

# Regulation of cellular growth by the *Drosophila* target of rapamycin *dTOR*

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The TOR protein kinases (TOR1 and TOR2 in yeast; mTOR/FRAP/RAFT1 in mammals) promote cellular proliferation in response to nutrients and growth factors, but their role in development is poorly understood. Here, we show that the *Drosophila* TOR homolog *dTOR* is required cell autonomously for normal growth and proliferation during larval development, and for increases in cellular growth caused by activation of the phosphoinositide 3-kinase (PI3K) signaling pathway. As in mammalian cells, the kinase activity of *dTOR* is required for growth factor-dependent phosphorylation of p70 S6 kinase (p70<sup>S6K</sup>) in vitro, and we demonstrate that overexpression of p70<sup>S6K</sup> in vivo can rescue *dTOR* mutant animals to viability. Loss of *dTOR* also results in cellular phenotypes characteristic of amino acid deprivation, including reduced nucleolar size, lipid vesicle aggregation in the larval fat body, and a cell type-specific pattern of cell cycle arrest that can be bypassed by overexpression of the S-phase regulator cyclin E. Our results suggest that *dTOR* regulates growth during animal development by coupling growth factor signaling to nutrient availability.

[Key Words: Rapamycin; TOR/FRAP; cell growth; cell cycle; *Drosophila*]

Received July 13, 2000; revised version accepted September 6, 2000.

During metazoan evolution, control of cell growth has evolved from a simple cell autonomous response to nutrient levels to a complex network of intercellular growth factor-mediated signals. Despite these additional layers of control, individual cells of higher eukaryotes have retained the ability to sense and respond directly to levels of nutrients such as amino acids (for review, see Kimball and Jefferson 2000). This more primal mode of regulation may serve as a checkpoint to gauge the appropriateness of intercellular growth signals, and coordination of these two types of regulatory inputs is likely to be essential for normal cell growth, proliferation, and survival.

The mammalian target of rapamycin (mTOR; also known as FRAP or RAFT1) has been implicated in such coordination. mTOR is a large protein kinase of the phosphoinositide kinase (PIK)-related family, whose other members include the DNA damage checkpoint proteins ATM, ATR, and DNA-PK (for review, see Keith and Schreiber 1995). Inactivation of mTOR with the macrolide rapamycin results in G<sub>1</sub> arrest and an attendant dephosphorylation of two of mTOR's targets, p70<sup>S6K</sup> and 4E-BP1 (for review, see Brown and Schreiber

1996; Dennis et al. 1999). These proteins regulate ribosome biogenesis and cap-dependent translation, respectively, and their phosphorylation is required to achieve the high levels of protein synthesis necessary for cell cycle entry.

Binding of growth factors such as insulin to their receptors in mammalian cultured cells causes rapid, PI3K-dependent phosphorylation of p70<sup>S6K</sup> and 4E-BP1 through a series of serine/threonine kinases including PDK1 and Akt/PKB (for a recent review, see Toker 2000). Although mTOR is required for PI3K-dependent p70<sup>S6K</sup> and 4E-BP1 phosphorylation, how it interacts with this signaling pathway is unclear. Akt can stimulate the kinase activity of mTOR immunoprecipitates and can phosphorylate mTOR in vitro (Scott et al. 1998; Navé et al. 1999; Sekulic et al. 2000). However, recent experiments have revealed that mutation of the Akt phosphorylation sites on mTOR has no effect on its kinase activity (Sekulic et al. 2000). Furthermore, addition of growth factors to serum-starved cells causes a robust increase in p70<sup>S6K</sup> and 4E-BP1 phosphorylation, but only a marginal increase in mTOR kinase activity (Burnett et al. 1998; Scott et al. 1998). In addition, specific truncation mutants of p70<sup>S6K</sup> have been identified whose PI3K-mediated phosphorylation is mTOR independent (Cheatham et al. 1995; Weng et al. 1995). These results suggest that signals from PI3K to p70<sup>S6K</sup> and 4E-BP1 do not pass through mTOR, and hence that mTOR and PI3K may converge on the same substrates through distinct pathways.

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Article and publication are at [www.genesdev.org/cgi/doi/10.1101/gad.835000](http://www.genesdev.org/cgi/doi/10.1101/gad.835000).

Loss of TOR activity in the yeast *Saccharomyces* causes a group of phenotypes nearly identical to those of cells starved for nutrients, including severely reduced protein synthesis, accumulation of glycogen, enlargement of the vacuole, induction of autophagy, and a specific pattern of gene expression (Kunz et al. 1993; Barbet et al. 1996; Noda and Ohsumi 1998; Cardenas et al. 1999; Hardwick et al. 1999). This has led to the idea that the yeast TOR proteins act as part of a nutrient-sensing mechanism. Remarkably, this function of TOR may also be conserved in higher eukaryotes. Withdrawal of amino acids in vitro or starvation in vivo results in rapid, reversible dephosphorylation of p70<sup>S6K</sup> and 4E-BP1, and rephosphorylation of these proteins after readdition of nutrients is blocked by rapamycin (Svanberg et al. 1997; Fox et al. 1998; Hara et al. 1998; Wang et al. 1998; Xu et al. 1998; Iiboshi et al. 1999). In contrast, activity of the PI3K/Akt or MAPK pathways is unaffected by amino acid levels in cell culture (Hara et al. 1998; Wang et al. 1998; Iiboshi et al. 1999). Furthermore, rapamycin-resistant mutants of p70<sup>S6K</sup> are also resistant to dephosphorylation by amino acid withdrawal (Hara et al. 1998). These data support a model in which full activation of p70<sup>S6K</sup> and eIF4E requires two distinct signals, one in response to growth factors and another from an amino acid sensing pathway, and thus provide a mechanism whereby individual cells can coordinate their responses to growth factors with nutrient availability. However, changes in TOR kinase activity in response to amino acids or other nutrients have not been observed (Kleijn and Proud 2000), and thus the mechanisms by which TOR is regulated remain unclear.

To date, studies of mammalian TOR have relied on inactivation by rapamycin, as TOR mutations have not yet been reported in multicellular organisms. Although rapamycin mimics many of the effects of TOR mutational inactivation in yeast, some essential functions of TOR2, such as polarization of the actin cytoskeleton, are not inhibited by rapamycin (Zheng et al. 1995; Schmidt et al. 1996). This may be the case in higher eukaryotes as well, as it has been shown that rapamycin does not fully inhibit the kinase activity of mTOR (Burnett et al. 1998; Peterson et al. 2000). Furthermore, the kinase domain of mTOR constitutes only ~10% of the total protein, and the in vivo function of the remainder is essentially unknown.

To initiate a genetic analysis of TOR in a multicellular organism, we have generated mutations in the *Drosophila* TOR homolog, *dTOR*, and have used these mutants to study the role of *dTOR* during development. The PI3K/Akt/p70S6K signaling module is conserved in *Drosophila*, where it acts to regulate cell, organ, and organismal growth (for review, see Coelho and Leivers 2000). Mutational inactivation of this pathway reduces cell size, hinders proliferation, and delays or arrests development, and its activation leads to autonomous increases in cell and organ size. We find that *dTOR* mutant phenotypes recapitulate aspects of both PI3K-dependent signaling and nutritional sensing, consistent with *dTOR* acting at the junction of these pathways.

## Results

### Identification of mutations in *dTOR*

The *Drosophila* genome encodes four members of the PIK-related family: *mei41*, which encodes an ATR/ATM homolog required for cell cycle arrest in response to DNA damage (Hari et al. 1995); *CG6535*, a second ATM-related gene of unknown function (Adams et al. 2000); *CG4549*, whose closest relative encodes the nonsense-mediated mRNA decay protein SMG-1 in *C. elegans* (O'Connor and Anderson 1999; Adams et al. 2000); and the *dTOR* gene described here. A fifth member of this family, the DNA-dependent protein kinase, is not found in *Drosophila* or *C. elegans*.

Using a combination of cDNA library screening and RACE (5' rapid amplification of cDNA ends; see Materials and Methods), we isolated overlapping cDNAs that together contain a large ORF of 2471 amino acids with strong similarity to mammalian mTOR and to TOR1 and TOR2 from budding yeast. Subsequently, the identical ORF was identified by computational analysis of the annotated *Drosophila* genome (CG5092, GenBank accession no. AE003638; Adams et al. 2000). Sequence comparisons revealed that the predicted protein, which we have named *dTOR*, is 56% and 38% identical to human mTOR and yeast TOR2, respectively, with the highest levels of identity in the carboxy-terminal region containing the putative kinase and rapamycin/FKBP12-binding domains (73% identity with mTOR over the carboxy-terminal 675 amino acids; Fig. 1). Additional structural motifs were also found to be well-conserved, including a series of HEAT repeats in the amino-terminal half of the protein, a domain shown to bind the peripheral membrane protein gephyrin (Sabatini et al. 1999), and a short sequence at the extreme carboxyl terminus of essential but unknown function that is highly conserved amongst PIK-related family members (Keith and Schreiber 1995; Peterson et al. 2000). Interestingly, previously described sites of autophosphorylation (Peterson et al. 2000) and phosphorylation by Akt/PKB (Scott et al. 1998; Navé et al. 1999; Sekulic et al. 2000) are not conserved in *dTOR* (Fig. 1). Genomic DNA blot analysis and in situ hybridization to larval polytene chromosomes revealed that *dTOR* is a single copy gene (data not shown), as was confirmed by *Drosophila* genomic sequencing (Adams et al. 2000).

