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The mTOR complex 2 is required for the development of prostate cancer induced by *Pten* loss in mice

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Abstract

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 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 the 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.

Significance—Small molecule inhibitors that compromise cancer but not normal cell functions would be valuable anti-cancer therapeutics. Identifying intracellular targets for this type of inhibitor is challenging. 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 a 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

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many human cancers. Our findings suggest that mTORC2 inhibitors could have broad clinical applications.

Keywords

mTOR; mTORC1; mTORC2; PTEN; Akt; PKB; prostate cancer; rapamycin

Introduction

The 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 PtdIns (3,4,5)P₃ accumulation at cell membranes, which serves 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 target of the drug rapamycin, assembles into at least two distinct complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each having unique substrates [Reviewed in (Guertin and Sabatini, 2007)]. In addition to mTOR, mTORC1 contains Raptor, PRAS40, and mLST8/GβL and regulates cell growth by controlling the activity of the S6 kinases and the 4E-BP proteins. mTORC2 contains mLST8/GβL and 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. However, 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 intense. 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 (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 lifespan (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). Expressing Cre recombinase in prostate epithelial cells of *Pten^{loxP/loxP}* mice results in *Pten* deletion and Akt hyper-phosphorylation in the prostate epithelium (Wang et al., 2003). These mice develop epithelial hyperplasia, which progresses to prostatic intraepithelial neoplasia (PIN) within 6 weeks of age, and to invasive adenocarcinoma and metastasis by 9-12 weeks in 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 anti-cancer drug is one that when inhibited has no effect on normal cells but compromises the proliferation and/or survival of cancer cells. We hypothesize that mTORC2 may be such a target in cancers driven by PI3K-activation because previous studies show it is not required for survival of mouse embryo fibroblasts or for the 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 which lacks PTEN expression; (2) in a spontaneous mouse model of cancer dependent upon *Pten* loss; and (3) in 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 tumor 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 if PTEN-deficient human prostate cancer cells require mTORC2 activity to form tumors, we asked if 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 shRNA hairpins 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 show that in vitro, shRictor¹ robustly decreases total Rictor protein while shRictor² reduces Rictor to an intermediate degree [Figure 1A]. Both Rictor hairpins reduce Akt^{S473} phosphorylation to levels consistent with the level of Rictor knockdown [Figure 1A]. In vitro, both shRictor¹ and shRictor² knockdown cells have a proliferation defect, the severity of which also correlates with the level of Rictor knockdown [Figure 1B].

To determine if Rictor depletion affects the ability of PC-3 cells to form solid tumors in vivo, we injected the 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 form tumors that are visibly larger than tumors formed by PC-3 cells expressing either shRictor¹ or shRictor² [Figure 1C]. The shLuc-expressing cells grow tumors to an average volume of 312 mm³ [Figure 1D]. In contrast, shRictor¹- and shRictor²-expressing cells grow tumors to 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 lifespan of *Pten*^{+/-} mice and can protect mice against prostate cancer

Our xenograft study suggests that mTORC2 activity may be important in prostate cancer development when the PTEN tumor suppressor is lost. We next asked if reducing mTORC1 and/or mTORC2 activity in a genetic model of cancer dependent upon *Pten* loss could extend lifespan. *Pten*^{+/-} mice spontaneously develop tumors in multiple organs resulting in a shortened lifespan (Di Cristofano et al., 1998; Freeman et al., 2006; Podsypanina et al., 1999; Suzuki et al., 1998a). We crossed *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 live longer than strain-matched *Pten*^{+/-} controls [Figure 2A]. In contrast, *Pten*^{+/-}*Raptor*^{+/-} mice show no difference in lifespan compared to *Pten*^{+/-} controls. *Pten*^{+/-}*Rictor*^{+/-} mice also tend to live longer than *Pten*^{+/-} mice but the effect is less pronounced due to a difference in the *Rictor*^{+/-} mouse strain composition (see Methods and (Freeman et al., 2006). In developing mouse embryos, only mTORC2 requires *mlst8* and *Rictor*, while mTORC1 requires *Raptor* (Guertin et al., 2006). Thus lifespan 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 lifespan.

