Determinants of Sensitivity and Resistance to Rapamycin-Chemotherapy Drug Combinations *In vivo*

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Abstract

The phosphatidylinositol-3-OH kinase [PI(3)K] pathway is frequently activated in human cancers and represents a rational target for therapeutic intervention. We have previously shown that enforced expression of Akt, which is a downstream effector of PI(3)K, could promote tumorigenesis and drug resistance in the Eμ-*myc* mouse lymphoma model, and that these tumors were particularly sensitive to inhibition of mammalian target of rapamycin (mTOR) with rapamycin when combined with conventional chemotherapy. We now show that reduced dosage of PTEN, a negative regulator of PI(3)K signaling, is sufficient to activate Akt, but has only a modest effect on lymphomagenesis in the same model. Nonetheless, loss of even one *PTEN* allele resulted in lymphomas that were resistant to conventional chemotherapy yet sensitive to rapamycin/chemotherapy combinations. These effects could be recapitulated by using RNA interference to suppress PTEN expression in lymphomas, which were previously established in the absence of PI(3)K lesions. Finally, the introduction of lesions that act downstream of mTOR (*eIF4E*) or disable apoptosis (*Bcl-2* and loss of *p53*) into *PTEN*+/− lymphomas promoted resistance to rapamycin/chemotherapy combinations. Thus, whether activation of the PI(3)K pathway confers sensitivity or resistance to therapy depends on the therapy used as well as secondary genetic events. Understanding these genotype-response relationships in human tumors will be important for the effective use of rapamycin or other compounds targeting the PI(3)K pathway in the clinic. (Cancer Res 2006; 66(15): 7639-46)

Introduction

Tumorigenesis involves a series of genetic events that disrupt or alter signaling networks controlling proliferation and survival. The precise order of genetic alterations and their combinations that can confer malignant characteristics is variable, thereby producing heterogeneity in tumor behavior. As one example, increased oncogenic signals activate tumor suppressor programs, including apoptosis and senescence, and their disruption is an obligate requirement during tumorigenesis (1, 2). Disruption of apoptotic programs in tumor development can occur in different ways, for example through loss of tumor suppressor genes like *ARF* and *p53*, or increased activity of dominant oncogenes like *Bcl-2* (3) and survival pathways like the phosphatidylinositol-3-OH kinase [PI(3)K] pathway or its effectors *Akt* and *eIF4E* (4–6). Importantly, some of the same pathways that block apoptosis during tumorigenesis also impinge on the apoptotic response to chemotherapeutic drugs. Thus, the nature of the genetic lesions incurred during tumorigenesis to disrupt apoptosis can influence treatment behavior to varying degrees (4, 7–10). Conversely, strategies to restore apoptosis to tumor cells, either by increasing proapoptotic signals, suppressing prosurvival signals, or by simultaneously achieving both, may prove effective for treating otherwise refractory tumors.

The PI(3)K pathway is implicated in cellular transformation and tumor development and contributes to the oncogenic activities of *Ras* and *Bcr-abl* [reviewed in ref. 11]. Concordantly, deregulation of this pathway is observed in many cancers, including lymphoma and leukemia, and most often involves inactivation of the negative regulator *PTEN* (refs. 12–14; reviewed in ref. 15). Also, *PTEN* heterozygous mice develop tumors in multiple tissues, sometimes in the absence of complete PTEN inactivation, indicating that in certain contexts *PTEN* can be haploinsufficient for tumor suppression (16–19). Activation of the PI(3)K pathway has myriad effects on cellular physiology by virtue of its ability to regulate effectors controlling translation, metabolism, and cell survival (20–25). Although it seems likely that all of these properties contribute to tumorigenesis and drug resistance, the ability of deregulated PI(3)K signaling to promote cell survival seems particularly important (4).

Owing to its "gain-of-function" mode of action, the PI(3)K pathway represents an attractive therapeutic target, and compounds targeting multiple components of the pathway are in preclinical and clinical development (26). One drug that targets PI(3)K signaling is rapamycin, which acts to inhibit specific mammalian target of rapamycin (mTOR) complexes, thereby modulating translation in response to survival signals, or nutrient or energy availability. Initially approved as an immunosuppressant, rapamycin and its analogues have antitumor activity in some preclinical models and are currently in clinical trials (4, 27–32). It is therefore important to identify mechanisms of sensitivity and resistance to these agents.

We have previously described the effects of aberrant Akt expression on tumorigenesis, chemotherapy responses, and rapamycin sensitivity in the Eμ-*myc* lymphoma model (4). Specifically, we have shown that Akt dramatically accelerated *myc*-induced tumorigenesis and promoted resistance to conventional chemotherapy. Rapamycin suppressed mTOR activity and synergized with chemotherapy in Akt-expressing lymphomas, leading to potent antitumor responses. Interestingly, eIF4E, a translational regulator acting downstream of mTOR, accelerated lymphomagenesis and