To begin a mutational analysis of *dTOR*, we searched the Berkeley *Drosophila* Genome Project P-element database and identified two independent homozygous lethal lines bearing P insertions in the *dTOR* gene (designated here as *dTOR*<sup>P1</sup> and *dTOR*<sup>P2</sup>). Mobilization of these elements restored viability to each chromosome, indicating that the insertions are responsible for the associated lethality. Comparison of the insertion sites of *dTOR*<sup>P1</sup> and *dTOR*<sup>P2</sup> with the *dTOR* transcription unit revealed that the P-elements are inserted at 24 and 74 bases downstream of the *dTOR* transcription start site, respectively (Fig. 2A), and would likely interfere with normal *dTOR* expression.

To generate additional *dTOR* alleles, a series of dele-

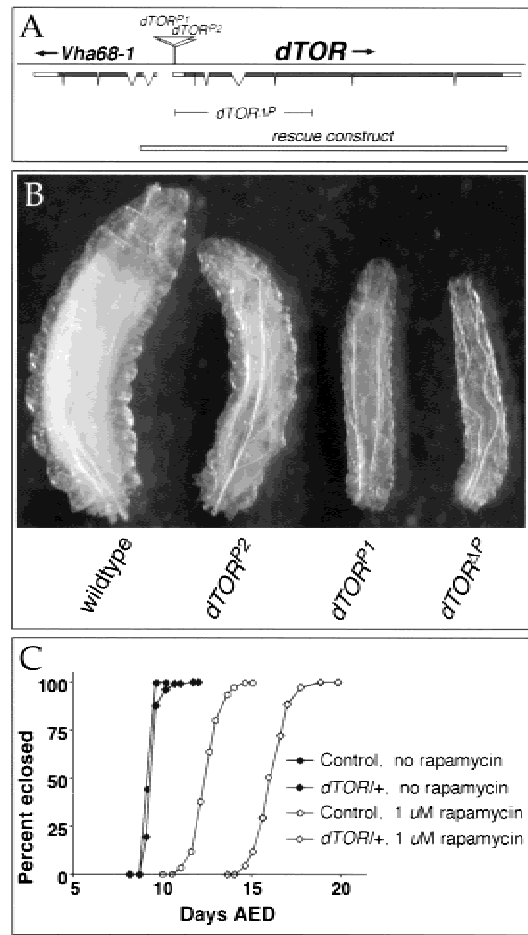
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dTOR 1 -----VSTISVQQVVMKSKPFRNRYQADTQDLFFKTELREMSQEE
mTOR 1 NLTGTGPAATTAATTSVNVQVQVQASGLKSKSEETRAAKKQVHVVHFRMSQEE
dTOR 46 AQTDFDFHLLTUNVALRDEKKGKAKKCLNHCISLTAQRKISPIVLRQRDLIL
mTOR 46 TRDFDQNLNHLTELVSSDQNEKKGKAKKCLNHCISLTAQRKISPIVLRQRDLIL
dTOR 106 DVSNVEEAAISVVKLHMPTSKGKASDFDQKQKQFVWQGERKRRKRSVILRELA
mTOR 106 DVSNVEEAAISVVKLHMPTSKGKASDFDQKQKQFVWQGERKRRKRSVILRELA
dTOR 166 LPTTFQPHLITFEVFEVLRPKQAFREISGCEKLLALVVAARESTQSSSPQWYIC
mTOR 166 LPTTFQPHLITFEVFEVLRPKQAFREISGCEKLLALVVAARESTQSSSPQWYIC
dTOR 206 VDFMGSFMADESSKQDQKQVDDRRRHGHLVWVNLFLQCAMATMEQRYTSIKTIFP-KT
mTOR 206 VDFMGSFMADESSKQDQKQVDDRRRHGHLVWVNLFLQCAMATMEQRYTSIKTIFP-KT
dTOR 285 VQVNLLEASSSSSPQLNIVYRLKLVFFDKLGS-ITHTGEGEHLQVAKFAS-----
mTOR 285 VQVNLCKDLMG--FQKPRHITFTISFQAQPPQSVALVGLGYSYQVIMGFASLSPSPA
dTOR 339 --HNVLESAVAQSEIHTDITSDQMLEGKSSKSPYQVALLLPLRANFRANVFC-KY
mTOR 339 KSTLEKCCCRDLEKEDQDQVWVVKCKRASSNSLDMTELLRFRALFRPSAATDITD
dTOR 387 QATQSRHVIQKCKEKRAVADITRHYAVAVQSAIEDLSSSTITSVVALSKDLTS
mTOR 387 QATQSRHVIQKCKEKRAVADITRHYAVAVQSAIEDLSSSTITSVVALSKDLTS
dTOR 457 PK--VDFPAPVATVCTLLARKESEADKQKLELQVFIQSPALVGRSESNVPL
mTOR 457 PK--VDFPAPVATVCTLLARKESEADKQKLELQVFIQSPALVGRSESNVPL
dTOR 515 SASITGEGHLSVWMAAILVYTAIPTAIDG-----MNRGQVITVVALTLGL
mTOR 515 KCDGGLKXISVWMAAILVYTAIPTAIDG-----MNRGQVITVVALTLGL
dTOR 570 VVEEQNLLIYQKQADVHVAEATEERVATCRLKLAQR--SESSEMSKLSST
mTOR 570 VVEEQNLLIYQKQADVHVAEATEERVATCRLKLAQR--SESSEMSKLSST
dTOR 628 VSHVRELLVITVDQDNRIRPILRSLDETFQKLAGPESNSFITHDFEIRELA
mTOR 628 VSHVRELLVITVDQDNRIRPILRSLDETFQKLAGPESNSFITHDFEIRELA
dTOR 688 VVTFGLSSNPAVMPKLTITDEITDQKYSNPKVQSAKMLQHVLSIPRISSTY
mTOR 688 VVTFGLSSNPAVMPKLTITDEITDQKYSNPKVQSAKMLQHVLSIPRISSTY
dTOR 748 VPIKALVKKKHEPSS--NPSVWVLRISQVLAQNGSSDELRADLLEGL
mTOR 748 VPIKALVKKKHEPSS--NPSVWVLRISQVLAQNGSSDELRADLLEGL
dTOR 805 VAGSPDQKVALNLSQLLSAIVKVVYKTPVLELILVFLKTEQRSSQREITRVLQ
mTOR 805 VAGSPDQKVALNLSQLLSAIVKVVYKTPVLELILVFLKTEQRSSQREITRVLQ
dTOR 866 LLGALDPPKKNKGLDTSQVNLVAYDGRVDE--QDLSAEELVNMGN--ALREYVA
mTOR 866 LLGALDPPKKNKGLDTSQVNLVAYDGRVDE--QDLSAEELVNMGN--ALREYVA
dTOR 924 VAVLALRLLRPLSTLHSSVWRAIFLQVSLKCFVCLVPLDNDVITADVW
mTOR 924 VAVLALRLLRPLSTLHSSVWRAIFLQVSLKCFVCLVPLDNDVITADVW
dTOR 984 EFLTQDLVAVVAVKLLSNGVFLMKEPITITPQVITLITLQDVALVGEGLD
mTOR 1010 EFLTQDLVAVVAVKLLSNGVFLMKEPITITPQVITLITLQDVALVGEGLD
dTOR 1044 VAEIPRIRLGRNKSQVITLQALRQVQSTVYVQVILSPVKKVDSVYVQ
mTOR 1070 VAEIPRIRLGRNKSQVITLQALRQVQSTVYVQVILSPVKKVDSVYVQ
dTOR 1184 VQVWVLELITNNAACQLPFDTSRSLRHPVVRDAEPELRDQANTLRSKAKLGGKRL
mTOR 1130 PFSKALELITDRTELEDFDIASTFLHPVVRDAEPELRDQANTLRSKAKLGGKRL
dTOR 1164 VVFNWVLTNHRITVDPPEEELSSKSKCSLADSYGAGES--ELRPSFKLWEPFVQD
mTOR 1190 VVFNWVLTNHRITVDPPEEELSSKSKCSLADSYGAGES--ELRPSFKLWEPFVQD
dTOR 1222 --RNSMNVQVWVNEVRIQVTVTRVSKDNEVLRSLISLLEKSSHAHARRSLADE
mTOR 1250 VETGPMKLVSTINQKGAARVSKDNEVLRSLISLLEKSSHAHARRSLADE
dTOR 1281 QVTLQDLFNAATSCHEVNEQVQKLLRSJELQVTSQDQVITLILNLAEFMCH
mTOR 1310 QVTLQDLFNAATSCHEVNEQVQKLLRSJELQVTSQDQVITLILNLAEFMCH
dTOR 1341 DQVETIK----LGLRMAQVAKALRKYBETLLREDSQVIFESLITLNMKQQRAR
mTOR 1370 DQVETIK----LGLRMAQVAKALRKYBETLLREDSQVIFESLITLNMKQQRAR
dTOR 1387 EGILTRYRMAANEINDRNYERKLVMDVAREHEINIKKSSD--BAR--GHNCLGALG
mTOR 1430 EGILTRYRMAANEINDRNYERKLVMDVAREHEINIKKSSD--BAR--GHNCLGALG
dTOR 1457 VESLSVTKHEEMFQTAKSAQPHAVVAVGQVQVAREVTRVRC--DQDQSYVYAV
mTOR 1490 VESLSVTKHEEMFQTAKSAQPHAVVAVGQVQVAREVTRVRC--DQDQSYVYAV
dTOR 1517 VAVVQDREIACQLIKRQLLELSMAGESNSRANGAMVAVNMALEEVYQKLV
mTOR 1550 VAVVQDREIACQLIKRQLLELSMAGESNSRANGAMVAVNMALEEVYQKLV
dTOR 1577 EEREPIKTVVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQV
mTOR 1610 EEREPIKTVVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQVQV
dTOR 1637 KRLVNLLEQDQKLPNPLPCQPMVYAVVQVAAANMLQAEGLQVHVSIVSLELS
mTOR 1670 KRLVNLLEQDQKLPNPLPCQPMVYAVVQVAAANMLQAEGLQVHVSIVSLELS
dTOR 1687 ---CLPPEALQDQKLPNPLPCQPMVYAVVQVAAANMLQAEGLQVHVSIVSLELS
mTOR 1730 HAALEEDQKQLHLNARQKQKQVPLNLOG--VNSTPKVIVSIVSIVSIVSIVSIVS
dTOR 1754 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 1789 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 1791 LQSLMIAR--VAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 1849 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 1830 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 1909 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 1890 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 1969 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 1950 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2029 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2010 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2089 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2070 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2149 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2130 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2209 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2190 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2269 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2250 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2329 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2310 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2389 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2370 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2446 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
dTOR 2427 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
mTOR 2506 VVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVVAVV
    
