**Supplemental Information**

# Inhibition of mTORC1 Kinase Activates Smads 1 and 5 but not Smad8 in Human Prostate Cancer Cells, Mediating Cytostatic Response to Rapamycin

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## Experimental Procedures

**Cell viability assay.** Cell viability was assessed by Trypan Blue exclusion under phase-contrast microscopy as before [1]. Cells were plated in 12-well dishes in low serum conditions at a density of 30,000-50,000 cells/1ml/well as previously described [2, 3]. In brief, cells were pre-treated with rapamycin (200 nmol/L) or vehicle control 2 h prior to addition of BMP4 (5 ng/ml) or control treatment for 24-72 h. Cells were detached via trypsinization, neutralized with DMEM/F12 + 5% FBS, spun at 500g for 5 min and cell pellet was resuspended in 100 µL DMEM/F12 + 5% FBS. Ten µl cell suspensions were combined with 10 µl of Trypan Blue dye solution and examined with a hemocytometer. Viable cells (dye excluded) were counted from a total of 300 cells examined.

**Hoechst 33258 staining.**. Briefly, cells were plated in 6 well dishes in at a density of 3-5x$10^4$ cells/well in 2 ml of DMEM/F12, 1% FBS, 15 mM HEPES (pH 7.4) (for LNCaP, C4-2, C4-2B, PC3, and DU145) or in GM3.1 (for NRP-152, NRP-154, and DP-153). Cells were treated with vehicle or rapamycin (200 nmol/L) 2 h prior to BMP4 (5 ng/ml) addition. After 24 h cells were

stained with 10 μg/ml Hoechst 33258 (Sigma) and apoptotic cells at each time point were enumerated by fluorescent microscopy. Three hundred cells were analyzed in triplicate [4].

**Flow Cytometry.** Cells were treated as previously described [5].

**Cell number assay.** 3-5 x $10^4$ cells/ml were seeded in 12-well dishes in medium described in Hoechst staining assay. The following day, cells were pre-treated ± rapamycin (200 nmol/L) 2 h prior to ± BMP4 (5 ng/ml) treatment for up to 72 h. Adherent cells were detached by trypsinization and enumerated with a Coulter Electronics counter.

**PCR primers.** The PCR primers were purchased from Operon Biotechnologies, Inc. A full description of all primers used was described previously [5].

**MTT Assay.** Cell viability was also assessed by use of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) which is cleaved at the tetrazolium ring by mitochondrial dehydrogenases, yielding purple insoluble formazan crystals**.** Cells were seeded in 96-well plates at 0.5-1 x $10^4$ cells/well in 100 μl of culture media. Next day, cells were treated with 0-1000 nM rapamycin or DMSO control for 72 h. Cultures were then treated with 10 μl of 5 mg/ml MTT (Sigma-Aldrich), incubated at 37°C for 3–4 hours in a humidified CO2 incubator, and colored product was solubilized with 100 μl of isoproponal-0.1 N HCl. Colored product was quantified within 1 h by absorbance at 570 nm – 690 nm (background subtraction) with a Tecan-mini spectrophotometer.

**Retrovirus.** Smad1, Smad5, and Smad8 with Flag tag was PCR amplified and subcloned into the proretroviral construct pLPCX (BD Biosciences Clontech, Palo Alto, California, USA). Replication-defective infectious retroviruses were generated by transfecting the retroviral plasmids into A-BOSC cells as previously described [6]. Exponentially growing LNCaP or PC3 cells were transduced with pLPCX control retrovirus or pLPCX-Smad1, -Smad5, or –Smad8 and were maintained under puromyocin selection (2 μg/ml) for one week. pLPCx-control/-Smad1/-Smad5/-Smad8 viral preparations to deliver desired functional protein was evaluated by

overnight infection (followed by 48 h recovery in DMEM/F12 medium containing 5% FBS) in either LNCaP or PC3 cells.

**Lentivirus.** The generation of stable shRNA expression lentiviruses harboring shLacZ, shSmad1, shSmad5, shSmad1/5, shSmad8 were used in LNCaP or PC3 cells (to drive LNCaP-shRNA or PC3-shRNA cells). The targeting sequences for these shRNA constructs were: shLacZ 5'-GTGACCAGCGAATACCTGT-3'[7], shSmad1#1: 5'-GGAAACAGGGCGATGAAGA -3', shSmad1#2: 5'-CGGAATTCCACTATTGAAA-3', shSmad5#1: 5'-GAGCTAAAGCCGTTGGATA -3', shSmad5 #2: 5'-TCAGATGGGTCAAGATAAT-3', shSmad8#1: 5'-GCAAGGAGATGAAGAGGAA-3', shSmad8#2: 5'-GCTTTGAAGTCGTGTATGA -3' (Designed by Dharmacon RNAi Technology and oligomers were purchased from Integrated DNA Technologies, Coralville IA).

**Real Time quantitative reverse transcriptase-PCR (RT-qPCR).** cDNA was designed as described previously [5] using 1 μg of total RNA. Predesigned gene specific probe sets for *Id1*were made for Taqman Gene Expression Assay (Applied Biosystems, Inc. Foster City, CA) .. RT-PCR was performed as specified in the manufacturer's protocol of the 7500 Fast Real Time PCR System (Applied Biosystems, Inc., Foster City, CA).

