Genetic analysis of the role of *pebble* during cytokinesis in *Drosophila*

A thesis submitted for the degree of Doctor of Philosophy

by

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ABSTRACT

The *pebble (pbl)* gene is required for cytokinesis in *Drosophila,* and encodes a guanine nucleotide exchange factor that activates Rho small GTPase family members. PBL is located in the nucleus of cells that are not dividing, and at cortical regions of dividing cells. The role of nuclear localised PBL is unknown, while it is likely that cortical PBL acts to reorganise the actin cytoskeleton during cytokinesis. Real-time imaging of *pbl* mutant cells, in this study, has revealed the formation of transient, partial cleavage furrows, suggesting a possible later role for PBL in contractile ring function than was initially thought.

Ectopic expression of PBL had no effect during embryogenesis, suggesting that PBL is not rate-limiting during cytokinesis. However, ectopic expression of PBLΔDH (unable to catalyse GDP/GTP exchange) resulted in an inhibition of cytokinesis, consistent with it functioning as a dominant negative form of PBL. Distinct rough eye phenotypes were observed when either PBL or PBLΔDH were ectopically expressed during eye development. Extra cells observed with *GMR>pbl* resulted from an inhibition of apoptosis, while *GMR>pblΔDH* inhibited cytokinesis, resulting in fewer cells in the eye.

Genetic interactions using the *GMR>pbl* and *GMR>pblΔDH* rough eye phenotypes, showed that PBL is acting predominantly through Rho1. Furthermore, downstream signalling of PBLRhoGEF activity could involve the activation of Diaphanous to reorganise the actin cytoskeleton, and Rho-kinase, for the activation of myosin. Genetic interactions with regulators of mitosis suggested that CDK1, together with CYC-B and/or CYC-B3, could also regulate PBL activity. This would provide a link between exit from mitosis and the onset of cytokinesis. However, *in vivo* studies showed that PBL is not regulated by phosphorylation at consensus CDK1 sites.

The genetic systems established here have also been used in an initial screen to identify components of PBL signalling pathways. Interactors identified to date include PP2A and WUN, phosphatases that could act in opposition to kinases downstream of PBL-activated Rho1. Further screening, using these systems, should enable a more comprehensive analysis of PBL signalling pathways during cytokinesis.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent for my thesis, when deposited in the University library, being made available for loan and photocopying.

Louise O'Keefe, December 2001

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Chapter 1: Introduction

Cell division is a fundamental process in the biology of all organisms. During mitosis, the duplicated genetic complement of a cell is distributed equally into two discrete nuclei. This nuclear division must be tightly coupled with the process of cellular division, or cytokinesis, where the cytoplasmic components are partitioned between the two daughter cells. Mitosis is predominantly driven by reorganisation of microtubule filaments to form the mitotic spindle. While cytokinesis requires a significant reorganisation of the networks of actin microfilaments that comprise an important part of the cytoskeleton. Correct regulation of cytokinesis necessitates spatial and temporal co-operation between the microtubule and microfilament networks within mitotic cells. The process of cytokinesis has been observed microscopically for many years in different organisms and, more recently, some of the molecular components of the cytokinetic machinery have been identified. However, the nature of the regulatory signals remains poorly understood.

1-1 Cytokinesis in animal and yeast cells

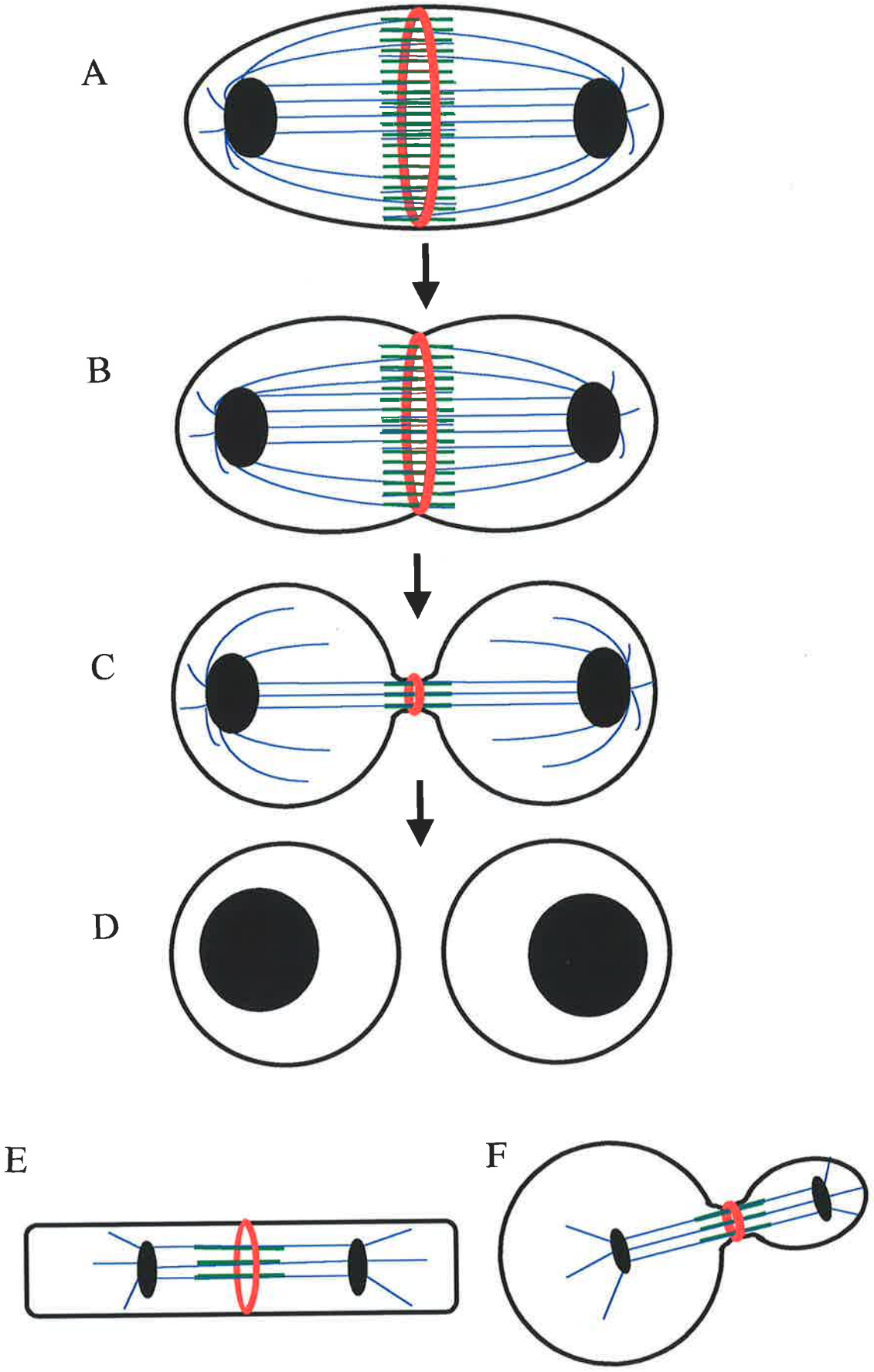
There are many similarities in the basic mechanism of cytokinesis in various animal and yeast cells, with many of the molecular components having been conserved. This has facilitated the identification of these components through genetic screens in different organisms. In particular, the yeasts have proven to be valuable experimental organisms in elucidating pathways involved in cytokinesis. The main similarities and differences between higher eukaryotic and yeast cytokinetic mechanisms will be outlined below, followed by a more extensive description of cytokinesis in animal cells.

1-1.1 A general mechanism for cytokinesis in animal cells

In animal cells, the initial determinant of cytokinesis is specification of the cleavage plane such that it forms at the right time and is correctly positioned to give rise to two cells, each with a newly divided nucleus and the appropriate distribution of cytoplasmic components. A contractile ring, predominantly comprised of actin filaments interspersed with myosin, then assembles below the cell cortex at the presumptive plane of cleavage (Fig. 1-1A, contractile ring shown in red). A specialized microtubule structure, termed the central spindle, also forms in the spindle midzone region (Fig. 1-1A, central spindle shown in green). Constriction of the contractile ring, and pulling in of the associated cell membrane, results in the formation of a cleavage furrow (Fig. 1-1B). Ingression of this cleavage furrow continues until the plasma membranes have been pulled in towards the microtubules forming the central spindle

Figure 1-1 Cytokinesis in animal and yeast cells

Schematic diagrams of cytokinesis in animal and yeast cells. Segregated nuclear material and cell outlines are shown in black, microtubules are shown in blue, contractile rings are shown in red and central spindle regions are shown in green. **A.** Specification of the cleavage furrow following segregation of nuclear material results in the formation of a contractile ring at the site of division. **B.** Constriction of the contractile ring and pulling in of the associated plasma membranes results in the formation of a cleavage furrow. **C.** After constriction of this ring, the two daughter cells are joined by a small bridge termed the midbody. **D.** Resolution of the midbody results in the separation of two complete daughter cells. **E.** *S. pombe* cells divide by fission, a contractile ring forms at the site of division, midway between the segregated nuclear material. **F.** *S. cerevisiae* cells divide by budding, a contractile ring forms at the site of the mother-bud neck, midway between the segregated nuclear material.


A
B
C
D
E
F

region. The resultant bridge structure joining the two daughter cells is referred to as the midbody (Fig. 1-1C). Final separation of the two daughter cells requires resolution of the midbody with deposition of new membrane to allow these to become two complete daughter cells (Fig. 1-1D).

1-1.2 Cell division in yeast cells

This general mechanism of cytokinesis is conserved to some extent in dividing yeast cells, in that an acto-myosin based contractile ring forms at the site of division. However there are also some differences. Firstly, specification of the cleavage furrow occurs at an earlier stage than in animal cells, although it remains coupled to nuclear events. Secondly, for final cell separation, constriction of the acto-myosin ring must be coupled to an additional mechanism termed septum formation, since yeast cells posess a rigid cell wall.

Cells of the yeast *Schizosaccharomyces pombe (S. pombe),* are rod shaped and divide by fission (see review by Gould and Simanis, 1997). The position of the cleavage plane is determined by the position of the pre-mitotic nucleus. A medial ring forms at this position which is comprised predominantly of F-actin (Fig. 1-1E). Following constriction of the medial ring, a division septum is deposited in a centripetal manner and brings about final separation of the two daughter cells. The Spg1 GTPase pathway has been identified as a key regulator of septum formation, with ectopic expression of Spg1p resulting in the formation of ectopic septa independent of nuclear division events (Schmidt et al., 1997).

In the yeast *Saccharomyces cerevisiae (S. cerevisiae),* cells divide by budding (for a review see Palmieri and Haarer, 1998). The site of bud formation is specified at the end of G1 phase of the cell cycle and an acto-myosin based contractile ring forms at the mother-bud neck. Constriction and pinching off of the daughter cell occurs following elongation of the mitotic spindle into the preformed bud during anaphase (Fig 1-1F). In *S. cerevisiae* a Tem1p GTPase pathway, homologous to that of Spg1p in *S. pombe,* is required for regulating exit from mitosis in addition to being required during cytokinesis for actin ring formation (Lippincott et al., 2001).

1-2 The mitotic spindle as the source of the stimulus for cytokinesis in animal cells

The mitotic spindle is thought to be the source of the stimulus for the initiation of cytokinesis. However, some controversy exists over which component of the mitotic spindle is responsible. Regulatory signal(s) originating from the spindle would allow for a precise spatial and temporal coordination of cytokinesis with the completion of mitosis. This would

ensure both the correct timing of cytokinesis and positioning of the cleavage furrow such that it forms between the two sets of chromosomes after successful separation.

1-2.1 Asters as the source of the cytokinetic stimulus

The mitotic spindle is comprised of microtubules (MTs), the minus ends of which emanate from microtubule organising centres (MTOCs) at polar regions of dividing cells. In eukaryotic cells, centrosomes are the major MTOCs. Various MTs are nucleated from these MTOCs; kinetochore MTs which attach to chromosomes, spindle MTs which form the mitotic apparatus and astral MTs which extend to other regions of the cell. These MTOCs are sometimes referred to as asters, since they resemble star-like structures or, alternatively, spindle pole bodies (SPBs) in yeast cells.

Early experiments carried out in sea urchin embryos supported a model where the asters were thought to be the origin of the stimulus for cytokinesis. This model resulted originally from an experiment in which cleavage furrows were observed following the juxtapositioning of two polar asters from different spindles within the same cell (Rappaport, 1961). In support of this model, removal of different components of the mitotic apparatus by microsurgical manipulation confirmed that cleavage furrows were only formed midway between two asters present within the same cell (Hiramoto, 1971).

From these early experiments it was proposed that a long-range signal, originating at polar regions of a dividing cell, was transported along astral MTs. This signal would terminate at equatorial regions, where some of these astral MTs are in close proximity to the cell cortex, and result in the formation of a cleavage furrow by the stimulation of active constriction (Devore et al., 1989). An alternative interpretation of these data is that signals from two adjacent asters could result in the relaxation of acto-myosin dynamics at polar regions, this could also result in increased contractility at equatorial regions and cleavage furrow formation. However, measurement of traction forces of dividing cells attached to an elastic substrata, by the size of wrinkles produced, found there was significant generation of force in equatorial regions, supporting the model for increased contractility at equatorial regions (Burton and Taylor, 1997).

1-2.2 The central spindle as the source of the stimulus

Contrary to early findings in sea urchin embryos, more recent experiments in mammalian tissue culture cells strongly favour the central spindle as the region of the mitotic apparatus that is responsible for the cytokinetic signal. Using well spread normal rat kidney (NRK) cells, precise perforations made between the spindle midzone and the cell cortex were found to prevent furrow formation while those made outside this region had no effect (Cao and

Wang, 1996). In addition, it was found that the formation of ectopic cleavage furrows between polar regions of adjacent spindles in tripolar NRK cells correlated strongly with the presence of overlapping microtubules in the midzone region, rather than simply the presence of two asters (Wheatley and Wang, 1996). This correlation was also observed in mitotic heterokaryons generated by fusion of human cultured cells (Eckley et al., 1997) and PtK1 cells (Savoian et al., 1999) and in NRK cells where the formation of the spindle midzone was prevented by inhibition of chromosome separation (Wheatley et al., 1998).

The different results reported from experiments carried out in sea urchin embryos and mammalian tissue culture cells may reflect differences between these cell types. Sea urchin embryos are large with a small central spindle region while mammalian tissue culture cells have a relatively large central spindle region. Thus, it may be necessary to have extra signal delivery via the astral MT network in sea urchin embryos, in addition to signals emanating from the central spindle region. Alternatively, it is more likely that the more recent data reflect an ability to perform more precise manipulations and to study the effects of various perturbations using cell biological techniques. The early observations in sea urchin embryos could also be explained as signals emanating from the spindle midzone, since the juxtapositioning of two asters could result in the formation of overlapping microtubules midway between the two asters. However, the issue of the source of the stimulus to divide has not yet been completely resolved.

Genetic studies of cytokinesis in *Drosophila* have provided support for the central spindle as the origin of the cytokinetic stimulus. Mutations have been identified in *asterless,* where male meiosis (Bonaccorsi et al., 1998) and embryonic neuroblast divisions (Giansanti et al., 2001) can occur in the absence of asters. Additionally, female meiosis in *Drosophila* normally occurs in the absence of prominent asters. Also a strong correlation between the presence of a central spindle and formation of a contractile ring during male meiosis has been confirmed in many different mutant backgrounds (Giansanti et al., 1998). Thus, strong genetic evidence exists which supports the model for the stimulus for cytokinesis arising from the central spindle region in these cell types, rather than from the asters.

1-2.3 Co-ordinating the timing of cytokinesis with the exit from mitosis

The timing of cytokinesis must be precisely coupled to the completion of mitosis such that each daughter cell receives a complete set of segregated chromosomes. Phosphorylation of various cellular proteins plays a major role in the regulation of mitotic events. A high level of cyclin-associated cyclin-dependent kinase (CDK) activity triggers entry into mitosis. Exit from mitosis is regulated by the anaphase promoting complex (APC)-mediated destruction of mitotic B-type cyclins and decreased levels of their associated CDK activity (see review by

Zachariae, 1999). Good evidence exists for a link between regulators of mitotic exit and regulators of cytokinesis (see introduction to chapter 6 and review by Nigg, 2001).

A strong correlation between nuclear division and cytokinesis has also emerged from studies in yeast (see review by McCollum and Gould, 2001). In *S. pombe,* members of the septation initiation network (SIN) pathway (including Spg1) have been identified primarily as regulators of septation. Cells mutant for members of this pathway form a medial acto-myosin ring which fails to constrict. Homologous proteins identified in *S. cerevisiae* form the mitotic exit network (MEN) pathway. Mutations in these genes result in a slightly different phenotype where levels of cyclin B remain high, cells fail to progress beyond anaphase and cytokinesis does not occur. Interestingly, most of the components of the SIN pathway localise to the SPB (Sawin, 2000), highlighting the importance of signals emanating from polar regions of the cell in the regulation of cell division.

1-3 The formation and activity of the contractile ring

Once the signal to divide has been sent, a contractile ring forms midway between the segregated nuclear material. This ring is predominantly comprised of actin filaments interspersed with myosin, which together provide the contractile activity. In addition, a number of other proteins have been found to concentrate at the contractile ring; actin binding proteins, septins and Formin homology (FH) proteins (Fig. 1-2A).

1-3.1 Actin fibre organisation at the contractile ring

Early ultrastructural studies identified filaments found in the cleavage furrow of dividing cells as actin, by the binding of heavy meromyosin (Schroeder, 1973). In addition, these filaments showed banding patterns that were similar to that of actin stress fibres, consistent with these filaments being able to generate the force necessary for constriction at the cleavage furrow (Sanger and Sanger, 1980).

Ultrastructural studies on mouse blastomeres revealed that these bundles of actin filaments were arranged in parallel arrays around the entire circumference of dividing cells. However, in equatorial regions corresponding to the position of the contractile ring, the accumulated actin filaments were also found to be perpendicular to the length of the mitotic spindle (Opas and SoÇtyÑska, 1978). This unexpected arrangement of actin filaments was confirmed by phalloidin staining of dividing cultured cells (Fishkind and Wang, 1993) and in *Dictyostelium* (Fukui and Inoue, 1991). Thus, the original model of the sliding of parallel actin fibres driving constriction of the contractile ring inwards was not sufficient to account for these observations. Rather, a more complex mechanism of interplay between actin filaments was invoked.

The generation of a cleavage furrow requires the accumulation of actin at the cleavage furrow. It has been shown that a fluorescently labelled cytoplasmic pool of pre-existing actin filaments was randomly recruited to cortical regions, redistributed towards equatorial regions and subsequently released back into the cytoplasm during division in cultured normal rat kidney cells (Cao and Wang, 1990). Thus, it appears that there is a constant recycling of actin filaments as the furrow progresses. This would be consistent with a proposed mechanism of cortical flow for the movement of cytoplasmic and cortically associated components towards the equator in many different cell types (Bray and White, 1988). This was also suggested by studies that tracked the movement of membrane localised particles during cell division, where significant movement of those particles towards equatorial regions was observed (Wang et al., 1994).

Finally, disruption to the MT networks within a cell resulted in disruptions to the reorganisation of the actin cytoskeleton at the onset of cytokinesis, providing strong evidence that these mechanisms must be co-ordinated during cytokinesis (Fishkind et al., 1996).

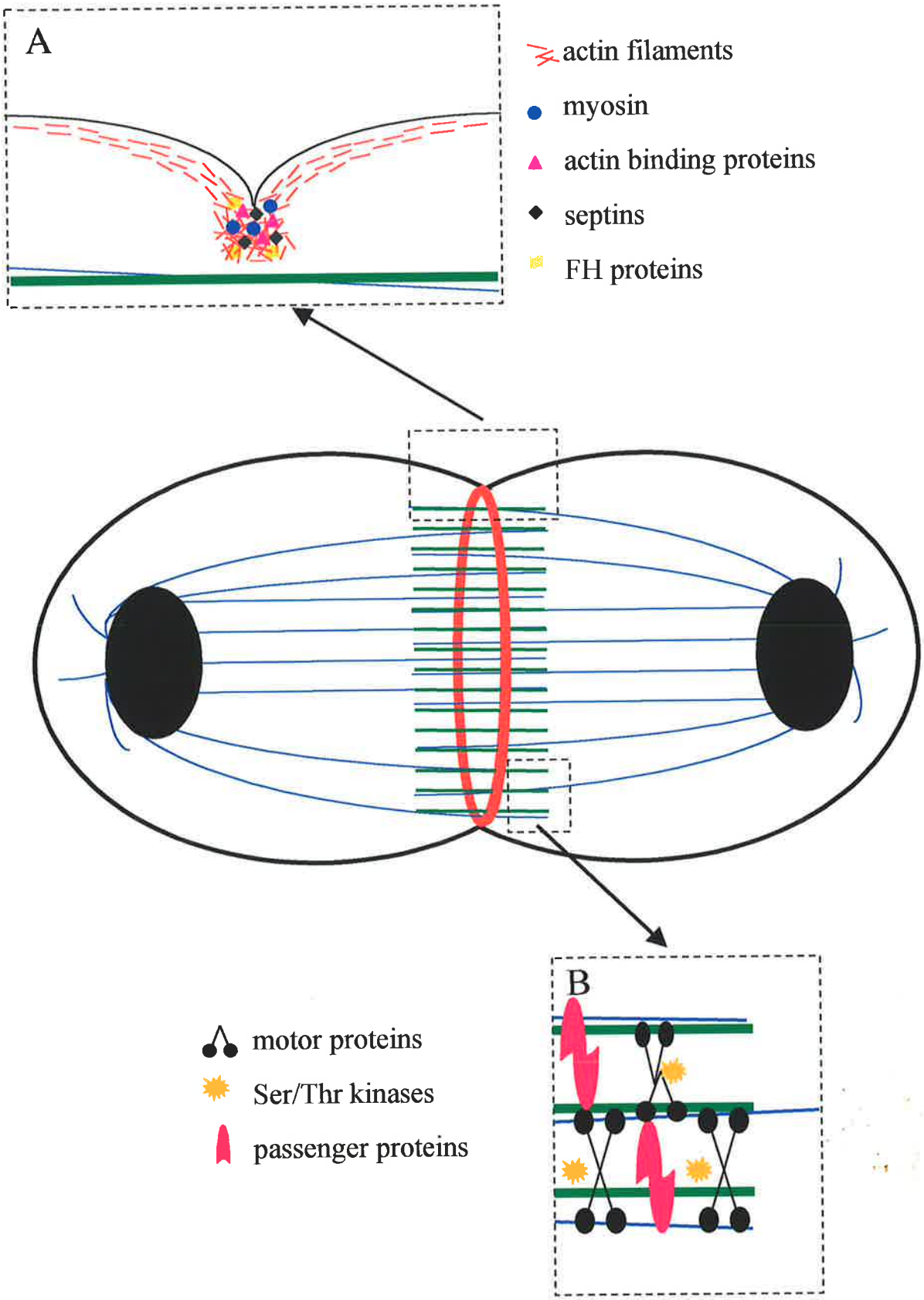
1-3.2 Myosin at the contractile ring

The first conclusive evidence of myosin localisation to the cleavage furrow came from the use of fluorescently labelled antibodies directed against myosin (Fujiwara and Pollard, 1978). A requirement for myosin activity during contractile ring function was first shown by injection of anti-myosin antibodies, which resulted in an inhibition of cytokinesis in starfish blastomeres (Mabuchi and Okuno, 1977). This was further supported by genetic analysis where *Drosophila* mutants in *spaghetti squash (sqh),* encoding the regulatory light chain (RLC) of myosin, resulted in cytokinetic defects in larval neuroblasts (Karess et al., 1991). Also, the nonmuscle myosin RLC gene, *mlc-4,* is required for embryonic cytokineses in *C. elegans* (Shelton et al., 1999). In fission yeast there are two myosins; Myo2p which localises to the contractile ring during anaphase and Myp2p which localises to the contractile ring slightly later (Bezanilla et al., 2000). Disruptions to *Myo2p* are lethal indicating an essential role during cytokinesis, although disruptions to *Myp2p* only affect cytokinesis under certain conditions. There is, therefore, strong genetic evidence from a variety of organisms for a requirement for myosin activity during cytokinesis.

Myosin activity is not always an absolute requirement during cytokinesis. In *S. cerevisiae* a ring of myosin forms at the presumptive bud neck, to which actin is later recruited to form a contractile ring. Constriction of this ring is then coupled to septum formation and final separation of mother and daughter cell. However, strains deleted for the single non-muscle myosin gene, *MYO1,* are viable indicating that myosin function is not essential for cytokinesis (Bi et al., 1998). In this modified division, actin is still recruited to

Figure 1-2 Components of the contractile ring and central spindle

A schematic diagram of a dividing cell at cleavage furrow formation. Segregated nuclear material and cell outlines are shown in black, microtubules are shown in blue, contractile rings are shown in red and central spindle regions are shown in green. **A.** A region of this dividing cell, highlighting the components of the contractile ring; actin, myosin, actin-binding proteins (Anillin, Cofilin, Profilin), septins and formin homology (FH) proteins. **B.** A region of this dividing cell, highlighting components that localise to the central spindle; motor proteins (PAV, KLP3A), Ser/Thr kinases (Polo, AuroraB) and chromosome passenger-proteins (INCENP and TD-60).


A
actin filaments
myosin
actin binding proteins
septins
FH proteins
B
motor proteins
Ser/Thr kinases
passenger proteins

the bud neck region and division presumably occurs by an alternate mechanism that is linked to septum formation. In the amoeba, *Dictyostelium discoidium,* cytokinesis can occur in cells either in suspension or attached to a substrate, and is accompanied by the formation of an acto-myosin contractile ring. However, cells mutant for myosin II cannot undergo cytokinesis in suspension. They can only divide in a cell-cycle dependent manner if they are attached to a solid substrate (Uyeda et al., 2000). This substrate-assisted division in the absence of myosin II function has been shown, at least partially, to be brought about by an increase in cortexillins, which accumulate in equatorial regions of dividing cells (Weber et al., 2000). Cortexillins are a family of proteins which are known to bundle actin filaments and are responsible for positioning the cleavage furrow in all *Dictyostelium* divisions (Weber et al., 1999). Thus, in the absence of myosin, additional mechanisms are employed by cells to bring about constriction and division.

1-3.3 Actin binding proteins at the contractile ring

The *Drosophila* gene *chickadee (chic)* encodes Profilin, a protein that binds actin monomers and modulates filament formation. *chic* mutants show cytokinetic defects during male and female meiosis (Giansanti et al., 1998; Verheyen and Cooley, 1994). Profilin had previously been identified in *S. pombe* as the product of the *cdc3* gene and shown to be required for formation of the contractile ring (Balasubramanian, et al, 1994). Thus, it is likely that Profilin activity is required for the reorganisation of actin filaments in response to the cytokinetic stimulus. However, from studies in *S. cerevisiae* cells mutant for Profilin, it is apparent that Profilin is not essential for cytokinesis, since these cells can survive and grow although some level of multinucleate cells was reported (Haarer et al., 1990).

Various other actin-binding proteins have also been identified as being essential for cytokinesis. Anillin binds to actin filaments and has been shown to accumulate at the contractile ring during cytokinesis of the *Drosophila* embryonic divisions (Field and Alberts, 1995). However mutations in this gene are not available to determine whether its activity is essential during cytokinesis. Functional analysis of a human homologue of Anillin revealed that injection of anti-Anillin antibodies into tissue culture cells resulted in the regression of cleavage furrows and formation of multinucleate cells, suggesting that Anillin is likely to perform a conserved function during cytokinesis (Oegema et al., 2000). Interestingly, both in *Drosophila* embryonic cells and human cell lines, Anillin is nuclear localised during interphase and relocalises to cortical regions in dividing cells. However, the function of this nuclear localisation is unknown.

Proteins of the actin depolymerizing factor (ADF)/cofilin family regulate actin dynamics by promoting the disassembly of actin filaments (Bamburg, 1999). Mutations in

twinstar, a *Drosophila* gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis (Gunsalus, et al 1995). A *Drosophila* orthologue of LIM-kinase phosphorylates cofilin and induces actin cytoskeletal reorganization (Ohashi et al., 2000). Cofilin has also been found to localise to the cleavage furrow during cytokinesis in mammalian cells (Nagaoka et al., 1995). Thus it appears that the activity of cofilin, together with Anillin, Profilin and possibly various other actin-binding proteins is required for the cytoskeletal reorganisation events necessary for contractile ring formation and function.

1-3.4 Septins at the contractile ring

The Septins comprise a family of proteins that were first described as components of the bud neck in *S. cerevisiae* but are now recognized as a highly conserved family of proteins required for cytokinesis in many organisms (for a review see Longtine et al., 1996). All Septins have a GTP-binding domain near their NH_2 terminus and most have a predicted coiled coil domain at the COOH terminus.

The *S. cerevisiae* genes *Cdc3, Cdc10, Cdc11, Cdc12* and *Shs1/Sep7* all encode septins, which assemble at the site of contractile ring formation (Cid et al., 2001). Localisation of septins and myosin to the contractile ring occurs prior to bud formation, and they are required for the recruitment of actin filaments. The septins then split into two rings comprised of polymerised filaments, and constriction of the actin ring occurs in the middle. *Cdc3, Cdc12* and *Shs1/Sep7* are required for cytokinesis, while cells mutant for *cdc10* or *cdc11* can continue to divide (Frazier et al., 1998). In these cases, cytokinesis proceeds in the absence of the polymerised filaments of septins that normally form, suggesting they may not be absolutely required to perform a structural role during cytokinesis. Another gene, *gin4,* was identified as a synthetic lethal with *cdc12* (Longtine et al., 1998). Gin4p is a protein kinase, required for organisation of various septins. Cytokinesis can proceed in *gin4* mutants, despite numerous morphological abnormalities that result. Gin4p is specifically activated during mitosis, raising the possibility of a role for septins in regulating the initiation of cytokinesis in a cell cycle dependent manner (Carroll et al., 1998).

peanut (pnut) encodes a member of the Septin family in *Drosophila.* PNUT has been shown to localise to the contractile ring and is required for cytokinesis during larval neuroblast divisions (Neufeld and Rubin, 1994). An earlier requirement during the late stages of cellularisation was revealed by the generation of germ-line clones for *pnut* (Adam et al., 2000). Two other Septins, SEP1 and SEP2 have also been identified in *Drosophila,* but in the absence of mutations in these genes, their role during cytokinesis has not been determined (Field et al., 1996). It has been shown that PNUT, SEP1 and SEP2 form a stable protein complex *in vitro* (Field et al., 1996). This complex displays nucleotide exchange activity and

can form filaments. Complex formation has also been shown for Nedd5, a mammalian septin (Kinoshita et al., 1997). Thus it is possible that these proteins may perform both a structural and a signalling role during cytokinesis.

Two genes encoding septins, *unc-59* and *unc-61* have been identified in *C. elegans* (Nguyen et al., 2000). The UNC-59 and UNC-61 proteins are co-dependent on each other for localisation to the contractile ring during cytokinesis. Although the activities of *unc-59* and *unc-61* are not required during embryonic divisions (in both single and double mutants), the post-embryonic mutant phenotype has, at least partially, been shown to be a result of a cytokinetic defect

Thus, multiple septins have been identified in many different species and they all show localisation to the contractile ring. However, a consensus view of their function is yet to be formed.

1-3.5 Formin homology proteins at the contractile ring

Formin homology (FH) proteins are characterised by various conserved domains. The FH1 domain is approximately 100 amino acids, is rich in proline residues and has been shown to bind to profilin. This implicates FH proteins as upstream regulators of actin reorganisation events. This direct interaction with profilin has been shown in *S. pombe* (Chang et al., 1997) and *S. cerevisiae* (Imamura et al., 1997). FH family members also have a conserved FH2 domain of approximately 130 amino acids and a region that binds to Ras homology (Rho) GTPase family members. Rho GTPases regulate reorganisation of the actin cytoskeleton during a number of different processes, including cytokinesis, that involve significant changes in cell shape.

Formin homology (FH) proteins have been shown to localise to contractile ring structures and are required for cytokinesis in many species. Bni1p (bud neck involved) and Bnr1p (bud neck related) from *S. cerevisiae* are required for cytokinesis and cell polarisation during bud formation (Evangelista et al., 1997; Imamura et al., 1997). Cdc12p is required for formation of the division septum during cell division in *S. pombe* and localises to the medial ring during cell division (Chang et al., 1997). *diaphanous (dia)* has been shown to be required for cytokinesis in the male germline and during embryonic development in *Drosophila* (Afshar et al., 2000; Castrillon and Wasserman, 1994). DIA in *Drosophila* colocalises with the contractile ring during cellular division and with acto-myosin structures that form as membranes invaginate during cellularisation. In mammalian systems, mDia colocalises with membrane ruffles which are induced by actin polymerisation events in cultured cells (Watanabe et al., 1997). mDia has been shown to localise to the mitotic spindle in dividing HeLa cells, consistent with a conserved role during cytokinesis (Kato et al., 2001).

FH proteins have been strongly implicated in the regulation of actin dynamics, but they also interact with the MT network in budding yeast (Miller et al., 1999), fission yeast (Chang et al., 1997) and mammalian cells (Ishizaki et al., 2001). Thus, FH proteins provide a functional link between these two filamentous networks and could be a major player in co-ordinating mitosis and cytokinesis.

1-4 The formation and function of the central spindle and midbody

The central spindle refers to the specialised MT based structure that forms in the spindle midzone region during cytokinesis (Fig. 1-1A, B, shown in green). The midbody refers to the structure that forms once the furrow has almost completely ingressed and cells are joined by just a small bridge of tightly bundled MTs surrounded by cell membrane (Fig. 1-1C, shown in green). Many of the proteins that localise to these structures are required for cytokinesis (Fig. 1-2B). Various chromosome-associated proteins relocate to the central spindle when chromosomes line up on the metaphase plate. Also, motor proteins are required for the correct organisation of microtubules and possibly to motor in other important cargo. One possible class of cargo is the various Serine/Threonine kinases, which have been found at the midbody and have been shown to be important during cytokinesis.

1-4.1 Microtubule structure of the midbody

Tight bundles of anti-parallel, interdigitating MTs are an integral component of the central spindle. It has been shown that these MTs are no longer attached to centrosomes (Mastronarde et al., 1993; Saxton and McIntosh, 1987). They have also been shown to have gamma tubulin, which is normally associated with minus ends, located at the lateral edges (Julian et al., 1993; Shu et al., 1995). Together, these observations argue that the specialised structures of the central spindle and midbody MTs are formed by de novo nucleation of MTs, not simply the overlap of the pre-existing spindle MTs.

The importance of this structure during cytokinesis was first shown directly by specific depolymerisation of the central spindle in mammalian tissue culture cells which resulted in an inhibition of cytokinesis (Wheatley et al., 1998). In addition, the inhibition of chromosome separation in NRK cells prevented the formation of the midzone microtubule structure and hence prevented furrowing (Wheatley et al., 1998). Thus, the central spindle region and midbody of dividing cells play an important role in the regulation of cytokinesis.

1-4.2 Chromosome passenger proteins at the midbody

It was further proposed, from the above experiments, that the central spindle-associated signal for cytokinesis actually originated from aligned metaphase chromosomes. After signal

delivery to the central spindle MTs, some of which were in close proximity to the equatorial cell cortex, this would result in cleavage furrow specification at this position. The timing of delivery of this signal was determined to be before the onset of anaphase, lending supporting to this model (Cao and Wang, 1996).

A number of so called "passenger proteins" had been identified which could be performing this regulatory role. Passenger proteins become concentrated at centromeric regions of chromosomes, before relocating to central spindle MTs and the cell cortex at the site of the future cleavage, and finally at the midbody. Inner centromere proteins (INCENPs) show this pattern of localisation (Earnshaw and Cooke, 1991). INCENPs have been shown to localise to the site of cleavage before furrowing occurs but also before the localisation of myosin. Thus, they are one of the earliest components recruited to the site of contractile ring formation (Eckley et al., 1997). TD60 is another of these passenger proteins. The localisation pattern of TD60 led to the proposal that there exists a "telophase disc" which extends through the equatorial cross section of a cell during anaphase. This disc can bind and organise myosin and is then responsible for constriction by interaction with cortical actin (Andreassen et al., 1991). Colocalisation has been observed between TD60 and INCENP across this equatorial disc region (Martineau-Thuillier et al., 1998). The formation of such a structure could explain how signals emanating from the central spindle region could extend out as far as the cell cortex. Ectopic expression of a truncated version of INCENP, which can bind to centromeres but not MTs, results in disruptions during the early stages of mitosis and late stages of cytokinesis, implicating its involvement in both of these processes (Mackay et al., 1998). INCENP knockout mice revealed more severe defects in chromosome segregation, microtubule bundling and nuclear bridging, such that an effect on cytokinesis could not be specifically identified (Cutts et al., 1999).

Although there is substantial evidence of a role for passenger proteins during cytokinesis, it has also been reported that in grasshopper spermatocytes, cytokinesis can occur in the absence of chromosomes (Zhang and Nicklas, 1996). This could suggest that passenger proteins were not essential for cytokinesis, rather that spindle associated proteins were sufficient to provide the stimulus for cytokinesis in these cells. Or alternatively, that in the absence of chromosomes to deliver the passenger proteins to the central spindle, there is an alternative source of these regulatory proteins.

1-4.3 Kinesin motor proteins at the midzone

Kinesins form a superfamily of microtubule based motor proteins which are involved in various cellular functions, including vesicle transport, and various aspects of mitosis and cytokinesis (Moore and Endow, 1996). Kinesin-like protein 3A (KLP3A) in *Drosophila* is

required for cytokinesis during male meiosis. It is located in the nucleus during interphase, expressed throughout the cell during metaphase and localises to the midbody during anaphase and telophase (Williams et al., 1995). KLP3A is proposed to be required for the bundling of microtubules at the midzone since this fails to occur in *klp3A* mutants.

pavarotti (pav) encodes a mitotic-KLP (MKLP) in *Drosophila* which has also been shown to be required for cytokinesis during embryonic divisions (Adams et al., 1998). PAVKLP localises to the centrosomes early in mitosis and to MTs of the midbody during telophase. It is also required for proper organisation of the central spindle and could provide a mechanism for delivery of signals from polar regions of diving cells to the central spindle region. In *C. elegans,* mutations in the *zen-4/MKLP* orthologue result in failure to form a spindle and disrupt cleavage furrow formation (Powers et al., 1998; Raich et al., 1998). The mammalian orthologue, MKLP1, localises to the central spindle and is required for proper organisation of this structure (Nislow et al., 1992). RB6K, a human KLP distantly related to MKLP also shows localisation to the midbody and is required during cytokinesis (Fontijn et al., 2001), suggesting that there could be many different KLP proteins involved in this process. Thus, MKLP proteins perform a conserved function during central spindle organisation.

1-4.4 Serine/Threonine kinases at the midbody

Polo and Aurora kinases are located at the midbody and are involved in the regulation of cytokinesis. The founding member of the family of Polo-like kinases was identified in *Drosophila,* where mitotic defects and abnormal spindle poles were observed in *polo* mutant embryos (Llamazares et al., 1991; Sunkel and Glover, 1988). Adult males homozygous for this original hypomorphic mutation in *polo* first revealed a specific requirement during cytokinesis, with a failure to properly form the spindle midzone region during cell divisions throughout spermatogenesis (Carmena et al., 1998). These mutant divisions resulted in an inability to localise PAV-KLP to the spindle midzone, and a subsequent failure to localise PNUT to the contractile ring. POLO also fails to localise to the poorly organised spindle midzone region in *pav* mutant cells, suggesting that they are co-dependent for their localisation (Adams et al., 1998). This is supported by their physical interaction *in vivo* in mammalian cells (Lee et al., 1995).

POLO function is also involved in earlier aspects of mitosis. Meiotic divisions in females homozygous for the same hypomorphic allele of *polo* revealed a failure to properly organise the MTOC (Riparbelli et al., 2000). In addition, stronger mutations revealed a defect during embyrogenesis with failure to undergo the metaphase/anaphase transition and a failure of centromere separation (Donaldson et al., 2001). Thus, POLO plays multiple roles during

mitosis and cytokinesis in *Drosophila*.

Mutations in genes encoding Polo-like kinases (Plks) have subsequently been identified in other organisms, with the phenotypes observed providing support for multiple roles during mitosis and cytokinesis (see review by Donaldson et al., 2001). *S. pombe* mutants in *plo1* result in a mitotic arrest with condensed chromosomes that are associated with monopolar spindles, in addition to a failure to form an actin ring or division septum (Ohkura et al., 1995). Plo1p has subsequently been shown to act together with another protein, Mid1, to ensure correct positioning of the actin ring (Bahler et al., 1998). Plo1p has also been shown to be rate limiting for septation in *S. pombe*, acting upstream of the SIN pathway which provides a direct link between mitotic exit and the regulation of cytokinesis (Tanaka et al., 2001). The function of *cdc5,* which encodes a Polo-like kinase in *S. cerevisiae*, is required during mitosis both for the activation of APC and to ensure mitotic exit (Charles et al., 1998). *cdc5* has also been shown to be essential during cytokinesis for the correct functioning of septins at the contractile ring (Song and Lee, 2001). In *C. elegans,* structural homologs of human *plk* and *prk* have been identified but their function is yet to be determined (Ouyang et al., 1999). Finally, ectopic Plk1 activity in mammalian cells results in multinucleate cells (Mundt et al., 1997).

The pattern of POLO localisation during cell division in a number of organisms supports its requirement at different stages of mitosis. The *in vivo* localisation of GFP-POLO during mitosis in early embryogenesis of *Drosophila* revealed a dynamic subcellular distribution, reflecting the proposed role in centrosome function and cytokinesis (Moutinho-Santos et al., 1999). *S. pombe* Plo1p localises to the SPB from early in mitosis until anaphase (Mulvihill et al., 1999) and subsequently localises to the actin ring (Bahler et al., 1998). In *S. cerevisiae* Cdc5 is found at the SPB at early stages of mitosis and to bud-neck filaments during contractile ring function (Song et al., 2000). In mammalian cells Plk associates with centrosomes early in mitosis and relocates to the central spindle as cells enter anaphase and, finally, to the midbody (Golsteyn et al., 1995; Lee et al., 1995).

Aurora kinases were first identified in *S. cerevisiae* where mutations in *increase-in-poloidy1* (*ipl1*) resulted in missegregation of chromosomes (Francisco et al., 1994). *Drosophila* mutations in *aurora* subsequently revealed a requirement during centrosome separation and the formation of a bipolar spindle (Glover et al., 1995). A second Aurora-like kinase (*Aurora B/IAL*) has also been identified in *Drosophila.* Mutations in this gene are not available, but depletion of the product of this gene using RNA interference revealed a requirement during chromosome condensation and segregation, in addition to a requirement during cytokinesis (Giet and Glover, 2001). Identification of mutants in *AIR-2,* an Aurora B

kinase from *C. elegans,* and experiments using RNA interference for *AIR-2,* also revealed a requirement during cytokinesis in addition to a role during chromosome segregation (Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000). AIR-2 behaves as a passenger protein and indeed was shown to interact with the INCENP proteins ICP-1 and-2 in *C. elegans* (Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000)). Thus AIR-2 is found associated with chromosomes early during mitosis, concentrated to centromeric regions and then transferred to the central spindle at later stages where it is shown to be required for correct organisation of the central spindle and localisation of PAV-KLP. Another protein, Survivin, which was originally identified as a potential inhibitor of apoptosis, is also in this complex of passenger proteins and is thought to play an important role in the regulation of cytokinesis (Skoufias et al., 2000; Uren et al., 2000).

Finally, Aurora and Ipl1-like midbody associated protein (AIM-1) is a Ser/Thr kinase isolated from rat and shown to be specifically required for cytokinesis, since dominant negative versions of this protein can block cytokinesis without affecting mitosis (Terada et al., 1998). Downregulation of AIM-1 kinase has been correlated with megakaryocytic polyploidization of human hematopoietic cells, suggesting it could normally play a role in regulating cytokinesis (Kawasaki et al., 2001).

Thus, Polo and Aurora ser/thr kinases (and possibly others) are playing multiple roles and interacting with multiple targets to ensure the correct regulation of mitosis and cytokinesis.

1-5 Final separation of the two daughter cells

Final separation of the two daughter cells requires resolution of the midbody and insertion of new membrane resulting in two complete new daughter cells.

1-5.1 New membrane insertion as furrow ingression occurs

The insertion of new membrane which is necessary for cleavage furrows ingression, and for the complete separation of daughter cells, has been suggested to occur by targeted vesicle insertion. The family of syntaxin proteins has been implicated in this process. Inhibition of syntaxin results in an inhibition of cytokinesis in *C. elegans* (Jantsch-Plunger and Glotzer, 1999) and sea urchin (Conner and Wessel, 1999). In *Drosophila,* inhibition of syntaxin activity resulted in an inhibition of cellularisation (Burgess et al., 1997). In mutants for *lavalamp,* which encodes a golgi-associated protein that links membrane insertion and furrow ingression, a similar inhibition of cellularisation was observed (Sisson et al., 1999). While, the functions of Syntaxin and Lavalamp are required for the acto-myosin events during cellularisation, a possible role during cytokinesis has not yet been determined. The Rab

family of small GTPases are required during vesicle trafficking. Rab6-KIFL, a human kinesin related protein that binds to Rab6, has been shown to accumulate at the central spindle and midbody, consistent with a role during cytokinesis (Hill et al., 2000).

