**Research Paper**

**A Drosophila Overexpression Screen for Modifiers of Rho Signalling in Cytokinesis**

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**KEY WORDS**

Rho signalling, cytokinesis, genetic screen, Pebble, Ect2, Drosophila

**ABBRévIATIONS**

FACS fluorescence activated cell sorting
GTPase guanosine triphosphatase
KLP kinesin like protein
dsRNA double stranded RNA
EMS ethylmethanesulfonate
GEF GTP exchange factor
DH Dbl homology

**ACKNOWLEDGEMENTS**

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**ABSTRACT**

To identify genes that modulate Rho signalling during cytokinesis we tested the effect of overexpressing a set of 2190 genes on an eye phenotype caused by defective Rho activation. The resulting 112 modifier loci fall into three main classes: cell cycle genes, signalling effectors and metabolic enzymes. We developed a further series of genetic tests to refine the interactors into those most likely to modify Rho signalling during cytokinesis. In addition to a number of genes previously implicated in the Rho pathway during cytokinesis, we identified four novel primary candidates: cdc4, Pitx4, PDK1 and thread/diap1. cdc4 and thread/diap1 orthologs have, however, been implicated in cytokinesis in other organisms, as have molecules related to Thread/Diap1. The identification of several modifiers that are genetically redundant paralogs highlights the ability of overexpression screens to identify genes that are refractory to traditional loss-of-function approaches. Overexpression screens and sensitized phenotypes, therefore, may help identify the many factors that are expected to be involved in cytokinesis but have not been discovered by previous genetic screens.

**INTRODUCTION**

The small GTPase RhoA plays a crucial role in the regulation of animal cell cytokinesis (reviewed by Gregory et al., ref. 1). In most cases Rho signalling is not required for entry into mitosis, establishment of a mitotic spindle or the separation of DNA but is required, during early anaphase, for formation of the acto-myosin contractile ring at the cell cortex.² RhoA is broadly localized to the cell equator at the onset of cytokinesis³ and is specifically activated at the site of constriction.⁴

An important clue to the activation of Rho at the equator began with the identification of Drosophila Pebble and its mammalian ortholog, Ect2, as proteins that are required for cytokinesis.⁵⁶ In Pebble (phb) mutants, embryonic cells fail to divide, but the cell cycle continues, with further rounds of DNA replication and mitosis leading to multinucleate cells. Phb was cloned and found to be a Rho GTP exchange factor.⁷ Proteins of this class typically activate Rho, which then binds to downstream effectors that mediate cytoskeletal reorganisation (reviewed by Schmidt and Hall, ref. 9). Generic and physical interactions with Rho confirmed that Phb, and its vertebrate ortholog, ECT2, activate Rho at the equator to bring about cytokinesis.⁸ Other Rho GTPase regulators, C. elegans Cyt-4 and its Drosophila ortholog RacGAP50C/Tumbleweed (Tum), have also been identified as a Rho regulatory protein required for cytokinesis.⁹ In addition to binding the Phb/Ect2 GEF, Tum/RacGAP50C and Cyt-4 also bind the plus-end directed microtubule motor, Drosophila Pavortto (Pav-KLP) and its C. elegans ortholog, Zen-4, respectively.¹⁰¹¹ This provides a link between Rho activation through Phb and equatorial localization of the Rho-activation complex through the Tum/Pav-KLP complex. Our model for positioning of the cytokinetic contractile ring, then, proposes that after the onset of anaphase, a complex of Pav-KLP/Tum/RacGAP50C and Phb localizes to overlapping microtubules at the equator to activate Rho specifically where cleavage is to occur.¹² Recent studies suggest that this mechanism also operates in mammalian cells.¹³¹⁴

Despite these advances in our understanding of the earlier events of cytokinesis, we still have no mechanistic model for what happens following the activation of Rho at the cell equator. A variety of molecules such as Diaphanous, Rho Kinase and Citron Kinase are predicted to bind to and be activated by Rho,¹⁶ but how their activation brings about the formation of a contractile ring of actin and myosin is unclear. There are many other unanswered questions about cytokinesis, including: how does Rho regulate myosin contraction,
how does Rho recruit actin into a ring, what connects the ring to the membrane, what turns off and disassembles the contractile apparatus and, finally, what is the molecular basis of abscission. Our hypotheses about Rho activity during cytokinesis are limited by uncertainties about the effector molecules involved. For example, it is still not certain which kinase or kinases are responsible for the activation of myosin contraction, a key event in cleavage.19

