SUPPLEMENTAL DATA

The Supplemental Data include ten supplemental figures and Supplemental Experimental Procedures and can be found with this article online at http://www.jci.org

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of *Bmi1*^{LSL} mice

Bmi1^{LSL} mice were generated as follows: we used a vector in which the CAG (CMV/chicken beta-actin) promoter is followed by the neomycin resistance cassette and three copies of the SV40 polyA signal (Neo Stop) flanked by LoxP sites. The full-length murine Bmi1 cDNA was inserted after the second LoxP site, upstream of an IRES-eGFP sequence. Upon excision of the Neo Stop element through Cre-mediated recombination, constitutive overexpression of the transgene is controlled by the ubiquitous CAG promoter. The Bmi1^{LSL} vector was targeted to the ROSA26 locus by homologous recombination in Ola129-derived E14 ES cells. Neomycin-resistant clones with a single, proper integration and showing significant Bmi1 overexpression upon transient expression of the Cre recombinase were injected into blastocysts to generate chimeric mice. Bmi1^{LSL} line was established through germline transmission and backcrossed (>9 generations) into FVB genetic background. Mice are available upon request to our Institution NKI-AVL reference strain #1343(FVB), #1656(C57BL/6) or to MGI http://www.informatics.jax.org/ #MGI43498910 Gt(ROSA)26Sor<tm1(CMV-Bmi1,-EGFP)Nki>, also named Bmi-CTS.

Purification of Recombinant Bmi1/Ring1B and Mel-18/Ring1B complexes

The murine recombinant Bmi1/Ring1B and Mel18/Ring1B complexes were expressed in insect Sf21 cells using the Bac-to-Bac technology (Invitrogen). The complexes were affinity purified using the His-tag on the C-terminus of Ring1B followed by a second purification step using gel filtration chromatography.

Retroviral, lentiviral vectors and procedures

pLZRS-HA.c-Myc-ires-GFP was a gift from Daniel Peeper. pMSCVblast-empty and pMSCVblast-HA.Bmi1-WT were gifts from Koen Braat. pMSCVblast-HA.Bmi1-3A was obtained through PCR-mediated site-directed mutagenesis using pMSCVblast-HA.Bmi1-WT as a template. For mouse cell infection, viruses were produced in Phoenix packaging cells by standard methods. For human cell infection, viruses were produced by transfecting HEK293T cells with 20 μg retroviral plasmid together with 5 μg pCL-Ampho (Imgenex Corporation). In both cases viral supernatants were collected 2 and 3 days after transfection, filtered through 45 μm strainers and frozen down to -80C until use.

The tet-shBmi1 lentiviral construct was obtained by inserting an shRNA against human Bmi1 (target sequence: GGAGGAGGTGAATGATAAA) into the FH1tUTG lentiviral vector, which constitutively expresses GFP (Herold et al., 2008). Lentiviral particles were produced in HEK293T cells according to standard procedures. LNCaP cells were infected with tet-shBmi1 lentivirus to generate LNCaP-tet-shBmi1 cells.

Western blot

Whole cell extracts were prepared in RIPA buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Equal amounts of protein, as determined by Micro BCA Protein Assay Kit (Pierce), were resolved by SDS-PAGE and transferred onto Nitrocellulose membranes. Membranes were blocked in PBS 0.1 % Tween-20 (PBST) 5% BSA for 1 hour and incubated with primary antibodies in PBST 1% BSA overnight at 4°C. Secondary antibodies coupled to HRP were incubated for 45 minutes in PBST 1% BSA. Primary antibodies: made-in-house Bmi1 (F6 clone); phospho-Akt (Ser 473) (D9E), PTEN (138G6), Akt1 (C73H10), Akt2 (D6G4) and pGSK3ß (Ser9) (Cell Signaling); β-tubulin (SDL.3D10) (Sigma-Aldrich).

Cell culture

MEFs and U2OS were maintained in DMEM (Gibco) with 10 % FBS (Sigma) and antibiotics (Gibco). LNCaP, PC3, DU145 and PNT1a cell lines were grown in RPMI-1640 (Gibco) with 10 % FBS and antibiotics.

For Bmi1 reconstitution in LNCaP, cells were first infected with the tet-shBmi1 lentivirus and superinfected with pMSCV-blast retroviruses encoding HA-tagged mouse Bmi1-WT or Bmi1-3A and were subjected to blasticidine selection. Positive clones were expanded and checked by Western blot for expression of exogenous Bmi1 and for knockdown of endogenous Bmi1 upon treatment with doxycycline. 2000 cells per well were then seeded onto 6-well plates and incubated for 8 days ± 100 ng/ml doxycycline (Sigma). Cells were

stained with crystal violet. Cell quantification was obtained by incubating the plates for 30 minutes in 500 µl acetic acid (10 %) followed by OD measurement at 590 nm.

Soft agar experiments were performed according to standard procedures. *Ink4a/Arf*$^{-/-}$ MEFs were infected with pMSCV-blast-empty, pMSCV-blast-HA-Bmi1-WT, or pMSCV-blast-HA-Bmi1-3A retroviruses and subjected to blasticidine selection. Resistant cells were then superinfected with pLZRS-HA-c-Myc-ires-GFP and 10^5 cells were seeded in soft agar. Macroscopic colonies were counted after 4 weeks. Results are mean ± standard deviation of two independent experiments performed in triplicate.

In vitro kinase assay

Recombinant active Akt (100 ng) (Cell Signaling Technology) was incubated with 0.8 µCi of [γ-^{32}P]ATP and 30 ng of recombinant Bmi1/Ring1B or Bmi1-3A/Ring1b complex in 30 µl of kinase buffer (5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl2, 0.05 mM DTT, 50 µM ATP). Reactions were incubated at 30°C for indicated times and terminated by addition of Laemmli SDS sample dilution buffer. Proteins were separated by 10% SDS-PAGE, and phosphorylation was visualized by autoradiography. Home made recombinant GST and GST-GSK3 crosstide (Cell Signaling #9237) were used as controls.