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tions spanning the *dTOR* gene was generated by imprecise mobilization of the P-elements. One such mutant, designated *dTOR<sup>ΔP</sup>*, was selected for further analysis. Sequence analysis revealed that the *dTOR<sup>ΔP</sup>* deletion originates at the *dTOR<sup>P2</sup>* insertion site and extends 3514

**Figure 1.** Comparison of *Drosophila* and human TOR proteins. The ClustalW 1.8 algorithm was used to align the amino acid sequences of dTOR and human mTOR/FRAP (GenBank accession no. 744518). Dark boxes indicate identical amino acids; gray boxes indicate similarity. The conserved FKBP12-rapamycin-binding domain (amino acids 2025–2114 in mTOR; Vilella-Bach et al. 1999) is underlined, and the essential serine in this domain is indicated by an asterisk. Sites of mTOR autophosphorylation (Ser-2481) and phosphorylation by Akt/PKB (Ser-2448) are marked with carats.



**Figure 2.** Mutations in *dTOR* inhibit larval growth. (A) The structure of the 8.0-kb *dTOR* transcript is shown. Coding sequence is indicated by filled boxes; 5' and 3' untranslated regions are represented by open boxes. Breaks in the boxes indicate introns. Sites of the two P-element insertions in *dTOR* are indicated, and the range of the *dTOR<sup>ΔP</sup>* deletion is shown. The stippled bar at the bottom of the figure indicates the DNA fragment used for genomic rescue. *Vha68-1* encodes a mitochondrial ATPase transcribed in the opposite direction as *dTOR*. (B) Size comparison of wild-type and *dTOR* homozygous mutant larvae at 126 h of development. Wild-type larvae pupate within 12 h of this time point, whereas *dTOR* mutants remain arrested in the larval stage with little or no further growth. (C) Heterozygosity for *dTOR* sensitizes larvae to rapamycin. On normal food, wild-type (black circles) and *dTOR*/+ larvae (black diamonds) grow at indistinguishable rates. Addition of 1 μM rapamycin delays development by ~3 d in wild-type (open circles) and ~6 d in *dTOR*/+ larvae (open diamonds).

bp downstream, removing the *dTOR* translation start site and amino-terminal 902 codons, and thus likely represents a null allele of *dTOR* (Fig. 2A). This was confirmed by the absence of detectable dTOR protein in immunoblots of *dTOR*<sup>ΔP</sup> larval extracts (data not shown). A 9.4-kb genomic rescue construct encompassing the *dTOR* gene and no other predicted transcription units (Fig. 2A) restored full viability and fertility to *dTOR*<sup>ΔP</sup> homozygotes. Therefore, the phenotypes described below are due to loss of *dTOR* function.

#### *dTOR* is required for normal growth

*dTOR*<sup>ΔP</sup> homozygotes were found to hatch at normal rates, but grew more slowly than normal and eventually arrested during larval development, reaching only 24% the mass of wild-type controls (Fig. 2B). Larvae homozygous for *dTOR*<sup>P1</sup> or *dTOR*<sup>P2</sup> alleles displayed a less severe phenotype, eventually growing to approximately 40% and 79% the mass of wild type, respectively, indicating that these alleles likely retain partial *dTOR* function. In each case, the mutants remained viable and active during an extended larval period of ~30 d, and eventually died without pupating. Larvae heterozygous for *dTOR* grew at a rate indistinguishable from wild-type controls under normal culture conditions, but were hypersensitive to low concentrations of rapamycin (Fig.

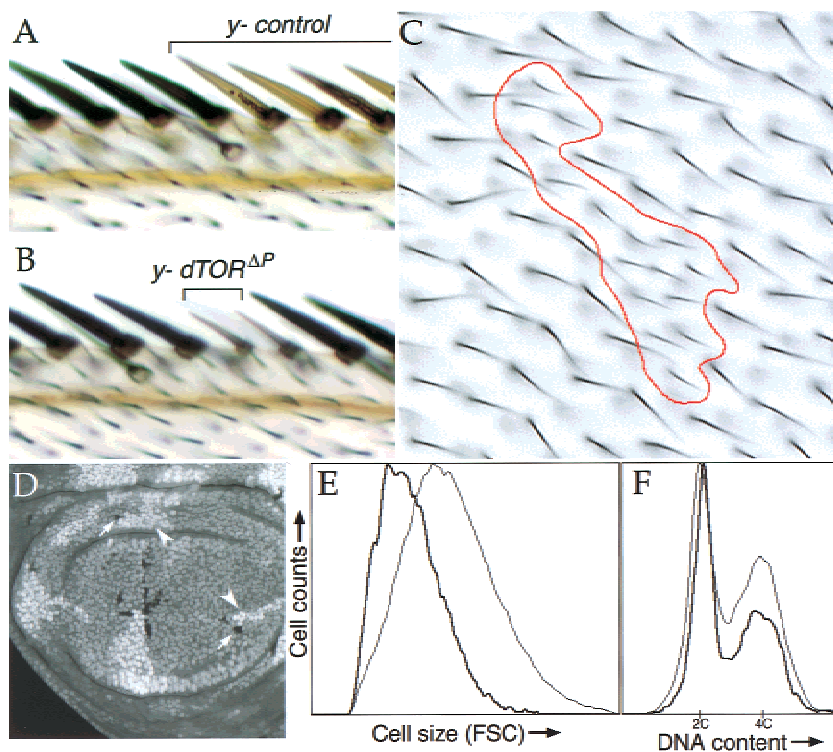
2C). Thus, *dTOR* encodes a rapamycin-sensitive protein required for normal growth during larval development.

#### Cells lacking dTOR are reduced in size and proliferative capacity

Overall growth of an organism is generally accompanied by increases in cell number (proliferation), cell size (hypertrophy), or both (for review, see Conlon and Raff 1999). To determine how mutations in *dTOR* inhibit growth, we examined these parameters in a number of tissues.

The effect of *dTOR* on cell size was analyzed in marked clones of *dTOR*<sup>ΔP</sup> homozygous cells, which were generated by FLP/FRT-mediated mitotic recombination in *dTOR*<sup>ΔP</sup> heterozygous animals. Examination of adult cuticular structures revealed that *dTOR* homozygous mutant cells were markedly reduced in size. For example, bristles of the wing margin that lack *dTOR* were both thinner and shorter than adjacent wild-type cells (Fig. 3, cf. A and B). Area measurements of mutant clones in the wing epithelium (Fig. 3C) showed that *dTOR*<sup>ΔP</sup> mutant cells were approximately half (56%) the size of controls ( $n = 498$  cells). Similar effects were observed in the eye, abdomen, and notum (data not shown).

