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Report

# Essential Role for Activation of the Polycomb Group (PcG) Protein Chromatin Silencing Pathway in Metastatic Prostate Cancer

**Olga P. Berezovska**[1]
**Anna B.Glinskii**[1]
**Zhijian Yang**[3]
**Xiao-Ming Li**[3]
**Robert M. Hoffman**[3]
**Gennadi V. Glinsky**[1,2,4]*

[1]Translational and Functional Genomics Laboratory; Ordway Cancer Center; Ordway Research Institute, Inc.; Center for Medical Sciences; Albany, NewYork USA

[2]Division of Urology; Albany Medical College; Albany, NewYork USA

[3]AntiCancer, Inc.; San Diego, California USA

[4]Sidney Kimmel Cancer Center; San Diego, California USA

*Correspondence to: Gennadi V. Glinsky; Translational and Functional Genomics Laboratory; Ordway Research Institute Cancer Center, Center for Medical Science; 150 New Scotland Avenue; Albany, New York 12208; Tel.: 518.641.6975; Fax: 518. 641.6303; Email: gglinsky@ordwayresearch.org

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**KEY WORDS**

Ezh2, BMI1, xenograft models, metastasis, prostate cancer, microarray analysis, stem cells, self-renewal, Polycomb proteins, survival predictor

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See page 1899.

## ABSTRACT

The Polycomb group (PcG) gene *BMI1* is required for the proliferation and self-renewal of normal and leukemic stem cells. Overexpression of *Bmi1* oncogene causes neoplastic transformation of lymphocytes and plays essential role in pathogenesis of myeloid leukemia. Another PcG protein, Ezh2, was implicated in metastatic prostate and breast cancers, suggesting that PcG pathway activation is relevant for epithelial malignancies. Whether an oncogenic role of the *BMI1* and PcG pathway activation may be extended beyond the leukemia and may affect progression of solid tumors as well remains unknown. Here we demonstrate that activation of the BMI1 oncogene-associated PcG pathway plays an essential role in metastatic prostate cancer, thus mechanistically linking the pathogenesis of leukemia, self-renewal of stem cells, and prostate cancer metastasis. To characterize the functional status of the PcG pathway in metastatic prostate cancer, we utilized advanced cell- and whole animal-imaging technologies, gene and protein expression profiling, stable siRNA-gene targeting, and tissue microarray (TMA) analysis in relevant experimental and clinical settings. We demonstrate that in multiple experimental models of metastatic prostate cancer both *BMI1* and *Ezh2* genes are amplified and gene amplification is associated with increased expression of corresponding mRNAs and proteins. For the first time, we provide images of human prostate carcinoma metastasis precursor cells isolated from blood and shown to overexpress both BMI1 and Ezh2 oncoproteins. Consistent with the PcG pathway activation hypothesis, increased BMI1 and Ezh2 expression in metastatic cancer cells is associated with elevated levels of H2AubiK119 and H3metK27 histones. Quantitative immunofluorescence colocalization analysis and expression profiling experiments documented increased BMI1 and Ezh2 expression in clinical prostate carcinoma samples and demonstrated that high levels of BMI1 and Ezh2 expression are associated with markedly increased likelihood of therapy failure and disease relapse after radical prostatectomy. Gene-silencing analysis reveals that activation of the PcG pathway is mechanistically linked with highly malignant behavior of human prostate carcinoma cells and is essential for in vivo growth and metastasis of human prostate cancer. We conclude that the results of experimental and clinical analyses indicate the important biological role of the PcG pathway activation in metastatic prostate cancer. Our work suggests that the PcG pathway activation is a common oncogenic event in pathogenesis of metastatic solid tumors and provides justification for development of small molecule inhibitors of the PcG chromatin silencing pathway as a novel therapeutic modality for treatment of metastatic prostate cancer.

## INTRODUCTION

The *Polycomb group (PcG)* gene *Bmi1* determines the proliferative potential of normal and leukemic stem cell[1] and is required for the self-renewal of hematopoietic and neural stem cells.[1-3] Self-renewal ability is one of the essential defining properties of a pluripotent stem cell-like phenotype distinguishing stem cells from many other cell types.[4] *Bmi1* oncogene is expressed in all primary myeloid leukemia and leukemic cell lines analyzed so far[1,5] and overexpression of *Bmi1* causes neoplastic transformation of lymphocytes.[6,7] Recent experimental observations documented an increased *Bmi1* expression in human nonsmall-cell lung cancer,[8] human breast carcinomas,[9,10] human medulloblastomas,[11] prostate carcinomas,[12] and colon cancer,[13] suggesting that an oncogenic role of the *Bmi1* activation may be extended beyond the leukemia and, perhaps, may affect progression of the epithelial malignancies and other solid tumors as well.

Increased expression of another PcG protein Ezh2 was documented in multiple types of human cancer, including hematological malignancies;[14-18] prostate carcinomas;[19-22]

breast carcinomas;[21,23-26] bladder carcinomas;[27,28] cutaneous melanomas;[21] bronchial squamous cell carcinomas;[29] multiple myelomas.[30] Initial experimental evidence supports the hypothesis that Ezh2 may function as a bona fide human oncogene.[23,31] Consistent with this idea, several studies demonstrated Ezh2 amplification in human cancer cell lines and clinical tumor samples.[22,31,32]

Recent experimental evidence support the hypothesis that increased expression of the *BMI1* oncogene is one of the key regulatory factors determining a cellular phenotype captured by the expression of a death-from-cancer signature in a broad spectrum of therapy-resistant clinically lethal malignancies.[12,33,34] A mouse/human comparative translational genomics approach was utilized to identify the 11-gene signature distinguishing stem cells with normal self-renewal function versus stem cells with diminished self-renewal ability due to the loss of the *BMI1* gene and similarly expressed in metastatic prostate tumors.[12,33,34] Distant metastatic lesions consistently display a stem cell-like expression profile of the 11-gene signature as revealed by the analysis of metastases and primary tumors from a transgenic mouse model of prostate cancer and cancer patients. Cancer patients with a stem cell-like expression profile of the 11-gene signature in primary prostate tumors had a significantly higher probability of disease recurrence after radical prostatectomy and diminished likelihood of the relapse-free survival after therapy.[12]

Kaplan-Meier analysis demonstrated that a stem cell-like expression profile of the 11-gene signature in tumors is a consistent powerful predictor of a short interval to disease recurrence, distant metastasis, and death after therapy in cancer patients diagnosed with multiple types of cancer.[12,33,34] These data suggest that a conserved *BMI1* oncogene-associated pathway, such as the PcG chromatin silencing pathway,[35-40] might be similarly activated in both normal stem cells and a highly malignant subset of human cancers diagnosed in a wide range of organs and uniformly exhibiting a marked propensity toward metastatic dissemination as well as a therapy resistance phenotype. Here we tested the validity of this concept with respect to the metastatic prostate cancer and demonstrated that PcG pathway appears activated in a majority of clinical samples of prostate adenocarcinoma and overexpression of both BMI1 and Ezh2 oncoproteins is essential for malignant behavior in vivo of human prostate carcinoma metastasis precursor cells.

## MATERIALS AND METHODS

**Clinical samples.** Two clinical outcome sets comprising 21 (outcome set 1) and 79 (outcome set 2) samples were utilized for analysis of the association of the therapy outcome with expression levels of the BMI1 and Ezh2 genes and other clinico-pathological parameters. Original gene expression profiles of the 21 clinical samples analyzed in this study were reported elsewhere.[41] Primary gene expression data files of clinical samples as well as associated clinical information can be found at www-genome.wi.mit.edu/cancer/.

Prostate tumor tissues comprising second clinical outcome set were obtained from 79 prostate cancer patients undergoing therapeutic or diagnostic procedures performed as part of routine clinical management at the Memorial Sloan-Kettering Cancer Center (MSKCC). Clinical and pathological features of 79 prostate cancer cases comprising validation outcome set are presented elsewhere.[12,42] Median follow-up after therapy in this cohort of patients was 70 months. Samples were snap-frozen in liquid nitrogen and stored at -80°C. Each sample was examined histologically using H&E-stained cryostat sections. Care was taken to remove non-neoplastic tissues from tumor samples. Cells of interest were manually dissected from the frozen block, trimming away other tissues. All of the microarray studies analyzed in this paper were previously published and were conducted under MSKCC Institutional Review Board-approved protocols. Overall, 146 human prostate tissue samples were analyzed in this study, including forty-six samples in a tissue microarray (TMA) format. TMA samples analyzed in this study were exempt according to the NIH guidelines.

**Cell culture.** Cell lines used in this study were previously described.[32,42-45] The LNCap- and PC-3-derived cell lines were developed by consecutive serial orthotopic implantation, either from metastases to the lymph node (for the LN series), or reimplanted from the prostate (Pro series). This procedure generated cell variants with differing tumorigenicity, frequency and latency of regional lymph node metastasis.[32,42-45] Except where noted, cell lines were grown in RPMI1640 supplemented with 10% FBS and gentamycin (Gibco BRL) to 70–80% confluence and subjected to serum starvation as described,[32,42-45] or maintained in fresh complete media, supplemented with 10% FBS.

**Anoikis assay.** Cells were harvested by 5-min digestion with 0.25% trypsin/0.02% EDTA (Irvine Scientific, Santa Ana, CA, USA), washed and resuspended in serum free medium.[12,43,44] Cells at concentration $1.7 \times 10^5$ cells/well in 1 ml of serum free medium were plated in 24-well ultra low attachment polystyrene plates (Corning Inc., Corning, NY, USA) and incubated at 37°C and 5% $CO_2$ overnight. Viability of cell cultures subjected to anoikis assays were >95% in Trypan blue dye exclusion test.

**Apoptosis assay.** Apoptotic cells were identified and quantified using the Annexin V-FITC kit (BD Biosciences Pharmingen, www.bdbisciences.com) per manufacturer instructions.[12,43,44] The following controls were used to set up compensation and quadrants: (1) Unstained cells; (2) Cells stained with Annexin V-FITC (no PI); (3) Cells stained with PI (no Annexin V-FITC). Each measurements were carried out in quadruplicate and each experiments were repeated at least twice. Annexin V-FITC positive cells were scored as early apoptotic cells; both Annexin V-FITC and PI positive cells were scored as late apoptotic cells; unstained Annexin V-FITC and PI negative cells were scored as viable or surviving cells. In selected experiments apoptotic cell death was documented using the TUNEL assay.

**Flow cytometry.** Cells were washed in cold PBS phosphate-buffered saline and stained according to manufacturer's instructions using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Flow analysis was performed by a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). Cell Quest Software was used for data acquisition and analysis. All measurements were performed under the same instrument setting, analyzing $10^3$–$10^4$ cells per sample [12,43,44].

