

mTOR Complex 2 Is Required for the Development of Prostate Cancer Induced by *Pten* Loss in Mice

David A. Guertin,^{1,2,3} Deanna M. Stevens,^{1,8} Maki Saitoh,^{1,2} Stephanie Kinkel,^{1,2,3} Katherine Crosby,⁴ Joon-Ho Sheen,^{1,2,3} David J. Mullholland,⁵ Mark A. Magnuson,⁶ Hong Wu,⁵ and David M. Sabatini^{1,2,3,7,*}

¹Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

²Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

³The David H. Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA 02139, USA

⁴Cell Signaling Technologies, Danvers, MA 01923, USA

⁵Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA

⁶Department of Molecular Physiology and Biophysics and Center for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

⁷The Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA

⁸Present address: Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA

*Correspondence: sabatini@wi.mit.edu

DOI 10.1016/j.ccr.2008.12.017

SUMMARY

mTOR complex 2 (mTORC2) contains the mammalian target of rapamycin (mTOR) kinase and the Rictor regulatory protein and phosphorylates Akt. Whether this function of mTORC2 is critical for cancer progression is unknown. Here, we show that transformed human prostate epithelial cells lacking PTEN require mTORC2 to form tumors when injected into nude mice. Furthermore, we find that *Rictor* is a haploinsufficient gene and that deleting one copy protects *Pten* heterozygous mice from prostate cancer. Finally, we show that the development of prostate cancer caused by *Pten* deletion specifically in prostate epithelium requires mTORC2, but that for normal prostate epithelial cells, mTORC2 activity is nonessential. The selective requirement for mTORC2 in tumor development suggests that mTORC2 inhibitors may be of substantial clinical utility.

INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is aberrantly active in many human cancers. The best characterized downstream target of PI3K activation is the Akt kinase, which influences cancer cell metabolism, survival, growth, proliferation, angiogenesis, and migration by phosphorylating a diverse array of substrates (reviewed in Manning and Cantley, 2007). Two common causes of aberrant PI3K activation include loss of the PTEN tumor suppressor and activating mutations in PI3K. Either event results in phosphatidylinositol (3,4,5)P₃ accumulation at cell membranes, which serve as a docking site for Akt

localization and activation. Akt requires phosphorylation on two sites for full activation (reviewed in Guertin and Sabatini, 2007; Hanada et al., 2004; Manning and Cantley, 2007). Following membrane recruitment, PDK1 phosphorylates Akt at one site (T308 in Akt1) in the kinase domain while mTOR phosphorylates Akt at another site (S473 in Akt1) in a C-terminal hydrophobic motif (reviewed in Guertin and Sabatini, 2007; Hanada et al., 2004; Manning and Cantley, 2007).

The mTOR kinase, which is the mammalian target of the drug rapamycin, assembles into at least two distinct complexes, called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each of which has unique substrates (reviewed in

SIGNIFICANCE

Small-molecule inhibitors that compromise cancer but not normal cell functions would be valuable anticancer therapeutics. However, identifying intracellular targets for this type of inhibitor is challenging. Here, we present genetic evidence that mTOR complex 2 (mTORC2) is a candidate target for such an inhibitor, as the development of invasive prostate cancer induced by *Pten* loss in mice requires mTORC2 activity. However, mTORC2 activity is dispensable for the development of normal prostate epithelium in mice and for the proliferation and survival of primary mouse fibroblasts in culture. PTEN loss activates the PI3K signaling pathway, which is inappropriately activated in many human cancers. Our findings suggest that mTORC2 inhibitors could have broad clinical applications.

Guertin and Sabatini, 2007). In addition to mTOR, mTORC1 contains Raptor, PRAS40, and mLST8/G β L and regulates cell growth by controlling the activity of the S6 kinases and the 4E-BP proteins. mTORC2 contains mLST8/G β L, two unique regulatory proteins named Rictor and SIN1, and a protein of unknown function called PROTOR/PRR5. When assembled into mTORC2, mTOR phosphorylates Akt (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005; Shiota et al., 2006). Rapamycin, despite being a potent inhibitor of mTORC1, does not appear to be a general inhibitor of mTORC2. In a subset of human cancer cells, rapamycin inhibits mTORC2 by preventing its assembly, but the determinants of this phenomenon are unknown (Sarbassov et al., 2006).

Efforts to develop PI3K inhibitors or inhibitors of downstream effectors like Akt are intensive. One Akt-regulated pathway receiving considerable interest from drug development enterprises is the mTORC1 growth pathway (reviewed in Abraham and Eng, 2008). Akt activates mTORC1 by phosphorylating and inhibiting TSC2, a GTPase-activating protein that negatively regulates mTORC1 activity (reviewed in Manning and Cantley, 2007). Akt also activates mTORC1 directly by phosphorylating the PRAS40 subunit (Vander Haar et al., 2007; Huang and Porter, 2005; Kovacina et al., 2003; Sancak et al., 2007). Other well-known targets of Akt include the FoxO transcription factors and the GSK3 kinase (reviewed in Manning and Cantley, 2007).

Genetic studies in mice have been crucial to understanding the role of PI3K activation in tumorigenesis (Salmena et al., 2008). *Pten* heterozygous mice spontaneously develop neoplasms in multiple organs, resulting in a shortened life span (Di Cristofano et al., 1998; Freeman et al., 2006; Podsypanina et al., 1999; Suzuki et al., 1998a). Reports demonstrating that mice expressing a hypomorphic allele of *Pdk1* or lacking *Akt1* (one of three *Akt* genes in mammals) are protected from tumor development induced by *Pten* loss highlight the importance of Akt activity (Bayascas et al., 2005; Chen et al., 2006).

Mice expressing conditional alleles of *Pten* have also been developed to study the role of PTEN in tissue-specific cancers (Backman et al., 2001; Trotman et al., 2003; Wang et al., 2003). Human prostate cancer in particular shows strong association with PTEN loss (Dahia, 2000; Sellers and Sawyers, 2002; Suzuki et al., 1998b). Expression of Cre recombinase in prostate epithelial cells of *Pten^{loxP/loxP}* mice results in *Pten* deletion and Akt hyperphosphorylation in the prostate epithelium (Wang et al., 2003). These mice develop epithelial hyperplasia, which progresses to murine prostatic intraepithelial neoplasia (mPIN) within 6 weeks of age and to invasive adenocarcinoma and metastasis by 9–12 weeks in a manner that recapitulates the disease progression in humans. Similar to human prostate cancer, tumors in these mice derive from the prostate epithelium, respond to androgen ablation, and exhibit similar gene expression changes, making them a useful model to study the disease.

An ideal target for an anticancer drug is one that, when inhibited, has no effect on normal cells but compromises the proliferation and/or survival of cancer cells. We hypothesized that mTORC2 may be such a target in cancers driven by PI3K activation because previous studies have shown that it is not required for survival of mouse embryonic fibroblasts (MEFs) or development of *Drosophila* embryos (Guertin et al., 2006;

Hietakangas and Cohen, 2007; Jacinto et al., 2006; Shiota et al., 2006). The role of mTORC2 in tumorigenesis cannot be tested pharmacologically because specific inhibitors of mTORC2 are currently unavailable. Therefore, to test our hypothesis, we investigated the role of mTORC2 activity in (1) a human prostate cancer epithelial cell line lacking *Pten* expression, (2) a spontaneous mouse model of cancer dependent upon *Pten* loss, and (3) a mouse model of prostate cancer initiated by *Pten* deletion specifically in prostate epithelial cells.

RESULTS

Rictor Is Required for PC-3 Cells to Form Tumors as Xenografts

Human prostate cancer is associated with loss of the PTEN tumor suppressor (Dahia, 2000; Sellers and Sawyers, 2002; Suzuki et al., 1998b). To test whether PTEN-deficient human prostate cancer cells require mTORC2 activity to form tumors, we investigated whether knocking down Rictor in the PC-3 cell line (a human prostate cancer cell line null for *PTEN*) impairs their ability to form tumors when injected into nude mice. Two independent short hairpin RNAs (shRNAs) targeting Rictor (shRictor¹ and shRictor²) or a control shRNA targeting luciferase (shLuc) were delivered by lentivirus and stably expressed in PC-3 cells. We selected hairpins that differentially decrease Rictor expression and showed that, in vitro, shRictor¹ robustly decreases total Rictor protein while shRictor² reduces Rictor to an intermediate degree (Figure 1A). Both Rictor hairpins reduced Akt^{S473} phosphorylation to levels consistent with the level of Rictor knockdown (Figure 1A). In vitro, both shRictor¹ and shRictor² knockdown cells had a proliferation defect, the severity of which also correlated with the level of Rictor knockdown (Figure 1B).

To determine whether Rictor depletion affects the ability of PC-3 cells to form solid tumors in vivo, we injected PC-3 cells expressing shLuc, shRictor¹, or shRictor² subcutaneously into nude mice and monitored tumor formation for 28 days, at which point tumors were removed and measured. PC-3 cells expressing shLuc formed tumors visibly larger than tumors formed by PC-3 cells expressing either shRictor¹ or shRictor² (Figure 1C). Tumors that developed from shLuc-expressing cells grew to an average volume of 312 mm³ (Figure 1D). In contrast, tumors that developed from shRictor¹- and shRictor²-expressing cells grew to an average volume of 10.1 mm³ ($p = 0.01$) and 39.6 mm³ ($p = 0.01$), respectively, with the difference in tumor volume again corresponding to Rictor knockdown levels (Figure 1D). Our findings indicate that PC-3 cells require mTORC2 activity to form tumors in vivo.

