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The Role Of Cpeb2 Alternative Splicing In TNBC Metastasis

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by

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DEDICATION

This dissertation is dedicated to my wonderful wife Danielle, whose support and encouragement have been with me through every aspect of this endeavor. Mere words cannot express the gratitude which fills me when I contemplate the gravity of her sacrifice through this process. I also dedicate this to my six incredible children, who have been a source of profound motivation to press on and demonstrate resilience in pursuing your goals.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3’UTR	3’ Untranslated Region
5’UTR	5’ Untranslated Region
AnR	Anoikis Resistance
AS	Alternative pre-mRNA Splicing
ASO	Antisense Oligonucleotide
BL1	Basal-like 1
BL2	Basal-like 2
BRCA	Breast Invasive Carcinoma Cohort
BS	Branch Site
CFL1	Cofilin1
CLIP-seq	Cross-Linking Immunoprecipitation – Next Generation Sequencing
Clk1/4	CDC-like Kinase 1 and 4
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
DNA	Deoxyribonucleic Acid
eEF2	Eukaryotic Elongation Factor 2
ECM	Extracellular Matrix
eIF3H	Eukaryotic Initiation Factor 3H
eIF4E	Eukaryotic Initiation Factor 4E
eIF4G	Eukaryotic Initiation Factor 4G

EMSA	Electrophoretic Mobility Shift Assay
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ESE	Exon Splicing Enhancer
ESS	Exon Splicing Silencer
FAK	Focal Adhesion Kinase
GTP	Guanosine Triphosphate
HER2	Human Epidermal Growth Factor Receptor 2
hnRNP	Heteronuclear Ribonuclear Protein
hnRNPR	Heteronuclear Ribonuclear Protein R
hnRNPF/H	Heteronuclear Ribonuclear Protein F/H
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
IRES	Internal Ribosome Entry Site
ITAF	IRES Trans-acting Factor
MSL	Mesenchymal Stem-like
mRNA	Messenger RNA
NGS	Next Generation Sequencing
NMD	Nonsense-mediated decay
PCR	Polymerase Chain Reaction
PIC	Pre-Initiation Complex
Pol II	RNA Polymerase II
PR	Progesterone Receptor

PTM	Post Translational Modification
RBD	RNA Binding Domain
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RRM	RNA Recognition Motif
RS	Arginine/Serine-Rich Domain
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBAP	Streptavidin Biotin Affinity Purification
SR	Serine/Arginine-Rich Protein
SRSF3	Serine/Arginine Splicing Factor 3
ss	Splice Site
TCGA	The Cancer Genome Atlas
TNBC	Triple-Negative Breast Cancer

ABSTRACT

Breast cancer is the second leading cause of cancer-related deaths for women in the U.S. Although the overall 5-year survival rate for breast cancer is 90%, this rate drops substantially for triple-negative breast cancer (TNBC) due to its high metastatic potential. Furthermore, there is a lack of targeted therapeutics for TNBC, and clinical trials have been largely unsuccessful. These characteristics validate the need for identifying novel therapeutic targets for the treatment of TNBC. The study of alternative splicing (AS) has emerged as a powerful tool to elucidate the molecular underpinnings driving cancer.

Our lab has identified cytoplasmic polyadenylation element-binding protein 2 (CPEB2), which has two main isoforms, CPEB2A and CPEB2B, which differ via the inclusion/exclusion of exon four in the mature mRNA transcript. These two isoforms have opposing functions as translational regulators of mRNA species implicated in metastatic progression. A shift in the spicing ratio favoring an increase in CPEB2B and a reduction in CPEB2A resulted in increased translation of HIF1α and TWIST1 mRNA, transcription factors important in the regulation of the hypoxic response and epithelial-to-mesenchymal (EMT) pathways and contributed to the acquisition of anoikis resistance (AnR) and metastasis in TNBC cells *in vivo*.

Increased levels of serine/arginine-rich factor 3 (SRSF3), an AS regulator, were identified in TNBC AnR cells. SRSF3 was also determined to be the *trans*-splicing factor responsible for regulating the inclusion/exclusion of exon four of CPEB2 by binding to a

consensus sequence within exon four. Mutation of the SRSF3 consensus sequence in exon four of CPEB2 ablated SRSF3 binding resulting in decreased inclusion of exon four. A minigene construct investigation of the SRSF3/CPEB2 exon four alternative splicing axis indicated that downregulation of SRSF3 via siRNA resulted in a CPEB2 alternative splicing ratio shift favoring the production of CPEB2A (exon for exclusion). However, in the siSRSF3 CPEB2 mutant minigene, the ratio shift was ablated. Furthermore, siRNA targeting SRSF3 decreased CPEB2B (exon four inclusion) and reduced AnR and survival in TNBC, which was “rescued” by the ectopic expression CPEB2B. Ultimately, these studies demonstrate the importance of CPEB2 AS via the *trans*-splicing factor SRSF3 in the acquisition of AnR and metastasis in TNBC.

We also propose a mechanism of HIF1α and TWIST1 translational regulation via CPEB2A and CPEB2B. We determined that the CPEB2A isoform bound to the CPE sites located in HIF1α and TWIST1 3'UTR mRNA and interacted with known polyadenylation complex proteins. Similarly, we found CPEB2B associated with polyadenylation complex proteins, albeit a weaker interaction, but did not bind CPE sites in HIF1α and TWIST1 3'UTR mRNA. We also found that CPEB2A and CPEB2B localize to both the nucleus and cytoplasm. Furthermore, we identified novel protein interactions for CPEB2B, specifically exon four, which is absent in the CPEB2A transcript. CPEB2B was shown to interact with proteins, specifically, the translation invitation factor eIF3H and heteronuclear-ribonuclear proteins hnRNPR and hnRNPF/H, which have been identified as IRES trans-acting factors (ITAFs) important in cap-independent translational activation of mRNAs in cellular stress events. Interestingly, we also identified a strong interaction between CPEB2A and CPEB2B proteins

suggesting a potential connection between the CPEB2 alternative splicing regulation and translational activation/inhibition of HIF1α and TWIST1. These novel interactions have never been described and provide evidence that alternative splicing inducing a ratio shift of CPEB2A to CPEB2B proteins results in translational activation of HIF1α and TWIST1 mRNA through an IRES-mediated cap-independent translational mechanism promoting the acquisition AnR and the metastatic phenotype in TNBC.

CHAPTER ONE: INTRODUCTION

Alternative mRNA splicing

According to the central dogma of molecular biology, deoxyribonucleic acid (DNA) is transcribed to ribonucleic acid (RNA), which is then translated into a polypeptide chain of amino acids called protein. Proteins are responsible for carrying out a host of diverse cellular functions. It is estimated that the proteome consists of well over 150,000 unique proteins [1]. Remarkably, this extensive proteome is produced from the human genome, consisting of only 20,000 genes. The reason that the cell can produce the extensive proteome from a smaller genome is partly due to a molecular process known as alternative RNA splicing (AS) [2].

Alternative RNA splicing is a highly regulated molecular process in which precursor-mRNA is modified to produce the mature mRNA transcript. This modification is accomplished by excising nucleic acid sequences termed (introns) and joining together remaining nucleic acid sequences, termed exons via a covalent phosphodiester linkage in a process called mRNA splicing [3]. Initially discovered by Phillip Sharp and Richard Roberts in 1977, this process is called splicing because it resembles the strategy through which a movie editor would cut and splice together film to produce the final edited version of a movie [4].

Since its discovery, research focused towards understanding AS regulation has led to many novel findings. It is now known that approximately ninety-five percent of all genes in the human gene undergo the AS process to produce the mature mRNA

transcript. Furthermore, in addition to constitutive splicing, different variations of AS have been discovered. One such example involves the inclusion or exclusion of exons. In this AS variation, an exon may be included "spliced in" or excluded "spliced out," adding to the potential of multiple proteins being produced by a single gene [5]. By modulating final mRNA transcripts through the AS mechanism, the cell can adjust the proteome in response to ever-fluctuating cellular demands ensuring cellular homeostasis. Dysregulation of this crucial process can lead to a plethora of diseases, including cancer. Small changes in AS (dysregulation) can have enormous consequences on the proteome, resulting in significant alterations in cellular signaling pathways and is often a process cancer cells use to their advantage. Numerous cellular pathways essential for cancer growth and development may become hyper- or hypoactive when dysregulation of AS occurs [6]. Some examples of these include angiogenesis, anoikis resistance, apoptosis resistance, cell proliferation, and cell migration.

Alternative Splicing Mechanism and Regulation

The molecular process of alternative splicing co-occurs with transcription. This is accomplished via direct interaction of the translational complex with components of the spliceosome. The transcriptional complex which is responsible for the process of pre-RNA splicing is a muti-megaDalton collection of ribonucleoproteins (RNPs) termed the spliceosome [7]. This complex catalyzes the removal of introns from newly synthesized pre-mRNA transcripts by RNA polymerase II (Pol II). The spliceosome consists of five major components, each containing a distinct collection of small nuclear RNAs

(snRNA). In conjunction with specific RNPs, these snRNAs form the basis of uridine-rich small nuclear ribonucleoprotein particles (snRNPs) [8]. For each splicing event, the assembly of the snRNPs occurs in an ordered and sequential manner and in conjunction with an extensive network of associated proteins, totaling over three hundred, to form the spliceosome complex and interacting with specific consensus RNA *cis*-elements in the growing pre-mRNA molecule [9].

The process of pre-mRNA maturation is tightly regulated and is dependent on specific cellular stimuli associated with shifts in physiological states [10]. For most genes, the synthesis of pre-mRNA is carried out by Pol II, beginning in the promoter region of the gene. The transcription process resembles a coordinated dance as numerous transcription factor proteins assemble at the promoter region, forming the preinitiation complex and ultimately recruiting Pol II. After assembly at the promoter region, pre-mRNA transcription proceeds as the complex encounters a transcription start site. Immediately upon the start of transcription, the pre-mRNA is stabilized via a modification at the 5' end in which a 7-methylguanosine cap is added. In addition to stabilizing the growing pre-mRNA, the newly added cap also prevents the pre-mRNA from exonuclease activity [11].

The mechanism of pre-mRNA splicing is enhanced through direct interaction with the cap-binding complex (U4/U6-U5 tri-snRNP) at the 5' intron site. The first step in the splice site selection involves the recognition of three conserved sequences near the exon/intron conjunction by the spliceosome. U1 snRNP binds to the pre-mRNA at the donor (5’ splice site, ss). The 3' splice site contains an adenine nucleotide also known as the branch site (BS) and is located just upstream of a variable-length of

polypyrimidines (polypyrimidine tract). The branch site (BS) is vital to the formation of the lariat structure during splicing [12].

Additional *cis*-elements located within both intronic and exonic regions can promote the inclusion or exclusion of exon and introns in multiple combinations, promoting significant isoform diversity. Therefore, these *cis*-elements can contribute to a variety of isoform variations, including constitutive splicing, selection of competing spicing sites, and can perform tissue-specific splicing. The mechanism of action for *cis*-elements within the intronic and exonic regions is to facilitate binding of spliceosome components and thereby initiate/block the splicing program [13]. Exon splicing enhancers (ESEs) and intron splicing enhancers (ISEs) reside in the exon and introns, respectively, and promote the inclusion of exons or introns. Exon splicing silencers (ESS) and intron splicing silencers (ISS) also reside within exon and introns, respectively, promoting exonic or intronic splicing. The RNA *cis*-elements promote the recruitment of non-spliceosome RNA *trans*-acting factors, which regulate the mechanism of exon/intron inclusion-exclusion. These *cis*-elements have been described as crucial for splice site selection [14].

