Gata3 antagonizes cancer progression in Pten-deficient prostates

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Loss of the tumor suppressor PTEN is a common occurrence in prostate cancer. This aberration leads to the ectopic activation of the PI3K-Akt pathway, which promotes tumor growth. Here, we show that the transcription factor Gata3 is progressively lost in Pten-deficient mouse prostate tumors as a result of both transcriptional down-regulation and increased proteasomal degradation. To determine the significance of this loss, we used conditional loss- and gain-of-function approaches to manipulate Gata3 expression levels in prostate tumors. Our results show that Gata3 inactivation in Pten-deficient prostates accelerates tumor invasion. Conversely, enforced expression of GATA3 in Pten-deficient tissues markedly delays tumor progression. In Pten-deficient prostatic ducts, enforced GATA3 prevented Akt activation, which correlated with the down-regulation of Pik3cg and Pik3c2a mRNAs, encoding respectively class I and II PI3K subunits. Remarkably, the majority of human prostate tumors similarly show loss of active GATA3 as they progress to the aggressive castrate-resistant stage. In addition, GATA3 expression levels in hormone-sensitive tumors holds predictive value for tumor recurrence. Together, these data establish Gata3 as an important regulator of prostate cancer progression.

INTRODUCTION

The primary cause of mortality from prostate cancer is the development of a recurrent and aggressive form of the disease. In the prostate, cancer recurrence is associated with the acquisition of castration-resistance following androgen depletion therapy. The lack of efficient targeted therapy against these tumors is due to our limited understanding of the molecular changes underlying this complex process.

One of the most frequent alterations in human prostate cancers is the inactivation of the PTEN tumor suppressor gene (1,2). PTEN loss results in ectopic PI3K/Akt activation, which regulates proliferation, survival, translation and cellular metabolism (3). In the prostate, the PTEN locus shows heterozygous loss in up to 68% and homozygous loss in up to 12% of primary tumors (4–6). The loss of a second allele of PTEN is observed in 40% of castration-resistant and aggressive forms of prostate cancer (7,8). In the mouse, prostate-specific inactivation of Pten similarly leads to invasive adenocarcinoma, associated with castration-resistance (9,10).

Gata3 is a member of the conserved Gata family of transcription factors (11). It plays a critical role in the development of several tissues, including the parathyroid, kidney, mammary gland and lymphoid system (12–19). Gata3 inactivation in breast cancer leads to cellular dedifferentiation and promotes tumor progression (20–23). In the prostate, GATA3 is
expressed in prostatic ducts but lost in prostate cancer cell lines (24). Furthermore, GATA3 binds the KLK3/PSA (prostate specific antigen) promoter, which promotes PSA activation by the androgen receptor (24).

In this report, we investigate the role of Gata3 in prostate cancer using both genetic loss- and gain-of-function mouse models and human prostate tissue microarrays (TMAs). We show that Gata3 is spontaneously lost through transcriptional down-regulation and proteasome-mediated degradation during the progression of Pten-deficient tumors. Importantly, acute Gata3 deletion in this model accelerates progression of prostatic intraepithelial neoplasia (PIN) to invasive carcinoma, while enforced expression of human GATA3 prevents tumor progression. This property of GATA3 is partly mediated through dampening of PI3K-Akt pathway activation. Finally, we show that progression to castration resistance in human prostate tumors correlates with a loss of GATA3 nuclear expression, which corroborates our findings in the mouse model.

