PDK1 regulates growth through Akt and S6K in *Drosophila*

Felix Rintelen*, Hugo Stocker*, George Thomas†, and Ernst Hafen*‡

*Zoologisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland; and †Friedrich Miescher Institute, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

The insulin/insulin-like growth factor-1 signaling pathway promotes growth in invertebrates and vertebrates by increasing the levels of phosphatidylinositol 3,4,5-triphosphate through the activation of p110 phosphatidylinositol 3-kinase. Two key effectors of this pathway are the phosphoinositide-dependent protein kinase 1 (PDK1) and Akt/PKB. Although genetic analysis in *Caenorhabditis elegans* has implicated Akt as the only relevant PDK1 substrate, cell culture studies have suggested that PDK1 has additional targets. Here we show that, in *Drosophila,* dPDK1 controls cellular and organism growth by activating dAkt and S6 kinase, dS6K. Furthermore, *dPDK1* genetically interacts with *dRSK* but not with *dPKN,* encoding two substrates of PDK1 *in vitro*. Thus, the results suggest that dPDK1 is required for dRSK but not dPKN activation and that it regulates insulin-mediated growth through two main effector branches, dAkt and dS6K.

Genetic studies in *Caenorhabditis elegans* and *Drosophila,* and biochemical analyses in vertebrate cell culture systems have led to the identification of key components of the insulin signal transduction pathway, including members of the phosphatidylinositol 3-kinase [PI(3)K] signaling pathway; the protein kinases PDK1, Akt, GSK3, and S6K and the 3-phosphatidylinositide phosphatase PTEN, which antagonizes the effects of PI(3)K by converting phosphatidylinositol 3,4,5-triphosphate (PIP_3) to phosphatidylinositol 4,5-biphosphate. In addition, studies *in vitro* and in vertebrate cell culture systems have implicated phosphoinositide-dependent protein kinase 1 (PDK1) as the critical regulator of T-loop phosphorylation in many members of the AGC family of kinases, which include Akt (1–6), S6K (7, 8), RSK (9, 10), PKN (11), and all isoforms of protein kinase C (12–15). PDK1 possesses two functional domains, a serine/threonine kinase domain located amino-terminally and a Pleckstrin-homology domain with a high affinity to PIP_3. Owing to this high affinity to PIP_3, PDK1 is located at the membrane even in resting cells and controls activity of its target kinases at the plasma membrane (4, 16). Consistent with PDK1 being a direct effector of Akt, S6K, and RSK, activation of all three kinases is blocked in $PDK1^{-/-}$-deficient embryonic stem cells (17). These findings imply that *in vivo* PDK1 has multiple targets and acts as a downstream branch point for PI(3)K signaling. However, despite these observations, genetic analyses in *C. elegans* and a recent study in *Drosophila* have implicated Akt as the only relevant target for PDK1 function (18, 19). In contrast, the detailed genetic analysis of dPDK1 function in *Drosophila* presented here indicates that PDK1 functions as a central regulator of cell growth by regulating two effector pathways controlled by the AGC kinases Akt and S6K, respectively.

Methods

Ethyl Methanesulfonate (EMS) Mutagenesis and Analysis of Mutants.
To generate mutations in *dPDK1, y w*; *EP(3)0837/TM2 y⁺* males were treated with EMS according to Lewis and Bacher (20) and mated to *y w*; *GMR-Gal4 UAS-dAkt/CyO*; *MKRS/TM2* females. A total number of 2,300 F_1 progeny of the genotype *y w*; *GMR-Gal4 UAS-dAkt/+*; *EP(3)0837/MKRS* or *TM2* was screened for a suppression of the big eye phenotype shown in Fig.

2d. Primary positives were retested, and stocks were established by balancing the potential *dPDK1* alleles with the *TM6B y⁺* balancer.

Genomic DNA was extracted from heterozygous flies, and coding exons of *dPDK1* were amplified by PCR. The PCR products were sequenced and analyzed with SEQUENCHER software for the appearance of double peaks in the sequence chromatogram, and compared with the published sequence (21). The nucleotide changes are: *dPDK1³* (GGT→AGT), *dPDK1⁴* (CCG→CTG), *dPDK1⁵* (CAG→TAG).

Clonal Analysis.
Clonal analysis of *dPDK1* loss-of-function alleles was performed by using the *Flp/FRT* and the *ey-Flp* systems as described (22, 23). To generate marked clones that express either *EP(3)0837* controlled *dPDK1* and/or *UAS-dAkt* in eye disk cells during the last cell cycle and subsequent differentiation, 24- to 48-h-old larvae containing a heat-shock-inducible *Flp* recombinase, a Flp-out transgene (*GMR>FRT w⁺ STOP FRT>Gal4*), the *EP(3)0837* element, and/or a *UAS-dAkt* construct were subjected to a heat shock for 1 h at 37°C. This procedure induces recombination between the *FRT* sites of *GMR>FRT w⁺ STOP FRT>Gal4* and removes the *w⁺* STOP cassette in clones, thus allowing expression of *dPDK1* and *dAkt* under the control of *GMR-Gal4*. Histological sections of the eyes were performed as described (24).

Plasmids and Germ-Line Transformation.
To generate *UAS-dPDK1,* the ORF coding for dPDK1 was amplified from the full-length cDNA clone LD16509 (obtained from Research Genetics, Huntsville, AL) by PCR by using the primers 5′-GGAATTCAT-GGCCAAGGAGAAAGCATC-3′ (ofr77) and 5′-GCTCTA-GACGTTTACTTAGACGCCGTC-3′ (ofr80), which introduced *Eco*RI and *Xba*I sites at the 5′ and 3′ ends, respectively. The PCR product was ligated into the pUAST *Drosophila* transformation vector (25), and the resulting plasmid *UAS-dPDK1* was used for transformation.

