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## PI3K regulation of the SKP-2/p27 axis through mTORC2

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### Abstract

The cyclin dependent kinase inhibitor p27 is a key regulator of cell cycle progression. Its expression and localization are altered in several types of malignancies, which has prognostic significance in cancers such as renal cell carcinoma (RCC). S-phase kinase associated protein 2 (SKP-2) is an F-box protein that is part of the SKP1/Cul1/F-box (SCF) ubiquitin ligase complex that targets nuclear p27 among many other cell cycle proteins for proteosomal degradation. Its overexpression has been observed in several tumor types. Signaling by phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) has previously been shown to regulate the SKP-2/p27 axis. Recent evidence suggests that PI3K signaling may activate mTOR complex 2 (mTORC2) activity. As PI3K signaling is known to regulate SKP-2 and p27, we sought to determine whether these effects were mediated by mTORC2. Here, we provide additional genetic evidence that PI3K signaling activates mTORC2 kinase activity. We also demonstrate a novel role for mTORC2 in the modulation of nuclear p27 levels. In particular, mTORC2 signaling promotes the reduction of nuclear p27 protein levels through the increased protein expression of SKP-2. These are the first data to demonstrate a role for mTOR in the regulation of SKP-2. In concordance with these findings, mTORC2 activity promotes cell proliferation of RCC cells at the G1-S interphase of the cell cycle. Collectively, these data implicate mTORC2 signaling in the regulation of the SKP-2/p27 axis, a signaling node commonly altered in cancer.

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#### Statistical analysis

Student's t-TEST was used to analyze the statistical significance.  $p < 0.05$  was considered to be significant.

**Conflict of Interest.** The authors declare there are no competing financial interests in relation to the work described.

## Keywords

p27; SKP-2; mTOR; RICTOR

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## Introduction

p27, a cyclin-dependent kinase inhibitor, negatively affects cell cycle progression through effects at the G1/S interphase. Studies demonstrated that p27 is often dysregulated in several cancer types (1). In the context of renal cancer, studies have established loss of p27 expression with increasing stage and grade of tumors(2–8). More recently, work by Kim *et al.* has identified cytoplasmic sequestration of p27 with increasing tumor grade(9). These data suggest that p27 levels and its localization may have biological significance in RCC. Therefore determining the cellular mechanisms that regulate p27 may indicate key signaling pathways in RCC as well as other malignancies such as breast and prostate cancer where p27 is dysregulated.

A major point of regulation in p27 is through signaling of the phosphoinositide 3-kinase (PI3K) axis. Genetic and pharmacologic approaches demonstrate that PI3K signaling regulates p27 expression and localization. AKT, a major downstream effector of PI3K signaling also has a role in p27 regulation. The PI3K/AKT signaling axis regulates p27 expression and localization through transcriptional as well as post-transcriptional mechanisms. Transcriptional regulation involves the Forkhead family of transcription factors(10). AKT phosphorylates p27 at multiple residues including S10, T157, and T198, which mediates p27 stability and/or localization depending on the cellular context (11). PI3K signaling also promotes p27 proteolysis through effects on the F-Box protein S-phase kinase associated protein 2(SK P-2). SKP-2 is a component of the SCF<sup>SKP-2</sup> (S-phase kinase associated protein 1 (SKP1)/Cullin/F-Box) ubiquitin ligase complex that mediates p27 proteolysis(12). Degradation of p27 by this complex is mediated, in part, by phosphorylation of p27 at Thr187 by CDK2(13). However, SKP-2 has also been shown to elicit proteolysis of p27 independent of phosphorylation at the Thr187 site of p27(14). Interestingly, mounting evidence supports a role for the SKP-2 in tumorigenesis.

Full activation of AKT requires phosphorylation of Thr308 and Ser473. Thr308 is phosphorylated by phosphoinositide kinase 1 (PDK1), which is immediately downstream of PI3K(15, 16). Until recently, the identity of the kinase responsible for Ser473 phosphorylation remained elusive, and was referred to as PDK-2. The Ser473 site is present in the hydrophobic motif (HM) present in members of the AGC family of kinases (protein kinase A/protein kinase G/protein kinase C-family). Complex 2 of the mammalian target of rapamycin (mTORC2) has been shown to demonstrate PDK-2 activity in a variety of cell types(17–20). The mechanisms by which growth factors lead to mTORC2 activation remain unclear at this time. Recent evidence suggests that PI3K signaling may directly mediate mTORC2 activity(21, 22). Gan *et al.* demonstrated that phosphatidylinositol 3,4,5-triphosphate (PIP3), the product of PI3K catalysis, can directly stimulate the kinase activity of mTORC2. Therefore the effects of PI3K signaling on p27 may be mediated by mTORC2.

Given that activated AKT can modulate p27 via SKP-2, we asked if mTORC2 has a role in the regulation of the SKP-2/p27 axis.

## RESULTS

### PI3K/AKT Regulates p27 protein expression in RCC Cells

Given a potential link between PI3K and mTORC2, we wanted to first establish the role of PI3K in the regulation of p27 in the context of RCC cells. Consistent with prior data in tumor cell types including RCC(9), we found that treatment with LY294002, a pharmacologic inhibitor of PI3K, increased p27 protein levels in both 786-O and A498 RCC cells (Fig. 1A). As LY294002 may impact other kinases, we used a genetic approach to determine the effect of PI3K signaling on p27 protein expression. 786-O cells are *PTEN* null. We therefore utilized 786-O cells stably transfected with a tet-inducible *PTEN* construct, hereafter referred to as 786-O/iPTEN. Treatment of 786-O/iPTEN cells with doxycycline resulted in a dose-dependent induction of PTEN protein with a concomitant decrease in AKT phosphorylation at Ser473 which is consistent with reduced AKT signaling (Fig. 1B). Doxycycline treatment of these cells also resulted in an increase in cellular p27 levels in a dose-dependent manner (Fig. 1C). As PI3K signaling may have several downstream effectors, we next determined the role of AKT, specifically AKT-1, in p27 regulation in RCC cells. We focused on this isoform of AKT as it was recently shown to preferentially bind with the mTORC2-specific subunit RICTOR(23). Knockdown of AKT-1 led to an increase in p27 levels in both 786-O and A498 cells (Fig. 1D) confirming the role of PI3K/AKT signaling in regulating p27 levels in RCC cells. We also determined the increase in nuclear p27 levels in RCC cells upon PI3K inhibition. Consistent with previous reports, treatment of RCC cells with LY294002 resulted in an increase in nuclear p27 levels in both 786-O and A498 cells as determined by immunofluorescence (Fig. 1E) and cell fractionation studies (data not shown). In agreement with these data, Dox treatment of 786-O/iPTEN cells resulted in an increase in nuclear p27 levels as determined by western blotting of nuclear fractions (Fig. 1F). Collectively, these data demonstrate the role of PI3K/AKT signaling in the regulation of p27 protein levels as well as localization in RCC cells.

