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Supplemental Information

Bmi-1 Is a Crucial Regulator of Prostate Stem Cell Self-Renewal and Malignant Transformation

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Inventory of supplemental information

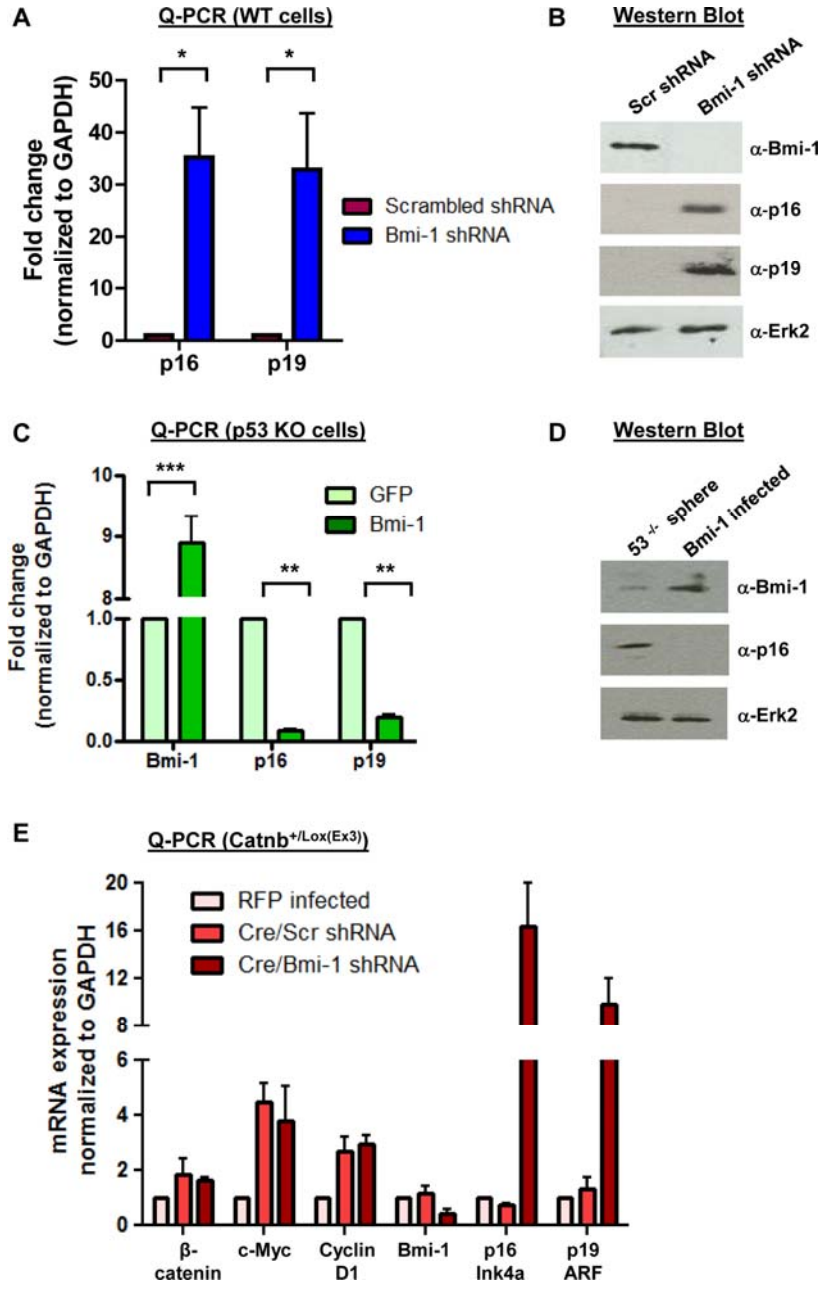

Figure S1. Bmi-1 modulates p16 and p19 expression. (A) Q-PCR data shows p16 and p19 mRNA levels in the scrambled shRNA and Bmi-1 shRNA infected 1st generation spheres. Values were normalized to GAPDH mRNA levels from each condition. Error bars represent duplicate samples from 3 separate experiments. (B) Western blots show protein expression levels of Bmi-1, p16, p19, and loading control Erk2 in the scrambled shRNA and Bmi-1 shRNA infected 1st generation spheres. Related to Figure 2. See also Figure S2. (C) Bar graphs show Bmi-1, p16, and p19 mRNA levels in control GFP and Bmi-1 infected spheres from generation 1 spheres. Values were normalized to GAPDH mRNA levels in each condition. Data represents Mean +/- SEM. (D) Western blot shows protein levels on Bmi-1, p16, and Erk2 in lysates from control GFP and Bmi-1 infected spheres. Related to Figure 2.