Partial Loss of mTORC2 Activity Extends the Life Span of *Pten^{+/-}* Mice and Can Protect Mice against Prostate Cancer

Our xenograft study suggested that mTORC2 activity may be important in prostate cancer development when the PTEN tumor suppressor is lost. We next asked whether reducing mTORC1 and/or mTORC2 activity in a genetic model of cancer dependent upon *Pten* loss could extend life span. *Pten^{+/-}* mice spontaneously develop tumors in multiple organs, resulting in a shortened life span (Di Cristofano et al., 1998; Freeman et al., 2006; Podsypanina et al., 1999; Suzuki et al., 1998a). We crossed

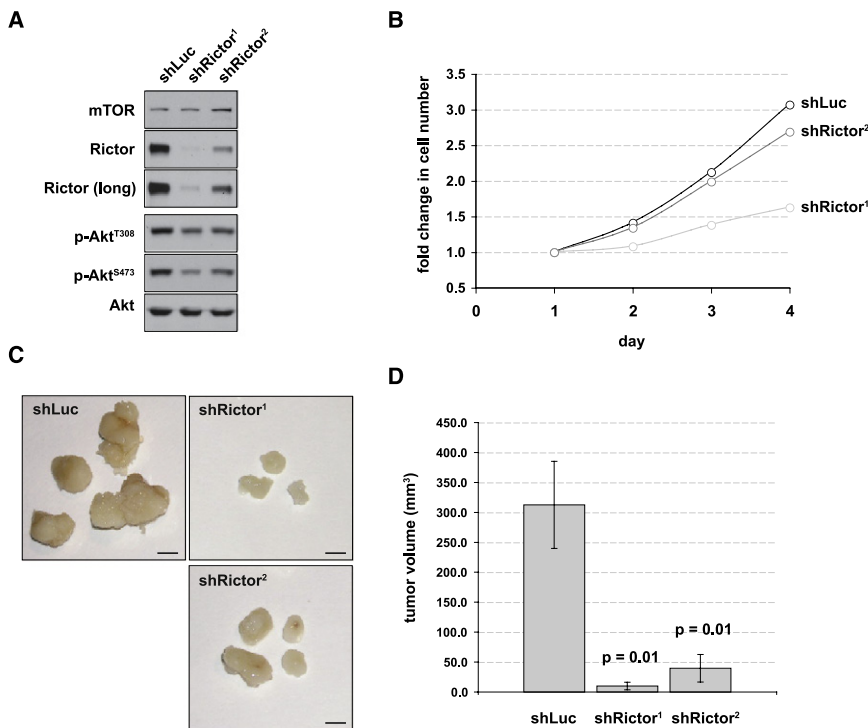


Figure 1. PC-3 Prostate Cancer Cells Require Rictor to Form Tumors as Xenografts

(A) Generation of PC-3 cells with stable knockdown of Rictor. Using a lentiviral delivery system, two independent shRNAs that silence *Rictor* expression (shRictor¹ and shRictor²) or a control shRNA targeting luciferase (shLuc) were stably expressed in PC-3 cells. Protein lysates prepared 6 days postinfection were probed with the indicated antibodies.

(B) Rictor knockdown impairs cell proliferation in vitro. PC-3 cells stably expressing shLuc, shRictor¹, or shRictor² were seeded in triplicate at equal density, and cell number was counted on four consecutive days. Fold change in cell number is shown.

(C) PC-3 cells require mTORC2 to form tumors as xenografts. PC-3 cells stably expressing shLuc, shRictor¹, or shRictor² were injected subcutaneously into nude mice. Tumors were dissected and photographed 28 days postinjection. Scale bar = 2 mm.

(D) Average tumor volume (in mm³) ± SEM.

Pten^{+/-} mice with mice heterozygous for the *mtor*, *Raptor*, *mlst8*, or *Rictor* genes, and the offspring were monitored for 52 weeks. Interestingly, *Pten*^{+/-}*mtor*^{+/-} ($p = 0.023$) and *Pten*^{+/-}*mlst8*^{+/-} ($p = 0.044$) mice lived longer than strain-matched *Pten*^{+/-} controls (Figure 2A). In contrast, *Pten*^{+/-}*Raptor*^{+/-} mice showed no difference in life span compared to *Pten*^{+/-} controls. *Pten*^{+/-}*Rictor*^{+/-} mice also tended to live longer than *Pten*^{+/-} mice, but the effect was less pronounced due to a difference in the *Rictor*^{+/-} mouse strain composition (see Experimental Procedures and Freeman et al., 2006). In developing mouse embryos, only mTORC2 requires *mlst8* and *Rictor*, while mTORC1 requires *Raptor* (Guertin et al., 2006). Thus, life-span extension of *Pten*^{+/-} mice doubly heterozygous for *mtor*, *mlst8*, or *Rictor* likely reflects a reduced capacity for mTORC2 signaling, although this does not rule out the possibility that reducing mTORC1 activity may also contribute to extending life span.

To confirm that deleting one *Rictor* gene reduces mTORC2 signaling, we examined Akt activity in *Rictor* heterozygous MEFs. We found that deleting one *Rictor* gene reduced the serum-stimulated in vitro kinase activity of Akt (Figure 2B) and that this correlated with reduced Akt^{S473} phosphorylation (Figure 2C). To generate MEFs null for *Pten* and also missing only one *Rictor* gene, we infected *Pten*^{loxP/loxP} and *Pten*^{loxP/loxP} *Rictor*^{loxP/+} MEFs with a control adenovirus or adenovirus expressing Cre recombinase (Adeno-Cre) and investigated whether partial loss of *Rictor* impairs *Pten* deletion-induced Akt^{S473} phosphorylation. We found that deleting one *Rictor* gene slightly reduced the increase in Akt^{S473} phosphorylation caused by *Pten* deletion and prevented insulin from further increasing the phospho-Akt^{S473} signal (Figure 2D). Moreover, loss of one *Rictor* gene reduced the expected Mendelian ratio at birth (Guertin et al., 2006) and decreased Akt^{S473} phosphorylation in liver tissue (Yang et al., 2006). Thus, *Rictor*

is a haploinsufficient gene, and deleting one copy diminishes mTORC2 activity.

Because we were interested in the role of mTORC2 in prostate cancer, we examined the prostate tissue of offspring born from *Pten*^{+/-} and *Rictor*^{+/-} crosses that had survived for 1 year. Five of nine *Pten*^{+/-} male mice had visible tumors, while only one of ten surviving *Pten*^{+/-}*Rictor*^{+/-} mice had a similar phenotype (Figure 2E). No tumors were detectable in any of the wild-type controls ($n = 10$). A comparison of hematoxylin and eosin (H&E)-stained sections generated from these samples indicated that *Pten*^{+/-} mice developed severe neoplasia (Figure 2F). Prostates from *Pten*^{+/-}*Rictor*^{+/-} mice exhibited signs of hyperplasia (Figure 2F). However, in contrast to the lesions observed in *Pten*^{+/-} mice, the lesions in the double-heterozygous mice appeared less severe and contained larger cells. There also were fewer proliferating cells in the *Pten*^{+/-}*Rictor*^{+/-} samples, as only 2.3% (19 of 836) of the cells in three representative images were positive for the proliferation marker Ki67, compared with 10.7% (203 of 1902) of the cells from *Pten*^{+/-} samples ($p < 0.001$) (Figure 2F). Thus, the haploinsufficiency associated with loss of one *Rictor* gene protects *Pten*^{+/-} mice from prostate cancer. Because of a difference in genetic background between *Rictor*^{+/-} mice and *mtor*, *Raptor*, and *mlst8* heterozygous mice, *Pten*^{+/-} controls born from crosses between *Pten*^{+/-} and *mtor*^{+/-}, *Raptor*^{+/-}, or *mlst8*^{+/-} parents are less susceptible to prostate cancer, and this precludes an analysis of the disease in these cohorts (Freeman et al., 2006; Guertin et al., 2006).

Rictor Is Required for *Pten* Deletion-Induced Akt Phosphorylation and Transformation of Prostate Epithelial Cells In Vivo

The observation that partial loss of *Rictor* protects *Pten*^{+/-} mice from prostate cancer prompted us to examine this role of