### PI3K Signaling mediates mTORC2 kinase activity

As noted, recent reports suggest a link between PI3K and mTORC2. However, these reports principally relied on pharmacologic treatments to demonstrate this connection. Doxycycline treatment of 786-O/iPTEN cells reduced AKT phosphorylation but did not result in any significant changes of mTOR (Fig. 2A). We next examined mTORC2 activity with the use of an *in vitro kinase* assay utilizing catalytically inactive AKT as the substrate (Fig. 2B). Consistent with prior work, ser473 phosphorylation of the substrate was barely detectable in IgG pulldown extracts and was due to a low basal level of substrate autophosphorylation(24). In contrast, immunoprecipitation of mTORC2 (with anti-Rictor Ab) from lysates prepared from untreated 786-O/iPTEN (i.e. without Dox) resulted in readily detectable levels of ser473 phosphorylation (Fig. 2B). In contrast, treatment of cells with Dox prior to lysate preparation and immunoprecipitation resulted in reduced mTORC2 kinase activity as demonstrated by low levels of *in vitro* AKT phosphorylation at ser473.

Moreover, the reduction in kinase activity was not associated with reduced levels of mTOR or RICTOR in immunoprecipitated mTORC2 (Fig. 2B). To confirm these results, we performed a similar experiment with mTORC2 immunoprecipitated with antibody to the mTORC2-specific subunit SIN1. Dox treatment of 786-O/iPTEN cells reduced the *in vitro* kinase activity of SIN1-immunoprecipitated mTORC2 without changes in the protein levels of mTOR, RICTOR, and SIN1 in the immunoprecipitate (Fig. 2C).

### mTORC2 kinase inhibition increases nuclear p27 protein levels in RCC cells

Prior studies demonstrated that RICTOR knockdown in RCC cells reduced AKT phosphorylation at Ser473(25). While RICTOR has previously been shown to phosphorylate Ser473 as part of an mTOR complex, it has also been shown to phosphorylate Ser473 as part of a complex with integrin-linked kinase (ILK)(26). We therefore determined if mTORC2 has PDK-2 activity in RCC cells. Pharmacologic inhibition of mTORC1 and mTORC2 using mTOR kinase inhibitor Torin1 (27) readily reduced pAKTser473 in 786-O and A498 RCC cells (Fig. 3A). Given the role of PI3K/AKT signaling in regulating p27 and our data demonstrating that PI3K modulates mTORC2, we next examined the role of mTORC2 in regulating p27 protein expression in RCC cells. mTOR inhibition with Torin1 increased p27 protein levels in 786-O and A498 cells (Fig 3A). We next examined effects on nuclear p27 levels as loss of nuclear p27 expression is a negative prognostic factor in many cancer types including RCC. We found that Torin1 treatment increased nuclear p27 levels in both 786-O and A498 cells (Fig. 3B). We confirmed these data in 786-O cells by immunofluorescence for nuclear p27 (Fig. 3C). siRNA knockdown of the mTORC2-specific subunits RICTOR and mSIN-1 also reduced ser473 phosphorylation of AKT in both 786-O and A498 cells (Figs. 3D and 3E) consistent with mTORC2 having PDK-2 activity in RCC. Similar to Torin1 treatment, mTORC2 inhibition via knockdown of either RICTOR or mSIN-1 resulted in increased p27 levels in both 786-O and A498 cells (Fig 3D and 3E). mTORC2 inhibition via RICTOR or mSIN-1 knockdown in 786-O and A498 cells led to an increase in nuclear p27 levels based on nuclear fraction western blotting (Fig. 3F) and immunofluorescence (Figs 3G and Supplemental Fig 1S). Collectively, these data demonstrate that mTORC2 plays a role in the regulation of both total and nuclear p27 protein levels.

### mTORC2 inhibition leads to decreased SKP-2 levels

We next wanted to determine the mechanism by which mTORC2 regulates nuclear p27 levels. As nuclear p27 degradation is mediated by the SCF<sup>SKP-2</sup> ubiquitin ligase complex, we next examined the effects of PI3K and mTORC2 signaling on SKP-2 levels. Treatment of 786-O and A498 RCC cells with LY-294002 (Fig. 4A), and dox treatment of 786-O PTEN inducible cells (Fig. 4B) resulted in a decrease in SKP-2 protein levels. Furthermore, AKT-1 knockdown also resulted in decreased SKP-2 levels in RCC cells (Fig. 4C). Torin1 treatment (Fig. 4D) and RICTOR and mSIN-1 knockdown (Fig. 4E) reduced SKP-2 protein levels in 786-O and A498 cells. We next stably knocked down RICTOR with shRNA in 786-O cells. Consistent with transient siRNA experiments, stable RICTOR knockdown resulted in increased p27 levels with a concomitant decrease in SKP-2 levels (Fig. 4F, compare lanes 1 and 3; Supplemental Fig. 2S). Overexpression of constitutively active myristolated AKT (myr AKT) in RICTOR knockdown cells reversed this phenotype (Fig.

4F, compare lanes 3 and 4) indicating that mTORC2 is upstream of AKT and that AKT is responsible for the effects on SKP-2 and p27.

Given these findings, we next determined whether SKP-2 mediated the effects of mTORC2 on p27 expression. First, we examined p27 protein half-life given SKP-2's role in regulating p27 protein stability. As noted before, stable RICTOR knockdown in 786-O cells increased basal p27 protein levels. In addition, stable RICTOR knockdown in 786-O cells increased p27 protein half-life relative to control vector cells (Fig. 5A). Transient overexpression of SKP-2 in RICTOR knockdown cells decreased p27 levels and reduced p27 protein half-life relative to control vector transfection consistent with SKP-2's role in p27 degradation (Fig. 5B, left and right panels). SKP-2 overexpression was confirmed by immunoblotting (data not shown). We next stably transfected A498 cells with control vector and myc-tagged SKP-2 construct. Similar to parental cells, control cells transiently transfected with siRICTOR increased p27 levels (Fig. 5C, compare lanes 1 and 2). As expected, SKP-2 transfected cells demonstrated lower basal levels of p27 (Fig. 5C, compare lanes 1 and 3). However unlike control cells, RICTOR knockdown in SKP-2 transfected cells did not increase p27 levels (Fig. 5C, compare lanes 3 and 4). Similar to RCC cells, the effect of RICTOR knockdown on p27 levels could be blocked via overexpression of HA tagged SKP-2 in HEK293 cells (Supplemental Fig. 3S). Collectively, these data demonstrate that mTORC2 mediates p27 protein levels through effects on SKP-2.

### PI3K regulates SKP-2 and p27 through mTORC2

Given our data linking PI3K signaling to mTORC2 kinase activity, we wanted to determine whether PI3K modulates SKP-2 and p27 levels through mTORC2. We therefore knocked down PTEN in A498 cells to activate PI3K signaling. In concordance with our prior findings, PTEN knockdown activated AKT signaling and resulted in increased SKP-2 and decreased p27 levels (Fig. 6, compare lanes 1 and 2). In contrast, cotransfection of A498 cells with both siPTEN and siRICTOR blocked AKT activation (Fig. 6A, compares lanes 2 and 3) consistent with our finding that PI3K regulates AKT through mTORC2.