(E) Q-PCR analysis of uninfected, Cre/scrambled shRNA, and Cre/Bmi-1 shRNA infected Catnb$^{+/Lox(Ex\ 3)}$ cells. Data shown as mean +/- SEM, and represent 3 independent experiments. Related to Figure 4.

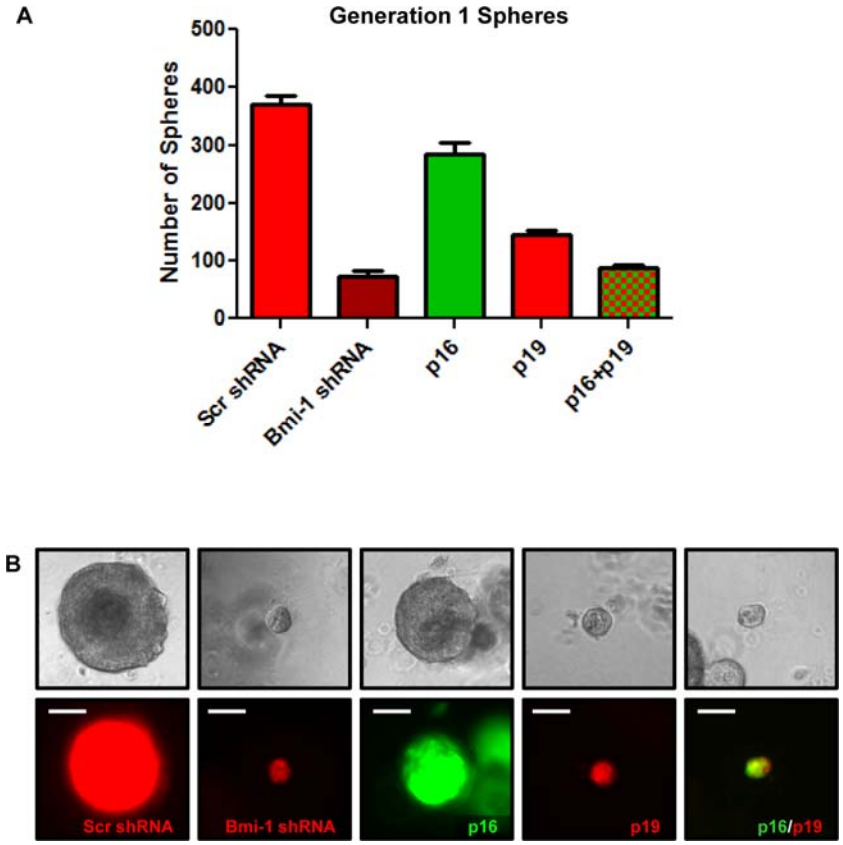

Figure S2. Over-expression of p16 and p19 together results in similar sphere inhibition as Bmi-1 loss. (A) Frequency of primary prostate spheres formed from infected cells. Cells were infected with scrambled shRNA, Bmi-1 shRNA, p16, p19, or co-infected with p16+p19 and plated in the sphere assay in triplicates. Graph represents 2 separate experiments, shown as mean +/- SEM. (B) Representative transilluminating (top) and fluorescence (bottom) images of spheres. Scale bar=100µm. Related to Figure 2 and Figure S1.