To determine whether loss of *dTOR* affected the size of actively proliferating cells, we examined *dTOR* mu-



**Figure 3.** *dTOR* clonal phenotypes. (A–C) Loss of *dTOR* reduces the size of cells in the adult wing. Clones of *y*-marked wild-type (A) or *dTOR*<sup>ΔP</sup> (B,C) cells were induced during the first larval instar by FLP/FRT-mediated mitotic recombination. Representative clones including bristles of the wing margin (A,B) and epithelial cells of the wing blade (C) are shown. The red trace in C outlines a *dTOR*<sup>ΔP</sup> mutant clone. Each hairlike structure is a trichome emanating from a single epithelial cell. Note the reduced size and increased density of *dTOR* cells. Genotype of A–C: *y, w, HS-FLP<sup>122</sup>/+; dTOR<sup>ΔP</sup> FRT40A/Py<sup>+</sup> FRT40A*. (D–F) Phenotypes of *dTOR* mutant cells during proliferation. (D) Confocal image of wing imaginal disc containing *dTOR*<sup>ΔP</sup> clones marked with ubiquitin–GFP. By 72 h after induction, *dTOR*<sup>ΔP</sup> mutant clones (indicated by arrows; cells lack marker) contain fewer cells than their wild-type twispot clones (indicated by arrowheads; cells carry two copies of GFP marker). (E,F) FACS histograms of dissociated cells from wing discs containing *dTOR*<sup>ΔP</sup> clones, as in D. *dTOR*<sup>ΔP</sup> cells and wild-type control cells are indicated by heavy and light traces, respectively. Cells lacking *dTOR* have reduced forward light scatter (FSC) values, indicating a smaller cell size (E). Populations of *dTOR* mutant cells also have a lower fraction of cells in S and G<sub>2</sub> phases of the cell cycle (F). Genotype of D–F: *y, w, HS-FLP<sup>122</sup>/+; dTOR<sup>ΔP</sup> FRT40A/Ubi-GFP FRT40A*.

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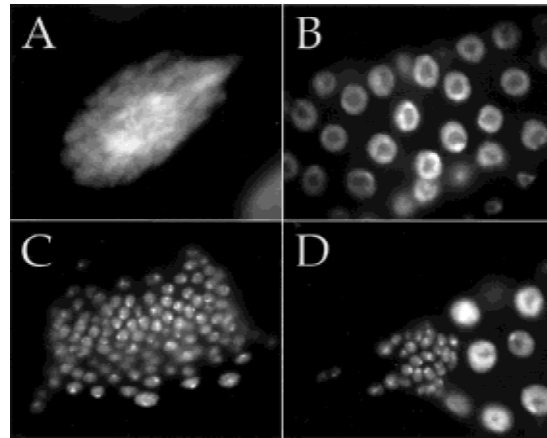
tant clones in the developing imaginal discs, epithelial primordia that proliferate mitotically to give rise to adult structures. Imaginal wing discs containing GFP-marked clones of *dTOR*<sup>ΔP</sup> homozygous cells (Fig. 3D) were dissociated into single cells, which were then analyzed by flow cytometry. The mean forward light scatter value (a measure of cell size) of *dTOR* mutant cells was decreased by 30% compared to wild-type control cells from the same discs (Fig. 3E). This decrease in cell size was observed in all phases of the cell cycle (data not shown). Thus, loss of *dTOR* causes a cell autonomous reduction in the size of both proliferating and postmitotic cells.

Fluorescence-activated cell sorter (FACS) analysis also revealed that the cell cycle phasing of *dTOR* cells differed significantly from that of controls (Fig. 3F), with relatively more cells in G<sub>1</sub>, and fewer in S and G<sub>2</sub> phases. This is consistent with the ability of rapamycin to induce G<sub>1</sub> arrest in yeast and in mammalian cell culture (Heitman et al. 1991; Chung et al. 1992). To measure proliferation rates of *dTOR* mutant cells, we compared the number of cells in *dTOR*<sup>ΔP</sup> clones with that of their wild-type sister clones (twin spots). Clones of *dTOR*<sup>ΔP</sup> mutant cells were similar in size to their twin spots at 48 h after induction (data not shown), but by 72–96 h they contained significantly fewer cells (Fig. 3D), indicating that loss of *dTOR* leads to a reduced rate of cell proliferation. In addition, lone twin spots lacking a corresponding mutant sister clone were occasionally observed at 96 h after induction, indicating that at some frequency *dTOR*<sup>ΔP</sup> homozygous cells were eliminated from the disc epithelium. Because *dTOR*<sup>ΔP</sup> cells remain viable for weeks in the context of a homozygous mutant animal (see above and Fig. 2B), the loss of *dTOR*<sup>ΔP</sup> mutant clones is likely the result of cell competition with adjacent wild-type cells, as has been described previously for cells with a growth disadvantage (Morata and Ripoll 1975).

Growth properties of cells in the salivary glands of homozygous *dTOR*<sup>ΔP</sup> larvae were also examined. The salivary gland is comprised of two cell types: polytene gland cells that undergo multiple rounds of endoreduplication to generate giant nuclei with a ploidy of up to 2048 C, and imaginal ring cells that remain diploid and cycle mitotically. Loss of *dTOR* affected both cell types. The endoreplicative cells in *dTOR*<sup>ΔP</sup> salivary glands underwent only four to five rounds of replication before entering quiescence (see below), reaching a ploidy of 16–32C and a size ~10% that of wild type (Fig. 4A,B). The imaginal rings in *dTOR*<sup>ΔP</sup> larvae contained approximately fivefold fewer cells than wild type (Fig. 4C,D). Together, our results indicate that *dTOR* is required to promote cell cycle progression in both mitotic and endoreplicative cells, and acts primarily at the G<sub>1</sub>/S transition.

#### Growth stimulation by the PI3K pathway requires dTOR

The cell autonomous reduction in the size of *dTOR* mutant cells is reminiscent of mutations in components of

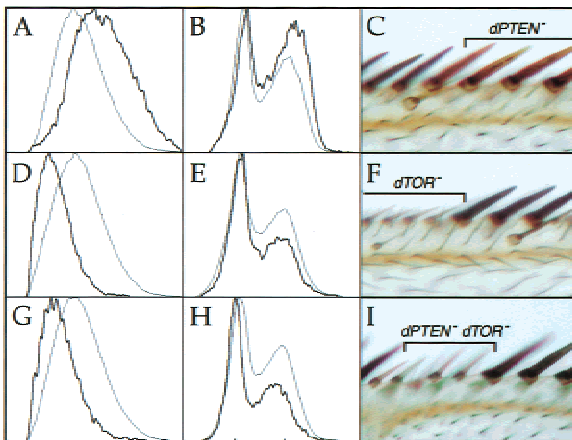


**Figure 4.** Both mitotic and endoreplicative cell cycles require *dTOR*. Salivary gland nuclei stained with Hoechst 33258 are shown for wild type (A,C) and *dTOR*<sup>ΔP</sup> (B,D) at 5–6 d of development. (A–D) Same magnification. (A) A single endoreplicative nucleus of ~1024 C from a wild-type salivary gland. Endoreplicative salivary gland nuclei from *dTOR*<sup>ΔP</sup> larvae reach a ploidy of only 16–32 C (B). Proliferative diploid cells of the imaginal ring reach ~fivefold greater numbers in wild-type (C) than *dTOR*<sup>ΔP</sup> (D) salivary glands.

the PI3K/S6K signaling pathway (Coelho and Leever 2000). Mutations in *dPTEN*, the fly homolog of the PTEN tumor suppressor, cause activation of this pathway, leading to increased cell growth. To determine whether *dTOR* is required for PI3K-dependent signaling, we examined the growth properties of cells lacking both *dPTEN* and *dTOR*. As we have shown previously (Gao et al. 2000), clonal loss of *dPTEN* caused enlargement of imaginal and adult cells, and increased the percentage of cells in the S and G<sub>2</sub> phases of the cell cycle (Fig. 5A–C). In contrast, cells carrying null alleles of both *dPTEN* and *dTOR* were indistinguishable from cells lacking *dTOR* alone, with a similar reduction in cell size and accumulation in G<sub>1</sub> (Fig. 5G–I). Loss of *dTOR* also prevented the increased proliferation caused by mutations in *dPTEN* (data not shown). Cells mutant for weaker alleles of *dPTEN* and *dTOR* (MGH1 and P2, respectively) were intermediate in size (data not shown), indicating that *dTOR*<sup>P2</sup> cells retain partial signaling function. We conclude that *dTOR* is epistatic to *dPTEN*, and therefore, that *dTOR* functions at a step downstream of or in parallel to PI3K signaling.