Although alternative splicing regulation is not fully understood, spice site selection is regulated by the interaction of *cis*-elements with *trans*-acting splicing factors (*trans*-factors). These *trans*-acting factors include serine/arginine (SR)-rich proteins, heteronuclear ribonuclear proteins (hnRNPs), among others. These *trans*-acting splice factors bind to the ESE/ISE or ESS/ISS *cis*-elements within pre-mRNA and regulate exon/intron exclusion or inclusion. SR proteins typically bind to ESE *cis*-elements and allow spliceosome complex members to bind to 3' and 5' splice sites allowing for the

inclusion of the exon [15]. Conversely, hnRNPs typically bind ESS *cis*-elements and inhibit spliceosome complex members from interacting with 3' and 5' splice sites, usually resulting in exon exclusion. In order to elicit their specific function, these proteins contain unique RNA recognition motifs (RRMs), which include quasi and pseudo-RRM variants, serine/arginine-rich (RS) domains, zinc finger, and K homology (KH) domains [16]. The domains of the proteins are usually arranged as tandem repeats. In this manner, splicing factors can recognize different RNAs with varying specificity and variability. Additionally, the potential for binding of these *trans*-factors to *cis*-elements contained within pre-mRNA is highly dependent upon the presence or absence of post-translational modifications [17].

Serine/Arginine-Rich Splicing Factor Proteins

Serine/Arginine-rich (SR) proteins are RNA-binding proteins that function as facilitators of spliceosome assembly, and are required to modulate pre-mRNA alternative processing, and have a role in regulating the fate of cytoplasmic mRNA. The SR-protein family consists of twelve family members [18] all of whom consist of one or two RRM domains in their n-terminal RNA binding domain (RBD). They also contain the characteristic namesake sequences of arginine and serine dipeptides located in their arginine/serine (RS) domain closer to the C-terminus. SR proteins are multifunctional and have been shown to be important regulators of transcription, alternative splicing, and translation. SR proteins link these pathways via interactions with Pol II, the spliceosome, and binding mature RNA in the cytoplasm [19]. These interactions are controlled by the phosphorylation status of serine residues in the RS

domain, ranging from non-phosphorylated to hyper-phosphorylation. This mechanism of post-translational modification is highly regulated and allows for stability, RNA binding specificity, and also enhances specific protein-protein interactions in the spliceosome formation process [20].

Although it is known that SR proteins regulate AS through interactions and recruitment of the spliceosome to RNA binding sites, a universal mechanism for SR proteins is as yet undescribed. This may be attributed to the pleiotropic functional nature of these proteins in cellular processes. In addition to their role in regulating alternative splicing, SR proteins have been implicated as functional contributors to a plethora of cellular processes such as chromatin interactions and cross-regulatory feedback of non-coding RNA (ncRNA) [21]. Since SR proteins are a crucial regulators of AS and are multifunctional RNA binding factors, aberrant expression of SR proteins can lead to numerous disease states [22]. This is partly due to SR proteins' vital role in regulating the transcriptome via RNA splicing regulation. Indeed, multiple laboratory groups have noted aberrant expression of SR proteins in multiple cancer types [23].

Cytoplasmic Polyadenylation Element Binding Protein 2 (CPEB2)

Cytoplasmic polyadenylation element-binding protein 2 (CPEB2) belongs to the CPEB family of proteins that mediate polyadenylation of mRNA targets in the cytoplasm. CPEB proteins recruit cytoplasmic polyadenylation machinery and regulate the repression and activation of translation of targeted mRNA [24]. The CPEB protein family consists of four family members (1-4). CPEB proteins are divided into two categories based on protein sequence alignment similarity [25]. CPEB1 is the most

distinct of the CPEB family members, whereas CPEB2, CPEB3, and CPEB4 are closely related. An initial examination of CPEB2-4 via SELEX analysis indicated that CPEB2-4 proteins interact with different RNA cis-regulatory elements than the CPE sequence identified for CPEB1 [26]. However, further studies revealed that the CPEB2-4 proteins will bind the CPE consensus sequence, but these interactions are weaker than the CPEB1 interaction [27]. In other studies, CPEB3 was also shown to bind the CPE consensus sequence [28]. The first of the CPEB family members to be discovered and characterized was CPEB1. In *Xenopus laevis* oocytes, CPEB1 was identified as a translational regulator of mRNA species important in oocyte maturation, embryonic and neuronal development [29]. CPEB2-4 regulate translation of mRNAs important in memory, learning and stress response pathways [30]. Since their initial discovery, CPEB proteins have been described as important regulators of translation in multiple cell types.

Structurally, all CPEB proteins contain three regions: an N-terminal domain characterized as intrinsically disordered, highly structured c-terminal domains consisting of two RNA recognition motifs (RRMs) in close proximity to each other and a cysteine-histidine-rich region that resembles a zinc finger at the end of the C-terminus. The RRMs of CPEB proteins bind a consensus cytoplasmic polyadenylation element (CPE) sequence, the most common of which is UUUUUAU, located in the 3' untranslated regions (UTR) of mature mRNA transcripts [31]. There is debate as to the manner in which the RRMs of CPEB proteins bind the CPEs of mRNA. Traditionally, the RRMs of CPEBs were thought to individually bind CPEs in mRNA UTRs; however, there is evidence that the RRMs may bind to a single CPE site in a manner termed "fly-trap"

[32]. Interestingly, evidence suggests that CPEB proteins, specifically CPEB1 and CPEB4, bind the CPEs of target mRNAs in the nucleus. The CPEB/mRNA complex is then exported to the cytoplasm [33]. In this manner, CPEB proteins can exhibit tight control of the expression of mRNAs which are crucial to stress responses.

As previously mentioned, the N-terminal end of CPEB proteins is characterized as intrinsically disordered. The N-terminal domain sequences of CPEB proteins vary greatly amongst family members. In CPEB1, the N-terminal region was shown to bind to proteins that belong to the polyadenylation complex, specifically GLD2 and PARN, as well as the cap-binding initiation factor eIF4E [34]. In this manner, CPEB1 regulates the translation of target mRNA. Specifically, Richter and colleges showed that CPEB1 binds to the 3'UTR CPE site in mRNA, interacts with the polyadenylation complex proteins GLD2 and PARN [35]. PARN, a deadenylating enzyme, outcompetes GLD2, a poly(A) polymerase, therefore inhibiting the polyadenylation of the mRNA. CPEB1 also interacts with eIF4E, a cap-binding protein, ultimately inhibiting the eIF4E/eIF4G interaction from recruiting the 43s preinitiation complex and halting translation initiation. Upon phosphorylation of CPEB1 via Aurora kinase, CPEB1 undergoes a conformational change and disassociation with PARN. This allows for polyadenylation of the mRNA by GLD2. Furthermore, this conformational change releases eIF4E, allowing interaction with eIF4G, and recruits the 43s preinitiation complex to the 5'cap initiating translation [36]. However, as mentioned earlier, the mechanism for regulating mRNA translation by the CPEB (2-4) family members differ from CPEB1 due, in part, to CPEB (2-4) proteins missing the Aurora kinase phosphorylation site in the N-terminal domain [37].

Although translational regulation of mRNA by CPEB2 differs for CPEB1, some progress towards understanding its mechanism has been reported. Chen and colleagues reported that CPEB2 inhibits translation of hypoxia-inducible factor one-alpha (HIF1α) by binding to its 3'CPE region in conjunction with binding to the eEF2 elongation factor in Neuro 2A (N2A) murine cells. Upon oxidative stress, a GTP/GDP reaction releases eEF2, and the ribosome is able to continue translation [38]. A mechanism of translational regulation by CPEB2 was also described for Twist Family BHLH Transcription Factor 1 (TWIST1) in MCF-10A non-malignant breast epithelial cells by Nairisgami and colleagues. They reported a mechanism in which CPEB2 and CPEB1 regulate TWIST1 translation by binding to CPE sites, of which there are two, within the TWIST1 3'UTR and allowing polyadenylation of TWIST1 mRNA. The binding of both CPE sites allows for the inclusion of miRNA regulatory binding sites in the 3'UTR, leading to reduced TWIST1 protein translation [39].

Epithelial-Mesenchymal Transition

A characteristic of epithelial cells is that they maintain apical-basal polarity, including contact with adjacent cells. The contact with adjacent cells is accomplished through tight junctions, desmosomes, and adherens junctions [40]. On the other hand, characteristics of mesenchymal cells include separation from surrounding cells, a lack of basal lamina, which separates them from adjacent tissue, and they do not contain distinctive apical-basolateral polarity that is characteristic of epithelial cells [41].

The epithelial-mesenchymal transition (EMT) is generally defined as the acquisition of mesenchymal characteristics by epithelial cells. This process occurs

naturally in several biological processes such as embryonic development and in tissue regeneration; however, an aberrant form of EMT occurs in cancer progression [42]. As a contributor to cancer progression, EMT can promote the malignant phenotype in primary solid tumors, allowing heightened invasiveness and metastasis [43]. However, secondary tumor histological characteristics tend to resemble that of the primary tumor [44]. The histological feature that connects the primary and secondary tumors is the EMT.

Furthermore, the reversibility of EMT has been demonstrated in mesenchymal tumor types and has been termed mesenchymal-epithelial transition (MET) [45]. The molecular events contributing to the advent of EMT are a loss of adherent junctions coinciding with the downregulation of cytokeratin and E-cadherin and increases in mesenchymal associated markers such as N-cadherin, vimentin, and fibronectin [46]. Furthermore, EMT promotes the increase in the invasive phenotype and is associated with anoikis resistance.

In cancer, EMT is a common mechanism driving the invasive and metastatic progression of tumors. Indeed, a role for EMT in tumorigenesis has been reported in numerous cancer types, including lung, prostate, liver, pancreatic, breast, and non-small cell lung cancers [47]. Widespread changes in ECM-related proteins, including collagens, integrins, and metalloprotease, which are activated by signaling cascades in Ras, Wnt/β-catenin, and Src pathways are the initial step in EMT [48]. Dismantling of the basement membrane by tumor cells at the stromal interface combined with activation of matrix contribute to the invasive phenotype in cancer. The process allows for increased cell motility and angiogenesis, setting the stage for systemic escape. For

the cell to induce motility during EMT, cytoskeletal rearrangements are necessary. This is accomplished through the upregulation of Cofilin 1 (CFL1). CFL1, in turn, binds F-actin filaments, and regulation of cytoskeleton dynamics is achieved [49]. The transcription factor TGFβ1, which is a known activator of EMT, induces CFL1 and regulates the assembly/disassembly of microfilaments necessary for cell motility [50]. vimentin is another filament protein essential in the EMT process which is upregulated in tumor cells. Vimentin acts to stabilize collagen RNAs which expression contribute to high collagen levels, a typical characteristic of mesenchymal cells [51]. Collectively, the shift towards the increased expression of these key proteins drives the migratory and invasive phenotype associated with EMT.

The activation of EMT, and the orchestrated increase in the proteins responsible, including the phenotypic changes, are dependent upon a plethora of activated transcription factors. One such transcription factor critical to the EMT process is TWIST1. Typically, TWIST1 orchestrates the mesoderm formation in embryotic maturation [52]. However, in EMT, TWIST1 regulates a necessary process termed "cadherin switching," in which E-cadherin is repressed, and N-cadherin is activated [53]. Notably, increased expression of TWIST1 is reported in numerous cancers and invasive cell lines. The high levels of TWIST1 are also associated with an increase in the aggressive phenotype, reduced survival, and higher incidences of recurrence [54].

Another important transcription factor that contributes to EMT is the hypoxia-inducible factor (HIF). The tumor microenvironment typically consists of hypoxic conditions because the aggressive growth of tumor cells often outpace the supply of oxygen available in the blood supply. To overcome the hypoxic conditions, the cell

responds with HIF activation. Activation of HIF is accomplished through the dimerization of the HIF1α and HIF1β subunits. The combined subunits create an activated transcription factor that targets hypoxia-related genes, regulating numerous pathways, including survival, motility, and angiogenesis [55]. The hypoxic response also contributes to the activation of EMT and the metastatic cascade in numerous cancers, including ovarian, breast, and glioblastoma [56].