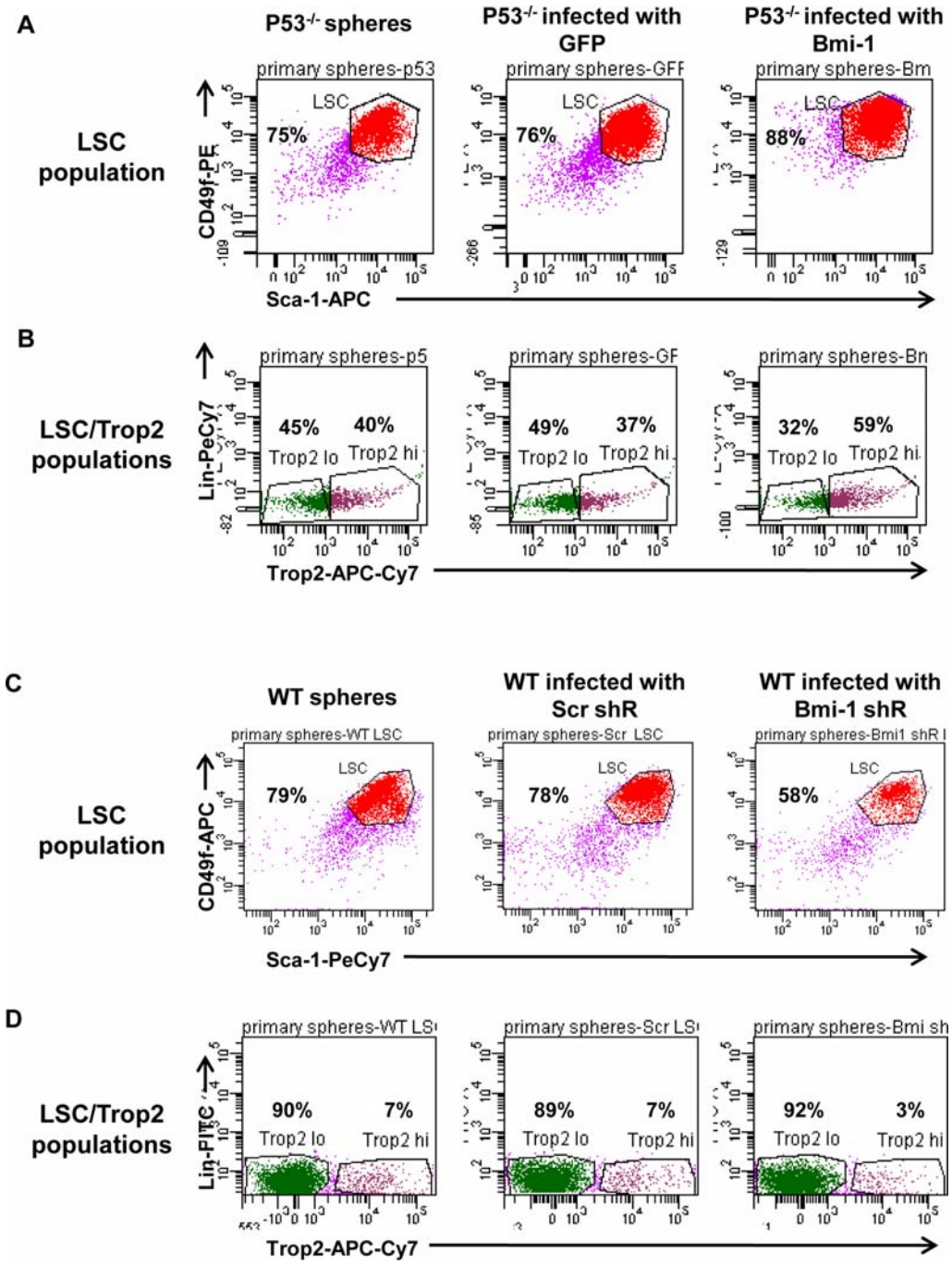

Figure S3. The LSC and LSCT stem cell populations increase upon Bmi-1 over-expression and decrease upon Bmi-1 knock-down in sphere cells. FACS plots show the $Lin^{-}Sca\text{-}1^{+}CD49f^{+}$ (LSC) stem cell population in (A) P53-/- uninfected, GFP, and Bmi-1 infected sphere cells and (C) C57/Bl6 uninfected, scrambled, and Bmi-1 shRNA infected sphere cells. FACS plots also show the $Trop2^{hi}$ and $Trop2^{lo}$ populations within the LSC fraction of (B) P53-/- uninfected, GFP, and Bmi-1 infected sphere cells and (D) C57/Bl6 uninfected, scrambled, and Bmi-1 shRNA infected sphere cells. Related to Figure 3.