In vitro Ubiquitin E3 ligase assay

Reactions (25 µl) were performed using Ubiquitin (20 µM), E1 (140 nM), Ubc5c (1,2 µM), purified E3 complexes (250 nM), and Hela-purified

nucleosomes (1,2 μM) in ubiquitination buffer (50mM Tris-HCl ph 8, 100 mM NaCl, 10 mM MgCl2, 1 μM ZnCl2, 1 mM DTT, 3 mM ATP) for 45 minutes at 32°C. Where indicated, complexes were dephosphorylated by treatment with 2U of AP (Roche) in phosphatase buffer for 1 hr at 37°C. Inactive AP (control) was obtained after 1hr of pre-incubation of active AP in phosphatase buffer supplemented with 10 mM Na3VO4. For the Ubiquination assay in the presence of Akt/inactive Akt, 600ng of Akt or inactive Akt were added to the reactions that were carried out for 15 minutes at 32°C.

UVA laser scissors

Laser Scissors: The cells were grown in LAB-TEK chamber slides, (Nalge Nunc International; Naperville,IL) and treated with 10 μM 5-iodo-2-deoxyuridine (IdU; Sigma; St. Louis, MO) for 24h prior to laser irradiation. For micro-irradiation, Cells were visualized under visible light of a Zeiss Axiovert 200 microscope integrated with the P.A.L.M Microlaser workstation (P.A.L.M. Laser Technologies, Bernried, Germany). Laser targeted nuclei selected using the supplied software (P.A.L.M Robo v3.2Software) were irradiated with a pulsed solid-state UVA-laser (30Hz, 337nm). Immunofluorescence experiments were done as described in (45).

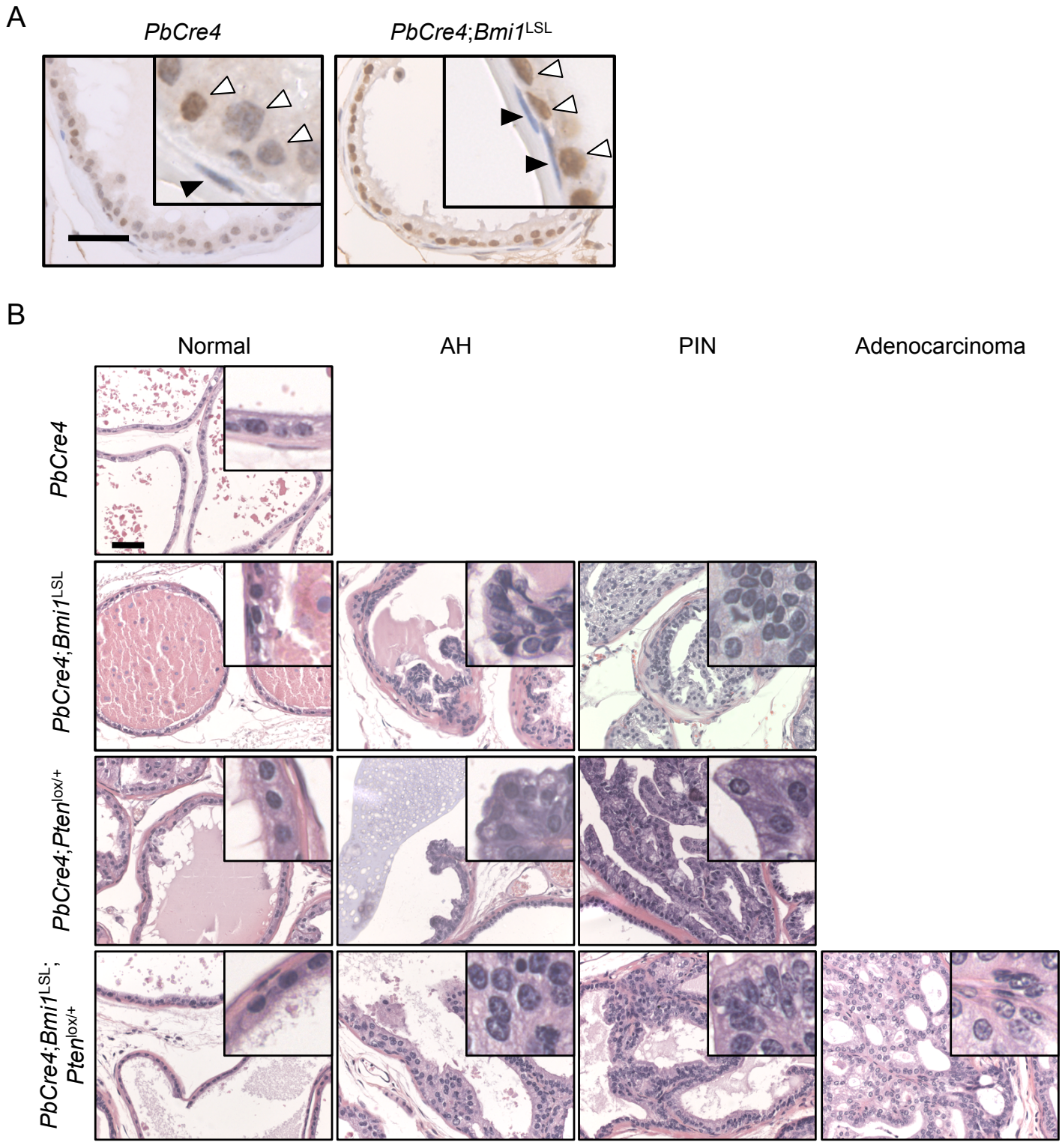
Fluorescence-based homologous recombination (HR) repair assay

HR repair assays were performed as described previously using the DR-U2OS cell line (DR-GFP) (47). To assay the rate of HR, DR-U2OS cells expressing mouse Bmi1-WT or Bmi1-3A were transfected with human Bmi1-specific siRNA, and then 48 hr later transfected with an I-SceI-encoding