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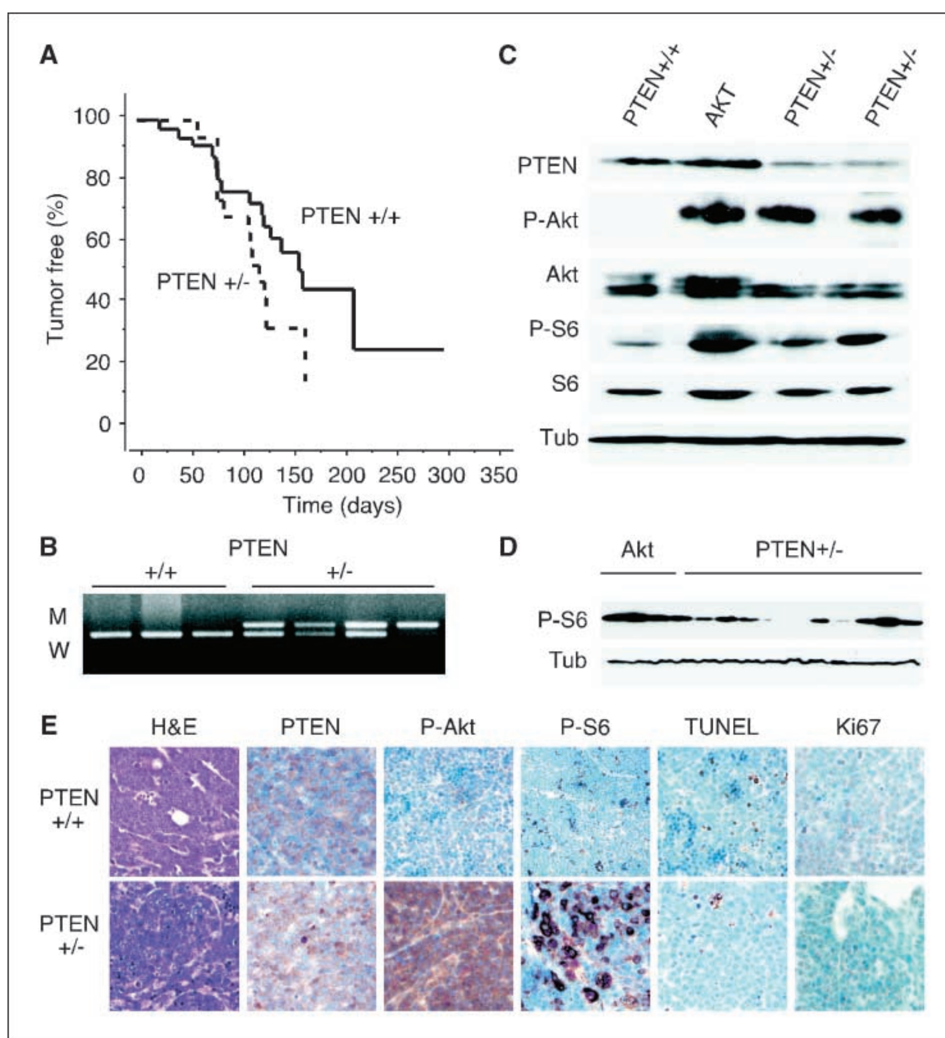

Figure 1. Effect of *PTEN* heterozygosity on *Eμ-myc* lymphomagenesis. *A,* Kaplan-Meier plot comparing tumor latencies in *Eμ-myc/PTEN+/+* (*solid line,* $n = 33$) and *Eμ-myc/PTEN+/−* (*dashed line,* $n = 19$) mice ($P = 0.06$). *B,* PCR to detect loss of heterozygosity at the *PTEN* locus (*M,* mutant allele, *W,* wild-type allele). *C,* lysates prepared from untreated *Eμ-myc* tumors of the indicated genotypes and probed for PTEN, phosphorylated and total Akt (*P-Akt* and *Akt*), and ribosomal S6 protein (*P-S6* and *S6*, respectively), and α-tubulin (*Tub*) as a loading control. *D,* immunoblot for phosphorylated ribosomal S6 protein and tubulin in Eμ-*myc* tumors expressing Akt (*lanes 1-3*) or heterozygous for *PTEN* (*lanes 4-14*). *E,* representative microphotographs of *Eμ-myc/PTEN+/+* or *Eμ-myc/PTEN+/−* tumors either H&E stained or immunohistochemically stained as indicated.

promoted drug resistance in a manner comparable with Akt, suggesting that a substantial portion of the Akt survival signal is transmitted through deregulated translation. Here, we studied the effects of reduced PTEN activity in the same genetic context and address the problem of rapamycin resistance, because this genetic lesion is the most prominent lesion that produces Akt activation in human cancers. We further evaluated genetic determinants of sensitivity and resistance cytotoxic agents, rapamycin, and combinations of both.

Materials and Methods

Generation of mice. *Eμ-myc* mice (C57BL/6 strain) and *PTEN+/−, ARF−/−, p53−/−* mice were crossed, and their offsprings were genotyped as described (17, 33). The animals were monitored for development of lymphoma and associated leukemia by biweekly palpation and blood counts, respectively. Upon the appearance of well-palpable lymphomas, the tumors were harvested and either fixed in formalin for histologic evaluation, rendered single-cell suspensions and frozen in 10% DMSO, or transplanted directly into C57Bl/6 mice for treatment studies (4). Loss of heterozygosity at the *PTEN* locus in tumor cells was determined by allele-specific PCR following brief (24 hours) *in vitro* culture (17).

Treatment studies. Treatment studies in mice were done as previously described (4). Briefly, 1×10^6 DMSO frozen or primary lymphoma cells were injected into the tail vein of 6- to 8-week-old female C57BL/6 mice. Upon the formation of palpable tumors, the animals were treated with rapamycin (4 mg/kg, i.p. ×5 days), doxorubicin (10 mg/kg, i.p.), cyclophosphamide (300 mg/kg, i.p.), or combinations. In combination studies, the cytotoxic agent was given on day 2 of the rapamycin protocol. Rapamycin (LC Labs, Woburn, MA) was initially dissolved in 100% ethanol, stored at −20°C, and further diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG 400 (final ethanol concentration, 2%) immediately before use. Doxorubicin (Sigma, St. Louis, MO) and cyclophosphamide (Sigma) were dissolved in water. In treatment studies, chemosensitive and chemoresistant control lymphomas were *Arf*-null tumors arising in a *Eμ-myc/Arf+/−* background and *p53*-null lymphomas arising in a *Eμ-myc/p53+/−* background, respectively (8). Treatment responses were monitored by twice weekly palpation and blood smears stained with Giemsa (Fisher Diagnostics, Middletown, VA). A "complete remission" was defined as the absence of any detectable tumor and leukemia. Tumor-free survival was defined as the time between treatment and reappearance of lymphoma or leukemia (4). Tumor-free and overall survival data were analyzed in the Kaplan-Meier format using the log-rank (Mantel-Cox) test for statistical significance.

Histopathology. Samples were fixed for 24 hours in 10% buffered formalin and embedded in paraffin. Thin sections (5 μm) were stained with H&E according to standard protocols. Detection of PTEN (99552, 1:100, Cell Signalling, Danvers, MA), phosphorylated Akt (9275, 1:100, Cell Signalling), phosphorylated ribosomal S6 protein (2215, 1:100, Cell Signalling), and Ki67 (1:100, NovoCastra, Newcastle upon Tyne, United Kingdom) was by standard avidin-biotin immunoperoxidase method, using biotinylated goat or rabbit specific immunoglobulins (Vector Labs,

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Burlingame, CA) at 1:500 and avidin-biotin peroxidase complexes (1:25, Vector Labs). Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. The apoptotic rate was analyzed by terminal deoxyribonucleotide transferase–mediated nick-end labeling assay (TUNEL) according to published protocols (34).