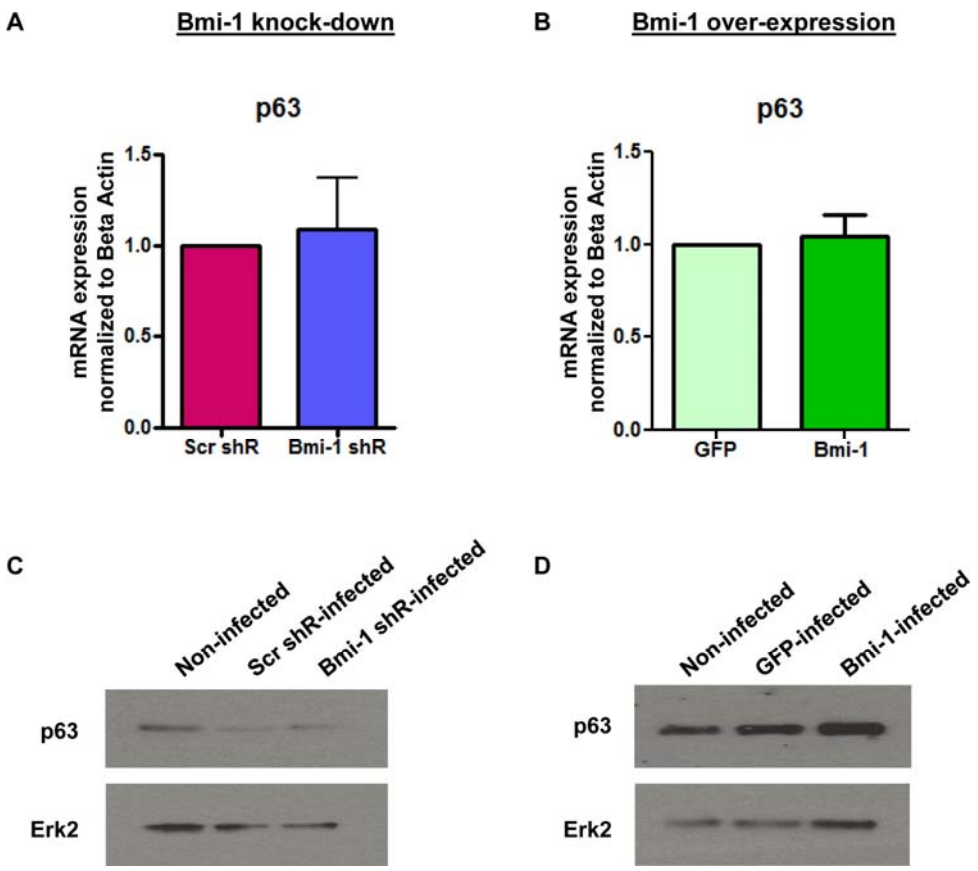

Figure S4. Quantification of p63 mRNA levels upon Bmi-1 knock-down and over-expression. Prostate cells were infected with (A and C) Scr or Bmi-1 shRNA, and (B and D) GFP or Bmi-1 lentivirus. Cells were harvested 5 days after infection to analyze mRNA and protein levels of p63. (A and B) Bar graphs show mRNA levels of p63, normalized to beta-actin expression. Data represents mean +/- SEM. (C and D) Western blot analysis of p63 and Erk2 in (C) Bmi-1 knock-down conditions and (D) Bmi-1 over-expression conditions. Related to Figure 3.

