Rapamycin resistance is linked to defective regulation of Skp2

Hana Totary-Jain¹, Despina Sanoudou³, Cula N. Dautriche¹, Hillary Schneller¹, Lester Zambrana¹, and Andrew R. Marks¹²

¹Department of Physiology and Cellular Biophysics, the Clyde and Helen Wu Center for Molecular Cardiology, ²Department of Medicine, Columbia University College of Physicians & Surgeons, New York, NY 10032; ³Department of Pharmacology, Medical School, University of Athens, Greece and Molecular Biology Division, Biomedical Research Foundation, Academy of Athens, Soranou Efesiou 4, Athens, 115 27, Greece

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Corresponding Authors:
Andrew R. Marks: 1150 St. Nicholas Ave., NY, NY 10032. Tel. 212 851-5340, Fax. 212 851-5345, Email: arm42@columbia.edu
Hana Totary-Jain: 1150 St. Nicholas Ave., NY, NY 10032. Tel. 212 851-5348, Fax. 212 851-5345, Email: ht2167@columbia.edu

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Abstract

The mammalian target of rapamycin (mTOR) plays a role in controlling malignant cellular growth. mTOR inhibitors including rapamycin (sirolimus) are currently being evaluated in cancer trials. However, a significant number of tumors are rapamycin resistant. In this study, we report that the ability of rapamycin to down-regulate Skp2, a subunit of the ubiquitin protein ligase complex, identifies tumors that are sensitive to rapamycin. RNAi-mediated silencing of Skp2 in human tumor cells increased their sensitivity to rapamycin in vitro and inhibited the growth of tumor xenografts in vivo. Our findings suggest that Skp2 levels are a key determinant of antitumor responses to mTOR inhibitors, highlighting a potentially important pharmacogenomic marker to predict sensitivity to rapamycin as well as Skp2 silencing strategies for therapeutic purposes.
Introduction

Dysregulated cell cycle progression is a hallmark of cancer. Cell cycle progression is normally regulated by the ubiquitin-mediated proteolysis of key regulatory proteins including the cyclins and cyclin-dependent kinase (CDK) inhibitors (1). The anaphase-promoting complex (APC) and the Skp1-Cul1-F-box (SCF) complex are the two main classes of E3 ubiquitin ligases. SKP2 is an F-box protein that constitutes the substrate recognition subunit of the SCFSKP2 ubiquitin ligase complex, and is the rate-limiting component required for the turnover of proteins involved in cell cycle control such as the CDK inhibitor p27 (2). Skp2 is considered to be a proto-oncogene as Skp2 over expression causes increased proliferation, invasion and inhibits apoptosis (3-5). Many clinical studies have indicated that up-regulation of Skp2 and loss of p27 are common in malignant tumors and are associated with poor prognosis (6).

The mechanisms that regulate Skp2 protein levels remain controversial. Meloche et al showed that phosphorylation of Skp2 on Ser-64 and Ser-72 increases its stability (7). Moreover, Lin et al. and Gao et al. observed that Akt-mediated phosphorylation of Skp2 at Ser-72 impairs its degradation and relocalizes it to the cytosol contributing to its oncogenic function (8, 9). More recently, conflicting reports from Boutonnet, et al. and Bashir, et al. showed that phosphorylation of Skp2 on Ser-72 does not control Skp2 binding to Skp1 and Cul1, has no influence on SCF (Skp2) ubiquitin ligase activity, and does not affect its subcellular localization (10, 11), but do not dispute its phosphorylation by Akt at S72. Interestingly, it has been shown that Akt phosphorylates CDK2 regulating its cellular localization, which is required for cell cycle progression (12). At the transcriptional level, the PI3K/Akt pathway regulates Skp2 through E2F1 binding to its promoter (13, 14). This finding implies a positive feedback loop comprised of Skp2, p27, cyclinE-CDK2 and Rb-
E2F termed the skp2 auto-induction loop that promotes progression through the restriction point (15, 16).

