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Journal Title: Journal of Genetics and Genomics
Volume: Volume 42, Number 7
Publisher: Elsevier | 2015-07-20, Pages 373-382
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1016/j.jgg.2015.06.004
Permanent URL: https://pid.emory.edu/ark:/25593/rn928

Final published version: http://dx.doi.org/10.1016/j.jgg.2015.06.004

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Accessed June 15, 2018 10:09 PM EDT
Additive effect of Zfhx3/Atbf1 and Pten deletion on mouse prostatic tumorigenesis

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Abstract

The phosphatase and tensin homolog (PTEN) and the zinc finger homeobox 3 (ZFHX3)/AT-motif binding factor 1 (ATBF1) genes have been established as tumor suppressor genes in prostate cancer by their frequent deletions and mutations in human prostate cancer and by the formation of mouse prostatic intraepithelial neoplasia (mPIN) or tumor by their deletions in mouse prostates. However, whether ZFHX3/ATBF1 deletion together with PTEN deletion facilitates prostatic tumorigenesis is unknown. In this study, we simultaneously deleted both genes in mouse prostatic epithelia and performed histological and molecular analyses. While deletion of one Pten allele alone caused low-grade (LG) mPIN as previously reported, concurrent deletion of Zfhx3/Atbf1 promoted the progression to high-grade (HG) mPIN or early carcinoma. Zfhx3/Atbf1 and Pten deletions together increased cell proliferation, disrupted the smooth muscle layer between epithelium and stroma, and increased the number of apoptotic cells. Deletion of both genes also accelerated the activation of Akt and Erk1/2 oncoproteins. These results suggest an additive effect of ZFHX3/ATBF1 and PTEN deletions on the development and progression of prostate neoplasia.

Keywords

ZFHX3; ATBF1; PTEN; prostate cancer; mPIN

INTRODUCTION

The development of invasive prostate cancer is a multi-stage process driven by genetic and epigenetic alterations in genes involved in the formation and maintenance of the prostate. Common alterations include the inactivation of tumor suppressor genes and activation of oncogenes by mutation or copy number change (Shen and Abate-Shen, 2010). Understanding the biologic function of such genes and their interaction is necessary for the
development of biomarkers and therapeutic targets for improved detection and treatment of prostate cancer.

One of the frequently mutated or deleted genes in human prostate cancer is the phosphatase and tensin homolog (PTEN) gene, which encodes a protein that functions as a lipid and protein phosphatase to inhibit the activation of the AKT oncoprotein by PDK1 (Salmena et al., 2008). Loss of one PTEN allele may occur in more than half of primary prostate tumors and is associated with metastasis (Dong, 2001). Loss of PTEN function results in constitutive activation of AKT and its downstream targets, contributing to a broad spectrum of cancers including prostate cancer (Hollander et al., 2011). In mice, loss of one Pten allele causes mouse prostatic intraepithelial neoplasia (mPIN), a precursor of prostate adenocarcinoma, whereas loss of both Pten alleles sometimes induces invasive prostate cancer (Di Cristofano et al., 1998; Wang et al., 2003; Backman et al., 2004; Ma et al., 2005; Ahmad et al., 2008; Svensson et al., 2011). A number of studies have shown that inactivation of another tumor suppressor gene in Pten-deficient mice, such as p27, Nkx3-1, Smad4 and Klf5, could promote prostatic tumorigenesis (Di Cristofano et al., 2001; Kim et al., 2002a; Abate-Shen et al., 2003; Ding et al., 2011; Bjerke et al., 2014; Xing et al., 2014).

