Drosophila PTEN Regulates Cell Growth and Proliferation through PI3K-Dependent and -Independent Pathways

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The control of cell and organ growth is fundamental to the development of multicellular organisms. Here, we show that *dPTEN*, a *Drosophila* homolog of the mammalian *PTEN* tumor suppressor gene, plays an essential role in the control of cell size, cell number, and organ size. In mosaic animals, *dPTEN*$^{-}$ cells proliferate faster than their heterozygous siblings, show an autonomous increase in cell size, and form organs of increased size, whereas overexpression of *dPTEN* results in opposite phenotypes. The loss-of-function phenotypes of *dPTEN* are suppressed by mutations in the PI3K target *Dakt1* and the translational initiation factor *eif4A*, suggesting that dPTEN acts through the PI3K signaling pathway to regulate translation. Although activation of PI3K and Akt has been reported to increase rates of cellular growth but not proliferation, loss of *dPTEN* stimulates both of these processes, suggesting that PTEN regulates overall growth through PI3K/Akt-dependent and -independent pathways. Furthermore, we show that *dPTEN* does not play a major role in cell survival during *Drosophila* development. Our results provide a potential explanation for the high frequency of *PTEN* mutation in human cancer.

Key Words: **cell size; cell cycle; tumor suppressor; translational control; Akt; cell death.**

INTRODUCTION

During animal development, patterning and growth must be intimately coordinated to generate organs of reproducible size and shape. The final size of an organ is a function of both cell number and cell size, and thus overall growth of an organ can result from changes in cell number, cell size, or both (Conlon and Raff, 1999). Since cell growth and cell proliferation contribute to cell size and cell number, respectively, it is important to understand the relationship between cell growth, cell proliferation, and organ size (overall growth in mass). Previous studies of *Drosophila* imaginal disc development suggest that the regulation of cell cycle alone is not sufficient to control organ size (Neufeld *et al.*, 1998; Weigmann *et al.*, 1997). Blocking cell division in the *Drosophila* wing results in normal-sized compartments consisting of fewer but larger cells, while accelerating cell division results in normal-sized compartments consisting of more and smaller cells. These studies suggest that overall growth is regulated by a mechanism that measures tissue mass rather than cell numbers and that cell proliferation alone does not drive overall growth.

It has long been appreciated that hormones, growth factors, and nutrition play an important role in the control of animal growth. In particular, insulin and insulin-like growth factor have been implicated in growth control. Biochemical studies in mammalian cell culture have elucidated multiple pathways that are activated by insulin/insulin-like receptors (reviewed in Proud and Denton, 1997). One of these pathways involves the phosphoinositide 3-kinase (PI3K) (reviewed in Cantley and Neel, 1999). Briefly, upon activation by growth factors, insulin receptor recruits PI3K to the membrane, either directly or through insulin receptor substrates as intermediates. Phosphorylation of the membrane lipid phosphatidylinositol 4,5-biphosphate (PIP_2) by PI3K produces the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 acti-

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vates two serine/threonine kinases, Akt and PDK1, which in turn control various downstream effectors in regulating cell cycle progression, cell death, and protein synthesis.

Recent studies in *Drosophila* imaginal discs have provided *in vivo* evidence that the insulin signal transduction pathway plays an essential role in the control of overall growth. Loss-of-function mutations in components of this pathway, including *Drosophila* insulin receptor (*Inr*), insulin receptor substrate (*chico*), PI3K subunits *Dp110* and *Dp60*, Akt (*Dakt1*), and S6 kinase (*dS6K*), all lead to a reduction of cell size and in some cases cause reduced cell numbers (Weinkove *et al.*, 1999; Böhni *et al.*, 1999; Montagne *et al.*, 1999; Chen *et al.*, 1996; Verdu *et al.*, 1999). A critical question arising from these studies concerns whether cell proliferation is directly regulated by this pathway or whether the observed reductions in cell number result indirectly from reduced growth rates. Importantly, overexpression of *Dp110* or *Dakt1* leads to increased cell size, but has no effect on cell number, indicating that activation of PI3K signaling may not be sufficient to promote cell proliferation (Weinkove *et al.*, 1999; Verdu *et al.*, 1999).

PTEN (phosphatase and tensin homolog deleted on chromosome ten), or *MMAC* (mutated in multiple advanced cancers), was initially identified as a tumor suppressor gene located at 10q23 (Li *et al.*, 1997; Steck *et al.*, 1997). Homozygous *PTEN* mutations are found predominantly in advanced glial and prostate tumors, as well as in endometrial, renal, and small cell lung carcinomas; melanomas; and meningiomas. Overall, *PTEN* is one of the most common targets of mutation in human cancer, with a mutation frequency approaching that of *p53* (reviewed in Cantley and Neel, 1999; Maehama and Dixon, 1999). Germ-line mutations in *PTEN* are responsible for three autosomal dominant hamartoma cancer syndromes: Cowden syndrome, Bannayan–Zonana syndrome, and Lhermitte–Duclos syndrome (Liaw *et al.*, 1997; Nelen *et al.*, 1997; Marsh *et al.*, 1998). These syndromes are characterized by hamartomas, benign tumors in which differentiation is normal but cells are highly disorganized.

The predicted protein of the *PTEN* gene has an amino-terminal domain with extensive similarity to tensin, a protein that interacts with actin filaments at focal adhesions, and a domain with homology to protein tyrosine phosphatases (Li *et al.*, 1997; Steck *et al.*, 1997). PTEN protein can dephosphorylate tyrosine-, serine-, and threonine-phosphorylated peptides (Myers *et al.*, 1997), and the focal adhesion kinase (FAK) has been shown to be a substrate of PTEN *in vitro* (Tamura *et al.*, 1998). In addition, recent biochemical studies have shown that PTEN can dephosphorylate the 3′ position of PIP_3 to generate PIP_2 (Maehama and Dixon, 1998). Such biochemical function suggests that PTEN may antagonize the activity of PI3K, which converts PIP_2 to PIP_3. Consistent with this model, PTEN-deficient cells show elevated level of PIP_3 (Stambolic *et al.*, 1998; Sun *et al.*, 1999), and several patient-derived mutations eliminate PTEN's lipid phosphatase activity but not its protein phosphatase activity (Myers *et al.*, 1998; Furnari *et al.*, 1998).

PTEN has been proposed to control several cellular functions, including proliferation (Li and Sun, 1998; Furnari *et al.*, 1998; Suzuki *et al.*, 1998; Podsypanina *et al.*, 1999), apoptosis (Stambolic *et al.*, 1998; Myers *et al.*, 1998; Li *et al.*, 1998), and migration (Tamura *et al.*, 1998). However, it is not clear how these potential functions are employed during normal development. *PTEN* mutant mice generated by gene targeting are embryonic lethal as homozygotes, while heterozygotes are predisposed for tumor formation (Suzuki *et al.*, 1998; Podsypanina *et al.*, 1999; Di Cristofano *et al.*, 1998). Cell lines derived from such animals show elevated PIP_3 level and increased Akt activity (Stambolic *et al.*, 1998; Sun *et al.*, 1999). *Daf-18*, a *Caenorhabditis elegans* homolog of *PTEN*, is involved in metabolic control, dauer larva arrest, and longevity. *Daf-18* mutants suppress the mutant phenotype of *daf-2*, which encodes an insulin-like receptor, and *age-1*, which encodes a PI3K (Ogg and Ruvkun, 1998; Gil *et al.*, 1999; Mihaylova *et al.*, 1999). In this paper, we present evidence that *dPTEN*, a *Drosophila* homolog of the mammalian *PTEN* gene, plays a critical role in the control of cell proliferation, cell size, and organ size during development. We find that *dPTEN* loss of function results in phenotypes distinct from those caused by PI3K activation, suggesting that PTEN acts through PI3K-dependent and -independent pathways.

MATERIALS AND METHODS

Molecular Biology

Southern blot analysis revealed DNA polymorphism in the *dPTEN* genomic region in *dj189* that is most consistent with the insertion of a 4.6-kb exogenous DNA. A plasmid library was made to clone the insertion breakpoint. Sequence analysis of the insertion breakpoint revealed the existence of an F-element within a coding exon at the 5′ end of the *dPTEN* gene (Fig. 1A). A 6-kb genomic fragment containing just the *dPTEN* transcription unit was cloned into Casperhs-1, a modified Casperhs vector (Pan and Rubin, 1997), for the rescue experiment.

A full-length *dPTEN* cDNA clone, LP04865, was obtained from Research Genetics. Sequence analysis showed that this clone contains a full-length *dPTEN* cDNA fused to an irrelevant cDNA after the poly(A) tail. The *dPTEN* portion of the clone was excised and cloned into the PUAST vector (Brand and Perrimon, 1993) to generate UAS-*dPTEN*.

