

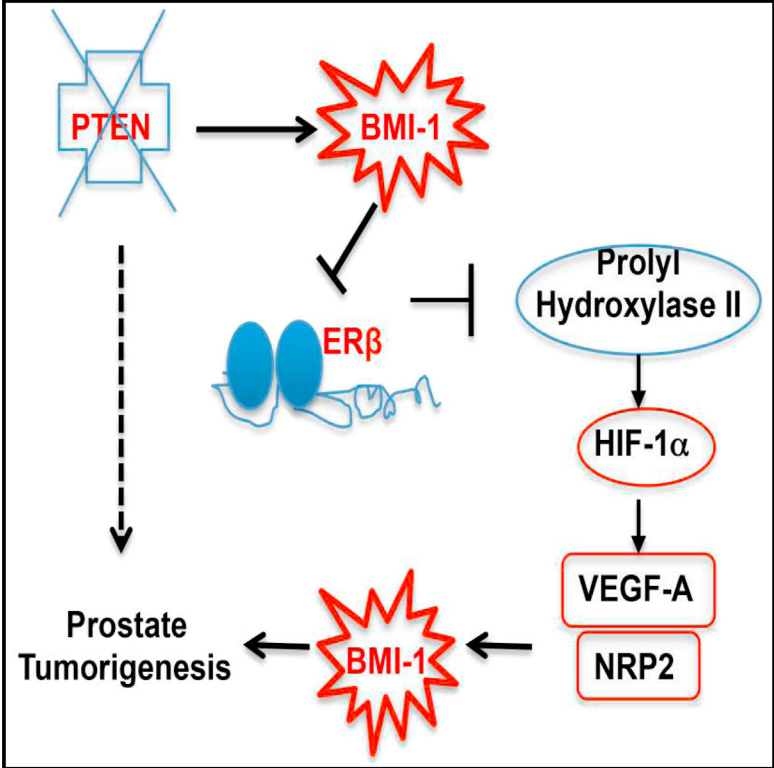
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## Prostate Tumorigenesis Induced by PTEN Deletion Involves Estrogen Receptor $\beta$ Repression

### Graphical Abstract



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### In Brief

A causal role for ER $\beta$  in prostate cancer has not been established. Mak et al. now show that loss of ER $\beta$  occurs as a consequence of prostate tumorigenesis induced by PTEN deletion, and that this loss is necessary for tumorigenesis because it enables HIF/VEGF signaling in tumor cells.

### Highlights

- Prostate tumorigenesis caused by PTEN deletion involves loss of estrogen receptor  $\beta$
- ER $\beta$  transcription is repressed by BMI-1, which is induced by PTEN deletion
- ER $\beta$  repression is needed for tumorigenesis because it enables HIF/VEGF signaling
- HIF/VEGF signaling sustains BMI-1 expression, resulting in a positive feedback loop

# Prostate Tumorigenesis Induced by PTEN Deletion Involves Estrogen Receptor $\beta$ Repression

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## SUMMARY

The role of ER $\beta$  in prostate cancer is unclear, although loss of ER $\beta$  is associated with aggressive disease. Given that mice deficient in ER $\beta$  do not develop prostate cancer, we hypothesized that ER $\beta$  loss occurs as a consequence of tumorigenesis caused by other oncogenic mechanisms and that its loss is necessary for tumorigenesis. In support of this hypothesis, we found that ER $\beta$  is targeted for repression in prostate cancer caused by PTEN deletion and that loss of ER $\beta$  is important for tumor formation. ER $\beta$  transcription is repressed by BMI-1, which is induced by PTEN deletion and important for prostate tumorigenesis. This finding provides a mechanism for how ER $\beta$  expression is regulated in prostate cancer. Repression of ER $\beta$  contributes to tumorigenesis because it enables HIF-1/VEGF signaling that sustains BMI-1 expression. These data reveal a positive feedback loop that is activated in response to PTEN loss and sustains BMI-1.

## INTRODUCTION

The role of estrogen receptors (ERs) in epithelial cell biology and cancer is an emerging area of considerable biological interest and pathological relevance. In the prostate, ER $\beta$  is expressed in epithelial cells, while ER $\alpha$  expression is confined to stromal cells (Kuiper et al., 1996; Leav et al., 2001; Thomas and Gustafsson, 2011). The contribution of ER $\beta$  to prostate cancer appears to be significant, but much remains to be learned (Christoforou et al., 2014; Dey et al., 2013). The inverse correlation between the expression of ER $\beta$  and differentiation (Gleason score) (Leav et al., 2001; Mak et al., 2010) is supported by mechanistic cell biology studies demonstrating that one function of ER $\beta$  is to impede an epithelial mesenchymal transition (EMT) (Mak et al., 2010, 2013). The mechanism involves the ability of ER $\beta$  to sustain prolyl hydroxylase 2 (PHD2) expression and subsequently promote HIF-1 $\alpha$  degradation and HIF-1-mediated EMT (Mak et al., 2013).

An important and timely issue is the contribution of ER $\beta$  to prostate tumorigenesis. Although the loss of ER $\beta$  is associated with a higher Gleason grade and more aggressive disease, a causal role for ER $\beta$  in impeding the formation of aggressive tumors has not been established. There is evidence that loss of ER $\beta$  can increase the incidence of poorly differentiated prostate carcinoma, but the mechanism is not known (Slusarz et al., 2012). This problem has been obscured by the analysis of ER $\beta$  knockout (BERKO) mice. These mice do not develop prostate cancer (Antal et al., 2008; Imamov et al., 2004), although some studies have observed prostate hyperplasia in older BERKO mice (Imamov et al., 2004). Furthermore, deletion of ER $\beta$  in the FGF8b transgenic model of prostate tumorigenesis did not increase tumor incidence, a finding that has been used to discount a tumor-suppressive function for ER $\beta$  (Elo et al., 2014).

We approached the problem of the potential role of ER $\beta$  in prostate tumorigenesis from a different perspective. Specifically, we hypothesized that ER $\beta$  loss occurs as a consequence of tumorigenesis caused by other oncogenic mechanisms and that its loss is necessary for this tumorigenesis. To test this hypothesis, we focused on prostate tumorigenesis induced by PTEN loss for several reasons. Inactivation or loss of PTEN is one of the most common genetic lesions in prostate cancer, and its frequency increases with Gleason grade and more aggressive disease (Cairns et al., 1997; Goel et al., 2012; McMenamin et al., 1999). Given that ER $\beta$  loss also increases with de-differentiated, aggressive disease (Mak et al., 2010), these observations suggest a causal relationship between loss of PTEN and loss of ER $\beta$  that may be significant for prostate tumorigenesis. The results presented in this study validate this hypothesis and provide a mechanism for how PTEN loss results in the transcriptional repression of ER $\beta$  that involves BMI-1, an oncogene that regulates cell proliferation and senescence through the ink4a locus (Jacobs et al., 1999) and has been implicated in prostate tumorigenesis (Lukacs et al., 2010). Importantly, we also establish that the loss of ER $\beta$  is necessary for tumorigenesis caused by PTEN loss because it enables autocrine VEGF signaling, which has been implicated in the genesis of several cancers including prostate (reviewed in Goel and Mercurio [2013]).