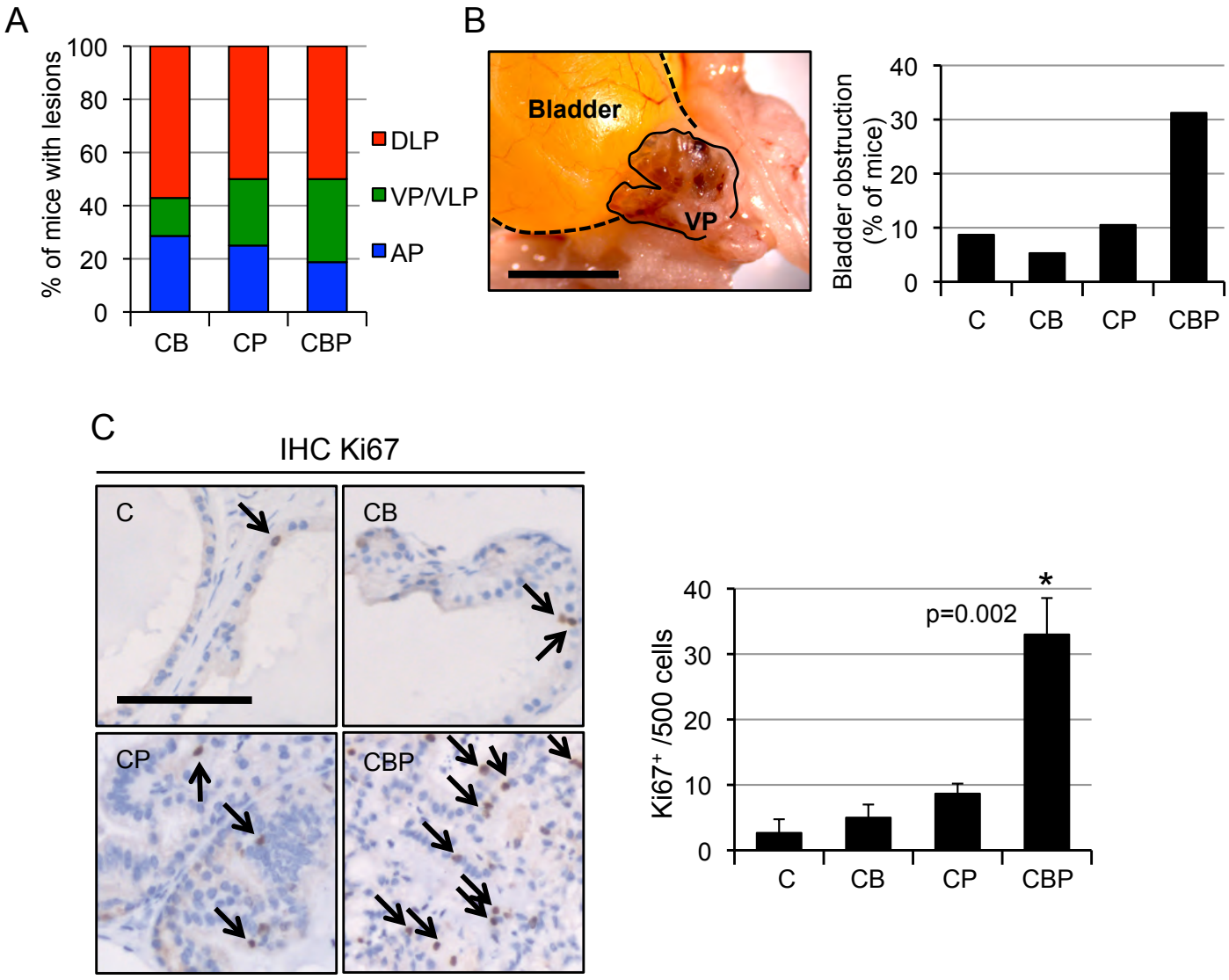
plasmid plus additional siRNA. After I-SceI transfections the cells were then grown for 3 days and harvested. The percentage of GFP-positive cells was measured using the Cytomics FC 500 Series flow cytometer (Beckman Coulter). The data were analyzed using software CXP (Beckman Coulter, CA).

Sister chromatid exchange (SCE) assay

LNCaP-tet-shBmi1 cells were grown in the presence of 10μM of bromodeoxyuridine (BrdU; Sigma) through two cell cycles (2 days) to achieve preferential labeling of sister chromatids. Colcemid (Karyomax; Invitrogen) was added at a final concentration of 0.2 μg/ml to accumulate mitotic cells 3 hr prior to harvesting cells by mitotic shake off. Metaphase preparations were made using standard methods. Slides were aged overnight and stained with 5μg/ml Hoechst 33258 (Invitrogen) for 12 min. Sister chromatid differentiation was performed by the fluorescence-plus-Giemsa technique according to the standard procedure. For Doxycycline (Dox) treatment, Dox was added to the culture at a final concentration of 100 ng/ml 3 days prior to - and during - BrdU treatment. For Akt inhibitor (Akti) treatment, cells were exposed to 1μM Akti (Akt inhibitor VIII; Calbiochem) during BrdU treatment. Digitally captured images of differentially stained metaphase chromosome spreads were scored for SCEs in a blinded fashion.