Genetic screens are frequently used to identify genes that act in signalling pathways. Several genetic screens aimed at identifying molecules involved in cytokinesis have been carried out.20-25 These have all been loss-of-function screens using either dsRNA expression or EMS mutagenesis in which the readout is failure or delay in cytokinesis. Confirming the value of these screens, previously unidentified loci were found in each screen, showing that relevant genes are still to be found. While these constitute an excellent approach to genetic screening, they nonetheless have some limitations. For example, they are unlikely to identify genes that act redundantly. Furthermore, in Drosophila, the tissues screened were either cultured cells or spermatoocytes, neither of which may reflect the process of cell division in other cell types. For example, genes such as Rob and fis were identified even though they have no cytokinetic phenotype in at least some normal mitotic divisions.22,24

We report here an alternative genetic screen aimed at identifying new modulators of Rho signalling during cytokinesis. Our screen differs from those described above in three significant ways. The first is that the screen utilizes a hypomorphic Rho pathway phenotype induced by expression of a dominant negative form of the Rho pathway activator, the RhoGEF Pbl. We have previously shown that expression of a dominant negative form of Pbl is able to reduce Rho signalling in the eye, block cytokinesis and produce a rough eye phenotype.8 Such a sensitized phenotype may enable us to identify genes that influence, but are not necessarily essential for, Rho signalling during cytokinesis. Secondly, we use the developing eye imaginal disc, a normal developing single cell layer epithelium, as our initial test tissue. The set of cell divisions that occur during eye organogenesis has been well characterised and the Drosophila eye is both sensitive to subtle disruption as well as dispensable if entirely eliminated. Thirdly, we avoid the problem of genetic redundancy by using overexpression of genes, rather than loss of expression, to screen for genetic modifiers. Several successful screens based on overexpression eye phenotypes have been reported.26-29

Using this approach, we report the identification of a set of 112 interactors, mostly affecting the cell cycle, signalling or phosphoinositide metabolism. We also describe additional tests designed to identify those modifiers most likely to be modulating cytokinesis. Finally, we discuss the advantages and disadvantages of this type of screen for cytokinetic components.

RESULTS

Reduction in Rho signalling by pblDpMO expression. Our primary screen was intended to identify genes that, when overexpressed, would modulate a phenotype caused by reduced Pbl-activated Rho signalling in cytokinesis. Our assay strain carried constructs that drive expression of a dominant-negative form of pbl, under the control of the eye-specific promoter, GMR, during the last rounds of cell division in the developing eye.8 In this dominant negative construct, the catalytic (DH) domain of Pbl is mutated, with the expectation that this form is unable to activate Rho. The result of pblDpMO expression in the developing eye is the appearance of binucleate cells in the late third instar eye imaginal disc30 and an obvious roughening of the normally ordered adult ommatidial array (compare Fig. 1A and B). Loss of the function of one allele of Rhoa or pbl significantly worsens the phenotype (Fig. 1C and Prokopenko et al., ref. 8), consistent with the effect of this construct being primarily to reduce Rho signalling and confirming that halving the dose of a relevant gene could modify the phenotype.
To test whether genes implicated in Rho function during cytokinesis, rather than other Rho functions, could modify the \( p_b^{ADH} \) phenotype, we tested overexpression of the kinase-like protein Pav-KLP, which is known to be required for the organisation of the cytokinetic central spindle and contractile ring. Loss of function in one allele of Pav-KLP enhances the phenotype of \( p_b^{ADH} \) expression in the eye (Fig. 1D), indicating that the phenotype is sensitive to dosage changes in proteins that mediate cytokinesis. We have also seen enhancement of the phenotype in flies heterozygous for \( diaphanous \), which encodes an actin modifier required for cytokinesis. These results indicated that the \( p_b^{ADH} \) phenotype generates a sensitised background suitable for screening mutations that affect cytokinesis.

It was also important to demonstrate that \( p_b^{ADH} \) phenotype could be suppressed. The central mitotic regulatory complex, the Cdk1-Cyclin B complex, is implicated in the suppression of Pbl/Ect2 and Pav/MKL1 activity during mitosis, so that removal of one copy of genes encoding components of these kinases should enhance Pbl/Ect2 activity and suppress the phenotype. As expected, removing one copy each of \( cyclin B \) and \( cyclin B3 \) leads to significant suppression of the \( p_b^{ADH} \) phenotype (Fig. 1E). Taken together these data indicate that the \( p_b^{ADH} \) phenotype is appropriate for use in an overexpression screen for modifiers of cytokinesis.