RESULTS

Gata3 is down-regulated during progression of Pten-deficient tumors in the mouse

To determine the role of Gata3 in the prostate, we initially defined its expression pattern. Immunostaining of Gata3 together with cell-specific markers for luminal cells (CK8/18), basal cells (CK5) or neuroendocrine cells (Synaptophysin) established that Gata3 expression is strongly expressed to the luminal cell compartment in the adult prostate, while its expression in the basal and neuroendocrine lineages was weak or absent (Fig. 1A). We next investigated Gata3 expression during prostate cancer progression by Cre-mediated inactivation of the tumor suppressor gene Pten in the prostate epithelium (PE) using the PbCre4 transgene (PtenPE/−/−). This model was previously shown to progress through all stages of prostate tumor development from hyperplasia and PIN to carcinoma (10,25). Immunostaining for Gata3 on control and Pten-deficient prostates revealed a down-regulation of Gata3 protein levels starting at 6 weeks of age, associated with the absence of Pten (Fig. 1B, Supplementary Material, Fig. S1A; P < 0.0005). Immunofluorescence staining and western blot analyses determined that Gata3 is completely lost by 12 weeks of age, when most Pten-deficient tumors have progressed to invasive carcinoma (Fig. 1B and C). To further validate the link between Pten loss and Gata3 down-regulation, we measured the cellular level of activated Akt (pAkt) in comparison to Gata3 protein expression levels in 6 weeks old prostates. This analysis revealed a strong correlation between ectopic Akt activation and Gata3 loss at the cellular level (Supplementary Material, Fig. S1B). To determine whether the down-regulation of Gata3 was occurring at the transcriptional level, we assessed Gata3 mRNA expression at 8 and 12 weeks of age by quantitative RT-PCR. Interestingly, Gata3 mRNA was already reduced by ~60% at 8 weeks and remained at this level at 12 weeks of age (Fig. 1D). This observation is in line with three independent microarray studies (Oncomine) of human prostate cancer samples, which reveal a progressive down-regulation of GATA3 mRNA as the tumors progress through benign lesions to carcinomas and metastases (Supplementary Material, Fig. S1C–E) (26–28). We next determined whether Gata3 protein stability was affected by Pten loss. For this, we treated primary cells from Pten-deficient prostates with the proteasome inhibitors MG132 and Lactacystin (Fig. 1E). As expected, Gata3 protein levels were reduced in Pten-deficient cells compared with wild-type cells. Remarkably, Gata3 levels were significantly increased by MG132 and Lactacystin treatment, suggesting that Gata3 loss is largely mediated by increased protein degradation in Pten-deficient cells. Together, these results indicate that Gata3 expression is down-regulated at the transcriptional as well as post-translational levels during progression from PIN to carcinoma in Pten-deficient prostates.

Acute loss of Gata3 accelerates progression of Pten-deficient prostate tumors

To better understand the consequence of Gata3 loss, we first inactivated Gata3 in conjunction with Pten in the PE. Gross morphological assessment of PtenPE−/−; Gata3PE−/− prostates did not reveal marked changes in tumor size compared with PtenPE−/− (Supplementary Material, Fig. S2A). However, histopathological analyses revealed invasive lesions at an earlier time point in PtenPE−/−; Gata3PE−/− compared with PtenPE−/− tumors (Fig. 2A–C and Supplementary Material, Fig. S2B). At 6 weeks, most PtenPE−/−; PtenPE−/− tumors examined had progressed to PIN but none had developed invasive carcinoma (Fig. 2B). In contrast, 60% of PtenPE−/−; Gata3PE−/− tumors showed invasive lesions at this stage, with the remaining 40% showing PIN lesions (Fig. 2B, P < 0.001). At 12 weeks, all PtenPE−/−; Gata3PE−/− tumors developed invasive carcinoma, whereas some PtenPE−/−; Gata3PE−/− prostates did not yet show invasive lesions at this stage (Fig. 2C, P < 0.009). These observations suggest that Gata3 loss accelerates tumor progression in Pten-deficient mice.

GATA3 antagonizes prostate tumor formation mediated by Pten-deficiency

To investigate further the significance of Gata3 loss during prostate cancer progression, we generated a conditional gain-of-function allele in which the human GATA3 cDNA is expressed under the ubiquitously expressed PSA (PtenPE−/−; R26G3/G3), independently of transcriptional modulation by Pten loss. The expression of GATA3 is activated by PbCre4-mediated deletion of a stop cassette located upstream of the GATA3 cDNA (Fig. 3A). Quantitative RT-PCR analysis confirmed the down-regulation of mouse Gata3 and expression of human GATA3 mRNA in these tumors (Supplementary Material, Fig. S3A). The expression pattern of GATA3 in PtenPE−/−; R26G3/G3 prostatic ducts came in two major variants. At 12 weeks, about 30% of prostatic ducts showed high GATA3 expression (type 1; Fig. 3B and Supplementary Material, Fig. S3B), while the remaining ducts of the same prostates had lost GATA3 (type 2; Fig. 3B and Supplementary Material, Fig. S3B). MG132 and Lactacystin treatment of primary tumor cells indicated that GATA3 expressed from the Rosa26 locus was also degraded.
by the proteasome (Supplementary Material, Fig. S3C). Quantitative RT-PCR analysis of Pten mRNA levels in these tumors confirmed an equivalent rate of Cre-mediated Pten inactivation in PtenPE2/2 and PtenPE2/2; R26G3/G3 samples (Supplementary Material, Fig. S3D).