To generate *UAS-PDK1^{A467V}* the point mutation C→T at *dPDK1* nucleotide position 2,032 was introduced with a Quick-Change Site-Directed Mutagenesis Kit from Stratagene. For PCR we used the primers 5′-GTTTATCAGATGATCGTCG-GCCTACCGCCATTC-3′ and 5′-GAATGGCGGTAGGC-CGACGATCATCTGATAAAC-3′ and the pBluescript SK(−) plasmid containing the cDNA clone LD16509 as a template. The resulting plasmid was used as a template for PCR with primers ofr77 and ofr80. The PCR product was digested with *Eco*RI and *Xba*I and cloned into the pUAST vector. The resulting plasmid *UAS-PDK1^{A467V}* was used for transformation.
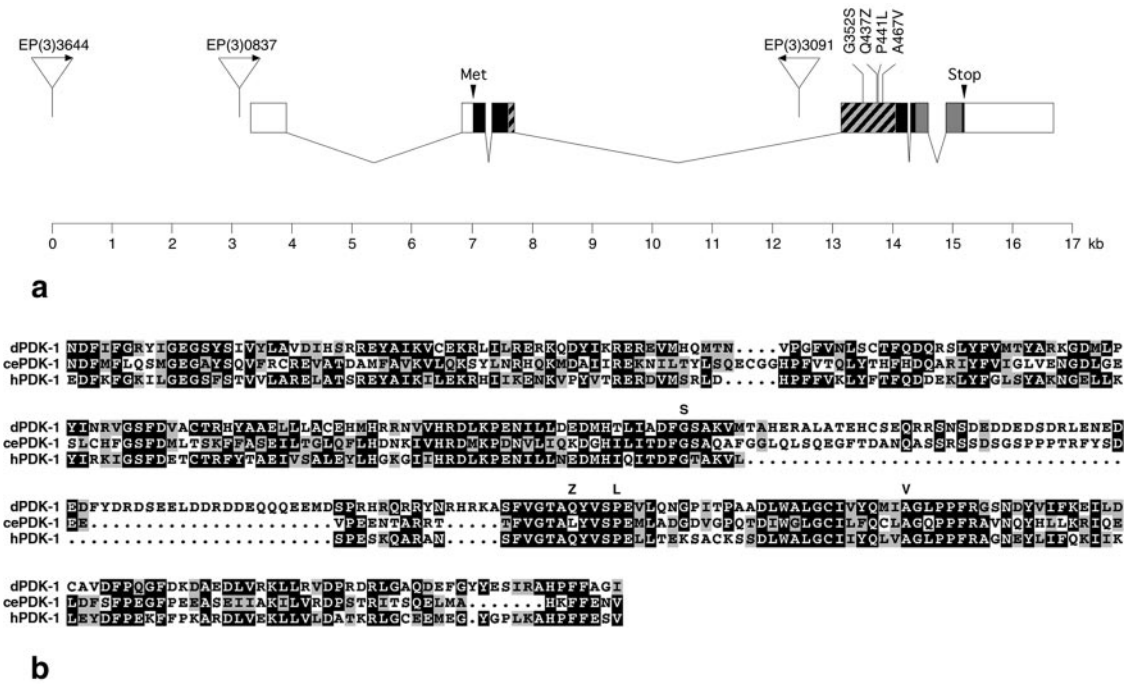
Fig. 1. Gain- and loss-of-function mutations in the *dPDK1* locus. (*a*) Genomic structure of the *dPDK1* locus. One of several reported transcripts (42) represented by the expressed sequence tag (EST) cDNA LD16509 is shown. Boxes represent exons. Dark boxes indicate the ORF; hatched and gray boxes represent the kinase and Pleckstrin-homology domains, respectively. EP insertions *EP(3)0837*, *EP(3)3091*, and *EP(3)3644* are shown as triangles, and the direction of transcription from the *UAS*-controlled promoter is marked by arrows. *EP(3)3644* inserted 7,081 and *EP(3)0837* 3,875 nt upstream of the putative start codon (Met). *EP(3)3091* inserted 710 bp upstream of the 5′ end of exon 3. The three EMS-induced loss-of-function mutations and the activating mutation A467V are shown above exon 4. (*b*) Kinase domain alignment of *Drosophila*, *C. elegans*, and human PDK1. Dark and gray boxes indicate amino acid identity and similarity, respectively. Amino acid changes in the *dPDK1³⁻⁵* and *dPDK1^A467V* mutants are shown above the dPDK1 sequence. Note that the amino acid substitutions in dPDK1³ (G352S) and dPDK1⁴ (P441L) are in highly conserved amino acid residues.

To generate *UAS-dPKN* and *UAS-dRSK* we performed PCR by using as a template double-stranded cDNA derived from 0- to 24-h-old *Drosophila* embryos (kindly provided by K. Nairz, Universität Zürich, Zürich, Switzerland) with the following primer pairs: 5′-CGGCGAATTAACGAGAAACC-3′ and 5′-GGC-CCGTTAGTAAATCCTTG-3′ for *dPKN* and 5′-AACAAAG-GAACCGCTAGGAG-3′ and 5′-AAGTAGTCGGACTATCT-GCC-3′ for *dRSK*. The PCR products were cloned by using the pCRII-TOPOTA vector system (Invitrogen). Subsequently, the *dPKN* and *dRSK* cDNAs were cut out with Asp-718 and *Not*I and ligated into the pUAST vector. The resulting plasmids *UAS-dPKN* and *UAS-dRSK* were used for transformation.

P element-mediated germ-line transformation was performed as described (26). The constructs were injected into *y w* embryos. Several independent transformant lines were established for all constructs.