**Immunohistochemistry and Tissue Microarray (TMA).** PR8011 tissue microarray (TMA) included 22 cases/cores of patients with prostate adenocarcinoma, including TNM, and pathology grade. Slides were processed using IHC standard protocol as described [5]. Sections were either stained with anti-phospho-Smad-1,5,8 antibody (Cell signaling Inc., Danvers, MA, Cat.#9511) at (1:100) dilution, Phospho-S6 Ribosomal Protein (Ser 235/236) antibody (Cell signaling Inc., Danvers, MA, Cat #2211) at (1:100) dilution, survivin (sc-10811) at (1:400) or normal rabbit IgG used as a negative control. All slides were reviewed independently by two investigators (R.W and K.S) who were blinded to the clinical and pathological data and reconfirmed by a second set of evaluations by one investigator (D.S) blinded to the interpretations of the first set of evaluations, as well as clinical and pathological data. Immunostaining results were validated by the consensus of all reviewers.

**Figure Legend**

**Supplementary Figure S1. Rapamycin-mediated phospho-Smad1/5/8 and *Id1* luciferase activity is time and dose-dependent in mediating cell death of human and rat prostate epithelial cell lines (PC3 and NRP-152).** A, DU145 cells were treated with rapamycin (200 nmol/L, 24 h) either in low serum (1% FBS) or higher serum (5% FBS); cells cultured in high serum were infected with control adenovirus for 24 h prior to addition of rapamycin and a analyzed for expression of phospho-Smad1/5/8. B, PC3 cells were treated with 200 nmol/L rapamycin or vehicle control for 0-72 h prior to Western blot analysis of phospho-Smad1/5/8 expression. C, NRP-152 cells were treated as described above prior to examining phospho-Smad1/5/8 expression by western blot analysis (left), or treated ± rapamycin (200 nmol/L) for a period of 5 days, and adherent cells were enumerated with a Coulter counter (right). D) NRP-152 cells were co-transfected with *Id1* promoter as described previously for 24 h prior to dose response (0-1000nmol/L) of rapamycin for 24 h (left) or a rapamycin (200 nmol/L) time-course for 0-72 h (right), and luciferase activity was measured as indicated. *Columns,* average of triplicate determinations; *bar*, ±S.E. Data is representative of three independent experiments.

**Supplementary Figure S2. Rapamycin-mediated cell death is enhanced in the presence of BMP4 as examined in human and rat prostate epithelial cell lines.** A, PC3 cells were treated with rapamycin (200nmol/L) vehicle control 2 h prior to treating the cells with BMP4 (5 ng/ml) for an additional 5 days, and cells were enumerated with a Coulter counter. B, NRP-152 cells were co-transfected with *Id1*-luciferase as described above for 24 h then treated with rapamycin (200 nmol/L) or vehicle 24 h to ±BMP4 (5 ng/ml) for an additional 4 h, and luciferase activity was then measured. C. LNCaP, NRP-152, and PC3 cells were treated with rapamycin (200nmol/L) or vehicle control 2 h prior to treating the cells with BMP4 (5 ng/ml) for 72 h prior to harvesting the cells and total adherent cells were enumerated with a Coulter counter. D, NRP-152 and PC3 cells were treated with rapamycin (200 nmol/L) or vehicle 2 h prior to ±BMP4 (5 ng/ml) for 72 h, stained with Hoechst dye, and examined under fluorescence and phase contrast microscopy at 200X. *Columns,* average of triplicate determinations; *bar*, ±S.E. Data is representative of three independent experiments.

**Supplementary Figure S3. mTOR inhibition in the presence of BMP4 enhance apoptosis in PC3 cells.** A, PC3 cells were treated ± rapamycin (200nmol/L) 2 h prior to treating cells with BMP4 (5 ng/ml) for an period of 0-72 h and cell fractions in either Sub G1 (apoptotic fraction), G1, or G2/M transition as examined by flow cytometry.

**Supplemental Figure S4. Silencing Smad1, Smad5, and/or Smad1/5 cells enhance rapamycin-mediated cell death.** A & B, Smads1, Smad5, Smad1/5 and Smad8 were stably silenced in both PC3 (A) and LNCaP cells (B) and treated with rapamycin (200 nmol/L) for 24 h prior to examining phospho-Smad1/5/8 and/or total Smads expression by western blot.

**Supplemental Figure S5. Silencing Smad1, Smad5, and/or Smad1/5 cells enhance rapamycin-mediated cell death.** LNCaP-shLacZ, LNCaP-shSmad1, LNCaP-shSmad5, and LNCaP-shSmad8 co-transfected with *Id1* promoter 24 h prior to rapamycin (200 nmol/L) treatment and BMP4 (5 ng/ml) was added 4 h before measuring luciferase activity. *Columns*, average of triplicate determinants; *bar*, ±S.E and experiments were run in triplicates.

**Supplemental Figure S6. Overexpression of Smad1 and Smad5 enhance rapamycin-mediated Smad activation, *Id1* promoter activity and cell death in PC3 cells.** A, PC3-pLPCX-control, -Smad-1,-5 or-8 cells were treated with rapamycin (200 nmol/L) for 24 h and examined by western blot analysis. B, Stably overexpressing PC3-Smad1, -Smad5, and/or -Smad8 were subjected to *Id1* promoter activity following treatment of cells with rapamycin (200 nmol/L) for 24 h. C, LNCaP-pLPCX-control or Smad1,-Smad5, and/or -Smad8 stably overexpressing cells were treated rapamycin (200 nmol/L) for 24 h prior to examining luciferase activity. *Columns*, average of triplicate determinants; *bar*, ±S.E and experiments were run in triplicates.

**Supplemental Figure S7. Rapamycin-induced *Id1* promoter activity and expression requires BMPRI.** A & B, LNCaP and NRP-152 cells were treated with various concentrations of LDN-193189 (nmol/L) 2 h prior to treatment with rapamycin (200 nmol/L, 3 days) to reverse rapamycin-mediated cell death. C, Stably silenced LNCaP-shGFP or LNCaP-sh*Id1* were treated with rapamycin (200 nmol/L) 2 h prior to BMP4 (5 ng/ml) stimulation for 24 h and *Id1*

expression was analyzed by western blot. Data are representative of three independent experiments.

