Supplemental Data

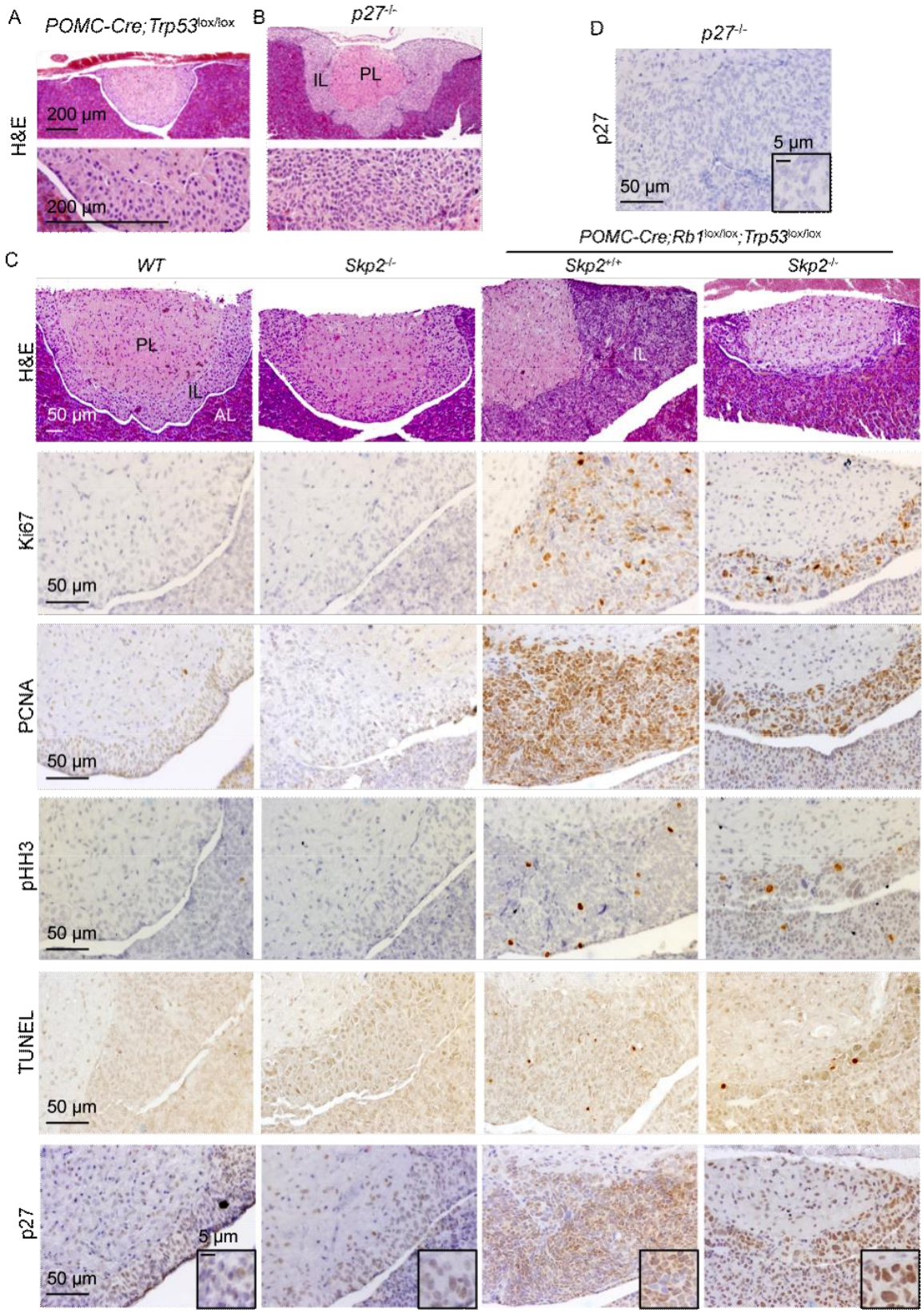


Figure S1. Related to Figure 1. (A and B) H&E staining of pituitary sections of 7 weeks old *POMC-Cre;Trp53*$^{lox/lox}$ (A) or *p27*$^{-/-}$ (B) mice. (C) Pituitary sections of the indicated genotypes were stained as indicated. (D) Pituitary sections of *p27*$^{-/-}$ mice stained with anti-p27.