The serine/threonine kinase mTOR plays a central role in controlling cell growth (17). mTOR exists in two complexes. mTOR complex 1 (mTORC1) regulates protein translation via modulation of S6K1 and 4EBP1, and mTOR complex 2 (mTORC2) regulates cell survival via phosphorylation of Akt on Ser-473, which is necessary for full activation of Akt, along with PDK1 phosphorylation of Thr308 (18). The immunosuppressant drug rapamycin, when bound to the FK506 binding protein 12 (FKBP12), inhibits the kinase activity of mTORC1 and significantly reduces the kinase activity of the cdk4/cyclin D and cdk2/cyclinE complexes by increasing the CDK inhibitor p27 leading to cell cycle arrest at G1 (19, 20). The FKBP12-rapamycin complex does not bind to mTORC2, however prolonged rapamycin treatment suppresses the assembly of mTORC2 and its ability to activate Akt in a cell-type specific manner (21). In many cell lines, active mTORC1 suppresses PI3K activity by a negative feedback loop mediated by S6K1. Inhibition of mTORC1 release this inhibitory feedback loop resulting in increased Akt activity (22).

Since mTOR activation is common in cancers, we investigated the role of Skp2 in the response of tumor cells to the mTOR inhibitor, rapamycin. Here we show that Skp2 is down-regulated upon rapamycin treatment in rapamycin sensitive human tumor cells, whereas Skp2 expression was not affected in rapamycin resistant tumor cells. Moreover, when tumor xenografts derived from rapamycin resistant cells were treated with rapamycin, the tumor volume was significantly increased. Silencing Skp2 in rapamycin resistant tumor cells using shRNA was sufficient to increase the sensitivity to rapamycin and to inhibit the growth of tumor xenografts.
Materials and Methods

Materials

Reagents were obtained from the following sources: DMEM, RPMI and McCoy’s from Invitrogen. FK12 from ATCC and FBS from Atlanta Biologicals. Rapamycin from Biovision, LY294002 from VWR and NVP-BEZ235 was a gift from Dr. Deng Shi-Xian (Columbia University, NY, USA). Skp2-shRNA and negative control sh-RNA (scrambled sequence) vectors were obtained from SuperArray Bioscience Corporation. Antibodies to phospho-S473, phospho-T308 Akt/PKB, Akt/PKB (all three Akt/PKB-directed antibodies recognize the three known Akt/PKB isoforms) and GAPDH from Cell Signaling Technologies; skp2 from Santa Cruz; Phospho-Ser-64-Skp2 was kindly provided by Sylvain Meloche (University of Montreal, Canada) and anti-Flag from Sigma.

Cell Lines and Culture

Cell lines were obtained from the American Type Culture Collection (Authenticated and characterized by STR analysis, Karyotyping, COI testing and are contamination-free). Cells were cultured in the following media: BC3H1, RR1 and RR3 in DMEM with 20% FBS. U937, LNCap and T47D in RPMI with 10% FBS; HeLa in DMEM with 10% FBS; PC3 in Fk12 with 10% FBS; MDA-MB-231 in McCoy’s with 10%FBS; All of the cell lines were passaged less than 6 months and cultured at a density that allowed cell division throughout the course of the experiment.

Cell Lysis and Immunoblotting

Cells growing in 10 cm diameter dishes were rinsed once with cold PBS and lysed for 20
min in ice-cold buffer A (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors [Roche]) containing 0.3% CHAPS. After clearing of the lysates by centrifugation at 13,000 × g for 10 min, samples containing 50 μg of protein were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting using infrared-labeled secondary antibodies (1:10,000, LICOR Biosystems). Band intensities were quantified using the Odyssey Infrared Imaging System (LICOR Biosciences). For in vitro dephosphorylation assay, 50 mg cell lysates (which excluded phosphatase inhibitors) were incubated at 30°C for 2 hr with 200 U of λ phosphatase in phosphatase buffer.

Quantitative real time-PCR

Total RNA (0.5 μg), was used to synthesize cDNAs using the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (NE BioLabs) according to the manufacturer's specifications. Real-time PCR was carried out using a LightCycler 1.5 (Roche) with the LightCycler Taqman Master mix. For Skp2 we used universal probe# 50 with 5′-GGTGCTCTGGGTTTCTGAAT forward primer and 5′-AAGTCAAAGCACCAGGAGAGA reverse primer, for p27 universal probe # 62 with 5′-GAGCAGTGCTCCAGGGATGAG forward primer and 5′-TCTGTTGGCCCTTTTGTTTT reverse primer and for Gapdh universal probe # 9 with 5′-AGCTTGTCATCAACGGGAAG forward primer and 5′-TTTGATGTTAGTGGGCTCTCG reverse primer.

