Identification of an adaptor for the *Drosophila* Ciass I_A phosphoinositide 3-kinase and the study of its function *in vivo* and *in vitro*

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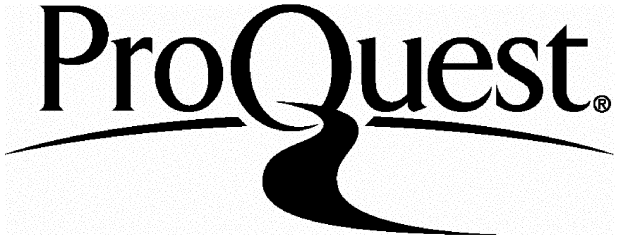

Abstract

Class I_A phosphoinositide 3-kinases (PI3Ks) regulate several cellular processes in response to receptor tyrosine kinase (RTK) activation. This response is mediated by the SH2 domain-containing adaptors for Class I_A PI3Ks, which, by binding to phosphotyrosines on activated RTKs or their substrates, bring Class I_A PI3Ks close to their lipid substrates at the membrane. This thesis describes the identification of p60, the adaptor for the *Drosophila* Class I_A PI3K, Dp110. p60 was isolated by affinity purification with a phosphopeptide derived from a human RTK, and the corresponding cDNA was cloned using peptide sequence data. Like the mammalian Class I_A PI3K adaptors, p60 possesses two SH2 domains, which enable receptor binding, and an inter-SH2 domain, which facilitates Dp110 binding. Biochemical analyses showed that the Dp110/p60 complex is widely expressed and possesses lipid and protein kinase activity. After determining the genomic structure of the *p60* region and characterising candidate mutations, two *p60* mutants were generated. Zygotic *p60* mutants, like *Dp110* mutants, showed a lethal phenotype resulting from defective larval development. Clones of *p60* or *Dp110* mutant cells survived and proliferated during imaginal disc development, but were reduced in both size and number. To address its *in vivo* function further, p60 was ectopically expressed in eye and wing imaginal discs. Previously, it was shown that ectopically-expressed Dp110 promotes imaginal disc growth. In contrast, p60 ectopic expression resulted in small wings and eyes. Expressed together, Dp110 and p60 had little effect on growth, even though the proteins were present at higher levels when coexpressed than when expressed alone. Together, these results demonstrate a specific function for Dp110/p60 in imaginal disc development. Models for the regulation of Dp110 by p60 and the control of organ growth by Dp110/p60 are proposed, and their implications for mammalian Class I_A PI3K function are discussed.

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Chapter 1: Introduction

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1.1 Overview

Communication between cells is essential for the development and viability of multicellular organisms. Intercellular communication is often achieved by one cell producing extracellular ligands that activate receptors on the surface of another cell. Activation of these receptors generates intracellular signals that modify the behaviour of the recipient cell. Thus, intracellular signalling is vital to the development and function of an organism and its disruption is a key factor in the pathogenesis of many diseases, including cancer.

The experiments described in this thesis investigate the function of Class I_A phosphoinositide 3-kinases (PI3Ks). These lipid kinases have been shown to respond to stimulated receptors and regulate several cellular processes including proliferation, protection from apoptosis, glucose metabolism, protein synthesis, membrane trafficking and cytoskeletal rearrangements. Many of the assigned functions of Class I_A PI3Ks have been inferred from the function of their upstream activators and downstream effectors, which will be described in Sections 1.3 and 1.4 before Class I_A PI3K function is addressed directly in Section 1.5. Most of the studies of Class I_A PI3Ks have not dealt with the consequences of Class I_A PI3K activation during the development and function of multicellular organisms. However, recent studies in *C. elegans*, *Drosophila* and mice provide interesting insights into Class I_A PI3K function and will be reviewed in Sections 1.6 and 1.7. Finally, in this thesis, Class I_A PI3K function is investigated in the developing *Drosophila* imaginal disc. Therefore, the development of imaginal discs and the cellular processes that might be regulated by Class I_A PI3Ks during imaginal disc development will also be discussed (Section 1.8).

1.2 The three classes of phosphoinositide 3-kinases

PI3Ks are specific lipid kinases that phosphorylate the 3' hydroxyl of the inositol ring of phosphoinositides (Stephens *et al.* 1993). All PI3Ks identified to date, with the exception of the PI3Ks in *Dictyostelium* (Zhou *et al.* 1995), have been grouped into three separate classes on the basis of their amino acid sequence and *in vitro* lipid substrate specificity (Figure 1.1, Vanhaesebroeck *et al.* 1997a). *In vitro*, Class I PI3Ks are able to produce phosphatidylinositol 3-phosphate (PtdIns(3)P) from phosphatidylinositol (PtdIns), PtdIns(3,4)P_2 from

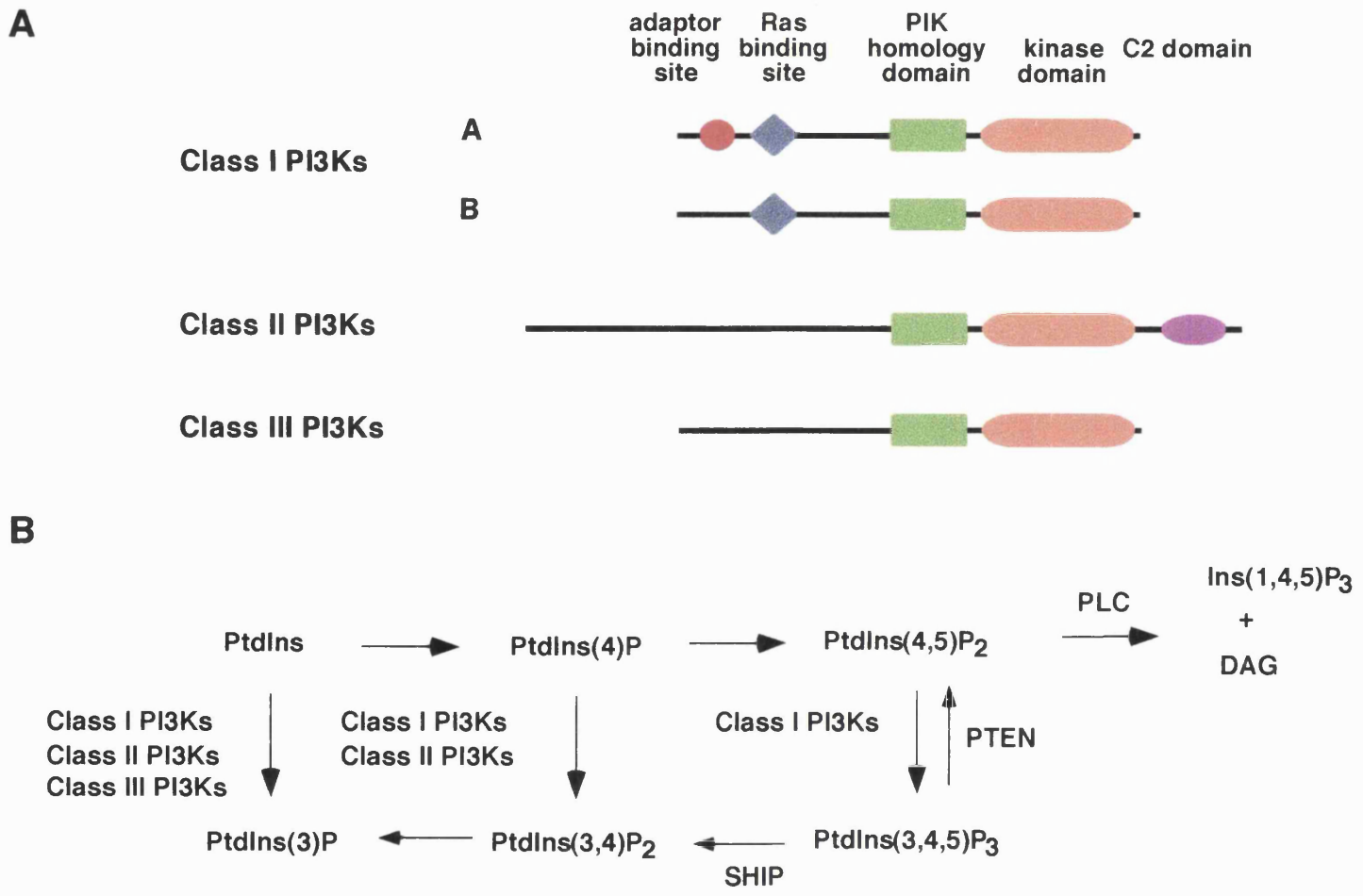


Figure 1.1. The three classes of PI3Ks and their roles in lipid metabolism. (**A**) Comparison of the domain structures of the three classes of PI3Ks, adapted from Vanhaesebroeck *et al.* 1997a. (**B**) A schematic showing pathways of phosphoinositide lipid metabolism that are revelant to the functions of PI3Ks and other proteins discussed in this thesis.

PtdIns(4)P, and PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2. In contrast, Class II PI3Ks are only able to produce PtdIns(3)P and PtdIns(3,4)P_2 (MacDougall *et al.* 1995; Domin *et al.* 1997; Arcaro *et al.* 1998) and Class III PI3Ks are only able to produce PtdIns(3)P (Schu *et al.* 1993; Volinia *et al.* 1995).

PI3Ks have been further classified on the basis of their binding partners and their proposed method of activation. Class I_A PI3Ks have been found in complex with src homology 2 (SH2) domain-containing adaptors, allowing them to be responsive to receptor tyrosine kinase (RTK) stimulation (Section 1.3). Class I_B PI3Ks, on the other hand, have been found in association with a 101 kD protein and have been shown to be stimulated by G-protein coupled receptors (Stoyanov *et al.* 1995; Stephens *et al.* 1997) and Class III PI3Ks have been shown to bind to a serine/threonine kinase (Panaretou *et al.* 1997). In contrast, no binding partners have yet been identified for Class II PI3Ks, and their method of activation has yet to be established, though there have been some suggestions that Class II PI3Ks and Class I PI3Ks can be activated by the same stimuli (Figure 1.1, Turner *et al.* 1998; Arcaro *et al.* 1999). Interestingly, all Class II PI3Ks possess a common carboxy-terminal (C-terminal) domain that is termed a C2 domain because of its homology with a family of domains that includes the 2nd conserved domain of the protein kinase C (PKC) family. The C2 domain family also includes the Ca^{2+} binding domain of synaptotagmin, a protein that mediates secretion at synaptic junctions. However, many C2 domains, including those of the Class II PI3Ks, lack the critical aspartate residues necessary to bind Ca^{2+} (Rizo and Sudhof 1998), and no function has yet been ascribed to the C2 domains of Class II PI3Ks.

PI3Ks from each of the above classes have been identified in several different species. Furthermore, in many species, more than one member of each class is present. Thus, so far it appears that mammals possess three Class I_A PI3Ks (p110α, β and δ), one Class I_B PI3K (p110γ), three Class II PI3Ks (PI3KC2α, β [possibly not in mice] and γ) and one Class III PI3K (Wymann and Pirola 1998). With the possible exception of the PI3Ks discovered in the slime mould *Dictyostelium* (Zhou *et al.* 1995), which are hard to classify by the scheme described above, Class I and II PI3Ks have only been found in metazoan organisms. The completed genome sequence of *Saccharomyces cerevisiae* reveals that the Class III PI3K, Vps34p, is the only PI3K in budding yeast (Mewes *et al.* 1997). In contrast, the completed genome sequence of *Caenorhabditis elegans* reveals one Class I_A PI3K, one Class II PI3K and one Class III PI3K (Ruvkun and Hobert 1998, Section 1.6). Similarly, only one PI3K from each class has been identified in *Drosophila* (Section 1.7, Figure 1.7).

The kinase domains of all PI3Ks are related in sequence to the kinase domains of all serine/threonine (S/T) protein kinases (Carpenter and Cantley 1998), and all PI3Ks tested so far display S/T protein kinase activity *in vitro.* Comparison of the amino acid sequences of the PI3Ks with the sequences of other kinases has allowed the PI3K family to be placed in a larger kinase superfamily (Hunter 1995). This family also includes the PI 4-kinase family as well as a family of high molecular weight protein kinases that includes the ataxia telangiectasia gene product (ATM), the DNA-dependent protein kinase (DNA-PK) and the targets of [the immunosuppressant] rapamycin (TORs, also called FRAP for FKBP12-rapamycin associated protein and RAFT for rapamycin and FKBP12 target).

1.3 PI3Ks may mediate many of the functions of receptor tyrosine kinases

RTKs have been shown to stimulate many cellular processes upon extracellular stimulation including differentiation, mitogenesis, changes in metabolic activity and cytoskeletal rearrangement (van der Geer *et al.* 1994). Early experiments demonstrated that the intracellular levels of PtdIns(3,4,5)P_3 increase rapidly when cells are treated with ligands that stimulate RTKs or with other classes of ligand that stimulate receptors coupled to intracellular tyrosine kinases or G-proteins (Auger *et al.* 1989; Stephens *et al.* 1993). In contrast, increases in PtdIns(3,4)P_2 levels occur more slowly and to a lesser extent than the increases in PtdIns(3,4,5)P_3 levels following receptor stimulation. Interestingly, the levels of PtdIns(3)P remain unchanged *in vivo* after receptor stimulation. Taken together, these discoveries suggested that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 act as second messengers for RTKs and because PI3Ks produce these lipids, they were implicated as the regulators of several of the cellular functions initiated by extracellular ligands. However, the fact that PtdIns(3)P is produced very efficiently by all PI3Ks *in vitro* but is not increased by RTK activation *in vivo* remains an enigma. (Lists of stimuli that increase PtdIns(3,4,5)P3 can be found in Stephens *et al.* 1993).

1.3.1 Signalling downstream of RTKs via proteins that recognise and bind phosphotyrosine

Upon the binding of extracellular ligands, RTKs (which are constitutive dimers or which dimerise upon stimulation) transphosphorylate on specific exposed tyrosine residues. In addition, certain RTKs also phosphorylate specific substrate proteins (Section 1.3.4). Signalling proteins that possess SH2

or phosphotyrosine-binding (PTB) domains, which recognise phosphotyrosines in particular amino acid sequence contexts, can then associate with the tyrosine-phosphorylated RTKs and RTK substrates (Pawson and Scott 1997). As well as SH2 or PTB domains, these downstream proteins also contain domains with enzymatic activity or contain domains that facilitate further protein: protein interactions. Signals are therefore transduced to the cellular machinery through the activities of enzymes, such as kinases, phosphatases and phospholipases, that are recruited to RTKs directly or indirectly, through interactions with other proteins.

Several signalling mechanisms triggered by activated RTKs have been well characterised (Pawson 1995). For example, the protein Grb2 binds to activated RTKs through its SH2 domain. The N-terminal src homology 3 (SH3) domain of Grb2 binds to proline-rich sequences in the guanine nucleotide exchange factor (GEF) Sos, which in turn catalyses the exchange of guanine nucleotide on inactive GDP-bound Ras to generate active GTP-bound Ras (Buday and Downward 1993; Reif *et al.* 1994). The generation of Ras-GTP at the plasma membrane causes the translocation and activation of the S/T kinase, Raf, which in turn triggers a cascade of S/T kinases and the phosphorylation of multiple targets proteins, including transcription factors, phosphatases and metabolic enzymes (Marshall 1994).

Together, the numerous pathways triggered by tyrosine phosphorylation of RTKs and their substrates have been proposed to mediate all RTK functions (Pawson and Scott 1997). This proposal has been well supported both by experiments in cultured cells overexpressing RTK mutants lacking various tyrosine motifs and, more recently, by genetic studies. For example, a mouse with a mutant Met RTK in which two tyrosines had been converted to phenylalanine had the same phenotype as a null mutation in the Met RTK (Maina *et al.* 1996).

1.3.2 Class I_A PI3Ks associate with activated RTKs through SH2 domain-containing adaptors

Class I_A PI3Ks have been shown to bind to tyrosine phosphorylated RTKs and their substrates through the SH2-domain containing adaptor proteins with which all Class I_A PI3Ks associate (Figure 1.2A). The association of Class I_A PI3Ks with RTKs has been proposed to transduce a signal by allowing Class I_A PI3Ks to reach the plasma membrane, where their phosphoinositide substrates are located. However, studies from several laboratories have suggested that the mechanism of activation of Class I_A PI3Ks is more complicated than this simple model (Section 1.3.3, Section 6.2).

1.3.2.1 The adaptors for Class I_A PI3Ks

In mammals, three genes have been identified that encode adaptors for the mammalian Class I_A PI3Ks: p85α, p85β and p55γ (or $p55^{PIK}$) (Figure 1.2B, Escobedo *et al.* 1991; Otsu *et al.* 1991; Skolnik *et al.* 1991; Pons *et al.* 1995; Dey *et al.* 1998). Four additional adaptors are encoded by splice variants of the p85α transcript: p55α, p50α, p85αAS53 and p55αAS53 (Antonetti *et al.* 1996; Fruman *et al.* 1996; Inukai *et al.* 1996). All of these adaptors contain two SH2 domains separated by a large domain, called the inter-SH2 domain, which has been shown to be responsible for the association of the adaptors with the Class I_A PI3Ks (Dhand *et al.* 1994a). The p85α and p85β adaptors also possess long N-terminal extensions that contain an SH3 domain and a breakpoint cluster region homology (BH) domain. In contrast, the p55γ, p55α and p55αAS53 adaptors have short N-terminal extensions (Figure 1.2B). In addition, p85α and p85β have a proline-rich, SH3 domain-binding stretch of amino acids (termed P1) that is situated between the SH3 and BH domains. Furthermore, all mammalian isoforms have a second proline-rich sequence (P2) located approximately 20 residues N-terminal of the N-terminal SH2 domain.

The existence of the BH and SH3 domains and the proline-rich sequences has raised the possibility that the adaptors for Class I_A PI3Ks may receive signals from, or transduce signals to, molecules besides RTKs and Class I_A PI3Ks. The BH domains have structural homology with GTPase activating proteins (GAPs) but have not been shown to possess any GAP activity; thus, their function is unclear. The SH3 domains of p85α and p85β recognise proline-rich peptide motifs and have been shown to bind a number of proteins *in vitro* (Gout *et al.* 1993; Harrison-Findik *et al.* 1995; Soltoff and Cantley 1996). The situation *in vivo* may be different, however, because the SH3 domains of p85α have also been shown to bind to the proline-rich sequences found in the adaptors themselves and, together with the BH domain, have been suggested to mediate the dimerisation of the p85 adaptors when they are in complex with Class I_A PI3Ks (Layton *et al.* 1998).

In spite of the structural diversity described above, there has been no experimental demonstration of selectivity of binding between different adaptors and the mammalian Class I_A PI3Ks, p110α, β or δ (Vanhaesebroeck *et al.* 1997b). However, there have been reports that the different adaptors can localise to different compartments within the cell (Shepherd *et al.* 1997), which may provide the basis for the functional specialisation of the various isoforms.

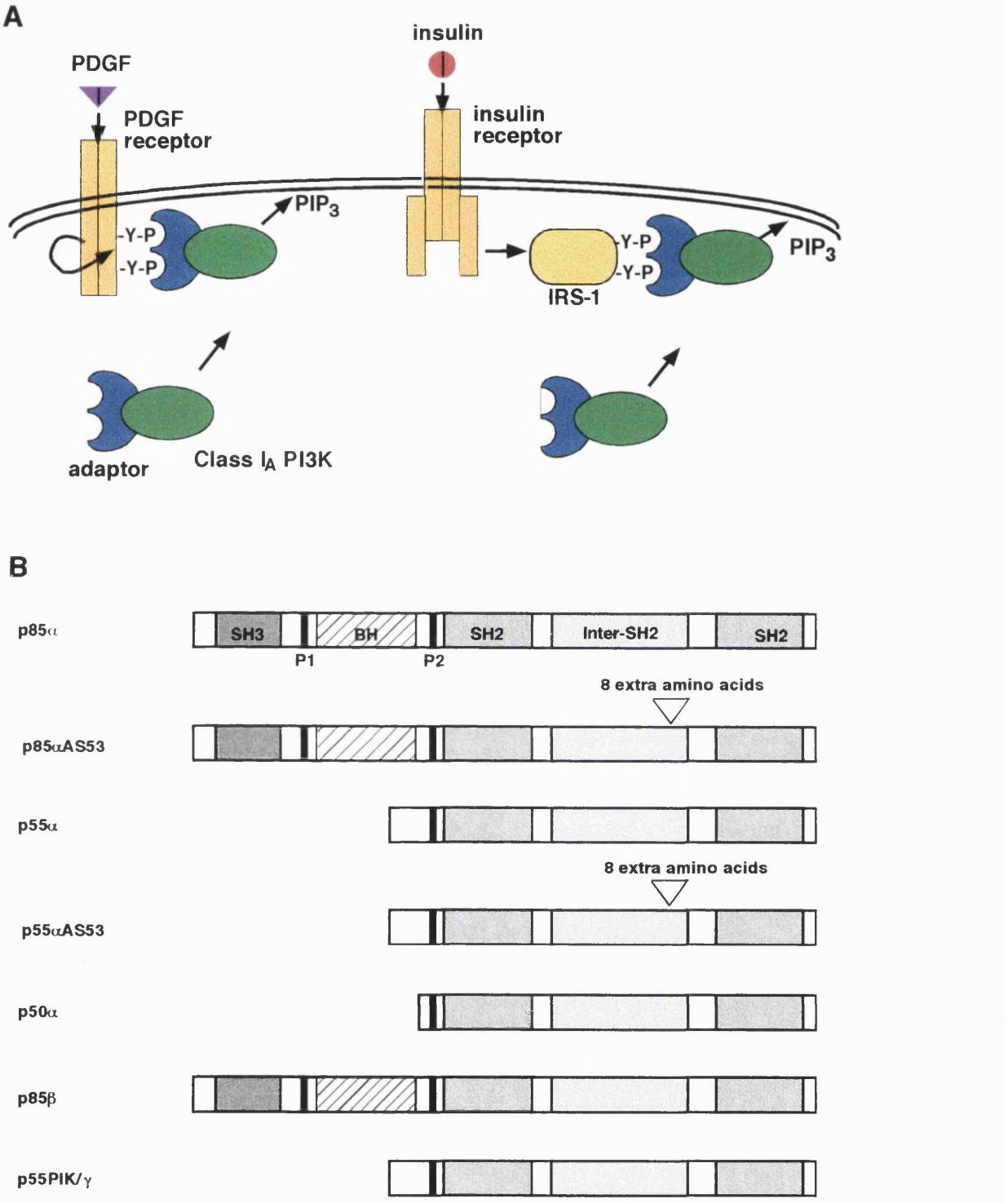


Figure 1.2. The role of the adaptor in Class I_A PI3Ks activation. (**A**) The SH2 domains of the adaptor allow the Class I_A PI3K to be translocated to the membrane by activated RTKs such as the PDGF receptor or by phosphorylated receptor substrate molecules such as IRS-1. (**B**) Seven adaptors are encoded by three genes. There are five splice variants of p85α.

1.3.2.2 The SH2 domains of the adaptors for Class I_A PI3Ks

The binding specificity of distinct SH2 domains lies in the ability of these domains to recognise phosphotyrosine in the context of particular amino acid sequences C-terminal to the phosphotyrosine. The preferred phosphotyrosine binding motifs of several different SH2 domains have been established using purified SH2 domains to screen a library of phosphotyrosine peptides (Songyang *et al.* 1993). This approach predicted that the SH2 domains of p85α would bind to phosphotyrosine in the context pYXXM where X can be any amino acid. Consistent with this prediction, peptides containing pYXXM motifs have been utilised to purify the adaptors in complex with Class I_A PI3Ks (Otsu *et al.* 1991; Fry *et al.* 1992, Chapter 3). Structural studies of many SH2 domains using X-ray crystallography and nuclear magnetic resonance have revealed that SH2 domains share a common fold consisting of a central beta sheet flanked by two alpha helices (Figure 1.3A, Waksman *et al.* 1993). All SH2 domains contain a "pocket" that can bind phosphotyrosine. Binding specificity is achieved by other, more variable regions of the domain that form binding surfaces or pockets for amino acids that lie C-terminal to the phosphotyrosine in the bound peptide. Structural studies of the N- and C-terminal SH2 domains of p85α in complex with pYXXM peptides have revealed that, in addition to the phosphotyrosine-binding pocket, both domains form a hydrophobic pocket that binds to the methionine residue situated three amino acids C-terminal of the phosphotyrosine in the bound peptide (Figure 1.3B, Breeze *et al.* 1996; Nolte *et al.* 1996).

1.3.3 The activation of Class I_A PI3Ks

Purified Class I_A PI3Ks in complex with their adaptors have been shown to be constitutively active *in vitro* (Hiles *et al.* 1992; Vanhaesebroeck *et al.* 1997b). These studies suggest that Class I_A PI3Ks are constitutively active in the cell and that the regulated production of 3' phosphoinositides is achieved *in vivo* by bringing the active enzymes to their substrates in the membrane. This model is supported by studies showing that downstream pathways are activated by overexpressed Class I_A PI3Ks targeted to the membrane by the addition of amino acid sequences that become farnesylated or myristylated or by the addition of transmembrane sequence of the protein, CD2 (Klippel *et al.* 1996; Leevers *et al.* 1996; Brennan *et al.* 1997). However, other studies have suggested that translocation to the membrane is not sufficient for full activation of Class I_A PI3Ks. For example, it has been shown that, although overexpressed membrane-targeted p110α activates downstream signalling pathways, its activity is further increased by fusing the inter-SH2 domain of

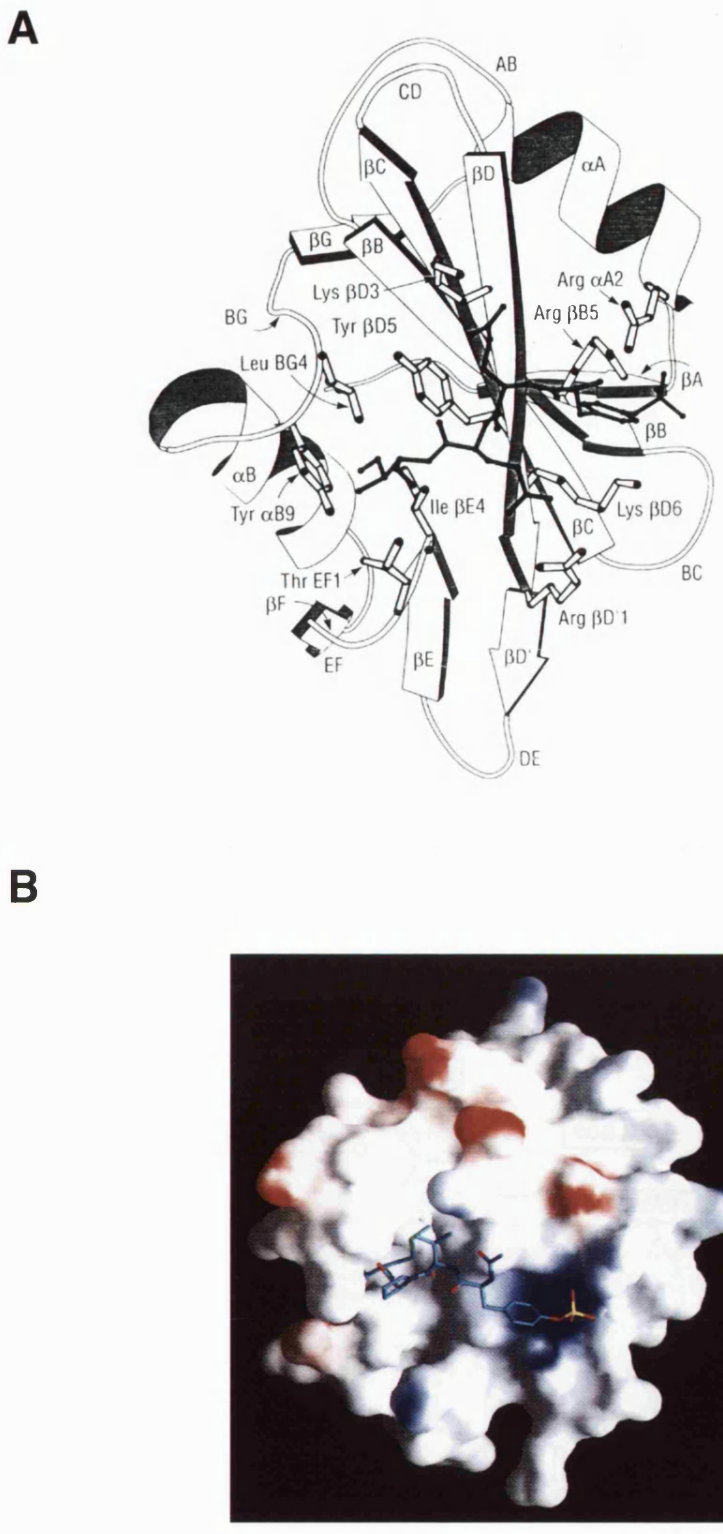


Figure 1.3. SH2 domain structure. (**A**) The structure of the Src SH2 domain showing the two alpha helices and the beta sheet that are conserved in all SH2 domains. Reproduced with permission from Waksman *et al.* 1993. (**B**) The C-terminal SH2 domain of p85α bound to a pYXXM peptide. Reproduced with permission from Breeze *et al.* 1996.

p85α to the N-terminus of p110α (Hu *et al.* 1995). Thus, the adaptors might upregulate Class I_A PI3K activity by a mechanism in addition to membrane localisation. Consistent with this notion, another study showed that the simple coexpression of the inter-SH2 domain of p85α with p110α resulted in a dramatic increase in PI3K activity (Frevert and Kahn 1997). Furthermore, several reports have demonstrated that the addition of pYXXM phosphopeptides to Class I_A PI3K/adaptor complexes increases lipid kinase activity 2-3 fold *in vitro* (Carpenter *et al.* 1993a; Rordorf Nikolic *et al.* 1995). In addition, pYXXM phosphopeptides can be used to activate Class I_A PI3Ks *in vivo* (Kotani *et al.* 1994; Derossi *et al.* 1998).

Recently, Yu *et al.* (1998b) showed that p110α overexpressed in a mammalian cell line was unstable at 37 °C but could be stabilised by reducing the temperature to 30 °C or by adding various extensions to the N-terminus. Therefore, these authors argued that the apparent activation of Class I_A PI3Ks by the addition of N-terminal extensions that had been reported by other investigators was actually a consequence of the increased stability of these fusion proteins compared with that of the untagged p110α (Yu *et al.* 1998b). Furthermore, it was demonstrated that the lipid kinase activity of p110α stabilised by an N-terminal tag and synthesised at 30 °C was inhibited by the adaptor, p85α. Experiments in which p110α and p85α were overexpressed in Sf9 insect cells were also used to show that p85α could inhibit p110α, and that this inhibition was relieved by the addition of pYXXM phosphopeptides (Yu *et al.* 1998b). Thus, the observed activation of Class I_A PI3Ks by the addition of phosphopeptides may, in reality, be a relief of inhibition by the adaptor. Consistent with this hypothesis, other experiments have shown that the adaptors can inhibit lipid kinase activity when in complex with Class I_A PI3Ks. Expression of a mutated version of p85α, which was truncated from the C-terminal end of the inter-SH2 domain through chromosomal rearrangement, was shown to cause an increase in PI3K activity *in vivo*, possibly because translocation of the Class I_A PI3K to the membrane was increased (Jimenez *et al.* 1998). Therefore, the C-terminal SH2 domain and/or the C-terminal region of the inter-SH2 domain may inhibit the activity of associated Class I_A PI3Ks by altering their intracellular location. However, *in vitro* experiments have shown that the N-terminal SH2 domain and the inter-SH2 domain are necessary and sufficient for the adaptor to inhibit Class I_A PI3K kinase activity (Yu *et al.* 1998a). Thus, it is possible that the adaptors can inhibit Class I_A PI3Ks by two distinct mechanisms, firstly, by directly inhibiting kinase activity by association and, secondly, by restricting the access of the associated Class I_A PI3Ks to their substrates at the membrane. The role of the adaptor in the

regulation of Class I_A PI3K function in the light of the results described in this thesis will be discussed in Section 6.2.

1.3.4 RTK specificity in the activation of Class I_A PI3Ks

Receptor tyrosine kinases form a large family of molecules that have specific and diverse functions in the control of cellular processes and the development of multicellular organisms (van der Geer *et al.* 1994). The remarkable phosphotyrosine-binding specificity of the adaptors for Class I_A PI3Ks has led to the prediction that all RTKs with YXXM motifs that become tyrosine-phosphorylated upon stimulation will activate Class I_A PI3Ks (Section 1.3.4).

However, this model is not completely reliable for a number of reasons. Firstly, pYXXM motifs are recognised by the SH2 domains of other signalling molecules including phospholipase Cγ (PLCγ, Bourette *et al.* 1997, Chapter 3) and the multidomain protein, Nck (Nishimura *et al.* 1993). Secondly, Class I_A PI3K/adaptor complexes have been shown to associate with the pYVXV motif of the Met receptor, albeit with a lower affinity than that for pYXXM motifs (Ponzetto *et al.* 1993). Thirdly, several RTKs that lack YXXM motifs, such as the epidermal growth factor receptor (EGF receptor or ErbB1) have also been shown activate Class I_A PI3Ks upon stimulation (Stephens *et al.* 1993). The translocation of Class I_A PI3Ks to the membrane upon activation of these RTKs is achieved by the adaptors binding to pYXXM motifs on receptor substrate molecules that are phosphorylated by the activated RTKs. In particular, activated insulin and insulin-like growth factor (IGF) receptors have been shown to tyrosine phosphorylate insulin receptor substrates (IRS 1-4), thereby, inducing the association of the substrate molecules with Class I_A PI3Ks (Backer *et al.* 1992; Yenush and White 1997). Other RTKs can phosphorylate the related substrate molecule, Gab1, which also contains YXXM motifs (Holgado-Madruga *et al.* 1996; Ingham *et al.* 1998). In addition, cytokine receptors without intrinsic tyrosine kinase activity have been shown to activate Class I_A PI3Ks through the activation of intracellular protein tyrosine kinases that phosphorylate YXXM motifs on other proteins (a comprehensive list of cytokines that activate PI3Ks can be found in Wymann and Pirola 1998). Finally, some RTKs such as the fibroblast growth factor (FGF) receptor are thought to stimulate Class I_A PI3Ks solely through the activation of Ras (van Weering *et al.* 1998, Section 1.3.5). Thus, although the presence of a phosphorylated YXXM motif on a receptor is a good indicator that it will activate Class I_A PI3Ks, it cannot be concluded that the motif will not also

activate other proteins or that an RTK without a pYXXM will not activate Class I_A PI3Ks.

1.3.5 Other means of activation of Class I_A PI3Ks

As well as being activated by their association with pYXXM motifs, Class I_A PI3Ks are also activated by other molecules. These molecules include tyrosine phosphorylated IκB, the inhibitor of the transcription factor NFκB (Beraud *et al.* 1999), and Ca^{2+}-bound calmodulin, which binds to the SH2 domains of p85α (Joyal *et al.* 1997). However, the only molecule that has been shown to be able to activate Class I_A PI3Ks independently of the adaptor is the small GTPase Ras (Section 1.3.1). Specifically, it has been shown that PI3K activity can be co-purified with Ras, and overexpression of constitutively active Ras increases the levels of 3' phosphorylated inositol lipids *in vivo* (Sjolander *et al.* 1991; Rodriguez-Viciana *et al.* 1994). In addition to Raf and Ral GDS (guanine nucleotide dissociation stimulator), active GTP-bound Ras is able to associate with and activate the Class I_A PI3K, p110α (Rodriguez-Viciana *et al.* 1994). Mutant forms of Ras that bind preferentially to one of these three effectors have been generated by the introduction of specific point mutations, which were first identified in a yeast two-hybrid screen for modifiers of Ras binding (White *et al.* 1995; Rodriguez-Viciana *et al.* 1997). Overexpression of constitutively active Ras with one of these effector mutations (V12 Ras C40) in COS cells increases Class I_A PI3K activity (though to a lesser extent than V12 Ras without the effector domain mutation) but does not activate Raf or Ral GDS (Rodriguez-Viciana *et al.* 1997). These studies provide a useful tool to activate Class I_A PI3Ks in cells as well as further evidence that Class I_A PI3Ks are direct effectors of Ras. In addition, the region of p110α that interacts with Ras has been mapped (Figure 1.1). Consistent with the proposal that the association of Ras with this region of p110α leads to activation of the kinase, a form of p110α with a point mutation in the Ras-binding region possesses increased lipid kinase activity *in vitro* (Rodriguez-Viciana *et al.* 1996). This discovery again provides a reagent that can be used to mimic Class I_A PI3K activation.

The ability of Ras-GTP to activate Class I_A PI3Ks in at least some experimental situations suggests that there is another mechanism in addition to direct association with the adaptors for Class I_A PI3Ks by which RTKs can increase 3' phosphoinositide production. However, the level of Ras-induced activation of Class I_A PI3Ks by the FGF receptor, which activates Ras but does not possess YXXM motifs, has been found to be significantly less than the level of Class I_A PI3K activation caused by RTKs that associate with Class I_A PI3K

adaptors via their YXXM motifs (van Weering *et al.* 1998). Interestingly, reactive free radical species generated by nitric oxide donors have been reported to induce the association of p110δ and p110β but not p110α with Ras, providing a mechanism for Class I_A PI3K activation that is independent of tyrosine kinase activation (Deora *et al.* 1998).

Together, these studies can be used to argue that Ras functions upstream of p110α. However, other studies have shown that overexpressed, activated p110α can increase the amount of Ras-GTP in NIH 3T3 cells (Hu *et al.* 1995). Furthermore, the cellular effects of activated p110α expression can be inhibited by the coexpression of dominant negative Ras and mimicked by the expression of activated Ras (Hu *et al.* 1995). These results are more consistent with Ras acting downstream of p110α. Another apparently conflicting report has shown that dominant negative Ras has no effect on Class I_A PI3K signalling in 3T3 L1 adipocytes (Gnudi *et al.* 1997). Therefore, the interaction between Ras and Class I_A PI3Ks is still controversial and may be cell type specific. Genetic studies are required to demonstrate the importance of the interaction in normal signalling processes.

1.3.6 Downregulation of Class I_A PI3K activity

In theory, there are several ways in which the downregulation of Class I_A PI3K activity might be achieved. The possibilities include 1) the spatial restriction of the enzyme away from its lipid substrates in the membrane; 2) the direct attenuation of its catalytic activity or, 3) the depletion of the 3' phosphorylated lipid products. As discussed in Section 1.3.3, the adaptors may utilise the first two mechanisms to inhibit Class I_A PI3Ks. The lipid kinase activity of p110α is also attenuated after it phosphorylates its associated p85α on serine 608 (Carpenter *et al.* 1993b; Dhand *et al.* 1994b). Interestingly, p110δ does not phosphorylate associated adaptors but does phosphorylate itself, which also leads to a reduction in lipid kinase activity (Vanhaesebroeck *et al.* 1999a).

There is good evidence for the third mechanism of downregulation of Class I_A PI3K activity, namely, the degradation of the 3' phosphoinositides. The cellular level of PtdIns(3,4,5)P_3 rises rapidly upon extracellular stimulation but is followed by a sharp decline (Auger *et al.* 1989; Stephens *et al.* 1993). The peak in the level of PtdIns(3,4,5)P_3 is consistently followed by a later peak in the level of PtdIns(3,4)P_2, suggesting that the increase in PtdIns(3,4)P_2 arises from the dephosphorylation of PtdIns(3,4,5)P_3 by a 5' phosphatase rather than from the phosphorylation of PtdIns(4)P by PI3Ks. If PtdIns(3,4,5)P_3 rather than PtdIns(3,4)P_2 is the important signalling product of Class I_A PI3K, such a 5'

phosphatase would be expected to downregulate the effects of Class I_A PI3Ks. The SH2 domain-containing 5' inositol phosphatase (SHIP) is one of a family of 5' inositol phosphatases and has been shown to dephosphorylate PtdIns(3,4,5)P_3 to generate PtdIns(3,4)P_2 (Woscholski and Parker 1997). Importantly, the overexpression of SHIP inhibits the activation of the downstream targets of Class I_A PI3Ks (Aman *et al.* 1998; Vollenweider *et al.* 1999).

The protein PTEN has been identified as a tumour suppressor both because cells from many human cancers have somatic loss of function mutations in PTEN, and because PTEN is mutated in the germline of patients with the cancer-related syndromes, Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (Maehama and Dixon 1999). Interestingly, PTEN possesses 3' lipid phosphatase activity and dephosphorylates PtdIns(3,4,5)P_3 to form PtdIns(4,5)P_2 (Maehama and Dixon 1998; Stambolic *et al.* 1998). PtdIns(3,4)P_2 and PtdIns(3)P are also dephosphorylated by PTEN but at a significantly lower rate (Maehama and Dixon 1998). Studies in which SHIP or PTEN have been inhibited, mutated or overexpressed suggest that these lipid phosphatases antagonise PI3K activity *in vivo* (Aman *et al.* 1998; Stambolic *et al.* 1998), making them excellent reagents for the study of Class I_A PI3K function (Section 1.5).

1.4 Downstream effectors of Class I_A PI3Ks

Although the association of Class I_A PI3Ks with RTKs and the sudden increase in PtdIns(3,4,5)P_3 upon RTK stimulation implicated Class I_A PI3Ks and their lipid products in the control of various cellular processes, a mechanism by which these lipids act as second messengers was also required. In other words, how do lipids with a particular chemical structure cause changes in cell behaviour? There have been suggestions that the 3' phosphoinositides alter cell behaviour through biophysical effects on membrane structure or by interfering with actin polymerisation. However, the existence of specific proteins that associate with the 3' phosphorylated inositol lipids is also an attractive model.

The search for proteins that transduce the signal from the 3' phosphorylated inositol lipids to the cellular machinery took two different routes. Firstly, RTK activated proteins that were implicated in regulating the same cellular processes as Class I_A PI3Ks were examined. Secondly, searches were performed for proteins that specifically bound the 3′ phosphorylated inositol lipids generated by Class I_A PI3Ks. Both of these approaches have

benefited enormously from the use of synthetic 3' phosphoinositides. Importantly, these lipids were synthesised and made available in both the naturally-occurring and non-naturally-occurring chiral forms, thus providing an excellent control for the non-specific binding of charged lipids to proteins (Gaffney and Reese 1997). Together, research based on these two approaches led to the discovery that certain proteins containing pleckstrin homology (PH) domains could bind specifically to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Figure 1.4, Lemmon *et al.* 1996). Interestingly, only a subset of PH domains have been shown to bind these lipids in preference to other phosphoinositides. Furthermore, other classes of domain may also bind specifically to the 3' phosphorylated inositol lipids (Section 1.4.6). (An extensive list of PtdIns(3,4,5)P3-binding proteins can be found in Shepherd *et al.* 1998).

Many of the methods used to investigate Class I_A PI3K function (Section 1.5.1) have also been used to establish that particular proteins are downstream targets of Class I_A PI3Ks. Once it has been established that a protein is directly activated (or inhibited) by Class I_A PI3Ks, investigation of the function of that protein can provide further evidence for the functional studies of Class I_A PI3Ks. Therefore, the best characterised downstream effectors will be discussed before addressing Class I_A PI3K function (Section 1.5).

1.4.1 The protein serine/threonine kinases, PKB and PDK1

The S/T kinase, protein kinase B (PKB, also known as Akt [the cellular form of the oncogene v-Akt] and RAC-PK [Related to A and C protein kinase]) has been shown to act downstream of Class I_A PI3Ks. The initial experiments that implicated PKB as a target of Class I_A PI3Ks demonstrated that PKB was activated by the RTK ligand platelet derived growth factor (PDGF) and that this activation was dependent not only on Class I_A PI3K activity, but also on the association of Class I_A PI3Ks with the PDGF receptor (Burgering and Coffer 1995; Franke *et al.* 1995; Kohn *et al.* 1995). The PH domain of PKB can bind selectively to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, and there have been suggestions that lipid binding can relieve the inhibition of PKB activity by its PH domain (James *et al.* 1996; Franke *et al.* 1997; Frech *et al.* 1997; Klippel *et al.* 1997). However, PKB is also activated by Class I_A PI3Ks through the action of 3' phosphoinositide-dependent kinase 1 (PDK1), which can also bind to PtdIns(3,4,5)P_3 through its PH domain (Alessi *et al.* 1997a; Stephens *et al.* 1998). PDK1 phosphorylates PKB on serine 308, one of the two sites on PKB that must be phosphorylated for its complete activation (Alessi *et al.* 1997b; Stokoe *et al.* 1997). An additional role of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the

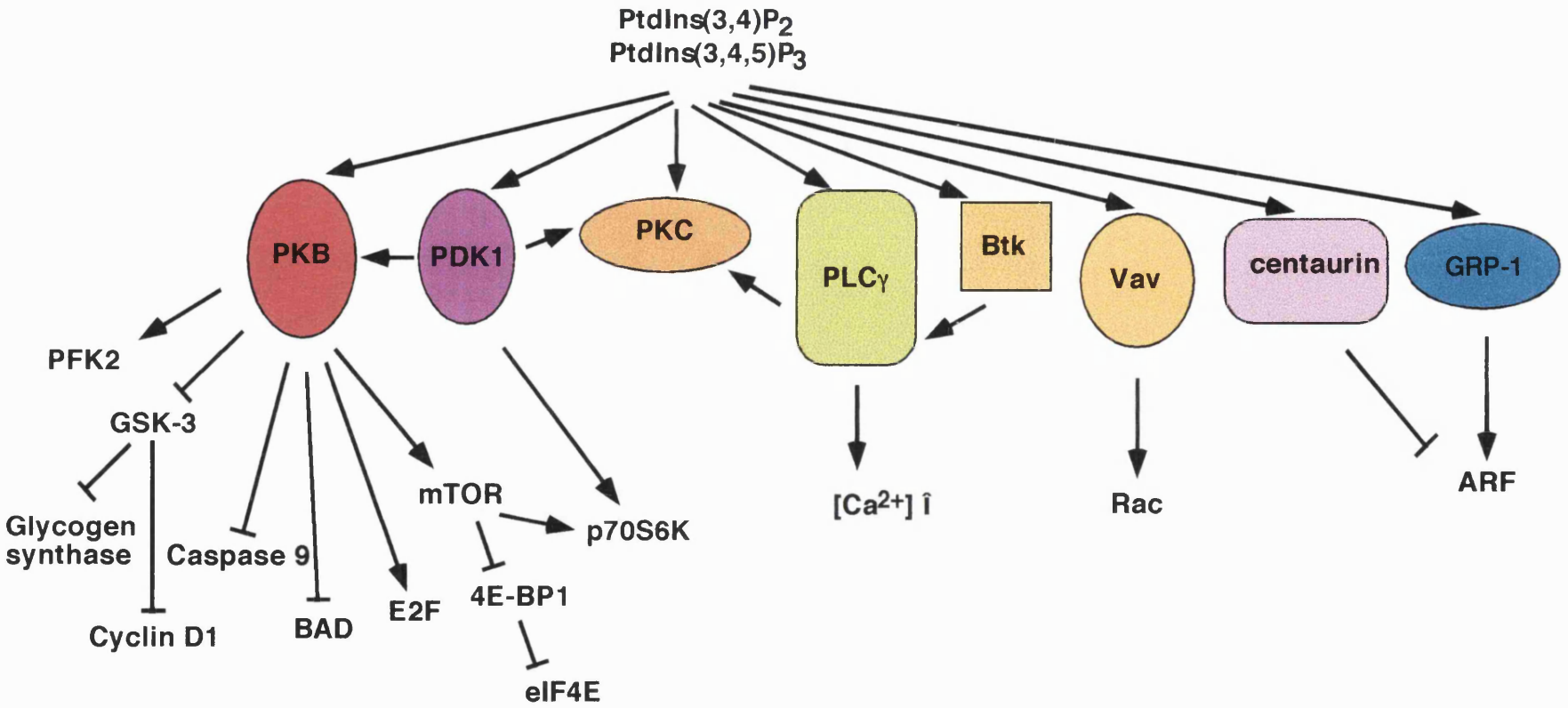


Figure 1.4. A diagram depicting the downstream targets of the 3' phosphoinoisitides and the some potential signalling pathways regulated by these targets. There is published evidence supporting all of these interactions, at least *in vitro*, though the functional importance of these pathways is not always clear.

activation of PKB may be to colocalise PDK1 and PKB. It has been suggested that another protein kinase, termed PDK2, phosphorylates the second site on PKB, threonine 473, to result in the full activation of PKB. PDK2 has yet to be identified, but integrin linked kinase, which can phosphorylate threonine 473 on PKB *in vitro*, is a proposed candidate (Coffer *et al.* 1998; Delcommenne *et al.* 1998).

There are several potential targets of PKB, many of which are proteins that were thought to act downstream of Class I_A PI3Ks but for which there was no established mechanism for their direct activation by Class I_A PI3Ks (Figure 1.4). These proteins include glycogen synthase kinase-3 (GSK-3), phosphofructokinase-2 (PFK-2) and mTOR, all of which will be discussed later in the context of the cellular functions that they are thought to regulate (Section 1.5). PKB has been implicated in the control of several cellular processes, including protection from apoptosis, insulin-induced changes in metabolism, progression through the cell cycle, protein synthesis and glucose uptake (Coffer *et al.* 1998, Section 1.5). It is so generally accepted that PKB is activated by Class I_A PI3Ks that PKB activation is often used as a measure of Class I_A PI3K activity. However, it is important to note that PKB can be activated independently of Class I_A PI3Ks (Yano *et al.* 1998). In addition, PDK1 has been shown to phosphorylate several substrates other than PKB, some of which might be non 3'-phosphoinositide dependent PDK1 targets (Alessi *et al.* 1998; Cheng *et al.* 1998; Chou *et al.* 1998; Le Good *et al.* 1998; Pullen *et al.* 1998).

1.4.2 Guanine nucleotide exchange factors

Guanine nucleotide exchange factors (GEFs) catalyse the activation of small GTPases by inducing the exchange of bound GDP for GTP. Several GEFs possess PH domains, including all GEFs for the Rho family GTPases. The PH domain of the Rac GEF, Vav, has been shown to bind to PtdIns(3,4,5)P_3 and this interaction increases the GEF activity of Vav towards Rac (Han *et al.* 1998). Consistent with this observation, Rac has been shown to be activated by Class I_A PI3Ks *in vivo* (Hawkins *et al.* 1995). Interestingly, the Ras GEF, Sos, also has a PH domain that binds selectively to PtdIns(3,4,5)P_3 (Rameh *et al.* 1997), suggesting that Ras family GTPases might also be activated via the stimulation of GEFs by PtdIns(3,4,5)P_3.

Recently, another family of PH domain-containing GEFs comprised of GRP-1, ARNO and cytohesin-1 has also been shown to bind PtdIns(3,4,5)P_3. These proteins have homology with the yeast protein Sec7, and are all GEFs for ADP-ribosylation factors (ARFs), which are GTPases involved in the regulation of various vesicular trafficking pathways and the activation of

specific phospholipase D isoforms (Moss and Vaughan 1998). ARNO was identified through its homology with the yeast ARF GEF, Gea1 (Chardin *et al.* 1996; Peyroche *et al.* 1996), whereas GRP1 was identified in a screen of an expression library for PtdIns(3,4,5)P_3-binding proteins (Klarlund *et al.* 1997). Independently of studies of ARNO and GRP1, cytohesin-1 was found in a yeast two-hybrid screen for proteins that associate with the cytoplasmic domain of an integrin found in T cells. Cytohesin-1 has been proposed to promote cellular adhesion to extracellular substrates by stimulating the exocytosis of integrins (Kolanus *et al.* 1996).

The PH domains of ARNO and cytohesin-1 bind to PtdIns(3,4,5)P_3 specifically and are membrane-localised upon stimulation (Nagel *et al.* 1998; Venkateswarlu *et al.* 1998b). Strikingly, when fused to green fluorescent protein (GFP), the ARNO-PH domain can be visualised translocating from the cytoplasm to the plasma membrane within seconds of stimulating live adipocytes with insulin (Venkateswarlu *et al.* 1998b). A similar effect was seen with a GRP1 PH domain GFP fusion protein in PC12 cells stimulated with NGF or EGF (Venkateswarlu *et al.* 1998a). Therefore, it has been postulated that the function of PtdIns(3,4,5)P_3 binding in ARF GEF activation is to localise these proteins to the appropriate cellular environment rather than to stimulate their GEF activity directly (Moss and Vaughan 1998).

