Zbtb7a suppresses prostate cancer through repression of a Sox9-dependent pathway for cellular senescence bypass and tumor invasion

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Zbtb7a has previously been described as a powerful proto-oncogene. Here we unexpectedly demonstrate that Zbtb7a has a critical oncosuppressive role in the prostate. Prostate-specific inactivation of Zbtb7a leads to a marked acceleration of Pten loss–driven prostate tumorigenesis through bypass of Pten loss–induced cellular senescence (PICS). We show that ZBTB7A physically interacts with SOX9 and functionally antagonizes its transcriptional activity on key target genes such as MIA, which is involved in tumor cell invasion, and H19, a long noncoding RNA precursor for an RB-targeting microRNA. Inactivation of Zbtb7a in vivo leads to Rb downregulation, PICS bypass and invasive prostate cancer. Notably, we found that ZBTB7A is genetically lost, as well as downregulated at both the mRNA and protein levels, in a subset of human advanced prostate cancers. Thus, we identify ZBTB7A as a context-dependent cancer gene that can act as an oncogene in some contexts but also has oncosuppressive-like activity in Pten-null tumors.

RESULTS

Zbtb7a loss promotes Pten null–driven prostate tumorigenesis

To assess the possible proto-oncogenic role of ZBTB7A in prostate cancer development, we generated a transgenic mouse with prostate-specific overexpression of ZBTB7A. Unexpectedly, we found that overexpression of ZBTB7A in the prostate epithelium was insufficient to trigger any sign of neoplastic transformation (Supplementary Fig. 1). At the same time, we also generated mice with conditional inactivation of Zbtb7a in the prostate (following a strategy described previously15), expecting the mice to show profound suppression of tumor development when crossed with mice harboring genetic deletion of known prostate tumor suppressors such as Pten. Specifically, we crossed Pbsn-cre4 transgenic mice (expressing Cre after puberty in the prostate epithelium16) with Zbtb7afl/fl, Ptenfl/fl or Ptenfl/fl; Zbtb7afl/fl mice to conditionally inactivate Zbtb7a and Pten in the prostate (generating Pbsn-cre4; Zbtb7afl/fl, Pbsn-cre4; Ptenfl/fl and Pbsn-cre4; Ptenfl/fl; Zbtb7afl/fl mutant mice). Inactivation of Zbtb7a alone in the

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prostate did not lead to pathological changes in any prostate lobe in 11-week-old mice (n = 10 mice) (Fig. 1a). However, histopathological analysis using hematoxylin and eosin (Fig. 1a, top), pan-cytokeratin (Fig. 1a, middle) and smooth muscle actin (SMA) (Fig. 1a, bottom) staining showed unexpected, highly penetrant invasive prostatic adenocarcinoma at as early as 11 weeks in the mutants with inactivation of both 
Pten and 
Pten in the prostate. In line with our previous report15, at this age, only high-grade prostatic intraepithelial neoplasia (PIN) was found in the mice with inactivation of only 
Pten (n = 10) (Fig. 1a,b).

To further analyze the consequences of 
Pten inactivation for prostate cancer driven by 
Pten loss, we followed cohorts of mice with conditional inactivation of 
Pten alone and in combination with 
Pten by monthly magnetic resonance imaging (MRI) analysis. In agreement with previous reports17, MRI detected the presence of tumors in the prostates of 6-month-old mice with prostate-specific inactivation of 
Pten (Fig. 1c). Tumors were significantly enlarged in the age-matched cohort that also had inactivation of 
Pten compared to tumors from mice with loss of 
Pten alone in terms of both tumor volume (Fig. 1c,d) and weight (Fig. 1e–g).

To test whether the acceleration in prostate tumorigenesis described in the mice with inactivation of both 
Pten and 
Pten affected long-term survival, we followed a further cohort of mutant mice over 80 weeks. Kaplan-Meier cumulative survival analysis showed that concomitant loss of 
Pten and 
Pten led to lethal prostate tumors at around 13 months (Fig. 1h–j). Double-mutant mice with inactivation of both 
Pten and 
Pten either died or were euthanized owing to extensive tumor burden (Fig. 1h,i), whereas most mutant mice with inactivation of only 
Pten survived beyond 13 months (Fig. 1j). None of the wild-type control mice or those with prostate-specific inactivation of 
Pten alone died during this period, suggesting that loss of 
Pten in combination with 
Pten deficiency has a profound effect on the survival of the mutant mice. Thus, loss of 
Pten markedly accelerates the progression of prostate tumors driven by 
Pten loss, leading to massive tumor growth, early stromal invasion and lethal prostate cancer. To further define whether 
Pten had tumor suppressive–like functions in prostate cancer, we followed a cohort of wild-type mice and mutant mice with prostate-specific loss of 
Pten over a period of 2 years. We found that 16- to 18-month-old mutants with inactivation of 
Pten developed PIN in ventral and dorsolateral lobes (~17%; data not shown). Even though low disease penetrance affected the statistical power of this analysis, overall, these results suggest that inactivation of 
Pten can favor both tumor initiation and progression in prostate cancer.
**Zbtb7a loss overcomes PICS and promotes tumor invasion**

