

# Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling

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**Tuberous sclerosis complex (TSC) is a genetic disorder caused by mutations in one of two tumor suppressor genes, *TSC1* and *TSC2*. Here, we show that absence of *Drosophila* *Tsc1/2* leads to constitutive dS6K activation and inhibition of dPKB, the latter effect being relieved by loss of dS6K. In contrast, the dPTEN tumor suppressor, a negative effector of PI3K, has little effect on dS6K, but negatively regulates dPKB. More importantly, we demonstrate that reducing dS6K signaling rescues early larval lethality associated with loss of dTsc1/2 function, arguing that the S6K pathway is a promising target for the treatment of TSC.**

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Hereditary cancers have revealed the existence of a number of tumor suppressor genes that function to control cell proliferation and maintain normal tissue homeostasis (Macleod 2000). Two tumor suppressors have been implicated in the PI3K-signaling pathway, PTEN (Cantley and Neel 1999) and more recently, a complex composed of two proteins, hamartin (*TSC1*) and tuberlin (*TSC2*; Montagne et al. 2001). The lipid phosphatase PTEN, which constrains PI3K signaling by dephosphorylating its product phosphatidylinositol 3,4,5-trisphosphate (PIP3), is found mutated in a number of cancers (Cantley and Neel 1999). Mutations in either *TSC1* or *TSC2* are associated with widespread medically distinct tumors of the brain, eyes, skin, heart, lungs, and kidneys (Young and Povey 1998). *TSC1* and *TSC2* contain putative coiled-coil domains, with *TSC1* having a predicted transmembrane domain and *TSC2* a region similar to that of small-GTPase-activating protein (GAP) domains (Montagne et al. 2001). Although little is known concerning the growth regulatory targets of *TSC1* and *TSC2*, genetic studies in *Drosophila* (Potter et al. 2001; Tapon et al. 2001) have led recently to the hypothesis that dTsc1/2 acts as a negative effector of dS6K or of a dS6K target (Potter et al. 2001). In these models, dS6K was

placed as a downstream effector of dPI3K, via dPKB (Potter et al. 2001). However, dS6K and dPI3K/dPKB appear to reside on parallel growth-promoting pathways rather than functioning in a linear-signaling cascade (Radimerski et al. 2002). Seemingly consistent with these findings, in *Drosophila* devoid of dPTEN, dPKB appears to be the sole critical target activated by elevated PIP3 levels (Stocker et al. 2002).

Here, we use double-stranded RNA mediated interference (dsRNAi) in *Drosophila Kc167* cultured cells to demonstrate that dTsc1/2 acts to suppress dS6K activation, whereas dPTEN negatively regulates dPKB activation but has little effect on dS6K activity. Similar findings are obtained in second instar larvae deficient for either dTsc or dPTEN function, whereas overexpression of either dTsc1/2 or dPTEN in second instar larvae selectively inhibits dS6K or dPKB activity, respectively. In addition, loss of dTsc1/2 function in *Kc167* cells or in larvae also leads to suppression of dPKB activity, an effect that is relieved by loss of dS6K. More strikingly, we demonstrate that a relative subtle pharmacological or genetic reduction in dS6K signaling is sufficient to rescue larval lethality associated with loss of dTsc function. These latter findings strongly suggest that the S6K pathway is a promising target for pharmaceutical intervention in tuberous sclerosis treatment.