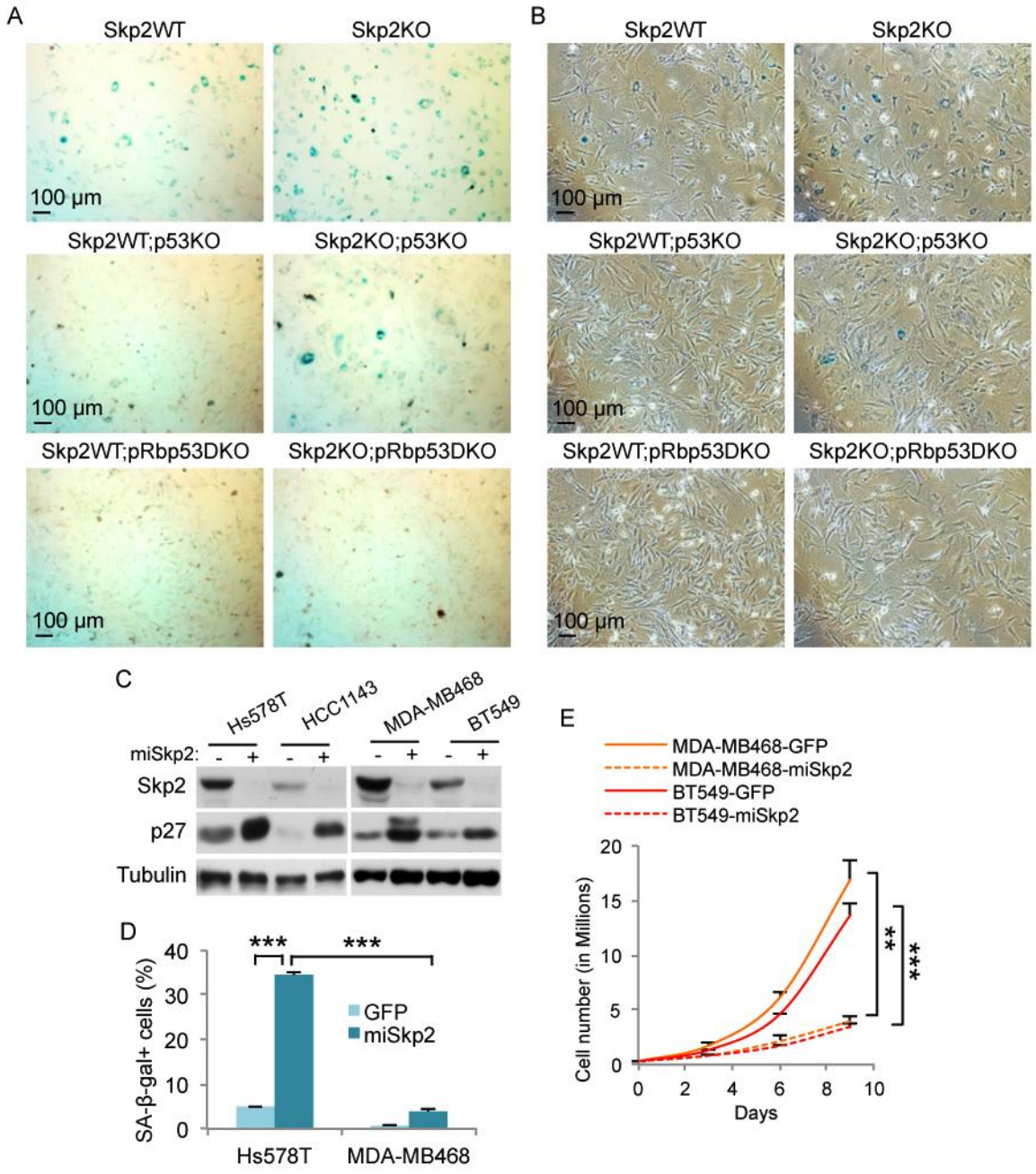


Figure S2. Related to Figure 2. (A and B) MEFs of the indicated genotypes were stained with SA-β-gal and photographed in bright field (A) or in phase contrast (B). (C) Indicated human breast cancer cell lines, with or without Skp2 knockdown (miSkp2) were subjected to Western blot. (D) Quantification of SA-β-gal stain in photographs shown in Figure 2B. (E) Actual cell proliferations for the indicated human breast cancer cells expressing GFP or miSkp2. Quantitative data are presented as average +/- SEM. Student's *t* test was used for statistical analysis. **, $p < 0.01$; ***, $p < 0.002$.