To confirm that deleting one *Rictor* gene reduces mTORC2 signaling, we examined Akt activity in *Rictor* heterozygous MEFs. We find that deleting one *Rictor* gene reduces the serum-stimulated in vitro kinase activity of Akt [Figure 2B] and that this correlates with reduced Akt^{S473} phosphorylation [Figure 2C]. To generate MEFs that are null for *Pten* and are 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 the Cre recombinase (Adeno-CRE) and asked whether partial loss of *Rictor* impairs *Pten*-deletion induced Akt^{S473} phosphorylation. We find that deleting one *Rictor* gene slightly reduces the increase in Akt^{S473} phosphorylation caused by *Pten*-deletion and prevents insulin from further increasing the phospho-Akt^{S473} signal [Figure 2D]. Moreover, loss of one *Rictor* gene reduces the expected Mendelian ratio at birth (Guertin et al., 2006) and decreases 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 are 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 one year. Five out 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 & eosin (H&E) stained sections generated from these samples indicates that *Pten*^{+/-} mice develop severe neoplasia [Figure 2F]. Prostates from *Pten*^{+/-}*Rictor*^{+/-} mice exhibit signs of hyperplasia [Figure 2F]. However, in contrast to the lesions observed in *Pten*^{+/-} mice, the lesions in the double heterozygous mice appear less severe and contain larger cells. There are also fewer proliferating cells in the *Pten*^{+/-}*Rictor*^{+/-} samples as only 2.3% (19/836) of the cells in three representative images are positive for the proliferation marker Ki67 compared with 10.7% (203/1902) of the cells from *Pten*^{+/-} samples ($p < 0.001$) [Figure 2F]. Thus, the haploinsufficiency associated with losing one *Rictor* gene protects *Pten*^{+/-} mice from prostate cancer. Because of a difference in genetic background between the *Rictor*^{+/-} mice and the *mtor*, *Raptor*, and *mlst8* heterozygous mice, *Pten*^{+/-} controls born from *mtor*^{+/-}, *Raptor*^{+/-}, or *mlst8*^{+/-} and *Pten*^{+/-} parents are less susceptible to prostate cancer and this precludes an analysis of prostate cancer 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 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 promoter (Wu et al., 2001) in conditional *Pten* mice (*Pten*^{loxP/loxP}*PB-Cre*⁺) induces post-natal 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 Cre-expressing adenoviruses (Adeno-Cre) and cultured for five 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 reduces PTEN expression, and elevates both Akt^{S473} and Akt^{T308} phosphorylation in serum-deprived cells [Figure 3A, lanes 1 & 3] and in insulin stimulated cells [Figure 3A, lanes 2 & 4]. In contrast, *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} MEFs infected with Adeno-CRE show no increase in Akt^{S473} phosphorylation despite reduced *Pten* expression [Figure 3A, lanes 5 & 7]. Furthermore, these MEFs exhibit only a small increase in S473 phosphorylation following insulin stimulation [Figure 3A, lanes 6 & 8]. This slight increase in S473 phosphorylation in insulin-stimulated *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} MEFs likely results from a subpopulation of cells that Adeno-Cre failed to infect as an immunoblot analysis clearly shows that not all Rictor is lost. As expected in MEFs, insulin regulation of Akt^{T308} phosphorylation and S6K^{T389} phosphorylation (a reporter for mTORC1 activity) are unaffected by *Rictor* deletion [Figure 3 and (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 that harbor either one or two conditional alleles of *Rictor* and the *PB-Cre*⁺ transgene indicates that recombination occurs 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 dosolateral prostate tissue of *wild-type*, *Pten*^{loxP/loxP} *PB-Cre*⁺, and *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} *PB-Cre*⁺ animals. By immunoblot analysis, we find that mTOR protein levels remain unchanged in all three genotypes [Figure 3C]. In contrast, total Rictor protein increases in *Pten*^{loxP/loxP} *PB-Cre*⁺ prostate tissue, but is mostly absent in *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} *PB-Cre*⁺ tissue [Figure 3C]. As expected, *Pten*-loss substantially elevates both Akt^{T308} and Akt^{S473} phosphorylation. Importantly, the increase in phospho-Akt^{S473} is suppressed in *Pten*^{loxP/loxP}*Rictor*^{loxP/loxP} *PB-Cre*⁺ tissue indicating that Akt^{S473} phosphorylation caused by *Pten* deletion requires mTORC2. Interestingly, we find that the increase in T308 phosphorylation induced by *Pten* loss also requires mTORC2. This is different to what we observe in MEFs [Figure 3A and (Guertin et al., 2006)] but is more similar to the situation in human cancer cells in which phosphorylation at T308 requires mTORC2 activity [Figure 1A and (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 with a Rictor antibody performed on dorsolateral prostate tissue indicates 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 and S1]. Consistent with

this observation, immortalized *Rictor*-null MEFs are smaller than *wild-type* MEFs in volume by ~10% while they proliferate at the same rate [Supplemental Figure S1]. We have examined the histology of prostate tissue from *Rictor^{loxP/loxP}PB-Cre⁺* mice as old as 16 weeks and find no obvious differences when 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 a normal prostate epithelium.

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

In contrast, prostatic ductules from *Pten^{loxP/loxP}Rictor^{loxP/loxP}PB-Cre⁺* exhibit a mixed phenotype containing mostly normal, organized epithelial cells and patches of large hyperplastic disorganized 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 indicate that normal cells deficient in Rictor protein expression are negative for Akt^{S473} phosphorylation, while atypical cells maintain Rictor protein expression and stain positively for phosphorylated S473 [Figure 3D & S2]. This is 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 see some PTEN-negative cells that are positive for phospho-Akt^{S473} and others that are negative for phospho-Akt^{S473} signal [Figure 3E]. Importantly, PTEN deficient phospho-Akt^{S473} negative cells appear normal. A recent study suggests that *Pten* loss down-regulates 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 is detectable in *Pten^{loxP/loxP} PB-Cre⁺* mice. Nearly all of the dorsolateral lobes at this age contain extensive malignant cells characterized by Akt^{S473} phosphorylation, especially at the membrane [Figure 4A]. Compared to *wild-type* tissue, the diseased glands are enlarged and the ductule boundaries are disorganized. Invasive Akt^{S473} positive cells are also detectable in the surrounding stroma, which is characterized by hypercellularity [Figure 4B]. Ki-67 staining indicates that the malignant cells are highly proliferative, with proliferating cells concentrating at the stromal interface 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. The trichrome stain, which marks collagen blue and fibrin pink, reveals a recruitment of connective tissues to the diseased areas [Figure 4C]. Rictor protein is clearly detectable in malignant cells, and notably, some cells at the stromal interface express much higher levels of Rictor protein [Figure 4C].