## Results and Discussion

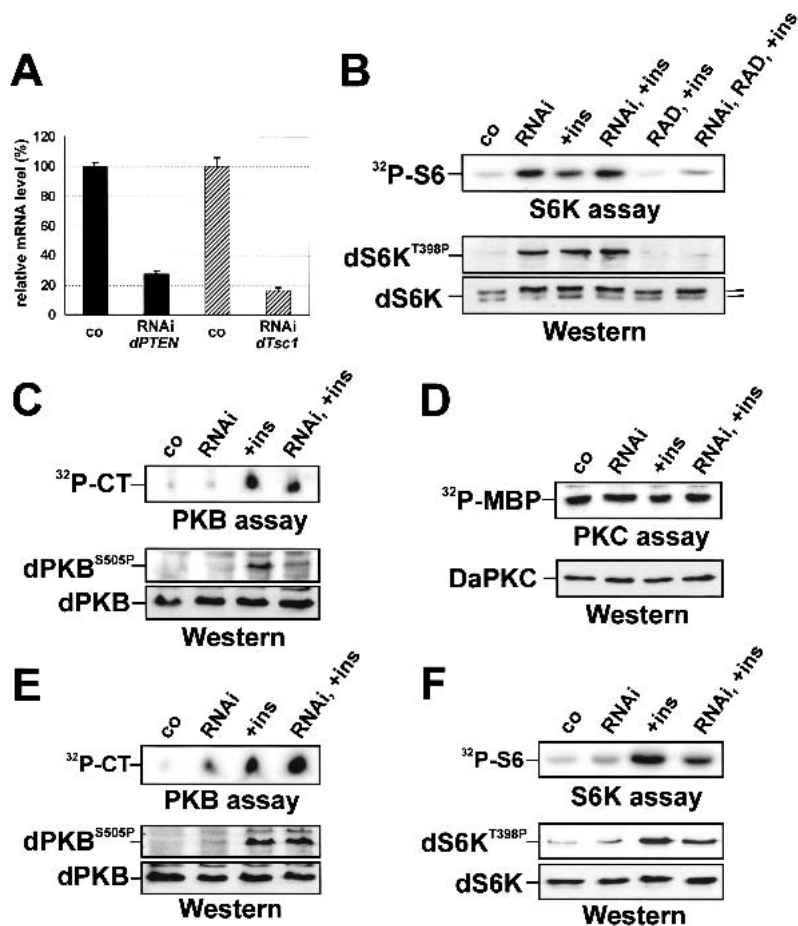
To determine whether loss of dTsc1/2 or dPTEN directly affected dS6K activity, each was depleted in *Drosophila Kc167* cells by dsRNAi (Clemens et al. 2000). Quantitative Real Time PCR showed that such treatment strongly reduced levels of both transcripts (Fig. 1A). Compared with control cells, depletion of dTsc1 increased dS6K activity and T398 phosphorylation, consistent with the reduced electrophoretic mobility of dS6K (Fig. 1B). These results are in agreement with recent findings in *TSC1* null mammalian cells (Kwiatkowski et al. 2002). Insulin treatment of either control cells or dTsc1-depleted cells did not significantly increase these responses beyond that of dTsc1 depletion alone (Fig. 1B), indicating that loss of dTsc function leads to full dS6K activation. RAD001, a rapamycin derivative (Radimerski et al. 2002), blocked dS6K activity in both control and dTsc1-depleted cells treated with insulin (Fig. 1B). However, it was consistently noted that the RAD001 block of insulin-induced dS6K activation was not as strong in dTsc1-depleted cells (Fig. 1B), suggesting that not all the effects of dTsc on dS6K are dependent on the *Drosophila* target of rapamycin, dTOR. Similar results to those described here were obtained by dTsc2 depletion (Supplementary Fig. 1). In addition, the effects appeared specific, as dTsc1 depletion had no effect on the basal activity of other AGC-kinase family members, such as dPKB or *Drosophila* atypical PKC (Fig. 1C,D). However, insulin-induced dPKB activation and S505 phosphorylation were repressed in dTsc1-depleted cells as compared with control cells (Fig. 1C), consistent with dS6K acting in a negative feedback loop to dampen dPKB signaling (Haruta et al. 2000). In contrast to loss of dTsc1, depletion of dPTEN had little effect on dS6K activity and T398 phosphorylation, whereas it led to elevated levels of both basal and insulin-stimulated dPKB activity and S505