The zinc finger homeobox 3/AT-motif binding factor 1 (ZFHX3/ATBF1, ZFHX3 hereafter) gene is another mutated or deleted gene in prostate cancer and the mutation also appears to occur in other cancers including those of breast, liver, stomach, and head and neck (Sun et al., 2005; Zhang et al., 2005; Cho et al., 2007; Kai et al., 2008; Kim et al., 2008; Sun et al., 2013). The ZFHX3 locus at 16q22 is frequently deleted, and ZFHX3 is the second most frequently mutated gene in metastatic human prostate cancer (Sun et al., 2005; Grasso et al., 2012). In addition, a germline variant of ZFHX3 appears to be associated with increased risk of sporadic prostate cancer (Xu et al., 2006), and reduced expression of Zfhx3 has been observed in mouse models of prostate cancer such as TRAMP-ARpe-T877AVY and ARR2PB-c-myc mice (Ellwood-Yen et al., 2003; Takahashi et al., 2011). Using recently generated mice with a floxed Zfhx3 allele (Sun et al., 2012), we demonstrated that deletion of Zfhx3 in mouse prostates caused mPIN lesions in all prostatic lobes, and these mPIN lesions harbored molecular and histological alterations characteristic of human PIN and prostate cancer, including enhanced cell proliferation, attenuated basal layer and surrounding smooth muscle layer, elevated levels of phosphorylated (p-) Erk1/2 and Akt, and aberrant glycosylation (Sun et al., 2014). While these results support a tumor suppressor function for Zfhx3, its deletion did not cause invasive prostate tumors in mice even at 24 months (Sun et al., 2014).

Alterations of multiple genes are often required to initiate and promote carcinoma. For example, deletion of the tumor suppressor gene Nkx3-1 or CDKN1B/p27 only induces mPIN in mouse prostates (Di Cristofano et al., 2001; Abate-Shen et al., 2003), while the deletion of either gene along with the deletion of Pten promotes mouse prostatic tumorigenesis (Di Cristofano et al., 2001; Kim et al., 2002a). In a recent genome-wide mutation and copy number change study, 12 of 61 (19.7%) metastatic human prostate cancers had deletion or mutation of ZFHX3, and interestingly, 10 of the 12 tumors with ZFHX3 alterations also had PTEN deletion or mutation (Grasso et al., 2012). We therefore hypothesized that inactivation of ZFHX3 together with that of PTEN initiates and promotes prostatic tumorigenesis. We tested this hypothesis by generating mice in which both Zfhx3
and Pten were concurrently deleted, and performing histological and molecular analyses. While deletion of one Pten allele alone caused low-grade (LG) mPIN, we found that concurrent deletion of Zfhx3 promoted the development of high-grade (HG) mPIN or early carcinoma, accompanied by increased cell proliferation, disrupted smooth muscle layer, and increased apoptosis. Deletion of the two genes also had an additive effect on the activation of Akt and Erk1/2 oncoproteins. These histopathological and molecular changes indicate the ability of ZFHX3 deletion and PTEN deletion to promote the development and progression of prostate cancer.

RESULTS

Zfhx3 inactivation promotes the progression of precancerous lesions induced by Pten deletion in mouse prostate

Deletion of both Pten alleles induces high-grade mPIN and, sometimes, invasive prostate tumors in mice (Wang et al., 2003; Hollander et al., 2011), which may conceal the effect of an interaction between Zfhx3 deletion and Pten deletion. We therefore focused on the deletion of one Pten allele, which causes mPIN (Backman et al., 2004; Ma et al., 2005; Svensson et al., 2011), together with Zfhx3 deletion in the development of prostate cancer. We generated five groups of mice with either wild-type Pten (Zfhx3
\textsuperscript{PE}+/+Pten
\textsuperscript{PE}+/+) and Zfhx3
\textsuperscript{PE}−/−Pten
\textsuperscript{PE}+/+, from mice Zfhx3
\textsuperscript{w/wt};Pten
\textsuperscript{wt/wt};PB-Cre and Zfhx3
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{fl}\textsuperscript{ox};Pten
\textsuperscript{w/wt};PB-Cre respectively) or heterozygous deletion of Pten (Zfhx3
\textsuperscript{PE}+/+Pten
\textsuperscript{PE}+/−, Zfhx3
\textsuperscript{PE}+/−Pten
\textsuperscript{PE}+/−, and Zfhx3
\textsuperscript{PE}−/−Pten
\textsuperscript{PE}+/−, from mice Zfhx3
\textsuperscript{w/wt};Pten
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{wt};PB-Cre, Zfhx3
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{wt};Pten
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{wt};PB-Cre, and Zfhx3
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{fl}\textsuperscript{ox};Pten
\textsuperscript{PE}+/−;PB-Cre respectively) (Fig. 1). We then collected and examined prostatic lobes with the deletion of Zfhx3, Pten, or their combination for morphological and molecular alterations.

