



# The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in *Drosophila*

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**Akt (or PKB) is an oncogene involved in the regulation of cell survival. Akt is regulated by phosphatidylinositol 3-OH kinase (PI3'K) signaling and has shown to be hyperactivated through the loss of the PTEN tumor suppressor. In *Drosophila*, insulin signaling as studied using the *Drosophila* IRS-4 homolog (*Chico*) has been shown to be a crucial regulator of cell size. We have studied *Drosophila* Akt (*Dakt1*) and have shown that it is also involved in the regulation of cell size. Furthermore we have performed genetic epistasis tests to demonstrate that in *Drosophila*, PI3'K, PTEN and Akt comprise a signaling cassette that is utilized during multiple stages of development. In addition, we show that this signaling cassette is also involved in the regulation of cell survival during embryogenesis. This study therefore establishes the evolutionary conservation of this signaling pathway in *Drosophila*.** *Oncogene* (2000) 19, 3971–3977.

**Keywords:** *Drosophila*; PKB; PI3K; PTEN; cell size; cell survival

## Introduction

As multicellular organisms grow, the cells within them proliferate, grow and undergo apoptosis in a highly coordinated manner (Conlon and Raff, 1999). De-regulation of these processes can result in dramatic alteration of normal development and homeostasis. In mammalian cells, the perturbation of these processes may lead to malignant transformation. Akt or protein kinase B (PKB) was first identified as an oncogene (Bellacosa *et al.*, 1991). Akt was also shown experimentally to act as a cell survival or protection factor. Upon withdrawal of survival signals, c-Myc overexpression (Kauffmann-Zeh *et al.*, 1997), or anoikis (Khwaja *et al.*, 1997) in mammalian cells, the resulting induction of apoptosis can be suppressed through the misexpression of Akt. Indeed, Akt appears to be a major cell protection factor downstream of several growth factors and cytokines (Downward, 1998). Moreover, the identification of a

pleckstrin homology (PH) domain in Akt has led to research demonstrating the activation of this kinase by phosphatidylinositol 3-OH kinase (PI3'K) (Datta *et al.*, 1997; Chang *et al.*, 1997). PI3'K phosphorylates phosphoinositides on the 3' hydroxyl group of the inositol ring. PKB/Akt has been shown to be activated by both the direct and indirect interaction of phosphatidylinositol-3,4-bisphosphate and -3, 4, 5-triphosphate with the PH domain *in vitro* as well as *in vivo* (Coffer *et al.*, 1998). This leads to the model whereby the activation of PI3'K by growth factors (such as insulin) leads to the activation of Akt (Downward, 1998; Coffer *et al.*, 1998). Another factor that has been implicated in the regulation of Akt during the regulation of proliferation and survival is the PTEN protein. Stambolic *et al.* (1998) have proposed that PTEN exerts its role as a tumor suppressor through the negative regulation of PI3'K/Akt signaling. It therefore appears that both PI3'K and PTEN regulate Akt in the regulation of homeostasis in mammalian cells.

Recent studies in *Drosophila* have implicated a role for an insulin-receptor-substrate (IRS) protein (encoded by the *chico* gene) and phosphatidylinositol-3-OH kinase (PI3'K) in the regulation of cellular growth (Böhni *et al.*, 1999; Leever *et al.*, 1996; Weinkove *et al.*, 1999). In *chico* mutants, all aspects of development are normal except for the diminutive size of homozygous *chico* flies (Böhni *et al.*, 1999; Lehner, 1999). It has therefore been postulated that insulin/PI3'K signaling in *Drosophila* is required for the regulation of cell growth (Lehner, 1999). Consistent with this hypothesis, *Drosophila* Akt (*Dakt1*) and *Drosophila* PTEN (DPTEN) have both been shown to play a role in cell growth during imaginal development (Verdu *et al.*, 1999; Huang *et al.*, 1999; Goberdham *et al.*, 1999). Our previous studies on *Dakt1* implicated a role for *Dakt1* signaling in the regulation of cell survival during *Drosophila* embryogenesis (Staveley *et al.*, 1998). In this study, we use biochemical and genetic tests to demonstrate the conservation of PI3'K/Akt signaling in *Drosophila*. We also show that PI3'K/PTEN/Akt signaling is required for both cell growth and survival during *Drosophila* development in a stage-specific manner. Our study therefore suggests the integration of these processes under the control of a single mechanism encoded by the PI3'K/PTEN/Akt signaling cassette.

