Serine/Arginine-Rich Splicing Factor 3 Modulates the Alternative Splicing of Cytoplasmic Polyadenylation Element Binding Protein 2


Abstract

Triple negative breast cancer (TNBC) has an unusually low 5-year survival rate linked to higher metastatic rates. Our laboratory recently delineated a role for the alternative RNA splicing (AS) of cytoplasmic polyadenylation element binding protein 2 (CPEB2), via inclusion/exclusion of exon 4, in the metastasis of TNBC. In these studies, the mechanism governing the inclusion/exclusion of exon 4 was examined. Specifically, the RNA trans-factor, SRSF3, was found to be explicitly associated with CPEB2 exon 4. A SRSF3 consensus sequence was identified in exon 4, and mutation of this sequence abolished the association of SRSF3. The expression of SRSF3 was upregulated in TNBC cells upon the acquisition of anoikis resistance correlating with a reduction in the CPEB2A/B ratio. Importantly, downregulation of SRSF3 in these cells by siRNA induced the exclusion of exon 4 in cells increasing the ratio of CPEB2A (exon 4 excluded) to CPEB2B (exon 4 included). Downregulation of SRSF3 also reversed the CPEB2A/B ratio of a wild-type CPEB2 exon 4 minigene and endogenous CPEB2 pre-mRNA, but not a mutant CPEB2 minigene with the SRSF3 RNA cis-element ablated. SRSF3 downregulation ablated the anoikis resistance of TNBC cells, which was "rescued" by ectopic expression of CPEB2B. Finally, analysis of The Cancer Genome Atlas database showed a positive relationship between SRSF3 expression and lower CPEB2A/B ratios in aggressive breast cancers.

Implications: These findings demonstrate that SRSF3 modulates CPEB2 AS to induce the expression of the CPEB2B isoform that drives TNBC phenotypes correlating with aggressive human breast cancer.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/17/9/1920/F1.large.jpg.

Introduction

Among the four main molecular subtypes of breast cancer, TNBC, which may also include the basal-like phenotype, is characterized histologically by a lack of estrogen receptor, progesterone receptor, and EGFR 2 (ERBB2, commonly referred to as HER2) expression (1). Compared with the hormone receptor-positive breast cancer subtypes, TNBC displays considerable genetic complexity and tumor heterogeneity making "targeted" therapies ineffective (2, 3). The standard-of-care for most patients

1Department of Biochemistry and Molecular Biology, Virginia Commonwealth University (VCU), Richmond Virginia. 2Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, Tampa, Florida. 3VCU Massey Cancer Center, Cancer Cell Signaling Program, VCU, Richmond Virginia. 4Research Service, Hunter Holmes McGuire Veterans Administration Medical Center, Richmond, Virginia. 5VCU Institute of Molecular Medicine, Richmond, Virginia. 6VCU Johnson Center for Critical Care and Pulmonary Research, Richmond, Virginia. 7Research Service, James A. Haley Veterans Hospital, Tampa, Florida. 8The Moffitt Cancer Center, Tampa, Florida.

J.T. DeLigio and S.C. Stevens contributed equally to this article.

Corresponding Authors: Margaret A. Park, University of South Florida, 4202 E. Fowler Avenue, IS56203, Tampa, FL 33620. Phone: 813-974-6657; Fax: 813-828-1473; E-mail: mapark@usf.edu; and Charles E. Chalfant, cechalfant@usf.edu


Published OnlineFirst May 28, 2019; DOI: 10.1158/1541-7786.MCR-18-1291

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Mol Cancer Res; 17(9) September 2019

Molecular Cancer Research
who present with TNBC is neoadjuvant chemotherapy and surgery, which, in many cases, initially provides a favorable pathologic response rate and outcome. However, the 5-year progression-free and survival rates for TNBC rank among the poorest of all breast cancer subtypes (4). The highly metastatic nature of TNBC and lack of known therapeutic targets make discovery of novel treatment options critical.

Increased histologic and molecular complexity in TNBC suggests very early-stage regulatory events in gene expression are involved in driving the underlying tumorigenic capacity of these cells (5–7). As such, the genomic alterations thought to influence breast cancer progression include alternative pre-RNA splicing (AS), which alters the coding regions of resulting proteins. While AS promotes novel and cancer-specific splice variants, the upregulation of RNA splicing factors in a broad scale occurs in TNBC, and thus, provides an additional layer to the complexity of exon assembly in this paradigm (8, 9). Indeed, the upregulation of specific RNA trans-activating factors alone can cause malignant transformation via dysregulated activation of downstream alternative splicing pathways (10–13).