## RESULTS

### ER $\beta$ Is Targeted for Repression in Prostate Tumorigenesis Induced by PTEN Loss

To assess the potential relationship between ER $\beta$  and PTEN, we evaluated ER $\beta$  expression in a transgenic model of prostate tumorigenesis mice induced by PTEN deletion (Pten<sup>loxP/loxP</sup>; PB-Cre<sup>+</sup> [referred to as Pten pc<sup>-/-</sup>]). Prostate-specific deletion of PTEN results in tumors that are invasive and aggressive (Wang et al., 2003). The normal glandular features of the wild-type prostates (control) and tumor formation in age-matched Pten pc<sup>-/-</sup> prostates are shown in Figure 1A. PTEN expression is apparent in wild-type prostates but absent in Pten pc<sup>-/-</sup> mice (Figure 1B). Importantly, ER $\beta$  expression is lost in Pten pc<sup>-/-</sup> mice, whereas the age-matched wild-type prostates exhibit ER $\beta$  expression (Figure 1C). We also observed that PTEN and ER $\beta$  expression correlate in human prostate tumors based on analysis of the cBioportal database (Figure 1D; Table S1). High Gleason grade tumors (primary Gleason grade 5) exhibit uniform loss of PTEN and ER $\beta$  (Cairns et al., 1997; Mak et al., 2010). Gleason grade 3 tumors are interesting in this regard because these tumors are characterized by heterogeneity in PTEN expression (McMenamin et al., 1999). Indeed, we quantified PTEN and ER $\beta$  mRNA expression in grade 3 tumors and observed a correlation between PTEN and ER $\beta$  expression (Figure 1E).

To investigate the relationship between ER $\beta$  and PTEN further, we depleted PTEN in PNT1a cells, an immortalized prostate epithelial cell line (Berthon et al., 1995). Depletion of PTEN caused an EMT consistent with previous reports (Mulholland et al., 2012; Song et al., 2009), and it also resulted in a decrease in ER $\beta$  mRNA and protein expression compared to control cells, indicating that PTEN regulates ER $\beta$  expression (Figure 1F). In contrast, ER $\beta$  does not appear to impact PTEN expression because depletion of ER $\beta$  did not alter PTEN levels (Figure 1G). Furthermore, two physiological ligands of ER $\beta$  (3 $\beta$ -Adiol and 17 $\beta$ -estradiol:E2) did not affect PTEN expression (Figure 1G). Interestingly, both a PI3K inhibitor (wortmannin) and Akt inhibitor (Akt Inhibitor VIII) reversed the mesenchymal phenotypes of PTEN-depleted PNT1a cells to an epithelial phenotype with a concomitant restoration of ER $\beta$  expression (Figure 1H).

To support a functional link between PTEN and ER $\beta$  expression, we examined the effect of expressing ER $\beta$  in PTEN-depleted PNT1a cells. The mesenchymal morphology of PTEN-depleted cells reverted to an epithelial morphology in response to expression of HA-ER $\beta$  with a concomitant decrease in mesenchymal markers (N-cadherin and vimentin) (Figure 2A). We also assessed the tumor-suppressive activity of ER $\beta$  in the context of PTEN by injecting control cells, PTEN-depleted cells, and PTEN-depleted cells that express ER $\beta$  into nude mice. Control cells did not form tumors after 9 weeks, whereas 71% of mice injected with PTEN-depleted cells had tumors at this time (Figure 2B). More importantly, tumor formation was greatly reduced (12% of mice had tumors) when mice were injected with PTEN-depleted cells expressing HA-ER $\beta$  (Figure 2B). This observation was substantiated by expressing ER $\beta$  in PC3-M cells, a highly tumorigenic, PTEN-prostate cancer cell line (Kozlowski et al., 1984) that expresses low levels of ER $\beta$  (Figure 2C).

Indeed, ER $\beta$  expression dramatically reduced the ability of these cells to form tumors (Figure 2C).

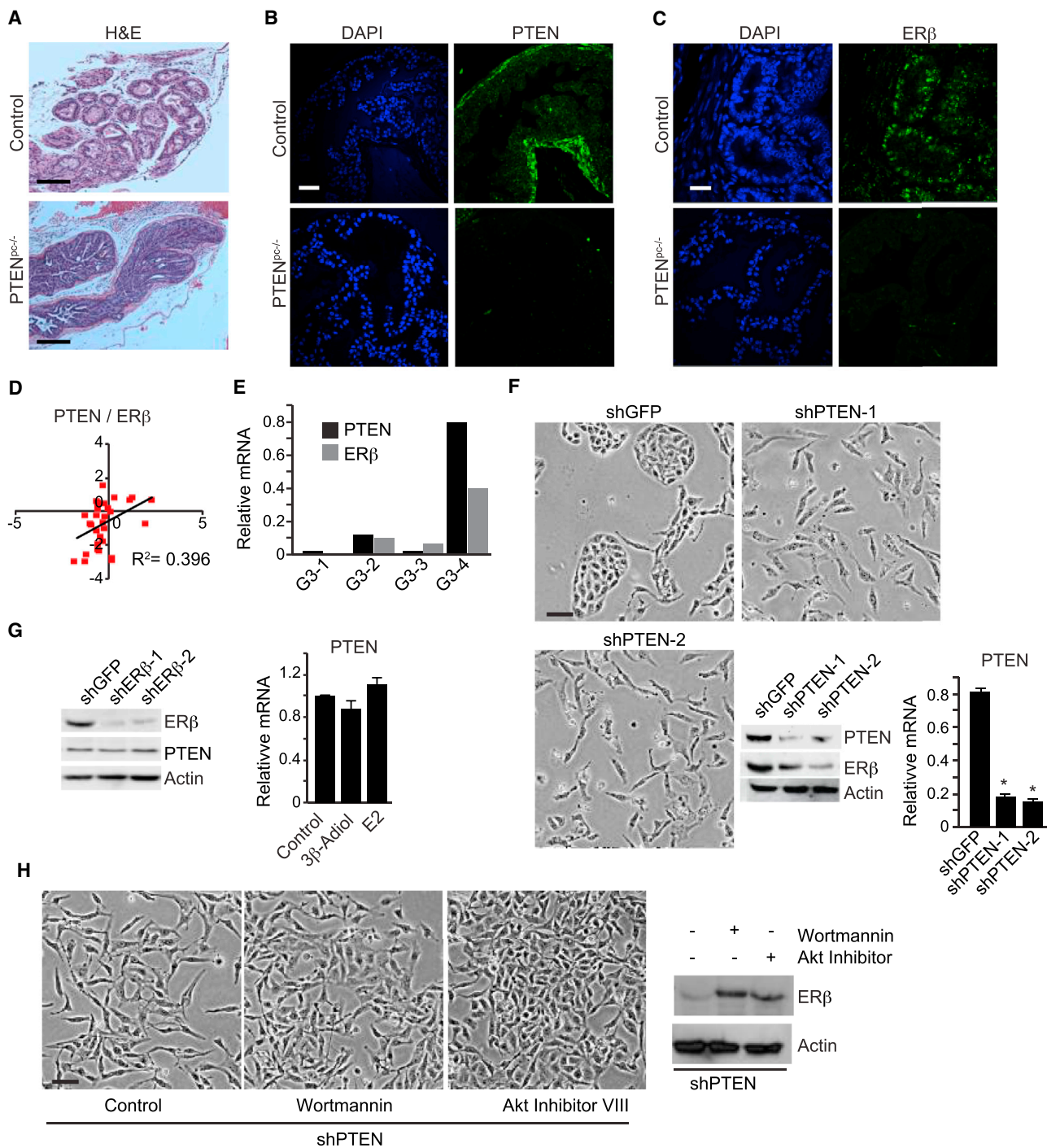
### BMI-1 Is a Transcriptional Repressor of ER $\beta$