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**Figure 1.** dS6K is constitutively activated in dTsc- but not dPTEN-depleted *Drosophila Kc167* cells. (A) Real-time PCR analysis following *dTsc1* and *dPTEN* dsRNAi. (B) Analysis of dS6K activity following *dTsc1* dsRNAi. Co, untreated control; RNAi, dsRNAi targeting *dTsc1*; +ins, insulin (100 nM) stimulation for 30 min; RAD, 15 min RAD001 (20 nM) pretreatment. (Top panel) In vitro dS6K activity. (Bottom panels) Western blot analysis of dS6K T398 phosphorylation (top) and dS6K band-shift (bottom). (C) Analysis of dPKB activity following *dTsc1* dsRNAi treatment. (Top panel) In vitro dPKB activity assay. CT, Crosstide. (Bottom panels) Western blot analysis of dPKB S505 phosphorylation (top) and total dPKB (bottom). (D) Analysis of atypical dPKC activity following *dTsc1* dsRNAi treatment. (Top) In vitro dPKC activity assay using myelin basic protein as substrate (MBP). (Bottom) Western blot of total atypical dPKC. (E) Analysis of dPKB activity following *dPTEN* dsRNAi treatment. Panels are as in C. (F) Analysis of dS6K activity following *dPTEN* dsRNAi treatment. The top panel is as in B, the bottom panels are Western blot analyses of dS6K T398 phosphorylation (top) and total dS6K (bottom).

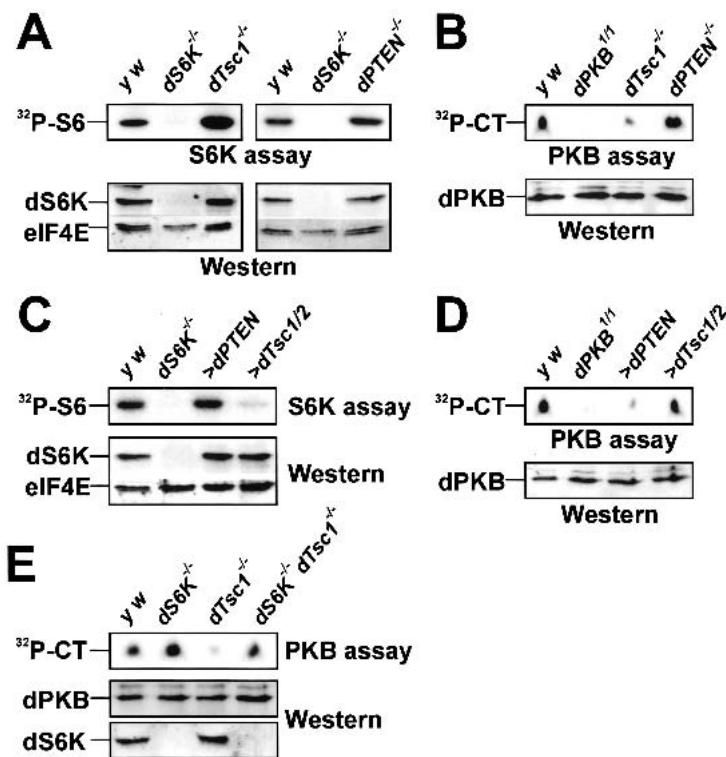
phosphorylation (Fig. 1, cf. E and F). Thus, loss of dTsc1/2, but not dPTEN, leads to constitutive dS6K activation.

To determine whether the findings above could be corroborated in the animal, dS6K activity was measured in extracts of *dTsc1*, *dPTEN*, and *dS6K* null larvae. The results show that dS6K activity in extracts derived from *dTsc1* null larvae was strongly increased over that of wild-type larvae, whereas it was slightly increased in larvae lacking *dPTEN* (Fig. 2A). The opposite was found for dPKB activity, which was strongly repressed in *dTsc1* null larvae, and up-regulated in *dPTEN*-deficient larvae (Fig. 2B). Hence, it cannot be excluded that reduced dPKB activity contributes to larval lethality of *dTsc* mutants.

Given that loss of dTsc function leads to increased dS6K activity, it was reasoned that ectopic expression of dTsc1/2, but not dPTEN, would inhibit dS6K activity. To test this hypothesis, both tumor suppressors were expressed ubiquitously in larvae using the GAL4/UAS system, such that the GAL4 promoter chosen in each case led to developmental arrest at late larval second instar. Extracts from larvae overexpressing dTsc1/2 display strongly reduced dS6K activity, whereas those from dPTEN overexpressing larvae have normal levels of dS6K activity (Fig. 2C). In contrast, dPKB activity is strongly suppressed in dPTEN overexpressing larvae and little affected in extracts from larvae overexpressing dTsc1/2 (Fig. 2D). These data corroborate previous findings that dS6K and dPKB act in parallel signal transduction pathways (Radimerski et al. 2002), and provide compelling evidence that they are negatively controlled by distinct tumor suppressor genes.