**Immunofluorescence microscopy.** Cells fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PFA/PBS) for 15 min were permeabilized with 0.5% Triton-X100 (Sigma, St.Louis, Mo, USA)/PBS for 5 min. After washing in PBS, cells were incubated in PBS containing 100 mM glycine for 10 min. Primary antibodies were diluted in 0.5% BSA/0.05% gelatin cold water fish skin/ PBS, and cells were incubated in this buffer for 10 min before antibodies were applied for 16 hrs at room temperature. After washing in PBS buffer, cells were incubated with secondary antibodies at 1:500 dilution. Coverslips were mounted in Prolong (Molecular Probes, Inc.). Images were collected on an inverted microscope (OlympusIX70) equipped with a DeltaVision imaging system using a x 40 objective. Images were processed by softWoRx v.2.5 software (Applied Precision

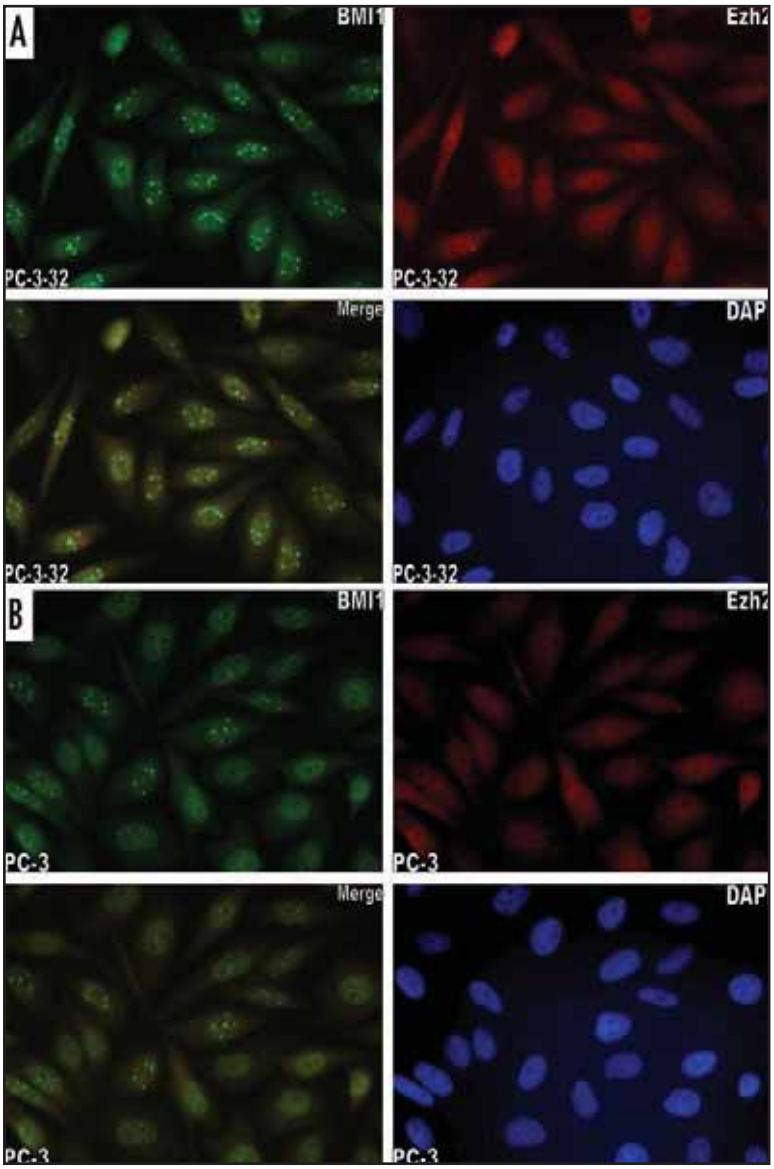



Figure 1. High expression levels of the BMI1 and Ezh2 oncoproteins in human prostate carcinoma metastasis precursor cells are associated with marked accumulation of a dual-positive high BMI1/Ezh2-expressing cell population and increased DNA copy number of the *BMI1* and *Ezh2* genes. (A–D) A quantitative immunofluorescence colocalization analysis of the BMI1 (mouse monoclonal antibody) and Ezh2 (rabbit polyclonal antibody) oncoproteins in PC-3-32 humna prostate carcinoma metastasis precursor cells and parental PC-3 cells. The protein expression differences and the accumulation of dual-positive high BMI1/Ezh2-expressing cells were confirmed using a second distinct combination of antibodies: rabbit polyclonal antibodies for BMI1 detection and mouse monoclonal antibodies for Ezh2 detection. (A) Immunofluorescent analysis of PC-3-32 cells; (B) Immunofluorescent analysis of PC-3 cells; (C) The histograms representing typical distributions of the BMI1 (top panels) and Ezh2 (bottom panels) expression levels in PC-3 and PC-3-32 cells; (D) The plots illustrating the levels of dual positive high BMI1/Ezh2-expressing cells in metastatic PC-3-32 cells (22.4%; top panel) and parental PC-3 cells (1.5%; bottom panel). The results of one of two independent experiments are shown. (E) A quantitative reverse-transcription PCR (Q-RT-PCR) analysis of DNA copy numbers of the *BMI1* and *Ezh2* genes in multiple experimental models of human prostate cancer. Note marked increase of the *BMI1* and *Ezh2* gene copy numbers in highly metastatic variants compared to the low metastatic counterparts in the multiple independently selected lineages. The results of one of two independent experiments are shown. (F) 3D-view of dual-positive high BMI1/Ezh2-expressing human prostate carcinoma cells in cultures of blood-borne metastasis precursor cells and parental cells. Adherent cultures of parental PC-3 (bottom three panels) and blood-borne PC-3-32 (top three panels) human prostate carcinoma cells were stained for visualization of the BMI1 and Ezh2 oncoproteins and analyzed using a multi-color fluorescent confocal microscopy. Note a higher proportion of cells with large discrete nuclear PcG bodies in the population of PC-3-32 human prostate carcinoma cells (typically, these cells contain six PcG bodies per nucleus). Blue, DNA; Green, BMI1; Red, Ezh2.

Inc., Issaquah, WA) and images were quantified with using ImageJ 1.29x software (http://rsb.info.nih.gov/ij).

Quantitative immunofluorescence analysis of the PcG protein expression was performed using human prostate cancer tissue microarrays (TMAs) representing 46 prostate tissue samples (thirty-nine cases of prostate cancer and seven cases of normal prostate). Analysis was carried-out on the prostate cancer TMAs from Chemicon (Temecula, CA; TMA #3202-4; four cancer cases and two cases of normal tissue; and TMA #1202-4; twenty five cases of cancer and five cases of normal tissue) and TMA of 10 cases of prostate cancer from the SKCC tumor bank (San Diego, CA). TMAs contain two 2.0 mm cores of each case and haematoxylin-and-eosin (H&E) sections which were used for visual selection of the pathological tissues, histological diagnosis, and grading by the pathologists of TMA providers.

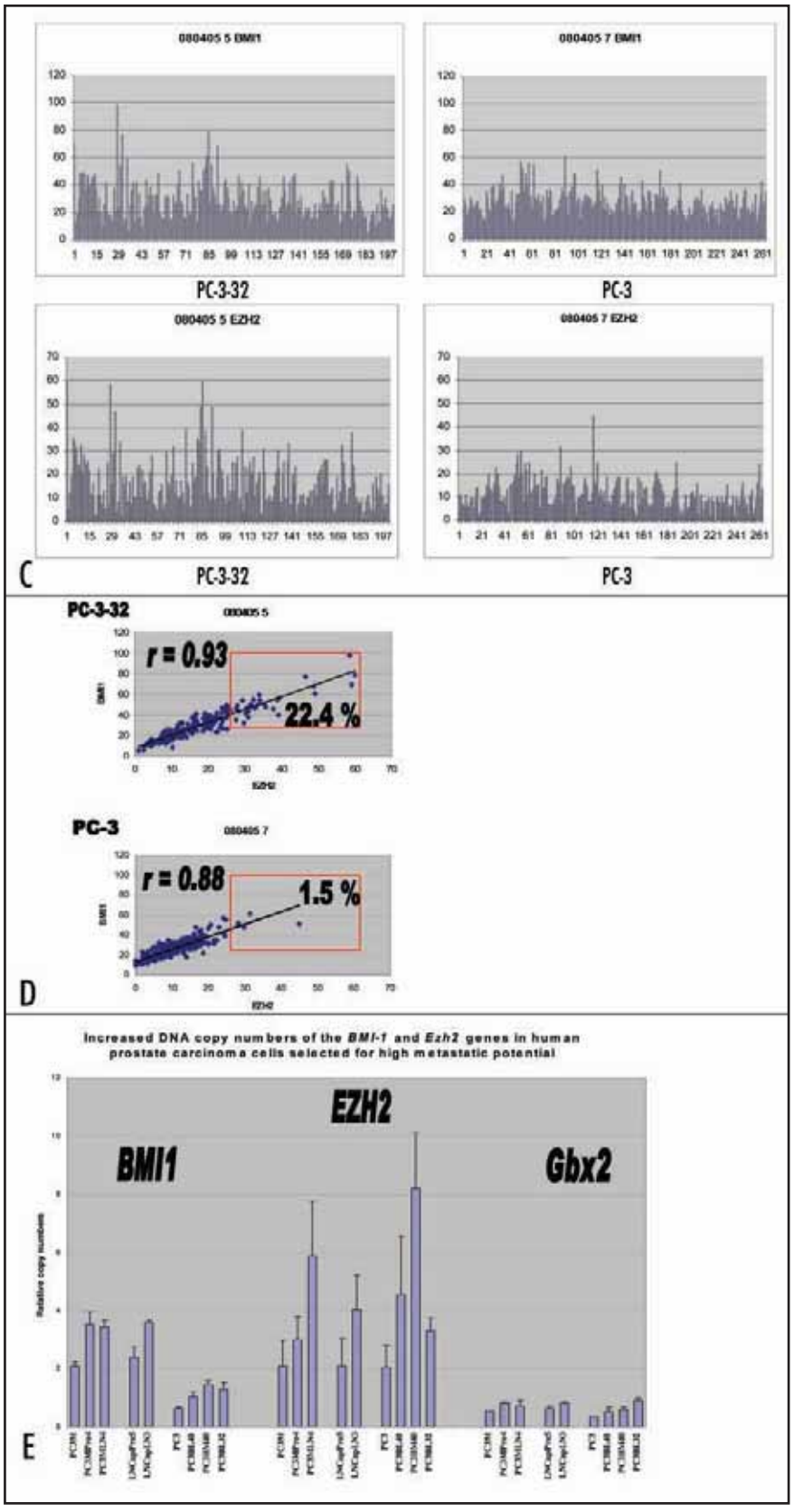
Four- or five-micrometer paraffin-embedded sections were baked at 56°C for 1 hour, allowed to cool for about 5 minutes, dewaxed in xylene, and rehydrated in a series of graded alcohols. Antigen retrieval was achieved by boiling slides in 10mM sodium citrate buffer, 0.05% Tween 20, pH 6.0 in a water bath for 30 minutes. The sections were washed with PBS, incubated in 100 mM glycine/PBS for 10 minutes, blocked in 0.5% BSA/0.05% gelatine cold water fish skin/PBS and incubated with primary antibody overnight.

Primary antibodies were EZH2 rabbit polyclonal antibody (1:50), BMI1 mouse monoclonal IgG1 antibody (1:50), ubiH2A mouse IgM (1:100), 3metK27 rabbit polyclonal antibody (1:100) (Upstate, Lake Placid, NY). Suz12 rabbit (1:50), AMACR rabbit (1:50) antibodies and Dicer mouse IgG1 (1:20) were purchased from Abcam (Cambridge, MA). BMI1 rabbit (1:50) and TRAP100 (1:50) goat antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1 rabbit polyclonal antibody (1:50) were from Biocare Medical (Concord, CA). EZH2 mouse monoclonal antibodies were kindly provided by Dr. A.P. Otte.

The primary antibodies were rinsed off with PBS and slides were incubated with secondary antibodies at 1:300 dilution for 1 hour at room temperature. Secondary antibodies (chicken antirabbit Alexa 594, goat antimouse Alexa 488, goat antimouse IgG1 Alexa 350, and donkey antigoat Alexa 488 conjugates) were from Molecular Probes (Eugene, OR). The slides were washed four times in PBS for five minutes each wash, rinsed in distilled water and the specimen were coversliped with Prolong Gold Antifade Reagent (Molecular Probes, Eugene, OR) containing DAPI. For negative controls, the primary antibodies were omitted. Three samples were excluded from analysis because one of the following reasons: core loss, unrepresentative sample, or sub-optimal DNA and antigen preservation.

