Identification of New Tumor Suppressor Genes in Triple-Negative Breast Cancer

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Abstract

Although genomic sequencing has provided a better understanding of the genetic landmarks in triple-negative breast cancer (TNBC), functional validation of candidate cancer genes (CCG) remains unsolved. In this study, we used a transposon mutagenesis strategy based on a two-step sleeping beauty (SB) forward genetic screen to identify and validate new tumor suppressors (TS) in this disease. We generated 120 siRNAs targeting 40 SB-identified candidate breast cancer TS genes and used them to downregulate expression of these genes in four human TNBC cell lines. Among CCGs, whose SB-mediated genetic mutation resulted in increased cellular proliferation in all cell lines tested, the genes ADNP, AP2B1, TOMM70A, and ZNF326 showed TS activity in xenograft studies. Subsequent studies showed that ZNF326 regulated expression of multiple epithelial–mesenchymal transition and cancer stem cell (CSC) pathway genes. It also modulated expression of TS genes involved in the regulation of migration and cellular invasion and was a direct transcriptional activator of genes that regulate CSC self-renewal. ZNF326 expression associated with TNBC patient survival, with ZNF326 protein levels showing a marked reduction in TNBC. Our validation of several new TS genes in TNBC demonstrate the utility of two-step forward genetic screens in mice and offer an invaluable tool to identify novel candidate therapeutic pathways and targets. Cancer Res; 77(15); 4089–101. ©2017 AACR.

Introduction

Breast cancer is one of the leading causes of cancer-related deaths in women worldwide. Triple-negative breast cancer (TNBC), which accounts for 10%–20% of all breast cancers, does not express estrogen or progesterone receptors and lacks HER2 amplification. Patients diagnosed with TNBC have a higher risk of disease relapse within 5 years than patients treated for other breast cancer subtypes (1, 2). Among breast cancer subtypes, TNBC is the most aggressive, with the highest relapse incidence and the most undifferentiated phenotype. Interestingly, residual breast cancer tumors after conventional therapy display mesenchymal and cancer stem cell (CSC) features (3).

Epithelial–mesenchymal transition (EMT) is a biological process that allows polarized epithelial cells to acquire a mesenchymal phenotype, which provides advantages for cell migration, invasion, and resistance to apoptosis, in addition to increases in the production of extracellular matrix proteins. In cancer, multiple transcription factors regulate the EMT pathway with a coordinated inactivation of tumor suppressor (TS) genes (4). Mesenchymal breast tumors contain a small cell population of CSCs that have the capacity to form mammospheres in vitro and maintain tumorigenic activity. It has now been established that a heterogeneous population of CSCs is responsible for tumor initiation and maintenance (5, 6). These CSCs are responsible for metastatic growth in breast cancer, which contributes to the majority of the breast cancer related morbidity and mortality (7, 8). To better understand the pathogenesis of TNBC, a comprehensive understanding of the genes and pathways that regulate EMT and CSCs in breast cancer is essential. Recent human cancer genome sequencing studies have provided a genomic mutation landscape of breast cancer (9), thus identification and functional validation of CCGs is a high priority.

Genome-wide insertional mutagenesis is an unbiased and high-throughput method with which to profile the landscape of driver genes in a mouse model system (10). The sleeping beauty (SB) transposon system has expanded the use of insertional mutagenesis for the study of many types of cancers (11–20); and discovered candidate cancer genes (CCG) involved in the EMT and CSC pathways (21–23). To better understand the genes and pathways involved in the pathogenesis of TNBC, we performed an siRNA validation study of 40 SB-identified breast cancer TS genes identified in an SB mutagenesis screen performed in Pten mutant mice (24). Among the seven CCGs that consistently resulted in
increased proliferation in four TNBC cell lines, four including ADNP, AP2B1, TOMM70A, and ZNF326 also showed TS activity in orthotopic tumor xenograft validation studies. Subsequent studies showed that ZNF326 is a TS gene that mediates the expression of DACH1, KLF17, and GATA3, and disrupts the EMT and CSC pathways. Our results show that two-step forward genetic screens in mice are invaluable tools for uncovering CCGs in different signaling pathways involved in TNBC.