Drosophila Strains

The GAL4 driver *MS1096*, *en-GAL4*, *apterous-GAL4*, and *arm-GAL4* were described previously (Capdevila and Guerrero, 1994; Montagne *et al.*, 1999; Brand and Perrimon, 1993; Sanson *et al.*, 1996). *UAS-Dp110* and *UAS-PI3KDN* were gifts from Sally Leevers (Weinkove *et al.*, 1999). The *armlacZ FRT40 A* chromosome was described previously (Pan and Rubin, 1995). The *MS1096 hsp-flp* chromosome was a gift from Jin Jiang (G. Wang *et al.*, 1999). *eif4A* mutant alleles were kindly provided by Galloni and Edgar (1999). *inr* mutants were kindly provided by Manfred Frasch. Clones of

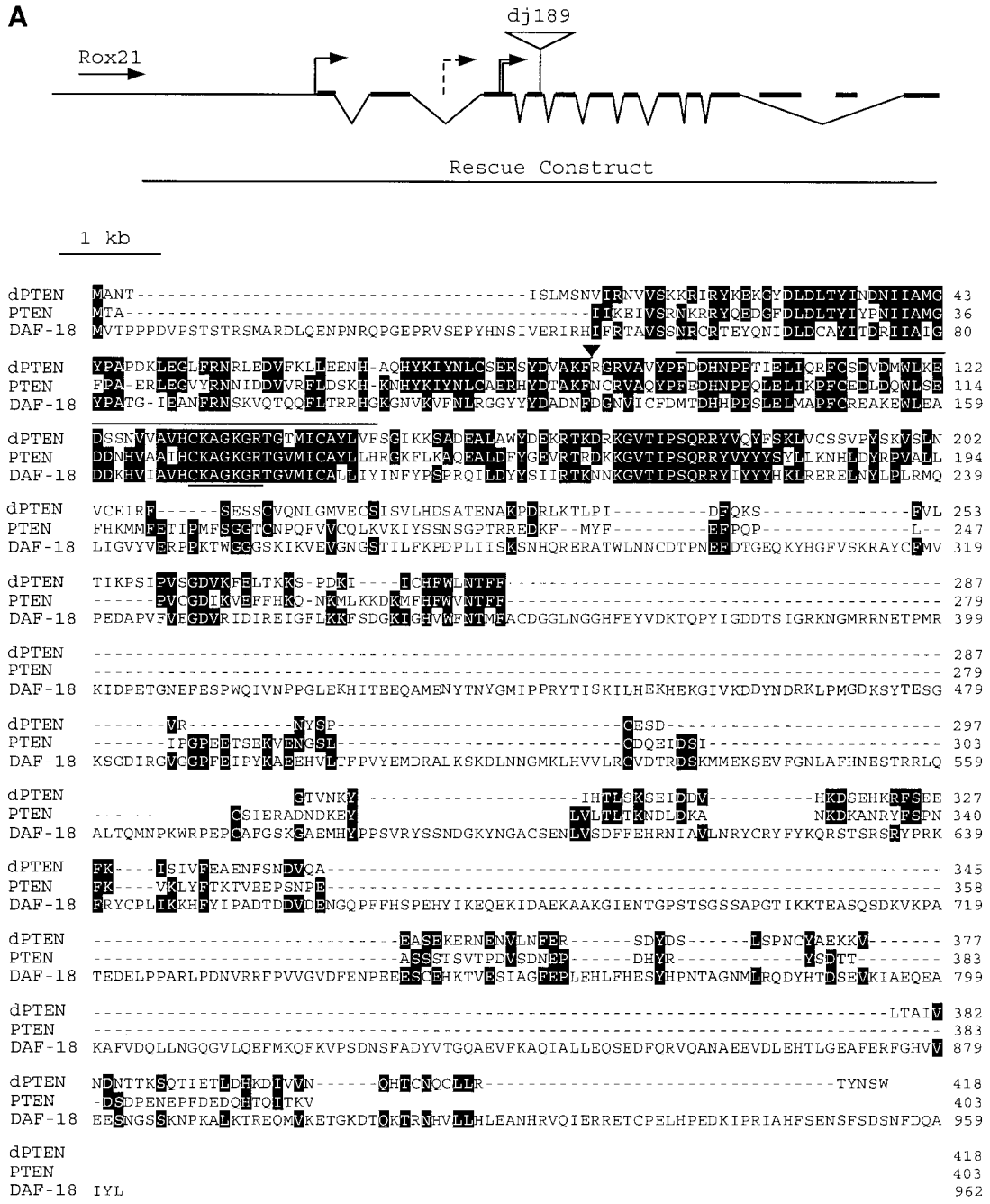


FIG. 1. Structure of the *dPTEN* locus and the predicted dPTEN protein sequence. (A) Genomic structure of the *dPTEN* locus. The *dPTEN* gene is located just distal to the *Rox21* gene on 2L. The exons are indicated with thick lines and the introns are shown as thin lines connecting adjacent exons. The transcription start site is indicated with a single line with an arrow at the end. The translation start site is indicated with double lines with an arrow at the end. An alternative transcriptional initiation site, corresponding to the cDNAs published by Smith *et al.* (1999), is marked by a dashed line with an arrow at the end. Two alternative exons at the 3′ end of the gene are also indicated. The usage of these alternative exons generates two additional cDNAs that encode proteins with different C-termini (Smith *et al.*, 1999). The position of the F-element insertion that resulted in the $dPTEN^{dj189}$ mutation is also indicated. The genomic DNA fragment used in the rescue construct is indicated. (B) Sequence alignment of predicted PTEN proteins from *Drosophila* (dPTEN), human (PTEN), and *C. elegans* (DAF-18). The phosphatase domain is indicated by a line above the sequence, and the phosphatase active site C-(X)5-R motif is indicated by a line below the protein sequence. The arrowhead marks the insertion site of the F-element in *dj189*.

mutant cells were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993). The stocks used for *ey-flp* experiments were kindly provided by Mike Brodsky in Gerry Rubin's lab. Genotypes for generating clones are as follows. (1) *dPTEN* mutant clones: *y w hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, y$^+$w$^+$* for adults; *w hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, armlacZ* for imaginal discs; and *w hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, ovoD2L* for germ-line clones. (2) *ey-Flp dPTEN:* y w ey-flp; *dPTEN^{dj189} FRT40A/FRT40A, M24.* (3) *eif4A* mutant clones: *w hsp-flp; eif4A FRT40A/FRT40A, armlacZ.* (4) *dPTEN eif4A* mutant clones: *w hsp-flp; dPTEN^{dj189} eif4A FRT40A/FRT40A, armlacZ.* (5) *dPTEN* mutant clones overexpressing PI3K subunit Dp110: *y w MS1096 hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, y$^+$w$^+$; UAS-PI3KDp110.* (6) *dPTEN* mutant clones overexpressing PI3KDN: *y w MS1096 hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, y$^+$w$^+$; UAS-PI3KDN.* (7) *dPTEN* mutant clones in *inr* mutant background: *y w hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, y$^+$w$^+$; inr^{35}/inr$^{l(3)05545}$.* (8) *dPTEN* mutant clones in *Dakt1* mutant background: *y w hsp-flp; dPTEN^{dj189} FRT40A/FRT40A, y$^+$w$^+$; Dakt1$^{l(3)04226}$.*

Flow Cytometry

FACS analysis of dissociated imaginal wing disc cells was performed as previously described (Neufeld *et al.,* 1998). *dPTEN* homozygous mutant clones induced by FLP/FRT-mediated recombination were marked by their lack of GFP expression using a ubiquitin–GFP FRT40A chromosome (a gift from Christina Martin-Castellanos). Clones of cells coexpressing *dPTEN* and GFP were generated using Act > CD2 > GAL4, UAS-GFP, and UAS-*dPTEN* transgenic flies. In both cases, clones were induced at 80 (±4) h AED, and larvae were dissected 40 h later. Cell cycle analysis of FACS data files was performed with ModFit V2.0.

TUNEL and Immunostaining

Double labeling of TUNEL and lacZ antibody was performed according the protocols of S. L. Wang *et al.* (1999). Germ-line clone embryos without paternal contribution can be distinguished from those with paternal contribution by the absence of a *Kr-lacZ* marker on the balancer chromosome. Images were taken on a Zeiss confocal microscope. A range of 80 μm in depth was scanned in 20 optical sections and projected into one focal plane using the NIH Image software.

Immunostaining of imaginal discs was done as described by Xu and Rubin (1993). Confocal images were collected using a Zeiss confocal microscope and processed using Adobe PhotoShop.