Further evidence for the role of vesicle fusion during cytokinesis can be found in plants. There are many similarities between the divisions of plant cells and cytokinesis in animal cells, despite the presence of rigid cell walls in plants (see review by Bowerman and Severson, 1999). In *Arabidopsis,* the *knolle* and *keule* genes have been shown to promote vesicle fusion during cytokinesis (Waizenegger et al., 2000). *Keule* is an orthologue of the *S. cerevisiae sec1*, a key regulator of vesicle trafficking and Keule can bind directly to the syntaxin, Knolle (Assaad et al., 2001). Ectopic cytokineses were not observed following ectopic expression of Knolle, suggesting these membrane targeting and insertion events require some cell cycle dependent mode of activation to ensure they are correctly orchestrated with the completion of cytokinesis (Volker et al., 2001).

1-5.2 Centrosomes and the late events of cytokinesis

The importance of the centrosome has again come to the fore more recently with real-time imaging of tissue culture cells using a centrin-GFP fusion protein (Piel et al., 2001). Each centrosome is comprised of a mother, and a newly syntheized daughter centriole, at each cell division. It was shown that in cells where furrow ingression had progressed as far as the midbody, final separation of the two daughter cells coincided with movement of one or other of the mother centrioles towards the midbody region. Thus, the use of sophisticated microscopic techniques has allowed valuable insight into the behaviour of centrosomes at late stages of division. As a result, some of the emphasis for the source of the cytokinetic stimulus has been placed back on the importance of structures found at the polar regions of dividing cells. Since many signalling proteins are known to localise to the centrosomes at some point during division, they are clearly important for cytokinesis, at least in some cell types.

1-6 Cytokineses in different cell types

Despite the highly conserved nature of cytokinesis, there are also many differences both between organisms and even within different cell types of the same organism. Cytokinesis has been extensively studied in a number of different cell types in *Drosophila,* with some variations to the general mechanism outlined above.

1-6.1 Cytokinesis in different cell types of *Drosophila*

The first 13 divisions during *Drosophila* embryogenesis are synchronous and occur in a syncytium. These nuclear divisions occur in the absence of cytokinesis, as nuclei oscillate between S-phase (DNA replication phase) and M-phase (mitosis). Although there is no cytokinesis per se, there is a high level of organisation of the cytoplasm surrounding these nuclei, by the formation of specialised acto-myosin structures (Foe et al., 1993). Once the syncitial divisions are complete, the nuclei are arrested in G2 phase of cycle 14 and are located at the periphery of the embryo where they become enclosed within a cell membrane. This process of cellularisation is an acto-myosin-dependent process similar to cytokinesis, in that pseudo-cleavage furrows form as cell membranes extend down from the periphery of the embryo to surround each nucleus. A contractile ring structure is also required for the constriction and formation of complete new cells.

Cell divisions during neurogenesis are asymmetric both with respect to the size of the daughter cells and the distribution of cytoplasmic components. The neural determinant, Prospero, is located basally within ventral epidermal cells. During epidermal divisions equal sized daughter cells receive the same amount of basally located Prospero. However during neurogenesis, cell division occurs perpendicular to the embryonic divisions and all of the Prospero is segregated to the smaller basal daughter cell. In these divisions the spindle is initially orientated as for embryonic divisions, but then rotates by 90 degrees. Elongation of the apical microtubules together with shortening of the basal microtubules ensures that the spindle is positioned to give rise to daughter cells of different sizes (Kaltschmidt et al., 2000).

Incomplete cytokineses are observed during male and female germ line divisions. Contractile rings form and constrict but then form stable intercellular bridges referred to as ring canals. These allow connected cells to share cytoplasmic components while the nuclei remain segregated. These ring canals are comprised of a number of different structural components found at the contractile ring, but their structural makeup differs somewhat between males and females (Hime et al., 1996; Robinson et al., 1994).

Thus, specialisation of structures required for cytokinesis allow for different cell types to undergo variant cytokineses according to their differing cellular needs.

1-7 Rho family GTPases and regulation of the actin cytoskeleton

The Ras homology (Rho) family of GTPases consists of multiple members, of which Rho, Rac and Cdc42 have been best characterised in a variety of systems. Once activated, they become localised to plasma membranes where they interact with downstream effector molecules to regulate acto-myosin dynamics. The Rho family of proteins is required for a

number of cellular processes where significant changes in cell shape are required, including cytokinesis.

1-7.1 Regulation of cytoskeletal dynamics by RhoGTPases

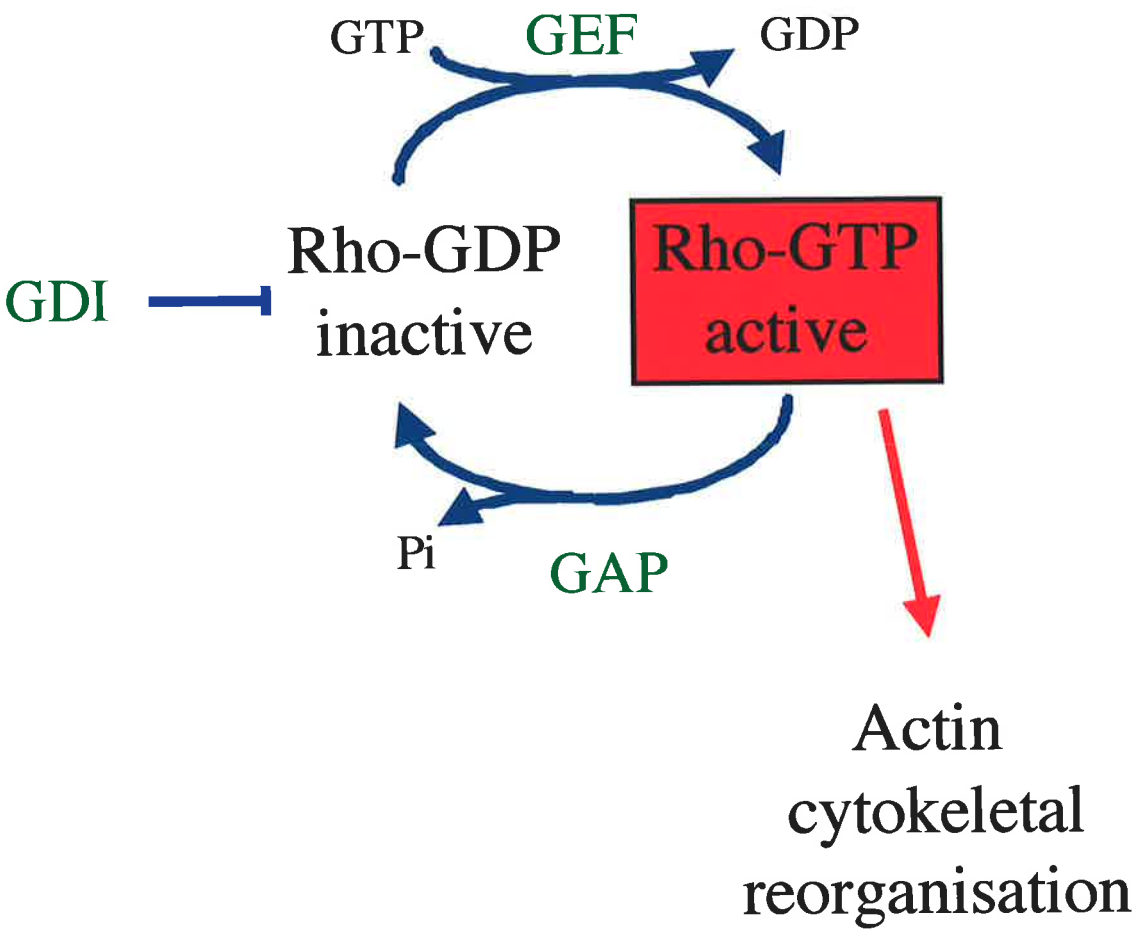
Distinct effects on morphology were observed after the activation of Rho, Rac and Cdc42 in mammalian tissue culture cells. Activation by Rho resulted in the bundling of actin fibres to form stress fibres, which are linked to the plasma membrane at focal adhesions (Ridley and Hall, 1992). Microinjection of Rac1 induced the accumulation of actin filaments at the plasma membrane to form membrane ruffles and lamellopodia, which was followed by a later response of Rho dependent actin stress fibre formation (Ridley et al., 1992). Cdc42 was shown to induce the formation of filopodia. These were later interspersed with lamellopodia which resulted in formation of the leading edge and was subsequently followed by the induction of stress fibre formation (Nobes and Hall, 1995). Thus, these Rho proteins form a regulatory cascade where Cdc42 activation and filopodia formation is followed by Rac activation and the formation of lamellopodia which in turn promotes Rho activation and the formation of stress fibres (Chant and Stowers, 1995).

1-7.2 Regulators of Rho family GTPases

Rho GTPases alternate between an active (GTP-bound) form and an inactive (GDP-bound) form (Fig. 1-3). Three different classes of regulators for these Rho GTPases have been identified, the RhoGEFs, RhoGAPs and RhoGDIs (see a review by Symons and Settleman, 2000). RhoGEFs promote the exchange of bound GDP for GTP thus increasing the amount of active Rho. RhoGEFs are characterised by two domains found in tandem, the Dbl oncogene homology (DH) and Pleckstrin homology (PH) domains (Cerione and Zheng, 1996). In addition, different GEF family members have well characterised structural motifs, many of which are characteristic of domains found in other signalling molecules (for example; cysteine-rich domains and SH2 and SH3 domains). Truncation of these GEF proteins, producing proteins with DH/PH but not other domains, results in their oncogenic activation, althgough the mechanism by which this occurs is not well understood. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho family members, resulting in an accumulation of the inactive GDP-bound form. Finally, guanine nucleotide dissociation inhibitors (GDIs) sequester RhoGDP to the cytosolic fraction, thus decreasing the amount of active RhoGTP localised to the plasma membrane. Regulated expression of these GEFs, GAPs and GDIs could provide a mechanism for the correct spatio-temporal regulation of activities of Rho family members.

Figure 1-3 Regulators of RhoGTPases

Rho family members oscillate between an inactive GDP-bound state and an active GTP-bound state. Correct regulation of the activity of these Rho GTPases is achieved by a number of different proteins; GEFs catalyse the exchange of GDP for GTP and hence promote the active form, GAPs catalyse the intrinsic GTPase activity of RhoGTP and hence promote the inactive state and GDIs prevent dissociation of GDP and hence sequester the RhoGTPase in its inactive form.


GTP
GEF
GDP
GDI
Rho-GDP
inactive
Rho-GTP
active
Pi
GAP
Actin
cytokeletal
reorganisation

1-7.3 Rho GTPases and cytokinesis

Evidence for a requirement for Rho family members during cytokinesis in metazoans first came from the use of C3 toxin, which specifically inactivates Rho, and by injection of constitutively active and dominant negative versions of various Rho family members. Rho was shown to be required for cytokinesis in sea urchin embryos (Mabuchi et al., 1993), *Xenopus* (Drechsel et al., 1997; Kishi et al., 1993) and human cultured cells (O'Connell et al, 1999). A requirement for Cdc42 during cytokinesis was demonstrated in *Xenopus* (Drechsel et al., 1997) and human cultured cells (Dutartre et al., 1996). In *Drosophila, Rho1* mutants display a cytokinetic defect (Prokopenko et al., 1999). Mutants are not available for Rac and Cdc42 family members in *Drosophila,* but ectopic expression of modified forms has not revealed a requirement during cytokinesis (Harden et al., 1995; Luo et al., 1994; Murphy and Montell, 1996). Knockout of gene function using RNAi in *C.elegans* revealed a requirement for RhoA but not Rac1 or Cdc42 during cytokinesis (Jantsch-Plunger et al., 2000). Rho and Cdc42 proteins have not been found in *Dictyostelium discoidium,* although a number of different Rac proteins have been identified. A requirement during cytokinesis has been demonstrated for Rac1A, Rac1B and Rac1C by ectopic expression of modified forms. (Dumontier et al., 2000; Palmieri et al., 2000). Mutants in RacE, a more highly diverged Rac family member in *Dictyostelium discoidium*, have also revealed a requirement for this GTPase during cytokinesis (Larochelle et al., 1996).

Various regulators of Rho GTPases have also been shown to be required for cytokinesis. Injection of RhoGDI in *Xenopus* resulted in cytokinetic defects (Kishi et al., 1993). CYK-4 is a Rho family GAP in *C.elegans* which is required for central spindle formation and cytokinesis (Jantsch-Plunger et al., 2000). The mouse orthologue of *cyk-4* has also been identified from mouse and shown to be required for cytokinesis (Hirose et al., 2001). ECT2, a RhoGEF is also required for cytokinesis (Tatsumoto et al., 1999). *Ect2* encodes the mammalian orthologue of *pbl,* the subject of this thesis, and will be discussed in more detail in section 1-8.

Thus, surprisingly there is no consensus between various organisms of a requirement for a particular Rho family member during cytokinesis. This could suggest either organism or cell type specific requirements of various RhoGTP signalling pathways for the correct regulation of cytokinesis.

1-7.4 Downstream signalling pathways activated by active Rho GTPases

Many downstream effectors of Rho family signalling pathways have been identified, mostly through yeast two hybrid and biochemical approaches (reviewed in Bishop and Hall, 2000).

These effectors fall into three classes; Ser/Thr kinases, scaffold proteins or lipid kinases and exert their effects on actin and/or myosin organisation resulting in the formation of actin stress fibres, lamellopodia or filopodia (Fig. 1-4). There is considerable overlap between the downstream effectors activated by Rac-GTP and Cdc42-GTP, while effectors of Rho-GTP are more distinct. Some of these effectors have also been shown to be required for cytokinesis in various systems (Fig. 1-4, highlighted in green). Thus, they represent potential candidates for downstream components of PBL-activated Rho family members during cytokinesis.

One downstream effector of Rho-GTP is Rho-asssociated kinase (Rho-kinase). Activation of Rho-kinase results in its specific accumulation at the cleavage furrow during cytokinesis (Kosako et al., 1999). Rho-kinase has also been shown to interact with a number of different proteins required for cytokinesis, suggesting it could play a significant role in the regulation of cytokinesis (see review by Amano et al., 2000). Firstly, Rho-kinase results in the direct phosphorylation and activation of the myosin regulatory light chain (RLC) (Amano et al., 1996). A functional role for this phosphorylation during mitosis and cytokinesis was demonstrated by the defects observed after injection of non-phosphorylatable myosin RLC into mammalian cells (Komatsu et al., 2000b). Rho-kinase also indirectly regulates myosin activity by phosphorylation, and inhibition, of the myosin binding subunit of myosin phosphatase, which normally acts to decrease the amount of active myosin RLC (Kimura et al., 1996).

These observations of Rho-GTP activation of myosin have also been proven genetically to be required *in vivo* for cell shape changes during early embryogenesis in *Drosophila* (Halsell et al., 2000) and *C. elegans* (Piekny et al., 2000; Wissmann et al., 1997). However, it has been shown that phosphorylation of myosin RLC does not regulate the timing of cytokinesis in fission yeast (McCollum et al., 1999). Also, it has been shown that the phosphorylation of myosin RLC in rats could be, at least in part, brought about by the activity of AIM-1 (Aurora) kinase (Murata-Hori et al., 2000). Thus, Rho-kinase may not be acting alone in the phosphorylation and activation of myosin RLC during cytokinesis.

Rho-kinase also regulates intermediate filament (IF) organisation during cytokinesis (see review by Goto et al., 2000). IFs are intermediate in size between the larger MTs and the smaller actin micro-filaments, and they perform a major structural role in cells. During cytokinesis there is a cleavage furrow-specific phosphorylation of the IFs, vimentin (Goto et al., 1998) and glial fibrillary acidic protein (GFAP), a type III IF protein (Kosako et al., 1997). In addition, GFAP has been shown to be directly phosphorylated by Rho-kinase and is required during cytokinesis (Yasui et al., 1998).

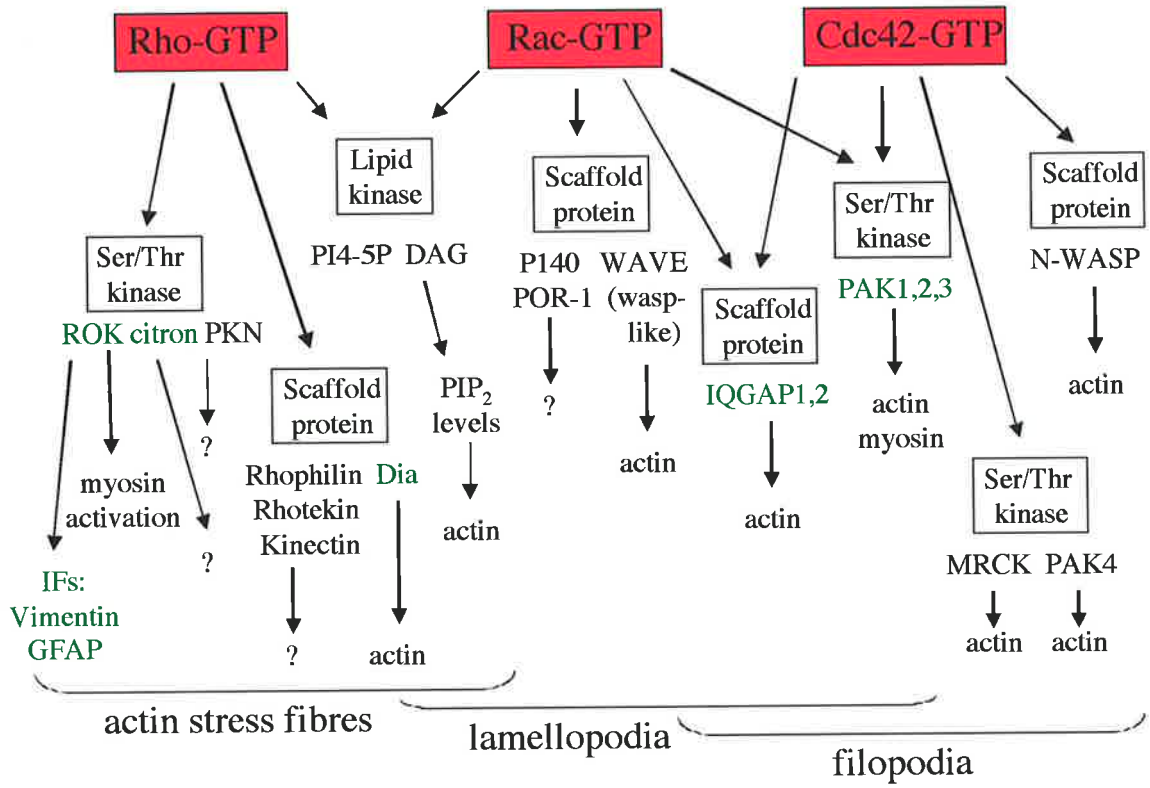
Despite the evidence for Rho-kinase regulation of myosin activity, a clear role for Rho-kinase during cytokinesis via genetic analysis, has not yet been determined. Expression of a dominant negative form of Rho-kinase resulted in an inhibition of cleavage furrow formation in *Xenopus* oocytes and multinucleated mammalian tissue culture cells (Yasui et al., 1998). However, identification and characterization of *rok,* the *Drosophila* orthologue of Rho-kinase, revealed that it was not required for cytokinesis, since somatic clones of *rok* were able to proliferate (Mizuno et al., 1999). Rho-kinase has been shown to link Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton (Winter et al, 2001). Embryos homozygous for mutations in the *C. elegans* Rho-kinase, *let-502,* exhibit an early morphological defect, where they fail to undergo an acto-myosin dependent elongation, but cytokinesis is not affected (Wissmann et al., 1997). Citron kinase is related to Rho-kinase and has been shown, in mammalian tissue culture cells, to be required for cytokinesis (Madaule et al., 1998), being recruited to the cleavage furrow of dividing cells in a Rho-GTP dependent manner (Eda et al., 2001). Citron kinase knockout mice displayed neurogenic defects characterised by altered cytokinesis and severe apoptosis, although not all tissues showed cytokinetic defects (Di Cunto et al., 2000), Orthologues in other species are yet to be identified. Another signalling pathway downstream of Rho GTPases and previously shown to be required during cytokinesis is Rho-mediated actin polymerisation via the activation of Profilin. The activity of this actin binding protein has already been discussed in section 1-3.3.

A family of proteins termed the IQGAPs, based on their homology to the calmodulin binding (IQ) domain and to GAP proteins, were first isolated from mammalian cells and shown to act downstream of Rac and CC42 (Kuroda et al., 1996). Although GAP activity has not been shown for these proteins, they act as downstream effectors of Rho GTPases and have been implicated in cytokinesis in a number of organisms. IQGAPs have been shown to be required for cytokinesis in *Dictyostelium* (Adachi et al., 1997) and *S. cerevisiae* (Epp and Chant, 1997). In *S. pombe* an IQGAP related protein, RNG2, has been shown to be required for the organisation of actin cables that accumulate at the medial regions prior to division (Eng et al., 1998). IQGAPs have also been shown to recruit actin to the cleavage furrow in *C. elegans* (Shannon and Li, 1999).

Other downstream effectors of Rac and Cdc42 have been implicated in cytokinesis. Null mutants of *paka,* which encodes a putative p21-activated Ser/Thr protein kinase (PAK) from *Dictyostelium*, has been shown to prevent cytokinesis of cells in suspension (Chung and Firtel, 1999). However, mutants in *Drosophila PAK* have not shown an effect on cytokinesis. Rather, PAK has been shown to act downstream of Trio RhoGEF-activated Rac, where it is required for axon guidance (Newsome et al., 2000).

Figure 1-4 Downstream signalling pathways activated by Rho, Rac and Cdc42 GTPases

The cellular targets of activated Rho, Rac and Cdc42 fall into three classes of proteins; Ser/Thr kinases, scaffold proteins and lipid kinases. Activation of these pathways in mammalian tissue culture cells results in changes in actin and/or myosin activity resulting in the formation of actin stress fibres, lamellopodia or filopodia. Components of these signalling pathways which have been shown to be required during cytokinesis are highlighted in green. (ROK: Rho kinase, Citron: Citron kinase, PKN: Protein kinase N, IF: internediate filament, GFAP: glial fibrillary acidic protein, Dia: Diaphanous, PI4-5K: Phosphatidylinositol-4-phosphate-5-kinase, PIP_2: phosphatidylinositol 4,5-biphosphate, DAG: Diacylglycerol kinase, POR-1: partner of Rac1, IQGAP: Protein with a calmodulin binding domain and a domain bearing homology to GAP proteins, PAK: p21-activated kinase, MRCK: Myosin regulatory chain kinase, WASP: Wiskott-Aldrich-syndrome protein).


Rho-GTP
Rac-GTP
Cdc42-GTP
Lipid kinase
PI4-5P DAG
Scaffold protein
P140 WAVE
POR-1 (wasp-like)
Ser/Thr kinase
PAK1,2,3
Scaffold protein
N-WASP
Ser/Thr kinase
ROK citron PKN
myosin activation
?
?
Scaffold protein
Rhophilin Dia
Rhotekin
Kinectin
PIP2 levels
actin
?
actin
Scaffold protein
IQGAP1,2
actin
actin myosin
actin
Ser/Thr kinase
MRCK PAK4
actin actin
IFs:
Vimentin
GFAP
?
actin
actin stress fibres
lamellopodia
filopodia

Thus, most of the evidence to date would support a major role for signalling pathways downstream of Rho-GTP in the correct regulation of acto-myosin structures during cytokinesis but Rac-GTP and Cdc42-GTP activated signalling pathways also appear to play some role.

1-7.5 Rho GTPases and signalling of nuclear events

In addition to the role of Rho GTPase signalling cascades in cytoskeletal reorganisation during various cellular processes, they have also been shown to signal to the nucleus. For example, signalling via Rho, Rac and Cdc42 has been shown to result in the trasncriptional activation of genes regulated by serum response factor (SRF) (Hill et al., 1995). Also, the DBL RhoGEF-mediated activation of transcription factor NF-κB has been shown to be necessary for the oncogenic activity of DBL (Sulciner et al., 1996). This activation can be mediated by Rho, Rac or Cdc42, but requires distinct downstream signalling pathways in each case. Also, nuclear signalling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the *Drosophila* eye (Fanto et al., 2000). Finally, the activities of Rho, Rac and Cdc42 are all required for G1 progression of fibroblasts (Olson et al., 1995). Thus their correct regulation is important during normal cell growth and their mis-regulation is an important factor during cellular transformation.

Thus, the activation of Rho proteins can result in quite diverse downstream signalling effects on cytoskeletal reorganisation, in addition to the activation of SRF transcription and cellular transformation. Mutations in distinct effector loops of Rho have been shown to distinguish between these signalling pathways (Sahai et al., 1998).

1-7.6 Rho GTPases in *Drosophila*

Rho family members were identified in *Drosophila* based on sequence similarity to their mammalian counterparts and, in the absence of mutations, overexpression studies were initially used to determine their functions during development. Dominant negative and constitutively active forms of these proteins were generated based on the analagous mutations made in mammalian Rho family members, which were then ectopically expressed in various tissue specific patterns. Mutant forms of Drac1 and Dcdc42 have been shown to have distinct effects on the processes of axon outgrowth and myoblast fusion (Luo et al., 1994), in addition to their ability to control different actin dependent processes in the imaginal wing (Eaton et al., 1995). Dominant negative mutations in *Drac1* have also been shown to interfere with the processes of dorsal closure, head involution and germ band retraction, implicating a role for Drac1 in the cell shape changes normally required for these morphogenetic processes (Harden et al., 1995). Cellularisation is disrupted by the inhibition of Rho activity, or by the activation

of Cdc42 function. (Crawford et al., 1998). Rho-like (RhoL) was identified and found to be important, together with Rac, Rho and Cdc42, for distinct functions during oogenesis (Murphy and Montell, 1996). RhoL/Drac3 is also thought to play a role in hemocyte differentiation (Sasamura et al., 1997) while the function of Drac2 is yet to be determined.

Hypomorphic mutations in *Rho1* have been shown to affect tissue polarity during early eye development (Strutt et al., 1997) and to affect morphogenesis and segmentation during early development (Magie et al., 1999). Clones of a *Rho1* null allele failed to proliferate (Strutt et al., 1997) and mutant embryos were subsequently shown to have defects duirng cytokinesis (Prokopenko et al., 1999). The function of *Rho1* has also been shown to be required for the proliferation of neuroblasts and dendritic cells but not for axonal morphogenesis. (Lee et al., 2000). Lethal mutations of *cdc42* have been isolated by a reverse genetic screen (Fehon et al., 1997) and functional analysis has revelaed a role in actin filament assembly, epithelial morphogenesis, and cell signaling but not during cytokinesis (Genova, et al 2000).

1-7.7 RhoGEFs and GAPs in *Drosophila*

Overexpression of wild-type Rho1 in the eye imaginal disc has no effect on the process of cell fate determination, but affects the later stages of development where cells undergo significant cell shape changes as they form the adult retina (Hariharan et al., 1995). *DRhoGEF2* was identified as a suppressor of the rough eye phenotype that results from ectopic expression of Rho1 (Barrett et al., 1997). *DRhoGEF2* was also isolated independently in a screen for maternal effect mutations that affected cell shape changes early during embryogenesis (Hacker and Perrimon, 1998). DRhoGEF2 is required for the cell shape changes necessary for gastrulation in the early embryo, and appears to act in response to the Folded Gastrulation (FOG) extracellular ligand through the Concertina (CTA) Gα subunit of a heterotrimeric G protein. DRhoGEF2 has a RGS (regulator of G protein signalling) domain which acts as a GTPase activating protein (GAP) for the Gα subunit of heterotrimeric G proteins. Thus, DRhoGEF provides a direct link between extracellular signals received by the large heterotrimeric G proteins and the subsequent effects on the Rho GTPases and actin cytoskeletal reorganisation. This communication between heterotrimeric G proteins and the Rho GTPases has also been shown for the mammalian protein, p115RhoGEF (Hart et al., 1998; Kozasa et al., 1998).

In addition to DRhoGEF2, six other RhoGEFs have been identified in *Drosophila.* PBL, which is the subject of this thesis (see section 1-8); DrtGEF (or DRhoGEF1), which is expressed throughout embryogenesis and oogenesis (Werner and Manseau, 1997); Still Life, of which truncated forms have been shown to interfere with the organisation of the actin

cytoskeleton in synaptic termini (Sone et al., 1997); DrhoGEF3, which exhibits a highly dynamic pattern of expression during embryogensis (Hicks et al., 2001); Trio which has two GEF domains, one of which has been shown to be responsible for the correct activation of Rac during axon guidance (Newsome et al., 2000) and finally, dPIX, which is required for regulation of the post synaptic structure of neuromuscular junctions (Parnas et al., 2001).

The functions of three RacGAPs have been identified in *Drosophila.* Rotund RacGAP, which has been shown to affect spermatid and retinal differentiation (Bergeret et al., 2001); P190RhoGAP, which has been shown by RNA interference analysis to regulate axon branch stability (Billuart et al., 2001) and DRacGAP, which has been shown to inhibit EGFR signalling in the imaginal wing disc through negative regulation of DRac1 and Cdc42 (Sotillos and Campuzano, 2000). In addition, another RhoGAP; d-CdGAPr has been identified but the function has not yet been determined (Sagnier et al., 2000).

The Rho family members, GEFs and GAPs discussed here are those that have been reported in the literature to date. However, the release of the *Drospohila* genome sequence, ha revealed many others that are yet to be characterised.

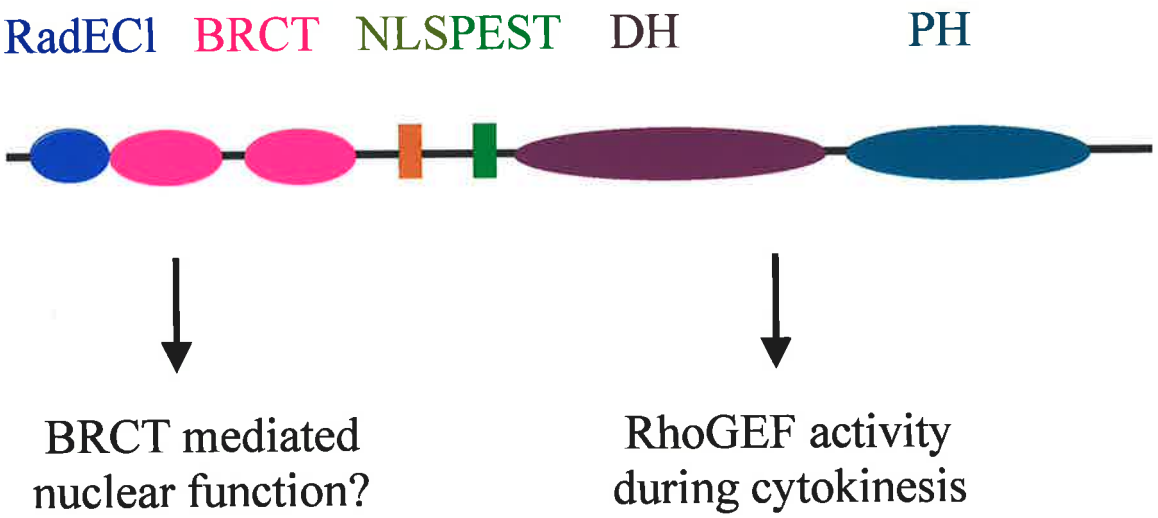
1-8 *pbl* as a regulator of cytokinesis in *Drosophila*

1-8.1 Genetic approaches for the identification of regulators of cytokinesis

Candidate genes affecting cytokinesis have been identified in screens for mutations that result in male sterility or lethality at embryonic or pupal stages. Mutations in genes affecting these processes do not affect the early embryonic divisions but manifest themselves later in development. Male sterile mutations have been identified as *P*-element insertions that disrupt regulatory regions of genes, resulting in germline specific effects (Castrillon et al., 1993). In the case of embryonic or pupal lethality, genes have been identified where the maternally provided gene product was sufficient for the early embryonic divisions but became limiting later in development, resulting in defects of neuroblast divisions (Gatti and Goldberg, 1991). The *pbl* gene was originally identified as a zygotic embryonic lethal mutation affecting the pattern of the larval cuticle in *Drosophila* (Jurgens et al., 1984). Further characterisation of this gene revealed a specific requirement during cytokinesis in all embryonic divisions (Hime and Saint, 1992) and (Lehner, 1992). The early phenotype observed with *pbl* mutant embryos, which has been shown to specifically inhibit cytokinesis, suggests the product of this gene may be a basic requirement during cytokinesis, and may play a regulatory role.

Figure 1-5 The domain structure of PBL

The RhoGEF activity of PBL is inferred by the presence of tandem Dbl-oncogene homology (DH) and Pleckstrin homology (PH) domains at the C terminus. Together these specify a Rho small GTPase regulatory activity, consistent with a role for PBL in cytoskeletal reorganisation necessary for cytokinesis. In addition, PBL has a RadECl domain which is novel and has been identified by homology to RAD4-like, ECT2 and CLB6. PBL also has two BRCT domains that were originally identified in the BRCA1 C-Terminal sequences. Together these RadECl and BRCT domains suggest a nuclear function for PBL, which is supported by the presence of a nuclear localisation sequence (NLS). PBL also contains PEST sequences that could regulate protein stability.



RadECl: Rad4-like, ECT2, Clb6 homology
BRCT: BRCA1 C-Terminal repeat
NLS: Nuclear localisation sequence
PEST: Degradation sequence
DH: Dbl oncogene homology domain
PH: Pleckstrin homology domain

1-8.2 *pbl* encodes a RhoGEF with BRCT domains and is required for cytokinesis

Cloning of the *pbl* gene was facilitated by the identification of *P*-element mutations affecting PNS development (Salzberg et al., 1997). Analysis of the open reading frame of PBL (Prokopenko et al., 1999) revealed a number of interesting domains in the encoded protein (Fig. 1-5). The tandem Dbl oncogene homology (DH) and pleckstrin homology (PH) domains are characteristic of guanine nucleotide exchange factors (GEFs), which act as positive regulators of the Ras homology (Rho) family of GTPases (Cerione and Zheng, 1996). This family of GTPase signalling molecules is required for reorganisation of the actin cytoskeleton during various cellular processes where significant cell shape changes are required (Hall, 1998). The RhoGEF activity of PBL could confer spatio-temporal specificity to the activation of Rho family GTPase(s) during cytokinesis. The localisation of PBL during cell division would support this hypothesis. PBL is concentrated around the cortex of the cell during late anaphase when reorganisation of cortical actin begins, it then becomes concentrated at the contractile ring during cytokinesis (Prokopenko et al., 1999). Thus PBL could provide localised activation of downstream effector molecules necessary for actin filament reorganisation and/or myosin activation, resulting in contractile ring formation and constriction.

In addition to the GEF domains, Pbl has a novel RAD4-like, ECT2, Clb6 (in this thesis referred to as the RadECl) domain and two BRCT domains at its amino terminus. These domains are found in proteins required for cell cycle checkpoints and DNA repair (Bork et al., 1997). Together with the nuclear localisation sequence (NLS), these domains suggest a possible nuclear function for PBL. PBL also has a predicted PEST sequence that may regulate stability of the protein product, perhaps during the cell cycle (Rechsteiner and Rogers, 1996). Thus, in addition to the predicted RhoGEF activity as a regulator of cytoskeletal dynamics, there may also be a nuclear role for PBL.

The *pbl* gene product is orthologous to the mouse proto-oncogene, *ect2*. *ect2* was originally identified by its ability to transform NIH3T3 cells, this transformation was only observed with an N-terminally truuncated form of *ect2* (Miki et al., 1993). It was further revealed that ECT2 was able to bind *in vitro* to members of the Rho family; RhoC and Rac1 however, no GDP-GTP exchange was demonstrated for these complexes (Miki et al., 1993). It was subsequently shown that full length ECT2, was required for cytokinesis, with *in vitro* exchange activity reported for Rho, Rac and Cdc42 (Tatsumoto et al., 1999). ECT2 activity was also shown to result in the accumulation of GTP-bound active RhoA at the cleavage furrow (Kimura et al., 2000). ECT2 has also been identified in humans and localised to 3q26.2-q26.3 on human chromosomes. Common chromosomal rearrangements in this region

are associated with acute myeloid leukaemia and myelodysplastic syndromes (Takai et al., 1998).

PBL and ECT2 are currently the only known RhoGEF family members to also have BRCT domains. The conservation of these two distinct types of domains in a single protein raises interesting questions about their roles in cellular signalling and cytokinesis.

1-9 This work

Although cytokinesis has been studied mechanistically for many years, the molecular nature of many of the regulatory mechanisms remains poorly understood. The subject of this thesis is the *pbl* gene of *Drosophila,* which has previously been shown to be required during cytokinesis. *pbl* encodes a RhoGEF, a putative activator of Ras homology (Rho) GTPase family members, and as such represents a good candidate for acting as a key regulator of cytokinesis. Various genetic approaches are undertaken in this thesis to address the role of PBL during cytokinesis and to identify other components of PBL signalling pathways.

Chapter 2: Materials and Methods

2-1 Materials

2-1.1 Chemical reagents

All reagents were of analytical grade, or the highest grade obtainable.

2-1.2 Enzymes

Restriction endonucleases : Boehringer Mannheim, New England Biolabs (NEB), Pharmacia

T4 DNA ligase : Boehringer Mannheim, NEB

Calf Intestinal Phosphatase (CIP): Boehringer Mannheim

RNase A and Lysozyme : Sigma

Pfu DNA polymerase : Stratagene

2-1.3 Kits

QIAquick gel extraction kit : Qiagen

QIAprep spin miniprep kit: Qiagen

Vectastain ABC kit : Vector Laboratories Inc.

Enhanced Chemiluminescence (ECL) kit : Amersham

BRESAspin plasmid mini kit : Geneworks

Western blot recycling kit : Alpha Diagnostics

2-1.4 Antibiotics

Ampicillin: Sigma

2-1.5 Molecular weight markers

DNA

λDNA was digested with *BstEII* and *SalI* to produce fragments of the following sizes (in kb) 14.14, 8.45, 7.24, 5.70, 4.82, 4.32, 3.68, 3.13, 2.74, 2.32, 1.93, 1.37, 1.26, 0.70, 0.50, 0.22 and 0.12.

Protein

High molecular weight markers (GIBCO BRL) sizes (in kDa) 205, 116, 97, 66, 45 and 29.

Prestained molecular weight markers (NEB) sizes (in kDa) 175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5.

Kaleidoscope prestained standards (Bio-Rad) sizes (in kDa) 216, 132, 78, 45.7, 32.5, 18.4 and 7.6.

2-1.6 Oligonucleotides

Oligonucleotides designed for *in vitro* mutagenesis were obtained from Genset. Those of 31bp length were reverse phase chromatography purified, while those of 39 bp were desalted and subsequently gel purified before using in the mutagenesis reaction.

Primers for inactivating the consensus CDK1 sites

S360Af: 5' cga ttg tac cac c**gc** **c**cc gga taa act gga g 3'

S360Ar: 5' ctc cag ttt atc cgg **ggc** ggt ggt aca atc g 3'

T380Vf: 5' ggc gag cga tgc c**gt** **c**cc agc caa gaa atc g 3'

T380Vr: 5' cga ttt ctt ggc tgg **gac** ggc atc gct cgc c 3'

T771Vf: 5' gct ttc tcc ttt aac aaa **gtc** ccc aac aaa ctg aaa cgg 3'

T771Vr: 5' ccg ttt cag ttt gtt ggg **gac** ttt gtt aaa gga gaa agc 3'

Primers for activating the consensus CDK1 sites

S360Df: 5' cga ttg tac cac c**ga** **c**cc gga taa act gga g 3'

S360Dr: 5' ctc cag ttt atc cgg **gtc** ggt ggt aca atc g 3'

T380Df: 5' ggc gag cga tgc c**ga** **c**cc agc caa gaa atc g 3'

T380Dr: 5' cga ttt ctt ggc tgg **gtc** ggc atc gct cgc c 3'

T771Df: 5' gct ttc tcc ttt aac aaa **gac** ccc aac aaa ctg aaa cgg 3'

T771Dr: 5' ccg ttt cag ttt gtt ggg **gtc** ttt gtt aaa gga gaa agc 3'

Primers for GFP-PBL

5'GFP: 5' cat gtc atg agt aaa gga gaa gaa ctt ttc act g 3'

3'GFP: 5' cat gtc atg att ttg tat agt tca tcc atg cca tgt gta atc cc 3'

2-1.7 Antibodies

All secondary and tertiary antibodies were reconstituted according to the manufacturers instructions.

Primary antibodies:

Actin (JLA20): mouse monoclonal (Developmental Studies Hybridoma bank) used at 1:5 on tissues.

β-Tubulin: mouse monclonal (Sigma), used at 1:1,000 on embryos.

PBL: rat polyclonal, crude sera (S. Prokopenko), used at 1:500 on tissues and 1:5,000 on western blots.

PBL: rat polyclonal, IgG purified sera, used at 1:250 on tissues and 1:2,500 on western blots.

Spectrin (3A9): rabbit polyclonal (D. Branton), used at 1:50 on tissues.

Lamin: mouse monoclonal (Y. Gruenbaum), used at 1:10 on tissues.

Myc (9E10): mouse monoclonal (Developmental Studies Hybridoma Bank, purified by the Instiutute of Medical and Veterinary Science, Adelaide, SA.(IMVS)), used at 1:250 on tissues and 1:2,500 on western blots.

PH3: rabbit poyclonal (Upstate Biotechnology), used at 1:300 on tissues.

ELAV (7E8A10): rat polyclonal (Developmental Studies Hybridoma Bank), used at 1:500 on tissues.

Secondary antibodies:

α-mouse-biotin (Jackson Laboratories), used at 1:200 on tissues.

α-rat-biotin (Jackson Laboratories), used at 1:200 on tissues.

α-rabbit-Cy5, (Jackson Laboratories) used at 1:200 on tissues.

α-mouse-HRP (Jackson Laboratories), used at 1:3,000 on western blots.

α-rat-HRP (Jackson Laboratories), used at 1:3,000 on western blots.

Tertiary complexes:

Strepavidin-Alexa 488 (Molecular Probes), used at 1:200 on tissues.

Strepavidin-Cy5 (Jackson Laboratories), used at 1:200 on tissues.

2-1.8 Media, Buffers and Solutions

Drosophila media

Fortified (F1) Drosophila medium: 1% (w/v) agar, 18.75% compressed yeast, 10% treacle, 10% polenta, 1.5% acid mix (47% propionic acid, 4.7% orthophosphoric acid), 2.5% tegosept (10% para-hydroxybenzoate in ethanol)

Grape juice agar plates: 0.3% agar, 25% grape juice, 0.3% sucrose, 0.03% tegosept mix

Bacterial Media

All media were prepared with distilled and deionised water and sterilised by autoclaving, except heat labile reagents, which were filter sterilised. Antibiotics were added from sterile stock solutions to the media after it had been autoclaved.

L-Broth:	1% (w/v) amine A, 0.5% yeast extract, 1% NaCl, pH 7.0.
SOC:	2% bactotyptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl,10mM $MgCl_2$, 10 mM $MgSO_4$, 20mM glucose.
Plates:	L-Broth with 1.5% (w/v) bactoagar supplemented with ampicillin (50mg/ml) where appropriate.

Buffers and solutions

Agarose gel loading buffer:	50% (w/v) glycerol, 50 mM EDTA, 0.1% (w/v) bromophenol blue
Embryo injecting buffer (1x):	5mM KCl, 0.1mM $NaPO_4$ pH 6.8
PBS:	7.5mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145mM NaCl
1 X PBT:	1 x PBS, 0.1% Tween 20 or Triton X-100
Protein gel transfer buffer:	48mM Tris-base, 39mM Glycine, 0.037% (w/v) SDS, 20% methanol
3 X sample buffer:	10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue, 12.5% 0.5M Tris-HCl pH6.8
Protein gel running buffer:	1.5% Tris-base, 7.2% Glycine, 0.5% SDS
STET:	50mM Tris-HCl pH8.0, 50mM EDTA, 8%w/v sucrose and 0.05% Triton X-100
TAE:	40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2
TBS:	20mM Tris-HCl pH 7.5, 150mM NaCl
10X HEN buffer:	1M HEPES, O.5M EGTA, 0.1% NP-40, pH to 6.9 and filter sterilised.

2-1.9 Plasmids

pBluescript KS+ (Statagene)

pUAST - UAS promoter (obtained from H. Richardson)

2-1.10 Bacterial strains

E. coli DH5α: F' f80 lacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK- mK+) supE44 relA1 deoR Δ(lacZYA-argF) U169

2-1.11 Drosophila strains

2-1.11.1 Wild-type

w^{1118} was used as the wild-type strain in immunohistological stains and for the generation of transgenic lines.