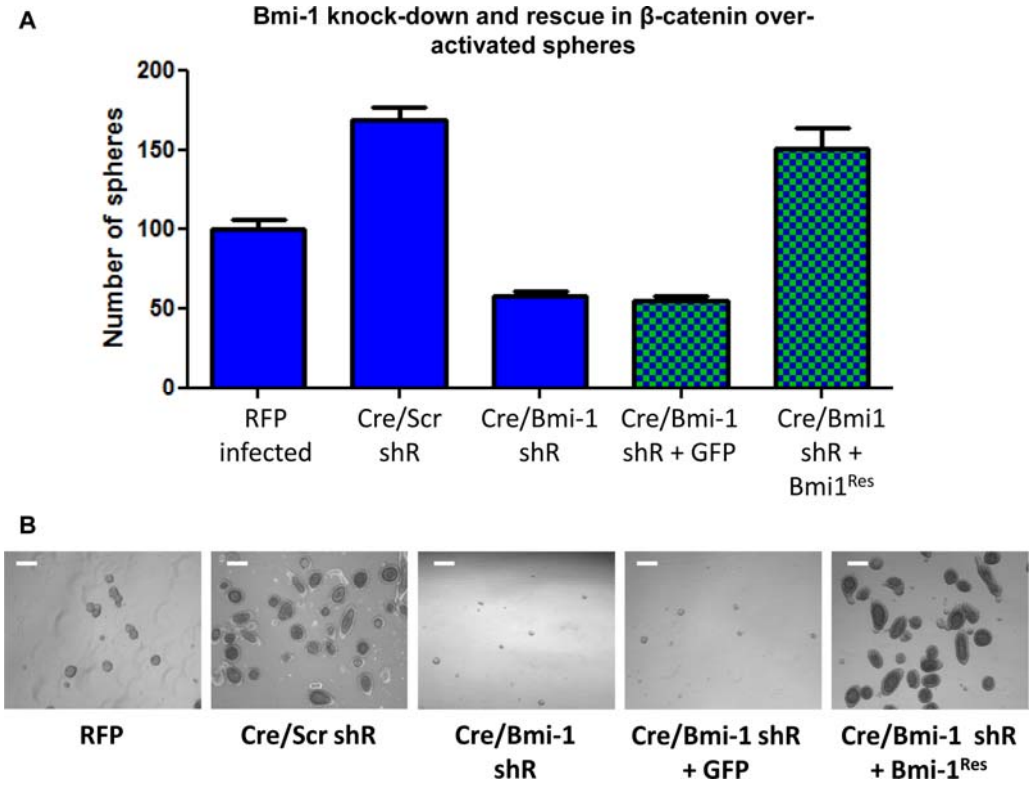

Figure S5. Reintroduction of Bmi-1Res into Bmi-1 shRNA infected beta-catenin over-activation spheres rescues sphere forming capacity. (A) Frequency of primary prostate spheres generated from equal numbers of Catnb$^{+/Lox(Ex\ 3)}$ cells infected with RFP, Cre/scrambled shRNA, Cre/Bmi-1 shRNA, or co-infected with Cre/Bmi-1 shRNA+GFP, or Cre/Bmi-1shRNA+Bmi-1Res. Graph represents triplicates from 2 separate experiments, shown as mean +/- SEM. (B) Representative transilluminating images of spheres from each condition after dispase treatment and release from Matrigel. Bar=200μm. Related to Figure 4.

