SPEN is a tumor suppressor in ERα positive breast cancers

**Title**

The estrogen receptor cofactor SPEN functions as a tumor suppressor and candidate biomarker of drug responsiveness in hormone-dependent breast cancers.

**Running Title**

SPEN is a tumor suppressor in ERα positive breast cancers

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**Keywords**

- Transcriptional corepressor
- Tumor suppressor gene
- Estrogen receptor positive breast cancers
- Endocrine resistance

**Financial support**

- Stéphanie Légaré
  - Supported by a McGill Integrated Cancer Research Training Program studentship (FRN53888) and a doctoral award from the Fonds de recherche du Québec – Santé.
- Isabelle Sirois
  - Recipient of the Eileen Iwanicki postdoctoral fellowship in Breast Cancer Research from the Canadian Institutes of Health Research in partnership with the Breast Cancer Society of Canada.

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- Mark Basik, Patricia N. Tonin and Sylvie Mader
  - Supported by grants from the Cancer Research Society and the FRQS Réseau de Cancer Axe Cancer du Sein et Ovaire as well as a grant from the Quebec Breast Cancer Foundation.

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**Other notes about the manuscript**

Number of words in text: 4294

Total number of figures and tables: 5 figures

Total number of supplementary figures and tables: 8 figures – 5 tables

**Conflict of interest**

The authors declare no conflict of interest. No competing financial interest exists.
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Abstract

The treatment of breast cancer has benefitted tremendously from the generation of estrogen receptor alpha (ERα)-targeted therapies, but disease relapse continues to pose a challenge due to intrinsic or acquired drug resistance. In an effort to delineate potential predictive biomarkers of therapy responsiveness, multiple groups have identified several uncharacterized cofactors and interacting partners of ERα, including Split Ends (SPEN), a transcriptional co-repressor. Here we demonstrate a role for SPEN in ERα-expressing breast cancers. SPEN nonsense mutations were detectable in the ERα-expressing breast cancer cell line T47D, and corresponded to undetectable protein levels. Further analysis of 101 primary breast tumors revealed that 23% displayed loss of heterozygosity at the SPEN locus and that 3-4% harbored somatically acquired mutations. A combination of in vitro and in vivo functional assays with microarray-based pathway analyses showed that SPEN functions as a tumor suppressor to regulate cell proliferation, tumor growth, and survival. We also found that SPEN binds ERα in a ligand-independent manner and negatively regulates the transcription of ERα targets. Moreover, we demonstrate that SPEN overexpression sensitizes hormone receptor-positive breast cancer cells to the apoptotic effects of tamoxifen, but has no effect on responsiveness to fulvestrant. Consistent with these findings, two independent datasets revealed that high SPEN protein and RNA expression in ERα-positive breast tumors predicted favorable outcome in patients treated with tamoxifen alone. Together, our data suggest that SPEN is a novel tumor suppressor gene that may be clinically useful as a predictive biomarker of tamoxifen response in ERα-positive breast cancers.
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Introduction

Approximately 70% of breast cancers express the estrogen receptor alpha (ERα) and/or its transcriptional target, the progesterone receptor (PgR) and are dependent on hormones for their growth, proliferation and survival (1). The ERα is a member of the nuclear hormone receptor family of ligand-dependent transcription factors that regulates gene transcription in the presence of its ligand by binding cis-regulatory motifs, also known as estrogen response elements, lying upstream of target genes or via tethering to DNA by other transcription factors, including AP1 and SP1. DNA-bound ERα recruits the basal transcriptional machinery and induces the expression of genes implicated in numerous cancer signaling pathways, a process tightly regulated by complex dynamic interactions with coactivators and corepressors (2, 3). Although the ERα has been shown to be one of the most important mitogenic drivers in breast cancer in a multitude of pre-clinical and clinical studies, genomic events affecting the ESR1 gene are only observed in metastatic breast cancers (4, 5). Interestingly, mutations and chromosomal aberrations appear to occur at higher rates in coactivators (e.g. AIB1) and corepressors (e.g GATA3, NCOR1 and NCOR2) of the ERα, suggesting that their regulation of the receptor’s genomic actions may be intimately linked to the development of hormone-dependent tumors.

With pro-tumorigenic functions affecting proliferation, growth and survival, the ERα is the main oncogenic driver in breast cancer and represents an important target for the treatment of hormone-responsive tumors. Therapies targeting the ERα in breast cancer include anti-estrogens, such as tamoxifen and fulvestrant, and inhibitors of estrogen biosynthesis (aromatase inhibitors). Tamoxifen is a selective estrogen receptor modulator that competes with estrogen for the ligand-binding domain of the estrogen receptor and induces an alternative conformation that prevents coactivator binding to the receptor but favors recruitment and interaction with corepressors (6). Although tamoxifen has been successfully used to treat both early and late hormone receptor positive breast cancers, more than 50% of patients with advanced hormone-responsive tumors will progress or experience disease relapse due to intrinsic and acquired resistance to tamoxifen (7). While many mechanisms of resistance to tamoxifen have been characterized in vitro, none have shown clinical utility as biomarkers guiding treatment with tamoxifen in patients with ERα- and/or PgR-positive tumors.

Over the last decades, a number of previously uncharacterized ERα partners and co-factors were identified, including Split Ends (SPEN), a protein with essential regulatory roles in transcriptional repression (8-12). SPEN,
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which is also known as SMRT/HDAC1 Associated Repressor Protein (SHARP), is a large nuclear protein of 402 kDa characterized by four N-terminal RNA-recognition motifs and a highly conserved C-terminal SPOC (Spen Paralog and Ortholog C-terminal) domain. In various model organisms, SPEN has been shown to be critical during embryogenesis and throughout development in part due to its regulation of the Notch, TCF/LEF and EGFR signaling pathways (8, 9, 13). SPEN has also been identified as an estrogen-inducible cofactor able to integrate nuclear hormone receptor activation and repression (10). Despite some evidence for SPEN being implicated in endocrine regulation and development, SPEN functions have not been investigated in breast cancer.

Using an innovative unbiased integrative genomic approach, we identified mutations in the SPEN gene in a breast cancer cell line and four primary breast tumors (14). We also found that SPEN inhibits tumor growth and modulates the transcription of ERα-target genes, including PGR and BCL2. Moreover, we demonstrate that SPEN expression predicts response to tamoxifen in vitro and in clinical samples. Together, our findings show that SPEN is a novel tumor suppressor gene and a candidate predictive biomarker of tamoxifen response in ERα-positive breast cancers.
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**Material and methods**

**Cell lines.** MCF-7 and T47D cells were obtained from the American Type Culture Collection (ATCC) and were cultured in DMEM supplemented with 10% FBS (Wisent). BT20 and MDA-MB-436 were obtained from ATCC and cultured in EMEM and RPMI 1640 from ATCC supplemented with 10% FBS respectively.

**Cell viability assay.** Cells were plated at a density of 2,500 cells per well in 96-well plate for experiments performed in DMEM supplemented with 10% FBS and 5,000 cells per well in 96-well plate for assays carried under 1% FBS conditions. At 1, 2, 4 and 7 days, cell viability was assessed by replacing the medium with a 10% Alamar Blue solution (Invitrogen) prepared in DMEM. Cells were incubated with the solution at 37°C for 4 hours and fluorescence measured with a plate reader, FLUOstar Optima, using 560 nm (Excitation) and 590 nm (Emission) filter settings.