Fluorescence-activated cell sorting analysis. Tumor cell suspensions of representative tumors of each genotype were stained with the indicated monoclonal antibodies (PharMingen, San Diego, CA and CalTag, Burlingame, CA) conjugated with phycoerythrin, TRI-COLOR, or biotin, developed with streptavidin-allophycocyanin (PharMingen) and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest Pro software.

Western blotting. Immunoblots were done from whole-cell lysates as previously described (35). Fifty micrograms of protein per sample were resolved on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Antibodies against PTEN (a gift from M. Myers, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), phosphorylated Akt (9275, 1:1,000, Cell Signalling), Akt (9272, 1:1,000, Cell Signalling), ribosomal S6 protein (2212, 1:1,000, Cell Signalling), phosphorylated ribosomal S6 (2215, 1:1,000 Cell Signalling), and α-tubulin (B-5-1-2, 1:5,000, Sigma) were used as probes and detected using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ; Lumilight, Roche, Nutley, NJ).

Competition experiments. Tumor cells, either *Eμ-myc/ARF−/−* or *Eμ-myc/PTEN+/−*, were transduced with retroviral vectors at a low multiplicity of infection to create mixed populations of cells containing or lacking each vector. The vectors were *MSCV-GFP* (the empty vector control), *MSCV-bcl-2-IRES-GFP* (10), *MSCV-eIF4E-IRES-GFP* (4), *MSCV-shPTEN-SV40-GFP, MSCV-shPTEN-puro^R-IRES-GFP, MSCV-p53C* (36), or *MSCV-p53D* (LMP-p53D; ref. 37). For *in vitro* experiments, the resulting mixed populations of infected and uninfected tumor cells were propagated in standard medium in the presence or absence of rapamycin (1 μmol/L) and then analyzed for green fluorescent protein (GFP) content by flow cytometry. For the *in vivo* studies, the mixed populations were transplanted by tail-vein injection into nontransgenic female C57BL/6 animals; upon tumor formation, these mice were treated. The mice were typically sacrificed 48 hours later and single-cell suspensions of residual tumors were analyzed for GFP content.

Results

Given the high frequency of *PTEN* alterations in human tumors (15), we wished to address how *PTEN* heterozygosity would effect *myc*-driven tumorigenesis *in vivo*. To this end, we generated crosses of *Eμ-myc* to *PTEN+/−* mice and monitored the progeny for lymphoma development. Kaplan-Meier analysis comparing tumor latencies in *Eμ-myc/PTEN+/−* and their *Eμ-myc/PTEN+/+* littermates revealed only a modest acceleration of disease onset in the heterozygous animals that did not achieve statistical significance (P = 0.06; Fig. 1*A*). Tumors in nontransgenic *PTEN+/−* animals were rare (data not shown). We analyzed DNA prepared from pure populations of tumor cells harvested from *Eμ-myc/PTEN+/−* animals by *PTEN* allele-specific PCR and found that the wild-type *PTEN* allele remained detectable in the majority of cases, with loss of heterozygosity only rarely observed (2 of 10 cases; Fig. 1*B*).

Immunoblotting analysis on lysates from primary tumors confirmed that PTEN protein expression was retained in *Eμ-myc/PTEN+/−* tumors. Nonetheless, these tumors displayed increased ratios of phosphorylated to total AKT and ribosomal S6 proteins, suggesting that loss of one *PTEN* allele was sufficient to deregulate the PI(3)K pathway (Fig. 1*C*). PI(3)K pathway activation was, however, more varied and typically to a lesser extend than observed in tumors expressing an activated form of Akt (Fig. 1*D*; see also ref. 19). Similar results were observed in tissue sections examined by immunohistochemistry (Fig. 1*E*). Consistent with their similar tumor latencies, lymphomas arising in *PTEN+/+* and *PTEN+/−* animals showed similar rates of apoptosis and proliferation by TUNEL and Ki67 staining, respectively. Immunophenotypically, *Eμ-myc/PTEN+/−* tumors exhibited a mature B-cell phenotype, showing surface expression of B220 (CD45R), CD19, and surface immunoglobulin (data not shown). This immunophenotype was similar to that observed in *Eμ-myc* mice without loss of *PTEN*, but contrasts with the immature nonlineage determined phenotype of *Eμ-myc* lymphomas overexpressing AKT. Interestingly, overexpression of eIF4E, like PTEN loss, led to mature B-cell tumors (4). Thus, although loss of one *PTEN* allele can activate the PI(3)K pathway, its effect on lymphomagenesis is minimal. Because the *PTEN−/−* mouse is an embryonic lethal, we could not examine the effects of complete PTEN loss on lymphoma development using this model.

Given the minor effects of *PTEN* heterozygosity on tumor development, we wondered if this alteration would affect treatment response. We therefore transplanted primary tumors arising in *Eμ-myc/PTEN+/−* mice into multiple wild-type recipients. Upon tumor formation, we initiated therapy with either cyclophosphamide or doxorubicin and tracked its effect by biweekly tumor palpation and blood smears. Of note, in the absence of a cooperating oncogene (e.g., *Akt* or *Bcl-2*) or preexisting loss of tumor suppressors (e.g., *PTEN*), *myc*-driven tumors are heterogeneous with *ARF* and *p53* being each inactivated in ~25% of tumors (38). We therefore chose to compare *PTEN+/−* tumors with genetically defined *Eμ-myc* lymphomas lacking either *ARF* or *p53*, which are chemosensitive and chemoresistant, respectively (8). Doxorubicin treatment at the maximum tolerated dose of 10 mg/kg induced complete responses in 90% of mice bearing *Eμ-myc/ARF−/−* tumors. By contrast, the response of *Eμ-myc/PTEN+/−* lymphomas was significantly worse and approached the poor response of *p53*-null lymphomas, with only 50% of mice