In contrast to *Pten^{loxP/loxP} PB-Cre⁺* mice, prostate epithelial cells from 9-10 week old *Pten^{loxP/loxP}Rictor^{loxP/loxP} PB-Cre⁺* mice are protected from transformation [Figure 4A & B]. Individual ductules in many cases are more similar in appearance to *wild-type* prostate tissue, with little evidence of hyperplasia. Nearly all of these cells are non-proliferating as determined by Ki-67 staining, and there is no desmoplastic response [Figure 4C]. Consistent with the inefficient *Rictor* deletion noted above, we again observe patches of Akt^{S473} positive epithelial cells [Figure 4A & B]. This often results in Akt^{S473} positive epithelial cells mixed among many

cells that do not stain for Akt^{S473} [Figure 4A & B]. In some instances we observe more extensive hyperplasia of large Akt^{S473} positive cells [Figure 4B]; however, the phenotype is less severe as the epithelial-stromal boundaries are intact and invasive cells are not detectable. A proliferation increase is associated with the patches of hyperplastic cells in the double deletion, but it is not to the extent as that seen in *Pten*^{loxP/loxP} *PB-Cre*⁺ tissue [Figure 4C]. Furthermore, connective tissue is not recruited to the surrounding stroma [Figure 4C]. As expected, the Akt^{S473} positive cells in the double deletion tissue co-stain for Rictor protein although the signal is less pronounced compared to *Pten*^{loxP/loxP} *PB-Cre*⁺ tissue [Figure 4C]. Perhaps in these cases, only one allele of *Rictor* is deleted.

A close inspection of Rictor protein expression in the *Pten*-deficient prostate epithelium indicates that Rictor is often localized to cell membranes, particularly in the luminal epithelial cells [Figure 4C and S2]. This suggests that a fraction of active mTORC2 co-localizes 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 GSK3 β suggesting that in these cells Akt retains activity (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 inhibits their activity by excluding them from the nucleus. Immunoblot analysis indicates that FoxO1^{T24} phosphorylation increases in tissue lysates prepared from *Pten*^{loxP/loxP} *PB-Cre*⁺ but not from *Pten*^{loxP/loxP} *Rictor*^{loxP/loxP} *PB-Cre*⁺ mice [Figure 3C]. We also examined FoxO1 localization. In *wild-type* prostatic epithelial cells, Akt^{S473} phosphorylation is undetectable, and a very faint FoxO1 signal is present both in the nucleus and cytoplasm of these cells [Figure 5A, left]. In *Pten*^{loxP/loxP} *PB-Cre*⁺ prostates, FoxO1 is excluded from the nucleus and concentrates in the cytoplasm [Figure 5A, middle]. In contrast to both *wild-type* and *Pten*^{loxP/loxP} *PB-Cre*⁺ prostate epithelial cells, we detect strong nuclear localization of FoxO1 in Akt^{S473} negative cells in *Pten*^{loxP/loxP} *Rictor*^{loxP/loxP} *PB-Cre*⁺ tissue [Figure 5A, right]. Importantly, FoxO1 is 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 GSK3 β and PRAS40 using antibodies to the Akt-dependent Ser9 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 GSK3 β and PRAS40 protein levels are unchanged [Figure 3C]. In *Pten*^{loxP/loxP} *PB-Cre*⁺ samples, *Pten* loss elevates GSK3 β ^{S9} and PRAS40^{T246} phosphorylation. However, the increases in GSK3 β ^{S9} and PRAS40^{T246} phosphorylation are greatly diminished in *Pten*^{loxP/loxP} *Rictor*^{loxP/loxP} *PB-Cre*⁺ tissue samples [Figure 3C]. By immunohistochemical analysis, GSK3 β ^{S9} phosphorylation is below the level of detection in *wild-type* sections [Figure 5B]. Consistent with the immunoblot results, GSK3 β ^{S9} phosphorylation, like Akt^{S473} phosphorylation, uniformly increases in all *Pten*^{loxP/loxP} *PB-Cre*⁺ prostate epithelial cells. However, in *Pten*^{loxP/loxP} *Rictor*^{loxP/loxP} *PB-Cre*⁺ prostate tissue GSK3 β ^{S9} phosphorylation is 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 a reporter for activation of the mTORC1