**Colony formation assay.** Cells were seeded at a density of 5,000 cells per well in 6-well plates in 1 mL of soft agar (0.3% soft agar in DMEM with 10% FBS), plated onto 2 mL of solidified agar (0.7% soft agar in DMEM with 10% FBS) and incubated at 37°C. 250 µL of medium was supplemented to each well twice weekly for 4 weeks. Colonies were scored electronically using an automated cell colony counter (GelCount, Oxford Optronix, Oxford, UK).

**Plasmids.** The full-length human SPEN cDNA cloned into the pDream2.1/MCS expression vector was purchased from GenScript. SPEN expressing vector and the empty control vector were transfected in T47D cells using Attractene (Qiagen) according to the manufacturer’s protocol. After 72 hours, cells were seeded in 6-well plates in DMEM with 10% FBS supplemented with neomycin (Life Technologies) at a concentration of 250 or 500 µg/mL. Clones were isolated and maintained in selection media throughout the culturing period.

**SPEN knockdown by RNA interference.** Two SPEN MISSIONshRNA plasmids with the following sequences: SPEN shRNA 1, 5’ CCGGCCTGTGGTAAAGGTGGTGTTTCTCGAGAAACACCACCTTTAC-CACAGGTTTTG 3’ (TRCN0000075165) and SPEN shRNA 2, 5’ CCGGCCTCCATCATCAATGACATCTCGAGAT-GTCATTGATGAT-GGAGCCGTTTTG 3’ (TRCN0000075166) were purchased from Sigma-Aldrich. pLKO.1-puro Non-Target control and SPEN-specific shRNA plasmids were transfected in MCF-7 cells using Attractene (Qiagen). After 72 hours, cells were seeded in 6-well plates in DMEM plus 10% FBS supplemented with puromycin (Life Technologies) at a concentration of 0.5, 1.0 or 2.0 µg/mL. Clones were isolated and maintained in selection media throughout the culturing period.
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**RNA isolation and qRT-PCR.** Total RNA was isolated from subconfluent cultures using the RNeasy Mini Kit (Qiagen Sciences) and reversed transcribed into cDNA with iScript cDNA Synthesis Kit (Bio-Rad). qRT-PCR were performed with TaqMan Gene Expression Assays (Applied Biosystems) and with the following probes: SPEN (Invitrogen, Hs00209232_m1), PGR (Invitrogen, Hs01556702_m1) and 18S (Invitrogen, Hs99999901_s1)

**DNA microarray expression profiling and analysis.** Expression profiling was conducted according to the manufacturer's instructions and following the One-Color Microarray-Based Gene Expression Analysis Protocol from Agilent Technologies. Briefly, integrity and concentration of the input RNA was evaluated with the Agilent 2011 Bioanalyzer. 100 nanograms of total RNA was reverse transcribed into cRNA, amplified and labeled with Cy3 dye. The resulting labeled cRNA was purified using RNeasy Mini Kit (Qiagen Sciences) according to the instructions of the manufacturer and hybridized to a Sure Print G3 Human GE 8X60K microarray (Agilent Technologies) for 17 hours at 65°C. The array was then washed and scanned on the Agilent DNA Microarray scanner at a resolution of 3 µM. Images were extracted and normalized with Feature Extraction software version 9.5. Expression values of three biological RNA replicates for each probe in the expression array were analyzed using Gene Spring GX (Agilent Technologies).

**Statistical analysis.** Microarray expression data was processed with GeneSpring GX software. Data were normalized to the 75th percentile of all values on the microarrays and to the median expression levels of all samples. The normalized gene expression data were filtered on flags and only those classified as detected were allowed to pass the filter and included in the analysis. The expression profiles of genes differentially expressed by more than 1.5-fold based on three biological replicates were compared using two tailed unpaired t-tests.

**Ingenuity Pathway Analysis.** Microarray data was analyzed using the Ingenuity Pathways Analysis software available at: [http://www.ingenuity.com](http://www.ingenuity.com). Genes up- or down-regulated by at least 1.50 fold (p-value < 0.05) in Spen 1, SPEN sh1 and SPEN sh3 compared to their respective controls were considered for further analyses. Functions (p-value < 0.05) predicted to be increased (z-score > 0.75) in SPEN sh1 and SPEN sh3 relative to the non-target shRNA transfected MCF-7 clone (NT2) and anticipated to be decreased (z-score < -0.75) in Spen 1 compared to its empty vector transfected T47D clone (CTL1) were analyzed. Functions consistently modulated were considered as regulated by SPEN. Similarly, an analysis of the upstream regulators predicted to be responsible for the observed gene expression changes was conducted. All molecule types were included in the analysis except for chemicals and
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miRNAs. Upstream regulators (p-value < 0.05) predicted to be activated (z-score > 0.75) in SPEN sh1 and SPEN sh3 relative to the non-target shRNA-transfected MCF-7 clone (NT2) and anticipated to be inhibited (z-score < -0.75) in Spen 1 compared to its empty vector T47D control (CTL1) were analyzed. Those common to all pairs of cell lines were considered as modulated by SPEN. The expression data of tumors from the TCGA database were also analyzed with IPA. For this analysis, a log-2 ratio of 2 was used to obtain a list of approximately 3000 differentially expressed genes between the tumor and normal-matched mammary sample. In each case, the predicted score of activation (z-score) was computed for the estrogen receptor (ER) and plotted against the log 2 ratio of SPEN RNA expression in tumors compared with normal-matched breast tissue samples. A log 2 ratio of -0.5 was used to stratify patients into high and low SPEN expressing groups.

**Fluorescence microscopy to quantify cells with chromatin condensation and membrane permeabilization.**

Cells were seeded at a density of 2.5 x 10^5 cells per well in 6-well plates and incubated at 37°C. 72 hours after plating, 20 µL of Hoechst 33342 (HO, 100 µg/mL, Sigma) was added to the culture medium and plates incubated at 37°C for 10 minutes. Cells were then stained with 2 mL of Propidium Iodide (PI, 5 µg/mL, Sigma) and analyzed by fluorescence microscopy on an Axiovert 40 CFL (Zeiss) microscope. The percentage of normal, apoptotic and necrotic cells were estimated in 6 random fields per condition. Apoptotic cells showed highly pyknotic nuclei stained with either HO or PI in the early and late phases of apoptosis, respectively.

**Survival assays.** For experiments with tamoxifen, cells were seeded at a density of 2,500 cells per well in 96-well plates in complete medium and incubated at 37°C. 24 hours after plating, the medium was replaced with 100 µL of hormone-depleted medium (DMEM without phenol red (Gibco) supplemented with 10% charcoal stripped fetal bovine serum (Gibco)). Another 24 hours later, tamoxifen or its vehicle was added to each well in 100 µL of hormone-depleted medium. Cell viability was measured 5 days later with a 10% Alamar Blue solution.