#### *p70*<sup>S6K</sup> is a critical effector of dTOR in vivo

In further tests of dTOR's role in PI3K/S6K signaling, we found that rapamycin inhibited the serum-dependent phosphorylation of *Drosophila* p70<sup>S6K</sup> (dS6K) expressed in S<sub>2</sub> cells (Fig. 6A, lane 2), as reported previously (Stewart et al. 1996). Dephosphorylation of dS6K by rapamycin was prevented by cotransfection of a *dTOR* point mutant containing a Ser<sup>1956</sup> to Thr substitution (*dTOR*<sup>RR</sup>; Fig. 6A, lane 6), which confers rapamycin resistance to mammalian and yeast TOR proteins. Expression of *dTOR*<sup>RR</sup> carrying an additional point mutation in a residue cru-



**Figure 5.** *dTOR* acts downstream of *dPTEN*. Clones of cells mutant for null alleles of *dPTEN* (A–C), *dTOR* (D–F), or *dPTEN* and *dTOR* (G–I) were induced by FLP/FRT-mediated mitotic recombination. Mutant cells were marked by the absence of GFP or *y+* as in Fig. 3. The leftmost columns depict histograms of wing imaginal disc cells analyzed by flow cytometry; dark traces indicate mutant cells, and light traces represent wild-type control cells from the same discs. The right column shows photomicrographs of mutant clones in the anterior margin of the adult wing. Loss of *dPTEN* causes cell enlargement (A,C), and increases the proportion of cells in the S and G<sub>2</sub> phases of the cell cycle (B). Loss of *dTOR* decreases cell size (D,E) and reduces the S and G<sub>2</sub> populations (F). Cells lacking *dTOR* and *dPTEN* are indistinguishable from cells lacking *dTOR* alone (G–I). Genotypes: (A,B) *y, w, HS-FLP<sup>122/+</sup>, dPTEN<sup>DJ189</sup> FRT40A/Ubi-GFP FRT40A*; (C) *y, w, HS-FLP<sup>122/+</sup>, dPTEN<sup>DJ189</sup> FRT40A/Py+ FRT40A*. (D,E) *y, w, HS-FLP<sup>122/+</sup>, dTOR<sup>ΔP</sup> FRT40A/Ubi-GFP FRT40A*; (F) *y, w, HS-FLP<sup>122/+</sup>, dTOR<sup>ΔP</sup> FRT40A/Py+ FRT40A*. (G,H) *y, w, HS-FLP<sup>122/+</sup>, dTOR<sup>ΔP</sup> dPTEN<sup>DJ189</sup> FRT40A/Ubi-GFP FRT40A*; (I) *y, w, HS-FLP<sup>122/+</sup>, dTOR<sup>ΔP</sup> dPTEN<sup>DJ189</sup> FRT40A/Py+ FRT40A*.

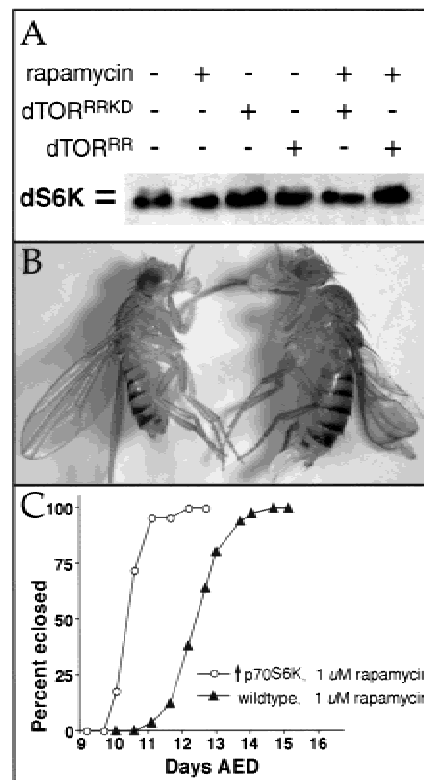
cial for kinase activity (*dTOR<sup>RRKD</sup>*; Fig. 6A, lane 5) failed to protect *dS6K* from rapamycin-induced dephosphorylation, indicating that the kinase function of *dTOR* is required to maintain *dS6K* phosphorylation.

To determine whether these biochemical interactions between *dTOR* and *dS6K* were relevant to their functions *in vivo*, we tested for genetic interactions between them. Remarkably, constitutive overexpression of *Drosophila dS6K* or human *p70<sup>S6K1</sup>* was able to rescue *dTOR<sup>P2/P2</sup>* and *dTOR<sup>P1/P2</sup>* flies to viability (Fig. 6B). The greatest degree of rescue was provided by a mutant version of *p70<sup>S6K1</sup>*, in which four mitogen-induced phosphorylation sites are mutated to aspartate and glutamate residues (mutant D4; Cheatham et al. 1995). Expression of this construct allowed 74% of expected *dTOR<sup>P1/P2</sup>* progeny to survive to adulthood, whereas no *dTOR<sup>P1/P2</sup>* animals survived in the absence of *S6K* overexpression. *dTOR* flies rescued by *S6K* overexpression were slightly smaller than wild-type controls (Fig. 6B), but were fertile and developed at a similar rate as wild type. Although overexpression of *S6K* did not rescue *dTOR<sup>ΔP</sup>* null mutants to adulthood, it did enable them to progress to the pupal stage (data not shown). Overexpression of *S6K* in wild-type larvae also conferred significant resistance to

rapamycin (Fig. 6C). Again, constitutively active *p70<sup>S6K1</sup>* provided the greatest degree of rapamycin resistance. Together, these results indicate that a major function of *dTOR* is to maintain levels of active *S6K* sufficient for normal growth.

#### Amino acid withdrawal phenocopies of *dTOR* mutant phenotypes

Like *dTOR* mutants, wild-type larvae deprived of amino acids enter an extended larval period with little or no growth (Britton and Edgar 1998). Amino acid deprivation also causes a series of distinctive cellular phenotypes including a reduction in nucleolar area, changes in morphology of the larval fat body, and a cell type-specific cell cycle arrest (Butterworth et al. 1965; Nanya and Bicudo 1995; Britton and Edgar 1998; Zinke et al. 1999). As TOR



**Figure 6.** *dTOR* interacts with *dS6K*. (A) Immunoblot of extracts from *Drosophila* S<sub>2</sub> cells transfected with HA-tagged *dS6K*, visualized with anti-HA antibodies. *dS6K* migrates as a doublet, and the slower migrating band (top), which represents phosphorylated *dS6K*, is abolished by treatment with rapamycin (lane 2; Stewart et al. 1996). Phosphorylation of *dS6K* is maintained in the presence of rapamycin when *dTOR<sup>S1956T</sup>* (*dTOR<sup>RR</sup>*, lane 6) but not kinase-inactive *dTOR<sup>S1956T</sup>* (*dTOR<sup>RRKD</sup>*, lane 5) is cotransfected with *dS6K*. (B) Constitutive expression of activated human *p70<sup>S6K1</sup>* rescues *dTOR* flies to viability. Genotypes: (Left) *dTOR<sup>P2</sup> UAS-p70<sup>S6K1-D4</sup>/dTOR<sup>P1</sup>; Act5c-Gal4/+*; (right) *dTOR<sup>P2</sup> UAS-p70<sup>S6K1-D4</sup>/CyO; Act5c-Gal4/+*. Note that the rescued *dTOR* fly (left) is smaller than the control. (C) Constitutive expression of *dS6K* provides rapamycin resistance. *UAS-dS6K/+; Act5c-Gal4/+* flies cultured with 1 μM rapamycin eclose ~3 d earlier than wild-type controls.

proteins have been proposed to be regulated in response to amino acid levels, we wished to examine whether loss of *dTOR* mimics these cellular effects.

The nucleolus is the major cellular site of ribosomal assembly, and its size has been shown to correlate with protein synthetic capacity and proliferation rate (Derenzini et al. 1998). To measure nucleolar size in wild-type and *dTOR* mutant cells, wing imaginal discs containing clones of *dTOR*<sup>ΔP</sup> homozygous cells were labeled with an antibody against the nucleolar protein fibrillar protein (Aris and Blobel 1988), and examined by confocal sectioning (Fig. 7A–C). We found that the nucleolar area in clones of *dTOR* mutant cells in the wing imaginal disc was approximately half that of surrounding wild-type cells (*dTOR* nucleoli, 27.9 ± 5.5 pixels<sup>2</sup>, *n* = 95; wild-type nucleoli, 52.3 ± 11.1 pixels<sup>2</sup>, *n* = 100), consistent with a role for *dTOR* in ribosome biogenesis.