Materials and Methods

Cell culture
Human breast cancer cell lines HCC70 (CRL-2315), HCC1569 (CRL-2330), MDA-MB-231 (HTB-26), MDA-MB-468 (HTB-132), and BT-549 (HTB-122) were obtained from the ATCC. The cell lines were cultured according to vendor instructions. All cell lines were purchased between 2012 and 2014 and were frequently tested for mycoplasma infection using MycoAlert Detection Kit (Lonza). All cell lines were propagated no more than 2 months or 10 passages after resuscitation from stocks and not further authenticated.

siRNA screening
siRNA oligonucleotides were purchased from Thermo Fisher Scientific. A list of siRNA oligonucleotides is provided in Supplementary Table S1. We generated pools of three siRNA oligos per gene. siRNA transfection was performed using the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, 4 x 10^5 cells were seeded into 96-well plates and cultured with medium containing 10% FBS. After 24 hours, cells were transfected with siRNA pooled oligos and incubated at 37°C for 96 hours. Cellular proliferation was then measured using the WST-1 cell proliferation assay system (Takara). WST-1 regent was added to each well and incubated for 2 hours at 37°C. The absorbance at 450 and 650 nm (reference) was then measured using an Infinite 200 pro multimode reader (TECAN).

Generation of stable shRNA-expressing cell lines
All lentiviral particles containing GIPZ shRNA or nontargeting control shRNA were purchased from Open Biosystems. A list of shRNAs is provided in Supplementary Table S2. Cells were plated at a density of 1 x 10^5 cells per 6-well plate 1 day before infection. The next day, the medium was changed into serum-free medium containing 8 μg/mL polybrene (Millipore) and transduced with three shRNA lentivirus-pool or single shRNA lentivirus clones at a multiplicity of infection of 6 or 1, respectively. After 48 hours, cells were supplemented with complete medium containing 1.0 to 1.5 μg/mL puromycin for 7 days (Thermo Fisher Scientific) to establish stable cell lines containing lentiviral integrations. For overexpression studies, all cell lines were selected with blasticidin 5 μg/mL, except for MDA-MB-231 (20 μg/mL), for 2 weeks.

Cell migration and invasion assays
Transwell migration assays were performed in a 24-multiwell insert system with a porous polycarbonate membrane (8-μm pore size) according to the manufacturer’s instructions (Cell Biolabs). Cells were allowed to grow to subconfluency (~75%–80%) and were then serum-starved for 24 hours. After detachment with trypsin, cells were washed with PBS, resuspended in serum-free medium, and 300 μL of cell suspension (5 x 10^5 cell/mL) was then added to the upper chamber. Five-hundred microliters of complete medium was added to the bottom wells of the chamber. The cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower face of the filters were fixed, stained, and quantified according to the manufacturer’s instructions (Cell Biolabs). Similar inserts coated with Matrigel were used in the invasion assays.

Mammosphere assays
In brief, 500 cells were seeded into ultra-low adherent six-well plates (Corning) and plated in MammoCult medium (Stem Cell Technologies) supplemented with hydrocortisone, heparin, amphotericin, and gentamicin (SIGMA) for 7 to 10 days. Mammosphere formation was visible at 7 to 10 days and photographs of comparable groups were captured using phase-contrast microscopy (EVOS XL, Thermo Fisher Scientific) at ×20 magnification. All experiments were done in triplicate.

Tumor xenografts
All mouse experiments were approved by the Animal Care and Use Committee (Houston Methodist Research Institute, Houston, TX). Female 6- to 7-week-old Crl:NIH(Ncr)-Foxn™ mice were purchased from Charles River Laboratories. Mammary fat pad injections into athymic nude mice were performed using 3 x 10^6 cells (HCC70 and MDA-MB-231). The human cancer cells were resuspended in 100 μL of a 1:1 mix of PBS and Matrigel (TREximaGEN). For HCC1569 human breast cancer cells, we injected 4 x 10^5 cells in 100 μL of a 1:1 mix of PBS and Matrigel. Injections were done into the fourth mammary gland. Tumors were measured using a digital caliper and the tumor volume was calculated using the following formula: volume (mm^3) = width x length x 1/2.

qRT-PCR
Total RNA was purified and DNase treated using the RNeasy Mini Kit (Qiagen). Synthesis of cDNA was performed using SuperScript VILO Master Mix (Life Technologies). Quantitative PCR analysis was performed on the QuantStudio 12K Flex System (Life Technologies). All signals were normalized to the levels of GAPDH TaqMan probes. TaqMan probes were obtained from Life Technologies (Supplementary Table S3). EMT (PAHS-090Z) and CSC (PAHS-176Z) RT-PCR arrays were purchased from Qiagen. The assays were performed according to the manufacturer instructions and the results were evaluated using the Qiagen data analysis center.