Recently, our laboratory (2015) demonstrated that AS of cytoplasmic polyadenylation element binding protein 2 (CPEB2) affects the metastatic potential of TNBC (14). Specifically, upregulation of the CPEB2 splice variant, which incorporates exon 4 into the final transcript, produced a phenotype that was resistant to detachment-induced cell death [i.e., anoikis resistance (AnR)], and primary tumors became highly metastatic targeting the lung (14). In contrast to the CPEB2 isoform, which promoted neoplastic transformation, the CPEB2A isoform applied an opposite effect on primary tumor growth and metastasis in TNBC notably through translational repression of the DNA trans-factors TWIST1 and HIF1α (15). While the splice variant isoform ratio for CPEB2A/CPEB2B remains generally high in normal and non-tumorigenic breast tissue, the CPEB2 splice variant was found more highly expressed, reversing the A/B ratio in human TNBC and cells selected for anchorage-independent growth (14).

CPEB2 belongs to the CPEB protein family (CPEB1-4), which shares a highly conserved RNA recognition motif at their C-terminal ends; however, the N-terminus is highly variable among family members (16). The canonical member, CPEB1, has been shown to regulate translation of nascent mRNA transcripts through binding at the 3′ UTR and preventing extension of the polyadenylate tail (17). Much is unknown about the remaining family members, although CPEB2 has been shown to be essential for successful mitotic cell division and, by our laboratories, as required for the acquisition of anoikis resistance (18).

As to plausible regulators of CPEB2 AS, the serine/arginine (SR)-rich family is a well-known family of RNA trans-activating factors that modulate splice site selection and exon inclusion/exclusion via association of a RNA recognition motif (RRM) and RNA binding domain (RBD) to exonic splicing enhancers (ESE) in pre-mRNA to mediate spliceosome assembly (for reviews see refs. 19–21). The SR protein family member, SRSF3, is reportedly upregulated during oxidative stress and hypoxia, conditions typically found within a tumor, where the trans-RNA splicing factor can exert wide influence in genes associated with cell cycle and proliferation (22–24). In this study, our laboratory demonstrated an increased occurrence of both SRSF3 and the CPEB2B isoform in TNBC, and identified a consensus binding motif for SRSF3 in the exon promoting the CPEB2B isoform. This evidence suggested a mechanistic link to the SRSF3-CPEB2B splicing paradigm. This study shows that the splicing event, which promotes expression of CPEB2B, is driven by SRSF3 as well as linked to aggressive growth in metastatic TNBC.

Materials and Methods

Cell culture and reagents

MDA-MB-231 and MDA-MB-468 cells were obtained from ATCC and maintained in RPMI (Invitrogen). The ATCC routinely authenticates cell lines using short-tandem repeat profiling. All cell lines were supplemented with 10% FBS (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen). Cells were maintained in a 95% air/5% CO2 incubator at 37°C. Cells were passaged once every 3 to 5 days (~90% confluence), and all experiments were performed during the first 12 passages.

Western blotting analysis

Total protein (5–20 μg) was electrophoretically separated on 7.5% to 12% SDS-PAGE. Samples were transferred electrophoretically to polyvinylidene difluoride membranes, then probed with the appropriate antibody as described previously (14, 15, 25). Antibodies were purchased from Cell Signaling Technology with the exception of SRSF3 (Thermo Fisher Scientific, Clone ID: 7B4).

qRT-PCR

Primer/probe sets were described previously (14). PCR was performed as described previously (25). cDNA was synthesized using the Superscript III Kit (Life Technologies) and manufacturer’s instructions (26–28). Samples were then amplified using a BioRad CFX Connect qPCR machine and calculated using the standard curve method.

siRNA treatment and plasmid transfection

All transfections were carried out in triplicate in 6-well tissue culture dishes. Validated Silencer Select siRNA toward SRSF3 (s12732 or s12733) or nontargeting control (Thermo Fisher Scientific) was utilized in this study at 30 nmol/L concentrations and transfected using Dharmafect 4 Transfection Reagent (Dharmacon) as described previously and according to the manufacturer’s specifications. Plasmid transfections were accomplished using the Effectene System (Qiagen) according to the manufacturer’s instructions as described previously (14, 25, 29, 30) and using 0.5 to 1.0 μg total DNA per well.