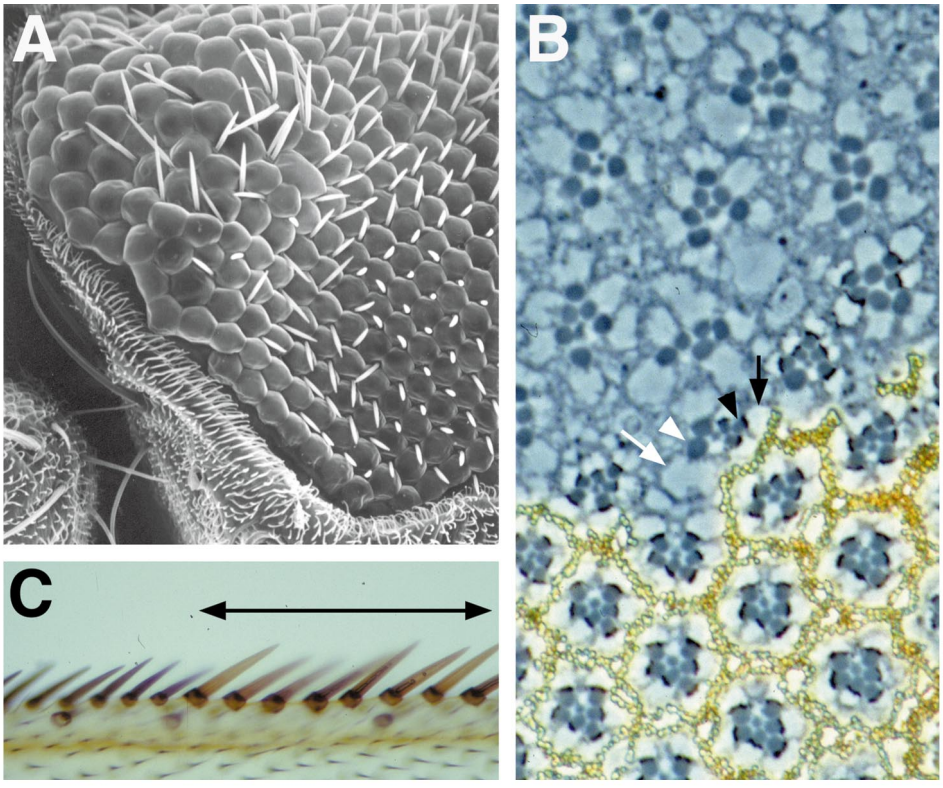
RESULTS

Isolation of a dPTEN Mutant

We used the FRT/FLP recombination system to screen the left arm of the second chromosome for mutations affecting the development of adult cuticular structures such as the eyes and the wings. One embryonic lethal mutation that produced enlarged cells in mutant clones in the eye and the wing was recovered. This mutation, *dj189,* was localized to the 31B1;31D8–11 region by deficiency mapping. Database search identified a *Drosophila* homolog of the mammalian *PTEN* gene (*dPTEN*) in this interval (Berkeley *Drosophila* Genome Project Database). Given the role of the insulin pathway in growth control, we investigated whether our mutation disrupted *dPTEN*. Genomic DNA from the wild-type *dPTEN* locus was used to probe Southern blots made from heterozygous *dj189* mutant and control flies. Such analysis revealed DNA polymorphism in the *dPTEN* genomic region in *dj189* mutant that is most consistent with the insertion of a 4.6-kb DNA species (data not shown). Further molecular analysis revealed that the 4.6-kb species corresponded to a previously described transposable element in *Drosophila* called the F-element (Di Nocera *et al.,* 1983). This F-element was inserted within a coding exon at the 5′ end of the *dPTEN* gene and disrupted the dPTEN open reading frame after amino acid 89 (Fig. 1A).

FIG. 2. Adult mutant phenotype of *dPTEN*. (A) Scanning electron micrograph of an eye carrying a clone of homozygous *dPTEN^{dj189}* cells. The mutant clone is located at the upper left of the picture. Note that *dPTEN* mutant ommatidia are larger and often bulge out of the eye surface. (B) Section through a *dPTEN^{dj189}* clone in the adult eye. The mutant clone is marked by the absence of pigment. Mutant photoreceptor cells are increased in area by about 140% compared with heterozygous photoreceptor cells (measured by NIH Image software). At the clone border, mosaic ommatidia containing normal-sized heterozygous cells (marked by the pigment granules at the base of the rhabdomere) and enlarged homozygous *dPTEN* mutant cells (marked by the absence of pigment granules at the base of the rhabdomere) can be seen, indicating that *dPTEN* controls cell size autonomously. The white arrow and arrowhead indicate the cell body and rhabdomere of a mutant photoreceptor cell, while the dark arrow and arrowhead indicate the cell body and rhabdomere of a wild-type photoreceptor cell. Note that both cell body and rhabdomere are enlarged in *dPTEN* mutant photoreceptor cells. (C) Wing margin carrying a *dPTEN^{dj189}* mutant clone. Note that the *dPTEN* mutant bristles (marked by *y*$^-$, indicated by a line above the wing margin) are thicker and longer than the wild-type bristles, which are marked with *y*$^+$ and have a dark color.

FIG. 3. *dPTEN* controls cell proliferation and cell size in the eye and the wing. (A) A light microscope image of an eye containing a homozygous *dPTEN^{dj189}* mutant clone induced at 24–36 h AED. The mutant clone is marked by the absence of pigment (arrow), and the twin spot is marked by the higher level of pigment (arrowhead). Quantitation of the number of ommatidia revealed that *dPTEN* mutant clones induced at 24–36 h AED contained on average 3.2 times as many cells compared to the twin spots (n = 10). (B–D) Confocal images of a portion of a third-instar eye disc. A homozygous mutant (*dPTEN*$^-$/*dPTEN*$^-$) clone (arrow) is marked by the absence of arm-lacZ staining (green). The adjacent area of darker green staining represents *dPTEN*$^+$/*dPTEN*$^+$ twin spot (arrowhead). The disc was also stained for the neuronal-specific nuclear Elav protein (red). Three images are shown, one of arm-lacZ staining (B), one of Elav staining (C), and one of superimposed lacZ and Elav staining (D). Note the increased size of the mutant cell nuclei and the dramatically increased area of the mutant clone compared to the twin spot. The increased spacing



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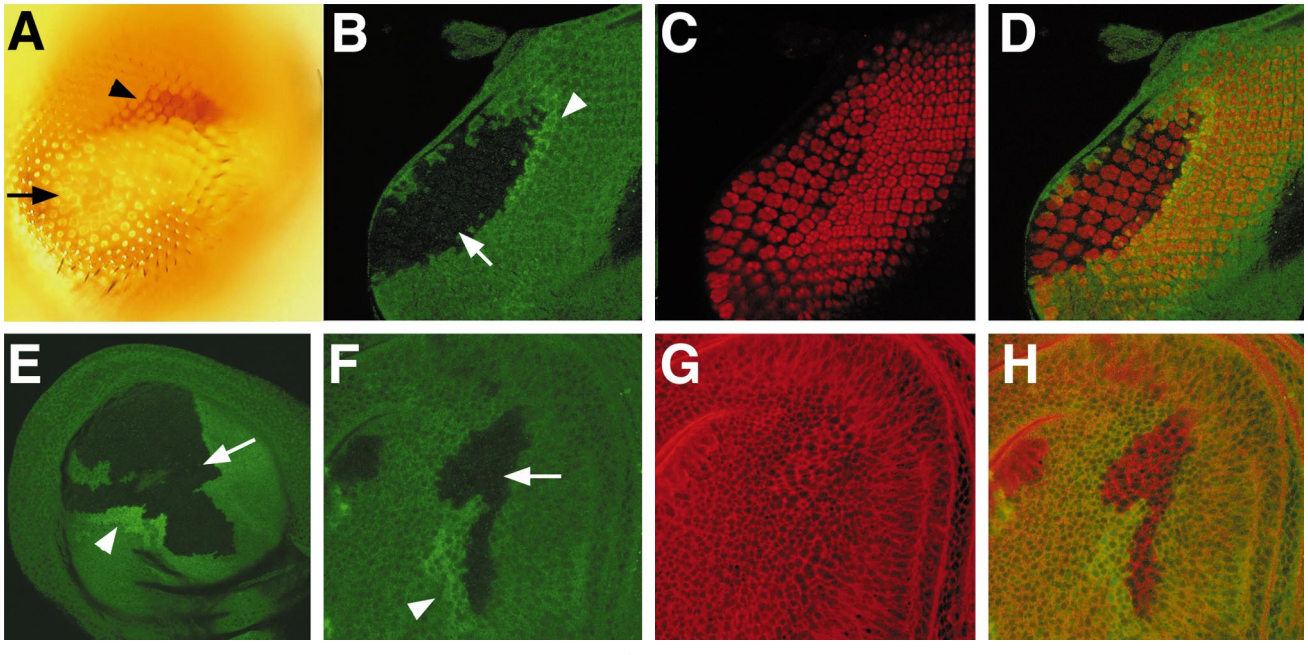

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between the mutant photoreceptor clusters suggests that the size of the intervening nonneuronal cells was also increased in the mutant clone. (E) Confocal image of a third-instar wing disc containing a large *dPTEN* mutant clone. The mutant clone and twin spot are marked as in B–D. Note that the mutant clone occupied a much larger area than the twin spot. (F–H) Confocal images of a portion of a third-instar wing disc containing a *dPTEN* mutant clone induced 12 h later than those in A–E. The wing disc is shown at higher magnification than in E. The mutant clone and twin spot are marked as in B–D. The disc was also stained for phalloidin (red), which highlights the outlines of the cells. Three images are shown, one of arm-lacZ staining (F), one of phalloidin staining (G), and one of superimposed lacZ and phalloidin staining (H). Note the increased size of mutant cells. Note that the increase in cell number in the mutant clone is less dramatic compared to the mutant clone in E. Clones in A–E were induced at 24–36 h AED. Clones in F–H were induced at 36–48 h AED.