Chromatin immunoprecipitation studies
To determine where ZNF326 binds within the promoter regions, first we retrieve the genomic sequence (www.ensembl.org) located upstream of the transcriptional start site of the gene including 200 bp downstream. Next, we design PCR primers in which the amplified products range between 250 and 300 bp and overlap 100 bp (Supplementary Table S4). HCC70 and MDA-MB-231 cells were fixed in 1% formaldehyde at 37°C for 10 minutes. Cells were washed twice with ice-cold PBS containing protease inhibitors, scraped, and centrifuged at 4°C. Cell pellets were resuspended in lysis buffer and sonicated (Covaris S220) to shear DNA with a fragment range of 300 to 400 bp. After sonication, the lysate was centrifuged and the supernatant was diluted 10-fold with ChIP dilution buffer (EZ-CHIP Kit, EMD-MILLIPORE). Rabbit anti-V5 tag antibody (Abcam, ab15828), or...
normal rabbit IgG control (Abcam, ab171870) were added to the diluted chromatin and incubated overnight at 4°C with rotation. The antigen–antibody complex was precipitated with protein A/G-agarose and washed sequentially with low-salt buffer, high-salt buffer, and lithium chloride wash buffer, and eluted with elution buffer (1% SDS, 0.1 M NaHCO₃, and 200 mmol/L NaCl). Reversal of crosslinking was performed by heating at 65°C overnight in the presence of NaCl. DNA was purified using DNA-binding columns provided by the EZ-ChIP Kit. The amount of immunoprecipitated DNA was quantified using high-sensitive detection DNA reagent (Q32851, Life Technologies) and measured using a Qubit 3.0 Fluorometer (Life Technologies). qPCRs were run in triplicate. The primer list is available in Supplementary Table S4.

**Statistical analysis**

All data are provided as mean ± SEM unless otherwise indicated. Statistical analyses were performed using an unpaired Student t test, using GraphPad Prism 6 software (Version 6.0).

**Results**

**A two-step forward genetic screen identifies multiple TNBC TS genes**

TNBC has the worst prognosis of any breast cancer subtype. To better understand the genetic forces driving TNBC, we performed a SB transposon mutagenesis screen in Pten-mutant mice. Pten was used as a sensitizing mutation in the screen because loss of PTEN has been implicated in breast cancer progression, is clonally selected in TNBC, and favors the activation of the EMT pathway to promote metastasis (7, 25).

The screen identified 12 candidate trunk drivers and a much larger number of progression genes, many of which were predicted to function as TS genes (24). To provide additional functional validation for these genes in TNBC, we generated 120 siRNAs targeting 40 SB-identified candidate TS genes (three siRNAs per gene) and then used them to downregulate the expression of these genes in four human TNBC cell lines (BT549, HCC70, MAD-MB-231, MDA-MB-468; Fig. 1A; Supplementary Table S5). We then asked whether downregulation of these genes increased cell proliferation, as this is one of the most commonly altered processes promoting tumorigenesis observed in tumors with mutations in TS genes (26). Downregulation of seven SB-identified candidate TS genes (ADNP, AP2B1, ARID1B, TOMM70A, ZC3H7A, ZCCHC7, and ZNF326) led to a >15% increase in cell proliferation with all three siRNAs tested, in each of the four cell lines assayed, suggesting a high probability of TS activity (Fig. 1B).