Images were collected on an inverted fluorescent microscope (LEICA DMIRE 2 or Olympus IX70) using an x 40 objective.

Figure 1C–E. See Legend, page e1887.




Images were processed by Leica FW4000 software and images were quantified with using ImageJ 1.29x software. Expression values were measured in at least 200 nuclei from two microscopic fields for each case.

The measurements were carried out in the nuclei of individual cells defined by DAPI staining both in experimental and clinical samples. For experimental samples, the comparison thresholds for each marker combination were defined at the 90–95% exclusion levels for dual positive cells in corresponding control samples (parental low metastatic cells). For clinical samples, the comparison thresholds for each marker combination were defined at the 99% or greater exclusion levels for dual positive cells in corresponding control samples (normal epithelial cells in TMA experiments). All individual immmunofluorescent assay experiments (defined as the experiments in which the corresponding comparisons were made) were carried out simultaneously using the same reagents and included all experimental samples and controls utilized for a quantitative analysis. Statistical significance of the measurements was ascertained and consistency of the findings was confirmed in multiple independent experiments, including several independent sources of the prostate cancer TMA samples.

**Orthotopic xenografts.** Orthotopic xenografts of human prostate PC-3 cells and prostate cancer metastasis precursor sublines used in this study were developed by surgical orthotopic implantation as previously described.[32,42-45] Briefly, 2 x $10^6$ cultured PC-3 cells or sublines were injected subcutaneously into male athymic mice, and allowed to develop into firm palpable and visible tumors over the course of 2–4 weeks. Intact tissue was harvested from a single subcutaneous tumor and surgically implanted in the ventral lateral lobes of the prostate gland in a series of ten athymic mice per cell line subtype as described earlier.[32,42-45] During orthotopic cell inoculation experiments, a single-cell suspension of 1.5 x $10^6$ cells was injected into mouse prostate gland in a series of ten athymic mice per therapy group.

**siRNA experiments.** The target siRNA SMART pools and chemically modified degradation-resistant variants of the siRNAs (stable siRNAs) for *BMI1, Ezh2*, and control luciferase siRNAs were purchased from Dharmacon Research, Inc. siRNAs were transfected into human prostate carcinoma cells according to the manufacturer's protocols. Cell cultures were continuously monitored for growth and viability and assayed for mRNA expression levels of *BMI1, Ezh2*, and selected set of genes using RT-PCR and Q-RT-PCR methods. Eight individual siRNA sequences comprising the SMART pools (four sequences for each gene, *BMI1* and *Ezh2*) were tested and a single most effective siRNA sequence was selected for synthesis in the chemically modified stable siRNA form for each gene. We designed the siRNA treatment protocol [two consecutive treatments of cells in adherent cultures with 100 nM (final concentration) of Dharmacon degradation-resistant siRNAs at day 1 and 4 after plating] causing only moderate reduction in the average BMI1 and Ezh2 protein expression levels (20–50% maximal effect)

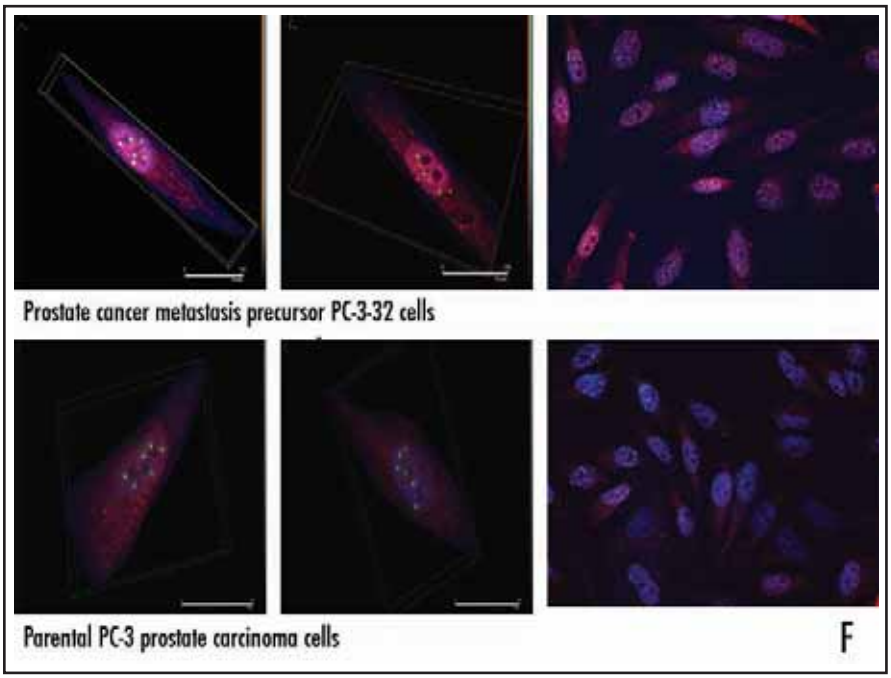



Figure 1F. See Legend, page e1887.

and having no or only marginal effect on cell proliferation in the adherent cultures (at most ~25% reduction in cell proliferation).

**Quantitative RT-PCR analysis.** The real time PCR methods measures the accumulation of PCR products by a fluorescence detector system and allows for quantification of the amount of amplified PCR products in the log phase of the reaction. Total RNA was extracted using RNeasy mini-kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. A measure of 1 μg (tumor samples), or 2 μg and 4 μg (independent preparations of reference cDNA and DNA samples from cell culture experiments) of total RNA was used then as a template for cDNA synthesis with SuperScript II (Invitrogen, Carlsbad, CA, USA). cDNA synthesis step was omitted in the DNA copy number analysis.[32] Q-PCR primer sequences were selected for each cDNA and DNA with the aid of Primer Express™ software (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed with the gene-specific primers.[12,32,42-45]

Q-PCR reactions and measurements were performed with the SYBR-Green and ROX as a passive reference, using the ABI 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Conditions for the PCR were as follows: one cycle of 10 min at 95°C; 40 cycles of 0.20 min at 94°C; 0.20 min at 60°C and 0.30 min at 72°C. The results were normalized to the relative amount of expression of an endogenous control gene *GAPDH*.

Expression of messenger RNA (mRNA) and DNA copy number for target genes and an endogenous control gene (*GAPDH*) was measured by real-time PCR method on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). For each gene at least two sets of primers were tested and the set-up with highest amplification efficiency was selected for the assay used in this study. Specificity of the assay for mRNA measurements was confirmed by the absence of the expected PCR products when genomic DNA was used as a template. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*: 5'- CCCTCAACGACCACTTTGTCA-3' and 5'-TTC-CTCTTGTGCTCTTGCTGG-3') was used as the endogenous RNA and cDNA quantity normalization control. For calibration and generation of standard curves, we used several reference cDNAs: cDNA prepared from primary in vitro cultures of normal human prostate epithelial cells (NPEC, refs. 12, 42 and 45), cDNA derived from the PC-3M human prostate carcinoma cell line,[12,32,42-45] and cDNA prepared from normal human prostate (NHP, refs. 12, 42 and 45). For DNA copy number analysis, human placental DNA was used as a normalization control.[32] Expression and DNA copy number analysis analysis of all genes was assessed at least in two independent experiments using reference cDNAs to control for variations among different Q-RT-PCR experiments. Prior to statistical analysis, the normalized gene expression values were log-transformed (on a base 10 scale) similarly to the transformation of the array-based gene expression data.

**Survival analysis.** The Kaplan-Meier survival analysis was carried out using the GraphPad Prism version 4.00 software (GraphPad Software, San Diego, CA; www.graphpad.com). The end point for survival analysis in prostate cancer was the biochemical recurrence defined by the serum PSA increase after therapy. Disease-free interval (DFI) was defined as the time period between the date of radical prostatectomy (RP) and the date of PSA relapse (recurrence group) or date of last follow-up (non-recurrence group). Statistical significance of the difference between the survival curves for different groups of patients was assessed using Chi square and Log-rank tests. To evaluate the incremental statistical power of the individual covariates as predictors of therapy outcome and unfavorable prognosis, we performed both univariate and multivariate Cox proportional hazard survival analyses. Clinico-pathological covariates included in this analysis were: preoperative PSA, Gleason score, surgical margins, extra-capsular invasion, seminal vesicle invasion, and age.

## RESULTS

**Activation of PcG protein chromatin silencing pathway in human prostate carcinoma metastasis precursor cells.** PcG pathway activation hypothesis implies that individual cells with activated chromatin silencing pathway would exhibit a concomitant nuclear expression of both BMI1 and Ezh2 proteins. Furthermore, cells with activated PcG pathway would manifest the increased expression levels of protein substrates targeted by the activation of corresponding enzymes to catalyze the H2A-K119 ubiquitination (BMI1-containing PRC1 complex) and H3-K27 methylation (Ezh2-containing PRC2 complex).[35-40] Observations that increased BMI1 expression is associated with metastatic prostate cancer[12] suggest that PcG pathway might be activated in metastatic human prostate carcinoma cells. Consistent with this idea, previous independent studies documented an association of the increased Ezh2 expression with metastatic disease in prostate cancer patients.[19] We therefore applied immunofluorescence analysis to measure the expression of protein markers of the PcG pathway activation in prostate cancer metastasis precursor cells isolated from blood of nude mice bearing orthotopic human prostate carcinoma xenografts.[43,44]

Immunofluorescence analysis reveals that expression of all four individual protein markers of PcG pathway activation is elevated in blood-borne human prostate carcinoma metastasis precursor cells