**Immunoprecipitation.** Cells were rinsed with ice cold PBS, harvested and lysed in lysis buffer (250 mM NaCl, 0.5% NP-40, 5 mM EDTA, 50 mM Tris) freshly supplemented with protease inhibitors (5 mM Sodium Fluoride, 1 mM Sodium Orthovanadate, 1 mM PMSF, 10 µg/mL Aprotinin and 10 µg/mL Leupeptin). After Micro-BCA quantification (Thermo Scientific), at least 250 µg of proteins were incubated overnight with 1 µg of anti-SPEN antibody (Sigma, HPA015825) or IgG control antibody (Abcam, ab46540-1). Protein A Dynabeads beads (Life Technologies) were added for 1.5 hours and washed three times with 400 µL of lysis buffer. Precipitated beads were then incubated at 95°C for 7
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minutes in 60 µL of Laemmli sample buffer followed by a centrifugation at 13,000 RPM for 1 minute. Proteins were ran in a pre-cast 4-20% gel (Bio-Rad) for 4 hours at 120V and transferred to a nitrocellulose membrane overnight at 33V. Membranes were blocked with 5% BSA in TBST and immunoblotted overnight with an anti-SPEN antibody (Sigma, HPA015825, 1:500).

**Western blotting.** Sub-confluent cells were collected by trypsinization, washed in ice-cold PBS and lysed in lysis buffer freshly supplemented with protease inhibitors. Lysates were then placed on a rocker machine for 30 minutes and centrifuged for 5 minutes at 4°C. Supernatants were subjected to Bradford quantification and 50 µg of proteins were loaded and ran by SDS-PAGE in a 8% gel for 1 hour. Proteins were transferred to nitrocellulose membranes and incubated with progesterone receptor (PgR, 1:1,000, Cell signaling) and alpha tubulin (1:10 000, Abcam) antibodies at 4°C overnight. Protein bands were detected using the Amersham ECL Western Blotting Detection reagent.

**Clonogenic assays.** Cells were seeded at a density of 2.5 X 10^4 cells per cm^2 and grown in DMEM supplemented with 10% FBS for 24 hours. Media was then replaced with 2 mL of hormone-depleted medium (DMEM without phenol red (Gibco) supplemented with 10% charcoal stripped FBS (Gibco)). Another 24 hours later, 5 µM or 10 µM of tamoxifen or its vehicle was added to the cells in hormone-depleted medium. Cells were stained with a fixing solution containing crystal violet 5 days after the addition of the drug.

**Stimulation with 17β-estradiol.** Cells were seeded at a density of 5,000 cells per well in 96-well plates in complete medium and incubated at 37°C. 24 hours after plating, the medium was replaced with 200 µL of hormone-depleted medium (DMEM without phenol red (Gibco) supplemented with 10% charcoal stripped FBS (Gibco)). Another 24 hours later, media was replaced with 1 nM 17β-estradiol in hormone-depleted DMEM plus 10% charcoal-stripped FBS for 24 hours. Cell viability was measured at day 0, 2, 5 and 7 with a 10% Alamar Blue solution and fold induction in proliferation by estradiol compared to 100% control was determined by dividing fluorescence values obtained in the presence of estrogen over that obtained with the vehicle.

**Patient cohort.** The Tamoxifen 50/50 tissue microarray cohort is a power series, derived from the Calgary Tamoxifen Breast Cancer Cohort (Cal-TBCC), which includes 50 patients alive 5 years post-diagnosis and 50 patients who died of breast cancer within 5 years of diagnosis. The Cal-TBCC is a retrospective database and contains demographic data, clinical data, and pathological data from a parent cohort of 819 breast cancer patients diagnosed between 1985 and 2000 at the Tom Baker Cancer Center in Calgary, Alberta, Canada. Inclusion criteria: confirmed diagnosis of
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invasive breast carcinoma and treatment with primary surgical intervention with or without post-operative local radiation therapy, followed by adjuvant tamoxifen endocrine therapy (20 mg p.o./day) for 5 years, regardless of ER or PgR status. HER2 status was not systematically performed at the time of diagnosis for the patients in this cohort. Exclusion criteria: if diagnostic biopsy or primary surgical tissue specimens were unavailable, if patients received prior or adjuvant chemotherapy. 534 cases met the criteria with a median follow-up time of 82.1 months. The clinical and initial pathology data were retrieved, data for progression free survival at 5 years were retrieved, and formalin-fixed paraffin-embedded (FFPE) tissue blocks were acquired and replicate 0.6 mm cores were built into tissue microarrays (TMAs). All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin according to standard procedures for the time period. Ethical approval for the use of human tissue samples was obtained from the Conjoint Health Research Ethics Board.

**Breast tumors.** One hundred consecutive breast tumors were collected as part of a government-funded (FRSQ) tumor bank at the Centre Hospitalier de l’Université de Montréal from 2000 to 2003. Patients had signed informed consent for breast tumor banking. Tissues sections for each tumor showed >70% tumor cells as determined by an H&E staining for each sample. DNA was extracted using the Qiagen DNAmp extraction kit according to the manufacturer’s instructions.

**aCGH of breast tumors.** DNA quality was assessed using a 2100 bioanalyzer using a DNA 12000 Lab ChIP kit (Agilent Technologies). aCGH was performed as previously reported (15).

**Loss of heterozygosity assessment using polymorphic microsatellite repeat markers.** Loss of heterozygosity (LOH) analysis for the region surrounding 1p36.1 was performed using the AFM217zc3a polymorphic marker (Table S4). PCR was performed in 12.5 ul volume containing 100 ng of genomic DNA, 1mCi of 35S[dATP], 1x PCR buffer, 200nm each of dCTP, dGTP and dTTP, 50 pmol of each primer, and 0.5 unit of Taq polymerase. The PCR conditions were as follow: 94°C for 5 min (1cycle), 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds (29 cycles) followed by an incubation at 72°C for 10 minutes (1cycle). Products were electrophoresed on denaturing gels and autoradiographed at room temperature for five days. LOH was scored based on the difference in the relative intensity of signals representing 2 alleles in tumor DNA samples. All samples positive for LOH or allelic imbalance at individual loci were analyzed twice in independent assays and Sanger sequenced.
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**Mutation analysis of SPEN.** Mutation analyses were performed as previously described (Mamo et al., 2012). Briefly, the genomic sequence of the protein-encoding region of SPEN was obtained from the UCSC Santa Cruz Genome Bioinformatics Site available at: http://genome.ucsc.edu. Primer pairs for PCR amplification and sequencing of SPEN were generated using the Primer3 software available at: http://bioinfo.ut.ee/primer3-0.4.0/ (Table S5). PCR products derived from breast tumors were purified as per manufacturer’s recommendations using QIAquick PCR purification kit (Qiagen Sciences) and sequenced at the McGill University and Genome Quebec Innovation Center and the TGEN DNA sequencing facility. Sequence chromatograms were aligned and analyzed with the Staden Package (http://staden.sourceforge.net/) and the Mutation surveyor software version 3.24 by using the full length SPEN reference sequence NM_015001.