pathway by IHC. In lysates from *wild-type* prostate tissue, S6^{S235/236} phosphorylation is undetectable, while in *Pten*^{loxP/loxP} *PB-Cre*⁺ prostate tissue phosphorylation increases [Figure 3C]. However, total S6 levels also increase and therefore it is 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 are comparable to what is detected in *wild-type* lysates. Thus, whatever mechanism is responsible for increasing total S6 protein requires mTORC2. By IHC, S6 phosphorylation is undetectable in *wild-type* prostate epithelial cells [Figure 5B]. Similar to the phospho-Akt^{S473} signal, all cells in the *Pten*^{loxP/loxP} *PB-Cre*⁺ tissue samples display and increase in the phospho-S6^{S235/236} signal, however, a subset of cells--particularly those near the stromal interface--exhibit 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 are 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 can 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, which 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 transformation by *Pten* deletion, but *Rictor* has no significant role by itself in maintaining the integrity of a 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)](Bozulic 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 *PTEN* 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 suggest that rapamycin is effective against tumors with *PTEN* loss (Bjornsti and Houghton, 2004; Rowinsky, 2004; Vignot et al., 2005). However, clinical studies show variable and unpredictable successes with rapamycin as an anti-cancer 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 towards 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; Sarbassov 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 (Sarbassov 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 (Sarbassov 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 show 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 may 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 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 may 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. a resistant pool of mTORCs) or it 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 anti-cancer therapeutic. We believe that mTORC2 is an attractive target for drug development for two reasons: (1) By targeting only mTORC2, there may not be feedback activation of Akt signaling since there would be no direct inhibition of mTORC1; (2) an mTORC2 inhibitor may be well tolerated since its activity is not required in normal prostate epithelial cells (or in cultured MEFs), but 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 muscle-specific deletion of *Rictor* in mice, while sufficient to block insulin stimulated Akt^{S473} phosphorylation, only results in 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 finds 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 up-regulation 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 PI3K activity in these cells.

It is currently unclear if 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 co-regulated 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 mTOR Complex 2 or 1 respectively, it seems likely 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 if 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 mTORC2 may have a broader role in regulating AGC kinases beyond hydrophobic motif phosphorylation and 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 observe that Rictor concentrates at cell membranes in *Pten*-deficient prostate epithelial cells. Perhaps disrupting mTORC2 localization might prevent it from phosphorylating Akt.

Co-regulation of Akt by PDK1 and mTORC2

One noteworthy finding from the 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 find that downstream targets of Akt require mTORC2 activity to be phosphorylated in *Pten*-deficient prostate epithelial cells. We also show that unlike the case in MEFs, mTORC2 is required for both Akt^{S473} and Akt^{T308} phosphorylation when *Pten* is deleted. 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 Supplemental Figure 3]. This intriguing observation suggests that co-regulation 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.

Methods

RNAi

PC-3 cells stably expressing the 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 post infection. 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, 1M PC-3 cells were injected on the back of pre-irradiated (4Gray) *ncr-nu/nu* male mice and tumors were monitored for 28 days.

MEFs

Rictor deficient MEFs were 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 d14 embryos. Adeno-Cre (U of Iowa) was added directly to the medium on two consecutive days. Five days after the initial infection, cells were starved 6 hours in serum free medium, stimulated with insulin for 10 minutes, then lysed.

