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PDGF/VEGF signalling controls cell size in Drosophila

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Abstract

Background

In multicellular animals, cell size is regulated by a limited set of conserved intracellular signalling pathways, which when deregulated contribute to tumourigenesis by enabling cells to grow outside their usual niche. To delineate the pathways controlling this process, we screened a genome-scale, image-based *Drosophila* RNAi dataset for dsRNAs that reduce the average size of adherent S2R+ cells.

Results

Automated analysis of images from this RNAi screen identified the receptor tyrosine kinase *Pvr*, Ras pathway components and several novel genes as regulators of cell size. Significantly, *Pvr*/*Ras* signalling also affected the size of other *Drosophila* cell lines and of larval haemocytes. A detailed genetic analysis of this growth signalling pathway revealed a role for redundant secreted ligands, *Pvf2* and *Pvf3,* in the establishment of an autocrine growth signalling loop. Downstream of *Ras1,* growth signalling was found to depend on parallel MAPK and PI3K signalling modules, as well as the *Tor* pathway.

Conclusions

This automated genome-wide screen identifies autocrine Pvf/Pvr signalling, upstream of Ras, MAPK and PI3K, as rate-limiting for growth in immortalised fly cells in culture. Since, *Pvf2/3* and *Pvr* show mutually exclusive *in vivo* patterns of gene expression, these data suggest that co-expression of this receptor-ligand pair plays a key role in driving cell autonomous growth during the establishment of *Drosophila* cell lines, as has been suggested to occur during tumour development.

Background

Tissue growth is regulated by a balance of cell growth, proliferation and apoptosis. In many systems, however, cell proliferation and the accumulation of individual cell mass (cell growth) have been shown to be regulated independently; including in mammalian cells [1], fly cells [2] and in yeast [3]. This is explained in part by the action of distinct signalling pathways [4]. *Ras-MAPK* signalling plays the predominant role in the promotion of cell proliferation in mammalian cells, while *PI3K* and *Tor* signalling pathways primarily control the ability of individual cells to accumulate mass, through the promotion of ribosome biogenesis and protein translation [5]. To maintain cell size during tissue growth it is therefore important that increases in cell proliferation and the rate of mass accumulation be coordinated. One way to do this is via pathway crosstalk, and there is increasing evidence for direct crosstalk between growth and proliferation signals during normal development [6, 7] and in diseased states [7-9]. Historically, genetic screens to identify genes controlling cell proliferation and growth have been carried out in a tissue context in the animal [10]. Since the discovery of RNAi [11], however, several groups have taken advantage of the ability to carry out systematic, genome-scale RNAi screens in *Drosophila* cell culture [12-15] to address this problem. Through the design of luciferase and FACS based RNAi screens, large numbers of genes have been identified that regulate overall population growth, cell cycle progression, cell size, cell viability and Ras-MAPK signalling [13-15]. In addition, methods have been developed to carry out high content cell-based RNAi screens in *Drosophila* cell culture [16]. Whilst the analysis of such data sets represents a challenge, computational tools have recently been developed that allow an automated analysis of phenotypes from cell images [17]. We have used an automated image analysis pipeline to screen images from a genome-scale, high-content RNAi screen (Sims *et al*, unpublished data) for genes that limit the average size of adherent haemocyte-derived S2R+ cells [16, 18]**.** In this way, we have identified a novel role for autocrine *Pvf/Pvr* signalling, upstream of both Ras and PI3K, as a rate-limiting step in the regulation of *Drosophila* cell size.

Results

A Genome-scale RNAi Screen Reveals Genes that Regulate Cell Size

To identify regulators of *Drosophila* cell size, a library of ~22,000 dsRNAs covering 91% of the *Drosophila* genome [14] was screened in 384-well plates. After five days of RNAi treatment, S2R+ cells were stained to visualise F-actin, microtubules and DNA and imaged by automated microscopy (Figure 1). The resulting images were computationally analysed to identify dsRNAs that led to a reduction in average cell area. First, regions of each image containing a monolayer of adherent cells were identified using an algorithm that removes cell clumps and non-cellular background. Individual nuclei within this region were identified, and average cell area calculated by dividing the monolayer area by the nuclear count (Figure 1). Next, scores were normalised using the CellHTS package [19] within the online RNAi database FLIGHT [20] (Figure 1). Results were then filtered to remove dsRNAs that have a profound affect on cell number (Figure 1), which included many housekeeping genes. Finally, manual curation was used to filter out dsRNAs displaying secondary phenotypes, including defects in cell adhesion. The remaining scores were then ranked based on normalised mean cell area, to reveal the 15 dsRNAs that act most potently to reduce S2R+ cell size (Table 1 & Additional data file 1), all of which are conserved between fly and human.

Network analysis of this putative hit list in the database FLIGHT [20] revealed a core set of genes that participate in the Ras/MAPK signalling pathway (*drk*/*Grb2*, *csw*/*SHP-2*, *Sos*, *Ras1, Dsor1/MEK* and *rl*/*ERK*) (Figure 1). In most cases, Ras/MAPK signalling is thought to be activated downstream of ligand binding to a receptor tyrosine kinase [21]. It was therefore notable that the screen identified a single receptor tyrosine kinase, *Pvr*, that exhibited a strong reduced cell size phenotype (Table 1). *Pvr* is the sole member of the *PDGF/VEGF* family of receptors in *Drosophila*, and has previously been implicated in a range of cellular functions including migration, proliferation and survival [22-31], but not thus far in the regulation of cell size.

Of the remaining putative hits, only *Rheb*, a component of the growth regulating *Tor* pathway [32], had a known signalling function. Novel hits were diverse in functions and included *CG9306*, a component of the electron transport machinery, *Nup44A*, a nuclear pore component and several transcription factors. The screen also identified a large number of housekeeping genes, such as ribosomal components *Rps8* and *Rps18* and proteosomal components *Prosalpha7* and *Pomp*, most of which led to a reduction in both cell size and number (see Additional data file 1). However, given our focus on cell growth, we limited our further analysis to delineation of the signalling pathway by which *Pvr* and *Ras1* regulate cell size.

Validation of Hits Affecting Cell Size

In order to reduce the likelihood of false positives resulting from sequence-specific off-target effects, two non-overlapping dsRNAs were used to validate each putative hit identified in the screen [33]. RNAi phenotypes for all components of the canonical Ras/MAPK signalling pathway (Figure 2a) were verified using a microscopy-based assay (Figure 2b) and by using an electronic cell counter to directly measure cell volumes (Figure 2c). This analysis revealed that dsRNAs targeting *Pvr*, *Grb2, Sos*, *Ras1*, *ERK* and *ksr* reduce cell size. Conversely, RNAi-induced silencing of *Gap1*, a *Ras1* GTPase activating protein (GAP) that is a negative regulator of the pathway, led to a significant increase in cell size. However, RNAi-induced silencing of *Raf* and *MEK,* previously described as members of the Ras/MAPK signalling pathway, failed to generate equivalent changes in cell size, even when targeted using different dsRNAs (data not shown). Why this might be the case is explored below.

Pvf2 **and** ***Pvf3*** **Redundantly Activate** ***Pvr*** **to Control Cell Size**

In order to identify the upstream signal(s) that trigger the *Pvr*-dependent increase in S2R+ cell size, we turned our attention to the previously described *Pvr* ligands, *Pvf1*, *Pvf2* and *Pvf3* [24]. Since, none of these three ligands were identified in the phenotypic screen (see Additional data file 2), we tested for functional redundancy between the 3 ligands using RNAi to silence the expression of Pvfs in combination. Whilst silencing of individual Pvfs failed to induce a change in cell size, a significant reduction in cell size was observed when *Pvf2* and *Pvf3* were silenced together (Figure 2d), suggesting that these two ligands act redundantly to activate *Pvr*. No such synergy was seen with *Pvf1* and the other ligands.