Expression vectors and stable transfection

The coding regions of human Skp2 were amplified by PCR using the forward primer 5′-
CTGAAGCTTATGCACAGGAAGCACCTCCAG and the reverse primer 5’-CTAGAATTCTCATAGACAACACTGGGCTTTTGCACTG that contain Hind III and EcoRI respectively, and cloned into p3XFLAG-CMV-10 (Sigma). The S64D and S72D mutations (DD-Skp2) were generated by Quikchage II XL site directed mutagenesis (Stratagene) using the following primers: S64D-forward 5’-GGGCCACCCGGAGGACCCCCACGGAAA. S64d-reverse 5’-TTTCCGTGGGGGGTCCTCCGGGTGGCCC. S72D-forward 5’-CCACGGAACGCTGAAAGGACAAAGGGAGTGACAAAGA and S72D-reverse 5’-TCTTTGTCACTCCCTTTGTCTTCAGCCGTTTCCGTGG. The myristoylated, constitutively active form of Akt (myr-Akt) was a gift from Dr. RA Roth (Addgene plasmid # 10841) and the S47D-Akt was a gift from Dr. J. Woodgett (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada). Vectors were transfected into the cells using Lipofectamine 2000 (Invitrogen) and selected with 0.8 mg/ml G418 (Invitrogen) or 5 μg/ml puromycin (Sigma-Aldrich).

Tumor Xenografts

PC3 cell lines stably expressing wild-type Skp2 or the empty vector, or HeLa cells stably transfected with Skp2-specific Sh-RNA or with empty vector were xenografted into 6-week-old immunodeficient mice (Ncr nu/nu mice; Harlan Laboratories). All animal studies were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. PC3 cells (3 X 10⁶) or HeLa cells (5 X 10⁶) were injected subcutaneously in the upper flank region of mice that had been anaesthetized with isoflurane. Mice were treated with rapamycin (10 mg/kg) or with NVP-BEZ235 (30 mg/kg in 10% NMP–90% PEG, given p.o. in 100 μL of volume) as described (23). At completion of all xenograft studies mice were sacrificed, the tumors were excised and tumor volumes estimated with
the following formula: \( \text{volume} = \frac{a^2 \times b}{2} \), where \( a \) = short and \( b \) = long tumor lengths, respectively, in millimeters.
Results and Discussion

Rapamycin resistance correlates with increased Skp2 and decrease in p27.

The potential role of Skp2 in the response of tumor cells to the mTOR inhibitor, rapamycin, was investigated by comparing Skp2 protein levels in a rapamycin sensitive murine cell line, BC3H1, derived from a mouse brain tumor, and two rapamycin resistant BC3H1 clonal cell lines (RR1 and RR3) developed in our laboratory (24). Rapamycin inhibited cell growth in the BC3H1 cells but not in RR1 or RR3 cells (Fig. 1A). Skp2 protein and mRNA levels were low in rapamycin sensitive BC3H1 cells, and rapamycin treatment or serum starvation resulted in a further reduction (Fig. 1B, C). In contrast, in RR1 and RR3 cells, Skp2 protein and mRNA levels were elevated more than 3-fold compared to the parental BC3H1 cells, and were unchanged by rapamycin treatment or serum starvation (Fig. 1B, C). Moreover, Skp2 protein levels were inversely correlated with p27 protein levels (Fig. 1B, C). These data are consistent with previous reports of decreased p27 levels in RR cells (24) and may suggest that low levels of p27 protein might be due to increased Skp2 expression. The increase in Skp2 at the transcriptional level is consistent with previous data showing persistent Rb hyperphosphorylation in RR cells (24), releasing the transcription factor E2F (13). In addition, down-regulation of p27, which activates CDK2 in RR cells, may increase the stability of Skp2 at the protein level by phosphorylating it at the conserved S64 site (7).

Rapamycin resistance is associated with defective regulation of Skp2 in human tumor cell lines

We next tested whether Skp2 and p27 protein levels correlated with the response of six different human tumor cell lines to rapamycin. Cells that were resistant to rapamycin
(HeLa, MDA-MB-231, LNcaP) (Fig. 2A) expressed high levels of Skp2 (both protein and mRNA) that was not affected by rapamycin treatment (Fig. 2B, C). Conversely, rapamycin sensitive cells (U937, T47D, PC3) (Fig. 2A) exhibited ~2-fold decrease in Skp2 protein levels and ~1.5-fold decrease in Skp2 mRNA levels upon rapamycin treatment (Fig. 2B, C). Notably, p27 and Skp2 protein levels were inversely correlated (Fig. 2B). These data agree with previous reports that low p27 and high Skp2 levels are associated with more aggressive tumors (25), and indicate that impaired down-regulation of Skp2 by rapamycin correlates with rapamycin resistance.