Correspondingly, cotransfection with siRICTOR blocked the effect of siPTEN on SKP-2 and p27 (Fig. 6, compares lanes 2 and 3). Collectively, these data demonstrate that PI3K regulates the SKP-2/p27 axis through mTORC2.

### mTORC2 regulates cell proliferation

Finally, we wanted to examine the biological effects of mTORC2 signaling on the proliferation of RCC cells given the effects on cell cycle regulators. First, we assessed cell cycle distribution of RCC cells following treatment with Torin1. Torin1 treatment blocked G1/S progression in both 786-O and A498 RCC cells (Fig. 7A). Next, we assessed cell cycle distribution in RCC cells following RICTOR knockdown (Fig. 7B-upper panel). Relative to control cells, RICTOR knockdown 786-O and A498 cells demonstrated a significant reduction in cells in S-phase with a concomitant increase in cells in G1 phase. These data are consistent with the role of SKP-2/p27 in regulating G1-S progression. We next examined proliferation via an assay to measure incorporation of the thymidine analogue EdU. Transient RICTOR knockdown with siRNA reduced cellular proliferation in both 786-O and A498 cells (Fig. 7B-lower panels). Similar data were obtained by measuring incorporation

of H3-thymidine (supplemental Fig. 4S). In accordance with these data, stable RICTOR knockdown in 786-O cells also inhibited G1/S progression and proliferation (supplemental Fig. 5S).

We next determined whether mTORC2 regulates proliferation through its effect on SKP-2 by examining the effects of RICTOR knockdown on cells overexpressing SKP-2 (myc-tagged, see Fig. 5C). A498 cells stably transfected with control vector (CV) cells demonstrated reduced proliferation following siRICTOR treatment similar to parental A498 cells (Fig. 7C). In contrast, SKP-2 overexpressing cells were resistant to the antiproliferative effects of siRICTOR (Fig. 7C). We next determined the contribution of p27 to the antiproliferative effects of mTORC2 inhibition in A498 cells. Consistent with prior results, RICTOR knockdown in A498 cells inhibited G1/S progression (Fig. 7D-upper panel) and proliferation (Fig. 7D-lower panel). However, concurrent knockdown of both RICTOR and p27 rescued cells from the antiproliferative phenotype of cells treated with siRICTOR alone as determined by cell cycle and EdU incorporation analyses (Fig. 7D). Collectively, these data demonstrate that mTORC2 regulates proliferation through its effects on the SKP-2/p27 axis.

## DISCUSSION

This report demonstrates a novel role for mTORC2 in the regulation of the SKP-2/p27 axis. p27 dysregulation has long been implicated in cancer. In the case of renal cancer, several studies have examined the expression and/or localization of p27. Decreased p27 expression in RCC has been shown to inversely correlate with tumor stage and grade. In addition loss of p27 expression has been linked to reduced cancer-specific survival rates. Cytoplasmic sequestration of p27 away from the nucleus has also been shown to correlate with increasing nuclear grade(2–8). Overall, the general consensus is that loss of p27 expression, as well as its nuclear exclusion, are associated with a poorer prognosis. More recent studies have focused on the role of SKP-2 in RCC as well as other malignancies given its established role in p27 regulation. SKP-2 has been shown to have negative prognostic significance in multiple tumor types including renal cancer. Recent studies demonstrating increasing SKP-2 expression with tumor size and stage (28). Moreover, SKP-2 expression is associated with lower disease-free survival in patients with renal cancer(6). Given emerging evidence that SKP-2 has oncogenic properties, elucidating the mechanisms by which this F-box protein is regulated will provide insights for therapeutic targeting.

The current study presents several novel findings. Our data are the first to demonstrate direct regulation of mTORC2 by PTEN, an antagonist of class I PI3K. *PTEN* is among the most commonly mutated tumor suppressor genes. In either RICTOR or SIN1 immunoprecipitated mTORC2, PTEN expression suppressed the *in vitro* kinase activity of mTORC2. Although implied, a direct link between PI3K signaling and mTORC2 is only now emerging. Gan *et al.* recently demonstrated that phosphatidylinositol 3,4,5-triphosphate (PIP3), the product of PI3K catalysis, was able to directly activate HM site phosphorylation (ser473) of AKT by immunoprecipitated mTORC2 complex(21). They propose that PIP3 can activate mTORC2 via 3 distinct mechanisms: 1) activation of mTORC2 kinase activity, 2) substrate recruitment and 3) enhancing the ability of molecules such as AKT to be substrates of



mTORC2 through conformational changes. Consistent with our findings are data from Huang *et al.* who demonstrated that pretreatment of cells with the PI3K inhibitor wortmannin diminished insulin-stimulated mTORC2 *in vitro* kinase activity(24). Collectively, these data point to PTEN's role in PI3K signaling, as opposed to its protein phosphatase activity, in the regulation of mTORC2. The precise mechanism by which PI3K modulates the kinase activity of mTORC2 remains to be elucidated. We suggest that PIP3 regulates mTORC2, perhaps through induction of conformational changes. There is precedent for a role in PIP3 in inducing conformation changes in proteins that contain pleckstrin homology (PH) domains(29). Interestingly, mSIN-1 possesses a PH domain at the C-terminus. While this domain may be critical to localizing mTORC2 with substrates, it may also be subject to regulation by PIP3 that can impact mTORC2 kinase activity.

The data presented are also the first to demonstrate a role for mTORC2 in the regulation of SKP-2, a particularly important finding given SKP-2's emerging role in transformation. Moreover, these are the first data to demonstrate that mTORC2's effect on proliferation is mediated by the SKP-2/p27 axis. Our data demonstrate that activated mTORC2 signaling leads to reduced levels of p27 via effects on SKP-2. As noted before, SKP-2 and p27 expression have prognostic significance in renal cancer as well as other malignancies. Hence, uncovering the signaling nodes that regulate the balance between SKP-2 and p27 may have biologic and clinical significance. Our study demonstrates that mTORC2 is a regulator of the SKP-2/p27 axis in RCC. Given that mTORC2 contributes to AKT activation in other cancer cell types, it is also likely to regulate this balance in other malignancies.