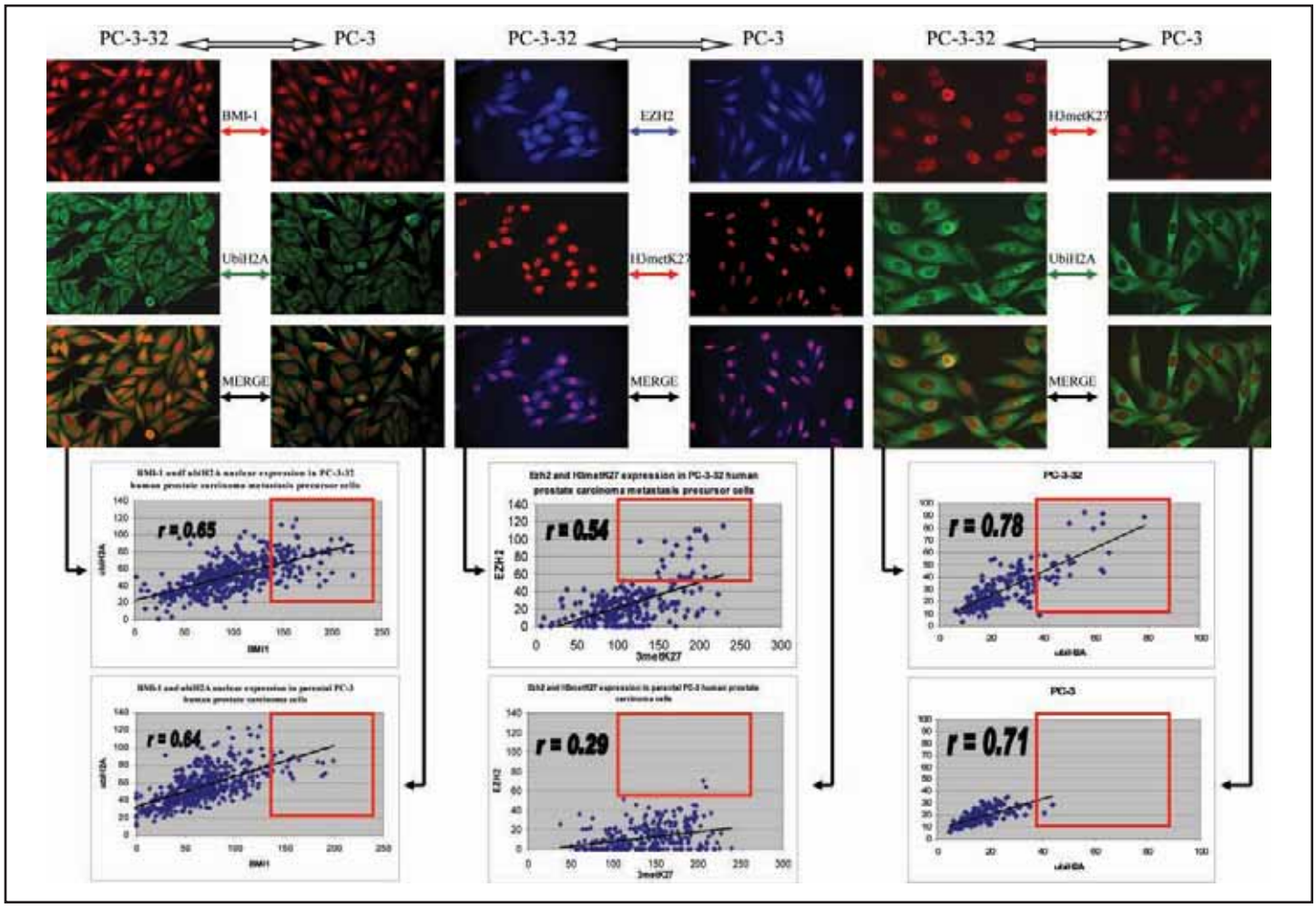



Figure 2. Activation of the PcG chromatin silencing pathway in metastatic human prostate carcinoma cells. A quantitative immunofluorescence colocalization analysis was utilized to measure the expression of the BMI1, Ezh2, H3metK27 and UbiH2A markers in human prostate carcinoma cells and calculate the numbers of dual-positive cells expressing various two-marker combinations. Note that high expression of the BMI1 and Ezh2 oncoproteins in PC-3-32 human prostate carcinoma metastasis precursor cells compared to parental PC-3 cells is associated with increased levels of histone H3 lysine 27 methylation (H3metK27), histone H2A lysine 119 ubiquitination (UbiH2A) and marked enrichment for dual-positive cell populations expressing high levels of BMI1/UbiH2A, Ezh2/H3metK27 and H3metK27/UbiH2A two-marker combinations.

compared to the parental cells comprising a bulk of primary tumors (Figs. 1 and 2). To document the PcG pathway activation in individual cells, we carried out the quantitative immunofluorescence colocalization analysis allowing for a simultaneous detection and quantification of several markers in a single cell. The quantitative immunofluorescence colocalization analysis demonstrates a marked enrichment of the population of blood-borne human prostate carcinoma metastasis precursor cells with the dual positive high BMI1/Ezh2-expressing cells (Fig. 1A). These results were confirmed using two different mouse/rabbit primary antibody combinations for BMI1 and Ezh2 protein detection as well as different secondary fluorescent antibodies. Similar enrichment for the PcG pathway activated cells in a pool of circulating metastasis precursor cells is evident for other two-marker combination panels as well (Fig. 2). In contrast to the protein markers of the PcG pathway activation, a significantly smaller fraction of cells expressing concomitantly high levels of the cytoplasmic AMACR/nuclear p63 proteins was detected in human prostate carcinoma metastasis precursor cells compared to the parental cell population (data not shown). Therefore, the results of a quantitative immunofluorescence colocalization analysis seems to indicate that measurements of several two-marker combinations demonstrate a significant enrichment of the population of prostate carcinoma metastasis precursor cells with the cells expressing high levels of the PcG pathway activation markers (Figs. 1 and 2). We and other previously documented that increased *BMI1* and *Ezh2* mRNA expression is associated with metastatic prostate cancer.[12,19-22] Taken together these data support the hypothesis that PcG chromatin silencing pathway is activated in blood-borne human prostate carcinoma metastasis precursor cells and might contribute to the ability of metastatic cancer cells to survive and grow at distant sites.

**Amplification of the *BMI1* and *Ezh2* genes in multiple experimental models of human prostate cancer.** Increased expression of oncogenes is often associated with gene amplification. In agreement with proposed oncogenic role of the BMI1 and Ezh2 overexpression in human prostate carcinoma cells, we documented a significant amplification of both *BMI1* and *Ezh2* genes in human prostate carcinoma cell lines representing multiple experimental models of metastatic prostate cancer (Fig. 1E). Notably, the level of gene amplification as determined by the measurement of DNA copy number for both *BMI1* and *Ezh2* genes is higher in metastatic cancer

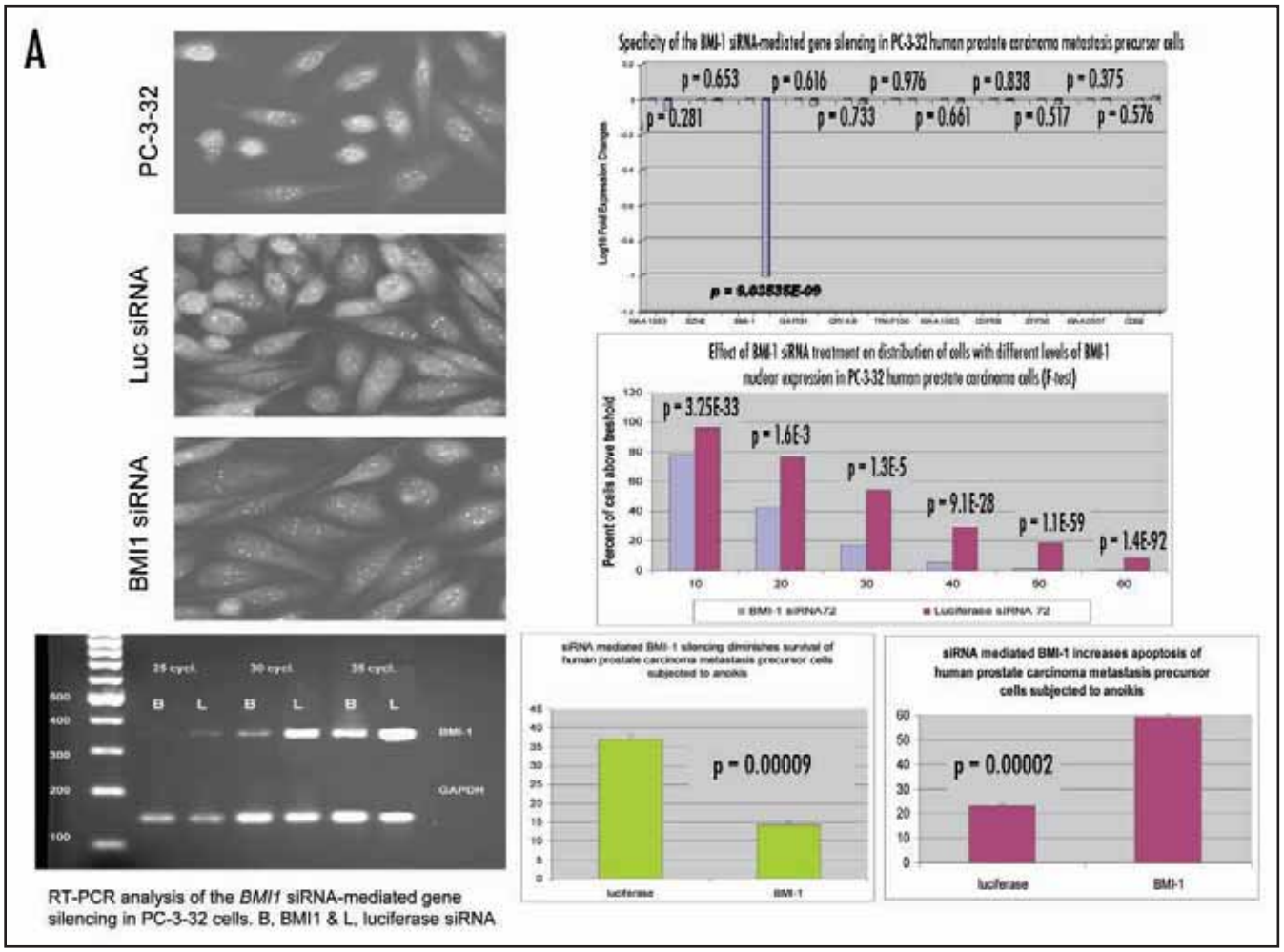



Figure 3. Targeted reduction of the BMI1 (A) or Ezh2 (B) expression increases sensitivity of human prostate carcinoma metastasis precursor cells to anoikis. Anoikis-resistant PC-3-32 prostate carcinoma cells[43,44] were treated with *BMI1*- or *Ezh2*-targeting siRNAs and continuously monitored for expression levels of the various mRNAs, BMI and Ezh2 oncoproteins, as well as cell growth and viability under various culture conditions. PC-3-32 cells with reduced expression of either BMI1 or Ezh2 oncoproteins acquired sensitivity to anoikis as demonstrated by the loss of viability and increased apoptosis compared to the control LUC siRNA-treated cultures growing in detached conditions. Note that targeted siRNA-mediated downregulation of expression of either BMI1 (A) or Ezh2 (B) oncoproteins causes similar loss of anoikis resistance in human prostate carcinoma metastasis precursor cells.

cell variants compared to the nonmetastatic or less malignant counterparts, suggesting that gene amplification may play a casual role in elevation of the BMI1 and Ezh2 oncoprotein expression levels and high BMI1/Ezh2-expressing cells may acquire a competitive survival advantage during tumor progression. These results are in agreement with the previous studies demonstrating amplification of the *Ezh2* gene in human cancer cell lines and clinical tumor samples.[22,31,32]

**PcG pathway activation renders circulating human prostate carcinoma metastasis precursor cells resistant to anoikis.** To ascertain the biological role of the PcG pathway activation in prostate cancer metastasis, human prostate carcinoma metastasis precursor cells were isolated from the blood of nude mice bearing orthotopic human prostate carcinoma xenografts,[43,44] transfected with *BMI1, Ezh2*, or control siRNAs, and continuously monitored for mRNA and protein expression levels of *BMI1, Ezh2*, and a set of additional genes and protein markers using inmmunofluorescence analysis, RT-PCR, and Q-RT-PCR methods. Q-RT-PCR and RT-PCR analyses showed that siRNA-mediated *BMI1*-silencing caused ~90% inhibition of the endogenous *BMI1* mRNA expression. The effect of siRNA-mediated *BMI1* silencing was validated at the protein expression level using immunofluorescence analysis (Fig. 3). The *BMI1* silencing was specific since the expression levels of nine un-related transcripts were not altered (Fig. 3). Consistent with the hypothesis that expression of genes comprising the 11-gene death-from-cancer signature is associated with the expression of the *BMI1* gene product, mRNA abundance levels of 8 of 11 interrogated *BMI1*-pathway target genes were altered in the human prostate carcinoma cells with siRNA-silenced *BMI1* gene.[12] For biological analysis we adopted the silencing protocol resulting in 80–100% reduction of the level of dual-positive BMI1/Ezh2 high-expressing metastasis precursor cells, thus yielding the cell population more closely resembling nontreated parental cells and markedly distinct from metastasis precursor cells treated with control siRNA (Figs. 3 and 4).