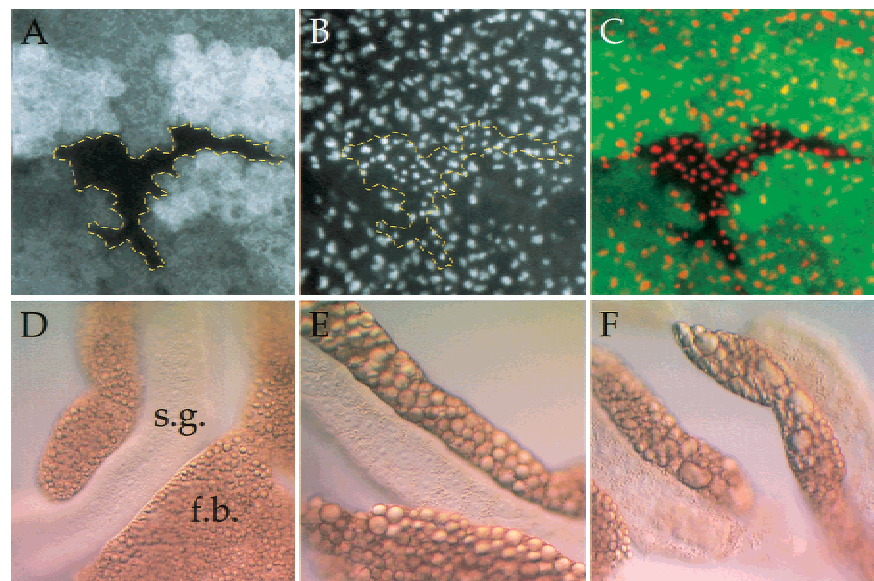
During metamorphosis or starvation, stores of protein, lipid, and glycogen are mobilized from adipose cells of the larval fat body and are used by other tissues as an energy source in place of dietary nutrients. These metabolic effects are visible as changes in appearance of fat body cells (Butterworth et al. 1965; Britton and Edgar 1998; Zinke et al. 1999). The major visible change in fat body cells in larvae deprived of amino acids is an aggregation of lipid vesicles (Fig. 7E), and this effect was indistinguishable from that caused by loss of *dTOR* (Fig. 7F).

Within 48–72 h of amino acid withdrawal, endoreplicative cells become quiescent, whereas mitotic neuroblasts of the central nervous system continue to cycle for at least 8 d in the absence of amino acids (Britton and Edgar 1998). This pattern of cell cycle responses is distinct from that caused by complete inhibition of protein synthesis, which causes all larval cells to arrest DNA synthesis (Britton and Edgar 1998). To determine whether loss of *dTOR* causes a cell cycle response similar to that elicited by starvation, we examined the cell

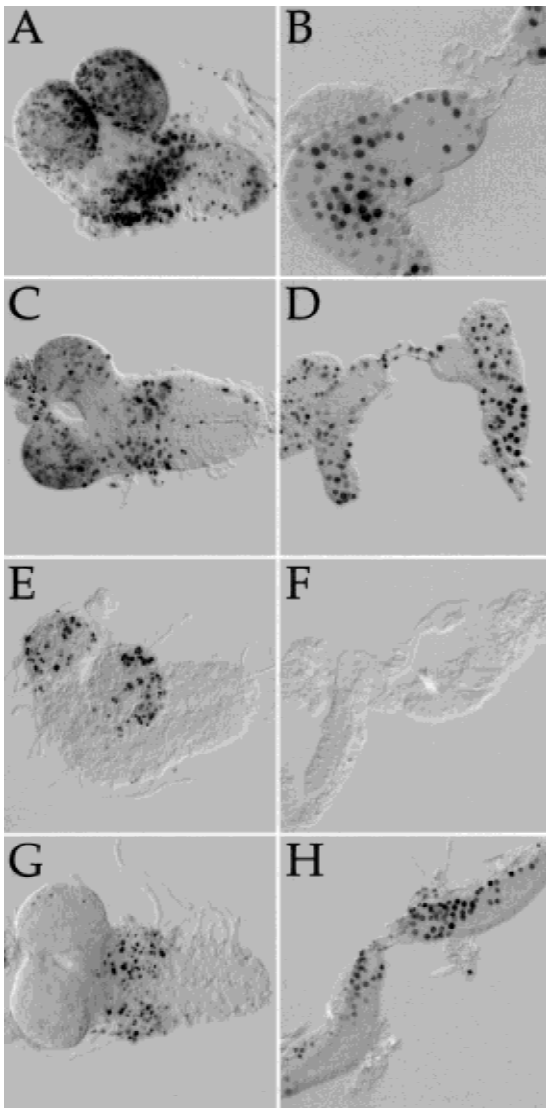
cycle behavior of these cell types in *dTOR*<sup>ΔP</sup> larvae at multiple stages of development. At 3–4 d after egg deposition (AED), both endoreplicative and mitotic tissues were found to cycle normally in *dTOR*<sup>ΔP</sup> homozygotes, as measured by incorporation of the nucleotide analog BrdU (Fig. 8A–D). In contrast, by 5–6 d AED all endoreplicative tissues including the gut (data not shown), fat body, and salivary glands failed to incorporate BrdU, whereas neuroblasts continued to cycle (Fig. 8E,F). A similar pattern was observed at 10 d AED (data not shown). Presumably the appearance of this cell cycle arrest at 4–5 d AED results from the perdurance of maternal stores of *dTOR* mRNA or protein until this time. We found that the cell cycle arrest of *dTOR* endoreplicative cells could be bypassed by overexpression of the G<sub>1</sub>/S regulators cyclin E (Fig. 8H) or dE2F/dDP (data not shown), as was demonstrated previously for endoreplicative cells arrested by amino acid withdrawal (Britton and Edgar 1998). Thus, amino acid insufficiency and loss of *dTOR* each cause similar growth arrests, changes in cell morphology, and cell type-specific patterns of G<sub>1</sub> arrest.

#### *dTOR* regulates G<sub>1</sub>/S progression through cyclin E

In budding yeast, TOR proteins govern S-phase entry in response to nutrient levels by regulating translation of the G<sub>1</sub>/S regulator Cln3 (Barbet et al. 1996; Polymenis and Schmidt 1997). *Drosophila* cyclin E has been proposed to play a role analogous to Cln3, and its abundance increases in response to growth stimuli such as overexpression of activated Ras (Johnston et al. 1999; Prober and Edgar 2000). Because cyclin E overexpression was able to bypass the cell cycle arrest in *dTOR* mutants (Fig. 8H), we examined whether loss of *dTOR* affected cyclin E expression. Immunoblot analysis of whole larval extracts revealed that the level of cyclin E protein was reduced ~30-fold in *dTOR*<sup>ΔP</sup> mutants compared to wild-type larvae of similar stage (Fig. 9A).



**Figure 7.** Loss of *dTOR* mimics starvation. (A–C) Wing imaginal disc containing a clone of *dTOR*<sup>ΔP</sup> cells marked by the absence of GFP (A) and stained with antifibrillar protein to highlight nucleoli (B). The extent of the *dTOR* mutant clone is outlined in yellow. (C) An overlay of GFP (green) and antifibrillar protein (red). Loss of *dTOR* causes a reduction in nucleolar area. (D–F) Differential interference contrast images of salivary glands (s.g.) and associated fat body (f.b.) from normally fed larvae (D), larvae that were deprived of amino acids for 4 d (E), and normally fed *dTOR*<sup>ΔP</sup> larvae (F). Starvation and loss of *dTOR* each cause aggregation of lipid vesicles in the fat body, as well as accumulation of proteinaceous granules in salivary gland cells.



**Figure 8.** Cell cycle behavior of *dTOR* cells. BrdU incorporation patterns in larval brains (A,C,E,G) and salivary glands (B,D,F,H). Darkly stained nuclei indicate cells that have incorporated BrdU and thus were in S-phase during the experiment. (A,B) Wild type at 3–4 d AED. (C,D) *dTOR*<sup>ΔP</sup> at 3–4 d AED. (E,F) *dTOR*<sup>ΔP</sup> at 5–6 d AED. Note that salivary gland cells no longer incorporate BrdU, whereas CNS neuroblasts continue to cycle. (G,H) *dTOR*<sup>ΔP</sup> carrying a single copy of a HS-cyclin E transgene at 6–7 d AED, after a 2-h heat shock. Ectopic expression of cyclin E drives quiescent *dTOR*<sup>ΔP</sup> salivary gland cells into S-phase.

We have shown previously that clonal induction of cells lacking cyclin E in the wing imaginal disc results in a G<sub>1</sub> arrest within one to two cell divisions (Neufeld et al. 1998). In contrast, cells mutant for *dTOR* continue to cycle slowly for several days, and give rise to clones containing multiple cells (see Fig. 3D), indicating that *dTOR* mutant cells retain at least partial cyclin E activity. Accordingly, we found that cyclin E protein was reduced but not eliminated in *dTOR* mutant clones in the wing disc. Although 72-h *dTOR*<sup>ΔP</sup> clones containing little or

no detectable cyclin E immunoreactivity were often observed (Fig. 9B–D, arrowhead; data not shown), we also found many *dTOR* clones containing cells with apparently normal cyclin E levels (Fig. 9B–D, arrow). We conclude that the observed reduction in cyclin E protein levels in *dTOR*<sup>ΔP</sup> larval extracts is due largely to reduced cyclin E levels in endoreplicating cells, which comprise the majority of larval mass, resulting in a G<sub>1</sub> arrest in this cell type. In contrast, *dTOR* may be required in imaginal cells to maintain normal rates of cyclin E accumulation, rather than absolute levels, consistent with the reduced rate of cell division and extended G<sub>1</sub> phase observed in *dTOR* mutant clones.