Strikingly, the re-expression of GATA3 was associated with a highly improved histology (type 1, Fig. 3C; Supplementary Material, Fig. S3E, \( P < 0.001 \)). PtenPE2/2; R26G3/G3 prostatic ducts expressing GATA3 were glandular and well differentiated even at 12 weeks of age, while PtenPE2/2 ducts presented with a hyperplastic and invasive phenotype (Fig. 3C). In contrast, prostatic ducts that lost GATA3 expression (type 2) were associated with a histology resembling PtenPE2/2 tumors (Fig. 3C). Together, these results establish GATA3 as a critical factor in preventing prostate cancer progression.

**Gata3 maintains cellular differentiation and organization in Pten-deficient prostates**

To determine the cellular consequences of Gata3 modulation, we assessed the prostates for hallmarks of cancer progression such as loss of cellular differentiation, polarity and organization. We first looked at the lineage markers CK8/18 and CK5 for luminal and basal cells, respectively. In normal prostatic ducts, CK8/18 marks a simple pseudo-stratified to columnar epithelium overlying a single layer of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells. In Pten-deficient tumors, the basal epithelium was disorganized with regions completely devoid of CK5+ cells, while others accumulated several layers of CK5+ basal cells.

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Enforced expression of GATA3 restored normal basal cell morphology and apically enriched CK8/18 localization in type 1 PtenPE2/2; R26G3/G3 prostates (Fig. 4B), while type 2 prostates resembled Pten-deficient tumors (Fig. 4B). The expression of epithelial marker E-cadherin in the basolateral compartment confirmed the disorganized tissue polarity in PtenPE2/2, a defect that was largely corrected in type 1, but not in type 2 PtenPE2/2; R26G3/G3 prostates (Fig. 4C). The
transcription factor Nkx3-1, normally expressed in the luminal layer of the prostate, was down-regulated in \( \text{Pten}^{PE/-} / \) tumors (Fig. 4D). Nkx3-1 expression was restored in type 1 but not in type 2 \( \text{Pten}^{PE/-} / \) ; \( \text{R26G3/G3} \) prostates (Fig. 4D). As for the androgen receptor, \( \text{Pten} \) inactivation resulted in an elevation of both cytoplasmic and nuclear AR levels (Fig. 4E). In accordance with the improved histology of type 1 \( \text{Pten}^{PE/-} / \) ; \( \text{R26G3/G3} \) prostates, re-expression of GATA3 essentially maintained a normal distribution of AR, while type 2 ducts failed to do so (Fig. 4E). Hence, GATA3 effectively prevents the loss of cellular organization, differentiation and polarity observed in \( \text{Pten} \)-deficient tumors.

**Gata3 dampens Akt pathway activation**

The observation that GATA3 can prevent several phenotypes characteristic of \( \text{Pten} \)-deficient tumors suggests that it may regulate a program impinging on the PI3K-Akt pathway. To address this possibility, we performed western blot analysis for the activated form of Akt (phospho-Akt S473 and T308). While the normal prostate did not express activated Akt, \( \text{Pten}^{PE/-} / \) tumors expressed very high levels of p-Akt S473 and p-Akt T308 (Fig. 5A). Strikingly, both p-Akt S473 and p-Akt T308 were markedly reduced in \( \text{Pten}^{PE/-} / \) ; \( \text{R26G3/G3} \) prostates.