To define the mechanism by which loss of PTEN diminishes ER $\beta$  expression, we focused on BMI-1, the key regulatory component of the polycomb repressive complex-1 that modulates chromatin structure and represses the transcription of a number of genes (Cao et al., 2005; Jacobs et al., 1999; Miyazaki et al., 2008), for several reasons. BMI-1 has been implicated in prostate hyperplasia and tumorigenesis (Lukacs et al., 2010; van Leenders et al., 2007). We also found that expression of BMI-1 in ER $\beta$ -expressing PTEN-depleted cells promoted tumor formation (Figure 2C). Furthermore, PTEN loss induces BMI-1 expression (Goel et al., 2012), as evident in PNT1a cells (Figure 2D). The ability of both wortmannin and Akt Inhibitor VIII to attenuate BMI-1 expression in shPTEN cells supports our previous finding on their ability to restore ER $\beta$  expression in these cells (Figure 2E). For these reasons, we evaluated the possibility that BMI-1 represses ER $\beta$ . Expression of BMI-1 in PNT1a cells suppressed ER $\beta$  compared to control cells (Figure 2F). Conversely, we depleted BMI-1 in PC3-M cells and observed an induction of ER $\beta$  expression compared to the control (Figure 2G). We also observed that 17 $\beta$ -estradiol (E2) had no effect on the expression of BMI-1 on PTEN-depleted cells, suggesting that ER $\alpha$  does not regulate BMI-1 (Figure 2H). The multiple bands observed in the BMI-1 immunoblots may represent phosphorylated forms of the protein (Nacerddine et al., 2012).

The inverse functional relationship between BMI-1 and ER $\beta$  prompted us to test the possibility that BMI-1 is a transcriptional repressor of ER $\beta$ . Therefore, we performed chromatin immunoprecipitation (ChIP) analysis on the ER $\beta$  promoter to assess BMI-1 binding. We examined eight regions within the 3 kb spanning from +331 to -2,996 base pairs and detected one major BMI-1 binding locus in region 1 proximal to the transcription start site (Figure 2I; Figure S1A). The impact of BMI-1 on ER $\beta$  promoter activity was evaluated by expressing luciferase reporter constructs containing two regions of the promoter (region 1 and regions 1 and 2) in control and BMI-1-depleted PC3-M cells. Diminishing BMI-1 expression resulted in a significant increase in luciferase activity in both constructs compared to the control (Figure 2J). Moreover, this activity was concentrated in region 1, supporting our ChIP data. Given that ER $\beta$  expression is regulated by two promoters, ON and OK (Hirata et al., 2001), we sought to determine which promoter is utilized by BMI-1 to exert its repressive function. The BMI-1 binding locus of region 1 in our ChIP assays lined up perfectly within the ON promoter, but not within the OK promoter (Figure S1B). These data indicate that ER $\beta$  transcription is repressed by BMI-1 on the ON promoter.

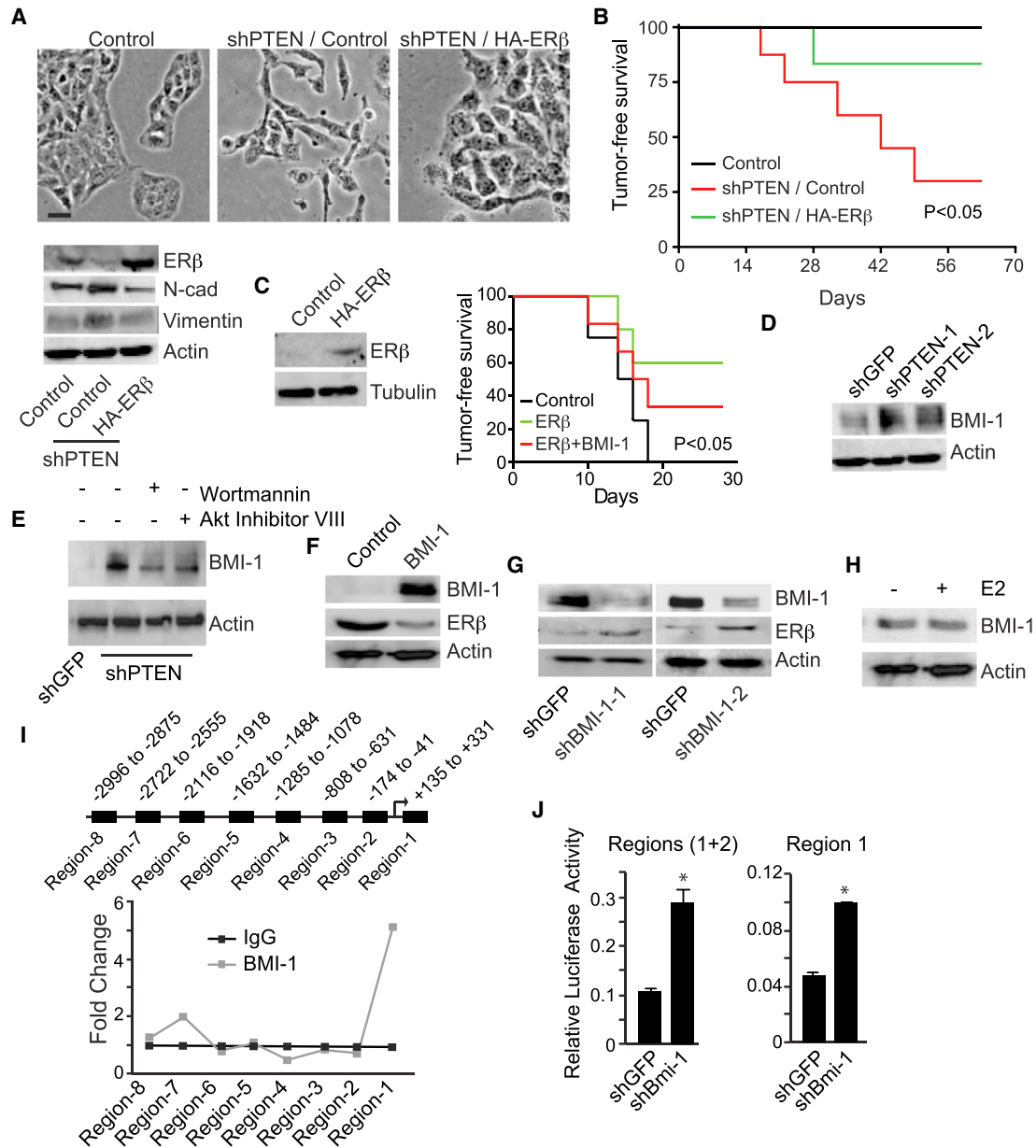
### Role of ER $\beta$ Repression in Prostate Tumorigenesis

The critical issue that arises from the foregoing observations is why ER $\beta$  repression is important for prostate tumorigenesis induced by PTEN loss (see Figure 1A). Previously, we reported that loss of ER $\beta$  stabilizes HIF-1 $\alpha$  and enables autocrine VEGF signaling in prostate cancer cells (Mak et al., 2010). This finding is relevant because autocrine VEGF signaling has emerged as an important component of tumorigenesis (Goel and Mercurio,



**Figure 1. ERβ Is Targeted for Repression in Prostate Tumorigenesis Induced by PTEN Loss**

(A) H&E staining of ventral prostates of wild-type (control) and aged-matched *Pten*  $pc^{-/-}$  mice is shown. (B and C) The expression of PTEN (B) and ERβ (C) in these tissues was examined by immunofluorescence microscopy. Scale bar, 50 μm. (D) A positive correlation between PTEN and ERβ expression in a cohort of 34 prostate tumors was determined from the cBioportal database. (E) Four different Grade 3 tumors were analyzed by qPCR for ERβ and PTEN mRNA expression. (F) PTEN-depleted PNT1a cells (shPTEN-1 and shPTEN-2) and control (shGFP) PNT1a cells were analyzed for morphology (phase contrast images) and expression of ERβ and PTEN (immunoblot). PTEN mRNA expression was also quantified by qPCR (bar graph). (G) Immunoblot shows PTEN expression in ERβ-depleted (shERβ-1 and shERβ-2) and control (shGFP) PNT1a cells. Bar graph depicts the lack of an effect of 3β-Adiol and estrogen (E2) treatment on PTEN mRNA expression. Data represent the average of three experiments (±SEM). (H) Effects of wortmannin and Akt Inhibitor VIII on cell morphology and ERβ expression in shPTEN PNT1a cells are shown. See also Table S1.