To verify this putative role for *Pvf2* and *Pvf3* in the control of S2R+ cell size, cells were transiently transfected with *Pvf* containing plasmids. *Pvf* expression was then induced and cell volumes were measured using the automatic cell counter. Significantly, the expression of either *Pvf2* or *Pvf3* was sufficient to induce a significant increase in the average size of S2R+ cells relative to a GFP control (Figure 2e). By contrast, *Pvf1* expression had no detectable effect on cell size (Figure 2e). Although it is unclear why one particular ligand should be non-functional in this specific context, previous studies have shown that different ligands operate in different settings *in vivo* [26-28, 30]. Importantly, the increase in cell size induced by *Pvf2/3* was observed across the population, even though transfection efficiencies remained at ~20%. This implies that secreted *Pvf2* and *Pvf3* are able to diffuse in the culture medium to trigger cell signalling in a paracrine fashion, as has been previously suggested [29]. To confirm that this effect of *Pvfs* on cell size was mediated by the *Pvr* receptor, an epistasis experiment was carried out in which *Pvr* RNAi cells were transfected with a construct expressing *Pvf3* (Figure 2e), or a control plasmid. As expected, no significant differences in cell size were observed between the experimental and control populations, confirming that *Pvfs* act via *Pvr* to alter cell size.

***Pvr* Signalling Controls Cell Growth**

Changes in cell size can occur in the absence of alterations in the rate of cell growth via an acceleration or delay of cell division [34, 35]. Such effects were clearly seen in the screen, where the silencing of *cdc25* (*string*) caused growing cells to arrest in G2, resulting in a large increase in cell size over time (yielding a mean cell area z-score of +13.51) and a concomitant reduction in cell number. Conversely, the acceleration of cell cycle progression induced by silencing a negative regulator of the cell cycle, *wee*, reduced cell size (yielding a mean cell area z-score of -1.53). Noticeably, however, this was not accompanied by a reduction in cell number like that seen following *Pvr* or *Ras* RNAi (data not shown) [2].

Because of this link between cell cycle progression and cell size, it was important to determine whether changes in cell cycle progression contribute to the effects of *Pvr/Ras* signalling on cell size. To do this, we used a FACS analysis to examine the cell cycle profile of cells compromised for *Pvr/Ras* signalling. This revealed a significant increase in the proportion of cells in G1 in cells treated with dsRNA targeting *Pvr* or *Ras* (Figure 3a). This could be the result of a delay in the progression of cells from G1 into S-phase or the arrest of a sub-population of cells at the G1/S transition. To determine which is likely to be the case, in a second experiment we used the incorporation of BrdU as a measure of the proportion of cycling cells. BrdU was added to *Pvr, Ras* and *Rheb* RNAi cells 3 days after dsRNA treatment. Cells were then fixed and permeabilised 24 hours later so that incorporated BrdU could be visualized (Figure 3b). In each case, the percentage of BrdU positive cells was similar to that of the GFP RNAi control (>50%). These data strongly suggest that *Pvr*/*Ras* silencing causes a shift in the relative timing of G1/S and G2/M progression, without inducing a cell cycle arrest.

We then combined dsRNA targeting *Pvr* or other components of the Ras/MAPK pathway (*Sos*, *Ras1*, *ksr*, *Raf*, *MEK* and *ERK*) with *string* dsRNA to determine whether *Pvr/Ras* is required for cell growth in S2R+ cells that are unable to cycle. In each case, the FACS profile revealed a large G2 peak (data not shown), and an accompanying reduction in BrdU incorporation between days 3-4 (Figure 3b), as expected for a *string* dsRNA-induced G2/M arrest. Significantly, however, dsRNAs targeting components of the *Pvr/Ras* pathway caused a significant reduction in the size of *string* RNAi cells (Figure

3c), indicating that the pathway is required for cell growth in cells arrested in G2, as it is in cycling cells. Taken together, these data suggest that the *Pvr/Ras* pathway is rate-limiting for the growth (accumulation of mass) of S2R+ cells and, either directly or indirectly, affects the relative time cells spend in G1 and G2.

Tor but not Insulin Signalling is Required for Growth of S2R+ Cells

Since *Pvr* has not been previously reported to control cell size, we examined the role of established growth regulatory pathways in the S2R+ cell line. Previous studies have identified the protein kinase *Tor* as a key regulator of cell growth in a wide variety of eukaryotic systems [32]. In the canonical Tor pathway, the small GTPase *Rheb* activates the *Tor/Raptor* complex which phosphorylates ribosomal S6-kinase to stimulate cell growth [32]. However, our genome-wide RNAi screen only identified a single member of the *Tor* pathway, *Rheb,* as a putative regulator of cell size. A closer examination of the screen data revealed that our failure to identify other components of the *Tor* signalling pathway was due in part to the stringent cut-off employed in the computational analysis to reduce the number of false positives. In fact, *Tor*, *Raptor* and *S6k* silencing was associated with a small, but measurable decrease in cell area ($z < -1.6$ in each case), suggesting that the *Tor* pathway does indeed play a role in the control of cell growth in S2R+ cells. To confirm this, we generated non-overlapping dsRNAs for each pathway component and directly measured cell sizes using the electronic cell analyser. All core members of the canonical Tor pathway displayed the expected RNAi phenotype. Silencing positive regulators of the pathway (*Rheb*, *Tor*, *Raptor* and *S6k*) led to a significant decrease in cell size (Figure 3d). Conversely, dsRNAs targeting either of the two negative regulators of the pathway, *Tsc1* and *Tsc2* (which together form a *Rheb* GAP), increased cell size (Figure 3d). Furthermore, FACS analysis revealed that *Rheb* or *Tor* RNAi leads to an increase in the proportion of cells in G1, similar to that seen in *Pvr* RNAi cells (Figure 3a). These results confirm that the canonical *Tor* pathway controls cell growth in S2R+ cells, as previously demonstrated in S2 cells [36, 37] and *in vivo* [10].

Tor has been shown to act downstream of insulin-induced RTK signalling to control *Drosophila* cell growth *in vivo* [10]. Moreover, insulin has been shown to stimulate the growth of *Drosophila* cells *in vivo* [38] and in fly cell culture [39]. This signal is mediated by the insulin receptor (*InR*). The activated receptor recruits the insulin receptor substrate (*IRS*) adaptor protein, which binds the regulatory (*p60*) subunit of class I phosphoinositide-3-kinase (PI3K), enabling the catalytic *(p110)* subunit to convert the phospholipid PIP2 to PIP3 in the membrane. PIP3 then recruits several downstream targets, most notably *PDK1,* to the membrane, to induce the phosphorylation and activation of *Akt/PKB*, which goes on to inactivate *Tsc1/2* to stimulate the *Tor* pathway. In analysing the role of insulin signalling in the growth of S2R+ cells, we first verified that insulin is able to alter their growth in cell culture. As expected for a cell line with an intact insulin signalling pathway, the addition of insulin to the medium of these cells increased the rate of proliferation and average cell volume (data not shown). This does not mean, however, that insulin signalling is required for normal S2R+ cell growth. To test whether or not this was the case, we measured cell size following RNAi-induced silencing of pathway components. Knocking down of upstream components *InR*, *IRS*, *p60* or *p110* had no effect on cell size (Figure 3d), even though this was sufficient to fully (*InR*, *IRS*, *p60*) or partially (*p110)* [40] attenuate the insulin-induced phospho-*Akt* response (data not shown), while *PDK1* or *Akt* silencing induced a small reduction in cell size (Figure 3d). These experiments suggest that while the insulin pathway is operational in S2R+ cells, it is not rate-limiting for size control in this cell line under normal cell culture conditions.