1.4.3 PLCγ

The phospholipase Cs are a family of enzymes that hydrolyse PtdIns(4,5)P_2 to generate the second messengers inositol (1,4,5) trisphosphate and diacylgycerol, molecules which in turn induce intracellular Ca^{2+} release and protein kinase C (PKC) activation (Lee and Rhee 1995). Thus, the PLCs share a substrate with Class I_A PI3Ks. In addition to their catalytic domains, PLCγ isoforms have two SH2 domains and a PH domain. The N-terminal SH2 domain of PLCγ2 has been shown to bind the same phosphotyrosine on the macrophage colony stimulating factor (M-CSF) receptor as the adaptors for Class I_A PI3Ks. Thus, as well as sharing a substrate with Class I_A PI3Ks, PLCγ isoforms can be recruited to the same RTKs (Bourette *et al.* 1997, see also Section 3.2.2). Furthermore, the PH domain and both SH2 domains of PLCγ1 have been shown to bind PtdIns(3,4,5)P_3 and mediate the translocation of PLCγ1 to the plasma membrane (Bae *et al.* 1998; Falasca *et al.* 1998). Consistent with the idea that these multiple interactions between Class I_A PI3K and PLCγ isoforms have physiological significance, overexpression of active Class I_A PI3K leads to an increase in PLC activity in COS cells (Bae *et al.* 1998). Therefore, it has been postulated that PLCγ isoforms are downstream targets

of Class I_A PI3Ks. Tyrosine phosphorylation of PLCγ isoforms by Tec family kinases may be another mechanism by which Class I_A PI3Ks activate PLCγ isoforms (Section 1.4.4). However, various reports have shown that tyrosine phosphorylation does not always correlate with PLCγ activation (Rhee and Bae 1997; Gratacap *et al.* 1998). It is also possible that the recruitment of PLCγ by PtdIns(3,4,5)P_3 to the same environment as Class I_A PI3Ks leads to the depletion of the substrate for Class I_A PI3Ks, resulting in an effective downregulation of PI3K activity.

1.4.4 Other downstream effectors

Other proteins that can bind to PtdIns(3,4,5)P_3 *in vitro*, and are therefore potential targets of PtdIns(3,4,5)P_3 *in vivo*, are certain PKC isoforms (Nakanishi *et al.* 1993; Toker *et al.* 1994; Le Good *et al.* 1998), centaurin-α, which has a PtdIns(3,4,5)P_3-binding PH domain and a domain with homology to ARF GAPs (Hammonds-Odie *et al.* 1996), and the Tec family of intracellular tyrosine kinases (August *et al.* 1997; Scharenberg *et al.* 1998). In the search for PH domains that selectively bind to PtdIns(3,4,5,)P_3, the PH domain from one of these kinases, Bruton's tyrosine kinase (Btk) was identified (Salim *et al.* 1996; Rameh *et al.* 1997). Mutations in human Btk are found in patients with X-linked agammaglobulinaemia, a disease arising from a deficiency in B cells (Mattsson *et al.* 1996). Importantly, a number of the point mutations that are associated with this disease map to the PH domain of Btk and disrupt the binding of PtdIns(3,4,5,)P_3 to Btk (Salim *et al.* 1996).

Interestingly, a newly-identified domain called the FYVE domain has shown to bind specifically to PtdIns(3)P (Burd and Emr 1998; Gaullier *et al.* 1998; Patki *et al.* 1998). Proteins containing these domains are potential downstream effectors of Class III PI3Ks. It is possible that there are as yet unidentified FYVE domains that bind to PtdIns(3,4,5)P_3. Finally, some SH2 domains have been shown to bind PtdIns(3,4,5)P_3, including the SH2 domain of p85α, the SH2 domain of Src and the SH2 domains of PLCγ (Rameh *et al.* 1995; Bae *et al.* 1998; Rameh *et al.* 1998). Clearly, these interactions create further possibilities for the downregulation of Class I_A PI3K activity and the cross-regulation of signalling pathways.

1.5 The cellular functions of Class I_A PI3Ks

As described above, early ideas about Class I_A PI3K function were based on the correlation of the regulation of cellular processes by RTKs with the appearance of the lipid products of PI3Ks and the recruitment of Class I_A PI3Ks

to these receptors (Stephens *et al.* 1993). However, a causal link between Class I_A PI3K activation and changes in cell behaviour had not been demonstrated. At that time, the only firm evidence that PI3K activation was necessary to mediate changes in cell behaviour came from studies of the *S. cerevisiae* Class III PI3K, Vps34, which is necessary for vesicular transport to the yeast vacuole (Schu *et al.* 1993). Thus, to investigate the relationship between Class I_A PI3K activation and the regulation of various cellular processes, several biochemical reagents have been developed that either inhibit or mimic the activation of Class I_A PI3Ks in cells

1.5.1 Methods of investigation of Class I_A PI3K function

The inhibition of Class I_A PI3K activity can be used to establish whether Class I_A PI3Ks are required for the regulation of specific cellular processes. Two chemical inhibitors of PI3K catalytic activity have been identified: wortmannin, a fungal metabolite, and the synthetic compound LY294002 (Vlahos *et al.* 1994; Ui *et al.* 1995). These compounds inhibit PI3Ks at nanomolar and micromolar concentrations, respectively. With the exception of the Class II PI3K, PI3KC2α, which is inhibited at higher concentrations (Domin *et al.* 1997), these compounds inhibit all other mammalian PI3Ks to a similar degree.

Other methods of inhibiting Class I_A PI3Ks have also been developed. These methods include the use of isoform-specific neutralising antibodies (Roche *et al.* 1994), RTK mutants that lack the YXXM motifs required to bind the adaptors for Class I_A PI3Ks (Valius and Kazlauskas 1993), and a mutant form of the p85α adaptor, Δp85. Δp85 has a small part of the inter-SH2 domain removed so that it can no longer bind Class I_A PI3Ks, and is thought to inhibit Class I_A PI3Ks by competing with endogenous Class I_A PI3K/adaptor complexes for pYXXM motifs on upstream RTKs (Kotani *et al.* 1994).

All of the reagents described above have potential problems with specificity. For example, wortmannin has been reported to inhibit other lipid kinases at higher concentrations (Hsuan *et al.* 1998). Neutralising antibodies might bind to other proteins or modify the function of the target protein in an unexpected way (Roche *et al.* 1994; McIlroy *et al.* 1997). The mutation of RTK YXXM motifs may affect the binding of SH2 domain-containing proteins other than the adaptors for Class I_A PI3Ks (Section 1.3.4) and similarly, the binding of an excess of Δp85 to an RTK may hinder the binding of other SH2 domain-containing proteins besides the adaptors for Class I_A PI3Ks.

Keeping the above caveats in mind, experiments in which Class I_A PI3K activity is inhibited can establish whether Class I_A PI3Ks are necessary for the regulation of a particular cellular process. However, it is also important to

investigate whether an increase in PI3K activity is sufficient to cause a change in the same cellular process. A number of artificially activated forms of Class I_A PI3Ks (Section 1.3.3) have been used in this type of experiment. These activated Class I_A PI3Ks have been shown to increase the levels of 3' phosphorylated lipids in the cell, but it is difficult to establish whether these lipids are produced in the quantities and at the cellular locations that are relevant to the signalling system being studied.

Separately and in combination, the different techniques outlined above have been used in various mammalian model systems (mostly cultured cell lines) to demonstrate a role for Class I_A PI3Ks in the control of several cellular processes (see below). Though each of the techniques has intrinsic problems of specificity, it has generally been argued that the use of a combination of these techniques provides strong evidence for the involvement of Class I_A PI3Ks in a particular process. In many cases, these claims seem justified. However, it is important to be wary of the use of multiple techniques which have similar problems with their specificity. For example, as discussed above, RTK YXXM mutants and Δp85 may inhibit the same SH2 domain-containing proteins, and so investigating a process solely with these two techniques cannot be considered comprehensive.

Another important caveat of these functional studies is that the investigator must distinguish direct effects of Class I_A PI3K activation from indirect effects. In other words, one cannot assume that a single cellular process is independent of all others and should therefore bear in mind that the cellular process being assayed may be affected by one or several other cellular processes. For example, it would be reasonable to assume that an increase in glucose metabolism in a cell would lead to enhanced protein synthesis, growth, cell division and cell migration (Figure 1.5). Therefore, if Class I_A PI3K activation directly enhances glucose metabolism, experimentation would also implicate it as a regulator of other, less direct, consequences of that effect, if those consequences were being monitored.

More recently, additional techniques and reagents have been developed to investigate the cellular functions of Class I_A PI3Ks, including extrapolation from the function of downstream effectors (Section 1.4), the use of lipid phosphatases as antagonists of Class I_A PI3K signalling (Section 1.3.6) and genetic analyses (Fruman *et al.* 1999; Suzuki *et al.* 1999, Section 1.6 and this study). The putative roles of Class I_A PI3Ks in the regulation of several cellular processes will now be discussed in turn (Figure 1.5).

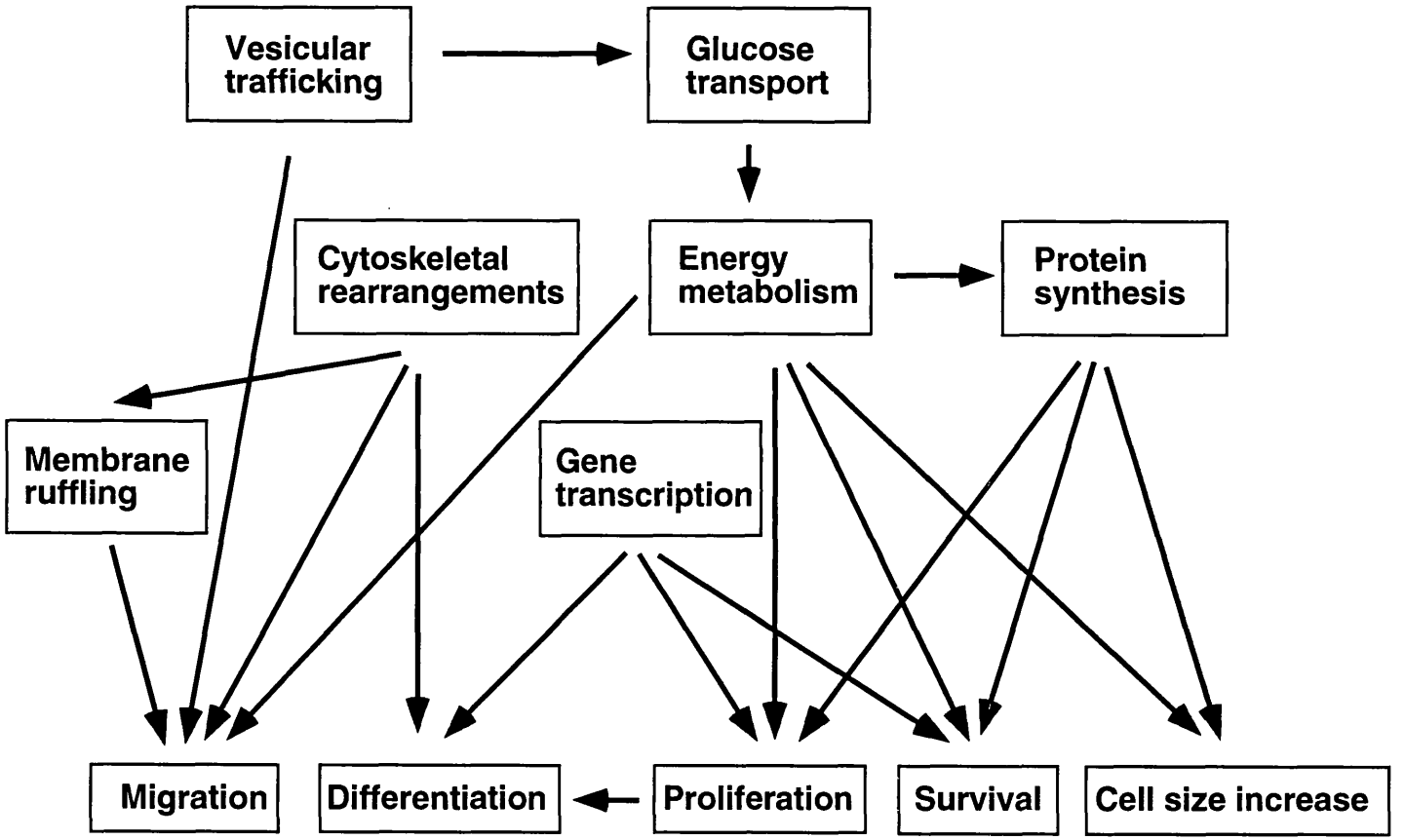


Figure 1.5. A diagram illustrating some of the possible relationships among the various cellular processes regulated by Class I_A PI3Ks. Most of these relationships are supported by published evidence cited in the text. Cell size regulation is discussed in Chapter 6.

1.5.2 Stimulation of proliferation by Class I_A PI3Ks

Cell proliferation can be defined as the increase in cell number that results from progression through the "cell cycle" - a complex series of interrelated events that leads to cell division. Proliferation can be monitored by measuring DNA synthesis, by directly observing cell division or by measuring increases in cell number, with the proviso that cell number can be affected by both cell division and cell death (Section 1.5.3). A number of experiments have implicated Class I_A PI3Ks in mediating the stimulation of proliferation by RTKs.

Various experiments have demonstrated that PDGF-stimulated DNA synthesis can be inhibited by the overexpression of PDGF receptors with mutated YXXM motifs (in HepG2 cells, Valius and Kazlauskas 1993), by the overexpression of Δp85, by wortmannin (in 3T3-L1 adipocytes, Frevert and Kahn 1997), or by treating cells with antibodies that neutralise p110α (in fibroblasts, Roche *et al.* 1994). In a study of the function of different mammalian Class I_A PI3K isoforms, microinjection of antibodies that neutralise p110α inhibited M-CSF-induced DNA synthesis in a macrophage cell line, whereas microinjection of neutralising antibodies to p110β and p110δ did not (Vanhaesebroeck *et al.* 1999b). In a similar study in fibroblasts, neutralising antibodies to p110β did not inhibit PDGF-induced DNA synthesis but did inhibit DNA synthesis induced by insulin (Roche *et al.* 1998). Overexpression of the 5' phosphatase SHIP in adipocytes also decreased insulin-stimulated DNA synthesis, suggesting that PtdIns(3,4,5)P_3 rather than PtdIns(3,4)P_2 was involved in mediating the response (Vollenweider *et al.* 1999).

Activated forms of p110α have been shown to stimulate DNA synthesis in a variety of cell types, demonstrating that Class I_A PI3K activation is sufficient, as well as necessary, to increase proliferation (Hu *et al.* 1995; Frevert and Kahn 1997; Klippel *et al.* 1998; Takuwa *et al.* 1999). Interestingly, mice embryos lacking the endogenous PTEN gene display a hyper-proliferation of cells in the cephalic and caudal regions (Stambolic *et al.* 1998). However, embryonic fibroblasts from the same mice proliferated normally. Instead, they showed increased resistance to apoptotic stimuli (Section 1.5.3). Therefore, this experiment suggests that PTEN, and by implication, Class I_A PI3Ks, may have a cell type-dependent effect on proliferation.

In agreement with results showing that Class I_A PI3Ks stimulate proliferation, targeted disruption of the *p85α* gene in mice causes, among other effects, a reduction in the number of B cells and reduced DNA synthesis in the remaining cells (Fruman *et al.* 1999; Suzuki *et al.* 1999, Section 1.6.2). This

phenotype was observed both in B cells from animals that were entirely mutant for *p85α* and in mutant hematopoietic cells from otherwise wild type mice (using the RAG system). Interestingly, this phenotype is reminiscent of the phenotype resulting from a loss of the Class I_A PI3K target, Btk, in mice (Section 1.4.4, Kerner *et al.* 1995). In contrast to the results in B cells, proliferation is unaffected in T cells lacking p85α, again suggesting that the effect of Class I_A PI3K activity on proliferation is cell type specific. It should be noted, however, that the disruption of one adaptor isoform does not necessarily decrease Class I_A PI3K activity (Terauchi *et al.* 1999, Sections 1.6.2 and 6.2).

The mechanism by which proliferation is induced by Class I_A PI3Ks is unknown, but there is some evidence that PKB is involved. The expression of activated PKB allows B cells to cycle in the absence of IL2 stimulation (Ahmed *et al.* 1997). Furthermore, in T cells, activated p110α or activated PKB leads to the activation of E2F, a cell cycle regulator and transcriptional activator (Brennan *et al.* 1997). In addition, the enhanced transcription of genes involved in cell cycle progression, such as *c-fos*, and cyclin D1, has been proposed to be a downstream effect of Class I_A PI3K activation (Hu *et al.* 1995; Klippel *et al.* 1998; Takuwa *et al.* 1999). Interestingly, the degradation of cyclin D1 is increased by its phosphorylation by GSK3, a kinase that is inhibited by PKB (Cross *et al.* 1995; Diehl *et al.* 1998). In summary, Class I_A PI3Ks appear to stimulate proliferation and are necessary for certain RTKs to stimulate proliferation. However, the mechanism by which Class I_A PI3Ks exert these effects is unclear.

1.5.3 Protection from apoptosis by Class I_A PI3Ks

Apoptosis, or programmed cell death, is an essential part of animal development and function. In the absence of specific extracellular stimuli, known as survival signals, almost all cells will undergo apoptosis through the activation of the cell death machinery, a cascade of cysteinyl aspartate-specific proteinases (Nicholson and Thornberry 1997; Raff 1998). For example, the RTK ligands, PDGF, nerve growth factor (NGF) and IGF-1 have been shown to delay apoptosis induced by various agents (Barres *et al.* 1992; Harrington *et al.* 1994).

A number of studies have implicated Class I_A PI3Ks in survival signalling. The protection of neuronal cells and fibroblasts from apoptosis by PDGF, NGF and IGF-1 is inhibited by wortmannin or LY294002 (Yao and Cooper 1995; Vemuri and McMorris 1996; Yao and Cooper 1996), suggesting a requirement for PI3K activity. The use of a PDGF receptor YXXM mutant has

further implicated Class I_A PI3Ks in mediating protection from apoptosis (Yao and Cooper 1995). In addition, activated forms of p110α and the Ras effector mutant that only stimulates p110α (Section 1.3.5) have been shown to protect fibroblasts and epithelial cells from apoptosis (Kauffmann-Zeh *et al.* 1997; Kennedy *et al.* 1997; Khwaja *et al.* 1997; Kulik *et al.* 1997). It is thought that the anti-apoptotic effects of Class I_A PI3Ks are mediated by PKB, which has been shown to protect neurons, fibroblasts and a hematopoietic cell line from apoptosis (Ahmed *et al.* 1997; Dudek *et al.* 1997; Kauffmann-Zeh *et al.* 1997; Kennedy *et al.* 1997; Eves *et al.* 1998), possibly by phosphorylating the pro-apoptotic protein, BAD (Datta *et al.* 1997), by phosphorylating caspase 9 (Cardone *et al.* 1998), by inducing the expression of the anti-apoptotic protein, Bcl2 (Ahmed *et al.* 1997) or by affecting a pathway upstream of the cell death machinery (Rohn *et al.* 1998).

However, Class I_A PI3K activity is not necessary for survival signals in all cell types. For example, in primary superior cervical neurons, neither LY294002 nor Δp85 inhibit protection from apoptosis by NGF even though an activated form of p110α did prevent apoptosis in the absence of NGF (Philpott *et al.* 1997). Thus, *in vivo*, Class I_A PI3Ks may only protect cells from apoptosis under certain circumstances.

1.5.4 Effects of Class I_A PI3Ks on glucose and lipid metabolism

Class I_A PI3Ks have been implicated in mediating many of the effects of insulin on cellular metabolism. Insulin stimulation leads to enhanced glycogen synthesis, glycolysis and fatty acid breakdown in number of cell types (Shepherd *et al.* 1998). Class I_A PI3Ks are thought to play a role in the control of these processes mostly because their upregulation by insulin has been shown to be inhibited by wortmannin and LY294002 (Moule *et al.* 1995; Hurel *et al.* 1996). Furthermore, GSK3, which inhibits the activity of many proteins including glycogen synthase, is inhibited when it is phosphorylated by PKB (Cross *et al.* 1995). In addition, PFK2, which stimulates glycolysis in response to insulin, is phosphorylated and activated by PKB *in vitro* (Deprez *et al.* 1997). However, the role of Class I_A PI3Ks in the control of these processes is controversial, because the overexpression of activated Class I_A PI3Ks does not always affect glucose metabolism (Frevert and Kahn 1997; Shepherd *et al.* 1998). The effects of Class I_A PI3Ks on glucose metabolism and glucose transport (Section 1.5.6) may be important in understanding some of the other effects of Class I_A PI3Ks. For example, glucose deprivation or the treatment of cells with a glycolytic inhibitor causes untransformed fibroblasts to arrest in the G_1 phase of the cell cycle, and transformed cells of several cell types to

undergo extensive apoptosis, an effect which is blocked by increased Bcl2 expression (Shim *et al.* 1998).

1.5.5. Regulation of protein synthesis by Class I_A PI3Ks

Treatment of quiescent cells with insulin and other extracellular stimuli leads to an increase in protein translation. Overall protein synthesis is increased by 2-3 fold by insulin, but a far greater increase is observed in the translation of a particular subset of transcripts, many of which produce proteins that are components of the translation machinery (Brown and Schreiber 1996; Proud and Denton 1997). All of the transcripts for which translation is upregulated in this way possess a terminal oligopyrimidine tract in the 5' untranslated region, termed a 5' TOP. Two proteins are thought to be responsible for the control of this form of translation: the protein 4E-BP1, which binds to and inhibits the translation initiation factor eIF4E, and p70S6 kinase, which phosphorylates the ribosomal protein, S6 (Proud 1996; Jefferies *et al.* 1997; Lawrence and Abraham 1997). Experiments utilising rapamycin, which prevents mTOR activation and translation of 5' TOPs, suggest that mTOR normally inhibits 4E-BP1 and activates p70S6 kinase through independent pathways (Brown *et al.* 1995; Brunn *et al.* 1996). The results of studies with wortmannin and rapamycin suggest that Class I_A PI3Ks affect protein synthesis through both mTOR-dependent and -independent pathways (Proud 1996). Consistent with this hypothesis, it has been shown that mTOR is stimulated by PKB and that p70S6 kinase is phosphorylated by PDK1 (Alessi *et al.* 1998; Pullen *et al.* 1998; Scott *et al.* 1998).

Interestingly, in *S. cerevisiae*, TOR1 has been shown to increase the levels of cellular proteins by mechanisms that include the upregulation of translation and amino acid uptake and the suppression of protein degradation (Berset *et al.* 1998; Schmidt *et al.* 1998; Dennis *et al.* 1999). It is possible that mTOR also has widespread effects in mammalian cells. Interestingly, TOR1 function is necessary for progression through the G_1 phase of the yeast cell cycle and this phenotype is solely due to a requirement for TOR1-induced protein synthesis rather than any further function of TOR1 (Barbet *et al.* 1996). Similarly, studies in mammalian cells have shown that rapamycin, the mTOR inhibitor, causes G_1 arrest, suggesting that mTOR-induced protein synthesis is also necessary for cell cycle progression in mammals (Brown *et al.* 1994). In support of this hypothesis, overexpression of eIF4E in mammalian HeLa cells leads to increased cell proliferation, changes in morphology and cell transformation (De Benedetti and Rhoads 1990; Lazaris-Karatzas *et al.* 1990; Lawrence and Abraham 1997). Thus, like the upregulation of glucose metabolism, the

upregulation of protein synthesis may also account for some of the other functions attributed to Class I_A PI3K activation.

1.5.6 Regulation of membrane trafficking by Class I_A PI3Ks

One of the first functional studies of a PI3K showed that the yeast Class III PI3K, Vps34p, is necessary for the vesicular transport of proteins to the yeast vacuole, a structure equivalent to the lysosome in higher eukaryotes (Schu *et al.* 1993). More recently, Class I_A PI3Ks have also been implicated in two specific modes of membrane trafficking: the translocation of GLUT4-containing vesicles to the plasma membrane upon insulin stimulation, and the transport of integrins to the plasma membrane. However, the degree of conservation of function between Class I_A PI3Ks and Class III PI3K is likely to be low as each signals to different downstream targets (Section 1.4). The ability of wortmannin and LY294002 to inhibit almost all PI3Ks to a similar degree makes it difficult to interpret experiments that only use these compounds to investigate membrane trafficking. A number of early reports, which relied solely on these compounds, will not be discussed here.

The best characterised membrane trafficking process that is mediated by Class I_A PI3Ks is insulin-stimulated glucose uptake. This process occurs through the translocation of GLUT4-containing vesicles to the plasma membrane 20-30 minutes after insulin stimulation (Pessin *et al.* 1999). Wortmannin, LY294002, Δp85 and SHIP inhibit this process whereas activated p110α can promote it (Frevert and Kahn 1997; Vollenweider *et al.* 1999). Once again, PKB is implicated in transducing the downstream signal from Class I_A PI3Ks to promote vesicle trafficking (Kohn *et al.* 1996; Ueki *et al.* 1998). In addition, it has recently been shown that a specific isoform of PKB, Akt-2, associates with the GLUT4-containing vesicles (Kupriyanova and Kandror 1999). However, it has also been proposed that PKCλ mediates insulin-stimulated, Class I_A PI3K-dependent glucose uptake in PKB-independent manner (Kotani *et al.* 1998).

The mechanism by which Class I_A PI3Ks ultimately regulate vesicle trafficking is unclear. However, ARF GEFs are recruited by PtdIns(3,4,5)P_3 and ARF is implicated in the control of membrane fusion events (Section 1.4.2, Moss and Vaughan 1998). For example, one of the ARF GEFs, cytohesin-1, has been proposed to stimulate T cell adhesion by promoting the translocation of integrins to the membrane (Nagel *et al.* 1998). Class I_A PI3Ks have also been shown to activate Rab4, another GTPase involved in the regulation of membrane trafficking (Shibata *et al.* 1997). Thus, ARF and Rab4 may be involved in the translocation of GLUT4-containing vesicles.

Despite the evidence described above, it has recently been argued that Class I_A PI3Ks are necessary but not sufficient to stimulate glucose uptake (Czech and Corvera 1999). For example, the activation of Class I_A PI3Ks by integrins or PDGF in adipocytes had no effect on glucose uptake (Nave *et al.* 1996; Guilherme and Czech 1998) whereas insulin did stimulate glucose uptake in the same cells. A proposed explanation for this discrepancy is that PDGF and insulin receptors signal in different compartments of the cell. The evidence for this hypothesis is that, in these experiments, PDGF stimulated PI3K activity was found in the plasma membrane fraction, whereas insulin stimulate PI3K activity was localised to the microsomal membrane fraction (Nave *et al.* 1996). It is also worth noting that there is evidence that Class I_A PI3Ks are not necessary for glucose uptake; namely, muscle contraction in skeletal muscle has been shown to stimulate glucose uptake independently of PI3K activity (Brozinick and Birnbaum 1998). Together, these experiments suggest that Class I_A PI3Ks can regulate certain types of membrane trafficking by a mechanism that is distinct from that utilised by Class III PI3Ks.

1.5.7 Cytoskeletal rearrangements mediated by Class I_A PI3Ks

Stimulation of cells with PDGF and several other ligands induces the formation of ruffles in the cell membrane, a process that involves actin reorganisation. Experiments using wortmannin, PDGF receptor mutants and Δp85 have shown that Class I_A PI3Ks are required for PDGF-induced membrane ruffling (Kotani *et al.* 1994; Wennstrom *et al.* 1994). Further experiments, that utilised activated p110α and the activated Ras effector mutant, which stimulates only Class I_A PI3Ks, revealed that Class I_A PI3K activation is also sufficient to induce membrane ruffling (Rodriguez-Viciana *et al.* 1997).

Various experiments suggest that Class I_A PI3Ks are likely to regulate membrane ruffling through the activation of the Rac GTPase. For example, activated Rac induces in membrane ruffling whereas a dominant negative version of Rac inhibits PI3K-mediated ruffling (Ridley *et al.* 1992; Rodriguez-Viciana *et al.* 1997; Welch *et al.* 1998). Furthermore, PDGF activates Rac in a Class I_A PI3K-dependent manner and Class I_A PI3Ks can activate the Rac exchange factor, Vav (Hawkins *et al.* 1995; Han *et al.* 1998, Section 1.4.3). In contrast to several other cellular processes regulated by Class I_A PI3Ks, activated PKB does not affect on membrane ruffling (Welch *et al.* 1998).

Class I_A PI3Ks have also been implicated in regulating M-CSF-induced migration of macrophages (Vanhaesebroeck *et al.* 1999b). Treating macrophages with CSF-1 induces the cells to migrate and undergo several

changes to the actin cytoskeleton (Allen *et al.* 1997). Both of these effects are inhibited by LY294002 as well as antibodies that neutralise p110β or p110δ but are not inhibited by antibodies that neutralise p110α. However, p110β and p110δ do not necessarily affect migration via effects on the actin cytoskeleton, because it has been shown that Class I_A PI3K activation can affect other cellular movements in ways that are independent of the actin cytoskeleton. For example, the inhibition of pseudopod extension in macrophages by wortmannin and LY294002 is due to the inhibition of membrane movements rather than actin polymerisation (Cox *et al.* 1999). Furthermore, neurite extension in PC12 cells caused by overexpression of an activated form of p110α is mediated by changes in tubulin structures, and the resulting extensions lack the actin structures found in NGF-stimulated extensions (Kobayashi *et al.* 1997). In summary, Class I_A PI3Ks can regulate certain cytoskeletal rearrangements, but the role of these rearrangements in the regulation of cell movement is not yet clear.

1.5.8 Class I_A PI3Ks and cell transformation

The transformation of cultured cells is characterised by morphological changes as well as anchorage-independent growth that is not inhibited by high cell densities or by a reduction in growth factors. Transformed cells resemble cells from cancers and cause tumours when injected into immunodeficient mice. Thus, cellular transformation has been used extensively as a model system in the investigation of cancer biology. Normal cellular genes that transform cells in tissue culture when activated by mutation are termed oncogenes. Many of these genes were isolated as activated forms encoded by the genomes of viruses that cause tumours in experimental animals. These genes encode protein that include several RTKs, their ligands and their downstream effectors. For example, PDGF, the EGF receptor and Ras are all oncogenes.

The first evidence that PI3Ks were involved in cellular transformation came from the observation that PI3K activity copurifies with the complex of the transforming protein, middle T antigen and the tyrosine kinase, Src. Furthermore, mutation of the phosphotyrosine residues on middle T antigen that facilitate binding of the adaptors for Class I_A PI3Ks leads to a failure to recruit PI3K kinase activity to this complex and the loss of its transforming activity (Whitman *et al.* 1985). Recently, an activated form of chicken p110α was isolated from a retrovirus that induces hemangiosarcomas in chickens and transforms chicken embryo fibroblasts in culture (Chang *et al.* 1997). The activation of p110α results from its fusion to the viral gag sequence. The viral gag myristylation signal presumably targets the encoded fusion protein to the membrane, a known

mechanism of Class I_A PI3K activation (Section 1.3.3). In addition, studies in mammalian cells have shown that activated p110α can act as a transforming agent in combination with activated forms of other signalling molecules such as the S/T kinase Raf (Rodriguez-Viciana *et al.* 1997; Jimenez *et al.* 1998). Furthermore, PKB was originally identified as a viral oncogene (Staal 1987; Bellacosa *et al.* 1991). Finally, Class I_A PI3Ks have been further implicated in carcinogenesis by the recent findings that the human p110α gene is highly amplified in 40% of ovarian cancers (Shayesteh *et al.* 1999), and that the 3' lipid phosphatase PTEN is deleted in a large number of human tumours (Sections 1.3.6 and 1.6.1). However, it remains to demonstrated that the inhibition of Class I_A PI3K activity can inhibit tumour growth.

1.5.9 Other roles of Class I_A PI3Ks

In addition to the cellular processes described above, there is evidence that Class I_A PI3Ks can mediate other RTK-regulated processes including the upregulation of gene transcription, cellular differentiation and the regulation of calcium channels. A number of studies have provided evidence that Class I_A PI3Ks can regulate transcription. Activation of Class I_A PI3Ks has been shown to stimulate IL2 transcription (Eder *et al.* 1998), the transcriptional regulator E2F (Brennan *et al.* 1997) and the transcription factor NFκB (Beraud *et al.* 1999). Furthermore, the phosphorylation of the transcription factor CREB, which alters gene expression, has been proposed to be a downstream event of PKB activation (Du and Montminy 1998). In addition, genetic evidence from *C. elegans* demonstrates that the inhibition of a forkhead-like transcription factor is a major downstream effect of Class I_A PI3K activation (Lin *et al.* 1997; Ogg *et al.* 1997, Section 1.6.1). Very recently, it has been shown that a similar forkhead transcription factor is phosphorylated and inhibited by PKB in a mammalian cell line (Brunet *et al.* 1999). Consistent with the proposed function of Class I_A PI3Ks in transcriptional regulation, PKB and another protein that binds PtdIns(3,4,5)P_3 have been found in the nucleus (Andjelkovic *et al.* 1997; Tanaka *et al.* 1999).

A number of reports suggest that Class I_A PI3Ks mediate the differentiation of adipocytes in response to insulin, the differentiation of PC12 cells in response to NGF, the formation of myotubes from myoblasts and the maturation of B cells (Magun *et al.* 1996; Kobayashi *et al.* 1997; Fruman *et al.* 1999; Jiang *et al.* 1999). However, Class I_A PI3Ks may exert their effects on these processes by distinct mechanisms because the various forms of differentiation are different. For example, differentiating PC12 cells may require Class I_A PI3Ks

for process formation whereas maturing B cells may require Class I_A PI3Ks to proliferate (Kobayashi *et al.* 1997; Fruman *et al.* 1999).

Finally, Class I_A PI3Ks have been shown to mediate IGF1 potentiation of calcium currents in cerebellar granule neurons (Blair and Marshall 1997). Thus, Class I_A PI3Ks may mediate IGF1-dependent differentiation and survival through calcium-dependent processes, although the effects and universality of Class I_A PI3K-activated calcium influx remains to be demonstrated.

1.6 The role of Class I_A PI3Ks in the whole organism

As discussed above, several diverse cellular processes are regulated by Class I_A PI3Ks in cultured mammalian cell lines (Figure 1.5). However, Class I_A PI3Ks do not have the same effects in all cell types. Furthermore, it is not clear how many of these processes are affected simultaneously, because, in most studies, only one or two cellular processes are monitored at a time. The reason that Class I_A PI3Ks exert different effects in different systems may be explained by the selective function of different Class I_A PI3K isoforms (Section 1.5.7, Vanhaesebroeck *et al.* 1999b), by the differential localisation or utilisation of the adaptors (Section 1.3.2.1, Shepherd *et al.* 1997) or by differences in the magnitude and duration of signalling through Class I_A PI3Ks. For example, both insulin and PDGF increase Class I_A PI3K activity in an adipocyte cell line, but only insulin stimulates glucose transport. This discrepancy may be explained by the differential localisation of Class I_A PI3K activity (Nave *et al.* 1996).

It is generally recognised that signalling pathways can induce distinct effects in different cell types and that these differential responses allow different cell types in a multicellular organism to serve diverse functions. For example, the activation of the *Drosophila* EGF receptor (DER) in cells with distinct developmental histories has been proposed to lead to the differentiation of a number of different cell types in the *Drosophila* eye (Freeman 1997). Alternatively, the diverse effects of one signalling pathway have been attributed to differences in the duration and intensity of signalling. For example, the activation of the MAPK pathway by similar RTKs leads to either proliferation or differentiation in PC12 cells and these differences correlate with the duration of signalling (Marshall 1995).

The situation is further complicated by cross-talk amongst the many signalling pathways downstream of RTKs (e.g. between Class I_A PI3K signalling and PLCγ signalling, Section 1.4.4). In spite of these complexities, multicellular organisms develop in a highly reproducible manner and contain

multiple cell types with specific functions. As Class I_A PI3Ks only appear to be present in metazoa, it is important to establish their role in the normal development and function of these organisms in order to make sense of the studies of Class I_A PI3K function in tissue culture systems. Therefore, the analysis of Class I_A PI3K function in the established model animals, *C. elegans*, mice and *Drosophila melanogaster* will now be described.

1.6.1 Genetic analysis of Class I_A PI3K function in *C. elegans*

Caenorhabditis elegans is a small nematode worm that serves as a powerful model organism, because, amongst other features, the position and lineage of every cell has been described and its genome has been completely sequenced. The genome sequence of *C. elegans* has revealed one Class I_A PI3K, one Class II PI3K and one Class III PI3K (Ruvkun and Hobert 1998, Section 1.2). Mutations in the gene encoding the *C. elegans* Class I_A PI3K can have two distinct effects; namely, null (loss of function) mutations in the Class I_A PI3K result in constitutive entry into the dauer stage of the *C. elegans* life cycle, whereas hypomorphic (reduced function) mutations result in a substantial increase in adult life span.

The dauer stage is an alternative to the normal third larval stage (Figure 1.6A) and is entered under conditions of starvation or overcrowding. Dauer larvae have a tougher exterior than normal larvae, and they do not eat. Furthermore, the dauer larval stage can persist for several weeks, whereas the normal life cycle takes 3 days and the adult life span is two to three weeks. When conditions improve, dauers can molt, enter the fourth larval stage and continue to progress through the normal life cycle. The decision to form a dauer or non-dauer third larval stage is made in the second larval stage of the *C. elegans* life cycle. A number of mutant screens for worms that are dauer-formation constitutive (daf-c, always becoming dauers) or dauer formation-defective (daf-d, never becoming dauers) have been performed. Two of the daf-c genes, *daf-2* and *daf-23*, have been shown to encode an IGF/insulin-like receptor and a Class I_A PI3K, respectively (Morris *et al.* 1996; Kimura *et al.* 1997). Genetic analyses have allowed the ordering of the daf genes into a complex branched pathway (e.g., Larsen *et al.* 1995). Although the precise ordering of some of the components remains controversial, in one arm of the pathway, it has been firmly established that *daf-23* acts downstream of *daf-2* and upstream of *daf-16*, a daf-d gene that was predicted to be inhibited by *daf-23* and shown to encode a forkhead-like transcription factor (Lin *et al.* 1997, Figure 1.6B; Ogg *et al.* 1997). In addition, two homologues of PKB have been shown to act between *daf-23* and *daf-16* (Paradis and Ruvkun 1998).

In a separate screen for mutant worms with increased adult life span, mutations were recovered in a novel gene that called *age-1* (Friedman and Johnson 1988). These mutations were subsequently shown to be hypomorphic rather than null mutations in the gene encoding the *C. elegans* Class I_A PI3K (Morris *et al.* 1996). Strikingly, *age-1* worms lived 2-3 times longer that the wild type. When tested for effects on longevity, worms with hypomorphic mutations in *daf-2* showed a similar increase in life span (Kenyon *et al.* 1993). *daf-2, age-1* worms lived no longer than *daf-2* worms and the addition the *daf-16* mutation eliminated the extension in life span. Thus, it appears that the same pathway that controls dauer formation can also control life span. Interestingly, the *C. elegans* gene product DAF-18 is a homologue of PTEN and antagonises the DAF-2 pathway (Ogg and Ruvkun 1998). However, unlike *age-1* worms (with null mutations), *age-1, daf-18* worms do not form dauers, suggesting that PTEN can antagonise the effect of a Class I_A PI3K in the absence of PtdIns(3,4,5)P_3.

Considering the proposed cellular functions of Class I_A PI3Ks described in Section 1.5, the involvement of Class I_A PI3K in the control of dauer formation and longevity appears surprising. Clearly, all gene products work on a cellular level in some way, so how does AGE-1 control dauer formation and longevity? An elegant study of worms mosaic for the *daf-2* gene revealed that the lack of DAF-2 function in only a small subset of cells was necessary for both the dauer formation and longevity phenotypes. In other words, this experiment suggests that the *daf-2* phenotype is not cell autonomous (Apfeld and Kenyon 1998). Thus, to control dauer formation, AGE-1 would be expected to control the production of an endocrine signal from a small number of neural cells that is used to instruct the cells of the worm to assume dauer-like properties.

Longevity is a difficult concept to understand in cellular terms. Cells could increase their life span by becoming more resistant to stress, particularly from oxygen free radicals, or by reducing their rate of metabolism. Although studies of *age-1* worms reveal no obvious differences in rates of metabolism or duration of development when compared to the wild type, these mutants do possess an increased resistance to UV stress (Friedman and Johnson 1988; Murakami and Johnson 1996). However, it is not clear that the longevity of *age-1* worms is solely explained by increased cellular stress resistance. Dietary restriction has also been shown to increase life span in several species, including mammals (Wachsman 1996). Thus, the regulation of responses to nutrition by insulin in mammals has led to the suggestion that disruptions to the insulin signalling pathway affect longevity in the same way as dietary restriction.

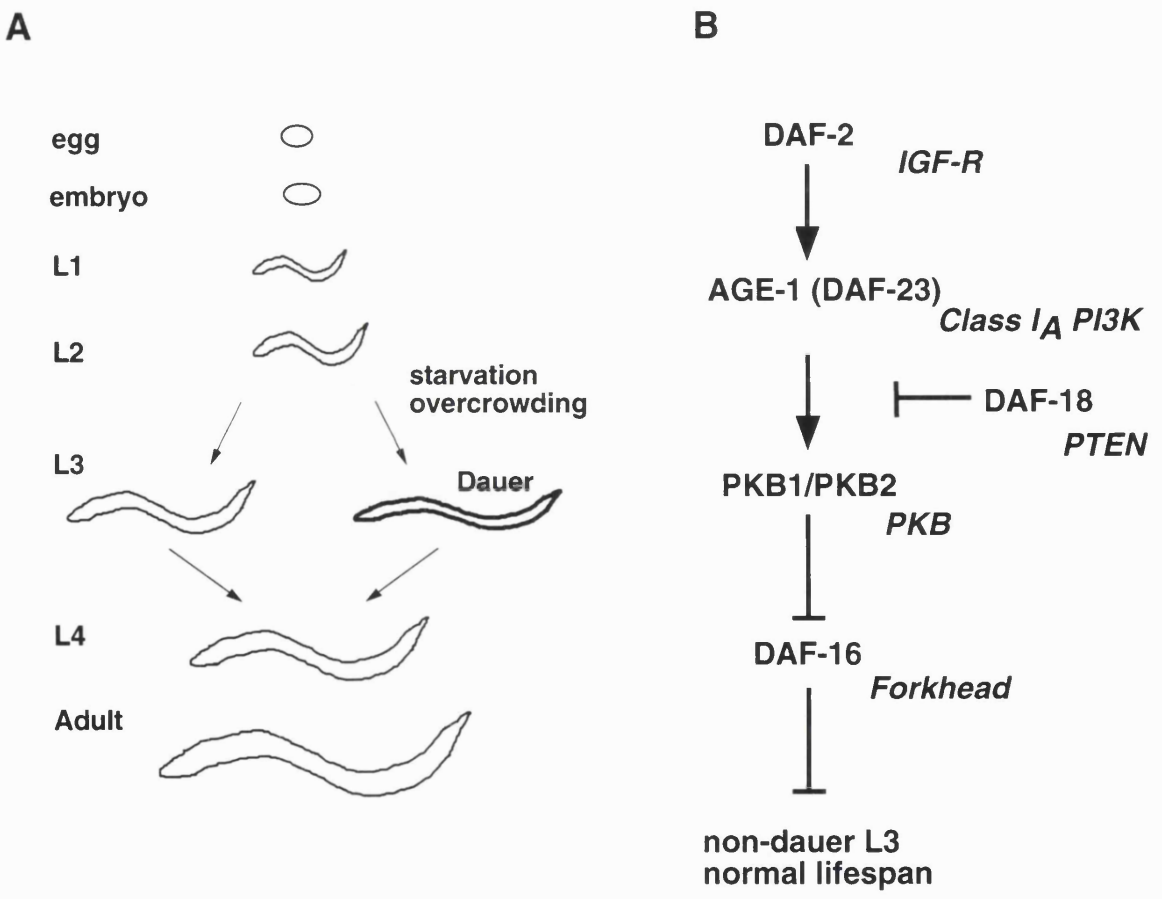


Figure 1.6. The *C. elegans* life cycle and the pathway involving the *C. elegans* Class I_A PI3K. (**A**) The different stages of the *C. elegans* life cycle, including the dauer stage, an alternative to L3 that is formed under conditions of starvation and overcrowding. (**B**) The gene products shown to be involved in a pathway downstream of DAF2 that controls both dauer formation and longevity. The mammalian homologues of these gene products are indicated.

1.6.2 Analysis of Class I_A PI3Ks in mice

Genetic studies of Class I_A PI3Ks and their adaptors in mice have been reported only recently. As described in Section 1.5, the result of experiments utilising two mutations of the endogenous *p85α* gene have been published. Homozygous disruption of the entire gene leads to lethality but the phenotype has not been described further and one mutant pup lived for 5 weeks before it was sacrificed by the investigators, suggesting that p85α is not absolutely necessary for development (Fruman *et al.* 1999). The other published mutation only partially disrupted the gene, eliminating the p85α splice variant but not p55α or p50α. Animals homozygous for this mutation developed into fully formed adult mice (Suzuki *et al.* 1999). However, among other defects, these mice had an impaired immune system due to a reduced number of B cells (Section 1.5.2). Interestingly, the mice were also hypoglycaemic due to increased glucose uptake in skeletal muscle and adipocytes in response to insulin (Terauchi *et al.* 1999). A possible explanation for this apparent increase in a process regulated by Class I_A PI3Ks may be that the lack of p85α was compensated for by the p50α adaptor and this adaptor may be less inhibitory than p85α (Section 1.3.3), allowing greater signalling through Class I_A PI3Ks.

The large number of possible combinations of Class I_A PI3Ks and their adaptors found in mammals (3x7=21) may make genetic analyses of their functions in mice difficult. Analysis of other genes involved in Class I_A PI3K signalling may prove more informative. As discussed in Section 1.5.2, mice lacking PTEN showed increased proliferation during development of cephalic and caudal regions but not in the development of other regions (Stambolic *et al.* 1998). Furthermore, mice heterozygous for PTEN developed spontaneous tumours through loss of heterozygosity (Di Cristofano *et al.* 1998), consistent with a loss of heterozygosity of PTEN in many human tumours. Thus, although only a few genetic studies of Class I_A PI3Ks have been undertaken in mice, our understanding of human disease states has already been furthered. However, targeted disruption of the genes encoding the Class I_A PI3Ks themselves and further investigation of the phenotypes of the existing mutants will be needed to fully understand the roles of Class I_A PI3Ks in mammals.

1.7 Using *Drosophila* to study Class I_A PI3Ks

Drosophila is an excellent system to study Class I_A PI3Ks for two reasons. Firstly, it is a system in which Class I_A PI3K function can be studied in the cell and in the whole organism simultaneously. Secondly, it provides a

powerful genetic system to analyse the interactions between components of Class I_A PI3K signalling pathways *in vivo*.

Polymerase chain reaction (PCR) amplification of *Drosophila* cDNA using degenerate primers based on the amino acid sequences of yeast Vps34 and bovine p110α has revealed the existence of three *Drosophila* PI3Ks, one from each class (MacDougall *et al.* 1995; Leevers *et al.* 1996, Figure 1.7A; Linassier *et al.* 1997). Thus, if these 3 genes encode the only PI3Ks in the fly, the complexity of genetic analyses in mammals that arises from the presence of multiple Class I_A PI3Ks, and their possible redundancies, do not pose a problem in *Drosophila*.

1.7.1 The *Drosophila* Class I_A PI3K, Dp110, plays a role in growth control

Prior to the work described in this thesis, the function of the *Drosophila* Class I_A PI3K, Dp110, was investigated by ectopic expression in the developing wing and eye imaginal discs (Section 1.8). Ectopic expression of either wild type or a membrane-targeted version of Dp110 was shown to result in wings and eyes that were bigger than wild type, but that were patterned normally (Leevers *et al.* 1996, Figure 1.7B, Chapter 5). Conversely, ectopic expression of a form of Dp110 with a putative inactivating mutation in the kinase domain resulted in wings and eyes that were smaller than wild type, but that were patterned normally. In the wing, these changes in final organ size were the result of changes in both cell size and cell number (Leevers *et al.* 1996). As these experiments imply a role for Dp110 in the regulation of imaginal disc growth, and because experiments described in Chapters 4 and 5 of this thesis investigate Class I_A PI3K function in imaginal discs, the development and growth of imaginal discs will now be discussed.

1.8 Imaginal disc growth

1.8.1 Imaginal disc development

Drosophila imaginal discs are monolayer epithelial organs derived from cells set aside during embryogenesis that proliferate during larval development and are re-organised during metamorphosis to generate adult epidermal structures such as eyes, wings and legs (Cohen 1993). For example, dramatic structural rearrangements of the wing imaginal disc take place during pupation as it is reorganised to form the adult wing blade, hinge and adjoining region of the thorax.

The wing imaginal disc is generated from approximately 50 primordial cells that start to proliferate in the first instar of larval development,

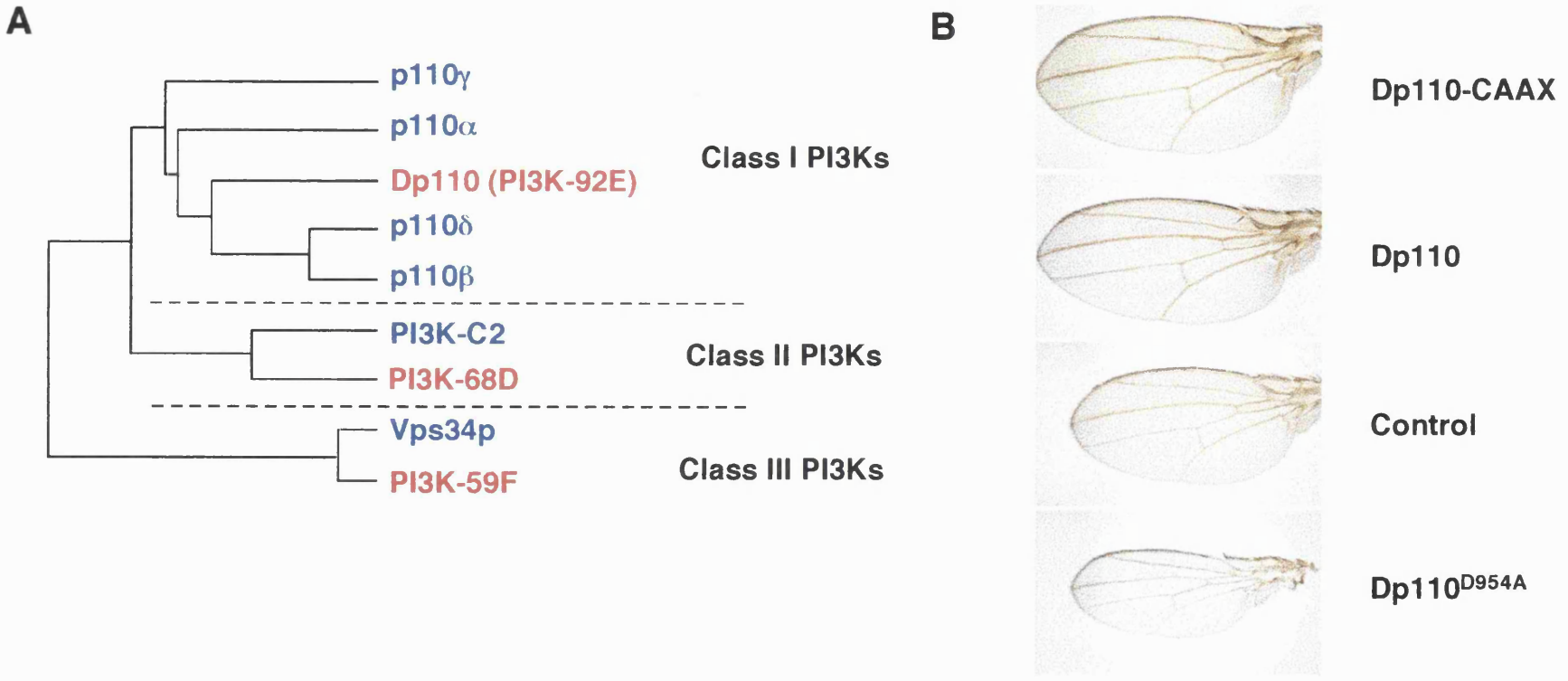


Figure 1.7. *Drosophila* possess one of each class of PI3K and the *Drosophila* Class I_A PI3K promotes growth in the wing imaginal disc. (**A**) A dendrogram representing the amino acid conservation of the mammalian and *Drosophila* PI3Ks, based on their kinase domains (MacDougall *et al.* 1995). (**B**) Ectopic expression of wild type or membrane targeted Dp110 in the wing imaginal disc causes the resulting wing to grow larger than wild type, whereas kinase dead Dp110 expression results in a smaller wing than wild type (Leevers *et al.* 1996, Chapter 5).

culminating in a structure consisting of approximately 50,000 cells by the end of the third, and final, larval instar (Madhavan and Scheiderman 1977). Throughout the development of the imaginal disc, the fates of the constituent cells are determined by the actions of the gene products expressed in restricted regions of the disc. The wing disc is divided into anterior and posterior compartments both by lineage restriction as well as by interactions between the gene products expressed each compartment. The disc is also divided into dorsal and ventral regions, again by interactions between several gene products (Serrano and O'Farrell 1997). The extracellular ligands, Decapentaplegic (Dpp) and Wingless (Wg), are expressed along the anterior/posterior boundary and the dorsal/ventral boundary, respectively (Figure 1.8A). Together, these two ligands have been proposed to direct both the growth and the patterning of the wing disc (Nellen *et al.* 1996; Zecca *et al.* 1996; Serrano and O'Farrell 1997). The vertebrate homologues of Wg (the Wnt family) and Dpp (the transforming growth factor β - TGFβ family) are also involved in the patterning of several organs including vertebrate limbs (Roelink 1996; Sasai and De Robertis 1997). In summary, the wing imaginal disc is an epithelial structure in which patterning and growth occur simultaneously and thus, resembles the development of many mammalian epithelial organs.