As previously observed for the context of wild-type Pten, deletion of Zfhx3 (Zfhx3
\textsuperscript{PE}−/−Pten
\textsuperscript{PE}+/+) resulted in hyperplasia and low-grade mPIN (LG-mPIN) predominantly in the dorsal prostate (DP) (Fig. 2A, a–f). Deletion of one Pten allele caused a slowly developing histopathological abnormality in the DP at younger ages; most Zfhx3
\textsuperscript{PE}+/+Pten
\textsuperscript{PE}+/− mice showed a normal histology at 8 months of age, while the majority developed hyperplasia at around 12 months (Fig. 2A, g and h; Table 1). By 18 months, Zfhx3
\textsuperscript{fl}\textsuperscript{ox}/\textsuperscript{fl}\textsuperscript{ox};Pten
\textsuperscript{PE}+/− mice developed LG-mPIN, characterized by tufted atypical luminal cells, hyperchromasia, and abundant pale cytoplasm (Fig. 2A, i). Deletion of one Pten allele had a milder effect in our group of mice than those in a previous study (Wang et al., 2003), likely due to different genetic background of mice.

In the context of deleting one Pten allele, Zfhx3 deletion significantly promoted the development of mPIN; high-grade mPIN (HG-mPIN) was frequently observed in the DP of mice with both the Zfhx3
\textsuperscript{PE}−/−Pten
\textsuperscript{PE}+/− and Zfhx3
\textsuperscript{PE}−/−Pten
\textsuperscript{PE}+/− genotypes (Fig. 2B, j–o; and Table 1). These HG-mPIN lesions consisted of cells with prominent nucleoli, increased nuclear/cytoplasmic ratio, pleomorphism, frequent mitotic figures, and apoptotic cells (Fig. 2A, o). Enhanced cell proliferation, as indicated by Ki67 staining, and interrupted epithelial/stromal boundary, as indicated by Sma staining, were also observed in the mPIN lesions (Figs. 3A and 4). These lesions could be characterized as HG-mPIN/early carcinoma.
(EC) as previously described for Nkx3.1/Pten double knockout mice (Kim et al., 2002a). As expected, more mice with the deletion of two Zfhx3 alleles (Zfhx3^PE−/−Pten^PE+/−) developed HG-mPIN/EC lesions compared to those with only one Zfhx3 allele deleted (Fig. 2B).

Some morphological changes were observed in other lobes of the prostate. Deletion of one Pten allele also induced hyperplasia or LG-mPIN lesions in the lateral prostate (LP) and the anterior prostate (AP), whereas deletion of both Zfhx3 and Pten (Zfhx3^PE−/−Pten^PE+/−) caused HG-mPIN/EC in both the LP and AP at 20 months of age in a small number of mice (Table 1). The ventral prostate (VP) was the least affected by the deletion of both Zfhx3 and Pten.

Deletion of both Zfhx3 and Pten increased the number of mitotic and apoptotic cells compared to either alone

To determine whether deletion of both Zfhx3 and Pten increased cell proliferation, we performed immunohistochemistry for Ki-67 in DP (Fig. 3A). Consistent with our previous study (Sun et al., 2014), normal prostates (Zfhx3^PE+/+Pten^PE+/+) rarely had Ki-67-positive cells (Fig. 3A, a and b), while deletion of Zfhx3 alleles increased the number of Ki67-positive cells in prostate epithelium (Fig. 3A, c and d). Deletion of one Pten allele (Zfhx3^PE+/+Pten^PE+/−) slightly increased Ki-67-positive cells (Fig. 3A, e and f), but concurrent knockout of Zfhx3 (Zfhx3^PE+/−Pten^PE+/− and Zfhx3^PE−/−Pten^PE+/−) resulted in a remarkable increase of Ki-67-positive cells (Fig. 3A, g and h). The percentages of Ki67-positive cells in DPs were estimated for each 18-month-old mouse examined. Compared to wild-type mice in which the Ki67-positive rate was 0.64%, deletion of one Pten allele increased the rate to 2.01% while deletion of both Zfhx3 alleles increased it to 1.88%, and both increases were statistically significant (P < 0.05). In prostates with one Pten allele deleted (Pten^PE+/−), deletions of one and both Zfhx3 alleles increased Ki67-positivity rates to 6.21% and 12.1%, respectively, and the latter increase was statistically significant (P < 0.01). These results indicate that loss of both the Zfhx3 and Pten genes promotes epithelial cell proliferation.