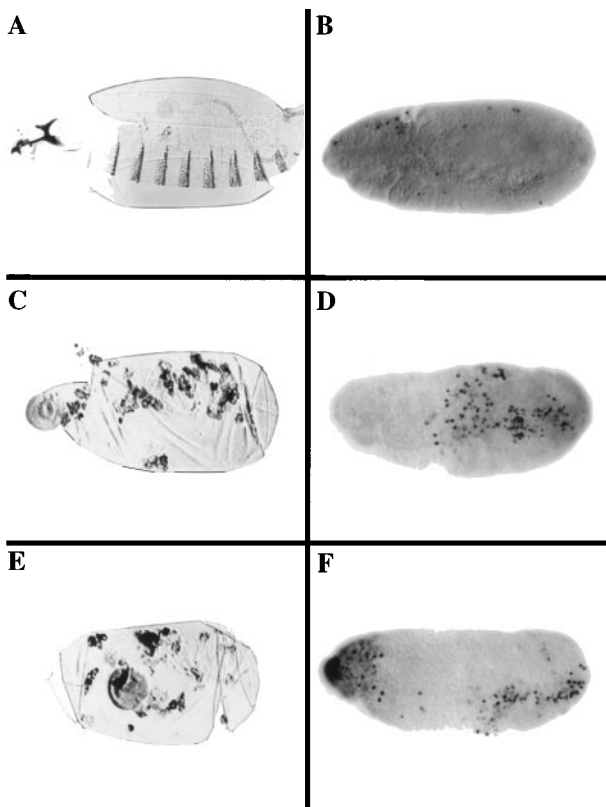
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## Results and discussion

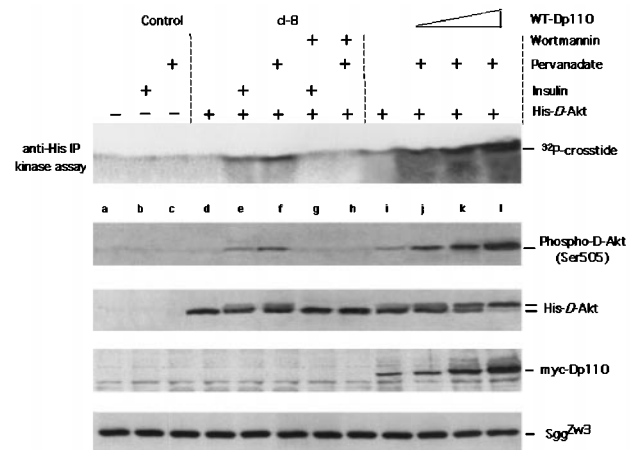
Our previous study described a null mutant allele of *Dakt1* that resulted in zygotic larval lethality (Staveley *et al.*, 1998). Germ line clone (GLC) analysis removing both maternal and zygotic expression of *Dakt1* resulted in embryos that exhibit ectopic and widespread apoptosis (Figure 1) (Staveley *et al.*, 1998). We also demonstrated that the ectopic apoptosis in *Dakt1* GLC embryos was independent of the *grim*, *reaper* and *hid* genes but could be suppressed through the expression of the baculoviral caspase inhibitor P35 (Staveley *et al.*, 1998). This maternal role for *Dakt1* prompted us to test for a potential role for PI3'K signaling in early *Drosophila* embryos. The ability of a dominant negative mutant of the catalytic subunit of *Drosophila* PI3'K (Dp110<sup>D954A</sup>) (Leever *et al.*, 1996) to affect cell viability in early embryos was therefore tested. Expression of high levels of Dp110<sup>D954A</sup> resulted in the extensive loss of cuticle (Figure 1e) and a high incidence of DNA fragmentation as assayed by TUNEL staining (Figure 1f) indicative of apoptosis. Furthermore, these embryos also showed high incidence of Acridine Orange (AO) staining (not shown), another accurate marker for apoptosis in *Drosophila* (Abrams *et al.*, 1993; Abrams, 1999). We also find that



**Figure 1** Embryonic phenotypes of *Dakt1* mutant embryos and embryos over expressing dominant negative Dp110. Comparison of the cuticle of a wild-type embryo (a) to that of embryos lacking *Dakt1* activity (c) and to that of an embryo expressing high levels of dominant negative Dp110 (Dp110<sup>D954A</sup>) (e). In the absence of either *Dakt1* or Dp110 activity, a significant amount of cuticle is missing leaving only 'scraps'. This loss of cuticle is reflected in the high incidence of DNA fragmentation in embryos lacking *Dakt1* (d) and *Dp110* (f) activity as indicated by the TUNEL assay. By comparison, wild-type embryos show very little TUNEL staining (b).

the ectopic expression of Dp110<sup>D954A</sup> can induce apoptosis at most times during embryogenesis (not shown). In fact both Dp110<sup>D954A</sup>-expressing and *Dakt1* GLC embryos exhibit ectopic apoptosis prior to any detectable sign of apoptosis in wild-type embryos (Figure 1b,d, and f). Also, the incidence of AO or TUNEL staining in these embryos does not seem to follow strict spatial distribution patterns. This is consistent with the analysis of cuticle secreted by these embryos. Thus, expression of Dp110<sup>D954A</sup> in embryos results in reduced cell viability due to apoptosis as in the case of *Dakt1* GLC embryos (Figure 1). These results suggest that *Dakt1* and *Dp110* activities may be required for cell survival during embryogenesis.