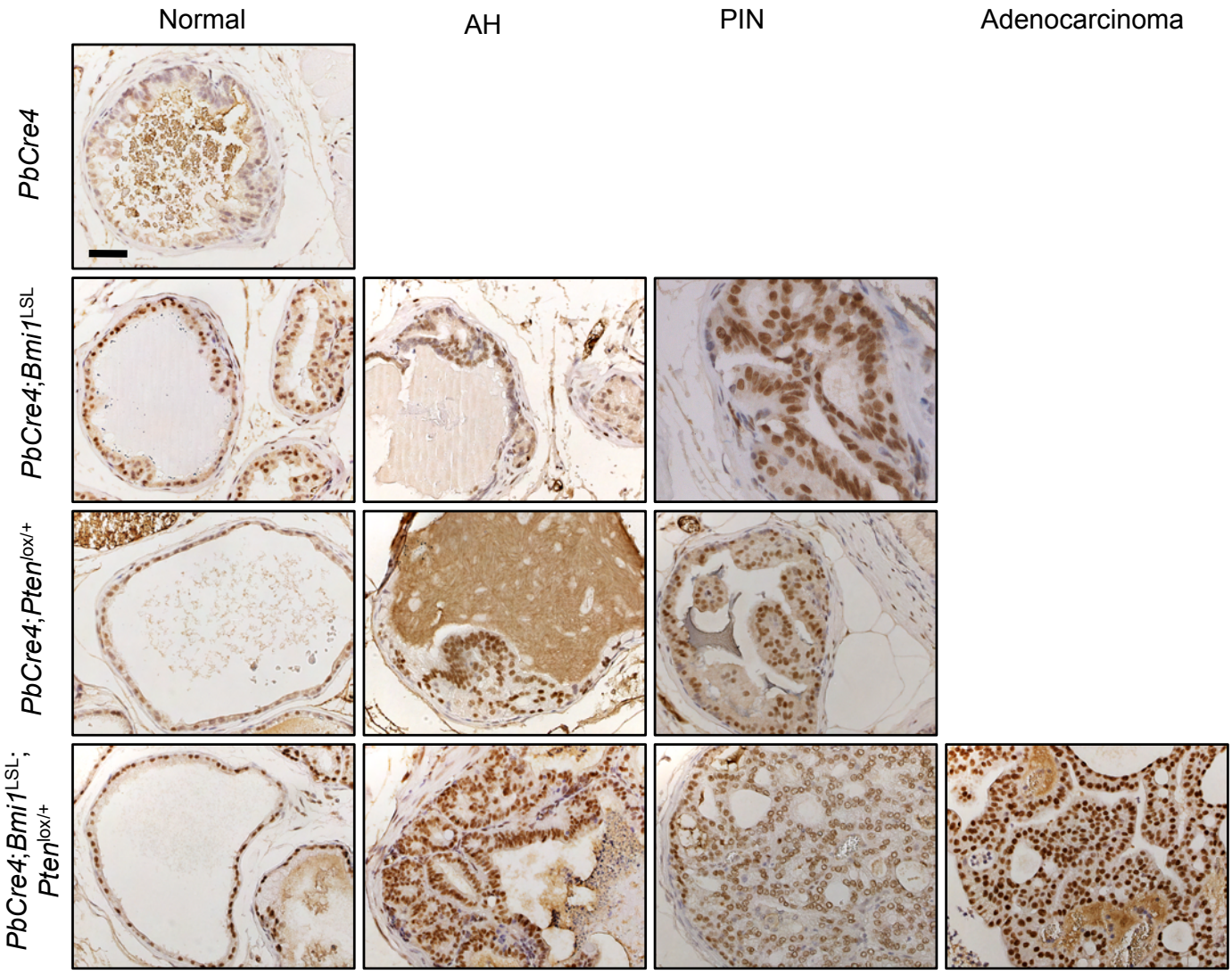


Supplemental Figure 1. A, Bmi1 is overexpressed in luminal (open arrows) but not in basal (closed arrows) prostate cells of *PbCre4*;*Bmi1*^{LSL} mice. B, Histological analysis of transgenic prostates. Tissue sections from 1 year-old males of the indicated genotypes were stained with H&E and scored for prostate lesions. The most advanced lesion in each mouse was taken into account to generate Figure 1B,C. Mice harboring a given type of lesion often displayed the whole panel of lower grade lesions. Images are from one representative mouse per genotype for consistency. Scale bar: 100 μm. Insets: 5-fold higher-power views.

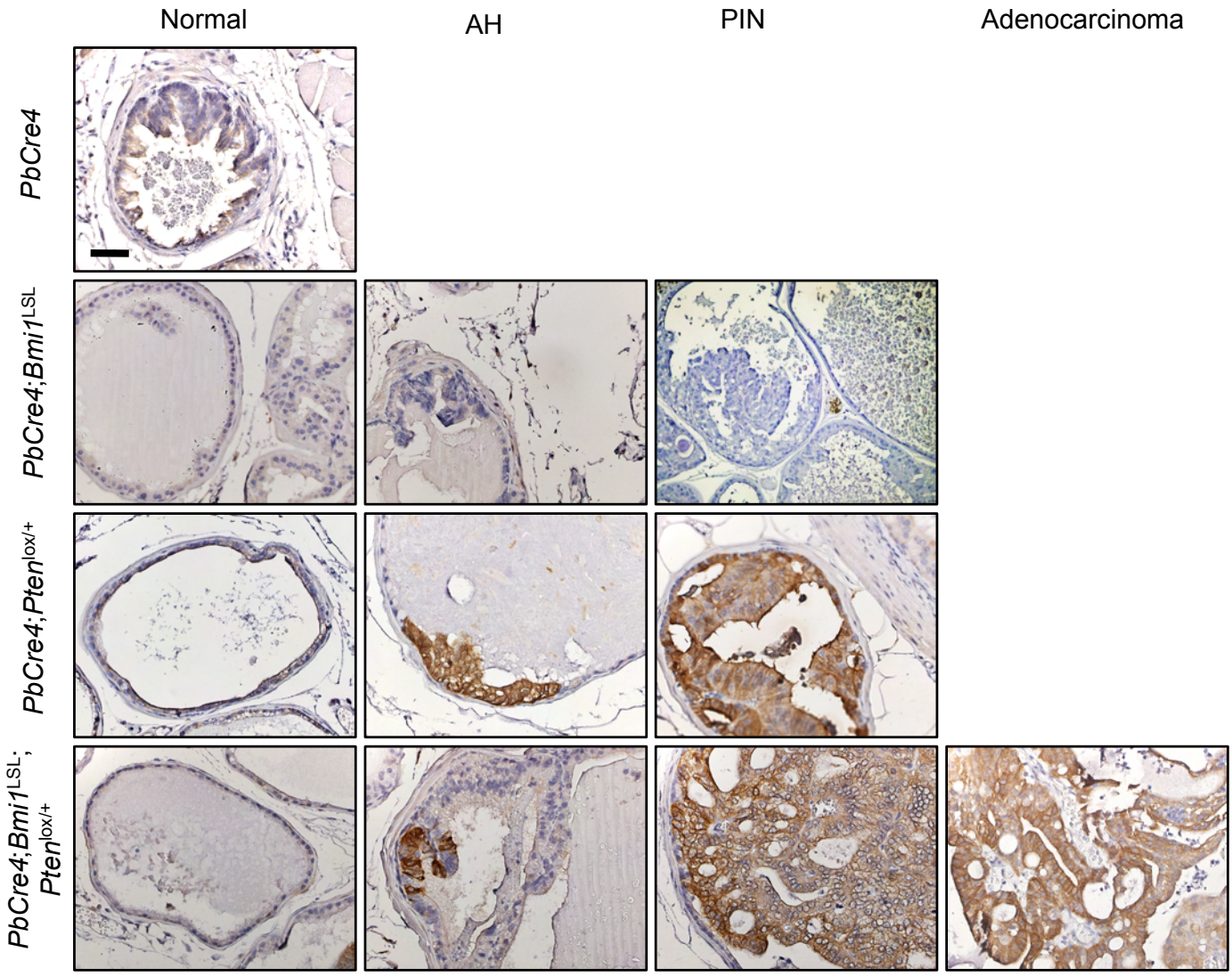


Supplemental Figure 2. Invasive Prostatic Adenocarcinoma in *PbCre4*;*Bmi1*^{LSL};*Pten*$^{loxP/+}$ Mice