To further confirm a role for these seven TS genes in TNBC, we used short hairpin RNAs (shRNA) delivered by lentivirus to silence the expression of these genes in HCC70 and MDA-MB-231 cells (Supplementary Figs. S2A and S2B). The cells were then orthotypically injected into the mammary fat pad of athymic nude mice and, after 45 days, the animals were necropsied and tumor volumes measured (Fig. 1C). The silencing of four TS genes (ADNP, AP2B1, ARID1B, and ZNF326) accelerated tumor growth for both cell lines tested following orthotopic transplant (Fig. 1D and E), confirming that they are TS genes.

**ZNF326 is a TS gene in TNBC**

Given ZNF326 had the most consistent increase in tumor growth in orthotopic transplants, we repeated the orthotopic transplants using two different shRNAs, in three different TNBC cell lines (Supplementary Figs. S3A–S3C) to confirm that off-target effects of the shRNA did not cause the increased tumor growth. After injecting the different tumor cell lines with different ZNF326 shRNA constructs, we observed a significantly accelerated tumor growth in nude mice (Fig. 2A–C), confirming that off-target effects of the shRNA did not cause the increased tumor growth. Finally, to determine whether ZNF326 overexpression would reduce tumor growth, we overexpressed ZNF326 in MDA-MB-231 cells using a lentivirus construct expressing the ZNF326 open reading fused to a V5 tag at the 3’ end (Supplementary Fig. S3D). Tumor cells were then injected into the mammary fat pad of nude mice, where we observed a statistically significant reduction in tumor growth in cells overexpressing ZNF326 (Fig. 2D). Collectively, these results show that ZNF326 functions as a TS gene in TNBC.

**ZNF326 regulates the expression of multiple genes in the EMT pathway**

ZNF326 encodes a protein containing two C2H2-type zinc finger protein motifs and a glutamic acid-rich domain in the C-terminus. It exhibits DNA-binding activity in a zinc-dependent manner. It can bind to the promoter regions of multiple genes, including those involved in the EMT pathway. This binding may regulate the expression of these genes, thereby contributing to the progression of TNBC.
Validation of candidate TNBC tumor suppressor genes identified by transposon mutagenesis.

A. Schematic representation of the siRNA proliferation screen used for candidate TS gene validation.

B. Top seven candidate tumor suppressor genes selected by WST-1 assay.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Number of oligos that show &gt;15% proliferation increase</th>
<th>Average proliferation ratio relative to negative control</th>
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<tbody>
<tr>
<td>ADNP</td>
<td>3</td>
<td>128.5 BT549, 126.3 HCC70, 134.6 MDA-MB-231, 125.3 MDA-MB-468</td>
</tr>
<tr>
<td>AP2B1</td>
<td>3</td>
<td>131.1 BT549, 126.2 HCC70, 128.8 MDA-MB-231, 129.1 MDA-MB-468</td>
</tr>
<tr>
<td>ARL6IP5</td>
<td>3</td>
<td>130.6 BT549, 126.8 HCC70, 132.1 MDA-MB-231, 132.5 MDA-MB-468</td>
</tr>
<tr>
<td>TOMM70A</td>
<td>3</td>
<td>127.2 BT549, 125.7 HCC70, 151.3 MDA-MB-231, 130.9 MDA-MB-468</td>
</tr>
<tr>
<td>ZC3H17A</td>
<td>3</td>
<td>129.4 BT549, 122.9 HCC70, 119.7 MDA-MB-231, 141.5 MDA-MB-468</td>
</tr>
<tr>
<td>ZCCHC7</td>
<td>3</td>
<td>124.3 BT549, 118.2 HCC70, 122.3 MDA-MB-231, 137.5 MDA-MB-468</td>
</tr>
<tr>
<td>ZNF326</td>
<td>3</td>
<td>127.7 BT549, 124.1 HCC70, 131.8 MDA-MB-231, 133.7 MDA-MB-468</td>
</tr>
</tbody>
</table>

C. Stable HCC70 and MDA-MB-231 cell lines were generated containing three lentiviral shRNAs for each candidate TS gene identified in B and orthotopically injected into transplant mice.

D and E. Orthotopic tumor xenografts validated ADNP, AP2B1, TOMM70A, and ZNF326 as novel TS genes in TNBC. n = 5 mice per group. Error bars, SEM, * P < 0.05. NC shRNA, negative control shRNA.