### Discussion

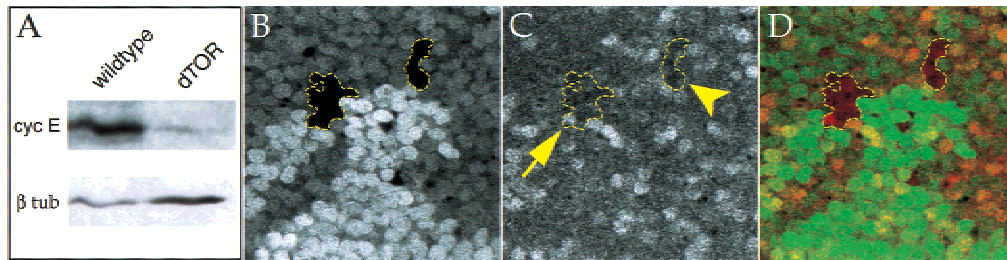
Studies in *Drosophila* have revealed that a critical role of the PI3K/Akt/p70<sup>S6K</sup> signaling pathway is to regulate size at the cellular, organ, and organismal levels (Coelho and Leever 2000). A role for this pathway in size control is conserved in mammals, as it was recently shown that PI3K regulates cell and organ size in mice (Shioi et al. 2000). The results presented here indicate that the relationship of TOR to this pathway is also conserved between flies and mammals. *dTOR* is required for phosphorylation of *dS6K* in cell culture, and mutant animals with reduced *dTOR* activity can be rescued to viability by overexpression of *dS6K*. Moreover, *dTOR* is required for the changes in cell size and cell cycle phasing caused by activation of PI3K signaling in vivo. Like PI3K pathway mutants, loss of *dTOR* function reduces cell size and proliferation, with little apparent disruption of normal patterning.

### Regulation of *dTOR* activity

Although these phenotypic similarities underscore the role of *dTOR* in PI3K signaling, *dTOR* mutants differ from the known PI3K pathway mutants in several important respects. First, the growth defects caused by loss of *dTOR* are more severe than those arising from mutations in components of the PI3K signaling pathway. Null mutations in the PI3K subunits *Dp110* or *p60* allow growth to the third instar larval stage, and *chico* (IRS-1) and *dS6K* mutants survive to adulthood (Bohni et al. 1999; Montagne et al. 1999; Weinkove et al. 1999). Although BrdU incorporation was not analyzed in these studies, the results imply that at least in the case of *chico* and *dS6K*, many cells are able to cycle normally throughout development. In contrast, animals lacking *dTOR* reach only the size of second instar larvae, at which point they undergo cell cycle arrest. Moreover, whereas overexpression of *Dp110*, *Akt*, or *dS6K* leads to increased growth rate and cell size (Montagne et al. 1999; Verdu et al. 1999; Weinkove et al. 1999), we find that *dTOR* overexpression inhibits growth and reduces cell size (T.P. Neufeld, unpubl.). Although variations in genetic background may partially account for some of these phenotypic differences, these results are inconsistent with *dTOR* acting as an integral component of a



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**Figure 9.** Cyclin E expression is reduced in *dTOR* mutants. (A) Immunoblot of total larval extracts from *dTOR*<sup>ΔP</sup> and wild-type second instar larvae, probed with anti-cyclin E. The blot was also probed with anti-β-tubulin as a loading control. (B–D) Shown is a region of a wing imaginal disc containing *dTOR*<sup>ΔP</sup> clones 72 h after induction, marked by the absence of GFP (B) and stained with anti-cyclin E antibody (C). *dTOR* mutant clones are outlined in yellow. These images are superimposed in D, with GFP shown in green and cyclin E in red. The arrow indicates a single *dTOR* mutant cell expressing cyclin E; the arrowhead indicates a *dTOR* clone lacking cyclin E expression.

linear PI3K/Akt/S6K pathway, and instead argue that *dTOR* may converge upon this pathway in response to a distinct set of cues.

In yeast, TOR1 and TOR2 regulate cell growth directly in response to levels of nutrients such as amino acids, rather than in response to intercellular signals (Barbet et al. 1996). Similarly, it has recently been shown that human mTOR may also be regulated by nutrient levels (Fox et al. 1998; Hara et al. 1998; Wang et al. 1998; Xu et al. 1998; Iiboshi et al. 1999). These considerations prompted us to compare the phenotypes of *dTOR* mutant animals with the physiological changes caused by nutrient deprivation. By the three criteria we examined—nucleolar size, fat body vesicle formation, and endoreplicative cell cycle arrest—loss of *dTOR* precisely mirrored the effects of starvation. An efficient explanation of these results is that *dTOR* is required for normal responses to changes in nutrient levels. This would be consistent with a model in which full activation of growth targets such as p70<sup>S6K</sup> requires two distinct inputs: growth factor-mediated intercellular signals through PI3K, and nutrient-sensing signals through TOR. In this view, TOR proteins may act as part of a checkpoint to attenuate growth factor signaling when local conditions are unfavorable for cell growth.

How TOR proteins might be regulated by nutrient levels is unclear. In the case of amino acids, recent evidence suggests that the primary signal may be uncharged tRNA, which increases in abundance when amino acid levels are low (Iiboshi et al. 1999). Interestingly, other members of the PIK-related kinase family such as ATM and DNA-PK also act as checkpoint proteins that are regulated by specific nucleic acid structures. In addition, a recently described member of this family, SMG-1, is involved in the degradation of aberrant mRNAs containing inappropriate nonsense codons (O'Connor and Anderson 1999). Thus, regulation by nucleic acid may be a common feature of this kinase family.

#### Effectors of *dTOR* function

Activation of p70<sup>S6K</sup> is a common response to virtually all mitogenic stimuli (Chou and Blenis 1995). Phos-

phorylation of the target of p70<sup>S6K</sup>, ribosomal protein S6, leads to the selective increase in translation of a subset of transcripts that contain an oligopyrimidine tract at their 5' termini (5' TOP). This class of messages encodes ribosomal proteins and translation elongation factors, and thus p70<sup>S6K</sup> activation leads to increased ribosomal biogenesis (for review, see Brown and Schreiber 1996). Our demonstration here that overexpression of S6K can rescue *dTOR* mutants to viability indicates that dS6K is a critical effector of *dTOR*, and that one essential function of *dTOR* is to regulate the activity of dS6K.

Despite the central role of *dS6K* in mediating the functions of *dTOR*, several lines of evidence indicate that *dTOR* has additional *dS6K*-independent roles. First, *dTOR* mutant phenotypes are more severe than those of *dS6K*; lack of *dTOR* results in growth arrest at the second instar larval stage, whereas *dS6K* mutant animals survive to adulthood, albeit with a delayed development and decreased body size (Montagne et al. 1999). Second, although S6K overexpression suppressed the lethality of *dTOR* mutants, this was only the case for hypomorphic *dTOR* allelic combinations; *dTOR* null animals expressing S6K advanced only to the pupal stage. Third, *dTOR* flies rescued by S6K did not grow to the full size of wild-type controls (Fig. 6B). Thus, some *dTOR* functions are not fully rescued by S6K. As noted above, in mammalian cells mTOR also stimulates translation through phosphorylation and inactivation of 4E-BP1, an inhibitory binding factor of the translation initiation factor eIF4E. *Drosophila* *eIF4E* mutants display a severe growth arrest phenotype (Zhang et al., unpubl.), and thus aspects of *dTOR* function that are not rescued by *dS6K* may reflect diminished *eIF4E* activity. However, neither overexpression of *eIF4E* nor mutations in *4E-BP1* detectably alleviated *dTOR* mutant phenotypes (data not shown).

In addition to effects on translation, studies in budding yeast have found that inactivation of TOR modulates the level of a number of growth-related or nutrient-regulated transcripts, including those encoding ribosomal proteins (Barbet et al. 1996; Zaragoza et al. 1998; Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999; Powers and Walter 1999). A transcriptional role for TOR in higher eukaryotes has been reported as well (Mahajan

1994). Moreover, recent studies have found that mTOR can interact with a number of additional signaling factors including STAT3, protein kinase C, *c-Abl*, and 14-3-3 (Parekh et al. 1999; Kumar et al. 2000a,b; Mori et al. 2000; Yokogami et al. 2000). Rapamycin has also recently been shown to disrupt microtubule assembly and function in yeast, independent of its effects on translation (Choi et al. 2000). Thus, the inability of S6K overexpression to fully supplant *dTOR* may be due to a requirement for *dTOR* in multiple cellular functions.