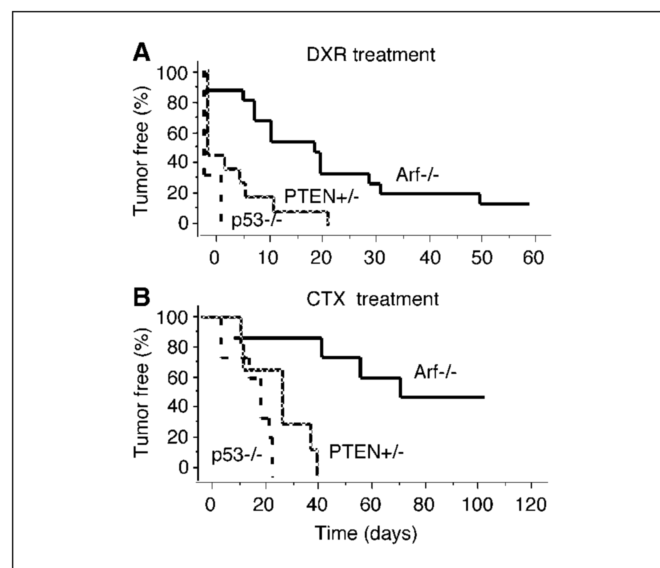

Figure 2. PTEN deficiency promotes chemoresistance *in vivo*. Kaplan-Meier plots detailing the time to relapse following treatment with doxorubicin (*DXR*; *A*) and cyclophosphamide (*CTX*; *B*). Mice bearing either chemosensitive *Eμ-Myc/ARF−/−* lymphomas (*solid line*; doxorubicin, n = 15; cyclophosphamide, n = 14), *Eμ-Myc/PTEN+/−* lymphomas (*hatched line*; doxorubicin, n = 11; cyclophosphamide, n = 6), or *Eμ-myc/p53−/−* lymphomas (*dashed line*; doxorubicin, n = 6; cyclophosphamide, n = 9) were monitored for tumor-free survival.

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achieving a complete remission and none lasting longer than 3 weeks (Fig. 2*A*). Cyclophosphamide therapy at the maximum tolerated dose of 300 mg/kg is more effective against *Eμ-myc* lymphomas and induced complete remissions in all animals irrespective of tumor genotype (Fig. 2*B*). However, both *Eμ-myc/p53−/−* and *Eμ-myc/PTEN+/−* tumors relapsed within 20 and 40 days, respectively, whereas half the animals bearing *Eμ-myc/ARF−/−* tumors did not relapse in the 100-day observation period. Thus, lymphomas with loss of only one *PTEN* allele display a resistance to common chemotherapeutic agents that is comparable with loss of *p53* or overexpression of Akt (4, 39).

In Akt-expressing tumors, rapamycin potently blocks mTOR activity *in vivo* and sensitizes these tumors to apoptosis induced by cytotoxic therapy (4). We therefore tested whether rapamycin would have similar effects in *PTEN+/−* tumors. Immunoblotting of lysates from *Eμ-myc/PTEN+/−* cells confirmed low levels of PTEN expression and subsequent activation of the PI(3)K/Akt pathway (Fig. 3*A*). These experiments also documented the ability of rapamycin to inhibit mTOR activity *in vivo*, as phosphorylation of ribosomal S6 protein was reduced 6 hours after rapamycin treatment relative to an untreated control. Doxorubicin did not affect S6 phosphorylation, and neither treatment affected phosphorylation of Akt (Fig. 3*A*). Immunohistochemistry on parallel tumors harvested 18 hours after rapamycin treatment revealed persistent mTOR inhibition and undetectable phosphorylated S6 protein. However, at this dose and schedule, rapamycin therapy only produced a modest increase in apoptosis, similar to that produced by chemotherapy alone (Fig. 3*B*). Although the combination of doxorubicin and rapamycin produced no further reduction in S6 phosphorylation compared with rapamycin alone, the tumors displayed substantially more apoptosis. Thus, rapamycin induces lasting mTOR inhibition and enhances the apoptotic response to DNA damage in *Eμ-myc/PTEN+/−* tumors.

We next asked whether the sensitization to apoptosis produced by rapamycin and chemotherapy would translate into a synergistic therapeutic effect *in vivo*. Indeed, cumulative analysis of "time-to-relapse" data from mice bearing *Eμ-myc/PTEN+/−* tumors revealed that the rapamycin/doxorubicin combination therapy induced complete remissions lasting at minimum 3 weeks and up to 45 days; by contrast, treatment with either drug alone achieved few responses and these were invariably short lived (rapamycin + doxorubicin versus rapamycin or doxorubicin: $P = 0.0002$; rapamycin versus doxorubicin: $P = 0.5$; Fig. 4*A*). The rapamycin/cyclophosphamide combination produced an even greater response and induced "cures" in almost 40% of animals (rapamycin + cyclophosphamide versus rapamycin or cyclophosphamide: $P \sim 0.02$; rapamycin versus cyclophosphamide: $P = 0.07$; Fig. 4*B*). This effect was highly specific for tumors with an activated PI(3)K pathway in that treatment of chemosensitive *Eμ-myc/ARF−/−* tumors with the same rapamycin and doxorubicin combination resulted in a reduced response compared with treatment with doxorubicin alone ($P = 0.0002$; Fig. 4*C*). Therefore, the synergistic antitumor activity of rapamycin-based combinations appears specific for tumors with PI(3)K/Akt pathway activating lesions. Surprisingly, sensitization to the effects of these agents requires loss of only one allele of *PTEN*.

These findings raise further questions regarding rapamycin sensitivity: (*a*) whether additional genetic lesions affect rapamycin sensitivity in *PTEN+/−* tumors and (*b*) whether it is important for PI(3)K activation to occur early in tumorigenesis to induce sensitivity to rapamycin. To address these issues, we established an *in vivo* competition assay. This approach allows us to generate genetically defined mixed tumor populations and examine the relative competitiveness of each subpopulation *in vivo* (Fig. 5*A*). Specifically, we infect a fraction of tumor cells from a defined genotype with a retroviral vector coexpressing a transgene or RNA interference (RNAi) construct together with GFP, creating a mixed