Another novel aspect of the current study is the mechanistic insight provided into PI3K's regulation of SKP-2 through AKT. Recent interest has focused on the events downstream of AKT. In particular, studies have focused on AKT-mediated phosphorylation of SKP-2. Lin *et al.*, found that AKT-mediated phosphorylation of SKP-2 at ser72 promotes SCF<sup>SKP-2</sup> formation as well as SCF<sup>SKP-2</sup> ubiquitin ligase activity. Additionally, ser72 phosphorylation was found to promote SKP-2 relocalization to the cytosol(30). Gao *et al.* also recently reported that AKT1 phosphorylates SKP-2 at ser72(31). In their studies, AKT phosphorylation of SKP-2 had two effects: 1) SKP-2 phosphorylation decreased interaction of SKP-2 with its ubiquitin ligase Cdh1 therefore leading to increased SKP-2 protein stability, and 2) promotes cytoplasmic translocation of SKP-2. However, recent studies by three independent groups demonstrate that phosphorylation at ser72 does not regulate SCF assembly, SCF ubiquitin ligase activity, or SKP-2 localization(32–34). Alternatively, AKT has recently been shown to promote SKP-2 mRNA translation as recently reported by Nogueira *et al.*(35). Our study is novel in that it examines the role of signaling molecules *upstream* of AKT to provide further insight into how PI3K regulates SKP-2. Our data demonstrate that PI3K's regulation of SKP-2 is mTORC2 dependent, and therefore further underscores the emerging role for mTORC2 signaling in cancer which is of recent interest.

Guertin *et al.* examined the role of mTORC2 signaling in a *PTEN* conditional knockout model of prostate cancer in mice(36). While *PTEN* knockout led to prostate cancer development, simultaneous knockout of RICTOR abrogated prostate cancer development. In addition to these studies, subcutaneous xenograft studies demonstrate a role of mTORC2 in tumorigenesis in the context of glioblastoma and colon cancer cells(37, 38). Given the

emerging role of mTORC2 in cancer biology, the elucidation of the downstream effectors of mTORC2 is warranted. While AKT was the first bona fide mTORC2 substrate, subsequent studies have led to the identification of PKC- $\alpha$  (in addition to other PKC family members) as well as SGK1 (serum and glucocorticoid inducible kinase) as substrates for mTORC2(18, 39). While our data specifically implicate AKT in the effects on SKP-2 and p27, these alternate substrates may have overlapping effects on this axis.

Recent evidence suggests that targeting PI3K may be therapeutically advantageous in RCC. Cho *et al.* recently reported on the efficacy of a dual PI3K/mTOR inhibitor (NVP-BEZ235) compared with mTOR inhibitor rapamycin in RCC(40). They found that dual PI3K/mTOR inhibition was more effective than rapamycin both *in vitro* and *in vivo*. Interestingly, they demonstrated that NVP-BEZ235, unlike rapamycin, was able to promote nuclear accumulation of p27. Although rapalogs are currently FDA-approved for the treatment of metastatic RCC, the response to these agent are limited and most patients will demonstrate progression of their disease while on therapy. Hence, it may be that targeting PI3K and/or mTORC2 may be more efficacious than current therapies as proposed by Cho *et al.*

In summary, this report demonstrates the role of mTORC2 as a downstream mediator of PI3K signaling in the regulation of the SKP-2/p27 axis, which is commonly altered in cancer. Our data demonstrate that PI3K signaling, through mTORC2, tips the balance between p27 and SKP-2 in favor of a high SKP-2/low p27 state that is conducive to tumor formation and/or progression (Fig. 8). Our findings therefore indicate a role for targeting mTORC2 in RCC as well as other malignancies where the SKP-2/p27 axis is biologically relevant.

## Methods

### Cells

A498 and 786-O, and HEK-293 cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The 786-O inducible PTEN line (7860/iPTEN) was created as follows. Human PTEN cDNA (kindly provided by Jack Dixon, UCSD) was cloned into multiple cloning site of pTRE2-pur from Clontech. pUHD/rtTA2sM2 (a kind gift of Hermann Bujard, University of Heidelberg) transcribes the reverse tetracycline transactivator. We cotransfected 786-O cells with pUHD/rtTA2sM2 and pTRE2-pur-PTEN at a 10:1 ratio and selected a puromycin resistant clone that most stringently expressed PTEN with doxycycline.

### Reagents and Chemicals

Expression plasmid of HA-tagged SKP-2 was a kind gift from Renee Yew (UTHSCSA). Myc-tagged SKP-2 construct was acquired from Y. Xiong (UNC) through Addgene. A498 cells overexpressing Myc-tagged SKP-2 were identified after G418 selection and were assessed for myc expression by immunoblotting. LY294002, doxycycline, and cycloheximide were purchased from Sigma. Torin1 was kindly provided by Nathanael Gray (Harvard, DFCI).



### **Nuclear Fractionation**

Cells were lysed in low salt lysis buffer (20 mM HEPES, 5 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% NP-40 supplemented with protease inhibitors). After centrifugation, the supernatant was removed (cytoplasmic fraction). The pellet (nuclei) was then lysed with high salt buffer (0.4 M NaCl, 1 mM EDTA, 20mM HEPES supplemented with protease inhibitors). After centrifugation, the nuclear lysate (supernatant) was removed followed by protein concentration determination.

### **Immunoblotting**

All immunoblot analyses (whole cell and nuclear) were performed as previously described prepared(41). Antibodies were obtained from the following commercial sources: Novus (GAPDH), Sigma ( $\alpha$ -Tubulin and Actin), Cell Signaling (Total p-27, mTOR, total AKT, AKT-1, PTEN, Myc, and Ser473 phospho AKT), Bethyl (RICTOR and Sin-1) and Invitrogen (SKP-2).

### **RNA Interference**

For AKT-1, RICTOR and Sin-1 knockdown, cells were transfected with pooled siRNA reagent (Thermo Fisher) with the Amaxa Nucleofector system according to the manufacturer's protocol. Cells were harvested at 24–72 hours following transfection. A non-targeting scramble siRNA pool was used as negative control (Thermo Fisher). shRNA to RICTOR and control vector were acquired from D. Sabatini through Addgene. Selection of stable transfectants was performed with puromycin.

### **Immunofluorescence**

After the indicated treatment, cells were washed once with PBS and fixed with 4% formaldehyde in PBS for 10 min at room temperature. Following PBS rinse, cells were then permeabilized with 0.2% Triton X-100 in PBS on ice for 10 min followed by blocking with 3% BSA/PBS for 30 min. p27 expression was detected with an anti-p27 antibody (1:1000), followed by addition of the Alexa 488 (Invitrogen) conjugated secondary antibody (green). Phalloidin (Invitrogen) 468 was used to stain the actin. Nuclear staining was performed with DAPI (4', 6-diamidno-2-phenylindole; Invitrogen, Carlsbad, CA). Images were acquired on an Olympus FV-500 laser-scanning confocal microscope.

### **Cell Cycle Analysis**

Cells were harvested and washed with PBS and fixed with 70% ethanol. Cells were then resuspended in 1 ml of propidium iodide solution (1.1 mg/ml sodium citrate, 46  $\mu$ g/ml propidium iodide, 0.01% NP40 and 10  $\mu$ g/ml RNase). Data was acquired on the FACS Calibur (using Cellquest software) and analyzed using ModFit software at the University of Texas Health Science Center at San Antonio flow cytometry core facility.

### **EdU Cell Proliferation Assay**

DNA synthesis was quantified with the Click-iT EdU Cell proliferation Assay (Life Technologies) according to the manufacture's protocol. EdU is a thymidine analog. EdU incorporation was detected by fluorescence (Alexa Fluor® 647). Data was acquired on the

FACS Calibur at the University of Texas Health Science Center at San Antonio flow cytometry core facility. A minimum of 10,000 events was scored to determine the proportion of EdU positive cells.