Identification of potential cytokinesis factors by a gain-of-function screen. We tested 2190 GeneSearch EP lines in a first-pass screen for genes that, when overexpressed in the eye, could affect the phenotype caused by \( p_b^{ADH} \) expression in the eye. Of these, a surprisingly high proportion modified the phenotype, 417 giving clear suppression and 344 giving clear enhancement (see Supplementary Table 1). We also found 72 EP lines that were lethal in this genetic background, most likely due to low levels of GMR-Gal4 driven EP expression in non-eye tissues. One obvious potential class of artificial modifiers are any proteins that affect the ability of GMR-Gal4 to drive \( p_b^{ADH} \). Global transcriptional activators or repressors or ribosomal components potentially fall into this class. To identify whether there were significant numbers of such non-specific modifiers in the GS collection we also screened 1500 of the inserts for modification of an adhesion defect in the wing caused by dominant negative integrin expression. We found only 7% of our hits modified both assays, suggesting that this potential problem is relatively minor (data not shown), and this was borne out by subsequent analysis of our most interesting candidates, none of which were nonspecific Gal4 modifiers (see below).

The number of insertions we found was even more than the two hundred genes identified in previous loss of function screens for cytokinesis failure. This may be due to the increased sensitivity of the hypomorphic phenotype and to the fact that overexpression of a gene that normally is not involved in the processes under study may nevertheless produce a protein whose biochemical activity modifies one of these pathways. These issues are discussed further below. Nonetheless, our screen has the advantage that in many cases the targeted locus is known, allowing prioritisation of modifiers on the basis of their annotated features, such as their potential to play a role in Rho signalling and/or cytokinesis. On this basis and on the strength of the genetic interaction, we retested a set of primary interactors, confirming 71 suppressor and 41 enhancer genes (Table 1). It should be noted that our analysis assumes that expression of the gene adjacent to the EP element modifies the phenotype. While this is the most likely explanation, it would need to be confirmed in each case by examining the effect of driving expression of the relevant cDNA. More than half of the known genes could be broadly classified as affecting cell cycle or cytoskeletal signalling, while another large group of suppressors were metabolic enzymes, particularly phospholipid regulators. The significance of these interactors is discussed below.

We further characterised 19 promising interactors by additional genetic tests (Table 2). The first assay determined whether overexpression of the candidate altered the rough eye phenotype caused by overexpression of wild-type Pbl in the eye. We have shown that overexpression of normal Pbl affects Rho signalling in the opposite manner to \( p_b^{ADH} \) so we reasoned that genuine modifiers of Rho signalling should affect both phenotypes in opposite ways. This assay also excludes suppressors that simply block Gal4-driven transcription, since members of this class should suppress both the dominant negative and overexpression phenotypes. Of the 19 genes chosen for further study, 16 affected the rough eye Pbble overexpression phenotype, and 11 of these gave the opposite modification to the original GMR > \( p_b^{ADH} \) assay (Table 2). We also tested to see whether the GMR-induced overexpression of the modifying gene in an otherwise wild type background disrupted eye development and found that all genes whose expression gave enhancement in both Pbl assay backgrounds also disrupted wild type eye development. This suggests that enhancement of the \( p_b^{ADH} \) phenotype by these genes is likely to be a consequence of cytokinesis-independent effects on eye development.

Wing-based assays for modification of a pbl RNAi-induced cytokinesis phenotype. One drawback in using the eye assay is that both too much and too little activation of Rho disrupt development of the eye. To clarify which is occurring in the enhancers and to test whether the interactions could be generalised to other dividing tissues, we extended our analysis to include a wing assay. We also chose a different approach to the reduction of Pbl activity. Rather than expressing dominant negative Pbl, we inhibited Pbl synthesis by expressing an RNAi construct in the posterior compartment of the developing wing using an \( en > GAL4 \) driver. This also blocks cytokinesis by reducing Rho activity, leading to a reduced wing size, more multinucleate cells and the appearance of cells with multiple wing hairs (Fig. 2 and Shandala et al., ref. 35). The number of wing cells with multiple hairs is a readily scored assay for cell division failure that is sensitive to reductions in Rho or downstream effectors such as Citron kinase.

Table 2 shows that 11 of 16 inserts tested were able to significantly modify the effect of reducing Pbl expression in the wing. For example, overexpression of Pav-KLP reduced by more than half the number of multiple wing hair cells in a \( p_b^{RNAi} \) background (Fig. 2C). Such suppressors represent good candidates for modifiers of Rho signalling in cytokinesis, since they consistently affect the process in two very different tissue types and using two methods to block Pbl-activated Rho signalling. There were three inserts that did not affect the wing assay and none that gave the opposite modification in the wing compared to the eye (Table 2).