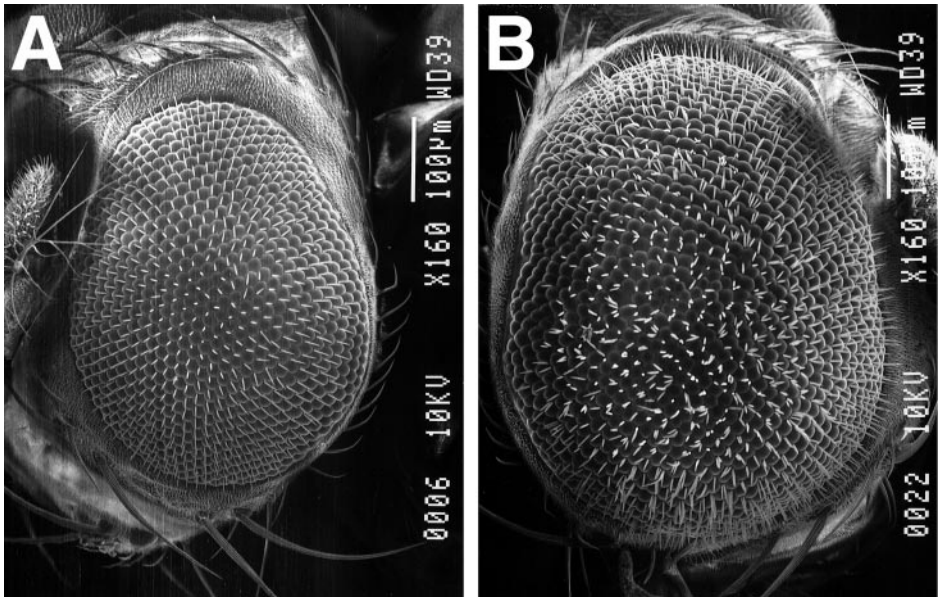


FIG. 4. *dPTEN* controls organ size. Scanning electron micrograph of (A) control eyes (*y w ey-Flp; FRT40A/M24 FRT40A*) and (B) eyes in which *dPTEN* function was selectively removed using the *ey-Flp* technique (*y w ey-Flp; dPTEN*^{dj189} *FRT40A/M24 FRT40A*). These images were taken under the same magnification (160×, see the side bars on these images).

Since the predicted phosphatase domain is located between aa 98 and aa 149, this insertion is predicted to eliminate the phosphatase activity. In addition, this insertion should truncate most of the tensin homology domain, which is located between aa 22 and aa 192. We therefore consider *dj189* a likely null allele of *dPTEN*. A construct containing just the *dPTEN* genomic DNA was able to fully rescue *dj189* homozygotes to viable and phenotypically wild-type animals, confirming that the mutation indeed corresponds to *dPTEN*. We will therefore refer to *dj189* as *dPTEN*^{dj189} throughout the rest of the paper.

A search of the *Drosophila* EST database identified a full-length *dPTEN* cDNA clone, LP04865. The sequence of this cDNA predicts a protein of 418 amino acids with 35%

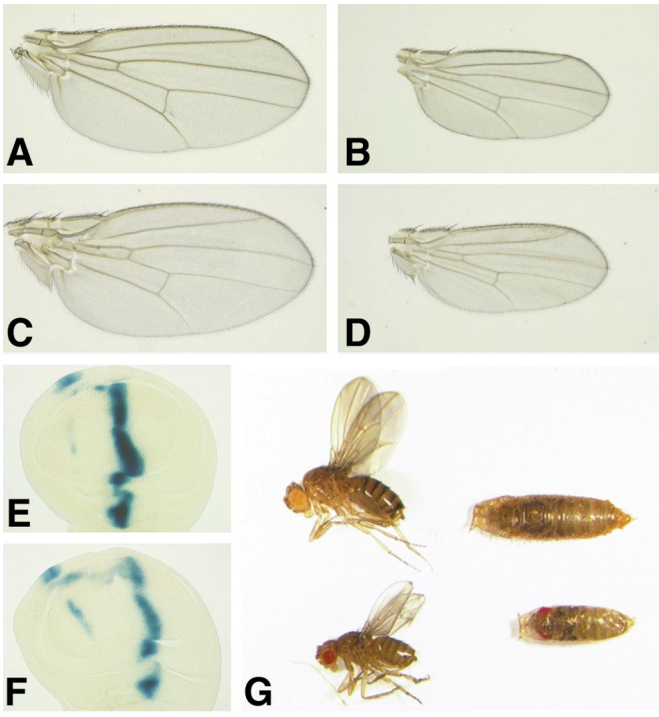


FIG. 5. Overexpression of *dPTEN* reduces organ size, compartment size, and organism size. (A–D) Wings from the wild-type flies (A) and flies expressing *MS1096/UAS-dPTEN* (B), *MS1096/UAS-P35* (C), and *MS1096/UAS-dPTEN; UAS-P35* (D). These images were taken under the same magnification. The relative wing sizes in A–D are wildtype (100%), *MS1096/UAS-dPTEN* (53%), *MS1096/UAS-P35* (103%), and *MS1096/UAS-dPTEN; UAS-P35* (53%). At least six wings of each genotype were measured using the NIH Image software. (E and F) A wild-type wing disc and a wing disc expressing *en-GAL4/UAS-dPTEN*, respectively. The A/P compartment border is indicated by the expression of *dpp-lacZ* (blue). Anterior is to the left and posterior to the right. The ratio of posterior to anterior compartment size is 0.76 for the wildtype and 0.44 for the *en-GAL4/UAS-dPTEN* flies ($n = 5$). (G) Wild-type fly and pupa (top row) and a fly and pupa expressing *arm-GAL4/UAS-dPTEN* (bottom row). Note the reduction in body size in flies overexpressing *dPTEN*.

identity with human PTEN (Fig. 1B). Homology between dPTEN and human PTEN is highest within the phosphatase domain (69% identical, 36/52 aa), especially at positions surrounding the $Cys(X)_5Arg$ active site. Comparison between dPTEN, human PTEN, and *C. elegans* PTEN (DAF-18) reveals that while the N-terminal region is highly conserved among these species, the C-terminal halves are much more divergent (Fig. 1B).

dPTEN Autonomously Controls Cell Size

Scanning electron micrographs revealed that the unit eyes (ommatidia) in $dPTEN^{dj189}$ mutant clones were larger than the surrounding wild-type ommatidia (Fig. 2A). Eye sections showed that the mutant photoreceptor cells, including their cell bodies and rhabdomeres, were enlarged by approximately 140% in area. However, the organization of ommatidia and the differentiation of various cell types were nearly normal, with occasional loss of photoreceptor cells (Fig. 2B). Careful examination of mosaic ommatidia consisting of genetically marked *dPTEN* mutant and non-mutant cells revealed that the effect of *dPTEN* mutation on cell size was strictly cell autonomous (Fig. 2B). A similar autonomous effect on cell size was also observed in the wing (Fig. 2C). Therefore, *dPTEN* autonomously controls cell size.

dPTEN Controls Cell Proliferation

In generating *dPTEN* mutant clones in the eye, we often recovered mutant (−/−) clones that were severalfold larger than their +/+ twin spots (Fig. 3A). In fact, we could generate mosaic eyes in which over 95% of the ommatidia were mutant for *dPTEN* when clones were induced early in the first-instar stage (data not shown). This behavior is very similar to the proliferative advantage of $Minute^+$ cells in a *Minute* background. Such an increase in clone size cannot be explained by a mere 140% increase in cell size. Therefore, we investigated whether *dPTEN* mutation also confers proliferative advantage in imaginal discs. Mutant clones were induced during first-instar larval stage and examined in the third-instar eye (Figs. 3B–D) and wing (Fig. 3E) imaginal discs. As seen in mosaic adults, *dPTEN* mutant clones were severalfold larger than their twin spots (Figs. 3B and 3E) and contained larger cells (Fig. 3C). In addition, *dPTEN* mutant clones contained more cells than their twin spots (the mutant clone in Figs. 3B–3D had three times as many cells as the twin spot; see also Fig. 3E). Since the mutant clones and twin spots originate from mitotic sister cells born at the same developmental time point, these results indicate that *dPTEN* controls cell number in addition to cell size. We noticed that the difference in cell number between the mutant clone and the twin spot was less dramatic when clones were induced later in development (Figs. 3F–3H; 1.5 times as many cells in the mutant clone compared to the twin spot). This may reflect the perdurance of the *dPTEN* gene product or, alternatively, that the effect on cell number requires the accumulation of many generations of cell divisions. We prefer the latter possibility, since despite a less dramatic increase in cell number, clones induced later in development (Figs. 3F–3H) showed a increase in cell size similar to that of early induced clones (Figs. 3B–3D). In addition, changes in cell size and cell number were seen in undifferentiated cells in the wing imaginal disc (Figs. 3E–3H) and undifferentiated cells anterior to the morphogenetic furrow in the eye imaginal disc (data not shown), suggesting that *dPTEN* controls cell number and cell size independent of cell differentiation.