Next, we attempted to define the cellular and molecular mechanisms through which Zbtb7a suppress tumor formation in mice with prostate-specific inactivation of Pten. We had previously reported that PICS represents an important fail-safe mechanism for counteracting tumor progression in the prostate. We therefore tested the cellular senescence response in double-mutant mice with inactivation of both Pten and Zbtb7a in the prostate. To this end, prostate sections with various genotypes were analyzed by senescence-associated β-galactosidase (SA-β-gal) staining. A strong cellular senescence response was observed in mice with inactivation of Pten alone yet was markedly reduced in the double-mutant mice with inactivation of both Pten and Zbtb7a (Fig. 2a), suggesting that loss of Zbtb7a in a Pten-null context can yield an unexpected bypass of the senescence response (Fig. 2b).

We then investigated the mechanisms that could explain this unexpected senescence bypass. Along with other laboratories, we have previously demonstrated that the PICS program in the prostate is dependent on induction of p53, p27 and Smad4 (refs. 15,19,20). We therefore compared the expression of these proteins in prostate tumors from mice with prostate-specific inactivation of Pten alone or double-mutant mice with inactivation of both Pten and Zbtb7a. Immunohistochemistry and protein blot analyses showed that p53, p27 and Smad4 protein amounts were similarly induced in Pten and Zbtb7a double-mutant and in single-mutant mice with loss of only Pten (Fig. 2c,d). Furthermore, to ensure that the ability of p53 to regulate its downstream target genes was not impaired in the double-mutant mice, we performed quantitative PCR (qPCR) analysis of Cdkn1a and Mdm2 mRNA levels as readout for p53 activity. Similar induction of both transcripts was observed in mice with inactivation of both Pten and Zbtb7a in those with inactivation of only Pten (Supplementary Fig. 2a,b), suggesting that p53 function was intact in the prostates of double-mutant mice and that evasion of the cellular senescence response in these mutants was attributable to a distinct cellular pathway. Additionally, in agreement with the previously reported role of ZBTB7A in ARF transcriptional repression, we did observe an induction of Arf in Zbtb7a and Pten double-null mouse embryonic fibroblasts (MEFs) (Supplementary Fig. 2c) and mouse prostates compared to their Pten-null counterparts (Fig. 2c,d).

These findings are fully coherent with those of previous studies in which we proved genetically that the Arf transcript does not have a tumor-suppressive function in the mouse prostate on its own or, critical to this study, with concomitant loss of Pten. This notion has been further corroborated by our own as well as other investigators’ previous analyses of human specimens, which have shown that complete loss of p14ARF is extremely rare in human prostate cancer and that greater ARF abundance unexpectedly correlates with disease aggressiveness. The senescence bypass observed in Pten
and Zbtb7a double mutants was also accompanied by a differential proliferation rate compared to the mutant with inactivation of Pten alone. There was a significant increase in the number of Ki-67–positive prostate tumoral cells in the Pten and Zbtb7a double mutants compared to the mutants with loss of Pten alone (Fig. 2c,f), providing evidence that loss of Zbtb7a confers a proliferative advantage in the Pten-deficient prostate. In addition, protein blot analysis for cleaved caspase-3 showed that apoptosis in prostate tumors from Pten and Zbtb7a double mutants was substantially suppressed compared to mutants with inactivation of only Pten (Fig. 2g). Thus, inactivation of Zbtb7a bypasses PICS, favoring proliferation, survival and invasion, even though p53, p27 and Smad are seemingly unaffected.