In HE-stained tissue sections, we noticed an increase in apoptosis in prostates with the deletion of both Zfhx3 and Pten (Zfhx3^PE−/−Pten^PE+/−). To further confirm increased apoptosis, we performed IHC staining for the cleaved form of caspase-3 (c-Casp3), a marker for apoptotic cells. Apoptotic cells were very rare in the wild-type group or the groups with deletion of either Zfhx3 or Pten (Fig. 3B, a–c). With the deletion of one Pten allele, however, apoptotic cells were occasionally seen in mouse prostates with one Zfhx3 allele deleted (Zfhx3^PE+/−Pten^PE+/−) (Fig. 3B, d) and were dramatically increased when both Zfhx3 alleles were deleted (Zfhx3^PE−/−Pten^PE+/−) (Fig. 3B, e).

Molecular characteristics of HG-mPIN/EC caused by Zfhx3 and Pten deletion

We examined the protein expression for different types of cells in the prostatic epithelium of lesions induced by the deletion of both Zfhx3 and Pten. In the normal prostate, cytokeratin 5 (CK5) is exclusively expressed in basal cells, while in Pten heterozygous (Pten^PE+/−) prostates, CK5 was detected in some luminal cells (Fig. 4A, a–c). In prostates with both Zfhx3 and Pten deleted (Zfhx3^PE−/−Pten^PE+/− and Zfhx3^PE−/−Pten^PE+/−), these cells became
more conspicuous (Fig. 4A, d and e). For the luminal cell marker CK18, the tufted cells in prostates with loss of Zfhx3, regardless of the deletion of Pten, showed strong staining for CK18 (Fig. 4A, f–j). Cells were also mostly positive in prostates with the deletion of Pten alone (Fig. 4A, h).

We also stained mouse prostate sections for synaptophysin (Syn), a marker for neuroendocrine cells. Syn-positive cells were rare in wild-type, Zfhx3 single knockout, and Pten single knockout (Zfhx3\(^{PE+/+}\) Pten\(^{PE+/+}\), Zfhx3\(^{PE−/−}\) Pten\(^{PE+/+}\) and Zfhx3\(^{PE+/−}\) Pten\(^{PE+/−}\)) prostates (Fig. 4A, k–m). However, when both Zfhx3 and Pten were knocked out (Zfhx3\(^{PE−/−}\) Pten\(^{PE−/−}\) and Zfhx3\(^{PE+/−}\) Pten\(^{PE+/−}\)), a small number of such cells were observed (Fig. 4A, n and o), suggesting a mildly enhanced neuroendocrine phenotype.

In staining prostates for Sma, the smooth muscle layer was intact in all wild-type mice or those with deletion of either Zfhx3 or Pten alone (Zfhx3\(^{PE−/−}\) Pten\(^{PE+/+}\) and Zfhx3\(^{PE+/+}\) Pten\(^{PE+/−}\)) as well as in the majority of mice with heterozygous Zfhx3 and Pten (Zfhx3\(^{PE+/−}\) Pten\(^{PE+/−}\)) (Fig. 4A, p–s); however, there was loss of Sma in Zfhx3-null and Pten-heterozygous mice (Zfhx3\(^{PE−/−}\) Pten\(^{PE+/−}\)) (Fig. 4A, t). To further analyze the interruption of smooth muscle layer, we performed dual-color immunofluorescent staining to detect both the androgen receptor (Ar), which is highly expressed in prostatic epithelial cells, and Sma in mouse prostates. As shown in Fig. 4B, prostate glands in mice with deletions of Zfhx3 and Pten (Zfhx3\(^{PE−/−}\) Pten\(^{PE+/−}\)) lost polarized structures and the muscular layer significantly, suggesting a potential invasion of the neoplastic lesion into the surrounding stroma (Fig. 4B). These results, along with the histopathologic changes, suggest that Zfhx3 and Pten deletions (Zfhx3\(^{PE−/−}\) Pten\(^{PE+/−}\)) cause early carcinoma.