To investigate the relationship between Gata3 and Akt activation, we performed immunostaining for both proteins in the different experimental genotypes. As expected, \( \text{Pten} \) inactivation led to a strong activation of p-Akt localized at the cell membrane (Fig. 5A). In contrast, type 1 \( \text{Pten}^{PE/-} / \) ; \( \text{R26G3/G3} \) prostates expressing high levels of GATA3 lacked Akt activation (Fig. 5B). Type 2 regions with low or no GATA3 expression had intermediate to high p-Akt signal (Fig. 5B). Hence, in this system, the presence of GATA3 inversely correlates with Akt activation. Importantly, an antibody recognizing all three isoforms of Akt (Akt1/2/3) revealed little change in total cellular Akt levels in the tumors compared with control (Fig. 5A and C and Supplementary Material, Fig. S4A–C).

As p-Akt T308 is a target site of the Pdpk1 kinase (29), the lack of p-Akt T308 activation in \( \text{Pten}^{PE/-} / \) ; \( \text{R26G3/G3} \) prostates suggested that the negative effect of GATA3 might be elicited by the regulation of PI3K signaling components upstream of Akt. To test this possibility, we determined the transcriptional levels of the Akt regulator Pdpk1 as well as class I and class II PI3K components (Pik3ca, Pik3cb, Pik3cd, Pik3cg, Pik3r1, Pik3r2, Pik3c2a and Pik3c2g) in wild-type and in \( \text{Pten} \)-deficient prostates with loss or gain of function of Gata3 (Supplementary Material, Fig. S4). Of these, subunits Pik3cg (p110y) and Pik3c2a were found increased by more than three folds in \( \text{Pten}^{PE/-} / \) and \( \text{Pten}^{PE/-} / \) ; \( \text{Gata3}^{PE/-} / \) prostates at 8 weeks.
of age and brought down near control expression levels by enforced GATA3 expression (PtenPE−/−; R26G3/G3) (Fig. 5D and E). A similar tendency was seen at 12 weeks of age, where re-expressing GATA3 strongly reduced Pik3c2a and Pik3cg expression levels. Together, these results indicate that Gata3 antagonizes PI3K/Akt signaling by dampening PI3K pathway amplification at the transcriptional level.

**GATA3 expression and predictive value in human prostate tumors**

To assess whether our findings in mouse models parallel human tumors, we examined a series of TMAs for GATA3 expression. These arrays included a total of 224 specimens, including 48 normal prostate, 54 non-neoplastic adjacent tissue, 30 prostatic intraepithelial neoplastic tissue, 63 hormone-sensitive tumors and 29 castration-resistant tumors. Blind scoring was performed for nuclear and cytoplasmic localization of GATA3 by two independent observers. Normal tissues from non-affected controls were found to harbor nuclear GATA3 staining (Fig. 6A and Supplementary Material, Fig. S5A). Similarly, tissue cores classified as hormone-sensitive tumors largely harbored nuclear GATA3 localization (Fig. 6D and Supplementary Material, Fig. S5D). Most remarkable, however, was the nearly complete absence of nuclear GATA3 staining in castration-resistant tumors, which was coupled with an increase in cytoplasmic GATA3 accumulation (Fig. 6E and Supplementary Material, Fig. S5E). Quantification for all TMA features revealed that castration-resistant tumors show nuclear GATA3 signal in only 15% of samples, which represent a 75% reduction compared with hormone-sensitive tumors (Fig. 6F).

We next asked whether the expression level of GATA3 in tumors prior to hormone-deprivation therapy had predictive value on clinical outcome. For this, GATA3 expression levels in hormone-sensitive tumors were assessed with respect to biochemical-recurrence as measured by PSA expression. This analysis revealed a significant association between low cellular levels of GATA3 and the risk of biochemical recurrence (log-rank P-value = 0.046) (Fig. 6G). Taken together, the expression of GATA3 in human prostate cancer tissue is in line with the loss of Gata3 expression observed in the mouse and further supports the critical role of active GATA3 in preventing prostate cancer progression.
DISCUSSION

A critical challenge in the management of prostate cancer is to identify the characteristics of tumors most likely to recur as aggressive castration-resistant and the mechanisms underlying recurrence. Here, we identified a role for Gata3 in preventing prostate tumor progression in the castration-resistant *Pten* deficient model. Enforced expression of a human GATA3 transgene in these tumors reveals that GATA3 antagonizes Akt signaling and maintains a differentiated prostatic duct phenotype. Analysis of human prostate tumor samples identified nuclear loss of GATA3 in castration-resistant tumors and revealed that high GATA3 expression levels in hormone-sensitive tumors are predictive of low tumor recurrence.