**Figure 2. ER $\beta$  Impedes Tumor Initiation Induced by PTEN Loss**

(A) HA-ER $\beta$  was expressed in PTEN-depleted cells and the impact on cell morphology (phase contrast images) and expression of ER $\beta$  (immunoblot) was evaluated. Scale bar, 50  $\mu$ m.

(B) The indicated cells ( $10^5$ ) were injected subcutaneously into *nu/nu* mice ( $n = 7$ ) and tumor formation was assessed by palpation. The curve comparison was done using log rank test ( $p < 0.05$ ).

(C) HA-ER $\beta$  was expressed in PC3-M cells, which are PTEN<sup>-</sup> and express a very low level of ER $\beta$ . The indicated cells ( $10^5$ ) were injected subcutaneously into *nu/nu* mice ( $n = 7$ ) and tumor formation was assessed by palpation. The curve comparison was done using log rank test ( $p < 0.05$ ).

(D) Effects of wortmannin and Akt Inhibitor VIII on BMI-1 expression in shPTEN PNT1a cells are shown.

(E) Expression of BMI-1 in PTEN-depleted (shPTEN) and control (shGFP) PNT1a cells is shown.

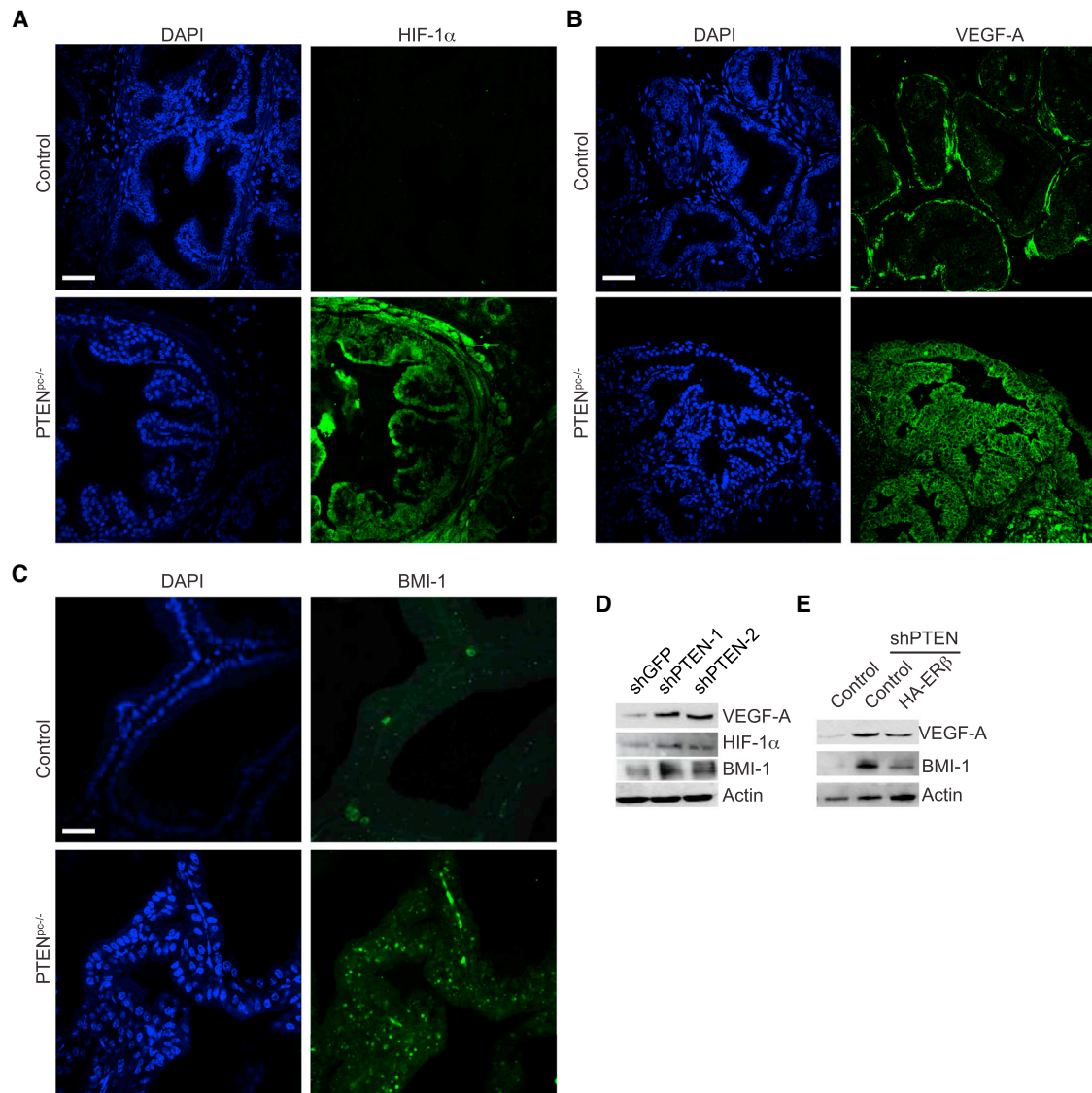
(F) BMI-1 was expressed in PNT1a cells and the effect on ER $\beta$  expression was evaluated by immunoblotting.

(G) BMI-1 expression was diminished in PC3-M cells using shRNAs (shBMI-1 and shBMI-2) and the expression of ER $\beta$  was assessed (immunoblots).

(H) Effect of estrogen (E2, 10 nM) on BMI-1 expression in shPTEN cells is shown.

(I) Schematic of the ER $\beta$  promoter shows the primer sets that were used to assess binding activity of BMI-1 in the eight regions depicted by the black boxes. ChIP was performed using a BMI-1 Ab and the line graph depicts the quantitation of the ChIP results by qPCR normalized to IgG. Data represent the average of two separate experiments.

(J) Luciferase reporter constructs containing regions 1 and 2 of the ER $\beta$  promoter (left graph) or region 1 (right graph) were expressed in PC3-M cells. Luciferase activity was normalized to Renilla ( $\pm$ SEM) and the experiment was repeated three times with similar results. See also Figure S1.



### Figure 3. Role of ER $\beta$ Repression in Prostate Tumorigenesis

(A–C) Ventral prostates of wild-type (control) and aged-matched Pten  $pc^{-/-}$  mice were stained for (A) HIF-1 $\alpha$ , (B) VEGF-A, and (C) BMI-1 and analyzed by immunofluorescence microscopy. Scale bar, 50  $\mu$ m.

(D) Immunoblot shows the expression of HIF1 $\alpha$ , VEGF-A, and BMI-1 in PTEN-depleted (shPTEN) and control (shGFP) PNT1a cells.