Figure 1.
manner and plays a role in regulating cell growth (27, 28). Recently, ZNF326 has been biochemically characterized as a core component of the DBIRD complex, a multiprotein complex that acts at the interface between core mRNP particles and RNA polymerase II (RNAPII), and integrates transcript elongation with the regulation of alternative splicing (29). Moreover, siRNA studies have shown that downregulation of ZNF326 in HEK cell lines increases the expression of several EMT pathway genes (29).

To determine whether ZNF326 regulates the expression of EMT genes in TNBC cells, we knocked down expression of ZNF326 in HCC70 and HCC1569 cells using three independent ZNF326 shRNAs, and then used an EMT PCR gene expression array to identify genes regulated by ZNF326. The EMT PCR array profiles the expression of 84 key genes that either change their expression during the process of EMT or regulate the expression of these genes. This analysis identified many EMT pathway genes that were deregulated by downregulation of ZNF326 in TNBC cells (Fig. 3A and B). As an example, we found an upregulation of Zeb1 and Zeb2 downstream targets like cadherin-2 and vimentin, supporting a role for ZNF326 in EMT (Fig. 3A and B). These results show that ZNF326 regulates multiple genes in the EMT pathway in TNBC cells.

To further explore the biological effects of these expression changes, we performed migration and invasion assays using TNBC cells that have downregulated ZNF326 expression. We observed increased migration and invasion in HCC70 and MDA-MB-231 cells with reduced ZNF326 expression compared with control parental cells (Fig. 3C and D). These results show that ZNF326 negatively regulates cell migration and invasion in addition to controlling the expression of multiple genes in the EMT pathway.

ZNF326 regulates the expression of multiple genes in the CSC pathway

Interestingly, ZNF326 was also identified in a transposon screen that identified genes, which can promote the immortalization of atroglial-like cells (22). Likewise, other studies in

Figure 2. ZNF326 is a tumor suppressor gene in TNBC. A–C, shRNA-induced inactivation of ZNF326 in HCC70, HCC1569, and MDA-MB-231 TNBC cells accelerated tumor growth in orthotopic xenograft assays. D, Ectopic overexpression of ZNF326 inhibited tumor growth in MDA-MB-231 orthotopic xenograft assays. n = 10 mice per group. Error bars, SEM. *, P < 0.05. NC shRNA, negative control shRNA.
Figure 3.
ZNF326 regulates the expression of multiple genes in the EMT pathway. A and B, shRNA knockdown of ZNF326 in HCC70 and HCC1569 TNBC cells resulting in the deregulation of multiple genes in the EMT pathway. Three independent ZNF326 shRNAs showed consistent mRNA expression levels over control. 1.5-fold gene expression difference over control was considered as significant. C and D, Migration (C) and invasion (D) assays of stable ZNF326-shRNA knockdown HCC70 and MDA-MB-231 tumor cells. Data represent means ± SEM of three independent experiments. *, P < 0.05. NC shRNA, negative control shRNA.
embryonic stem cells have shown that ZNF326 forms complexes with Nanog–Sox2, which plays an important role in stem cell self-renewal (30). On the basis of these findings, we asked whether ZNF326 might also regulate genes linked to CSC and thereby promote CSC self-renewal. We used two different ZNF326 shRNAs to downregulate ZNF326 expression in MDA-MB-231 and HCC70 cells, and then used a CSC PCR array that profiles the expression of 84 genes linked to CSCs, to ask whether ZNF326 downregulation affects the expression of any of these genes. We observed a significant upregulation of CXCL8 and THY1 expression, and a significant downregulation of DACH1, DKK1, DLL1, GATA3, and KLF17 expression in ZNF326 shRNA-expressing cells (Fig. 4A and B, top; Supplementary Figs. S4A and S4B). These results indicate that ZNF326 regulates multiple genes linked to CSCs.

To further explore the biological effects of these expression changes, we performed mammosphere assays using TNBC cells with deregulated ZNF326 expression. We observed an increased number of mammospheres in HCC70 tumor cells, respectively. The values are the means ± SEM from three independent experiments. *, P < 0.05.