Phenotypic Analysis. Unless indicated otherwise, all phenotypic analyses were done in females. Measurements of cell number, cell size, and body weight were performed as described (27). National Institutes of Health IMAGE 1.61 was used to quantify the size of ommatidia and rhabdomeres by measuring the corresponding area.

***Drosophila* Strains.** *EP(3)0837*, *EP(3)3091*, and *EP(3)3644* flies were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). Genomic sequences flanking the 3′ end of the enhancer–promoter (EP) elements was isolated by plasmid rescue (28), sequenced, and analyzed with use of the Berkeley *Drosophila* Genome Project database (Berkeley, CA). The *Gal4* driver *GMR-Gal4* was a gift of M. Freeman (MRC Laboratory of Molecular Biology, Cambridge, U.K.), *ap-Gal4* was described

in ref. 29, and *arm-Gal4* was obtained from the Bloomington *Drosophila* Stock Center. The following alleles were used for genetic interaction studies: *DPTEN^dj189* [a putative null mutation caused by the insertion of an F element in a coding exon which disrupts the *dPTEN* ORF after amino acid 89 (30)], *dPTEN^c494* [encoding a strong hypomorph of *dPTEN* caused by an EMS-induced amino acid exchange (G135E) in the active-site motif of the catalytic domain required for phosphatase activity (31)], *dS6K^l-1* [a putative null mutation generated by imprecise excision of a P element insertion in the *dS6K* gene, which removed part of the first exon, including a portion of the catalytic domain (32)], and *dAkt¹* [encoding a kinase dead version of dAkt carrying a single amino acid substitution (F327I) in the DFG motif in kinase domain VII (33)]. *ap-Gal4 UAS-dS6K* flies are described in ref. 32. The construction of *UAS-dAkt* flies will be described elsewhere. For overexpression studies in which the EMS-induced *dPDK1* alleles *dPDK1⁴* and *dPDK1⁵* were used, we induced the jump-out of the EP element *EP(3)0837* from fly stocks *dPDK1⁴* and *dPDK1⁵* to avoid overexpression of mutant dPDK1 proteins in a background where *Gal4* is expressed. P element mobilization was achieved by standard genetic techniques.

Results and Discussion

To analyze the function of dPDK1 in *Drosophila*, we aimed to generate both gain- and loss-of-function alleles of the kinase. *Drosophila* contains a single gene that encodes a kinase that is highly homologous to PDK1 in its primary sequence and its domain structure (2). Initially, we identified two EP transposable elements in the 5′ region of the endogenous *Drosophila PDK1* gene *dPDK1* (Fig. 1*a*). These EP elements drive expression of dPDK1 under the control of the *Gal4* system (25, 34), allowing us to test whether dPDK1 and dAkt cooperate in promoting
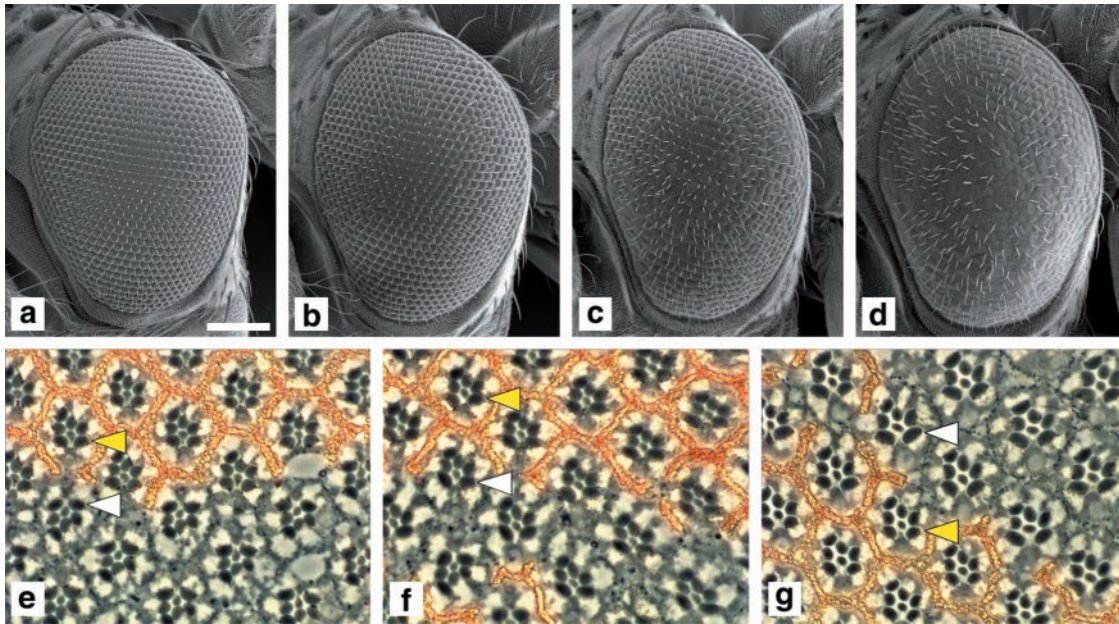
Fig. 2. Simultaneous overexpression of dPDK1 and dAkt with *GMR-Gal4* increases eye and cell size. (*a–d*) Simultaneous overexpression of dPDK1 and dAkt in the developing third instar eye imaginal disk results in the formation of larger eyes. Scanning electron micrographs of adult eyes of the following genotypes: (*a*) *OregonR*, wild type; (*b*) *y w*; *GMR-Gal4/+*; *EP(3)0837/+*; (*c*) *y w*; *GMR-Gal4 UAS-dAkt/+*; *TM2/+*; (*d*) *y w*; *GMR-Gal4 UAS-dAkt/+*; *TM2/EP(3)0837*. Although the overexpression of dPDK1 or dAkt alone results only in a slight, but in the case of dPDK1, significant increase in eye size (*b* and *c*), simultaneous expression of dPDK1 and dAkt causes a substantial increase in eye size (*d*). The area of at least 29 ommatidia in 3–6 eyes was measured for each genotype. Because the size of ommatidia of the genotypes *y w*; *GMR-Gal4 UAS-dAkt/+*; *TM2/+* and *y w*; *GMR-Gal4 UAS-dAkt/+*; *TM2/EP(3)0837* is variable, only the values of the 30% largest ommatidia were included in the calculation. We used flies of the following genotype, *y w*; *GMR-Gal4/UAS-lacZ*, as a control. The means of these values are (normalized to a value of $100 \pm$ SD): 100 ± 3 (control); 113 ± 3 (*b*); 108 ± 6 (*c*); 131 ± 10 (*d*). (*e–g*) dPDK1 and dAkt act synergistically to increase cell size in a cell-autonomous manner. Tangential sections through adult eyes containing clones in which dPDK1 and/or dAkt were overexpressed: (*e*) *y w hs-Flp/y w*; *GMR>FRT w⁺ STOP FRT>Gal4*; *EP(3)0837/+*; (*f*) *y w hs-Flp/y w*; *GMR>FRT w⁺ STOP FRT>Gal4/UAS-dAkt*; (*g*) *y w hs-Flp/y w*; *GMR>FRT w⁺ STOP FRT>Gal4/UAS-dAkt*; *EP(3)0837/+*. Clones are marked by the lack of red pigment. No increase in cell size is observed by overexpressing dPDK1 or dAkt alone (*e* and *f*), but simultaneous overexpression of dPDK1 and dAkt slightly increases cell size (*g*). For quantification, the area of the R6 rhabdomere for 15 photoreceptors in clones overexpressing dPDK1 and/or dAkt (white arrowhead) were compared with the corresponding value for the sister control clone in the same section (yellow arrowhead): The values were normalized to $100 \pm$ SD for the sister control clone and compared with the value in the overexpression clone: 100 ± 6 vs. 110 ± 7 (*e*); 100 ± 7 vs. 113 ± 7 (*f*); 100 ± 6 vs. 155 ± 10 (*g*). At the border of the clones, ommatidia composed of wild-type cells and cells overexpressing dPDK1 and/or dAkt are visible, indicating that the increase in cell size in *g* is cell-autonomous. (Bar = 100 μm.)