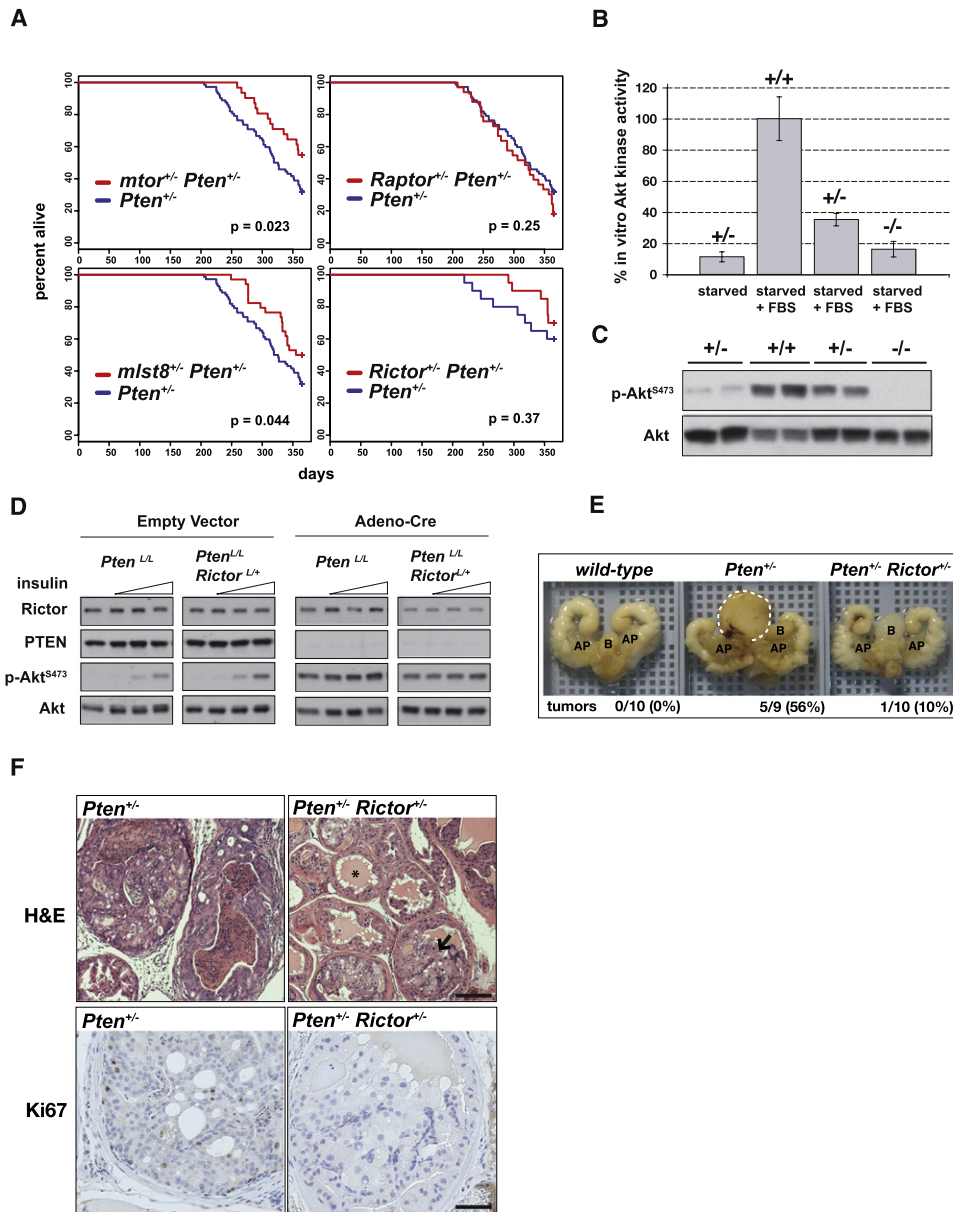


Figure 2. Phenotypes Associated with *Pten* Heterozygosity Require mTORC2

(A) *Pten*^{+/-} mice lacking one *mtor*, *mlst8*, or *Rictor* gene tend to live longer. Survival was monitored for 1 year and is displayed using Kaplan-Meier plots. (B and C) *Rictor*^{+/-} mouse embryonic fibroblasts (MEFs) have reduced Akt activity. (B) Akt was isolated from serum-deprived (starved) or stimulated (+FBS) MEFs that were wild-type or deleted for one or both *Rictor* genes. The ability of immunopurified Akt to incorporate radiolabeled phosphate into a synthetic substrate was measured. Error bars indicate standard error of the mean. (C) Immunoblot corresponding to (B), showing phospho-Akt^{S473} and total Akt levels. (D) Adeno-Cre was added to MEFs harboring conditional alleles of *Pten* and *Rictor* (*Pten*^{L/L} and *Pten*^{L/L}*Rictor*^{L/+}) to generate *Pten* null MEFs lacking only one *Rictor* gene. Empty vector was added to generate control cells. Lysates were prepared from serum-deprived cells that were stimulated with 0, 1, 10, or 100 nm insulin for 10 min and probed with the indicated antibodies. (E and F) *Pten*^{+/-} *Rictor*^{L/+} mice are protected against prostate tumor development. (E) Genitourinary tracts of wild-type, *Pten*^{+/-}, and *Pten*^{+/-} *Rictor*^{L/+} mice that survived to 1 year were dissected and photographed. Bladder (B) and anterior prostate (AP) are indicated for orientation. An example of a large tumor associated with a *Pten*^{+/-} prostate is circled with a dashed white line. (F) *Pten*^{+/-} and *Pten*^{+/-} *Rictor*^{L/+} prostate tissue was sectioned and stained with hematoxylin and eosin (H&E, top) or labeled with Ki67 antibody (bottom). Representative images are shown. The asterisk in the upper right image indicates a normal prostate ductule, and the arrow points to a ductule containing large hyperplastic cells. Scale bars = 50 μ m (top) and 25 μ m (bottom).

mTORC2 more rigorously. Conditional deletion of *Pten* specifically in the prostate epithelium leads to prostate cancer with short latency and a histological pattern of disease progression modeling that of human patients with prostate cancer (Wang et al., 2003). In this model, expression of Cre recombinase from a modified rat probasin (*PB*) promoter (Wu et al., 2001) in *Pten* conditional mice (*Pten^{loxP/loxP}PB-Cre⁺*) induces postnatal deletion of *Pten* in prostate epithelial cells (Wang et al., 2003; Wu et al., 2001). *Pten^{loxP/loxP}PB-Cre⁺* mice develop mPIN by 6 weeks of age, which progresses to invasive adenocarcinoma by 9 weeks and then to metastatic cancer by 12 weeks (Wang et al., 2003). To determine the role of mTORC2 in the progression of cancer in this model, we used conditional alleles to delete *Rictor* in combination with *Pten*.

To test the efficiency of the double-knockout strategy, primary MEFs derived from *Pten^{loxP/loxP}* or *Pten^{loxP/loxP}Rictor^{loxP/loxP}* embryos were infected with Adeno-Cre and cultured for 5 days. PTEN and Rictor protein and Akt phosphorylation levels were then measured by western analysis. As expected, infection of *Pten^{loxP/loxP}* MEFs with Adeno-Cre reduced PTEN expression and elevated both Akt^{S473} and Akt^{T308} phosphorylation in serum-deprived cells (Figure 3A, lanes 1 and 3) and in insulin-stimulated cells (Figure 3A, lanes 2 and 4). In contrast, *Pten^{loxP/loxP}Rictor^{loxP/loxP}* MEFs infected with Adeno-Cre showed no increase in Akt^{S473} phosphorylation despite reduced *Pten* expression (Figure 3A, lanes 5 and 7). Furthermore, these MEFs exhibited only a small increase in S473 phosphorylation following insulin stimulation (Figure 3A, lanes 6 and 8). This slight increase in S473 phosphorylation in insulin-stimulated *Pten^{loxP/loxP}Rictor^{loxP/loxP}* MEFs likely resulted from a subpopulation of cells that Adeno-Cre failed to infect, as an immunoblot analysis clearly showed that not all Rictor was lost. As expected in MEFs, insulin regulation of Akt^{T308} phosphorylation and S6K^{T389} phosphorylation (a reporter for mTORC1 activity) was unaffected by *Rictor* deletion (Figure 3; Guertin et al., 2006).

We then asked whether conditional deletion of *Rictor* inhibits the increase in Akt^{S473} phosphorylation induced by *Pten* deletion in the prostate. PCR analysis of prostate tissue samples from *Pten* conditional mice harboring either one or two conditional alleles of *Rictor* and the *PB-Cre⁺* transgene indicated that recombination occurred simultaneously in the prostate epithelium, but not in tail or bladder tissue prepared from the same animals (Figure 3B). Next, we generated protein lysates from dorsolateral prostate tissue of wild-type, *Pten^{loxP/loxP}PB-Cre⁺*, and *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* animals. By immunoblot analysis, we found that mTOR protein levels remained unchanged in all three genotypes (Figure 3C). In contrast, total Rictor protein increased in *Pten^{loxP/loxP}PB-Cre⁺* prostate tissue but was mostly absent in *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* tissue (Figure 3C). As expected, *Pten* loss substantially elevated both Akt^{T308} and Akt^{S473} phosphorylation. Importantly, the increase in phospho-Akt^{S473} was suppressed in *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* tissue, indicating that Akt^{S473} phosphorylation caused by *Pten* deletion requires mTORC2. Interestingly, we found that the increase in T308 phosphorylation induced by *Pten* loss also requires mTORC2. This is different from what we observed in MEFs (Figure 3A; Guertin et al., 2006) but is more similar to the situation in human cancer cells, wherein phosphorylation at T308 requires

mTORC2 activity (Figure 1A; Hresko and Mueckler, 2005; Sarbassov et al., 2005).

Genitourinary tracts were isolated from 7-week-old wild-type, *Rictor* conditional, *Pten* conditional, or doubly conditional *PB-Cre⁺* mice. H&E staining and immunohistochemistry (IHC) with a Rictor antibody performed on dorsolateral prostate tissue indicated that, compared to wild-type epithelial cells, *Rictor^{loxP/loxP}PB-Cre⁺* cells are slightly smaller, but the overall architecture of the prostate epithelial cell layer is normal (Figure 3D; see also Figure S1 available online). Consistent with this observation, immortalized *Rictor* null MEFs were smaller than wild-type MEFs in volume by ~10%, while proliferating at the same rate (Figure S1). We have examined the histology of prostate tissue from *Rictor^{loxP/loxP}PB-Cre⁺* mice as old as 16 weeks and have found no obvious differences compared to age-matched wild-type tissue (data not shown). In both wild-type and *Rictor^{loxP/loxP}PB-Cre⁺* tissue, Akt^{S473} phosphorylation is undetectable (Figure 3D). Thus, Rictor, and by extension mTORC2, is not required to maintain the integrity of normal prostate epithelium.

Consistent with previous studies, *Pten^{loxP/loxP}PB-Cre⁺* mice developed mPIN, which is characterized by extensive epithelial cell hyperplasia within preexisting ductules (Figure 3D), by 7 weeks of age. In general, the individual prostate ductules were enlarged but remained intact with no signs of a desmoplastic response in the surrounding stromal tissue. The individual atypical epithelial cells were clearly larger than wild-type cells and were positive for Akt^{S473} phosphorylation, with the most intense signal at the cell membrane. *Pten* deletion also appeared to increase total Rictor protein level, consistent with the immunoblot data in Figure 3C, and suggests possible feedback activation of *Rictor* gene expression.