The retina of the adult eye is composed of a hexagonal array of repeating units termed ommatidia. The development of the eye and the imaginal disc from which it arises is also used as model for organogenesis. However, although Wg and Dpp are also involved, the determination of the final cell fates in the eye imaginal disc occurs in a very different way from the patterning of the wing imaginal disc (Wolff 1993; Treisman and Heberlein 1998). The cells of the eye imaginal disc also proliferate during larval development but early in the third instar, cells at the posterior edge of the disc begin to differentiate and organise into ommatidial precursors while cells in the rest of the disc are still proliferating (Figure 1.8B). As the eye imaginal disc cells stop proliferating, they form a dorsal-ventral indentation in the apical surface of the disc called the morphogenetic furrow. The morphogenetic furrow moves from the posterior to anterior of the disc as the cells are progressively recruited into structures known as preclusters. These cells differentiate in a stepwise manner to form the precursors of the photoreceptors, cone cells and pigment cells that make up each adult ommatidium. The correct differentiation of these cells depends upon, amongst other factors, the activation of the RTKs DER and Sevenless, which in turn

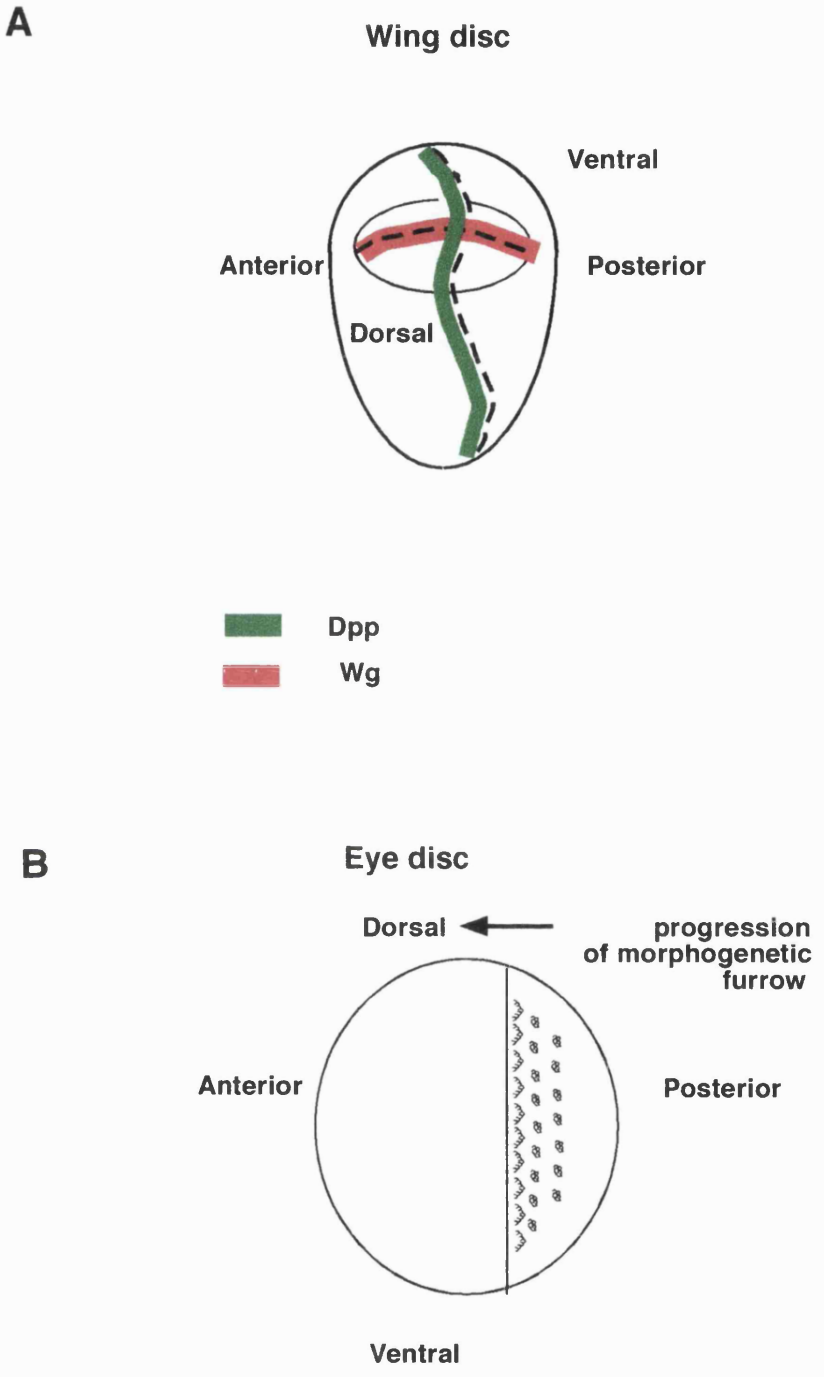


Figure 1.8. Basic features of wing and eye imaginal disc development. (**A**) The wing imaginal disc is divided into anterior and posterior compartments and dorsal and ventral regions. Dpp is expressed along the A/P boundary and Wg is expressed along the D/V boundary or wing margin. Together, Wg and Dpp direct much of the growth and patterning of the disc. (**B**) Cells of the eye imaginal disc form the morphogenetic furrow as they stop proliferating and start to differentiate into ommatidial cell precursors.

activate the Grb2/Sos/Ras/Raf/MAPK signalling pathway (Dickson and Hafen 1993; Freeman 1996; Dominguez *et al.* 1998, Section 1.3.1). Other proteins have also been implicated in signalling downstream of DER and Sevenless; namely, Dos, an RTK substrate, Ksr, a S/T kinase related to Raf and more recently, PLCγ (Therrien *et al.* 1995; Raabe *et al.* 1996; Thackeray *et al.* 1998). However, it has been proposed that all of these proteins ultimately affect the same pathway and that the activation of MAPK is the only signal necessary for DER- and Sevenless-induced photoreceptor differentiation (Freeman 1997).

1.8.2 The regulation of growth during development

As described in Section 1.5, Class I_A PI3Ks have been implicated in the regulation of cell proliferation, survival, shape, differentiation and metabolism, all of which are involved in some way in imaginal disc development. Because the ectopic expression of Dp110 affects imaginal disc growth, the following discussion will focus on the control of growth during imaginal disc development and the various cellular processes that may affect growth.

1.8.2.1 Intrinsic and extrinsic controls of imaginal disc growth

Imaginal disc growth is thought to be both under the control of factors intrinsic to the disc as well as to be responsive to extrinsic, systemic factors (Bryant and Simpson 1984). There are a large number of *Drosophila* mutants in which the adult flies are smaller than wild type. Systemic growth factors are probably disrupted in many of these mutants. For example, mutation of the Ras GAP, NF1, leads to small flies. However, imaginal disc cells that are mutant for NF1 grow normally, suggesting that the function of NF1 is to produce a systemic factor that affects the growth of the entire organism (The *et al.* 1997).

Evidence that imaginal disc growth is also under intrinsic control has come from various experiments. For example, larval discs transplanted into an adult host have been shown to grow to the same size regardless of the developmental stage of the transplanted disc or the host (Garcia-Bellido 1965). In addition, the growing disc is remarkably resistant to perturbation, providing further evidence that discs have an intrinsic mechanism to compensate for external effects on growth. For example, when small regions are ablated early in development, discs of wild type size are still produced (Haynie and Bryant 1977; Milan *et al.* 1997). Notably, physical disruption of an imaginal disc during the third instar results in a delay before pupariation

while the damaged imaginal disc is repaired (Simpson *et al.* 1980). However, the extension to the third larval instar caused by this process does not alter the size of the remaining imaginal discs, suggesting that growth halts when the disc reaches a particular size that is regulated by mechanisms intrinsic to the discs themselves. Transplantation studies have shown that many mammalian organs also have intrinsic mechanisms that regulate their size. For example, if an infant rat heart or kidney is transplanted into an adult rat it will grow to the normal adult size (Bryant and Simpson 1984).

Plotting the growth of various organs (including *Drosophila* imaginal discs) against time produces a sigmoidal curve (Madhavan and Scheiderman 1977; Bryant and Simpson 1984). One interpretation of this observation is that there is an intrinsic mechanism that slows down growth when the organ approaches its final size. Interestingly, studies in which cell division, cell survival and protein synthesis have been disrupted experimentally provide further evidence for the existence of an intrinsic control of imaginal disc growth. These experiments and the involvement of cell division, cell survival and protein synthesis in imaginal disc development are described below.

1.8.2.2. The involvement of proliferation, apoptosis and protein synthesis in imaginal disc development

Cell proliferation in *Drosophila* imaginal discs has been studied in two ways. Firstly, marked clones of cells arising from a single mitotic event have been generated (e.g. by X-ray irradiation or by the use of the FRT-FLP system, see Chapter 4). The size and shape of the resulting clones and associated twin spots, which can be observed through the use of specific genetic markers, reveals information about the extent and patterns of proliferating cells in the developing imaginal disc (e.g. Gonzalez-Gaitan *et al.* 1994). Clones of cells never cross the boundary between the anterior and posterior compartments, but, apart from this restriction, the distribution of clonal populations does not correspond to patterned regions of the organs. Thus, aside from the lineage restriction that originally defined the anterior and posterior compartments, cell lineage does not determine cell fate in the wing imaginal disc (Garcia-Bellido *et al.* 1973).

A second experimental strategy to monitor cell proliferation in the developing wing imaginal disc has been to assess BrdU incorporation into newly synthesised DNA (Milan *et al.* 1996). These studies show that the cells of the wing imaginal disc divide in small clusters that are not related by lineage. It has been suggested that this local coordination is mediated by gap junctions

between imaginal cells, structures that have been visualised in dye-filling experiments (Fraser and Bryant 1985; Bryant 1996).

Interestingly, the activation of cell division in one compartment of the wing imaginal disc by the overexpression of E2F, or the inhibition of cell division with a temperature sensitive mutation in Cdc2, results in increased or reduced cell number, respectively, but final compartment or disc size is not affected (Weigmann *et al.* 1997, Figure 1.9A; Neufeld *et al.* 1998). Rather, those compartments contain more, smaller cells or fewer, bigger cells. In summary, studies of proliferation in the wing imaginal disc show, firstly, that, with the exception of the compartment boundary restriction, patterns of proliferation in the disc are not directly connected to the determination of cell fate and, secondly, that inhibition or activation of cell division has no effect on the size of the imaginal disc (Su and O'Farrell 1998).

Cells undergoing apoptosis have been monitored in *Drosophila* imaginal discs by various methods, including, acridine orange staining, which is specific for apoptotic cells, Hoechst dye, which stains condensed nuclei, and the TUNEL technique, in which the DNA fragments found in apoptotic cells are recognised by antibodies (Milan *et al.* 1997). These experiments indicate that apoptosis takes place in single cells as well as in small clusters of cells in the wing imaginal disc. During the first and second instar, apoptosis occurs randomly throughout the disc, whereas during the third instar and pupal stage, apoptosis occurs less often and in non-random locations such as the border between the regions that will become the wing and the notum. Interestingly, the induction of apoptosis in one region of the disc by ectopic expression of ricin, a cytotoxin, also increased apoptosis in other regions of the disc (Milan *et al.* 1997). However, the loss of cells through apoptosis is compensated for by increased proliferation, resulting in a disc of wild type size, consistent with earlier experiments in which X-ray irradiation was used to kill cells (Haynie and Bryant 1977, Figure 1.9B). Induction of apoptosis by ricin expression for longer periods of time does reduce the size of the final disc, but the disc remains patterned correctly, again suggesting that there are intrinsic mechanisms that resist disruption to imaginal disc development (Milan *et al.* 1997).

The use of mutations in a class of *Drosophila* genes, termed *Minutes*, some of which have been shown to encode components of the ribosomal machinery, allows protein synthesis to be disrupted during imaginal disc development. The mutational inactivation of only one copy of a *Minute* gene is sufficient to have effects on the adult fly (i.e. Minute mutations are genetically dominant). Furthermore, cells homozygous for *Minute* mutations do not

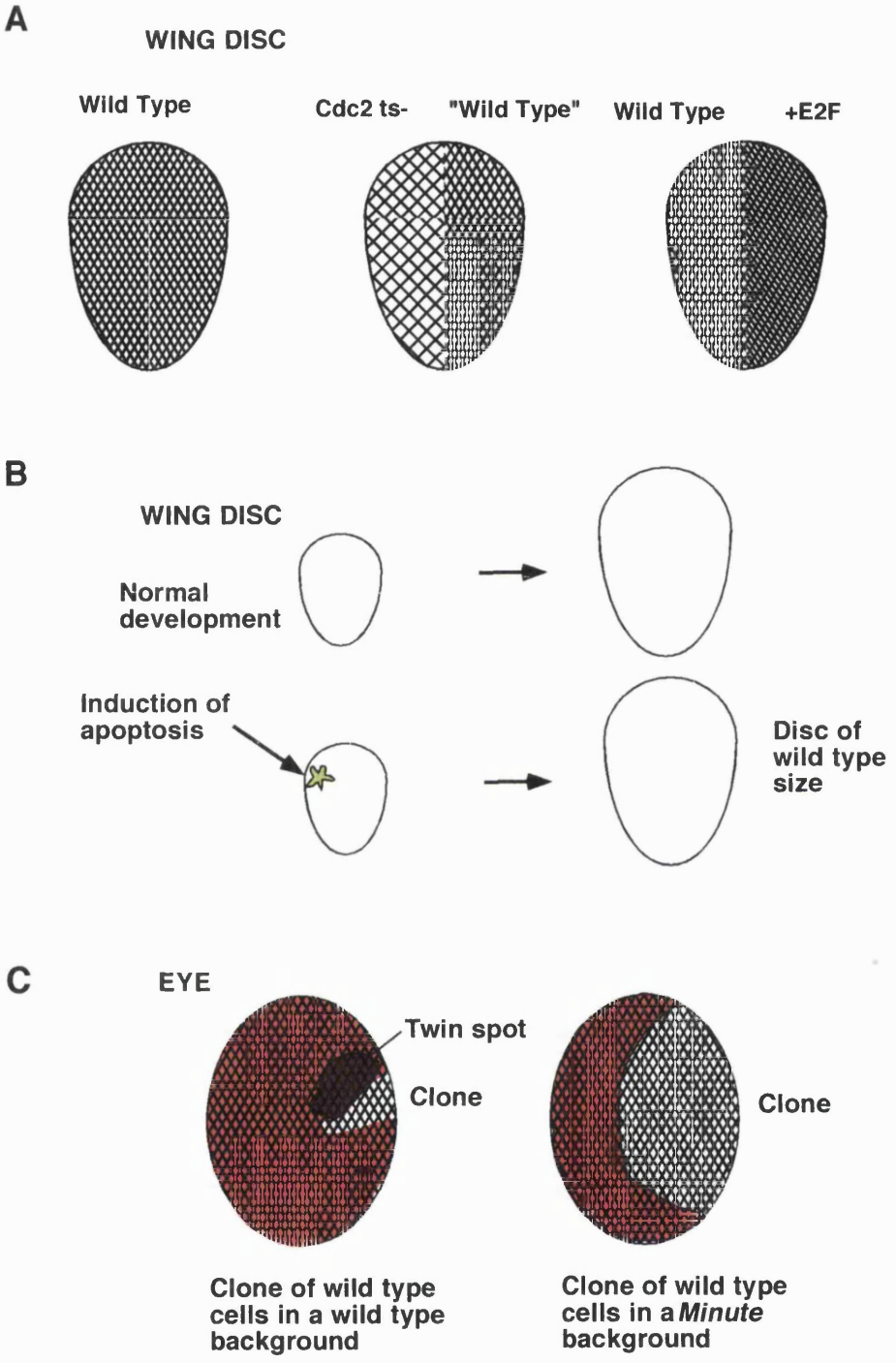


Figure 1.9. Various perturbations to cell division, cell survival and protein synthesis have no effect on the size of the resulting organ. (**A**) Inhibition of cell division with a temperature-sensitive mutation of Cdc2 in one compartment of the wing imaginal disc, or activation of cell division in one compartment by ectopically expressing E2F, changes cell number and size but has no effect on disc size. (**B**) Induction of apoptosis in a small region of the wing imaginal disc early in development has no effect on the final size of the disc. (**C**) Clones of wild type cells in a *Minute* background take over a large region of the organ but do not affect its final size. (Also, they have no twin spot).

survive. *Minute* flies have slender bristles, but are not generally smaller than wild type. Clones of wild type imaginal disc cells in a *Minute* background proliferate more than their neighbours, taking over large regions of the disc and final organ (Figure 1.9C). However, these clones do not affect the final size or shape of the disc (Morata and Ripoll 1975). Thus, these experiments demonstrate, firstly, that cells with enhanced protein synthesis can outgrow their neighbours, and secondly, that this increase in growth is compensated for by a reduction in growth in the other cells in the disc, resulting ultimately in a disc of wild type size.

In summary, while clearly necessary for growth, the processes of cell proliferation, cell survival and protein synthesis can be substantially disrupted without having an effect on the size of *Drosophila* imaginal discs. In fact, there is evidence that the disruption of one of these process can result in compensating effects in another of these processes. As work in mammalian systems clearly shows a role for Class I_A PI3Ks in the regulation of cell proliferation, cell survival and protein synthesis, the *Drosophila* Class I_A PI3K, Dp110, might be expected to regulate growth via any or all of these processes. The mechanism by which the ectopic expression of Dp110 promotes growth will be discussed further in the light of the results presented in this thesis (Section 6.3).

1.8.2.3 The coordination of growth and patterning during imaginal disc development

In contrast to the results described above, other interventions in imaginal disc development are able to affect final organ size, but they are accompanied by a disruption in patterning. It has already been mentioned that the extracellular ligands, Dpp and Wg can affect both growth and cell fate. For example, ectopic expression of Dpp or Wg in the developing wing imaginal disc generates duplicated structures and changes final organ size (Struhl and Basler 1993; Nellen *et al.* 1996; Zecca *et al.* 1996). Similar results are seen when Dpp production is induced by the creation of an ectopic anterior/posterior boundary (Tabata *et al.* 1995). Thus, the patterning elements of an organ may determine the final size required by the intrinsic controls of growth.

Hyperplastic growth in imaginal discs can also be achieved by certain interventions but this effect is always accompanied by a loss of patterning. For example, a number of genes, termed tumour suppressor genes, have been identified that cause overgrowth when both copies of the genes are removed in a mitotic clone (Bryant *et al.* 1993; Xu *et al.* 1995). Although the constituent cells of these overgrowths are often differentiated, the resulting structures do not

retain the pattern of the organ from which they originated. Most of the tumour suppressor genes cloned to date encode proteins involved in the formation of intercellular junctions, suggesting that direct cell-to-cell contacts are involved in growth control. Ectopic expression of activated Ras in the *Drosophila* eye imaginal disc can also induce chronic over-proliferation, but this effect is also accompanied by a loss of structure (Karim and Rubin 1998). Together, the experiments described thus far demonstrate that interventions to imaginal disc growth have one of two effects: either the final size and patterning of the disc is not affected or both size and patterning are affected. Strikingly, however, ectopic expression of Dp110 appears to be able to increase disc size without affecting patterning, an effect that has never been reported for other proteins ectopically expressed in the imaginal disc.

The experiments presented in this thesis describe the identification of p60, the SH2 domain-containing adaptor for Dp110 along with the characterisation of its function in complex with Dp110 using biochemical and genetic methods. The results of these experiments provide important insights into A) the interactions between Class I_A PI3Ks and their adaptors, B) the cellular roles of Class I_A PI3Ks and, C) the role of Class I_A PI3Ks in the control of organ growth and final size.

Chapter 2: Experimental procedures

Chapter 2: Experimental procedures

2.1 Culture of cells and flies

2.1.1 Cell culture

Drosophila S2 cells were grown in Schneider's *Drosophila* medium with 10% (v/v) foetal bovine serum, 1% (v/v) fungizone and 0.1% (w/v) gentamycin (all from Gibco) at 25-27°C in 175 cm^2 tissue culture flasks. The cells were split using a 1:5 ratio of culture to fresh medium every 3-4 days. Human cell lines were grown in suspension in standard medium supplemented with 5% (v/v) foetal bovine serum.

2.1.2 Fly culture

Flies were raised in tubes and bottle containing standard fly food (0.8% [w/v] agar, 8% [w/v] yeast, 5% [w/v] corn meal, 7% [w/v] sucrose, 0.4% [w/v] nipagin and 0.4% [v/v] proprionic acid) at 18°C or 25°C depending on the experimental requirements.

2.2 Biochemical techniques

All steps were performed at 4°C or on ice, unless indicated

2.2.1 Preparation of lysates

To prepare S2 cell lysate, confluent, but not overgrown, S2 cells were loosened with a sharp tap on the flask, poured into 50 ml Falcon tubes and centrifuged at 400 *g* for 7 min. The supernatant was removed and the pellet was washed once with cold phosphate-buffered saline (PBS). The centrifugation was repeated and the supernatant was removed completely by aspiration. 1 ml lysis buffer (1% [v/v] Triton X-100, 50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM dithiothreitol, 0.17 mg/ml phenylmethylsulphonyl fluoride, 2 µg/ml leupeptin, 1% [w/v] aprotinin, and 5 mM benzamidine [all from Sigma]) was added for each 175 cm^2 flask of cells used, and incubated for 20 -30 min on ice and vortexed occasionally. The lysate was clarified by centrifugation at 15000 *g* for 20 min, and the supernatant was used for further experiments. Detergent lysates of *Oregon R* third instar larvae, pupae and adult flies were prepared in lysis buffer, supplemented with 5 µM diisoproproylfluorophosphate (ICN) and 15 µM N-α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma), by homogenisation with a polytron (2 x 10 seconds, on ice). The homogenate was clarified by centrifugation at 20000 *g* for

1 h, then at 20000 *g* for 30 min. Care was taken to avoid the floating lipid fraction when the supernatant was removed for experimentation.

For the experiments described in Chapter 5, flies were heat-shocked (20 min at 33°C, 25°C for 20 min, 20 min at 33°C), then homogenised in lysis buffer (20 µl per fly) by using a small pestle in an eppendorf tube. The lysate was then centrifuged at 15000 *g* for 1 h and the supernatant removed for use in experiments.

2.2.2 Affinity purification

Affinity purification was performed with phosphopeptides coupled to Actigel beads (Sterogene). The peptide was coupled to Actigel-ALD Superflow resin as described (1 mg peptide/ml of 50% [v/v] slurry, Fry *et al.* 1992). For each purification, 2-5 µl of a 50:50 slurry of beads and lysis buffer was added per ml lysate, and incubated with constant tumbling for 1 h. The beads were washed 5 times in lysis buffer except for when the products were to be used for lipid and protein kinase assays; in this case, they were washed three times in lysis buffer and twice in kinase buffer (20 mM Tris HCl pH 7.4, 100 mM NaCl and 0.1 mM EGTA).

2.2.3 Immunoprecipitation

Immunoprecipitation with polyclonal antibodies was performed by incubating 5 µl antisera per ml lysate for 1 h at 4°C then adding 10 µl 50:50 slurry of lysis buffer and protein A Sepharose beads (Pharmacia) and incubating for a further 30 min. The beads were washed in the same manner as the peptide-coupled beads (Section 2.2.2). Immunoprecipitation with monoclonal antibodies was performed in a similar manner to immunoprecipitation with polyclonal antibodies but Protein G Sepharose beads (Pharmacia) were used instead of Protein A Sepharose beads. The concentrations of antibodies used were 10µl/ml lysate for αHA (0.4 mg/ml stock solution, Boehringer Mannheim) or 5µl/ml lysate for αmyc (9E10, ascites, Evan *et al.* 1985).

2.2.4 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (PAGE) was performed using a procedure modified from that of Laemmli (1970). Gels of width 1.5 mm or 0.8 mm were run using Life technologies apparatus. Resolving gels contained 7.5% (w/v) or 10% (w/v) acrylamide: bisacrylamide in a ratio of 37.5:1 (Scotlab), 375 mM Tris HCl pH 8.8 and 0.1% (w/v) SDS . Stacking gels consisted of 125 mM Tris HCl pH 6.8 and 4% acrylamide: bisacrylamide, and

0.1% (w/v) SDS. Both resolving and stacking gels were polymerised by the addition of ammonium persulphate and TEMED.

2.2.4.1 Staining SDS PAGE gels

Gels were stained with 0.1% (w/v) Coomassie brilliant blue R250 in 45% methanol/45% water/10% (v/v) acetic acid and then destained in the same solution. Alternatively, gels were silver-stained by the following procedure: thin gels (0.8 mm) were placed for 5 min in 50% (v/v) methanol, 12% (w/v) trichloroacetic acid and 2% (w/v) $CuCl_2$ and then washed for 5 min in solution A (10% [v/v] ethanol and 5% [v/v] acetic acid) followed by 5 min in 0.005% (w/v) $KMnO_4$, 1 min in solution A, 5 min in 10% (v/v) ethanol, 5 min in water and 5 min in 0.1% (w/v) $AgNO_3$. For developing, gels were rinsed for 20 sec in water, placed in 10% (w/v) K_2CO_3 for 1 min and then in a solution containing 2% (w/v) K_2CO_3 and 0.01% (v/v) formaldehyde until protein bands were visible. The reaction was stopped by placing the gel in solution A for 2 min and then rinsing with water. All the solutions were freshly made, and the gel was gently agitated on a rotary shaker throughout. The duration of each step was increased approximately 4-fold to stain thick gels (1.5 mm).

2.2.5 Western blotting

To perform a Western blot, the protein content of a gel was transferred to a polyvinylidene membrane (Immobilon-P, Millipore) by the "semi dry" method for 60-90 min at 200-250 mA. Transfer was performed in transfer buffer: 2.9 g/l glycine, 5.8 g/l Tris base, 0.0375% (w/v) SDS and 20% (v/v) methanol. To probe the resulting blot, the membrane was first blocked for 1 h in Tris-buffered saline (20 mM Tris pH 7.4, 150 mM NaCl) containing 0.1% (v/v) Tween-20 (TBS-T) and 5% (w/v) powdered milk (Marvel) with agitation on a rotary shaker. Antibodies were then diluted in this solution using the appropriate ratio. Standard dilutions were 1:2000 for αp60C, 1:1000 for αp60N and αDp110 (Leevers *et al.* 1996), 1:3000 for αHA (0.4 mg/ml stock solution, Boehringer Mannheim) and 1:10000 for αmyc (9E10, ascites, Evan *et al.* 1985). The membrane was agitated in the antibody solution for 1 h and then washed 3 times for 10 min each in TBS-T. The blot was next incubated with the appropriate secondary horse radish peroxidase-conjugated antibody diluted 1 in 2000 in this solution and washed again for 3 x 10 min in TBS containing 0.1% Tween-20. The blot was subsequently blotted dry, placed in enhanced chemiluminescence reagents (Amersham) for 1 min and then dried and exposed on X-ray film (Fuji).

2.2.6 Peptide sequencing

Proteins were affinity purified from approximately 10^{10} S2 cells (from 20 triple 175 cm^2 flasks), then resolved by SDS-PAGE and stained with Coomassie blue R250. Each band was excised and digested with lysyl endopeptidase C (Wako Chemicals). The resulting peptides resolved by passage through an AX-300 pre-column followed by a C18 (150 x 1 mm) column (Relasil). Selected peptides were subsequently sequenced at the 500 femtomole level using an ABI Procise system. These procedures were carried out by Alistair Sterling, Hans Hansen and Nick Totty.

2.2.7 Making antisera

Peptides with a CGG coupling linker at the N-terminus (Section 3.4.1) were dissolved whenever possible in PBS alone; DMSO was added where necessary to achieve solubility. The dissolved peptides were incubated for 2 h with maleimide-activated keyhole limpet haemocyanin as directed (Pierce). The resulting mixtures were dialysed against PBS overnight, then combined into pools of N-terminal (p60N) and C-terminal antigens (p60C) and aliquoted for immunisation. Two rabbits were immunised with each pool by Eurogentec, Belgium (rabbits SK155, SK156 to make αp60N and SK157, SK158 to make αp60C). The immunisation protocol was as follows. After, taking a preimmune bleed, rabbits were inoculated three times over two weeks then bled after another week. After this program, the rabbits were injected with antigen and bled every 28 days. This procedure was repeated until five bleeds had been collected. Most experiments performed with these antisera utilised the third bleed, with the appropriate preimmune sera as a control.

2.2.8 Protein kinase assays

For the lipid and protein kinase assays, the beads from experimental protein purifications were washed three times in lysis buffer and twice in 20 mM Tris HCl pH 7.4, 100 mM NaCl, and 0.1 mM EGTA. Protein kinase assays were performed in 30 µl of 20 mM Tris HCl pH 7.4, 100 mM NaCl, 0.1 mM EGTA, 2.5 mM $MgCl_2$, 100 µM ATP containing 2.5 µCi [γ-^{32}P] ATP for 30 min at room temperature and resolved by SDS-PAGE on 7.5% polyacrylamide gels. To visualise the results of the assays, the gels were dried and exposed on X-ray film (Hyperfilm, Amersham).

2.2.9 Lipid kinase assays

Lipid kinase was assayed essentially as described (Whitman *et al.* 1985) in 60 µl of 20 mM Tris HCl pH 7.4, 100 mM NaCl, 0.1 mM EGTA, 2.5 mM

$MgCl_2$, 100 µM ATP containing 2.5 µCi [γ-^{32}P] ATP and 200 µM sonicated phosphatidylinositol (Sigma). The reaction was incubated for 30 min at room temperature and terminated with acidified chloroform; next, the lipids were extracted and resolved by thin layer chromatography (TLC) with chloroform: methanol: 4 M ammonium hydroxide (45:35:10). After resolving for approximately two hours, the TLC plate was dried and exposed on X-ray film (Hyperfilm, Amersham).

2.3 Molecular biology techniques

Basic molecular biology techniques were performed essentially as described in Sambrook *et al.* (1989). All restriction enzymes, other modifying enzymes and kits were used according to the manufacturer's instructions. Only significant differences from standard techniques will be described in this section.

2.3.1 Transforming *E. coli* with plasmid DNA.

Competent XL1-Blue cells for use in all the ligations described in this thesis were prepared by a procedure modified from Hanahan (1983). A single colony from an agar plate containing LB (1% [w/v] bacto-tryptone, 0.5% [w/v], and 1% [w/v] NaCl) and tetracycline was inoculated into 20 ml TYM broth (2% [w/v] bacto-tryptone, 0.5% [w/v] yeast extract, 0.1 M NaCl, and 10 mM $MgSO_4$) and shaken at 37°C in a 250 ml flask until the A_{600} was 0.2–0.8. The culture was transferred to a 2 l flask containing 100 ml TYM broth and agitated at 37°C until the A_{600} reached 0.5–0.9, then a further 500 ml TYM broth was added and the incubation continued until the A_{600} reached 0.6. The culture was cooled rapidly by swirling the flask in an ice bath, then the bacteria were pelleted (5,000 *g*, 15 min), resuspended in 100 ml ice cold TfBI (30 mM KOAc, 50 mM $MgCl_2$, 100 mM KCl, 10 mM $CaCl_2$, and 15% [v/v] glycerol) by gentle shaking/vortexing. The bacteria were re-pelleted (5,000 *g*, 8 min) and resuspended in 20 ml TfBII (10 mM NaMOPS pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, and 15% [v/v] glycerol), then 0.6 ml aliquots were 'snap-frozen' in liquid nitrogen and stored at -70°C. Cells were transformed by the method described in (Pope and Kent 1996), in which the DNA is mixed with the cells on ice, incubated for 5 min and spread directly onto warm LB agar plates, without heat-shock.

2.3.2 Preparation of genomic DNA

To make genomic DNA for Southern blotting, 25 males and 25 females of each genotype were homogenised on ice in 400 µl solution A (0.1 M Tris HCl pH 8.8, 0.1 M EDTA, and 1% [w/v] SDS) in an eppendorf tube using a small pestle. The homogenate was then incubated at 70°C for 30 min. Next, 56 µl 8 M potassium acetate was added, and the lysate was incubated on ice for 30 min. The tube was centrifuged at full speed in a bench-top microfuge at room temperature and the resulting supernatant was transferred to a fresh tube. 200 µl isopropanol was then added and, after mixing, the tube was centrifuged again at full speed for 5 min at room temperature. The supernatant was next carefully aspirated, and the pellet was washed with 500 µl 70% (v/v) ethanol. After drying in a vacuum chamber, the pellet was resuspended in 100 µl water.

2.3.3 Preparation of mRNA and first strand cDNA

Poly A+ mRNA was prepared from guanidine isocyothionate lysates of S2 cells, third instar larvae, pupae and adult flies using oligo (dT) cellulose as directed (Stratagene). The resulting RNA was used both as a template to synthesise first strand cDNA with Moloney Murine Leukemia Virus reverse transcriptase as directed (Pharmacia) and for Northern blotting (Section 2.3.6).

2.3.4. Radioactive probe labelling

To perform Southern blotting, Northern blotting and library screening, radioactive DNA probes with incorporated [γ-^{32}P] dCTP were generated using random priming of the template and extension with Klenow polymerase as directed (Multiprime kit, Amersham). The probes were purified from unincorporated radionucleotides on a Nuc trap column (Stratagene) and eluted with 200 µl STE pH 8 (10 mM Tris HCl pH 8, 1 mM EDTA, and 100 mM NaCl).

2.3.5 Southern blotting

Lambda DNA was digested and prepared as described (Sambrook *et al.* 1989). Genomic DNA from flies was digested as follows: 40 µl DNA, prepared as described in Section 2.3.2.1 was gently stirred on ice with 2 µl restriction enzyme (10 U/µl) in a total volume of 100 µl with the appropriate buffer. The mixture was then incubated for 2 h at 37°C. 10 µl 3M sodium acetate pH 5.2 and 250 µl ethanol were added and, after further mixing, the tube was incubated on ice for 15 min and then centrifuged at full speed in a bench-top microcentrifuge for 15 min at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and resuspended in 20 µl water.

To optimise resolution, TBE (45 mM Tris borate and 1 mM EDTA) gels were used for Southern blots of genomic and lambda DNA. DNA samples and DNA markers were incubated at 56°C for 15 min before resolving overnight by agarose gel electrophoresis using a potential difference of 15-20 V. Before transferring to a membrane, the gel was photographed with a ruler, incubated in 200-400 ml denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 45 min, rinsed with distilled water and incubated in neutralising solution (200-400 ml 1.5 M NaCl, 1.0 M Tris HCl pH 7.4). Capillary transfer to HyBond N+ nylon filters (Amersham) was then set up as described (Sambrook *et al.* 1989) using 10x SSC (0.15 M Tris-sodium citrate, 1.5 M NaCl) as the buffer. After transferring overnight, the membrane was baked under vacuum at 70-80°C for 2 h.

To probe the blot, it was first prehybridised for 1 h at 65°C in Church Buffer (0.5 M sodium phosphate pH 7.2, 7% (w/v) SDS, 1 mM EDTA pH 8.0). The probe was denatured by boiling and then added to 10 ml Church buffer and hybridised to the blot at 65°C overnight. The blot was then washed 3 times with 2x SSC at room temperature and then 3 or 4 times in 0.1x SSC, 0.5% (w/v) SDS at 65°C over 1 h. The blot was then placed in cling-film and exposed on X-ray film.

2.3.6 Northern blotting

For the preparation and handling of RNA, all solutions were DEPC treated. mRNA samples prepared as described in Section 2.3.3 were resolved on a formaldehyde agarose gel and transferred to HyBond N membranes (Amersham) by standard methods (Sambrook *et al.* 1989), and probed as described for Southern blotting (Section 2.3.5).

2.3.7 Library screening

To isolate cDNAs encoding p60, a λgt10 eye imaginal disc cDNA library (kindly provided by Markus Noll) transformed into Y1090 cells was screened by probing duplicate HyBond N filters with the probe described in Section 3.2.2. After transferring the plaque DNA to the filters by contact with the petri dish, the filters were placed in denaturing solution for 1 min, then neutralising solution for 1 min and finally washed in 2x SSC for 2 min. The filters were probed as described for Southern blots (Section 2.3.5). Clones positive on the duplicate filters were purified by two further rounds of replating and screening. The cDNA was recovered from the isolated phage by standard methods (Sambrook *et al.* 1989).

2.3.8 PCR: standard, degenerate, long range

PCR amplifications with degenerate primers based on the peptide sequences derived from p60 (Table 3.1) were performed using first strand cDNA as a template and 0.025 U/µl Taq, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 µM each primer in a total volume of 50 µl. 40 cycles of amplification (94°C denaturation for 30 s, 50°C annealing for 30 s and 72°C extension for 60 s) were performed. A similar method was used for standard PCR amplification, except that 25-30 cycles of amplification were utilised and the annealing temperature and the time at 72°C were varied according to the primers used and length of product required, respectively. For long range PCR amplification, genomic DNA prepared as described in Section 2.3.2.1 was used as the template and the polymerase mix (Taq + Pwo) was used as directed (Expand^{TM}, Boehringer Mannheim).

2.3.9 DNA sequencing

Sequencing reactions were carried out using the PCR-amplification dye termination method as directed (Perkin Elmer) and were resolved on an ABI 373 automated DNA sequencer. To sequence the p60 cDNA, each fragment was sequenced in both directions with T3, T7 and p60-specific primers .

2.3.10 Subcloning and plasmid construction

A cassette encoding p60 tagged at the N-terminus with the HA epitope was generated in the following way. Firstly, a Vent DNA polymerase PCR product was amplified from pDW1 (pBluescript SK containing a 900 bp p60 cDNA fragment that includes the 5' UTR and the first 439 bp of the p60 open reading frame) using a T7 primer and a primer containing a NotI site, initiation codon, BamHI site and codons 2-6 of p60 (TCCCCGCGGTGGCGGCCGCACCACCATGGGATCCCAACCCTCCCCGCTCC). The product was digested with NotI and KspI and subcloned into the NotI/KspI-digested pDW1 to generate pDW2. pDW2 was linearised with BamHI and annealed oligonucleotides encoding YPYDDVPDYA (an unintentionally modified version of the HA epitope tag) flanked by cohesive BamHI ends were inserted to generate pDW3 (sense oligonucleotide, GATCCTATCCATATGATGACGTACCAGATTACGCTG, antisense oligonucleotide, GATCCAGCGTAATCTGGTACGTCATCATATGGATAG). An XbaI site was then inserted into the XhoI site of the pDW3 polylinker with two annealed oligonucleotides to generate pDW4 (sense oligonucleotide TCGACACCGCTCTAGAACG, antisense oligonucleotide, TCGACGTTCTAGAGCGGTG). A full-length p60 cDNA was then generated

by linearising pDW4 with EcoRI and XbaI and inserting an EcoRI/XbaI fragment of the p60 cDNA containing the remainder of the coding sequence and 102 bp of 3' UTR, thereby creating pBluescript-HAp60. pGMR-HAp60 was generated by digesting pBluescript-HAp60 with NotI/XbaI, blunt-ending the insert with Klenow DNA polymerase, and ligating it into EcoRI-digested, blunt-ended pGMR. pUAST-HAp60 was generated by subcloning the same blunt-ended HAp60 insert into a modified version of pUAST, which had been cut with XhoI/XbaI and blunt ended.

2.4 Molecular Genetics

2.4.1 Mapping P element insertion sites and deletion breakpoints

In addition to the Southern blot-based mapping described in Section 4.3.2, the insertion point of the P element *P[l(2)k14504]* was mapped by long range PCR amplification (Section 2.3.8). A 5.5 kb PCR product was obtained using DNA from this line as a template, a P element-specific primer and a primer from the first p60 exon. The identity of this PCR product was confirmed by Southern blotting.

In addition to the PCR-based mapping of the breakpoints of $p60^A$ described in Section 4.3.3 both $p60^A$and $p60^B$ were characterised by digestion of the appropriate genomic DNA with BamHI and EcoRI followed by Southern blotting and hybridisation with the probes shown in Figure 4.3 All these probes were PCR products generated using genomic primers and wild type DNA as a template except for the probes 946/P and 947/P which were PCR products generated using the genomic primers 946 or 947 and a primer based on the P element termini with DNA from $p60^A$/ *SM6a* flies as template (Section 4.3.3). The analysis of the results of these Southern blots firstly confirmed the deletion breakpoints that has been determined by PCR and sequencing and secondly, demonstrated that $p60^A$ contained an insertion of P element-derived DNA of approximately 6-7 kb. Finally, the Southern blot analysis allowed the breakpoints of $p60^B$ to be placed within the boundaries specified in Figure 4.3.

2.5 Genetics

2.5.1 Making transgenic flies

Transgenic flies were generated by P element-mediated germline transformation. For injection with DNA, *yw* embryos were prepared as follows: eggs laid on apple-juice agar plates during a 30 min period were washed, dechorionated by incubation for 1 min in a 50: 50 mix of household

bleach and water and washed again thoroughly. After arranging the embyros in a line of 50-100 embyros orientated in the same anterior: posterior direction, they were dried by incubation in jar containing silica gel. The dried embryos were covered with a layer of Voltalef 10S oil (Elf Atochem) and injected with DNA at the posterior end using a inverted microscope and a glass capillary needle. The arranging, drying and injecting of embyros were carried out at 18°C. The plasmid DNAs to be injected were produced using a Qiagen midi prep kit and co-precipitated with pTurbo, a transposase-encoding plasmid with disabled P element repeats (D. Rio, Flybase), which was also purified with a Qiagen midi prep kit. Syncitial embryos were injected with a solution containing 0.8 µg/µl transgene plasmid and 0.2 µg/µl pTurbo. Cellularised embryos were killed with the injecting needle. After injection, the embryos were incubated at 18°C until they hatched. Hatched larvae were placed in standard fly food and allowed to develop to adulthood. Adults (G_0s) were crossed in groups of two or three balancer flies and their progeny (F_1s) scored for red eyes (w^+), which indicated germline integration of the transgene.

Flies carrying the transgene were mapped by further crosses to flies with balancer chromosomes. Lines derived from F_1s from the same tube, that mapped to the same chromosome and had the same eye colour intensity were considered to be derived from the same insertion and in this case, only one line was maintained.

2.5.2 Lines used in experiments

The *yw* line originating from the Ernst Hafen's laboratory was used as wild type controls and the background for transgenic lines. *Oregon R* was used as wild type control line where indicated.

Name	Line(s)	Synonym	Source	Ref/Note
Chapter 4				
Df(2L)al	*Df(2L)al / CyO*	U-45110	Umea	
Df(2L)net -PMF	*Df(2L)net -PMF/SM6a*	B-3638	Bloomington	
P[l(2)k14504]	*yw; P[w+ l(2)k14504] /CyO*	k14504	BDGP	(Torok *et al.* 1993)
P[l(2)k00616]	*yw; P[w+ l(2)k00616] /CyO*	k00616	BDGP	(Torok *et al.* 1993)
P[l(2)06751]	*P[ry+ l(2)06751] /CyO*	P06751 B-2323	Bloomington	(Rudner *et al.* 1996)

mbm^{1}			Martin Heisenberg	(de Belle and Heisenberg 1996)
p60^{A}	*p60*^{A} */SM6a*	PA5	Martin Heisenberg	
p60^{B}	*p60*^{B}*/CyO*	E8	Donald Rio	(Rudner *et al.* 1996)
P[gR10]	*w; P[w+ gR10]*		Joan Hooper	
P[gp60]	1.12 on III			Lost
	2.24a on II	DW21		
	2.24b on II	DW22		
	2.3a on III	DW20		No rescue
P[gH] ,Dp110^{A}			Sally Leevers	
P[gH] ,Dp110^{B}			Sally Leevers	
y w P[hs-FLP ry^{+}*]; P[w*$^{+}$ *hs-π–myc] FRT40A/CyO*)			Ernst Hafen	

Chapter 5

GMR-HAp60	94.1a on II	DW1
	94.2a on I	DW2
	94.2b on II	DW3
	94.3b on I	DW4
GMR-HAΔp60	95.2c on III	DW5
	95.5c on I	DW6
	95.6b on III	DW7
	95.9c on I	DW8
UAS-HAp60	92.1b on II	DW9
	92.2c on II	DW10
	92.3c on III	DW11
	92.4a on I	DW12
	92.5a on II	DW13
UAS-HAΔp60	93.1a on II	DW14
	93.2c on II	DW15
	93.2d on III	DW16
	93.3b on III	DW17
	93.3c on III	DW18

	93.6a on III	DW19		
GMR-mycDp110	SJ68		Sally Leevers	
	SJ69		Sally Leevers	
GMR-mycDp110^{D954A}	Sj83		Sally Leevers	
	SJ86		Sally Leevers	
UAS-mycDp110	SJ9		Sally Leevers	(Leevers *et al.* 1996)
UAS-mycDp110^{D954A}	SJ17		Sally Leevers	(Leevers *et al.* 1996)
GMR-GAL4			Matthew Freeman	
MS1096				(Capdevila and Guerrero 1994)
hsGAL4			Ernst Hafen	

The FRT line for $p60^B$ was made by recombining a $p60^B$ chromosome with an *FRT40A*, w^+ chromosome and screening for w^- flies containing a chromosome with the FRT, on the basis of the geneticin resistance that it confers, and $p60^B$, on the basis of non-complementation with $p60^A$.

2.6 Phenotypic analysis

2.6.1 Light microscopy and CCD camera

All light microscopy images were recorded with a Leaf CCD camera (Entwistle 1999), and using either a Zeiss Axiophot microscope or a Leica dissecting microscope.

2.6.2 Preparation of eye sections

Heads were removed from adult flies using fine forceps, then bisected with a razor blade and placed in Ringer's solution on ice. The heads were next fixed on ice in 1% osmium tetraoxide, 2% glutaraldehyde in a cacodylate buffer for 30 min, then transferred to 2% osmium tetraoxide for a further 90-120 min. The eyes were serially dehydrated in acetone, then placed in 1:1 acetone: Spurr (24% [w/w] 4-vinylcyclohexenedioxide, 63% [w/w] 2 nonen 1-ylsuccinic anhydride, 12% DER [w/w], 1% [w/w] dimethylaminoethanol [all from Fluka]) and left overnight. The solution was exchanged for 100% Spurr,

and left for 4 h, then the eyes were orientated and the resin was polymerised by incubation at 80°C for 24 h. The eyes were then sectioned using a microtome with glass knives. The sections were 1-2 μm in width and were collected by floating onto water and then placing on a slide. The sections were dried onto the slide by placing it on a hot heat block, then stained with 0.5% (w/v) Azur II, 0.5% (w/v) methylene blue and 0.5% (w/v) sodium tetraborate, mounted in DPX mountant (Fluka) and flattened by placing weights on the coverslip and incubating at approximately 60°C overnight.

2.6.3 Mounting wings

Wings were dehydrated in ethanol, mounted in euparal (Agar Scientific) and allowed to harden at around 60°C overnight.

2.6.4 Scanning electron microscopy

Adult flies were prepared for SEM either by standard methods in which acetone is used for dehydration or by the following method devised to preserve eye structure. Adult flies were first fixed in 5% (w/v) glutaraldehyde for 4 h, then after performing a serial dehydration into 100% ethanol, and incubating in 100% amyl acetate for 1 h, they were critical-point dried and gold coated.

SEM at 10 kV or 15 kV was performed according to standard procedures using a JEOL 4500LV scanning electron microscope. To allow the comparison of size, the voltage and current were kept constant and the images focused by manual manipulation of the specimen stage. The resulting images were analysed using Photoshop and NIH Image. Specifically, ommatidia were counted by using a extra layer in Photoshop to put a coloured dot on each ommatidia. These dots were counted by exporting the layer into NIH Image.

Chapter 3: Identification and characterisation of p60, an adaptor for the *Drosophila* Class I_A PI3K

Chapter 3: Identification and characterisation of p60, an adaptor for the *Drosophila* Class I_A PI3K

3.1 Introduction

The aim of the research described in this thesis is to develop and utilise *Drosophila* as a model system to study Class I_A PI3K function *in vivo*. The essential foundation for this approach was to identify and characterise the adaptor for the *Drosophila* Class I_A PI3K, Dp110, for three reasons. Firstly, ascertaining whether Dp110, like mammalian Class I_A PI3Ks, possesses an SH2 domain-containing adaptor would support the relevance of using *Drosophila* as a model for the study of Class I_A PI3Ks. Secondly, knowledge of the adaptor would be essential to understand the structure/function relationships and *in vivo* regulation of the *Drosophila* Class I_A PI3K. Finally, the identification of an adaptor would allow the generation of genetic and biochemical reagents to investigate *in vivo* function that would be complementary to, yet independent of, similar reagents created for Dp110.

When this research was initiated in 1995, it was already known that *Drosophila* possess a Class I_A PI3K, Dp110, as well as a Class II and a Class III PI3K (MacDougall *et al.* 1995). Although there is moderate conservation between the amino acid sequence of the adaptor binding region in Dp110 and that of the mammalian Class I_A PI3Ks (20% amino acid identity, Leevers *et al.* 1996), Dp110 had not been shown to bind to an SH2 domain-containing adaptor. One possible strategy to identify an adaptor for Dp110 would have been to design degenerate DNA primers or probes based on the amino acid sequences of the mammalian adaptors and use PCR or low stringency hybridisation to identify the gene. However, adaptors for Class I_A PI3Ks had only been identified in mammals, so it was not clear whether there would be sufficient homology for this strategy to work. Even if there was sufficient homology, it would be difficult to predict which amino acids would be conserved. Furthermore, upon identification of a candidate adaptor, it would still be necessary to demonstrate that the encoded protein associated with Dp110.

Therefore, the selected approach was to use biochemical methods to purify the adaptor for Dp110 from lysates prepared from the *Drosophila* S2 cell line. Neither immunoprecipitation with monoclonal antibodies against the mammalian adaptors nor affinity purification using GST-Dp110 as a "bait" was successful, so I next tried another technique that had previously been used to purify mammalian Class I_A PI3Ks. This chapter describes the affinity purification

of Dp110 in complex with an SH2 domain-containing adaptor using a pYXXM phosphopeptide affinity matrix.

3.2 Identification of an adaptor for Dp110

3.2.1 Affinity purification of p60 using pYXXM phosphopeptides

Phosphopeptides, containing either one or both of the pYXXM motifs found at positions 740 and 751 of the human PDGFβ receptor, have been shown to bind selectively to the mammalian Class I_A PI3K adaptors p85α and β (Panayotou *et al.* 1993, Section 1.3.2.2). When coupled to agarose beads, these phosphopeptides can be used to affinity purify heterodimeric complexes containing the adaptors bound to mammalian Class I_A PI3Ks (Otsu *et al.* 1991; Fry *et al.* 1992). The same approach was taken to purify the adaptor for Dp110. The tyrosine-phosphorylated peptide GGYMDMSKDESVDpYVPML (Y751) was coupled to agarose beads and used to purify proteins from a *Drosophila* S2 cell lysate and a human U937 cell lysate (Sections 2.1.1, 2.2.1, 2.2.2). The purified products were resolved by SDS-PAGE and visualised by silver staining (Figure 3.1, Section 2.2.4). The three major proteins purified from the human cell line had been previously identified as various isoforms of p85, p110 and PLCγ (Ivan Gout, personal communication). Three proteins of approximately 145, 120 and 60 kD were purified from the *Drosophila* cell line (designated p145, p120 and p60). The same proteins were purified regardless of whether the peptide was phosphorylated on tyrosine 740, tyrosine 751 or on both tyrosine 740 and tyrosine 751 (Y740, DPY, Figure 3.1B).