**Zbtb7a deficiency promotes Sox9 oncogenic activity**

We therefore hypothesized that Zbtb7a inactivation could transcriptionally perturb yet another pathway that promotes senescence, and we performed transcriptome analysis using prostates from 12-week-old wild-type mice and mice with prostate-specific inactivation of Zbtb7a and Pten, alone and in combination (three mice per genotype). We found 567 genes whose expression was significantly upregulated and 482 genes whose expression was significantly downregulated at least 1.5-fold (P < 0.01) in Pten and Zbtb7a double mutants compared to mutants with inactivation of only Pten (Supplementary Table 1). Mia (encoding melanoma inhibitory activity) and Dmbt1 (encoding deleted in malignant brain tumors 1) were two of the most substantially upregulated genes in the ventral and anterior lobes of prostates from Pten and Zbtb7a double-mutant mice (Supplementary Fig. 3a).

Notably, both genes are well-characterized transcriptional targets of Sry (sex-determining region Y)-box 9 (Sox9)23–25, although no increase in the levels of Sox9 mRNA was detected in mice with only Pten inactivation compared to those with inactivation of both Pten and Zbtb7a (Fig. 3a). We additionally performed unbiased oPOSSUM analysis26–28, Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA)29,30 on our transcriptome data to gain mechanistic insights into the function of Zbtb7a in prostate tumorigenesis. oPOSSUM analysis indicated that Sox9 was one of the most significantly upregulated genes in the ventral and anterior lobes of prostates from double-mutant mice (WT, and Pten and Sox9) compared to mutants with inactivation of only Pten (Fig. 2g). Thus, inactivation of Zbtb7a in combination with Pten leads to an increase in Sox9 and Zbtb7a from PC3 cells, RWPE-1 cells and mouse prostate. IP, immunoprecipitation. (Fig. 3f).

**Figure 3** Hyperactivation of Sox9 in Pten and Zbtb7a double-null prostate tumors downregulates Rb expression through H19 and miR-675.

(a) Quantitative RT-PCR analysis of Mia, Dmbt1 and Sox9 expression in prostates from 12-week-old wild-type mice (black) and mice with inactivation of Zbtb7a (orange), Pten (red) and Zbtb7a and Pten (blue) (n = 3 per genotype). (b) Staining for Sox9 of prostates from wild-type mice and mice with inactivation of Zbtb7a and Pten, alone and in combination. Scale bars, 0.1 mm. (c) Protein blot analysis of human prostate cell lines for ZBTB7A expression. (d) Dual-luciferase assay with a SOX9 reporter in PC3 cells. Data are shown in relative luciferase units (RLU). (e) Coimmunoprecipitation of Sox9 and ZBTB7A from PC3 cells, RWPE-1 cells and mouse prostate. IP, immunoprecipitation. (f) shRNA targeting ZBTB7A (gray) in RWPE-1 cells leads to an increase in Mia and DMBT1 mRNAs expression, shRNA for GFP was used as control (magenta). (g) ChIP analysis in RWPE-1 cells of MIA and DMBT1 promoters using antibodies to ZBTB7A (yellow) and SOX9 (green) or rabbit IgG (white). (h) H19 and miR-675 expression in prostates from wild-type mice (black) and mice with inactivation of Zbtb7a (orange), Pten (red) and Zbtb7a and Pten (blue) (n = 3 per genotype). (i) shRNA targeting ZBTB7A (gray) in RWPE-1 cells leads to an increase in H19 mRNA expression, shRNA for GFP was used as control (magenta). (j) Both ZBTB7A (yellow) and SOX9 (green) bind to the H19 promoter as shown by ChIP analysis in RWPE-1 cells. Rabbit IgG was used as control (white). All data are presented as the mean of three independent experiments ± s.d. (k,l) Rb protein expression in prostates from wild-type mice and mice with inactivation of Pten alone and in combination with Zbtb7a as shown by protein blot analysis (k) and immunohistochemistry analysis (l). Scale bars in l, 0.05 mm.
the top ten transcription factors with over-represented binding sites in coexpressed genes (Supplementary Table 2), an observation that is consistent with our hypothesis. In addition, by using two other independent bioinformatics tools, IPA and GSEA, we found that the most significantly enriched gene categories in the signature of mice with inactivation of both Pten and Zbtb7a were cellular movement, cell death and survival, and cellular growth and proliferation (Supplementary Tables 3 and 4), a finding that is consistent with the aggressive nature of the prostate tumors in mice with this genotype that we observed by histopathological and molecular analyses (Figs. 1a,b and 2e,g).