In contrast, prostatic ductules from *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* mice exhibited a mixed phenotype containing mostly normal, organized epithelial cells and patches of large, disorganized hyperplastic cells (Figure 3D). We suspected that *Rictor*, which is required for Akt^{S473} phosphorylation, might be inefficiently deleted in these atypical cells. Indeed, comparison of Rictor protein levels and the phospho-Akt^{S473} signal indicated that normal cells deficient in Rictor protein expression were negative for Akt^{S473} phosphorylation, while atypical cells maintained Rictor protein expression and stained positively for phosphorylated S473 (Figure 3D; Figure S2). This was consistent with the immunoblot data showing that Akt^{S473} phosphorylation is not completely lost in *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* tissue (Figure 3C). Also consistent with inefficient *Rictor* deletion, we clearly observed some PTEN-negative cells that were positive for phospho-Akt^{S473} and others that were negative for phospho-Akt^{S473} signal (Figure 3E). Importantly, PTEN-deficient phospho-Akt^{S473}-negative cells appeared normal. A recent study has suggested that *Pten* loss downregulates the probasin promoter, which could explain the recombination inefficiency (Jiao et al., 2007; Lei et al., 2006).

By 9–10 weeks of age, prostate adenocarcinoma was detectable in *Pten^{loxP/loxP}PB-Cre⁺* mice. Nearly all of the dorsolateral lobes at this age contained extensive malignant cells characterized by Akt^{S473} phosphorylation, especially at the membrane (Figure 4A). Compared to wild-type tissue, the diseased glands were enlarged and the ductule boundaries disorganized. Invasive

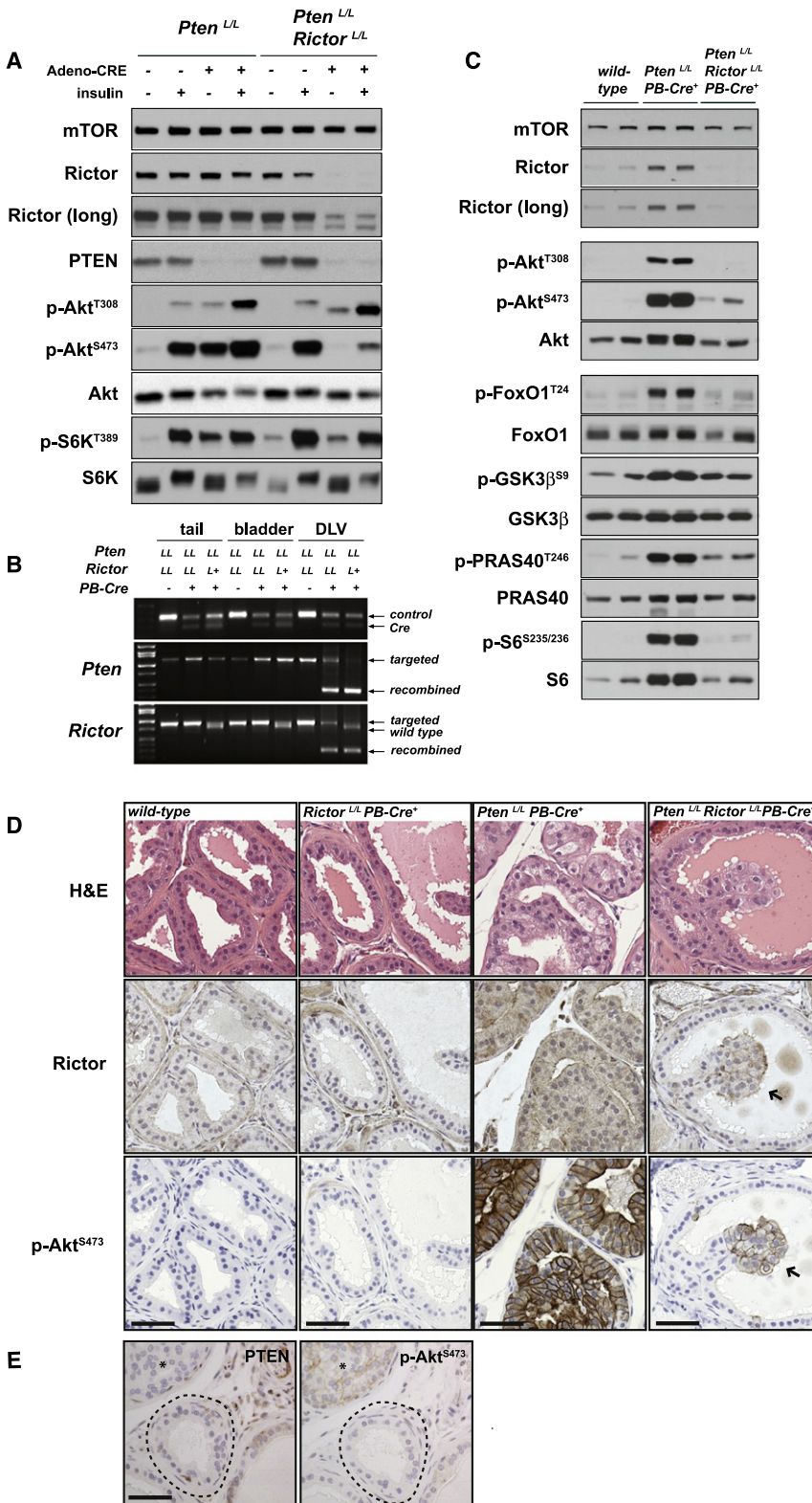


Figure 3. *Pten* Deletion-Induced Phosphorylation of Akt^{S473} in MEFs and Prostate Epithelial Cells Requires Rictor

(A) Deleting *Rictor* in combination with *Pten* blocks hyperphosphorylation of Akt^{S473} in MEFs. *Pten*^{LoxP/LoxP} or *Pten*^{LoxP/LoxP}*Rictor*^{LoxP/LoxP} MEFs were infected with Adeno-Cre virus. After 5 days, cells were starved and stimulated with 300 nm insulin for 15 min. Protein lysates were prepared and probed with the indicated antibodies. For the Rictor immunoblot, “long” indicates longer exposure.

(B) The *PB-Cre* transgene induces recombination of *Pten* and *Rictor* conditional alleles only in prostate tissue, and not in bladder or tail tissue. A sample set of PCR reactions is shown. “DLV” indicates pooled tissue from dorsal, lateral, and ventral prostate.

(C) Deleting *Rictor* in combination with *Pten* inhibits Akt^{S473} phosphorylation and activity toward downstream substrates in vivo. Protein lysates were prepared from dorsolateral prostates dissected from wild-type, *Pten*^{LoxP/LoxP}*PB-Cre*⁺, or *Pten*^{LoxP/LoxP}*Rictor*^{LoxP/LoxP}*PB-Cre*⁺ mice and probed with the indicated antibodies.

(D and E) *Rictor* deletion does not affect normal prostate architecture but is required for Akt^{S473} phosphorylation induced by *Pten* deletion.

(D) Serial sections of 7-week-old prostate tissue stained with H&E or labeled with antibodies to Rictor or phospho-Akt^{S473} are shown. Arrows point to a patch of cells in which inefficient *Rictor* deletion results in abnormal Akt^{S473}-positive cells. Scale bars = 50 μm.

(E) Cells lacking PTEN and phospho-Akt^{S473} appear normal (circled area), consistent with loss of mTORC2 activity blocking transformation. For comparison, this sample includes a nearby section of hyperplastic cells (indicated by asterisk) that stain negative for PTEN and positive for Akt^{S473}, indicative of inefficient *Rictor* deletion. Scale bar = 50 μm.

face and invading into the stromal region (Figure 4C). Invasive adenocarcinoma induces a phenomenon called the desmoplastic response, in which collagenous material is deposited in the surrounding stromal tissue. Trichrome stain, which marks collagen blue and fibrin pink, revealed a recruitment of connective tissues to the diseased areas (Figure 4C). Rictor protein was clearly detectable in malignant cells, and notably, some cells at the stromal interface expressed much higher levels of Rictor protein (Figure 4C).

In contrast to prostate epithelial cells from *Pten*^{loxP/loxP}*PB-Cre*⁺ mice, those from 9- to 10-week-old *Pten*^{loxP/loxP}

Akt^{S473}-positive cells were also detectable in the surrounding stroma, which was characterized by hypercellularity (Figure 4B). Ki67 staining indicated that the malignant cells were highly proliferative, with proliferating cells concentrating at the stromal inter-

Rictor^{loxP/loxP}*PB-Cre*⁺ mice were protected from transformation (Figures 4A and 4B). Individual ductules in many cases were more similar in appearance to wild-type prostate tissue, with little evidence of hyperplasia. Nearly all of these cells were