### Immunoprecipitation and kinase assays

Immunoprecipitation of the mTORC2 complex was performed as previously described(42, 43). Four  $\mu\text{g}$  of anti-RICTOR antibody were added to the cleared cellular lysates and incubated with rotation for 90-min. Twenty-five  $\mu\text{l}$  of a 50% slurry of protein G-sepharose was then added and the incubation continued for 1 h. Immunoprecipitates captured with protein G-sepharose were washed four times with the CHAPS Lysis Buffer and once with the kinase buffer (25 mM Hepes pH 7.5, 100 mM potassium acetate, 1 mM  $\text{MgCl}_2$ ). For kinase reaction immunoprecipitates were incubated in a final volume of 15  $\mu\text{l}$  for 20 min at 37 °C in kinase buffer containing 100 ng inactive Akt1/PKB1 (Akt1/PKB1, Upstate Biotechnology, #14-279) and 500  $\mu\text{M}$  ATP. The reaction was stopped by the addition of sample buffer followed by analysis by immunoblotting.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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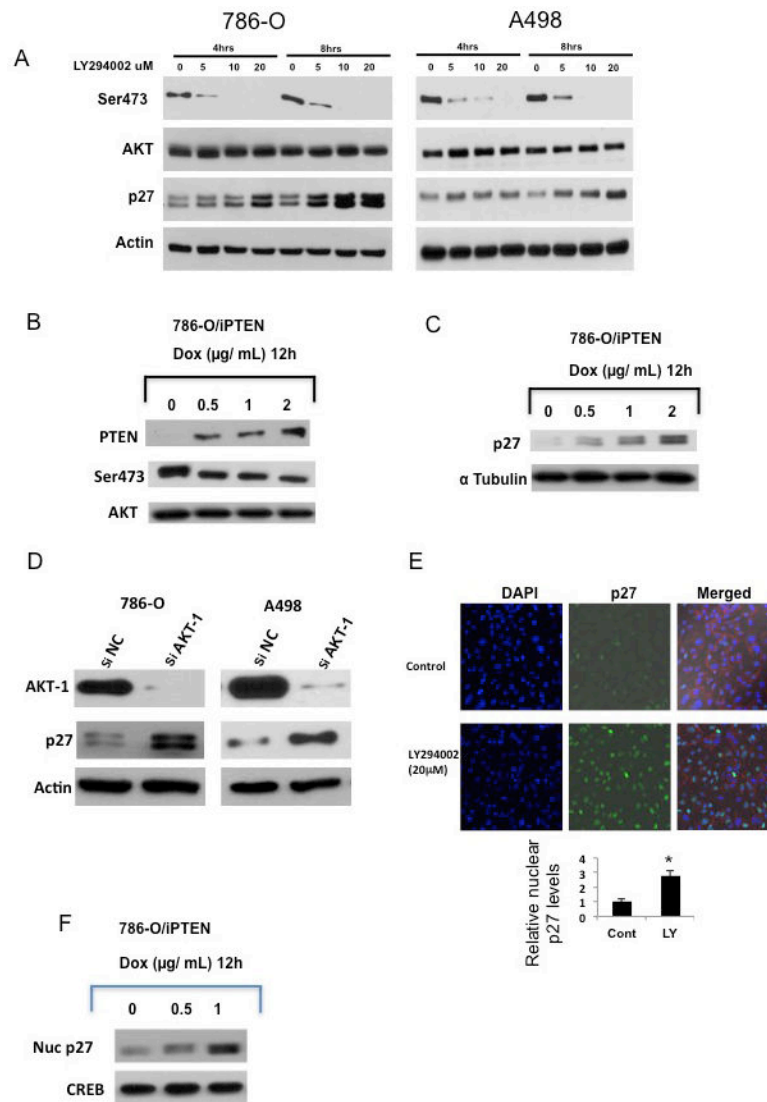
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**Figure 1.**

PI3K/AKT regulates p27 protein expression in RCC cells. (A) 786-O and A498 cells were treated with increasing concentration of LY294002 for the indicated times. Protein lysate was immunoblotted for p27, Ser473 phospho AKT, and total AKT. Actin was used as loading control. (B) 786-O/iPTEN (inducible PTEN) cells were treated with increasing concentrations of doxycycline for 12h and protein lysate was analyzed for levels of PTEN, Ser473 phospho AKT, and total AKT. (C) 786-O/iPTEN (inducible PTEN) cells were treated with increasing concentrations of doxycycline for 12h and protein lysate was analyzed for levels of p27.  $\alpha$  Tubulin was used as loading control. (D) 786-O and A498 cells were transfected with siRNA to AKT-1 and scramble control (si NC) and protein lysates were immunoblotted for AKT-1 and p27 along with Actin as loading control. (E) 786-O cells treated with 10  $\mu$ M LY 294002 for 8h. 786-O cells were fixed and stained with antibody against p27 (green), phalloidin for actin (red) along with nuclear counter stain, DAPI (blue). Images obtained with confocal microscopy (40x). Relative levels of nuclear p27 expression for control and LY294002 cells are shown below. (\* = statistically



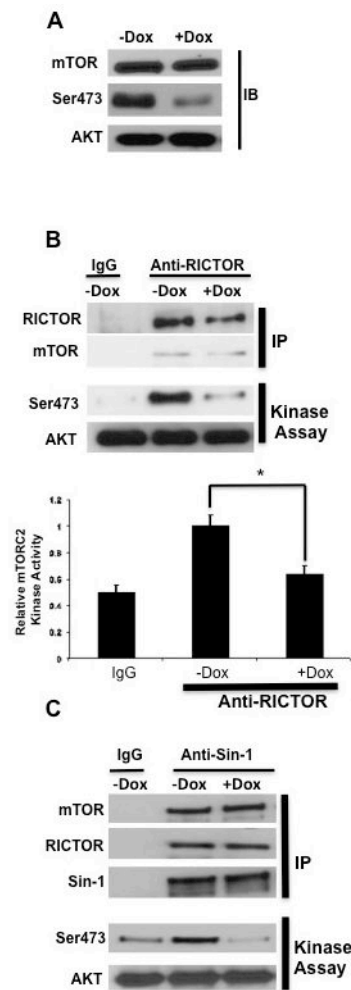
significant difference relative to control treatment:  $p < 0.05$ ). (F) p27 expression in nuclear extracts from 786-O/iPTEN cells treated with doxycycline for 12h. CREB expression was used as loading control.