**Fluorescence activated cell sorting (FACS).** For cell cycle analyses, cells were detached with trypsin, washed in PBS supplemented with 5 mM EDTA, suspended in a fixing solution (1 mL of PBS, 5 mM EDTA for 3 mL of 100% ethanol) and incubated at -20°C for at least 24 hours. Then, cells were washed with PBS/EDTA and resuspended in 2 mL of staining solution (PBS, Propidium Iodide (PI, 50 µg/mL), RNAse A (20 µg/mL)). For Annexin V/PI staining experiments, cells were detached with trypsin and washed once with PBS. Then, they were resuspended in 200 µL of Annexin V binding solution (1X) and stained with 4 µL of PI and 4 µL of Annexin V. For both assays, cell fluorescence signals were determined immediately after staining using a FACSscalibur flow cytometer (Becton Dickinson). The analysis was performed using the BD CellQuest (Becton Dickinson), ModFit (Becton Dickinson) and FlowJo softwares.

**Intersection probabilities.** To determine the statistical significance of intersection between two lists of genes, we assessed the probability of this intersection to occur by performing 10,000 independent simulations with randomly selected lists of genes of the same size. P-values were calculated using an hypergeometric test.

**Chromatin immunoprecipitation.** Cells were plated (1.2 x 10^6 cells in 150 mm culture dishes) and grown for 72h in DMEM containing 10% FBS. Cells were fixed at room temperature for 30 min. in 2 mM EGS (16) followed with 10 min. in 1% formaldehyde. Glycine was added (125 mM final concentration) and left at room temperature for 5 min. to quench formaldehyde. Cells were rinsed twice and scraped in ice cold PBS. Collected cells were then centrifuged at 1400 RPM for 5 min. and lysed into lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS) freshly supplemented with protease inhibitors. Samples were incubated on ice for 10 min. and sonicated to obtain fragments of 300-1000 bp in size (setting: 25%, 10 times 15 sec.).
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After centrifugation (15 min. 13 000 RPM, 4°C), the sheared chromatin was diluted in dilution buffer (1% Triton X-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH 8, 150 mM) freshly supplemented with protease inhibitors. Samples were then incubated with 1.5 μg of antibody (SPEN: HPA015825, Sigma ERα: sc-543, Santa Cruz Biotechnology, IgG: ab46540-1, Abcam) at 4°C with rotation overnight in a final volume of 1 ml. DNA-protein interactions were washed 3 times for 10 min. with wash buffer (0.1%, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 150 mM NaCl) and once for 10 min. with final wash buffer (0.1%, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 500 mM NaCl). Chromatin was eluted with 350 μL of elution buffer (1% SDS, 100 mM NaHCO₃) by rotation for 15 min. and reverse cross-linked with 4 μL of proteinase K solution at 55°C for 90 min. followed by an overnight incubation at 65°C. DNA was purified using DNeasy Mini Spin columns (Qiagen) and following the manufacturer’s instructions. PCR amplification of a DNA fragment, 311 kb upstream of the PGR transcription start site was performed using the following primers: FW 5’-CCA CTT TGC CAC ATG ACA TC REV-3’ REV 5’-AAC TCC CAA GGG ACC ATT TC-3’.
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Results

SPEN is mutated and under-expressed in breast tumors.

Using an integrative genomic approach, we previously identified a number of genes containing nonsense mutations and mapping to regions exhibiting loss of heterozygosity (LOH) in breast cancer cell lines (14, 17). Briefly, this approach involved the profiling of cell lines treated with emetine, an inhibitor of the nonsense-mediated RNA decay (NMD) pathway (17). Genes whose transcript levels were increased by emetine treatment potentially contained a nonsense mutation and were prioritized based on those that aligned to regions of LOH or deletion as assayed by genotyping with single nucleotide polymorphisms (SNP) arrays. Using this strategy, we identified and further established the AT-rich interactive domain 1a (ARID1A) as a candidate tumor suppressor gene in breast cancer, a finding that has since been confirmed by the discovery of ARID1A mutations in breast tumors by several groups (16, 18). Similarly, we identified an insertion/truncation mutation at nucleotide 1184 in the Split Ends (SPEN) gene and LOH at the SPEN locus (Chromosome 1p36) resulting in undetectable SPEN protein levels in the ERα-positive, T47D breast cancer cell line (Supplementary Fig. S1A-C).

To assess the prevalence of somatically acquired genomic aberrations and mutations affecting SPEN in breast cancer, we performed array comparative genomic hybridization (aCGH) or microsatellite polymerase chain reactions (PCR) at the AFM217zc3a polymorphic marker on a cohort of 101 primary breast tumors (Figure 1A and Table S1). We found that 22.8% (23/101) of tumors had LOH or a copy number loss at the SPEN locus. Sequencing of the protein-encoding exons and splice-site junctions of SPEN revealed that 17.4% (4/23) of tumors with LOH harbored a somatic mutation within the gene. Two missense mutations (P2158A, A3327T) were detected in 2 different samples, whereas the same nonsense mutation (Q3141X) was found in two other tumors (Figure 1B-1C).

To corroborate the prevalence of genomic and genetic alterations affecting SPEN, we then surveyed data from The Cancer Genome Atlas (TCGA) and the catalogue of somatic mutations in cancer (COSMIC). We found that SPEN RNA expression is down-regulated in invasive breast carcinoma compared to normal breast tissue (P=1.43E-07) and that SPEN is homo- or heterozygously deleted in 27.2% of breast cancer samples (Figure S2A and S2B) (19-22), similar to the prevalence of LOH in our own tumor set. Data analysis also revealed that tumors harboring nonsense mutations in SPEN express low to very low SPEN mRNA levels, even in the absence of copy number loss and that breast cancer samples with LOH at the 1p36 locus express significantly lower SPEN transcript levels.
SPEN is a tumor suppressor in ERα positive breast cancers compared to tumors without copy number alterations (Figure S2B) (20, 22). In both databases, somatic mutations in *SPEN* were reported in 2-3% of breast cancers as well as in many other cancer types, with a prevalence reaching 14.3% and 11% in cervical and endometrial cancers, respectively (Figure S2C) (23).

**SPEN acts as a tumor suppressor gene in ER-positive breast cancer cells**

Given the suggested role of SPEN in endocrine regulation and our identification of an insertion-truncation mutation in *SPEN* in the ERα-positive T47D breast cancer cell line, we attempted to investigate the functions of SPEN in the context of ERα-positive breast cancers. We first transfected a SPEN expressing vector in T47D cells and isolated two stable clones (T47D-Spen 1-2) (Figure 2A and 2B), with varying degrees of restored SPEN expression. T47D-SPEN cells displayed a marked reduction of proliferation in both normal and low serum conditions (Figure 2C and Figure S3A) and exhibited increased sensitivity to apoptosis under serum starved growth conditions (Figure S3B). Re-introduction of SPEN into T47D cells did not lead to their accumulation in G1 or G2 either in 10% or 1% fetal bovine serum conditions, suggesting that the observed effects are not due to cell cycle arrest (Figure S3C and S3D). A soft agar assay revealed that restoration of SPEN levels in T47D cells abrogates anchorage-independent growth (Figure 2D). To further establish SPEN as a tumor suppressor gene in breast cancer, in vivo xenografts studies were performed with BALB-c nude mice implanted with 60 days slow release 17β-estradiol pellets and injected with T47D control cells in one mammary fat pad and T47D-SPEN cells injected contralaterally. Of a total of 8 mice that developed palpable tumors, 7 developed tumors from control cells without tumors arising from contralateral SPEN-overexpressing clones (Figure 2E and Figure S3E and S3F). Notably, only one tumor derived from T47D-SPEN cells was collected after completion of the study and its volume was much smaller than control tumors. Together, these *in vitro* and *in vivo* results support the tumor suppressive functions of SPEN in breast cancer.