**Supplementary Figure S8. Silencing *Id1* enhanced rapamycin-mediated cell death in LNCaP cells.** A, LNCaP-shGFP or LNCaP-sh*Id1*#1 cells were treated with rapamycin (200 nmol/L) or vehicle 2 h prior to BMP4 (5 ng/ml) stimulation for an additional 70 h where morphological alterations of cells were examined by phase contrast microscope at 200x.

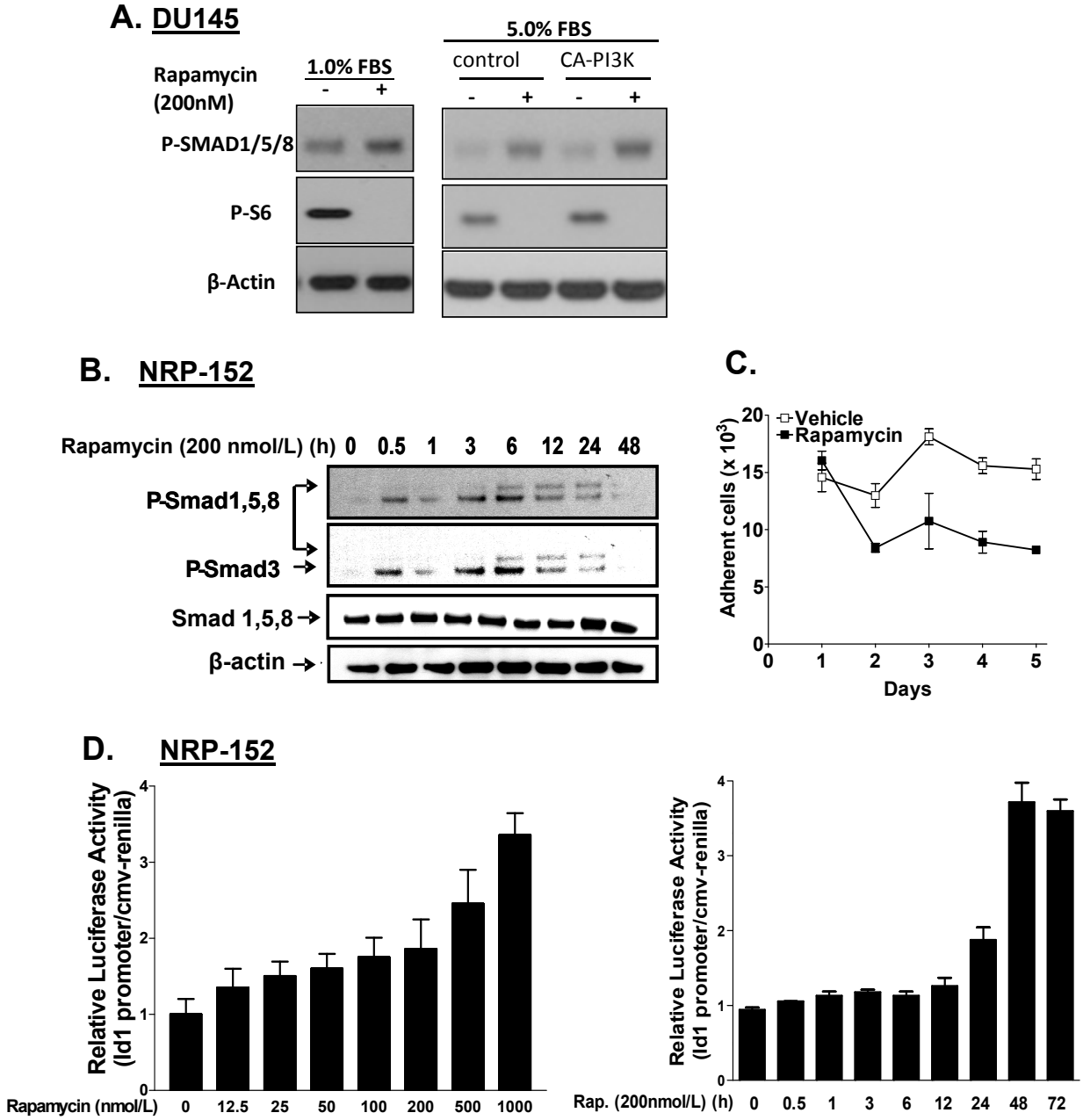
**Supplementary Figure S9. Smad1/5/8 expression is enhanced in RAD001 treatment compared to non treated controls *in vivo.*** A, Immunostaining of normal prostate gland and prostate adenocarcinoma (stage III) stained with H&E, phospho-Smad1/5/8, PTEN, or phospho-S6. B&C, Randomized phase II study of two different does of RAD001 (Everolimus) (5mg or 10 mg) as neo-adjuvant therapy in patients with localized prostate cancer radical prostatectomy (n=6) were stained with phospho-Smad1/5/8 or phospho-S6 and analyzed at 200x.
A total of six patients were initially enrolled on this clinical trial. H-score analysis was used to examine the relative fold expression of phospho-Smad1/5/8 and phospho-S6 in the RAD001 trial section against a larger cohort (22 biopsy cores) of non-treated localized prostate adenocarcinoma stages II-III (from a human prostate tissue microarray {PR8011 series} obtained from US BioMax, Inc) (A, adenocarcinoma; H, hyperplasia). Examined individually (see above Panels), all patients responded to RAD001 (by loss of pS6 relative to untreated group). In this analysis, patients who demonstrated the greatest response exhibited highest elevation of phospho-Smad. This data supports that suppression of mTOR by RAD001 enhanced phospho-Smad1/5/8 expression.