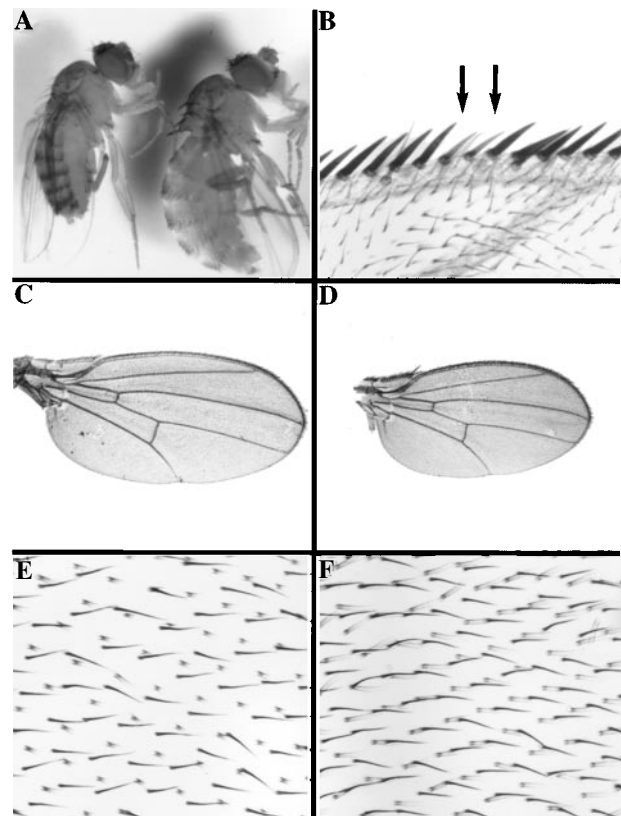
To gain mechanistic insight into the regulation of *Dakt1* protein (D-Akt) by PI3'K, we utilized a *Drosophila* imaginal disc cell line (cl-8) (Cullen and Milner, 1991). Incubation of cl-8 cells with the known PI3'K agonists insulin or pervanadate (Anjelkovic *et al.*, 1996; Alessi *et al.*, 1997) resulted in an increase in kinase activity of transiently transfected hexahistidine tagged D-Akt (His-D-Akt) (Figure 2, lanes e and f). Insulin or pervanadate treatment caused a 3- and 10-fold increase in wild-type His-D-Akt activity respectively, but did not affect the kinase activity of a mutant D-Akt (data not shown). Activation by insulin or pervanadate also led to the appearance of His-D-Akt forms with reduced electrophoretic mobility (Figure 2, lanes e and f), likely reflecting differences in protein phosphorylation. Phosphatase treatment collapsed



**Figure 2** Regulation of D-Akt by PI3'K/Dp110. *Drosophila* imaginal disc cells expressing empty plasmid or plasmid inducibly expressing myc-tagged, wild-type Dp110 were transfected with 20  $\mu$ g hexahistidine-tagged D-Akt as indicated (lanes d-l). Expression of Dp110 was induced for 0, 4, and 8 h (lanes j-l, respectively). The cells were treated with 1  $\mu$ M bovine insulin, 100  $\mu$ M pervanadate with or without 500 nM wortmannin as indicated. D-Akt activity was determined by immunoprecipitation/kinase assay using Crosstide as substrate. The degree of phosphorylation of Serine 505 of D-Akt was determined by immunoblotting with phospho-Akt/PKB (Ser-473) antibodies (NEB). The bottom three panels show immunoblots measuring the amount of D-Akt, Dp110 and a loading control (Sgg<sup>Zw3</sup>) using antibodies to polyhistidine, the myc epitope and Sgg<sup>Zw3</sup>, respectively. Both insulin and pervanadate induce D-Akt activation as determined by Ser 505 phosphorylation, Crosstide kinase activity and D-Akt gel retardation (lanes e and f); this activation is inhibited by wortmannin (lanes g and h). Increased expression of Dp110 causes a corresponding increase in activation of D-Akt (lanes i-l).