Then, to further characterize SPEN functions in ERα-positive breast cancer cells, we silenced its expression in another hormone-dependent breast cancer cell line, MCF-7, which express high endogenous levels of the protein (Figure S1B and S1C). Using two different short-hairpin RNAs targeting SPEN, four MCF-7-shRNA-SPEN clones showing decreased SPEN expression were generated (Figure 3A and 3B and Figure S3G-S3I). shRNA-mediated knockdown of SPEN slightly increased proliferation (Figure S3J) but markedly increased colony formation in soft agar assays (Figure 3C and Figure S3K) and reduced the rate at which MCF-7 cells undergo apoptosis under low serum conditions (Figure S3L). As in T47D-SPEN clones, no significant difference in cell cycle distribution was observed.
SPEN is a tumor suppressor in ERα positive breast cancers between MCF-7-shRNA-SPEN clones and their control (data not shown). The results of the functional assays performed with MCF-7 cells are complementary to those obtained with T47D cells and demonstrate that SPEN regulates proliferation, tumor growth and survival in ERα-positive breast cancer cells. Interestingly, using a cohort of 1784 breast cancer patients with luminal A tumors, we assessed the clinical significance of SPEN expression and found that high SPEN RNA levels were significantly associated with good survival (HR=0.78, P=0.005) over 20 years (Figure 3D) (24).

Then, to evaluate whether the tumor suppressive function of SPEN extends to estrogen receptor negative breast cancer cells, we silenced its expression using siRNAs in BT20 and MDA-MB-436, two triple negative breast cancer cell lines expressing high RNA and protein levels of SPEN (Figure S4A and S4B), and measured SPEN effects on proliferation. Contrary to our expectations, we found that SPEN knockdown limited the proliferation of both cell lines in proliferation assays, suggesting that SPEN may have pro-proliferative functions in ERα-negative breast cancer cells (Figure S4C-S4F). Consistent with these findings is the observation that SPEN RNA expression levels are predictive of poor prognosis (HR=1.49, P=0.016) in a cohort of 581 patients with basal breast tumors (Figure S4G) (24). Although further experiments are required to define the roles of SPEN in basal breast cancers, our results suggest that SPEN has opposing functions in ERα-positive and ERα-negative cancer cells while its expression may serve as a useful marker for patient prognostication and stratification in both subtypes.

**SPEN regulates the expression of genes related to cell death.**

To delineate the transcriptional program regulated by SPEN in ERα-positive breast cancers, gene expression profiling using DNA microarrays was conducted on RNA from one of the two clones generated with the SPEN expression vector (Spen 1, the highest expressor), as well as one of the two clones generated with each of the shRNA hairpin vectors, SPEN shRNA1 (SPEN sh1) and SPEN shRNA2 (SPEN sh3) along with their respective controls. Microarray results confirmed the knockdown or overexpression of SPEN in each stably transfected clone (Table S2). Ingenuity Pathway analyses (IPA) performed with genes significantly (P<0.05) altered (≥1.5-fold) in each clone compared with its control revealed that the largest proportion of genes (32-37%) in our dataset are associated with “cell death and survival” (Figure S5A). Using the DeathBase database, we found that 31% of genes reported to be involved in cell death (27/86) are differentially expressed in SPEN clones compared to their controls (Figure S5B-S5D) (25). Notably, most of these differentially expressed genes implicated in cell death and survival (e.g. BCL2, BMF and
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 Bik) are localized at the mitochondrial membrane and participate in apoptosis induced by intracellular signals. We also found that the expression of BCL2, an estrogen-target gene that has strong anti-apoptotic functions, is significantly down-regulated in both SPEN-overexpressing T47D clones (Figure S5F), a finding that may explain, at least in part, the increased susceptibility of T47D-Spen 1 and T47D-Spen 2 to apoptosis. Then, to better define the tumor suppressive activity of SPEN in breast cancer, we focused on functions predicted to be increased in MCF-7-SPEN-sh1 and MCF-7-SPEN-sh3 silenced cells and decreased in T47D-Spen 1. With these criteria, only the biological function of “cell survival” was identified as consistently repressed by SPEN expression (Figure S5E). The data obtained from transcriptome analyses thus point to a role for SPEN in the regulation of cell viability and these results are consistent with our findings of cells grown under low serum and anchorage-independent conditions.

Transcriptional regulation of the ERα targets by SPEN

Further analyses with IPA revealed that most upstream regulators affected by the modulation of SPEN expression are involved in the regulation of transcription (Figure S6A), providing additional evidence that SPEN participates in the organization of transcriptional programs. In addition, we found that the estrogen receptor is the only upstream regulator whose predicted activation is inversely correlated with SPEN expression (Figure 4A). To strengthen this relationship between SPEN and the estrogen receptor, we then cross-referenced our microarray data with two publicly available lists of 7095 and 5342 genes bound by ERα and ERβ respectively, within 25 kb of their transcription start site as assayed by ChIP-seq experiments in MCF-7 cells treated with 17β-estradiol (26, 27). In both cases, almost half the number of genes significantly up-regulated in MCF-7-shRNA-SPEN cells compared to their control were included in these lists (P=0.00003) (Figure S6B-S6E) (28). Interestingly, no such enrichment was observed with the lists of down-regulated genes in MCF-7-shRNA-SPEN cells. Then, because ERα and ERβ often dimerize with one another, we next examined whether the observed enrichments were specific for genes bound by both receptors and/or uniquely bound by ERα or ERβ. Interestingly, we found a significant overlap between our microarray data and genes bound by both receptors or reported to be uniquely bound by ERα upon stimulation with estrogen. No significant overlap with genes solely bound by ERβ was observed, suggesting that our data is mainly enriched for ERα but not ERβ target genes. In line with these results, we also observed that restoration of SPEN levels significantly dampens estradiol-induced proliferation in T47D cells (Figure 4B) (P=0.002). Next, to evaluate whether SPEN mediates its effects on the ERα through an interaction with the receptor complex, co-
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immunoprecipitation studies were conducted under conditions of hormone depletion and stimulation with 17β-estradiol. Our data showed that SPEN interacts with both liganded and unliganded ERα (Figure 4C). However, a larger fraction of ERα co-immunoprecipitated with SPEN under hormone-depleted conditions, a finding that is consistent with corepressors interacting more strongly with unbound nuclear receptors. Interestingly, no interaction between SPEN and ERβ was detected under these conditions, suggesting that SPEN preferentially interacts with ERα over ERβ, consistent with our aforementioned transcriptomic analysis. Then, to further support a role for SPEN in the repression of ERα-dependent transcription, the expression of the progesterone receptor (PgR), a primary ERα target gene, was measured in our SPEN transfectants (29). We observed an inverse relationship between SPEN and PgR expression, at both mRNA and protein levels (Figure 4D-4G and Table S2). In addition, chromatin immunoprecipitation experiments performed with MCF-7 and T47D cells revealed that SPEN modestly but consistently interacts with DNA upstream of the PgR gene at a site also bound by the ERα in MCF-7 cells but not in T47D cells (Figure 4H; Figure S6G). Interestingly, SPEN interaction with DNA at this genomic site was restored by its re-expression in T47D cells (Figure S6H). Together, these results provide evidence for SPEN as a negative regulator of the in ERα-dependent transcriptional program in ERα-positive breast cancer cells.