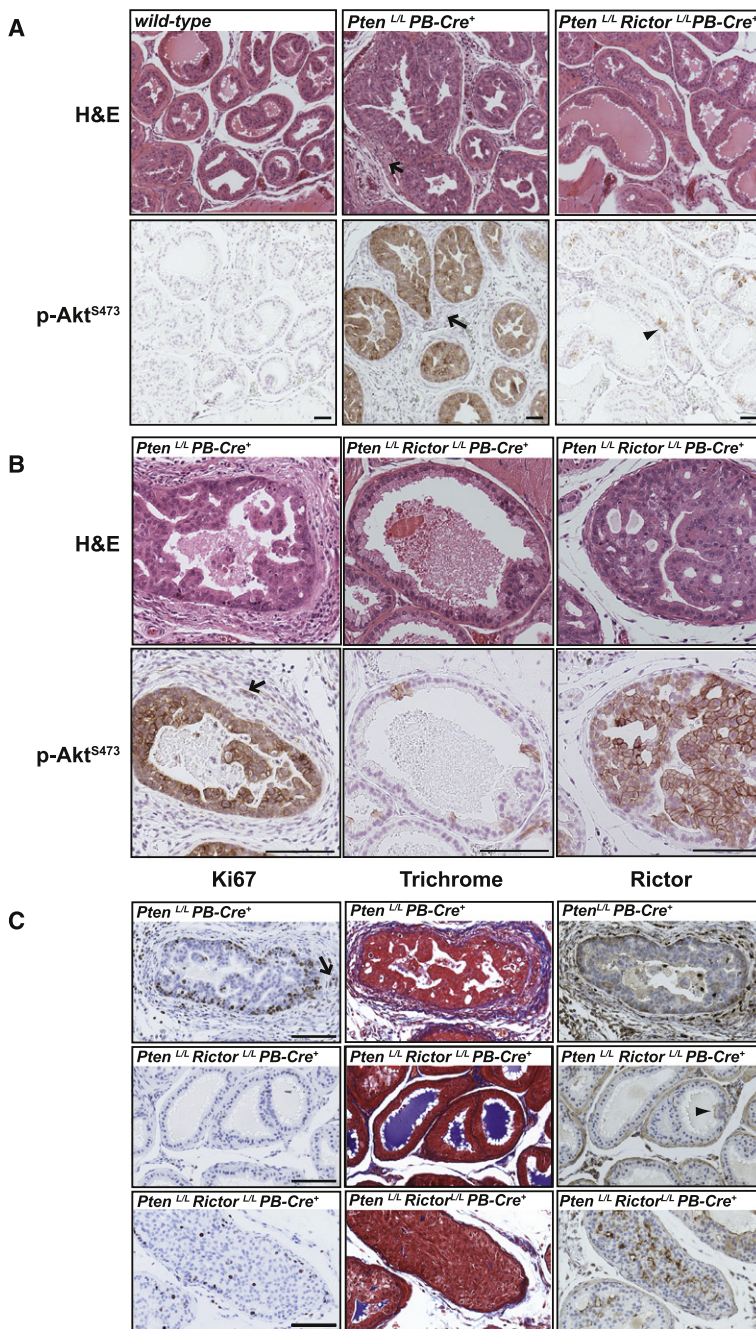


Figure 4. *Pten* Deletion-Induced Invasive Adenocarcinoma Requires Rictor

(A) Serial prostate tissue sections from 9-week-old mice were stained with H&E or labeled with a phospho-Akt^{S473} antibody and imaged at 10×. Arrows point to changes in the stroma; arrowhead indicates a patch of phospho-Akt^{S473}-positive cells. Scale bars = 50 μm.

(B) Higher-magnification images (20×) of serial sections stained with H&E or labeled with a phospho-Akt^{S473} antibody. Invasive phospho-Akt^{S473}-positive cells are indicated by the arrow. Scale bars = 50 μm.

(C) Labeling with Ki67 antibody (left), trichrome stain (middle), and Rictor antibody (right). Arrow indicates proliferating (Ki67-positive) cells in the stroma. Arrowhead points to a small patch of cells that have not lost Rictor expression. Scale bars = 50 μm.

to the extent seen in *Pten*^{loxP/loxP} PB-Cre⁺ tissue (Figure 4C). Furthermore, connective tissue was not recruited to the surrounding stroma (Figure 4C). As expected, the Akt^{S473}-positive cells in the double-deletion tissue costained for Rictor protein, although the signal was less pronounced compared to that in *Pten*^{loxP/loxP} PB-Cre⁺ tissue (Figure 4C). Perhaps in these cases, only one allele of *Rictor* was deleted.

A close inspection of Rictor protein expression in *Pten*-deficient prostate epithelium indicated that Rictor was often localized to cell membranes, particularly in the luminal epithelial cells (Figure 4C; Figure S2). This suggests that a fraction of active mTORC2 colocalizes with Akt at cell membranes. Collectively, these results argue that loss of *Rictor* expression does not impair development of the prostate epithelium but protects cells from transformation caused by *Pten* deletion.

Rictor Is Required for Akt Signaling

Deletion of *Rictor* in MEFs ablates Akt^{S473} phosphorylation, and this partly impairs the ability of Akt to phosphorylate FoxO, but not TSC2 or GSK3β, suggesting that Akt retains activity in these cells (Guertin et al., 2006; Jacinto et al., 2006). Therefore, we asked whether Akt is still capable of phosphorylating downstream substrates in *Pten*^{loxP/loxP} Rictor^{loxP/loxP} PB-Cre⁺ tissue. Akt phosphorylates the FoxO transcription factors, and this

nonproliferating as determined by Ki67 staining, and there was no desmoplastic response (Figure 4C). Consistent with the inefficient *Rictor* deletion noted above, we again observed patches of Akt^{S473}-positive epithelial cells (Figures 4A and 4B). This often resulted in Akt^{S473}-positive epithelial cells mixed among many cells that did not stain for Akt^{S473} (Figures 4A and 4B). In some instances, we observed more extensive hyperplasia of large Akt^{S473}-positive cells (Figure 4B); however, the phenotype was less severe, as the epithelial-stromal boundaries were intact and invasive cells were not detectable. A proliferation increase was associated with the patches of hyperplastic cells in the double deletion, but not

inhibits their activity by excluding them from the nucleus. Immunoblot analysis indicated that FoxO1^{T24} phosphorylation was increased in tissue lysates prepared from *Pten*^{loxP/loxP} PB-Cre⁺ mice, but not *Pten*^{loxP/loxP} Rictor^{loxP/loxP} PB-Cre⁺ mice (Figure 3C). We also examined FoxO1 localization. In wild-type prostate epithelial cells, Akt^{S473} phosphorylation was undetectable, and a very faint FoxO1 signal was present in both the nucleus and cytoplasm of these cells (Figure 5A, left). In *Pten*^{loxP/loxP} PB-Cre⁺ prostates, FoxO1 was excluded from the nucleus and concentrated in the cytoplasm (Figure 5A, middle). In contrast to both wild-type and *Pten*^{loxP/loxP} PB-Cre⁺ prostate epithelial cells, we detected strong nuclear localization of FoxO1 in

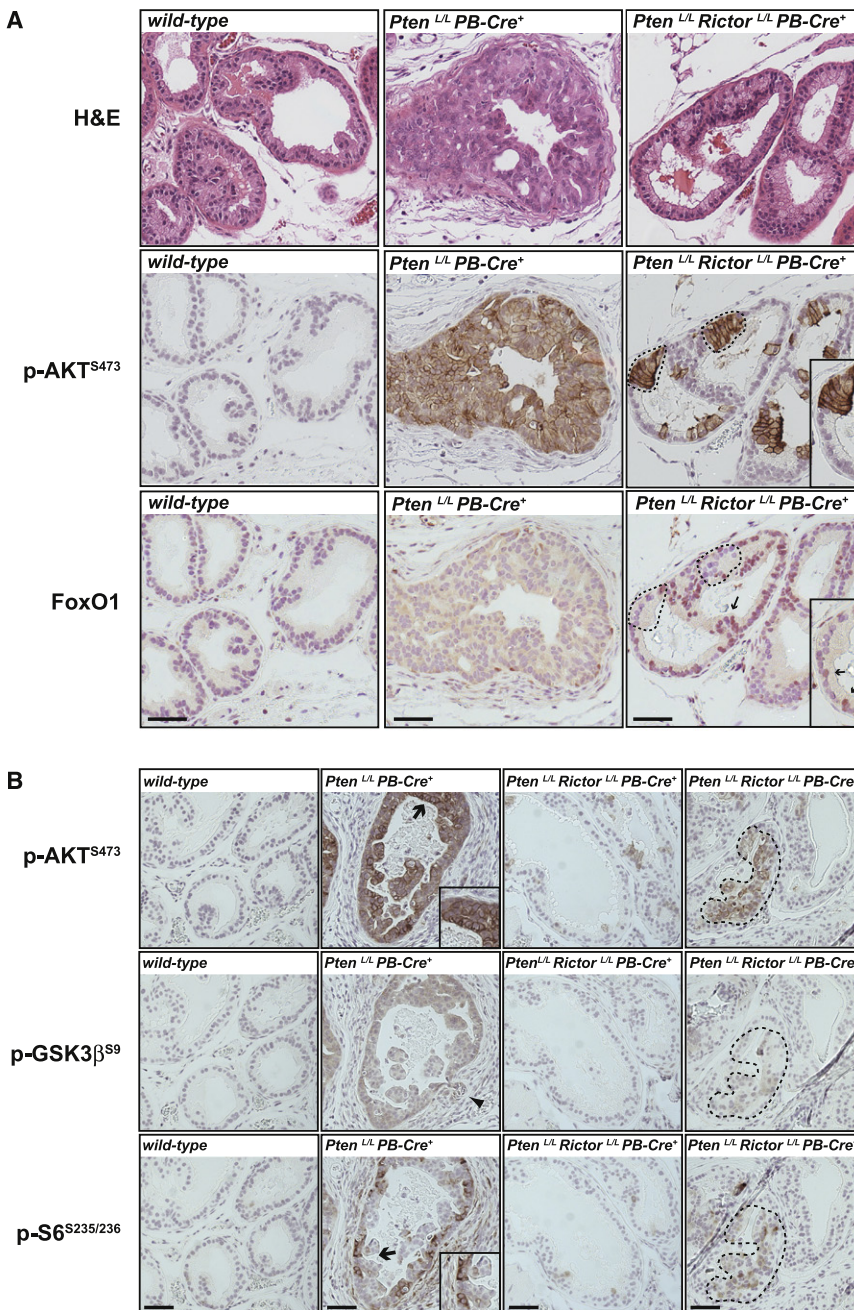


Figure 5. Akt Activity toward Downstream Substrates in *Pten*-Deficient Prostate Epithelial Cells Requires Rictor

(A) Serial sections from wild-type, *Pten*^{LoxP/LoxP} PB-Cre⁺, or *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ tissue stained with H&E or labeled with antibodies to phospho-Akt^{S473} or FoxO1. Dotted circles indicate phospho-Akt^{S473}-positive cells in which FoxO1 is excluded from the nucleus. Arrows point to phospho-Akt^{S473}-negative cells in which FoxO1 concentrates in the nucleus. Boxed inserts at right show enlarged sections. Scale bars = 25 μm. (B) Serial sections from wild-type, *Pten*^{LoxP/LoxP} PB-Cre⁺, or *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ tissue labeled with antibodies to phospho-Akt^{S473}, phospho-GSK3β^{S9}, or phospho-S6^{S235/236}. Arrows indicate regions highlighted in the boxed inserts. Arrowhead points to invasive cells. The encircled area in the right panels indicates a patch of phospho-Akt^{S473}-positive cells. Scale bars = 25 μm.