**Dephosphorylation of Skp2 and Akt correlates with human tumor cell line sensitivity to rapamycin**

In addition to the transcriptional dysregulation of Skp2 in rapamycin resistant cells, aberrant regulation of Skp2 protein stability may also play a critical role in determining rapamycin sensitivity. Phosphorylation of Skp2 at S64 and S72 by CDK2 and Akt respectively were shown to increase Skp2 stability by interfering with its association with Cdh1 (7-9).

Since prolonged rapamycin treatment (24 hr) inhibited the phosphorylation and activation of Akt in certain tumor cells (21), we hypothesized that cell lines with rapamycin sensitive Akt phosphorylation would have low amounts of Skp2 after rapamycin treatment. Indeed, cell lines with rapamycin sensitive Akt phosphorylation (U937, PC3, T47D) exhibited decreased Skp2 protein levels upon rapamycin treatment (Fig. 3A). In contrast, cell lines with rapamycin insensitive Akt phosphorylation (LNCaP, HeLa, MDA-MB-231) showed no change in Skp2 levels upon rapamycin treatment (Fig. 3A).

To test the effect of Akt on Skp2 expression, we treated the same tumor cell lines
with the PI3K inhibitor LY294002, or the dual inhibitor of PI3K and mTOR, NVP-BEZ235 (26). LNCaP, HeLa and MDA-MB-231 cells treated with LY294002 or NVP-BEZ235 exhibited inhibition of Ser-473-Akt phosphorylation and ~2-fold decrease in Skp2 expression (Fig. 3B, C). Moreover, the decrease in Skp2 expression correlated with the increased sensitivity of cells to LY294002 or NVP-BEZ235 (Fig. 3D). In addition, treatment of these cells with the dual PI3K and mTOR inhibitor increased the expression of p27 (Fig. 3C). These data show a direct correlation between the degree of Akt phosphorylation, Skp2 protein levels, and the cellular response to rapamyicin, LY294002 and to the dual inhibitor NVP-BEZ235. These data are supported by Sarbassov, et al. who showed that tumor xenografts of PC3 cells stably transfected with S473D-Akt but not wild-type Akt were resistant to rapamycin induced decrease in tumor size (21).

**Down-regulation of Skp2 by rapamycin is phosphorylation-dependent.**

Recent reports have suggested that phosphorylation of Skp2 at Ser-64 and/or Ser-72 by CDK2 and/or Akt, increases its stability (7-9). Since both CDK and Akt are downstream of mTOR, we tested whether the down-regulation of Skp2 in the rapamycin sensitive cells is phosphorylation-dependent.

We therefore replaced both Ser-64 and Ser-72 with the phosphomimetic amino acid aspartic acid using a flag-tagged Skp2 expressing plasmid (DD-Skp2). Upon rapamycin treatment, the rapamycin sensitive cells (PC3, T47D) down-regulated the endogenous Skp2 and the WT-Flag-Skp2 but not the DD-Flag-Skp2 (Fig. 4A, B). The down-regulation of Skp2 correlated with the rapamycin sensitive decrease in Akt phosphorylation. Interestingly, rapamycin resistant cells (HeLa), showed no change in the expression levels of the endogenous Skp2, the WT-Flag-Skp2 or the DD-Flag-Skp2 upon rapamycin treatment, and correlated with the rapamycin insensitive Akt phosphorylation (Fig. 4C).
These data demonstrate that dephosphorylation of Skp2 at Ser-64 and/or Ser-72 is critical for Skp2 down-regulation by rapamycin.

**Skp2 determines tumor xenograft sensitivity to rapamycin.**

We further investigated the biological significance of Skp2 expression on the cellular response to rapamycin, by ectopically over expressing WT-Flag-Skp2 in rapamycin sensitive cells (PC3). Stably transfected PC3 cells with WT-Flag-Skp2 exhibited reduced sensitivity to rapamycin (Fig. 5A, B). To determine the role of Skp2 in the response of tumors to rapamycin in vivo, control or WT-Flag-Skp2 stably transfected PC3 cells were xenografted onto nude mice. Rapamycin inhibited the growth of tumors derived from control PC3 cells by 85%, whereas tumors derived from WT-Flag-Skp2 over expressing PC3 cells were relatively resistant to rapamycin exhibiting less than 50% reduction in tumor growth (Fig. 5C, D).