(E) Expression of VEGF-A and BMI-1 in ER $\beta$ -expressing PTEN-depleted cells (shPTEN-2 + HA-ER $\beta$ ) was compared to PTEN-depleted cells (shPTEN).

2013). Of particular relevance, we reported that autocrine VEGF signaling results in the enhancement of BMI-1 expression by a mechanism that involves Neuropilin-2 (NRP2) and Gli-1 (Goel et al., 2012). Together, these observations support the hypothesis that ER $\beta$  repression is important for prostate tumorigenesis induced by PTEN loss because it enables autocrine VEGF signaling via HIF-1 $\alpha$  stabilization that sustains BMI-1 expression. Initially, we assessed HIF-1 $\alpha$  and VEGF expression in wild-type and Pten  $pc^{-/-}$  prostates. Deletion of PTEN induced the expression of HIF-1 $\alpha$ , VEGF, as well as BMI-1 (Figures 3A–3C), in tumor cells. This observation is supported by the analysis of PTEN-depleted PNT1a cells, which express high levels of HIF-1 $\alpha$ ,

VEGF-A, and BMI-1 compared to control cells (shGFP) (Figure 3D). To demonstrate that the increase in VEGF and BMI-1 expression observed in response to PTEN deletion is dependent on ER $\beta$  repression, we expressed ER $\beta$  in PTEN-depleted PNT1a cells and observed a reduction in VEGF and BMI-1 expression compared to controls (Figure 3E).

The results reported so far indicate that PTEN loss results in the BMI-1-mediated repression of ER $\beta$  and that repression of ER $\beta$  enables VEGF signaling that sustains BMI-1 expression. In essence, the data reveal a positive feedback loop that functions to maintain BMI-1 expression and is activated in response to PTEN loss. This hypothesis infers that loss of ER $\beta$  should induce

BMI-1 expression by a mechanism that involves HIF-1/VEGF signaling. Indeed, depletion of ER $\beta$  in PNT1a cells induced HIF-1 $\alpha$ , VEGF-A, and BMI-1 expression compared to the controls (Figure 4A). We also observed an induction of NRP2 in ER $\beta$ -depleted cells with a concomitant increase in BMI-1 (Figure S2). Similar results were obtained by depleting PHD2, which is sustained by ER $\beta$  (Mak et al., 2013; Figure 4B). The PHD2-depleted cells also had high expression levels of N-cadherin and vimentin compared to the control cells, supporting our previous observation that loss of PHD2 induced an EMT in PNT1a cells (Mak et al., 2013). To establish a causal role for HIF-1 $\alpha$  in regulating BMI-1 expression, we knocked down HIF-1 $\alpha$  in ER $\beta$ -depleted cells and observed a substantial decrease in VEGF-A and BMI-1 expression compared to ER $\beta$ -depleted cells alone (Figure 4C). These expression differences also were manifested in cell morphology. Control (shGFP) and shER $\beta$ /shHIF-1 $\alpha$  cells exhibited an epithelial morphology compared to the mesenchymal morphology of shER $\beta$ /shGFP cells (Figure 4C). Based on these data, the possibility existed that BMI-1 is a HIF-1 $\alpha$  target gene. However, promoter activity analyses did not support this possibility (data not shown). For this reason, we focused on the role of VEGF-A signaling in regulating BMI-1 in the context of ER $\beta$ . Specifically, knocking down VEGF-A in ER $\beta$ -depleted cells attenuated BMI-1 expression and induced an epithelial morphology (Figure 4D).

Subsequently, we analyzed the prostates of BERKO mice to assess the impact of ER $\beta$  loss on HIF-1 $\alpha$ , VEGF, and BMI-1 expression. Ventral prostates of wild-type mice (control) exhibited normal glandular structure with ER $\beta$  expression in epithelial cells and undetectable HIF-1 $\alpha$  and BMI-1 and very low VEGF-A expression (Figure 4E). In contrast, BERKO mice of the same age and genetic background exhibited hyperplasia and decreased epithelial differentiation with high HIF-1 $\alpha$ , VEGF-A, and BMI-1 expression in the absence of ER $\beta$  (Figure 4E). Interestingly, both control and BERKO prostates exhibited a similar intensity of PTEN staining (Figure 4E). Five BERKO mice and their control counterparts were examined with similar findings. These observations were substantiated by the observation that a negative correlation between ER $\beta$  and BMI-1 expression exists in a cohort of 87 human prostate tumors, based on analysis of the cBioportal database (Figures 4F; Table S2).

## DISCUSSION

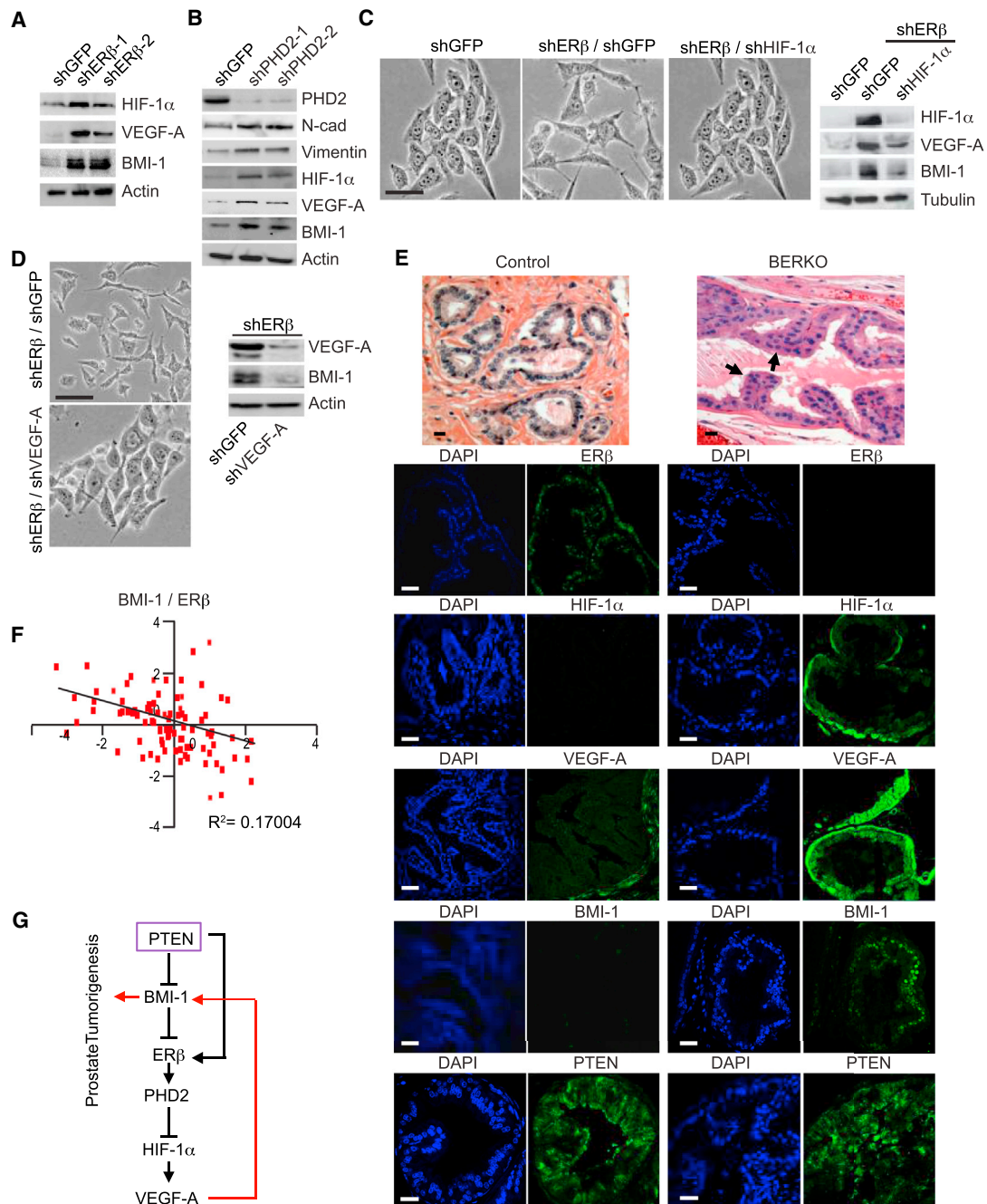
This study provides insight into the role of ER $\beta$  in prostate tumorigenesis and the mechanisms that regulate its expression. First and foremost, we demonstrate that prostate tumorigenesis caused by PTEN deletion involves BMI-1-mediated repression of ER $\beta$  and that repression of ER $\beta$  enables HIF-1/VEGF signaling that sustains BMI-1 expression. These findings should help to clarify the issue of why prostate cancer has not been observed in BERKO mice. Specifically, we argue that loss of ER $\beta$  is not sufficient to promote tumorigenesis in the absence of an oncogenic stimulus, despite the fact that BMI-1 expression is increased. This hypothesis is consistent with the report that BMI-1 inhibition slows the growth of PTEN-deletion-induced prostate cancer, but it does not prevent tumorigenesis (Lukacs et al., 2010). In fact,