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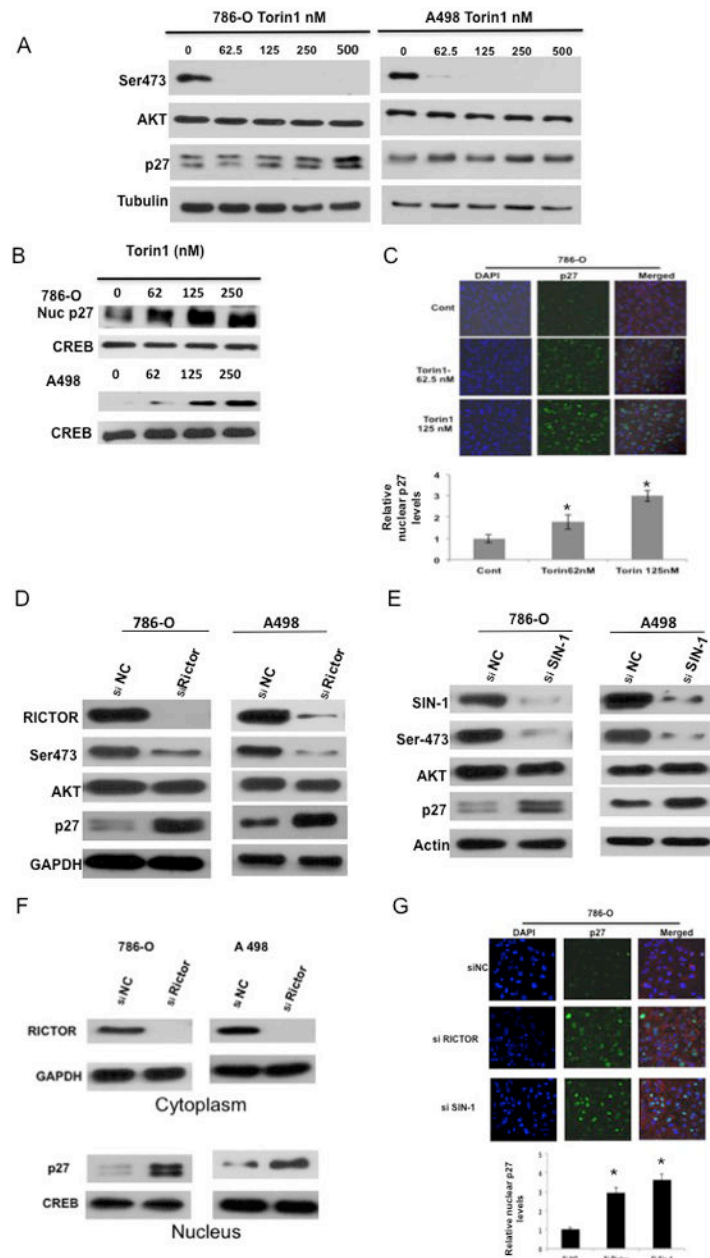
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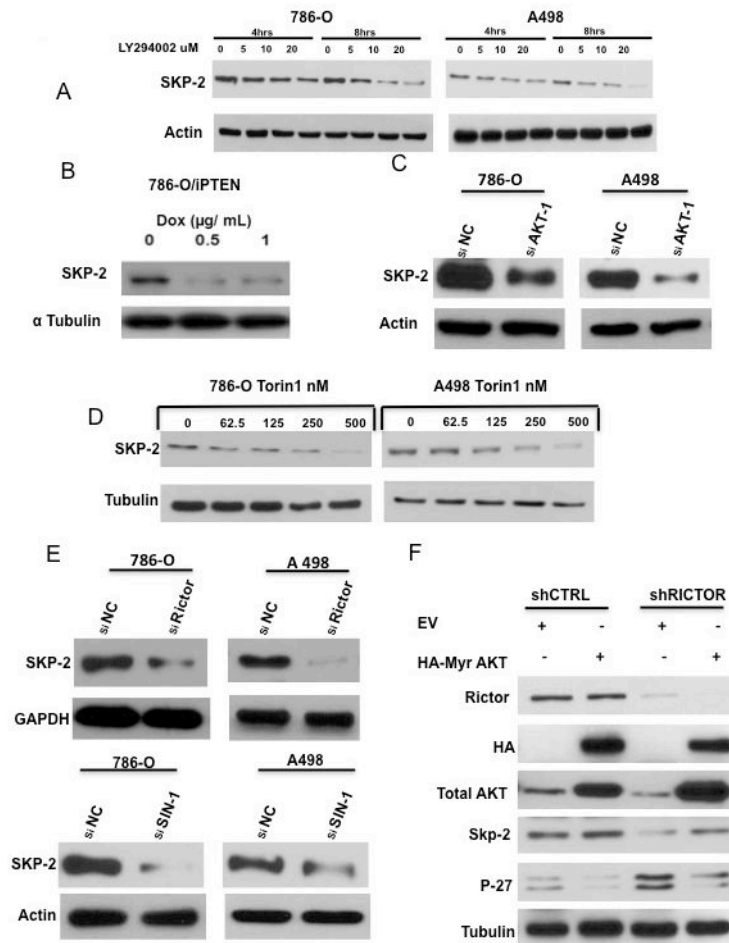
**Figure 2.**

PI3K signaling mediates mTORC2 kinase. (A) Immunoblot analysis in 786-O/iPTEN cells treated with doxycycline (1 µg/ml) for 12 h. (B) mTORC2 was immunoprecipitated from cells treated without and with doxycycline with anti-RICTOR antibody (in addition to IgG control). Immunoprecipitates were probed for levels of RICTOR and mTOR. In addition, immunoprecipitates were used to phosphorylate inactive AKT at Ser473 in an *in vitro* kinase assay. Relative levels of mTORC2 *in vitro* kinase activity are shown below. (C) mTORC2 was immunoprecipitated from cells treated without and with doxycycline with anti-SIN1 antibody (in addition to IgG control). Immunoprecipitates were probed for levels of SIN1, RICTOR, and mTOR. In addition, immunoprecipitates were used to phosphorylate inactive AKT at Ser473 in an *in vitro* kinase assay.