The enhancement of the pbl RNAi wing phenotype could be quite strong, for example by \( cdc42 \) (GS line 9041) (Fig. 2D) and some inserts could not be assayed due to earlier lethality when overexpressed with the \( en > GAL4 \) driver. For some of these, reducing expression levels by rearing at 18°C allowed enhancement to be tested, though the pbl RNAi phenotype at this temperature is too mild for suppression to be reliably scored.

To further test the possibility that the genetic modifications represented modification of the cytokinesis phenotypes, we used flow
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<td>as-chromosoma segregation/humoral immunity inducer</td>
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<td>Serine/threonine kinase, string regulator, cell cycle control</td>
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<td>Ew</td>
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<td>Myosin binding phosphatase, affects M phase</td>
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### Cell Cycle progression

- Cyclin-dependent protein kinase activating kinase
- Cytoskeletal and cell cycle regulator
- Cytokinesis (Drosophila) Phosphoinositide transporter
- Cyclin dependent kinase
- Cdc25 phosphatase, M phase entry
- Aurora kinase, centrosome separation
- Cytokinesis (Drosophila) Upstream as-Pi4 kinase
- Cyclin dependent kinase 4/8

### Cytoskeletal signalling

- Frizzled ligand Affects cell migration/axion guidance
- Dynein-binding-like/ribosomal protein
- Ack - putative effector kinase for cdc42/ as-ferminin 1 adhesion molecule
- Expression suppresses RacDN, required for dorsal closure

### Other

- Wnt5
- rab37BC/Rpl30
- Ack/asCG14991
- as-fishel
- fringe
- Rho188/ide
- CG5973
- frpsr
- numb
- as-coro
- nuclear fallout
- crossveinless-2
- foraging
- Marin 1
- as-Myc31DF
- Rso
- Gs-Galp673B
- Dlc90F
- CG17283/Sur-8
- Calmodulin
- fze
- as-Dad
- as-Mic2
- Roughened
- as-sip1
- Roc2
- Moeisn
- scabrous
- wnderborst
- Trk
- Pphmeg
- spitz
- tso
- glgmesh
- shot
- centaurin beta 1A

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</table>

Lines are grouped by broad classification of the predicted function of the overexpressed gene, thus by strength and direction of modification of the eye phenotype and finally by Gene Search stock number. The abbreviations for modified phenotypes are: SS strong, invetable suppression, S moderate or slightly variable suppression, Sw weak, variable suppression, SE some aspect of the phenotype suppressed, some enhanced, EE strong, invetable enhancement, E moderate or slightly variable enhancement, Ew weak, variable enhancement. The number of hits refers to the number of randomly screened GS lines that effected this locus and give the same modification. The gene is listed as "as" if the insert drives antisense transcription from the locus and as "in" if it begins inside the coding region. All other inserts drive transcription in the same direction starting upstream of the coding region. Any assigned genes affecting the same locus but with a different effect are listed in the comments, with "as" if it had no effect. The comments include the predicted function or homology of the locus being overexpressed on the basis of the Gene Search and FlyBase (http://flybase.com) entries. A question mark indicates either that no nearby transcripts give any indication of homology or function, or that the listed function is still speculative.
cytometry analysis of dividing wing primordia to directly assess the
effect of several inserts on cell cycle progression. Expression of pbl
RNAi in the posterior half of the wing resulted in a reduction of the
fraction of cells in G₂, and an increase in hypodermal cells, consistent
with our observation that pbl RNAi induces binucleate cells (Fig.
3A and D and Shandala et al., ref. 35). Overexpression of PDK1
suppressed both the G₂ and hypodermal cell effects of the pbl RNAi
phenotype (Fig. 3C and D), making it an excellent candidate for a
regulator of Rho signaling in cytokinesis. Overexpression of Cdc14
strongly enhanced the hypodermal fraction and reduced the fraction
of cells in G₂ (Fig. 3B and D). This is the first report of a function
for Cdc14 in Drosophila, and is consistent with evidence that Cdc14
is a rate-limiting inhibitor of mitotic exit in several other species, as
well as having a direct role in cytokinesis in some.36

We also used flow cytometry to test whether we could discrimi-
nate effects on cytokinesis from effects on apoptosis or G₂/M
progression. Overexpression of the baculovirus inhibitor of apoptosis
p35, which suppresses the pbl RNAi phenotype (Fig. 1F), enhanced
the proportion of hypodermal cells (Fig. 3D), suggesting that apo-
ptosis normally reduces these cells. In contrast, overexpression of
thread/diap1 reduced the hypodermal fraction (Fig. 3D), suggesting
that Thread/Diap1 is able to suppress the primary phenotype of cyto-
kinesis failure, although it is also an anti-apoptotic protein.37 This
assay therefore effectively discriminates between apoptosis blockers,
which suppress in the eye assay but enhance in the FACS assay, from
cytokinesis mediators, which suppress both.

