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.

Keywords: growth; TSC; S6K; TOR; PTEN; PKB

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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 tuberin (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 (Macleod 2000). 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 (Clement 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 signalings [Handa 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 phosphorylation, in agreement with the reduced electrophoretic mobility of dS6K [Fig. 1B].

Here, we show that the S6K pathway is a promising target for pharmaceutical intervention in tuberous sclerosis treatment.
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 promotor 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 [Pottet et al. 2001]. In contrast, removal of dPTEN in the eyes of dS6K null flies still induces overgrowth of ommatidia with larger 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.
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 semiferile 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 dTsc2 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, heterorallelic 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 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 larval 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

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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 cross-talk 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 pharmacological agents in the treatment of tumors arising from TSC.

Materials and methods

Fly strains

The following null alleles were used: dS6K-2 (Montagne et al. 1999), dTsc11q87X (Tapon et al. 2001), and dPTEN117 (Stocker et al. 2002). dS6K-1 and dTsc11q87X alleles were combined on the same chromosome by recombination. dTOR2L1 (Oldham et al. 2000) and dPKB1 (Staveley et al. 1998) encode kinase mutant proteins. UAS-dPTEN and UAS-dTsc1 UAS-dTsc2 strains were described previously (Coberdhan et al. 1999; Tapon et al. 2001). For ubiquitous GAL4 expression, act5C-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 (dsRNA) 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). Primers were designed starting with the T7 RNA polymerase binding site as follows: 5’-TTATACGACTACTATGGGAGA-3’. dTsc1, accession no.

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 |
|----------|----------------------------------|----------------------------------|
| dTsc11q87X/dTsc11q87X | 0% | 0% [n = 80] |
| dS6KΔ1/dTsc11q87X + dTsc11q87X | 45% | 0% [n = 120] |
| dTOR2L1/dTsc11q87X + dTsc11q87X | 82.5% | 18.5% [n = 114] |
| dTOR2L1/dS6KΔ1/dTsc11q87X + dTsc11q87X | 93% | 62% [n = 125] |
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; −/−, dTsc1Q87X/dTsc1Q87X larvae (lethality occurs at late second instar stages); −/−/+; dTsc1Q87X/dTsc1Q87X third instar wandering larvae fed 30 µM RAD001. (B) Dorsal view of pupal cases. Genotypes: (Pupae 1) y w; (pupae 2) dS6Kl1/dS6Kl1; (pupae 3) dS6Kl1/dS6Kl1 dTsc1Q87X/dTsc1Q87X; (pupae 4) dS6Kl1/dS6Kl1 dTsc1Q87X/dTsc1Q87X. (C) Rescue of dTsc1 loss-of-function lethality by genetic reduction of dTOR/dS6K signaling. (Left) Upper fly, y w female; lower fly, dTOR2[1]/+, dS6Kl1/dTsc1Q87X/dTsc1Q87X female. (Right) Upper fly, y w male; lower fly, dTOR2[1]/+, dS6Kl1/dTsc1Q87X/dTsc1Q87X male. Bars, 1 mm.

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 [Radmireski et al. 2002]. Drosophila atypical PKC was immunoprecipitated with the nPKC1 (C-20) antibody [Santa Cruz Biotechnology] and activity assayed as for dS6K except for use of myelin basic protein (0.5 µg) as substrate.

RAD feeding

RAD was prepared freshly from a 20-mM stock in ethanol by diluting to 30 µM in water, and 100 µL were distributed over the surface of standard Drosophila corneal–molasses medium (10 mL), onto which flies had previously deposited roughly 150 eggs in short-term collections.

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