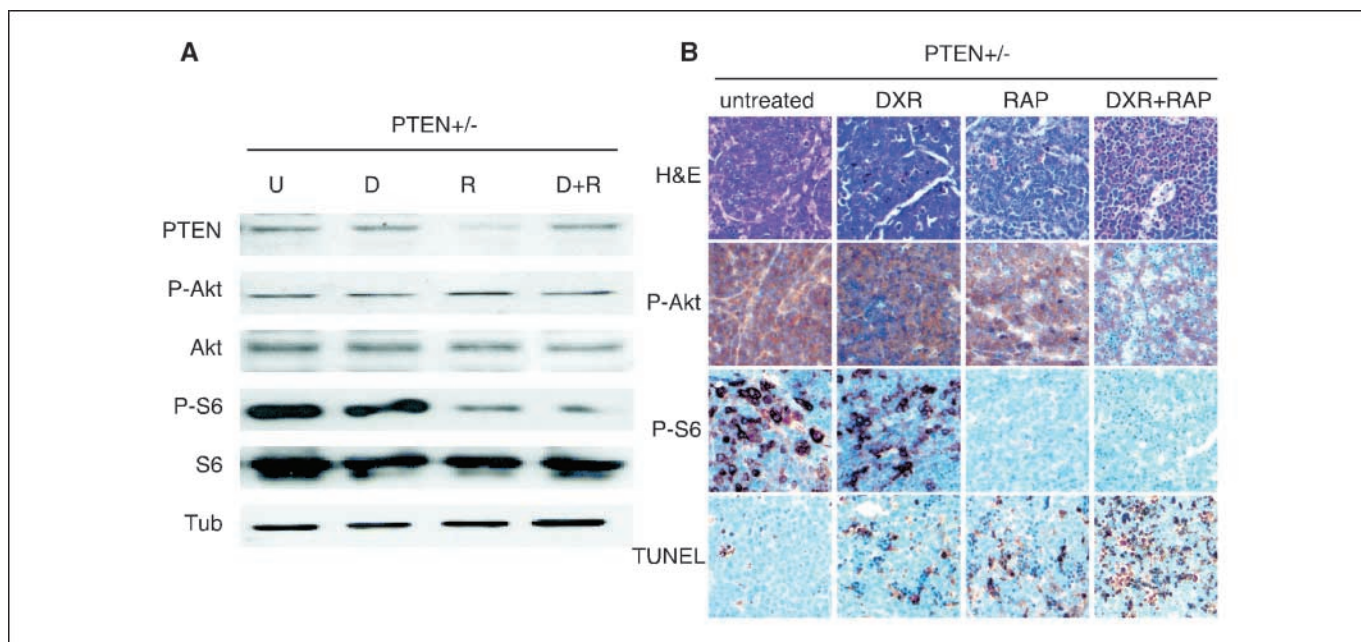

Figure 3. Rapamycin inhibits mTOR activity *in vivo* and promotes apoptosis in response to chemotherapy. *A,* lysates prepared from *Eμ-myc/PTEN+/−* tumors either left untreated (*U*) or 6 hours following *in vivo* treatment with doxorubicin (*D*), rapamycin (*R*), or doxorubicin and rapamycin (*D + R*), and probed for PTEN, phosphorylated and total Akt, and ribosomal S6 protein, and α-tubulin. *B,* representative micrographs of *Eμ-myc/PTEN+/−* tumors untreated or 18 hours after treatment with doxorubicin, rapamycin, or doxorubicin + rapamycin and stained with H&E or immunohistochemical probing detecting the phosphorylated forms of Akt and ribosomal S6 protein or TUNEL.

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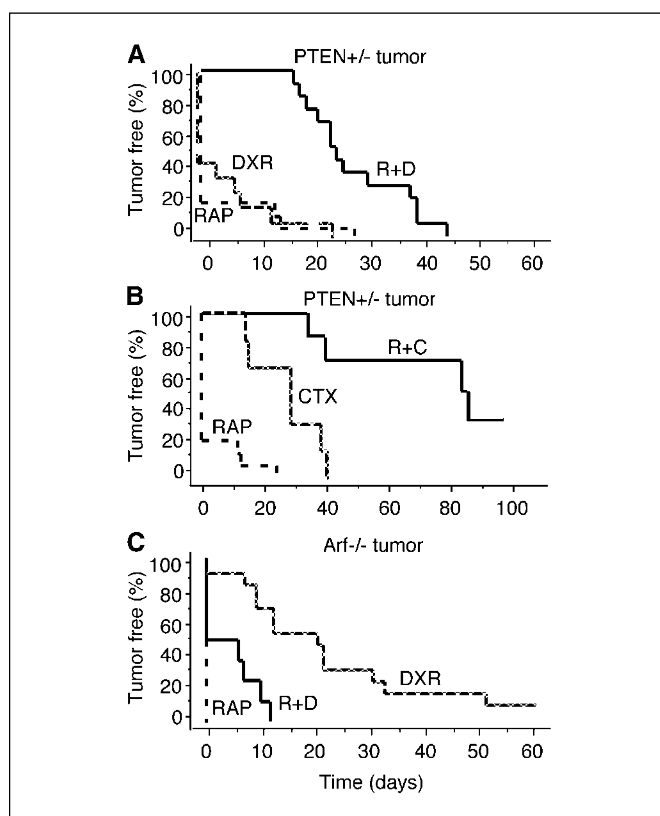

Figure 4. Rapamycin reverses chemoresistance in PTEN-deficient tumors *in vivo*. Kaplan-Meier analyses of tumor-free survival in *Eμ-myc/PTEN+/−* lymphomas following treatment with (*A*) doxorubicin (*hatched line, n* = 11), rapamycin (*RAP*; *dashed line, n* = 13) and doxorubicin + rapamycin (*solid line, R + D, n* = 13). *B,* following treatment with cyclophosphamide (*n* = 6, *hatched line*), rapamycin (*n* = 13, *dashed line*), or cyclophosphamide + rapamycin (*C + R, n* = 7, *solid line*). *C,* time to relapse following treatment of control tumors (*Eμ-myc/ARF−/−*) with doxorubicin (*n* = 15; *hatched line*), rapamycin (*n* = 11, *dashed line*), doxorubicin + rapamycin (*D + R, n* = 8, *solid line*).

population of cells expressing or lacking the provirus. These populations are reintroduced into several recipient animals and, upon lymphoma manifestation, the tumor-bearing mice are either left untreated or treated with a drug or drug combination. Later, residual tumor cells are harvested and subjected to flow cytometry to determine the percentage of GFP-positive cells. Enrichment of GFP-positive cells following therapy (relative to the GFP fraction in untreated tumors) indicates that the construct enhances resistance to the therapy, whereas depletion of GFP suggests a sensitizing effect. Such internally controlled experiments provide a simple and sensitive assay to assess whether a particular lesion influences treatment response *in vivo*.