We also anticipated that this assay would identify any modifiers
that just push cells through the cycle, since these should not be able
to suppress the formation of hypodermal cells although there might be
more cells in total. Expression of the Schizosaccharomyces pombe cdc25
and string results in clear suppression of the eye and wing phenotypes
(Table 2), but in the FACS assay it shows no significant suppression
of the number of hypodermal cells, though it does strongly push cells
from the G₂ to G₁ fraction (Fig. 3D). This is consistent with the
known role for string in G₂/M progression,38 and suggests it has no
additional effect on cytokinesis.

Other aspects of the overexpression screen. The intention of this
screen was to use inserts in which the promoter drives overexpression
of an adjacent gene. However in 14 of the 112 confirmed modifiers,
the inserted promoter would be expected to transcribe the nonsense
strand, and lead to a loss-of-function phenotype. It is interesting
to note that nine of these were suppressors, suggesting that there
may be a significant number of factors that downregulate or titrate
away Rho signalling in the eye. A good example of this is Myosin
31DF, in which three independent antisense inserts all suppressed
the pblM07 phenotype. This gene is also an example of the set where
both a sense and an antisense insert were isolated for the same gene,
in this case giving opposite effects. It is not necessarily to be expected
that an insertion in the antisense strand should give the opposite
modification to overexpression of a gene in this assay, since both an
increase or decrease in a factor that influences dynamic cytoskeletal
structures can disrupt eye development, as we have observed for pbl.8
In our screen, we found ten cases where both sense and antisense
transcription of a gene modified the phenotype, and of these five gave
opposite effects and in five both suppressed (Table 1).

In a few cases, the insert was in the coding region and produced
a truncated sense transcript. In Rho-1, for example, the insert falls at
+597, corresponding to the coding for amino acid 73. These may
represent a class of alleles that modify the phenotype via the expres-
sion of dominant negative or activated forms of the protein. Such


tables are grouped by original screen phenotype then by results from subsequent assays. The abbreviations
for modified phenotypes are as in Table 1, with the addition of L, overexpression of the gene is lethal in
this background, so no modification of the phenotype. Where two phenotypes are listed for the wing assay,
the first refers to a cross kept at 25°C and the second at 18°C. The first column of phenotypes is the original
screen phenotype from Table 1. The second column shows the effect of overexpressing the gene in eyes
overexpressing wild type Pelle. The third column shows the effect of overexpressing the gene by itself in
an otherwise wild type eye. The fourth column shows the effect of overexpressing the gene in the posterior
half of wings that also express a pelleRNAi construct.

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<th>Gene</th>
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<th>Extra Pbl</th>
<th>wt</th>
<th>Pbl RNAi</th>
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<td>L</td>
<td>rough</td>
<td>n/t</td>
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Lines are grouped by original screen phenotype then by results from subsequent assays. The abbreviations
for modified phenotypes are as in Table 1, with the addition of L, overexpression of the gene is lethal in
this background, so no modification of the phenotype. Where two phenotypes are listed for the wing assay,
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an otherwise wild type eye. The fourth column shows the effect of overexpressing the gene in the posterior
half of wings that also express a pelleRNAi construct.

Discussion

To identify genes that modulate Rho signalling during cyto-
kinesis, we have carried out an initial screen for genes that, when
overexpressed, modify the effect of a hypomorphic phenotype caused
by reduction of Pbl-activated Rho signalling in the developing eye.
We used subsequent genetic tests, such as suppression of Pbl over-
expression, to discriminate interesting enhancers of the Rho phenotype
from genes that simply disrupt eye development. An adult wing assay
was then used as an independent method for confirming an effect on
cell division, and a FACS assay was used to show whether the modi-
fication affected cytokinesis or some other cell cycle process. These
tests enabled us to define modifiers that were likely to be involved
in cytokinesis, cell proliferation or apoptosis, the three aspects of eye
development that we expected to be able to modify the Rho loss of
activity phenotype.