The PI3K and MAPK Pathways Act in Parallel to Relay the *Pvr* Growth Signal

Having established important roles for *Pvr* and *Ras1* in the regulation of S2R+ cell growth, an RNAi epistasis analysis was used in an attempt to delineate the downstream signal events in more detail, and to understand the reason for the minor phenotypic consequences of using RNAi to deplete several well-established *Ras* targets. To begin this analysis we used the negative regulators of cell growth signalling in this system, *Gap1* and *Tsc2*, as genetic landmarks to position positive regulators within the pathway.

We began by using RNAi to modify the *Gap1*, phenotype. As expected, the *Gap1* RNAi induced increase in cell size could be suppressed by *ERK* RNAi, and reversed by dsRNA-mediated silencing of *Ras1*, and by reductions in the expression of downstream components of the Tor signalling pathway, *Rheb*, *Tor* and *S6K* (Figure 4a). Interestingly, however, direct targets of *Ras1*, *Raf* and *p110*, only partially suppressed the effects of *Gap1* RNAi (Figure 4a), mirroring the results of single *Raf* and *p110* RNAi experiments (Figures 2c and 3c). These results suggest a contribution of both the MAPK and PI3K pathways to the communication of the growth signal downstream of *Ras1*.

We then repeated this epistasis analysis, in a background in which the *Rheb* GAP *Tsc2* was silenced, deregulating *Rheb* activity to increase cell growth (Figure 3c). Once again, although several dsRNAs (*Ras1*, *p110* or *Akt*) reduced the extent of the cell size increase seen following *Tsc2* RNAi, the *Tsc2* phenotype dominated in each of these cases (Figure 4b), placing these genes genetically upstream of *Tsc2*. Although different pathway members (e.g. *Pvr* versus *Ras1*) exhibited minor differences in their ability to suppress the Tsc2 phenotype, we believe that this is likely to reflect the fact that epistasis experiments are inherently sensitive to gene-specific differences in the kinetics of RNAi knockdown. Only *Rheb* and *S6k* strongly attenuated the *Tsc2* phenotype, implying that they function downstream of *Tsc2,* as previously reported [32]**.** Taken together (compare Figure 4a and 4b), these results suggest that *Pvr/Ras* signalling is likely to operate upstream of the *Tor* pathway in controlling cell growth in S2R+ cells, although we cannot exclude the possibility that *Ras* and *Tor* signalling operate in parallel.

These results focused our attention on the function of intermediate pathway components that have a minor impact on cell growth when targeted using RNAi (Figures 2c and 3d). Since *Ras* has been shown to signal directly to both PI3K and *Raf* in other systems [41], we decided to use combinatorial RNAi experiments to test whether *p110* and *Akt* might cooperate with *Raf* in relaying the growth signal downstream of *Ras1* in S2R+ cells (Figure 4c). This analysis revealed a set of additive and synergistic interactions between components of MAPK and PI3K pathways (Figure 4c and 4d). This was clearest for *p110*, since the reduction in cell size observed following silencing of *p110* together with either *Raf*, *ksr* or *ERK* was equal to or greater than the sum of phenotypes observed in

RNAi experiments targeting these genes independently (Figure 4c, 4d and data not shown). In addition, there was an additive effect of targeting *Akt* and these components of the MAPK pathway. Since *InR* RNAi failed to enhance the effect of *Raf* silencing (Figure 4c), this synergy between *Raf* and *p110/Akt* is unlikely to be the result of a parallel input from insulin signalling. Instead, because *p110/Akt* RNAi did not synergise with *Pvr* and *Ras1* RNAi (Figure 4d), the PI3K pathway likely functions downstream of *Ras1* in this growth assay, as has been described in other systems [41]. Taken together, these results suggest that signals relayed by both the MAPK and PI3K pathways cooperate in growth signalling. Indeed, both *Akt* and *ERK* have been shown to phosphorylate and inactivate *Tsc2* in mammalian systems [42-45]. Thus, the *Tsc1/Tsc2* complex may serve as a hub to integrate growth signals.

Pvr **Controls Cell Size in Other** ***Drosophila*** **Cell Lines and in Larval Haemocytes**

Having identified a *Pvr* signalling pathway that is rate-limiting for the growth of the S2R+ haemocyte-derived *Drosophila* cell line, we extended this analysis to investigate possible implications for the growth of other cells. First, we examined the effects of *Pvr* silencing in a variety of other *Drosophila* cell lines. *Pvr* showed a strong cell size phenotype in both the S2 haemocyte cell line and the neuronal cell line ML-DmBG3-c2 (Figure 5a), implying that *Pvf/Pvr* autocrine signalling is a common feature of the growth of *Drosophila* cell lines in culture.

To test whether *Pvr* might play a similar role in the regulation of cell size *in vivo*, we extracted haemocytes from *Drosophila* larvae containing *Pvr* or *Ras* RNAi constructs under the control of two different haemocyte drivers (Hml-Gal4 and Cg-Gal4). In both cases, we used UAS-GFP as a marker to confirm that transgenes were being expressed in these primary cells. In order to estimate cell size, mean cell area was measured after GFP-labelled cells had been given time to spread on an adhesive concanavilin A-coated surface. As controls, we also measured the spread area of cells lacking either the driver (data not shown) or the RNAi hairpin. In this experiment, *Pvr* silencing or the expression of a dominant negative *Pvr* or *Ras* construct led to a significant reduction in the size and number of haemocytes relative to control experiments (Figure 5b & data not shown). Although *Ras1* over-expression has previously been shown to cause an increase in larval haemocyte number [46], which necessarily requires coincident cell growth and division, these data suggest that Ras/MAPK signalling also plays a role in mass accumulation. Thus, *Pvr* and *Ras1* control the growth, proliferation [29] and viability [22] of *Drosophila* haemocytes *in vivo*.

Discussion

In this study, we have used an automated image analysis pipeline to screen through images from a high-content, genome-wide RNAi screen for genes whose activity is rate-limiting for the growth of *Drosophila* cells in culture. In doing so, we identified a number of known and novel genes regulating cell size. Interestingly, this screen identified a novel role for autocrine signalling through *Pvfs* and the receptor tyrosine kinase *Pvr* in the control of the autonomous growth of *Drosophila* cells in culture. Previous studies have suggested roles for *Pvf/Pvr* signalling in the control of cell migration [23, 27, 30], morphogenesis [25, 26, 31], cell viability [22] and proliferation [28, 29]. However, to our knowledge this is the first clear example of this pathway controlling cell size. This reduction in the size of *Pvr* RNAi cells was accompanied by a reduction in cell proliferation, as revealed by reduced cell numbers in the absence of significant apoptosis (data not shown), and by a delay in the passage of cells through G1 and into S phase. One interpretation for these observations is that Pvr knockdown might trigger autophagy in S2R+ cells. Indeed, one of the first responses that cells exhibit when confronted with unfavourable growth conditions is to shrink in size and trigger autophagy to meet their energy demand during poor nutritional conditions. It is also possible that a loss of cell mass following *Pvr/Ras* RNAi contributes to the small cell phenotype, should this growth signalling pathway regulate both growth and autophagy in S2R+ cells.

Next, we used an RNAi epistasis analysis to help delineate this *Pvr* growth-signalling pathway. Interestingly, whilst the effects of dsRNAs targeting upstream components (*Pvr, Sos* and *Ras)* of the Pvr/Ras pathway was much greater than the effects of targeting individual downstream components (*Raf, Ksr* and *ERK*), dramatic reductions in cell size were observed when dsRNAs were combined that target both PI3K and MAPK arms of the downstream signalling pathway (Figure 4d). These data suggest that the growth signal downstream of *Ras* requires the combined activity of downstream PI3K and MAPK signalling modules. Our data also suggest the possibility that these signalling pathways act upstream of the *Tor* pathway in S2R+ cells, as shown in other systems [42-45]. Although this type of growth signal integration has not been previously reported in *Drosophila*, *Ras1* has been shown to influence both growth and G1/S cell cycle progression *in vivo* [47] and *Ras1* has been shown to crosstalk to *dp110* in the control of *in vivo* cell and tissue growth [6].