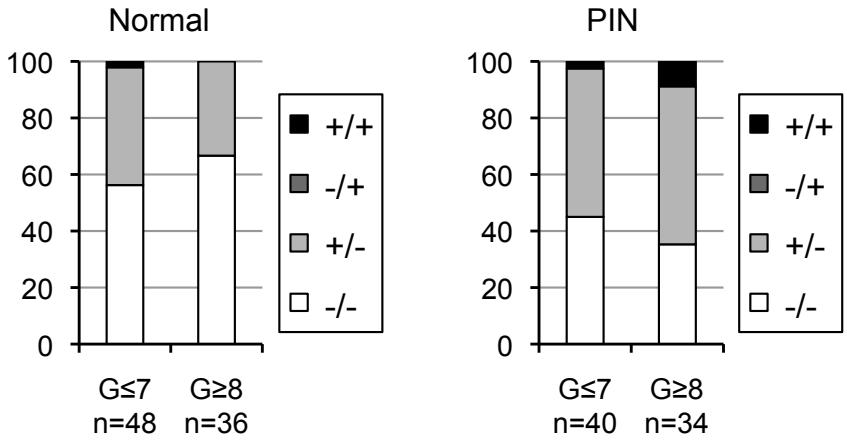
A, Lobe distribution of prostatic lesions found at 9 and 12 months in CB (n=7); CP (n=8) and CBP (n=16) mice. DLP: dorsolateral prostate, VP/VLP: ventral and ventrolateral prostate, AP: anterior prostate. B, Left panel: One year-old *PbCre4*;*Bmi1*^{LSL};*Pten*$^{loxP/+}$ representative male presenting a ventral prostate (VP) tumor with bladder outlet obstruction. Bar: 1 cm. Right panel: quantification of bladder obstructions (diameter >2 cm) in C (n=23); CB (n=19); CP (n=21) and CBP (n=17) mice. C, Left panels: Ki-67 immunohistochemical labeling of prostatic proliferative cells in C, CB, CP and CBP mice Bar: 100 μm. Arrows point Ki-67^{+} cells. Right panel: quantification of Ki-67^{+} cells from tissue sections of the aforementioned animals. The CBP mice show significantly more Ki-67^{+} cells than the other groups (p=0,002). Throughout the figure: C: *PbCre4*; CB: *PbCre4*;*Bmi1*^{LSL}; CP: *PbCre4;Pten*$^{loxP/+}$ and CBP: *PbCre4*;*Bmi1*^{LSL};*Pten*$^{loxP/+}$.



Supplemental Figure 3. Assessment of Bmi1 expression status in normal and diseased prostates of different genotypes. 1 year-old mouse prostates of the indicated genotypes were stained with an anti-Bmi1 antibody (see Materials and Methods). Scale bar 100μm.

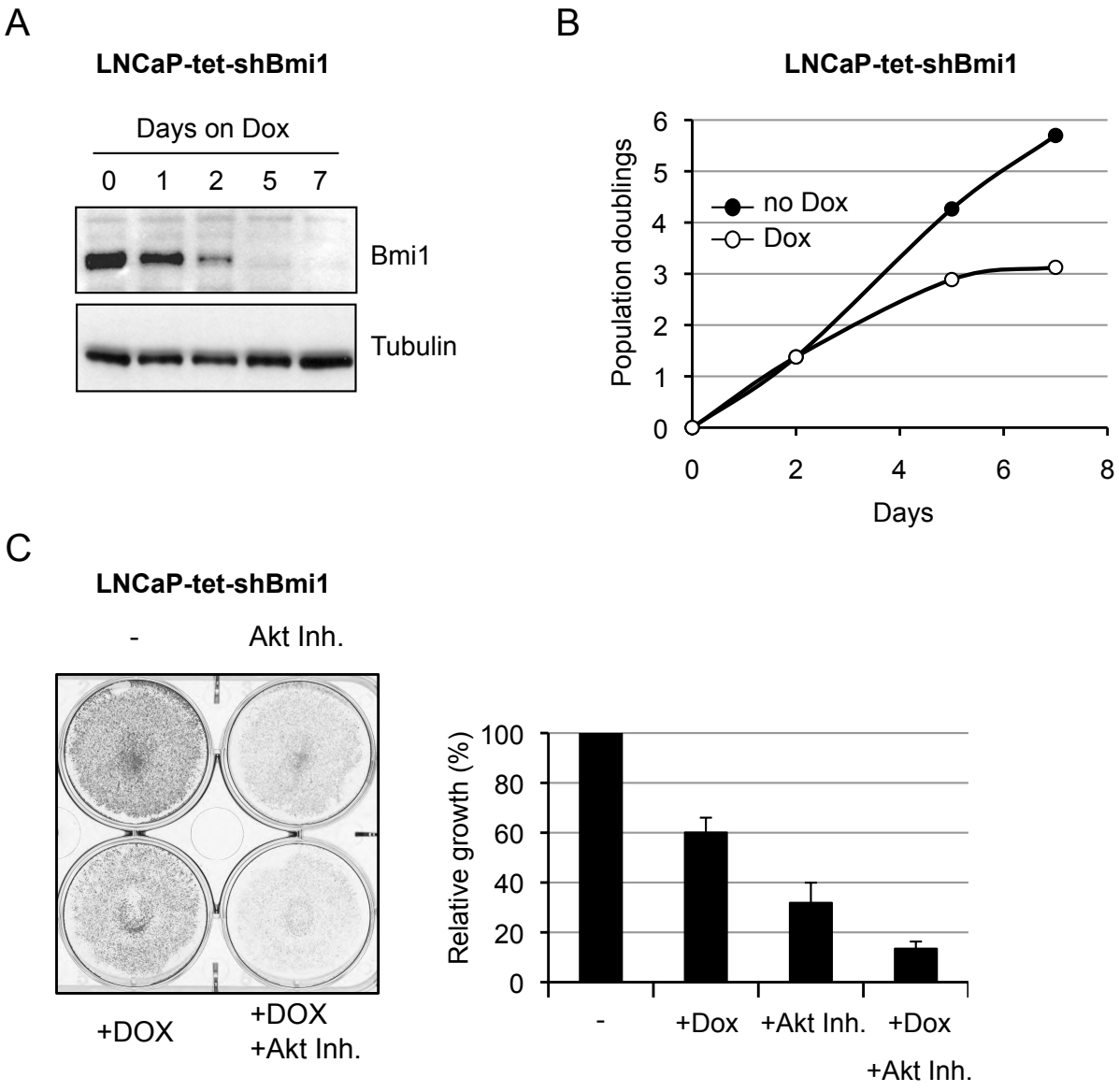


Supplemental Figure 4. Assessment of pAkt expression status in normal and diseased prostates of different genotypes. 1 year-old mouse prostates of the indicated genotypes were stained with an anti-pAkt antibody (see Materials and Methods). Scale bar: 100μm.