We first wished to determine whether deregulation of translational initiation downstream of mTOR would promote resistance to rapamycin/chemotherapy combinations in *PTEN+/−* tumors. eIF4e is a translation factor that is inhibited by 4E-BPs, which, in turn, are negatively regulated by mTOR-mediated phosphorylation (40). We therefore infected a subset of *Eμ-myc/PTEN+/−* tumor cells with a vector encoding eIF4E. As occurs in Akt-expressing lymphomas (4), eIF4E-expressing cells were enriched in *PTEN+/−* tumors 48 hours following rapamycin treatment or rapamycin/doxorubicin combinations (Fig. 5*B, left*), indicating that eIF4E enhances resistance to this drug combination. Furthermore, despite their initial poor response to conventional chemotherapy, *PTEN+/−* tumor cells expressing eIF4E were also enriched following treatment with doxorubicin alone. Apparently, deregulation of translation beyond that produced by *PTEN* heterozygosity alone can further enhance chemoresistance (Fig. 5*B, left*).

Although PI(3)K lesions can contribute to sensitization to rapamycin therapy, the extent of this sensitization may be influenced by factors that act downstream (e.g., eIF4E) or in parallel to the oncogenic signaling pathway itself. For example, some studies suggest that rapamycin/chemotherapy combinations may require *p53* tumor suppressor function, although the importance of *p53* to rapamycin-based therapies seems variable (41–43). In our lymphoma model, mTOR inhibition, particularly in combination with chemotherapy, is associated with apoptosis induction, suggesting that an intact apoptotic program might be required for an optimal antitumor response. To determine whether *p53* and/or apoptosis contribute significantly to the therapeutic responses in *PTEN+/−* tumors, we transduced short hairpin RNAs (shRNA) capable of suppressing p53 (36, 37), or a retrovirus overexpressing Bcl-2, into *PTEN+/−* lymphomas, and did *in vivo* competition assays. As expected, cells expressing *p53* shRNAs were more resistant to doxorubicin, confirming the effectiveness of RNAi in this context (Fig. 5*B, middle*). More importantly, lymphoma cells expressing *p53* shRNAs were also enriched following treatment with either rapamycin or combined treatment with doxorubicin and rapamycin, implying *p53* function is also needed for optimal responses to these agents. Similar results were obtained in lymphoma populations harboring Bcl-2-expressing cells, which were more resistant to all three therapies (Fig. 5*B, right*; refs. 10, 44). Thus, genetic lesions acting downstream of mTOR or affecting parallel signaling networks can modulate rapamycin sensitivity in tumors *in vivo*.

In human cancer, PTEN inactivation is often a late event and associated with advanced disease (45). In the *PTEN+/−* tumors we studied, reduced PTEN expression and elevated PI(3)K signaling were presumably present during tumorigenesis (46). To determine whether acute ablation of PTEN in an established tumor could produce similar effects, we used RNAi to suppress PTEN function in ARF-deficient tumor cells, which show no evidence of PI(3)K pathway deregulation and are not sensitive to rapamycin. We generated a *PTEN* shRNA that could achieve a significant, albeit incomplete, knockdown of PTEN in pure populations of infected cells (Fig. 6*A*). Mixed populations of *Eμ-myc/ARF−/−* cells harboring this shPTEN construct or a vector control were treated with rapamycin *in vitro*. Seven days later, the cultures were subjected to flow cytometry to examine the percentage of GFP-expressing cells. Cells expressing PTEN shRNAs showed a marked competitive disadvantage, which was not observed in cells harboring the control vector (Fig. 6*B*). This implies that acute suppression of PTEN sensitizes cells to rapamycin, at least *in vitro*.

In parallel experiments, the mixed populations of the tumor cells described above were transplanted into recipient animals for *in vivo* treatment. Consistent with the ability of deregulated PI(3)K signaling to promote doxorubicin resistance (see Fig. 2*A*), tumor cells harboring *PTEN* shRNAs were enriched to near purity within 48 hours of doxorubicin administration (Fig. 6*C*). By contrast, treatment with rapamycin or the rapamycin/doxorubicin combination dramatically depleted the shPTEN-expressing cell population from mixed tumors *in vivo*. Thus, RNAi-mediated depletion of PTEN in established tumors can sensitize tumor cells to rapamycin-based therapies in a manner that is similar to tumors arising in an *Eμ-myc/PTEN+/−* background. These data also