2-1.11.2 ***pbl* mutants**

pbl^{2} and pbl^{3} alleles (originally referred to as pbl^{5D} and pbl^{7O} respectively in (Jurgens et al., 1984) correspond to nonsense mutations that result in truncated protein products of 37 and 185 amino acids respectively (Prokopenko et al., 1999).

$Df(3L)pbl^{X25}$ was obtained from R Saint.

UAS-$pbl^{EP(3)3415}$ was obtained from Bloomington Stock centre.

2-1.11.3 ***GAL4/UAS* stocks**

GMR-GAL4, *hsp70-GAL4*, *prd-GAL4* and *sev-GAL4* were all obtained from Bloomington Stocks centre. *sev*-GAL4* was isolated in our laboratory following EMS mutagenesis of flies carrying *sev-GAL4* and *UAS-dri* in a screen for suppression of the lethality associated with ectopic expression of DRI (A. Lumsden, Pers. Comm.). It was determined that the suppression was most likely due to a mutation in the promoter region of *sev-GAL4* resulting in decreased levels of expression, predominantly in salivary glands whilst maintaining ectopic expression during eye development. *UAS-GFP* was obtained from Bloomington Stock centre. *UAS-ect2* was obtained from S. Prokopenko, which contains the full length cDNA encoding ECT2.

2-1.11.4 **Cell cycle mutants and apoptosis stocks**

$cycA^{C8LR1}$, *cycB2* and *cycB31* were obtained from P. O'Farrell. $cdk1^{E1.9}$ $cycE^{AR95}$, $cdc2c^{JS}$ and $E2F^{91}$, *GMR-P53* and *Df(3)H99* were obtained from H. Richardson.

2-1.11.5 **Deficiency and *P*-transposon stocks**

Deficiency stocks of *DK2* were obtained from the Bloomington Stock centre, however this collection has been updated and many stocks have changed since the screen of *DK2* described in this work was performed. A collection of 499 unverified *P*-transposon mutations were obtained from A. Spradling. Some of these have been verified and are available from Bloomington Stock centre, the remainder have been discarded. Independent alleles or deficiency stocks to verify *P*-transposon interactors were all obtained from Bloomington Stocks Centre unless otherwise specified.

2-1.11.6 ***Rho* family members**

$Rho1^{72R}$ and $Rho1^{72O}$ null alleles were obtained from M. Mlodzik. $Cdc42^{3}$ and $Cdc42^{4}$ lethal alleles were obtained from R. Fehon. The deficiency stock *Df(3L)Ar14-8* was used as an allele of *Rac1*. The deficiency stock *Df(3L)RM5-2* was used as an allele of *Rac2*. Since this stock also removes *pbl*, a recombinant chromosome was generated with the *cos34* transposon,

one copy of which is sufficient to rescue *pbl* mutant embryos to adulthood (J. Wong, Pers. Comm.). *ΔMtl* flies were obtained from B. Dickson. The deficiency stock *Df(3R)by10* was used as an allele of *Rac3*.

2-1.11.7 Downstream Rho effectors and genes required for cytokinesis

dia^{2} was provided by S. Wasserman. *rok*^{2} and *gek*^{D28} were provided by L. Luo. *polo*^{9} and *polo*^{10} stocks were provided by D. Glover. *chic*^{221} was provided by L. Jones, *act-up* by J. Treismann and *sktl*$^{\Delta 20}$ was obtained from H. Bellen. *pav*^{B200}, *PKN* $^{l(2)06736}$ and *pnut*^{XP} were obtained from Bloomington stocks centre. *sqh*^{1} was obtained from Umea stock centre.

2-2 Methods

Standard molecular genetic techniques were performed as described in (Sambrook et al., 1989) or (Ausubel et al., 1994).

2-2.1 Generation of recombinant plasmids

Dephosphorylation of vector DNA

After the vector DNA was linearized by restriction enzyme digestion, 1-2 units (U) of CIP was added to the reaction and incubated at 37°C for at least 1 hour.

Purification of DNA from agarose gels

QIAquick gel extraction kit was used to purify DNA bands from agarose gels, using the manufacturers protocol.

Ligation

DNA fragments to be ligated were placed in a mix (total volume 20µl) containing 1U of T4 DNA ligase, and 1x ligation buffer and incubated at 18°C overnight. 15% PEG was included in the ligation mix when DNA fragments to be ligated had blunt ends. For transformation by electroporation, the ligation was phenol/chloroform extracted, precipitated by adding 1 µl glycogen, 1/10 volume 3 M NaAcetate pH 5.2 and 2.5 volumes ethanol, then washed in 70% ethanol prior to resuspension in 10µl Milli Q water (MQ H_2O).

2-2.2 Transformation of Bacteria

500ml of L-broth was innoculated with 5ml of an overnight culture of *E. coli DH5α* cells and grown to an OD_{A600} of 0.5-0.6. The culture was then chilled in an ice slurry for 15 to 30 minutes and the cells harvested by centrifugation at 4000g for 15 minutes. The cells were then resuspended in 500ml of ice-cold MQ H_2O, pelleted at 5000g, resuspended in 250ml of ice-cold MQ H_2O, pelleted at 4000g, resuspended in 10ml of ice-cold 10% glycerol, re-

pelleted at 3000g and finally resuspended in 1ml of ice-cold 10% glycerol. The competent cells were then snap frozen in liquid nitrogen and stored as 45µl aliquots at -80°C.

For transformation, cells were thawed at RT, added to ligation reaction mixture and incubated on ice for at least 30 seconds. Cells were then transferred to an ice-cold 2mm electroporation cuvette and electroporated in a Bio-Rad Gene Pulser set to 2500V, 25 µFD capacitance and Capacitance Extender set to 500 µFD. The cuvette was immediately washed out with 1ml of SOC, and the suspension incubated at 37°C for 30 minutes. Cells were then pelleted for 8 seconds at 14 000rpm, then 800µl of the supernatant was removed, and the cells gently resuspended in the remaining SOC. The cell suspension was plated onto L-agar plates supplemented with 50µg/ml ampicillin and incubated at 37°C overnight. If selection for β-GALactosidase activity (blue/white colour selection) was required, 10µl of 10% IPTG and 10µl of 20% BCIG were added prior to plating.

2-2.3 Isolation of plasmid DNA

Small scale preparation- Rapid boiling lysis method:

A 2ml culture supplemented with the appropriate antibiotics, was incubated overnight at 37°C, with shaking. Cells were harvested by centrifugation at 14,000 rpm in a microcentifuge for 15 seconds. The bacterial pellet was resuspended in 200µl of STET, followed by addition of 10µl of lysozyme (10mg/ml). The suspension was heated at 100°C for 45 seconds and centrifuged at 14,000 rpm for 15 min. The pellet was removed with a toothpick. Plasmid DNA was then precipitated with 240µl of isopropanol, centrifuged, and washed with 70% ethanol. The pellet was then dried and resuspended in 20µl MQ H2O.

Large scale preparation:

Alkaline lysis midi-preps were prepared from 25-50 ml cultures following standard protocols (Sambrook et al., 1989).

High quality plasmid DNA, for microinjection of *Drosophila* embryos and for dye terminator sequencing reactions, was prepared using Qiagen midi columns, according to manufacturers instructions.

2-2.4 PCR amplification of DNA

Pfu polymerase was used in all of the reactions according to the manufacturers instructions. PCR conditions were using 0.5 U *Pfu* polymerase, 1 ng template DNA, 0.1 ng primers and 0.2 mM dNTPs in *Pfu* PCR buffer (20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, pH 8.75). Reactions were performed using a

MJ Research, PTC-200 peltier, thermal cycler, with the following conditions: 35 cycles of 95°C for 30 sec, 55°C for 1 min and 72°C for 1 min 30.

2-2.5 *In vitro* mutagenesis

The design of the primers used in the site directed mutagenesis followed the instructions provided in Stratagene's QuikChange Site-Directed Mutagenesis kit. The reaction was carried out in a 20µl volume. To each reaction the following was added

2µl of 10x reaction buffer
50ng of dsDNA template (pBS-Pcl Sac/Not)
125ng of the forward primer
125ng of the reverse primer
1µl of 10mM dNTP mix (2.5mM each dNTP)
Sterile MQ H_2O to a final volume of 19µl
1µl of *Pfu* Turbo DNA polymerase (2.5U/µl) was added last

The reaction was cycled according to the manufacturers instructions in a MJ Research, PTC-200 peltier, thermal cycler. The reaction was transferred to a new microfuge tube, 1µl of *DpnI* (10U/µl) added to the reaction, mixed and allowed to incubate at 37°C for 1 hour to digest the parental (nonmutated) dsDNA. The reaction was phenol/$CHCl_3$ extracted, ethanol precipitated and resuspended in 10µl of MQ H_2O. 10µl was then transformed into *DH5α* using electroporation. Restriction analysis was used to identify mutated clones where possible but all mutations were confirmed by sequence analysis before use in subsequent experiments.

2-2.6 Automated sequencing

DNA was sequenced using the ABI Prism™ Dye Teminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), essentially as descibed in the manufacturer's protocol with the modication of using half the descibed amount of reaction mix. Double-stranded DNA was used as a template and, in general, primers were de-salted. Reactions were performed on a Corbett Research FTS-1S Capillary Thermal Sequencer, with the following conditions: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes with a temperature ramp setting of 2.

Running and analysis of Dye Terminator gels was conducted by the Sequencing Centre at the IMVS.

2-2.7 Protein gel electrophoresis

A Bio-Rad mini-Protean II gel electrophoresis system was used with 0.8mm analytical gels prepared according to manufacturer's instructions. Protein samples were electrophoresed at 200V for 45 min.

2-2.8 Western blotting

Western blotting of proteins onto nitrocellulose membrane was performed using a Bio-Rad Transblot Semi-dri transfer cell according to manufacturer's guidelines. In general, a current of 3 mA per cm^2 of gel was applied for 30 min. Nitrocellulose blots were washed thoroughly with 1 X PBT and then blocked for 1 hour in 5% Skm milk powder (Blotto). Primary antibody incubations were carried out overnight at 4°C and secondary antibody incubations for 2hr at RT, with the appropriate dilutions of antibody in Blotto. The secondary antibodies were HRP conjugated and detected by ECL.

2-2.9 *Drosophila* cultures

Flies were raised at 18°C or 25°C, with 70% humidity, on F1 medium.

2-2.10 *P*-element mediated Transformation of *Drosophila*

Micro-injection: DNA for injection was prepared using the Qiagen kit according to the suppliers protocol. An injection mix was prepared to a concentration of 0.5-1μg/μl transformation vector plasmid DNA and 0.3μg/μl of pπ25.7wc (Δ2-3 transposase) plasmid in 1x Embryo injecting buffer. The injection needle was back filled using a drawn out capillary containing 2μl of the injection mix which had been centrifuged briefly to remove any particulate matter. w^{1118} embryos to be injected were collected from 30 min lays on grape juice agar plates at 25°C, dechorionated in 50% bleach for 3 min, and rinsed thoroughly in MQ H_20. Embryos were then aligned along a strip of non-toxic rubber glue such that their posterior ends would face the needle. A drop of liquid parafin was placed over the embryos and the slide placed on the stage of an inverted microscope. A micromanipulator was used to position the needle, with injection carried out by moving the microscope stage to bring the embryos to the needle, such that a very small amount of DNA was injected into the posterior cytoplasm.

Identification of transformants: The slides of microinjected embryos were then placed in a petri dish containing moist tissue paper with a small amount of yeast paste surrounding the embryos and kept at 18°C to allow the embryos to hatch. Larvae were collected after 2 days using strips of Whatman paper and placed in a fly food vial where they developed into adult flies. The flies were then crossed to w^{1118} flies to identify transformants on the basis of the

mini-white^{+} eye pigmentation phenotype. Numerous independent transformants were mapped to determine the chromosome of insertion, using the dominantly-marked balancer chromosomes CyO and TM6B in the stocks *w ; Sco / CyO* and *w ; Df(3R)roXB3 / TM6B Hu,* and homozygous lines generated using these balancers.

2-2.11 Clones and Stocks generated

2-2.11.1 ***GMR>pbl***

The cloning of the *pbl1A* cDNA into pUAST and subsequent generation of three *UAS-pbl* transgenic lines was performed by L. Prior. *UAS-pbl3.2* was the only one of these lines that resulted in a rough eye phenotype when expressed with *GMR-GAL4*. A recombinant chromosome was generated between *UAS-pbl3.2* and *GMR-GAL4* and balanced with CyO, thus generating the *GMR>pbl/CyO* stock.

2-2.11.2 ***GMR>pbl>pbl***

Mobilisation of *UAS-pbl3.2* was achieved by introducing a source of Δ2-3 transposase. Mapping of new insertions identified one on the third chromosome (*UAS-pbl3.2C*). A recombinant chromosome was generated between *UAS-pbl3.2C* and *UAS-pbl4-11*, one of the original insertions that was also mapped to the third chromosome, and balanced with TM6B thus generating the *UAS-pbl4-11, UAS-pbl3-2C / TM6B* stock. Co-expression of this stock with *GMR-GAL4* resulted in a rough eye phenotype which is referred to as *GMR>pbl>pbl.*

2-2.11.3 ***GMR>pblΔDH***

The *GMR>pblΔDH/CyO* stock was generated by A. Brumby. The *pbl1A* cDNA was digested with *ScaI* and *SpeI*. The *SpeI* site was end-filled and religated with *ScaI* which resulted in a 53 amino acid deletion in the catalytic portion of translated product of the *pbl1A* cDNA. This mutant form of PBL (PBLΔDH) would be unable to catalyse the exchange of GDP for GTP. This was cloned into pUAST and used to generate multiple *UAS-pblΔDH* transgenic lines. A recombinant chromosome was generated between two of these lines resulting in the *UAS-pblΔDH5B,UAS-pblΔDH6A/CyO* stock. A triple recombinant was then generated between *GMR-GAL4* and these two copies of *UAS-pblΔDH* resulting in a stock referred to as *GMR>pblΔDH/CyO*. This stock resulted in a temperature dependent rough eye phentoype that was visible at 25°C but not at 18°C.

2-2.11.4 ***GMR>pblΔL and GMR>pblΔDHΔL***

The *GMR>pblΔL/CyO* and *GMR>pblΔDHΔL/CyO* stocks were generated by A. Brumby. PCR primers were designed to amplify (Elongase, GIBCO BRL), the coding regions of *pbl1A* and *pbl1AΔDH*. These fragments were cloned separately into pUAST resulting in clones of *pbl1A* and *pbl1AΔDH* that were missing all upstream untranslated sequences and used to generate multiple *UAS-pblΔL* and *UAS-pblΔDHΔL* transgenic lines. Recombinant chromosomes were generated between *GMR-GAL4* and *UAS-pblΔL* resulting in the *GMR>pblΔL/CyO* stock and between *GMR-GAL4* and *UAS-pblΔDHΔL* resulting in the *GMR>pblΔDHΔL/CyO* stock.

2-2.11.5 ***GMR>pblΔ325***

UAS-pblΔ325 transgenic lines were generated by A. Brumby. A fragment of the *pbl1A* cDNA corresponding to amino acids 325-853 was amplified (Elongase, GIBCO BRL), cloned into pUAST and used to generate *UAS-pblΔ325* transgenic lines. Great difficulty was encountered in generating these transgenic lines indicating the increased lethality associated with ectopic expression of this oncogenic form of PBL. The construct was also sent to our collaborators in the Bellen Lab. They generated *UAS-pblΔ325*$^{S4\text{-}57B}$ which is the stock described in these studies.

2-2.11.6 ***UAS-myc-pbl***

To generate a MYC tagged PBL, *pbl1A* was digested with *NcoI* and end-filled prior to ligation with 6 myc epitope tag as a *DraI-XhoI* end-filled fragment from pCS2 (from S. Prokopenko). The *myc-pbl1A* construct was then subcloned into pUAST and used to generate multiple *UAS-myc-pbl* transgenic lines.

2-2.11.7 ***UAS-GFP-pbl***

A GFP tagged PBL was generated by insertion of GFP as a *BspHI* fragment, generated by PCR from pBD1010 (GFP in pUAST from B.Dickson) using primers 5'GFP and 3'GFP, into *pbl1A* digested with *Nco1*. The *GFP-pbl1A* construct was then subcloned into pUAST and used to generate multiple *UAS-GFP-pbl* transgenic lines.

2-2.11.8 ***UAS-pbl^{T771V} and UAS-pbl$^{3*A/V}$***

UAS-pbl^{T771V} was generated by site-directed mutagenesis using T771Vf primer and T771Vr primer with *pbl1A* as the template then subcloned into pUAST and used to generate multiple *UAS-pbl*^{T771V} transgenic lines.

UAS-pbl$^{3*A/V}$ was generated by site-directed mutagenesis from *pbl1A*^{T771} template using primers S360Af, S360Ar, T380Vf and T380Vr then subcloned into pUAST and used to generate multiple *UAS-pbl*$^{3*A/V}$ transgenic lines.

2-2.11.9 ***UAS-pbl^{T771D} and UAS-pbl^{3*D}***

UAS-pbl^{T771D} was generated by site-directed mutagenesis using T771Df primer and T771Dr primer with *pbl1A* as the template then subcloned into pUAST and used to generate multiple *UAS-pbl*^{T771D} transgenic lines.

UAS-pbl^{3*D} was generated by site-directed mutagenesis from *pbl1A*^{T771D} template using primers S360Df, S360Dr, T380Df and T380Dr then subcloned into pUAST and used to generate multiple *UAS-pbl*^{3*D} transgenic lines.

2-2.11.10 ***Stocks generated for rescue assays***

A recombinant chromosome was generated between *prd-GAL4* and *pbl*^{2} and balanced with *TM3Sb*. This *prd-GAL4,pbl2/TM3Sb* stock was used in all rescue experiments. Recombinant chromosomes were also generated between *pbl*^{3} and various *UAS-pbl* lines on the third chromosome and balanced with *TM3Sb*. The stocks generated are; *UAS-pbl3.2C,pbl*^{3}*/TM3Sb, UAS-myc::pbl,pbl*^{3}*/TM3Sb, UAS-GFP::pbl,pbl*^{3}*/TM3Sb UAS-pblΔDH,pbl*^{3}*/TM3Sb, UAS-pblΔL,pbl*^{3}*/TM3Sb, UAS-pbl*$^{3*A/V}$*,pbl*^{3}*/TM3Sb* and *UAS-pbl*^{3*D}*,pbl*^{3}*/TM3Sb*. A recombinant chromosome was also generated between *pbl*^{3} and *UAS-ect2* line on the third chromosome and balanced with *TM3Sb*, thus generating *UAS-ect2,pbl*^{3}*/TM3Sb*.

2-2.12 Growth conditions

Fly stocks were maintained on standard media at 18°C. Experimental fly crosses were performed at 25°C, this was to ensure consistency in the GAL/UAS induced levels of expression and resultant rough eye phenotypes. The roughening of *GMR>pblΔDH* adult eyes was especially sensitive to temperature. Egg lays were also performed at 25°C on grape juice agar plates.

2-2.13 Heat shock induction

Heat-shock induction of *pbl (hs>pbl)* at 24-25, 26-27, 28-29h APF was carried out by collecting aged pupae in a microfuge tube with a piece of wet "kim-wipe" and some holes in the lid. The times were adjusted for development at 18°C, and heat shocks were performed by incubating the tube in a 37°C water bath for 1hr at a time. The larvae were left to recover at 18°C until 42hr APF, after which they were then dissected in 1 X PBT and stained with anti-Armadillo antibodies.

2-2.14 *Drosophila* protein extracts

For western analysis of endogenous PBL and MYC-PBL protein, *Drosophila* embryonic extracts were prepared. Embryos were collected in homogenisation buffer (10mM Tris pH7.8, 1mM EDTA, 1.0% NP-40, protease inhibitor cocktail tablet, 1mN Na_3VO_4, 1mM NaF, 1mM PMSF and 1mM DTT) and stored at -80°C. Samples were thawed on ice and homogenised. Samples were spun for 10 mins at 4°C, supernatant taken and spun again for 10 mins at 4°C. Extracts were incubated for 10 mins at 30°C before addition of CIP (or an equivalent volume of homogenisation buffer) and incubated for a further 10 mins at 30°C. Protein sample buffer was added, samples were placed at 100°C for 10 minutes, then vortexed thoroughly and centrifuged before being loaded onto a SDS-polyacrylamide gel and subsequently analysed by western blot.

2-2.15 Collection and fixation of *Drosophila* embryos

Embryos were collected on grape juice agar plates (25% grape juice,3% sucrose, 3% tegosept and 3% agar) smeared with yeast. They were then harvested and washed thoroughly in a basket using 0.7%NaCl, 0.15%Triton X-100. The basket was then transferred into a container with 50% commercially available bleach (2% sodium hypochlorite) for 3 minutes to de-chorionate the embryos. The embryos were once again washed thoroughly in the basket using 0.7%NaCl, 0.15%Triton X-100. They were then transferred to a glass scintillation vial containing a two-phase mix of 4 ml of 4% formaldehyde in 1 x HEN and 4 ml of heptane. The vial was then shaken on an orbiting platform such that the interface between the liquid phases was disrupted and the embryos were bathing in an emulsion, for 20 minutes to 'fix' the embryos. The bottom phase (aqueous) was drawn off and replaced with 4 ml of methanol and the vial was shaken vigorously for 30 seconds to de-vitellinise the embryos. De-vitellinised embryos sink from the interface and were collected from the bottom phase (methanol). Embryos were rinsed thoroughly in methanol at which point they were either processed for whole mount immuno-staining or storage at -20°C in methanol. For subsequent immunostaining with anti-β-Tubulin, taxol was added to the fixation.

2-3.17 Whole mount immuno-staining of *Drosophila* embryos

The methanol was removed from embryos in a microfuge tube and replaced with 50% Methanol/1 x PBT. Several rinses were then done using 1 x PBT followed by a single wash for 30 minutes. The embryos were then 'blocked' in 200µl of 1 x PBT containing 0.1% bovine serum albumin (BSA) and 5% normal goat serum (NGS) for at least 1 hour. The blocking solution was removed and primary antibody diluted in fresh blocking solution was added (usually 50-200µl). The embryos were routinely incubated with gentle rocking at 4°C

overnight. The next day, the antibody solution was removed and the embryos were washed extensively in 1 x PBT (several changes of buffer over a 2 hour time period). The embryos were then incubated with secondary antibody diluted in fresh blocking solution for at least 2 hours at room temperature with gentle rocking. Following a period of washing, as for the primary antibody, the embryos were incubated with a tertiary complex for two hours and then washed extensively for two hours in 1 x PBT. Embryos were then stained for 2 mins with Hoechst and washed in 1 x PBT on the nutator overnight. The embryos were mounted in PBS/80% glycerol onto a slide under a coverslip supported by two pieces of double sided tape and coverslips were sealed to the glass using commercially available clear nail varnish.

2-2.16 Immunolocalisation of *Drosophila* larval eye discs

Larval eye discs were dissected in 1 x PBS and fixed in a mixture of 3 parts 1.5 x Brower fix (1.5 Brower fix: 0.15M PIPES, 3mM $MgSO_4$, 1.5mM EGTA, 1.5% NP-40 pH6.9), 0.5 part 16% Formaldehyde and 0.5 parts water for 30 minutes on ice. Discs were washed in 1 x PBT-BSA (1 x PBS, 0.3% triton-X100, 1mg/ml BSA) and blocked with a solution of 1 x PBS, 0.1% Tween-20, 5mg/ml BSA for at least 1 hour on ice. The primary antibody was added directly to the blocking solution and incubated at 4°C overnight. The next day, discs were washed extensively in 1 x PBT-BSA before adding the secondary antibody conjugated to biotin diluted in 1 x PBT-BSA. After several washes at 4°C, the tertiary complex was added and incubated with the discs at 4°C for at least 2 hours. After further washes, the discs were then stained for 2 mins with Hoechst and washed in 1 x PBT on the nutator overnight. For fluorescence microscopy the eye discs were dissected in 30% glycerol, flattened and mounted directly on the slide with a coverslip carefully lowered down. For colour detection the eye discs were incubated in a solution of 0.5 mg/ml DAB and 0.045% H_2O_2 until the staining had developed (as assayed on a dissecting microscope), and then rinsed thoroughly using PBT prior to dissection and mounting in PBS/80% Glycerol.

2-2.17 Immunolocalisation of *Drosophila* pupal retinas

White pre-pupae were picked and aged to the appropriate stage. Retinas were dissected in 1 x PBS and fixed in 4% formaldehyde in 1 x HEN for 30 mins at room temp. All fixation and washing steps were performed in a cut off eppendorf lid, using a drawn out pasteur pipette whilst looking down a dissecting microscope. Retinas were washed in 1 x PBS + 0.1% Saponin and blocked for 1hr with 5% normal goat serum. Incubations with primary antibodies were performed overnight at 4°C. While secondary antibodies and tertiary complexes were incubated for 2hr at room temp.

2-2.18 Acridine orange staining of pupal retinas

Pupae were aged to 28-29hr APF and their retinas dissected in 1 x PBS Retinas were stained for 5 mins in a drop of 1.6 x 10-6 M acridine orange in 1 x PBS (1.6×10^{-3} M stock of AO was made up in ethanol and stored at -20°C for convenience). Retinas were mounted directly in this drop of AO by flattening out the retina and carefully lowering a coverslip. Each sample was analysed, within 5 mins of staining, by fluorescence microscopy.

2-2.19 Dissociation of eye disc cells

Eye discs were dissected in 1 x PBS and incubated in 200µl of PBTH (55µl 10 x PBS, 100µl 2.5% trypsin, 0.5µl Hoechst, 395µl MQ H_2O) for 3 hr at room temp. with gentle rocking. Approx 30µl of this solution was mounted directly onto a slide for fluorescent microscopic analysis.

2-2.20 Scanning electron micrographs

Adult eyes were dehydrated progressively through an acetone series (25%, 50%, 75%, 100%), critical point dried, coated with carbon/gold and viewed with a field emission scanning electron microscope (Phillips, at CEMMSA, Adelaide University). Digital images were collected and analysed in Adobe Photoshop 6.0.

2-2.21 Transverse sections

Adult eyes were incubated in periodate/lysine/paraformaldehyde for 30 mins, fixed in 2.5% gluteraldehyde/0.1M sodium phosphate (pH7.2) overnight, post-fixed in 2% osmium tetroxide, washed in water, and dehydrated in acetone. Specimens were mounted in epoxy resin, sectioned at 2µm, and stained with methylene blue.

2-2.22 Light and Fluorescence microscopy

Light microscopy was performed on a Zeiss Axiophot light microscope with DIC optics. Images were collected digitally with Photograb and Adobe Photoshop 6.0 was used for image preparation. Fluorescence microscopy was performed on a Olympus AX70. Digital images were collected with a cooled CCD camera and Adobe Photoshop 6.0 was used for image preparation.

2-2.23 Regulatory considerations

All manipulations involving recombinant DNA were carried out in accordance with the regulations and approval of the Genetic Manipulation Advisory Committee and the University Council of Adelaide University.

Chapter 3: The role of Pebble during cytokinesis

3-1 Introduction

pbl is required for cytokinesis during *Drosophila* development (Hime and Saint, 1992; Lehner, 1992). It is likely that the putative RhoGEF activity of PBL is involved in some aspect of the actin reorganisation events necessary for formation and/or function of the contractile ring. However, the precise role of PBL during cytokinesis is not known. To address this, real-time imaging was used to more carefully determine whether any contractile activity could be observed at the completion of mitosis in *pbl* mutant cells. This analysis has provided new insight into the function of PBL during cytokinesis. In addition, the localization of PBL within the cell was determined and correlated with the timing of cytokinesis. The work presented in this chapter represents my contribution to the early characterisation of PBL, which was carried out in collaboration between our laboratory and the laboratory of Hugo Bellen. The major outcomes of this characterisation have been published (Prokopenko et al., 1999).

3-2 Results

3-2.1 *pbl* is required for cytokinesis

The first 13 nuclear divisions during *Drosophila* embryogenesis occur in the absence of cytokinesis as nuclei oscillate between S and M phases of the cell cycle. Once these divisions are complete, the nuclei are arrested in G2 phase of cycle 14 and move out towards the periphery of the embryo where the acto-myosin dependent process of cellularisation ensures that each nucleus is enclosed within a cell membrane. Distinct patches of cells, termed mitotic domains, then enter mitosis of cycle 14 in a highly regulated fashion. The cycle 14 mitoses are the first nuclear divisions to be accompanied by cytokinesis (Foe, 1989).

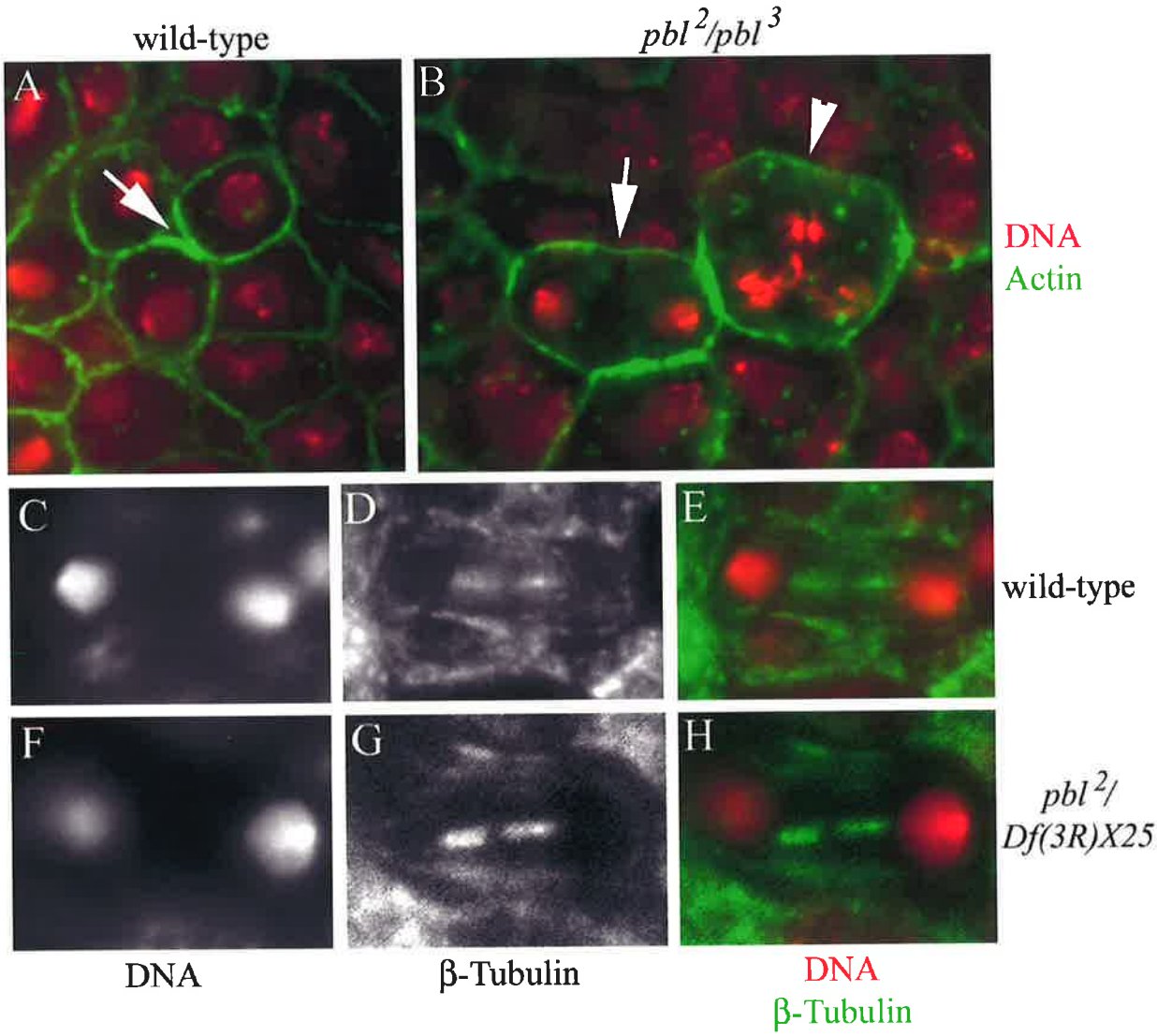
Staining of wild-type cycle 14 mitotic cells with anti-Actin revealed the constriction that is formed midway between the segregated nuclear material as cells undergo cytokinesis (Fig. 3.1A). In contrast, cells undergoing mitosis 14 in a *pbl* mutant embryo showed no such constriction between pairs of telophase nuclei (Fig. 3.1B, arrow). Other contractile ring components, Peanut and Anillin, also fail to relocalize from the cell cortex to the cleavage furrow in cycle 14 divisions of *pbl* mutant embryos (Prokopenko et al., 1999).

In wild-type embryos, most cells undergo two more cell division cycles. Remarkably, *pbl* mutant cells that failed cytokinesis in cycle 14 still undergo the characteristic cycle 15

Figure 3-1 Cytokinesis fails but the central spindle is formed in *pbl* mutant cells

Wild-type embryos (A) or *pbl* mutant (pbl^2/pbl^3) embryos (B) were stained by immunofluorescence using antibodies against Actin (green) and the Hoechst 33258 DNA stain (red). **A.** An example of a wild-type cycle 14 division showing a pair of telophase nuclei (red). Actin (green) labels the cortical membrane of this cell, constriction is occurring midway between the nuclei (arrow). **B.** A pair of cycle 14 telophase nuclei in a *pbl* mutant cell show no such constriction (arrow indicates absence of constriction). *pbl* mutant cells continue into cycle 15. DNA (red) shows two sets of segregating chromosomes within the one cell (arrowhead).

Wild-type (C-E) or PBL deficient ($pbl^2/Df(2R)X25$) embryos (F-H) were stained by immunofluorescence using antibodies against β-Tubulin (green) and the DNA stain Hoechst 33258 (red) and viewed with epifluorescence microscopy. **C.** A pair of telophase nuclei from a dividing cell in a wild-type embryo. **D.** A condensed array of spindle microtubules is formed midway between the separating nuclear material. **E.** A merged image with DNA shown in red and β-Tubulin shown in green. **F.** A pair of telophase nuclei from a cycle 14 mitotic cell in a *pbl* mutant embryo. **G.** The microtubule network that forms during mitosis of this cell, with a condensed array of spindle microtubules formed midway between the separating nuclear material. **H.** A merged image with DNA shown in red and β-Tubulin shown in green.


wild-type
pbl2/pbl3
A
B
DNA
Actin
C
D
E
wild-type
F
G
H
pbl2/
Df(3R)X25
DNA
β-Tubulin
DNA
β-Tubulin

mitoses (Fig. 3.1B, arrowhead). This highlights the specificity of the *pbl* mutant phenotype, as cells continue to cycle in response to developmental cues but they become increasingly disrupted in the absence of intervening cytokineses. Thus, from immunofluorescence microscopy of fixed embryos, we have evidence for the failure of formation of a contractile ring in *pbl* mutant cells.

3-2.2 *pbl* mutant cells initiate constriction

Real-time imaging was used to directly visualize the behaviour of *pbl* mutant cells *in vivo* during the late stages of mitosis, to determine if any constriction was occurring. *spaghetti-squash (sqh)* encodes the regulatory light chain (RLC) of non-muscle Myosin, which has been shown to be required for cytokinesis in *Drosophila* neuroblasts (Karess et al., 1991). Using time-lapse fluorescence video microscopy we were able to directly visualise the dynamic localization of a *sqh-gfp* fusion product to the contractile ring during cell division, thus allowing a direct readout of contractile ring activity (microscopy was carried out by A. Royou in the laboratory of R. Karess, CNRS, France). Injection of rhodamine-tubulin allowed the identification of cells that were undergoing mitosis and about to enter cytokinesis. Wild-type dividing cells were readily identified as enlarged cell outlines, highlighted by an accumulation of SQH-GFP with a weakly staining mitotic spindle (Fig. 3.2A). At a slightly later stage, this same cell started to accumulate SQH-GFP in the equatorial region as contractile ring formation began (Fig. 3.2B). This accumulation clearly showed constriction between the two daughter cells as cytokinesis progressed (Fig. 3.2C).

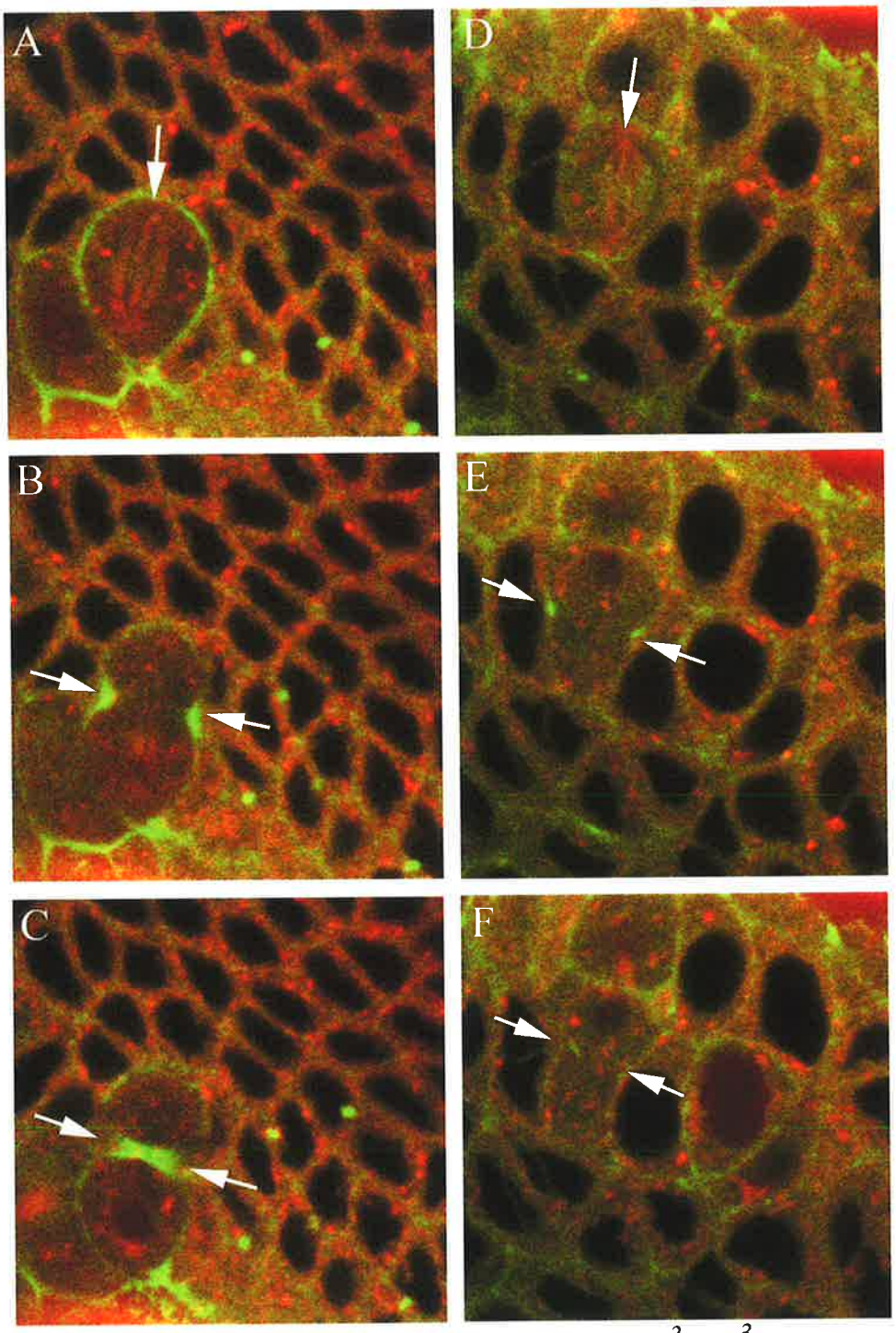
In *pbl* mutant cells, a decreased concentration of SQH-GFP was observed around the cortex of dividing cells (Fig. 3.2D), compared to wild-type cells. At a slightly later stage, this same cell showed some concentration of SQH-GFP at the cleavage furrow as the initial stages of constriction were observed (Fig. 3.2E). However, at later stages there was no evidence of further constriction (Fig. 3.2F). Thus, direct visualisation of *pbl* mutant cells using real-time imaging has shown that a contractile ring can form and constriction is initiated in *pbl* mutant cells. However, this constriction eventually fails since all cells in *pbl* mutant embryos are multinucleate.

3-2.3 The central spindle is formed in *pbl* mutant cells

A strong correlation has been observed between the presence of the central spindle and the activity of the contractile ring in dividing cells (Gatti et al., 2000). Since some contractile activity was observed in *pbl* mutant cells, it is possible that the central spindle also formed in these cells. However, it was not possible to visualise this in the time-lapse videos due to the weak staining of the mitotic spindles. Therefore, fixed samples of wild-type and

Figure 3-2 Real-time imaging of *pbl* mutant cells

Wild-type embryos (A-C) or *pbl* mutant (pbl^3/pbl^3) embryos (D-F) carrying two copies of *sqh-gfp* transgene were injected with rhodamine-Tubulin and visualised by time-lapse video microscopy (performed by Anne Royou in the laboratory of Roger Karess, CNRS, France). Dark areas are non-dividing nuclei whilst SQH-GFP (green) marks the outline of dividing cells. **A.** A wild-type dividing cell is readily identified by its enlarged size and outlined by the accumulation of SQH-GFP in green. Inside this cell, the mitotic spindle is weakly stained in red (arrow indicates one pole of the mitotic spindle). **B.** The same cell later in division shows accumulation of SQH-GFP at the point of constriction (arrows). **C.** As cytokinesis progressed further, a strong concentration of SQH-GFP was clearly seen at the plane of division between these two daughter cells (arrows). **D.** A *pbl* mutant cell undergoing mitosis (arrow indicates one pole of the mitotic spindle stained weakly in red). SQH-GFP (green) is not as strongly localised to the cortical regions as in the wild-type cell. **E.** The same cell later in division shows some accumulation of SQH-GFP (green) at the plane of constriction (arrows). **F.** At the completion of mitosis, the accumulation of SQH-GFP is no longer visible and there is no longer any evidence of constriction (arrows).


A
D
B
E
C
F
wild-type
pbl3/pbl3
SQH-GFP Tubulin

pbl mutant embryos were examined by immunofluorescence using antibodies directed against β-Tubulin. Pairs of telophase nuclei from dividing cells in a wild-type embryo (Fig. 3.1C), were associated with an organised array of spindle microtubules (Fig. 3.1D). The merged image (Fig. 3.1E) confirmed that the central spindle was formed between the separating nuclear material. Organised arrays of spindle microtubules were also observed between pairs of telophase nuclei in dividing cells of *pbl* mutant embryos (Fig. 3.1F, G). The merged image (Fig. 3.1H) clearly shows that the central spindle was formed in *pbl* mutant cells. This is consistent with our observations of some contractile activity using real-time imaging of *pbl* mutant cells and the reported co-operativity between the central spindle and the contractile ring.

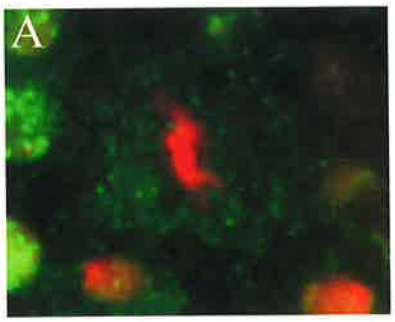
3-2.4 Dynamic localization of PBL during the cell cycle

Polyclonal antibodies were generated to most of the open reading frame of PBL (amino acids 96-853), as previously reported (Prokopenko et al., 1999). Immunohistochemical stains on wild-type embryos revealed a dynamic pattern of localization during cycle 14 divisions. During metaphase (Fig. 3.3A), early anaphase (Fig. 3.3B) and late anaphase (Fig. 3.3C) a small amount of particulate, cytoplasmic PBL staining was observed. However, later during telophase a very strong nuclear staining of PBL was observed (Fig. 3.3D). This nuclear staining persisted throughout interphase where PBL was diffusely distributed throughout the nucleus and was speckled in appearance (Fig. 3.3E). Thus, PBL has a dynamic pattern of expression throughout mitosis, the clearest expression being a nuclear accumulation of the PBL protein once cells have entered telophase. This nuclear staining persisted throughout interphase and took on a distinct speckled appearance.