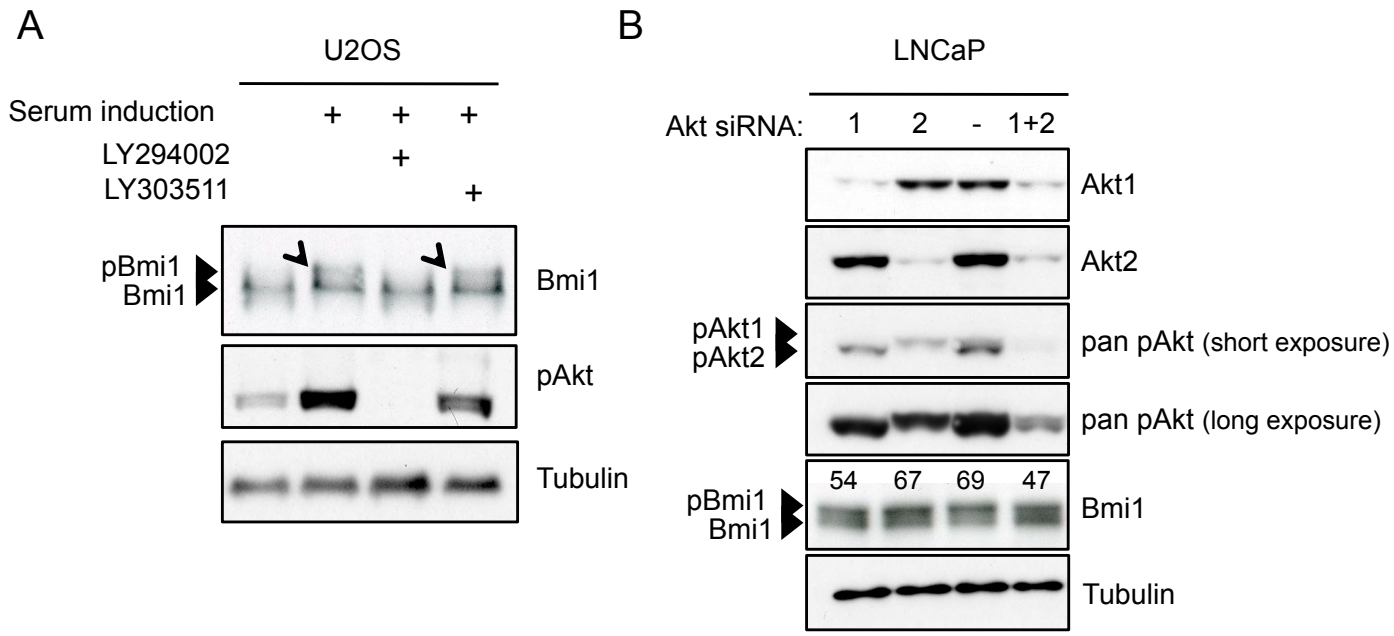
Supplemental Figure 5. Assessment of Bmi1 and pAkt Expression in PINs and non-Diseased Tissue in Prostate Cancer Clinical Samples.

Patients for whom tumor biopsies also harboured both PIN lesions and normal tissue were sorted according to Gleason score and the expression of Bmi1 and pAkt was assessed as in Figure 3. Note that the number of samples is lower than in Figure 3 because not all tumor samples exhibited both PINs and normal glands.



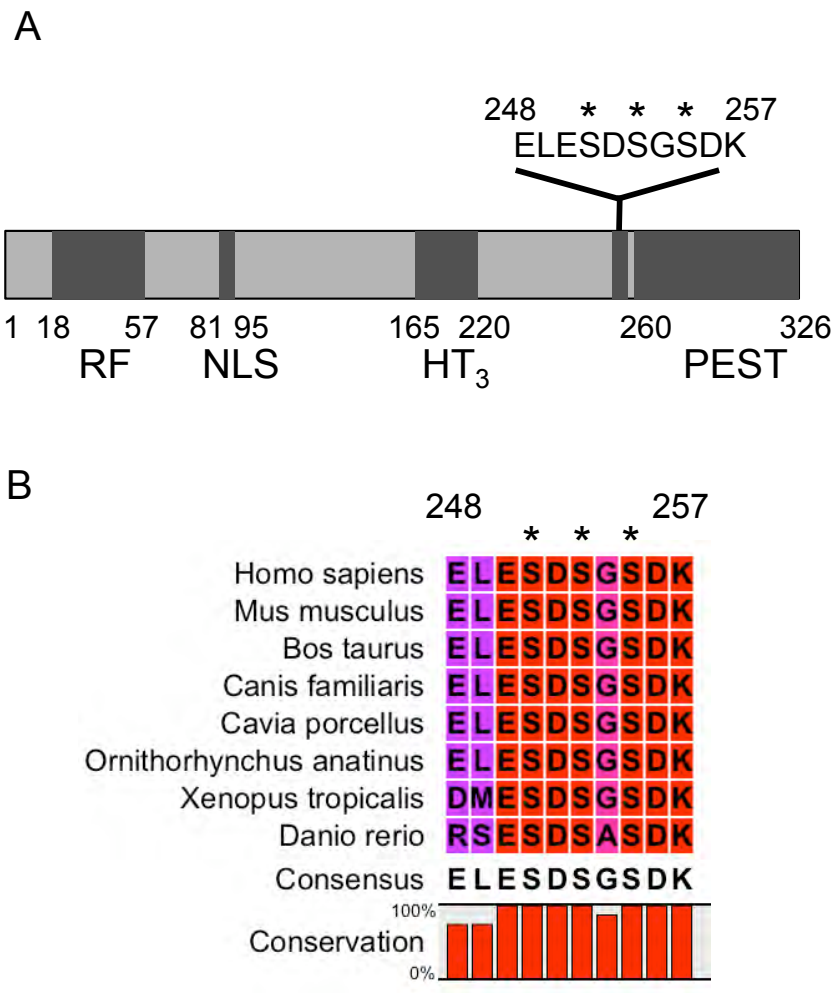
Supplemental Figure 6. Bmi1 Knockdown Impairs LNCaP Growth

A, LNCaP cells expressing a doxycycline-inducible Bmi1 shRNA (LNCaP-tet-shBmi1) were treated with doxycycline (Dox), 100 ng/ml, for the indicated duration and Bmi1 protein levels were determined by Western blot on whole cell lysate. B, Growth curves showing population doublings of LNCaP-tet-shBmi1**.** A dramatic decrease in growth upon Bmi1 knockdown is observed after 4 days of doxycycline treatment. C, Left panel: crystal violet staining of LNCaP-tet-shBmi1 cells either left untreated, treated with 100 ng/ml doxycycline for 3 days or/and 5μM Akt inhibitor VIII (Calbiochem) for the last 16 hours. Right panel: OD quantification of the crystal violet at 590nm, Data are means ± standard deviation of three independent experiments.