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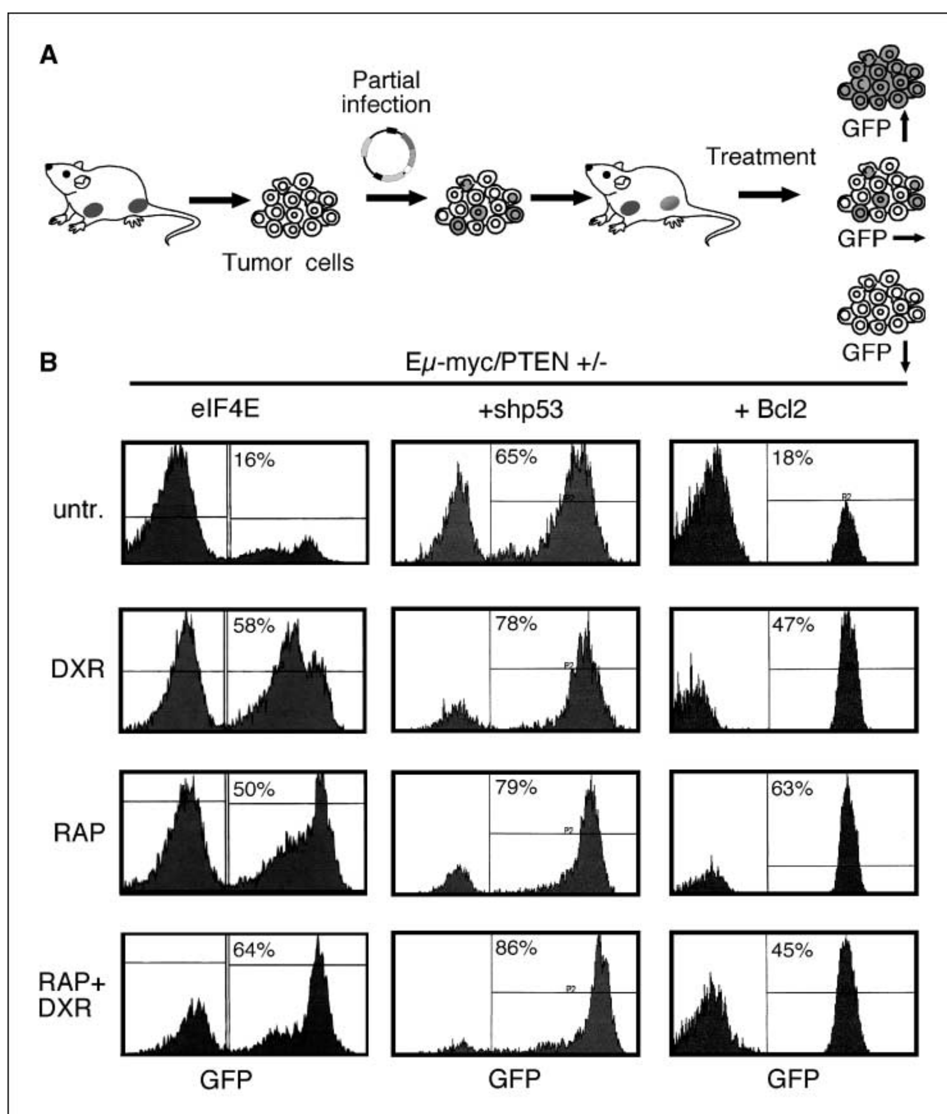

Figure 5. Genetic modifiers of rapamycin sensitivity *in vivo*. *A,* schematic of the *in vivo* competition experimental design. *B,* representative fluorescence-activated cell sorting (FACS) analyses of GFP expression in *Eμ-myc/PTEN+/−* tumors expressing either eIF4E/GFP, shp53/GFP, or Bcl-2/GFP in a fraction of tumor cells harvested 48 hours after the indicated treatment.

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provide the proof of concept for the feasibility of *in vivo* RNAi screens to identify modulators of drug response *in vivo*.

Discussion

The PI(3)K pathway is activated in many tumors, for example, through mutations that target the *PI3KA, PTEN, Akt*, or *TSC1* or *TSC2* genes (reviewed in refs. 11, 47). PI(3)K activation can produce diverse changes in cell physiology and inhibits apoptosis, indicating that PI(3)K pathway mutations may have broad consequences on tumorigenesis and treatment responses. Owing to its gain-of-function mode of action, the pathway is also a compelling target for new therapeutics. Nonetheless, whether mutations in different components of the PI(3)K pathway produce the same effects on tumor physiology is not clear. We had previously described the effects of Akt expression on lymphomagenesis and treatment response in the *Eμ-myc* lymphoma model, and observed a dramatic effect of Akt in promoting tumorigenesis, resistance to conventional therapy, and sensitivity to rapamycin/chemotherapy combinations (4). However, lesions that activate PI(3)K signaling at the level of PTEN are more common in human cancer (15), and so we wished to determine how suppression of PTEN would affect tumor phenotypes in the *Eμ-myc* system.

Although our previous studies showed that Akt could dramatically accelerate *myc*-induced lymphomagenesis, *Eμ-myc* transgenic mice harboring loss of one *PTEN* allele showed little, if any, acceleration and most tumors examined retained the wild-type *PTEN* allele. However, the PI(3)K pathway is clearly activated in *PTEN+/−* lymphomas, although to a lesser and more varied extent than in tumors expressing an activated form of Akt (Fig. 1*D*). Likely, tumorigenesis in the presence of Myc requires a high flux through the Akt pathway (19). It remains possible, however, that high levels of Akt may have broader consequences for tumorigenesis than PTEN loss, either through "off-target" effects on other signaling networks or by differentially affecting various feedback loops. In this regard, the *PTEN* knockout mouse dies during embryonic development, precluding study of a complete ablation of PTEN on tumorigenesis in these animals. More recently, conditional PTEN knockout mice have been developed, and at least in a glioma model, complete loss of PTEN mimics Akt action during tumorigenesis (48).

Despite the relatively minor effects of *PTEN* heterozygosity on tumorigenesis, we noted marked responses on the response of

tumors to conventional or targeted therapy. Thus, loss of even one allele of *PTEN* was sufficient to produce chemoresistant tumors, a phenomenon also observed in lymphomas produced by over-expressing Akt. In principle, such resistance could represent a secondary consequence of other mutations arising in the *PTEN+/−* tumors, but we see that acute suppression of PTEN in an established tumor was sufficient to promote resistance to doxorubicin, and that rapamycin (which targets mTOR downstream of PTEN) restores drug-induced apoptosis to *PTEN+/−* tumors. Such a haploinsufficient effect of *PTEN* has been noted in tumorigenesis in a mouse prostate cancer model (19). Here, we see that reduced PTEN dosage can have a varied effect on tumor phenotypes, affecting treatment sensitivity to a greater degree than tumorigenesis in our model.

Mice harboring *PTEN+/−* lymphomas could be effectively treated using rapamycin/chemotherapy combinations, leading to durable responses that were similar to mice harboring Akt lymphomas. Also, as observed in mice harboring Akt lymphomas, mice with *PTEN+/−* lymphomas treated did not respond well to rapamycin therapy at our dose and schedule, despite the ability of the drug to efficiently inhibit mTOR activity. Nevertheless, previous studies indicate that *PTEN* loss can be sufficient to confer rapamycin sensitivity in some contexts (28, 29, 31), and we see that suppression of PTEN confers a selective disadvantage to tumor cells treated with rapamycin in culture. Thus, chemotherapy combinations are not required for rapamycin to have an antitumor effect, but potentiate the consequences of mTOR inhibition. Together, these data indicate that even modest changes in flux through the PI(3)K pathway can have profound effects on treatment behavior.