In the context of development, crosstalk between signalling pathways, like that seen in our analysis, could help to integrate information from different types of intrinsic and extrinsic cue in order to aid cellular decision making. Alternatively, as seen in this study, the use of parallel signalling modules (PI3K and MAPK in this case) may serve to buffer cellular behaviour from changes in the relative levels of different input signals. However, *in vivo*, we would not expect to observe many instances of autocrine growth signalling, since this is inherently hard to regulate. Indeed, *in situ* hybridisation studies in *Drosophila* embryos suggest that receptors such as *Pvr* are expressed in specific populations of cells, such as haemocytes, that do not express any of the corresponding ligands *Pvf1-3* [23]. Similarly, Pvf secretion is restricted to particular compartments in pupal stages, and when deregulated can cause tumorous growth [29]. These data suggest that mutations leading to *Pvr* and *Pvfs* co-expression may contribute to the establishment of autonomous cell growth during the establishment of *Drosophila* cell lines in media based on bovine serum, which lacks strong activators of ERK and PI3K signalling [40]. In the future it will therefore be interesting to investigate the mechanisms used to ensure that the *in vivo* expression of ligand receptor pairs, like *Pvfs* and *Pvr,* remains mutually exclusive, and to reveal how these controls are deregulated during the establishment of a *Drosophila* cell line. We would expect this information to be useful in the establishment of new cell lines in the future, and in furthering our understanding of the processes leading to the deregulated expression of ligand-receptor pairs during the development of a variety of cancers [48, 49].

Conclusions

In summary, this study presents evidence for a novel role for autocrine Pvf/Pvr signalling in cell growth, both in cell culture and *in vivo*. The *Drosophila* homologue of the mammalian PDGF/VEGF receptor family acts upstream of Ras, and diverging MAPK and PI3K signalling modules. Since, *Pvf2/3* and *Pvr* show mutually exclusive patterns of gene expression *in vivo*, these data suggest that co-expression of this receptor-ligand pair plays a key role in driving cell autonomous growth during the establishment of *Drosophila* cell lines, as has been suggested to occur during tumour development.

Materials and Methods

Drosophila **Cell Culture**

S2R+ cells were grown in Schneider's medium (Invitrogen) or Shields and Sang M3 insect medium (Sigma) with 10% heat-inactivated fetal bovine serum (JRH Biosciences) and penicillin-streptomycin (Sigma). S2 cells were grown in InsectExpress media with L-Glutamine (PAA Laboratories). ML-DmBG3-c2 cells were cultured in M3 media supplemented with FBS, antibiotics and 10µg/ml bovine insulin (Sigma). *Drosophila* S2R+ cells were transiently transfected using the CellFectin (Gibco) lipid transfection reagent according to the manufacturer's protocol. Where necessary, gene expression was induced by addition of 1mM Cu SO_4 solution.

RNAi

dsRNA templates were amplified by PCR from genomic DNA using pairs of gene-specific primers. dsRNA synthesis was performed using the T7 Megascript kit (Ambion). RNA preparations were purified using PCR96 cleanup plates (Millipore) attached to a vacuum pump. Purified RNAs were resuspended in TE and annealed by heating at 65°C for 10 minutes and cooling slowly. Typically, cells suspended in serum-free medium were mixed with dsRNA to give a final concentration of 30μg/ml then plated into tissue culture dishes and incubated at 24°C for 30 minutes. Subsequently, three volumes of complete medium was added and cells were grown for 5-7 days at 24ºC to allow for protein turnover [50].

Cytoskeletal Staining and Image Acquisition

Cells in 384-well plates were washed with phosphate buffered saline (PBS) and fixed for 10 minutes in 4% formaldehyde (Polyscience). After fixation cells were permeablised by washing with PBS containing 0.1% Triton-X-100 (PBS-T), then blocked with 5% bovine serum albumin (Sigma) in PBS-T for 20 minutes. For staining, cells were first incubated with 1:500 α-Tubulin antibody (Sigma) in PBS-T containing 1% BSA overnight at 4°C. Cells were then washed twice with PBS-T and incubated with FITC anti-mouse IgG secondary antibody (Jackson labs) combined with TRITC-Phalloidin (Sigma) and DAPI (Sigma) for two hours. For BrdU experiments, BrdU was added to the culture medium 3 days after the addition of RNAi. Cells were then fixed, acid washed and stained 24 hours later, using TRITC-labelled anti-BrdU antibodies to reveal the extent of BrdU incorporation into DNA. In each case, fluorescent images were acquired using an automated Nikon TE2000E microscope with a 20x objective and HTS (high throughput screening) MetaMorph software (Universal Imaging) running an automated stage and shutter (Prior), and a Roper CoolSNAP cooled-coupled device camera.

Computational Image Analysis and Score Normalisation

Image analysis was performed using the image analysis toolkit in Matlab (Mathworks). F-actin and microtubule stained images were processed to remove cell clumps and background, leaving the cell monolayer. DNA stained images were segmented to identify nuclei in the cell monolayer. Mean cell area was calculated as the area of the monolayer divided by the number of nuclei. Raw scores from image analysis were normalised to correct for systematic differences between assay plates. Normalisation was performed using the CellHTS package [19], part of the Bioconductor suite of biological data analysis packages for the R statistical computing environment. Briefly, mean cell area scores were normalised using median centring per plate, and screen z-scores were calculated using the screen median and the MAD (median absolute deviation). Replicate scores from different image sites in the same well were summarised using the closest to zero function (equivalent to taking the minimum, independent of sign) to calculate a single z-score for each screen well. The proportion of nuclei that had undergone division was established by computational image analysis of BrdU and DAPI images.

Cell Size & Cell Cycle Measurements

Cell volume was measured using a CASY cell counter and analysis system (Scharfe System). Cells diluted 1:101 in CasyTon reagent (Scharfe System) were measured in triplicate. The mean cell volume for each treatment was calculated as the average peak volume from three independent readings. For each experiment the peak cell volume (the peak in the histogram of individual cell volumes) for at least ten control wells measured in triplicate was used to establish a solid baseline for comparison. Since control cell size varied between experiments it was necessary to normalise scores for each experiment before summarisation. Thus, volumes were converted to the percentage of mean control cell volume. Percentage of mean control cell volume from at least two independent experiments were averaged and used to construct bar charts. For cell cycle profiles, the cells were fixed in 70% ethanol at -20C and subsequently resuspended in PBS containing 50 ug/ml propidium iodide and 60 ug/ml RNaseA. The profiles were acquired on a FACSanto anlyzer, using FACSDiva software (Becton Dickinson). All cells were in log growth phase during the course of the experiments.

In vivo **Methods**

Cg-Gal4, Hml-Gal4, UAS-GFP and *UAS-RasDN* lines were obtained from the Bloomington stock centre. The *UAS-PvrDN* line was a gift from P. Rorth and *Pvr RNAi* lines were gifts from Benny Shilo. Late third instar larvae were washed and the integument was disrupted in the latero-posterior region without organ disruption. The circulating haemocytes were directly collected in M3 medium. In each case, pooled haemocytes from several larvae were plated on conconavalin A (ConA) coated 384-well plates and allowed to spread flat on this substrate for 2 hours. Attached haemocytes were fixed, stained and imaged as above and cell area was measured computationally. UAS-GFP was used to confirm Gal4 expression in larval haemocytes.