Our experiments demonstrate that the loss of functional Gata3 is linked to tumor progression in both mouse and human samples. In the *Pten*-deficient model, the effect of GATA3 expression was striking in terms of general tissue histology, cellular polarity, proliferation and differentiation. The strong effect of enforced GATA3 expression on activated

**Figure 4.** GATA3 maintains cellular differentiation, polarity and organization in *Pten*-deficient prostates. (A and B) CK8/18 (green) and CK5 (red) co-immunofluorescence staining on control, *Pten*^PE−/−^, type 1 and type 2 *Pten*^PE−/−^, *R26*^G3/G3^ dorso-lateral prostate sections of 6-week-old mice. Arrows point to CK5 and CK8/18 double positive cells. (C) Immunofluorescence staining of E-Cadherin on sections as in (A). Arrows point to cells without E-cad negative apical domain. Arrow heads point to cells with E-cad negative apical domain. (D) Nkx3-1 immunohistochemistry staining on sections as in (A). Arrows point to Nkx3-1 positive cells. (E) Immunofluorescence staining of the androgen receptor (AR) on dorso-lateral prostate sections of 12-week-old mice of the indicated genotypes. Arrows point to cells with nuclear AR, and arrowheads point to cytoplasmic AR.
Akt suggests that a major part of Gata3 anti-oncogenic activity is performed through the regulation of PI3K-Akt signaling. The differential phosphorylation of Akt at position T308, a site phosphorylated by the S/T kinase Pdpk1 (29), further suggested that Gata3 played a role in PI3K-Akt regulation upstream of Akt. Of the PI3K-Akt components tested, we found that *Pik3cg* (*p110γ*) and *Pik3c2a* expression levels were reduced by *Gata3* expression. Of interest, *Pik3c2a* was

**Figure 5.** Negative regulation of Akt signaling by GATA3. (A) Immunoblot of p-Akt T308, p-Akt S473, Akt and beta-Actin on protein extract of dorso-lateral prostates from 12-week-old mice of the indicated genotype. (B) Phospho-Akt S473 and Gata3 co-immunofluorescence staining on dorso-lateral prostate sections of 6-week-old control, *Pten*<sup>PE−/−</sup>, type 1 and type 2 *Pten*<sup>PE−/−</sup>; *R26*<sup>G3/G3</sup> mice. Arrows point to p-Akt S473 positive, Gata3 negative cells. (C) Co-immunofluorescence staining of Akt and Gata3 on sections as in (B). Arrows point to cells with membrane-positive Akt expression. (D and E) Quantitative RT-PCR analysis of *Pik3cg* (D) and *Pik3c2a* (E) on dorso-lateral prostate samples of 8- and 12-week-old mice of the indicated genotypes. Data are presented as ratios of mutant over control after normalization to *Ppia* [mean ± SD (n = 4); *P*-value ≤ 0.005; **P**-value ≤ 0.0005; ***P**-value ≤ 0.00005].
found upregulated in several cancers (30), while p110γ was shown to act as a potent oncogene when overexpressed in its wild-type form (31). Hence, the transcriptional regulation of PI3K components Pik3cg and Pik3c2a by Gata3 likely contribute to its negative effect on PI3K-Akt signaling.