However, the increases in GSK3β^{S9} and PRAS40^{T246} phosphorylation were greatly diminished in *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ tissue samples (Figure 3C). By IHC analysis, GSK3β^{S9} phosphorylation was below detectable levels in wild-type sections (Figure 5B). Consistent with the immunoblot results, GSK3β^{S9} phosphorylation, like Akt^{S473} phosphorylation, uniformly increased in all *Pten*^{LoxP/LoxP} PB-Cre⁺ prostate epithelial cells. However, in *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ prostate tissue, GSK3β^{S9} phosphorylation was only detectable in the subpopulation of Akt^{S473}-positive cells. We were unable to achieve reliable detection of PRAS40^{T246} phosphorylation by IHC.

Finally, we examined the activity of the mTORC1 pathway. TSC2, which Akt phosphorylates and inhibits, suppresses mTORC1 activity. Phosphorylation of S6^{S235/236}, a target of the mTORC1 substrate S6K1, is commonly used as

Akt^{S473}-negative cells in *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ tissue (Figure 5A, right). Importantly, FoxO1 was excluded from the nucleus in neighboring Akt^{S473}-positive cells in the same tissue. Thus, the absence of Akt phosphorylation in *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ cells reduces FoxO1 phosphorylation and promotes FoxO1 accumulation in the nucleus.

We next examined phosphorylation of the Akt substrates GSK3β and PRAS40 using antibodies to the Akt-dependent S9 or T246 phosphorylation sites, respectively. In prostate tissue lysates from wild-type, *Pten*^{LoxP/LoxP} PB-Cre⁺, or *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ mice, total GSK3β and PRAS40 protein levels were unchanged (Figure 3C). In *Pten*^{LoxP/LoxP} PB-Cre⁺ samples, *Pten* loss elevated GSK3β^{S9} and PRAS40^{T246} phosphorylation.

a reporter for activation of the mTORC1 pathway by IHC. In lysates from wild-type prostate tissue, S6^{S235/236} phosphorylation was undetectable, while in *Pten*^{LoxP/LoxP} PB-Cre⁺ prostate tissue, phosphorylation increased (Figure 3C). However, total S6 levels also increased, and therefore it was unclear to what extent *Pten* loss affects the catalytic activity of mTORC1. Both S6^{S235/236} phosphorylation and total S6 levels in the *Pten*^{LoxP/LoxP} Rictor^{LoxP/LoxP} PB-Cre⁺ prostate tissue lysates were comparable to that detected in wild-type lysates. Thus, whatever mechanism is responsible for increasing total S6 protein requires mTORC2. By IHC, S6 phosphorylation was undetectable in wild-type prostate epithelial cells (Figure 5B). Similar to the phospho-Akt^{S473} signal, the phospho-S6^{S235/236} signal was increased in all cells

in the *Pten*^{loxP/loxP}*PB-Cre*⁺ tissue samples; however, a subset of cells—particularly those near the stromal interface—exhibited a greater increase in phospho-S6 intensity. This variability may reflect the fact that mTORC1 activity is additionally sensitive to nutrient and oxygen availability. Again, consistent with the immunoblots, S6^{S235/236} phosphorylation levels were comparable to those of wild-type cells in prostate tissue from *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP}*PB-Cre*⁺ mice, and only in patches of Akt^{S473} positive cells could we detect phospho-S6 signal (Figure 5B). These findings support a model in which Akt requires mTORC2-dependent phosphorylation of Akt^{S473} in order to phosphorylate downstream substrates when *Pten* is deleted in prostate epithelial cells.

DISCUSSION

In this report, we investigate the role of mTORC2 in prostate cancer caused by *Pten* loss. First, we show that the *Pten*-deficient PC-3 cell line requires Rictor, an essential component of mTORC2, to form tumors in nude mice. Second, we find that the *Rictor* gene is haploinsufficient in mice and that *Pten* heterozygous mice prone to develop prostate cancer are more resistant to the disease when expressing only one copy of the *Rictor* gene. Finally, we show that in vivo prostate epithelial cells require *Rictor* to be transformed by *Pten* deletion but that *Rictor* has no significant role by itself in maintaining the integrity of normal prostate epithelium. We propose that cancers driven by PTEN loss or aberrant PI3K activation require mTORC2 signaling and that targeting mTORC2 in these cancers could be a promising therapeutic strategy.

In cultured cells, mTOR, when assembled into mTORC2, is an Akt^{S473} kinase (Sarbasov et al., 2005). Genetic studies in mice confirm that in the developing embryo, mTORC2 is the critical Akt^{S473} kinase complex (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Other Akt^{S473} kinases have been described (reviewed in Bhaskar and Hay, 2007; Bozovic et al., 2008), leaving open the possibility that when cells become transformed, kinases other than mTOR could phosphorylate Akt at S473. Our results argue that mTORC2 is the primary kinase that phosphorylates Akt^{S473} in a *Pten* deletion-dependent model of prostate cancer.

The function of TORC2 as an Akt^{S473} kinase is conserved in *Drosophila* and *Dictyostelium* (Hietakangas and Cohen, 2007; Lee et al., 2005; Sarbasov et al., 2005). Unlike developing mouse embryos, *Drosophila* embryos lacking mTORC2 activity are viable and display only minor growth defects (Guertin et al., 2006; Hietakangas and Cohen, 2007; Shiota et al., 2006). However, tissue overgrowth in the eye caused by *dPten* loss requires dTORC2 (Hietakangas and Cohen, 2007). Thus, the genetic requirement for TORC2 under conditions of high PI3K activity, but not necessarily under normal conditions, is conserved.

Inhibition of mTOR in Cancer Therapy

The most developed mTOR inhibitor for use in oncology is rapamycin. Initial studies suggested that rapamycin is effective against tumors with PTEN loss (Bjornsti and Houghton, 2004; Rowinsky, 2004; Vignot et al., 2005). However, clinical studies have shown variable and unpredictable successes with

rapamycin as an anticancer agent (Chiang and Abraham, 2007). In cells, rapamycin binds to its intracellular receptor FKBP12, and together, the rapamycin-FKBP12 complex binds to mTOR adjacent to the kinase domain, inhibiting its in vivo activity toward S6K1 at nanomolar concentrations. Numerous studies show that S6K1^{T389} phosphorylation, but not Akt^{S473} phosphorylation, is potently inhibited by rapamycin in most cell types, suggesting that rapamycin is an mTORC1-specific inhibitor.

It is now appreciated that prolonged exposure to rapamycin inhibits both S6K1^{T389} and Akt^{S473} phosphorylation in a subset of cell types (Phung et al., 2006; Sarbasov et al., 2006; Zeng et al., 2006). The mechanism of mTORC2 inhibition by rapamycin is under investigation, as rapamycin-FKBP12 binds mTORC1 but not mTORC2 (Sarbasov et al., 2004). However, rapamycin-FKBP12 also binds newly synthesized mTOR, and in some cells this appears to block mTORC2 activity by preventing the assembly of new mTORC2 complexes (Sarbasov et al., 2006). Why this phenomenon is not more ubiquitous is a mystery. Part of the reason could be that in many cells, a negative feedback loop masks this effect. Studies have shown that inhibiting mTORC1 releases PI3K signaling from negative feedback inhibition, resulting in strong Akt activation (Chiang and Abraham, 2007; Manning, 2004). According to this model, rapamycin would inhibit both mTORC1 and mTORC2 but push upstream Akt signaling in two directions: inhibiting Akt by blocking mTORC2 assembly, and activating Akt by releasing PI3K from mTORC1 negative feedback inhibition. This would generate counteractive signals and might result in no net observable difference in Akt phosphorylation levels. There are currently no cancer cell biomarkers predictive of rapamycin sensitivity. Interestingly, rapamycin inhibits mTOR at micromolar concentrations independently of FKBP12 (Shor et al., 2008). This phenomenon, termed the “high-dose effect,” broadens the effectiveness of rapamycin and correlates with inhibition of both mTORC1 and mTORC2.

Next-generation mTOR inhibitors will likely target the catalytic domain of mTOR and thus achieve universal inhibition of both mTOR complexes. It has been suggested that Akt-driven transformation of some cell types preferentially requires mTORC1 activity (Skeen et al., 2006). An mTOR kinase inhibitor would therefore target both an upstream activator and a downstream target of Akt, the major caveat being that such a drug could exhibit considerable toxicity in vivo. In theory, inhibiting the catalytic activity of both complexes might circumvent issues with feedback activation of Akt following mTORC1 inhibition since mTORC2 would also be inactive. However, if mTOR activity is not completely inhibited for technical reasons (i.e., due to a resistant pool of mTORCs) or cannot be inhibited for dose-dependent toxicity reasons, then a negative feedback loop in some cells could still be problematic. Until such a class of drugs is available, we can only speculate on their effectiveness.