As anticipated, we found several cell cycle genes that are known to
directly affect Rho signalling and cytokinesis, including diaphanos,
citron kinase, and four wheel drive. Diaphanos is a Rho effector
protein required to organise actin in the contractile ring.39 Citron
kinase is also essential for cytokinesis and acts downstream of Rho
to regulate targets such as Myosin II,22,35,40, 41 Four wheel drive is a
phosphoinositide-4 kinase and is required for cytokinesis that occur during spermatogenesis.\textsuperscript{41}

More significantly, we also found that the disruption to cytolysis caused by decreased Pbl activity was modified by overexpression of thread/diap1, cdc14, Pitrk and PDKI, none of which have been previously identified by conventional genetic screens for cell division genes in \textit{D. melanogaster}. All of these genes are good candidates for mediating Rho signalling in cytokinesis. Although Cdc14 phosphatase has not previously been implicated in \textit{Drosophila} cytokinesis, it is a rate-limiting inhibitor of mitotic exit in several other species, such as yeast, as well as having a direct role in cytokinesis in \textit{C. elegans}.\textsuperscript{46}

The identification of thread/diap1 as a modifier of cytokinesis is particularly interesting since it is best known as an apoptosis inhibitor. However, Thread is also known to mediate Rac signalling in \textit{Drosophila}\textsuperscript{42} and DnlKKe regulation of Thread/Diap1 stability has been shown to control F actin assembly and turnover in a non-apoptotic context.\textsuperscript{43} Furthermore, related IAP proteins are required for cytokinesis in \textit{C. elegans}.\textsuperscript{44} The cdk11 homolog Pitsil has been shown to block cytokinesis when overexpressed in CHO cells,\textsuperscript{45} and removal of Pitsil by RNAi gives spindle defects, at least in some \textit{Drosophila} cells.\textsuperscript{46,47} The phosphoinositide-dependant kinase PDK1 has previously only been implicated in control of cell growth in \textit{Drosophila}, but recent evidence from yeast suggests it may also play a direct role in cytokinesis.\textsuperscript{48}

Eight modifier genes identified in this screen constitute a group of regulators of phosphoinositide turnover (rdgB, giuto, raspberry, mir14, wanen, CG6718, CG16708, PIP5K and four wheel drive). There is now good evidence that regulation of phosphoinositide signalling is essential for cytokinesis\textsuperscript{41,49-53} and that phosphoinositides directly affect Rho signalling.\textsuperscript{54} Some of our modifiers, such as the Phosphoinositide Transport Protein (PTP) gene rdgB, have a restricted pattern of expression and mutant phenotype, and so are unlikely to be the crucial enzyme themselves in most cytokineses, however they may point to the essential enzymatic activity of a related gene (the PTP gene giuto)\textsuperscript{51}. This is an area where redundancy is particularly strong. For example, there are four \textit{Drosophila} PIP5 kinases and seven diacylglycerol kinases, so it is perhaps not surprising that most of our modifiers from this group have not previously been identified. The remaining genes of this group are less obviously related to cytokinesis; we speculate that they may affect cell growth rates, thus altering proliferation, F-actin affecting the phenotype, as discussed for cell cycle regulators in the next section.

As a block in cytokinesis and subsequent apoptosis of polyploid cells does not occur in every dividing cell expressing dominant negative Pebble, coexpression of any gene that can promote cell division will tend to restore the number of ommatidia and suppress the phenotype. We have confirmed this for known cell cycle regulators, such as \textit{string}. The available evidence suggests that Sec64B (GS 9618) also acts in this manner.\textsuperscript{55} The effect of altering cell cycle progression was quite marked in this screen with the isolation of at least eight other genes that we would expect to affect proliferation in a more way (Cyclin H, cdk4, grapes, E2f, Pitrk, tribbles, rifts, CG12690).

In a related screen, Zheng and Hariharan identified a set of genes which, when overexpressed, blocked cell division in the eye.\textsuperscript{27} A comparison of the genes identified in our screen with theirs showed

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Modification of the wing phenotype caused by reducing Pebble with RNAi: (A) Wild type wing with elongation of area counted for multiple hair cells. (B) Wing expressing Pebble\textsuperscript{RNAi} under the control of the engrailed driver shows reduction of size in the affected posterior half of the wing and many multiple hair cells (avg 70±/6%). (C) Wing expressing both Pebble\textsuperscript{RNAi} and Pitsil/Cdc11 (GS line 30033) shows significant suppression of the multihair cell phenotype (avg 34±/5%). (D) Wing expressing both Pebble\textsuperscript{RNAi} and cdc14 (GS line 9041) shows strong enhancement of the multihair phenotype.}
\end{figure}
seven common hits (Rac2, fringe, esagast, His39, schmurre, CG13791 and CG6701). We might have expected all of these to enhance our assay phenotype, since they reduce eye size by themselves when overexpressed. Two, however, were suppressors of pBPDN (fringe, CG6701). Differences in the screens may explain these unexpected interactions. In particular, Tsieng and Harlizius used an eyGAL4 driver, which expresses during the growth phase of the eye disc, while expression here was driven by GMGAL4, which should affect only the final cell division required to generate enough cells to form complete ommatidial sets of cells. Alternatively, these interactions may be examples of antagonistic epistasis, where the deleterious effect of overexpressing either pBPDN or the modifier is lost when they are coexpressed, though it is premature to speculate on a molecular mechanism.