The importance of Gata3 loss for tumor progression is further highlighted by the fact that it occurs at several levels. Our results show that Gata3 mRNA expression is significantly reduced during a critical stage of tumor progression (age 8–12 weeks) in the Pten-deficient mouse model. This observation is in line with our analysis of three independent human prostate cancer expression microarrays available through the Oncomine resource, which reveals a progressive down-regulation of GATA3 mRNA as the tumors progress through benign lesions to carcinomas and metastases. Furthermore, as Gata3 protein expression is completely lost in Pten-deficient tumors in spite of some mRNA expression remaining, we explored the mechanisms of Gata3 protein degradation in primary tumors. This analysis revealed that Gata3 protein loss could be reverted by proteasome inhibition. The levels reached upon proteasome inhibition of tumor cells were in fact higher than in wild-type cells, suggesting that Gata3 is degraded through the proteasome in normal cells but that Pten-deficient tumors accelerate the degradation rate. As expected, enforced expression of GATA3 was also submitted to proteasome degradation but the sustained transcription of GATA3 was sufficient to counteract this effect in type 1 prostates. The fact that proteasome inhibition allows high Gata3 protein accumulation argues against a regulation of Gata3 at the translational level as the reduced Gata3 mRNA expression present in tumor cells is clearly competent for translation in this context. The other interesting mode of Gata3 regulation

Figure 6. GATA3 localization and predictive value in human prostate tumors. (A–E) Immunohistochemistry of GATA3 on human TMAs. TMAs consist of normal prostate (A), non-neoplastic adjacent tissue (B), prostatic intraepithelial neoplastic tissue (PIN) (C), hormone-sensitive (HS) carcinoma (D) and castration-resistant (CR) tumors (E). Arrows point to GATA3 positive nuclei and arrowheads point to GATA3 negative nuclei. (F) Quantitative mean percentage of cells with positive nuclear GATA3 and corresponding cytoplasmic GATA3 intensity of each TMA group. (G) Recurrence-free survival curves were plotted using Kaplan–Meier analysis for GATA3 high (n = 20) versus GATA3 low (n = 43) expression. Censored points refer to end of clinical follow-up. Overall, 65% of GATA3-high patients remained recurrence-free as opposed to 35% of GATA3-low patients (log-rank = 3.958; P = 0.047).
we observed is a cytoplasmic protein accumulation, seen in human castration-resistant tumors. Although the mechanism of GATA3 subcellular localization is still elusive, the observation that human tumors use an alternative mechanism of GATA3 functional inactivation reinforces the importance of GATA3 loss for tumor progression.

A current limitation in prostate cancer management is to determine which tumors will progress to castration-resistant state. Our analysis of human prostate cancer samples shows that high GATA3 cellular level at the hormone-sensitive stage correlates with a reduced probability of tumor recurrence. Hence, GATA3 may be a useful addition to existing markers being developed to improve prognostic accuracy. Importantly, the fact that GATA3 remains expressed in human prostate tumors opens the doors to possible therapeutic approaches based on the redirection of GATA3 to the nucleus.

MATERIALS AND METHODS

Mice

All experimental mice were kept in a C57Bl/6 background. To generate prostate-specific Pten mutant mice (Pten°Flox°Flox), PbCre4 (32) transgenic mice were crossed with Pten°Flox mouse (25). Double Pten; Gata3 mutant mice (Pten°Flox°Flox; Gata3°Flox°Flox) were generated by breeding PbCre4; Pten°Flox mice with Gata3°Flox mice (14). Rosa26GATA3 mice were generated as previously described (33) (Supplementary Material, Fig. S6). Pten mutant, GATA3 rescue mice (Pten°Flox°Flox; R26GATA3) were generated by breeding PbCre4; Pten°Flox mice with Rosa26GATA3 mice. All animal procedures were approved by McGill University Animal Care Committee according to the Canadian Council on Animal Care guidelines for use of laboratory animals in biological research.

Histopathological analysis

For Pten°Flox°Flox; Gata3°Flox°Flox experiments, control and experimental mice were sacrificed at age 4, 6, 8 and 12 weeks to follow tumor progression. Tissues were processed for frozen section, paraffin section or flash frozen for RNA and protein. For pathological analysis, tissues were dehydrated in a gradient of ethanol and xylene. Dehydrated tissues were embedded in paraffin and sectioned at 4 μm. Hematoxylin and eosin (H&E) stained sections were analyzed by veterinary pathologist M.P.

Quantitative RT-PCR

Total RNA was extracted from frozen tissues of dorso-lateral prostate using RNAeasy mini kit (Qiagen). One microgram of RNA was reversed transcribed in a total of 20 μl reaction using SuperScript III (Invitrogen). Real-time quantitative PCR was done using IQ SYBR Green supermix (Biorad) on Q-Plex PCR machine (Eppendorf). Primers used are detailed in Supplementary Material, Table S1.