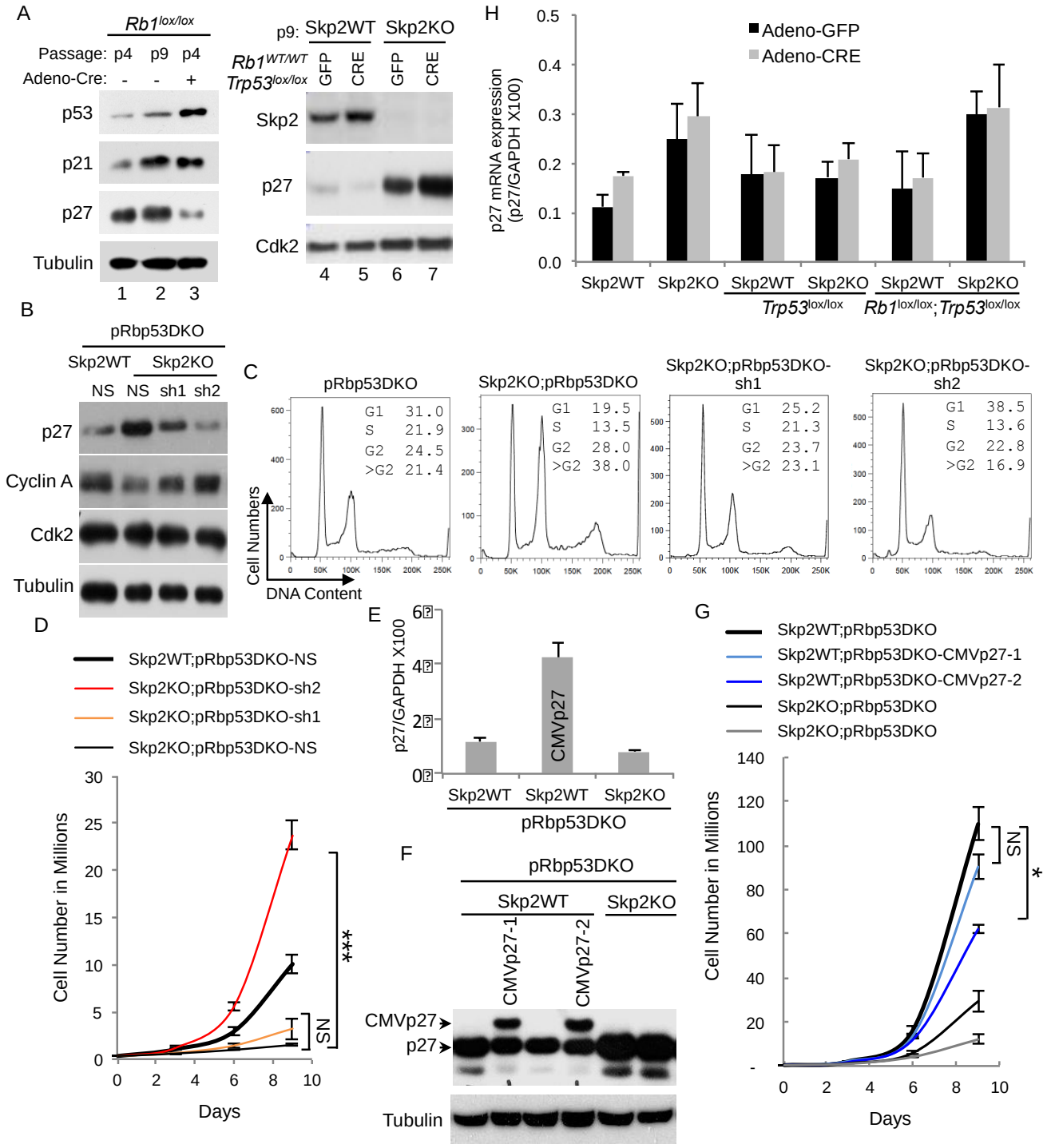
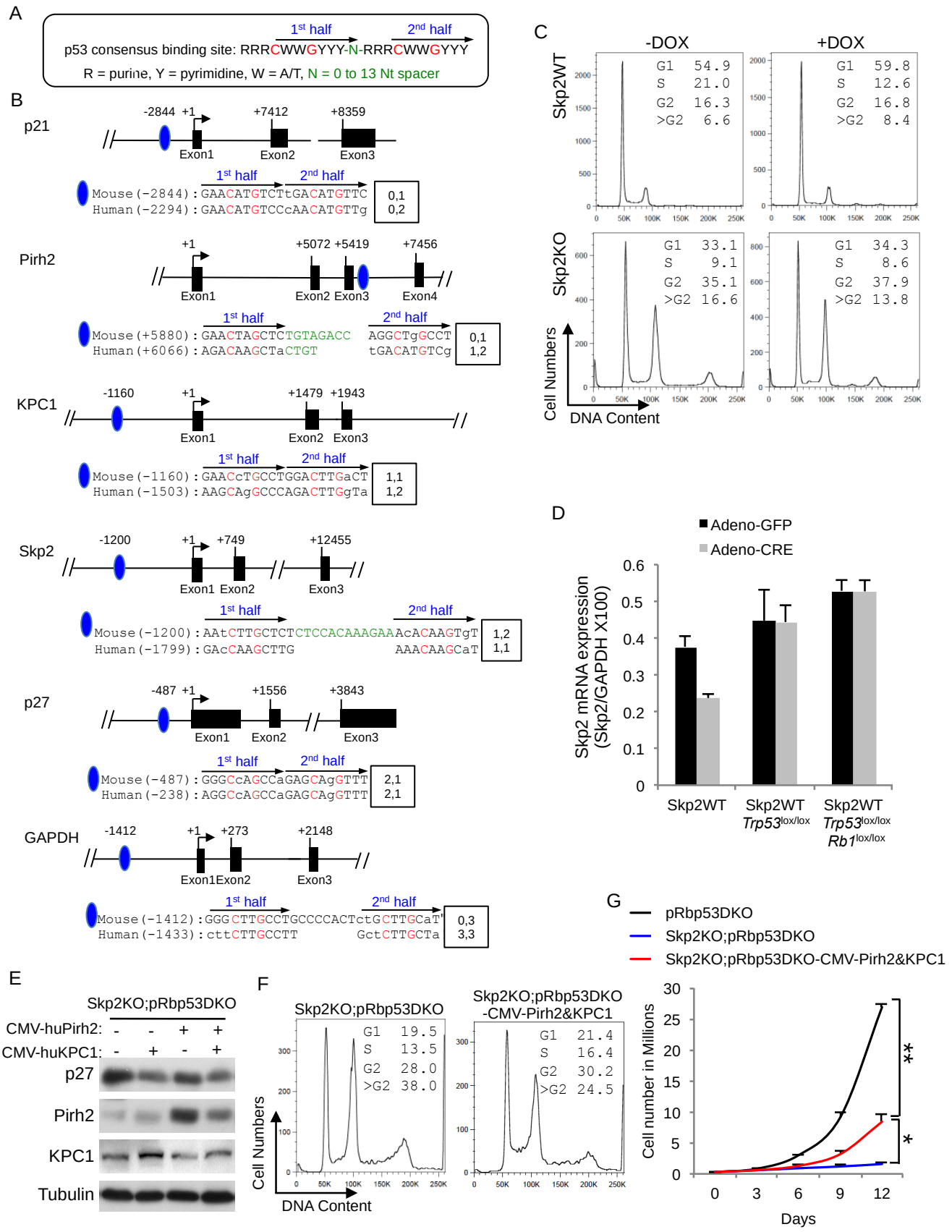