dPTEN Autonomously Controls Organ Size

Given our observation that *dPTEN* controls cell number and cell size in imaginal discs, we tested whether it regulates organ size. Since $dPTEN^{dj189}$ is embryonic lethal (data not shown), we selectively removed *dPTEN* function in the eye imaginal disc using *eyeless*-FLP, which selectively induces mitotic clones in the eye progenitor cells. Using this technique, we were able to generate at high frequency eyes in which over 95% of the cells were mutant for *dPTEN*. Using scanning electron microscopy, we measured the area as well as the number of ommatidia in $dPTEN^-$ eyes compared to controls (Fig. 4). While the control flies had approximately 750 ommatidia per eye, $dPTEN^-$ eyes had about 1075 ommatidia per eye ($n = 9$), corresponding to an increase of 43% in cell numbers. This result is consistent with the increased cell proliferation observed in imaginal discs (Fig. 3). We estimated the size of the eye by measuring the area occupied by the eye in scanning electron micrographs. Such analysis revealed that the $dPTEN^-$ eyes were increased by 55% in area ($n = 9$). This is likely to be an underestimation, since $dPTEN^-$ eyes were more bulged than the wild-type eyes (Fig. 4). Since the increase in eye size could not be accounted for by the increase in cell number alone, it follows that the rest of the increase in eye size was due to an increase in the average size of each cell. We conclude that *dPTEN* autonomously controls organ size, such that $dPTEN^-$ eyes are larger in overall size and consist of increased number of cells with increased cell size.

Overexpression of dPTEN Reduces Organ Size, Compartment Size, and Organism Size

We used the *GAL4-UAS* system to assess the effect of overexpressing *dPTEN* on the control of overall growth. *dPTEN* was expressed from a *UAS-dPTEN* transgene under the control of the *GAL4* driver line *MS1096,* which expresses high levels of GAL4 near-uniformly in the wing pouch region (Capdevila and Guerrero, 1994). *MS1096; UAS-dPTEN* flies showed dramatically reduced wing size (compare Figs. 5A and 5B). Close examination revealed that 18% of the reduction of wing size was due to a decrease in cell number, and the rest was due to a decrease in cell size. To test whether the decreased wing size was due to induction of cell death, we coexpressed with *dPTEN* the viral cell

death inhibitor *P35,* which has been shown to potently block cell death in *Drosophila* (Hay *et al.,* 1994). We found that *P35* overexpression did not suppress the decreased wing size resulting from *dPTEN* overexpression (compare Figs. 5B and 5D).

The *Drosophila* wing is divided into anterior/posterior and dorsal/ventral compartments. Previous studies have shown that activation or inhibition of cell division in one compartment increases or decrease cell number without affecting the size of that compartment (Neufeld *et al.,* 1998; Weigmann *et al.,* 1997). Rather, the increase or decrease in cell number is compensated for by decrease or increase in cell size such that the size of the compartment is unchanged. These studies suggest that compartment size is controlled by an intrinsic mechanism that is dominant over cell cycle control. Since *dPTEN* is required for the control of organ size, we investigated whether it might also be required for the control of compartment size. *dPTEN* was overexpressed from a *UAS-dPTEN* transgene under the control of the *en-GAL4* driver line, which specifically expresses GAL4 in the posterior compartment. The size of the posterior compartment was significantly reduced by *dPTEN* overexpression in *en-GAL4; UAS-dPTEN* flies (compare Figs. 5E and 5F). Similarly, overexpression of *dPTEN* in the dorsal compartment driven by *apterous-GAL4* resulted in reduced size of the dorsal compartment, generating wings that were bent toward the dorsal side (data not shown). Thus, manipulation of *dPTEN* activity can override the intrinsic control mechanism that regulates both compartment size and organ size.

To examine the effect of perturbing normal *dPTEN* function at the organism level, we overexpressed *dPTEN* throughout development using a ubiquitously expressed *armadillo* (*arm*)-*GAL4* driver. Most of the *arm-GAL4; UAS-dPTEN* flies died as larvae and pupae. The few surviving flies eclosed after a 6-day delay and showed reduction in body size (Fig. 5G). Thus, ubiquitous overexpression of *dPTEN* results in severe growth retardation of the whole organism.

Cell Cycle Phasing Is Altered in dPTEN Mutant Cells

The observation that clones of *dPTEN* mutant cells had a proliferative advantage over their wild-type twin spots (Fig. 3) indicated that *dPTEN* controls progression through the cell division cycle. To determine which phase(s) of the cell cycle *dPTEN* regulates, we used FACS analysis to examine the DNA content of dissociated wing imaginal disc cells containing clones of *dPTEN* mutant cells (Neufeld *et al.,* 1998). Loss of *dPTEN* resulted in a decrease in the percentage of cells in the G1 phase of the cell cycle and a relative increase in the S and G2 population (Fig. 6). Clonal overexpression of *dPTEN* had a complementary effect, causing a slight decrease in the number of S-phase cells (Fig. 6). FACS analysis also revealed complementary changes in cell size in response to *dPTEN* levels: loss of *dPTEN* caused an increase in average cell size, while *dPTEN* overexpression decreased it (Fig. 6). Similar effects were observed throughout all phases of the cell cycle and at multiple developmental stages (data not shown).

The cell size and cell cycle FACS profile of *dPTEN* mutant cells is remarkably similar to that of cells overexpressing *Drosophila* PI3K (Weinkove *et al.,* 1999). In both cases, the percentage of cells in G1 is reduced, indicating an acceleration of this phase of the cell cycle. Since overexpression of PI3K does not increase proliferation rates, this shortened G1 appears to be balanced by a commensurate lengthening in the duration of S and/or G2 phases. A similar phenomenon has been described for cells whose G1 phase is accelerated by overexpression of cyclin E, dMyc, or activated Ras (Neufeld *et al.,* 1998; Johnston *et al.,* 1999; Prober and Edgar, 2000). In contrast, the rapid proliferation rate of *dPTEN* mutant cells indicates that S and G2 phases do not lengthen in response to the abbreviated G1 and thus suggest that PTEN regulates multiple phases of the cell cycle.

dPTEN Antagonizes Signaling through PI3K, Inr, and Akt

We examined genetic interactions between *dPTEN* and *PI3K,* a component of the insulin signaling pathway in *Drosophila* (Weinkove *et al.,* 1999). It has been shown previously that overexpression of the PI3K catalytic subunit, Dp110, results in increased wing size (Weinkove *et al.,* 1999; Fig. 7B), while overexpression of a dominant negative Dp110 construct (PI3KDN) results in the opposite phenotype (Weinkove *et al.,* 1999; Fig. 7C). These phenotypes are due to changes in cell size (Weinkove *et al.,* 1999; see also Figs. 7I and 7J). We found that coexpression of *dPTEN* with PI3KDN further reduced wing size (Fig. 7F). In addition, overexpression of *dPTEN* suppressed the increased wing size resulting from PI3K overexpression (compare Figs. 7E and 7B). To further examine the genetic interaction between *dPTEN* and *PI3K,* we examined *dPTEN* mutant cells in a genetic background of overexpressing *PI3K* or *PI3KDN*. We found that when *PI3K* was overexpressed, *dPTEN* mutant cells are indistinguishable in cell size from their nonmutant siblings (Fig. 7I), suggesting that overexpression of *PI3K* results in increased PIP_3 levels and increased signaling that cannot be further activated by removing *dPTEN*. Conversely, overexpression of *PI3KDN* partially suppressed the increased cell size of *dPTEN* mutant cells (Fig. 7J). Thus, *PTEN* and *PI3K* antagonize each other in regulating cell size.

Certain combinations of loss-of-function alleles of the *Drosophila* insulin receptor (*inr*), such as $inr^{35}/inr^{l(3)05545}$, result in flies with a decreased cell size (Fernandez *et al.,* 1995; Böhni *et al.,* 1999). This provided us with an opportunity to examine the genetic epistasis between *dPTEN* and *inr*. We found that the increased cell size of *dPTEN* mutant cells could not be reversed in $inr^{35}/inr^{l(3)05545}$ animals (Fig. 7K), and thus loss of *dPTEN* function is epistatic to mutations in *inr*.

Previous studies have shown that loss of *PTEN* function promotes cell survival in mammals through activation of *Akt* (Stambolic *et al.*, 1998; Myers *et al.*, 1998; Li *et al.*, 1998). In addition, *PTEN* acts through *Akt* in metabolic and longevity control in *C. elegans* (Ogg and Ruvkun, 1998). *Dakt1*, a *Drosophila* homolog of *Akt*, has been suggested to play a role in cell survival in embryogenesis (Staveley *et al.*, 1998) and, more recently, cell size control (Verdu *et al.*, 1999). Recently, a hypomorphic allele of *Dakt1* was identified in the large-scale gene disruption project carried out by the Berkeley *Drosophila* Genome Project (Spradling *et al.*, 1999). This allele, $Dakt1^{l(3)04226}$, is semilethal, and homozygous survivors show reduced body size and cell size, consistent with a role of *Dakt1* in growth control (data not shown). To examine whether *PTEN* controls cell size through regulating *Akt* activity, we generated *dPTEN* mutant clones in $Dakt1^{l(3)04226}$ animals. We found that $Dakt1^{l(3)04226}$ completely suppressed the increase of cell size associated with the *dPTEN* mutation (Fig. 7L). This result provides strong *in vivo* evidence that *Dakt1* functions downstream of (or in parallel to) *dPTEN* in the control of cell size. Taken together, these genetic interactions suggest that the role of dPTEN in opposing signaling through the PI3K/Akt pathway is conserved between flies and vertebrates.