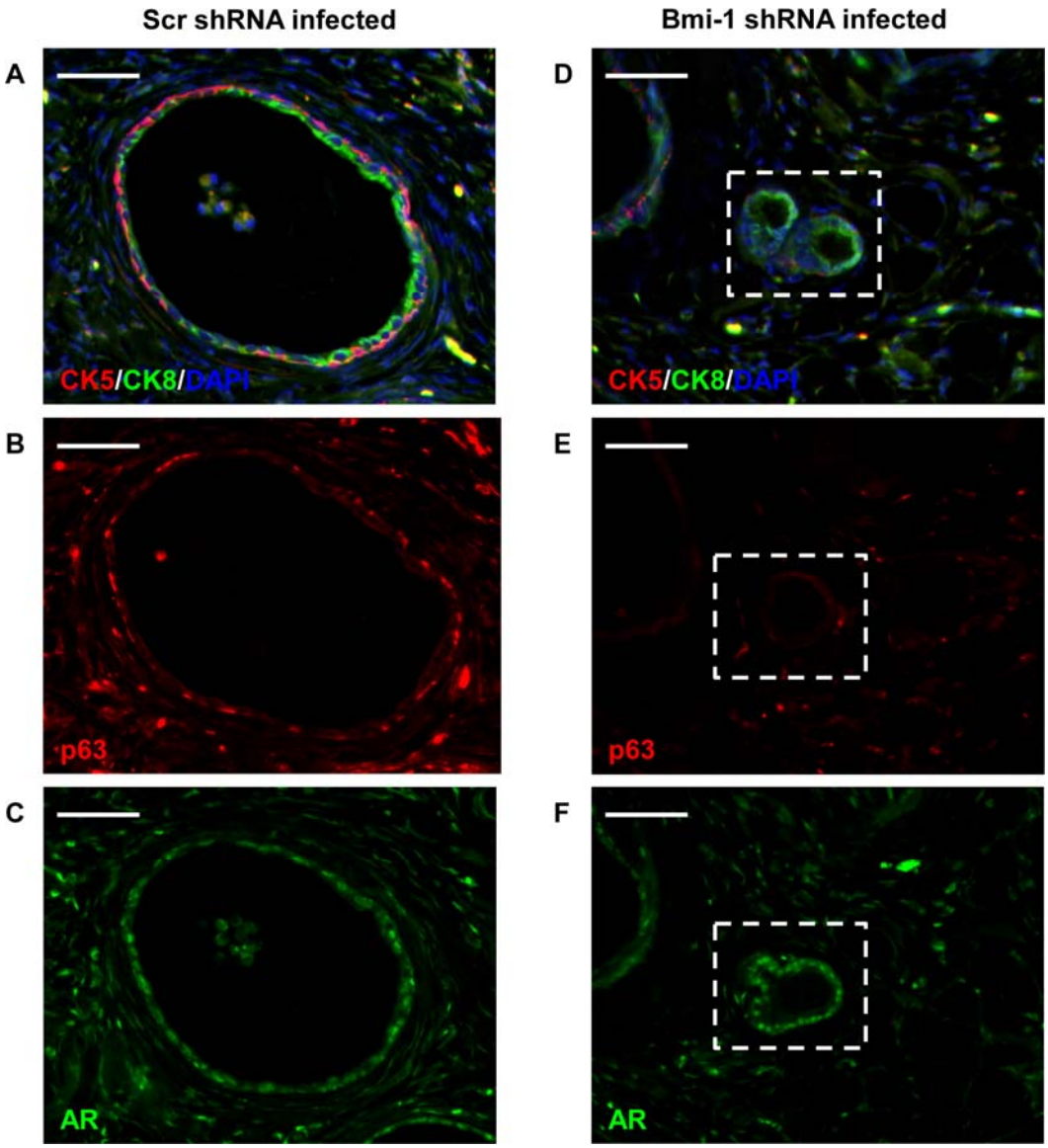

Figure S6. Subcutaneous regenerated tubules from Bmi-1 shRNA infected cells have a more differentiated phenotype. Images show (A) Cytokeratin 5 and 8 co-expression, (B) p63, and (C) AR expression in serial sections of Scrambled shRNA infected tubules. Images show (D) Cytokeratin 5 and 8 co-expression, (E) p63, and (F) AR expression in serial sections of Bmi-1 shRNA infected tubules. Bar=100μm. Related to Figure 5.