The affinity-purified complex possessed lipid and protein kinase activities, and immunoblotting with αDp110 antisera showed that the 120 kD protein was Dp110 (Section 3.4). Dp110 has no SH2 or PTB domains with which to bind the peptide directly so either p60 or p145 was likely to be the adaptor. To identify the protein that was necessary for Dp110 to bind to the pYXXM peptide, the complex was washed with lysis buffer containing increasing concentrations of sodium chloride (Section 2.2.2). Washing with lysis buffer containing 2 M NaCl removed the majority of the 145 kD protein, and had no effect on the staining intensity of the 120 kD or 60 kD proteins, suggesting that p60 was the adaptor (Figure 3.1C). At the time that this experiment was performed, p85α and p85β were the most commonly recognised adaptors in mammals and, even though $p55^{PIK}$ had been identified (Pons *et al.* 1995), it was expected that an 85 kD protein would associate with Dp110. However, it is now known that the p85α gene encodes 55 kD and 50

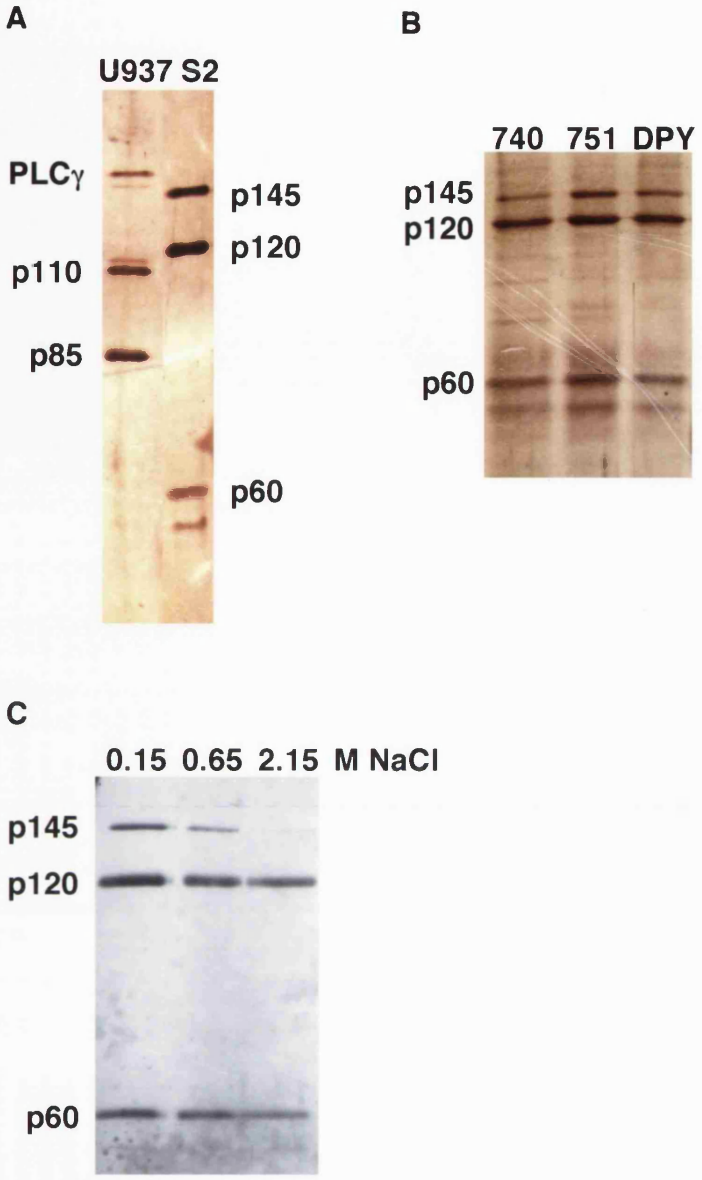


Figure 3.1. Affinity purification with pYXXM phosphopeptides implicates p60 as the adaptor for Dp110. (**A**) Y751 coupled to agarose beads was used to purify three major proteins from the human U937 cell line, and p60, p120 and p145 from the *Drosophila* S2 cell line. (**B**) The same three proteins were purified from S2 cells using Y740, Y751 and DPY (**C**) p145 was removed by washing with increasing concentrations of salt. The final washes were carried out in buffer containing the indicated concentrations of NaCl. Lysates from approximately 10^8 cells were used for each sample, resolved by SDS -PAGE (A and C , 7.5%, B,10% acrylamide gel) and silver stained.

kD splice variants that also associate with Class I_A PI3Ks (Section 1.3.2.1, Antonetti *et al.* 1996; Fruman *et al.* 1996; Inukai *et al.* 1996).

In order to identify the affinity-purified proteins and obtain peptide sequence information for cloning, a large-scale affinity purification was undertaken using approximately 10^{10} S2 cells. Alistair Sterling, Hans Hansen and Nick Totty resolved the purified product by SDS-PAGE and sequenced the peptides derived from digestion of p60, p120 and p145 by the Edman degradation method (Section 2.2.6). Three of the peptide sequences obtained from p120 confirmed that it was Dp110 (LMANYTGL, EYQVYGISTFN and LHVLE, Leevers *et al.* 1996). Two peptides sequences from p145, FMEXIYTDVR and FXNNXXCGYIL, revealed that this protein was *Drosophila* phospholipase Cγ (PLCγD, Emori *et al.* 1994). This result is consistent with human PLCγ1 also being affinity purified from human cell lines using the same pYXXM phosphopeptide (Figure 3.1A, see above). Furthermore, the C-terminal SH2 domain of mammalian PLCγ1 has been shown to interact with pYXXM motifs, although it preferentially binds to phosphotyrosines in other sequence contexts (Panayotou *et al.* 1993; Songyang *et al.* 1993). Nine peptide sequences were obtained from p60 (Table 3.1).

3.2.2 Cloning of the p60 cDNA through the use of degenerate PCR

In order to clone the cDNA that encoded p60, degenerate primers for use in PCR amplification were designed, based on the amino acids derived from the sequenced peptides (Table 3.1). When designing the primers, an attempt was made to maximise the theoretical melting temperature (Tm) and to minimise degeneracy. Tm was maximised by using the longer peptides to design long primers and, wherever possible, by using amino acids with GC-rich codons. To minimise degeneracy, the primers were based on amino acids represented by the fewest codons (e.g. methionine or tryptophan), particularly at the 3' end of the primer. Furthermore, at the 5' end, where specificity was less important, deoxyinosine was used instead of highly degenerate nucleotides, because inosine is thought to base-pair with A, T, G, and C, and has been successfully used for cloning by others (e.g. Aarts *et al.* 1991).

To make a template for PCR amplification, mRNA was purified from S2 cells and reverse-transcribed to generate first strand cDNA (Section 2.3.3). PCRs were carried out with all possible primer pairs using this cDNA as a template (Section 2.3.8). The primers based on peptides 6 and 9 yielded a 200 bp product that was cloned into pGEM-T, sequenced, and found to encode peptides 7 and 8. This fragment was used to screen a λgt10 eye imaginal disc cDNA library (Section 2.3.4). Four positive clones were isolated. These clones

Peptide Sequence	Degenerate Primers	Sequence from p60 cDNA
1.(K)XILHGK		KSILHGK (43-49)
2.(K)XNSFNAIREXXK		KRNSFNAIREELQEK (164-179)
3.(K)LGQSVFGNTEK	MW405 sense (K) L G Q S V F G N T E K AARYTIGGICARWSIGTITTYGG	KLHQSVFGNTEK (180-191)
4.(K)XPWNQIK	MW411 antisense K I Q N W P X K TCRTTYTTIATYTGRTTCAANGG	KIFRNQIK (191-198)
5.(K)ANVSNMK	MW406 sense K A N V S N M K AARGCIAAYGTIWSIAAYATGWA	KLNESFMK (198-205)
6.(K)LQELFHYMENK	MW407 sense (K) L Q E L F H Y M E N K: CARGARYTITTYCAYTAYATGGA MW408 antisense K N E M Y H F L E Q L K TCCATRTARTGRAAIARYTCYTG	KGQVLNHYMENK (255-266)
7.(K)XELQILQLRK	MW409 antisense K R L Q L I Q L E CIIARYTGIARDATYTGIARYTC	KPELQILQLRK(281-291)
8.(K)YIERLK		KYIERLK (293-299)
9.(K)XQQLYETVSNQ..	MW410 antisense Q N S V T E Y L Q Q SWIACIGTYTCRTAIARYTGYTG	KWQQLYETVSNQ (318-329)

Table 3.1. Peptides and primers used to isolated the p60 cDNA. A list of peptides from p60, indicating their sequence as predicted by Edman degradation, the degenerate primers that were designed based on their sequence and their predicted sequence based on the p60 cDNA (Figure 3.2A). The peptides were created by digestion with lysl-endopeptidase, so they were expected to be preceded by a lysine residue. Similarly, when a lysine was recovered from Edman degradation, it indicated the end of the peptide. Peptides 5 and 6 came from the same peptide fraction and the cDNA-derived sequence showed that some residues were assigned to the wrong peptide. For the degenerate primers: R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, N=A+C+G+T, V=G+A+C, I=inosine.

were restriction-mapped and, after subcloning into pBluescript SKII, the 5' and 3' ends of the restriction fragments were sequenced (Section 2.3.9). This analysis demonstrated that all four clones were identical, so one clone was chosen for further analysis. To establish the p60 cDNA sequence, the 0.9 kb and 2.2 kb EcoRI fragments generated from the digestion of this clone were sequenced in both directions with sequence specific primers as well as with T3 and T7 (Bluescript primers, Section 2.3.9).

Sequencing revealed that the EcoRI site between the two subcloned fragments is situated in the open reading frame of the cDNA. Because the original cDNA might have contained a small EcoRI fragment between these two sequenced fragments, which could have been lost during the subcloning, sequence-specific primers were used to PCR amplify across the internal EcoRI site using the original lambda clone as a template. The product was sequenced directly and this analysis revealed that the two isolated EcoRI fragments were adjacent in the original cDNA. The sequence derived from these two fragments contained an open reading frame that was predicted to encode a 57.5 kD protein containing all nine of the peptide sequences recovered from p60 (Table 3.1 and Figure 3.2).

3.3 Analysis of the p60 amino acid sequence

3.3.1 Homology with mammalian adaptors

The predicted amino acid sequence of p60, like the sequences of the mammalian adaptors for Class I_A PI3Ks, includes two SH2 domains and an inter-SH2 domain. However, the SH3 and BH domains found in p85α and β, the N-terminal extensions of $p55^{PIK}$ and the p55α splice variants, and the proline-rich SH3 domain-binding motifs found in all mammalian adaptors, are absent from p60 (Figure 3.3, Section 1.3.2.1). Instead, p60 has a short N-terminus (similar in size to the N-terminus of p50α, a splice variant of p85α) and a unique C-terminus of 70 amino acids that shows no significant similarity to other known proteins. When the amino acid sequences of the core SH2–inter-SH2–SH2 regions of p60, p85α, p85β and $p55^{PIK}$ were compared, p60 showed the same degree of similarity to all three mammalian adaptors (Figure 3.2, data not shown for p85β).

3.3.2 The SH2 domains of p60

In comparison to the mammalian adaptors, the N-terminal and C-terminal SH2 domains are the most conserved regions of p60, possessing 58% and 48% amino acid identity with the respective domains of bovine p85α

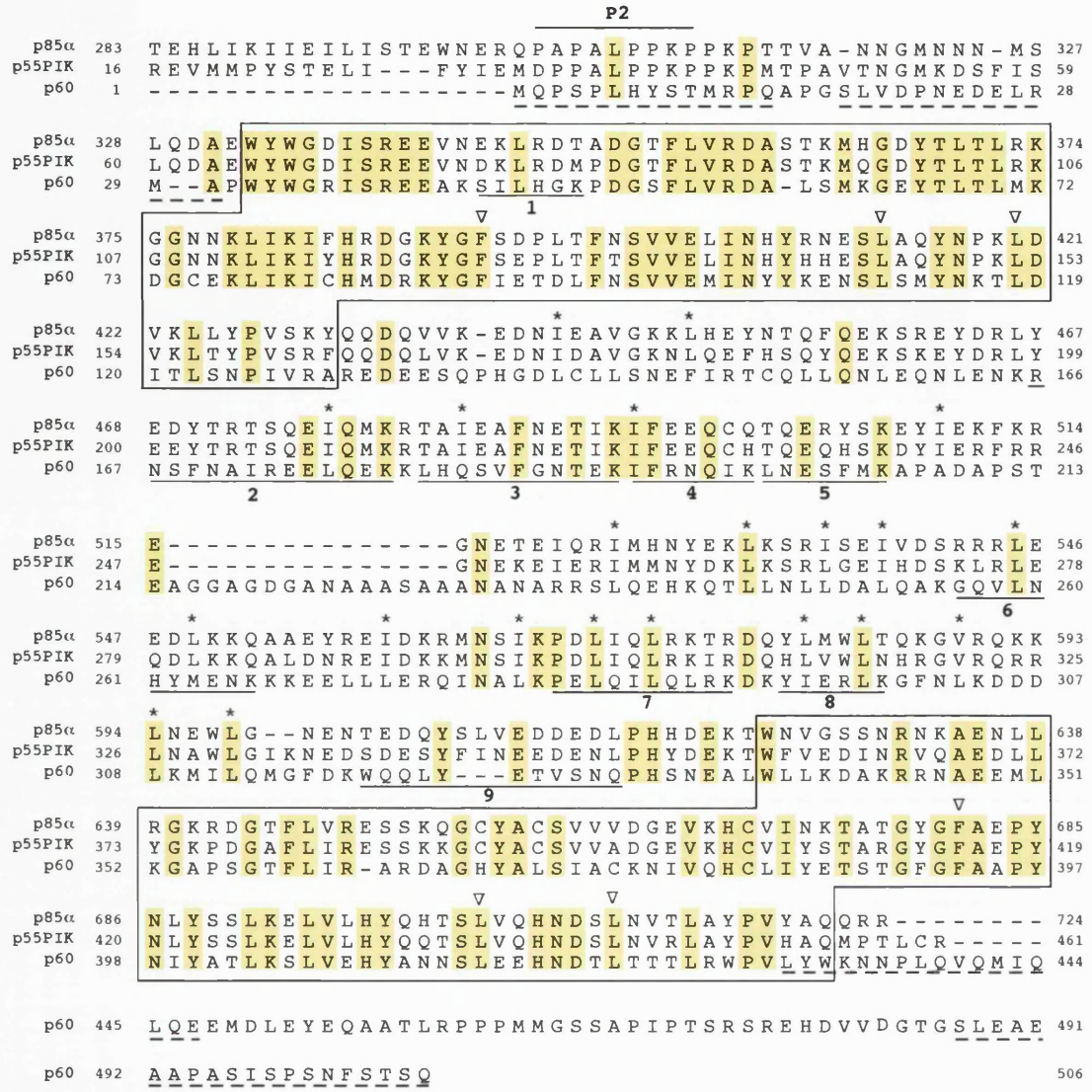


Figure 3.2. Amino acid sequence comparison of p60 with the mammalian adaptors. The amino acid sequence of p60 aligned with the homologous regions of bovine p85α (Otsu *et al.* 1991) and mouse p55PIK (Pons *et al.* 1995). The alignment was generated with the pileup algorithm of GCG (Devereux *et al.* 1984). Amino acids identical in all three sequences are shaded. The SH2 domains are boxed. The nine peptides sequences obtained are underlined by solid lines. The sequences used to design antigenic peptides are underlined by dashed lines. Conserved hydrophobic residues in the inter-SH2 domains predicted to be involved in coiled-coil structures are marked with an asterisk. The phenylalanine and leucine residues implicated in the SH2 domain binding specificity are marked with a triangle. The proline rich motif found in the mammalian adaptors is indicated as P2.

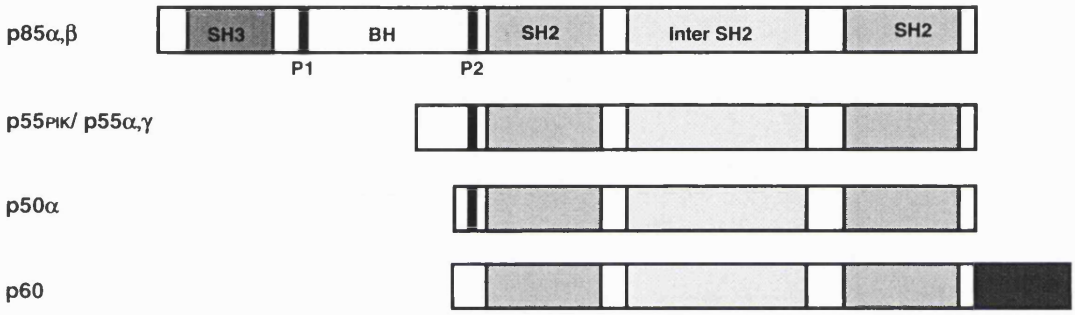


Figure 3.3. Comparison of the domain structure of p60 with mammalian adaptors for Class I_A PI3Ks. The relative positions of the SH3, BH, SH2 and inter-SH2 domains are shown for p85α/β. The proline-rich motifs are indicated as P1 and P2. The C-terminal extension of p60 is shaded.

(Figure 3.2). The three-dimensional structures of the N-terminal and C-terminal SH2 domains of p85α in complex with pYXXM phosphopeptides have been determined by X-ray crystallography and nuclear magnetic resonance (Breeze *et al.* 1996, Section 1.3.2.2; Nolte *et al.* 1996). These studies revealed that both the N-terminal and the C-terminal SH2 domains form a structure containing two binding "pockets". One pocket, common to all SH2 domains, has been shown to bind the phosphotyrosine residue of the target protein. The other pocket, which is unique to the SH2 domains of the adaptors for Class I_A PI3Ks, provides a hydrophobic environment that allows the specific binding of methionine three residues C-terminal to the phosphotyrosine. In p60, the amino acids involved in forming both these pockets are conserved. Specifically, the p60 sequence contains the phenylalanine of the beta strand E and the leucines of the loop between the alpha helix B and the beta strand G, indicated by triangles in Figure 3.2, which together define the methionine-binding pocket (Figure 1.3, Breeze *et al.* 1996; Nolte *et al.* 1996).

3.3.3 The inter-SH2 domain of p60

The inter-SH2 domain of mammalian p85α mediates binding to the Class I_A PI3K, p110α. Modelling studies of this inter-SH2 domain predict a two or four helix antiparallel coiled-coil structure similar to that revealed by X-ray crystallographic analysis of the inter-SH2 domain of ZAP-70 (Marketa Zvelebil personal communication, Dhand *et al.* 1994a; Hatada *et al.* 1995). The inter-SH2 domain is highly conserved among the mammalian adaptors (70% amino acid identity or greater, depending on the region examined). In contrast, the inter-SH2 domain of p60 is approximately 20% identical to the corresponding region of the mammalian adaptors. In spite of this low homology, this region of p60 is likely to form a similar structure because it contains the leucine-rich heptad repeats characteristic of coiled-coil alpha-helical bundles (Figure 3.2A). Furthermore, BLITZ and BLASTP database searches show that this inter-SH2 region is significantly homologous to the coiled-coil regions of proteins that form stable heterodimers (Table 3.2, Smith and Waterman 1981; Altschul *et al.* 1990). Thus, the sequence analysis of p60 further supports the prediction that the inter-SH2 domains of the adaptors for Class I_A PI3Ks form a coiled-coil structure.

			High Score	Smallest Sum Probability P(N)	N
Sequences producing High-scoring Segment Pairs:					
1	**pir:A55094**	**chromosomal protein XCAP-C - African claw...**	**77**	**0.00017**	**3**
2	sp:P115_RAT	GENERAL VESICULAR TRANSPORT FACTOR P115 (...	64	0.0029	4
3	pir:A55913	transcytosis-associated protein p115 - ra...	63	0.0054	4
4	pir:S49461	synaptonemal complex protein 1 - mouse>pr...	62	0.0073	2
5	gp:MUSTP_1	testicular protein [Mus musculus]	62	0.0076	2
6	sp:MLP1_YEAST	MYOSIN-LIKE PROTEIN MLP1.>pir:S38173 myos...	74	0.014	3
7	pir:A40937	bullous pemphigoid antigen 1 precursor - ...	53	0.014	4
8	prf:1717295A	bullous pemphigoid antigen BPAG1 - Homo s...	53	0.014	4
9	gp:HUMBPAG1B_1	bullous pemphigoid antigen [Homo sapiens]	53	0.014	4
10	sp:DMD_CHICK	DYSTROPHIN.>pir:S02041 dystrophin, muscle...	52	0.018	4
11	prf:1504190A	dystrophin - Gallus gallus (chicken)	52	0.018	4
12	pir:A42771	reticulocyte-binding protein 1 - Plasmodi...	57	0.023	3
13	sp:RBP1_PLAVB	RETICULOCYTE BINDING PROTEIN 1 PRECURSOR....	57	0.023	3
14	gpu:MMU40342_1	ninein [Mus musculus]	66	0.025	3
15	prf:2102219A	Golgi complex 372kD protein - Homo sapien...	62	0.032	4
16	pir:A56539	giantin - human>pir:S37536 macrogolgin - ...	62	0.033	4
17	prf:1911377A	myosin-like protein - Saccharomyces cerev...	71	0.035	3
18	pir:S24577	ovarian protein - fruit fly (Drosophila m...	57	0.042	2
19	gp:HAMSYN1A_1	synaptonemal complex protein [Mesocricetu...	57	0.053	2
20	pir:JH0820	160K golgi antigen - human (fragment)	69	0.11	2
.					
.					
.					
.					
65	**sp:P85A_BOVIN**	**PHOSPHATIDYLINOSITOL 3-KINASE REGULATORY ...**	**52**	**0.63**	**2**
66	**sp:P85A_HUMAN**	**PHOSPHATIDYLINOSITOL 3-KINASE REGULATORY ...**	**52**	**0.63**	**2**
67	**sp:P85A_MOUSE**	**PHOSPHATIDYLINOSITOL 3-KINASE REGULATORY ...**	**52**	**0.63**	**2**
68	**sp:P85B_BOVIN**	**PHOSPHATIDYLINOSITOL 3-KINASE REGULATORY ...**	**52**	**0.63**	**2**
69	**pir:A38747**	**phosphatidlyinositol 3-kinase (EC 2.7.1.-...**	**52**	**0.63**	**2**
70	**prf:1710141A**	**p85 protein - Mus musculus (house mouse)**	**52**	**0.63**	**2**
71	**prf:1710142A**	**PI3 kinase assocd p85 protein - Homo sapi...**	**52**	**0.63**	**2**
72	**prf:1710143B**	**p85beta protein - Bos primigenius taurus ...**	**52**	**0.63**	**2**
73	sp:CUT3_SCHPO	CHROMOSOME SEGREGATION PROTEIN CUT3.>pir:...	71	0.63	2
74	sp:YM92_CAEEL	HYPOTHETICAL 83.2 KD PROTEIN M01A8.2 IN C...	41	0.64	4
75	gp:HUMEEAP_1	endosome-associated protein [Homo sapiens]	55	0.65	4
76	pir:PW0009	myosin heavy chain rod, breast skeletal m...	62	0.69	2
77	prf:1814420D	fast myosin:SUBUNIT=heavy chain:ISOTYPE=C...	63	0.69	2
78	pir:S39081	myosin heavy chain, adult - chicken (frag...	63	0.69	2
79	sp:KINH_HUMAN	KINESIN HEAVY CHAIN.>pir:A41919 kinesin h...	51	0.70	3
80	sp:MYS3_HYDAT	MYOSIN HEAVY CHAIN, CLONE 203 (FRAGMENT)....	57	0.73	2
81	sp:LMB1_HUMAN	LAMININ BETA-1 CHAIN PRECURSOR (LAMININ B...	62	0.74	2
82	prf:1310285A	laminin B1 - Homo sapiens (man)	62	0.74	2
83	**gpu:RATP3KD_1**	**phosphatidylinositol 3-kinase [Rattus sp.]**	**50**	**0.76**	**2**

Table 3.2. BLAST analysis of the inter-SH2 domain of p60. BLAST search showing the low, but significant homology of the p60 inter-SH2 domain to several proteins that form coiled coil structures. For example, XCAP-C forms a heterodimer with XCAP-E through its coiled-coil domain (Hirano and Mitchison 1994). The mammalian adaptors were also identified in the search but with very low scores.

3.4 Biochemical analysis of Dp110/p60

3.4.1 Generation of p60-specific antisera

In order to confirm biochemically that the cloned cDNA did encode the protein purified with pYXXM peptides as well as to provide reagents for further study, peptides were designed to use as antigens to generate p60-specific antisera. The peptides were based on the N terminus (CGGMQPSPLHYSTMRPQ and CGGSLVDPNEDELRMA) and the C terminus (CGGLYWKNNPLQVQMIQLQE and CGGSLEAEAAPASISPSNFSTSQ) of p60 (Figure 3.2). An attempt was made to use sequences most likely to be exposed *in vivo* and less likely to be buried in the protein core. The sequences were chosen by the following criteria. Firstly, alpha helical structures, which can be predicted by the peptide structure algorithm of the Genetics Computer Group, Wisconsin (GCG), are more likely than other structures to have an exposed surfaces. Secondly, the N- and C-termini of p60 are also likely to be exposed. A further advantage of antisera directed to the termini is that are less likely to interfere with the functions of the SH2 and inter SH2 domains. A N-terminal cysteine was included in each peptide to enable coupling to maleimide-activated keyhole limpet haemocyanin (KLH), and two glycine residues were added to provide an N-terminal spacer. After coupling, the peptide/KLH complexes were combined to generate N-terminal and C-terminal pools, and each pool was used to immunise two rabbits (Eurogentec, Belgium, Section 2.2.7).

This technique of pooling peptides, developed by Kyo Higashi, maximises the chance of obtaining useful antisera. The resulting antisera can be affinity purified to resolve different immunoreactivities or can be used as antisera that might be directed against more than one site on the protein. In order to test the antisera raised to the p60 peptides, they were used for Western blot analysis of an S2 cell lysate and an affinity purification with the DPY phosphopeptide from an S2 cell lysate (Section 2.2.5). Antisera raised to the N-terminal peptides recognises affinity-purified p60 but does not recognise p60 in an S2 whole cell lysate, whereas antisera raised to the C-terminal peptides recognises p60 both in the S2 cell lysate as well as in the affinity purification (Figure 3.4). These findings confirmed that the adaptor for Dp110 has been cloned and that the antisera (designated αp60N and αp60C, respectively) would be useful for further experiments.

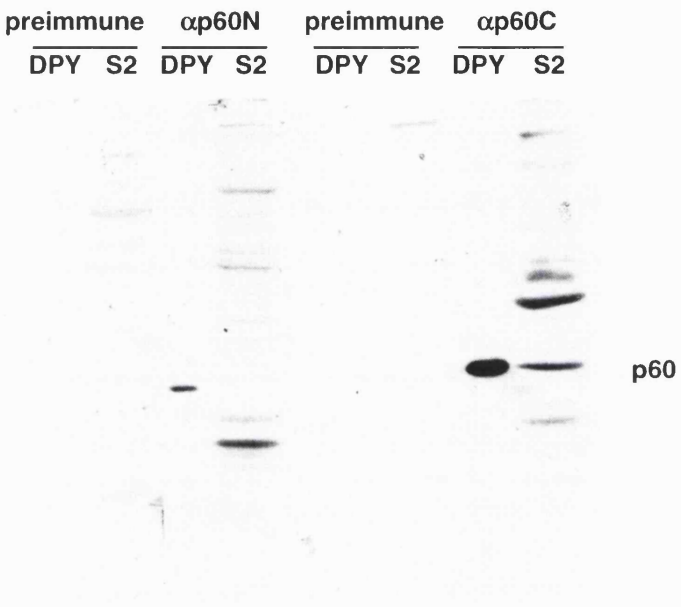


Figure 3.4. Testing αp60 antisera. Individual Western blots demonstrate that the antisera from rabbits injected with the N terminal peptides and C terminal peptides recognise a 60 kD protein in an S2 whole cell lysate and in an pYXXM affinity purification (DPY).

3.4.2 Affinity purification of p60 and Dp110 from different stages of the *Drosophila* life cycle

The antisera described above allow the unambiguous identification of p60, so they were next used to characterise the expression of p60 at different stages of the *Drosophila* life cycle. p60 was initially purified from the S2 cell line, an embryonically derived cell line that may no longer express proteins in manner that is representative of real *Drosophila* tissues. To check whether p60 is expressed in the intact organism, lysates of third instar larvae, pupae and adult flies were used for affinity purification with pYXXM phosphopeptide beads (Sections 2.2.1 and 2.2.2). The purified proteins were resolved by SDS-PAGE and either silver stained or Western blotted (Sections 2.2.4 and 2.2.5). The Dp110/p60 complex was present in all the lysates, although lower levels were consistently recovered from larvae than from the other stages (Figure 3.5). Immunoblotting with αp60N and αp60C and antisera against Dp110 confirmed the presence of these proteins (Figure 3.5B, Leevers *et al.* 1996).

An additional 55 kD protein is detected by αp60N, but not by αp60C (p60*, Figure 3.5). This protein might be a form of p60 that has been degraded from the C-terminus, a splice variant of p60 lacking the C-terminus, or the product of another gene that cross-reacts with αp60N. It seems most likely that this protein is a degraded form of p60 because its appearance coincides with lower levels of full length p60 and because lysate preparation in the absence of certain protease inhibitors or following the freezing and thawing of samples resulted in increased levels of this smaller protein. Furthermore, ectopically expressed, epitope-tagged p60 was found to degrade in a similar way (Section 5.4.1). In addition, the exon/intron structure of the p60 gene indicates that the 55 kD band is unlikely to be encoded by an alternatively spliced transcript (Section 4.2.2). Further experiments will be needed to ascertain whether the degraded form of p60 exists *in vivo* and if so, whether the processing of p60 has a physiological function.

In order to assess the expression of p60 mRNA level and to investigate the existence of possible splice variants or closely related genes, a Northern blot was performed (Section 2.3.6). Using the 5' EcoRI fragment of the p60 cDNA as a template for the probe, a single band was recognised in mRNA prepared from the S2 cell line, third instar larvae, pupae and adult flies (Figure 3.5C). Taken together, these data suggest that p60 is expressed, and found in complex with Dp110, in larvae, pupae and flies.

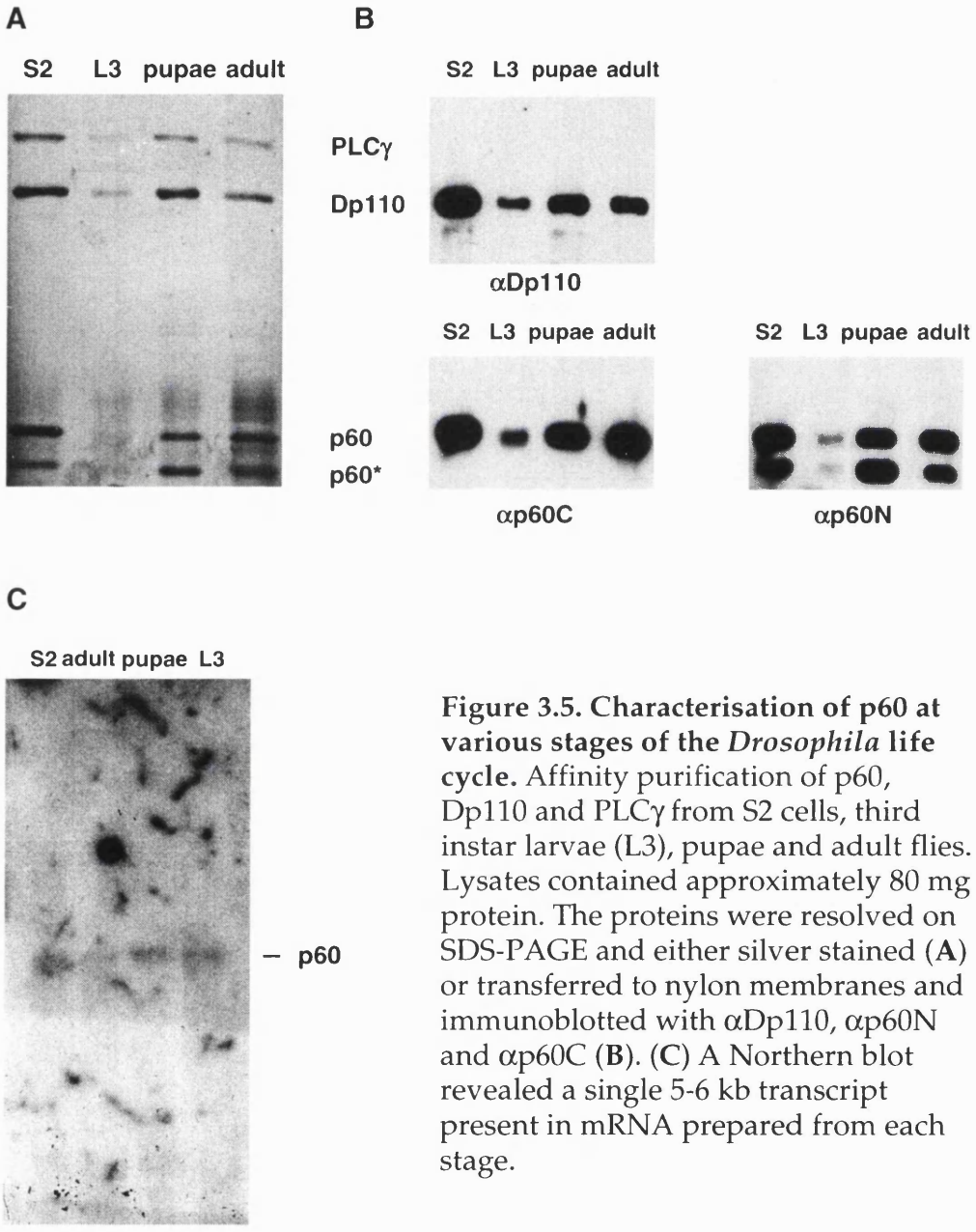


Figure 3.5. Characterisation of p60 at various stages of the *Drosophila* life cycle. Affinity purification of p60, Dp110 and PLCγ from S2 cells, third instar larvae (L3), pupae and adult flies. Lysates contained approximately 80 mg protein. The proteins were resolved on SDS-PAGE and either silver stained (**A**) or transferred to nylon membranes and immunoblotted with αDp110, αp60N and αp60C (**B**). (**C**) A Northern blot revealed a single 5-6 kb transcript present in mRNA prepared from each stage.

3.4.3 Immunoprecipitation of p60 and Dp110

The above results demonstrate that affinity purification with pYXXM phosphopeptide beads is a robust and reliable method of purifying Dp110/p60. However, any conclusions drawn about the activities or binding partners of Dp110/p60 using this technique are limited because other proteins may bind the phosphopeptides independently (e.g. PLCγD). To establish whether antisera could be used to purify Dp110/p60, αp60N and αp60C were used to immunoprecipitate proteins from S2 cell lysates, in parallel with immunoprecipitation (IP) using pooled preimmune sera as a control (Section 2.2.3). The proteins purified from these IPs were then compared to proteins isolated by affinity purification with the DPY phosphopeptide. SDS-PAGE followed by silver staining and immunoblotting revealed that both αp60N and αp60C can immunoprecipitate the Dp110/p60 complex, although αp60C is more efficient than αp60N (Figure 3.5A and B). Thus, this experiment shows that the Dp110 and p60 can be purified by two independent methods, and confirms that these proteins form a complex *in vivo*.

These independent methods of purification could also be used to identify proteins that bind to p60 or Dp110. For example, a pYXXM-containing protein might be expected to bind p60 and to be purified by αp60N and αp60C but not to be purified by affinity purification with a pYXXM phosphopeptide. One weakly detected protein that fits this criteria (p180, Figure 3.6A). However, in contrast to the relative levels of p60 purified by αp60N and αp60C, a larger quantity of this protein was purified by IP with αp60N than with αp60C. Interestingly, a protein with the same electrophoretic mobility as PLCγD was purified by αp60C, but at a much lower stoichiometry than PLCγD purified by pYXXM phosphopeptides. Thus, PLCγD may bind p60 or Dp110 directly, alternatively, PLCγD and p60 may bind to a common phosphotyrosine-containing protein. Western blot analysis with PLCγD specific antisera and/or performing similar experiments using flies deficient in PLCγD (Section 6.3.3) could be used to determine whether this protein is PLCγD.

3.4.4 Dp110 possesses lipid and protein kinase activities

The three methods of purifying the Dp110/p60 complex described above were next used to investigate the lipid and protein kinase activity of Dp110. Because these methods are independent of one another, they provide an effective control for contaminating kinases. The Dp110/p60 complex was immunoprecipitated and affinity purified with DPY; subsequently, the

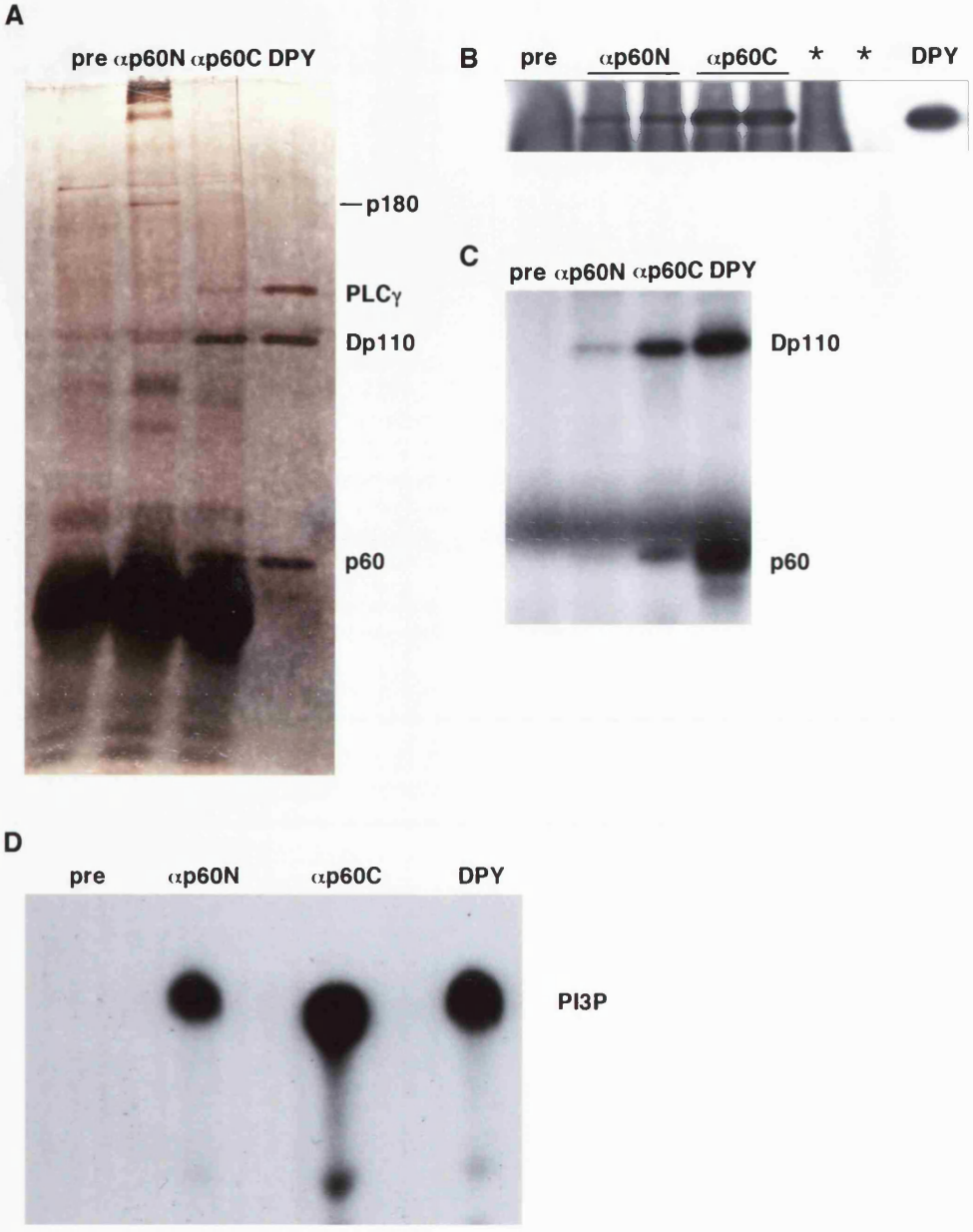


Figure 3.6. The Dp110/p60 complex can be purified by immunoprecipitation or with the DPY phosphopeptide and possesses protein and lipid kinase activity. Affinity purifications were performed from equal volumes of S2 cell lysate. Protein kinase assays were resolved by SDS-PAGE, silver stained(**A**) and autoradiographed(**C**). (**B**) A Western blot of IPs by αp60N and αp60C and purification using DPY, probed with αp60C. Immunoprecipitations not relevant to this figure are marked (*). (**D**) Lipid kinase assays were performed using PI as a substrate, resolved by TLC and autoradiographed.

purified products were divided into two and assayed for lipid or protein kinase activity (Sections 2.2.8 and 2.2.9).

Both immunoprecipitated Dp110/p60 complex and the affinity purified complex possessed lipid kinase activity as assessed by the conversion of PtdIns to PtdIns(3)P (Figure 3.6D), consistent with similar assays performed on other Class I_A PI3Ks (e.g. (Vanhaesebroeck *et al.* 1997b)). The product was confirmed to be PtdIns(3)P rather than PtdIns(4)P by high pressure liquid chromatography analysis performed by Rob Stein. Furthermore, Sally Leevers has shown that Dp110 purified using a pYXXM phosphopeptide can phosphorylate PtdIns(4)P to PtdIns(3,4)P_2 and PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3.

Protein kinase assays revealed that, like human p110δ (Vanhaesebroeck *et al.* 1997b), Dp110 is able to autophosphorylate, regardless of whether it is immunoprecipitated or affinity purified, (Figure 3.6C). In addition, however, Dp110 also transphosphorylates p60, which is reminiscent of the phosphorylation of p85α by mammalian p110α (Dhand *et al.* 1994b). Interestingly, Dp110 contains a C-terminal serine homologous to the phosphorylated serine in p110δ. Thus, autophosphorylation on this residue may downregulate the lipid kinase activity of Dp110 in the same way that p110δ activity is downregulated by autophosphorylation (Vanhaesebroeck *et al.* 1999a). In contrast, p60 does not contain a phosphorylation site homologous to serine 608 in p85α, which, when phosphorylated, leads to the downregulation of p110α (Dhand *et al.* 1994b). However, p60 does have a threonine and a serine a few amino acids C-terminal to the equivalent position of serine 608 in p85, so these residues may be targets of phosphorylation (Figure 3.2). In summary, by using three independent methods of purification, it has been demonstrated that Dp110 is a lipid and protein kinase.

3.5 Discussion

This chapter describes the identification of p60, the adaptor for the *Drosophila* Class I_A PI3K, by biochemical means. The cDNA encoding p60 has been cloned and the encoded protein has been characterised. This work was necessary to generate reagents for the experiments described in Chapters 4 and 5 and to facilitate the interpretation of the results of these experiments. However, conclusions about the function of p60 and the evolution of the adaptors for Class I_A PI3K can be drawn from the data presented in this chapter. Firstly, it is remarkable that p60 can be purified by a phosphopeptide from a human RTK and, in addition, that the pYXXM binding specificity of both SH2 domains appears to be conserved. A similar technique could also be

used to purify homologues of other mammalian SH2 domain proteins in *Drosophila* and other metazoa.

Secondly, the domain structure of p60 implies that the Class I_A PI3K adaptor of the common ancestor of vertebrates and flies consisted of the core SH2–inter-SH2–SH2 region. Interestingly, inspection of genomic sequence data for the adaptor for the *C. elegans* Class I_A PI3K reveals that this protein has a similar domain structure to p60 (D.W. unpublished data). However, while SH3 domains are found in other *Drosophila* and *C. elegans* signalling molecules, for example, the Grb2 homologues: Drk (Olivier *et al.* 1993) and SEM5 (Clark *et al.* 1992), the SH3 (and BH) domains found in mammalian p85α and β must be the result of more recent evolution because p60 has no SH3 domains. For the same reason, the SH3 domain-binding, proline-rich sequences found in all mammalian adaptors for Class I_A PI3Ks may also be involved in a more recently evolved mode of regulation. Thus, it is possible that the function of the proline-rich sequences is related to the presence of the SH3 domain found in p85α and β. Consistent with this hypothesis, the SH3 domain of p85α has been shown to bind one of the proline-rich sequences in p85α, and this interaction may be involved in p85α dimerisation (Harpur *et al.* 1999).

In conclusion, the identification and biochemical characterisation of p60 described in this chapter has allowed insights to be made into the structure and function of all adaptors for Class I_A PI3Ks. Furthermore, important reagents have been created that will allow the function of p60 to be investigated *in vivo* by both mutation of the endogenous gene, and by ectopic expression of p60. These studies will be described in Chapters 4 and 5, respectively.

Chapter 4: Understanding p60 function using genetics

Chapter 4: Understanding p60 function using genetics

4.1 Introduction

This chapter describes the use of reverse genetics to investigate p60 function *in vivo*. Essentially, the reverse genetic approach is to use sequence information from a cloned cDNA to derive an organism with a mutation in the corresponding endogenous gene. This mutant organism can then be compared with the wild type organism. Any differences between the mutant and wild type organism (the phenotype) can be used to make conclusions about the function of the endogenous gene. One problem with this approach is that once a mutation has been identified in the gene of interest, the observed phenotype can be uninformative if, for example, there is no phenotype in the organism heterozygous for the mutation and organisms homozygous for the mutation are not viable. In this situation, it can only be concluded that the gene is essential for viability. However, the reason for the death of the homozygous organism can sometimes be investigated further and may provide useful information about the function of the gene. Additionally, in *Drosophila* at least, one can produce somatic clones of cells homozygous for the mutation at various stages of development and examine their phenotype.

Firstly, however, pre-existing mutations in the endogenous gene have to be identified or new mutations generated. The ease of identification or generation of mutants can vary greatly from gene to gene, particularly in organisms such as *Drosophila*, in which mutagenesis cannot be directed by homologous recombination. Important factors in the identification and generation of mutations are the size and structure of the gene, the proximity and characteristics of neighbouring genes and, crucially, previous molecular and genetic characterisation of the region and the availability of reagents.

Having purified p60 as the adaptor for Dp110 and cloned the cDNA encoding p60, I next sought to generate mutations in the *p60* gene and to characterise the phenotypic effects of loss of *p60* function. The first part of this chapter describes the chromosomal localisation of the *p60* gene, the characterisation of the *p60* genomic region and the generation of mutants in *p60*. This process was greatly assisted by previous research on genes in the region and the availability of reagents and information from other research groups as well as the Berkeley Drosophila Genome Project (BDGP). The second part of this chapter describes experiments that utilised mutations in *p60* to investigate the function of p60 *in vivo*.

4.2 Characterisation of the *p60* region

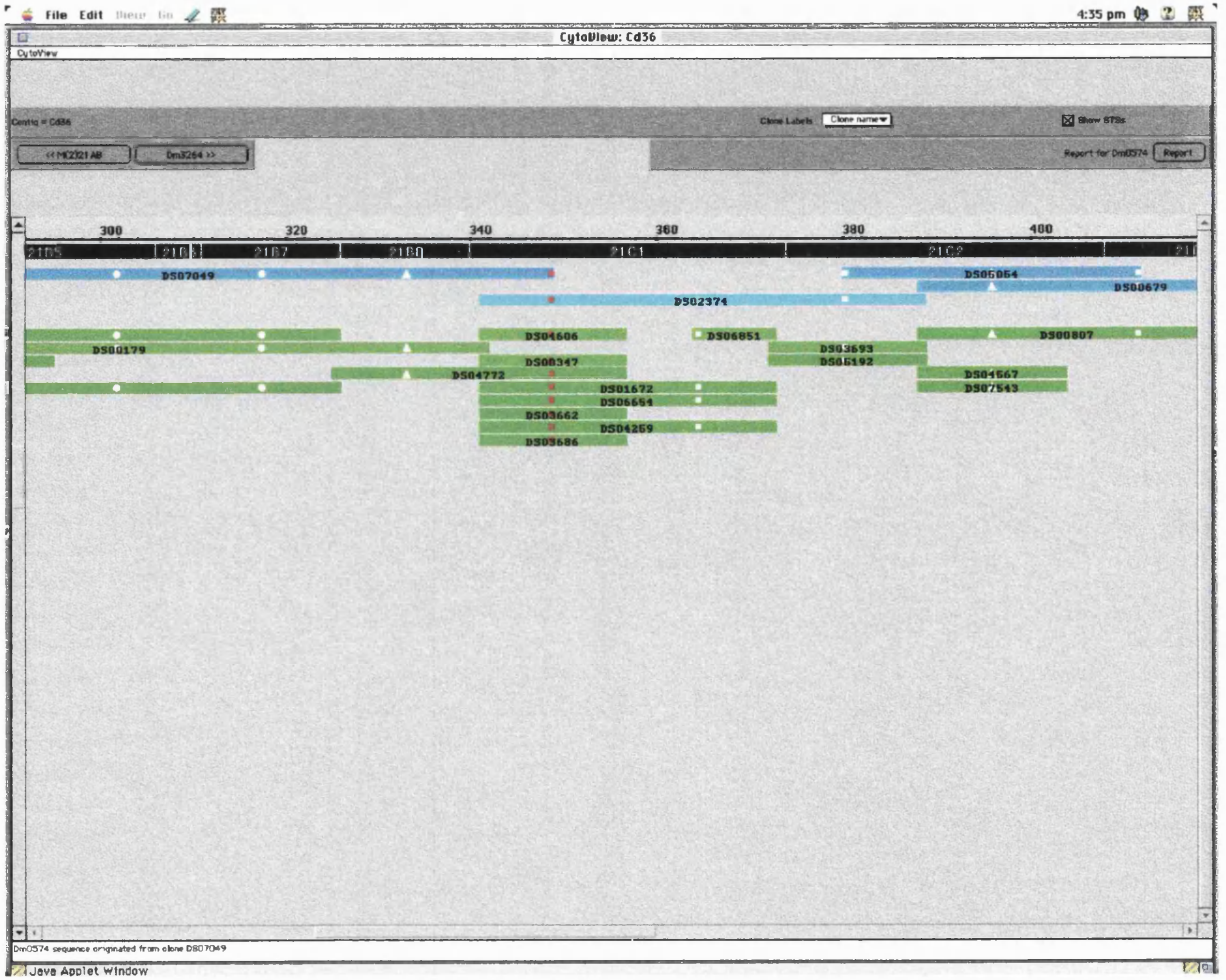
In order to identify and characterise potential mutations in a gene of interest, the chromosomal region containing that gene has to be identified and characterised molecularly. As the first stage in the generation of mutations in the *p60* gene, this section describes the characterisation of the *p60* genomic region.

4.2.1 Localisation of the *p60* gene

The conventional method for finding the genomic location of a cloned *Drosophila* gene has been to perform *in situ* hybridisation on squashes of polytene chromosomes from the salivary glands, using the cDNA as a template to generate the probe. More recently, it has been possible to determine the genomic region of a gene by utilising the information and reagents provided by the BDGP. The BDGP have generated a collection of bacteriophage P1 genomic clones that are 50-100 kb in length and together are estimated to encompass at least 85% of the *Drosophila* euchromatic genome (Hartl *et al.* 1994). They have arranged these clones into contiguous arrays (contigs: arrangements of overlapping clones) by the following method. Firstly, sequence tagged sites (STSs) have been generated by sequencing the ends of the P1 genomic clones and using this information to design a set of PCR primers that reliably amplify a specific product from a genomic DNA template. Secondly, overlapping P1 clones have been identified by using each clone as a template for each STS primer pair. Thirdly, after being grouped into contigs, the P1 clones have been localised to chromosomal regions by performing *in situ* hybridisations on polytene chromosomes using fragments from some of the P1s as probes (Hartl *et al.* 1994). Finally, the sequence of some STSs correspond to previously mapped genes, allowing the contigs to be localised more precisely. The investigator can now determine the chromosomal location of a cloned gene by hybridising the cDNA to a gridded filter of the BDGP P1 clones or by finding identity between the cDNA sequence and sequence derived from the P1 clones, such as STS sequence.

In the case of *p60*, a BLASTN database search with the p60 cDNA sequence identified an STS (Dm0574) from the BDGP that was identical in sequence to the 3' untranslated region (UTR) of the p60 cDNA. This STS was derived by sequencing one end of the P1 clone, DS07049, which has been mapped to the genomic region, 21B-C. The presence of Dm0574 in other P1s in the contig placed *p60* in the genomic interval 21B6-C2 (Figure 4.1).

Figure 4.1. P1 clones and STSs in the *p60* region. A screen shot of the ChromoView Java display of the *Cd36* contig from the BDGP. The STS Dm0574, which contains sequence from the 3' UTR of *p60*, is highlighted in red. The information derived from the mapping of the clones allowed p60 to be localised to the chromosomal region 21B6-C2. The physical sizes of the clones are not represented to scale on this map.



P1 clones are freely available from the BDGP but are 50-100 kb in length, so extensive analysis would be required to generate a restriction map and determine their sequence. Fortunately, a contig of bacteriophage lambda genomic clones from the region had already been generated by Markus Noll's laboratory to facilitate the cloning of the *aristaless* (al, Schneitz *et al.* 1993) and *smoothened* genes (smo, Alcedo *et al.* 1996). The lambda clones of this contig (the *al* contig) are approximately 15 kb in length and the EcoRI restriction sites of these clones have been mapped. To map the *p60* gene more precisely and characterise its structure, DNA fragments from several of the lambda clones from the *al* contig were analysed by Southern blot using fragments of the p60 cDNA as probes (Section 2.3.5). As described in Section 3.2.2, the p60 cDNA was cloned as two EcoRI fragments. The 5' EcoRI fragment of p60 cDNA (Section 3.2) hybridised to the 10 kb EcoRI fragment of BPalI whereas the 3' EcoRI fragment hybridised to the 1 kb EcoRI fragment of BPalI and the 3 kb EcoRI fragment present in both BPalI and BPal14 (Figure 4.2). The identification of these genomic fragments, which will be referred to as R10, R1 and R3 from now on, allowed the *p60* gene to be mapped precisely with respect to the published EcoRI restriction map of the *al* contig. Furthermore, the position of these fragments and the hybridisation data described above revealed that the gene is transcribed in the distal to proximal direction (Figure 4.3).