Abbreviations

RNAi: RNA interference; dsRNA: double-stranded RNA; MAPK: Mitogen activated protein kinase; PI3K: Phospho-inositide-3-kinase; FACS: fluorescence activated cell sorting; BrdU: Bromodeoxyuridine; GFP: green fluorescent protein; UAS: Upstream Activation Sequence; ConA: conconavalin A; MAD: median absolute deviation; GAP: GTPase activating protein.

Authors contributions

DS performed the computational RNAi screen analysis and the subsequent hit validation and epistasis analysis. PD performed a visual analysis of the RNAi screen and the over-expression and FACS studies. DS and BB carried out the *in vivo* analysis. DS and BB designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Additional data files

The following additional data are available with the online version of this paper. Additional data file 1 describes in full the top cell size hits from computational analysis of images from a high-content genome-wide RNAi screen. This list was generated using a mean cell area z-score threshold of <-2. dsRNAs giving rise to visually confirmed phenotypes are highlighted in yellow. Columns J-L: ‘Y’ indicates a hit, ‘N’ indicates that a dsRNA was screened but not hit and '-' indicated that no dsRNA targeting the gene in question was screened. Additional data file 2 provides details of computational image analysis scores for all MAPK, Tor and insulin pathway components. Columns I-K: ‘Y’ indicates a hit, ‘N’ indicates that a dsRNA was screened but not hit and '-' indicated that no dsRNA targeting the gene in question was screened. All image analysis scores along with original images, and dsRNA primer & amplicon details from the entire screen are available online in the FLIGHT database [20].

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Figure legends

Figure 1. Workflow of the computational analysis of images from a high-content, genome-wide RNAi screen in *Drosophila* cell culture.

Raw images of S2R+ cells stained for F-actin, microtubules and DNA were analysed computationally to calculate the total monolayer area and mean cell area in each image (see methods for details). Image analysis scores were then normalised across screen plates to create z-scores (see methods for details). Hits were selected using a z-score threshold of -2 for mean cell area, and a monolayer area z-score of -2 to exclude images with low cell number where small cell size may reflect viability defects (green squares). This, approach yielded 73 putative hits (red triangles) which were examined for known physical and genetic interactions in FLIGHT [20].

Figure 2. *Pvr, Ras1* and the MAPK pathway control cell size in a *Drosophila* haemocyte-like cell line.

(A) Schematic of the canonical Ras/MAPK signalling. (B) Control and RNAi-treated S2R+ cells fixed and stained for microtubules (20x magnification). *Pvr* and *Ras1* RNAi cause a decrease in cell area, whereas *Gap1* RNAi causes an increase in cell area compared to control. (C-E) Charts of the mean percentage change in volume of RNAi treated or *Pvf* transfected S2R+ cells relative to control cells. (C) Silencing of positive regulators of the Ras/MAPK pathway causes a reduction in cell diameter, whereas silencing of *Gap1*, a negative regulator of *Ras1* signalling, causes an increase in cell diameter. (D) Silencing of *Pvf1, 2 or 3* individually has no effect on cell volume, but silencing of *Pvf2* & *Pvf3* in combination significantly reduces cell size. (E) Over-expression of *Pvf2* or *Pvf3*, but not *Pvf1* leads to an increase in cell diameter. This affect is dependent on *Pvr*.

Figure 3. *Pvf/Pvr* signalling controls cell growth and G1/S progression.

(A) FACS analysis of RNAi-treated S2R+ cells. Control cells typically exhibit a large G2 peak and a much smaller G1 peak. However, treatment with dsRNA to *Pvf2/3*, *Pvr, Ras1* or *Rheb* causes a significant increase in the G1 peak with a concurrent decrease in the G2 peak. (B) BrdU labelling of cells treated with dsRNA targeting the Pvr/Ras pathway suggests that cells are still cycling. However, parallel silencing of Cdc25 expression completely blocks the cell cycle. (C) *Cdc25* (*String*) RNAi causes a significant increase in cell volume. This increase is suppressed by simultaneous silencing of *Pvr* or members of the Ras/MAPK pathway. (D) Chart of the mean percentage change in cell volume of RNAi treated S2R+ cells relative to control (LacZ RNAi) cells. Upstream components of the insulin signalling pathway do not affect cell size. However, downstream components (*PDK1*, *Akt*) exhibit a small effect. Silencing of negative regulators of both insulin (*Pten*) and *Tor* signalling (*Tsc1/Tsc2*) results in a significant increase in cell size. Conversely, silencing of positive regulators of *Tor* signalling reduces cell size.

Figure 4. *Pvf/Pvr* signalling activates a network of signalling modules upstream of the *Tor* pathway.

(A) *Gap1* RNAi epistasis. *Ras1*, *Rheb, Tor* and *S6k* dominate in their effect on cell size, *ERK* suppresses the *Gap1* RNAi phenotype, whereas *Raf*, *p110* and *Akt* only partially ameliorate the large increase in cell size seen following *Gap1* RNAi. The insulin receptor has little effect in this assay. (B) *Tsc2* RNAi epistasis. *Rheb* and *S6k* dominate, placing them genetically downstream of *Tsc2*. *Pvr*, *Ras1* and members of the MAPK and PI3K pathways fail to have a dramatic impact on the *Tsc2* RNAi phenotype. (C) *Raf* (*pole hole*) RNAi epistasis. Silencing of *Raf* leads to a minor reduction in cell size. However, silencing of *Raf* in conjuction with *p110* or *Akt1* causes a large reduction in cell size, like that seen in *Pvr, Ras, Tor, Rheb, S6K* RNAi experiments. (D) MAPK and PI3K pathway genetic interactions. *Raf, ERK* and *Ksr* all show additive or synergistic genetic interactions with *p110* and *Akt1*, but not with *InR*. Furthermore, these genetic interactions are stronger than those seen when combining *p110* or *Akt1* dsRNA with dsRNA targeting upstream or downstream pathway components of this putative growth signalling network (*Pvr*/*Ras1* and *Tor*/*S6k* respectively).

Figure 5. *Pvr* controls cell growth in a variety of cell lines and in *Drosophila* haemocytes.

(A) F-actin staining of *Drosophila* S2 and ML-DmBG3-c2 (neuronal) cells treated with dsRNA to *LacZ* or *Pvr* reveals a reduction in cell size in *Pvr* RNAi cells relative to control. (B) *Pvr* and *Ras1* dominant negative constructs were expressed in larval haemocytes *in vivo* using the Cg-Gal4 driver. Haemocytes were extracted, allowed to adhere to ConA and then fixed and stained for F-actin. Cell area was measured from images of haemocytes (see methods). Both *Pvr* and *Ras1* DN constructs caused a significant reduction in haemocyte cell area relative to wild-type. (C) Driving expression of Pvr RNAi constructs in haemocytes using either Cg-Gal4 or Hml-Gal4 causes a significant decrease in cell size relative to control (driver or hairpin only) cells. Cells are stained for F-actin. Control and experimental images in B and C were taken at the same magnification.

Tables

Table 1. List of top hits from computational analysis of mean cell area in images from a high-content genome-wide RNAi screen.