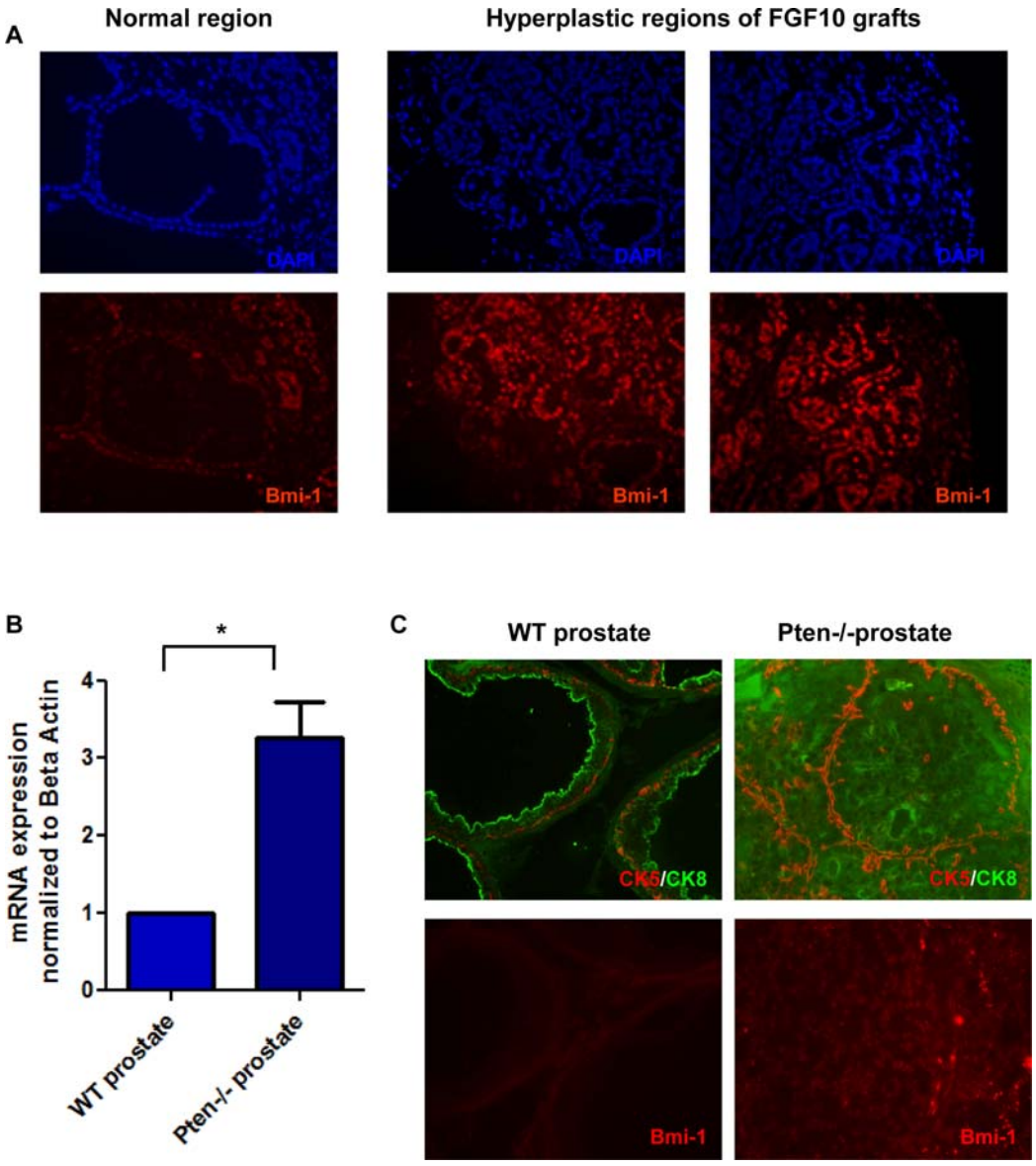

Figure S7. Bmi-1 is over-expressed in the hyperplastic epithelial regions of FGF10 induced transformation grafts and in Pten-null prostates. (A) C57/Bl6 prostate cells were combined with FGF10 over-expressing UGSM cells to produce grafts with hyperplastic regions. Immunohistochemical stains show DAPI staining (blue - top row) and the corresponding Bmi-1 expression (red - bottom row). Related to Figure 6. (B) Graph shows mRNA expression levels of Bmi-1 in prostates of 12-week old WT and age-matched Pten-null mouse. Data represented as mean +/- SEM. (C) Images show cytokeratin 5 and 8 (top) and Bmi-1 (bottom) staining in 6 month old WT and age-matched Pten-null prostate. Related to Figure 7.

Table S1. Quantification of the number of daughter spheres formed from single spheres with different Bmi-1 levels. Related to Figure 3.

	# of spheres that can generate daughter spheres	Average # of spheres
Scr shRNA	11 out of 12	**~25**
Bmi-1 shRNA	4 out of 12	**~1**
GFP	13 out of 15	**~26**
GFP-Bmi-1	14 out of 15	**~37**

Table S2. Weight and incidence of grafts regenerated from scrambled and Bmi-1 shRNA infected prostate cells. Related to Figure 5.

	Scr shRNA	Bmi-1 shRNA
Average weight of regenerated grafts (mg)	**5.68 ± 0.62**	**3.58 ± 0.34**
Incidence of grafts containing (red) tubules	**9/9**	**7/9**

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Vector production and viral packaging:

Murine Bmi-1 was PCR amplified from a pGEM vector containing the open reading frame of Bmi-1 (a generous gift from Dr. Sean Morrison), and XbaI restriction digest sites were added to the ends. The amplification product was subcloned into the XbaI site of FUCGW, directly downstream of the Ubiquitin promoter.

Oligonucleotides were designed to target the mouse *Bmi-1* ORF (TAAAGGATTACTACACGCTAATG) followed by the loop sequence (TTCAAGAGA) and the reverse compliment of the targeting sequence. Two protecting mutations were introduced into the sense region (TA**G**AGGATTACTACA**T**GCTAATG). Oligos were annealed and ligated into the psiRNA1.4 vector immediately downstream of the H1 promoter and before the termination sequence (Ax6). A scrambled shRNA was designed by mutating 3 of the anti-sense nucleotides in the Bmi-1 shRNA sequence (C**G**TTAGCGTGTAGT**T**AT**G**CTTTA). The H1/shRNA cassettes were subcloned into PacI site of the FUCRW vector. Knock down efficiencies of the shRNA vectors were tested in 293T cells by co-transfecting each shRNA vector with Bmi-1-FUCGW. Lentivirus was made and endogenous protein knock-down efficiency was tested by infecting 3T3 cells with this virus.