4.2.2 Determination of the exon/intron structure of the *p60* gene

To determine the exon/intron structure of a gene, the genomic DNA sequence must be compared to the cDNA sequence(s). The exon/intron structure of the *p60* gene was determined by comparing the sequence of the genomic clones from the *al* contig, which was determined by sequencing subcloned and PCR-amplified genomic fragments, with the p60 cDNA sequence. This analysis showed that the gene encoding p60 has three exons (Figure 4.3). The first exon consists entirely of 5' UTR and ends with the adenine of the initiation codon. The second exon consists entirely of coding sequence, from the thiamine of the initiation codon through to the third base of the codon for amino acid 296 (in the inter SH2 domain). The third exon contains the rest of the coding region and the 3' UTR. The third exon is likely to be longer than represented in Figure 4.3 because a polyadenylation signal was not identified in the cDNA sequence. The presence of an extended 3' UTR would account for the size discrepancy between the p60 cDNA and the mRNA identified on a Northern blot (Figure 3.5). The third exon is likely to be the last exon of *p60* because work by others has demonstrated that the last exon of a

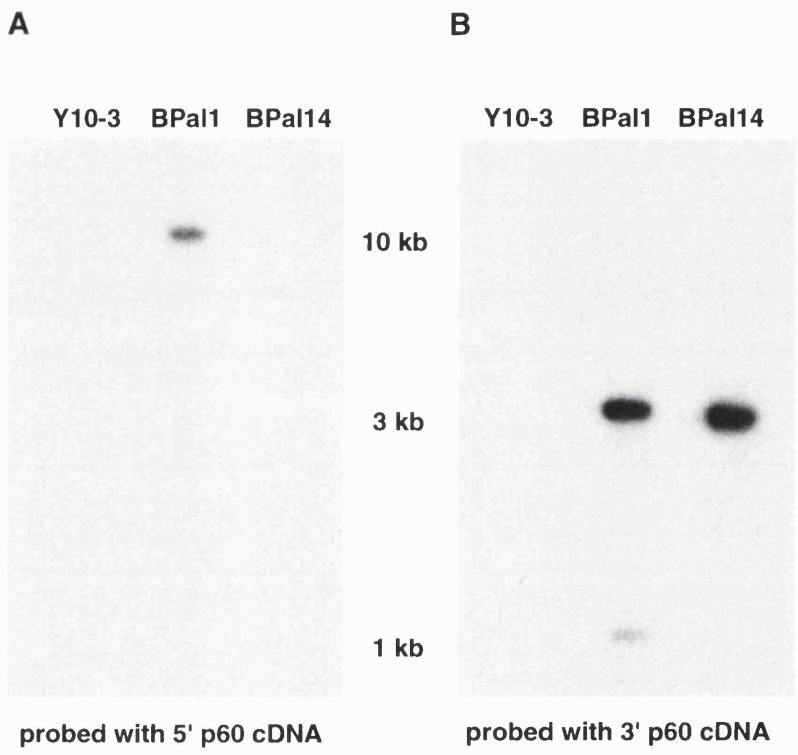


Figure 4.2. Southern blots of genomic clones of the *al* contig digested with EcoRI demonstrate the localisation and direction of transcription of *p60*. (**A**) A 10 kb fragment of BPalI clone was recognised by a probe made from the 5' EcoRI fragment of the p60 cDNA. (**B**) A 3 kb EcoRI fragment present in the BPalI and BPal14 clones and a 1 kb EcoRI fragment of BPal1 were recognised by a probe made from the 3' EcoRI fragment of the p60 cDNA. As well as the clone Y10-3 indicated, the clones Y12-3 and Y11-7 were analysed on the same Southern blot but displayed no hybridisation to the probe.

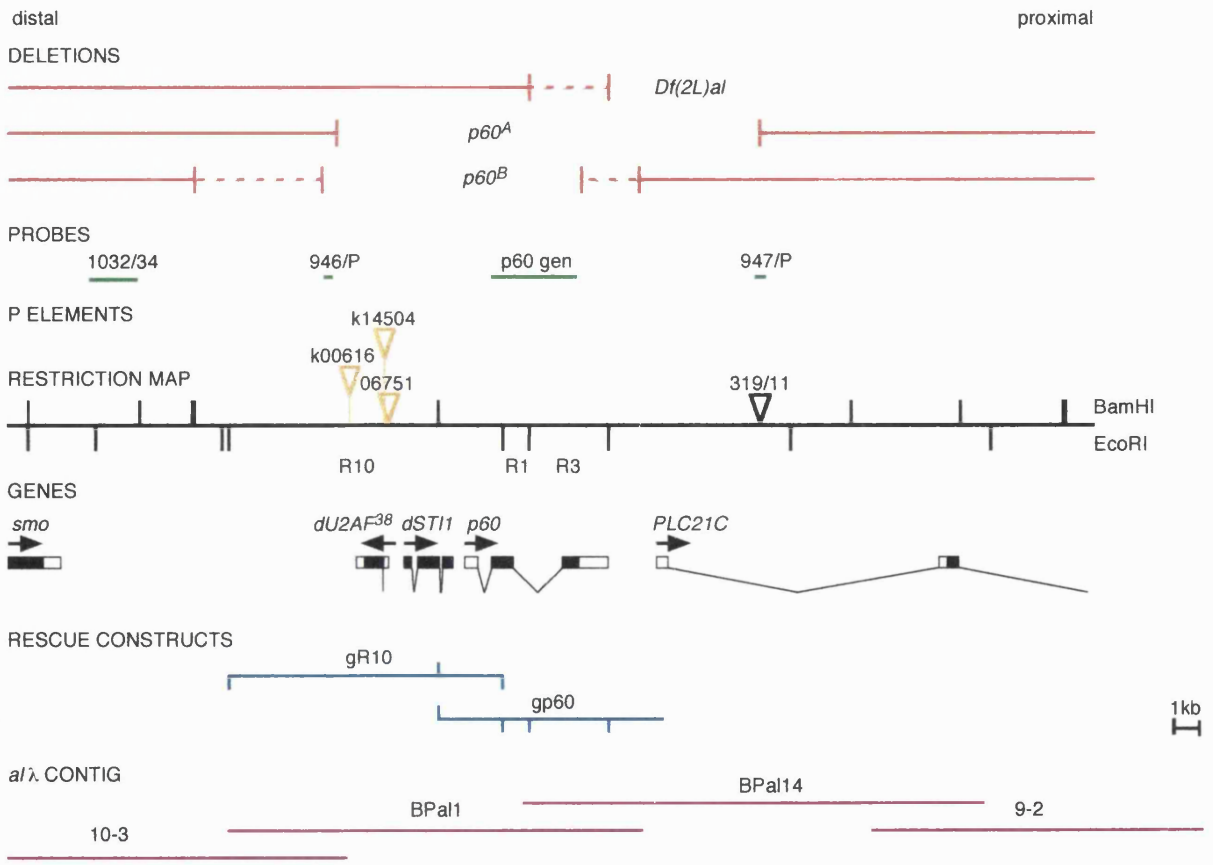


Figure 4.3. The p60 genomic region. A BamHI and EcoRI restriction map showing the deletions $p60^{A}$ and $p60^{B}$; the distal breakpoint of *Df(2L)al*; the genomic probes used for Southern blot analysis (Section 2.4); the P element insertions *k00616, k14504, l(2)06751* and *319/11*; the genes *p60, smo, dU2AF*^{38}, *dSTI1*, and *PLC21C*; the genomic fragments included in *P[gR10]* and *P[gp60]*; and the lambda genomic clones of the *al* contig. The restriction fragments R10, R3 and R1 are also indicated. For each gene, the exon/intron structure, coding sequence (closed boxes) and non-coding sequence (open boxes) are indicated. The exon/intron structure of *dSTI1* was extrapolated from the open reading frames identified by a BLAST search and therefore does not contain any UTRs. The genomic sequence revealed a small intron in *dU2AF*^{38} that was not reported by Rudner *et al.* 1996.

gene usually contains the stop codon and is usually the longest because it contains all of the 3' UTR (Zhang 1998). Therefore, although the existence of an extra exon containing additional 5' UTR cannot be excluded, and the first and the third exons described above are likely to be longer than is represented by the p60 cDNA, it appears that all the exons containing coding sequence of p60 have been identified.

None of the possible alternative splice variants of the three identified exons could encode a functional protein. Consistent with this observation, only one band was seen on a Northern blot probed with the p60 cDNA (Section 3.4.2, Figure 3.5). Albert *et al.* (1997), who cloned *p60* in the search for the *mushroom body miniature (mbm)* gene (Section 4.3.2), also observed a single band of around 5 kb (4.6 kb) on a Northern blot of mRNA from larvae, pupae and adults as shown in Figure 3.5. However, they also reported additional bands on a Northern blot of mRNA from embryos and adult females that may represent splice variants of *p60* in these tissues. Alternatively, because they used the R3 genomic fragment rather than the p60 cDNA as a template for the radiolabelled probe, the extra bands that they observed may have been due to hybridisation of the probe to transcripts from an exon of *PLC21C* or of another, unidentified, gene within R3 (See Section 4.2.3 for a discussion of other genes in the region). Consistent with this suggestion, Albert *et al.* (1997) reported that the first exon of *PLC2IC* is in R3, although a BLAST search did not reveal any homology between the published PLC2IC cDNA sequence and the sequence of R3 (Section 4.2.3).

In May 1997, the BDGP released the sequence of the P1s DS02374 (21B5-21C4, 80101 kb in length) and DS07049 (21B2-21B8, 65196 kb in length, Figure 4.1). These sequences included the genomic sequence of p60, which is more than 99% identical to that obtained from the genomic and cDNA clones for *p60*, confirming the localisation and exon/intron structure of *p60* shown in Figure 4.3.

4.2.3 Characterisation of other genes in the *p60* region

By subcloning and sequencing fragments of the BPal1 clone of the *al* contig, I revealed the existence of other genes close to *p60*: *dU2AF*^{38}, *dSTI1* and *PLC21C* (Figure 4.3). *dU2AF*^{38} encodes the *Drosophila* homologue of the human U2 splicing factor small subunit, hU2AF^{35} (Rudner *et al.* 1996). *dSTI1* is a gene that has not been previously reported, but here has been given the name *dSTI1* because the predicted amino acid sequence shows high similarity to the STI1 protein found in yeast, human and mouse (Blatch *et al.* 1997). STI1 was first isolated as a stress-inducible protein containing tetratricopeptide repeats

(TPRs) in *Saccharomyces cerevisiae* (Nicolet and Craig 1989), and homologues were subsequently identified in mouse (mSTI1, Blatch *et al.* 1997) and in human fibroblasts, in which hSTI1 is upregulated upon transformation (Honore *et al.* 1992). *PLC21C* encodes a PLCβ homologue (Shortridge *et al.* 1991) and contains several exons that span at least 50 kb of genomic DNA (Albert *et al.* 1997, Figure 4.3). The identification of these genes is essential for the subsequent analysis of the *p60* gene.

4.3 Characterisation of mutations and other chromosomal aberrations in the *p60* region

The previous sections described the molecular characterisation of the genomic region containing *p60*. In order to identify *Drosophila* lines with mutations in *p60*, lines with mutations and aberrations mapped to the chromosomal region containing *p60* were obtained and analysed both molecularly and genetically. Genomic DNA from these flies was examined using a combination of Southern blotting, PCR and sequencing. The genetic analysis relied mainly on complementation. The basic principle of complementation analysis is that two mutations that affect two different genes will complement each other (not show the mutant phenotype when the two different chromosomes are combined in the same organism as the result of an experimental cross). In contrast, two mutations that affect the same gene will not complement each other (show the mutant phenotype when the two different chromosomes are combined). Complementation analysis allows mutations to be distinguished and grouped, and thus provided a powerful tool in the search for a mutation in *p60*. All the mutations in the *p60* region that were assessed by complementation analysis had a recessive lethal phenotype. The mutations were maintained in lines in which the mutations were heterozygous to balancer chromosomes. Balancer chromosomes suppress recombination and are marked with dominant mutations with clearly distinguishable phenotypes. Thus, complementation of two recessive lethal mutations is indicated by the presence of flies amongst the progeny of the test cross that lack the dominant phenotypes of the balancer chromosomes. The results of the complementation analysis of lines carrying mutations and aberrations in the *p60* region are summarised in Table 4.1, and are described below.

	net	*a l*	*06751*	*14504*	*00616*	*p60^{A}*	*p60^{B}*	*p60$^{A;}$ R10*	*p60$^{B;}$ R10*	*p60$^{A;}$ gp60*	*p60$^{B;}$ gp60*
net	X		✓	✓	✓	-	-	-	-	-	-
a l		X	✓	✓	✓	✓	✓	-	-	-	-
06751			X	X	✓	X	X	✓	✓	-	-
14504				X	X	X	X	✓	✓	-	-
00616					X	X	X	✓	✓	-	-
p60^{A}						X	X	X	X	X	X
p60^{B}							X	X	X	X	X
p60$^{A;}$ R10								X	**X**	✓	✓
p60$^{B;}$ R10									X	✓	✓
p60$^{A;}$ gp60										X	-
p60$^{B;}$ gp60											X

Table 4.1. Results of complementation analysis of *Drosophila* lines with mutations or other aberrations in the *p60* region. Flies heterozygous for the deficiencies *Df(2L)al* and *Df(2L)net-PMF*, the P element insertions *P[l(2)06751]*, *P[l(2)k14504]*, and *P[l(2)k00616]* and the small deletions p60^{A} nor *p60^{B}* on their own and in combinations with the genomic rescue constructs *P[gR10]* and *P[gp60]* were crossed to each other The progeny were assessed for complementation of the recessive lethal phenotype. The results were either full complementation (✓) with the expected Mendelian number of progeny without balancer chromosomes or complete non-complementation (X) with no progeny without balancer chromosomes. Crosses marked (-) have not been tested because they were not relevant to this study. The crosses used to demonstrate that a *p60* mutant had been found are underlined. Larvae from the cross highlighted in bold were used to study the *p60* mutant phenotype.

4.3.1 Large deficiencies in the *p60* region

There are two large, publicly available deficiencies (deletions) in the *p60* region: *Df(2L)al* (21B8-C1- 21C8, Schneitz *et al.* 1993) and *Df(2L)net-PMF* (21A1-21B7-8, Caggese *et al.* 1988). However, there is a region in between these deficiencies, termed the *smo* region (Alcedo *et al.* 1996), for which there are no publicly available deficiencies. The deficiency $Df(2L)dpp^{S4L}\ Tg^{R}$ [21B; 21C6] covers this region but can only be maintained in the presence of the duplication $Dp(2;f)ush^{vx21}$ [21A; 21C6] that covers a haplolethality of the deficiency (Alcedo *et al.* 1996), leading others to suggest that there is a haplolethal gene in the *smo* region. *p60* is located at least 20 kb from the proximal breakpoint of *Df(2L)net-PMF* but is likely to overlap with the distal breakpoint of *Df(2L)al*, which has been mapped to somewhere in the R3 fragment that contains the third exon of *p60* (Figure 4.3, Schneitz *et al.* 1993).

4.3.2 Mutations in the *p60* region

To find a mutation in a gene, existing mutations that have been mapped to the relevant region must be assessed to ascertain whether the gene of interest is disrupted. Mutations in *Drosophila* can be classified into two categories: those that have been isolated in screens for particular phenotypes, or modifiers of phenotypes, and those that have been isolated in large scale screens for essential genes. Mutagenesis in *Drosophila* is achieved by one of three methods: 1) feeding flies a chemical mutagen such as ethylmethane sulphonate (EMS), which creates point mutations and small deletions; 2) irradiating flies with X-rays, which creates larger deletions and chromosomal rearrangements; or 3) the use of P-element transposons, which can disrupt genes by insertion into the genome. It is easier to identify genes disrupted by P elements than by other methods of mutagenesis, so P element insertion is often the favoured method of mutagenesis when large-scale screens for essential genes are performed. However, there are two disadvantages of using P elements. Firstly, because they do not insert completely randomly throughout the genome, some genes are much more likely to be disrupted than others. Secondly, because P elements preferentially insert into 5' UTRs, the gene product is not always eliminated by such insertion.

Several candidate mutations that might disrupt *p60* were identified, and the lines carrying these mutations were collected from stock centres (Section 2.5.2). Two P element insertions derived from a screen for recessive lethal mutations on the second chromosome were of particular interest: *P[l(2)k14504]* and *P[l(2)k00616]* (Torok *et al.* 1993). These insertions had both been localised to 21B7-8 by *in situ* hybridisation to polytene chromosomes, and

complemented *Df(2L)al*. This characterisation was carried out by the BDGP, who also maintain and provide the lines. The P element *P[l(2)06751]* partially disrupts $dU2AF^{38}$ and was useful both for mapping as well as for the generation of small deletions (Section 4.3.3, Rudner *et al.* 1996). Other P element lines that contain insertions in the region were also obtained from stock centres.

To investigate whether the P elements in the collected lines were inserted near *p60*, genomic DNA was prepared from flies heterozygous for these insertions, digested with EcoRI and analysed by Southern blot (Figure 4.4, Sections 2.3.2 and 2.3.5) using a fragment of genomic DNA containing the *p60* gene as a probe (p60gen, Figure 4.3). Hybridising bands representing R10, R3 and R1 were observed in all the samples (Section 4.2.2). An additional band of approximately 6 kb was observed in the majority of the samples. This band may have been the result of the probe hybridising to genomic DNA with a similar sequence located elsewhere in the *Drosophila* genome. In the lanes containing DNA from flies heterozygous for the P elements, *P[l(2)k14504]*, *P[l(2)k00616]* or *P[l(2)06751]*, extra hybridising bands were observed, indicating that all three P elements disrupted genomic DNA close to *p60*. The size of the extra bands and the apparent reduction in signal intensity of R10 in these samples suggested that the P elements were inserted in this fragment of DNA. This suggestion was confirmed by stripping the blot and reprobing with the 5' EcoRI fragment of the p60 cDNA. Thus, all three P elements were located 5' of, or within, the *p60* gene. The P element insertion sites were mapped more precisely from further analysis of the Southern blot data, the results of long range PCR amplification (for *P[l(2)k15404]*, Section 2.4.1) and data from Rudner *et al.* 1996 (Figure 4.3).

Complementation analysis revealed that *P[l(2)k14504]* did not complement *P[l(2)06751]* whereas *P[l(2)k00616]* did complement *P[l(2)06751]* (Table 4.1). Surprisingly, *P[l(2)k14504]* did not complement *P[l(2)k00616]*, suggesting that *P[l(2)k00616]* and *P[l(2)06751]* disrupted two different genes and that *P[l(2)k14504]* disrupted both of those genes.

The only candidate *p60* mutants that displayed a phenotype other than recessive lethality were flies homozygous for alleles of a gene called *mushroom body miniature (mbm)*. Mutations in this gene affect the morphology of mushroom bodies (structures in the fly brain) and the learning ability of flies and larvae but the gene has not been cloned (de Belle and Heisenberg 1996). The gene has been mapped to the region but the *mbm* phenotype was not thought to be due to mutations in *PLC21C* (Heisenberg 1995). Despite this finding, Thomas Twardzik in Martin Heisenberg's laboratory has mapped a P

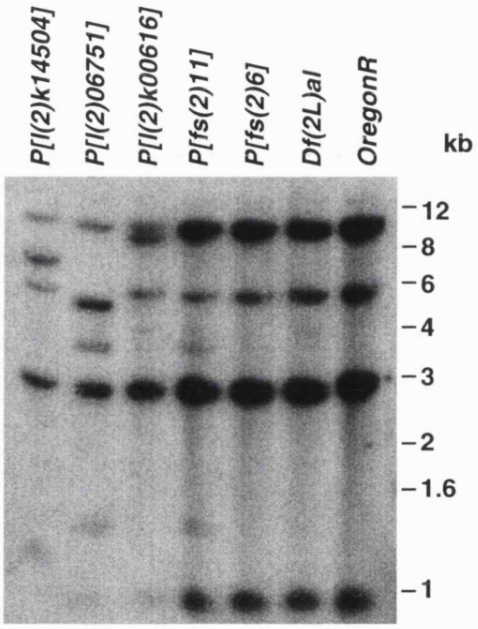


Figure 4.4. Southern blot of DNA from P element insertions demonstrates that *P[l(2)k14504]*, *P[l(2)06751]* and *P[l(2)k00616]* are inserted near the *p60* gene. The probe p60gen generated from *p60* genomic DNA (Section 2.4) recognised the R10, R3 and R1 genomic fragments containing the *p60* gene (See Figures 4.2 and 4.3). An additional fragment of 6 kb was also recognised. In the samples from *P[l(2)k14504]*, *P[l(2)06751]* and *P[l(2)k00616]*, amongst other differences, DNA fragments of 7 kb, 5 kb and 8-9 kb were observed, respectively.

element allele of *mbm*, *P[319/11]*, to a intron of *PLC21C* (Figure 4.3). A collaboration was established between this laboratory and our laboratory to investigate the possibility that the *mbm* phenotype was caused by the disruption of *p60*; the results are described below.

4.3.3 Small deletions in the *p60* region: *mbm* is not *p60*

To investigate whether the *mbm* phenotype is due to mutations in the *p60* gene, a small deletion that deleted *p60* was generated by Thomas Twardzik. To create this deletion, the P element *P[319/11]*, which was located proximal to *p60*, was mobilised to generate a duplication that consisted of the original *P[319/11]* and a second P element insertion 18 kb from *P[319/11]* and distal to *p60* (Figure 4.3). Deletion of the intervening region was achieved by *cis*-mediated excision (Cooley *et al.* 1990). I then mapped the breakpoints of the deletion by PCR amplification with P element-specific primers and primers based on the genomic sequence (primers 946 and 947, Figure 4.3), using DNA from flies heterozygous for the deletion as a template. The resulting products were directly sequenced and the results demonstrated that the deletion, termed $p60^A$, removed 18 kb of genomic DNA, as predicted. Southern blotting of EcoRI- and BamHI- digested DNA from flies heterozygous for $p60^A$, using fragments of genomic DNA as probes, was used to confirm the size of the deletion (Figures 4.5 and 4.3, Section 2.4.1).

We next undertook a combination of genetic and biochemical analyses to demonstrate that *mbm* was not *p60*. Thomas Twardzik showed that $p60^A$ complemented *mbm*. In addition, I performed protein kinase assays on Dp110/p60 affinity purified from mbm^1 and wild type flies (Figure 4.6, Section 3.4). The results of this experiment demonstrated that neither the size, the abundance nor the biochemical properties of p60 were altered in homozygous mbm^1 flies when compared to the wild type.

In the study of $dU2AF^{38}$, Rudner *et al.* created four deletions from the imprecise excision of *P[l(2)06751*. They provided me with these stocks for further analysis. Complementation analysis demonstrated that one deletion (*E8*) was likely to delete *p60* (Section 4.4.1). Southern blot analysis revealed that this deletion, renamed $p60^B$, removes 11-19 kb of genomic DNA (Figure 4.5). Neither $p60^A$ nor $p60^B$ complemented *P[l(2)06751]*, *P[l(2)k14504]* or *P[l(2)k00616]*, but both deletions complemented *Df(2L)al*. In summary, *p60* is likely to be an essential gene that is not disrupted by *Df(2L)al*. Additionally, two small deletions that have been created that delete a chromosomal region that is not deleted by any publicly available deficiencies and thus will be

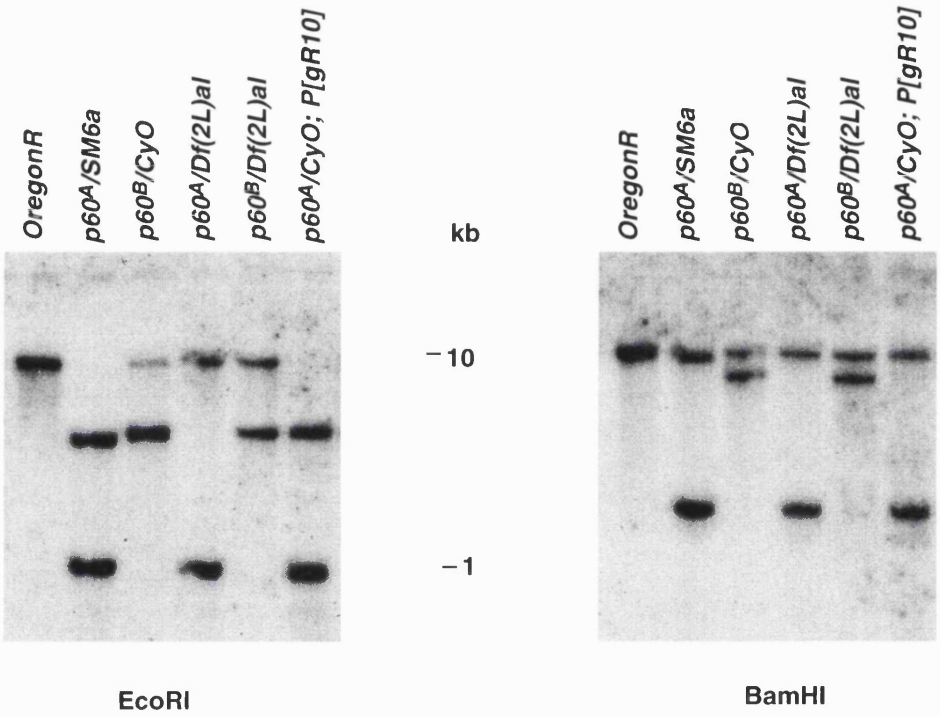


Figure 4.5. An example of the Southern blots used to determine the breakpoints of the small *p60* deletions. DNAs from flies heterozygous for the $p60^A$, $p60^B$ and *Df(2L)al* deletions were digested with either EcoRI (**A**) or BamHI (**B**) and probed with the probe 947/P (Figure 4.3, Section 2.4). The nature of *P[gR10]* was also confirmed.

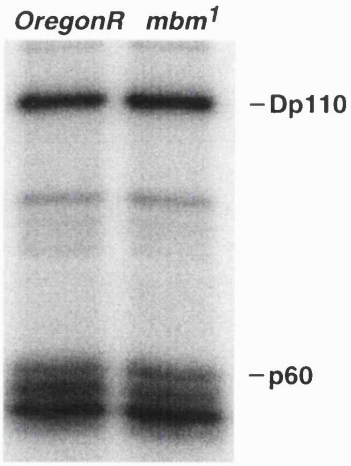


Figure 4.6. p60 purified from *mbm*^{1} flies binds to Dp110, is phosphorylated, and is the same size as p60 purified from wild type (*Oregon R*) flies. Affinity purifications with the DPY phosphopeptide from Triton X-100 lysates of homogenised adult flies were incubated with ^{32}P-γATP to assess protein kinase activity, resolved by SDS-PAGE and autoradiographed. See Section 3.4.4 for details of this assay.

useful tools for genetic analysis. Finally, the analysis presented above predicts that both deletions disrupt *p60*.

4.4 Construction and analysis of genomic rescue constructs for genes in the *p60* region and the generation of *p60* mutants

4.4.1 Analysis of *P[gR10]*, a genomic rescue construct for *dU2AF^{38}* and *dSTI1*

In order to use the deletions *p60^{A}* or *p60^{B}* for the genetic analysis of p60, transgenes must be generated that rescue the activity of the other genes that are disrupted by the deletions. The results presented above demonstrate that the genomic fragment R10 contains *dU2AF^{38}*, *dSTI1* and the 5' UTR exon of *p60* (Figure 4.3). The P element *P[gR10]* (the R10 genomic fragment subcloned into pCaSpeR4 and inserted on the third chromosome) was generated and provided by Joan Hooper. Complementation analysis was performed using this line to investigate the genetics of the mutations described above (Table 4.1). *P[gR10]* rescued the lethality of *P[l(2)06751]*, *P[l(2)k14504]* and *P[l(2)k00616]* in *trans* with *p60^{A}* or *p60^{B}* but did not rescue the lethality of *p60^{A}/p60^{A}*, *p60^{B}/p60^{B}* or *p60^{A}/p60^{B}*. In fact, *P[gR10]* rescued the lethality of *p60^{A}* in *trans* with the other deletions provided by Rudner *et al.*, allowing these deletions to be discarded in favour of *p60^{B}* (Section 4.3.3). The results of the complementation analysis described above were consistent with *P[l(2)06751]* disrupting *dU2AF^{38}* (Rudner *et al.* 1996) and strongly suggested that *P[l(2)k00616]* disrupted *dSTI1* and that *P[l(2)k14504]* disrupted both *dU2AF^{38}* and *dSTI1*. Assuming that *p60* is an essential gene, these experiments also confirmed that *P[l(2)06751]*, *P[l(2)k14504]* and *P[l(2)k00616]* do not disrupt *p60*, because *P[gR10]* does not contain enough sequence to produce a functional p60 protein. However, the fact that *P[gR10]* rescued the lethality of two genes that are disrupted by *p60^{A}* and *p60^{B}* suggests that *p60* would be the only essential gene disrupted in an organism with the genotype *p60^{A}/p60^{B}* ; *P[gR10]*.

4.4.2 Construction of a genomic rescue construct for *p60* and the generation of *p60* mutants

To test the possibility that a fly with the genotype *p60^{A}/p60^{B}; P[gR10]* is disrupted solely in *p60*, it was necessary to make a genomic construct that contained *p60*, which could be used to rescue the putative *p60* phenotype (lethality). To ensure that only the *p60* gene is rescued by such a construct, it must not contain sequences that allow the expression of any other genes. In

order to design the *p60* genomic rescue construct, the genomic structure of the *p60* gene was examined carefully. The promoter for *p60* is likely to lie in the region of less than 500 bp between the predicted coding region of *dSTI1* and the first predicted exon of *p60* (Figure 4.3). The BamHI-SacI fragment of the lambda clone BPal1(gp60, Figure 4.3) contains this region and the entire *p60* gene, so it was subcloned into pCaSpeR4 to generate pP[gp60]. gp60 also contains part of the *dSTI1* gene but lacks the promoter, initiation codon and the majority of the coding sequence of the gene, so it is unlikely to encode a functional protein.

pP[gp60] DNA was injected into early *Drosophila* embryos (See Section 2.5.1 for details of the procedure). P element-mediated transformation resulted in the generation of four lines of transgenic flies carrying independent insertions of *P[gp60]*(Section 2.5.2). To test whether $p60^A/p60^A$, $p60^B/p60^B$ or $p60^A/p60^B$ in combination with *P[gR10]* were *p60* mutants, lines were constructed containing each *P[gp60]* inserted into flies with these genetic backgrounds. Complementation analysis demonstrated that three out of four insertion rescued the lethality of these mutants, resulting in viable, healthy adult progeny (Table 4.1, Section 2.5.2). These results demonstrate that *p60* is an essential gene and that $p60^A/p60^B$; *P[gR10]*, $p60^A/p60^A$; *P[gR10]* and $p60^B/p60^B$; *P[gR10]* are null *p60* mutants. The complementation of $p60^A$ and $p60^B$ with *Df(2L)al* and the viability of the $p60^A$ or $p60^B$ with *P[gR10]* and *P[gp60]* suggest that either *PLC21C* is not an essential gene or that alternative transcripts of this gene can be produced when the region between the distal breakpoint of *Df(2L)al* and the proximal breakpoint of $p60^A$ is deleted (Figure 4.3). Therefore, the combination of *P[gR10]* with either $p60^A$ or $p60^B$ results in the generation of two independent null mutations in *p60*.

4.5 Analysis of the *p60* mutant phenotype and comparison with the *Dp110* mutant phenotype

4.5.1 Phenotypic analysis of $p60^-$ larvae

Having demonstrated that *p60* is an essential gene, it was now possible to study the *p60* phenotype, in order to investigate the *in vivo* function of p60. When only one allele of a gene is utilised for such an analysis, it is possible that part or all of the apparent phenotype of the homozygous mutant is due to accumulated additional mutations on the chromosome and not due to the mutation being studied. Because the lethality of $p60^A$; *P[gR10]* and $p60^B$; *P[gR10]* could be rescued by *P[gp60]*, it can only be explained by the absence of *p60* (Table 4.1). However, accumulated mutations in other genes could affect the

stage of lethality and/or the phenotype before death. To avoid this problem, the progeny of the cross between $p60^A$/*Bc, Gla, bw; P[gR10]* and $p60^B$/*Bc, Gla, bw; P[gR10]* with the zygotic phenotype $p60^A/p60^B$; *P[gR10]* were examined. These homozygous *p60* mutants could be distinguished from their heterozygous siblings by the absence of the dominant *Bc* marker.

Embryos with the phenotype $p60^A/p60^B$; *P[gR10]* hatched into larvae and developed normally until the third instar and with the same timing as wild type and heterozygous controls. At the end of the third instar, 5 days after egg laying (AEL), wild type larvae begin to wander out of the food. The $p60^-$ larvae were delayed during third instar development, only beginning to wander 10 days AEL. The wandering $p60^-$ larvae appeared thinner and more transparent than the wild type (Figure 4.7A). Dissection of these larvae revealed that their fat bodies and imaginal discs were drastically reduced in size when compared to the wild type (Figure 4.7B). There were no obvious differences in the other organs. Some of the $p60^-$ larvae attempted to pupariate and formed pupa-like structures. However, all of these animal died without any indications of significant pupal development. In summary, while the contribution of maternal *p60* has not been investigated, zygotic *p60* is not necessary for embryogenesis or the development of the first two larval instars. However, zygotic *p60* is necessary for third instar larval development and the phenotype is consistent with a role for p60 in growth control.

4.5.2 Comparison of the $p60^-$ and $Dp110^-$ larvae

Dp110 mutants were generated by Sally Leevers using a similar strategy as that used to generate the *p60* mutants described above (utilising a combination of small deletions and genomic rescue constructs). Like mutants in *p60*, *Dp110* zygotic mutants completed embryogenesis and the first two larval instars normally but their development was impaired in the third instar. However, the *Dp110* phenotype was more severe than the *p60* phenotype (Figure 4.7). *Dp110* larvae did not grow beyond the early third instar. While they moved around in the food and survived for approximately 20 days, they did not increase in size, wander or attempt to pupariate. Furthermore, no imaginal discs could be found by dissection.

The similarity between the *Dp110* and *p60* phenotypes was consistent with the biochemical association of Dp110 and p60. However, the *Dp110* phenotype was consistently more severe (See also Section 4.6.3). There are several possible explanations for this difference; these are discussed in Chapter 6. To provide further data to assess these possibilities, a *Dp110/p60* double mutant was created. These larvae had a similar appearance to *Dp110* larvae

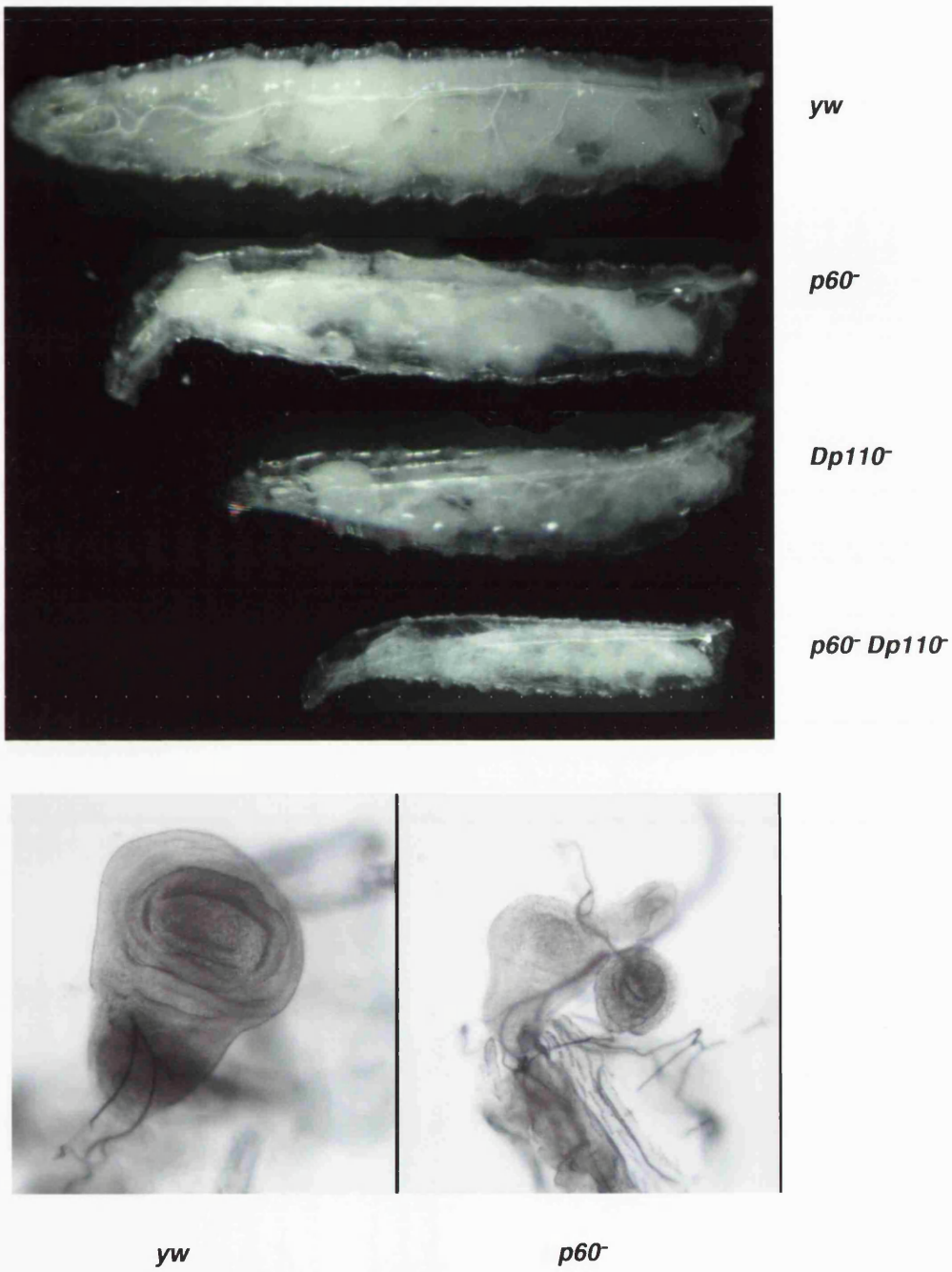


Figure 4.7. The zygotic phenotypes of *p60* mutants, *Dp110* mutants and *Dp110 p60* double mutants. (**A**) Third instar larvae with the following genotypes: ***p60***$^{-}$: $p60^{A}/p60^{B}$; *P[gR10]*. ***Dp110***$^{-}$: *P[gH], Dp110*^{A}*/P[gH], Dp110*^{B}. ***p60***$^{-}$ ***Dp110***$^{-}$: $p60^{A}$; *P[gR10], P[gH], Dp110*^{A} (**B**) A comparison of wing imaginal discs removed from a wild type third instar larva and a p60 mutant third instar larva, both viewed at the same magnification. No imaginal discs could be recovered from *Dp110* mutant larvae.

(Figure 4.7A). As well as being consistent with the function of p60 being totally dependent on Dp110, this result is consistent with Dp110 retaining the ability to function in the absence of p60 (Section 6.2).

4.6 Analysis of *p60⁻* and *Dp110⁻* cells

4.6.1 Generation of clones of *p60⁻* cells in the eye

The zygotic phenotype of *p60* mutant larvae is consistent with a role for Class I_A PI3Ks and their adaptors in the control of growth in the developing organism. In particular, p60 and Dp110 are necessary for imaginal disc growth. However, it could not be determined by examination of the mutant larvae whether the reduction of growth in the imaginal discs was due to a reduction in the intrinsic ability of imaginal disc cells to grow or due to the cells lacking certain extracellular stimuli and/or nutritional input from other organs of the larva. Furthermore, both intrinsic and extrinsic determinants of imaginal disc growth might be affected by the lack of p60.

These possibilities can be addressed by generating small clones of homozygous *p60⁻* cells in a background of heterozygous and wild type cells. The FRT-FLP system is the most convenient and widely-used method of generating mitotic clones, which result from inappropriate recombination or abnormal segregation of chromosomes during a mitotic cell division (Figure 4.8, Xu and Rubin 1993). The FRT-FLP system requires the construction of a chromosome containing an FRT repeat between the centromere and the mutation of interest. This chromosome is used in *trans* with a chromosome containing both an FRT repeat located at the same chromosomal location as the first FRT, and a marker gene. Recombination in a single cell, which is occasionally induced by expression of the FLP recombinase, results in the creation of a clone of mutant cells that can be identified by the loss of the marker gene. A sister clone or 'twin spot' is descended from the same recombination event. The twin spot contains two wild type copies of the gene being studied and can be identified by the presence of two copies of the marker gene.

In order to make clones of *p60⁻* cells, flies with the phenotype *y w; $p60^B$, FRT40A/ CyO; P[gR10]* were generated by recombination (Section 2.5.2). Sally Leevers crossed male flies with this phenotype to females containing a *w+* marker gene with the same FRT (*y w P[hs-FLP ry⁺]; P[w⁺ hs-π–myc] FRT40A/CyO*) to create flies with the genotype *y w P[hs-FLP ry⁺]/+; $p60^B$ FRT40A/P[w⁺ hs-π–myc] FRT40A; P[gR10]/+*. These flies were heat shocked 60 ±12 h AEL. I could identify clones of *p60⁻* cells in the adult eyes of these flies as

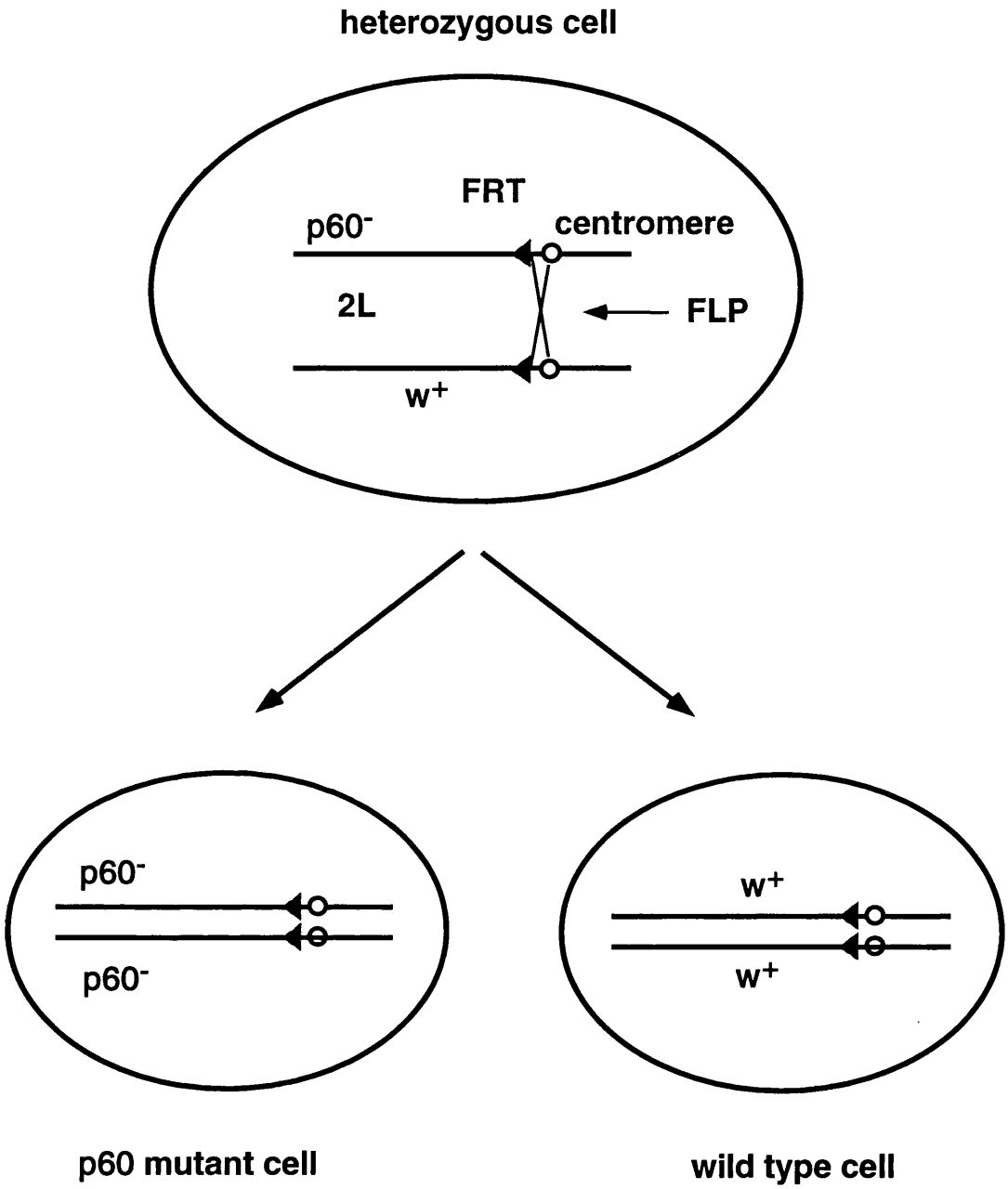


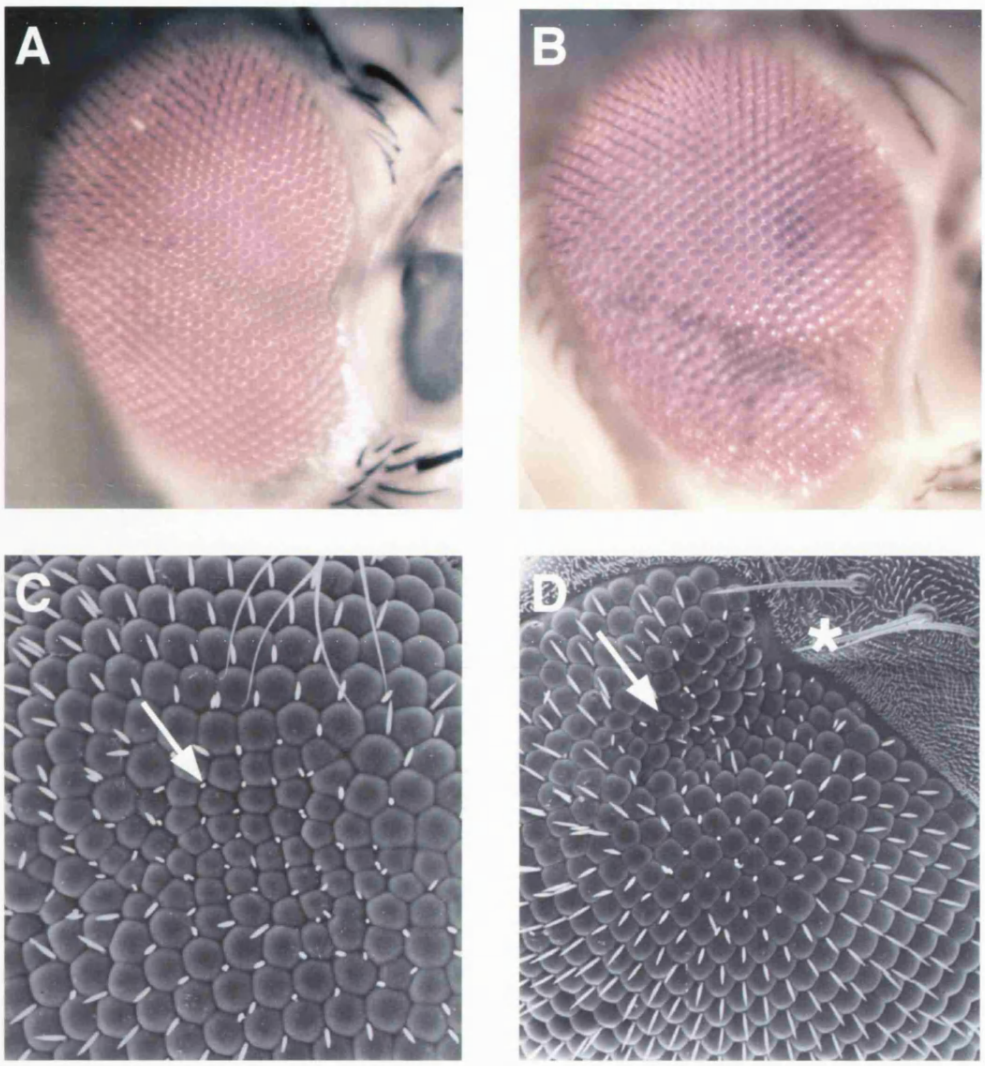
Figure 4.8. The FRT/FLP system. A diagram illustrating how clones of mutant *p60*$^{-}$ cells are generated with the FRT/FLP system. The FLP recombinase induces a cross-over of the chromosome arms at the site of an FRT repeat in a random somatic cell. This event gives rise to a clone of homozygous mutant cells and clone of cells that are wild type at that locus. The cells from this event can be distingusihed by a loss of gain of a marker gene such as w^{+}.

w^- (white) patches in a heterozygous w^+ (red) background. The mutant ommatidia were not completely white because the *P[gR10]* insertion contains a weakly-expressing w^+ marker. Despite the presence of this background colour, the twin spots could be recognised as patches of even darker cells (because they were homozygous for the w^+ marker). The eyes were examined using light microscopy and recorded with a CCD camera (Figure 4.9A, Section 2.6.1). The mutant ommatidia were significantly smaller than the surrounding wild type and heterozygous ommatidia; furthermore, the surface formed by the mutant ommatidia was recessed from the surface of the rest of the eye. These phenotypes were confirmed by examining the mutant ommatidia by scanning electron microscopy (SEM, Figure 4.9C, Section 2.6.4).

Interestingly, mutant clones were consistently smaller in area than their wild type twin spots Additionally, some eyes contained a visible twin spot but no detectable sign of a clone of mutant cells. However, the survival of large clones of *p60*$^-$ cells in several adult eyes demonstrates that p60 is not essential for proliferation or protection from apoptosis.

4.6.2 Analysis of *p60*$^-$ cells in tangential sections of the eye

Ommatidia consisting of *p60*$^-$ or *Dp110*$^-$ cells, though smaller than wild type, appeared to form ordered structures amongst on the surface of the eye (Figure 4.9). To investigate the effect of the mutation in *p60* on the constituent cells of the ommatidia more carefully, eyes containing clones were embedded in resin, sectioned with a microtome, stained and examined under a microsope (Figure 4.10A, Section 2.6.2). Clones of *p60*$^-$ cells could be identified by a reduction in red pigment granules as described above. Examination of these clones revealed that mutant photoreceptors could still differentiate and re-organise their internal membranes to form rhabdomeres (which stain dark blue). However, *p60*$^-$ cells were significantly smaller in cross-section and longitudinal-section than those in the surrounding heterozygous and wild type tissue. Although reduced in size, almost every ommatidium contained the wild type number of photoreceptors. Furthermore, the characteristic trapezoid arrangement of photoreceptor rhabdomeres within each ommatidium and the orientation of ommatidia relative to each other was generally wild type. These results demonstrate that although p60 is essential for cells in the adult eye to grow to their wild type size, it is not absolutely required for cell survival, proliferation or differentiation.



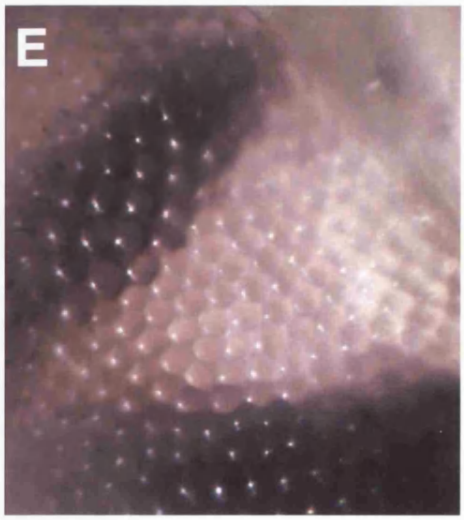


Figure 4.9. *Dp110*⁻ and *p60*⁻ ommatidia and cells in the adult eye are reduced in size. The FLP-FRT recombination system was used to generate mitotic clones 60h ±12h AEL and adult eye *p60*⁻ and *Dp110*⁻ phenotypes, respectively were examined by light microscopy (**A** and **B**) and scanning electron microscopy (**C** and **D**). *p60*⁻ and *Dp110*⁻ ommatidia were indented (*) and reduced in size (arrows). A close-up of the *Dp110*⁻ ommatidia is shown (**E**).