**SPEN genomic content and expression levels predict estrogen receptor activation**

To begin to evaluate the clinical significance of our findings that suggest a tumor suppressive role for SPEN in ERα-positive breast cancers due to its regulation of ERα-dependent transcription, the expression profiles of 60 luminal A breast tumors and their normal-matched counterpart were extracted from the TCGA database and subjected to pathway analysis using IPA. A pairwise comparison of SPEN RNA expression in this subset of 60 tumors showed that low SPEN expression predicts higher estrogen receptor activation (Figure 4I). As anticipated, no such correlation was observed in a subset of 40 basal breast tumors (data not shown). Taken together, our data suggest that SPEN represses estrogen receptor-driven transcription in ERα positive but not hormone receptor negative breast tumors and that ERα activity is strongly enhanced in breast cancer cells expressing low SPEN mRNA levels.

**SPEN sensitizes ERα-positive breast cancer cells to tamoxifen**

Having shown that SPEN represses the transcription of genes downstream of the ERα, we hypothesized that SPEN may affect cellular responses to the anti-estrogen, tamoxifen. Using pharmacologically relevant tamoxifen
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centrations, we found that SPEN silencing confers resistance to the drug in cell viability and clonogenic assays while its overexpression substantially increases sensitivity to tamoxifen (Figure 5A and 5B; Table S3; Figure S6A-S6E). Interestingly, induction of apoptosis following tamoxifen treatment was considerably reduced in MCF-7-shRNA clones and 2 to 3-fold higher in T47D-SPEN cells than in their respective controls (Figures 5C and 5D; Figure S6F-S6H), indicating that SPEN sensitizes cells to tamoxifen-induced apoptosis. Notably, modulation of SPEN expression did not affect cells sensitivity to ICI 182780 (fulvestrant) (Figure S8A-S8D), a pure ERα antagonist that induces ERα degradation, suggesting that SPEN’s interaction with an intact ERα may be critical to predispose cells to apoptosis in response to tamoxifen treatment.

The clinical significance of SPEN expression levels on tamoxifen response was determined by assessing its nuclear staining in tissue microarrays containing triplicate core biopsies from 100 early stage breast cancer patients treated exclusively with adjuvant tamoxifen. This cohort was comprised of 50 patients who had disease recurrence and died from breast cancer and 50 patients without recurrence or death from the disease. Tissues were scored according to SPEN nuclear staining intensity and percentage of SPEN-positive tumor cells. Kaplan-Meier survival analyses of the 65 hormone-responsive (ERα+PgR+HER2-) breast tumors revealed that high SPEN protein expression was significantly predictive of favorable clinical outcome (P=0.029) (Figure 6E). Moreover, high SPEN RNA expression was strongly correlated with good relapse-free survival (H.R.=0.55, P=0.0055) in an independent cohort of 424 luminal A ER-positive patients treated with tamoxifen alone (Figure 6F) (24, 30). This prognostic effect was not observed in patients with ERα-negative tumors (Figure S7I), suggesting that SPEN expression levels may serve as a predictive biomarker of tamoxifen response in hormone-sensitive breast cancers.
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Discussion

One of the hallmarks of cancer is the accumulation of genetic mutations in tumor cell DNA, resulting in the activation of oncogenes and the loss of tumor suppressor genes (31). Although their expression is usually lost in many tumors compared with normal tissues due to genetic and epigenetic events, the limited expression of tumor suppressor genes may nevertheless be of clinical relevance. Our identification of SPEN as a tumor suppressor gene and candidate predictive marker of tamoxifen response, due to its repressive activity of ERα-dependent transcription in breast cancer, demonstrates that the study of tumor suppressor genes might uncover novel mechanisms of drug resistance and biomarkers of drug response.

Using a genomic approach, we discovered LOH at the SPEN locus in 23% of breast cancers. Sequencing of SPEN in tumors with LOH at the 1p36 locus identified 4 nonsynonymous mutations, including a nonsense mutation recurrent in two independent tumors, adding to the 29 mutations in breast tumors reported in the COSMIC database (23). This finding, in addition to our observation that samples from TCGA with nonsense mutations in SPEN express low SPEN mRNA levels, suggest that SPEN gene expression might be regulated by the nonsense-mediated RNA decay pathway in breast cancer cells, a mechanism that we have shown to be responsible for undetectable SPEN protein levels in T47D cells.

To establish SPEN as a tumor suppressor gene and define its functions in breast cancer, we used an in vitro model with MCF-7 and T47D cells, two ERα-positive breast cancer cell lines expressing high and very low SPEN protein levels, respectively. The forced expression of SPEN in T47D cells and its knockdown in MCF-7 cells unmasked an inhibitory effect on cell proliferation, growth and survival. In addition, gene expression analyses of MCF-7 and T47D clones identified “cell survival” as the major biological function affected by the modulation of SPEN expression. The enrichment of our SPEN-regulated gene expression profiles for pro- and anti-apoptotic genes provides a molecular mechanism for the altered response of SPEN-silenced MCF-7 and SPEN-overexpressing T47D cells to apoptotic stimuli, such as suspension in soft agar and growth under serum-deprived conditions.

Further analysis of our microarray data revealed that SPEN regulates the expression of a number of genes downstream of the estrogen receptor, confirming its role as a repressor of the ERα-dependent signalling pathway in breast cancer cells. These results extend those of Shi et al., who demonstrated that SPEN inhibits estradiol-induced
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ERα-dependent transcription in a luciferase assay (10). Although not derived from experiments conducted with breast cancer cells, their results were highly suggestive of SPEN having repressive effects on ERα transcription. In a yeast-two hybrid assay, they also showed that SPEN interacts with other ERα co-factor, including SRA, an RNA molecule with co-activating functions, and SMRT and NCoR, two ERα corepressors. Our observation that SPEN represses the expression of ERα target genes, including the transcription of the PGR, and that it co-immunoprecipitates with the ERα complex in the presence of estradiol, demonstrates that endogenous SPEN can bind and repress ligand-bound ERα. While most corepressors bind unliganded receptors, we have demonstrated that SPEN acts on both estradiol-bound and unbound receptors, a property unique to few ERα co-regulators. This function may be particularly important in limiting the genomic effects of 17β-estradiol and preventing uncontrolled cell proliferation and survival between successive hormonal cycles in the normal breast. Indeed, our finding that SPEN knockdown potentiates the proliferation of the normal-like epithelial cell line, MCF10A, exposes a possible role for SPEN in preventing cellular transformation. Genetic aberrations affecting SPEN in normal and pre-malignant breast epithelial cells could therefore potentiate ERα transcriptional activity, especially during the follicular and estrogen-driven phase of the menstrual cycle, and lead to the expression of estrogen-responsive genes, including the progesterone receptor as well as anti-apoptosis-related genes, all of which may contribute to cell survival and cancer development.