An alternative process that could be affected to suppress the pBPDN phenotype is apoptosis. There is normally a wave of apoptosis that removes excess cells in the developing eye, so we expected that genes that could block these apoptoses and also prevent multinucleate cells from being cleared, might restore the loss of cells seen with pBPDN expression. Our isolation of thread/diap1 (GS 3057) and testing with the apoptosis inhibitor p35 (Fig. 1F), suggest that blocking apoptosis does suppress the eye phenotype. However as noted above, thread/Diap1 has also been found to interact with another Rho family member, Rac, in cell migration and to be involved in the regulation of F-actin, also in a non-apoptotic context. Consistent with these observations, our FACS analysis supports a cytokinetic role for thread.

We did not identify many directly anti-apoptotic genes, however we did find five modifiers from the Notch pathway (fringe, Furin 1, manb, tramtrack and sebraut), suggesting that these interactions are related to the role of Notch in patterning the eye by apoptosis. We found only one other confirmed suppressor directly related to inhibition of apoptosisthe non-coding microRNA mir-1, which also affects lipid metabolism. Lipopopposis is yet to be characterized in detail in Drophiulus, but it would be interesting to determine whether any of the metabolic genes we found affect this process. Importantly for our focus on cytokinesis, we showed that FACS analysis can separate such modifiers from those that actually suppressed cytokinesis failure.

A quarter of our confirmed hits did not fall into any obvious group. Some of these are well characterized but unexpected: for example it was not immediately clear why hits in the RNA binding proteins Pumilio and Nanos should affect cell cycle progression. However, this RNA processing pathway has been shown to affect the localized translation of Aurora, cdc2, and Moezin, providing a means by which they could be affecting proliferation and/or cytokinesis. Some, like CG8776, have an unknown role, but have plausible protein interactions (Par1, Cyclin K) that could explain their effect on cell division while for others this screen represents the first data about the function of the locus.

Strengths and Limitations of the Screen. A clear advantage of this approach over loss of function approaches is that it overcomes the problem of genomic redundancy. This is particularly useful in the study of cytokinesis, which appears to involve a very robust mechanism with multiple backups that have hampered conventional genetic analyses. We also did not find any cases where overexpression of a known cytokinesis gene had no effect on our assay, suggesting that the initial screen has a very low rate of false negatives.

A limitation of this screen is that we only tested approximately 10% of the annotated genes in Drophiulus. As more overexpression stocks become available, it will be possible to carry out more comprehensive screens. A further limitation is that we selected strains for further analysis based in part on the predicted molecular characteristics of their protein products. It is likely that some relevant loci identified in the primary screen will have been overlooked because of this. Another limitation is the very high rate at which we observed modification of our test phenotype. Nearly one in three of the lines tested showed some modification of the phenotype. One reason for the large number of enhancers is that overexpression of any gene that disrupts eye development will act as an enhancer. Consistent with this, our subsequent testing indicated that, as expected, many of the enhancers also disrupted eye development in a wild type background. In addition, overexpression of any gene that normally binds Rho or its effectors for a different purpose (such as migration) is likely to
disrupt or titrate away Rho signalling in cytogenesis. Roughened (GS 10082) and gigarmesh (GS 10076) may be examples of this kind of interactor, since they are involved in cytoskeletal reorganizations other than cytogenesis. Our interpretation of the number of suppressors is more complex: we found that the pbbRDNA phenotype is suppressed by the expression of genes that generate increased cell numbers, for example by enhancing proliferation or suppressing apoptosis.

Even given these classes of modifiers and the fact that we might expect the expression of a substantial number of genes to modulate processes as intricate as cytogenesis, we must also be dealing with a phenotype that is exquisitely sensitive to changes in cell physiology. This is probably not a unique feature of the phenotype used here. A significant rate of false positives was also observed in previous overexpression screens.26-29 Subsequent screens, such as those used in this study, are therefore very important in ruling out possible artefacts and determining which candidate should be followed up. A particularly important secondary screen described here was the use of flow cytometry to identify modifier phenotypes that specifically affected cytogenesis. Ultimately, of course, the analysis of loss of function alleles or, in the cases of genetic redundancy, combinations of loss of function mutations is required to dissect the role of the candidate genes in Rho signalling and/or cytogenesis.