growth in *Drosophila*. Overexpression of either kinase in the eye imaginal disk during the last cell division cycle and subsequent differentiation showed little effect on the size or the structure of the eye (Fig. 2 *b* and *c*). Co-overexpression of dAkt and dPDK1, however, led to a significant increase in eye size (Fig. 2*d*). Furthermore, analysis of clones of cells in the eye overexpressing dPDK1 and/or dAkt revealed that the observed effect on cell size is strictly autonomous (Fig. 2 *e–g*). These results indicate that overexpression of dPDK1 does not interfere with the normal differentiation of eye disk cells and that it promotes local growth through dAkt activation.

To generate loss-of-function alleles of *dPDK1*, the dominant eye size phenotype caused by co-overexpression of dPDK1 and dAkt was reverted by using EMS mutagenesis, leading to three partial or complete loss-of-function mutations. *dPDK1³* causes a G(352) to S substitution in the conserved DFG motif in the kinase subdomain VII (Fig. 1 *a* and *b*). The D residue in this motif is essential for kinase activity by orienting the ATP-Mg²⁺ complex for phosphotransfer (35–37). *dPDK1⁴* causes a P(441) to L substitution in a conserved residue in kinase subdomain VIII. In the *dPDK1⁵* allele, a Q codon at position 437 in kinase subdomain VIII is mutated to a STOP codon. Because this latter mutation results in the formation of a truncated dPDK1 protein lacking part of the kinase domain and the Pleckstrin-homology domain, *dPDK1⁵* is likely to be a null mutation. A fourth allele *EP(3)3091* (*dPDK1¹*), from the Berkeley *Drosophila* Genome Project, has an EP element located in the third intron of *dPDK1*

(Fig. 1*a*) and is homozygous lethal. It failed to complement *dPDK1⁵* (data not shown), and the lethality was reversed by EP element excision.