Anoikis resistance assay

Cells were transfected with the indicated siRNA and DNA plasmid using Dharmafect Duo Transfection Reagent (Dharmacon). After 48 hours, cells were washed, trypsinized, and added to each well of either normal or polyHEMA-coated 6-well tissue culture plates. The cells were incubated for 6 hours and then collected for analysis via Western blotting.

Competitive qRT-PCR

cDNA was synthesized as described previously (30), and then cDNA samples were subjected to competitive PCR using the following primers: endogenous CPER2A or CPER2B isoform amplification forward primer, 5′-GCAGCAGAGACAATCCTCTA-TAAC-3′ and reverse primer, 5′-CAGAGTGCATATTTCACTCAG-3′; miogenie specific CPER2A or CPER2B isoform...
amplification forward primer, 5'-CAGAACAGCAACAATAGTAATACACCTC-3' and reverse primer, 5'-AGGCGGCAACACAGATGTG-3'. PCR conditions for endogenous gene amplification consisted of an initial denaturing step at 98°C for 30 seconds followed by 25 cycles of 98°C denaturing for 10 seconds, 50°C annealing for 30 seconds, 72°C extension for 1 minute, and final extension step at 72°C for 5 minutes. Minigene-specific amplification conditions were identical and used 20 cycles. All PCR reactions were amplified using Standard Taq Polymerase (New England BioLabs) with products run on 5% polyacrylamide-TBE and stained with SYBR Gold (Thermo Fisher Scientific).

RNA-binding assays
Full length wild-type (WT) biotinylated RNA CPEB2 exon sequence (5'-CIGACAGACAGCCATTCCGCCCCCCTGCCCTCTATAATTACACCTC-3') and reverse primer, 5'-CTACGCTGTCTATCTGACTCTTCGGCCTGAGCT-3' was verified using an SRSF3-specific antibody for cross-linking immunoprecipitation coupled with qRT-PCR (CLIP-qRT-PCR, Fig. 1C). SRSF3 was shown to interact with the CPEB2 exon 4 coding sequence (Bi:5'-GUGAGAUCUAGUUUGCAGUUGCCAGCUUG-3') or mutant sequence (MUT, Bi:5'-GUGAGAUCUAGUUUGCAGUUGCCAGCUUG-3').

Electrophoretic mobility shift assay
FITC-conjugated full length (Fig. 1A) or partial (Fig. 1E) WT or mutant CPEB2 RNA sequences were subjected to EMSA as described previously (27).

Minigene construct and plasmids
The genomic region spanning exons 3, 4, and 5 of the CPEB2 gene was analyzed in this study. Template DNA was amplified from the RPCI-11 HS BAC Clone (Thermo Fisher Scientific, Clone ID: 629A7) in two different 1.7 kilobase fragments. PCR reactions used forward and reverse primers to amplify exon 3, intron 3, exon 4, and a partial segment of intron 4 with sequences 5'-AAACGGGCCCTCTAGATTTCCCTAGCCTCTTCTGA-3' and 5'-GGAAGGATGCTGATGACCCGGTTCTTCCGATGAG-3'. The second fragment of DNA was amplified using forward and reverse primers to amplify a region of intron 4 directly upstream exon 5 and all of exon 5 except the last three codons. Forward and reverse PCR primers consisted of sequences 5'-TCTAGACATCCTTCCGCTCA-3' and 5'-TACCAGAGCTGAGCCCGCATGCTTGCTC-3'. Genomic DNA fragments were amplified using standard PCR conditions and proofreading Taq polymerase (Phusion High Fidelity DNA Polymerase, New England BioLabs). Standard PCR conditions consisted of an initial denaturing step at 98°C for 30 seconds followed by 30 cycles of 98°C denaturing for 10 seconds, 60°C annealing for 30 seconds, 72°C extension for 30 seconds, and final extension step at 72°C for 10 minutes. Fusion of amplified genomic material with the pcDNA3.1 mammalian expression vector (Invitrogen) was carried out using the In-Fusion HD Cloning Reaction (ClonTech). At each step of PCR amplification and plasmid generation, the CPEB2 minigene sequence was verified by Sanger dideoxy method (GenScript). The CPEB2 minigene insert was designed to retain the XbaI and BamHI restriction sites at the 5' and 3' ends, respectively. All primers used in cloning and analysis were synthesized by Integrated DNA Technologies.

Site-directed mutagenesis was carried out using the Quick-Change II XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions and primers containing the mutant CPEB2 ESE with forward and reverse sequences 5'-

Biostatistics
Biostatistical analyses were performed using either SPSS or R. Statistical tests used included one-way ANOVA/pooled t test (in the case of only two samples), ANOVA (in the case of multiple samples), and an FDR-adjusted P value with Tukey HSD post hoc calculation.