Despite the fact that dS6K and dPKB act in parallel signaling pathways, loss of dTsc1/2 function leads to inhibition of dPKB activity (Figs. 1C, 2B; Supplementary Fig. 1B), suggesting cross-talk between the two pathways. Compatible with such a model, recent studies have shown that rapamycin treatment of adipocytes inhibits a negative feedback loop, which normally functions to dampen insulin-induced PKB activation (Haruta et al. 2000). As RAD001 inhibits dS6K activity (Fig. 1B; Radimerski et al. 2002) and increases dPKB activity (Radimerski et al. 2002), it raised the possibility that the effects of *dTsc* mutants on dPKB are mediated through dS6K. Consistent with this hypothesis, inhibition of dPKB activity due to loss of dTsc function was relieved in the absence of *dS6K* (Fig. 2E). Similar results were obtained by using dsRNAi in cell culture (see Supplementary Fig. 2). Thus, the suppression of dPKB by loss of dTsc function requires dS6K.

To genetically test the specificity of *dTsc1/2* and *dPTEN* tumor suppressor function, either *dTsc1* or *dPTEN* were removed in cells giving rise to the adult eye structure, by inducing mitotic recombination with the *FLP/FRT* system under the control of the *eyeless* promoter (Newsome et al. 2000). In a wild-type genetic background, loss of either *dTsc1* or *dPTEN* within the developing eye causes strong overgrowth of the head (Fig. 3, cf. A–C; Gao et al. 2000; Gao and Pan 2001). Eye overgrowth by removal of *dTsc1* is strongly suppressed in a genetic background null for *dS6K*, as is ommatidia size (Fig. 3, cf. D and E), in agreement with a previous report analyzing double mutant clones of *dTsc2* and *dS6K* in the eye (Potter et al. 2001). In contrast, removal of *dPTEN* in the eyes of *dS6K* null flies still induces overgrowth of clones with enlarged ommatidia (Fig. 3, cf. D and F). These findings are supported by results showing that eye overgrowth by removal of *dTsc1* is still observed in clones devoid of dPKB function



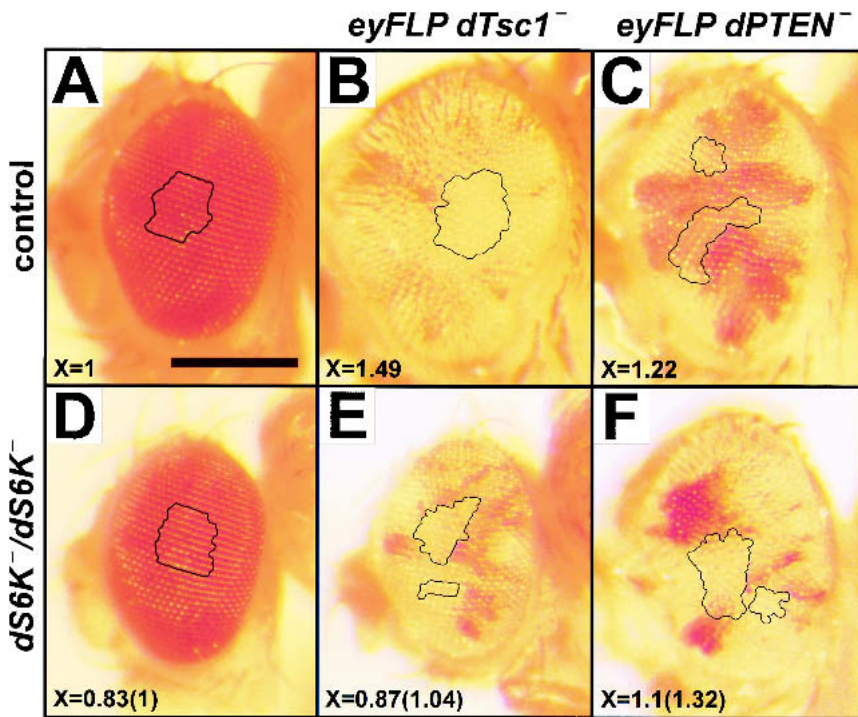
**Figure 2.** dTsc controls dS6K activity in vivo. (A) Analysis of dS6K activity from second instar larvae lacking either *dTsc1* (left) or *dPTEN* (right) compared with wild-type larvae (*y w*) and *dS6K* null larvae. (Top panels) In vitro dS6K kinase assay. (Bottom panels) Western blot detection of dS6K and eIF4E as loading control. (B) Analysis of dPKB activity from second instar *dTsc1* or *dPTEN* null larvae or larvae harboring a kinase inactive *dPKB*. (Top) In vitro dPKB kinase assay. CT, Crosstide. (Bottom) Western blot detection of dPKB. (C) Analysis of dS6K activity following either ubiquitous *dPTEN* (*da-GAL4>UAS-dPTEN*) or *dTsc1/2* overexpression (*act5C-GAL4>UAS-dTsc1/2*) in larvae. Panels are as in A. (D) Analysis of dPKB activity following either ubiquitous *dPTEN* (*da-GAL4>UAS-dPTEN*) or *dTsc1/2* overexpression (*act5C-GAL4>UAS-dTsc1/2*) in larvae. Panels are as in B. (E) Analysis of dPKB activity in larvae null for *dS6K*, null for *dTsc1*, and null for both *dS6K* and *dTsc1*. Panels are as in B. (Bottom) Western blot detection of total dS6K.