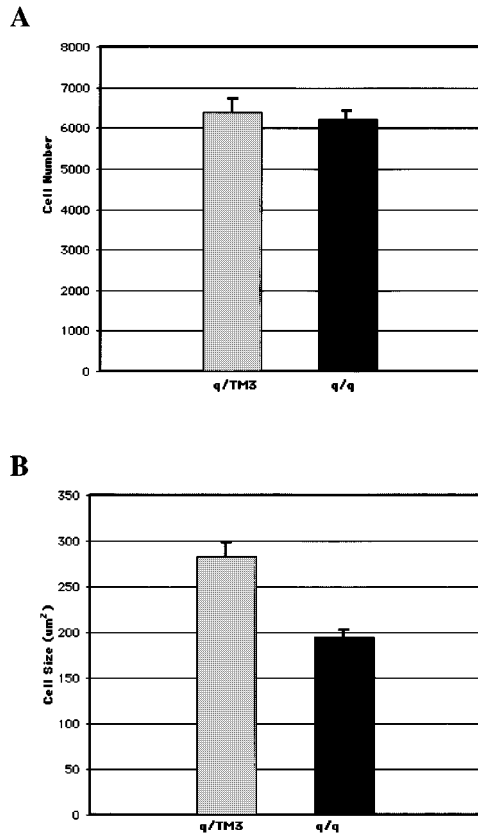
these bands into faster migrating species further suggesting that the mobility shift in His-D-Akt is due to phosphorylation (data not shown). Finally, the reduced electrophoretic mobility of His-D-Akt was accompanied by increased antigenicity of His-D-Akt towards a rabbit polyclonal antibody raised against the phosphorylated form of mammalian Akt (anti-PS473; NEB). This antibody recognizes the phosphorylated form of S505 of D-Akt, a residue corresponding to mammalian S473. Activation of His-D-Akt kinase activity by insulin or pervanadate could be inhibited by the pre-treatment of these cells with the PI3'K inhibitor, wortmannin (Figure 2, lanes g and h). This suggests that D-Akt is a target of PI3'K signaling *in vivo*. To assess this further, the ability of the catalytic subunit of PI3'K (Dp110) to directly activate D-Akt was analysed. For these experiments, cl-8 cell lines expressing wild-type Dp110 (WT Dp110) (Leevers *et al.*, 1996) under the control of an inducible metallothionein (MT-1) promoter were used. Treatment of these cells with CuSO<sub>4</sub> resulted in a 3–5-fold increase in WT-Dp110 protein levels (Figure 2, lanes j–l: Dp110 blot). Cl-8 cells expressing WT-Dp110 as well as parental cl-8 cells were transfected with His-D-Akt and treated with pervanadate. WT-Dp110 over expression in cl-8 cells resulted in a greater activation of His-D-Akt activity than in parental cl-8 cells (Figure 2, compare lanes f with k and l). This activation of His-D-Akt activity was proportional to the expression levels of WT-Dp110 (Figure 2, lanes j–l). Thus, D-Akt activity is regulated by PI3'K signaling *in vivo* during *Drosophila* development. PI3'K is also able to activate D-Akt kinase activity in response to insulin.

Loss of Dp110 activity in larval imaginal discs has been shown to result in the decrease of cell size and number (Leevers *et al.*, 1996; Weinkove *et al.*, 1999). As expression of dominant-negative Dp110 in embryos resulted in a phenotype similar to the loss of *Dakt1* activity, we decided to test the role of *Dakt1* during larval development. Ectopic expression of *Dakt1* had been used to suggest a role for *Dakt1* in the regulation of cell size (Verdu *et al.*, 1999). In this study, it was suggested that *Dakt1* regulates cell growth through the regulation of cell size and not cell number. However, the critical experiments to demonstrate this point were performed via ectopic expression of *Dakt1* (Verdu *et al.*, 1999). As *Dakt1* null flies are larval lethal during the first larval instar, we decided to generate *Dakt1* mutant animals at various stages of development in order to carry out a more detailed analysis. We used a heat-inducible *Dakt1* transgene (*HsDakt1*) to rescue the larval lethality of null *Dakt1* homozygous flies (see Materials and methods). With this methodology, we were able to generate homozygous *Dakt1* animals at all subsequent stages. In all cases tested, *Dakt1* larvae, pupae (not shown) or adult flies (Figure 3a) were significantly smaller in size than *Dakt1*/+ (heterozygous) animals. We also used clonal analysis through somatic recombination to generate homozygous *Dakt1* cells in a heterozygous (*Dakt1*/+) background. At the wing margin, homozygous *Dakt1* mutant sensory bristles marked with *yellow* (*y*) show a decrease in size as compared to their immediate *y* *Dakt1*/+ neighbors (Figure 3b). This is a cell autonomous effect as only *y*-marked (and thus *Dakt1* homozygous mutant) bristles show a decrease in size. Interestingly,



**Figure 3** Loss of *Dakt1* gene function during imaginal development affects cell size. (a) Comparison of *Dakt1*/+ (right) and *Dakt1/Dakt1* (left) adult flies showing a dramatic decrease in the size of *Dakt1* homozygous mutant flies. (b) Homozygous *Dakt1* mutant sensory bristles at the wing margin. These bristles appear gray because they have been marked by the *yellow* mutation (arrows) and therefore have a lighter color compared to wild-type bristles. Wings from wild-type (c) and *Dakt1* homozygous mutant (d) flies. (e) shows a higher magnification of a region of wild-type wing and (f) shows a higher magnification of a similar sized region of *Dakt1* mutant wing