Reduction of the *BMI1* mRNA and protein expression in human prostate carcinoma metastasis precursor cells did not alter significantly the viability of adherent cultures grown at the optimal growth condition and in serum starvation experiments. siRNA treatment had only modest inhibitory effect on proliferation causing ~25% reduction in the number of cells. However, the ability of human prostate carcinoma cells to survive in nonadherent state was severely

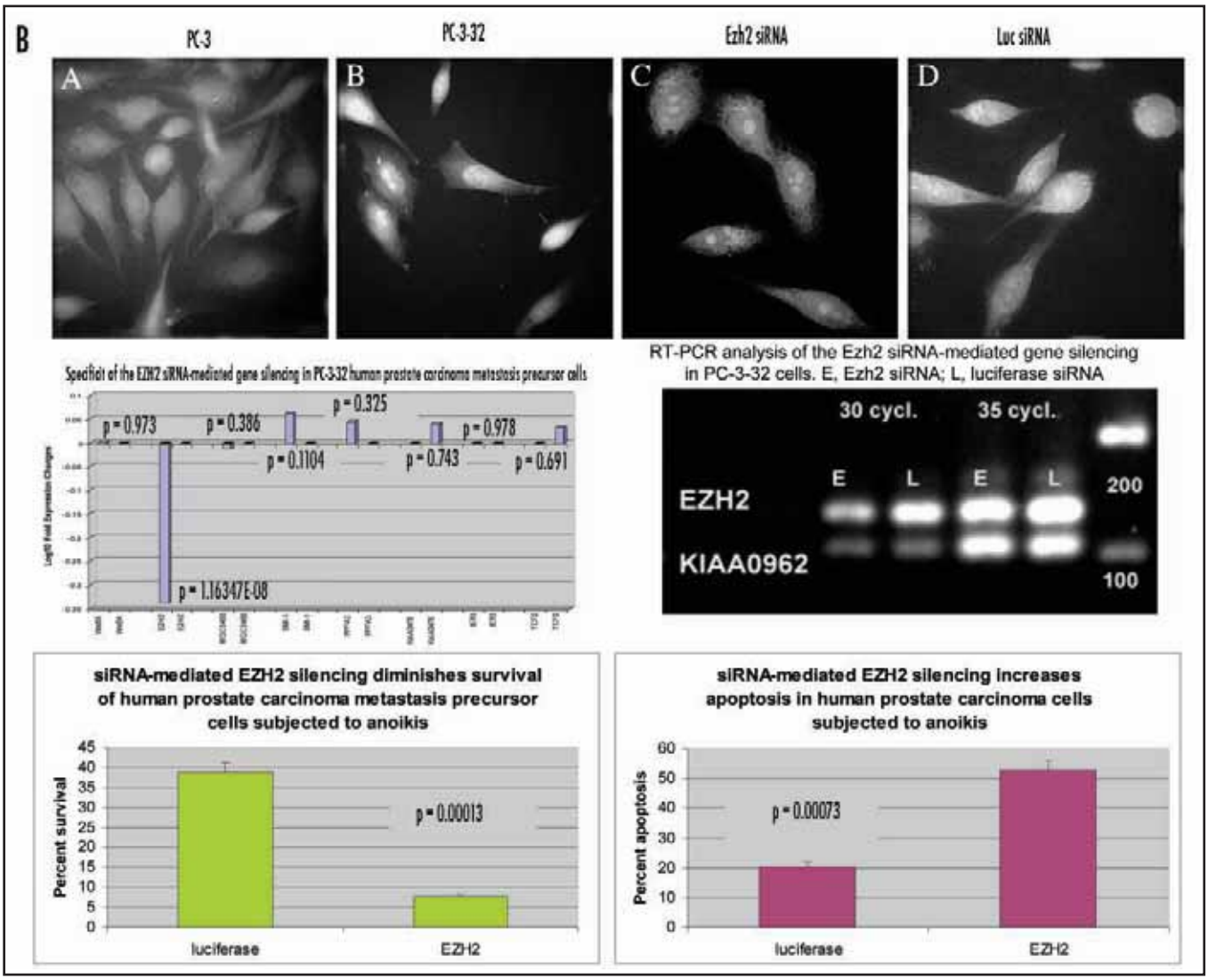



Figure 3B. See Legend, page e1892.

affected after siRNA-mediated reduction of the *BMI1* expression (Fig. 3). FACS analysis revealed ~3-fold increase of apoptosis in the *BMI1* siRNA-treated human prostate carcinoma cells cultured in nonadherent conditions (Fig. 3). These data suggest that human prostate carcinoma cells expressing high level of the BMI1 protein are more resistance to apoptosis induced in cells of epithelial origin in response to attachment deprivation (anoikis). It is likely that these anoikis-resistant cancer cells would survive better in blood or lymph during metastatic dissemination thus forming a pool of circulatory stress-surviving metastasis precursor cells.[12,43,44] Similar results were obtained when *Ezh2* silencing experiments were performed (Fig. 3), suggesting that targeting of either PRC1 or PRC2 complexes is sufficient for interference with the PcG pathway activity and inhibition of anoikis-resistance mechanisms in metastatic prostate carcinoma cells. Thus, downregulation of either *BMI1* or *Ezh2* expression in metastatic human prostate carcinoma cells is associated with diminished resistance to anoikis and increased apoptosis in non-adherent cultures (Fig. 3), suggesting that dual-positive high BMI1/Ezh2-expressing carcinoma cells may acquire a selective survival advantage during the passage through lymph and/or blood compartments.

**Targeted depletion of human prostate carcinoma cells with activated PcG pathway creates population of cancer cells with dramatically diminished malignant potential in vivo.** Results of our experiments demonstrate that a population of highly metastatic prostate carcinoma cells is markedly enriched for cancer cells expressing increased levels of multiple markers of the PcG pathway activation. These data suggest that carcinoma cells with activated PcG pathway may manifest a highly malignant behavior in vivo characteristic of cancer cell variants selected for increased metastatic potential. To test this hypothesis, we treated blood-borne human prostate carcinoma metastasis precursor cells with chemically modified stable siRNA targeting either *BMI1* or *Ezh2* mRNAs to generate a cancer cell population with diminished levels of dual positive high BMI1/Ezh2-expressing carcinoma cells. Stable siRNA-treated prostate carcinoma cells continue to grow in adherent culture in vitro for several weeks allowing for expansion of siRNA-treated cultures in quantities sufficient for in vivo analysis. These observations also indicate that treatment protocol was well-tolerated and was not detrimental for the general growth properties of a cancer cell population. Quantitative immunofluorescence colocalization analysis demonstrated that carcinoma cells after treatment with the *BMI1*- or *Ezh2*-targeting stable siRNA continue to express significantly lower

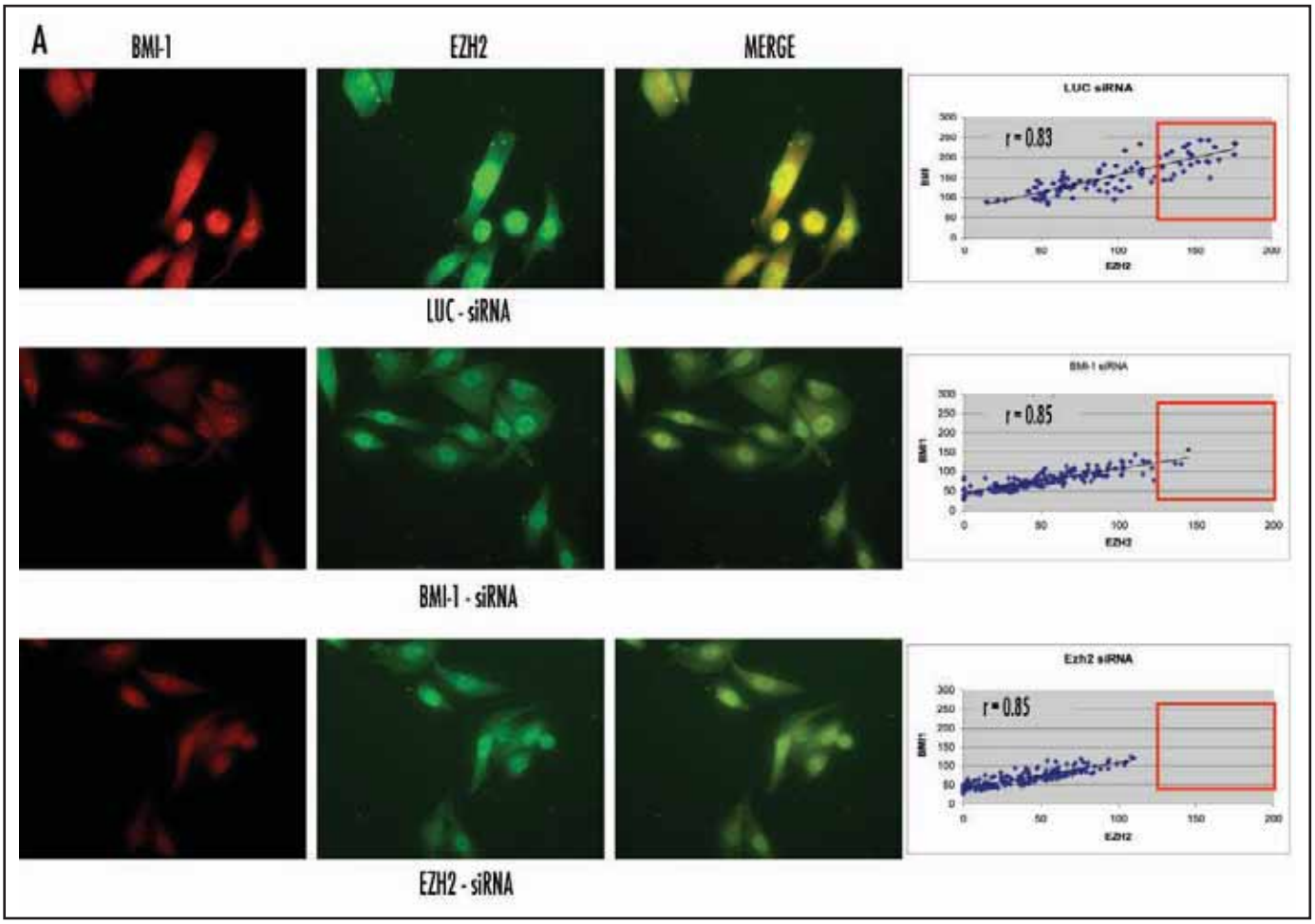
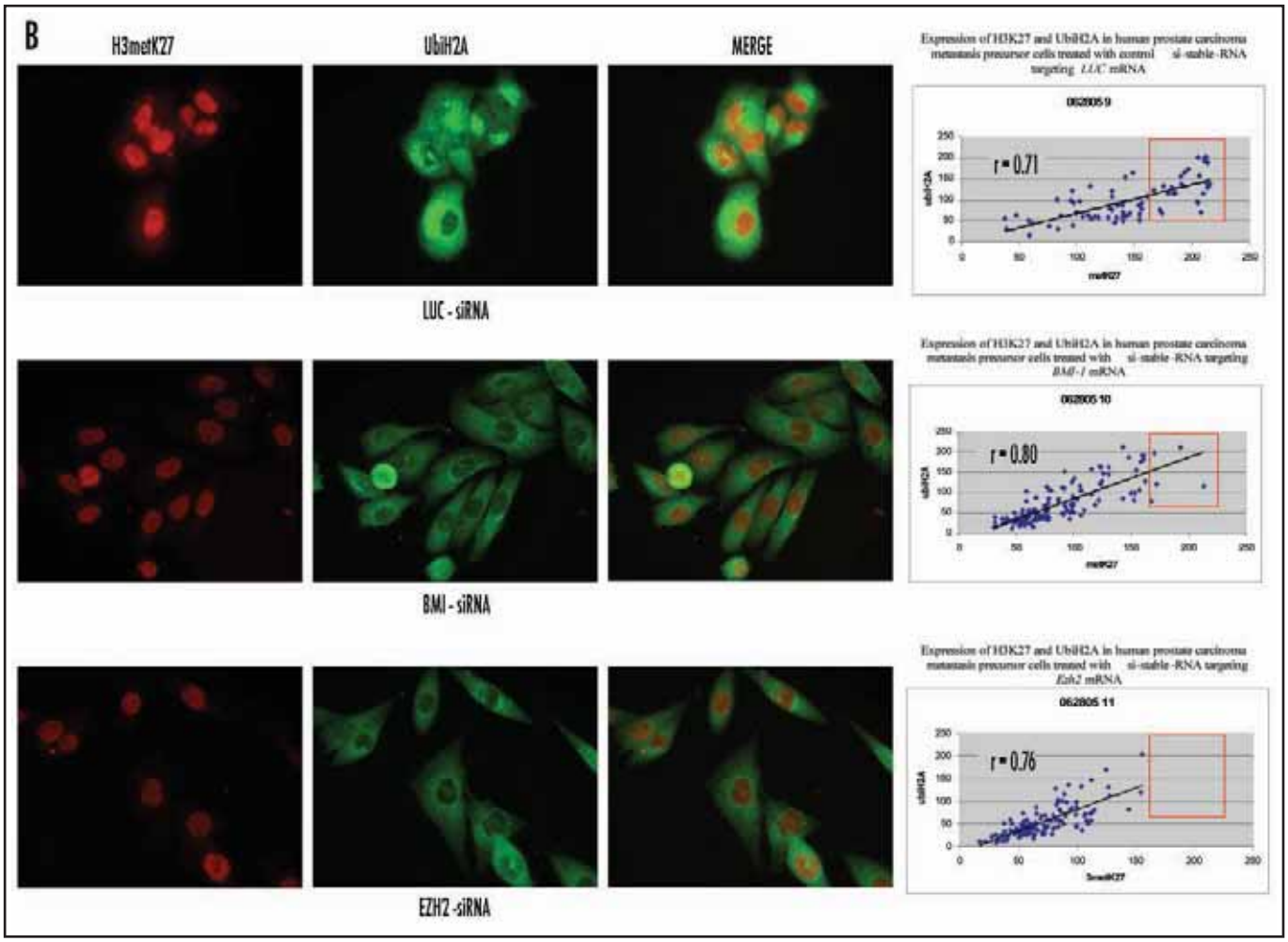