Mice

Pten^{+/-} mice (Podsypanina et al., 1999) that are 129/B6 were crossed with *mtor^{+/-}*, *Raptor^{+/-}*, or *mlst8^{+/-}* mice of the same strain background or *Rictor^{+/-}* mice that are of a mixed 129/B6/BalbC 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-Cre4⁺* 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 Cre expression highest in the dorsolateral (DL) and ventral (V) lobes. All procedures are 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 seconds in detergent free buffer using a Brinkmann Homogenizer and immediately after, detergent was added to the final concentrations of 0.1% SDS/1.0% Sodium Deoxycholate/1.0% Triton. Primary antibodies are as follows: From CST, 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); and 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 10mM 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# 4858) or SignalStain (R) Antibody Diluent (CST #8112) and incubated overnight at 4 degrees (CST# 4060, 2880, 9323). Detection was performed using Vector ABC Elite (Vector Laboratories) and NovaRed (Vector Laboratories). Rictor antibody (Santa Cruz #50678) was detected using a goat secondary probe/goat polymer system (Biocare Meidcal #ghp516) followed by DAB. Ki-67 (BD Pharmingen #550609) IHC was done using the DakoCytomation ARK kit (#K3954).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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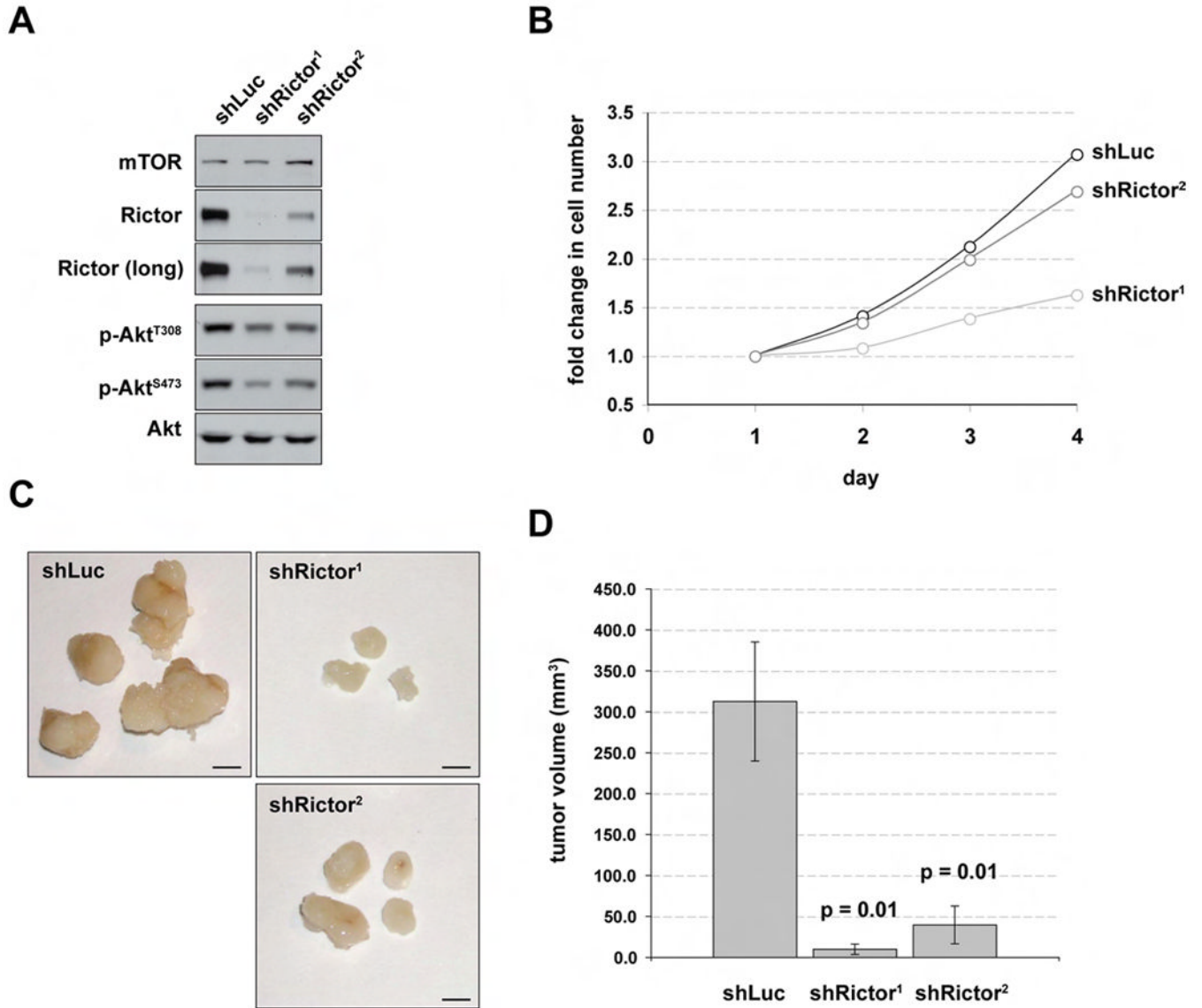


Figure 1. PC-3 prostate cancer cells require Rictor to form tumors as xenografts

(A) Generation of PC-3 cells with stable knockdown of Rictor. Using a lentiviral delivery system, two independent shRNAs (shRictor¹ or shRictor²) that silence Rictor expression or a control shRNA targeting luciferase (shLuc) were stably expressed in PC-3 cells. Protein lysates prepared 6 days post-infection 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 post injection. Scale bar = 2mm (D) Average tumor volume (mm³) and SEM is plotted.