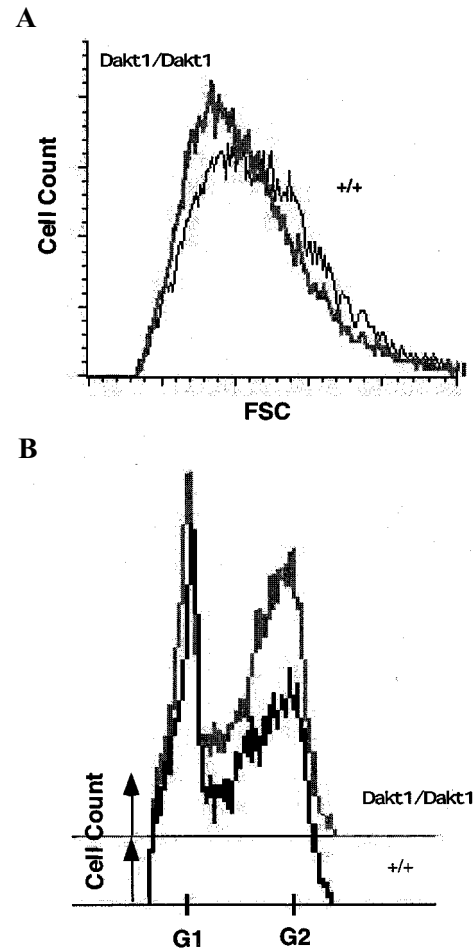
*y* *Dakt1* clones can be seen infrequently and are usually only 2–3 bristles in size at the wing margin. This is in sharp contrast to a variety of mutants tested in our laboratory using similar conditions. It is therefore possible that *Dakt1* mutation also has an effect on cell number as in the case of Dp110 (Leevers *et al.*, 1996; Weinkove *et al.*, 1999). These results are consistent with somatic clonal experiments in the eye using the same *Dakt1* allele (Verdu *et al.*, 1999). To determine a potential effect on cell number, we counted the number of cells in *Dakt1* mutant (Figure 3d,f) and wild-type wings (Figure 3c,e). We counted single wing hairs (trichomes) reflecting single epidermal wing cells within defined areas of wild-type and *Dakt1* mutant adult wings. The results were tabulated and calculated as in Montagne *et al.* (1999) and are depicted graphically in Figure 4. These results seem to indicate that *Dakt1* has a role in cell size and not cell number during the development of wings in *Drosophila*. We dissociated wing imaginal disc cells from wild-type and *Dakt1* mutant larvae, stained the cells with the DNA dye Hoechst and examined them using flow cytometry. This method allows us to determine the relative size of a population of cells by forward scatter (FSC) distribution (Neufeld *et al.*, 1998). Dissociated



**Figure 4** Quantitation of wing cell size. Tabulation of calculated number of inter-vein cells in the dorsal wing blade. Values are average of eight different wing samples for each genotype (see Materials and methods)

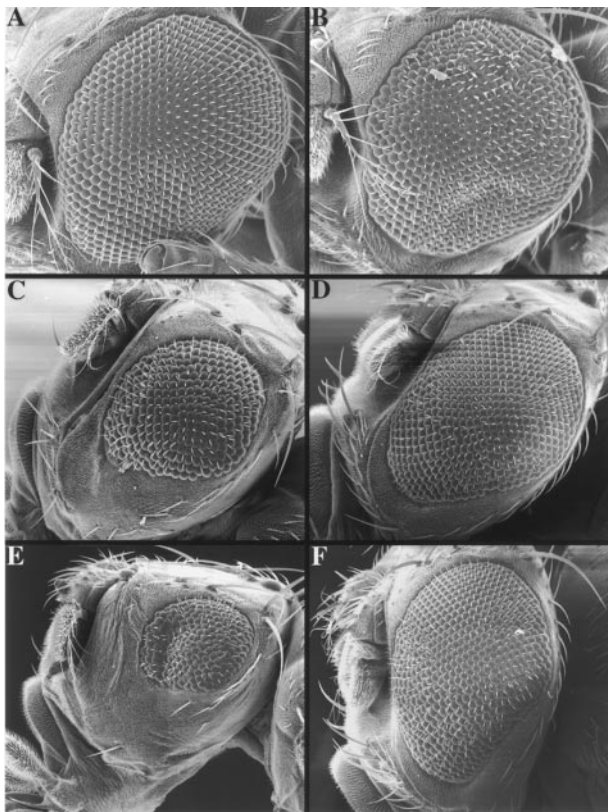
*Dakt1* mutant cells appear smaller than similarly treated wild-type cells in the FSC distribution (Figure 5a). Analysis of the same single cells using fluorescence-activated cell sorter (FACS) did not indicate a difference between the distribution of *Dakt1* mutant and wild-type cells within each phase of the cell cycle (Figure 5b). Therefore it appears that *Dakt1* mutant cells have a cell size phenotype alone.