**Figure 3.**

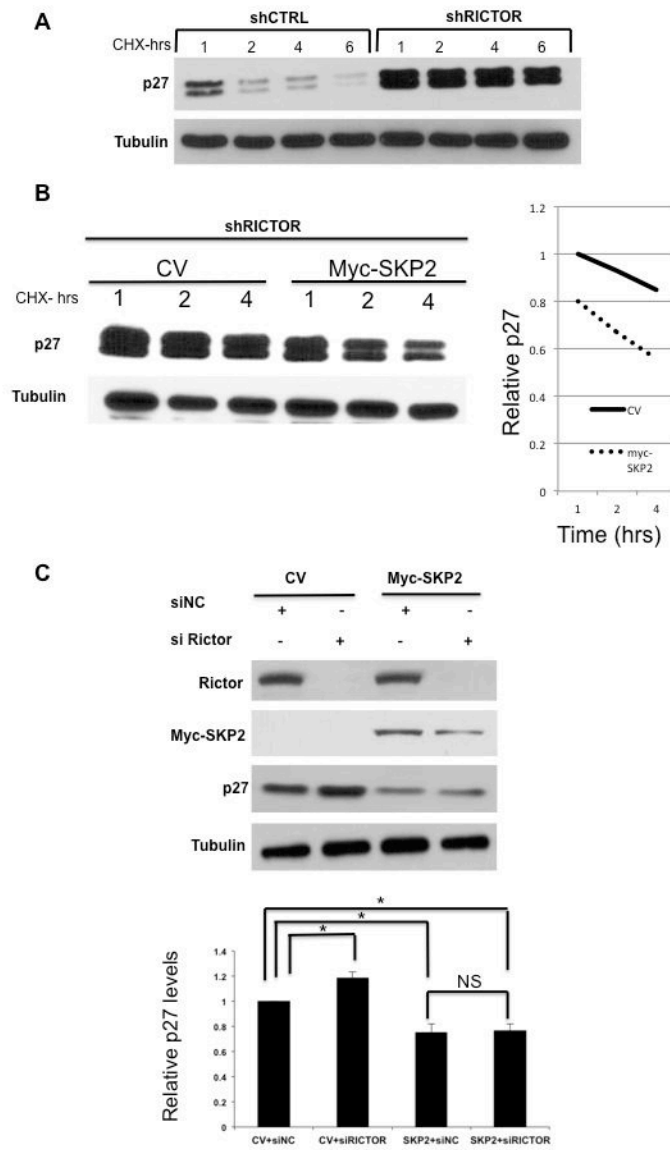
mTORC2 kinase inhibition increases p27 protein levels in RCC cells. (A) 786-O and A498 cells were treated with Torin1 at the indicated concentration for 6 hr. Lysates were immunoblotted for Ser473 phospho AKT, total AKT, p27, and tubulin. (B) 786-O and A498 cells treated with Torin1 at the indicated concentration for 6h. Nuclear lysates were then analyzed by Western for p27 and CREB (loading control). (C) Confocal images (40X oil immersion) demonstrating staining for p27 (green) in Torin1 treated 786-O cells compared to control. The cells were stained with phalloidin (red) to show actin and counter-stained with DAPI (blue) to show the nucleus. Nuclear p27 levels were quantified and relative levels are graphically displayed below (\* = statistically significant difference relative to control

treatment:  $p < 0.05$ ). (D) 786-O and A498 cells were transfected with siRNA to RICTOR and scramble negative control (si NC). Cell lysates were immunoblotted for the indicated proteins with GAPDH as a loading control. (E) 786-O cells and A498 cells were transfected with siRNA to Sin1 and si NC followed by western analysis. (F) 786-O and A498 cells were transfected with siRNA to RICTOR and si NC. Cytoplasmic and nuclear lysates were then analyzed by Western for the indicated proteins. (G) Confocal images (40X oil immersion) of RICTOR and Sin1 siRNA transfected 786-O cells showing staining of p27 (green) compared to scramble control (si NC). Cells were stained with phalloidin (red) for actin and counter stained with DAPI (blue). Nuclear p27 levels were quantified and relative levels are graphically displayed below (\* = statistically significant difference relative to control treatment:  $p < 0.05$ ).



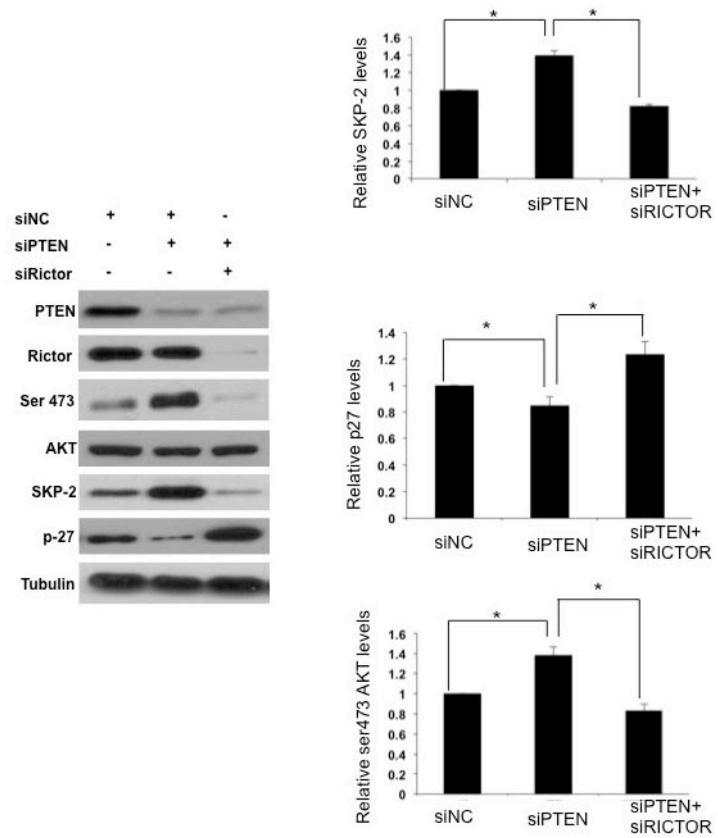
**Figure 4.**

PI3K/mTORC2 inhibition reduces SKP-2 protein levels in RCC cells. (A) 786-O and A498 cells treated with increasing concentration of LY294002. After the indicated time points protein lysates were prepared and analyzed by western blotting. (B) 786-O/iPTEN cells were treated with doxycycline and analyzed by Western for SKP-2 protein levels. (C) Western analysis for SKP-2 in 786-O and A498 cells transfected with siAKT and si NC. Actin was used as loading control. (D) 786-O and A498 cells treated with Torin1 for 6h at the indicated concentration. Immunoblots for SKP-2 and actin are shown. (E) Western blotting of lysates of 786-O and A498 cells transfected with siRICTOR compared to si NC (upper panel). Western blotting of lysates of 786-O and A498 cells transfected with siSIN1 compared to si NC (lower panel). (F) 786-O cells were stably transfected with control vector (shCTRL) or shRICTOR. Stable transfectants were then transiently transfected with either empty vector (EV) or HA-tagged constitutively active myristoylated AKT (HA-Myr AKT). Lysates were harvested and immunoblotted for the indicated proteins.