Potential Utility of an mTORC2-Specific Inhibitor

In this report, we present evidence that an mTORC2-specific inhibitor might be a promising anticancer therapeutic. We believe that mTORC2 is an attractive target for drug development for two reasons. First, targeting only mTORC2 may not result in feedback activation of Akt signaling since there would

be no direct inhibition of mTORC1. Second, an mTORC2 inhibitor may be well tolerated since mTORC2's activity is not required in normal prostate epithelial cells (or in cultured MEFs); rather it is required only for prostate epithelial cell transformation when *Pten* is deleted. This may be evidence for a therapeutic window in which inhibition of mTORC2 could be more deleterious to cancer cells than to normal cells. Because little is known about the physiological roles of mTORC2, it is difficult to predict what effects a theoretical mTORC2 inhibitor could have on other processes. Interestingly, a recent report indicates that muscle-specific deletion of *Rictor* in mice, while sufficient to block insulin-stimulated Akt^{S473} phosphorylation, results in only a mild impairment in glucose tolerance (Kumar et al., 2008).

The extent to which other cancers require mTORC2 is unclear, although we expect that cancers driven by mutations directly promoting Akt signaling, such as activating mutations in PI3K, may also require mTORC2 activity. A recent study found that in some glioma cell lines, Rictor expression is increased, and this elevates mTORC2 activity and promotes anchorage-independent growth and tumor formation in xenografts (Masri et al., 2007). The increase in mTORC2 activity does not correlate with PTEN status, suggesting that aberrant upregulation of mTORC2 activity might occur independently of PI3K activity. This might define another class of cancers preferentially susceptible to an mTORC2 inhibitor. However, it was not determined whether other mutations activate the PI3K pathway in these cells.

It is currently unclear whether Akt is the only relevant mTORC2 target in cancer. The three mammalian Akt proteins (Akt1, 2, and 3) belong to a family of structurally related kinases called the AGC family, which in addition to Akt includes the S6K, PKC, SGK, and RSK kinases (Hanada et al., 2004). AGC kinases are coregulated by PDK1 phosphorylation and phosphorylation by a hydrophobic motif kinase. Because mTOR phosphorylates the hydrophobic motif sites of Akt (S473) and S6K (T389) when assembled into mTORC2 or mTORC1, respectively, it seems likely that other AGC kinases could be targeted by mTOR. The SGKs are good candidates for mTORC2 regulation in cancer because they are structurally very similar to Akt, are regulated by PI3K signaling, and have cell functions that both overlap with and are distinct from Akt (Tessier and Woodgett, 2006). It was recently shown that mTORC2 controls hydrophobic motif phosphorylation of SGK1, suggesting that tumors with SGK activation might also be good candidates for treatment with mTORC2 inhibitors (Garcia-Martinez and Alessi, 2008). mTORC2 also regulates phosphorylation of the hydrophobic motif site in PKC α (S657), but it is not known whether this is a direct effect (Guertin et al., 2006; Sarbassov et al., 2004).

In addition to directly phosphorylating the hydrophobic motif of Akt, mTORC2 is required for the growth-factor-independent phosphorylation of Akt and PKC on the turn motif site, although the exact role of mTORC2 in this event is unclear (Facchinetti et al., 2008; Ikenoue et al., 2008). This constitutive phosphorylation event occurs during or shortly after translation and is important for maintaining protein stability. Interestingly, Hsp90 chaperones prevent Akt from degrading in mTORC2-deficient cells, in which Akt lacks both hydrophobic and turn motif phosphorylation (Facchinetti et al., 2008). These findings suggest that mTORC2 may have a broader role in regulating AGC kinases

beyond hydrophobic motif phosphorylation and that combining mTORC2 inhibition with chaperone inhibitors could be an effective therapeutic strategy.

To develop mTORC2 inhibitors, we need detailed knowledge about the structure and assembly of the mTOR complexes. One possible strategy is to disrupt protein-protein interactions required for mTORC2 activity. For instance, dissociation of Rictor, mSIN, or mLST8 from mTOR results in disassembly and inactivation of the complex (Frias et al., 2006; Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005). Another possible strategy is to inhibit kinase-substrate interactions, for example by disrupting the intracellular localization of the complex. We observed that Rictor concentrates at cell membranes in *Pten*-deficient prostate epithelial cells. Perhaps disrupting mTORC2 localization might prevent it from phosphorylating Akt.

Coregulation of Akt by PDK1 and mTORC2

One noteworthy finding from genetic studies of mTORC2 is that Akt has different sensitivities to mTORC2 inhibition in normal cells when compared to cancer cells. For example, Akt retains activity in *Rictor* null MEFs despite the absence of hydrophobic motif phosphorylation on S473 (this study; Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Hydrophobic motif phosphorylation is a prerequisite for PDK1 to phosphorylate S6K, SGK, and RSK, but this may not be the case for Akt even though full Akt activity in vitro requires phosphorylation at both sites (Alessi et al., 1996; Biondi et al., 2001; Collins et al., 2003). In MEFs, PDK1-dependent phosphorylation of Akt^{T308} is unaffected by *Rictor* deletion, supporting the idea that loss of S473 phosphorylation is not sufficient to block Akt activity in some cells (this study; Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Interestingly, we found that downstream targets of Akt require mTORC2 activity to be phosphorylated in *Pten*-deficient prostate epithelial cells. We also found that, unlike in MEFs, mTORC2 is required for both Akt^{S473} and Akt^{T308} phosphorylation when *Pten* is deleted in prostate epithelial cells. This is similar to what is observed in human cancer cells, in which knocking down Rictor decreases both S473 and T308 phosphorylation (Hresko and Mueckler, 2005; Sarbassov et al., 2005). We are developing Akt^{T308} antibodies for IHC, and our preliminary results are consistent with this finding (see Figure S3). This intriguing observation suggests that coregulation of Akt by mTORC2 and PDK1 is tethered in cancer cells, while in otherwise "normal" cells, these two inputs are uncoupled. This could explain why inhibiting mTORC2 in cancer cells is more deleterious to Akt activity than in normal cells.

EXPERIMENTAL PROCEDURES

RNAi

PC-3 cells stably expressing control or Rictor shRNAs were generated as described previously using a lentiviral system (Sarbassov et al., 2005). PC-3 cell lysates for immunoblot analysis were prepared 6 days postinfection. For proliferation analysis, cells were seeded at equal density onto 12-well cell culture dishes immediately following puromycin selection and counted each day with a Coulter counter. For xenografts, 1×10^6 PC-3 cells were injected in the back of preirradiated (4 Gy) Ncr-nu/nu male mice, and tumors were monitored for 28 days.

MEFs

Rictor-deficient MEFs have been described previously (Guertin et al., 2006). Akt activity was measured in MEFs using the Akt1/PKB α Immunoprecipitation Kinase Assay Kit (Upstate). To generate *Pten* null *Rictor* heterozygous MEFs, we crossed *Pten*^{loxP/loxP}*Rictor*^{LoxP/+} males with *Pten*^{LoxP/LoxP}*Rictor*^{LoxP/LoxP} females and prepared MEFs from E14 embryos. Adeno-Cre (Gene Transfer Vector Core, University of Iowa) was added directly to the medium on two consecutive days. Five days after initial infection, cells were starved for 6 hr in serum-free medium, stimulated with insulin for 10 min, and then lysed.

Mice

129/B6 *Pten*^{+/-} mice (Podszypanina et al., 1999) were crossed with *mtor*^{+/-}, *Raptor*^{+/-}, or *mlst8*^{+/-} mice of the same strain background or *Rictor*^{+/-} mice on a mixed 129/B6/BALB/c background (Guertin et al., 2006). The severity and latency of tumor development in *Pten*^{+/-} mice varies depending on the strain composition (Freeman et al., 2006). To generate mice with conditional alleles, *Pten*^{loxP/+}*Rictor*^{loxP/+}*PB-Cre*⁺ mice were crossed with *Pten*^{loxP/+}*Rictor*^{loxP/+} or *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} mice (all on a mixed background) (Shiota et al., 2006; Wang et al., 2003; Wu et al., 2001). Cre is expressed in all four lobes of the prostate, with expression being highest in the dorsolateral and ventral lobes. All procedures were approved by the Massachusetts Institute of Technology Division of Comparative Medicine's Committee on Animal Care and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals.

Protein Biochemistry

Cell lysates were prepared using conditions described in Kim et al. (2002). For tissue lysates, frozen samples were homogenized for 8 s in detergent-free buffer using a Brinkmann homogenizer, and detergent was added immediately afterward to the final concentrations of 0.1% SDS/1.0% sodium deoxycholate/1.0% Triton X-100. Primary antibodies were mTOR (#2893), Rictor (#2114), Akt^{T308} (#2965), Akt^{S473} (#4060), Akt (#4685), PTEN (#9559), S6K^{T389} (#9206), S6K (#9202), FoxO1 (#2880), GSK3 β ^{S9} (#9336), GSK3 (#9315), S6^{S235/236} (#4856), S6 (#2217) (all from Cell Signaling Technology); Foxo1^{T24} (Upstate #06-952); PRAS40^{T246} (BioSource #44-1100G); and PRAS40 (Upstate #05-988).

Immunohistochemistry

Slides were deparaffinized in three changes of xylene and rehydrated through graded ethanols. Antigen retrieval was performed using 10 mM citrate buffer (pH 6.0). Slides were quenched in 3% hydrogen peroxide and blocked with TBST/5% normal goat serum. Primary antibodies were diluted in TBST/5% normal goat serum (CST #858) or SignalStain Antibody Diluent (CST #8112) and incubated overnight at 4°C (CST #4060, 2880, 9323). Detection was performed using a Vector ABC Elite kit (Vector Laboratories) and NovaRed (Vector Laboratories). Rictor antibody (Santa Cruz #50678) was detected using a goat secondary probe/goat polymer system (Biocare Medical #GHP516) followed by diaminobenzidine. IHC for Ki67 (BD Pharmingen #550609) was performed using a DakoCytomation ARK kit (#K3954).