### Additive effect of Zfhx3 and Pten deletions on the activation of the Akt and Erk oncoproteins

Pten is known to inhibit the activation of Akt, while loss of Pten increases the expression of phospho-Akt (p-Akt). Pten deletion (Zfhx3\(^{3PE+/+}\) Pten\(^{PE+/−}\)) led to increased expression of p-Akt in mPIN lesions, particularly at 18 months of age (Fig. 5, a, c, f, h). While deletion of Zfhx3 alone (Zfhx3\(^{3PE−/−}\) Pten\(^{PE+/+}\)) also increased p-Akt expression in a few mice (Fig. 5, b and g), simultaneous deletion of both Zfhx3 and Pten (Zfhx3\(^{3PE−/−}\) Pten\(^{PE+/−}\)) caused a marked increase in p-Akt expression at both 12 and 18 months of age (Fig. 5, d, e, i, j).

In our previous study, we observed that deletion of Zfhx3 alone increased the expression of phospho-Erk1/2 (p-Erk1/2) (Sun et al., 2014), and this was confirmed in the current study (Fig. 5, k, p, l, q). Deletion of Pten alone, on the other hand, did not result in an overt increase in p-Erk1/2 expression (Fig. 5, m and r). When both Zfhx3 and Pten were deleted, a further increase in p-Erk1/2 expression was detected (Fig. 5, n, o, s, t), indicating that deletion of both Zfhx3 and Pten promotes p-Erk1/2 activation.

### DISCUSSION

Our data showed an additive effect of Zfhx3 deletion and Pten deletion on prostatic tumorigenesis. Although deletion of Zfhx3 or one Pten allele alone only caused LG-mPIN, their concurrent deletion promoted HG-mPIN and led to earlier and more frequent formation of HG-mPIN.
of LG-mPIN. Concurrent deletion of Zfhx3 and Pten also caused or enhanced a series of cellular and molecular alterations, which include an increase in mitotic cells and the interruption of the epithelial-stromal boundary with loss of the smooth muscle layer. The activation of Akt and Erk signaling was also enhanced when both Zfhx3 and Pten were deleted compared to the deletion of either gene alone. Activation of Akt and Erk1/2 signaling pathways frequently occurs in both human prostate cancer and mouse models of prostate cancer (Oka et al., 2005; Gao et al., 2006; Jeong et al., 2008; Ouyang et al., 2008).

Our previous study demonstrated that deletion of Zfhx3 alone attenuates basal cells but enhances the luminal characteristics in mPIN cells (Sun et al., 2014). Conversely, deletion of Pten alone has been shown to expand basal cell types including the transient amplifying cells (Wang et al., 2003; Wang et al., 2006; Lu et al., 2013). When both Zfhx3 and Pten were deleted in the current study, we found that the numbers of both basal and luminal cell types were increased, as indicated by the expression of CK5 and CK18. These results may suggest that Zfhx3 deletion and Pten deletion initiate and promote prostatic tumorigenesis by distinct pathways (Wang et al., 2003; Wang et al., 2006; Sun et al., 2014). Consistent with this idea, among the hundreds of genes dysregulated by Zfhx3 deletion (Sun et al., 2014) or by Pten deletion (Wang et al., 2003), only 17 (12 upregulated and 5 downregulated) were affected by both Zfhx3 deletion and Pten deletion.

Both ZFHX3 and PTEN show frequent mutations and deletions in human prostate cancer. For example, an analysis of 258 localized prostate cancers in The Cancer Genome Atlas (TCGA) database (www.tcga-data.nci.nih.gov) by the cBioPortal platform (Cerami et al., 2012; Gao et al., 2013) revealed deletion and mutation of ZFHX3 in 28 (11%) tumors, and that of PTEN in 60 of 258 (23%) tumors. The frequency of deletion and mutation occurred more often in metastatic prostate cancers, with deletion and mutation of ZFHX3 occurring in 12 of 61 (20%) of such tumors and that of PTEN in 29 of 61 (48%) (Grasso et al., 2012). In fact, in the 61 metastatic prostate cancers, ZFHX3 was the second most frequently mutated gene (after TP53) and PTEN deletion was the second most frequent copy number change among all human genes (Grasso et al., 2012). In addition, although only 4 of the 258 (1.6%) localized prostate cancers had deletion and mutation in both ZFHX3 and PTEN, both genes were altered in 9 of the 61 (15%) metastatic tumors with a significantly higher frequency ($P < 0.001$).