Fly gene	**Human Homologue**	**Z-score**	**Function**
Sos	**SOS1**	**-4.09**	**Ras signalling**
drk	**GRB2**	**-3.73**	**Ras signalling**
CG11294	CART1	-3.07	Transcription Factor
gfzf	GSTT1	-3.02	Glutathione transferase
Pvr	**PDGF/VEGF**	**-2.92**	**Receptor Tyrosine Kinase**
rl	**MAPK1**	**-2.80**	**Ras signalling**
Sec61alpha	SEC61A2	-2.66	Protein secretion
Rheb	RHEB	-2.52	Tor signalling
Nup44A	SEH1L	-2.47	Nuclear pore
Ras85D	**KRAS**	**-2.30**	**Ras signalling**
csw	**PTPN11 (SHP-2)**	**-2.22**	**Ras signalling**
CG9306	NDUFB9	-2.20	Mitochondrial electron transport
CG9300	NOL11	-2.15	Sugar transporter
fax	C6orf168	-2.04	Axonogenesis
Dsor1	**MAP2K1**	**-2.02**	**Ras signalling**

This hit list was derived from an initial hit list (see Additional data file 1) following i) the confirmation of phenotypes by visual inspection and ii) the exclusion of genes causing other cell shape phenotypes. Genes participating in the Ras signalling pathway are highlighted in bold.

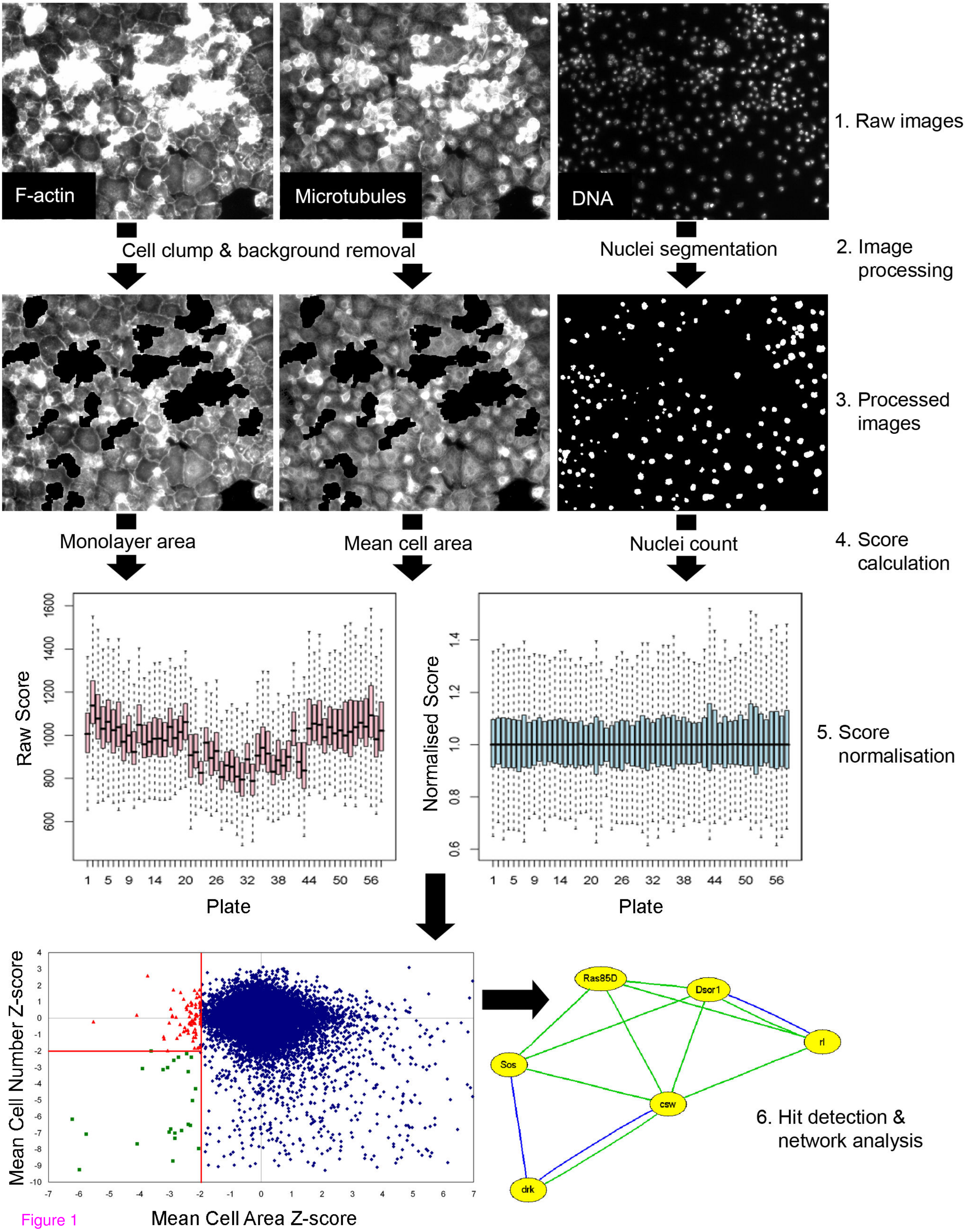

F-actin
Microtubules
DNA
1. Raw images
Cell clump & background removal
Nuclei segmentation
2. Image processing
3. Processed images
Monolayer area
Mean cell area
Nuclei count
4. Score calculation
Raw Score
600
800
1000
1200
1400
1600
Plate
1 5 9 14 20 26 32 38 44 50 56
Normalised Score
0.6
0.8
1.0
1.2
1.4
Plate
1 5 9 14 20 26 32 38 44 50 56
5. Score normalisation
Mean Cell Number Z-score
4 3 2 1 0 -1 -2 -3 -4 -5 -6 -7 -8 -9 -10
Mean Cell Area Z-score
-7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7
Ras85D
Dsor1
rl
Sos
csw
drk
6. Hit detection & network analysis