Anoikis Resistance

Perhaps the deadliest clinic-pathological phenotype in breast cancer is metastasis. It is estimated that ninety percent of all cancer-related deaths are attributed to metastasis [57]. An essential precursor in the early stages of metastatic transformation is the acquisition of Anoikis resistance (AnR). Typically, cells will undergo apoptosis after they lose contact with their extracellular matrix or their neighboring cells. This cell death process is called "anoikis," a term coined in 1994 by Frisch and Francis and means the "state of being without a home" [58]. Tumor cells that acquire malignant potential have developed mechanisms to resist anoikis and thereby survive after detachment from the primary tumor. Anoikis-resistant cells also demonstrate a hyper-activation of mitogenic signaling and EMT [59]. Anoikis regulation is dependent upon crosstalk between integrin-ECM attachment and signaling of growth factors critical to EMT. The role of integrins is to act as intermediaries in signaling cascades that link the extracellular matrix with the intercellular network through the combining of integrin-activated signaling such as focal adhesion kinase (FAK), ERK1/2, MAPK, and phosphatidylinositol-3-kinase (PI3K) [60]. The survival of cancer cells

during the acquisition of AnR is also dependent upon changes to the integrin profile. As a promoter of apoptosis, integrin αvβ5 is downregulated while an increase in the expression of αvβ6 activates pathways such as PI3K-AKT, which promote survival [61].

As an additional level of AnR regulation, autophagy, a process characterized by the degradation of proteins and organelles via lysosomes in response to cellar stresses and promoting survival, can mitigate AnR [62]. Pre-metastatic migrating tumor cells which demonstrate autophagy can delay apoptotic activation. Activation of this autophagic pathway allows temporary protection to the tumor cell, during which circulating tumor cells happen upon their destination and activates ECM reattachment [63]. Although tumors shed a large number of cells, only those that have acquired AnR and molecular plasticity will survive their hostile detachment and settle at distant sites and reactivate growth [64].

Cap-Independent mRNA translation

The cap-dependent mechanism of translation is the most common form of mRNA translation in eukaryotes. During transcription, an m7G (7-methyl guanosine) cap is added to the 5' end of the pre-mRNA [65]. During cap-dependent translation initiation, the m7G cap provides a recognition site for eukaryotic initiation factor 4E (eIF4E). This initiation factor is part of a larger eIF4F complex consisting of several initiation factors, including eIF4E, eIF4G, and eIF4A. The binding of eIF4E, and subsequently the eIF4F complex, facilitates the recruitment of another large complex, namely the pre-assembled 43S preinitiation complex (PIC) [66]. Components of the 43S PIC complex include the 40S ribosomal subunit, the initiation factors eIF1, eIF1A, eIF3, and eIF5 [67].

Additionally, the eIF4F complex recruits the eIF2/Met-tRNAi/GTP ternary complex. Upon assembly of the complexes, the 43S PIC complex scans the 5' untranslated region (UTR) of the mRNA until it encounters the start codon and recruitment of the 60S large ribosomal subunit is initiated. Combined, the 43 PIC complex and the 60S larger ribosomal subunit from the 80S ribosome and peptide synthesis occurs [68].

Some cellular conditions are not conducive to the cap-dependent mechanism of mRNA translation. One such example is during viral infection, as a variety of viral mRNAs are robustly translated by the host cell despite the absence of a 5' m7G cap structure [69]. In these instances, the alternative mechanism known as cap-independent translation can allow for translation using what is known as internal ribosome entry site (IRESs). Genes that allow the cap-independent translation are typically involved in cellular stress or viral infection, which suggests a role for IRES-mediated cap-independent translation under these cellular events [70].

Internal ribosome entry sites were first discovered in viruses of the *Picornaviridae* family, including the encephalomyocarditis virus and poliovirus (PV) [71]. Future discoveries of IRESs were later found in pathogenic viruses such as the hepatitis C virus (HCV) and human immunodeficiency virus (HIV) [72]. Many of these viral IRESs contain both similar secondary structures and can mechanisms of translation. However, the functional activity of these IRESs is often dependent upon other factors called IRES-transacting factors (ITAFs) [73]. The IRES sites are typically located in the 5' UTR of mRNA, downstream from the 5' cap structure. Although the mechanism of translational initiation is unclear, there is evidence that IRESs whose secondary structures differ require different ITAFs to associate with the 40s ribosomal subunit [74]. In general, the

activation of IRES cap-independent translation forgoes the formation of the eIF4E cap-binding and PIC recruitment to the 5' m7G cap allowing for the recruitment of the 40s ribosomal subunit to the IRES site by ITAFs, therefore, initiating peptide synthesis [75].

In eukaryotes, IRESs were also found in cellular mRNA. In fact, it is now estimated that ten percent of cellular mRNAs contain IRES sites [76]. Many of these mRNAs are essential in stress response pathways such as mitosis, hypoxia, and apoptosis [77]. Compared to viral IRESs, cellular IRESs have fewer RNA structures and appear to contain very little sequence conservation. Cellular IRESs are typically divided into two types depending on the mechanism in which the ribosome is recruited. The first type, type I, ribosomal interaction is dependent upon ITAFs bound to N-6-methadenosine (m^6A) and RNA binding motif *cis*-elements. Type II IRESs differ from type I because they contain a short *cis*-element that joins the 18S rRNA for ribosome recruitment [78].

Almost all known cellular IRESs are dependent upon the assistance of numerous ITAFs, which are typically RNA binding proteins, for recruitment of ribosomes and the initiation of translation. Most ITAF proteins are either nuclear or both nuclear and cytoplasmic shuttling proteins, which suggests that ITAFs engage in crosstalk between the transcription and translation processes [79]. An example of one such ITAF is the polypyrimidine tract-binding protein (PTB) which promotes the activity of numerous cellular IRESs. Functionally, the PTB protein promotes the ideas of crosstalk by ITAFs in the transcription and translation process as PTB is described as a regulator of mRNA splicing and transport [80]. The mechanism in which describe how ITAFs facilitate cap-independent IRES translation remains unclear. There is evidence that ITAFs may

function in an RNA chaperone capacity, remodeling the structure around the IRES, which enhances the ability of ribosomes to bind to the IRES [81].

It is known that under numerous cellular conditions such as hypoxia, heat shock, mitosis, DNA damage, nutrient depletion, and apoptosis, global translation is downregulated. These cellular conditions also coincide with characteristics of tumor progression, such as metastasis. However, tumors also need the active translation of specific proteins to cope with stresses associated with tumor progression. Many of the precursor mRNAs that code for these proteins contain IRESs, which suggest that cap-independent IRES-mediated translation plays a role in tumor progression. Indeed, there is evidence to support this theory as IRES-mediated translation was shown to promote the survival of tumor cells in inflammatory breast cancer and ovarian cancer [82]. In a 3D ovarian cell culture, treatment with a PI3K/mTOR inhibitor (downregulates cap-dependent peptide synthesis) leads to high cell death via apoptosis. However, cells that exhibited resistance to the inhibitor were shown to overexpress IRES-containing proteins [83]. These studies demonstrate that cap-independent IRES translation is crucial for tumor progression.

Triple-Negative Breast Cancer (TNBC)

Breast cancer is currently the most diagnosed cancer in women accounting for more than 1:8 new cancer diagnoses each year [84]. It is also currently the second leading cause of cancer-related deaths in women [85]. In 2021, there were roughly 330,000 cases and over 43,000 deaths in the US [86]. In 2020, there were approximately 2.3 million diagnosed cases of breast cancer and over 500,000 deaths

from this disease [87]. Triple-negative breast cancer (TNBC) is a highly aggressive and deadly form of breast cancer. TNBC accounts for roughly 15% of all diagnosed breast cancer cases each year. Also, the five-year survival rate of TNBC is substantially lower than all other types of receptor-positive breast cancers [88]. TNBC is also more common in women under forty and African American women [89]. TNBC also has higher growth and metastatic potential and a higher incidence of recurrence and thus poorer outcomes compared to receptor-positive subsets [90].

The term "triple-negative" refers to the surface receptor expression pattern of the cancer cells. It indicates that the cells lack the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (ERBB2, commonly referred to as HER2) [91]. TNBC belongs to the basal-like subclass of breast cancers characterized by an expression profile similar to that of myoepithelial normal mammary cells. Basal tumors frequently assimilate to triple-negative (TN) breast cancers. They display epidemiological and pathological features distinct from other subtypes. Furthermore, another subset of the basal classification is termed "claudin-low" [92]. Claudins are tetra-span transmembrane proteins of tight junctions. They are important in determining the barrier properties of cell-cell contact existing between the plasma membranes of two neighboring cells [93]. Compared to hormone receptor-positive breast cancer subtypes, TNBC displays considerable genetic complexity and tumor heterogeneity, leading some to suggest that "triple-negative breast cancers" is a more suitable name [94].

Genetically, TNBC is typically heterozygotes for P53, although there is evidence that advanced stages of the disease are p53 null [95]. About 70% of the triple-negative breast cancer cases are BRCA1 null [96]. Since TNBC lacks traditional

chemotherapeutic targets, treatment for TNBC has mainly been unsuccessful, leading to poorer outcomes. Although some chemotherapeutics, such as taxens, platinum agents, and PDL1 inhibitors, show initial success, TNBC tends to acquire resistance leading to the lowest overall five-year survival rate of all breast cancer subtypes [97]. Furthermore, TNBC's high propensity towards metastasis, in conjunction with lacking known therapeutic targets, makes the discovery of novel treatment options imperative.

Alternative mRNA Splicing of CPEB2 in TNBC

Although the metastatic potential of TNBC provides unique treatment challenges, it can also offer opportunities to identify novel therapeutic targets since metastasis requires significant transcriptome alterations to drive the drastic phenotypic changes [98]. The transcriptome alterations influencing TNBC progression result in shifts in alternative pre-RNA splicing (AS). Changes in AS alter the transcriptome profile leading to significant changes in the proteome. Alternative splicing of CPEB2 produces two main isoforms: CPEB2A (lacking exon 4) is constitutively expressed, is anti-neoplastic, and has been shown to inhibit translation of hypoxia-inducible factor 1-alpha (HIF1α) and twist related protein 1 (TWIST1), and CPEB2B (exon 4 included) which activates the translation of HIF1α and TWIST1 mRNA species driving anoikis resistance (AnR) and metastasis [99]. The studies presented herein are meant to elucidate critical aspects of AS dysregulation responsible for the aggressive metastatic potential of TNBC in the hope of furthering the identification and development of novel strategies targeting TNBC progression. Elucidation of the effects of AS dysregulation, and mechanisms governing such, in conjunction with continued marked advances in modern

medicine, provide the measured optimism towards improving TNBC treatment and outcomes.

Hypothesis and Specific Aims

The research presented in this dissertation two important scientific questions. First, what RNA *cis* and *trans*-splicing regulatory elements are responsible for regulating CPEB2 alternative splicing? Secondly, by what mechanism do the CPEB2A/B proteins regulate the translation of targeted mRNA species? Two hypotheses were examined to investigate these questions. For the first question, our studies investigated whether SRSF3 is the trans-splicing factor responsible for modulating the expression of CPEB2B via binding the pre-mRNA exon splicing enhancer *cis*-element located in exon four of CPEB2B. To address the second question, this research proposed that CPEB2A and CPEB2B regulate translation of mRNAs via interaction with CPE binding sites within the 3'UTR of mRNA and recruitment of polyadenylation complex proteins. The mechanism of increased regulation by CPEB2B results in novel interactions of exon four with ITAF promoting cap-independent IRES-mediated peptide synthesis.

There are two specific aims contained in this dissertation. The first specific aim attempts to characterize the mechanism for the inclusion of exon four in the CPEB2B isoform via splicing regulation by SRSF3. The second is to elucidate the mechanism of translational control of mRNA by the CPEB2A and CPEB2B isoforms. A rigorous effort has been made to determine well-described connections among the proposed hypotheses and specific aims to reveal a prescribed mechanism influencing the

alternative splicing of CPEB2 and its importance in biological events in TNBC progression.