we found that BERKO prostates retain PTEN expression. A reasonable hypothesis going forward is that PTEN loss involves additional events, such as the enhancement of PI3K/Akt signaling, that are essential for tumorigenesis (Worby and Dixon, 2014).

The fact that ER $\beta$  expression is lost during tumorigenesis caused by PTEN deletion is significant and relevant to other studies that have investigated the consequences of ER $\beta$  loss in the prostate. Specifically, it was reported recently that deletion of ER $\beta$  in the FGF8b transgenic model of prostate tumorigenesis did not increase tumor incidence (Elo et al., 2014). Although the authors discounted a tumor-suppressive role for ER $\beta$  based on these data, this conclusion should be tempered by the likely possibility that FGF8b-mediated tumorigenesis involves repression of ER $\beta$ , similar to our finding with prostate tumorigenesis caused by PTEN deletion. For this reason, deleting ER $\beta$  in either the FGF8b or PTEN models would not be expected to increase tumor incidence.

This study also addresses the mechanism by which ER $\beta$  is regulated in prostate cancer. Several studies have observed an inverse correlation between ER $\beta$  expression and differentiation (Gleason grade), but the mechanisms that contribute to the loss of ER $\beta$  in high-grade cancers are not well understood. Some reports indicated that hypermethylation of the ER $\beta$  promoter is associated with loss of expression (Lau et al., 2000; Zhu et al., 2004). Although our data do not discount the contribution of promoter methylation, compelling evidence now exists that BMI-1 expression correlates with Gleason grade and that BMI-1 is induced as a direct consequence of PTEN loss or inactivation (Goel et al., 2012). Moreover, we detected an inverse correlation between ER $\beta$  and BMI-1 in a cohort of human prostate tumors. These observations, coupled with our demonstration that BMI-1 can bind to the ER $\beta$  ON promoter and repress transcription, strongly implicate BMI-1 in the repression of ER $\beta$  in prostate cancer. Paradoxically, ER $\beta$  is expressed in prostate cancer metastases (Fixemer et al., 2003; Lai et al., 2004). It is tempting to speculate that this ER $\beta$  expression is regulated by the OK promoter, which is not repressed by BMI-1. From a different perspective, these findings add a new dimension to our understanding of how BMI-1 contributes to prostate tumorigenesis. Although BMI-1 also has been reported to suppress PTEN expression in nasopharyngeal epithelial cells (Song et al., 2009), we did not observe this phenomenon in the prostate epithelial and carcinoma cells that we analyzed.

Our finding that ER $\beta$  functions to suppress BMI-1 is significant because it forms the basis of our hypothesis that a positive feedback loop exists that maintains BMI-1 expression. Although ER $\beta$  has been implicated as a gatekeeper that impedes prostate tumorigenesis (Dey et al., 2013; Hussain et al., 2012; Slusarz et al., 2012), the mechanisms involved are not known. Clearly, its ability to repress BMI-1 is one such mechanism. Moreover, these findings add to our understanding of how BMI-1 is regulated in prostate cancer. Previous work by our group demonstrated that autocrine VEGF signaling in tumor cells sustains BMI-1 expression (Goel et al., 2012), but it was not apparent that this pathway is subject to inhibition by ER $\beta$ . As mentioned, autocrine VEGF signaling in tumor cells is emerging as an



**Figure 4. ERβ Represses BMI-1 by an HIF-1α/VEGF-Mediated Mechanism**

(A) Expression of HIF-1α, VEGF-A, and BMI-1 in ERβ-depleted PNT1a cells (shERβ-1 and shERβ-2) and control cells (shGFP) was assessed by immunoblotting. (B) Expression of N-cadherin, vimentin, HIF-1α, VEGF-A, and BMI-1 in PHD2-depleted cells (shPHD2-1 and shPHD2-2) compared to control cells (shGFP) was assessed by immunoblotting.

(C) HIF-1α expression was diminished in ERβ-depleted PNT1a cells using shRNA, and the impact on cell morphology and expression of HIF-1α, VEGF-A, and BMI-1 was determined. Scale bar, 50 μm.

(D) VEGF-A expression was diminished in ERβ-depleted PNT1a cells using shRNA, and the impact on cell morphology (phase contrast images) and expression of VEGF and BMI-1 was determined by immunoblotting.

(E) H&E staining of ventral prostates from 10-month-old wild-type (control) and BERKO mice. Arrows indicate areas of hyperplasia. These tissues were stained for ERβ, HIF-1α, VEGF-A, BMI-1, and PTEN and analyzed by immunofluorescence microscopy. Scale bar, 50 μm.

(F) An inverse correlation between BMI-1 and ERβ in a cohort of 87 prostate tumors was determined from analysis of the cBioportal database (Figure S1B; Taylor et al., 2010).

(G) Schematic summarizes the major conclusions of the study. See also Figure S2 and Table S2.

important mechanism that sustains the function of cancer stem cells and promotes tumor initiation, as evidenced by data obtained from several different cancers including prostate (Goel and Mercurio, 2013). Moreover, the ability of VEGF signaling to sustain BMI-1 expression accounts for how this pathway contributes to de-differentiation and tumorigenesis. The ability of ER $\beta$  to promote HIF-1 $\alpha$  degradation and, consequently, repress VEGF expression and signaling provides a mechanism for suppressing the tumorigenic potential of BMI-1. Indeed, the induction of HIF-1 $\alpha$ , VEGF, and BMI-1 expression in BERKO mice, in concert with the inverse correlation observed between ER $\beta$  and these molecules in human prostate tumors, provides support for this hypothesis. These findings also reinforce the hypothesis that loss of ER $\beta$  in prostate cancer mimics hypoxia by enabling HIF-1 $\alpha$ /VEGF signaling. Interestingly, a tumor-suppressive function for ER $\beta$  in breast cancer was reported recently (Yuan et al., 2014).

In summary, the data we report advance our understanding of how ER $\beta$  functions in prostate cancer as both a gatekeeper of epithelial differentiation and tumorigenesis and a target of oncogenic stimuli, as depicted in Figure 4G.