The shRNA resistant clone of murine Bmi-1 was made by introducing 3 silent mutations into the shRNA target region of Bmi-1 (TAAAAGACTACTACAC**CT**T**G**ATG). The resistant clone was subcloned into the XbaI site of FUCGW. The vector was co-transfected into 293T cells along with Bmi-1 shRNA-FUCRW to test the resistance against knock-down.

Cre/shRNA vectors were created by first replacing the CMV-GFP cassette of the FUCGW vector with the Cre-GFP fusion construct by subcloning it into the XbaI sites, downstream of the Ubiquitin promoter. Cre-RFP non-fused vectors were also created. The H1/scrambled or H1/Bmi-1 shRNA cassette was subcloned into the PacI site of these vectors. Lentivirus was produced, and Cre expression and Bmi-1 knock-down efficiencies were tested by infecting 3T3 cells with the virus and running western blot analyses.

Both p16 and p19 were generous gifts from Dr. Charles Sherr. The ORFs of both genes were cut out of the original vector with EcoRI and re-cloned into the FUCGW (p16) and FUCRW (p19) vectors into the EcoRI site, directly downstream of the Ubiquitin promoter.

Immunohistochemistry:

Sections (4μm) were stained with antibodies against Bmi-1 (Clone F6, Millipore; 1:200), p63 (Clone 4A4, Santa Cruz; 1:200), AR (Clone N-20, Santa Cruz; 1:200), Ki67 (VP-K451, Vector; 1:400), or In Situ Cell Death Detection Kit (Roche Applied Sciences). For visualization anti-rabbit-Alexa488 or –Alexa594 (Invitrogen; 1:1000) was used for AR and Ki67; anti-mouse-biotin (Dako; 1:250) was used for Bmi-1 and p63, followed by SA-Alexa594 (Invitrogen; 1:500). Coverslips were mounted and sections were counterstained with DAPI (Vector), and sections were visualized with fluorescence microscopy.

Primers used for Q-PCR:

CUSTOM DESIGNED

Gene	5' primer	3' primer
GAPDH	TGTTCCTACCCCCAATGTGT	GGTCCTCAGTGTAGCCCAAG
c-Myc	CCTACCCTCTAACGACAGC	CTCTGACCTTTTGCCAGGAG
Cyclin D1	ATGGTAGCTGCTGGGAGCGTGGT	CTTTGCGGGTGCCACTACTTGGT
p16	GTGTGCATGACGTGCGGG	GCAGTTCGAATCTGCACCGTAG
p19	GCTCTGGCTTTCGTGAACATG	TCGAATCTGCACCGTAGTTGAG

COMMERCIAL

Gene	Company	Catalogue Number
Bmi-1	SABiosciences	PPM37679E-200
β-catenin	SABiosciences	PPM03384A-200

Immunoblotting:

Dissociated sphere cells were lysed in RIPA buffer composed of 50mM Tris-HCl (pH 8.0), 150mM NaCl, 0.1%SDS, 0.5% SD1% NP-40, 1mM EDTA, 1mM PMSF, and cocktail protease inhibitor (Roche). Equal amounts of protein lysates (5-20 μg/lane) were separated by SDS-PAGE (Invitrogen), transferred onto nitrocellulose membranes, which were blocked in 5% milk for 1 hour at RT and blotted with antibody overnight at 4°C: Bmi-1 (Clone F6, Millipore; 1:1000), p63 (Clone 4A4, Santa Cruz; 1:300), β-catenin (Clone 14; BD Biosciences; 1:1000), Cyclin D1 (Clone DCS6, Cell Signaling; 1:1000), c-Myc (Clone 9E10 Santa Cruz; 1:1000), p19

(ab-80, Abcam; 1:500), p16 (M-156, Santa Cruz; 1:200) and Erk2 (Clone C-14, Santa Cruz; 1:1000). Peroxidase-conjugated 2° antibodies (BioRad) were used at 1:10,000 for 1hr at RT. ECL (Millipore) was the chemiluminescent substrate and blots were developed with Kodak Film.