Given our results showing that SPEN expression establishes a proclivity to apoptosis in ERα-positive breast cancer cells, and that it represses the transcriptional activity of the ERα, we evaluated the sensitivity of SPEN-silenced MCF-7 and SPEN-overexpressing T47D cells to the anti-estrogen, tamoxifen, a drug that antagonizes the ERα. Whereas several mechanisms of resistance to tamoxifen are known, such as ERα phosphorylation at S167, HER2 overexpression and hyperactivation of the PI3K pathway, no predictive biomarker for tamoxifen response is in current clinical use other than the ERα and the PgR (21, 32-35). Using an in vitro breast cancer model with MCF-7 and T47D cells, we showed that SPEN affects tamoxifen but not fulvestrant sensitivity in ERα-positive breast cancer cells. SPEN protein and RNA expression in two patient cohorts that had received tamoxifen therapy alone in the adjuvant setting support our findings, with significantly better outcomes for patients with breast tumors showing high SPEN expression than for those with low/moderate SPEN expression. Although further clinical validation is needed, our findings suggest that a subpopulation of ERα-positive early stage breast cancer patients may not benefit from adjuvant tamoxifen. In addition, our observation that SPEN expression does not affect cells sensitivity to ICI182780, a drug that induces ERα
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degradation, suggests that SPEN interaction with the ERα may be critical to establish a proclivity for cell death in response to tamoxifen treatment. It will thus be important in future studies to also evaluate the effect of SPEN expression on sensitivity to other endocrine therapies, such as aromatase inhibitors, which do not alter intracellular ERα levels. In addition to having potential clinical applications, such studies may provide new insights into SPEN functions in the context of anti-estrogen drug response. Taken together, our data indicates that breast cancers expressing SPEN may be more sensitive to tamoxifen-induced apoptosis and that SPEN expression could serve as a marker of tamoxifen response in ERα-positive breast cancers.

It is noteworthy that modulation of SPEN expression consistently affected serum deprivation and tamoxifen-induced apoptosis in both T47D and MCF-7 breast cancer cell lines, given that they have different defects in apoptotic mechanisms: T47D cells contain a p53 mutation and MCF-7 cells do not express the apoptotic mediator caspase 3 (36, 37). With genomic and non-genomic actions affecting proliferation, migration and apoptosis, the ERα has a central role in the biology of breast cancer. Indeed, prior studies have demonstrated that the ERα can protect breast cancer cells from program-induced cell death, in part by modulating the expression of apoptosis-related genes, such as BCL2, BIK and BMF (16, 38, 39). Whereas very few of the well-established ERα-target genes (i.e. GREB1, TFF1, CCND1, etc.) were differentially expressed in our microarray data besides PGR, the modulation of SPEN expression in MCF-7 and T47D cells affected the expression of a number of apoptosis-related genes, including BCL2, BIK and BMF. Our finding that approximately 35% of genes involved in “cell death and survival” and regulated by SPEN in each of our SPEN clones are also downstream of the estrogen receptor (P<0.0001) (Figure S6F) suggests that the anti-apoptotic transcriptional program regulated by the ERα is likely controlled by SPEN (28).

Generally, our findings suggest that the inactivation of SPEN by deletion and/or intragenic mutation may contribute to breast tumor formation and progression. As SPEN mutations were reported in other cancer types, we can speculate that SPEN tumor suppressive functions extend to many other tissues. Future studies should therefore seek to evaluate the functions of SPEN in other cancers, such as cervical and endometrial cancer, in which the occurrence of SPEN mutations is very high (14% and 11%, respectively). In conclusion, our results establish SPEN as a regulator of ERα-dependent transcription of apoptosis-related genes in breast cancer and provide functional and clinical evidence for SPEN as a tumor suppressor gene and a candidate predictive biomarker of tamoxifen response in ERα-positive breast cancers.
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Author Contribution

L.C. and A.M. conducted preliminary experiments that led to the identification of SPEN as a candidate tumor suppressor gene in breast cancer, with the assistance of M.B. and P.T. Clinical sample processing was provided by M.B. The mutation screen was performed by L.C. S.L. generated the in vitro model, designed and performed all in vitro experiments, with guidance from C.C., I.S., S.M. and M.B. In vivo experiments were performed by S.L and I.S. Gene expression profiling and all microarray analyses were performed by S.L Tissue microarrays were stained by D.K., scored by O.A. and M.B. and data analyzed by S.H., A.M. and A.K. Statistical analyses were carried by D.L. M.B. directed the study and wrote the manuscript with S.L. All co-authors reviewed and edited drafts of the manuscript.

Funding

This work was supported by grants from the Cancer Research Society and the FRQS Réseau de Cancer Axe Cancer du Sein et Ovaire as well as a grant from the Quebec Breast Cancer Foundation to M.B., P.T. and S.M S.L. was supported by a McGill Integrated Cancer Research Training Program studentship (FRN53888) and a doctoral award from the Fonds de recherche du Québec - Santé. I.S. is the recipient of the Eileen Iwanicki postdoctoral fellowship in Breast Cancer Research from the Canadian Institutes of Health Research in partnership with the Breast Cancer Society of Canada.

Acknowledgement

We thank past and current members of the M.B. laboratory, especially Banujan Balachandran and Elaheh Ahmadzadeh for technical assistance, as well as Isabelle Royal, Vincent Giguère and Volker Blank for assistance and discussion. We also acknowledge the help of Kathy Ann Forner and Christian Young from the animal care and flow cytometry facility, respectively, of the Lady Davis Institute for medical research.
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References

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Figure Legends

**Figure 1. SPEN genomic alterations and mutations in breast cancer.** (A) Representative image depicting copy number anomalies of chromosome 1 in a breast tumor sample measured using the 244K aCGH microarray. Individual probe values are represented as normalized log2 ratios. Red dots represent probes with increased copy number values (log2 ratio > 1) and green dots represent probes with decreased copy number values (log2 ratio < 1). DNA copy number losses are apparent at the telomeric end of the 1p arm, including the genomic locus containing SPEN. (B) Sequencing chromatograms of SPEN at the site of mutation in the four primary tumour samples (bottom panel) and their corresponding normal tissues (top panel). Arrows point at mutation sites in tumor samples. (C) Schematic diagram of SPEN protein domains (RRM – RNA Recognition Motif, NLS — Nuclear Localization Sequence, RID — Receptor Interaction Domain, SPOC — SPEN paralog and ortholog C-terminal domain), and locations of somatically acquired mutations resulting in the amino acid alterations, P2158A, Q3141X and A3327T found in the sequence analysis of breast tumors.

**Figure 2. SPEN regulates cell growth and survival in T47D cells.** (A) Representative blot showing immunoprecipitated SPEN protein levels in SPEN-overexpressing clones (Spen 1 and 2) and the control clone (CTL1). Immunoprecipitation with nonspecific rabbit IgG in Spen 1 cells was done as a negative control. (B) qRT-PCR of SPEN expression in SPEN-overexpressing T47D cells (Spen 1- 2) relative to control clones (CTL1-2). Represented is the mean (± s.e.m.) expression value of SPEN in four biological replicates, normalized to the control clone (CTL1) (p-value ***<0.005, **<0.01, *<0.05). (C) Growth curves of SPEN-overexpressing T47D clones (Spen 1-2) and control clones (CTL1-2) grown in DMEM plus 1% fetal bovine serum. Data points represent mean fluorescence values (± s.e.m.) of four experiments performed in quadruplicates. (D) Representative images of soft agar colony assays performed with T47D Spen 1 and Spen 2 clones and the control clones. Bar graphs represent the mean (± s.e.m.) number of colonies formed per well in four experiments performed in triplicates. (E) Tumor volume in fat pads of nude mice injected bilaterally with CTL1 and SPEN-overexpressing T47D clones. Data points represent the mean tumor volume (± s.e.m) of 7 CTL1 and 1 Spen 1 primary tumors.