To examine the role of *Dakt1* in the development of the eye, we utilized the *eyeless* promoter to make *Dakt1* homozygous mutant eyes (Figure 6b). As a comparison, we also utilized the same promoter to express *Dp110<sup>D954</sup>* to repress *Dp110* activity in the eye (Figure 6c). In both experiments, the resulting eyes were smaller in size as compared to wild-type eyes (Figure 6a). Experiments on *Dp110* and the *pto* subunit of PI3'K in *Drosophila* have clearly demonstrated that PI3'K signaling is required for the regulation of both cell size and number (Leever *et al.*, 1996; Weinkove *et al.*, 1999). Our results here as well as previously published results (Verdu *et al.*, 1999) have defined a role for *Dakt1* in the regulation of cell size alone. We therefore decided to test the capacity of ectopic *Dakt1* (using *HsDakt1*) to suppress the ectopic *Dp110<sup>D954</sup>* phenotype. The ectopic *Dp110<sup>D954</sup>* phenotype results in a dramatic decrease in cell number and the resulting eyes (Figure 6c) are much smaller than wild-type eyes (Figure 6a). However, ectopic *Dakt1* expression is able to significantly suppress the *Dp110<sup>D954</sup>* phenotype (Figure 6d). Ectopic expression of *Drosophila* PTEN (*DPTEN*) using the *eyeless* promoter also results in the decrease of eye size (Figure 6e) (Huang *et al.*, 1999;

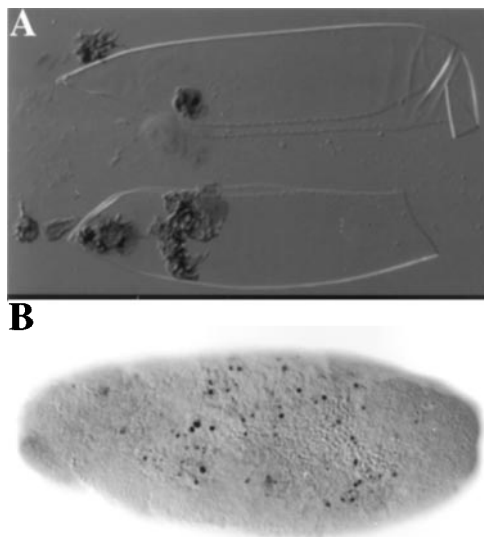


**Figure 5** Flow cytometry of wing imaginal disc cells. (a) Imaginal disc cell size as measured by Forward Scatter (FSC). The results from wild-type cells are denoted by the black line and the results from *Dakt1* mutant cells are denoted by the gray line. (b) DNA content of wild-type (black line) and *Dakt1* mutant (gray line) cells as measured by Hoechst uptake and FACS analysis

Goberdham *et al.*, 1999). Loss of function tests for *DPTEN* have confirmed that *DPTEN* has a negative role in cell growth but also proliferation (Huang *et al.*, 1999). Again, *HsDakt1* is able to suppress this phenotype to a great extent (Figure 6f). These genetic epistasis experiments indicate that *Dakt1* is downstream of both *Dp110* and *DPTEN*. Therefore, even though *Dakt1* is not required for cell number (or proliferation), the ectopic expression of *Dakt1* modifies the function of both *Dp110* and *DPTEN*. Finally, we ectopically expressed *DPTEN* in embryos, which resulted in the extensive loss of cuticle at first instar (Figure 7a). Ectopic *DPTEN* was able to induce extensive apoptosis as assayed by TUNEL staining (Figure 7b) and AO staining (not shown). This result is not unexpected because *PTEN* has been shown to be a potent inhibitor of Akt/PKB function (Stambolic *et al.*, 1998). As in the case of *Dp110<sup>D954A</sup>*, ectopic *PTEN* is able to induce apoptosis during a wide range of stages during development and at stages prior to the stage when the wild-type pattern of apoptosis begins. Therefore, both *Dp110* and *DPTEN* are potential regulators of cell survival during *Drosophila* embryogenesis.



**Figure 6** Epistatic relationship between *Dp110*, *DPTEN* and *Dakt1*. SEMs of eyes from wild-type (a) and *eyeless* FLPase induced *Dakt1* homozygous eye clone (b). Eye from *eyeless GAL4/UAS Dp110<sup>D954A</sup>* (c) and an eye with *eyeless GAL4/UAS Dp110<sup>D954A</sup>* with *HsDakt1* (d). Eye from *eyeless GAL4/UAS DPTEN* (e) and an eye from *eyeless GAL4/UAS DPTEN* with *HsDakt1* (f)



**Figure 7** Ectopic expression of *DPTEN* results in apoptosis in embryos. Effects of ectopic expression of *DPTEN* (*HsGAL4/UAS DPTEN*) in embryos on cuticle phenotype (a) and TUNEL staining (b)