Table 1. Tumor incidence in limiting dilution assay

<table>
<thead>
<tr>
<th>Cells from mammospheres injected</th>
<th>100</th>
<th>1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231 nontargeted control</td>
<td>0/10</td>
<td>4/10</td>
</tr>
<tr>
<td>MDA-MB-231 ZNF326 shRNA1</td>
<td>3/10</td>
<td>10/10</td>
</tr>
<tr>
<td>MDA-MB-231 ZNF326 shRNA2</td>
<td>6/10</td>
<td>10/10</td>
</tr>
<tr>
<td>MDA-MB-231 control</td>
<td>1/10</td>
<td>6/10</td>
</tr>
<tr>
<td>MDA-MB-231 ZNF326 ORF</td>
<td>0/10</td>
<td>1/10</td>
</tr>
</tbody>
</table>

NOTE: Tumor incidence/number of injections.

Figure 4. ZNF326 controls the expression of genes important for CSC self-renewal. CSC gene expression studies in ZNF326 knockdown MDA-MB-231 (A) and HCC70 (B) cells. Three independent shRNA ZNF326 clones showed consistent mRNA expression level changes relative to control cells. *, 1.5-fold gene expression difference over control was considered as significant. C and D, number of mammosphere-forming cells in control and ZNF326 shRNA knockdown and ZNF326 overexpression MDA-MB-231 and HCC70 tumor cells, respectively. The values are the means ± SEM from three independent experiments. *, P < 0.05.

Representative microphotographs of the mammospheres formed by 500 control and 500 ZNF326 shRNA knockdown and ZNF326 overexpression tumor cells. Scale bar, 100 μm. NC shRNA, negative control shRNA.
Figure 5.
ZNF326 binds to the promoter regions of $DACH_1$, $KLF17$, and $GATA3$. ChIP assays confirm that ZNF326 binds to the promoter regions of $DACH_1$ (A), $KLF17$ (B), and $GATA3$ (C). Top panels show the promoter regions (B1–B4) that were assayed for ZNF326 binding, while the bottom panels show the extent of ZNF326 binding to each of the promoter regions. r, cells expressing recombinant ZNF326-V5 protein; p, parental cells lacking the recombinant ZNF326-V5 protein. In all experiments, data represents means ± SEM of three independent experiments.
ZNF326 expression (52 vs. 7; Fig. 4C and D). We then used orthologous transplants to determine the relationship between mammosphere formation, tumorigenicity, and ZNF326 expression. We found that downregulation of ZNF326 expression in MDA-MB-231 mammospheres greatly increased tumor formation in orthologous transplants, whereas ZNF326 overexpression in mammospheres decreased tumor formation (Table 1). These results support the hypothesis that ZNF326 is a TS gene that regulates CSC self-renewal by controlling the expression of genes important for CSC self-renewal.

ZNF326 is a direct transcriptional activator of genes that regulate CSC self-renewal

To determine whether ZNF326 is a direct transcriptional activator of genes that regulate CSC self-renewal, we asked whether ZNF326 could bind to the promoter regions of DACH1, KLF17, and GATA3 in chromatin immunoprecipitation (ChIP) assays. ChIP assays using a V5 tag antibody showed binding to multiple sites within the promoter regions of DACH1, KLF17, and GATA3 in HCC70 and MDA-MB-231 cells expressing recombinant ZNF326-V5 tag protein, but not in parental cells that lacked the recombinant protein (Fig. 5). To further examine the transcriptional effects of this binding, we performed luciferase reporter assays. DACH1, KLF17, and GATA3 promoter-luciferase DNA constructs were transfected into ZNF326 knockdown or ZNF326-overexpressing tumor cells. The promoter constructs contained 1 kb upstream of the transcriptional start site and cloned into the pLightSwitch reporter vector. Subsequent quantification of luciferase activity showed that DACH1, KLF17, and GATA3 luciferase activity was significantly decreased in ZNF326 knockdown HCC70 or MDA-MB-231 cells compared with control parental cells. Conversely, DACH1, KLF17, and GATA3 luciferase activity was increased in tumor cells overexpressing ZNF326 (Fig. 6). These data support the hypothesis that ZNF326 is a direct transcriptional activator of genes that regulate CSC self-renewal.