Precisely how rapamycin and chemotherapy synergize to produce antitumor responses in *Eμ-myc* lymphomas or other cancers remains to be determined, but our studies provide insights into its mode of action. One factor seems to be the ability of rapamycin to inhibit translation, as enforced expression of the translation initiation factor eIF4E in *PTEN+/−* tumors enhanced resistance to chemotherapy or rapamycin/chemotherapy combinations. Another factor seems to be the presence of a PI(3)K pathway lesion, leading to the deregulation of PI(3)K signaling. Thus, tumors overexpressing Akt (4) or with reduced PTEN expression were sensitive to this combination and, at the same dose and schedule, ARF-deficient tumors [which show no evidence for PI(3)K involvement] showed a worse response to the combined therapy than to chemotherapy alone. Interestingly, these lesions did not have to be present during tumorigenesis, because acute suppression of PTEN in established tumors using RNAi could enhance rapamycin sensitivity.

Although a PI(3)K pathway lesion may confer sensitivity to rapamycin-based therapies, our data indicate that other pathways also influence rapamycin responses. Thus, disruption of *p53* or over-expression of Bcl-2 in *PTEN+/−* tumors produced tumor cells that were more resistant to rapamycin/chemotherapy combinations. Interestingly, the role of *p53* in influencing rapamycin responses is controversial, with studies differing on whether *p53*-deficient cells are more sensitive or resistant to rapamycin therapy *in vitro* (41–43, 49, 50). One

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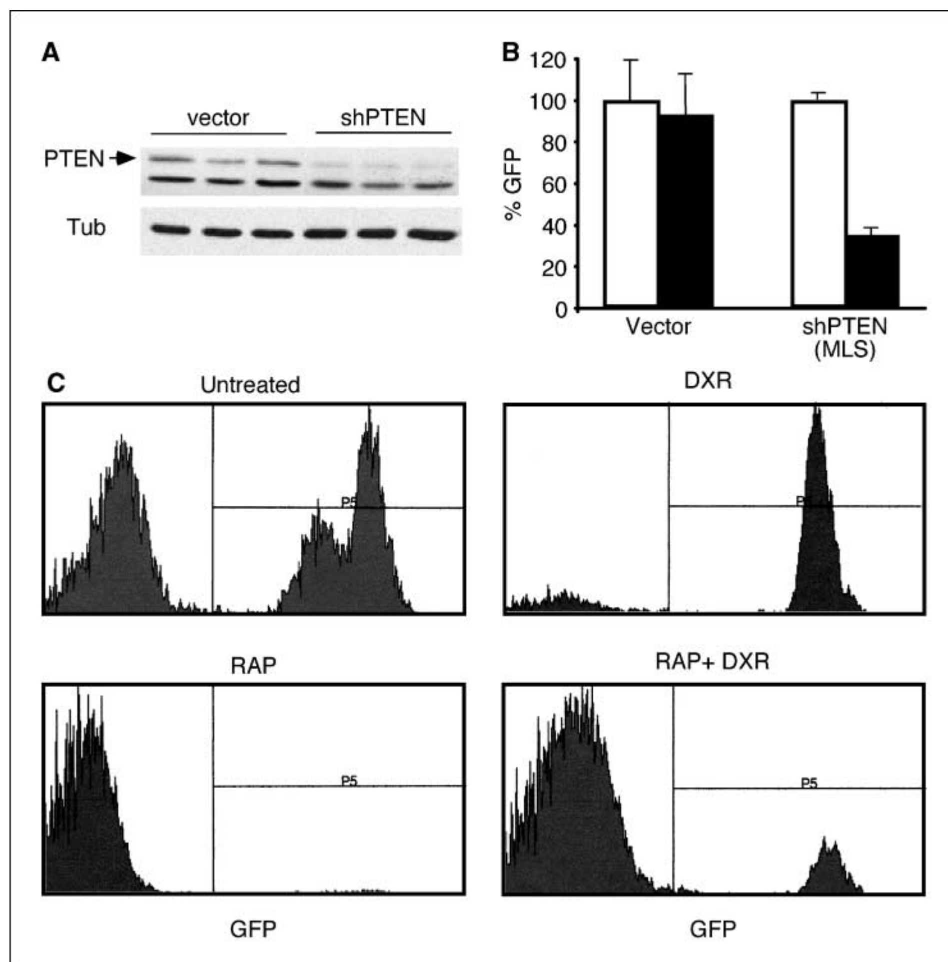

Figure 6. PTEN knockdown sensitizes established tumors to rapamycin *in vitro* and *in vivo. A,* immunoblot of lysates prepared from separate batches of Ba/F3/p210 cells transduced with either a control vector (*vector*; *lanes 1-3*) or an RNAi vector targeting PTEN (*shPTEN*; *lanes 4-6*), probed with antibodies against PTEN and tubulin as loading control. *B, in vitro* competition experiment. Summary of FACS analyses of GFP expression in *Eμ-myc/ARF−/− tumors* partially transduced with an RNAi-GFP vector targeting PTEN (*shPTEN*) or a control vector-GFP (*Vector*) and either left untreated (*white columns*) or treated with 1 μmol/L rapamycin for 7 days (*black columns*), data are normalized to the untreated control (100%). *Columns,* mean; *bars,* SD. *C,* FACS analysis of *Eμ-myc/ARF−/−* tumors partially transduced with an RNAi-GFP construct targeting PTEN (shPTEN) and harvested 48 hours after the indicated treatments.

recent report indicates that the antitumor activity of combination therapy with rapamycin and cisplatin depends on an intact *p53* response in cultured tumor cells (43), and our study indicates that *p53* is important for other rapamycin/chemotherapy combinations *in vivo*. However, the role of *p53* in affecting rapamycin-based therapies is not restricted to combinations involving cytotoxic drugs, as we see that disruption of *p53* can produce a selective advantage to tumor cells treated with rapamycin alone. This suggests that *p53* may influence how a tumor cell responds to mTOR inhibition, and is consistent with our observation that *p53* mutations promote resistance to imatinib in a mouse leukemia model initiated by *Bcr-abl*, an imatinib target (51). However, these observations do not rule out *p53*-independent modes of action, and conceivably the relative contributions of *p53* and apoptotic mechanisms to rapamycin action may depend on the cellular context. Understanding the precise molecular contexts whereby PI(3)K pathway lesions promote sensitivity to mTOR inhibition and the parallel pathways that can modulate its effects will be important for the effective use of mTOR inhibitors in the clinic.

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