Figure S3. Related to Figure 3. (A) Western blots of indicated MEFs. MEF cultures established from dissected embryos on previous days are "Passage" 1 (p1). (B) Western blots of indicated MEFs transduced with indicated knockdown hairpins. NS, non-specific random sequence hairpins. (C) DNA content FACS. (D) Same cells as in (C) were counted to determine actual cell proliferation. (E) RT-qPCR of p27 mRNA from indicated MEFs. (F) Western blot of cell in (E). (G) Proliferation curves of the indicated cells. (H) RT-qPCR of p27 mRNA. Quantitative data are presented as average +/- SEM. Student's *t* test was used for statistical analysis. *, $p < 0.05$; ***, $p < 0.002$; NS, $p > 0.05$.



<< Continued on the next page >>

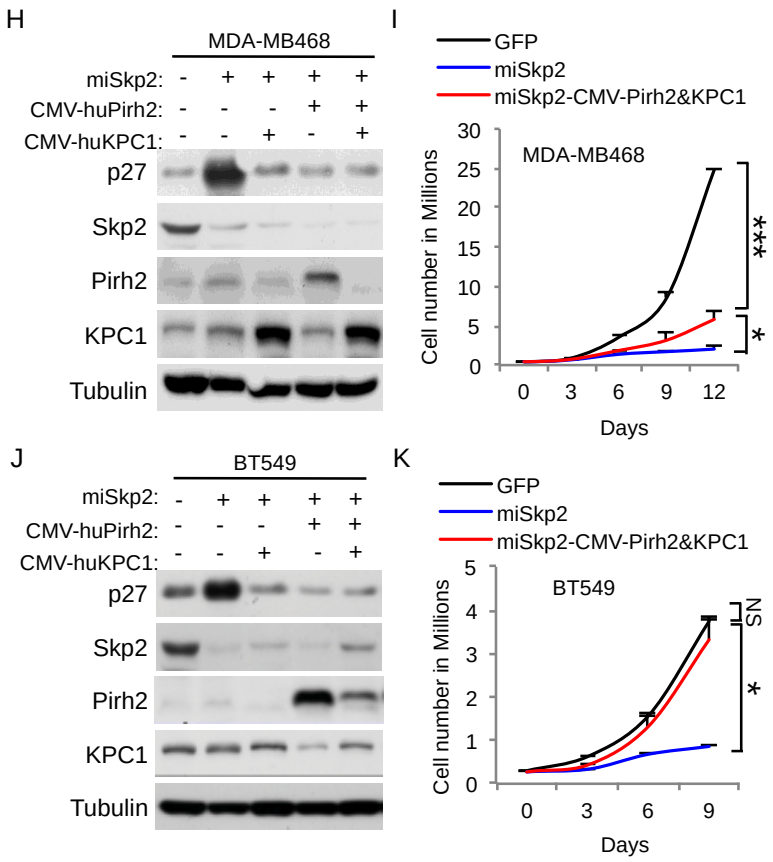


Figure S4. Related to Figure 4. (A) The consensus p53 binding site. (B) The best matching sequence to the consensus p53 binding site for each genes. Conservations between mouse and human are indicated. (C) DNA content FACS showing cell cycle profiles of WT and Skp2KO MEFs before and after DOX treatment, as in Figure 4A to 4C. (D) Effects of deleting *Trp53* alone or together with *Rb1* on mRNA levels of Skp2 is measured by RT-qPCR. (E to G) Expression of human Pirh2 and KPC1 from a CMV promoter was examined by western blots (E), their effects on cell cycle profiles were determined by DNA content FACS (F), and their effects on actual cell proliferation by cell number counts for 12 days (G). (H to K) Expression of CMV-huPirh2 and CMV-huKPC1 in human breast cancer cell lines were examined by Western blots (H and J) and their effects on cell proliferation by cell number counts (I and K). Quantitative data are presented as average +/- SEM. Student's *t* test was used for statistical analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.002$; NS, $p > 0.05$.