ZNF326 expression levels are clinically associated with patient survival

To determine whether ZNF326 expression levels are clinically associated with patient survival, we queried a publically available breast cancer database (31). We found that high ZNF326 expression is associated with increased relapse-free survival in luminal A and luminal B breast cancer patients (Fig. 7A). In TNBC, we also found a similar trend, but the level did not reach statistical significance. To further explore the expression of ZNF326 protein in breast cancer, we used commercially available TMAs...
(Pantomics). We compared 48 IDC ER\textsuperscript{+}, and 48 IDC TNBC tissue samples, in duplicate, and compared them with adjacent normal tissue. Interestingly, immunohistochemical analysis showed that IDC ER\textsuperscript{+}, which are the most predominant luminal A and B breast cancer subtypes, express high levels of ZNF326 protein compared with normal breast tissue (Fig. 7B and C). In contrast, we observed a marked reduction in ZNF326 protein levels in TNBC tumors (Fig. 7C), consistent with ZNF326 being an important TS in this breast cancer subtype. Similarly, DACH1 and GATA3 expression is dramatically reduced in basal-like TNBC (Supplementary Figs. S5A and S5B), in agreement with the reduction in ZNF326 expression we observed in TNBC in our IHC studies.

**Figure 7.** ZNF326 expression levels are clinically associated with patient survival. **A**, Kaplan–Meier plots depicting the recurrence-free survival probability of patients with different breast cancer subtypes based on ZNF326 expression levels. Luminal A (n = 1764), luminal B (n = 1002), HER2 (n = 208), and TNBC (n = 580). HR, hazard ratio. Log-rank test was used in the analysis. **B**, ZNF326 expression levels in ER\textsuperscript{+} and TNBC. Percentage distribution of ZNF326 protein expression determined by IHC score. **C**, Representative ZNF326 expression in normal breast epithelium, IDC, in situ lobular carcinoma, and TNBC using ZNF326-specific antibodies. Scale bar, 200 \( \mu m \).
Discussion

SB mutagenesis provides a powerful tool for identifying new cancer genes, tumor biomarkers, and therapeutic targets. In previous experiments, we used SB mutagenesis to identify candidate TS genes that could cooperate with mutant Pten in breast cancer progression (24). Here, we functionally validated four of these genes (ADNP, AP2B1, TOMM70A, and ZNF326) in TNBC using a combination of in vitro siRNA screening and orthotopic tumor xenografts. Homeobox-containing, activity-dependent, neuroprotective protein, ADNP, regulates OCT4 and PAX6 expression, and is essential for brain development (32, 33). ADNP interacts with components of the BAF complex, the eukaryotic equivalent of the SWI/SNF complex in yeast, which is involved in the regulation of gene expression (34). In cancer cells, ADNP regulates cell growth and differentiation and, under stress conditions, exhibits expression changes leading to cancer progression (35). In contrast, in intestinal cancer cells, downregulation of ADNP by antisense oligodeoxynucleotides upregulates the TS p53 and reduces the viability of intestinal cancer cells (36). ADNP may therefore also act as an oncogene in certain cellular contexts. AP2B1 is a subunit of the AP2 coat assembly protein complex involved in clathrin-mediated endocytosis. Knockdown studies in breast cancer cells have demonstrated an increase in the number of matrix degrading invadopodia, adhesion structures linked to invasive migration in cancer cells (37), suggesting that AP2B1 may control the balance between the formation of normal cell adhesions and invasive adhesion structures. Translocase of outer mitochondria membrane 70A, TOMM70A is part of a multisubunit complex involved in the recognition, unfolding and translocation of preproteins into the mitochondria. Under normal conditions, TOMM70A negatively regulates the levels of reactive oxygen species (ROS), but in tumor cells, ROS drives cancer progression and promotes inflammation (38).