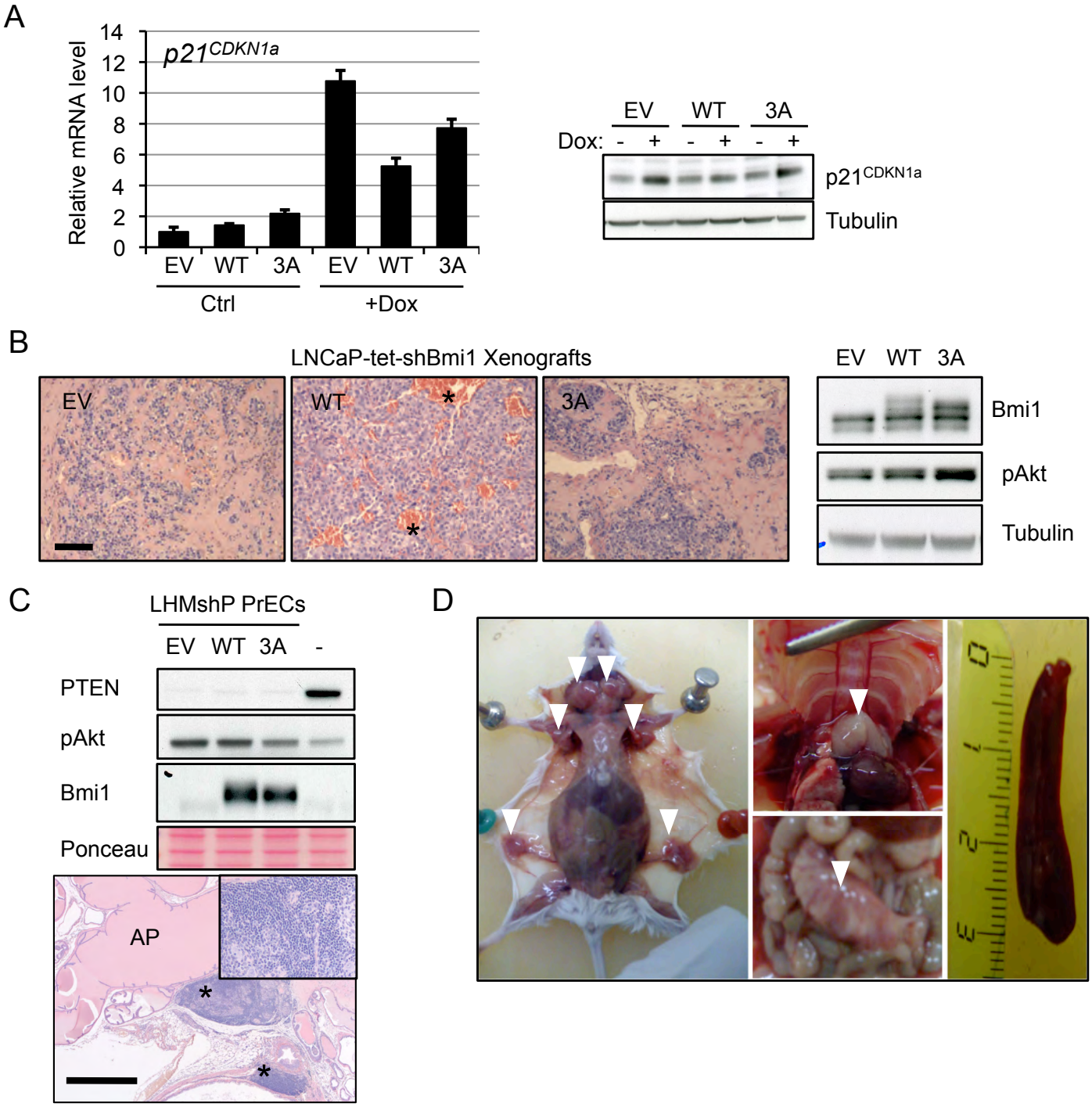
Supplemental Figure 7. Bmi1 and PTEN-PI3K/Akt Regulation

A, Phosphorylation of endogenous Bmi1 in response to serum-induced PI3K/Akt activation. U2OS cells were deprived of serum for 24 h and then stimulated in the presence of 15 % FBS for 30 min. 1 h prior and during serum stimulation cells were either left untreated, pretreated with 25 μM of LY294002 PI3K inhibitor or with 25 μM of LY303511 inactive analogue. Phosphorylated Bmi1 (arrowheads), phosphorylated Akt (pAkt), and Tubulin (loading control) are shown. B, LNCaP cells were transfected with isoform-specific siRNAs against Akt1 (1), Akt2 (2), Akt1 and Akt2 (1+2) or non-targeting pool siRNA (-). 48 hours post-transfection, cells were lysed and protein extracts were analyzed by Western-blot with antibodies against total Akt1, total Akt2, pan p(S473)Akt, Bmi1, and Tubulin as a loading control. The percentage of the Bmi1 phosphorylated species (pBmi1) over total Bmi1 was quantified using ImageJ software and appears within the blot. Specific knockdown of Akt1 and Akt1+Akt2 reduced the overall Bmi1 phosphorylation from 69 % to 54 % and 47 % respectively. Specific knockdown of Akt2 had no significant effect on the proportion of the phosphorylated-Bmi1 species (67 %).