Figure 4. Treatment of human prostate carcinoma metastasis precursor cells with stable siRNAs targeting either *BMI1* or *Ezh2* gene products depletes a sub-population of dual positive high BMI1/Ezh2-expressing cells. Blood-borne PC-3-32 prostate carcinoma cells were treated with chemically modified resistant to degradation *LUC-*, *BMI1-* or *Ezh2*-targeting stable siRNAs and continuously monitored for expression levels of the BMI and Ezh2 oncoproteins. Two consecutive applications of the stable siRNAs caused a sustained reduction of the BMI1 and Ezh2 expression and depletion of the sub-population of dual positive high BMI1/Ezh2-expressing carcinoma cells (A). Consistent with selective targeting of the carcinoma cells with activated Polycomb pathway, similar depletion of the sub-population of dual-positive high H3metK27/ubiH2A-expressing cells was documented (B).The results at the 11-day post-treatment time point are shown.

levels of targeted proteins for extended period of time (~30–50% reduction at the 11 days post-treatment time point) compared to the cells treated with the control *LUC* siRNA (Fig. 4). Importantly, the siRNA-treated human prostate carcinoma cell populations were essentially depleted for dual positive high BMI1/Ezh2-expressing carcinoma cells (Fig. 4) thus setting up the stage for critical in vivo analysis using a fluorescent orthotopic model of human prostate cancer metastasis in nude mice.[43,44]

Remarkably, highly malignant human prostate carcinoma cell populations depleted for dual positive high BMI1/Ezh2-expressing cells demonstrated markedly diminished tumorigenic and metastatic potential in vivo (Fig. 5). Within three weeks after inoculation of the 1.5 x $10^6$ of tumor cells, 100% of control animals developed rapidly growing highly invasive and metastatic carcinomas in the mouse prostate and all animal died within 50 days of the experiment (Fig. 5). In contrast, only 20% of animals in both *BMI1-* and *Ezh2*-targeting therapy groups developed seemingly less malignant tumors causing death of hosts 78–87 days after tumor cell inoculation (Fig. 5). Significantly, 150 days after tumor cell inoculation 83% and 67% of animals remain alive and disease-free in the therapy groups targeting the BMI1 and Ezh2 proteins, respectively (Fig. 5; p = 0.0007, Log rank test).

**Increased levels of dual positive high BMI/Ezh2-expressing cells indicate activation of the PcG pathway in a majority of human prostate adenocarcinomas.** To validate the significance of our findings for human disease, we applied the quantitative immunofluorescence colocalization analysis for measurements of the expression of BMI1 and Ezh2 proteins and detection of dual positive high BMI/Ezh2-expressing carcinoma cells in clinical samples obtained from patients diagnosed with prostate adenocarcinomas. The results of this analysis demonstrate that a majority (79–91% in different cohorts of patients) of human prostate tumors contains dual positive high BMI1/Ezh2-expressing carcinoma cells exceeding the threshold expression level in prostate samples from normal individuals (Fig. 6). Interestingly, a panel of adenocarcinoma samples appears quite heterogeneous with respect to the relative levels of dual positive high BMI1/Ezh2-expressing cells (Fig. 6). While in 50–74% of prostate tumors the level of high BMI1-, high Ezh2- or dual positive high BMI1/Ezh2-expressing cells was only slightly elevated (<15% of positive cells), a significant fraction (17–29%) of prostate adenocarcinomas demonstrates a marked enrichment for dual positive high

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BMI1/Ezh2-expressing cells (>15% of positive cells).

**Increased BMI1 and Ezh2 expression is associated with high likelihood of therapy failure in prostate cancer patients after radical prostatectomy.** Microarray analysis demonstrates that cancer patients with high levels of *BMI1* and *Ezh2* mRNA expression in prostate tumors have a significantly worst relapse-free survival after radical prostatectomy (RP) compared with the patients having low levels of BMI1 and Ezh2 expression (Fig. 7), suggesting that more profound alterations of the PcG protein chromatin silencing pathway in carcinoma cells are associated with therapy resistant clinically lethal prostate cancer phenotype. We carried out the multivariate Cox proportional hazards survival analysis to ascertain the prognostic power of measurements of *BMI1* and *Ezh2* expression in combination with known clinical and pathological markers of prostate cancer therapy outcome such as Gleason score, surgical margins, extra-capsular invasion, seminal vesicle invasion, serum PSA levels, and age. Of note, *BMI1* expression level remains a statistically significant prognostic marker in the multivariate analysis (Table 1). Application of the 8-covariate prostate cancer recurrence model combining the incremental statistical power of individual prognostic markers appears highly informative in stratification of prostate cancer patients into sub-groups with differing likelihood of therapy failure and disease relapse after radical prostatectomy (Fig. 7). One of the distinctive features of this model is that it identifies a sub-group of prostate cancer patients comprising bottom 20% of recurrence predictor score and manifesting no clinical or biochemical evidence of disease relapse (Fig. 7). In contrast, 80% of patients in a sub-group comprising top 20% of recurrence predictor score failed therapy within five year period after radical prostatectomy.

## DISCUSSION

Increasing experimental evidence suggest that an oncogenic role of the *BMI1* activation may be extended beyond the leukemia and, perhaps, play a key role in progression of the epithelial malignancies and other solid tumors as well (see Introduction). One of the compelling examples revealing an association of the activated BMI1 ocoprotein-driven pathway (s) with clinically lethal therapy-resistant malignant phenotype in patients diagnosed with multiple types of cancer is identification of a death-from-cancer gene expression signature.[12,33,34] An 11-gene signature distinguishes stem cells with normal self-renewal function versus stem cells with drastically diminished self-renewal ability due to the loss of the *BMI-1* oncogene and similarly expressed in metastatic prostate tumors.[12] To date, the prognostic power of the 11-gene signature was validated in multiple independent therapy outcome sets of clinical samples obtained from more than 2,500 cancer patients diagnosed with 12 different types of cancer, including six epithelial (prostate; breast; lung; ovarian; gastric; and bladder cancers) and five nonepithelial (lymphoma; mesothelioma; medulloblastoma; glioma; and acute myeloid

Table 1 **8-covariate prostate cancer recurrence predictor model**

| Covariate | Coefficient | SE | Significance, P | Confidence interval, low 95% | Confidence interval, high 95% |
|---|---|---|---|---|---|
| *BMI1* | 4.7732 | 1.5179 | 0.0017 | 1.798 | 7.7483 |
| *Ezh2* | 0.4345 | 0.8215 | 0.5969 | -1.1756 | 2.0446 |
| PRE RP PSA | 0.0236 | 0.023 | 0.3054 | -0.0215 | 0.0686 |
| RP GLSN SUM | 0.2809 | 0.1955 | 0.1508 | -0.1023 | 0.6642 |
| Capsular Inv | 1.4752 | 0.7593 | 0.052 | -0.0131 | 2.9634 |
| SM | 0.7786 | 0.4641 | 0.0934 | -0.1311 | 1.6883 |
| Sem Ves Inv | 0.5876 | 0.4419 | 0.1836 | -0.2785 | 1.4538 |
| AGE | 0.041 | 0.0335 | 0.2214 | -0.0247 | 0.1066 |

RP, radical prostatectomy; PSA, prostate-specific antigen; GLSN SUM, Gleason sum; SM, surgical margins; Sem Ves Inv, seminal vesicle invasion; Capsular Inv, capsular invasion. Overall model fit: Chi Square = 40.1250; df = 8; $p < 0.0001$.