SUPPLEMENTAL DATA

The Supplemental Data include three figures and can be found with this article online at [http://www.cancer-cell.org/supplemental/S1535-6108\(08\)00436-4](http://www.cancer-cell.org/supplemental/S1535-6108(08)00436-4).

ACKNOWLEDGMENTS

We thank K. Cormier (MIT Division of Comparative Medicine) and M. Brown (MIT Koch Institute) for histological support and R. Wetzel, W. Cheung, and K. Zuberek (Cell Signaling Technology) for reagents. This work was supported by grants from the National Institutes of Health to D.M. Sabatini (R01 CA103866 and R01 AI04389), H.W. (R01 CA107166), and D.A.G. (K99 CA1296613-01A1); an award from the W.M. Keck Foundation to D.M. Sabatini; and a fellowship from the Damon Runyon Cancer Research Foundation and a Career Development Award from the Leukemia & Lymphoma Society to D.A.G. D.M. Sabatini is an Investigator of the Howard Hughes Medical Institute.

Received: June 27, 2008

Revised: October 22, 2008

Accepted: December 18, 2008

Published: February 2, 2009

REFERENCES

- Abraham, R.T., and Eng, C.H. (2008). Mammalian target of rapamycin as a therapeutic target in oncology. *Expert Opin. Ther. Targets* 12, 209–222.
- Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 15, 6541–6551.
- Backman, S.A., Stambolic, V., Suzuki, A., Haight, J., Elia, A., Pretorius, J., Tsao, M.S., Shannon, P., Bolon, B., Ivy, G.O., and Mak, T.W. (2001). Deletion of *Pten* in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nat. Genet.* 29, 396–403.
- Bayascas, J.R., Leslie, N.R., Parsons, R., Fleming, S., and Alessi, D.R. (2005). Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/-) mice. *Curr. Biol.* 15, 1839–1846.
- Bhaskar, P.T., and Hay, N. (2007). The two TORCs and Akt. *Dev. Cell* 12, 487–502.
- Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., and Alessi, D.R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* 20, 4380–4390.
- Bjornsti, M.A., and Houghton, P.J. (2004). The TOR pathway: a target for cancer therapy. *Nat. Rev. Cancer* 4, 335–348.
- Bozulic, L., Surucu, B., Hynx, D., and Hemmings, B.A. (2008). PKB α /Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. *Mol. Cell* 30, 203–213.
- Chen, M.L., Xu, P.Z., Peng, X.D., Chen, W.S., Guzman, G., Yang, X., Di Cristofano, A., Pandolfi, P.P., and Hay, N. (2006). The deficiency of Akt1 is sufficient to suppress tumor development in *Pten*^{+/-} mice. *Genes Dev.* 20, 1569–1574.
- Chiang, G.G., and Abraham, R.T. (2007). Targeting the mTOR signaling network in cancer. *Trends Mol. Med.* 13, 433–442.
- Collins, B.J., Deak, M., Arthur, J.S., Armit, L.J., and Alessi, D.R. (2003). In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* 22, 4202–4211.
- Dahia, P.L. (2000). PTEN, a unique tumor suppressor gene. *Endocr. Relat. Cancer* 7, 115–129.
- Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P.P. (1998). *Pten* is essential for embryonic development and tumour suppression. *Nat. Genet.* 19, 348–355.
- Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q., et al. (2008). The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J.* 27, 1932–1943.
- Freeman, D., Lesche, R., Kertesz, N., Wang, S., Li, G., Gao, J., Groszer, M., Martinez-Diaz, H., Rozengurt, N., Thomas, G., et al. (2006). Genetic background controls tumor development in PTEN-deficient mice. *Cancer Res.* 66, 6492–6496.
- Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A., and Sabatini, D.M. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr. Biol.* 16, 1865–1870.
- Garcia-Martinez, J.M., and Alessi, D.R. (2008). mTOR complex-2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum and glucocorticoid induced protein kinase-1 (SGK1). *Biochem. J.* 416, 375–385.
- Guertin, D.A., and Sabatini, D.M. (2007). Defining the Role of mTOR in Cancer. *Cancer Cell* 12, 9–22.
- Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., and Sabatini, D.M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Dev. Cell* 11, 859–871.

- Hanada, M., Feng, J., and Hemmings, B.A. (2004). Structure, regulation and function of PKB/AKT—a major therapeutic target. *Biochim. Biophys. Acta* 1697, 3–16.
- Hietakangas, V., and Cohen, S.M. (2007). Re-evaluating AKT regulation: role of TOR complex 2 in tissue growth. *Genes Dev.* 21, 632–637.
- Hresko, R.C., and Mueckler, M. (2005). mTOR/RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* 280, 40406–40416.
- Huang, B., and Porter, G. (2005). Expression of proline-rich Akt-substrate PRAS40 in cell survival pathway and carcinogenesis. *Acta Pharmacol. Sin.* 26, 1253–1258.
- Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J.* 27, 1919–1931.
- Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 127, 125–137.
- Jiao, J., Wang, S., Qiao, R., Vivanco, I., Watson, P.A., Sawyers, C.L., and Wu, H. (2007). Murine cell lines derived from *Pten* null prostate cancer show the critical role of PTEN in hormone refractory prostate cancer development. *Cancer Res.* 67, 6083–6091.
- Kim, D.-H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110, 163–175.
- Kovacina, K.S., Park, G.Y., Bae, S.S., Guzzetta, A.W., Schaefer, E., Birnbaum, M.J., and Roth, R.A. (2003). Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. *J. Biol. Chem.* 278, 10189–10194.
- Kumar, A., Harris, T.E., Keller, S.R., Choi, K.M., Magnuson, M.A., and Lawrence, J.C., Jr. (2008). Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances basal glycogen synthase activity. *Mol. Cell. Biol.* 28, 61–70.
- Lee, S., Comer, F.I., Sasaki, A., McLeod, I.X., Duong, Y., Okumura, K., Yates, J.R., 3rd, Parent, C.A., and Firtel, R.A. (2005). TOR complex 2 integrates cell movement during chemotaxis and signal relay in *Dictyostelium*. *Mol. Biol. Cell* 16, 4572–4583.
- Lei, Q., Jiao, J., Xin, L., Chang, C.J., Wang, S., Gao, J., Gleave, M.E., Witte, O.N., Liu, X., and Wu, H. (2006). NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss. *Cancer Cell* 9, 367–378.
- Manning, B.D. (2004). Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *J. Cell Biol.* 167, 399–403.
- Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. *Cell* 129, 1261–1274.
- Masri, J., Bernath, A., Martin, J., Jo, O.D., Vartanian, R., Funk, A., and Gera, J. (2007). mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor. *Cancer Res.* 67, 11712–11720.
- Phung, T.L., Ziv, K., Dabydeen, D., Eyyah-Mensah, G., Riveros, M., Perruzzi, C., Sun, J., Monahan-Earley, R.A., Shiojima, I., Nagy, J.A., et al. (2006). Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell* 10, 159–170.
- Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E., and Parsons, R. (1999). Mutation of *Pten/Mmac1* in mice causes neoplasia in multiple organ systems. *Proc. Natl. Acad. Sci. USA* 96, 1563–1568.
- Rowinsky, E.K. (2004). Targeting the molecular target of rapamycin (mTOR). *Curr. Opin. Oncol.* 16, 564–575.
- Salmena, L., Carracedo, A., and Pandolfi, P.P. (2008). Tenets of PTEN tumor suppression. *Cell* 133, 403–414.
- Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol. Cell* 25, 903–915.
- Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* 14, 1296–1302.
- Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098–1101.
- Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* 22, 159–168.
- Sellers, W.R., and Sawyers, C.L. (2002). *Somatic Genetics of Prostate Cancer: Oncogenes and Tumor Suppressors* (Philadelphia: Lippincott Williams & Wilkins).
- Shiota, C., Woo, J.T., Lindner, J., Shelton, K.D., and Magnuson, M.A. (2006). Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev. Cell* 11, 583–589.
- Shor, B., Zhang, W.G., Toral-Barza, L., Lucas, J., Abraham, R.T., Gibbons, J.J., and Yu, K. (2008). A new pharmacologic action of CCI-779 involves FKBP12-independent inhibition of mTOR kinase activity and profound repression of global protein synthesis. *Cancer Res.* 68, 2934–2943.
- Skeen, J.E., Bhaskar, P.T., Chen, C.C., Chen, W.S., Peng, X.D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. *Cancer Cell* 10, 269–280.
- Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., et al. (1998a). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr. Biol.* 8, 1169–1178.
- Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B., and Bova, G.S. (1998b). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.* 58, 204–209.
- Tessier, M., and Woodgett, J.R. (2006). Serum and glucocorticoid-regulated protein kinases: variations on a theme. *J. Cell. Biochem.* 98, 1391–1407.
- Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., et al. (2003). *Pten* dose dictates cancer progression in the prostate. *PLoS Biol.* 1, E59.
- Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat. Cell Biol.* 9, 316–323.
- Vignot, S., Faivre, S., Aguirre, D., and Raymond, E. (2005). mTOR-targeted therapy of cancer with rapamycin derivatives. *Ann. Oncol.* 16, 525–537.
- Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., et al. (2003). Prostate-specific deletion of the murine *Pten* tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 4, 209–221.
- Wu, X., Wu, J., Huang, J., Powell, W.C., Zhang, J., Matusik, R.J., Sangiorgi, F.O., Maxson, R.E., Sucov, H.M., and Roy-Burman, P. (2001). Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation. *Mech. Dev.* 101, 61–69.
- Yang, Q., Inoki, K., Ikenoue, T., and Guan, K.L. (2006). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev.* 20, 2820–2832.
- Zeng, Z., Sarbassov, D.D., Samudio, I.J., Yee, K.W., Munsell, M.F., Jackson, C.E., Giles, F.J., Sabatini, D.M., Andreeff, M., and Konopleva, M. (2006). Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML. *Blood* 109, 3509–3512.