Silencing the endogenous Skp2 using Skp2-specific shRNA in rapamycin resistant cells (HeLa) increased the expression of p27 compared to control cells by >2-fold (Fig. 6A). When treated with rapamycin, Sh-Skp2 transfected cells showed a further increase in the expression of p27 while control cells showed a slight decrease (Fig. 6A). Moreover, HeLa cells transfected with Sh-Skp2 had a reduced proliferation rate and exhibited increased sensitivity to rapamycin (Fig. 6B, C). These data are in agreement with previous reports showing that mouse embryonic fibroblasts (MEFs) derived from Skp2<sup>−/−</sup> embryos and other organs from Skp2<sup>−/−</sup> mice exhibited increased levels of p27 protein, indicating that Skp2 regulates p27 protein degradation. Moreover, Skp2<sup>−/−</sup> MEFs proliferate slowly in culture (27). Notably, tumor xenografts of Hela cells stably transfected with Sh-control vector exhibited a 2-fold increase in tumor size upon rapamycin treatment (Fig. 6D). These
data are in agreement with reports of acceleration in tumor growth after the use of mTOR inhibitors, which may be due to the release of the negative feedback loop by rapamycin (28).

Remarkably, tumor xenografts of HeLa cells stably transfected with Skp2-shRNA (designed to mimic the low Skp2 expression and low phospho-Ser-473-Akt response of sensitive cells to rapamycin) exhibited >90% inhibition of tumor growth (Fig. 6D). However, when tumor xenografts of HeLa cells transfected with Skp2-shRNA were treated with rapamycin, which increased phospho-Ser-473-Akt, tumor growth was increased by ~4-fold compared to non treated HeLa cells transfected with Skp2-shRNA (Fig. 6D). We used the dual PI3K and mTOR inhibitor to test whether the detrimental effect of rapamycin observed in these tumors is due to rapamycin insensitive Akt. HeLa cells transfected with Skp2-shRNA or with Sh-control exhibited ~80% inhibition of tumor growth compared to non-treated HeLa cells transfected with Sh-control upon treatment with the dual PI3K and mTOR inhibitor NVP-BEZ235.

The existence of the negative feedback loop downstream of mTORC1 may contribute to rapamycin resistance observed in HeLa cells. Therefore, when the dual PI3K and mTOR inhibitor was used, it exhibited greater antiproliferative effect and reduction in tumor volume compared to that of rapamycin (Fig. 3D and Fig. 6D). Moreover, these data show that targeting Skp2 alone is sufficient to inhibit the growth of a tumor derived from rapamycin insensitive Akt in mouse xenografts. However, the combination of rapamycin with Skp2 inhibitors would not have a beneficial effect in tumors that have rapamycin insensitive Akt phosphorylation.

Our data are in agreement with recent reports showing that the combined knockout of Skp2 and Pten in mice inhibits the tumor formation which was observed in Pten
knockout mice (29), although tumors with PTEN loss are particularly susceptible to mTOR inhibitors (30). However, PTEN loss alone is neither necessary nor sufficient to result in rapamycin sensitive regulation of Akt phosphorylation (21). In fact, in the present study we chose two prostate cancer cell lines that do not express PTEN, the PC3 cells are sensitive to rapamycin while LNCaP are resistant, and of the two breast cancer cell lines, both of which express PTEN, the T47D cells were sensitive while MDA-MB-231 cells were resistant.

The present study examined whether Skp2 levels can be used to determine the response of tumors to mTOR inhibitors. We propose that rapamycin treatment is effective primarily in tumors that exhibit rapamycin sensitive dephosphorylation of Akt at Ser-473 and consequently decreased Skp2 expression. Upon validation in the clinical setting, Skp2 expression levels could serve as a marker for predicting the response of tumors to mTOR inhibitors. For tumors resistant to mTOR inhibitors in which rapamycin fails to inhibit or increases Ser-473 phosphorylation of Akt, targeting Skp2 might be a therapeutic alternative.
Acknowledgements

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Author Contributions

H.T.J. and A.R.M. conceived the project. H.T.J. designed the experiments. H.T.J., C.D., L.Z. and H.S. performed the experiments. Data were analyzed by H.T.J., D.S. and A.R.M. The manuscript was written by H.T.J. and edited by D.S. and A.R.M. The authors have no conflicts to declare. Correspondence and requests for materials should be addressed to H.T.J. and A.R.M.
References


Figures Legends

Figure 1 Rapamycin resistance correlates with increased Skp2 and decreased p27.