Given that SOX9 has been shown to have important oncogenic functions in the prostate both in vitro and in vivo31,32, we tested whether Sox9 and Zbtb7a functionally interact during prostate cancer development (Fig. 3). We first performed qPCR analysis of Mia and Dmbt1 mRNAs to confirm the findings of the transcriptome analysis. Indeed, Mia and Dmbt1 mRNAs were highly upregulated in Pten and Zbtb7a double mutants compared to mice with inactivation of only Pten (Fig. 3a). Because Sox9 expression is known to be markedly induced in Pten-deficient prostate31, we performed qPCR, protein blot and immunohistochemistry analyses to determine whether the concomitant loss of Zbtb7a and Pten affected Sox9 expression. Sox9 protein was expressed in both basal cells and luminal cells of the normal mouse prostate but was comparably induced in the mutant mice with loss of only Pten and double mutants with loss of both Pten and Zbtb7a (Fig. 3a,b,k). On the basis of this observation, we hypothesized that the profound and differential increase in expression of Sox9 target genes observed in Pten and Zbtb7a double-null prostates was due to an increase in Sox9 transcriptional activity rather than its expression level.

ZBTB7A mainly acts as a transcriptional repressor1, whereas SOX9 mainly acts as a transcriptional activator22. We therefore tested whether ZBTB7A opposes the transcriptional activity of SOX9. To this end, we performed transactivation assays with a luciferase reporter construct containing multiple SOX9-binding sites33 in PC3 and Du145 human prostate cancer cell lines that express low levels of endogenous ZBTB7A (Fig. 3c). ZBTB7A efficiently abrogated the ability of SOX9 to transactivate the reporter gene in a dose-dependent manner in both cell lines (Fig. 3d and Supplementary Fig. 3c). Because the multimeric SOX9 reporter is an artificial responsive element devoid of ZBTB7A-binding sites, we next determined whether ZBTB7A repressed SOX9 transcriptional activity through direct physical interaction. To this end, we performed an immunoprecipitation assay in PC3 and RWPE-1 cells using an antibody to SOX9 and control immunoglobulin G (IgG). Antibody to SOX9 specifically pulled down endogenous ZBTB7A in both cell lines (Fig. 3e), suggesting a physiological interaction between SOX9 and ZBTB7A in human prostate cells. Furthermore, immunoprecipitation using prostates from mice with inactivation of Pten that expressed high levels of both Zbtb7a and Sox9 also showed direct interaction of Zbtb7a and Sox9 in mouse primary prostate tumor cells (Fig. 3e).

To determine whether SOX9 target genes, such as MIA and DMBT1, were also regulated by ZBTB7A in human prostate cells, we inactivated ZBTB7A with a short hairpin RNA (shRNA) in RWPE-1 cells, which express high levels of endogenous ZBTB7A (Fig. 3c). Subsequent qPCR analysis showed that MIA and DMBT1 mRNA levels were upregulated in cells in which ZBTB7A was inactivated compared to those transfected with the control shRNA (Fig. 3f). Notably, chromatin immunoprecipitation (ChIP) assays with antibodies to ZBTB7A and SOX9 confirmed that both proteins specifically bound the promoters of MIA and DMBT1 (Fig. 3g). These data demonstrate that MIA and DMBT1 are also SOX9 target genes in human prostate cells and that their expression depends on the relative amounts of ZBTB7A and SOX9.

Thus, the exacerbation of SOX9 oncogenic activities in prostate cancer as a consequence of ZBTB7A loss could drive hyperproliferation as well as enhance invasiveness through the activities of target genes such as MIA and DMBT1 (refs. 34–44). On the basis of this idea, we speculated that Sox9 hyperactivity in Pten and Zbtb7a double-mutant mice might also be responsible for senescence bypass. With respect to this hypothesis, we noticed that the Sox9 target gene H19, which encodes a precursor noncoding RNA for the miR-675 microRNA (miRNA)45, was strongly upregulated in the double-mutant mice compared to those with inactivation of only Pten (Fig. 3h, left and Supplementary Fig. 3a). Given that miR-675 is known to target the tumor suppressor retinoblastoma (Rb)46, a key regulator of the cellular senescence response47, we tested whether miR-675 expression was concomitantly increased in Pten and Zbtb7a double-mutant mice. Indeed, expression levels of miR-675 were far higher in the prostates of the double-mutant mice than in mice with inactivation of Pten alone (Fig. 3h, right and Supplementary Fig. 4). Accordingly, H19 miRNA was also strongly induced when ZBTB7A was transiently inactivated in human RWPE-1 cells (Fig. 3i). Furthermore, both SOX9 and ZBTB7A were found to bind to the H19 promoter, as shown by ChIP assay (Fig. 3j), suggesting that H19 is a direct target for both of these transcription factors in prostate cells. Protein blot and immunohistochemistry analyses confirmed that Rb protein expression was strongly downregulated in prostate tissue from Pten and Zbtb7a double-mutant mice (Fig. 3k,l). As expected, lower amounts of Rb in prostates from Pten and Zbtb7a double-null mice coincided with the upregulation of a panel of proven E2f transcriptional target genes (Supplementary Table 5), although it has been shown recently that the regulation of senescence by Rb in mouse prostate is at least partly independent of E2f protein function48. Although the Rb-E2f pathway was clearly affected in prostate cancer with loss of both Pten and Zbtb7a, we cannot rule out the possibility that loss of Zbtb7a may also affect PICS by perturbing additional, unknown pathways that promote senescence.