**Supplementary Figure S10. Regulation of BMPRII and androgen-responsive gene expression by rapamycin.** LNCaP cells were treated ± rapamycin (200 nmol/L, 24 h) were analyzed for the expression of the indicated genes by semi-quantitative RT-PCR.

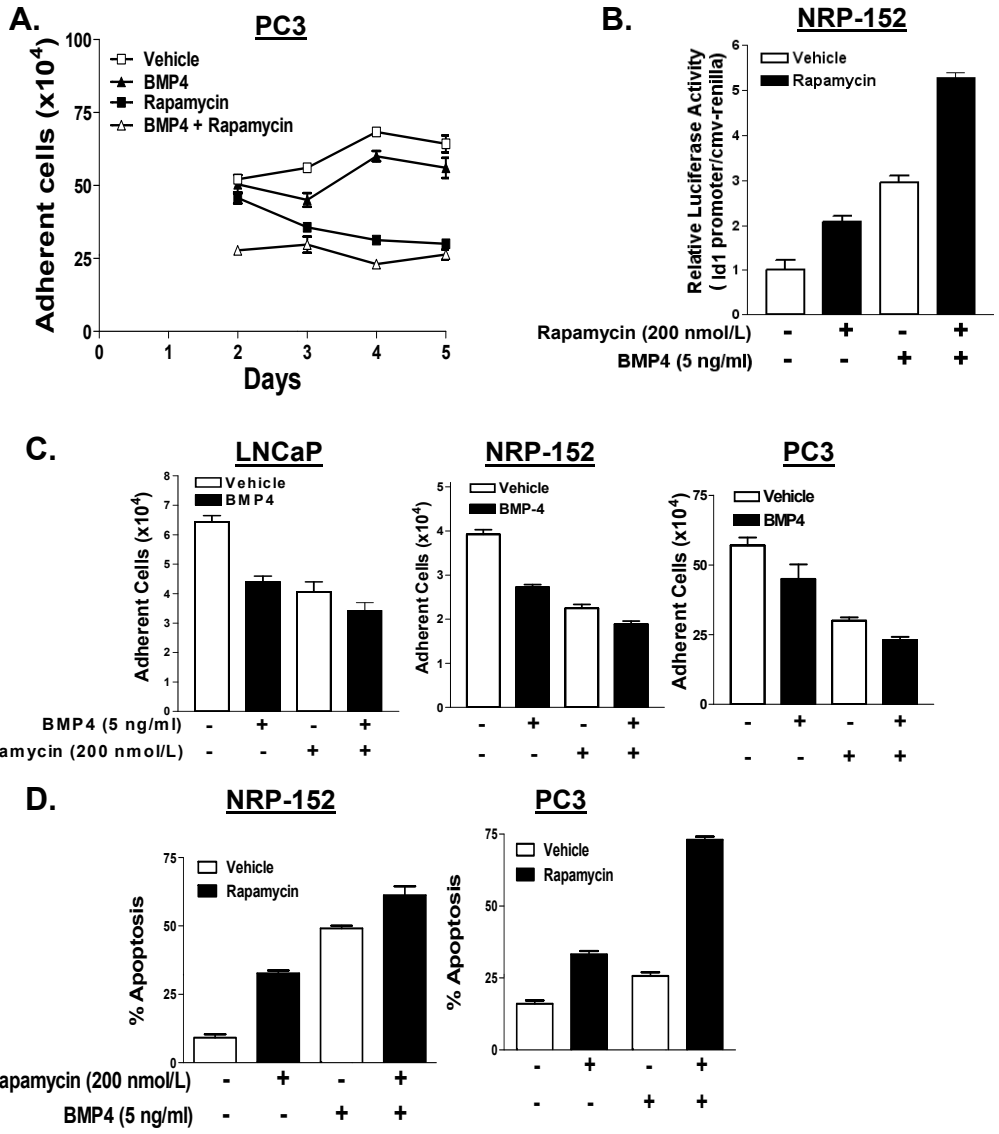
References:


1. Strober, W., *Trypan blue exclusion test of cell viability.* Curr Protoc Immunol, 2001. **Appendix 3**: p. Appendix 3B.
2. Song, K., et al., *Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation.* Embo J, 2006. **25**(1): p. 58-69.
3. Song, K., et al., *Androgenic control of transforming growth factor-beta signaling in prostate epithelial cells through transcriptional suppression of transforming growth factor-beta receptor II.* Cancer Res, 2008. **68**(19): p. 8173-82.
4. Gama, V., et al., *Hdm2 is a ubiquitin ligase of Ku70-Akt promotes cell survival by inhibiting Hdm2-dependent Ku70 destabilization.* Cell Death Differ, 2009. **16**(5): p. 758-69.
5. Wahdan-Alaswad, R.S., et al., *Insulin-Like Growth Factor I Suppresses Bone Morphogenetic Protein Signaling in Prostate Cancer Cells by Activating mTOR Signaling.* Cancer Res, 2010.
6. Pear, W.S., et al., *Production of high-titer helper-free retroviruses by transient transfection.* Proc Natl Acad Sci U S A, 1993. **90**(18): p. 8392-6.
7. Qin, X.F., et al., *Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5.* Proc Natl Acad Sci U S A, 2003. **100**(1): p. 183-8.