(A) BC3H1 cells were plated in 24 well plates (10^4 cells/well) and treated with 100 nM rapamycin or RR1 or RR3 grown without rapamycin for 1 week were treated with 1 µM or 100 nM rapamycin respectively. Cell counts were determined after 6 days incubation. Data shown are mean + SD (n=3), * compared to DMSO treated cells (p<0.01). (B) Representative immunoblots for Skp2, p27 and GAPDH and quantification bar graph for Skp2 normalized to GAPDH from lysates from the indicated cells treated with DMSO or with 100 nM rapamycin (Rap) or serum starved (Ser) for 24 hrs. Results presented as mean + S.D. (n=3), *compared to DMSO treated cells (p<0.01). (C) RT-PCR using total RNA extracted from BC3H1, RR1 and RR3 cells treated with DMSO or with 100 nM rapamycin (Rap) for 24 hrs. Data shown are mean + SD (n=3), * compared to DMSO treated cells (p<0.01).

Figure 2 Impaired down-regulation of Skp2 by rapamycin correlates with rapamycin resistance.

(A) Indicated cells were plated (10^4 cells/well) in 24 well, treated with DMSO, 10 nM or 100 nM rapamycin for 72 hr followed by cell counts. Results presented as mean + S.D. from a representative experiment performed in triplicate. *compared to DMSO treated cells (p<0.01). (B) Representative immunoblots for Skp2, p27 and GAPDH and quantification bar graph for Skp2 normalized to GAPDH from lysates from the indicated cells treated with DMSO or with 100 nM rapamycin (Rap) or serum starved (Ser) for 24 hrs. Results presented as mean + S.D. (n-3), *compared to DMSO treated cells (p<0.01). (C) RT-PCR using total RNA extracted from the indicated cells treated with DMSO or with 100 nM
rapamycin (Rap) for 24 hrs. Data shown are mean + SD (n=3), * compared to DMSO treated cells (p<0.01).

**Figure 3 Rapamycin sensitivity correlates with down-regulation of Skp2 and Akt phosphorylation (A-C)** Representative immunoblots and quantification bar graph for the indicated proteins and phosphorylation states from the indicated cells lines treated with DMSO, 100 nM rapamycin (Rap), 20 μM Ly 294002 (Ly) or 100 nM NBP-EZ235 for 24 hr. (D) Indicated cells were plated (10^4 cells/well) in 24 well plates, treated as in (A-C) for 72 hr followed by cell counts. Results presented as mean + S.D. from a representative experiment performed in triplicate. *compared to DMSO treated cells (p<0.01).

**Figure 4 Down-regulation of Skp2 by rapamycin is phosphorylation-dependent.** (A-C) Indicated cells stably expressing empty vector (EV), wild-type Flag-Skp2 (WT) or DD Flag-Skp2 (DD) were treated with DMSO or 100 nM rapamycin (Rap) for 24 hr, and cell lysates were analyzed by immunoblotting for the indicated proteins and phosphorylation states. Bar graphs indicate the quantification of the levels of endogenous Skp2 (Endo) and the Flag-Skp2. Data are presented as means S.D (n=3). *compared to DMSO treated cells (p<0.01).

**Figure 5 Overexpression of Skp2 increases cellular resistance to rapamycin.** (A) Representative immunoblots for indicated proteins from PC3 cells stably expressing empty vector (EV) or wild-type Flag-Skp2 (WT) treated with DMSO or with 100 nM rapamycin for 24 hr. (B) PC3 cells described in (A) were plated (10^4 cells/well) in 24 well plates, treated with DMSO or rapamycin as indicated for 72 hr followed by cell counts.
Results presented as mean + S.D. (n=3), * and # compared to DMSO treated cells or rapamycin treated cells respectively (p<0.01). (C, D) Mice were injected with 3 x 10^6 PC3 cells described in (A) and were allowed to grow to at least 50 mm^3 in size and then treated with rapamycin or vehicle for 4 executive days. After 14 days, the volumes of tumor xenografts were determined. Results presented as mean + S.D. for tumor volume. * compared to the percentage of vehicle-treated mice (n = 6 per condition, p< 0.01).