CHAPTER 2: SERINE/ARGININE-RICH SPLICING FACTOR 3 MODULATES ALTERNATIVE SPLICING OF CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEIN 2

Abstract

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer with a low 5-year survival rate and high metastatic rate. Our laboratory has elucidated a role for which the alternative RNA splicing (AS) of cytoplasmic polyadenylation element-binding protein 2 (CPEB2) contributes to the metastatic phenotype in TNBC. The alternative spliced variants of CPEB2 differ via the inclusion/exclusion of exon four. In these studies, the mechanism governing the regulation of exon four inclusion/exclusion was investigated. Specifically, we found that the RNA trans-acting factor serine-arginine splicing factor 3 (SRSF3) directly interacted with exon four of CPEB2. We also identified a consensus sequence for SRSF3 in exon four, which ablated SRSF3 exon four interaction when mutated. Increased expression of SRSF3 protein was also determined in TNBC in conjunction with the acquisition of anoikis resistance (AnR). This finding also correlated with a reduced CPEB2A/B expression profile favoring an increase in the CPEB2B isoform. Additionally, SRSF3 knock-down via siRNA resulted in decreased exon four inclusion, namely an increase in the CPEB2A isoform, corresponding to a decrease in CPEB2B (exon4 inclusion). These findings were consistent with siSRSF3 treatment in a wild-type CPEB2 exon four minigene and a

mutant CPEB2 minigene containing an ablated SRSF3 RNA *cis*-element. Also, downregulation of SRSF3 resulted in reacquisition of anoikis sensitivity in TNBC and ectopic expression of CPEB2B "rescued" this phenotype. Notably, The Cancer Genome Atlas (TCGA) analysis indicated a positive correlation between SRSF3 expression and reduced CPEB2A/B ratios in the most aggressive forms of breast cancer. These findings suggest that SRSF3 is at least partially responsible for regulating CPEB2 AS and promoting an aggressive phenotype in TNBC.

Introduction

TNBC is one of the four major breast cancer subtypes and is characterized histologically based upon its lack of the epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) [100]. Compared to the breast cancers subclass, which express these hormone receptors, TNBC exhibits heightened genetic complexity and tumor heterogenicity, rendering treatment with targeted therapies ineffective [101]. Traditional treatment options for TNBC include neoadjuvant chemotherapy and surgery, which initially prove relatively successful. However, TNBC commonly acquires resistance to chemotherapies resulting in the lowest five-year progression-free and survival rates of all the breast cancer subtypes [102]. This characteristic, in conjunction with the aggressive nature of TNBC, underlines the necessity of the discovery of novel targets and therapeutic strategies.

TNBCs characteristic molecular and histological complexity suggests early stage shifts in gene expression may contribute to the tumorigenic propensity of the cells [103]. Regulatory pathways which can contribute to the genetic alterations promoting breast

cancer progression include changes in alternative pre-mRNA splicing (AS). While AS is well documented to promote the expression of cancer-specific variants, alterations in the proteins which regulate AS, mRNA splicing factors occur on a broad scale in TNBC [104]. These alterations contribute to the increased complexity of exon assembly during AS events. Indeed, increased expression of mRNA trans-splicing factors can contribute to the malignant phenotypes in cancer via altered regulation of downstream AS pathways [105].

Our lab recently showed that AS of cytoplasmic polyadenylation element-binding protein 2 (CPEB2) directly regulates TNBC metastasis [106]. Specifically, a splicing ratio shift favoring increased expression of the CPEB2B isoform, which contains exon four, contributed anoikis resistance (AnR), detachment-induced cell death, promoting tumor metastasis to the lung. The pro-neoplastic transformative role for CPEB2B differs from the CPEB2A variant, which demonstrated reduced tumor growth and metastasis in TNBC via translational repression of the TWIST1 and HIF1α transcription factors. In non-tumorigenic breast cancer tissue, the CPEB2A/B isoform ratio is usually high, favoring the CPEB2A transcript. However, in TNBC, including cells that acquired the AnR phenotype, the ratio was reduced, coinciding with increased CPEB2B transcription [107].

Cytoplasmic polyadenylation element-binding protein 2 (CPEB2) belongs to the CPEB family of proteins that mediate polyadenylation of mRNA targets in the cytoplasm. The CPEB protein family consists of four family members (1-4). CPEB proteins are divided into two categories based on protein sequence alignment similarity. CPEB1 is the most distinct of the CPEB family members, whereas CPEB2, CPEB3, and

CPEB4 are closely related [108]. Structurally, all CPEB proteins contain three regions. They have an N-terminal domain characterized as intrinsically disordered, highly structured c-terminal domains consisting of two RNA recognition motifs (RRMs) in close proximity to each other and a cysteine-histidine region that resembles a zinc finger [109]. The RRMs of CPEB proteins bind a consensus cytoplasmic polyadenylation element (CPE) sequence, the most common of which is UUUUUAU, located in the 3' untranslated regions (UTR) of mature mRNA transcripts [110]. The most studied and well described member of the CPEB family of protein is CPEB1 which has been shown as a translational regulator targeted mRNA species. Specifically, Richter and colleges showed that CPEB1 binds to the 3'UTR CPE site in mRNA, interacts with the polyadenylation complex proteins GLD2 and PARN. PARN, a deadenylating enzyme, outcompetes GLD2, a poly(A) polymerase, therefore inhibiting the polyadenylation of the mRNA [111]. There is limited research pertaining to the other CPEB family members; however, CPEB2 has been described as essential for mitotic cell division [112].

Possible AS regulators which modulate the AS of CPEB2 include the serine/arginine (SR)-rich protein family of proteins. SR proteins are RNA trans-acting factors that regulate splice site selection via exon exclusion/inclusion [113]. This is dependent on the binding affinity of the RNA recognition motif (RRM) and binding domain contained in the SR protein to mRNA *cis*-elements, termed exon splicing enhancers (ESE), located within exons, and mediation of spliceosome assembly [114]. An important and well described member of the SR protein family, SRSF3, has been reported in increased levels during hypoxic and oxidative stress events [115]. These

events are also commonly found in tumors in which *trans*-splicing mRNA factors can regulate the AS of genes important in cellular processes such as cell cycle progression and proliferation [116].

In this study, our laboratory identified an increase in the occurrence of SRSF3 and the CPEB2 variant in TNBC. Our lab has also identified an SRSF3 consensus binding motif in exon four of CPEB2, in which inclusion promotes the CPEB2B isoform in TNBC. These findings provide evidence indicating an SRSF3 mediated splicing event promotes increased CPEB2 isoform expression linked to AnR and metastasis in TNBC.

Materials and Methods

Cell Culture and Reagents:

MDA-231, MDA-468, and BT549 cells were obtained from ATCC (Manassas, VA) and maintained in RPMI (Invitrogen). The cell lines were supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% Penicillin / Streptomycin (Invitrogen). Cell lines were maintained in a 95% air / 5% CO2 incubator at 37°C. Cells were passaged once every 3-5 days (~90% confluence), and all experiments were performed during the first 12 passages.

Western Blotting:

Total protein (5-10 µg) was electrophoretically separated on 7.5% or 12% SDS-polyacrylamide gels. Samples were transferred electrophoretically to PVDF membranes, then probed with the appropriate antibody as described previously [117].

Antibodies were purchased from Cell Signaling with the exception of SRSF3 (ThermoFisher, Clone ID: 7B4).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR):

Primer/probe sets were designed for CPEB2A (forward, 5′-GTGTTCAGAACAGACAACAATAG-3′; reverse, AATATCGATAAGGGAATTTTCC; Probe, 5′-CCCTTACAGGATCGAAGTAGAATGTATGACAG-3′) and CPEB2B (forward, 5′-CCTGGTCTATTCTGGATGTTCC-3′; reverse, 5′-ACCCTTACAGGTGAGATCTAGT-3′; probe, 5′-TCACTCCAAGATAGTTGGTGCACTGC-3′) and purchased from Integrated DNA Technologies. PCR was performed as described [117]. cDNA synthesis was accomplished using the Superscript III kit (Life Technologies) according to the manufacturer's instructions. Samples were amplified with the Bio-Rad CFX Connect qPCR machine and then calculated using the standard curve method.

siRNA Treatment and Plasmid Transfection:

All transfections were performed in triplicate with 6-well tissue culture dishes. Validated Silencer Select siRNA towards SRSF3 (s12732 or s12733) or non-targeting control (ThermoFisher Scientific) were utilized in this study at 30 nM concentrations and transfected using Dharmafect 4 transfection reagent (Dharmacon) as described previously and to manufacturer's specifications. The plasmid transfections were accomplished using the Effectene system (Qiagen) according to the manufacturer's instructions and using 0.5 to 1.0 μg total DNA per well.

Anoikis Resistance Assay:

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

Competitive Quantitative RT-PCR:

cDNA was synthesized as previously described [118], and then competitive PCR was performed on the cDNA samples using the following primers: endogenous CPEB2A or CPEB2B isoform amplification FWD primer 5’-GCAGCAGAGGAACTCCTATAAC-3' and reverse primer 5’-CAAAGAGTGCATATTCAAACTGTCA-3', minigene specific CPEB2A or CPEB2B isoform amplification forward primer 5’-CAGAACAGACAACAATAGTAATACACTC-3' and reverse primer 5’-AGGGGCAAACAACAGATGG-3'. PCR conditions for the endogenous gene amplification consisted of a denaturing step, 98 °C for 30 seconds, followed by 25 cycles of a second denaturing step at 98 °C 10 sec., 50 °C annealing for 30 sec., 72 °C extension for 1 min., and final extension step at 72 °C for 5 mins. Minigene-specific amplification conditions were identical, and 20 cycles were used. All PCR reactions were amplified with standard Taq polymerase (New England Biolabs) with products run on 5% polyacrylamide-TBE and stained with SYBRgold (ThermoFisher Scientific).

RNA Binding Assays:

Full length Wild-type biotinylated RNA CPEB2 exon sequence (Bi: 5’-GTGAGATCTAGTTTGCAGTTGCCAGCTTGGGGCTCAGATTCACTCCAAGATAGTTGGTGCACTGCAGCCGGAACATCCAGAATAGACCAG-3') or mutant sequence (MUT, see Fig.1) were incubated with recombinant SRSF3 (lsBio) and RNA-bound proteins were precipitated as described [118]. Samples were subjected to immunoblotting with an SRSF3 antibody.

Electrophoretic Mobility Shift Assay:

FITC conjugated full length (Fig.1A) or partial (Fig.1E) wild-type or mutant CPEB2 RNA sequences were subjected to EMSA as described [118].

Construction of Minigene Plasmids:

Genomic regions of CPEB2 spanning exons 3,4 and 5 were investigated in this study. The template DNA was amplified from the RPCI-11 HS BAC Clone (ThermoFisher, Clone ID: 629A7) using two different 1.7 kilobase fragments. PCR reactions utilized forward and reverse primers in order to amplify the exon 3, intron 3, exon 4, and partial segment of intron 4 regions with the following sequences 5’-AAACGGGCCCTCTAGATTTCCCTAGCCTCTTCTGA-3' and 5’-GGAAGGAATGCTAGATGACTAACGGTTTCTCCATA-3'. The second fragment was amplified with forward and reverse primers targeting a region of intron 4 directly upstream of exon 5, including all but the last three codons of exon 5. The forward and reverse PCR primers used consisted of sequences 5’-TCTAGCATTCCTTCCGTCA-3'

and 5’-TACCGAGCTCGGATCCGGATCATGCTCTGCTCTC-3'. The genomic DNA fragments were amplified using standard PCR conditions and proofreading Taq polymerase (Phusion High Fidelity DNA Polymerase, New England Biolabs). The PCR conditions consisted of a denaturing step of 98 °C for 30 seconds followed by 30 cycles, 98 °C denaturing for 10 sec., 60 °C annealing for 30 seconds, 72 °C extension for 30 sec., and final extension step at 72 °C for 10 min. Fusion of amplified genomic DNA material with the pcDNA3.1$^{(-)}$ mammalian vector (Invitrogen) was accomplished using the In-Fusion HD cloning reaction (Clontech). With each step of PCR amplification and plasmid generation, CPEB2 minigene sequences were verified by Sanger dideoxy method (GenScript). The CPEB2 minigene insert was designed retaining the XbaI, and BamHI restriction sites at the 5' and 3' ends respectively. All of the primers used in cloning and analysis were synthesized by Integrated DNA Technologies. Site-directed mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's instructions along with primers containing the mutated CPEB2 ESE with forward and reverse sequences 5’-GGTGCACTGCAGCCGGAAGAGTCAGAATAGACCAGGTAGG-3' and 5'-CCTACCTGGTCTATTCTGACTCTTCCGGCTGCAGTGCACC-3'.

The CPEB2B-flag plasmid construct was previously reported.