# Supplementary Figure 1



# Supplementary Figure S2



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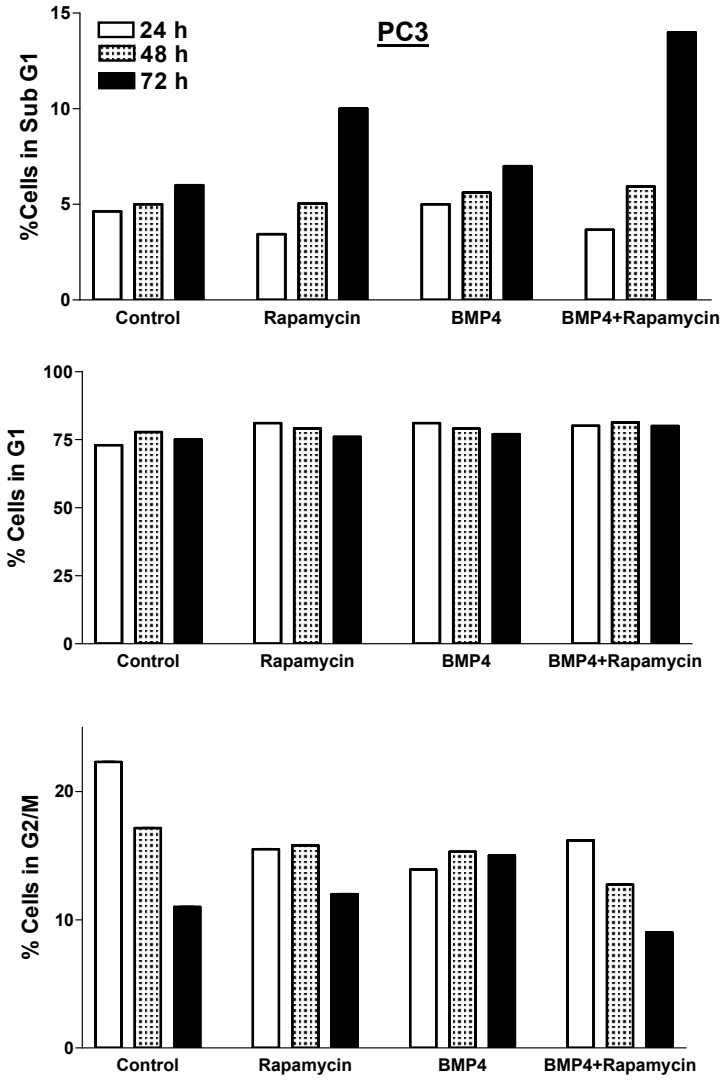

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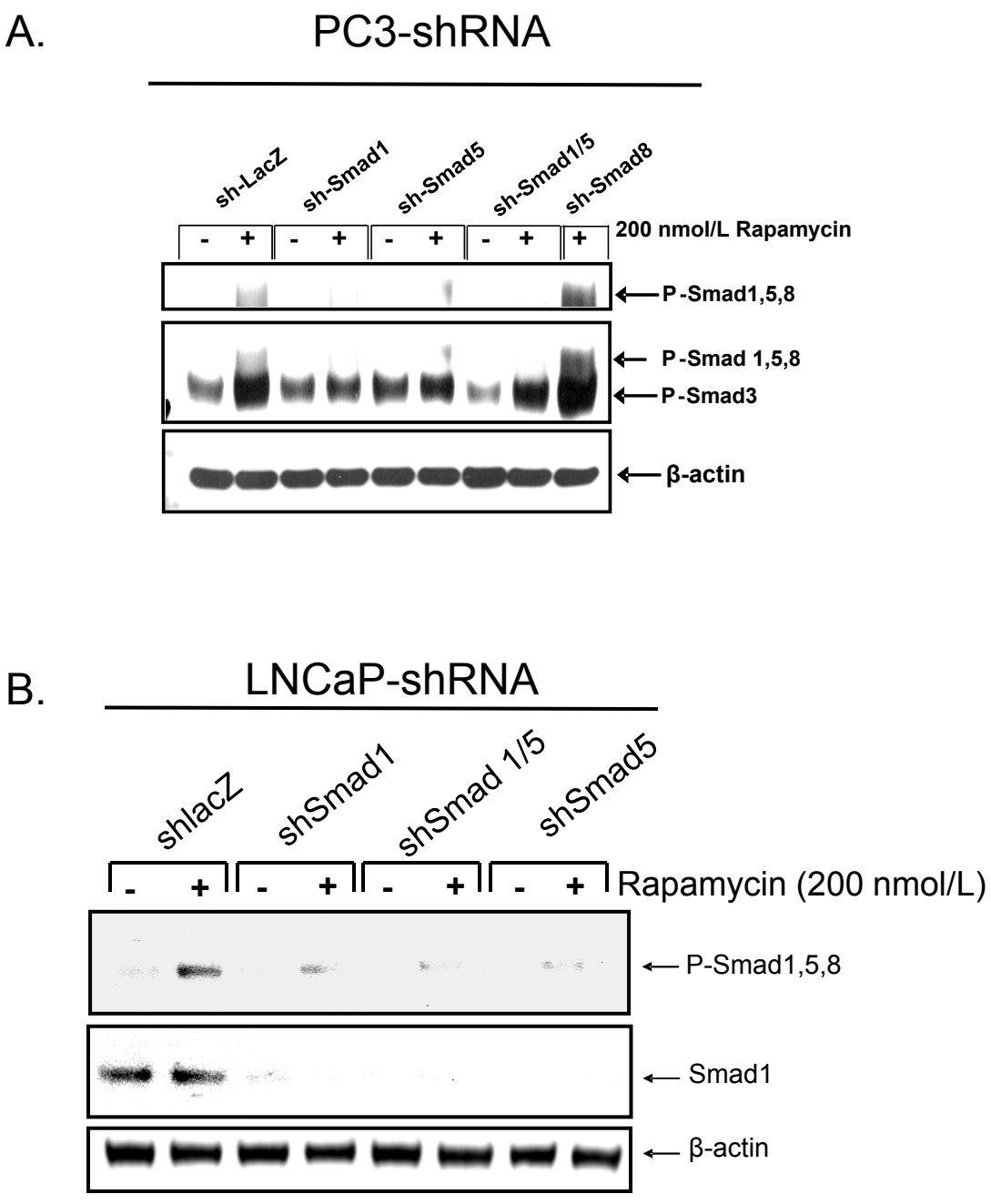