Inactivation of Zfhx3 alone induced mPIN but was insufficient to induce invasive prostate carcinoma in our previous study (Sun et al., 2014). This result is similar to the observations for most tumor suppressor genes in prostate cancer including p53, Rb, Nkx3.1, maspin, Brca2, and p27Kip1, as inactivation of any one of these genes alone only causes mPIN lesions (Di Cristofano et al., 2001; Kim et al., 2002b; Maddison et al., 2004; Zhou et al., 2006; Shao et al., 2008; Francis et al., 2010). Concurrent deletion of Pten with one of these genes has also been shown to promote prostate tumorigenesis (Di Cristofano et al., 2001; Kim et al., 2002a; Chen et al., 2005).

In summary, we found that concurrent deletion of Zfhx3 and Pten induced HG-mPIN/early carcinoma which included an increase in proliferating cells and apoptotic cells, intraepithelial blood vessels, and an interrupted smooth muscle layer between epithelium
and stroma. In addition, deletion of both Zfhx3 and Pten led to an enrichment of both CK5-positive basal cells and CK18-positive luminal cells. Activation of Akt and Erk1/2 oncogenic signaling was also enhanced in lesions with concurrent deletion of Zfhx3 and Pten. Taken together with frequent mutations and/or deletions of ZFHX3 and PTEN in human prostate cancer, particularly metastatic prostate cancer, our findings are additional evidence for that simultaneous inactivation of ZFHX3 and PTEN genes contributes to the development and progression of human prostate cancer.

**METHODS AND MATERIALS**

**Mouse breeding**

All animal experiments were performed in compliance with relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of Emory University. The Zfhx3 floxed mouse strain (Zfhx3^{flox/wt}) was generated in our previous study (Sun et al., 2012). The PB-Cre4 transgenic mouse line, in which the Cre gene is driven by a derivative of the rat probasin promoter that is transcriptionally active only in prostatic epithelium, was obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium (MMHCC, Frederick, MD, USA). Floxed Pten mice were generated by Dr. Hong Wu’s group at the University of California at Los Angeles and kindly provided by Dr. David Martin (Emory University). The breeding strategy is shown in Fig. 1. Briefly, female Zfhx3^{flox/wt};Pten^{flox/flox} mice were obtained by first breeding Zfhx3^{flox/wt} and Pten^{flox/flox} mouse lines, and then inbreeding the F1 mice (Fig. 1). Male Zfhx3^{flox/wt};PB-Cre^+ mice were obtained by breeding PB-Cre4 male mice with Zfhx3^{flox/wt} female mice. Subsequently, Zfhx3^{flox/wt};PB-Cre^+ male mice were crossed with Zfhx3^{flox/wt};Pten^{flox/flox} female mice to obtain mice with the following three genotypes: Zfhx3^{wt/wt};Pten^{flox/wt};PB-Cre^+(designated as Zfhx3^{PE−/−};Pten^{PE−/−}), Zfhx3^{flox/wt};Pten^{flox/wt};PB-Cre^+(designated as Zfhx3^{PE−/−};Pten^{PE−/−}), and Zfhx3^{flox/flox};Pten^{flox/wt};PB-Cre^+(designated as Zfhx3^{PE−/−};Pten^{PE−/−}). Mice with wildtype Pten, including Zfhx3^{wt/wt};Pten^{wt/wt};PB-Cre^+(designated as Zfhx3^{PE−/−};Pten^{PE−/−}) and Zfhx3^{flox/flox};Pten^{wt/wt};PB-Cre^+(designated as Zfhx3^{PE−/−};Pten^{PE−/−}), were obtained by breeding female Zfhx3^{flox/wt} mice and Zfhx3^{flox/wt};PB-Cre^+ male mice as previously described (Sun et al., 2014). All mice were maintained on a mixed background of C57BL/6J and 129Sv/J.