Results
Unbiased proteomic analysis identified SRSF3 associated with exon 4 of CPEB2
Previously, our laboratory reported that the acquisition of anoikis by TNBC cells required the inclusion of exon 4 into the mature CPEB2 mRNA to produce the CPEB2 isoform (14, 15). These findings led to the investigation of the mechanism governing the inclusion of exon 4 promoting mRNA maturation toward the CPEB2 isoform. To this end, electrophoretic mobility shift assays (EMSA) analysis was employed using nuclear extract from MDA-MB-231 cells to examine protein complexes associated with exon 4 of CPEB2. Unbiased proteomic analysis of complexes bound to CPEB2 exon 4 identified SRSF3, hnRNP F, and hnRNP H1 bound to exon 4 of CPEB2 (Fig. 1A; Table 1). Of the four RNA trans-factors associated with exon 4, SRSF3 was validated using an SRSF3-specific antibody for cross-linking immunoprecipitation coupled with qRT-PCR (CLIP-qRT-PCR, Fig. 1C). SRSF3 was shown to cross-link tRNA in either MDA-MB-231 parental (Par) or AnR cells via immunoprecipitation, and was shown to interact with Exon 4 of CPEB2 in both Par versus AnR cells through qRT-PCR amplification specific to CPEB2 Exon 4. A significant increase in RNA association of SRSF3 with CPEB2 Exon 4 was also observed in the AnR cells (Fig. 1B). Importantly, the levels of SRSF3 were increased in TNBC cells that acquire AnR (Fig. 1B).

Examination of exon 4 elucidated a consensus sequence for SRSF3 (C/U)(A/C/U)(U/A)(C/A/U)(A/C/U) (Fig. 1C), and to examine the association of SRSF3 with this sequence, a streptavidin–biotin affinity purification (SBAP) assay was developed using CPEB2 exon 4 RNA as "bait" and combined with recombinant SRSF3. Mutation of the SRSF3 consensus sequence led to no association of SRSF3 with exon 4. Mutation of this sequence abolished the association of SRSF3. This assay revealed that SRSF3 specifically binds to CPEB2 exon 4, with competition for SRSF3 binding achieved with unlabeled CPEB2 exon 4 RNA at excess concentration (100×), while nonspecific competitor RNA showed no effect on the RNA: protein complex (Fig. 1C–E). Importantly, mutation of the SRSF3 consensus sequence (CAUCC → GAGUC) abolished the association of SRSF3 (Fig. 1C–E). Importantly, reduction of the levels of SRSF3 induced a dramatic decrease in the amount of SRSF3 bound to the SRSF3 consensus sequence in TNBC cells (Fig. 1F). These data demonstrate that SRSF3 associates with exon 4 of CPEB2 in a specific manner via the SRSF3 consensus sequence, CAUCC.