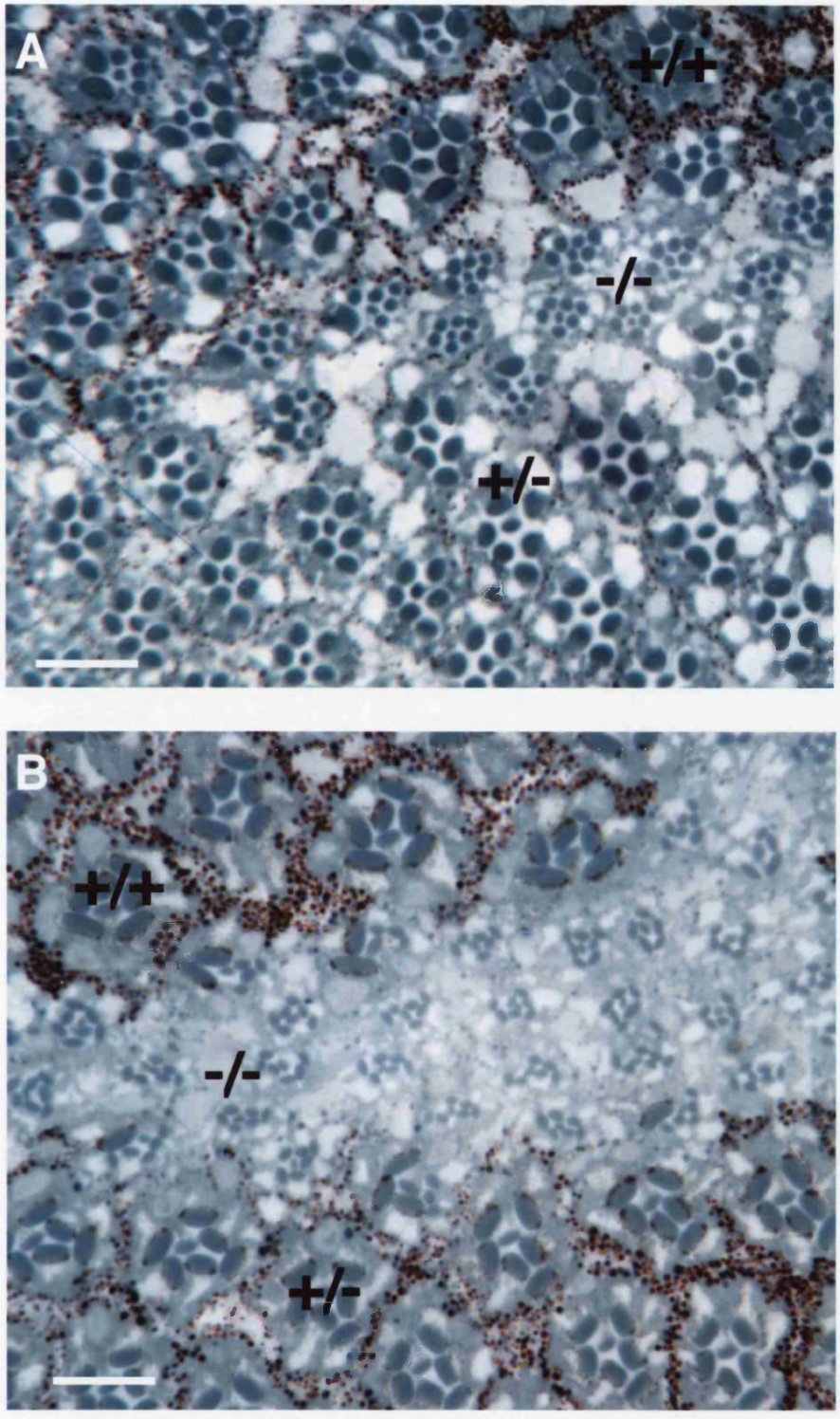


Figure 4.10. Tangential sections of *p60*⁻ and *Dp110*⁻ clones in the eye. Tangential sections through (**A**) *p60*⁻ and (**B**) *Dp110*⁻ clones (-/-) which contained smaller cells and photoreceptor rhabdomeres than their associated twin spots (+/+) and neighbouring heterozygous tissue (+/-). *p60*⁻ and *Dp110*⁻ adult eye clones were marked by a reduction in, or absence of pigment, respectively. Bar 10 μm.

4.6.3 Comparison of *p60* and *Dp110* cellular phenotypes

To investigate the relationship between p60 and Dp110 further, clones of *Dp110*$^-$ cells were examined in the same way as clones of *p60*$^-$ cells. Figure 4.9 shows that *Dp110*$^-$ ommatidia are small and recessed in the same way as *p60*$^-$ ommatidia. The clones shown in Figure 4.9 were created with a *Minute* gene on the marker FRT chromosome, providing the clone with a growth advantage to the neighbouring cells. However, *Dp110*$^-$ cells also survived in a wild type background. Tangential sections of *Dp110*$^-$ mutant ommatidia from such a clone revealed a phenotype that was similar but again more severe than the *p60* phenotype (Figure 4.10B). The rescue construct used to created *Dp110* mutants had a *ry*$^+$ rather than a *w*$^+$ marker (Section 2.5.2), so the mutant cells clones could be distinguished unambiguously from their heterozygous and wild type neighbours. As well as being clearly observable in pigment cells, the *w+* marker can be detected in photoreceptor cells by the presence of a small speck of pigment on the edge of the rhabdomere. Thus, the rhabdomere can be used a clear indication of both cell size and genotype. Wild type photoreceptors surrounded by *Dp110*$^-$ cells appear wild type in size, indicating that to achieve their normal size, wild type cells do not require Dp110 function in the surrounding cells (Figure 4.10B). In mixed ommatidia, the rhabdomeres of *Dp110*$^-$ cells are smaller than wild type, but larger than rhabdomeres in ommatidia composed entirely of mutant cells. These results show that photoreceptors need endogenous *Dp110* to achieve their normal size, but when these cells are growing in close proximity to *Dp110*$^+$ cells, there is a partial rescue of this growth defect.

4.7 Discussion

In this chapter, the *p60* genomic region has been identified and characterised. As well as being essential for the understanding of the genetics of *p60*, this information will assist the investigation of other genes in the region, particularly *dSTI1*, which had not been characterised previously. In addition, the mutations that have been characterised in the study will be useful reagents for further study of genes in the region.

Most importantly, the objective of this molecular genetic study has been achieved; null mutations in *p60* have been generated through a combination of deletions and rescue constructs. These mutations have allowed the endogenous function of p60 to be investigated, demonstrating that p60 is essential for viability and plays an essential role in imaginal disc tissue growth.

At the time, this study was the first genetic analysis of an adaptor for a Class I_A PI3K.

The generation of mitotic clones of mutants cells allows the investigation of the requirement for p60 at the cellular level. These studies reveal that both p60 and Dp110 are required for imaginal disc cells to achieve their normal size. However, the existence of clones that survive to adulthood demonstrates that neither p60 nor Dp110 are essential for proliferation or survival. These results reveal new aspects to the function of Class I_A PI3Ks and their adaptors that have not been suggested by studies using cultured mammalian cells.

The results of the genetic experiments described in this chapter correlate well with the results of previous experiments that utilised the ectopic expression of Dp110 in imaginal discs to investigate function. Therefore, as well as providing information about the cellular function of p60 and Dp110, these genetic experiments validate the experiments described in Chapter 5, which use the ectopic expression of p60 and Dp110 to further investigate the *in vivo* function of these proteins.

Chapter 5: Investigation of the *in vivo* function and regulation of p60 and Dp110 using ectopic expression

Chapter 5: Investigation of the *in vivo* function and regulation of p60 and Dp110 using ectopic expression

5.1 Introduction

Chapters 3 and 4 of this thesis describe the identification, biochemical characterisation and genetic analysis of p60, the adaptor for the *Drosophila* Class I_A PI3K, Dp110. These studies established two important points, firstly that Dp110 and p60 share several conserved features with mammalian Class I_A PI3Ks and their adaptors and secondly, that Dp110 and p60 are essential for normal imaginal disc growth. In addition to supporting a role for Dp110/p60 in normal disc growth, the demonstration that clones of cells deficient in Dp110 or p60 can be generated during imaginal disc development, and that those clones have a characteristic cell growth phenotype, supports the use of the *Drosophila* imaginal disc as model system to study Class I_A PI3K function in the context of a developing organ. The experiments described in this chapter extend the investigation of *in vivo* function by utilising ectopic expression of recombinant p60 during imaginal disc development. Furthermore, in attempt to more thoroughly address the functional relationship between p60 and Dp110, the effects of coexpressing various forms of the two proteins are investigated.

Ectopic expression, an established technique for investigating the *in vivo* function of *Drosophila* proteins (Phelps and Brand 1998), results in the expression of a protein at higher levels and/or in different tissues or at different stages of development than the endogenous protein. In addition to using the wild type protein, mutated forms of the protein that are either constitutively activated (dominant active) or interfere with the function of the endogenous protein (dominant negative) can also be ectopically expressed to investigate the role of the endogenous protein.

Ectopic expression of Dp110 has previously been shown to promote imaginal disc growth without affecting patterning (Leevers *et al.* 1996, Section 1.7.1). In contrast, the kinase-dead form of Dp110 reduces growth when it is ectopically expressed. Thus, in combination with the genetic analysis of Dp110 described in Chapter 4, these results demonstrate that Dp110 plays a role in promoting the growth of imaginal discs. This chapter firstly describes the effects of ectopic p60 expression. Secondly, these results are compared with the effects of ectopically expressing Dp110 both alone and in combination with p60. Finally, to assist the interpretation of these results, biochemical analyses

of the recombinant proteins are described. Together, the aim of these experiments is to increase our understanding of the *in vivo* interactions between a Class I_A PI3K and its adaptor.

5.2 Analysis of the effects of ectopic p60 and Δp60 expression

5.2.1 Generation of constructs to ectopically express HAp60 and HAΔp60 in *Drosophila*

Transgenes can be used to direct protein expression in a particular spatiotemporal pattern during development either by including specific promoter sequences upstream of the protein coding sequences or by using of the GAL4/UAS system (Figure 4.1, Phelps and Brand 1998). The latter system requires the construction and integration of a transgene in which DNA encoding the protein of interest is placed under the control of a promoter containing GAL4-responsive upstream activating sequences (UASs). The expression of the recombinant protein can be activated by crossing the transgenic animal with an animal containing one of a variety of GAL4-expressing transgenes, thereby allowing the protein to be expressed in one of several spatiotemporal patterns (Brand and Perrimon 1993). An additional benefit of this system is that detrimental effects caused by ectopically expressing the protein do not interfere with the generation of transgenic animals because there is minimal expression from the UAS promoter in the absence of GAL4.

The previous analysis of Dp110 function utilised GAL4 transgenes to ectopically express forms of Dp110 in the wing and eye imaginal discs (Leevers *et al.* 1996). Therefore, to be able to compare the results of ectopic expression of p60 with this analysis, UAS constructs were designed for p60. However, one of the disadvantages of the GAL4/UAS system is that GAL4 expression can have effects on its own, so to eliminate these background effects, constructs containing p60 under the control of the GMR (glass multimer reporter, Hay *et al.* 1994) promoter were also designed. In parallel, GMR-Dp110 constructs were made by Sally Leevers. The GMR promoter, which directs expression within and posterior to the morphogenetic furrow of the developing eye imaginal disc (Ellis *et al.* 1993), was chosen because, previously, GMR-GAL4 transgenes have been used to direct Dp110 ectopic expression (Leevers *et al.* 1996).

In order to make transgenic flies that ectopically express p60, the p60 cDNA was modified in the following manner (See Section 2.3.10 for details). Firstly, to ensure the reliable expression of the recombinant protein, the

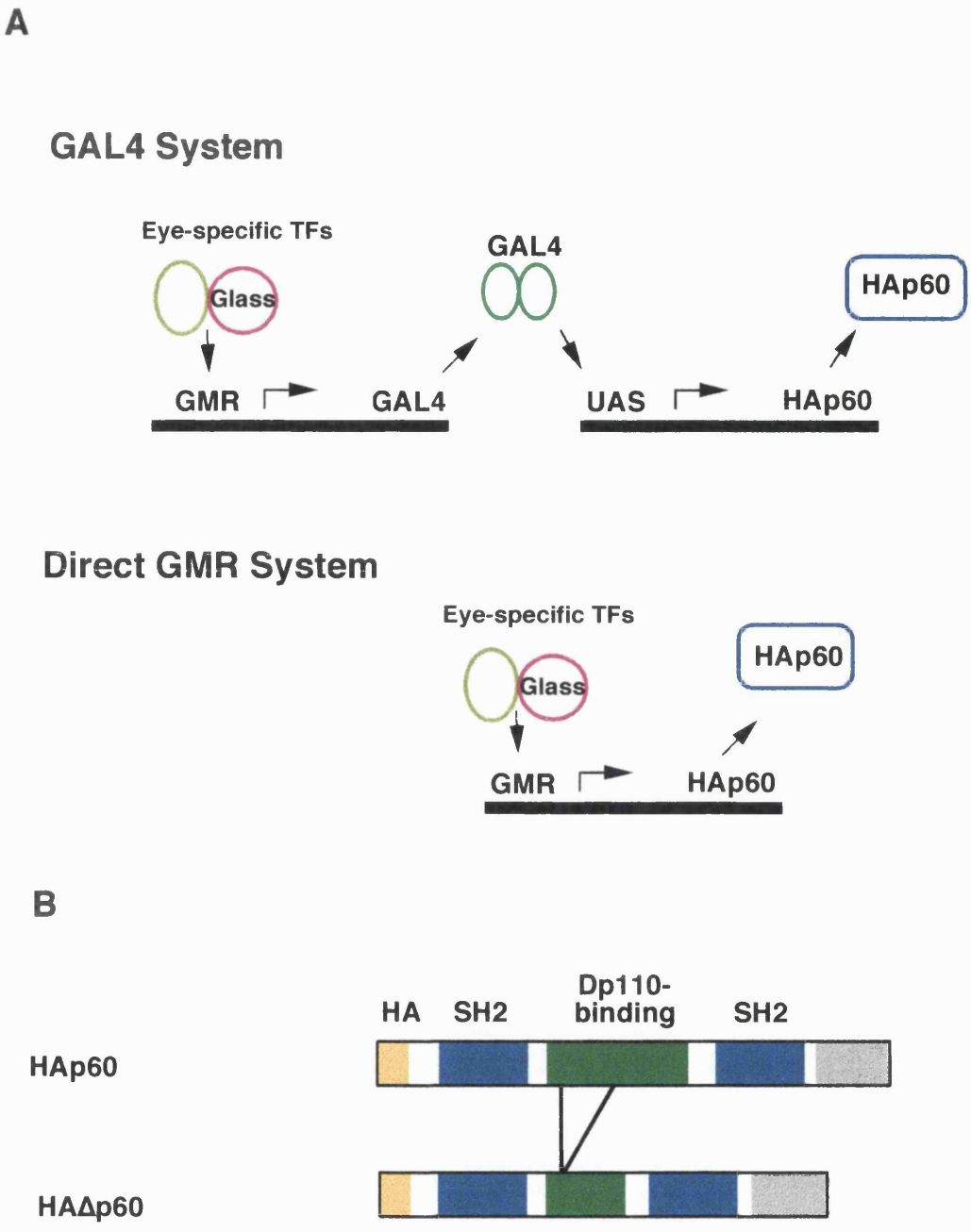


Figure 5.1. Ectopic expression strategies. (**A**) A schematic of the GAL/UAS system and a comparison with the strategy of the direct GMR promoter system. The UAS system allows ectopic expression in several different spatiotemporal patterns during development. However, the use of the direct promoter avoids the potential for artefacts of GAL4 expression. (**B**) Recombinant p60 is HA-tagged. HAΔp60 lacks amino acids 176 to 238 of p60 (Figure 3.2).

majority of the 5' UTR was removed from the cDNA, the 3' UTR was shortened, and the initiation codon was placed in the same optimal Kozak sequence context that was successfully used in the construction of Dp110 transgenes (Leevers *et al.* 1996). Next, a hemagglutinin tag (HA, Field *et al.* 1988) was added to the N-terminus of p60 (Figure 5.1), which was desirable for two reasons; namely, αHA antibodies would be able to both detect and purify HAp60, and the HA tag would distinguish HAp60 both from endogenous p60 and as well as from recombinant Dp110, which contains a myc epitope tag at the N-terminus (Evan *et al.* 1985; Leevers *et al.* 1996).

In addition to the wild type p60, a potentially dominant negative form of p60 was designed by analogy to Δp85, the dominant negative form of the mammalian Class I_A PI3K adaptor, p85α. This mutated form of p85α cannot bind to p110α because it lacks a small section of the inter SH2 domain (Section 1.5.1). The equivalent section of the p60 inter-SH2 domain was removed to generate HAΔp60 (Figure 3.2, Dhand *et al.* 1994a).

Finally, the plasmids encoding HAp60 and HAΔp60 were subcloned into pPUAST to make the UAS construct and pPGMR to make the GMR-directed construct. To make transgenic flies, these constructs were next injected into *Drosophila* embryos (Section 2.5.1). Several independent insertion lines for each transgene were recovered. The final stages in the subcloning of the plasmids were carried out by Sally Leevers, and the injections were carried out by Klaus Dücker in the laboratory of Ernst Hafen. The following section describes how the resulting transgenic lines were utilised to examine the effects of HAp60 and HAΔp60 ectopic expression.

5.2.2 The effect of HAp60 and HAΔp60 ectopic expression in the eye and wing disc

The effects of HAp60 and HAΔp60 ectopic expression were first investigated by examining the eyes of flies carrying the transgenes under the control of the GMR promoter. This promoter drives expression during the third larval instar in all eye cells both in, and posterior to, the morphogenetic furrow (Section 1.8.1). Furthermore, expression driven by the GMR promoter continues during pupation. Light microscopic examination of flies containing several independent insertion of the two transgenes revealed, firstly, that adult *GMR-HAp60* and *GMR-HAΔp60* flies possessed small eyes. Secondly, the eyes of *GMR-HAΔp60* flies were consistently smaller than the eyes of the *GMR-HAp60* flies. Finally, very similar phenotypes were observed in *GMR-GAL4/UAS-HAp60* and *GMR-GAL4/UAS-HAΔp60* flies. Thus, ectopic expression of both HAp60 and HAΔp60 appears to inhibit eye growth.

The eyes of *GMR-HAp60* and *GMR-HAΔp60* flies were next examined more closely by using SEM (Figure 5.2). Notably, the eyes of *GMR-HAΔp60* flies consistently collapsed during fixation, dehydration and critical point drying in the preparation for SEM (Figure 5.2, Section 2.6.4) whereas the eyes of *GMR-HAp60* flies did not. When the milder solvents, ethanol and amyl acetate, were used instead of the conventional acetone for the dehydration and critical point drying of the flies, some of the *GMR-HAΔp60* eyes remained intact (Figure 5.2, Section 2.6.4). These results suggest that the retinas of *GMR-HAΔp60* eyes were thinner than those of *GMR-HAp60* eyes.

The eyes of *GMR-HAp60* and *GMR-HAΔp60* flies were clearly smaller than wild type at a gross level, but it was not immediately clear whether this reduction in size was the result of these eyes possessing smaller ommatidia, fewer ommatidia, or both smaller and fewer ommatidia than wild type. Using images obtained with SEM to count the ommatidia (Section 2.6.4), it was revealed that the eyes of *GMR-HAp60* flies and *GMR-HAΔp60* flies contained 5% and 10% less ommatidia than wild type eyes, respectively (740±12 and 661±29 compared to 778±8, Figure 5.3). A rough estimate of the size of the ommatidia could be achieved through further analysis of the SEM images, revealing that ommatidia in the eyes of *GMR-HAp60* and *GMR-HAΔp60* flies were approximately 85% and 80% smaller in diameter than wild type ommatidia, respectively (23 units and 21 units compared to 27 units). Taken together, these data suggest that the ectopic expression of HAp60 and HAΔp60 reduces both cell number and cell size.

To examine whether the differentiation of the photoreceptors, cone cell and pigment cells were disrupted in *GMR-HAp60* eyes, tangential sections of these eyes were examined (Figure 5.2). While the size of the ommatidia appeared significantly smaller than wild type, the morphology and arrangement of the ommatidial cells appeared normal. Thus, ectopic expression of HAp60 did not affect patterning, suggesting that HAp60 does not interfere with the signal transduction pathway downstream of RTKs necessary for the correct differentiation of photoreceptors (Section 1.8.1). A similar analysis of *GMR-HAΔp60* eyes was attempted but while examination of tangential sections provided further evidence that the retinas were severely reduced in these eyes, the thinness of these retinas prevented detailed analysis of the differentiation of the constituent cells.

The above results are consistent with the ectopic expression of HAp60 or HAΔp60 inhibiting growth. These results are in marked contrast to the effect of ectopic mycDp110 expression, which promotes growth. To ensure that the effects of HAp60 and HAΔp60 ectopic expression were not due to a peculiarity of eye

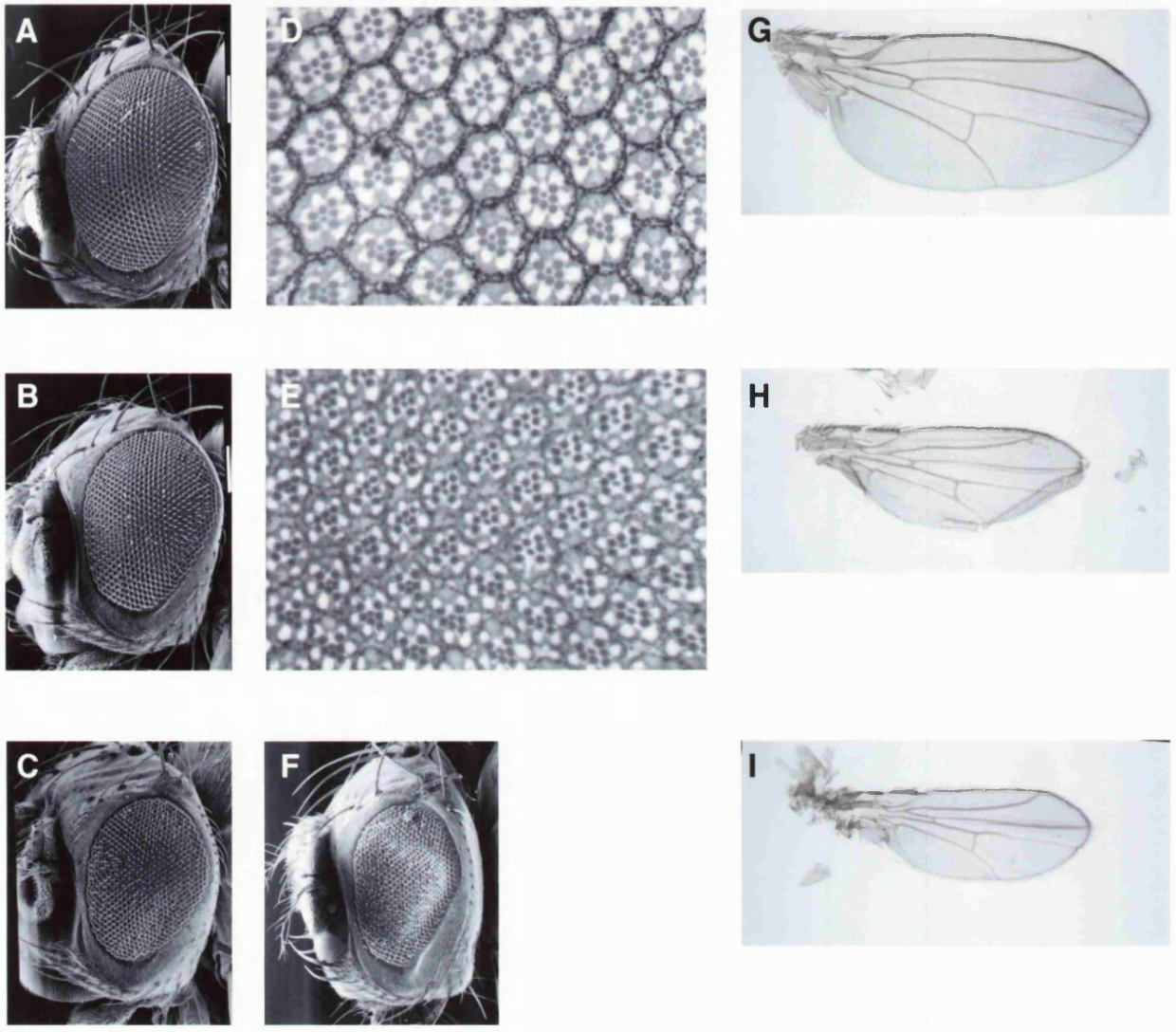


Figure 5.2. Ectopic expression of HAp60 and HAΔp60 in the eye or the wing imaginal disc reduces the size of the adult organs. SEMs of eyes from (**A**) wild type, (**B**) *GMR-HAp60* and (**C**) *GMR-HAΔp60* flies. Sections of (**D**) wild type and (**E**) *GMR-HAp60* eyes demonstrate that photoreceptor differentiation is unaffected by HAp60 ectopic expression. (**F**) Retinas of *GMR-HAΔp60* eyes were too thin to generate meaningful sections and often collapsed during SEM preparation . Wings from flies containing (**G**) *MS1096-GAL4* alone and in combination with (**H**) *UAS-HAp60* and (**I**) *UAS-HAΔp60*, together demonstrate that ectopic expression of HAp60 or HAΔp60 in the wing imaginal disc are consistent with results in the eye imaginal disc.

development, *UAS-HAp60* and *UAS-HAΔp60* flies were crossed to the *MS1096-GAL4* line, which had been used in previous experiments to direct mycDp110 expression in the dorsal pouch of the wing imaginal disc (Leevers *et al.* 1996). Consistent with the results obtained in the eye, the wings of *MS1096; UAS -HAp60/+* and *MS1096; UAS -HAΔp60/+* adult flies were considerably smaller than the wings of *MS1096/+* flies (Figure 5.2C). Furthermore, the dorsal expression pattern of MS1096 caused the resulting wings to curl upwards, the opposite effect to that seen when mycDp110 is expressed with MS1096 (Leevers *et al.* 1996). Thus, in both the wing and eye imaginal discs, ectopically expressed HAp60 and HAΔp60 have the opposite effect from that of mycDp110 ectopic expression.

5.3. Investigation of the relationship between Dp110 and p60

5.3.1 Ectopic expression of mycDp110 and HAp60 in the eye

The comparison of the effects of HAp60 and HAΔp60 ectopic expression (Section 5.2) with both the results of mycDp110 ectopic expression (Leevers *et al.* 1996) as well as the genetic analysis of Dp110/p60 function (Chapter 4) suggest that both HAp60 and HAΔp60 act as dominant-negative proteins. A possible explanation for these effects is that overexpressed HAp60 and HAΔp60 compete with endogenous Dp110/p60 complexes for upstream activators, which are likely to be pYXXM-containing RTKs or their substrates. However, an alternative explanation is that HAp60 and/or HAΔp60 interfere with a distinct growth-promoting pathway from that activated by Dp110. To provide more data to distinguish between these two possibilities, recombinant forms of p60 and Dp110 were ectopically coexpressed. For this series of experiments, *GMR-HAp60* and *GMR-HAΔp60* flies were crossed with flies containing *GMR-mycDp110*, *GMR-mycDp110*^{D954A} or *GMR-mycDp110-CAAX*. Each line used in these experiments was also crossed to the *yw* line that was the genetic background for the transgenic lines. Thus, flies carrying one copy of a p60 transgene and/or one copy of a Dp110 transgene could be compared. The experiments were repeated twice using two different lines for each insertion and examination with a light microscope confirmed that the results were reproducible and did not vary significantly between lines. Therefore, the experiment was repeated two more times with only one line for each insertion. To record and quantitate the results of these experiments, flies from the final experiment were examined by SEM; where possible, a number of flies were used for each point of the experiment (Figure 5.3).

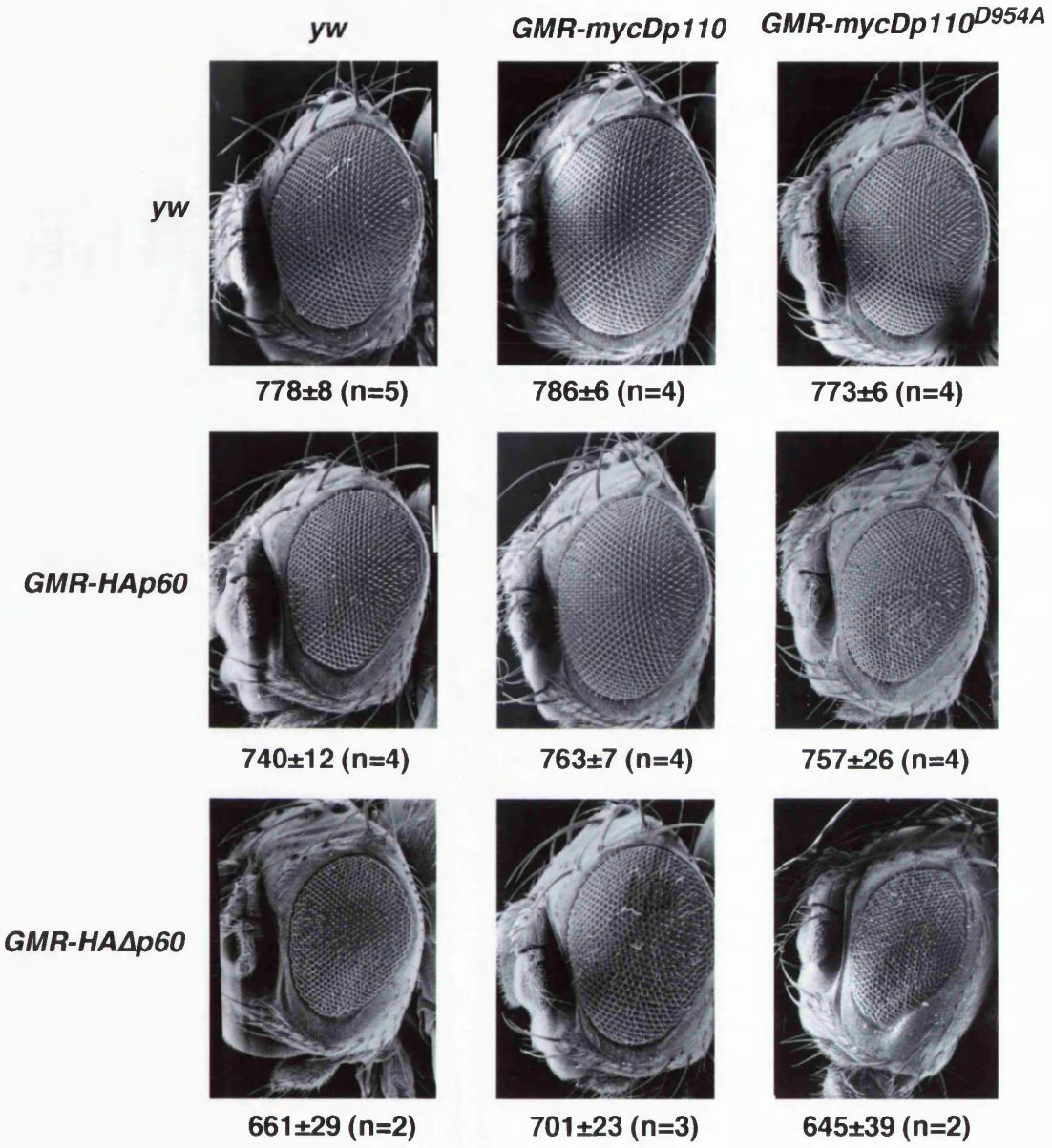


Figure 5.3. The combination of HAp60 and mycDp110 ectopic expression in the eye imaginal disc. SEMs of eyes from flies with insertions of *GMR-HAp60*, *GMR-HAΔp60 GMR-mycDp110*, or *GMR-mycDp110^{D954A}* on their own and in combinations. For each combination, the number of ommatidia are shown, in and in parentheses are indicated the number of eyes used for counting.

Before the experiments combining p60 and Dp110 overexpression could be interpreted, it was important to demonstrate that mycDp110 had a consistent effect regardless of whether it was expressed by using GMR-GAL4 or by using a direct GMR promoter. Indeed, like *GAL4/UAS-mycDp110* flies, *GMR-mycDp110* flies did have larger than wild type eyes (Figure 5.3). However, unlike *GMR-GAL4/UAS-mycDp110* flies, which had slightly rough eyes (Leevers *et al.* 1996), *GMR-mycDp110* eyes were perfectly smooth as assessed by SEM. One explanation for this discrepancy is that GAL4 expression, which causes minor roughness on its own, synergises with mycDp110 overexpression to cause roughness in the eye, but this hypothesis has not been explored further.

The eyes of *GMR-mycDp110/GMR-HAp60* flies were indistinguishable from the wild type (Figure 5.3), displaying no visible differences in size or appearance. In contrast, the eyes of *GMR-mycDp110^{D954A}/GMR-HAp60* flies appeared small, similar to the eyes of *GMR-HAp60* flies. These results demonstrate that the inhibition of growth by HAp60 overexpression is rescued by coexpression of mycDp110. Ectopic expression of mycDp110 also appeared to rescue the growth-inhibitory effects of HAΔp60 ectopic expression, although the resulting eyes had a slightly rough appearance (Figure 5.3). Because HAΔp60 is not predicted to bind to mycDp110, this result is interesting and will be discussed further in Section 6.3. In summary, The hypothesis that HAp60 and HAΔp60 have a dominant negative effect on endogenous Dp110 function is supported by the results of combining the ectopic expression of various forms of Dp110 and p60 in the eye.

5.3.2 Ectopic expression of Dp110 and p60 in the wing

To assess whether the results described above were applicable to imaginal disc growth in general, the experiments were repeated using the wing imaginal disc. In addition to providing a second independent disc type, analysing wings overcomes of one of the disadvantages of the eye; namely, that, while GMR-driven ectopic expression produced very reproducible results, it was very difficult to quantify these results, because the SEM images produced slightly distorted, two-dimensional representations of three-dimensional effects. In contrast, wing imaginal disc growth can be reliably assessed by examining a two-dimensional image of the adult wing. Preliminary experiments were performed by creating lines in which the *UAS-mycDp110* and *UAS-HAp60* transgenes were recombined on the same chromosome and then crossed to flies containing the MS1096-GAL4 driver (Section 2.5.2). The results of these experiments were generally consistent with

those obtained from ectopic expression in the eye (Figure 5.4). In particular, the coexpression of HAp60 with mycDp110 resulted in a correctly patterned wing that was either slightly smaller than or the same size as the wild type. Interestingly, when mycDp110 was coexpressed with HAΔp60, the wing was wild type in size but possessed a highly disrupted pattern of veins, suggesting that mycDp110 could the rescue of the growth inhibition caused by HAΔp60, it could not fully restore normal development of the wing (Figure 5.4). Despite the possibility of artefacts arising from GAL4 expression, experiments using *MS1096-GAL4* provided a more practical and quantitative system for assessing the effects of ectopic expression on growth and patterning than the use of GMR transgenes. Thus, while the analysis presented here utilising the MS1096 GAL4/UAS system is not as extensive as subsequent experiments performed in the laboratory using the same system, a number of conclusions can be drawn. Firstly, the growth inhibition by HAp60 can be rescued, if not completely, almost completely, mycDp110 without any effects on patterning. Secondly, MycDp110^{D954A} cannot rescue growth inhibition by HAp60. Finally, the combination of HAΔp60 and mycDp110 does not produce a normal wing.

5.4. The biochemical properties of ectopically expressed p60 and Dp110

5.4.1 Development of the hsGAL4/UAS system as a protein expression system

To aid in the interpretation of the results described above, I wanted to assess the relative expression levels and binding properties of the ectopically expressed forms of mycDp110 and HAp60 using biochemical techniques. As it was thought that the level of recombinant protein expressed in the imaginal discs using the GAL4 drivers described above, would not produce enough protein for these experiments, an alternative strategy of studying recombinant proteins in *Drosophila* was undertaken[1]. In order to generate lines for the large-scale production of HAp60 and mycDp110, the *hs-GAL4* line, which expresses GAL4 ubiquitously upon heat shock, was crossed to *UAS-HAp60* and *UAS-mycDp110*. Very few progeny with the desired phenotype emerged from these crosses, even when the progeny were reared at 18°C, indicating that the hsGAL4 transgene produced some GAL4 expression in the absence of heat shock and that the resulting HAp60 or mycDp110 expression caused lethality.

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[1]However, it has been shown subsequently by Krishna Pitrola and Nicole Mathon that Western blotting can be used to reveal *MS1096-GAL4*- driven *UAS-mycDp110* or *UAS-HAp60*.

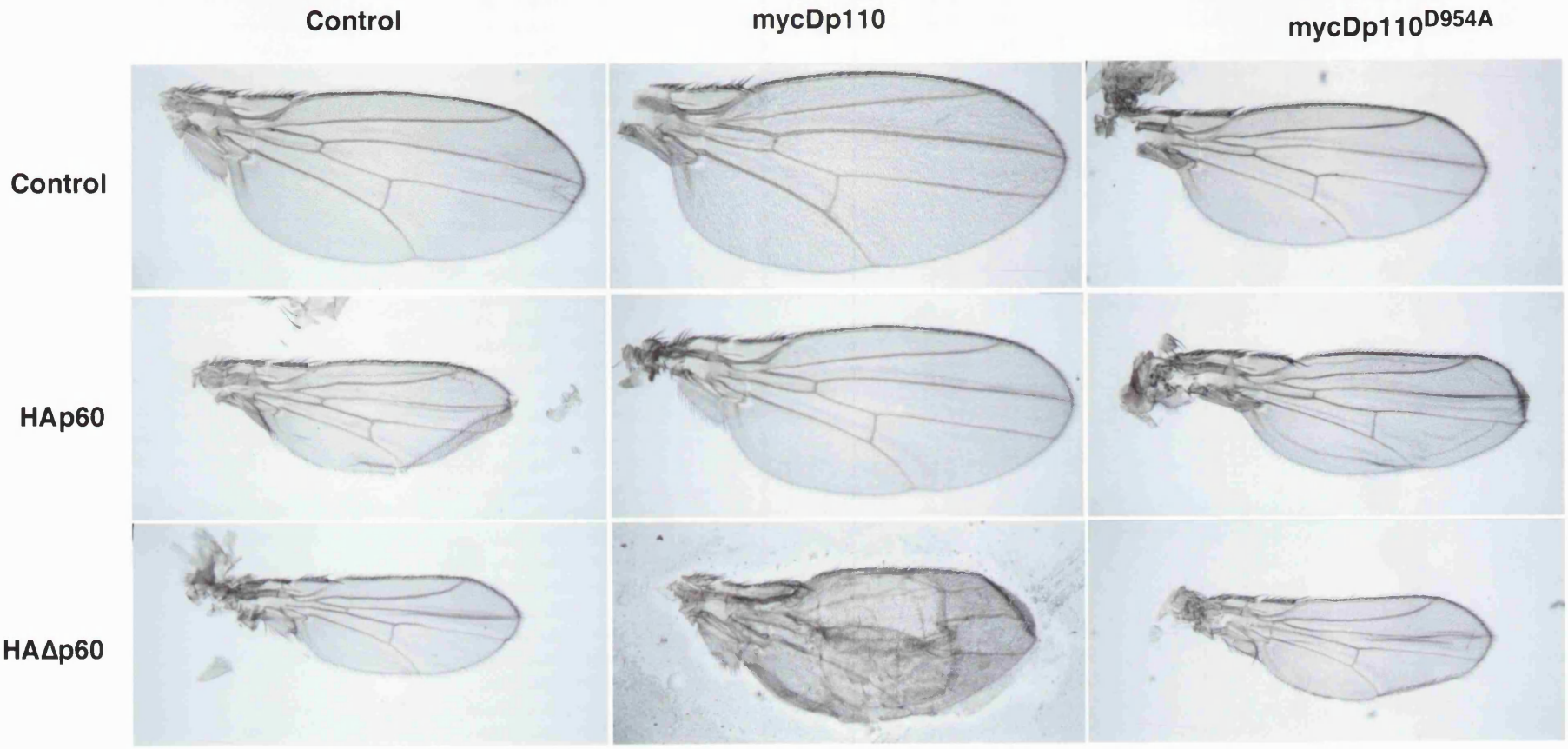


Figure 5.4 . The combination of HAp60 and mycDp110 ectopic expression in the wing imaginal disc. Wings from *MS1096-GAL4* / + females containing the indicated *UAS*- transgenes.

The low survival rate of the flies made it impossible to generate a viable line of flies containing either both *hsGAL4* and *UAS-HAp60* or both *hsGAL4* and *UAS-mycDp110*. Such a line could be used to generate flies expressing both proteins by crossing it to a line containing transgene for the other protein. Instead, flies containing *UAS-mycDp110* and *UAS-HAp60* recombined onto the same chromosome, described in the previous section, were crossed to *hsGAL4* flies. Interestingly, the *hsGAL4/UAS-mycDp110, UAS-HAp60* progeny appeared perfectly healthy, emerging with expected Mendelian frequency. This result suggests, firstly, that when mycDp110 and HAp60 are ubiquitously overexpressed together, they cancel out the activity that causes lethality when either protein is expressed alone. Secondly, as a parallel to the effects of mycDp110 and HAp60 ectopic expression in imaginal discs, which are also cancelled out when the two proteins were coexpressed, this results suggests that the lethality caused by hsGAL4-driven mycDp110 and HAp60 is due to the perturbation of the endogenous Dp110/p60 signalling during development rather than to non-specific toxicity.

For the biochemical analysis, flies with the genotype *hsGAL4/UAS-mycDp110, UAS-HAp60* were heat shocked and then either homogenised immediately or placed at 25°C and homogenised after 1, 2, 3, or 4 hours (Section 2.2.1). The resulting whole fly lysates were analysed by Western blotting with αHA and αDp110 antibodies. This experiment reveals that both HAp60 and mycDp110 were detectable in flies homogenised immediately after heat shock, but that the level of recombinant protein expression increased with time (Figure 5.5A). Thus, for subsequent experiments, flies were homogenised four hours after heat shock. Interestingly, αHA recognised three closely-migrating proteins on the Western blot, one with a slightly larger apparent size than endogenous p60 and two others with a slightly smaller apparent size than p60. The larger protein is likely to be full length HAp60, while the two smaller proteins may be degradation products. Consistent with this hypothesis, the two smaller proteins were not recognised by Western blotting with αp60C (Figure 5.5B), suggesting that like the C-terminus of endogenous p60 (Section 3.4.2), the C-terminus of HAp60 is prone to degradation.

To examine the relative expression levels of Dp110 and p60 with different combinations of transgenes, flies containing either *UAS-HAp60* or *UAS-HAΔp60* alone or with these transgenes recombined with either *UAS-mycDp110* or *UAS-mycDp110*^{D954A} were crossed on a large scale with *hsGAL4* flies. The progeny were reared at 18°C to minimise lethality cause by leaky expression of GAL4 driven by the heat shock promoter. Only the cross that resulted in progeny coexpressing mycDp110 and HAp60 produced large

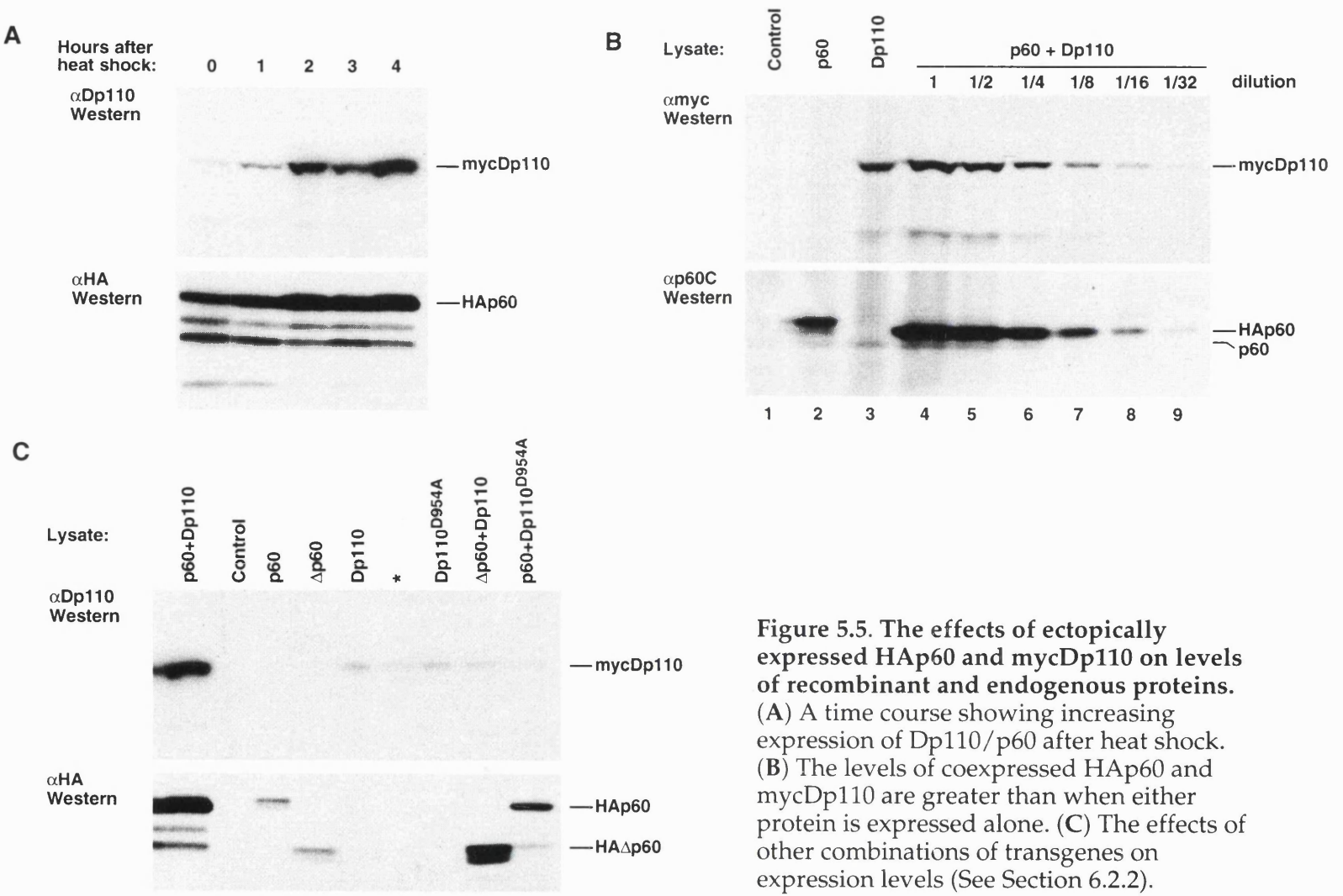


Figure 5.5. The effects of ectopically expressed HAp60 and mycDp110 on levels of recombinant and endogenous proteins. (**A**) A time course showing increasing expression of Dp110/p60 after heat shock. (**B**) The levels of coexpressed HAp60 and mycDp110 are greater than when either protein is expressed alone. (**C**) The effects of other combinations of transgenes on expression levels (See Section 6.2.2).

numbers of flies with the desired genotype. However, the remainder of the crosses produced enough flies to analyse biochemically. Flies of each genotype that were not homogenised after heat shock survived for several days. Thus, a system has been established to produce recombinant forms of mycDp110 and HAp60 in *Drosophila* in sufficient quantities for biochemical analysis.

5.4.2 The expression levels of HAp60 and mycDp110 when both proteins are ectopically coexpressed

One possible explanation for the apparent wild type phenotype that results from mycDp110 and HAp60 coexpression is the expression of both proteins is reduced when they are coexpressed, thus eliminating any effects they might exert. To test this possibility, expression levels were assessed by Western blotting with αmyc and αHA after mycDp110 and HAp60 expression was induced in adult flies using hs-GAL4, as described above (Figure 5.5B). Because Western blots developed with enhanced chemiluminescence (ECL) reagents and exposed on autoradiography film are very not quantitative, a two-fold dilution series was performed. This experiment showed that the amount of mycDp110 and HAp60 was not reduced by co-expression – in fact, when co-expressed, the amount of mycDp110 and HAp60 was found to be approximately 4-fold higher than when either transgene was expressed alone (Figure 5.5B, compare lanes 2, 3 and 4 with lane 6). When coexpressed with mycDp110, the level of HAp60 expression was at least 32 times the level of endogenous p60 expression and therefore around 8 times the endogenous level when expressed on its own. Thus, the effect, or lack of effect, of HAp60 and mycDp110 coexpression cannot be explained by a reduction in the levels of both proteins. Instead, the reason why increased levels of mycDp110 and HAp60 has no effect needs to be addressed.

5.4.3 The association of recombinant Dp110 and p60

One possible explanation for increase in HAp60 and mycDp110 levels when both proteins are expressed is that mycDp110 and HAp60 can co-stabilise one another. To test this possibility, it was important to determine whether, like endogenous Dp110 and p60 (Section 3.4.2), the recombinant proteins can co-associate. Immunoprecipitations with αmyc and αHA were performed from lysates of flies expressing both proteins and compared to affinity purification of the lysates using pYXXM phosphopeptide beads (Section 2.2). MycDp110 and HAp60 could be co-purified by all of these methods, demonstrating that the recombinant proteins did associate with one another (Figure 5.6A). In contrast, immunoprecipitation with either αmyc or

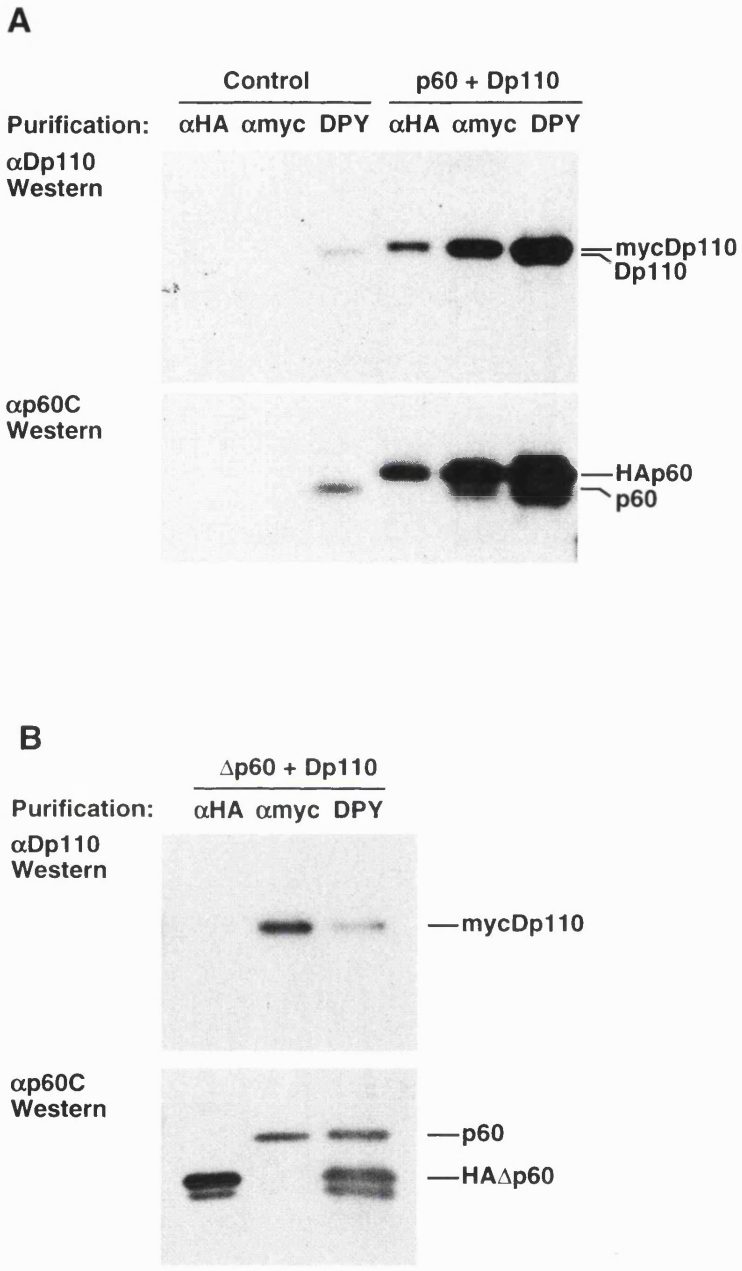


Figure 5.6. The biochemical association between Dp110 and p60 (both recombinant and endogenous). Immunoprecipitation with αHA, αmyc and affinity purification with a DPY phosphopeptide from lysates from heat shocked flies with the genotype: (**A**) *hsGAL4* and *hsGAL4/UAS-HAp60, UAS-mycDp110* and (**B**) *hsGAL4/UAS-HAΔp60, UAS-mycDp110.*

αHA failed to purify a complex of mycDp110 and HAΔp60, even though each protein could be purified on its own and HAΔp60 could be purified with pYXXM phosphopeptide beads (Figure 5.6B), demonstrating that, as predicted, HAΔp60 does not bind to mycDp110. These experiments also revealed that mycDp110 could bind to endogenous p60, which could be distinguished from HAp60 and HAΔp60 by a difference in electrophoretic mobility (Figure 5.6). It is also likely that HAp60 could bind endogenous Dp110, but mycDp110 could not be clearly resolved from endogenous Dp110 using a 7.5% acrylamide gel (Figure 5.6). In summary, taken together with the results of ectopic expression in the eye and wing imaginal discs, these results are consistent with the hypothesis that ectopically expressed mycDp110 and HAp60 affect the same signalling pathway, in opposing ways, when expressed alone. However, when coexpressed, mycDp110 and HAp60 form a complex that is neither activatory or inhibitory. The increased level of this complex, compared to the levels of expression observed when either protein is expressed alone, raises the possibility that, when complexed to HAp60, mycDp110 is only responsive to endogenous signals. However, this scenario is only one explanation of the data, which will be comprehensively discussed in Section 6.3.

5.5 Discussion

This chapter describes how the ectopic expression of p60 was utilised to further investigate the *in vivo* function of the protein. Ectopic expression of HAp60 in the developing eye or wing imaginal disc inhibits growth. While the mechanism by which this inhibition occurs is still unclear, the results of the HAp60 and Dp110 coexpression studies strongly suggest that HAp60 has a dominant negative effect on the Dp110 pathway. Thus, like the generation of clones of *p60* and *Dp110* mutant cells, the ectopic expression of HAp60 can be utilised to inhibit Dp110 experimentally during imaginal disc development. Furthermore, such inhibition can be directed to a defined spatiotemporal pattern by utilising specific GAL4 drivers.

The experiments described in this chapter demonstrate the importance of developing a method for studying the ectopically-expressed HAp60 and mycDp110 biochemically. These studies have revealed a number of possibilities for the regulation of p60 and Dp110, including the possibility that Dp110 and p60 can stabilise each other, and that p60 can inhibit associated Dp110, thereby allowing it to be activated only by endogenous upstream activating signals. These ideas and the implications for the understanding of Class I_A PI3K function in general will be discussed further in Chapter 6.