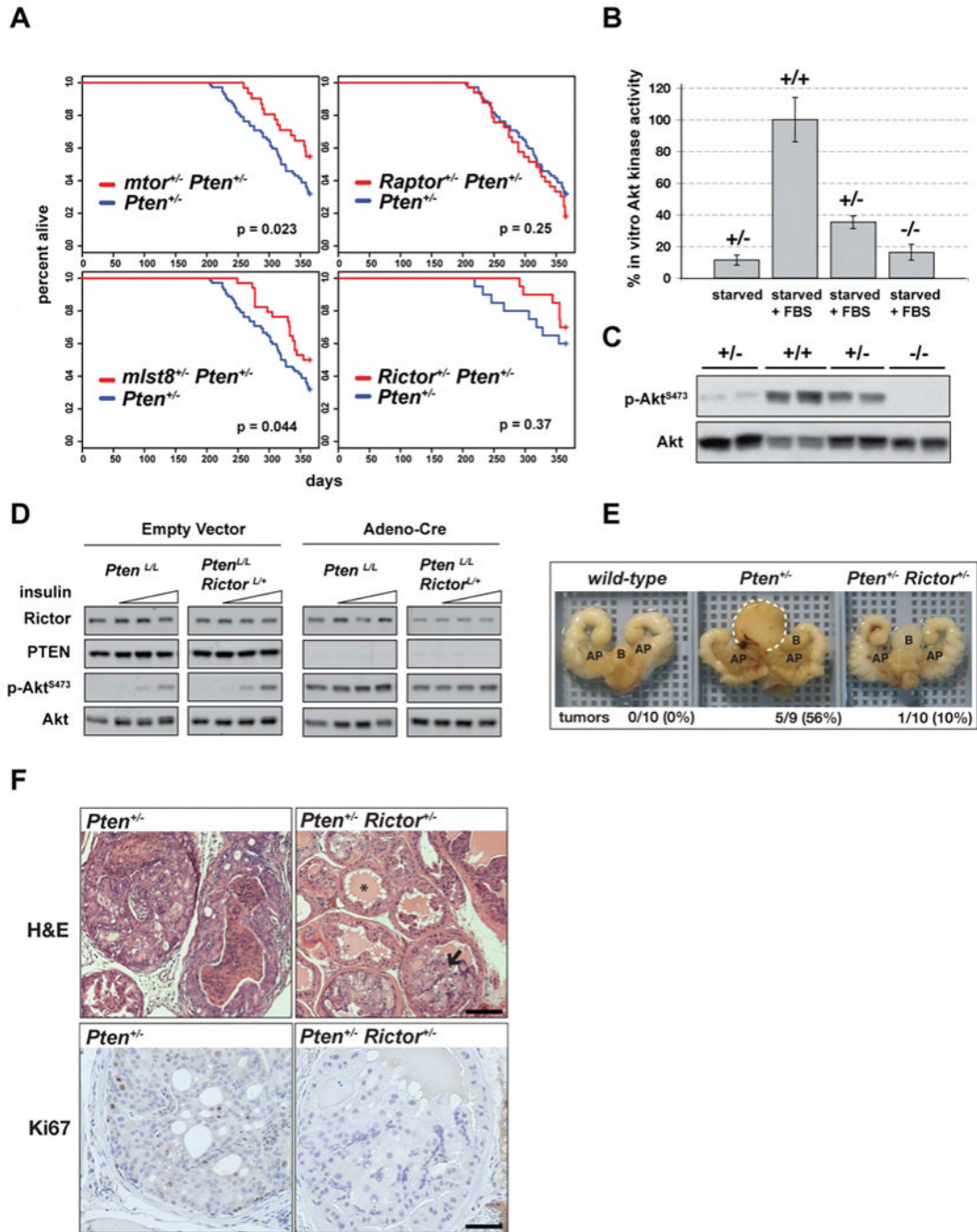


Figure 2. Phenotypes associated with *Pten* heterozygosity require mTORC2

(A) *Pten*^{+/-} mice lacking one *mtor*, *mlst8*, or *Rictor* gene tend to live longer. Survivability was monitored for 1 year and displayed using Kaplan-Meier plots. (B-C) *Rictor*^{+/-} 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. Bars indicate standard error. (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 100nm insulin for 10 minutes and probed with the indicated antibodies. (E-F) *Pten*^{+/-}*Rictor*^{+/-} mice are protected against prostate tumor development. (E) GU tracts of *wild-type*, *Pten*^{+/-} and *Pten*^{+/-}*Rictor*^{+/-} 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 white dotted line. (F) *Pten*^{+/-} and *Pten*^{+/-}*Rictor*^{+/-} prostate tissue was sectioned and stained by Hematoxylin & Eosin (top) or labeled with Ki-67 antibody (bottom). Representative images are shown. The star (*) indicates a normal prostate ductule and the arrow points to a ductule containing large hyperplastic cells. Scale bar = 50μm (top) and 25μm (bottom).