## Conclusions

During imaginal development, activation of *Dakt1* by insulin and PI3'K results in growth. *DPTEN* is a negative regulator of this process. *Dp110* (PI3'K signaling) and *DPTEN* influence growth by regulating

both cell size and cell number. Experiments on *Dakt1* indicate a role for this gene in the regulation of cell size alone. However, *Dakt1* must be able to influence cell number as well since ectopic *Dakt1* expression is able to suppress the phenotypes of both *Dp110* (*D954A*) and *DPTEN* overexpression. As the *Drosophila* homolog of S6 kinase has also been shown to have a cell size phenotype, it is possible that *Dakt1* may regulate cell size through the regulation of S6 kinase (Lehner, 1999). Studies on *Drosophila* *IRS-4*, *chico* have also demonstrated a role for *chico* in cell growth. Our results complete a mechanistic picture whereby insulin activates D-Akt in the regulation of cell growth. It therefore appears that the insulin signal transduction pathway is involved in the regulation of cell size in *Drosophila* whereby growth response to nutrient conditions may be regulated by insulin. During *Drosophila* embryogenesis, we have identified a different role for insulin signaling components. We have shown evidence that *Dakt1*, *Dp110* and *DPTEN* are involved in the regulation of cell survival. However, by contrast to PI3'K and *Dakt1*, *chico* does not have a role in cell survival during embryogenesis (Böhni et al., 1999). Our results therefore indicate that *Dp110/DPTEN/Dakt1* comprise a conserved signaling cassette in *Drosophila* with multiple roles depending on the stage of development. We believe that the regulation of PI3'K by *chico* is restricted to the cell growth function of this pathway during larval development. We are currently conducting genetic screens to identify genetic components that regulate the *Dp110/DPTEN/Dakt1* signaling module during embryogenesis for the cell survival function of this pathway.

## Materials and methods

### *Drosophila* strains and genetic experiments

Germ line clones (GLCs) of *Dakt1* were generated as described in (Staveley et al., 1998). In all experiments, we used the *q* allele of *Dakt1* (Staveley et al., 1998). We also used a P-element induced allele of *Dakt1* (*P1627*). *P1627* (*l(3)04226*) (Bloomington Stock Center) was demonstrated to be an allele of *q* using genetic complementation as well as rescue to viability by a heat-inducible transgene of *Dakt1* (*HsDakt1*). GLC embryos of *P1627* have the same phenotype as GLC embryos of *q*. To generate embryos expressing ectopic *Dp110<sup>D954A</sup>*, *HsGAL4* females were crossed to *UAS Dp110<sup>D954A</sup>* (Weinkove et al., 1999) males and the progeny were heat shocked for 8 min at 36°C during 0–4 h of embryogenesis. For the expression of ectopic *DPTEN*, *HsGAL4* females were crossed to *UAS DPTEN* males and the above procedure was followed. Embryos were then allowed to recover at 25°C for AO or cuticle analysis. For TUNEL stainings, a time course of fixations after the induction were carried out. We found the incidence of ectopic TUNEL staining after a mere 1 h after the initiation of the heat shock for both *HsGAL4/UAS Dp110<sup>D954A</sup>* and *HsGAL4/UAS DPTEN* (not shown). Cuticle analysis and TUNEL or AO staining procedures were followed as previously described (Staveley et al., 1998). We also tested the expression of *reaper* and *grim* in *HsGAL4/UAS Dp110<sup>D954A</sup>* and *HsGAL4/UAS DPTEN* embryos using DIG labeled probes to detect mRNA according to standard methodology (Manoukian, 1997).

To generate *Dakt1* homozygous adults, *HsDakt1*, *q/TM3*, *Sb* flies were allowed to lay eggs and the progeny were heat-shocked daily for 10–30 min at 35°C. The heat-shock regimen

was stopped at various times during development of *HsDakt1*, *q/TM3*, *Sb* progeny. Using this method, *q* homozygous individuals were rescued to various stages beyond first instar larvae. At all subsequent stages, the progeny demonstrated a dramatic decrease in size as compared to *Dakt1/+* homozygous individuals. Cell size of inter-vein cells in *q* and wild-type wings was calculated by counting the number of hairs in a rectangular area of 0.025 mm<sup>2</sup>, located between veins 3 and 4 of the dorsal wing blade. Eight different wing samples for each genotype were used for the analysis. The 0.025 mm<sup>2</sup> area was then divided by the average number of hairs in the designated area. The total number of dorsal wing blade inter-vein cells for the sample of wings was calculated by multiplying the average number of hairs in a 0.025 mm<sup>2</sup> rectangle by total average area of the dorsal wing blade from the eight wing samples divided by 0.025 mm<sup>2</sup> (Montagne *et al.*, 1999; Neufeld *et al.*, 1998).

For FSC and cell cycle analysis, we used the *HsDakt1*; *q/TM6b*, *Tb* line and the above mentioned heat shock regimen to generate and identify *q* homozygous larvae. Forty to 50 wing imaginal discs from each genotype were dissected and placed into a tube containing 200  $\mu$ l of cl-8/fetal calf serum media on ice. Fifteen  $\mu$ l of trypsin (10%) and 4  $\mu$ l of hoechst (0.25 mg/ml) was added along with 181  $\mu$ l of cl-8-fetal calf serum media to give a final volume of 400  $\mu$ l. The disc cells were disassociated for 3 h at room temperature while shaking slowly. Tubes were spun at RT for 10 min at 1000 r.p.m. to pellet the cells, and the supernatant was discarded. Cells were then re-suspended in fresh cl-8-fetal calf serum medium and analysed by FSC. Cell cycle analysis using hoechst uptake was performed with FACS Vantage (Becton Dickinson, Mountain View, CA, USA) and Cell Quest software, using Coherent Enterprise Laser II (Coherent, Santa Clara, CA, USA) at a power setting of 40 mW UV. Clonal analysis of *Dakt1* was carried out using the Flip recombinase system (Xu and Harrison, 1994). For this experiment, *yw HsFLP*; *FRT 82 $\beta$  Dakt1q/TM3* flies were crossed to *yw*; *FRT 82 $\beta$  y<sup>+</sup>* flies and heat-shocked to generate *y Dakt1* somatic clones.