## EXPERIMENTAL PROCEDURES

### Cells and Reagents

PNT1a cells were obtained from M. Littmann (Baylor College of Medicine). The human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (ATCC). PC3-M cells were obtained from R.C. Bergan (Northwestern University). 3 $\beta$ -androstane-diol (3 $\beta$ -adiol) and 17 $\beta$ -estradiol (E2) experiments were performed by incubating cells with 3 $\beta$ -adiol (5  $\mu$ M; Sigma-Aldrich) or E2 (10 nM; Sigma-Aldrich) for 2 to 3 days. Wortmannin and Akt inhibitor VIII were obtained from Calbiochem. Cells were incubated with these inhibitors (5  $\mu$ M) for 18–20 hr prior to subsequent analyses. The generation of ER $\beta$  and PHD2-ablated PNT1a cells using small hairpin RNAs (shRNAs) has been described previously (Mak et al., 2013). Lentiviruses (pLKO.1) containing BMI-1 shRNA oligonucleotides (TRCN0000020154, TRCN0000020156, and TRCN0000012565), VEGF-A shRNA oligonucleotides (TRCN000003343), HIF-1 $\alpha$  shRNA (TRCN0000054449), PTEN shRNA (TRCN0000028989 and TRCN0000028990), or pLKO-shGFP control were purchased from Open Biosystems and used to infect cells following standard protocols. Stable cell transfectants were generated by puromycin or hygromycin selection (0.5  $\mu$ g/ml for PNT1a and 2  $\mu$ g/ml for PC3-M cells). The resultant ER $\beta$ -, PHD2-, BMI-1-, HIF-1 $\alpha$ -, or VEGF-A-ablated cells were used for subsequent experiments. A lentiviral plasmid (FUGW) expressing BMI-1 and the control vector (FUGW) carrying the empty vector (EV) were obtained from Addgene.

### Biochemical Analyses

For immunoblotting, the following Abs were used: ER $\beta$  and PTEN (GeneTex), BMI-1 (Cell Signaling Technology), vimentin (Dako), HIF-1 $\alpha$  (Novus Biologicals), PHD2 (Abcam), and  $\alpha$ -tubulin and  $\beta$ -actin (Sigma-Aldrich). Immune complexes were detected using enhanced chemiluminescence (ECL) (Pierce). For quantitative real-time RT-PCR (qPCR), total RNA was extracted from cells using the TRI reagent (Sigma-Aldrich) and was reverse transcribed using reverse transcription reagents (Applied Biosystems), and then analyzed by SYBR Green Master (Rox) (Roche) using a real-time PCR system (ABI PRISM 7900HT Sequence Detection System, PE Biosystems). The expression of target genes was normalized to 18 s RNA and analyzed by the comparative cycle threshold method ( $\Delta\Delta$ Ct).

ChIP was performed using the ChIP-IT Express kit (53008, Active Motif). Briefly, the attached cells were cross-linked using 1% formaldehyde for 15 min at room temperature (RT) with rotation. Subsequent steps for ChIP analysis were performed according to the manufacturer's protocol. For chro-

matin precipitation, 3  $\mu$ g BMI-1 antibody (Cell Signaling Technology) or human isotype IgG (16-4301-81, eBioscience) was used. End-point real-time PCR was performed using the primer pairs listed in Figure S1E. For luciferase assays, PC3-M cells were transfected with the desired plasmids and the Renilla luciferase construct to normalize for transfection efficiency. Luciferase assays were performed using Dual Glo luciferase assay system (Promega). Relative luciferase activity was calculated as the ratio of firefly luciferase to Renilla luciferase activity.

### Xenograft Experiments

Cells were mixed with Matrigel (30%) and injected subcutaneously into *nu/nu* mice (6 weeks old, Jackson ImmunoResearch Laboratories) using a single dose as follows: PNT1a ( $10^6$ ) and PC3-M ( $10^5$ ). Animals were monitored three times per week for tumor formation by palpation. All animal experiments were in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

### Transgenic Mice

ER $\beta$  knockout (BERKO) mice were generated by the Korach laboratory (Krege et al., 1998) and were purchased from The Jackson Laboratory. The knockout allele was maintained on a C57BL/6 background. The mice used in these studies were 10 months old. Sections from these prostates and age-matched controls were processed for immunostaining as described below. A similar approach was used for specimens obtained from prostate tissue obtained from Pten<sup>loxP/loxP</sup>; PB-Cre<sup>+</sup> mice (prostate cancer) and age-matched Pten<sup>+/+</sup>; PB-Cre<sup>+</sup> mice (normal prostate) (Hübner et al., 2012).

### Immunostaining

Murine prostate specimens from transgenic mice (see above) and human prostate cancer specimens, which were obtained from the Tissue Bank at the University of Massachusetts Medical School, were fixed in paraformaldehyde (4%), embedded in paraffin, sectioned (5  $\mu$ M), and used for H&E and immunofluorescence staining. Immunofluorescence staining was conducted according to the manufacturer's instructions (Invitrogen and Life Sciences). After antigen unmasking, the specimens were incubated in 10% serum in PBS for 30 min, washed for 3 min in PBST, and incubated with rabbit polyclonal ER $\beta$  antibody (GTx 112927, GeneTex) or rabbit BMI-1 mAb (5856S, Cell Signaling Technology) overnight at 4°C. The slides were washed 5 min with PBST and incubated 45 min in a dark chamber with the fluorochrome-conjugated secondary antibody (goat anti-rabbit conjugated Alexa Fluor 488, A-11008, Life Sciences). Slides were washed and counterstained in the dark with DAPI (Invitrogen) for 10 min, washed with three changes of PBST, and mounted under coverslips with aqueous mounting medium (Thermo Electron). Results were analyzed with an LSM 710 Meta confocal microscope (Carl Zeiss).

### Statistical Analysis

Data are presented as the mean from three separate experiments  $\pm$  SD. The Student's *t* test was used to determine the significance of independent experiments. The criterion *p* < 0.05 was used to determine statistical significance.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.02.063>.

## AUTHOR CONTRIBUTIONS

P.M. designed, executed, and analyzed all experiments and wrote the manuscript. J.L. designed, executed, and analyzed experiments. S.S. performed the ChIP experiments. C.C. performed the database analyses and molecular cloning. D.J.J. provided the ER $\beta$  knockout mice. R.J.D. provided the PTEN<sup>pc-/-</sup> mice. I.L. evaluated the pathology of all tissue specimens and contributed to the overall focus of the study. A.M.M. supervised the study and wrote the manuscript together with P.M.