Figure 1

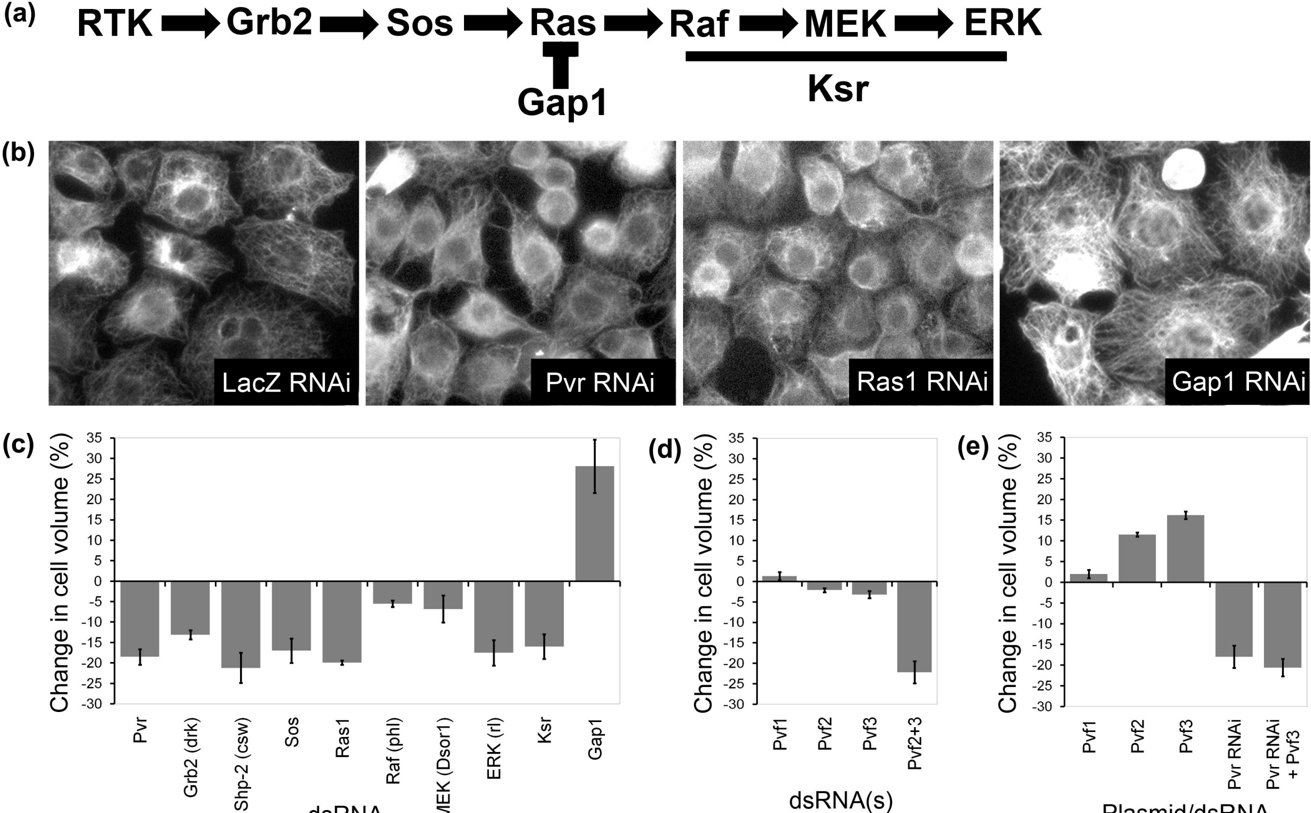

(a)
RTK
Grb2
Sos
Ras
Raf
MEK
ERK
Gap1
Ksr
(b)
LacZ RNAi
Pvr RNAi
Ras1 RNAi
Gap1 RNAi
(c)
Change in cell volume (%)
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
Pvr
Grb2 (drk)
Shp-2 (csw)
Sos
Ras1
Raf (phl)
MEK (Dsor1)
ERK (rl)
Ksr
Gap1
dsRNA
(d)
Change in cell volume (%)
Pvf1
Pvf2
Pvf3
Pvf2+3
dsRNA(s)
(e)
Change in cell volume (%)
Pvf1
Pvf2
Pvf3
Pvr RNAi
Pvr RNAi + Pvf3
Plasmid/dsRNA

Figure 2

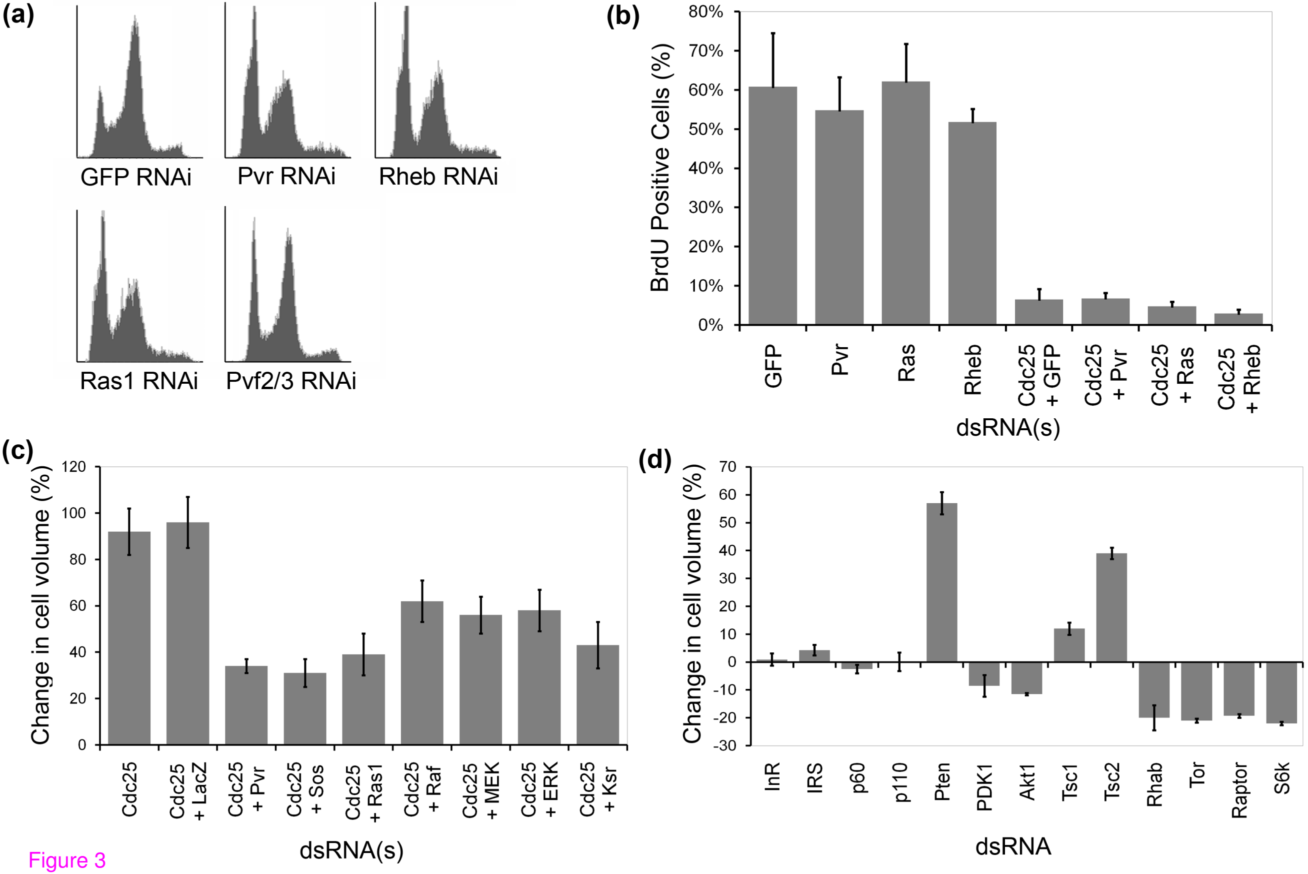
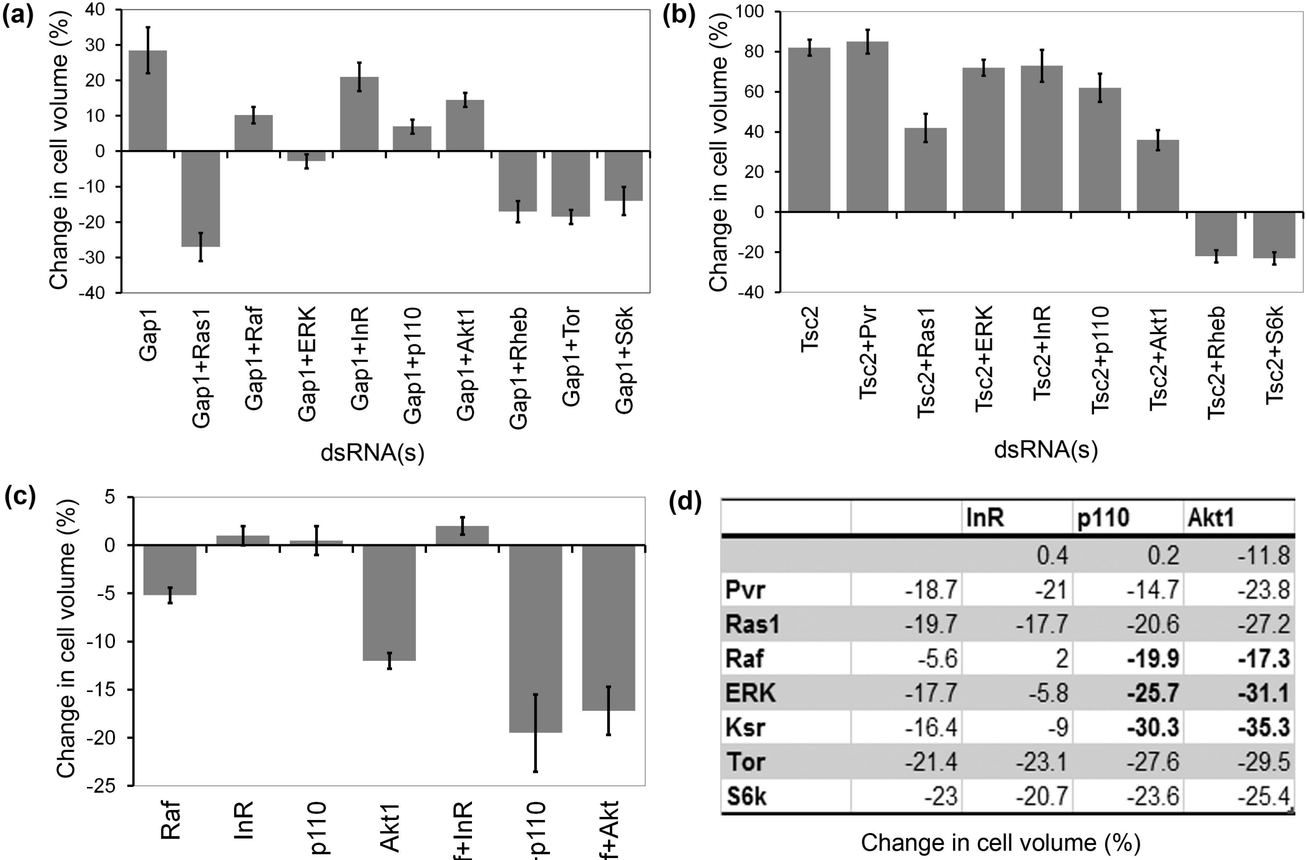