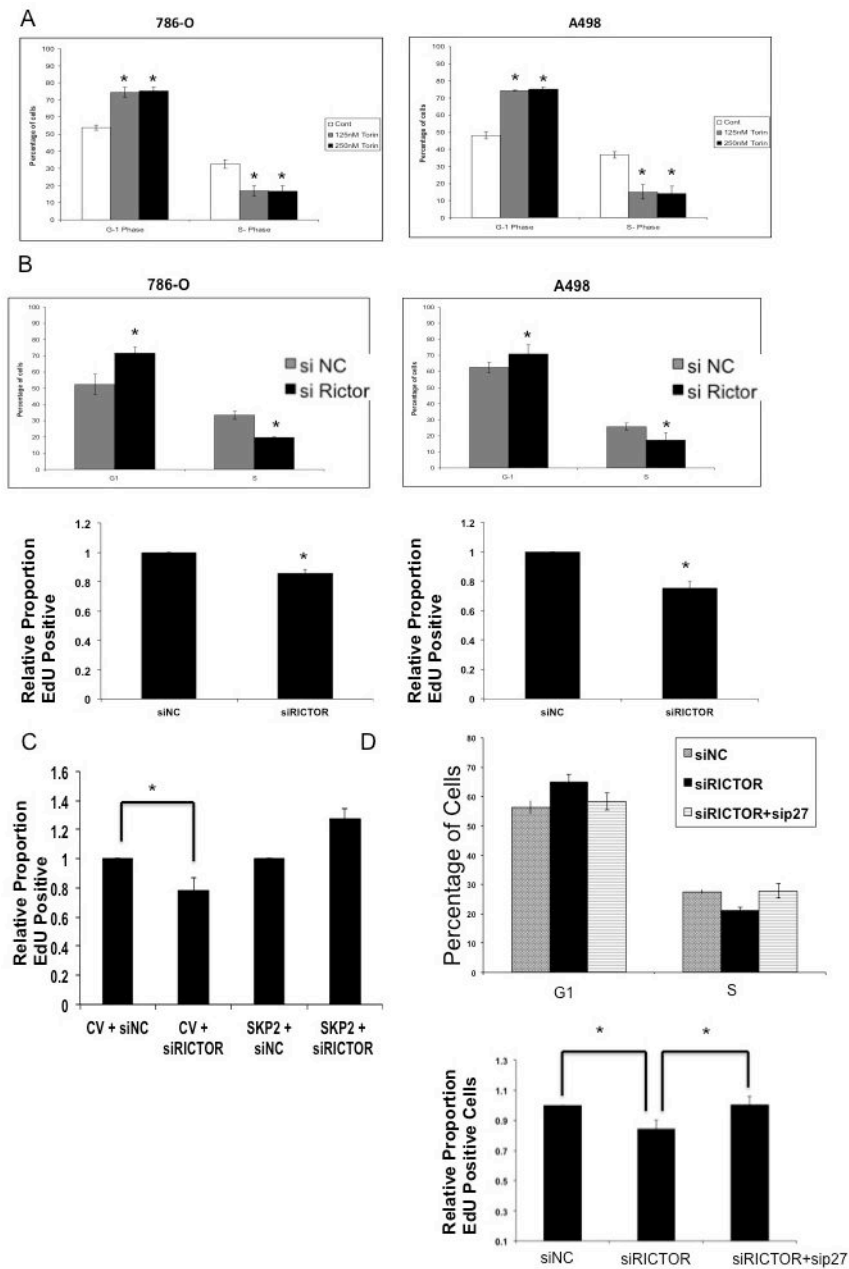
**Figure 5.**

SKP-2 mediates mTORC2 effects on p27. (A) 786-O transfectants (shCTRL and shRICTOR) were treated with the protein synthesis inhibitor cycloheximide (CHX) for the indicated time points. Protein lysates were harvested and immunoblotted for p27. (B) 786-O shRICTOR cells were transiently transfected with myc-SKP2 or CV control. Transfected cells were treated with the protein synthesis inhibitor cycloheximide (CHX) for the indicated time points. Protein lysates were harvested and immunoblotted for p27 and tubulin (left panel). The relative levels of p27 protein are graphically displayed (right panel). (C) A498 cells were stably transfected control vector (CV) or Myc-SKP2. Stable transfectants were then transiently transfected with siNC or siRICTOR. Lysates were immunoblotted for the indicated proteins. Levels of p27 protein were quantified and are shown below.





**Figure 6.** PI3K modulates SKP-2 protein levels through mTORC2. A498 cells were transiently transfected with the indicated siRNA and lysates were immunoblotted for the indicated proteins. Levels of SKP-2, p27, and ser473 were quantified and are graphically displayed.



**Figure 7.** mTORC2 regulates proliferation of RCC cells. (A) Cell cycle analysis in Torin1 treated 786-O (12h) and A498 cells (24h) at two different doses were compared to control in both cell lines. (B) 786-O and A498 cells were transfected with siRICTOR and siNC, and cells were then analyzed by flow cytometry for cell cycle distribution in G1 and S phases (upper panel). Cells were also analyzed for proliferation by measuring incorporation of the thymidine analogue EdU (lower panel). (C) A498 cells stably analyzed with control vector (CV) and myc-SKP-2 were analyzed for EdU incorporation following transient transfection with either control siRNA (siNC) or siRICTOR to determine the effect on proliferation as determined by EdU incorporation. (D) A498 cells were transiently transfected with the

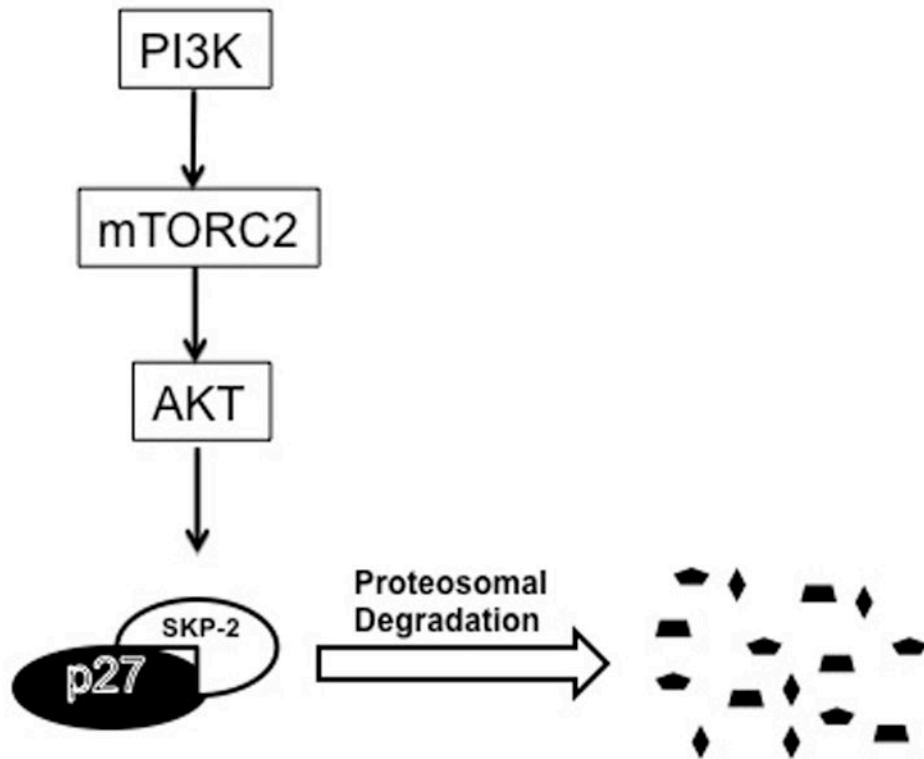
indicated siRNA. Upper panel-Cells were then analyzed for cell cycle distribution by flow cytometry. Data represent the mean  $\pm$  SE from 3 independent experiments. Lower panel-Cells were analyzed for proliferation based on EdU incorporation assay.

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**Figure 8.** PI3K regulates mTORC2 activity to alter the balance between SKP-2 and p27.