For the analysis of *Dakt1* and *Dp110* in the eye, *eyeless* FLPase; *FRT 82 $\beta$ /FRT 82 $\beta$  Dakt1* flies were generated and *eyeless GAL4* flies were crossed to *UAS Dp110* flies (respectively). *HsDakt1*; *eyeless GAL4/UASDp110<sup>D954A</sup>* and *HsDakt1*, *eyeless GAL4/UAS DPTEN* embryos were collected for 12 h at 25°C. One hour heat shocks at 37°C were then performed at 40 h (mid-1st instar), 56 h (mid-2nd instar) and 86 h (3rd instar) after egg collection. The resulting larvae were then allowed to develop until adulthood. Adult eyes were analysed using Scanning Electron Microscopy (SEM).

#### Transfections and cell culture

*Drosophila* cl-8 cells were maintained as described (Yanagawa *et al.*, 1995). Selection of stable transformed cl-8 cell lines was performed using hygromycin. The expression vector pMT-V5 (Invitrogen) is designed to express proteins under control of the metallothionein promoter. The 3.2 kb *KpnI-XbaI* fragment of wild-type *c-myc*-tagged Dp110 corresponding to the entire coding region was cloned into the *KpnI-XbaI* sites of pMT V5. The Dp110/pMT-V5 vectors were introduced into cl-8 cells by cotransfection with a second vector, pCoHygro,

carrying a selectable hygromycin B phosphotransferase gene, which confers resistance to hygromycin (300  $\mu$ g/ml). Transformed cells were maintained between  $1 \times 10^6$  and  $1 \times 10^7$  cells/ml and examined for metal-inducible expression (by addition of 0.5 mM CuSO<sub>4</sub>) by immunoblotting. Where added, wortmannin was used at a final concentration of 500 nM, insulin at 1  $\mu$ M and pervanadate (100  $\mu$ M sodium vanadate + 200  $\mu$ M H<sub>2</sub>O<sub>2</sub>) at 100  $\mu$ M. All treatments were for 15 min prior to cell lysis.

#### Immunoprecipitation and D-Akt kinase assays

For expression in cl-8 cell lines, a D-Akt fragment encoding aa 1-531 (amplified by PCR) was subcloned into pPAC3-His6 vector in frame with the histidine epitope at the N-terminus. Transfected cells were washed with PBS and lysed in Gentle Soft buffer (Stambolic *et al.*, 1996). To assess D-Akt kinase activity, His-D-Akt was immunoprecipitated and its activity was measured using a peptide substrate representing the amino-terminal phosphorylation site (serine 9) present in GSK-3 $\beta$  (Staveley *et al.*, 1998; Burgering and Coffey, 1995). For His-D-Akt immunoprecipitation, 20  $\mu$ l monoclonal anti-polyhistidine-agarose (Sigma) were added to the clarified cell lysates at 4°C for 2 h. Immunocomplexes were washed four times with lysis buffer. *In vitro* His-D-Akt kinase assays were performed for 30 min as described previously (Staveley *et al.*, 1998). Phosphorylated peptide was separated from unincorporated [<sup>32</sup>P]-ATP by tricine-SDS-PAGE (Stambolic *et al.*, 1996) and quantified using a phosphorimager. The phospho-serine-473 antibody raised against mammalian Akt peptides is also able to recognize phospho-serine-505 in D-Akt due to the conservation of the peptide used to generate the antibody (NEB). This antibody is therefore a good assay for the phosphorylation status of D-Akt at serine-505.

#### Abbreviations

PKB, Protein Kinase B; PI3'K, phosphatidylinositol 3-OH kinase; IRS, insulin receptor substrate; *Dakt1*, *Drosophila* Akt; DPTEN, *Drosophila* PTEN; *Dp110*, *Drosophila* PI3'K catalytic subunit; TUNEL, TdT-mediated dUTP nick end labeling; AO, Acridine orange; D-Akt, *Dakt1* protein; His-D-Akt, Hexahistidine tagged D-Akt; cl-8, Clone 8 *Drosophila* imaginal disc cell line; *HsDakt1*, Heat shock promoter *Dakt1* cDNA transgene; *y*, *Drosophila yellow* gene; FACS, fluorescence-activated cell sorter; FSC, Forward Scatter; SEM, Scanning electron microscopy; DIG, digoxigenin

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