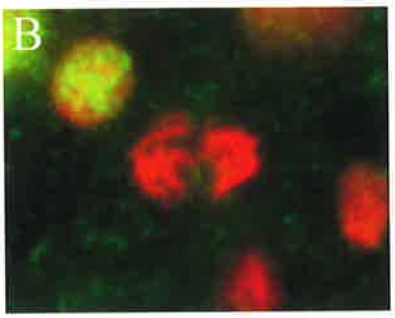
Is the strong nuclear accumulation of PBL during telophase necessary for signalling cytokinesis? In order to address this, wild-type embryos were stained with antibodies directed against Lamin and Spectrin to accurately determine the timing of constriction with respect to the progression of nuclear events. DNA stains revealed a pair of nuclei undergoing late anaphase (Fig. 3.4A). Anti-Lamin staining revealed that reformation of the nuclear envelope had just begun, signalling the completion of anaphase in this cell (Fig. 3.4B). Anti-Spectrin stains revealed that significant constriction had already begun at this stage (Fig. 3.4C). A merge of these stains is shown in Fig. 3.4D. This mitotic stage is significantly earlier, as judged by nuclear morphology, than the time at which nuclear accumulation of PBL was observed (Fig. 3.3D). Thus, it appears that the timing of the nuclear accumulation of PBL during telophase would be too late to provide an instructive signal for cytokinesis. More careful examination of PBL staining in cells that were undergoing cytokinesis provided further insights into this problem. Dividing cells at late stages of anaphase (Fig. 3.5A)

Figure 3-3 Dynamic localization of PBL during the cell cycle

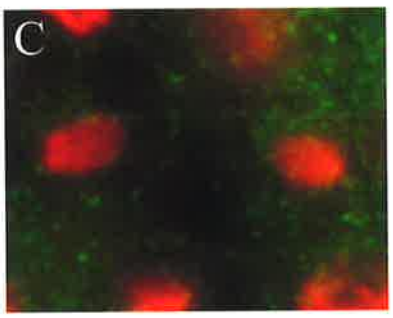
Wild-type embryos (A-E) were stained using antibodies against PBL (green) and the DNA stain Hoechst 33258 (red) and viewed with epifluorescence microscopy. Examples of various stages of the mitotic cell cycle are shown. **A-C** During metaphase (A), early (B) and late (C) anaphase, only very weak cytoplasmic staining of PBL was observed. **D.** Strong nuclear accumulation of PBL was observed during telophase. **E.** During interphase, this nuclear PBL persists but becomes more diffuse with a speckled appearance.



metaphase



early anaphase



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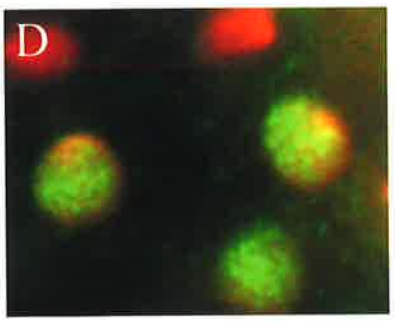
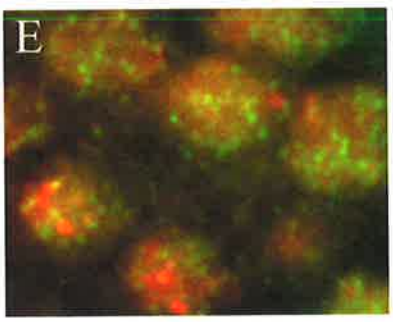


telophase

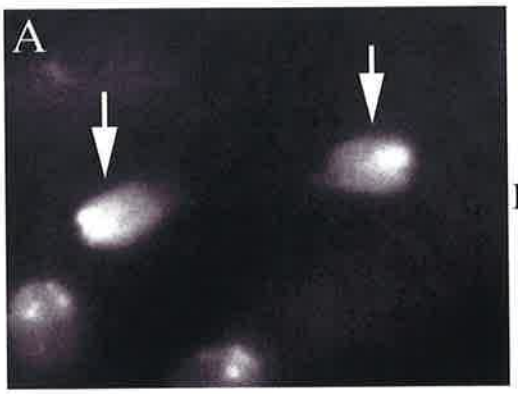


interphase

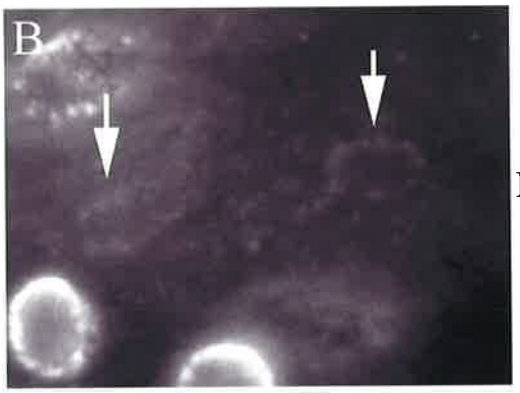
DNA Pebble

Figure 3-4 The timing of constriction with respect to the exit from mitosis

Wild-type embryos (A-D) were stained using antibodies against Lamin and Spectrin and a Hoechst 33258 DNA stain and viewed with epifluorescence microscopy. **A.** DNA stain revealed a cell undergoing the late stages of anaphase (indicated by arrows). **B.** Anti-Lamin revealed that nuclear envelope reformation has just begun (faint outline of nulcei indicated by arrows), signalling the exit from mitosis. **C.** Anti-Spectrin revealed significant constriction had already occurred at this stage. **D.** A merge of these images showing DNA in blue, Lamin in red and Spectrin in green.

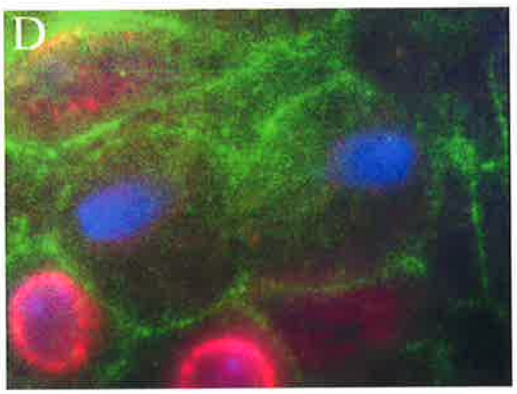


DNA



Lamin

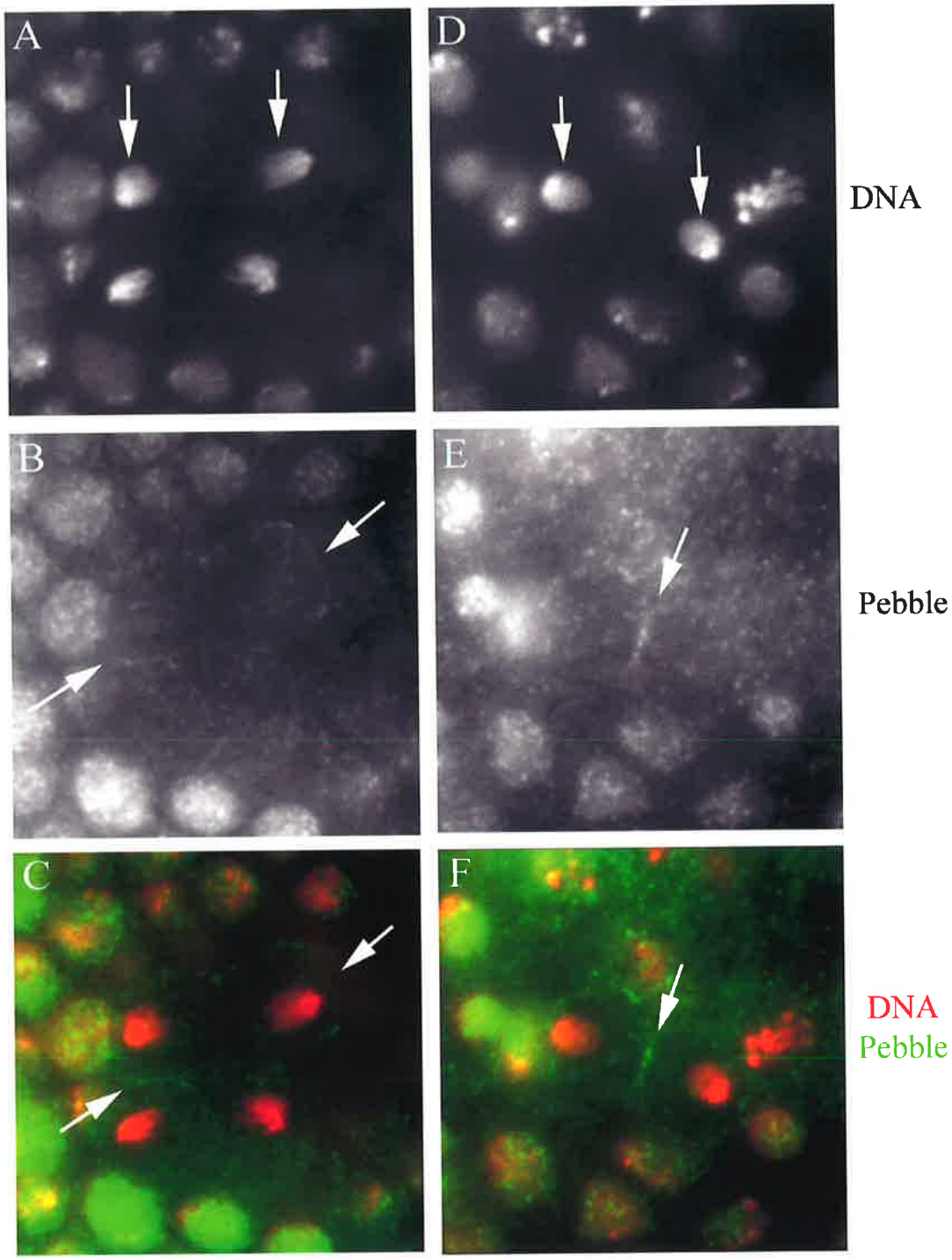
Spectrin



DNA
Lamin
Spectrin

Figure 3-5 PBL localizes to the cell membrane at the time of cytokinesis

Wild-type embryos were stained using antibodies against PBL (B, E) and a Hoechst 33258 DNA stain (A, D) and viewed with epifluorescence microscopy. **A.** 2 pairs of dividing nuclei from a dividing cell in a wild-type embryo, the upper pair represents an example of late anaphase (arrows). **B.** Weak cortical staining of PBL was observed outlining the cell membrane of this dividing cell (arrows). **C.** A merged image of the late anaphase cell with DNA shown in red and PBL, shown in green. **D.** A pair of early telophase nuclei (arrows), their morphology is more rounded than those in late anaphase. **E.** An accumulation of anti-PBL was visible midway between these nuclei, consistent with the position of a contractile ring. **F.** A merged image of the early telophase cell with DNA shown in red and PBL shown in green.


A
D
DNA
B
E
Pebble
C
F
DNA
Pebble
late anaphase
early telophase

revealed a weak accumulation of PBL outlining cortical regions of dividing cells (Fig. 3.5B, C). Dividing cells at early stages of telophase (Fig. 3.5D) revealed an accumulation of PBL staining midway between the two daughter nuclei, coincident with the position of the contractile ring (Fig. 3.5E, F). Following on from these initial observations, confocal analysis revealed clear relocalization of cortical PBL to the constricting furrow at the time of cytokinesis (Prokopenko et al., 2000). Thus, it would appear that there are two pools of PBL during cell division. A cytoplasmic pool of PBL associated with cortical regions, which accumulates at the contractile ring during cytokinesis in addition to a nuclear pool of PBL, which accumulates during telophase and persists throughout interphase.

3-3 Discussion

3-3.1 Does PBL function after the initiation of contractile ring formation?

Cytokinesis fails in all cells of *pbl* mutant embryos. Initial observations of immunohistochemical stains on fixed embryos revealed no evidence of any contractile ring formation in homozygous pbl^2, pbl^5 or heterozygous pbl^2/pbl^3 mutant cells. However, real-time imaging revealed that cleavage furrows began to form in cells homozygous for pbl^3. The pbl^3 allele results in a truncated PBL product of 185 amino acids which encodes the RadECl domain and most of the first BRCT domains. This truncated protein could act in a dominant negative fashion by soaking up other proteins that normally interact with PBL, but, in the absence of any RhoGEF activity it should not be able to function to reorganise the actin cytoskeleton. Ideally these same real-time imaging experiments should be carried out with embryos homozygous for the pbl^2 allele. This results in a truncated PBL product of 37 amino acids which disrupts the RadECl domain and would rule out any dominant negative activity of the pbl^3 truncated gene product. However the results observed with real-time imaging of pbl^3 mutant embryos indicate that, contrary to our initial conclusions (section 3.2.1), the initiation of contractile ring formation is not blocked in *pbl* mutant cells. Rather, the *pbl* mutant phenotype appears to be the result of failure of a later stage of the cytokinetic process. The presence of the central spindle in *pbl* mutant cells (section 3.2.3) is also consistent with these observations.

An alternative possibility for the observed initiation of constriction, given that the real-time imaging showed some variability between cells in the degree of contraction, is that residual maternal PBL may be sufficient to initiate actin reorganisation of the contractile ring but not sufficient to complete constriction. It is not possible to remove this maternal contribution by classical genetic means of generating germline clones, due to a requirement for PBL activity during oogenesis (data not shown). An alternative approach would be

injection of anti-PBL antibodies directly into syncytial embryos, prior to injection of rhodamine-tubulin and direct visualisation of SQH-GFP in dividing cells. Conversely, the dose of maternally provided PBL could be increased to determine whether this would allow constriction to proceed further. This could be achieved by introducing copies of Cos34, a cosmid *P*-transposon bearing the genomic region of *pbl,* into the maternal genotype. One copy of Cos34 rescues *pbl* mutant embryos to viability (J. Wong, Pers. Comm.). Thus, the level of constriction could be assayed by real-time imaging of embryos from females carrying one or multiple copies of this transposon however, these experiments have not been attempted as yet.

It is surprising that this initiation of contractile ring activity was not observed in immunohistochemical stains on fixed tissue. In addition, since Anillin is one of the first components to localize to the contractile ring it is also surprising that Anillin was not observed at the incomplete furrows that form in *pbl* mutant embryos. The observation that the central spindle is formed in fixed samples is consistent with an ability to initiate constriction in *pbl* mutant cells. However, the transient nature of the constrictions that occur could explain why they were not represented in the samples studied. In addition, the cortical distribution of Anillin and other contractile ring components during cytokinesis in embryonic cells may have masked a minor transient relocalization. Alternatively, it could be that the fixation conditions in some way destroy any fragile structures that do form and hence they can only be visualized with real-time imaging.

In terms of a possible role for PBL after the initiation of contractile ring formation, PBL might be required for the continued actin reorganisation events that accompany constriction of the contractile ring. A requirement for the RhoGEF activity of PBL after initiation of contractile ring formation is consistent with certain observations in other systems. Inhibition of Rho activity during cytokinesis in *Xenopus* revealed a requirement during furrow ingression (Drechsel et al., 1997). Cleavage furrows were specified normally and in some cases a small amount of ingression was observed. In contrast, inactivation of *RhoA* by RNAi in *C.elegans* completely inhibited furrow formation and ingression (Jantsch-Plunger et al., 2000). In both of these cases, cytokinesis was followed by time-lapse video microscopy of living embryos, negating any effects due to the fixation procedures. Thus, a conserved role for Rho in the initiation of contractile ring formation has not been established. Alternatively it could be that in *pbl* mutant cells, the contractile ring forms normally and can begin constriction, but that Myosin is not properly activated, so that constriction can only partially progress before the contractile ring becomes unstable and falls apart. In *Dictyostelium* it has been shown that cytokinesis can proceed in the complete absence of Myosin, provided the

cells are attached to a solid support (Uyeda et al., 2000). Dividing cells within the ectodermal context of *Drosophila* embryos would have such a surrounding support. Possibly this allows initiation of contractile ring formation in the absence of PBL-mediated actin cytoskeletal reorganisation activity.

3-3.2 Is PBL resynthesized during each cell cycle?

High levels of nuclear PBL were observed at telophase and persisted throughout interphase. However, only low levels of cytoplasmic PBL were observed during metaphase and anaphase, followed by a weak cortical accumulation corresponding to the time of cytokinesis. Is nuclear PBL degraded as cells enter mitosis and then newly synthesized prior to the cortical localization during each cell cycle? In order to address this question directly, a *UAS-gfp-pbl* construct was generated to follow the cell cycle dependent pattern of localisation of a GFP-PBL fusion product using time-lapse video microscopy of living embryos. Ectopic expression of *UAS-gfp-pbl* with *GMR-GAL4* resulted in high levels of GFP-PBL expression in non-dividing cells of the eye disc (data not shown). In addition, GFP-PBL was able to rescue cytokinesis in *pbl* mutant embryos (data not shown). However, it was not possible to directly detect this GFP-PBL fusion product in embryos in which the expression was driven by various enhancers. These results suggest a functional GFP-PBL fusion product is expressed but so far it has not been possible to directly visualize its localization at different stages of the cell cycle.

An alternative approach to this problem would be to perform western analysis on timed extracts from a synchronised population of cells and so determine the level of PBL expression througout the cell cycle. Although synchronisation of *Drosophila* tissue culture cells is yet to be achieved, it is possible to synchronise embryos at various stages of the cell cycle. This can be achieved by pulses of heat shock expression of the mitotic regulator String (STG) in *stg* mutant embryos, such that the normal pattern of mitotic domains is disrupted and all cells enter mitosis of cycle 14 at the same time (Edgar and O'Farrell, 1990). By this method cells can be fixed a short time after the pulse and individually selected, based on nuclear morphology, for different stages of the cell cycle. Extracts prepared from these embryos would then provide an accurate readout for the level of PBL at various stages of the cell cycle. Specifically, it could be determined whether levels of PBL decline during metaphase to early anaphase and then increase during late anaphase and telophase, or whether there is a constant level of PBL that is dyamically distributed throughout the cell cycle. Alternatively, tissue culture cells could be pulse labelled and immunoprecipitation used to follow the behaviour of PBL protein in these cells to see if it can survive successive cell divisions or is newly synthesized during each round of division.

If PBL was found to be resynthesized during each cell cycle, this would explain the requirement for zygotic PBL expression during cycle 14 in *pbl* mutant embryos. PBL is provided maternally, with strong nuclear staining seen throughout syncytial and pole cell divisions (Prokopenko et al., 2000). The requirement for zygotic expression would suggest the specific degradation of maternal stores prior to the first cellular division. The PEST sequence in PBL may play some role in regulating the stability of PBL once the nuclear envelope breaks down and cells enter the early stages of mitosis. Alternatively there may be a yet to be discovered zygotic specific isoform of PBL, which could account for the requirement for zygotic *pbl* function.

3-3.3 What are the roles of nuclear and cytoplasmic PBL?

There are two pools of PBL that localize dynamically in a cell cycle dependent manner. The cortical localization of PBL and relocalization to the constricting furrow is consistent with a direct role in the activation of Rho-mediated cytoskeletal reorganisation events necessary for the execution of cytokinesis. Thus, PBL could provide spatio-temporal specificity for the activation of Rho family GTPases. However, the role of the nuclear localized PBL is less apparent. The nuclear localization of PBL on completion of cytokinesis could simply be a mechanism to sequester it from the cytoplasm when cells are no longer dividing, thus preventing ectopic activation of cytokinesis. However, the presence of the conserved BRCT domains, known only to play nuclear roles, favours a specific nuclear role for PBL.

3-3.4 Conservation and divergence of PBL RhoGEF function

ECT2, the mammalian orthologue of PBL, is required for cytokinesis and shows nuclear staining at telophase, that persists throughout interphase, as seen for PBL (Tatsumoto et al., 1999). However, the cytoplasmic pool of ECT2 becomes localized to the mitotic spindle while the cell is undergoing mitosis and to the midbody at the completion of cytokinesis. PBL shows no such accumulation in the presence of taxol stabilised microtubules (data not shown). Thus it would seem that PBL and ECT2 could provide conserved regulatory signals from slightly different locations within the cell at the time of cytokinesis.

Chapter 4: Ectopic expression of PBL, and mutant forms of PBL, during eye and embryonic development

4-1 Introduction

To gain a better understanding of the role of PBL during cytokinesis, and possibly other processes, the effect of ectopic expression of various forms of the *pbl* cDNA during eye and embryonic development was investigated. In addition to providing information on the function of PBL, ectopic expression during eye development could also result in adult rough eye phenotypes, providing the basis for a genetic screening system to identify components of PBL signalling pathways.

Patterning of the adult eye initiates with a wave of differentiation that passes from the posterior to the anterior of the developing eye imaginal disc during the third larval instar (Wolff and Ready, 1993). The pattern of cell divisions in the developing eye disc has been well characterised, thus providing a good system to study potential disruptions to cytokinesis by ectopic expression of various forms of the *pbl* cDNA. At the front of the wave of differentiation, cell shape changes result in the formation of a distinct indentation termed the morphogenetic furrow (MF). Cells in the anterior region of the eye disc (ahead of the MF) are dividing asynchronously and are undifferentiated, whilst those posterior to the MF are at progressively later stages of differentiation. Immediately anterior to the MF, cells become synchronised such that they are all arrested in G1-phase on entry into the MF. From this pool of cells, the first five photoreceptor cells are specified, forming incomplete ommatidial clusters that emerge after the passing of the MF. All of the remaining uncommitted cells enter an additional round of S-phase as part of the second mitotic wave, so named to differentiate them the other divisions that occur anterior to the MF which collectively belong to the first mitotic wave. However, not all of these cells proceed directly through mitosis on completion of S-phase. The EGF receptor has been shown to be required for determining the G2/M progression of these cells and to ensure their survival (Baker and Yu, 2001). Most mitotic figures can be visualised 2-8 rows posterior to the MF, so this is the region in which cytokinesis should be observed. However, a few divisions occur randomly in more posterior regions of the developing eye disc. Following the second mitotic wave, the remaining photoreceptor cells are specified by cell-cell interactions from the resultant increased pool of cells. Subsequent patterning events during pupal retinal development ensure the complete and precise, repeated array of ommatidial clusters of the adult eye.

Ectopic expression experiments presented in this chapter were performed using the

GAL4/UAS system (Brand and Perrimon, 1993). The GMR (Glass minimal region) promoter drives expression in cells within, and posterior to, the morphogenetic furrow of the developing eye imaginal disc (Hay et al., 1994). Thus, *GMR-GAL4* will drive expression of various *UAS-pbl* constructs prior to the second mitotic wave and any resultant cytokinetic defects could disrupt the precise patterning of the adult eye. The effect of ectopic expression during embryonic development was also studied. The *paired (prd)* promoter drives expression in alternating stripes along the length of the embryo during embryonic divisions (Yoffe et al., 1995). This provides a good system to study the effects of ectopic expression of the various forms of PBL in a different cellular context. The time of expression of *prd* correlates with the timing of embryonic ectodermal division cycles 14-16. In addition, expression occurs in alternating stripes providing an internal control for the effects of ectopic expression.

4-2 Results

4-2.1 Ectopic expression constructs

At the time this work was initiated, a single cDNA (*pbl1A)* was identified for the *pbl* gene. *pbl1A* was cloned into *pUAST,* thus generating *UAS-pbl* (Fig. 4-1A). A construct was also generated to express a mutant form of *pbl* in which part of the Dbl homology domain was deleted, thus generating *UAS-pblΔDH* (Fig. 4-1B). This mutant form should be unable to catalyse GDP/GTP exchange and could function as a dominant negative form of PBL (PBLΔDH) by sequestering other components away from endogenous PBL. Constructs were also generated to express cDNAs encoding *pbl* and *pblΔDH* without 448bp of 5' untranslated region (5'UTR) sequence present in both the original clones. The resultant clones, which are missing this leader sequence, are referred to as *UAS-pblΔL* (Fig. 4-1C) and *UAS-pblΔDHΔL* (Fig. 4-1D). Finally, a construct was generated that is analagous to the truncated form of ECT2, the mammalian orthologue of PBL, which was originally isolated as a result of its ability to transform cells (Miki et al., 1993). This construct, encoding the truncated form of PBL, is referred to as *UAS-pblΔ325* (Figure 4-1E).

4-2.2 Phenotypes generated by ectopic expression during eye development

One of the three *UAS-pbl* lines initially generated resulted in a rough eye phenotype when expressed together with *GMR-GAL4* (Fig. 4-2D). A chromosome was generated carrying *GMR-GAL4* and this line of *UAS-pbl* which is hereafter referred to as *GMR>pbl.* The *GMR>pbl* adult rough eyes were characterised by disrupted ommatidial shape and an increased number of bristles (Fig. 4-2E). Transverse sections through these eyes revealed

rhabdomeres that were significantly disrupted in their shape and organisation, in addition to a striking increase in the amount of pigment surrounding each ommatidial cluster (Fig. 4-2F).

It was necessary to express two copies of various *UAS-pblΔDH* lines generated, together with *GMR-GAL4,* for the generation of an adult rough eye phenotype (Fig. 4-2J). A chromosome was generated carrying *GMR-GAL4* together with two independent insertions of *UAS-pblΔDH* and will be referred to as *GMR>pblΔDH.* This dose-dependent roughening was most prominent in the dorsal region of the eye (Fig. 4-2J). The roughness was characterised by fusions of ommatidia and loss of some bristles, which was quite mild in ventral regions (Fig. 4-2K). Transverse sections through these adult eyes revealed relatively mild disruptions to the shape and organisation of rhabdomeres, in addition to some loss of pigment between the ommatidial clusters (Fig. 4-2L).

Ectopic expression of one copy of each of the constructs lacking the upstream regulatory regions with *GMR-GAL4* resulted in strong rough eye phenotypes. *GMR>pblΔL* resulted in a severe roughening of the adult eye with significant disruptions to ommatidial shape and greatly increased numbers of bristles (Fig. 4-2G, H). Transverse sections revealed significant disruptions to the internal organisation of the adult eye (Fig. 4-2I). Clusters of grossly misshapen photoreceptors were barely identifiable and it was difficult to find a region of ommatidia where there was no lens structure (clear circular areas) indicating collapse of the overlying lens into the regions of ommatidia. *GMR>pblΔDHΔL* also resulted in a severe rough eye phenotype with significant ommatidial fusions and complete absence of bristles (Fig. 4-2M, N). Transverse sections revealed the presence of photoreceptor clusters but complete absence of any pigment (Fig. 4-2O).

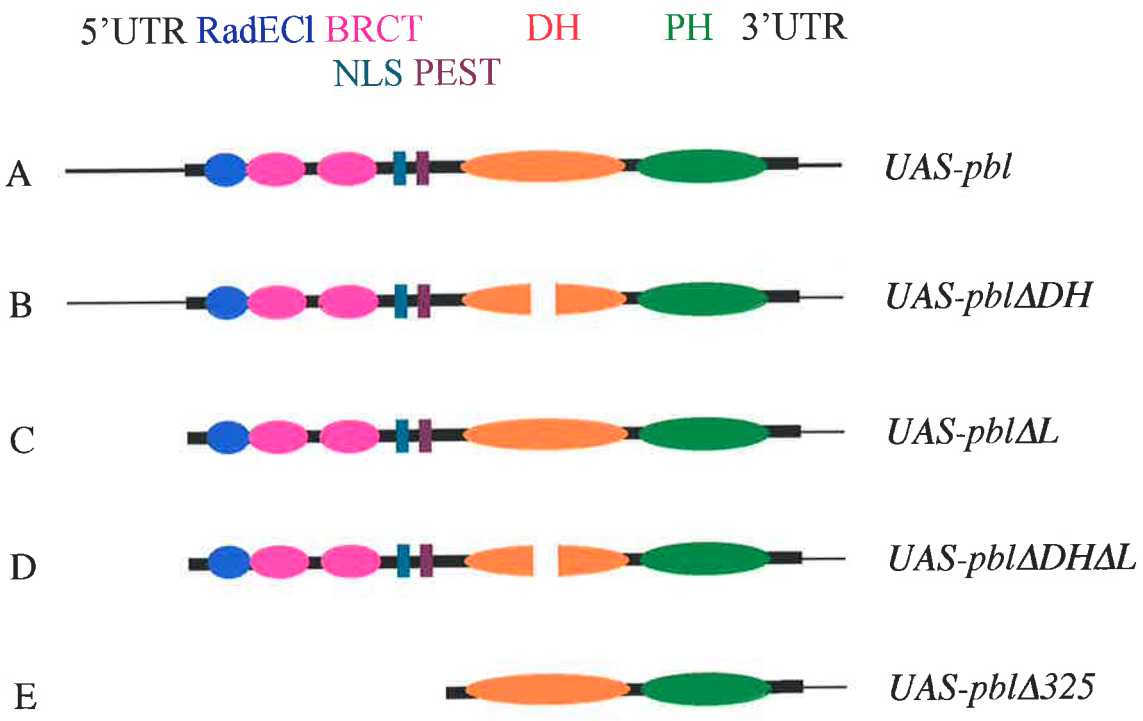
Ectopic expression of *UAS-Δ325*, the truncated form of PBL, was embryonic lethal in combination with *GMR-GAL4,* suggesting that this line drives expression in regions other than the eye, preventing characterisation of the eye phenotype.

4-2.3 Ectopic expression of PBL induced a dose dependent roughening of the adult eye

The *GMR>pbl* rough eye phenotype was only observed with one of three lines of UAS-*pbl* tested and may result from a higher level of expression in this line. Alternatively, disruption of an unrelated gene at the position of insertion of this *P*-element may be contributing to the rough eye phenotype. To distinguish between these possibilities, the *P*-element was mobilised and four new lines of *UAS-pbl* were generated. None of these new lines showed a rough eye phenotype when expressed together with GMR-*GAL4.* However, ectopic expression of two independent insertions of UAS-*pbl* resulted in a rough eye phenotype when expressed with *GMR-GAL4* and is hereafter referred to as *GMR>pbl>pbl* (Fig. 4-3G, H).

Figure 4-1 Ectopic expression constructs

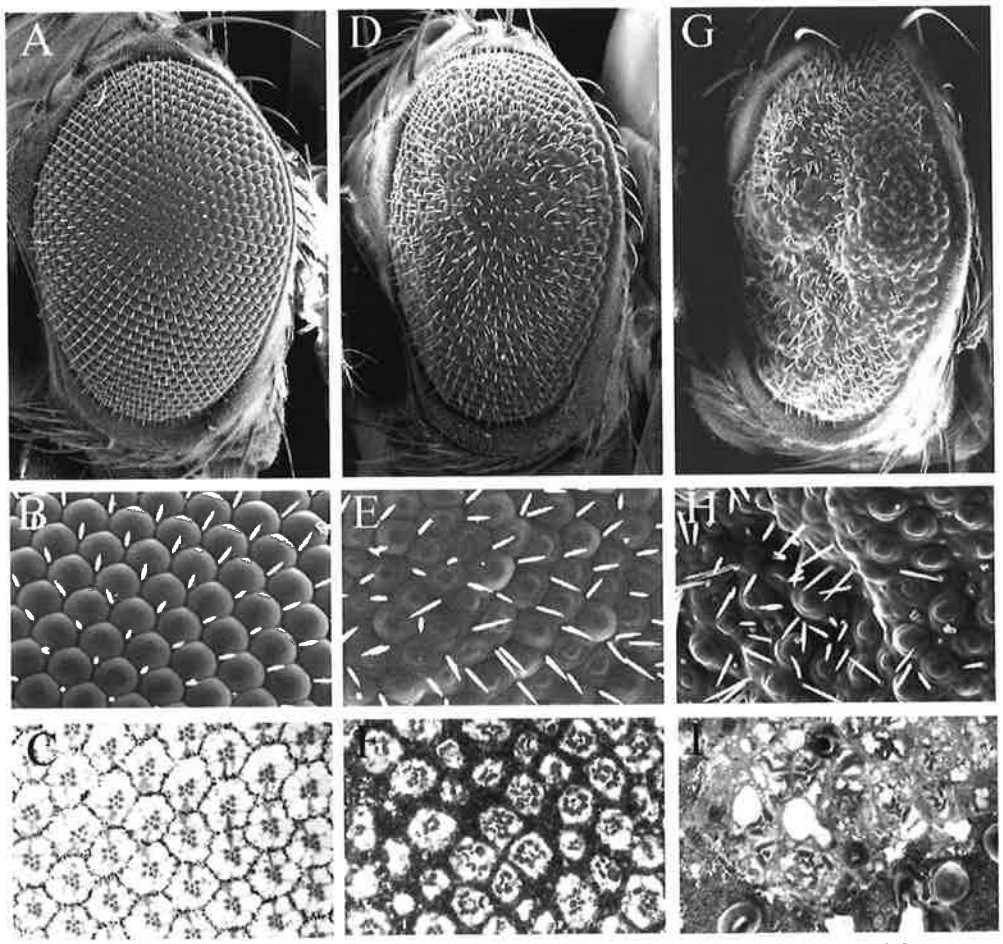
Various forms of *pbl1A* cDNA were cloned into *pUAST* to generate constructs for ectopic expression experiments. **A.** *UAS-pbl* encodes a fully functional form of the PBL protein. **B.** *UAS-pblΔDH* encodes a mutant form of the PBL protein that is unable to catalyse the exchange of bound-GDP for GTP, thus acting as a dominant negative form of PBL. **C.** *UAS-pblΔL* is missing the 5'UTR sequence of the original *pbl* cDNA. **D.** *UAS-pblΔDHΔL* encodes the mutant form of PBL and is missing the 5'UTR sequence. **E.** *UAS-pblΔ325* encodes a truncated version of PBL, equivalent to that of oncogenic ECT2.



DH: Dbl oncogene homology domain
PH: Pleckstrin homology domain
PEST: Degradation sequence
RadECl: Rad4-like, ECT2, Clb6 homology
BRCT: BRCA1 C-Terminal repeat
NLS: Nuclear localisation sequence
UTR: Untranslated region

Figure 4-2 *pbl* ectopic expression phenotypes

Scanning electron micrographs at low power (A, D, G, J, M) and high power (B, E, H, K, N) and transverse sections (C ,F ,I ,L ,O) of wild-type (A-C), *GMR>pbl* (D-F), *GMR>pblΔL* (G-I), *GMR>pblΔDH* (J-L) and *GMR>pblΔDHΔL* (M-O) adult eyes. **A, B.** The surface of a wild-type adult eye is comprised of precisely patterned rows of rounded ommatidia, each of which is in contact with three bristles. **C.** The internal structure of a wild-type adult eye. Each ommatidial cluster is comprised of seven visible rhabdomeres arranged in a trapezoidal pattern and surrounded by clear regions of cell bodies. These ommatidia are then separated by thin regions of dark staining pigment. **D.** Ectopic expression of PBL resulted in a roughening of the adult eye. **E.** *GMR>pbl* eyes were characterised by a disruption of ommatidial shape and an increased number of bristles. **F.** A disruption of the internal organisation of *GMR>pbl* eyes was also observed, with rhabdomeres significantly disrupted in their number, shape and arrangement and a significant increase in the amount of pigment separating ommatidial clusters. **G, H.** *GMR>pblΔ*L adult eyes were severely disrupted in their ommatidial arrangement and had increased numbers of bristles. **I.** Transverse sections revealed major disruption to the internal organisation of the adult eye. Clusters of grossly misshapen photoreceptors were barely identifiable with the collapse of multiple regions of the overlying lens throughout the section. **J.** Ectopic expression of PBLΔDH (a catalytically inactive form of PBL) in the eye resulted in disruption of the ordered array of ommatidia, predominantly in the dorsal region of the adult eye. **K.** This disruption was mild in ventral regions of the eye and characterised by fusions of ommatidia and loss of some bristles. **L.** A disruption of the internal organisation was also observed, with some disorganisation of rhabdomeres and a decreased amount of pigment separating ommatidial clusters. **M, N.** *GMR>pblΔDHΔ*L adult eyes showed a severe roughening of the adult eye with ommatidial fusions and complete loss of bristles. **O.** Transverse sections revealed the presence of photoreceptor clusters but complete absence of pigment.

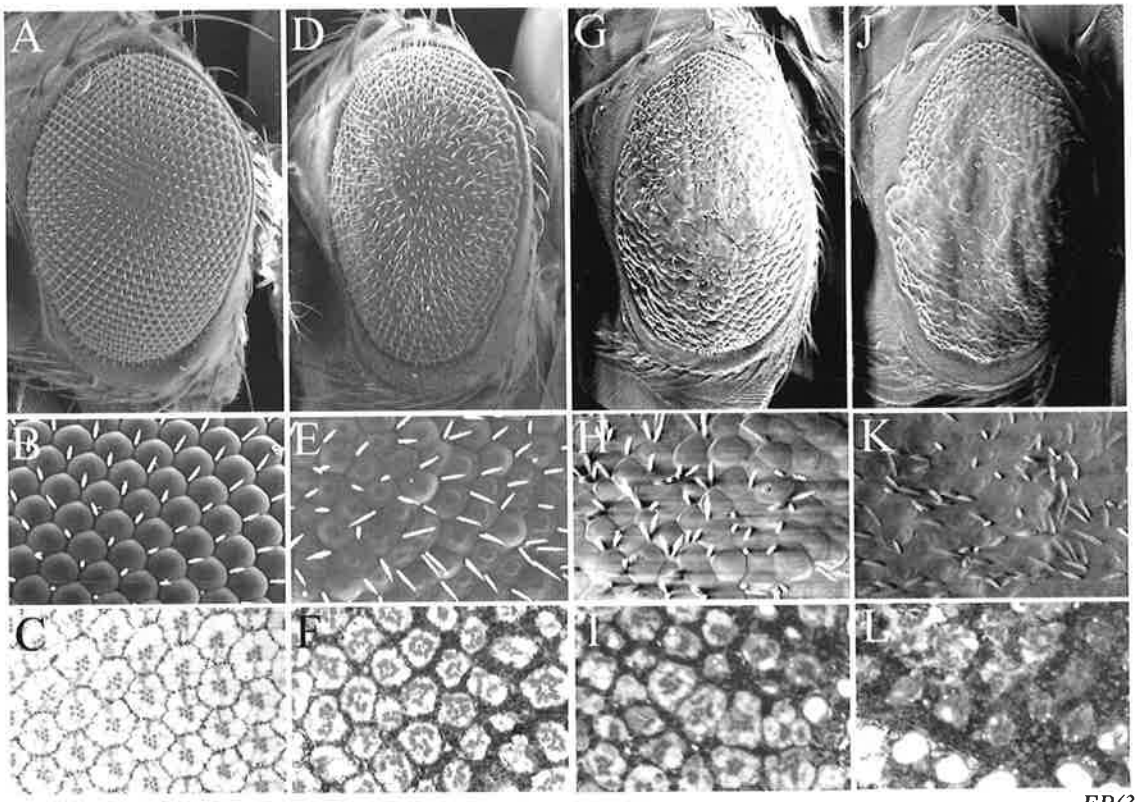


wild-type *GMR>pbl* *GMR>pblΔL*

GMR>pblΔDH *GMR>pblΔDHΔL*

Figure 4-3 Ectopic expression of PBL resulted in a dose-dependent rough eye phenotype

Scanning electron micrographs at low power (top row) and high power (middle row) and transverse sections (bottom row) of wild-type (A-C), GMR>*pbl* (D-F), GMR>*pbl*>*pbl* (G-I) and *GMR>EP(3)3415* (J-L) adult eyes. **A, B.** The regular patterning of the external surface of a wild-type eye. **C.** Transverse sections show the regular pattern of ommatidial arrays of a wild-type eye. **D, E.** The adult rough eye phenotype of GMR>*pbl.* **F.** The internal structure of GMR>*pbl* eyes. **G, H.** GMR>*pbl*>*pbl* resulted in a slightly stronger rough eye phenotype than previously observed with GMR>*pbl,* with similar disruptions to ommatidial shape and increased numbers of bristles. **I.** Transverse sections through the GMR>*pbl*>*pbl* adult eyes revealed significant disruptions to the internal organisation of ommatidia and an increased amount of pigment separating ommatidial clusters. **J, K.** *GMR>EP(3)3415* also resulted in a stronger roughening of the adult eye than previously observed with *GMR>pbl*, with disruptions to ommatidial shape and organisation and an increased numbers of bristles. **L.** Transverse sections through *GMR>EP(3)3415* adult eyes revealed severe disruptions to the internal organisation, with ommatidial clusters barely formed and a greatly increased amount of pigment. In addition, it was not possible to photograph these structures without intrusion of the overlying lens (clear circular areas at the bottom of the field) indicating a collapse of the eye structure.



wild-type *GMR>pbl* *GMR>pbl>pbl* *GMR>pbl*$^{EP(3)3415}$

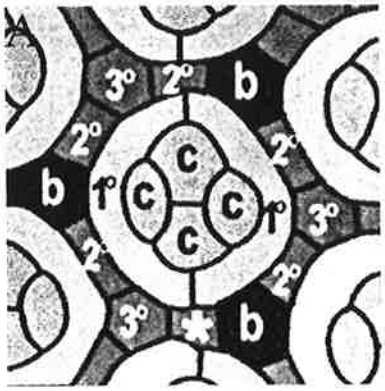
This roughness was more disorganised than that observed for *GMR>pbl*, but was characterised by similar disruptions to ommatidial shape and increased bristle numbers. Transverse sections also revealed a similar but stronger phenotype to GMR>*pbl* with major disruptions to ommatidial organisation and an increased amount of pigment (Fig. 4-3I). Together these phenotypes argue for a dose-dependent rough eye phenotype when the *pbl1A* cDNA is ectopically expressed with GMR-*GAL4* during eye development. In addition to the *UAS-pbl* constructs generated, an enhancer trap *P*-insertion (*EP)* line (Rorth, 1996) was available where the insertion point was 457 nucleotides upstream of the ATG for the *pbl* gene ($pbl^{EP(3)3415}$). $pbl^{EP(3)3415}$ failed to complement null alleles of *pbl*. In this insertion line, *UAS* sites are present upstream of the endogenous *pbl* gene. Ectopic expression of *UAS-*$pbl^{EP(3)3415}$ with *GMR-GAL4* (*GMR>*$pbl^{EP(3)3415}$) resulted in a more severe disruption of the adult eye than that observed with *GMR>pbl* (Fig. 4-3J). The external structure of the ommatidia was severely disrupted and there were numerous ectopic bristles (Fig. 4-3K). Transverse sections through these adult eyes revealed major disruptions, with ommatidia significantly disrupted and a greatly increased amount of pigment (Fig. 4-3L). In addition there were some regions where the overlying lens had collapsed (clear circles). The effect observed with this *EP* line was stronger than that observed with *GMR>pbl.* This could result from ectopic expression of different isoforms of PBL which have been identified by EST database searches (T. Brumby and J. Wong, Pers. Comm.) or from a higher level of *pbl* expression.

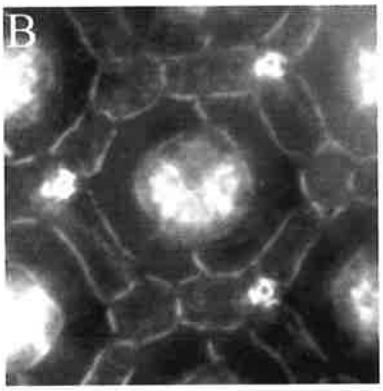
4-2.4 PBL and PBLΔDH have opposite effects on bristle and pigment cell numbers

Ectopic expression of PBL and a mutant form of PBL (PBLΔDH) during eye development resulted in distinct roughening of the adult eye, with opposite effects on numbers of bristles and the amount of pigment separating ommatidial clusters. Bristle and pigment cells are specified from a common pool of interommatidial precursor cells (IPCs) during pupal retinal development. For each developing ommatidium, four cone cells (c) and two primary pigment cells (1^{o}) are first specified from the single layered retinal epithelium. From the remaining pool of IPCs, six secondary pigment cells (2^{o}), which contact two adjacent ommatidia, three tertiary pigment cells (3^{o}), which contact three adjacent ommatidia and three bristle cells (b) are specified. The final result of these patterning events can be seen in wild-type retinas at 42hr after puparium formation (APF) as shown schematically in Fig. 4-4A. Anti-Armadillo (Arm) stains of wild-type retinas at this stage revealed the correct number and pattern of IPCs surrounding the central cone cells and 1^{o} pigment cells (Fig. 4-4B). *GMR>pbl* retinas at the same stage revealed the correct number and organisation of cone cells and 1^{o} pigment cells, but increased numbers of IPCs were observed (Fig. 4-4C). *GMR>pblΔDH* retinas at the same

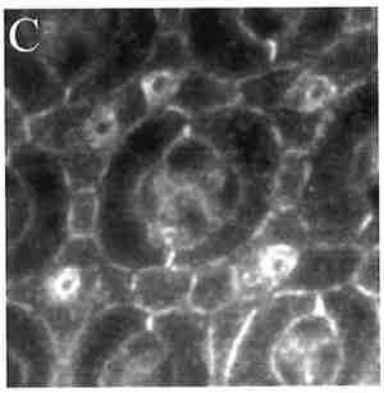
Figure 4-4 Ectopic PBL and PBLΔDH have opposite effects on bristle and pigment cell numbers

Retinas were dissected from wild-type (B), *GMR>pbl* (C) and *GMR>pblΔDH* (D) pupae at 42hr APF. Tissues were stained with antibodies directed against Armadillo (B-D) and visualised using immunofluorescence. **A**. A schematic of the normal pattern of cells specified from the IPCs; cone cells (c), primary pigment cells (1^{o}), secondary pigment cells (2^{o}), tertiary pigment cells (3^{o}) and bristle cells (b). In this schematic, the " * " represents a secondary pigment cell (taken from Miller and Cagan, 1998). **B**. The normal pattern of Arm-staining cells observed in wild-type retinas, three bristle cells are brightly stained. **C**. *GMR>pbl* retinas resulted in additional cells specified from the IPCs that form the lattice surrounding the cone cells and primary pigment cells, six bristle cells are present in this example. **D**. *GMR>pblΔDH* retinas resulted in fewer numbers of cells specified from the IPCs that form the lattice surrounding the cone cells and primary pigment cells. One bristle cell can be seen in this example.

