokine-activated JAK/STAT pathway signalling and Rho-dependent cytoskeletal dynamics and migratory behaviour is functionally conserved. If so, targeting the Gz subunits pharmacologically may represent a valuable approach to control metastatic invasion.

**MATERIALS AND METHODS**

**Fly stocks and genetics**

We used the following lines obtained from Bloomington Stock Center (USA), Vienna Drosophila RNAi Center (Austria), and the fly community: y*116, P(BAC1PB)Galapha[\(\text{f05574}\)], UAS-Rho[\(\text{t14}\)], UAS-Rho[199], Rho1-GFP, Rho[\(\text{B3}\),Cyo]. P(XP)Galaph[73B][\(\text{d01095}\)], P(XP)Baldsport[\(\text{d08619}\)]. ush-RNAi, stat-92E RNAi, Rho1-RNAi, hop\(\text{tum}^\text{tum}\)P[G5] (Bina et al., 2010), hop\(\text{tum}^\text{tum}\) (Luo et al., 1997), stat92E[\(\text{d08619}\]) (Silver and Montell, 2001), stat92E[\(\text{d01095}\]) (Baksa et al., 2002), srp-Gal4 and srp-Gal4.UAS-mCherry-Moesin (Stramer et al., 2010), pUAS-PKNG58AeGFP (Simões et al., 2006).

The Gal4/UAS system (Brand and Perrimon, 1993) was used to drive expression of UAS transgenes for overexpression and RNAi. Overexpression constructs for Gz73B were generated by cloning the full-length gene into a pUAS vector using the Gateway\(\text{®} System (Invitrogen, USA). Vectors were obtained from the Drosophila Genomics Resource Center, Indiana, USA. Constructs were injected into Drosophila embryos (BestGene, USA).

In order to create flies mutant for Gz73B, we used FLP/FRT-mediated recombination (Golic et al., 1997) to delete the region between the two P-elements P(XP)Baldsport[\(\text{d08619}\]) and P(XP)Galaph[73B][\(\text{d01095}\]) Loss of the genomic region was verified by PCR. As this recombination also removes part of the 3′ untranslated region of baldspot, we introduced a rescue construct (Jung et al., 2007), which partially reversed the embryonic lethality.

**In vivo tumour assays:** Heterozygous hop\(\text{tum}^\text{tum}\)P[G5] recombinants or hop\(\text{tum}^\text{tum}\) virgins were crossed to males carrying the UAS construct or mutation of interest. Eggs were collected for 24 hours at 19°C, incubated for another 24 hours at 19°C and then shifted 25°C. Adult females heterozygous for the hop allele and the UAS construct or mutation were scored for tumours using a tumour index as described by Shi et al., and validated by Student’s t-test. Error bars indicate ± standard error of the mean (s.e.m.) (Shi et al., 2008).

**Immunohistochemistry**

Third instar haemocytes were isolated by opening the larvae with forceps and bleeding the haemolymph into 30 \(\text{mL}\) phosphate buffered saline (PBS) onto a coverslip. Haemocytes were left to settle for 30 minutes at room temperature (rt), and were then fixed in 4% formaldehyde (Agar Scientific, UK) in PBS for 12 minutes. After three washes in 1× PBS haemocytes were blocked in 1× PBS, 0.1% Triton X-100 (Sigma-Aldrich, USA), 1% bovine serum albumin (BSA; Sigma-Aldrich, USA) for 1 hour at rt, and incubated with primary antibody in blocking solution overnight at 4°C. Haemocytes were incubated with secondary antibodies in PBS at rt. Coverslips were mounted onto slides with PermaFluor (Thermo Scientific, USA). All confocal images were acquired on a Leica SP1 (Leica, Germany).
Lamellocytes and tumours were isolated as above, but using ConAvalin A (Sigma-Aldrich, USA)-coated coverslips. Cells were left to settle for 30 minutes at rt, and fixed in acetone for 6 minutes, air dried, and rehydrated in 1x PBS for 10 minutes. After blocking in 1x PBS, 0.1% BSA, haemocytes were incubated in L1 cell supernatant supplemented with other primary antibodies overnight at 4°C. Washes were performed in 1x PBS at rt, and secondary antibodies were applied in 1x PBS for several hours at rt. Coverslips were mounted as above.

Dissections and immunostainings of lymph glands were as described previously (Krzemien et al., 2007). Antibodies used: AF488-α-tubulin 1:60 (Invitrogen, USA), L1 undiluted and P1 1:30 [a gift from I. Ando, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary (Kurucz et al., 2007)], anti-Collier 1:50 and anti-PSA 1:200 [a gift from M. Crozatier, Université Paul Sabatier, Toulouse, France (Krzemien et al., 2007)], ProPO 1:200 (gift from H. M. Müller via M. Crozatier), FITC-GFP 1:500 (Abcam, UK) and phallolidin 1:50 (Invitrogen, USA). To-Pro3 was used to stain DNA (Molecular Probes, USA).

Quantitative PCR
Total RNA extracted using TriReagent® (Sigma-Aldrich, USA) from Kc167 cells, third instar larval haemocytes or whole flies was used for Q-PCR as indicated in the figure legends. Upd (JAK/STAT pathway ligand)-conditioned media were prepared as described previously (Harrison et al., 1998). Kc167 cells were treated with Upd- or mock-conditioned medium for 30 minutes, washed, and total RNA was extracted after 4 hours. Haemocytes were isolated and put on coverslips as described above, and incubated for 30 minutes at rt before RNA extraction.