#### Coordination of cell division and growth

The reduced growth of *dTOR* mutants reflects reductions in both cell size and cell number. Because direct inhibition of proliferation increases rather than decreases cell size (Neufeld and Edgar 1998; Neufeld et al. 1998), we propose that the primary function of *dTOR* is to promote cell growth, and that the decreased proliferation of *dTOR* mutant cells is a secondary effect in response to their reduced rate of growth. The accumulation of *dTOR* mutant cells in the G<sub>1</sub> phase of the cell cycle suggests that the G<sub>1</sub>/S transition is particularly sensitive to growth rate. This is consistent with previous observations that stimulation of cell growth by overexpression of dMyc, PI3K, or activated Ras promotes progression through the G<sub>1</sub>/S but not G<sub>2</sub>/M transition (Johnston et al. 1999; Weinkove et al. 1999; Prober and Edgar 2000).

A factor likely to be involved in coupling growth and division rates is the G<sub>1</sub>/S regulator cyclin E. Cyclin E is rate limiting for G<sub>1</sub>/S progression in imaginal discs (Neufeld et al. 1998), and its levels increase by a post-transcriptional mechanism in response to stimulation of cell growth (Prober and Edgar 2000). Our demonstration that *dTOR* is required for normal accumulation of cyclin E suggests that translational control is likely to play an important role in cyclin E regulation. In budding yeast, translation of the G<sub>1</sub> cyclin Cln3 is regulated by a leaky scanning mechanism involving a small upstream ORF (Polymenis and Schmidt 1997). In this regard, it is interesting to note that the 5'-untranslated region of the *Drosophila* cyclin E message contains multiple upstream ORFs (Richardson et al. 1993). Thus, the mechanisms connecting G<sub>1</sub>/S progression to cell growth may be conserved between yeast and multicellular organisms.

## Materials and methods

### Fly strains

*w; P[lacw]l(2)k17004/CyO (dTOR<sup>P1</sup>)* and *w; EP(2)2353/CyO (dTOR<sup>P2</sup>)* are from the Bloomington *Drosophila* Stock Center and Berkeley *Drosophila* Genome Project, respectively. UbiGFP, FRT40A was a gift of Cristina Martin-Castellanos (Fred Hutchinson Cancer Research Center, Seattle, WA). UAS-dS6K and UAS-human p70<sup>S6K1</sup> were gifts of Kelly Watson (Harvard University, Cambridge, MA). hs-cyclin E (Richardson et al. 1995), hs-dE2F hsdDP (Duronio et al. 1996), and dPTEN<sup>D1189</sup> FRT40A (Gao et al. 2000) are as described. The Act5C-Gal4 line

used for constitutive Gal4 expression was derived from Act5C>CD2>Gal4 (Pignoni and Zipursky 1997) by FLP/FRT-mediated germ-line excision of the CD2 insert.

*dTOR* deletion alleles were generated by imprecise mobilization of the *dTOR<sup>P1</sup>* and *dTOR<sup>P2</sup>* inserts. For each line, 100 potential excisions identified by loss of the *w<sup>+</sup>* marker present on the P-element were tested for complementation with the starting P lines. Genomic DNA from lines that remained mutant for *dTOR* was used in PCR reactions (Expand Long Template PCR System, Roche Molecular Biochemicals) with primers flanking the P-insertion sites at -2970, -350, +2550, and +8470 relative to the *dTOR<sup>P1</sup>* insertion site. Ten lines bearing deletions in the *dTOR* gene were identified by increased electrophoretic mobility of PCR products. Sequence analysis revealed that these deletions each originate at the P-insertion site and extend downstream into the *dTOR* ORF, ranging in size from a 472-bp deletion that removes only the first exon, to an 8.0-kb deletion that removes all but the last 358 nucleotides of coding sequence.

### Molecular biology

A small segment of *dTOR* was amplified by PCR using degenerate primers designed against conserved regions of mTOR and yeast TORs. This PCR fragment was then used to screen the LD *Drosophila* cDNA library (a gift of Ling Hong, Berkeley *Drosophila* Genome Project). Several overlapping cDNA clones were isolated, but did not represent the complete *dTOR* transcription unit. The remaining 5' end of the *dTOR* transcript was amplified by RACE, using primers designed from the 5' end of the assembled cDNA. Two independent RACE reactions gave the same size product. The assembled *dTOR* cDNA includes 284 bp of 5' leader sequence, which contains an in-frame stop codon upstream of the *dTOR* ORF.

The *dTOR* genomic rescue construct was made as follows. The *dTOR* genomic region was subcloned as an 11.8-kb *NsiI* fragment from BAC48D10 (Berkeley *Drosophila* Genome Project) into the *PstI* site of pBluescript, to give pBS-dTOR-11.8. A 1.1-kb sequence upstream of the *dTOR* coding region was amplified by PCR and ligated into pET28a as an *XbaI/SalI* fragment to give pET-dTOR-1.1. An 8.3-kb *SalI* fragment from pBS-dTOR-11.8. was ligated into the *SalI* site of pET-dTOR-1.1., to give pET-dTOR-9.4. The 9.4-kb insert was liberated in an *XbaI/XhoI* partial digest, and transferred to pCasper4 to give pCasper-dTOR-9.4. This construct was injected into *dTOR<sup>ΔP</sup>/CyO* embryos by standard techniques.

### Larval culture

To measure the effect of rapamycin on developmental timing, pools of 100 embryos from 4-h collections were placed in vials containing standard *Drosophila* medium with or without 1 μM rapamycin (Drug synthesis and chemistry branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute), and cultured at 25°C until eclosion. Each experiment was conducted in triplicate, and the results averaged. For starvation experiments, larvae were cultured in 20% sucrose as described (Britton and Edgar 1998).

### Histology

Clones of cells mutant for *dTOR* were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin 1993), and identified using a *y<sup>+</sup>* transgene for adult bristles, and a ubiquitin-GFP marker in imaginal discs. Hair cell clones in the adult wing were identified on the basis of their small cell phenotype, and

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clonal areas were measured as described [Neufeld et al. 1998]. For FACS analysis, mutant clones were induced at 48 h AED with a 2-h heat shock at 37°C. Wing imaginal disc cells were dissociated 72 h later and analyzed by flow cytometry as described [Neufeld et al. 1998]. Cyclin E was detected on immunoblots with anti-*Drosophila* cyclin E monoclonal antibody (a gift of Helena Richardson, Adelaide University, South Australia), and in wing imaginal disc whole mounts with a guinea pig anti-*Drosophila* cyclin E polyclonal serum (a gift of Terry Orr-Weaver, MIT, Cambridge, MA).

For BrdU labeling, staged larvae were dissected in PBS and incubated for 9 h in M3 insect media (Sigma) containing 100 µg/mL BrdU (Sigma), then fixed on ice in 8% paraformaldehyde in PBS. For ectopic expression experiments, *Drosophila* cyclin E or dE2F/dDP were induced from heat shock-responsive transgenes with a 2-h heat shock at 37°C followed by 2 h of recovery at room temperature before dissection. BrdU incorporation was detected with mouse anti-BrdU primary (PharMingen) and Texas Red-conjugated goat anti-mouse secondary antibodies (Jackson Immunolabs). Tissues were mounted in Fluorogard (Bio-Rad) and photographed under DIC optics on a Zeiss Axio-scope 2 microscope.

To estimate ploidy of salivary gland polytene nuclei, tissues were fixed in 4% paraformaldehyde in PBS and stained with Hoechst 33258. Images were captured with a Zeiss AxioPlan 2 microscope equipped with a CARV spinning disc optical module. Nuclear DNA content was quantitated by measuring fluorescent intensity in Adobe Photoshop.

#### dS6K phosphorylation

*Drosophila* S<sub>2</sub> cells were cultured in Schneider's medium supplemented with 10% heat-inactivated FBS and antibiotics. S<sub>2</sub> cells were seeded in a six-well plate at 75% confluency for 3 h (2 mL of medium in each well). Cells were rinsed twice with Schneider's medium without serum before transfection. DNA (10 mg) was incubated at room temperature for 20–30 min with 50 mg of lipofectin (GIBCO BRL) in 1 mL of Schneider's medium, then added to cells for 6–8 h. After recovering for 1 d in complete medium, cells were starved in serum-free medium for 4 h, then pretreated with 50 nM rapamycin for 20 min. Medium containing 20% serum was then added for 30 min, after which cells were lysed for band-shifting assays. For cotransfection experiments, 9 mg of dTOR variants and 1 mg of dS6K were used.

#### Acknowledgments

We are grateful to Kelly Watson, Cristina Martín-Castellanos, and Todd Lavery for fly stocks, to John Aris, Terry Orr-Weaver, and Helena Richardson for antibodies, to Jean-Karim Heriche for advice on rapamycin treatment, and to Mike O'Connor for support. We thank Doujia Pan, Bruce Edgar, David Prober, and Jessica Britton for comments on the manuscript. We also gratefully acknowledge the Drug Synthesis and Chemistry Branch of the National Cancer Institute (NCI) for rapamycin, and the University of Minnesota Cancer Center Flow Cytometry Core Facility for assistance with FACS analysis (NCI Grant P30 CA77598).

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*Genes Dev.* 2000, **14**:

Access the most recent version at doi:[10.1101/gad.835000](https://doi.org/10.1101/gad.835000)

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