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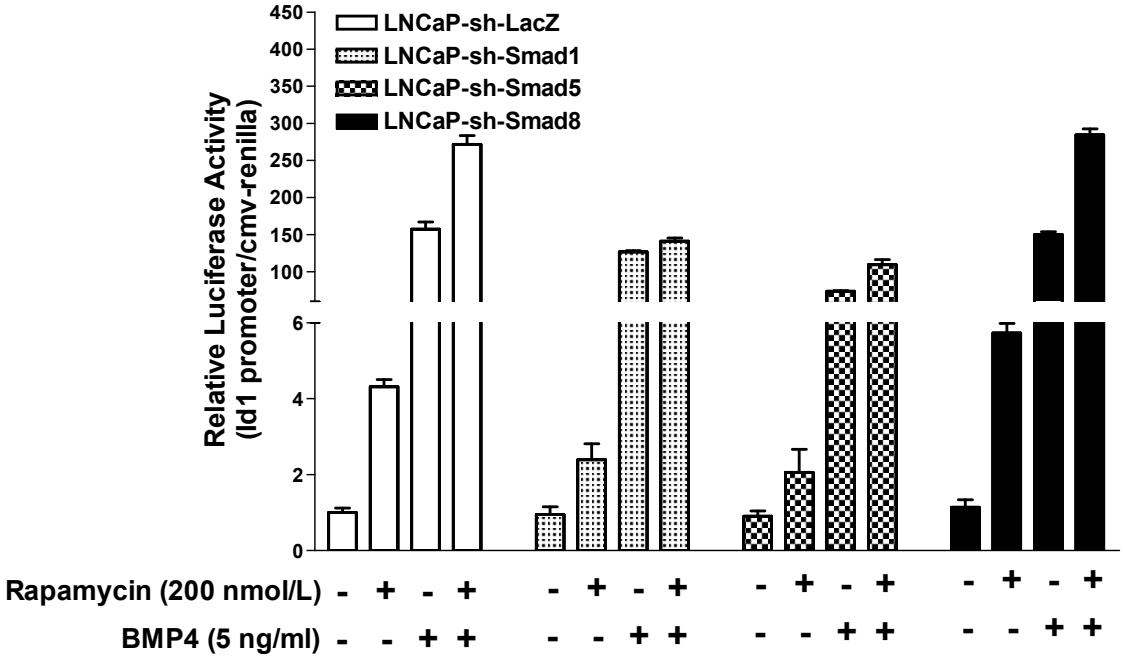

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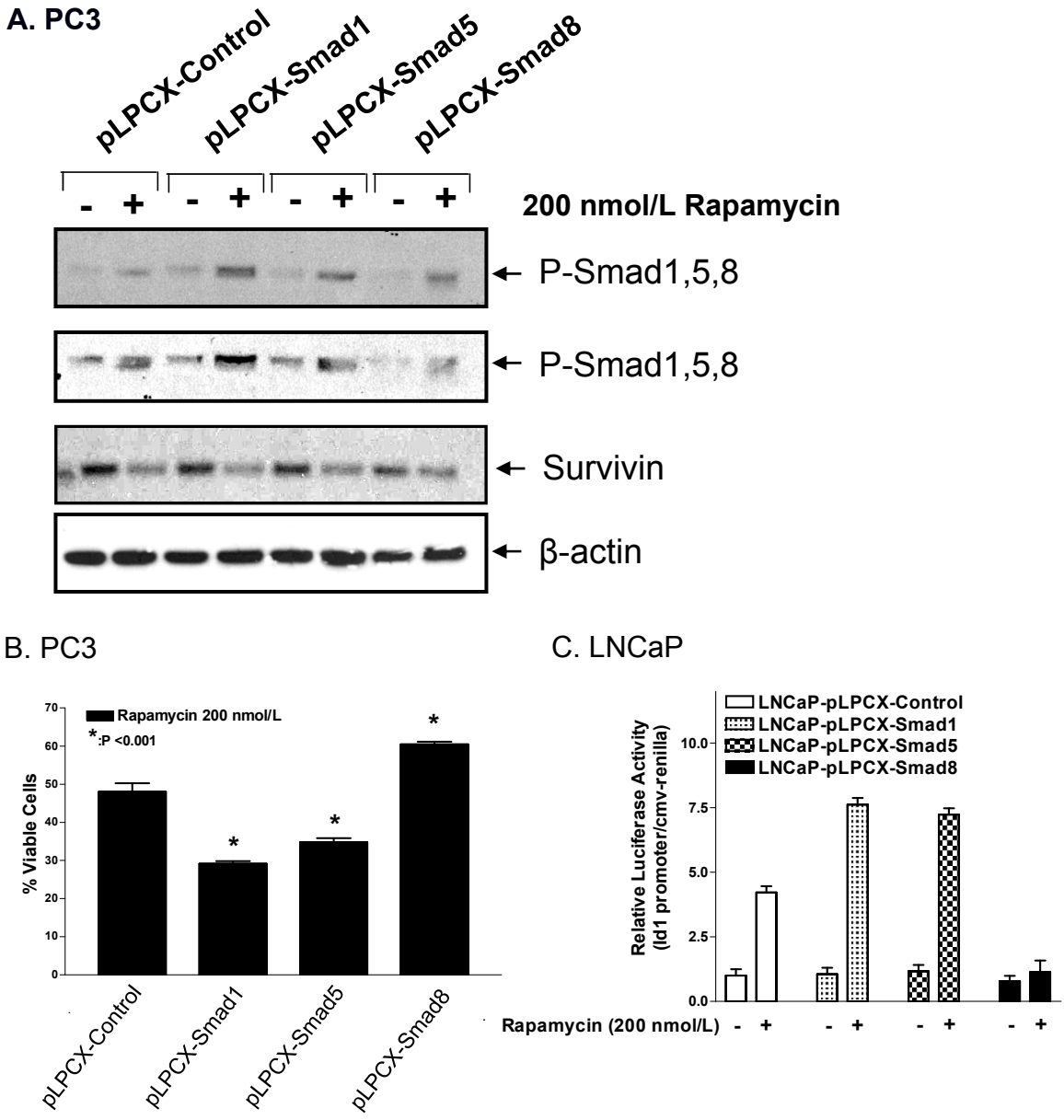