CONCLUSION

We have used an overexpression screen to identify genes that modulate a cytogenesis failure phenotype caused by a reduction in Pbl-activated Rho GTPase signalling. Further genetic testing revealed three genes (diaphanosus, citron kinase, and four wheel drive) that have previously been implicated in cytogenesis,5,29,41 as well as four genes (threadDisclaimer, cdc 13, Pidbr and PDK1) that have not been previously implicated in cytogenesis in Drosophila. Although they have limitations, overexpression approaches are therefore likely to be useful in identifying additional cytogenesis factors that have escaped detection by other approaches.

MATERIALS AND METHODS

Drosophila strains and genetics. All strains used in this analysis were obtained from the Bloomington Drosophila stock centre and grown on standard medium at 25°C unless otherwise indicated. Alleles used to test the assay strain were: parPROM (FBal0050242) RhoAATG (FBal0061660, obtained from M. Modzik), cycB2 (FBal0094855) and cycB3 (FBal0116683, obtained from P. O’Farrell). The assay strain used in the primary screen was GMR-Gal4 UAS>pebbleDDM1 / CyO S, using the dominant negative Pebble construct (FBal0101238) described by Prokopcean et al.8 The dominant negative integrin strain used for comparison was Cy2 > Gal4 UAS > Tav2-myosin heavy CyO S, derived from Martin-Bermudo and Brown (FBtp0016089).61 We screened a random subset of the GeneSearch collection of EP insert strains.34 At least ten flies carrying both the assay chromosome and the EP chromosome were scored for each strain. Eyes were classified as strong suppressors if every eye was close to wild type, moderate if all were clearly better than the assay strain or if some were strong but there was variation, and weak if only some eyes were noticeably better than the assay strain. Enhancers were similarly classified on the basis of strength and consistency of effect. Plausible modifiers were immediately retested and the adjacent gene checked for additional hits in our screen (http://gbsd.biol.metro-u.ac.jp/p67/Edculti/). The closest transcript downstream of the insert promoter is listed as the affected locus, though in some cases overlapping divergent transcripts made this assignment uncertain. In addition, the early GS lines from this collection have promoters on both ends, often giving two possible genes that may affect the phenotype. In these cases, the most plausible is listed first and used for classification of the hit. Where multiple hits resolved which gene was the modifier, the irrelevant gene was not listed.

To test the effect on Pebble overexpression, EP lines of interest were crossed to GMR > Gal4 UAS > pbbCyO (FBal0101231) as described in Prokopcenc et al.8 and scored as above. The pbbRNAi background used to test interactions in the wing was en > Gal4 UAS > CD8-GFP UAS > pbbleRNAi/Cyo (FBal0178473), as described in Shandala et al.34 We have tested constructs expressing dsRNA to a nonoverlapping region of pbble to confirm the specificity of this phenotype (data not shown). At least six female wings of the correct genotype and at least 150 cells per wing from a defined region between veins L4 and L5 (see Fig. 2) were scored for each cross. A significant interaction was indicated for any cross that averaged a multiple wing hair count of more than two standard deviations away from the assay strain cross to w (typically average 70%, standard deviation 6%).

Flow cytometry. The genotype used as the background for flow cytometry was en > Gal4 UAS > CD8-GFP UAS > pbbleRNAi/Cyo; UAS > Gal4. This marks with GFP the posterior compartment disc cells expressing pbbleRNAi while the anterior compartment cells were used as controls with a normal cell cycle. The UAS > Gal4 element (FBal0017417) was added to increase the transcription of the RNAi construct as soon as possible after the engrailed driver became active during wing development. It made the adult wing phenotype significantly more severe, as expected (data not shown). For flow cytometry, larvae were grown at 25°C for four days after laying, then wing imaginal discs were dissected and processed as described in Neufeld et al.38 For each GS line at least 20 discs were collected from each of at least three crosses to the assay strain. Cells were run on a BD LSRII machine and analysed using Wessel 2.3 (www.biotechcentre.net.au/cytometry/index.html). For each 20 disc sample, the fractions of GFP-positive cells with GI, G2, and hyperploid DNA contents were compared with those of the GFP-negative control cells from the same sample to assess the change in cell cycle profile caused by expressing pbbleRNAi plus the modifier.

Note

Supplementary Table 1 can be found at: www.landesbioscience.com/supplement/gregoryFLY1-1-sup.pdf

References


Fly 2007; Vol. 1 Issue 1