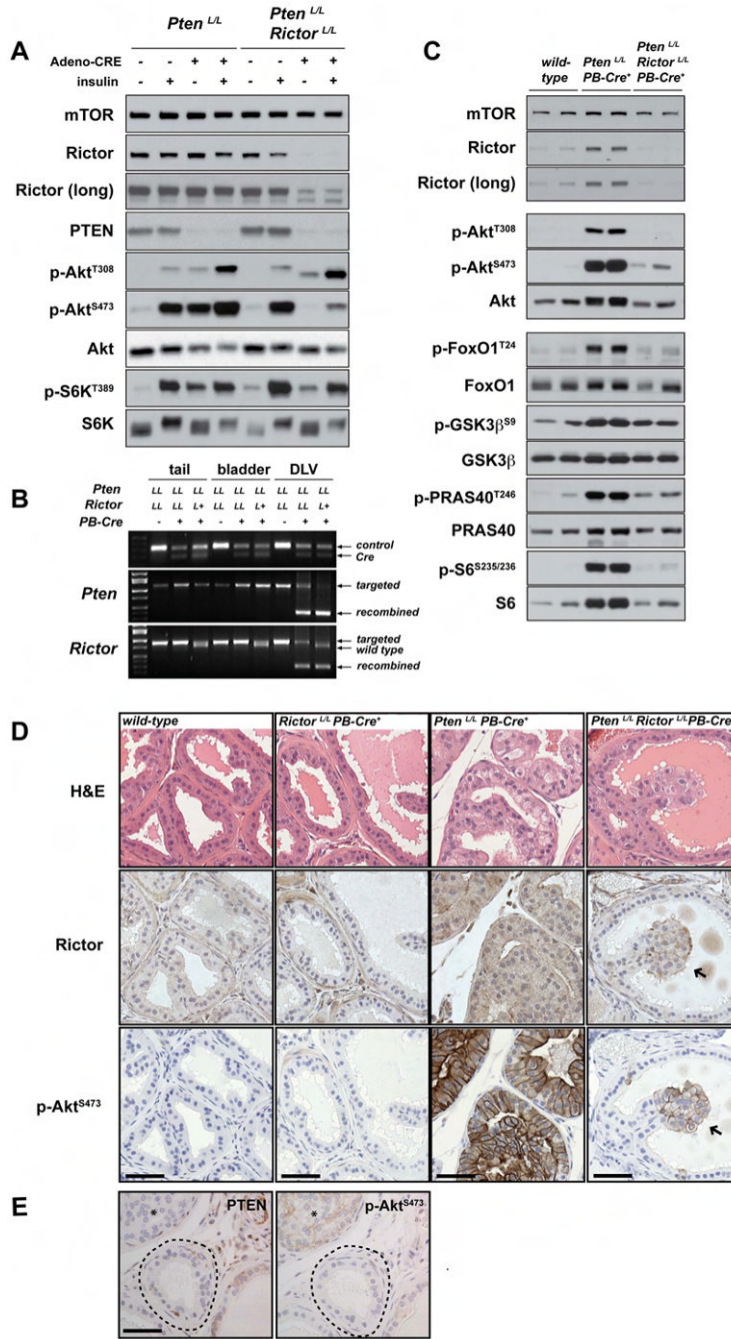


Figure 3. *Pten*-deletion-induced phosphorylation of Akt^{S473} in MEFs and in prostate epithelial cells requires *Rictor*

(A) Deleting *Rictor* in combination with *Pten* blocks hyper-phosphorylation 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 300nm insulin for 15 minutes. Protein lysates were prepared and probed with the indicated antibodies. For the Rictor immunoblot, “long” indicates longer exposure. (B) The *PB-Cre4* 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 towards 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-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 by 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 bar = 50μM. (E) Cells lacking PTEN and phospho-Akt^{S473} appear normal (circled area), consistent with loss of mTORC2 activity blocking transformation. For comparison, this example includes a nearby section of hyperplastic cells (indicated by the star) that stain negative for PTEN and positive for Akt^{S473}, which is indicative of inefficient *Rictor* deletion. Scale bar = 50μm.

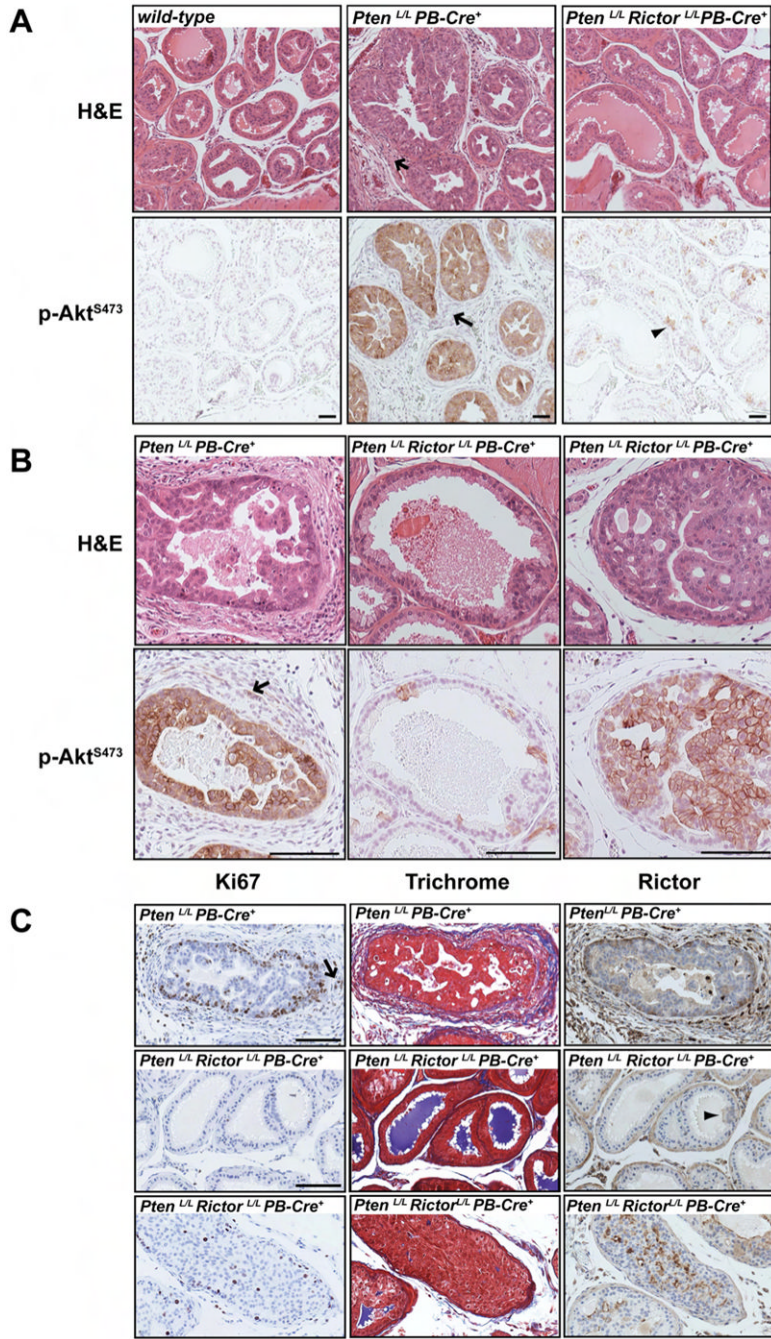


Figure 4. *Pten*-deletion-induced invasive adenocarcinoma requires *Rictor*

(A) Prostate tissue serial sections from 9-week old mice were stained by H&E or labeled with a phospho-Akt^{S473} antibody and imaged at 10X. The arrows point to changes in the stroma. The arrowhead indicates a patch of phospho-Akt^{S473} positive cells. Scale bar = 50µm. (B) Higher magnification images (20X) of serial sections stained by H&E or labeled with a phospho-Akt^{S473} antibody. Invasive phospho-Akt^{S473} positive cells are indicated with an arrow. Scale bar = 50µm. (C) Labeling with Ki-67 antibody (left), trichrome stain (middle) and Rictor antibody (right) are shown. The arrow indicates proliferating (Ki-67 positive) cells in the stroma. The arrowhead points to a small patch of cells that have not lost Rictor expression. Scale bar = 50µm.