Figure 6 Silencing of Skp2 increases cellular sensitivity to rapamycin and inhibits tumor growth.

(A) Representative immunoblots for indicated proteins from HeLa cells stably expressing empty vector (EV) or Skp2-Sh-RNA (Sh- Skp2) treated with DMSO or with 100 nM rapamycin for 24 hr. (B) HeLa cells as described in (A) were plated (10^4 cells/well) in 24 well plates. Cells were counted at the indicated time. Results are presented as the mean ± S.D, * compared to DMSO treated cells (p<0.01), (C) HeLa cells described in (A) were plated as in Fig 5B. Results presented as mean ± S.D (n=3), * and # compared to DMSO treated cells or rapamycin treated cells respectively (p<0.01). (D) Mice were injected with 5 x 10^6 HeLa cells as described in (A), at the same time and for an additional 5 days post-injection of cells, mice were treated with rapamycin or with NVP-BEZ235, and sacrificed 11 days after xenografting of cells. Results are presented as the mean ± S.D. for tumor volume. * and ** compared to the percentage of vehicle-treated mice (n = 6 per condition, *p< 0.01, ** p< 0.05).
Figure 1

A

\[
\begin{align*}
\text{BC3H1} & \quad \text{RR1} & \quad \text{RR3} \\
\text{DMSO} & \quad \text{Rapamycin} \\
\%	ext{Cell number} & \quad & \\
0 & \quad 50 & \quad 100 \\
\end{align*}
\]


B

\[
\begin{array}{ccc}
\text{BC3H1} & \text{RR1} & \text{RR3} \\
\text{Rap:} & - & + & - & - & + \\
\text{Ser:} & + & - & + & - & + \\
\text{27 kDa} & \text{p27} & \text{Skp2} & \text{GAPDH} \\
\text{45 kDa} & & & \\
\text{37 kDa} & & & \\
\end{array}
\]

C

\[
\begin{align*}
\text{mRNA levels (AU)} & \quad \text{Relative Skp2 expression (AU)} \\
\text{p27} & \quad & \text{BC3H1} \\
\text{Skp2} & \quad & \text{RR1} & \quad \text{RR3} \\
\text{BC3H1} & \quad & + & \quad - & \quad - \\
\text{BC3H1+Rap} & \quad & + & \quad + & \quad - \\
\text{RR1+Rap} & \quad & - & \quad + & \quad + \\
\text{RR3+Rap} & \quad & - & \quad - & \quad + \\
\end{align*}
\]
Figure 2

A

% Cell number

DMSO 10 nM Rap 100 nM Rap

U937 Hela T47D MDA-MB 231 PC3 LNCap

B

Relative Skp2 expression (AU)

DMSO 100 nM Rap

U937 Hela T47D MDA-MB 231 PC3 LNCap

C

Skp2 mRNA levels (AU)

DMSO 100 nM Rap

U937 Hela T47D MDA-MB 231 PC3 LNCap
Figure 4

A

B

C

EGF2 Expression
(arbitrary units)

0.0 0.5 1.0

EV FLAG-WT-SKP2 FLAG-DD-SKP2

EGF2 Expression
(arbitrary units)

0.0 0.5 1.0

EV FLAG-DD-SKP2

EGF2 Expression
(arbitrary units)

0.0 0.5 1.0

EV FLAG-DD-SKP2
Figure 6

A

B

C

D

Graph A shows a western blot analysis with bands for various molecular weights (60 kDa, 45 kDa, 37 kDa, 27 kDa) labeled as Sh-C, pS473-Akt, Akt, Skp2, and GAPDH. The bands are compared for Sh-C and Sh-Skp2 conditions.

Graph B depicts the cell number over time (days) with Sh-Control and Sh-Skp2 conditions, showing an increase in cell number over time.

Graph C shows a bar graph for Cell number (%) with different Rapamycin concentrations (0, 0.01, 0.1, 1, 10, 100 nM) for Sh-C and Sh-Skp2 conditions.

Graph D illustrates the % Tumor Volume for sh-control and sh-Skp2 conditions with treatments of DMSO, Rapamycin, and NVP-BEZ235.