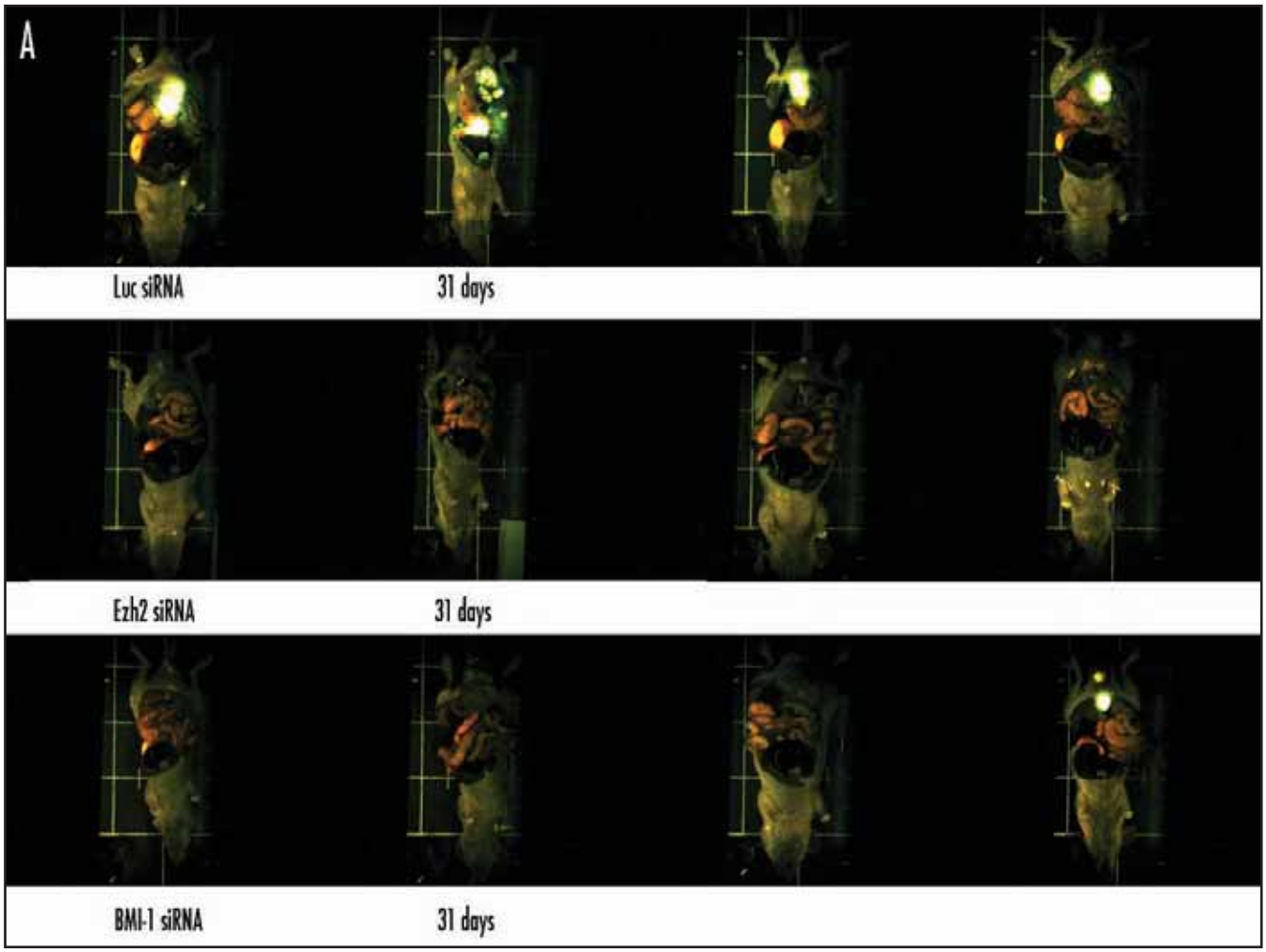


Figure 5. Human prostate carcinoma metastasis precursor cells depleted for a sub-population of dual positive high BMI1/Ezh2-expressing cells manifest a dramatic loss of malignant potential in vivo. Adherent cultures of blood-borne PC-3-GFP-39 prostate carcinoma cells[43,44] were treated with chemically modified degradation-resistant stable siRNAs targeting *BMI1* or *Ezh2* mRNAs or control LUC siRNA. Twenty-four hours after second treatment, 1.5 x $10^6$ cells were injected into prostates of nude mice. Note that all control animals developed highly aggressive rapidly growing metastatic prostate cancer and died within 50 days of experiment (A). Only 20% of mice in the BMI1- and Ezh2-targeting therapy groups developed less malignant more slowly growing tumors. One hundred fifty days after tumor cell inoculation, 83% and 67% of animals remain alive and disease-free in the therapy groups targeting the BMI1 and Ezh2 oncoproteins, respectively ($p = 0.0007$; log-rank test) (B). Six animals per group were monitored for survival.

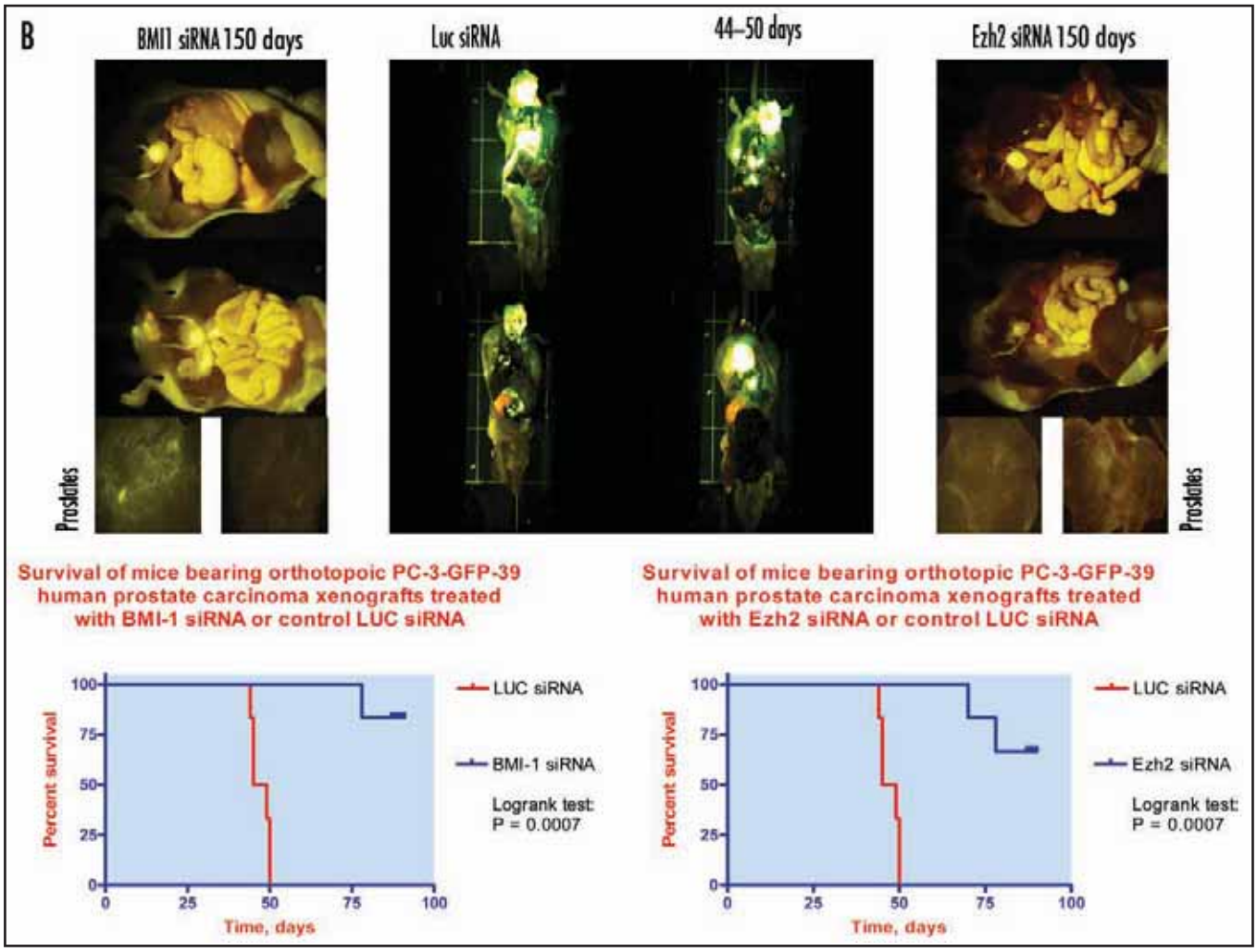


Figure 5B. See Legend, page 1896.

leukemia, AML) malignancies.[12,33,34] These data suggest the presence of a conserved *BMI1* oncogene-driven pathway, which is similarly activated in both normal stem cells and a highly malignant subset of human cancers diagnosed in a wide range of organs and uniformly exhibiting a marked propensity toward metastatic dissemination as well as a therapy resistance phenotype. Taken together with the results of the present study these data support the hypothesis that activation of the PcG chromatin silencing pathway is one of the key regulatory factors determining a cellular phenotype captured by the expression of a death-from-cancer signature in therapy-resistant clinically lethal malignancies.

Cancer cells with activated PcG pathway would be expected to exhibit a concomitantly high expression of both BMI1 and Ezh2 proteins. Furthermore, cells with activated PcG pathway would manifest the increased expression levels of protein substrates targeted by the activation of corresponding enzymes to catalyze the H2A-K119 ubiquitination (BMI1-containing PRC1 complex) and H3-K27 methylation (Ezh2-containing PRC2 complex). In this study we experimentally tested the relevance of this concept for metastatic prostate cancer. We applied a quantitative colocalization immunofluorescence analysis to measure the expression of four distinct protein markers of the PcG pathway activation and demonstrated a concomitantly increased expression of all four markers in a sub-population of human prostate carcinoma metastasis precursor cells isolated from the blood of nude mice bearing orthotopic metastatic human prostate carcinoma xenografts. Presence of dual positive high BMI1/Ezh2-expressing cells appears essential for maintenance of tumorigenic and metastatic potential of human prostate carcinoma cells in vivo, since targeted depletion of dual positive high BMI1/Ezh2-expressing cells from a population of highly metastatic human prostate carcinoma cells treated with stable siRNAs generates a cancer cell population with dramatically diminished malignant potential in vivo.

The BMI1 and Ezh2 proteins are members of the Polycomb group protein (PcG) chromatin silencing complexes conferring genome scale transcriptional repression via covalent modification of histones.[35] The BMI1 PcG protein is a component hPRC1L complex (human Polycomb repressive complex 1-like) which was recently identified as the E3 ubiquitin ligase complex that is specific for histone H2A and plays a key role in Polycomb silencing.[36] Ubiquitination/deubiquitination cycle of histones H2A and H2B is important in regulating chromatin dynamics and transcription mediated, in part, via 'cross-talk' between histone ubiquitination and methylation.[37] Importantly, one of the upregulated genes in the 11-gene death-from-cancer signature profile (Rnf2) plays a central role in the PRC1 complex formation and function[36] thus complementing the BMI-1