Chapter 6: Discussion

Chapter 6: Discussion

6.1 Overview

This thesis describes the identification of p60, the adaptor for the *Drosophila* Class I_A PI3K, Dp110, and its subsequent characterisation by biochemical and genetic methods. At the end of Chapters 3, 4 and 5, the immediate conclusions of the results presented in those chapters have been discussed. In this chapter, I would like to bring all of these results together to discuss both the conclusions that can be drawn about the functions of p60 and Dp110, as well as the relevance of those conclusions to the wider study of metazoan Class I_A PI3Ks and their adaptors. Firstly, the role of p60 and its relationship to Dp110 will be compared with the results of studies in mammalian systems. Secondly, the biological function of Dp110 and p60 will discussed in the light of functional data from other model systems. Finally, the implications of these studies in the understanding of imaginal disc growth and size control will be discussed.

6.2 The role of p60 as the adaptor for Dp110

The results presented in this thesis show that p60 binds to Dp110, that *p60* mutants have a similar phenotype to *Dp110* mutants, that ectopic expression of p60 appears to inhibit Dp110 function and that coexpression of Dp110 and p60 leads to an increase in the levels of both proteins. Thus, supported by studies in mammalian systems, these data fit with a model in which the adaptor acts as a signal transducer, inhibitor and stabiliser of Class I_A PI3Ks. These potential roles of p60 will be discussed in turn.

6.2.1 p60 as a signal transducer

The experiments described in Chapter 3 clearly demonstrate that p60 associates with both with the Class I_A PI3K, Dp110 and with pYXXM phosphopeptides. Furthermore, analysis of the amino acid sequence of p60 demonstrates homology to both the SH2-domain and inter-SH2-domain regions of the mammalian adaptors. Thus, both structural and functional evidence suggests that, the role of p60 in signal transduction is to facilitate the translocation of Dp110 to pYXXM motifs on activated RTKs and their substrates.

Comparison of the sequence data predicts that the pYXXM binding specificity of the mammalian adaptor SH2 domains is conserved in both of the SH2 domains of p60. Thus, it seems that all adaptors for Class I_A PI3Ks possess

two SH2 domains with the same apparent binding specificity. Why is this conservation of binding specificity in both domains so important? It has been argued that the presence of two SH2 domains in PLCγ, SHP-2, ZAP-70 and the adaptors for Class I_A PI3Ks allows these proteins to bind with high specificity to tandem phosphotyrosine peptides (Ottinger *et al.* 1998). In support of this idea, a number of RTKs and associated substrate molecules, including the PDGF receptor and IRS-1, have multiple phosphorylated YXXM motifs. Similarly, the spacing of the phosphotyrosines in the target peptide is vital for the binding of ZAP70 via its two SH2 domains (Herbst *et al.* 1994; Ottinger *et al.* 1998). However, other RTKs, such as the M-CSF and c-kit receptors, bind to Class I_A PI3K adaptors through only one pYXXM motif (Reedijk *et al.* 1992; Serve *et al.* 1995). Furthermore, it has been shown that the spacing of the tandem adaptor-binding phosphotyrosines does not affect the ability of p85α to bind to them (Herbst *et al.* 1994). Furthermore, p60 binds as well to an excess of singly phosphorylated pYXXM phosphopeptide as it does to an excess of doubly phosphorylated peptide (Figure 3.1). It is possible that, in these experiments, the presence of two SH2 domains allowed p60 to bind to more than one phosphopeptide simultaneously. Thus, it is still unclear whether the two SH2 domains found in the adaptors for Class I_A PI3Ks serve to increase binding affinity for activated RTKs and their substrates.

To address the question of why p60 has two SH2 domains, I generated p60 constructs with mutations that were predicted to abolish binding to phosphotyrosine in either or both SH2 domain (R57M, R362M, Chapter 3, Zvelebil *et al.* 1995). These constructs were subcloned into the appropriate vectors to make transgenic flies by Niki Scaplehorn and this work was continued by Krishna Pitrola and Nicole Mathon. Preliminary results suggest that mutation of either SH2 domain diminishes the inhibitory effect on growth caused by ectopic expression of p60; furthermore, mutation of both SH2 domains almost completely eliminates this inhibitory effect (Section 6.2.3). Thus, a simple increase in the affinity for pYXXM motifs is still a plausible reason for the presence of two SH2 domains in the adaptors for Class I_A PI3Ks (Section 6.2.3). Similar results were obtained when analogous mutations were made in p85α and the activity of the associated p110α was assessed *in vitro* (Rordorf Nikolic *et al.* 1995). To further test the requirement for two SH2 domains, it will interesting to examine whether the mutant forms of p60 with only one functional SH2 domain can be affinity purified with a pYXXM phosphopeptide.

The similarity between the *Dp110* and *p60* mutant phenotypes is consistent with the proposal that the sole function of p60 is to mediate Dp110 function. However, the *Dp110* phenotypes are consistently more severe than the

p60 phenotypes (Section 4.6). There are several possible explanations for this difference. In the case of the larval phenotype, the maternal contribution of mRNA/protein in the case of p60 may be greater than of the case of Dp110 and/or the p60 protein or mRNA may be more stable than that of Dp110 (Section 6.2.2). Alternatively, it may be that in *Dp110* mutants, free p60 inhibits growth in a Dp110-independent manner. A third possibility is that, in *p60* mutants, Dp110 may be active on its own or activated by an alternative mechanism such as through an interaction with Ras (Sections 1.3.5 and 6.3.3). The *Dp110, p60* double mutants have a similar or more severe zygotic phenotype than that of the *Dp110* mutants (Figure 4.7), demonstrating that free p60 does not inhibit growth in the *Dp110* mutant and that, as far as can be assessed, p60 has no role other than the regulation of Dp110. Therefore, this result is consistent with Dp110 possessing some activity in the absence of p60. The growth-promoting effect of ectopically expressing mycDp110 alone further supports this suggestion. However, the severity of the *p60* phenotype demonstrates that Dp110 is highly dependent upon p60 to fulfil its wild type biological function.

6.2.2 p60 as a stabiliser of Dp110

The results presented in Figure 5.5 show that coexpression of HAp60 with mycDp110 leads to an increase in the levels of both recombinant proteins, suggesting that the proteins can stabilise one another through association. Similarly, it has been shown that mammalian p110α is stabilised by the presence of an adaptor (Layton *et al.* 1998; Yu *et al.* 1998b, Section 1.3.3). While there has been no report of a Class I_A PI3K stabilising its adaptor, such an effect may not have been investigated previously because the recombinant adaptors are essentially stable on their own; furthermore, they are much more stable than the Class I_A PI3Ks. An alternative explanation for the increase in the level of HAp60 seen in Figure 5.5 is that it results from a mycDp110-induced increase in cellular protein synthesis and/or a reduction in protein degradation. When performing experiments to investigate the levels of ectopically expressed proteins described in Section 5.4.2, a number of interesting, albeit preliminary, observations were made that are relevant to this model. Firstly, while mycDp110^{D954A} was expressed at a similar level to mycDp110 when either protein was induced alone, its expression level was not enhanced by the coexpression of HAp60 (Figure 5.5C). This result suggests that the upregulation of mycDp110 by coexpression with HAp60 is dependent upon its kinase activity. Secondly, the coexpression of wild type Dp110 increased the amount of HAΔp60 protein (Figure 5.5C), suggesting that the upregulation of HAp60 may not be entirely dependent upon association. Finally, however, the level of HAp60 was increased by coexpression

with mycDp110^{D954A}, although not to the same degree as with wild type Dp110 (Figure 5.5C). This observation suggests that, to some extent, association does contribute to the increased levels of HAp60. In summary, the experiments presented in this thesis have demonstrated that the *in vivo* protein expression levels of a Class I_A PI3K and its adaptor are interdependent. Future studies, such as pulse-chase experiments, should determine the extent and physiological role of this interdependence.

6.2.3 p60 as an inhibitor of Dp110

This report demonstrates that overexpression of HAp60 inhibits endogenous Dp110 activity, resulting in an inhibition of growth (Chapter 5). There are at least three possible mechanisms by which p60 could inhibit Dp110: by competing with endogenous Dp110/p60 complexes for pYXXM motifs, by directly inhibiting kinase activity upon association with Dp110 or by taking Dp110 to a location that prevents access to its substrate (Figure 6.1A). A combination of some or all of these effects is also possible. However, the best explanation of the data is the first one because the other two mechanisms require protein: protein interaction between p60 and Dp110, whereas Δp60, which does not bind Dp110, still inhibits growth.

Interestingly, coexpression of mycDp110 can at least partially rescue the growth inhibition caused by Δp60 (Figure 5.4). This result suggests that mycDp110 can reach its substrate and transduce a growth-promoting signal without associating with the receptor through p60, consistent with mycDp110 promoting growth when ectopically expressed alone (Figure 6.1B). Interestingly, when mycDp110 and HAp60 are coexpressed, there is little or no effect on growth (Figures 5.3 and 5.4). Two possibilities that account for this result are presented as two models describing how mycDp110 and HAp60 interact when they are coexpressed (Figure 6.2). Distinguishing between the two models will determine whether endogenous p60 can acts an inhibitor of associated Dp110.

Both models assume that where possible, mycDp110 and HAp60 associate with one another. However, the first model proposes that when HAp60 and mycDp110 are coexpressed, there is an excess of free HAp60 (e.g. because it is more stable than mycDp110), which inhibits receptor signalling by competing with Dp110/p60 complexes for pYXXM motifs (Figure 6.2A). This inhibition of endogenous Dp110 signalling is overcome by the coexpressed mycDp110, which, although associated with HAp60, can still reach its substrate without being translocated to pYXXM motifs on activated RTKs or their substrates. Despite the large numbers of mycDp110 and HAp60 molecules present when both proteins are coexpressed, their two opposing effects on growth cancel out. Thus, in this

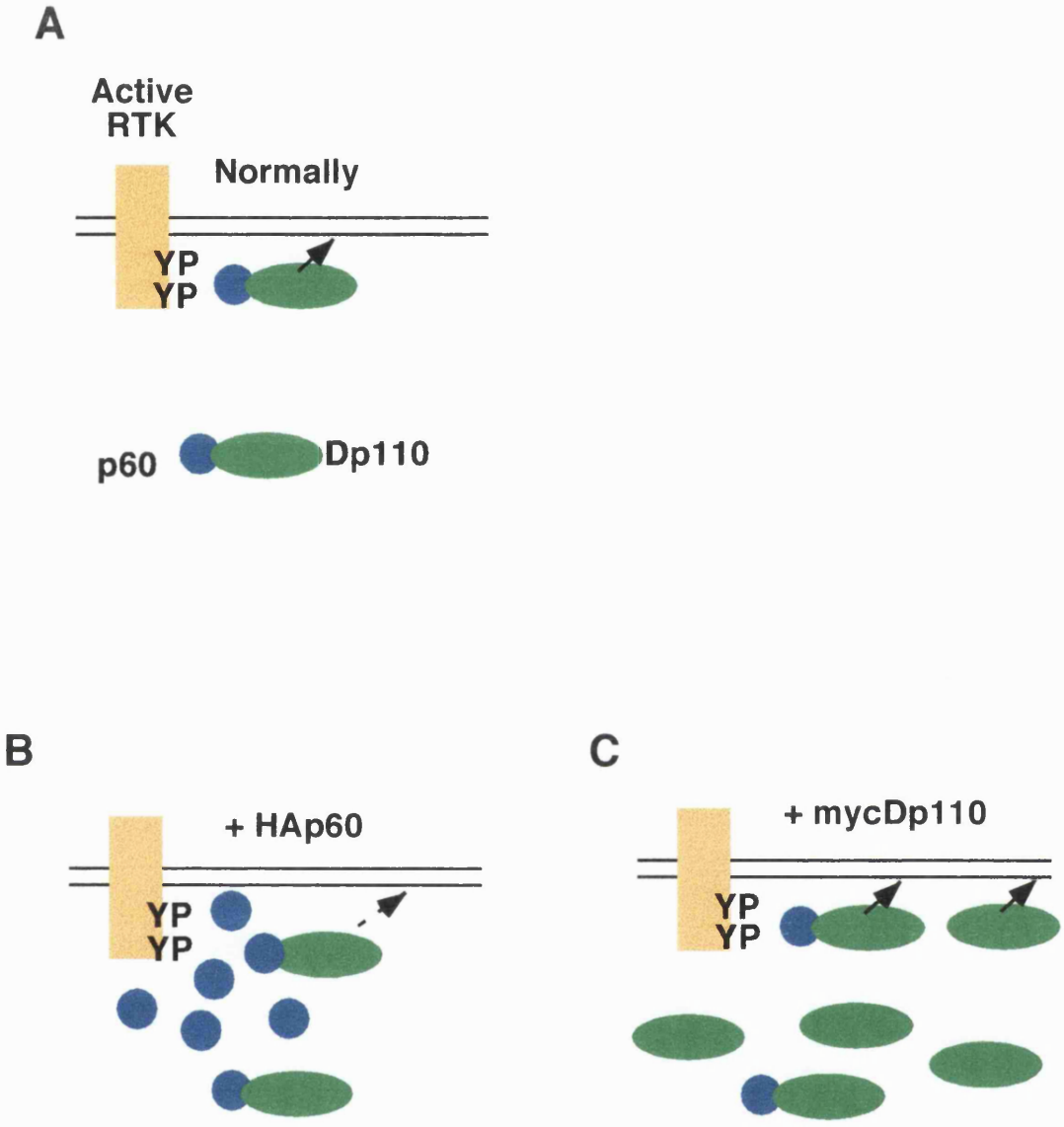
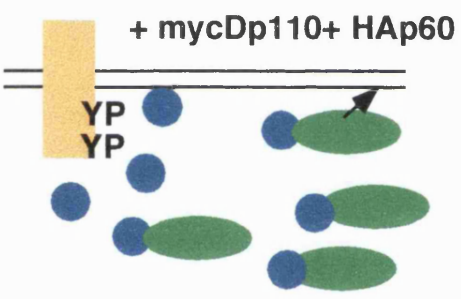


Figure 6.1. Possible modes of action for ectopically expressed HAp60 and mycDp110. (**A**) A simplified model of endogenous Dp110 / p60 activation. (**B**) Ectopically expresssed HAp60 competes with Dp110/p60 complexes for YXXM motifs. (**C**) Some ectopically expressed mycDp110 reaches the membrane without association with the active RTK.

Model 1

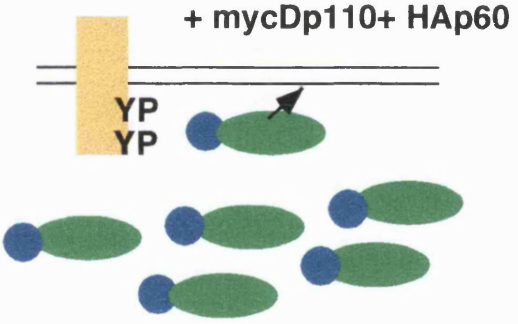
An excess of HAp60 is produced which competes with Dp110/p60 for YXXM motifs. This inhibitory effect is compensated by a fraction of mycDp110/HAp60 reaching the membrane without RTK association



THUS:
endogenous Dp110/p60 can produce a small signal without RTK activation

Model 2

An equal stoichiometry of HAp60 and mycDp110 is maintained. The resulting complexes are only activated by RTK activation.



THUS:
p60-bound Dp110 is kept inactive and is only activated by RTK activation

Figure 6.2. Two possible models describing the interactions between Dp110 and p60 The predictions for the effects of coexpressing mycDp110 and HAp60 are followed by the functional implications for the endogenous proteins.

model, it is not necessary for a p60 molecule to have the ability to inhibit its associated Dp110 molecule.

The second model proposes that there are stoichiometrically similar numbers of Dp110 and p60 molecules present when both proteins are coexpressed (recombinant and endogenous). In this model, the resulting Dp110/p60 complexes are only activated by endogenous receptor signalling because p60 prevents inappropriate activation of associated Dp110 in the absence of endogenous signalling (Figure 6.2B). The possible mechanisms of this inhibition is a secondary question and is discussed below.

One possible way to distinguish between these two models is to measure the relative levels of Dp110 and p60 when the two proteins are coexpressed. These relative levels cannot be measured by comparing the levels of recombinant and endogenous proteins because there is not an αDp110 antibody that can distinguish endogenous Dp110 in a whole cell lysate (Figure 5.5). Even if this comparison were technically possible, the natural stoichiometry of the endogenous proteins is unknown. An alternative way to address this problem is to determine whether there is free mycDp110 or HAp60 when both proteins are coexpressed. This can be achieved by purifying mycDp110 and HAp60 by four independent means: immunoprecipitation with αmyc, αHA and αp60C and affinity purification with a pYXXM phosphopeptide. Using serial titrations to normalise the levels of mycDp110 purified with each method would reveal the presence of unassociated HAp60, which would be detected as an increased amount of HAp60 purified by αHA compared to HAp60 purified by αmyc. Alternatively, αp60C or affinity purification with pYXXM phosphopeptides could substituted for αHA. One could also normalise the levels of HAp60 and compare the levels of mycDp110 purified by each method.

Both of the models presented above have interesting predictions. If the relative levels of Dp110 and p60 in a cell determine the magnitude of PI3K signalling, then the regulation of Dp110 and p60 may be an *in vivo* mechanism to control downstream signalling, leaving a only minor role for RTKs in the control of growth. Alternatively, if interactions between Dp110 and p60 normally maintain an equal stoichiometry, RTK activation would modulate signalling in the wild type organism. Such a balance between the levels of Dp110 and p60 may be achieved by mutual stabilisation of the proteins or, alternatively, by a feedback mechanism in which Dp110 activity regulates p60 and/or Dp110 protein expression (Section 6.2.2). Interestingly, the effects of the ectopic expression of mycDp110 or HAp60 are unaffected by the loss of one endogenous copy of either gene (D.W., Carmen Coelho and Sally Leevers, unpublished). Thus, if the relative stoichiometry of Dp110 and p60 is important, it is not

affected by gene dosage. If an intrinsic mechanism does exist to maintain an equal stoichiometry between Dp110 and p60, and this mechanism is still maintained when HAp60 and mycDp110 are coexpressed, the second model for the regulation of Dp110/p60 would supported for the following reason. The first model does not explain why, without an excess of HAp60 in the cell, the coexpression of mycDp110/HAp60 does not have the same effect as the expression of mycDp110 alone.

The second model proposes that p60 can prevent the inappropriate activation of Dp110, either by a directly affecting Dp110 kinase activity or by changing the localisation of Dp110 to prevent access to its lipid substrates. As discussed in Section 1.3.3, studies of mammalian Class I_A PI3Ks and their adaptors suggest that both methods of inhibition exist. Inhibition of Class I_A PI3Ks through their association with the adaptor is dependent on the N-terminal SH2 domain and the inter-SH2 domain but not on the phosphotyrosine-binding function of the N-terminal SH2 domain (Yu *et al.* 1998a). In contrast, inhibition by localisation appears to be dependent on the C-terminal SH2 domain and/or the C-terminal portion of the inter-SH2 domain (Jimenez *et al.* 1998). It could be that the C-terminal SH2 domain of the adaptor inhibits the Class I_A PI3K by binding to phosphotyrosine residues in a cellular location that is removed from the membrane. For example, it has been shown that the cytoskeleton can become highly phosphorylated by activated Src family tyrosine kinases and thus may sequester SH2 domain-containing proteins such as the adaptors for Class I_A PI3Ks (Cooley 1998; Kim and Wong 1998; Kapus *et al.* 1999). Thus, as discussed in Section 1.3.2, the adaptor localisation may be important for Class I_A PI3K regulation.

The experiments in which the SH2 domains of p60 were mutated were intended to establish whether the phosphotyrosine-binding ability of the C-terminal SH2 domain was necessary for inhibition by p60. Preliminary results from experiments in which HAp60 containing different SH2 domain mutations were coexpressed with mycDp110 suggest that both SH2 domains are necessary for HAp60 to inhibit the activity of mycDp110 (Niki Scaplehorn, Krishna Pitrola and Nicole Mathon). However, none of the combinations of mycDp110 and the various mutant forms of p60 had a greater stimulatory effect on growth than did mycDp110 alone. The relative levels of the HAp60 and mycDp110 have yet to be established in these experiments, but the results are consistent with the first model of Dp110/p60 regulation. In other words, the reduced ability of the mutants to inhibit Dp110 may simply reflect a reduced ability of the ectopically expressed HAp60 mutants to compete with endogenous Dp110/p60 complexes for pYXXM motifs on activated receptors and their substrates.

In summary, there are two possible models that fit the data obtained so far from experiments with Dp110 and p60. Measurement of the relative levels of each protein when coexpressed and in other situations should distinguish which of the two models more accurately reflects the endogenous mechanism. If the second model is shown to be more accurate, it could be inferred that p60 does inhibit Dp110, either directly or through localisation, in agreement with studies of mammalian Class I_A PI3Ks and their adaptors. Further mutational analysis of p60 and examination of intracellular location will then be necessary to determine the mechanism by which p60 inhibits Dp110.

6.3. The cellular functions of Dp110 and p60

The data presented here and in previous studies demonstrate that Dp110 and p60 are necessary for imaginal disc cells to achieve their normal size, and also that ectopic expression of forms of Dp110 and/or p60 in the eye or wing imaginal disc can alter the size of the final organ without affecting patterning (Leevers *et al.* 1996, Chapters 4 and 5). The data further suggest that Dp110 and p60 are essential for imaginal disc cells to achieve the correct number (Section 6.3.2). The significance of these results in the study of organ size control will be discussed in Section 6.4. Here, the implications of these observations for the study of the cellular functions of Class I_A PI3Ks will be discussed.

The appearance of *Dp110*$^-$ and *p60*$^-$ cells in tangential sections of the adult eye demonstrate that neither Dp110 nor p60 is needed for the differentiation or cytoskeletal rearrangements involved in the formation of normal ommatidia. Furthermore, though reductions in cell number were observed, large clones of mutant cells could be generated, demonstrating that neither Dp110 nor p60 function is essential for proliferation or for protection from apoptosis. However, in this thesis, the results of ectopic expression or mutation of the endogenous genes in imaginal discs cells have only been examined in the final adult organ. To further understand the cellular changes that must have taken place to lead to the adult phenotypes, Sally Leevers examined third instar imaginal disc cells directly using confocal microscopy and found that, compared to the wild type, a small reduction in both cell size and number could be detected in clones of cells mutant in *p60* or *Dp110* (unpublished data). However, in all other respects the cells appeared normal. Immunostaining with an anti-armadillo antibody revealed that the mutant cells in the eye were able to form and maintain adherens junctions as they differentiated posterior to the morphogenetic furrow (Section 1.8.1). Thus, the major effects of perturbing Dp110/p60 function during

imaginal disc development are on cell size and cell number. How these properties might be regulated Dp110 will be discussed in the following sections.

6.3.1 Cellular processes implicated in causing changes in cell size

Compared with the numerous studies of the regulation of cell number, there have been relative few studies of the regulation of cell size (Conlon and Raff 1999). With the exception of cell size increase caused by osmotic swelling, an increase in cell size would require an increase in biosynthesis. Thus, of the cellular processes thought to be activated by Class I_A PI3K, the upregulation of protein synthesis, glycogen synthesis and glucose uptake are the processes that have the potential to lead to an increase in cell size (Figure 1.5). In support of protein synthesis being a key determinant of cell size, loss of NCL-1, an inhibitor of ribosomal RNA in *C. elegans*, leads to an increase in protein synthesis and results in a worm with bigger cells than those of the wild type (Frank and Roth 1998). Interestingly, NGF, a ligand that can activate Class I_A PI3K through a RTK, has been shown to regulate neuron size (Purves *et al.* 1988). It has been proposed that NGF controls cell size through the control of both protein synthesis and degradation (Franklin and Johnson 1998) and that the stabilisation of protein levels by NGF is a mechanism by which NGF protects neurons from apoptosis.

However, increased protein synthesis may not be sufficient increase in cell size in *Drosophila* imaginal discs. Wild type imaginal disc cells in clones generated in a *Minute* background are not larger than the neighbouring *Minute* cells, in which protein synthesis is impaired (Section 1.8.2.2, Neufeld *et al.* 1998; Ito and Rubin 1999). Thus, the increase in cell size caused by the ectopic expression of Dp110 is unlikely to be attributable solely to a general increase in protein synthesis. It is possible that Dp110 may increase cell size by increasing the specific translation of transcripts that contain 5' TOPs (Section 1.5.5). Alternatively, Dp110 may control cell size by regulating glucose uptake or glucose metabolism, thereby increasing the energy available to the cell. As biosynthesis requires energy, energy supply could be the limiting factor in the increase of cell size. Consistent with this proposal, it has been shown that glucose metabolism can be the limiting factor in tumour growth, which may require increases in cell size as well as cell number (Dang and Semenza 1999). In summary, the increase in the synthesis of specific proteins and/or the upregulation of glucose metabolism are good candidates for the mechanism by which Dp110 increases cell size. How these suggestions can be tested experimentally will be discussed in Section 6.4.2.

6.3.2 Cellular processes implicated in causing changes in cell number

The ectopic expression of mycDp110 in the wing imaginal disc leads to an increase in cell number (Leevers *et al.* 1996). In contrast, in the same study, GMR-GAL4 driven expression of mycDp110 in and posterior to the morphogenetic furrow of the eye imaginal disc has no effect on ommatidial number (Leevers *et al.* 1996). Only limited proliferation and apoptosis take place amongst the differentiating cells in the GMR-GAL4 expression domain during normal development, so one possible interpretation of this result is that these cells are less able to respond to the proliferative or anti-apoptotic effects of increased Dp110 activity (Wolff and Ready 1991; Wolff 1993). However, cells posterior to the morphogenetic furrow can be protected from apoptosis by the ectopic expression of the baculovirus inhibitor of apoptosis, p35, under the control of the GMR promoter, which results in an eye that is bigger than wild type (Hay *et al.* 1994). The increased size of these eyes is a consequence of the survival of cells in between the ommatidia that are normally eliminated during development. The survival of these cells gives the eyes of *GMR-p35* flies a rough appearance, which is not seen in the eyes of *GMR-mycDp110* flies (Figure 5.3). Thus the *GMR-mycDp110* phenotype does not correspond with a simple inhibition of apoptosis.

In order to investigate whether the reduction in the number of ommatidia in the eyes of *GMR-HAp60* and *GMR-HAΔp60* flies could be attributed to an increase in apoptosis, the eyes of these flies were compared with the eyes of flies containing the same transgenes but in combination with *GMR-p35*. Similar experiments were carried out with *GMR-mycDp110* and *GMR mycDp110-CAAX* flies. Observation through a dissecting microscope revealed that p35 coexpression increased the size of all of the eyes but that the *GMR-HAp60/GMR-p35*, *GMR-HAΔp60/GMR-p35* eyes were still smaller than those of the wild type. This result suggests that an increase in apoptosis cannot entirely account for the reduction in the size of the eye by GMR-HAp60 and GMR-HAΔp60. As described in Section 5.2.2, ectopically expressed HAp60 can also reduce cell size. Furthermore, HAp60 may reduce cell proliferation.

It will be interesting to determine whether proliferation is reduced and/or apoptosis is increased in clones of *Dp110*$^-$ or *p60*$^-$ imaginal disc cells when compared to wild type cells in their associated twin spots. This question has been difficult to investigate experimentally because the differences in cell number between the clones and the twin spots are small, and both proliferation and apoptosis in imaginal discs occur in small, random patches (Bryant 1996; Milan *et al.* 1997, Section 1.8.2.2).

It is possible that both proliferation and cell survival may be affected simultaneously and indirectly by Dp110 through the regulation of another

cellular process. For example, it has been described that decreases in glucose metabolism can lead to cell cycle arrest and apoptosis (Section 1.5. Shim *et al.* 1998). Similar effects have been observed when protein synthesis is inhibited in certain systems (Lawrence and Abraham 1997; Franklin and Johnson 1998). Therefore, there are a number of possible cellular effects of perturbing Dp110 activity that may explain the observed effects on growth.

6.3.3 Potential upstream regulators and downstream effectors of Dp110/p60

As discussed in Section 1.4, the identification of upstream activators and downstream effectors of Dp110 will aid the study of its function. The demonstration that p60 binds to pYXXM motifs makes it possible to identify potential upstream activators by the presence of YXXM motifs that might be phosphorylated upon RTK activation (Section 1.3.4). The best candidates are the *Drosophila* insulin receptor homologue, Inr, and the RTK substrate proteins Dos and Chico, which are *Drosophila* homologues of Gab1 and IRS-1, respectively (Section 1.3.4, Fernandez *et al.* 1995; Raabe *et al.* 1996, Ernst Hafen, personal communication). The C-terminal region that resembles a mammalian IRS and, like IRS-1, contains three YXXM motifs. Far Western blotting has shown that Inr can associated with the N-terminal SH2 domain of p85α (Fernandez *et al.* 1995). Furthermore, a chimeric receptor containing the cytoplasmic domain of Inr and the extracellular domain of the human insulin receptor can bind to and activate Class I_A PI3Ks when overexpressed in mammalian cell lines (Yamaguchi *et al.* 1995; Yenush *et al.* 1996). Although homozygous *inr* mutants die during embryogenesis, adult flies with transheterozygous combinations of hypomorphic mutations in *inr* are viable but smaller than wild type flies (Fernandez *et al.* 1995; Chen *et al.* 1996). Thus, both biochemical and phenotypic evidence are consistent with Inr acting upstream of Dp110/p60 in *Drosophila* development.

Dos and Chico are both receptor substrate molecules with one YXXM motif each (Raabe *et al.* 1996, Ernst Hafen, personal communication). Dos is necessary for photoreceptor differentiation in response to Sevenless and functions downstream of several RTKs in *Drosophila* development (Raabe *et al.* 1996). However, it has been shown that mutation of the YXXM motif in Dos has no effect on Dos function (Thomas Raabe, personal communication). Homozygous null *chico* mutants do become viable adult flies but they are considerably smaller than wild type; also, *chico*$^-$ cells in mitotic clones are smaller than wild type (Ruth Böhni, Juan Riesgo-Escovar, Hugo Stocker and Ernst Hafen, personal communication). The phenotypes of *inr* and *chico* mutants are

consistent with the products of these genes functioning upstream of Dp110 and p60. Neither ablating *Dp110* and *p60* from clones of cells in the eye, nor ectopically expressing HAp60 in the eye, affected photoreceptor differentiation, suggesting that Dp110 and p60 are not necessary for the function of the RTKs DER and Sevenless in this process (Figure 4.10, Figure 5.2, Section 1.8.1). Therefore, as in *C. elegans*, the *Drosophila* Class I_A PI3K may function downstream of an insulin-like receptor (and its substrate) and not downstream of other characterised RTKs in *C. elegans* or *Drosophila*. However, the *Inr* and *chico* phenotypes do not completely match the Dp110 and p60 phenotypes; in addition, the interaction between Inr/Chico and Dp110/p60 has yet to be demonstrated, either biochemically or genetically. Thus, though Inr and chico are likely to be important activators of Dp110/p60, further experiments are need to understand the relationships among these signalling molecules.

Ras has been shown to activate mammalian Class I_A PI3Ks in several studies, although the physiological role of this interaction is still unclear (Section 1.3.5). Interestingly, ectopic expression of an activated version of the *Drosophila* homologue, Ras1, early in the development of the wing or eye imaginal discs leads to the overgrowth of those discs (Karim and Rubin 1998). However, the patterning of the resulting discs is severely disrupted, unlike the consequences of the ectopic expression of mycDp110. Also in contrast to the results obtained from the genetic manipulation of *Dp110* and *p60*, clones of *Ras1*$^-$ cells in the eye imaginal disc do not survive posterior to the morphogenetic furrow (Dominguez *et al.* 1998). Furthermore, the ectopic expression of Ras1 disrupts normal photoreceptor differentiation, whereas the ectopic expression of Dp110 or p60 does not (Fortini *et al.* 1992; Leevers *et al.* 1996, Figure 5.2). Thus, although Ras1 may activate Dp110, the activation of Dp110 does not account for many of the functions of Ras1.

Clearly, the *Drosophila* homologues of identified downstream effectors of mammalian Class I_A PI3Ks are candidates for downstream effectors of Dp110. Mutation of the gene encoding the *Drosophila* homologue of PKB, *Dakt1*, causes extensive apoptosis in the embryo (Staveley *et al.* 1998). However, the function of Dakt1 in imaginal disc development has not been established. The *Drosophila* homologue of p70S6 kinase, Dp70S6K, has also been identified (Stewart *et al.* 1996). Mutation in the gene encoding this kinase leads to a reduction in size of the fly, as well as to other phenotypes that are reminiscent of the *Minute* phenotypes that are thought to result from a disruption to protein synthesis (M. Stewart, J. Montagne and G. Thomas, personal communication, Section 1.8.2.2). Thus, it is possible that both Dakt1 and Dp70S6K act downstream of Dp110.

This reports show that PLCγD, the *Drosophila* homologue of PLCγ, a potential downstream target of Class I_A PI3Ks, can be purified with the same pYXXM phosphopeptide that can be used to purify PLCγ from mammalian cells (Figure 1.1). Thus, as in the case of p60, the binding specificity of the PLCγ SH2 domain(s) is conserved between mammals and flies. Interestingly, homozygous null mutations in the gene encoding PLCγD lead to viable flies with small wings (hence the gene is also called small wing, Thackeray *et al.* 1998). However, the reduction in wing size is a consequence of a reduction in cell number, not cell size. Furthermore, these mutants have defects in wing vein patterning and in photoreceptor differentiation, suggesting that PLCγD is a downstream target of the Sevenless and DER RTKs (Thackeray *et al.* 1998). Thus, it is unlikely that PLCγD functions downstream of Dp110, but some form of cross-talk between the two proteins cannot be ruled out.

In summary, it likely that, as has been shown for AGE-1 in *C. elegans*, p60 and Dp110 act in a pathway downstream of an insulin-like receptor and its substrate and upstream of a PKB homologue (Figure 6.3). However, as yet, no direct evidence for the existence of this pathway has been published. Furthermore, most of the alternative pathways (Figure 1.4) that have been suggested by studies in mammalian systems have not been investigated in *Drosophila*. In order to identify potential upstream regulators and downstream effectors of Dp110/p60, a screen has been performed for enhancers and suppressors of the growth inhibition that results from the ectopic expression of mycDp110^{D954A} in the wing (Carmen Coelho and Sally Leevers, unpublished).

6.4. The function of Dp110 and p60 in the whole organism

As described above, flies with mutations in *inr*, *chico* or *Dp70S6K* are smaller than wild type. Interestingly, starvation and over-crowding during larval development also result in smaller-than-wild type flies with fewer and smaller cells (Held 1979). This response may enable *Drosophila* to survive through periods of low nutrition, because the resulting small flies are still fertile. Therefore, Dp110 may be involved in a pathway that senses the larval nutritional environment and modulates growth to optimise the survival of the organism. Such a role has a striking parallel in *C. elegans*, for which starvation, over-crowding or mutation of the genes encoding the *C. elegans* Class I_A PI3K, AGE-1, or the insulin receptor homologue, DAF-2, induce the formation of dauer larvae, thereby increasing the survival of the organism in unfavourable conditions (Section 1.6.1). It is known that mammalian Class I_A PI3Ks are activated in response to feeding in insulin-responsive tissues, thereby

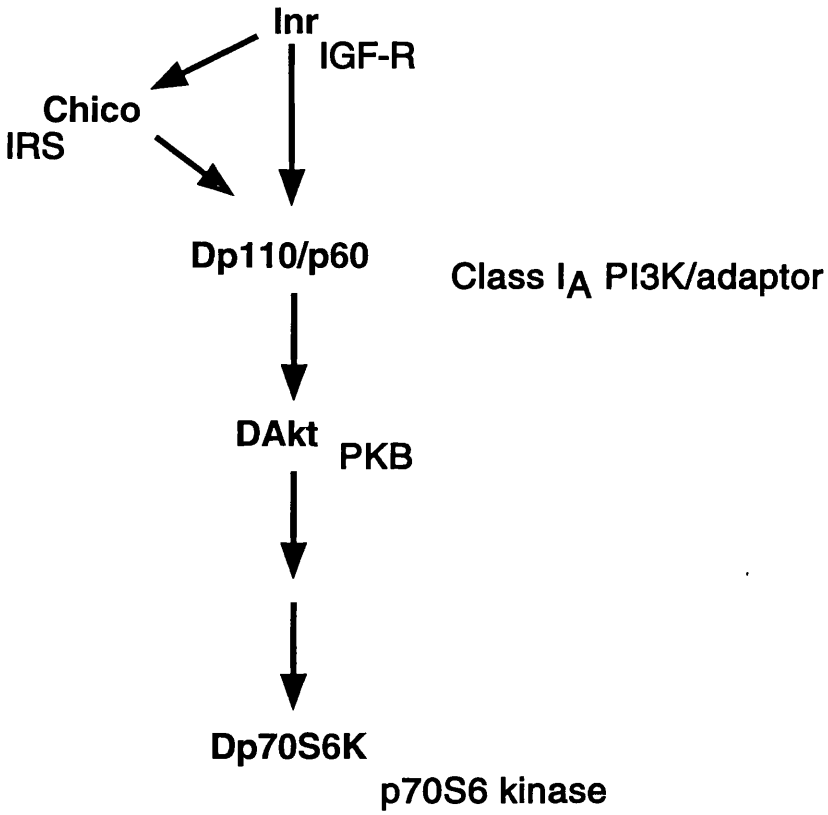


Figure 6.3. Possible pathway upstream and downstream of Dp110/p60. Candidate members of a Dp110/p60 pathway and their mammalian counterparts.

maintaining blood glucose homeostasis. Thus, a general function of Class I_A PI3Ks may be to mediate the response of the organism to its nutritional environment.

The mechanism by which Dp110 may influence the growth of the whole animal is unknown. Experiments have suggested that the *C. elegans* Class I_A PI3K, AGE-1, can affect dauer formation and longevity through the regulation of systemic endocrine factors (Section 1.6.1, Apfeld and Kenyon 1998). It has been suggested that this endocrine signal is in fact a steroid hormone that is recognised by the steroid hormone receptor, DAF-12 (Apfeld and Kenyon 1998). Interestingly, the steroid hormone ecdysone controls the insect life cycle and is produced in the prothoracic glands in response to an insulin-like ligand, the prothoracicotropic hormone (PTTH), which was first identified in the silkworm, *Bombyx mori* (Jhoti *et al.* 1987). Furthermore, PTTH has been shown to induce S6 phosphorylation in the tobacco worm, suggesting the involvement of Class I_A PI3Ks (Song and Gilbert 1997, Section 1.5.5). Therefore, although cell autonomous phenotypes are observed in clones of *Dp110*$^-$ or *p60*$^-$ cells, a non-autonomous effect of Dp110/p60 is also possible. Interestingly, as well as being defective in growth, *Dp110*$^-$ larvae fail to wander, a process induced by ecdysone (Section 4.5.2). Thus, studies in insects and *C. elegans* suggest that the endocrine role of Class I_A PI3Ks is conserved in many animals.

6.4.1 The control of imaginal disc size and the involvement of Dp110/p60

As discussed in Section 1.8.2, the size of imaginal discs is determined by factors intrinsic and extrinsic to the disc. Dp110/p60 may play a role in mediating the intrinsic control of disc size, because manipulation of Dp110 activity in a disc using transgenes can change the size of the disc and the resulting adult organ without affecting patterning (Chapter 5). Furthermore, experiments using clones of *Dp110*$^-$ and *p60*$^-$ cells show that the loss of these genes has a cell autonomous effect on growth (Chapter 4). In other words, Dp110 and p60 are needed in the cells of an imaginal disc if it is to reach its normal size.

One proposed explanation for the intrinsic control of organ growth is the polar coordinate model of development (Bryant and Simpson 1984). This model arose from observations made using grafting experiments and other manipulations of organs from various species including the imaginal discs of *Drosophila* (French *et al.* 1976; Haynie and Bryant 1976). The key observation of these experiments was that the disruption of organ patterning by physical manipulation leads to duplications or regeneration of the missing parts of the pattern. The polar coordinate model proposes that the different components of

the pattern have different positional values according to their location. The juxtaposition of cells that are not normally adjacent to one another is predicted to lead to a discontinuity in positional values and, therefore, the stimulation of growth and the consequent replacement of the intervening structures. This process is termed intercalation. The polar coordinate also predicts that normal growth is controlled by a similar mechanism. Specifically, the cells of an organ are stimulated to divide by a gradient of positional values between two ends of an organ, which are defined early in development. Growth and patterning continue until the gradient becomes too shallow for the cells to sense it. It has been argued that the sigmoidal shape of a curve derived when growth is plotted against time can be explained by this model because as the organ nears its final size, the gradient is less pronounced, slowing growth (Section 1.8.2, Bryant and Simpson 1984).

The molecular nature of the gradient was not specified in the original polar coordinate model (French *et al.* 1976) but is likely to consist of either an extracellular concentration gradient of a diffusible molecule or a gradient created by a relayed cascade of cell-to-cell interactions. In favour of the first possibility, it has been proposed that extracellular concentrations of the diffusible ligands, Wg and Dpp, could be responsible for establishing positional values in the wing imaginal disc (Bryant 1993; Couso *et al.* 1993; Serrano and O'Farrell 1997). In agreement with such a role for Wg and Dpp, both molecules have been shown to act as morphogens because they stimulate different cell fates at different extracellular concentrations, and this effect is dependent on the intracellular signalling pathways that are stimulated by Wg and Dpp (Burke and Basler 1996; Nellen *et al.* 1996; Zecca *et al.* 1996).

While attractive, the polar coordinate model is insufficient for a number of reasons. Firstly, it is still controversial whether the predictions of the polar coordinate model fit the patterning events observed in imaginal disc development (Cohen 1993). Secondly, as a explanation of growth control the model is flawed because its basic assumption is that the gradient of positional values triggers cell division, and that organ growth depends only upon cell number and not cell size. This assumption is not supported by experimentation because it has been shown that when the cell cycle in the developing wing imaginal disc is perturbed, there is no effect on organ growth (Weigmann *et al.* 1997, Section 1.8.2; Neufeld *et al.* 1998). Finally, there is no relationship between rates of growth in the imaginal discs and the expression domains of Wg/Dpp and their downstream targets (Section 1.8.2).

Here, I would like to put forward a further revised version of the polar coordinate model that takes into account both the results of the investigation of

Dp110 and p60 function and the results of experiments on imaginal disc growth described in Section 1.8. Firstly, this revised model predicts that the effect of the gradient of positional values would be to increase tissue mass rather than to directly increase cell number. The cell cycle control mechanisms operating in individual cells would determine whether an increase in mass would involve an increase in cell number, cell size or both. For example, the cells of the *Drosophila* salivary gland endoreplicate, so growth of these organs results in an increase in cell size. In contrast, tissues such as the imaginal discs maintain a fairly constant cell size during normal development and thus growth of these tissues chiefly results from an increase in cell number. The second prediction of my model is that p60 and Dp110 mediate a signalling process that is able to respond to the gradient of positional values. Thus, the interpretation of the results of ectopic expression of mycDp110 or HAp60 is that mycDp110 overexpression leads to an increase in sensitivity to the gradient, whereas HAΔp60 overexpression leads to a decrease in sensitivity.

It is difficult to integrate the control of growth by Wg and Dpp into this model, because, unlike Wg and Dpp, Dp110 and p60 have no effect on cell fate and there is no evidence that the mammalian Class I_A PI3Ks act downstream of Wnt or TGFβ family members. Instead, it seems likely that Dp110/p60 promotes growth in response to an insulin-like ligand. In support of the involvement of a such a ligand in organ size control, the ectopic expression of IGFII in particular organs of the mouse leads to an increase in the size of those organs without affecting patterning (Ward *et al.* 1994). As well as an increase in cell number, those organs also display an increase in the ratio of tissue mass to DNA, suggesting that cell size is also increased. Thus, perhaps, cell signalling induced by Dpp/Wg cooperates with the activators of Dp110/p60 to promote growth.

Interestingly, to explain the lack of relationship between regions of Wg/Dpp expression and growth into account, Serrano and O'Farrell (1997) invoke the gradient of a growth inhibitor across the disc in their version of the polar coordinate model. This gradient is proposed to counter the growth promoting effects of Wg and Dpp, resulting in evenly distributed growth. Alternatively, this gradient could be replaced conceptually by a counter-gradient of a growth activator or a gradient of cellular responsiveness. Thus, Dp110/p60 may be involved in integrating the growth response to positional information by responding to this system of counter-gradients, especially if a gradient of an insulin-like ligand is involved. In support of such an integrative role for Dp110/p60, it has been shown that while TGFβ treatment of human airway smooth muscle cells has no effect on basal PI3K activity, it does cause an increase in insulin-stimulated PI3K activity (Krymskaya *et al.* 1997). A similar synergy has

been described for the effects of glucose and insulin on the growth of pancreatic beta cells (Hugl *et al.* 1998) and, importantly, for the effects of insulin and a family of chitinases on the growth of cultured imaginal disc cells (Kawamura *et al.* 1998). Thus, the integration of patterning and growth in imaginal discs may be achieved by an integration of various extracellular growth factors that results in the appropriate activation of Dp110 in every cell.

6.4.2 Implications of the new model for the function of Class I_A PI3Ks

The beginnings of a model for the regulation of imaginal disc growth have been described above. If this model is accurate, there are some interesting predictions for the function of Dp110 and perhaps for Class I_A PI3Ks in general. Firstly, the model suggests that the mechanisms that activate Dp110 respond to tissue volume rather than to the number or the size of cells and that the function of Dp110 is to increase tissue volume rather than to increase either cell size or cell number specifically. Therefore, a key prediction of this model is that Dp110 upregulates a central cellular property that can increases tissue mass, whether that be by increasing cell size, by protecting cells from apoptosis or by stimulating proliferation (Figure 6.4). Good candidates for this central property are energy metabolism and protein synthesis, both of which are activated by Class I_A PI3K activation and when upregulated have been shown to increase both cell size and cell number. The opposing model would be that, together, the several different pathways and cellular functions which have been shown to be stimulated by mammalian Class I_A PI3Ks, could produce the effects on growth observed when Dp110 activity is manipulated. Most of these cellular functions appear to be regulated through PKB, which could allow their coordination. However, it seems more likely that, to achieve the regulation of the intrinsic control of organ size without affecting patterning and without major restraints on cell size or cell number, Dp110 would have to regulate only a single cellular property.

A second prediction of the revised polar coordinate growth model presented here is that Dp110 activity should increase in the surrounding cells when disc growth is disrupted by damage. Furthermore, when the intrinsic control of imaginal disc growth is overcome by the loss of tumour suppressor genes, Dp110 activity should increase in the constituent cells of the tumorous growth. These predictions could be tested if Dp110 activity could be monitored *in vivo*. Possibilities for the *in vivo* measurement of Dp110 activity include the use of phosphospecific antibodies that recognise activated PKB, the observation of ectopically-expressed GFP-PH domain fusion proteins that translocate to the membrane in response to the generation of 3'

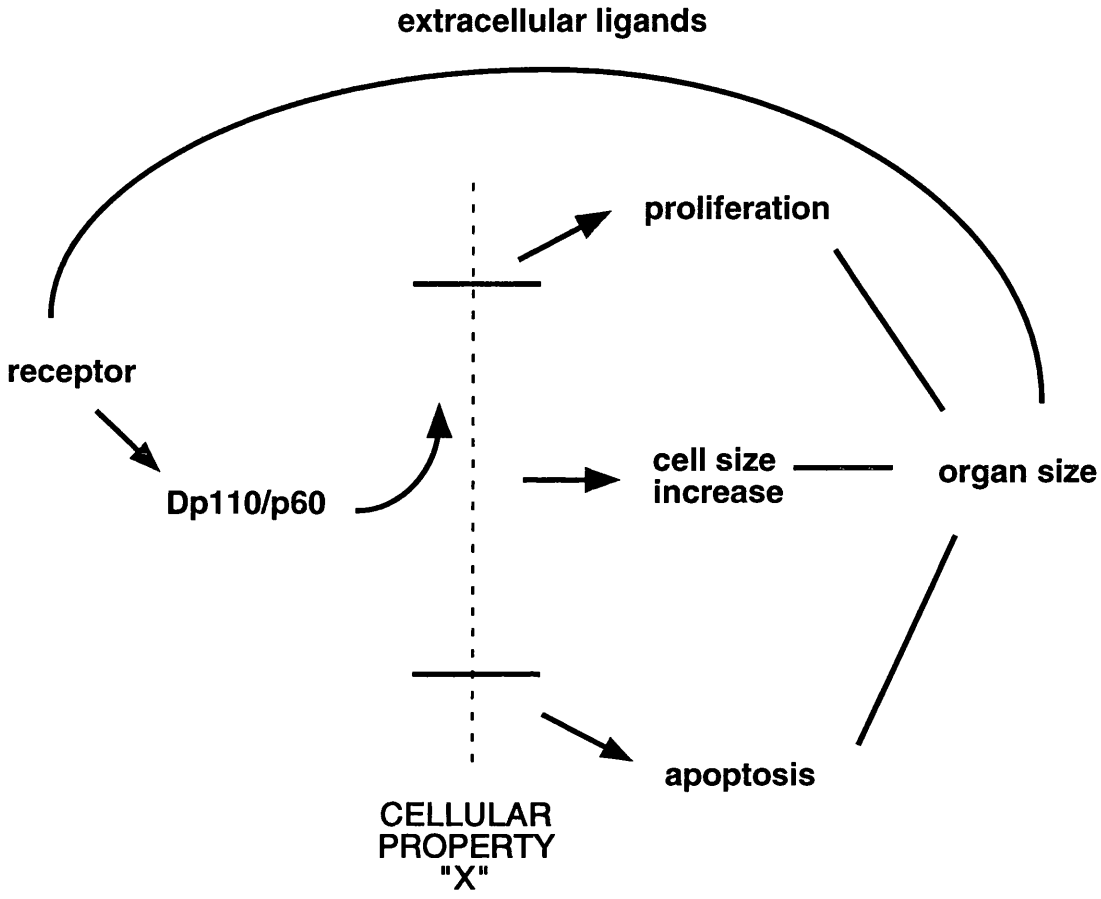


Figure 6.4. A model for the role of Dp110/p60 in organ size control. Dp110 activation can upregulate a cellular property "X". The upregulation of this property increase proliferation, reduces apoptosis or increases cell size, depending on the state of the cell. The integration of these effects on cell number and cell size determines organ size. There is likely to be a feedback system that sense organ growth and influences Dp110/p60 activation.

phosphorylated inositol lipids, and the measurement of Dp110-regulated cellular properties. For example, glucose metabolism has been measured in individual pancreatic islet cells by monitoring NADPH autofluorescence with two photon microscopy, and this technique may be applicable to imaginal discs (Piston *et al.* 1999).

Thirdly, it is clear that *Drosophila* imaginal discs are resistant to changes in cell proliferation and cell death, processes that are thought to be altered in tumorous cells. Therefore, it would be predicted that the intrinsic control of size is overcome by a neoplastic overgrowth and further, that Dp110 is activated in its constituent cells. To test the requirement for Dp110 in overgrowth, double mutant clones of cells lacking a tumour suppressor gene product and Dp110 or p60 could be generated.

Finally, it has been proposed in this thesis that Dp110 and p60 play a part in the mechanism that regulates *Drosophila* imaginal disc growth. If Class I_A PI3Ks play a similar role in the regulation of mammalian organ growth, the inhibition of Class I_A PI3Ks may prevent overgrowth in human disease. In support of this hypothesis, it has been shown that loss of the 3'-phosphatase, PTEN is involved in the generation of many human tumours. Further studies of Dp110 and p60 function may therefore shed light on a variety of biological problems, including not only the regulation of Class I_A PI3Ks and their downstream targets, but the control of imaginal disc growth and the nature of human carcinogenesis.

Abbreviations

The abbreviations in this thesis follow the guidelines summarised in *Biochemical Journal* 321, 1-16 (1997). In addition the following abbreviations are used:

AEL	after egg laying
ARF	ADP-ribosylation factor
ATM	ataxia telangiectasia gene product
BDGP	Berkeley Drosophila Genome Project
BH	breakpoint cluster region homology
BLAST	basic local alignment search tool
CSF-1	colony-stimulating factor-1
DNA-PK	DNA-dependent protein kinase
ECL	enhanced chemiluminesence
EGF	epidermal growth factor receptor
EMS	ethylmethane sulphonate
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GMR	glass multimer reporter
IGF	insulin-like growth factor
M-CSF	macrophage colony stimulating factor
NGF	nerve growth factor
NMR	nuclear magnetic resonance
ORF	open reading frame
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PDK1	3' phosphoinositide-dependent kinase 1
PFK-2	phosphofructokinase-2
PtdIns	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PH	pleckstrin homology
PTB	phosphotyrosine-binding
RTK	receptor tyrosine kinase
SEM	scanning electron microscopy
SH2	src homology 2

SH3	src homology 3
SHIP	SH2 domain-containing 5' inositol phosphatase
S/T	serine/threonine
STS	sequence tagged site
TGFβ	transforming growth factor β
TOR	target of rapamycin
UAS	upstream activating sequence
UTR	untranslated region

Genetic abbreviations:

al	*aristaless*
smo	*smoothened*
mbm	*mushroom body miniature*
Dpp	Decapentaplegic
Wg	Wingless

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References

Aarts, J. M., Hontelez, J. G., Fischer, P., Verkerk, R., van Kammen, A., and Zabel, P. (1991). Acid phosphatase-1(1), a tightly linked molecular marker for root-knot nematode resistance in tomato: from protein to gene, using PCR and degenerate primers containing deoxyinosine. *Plant Mol Biol*, 16(4), 647-61.