Downregulation of SRSF3 reduced the inclusion of exon 4 into the mature CPEB2 mRNA transcript
To determine whether SRSF3 regulated the inclusion/exclusion of CPEB2 exon 4, multiple siRNA sequences were utilized to
Figure 1. SRSF3/SRp20 binds specifically to exon 4 in the CPEB2 pre-mRNA. A, MDA-MB-231 nuclear extract was incubated with either FITC-conjugated CPEB2 exon 4 sequence + "cold" nonspecific competitor (FI-CP) or preincubated with 100× "cold" CPEB2ex4 as a specific competitor (+ SC). Samples were then electrophoresed and bound proteins were extracted and subjected to proteomic analysis. B, Lysates of Par and AnR MDA-MB-231 cells were immunoblotted and probed for the indicated antibodies. C, SRSF3-specific antibody was used for CLIP-qRT-PCR to detect CPEB2 levels in either MDA-MB-231 Par or AnR cells. RT-PCR to CPEB2 at exon 4 was evaluated (data represented as n = 3 ± SD, *, P < 0.05). IP, immunoprecipitated fraction. Non-IP, nonimmunoprecipitated fraction. D, The consensus sequence for SRSF3 and a partial sequence of exon 4 highlighting the proposed SRSF3-binding site. E, SBAP assay was used to detect SRSF3 bound to exon 4 of CPEB2. Recombinant SRSF3 was incubated with biotinylated CPEB2 exon 4 RNA oligos with WT or mutant SRSF3 ESE cis-element. Samples were incubated with either biotin-labeled CPEB2 exon 4 sequence + "cold" nonspecific competitor (NSC) or preincubated with 100× "cold" unlabeled CPEB2ex4 as a specific competitor (+ SC). F, EMSA analysis of siRNA-depleted expression of SRSF3 in MDA-MB-231 cells. EMSA analysis of siRNA-depleted expression of SRSF3 in MDA-MB-231 cells. EMSA labels correspond to MDA-MB-231 cells treated with siRNA control and then total protein lysates incubated with WT CPEB2 exon 4 ESE RNA (si0-WT), or siRNA to SRSF3-treated cell lysates incubated with WT CPEB2 exon 4 ESE RNA (siSF3-MUT). Sequences are indicated in the Materials and Methods section. Control samples were incubated with nonspecific IgG.
Downregulate SRSF3 as well as the other siRNA trans-factors identified in the unbiased proteomic screen (Fig. 2; Table 1). Downregulation of ≥75% of each protein was achieved in MDA-MB-468 and MDA-MB-231 cells (Table 1) using SRSF3-specific siRNA as compared with siRNA controls. Downregulation of SRSF3 induced a significant increase in the CPEB2A/B mRNA and protein ratio demonstrating a decrease in the inclusion of exon 4 into the mature CPEB2 mRNA transcript (Fig. 2A and B). Reducing the SRSF3 levels in MDA-MB-468 cell line, which inherently expresses more of the CPEB2B isoform versus MDA-MB-231 (ratio CPA/CPB 2.4 vs. 3.3, respectively), also increased the CPA/CPB ratio (Fig. 2A and B). These data demonstrate that SRSF3 is an RNA trans-factor that functions to enhance the inclusion of exon 4 into the mature mRNA transcript.

The consensus cis-element for SRSF3 is essential for the inclusion of CPEB2 exon 4

The consensus pentamer motif for SRSF3 association is contained within CPEB2 exon 4, and this motif has previously been reported to promote alternative splicing of target pre-RNAs including coding and noncoding species (31). To interrogate the SRSF3-binding site in CPEB2 exon 4 for regulation of CPEB2 AS, a minigene reporter system was generated for mutational analysis of this RNA cis-element (Fig. 3A; refs. 26, 32, 33). Specifically, CPEB2 exon 3, intron 3, exon 4, part of intron 4, and most of exon 5 were cloned into a mammalian expression vector under a CMV promoter (pcDNA3.1(−)). Utilizing competitive RT-PCR, the inclusion/exclusion of exon 4 into the minigene mRNA was assayed with a plasmid-specific, reverse primer to ensure the assay remains reporter-specific rather than amplifying endogenous splicing events (Fig. 3A). Both MDA-MB-231 Par and AnR cells were evaluated for their minigene splicing levels to test whether increased SRSF3 expression in AnR cells directly correlated with CPEB2 splicing in TNBC. Importantly, expression of the CPEB2 minigene in TNBC cells demonstrated a similar CPEB2 A/B mRNA ratio as endogenous CPEB2 mRNA (2.3/0.11 for the MG vs. 2.5/0.09 for endogenous) in MDA-MB-231 Par cells (Fig. 3B), while WT CPEB2 minigene splicing in MDA-MB-231 AnR cells followed a similar trend (1.3/0.02 for the MG vs. 1.7/0.07 for endogenous; Fig. 3B).

To determine whether SRSF3 expression affected the inclusion/exclusion of CPEB2 exon 4, siRNA downregulation of SRSF3 using siRNA induced a significant increase in the CPEB2A/B minigene mRNA ratio mimicking the effects observed on endogenous levels (Fig. 3C–F). Reductions in CPEB2B splicing were significantly
Figure 3.
Modulating levels of SRSF3 in TNBC cells affects the CPEB2 splice isoform ratio at exon 4. A, Schematic of CPEB2 exon 3-4/5 minigene. Genomic DNA was amplified from RPCI-11 Hs BAC Clone using primers, which spanned the entirety of exon 3, 4, and the majority of exon 5. The complete sequence for intron 3 was included, and partial sequence of intron 4 was included. Primers specific to the minigene were used to detect splicing events in RT-PCR analysis. B, MDA-MB-231 Par cells were compared with MDA-MB-231 AnR cells for basal levels of minigene splicing for the MG-specific CPA/CPB (MG) ratio and compared with endogenous CPEB2 splicing (endo). MDA-MB-231 Par (C) and MDA-MB-231 AnR (D) cells were treated with a combination of two siRNA sequences targeting SRSF3 (shown in Fig. 1). CPEB2 minigene splicing and endogenous CPEB2 splicing was detected via RT-PCR. MDA-MB-231 Par (E) and MDA-MB-231 AnR (F) SRSF3 protein levels were detected after siRNA treatment as indicated by Western blot analysis. Representative images from 3 independent experiments are shown, and for all quantitation $n = 3 \pm SD$ via densitometry. Statistical significance is reported as $P$ value from one-way ANOVA pooled t test of the MG or endo CPA/CPB ratio. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
different in siRNA treatment to SRSF3 compared with control siRNA and was observed to be approximately 1.3–1.4-fold less in both the Par and AnR cell lines ($P = 0.0023$ for Par and $P = 0.0003$ for AnR cells).