Primary sequences are available on request. Q-PCR was performed using SYBR-Green® Jump Start® TaqReadyMix® (Sigma-Aldrich, USA) on a Bio-Rad MyIQ® system or on a Bio-Rad CFX96® Real-Time System according to manufacturer’s’s (Bio-Rad, UK) recommendations. PCR was performed in triplicate and analysed by the ΔΔCt method, and normalized to rpL32 expression. Statistical validation was performed using Student’s t-test. Error bars indicate ±s.e.m.

Haemocyte analysis
Haemocyte counts were performed as described previously (Zinyk et al., 1993). The differentiation index was determined from haemocytes isolated, fixed and stained with L1 as described above.

The number of filopodia, and cell perimeter and area were determined for haemocytes isolated and fixed as described above. Perimeter and area measurements were performed using ImageJ. Statistical validation was performed using Student’s t-test; error bars indicate ±s.e.m.

For time-lapse imaging, wild-type or G73B mutant haemocytes carrying the srf-Gal4>moesin-mCherry insertion were isolated into PBS in a Lab-Tek® Chambered Borosilicate Coverglass System (Nalgene-Nunc, USA), incubated for up to 30 minutes and imaged for 5 minutes on a Perkin-Elmer (USA) UltraViewVox Spinning Disc system using Velocity software. Z-stack assembly and kymograph imaging was performed using ImageJ. Statistical validation was performed using Student’s t-test; error bars indicate ±s.e.m.

For PKNG58acGFP analysis, haemocytes were isolated, fixed and stained as described above. Confocal images were acquired at constant settings, and GFP intensity was determined using ImageJ. Statistical validation was done using one-way ANOVA. Error bars indicate ±s.e.m.

Tissue culture
Drosophila S2R+ cells were cultured under standard conditions and transfected with Gateway®-derived full-length FLAG-tagged-G73B expressed from an actin-derived promoter using Effectene according to manufacturer’s’s recommendations (Qiagen, Netherlands). Immunostainings were performed as described for haemocytes. Antibodies used were rabbit anti-FLAG and mouse anti-Rho1 (DSHB, USA).

Acknowledgements
The authors are indebted to M. Crozatier for her generosity and assistance throughout this project. In addition, we wish to thank M. Schäfer, I. Ando, D. Harrison, R. Schultz, D. Montell, W. Wood, A. Jacinto, C. Dearolf, L. Dubois, H. Strutt and N. Shepherd for fly stocks, antibodies, technical assistance and helpful comments. Imaging was undertaken at the University of Sheffield Wellcome Trust Light Microscopy Facility and at the Centre de Biologie du Development, Universite Paul Sabatier, Toulouse, France. MPZ is a Cancer Research UK Senior Cancer Research Fellow.

Competing interests
The authors declare no competing interests.

Author contributions
N.B. devised and undertook all experimental work, analysed the data and wrote the paper. M.P.Z. devised experiments, analysed data and wrote the paper.

Funding
This work was supported by a Cancer Research UK Senior Cancer Research Fellowship [number C24089/A8301 to M.Z.], and a Cancer Research UK Research Travel Award [number C43953/A14288 to N.B.]. Deposited in PMC for immediate release.

Supplementary material
Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.132852/-/DC1

References


Lu, Y. and Settleman, J. (2005). Cell invasion of highly metastatic MTLn3 cancer cells is dependent on phospholipase D2 (PLD2) and Janus kinase 3 (JAK3). *J. Mol. Biol.* 408, 850-862.


**Fig. S1.** Crystal cell differentiation. Wild-type *w^{1118}* (A) and *Ga73B^{4B7}* homozygous (B) third instar larvae showing the presence of crystal cells as visualised by heating to 60°C for 10 min in PBS. Arrows highlight individual crystal cells present in both larvae.

**Fig. S2.** *Ga73B* expression does not alter haemocyte differentiation. Haemocytes isolated from third instar larvae heterozygous for the *serpent-Gal4 (srp>)* insertion expressing either GFP (A), *Ga73B* tagged with Venus fluorescent protein (B) or *Ga73B* alone (C). Haemocytes are stained with anti-GFP (green) and phalloidin (magenta) to visualise F-actin.
Fig. S3. Quantification of haemocyte morphology phenotypes. (A) Still image from Movie 1 showing the positions of segments (green lines) used to generate the Kymographs which were used to measure filopodial extension frequency and length (shown in Fig 5). (B,C) Representative Kymograph showing the approach used to measure the number of filopodial extensions (B) and their length (C).
Movie 1. Haemocytes from $w^{1118}$ wild-type larvae expressing $srp$-Gal4, $UAS-mCherry-Moesin$ to visualise live filopodial dynamics. Movie represents a 5-minute time course with each frame showing a maximum projection of the original Z-stack dataset.

Movie 2. Haemocytes from $Ga^{73B4B7}$ larvae expressing $srp$-Gal4, $UAS-mCherry-Moesin$ to visualise live filopodial dynamics. Movies represent a 5-minute time course with each frame showing a maximum projection of the original Z-stack dataset.