Combinations of loss-of-function alleles provided mutants of varying strengths. Larvae homozygous for the *dPDK1⁵* null allele or larvae of the *dPDK1¹/⁵* heteroallelic combination die during the second instar stage. A less severe reduction in dPDK1 function (*dPDK1⁴/⁵*) permits development of viable *dPDK1* mutant flies that are delayed 1 day in development and smaller than their heterozygous siblings, having an 18% reduction in body weight (Fig. 3 *a* and *d*). By measuring the cell density in the wing, the reduction in size and weight apparently is primarily caused by a decrease in cell size, because cell number is only slightly affected (Fig. 3*d*). The lethality associated with the *dPDK1* null allele and the size defect of *dPDK1* hypomorphs was rescued by ubiquitous expression of a wild-type *dPDK1* transgene with *armadillo (arm)-Gal4* as a driver. *dPDK1⁴/⁵* male flies are almost completely sterile, although they show no obvious defect in sperm morphology and motility and in mating behavior (data not shown). That loss of zygotic dPDK1 function results in larval lethality is in contrast to a recent analysis of two *dPDK1* mutations caused by the EP insertion *EP(3)3091* (*dPDK1¹*) or a 10-kb deletion (*dPDK1²*), which were homozygous embryonic lethal (19). It is possible that the embryonic lethality observed by Cho *et al.* (19) is not caused by loss of dPDK1 function but by a linked lethal mutation on the same chromosome, because no rescue was attempted, and the phenotype was only analyzed in
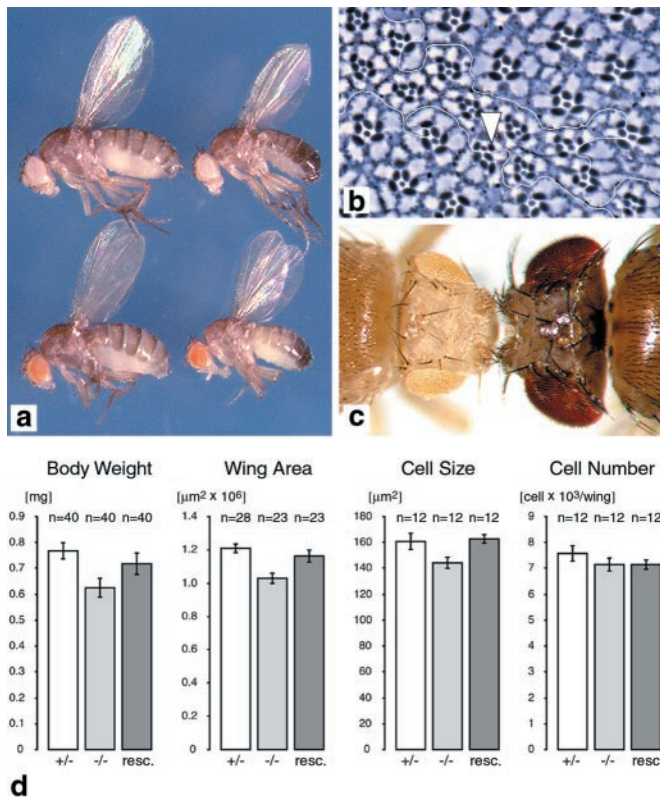
Fig. 3. dPDK1 loss-of-function phenotypes. (*a*) Body size reduction of hetero-allelic mutant flies. Males (*Right*) and females (*Left*) of the following geno-types are shown: *y w; dPDK1⁵/+* (*Top*); *y w; dPDK1⁴/dPDK1⁵* (*Bottom*). (*b*) Tangential section through an eye containing a *dPDK1⁵/⁵* clone. Within the clone, all photoreceptor cells are reduced in size compared with wild-type photoreceptor cells. At the border of the clone, ommatidia composed of phenotypically wild-type and mutant cells (arrowhead) are visible, indicating that dPDK1 controls cell size autonomously. The genotype is as follows: *y w ey-Flp/y w; dPDK1⁵ FRT80B/FRT80B.* (*c*) Selective removal of dPDK1 function from the eye imaginal disk results in a reduction of head and eye size. *y w ey-Flp/y w; dPDK1⁵ FRT80B/M(3)67c⁴ FRT80B* (*Left*); *OregonR*, wild type (*Right*). (*d*) Quantification of body and organ size in *dPDK1* heteroallelic mutant male flies compared with heterozygous and rescued flies, which overexpress a wild-type *dPDK1* cDNA under the control of the ubiquitous *arm-Gal4* driver. Values of *y w; dPDK1⁵/+* (+/−), *y w; dPDK1⁴/dPDK1⁵* (−/−), and *y w; arm-Gal4/UAS-dPDK1; dPDK1⁴/dPDK1⁵* flies (resc.) are shown. Values are the mean ± SD.

homozygotes. Consistent with this observation, larvae homozygous for a *dPDK1¹* mutant chromosome, which has been cleaned from second hits by recombination, die during the second instar stage. Although it is very likely that dPDK1 functions during embryogenesis, like dAkt (33), maternal transcripts may be sufficient to support embryonic development.

To determine whether the effects of loss of dPDK1 function on cell growth and organ development are autonomous events, we analyzed loss of dPDK1 in clones of cells by using the *FRT* mitotic recombination system (22). In contrast to organism lethality, clones of cells homozygous for the *dPDK1* null allele *dPDK1⁵* survive to adulthood. These cells show no defect in their ability to differentiate into photoreceptor cells or accessory cells, but mutant photoreceptor cells are ≈30% smaller than the heterozygous cells outside the clone (Fig. 3*b*), a strictly cell autonomous effect. To test whether an entire body part could develop in the absence of dPDK1 function, dPDK1 was selectively removed in much of the head primordium by using the *ey-Flp* system (23). Heads homozygous mutant for anyone of the three alleles, *dPDK1³*, *dPDK1⁴*, and *dPDK1⁵*, are reduced in size (Fig. 3*c*; data not shown), which indicates that entire

organs differentiate and develop in the absence of dPDK1 function, but that the final size of these organs autonomously depends on the amount of dPDK1 activity. The reduction in head size was most severe with *dPDK1⁵* followed by *dPDK1⁴* and *dPDK1³*, with the complete removal of dPDK1 function similar to that observed for loss-of-function mutations in the *Drosophila* insulin receptor (*dInr*), *Dp110/PI(3)K*, and *dAkt* (ref. 27; H.S. and E.H., unpublished work).

The pronounced effect of loss of dPDK1 function on head size suggested that it is a dominant constituent in the dInr pathway. To test this possibility, we examined the ability of complete and partial loss-of-function alleles of *dPDK1* to reverse phenotypes caused by either overexpression of dInr or by mutations in *dPTEN*, the 3-phosphatidylinositide phosphatase. Overexpression of a wild-type *dInr* cDNA under the control of *GMR-Gal4* led to a marked increase in eye size and a slightly rough eye surface (27), an effect dominantly suppressed by removing one copy of *dPDK1* (Fig. 4*a*). Further reduction of dPDK1 function by the *dPDK1¹/⁴* heteroallelic combination reduced the eye to almost wild-type size (Fig. 4*b*), suggesting that the amount of dPDK1 protein is rate-limiting for the dInr overgrowth phenotype. Null mutations in *dPTEN* cause lethality, and removal of dPTEN function in clones stimulates cell autonomous growth (30, 31, 38), suggesting that increased levels of PIP₃ promote growth and are the likely cause of lethality. Thus, if dPDK1 is an essential target of PIP₃, mutations in *dPDK1* may suppress the *dPTEN* phenotype. Surprisingly, some *dPTEN/dPDK1* double mutant flies survive to adulthood (Fig. 4*c*), indicating that the presumed PIP₃-induced lethality is primarily caused by the hyperactivation of dPDK1 or of one of its targets.