Biostatistics:

Biostatistical analyses were carried out using either SPSS or R. Statistical tests used include 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 Association with Exon four of CPEB2.

Our lab previously reported that AnR acquisition in TNBC required the AS variant of CPEB2, CPEB2B, which includes exon four in the mature mRNA [119]. This discovery prompted an investigation into the mechanism which governs exon four inclusion promoting CPEB2B isoform production. To accomplish this goal, the employment of electrophoretic mobility shift assays (EMSAs) analysis, using MDA-231 cell extract, was used to investigate protein complexes that associate with exon four of CPEB. Using an unbiased proteomic approach analyzing complexes that bind exon four of CPEB2 identified the several RNA *trans*-acting factors, including SRSF3 and numerous hnRNP proteins including hnRNPF and hnRNPH1 (Fig. 1A; Table 1). Or these, SRSF3 was validated by employing an anti-SRSF3 antibody and cross-linking immunoprecipitation combined with quantitative real-time PCR (Clip-qRT-PCR). The SRSF3/exon four interaction was observed in MDA-231 parental (Par) and in the MDA-231 anoikis resistant (AnR) cells (Fig. 1B). Additionally, increased SRSF3 protein levels were detected in the TNBC cells, which acquired the AnR phenotype (Fig. 1B).

Crosslinking of total RNA by SRSF3 was seen in both MDA-231 Par and AnR cells. Additionally, SRSF3 interaction with exon four was seen in both Par and AnR cells via qRT-PCR, specifically targeting exon four of CPEB2. The SRSF3/exon four

interaction was significantly increased in the AnR cells (Fig. 1B). Furthermore, SRSF3 protein levels were increased in TNBC AnR cells (Fig. 1B).

Examination of consensus sequences contained in exon four elucidated the (C/U)(A/C/U)(U/A)(C/A/U)(A/C/U) sequence (Fig. 1C), and to investigate this association, a streptavidin-biotin affinity purification (SBAP) was employed using exon four of CPEB2 as "bait" and incubated with recombinant SRSF3 protein confirmed this interaction. However, mutation of the consensus sequence abolished the SRSF3 interaction. This assay demonstrated that SRSF3 specifically binds exon four of CPEB2 while competition for SRSF3 binding being achieved with non-biotinylated exon 4 RNA in excess concentration (100X); with no RNA/protein interaction observed with a nonspecific competitor RNA (Fig. 1C-D). Furthermore, mutation of the consensus SRSF3 sequence (CAUCC -> GAGUC) ablated the SRSF3/exon four association (Fig. 1C-D). Importantly, reduced levels of SRSF3 drastically reduced the amount of SRSF3, which bond the consensus SRSF3 sequence in TNBC cells (Fig. 1E). Together, these data show that SRSF3 associates specifically with the CPEB2 exon four SRSF3 consensus sequence, CAUCC.

Table 1: RNA trans-splicing factor candidate screen utilizing siRNA for CPEB2A/B Protein Ratio

Protein	CPEB2A/B ratio
No Treatment	7.40
Non-targeting control	6.96
SRSF3	19.0
hnRNP H1	9.78
hnRNP F	7.06
hnRNP H1/F	3.58

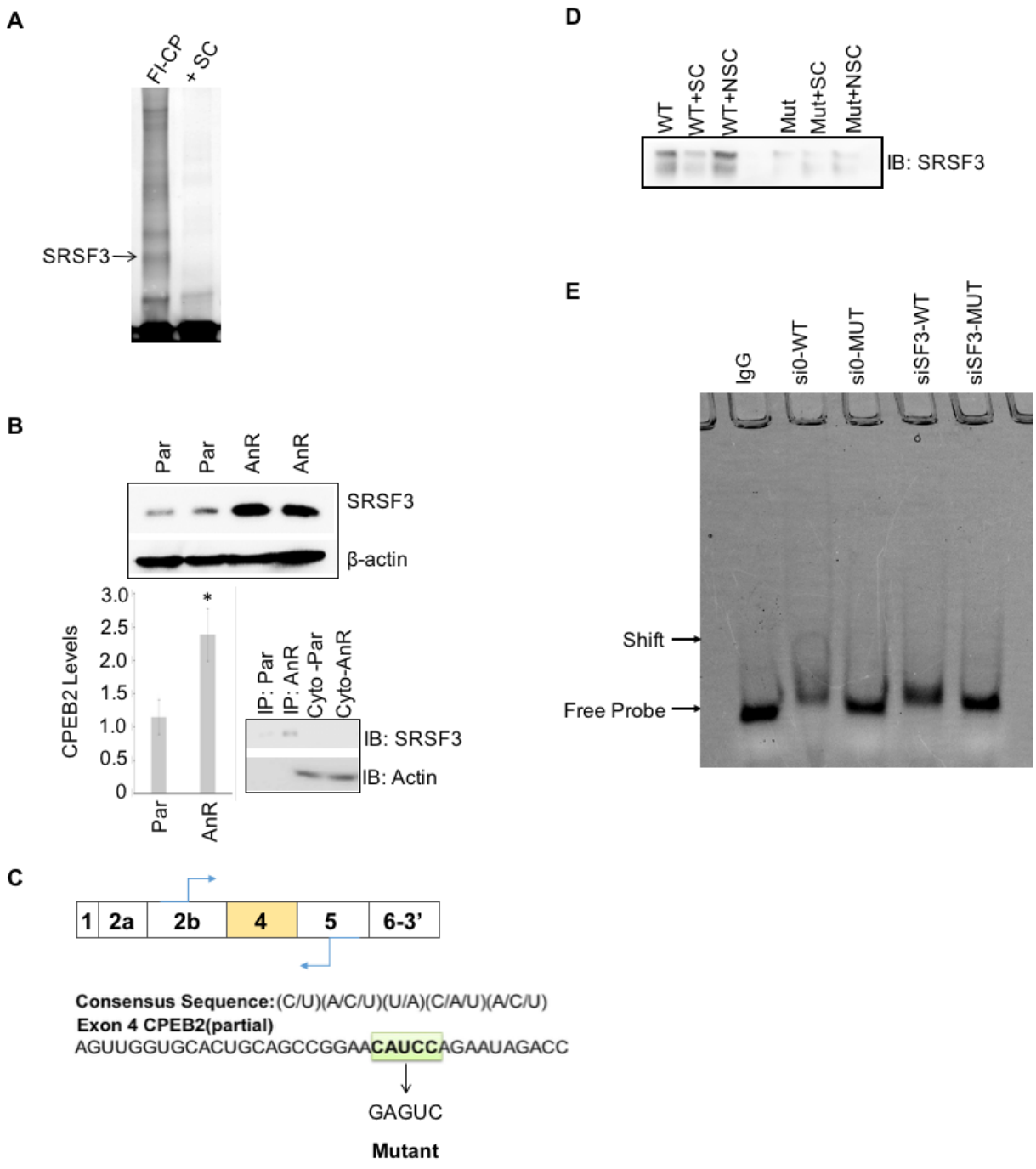

Figure 1: SRSF3/SRp20 specifically binds to exon four in CPEB2 pre-mRNA.

A) MDA-231 nuclear extract was incubated with either FITC-conjugated CPEB2 exon four sequence + "cold" nonspecific competitor (FI-CP) or pre-incubated with 100X "cold"

Figure 1 (continued): CPEB2ex4 as a specific competitor (+SC). Samples were then electrophoresed, and nuclear proteins bound to CPEB2 exon four RNA were extracted and subjected to proteomic analysis. B) SRSF3-specific antibody was used for CLIP-qRT-PCR to detect CPEB2 levels in either MDA-231 parental or AnR cells. Real-time PCR to CPEB2 at exon four was evaluated (data represented as n = 3 ± standard deviation (sd), * = $p < 0.05$). C) The consensus sequence for SRSF3 and a partial sequence of exon four highlighting the proposed SRSF3 binding site. D) SBAP assay was employed to detect SRSF3 bound to CPEB2 exon four. Recombinant SRSF3 was incubated with biotinylated exon four CPEB2 RNA oligos with either WT or the mutant SRSF3 ESE *cis*-element. The samples were incubated with either biotin-labeled CPEB2 exon four sequence + "cold" nonspecific competitor (NSC) or pre-incubated with 100X "cold" unlabeled CPEB2/exon4 as a specific competitor (+SC). E) An EMSA analysis of siRNA-depleted expression of SRSF3 in MDA-231 cells. EMSA labels correspond to MDA-231 cells treated with siRNA control, and then total protein lysates incubated with wild type CPEB2 exon four ESE RNA (si0-WT), siRNA control-treated cell lysates incubated with mutant exon four CPEB2 ESE RNA (si0-MUT), siRNA to SRSF3 treated cell lysates incubated with the wild type CPEB2 exon four ESE RNA (siSF3-WT), or siRNA to SRSF3 treated cell lysates incubated with mutant CPEB2 exon four ESE RNA (siSF3-MUT). Control samples were incubated with nonspecific IgG. The arrows indicate the electrophoretic shift of proteins bound by the anti-SRSF3 antibody or the presence of free FITC-conjugated CPEB2 exon four RNA probe as labeled.

Downregulation of SRSF3 Resulted in Reduced Inclusion of Exon Four into the Mature CPEB2 mRNA Transcript.

To investigate if SRSF3 regulated the exclusion/inclusion of exon four of CPEB2, siRNA targeting SRSF3, and the other *trans*-acting splicing factors which were elucidated via the unbiased proteomic screen, were employed (Fig. 2, Table 1). A total downregulation of ≥ 75% for each of the proteins was achieved in both the MDA-231 and MDA-468 cell lines (Fig. 2B & 2D, Table 1) with siRNA compared to the non-targeting siRNA controls. The downregulation of the RNA *trans*-acting factor SRSF3 resulted in a significant increase in the ratio of CPEB2A/B mRNA and proteins, favoring a decrease of inclusion of exon four in the mature CPEB2 mRNA (Fig. 2A & 2C). The reduction of SRSF3 protein in the MDA-468 cells, which characteristically express more endogenous CPEB2B isoform compared to MDA-231 (ratio of CPEB2A/CPEB2B 2.4 vs. 3.3, respectively), also resulted in an increase in the CPEB2A/CPEB2B ration (Fig. 2A & 2C). Together this data demonstrates that the RNA *trans*-acting factor SRSF3 enhances the inclusion of exon four in the mature CPEB2 mRNA transcript.