Taken together, these results show that, during prostate tumorigenesis, the oncosuppressive function of Zbtb7a directly impinges on the oncogenic activity of Sox9 and that Zbtb7a loss in the prostate favors (i) senescence bypass, (ii) increased proliferation rate, (iii) resistance to apoptosis and (iv) greater invasive potential.

Loss of ZBTB7A expression in human prostate cancer

We next investigated the status of ZBTB7A in human prostate cancer by first analyzing the expression of ZBTB7A mRNA and protein, respectively, in the Oncomine database and a customized human prostate cancer tissue microarray (TMA) (see also ref. 49). In line with our mouse data, our analysis of prostate cancer expression profile data sets50–54 showed that ZBTB7A was underexpressed in prostate cancer and downregulated upon tumor progression to high Gleason score and metastasis (Fig. 4a–d and Supplementary Fig. 5; see also ref. 49). Additionally, we analyzed human prostate cancer expression array data from men with prostate cancer stratified by prostate cancer recurrence55 (Fig. 4d), finding that ZBTB7A transcript levels were significantly lower in tumors demonstrating greater propensity for recurrence (Fig. 4d). As the ZBTB7A transcript is extremely GC rich (70%), which may affect specificity in annealing to the probe, we analyzed multiple prostate cancer expression profile data sets (11 in total)50–60; of these, 6 clearly showed downregulation of ZBTB7A in human prostate cancer and metastases, whereas 3 did not permit
**Figure 4** ZBTB7A downregulation in human prostate cancers correlates with tumor progression and metastasis. (a–d) ZBTB7A mRNA is significantly downregulated in different primary prostate cancer data sets reported by Luo et al.50 (a) and Liu et al.51 (b), especially in tumor progression (c) and recurrence (d). Data are presented as box plots, where the whiskers indicate the range of the data and the horizontal bar represents the median. (e) Expression analysis of miR-20a, miR-106b and miR-93 in normal prostate epithelium (gray) compared to prostate cancer tissue (red). Data are presented as mean ± s.d. (f) Immunohistochemistry analysis of PTEN and ZBTB7A protein expression in human prostate cancers with high Gleason score showing that loss of ZBTB7A is strongly associated with loss of PTEN. Scale bars, 0.1 mm. (g) Distribution of PTEN and ZBTB7A expression in samples in the cohort of men with prostate cancer analyzed. (h) Model for the role of ZBTB7A in prostate cancer progression.

To further define the cause of ZBTB7A loss in human cancer, we employed a multifaceted approach. Notably, array–comparative genomic hybridization (aCGH) analysis for genetic alterations involving the region of ZBTB7A on chromosome 19 showed that 18% of individuals with advanced prostate cancer (10 of 55) harbored monoallelic loss of ZBTB7A (Supplementary Fig. 6a; see also ref. 49). Additionally, we recently found that ZBTB7A levels are strongly downregulated by the mir-17 family (including miR-20a, miR-93 and miR-106b61, Supplementary Fig. 6b–e), which regulates ZBTB7A mRNA translation more than transcript degradation63 and also targets PTEN62. The MIR106B–25 cluster is located in intron 13 of the MCM7 gene (encoding minichromosome maintenance complex component 7), one of the most genetically amplified loci in human advanced prostate cancer63. Accordingly, we found this miRNA family markedly upregulated in human prostate cancer (Fig. 4e)53, and immunohistochemistry analysis with antibody to ZBTB7A of a TMA that included 50 human prostate samples of primary prostate cancer (detailed information given in Supplementary Table 6) confirmed loss of ZBTB7A protein in approximately 50% (22/42) of the biopsied specimens analyzed (Fig. 4f; 8 out of 50 prostate cancer samples were excluded from the analysis for technical reasons). Notably, we also observed significant association between ZBTB7A and PTEN protein loss in these 42 prostate cancer specimens ($\chi^2$ test $P = 0.0123$) (Fig. 4g), further suggesting cooperative oncosuppressive roles for ZBTB7A and PTEN in human prostate cancer (see also ref. 49). Lastly, we performed immunohistochemistry analysis of ZBTB7A and SOX9 on the same TMA and found that 20 out of 36 human advanced prostate tumors (with Gleason scores of 8–10) (detailed information given in Supplementary Table 7) were characterized by high levels of SOX9 expression (Supplementary Fig. 7a,b). Notably, 12 of these 20 tumors also had concomitant loss of ZBTB7A protein (Supplementary Fig. 7a,b).