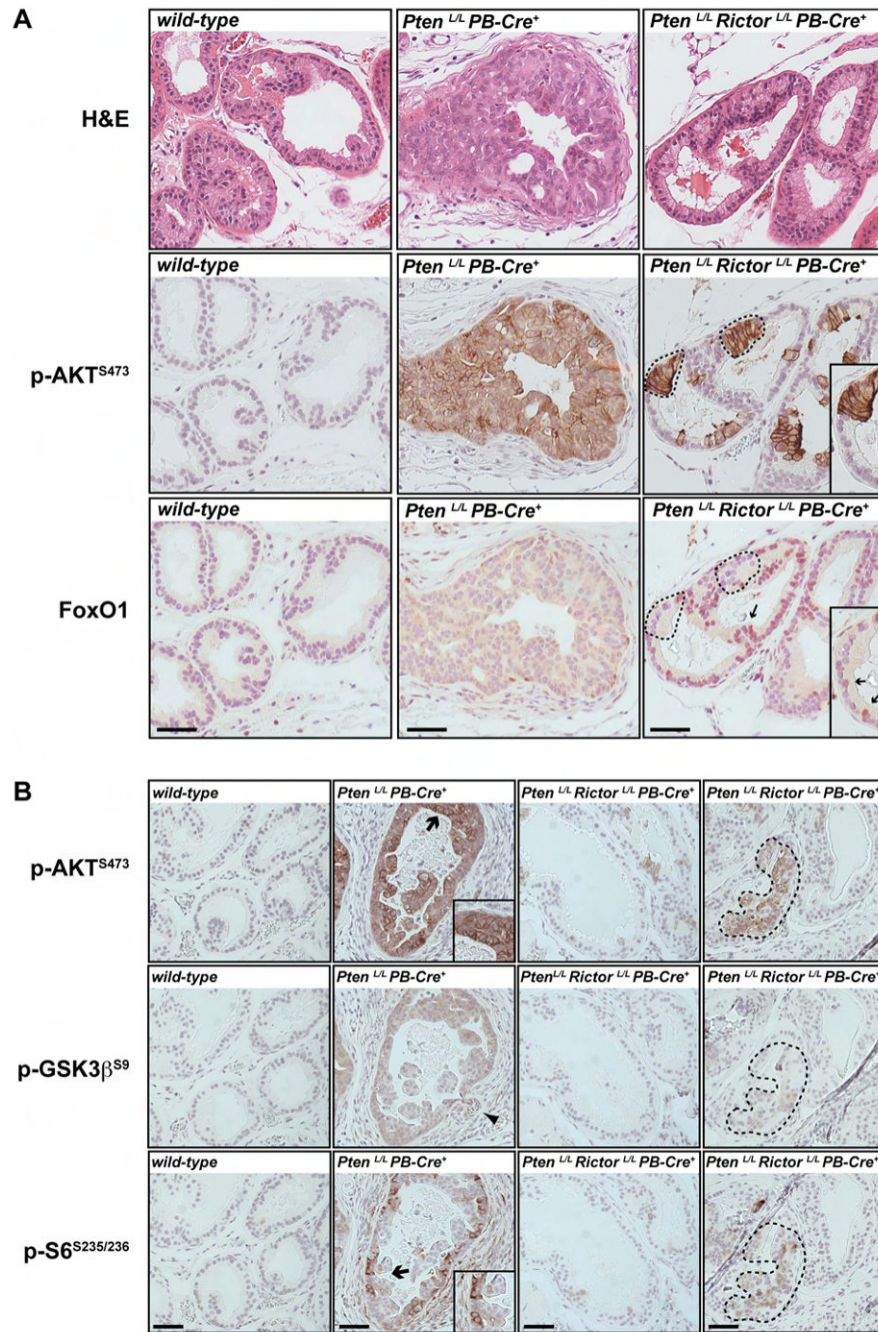


Figure 5. Akt activity towards downstream substrates in *Pten*-deficient prostate epithelial cells requires *Rictor*

(A) Serial sections from *wild-type*, *Pten*^{LoxP/LoxP} *PB-Cre*⁺ or *Pten*^{LoxP/LoxP} *Rictor*^{LoxP/LoxP} *PB-Cre*⁺ tissue stained by H&E, or labeled with antibodies to phospho-Akt^{S473} or to FoxO1 are shown. The 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. An enlarged section is shown in the boxed insert. Scale bar = 25μm. (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} are shown. Arrows indicate regions highlighted in the boxed

inserts. The arrowhead points to invasive cells. The encircled area in the right panels indicates a patch of phospho-Akt^{S473} positive cells. Scale bar = 25µm.