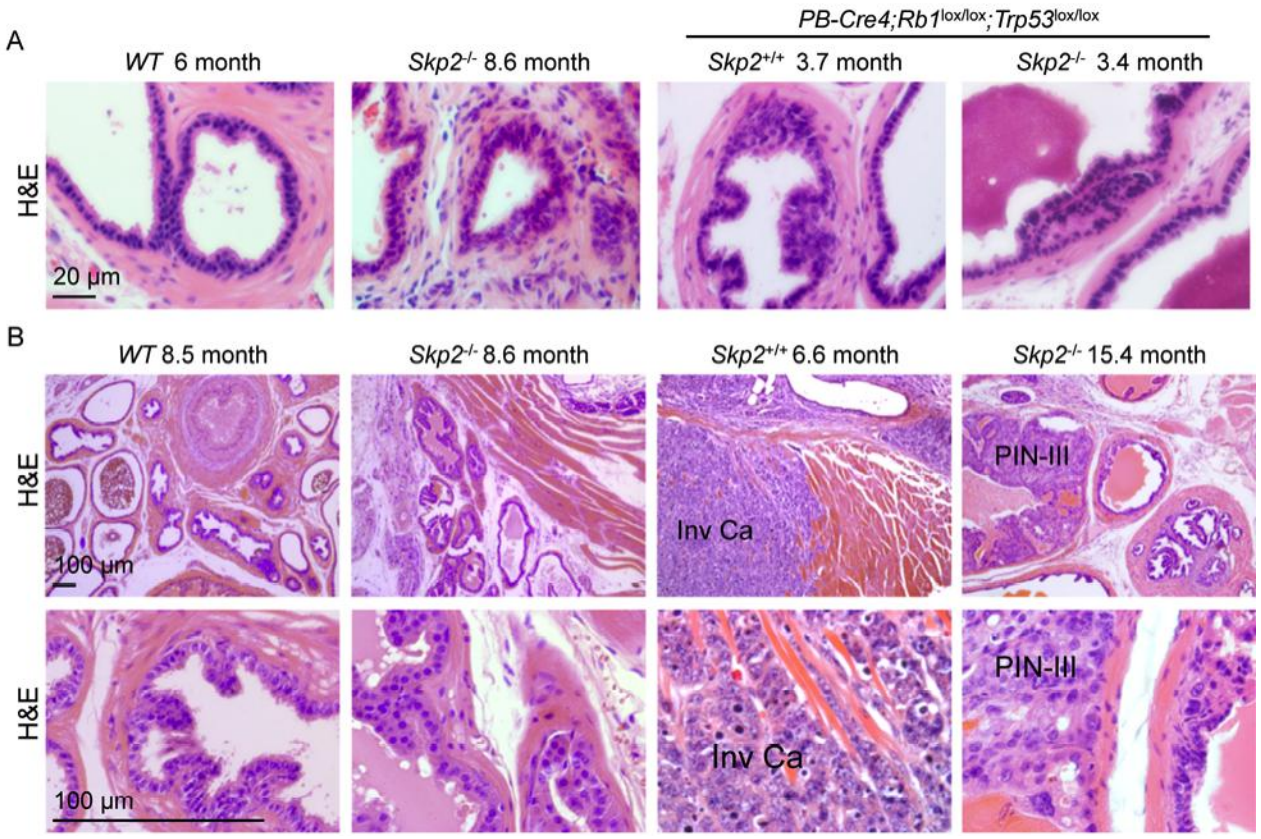


Figure S5. Related to Figure 5. (A) PINs at 3 months. (B) Invasive carcinoma in the presence of Skp2 at 6.6 month, and PIN-III in the absence of Skp2 at 15.4 month. Two magnifications are shown.