(Potter et al. 2001) and overgrowth by removal of *dPTEN* is suppressed in a viable *dPKB* mutant genetic background (Stocker et al. 2002). Thus, dTsc1/2 appears to be specific for the dS6K-signaling pathway, whereas *dPTEN* antagonizes PI3K signaling to counteract *dPKB* activation by decreasing PIP3 levels (Stocker et al. 2002).

As dTsc1/2 loss-of-function overgrowth in clones is suppressed by removing *dS6K* (Fig. 3E), it was reasoned that reducing increased dS6K activity in *dTsc1* loss-of-function larvae (Fig. 2A) might rescue second larval instar lethality. Consistent with this, feeding *dTsc1* null larvae low doses of RAD001, which induces a developmental delay of 3 d in wild-type larvae, allowed them to reach late wandering third larval instar (Fig. 4A). The *dTsc1* null larvae died shortly after pupation, presumably because wandering third instar larvae stop feeding and thus fail to receive the drug during pupal stages. To circumvent the problem of feeding, we attempted to reduce dS6K signaling by reducing the dosage of the gene. Compared with wild-type pupae (Fig. 4B, pupae 1), *dS6K*

null larvae are significantly reduced in size (Fig. 4B, pupae 2), and lack of one allele of *dTsc1* in this background had no significant effect on the *dS6K* null phenotype (Fig. 4B, pupae 3). Strikingly, the second instar lethality caused by lack of both *dTsc1* alleles was rescued to early pupal stages in the *dS6K* null background (Fig. 4B, pupae 4); however, these larvae were still small and severely delayed in development. In contrast, larvae in which one allele of *dS6K* had been removed in a *dTsc1* null background developed to early pupal stages with little developmental delay, although they were now significantly larger than wild type (Fig. 4B, pupae 5). On the basis of these latter findings, it was reasoned that further reduction of dS6K signaling, but not its abolishment, may allow *dTsc1* null animals to develop beyond early pupal stages. To test this in the *dTsc1* null background, we used either one allele of *dTOR* bearing a mutation in the kinase domain alone or in combination with one null allele of *dS6K*. *dTsc1* null larvae bearing one kinase mutant *dTOR* allele survived with higher frequency to pupae than animals with one null allele of *dS6K*, with a few emerging as adults (Table 1). However, genetically lowering dS6K signaling further by combining the *dTOR* and *dS6K* loss-of-function alleles, resulted in more than 60% of animals surviving to the adult stage (Table 1). The rescued females and males were slightly larger than wild-type flies, with overall patterning appearing normal (Fig. 4C). Furthermore, the rescued females were semifertile when crossed to wild-type males, whereas the rescued males were fully fertile when crossed to wild-type females (data not shown). Similarly, animals lacking *dTsc2* function (Canal et al. 1998; Ito and Rubin 1999) were rescued to viability (data not shown) by the same genetic approach applied above. Importantly, flies lacking one *dS6K* allele and bearing one kinase mutant *dTOR* allele display no obvious mutant phenotype (data not shown). Therefore, lowering but not abolishing dS6K signaling is sufficient to allow development of *Drosophila* lacking dTsc function.