(a)
GFP RNAi
Pvr RNAi
Rheb RNAi
Ras1 RNAi
Pvf2/3 RNAi
(b)
BrdU Positive Cells (%)
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP
Pvr
Ras
Rheb
Cdc25 + GFP
Cdc25 + Pvr
Cdc25 + Ras
Cdc25 + Rheb
dsRNA(s)
(c)
Change in cell volume (%)
120
100
80
60
40
20
0
Cdc25
Cdc25 + LacZ
Cdc25 + Pvr
Cdc25 + Sos
Cdc25 + Ras1
Cdc25 + Raf
Cdc25 + MEK
Cdc25 + ERK
Cdc25 + Ksr
dsRNA(s)
(d)
Change in cell volume (%)
70
60
50
40
30
20
10
0
-10
-20
-30
InR
IRS
p60
p110
Pten
PDK1
Akt1
Tsc1
Tsc2
Rhab
Tor
Raptor
S6k
dsRNA

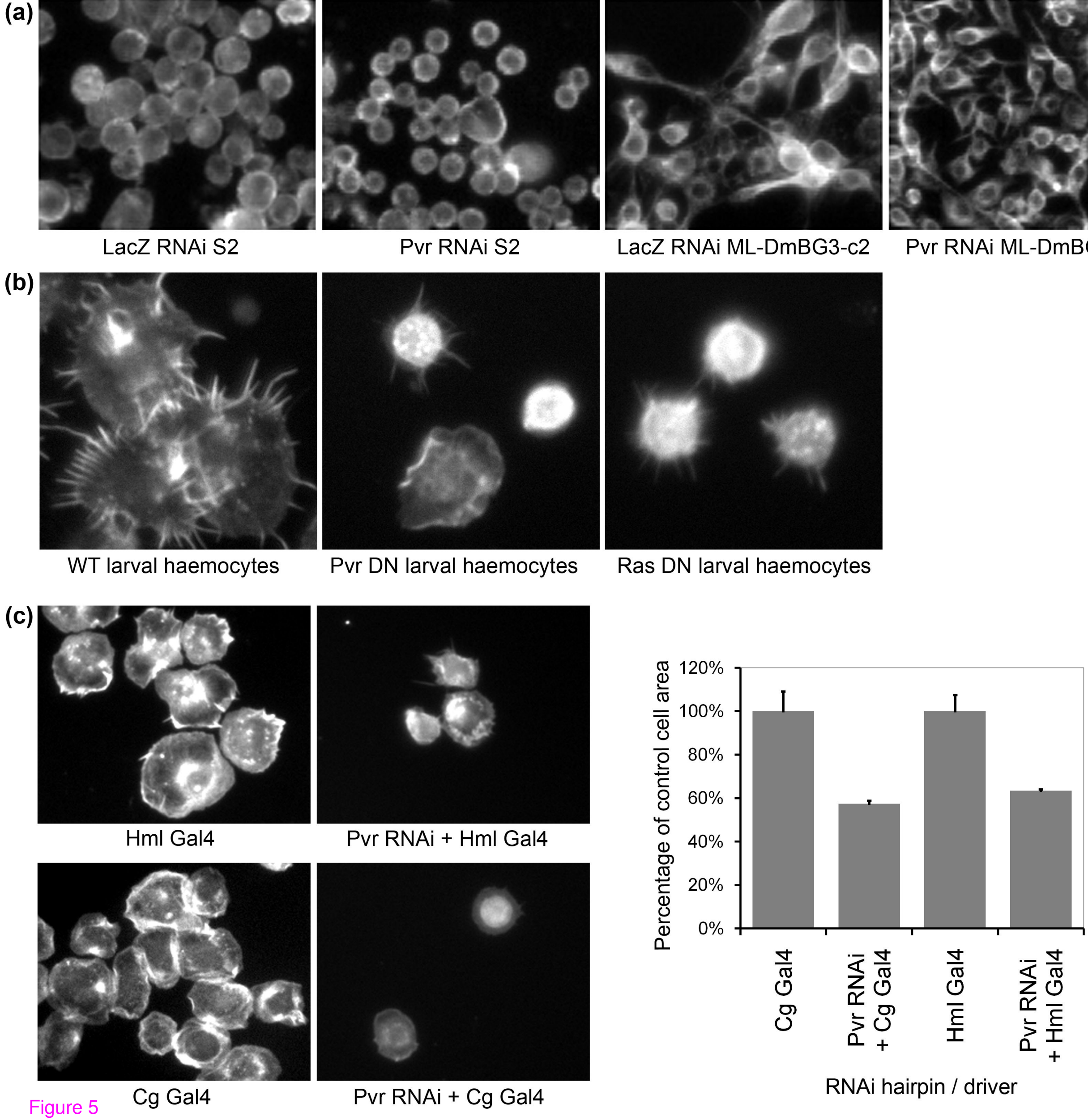
Figure 3



		InR	**p110**	**Akt1**
		0.4	0.2	-11.8
Pvr	-18.7	-21	-14.7	-23.8
Ras1	-19.7	-17.7	-20.6	-27.2
Raf	-5.6	2	**-19.9**	**-17.3**
ERK	-17.7	-5.8	**-25.7**	**-31.1**
Ksr	-16.4	-9	**-30.3**	**-35.3**
Tor	-21.4	-23.1	-27.6	-29.5
S6k	-23	-20.7	-23.6	-25.4

Change in cell volume (%)

Figure 4



LacZ RNAi S2

Pvr RNAi S2

LacZ RNAi ML-DmBG3-c2

Pvr RNAi ML-DmBG3-c2

WT larval haemocytes

Pvr DN larval haemocytes

Ras DN larval haemocytes

Hml Gal4

Pvr RNAi + Hml Gal4

Cg Gal4

Pvr RNAi + Cg Gal4

Figure 5

Additional files provided with this submission:

Additional file 1: sims, duchek & baum additional data file 1.xls, 69K
http://genomebiology.com/imedia/7085084432534603/supp1.xls
Additional file 2: sims, duchek & baum additional data file 2.xls, 38K
http://genomebiology.com/imedia/1182050466253460/supp2.xls