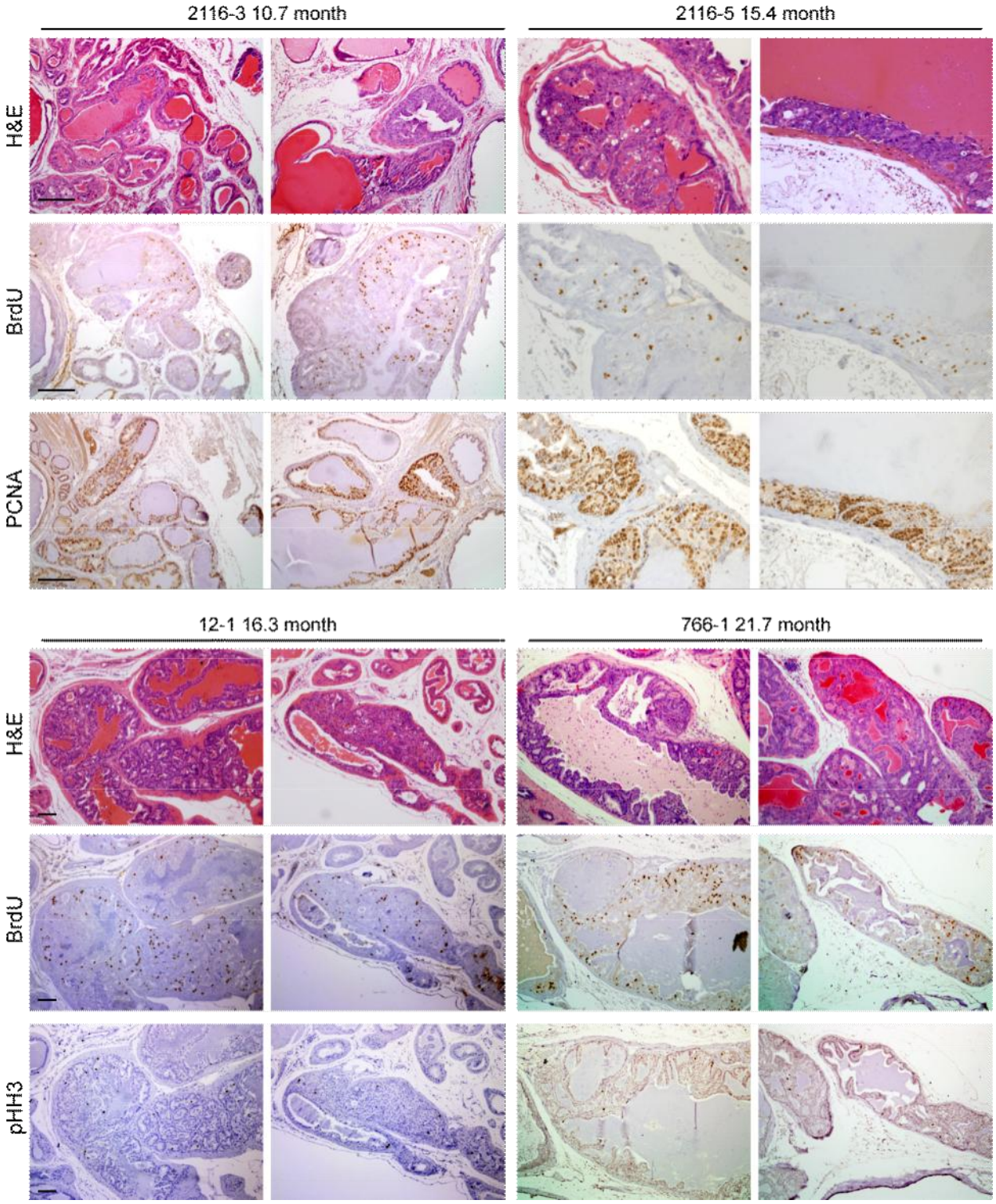


Figure S6. Related to Figure 7. Prostate sections from *Skp2*$^{-/-}$*;PB-Cre4;Rb1*$^{lox/lox}$*;Trp53*$^{lox/lox}$ mice in the 10-22 months age group (with their IDs) were analyzed with H&E, BrdU (2 hr labeling), PCNA, and pHH3 staining, as indicated. Scale bars = 100 μm.

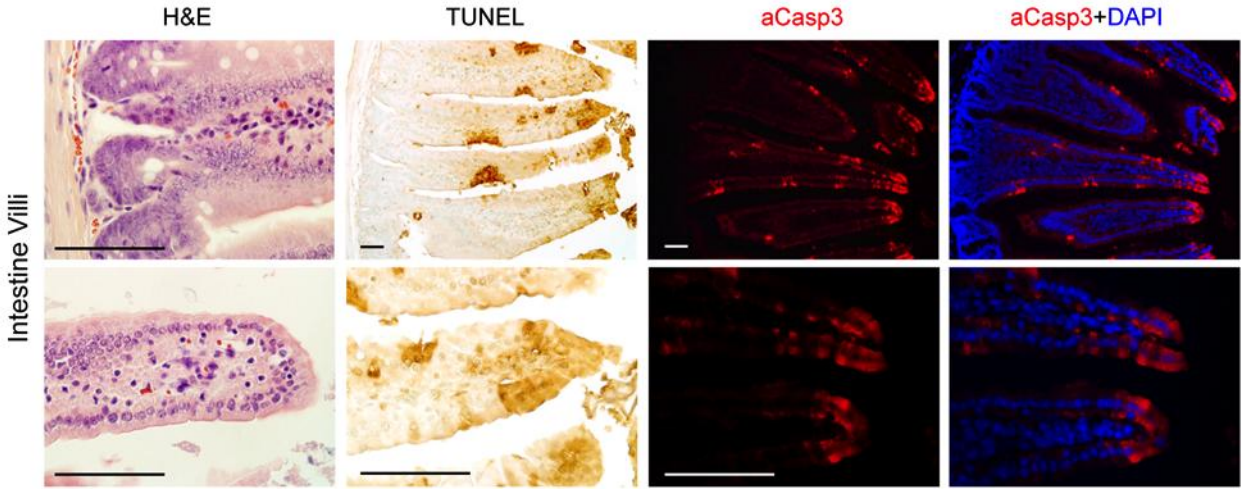


Figure S7. Related to Figure 8. Validation of TUNEL and aCasp3 staining for apoptosis with the characteristic distribution of apoptotic cells in intestine villi. Scale bars = 50 μm.

Supplemental Experimental Procedures

Mouse genotyping. For *POMC-Cre* transgenic mice: POMC-Cre-F: 5'-TGGCTCAATGTCCTTCCTGG-3' and POMC-Cre-R: 5'-GAAATCAGTGCGTTCGAACGCTAGA-3'. The transgene yields a PCR product of 400bp. For *Rb1*$^{lox/lox}$ mice: Rblox-F: 5'-CTCTAGATCCTCTCATTCTTC-3' and Rblox-R: 5'-CCTTGACCATAGCCCAGCAC-3'. The Rblox allele yields a PCR product of 310bp while the wild type allele a 250bp PCR product. For *Trp53*$^{lox/lox}$ mice: p53lox-F: 5'-CACAAAAACAGGTTAAACCCAG-3' and p53lox-R: 5'-AGCACATAGGAGGCAGAGAC-3'. The p53lox allele yields a PCR product of 370bp while the wild type allele a 288bp PCR product. For *Skp2*$^{+/-}$ mice: wild type allele, KN3: 5'-AGAGTGGAAGAACCCAGGCAGGAC-3' and KN4: 5'-CCCGTGGAGGGAAAAAGAGGGACG-3', yielding a 430bp PCR product, and KO allele: KN13: 5'- GCATCGCCTTCTATCGCCTTCTTG-3' and KN38: 5'-TTCCCACCCCCACATCCAGTCATT-3' yielding a PCR product of 500bp. For *PB-Cre4* mice: PB-CRE4-F: 5'-CTGAAGAATGGGACAGGCATTG-3' and PB-CRE4-R: 5'-CATCACTCGTTGCATCGACC-3'. The PB-Cre4 transgene yields a PCR product of 393bp. For *p27*$^{+/-}$ mice: WT-F: 5'-GATGGACGCCAGACAAGC-3' and WT-R: 5'-AGGGGCTTATGATTCTGAAAGTCG-3', p27KO-F: 5'-CCTTCTATCGCCTTCTTG-3' and p27KO-R: 5'-TGGAACCCTGTGCCATCTCTAT-3'. WT allele yields a PCR product of 166bp, KO allele a 500bp PCR product.