Taken together, our results demonstrate that the tumor suppressor dTsc1/2 is a critical component in controlling dS6K activation. Interestingly, this effect may be dTOR independent, as insulin-induced dS6K activation is more elevated in dTsc1/2-depleted cells pretreated with RAD001 than in control cells (Fig. 1B; Supplementary Fig. 1), and in preliminary studies, clonal overgrowth in the eye induced by loss of *dTsc1* is not suppressed in a semiviable, heteroallelic *dTOR* mutant background. We further show that overexpression of dTsc1/2 selectively suppresses the dS6K-signaling pathway, whereas *dPTEN* operates on the dPI3K-signaling pathway (Fig. 2). It was shown recently that double mutations for *dPTEN* and *dTsc1* are additive for clonal overgrowth (Gao and Pan 2001), compatible with dS6K and dPKB independently mediating growth. Nevertheless, inhibition of dPKB by loss of dTsc function shows that there is negative cross-talk between the two signaling pathways (Fig. 2). Given this negative cross-talk, the observation that in double mutant clones growth is additive, suggests that in the absence of *dPTEN*, inhibition of dPKB by loss of dTsc is circumvented. However, despite



**Figure 3.** Overgrowth in eye imaginal tissues mediated by loss of *dTsc1*, but not by loss of *dPTEN*, requires *dS6K*. (A–F) Lateral views of eyes. Clusters of pigmented ommatidia correspond to heterozygous tissue. Absence of red pigment represents homozygous mutant tissue in which mitotic recombination occurred. Relative ommatidia size, X, was determined from either control or mutant tissues as described in Materials and Methods. The value in parenthesis represents relative ommatidia size in the *dS6K* null background. Genotypes: (A) *ey-FLP; FRT82B 3R3.7 P(w<sup>+</sup>)/+*, (B) *ey-FLP; FRT82B dTsc1<sup>Q87X</sup>/FRT82B 3R3.7 P(w<sup>+</sup>)*, (C) *ey-FLP; dPTEN<sup>117</sup> FRT40/P(w<sup>+</sup>) l(2)2L-3.1 FRT40*, (D) *dS6K<sup>11</sup> FRT82B dTsc1<sup>Q87X</sup>/dS6K<sup>11</sup> FRT82B 3R3.7 P(w<sup>+</sup>)*, (E) *ey-FLP; dS6K<sup>11</sup> FRT82B dTsc1<sup>Q87X</sup>/dS6K<sup>11</sup> FRT82B 3R3.7 P(w<sup>+</sup>)*, (F) *ey-FLP; dPTEN<sup>117</sup> FRT40/P(w<sup>+</sup>) l(2)2L-3.1 FRT40; dS6K<sup>11</sup>/dS6K<sup>11</sup>*. Bar, 0.25 mm.

the observation that double mutations for *dPTEN* and *dTsc1* are additive for clonal overgrowth, overgrowth induced by absence of *dPTEN* is suppressed in clones mutant for *dTOR* (Oldham et al. 2000; Zhang et al. 2000). As *dS6K* does not prevent such overgrowth (Fig. 3), it is possible that this suppression actually represents an intermediate phenotype, or that *dPTEN* negatively acts on a *dTOR* target distinct from *dS6K*. At this point, it is important to gain a deeper knowledge of the molecular mechanisms by which *dTsc1/2* acts to suppress *dS6K* function and how the signaling components of these two pathways crosstalk with one another.