ZNF326 was initially identified as a transcriptional activator involved in neuronal differentiation (28). Recently, ZNF326 also called ZNF-protein interacting with nuclear messenger ribonucleoproteins and DBC1 (ZIRD)) was also shown to bind deleted in breast cancer 1 [DBC1] and form part of a protein complex named DBIRD that binds directly to RNAPII (29). DBIRD regulates alternative splicing of a large set of exons embedded in (A+T)-rich DNA, and is present at the affected exons. Analysis of ZNF326-deleted cells showed a >1.5-fold increase in exon inclusion in >2,800 cases, whereas exon exclusion was observed in only 390 cases. Similar results were observed in DBC1-deleted cells, supporting a close functional relationship between these two proteins. The effect of the depletion was at the level of alternative splicing, as depletion of ZNF326 or DBC1 only affected the expression level of a small number of genes. Although the oncogenic effects, if any, of these alternative splicing defects remain to be determined, it would not be surprising if some of the oncogenic effects seen in ZNF326 knockdown TNBC cells were promoted through these splicing defects.

We also showed that knockdown of ZNF326 in TNBC cells resulted in the deregulation of multiple genes involved in EMT, similar to what has previously been observed in ZNF326 HEK knockdown cells (29). In addition, we observed an increase in cell migration and invasion in ZNF326 TNBC knockdown cells, both biological signatures of EMT activation.

Insertional inactivation of ZNF326 has also been associated with the immortalization of astroglial-like neural stem cells in SB mutagenesis studies (22). Moreover, in embryonic stem cells, ZNF326 has been shown to form complexes with NANOG and SOX2 and play an important role in stem cell self-renewal (30), suggesting that ZNF326 might also function in CSC self-renewal. Consistent with this, we found that ZNF326 is a direct transcriptional activator of genes regulating CSC self-renewal, including DACH1, KLF17, and GATA3. We also observed an increased number and size of mammosphere formation in HCC70 and MDA-MB-231 TNBC cells with reduced ZNF326 expression, and decreased mammosphere formation and size in TNBC cells with increased ZNF326 expression. Downregulation of ZNF326 expression in MDA-MB-231 mammospheres also greatly increased tumor formation in orthologous transplants, whereas ZNF326 overexpression decreased tumor formation. These results strongly support the hypothesis that ZNF326 is a TS gene that regulates CSC self-renewal by directly controlling the expression of genes important for CSC self-renewal. Additional support for this hypothesis has been provided by other laboratories who have shown that DACH1 inactivation increases the number of CSCs in glioma and breast cancer, whereas Gata3 negatively regulates CSCs from luminal progenitors (39–41). Clinical studies have also demonstrated a correlation between poor prognosis in breast cancer patients and reduced DACH1 expression (42). Similar to what we observed for ZNF326 in TNBC cells, forced expression of DACH1 in glioma cell lines also resulted in decreased proliferation in vitro and tumor growth in vivo (39). Likewise, glioma-DACH1-low cells form spheroids in an immunodeficient medium whereas parental glioma cells do not. Compared with spheroid-forming glioma-DACH1-low cells, glioma-DACH1-high cells also displayed lower tumorigenicity, indicating that DACH1 decreases the number of tumor-initiating cells. KLF17 is a negative regulator of EMT and metastasis in breast cancer and a TS in lung, liver, and esophageal cancer (43–46), whereas GATA3 is a regulator of mammary gland morphogenesis and luminal differentiation in breast tissue (47, 48). In pathological settings, GATA3 is a marker of estrogen receptor (ER)–positive breast cancer in which high levels of GATA3 correlate with high ER expression, whereas low levels are strongly associated with ER-negative, higher histologic grade, lymph node positivity, and a higher propensity for metastasis (49). Finally, our immunohistochemical tissue array studies showed high ZNF326 protein levels in luminal A and B ER+ breast cancer subtypes, in contrast to low ZNF326 protein levels in TNBC tumors, consistent with ZNF326 being an important TS gene in this breast cancer subtype. In agreement with the reduced ZNF326 expression we observed in TNBC in tissue array studies, we also showed that DACH1 and GATA3 expression is dramatically reduced in basal-like breast cancers.

In summary, using a combination of siRNA screening and orthologous tumor xenografts, we have identified multiple new TNBC tumors suppressor genes and provided evidence that one of these genes, ZNF326, is a multifunctional TS gene that controls tumor cell growth through effects on RNA splicing, EMT, and CSC self-renewal. Our studies demonstrate the power of SB mutagenesis when combined with in vitro and in vivo functional validation for identifying new human TNBC TS genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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