To show SRSF3 binding to CPEB2 exon 4 is required to mediate enhancement of exon 4 inclusion, site-directed mutagenesis was performed on the putative SRSF3 ESE site in exon 4 (Fig. 4A; refs. 31, 34). Mutations using nucleotide

![Figure 4. Mutational analysis of SRSF3 RNA cis-element indicates the pentameric ESE is essential for CPEB2 exon 4 inclusion. A, Schematic representation of the mutant ESE minigene. Red bar indicates the position of the RNA cis-element is located near the 5' splice site. Genomic coordinates indicate the first nucleotide base in the RNA cis-element and were extracted from Genome Reference Consortium Human Build 38 (GRCh38.p12) B, RT-PCR analysis of MDA-MB-231 Par cells for the WT CPEB2 ESE compared with mutant (Mut) MG-specific CPA/CPB ratio. C, RT-PCR analysis of MDA-MB-231 AnR cells for the WT CPEB2 ESE compared with Mut MG-specific CPA/CPB ratio. Representative images from 3 independent experiments are shown. All quantitation shown as $n = 3$ ± SD via densitometry. Statistical significance is reported as $P$ value from one-way ANOVA pooled t test of the MG CPA/CPB ratio. ($* P < 0.05; ** P < 0.01; *** P < 0.001$).](#)

![Figure 5. SRSF3 modulates TNBC cells' sensitivity to cell death due to anoikis via expression of CPEB2B. siRNA treatment was applied for a 48-hour cycle, then incubated for 6 hours on adherent substrate or poly-HEMA substrate, which forced cells into suspension. After incubation, early-stage apoptosis was evaluated using Western blot to probe for cleaved PARP (clv-PARP) and cleaved caspase-3 (clv-CASP3). Antibodies for apoptotic markers detected full-size PARP (116 kDa) and cleaved PARP (89 kDa), and both large fragments of activated cleaved caspase-3 (17/19 kDa doublet). A, MDA-MB-231 AnR cells were treated as indicated in the plus/minus graphical organizer with nonspecific siRNA control (si0), pcDNA3.1(-) empty vector (pcDNA), siRNA to SRSF3 (siSF3), CPEB2B-Flag overexpression plasmid (CPEB2B), and poly-HEMA–coated substrate (p-HEMA). Samples shown representative of experiments done in triplicate for each treatment. B, MDA-MB-231 Par cells were treated identically to the cells described in A.](#)
coupled to our reports that CPEB2B is highly expressed in human like breast cancer. Expression of SRSF3 is enhanced in human TNBC and basal-like breast cancer. The proto-oncogene, SRSF3, is shown to promote the pro-metastatic CPEB2B isoform. We have identified AS of CPEB2 driven through the trans-RNA splicing factor SRSF3 in TNBC. Specifically, SRSF3 expression is upregulated in hypoxic conditions found in the tumor microenvironment typical of solid and malignant breast tumors. Previously, we reported two isoforms of CPEB2, CPEB2A and CPEB2B, exert opposing roles in the transformation from primary to metastatic phenotype in TNBC through acquisition of AnR (15). For the first time, the proto-oncogene, SRSF3, is shown to promote the pro-metastatic CPEB2B isoform. We have identified AS of CPEB2 precursor-mRNA is facilitated through SRSF3 by binding to a pyrimidine-rich, pentameric ESE found at the distal 3′ end of coding exon 4. This interaction promotes inclusion of the characteristically weak CPEB2B coding exon 4 into the mature transcript and leads to activation of signaling pathways, which initiate apoptosis in cancer metastasis.