MEF preparation and infection. Embryos from timed pregnancies were harvested between E12.5-E14.5. Heads, livers, and blood clots were removed and the rest of tissues were minced and put into 1 ml Trypsin (25300-054, Gibco) for 10 min at 37°C. The tissue and Trypsin mixture was pipetted up and down several times and the dissociated cells were cultured in DMEM (11965-092, Giboco) containing 10% FBS (S11550, Atlanta Biologicals) and 1% Pen/Strep (15140-122, Giboco). Cells were split every 3-4 days during early passages, and MEFs at passage 2 or 3 were used for infection to delete, knockdown or overexpress specific proteins. Adeno-GFP and Adeno-CRE viruses were from Einstein Gene therapy Core, lentivirus vectors expressing p27, p53, or shRNAs from Einstein shRNA Core facility. Lentiviral helper constructs were from L. Naldini and A. Follenzi (Follenzi et al., 2000) and Xia Wang of Einstein Gene therapy Core. Lentivirus stocks were generated and concentrated as described previously (Sun et al., 2006).

SA-β-gal stain. To determine cellular senescence, MEFs and human breast cancer cell lines were plated in triplicate 6 cm plates for 3 days, and were fixed with 0.2% glutaraldehyde (G5882, Sigma-Aldrich) for 15 minutes at room temperature. SA-β-gal activity was determined by staining cells with SA-β-gal staining solution (5 mM Potassium Ferrocyanide, 5 mM Potassium Ferricyanide, 2 mM $MgCl_2$, 150

mM NaCl, 40 mM citric acid/sodium phosphate buffer pH6.0, and 1 mg/ml X-gal) for 16 hr (MEFs) and 5 hr (human cancer cell lines) at 37°C. Results were quantified under microscope.

FACS. Cells were washed and fixed by adding 6 ml ice-cold 80% EtOH (~70% EtOH final) while vortexing. Cells were then washed with 5 ml PBS and resuspended in 0.5 ml of 0.25 mg/ml RNase A and 10 µg/ml propidium iodide in PBS for FACS analysis. BrdU labeling was for 30 min in 10 µM BrdU. After EtOH fixation for 30 minutes in 4˚C, cells were incubated in 1 ml 2 M HCl, 0.5% Triton X-100 for 30 min at room temperature, neutralized in 1 ml 0.1 M Sodium Borate pH9.0, and resuspended in 100-200 µl 0.2% BSA/0.5% Tween 20/PBS for 5 min. We used 20 µl anti-BrdU-FITC per 10^6 cells and incubated for 30-60 min at room temperature. Staining for pHH3 was performed with the same steps as anti-BrdU. FACS and data analysis were performed together with the Einstein FACS Core.

Cell proliferation assay. $3X10^5$ cells were plated in 6 cm plates in triplicate, and cell numbers were counted every 2 or 3 days under the microscope with hemocytometers for indicated days.

Western blot. MEF lysates were prepared with RIPA buffer (50 mM Tris-HCl pH7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, and standard protease inhibitors). Prostate tissues or tumors were snap-frozen in dry ice and stored in −80 °C. Frozen tissues were homogenized with Dounce glass homogenizer in tissue lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20, 1 mM dithiothreitol and standard protease inhibitors). Debris was removed by centrifugation for 10 min at 14,000 r.p.m. in an Eppendorf Centrifuge 5415C at 4 °C. Protein concentrations of the extracts were determined by Bio-Rad protein assay kit, and equal amounts of protein samples were loaded on 10% SDS gels and blotted onto polyvinylidene fluoride membrane.

RT-qPCR. RNA was extracted by Trizol reagent (Invitrogen). Oligo-dT and SuperScript II (Invitrogen) were used for the synthesis of the first-strand cDNA at 42 °C for 60 min. The qPCR primers for mouse Skp2, p21, p27, KPC1, Pirh2 and GAPDH are listed below. SYBR Green PCR Master Mix (4309155, ABI) and the standard program of ABI 7500 Fast real-time PCR were used.

ChIP and qPCR. MEFs were cross-linked with 1% formaldehyde (#252549, Sigma-Aldrich) in culture medium for 10 min at room temperature. Cross-link was stopped by addition of glycine to a final concentration of 0.125 M for 5 min at room temperature. Fixed cells were scraped off the plates, washed twice with PBS, pelleted and stored. Cell pellets were re-suspended in 1 ml of cell lysis buffer containing PMSF (5 mM HEPES pH8.0, 85 mM KCl, 0.5% Triton X-100,) and put on ice for 20 min before being pelleted again by centrifugation at 2500g for 5 minutes. Pellets were re-suspended with 600 µl nuclear lysis buffer containing PMSF (50 mMTris-HCl pH8.0, 10 mM EDTA, 1% SDS). DNA was sheared by sonication for 15 s x 4 in a 60 Sonic Dismembrator (Fisher), at a power setting of 10. The resultant genomic DNA fragments with a bulk size of 100–1000 bp were precleared by addition of 20 µl of blocked protein A-Agrose beads (P9269, Sigma). The beads were blocked with tRNA and Salmon sperm DNA. The precleared chromatin were incubated overnight at 4°C with 2 µg antibody (for p53, SC-71817X, Santa Cruz Biotechnology). The chromatin and antibody mixture were then incubated with blocked protein A-Agrose beads at 4°C for 4 hr with rotation and followed by successive 10-minute washes in 1 ml of IP dilution buffer (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, and 0.01% SDS), dialysis buffer (2 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.2% Sarkosyl), TSE-500 buffer (2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, and 0.1% SDS), LiCl detergent buffer (100 mM Tris-HCl pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 500 mM LiCl), and TE buffer. After washing, the samples were eluted with elution buffer (50 mM $NaHCO_3$, 1% SDS). The eluted material was purified by Qiagen PCR purification kit for qPCR.