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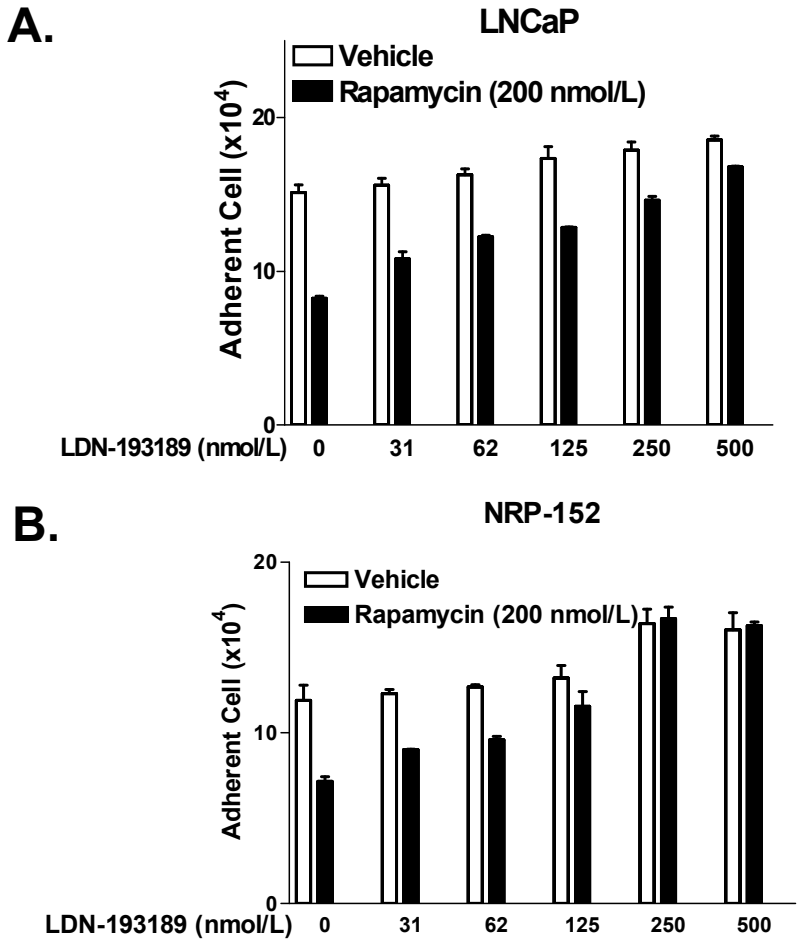


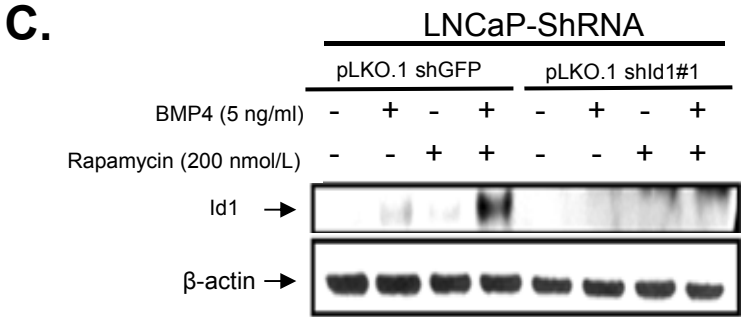

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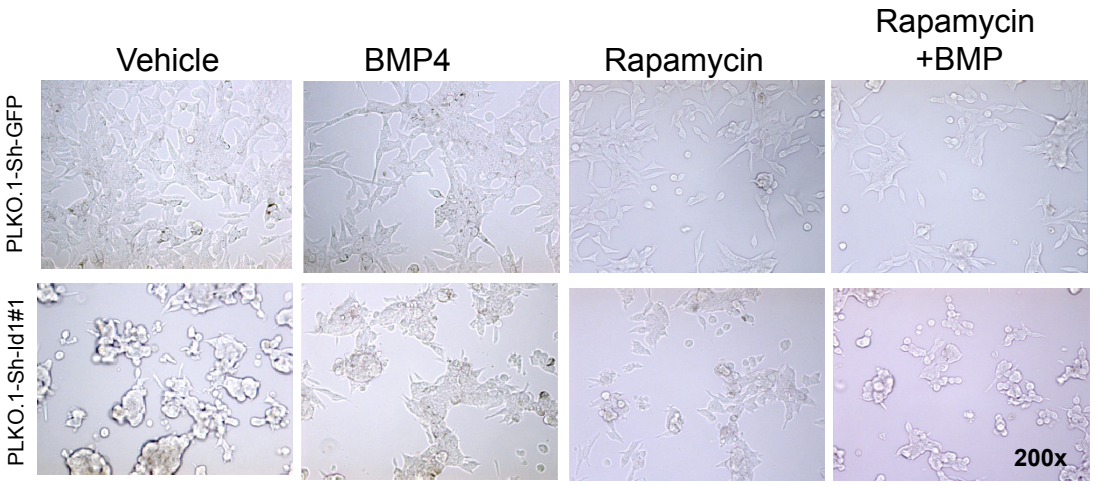
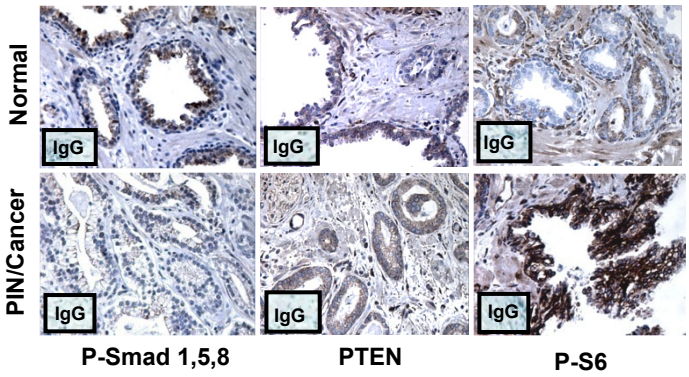