Expression of SRSF3 is enhanced in human TNBC and basal-like breast cancer

This mechanistic study, linking SRSF3 with CPEB2 expression coupled to our reports that CPEB2B is highly expressed in human TNBC and regulates TNBC metastasis, forms the premise that SRSF3 expression will correlate with human TNBC and the aggressiveness of human breast cancers. To examine the validity of this premise, sequence data contained in The Cancer Genome Atlas (TCGA) breast invasive carcinoma (BRCA) dataset of unique patient cases with both RNAseq and clinical data associated were evaluated for mRNA levels of SRSF3 (35). The mRNA z-scores were extracted for each PAM50 gene expression–based subtyping. TNBC and basal-like subtypes showed the highest expression of SRSF3, while Luminal A, Luminal B, and HER2+ subtypes all expressed significantly less (Fig. 6A). Using the R coding platform to interrogate the TCGA survival data (36), we observed stratifying patients by SRSF3 expression was predictive of survival probability (Fig. 6B). These data support the prediction that in TNBC with high SRSF3 expression, the CPEB2A/B isoform ratio will be low, and provide further insight into the powerful role of dysregulated AS in cancer progression and metastasis.

Discussion

Herein, our laboratory provides evidence that describes the mechanism for AS of CPEB2 driven through the trans-RNA splicing factor SRSF3 in TNBC. Specifically, SRSF3 expression is upregulated in hypoxic conditions found in the tumor microenvironment typical of solid and malignant breast tumors. Previously, we reported two isoforms of CPEB2, CPEB2A and CPEB2B, exert opposing roles in the transformation from primary to metastatic phenotype in TNBC through acquisition of AnR (15). For the first time, the proto-oncogene, SRSF3, is shown to promote the pro-metastatic CPEB2B isoform. We have identified AS of CPEB2 precursor-mRNA is facilitated through SRSF3 by binding to a pyrimidine-rich, pentameric ESE found at the distal 3′ end of coding exon 4. This interaction promotes inclusion of the characteristically weak CPEB2B coding exon 4 into the mature transcript and leads to activation of signaling pathways, which initiate apoptosis in cancer metastasis. 
epithelial-to-mesenchymal transition (EMT) pathology (Visual Overview). Importantly, SRSF3-mediated splicing of CPEB2 exon 4 is dependent on the fidelity of its RNA cis-element ESE in exon 4. Indeed, we observe a reversal of the CPEB2A/B ratio by substitution mutation in the CPEB2 minigene reporter at the SRSF3 ESE.

Finally, modulating the expression of SRSF3 through siRNA to deplete expressed protein levels led to a decrease in CPEB2B transcript levels, thus reversing the low CPA/CPB ratio to the higher ratio observed in cells sensitive to anoikis. Reversing this ratio also caused increased apoptosis in the biological phenotype, especially for the non-AnR cell line. The biological phenotype observed did not, however, show robust rescue by forced CPEB2B expression in the AnR cells as predicted in our original hypothesis. Levels of cleaved PARP were not attenuated by circumventing SRSF3 activity with the CPEB2B transient transfection in the splicing factor–depleted group. These data suggest that the maintenance of survival after detachment is dependent on SRSF3 expression.

SRSF3 plays a substantial role in regulation of AS, and increasing levels of SR proteins bound to ESE prevents exon skipping (37). Recent studies reveal that SRSF3 may play an antagonistic role compared with other SR-family proteins where SRSF3 acts to promote exon inclusion leading to tumor initiation, progression, and/or resistance (10, 22, 38). Indeed, data suggest SRSF3 is a potential master regulator of the transcriptome by the observation that it binds multiple SR-family proteins and promotes "poison cassette exon" autoregulatory feedback loops through nonsense-mediated decay (31). Although this research shows CPEB2A is tightly connected to SRSF3 binding at the ESE of exon 4 in precursor mRNA, this alone may not be the sole regulatory mechanism for CPEB2 splicing. For example, we observed short-term transient overexpression of SRSF3 cDNA in MDA-MB-231 cells did not lead to increases in CPEB2B levels as would be predicted (data not shown). In fact, protein levels of CPEB2B in MDA-MB-231 cells did not lead to increases in CPEB2B levels as observed short-term transient overexpression of SRSF3 cDNA in MDA-MB-231 cells. Further investigation into the activity of SRSF3 under various posttranslational modifications may uncover the influence this splicing factor exerts in driving the CPEB2A/B isoform ratio lower seen in AnR and TNBC.