**Genotyping**

All mice were genotyped by the PCR approach using genomic DNA isolated from tail biopsies (Sun et al., 2012). Alleles of Zfhx3^{flox} (1248 bp) and Zfhx3^{wt} (1071 bp) were distinguished based on the size of the PCR products using primers 5’GGCCCTTTGACTGATTTCTTTCCT-3’ and 5’ATTCGTTAATGGGAAGGTGTCAGA-3’. The Pten gene was genotyped with primers 5’ACTCAAGGCAGGGATGAGC-3’ and 5’GCCCCGATGCAATAAATATG-3’. Other primers used for genotyping included those for Cre (5’-CGGTGATGCAACGAGTGAT-3’ and 5’-CCACCGTCAGTACGTGAGAT-3’), II2 (5’-CTAGGGCCACAAATTGAAGATCT-3’ and 5’-GTAGGGAAATTCTGACATCC-3’), and Il2 (5’-CGGTGATGCAACGAGTGAT-3’ and 5’-CCACCGTCAGTACGTGAGAT-3’).
Histological analysis, immunohistochemistry, and immunofluorescent staining

Prostatic tissues were surgically harvested and fixed in 4% formaldehyde overnight. Prostatic lobes were then dissected, embedded in paraffin and sectioned at 5-μm thickness. HE staining was performed for histological analysis. All cases were reviewed following the criteria established previously (Ittmann et al., 2013).

For immunohistochemistry, tissue sections were deparaffinized, rehydrated, and washed in PBS. Antigen retrieval was done by heating slides in a microwave oven for 15 min or in a pressure cooker for 3 min in citrate buffer (pH 6.0, 10 mmol/L trisodium citrate). After blocking with 5% normal goat serum in Tris-buffered saline with 0.1% Tween-20 (TBST), tissue sections were incubated with primary antibodies at 4°C overnight, followed by incubation with EnVision Polymer-HPR secondary antibodies (Dako, Glostrup, Denmark) at room temperature for 40 min. DAB was used as the chromogen. Representative slides were scanned with the Nanozoomer 2.0HT scanner (Hamamatsu, Bridgewater, NJ, USA), and images were captured using the NDP.view computer program (Hamamatsu).

For immunofluorescent staining, tissue slides were prepared and primary antibodies were incubated as described above. The appropriate Alexa Fluor® fluorochrome-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used, and nuclei were counterstained with DAPI for 5 min. Slides were mounted and visualized with a fluorescent microscope (Zeiss, Thornwood, NY, USA). Primary antibodies used for immunohistochemistry and immunofluorescent included the following: anti-Ki-67 (Lab Vision, Fremont, CA, USA), anti-cytokeratin 5 (Covance, Princeton, NJ, USA), anti-cytokeratin 18 (Abcam, Cambridge, MA, USA), anti-androgen receptor (Ar), anti-Sma (Sigma-Aldrich, St. Louis, MO, USA), anti-synaptophysin (Invitrogen, Carlsbad, CA, USA), anti-cleaved Caspase-3 (c-Casp3), anti-phospho-Erk1/2, and anti-phospho-Akt (Cell Signaling Technology, Billerica, MA, USA).

Proliferation assay

Cell proliferation rate was estimated by detecting the expression of Ki67, a proliferation marker, by IHC staining in tissue sections of mouse prostates. For each individual mouse at 18 months, 6 or 7 representative fields in the dorsal prostate (DP) were selected, and both Ki67-positive and negative epithelial cells were counted. The percentage of Ki67-positive cells was then calculated. Student’s t-test was performed to evaluate the statistical significance for differences among different genotypes.

Acknowledgments

This work was supported in part by grant R01CA121459 from the National Cancer Institute, National Institutes of Health. It was also supported in part by the Integrated Cellular Imaging Shared Resource of Winship Cancer Institute of Emory University and the NIH/NCI under award number P30CA138292.

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J Genet Genomics. Author manuscript; available in PMC 2016 May 20.