**DISCUSSION**

In this work, we have unexpectedly demonstrated that loss of Zbtb7a accelerates the progression of Pten loss–driven tumors in the prostate. This notion is further corroborated by the lack of tumorigenesis and any discernable abnormal phenotype in mice that overexpressed ZBTB7A specifically in the prostate epithelium (Pbsn-ZBTB7A transgenic lines; Supplementary Fig. 1). ZBTB7A therefore has an important role in tumorigenesis, not only when overexpressed but also when downregulated, depending on its cellular and genetic milieu. In mechanistic terms, we show that Zbtb7a loss greatly accelerates the progression of Pten loss–driven prostate tumors by strongly activating the oncogenic transcriptional activity of Sox9, a key step that in prostate cancer leads to bypass of PICS and the activation of Sox9-dependent transcriptional signatures that promote proliferation, survival and invasion (Fig. 4h).

Notably, we determined that ZBTB7A is also genetically lost and transcriptionally and post-transcriptionally downregulated in human prostate cancers and that loss of ZBTB7A protein strongly correlates with loss of PTEN protein in advanced prostate cancer. We showed that ZBTB7A and PTEN transcripts are both targets of miRNAs encoded by the MIR106B–25 cluster located in intron 13 of MCM7 (ref. 62). MCM7 is one of the most amplified oncogenes in human prostate cancer, and its expression is highly correlated with tumor progression, biochemical recurrence and distant metastases63. In addition, it has been shown that RB negatively regulates DNA replication through direct interaction with MCM7 (ref. 64). Thus, downregulation of RB due to concomitant inactivation of ZBTB7A and PTEN may also promote the oncogenic activities of MCM7. This ability to simultaneously downregulate PTEN and ZBTB7A therefore defines a new causal link between MCM7 and MIR106B–25 amplification and prostate tumorigenesis. Other important genetic events such as TMPRSS2–ERG fusion or TP53 mutation and loss have
been identified as frequent events in human prostate cancer63–69 and are strongly associated with loss of PTEN. Robust evidence of functional cooperation between these genetic lesions has been provided by a plethora of different studies, particularly by those using specific mouse models15,20,71. Given that loss or downregulation of ZBTB7A is also associated with PTEN loss in advanced prostate tumors, it is conceivable that ZBTB7A might have important onc suppressive functions in the context of TMPRSS2-ERG fusion or TP53 loss or mutation in counteracting the progression of prostate tumors as well as the response to specific treatments49.

Thus, genetic analysis in mouse models has identified an unpredicted dual role of ZBTB7A in oncogenesis through its ability to control major tumor pathways such as ARF-p53, RB and SOX9, in a context- and tissue-dependent manner and has defined a new oncogenic pathway triggered by ZBTB7A loss that is pathogenetically linked to the amplification of the MCM7 locus in advanced human prostate cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. Microarray data have been accessioned with the Gene Expression Omnibus ( GEO) under series GSE46473.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.W., A.L. and P.P. designed, realized and analyzed the experiments. W.L.G., B.C., U.A. and J.Z. conducted the human genetic analysis. K.A.W. and A.E. performed the immunohistochemistry on mouse prostate samples. R.T., W.P.K., Z.C., D.R.S., Y.T., E.G.-B., X.-S.L. and C.N. helped with the experiments. C.C.-C. and S.S. reviewed all mouse pathology. G.W., A.L. and P.P. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of Pten and Zbtb7a mutant mice. Transgenic mice with Ptenfl/fl, Zbtb7afl/fl and Pbn-cre+ were maintained as described.15 To generate prostate-specific deletion of Pten and Zbtb7a, female Ptenfl/fl, Zbtb7afl/fl mice were crossed with male Pbn-cre+ transgenic mice.46 For genotyping, tail DNA was subjected to PCR analysis (detailed information is provided in Supplementary Table 8). To generate Ptenfl/fl, Zbtb7afl/fl mice, primers PtenLoxP_Fwd and PtenLoxP_Rev were used. To generate Zbtb7afl/fl mice, primers PCFW1, PCFW2, PCFW3, PCVR and PCNeo were used. All experimental animals were kept in a mixed genetic background of C57BL/6J X 129/Sv. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Plasmids and cell lines. The pcDNA3.1-XP-ZBTB7A plasmid, which expresses Xpress-tagged ZBTB7A, was generated by cloning the full-length human ZBTB7A cDNA into the pcDNA3.1/HisC plasmid. The pcDNA3-Flag-SOX9 plasmid27 was a gift from P. Berta, and the SOX9-regulated luciferase reporter (4x48-p89-luciferase)33 was provided by B. de Crombrugghe. PC3, Du145, LNCaP, RWPE-1 and PWR-1E cells were obtained from the American Type Culture Collection. PPC1 and TSU-PR1 cells were a gift from A. Ullrich. PC3, Du145, PPC1, TSU-PR1 and LNCaP cells were maintained in Keratinocyte Serum-Free Medium (KSFM) supplemented with 5% FBS. PC3, Du145, LNCaP, RWPE-1 and PWR-1E cells were cultured in Keratinocyte Serum-Free Medium (KSFM) supplemented with bovine pituitary extract (BPE) and recombinant human epidermal growth factor (EGF).