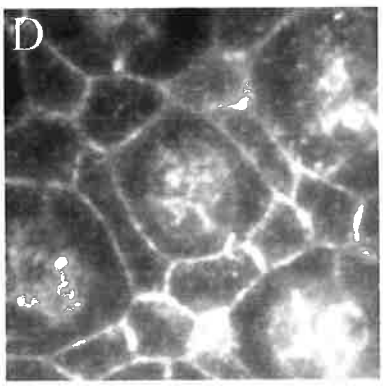




wild-type



GMR>pbl



GMR>pblΔDH

stage also revealed the correct number and organisation of cone cells and primary 1° cells, but decreased numbers of the surrounding IPCs (Fig. 4-4D). Thus, the opposite effects observed on bristle numbers and the amount of pigment in *GMR>pbl* and *GMR>pblΔDH* adult eyes can be directly attributed to the opposite effects on the number of bristle and pigment cells specified following ectopic expression of PBL and PBLΔDH during eye development.

4-2.5 PBL expression in wild-type, *GMR>pbl* and *GMR>pblΔDH* developing eyes

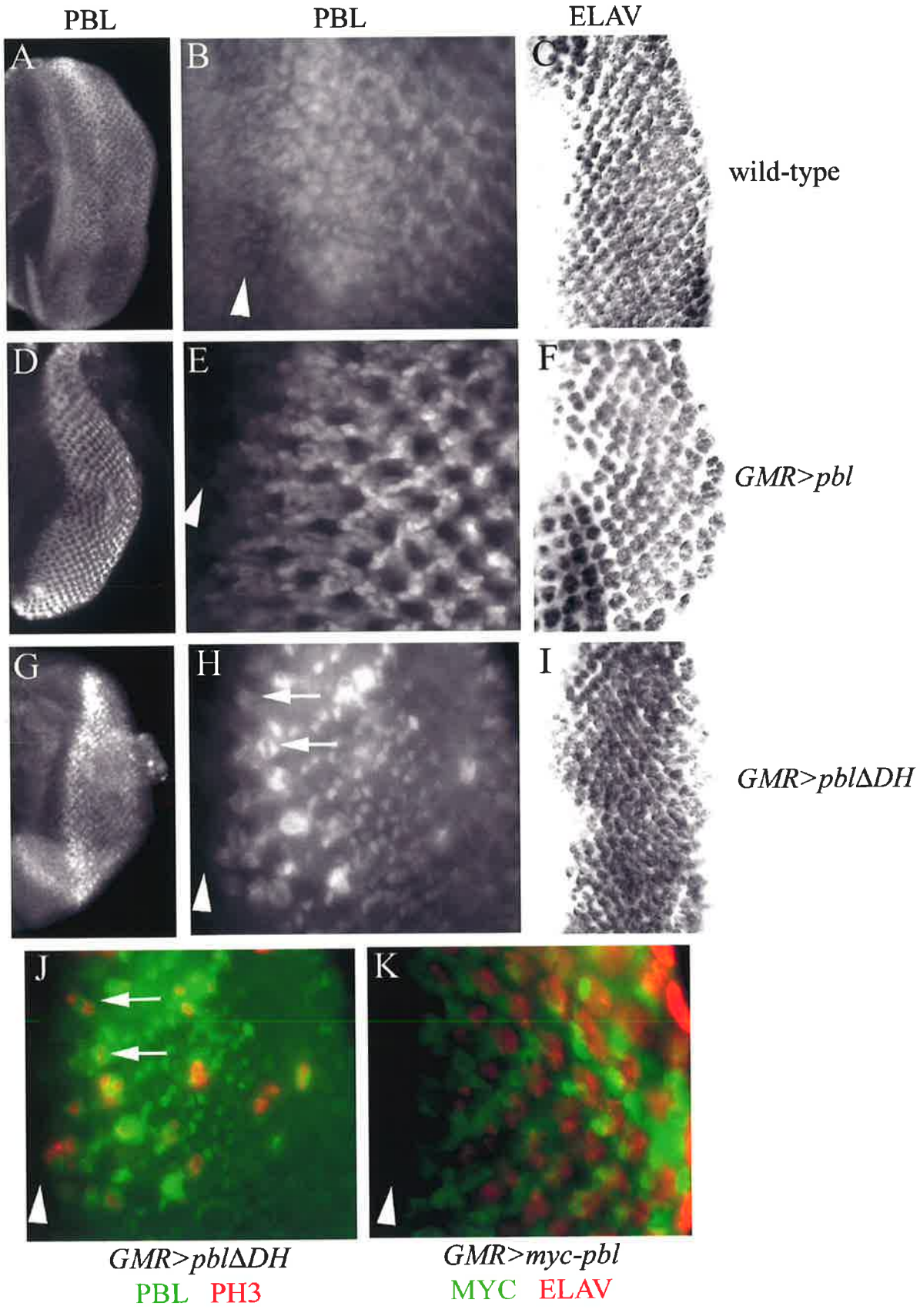
To develop an understanding of the adult *GMR>pbl* and *GMR>pblΔDH* phenotypes, the pattern of ectopic PBL expression, and any disruptions to the normal patterning, was determined in developing eye discs. Endogenous expression of PBL was detected at low levels in wild-type eye imaginal discs (Fig. 4-5A). Greatly increased levels of PBL were observed posterior to MF in *GMR>pbl* (Fig. 4-5D) and *GMR>pblΔDH* discs (Fig. 4-5G). A higher magnification of these eye discs revealed a honeycomb pattern of expression posterior to the MF in wild-type (Fig. 4-5B) and, more prominently, in *GMR>pbl* (Fig. 4-5E). However, only a subset of these cells appeared to be staining in *GMR>pblΔDH* discs, with cells staining at high levels immediately posterior to the MF (Fig. 4-5H). Thus, the mutant form of PBL appeared not to be as stable as the wild-type form in a subset of cells posterior to the MF. Alternatively, some of these PBL expressing cell types could be absent in *GMR>pblΔDH.*

Some of the PBL staining cells posterior to the MF in *GMR>pblΔDH* eye discs appeared significantly larger than others, with an apparent cytoplasmic accumulation of PBL (Fig. 4-5H, arrows). Double staining for anti-phophoshistoneH3 (PH3), which labels the chromosomes of mitotic cells, confirmed that these enlarged cells were undergoing various stages of mitosis (Fig. 4-5J, upper arrow indicates a cell undergoing anaphase, lower arrow indicates a cell in metaphase). These enlarged cells with cytoplasmic PBL accumulation were also visible, but to a lesser extent in *GMR>pbl* eye discs, and confirmed as cells undergoing various stages of mitosis by double labelling with anti-PH3 (data not shown). Thus, ectopic PBL and PBLΔDH accumulate in the cytoplasm of mitotic cells of the developing eye disc, and it appears that this accumulation is more prominent in *GMR>pblΔDH* eye discs.

Antibodies directed against ELAV, a nuclear neuronal marker, stain clusters of differentiating photoreceptors in the posterior half of wild-type developing eye discs (Fig. 4-5C). These neuronal cells are recruited progressively as cells emerge from the passing of the MF. Neuronal differentiation was not adversely affected in *GMR>pbl* (Fig. 4-5F) and *GMR>pblΔDH* eye discs (Fig. 4-5I). Clusters of neuronal fated cells were observed in the posterior half of these eye discs, although some difference was observed in the spacing

Figure 4-5 Ectopic expression of PBL and PBLΔDH during eye development

Eye discs were dissected from wild-type (A-C), *GMR>pbl* (D-F), *GMR>pblΔDH* (G-I,J) and *GMR>myc-pbl* (K) third instar larvae. Tissues were stained with antibodies directed against PBL (A, B, D, E, G, H, J), PhosphohistoneH3 (PH3) (J), ELAV (C, F, I, K) and MYC (K) and visualised using immunofluorescence. Low power views show the entire eye disc (A, D, G) or the region posterior to the MF (C, F, I). High power views show the region spanning, and immediately posterior to, the MF (B, E, H, J, K, the position of the MF is marked with an arrowhead). In all figures, the anterior region of the disc is to the left and the posterior, more differentiated, region is to the right. **A**. PBL is expressed at low levels in wild-type eye imaginal discs. **B**. A slightly increased level of endogenous PBL expression was observed immediately posterior to the MF, with a weak honeycomb pattern of expression in more posterior regions. The time of exposure for A and B was significantly increased compared to D, E, G and H, consistent with relatively low levels of endogenous PBL compared to high levels following ectopic expression with *GMR-GAL4*. **C**. The wild-type pattern of neuronal cell clusters that differentiate posterior to the MF. **D**. A significantly increased level of PBL expression was observed posterior to the MF in *GMR>pbl* eye discs, consistent with ectopic expression of *UAS-pbl* with *GMR-GAL4*. **E.** In *GMR>pbl* eye discs, a strong honeycomb pattern of PBL expression was observed posterior to the MF. **F**. Neuronal clusters were observed in *GMR>pbl* eye discs indicating that neuronal differentiation was not affected. However, the spacing between clusters appeared to be increased compared to wild-type. **G.** Increased levels of PBL were also observed posterior to the MF in *GMR>pblΔDH* eye discs. **H**. In *GMR>pblΔDH* eye discs, there seemed to be an increased level of PBL expression immediately posterior to the MF, and in a subset of the honeycomb pattern of cells in regions posterior to the MF. High levels of PBL staining, which appeared to be cytoplasmic, was observed in large cells (a few examples are indicated by the arrows). **I**. Neuronal clusters were also observed in *GMR>pblΔDH* eye discs indicating that neuronal differentiation was not affected. However, they were more disorganised and the spacing between the clusters appeared to be decreased compared to wild-type. **J**. *GMR>pblΔDH* eye discs stained for PBL (green) and PH3 (red) revealed that the large cells with cytoplasmic accumulation of PBL are undergoing various stages of mitosis (upper arrow indicates a cell underoing anaphase and the lower arrow indicates a cell in metaphase). **K**. *GMR>myc-pbl* eye discs stained with anti-MYC (green) and anti-ELAV (red) revealed that the honeycomb pattern of cells expressing PBL were complementary to the neuronal cell clusters.

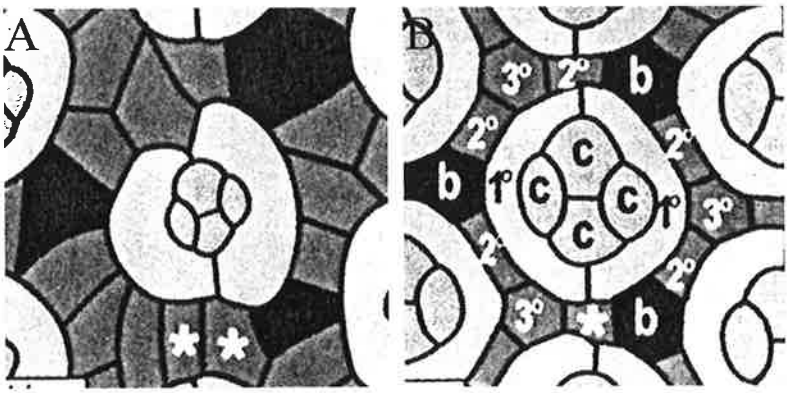

PBL
PBL
ELAV
A
B
C
wild-type
D
E
F
GMR>pbl
G
H
I
GMR>pblΔDH
J
K
GMR>pblΔDH
PBL PH3
GMR>myc-pbl
MYC ELAV

between these clusters, which was increased in *GMR>pbl* and decreased in *GMR>pblΔDH* compared to wild-type. This is consistent with the opposite effects observed on the number of IPCs in *GMR>pbl* and *GMR>pblΔDH* (Fig. 4-4) which correspond to the regions between the neuronal clusters. The honeycomb pattern of PBL staining cells in wild-type and *GMR>pbl* eye discs appeared complementary to the clusters of neuronal fated cells observed with anti-ELAV, suggesting that PBL persists in cells that have not yet begun to differentiate as neurons. Double immunofluorescence stains, to determine if this was the case, could not be carried out directly as the PBL and ELAV antibodies were both raised in rats. For this reason, an alternative approach was used. Eye discs from flies carrying two copies of a MYC tagged form of PBL (*UAS-myc-pbl*) and the *GMR-GAL4* driver (hereafter referred to as *GMR>myc-pbl)* were stained using antibodies directed against ELAV (rat polyclonal) and MYC (mouse monoclonal). Anti-MYC stains revealed a pattern similar to the honeycomb pattern observed with *GMR>pbl* (Fig. 4-5K, green), showing that the MYC tag does not affect PBL localisation. Anti-ELAV stains revealed the clusters of neuronal fated cells in these discs (Fig. 4-5K, red). The merged image between the anti-MYC and anti-ELAV stains revealed that indeed these populations of cells are complementary. Thus, PBL accumulates at highest levels in non-neuronal cells, which are undifferentiated and some of which will have not completed the divisions of the second mitotic wave. Conversely, PBL failed to accumulate in neuronal cells despite the fact that the *GMR* enhancer continues to drive expression in these cells throughout eye development.

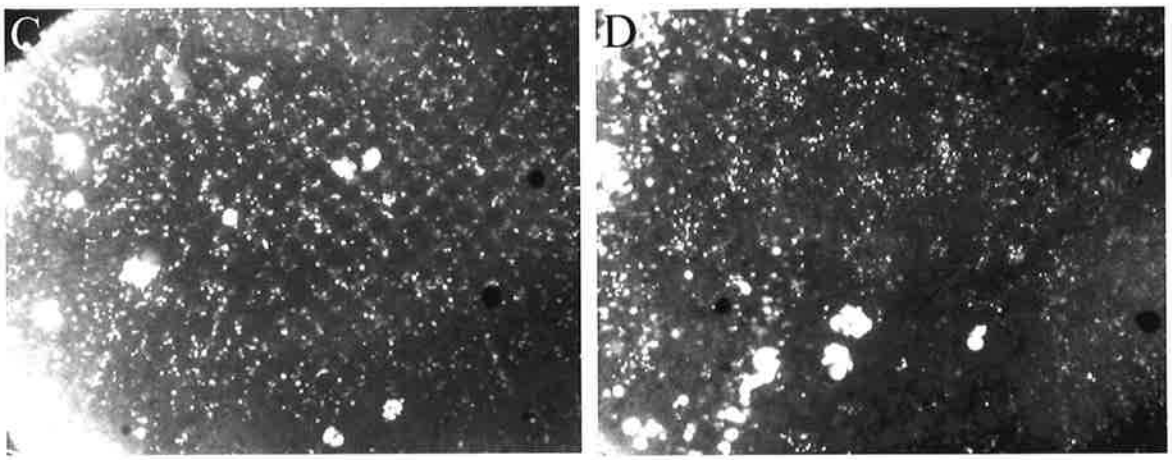
4-2.6 Ectopic expression of PBL inhibits some, but not all, apoptoses in retinal cells

GMR>pbl resulted in extra cells specified from the pool of IPCs during pupal retinal development. Apoptosis plays a vital role in the final patterning of the retina during pupal development (Cagan and Ready, 1989). In the pool of IPCs from which the pigment and bristle cells are specified, there are approximately two to three additional cells per ommatidium than the final number required. After all cell types have been specified, these excess cells are eliminated by a synchronous wave of apoptosis that occurs across the retina at 28-29hr after puparium formation (APF). These extra cells are present at 24hr APF, but have been eliminated by 42hr APF. This is shown schematically in Figure 4-6 where the two cells labelled with a "*" at 24hr APF (Fig. 4-6A) correspond to just a single cell, also labelled with a "*", at 42hr APF (Fig. 4-6B). The synchronous apoptoses can be directly observed by staining retinas at 29hr APF with acridine orange (AO), a dye which is only taken up by cells that are dying and have lost their ability to exclude the dye. The pattern of apoptosis in a wild-type retina can be seen as a regular lattice of AO staining cells, representing the extra

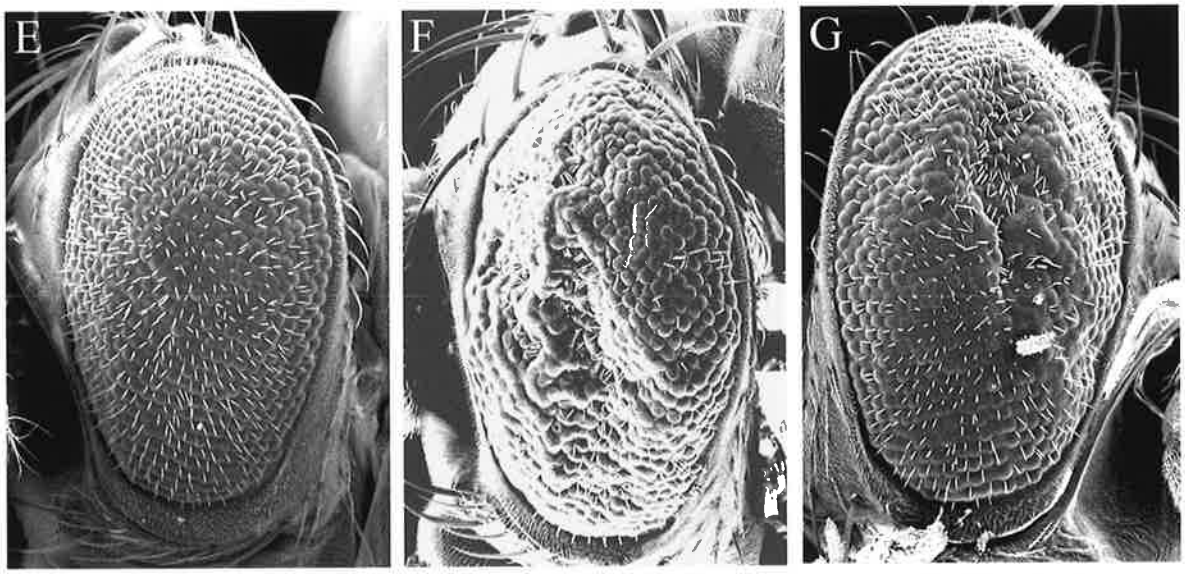
Figure 4-6 Ectopic expression of PBL inhibits some, but not all, apoptoses in retinal cells
A, B. Schematic diagrams of the wild-type cellular pattern of pupal retinas at 24hr and 48hr APF. Cells labelled with a "*" at 24hr APF correspond to a singe cell, also labelled with a "*", at 42hr APF following the wave of apoptosis that occurs at 28-29hr APF (from Miller and Cagan, 1998). Acridine orange stains were performed on wild-type (C) and *GMR>pbl* (D) pupal retinas at 29hr APF. **C**. The wild-type pattern of apoptoses that occur in pupal retinas. The lattice pattern represents apoptosis of IPCs which surround the central cone cells and primary pigment cells of each developing ommatidium. **D**. A decreased level of apoptosis at 29hr was observed in *GMR>pbl* retinas, with the lattice pattern no longer evident.Scanning electron micrographs of *GMR>pbl* (E), *GMR>pbl/GMR*-P35 (F) and *GMR>pbl/+;Df(3R)H99/+* (G) adult eyes. **E.** The *GMR>pbl* rough eye phenotype. **F.** A mild enhancement of the *GMR>pbl* rough eye phenotype was observed with introduction of one copy of *GMR-P35*, an inhibitor of apoptosis. **G.** A mild enhancement of the *GMR>pbl* rough eye phenotype was also observed with introduction of one copy of *Df(3R)H99* which removes one endogenous copy of three genes known to be required for apoptosis in *Drosophila.*



wild-type 24hr APF wild-type 42hr APF



wild-type *GMR>pbl*



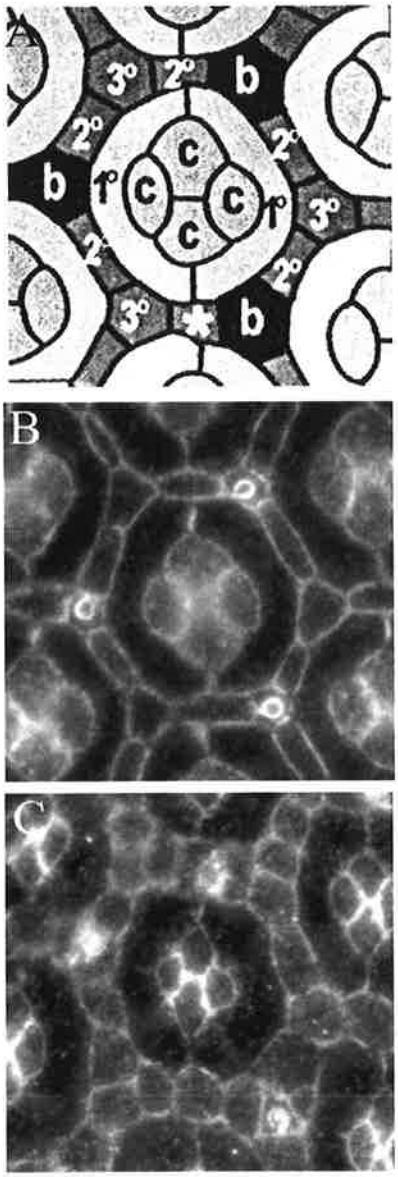
GMR>pbl *GMR>pbl/ GMR-P35* *GMR>pbl/+; Df(3R)H99/+*

IPCs surrounding each ommatidium (Fig. 4-6C). Some apoptosis was observed in *GMR>pbl* retinas but at a reduced level compared to wild-type, with only remnants of the regular lattice of AO staining cells visible (Fig. 4-6D). Thus, ectopic expression of PBL resulted in the inhibition of some, but not all, apoptoses in the developing retina. This was confirmed by co-expression of P35, an inhibitor of apoptosis, under direct control of the GMR promoter (Hay et al., 1994). One copy of *GMR-P35* does not result in a rough eye phenotype. However, co-expression of *GMR-P35* with *GMR>pbl* resulted in a mild enhancement of the *GMR>pbl* rough eye phenotype (Fig. 4-6F). A mild enhancement of *GMR>pbl* was also observed when one mutant copy of *Df(3)H99* was introduced (Fig 4-6G). This deficiency removes three genes required for apoptosis in *Drosophila*; *grim, reaper* and *head involution defective.* Removal of one copy alone has no effect on the adult eye phenotype. Thus, ectopic PBL expression resulted in decreased levels of apoptosis, observed both by direct visualisation and indirectly through genetic interactions.

4-2.7 Ectopic expression of PBL specifically affects numbers of IPCs specified

In addition to the supernumerary bristle and pigment cells seen with *GMR>pbl,* there were also significant disruptions to photoreceptor shape, number and distribution. Although the specification of cone cells and primary pigment cells was not affected by ectopic PBL expression, the anti-apoptotic effect of PBL expression could have been a secondary consequence of disruptions during earlier stages of photoreceptor specification and differentiation. Thus, it was necessary to show that PBL expression was directly inhibiting apoptosis of IPCs during retinal development. To address this, PBL was ectopically induced using *hsp70-GAL4* together with two copies of *UAS-pbl* (hereafter referred to as *hs>pbl).* Ectopic expression was induced at approximately 24-25hr, 26-27hr and 28-29hr APF. These time points immediately precede and overlap with the period where peak levels of apoptosis occur in the developing retina. The final patterning of these cells was then visualised at 42hr to determine the effects of ectopic expression of PBL specifically at this time. *hs>pbl* retinas, in the absence of heatshock, had a pattern that was indistinguishable from wild-type (Fig. 4-7B). Following heat shock induction, a dramatic increase in the number of IPCs was observed (Fig. 4-7C). These additional cells appeared more rounded than those observed in the absence of heat-shock, which could reflect an inability to undergo cell shape changes following ectopic PBL expression. Thus, ectopic PBL expression is directly affecting the final number of IPCs by inhibiting apoptosis of some, but not all, of these cells. It was difficult to accurately determine the number of extra IPCs surrounding each ommatidium following ectopic expression of PBL, either using the *hs* promoter (Fig. 4-7C) or the *GMR* promoter (Fig. 4-4C) as it was difficult to be sure of the number of bristle cells associated

Figure 4-7 Ectopic expression of PBL specifically affects numbers of IPCs specified
Pupal retinas were dissected after 42hr APF, then stained with anti-Arm and viewed by epifluorescene microscopy. **A**. A schematic of the wild-type pattern of cells observed in pupal retinas at 42hr APF. **B**. *hs>pbl* retinas in the absence of heat shock resulted in a pattern of cells that was indistinguishable from wild-type. **C**. *hs>pbl* retinas following heat shock at 24-25, 26-27 and 28-29hr APF resulted in an increased number of IPCs. In addition, these cells appeared more rounded than in wild-type.



wild-type

hs>pbl
-hs

hs>pbl
+hs

with each ommatidium. However, the approximate number of extra cells observed could be consistent with inhibition of apoptosis of 2-3 cells per ommatidium in addition to the 12 cells that are normally present. Thus, although the adult rough eye phenotypes observed following ectopic expression of PBL result from excess numbers of bristle and pigment cells specified during pupal retinal development, it appears that these extra cells arise as a consequence of an inhibition of apoptosis rather than from a direct effect on cytokinesis.

4-2.8 *GMR>pblΔDH* is acting as a dominant negative form of PBL

Although the increased cell numbers observed with *GMR>pbl* were not attributable to a cytokinetic effect, the decreased cell numbers observed with *GMR>pblΔDH* could result from a cytokinetic defect if this mutant form of PBL was acting in a dominant negative fashion. This was strongly supported by the genetic interaction of *GMR>pblΔDH* with *pbl,* since removal of one copy of endogenous *pbl* resulted in a significant enhancement of the *GMR>pblΔDH* rough eye phenotype. In the *GMR>pblΔDH/pbl* flies, significant roughening was observed over the entire surface of the adult eye (Fig. 4-8E), rather than being most prominent in dorsal regions as was observed with *GMR>pblΔDH* (Fig. 4-8C). This enhanced roughening was characterised by extensive ommatidial fusions and a further reduction in the number of bristles. This was especially evident in ventral regions of the eye that were only mildy affected in *GMR>pblΔDH* (Fig. 4-8D) but were significantly enhanced by removal of one copy of endogenous *pbl* (Fig. 4-8F). This genetic interaction indicates that this mutant form of PBL exerts a dominant negative effect during eye development.

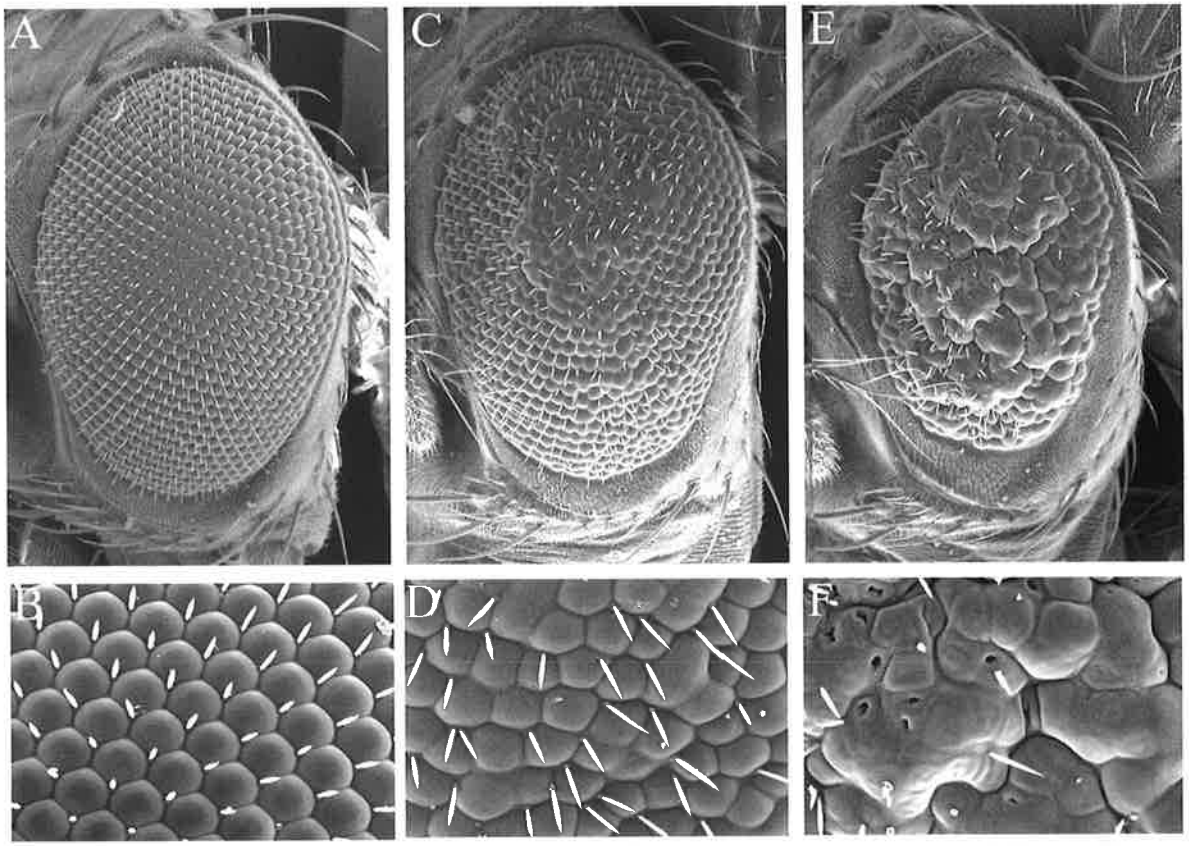
4-2.9 PBLΔDH specifically inhibits cytokinesis during eye and embryonic development

To determine whether the dominant negative effect of PBLΔDH resulted in an inhibition of cytokinesis, developing eye discs from larvae carrying the *GMR>pblΔDH* constructs were dissociated to yield single cells, which were stained for DNA. Cells ectopically expressing PBLΔDH were identified by co-expression of a cytoplasmic GFP marker. 412 GFP expressing cells were analysed, 33 of these were found to be enlarged (Fig 4-9A) and contained two nuclei (Fig. 4-9B, merge shown in Fig. 4-9C), indicating that cytokinesis had been blocked. Thus, in 8.0% of cells ectopically expressing PBLΔDH, an inhibition of cytokinesis was observed.

An inhibition of cytokinesis was also observed when this mutant form of PBL was expressed during embryonic development using the *paired (prd)* enhancer. *prd-GAL4* together with two copies of *UAS-pblΔDH* (*prd>pblΔDH)* resulted in ectopic expression of PBLΔDH in alternating stripes along the length of the developing embryo. *prd>pblΔDH*

Figure 4-8 *GMR>pblΔDH* is acting as a dominant negative form of *pbl*

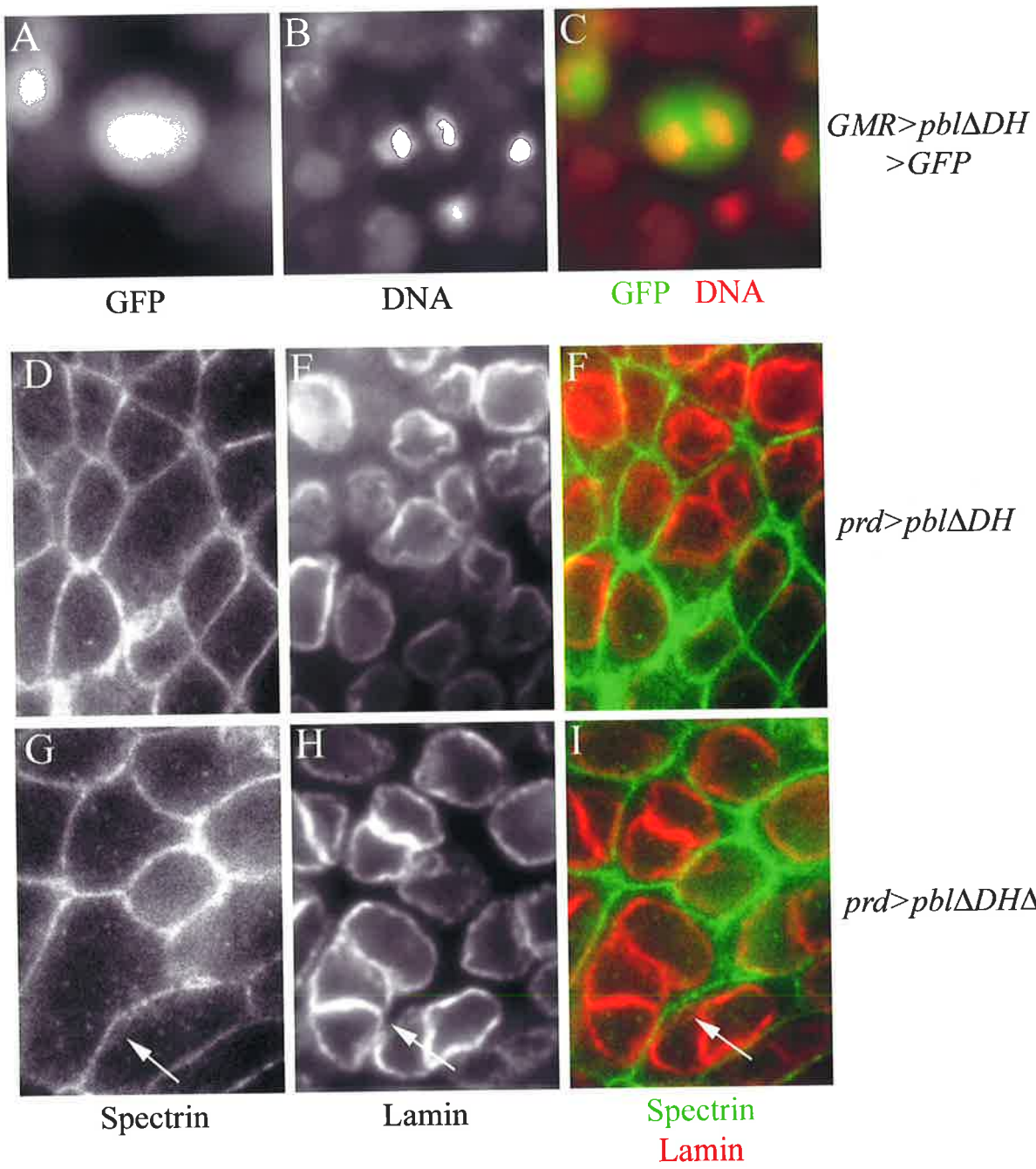
Scanning electron micrographs at low (A, C, E) and high power (B, D, F) of wild-type (A, B), *GMR>pblΔDH* (C, D) and *GMR>pblΔDH/pbl*^{2} (E, F) adult eyes. **A. B**. The regular pattern of ommatidia on the surface of a wild-type adult eye. **C**. The *GMR>pblΔDH* rough eye phenotype, roughening is most prominent in the dorsal region of the eye. **D.** Ventral regions of *GMR>pblΔDH* adult eyes were characterised by fusions of ommatidia and loss of some bristles. **E.** The adult rough eye phenotype of *GMR>pblΔDH* was strongly enhanced in a dominant fashion by mutations in *pbl*, with an enhanced roughening over the entire surface of the adult eye. **F.** This roughening was characterised by extensive ommatidial fusions and significantly decreased numbers of bristles.



wild-type | *GMR>pblΔDH* | *GMR>pblΔDH/+; pbl^2/+*

Figure 4-9 *GMR>pblΔDH* specifically inhibits cytokinesis

Dissociated eye disc cells (A-C) of *GMR>pblΔDH>gfp* third instar larvae were stained with the DNA stain Hoechst 33258 and GFP was viewed directly by epifluorescence microscopy. **A.** An example of an enlarged cell as visualised by cytoplasmic GFP stain. **B**. Two nuclei were present within this one cell. **C.** A merged image with DNA shown in red and GFP shown in green.*prd>pblΔDH* and *prd>pblΔDHΔL* embryos were immunostained with antibodies directed against Spectrin (D, F, G, I) and Lamin (E, F, H, I) and viewed by epifluorescence microscopy. **D.** Anti-Spectrin stains revealed the outline of a field of cells in an affected stripe of a *prd>pblΔDH* embryo. One cell is significantly larger than those surrounding it. **E.** Anti Lamin stains reveal the outline of nuclei in this same field of cells. **F.** The merged image shows that this enlarged cell contains two nuclei while the surrounding smaller cells contain a single nucleus (Lamin in red and Spectrin in green). **G.** Anti-Spectrin stains reveal the outline of a field of cells in an affected stripe of a *prd>pblΔDHΔL* embryo. **H.** Anti Lamin stains reveal the outline of nuclei in this same field of cells. **I.** The merged image with Lamin in red and Spectrin in green shows many of the cells in this stripe contain two nuclei, indicating that one round of cytokinesis has been blocked. There is also an example of a cell with at least three nuclei (indicated by arrows) indicating that successive rounds of cytokinesis have been blocked.


A
B
C
GMR>pblΔDH
>GFP
GFP
DNA
GFP DNA
D
E
F
prd>pblΔDH
G
H
I
prd>pblΔDHΔL
Spectrin
Lamin
Spectrin
Lamin

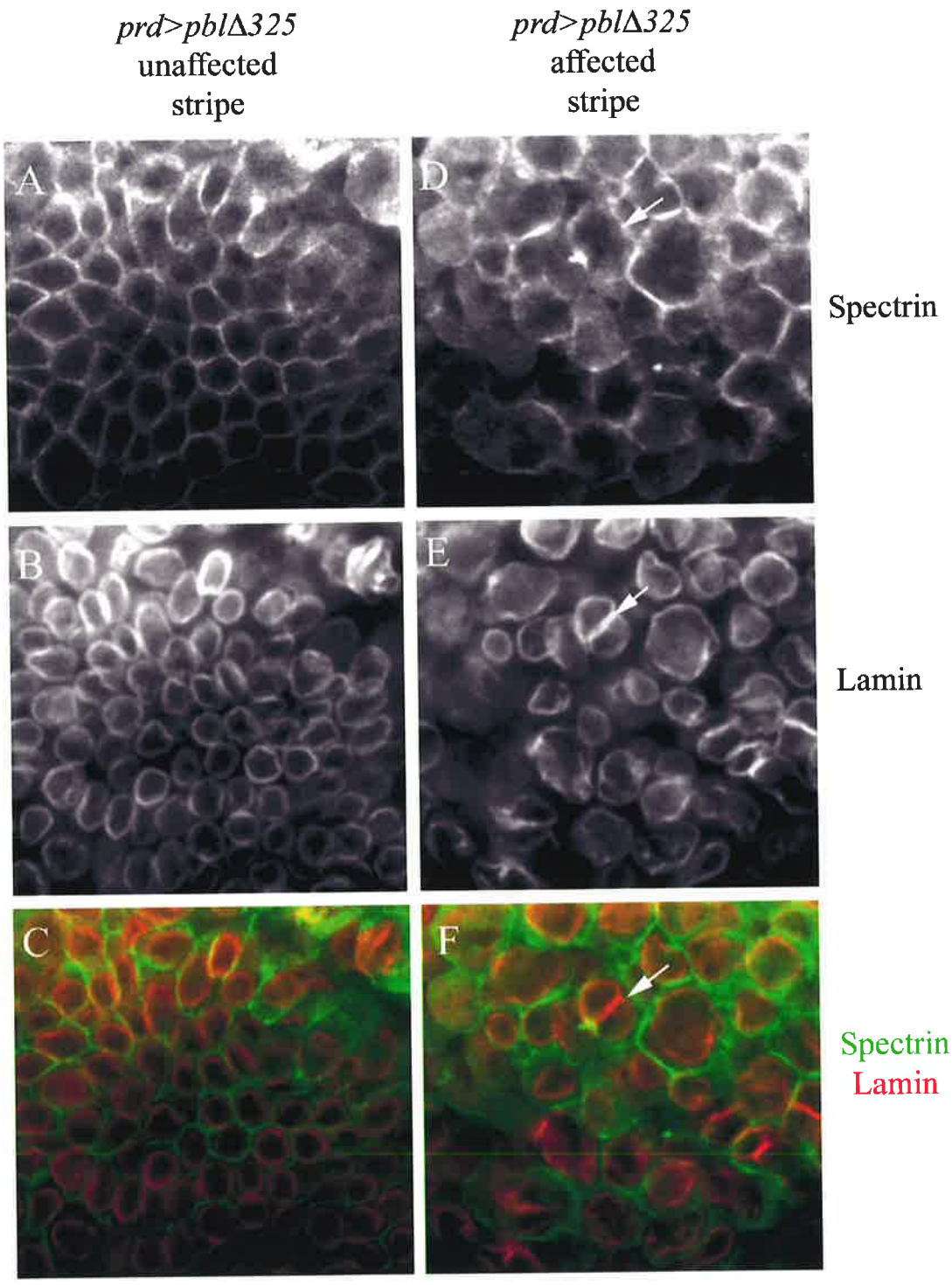
embryos were stained with antibodies directed against Spectrin to outline cell membranes and Lamin to highlight nuclear membranes. In alternating stripes of a *prd>pblΔDH* embryo, a few examples were observed of an enlarged cell outline (Fig. 4-9D) in which two nuclei were present (Fig. 4-9E, F). These binucleate cells were observed within a field of smaller cells, each with just a single nucleus. Binucleate cells were not observed in wild-type embryos nor in the adjacent intervening, non-expressing stripes of *prd>pblΔDH* embryos (data not shown). This indicates that ectopic expression of PBLΔDH during embryonic development resulted in an inhibition of cytokinesis, although the exact proportion of cells showing this effect was not determined. These stains would need to be repeated in the presence of either a *UAS-GFP* construct to confirm the identity of cells ectopically expressing PBLΔDH, or triple stained with antibodies directed against PBL to detect the increased levels of PBLΔDH over endogenous levels of expression.

A much stronger effect was observed following ectopic expression of a single copy of *UAS-pblΔDHΔL* with *prd-GAL4*. Anti-Spectrin stains of *prd>pblΔDHΔL* embryos revealed numerous enlarged cell outlines in alternating stripes along the length of these embryos (Fig. 4-9G). Each of these enlarged cells was shown to contain more than one nucleus (Fig. 4-9H, I). Thus, ectopic expression of PBLΔDHΔL during embryonic development had a much more dramatic effect than PBLΔDH on the inhibition of cytokinesis. In most cases this disruption was to inhibit a single round of cytokinesis, although some cells were also detected with more than two nuclei (Fig. 4-9G-I, arrow) indicating that successive rounds of cytokinesis could also be inhibited. To accurately determine the penetrance of this phenotype, these stains would need to be repeated with an appropriate marker for cells that are ectopically expressing PBLΔDHΔL.

4-2.10 PBLΔ325 disrupts cellular morphology during embryonic development

GMR-driven ectopic expression of *UAS-pblΔ325*, the *pbl* equivalent of the oncogenic form of mouse *ect2,* resulted in embryonic lethality. Thus, the effects of ectopic expression of this oncogenic form of PBL during eye development could not be determined. However, ectopic expression during embryonic development using *prd-GAL4* had a substantial effect on cellular morphology. Cells in the unaffected stripe of a *prd>pblΔ325* embryo were stained for anti-Spectrin, which revealed the regular pattern of cell outlines (Fig. 4-10A). Each cell contained a single nucleus, as shown by staining for Lamin (Fig. 4-10B), with the merged image shown in Fig. 4-10C. However, Spectrin stains of cells in the affected stripe revealed cell outlines

Figure 4-10 PBLΔ325 disrupts the actin cytoskeleton during embryonic development
prd>pblΔ325 embryos were immunostained with antibodies directed against Spectrin (A, C, D, F) and Lamin (B, C, E, F) and viewed by epifluorescence microscopy. **A.** Anti-Spectrin stains revealed the outline of a field of cells in an unaffected stripe of a *prd>pblΔ325* embryo. **B.** Anti Lamin stains revealed the outline of nuclei in this same field of cells. **C.** Merge with Spectrin shown in red and Lamin shown in green. **D.** Anti-Spectrin stains revealed the highly disorganised outlines of a field of cells in an affected stripe of a *prd>pblΔ325* embryo. **E.** Anti Lamin stains revealed the disrupted outline of nuclei in this same field of cells. **F.** Merge with Spectrin shown in red and Lamin shown in green, arrow indicates an example of a binucleate cell.


prd>pblΔ325
unaffected
stripe
prd>pblΔ325
affected
stripe
A
D
Spectrin
B
E
Lamin
C
F
Spectrin
Lamin

that were predominantly enlarged and highly disorganised (Fig. 4-10D). Lamin stains revealed that nuclear morphology was also significantly disrupted (Fig. 4-10E). Some examples of binucleate cells can be seen in the merged image (Fig. 4-10F, arrows). Thus, ectopic expresson of the truncated form of PBL (PBLΔ325) resulted in significant disruptions to the organisation of cellular architecture and some inhibition of cytokinesis.

4-3 Discussion

4-3.1 Do ectopic PBL and PBLΔDH have opposite effects on cytokinesis?

Distinct rough eye phenotypes were observed following ectopic expression of PBL (*GMR>pbl)* and a catalytically inactive form of PBL *(GMR>pblΔDH*). These phenotypes resulted from opposite effects on the numbers of IPCs, the cells that give rise to bristle and pigment cells, during pupal retinal development. However, further analysis revealed that these opposite effects on cell numbers appeared to arise by distinct mechanisms.