# Supplementary Figure S9

**A.**

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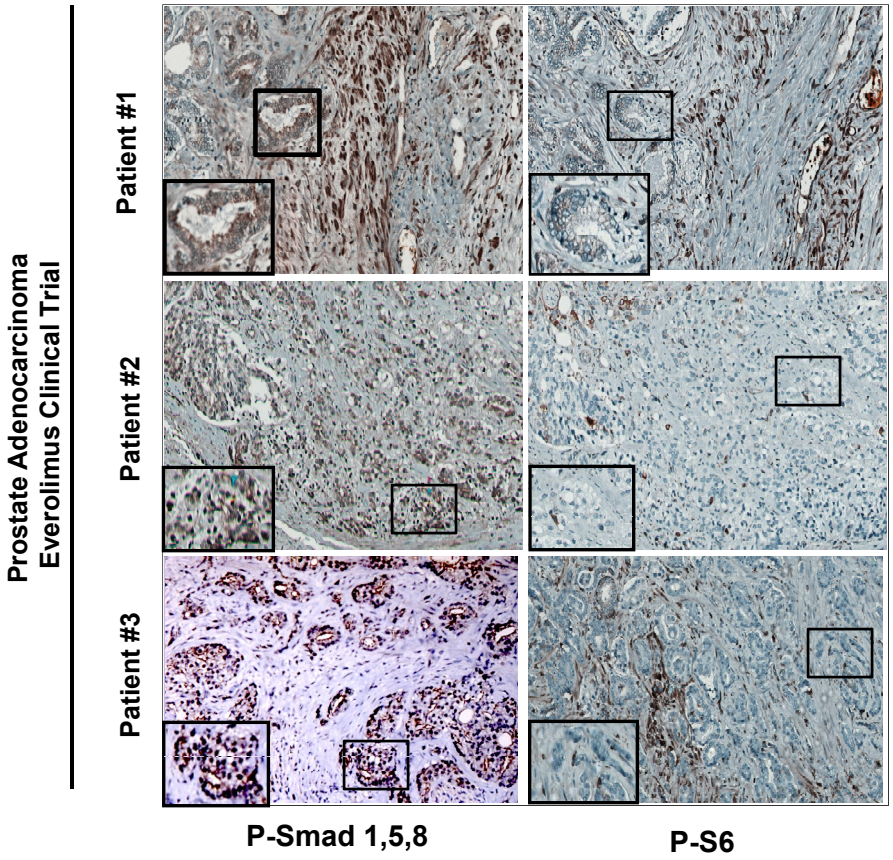
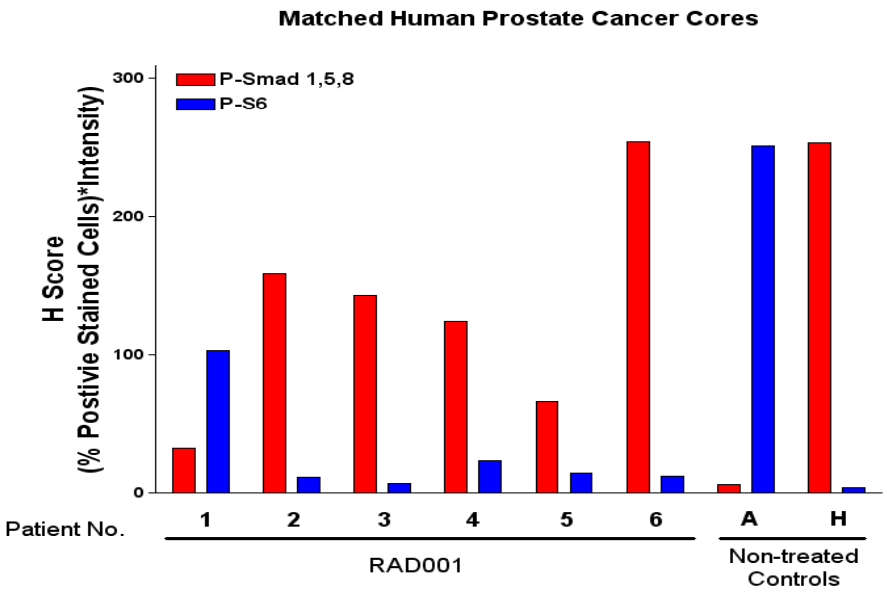


# Supplementary Figure S9

C.



# Supplementary Figure S10