shRNA constructs and lentiviral production. shRNA constructs were obtained from Open Biosystems in lentiviral cassettes. An shRNA with high homology to ZBTB7A knockdown efficiency was used, with an shRNA for GFP used as a control. As described previously, lentivirus was made using a three-plasmid packaging system. Briefly, shRNAs in the pLKO.1-puro vector were cotransfected into 293T cells along with expression vectors containing the packaging system. Briefly, shRNAs in the pLKO.1-puro vector were cotransfected into 293T cells along with expression vectors containing the packaging system. Briefly, shRNAs in the pLKO.1-puro vector were cotransfected into 293T cells along with expression vectors containing the packaging system. Briefly, shRNAs in the pLKO.1-puro vector were cotransfected into 293T cells along with expression vectors containing the packaging system.

Senescence and apoptosis assays. SA-β-gal activity in prostate tissue was measured with the senescence detection kit (Calbiochem) on frozen sections 6 μm in thickness. For apoptosis analysis, dexamethasone and rehydrated paraffin sections were treated with the In Situ Cell Death Detection kit (Roche), and apoptotic cells were identified by positive TUNEL staining.

Protein blotting, immunoprecipitation and immunohistochemistry. For protein blotting, cell lysates were prepared with RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche)). The following antibodies were used for protein blotting: rat monoclonal antibody to p19Arf (5-C3-1, Calbiochem; 1:1,000 dilution), rabbit polyclonal antibody to p53 (CM5, Novocastra; 1:1,000 dilution), rabbit polyclonal antibody to p21 (C-19, Santa Cruz Biotechnology; 1:2,000 dilution), mouse monoclonal antibody to p27 (57/Kip1/p27, BD Transduction Laboratories), rabbit polyclonal antibody to AR (PG21, Millipore; 1:2,000 dilution), rabbit monoclonal antibody to SOX9 (AB5355, Millipore; 1:2,000 dilution), mouse monoclonal antibody to β-actin (A5316, Sigma-Aldrich; 1:5,000 dilution), mouse monoclonal antibody to HSP70 (610607, BD Transduction Laboratories; 1:3,000 dilution), rabbit polyclonal antibody to total caspase-3 (9662, Cell Signaling Technology; 1:1,000 dilution), rabbit monoclonal antibody to cleaved caspase-3 (9662, Cell Signaling Technology; 1:1,000 dilution), rabbit monoclonal antibody to GAPDH (14C10, Cell Signaling Technology; 1:6,000 dilution), mouse monoclonal antibody to Rb (554136, BD Bioscience; 1:1,000 dilution), rabbit polyclonal antibody to PTEN (1386G, Cell Signaling Technology; 1:1,000 dilution), rabbit polyclonal antibody to phosphorylated AKT (Ser473) (9272S, Cell Signaling Technology), rabbit polyclonal antibody to phosphorylated SMA2D (32101S, Cell Signaling Technology; 1:2,000 dilution) and hamster monoclonal antibody to ZBTB7A (clone 13E9; 1:3,000 dilution). For immunoprecipitation, lysates were prepared in EBC buffer (50 mM Tris, pH 7.5, 120 mM NaCl and 0.5% NP-40) and subjected to immunoprecipitation with 2 μg of antibody to Sox9 (AB5535, Millipore). Immunoprecipitates were washed with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) and analyzed by SDS-PAGE. For immunohistochemistry, tissues were fixed in 10% formalin and embedded in paraffin in accordance with standard procedures. Sections were stained for PTEN (Ab-2, NeoMarkers; 1:250 dilution), ZBTB7A (clone 13E9; 1:200 dilution), Rb (10048-2-Ig, ProteinTech Group; 1:200 dilution), Sox9 (AB8509, Millipore; 1:500 dilution), Ki-67 (SP6, ThermoScientific; 1:200 dilution), p19Arf (5-C3-1, Calbiochem; 1:1,000 dilution), p27 (57/Kip1/p27, BD Transduction Laboratories; 1:1,000 dilution), p53 (FL-393, Santa Cruz Biotechnology; 1:500 dilution), SMA (1A4, Dakocytomation; 1:1,000 dilution) and pan-cytokeratin (PKC-6, Sigma-Aldrich; 1:2,500 dilution).