Fig. 1. A schematic of the breeding strategy

Mouse lines of Zfhx3\(^{\text{floxed}}\)/wt, PB-Cre\(^{4}\), Zfhx3\(^{\text{floxed}}\)/floxed, and Pten\(^{\text{floxed}}\)/floxed were bred to generate the 5 desired groups of mice (and prostate tissues with desired Zfhx3 or Pten deletion):

- Zfhx3\(^{\text{w/wt}}\);Pten\(^{\text{w/wt}}\);PB-Cre\(^{+}\)
- Zfhx3\(^{\text{flox/flox}}\);Pten\(^{\text{w/wt}}\);PB-Cre\(^{+}\)
- Zfhx3\(^{\text{flox/flox}}\);Pten\(^{\text{flox/flox}}\);PB-Cre\(^{+}\)
- Zfhx3\(^{\text{flox/flox}}\);Pten\(^{\text{flox/flox}}\);PB-Cre\(^{+}\)
- Zfhx3\(^{\text{flox/flox}}\);Pten\(^{\text{flox/flox}}\);PB-Cre\(^{+}\)
Fig. 2. Concurrent deletion of Zfhx3 and Pten induces high grade mPIN (HG-mPIN) and early carcinoma in mouse prostates

A: Representative HE-stained mouse dorsal prostates (DPs) with different deletion status of Zfhx3 and Pten (indicated at the top) at various ages (indicated at the left). Hyperplasia in panel j is marked by an arrow, whereas arrows in panels e, f, i, k, l, m, and n indicate low grade mPIN (LG-mPIN). Panel o represents a HG-mPIN/EC lesion (arrow), where stars and the arrowhead mark apoptosis and small intraepithelial blood vessels respectively. All scale bars are 100 μm.

B: Distribution of histopathologic abnormalities, including hyperplasia, LG-mPIN and HG-mPIN caused by concurrent deletion of Zfhx3 and Pten in mouse prostates. Deletion status of Zfhx3 and Pten is shown at the bottom. “+” and “−” indicate the

Sun et al.  Page 13
presence or absence of an allele respectively. Also shown at the bottom are the number and age of mice for each genotype.
Fig. 3. Detection of proliferating and apoptotic cells in mouse prostatic lesions induced by Zfhx3 and Pten deletion

IHC staining was used detect the proliferation marker Ki67 (A) and apoptosis marker c-Casp3 (B). Deletion status of Zfhx3 and Pten is shown at the top, and ages of mice at the left. Ki-67-positive cells are indicated by arrows in panels a–g and i of A but not in panels h and j where the number of Ki-67-positive cells was very large. All scale bars are 100 μm.
Fig. 4. Molecular characterization of mPIN lesions induced by Zfhx3 and Pten deletion

A: IHC staining of the basal cell marker CK5 (a–e), luminal cell marker CK18 (f–j), neuroendocrine cell marker synaptophysin (Syn) (k–o), and fibromuscular marker smooth muscle actin (Sma) (p–t) in mPIN lesions at 18 months. Magnified regions showing detailed CK5 staining patterns are at the upper right corners of panels a and e. Deletion status of Zfhx3 and Pten is indicated at the top, and protein names at the left. All scale bars are 100 μm.

B: Loss of the smooth muscle layer in mPIN lesions induced by Zfhx3 and Pten deletion, as confirmed by immunofluorescent staining of Sma (red) with androgen receptor (Ar, green) as a control, in 18 month old mice. Nuclei were counterstained with DAPI (blue). Deletion status of Zfhx3 and Pten is indicated at the left, and protein name and DAPI shown at the bottom.
Fig. 5. Enhanced activation of Akt and Erk1/2 by Zfhx3 and Pten deletion in mouse prostates

IHC staining was performed to detect the expression of p-Akt and p-Erk1/2 in mouse dorsal prostates at 12 and 18 months of age. Arrows in panels d and g point to occasional p-Akt-positive prostatic ducts, and arrows in panels l, o, q and s point to p-Erk1/2-positive cells. Deletion status of Zfhx3 and Pten is indicated at the top, and protein names and mouse ages at the left. All scale bars are 100 μm.
### Table 1
Summary of morphological abnormalities in different mouse prostate lobes induced by concurrent deletion of *Zfhx3* and *Pten*.

<table>
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<th>Prostatic lobe</th>
<th>Age (months)</th>
<th>Zfhx3 genotype</th>
<th>Phenotype</th>
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For all mice, one allele of Pten was deleted. N, number of mice. mPIN, mouse prostatic intraepithelial neoplasia. LG-mPIN, low-grade mPIN. HG-mPIN, high-grade mPIN. EC, early carcinoma. For Zfhx3 or Pten genotypes, “+” and “−” indicate the presence and absence of an allele respectively.