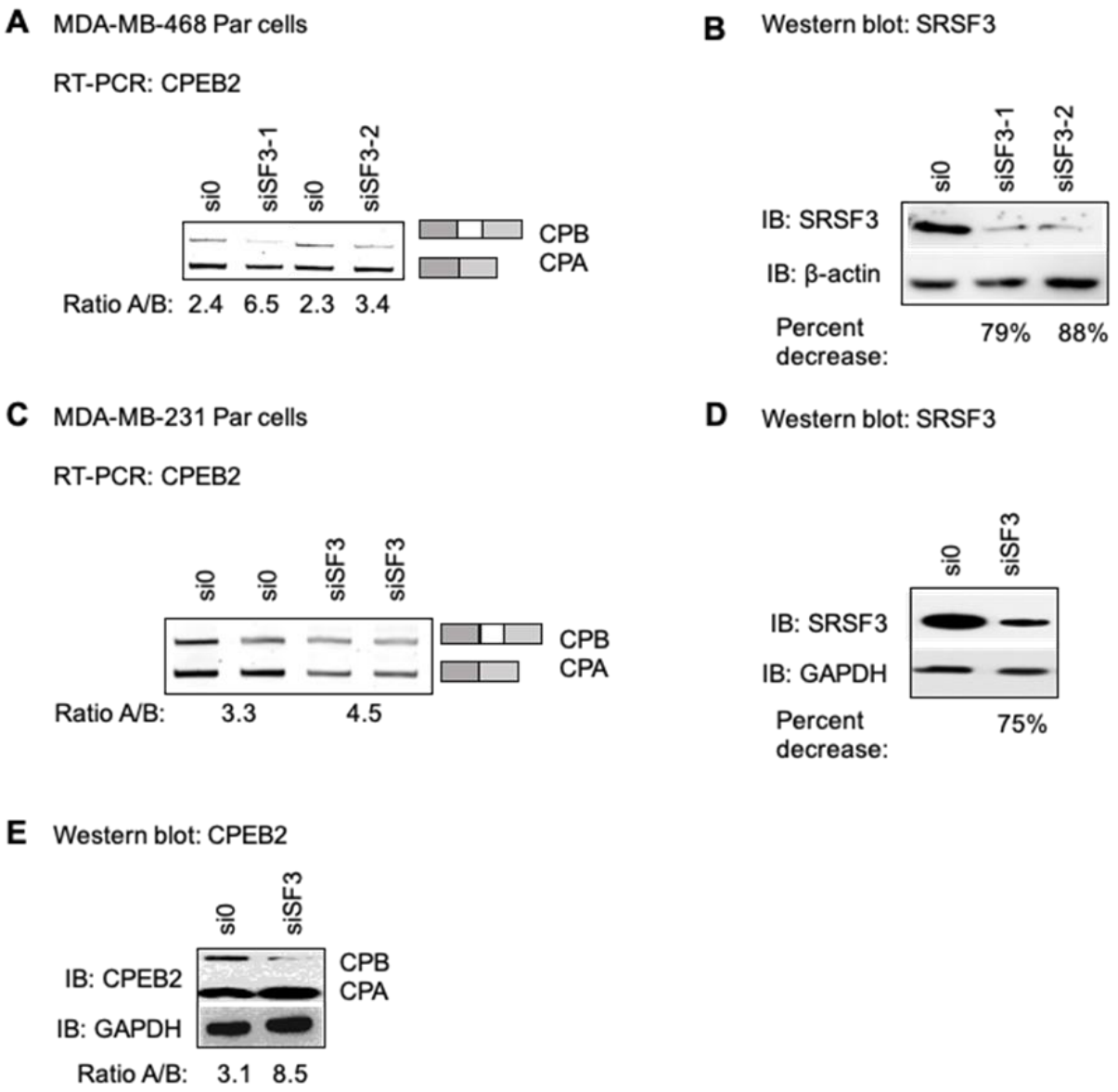

Figure 2: Downregulation of SRSF3 decreases exon four inclusion in endogenous CPEB2 transcripts and correlates to a loss of CPEB2B protein expression.

A) MDA-468 cells were subjected to nonspecific siRNA treatment (si0) or two different siRNA specific to SRSF3 (siSF3-1 and siSF3-2), and the endogenous levels of either CPEB2A (CPA) or CPEB2B (CPB) mature mRNA transcripts were detected by RT-PCR with primers spanning exon four. B) Western blot analysis of the siRNA treated MDA-

Figure 2 (continued): 468 cells indicates expression of SRSF3 in siRNA depleted samples. C) MDA-231 cells were treated with siRNA treatment to SRSF3 in duplicate, then RT-PCR products for endogenous CPEB2 isoform mRNA was quantified via densitometry. D) Western blot analysis of the siRNA treated samples in MDA-231 cells indicates expression of SRSF3 in siRNA depleted samples. Percent decrease was calculated by setting control at 100% then subtracting the signal in the SRSF3 depleted sample. E) Western blot analysis of the siRNA treated samples in MDA-231 cells indicates expression of CPEB2 in siRNA depleted samples.

The Consensus RNA *cis*-element for SRSF3 is Essential for the Inclusion of Exon Four in CPEB2.

A consensus pentamer nucleotide motif is contained within exon four of CPEB2. This motif has been implicated as promoting the alternative splicing of pre-mRNAs for both coding and non-coding transcripts. To investigate the binding of SRSF3 to the RNA *cis*-element contained in exon four, a minigene reporter system was designed for mutational analysis (Fig. 3A). More specifically, exon three, intron three, exon four, partial intron four, and most of exon five were into the (pcDNA 3.1(-)) mammalian expression vector containing a CMV promoter. A competitive RT-PCR assay was used to analyze the exclusion/inclusion of exon four into the minigene mRNA. To ensure specificity avoiding the amplification of endogenous splicing events, a plasmid-specific reverse primer was used (Fig. 3A). MDA-231 parental (Par) and anoikis resistant (AnR) cells were analyzed for their minigene splicing profiles to determine whether the observed increase in SRSF3 protein levels in AnR cells directly correlated with CPEB2 AS in TNBC. Notably, the minigene expression of CPEB2 in TNBC cells were similar to

the CPEB2A/CPEB2B mRNA endogenous ratio (2.3±0.11 for the minigene vs. 2.5±0.09 for endogenous) in MDA-231 Par cells, and the wild-type CPEB2 minigene splicing in MDA-231 AnR resulted in a similar ratio trend (1.3±0.02 for the minigene vs. 1.7±0.07 for endogenous).

To investigate if SRSF3 expression affected inclusion/exclusion of exon four of CPEB2, siRNA targeting SRSF3 was used and resulted in a significant increase in the CPEB2A/CPEB2B minigene mRNA ratio, which mimic the effects which were observed for the endogenous levels (Fig. 3C-F). The reductions in CPEB2 AS were significant in the siSRSF3 treatment compared to the siRNA control and equated to an approximate 1.3-1.4-fold decrease in both the Par and AnR cells (p-value = 0.0023 for parental and p-value = 0.0003 for the anoikis resistant cells).

To demonstrate SRSF3 binding to exon four of CPEB2 is required for the inclusion of exon four, site-directed mutagenesis was used targeting the punitive SRSF3 *cis*-element located in exon four (Fig. 4A). Mutations were introduced using nucleotide substitution with residues which were predicted to abolish binding (see Fig. 1C & D). Upon mutation of the SRSF3 consensus sequence in exon four, a basal reduction of the CPEB2A/CPEB2B minigene mRNA ratio was observed for both the MDA-231 Par and AnR cell lines, with slight variation in amounts (6.0±0.70 and 2.8±0.42, respectively (Fig. 4B & C). Mutation of the *cis*-acting mRNA factor specific to SRSF3 binding resulted in a significant reduction of the CPEB2B isoform in the Par and AnR cells (p-value = 0.0007 and 0.0053, respectively). Taken together, these data demonstrate SRSF3 is the *trans*-acting factor regulating the inclusion of exon four via association with the mRNA *cis*-element, CAUCC in TNBC.

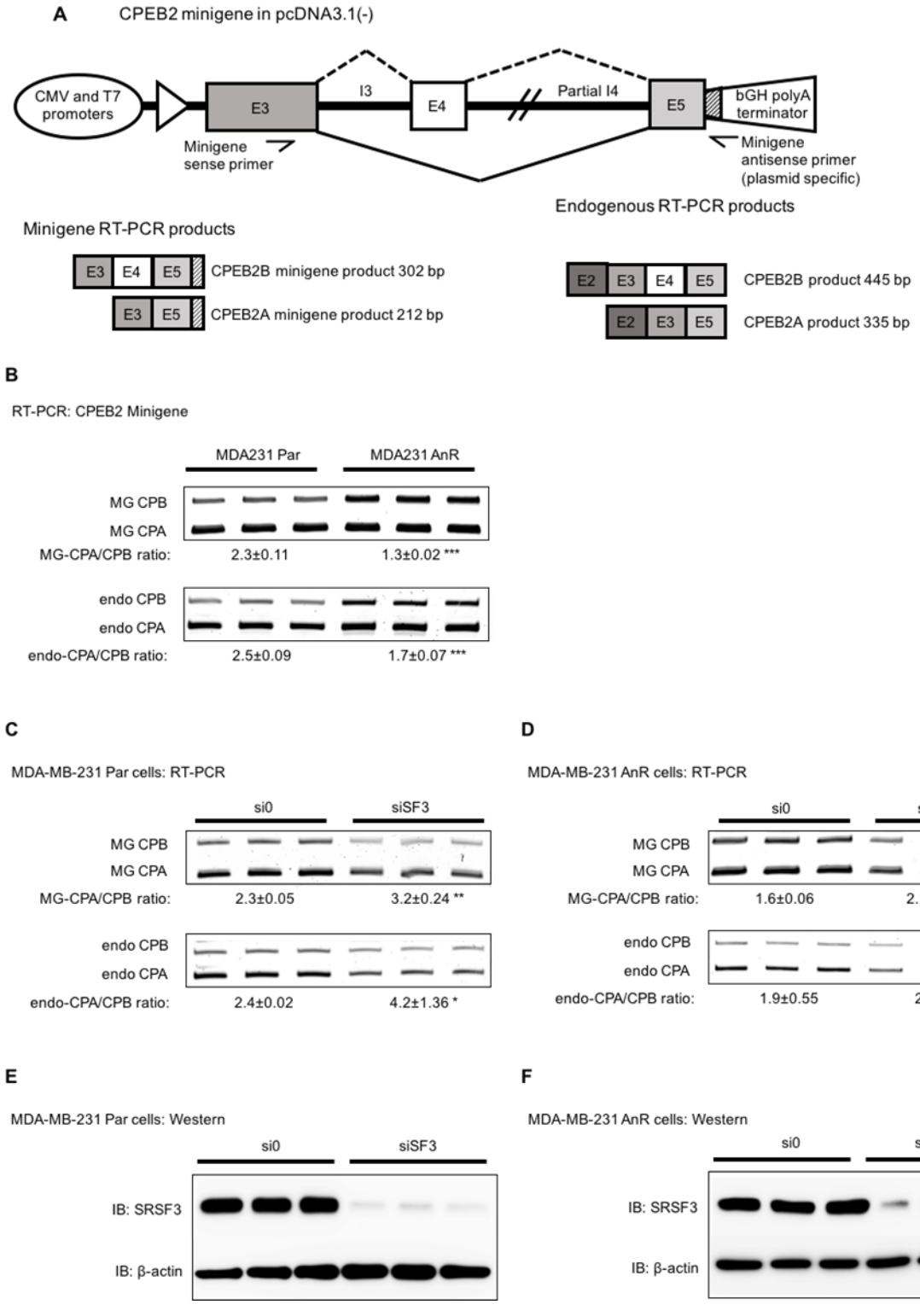

Figure 3: Varying levels of SRSF3 in TNBC cells affects the CPEB2 mRNA isoform ratio at exon four.

Figure 3 (continued): A) Schematic of the CPEB2 exon 3-4/5 minigene. Genomic DNA was amplified from the RPCI-11 Hs BAC Clone using primers that spanned all of exon 3 and 4, and partial of exon 5. The complete intron 3 sequence was included, and partial amplification of intron 4 was included. Primers that were specific to the minigene were used to detect splicing events in RT-PCR analysis. B) MDA-231 Par cells were compared to MDA- 231 AnR cells analyzing basal levels of minigene splicing for the minigene-specific CPEB2A/CPEB2B minigene ratio and compared to endogenous CPEB2 splicing (endo). C-D) MDA-231 Par (C) and MDA-231 AnR (D) cells were treated with siRNA targeting SRSF3. CPEB2 minigene splicing and endogenous CPEB2 splicing was detected via RT-PCR. E-F. MDA-231 Par (E) and MDA-231 AnR (F) SRSF3 protein levels were detected after siRNA treatment as indicated by Western blot. The representative images from the three independent experiments are presented, and for all quantitation n = 3 ± standard deviation (sd) via densitometry. Statistical significance is reported as a p-value from oneway ANOVA pooled t-test of the MG or endo CPEB2A/CPEB2B ratio. (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).