Transfection and luciferase reporter assays. One day before transfection, cells were plated into 24-well plates at 70–80% confluency. Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) for 24 h according to the manufacturer’s recommendations. Forty-eight hours after transfection, cells were lysed with Passive Lysis Buffer and analyzed for luciferase activity using the Dual-Luciferase Assay system (Promega). pRL-SV40-Renilla was used as a control for transfection efficiency.

ChIP assays. ChIP assays were performed as described previously. Briefly, formaldehyde was added to a final concentration of 1% to PC3 cells or RWPE-1 cells for 5 min to cross-link proteins and DNA. Cells were then washed and resuspended in lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl at pH 8.1) with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml pepstatin-A added. After brief sonication, cell lysates were cleared by centrifugation and were diluted tenfold with dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl at pH 8.1 and 167 mM NaCl) containing protease inhibitors. Antibodies (5 μg) to Sox9 (C-20, Santa Cruz Biotechnology) and ZBTB7A (A300-549A, Bethyl Laboratory) or control IgG was incubated with lysates at 4 °C overnight with rotation. Immunoprecipitated complexes were collected using Protein G Dynabeads (Invitrogen) and were washed with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LICl immune complex wash buffer and TE buffer. NaCl (5 M) was added to the resulting precipitants to reverse the formaldehyde cross-linking by heating at 65 °C for 6 h. After phenol-chloroform extraction and precipitation with ethanol, DNA pellets were resuspended in TE buffer and were subjected to qPCR analysis using forward and reverse primers selected from the H19, MIA and DMBT1 promoter sequences (detailed information is given in Supplementary Table 8). qPCR was used to amplify immunoprecipitated DNA with the Quantitect SYBR Green PCR kit (Qiagen). Relative enrichment of chromatin precipitated with antibodies to ZBTB7A and Sox9 was normalized to the amount of DNA precipitated with IgG control using the comparative Ct method.

RNA isolation, gene expression profiling and qPCR analysis. Total RNA was purified from anterior or ventral prostate or from the indicated cell lines using the RNeasy Mini kit (Qiagen) and treated with RNase-free DNase (Qiagen). For gene expression profiling experiments, RNA from anterior or ventral lobes E hybridized with HT Mouse Genome 430 PM arrays by the Beth Israel Deaconess Medical Center Genomics and Proteomics Center. For qPCR analysis, 2 μg of total RNA was reverse transcribed into cDNA using the Transcriptor First-Strand cDNA Synthesis kit (Roche Applied Science). TaqMan qPCR analysis (Applied Biosystems) was performed at the Biopolymers Facility at Harvard Medical School using an Applied Biosystems 7900 HT Fast instrument according to the manufacturer’s protocol. Each target was run in triplicate, and expression levels were normalized to those of mouse glucuronidase β (Gusb) or human β-actin (ACTB). For miRNA profiling, total RNA was isolated from the anterior lobes of prostates and subjected to global miRNA profiling with Nanostring technology. A total of 578 miRNAs were evaluated using the nCounter Mouse miRNA Expression Assay kit.

TMAs. The TMAs used in this study were constructed at the Memorial Sloan-Kettering Cancer Center (MSKCC) or purchased from US Biomax and were stained at the Pathology and Molecular Cytology Core Facilities as described previously.
Statistical analysis. Data were analyzed using unpaired t tests (GraphPad Prism, GraphPad Software). P values of <0.05 were considered to be statistically significant. Means ± s.d. of three or more independent experiments are reported.

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