Inhibition of cytokinesis was observed following ectopic expression of PBLΔDH during both eye and embryonic development, suggesting that ectopic PBLΔDH is acting in a dominant negative manner with respect to cytokinesis. In *GMR>pblΔ*DH eye discs, an inhibition of cytokinesis in 8% of cells posterior to the MF could account for the observed decrease in numbers of IPCs giving rise to the adult rough eye phenotype. This would result if there were insufficient numbers of cells in the pool of precursors for the normal complement of IPCs to be specified. Whether the binucleate cells that result from ectopic expression of PBLΔDH can be specified as IPCs or whether they are recognised as aberrant and removed by apoptosis is yet to be determined. During embryonic development it is clear that multinucleate cells are not removed by apoptosis and that these cells are able to differentiate, since staining for 22C10, a neuronal marker, has revealed the presence of neuronal cells in pbl mutant embryos (Hime and Saint, 1992). However, this has not been determined for multinucleate cells formed as a result of ectopic PBLΔDH expression.

The extra cells that were observed following ectopic expression of PBL during eye development, however, do not seem to result from a cytokinetic effect. If PBL was rate limiting for cytokinesis, ectopic expression of PBL would be expected to result in increased cell numbers. Some of the excess cells would be expected not to have a nucleus, since ectopic cytokineses would be uncoupled from mitosis. Our evidence supports that the extra cells observed following ectopic expression of PBL during eye development resulted from an inhibition of apoptosis in a subset of cells in the developing retina. No disruption to nuclear or cellular morphology was observed following ectopic expression of PBL during

embryogenesis (data not shown), although staining with AO to detect any decrease in levels of apoptosis is yet to be carried out on these embryos. These results could suggest developmental differences between cells in embryos and eye discs. These differences could possibly reflect cell cycle differences between these different cell types.

4-3.2 Is PBL ectopically activating a Rho family member during eye development

In addition to differences in numbers of IPCs, significant disruptions were also observed in the number, shape and organisation of rhabdomeres within each ommatidium of the rough eye phenotypes examined. The differences in the number of rhabdomeres varied in both *GMR>pbl* and *GMR>pblΔDH* flies, with examples of missing and supernumerary rhabdomeres in both cases. The disruption to rhabdomere shape is much more severe with *GMR>pbl* than with *GMR>pblΔDH* and may result from Rho-GTP mediated effects on cell shape during eye development. It has previously been reported that ectopic expression of Rho *(GMR-Rho)* affects cell shape during eye development (Hariharan et al., 1995). Another RhoGEF, encoded by *dRhoGEF2,* has been identified as a major regulator of Rho activity for the significant cell shape changes required during eye development and for gastrulation during embryogenesis (Barrett et al., 1997; Hacker and Perrimon, 1998). However, ectopic expression of PBL RhoGEF activity could also result in ectopic activation of Rho and/or other Rho family members which results on the observed effect on cell shape during eye development.

4-3.3 Inhibiton of apoptosis by a Rho effector

Inhibition of some, but not all, apoptoses was observed in developing *GMR>pbl* adult eyes. This could result from activation of a Rho family member that is not normally activated in these cells at this time. The involvement of Rho family members during apoptosis is controversial, with conflicting reports on their action. Rac has been shown both to inhibit and promote apoptosis in different studies (Coniglio et al., 2001; Embade et al., 2000). However, in one study it was shown that cell survival is dependent on Rho but not Rac or Cdc42 (Moorman et al, 1999). Thus, PBL activation of Rho-GTP could provide a survival signal for IPCs that would normally undergo apoptosis during patterning of the retina. This could be directly tested by ectopic expression of an activated form of Rho just prior to, and overlapping with, the normal wave of apoptosis (29hr APF), to determine any effect on the final number of IPCs specified.

An alternate possibility is that the effect on cell shape observed after ectopic PBL expression is having an indirect effect on cell survival. This possibility arises from a model of apoptosis in retinas, whereby cell shape changes of secondary pigment cells are necessary

to "push" excess cells into particular regions of the interommatidial space, termed death zones, where they are eliminated (C. Brachmann, Pers. Com.). Ectopic PBL could result in a disruption to cell shape changes such that they cannot be pushed into the death zones and therfore do not undergo apoptosis. Live membrane GFP staining of *hs>pbl* retinas could be used to determine whether cells actually make it to the death zone in the presence of ectopic PBL expression.

Finally, ectopic expression of PBL could have a direct role on the inhibition of apoptosis. A link between cytokinesis and apoptosis has already been established by the isolation of a family of inhibitors of apoptosis (IAPs) (reviewed by (Reed and Bischoff, 2000). IAPs were originally identified from baculovirus as inhibitors of cell death, but members of the family in *C. elegans* have subsequently been shown to also be required for cytokinesis (Fraser et al., 1999). Baculovirus IAP repeat (BIR-1) in *C. elegans* was shown to localise Aurora-like kinase (AIR-2) to chromosomes and subsequently to the spindle midzone, where AIR-2 phosphorylates targets that regulate chromosome behaviour and spindle organisation (Speliotes et al., 2000). More recently, Survivin-type IAPs have been proposed to invoke a survival signal, through association with the mitotic spindle, to prevent cells from entering into a default pathway of apoptosis during each mitosis (Li et al., 1998). *deterrin* encodes a *Drosophila* orthologue of Survivin but mutants are not yet available to test for a role during cytokinesis (Jones et al., 2000). Thus, a link has already been established between the regulation of cell division and apoptosis by the IAPs. PBL may also be involved in this process. This possibility could initially be tested by looking for genetic interactions between the PBL-induced rough eye phenotypes and mutations in the genes encoding various IAPs identified in *Drosophila.*

4-3.4 Is PBL no longer required once cells have begun to terminally differentiate?

Ectopic PBL expression was not detected in cells of the eye disc that had begun to terminally differentiate, even though *GMR-GAL4* would continue to drive *UAS-pbl* expression in these cells. This suggests that once these cells have begun neuronal differentiation, PBL is no longer stable. This would not be surprising, as these cells are no longer cycling and have no further need for PBL activity during cytokinesis. However, ectopic and endogenous PBL expression was detected in the undifferentiated cells of the developing eye. Only a few of these cells have not completed their divisions and would require PBL activity for cytokinesis at a later stage. Thus, PBL function may be required for other purposes in these cells. For example, a role in a DNA repair process, suggested by the RadECl and BRCT domains, could be essential to ensure the integrity of these undifferentiated cells. Alternatively it may be that PBL is specifically degraded in differentiating neuronal cells to allow cells to respond to a

different form of Rho regulation, possibly required for cell shape changes associated with axon guidance of developing neurons to reach their appropriate targets in the developing brain.

4-3.5 PBL activity is regulated at the post-transcriptional level

Removal of the 5'UTR sequences from the various *pbl* cDNA clones used for ectopic expression resulted in a significant increase in the severity of the phenotypes observed. This suggests that the 5'UTR contains sequences that are involved in post-transcriptional regulation of *pbl* expression. For example, these sequences could ensure the correct regulation of PBL translation with respect to the various stages of the cell cycle. Deletion constructs of this region could be generated to further define the sequences involved in this regulation. Further analysis would require the identification of factors that bind to these sequences.

Chapter 5: Genetic interactions of candidate genes with *GMR>pbl* and *GMR>pblΔDH*

5-1 Introduction

Many questions remain unanswered about the exact role of PBL RhoGEF activity during cytokinesis in *Drosophila*. Which Rho family member(s) are activated by PBL-mediated RhoGEF activity? Which GAP(s) and GDI(s) act together with PBL to ensure signalling is switched off once cytokinesis is complete? What controls PBL activity to ensure cytokinesis occurs at the correct time? How is the cytokinetic activity of Rho inactivated while cells are not dividing? Which downstream effector molecules are required for the actomyosin reorganisation events specific for the execution of cytokinesis?

One way to answer these questions is to use genetic analysis to identify components of the PBL-activated RhoGEF signalling pathway. The *Drosophila* eye provides a powerful system for the study of genetic interactions. Loss of function or ectopic expression of a particular gene product can lead to a disruption of the precise patterning of the adult eye. The resultant rough eye phenotype is sensitive to alterations in expression levels of other gene products in the same pathway. The value of this kind of approach was first shown by the identification of components of the Ras signalling pathway (Simon et al., 1991). Subsequent eye screens have provided important insights into many different signalling pathways (Bergmann et al., 1998; Huang and Rubin, 2000; Lane et al., 2000; Rebay et al., 2000).

The dose dependency of the rough eye phenotypes observed with *GMR>pbl* and *GMR>pblΔDH* suggested that they could provide the basis for a sensitised genetic screening system, such that mutations in genes required for normal PBL signalling could dominantly modify these phenotypes. Although these distinct rough eye phenotypes were shown to have opposite effects on cell numbers, it was subsequently shown that these result from distinct mechanisms. The effect of ectopic PBLΔDH was specifically to inhibit cytokinesis while the effect of ectopic PBL appeared to be a developmental-specific inhibition of apoptosis. Thus, genetic interactors of *GMR>pblΔDH* could be more informative for the identification of other factors specifically involved in cytokinesis. However, interactors of *GMR>pbl* or both *GMR>pbl* and *GMR>pblΔDH* could still provide important information on the downstream components of Rho signalling pathways which may or may not be involved in cytokinesis. In this study, various candidate genes that encode Rho family members, components of Rho downstream signalling pathways, or gene products that have previously been shown to play a role during cytokinesis, have been tested for their ability to modify *GMR>pbl* and/or

GMR>pblΔDH.

5-2 Results

5-2.1 PBL acts primarily on Rho1

Mutations in various Rho family members were tested as candidates for modification of the rough eye phenotypes. Six members have been described in Drosophila: *Rho1, Rac1, Rac2, Cdc42, RhoL* and *Mig2-like (Mtl)* (Harden et al., 1995; Hariharan et al., 1995; Luo et al., 1994; Murphy and Montell, 1996; Newsome et al., 2000). Flies heterozygous for a mutant allele of *Rho1* (Strutt et al., 1997), exhibited a marked suppression of the *GMR>pbl* rough eye (Fig. 5-1G, H). Transverse sections of these eyes revealed rhabdomeres of a more normal shape, number and arrangement, in addition to a more regular array of pigment cells (Fig. 5-1I). Halving the dosage of *Rho1* also resulted in an enhancement of the *GMR>pblΔDH* rough eye, with the entire surface of the eye showing significant fusions of ommatidia and decreased numbers of bristles (Fig. 5-1M, N). Also, a further decrease in the amount of pigment and complete absence of rhabdomeres was observed in transverse sections (Fig. 5-1O). Thus, the activation of *Rho1* in response to ectopic expression of the RhoGEF activity of PBL is, at least partially, responsible for the *GMR>pbl* rough eye phenotype. Similarly, the enhancement of the *GMR>pblΔDH* phenotype by decreasing the amount of *Rho1,* suggests that the already compromised level of PBL RhoGEF activity is also acting, to some extent, through *Rho1.*

Such a striking interaction was not seen with mutations in other Rho family members (see Table 5-1). Lethal mutations have been identified for *Cdc42* (Fehon et al., 1997). Decreasing the dosage of *Cdc42* had no effect on *GMR>pbl,* and either had no effect or, in some cases, mildly enhanced *GMR>pblΔDH.* This variation most likely reflects slight fluctuations in temperature at which the crosses were performed on different occasions. Specific mutations in *mtl* (B. Dickson, unpublished observations) showed no significant modification of either rough eye phenotype. Deficiency stocks were used to test other Rho family members, because specific mutations were not available. A deficiency which removes *Rac1* had no significant effect on either *GMR>pbl* or *GMR>pblΔDH.* Deficiency stocks that remove *Rac2,* also remove *pbl,* so any effect due to loss *of Rac2* function was masked by loss of *pbl* function, which independently enhances *GMR>pblΔDH* (shown in Fig. 4-8E, F). This problem was overcome by generation of a chromosome carrying the deficiency that removes *Rac2* and *pbl,* and carries Cos34, a transposon insertion bearing the genomic region of *pbl.* One copy of Cos34 rescues *pbl* mutant embryos to viability (J. Wong, Pers. Comm.).

Table 5-1

Genetic Interaction of Rho family members with *GMR>pbl* and/or *GMR>pblΔDH*

Rho family member	Allele/Deficiency	*GMR>pbl*	*GMR>pblΔDH*
Rho1 (52E11-F1)	$Rho1^{720}$	S+++	E++
Cdc42 (18D13-E1)	$Cdc42^{4}$	no effect	no effect/E+
Rac1 (61F5-6)	*Df(3L)Ar14-8*	no effect	no effect
Rac2 and *pbl*	*Df(3L)RM5-2 (Rac2,pbl)*	no effect	E++
pbl (66A18-20)	pbl^{2}	no effect	E++
Rac2 (65F10-11)	*Df(3L)RM5-2,Cos34 (Rac2,pbl* and Pbl^{+}*)*	no effect	S++
RhoL/Rac3 (85D24-25)	*Df(3R)by10 (RhoL* and *Ras85D)*	E+++	no
effect*Ras85D* (85D25-26)	$Ras85D^{e1B}$	no effect	E+
mtl	*Δmtl*	no effect	no effect

E+, E++, E+++; mild, medium and strong enhancement
S+, S++, S+++; mild, medium and strong suppression

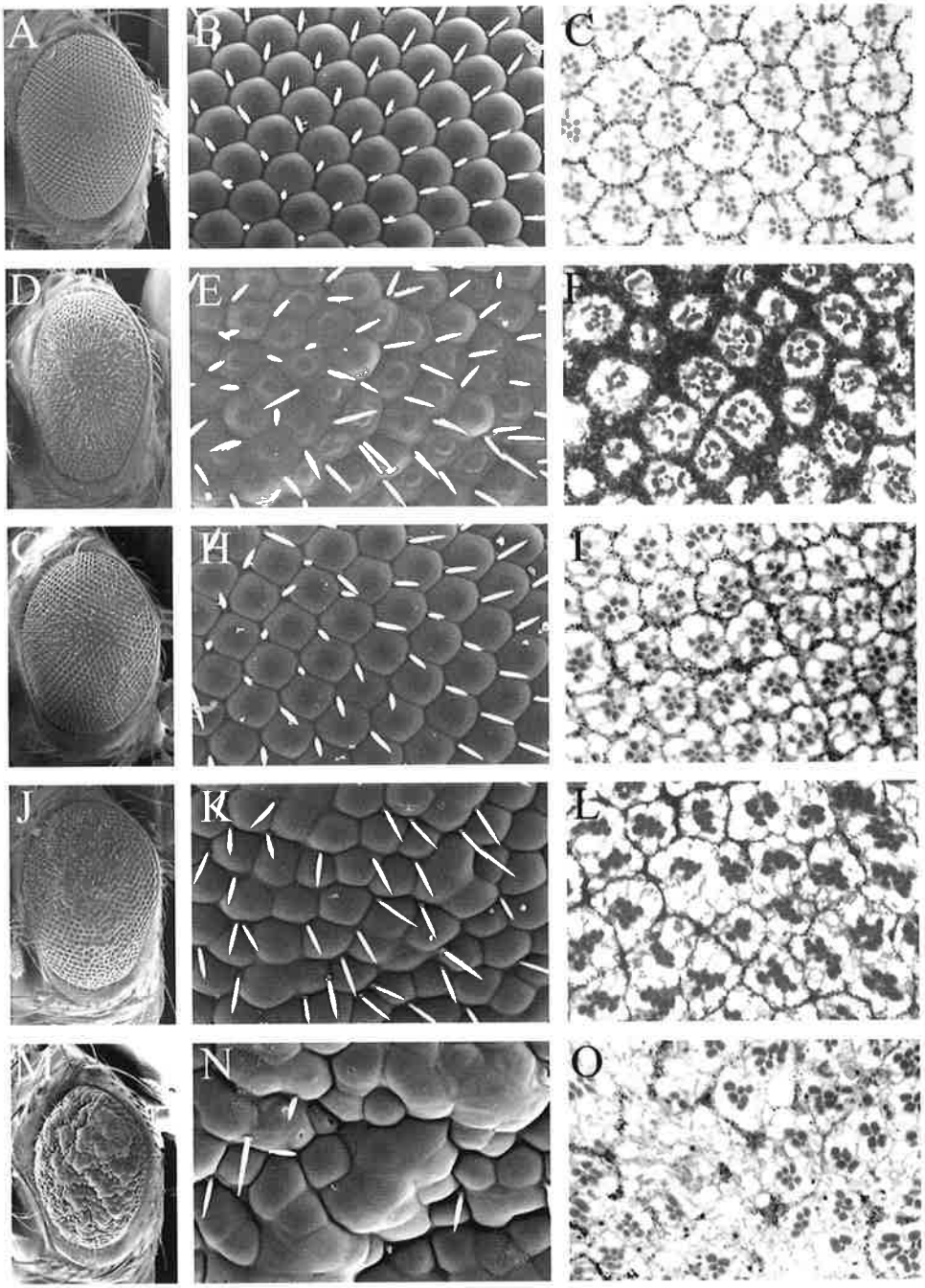
The combination of the deficiency which removes one copy each of *Rac2* and *pbl*, and the transgene that restores one copy of *pbl* did not significantly modify the *GMR>pbl* phenotype, but suppressed *GMR>pblΔDH*. Deficiencies that uncover the divergent Rho family member, *RhoL* also uncover *Ras85D*. Mutations in *Ras85D* alone resulted in modification of the *GMR>pblΔDH* rough eye phenotype. However, the deficiency removing *RhoL* and *Ras85D* resulted in a strong enhancement of *GMR>pbl*, which was not seen with *Ras85D* alone. This is inconsistent with the possibility that PBL activates RhoL. Thus, the RhoGEF activity of PBL is acting specifically through *Rho1* in this system.

5-2.2 Ectopic expression of PBLΔ325 is suppressed by *Rho1*

Ectopic embryonic expression of PBLΔ325, the mutated form of PBL equivalent to the mouse ECT2 oncogenic form, during embryogenesis resulted in a significant disruption to cellular architecture with some inhibition of cytokinesis (Fig. 4-10D-F). However, it could not be determined whether this extreme phenotype resulted from ectopic activation of a Rho family member or from a dominant negative effect. Genetic interaction experiments can be used to differentiate between these possibilities. The effect of ectopic expression of the truncated form of PBL (PBLΔ325) during eye development could not be studied using *GMR-GAL4* due to the embryonic lethality observed with *GMR>PBLΔ325*. However, using a different driver that is expressed in a more restricted spatio-temporal pattern in eye discs it was possible to generate a rough eye phenotype following ectopic expression of PBLΔ325.

Figure 5-1 *pbl* interacts genetically with *Rho1*

Scanning electron micrographs at low (left column) and high power (middle column) and transverse sections (right column) of wild-type (A-C), *GMR>pbl* (D-F), *GMR>pbl/Rho1*^{720} (G-I), *GMR>pblΔDH* (J-L) and *GMR>pblΔDH/Rho1*^{720} (M-O) adult eyes. **A, B.** The regular patterning of the external surface of a wild-type eye. **C.** Transverse sections show the regular pattern of ommatidial arrays of a wild-type eye. **D, E.** The *GMR>pbl* rough eye phenotype. **F.** Transverse sections of *GMR>pbl* adult eyes. **G, H.** A significant suppression of the external roughening of *GMR>pbl* was observed with removal of one copy of endogenous *Rho1*. **I.** The effect on the internal organisation was also markedly suppressed by the removal of one copy of endogenous *Rho1,* with a more regular array of ommatidia separated by a decreased amount of pigment. **J, K.** The *GMR>pblΔDH* rough eye phenotype. **L.** Transverse sections of *GMR>pblΔDH* adult eyes. **M, N.** The adult rough eye phenotype of *GMR>pblΔDH* was strongly enhanced by removing one copy of the endogenous *Rho1* gene, resulting in significant roughening over the entire surface of the eye. **O.** The effects on the internal organisation were also markedly enhanced by removal of one copy of endogenous *Rho1*.


A
B
C
wild-type
D
E
F
GMR>pbl
G
H
I
GMR>pbl/
Rho1 720
J
K
L
GMR>pblΔDH
M
N
O
GMR>pblΔDH/
Rho1 720

Sevenless (sev) is expressed transiently in a subset of photoreceptor cells (R3, R4 and R7). However, a mutated version of *sev-GAL4* that was isolated in our laboratory, *sev*-GAL4* (A. Lumsden, Pers. Comm.) resulted in a rough eye phenotype when expressed together with *UAS-pblΔ325* (Fig. 5-2B). This phenotype was significantly suppressed when one mutant copy of *Rho1* was introduced (Fig. 5-2C). This provides strong evidence that ectopic expression of the truncated, "oncogenic" form of PBL resulted in constitutive activation of Rho1 resulting in the severe adult eye roughening. However, the possibility of ectopic activation of other Rho family members following ectopic expression of PBLΔ325 is yet to be determined.

5-2.3 Genetic interactions of *pbl* with candidate genes encoding downstream effectors of Rho and genes known to be required during cytokinesis

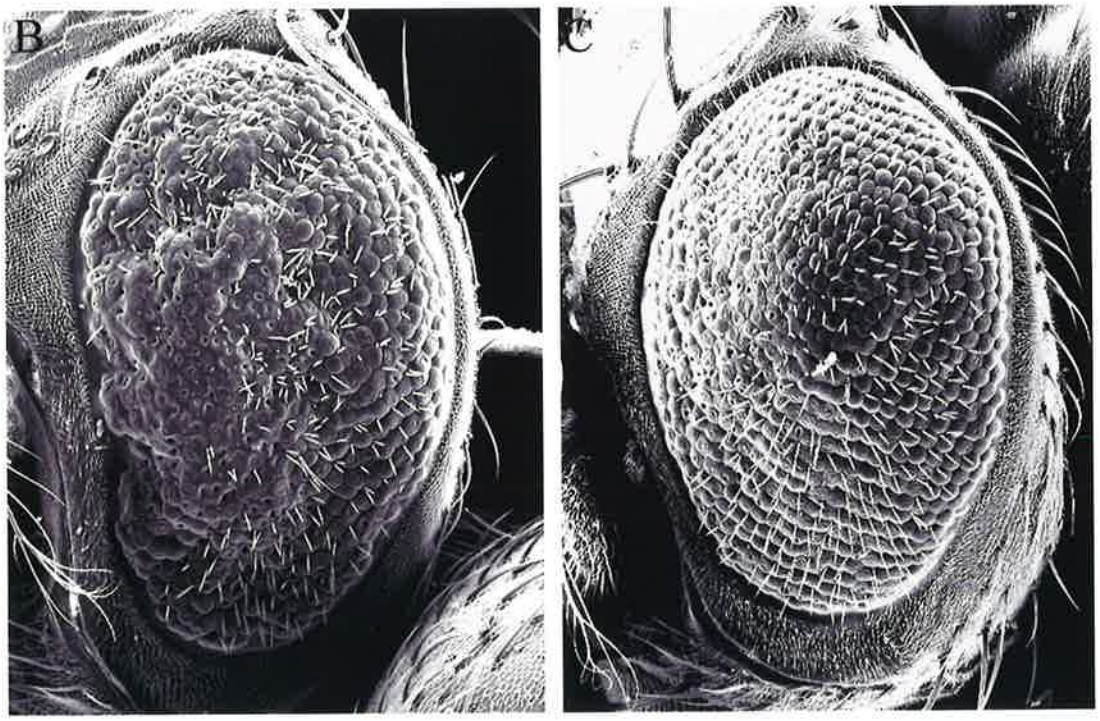
Many downstream effectors of Rho proteins have been identified in other systems, some of which have previously been shown to be required for cytokinesis (Fig. 1-5). Mutations in *Drosophila* orthologues (where they were available) of these downstream signalling components were tested for modification of *GMR>pbl* and/or *GMR>pblΔDH*.

Alleles of *Drosophila rho kinase* (*rok*) resulted in mild suppression of *GMR>pbl*. This suppression was seen as a slight increase in the size of the eye and decrease in the overall roughness (Table 5-2, Fig. 5-3B). This suggests that ectopic expression of PBL results in ectopic activation of Rho1 and subsequently some *increase in the level of Rho kinase activity. A mild enhancement of GMR>pblΔDH* was also observed by introducing one mutant copy of *rok*, with the fusions of ommatidia extending over a slightly larger region of the surface of the eye (Table 5-2, Fig. 5-3D). These results suggest that activation of ROK as a downstream effector of Rho signalling might play only a minor role in the regulation of cytokinesis, or may not be rate limiting for this process.

Mutations in *diaphanous (dia)* resulted in a medium enhancement of the *GMR>pblΔDH* phenotype but had no effect on *GMR>pbl* (Table 5-2, Fig. 5-3E). DIA is required for actin reorganisation and has previously been shown to be required for cytokinesis, so it could be a component of the PBL-activated Rho pathway during cytokinesis (Castrillon and Wasserman, 1994). *chickadee (chic)* encodes Profilin which has been shown to act downstream of DIA during actin reorganisation events (Verheyen and Cooley, 1994). *chic* mutations resulted in a mild suppression of both phenotypes. This could be consistent with a downstream effect on actin reorganisation that affects both eye phenotypes. *act-up (acu)* has been shown to act in the opposite direction to *chic* (Benlali et al., 2000), but *acu* mutations had no effect on either of the rough eye phenotypes.

Figure 5-2 Ectopic expression of PBLΔ325 is suppressed by *Rho1*

A. A schematic of *UAS-pbl,* encoding full length PBL, and *UAS-pblΔ325,* encoding the truncated, "oncogenic" form of PBL. Scanning electron micrographs of *sev***>pblΔ325* (A) and *sev***>pblΔ325/Rho1*^{720} (B) adult eyes. **B.** *sev***>pblΔ325* resulted in a severe adult rough eye phenotype. **C.** A significant suppression of the external roughening of *sev***>pblΔ325* was observed with removal of one copy of endogenous *Rho1.*



sev>pblΔ325* *sev*>pblΔ325/Rho1^{720}*

Table 5-2

Genetic interactions with *GMR>pbl* and/or *GMR>pblΔDH*

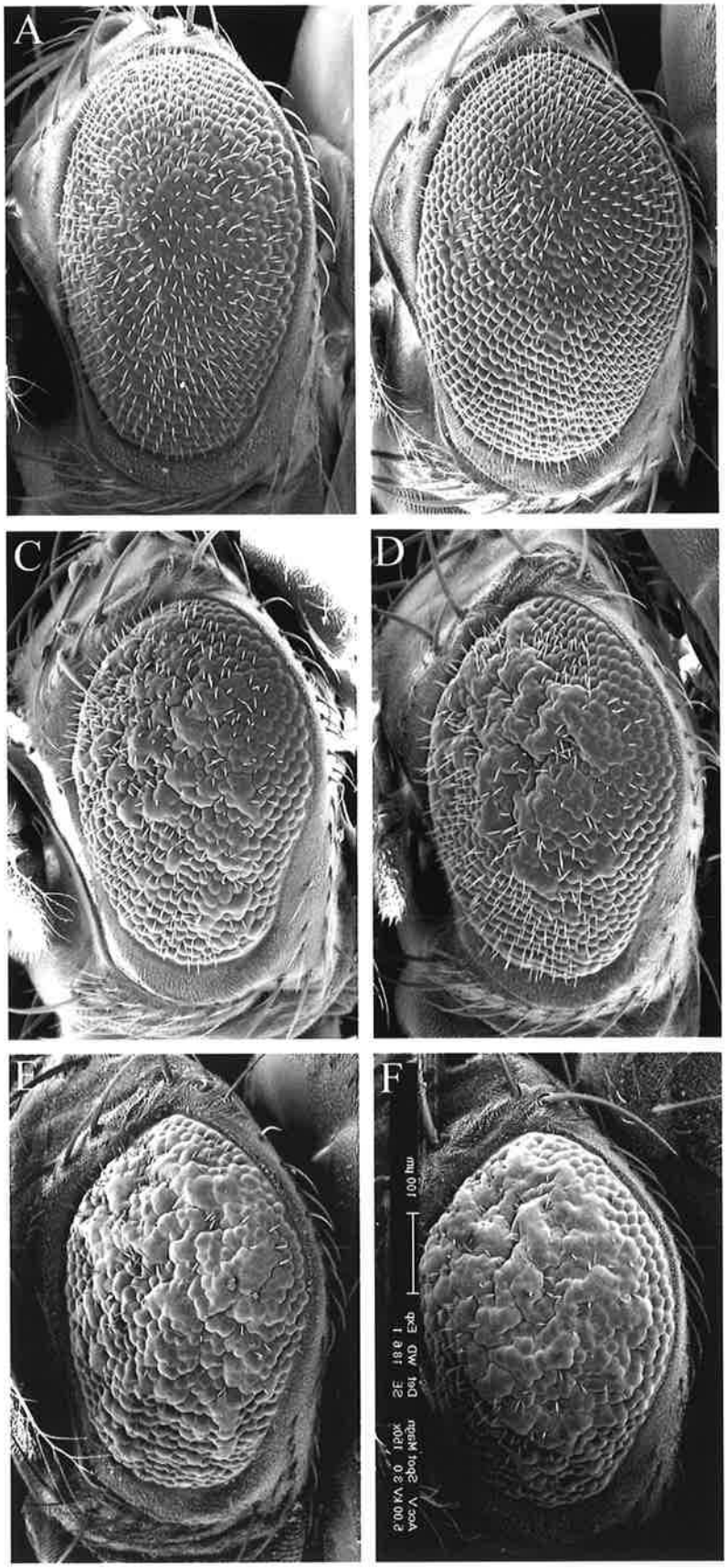
Rho effector	Allele/Deficiency	*GMR>pbl*	*GMR>pblΔDH*
Rho effectors			
Rho-kinase	rok^{2}	S+	E+
Protein kinase N	$PKN^{l(2)06736}$ (amorph)	no effect	no effect
Diaphanous	dia^{2} (amorph)	no effect	E++
profilin	$chic^{221}$	S+	S+
act-up	*acu*	no effect	no effect
PI-4-P5K (skittles)	$sktl^{\Delta 20}$	no effect	no effect
Rac/Cdc42 effectors			
MRCK	gek^{D23}	no effect	no effect
Genes requierd during cytokinesis			
pavarotti	pav^{B200}	no effect	E++
peanut	$pnut^{XP}$	no effect	no effect
spaghetti-squash	sqh^{1}	S++	S++
polo kinase	$polo^{9}$ (stromg hypomorphic, *P*-insertion affects both transcripts)	no effect	S+
	$polo^{10}$ (strong hypomorphic, *P*-insertion affects a single transcript)	no effect	S+
	Df(3L)rdgC-co2 (*polo* and *fbl)*	no effect	E+
	fbl	no effect	E+
aurora-B (IAL)	*Df(2L)J39*	no effect	S+++

E+, E++, E+++; mild, medium and strong enhancement
S+, S++, S+++; mild, medium and strong suppression

Pavarotti (pav) resulted in a medium enhancement of the *GMR>pblΔDH* phenotype (Table 5-2, Fig. 5-3F). *pav* encodes a kinesin like protein (KLP) that has previously been shown to be required for organisation of the central spindle and hence is necessary for cytokinesis (Adams et al., 1998). PAV was also shown to be responsible for the localisation of POLO to the central spindle (Adams et al., 1998). However, modifications of *GMR>pblΔDH* with strong hypmorphic alleles of *polo* resulted in a mild suppression of *GMR>pblΔDH.* Since these were not null alleles of *polo,* a deficiency stock which removes *polo* was also tested for its genetic interaction with *GMR>pbl* and *GMR>pblΔDH. Df(3L)rdgC-co2* had no effect on *GMR>pbl* but resulted in a mild enhancement of *GMR>pblΔDH.* This deficiency takes out a number of genes in addition to *polo.* One of these genes is *fumble (fbl),* which alone results in an enhancement of *GMR>pblΔDH. fbl* encodes a panthotenate kinase, which has been shown to be required for cytokinesis in *Drosophila,* and is though to play a role in the formation of new membrane as the furrow constricts (Afshar et al., 2001).

Figure 5-3 Genetic interactions of *pbl* with genes encoding downstream effectors of PBL-activated Rho-GTP and genes required for cytokinesis

Scanning electron micrographs of (A) *GMR>pbl/+*, (B) *rok*^{2}*/+;GMR>pbl/+*, (C) *GMR>pblΔDH/+*, (D) *rok*^{2}*/+;GMR>pblΔDH/+*, (E) *GMR>pblΔDH/dia*^{2} and (F) *GMR>pblΔDH/+;pav*^{B200}*/+* adult eyes. **A.** *GMR>pbl* adult rough eye phentoype. **B.** Mutations in *rok* resulted in a mild suppression of the *GMR>pbl* rough eye phenotype, with a slight increase in the size of the eye and decrease in overall roughness. **C.** The *GMR>pblΔDH* rough eye phenotype. **D.** Mutations in *rok* resulted in mild enhancement of the *GMR>pblΔDH* rough eye phenotype, with ommatidial fusions covering a slightly larger region of the surface of the eye. **E.** Mutations in *dia* resulted in a medium enhancement of *GMR>pblΔDH*, with extensive ommatidial fusions covering the entire surface of the eye. **F.** Mutations in *pav* also resulted in a dominant enhancement of *GMR>pblΔDH*, with extensive ommatidial fusions covering the entire surface of the eye.



GMR>pbl/+

rok^{2}*/+; GMR>pbl/+*

GMR> pblΔDH/+

rok^{2}*/+; GMR> pblΔDH/+*

GMR> pblΔDH/ dia

GMR> pblΔDH/+; pav^{B200}*/+*

5-3 Discussion

5-3.1 *pbl* is acting specifically through *Rho1*

The results reported in this chapter show that *pbl* interacts genetically with *Rho1* in this ectopic expression system. This specificity of the interaction between PBL and Rho was also observed in a yeast two-hybrid assay system, where a physical interaction between PBL and Rho1 but not Rac1, Rac2 or Cdc42 was detected (Prokopenko et al., 1999). In terms of the activation of cytokinesis, it is likely that the exchange factor activity of PBL, which localises to the cell cortex and contractile ring, could provide spatio-temporal activation of Rho1 and hence bring about the cytoskeletal reorganisation required for cytokinesis. A direct role for *Rho1* during cytokinesis in *Drosophila* had not previously been described. As a result of the work described here, *Rho1* mutants were analysed and cytokinetic defects were observed in the head region of mutant embryos (Prokopenko et al., 1999). *pbl* may also be interacting to some extent with *Rac2* and the more highly diverged *RhoL/Rac3*. However, in the absence of specific mutations this remains to be confirmed. Also, there may be redundancy between the various Rac family members that could mask an interaction. Interestingly, the interactions that were observed for *Rac* are in the opposite direction to that observed with *Rho1*. There is some evidence for the opposing activities of Rho and Rac in other systems (Leeuwen et al., 1997). Thus, the RhoGEF activity of PBL appears to be specifically promoting Rho1 GTPase activity in this system. PBL also appears to be having some inhibitory activity towards Rac2 and RhoL/Rac3, which may occur by crosstalk between Rho and Rac signalling cascades. There does not seem to be any major effects on Cdc42 GTPase signalling. Rho, Rac and Cdc42 are the most extensively characterised Rho family members. However, many others have been identified now that the *Drosophila* genome sequence has been determined and these also need to be considered as potential targets of PBL RhoGEF activity.

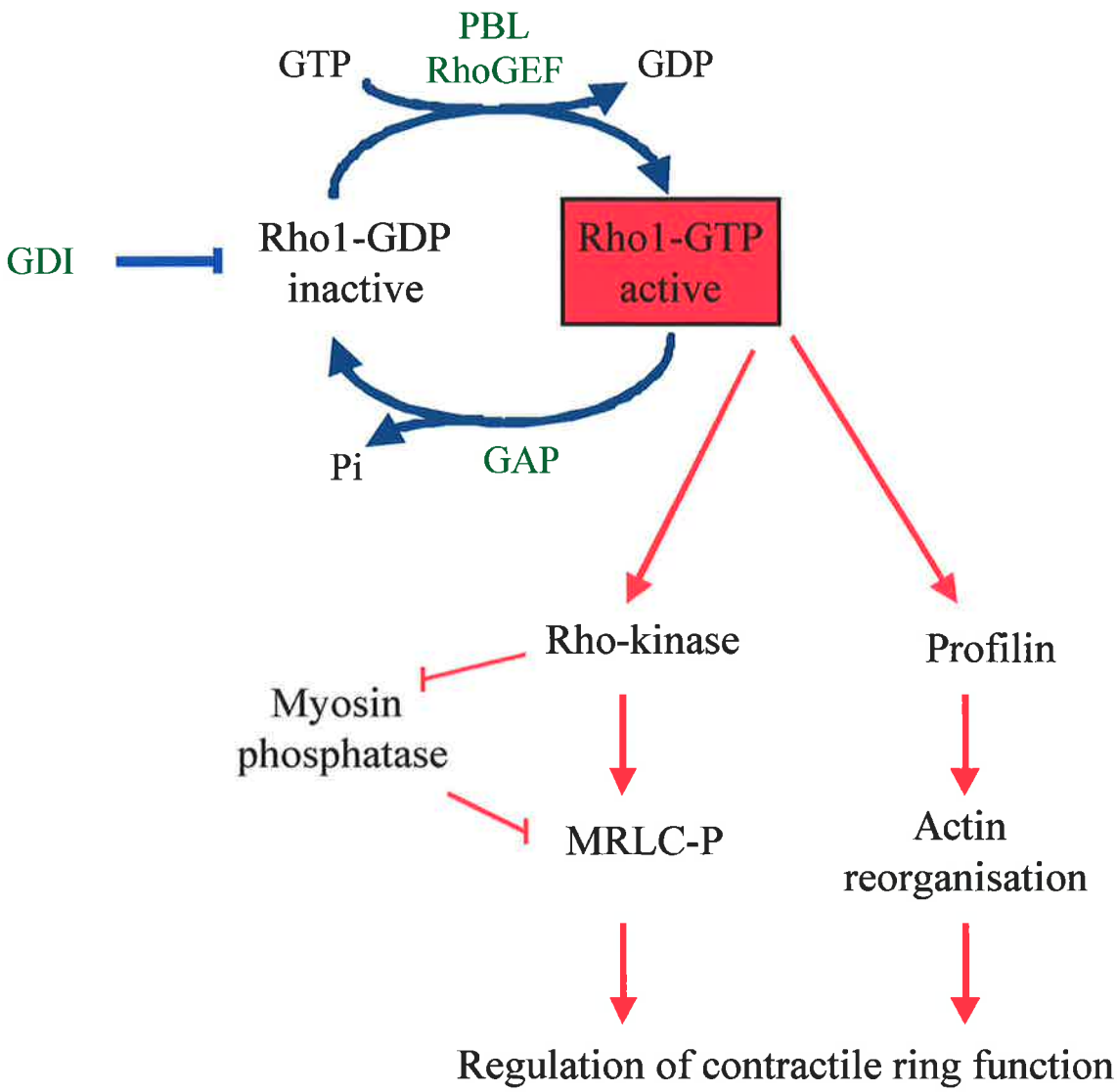
PBL mediated activation of Rho1 activity is the most likely consequence of ectopic PBL expression. However, it is also possible that Rho acts upstream of PBL and may be required for the correct localisation and/or activation of PBL activity at the time of cytokinesis. In this situation, the RhoGEF activity of PBL could be directed against another, as yet unidentified, Rho family member.

1-1.2 Truncated PBL results in constitutive activation of Rho1

The suppression of *sev***>pblΔ325* by introduction of one mutant copy of *Rho1* suggests that the truncated form of PBL results in ectopic activation of Rho1. However, it remains to be tested if this truncated form of PBL retains specificity for Rho1 or whether it can also lead to ectopic expression of other Rho family members. Ectopic expression of this truncated form

Figure 5-4 A model of PBL-mediated activation of Rho1 and contractile ring activity

The putative RhoGEF activity of PBL could result in increased levels of active Rho1-GTP, resulting in activation of downstream signalling components. Profilin is responsible for actin reorganisation events and Rho-kinase could result in activation of myosin, either by direct phosphorylation of the regulatory light chain or indirectly via inhibtion of an inhibitory myosin phosphatase. Both of these activities could be required for correct regulation of the contractile ring function. It is not known which GAP acts in opposition to PBL RhoGEF activity nor if there are any GDIs involved in correct regulation of contractile ring function.



GEF: guanine nucleotide exchange factor
GAP: GTPase activating protein
GDI: guanine nucleotide dissociation inhibitor
MRLC: myosin regulatory light chain

of PBL may lead to an inhibition of cytokinesis in some cases. However, this is not a dominant negative effect. Rather, it probably reflects an inability of the actin cytoskeleton to overcome the significant disruptions resulting from ectopic Rho1 activation. The severe phenotype observed following ectopic expression of PBLΔ325 is consistent with constitutive activation that results from truncated versions of other DH family members (Cerione and Zheng, 1996).

5-3.3 How does the RhoGEF activity of PBL mediate contractile ring activity?

Genetic interactions suggest that components downstream of PBL-activated Rho1 could be responsible for actin reorganisation and/or myosin function necessary for proper contractile ring activity. *rok* was identified as a mild suppresor of *GMR>pbl* and enhancer of *GMR>pblΔDH.* ROK activity is not absolutely required for cytokinesis since *Rok* somatic clones revealed no proliferation defects (Winter et al., 2001). However, ROK activity was shown to be required for phosphorylation of myosin regulatory light chain (RLC) specifically at the cleavage furrow in cultured cell lines (Kosako et al., 1999). This suggests that PBL mediated activation of ROK could also be required for the correct activation of myosin during contractile ring activity in *Drosophila,* but that this is not absolutely required during cytokinesis.

dia encodes a member of the formin homology (FH) family of proteins and has previously been shown to be required for cytokinesis in *Drosophila* (Castrillon and Wasserman, 1994). The observed genetic modification suggests that PBL-activated RhoGTP could also mediate its actin reorganisation events, resulting in formation and/or function of the contractile ring, primarily through the activation of DIA. Profilin, which is encoded by *chickadee (chic*) acts downstream of DIA and has been shown to play a role in many actin-dependent processes (Verheyen and Cooley, 1994). Mild suppression of both eye phenotypes was observed with *chic* alleles. This suggests that limiting the amount of actin regulators can have some effect on reorganisation events seen with both eye phenotypes. Thus, genetic interactions observed between candidate genes previously known to be involved in the regulation of cytokinesis and the *GMR>pbl* and/or *GMR>pblΔDH* rough eye phenotypes, provide a framework for a model for the downstream events of PBL-activated Rho1 at the time of cytokinesis (Fig. 5-4).

pav alleles were also observed to result in an enhancement of *GMR>pblΔDH.* This could result from additive effects since the KLP activity of PAV has previously been shown to be required for cytokinesis (Adams et al., 1998). Reducing the dosage of *pav* in *GMR>pblΔDH,* where the process of cytokinesis had already been compromised, could result in a further

impediment to cytokinesis and enhancement of the rough eye phenotype. However, the relationship may be more direct if PAV is necessary to shuttle a specific cargo in to the central spindle region such that it could then communicate with PBL at surrounding cortical regions of the contractile ring. POLO is one such cargo, for which a weak genetic interaction with *GMR>pblΔDH* was observed, but others presumably exist. A RacGAP has been identified in *C. elegans* (*cyk-4*) and mouse (*mgcRacGAP)* and shown to be required during cytokinesis (Hirose et al., 2001; Jantsch-Plunger et al., 2000). CYK-4 has been shown to interact directly with PAV and is required for organisation of the central spindle and cytokinesis. This RacGAP was shown to have highest GAP activity for Rac and Cdc42 with limited activity for Rho1. However, inactivation of *Rho1* but not *Rac1* or *Cdc42* resulted in cytokinetic defects in early divisions of *C.elegans*. Thus, it was proposed that the RacGAP activity of CYK-4/MgcRacGAP could still be acting on Rho1 (Jantsch-Plunger et al., 2000). If so, PAV could be responsible for localizing RacGAP to counter the RhoGEF activity of PBL at the time of cytokinesis. Alternatively, since the observed genetic interactions of *pbl* with *Rho1* were in the opposite direction to those observed with *pbl* and various *Rac* family members, it is possible that CYK-4/Mgc RacGAP activity may be required to decrease Rac signalling. This RacGAP has also been identified in *Drosophila (DRacGAP)* and shown to play a role in proliferation and patterning of the wing disc, however there was no reported effect on cytokinesis (Sotillos and Campuzano, 2000). These experiments were performed by ectopic expression of dominant negative forms of RacGAP however, specific mutations that would allow adequate assessment of a requirement during cytokinesis do not exist.

Chapter 6: Cdk1 phosphorylation and RhoGEF activity of Pebble during cytokinesis

6-1 Introduction

The precise coupling of mitosis and cytokinesis is crucial to ensure the integrity of the resultant daughter cells. Regulators of mitosis are well established, but the signals responsible for regulating cytokinesis remain largely unknown. The *GMR>pblΔDH* rough eye phenotype was used to test for a genetic interaction between *pbl* and various regulators of mitosis that might suggest a regulatory relationship.