The fact that the growth phenotypes of *dPDK1* mutations are similar to those caused by mutations in genes coding for dS6K (32), and dAkt (refs. 39 and 40; H.S. and E.H., unpublished work), and that S6K1 is a mammalian PDK1 substrate, raised the possibility that dPDK1 may independently control growth through dS6K. This possibility was tested in the wing, which is composed of a dorsal and a ventral epithelial sheet that are tightly attached to each other through extracellular matrix. We have shown that selective overexpression of a wild-type *dS6K* cDNA in the dorsal wing epithelium with the *apterous* (*ap*)-*Gal4* driver leads to a bending down of the wing blade, probably because of a cell-size increase in the dorsal surface (32). This phenotype was suppressed by a reduction of dPDK1 function (Fig. 5 *a–c*). Although *ap-Gal4* induced overexpression of wild-type dPDK1 alone had little effect on wing morphology (data not shown), overexpression of a dPDK1^A467V variant was sufficient to cause a bent-wing phenotype (Fig. 5*d*). The corresponding amino acid substitution in the *C. elegans* PDK1 is thought to cause a hyperactivation of the kinase (18). The dPDK1^A467V-induced bent wing phenotype depends on normal levels of dS6K and dAkt, because null mutations in either of the corresponding genes dominantly suppress the phenotype (Fig. 5 *e* and *f*). Together with the biochemical evidence in cultured cells and *in vivo* that dPDK1 controls the activity of dAkt and dS6K (ref. 19; T. Radimerski, J. Montagne, F. R. J. van der Kaay, C. P. Downes, E.H., and G.T., unpublished work) these results provide functional evidence that dPDK1 is a key regulator in the control of growth and cell size by regulating the activity of two AGC kinases, dAkt and dS6K.