Ahmed, N. N., Grimes, H. L., Bellacosa, A., Chan, T. O., and Tsichlis, P. N. (1997). Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc Natl Acad Sci U S A*, 94(8), 3627-32.

Albert, S., Twardzik, T., Heisenberg, M., and Schneuwly, S. (1997). Isolation and characterization of the droPIK57 gene encoding a new regulatory subunit of phosphatidylinositol 3-kinase from Drosophila melanogaster. *Gene*, 198(1-2), 181-9.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J. (1996). The Drosophila *smoothened* gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell*, 86, 221-232.

Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A., and Bownes, M. (1997a). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol*, 7(10), 776-89.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997b). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol*, 7(4), 261-9.

Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998). 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr Biol*, 8(2), 69-81.

Allen, W. E., Jones, G. E., Pollard, J. W., and Ridley, A. J. (1997). Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J Cell Sci*, 110(Pt 6), 707-20.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol*, 215, 403-410.

Aman, M. J., Lamkin, T. D., Okada, H., Kurosaki, T., and Ravichandran, K. S. (1998). The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem*, 273(51), 33922-8.

Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997). Role of translocation in the activation and function of protein kinase B. *J Biol Chem*, 272(50), 31515-24.

Antonetti, D., Algenstaedt, P., and Kahn, C. (1996). Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. *Molecular and Cellular Biology*, 16, 2195-2203.

Apfeld, J., and Kenyon, C. (1998). Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span [In Process Citation]. *Cell*, 95(2), 199-210.

Arcaro, A., Volinia, S., Zvelebil, M. J., Stein, R., Watton, S. J., Layton, M. J., Gout, I., Ahmadi, K., Downward, J., and Waterfield, M. D. (1998). Human Phosphoinositide 3-Kinase C2beta, the Role of Calcium and the C2 Domain in Enzyme Activity. *J Biol Chem*, 273(49), 33082-33090.

Arcaro, A., Zvelebil, M. J., Wallasch, C., Ullrich, A., Waterfield, M. D., and Domin, J. (1999). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *unpublished.*

Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell*, 57(1), 167-75.

August, A., Sadra, A., Dupont, B., and Hanafusa, H. (1997). Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc Natl Acad Sci U S A*, 94(21), 11227-32.

Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, Y., Schlessinger, J., and White, M. F. (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J*, 11, 3469-3479.

Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S., and Rhee, S. G. (1998). Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5- trisphosphate. *J Biol Chem*, 273(8), 4465-9.

Barbet, N. C., Schneider, U., Helliwell, S. B., Stansfield, I., Tuite, M. F., and Hall, M. N. (1996). TOR controls translation initiation and early G1 progression in yeast. *Mol Biol Cell*, 7(1), 25-42.

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*, 70(1), 31-46.

Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, 254(5029), 274-7.

Beraud, C., Henzel, W. J., and Baeuerle, P. A. (1999). Involvement of regulatory and catalytic subunits of phosphoinositide 3- kinase in NF-kappaB activation. *Proc Natl Acad Sci U S A*, 96(2), 429-434.

Berset, C., Trachsel, H., and Altmann, M. (1998). The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, 95(8), 4264-9.

Blair, L. A., and Marshall, J. (1997). IGF-1 modulates N and L calcium channels in a PI 3-kinase-dependent manner. *Neuron*, 19(2), 421-9.

Blatch, G. L., Lassle, M., Zetter, B. R., and Kundra, V. (1997). Isolation of a mouse cDNA encoding mSTI1, a stress-inducible protein containing the TPR motif. *Gene*, 194(2), 277-82.

Bourette, R. P., Myles, G. M., Choi, J. L., and Rohrschneider, L. R. (1997). Sequential activation of phoshatidylinositol 3-kinase and phospholipase C-gamma2 by the M-CSF receptor is necessary for differentiation signaling. *Embo J*, 16(19), 5880-93.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, 118(2), 401-15.

Breeze, A., Kara, B., Barratt, D., Anderson, M., Smith, J., Luke, R., Best, J., and Cartlidge, S. (1996). Structure of a specific peptide complex of the carboxy terminal SH2 domain from the p85a subunit of phosphatidylinositol 3-kinase. *EMBO J.*, 15, 3579-3589.

Brennan, P., Babbage, J. W., Burgering, B. M., Groner, B., Reif, K., and Cantrell, D. A. (1997). Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity*, 7(5), 679-89.

Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, 369(6483), 756-8.

Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo [published erratum appears in Nature 1995 Dec 7;378(6557):644]. *Nature*, 377(6548), 441-6.

Brown, E. J., and Schreiber, S. L. (1996). A signaling pathway to translational control. *Cell*, 86(4), 517-20.

Brozinick, J. T., Jr., and Birnbaum, M. J. (1998). Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. *J Biol Chem*, 273(24), 14679-82.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell*, 96(6), 857-868.

Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., Jr., and Abraham, R. T. (1996). Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *Embo J*, 15(19), 5256-67.

Bryant, P. J. (1993). The polar coordinate model goes molecular [comment]. *Science*, 259(5094), 471-2.

Bryant, P. J. (1996). Cell proliferation control in Drosophila: flies are not worms. *Bioessays*, 18(10), 781-4.

Bryant, P. J., and Simpson, P. (1984). Intrinsic and extrinsic control of growth in developing organs. *Q Rev Biol*, 59(4), 387-415.

Bryant, P. J., Watson, K. L., Justice, R. W., and Woods, D. F. (1993). Tumor suppressor genes encoding proteins required for cell interactions and signal transduction in Drosophila. *Dev Suppl*, 239-49.

Buday, L., and Downward, J. (1993). Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*, 73(3), 611-20.

Burd, C. G., and Emr, S. D. (1998). Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol Cell*, 2(1), 157-62.

Burgering, B. M., and Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction [see comments]. *Nature*, 376(6541), 599-602.

Burke, R., and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. *Development*, 122(7), 2261-9.

Caggese, C., Caizzi, R., Bozzetti, M. P., Barsanti, P., and Ritossa, F. (1988). Genetic determinants of glutamine synthetase in Drosophila melanogaster: a gene for glutamine synthetase I resides in the 21B3-6 region. *Biochem Genet*, 26(9-10), 571-84.

Capdevila, J., and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. *Embo J*, 13(19), 4459-68.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science*, 282(5392), 1318-21.

Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L. C. (1993a). Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. *J Biol Chem*, 268(13), 9478-83.

Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hou, W. M., Schaffhausen, B., and Cantley, L. C. (1993b). A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. *Mol Cell Biol*, 13(3), 1657-65.

Carpenter, C. L., and Cantley, L. C. (1998). A flattened face for membranes [news]. *Nat Struct Biol*, 5(10), 843-5.

Chang, H. W., Aoki, M., Fruman, D., Auger, K. R., Bellacosa, A., Tsichlis, P. N., Cantley, L. C., Roberts, T. M., and Vogt, P. K. (1997). Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science*, 276(5320), 1848-50.

Chardin, P., Paris, S., Antonny, B., Robineau, S., Beraud-Dufour, S., Jackson, C. L., and Chabre, M. (1996). A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature*, 384(6608), 481-4.

Chen, C., Jack, J., and Garofalo, R. S. (1996). The Drosophila insulin receptor is required for normal growth. *Endocrinology*, 137(3), 846-56.

Cheng, X., Ma, Y., Moore, M., Hemmings, B. A., and Taylor, S. S. (1998). Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. *Proc Natl Acad Sci U S A*, 95(17), 9849-54.

Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998). Regulation of protein kinase C z by PI 3-kinase and PDK-1. *Curr Biol*, 8(19), 1069-77.

Clark, S. G., Stern, M. J., and Horvitz, H. R. (1992). C. elegans cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains [see comments]. *Nature*, 356(6367), 340-4.

Coffer, P. J., Jin, J., and Woodgett, J. R. (1998). Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J*, 335(Pt 1), 1-13.

Cohen, S. (1993). "Imaginal disc development." The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 747-842.

Conlon, I., and Raff, M. (1999). Size control in animal development [In Process Citation]. *Cell*, 96(2), 235-44.

Cooley, L. (1998). Drosophila ring canal growth requires Src and Tec kinases. *Cell*, 93(6), 913-5.

Cooley, L., Thompson, D., and Spradling, A. C. (1990). Constructing deletions with defined endpoints in Drosophila. *Proc Natl Acad Sci U S A*, 87(8), 3170-3.

Couso, J. P., Bate, M., and Martinez-Arias, A. (1993). A wingless-dependent polar coordinate system in Drosophila imaginal discs [see comments]. *Science*, 259(5094), 484-9.

Cox, D., Tseng, C. C., Bjekic, G., and Greenberg, S. (1999). A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J Biol Chem*, 274(3), 1240-7.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, 378(6559), 785-9.

Czech, M. P., and Corvera, S. (1999). Signaling mechanisms that regulate glucose transport. *J Biol Chem*, 274(4), 1865-8.

Dang, C. V., and Semenza, G. L. (1999). Oncogenic alterations of metabolism. *Trends in Biochemical Sciences*, 24, 68-72.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. *Cell*, 91(2), 231-41.

de Belle, J. S., and Heisenberg, M. (1996). Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc Natl Acad Sci U S A*, 93(18), 9875-80.

De Benedetti, A., and Rhoads, R. E. (1990). Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. *Proc Natl Acad Sci U S A*, 87(21), 8212-6.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci U S A*, 95(19), 11211-6.

Dennis, P. B., Fumagalli, S., and Thomas, G. (1999). Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr Opin Genet Dev*, 9(1), 49-54.

Deora, A. A., Win, T., Vanhaesebroeck, B., and Lander, H. M. (1998). A Redox-triggered Ras-Effector Interaction. Recruitment of phosphatidylinositol 3'-kinase to ras by redox stress. *J Biol Chem*, 273(45), 29923-29928.

Deprez, J., Vertommen, D., Alessi, D. R., Hue, L., and Rider, M. H. (1997). Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *J Biol Chem*, 272(28), 17269-75.

Derossi, D., Williams, E. J., Green, P. J., Dunican, D. J., and Doherty, P. (1998). Stimulation of mitogenesis by a cell-permeable PI 3-kinase binding peptide. *Biochem Biophys Res Commun*, 251(1), 148-52.

Dey, B. R., Furlanetto, R. W., and Nissley, S. P. (1998). Cloning of human p55 gamma, a regulatory subunit of phosphatidylinositol 3-kinase, by a yeast two-hybrid library screen with the insulin-like growth factor-I receptor [published erratum appears in Gene 1998 May 28;212(1):155]. *Gene*, 209(1-2), 175-83.

Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994a). PI 3-kinase: structural and functional analysis of intersubunit interactions. *Embo J*, 13(3), 511-21.

Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S. A., and Waterfield, M. D. (1994b). PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. *EMBO J*, 13(3), 522-33.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression. *Nat Genet*, 19(4), 348-55.

Dickson, B., and Hafen, E. (1993). "Genetic dissection of eye development in *Drosophila*." The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1327-1362.

Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*, 12(22), 3499-511.

Domin, J., Pages, F., Volinia, S., Rittenhouse, S. E., Zvelebil, M. J., Stein, R. C., and Waterfield, M. D. (1997). Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. *Biochem J*, 326(Pt 1), 139-47.

Dominguez, M., Wasserman, J. D., and Freeman, M. (1998). Multiple functions of the EGF receptor in Drosophila eye development [In Process Citation]. *Curr Biol*, 8(19), 1039-48.

Du, K., and Montminy, M. (1998). CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem*, 273(49), 32377-9.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt [see comments]. *Science*, 275(5300), 661-5.

Eder, A. M., Dominguez, L., Franke, T. F., and Ashwell, J. D. (1998). Phosphoinositide 3-kinase regulation of T cell receptor-mediated interleukin-2 gene expression in normal T cells. *J Biol Chem*, 273(43), 28025-31.

Ellis, M. C., O'Neill, E. M., and Rubin, G. M. (1993). Expression of Drosophila glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. *Development*, 119(3), 855-65.

Emori, Y., Sugaya, R., Akimaru, H., Higashijima, S., Shishido, E., Saigo, K., and Homma, Y. (1994). Drosophila phospholipase C-gamma expressed predominantly in blastoderm cells at cellularization and in endodermal cells during later embryonic stages. *Journal of Biological Chemistry*, 269, 19474-19479.

Entwistle, A. (1999). A comparison between the use of a high-resolution CCD camera and 35mm film for obtaining colour micrographs. *Journal of Microscopy*.

Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A., and Williams, L. T. (1991). cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell*, 65(1), 75-82.

Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto- oncogene product. *Mol Cell Biol*, 5(12), 3610-6.

Eves, E. M., Xiong, W., Bellacosa, A., Kennedy, S. G., Tsichlis, P. N., Rosner, M. R., and Hay, N. (1998). Akt, a target of phosphatidylinositol 3-kinase, inhibits apoptosis in a differentiating neuronal cell line. *Mol Cell Biol*, 18(4), 2143-52.

Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998). Activation of phospholipase C gamma by PI 3-kinase-induced PH domain- mediated membrane targeting. *Embo J*, 17(2), 414-22.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *Embo J*, 14(14), 3373-84.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988). Purification of a RAS-responsive adenylyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. *Mol Cell Biol*, 8(5), 2159-65.

Fortini, M. E., Simon, M. A., and Rubin, G. M. (1992). Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation [see comments]. *Nature*, 355(6360), 559-61.

Frank, D. J., and Roth, M. B. (1998). ncl-1 is required for the regulation of cell size and ribosomal RNA synthesis in Caenorhabditis elegans. *J Cell Biol*, 140(6), 1321-9.

Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate [see comments]. *Science*, 275(5300), 665-8.

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, 81(5), 727-36.

Franklin, J. L., and Johnson, E. M. (1998). Control of neuronal size homeostasis by trophic factor-mediated coupling of protein degradation to protein synthesis. *J Cell Biol*, 142(5), 1313-24.

Fraser, S. E., and Bryant, P. J. (1985). Patterns of dye coupling in the imaginal wing disk of Drosophila melanogaster. *Nature*, 317(6037), 533-6.

Frech, M., Andjelkovic, M., Ingley, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1997). High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J Biol Chem*, 272(13), 8474-81.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell*, 87(4), 651-60.

Freeman, M. (1997). Cell determination strategies in the Drosophila eye. *Development*, 124(2), 261-70.

French, V., Bryant, P. J., and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science*, 193(4257), 969-81.

Frevert, E. U., and Kahn, B. B. (1997). Differential effects of constitutively active phosphatidylinositol 3- kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol Cell Biol*, 17(1), 190-8.

Friedman, D. B., and Johnson, T. E. (1988). A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics*, 118(1), 75-86.

Fruman, D., Cantley, L., and Carpenter, C. (1996). Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85a gene. *Genomics*, 37, 113-121.

Fruman, D. A., Snapper, S. B., Yballe, C. M., Davidson, L., Yu, J. Y., Alt, F. W., and Cantley, L. C. (1999). Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science*, 283(5400), 393-7.

Fry, M. J., Panayotou, G., Dhand, R., Ruiz Larrea, F., Gout, I., Nguyen, O., Courtneidge, S. A., and Waterfield, M. D. (1992). Purification and characterization of a phosphatidylinositol 3-kinase complex from bovine brain by using phosphopeptide affinity columns. *Biochem J*, 288(Pt 2), 383-93.

Gaffney, P. R. J., and Reese, C. B. (1997). Synthesis of 1-[(1-O-Stearoyl-2-O-arachidonoyl-sn-glycer-3-yl)-phosphoryl]-D-myo-inositol 3,4,5-Trisphosphate [PtdIns(3,4,5)P3] and its Stereoisomers. *Bioorg Med Chem Lett*, 7, 3171-3176.

Garcia-Bellido, A. (1965). Larvalentwicklung Transplantierter Organe von *Drosophila melanogaster* im Adultmilieu. *J. Insect Physiol.*, 11, 1071–1078.

Garcia-Bellido, A., Ripoll, P., and Morata, G. (1973). Developmental compartmentalisation of the wing disk of Drosophila. *Nat New Biol*, 245(147), 251-3.

Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998). FYVE fingers bind PtdIns(3)P. *Nature*, 394(6692), 432-3.

Gnudi, L., Frevert, E. U., Houseknecht, K. L., Erhardt, P., and Kahn, B. B. (1997). Adenovirus-mediated gene transfer of dominant negative ras(asn17) in 3T3L1 adipocytes does not alter insulin-stimulated P13-kinase activity or glucose transport. *Mol Endocrinol*, 11(1), 67-76.

Gonzalez-Gaitan, M., Capdevila, M. P., and Garcia-Bellido, A. (1994). Cell proliferation patterns in the wing imaginal disc of Drosophila. *Mech Dev*, 46(3), 183-200.

Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., and et al. (1993). The GTPase dynamin binds to and is activated by a subset of SH3 domains. *Cell*, 75(1), 25-36.

Gratacap, M. P., Payrastre, B., Viala, C., Mauco, G., Plantavid, M., and Chap, H. (1998). Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J Biol Chem*, 273(38), 24314-21.

Guilherme, A., and Czech, M. P. (1998). Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by beta1-integrin signaling in rat adipocytes. *J Biol Chem*, 273(50), 33119-22.

Hammonds-Odie, L. P., Jackson, T. R., Profit, A. A., Blader, I. J., Turck, C. W., Prestwich, G. D., and Theibert, A. B. (1996). Identification and cloning of centaurin-alpha. A novel phosphatidylinositol 3,4,5-trisphosphate-binding protein from rat brain. *J Biol Chem*, 271(31), 18859-68.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. (1998). Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science*, 279(5350), 558-60.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol*, 166(4), 557-80.

Harpur, A. G., Layton, M. J., Das, P., Bottomley, M. J., Panayotou, G., Driscoll, P. C., and Waterfield, M. D. (1999). Intermolecular interactions of the p85alpha regulatory subunit of phosphatidylinositol 3-kinase [In Process Citation]. *J Biol Chem*, 274(18), 12323-32.

Harrington, E. A., Bennett, M. R., Fanidi, A., and Evan, G. I. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *Embo J*, 13(14), 3286-95.

Harrison-Findik, D., Susa, M., and Varticovski, L. (1995). Association of phosphatidylinositol 3-kinase with SHC in chronic myelogeneous leukemia cells. *Oncogene*, 10(7), 1385-91.

Hartl, D. L., Nurminsky, D. I., Jones, R. W., and Lozovskaya, E. R. (1994). Genome structure and evolution in *Drosophila*: Applications of the framework P1 map. *Proc. Natl. Acad. Sci.*, 91, 6824-6829.

Hatada, M. H., Lu, X., Laird, E. R., Green, J., Morgenstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Theriault, K., Zoller, M. J., and Karas, J. L. (1995). Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor [see comments]. *Nature*, 376(6544), 32-8.

Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M., and et al. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr Biol*, 5(4), 393-403.

Hay, B. A., Wolff, T., and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development*, 120(8), 2121-9.

Haynie, J. L., and Bryant, P. J. (1976). Intercalary regeneration in imaginal wing disc of *Drosophila melanogaster*. *Nature*, 259, 132-134.

Haynie, J. L., and Bryant, P. J. (1977). The effects of X-rays on the proliferation dynamics in the imaginal wing disc of *Drosophila melanogaster*. *Wilhelm Roux Archives*, 183, 85–100.

Heisenberg, M. (1995). Archived, Flybase FBrf0079811. .

Held, L. I. (1979). Pattern as a function of cell number and cell size on the second-leg basitarsus of *Drosophila*. *Wilhelm Roux Archives*, 187, 105–127.

Herbst, J. J., Andrews, G., Contillo, L., Lamphere, L., Gardner, J., Lienhard, G. E., and Gibbs, E. M. (1994). Potent activation of phosphatidylinositol 3'-kinase by simple phosphotyrosine peptides derived from insulin receptor substrate 1 containing two YMXM motifs for binding SH2 domains. *Biochemistry*, 33(32), 9376-81.

Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*, 70(3), 419-29.

Hirano, T., and Mitchison, T. J. (1994). A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. *Cell*, 79(3), 449-58.

Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996). A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*, 379(6565), 560-4.

Honore, B., Leffers, H., Madsen, P., Rasmussen, H. H., Vandekerckhove, J., and Celis, J. E. (1992). Molecular cloning and expression of a transformation-sensitive human protein containing the TPR motif and sharing identity to the stress- inducible yeast protein STI1. *J Biol Chem*, 267(12), 8485-91.

Hsuan, J. J., Minogue, S., and dos Santos, M. (1998). Phosphoinositide 4- and 5-kinases and the cellular roles of phosphatidylinositol 4,5-bisphosphate. *Adv Cancer Res*, 74, 167-216.

Hu, Q., Klippel, A., Muslin, A. J., Fantl, W. J., and Williams, L. T. (1995). Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science*, 268(5207), 100-2.

Hugl, S. R., White, M. F., and Rhodes, C. J. (1998). Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem*, 273(28), 17771-9.

Hunter, T. (1995). When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell*, 83(1), 1-4.

Hurel, S. J., Rochford, J. J., Borthwick, A. C., Wells, A. M., Vandenheede, J. R., Turnbull, D. M., and Yeaman, S. J. (1996). Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis. *Biochem J*, 320(Pt 3), 871-7.

Ingham, R. J., Holgado-Madruga, M., Siu, C., Wong, A. J., and Gold, M. R. (1998). The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor. *J Biol Chem*, 273(46), 30630-7.

Inukai, K., Anai, M., Van Breda, E., Hosaka, T., Katagiri, H., Funaki, M., Fukushima, Y., Ogihara, T., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1996). A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinasestructurally similar to p55PIK is generated by alternative splicing of the p85 alpha gene. *J. Biol. Chem.*, 271, 5317-5320.

Ito, N., and Rubin, G. M. (1999). gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. *Cell*, 96(4), 529-39.

James, S. R., Downes, C. P., Gigg, R., Grove, S. J., Holmes, A. B., and Alessi, D. R. (1996). Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem J*, 315(Pt 3), 709-13.

Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997). Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *Embo J*, 16(12), 3693-704.

Jhoti, H., McLeod, A. N., Blundell, T. L., Ishizaki, H., Nagasawa, H., and Suzuki, A. (1987). Prothoracicotrophic hormone has an insulin-like tertiary structure. *FEBS Lett*, 219(2), 419-25.

Jiang, B. H., Aoki, M., Zheng, J. Z., Li, J., and Vogt, P. K. (1999). Myogenic signaling of phosphatidylinositol 3-kinase requires the serine- threonine kinase Akt/protein kinase B. *Proc Natl Acad Sci U S A*, 96(5), 2077-2081.

Jimenez, C., Jones, D. R., Rodriguez-Viciana, P., Gonzalez-Garcia, A., Leonardo, E., Wennstrom, S., von Kobbe, C., Toran, J. L., L, R. B., Calvo, V., Copin, S. G., Albar, J. P., Gaspar, M. L., Diez, E., Marcos, M. A., Downward, J., Martinez, A. C., Merida, I., and Carrera, A. C. (1998). Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *Embo J*, 17(3), 743-53.

Joyal, J. L., Burks, D. J., Pons, S., Matter, W. F., Vlahos, C. J., White, M. F., and Sacks, D. B. (1997). Calmodulin activates phosphatidylinositol 3-kinase. *J Biol Chem*, 272(45), 28183-6.

Kapus, A., Szaszi, K., Sun, J., Rizoli, S., and Rotstein, O. D. (1999). Cell shrinkage regulates Src kinases and induces tyrosine phosphorylation of cortactin, independent of the osmotic regulation of Na+/H+ exchangers. *J Biol Chem*, 274(12), 8093-102.

Karim, F. D., and Rubin, G. M. (1998). Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues. *Development*, 125(1), 1-9.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature*, 385(6616), 544-8.

Kawamura, K., Shibata, T., Saget, O., Peel, D., and Bryant, P. J. (1998). A new family of growth factors produced by the fat body and active on Drosophila imaginal disc cells. *Development*, 126(Pt 2), 211-219.

Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N., and Hay, N. (1997). The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev*, 11(6), 701-13.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type [see comments]. *Nature*, 366(6454), 461-4.

Kerner, J. D., Appleby, M. W., Mohr, R. N., Chien, S., Rawlings, D. J., Maliszewski, C. R., Witte, O. N., and Perlmutter, R. M. (1995). Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity*, 3(3), 301-12.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J*, 16(10), 2783-93.

Kim, L., and Wong, T. W. (1998). Growth factor-dependent phosphorylation of the actin-binding protein cortactin is mediated by the cytoplasmic tyrosine kinase FER. *J Biol Chem*, 273(36), 23542-8.

Kimura, K. D., Tissenbaum, H. A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans [see comments]. *Science*, 277(5328), 942-6.

Klarlund, J. K., Guilherme, A., Holik, J. J., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997). Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains [see comments]. *Science*, 275(5308), 1927-30.

Klippel, A., Escobedo, M. A., Wachowicz, M. S., Apell, G., Brown, T. W., Giedlin, M. A., Kavanaugh, W. M., and Williams, L. T. (1998). Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. *Mol Cell Biol*, 18(10), 5699-711.

Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997). A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol Cell Biol*, 17(1), 338-44.

Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996). Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol Cell Biol*, 16(8), 4117-27.

Kobayashi, M., Nagata, S., Kita, Y., Nakatsu, N., Ihara, S., Kaibuchi, K., Kuroda, S., Ui, M., Iba, H., Konishi, H., Kikkawa, U., Saitoh, I., and Fukui, Y. (1997). Expression of a constitutively active phosphatidylinositol 3-kinase induces process formation in rat PC12 cells. Use of Cre/loxP recombination system. *J Biol Chem*, 272(26), 16089-92.

Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995). Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. *Embo J*, 14(17), 4288-95.

Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem*, 271(49), 31372-8.

Kolanus, W., Nagel, W., Schiller, B., Zeitlmann, L., Godar, S., Stockinger, H., and Seed, B. (1996). Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell*, 86(2), 233-42.

Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakaue, H., Hino, Y., Miyake, K., Sano, W., Akimoto, K., Ohno, S., and Kasuga, M. (1998). Requirement of atypical protein kinase clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol*, 18(12), 6971-82.

Kotani, K., Yonezawa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, F., and et al. (1994). Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. *Embo J*, 13(10), 2313-21.

Krymskaya, V. P., Hoffman, R., Eszterhas, A., Ciocca, V., and Panettieri, R. A., Jr. (1997). TGF-beta 1 modulates EGF-stimulated phosphatidylinositol 3-kinase activity in human airway smooth muscle cells. *Am J Physiol*, 273(6 Pt 1), L1220-7.

Kulik, G., Klippel, A., and Weber, M. J. (1997). Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol*, 17(3), 1595-606.

Kupriyanova, T. A., and Kandror, K. V. (1999). Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *J Biol Chem*, 274(3), 1458-64.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(259), 680-5.

Larsen, P. L., Albert, P. S., and Riddle, D. L. (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. *Genetics*, 139(4), 1567-83.

Lawrence, J. C., Jr., and Abraham, R. T. (1997). PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem Sci*, 22(9), 345-9.

Layton, M. J., Harpur, A. G., Panayotou, G., Bastiaens, P. I. H., and Waterfield, M. D. (1998). Binding of a diphosphotyrosine-containing peptide that mimics activated platelet-derived growth factor receptor beta induces oligomerization of phosphatidylinositol 3-kinase [In Process Citation]. *J Biol Chem*, 273(50), 33379-85.

Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature*, 345(6275), 544-7.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science*, 281(5385), 2042-5.

Lee, S. B., and Rhee, S. G. (1995). Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol*, 7(2), 183-9.

Leevers, S. J., Weinkove, D., MacDougall, L. K., Hafen, E., and Waterfield, M. D. (1996). The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J.*, 15, 6584-6594.

Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996). PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, 85(5), 621-4.

Lin, K., Dorman, J. B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans [see comments]. *Science*, 278(5341), 1319-22.

Linassier, C., MacDougall, L. K., Domin, J., and Waterfield, M. D. (1997). Molecular cloning and biochemical characterization of a Drosophila phosphatidylinositol-specific phosphoinositide 3-kinase. *Biochem J*, 321(Pt 3), 849-56.

MacDougall, L. K., Domin, J., and Waterfield, M. D. (1995). A family of phosphoinositide 3-kinases in Drosophila identifies a new mediator of signal transduction. *Curr. Biol.*, 5, 1404–1415.

Madhavan, M. M., and Scheiderman, H. A. (1977). Histological analysis of the dynamicas of growth of the imaginal discs and histoblast nests during larval development of *Drosophila melanogaster*. *Wilhelm Roux's Archives*, 183, 269-305.

Maehama, T., and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*, 273(22), 13375-8.

Maehama, T., and Dixon, J. E. (1999). PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends in Cell Biology*, 9, 125-128.

Magun, R., Burgering, B. M., Coffer, P. J., Pardasani, D., Lin, Y., Chabot, J., and Sorisky, A. (1996). Expression of a constitutively activated form of protein kinase B (c- Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. *Endocrinology*, 137(8), 3590-3.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R., and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. *Cell*, 87(3), 531-42.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev*, 4(1), 82-9.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, 80(2), 179-85.

Mattsson, P. T., Vihinen, M., and Smith, C. I. (1996). X-linked agammaglobulinemia (XLA): a genetic tyrosine kinase (Btk) disease. *Bioessays*, 18(10), 825-34.

McIlroy, J., Chen, D., Wjasow, C., Michaeli, T., and Backer, J. M. (1997). Specific activation of p85-p110 phosphatidylinositol 3'-kinase stimulates DNA synthesis by ras- and p70 S6 kinase-dependent pathways. *Mol Cell Biol*, 17(1), 248-55.

Mewes, H. W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S. G., Pfeiffer, F., and Zollner, A. (1997). Overview of the yeast genome [published erratum appears in Nature 1997 Jun 12;387(6634):737]. *Nature*, 387(6632 Suppl), 7-65.

Milan, M., Campuzano, S., and Garcia-Bellido, A. (1996). Cell cycling and patterned cell proliferation in the wing primordium of Drosophila. *Proc Natl Acad Sci U S A*, 93(2), 640-5.

Milan, M., Campuzano, S., and Garcia-Bellido, A. (1997). Developmental parameters of cell death in the wing disc of Drosophila. *Proc Natl Acad Sci U S A*, 94(11), 5691-6.

Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. *Dev Biol*, 42(2), 211-21.

Morris, J. Z., Tissenbaum, H. A., and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature*, 382(6591), 536-9.

Moss, J., and Vaughan, M. (1998). Molecules in the ARF orbit. *J Biol Chem*, 273(34), 21431-4.

Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G., and Denton, R. M. (1995). Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells. *Biochem J*, 311(Pt 2), 595-601.

Murakami, S., and Johnson, T. E. (1996). A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. *Genetics*, 143(3), 1207-18.

Nagel, W., Zeitlmann, L., Schilcher, P., Geiger, C., Kolanus, J., and Kolanus, W. (1998). Phosphoinositide 3-OH kinase activates the beta2 integrin adhesion pathway and induces membrane recruitment of cytohesin-1. *J Biol Chem*, 273(24), 14853-61.

Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*, 268(1), 13-6.

Nave, B. T., Haigh, R. J., Hayward, A. C., Siddle, K., and Shepherd, P. R. (1996). Compartment-specific regulation of phosphoinositide 3-kinase by platelet-derived growth factor and insulin in 3T3-L1 adipocytes. *Biochem J*, 318(Pt 1), 55-60.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell*, 85(3), 357-68.

Neufeld, T. P., de la Cruz, A. F., Johnston, L. A., and Edgar, B. A. (1998). Coordination of growth and cell division in the Drosophila wing. *Cell*, 93(7), 1183-93.

Nicholson, D. W., and Thornberry, N. A. (1997). Caspases: killer proteases. *Trends Biochem Sci*, 22(8), 299-306.

Nicolet, C. M., and Craig, E. A. (1989). Isolation and characterization of STI1, a stress-inducible gene from Saccharomyces cerevisiae. *Mol Cell Biol*, 9(9), 3638-46.

Nishimura, R., Li, W., Kashishian, A., Mondino, A., Zhou, M., Cooper, J., and Schlessinger, J. (1993). Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol*, 13(11), 6889-96.

Nolte, R., Eck, M., Schlessinger, J., Shoelson, S., and Harrison, S. (1996). Crystal structure of the PI 3-kinase p85 amino-terminal SH2 domain and its phosphopeptide complexes. *Nature Structural Biology*, 3, 364-373.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature*, 389(6654), 994-9.

Ogg, S., and Ruvkun, G. (1998). The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol Cell*, 2(6), 887-93.

Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993). A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell*, 73(1), 179-91.

Otsu, M., Hiles, I., Gout, I., Fry, M., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell*, 65, 91-104.

Ottinger, E. A., Botfield, M. C., and Shoelson, S. E. (1998). Tandem SH2 domains confer high specificity in tyrosine kinase signaling. *J Biol Chem*, 273(2), 729-35.

Panaretou, C., Domin, J., Cockcroft, S., and Waterfield, M. D. (1997). Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. *J Biol Chem*, 272(4), 2477-85.

Panayotou, G., Gish, G., End, P., Truong, O., Gout, I., Dhand, R., Fry, M. J., Hiles, I., Pawson, T., and Waterfield, M. D. (1993). Interactions between SH2 domains and tyrosine-phosphorylated platelet-derived growth factor beta-receptor sequences: analysis of kinetic parameters by a novel biosensor-based approach. *Mol Cell Biol*, 13(6), 3567-76.

Paradis, S., and Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev*, 12(16), 2488-98.

Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V., and Chawla, A. (1998). A functional PtdIns(3)P-binding motif. *Nature*, 394(6692), 433-4.

Pawson, T. (1995). Protein modules and signalling networks. *Nature*, 373(6515), 573-580.

Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science*, 278(5346), 2075-80.

Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., and Okada, S. (1999). Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! location! [In Process Citation]. *J Biol Chem*, 274(5), 2593-6.

Peyroche, A., Paris, S., and Jackson, C. L. (1996). Nucleotide exchange on ARF mediated by yeast Gea1 protein. *Nature*, 384(6608), 479-81.

Phelps, C. B., and Brand, A. H. (1998). Ectopic gene expression in Drosophila using GAL4 system. *Methods*, 14(4), 367-79.

Philpott, K. L., McCarthy, M. J., Klippel, A., and Rubin, L. L. (1997). Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. *J Cell Biol*, 139(3), 809-15.

Piston, D. W., Knobel, S. M., Postic, C., Shelton, K. D., and Magnuson, M. A. (1999). Adenovirus-mediated knockout of a conditional glucokinase gene in isolated pancreatic islets reveals an essential role for proximal metabolic coupling events in glucose-stimulated insulin secretion. *J Biol Chem*, 274(2), 1000-4.

Pons, S., Asano, T., Glasheen, E., Miralpeix, M., Zhang, Y., Fisher, T. L., Myers, M. G., Jr., Sun, X. J., and White, M. F. (1995). The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol*, 15(8), 4453-65.

Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M. D., and Comoglio, P. M. (1993). A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. *Mol Cell Biol*, 13(8), 4600-8.

Pope, B., and Kent, H. M. (1996). High efficiency 5 min transformation of Escherichia coli. *Nucleic Acids Res*, 24(3), 536-7.

Proud, C. G. (1996). p70 S6 kinase: an enigma with variations. *Trends Biochem Sci*, 21(5), 181-5.

Proud, C. G., and Denton, R. M. (1997). Molecular mechanisms for the control of translation by insulin. *Biochem J*, 328(Pt 2), 329-41.

Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998). Phosphorylation and activation of p70s6k by PDK1 [see comments]. *Science*, 279(5351), 707-10.

Purves, D., Snider, W. D., and Voyvodic, J. T. (1988). Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature*, 336(6195), 123-8.

Raabe, T., Riesgo-Escovar, J., Liu, X., Bausenwein, B., Deak, P., Maröy, P., and Hafen, E. (1996). Dos, a novel pleckstrin homology domain-containing protein required for signal transduction between sevenless and ras1 in Drosophila. *Cell*, 85, 911–920.

Raff, M. (1998). Cell suicide for beginners [news]. *Nature*, 396(6707), 119-22.

Rameh, L. E., Arvidsson, A., Carraway, K. L., 3rd, Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D. S., Chen, C. S., and Cantley, L. C. (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J Biol Chem*, 272(35), 22059-66.

Rameh, L. E., Chen, C. S., and Cantley, L. C. (1995). Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*, 83(5), 821-30.

Rameh, L. E., Rhee, S. G., Spokes, K., Kazlauskas, A., Cantley, L. C., and Cantley, L. G. (1998). Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling. *J Biol Chem*, 273(37), 23750-7.

Reedijk, M., Liu, X., van der Geer, P., Letwin, K., Waterfield, M. D., Hunter, T., and Pawson, T. (1992). Tyr721 regulates specific binding of the CSF-1 receptor kinase insert to PI 3'-kinase SH2 domains: a model for SH2-mediated receptor-target interactions. *Embo J*, 11(4), 1365-72.

Reif, K., Buday, L., Downward, J., and Cantrell, D. A. (1994). SH3 domains of the adapter molecule Grb2 complex with two proteins in T cells: the guanine nucleotide exchange protein Sos and a 75-kDa protein that is a substrate for T cell antigen receptor-activated tyrosine kinases. *J Biol Chem*, 269(19), 14081-7.

Rhee, S. G., and Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem*, 272(24), 15045-8.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, 70(3), 401-10.

Rizo, J., and Sudhof, T. C. (1998). C2-domains, structure and function of a universal Ca2+-binding domain. *J Biol Chem*, 273(26), 15879-82.

Roche, S., Downward, J., Raynal, P., and Courtneidge, S. A. (1998). A function for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in fibroblasts during mitogenesis: requirement for insulin- and lysophosphatidic acid-mediated signal transduction. *Mol Cell Biol*, 18(12), 7119-29.

Roche, S., Koegl, M., and Courtneidge, S. A. (1994). The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. *Proc Natl Acad Sci U S A*, 91(19), 9185-9.

Rodriguez-Viciana, P., Warne, P., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M., Waterfield, M., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*, 370, 527-532.

Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell*, 89(3), 457-67.

Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996). Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *Embo J*, 15(10), 2442-51.

Roelink, H. (1996). Tripartite signaling of pattern: interactions between Hedgehogs, BMPs and Wnts in the control of vertebrate development. *Curr Opin Neurobiol*, 6(1), 33-40.

Rohn, J. L., Hueber, A. O., McCarthy, N. J., Lyon, D., Navarro, P., Burgering, B. M., and Evan, G. I. (1998). The opposing roles of the Akt and c-Myc signalling pathways in survival from CD95-mediated apoptosis. *Oncogene*, 17(22), 2811-8.

Rordorf Nikolic, T., Van Horn, D. J., Chen, D., White, M. F., and Backer, J. M. (1995). Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit. *J Biol Chem*, 270(8), 3662-6.

Rudner, D. Z., Kanaar, R., Breger, K. S., and Rio, D. C. (1996). Mutations in the small subunit of the Drosophila U2AF splicing factor cause lethality and developmental defects. *Proc Natl Acad Sci U S A*, 93(19), 10333-7.

Ruvkun, G., and Hobert, O. (1998). The taxonomy of developmental control in Caenorhabditis elegans [see comments]. *Science*, 282(5396), 2033-41.

Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *Embo J*, 15(22), 6241-50.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sasai, Y., and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev Biol*, 182(1), 5-20.

Scharenberg, A. M., El-Hillal, O., Fruman, D. A., Beitz, L. O., Li, Z., Lin, S., Gout, I., Cantley, L. C., Rawlings, D. J., and Kinet, J. P. (1998). Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase- dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *Embo J*, 17(7), 1961-72.

Schmidt, A., Beck, T., Koller, A., Kunz, J., and Hall, M. N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease [In Process Citation]. *Embo J*, 17(23), 6924-31.

Schneitz, K., Spielmann, P., and Noll, M. (1993). Molecular genetics of *aristaless*, a *prd*-type homeo box gene involved in the morphogenesis of proximal and distal pattern elements in a subset of appendages in Drosophila. *Genes and Development*, 7, 114-129.

Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science*, 260(5104), 88-91.

Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A., and Lawrence, J. C., Jr. (1998). Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc Natl Acad Sci U S A*, 95(13), 7772-7.

Serrano, N., and O'Farrell, P. H. (1997). Limb morphogenesis: connections between patterning and growth. *Curr Biol*, 7(3), R186-95.

Serve, H., Yee, N. S., Stella, G., Sepp-Lorenzino, L., Tan, J. C., and Besmer, P. (1995). Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor- mediated proliferation, survival and cell adhesion in mast cells. *Embo J*, 14(3), 473-83.

Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B., and Gray, J. W. (1999). PIK3CA is implicated as an oncogene in ovarian cancer [In Process Citation]. *Nat Genet*, 21(1), 99-102.

Shepherd, P. R., Nave, B. T., Rincon, J., Nolte, L. A., Bevan, A. P., Siddle, K., Zierath, J. R., and Wallberg-Henriksson, H. (1997). Differential regulation of phosphoinositide 3-kinase adapter subunit variants by insulin in human skeletal muscle. *J Biol Chem*, 272(30), 19000-7.

Shepherd, P. R., Withers, D. J., and Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling [published erratum appears in Biochem J 1998 Nov 1;335(Pt 3):711]. *Biochem J*, 333(Pt 3), 471-90.

Shibata, H., Omata, W., and Kojima, I. (1997). Insulin stimulates guanine nucleotide exchange on Rab4 via a wortmannin- sensitive signaling pathway in rat adipocytes. *J Biol Chem*, 272(23), 14542-6.

Shim, H., Chun, Y. S., Lewis, B. C., and Dang, C. V. (1998). A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc Natl Acad Sci U S A*, 95(4), 1511-6.

Shortridge, R. D., Yoon, J., Lending, C. R., Bloomquist, B. T., Perdew, M. H., and Pak, W. L. (1991). A Drosophila phospholipase C gene that is expressed in the central nervous system. *J Biol Chem*, 266(19), 12474-80.

Simpson, P., Berreur, P., and Berreur-Bonnenfant, J. (1980). The initiation of pupariation in Drosophila: dependence on growth of the imaginal discs. *J Embryol Exp Morphol*, 57, 155-65.

Sjolander, A., Yamamoto, K., Huber, B. E., and Lapetina, E. G. (1991). Association of p21ras with phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A*, 88(18), 7908-12.

Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A., and Schlessinger, J. (1991). Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell*, 65(1), 83-90.

Smith, T. F., and Waterman, M. S. (1981). Identification of common molecular subsequences. *J. Mol. Biol.*, 147, 195-197.

Soltoff, S. P., and Cantley, L. C. (1996). p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. *J Biol Chem*, 271(1), 563-7.

Song, Q., and Gilbert, L. I. (1997). Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. *J Biol Chem*, 272(7), 4429-35.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell*, 72(5), 767-78.

Staal, S. P. (1987). Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc Natl Acad Sci U S A*, 84(14), 5034-7.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN [In Process Citation]. *Cell*, 95(1), 29-39.

Staveley, B. E., Ruel, L., Jin, J., Stambolic, V., Mastronardi, F. G., Heitzler, P., Woodgett, J. R., and Manoukian, A. S. (1998). Genetic analysis of protein kinase B (AKT) in Drosophila. *Curr Biol*, 8(10), 599-602.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5- trisphosphate-dependent activation of protein kinase B [see comments]. *Science*, 279(5351), 710-4.

Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P., and Hawkins, P. T. (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell*, 89(1), 105-14.

Stephens, L. R., Jackson, T. R., and Hawkins, P. T. (1993). Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)- trisphosphate: a new intracellular signalling system? *Biochim Biophys Acta*, 1179(1), 27-75.

Stewart, M. J., Berry, C. O., Zilberman, F., Thomas, G., and Kozma, S. C. (1996). The Drosophila p70s6k homolog exhibits conserved regulatory elements and rapamycin sensitivity. *Proc Natl Acad Sci U S A*, 93(20), 10791-6.

Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B [see comments]. *Science*, 277(5325), 567-70.

Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., and et al. (1995). Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*, 269(5224), 690-3.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell*, 72(4), 527-40.

Su, T. T., and O'Farrell, P. H. (1998). Size control: cell proliferation does not equal growth. *Curr Biol*, 8(19), R687-9.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., and Koyasu, S. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science*, 283(5400), 390-2.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z., and Kornberg, T. B. (1995). Creating a Drosophila wing de novo, the role of engrailed, and the compartment border hypothesis. *Development*, 121(10), 3359-69.

Takuwa, N., Fukui, Y., and Takuwa, Y. (1999). Cyclin D1 expression mediated by phosphatidylinositol 3-kinase through mTOR-p70(S6K)-independent signaling in growth factor-stimulated NIH 3T3 fibroblasts. *Mol Cell Biol*, 19(2), 1346-58.

Tanaka, K., Horiguchi, K., Yoshida, T., Takeda, M., Fujisawa, H., Takeuchi, K., Umeda, M., Kato, S., Ihara, S., Nagata, S., and Fukui, Y. (1999). Evidence that a phosphatidylinositol 3,4,5-trisphosphate-binding protein can function in nucleus [In Process Citation]. *J Biol Chem*, 274(7), 3919-22.

Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., Inukai, K., Asano, T., Kaburagi, Y., Ueki, K., Nakajima, H., Hanafusa, T., Matsuzawa, Y., Sekihara, H., Yin, Y., Barrett, J. C., Oda, H., Ishikawa, T., Akanuma, Y., Komuro, I., Suzuki, M., Yamamura, K.,

Kodama, T., Suzuki, H., Kadowaki, T., and et al. (1999). Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. *Nat Genet*, 21(2), 230-5.

Thackeray, J. R., Gaines, P. C., Ebert, P., and Carlson, J. R. (1998). small wing encodes a phospholipase C-(gamma) that acts as a negative regulator of R7 development in Drosophila. *Development*, 125(24), 5033-42.

The, I., Hannigan, G. E., Cowley, G. S., Reginald, S., Zhong, Y., Gusella, J. F., Hariharan, I. K., and Bernards, A. (1997). Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. *Science*, 276(5313), 791-4.

Therrien, M., Chang, H. C., Solomon, N. M., Karim, F. D., Wassarman, D. A., and Rubin, G. M. (1995). KSR, a novel protein kinase required for RAS signal transduction [see comments]. *Cell*, 83(6), 879-88.

Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J Biol Chem*, 269(51), 32358-67.

Torok, T., Tick, G., Alvarado, M., and Kiss, I. (1993). P-lacW insertional mutagenesis on the second chromosome of Drosophila melanogaster: isolation of lethals with different overgrowth phenotypes. *Genetics*, 135(1), 71-80.

Treisman, J. E., and Heberlein, U. (1998). Eye development in Drosophila: formation of the eye field and control of differentiation. *Curr Top Dev Biol*, 39, 119-58.

Turner, S. J., Domin, J., Waterfield, M. D., Ward, S. G., and Westwick, J. (1998). The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K- C2alpha. *J Biol Chem*, 273(40), 25987-95.

Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998). Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J Biol Chem*, 273(9), 5315-22.

Ui, M., Okada, T., Hazeki, K., and Hazeki, O. (1995). Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci*, 20(8), 303-7.

Valius, M., and Kazlauskas, A. (1993). Phospholipase C-gamma 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell*, 73(2), 321-34.

van der Geer, P., Hunter, T., and Lindberg, R. A. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol*, 10, 251-337.

van Weering, D. H., de Rooij, J., Marte, B., Downward, J., Bos, J. L., and Burgering, B. M. (1998). Protein kinase B activation and lamellipodium formation are independent phosphoinositide 3-kinase-mediated events differentially regulated by endogenous Ras. *Mol Cell Biol*, 18(4), 1802-11.

Vanhaesebroeck, B., Higashi, K., Raven, C., Welham, M., Anderson, S., Brennan, P., Ward, S. G., and Waterfield, M. D. (1999a). Autophosphorylation of p110delta phosphoinositide 3-kinase: a new paradigm for the regulation of lipid kinases in vitro and in vivo. *EMBO J.*, 18(5), 1292-1302.

Vanhaesebroeck, B., Jones, G. E., Allen, W. E., Zicha, D., Hooshmand-Rad, R., Sawyer, C., Wells, C., Waterfield, M. D., and Ridley, A. J. (1999b). Distinct PI 3-kinases mediate mitogenic signalling and cell migration in macrophages. *Nature Cell Biology*, in press.

Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. (1997a). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci*, 22(7), 267-72.

Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J., and Waterfield, M. D. (1997b). P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A*, 94(9), 4330-5.

Vemuri, G. S., and McMorris, F. A. (1996). Oligodendrocytes and their precursors require phosphatidylinositol 3- kinase signaling for survival. *Development*, 122(8), 2529-37.

Venkateswarlu, K., Gunn-Moore, F., Oatey, P. B., Tavar, J. M., and Cullen, P. J. (1998a). Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. *Biochem J*, 335(Pt 1), 139-146.

Venkateswarlu, K., Oatey, P. B., Tavare, J. M., and Cullen, P. J. (1998b). Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr Biol*, 8(8), 463-6.

Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*, 269(7), 5241-8.

Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L. K., Stein, R., Zvelebil, M. J., Domin, J., Panaretou, C., and Waterfield, M. D. (1995). A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *Embo J*, 14(14), 3339-48.

Vollenweider, P., Clodi, M., Martin, S. S., Imamura, T., Kavanaugh, W. M., and Olefsky, J. M. (1999). An SH2 domain-containing 5' inositolphosphatase inhibits insulin- induced GLUT4 translocation and growth factor-induced actin filament rearrangement. *Mol Cell Biol*, 19(2), 1081-91.

Wachsman, J. T. (1996). The beneficial effects of dietary restriction: reduced oxidative damage and enhanced apoptosis. *Mutat Res*, 350(1), 25-34.

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D., and Kuriyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell*, 72(5), 779-90.

Ward, A., Bates, P., Fisher, R., Richardson, L., and Graham, C. F. (1994). Disproportionate growth in mice with Igf-2 transgenes. *Proc Natl Acad Sci U S A*, 91(22), 10365-9.

Weigmann, K., Cohen, S. M., and Lehner, C. F. (1997). Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase. *Development*, 124(18), 3555-63.

Welch, H., Eguinoa, A., Stephens, L. R., and Hawkins, P. T. (1998). Protein kinase B and rac are activated in parallel within a phosphatidylinositide 3OH-kinase-controlled signaling pathway. *J Biol Chem*, 273(18), 11248-56.

Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L., and Stephens, L. (1994). Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr Biol*, 4(5), 385-93.

White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995). Multiple Ras functions can contribute to mammalian cell transformation. *Cell*, 80(4), 533-41.

Whitman, M., Kaplan, D., Schaffhausen, B., Cantley, L., and Roberts, T. (1985). Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature*, 315, 239-242.

Wolff, T. (1993). "Pattern formation in the *Drosophila* retina." The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1277-1326.

Wolff, T., and Ready, D. F. (1991). Cell death in normal and rough eye mutants of Drosophila. *Development*, 113(3), 825-39.

Woscholski, R., and Parker, P. J. (1997). Inositol lipid 5-phosphatases--traffic signals and signal traffic. *Trends Biochem Sci*, 22(11), 427-31.

Wymann, M. P., and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta*, 1436(1-2), 127-50.

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development*, 117(4), 1223-37.

Xu, T., Wang, W., Zhang, S., Stewart, R. A., and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development*, 121(4), 1053-63.

Yamaguchi, T., Fernandez, R., and Roth, R. A. (1995). Comparison of the signaling abilities of the Drosophila and human insulin receptors in mammalian cells. *Biochemistry*, 34(15), 4962-8.

Yano, S., Tokumitsu, H., and Soderling, T. R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*, 396(6711), 584-7.

Yao, R., and Cooper, G. M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*, 267(5206), 2003-6.

Yao, R., and Cooper, G. M. (1996). Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of pp70S6K activity. *Oncogene*, 13(2), 343-51.

Yenush, L., Fernandez, R., Myers, M. G., Jr., Grammer, T. C., Sun, X. J., Blenis, J., Pierce, J. H., Schlessinger, J., and White, M. F. (1996). The Drosophila insulin receptor activates multiple signaling pathways but requires insulin receptor substrate proteins for DNA synthesis. *Mol Cell Biol*, 16(5), 2509-17.

Yenush, L., and White, M. F. (1997). The IRS-signalling system during insulin and cytokine action. *Bioessays*, 19(6), 491-500.

Yu, J., Wjasow, C., and Backer, J. M. (1998a). Regulation of the p85/p110alpha phosphatidylinositol 3'-kinase. Distinct roles for the n-terminal and c-terminal SH2 domains. *J Biol Chem*, 273(46), 30199-203.

Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G. A., and Backer, J. M. (1998b). Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol*, 18(3), 1379-87.

Zecca, M., Basler, K., and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell*, 87(5), 833-44.

Zhang, M. Q. (1998). Statistical features of human exons and their flanking regions. *Hum Mol Genet*, 7(5), 919-32.

Zhou, K., Takegawa, K., Emr, S., and Firtel, R. (1995). A phosphatidylinositol (PI) kinase gene family in Dictyostelium discoideum: biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development. *Mol. Cell. Biol.*, 15, 5645-5656.

Zvelebil, M. J., Panayotou, G., Linacre, J., and Waterfield, M. D. (1995). Prediction and analysis of SH2 domain-phosphopeptide interactions. *Protein Eng*, 8(6), 527-33.