Phosphorylation plays a major role in the regulation of mitosis. A high level of Cyclin/ Cyclin dependent kinase (CDK) activity triggers entry into mitosis (Ohi and Gould, 1999). Exit from mitosis is regulated by Anaphase Promoting Complex (APC) mediated destruction of mitotic B-type cyclins and decreased levels of associated CDK activity (reviewed by Zachariae, 1999). The introduction of stable forms of cyclin B (CYC-B) into cells in many systems has shown that degradation of B-type Cyclins is essential not only to allow the late stages of mitosis to occur, but also for cytokinesis to proceed (Murray et al., 1989; Parry and O'Farrell, 2001; Rimmington et al., 1994; Sigrist et al., 1995; Surana et al., 1993; Yamano et al., 1996). Similarly it has been shown that specific inactivation of CDK1 activity at late stages of mitosis is essential for regulating spindle dynamics and allowing cytokinesis to proceed (Wheatley et al., 1997). Thus, there is evidence that a relationship exists between regulators of mitosis and cytokinesis.

In *Drosophila*, sequential destruction of the mitotic cyclins, CYC-A, B and B3, occurs during mitosis (Sigrist et al., 1995). Consistent with distinct functions reflected in the temporal pattern of destruction, real-time imaging of cells expressing stable forms of these three cyclins revealed mitotic phenotypes at progressively later stages (Parry and O'Farrell, 2001). In addition to a mitotic arrest, cytokinesis was blocked following ectopic expression of stable CYC-A and CYC-B. However, the onset of anaphase and cytokinesis was observed to proceed normally following ectopic expression of stable CYC-B3. This suggests that the degradation of CYC-B is likely to be a determinant of the onset of cytokinesis. In addition, cytokinetic furrows have been shown to form prematurely in cells of *cycB* mutant *Drosophila* embryos, further supporting the hypothesis that CYC-B negatively regulates cytokinesis (Knoblich and Lehner, 1993).

As a key regulator of cytokinesis, PBL is an ideal candidate for negative regulation by CYC-B/CDK1 complexes. Phosphorylation by CYC-B/CDK1 could suppress PBL activity

until the onset of anaphase, allowing it to become active right at the time when cytokinesis is initiated. The spatio-temporal pattern of CYC-B at the end of mitosis during syncitial mitoses is tightly regulated. CYC-B, associated with mitotic spindles, disappears as a wave that begins at the centrosomes then moves in to the spindle midzone region where it is degraded as cells enter anaphase (Huang and Raff, 1999). If this same pattern of CYC-B degradation occurs during the cellular divisions, then negative regulation of cortical PBL by midzone associated CYC-B/CDK1 until cells enter anaphase would provide an ideal regulatory relationship. This would rely on communication between the spindle midzone and cortical regions, which has previously been shown to be necessary for cytokinesis (Gatti et al., 2000).

Evidence against a role for Cyclin/CDK in the repression of PBL activity has come from studies of ECT2, the mammalian homologue of PBL. ECT2 is phosphorylated on Ser/Thr residues at the G2/M transition (Tatsumoto et al., 1999). This could be consistent with phosphorylation by CDK1/CYC-B complexes. However, *in vitro* assays of ECT2 RhoGEF activity indicate that the phosphorylated form is catalytically active, not inactive as the above model for PBL would predict. As a result, a second hypothesis could be that PBL is a direct target of CDK1/CYC-B complexes, but that these complexes act as essential activators of PBL for a requirement at an earlier stage during mitosis. In this chapter I use genetic analysis to test these conflicting hypotheses.

6-2 Results

6-2.1 *cdk1, cycB* and *cycB3* interact genetically with *GMR>pblΔDH*

To address a possible role for the regulation of PBL activity by CYC-B/CDK1 phosphorylation, a number of candidate genes were tested for their ability to dominantly modify the *GMR>pblΔDH* rough eye phenotype. The cellular basis of which is, at least partially, a result of an inhibition of cytokinesis (see chapter 4). Flies heterozygous for a mutation in *cdk1* exhibited a strong suppression of the *GMR>pblΔDH* phenotype (Fig. 6-1B). Suppression of *GMR>pblΔDH* was not observed with mutations in *cycA* (Fig. 6-1C), *cycE* (data not shown) or *cycB* (Fig. 6-1C,D), whilst mutations in *cycB3* resulted in a medium level of suppression (Fig. 6-1E). The function of the products of *cycB* and *B3* are redundant, since flies carrying either of these mutations are viable but double mutants cannot survive (Jacobs et al., 1998). Reducing the dosage of both *cycB* and *cycB3* resulted in a strong suppression of *GMR>pblΔDH* (Fig. 6-1F). Thus, removal of one copy of *cdk1,* or both *cycB* and *cycB3,* resulted in strong suppression of the *GMR>pblΔDH* dominant negative phenotype. These genetic interactions indicate, therefore, that *cdk1* and *cycB* and/or *cycB3* are acting as negative

regulators of PBL activity. These observations are consistent with the hypothesis that CYC-B/CDK1 complex acts as a suppressor of PBL activity via phosphorylation until shortly after the onset of anaphase. However, they do not support the alternative hypothesis that CYC-B/CDK1 is required to phosphorylate and activate PBL.

6-2.2 Conservation of CDK1 phosphorylation sites in PBL and ECT2

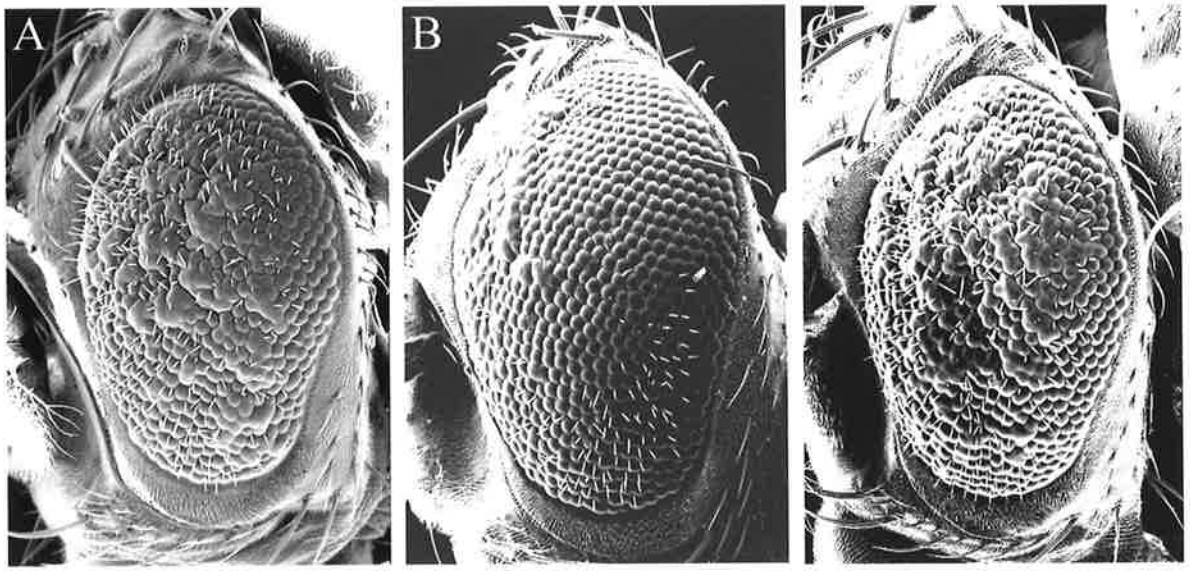
Examination of the PBL protein sequence revealed the presence of three consensus CDK1 phosphorylation sites (S/T P X K/R). These are S360, T380 and T771 (Fig. 6-2, highlighted in red). Comparison of the PBL sequence with its mammalian homologue, ECT2, revealed that one of these consensus sites (T771) is conserved, corresponding to T815 in the ECT2 sequence (Fig. 6-2, highlighted in green). The presence of these sites in PBL and the conservation of at least one of these sites with the mammalian orthologue supports the hypothesis that phosphorylation at these sites could play a role in the regulation of PBL activity.

6-2.3 PBL phosphorylation in proliferating cells

Phosphorylation of proteins can alter their mobility when analysed by SDS-PAGE, most often resulting in forms that migrate more slowly. To assess whether or not PBL was phosphorylated in proliferating cells *in vivo*, gel electrophoresis and western blot analysis was used to analyse the mobility of endogenous PBL protein in 3-6hr embryos. Most cells in the embryo are undergoing the cycles 14-16 at this stage of development. A predominant band of approximately 125kDa was observed, with no forms of endogenous PBL showing obvious differing mobilities (Fig. 6-3A, lane 1). However, the level of background was quite high using the anti-PBL antibody despite trying various blocking agents. To overcome this problem, a MYC tagged version of the PBL protein (MYC-PBL) was also analysed. *UAS-myc-pbl* was able to rescue cytokinesis in alternating stripes of *pbl* mutant embryos when ectopically expressed with *prd-GAL4* (see Fig. 6-5A-C). Western analysis was used to determine whether this form of PBL, which we know to be functional as it can rescue cytokinesis, was present in differentially phosphorylated forms. Individual rescued embryos at the stage of germ band extension were selected, based on their alternating stripes of nuclei of differing morphology. At this stage most of the cells in the embryos are proliferating, embryos were pooled and extracts prepared. Western analysis revealed a single band of MYC-PBL at approx 135KDa (Fig. 6-3B, lane 1). The increased size of MYC-PBL was consistent with the 10kDa size of the MYC-tag. Thus, both endogenous PBL and ectopically expressed MYC-PBL showed no evidence of differentially phosphorylated forms in proliferating embryos. In addition, preincubation of these embryonic extracts with a

Figure 6-1 *GMR> pblΔDH* interacts genetically with *cdk1, cycB and cycB3*

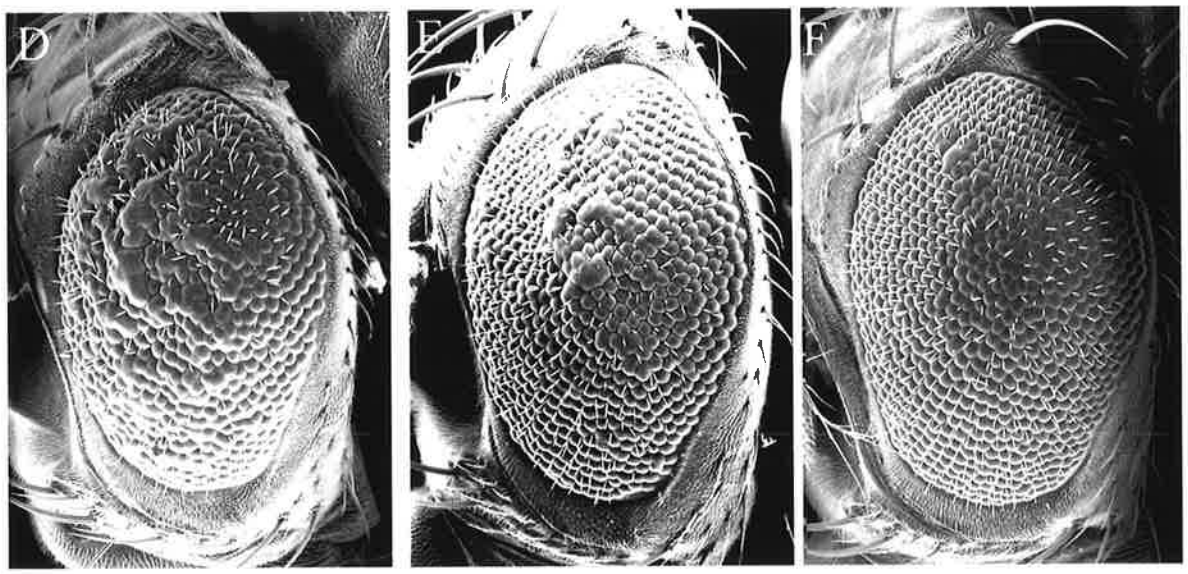
Scanning electron micrographs of (A) *GMR>pblΔDH/+*, (B) *GMR>pblΔDH/cdk1*$^{E1.9}$, (C) *GMR>pblΔDH/+;cycA*^{C8LR1}*/+*, (D) *GMR>pblΔDH/cycB*^{2}, (E) *GMR>pblΔDH/+;cycB3*^{1}*/+* and (F) *GMR>pblΔDH/cycB*^{2}*;cycB3*^{1}*/+* adult eyes. **A.** The *GMR>pblΔDH* adult rough eye phenotype. **B.** Strong suppression of *GMR>pblΔDH* was observed following the introduction of one mutant copy of *cdk1* (the absence of bristles in this eye reflects loss of bristles during preparation of the sample and not a developmental absence of bristles). **C.** No modification of *GMR>pblΔDH* was observed with a mutation in *cycA*. **D.** No modification of *GMR>pblΔDH* was observed with a mutation in *cycB*. **E.** Medium suppression of *GMR>pblΔDH* was observed with a mutation in *cycB3*. **F.** Strong suppression of *GMR>pblΔDH* was observed with mutations in both *cycB* and *cycB3*.



GMR>pblΔDH/+

GMR>pblΔDH/ cdk1 $^{E1.9}$

GMR>pblΔDH/+; cycA ^{C8LR1}/+



GMR>pblΔDH/ cycB ^{2}

GMR>pblΔDH/+; cycB3 ^{1}/+

GMR>pblΔDH/cycB ^{2}; cycB3 ^{1}/+

Figure 6-2 Conservation of CDK1 phosphorylation sites in PBL and ECT2

Alignment of the predicted amino acid sequences showing the homology between PBL and ECT2. Identity between amino acids in PBL and ECT2 is denoted by a ":" whilst similarity is denoted by a ".". PBL has three consensus CDK1 phosphorylation sequences (S/T P X K/R); SPDK (S360), TPAK (T380) and TPNK (T771) shown in red. ECT2 has a single consensus CDK1 phosphorylation site; TPKR (T815) shown in green, the position of which is conserved with T771 in PBL.

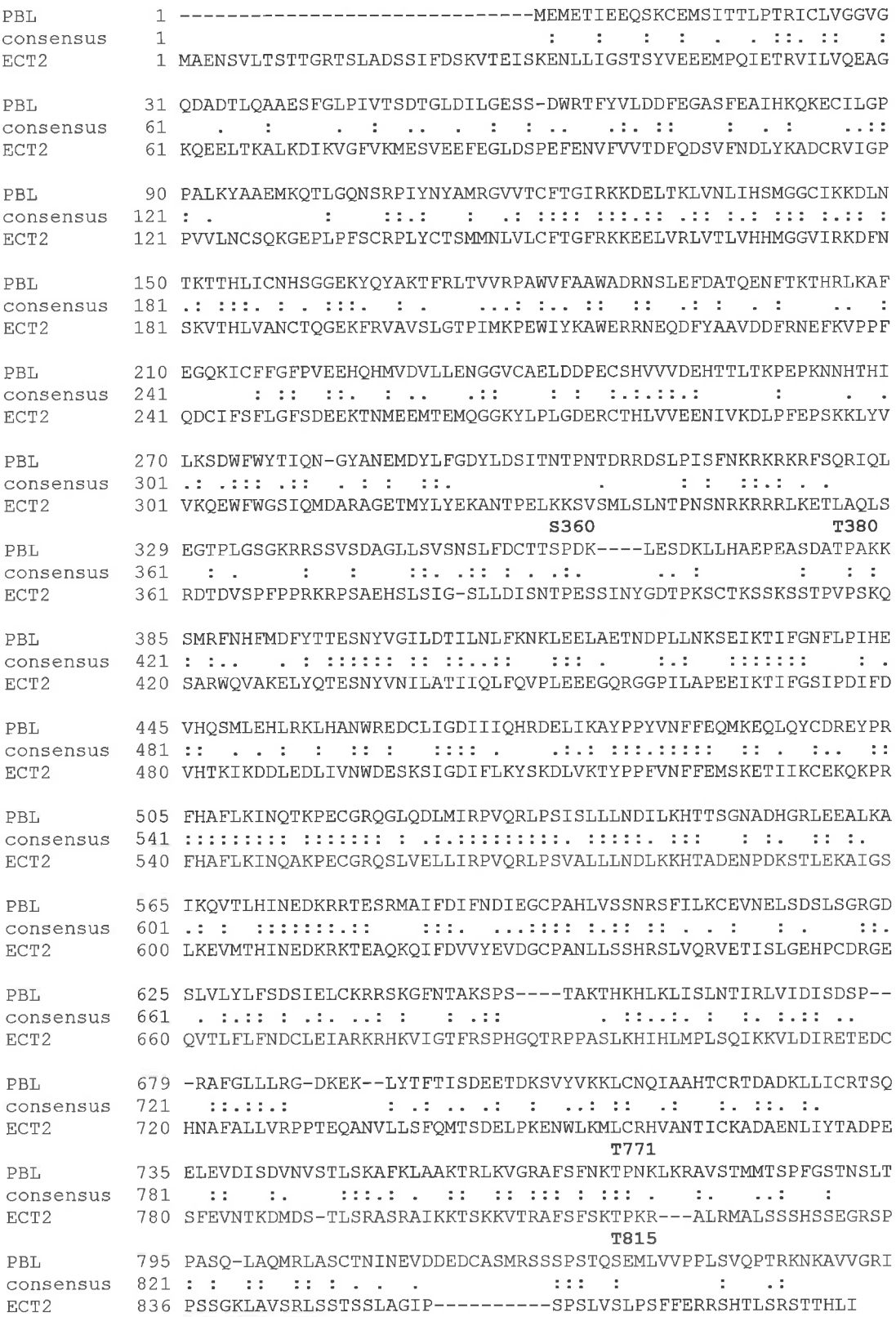
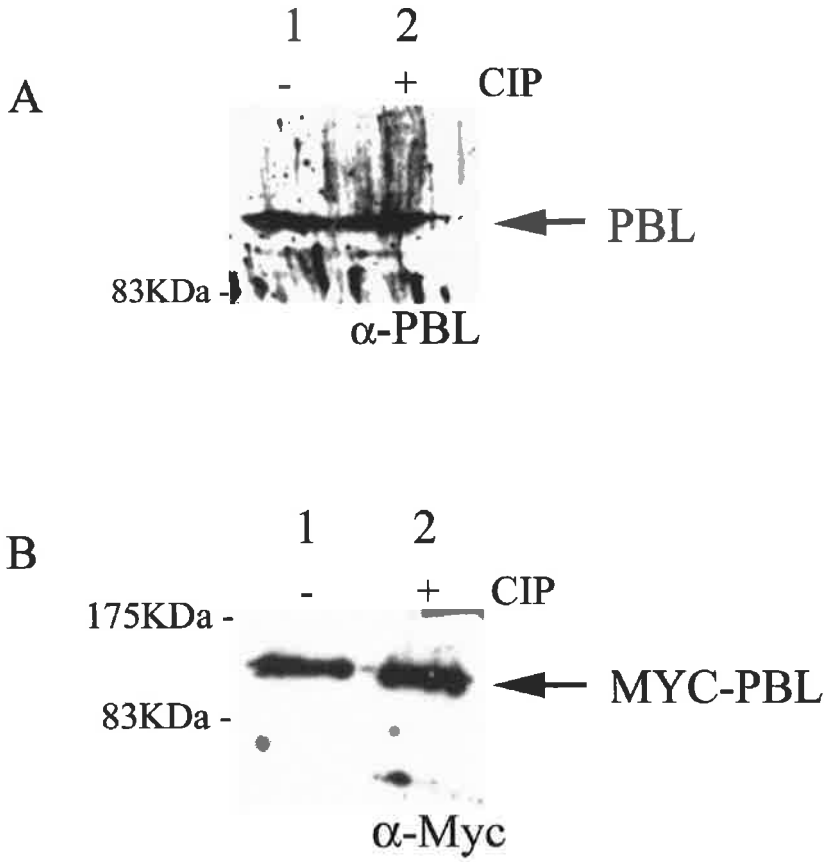

PBL 1 ----------MEMETIEEQSKCEMSITTLPTRICLVGGVG
consensus 1
ECT2 1 MAENSVLTSTTGRTSLADSSIFDSKVTEISKENLLIGSTSYVEEEMPQIETRVILVQEAG
PBL 31 QDADTLQAAESFGLPIVTSDTGLDILGESS-DWRTFYVLDDFEGASFEAIHKQKECILGP
consensus 61
ECT2 61 KQEELTKALKDIKVGFVKMESVEEFEGLDSPEFENVFVVTDFQDSVFNDLYKADCRVIGP
PBL 90 PALKYAAEMKQTLGQNSRPIYNYAMRGVVTCFTGIRKKDELTKLVNLIHSMGGCIKKDLN
consensus 121
ECT2 121 PVVLNCSQKGEPLPFSCRPLYCTSMMNLVLCFTGFRKKEELVRLVTLVHHMGGVIRKDFN
PBL 150 TKTTHLICNHSGGEKYQYAKTFRLTVVRPAWVFAAWADRNSLEFDATQENFTKTHRLKAF
consensus 181
ECT2 181 SKVTHLVANCTQGEKFRVAVSLGTPIMKPEWIYKAWERRNEQDFYAAVDDFRNEFKVPPF
PBL 210 EGQKICFFGFPVEEHQHMVDVLLENGGVCAELDDPECSHVVVDEHTTLTKPEPKNNHTHI
consensus 241
ECT2 241 QDCIFSFLGFSDEEKTNMEEMTEMQGGKYLPLGDERCTHLVVEENIVKDLPFEPSKKLYV
PBL 270 LKSDWFWYTIQN-GYANEMDYLFGDYLDSITNTPNTDRRDSLPISFNKRKRKRFSQRIQL
consensus 301
ECT2 301 VKQEWFWGSIQMDARAGETMYLYEKANTPELKKSVSMLSLNTPNSNRKRRRLKETLAQLS
S360 T380
PBL 329 EGTPLGSGKRRSSVSDAGLLSVSNSLFDCTTSPDK----LESDKLLHAEPEASDATPAKK
consensus 361
ECT2 361 RDTDVSPFPPRKRPSAEHSLSIG-SLLDISNTPESSINYGDTPKSCTKSSKSSTPVPSKQ
PBL 385 SMRFNHFMDFYTTESNYVGILDTILNLFKNKLEELAETNDPLLNKSEIKTIFGNFLPIHE
consensus 421
ECT2 420 SARWQVAKELYQTESNYVNILATIIQLFQVPLEEEGQRGGPILAPEEIKTIFGSIPDIFD
PBL 445 VHQSMLEHLRKLHANWREDCLIGDIIIQHRDELIKAYPPYVNFFEQMKEQLQYCDREYPR
consensus 481
ECT2 480 VHTKIKDDLEDLIVNWDESKSIGDIFLKYSKDLVKTYPPFVNFFEMSKETIIKCEKQKPR
PBL 505 FHAFLKINQTKPECGRQGLQDLMIRPVQRLPSISLLLNDILKHTTSGNADHGRLEEALKA
consensus 541
ECT2 540 FHAFLKINQAKPECGRQSLVELLIRPVQRLPSVALLLNDLKKHTADENPDKSTLEKAIGS
PBL 565 IKQVTLHINEDKRRTESRMAIFDIFNDIEGCPAHLVSSNRSFILKCEVNELSDSLSGRGD
consensus 601
ECT2 600 LKEVMTHINEDKRKTEAQKQIFDVVYEVDGCPANLLSSHRSLVQRVETISLGEHPCDRGE
PBL 625 SLVLYLFSDSIELCKRRSKGFNTAKSPS----TAKTHKHLKLISLNTIRLVIDISDSP--
consensus 661
ECT2 660 QVTLFLFNDCLEIARKRHKVIGTFRSPHGQTRPPASLKHIHLMPLSQIKKVLDIRETEDC
PBL 679 -RAFGLLLRG-DKEK--LYTFTISDEETDKSVYVKKLCNQIAAHTCRTDADKLLICRTSQ
consensus 721
ECT2 720 HNAFALLVRPPTEQANVLLSFQMTSDELPKENWLKMLCRHVANTICKADAENLIYTADPE
T771
PBL 735 ELEVDISDVNVSTLSKAFKLAAKTRLKVGRAFSFNKTPNKLKRAVSTMMTSPFGSTNSLT
consensus 781
ECT2 780 SFEVNTKDMDS-TLSRASRAIKKTSKKVTRAFSFSKTPKR---ALRMALSSSHSSEGRSP
T815
PBL 795 PASQ-LAQMRLASCTNINEVDDEDCASMRSSSPSTQSEMLVVPPLSVQPTRKNKAVVGRI
consensus 821
ECT2 836 PSSGKLAVSRLSSTSSLAGIP----------SPSLVSLPSFFERRSHTLSRSTTHLI

Figure 6-3 Western analysis of PBL and MYC-PBL in proliferating embryos

Embryonic extracts were prepared from (A) wild-type embryos collected and aged to 3-6hr and (B) *prd-GAL4, pbl*^{2}*/UAS-myc-pbl,pbl*^{3} mutant embryos individually selected at the stage of germband extension, based on nuclear morphology, These extracts were each incubated in the absence (-) or presence (+) of a phosphatase (CIP). SDS-PAGE and western analysis were performed using antibodies directed against PBL (A) and MYC (B) to detect the endogenous and tagged PBL proteins respectively. **A.** Endogenous PBL was detected as a band at approx 125 kDa (lane 1), with no alteration in mobility after the addition of CIP (lane 2). High levels of background were observed using this antibody. **B.** MYC-PBL was detected as a single band at approx. 135 kDa (lane 1, this is consistent with the expected size increase associated with the additional 10 kDa MYC tag). No alteration in mobility was observed after addition of CIP (lane 2).


A
1
2
-
+
CIP
PBL
83KDa -
α-PBL
B
1
2
-
+
CIP
175KDa -
MYC-PBL
83KDa -
α-Myc

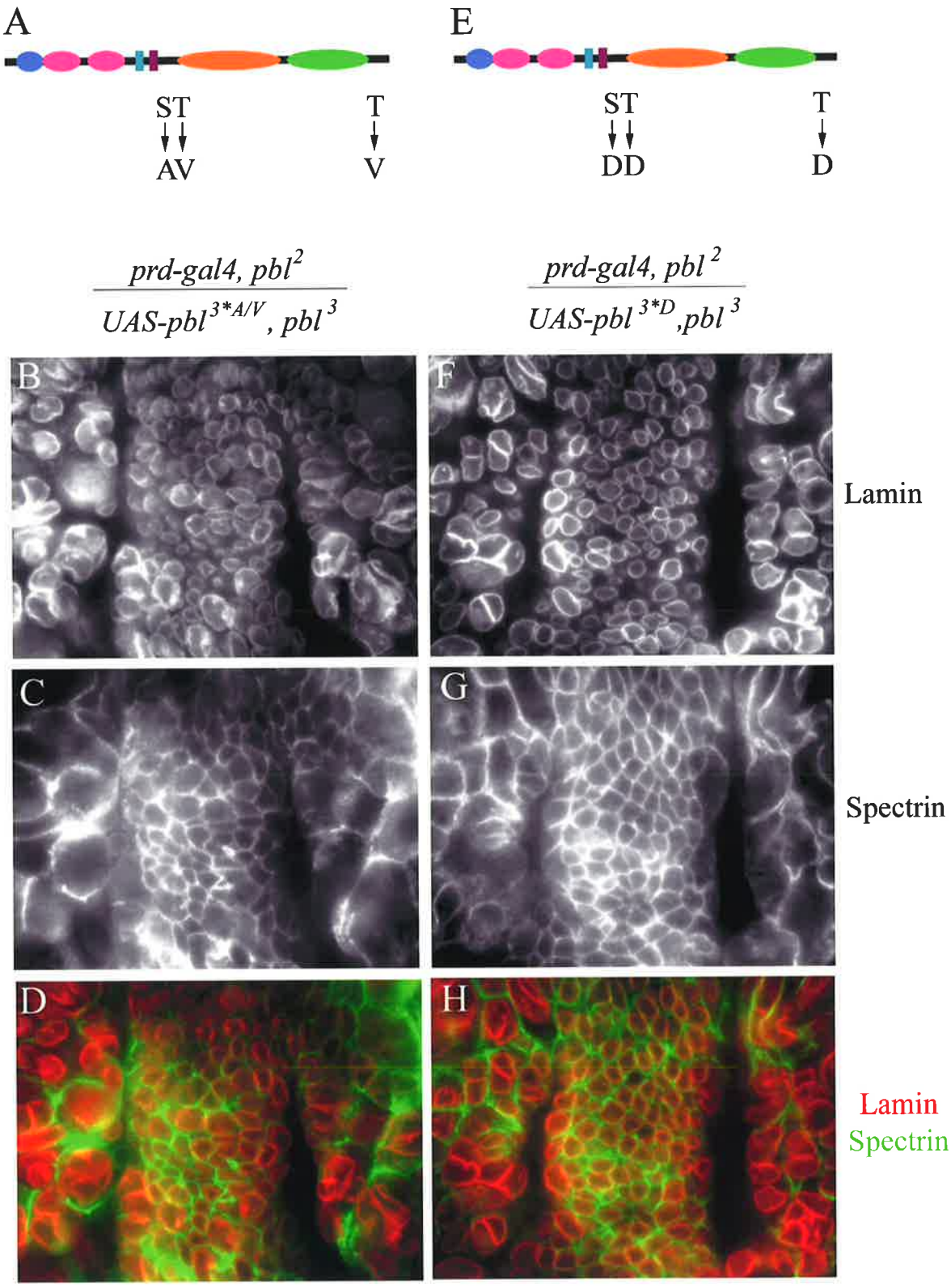
phosphatase has no effect on the mobility of these bands (Fig. 6-3A, B, lanes 2). However, this is inconclusive in the absence of controls to show that the conditions for preparation of extracts were suitable to preserve phosphorylated forms of proteins and that the CIP treatment is effective. The absence of a detectable phosphorylated form of PBL contrasts with clear phosphorylated forms observed for ECT2 (Tatsumoto et al., 1999). It is possible that the relatively large size of PBL makes it difficult to observe the phosphorylated forms. Alternatively, it could be that the phosphorylated form of PBL is only present transiently or in a spatially restricted region and hence is not readily detected in a non synchronised population of cells. The presence of the majority of PBL in the nucleus, as opposed to the cleavage furrow, in non-synchronous cells (Prokopenko et al., 1999), makes this a distinct possibility. As it was not possible to resolve this issue by western analysis of the protein product, it was decided to carry out a specific test of the role of the CDK1 phosphorylation sites in PBL function *in vivo*.

6-2.4 Inactivation of CDK1 phosphorylation sites has no effect on PBL function *in vivo*

To directly address the role of the consensus CDK1 phosphorylation sites of PBL *in vivo,* site directed mutagenesis was used to generate expression constructs, with codons for serine and threonine residues of the consensus CDK1 sites specifically altered to encode residues that could not be phosphorylated (serine to alanine and threonine to valine). Constructs were generated where the conserved site alone was mutated, *UAS-pbl*^{T771V} or where all three consensus sites were mutated *UAS-pbl*^{S360A,S380V,T771V} (*UAS-pbl*$^{3*A/V}$). If phosphorylation of PBL at any or all of these sites during mitosis prevents its premature activation, we would expect these mutated forms to be constitutively active. This could result in dramatic effects on actin rearrangement, which may include premature or ectopic cytokineses. Co-expression of one copy of each of these constructs with *GMR-GAL4* did not result in a rough eye phentoype (data not shown) which was consistent with this mutated form of PBL not having a dramatic effect on the actin cytoskeleton. In addition co-expression of two copies of each of these constructs with *GMR-GAL4* resulted in a rough eye phenotype that was comparable to *GMR>pbl>pbl* (data not shown and see Fig 4-3G, H). In addition, ectopic expression of two copies of each of these constructs with *prd-GAL4* in a wild-type background had no detectable effect on cytokinesis, as observed with *prd>pbl>pbl* (data not shown). Thus, inactivation of one or all of the CDK1 consensus sites in PBL did not result in a significant disruption to the function of ectopic PBL during eye or embryonic development. These results suggest that phosphorylation of PBL at any or all of these sites does not appear to be responsible for preventing the premature activation of PBL during the cell cycle to ensure correct the regulation of cytokinesis.

Figure 6-4 Mutation of CDK1 phosphorylation sites has no effect on PBL function *in vivo*

Site directed mutagenesis was used to specifically mutate the three consensus CDK1 phosphorylation sites in *UAS-pbl* so that they could not longer be phosphorylated (*UAS-pbl*$^{3*A/V}$, shown schematically in Fig. 6-4A) or so that they were constitutuvely activated (*UAS-pbl*^{3*D}, shown schematically in Fig. 6-4E). Embryos ectopically expressing *prd-GAL4* and either *UAS-pbl*$^{3*A/V}$ (Fig. 6-4B-D) or *UAS-pbl*^{3*D} (Fig. 6-4F-H) in a *pbl* mutant background (*pbl*^{2}/*pbl*^{3}) were stained with anti-Lamin (B, D, F, H) and anti-Spectrin (C, D, G, H) antibodies and viewed by epifluorescence microscopy. **B**. Anti-Lamin stains of *prd-GAL4,pbl*^{2}/*UAS-pbl*$^{3*A/V}$,*pbl*^{3} embryos revealed stripes of small regular nuclei, characteristic of those found in wild-type embryos, which were flanked by stripes of larger, irregular nuclei resembling those seen in *pbl* mutant embryos. **C.** Anti-Spectrin stains revealed stripes of small, regular cell outlines flanked by stripes of larger cell outlines. **D.** The merged image confirmed that cells in the central rescued stripe each contained a single nucleus whilst those in the flanking non-rescued stripes were multinucleate, characteristic of the *pbl* mutant background of these embryos. **F.** Anti-Lamin, **G.** Anti-Spectrin and **H.** the merged image of these stains, confirmed the rescue of cytokinesis in *prd-GAL4,pbl*^{2}/*UAS-pbl*^{3*D},*pbl*^{3} embryos. A central rescued stripe, where each cell contains a single nucleus, is flanked by stripes of multinucleate cells characteristic of the *pbl* mutant background.


A
ST
AV
T
V
E
ST
DD
T
D
prd-gal4, pbl2
UAS-pbl3*A/V, pbl3
prd-gal4, pbl2
UAS-pbl3*D,pbl3
B
F
C
G
D
H
Lamin
Spectrin
Lamin
Spectrin

Conversely, if phosphorylation at any or all of the consensus sites was required for the activation of PBL, an inability to phosphorylate these residues would result in a protein that would not be able to function during cytokinesis. In this case, these mutated forms of PBL would be unable to rescue cytokinesis in *pbl* mutant embryos. Embryos expressing *prd-GAL4* and *UAS-pbl*$^{3*A/V}$ in a *pbl* mutant background were examined (Fig. 6.4A-C). Lamin stains revealed these embryos had stripes of small, regular nuclei, characteristic of those found in wild-type embryos which were flanked by stripes of large, irregular nuclei, characteristic of those found in *pbl* mutant embryos (Fig. 6-4A). Spectrin stains revealed different cell sizes in these alternating stripes, with stripes of small cell outlines flanked by stripes of much larger cell outlines (Fig. 6-4B). The merged image confirms that the cells containing the large irregular nuclei were multinucleate, as expected for the *pbl* mutant background, while the smaller cells each contained a single nucleus.

This shows that cytokinesis had been rescued in these central stripes (Fig. 6-5C). This rescue was indistinguishable from that observed with ectopic expression of the fully functional form of PBL (data not shown) or of a MYC tagged form of PBL (Fig. 6-5A-C). Since this mutant form of PBL was functional and there was no evidence for premature or ectopic cytokineses in the rescued stripes, neither of the hypotheses for direct regulation of PBL by CDK1 phosphorylation was supported by these observations.

6-2.5 Activation of CDK1 phosphorylation sites results in a functional protein

Constructs were also generated in which the serine and threonine residues at the consensus CDK1 sites were mutated to aspartate residues, which are positively charged to mimic the phosphorylated state. Constructs were generated in which the conserved site alone was altered (*UAS-pbl*^{T771D}) and where all three consensus sites were altered (*UAS-pbl*^{S360D,S380D,T771D} = *UAS-pbl*^{3*D}). If phosphorylation of PBL is required for activation, these constructs should encode constitutively activated forms of PBL and ectopic expression should result in dramatic effects, as cytokinesis would be uncoupled from the late stages of mitosis. However, these predicted dramatic effects were not observed ,as *GMR-GAL4* and two copies of *UAS-pbl*^{3*D} resulted in phenotypes that were comparable to *GMR>pbl>pbl* (data not shown and see Fig. 4-3G, H). Also, ectopic expression of two copies of *UAS-pbl*^{3*D} with *prd-GAL4* in a wild-type background had no effect on cytokinesis (data not shown). Taken together, these results argue that this mutant form of PBL does not appear to be having dramatic effects on the actin cytoskeleton.

If dephosphorylation of PBL at any or all of these sites at the completion of mitosis is essential for its activity, this modified PBL^{3*D} should not be able to function during cytokinesis. However, *UAS-pbl*^{3*D} was also found to rescue the cytokinesis defect in

alternating stripes of *pbl* mutant embryos when expressed with *prd-GAL4* (Fig. 6-4F-H). Anti-Lamin stains confirmed the presence of stripes of small, regularly shaped nuclei in the rescued stripe which were flanked by stripes of larger, more irregularly shaped nuclei that were often clumped together (Fig. 6-4F). Anti-Spectrin stains revealed stripes of more regular shaped cell outlines flanked by stripes of large irregular shaped cell outlines (Fig. 6-5G). The merged image confirms that in the central rescued stripe, each cell contains a single nucleus and this is flanked by non-rescued stripes where each cell contains the multiple nuclei expected for the *pbl* mutant background (Fig. 6-5G). Taken together, these results support the conclusion that the regulation of PBL activity does not occur directly via CYC-B/CDK1 phosphorylation.

6-2.6 ECT2 fails to rescue cytokinesis in *pbl* mutant embryos

These results suggest that PBL activity is not regulated by phosphorylation at these CDK1 sites. This could highlight differences in the regulation of PBL and ECT2, if indeed ECT2 is directly regulated by CYC-B/CDK1 activity. Thus, ectopic expression of ECT2 was tested for its ability to replace the function of PBL in the embryonic rescue assay system. Embryos expressing *prd-GAL4* and *UAS-ect2* in a *pbl* mutant background did not result in rescue of cytokinesis in alternating stripes along the length of the embryo. Staining with anti-Lamin (Fig. 6-5C) revealed that all nuclei were large and irregular. Anti-Spectrin stains revealed that all cells along the length of the embryos are enlarged (Fig. 6.5D). The merged image confirms that each cell contained multiple nuclei (Fig. 6-5D, merge). Thus ECT2 was unable to substitute for PBL in this assay, suggesting that they may be regulated differently. However, since this form of ECT2 was not tagged and antibodies to ECT2 were unavailable, it was not possible to ensure that ECT2 was being expressed in these embryos.

6-3 Discussion

6-3.1 PBL RhoGEF activity during cytokinesis is not directly regulated by CDK1

Two hypotheses were tested in this study. In the first, CDK1 activity maintains a phosphorylated, inactive form of PBL throughout mitosis. This would provide an ideal mechanism for correctly timing the activation of cytokinesis with the completion of mitosis when CDK1 activity is downregulated by the degradation of CYC-B. Genetic interactions of *GMR>pblΔDH* with various mitotic regulators provided strong support for this. In the second hypothesis, CDK1 is responsible for phosphorylation, and activation of PBL throughout mitosis. This possibility was raised by the observation that ECT2 is active as a RhoGEF when phosphorylated at serine and/or threonine residues, although the kinase responsible for

this is unknown. Neither of these hypotheses is supported by the data, since inactivation of CDK1 consensus phosphorylation sites had no effect on PBL activity during cytokinesis *in vivo*, nor on its ability to rescue the *pbl* mutant phenotype. Similarly, altering the CDK1 consensus phosphorylation sites to aspartate residues, and therefore mimicking the phosphorylated form of the protein, also produced a protein that behaved normally during cytokinesis.

6-3.2 Is PBL differentially phosphorylated during the cell cycle?

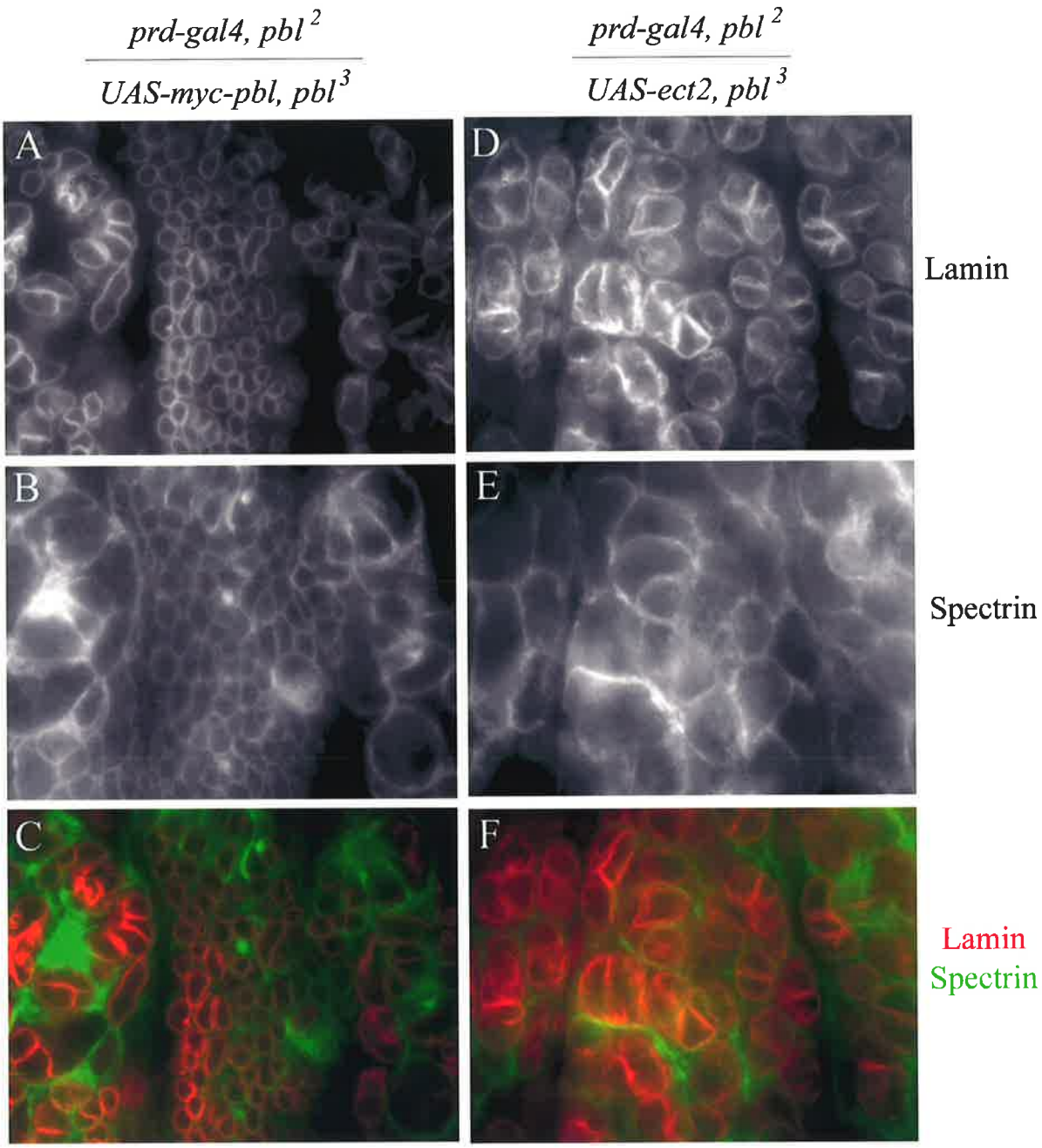
By western analysis it was not possible to detect bands of differing mobility for endogenous PBL or MYC-PBL in extracts prepared from proliferating embryos. However, this is by no means conclusive and alternative approaches could be used to determine the phosphorylation status of PBL. Labelling of tissue culture cells with radioactive phosphate and immunoprecipitation with anti-PBL antibodies could be used to determine directly whether PBL present within these cells can be phosphorylated. Due to the problems encountered with the anti-PBL antibodies in the course of this work, this experiment could also be carried out with cells ectopically expressing MYC-PBL and immunoprecipitation using anti-MYC antibodies. Better approaches could be to perform western analysis on immunoprecipitated PBL or MYC-PBL from tissue culture cells or embryos using antibodies specifically directed against phosphorylated Ser, Thr or Tyr residues. Or, alternatively, 2D gel electrophoresis could be performed on immunoprecipitated PBL or MYC-PBL to determine if there are differentially modified forms of PBL in embryos that could correspond to phosphorylated forms.