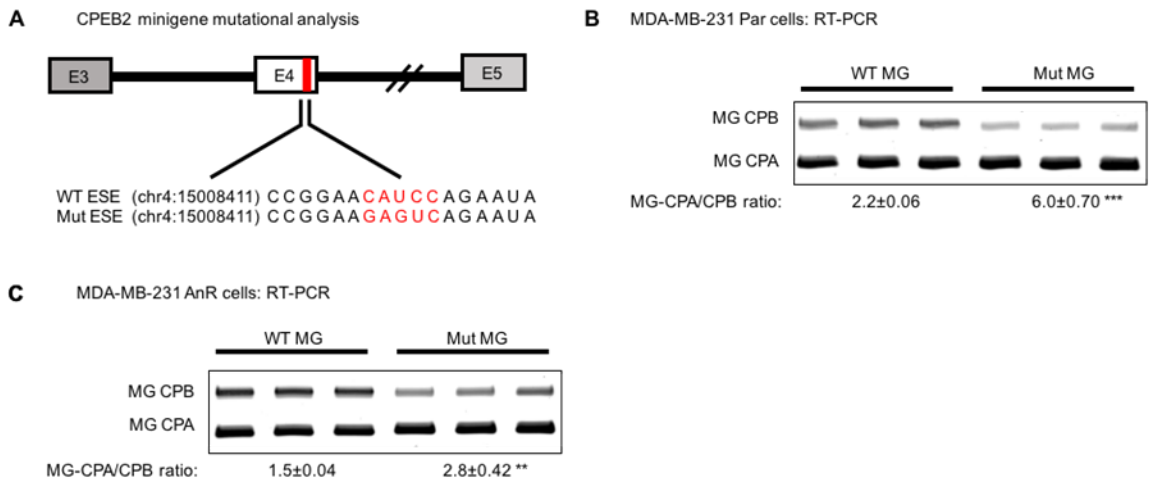

Figure 4: A Mutational analysis of SRSF3 RNA *cis*-element indicates the pentameric cis-element is essential for the inclusion of exon four in CPEB2.

A) Schematic representation of the mutant minigene. The red bar indicates that the RNA *cis*-element is located near the 5' splice site. Genomic coordinates showed the first nucleotide base in the RNA *cis*-element and were retrieved from Genome Reference Consortium Human Build (GRCh38.p12). B) RT-PCR analysis of MDA- 231 Par cells for the wild type (WT) CPEB2 *cis*-element compared to mutant (Mut) minigene-specific CPEB2A/CPEB2B ratio. C) An RT-PCR analysis of MDA-231 AnR cells for the WT CPEB2 *cis*-element compared to Mut minigene-specific CPEB2A/CPEB2B ratio. Representative images of three independent experiments are presented. All quantitation is shown as n = 3 ± standard deviation (sd) via densitometry. Statistical significance is reported as a p-value from one way ANOVA pooled t-test of the MG CPA/CPB ratio. (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).

SRSF3 regulates anoikis sensitivity in TNBC cells by enhancing the inclusion of exon four in CPEB2 mRNA.

To investigate whether SRSF3 is a key regulatory mediator in acquiring anoikis resistance (AnR) via CPEB2 AS, downregulation of the SRSF3 protein was analyzed in both MDA-231 and MDA-469 AnR cell lines. Interestingly, with SRSF3 downregulation, an increase in basal apoptosis and detached induced cell death was observed in both AnR cell lines (Fig. 5A-B). Notably, the ectopic expression of CPEB2B (exon four included) ablated the effect of SRSF3 downregulation (Fig. 5A). Together, these data establish a link between the mRNA *trans*-factor SRSF3 binding exon four of CPEB2 and the acquisition of the AnR phenotype in TNBC. This study provides clear evidence that the inclusion of exon four, regulated by the SRSF3/CPEB2 AS axis, induces AnR in TNBC by promoting the CPEB2B mRNA transcript expression.

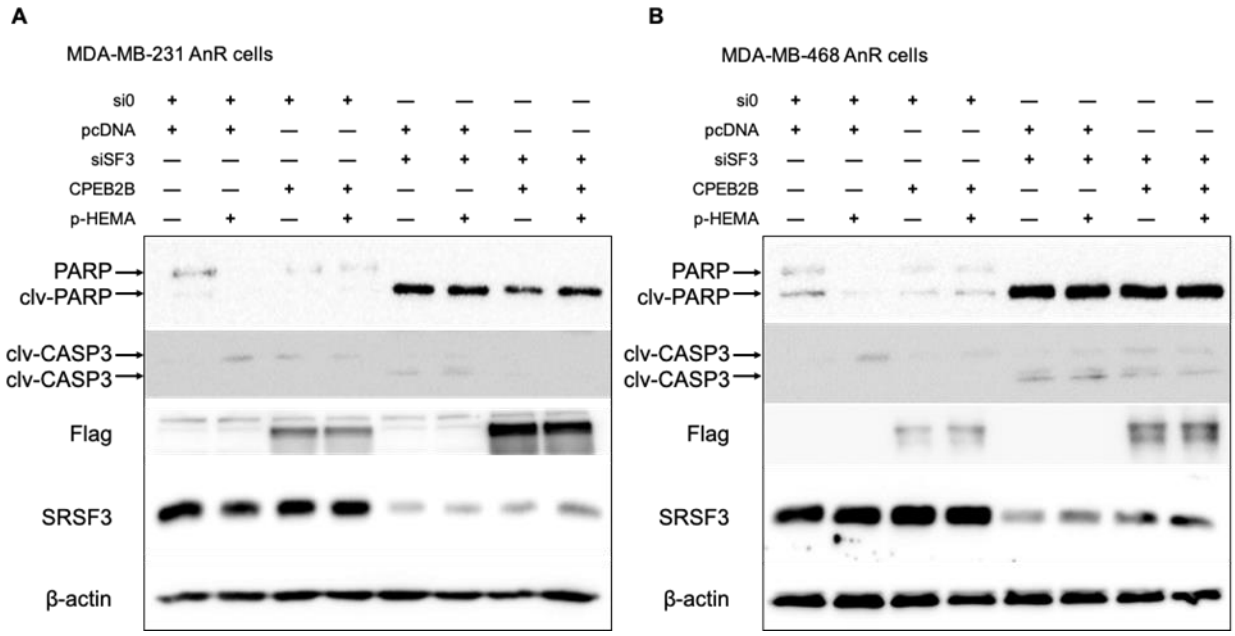

Figure 5: SRSF3 modulates TNBC sensitivity to anoikis-mediated cell death due to expression of CPEB2B.

siRNA treatment was applied for 48-hours then incubated for 6 hours on regular substrate or poly HEMA substrate, which forced cells into suspension. After incubation, early-stage apoptosis was analyzed using Western blot to probe for cleaved PARP (clv-PARP) and cleaved Caspase 3 (clv-CASP3). Using 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 -231 AnR cells were treated as indicated in 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-468 AnR cells were treated identically to the cells described in panel A.

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

The study linking SRSF3 AS regulation of CPEB2 promoting the CPEB2B isoform in conjunction with our previous reports of CPEB2B high expression levels in TNBC promote TNBC metastasis, collectively postulate the premise that SRSF3 expression levels will correlate with the aggressiveness of TNBC and other breast cancer subtypes. To investigate this premise, sequence data obtained from the TCGA Breast Invasive Carcinoma (BRCA) dataset was examined, looking at unique patient cases data that contained RNAseq and clinical data for SRSF3 mRNA levels. The corresponding mRNA z-scores were deduced for each of the PAM50 gene-expression-based subtypings. The TNBC and also the basal-like subtypes both indicated the highest SRSF3 mRNA expression levels, while the HER2+, Luminal A, and Luminal B subtypes expressed significantly less SRSF3 mRNA (Fig. 6A). The R coding platform was used to interrogate the TCGA data for survival which revealed that stratifying patients according to SRSF3 mRNA expression was indicative of survival (Fig. 6B). This data supports the premise that in TNBC with high SRSF3 mRNA expression, the CPEB2A/CPEB2B isoform ratio will be low. This provides further insight into the vital role of dysregulated alternative splicing in the progression and metastasis of cancer.

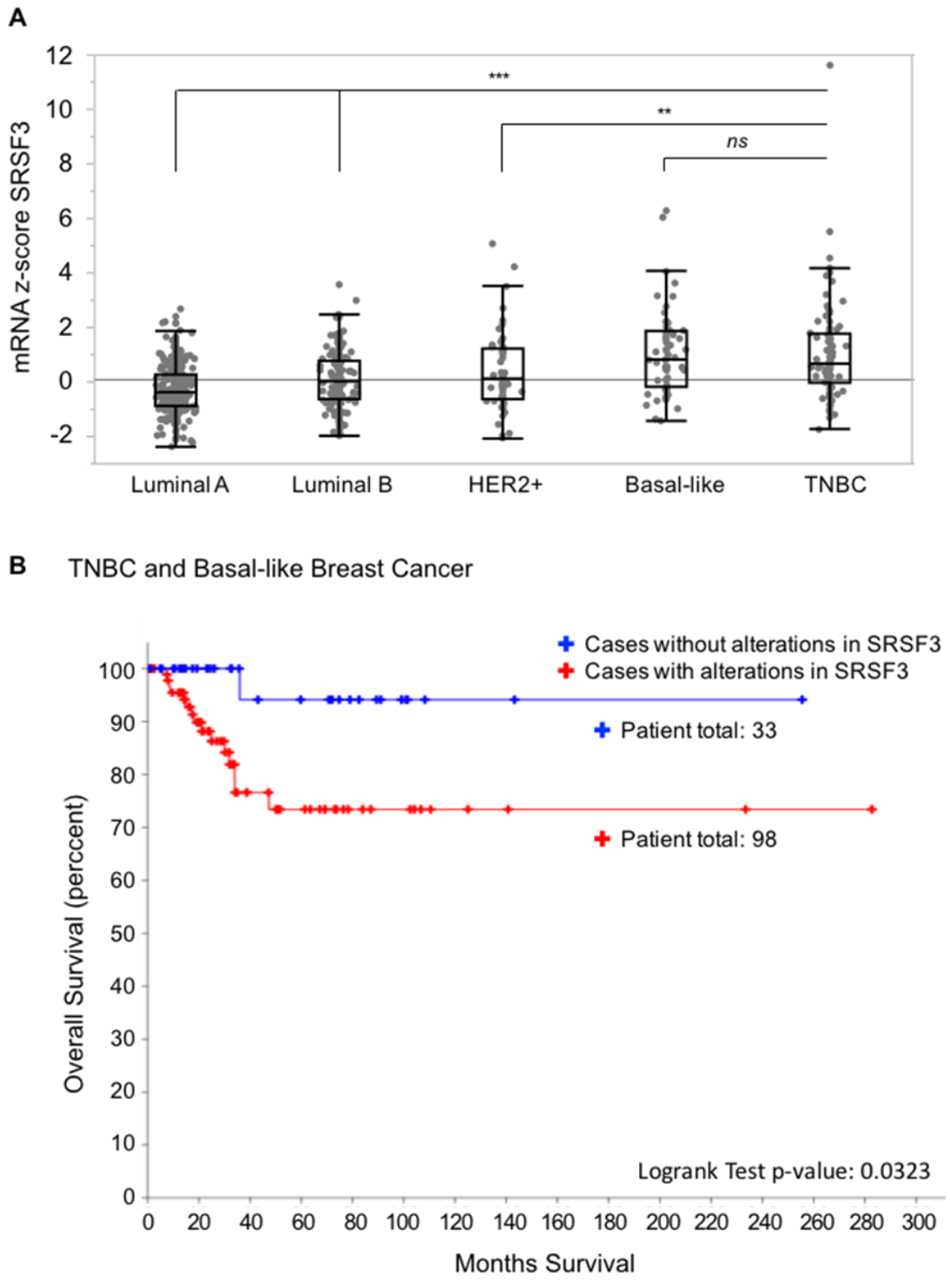

Figure 6: SRSF3 is over-represented in the most aggressive and metastatic breast cancers in The Cancer Genome Atlas.

Figure 6 (continued): A) The mRNA z-scores were derived from patient samples with both clinical breast cancer subtypes based on PAM50, histopathology, and RNASeq data in the Breast Invasive Carcinoma (TCGA, Cell 2015) data set containing 1105 patient samples. SRSF3 mRNA expression data across the main breast cancer subtypes was mined from cBioPortal and evaluated using ANOVA with posthoc Tukey HSD (** = p-value < 0.01, *** = p-value < 0.0001). B) Clinical overall survival data for BRCA data set was extracted from TCGA for combined Basal-like and TNBC cohorts, consisting of 131 patients. Survival probability based on SRSF3 mRNA expression levels were plotted as a Kaplan-Meyer curve using the cBioPortal. The cases with alterations are defined as deviating based on mRNA z-scores in the both upper or lower quantiles of the population. Calculated statistical significance is reported as log-rank test analysis (p-value = 0.0323).