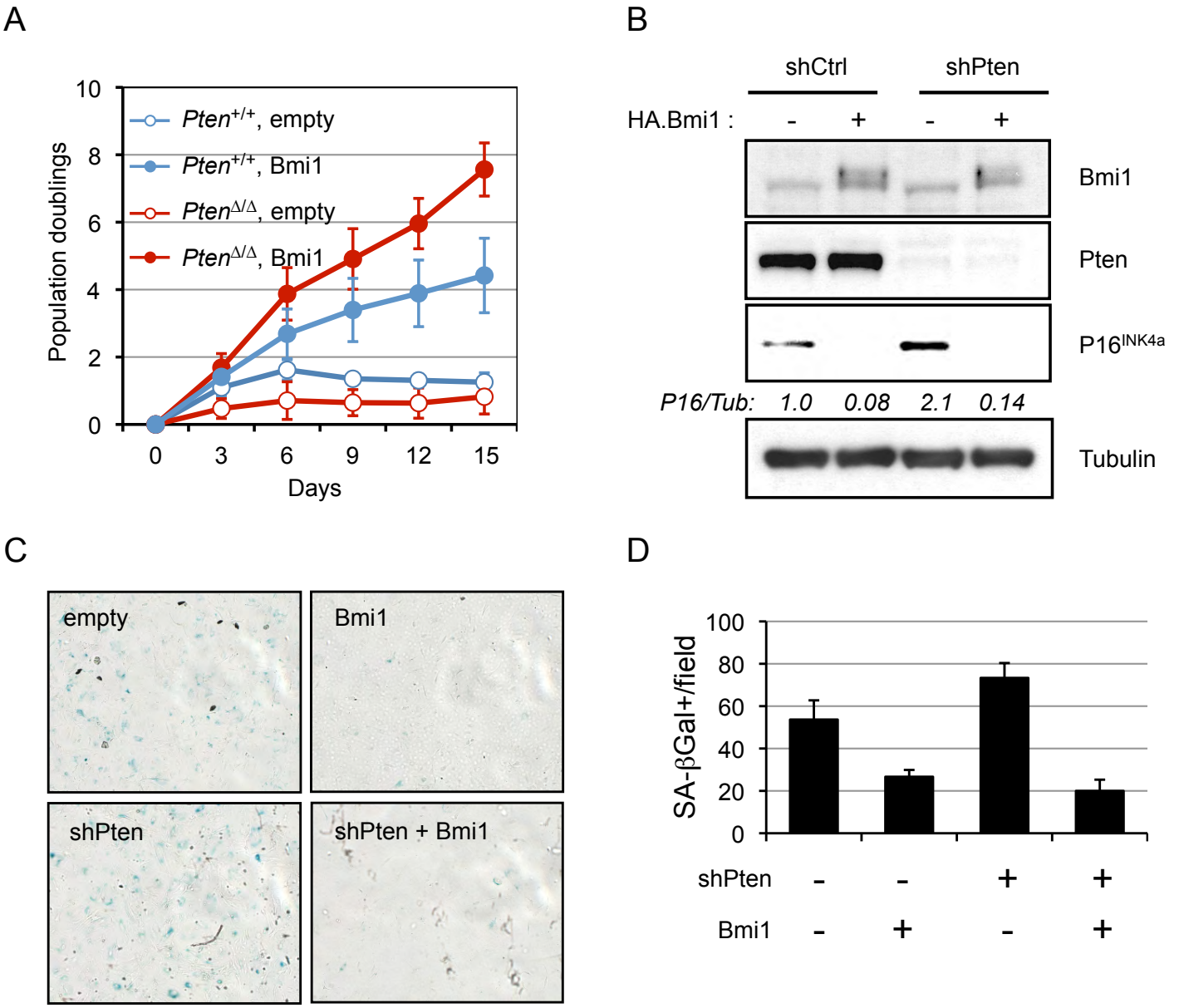
Supplemental Figure 8. Bmi1 Phosphorylation Occurs on a Conserved C-terminal Serine Triad.

A, Schematic representation of Bmi1 protein showing the RING finger domain (RF), the nuclear localization signal (NLS), the helix-turn-helix-turn-helix-turn (HT_3) and the proline/serine rich domains. Serine residues that have been identified by mass spectrometry analysis as being phosphorylated (22-30) are shown (asterisks). B, Bmi1 protein sequence alignment (CLC Free Workbench, CLC Bio software) showing the species conservation of Serine 251, 253 and 255 (asterisks) and flanking residues.



Supplemental Figure 9. Supporting Data for Figures 9B-E

A, qRT-PCR analysis showing *p21*^{CDKN1a} expression in LNCaP-tet-shBmi1 cells that were infected with either control, Bmi1-WT or Bmi1-3A retroviruses and treated with mock or 100 ng/ml Dox for 4 days. *p21* mRNA was quantified relative to β-actin. Data are means +/- sd of 3 independent experiments. B, *Left*: Hematoxylin and eosin staining of LNCaP xenograft subcutaneous tumors expressing control empty vector (EV), Bmi1-WT (WT) and Bmi1-3A (3A) retroviral constructs, Bmi1-WT tumors have increased cell density and size as well as a pronounced vascularization (asterisks); Bar: 100 μm. *Right*: western blot showing expression of endogenous and exogenous Bmi1 in cells used in left panel. C, Expression levels of PTEN, pAkt, HA-tagged Bmi1-WT and Bmi1-3A in immortalized human prostate epithelial cells (hPrECs) expressing SV40 LT, hTERT, c-Myc and shPTEN (LHMshP) prior to their orthotopic transplantation into the anterior prostate of nude mice, lower panel shows 2 sites (*) of Bmi1-WT-driven tumors (Bar: 500 μm). Parental hPrECs are shown as controls. D, Example of disseminated lymphoma arising in lethally irradiated recipient mice reconstituted with Eμ-Myc;Bmi1 fetal liver cells. Arrows point to the enlarged superficial cervical, axillary, and inguinal lymph nodes (left panel), thymus (middle upper panel), mesenteric lymph node (middle lower panel) and spleen (right panel).



Supplemental Figure 10. Bmi1 Bypasses Senescence Induced by Acute Loss of Pten in MEFs.

A, MEFs of the indicated genotype were infected with Bmi1-encoding or control retroviruses and superinfected with an Adeno-Cre virus at a MOI of 50 (Day 0). Growth was measured by a 3T3 serial passaging protocol over the indicated period. B, Bmi1 overexpression prevents the upregulation of Ink4a/Arf upon loss of Pten. MEFs were infected with retroviruses encoding HA-tagged Bmi1 and shRNA against Pten. Whole cell extracts were subjected to western blot. The p16/Tubulin expression ratio was measured using ImageJ software and was normalized to uninfected control cells. C, Bmi1 overexpression bypasses senescence upon loss of Pten. MEFs were infected as in C and were subjected to senescence-associated beta-galactosidase staining 7 days post-infection. D, Quantification of senescence-associated beta-galactosidase positive cells. Data are means ± standard deviation of 3 independent experiments.