Recently, a successful Phase I clinical trial was completed for a rapamycin analog in the treatment of solid tumors. The results of the trial demonstrated that the drug was efficacious at subtoxic doses, and suggested that specific tumor types may be more sensitive to inhibition by rapamycin than others (Hidalgo and Rowinsky 2000). The question that arose from the trial is, which tumors would be susceptible to rapamycin treatment? Here, we demonstrate for the first time in vivo that a mild reduction in *dS6K* signaling, which alone has no blatant phenotype, is sufficient to restore viability of flies devoid of *dTsc* function. Thus, these findings imply that rapamycin or its derivatives might be very promising pharmaceutical agents in the treatment of tumors arising from TSC.

**Table 1.** Reduction of *dS6K* signaling rescues the lethality of the *dTsc1* loss-of-function mutation

Genotype	Animals surviving to pupal stage	Animals surviving to adult stage	
$\frac{dTsc1^{Q87X}}{dTsc1^{Q87X}}$	0%	0%	(n = 80)
$\frac{dS6K^{11} dTsc1^{Q87X}}{+ dTsc1^{Q87X}}$	45%	0%	(n = 120)
$\frac{dTOR^{2L1}; dTsc1^{Q87X}}{+ dTsc1^{Q87X}}$	82.5%	18.5%	(n = 114)
$\frac{dTOR^{2L1}; dS6K^{11} dTsc1^{Q87X}}{+ + dTsc1^{Q87X}}$	93%	62%	(n = 125)