The Proliferative Advantage of dPTEN Mutant Cells Requires Translation Initiation Factor eif4A

eif4A is an ATP-dependent RNA helicase that is an essential component of the eif4F translation initiation complex. Previous studies have identified an allelic series of *eif4A* mutants that affects larval growth, DNA replication, and cell proliferation (Galloni and Edgar, 1999). Since the defect in DNA synthesis in *eif4A* mutants can be bypassed by overexpressing the E2F transcription factor, it was suggested that *eif4A* preferentially regulates a specific set of cell-cycle regulatory genes (Galloni and Edgar, 1999). To examine whether the proliferative advantage of *dPTEN* mutant cells requires the same set of cell-cycle regulatory genes as those controlled by *eif4A*, we examined *dPTEN eif4A* double-mutant clones. We used a hypomorphic allele, $eif4A^{1069}$, and a stronger allele, $eif4A^{1006}$. The weaker allele, $eif4A^{1069}$, confers proliferation disadvantage to the cells, resulting in mutant clones that are smaller than the twin spots without affecting cell size (Galloni and Edgar, 1999; Figs. 8A and 8B). We found that $eif4A^{1069}$ can partially suppress the overproliferation of *dPTEN* mutant cells (Fig. 8C), but the increased cell size of *dPTEN* mutant cells was not suppressed (Fig. 8D). The stronger $eif4A^{1006}$ allele completely suppressed the proliferation of *dPTEN* mutant cells. *dPTEN* $eif4A^{1006}$ double-mutant clones were undetectable (Fig. 8F), as has been observed in the $eif4A^{1006}$ single mutant (Galloni and Edgar, 1999; Fig. 8E). We suggest that modulation of translation initiation is an important aspect of *dPTEN* function in regulating cell proliferation.

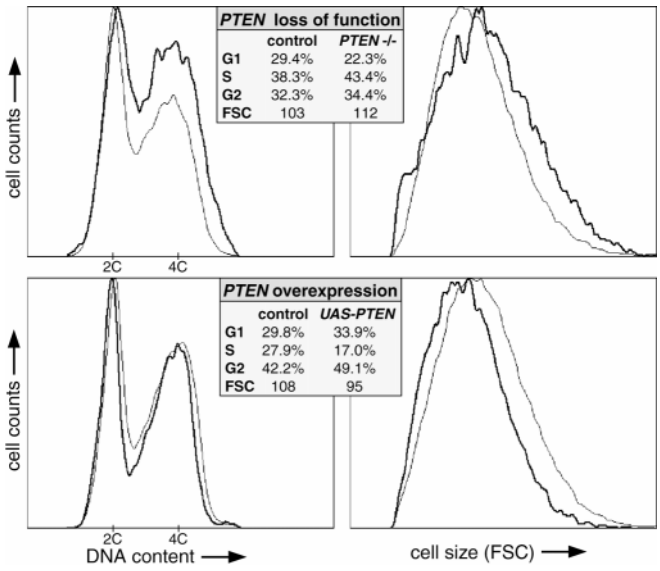
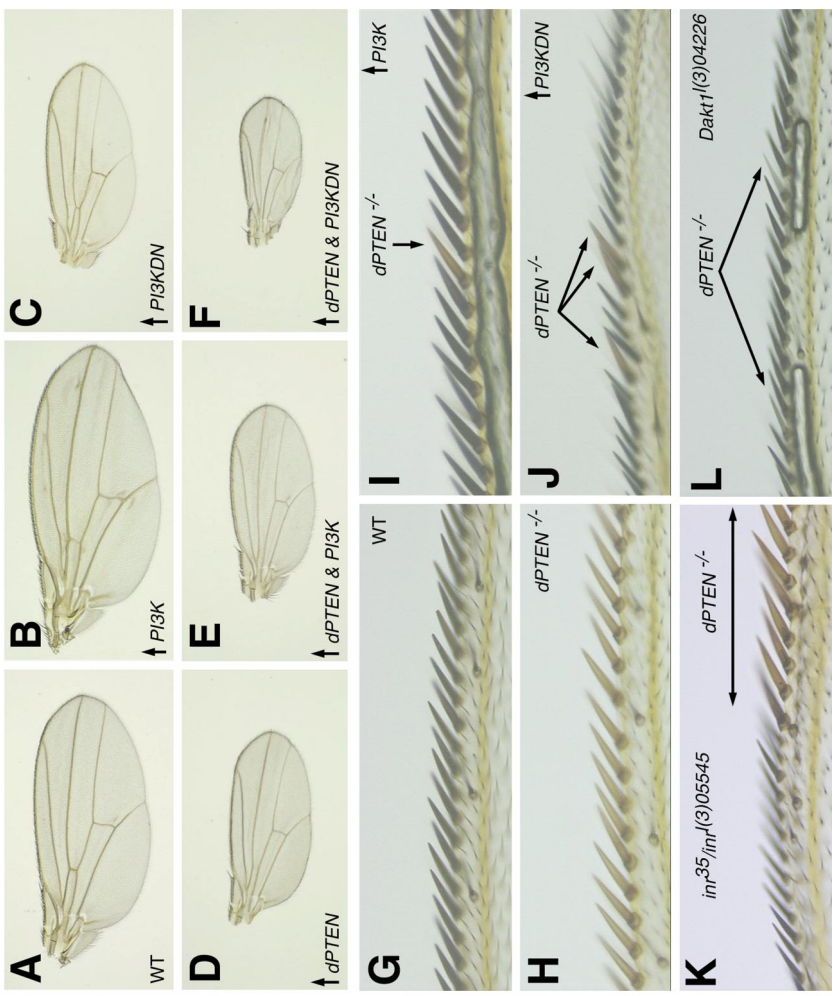


FIG. 6. Cell cycle phase and cell size alterations in PTEN mutant cells. Flow cytometric analysis of dissociated wing imaginal discs containing clones of *PTEN* loss-of-function (top) and overexpressing (bottom) cells. Left: Histograms showing DNA content of *PTEN* mutant cells (heavy traces) and control cells (light traces). Right: Histograms of forward light scatter value (FSC), a measure of cell size. Insets show the percentage of cells in each cell cycle phase and median forward scatter values for control and *PTEN* mutant cells. Genotypes: top, *hs-FLP; Ubi-GFP FRT40A/dPTEN*^{dj189} *FRT40A;* bottom, *hs-FLP; UAS-PTEN/Act > CD2 > GAL4 UAS-GFP.* For each genotype, 10,000 or more cells were analyzed.

dPTEN Does Not Promote Cell Death in Normal Development

Studies of the *PTEN* tumor suppressor gene in mammals have suggested a role of *PTEN* in promoting cell death (Stambolic *et al.*, 1998; Myers *et al.*, 1998; Li *et al.*, 1998). If this function is conserved, we might expect the *dPTEN* mutation to reduce or eliminate cell death in *Drosophila.* In theory, this may contribute to the increased number of cells in *dPTEN* mutant clones compared to twin spots. We think this is unlikely for the following reasons. First, in the FACS analysis of *dPTEN* mutant cells in imaginal discs, we did not detect any significant difference in the apoptotic sub-G1 fraction of *dPTEN* mutant cells compared with heterozygous cells (Fig. 6). Second, eye imaginal discs composed almost exclusively of *dPTEN* mutant cells (generated by eyeless-FLP) did not show a significant difference in acridine orange staining (data not shown). Third, the decreased wing size resulting from *dPTEN* overexpression was not suppressed by *P35* expression (Fig. 5).