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## REFERENCES

- Antal, M.C., Krust, A., Chambon, P., and Mark, M. (2008). Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant. *Proc. Natl. Acad. Sci. USA* *105*, 2433–2438.
- Berthon, P., Cussenot, O., Hopwood, L., Leduc, A., and Maitland, N. (1995). Functional expression of sv40 in normal human prostatic epithelial and fibroblastic cells - differentiation pattern of nontumorigenic cell-lines. *Int. J. Oncol.* *6*, 333–343.
- Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jen, J., Isaacs, W.B., Bova, G.S., and Sidransky, D. (1997). Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res.* *57*, 4997–5000.
- Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol. Cell* *20*, 845–854.
- Christoforou, P., Christopoulos, P.F., and Koutsilieris, M. (2014). The role of estrogen receptor  $\beta$  in prostate cancer. *Mol. Med.* *20*, 427–434.
- Dey, P., Barros, R.P., Warner, M., Ström, A., and Gustafsson, J.A. (2013). Insight into the mechanisms of action of estrogen receptor  $\beta$  in the breast, prostate, colon, and CNS. *J. Mol. Endocrinol.* *51*, T61–T74.
- Elo, T., Yu, L., Valve, E., Mäkelä, S., and Härkönen, P. (2014). Deficiency of ER $\beta$  and prostate tumorigenesis in FGF8b transgenic mice. *Endocr. Relat. Cancer* *21*, 677–690.
- Fixemer, T., Remberger, K., and Bonkhoff, H. (2003). Differential expression of the estrogen receptor beta (ERbeta) in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. *Prostate* *54*, 79–87.
- Goel, H.L., and Mercurio, A.M. (2013). VEGF targets the tumour cell. *Nat. Rev. Cancer* *13*, 871–882.
- Goel, H.L., Chang, C., Pursell, B., Leav, I., Lyle, S., Xi, H.S., Hsieh, C.C., Adisetiyo, H., Roy-Burman, P., Coleman, I.M., et al. (2012). VEGF/neuropilin-2 regulation of Bmi-1 and consequent repression of IGF-IR define a novel mechanism of aggressive prostate cancer. *Cancer Discov.* *2*, 906–921.
- Hirata, S., Shoda, T., Kato, J., and Hoshi, K. (2001). The multiple untranslated first exons system of the human estrogen receptor beta (ER beta) gene. *J. Steroid Biochem. Mol. Biol.* *78*, 33–40.
- Hübner, A., Mulholland, D.J., Standen, C.L., Karasarides, M., Cavanagh-Kyros, J., Barrett, T., Chi, H., Greiner, D.L., Tournier, C., Sawyers, C.L., et al. (2012). JNK and PTEN cooperatively control the development of invasive adenocarcinoma of the prostate. *Proc. Natl. Acad. Sci. USA* *109*, 12046–12051.
- Hussain, S., Lawrence, M.G., Taylor, R.A., Lo, C.Y., Frydenberg, M., Ellem, S.J., Furic, L., and Risbridger, G.P.; APC BioResource (2012). Estrogen receptor  $\beta$  activation impairs prostatic regeneration by inducing apoptosis in murine and human stem/progenitor enriched cell populations. *PLoS ONE* *7*, e40732.
- Imamov, O., Morani, A., Shim, G.J., Omoto, Y., Thulin-Andersson, C., Warner, M., and Gustafsson, J.A. (2004). Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. *Proc. Natl. Acad. Sci. USA* *101*, 9375–9380.
- Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* *397*, 164–168.
- Kozlowski, J.M., Fidler, I.J., Campbell, D., Xu, Z.L., Kaighn, M.E., and Hart, I.R. (1984). Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res.* *44*, 3522–3529.
- Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.A., and Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. USA* *95*, 15677–15682.
- Kuiper, G.G., Enmark, E., Peltö-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* *93*, 5925–5930.
- Lai, J.S., Brown, L.G., True, L.D., Hawley, S.J., Etzioni, R.B., Higano, C.S., Ho, S.M., Vessella, R.L., and Corey, E. (2004). Metastases of prostate cancer express estrogen receptor-beta. *Urology* *64*, 814–820.
- Lau, K.M., LaSpina, M., Long, J., and Ho, S.M. (2000). Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. *Cancer Res.* *60*, 3175–3182.
- Leav, I., Lau, K.M., Adams, J.Y., McNeal, J.E., Taplin, M.E., Wang, J., Singh, H., and Ho, S.M. (2001). Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. *Am. J. Pathol.* *159*, 79–92.
- Lukacs, R.U., Memarzadeh, S., Wu, H., and Witte, O.N. (2010). Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. *Cell Stem Cell* *7*, 682–693.
- Mak, P., Leav, I., Pursell, B., Bae, D., Yang, X., Taglienti, C.A., Gouvin, L.M., Sharma, V.M., and Mercurio, A.M. (2010). ERbeta impedes prostate cancer EMT by destabilizing HIF-1alpha and inhibiting VEGF-mediated snail nuclear localization: implications for Gleason grading. *Cancer Cell* *17*, 319–332.
- Mak, P., Chang, C., Pursell, B., and Mercurio, A.M. (2013). Estrogen receptor  $\beta$  sustains epithelial differentiation by regulating prolyl hydroxylase 2 transcription. *Proc. Natl. Acad. Sci. USA* *110*, 4708–4713.
- McMenamin, M.E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W.R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.* *59*, 4291–4296.
- Miyazaki, M., Miyazaki, K., Itoi, M., Katoh, Y., Guo, Y., Kanno, R., Katoh-Fukui, Y., Honda, H., Amagai, T., van Lohuizen, M., et al. (2008). Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. *Immunity* *28*, 231–245.
- Mulholland, D.J., Kobayashi, N., Ruscetti, M., Zhi, A., Tran, L.M., Huang, J., Gleave, M., and Wu, H. (2012). Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. *Cancer Res.* *72*, 1878–1889.
- Nacerddine, K., Beaudry, J.B., Ginja, V., Westerman, B., Mattioli, F., Song, J.Y., van der Poel, H., Ponz, O.B., Pritchard, C., Cornelissen-Steijger, P., et al. (2012). Akt-mediated phosphorylation of Bmi1 modulates its oncogenic potential, E3 ligase activity, and DNA damage repair activity in mouse prostate cancer. *J. Clin. Invest.* *122*, 1920–1932.
- Slusarz, A., Jackson, G.A., Day, J.K., Shenouda, N.S., Bogener, J.L., Browning, J.D., Fritsche, K.L., MacDonald, R.S., Besch-Williford, C.L., and Lubahn, D.B. (2012). Aggressive prostate cancer is prevented in ER $\alpha$ KO mice and stimulated in ER $\beta$ KO TRAMP mice. *Endocrinology* *153*, 4160–4170.
- Song, L.B., Li, J., Liao, W.T., Feng, Y., Yu, C.P., Hu, L.J., Kong, Q.L., Xu, L.H., Zhang, X., Liu, W.L., et al. (2009). The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J. Clin. Invest.* *119*, 3626–3636.
- Taylor, B.S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B.S., Arora, V.K., Kaushik, P., Cerami, E., Reva, B., et al. (2010). Integrative genomic profiling of human prostate cancer. *Cancer Cell* *18*, 11–22.
- Thomas, C., and Gustafsson, J.A. (2011). The different roles of ER subtypes in cancer biology and therapy. *Nat. Rev. Cancer* *11*, 597–608.
- van Leenders, G.J., Dukers, D., Hessels, D., van den Kieboom, S.W., Hulsbergen, C.A., Witjes, J.A., Otte, A.P., Meijer, C.J., and Raaphorst, F.M. (2007). Polycomb-group oncogenes EZH2, BMI1, and RING1 are overexpressed in

prostate cancer with adverse pathologic and clinical features. *Eur. Urol.* 52, 455–463.

Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., et al. (2003). Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 4, 209–221.

Worby, C.A., and Dixon, J.E. (2014). Pten. *Annu. Rev. Biochem.* 83, 641–669.

Yuan, B., Cheng, L., Chiang, H.C., Xu, X., Han, Y., Su, H., Wang, L., Zhang, B., Lin, J., Li, X., et al. (2014). A phosphotyrosine switch determines the antitumor activity of ER $\beta$ . *J. Clin. Invest.* 124, 3378–3390.

Zhu, X., Leav, I., Leung, Y.K., Wu, M., Liu, Q., Gao, Y., McNeal, J.E., and Ho, S.M. (2004). Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. *Am. J. Pathol.* 164, 2003–2012.