With regard to PTMs of SRSF3, NEDDylation, a modification covalently linking the small ubiquitin-like protein NEDD8 to Lys85 of SRSF3 has been shown to promote stress granule (SG) assembly during times of oxidative stress (40). This metabolically efficient strategy is coopted by tumor cells experiencing persistent stress to keep nascent mRNA and proteins compartmentalized and ready to regulate signaling pathway activity (41). Not only does SRSF3 localize to SG, but both CPEB2A and B proteins contain a low complexity domain (LCD) immediately distal to the amino acids encoded by exon 4 in the primary amino acid sequence of CPEB2B. The CDR of CPEB2 and other RNA-binding proteins contribute dynamically to the formation of ordered suborganelle compartments such as the SG (42). Furthermore, extensive phosphorylation of SRSF3 at the RS domain is crucial for spliceosomal assembly, while AS catalysis is dependent on subsequent dephosphorylation. The cycle of phosphorylation/dephosphorylation also allows SRSF3 to interact with the nuclear export factor, NXf1 to couple AS and polyadenylation during NXf1-mediated mRNA export (43). In the context of TNBC, where SRSF3 levels are inherently high, one can hypothesize that the metastatic potential of primary tumors may be activated by CPEB2 precursor mRNA bound by highly phosphorylated SRSF3 at exon 4 and shuttled to SG in the cytoplasm, thereby increasing cytoplasmic abundance of the CPEB2B transcript via promotion of exon inclusion and activating the EMT pathway DNA trans-factors TWIST1 and HIF1α (Visual Overview).

Regarding other proteins in the SR family, it is well established in the literature that these trans-factors regulate a variety of apoptosis/survival proteins and thereby hallmarks of cancer (reviewed in 44). For example, SRSF1 is involved in both breast and lung cancer pathogenesis and SRSF6 has been shown to regulate Bim expression in melanoma (44). Hence, our data will not "rule out" other SR-dependent pathways, which may induce breast cancer metastasis.

Defective AS frequently impacts dysregulation in many of the hallmarks of cancer, which have potential to promote primary tumor metastasis (45). In the case of TNBC, the higher level of genetic heterogeneity may be attributed to defects in pre-mRNA splicing and the resulting structural protein variants, which contribute to therapeutic resistance (46). Increased interest in targeting not only the products of aberrant splicing in cancer, but also trans-AS splicing factors, namely SRSF3, using antisense oligonucleotide (ASO)-mediated inhibition has emerged and appears to be a promising step in development of novel therapeutic targets in cancer (47, 48). Certainly, the feasibility of targeting the SRSF3: CPEB2B splicing paradigm is worth pursuing in the context of TNBC.

In summary, we have identified SRSF3 as the trans-AS splicing factor responsible for mediating AS of CPEB2 through inclusion of exon 4 via binding at the cis-RNA pentameric ESE. Our study demonstrates that the SRSF3:CPEB2B splicing paradigm is essential for the acquisition of AnR in TNBC. We also show that siRNA-mediated depletion of SRSF3 results in a loss of resistance to anchorage-independent growth and reverts the CPEB2A/B ratio to that of nontumorigenic breast tissue. Further studies will be needed to derive the activation of this novel splicing ontology and its potential to translate into a tangible clinical target for human breast carcinoma.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Disclaimer

The contents of this manuscript do not represent the views of the Department of Veterans Affairs or the United States Government.

### Authors' Contributions

Conception and design: J.T. DeLigio, S.C. Stevens, C.E. Chalfant, M.A. Park
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.T. DeLigio, S.C. Stevens, H.P. MacKnight, K.K. Doe, M.A. Park
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.T. DeLigio, S.C. Stevens, H.P. MacKnight, C.E. Chalfant
Writing, review, and/or revision of the manuscript: J.T. DeLigio, S.C. Stevens, H.P. MacKnight, C.E. Chalfant
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.C. Stevens, C.E. Chalfant, M.A. Park

Study supervision: C.E. Chalfant, M.A. Park

Acknowledgments

The authors would like to heartily thank Dr. Binks Wattenberg for his assistance and critical review in this effort. This work was supported by research grants from the American Cancer Society (ACS), IRG-17-173-22 (to M.A. Park), U.S. Department of Veterans Affairs (VA Merit Review, I BX001792; to C.E. Chalfant), a Research Career Scientist Award (13F-RCS-002, to C.E. Chalfant), the NIH via TR008057-04 (to M.A. Park), U101HD087196-01 (to C.E. Chalfant), CA117950 (to C.E. Chalfant), CA154314 (to C.E. Chalfant), and AI139072 (to C.E. Chalfant).

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Received December 3, 2018; revised April 17, 2019; accepted May 21, 2019; published first May 28, 2019.

References


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