6-3.3 CDK1 may be responsible for indirectly regulating the activity of PBL

If PBL is not directly regulated by CDK1, how can the observed genetic interactions with *cdk1, cycB* and *cycB3* be explained? It is possible that mutation of one copy of these genes slows the cell cycle sufficiently for cells to compensate for decreased PBL activity, thus resulting in suppression of the rough eye phenotype. However, the observation that mutations in other cyclins; *cycE* and *cycA*, do not shown this same modification argues against this. The other possibility is that CDK1, together with either CYC-B or B3 indirectly regulates the level of PBL activity. Although we could not detect phosphorylated forms of PBL, we could not rule out the possibility that such forms exist. It is possible, therefore, that other kinases required for mitosis, and now also shown to play roles during cytokinesis, are candidates for regulation of PBL activity. POLO and AuroraB kinases are potential candidates (as discussed in the introduction). Lethal alleles of *polo* mildly suppress the *GMR>pblΔDH* rough eye phenotype, while a deficiency that removes *polo* resulted in a mild

Figure 6-5 ECT2 fails to rescue cytokinesis in *pbl* mutant embryos

Embryos ectopically expressing *prd-GAL4* and either *UAS-myc-pbl* (A-C) or *UAS-ect2* (D-F) in a *pbl* mutant background (pbl^2/pbl^3) were stained with anti-Lamin (A, C, D, F) and anti-Spectrin (B, C, E, F) antibodies and viewed by epifluoescence microscopy. **A.** Anti-Lamin stains of *prd-GAL4,* pbl^2*/UAS-myc-pbl,* pbl^3 embryos revealed stripes of small regular nuclei, characteristic of those found in wild-type embryos, which were flanked by stripes of larger, irregular nuclei resembling those seen in *pbl* mutant embryos. **B.** Anti-Spectrin stains revealed stripes of small, regular cell outlines flanked by stripes of larger cell outlines. **C.** The merged image confirmed that cells in the central rescued stripe each contained a single nucleus whilst those in the flanking non-rescued stripes were multinucleate, characteristic of the *pbl* mutant background of these embryos. **D.** Anti-Lamin revealed that all nuclei in *prd-GAL4,* pbl^2*/UAS-ect2,* pbl^3 embryos were large and irregular, characteristic of those found in *pbl* mutant embryos. **E.** Anti-Spectrin revealed that all cells in these embryos were large and irregular. **F.** The merged image of these stains confirmed that all cells in these embryos were multinucleate, indicating that ectopic expression of *UAS-ect2* was not able to rescue cytokinesis in a *pbl* mutant background.


prd-gal4, pbl 2
UAS-myc-pbl, pbl 3
prd-gal4, pbl 2
UAS-ect2, pbl 3
A
D
B
E
C
F
Lamin
Spectrin
Lamin
Spectrin

enhancement of *GMR>pblΔDH* (Table 5-2). Although specific alleles are not yet available, a deficiency stock removing *IAL/auroraB* strongly suppresses the *GMR>pblΔDH* rough eye phenotype (Table 5-2). These results provide preliminary support for either of these kinases to regulate the activity of PBL during cytokinesis. However, it may also be that a modification other than phosphorylation, such as the subcellular localisation of PBL at different stages of the cell cycle, may regulate the activity of PBL.

6-3.4 Are PBL and ECT2 regulated differently during cytokinesis?

Our observations are also inconsistent with the possibility that PBL is regulated in the same way as that suggested for ECT2, whereby phosphorylation of ECT2 by CYC/CDK complexes was proposed to activate this RhoGEF (Tatsumoto et al., 1999). Our results suggest that other mechanisms downstream of CDK1 kinase activity are likely to be responsible for the regulation of PBL activity during cytokinesis. Alternatively it may be that PBL and ECT2 are regulated differently. It was shown that ectopic expression of ECT2 in a *pbl* mutant background was unable to rescue the cytokinetic defect. In addition, they have different cellular localisations which could explain the differences, as discussed previously in section 3-3.4.

Chapter 7: A genetic screen to identify novel components of Pebble signalling pathway(s)

7-1 Introduction

In this chapter, the *GMR>pbl* and *GMR>pblΔDH* rough eye phenotypes have been used as the basis for a dominant modifier screen in an attempt to identify novel components of PBL signalling pathways. A significant advantage of such a genetic approach is that no assumptions are made about the biochemical roles of components of the pathways. The effect of *GMR>pblΔDH* has been shown to be an inhibition of cytokinesis during eye and embryonic development. However, the effect of *GMR>pbl* does not seem to be a cytokinetic defect, rather a specific inhibition of apoptosis during pupal retinal development. Thus, the most relevant interactors identified with respect to cytokinesis would be expected to be those that modify *GMR>pblΔDH*. However, the opposite effect of *Rho1* mutations on the modification of both *GMR>pbl* and *GMR>pblΔDH* suggest that modifiers of *GMR>pbl* could still provide a counter screen for interactors identified by modification of *GMR>pblΔDH*. Alternatively, modifiers of *GMR>pbl* may provide insight into the downstream effects of ectopic PBL-activated Rho-GTP which may be involved in processes other than cytokinesis. A collection of deficiency stocks and *P*-transposon lines were screened to assess the feasability of this approach. A number of candidates were identified whose gene products may play a role in PBL signalling pathways.

7-2 Results

7-2.1 Deficiency stocks that interact genetically with *pbl*

Screening of deficiency collections allows large regions of chromosomes, covering multiple genes, to be tested for their ability to modify the rough eye phenotypes. One disadvantage of this approach is that the effects of one gene under that deficiency could mask the interaction of another. However, these data can give a rough idea of the number of interactors that are likely to be identified and hence determine the feasibility of a modifier screen. The second chromosome deficiency kit was screened for genomic regions that could dominantly modify either or both of the *GMR>pbl* and *GMR>pblΔDH* rough eye phenotypes (Table 7-1).

Only one of the deficiency stocks suppressed the *GMR>pbl* phenotype. This stock also enhanced the *GMR>pblΔDH* phenotype. A strong candidate gene in this region is *Rho1*, mutations in which have already been shown to have the same effect (Fig. 5-1). In contrast,

Table 7-1

Deficiency stocks that interact genetically with *GMR>pbl* and/or *GMR>pblΔDH*

Deficiency	Region deleted	Candidate gene	*GMR>pbl*	*GMR>pblΔDH*
Df(2R)Jp8	052F05-09;052F10-53A01	*RhoA*	S+++	E+++
Df(2L)net-PMF	021A01;021B07-08		E+	S+
Df(2L)al	021B08-C01;021C08-D	*aop, S*	E+	S++
Df(2L)sc19-8	024C02-08;025C08-09		E+	S+
Df(2L)J39	031C-D;032D-E	*cdc2, IAL*	E++	S+++
Df(2R)cn9	042E;044C	*raf*	E++	S+++
In(2R)bw	041A-B;042A02-03		E++	S+++
Df(2R)017	056F05;056F015		E+	S++
Df(2R)X58-12	058D01-02;059A	*cycB*	E+	S+++
Df(2L)C144,	023A01-02;023C03-05		no effect	S+++
Df(2L)cl-h3	025D02-04;026B02-05		no effect	S
Df(2L)Mdh	030D-30F;031F	*cdc2*	no effect	S++
Df(2L)H20	036A08-09;036E01-02	*grp*	no effect	S+
Df(2R)X1	046C;047A01	*14-3-3ζ*	no effect	S++
Df(2R)trix	051A01-02;051B06	*phyl*	no effect	S+++
Df(1)w67c23	028DE (within)		no effect	E+
Df(2L)N22-5	029D01-02;030C04-D01	*pelo*	no effect	E+
Df(2L)b87e25	034B12-C01;035B10-C01		no effect	E++
Df(2L)osp29	035B01-03;035E06	*cycE, gft*	no effect	E+
Df(2L)TW161	038A06-B01;040A04-B01	*Dia, DRhoGEF2*	no effect	E++
Df(2R)B5	046A;046C		no effect	E+
Df(2R)en30	048A03-04;048C06-08		no effect	E+
Df(2R)Jp1	051D03-08;052F05-09		no effect	E++
Df(2R)CX1	049C01-04;050C23-D02	*Dp, DRacGAP*	lethal	E++ (semi-lethal)
Df(2L)JS32	023C03-05;023D01-02		E++	no effect
Df(2L)N22-3	030A01-02;030D01-02		E+++	no effect
Df(2L)TW137	036C02-04;037B09-C01		E++	no effect
Df(2L)TW50	036E04-F01;038A06-07		E++	no effect
Df(2R)nap9	042A01-02;042E06-F01		E++	no effect
Df(2R)en-A	047D03;048B02		E+++	no effect
Df(2R)or-BR6	059D05-10;060B03-08		E+	no effect
Df(2R)Px2	060C05-06;060D09-10		E+	no effect
Df(2L)E110	025F03-026A01;026D03-11	*chic*	S++	S+++
Df(2L)J-H	027C02-09;028B03-04		E+++	E
Df(2L)30A-C	029F07-030A01;030C02-05		E+++	E+++
Df(2L)Prl	032F01-03;033F01-02		E++	E
Df(2L)prd1.7	033B02-03;034A01-02		E++	E
Df(2R)ST1	042B03-05;043E15-18	*raf*	E+	E
Df(2R)AA21	056F09-17;057D11-12		E+++	E
Df(2R)Pu-D17	057B04;058B	*DER*	E+++	E+
Df(2R)ES1	060E06-08;060F01-02		E	E

E+, E++, E+++; mild, medium and strong enhancement S+. S++, S+++; mild, medium and strong suppression

E; enhancement scored but strength not recorded, S; suppresion scored but strength not recorded

Table 7-1 (continued)

Deficiency stocks that did not interact genetically with *GMR>pbl* and/or *GMR>pblΔDH*

Deficiency	Region deleted	Candidate gene	*GMR>pbl*	*GMR>pblΔDH*
Df(2L)ast2	021D01-02;022B02-03		no effect	no effect
BL-627	25A4-5;25D5-7		no effect	no effect
Df(2L)spd	027D-E;028C		no effect	no effect
In(1)w[m4h]	028E04-07;029B02-C01		no effect	no effect
Df(2R)pk78s	042C01-07;043F05-08	*raf*	no effect	no effect
Df(2R)H3C1	043F;044D03-08	*pnut*	no effect	no effect
Df(2R)H3E1	044D01-04;044F12		no effect	no effect
Df(2R)stan2	046F01-02;047D01-02		no effect	no effect
Df(2R)vg135	049A-B;049D-E		no effect	no effect
Df(2R)vg-C	049A04-13;049E07-F01		no effect	no effect
Df(2R)Pcl7B	054E08-F01;055B09-C01		no effect	no effect
Df(2R)PC4	055A;055F		no effect	no effect
Df(2R)P34	055E02-04;056C01-11		no effect	no effect
Df(2R0X58-7	058A01-02;058E04-10		no effect	no effect
Df(2R)M60E	060E02-03;060E11-12		no effect	no effect

E+, E++, E+++; mild, medium and strong enhancement S+. S++, S+++; mild, medium and strong suppression

E; enhancement scored but strength not recorded, S; suppresion scored but strength not recorded

numerous deficiency stocks were identified as suppressors of the *GMR>pblΔDH* phenotype. The difference in the numbers of suppressors identified suggests that the *GMR>pblΔDH* rough eye could be closer to the phenotypic threshold resulting from ectopic expression and hence is quite sensitive to changes in the dosage of many other genes. However, some suppressors of *GMR>pblΔDH* also showed enhancement of *GMR>pbl*, suggesting they may be more likely to represent components of PBL signalling pathway(s). Some candidate genes uncovered by these deficiency stocks are also shown (Table 7-1).

A number of the deficiency stocks showed modification of one of the phenotypes but had no effect on the other. This is not unexpected due to the differences in the cellular basis of these phenotypes. An example of this is *Df(2L)TW161,* which resulted in an enhancement of *GMR>pblΔDH* but had no effect on *GMR>pbl.* A candidate gene uncovered by this deficiency is *diaphanous (dia),* which has independently been shown to be required for cytokinesis in *Drosophila* (Castrillon and Wasserman, 1994). Mutant alleles of *dia* resulted in enhancement of *GMR>pblΔDH*, while having no effect on *GMR>pbl* (Table 5-2 and Fig. 5-3H). Those deficiency stocks that showed enhancement (or suppression) of both phenotypes may result from non-specific effects, either directly on the level of GAL4 expression or indirectly on some unrelated aspect of eye development. Alternatively they may represent true genetic interactors of *pbl.* An example of this is *Df(2L)E110*, which deletes the candidate gene *chic.* Mutations in *chic* were independently found to weakly suppress both *GMR>pbl* and *GMR>pblΔDH* (Table 5-2). Thus, reducing the dosage of *chic*

appears to affect the dynamics of the actin cytoskeleton such that both *GMR>pbl* and *GMR>pblΔDH* rough eye phenotypes are suppressed.

7-2.2 *P*-element stocks that interact genetically with *pbl*

A more direct approach towards the identification of novel genes involved in PBL function relies on the generation of random mutations in single genes throughout the genome. The main advantage of screening *P*-induced mutations is that they are "tagged" such that the DNA flanking the point of insertion can be isolated and the sequence determined. Information provided by the analysis of the completed *Drosophila* genome sequence has allowed the rapid identification of the nearest gene that is the likely target of *P*-element disruption. One disadvantage is that insertion of these elements tends to be in 5' regulatory regions of genes where they may not decrease activity sufficiently to cause a dominant modification. A further significant disadvantage is that *P*-element mutant alleles are available for only approximately 20% of all essential genes, as a result of their preference for certain genomic regions. However, the availability of collections of *P*-element stocks and the relative ease of identification of the disrupted gene make them an attractive source of mutations for screening. A collection of 617 *P*-element insertion stocks was obtained from A. Spradling. This screen was carried out in collaboration with A. Harley, with the interactors from my half of the screen summarised in Table 7-2, the others are to be reported elsewhere (A. Harley, unpublished observations). Suppression of *GMR>pbl* was not observed with any of the 617 stocks tested (*Rho1* was not represented in these mutations). Many suppressors of *GMR>pblΔDH* were identified, five of which showed a strong suppression of the rough eye phenotype (almost to wild-type) and a further ten stocks showed a medium level of suppression. Two of these medium suppressors also showed a mild enhancement of *GMR>pbl.* Nine stocks were also identified as enhancers of *GMR>pblΔDH,* with no effect on *GMR>pbl.*

At the time this work was initiated, most of the *P*-element insertion stocks were not characterised and the disrupted gene had not been identified. As a result of continuing work from the Berkeley Drosophila Genome Project (Spradling et al., 1999) and the recent release of the complete Drosophila genome sequence (Adams et al., 2000), it has been possible to determine the exact point of insertion for most of these *P*-element stocks. In some cases the insertions were found to correspond to a gene for which an independent allele was available to confirm or discount the interaction. Deficiency stocks covering the point of insertion of the *P*-element were used for those genes where independent alleles were not available. Finally, in some cases the site of insertion of the transposon was not identified. In these cases, the genetic interactions could not easily be confirmed or discounted.

Table 7-2
P*-element suppressors of *GMR>pblΔDH

Gene	allele/Deficiency	*GMR>pbl*	*GMR>pblΔDH*
Enhancers of *GMR>pbl* and suppressors of *GMR>pblΔDH*			
hid	*l(3)05014* (embryonic lethal)	E+	S++
	W (hypomorphic, viable)	no effect	no effect
	Df(3L)H99	E+	S+++
stg	*l(3)05473* (lethal recessive)	E+	S++
	stg^4 (amorph)	no effect	E+
Strong suppressors of *GMR>pblΔDH*			
wun	*l(2)03497* (lethality not associated with *P* insertion)	no effect	S+++
	wun^{K10201} (lethal recessive)	no effect	S++
tws	*l(3)06848* (unknown phenotype)	no effect	S+++
	l(3)02414 (lethal recessive)	no effect	S++
l(3)05842	*l(3)06656* (unknown phenotype)	no effect	S+++
	l(3)05842 (lethal recessive)	no effect	no effect
?	*l(2)01462* (discarded)	no effect	S+++
?	*l(3)08050* (discarded)	no effect	S+++
Medium suppressors of *GMR>pblΔDH*			
14-3-3ζ	*l(2)07103* (hypomorph)	no effect	S++
	Df(2R)X1	no effect	S++
CG13880	*l(3)06240* (semi-lethal)	no effect	S++
	l(3)rH321 (lethal recessive)	no effect	no effect
CG7552	*l(3)07310* (lethal recessive)	no effect	S++
	l(3)02404 (lethal recessive)	no effect	no effect
CG12972	*l(3)04063* (lethal recessive)	no effect	S++
	Df(3L)Pc-MK	no effect	no effect
l(3)02733	*l(3)02733* (lethal recessive)	no effect	S++
	l(3)10619 (lethal recessive)	E+	S+++
E2F	*l(3)07172* (loss of function)	no effect	S++
	$E2F^{91}$ (loss of function)	no effect	E++
	$E2F^{RM729}$ (loss of function)	no effect	E+
hairy	*l(3)02537* (lethal recessive)	no effect	S++
	h^{22} (amorph)	no effect	S+
emc	*l(3)05592* (semi-lethal)	no effect	S++
	emc^1 (lethal recessive)	no effect	E+

E+, E++, E+++; mild, medium and strong enhancement
S+, S++, S+++; mild, medium and strong suppression

Table 7-3
P*-element enhancers of *GMR>pblΔDH

Gene	allele/Deficiency	*GMR>pbl*	*GMR>pblΔDH*
l(3)03691	*l(3)03691* (discarded)	no effect	E+
Mi-2	*l(3)01058* (semi lethal)	no effect	E+
	$Mi\text{-}2^{i3D4}$ (recessive lethal)	no effect	E+
l(3)07615	*l(3)07615* (recessive lethal)	no effect	E+
	Df(3L)Pc-MK	no effect	no effect
l(3)03806	*l(3)03806* (recessive lethal)	no effect	E+
mod(mdg4)	*l(3)07038* (recessive lethal)	no effect	E+
	l(3)03852 (recessive lethal)	no effect	no effect
	$mod(mdg4)^{L3101}$ (recessive lethal)	no effect	S++
ttk	*l(3)02667* (semi-lethal)	no effect	E+
	ttk^{lEI1} (loss of function, affects p69 and p88)	no effect	no effect
	ttk^{l} (loss of function, affects p69 only)	no effect	no effect/E+
?	*l(3)03733* (discarded)	no effect	E+
?	*l(3)06434* (discarded)	no effect	E+

E+, E++, E+++; mild, medium and strong enhancement
S+, S++, S+++; mild, medium and strong suppression

Of the fifteen stocks identified as suppressors of *GMR>pblΔDH,* independent alleles of *wunen (wun), twins (tws), l(3)02733* and *hairy(h)* confirmed these as genetic interactors of *pbl.* However, independent alleles of *string (stg), l(3)05842, CG13880, CG7552, E2F and emc* suggested that the original *P* insertions in these genes were not responsible for the observed modification. *head involution defective (hid)* is allelic to *Wrinkled (W).* Hypomorphic, viable *W* alleles did not show any modification but a deficiency stock covering *hid* did modify *GMR>pblΔDH,* providing support for the observed *P*-element interaction. Similarly, a deficiency stock covering *14-3.3ζ/leonardo(leo)* also provided evidence of a genetic interaction. However, a deficiency stock covering *CG12972* did not show the same genetic interaction as the *P*-transposon, suggesting these interactions might not be caused by the *P*-insertion. The genes disrupted by *l(2)01462* and *l(3)08050* have not been identified and thus their genetic interaction with *pbl* has not been confirmed.

Of the nine stocks identified as enhancers of *GMR>pblΔDH,* only *Mi-2* was confirmed by the interaction of an independent allele. Independent alleles of *CG7552, mod(mdg4)* and *tramtrack (ttk)* suggested the original *P*-insertions in these genes were not responsible for the observed modification. Similarly, deficiencies covering *CG12972* and *l(3)07615* did not show the same genetic interaction as the *P*-transposon, suggesting that these

interactions might not be due to the *P*-insertion. Deficiency stocks covering *l(3)03691* and *l(3)03806* still need to be tested for their ability to modify the rough eye phenotypes. The position of insertion, and hence the genes disrupted by, *l(3)03733* and *l(3)06434* have not been identified and thus their genetic interaction with *pbl* has not been confirmed.

7-3 Discussion

7-3.1 Screening of deficiency stocks

The *GMR>pbl* and *GMR>pblΔDH* rough eye phenotypes formed the basis of a preliminary study to determine their effectiveness for identifying novel genetic interactors of *pbl.* Screening of deficiency stocks was first used to determine the likely number of interactors present in the genome and assess the feasibility of the screen. A single suppressor of *GMR>pbl* was identified in this screen indicating that very few would be expected on a genome wide basis. However, fourteen suppressors of *GMR>pblΔDH* were identified, eight of which also showed enhancement of *GMR>pbl.* The coverage provided by the second chromosome deficiency stocks is approximately 22.5% of the total euchromatic DNA. This number suggests the total number of genetic interactors affecting either or both rough eye phenotypes would not be excessive. In addition to providing information on the numbers of modifiers, the deficiencies could be used to test candidate genes, some of which are shown in Table 7-1, for their ability to modify the rough eye phenotypes.

7-3.2 *P*-element screen

P-element insertion stocks were used to identify specific genes, mutations in which modified either or both of the rough eye phenotypes. As expected, multiple suppressors of *GMR>pblΔDH* were identified, two of which were also enhancers of *GMR>pbl.* Only one of these, *head involution defective (hid)*, was supported as a possible true genetic interactor of *pbl* by the analysis of a deficiency stock covering this gene. In the absence of independent null alleles, the best way to determine the validity of the interaction would be to generate *P*-element revertants and test for reversion of the genetic interaction. *P*-element reversion could also be used to generate deletion mutants, providing stronger alleles for further study. *hid* is required for cell death during *Drosophila* development (Grether et al., 1995). Given the anti-apoptotic effect observed with *GMR>pbl,* it is not surprising that reducing the dosage of a gene required for this process would have a more severe effect, resulting in an enhancement of *GMR>pbl.* A similar enhancement of *GMR>pbl* was also observed with *Df(3)3LH99* (Fig. 4-6G). This stock removes *hid* in addition to *grim* and *reaper,* all three of which are known to be involved in apoptosis during *Drosophila* development. The observed suppression of

GMR>pblΔDH suggests either that apoptosis of multinucleate cells in these eye discs could be responsible for decreasing cell numbers or alternatively that PBL normally plays a role during apoptosis. However these possibilities have not been tested directly.Of the strong suppressors of *GMR>pblΔDH, twins (tws)* and *wunen (wun)* appeared to be true genetic interactors of *pbl* as confirmed by independent alleles. The *tws* locus was first described as *abnormal anaphase resolution (aar),* which was shown to be required for progression through anaphase in syncitial and late larval divisions (Gomes et al., 1993). *P*-element mutations in the same locus resulted in mirror image duplications of imaginal discs (Uemura et al., 1993). *aar/tws* has since been shown to encode a protein phosphatase type 2A (PP2A) regulator (Spradling et al., 1999). In yeast and mammalian systems, PP2A negatively regulates CYC-B/CDK1 activity and has been shown to play multiple roles during cell growth and in many cellular signalling pathways (see review by Janssens and Goris, 2001). The effects of CYC-B/CDK1 on the regulation of PBL activity have already been investigated (see chapter 6). These results suggest that negative regulation of PBL could be affected indirectly by the activity of CYC-B/CDK1 complexes. However, the direction of the observed interaction with *tws/PP2A* was the same as that observed with *cdk1* and *cycB* or *cycB3* (Fig. 6-1), which would not be expected if PP2A acts as a negative regulator of CYC-B/CDK1. In addition, PP2A in *Xenopus* has been shown to act antagonistically with Polo kinase in the regulation of CYC-B/CDK1 auto-amplification (Karaiskou et al., 1999). However, again the modifications of *GMR>pblΔDH* observed with *tws/PP2A* and *polo* (Table 5-2) are in the same direction, which would not be expected if the same regulatory relationship of these genes were true in flies. This antagonistic regulation of CYC-B/CDK1 by PP2A and Polo occurs through their opposing activities on CDC25, a phosphatase that activates CYC-B associated CDK1. The *string (stg)* gene encodes the *Drosophila* CDC25 phosphatase and, although a *P*-element mutation in *stg* originally showed suppression of *GMR>pblΔDH,* a null allele for *stg* had the opposite effect, resulting in a mild enhancement of *GMR>pblΔDH.* Thus, while the genetic interactions observed between *pbl* and many components of the complex array of mitotic regulatory signals suggest PBL could be involved in these pathways, a clear mechanism cannot be invoked from these results.

The second of the confirmed interactors is *wun* which encodes phosphatidate phosphatase, a lipid phosphatase located at the plasma membrane (Zhang et al., 1996; Zhang et al., 1997). Mutations in *wun* were first identified as a result of germ cell migration defects, where germ cells failed to make appropriate cell extensions and respond to repulsive cues necessary for their correct guidance to reach the gonad. It was subsequently determined that there was an additional gene, *wunen2 (wun2)* located adjacent to *wun,* and that the products of

these two genes were redundant with respect to their function during germ cell migration (Starz-Gaiano et al., 2001). Additional alleles to those used in this study, affecting both *wun* and *wun2* are available and should be tested in this genetic assay (Starz-Gaiano et al., 2001). Analysis of the zygotic mutant phenotype of these alleles did not reveal a requirement during cytokinesis. However it would be necessary to remove the maternal contribution by the generation of germ-line clones to determine whether cytokinesis is affected in the absence of both of these gene products.

These candidate interactors encoding ser/thr (*tws)* and lipid (*wun*) phosphatases could be involved in counteracting the activity of unknown ser/thr and lipid kinases acting as downstream effectors of Rho-GTP (Fig. 1-5). This is supported by the nature of the interactions observed. Decreasing the activity of a phosphatase that would normally counter the activity of a kinase downstream of Rho-GTP would result in increased kinase activity. This could result in suppression of the *GMR>pblΔDH* rough eye phenotype. Thus, there is a clear mechansim by which these phosphatases could be acting in the same pathway as PBL. No candidates for such kinases were identified as enhancers of *GMR>pblΔDH* in this initial screen. Further genetic analysis may lead to their identification as enhancers of *GMR>pblΔDH.* An alternative approach could be to screen *EP* collections to determine gene products which, when over expressed, could result in an enhancement of *GMR>pblΔDH* and/or suppression of *GMR>pbl.*

Of the medium suppressors of *GMR>pblΔDH; 14-3.3ζ, l(3)02733* and *hairy (h)* appeared to be true genetic interactors of *pbl. 14-3.3ζ*or *leonardo (leo),* encodes a tryptophan hydroxylase activator which has been shown to be required for the negative regulation of RAS small GTPase signal transduction by binding directly to Raf kinase (Rommel et al., 1997). *leo* is also required during memory and learning (Broadie et al., 1997). *hairy (h)* encodes a specific RNA polymerase II transcription factor which is known to act as a transcriptional repressor (Barolo and Levine, 1997; Carroll and Whyte, 1989). Thus, the medium suppressors of *GMR>pblΔDH* identified candidate genes that could be more indirect in their genetic interactions with *pbl,* either by modifying cross-talk between Rho and Ras signalling pathways, or by regulating transcription of a component required for PBL signalling. The insertion point of *l(3)02733* is 5023 bp 3' to *hus1-like,* a gene required for DNA repair (Komatsu et al., 2000a) and 1353 bp 5' to *CG14656,* which encodes an unknown gene with no significant homology to any known proteins. Thus, it is unclear which of these genes is being affected by this insertion. Identification of the insertion point of *l(3)10619,* which fails to complement *l(3)02733* and showed modification of both *GMR>pbl* and *GMR>pblΔDH,* would help to distinguish between these possibilities.

The *P*-element enhancers of *GMR>pblΔDH* identified in this screen were weak. From the testing of independent alleles or deficiency stocks to date, *Mi-2* appeared to be the only one that could represent a true genetic interactor of *pbl*. *Mi-2* encodes a DNA binding, ATP dependent helicase found in the nucleus and required for chromatin remodelling (Brehm et al., 2000). The weakness of the *GMR>pblΔDH* enhancers could reflect the hypomorphic nature of these mutations or the lesser role of the genes represented in this study, since a much stronger enhancement of *GMR>pblΔDH* was generated by the other interactors.

7-3.3 Future Screens

The 617 *P*-element stocks represented mutations in 499 genes (157 on II and 342 on III (Spradling et al., 1999). The total collection of *P*-element mutations currently available covers 1052 of the 3600 *Drosophila* genes that are essential for adult viability. Screening the remainder of these *P* stocks would yield few, if any, suppressors of *GMR>pbl* and multiple suppressors of *GMR>pblΔDH,* based on the number of interactors identified in this pilot screen. Also of concern is the significant number of cases where the modification observed with a *P*-transposon was not verified by a null mutation in the same gene. A better approach for the identification of interactors would be to screen mutations generated by chemical methods, which generally result in stronger mutations and would allow screening to saturation. At the time this work was initiated, a major disadvantage in this approach was the difficulty in identifying the gene disrupted by such mutations. This is no longer such an obstacle due to the development of genome wide polymorphic markers available for precise mapping of these mutations (Louis et al., 1997). Screening in this way for suppressors of *GMR>pbl* should yield only a few interactors, but should allow downstream interactors, in addition to *Rho1* and *Rok*, to be identified. Enhancement of *GMR>pblΔDH* has been observed with null mutations in a number of genes previously known to be required for cytokinesis; *Rho1, dia* and *pav* (Fig. 4-3C,H,M and Fig. 4-4E,F). Identification of stronger enhancers of *GMR>pblΔDH* than those identified in the *P*-element screen could be a good way to identify new genes interacting with *pbl* during cytokinesis.

In addition to improving the quality of the mutations screened, it would also be ideal to have a secondary screen against any non-specific interactors, in order to concentrate on those specifically required during cytokinesis. One way to do this would be to generate dsRNA of candidate interactors and perform RNAi on *Drosophila* tissue culture cells. This has been shown to be a rapid and successful means of knocking out gene function of *pbl* and other genes, required for cytokinesis (G. Somers. Pers. Comm).

Chapter 8: Final Discussion

8-1 Introduction

In this thesis the role of PBL as a regulator of cytokinesis has been explored. Initially this involved characterisation of the function of PBL during cytokinesis. Further experiments were performed to study the effects of ectopic expression of various forms of wild-type and mutated *pbl* cDNA constructs. The resultant adult rough eye phenotypes then formed the basis of a screening system for genetic interactors, towards identifying components of PBL signalling pathways during cytokinesis, and possibly, other cellular processes.

8-2 The role of PBL during cytokinesis

pbl encodes a RhoGEF and is required for cytokinesis in *Drosophila* (Prokopenko et al., 1999). In this study, it was shown that the RhoGEF activity of PBL is acting predominantly through the activation of Rho1. Also, that the downstream signalling of PBL-activated Rho1 could involve the activation of Diaphanous (DIA) and therefore regulation of actin dynamics, in addition to the activation of Rho-kinase which both directly, and indirectly, activates myosin (see Fig. 5-4). We propose that DIA and Rho-kinase could represent key components of signalling pathways necessary for the cytoskeletal reorganisation events during contractile ring formation and/or function.

A novel requirement for PBL function during the later stages of cytokinesis was identified in these studies. Previous work using fixed tissue had shown that the contractile ring failed to form in dividing cells of *pbl* mutant embryos. However, real-time imaging revealed that *pbl* mutant cells initiate but cannot complete constriction, resulting in a failure of cell division. In addition, it was determined that the central spindle can form in *pbl* mutant cells. This is in agreement with the co-dependency that has been observed between central spindle formation and contractile ring function in various systems (Gatti et al., 2000). Thus, it is possible that PBL activity could be required at a later stage in cytokinesis than originally thought and may play a role in continued reorganisation of the actin cytoskeleton as constriction of the contractile ring proceeds, rather than an early role in formation of the contractile ring. However, this must be qualified since there may be some maternally deposited PBL present in these cells which is sufficient to organise the central spindle region and initiate contractile activity. This could be resolved by repeating these experiments following depletion of maternal PBL, which could be achieved by injection of embryos with antibodies directed against PBL prior to time-lapse video recordings.

Antibodies generated against PBL were used to analyse the distribution of the protein in fixed tissue. Initial studies identified a dynamic cell cycle dependent localization pattern with the most prominent staining being a nuclear accumulation of PBL during telophase. However, this occurred at a stage that would be too late to give an instructive signal for cytokinesis. After careful observation of cells at late stages of anaphase/early telophase, a much weaker accumulation of PBL was observed at cortical regions of these dividing cells. This cortical localisation of PBL would be consistent with a role in reorganisation of the actin cytoskeleton at the time of cytokinesis. However, it is still unclear if this dynamic localisation results from a pool of PBL that shuttles between the nucleus and cytoplasm at different stages of the cell cycle, or whether PBL is destroyed and resynthesized before each division. Post-transcriptional processing could play a role in such a form of regulation, as suggested by the dramatic phenotypes observed when 5'UTR sequences were deleted from the *pbl* cDNA ectopic expression constructs.

Clearly PBL plays a role during cytokinesis, but does PBL function prior to the cycle 14 divisions? PBL is provided maternally, with strong nuclear staining observed during syncitial and pole cell divisions (Prokopenko et al., 2000). Is PBL required for the actomyosin dependent processes that organise the cytoplasm surrounding the syncitial divisions? Furthermore, is PBL required for the actomyosin dependent processes involved in membrane invagination during cellularisation? An attempt was made to answer these questions by the generation of germline *pbl* mutant clones. However, this approach revealed a requirement for PBL function during oogenesis, preventing the analysis of these early processes in the absence of *pbl* activity. Decreasing the amount of PBL in early embryos by the injection of antibodies directed against PBL, or the use of RNA interference for *pbl* (Carthew, 2001) would allow these questions to be addressed.

8-3 Is there a nuclear role for PBL?

A major unresolved question is whether there is a specific nuclear role for PBL or whether this nuclear localisation is simply a mechanism to remove it from the cytoplasm of cells while they are not dividing to prevent ectopioc cytokineses. There is some preliminary evidence for a requirement for PBL activity in response to DNA damage (A. Harley Pers. Comm.). This would be consistent with the presence of the conserved BRCT domains in PBL, which are found in other proteins that play a role in DNA repair pathways. However, whether this activity is dependent on an interaction with a Rho family member, and whether such a role in DNA repair would be independent of cytokinesis, remains to be determined. There is no yeast protein that contains the DH/PH domains (characteristic of GEF proteins) together with

the BRCT/RadECl domains, but there are clear orthologues of PBL in *C. elegans* and mammals. Thus a nuclear role, possibly in response to DNA damage, that is co-ordinated with cytokinetic function could represent a specialisation of metazoan cells.

Although there is no orthologue of *pbl* in *S. cerevisiae*, *cdc24* encodes a GEF (without RadECl/BRCT domains) for Cdc42p that is required for actin-dependent processes and shows a strikingly similar pattern of localization to PBL (Toenjes et al., 1999). Cdc24p is localised to the plasma membrane at polarized growth sites, the mother-bud neck to regulate actin cytoskeletal reorganisation events, and to the nucleus during G1 phase. Cdc24p is sequestered in the nucleus, by binding to the cyclin dependent kinase inhibitor, Far1p, and nucleocytoplasmic shuttling of Cdc24p has been shown in response to different stimuli (Nern and Arkowitz, 2000). Entry into mitosis triggers degradation of Far1p and export of Cdc24p from the nucleus to localise at the developing mother-bud neck. However during G1 phase, in response to pheromone, pre-formed complexes of Far1p and Cdc24p are exported from the nucleus and localise to sites of polarized cell growth.

A similar regulated mechanism of nucleocytoplasmic shuttling in response to a cell cycle regulatory signal may be required for the correct localisation of PBL to the plasma membrane for activation of Rho GTPases at the time of cytokinesis. However, Far1-like proteins have not yet been identified in other species.

8-4 Ectopic expression of PBL and PBLΔDH

Ectopic expression of PBL had no detectable effect during embryogenesis, suggesting that PBL is not rate limiting in the regulation of cytokinesis. However, ectopic expression of PBL during eye development did result in increased numbers of bristle and pigment cells, resulting in a rough eye phenotype. The extra cells observed resulted from an inhibition of some apoptoses in the developing pupal retina, rather than an effect on cytokinesis. It is not clear whether this is a direct effect, where PBL is interacting in an apoptotic pathway, or a secondary consequence of an inability of cells to undergo cell shape changes such that they can no longer move into the regions where apoptosis is occurring. A direct effect in an apoptotic pathway could be tested, in the first instance, by testing for genetic interactions between *pbl* and mutants of components of these pathways. The possibility of a more indirect effect could be tested, by performing time-lapse video recordings on retinas ectopically expressing PBL in the presence of a membrane GFP marker. This would allow these cells to be followed in real-time to determine whether they are able to undergo the cell shape changes necessary for movement to regions where cell death is occurring during retinal development (C. Brachmann. Pers. Comm).

Ectopic expression of PBLΔDH (which is defective for GTP exchange) resulted in decreased cell numbers during embryogenesis, as a direct result of an inhibition of cytokinesis. Ectopic expression during eye development also resulted in decreased numbers of cells and a rough eye phentoype which was distinct from the roughening observed following ectopic expression of PBL. The decrease in cell numbers in the eye was shown, at least in part, to result from an inhibition of cytokinesis. Thus, this mutant form of PBL was able to act in a dominant negative manner to specifically inhibit cytokinesis in both the eye and embryonic tissues. Ectopic expression of PBLΔDH during eye development also resulted in a rough eye phenotype.

8-5 Genetic dissection of PBL signalling pathways

Although ectopic expression of PBL and PBLΔDH resulted in rough eye phenotypes, and had opposite effects on bristle and pigment cell numbers, the phenotypes were not the result of opposite effects on cytokinesis. Despite the differences in the cellular bases of these rough eyes, each of the phenotypes appeared to arise by an effect on Rho1 activity and not on other members of the Rho family. This specificity between PBL and Rho1 was also observed by direct interaction in a yeast two hybrid system (Prokopenko et al., 1999).

The *GMR>pbl* and *GMR>pblΔDH* rough eyes provided the basis for testing genetic interactions of *pbl* with the aim being to identify genes encoding other components of PBL signalling pathways. A number of candidate genes required for cytokinesis or components of signalling pathways downstream of Rho1 were tested for their genetic interaction in this system. These interactions suggested a mechanism for PBL-activated Rho signalling during contractile ring formation and function (see Fig. 5-4). Activation of Diaphanous could be responsible for the actin reorganisation events necessary for contractile ring formation and possibly for the continued reorganisation that would be required as constriction progresses. Also, the activation of Rho-kinase could result in myosin activation at the contractile ring. However, this can not be an essential function since a cytokinetic defect was not observed in clones of *rok* from *Drosophila* (Winter et al., 2001). In addition to actin and myosin, there are many other contractile ring components. PBL may likewise be responsible for their localisation and activation, through the activation of Rho1, or other, as yet uncharacterised, Rho family members. Some of these could be identified using the genetic system described in this thesis.

8-6 PBL is not directly regulated by CDK1 phosphorylation

There is ample evidence that regulators of mitosis can also regulate cytokinesis (Nigg, 2001). Genetic interactions between *pbl* and genes encoding the mitotic regulators, *cdk1, cycB* and *cycB3,* identified in this study, lend further support to this possibility. *In vitro,* studies suggest that ECT2, the mammalian orthologue of PBL, is directly regulated by phosphorylation (Tatsumoto et al., 1999). CDK1 was suggested as the best candidate to carry out his phosphorylation, and this is supported by the presence of a conserved CDK1 phosphorylation site in PBL and ECT2. To determine whether the genetic interactions were indicative of a direct biochemical interaction, the *in vivo* consequences of inactivation or constitutive activation of potential CDK1 phosphorylation sites in PBL were investigated. However, it was found that regulation of PBL activity does not occur via direct phosphorylation of these CDK1 sites. This might also suggest that ECT2 activity, likewise, is not regulated directly by CDK1 phosphorylation. However, differences were observed between PBL and ECT2, which should caution against any conclusions made between the two proteins. For example, the subcellular localization of PBL is different to that of ECT2 during cell division. Differentially phosphorylated forms of PBL were not detected in this work while they have been previously described for ECT2, although this analysis is far from conclusive. Also, ECT2 failed to rescue cytokinesis in *pbl* mutant embryos, suggesting that PBL and ECT2 may behave differently in dividing cells. This observation must be qualified however, by saying that it has not been demonstrated whether ECT2 was being expressed in this assay system.

Although it was shown that PBL is not directly regulated by CDK1 phosphorylation, the genetic interactions observed between *pbl* and *cdk1* and *pbl* and *cycB* and/or *cycB3,* would suggest that they could be indirectly regulated by genes that regulate mitosis. Again, some of these genes could be identified using the genetic system described in this thesis.

8-7 A dominant modifier screen using *GMR>pbl* and *GMR>pblΔDH*

The rough eye phenotypes generated by ectopic expression of PBL and PBLΔDH were used as the basis for a genetic screen to identify novel genetic interactors of *pbl.* Two interactors encoding known genes, *wunen* and *PP2A,* were the most promising candidate interactors identified in this pilot screen. These encode a phosphatidate phosphatase and a ser/thr phosphatase respectively. These phosphatases clearly could act in opposition to kinases activated downstream of PBL-activated Rho1 signalling. The identity of these kinases are unknown at present, but a more thorough genetic analysis could allow their identification. In terms of WUN and PP2A function, it remains to be determined whether defects in cytokinesis

arise in embryos from which the maternal and zygotic contributions of these gene products have been removed.

Independent alleles of a third interactor, *l(3)02733/l(3)10619* were also identified, but it is not yet clear which of two transcripts adjacent to *l(3)02733* is being affected. One transcript corresponds to *hus1-like,* a gene required for DNA repair (Komatsu et al., 2000a). This would be an ideal candidate for a PBL interactor that could play a nuclear role. However, in the absence of specific alleles of *hus1-like* this remains to be confirmed. The second transcript corresponds to *CG14656,* which encodes an unknown gene with no significant homology to any known proteins. This would be the more likely of the two candidates, based in the position of insertion of *l(3)02733.* However, determination of the insertion position of *l(3)10619* by inverse PCR would help to clarify this. The identification of independent alleles of this locus strongly supports the argument that the gene affected represents a true genetic interactor of *pbl.*

Although some promising interactors were identified in this pilot screen of *P*-element induced mutations, it is clear that this collection of *P*-elements was not the best source of mutations for a dominant modifier screen. Independent, often null, alleles failed to show the same interaction as the original *P*-element insertion, suggesting that alleles independent of the *P*-insertion alleles were responsible for the genetic modifications. A better approach for future screens could be to use collections of confirmed *P*-element stocks, where reversion of the *P*-element insertion resulted in reversion of the lethality. Alternatively, chemically induced mutations could be used to screen only for strong interactors of either or both of the rough eye phenotypes. Of significant interest would be the identification of suppressors of *GMR>pbl* since so few suppressors were identified in this screen. Although these would not necessarily identify components of PBL signalling pathways during cytokinesis, downstream components of PBL-activated Rho signalling pathways could provide valuable insight into other roles for PBL. It would also be ideal to incorporate a secondary screen, such as using RNA interference on interacting gene on tissue culture cells (Clemens et al., 2000). This would provide a quick and efficient confirmation of whether any suppressors of *GMR>pbl,* and/or enhancers of *GMR>pblΔDH* play a role during cytokinesis.

8-8 Summary

The RhoGEF activity of PBL was shown to be acting predominantly by the activation of Rho1 and downstream signalling pathways required for contractile ring function during cytokinesis. Genetic evidence suggests this could be through the activation of Diaphanous (a FH protein) to reorganise the actin cytoskeleton, as well as through the activation of Rho-

kinase which results in the phosphorylation, and activation, of myosin. This work has also provided novel insights into the role of PBL during cytokinesis, particularly highlighting a possible role during contractile ring function that is at a later stage than previously thought. Genetic interaction screens were employed to identify regulators of PBL activity during cytokinesis. CDK1 was identified genetically as a candidate for regulating PBL activity, but functional studies *in vivo* showed that this regulation was not by direct phosphorylation of the PBL consensus CDK1 sites tested. Further screening using the system described in this study has identified other possible components of PBL signalling pathways, but a role during cytokinesis for these interactors remains to be confirmed. The eye phenotypes described in this thesis provide ideal systems for the identification of components of PBL signalling pathways in *Drosophila.* The high level of conservation in the mechanism of cytokinesis from yeast to mammals would also suggest that interactors identified in these studies would most likely represent components of cytokinesis pathways in all eukaryotes.

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Amendments to Thesis

Typographical errors:

Page 19: section 1-7.1

"lamellopodia" should be "lamellipodia" in several sentences

Page 19: section 1-7.2

"althgough" should be "although"

Page 26: section 1-7.6

"duirng" should be "during"

Reference errors:

Page 21: section 1-7.3

Clostridium botulinum C3 ribosyltransferase toxin specifically inactivates Rho by ADP ribosylating its Asn_{41} residue, as described in Sekine A., et al. (1989) "Asparagine residue in the rho gene product is the modification site for *botulinum* ADP-ribosyltransferase". J Biol Chem. *264(*15):8602-5.

Page 45: section 2-2.21

Transverse eye sections were performed as described in Tomlinson A. and Ready, D.F. (1987). "Cell fate in the *Drosophila* ommatidium". Dev. Biol. *123:* 264-275.

Page 122: section 7-3.2

aar/tws encodes a protein phosphatase type 2A (PP2A) regulator, as first shown by Mayer-Jaekel R.E. et al., (1993). "The 55 kd regulatory subunit of *Drosophila* protein phosphatase 2A is required for anaphase" Cell. *72*(4):621-33.

Clarity of statement

Page 5: last paragraph of section 1-2.2

Female meiosis occurs in the absence of prominent asters however, female meiosis does not require cytokinesis and hence lends no support to the argument that it is the central spindle that acts as the origin of the cytokinetic stimulus rather than the asters.