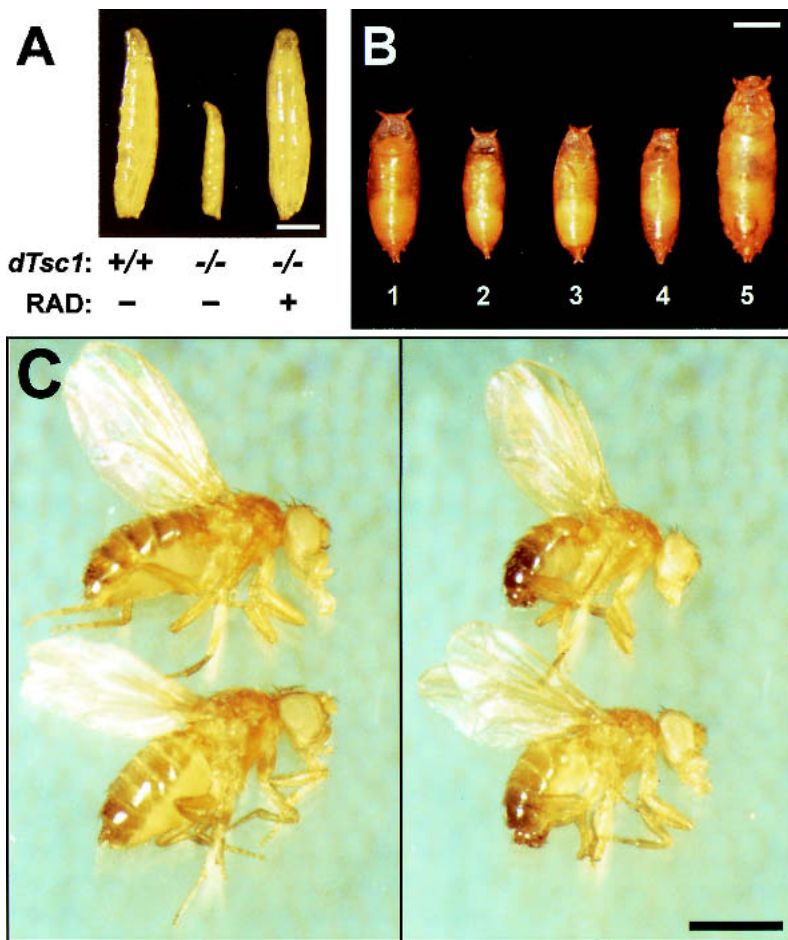
## Materials and methods

### Fly strains

The following null alleles were used: *dS6K<sup>11</sup>* (Montagne et al. 1999), *dTsc1<sup>Q87X</sup>* (Tapon et al. 2001), and *dPTEN<sup>117</sup>* (Stocker et al. 2002). *dS6K<sup>11</sup>* and *dTsc1<sup>Q87X</sup>* alleles were combined on the same chromosome by recombination. *dTOR<sup>2L1</sup>* (Oldham et al. 2000) and *dPKB<sup>1</sup>* (Staveley et al. 1998) encode kinase mutant proteins. *UAS-dPTEN* and *UAS-dTsc1* *UAS-dTsc2* strains were described previously (Goberdhan et al. 1999; Tapon et al. 2001). For ubiquitous GAL4 expression, *actin5C-GAL4* (*act-GAL4*) and *daughterless-GAL4* (*da-GAL4*) lines were used. Experiments were performed in the *y w* genetic background, crosses were set at 25°C, and adult phenotype analysis was done in females. Mosaic clones within the developing eye were generated by mitotic recombination using the *ey-FLP FRT* system as described (Newsome et al. 2000). Relative size of ommatidia was determined by measuring the area inscribed within the black outlines of individual eyes with the Adobe Photoshop 4.0 histogram function and dividing this value by the number of ommatidia within each outlined area. At least 50 ommatidia were counted for each genotype.

### Double-stranded RNA mediated interference

Double-stranded RNA (dsRNAi) was performed essentially as described (Clemens et al. 2000) with an incubation time of 1 wk. Cells were maintained and treated as reported (Radimerski et al. 2002). All primers were designed starting with the T7 RNA polymerase binding site as follows: 5'-TTAATACGACTCACTATAGGGAGA-3'. *dTsc1*; accession no.



**Figure 4.** Reduction of dS6K signaling rescues lethality caused by loss of dTsc1 function. (A) From left to right: +/+, y w third instar wandering larvae; -/-, dTsc1<sup>Q87X</sup>/dTsc1<sup>Q87X</sup> larvae (lethality occurs at late second instar stages); -/-/+, dTsc1<sup>Q87X</sup>/dTsc1<sup>Q87X</sup> third instar wandering larvae fed 30  $\mu$ M RAD001. (B) Dorsal view of pupal cases. Genotypes: (Pupae 1) y w; (pupae 2) dS6K<sup>11</sup>/dS6K<sup>11</sup>; (pupae 3) dS6K<sup>11</sup>/dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>; (pupae 4) dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>/dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>; (pupae 5) dTsc1<sup>Q87X</sup>/dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>. (C) Rescue of dTsc1 loss-of-function lethality by genetic reduction of dTOR/dS6K signaling. (Left) Upper fly, y w female; lower fly, dTOR<sup>2L1</sup>/+; dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>/dTsc1<sup>Q87X</sup> female. (Right) Upper fly, y w male; lower fly, dTOR<sup>2L1</sup>/+; dS6K<sup>11</sup> dTsc1<sup>Q87X</sup>/dTsc1<sup>Q87X</sup> male. Bars, 1 mm.

AF173560, sense-primer 436–453, antisense-primer 1081–1098, dTsc2; accession no. AF172995, sense primer 591–608, antisense primer 1371–1388, dPTEN; accession no. AF144232, sense primer 205–222, antisense primer 895–912. Total RNA was isolated with the RNeasy kit (Qiagen) according to the supplier's protocol and dTsc1, dTsc2, and dPTEN mRNA levels were analyzed by Quantitative Real Time PCR using the following probes: sense-primer 1640–1664; Taq 1667–1694; antisense-primer 1696–1716 of AF144232, sense-primer 1831–1850; Taq 1857–1874; antisense-primer 1878–1896 of AF172995, and sense-primer 1120–1138; Taq 1144–1166; antisense-primer 1179–1197 of AF173560.

#### In vitro kinase activity assays

Protein extracts of cultured cells or of larvae were prepared as reported (Oldham et al. 2000). Kinase activity of dS6K and dPKB was measured essentially as described (Radimerski et al. 2002). *Drosophila* atypical PKC was immunoprecipitated with the nPKC $\zeta$  (C-20) antibody (Santa Cruz Biotechnology) and activity assayed as for dS6K except for use of myelin basic protein (0.5  $\mu$ g) as substrate.

#### RAD feeding

RAD was prepared freshly from a 20-mM stock in ethanol by diluting to 30  $\mu$ M in water, and 100  $\mu$ L were distributed over the surface of standard

*Drosophila* cornmeal–molasses medium (10 mL), onto which flies had previously deposited roughly 150 eggs in short-term collections.

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