Western blot

Total protein lysate was extracted from frozen tissues in phospho-stable lysis buffer as previously described (34). For proteasome inhibitors treatment, dorso-lateral prostates were dissociated and kept at 37°C for 4 h in the PrEGM medium (Clonetics, Lonza) containing 20 μM MG132 (Calbiochem) or 20 μM Lactacystin (Calbiochem) followed by total protein extraction in Laemmli buffer (58.3 mM Tris–HCl pH 6.8, 1.6% SDS, 5% glycerol, 38.3 mM β-mercaptoethanol) heated at 100°C. Fifty micrograms of protein sample was run on SDS–PAGE and transferred to PVDF membrane. Antibodies and conditions used are detailed in Supplementary Material, Table S2.

In situ hybridization

Tissues were fixed in 4% paraformaldehyde and passed through a gradient of 15 and 30% sucrose. Equilibrated tissues were embedded in optimal cutting temperature and sectioned at 12 mm. Generation of cRNA probe and in situ hybridization procedure were performed as previously described (14).

Immunofluorescence and immunohistochemistry

Immunofluorescence (IHF) staining was performed on paraffin sections. Briefly, sections were rehydrated through a gradient of xylene and ethanol. Rehydrated sections were antigen-retrieved by microwave treatment in 10 mM citrate or 1 mM EDTA buffer for 20 min. Tissues were blocked for 1 h at room temperature in 10% serum, primary antibodies were added to sections and incubated at 4°C overnight. Primary antibodies used for immunofluorescence are listed in Supplementary Material, Table S2. Alexa-conjugated secondary antibodies (1:500) (Invitrogen) were incubated on sections for 1 h at RT. Sections were mounted with Slow fade anti-fade reagent (Invitrogen). Immunohistochemistry (IHC) was performed according to the manufacturer’s instruction (Dako). Stained sections were counterstained with 50% hematoxylin, dehydrated and mounted with Permount medium.

Human prostate TMA

Construction of human prostate TMA was described previously (35). Epithelial zones were scored according to the staining intensity of the cytoplasm (value of 0 for absence, 1 for weak, 2 for moderate, 3 for moderately high, 4 for high intensity). The percentage of cancer cells exhibiting moderate to strong nuclear staining was also counted. In cores where staining was of variable intensity, the average intensity was reported. Each array was independently analyzed in a blinded fashion by two independent observers. Inter-rating correlation was >75%. When strong differences in scoring between the two observers (more than 1 unit per core) occurred, the core was re-evaluated to reach a concordant scoring between the two observers. The average of all cores with cancer from the same patient was used for analysis. No decrease in staining intensity on older paraffin blocks was observed.
Microscopy
H&E and immunohistochemistry bright field images were acquired with an Axioplan 2 microscope (Zeiss). Immunofluorescence images were acquired with a LSM 510 Meta Confocal Microscope (Zeiss). Images were acquired at 2 mm, 1.2 mm and 0.8 mm thickness using ×40, ×63, and ×100 objectives, respectively. High-throughput image acquisition of TMA was performed using an Aperio XT Slide Scanner.

Statistical analysis
Statistical analysis was performed using SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). The median score of normal tissues, normal adjacent tissues and tumor tissues was used as cutoff values. The median marker expression was used to categorize data in dichotomized variables (positive or negative). Since not all marker expression had normal distribution and equal variance, the non-parametric Kruskal–Wallis test was used to determine significance between the normal, normal adjacent, PIN, HS and CR groups. Correlations between markers and with clinicopathologic variables were done using the non-parametric Spearman rho correlation test (two tailed). Survival curves were plotted using the Kaplan–Meier analysis and the log-rank test was used to test for significant differences.

AUTHORS’ CONTRIBUTION
A.H.T.N., M.T. and M.B. conceived the project and designed the experiments. A.H.T.N., M.T. and I.H.K carried out the experiments. M.P. performed the histopathological analysis. P.P.P., K.H. and J.J.H. generated animals and provided expertise. A.-M.M.-M. and F.S. generated and supervised tissue microarray analysis. A.H.T.N., M.T. and M.B. wrote the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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