**Figure 3. SPEN regulates cell growth and survival in MCF-7 cells.** (A) Representative blot showing immunoprecipitated SPEN protein levels in MCF-7 (SPEN sh1-sh4) clones and their control (NT refers to Non-Target shRNA-transfected control). Immunoprecipitation of SPEN in T47D cells was done as a negative control. (B)
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qRT-PCR of SPEN expression in SPEN-silenced MCF-7 clones (SPEN sh1-sh4) relative to their control. Represented is the mean (± s.e.m.) expression value of SPEN in four biological replicates, normalized to the control (NT2). (C) Representative images of soft agar colony assays performed with MCF-7 clones and their control (NT1). Bar graphs represent the mean (± s.e.m.) number of colonies formed per well in five experiments performed in triplicates. (p-value ***<0.005, **<0.01, *<0.05) (D) Kaplan-Meier graphs depicting progression free survival of 1784 patients with luminal A breast tumors, stratified according to high and moderate/weak (black) expression of SPEN.

**Figure 4. SPEN represses ERα transcriptional activity.** (A) Ingenuity pathway analysis showing that the Estrogen Receptor is the only upstream regulator whose activation is inversely correlated with SPEN expression in the SPEN-overexpressing clone, Spen 1, and SPEN-silenced clones, SPEN sh1 and SPEN sh3. (B) Alamar blue growth assay with the parental T47D cells, SPEN-overexpressing clones (Spen 1-2) or the control clone (CTL1) treated with vehicle (ethanol) or 1 nM 17β-estradiol for 7 days. Cell proliferation is expressed as fold induction in proliferation (± s.e.m.) relative to vehicle-treated cells in three experiments performed in quadruplicates. (C) Co-immunoprecipitation of ERα with SPEN in MCF-7 cells grown in DMEM plus 10% fetal bovine serum or phenol-and hormone-free DMEM plus 10% charcoal-stripped fetal bovine serum (HD), supplemented or not with 1 nM 17β-estradiol (E2) or 10 µM tamoxifen (Tam) for 24 hours. Immunoprecipitation with nonspecific rabbit IgG was done as a negative control. (D and E) Western blots showing baseline expression of PgR in Spen 1-2 and SPEN sh1-sh4 clones compared to their respective controls (NT refers to Non-Target shRNA-transfected clone). (F and G) qRT-PCR of PGR gene expression in T47D (Spen 1-2) and MCF-7 (SPEN sh1-sh4) clones compared with their respective controls. Represented is the mean (± s.e.m.) expression value of PGR in four (F) and three (G) biological replicates, normalized to the control (p-value ***<0.005, **<0.01, *<0.05). (H) Chromatin immunoprecipitation studies of SPEN and ERα binding to DNA, 311 kB upstream of the PGR transcription start site, in MCF-7 and T47D cells, indicating that SPEN interacts with the PGR promoter in MCF-7 but not in T47D cells. Bar graphs represent the fold signal enrichment (± s.e.m.) of SPEN and ERα relative to IgG, in three independent experiments. (I) Box plot depicting the relationship between SPEN expression in 60 hormone-sensitive tumors and Estrogen Receptor activation score, as predicted by IPA analysis of each patient samples transcriptional profile.
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**Figure 5. SPEN regulates ERα-positive breast cancer cells response to tamoxifen.** (A and B) Alamar blue survival assay performed with T47D (Spen 1-2) and MCF-7 (SPEN sh1-sh4) clones treated with tamoxifen (T47D: 8 µM, MCF-7: 6 µM) or its vehicle for 5 days. Bar graphs represent the mean % survival (± s.e.m.) of tamoxifen-treated relative to vehicle-treated cells in three (T47D) and at least four (MCF-7) experiments performed in quadruplicates. (C and D) Annexin-V/PI flow cytometric analyses performed with T47D (Spen 1-2) and MCF-7 (SPEN sh1-sh4) clones treated with tamoxifen or its vehicle for 5 days. Bar graphs represent the percentage of PI-positive (PI+) cells (± s.e.m.) collected after treatment with tamoxifen or its vehicle in five experiments (p-value ***<0.005, **<0.01, *<0.05). (E and F) Kaplan-Meier graphs depicting progression free survival of ERα+/PgR+/HER2–patients treated with tamoxifen alone, stratified according to high and moderate/weak (blue-black) expression of SPEN.
Figure 1

A

B

Patient 1  
C9625T  

Patient 2  
C9625T  

Patient 3  
G10183A  

Patient 4  
C6676G

Normal

Tumor

C

1184insA (T47D)

RRM

1 2 3 4

NLS NLS NLS

P2158A

RID

NLS NLS

A3327T

SPOC

7 336 810 1037 1230

2065

2707

3417

3664
Figure 2

A. Western blot analysis showing ~400 kDa bands for SpEN.

B. Gene expression fold change of SpEN.

C. Graph showing fluorescence (RFU) over 5 days for CTL1, CTL2, Spen 1, and Spen 2.

D. Colony formation assay for CTL1, CTL2, Spen 1, and Spen 2.

E. Tumor volume (mm³) over 60 days for CTL1 and Spen 1.
Figure 4

A. Venn diagram showing the overlap of genes between Spen, SPEN sh1, and SPEN sh3. The table lists the Z-score and p-value for the estrogen receptor's fold induction in proliferation by E2:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Z-score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spen</td>
<td>-1.292</td>
<td>0.007</td>
</tr>
<tr>
<td>SPEN sh1</td>
<td>2.920</td>
<td>&lt; 0.0000001</td>
</tr>
<tr>
<td>SPEN sh3</td>
<td>0.922</td>
<td>&lt; 0.0000001</td>
</tr>
</tbody>
</table>

B. Graph showing the fold induction in proliferation by E2 over time for T47D, CTL1, Spen 1, and Spen 2.

C. Western blot images showing proteins detected with antibodies (IB) for SPEN, ERα, and ERβ.

D. Western blot images showing proteins detected with antibodies (IB) for PgR and Tubulin.

E. Western blot images showing proteins detected with antibodies (IB) for PgR and Tubulin.

F. Graph showing the gene expression of PGR for CTL1, CTL2, Spen 1, and Spen 2.

G. Graph showing the gene expression of PGR for NT, SPEN sh1, SPEN sh2, SPEN sh3, and SPEN sh4.

H. Graph showing the ChIP signal for SPEN, ERG, and IgG.

I. Box plot showing the ER activation (z-score).
The estrogen receptor cofactor SPEN functions as a tumor suppressor and candidate biomarker of drug responsiveness in hormone-dependent breast cancers.

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Cancer Res  Published OnlineFirst August 21, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-3475

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