Tissue preparation and staining. Tissues were fixed in 10% Formalin (SF 100-4, Fisher Scientific), embedded in paraffin wax and sectioned. Paraffin sections were processed according to protocols for the SuperPicture™ kit (879263 and 879163, Invitrogen). Secondary antibodies for IHC were HRP polymer conjugated anti-rabbit or anti-mouse IgG. Color development was by DAB chromogen supplied in the SuperPicture™ kit. Sections were counterstained by Harris Hematoxylin (S212, Poly Scientific). Immunofluorescence detection was by Rhodamine conjugated goat anti-rabbit-IgG (SC-2091, Santa Cruz Biotechnology) or Fluorescein conjugated horse-anti-mouse-IgG (FI-2000, Vector laboratory).

TSA™PLUS Fluorescence Kit (NEL741001KT, PerkinElmer) was used to amplify signals when indicated. DNA was stained with DAPI. Feulgen staining was done with Schiff's reagent (s272, Poly Scientific R&D Corp.), and counter stained with fast green (s2114, Poly Scientific R&D Corp.).

DNA content analysis with tissue sections. DAPI stained prostate sections were scanned and analyzed by the iCys® Research Imaging Cytometer and iCys® Cytometric Analysis Software (CompuCyte Corporation). At least 500 prostate epithelial cells were analyzed for each genotype.

Primer sequences

qPCR primers	
GAPDH	
GAPDH-F	5'-AATGTGTCCGTCGTGGATCT-3'
GAPDH-R	5'-GGTCCTCAGTGTAGCCCAAG-3'
p27	
p27-F	5'-GCGGTGCCTTTAATTGGGTCT-3'
p27-R	5'-GGCTTCTTGGGCGTCTGC T-3'
p21	
p21-F	5'-CCCGAGAACGGTGGAACTT-3'
p21-R	5'-TGCAGCAGGGCAGAGGAA-3'
Skp2	
Skp2-F	5'-CCAGCAAGACTTCTGAACTGC-3'
Skp2-R	5'-GAGGCACAGACAGGAAAAGA-3'
KPC1	
KPC1-F	5'-GAAGTCCAGGGTCACAGGCA-3'
KPC1-R	5'-GGTTATGGAAGTTTAGCGGTT T-3'
Pirh2	
Pirh2-F	5'-GCCTTAGACATGACTCGGTAC-3'
Pirh2-R	5'-CTGCTGATCCACTGGCACTCT-3'
ChIP primers	
GAPDH	
GAPDH-F	5'-GAGTTCTGGGAGTCTCGTGG-3'
GAPDH-R	5'-CTCTTCGGGTGGTGGTTCA-3'
p21	
p21-F	5'- TTCAGTGCAGGGTGGTGGA-3'
p21-R	5'- ATTCTGCTGGCAAAGTGG G-3'
Pirh2	
Pirh2-F	5'- CATTTCTTCCCTCCGAACCCT-3'
Pirh2-R	5'- CTAGTTCCAGGACAGCCAAAG C-3'
KPC1	
KPC1-F	5'-GTAGGCAGGACTTAGGAGGGT-3'
KPC1-R	5'-GGATAGATGGTGGCAGGAAG-3'
Skp2	
Skp2-F	5'-TCTCCCCTGTTGCACAGTTT-3'
Skp2-R	5'-TGATGAGTCTCCCAAATACCA-3'
p27	
p27-F	5'-ACCGCCATATTGGGCAACTAAA-3'
p27-R	5'-GTGGCAAACAGTCGGAGCGTA-3'

Knockdown and overexpression vectors

Constructs	Packaging plasmids	Mammalian selection	Sequences
pGIP2-shp27-1	pCMV-dR8.91; pMD2-VSVG	Puromycin (10ug/ml)	5'-AGAAGATTCTTCTTCGCAA-3'
pGIP2-shp27-2	pCMV-dR8.91; pMD2-VSVG	Puromycin (10ug/ml)	5'-ACAATAACACTAAAATTTT-3'
Lenti-CMV-miSkp2	pMDLg/pRRE, pRSV-REV, and pMD2-VSVG	N/A	5′-CCTTAGACCTCACAGGTAA-3′
pLOC-p27	pCMV-dR8.91; pMD2-VSVG	Blasticidin (10ug/ml)	Human p27 ORF
pLX304-p53	pMDLg/pRRE,	Blasticidin	Human p53 ORF

	pRSV-REV, and pMD2-VSVG	(10ug/ml)	
pLOC-RNF123	pCMV-dR8.91; pMD2-VSVG	Blasticidin (10ug/ml)	Human KPC1 ORF
pLOC-RCHY1	pCMV-dR8.91; pMD2-VSVG	Blasticidin (10ug/ml)	Human Pirh2 ORF

Supplemental Reference

Follenzi, A., Ailles, L.E., Bakovic, S., Geuna, M., and Naldini, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet *25*, 217-222.