To further examine a possible role of *dPTEN* in cell survival in *Drosophila,* we generated *dPTEN* germ-line clone mutant embryos that lack both paternal and maternal gene product. We assayed cell death in such embryos using terminal deoxynucleotidyl transferase-mediated dUTP nick



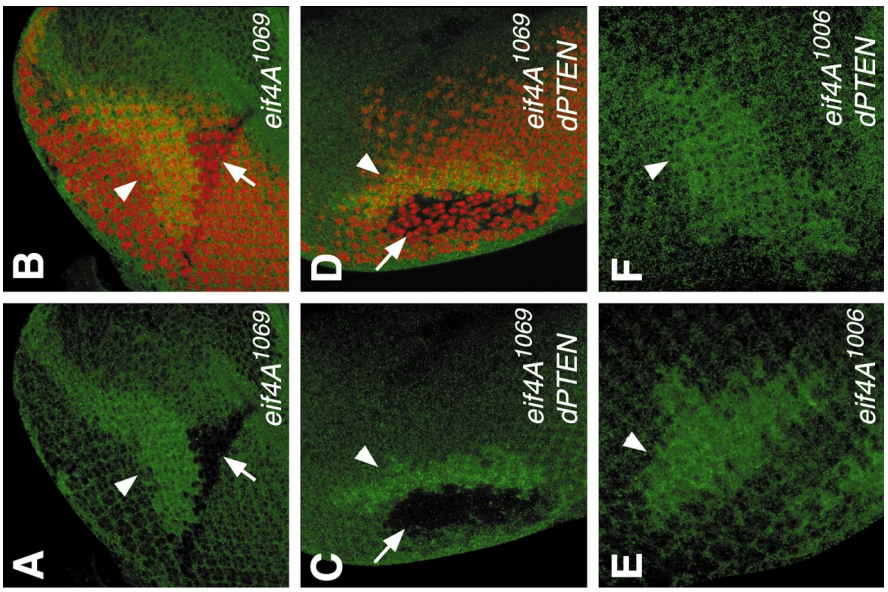


FIG. 7. Genetic interactions of *dPTEN* with *PI3K*, *inr*, and *Dakt1*. (A) A wild-type wing. (B–F) Wings expressing *MS1096/UAS-PI3KDp110*, *MS1096/UAS-PI3KDN*, *MS1096/UAS-dPTEN*, *MS1096/UAS-dPTEN; UAS-PI3KDp110*, and *MS1096/UAS-dPTEN; UAS-PI3KDN*, respectively. These images were taken under the same magnification. The relative wing sizes in A–F are wild type (100%), *MS1096/UAS-PI3KDp110* (115%), *MS1096/UAS-PI3KDN* (59%), *MS1096/UAS-dPTEN* (53%), *MS1096/UAS-dPTEN; UAS-PI3KDp110* (54%), and *MS1096/UAS-dPTEN; UAS-PI3KDN* (29%). At least six wings of each genotype were measured using the NIH Image software. (G–L) Wing margin of various genotypes. All images were taken under the same magnification. (G) Wildtype. (H) A large *dPTEN* mutant clone on the wing margin. The increase in cell size is reflected by the increase in the absolute size of the bristles and a reduction in the number of wing margin bristles within a given length (16 *dPTEN* mutant bristles instead of 21 in wildtype wing margin of the same length (see G)). (I) An *MS1096/UAS-PI3KDp110* wing containing a *dPTEN* mutant bristle. The *dPTEN* mutant bristle is indistinguishable in size from its nonmutant siblings. (J) An *MS1096/UAS-PI3KDN* wing containing three *dPTEN* mutant bristles. The *dPTEN* mutant bristles are larger than surrounding *dPTEN*$^{+}$ bristles in this genetic background. (K) An $inr^{35}/inr^{l(3)05545}$ wing containing *dPTEN* mutant bristles . Note that the homozygous *dPTEN* mutant bristles are much larger than surrounding *dPTEN*$^{+}$ cells in this genetic background. (L) A $Dakt1^{l(3)04226}$ wing containing two *dPTEN* mutant bristles. Note that the homozygous *dPTEN* mutant bristles are indistinguishable in size from their nonmutant siblings.

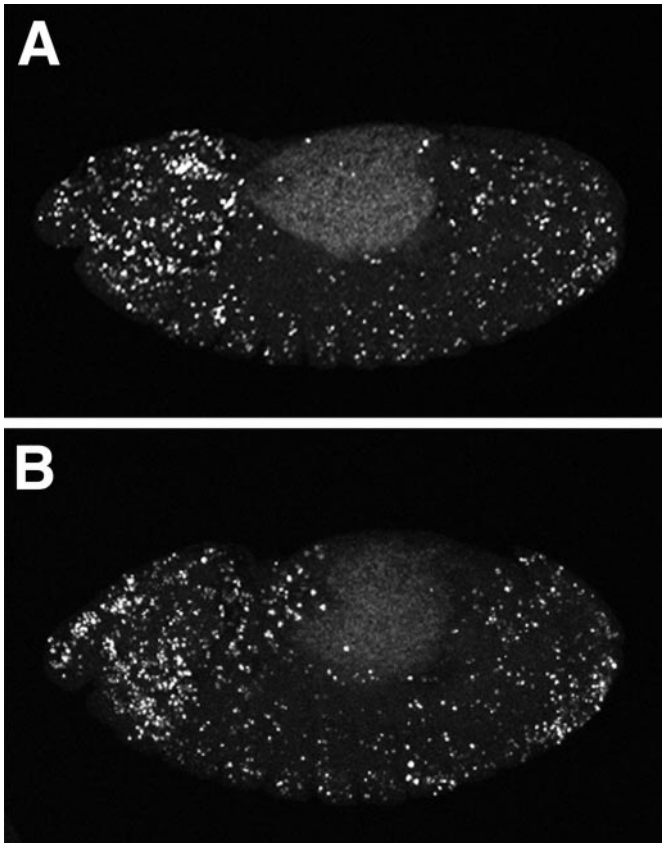


FIG. 9. *dPTEN* mutant embryos do not show reduced cell death. Confocal images of a wild-type embryo (A) and a germ-line clone mutant embryo lacking both paternal and maternal *dPTEN* gene product (B), labeled for TUNEL staining. No significant difference was detected between the mutant and the wild-type embryos.

end labeling (TUNEL). As seen in Fig. 9, no significant differences were observed between *dPTEN* mutant embryos and wild-type control embryos. We conclude that *dPTEN* does not have a major role in cell death in the developmental contexts we have examined.

DISCUSSION

The mechanisms of how body and organ size are regulated are largely unknown (reviewed in Conlon and Raff, 1999). Recent genetic studies in *Drosophila* suggest that the insulin pathway may coordinately control both cell growth and cell proliferation and in turn regulate organ size. Here, we have provided evidence that *dPTEN*, a *Drosophila* homolog of the mammalian *PTEN* tumor suppressor gene, plays an essential role in the control of cell size, cell number, and organ size. In mosaic animals, $dPTEN^{-}$ cells proliferate faster than their heterozygous siblings, show an autonomous increase in cell size, and form organs of increased size, whereas overexpression of *dPTEN* results in opposite phenotypes. Since tumor growth occurs by increase in both mass and cell number, our observation that PTEN regulates both of these functions provides a potential explanation for its role as a tumor suppressor. During the preparation and review of this article, Huang *et al.* (1999) and Goberdhan *et al.* (1999) independently reported studies of *dPTEN* function in *Drosophila*. We will discuss the distinctions between our studies and these two recent papers.

PTEN and Cell Size Control

Our studies of *dPTEN* function in *Drosophila* imaginal disc development have revealed a novel function of this gene in the control of cell size. An increase in cell size has not been observed in human tumors carrying *PTEN* mutations. This may simply reflect a technical difficulty in assessing cell size in a heterogeneous population of cells or, alternatively, a fundamental difference in the mechanisms controlling cell size between *Drosophila* and human. Given the conservation of many signaling pathways between *Drosophila* and human, the latter possibility is less likely. Our results suggest that PTEN regulates cell size by antagonizing PI3K. Loss of function *dPTEN* mutation resulted in a cell size phenotype opposite to that of loss-of-function PI3K mutations, while overexpression of *dPTEN* suppressed the phenotype of overexpressing PI3K. Huang *et al.* (1999) and Goberdhan *et al.* (1999) have reached similar conclusions.

FIG. 8. *eif4A* is epistatic to *dPTEN* in the control of cell proliferation. (A and B) Confocal images of a portion of a third-instar eye disc containing an $eif4A^{1069}$ mutant clone. Homozygous mutant ($eif4A^{1069-}/eif4A^{1069-}$) clone (arrow) was marked by the absence of arm-lacZ staining (green). The adjacent area of darker green staining represented $eif4A^{1069+}/eif4A^{1069+}$ twin spot (arrowhead). The disc was also stained for the neuronal-specific Elav protein (red). Two images are shown, one of arm-lacZ staining (A) and the other of superimposed lacZ and Elav staining (B). Note that $eif4A^{1069}$ mutation does not affect cell size, but confers proliferative disadvantage, resulting in mutant clones that are smaller than their twin spots. (C and D) Confocal images of a portion of a third-instar eye disc containing an $eif4A^{1069}$ *dPTEN* double-mutant clone. The mutant clone and twin spot are marked as in A and B. The disc was also stained for the neuronal-specific Elav protein (red). Two images are shown, one of arm-lacZ staining (C) and the other of superimposed lacZ and Elav staining (D). Note that the $eif4A^{1069}$ *dPTEN* double-mutant photoreceptor cells and clusters are larger than their nonmutant siblings. The double-mutant clone is similar in size to the twin spot, a phenotype that is intermediate between each single mutant (compare C to A and Fig. 3B). (E and F) Confocal images of a portion of a third-instar eye imaginal disc in which homozygous $eif4A^{1006}$ (E) and $eif4A^{1006}$ *dPTEN* double-mutant (F) clones were induced at the first-instar stage. In both cases, homozygous mutant (−/−) cells, marked by the absence of arm-lacZ staining (green), were undetectable due to the their severe growth disadvantage, while +/+ twin spot, marked by the darker green staining (arrowhead), can be observed.