The effects of dPDK1 on dS6K raised the possibility that dPDK1 controls the activity of other AGC kinases *in vivo*, such as dRSK and dPKN, which have been implicated as mammalian PDK1 substrates. Because the developing eye depends on endogenous levels of dPDK1, we examined whether lowering the dose of dPDK1 was sufficient to suppress dominantly the rough eye phenotype caused by overexpression of dRSK and dPKN under *GMR-Gal4* control (Fig. 5*g*; data not shown). Reduction of dPDK1 activity in a viable *dPDK1* mutant combination was sufficient to suppress the rough eye phenotype of dRSK but not
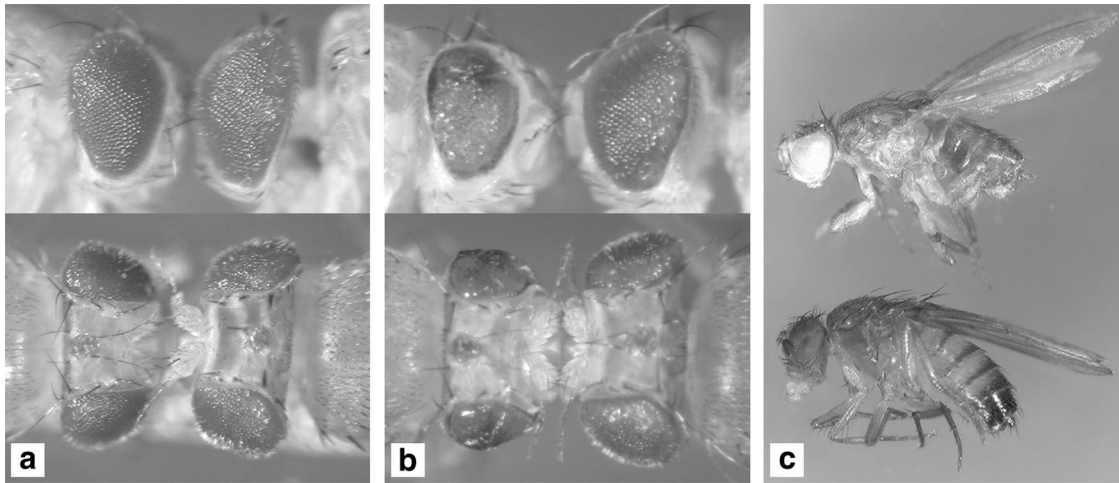
Fig. 4. dPDK1 loss-of-function mutations suppress *dInr* and *dPTEN* mutant phenotypes. (*a* and *b*) The eye phenotype caused by overexpression of *UAS-dInr* with the *GMR-Gal4* driver is dominantly suppressed by removing one copy of *dPDK1*, and the eye size is almost completely restored to wild-type size in a *dPDK1¹ᐟ⁴* heteroallelic mutant background, although eye roughness is increased. The reason for this latter observation is unclear. (*a*) *y w*; *GMR-Gal4 UAS-dInr*/+; *dPDK1⁵*/+ (*Left*); *y w*; *GMR-Gal4 UAS-dInr*/+; *MKRS*/+ (*Right*); (*b*) *y w*; *GMR-Gal4 UAS-dInr*/+; *dPDK1¹*/*dPDK1⁴* (*Left*); *y w*; *GMR-Gal4 UAS-dInr*/+; *dPDK1⁴*/+ (*Right*). (*c*) The lethality caused by mutations in *dPTEN* is rescued in a *dPDK1* heteroallelic mutant background: Some *dPTEN, dPDK1* double-mutant flies survive to adulthood, although they display mutant phenotypes like an unproportionally reduced size of the abdomen and deformed leg structures. Similar phenotypes have been observed in partial loss-of-function mutations for *dTOR* (S. Oldham and E.H., unpublished work). Flies of the following genotypes are shown: *y w dPTENᵈʲ¹⁸⁹*/*dPTEN⁴⁹⁴*; *dPDK1⁴*/*dPDK1⁵* (*Upper*), *OregonR,* wild-type (*Lower*).
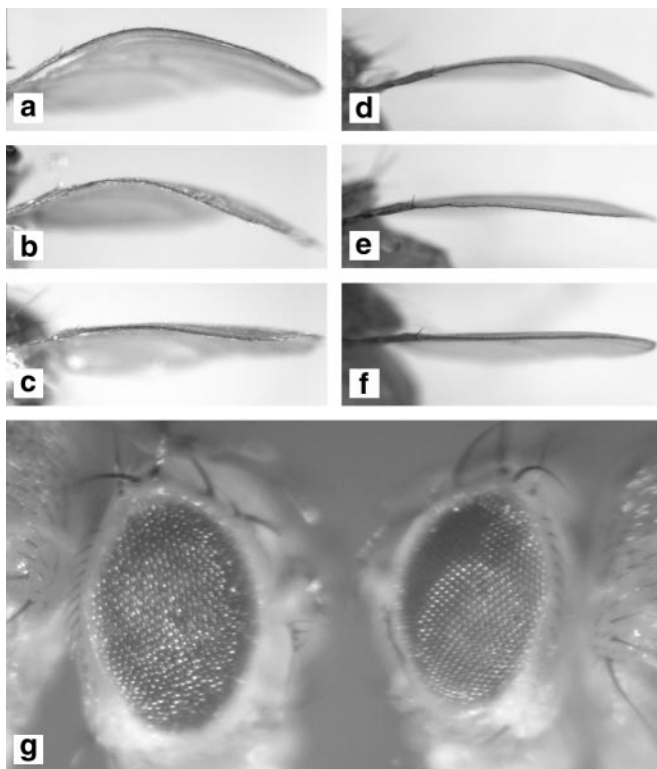
Fig. 5. Genetic interaction of dPDK1 with the AGC kinases dAkt, dS6K, and dRSK. (*a–c*) Mutations in *dPDK1* suppress the *ap-Gal4 UAS-dS6K* bent-wing phenotype. (*a*) *y w*; *ap-Gal4 UAS-dS6K*/+; *MKRS*/+; (*b*) *y w*; *ap-Gal4 UAS-dS6K*/+; *dPDK1⁵*/+; (*c*) *y w*; *ap-Gal4 UAS-dS6K*/+; *dPDK1⁴*/*dPDK1⁵*. (*d–f*) Null mutations in *dS6K* and *dAkt* dominantly suppress the *ap-Gal4 UAS-dPDK1ᴬ⁴⁶⁷ⱽ* bent-wing phenotype. (*d*) *y w*; *ap-Gal4 UAS-dPDK1ᴬ⁴⁶⁷ⱽ*/+; (*e*) *y w*; *ap-Gal4 UAS-dPDK1ᴬ⁴⁶⁷ⱽ*/+; *dS6Kˡ⁻¹*/+; (*f*) *y w*; *ap-Gal4 UAS-dPDK1ᴬ⁴⁶⁷ⱽ*/+; *dAkt¹*/+. (*g*) Mutations in *dPDK1* suppress the rough eye phenotype caused by overexpression of *UAS-dRSK* under *GMR-Gal4* control. *y w*; *GMR-Gal4 UAS-dRSK*/+; *dPDK1⁵*/+ (*Left*); *y w*; *GMR-Gal4 UAS-dRSK*/+; *dPDK1⁴*/*dPDK1⁵* (*Right*).

of dPKN overexpression (Fig. 5*g*; data not shown). These results suggest that at least in this *in vivo* assay, dRSK activity critically depends on dPDK1 function, whereas dPKN activity is not changed by a reduction in dPDK1 levels. This idea is in line with the recent finding that in *PDK1⁻/⁻* embryonic stem cells the protein kinase C-related protein kinase PRK2, which shares extensive homology with PKN, is still partially phosphorylated at its T loop residue (41), indicating that PDK1-independent mechanisms for the phosphorylation of the T loop of certain AGC kinases including dPKN may exist.

Our results show that dPDK1 is an essential component in the insulin signaling pathway in the control of cell growth and body size through its two substrates, dAkt and dS6K. These results are distinct from the genetic evidence in *C. elegans* where Akt is the primary target of PDK1 in dauer formation. Because mutations in the insulin signaling pathway do not show an autonomous alteration of cell size in *C. elegans*, the regulation of the rate of protein synthesis through S6K does not seem to be a primary target of this pathway. However, that dPDK1 may yet have additional substrates is suggested by the genetic interaction with *dRSK* gain-of-function mutations and because viable *dPDK1* males are almost completely sterile. Although mutations in components of the insulin signaling pathway such as *dInr, chico, Dp110/PI(3)K,* and *dAkt* cause female sterility, male sterility is not observed. Further genetic dissection of dPDK1 function is required to determine the role of dPDK1 in male fertility. Our findings in *Drosophila* are consistent with the absence of insulin growth factor-1-induced activation of S6K, Akt, and RSK in mammalian *PDK1⁻/⁻* embryonic stem cells (17), and therefore provide evidence for the functional conservation of branch points in kinase networks during evolution.

1. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. & Cohen, P. (1997) *Curr. Biol.* **7,** 261–269.
2. 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., *et al.* (1997) *Curr. Biol.* **7,** 776–789.
3. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. & Hawkins, P. T. (1997) *Science* **277,** 567–570.
4. 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., *et al.* (1998) *Science* **279,** 710–714.
5. Belham, C., Wu, S. & Avruch, J. (1999) *Curr. Biol.* **9,** R93–R96.
6. Peterson, R. T. & Schreiber, S. L. (1999) *Curr. Biol.* **9,** R521–R524.
7. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. & Avruch, J. (1998) *Curr. Biol.* **8,** 69–81.
8. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. & Thomas, G. (1998) *Science* **279,** 707–710.
9. Jensen, C. J., Buch, M. B., Krag, T. O., Hemmings, B. A., Gammeltoft, S. & Frodin, M. (1999) *J. Biol. Chem.* **274,** 27168–27176.
10. Frodin, M., Jensen, C. J., Merienne, K. & Gammeltoft, S. (2000) *EMBO J.* **19,** 2924–2934.
11. Dong, L. Q., Landa, L. R., Wick, M. J., Zhu, L., Mukai, H., Ono, Y. & Liu, F. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5089–5094. (First Published May 2, 2000; 10.1073/pnas.090491897)
12. Dutil, E. M., Toker, A. & Newton, A. C. (1998) *Curr. Biol.* **8,** 1366–1375.
13. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. & Parker, P. J. (1998) *Science* **281,** 2042–2045.
14. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S. & Toker, A. (1998) *Curr. Biol.* **8,** 1069–1077.
15. Dong, L. Q., Zhang, R. B., Langlais, P., He, H., Clark, M., Zhu, L. & Liu, F. (1999) *J. Biol. Chem.* **274,** 8117–8122.
16. Anderson, K. E., Coadwell, J., Stephens, L. R. & Hawkins, P. T. (1998) *Curr. Biol.* **8,** 684–691.
17. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. & Alessi, D. R. (2000) *Curr. Biol.* **10,** 439–448.
18. Paradis, S., Ailion, M., Toker, A., Thomas, J. H. & Ruvkun, G. (1999) *Genes Dev.* **13,** 1438–1452.
19. Cho, K. S., Lee, J. H., Kim, S., Kim, D., Koh, H., Lee, J., Kim, C., Kim, J. & Chung, J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 6144–6149. (First Published May 8, 2001; 10.1073/pnas.101596998)
20. Lewis, E. B. & Bacher, F. (1968) *Drosophila Inf. Serv.* **43,** 193–194.
21. MacDougall, C. N., Clyde, D., Wood, T., Todman, M., Harbison, D. & Bownes, M. (1999) *Eur. J. Biochem.* **262,** 456–466.
22. Xu, T. & Rubin, G. M. (1993) *Development (Cambridge, U.K.)* **117,** 1223–1237.
23. Newsome, T. P., Asling, B. & Dickson, B. J. (2000) *Development (Cambridge, U.K.)* **127,** 851–860.
24. Basler, K. & Hafen, E. (1988) *Cell* **54,** 299–311.
25. Brand, A. H. & Perrimon, N. (1993) *Development (Cambridge, U.K.)* **118,** 401–415.
26. Basler, K., Christen, B. & Hafen, E. (1991) *Cell* **64,** 1069–1081.
27. Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R. & Hafen, E. (2001) *Curr. Biol.* **11,** 213–221.
28. Hamilton, B. A., Palazzolo, M. J., Chang, J. H., VijayRaghavan, K., Mayeda, C. A., Whitney, M. A. & Meyerowitz, E. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2731–2735.
29. Calleja, M., Moreno, E., Pelaz, S. & Morata, G. (1996) *Science* **274,** 252–255.
30. Gao, X., Neufeld, T. P. & Pan, D. (2000) *Dev. Biol.* **221,** 404–418.
31. Huang, H., Potter, C. J., Tao, W., Li, D. M., Brogiolo, W., Hafen, E., Sun, H. & Xu, T. (1999) *Development (Cambridge, U.K.)* **126,** 5365–5372.
32. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. & Thomas, G. (1999) *Science* **285,** 2126–2129.
33. Staveley, B. E., Ruel, L., Jin, J., Stambolic, V., Mastronardi, F. G., Heitzler, P., Woodgett, J. R. & Manoukian, A. S. (1998) *Curr. Biol.* **8,** 599–602.
34. Rorth, P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12418–12422.
35. Moran, M. F., Koch, C. A., Sadowski, I. & Pawson, T. (1988) *Oncogene* **3,** 665–672.
36. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S. & Zoller, M. J. (1993) *Trends Biochem. Sci.* **18,** 84–89.
37. van der Geer, P., Hunter, T. & Lindberg, R. A. (1994) *Annu. Rev. Cell Biol.* **10,** 251–337.
38. Goberdhan, D. C., Paricio, N., Goodman, E. C., Mlodzik, M. & Wilson, C. (1999) *Genes Dev.* **13,** 3244–3258.
39. Verdu, J., Buratovich, M. A., Wilder, E. L. & Birnbaum, M. J. (1999) *Nat. Cell Biol.* **1,** 500–506.
40. Scanga, S. E., Ruel, L., Binari, R. C., Snow, B., Stambolic, V., Bouchard, D., Peters, M., Calvieri, B., Mak, T. W., Woodgett, J. R., *et al.* (2000) *Oncogene* **19,** 3971–3977.
41. Balendran, A., Hare, G. R., Kieloch, A., Williams, M. R. & Alessi, D. R. (2000) *FEBS Lett.* **484,** 217–223.
42. Clyde, D. & Bownes, M. (2000) *J. Endocrinol.* **167,** 391–401.

DEVELOPMENTAL BIOLOGY