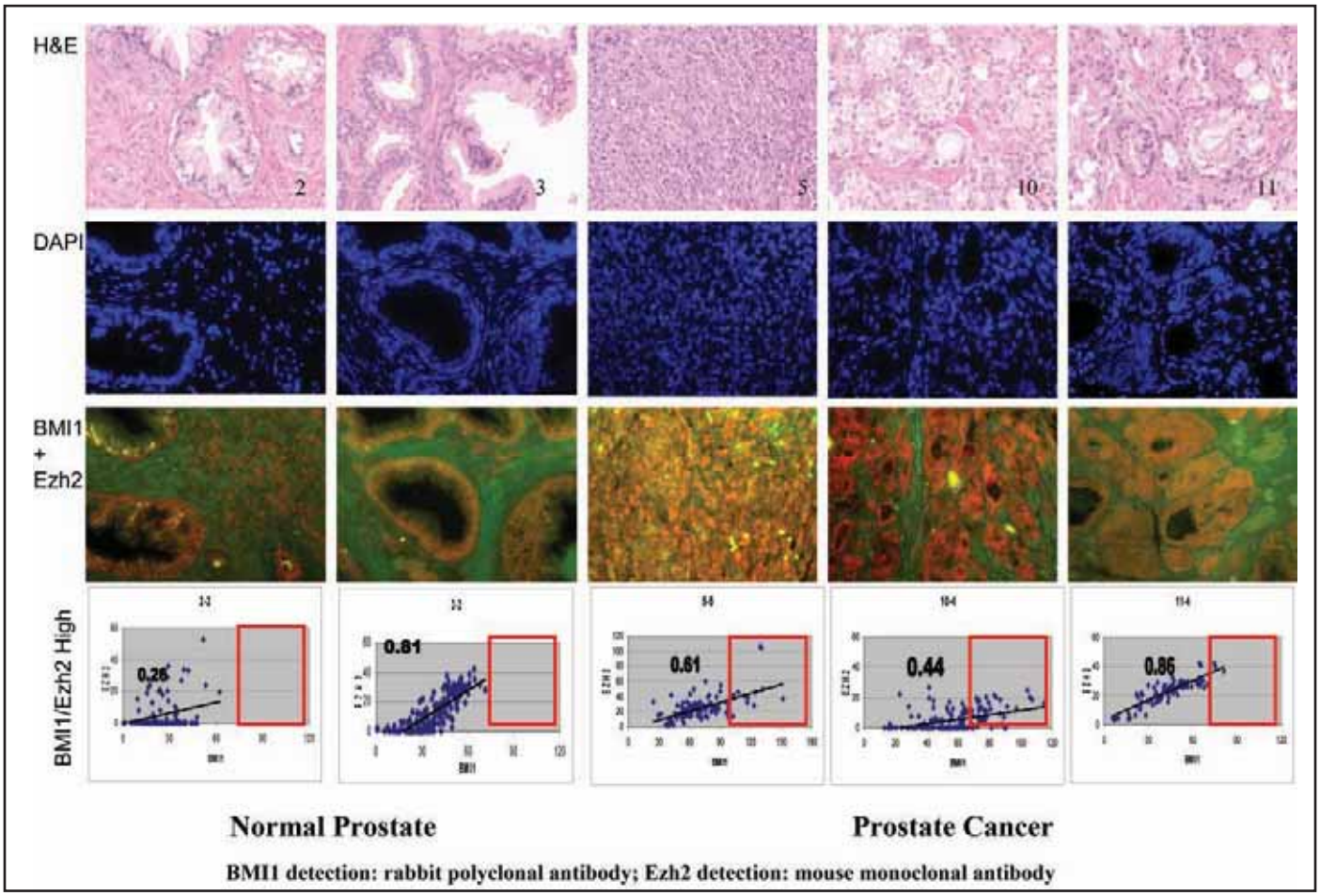


Figure 6. Tissue microarray analysis (TMA) of primary prostate tumors from patients diagnosed with prostate adenocarcinomas reveals increased levels of dual-positive BMI1/Ezh2 high-expressing cells. BMI1 and Ezh2 oncoprotein expression were measured in prostate TMA samples from cancer patients and healthy donors using a quantitative colocalization immufluorescence method and the number of dual positive high BMI1/Ezh2-expressing nuclei was calculated for each sample. Note that primary prostate tumors from patients diagnosed with prostate adenocarcinomas manifest a diverse spectrum of accumulation of dual positive BMI1/Ezh2 high-expressing cells and patients with higher levels of *BMI1* or *Ezh2* expression in prostate tumors manifest therapy-resistant malignant phenotype (Fig. 7). A majority (79–92% in different cohorts of patients) of human prostate tumors contains dual positive high BMI1/Ezh2-expressing cells exceeding the threshold expression levels in prostate samples from normal individuals.

function in the PRC1 complex. Rnf2 expression plays a crucial non-redundant role in development during a transient contact formation between PRC1 and PRC2 complexes via Rnf2 as described for Drosophila.[38,39]

The Ezh2 protein is a member of the Polycomb PRC2 and PRC3 complexes with a histone lysine methyltransferase (HKMT) activity that is associated with transcriptional repression due to chromatin silencing. The HKMT-Ezh2 activity targets lysine residues on histones H1 and H3 (H3-K27 or H1-K26). H3-K27 methylation conferred by an active HKMT-Ezh2-containing complex is one of the key molecular events essential for chromatin silencing in vivo. Taken together, these data imply that in vivo Polycomb chromatin silencing pathway in distinct cell types would require a coordinate activation of multiple distinct PRC complexes. For example, Ezh2 associates with different EED isoforms thereby determining the specificity of histone methyltransferase activity toward histone H3-K27 or histone H1-K26.[40] Collectively, these results suggest that coherent function of the PcG chromatin silencing pathway would require a concomitant coordinated activation of multiple protein components of PRC1, PRC2, and PRC3 complexes implying a coordinate regulation of expression of their essential components such as BMI1 and Ezh2 oncoproteins. It follows that dual positive high BMI1/Ezh2-expressing carcinoma cells with elevated expression of the H2AubiK119 and H3metK27 histones should be regarded as cells with activated PcG protein chromatin silencing pathway.

In human cells the BMI1-containing PcG complex forms a unique discrete nuclear structure that was termed the PcG bodies,[46] the size and number of which in nuclei significantly varied in different cell types. Of note, the nuclei of dual positive high BMI1/Ezh2-expressing cells almost uniformly contain six prominent discrete PcG bodies, perhaps, reflecting the high level of the BMI1 expression and indicating the active state of the PcG protein chromatin silencing pathway. It has been shown recently that in cancer cells expressing high level of the Ezh2 protein the new type of the PcG chromatin silencing complex is formed containing the Sirt1 protein.[47] These data suggest that in high Ezh2-expressing carcinoma cells a distinct set of genetic loci could be repressed due to activation of the Ezh2/Sirt1-containing PcG chromatin silencing complex.

One of the notable features of dual positive high BMI1/Ezh2-expressing carcinoma cells is a prominent cytosolic expression of the Ezh2 oncoprotein (Fig. 1). Recent evidence revealed the existence of the cytosolic Ezh2-containing methyltransferase complex

regulating actin polymerization and extra-nuclear signaling processes in various cell types.[48] It is possible that both nuclear and extra-nuclear functions of the Ezh2-containing methyltransferase complex may play an important role in determining the malignant behavior of metastatic human prostate carcinoma cells. Recent observations directly demonstrated that the PcG repressive complexes PRC1 and PRC2 cooccupied a large set of genes in human and murine genomes, many of which are transcriptional developmental regulators.[49,50] These data suggest that repression of multiple developmental and differentiation pathways by Polycomb complexes may be required for maintaining stem cell pluripotency and add further support to the idea that repression of critical developmental regulators by PcG proteins may play a crucial role in tumor progression and metastasis.

The results of our experiments indicate that PcG pathway is frequently activated in human prostate tumors and is mechanistically linked to the highly malignant behavior of human prostate carcinoma cells in a xenograft model of prostate cancer metastasis. It remains to be elucidated whether similarly to the xenograft model of human prostate cancer metastasis in nude mice the PcG pathway activation is mechanistically associated with metastatic disease in prostate cancer patients as well. It will be of interest to study whether the level of enrichment of primary prostate tumors with dual positive high BMI1/Ezh2-expressing cancer cells would correlate with a degree of PcG pathway activation and would be informative in predicting the clinical behavior of prostate cancer in patients. Follow-up studies would be required to determine whether human prostate tumors manifesting markedly increased levels of dual positive high BMI1/Ezh2-expressing cells represent a therapy resistant clinically lethal type of prostate adenocarcinomas. Finally, this work sets the stage for development of small molecule inhibitors of the PcG protein chromatin silencing pathway as a novel therapeutic modality for treatment of metastatic prostate cancer.

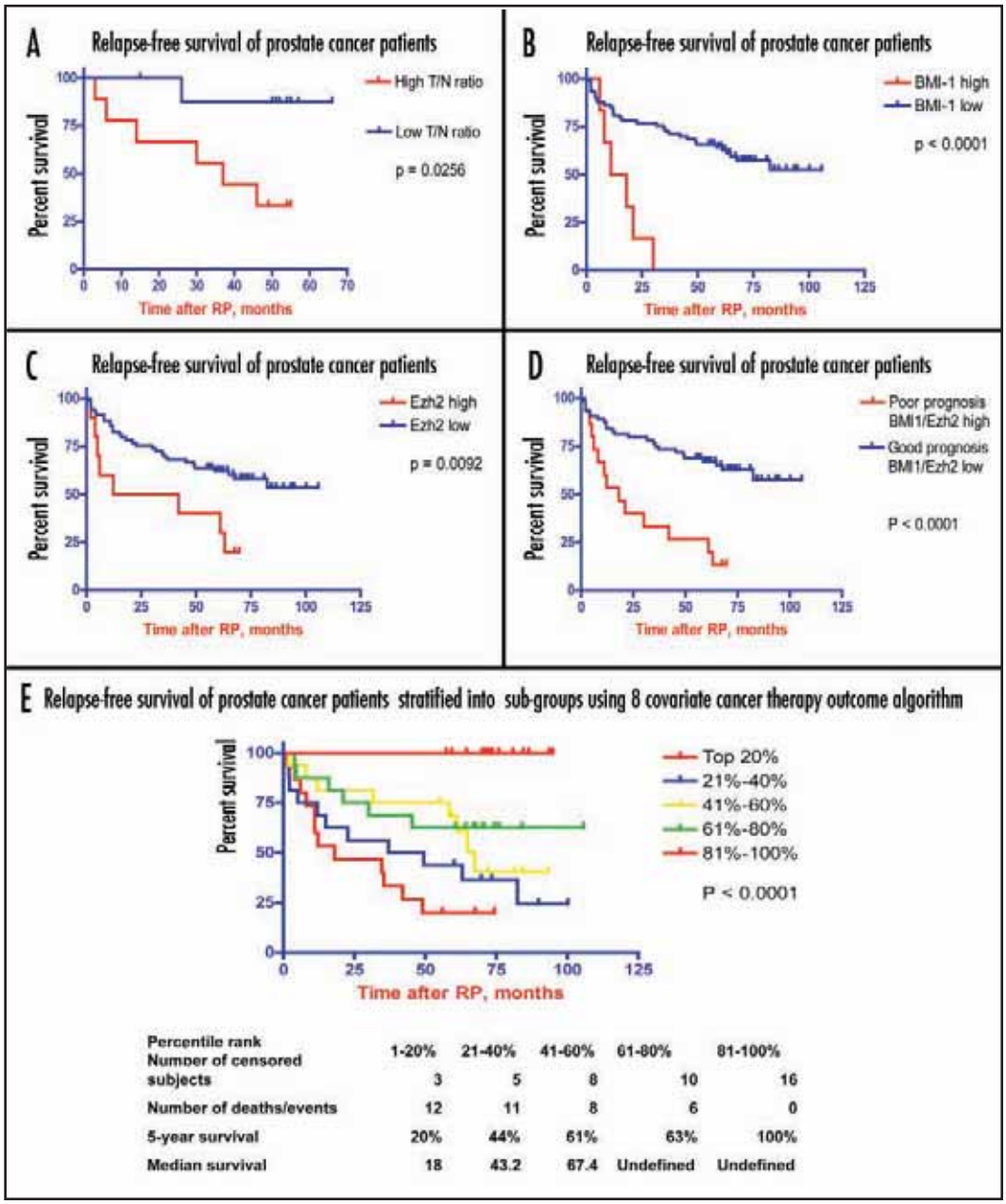


Figure 7. Increased *BMI1* and *Ezh2* expression is associated with high likelihood of therapy failure and disease relapse in prostate cancer patients after radical prostatectomy (E). (D) Shows the Kaplan-Meyer survival analysis taking into account of the expression levels of both BMI1 ans Ezh2 genes. Kaplan-Meier survival analysis demonstrates that cancer patients with more significant elevation of the *BMI1* and *Ezh2* expression [having higher tumor (T) to adjacent normal tissue (N) ratio, T/N (A); or having tumors with higher levels of *BMI1* (B) or *Ezh2* (C) expression] are more likely to fail therapy and develop a disease recurrence after radical prostatectomy. (E) shows the Kaplan-Meier survival analysis of 79 prostate cancer patients stratified into five sub-groups using eight-covariate cancer therapy outcome (CTO) algorithm (Table 1). CTO algorithm integrates individual prognostic powers of *BMI1* and *Ezh2* expression values and six clinicopathological covariates (preoperative PSA, Gleason score, surgical margins, extra-capsular invasion, seminal vesicle invasion, and age).

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### Conflicts of interest

The authors have no conflicting financial interests.

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