We further showed that the increased cell size of *dPTEN* mutant cells was suppressed by mutations in *Drosophila Dakt1*, suggesting that *dPTEN* controls cell size by regulating *Akt* activity. A further downstream target of *Akt* in cell size control is likely to be the 40S ribosomal protein S6 kinase, which has been shown to regulate cell size without impinging on cell number (Montagne *et al.*, 1999).

dPTEN Controls both Cell Growth and Cell Proliferation

Previous studies have found that when cell division rates in imaginal discs were altered by manipulating expression of cell cycle regulators, cell growth rates were unaffected (Neufeld *et al.*, 1998; Weigmann *et al.*, 1997). The resulting disparity between rates of growth and cell division led to changes in cell size. Overexpression of the transcription factor dE2F caused cells to divide faster than they grew, resulting in diminished cell size, whereas overexpression of the retinoblastoma homolog RBF decreased cell division rates and caused cell enlargement (Neufeld *et al.*, 1998). In this report, we show that loss of *PTEN* function caused an increase both in the rate of cell division and in cell size, whereas overexpression of *PTEN* inhibited proliferation and caused a reduction in cell size. These data are inconsistent with *PTEN* acting simply as a cell cycle regulator and instead suggest that *PTEN* coordinately regulates cell growth and cell cycle progression. Huang *et al.* reported a point mutation in *dPTEN*, $dPTEN^{c494}$, which affects cell size but not cell proliferation (Huang *et al.*, 1999). The difference in phenotypes between *dj189* and *c494* is likely due to the fact that *dj189* is a null allele, while the point mutation in *c494*, G135E, may not inactivate all the biochemical function of dPTEN (see below).

Vertebrate PTEN has been shown to possess a phosphoinositide 3-phosphatase activity and thus to oppose the activity of PI3K (Maehama and Dixon, 1998). Imaginal disc cells lacking *Drosophila dPTEN* have a cell enlargement phenotype strikingly similar to that of cells overexpressing the *Drosophila* PI3K Dp110 subunit (Fig. 6; Weinkove *et al.*, 1999). Moreover, the genetic interactions between *dPTEN*, *PI3K*, and the *PI3K* target *Dakt1* (Fig. 7) are consistent with dPTEN antagonizing PI3K signaling. However, although overexpression of *Dp110* or *Dakt1* in imaginal discs causes an increased rate of cell growth, recent studies have shown that this does not affect the rate of cell proliferation (Weinkove *et al.*, 1999; Verdu *et al.*, 1999). *Dp110* overexpression results in an abbreviated G1 phase followed by an expanded G2 period, and so overall cell division rates are unchanged. Thus *Dp110* activity appears to drive progression through the G1/S but not the G2/M transition of the cell cycle. Similar conclusions were reached for *Drosophila myc*, which when overexpressed causes dramatically increased growth rates and promotes G1/S progression, but fails to accelerate cell division (Johnston *et al.*, 1999). Moreover, activation of vertebrate PI3K in rat embryo fibroblasts promotes entry into S phase, but is not sufficient to provide for progression through the entire cell cycle (Klippel *et al.*, 1998). Thus, acceleration of growth is not necessarily sufficient to increase rates of cell proliferation.

In addition to a defect in cell growth, loss-of-function mutations in *inr, chico, PI3K, Dakt1,* and *S6K* result in a decrease in cell number in the mutant clones (Weinkove *et al.*, 1999; Böhni *et al.*, 1999; Montagne *et al.*, 1999; Chen *et al.*, 1996; Verdu *et al.*, 1999). This decrease in cell number is not sufficient to suggest a direct role for these genes in cell proliferation, since it could be caused as a secondary consequence of the cell growth phenotype. Rather, gain-of-function experiments are required to identify the primary role of these genes in cell growth or proliferation. Such analyses revealed that the primary role of PI3K and Dakt1 is promoting cell growth, not proliferation (Weinkove *et al.*, 1999; Verdu *et al.*, 1999). Overexpression of *Drosophila insulin receptor* (*inr*) does lead to an increased proliferation (Huang *et al.*, 1999). Since such phenotype is not induced by activation of PI3K, the increased proliferation resulting from *inr* overexpression is likely to be mediated by other pathways activated by the insulin receptor.

We demonstrate here that a null mutation in *dPTEN* causes a significant increase in the rate of both cell division and cell growth. Cells lacking *dPTEN* activity proliferate at a greater rate and to a greater extent than wild-type cells (Fig. 3). Thus, our findings demonstrate that loss of *dPTEN* function is not simply equivalent to activation of PI3K. One possibility is that overexpression of PI3K does not fully activate its biochemical function. Loss of *dPTEN* may stimulate growth to a greater degree than does PI3K activation, and perhaps this high rate of growth is necessary to increase cell division rates. However, we consider this unlikely, since overexpression of *PI3K* results in an increase in cell size similar to loss of *dPTEN* function (Weinkove *et al.*, 1999; this study), and *dmyc* overexpression causes a greater degree of cell enlargement than *dPTEN* mutation without increasing proliferation (Johnston *et al.*, 1999). Instead, we propose that PTEN regulates cell proliferation by multiple mechanisms, both PI3K-dependent and -independent.

One potential PI3K-independent mechanism is suggested by the domain in PTEN related to tensin, an actin filament capping protein that localizes to focal adhesions. It was recently shown that overexpression of tensin can suppress anchorage-independent proliferation of Ras-transformed 3T3 cells (Tikoo *et al.*, 1999), and therefore this domain may provide a growth-regulatory function in PTEN as well. Moreover, in addition to its role as a lipid phosphatase, PTEN also possesses a dual-specificity protein phosphatase activity. PTEN has been shown to bind and dephosphorylate the focal adhesion kinase FAK and to down-regulate the formation of focal adhesions (Tamura *et al.*, 1998). Such cell contacts play a critical role in regulating proliferation in *Drosophila*, and the gene products of several *Drosophila* tumor suppressors such as *expanded*, *fat*, and *l(2) discs large* all localize to adherens or septate junctions (Woods *et al.*, 1997). Our results are thus consistent with a model in

which PTEN suppresses cell growth and G1/S progression by down-regulating the PI3K/Akt pathway and inhibits the G2/M transition through an alternative mechanism, perhaps involving regulation of the cytoarchitecture. The ability to regulate both growth and cell division may explain why PTEN is such a common target in advanced tumors. This model is also consistent with the different mutant phenotypes between the null *dPTEN* allele *dj189* and the *c494* allele that carries the G135E point mutation (Huang *et al.*, 1999). While the G135E mutation changed an invariant amino acid within the phosphatase active site and is likely to inactivate the lipid phosphatase activity, the other domains of PTEN are still intact. Characterization of PTEN mutants that are specifically defective in cell growth or proliferation may shed further light on its role in the control of overall growth.

The Role of Insulin Signaling in Cell Survival

Recent studies in *Drosophila* imaginal discs have provided *in vivo* evidence that the insulin signal transduction pathway plays an essential role in the control of overall growth. Loss-of-function mutations in positive regulators of the insulin pathway, including the insulin receptor (*inr*), insulin receptor substrate (*chico*), and *PI3K*, all result in decreased cell number and cell size (Weinkove *et al.*, 1999; Böhni *et al.*, 1999; Chen *et al.*, 1996). While these studies firmly established a role for the insulin pathway in cell size control, it was more difficult to pinpoint whether the decrease in cell number was due to slowed cell proliferation or increased cell death. Distinguishing between these two possibilities is especially important given that mammalian *PI3K* and *PI3K* target *Akt* have been implicated in both cell survival and cell proliferation. In the case of *chico*, the authors did not observe an effect of *chico* mutation on cell death, suggesting that the decrease in cell number is not due to increased cell death (Böhni *et al.*, 1999).

Our analysis of *dPTEN* mutant phenotypes provides further evidence that *dPTEN* and hence the insulin pathway do not play a major role in regulating cell survival in *Drosophila* imaginal disc development. First, loss-of-function *dPTEN* mutant cells in the imaginal discs did not show decreased cell death as assayed by acridine orange staining (data not shown) and FACS (Fig. 6), as would be predicted from studies in the mammalian systems (Stambolic *et al.*, 1998; Myers *et al.*, 1998; Li *et al.*, 1998). Second, decreased wing size resulting from *dPTEN* overexpression was not suppressed by *P35* expression (Fig. 5). Third, TUNEL analysis did not reveal significant difference between *dPTEN* germ-line clone mutant embryos that lack both paternal and maternal gene product and wild-type control embryos (Fig. 9). Since neither positive (*chico*) or negative (*dPTEN*) regulators of the insulin pathway obviously affect cell death, we favor the hypothesis that the insulin pathway controls overall growth in *Drosophila* by regulating cell growth and cell proliferation, not by directly regulating cell survival. The apparent difference between *Drosophila* imaginal discs and mammalian cell culture implies that the effect of insulin signaling on cell survival is context-dependent. Consistent with this hypothesis, overexpression of *dPTEN* has been shown to trigger cell death in differentiating cells but not in proliferating cells in *Drosophila* imaginal discs (Huang *et al.*, 1999).

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