Discussion

Our laboratory has provided evidence describing a mechanism for CPEB2 AS regulation by the RNA *trans*-splicing factor SRSF3 in TNBC. Importantly, SRSF3 is shown to be upregulated during hypoxia in the TME associated with solid and malignant breast tumors. Previously, we reported that the two isoforms, CPEB2A and CPEB2B, of CPEB2, exert opposing roles for TNBC from primary to the metastatic phenotypes via AnR acquisition. Here we describe, for the first time, that the pro-oncogene SRSF3 promotes the expression of the pro-metastatic CPEB2B mRNA isoform. We have also identified that the alternative splicing of CPEB2 pre-mRNA is regulated by the binding of SRSF3 to the pyrimidine-rich, pentameric *cis*-element found in the distal 3' of exon four.

This association promotes the inclusion of exon four into the CPEB2B mRNA transcript leading to activation of signaling pathways important in the initiation of epithelial to mesenchymal transition (EMT) (Fig. 7). Importantly, SRSF3 splicing regulation of exon four of CPEB2 is dependent on RNA cis-element sequence fidelity in exon four. Indeed, we observed a reversal of the CPEB2A/CPEB2B mRNA ratio with a substitution mutation at the SRSF3 consensus sequence in the CPEB2 minigene. Finally, modulation of SRSF3 protein levels via siRNA targeting SRSF3 mRNA led to a decrease in CPEB2B mRNA transcript levels resulting in a reversal of the low CPEB2A/CPEB2B ratio to the higher ratio observed in the AnR cells. This reversed ratio resulted in an increase in early apoptosis of the TNBC AnR cells. Introduction of exogenous CPEB2B expression with the cDNA vector "rescued" AnR by forced growth in suspension, which is representative of early phenotypic adaptations in cells during the acquisition of AnR important in the metastasis of TNBC. However, a robust rescue was not observed in the biological phenotype as predicted by the original hypothesis as cleaved-PARP levels were not attenuated by circumvention SRSF3 activity via CPEB2 exogenous expression in the siSRSF3 treatment group. This suggests that although SRSF3 is necessary for CPEB2B isoform generation, the CPEB2B isoform alone is not sufficient to completely rescue the biological phenotype.

Globally, SRSF3 plays a crucial role in regulating AS events, and increased levels of SR proteins bound to exon splicing enhancer *cis*-elements in pre-mRNA prevent exon skipping [120]. Recent studies elucidate an antagonistic role for SRSF3 that differs from other SR proteins in that SRSF3 promotes exon inclusion during events such as tumor initiation, progression, resistance to detached induced cell death [121].

Indeed, these data indicate that SRSF3 is potentially a master transcriptome regulator due to evidence that SRSF3 can bind with several SR protein family members and promote a "poison cassette exon" autoregulatory feedback loop via nonsense-mediated decay (NMB) [122]. While current research shows that CPEB2 AS is connected to SRSF3 association with an RNA *cis*-element in exon four of CPEB2 pre-mRNA, this may not be the only regulatory mechanism for CPEB2 AS. For example, we found short term transient upregulation of SRSF3 cDNA did not increase CPEB2B mRNA levels in MDA-231 cells as might be predicted (data not shown). In fact, increased SRSF3 protein levels may be due to chromosomal amplifications in the MDA-231 cell line, which is pentameric at the SRSF3 6p21.1 locus (MDA-231 SKY/M-FISH SKYGRAM) [123]. A possible explanation is that extensive post-translational modifications (PTMs) of oncogenic pathways promote hyperactivation of SRSF3, providing resistance to proteolytic degradation in TNCB cells. Continued investigation into SRSF3 activity during various PTMs may elucidate the influence that SRSF3 exerts on lowering the CPEB2A/CPEB2B isoform ratio seen in TNBC AnR.

Regarding SRSF3 PTMs, neddylation, which is a modification that covalently links the small ubiquitin-like protein NEDD8 to Lys85 in SRSF3, was shown to promote stress granule (SG) assembly during oxidative stress [124]. This strategy is co-opted by tumor cells which are subject to persistent stress to ensure nascent mRNAs and proteins are compartmentalized and readily available to regulate activation of signaling pathways. Interestingly, not only is there evidence that SRSF3 localizes to SGs [125], both CPEB2A and CPEB2B protein contain a low complexity domain (LCD) in their N-terminal intrinsically domains [126]. These LCDs, which are found in other RNA binding

proteins, may contribute to the formation of sub-organelle compartments like SGs [127]. Additionally, the phosphorylation of SRSF3 in the RS domain is significantly important to the regulation of spliceosome assembly, and catalysis of AS events are dependent on dephosphorylation of the SRSF3 RS domain [128]. This phosphorylation/dephosphorylation cycle also contributes to SRSF3s ability to interact with nuclear export factor NXF1 to couple the AS and polyadenylation of mRNA during NXF1-mediated export [129]. In TNBC, where SRSF3 protein levels are high, it may be hypothesized that CPEB2 activates the acquisition of metastasis by primary tumors if CPEB2B mRNA is bound at exon four by highly phosphorylated SRSF3 resulting in CPEB2B shuttling to SGs in the cytoplasm, thereby increasing CPEB2B abundance and promoting the translation of the HIF1α and TWIST1 transcription factors important in the activation of EMT.

Defects in AS often impact dysregulation in numerous hallmarks of cancer, promoting the metastasis of primary tumors. In TNBC, the increased genomic heterogenicity may result from defects in AS resulting in structural protein variants which increase therapeutic resistance. Interest in targeting the protein products of aberrant splicing in cancer and the RNA trans-splicing factors, particularly SRSF3, using antisense oligonucleotides (ASOs), has grown and may be a promising novel therapeutic option in cancer treatment [130]. Most certainly, targeting the SRSF3/CPEB2 paradigm in TNBC is worth pursuing.

In conclusion, we have identified the RNA *trans*-splicing factor SRSF3 as the AS regulator responsible for mediating the alternative splicing of CPEB2 via association with the RNA *cis*-element in exon four. Our study describes the importance of the

SRSF3/CPEB2B splicing paradigm in acquiring AnR in TNBC. Additionally, we provide evidence that depletion of SRSF3 via siRNA results in a loss of AnR and reverts the CPEB2A/CPEB2B ratio to resemble that of non-tumorigenic breast tissue. Future studies are necessary to elucidate the activation of this novel splicing event and the potential therein to translate into a potential target for breast cancer carcinoma.

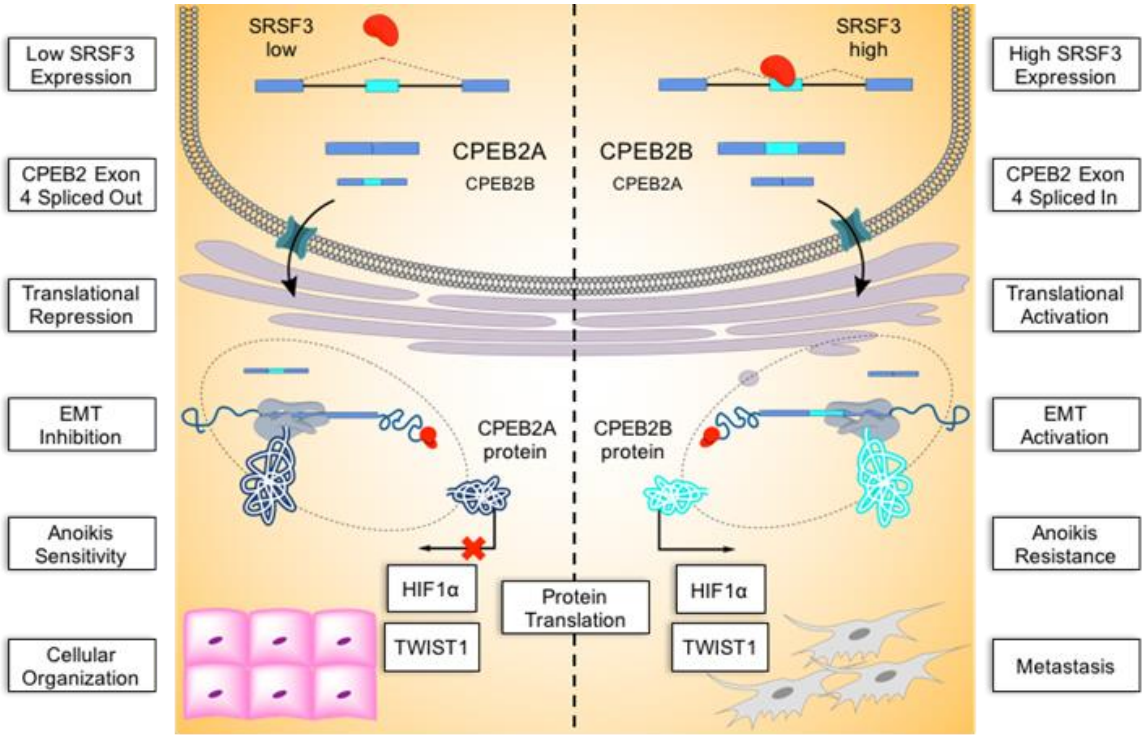

Figure 7: Proposed pathway for the mechanism of SRSF3 and CPEB2 splicing in the SRSF3/CPEB2B splicing paradigm constituting progression towards metastatic TNBC.

CHAPTER 3: THE MECHANISM OF TRANSLATIONAL REGULATION OF HIF1α AND TWIST1 MRNA VIA CPEB2A AND CPEB2B ISOFORMS

Abstract

Triple-negative breast cancer (TNBC) remains the most deadly and aggressive subtype of breast cancer. Furthermore, TNBC is more common in younger women and the African American population. Our lab has recently elucidated a role for which the alternative spliced isoforms of cytoplasmic polyadenylation element-binding protein 2 (CPEB2), CPEB2A, and CPEB2B, regulate the acquisition of anoikis resistance (AnR) and metastasis in TNBC via translational regulation of hypoxia-inducible factor-alpha (HIF1α) and twist-related transcription factor (TWIST1). Specifically, The CPEB2A protein isoform inhibits translation of HIF1α and TWIST1 and the CPEB2B isoform enhances translation of these transcription factors contributing to the metastatic cascade. In this study, the mechanism of HIF1α and TWIST1 translational regulation via CPEB2A and CPEB2B was examined. We determined that the CPEB2A isoform bound to the CPE sites located in HIF1α and TWIST1 3'UTR mRNA and interacted with known polyadenylation complex proteins. Similarly, we found CPEB2B associated with polyadenylation complex proteins, albeit a weaker interaction, but did not bind CPE sites in HIF1α and TWIST1 3'UTR mRNA. We also found that CPEB2A and CPEB2B localize to both the nucleus and cytoplasm. Furthermore, we identified novel protein interactions for CPEB2B, specifically exon four, which is absent in the CPEB2A

transcript. CPEB2B was shown to interact with proteins, specifically, the translation invitation factor eIF3H and heteronuclear-ribonuclear proteins hnRNPR and hnRNPF/H, which have been identified as IRES trans-acting factors (ITAFs) important in cap-independent translational activation of mRNAs in cellular stress events. Interestingly, we also identified a strong interaction between CPEB2A and CPEB2B proteins suggesting a potential connection between the CPEB2 alternative splicing regulation and translational activation/inhibition of HIF1α and TWIST1. These novel interactions have never been described and provide evidence that alternative splicing inducing a ratio shift of CPEB2A to CPEB2B proteins results in translational activation of HIF1α and TWIST1 mRNA through an IRES-mediated cap-independent translational mechanism promoting the acquisition AnR and the metastatic phenotype in TNBC.

Introduction

TNBC is a breast cancer subtype that is characterized histologically by its lack of the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2) [131]. Compared to other breast cancer subclasses containing these hormone receptors, TNBC exhibits significant genetic complexity and tumor heterogenicity, which renders treatment with targeted chemotherapeutics ineffective [132]. Common treatment options for TNBC, such as neoadjuvant chemotherapy, may initially prove relatively successful; however, TNBC commonly acquires resistance to chemotherapeutics contributing to the lowest five-year progression-free and survival rates of all breast cancer subtypes [133]. TNBCs aggressive nature, combined with its poor prognosis, makes the discovery of novel treatments imperative.

Investigation of CPEB2 has uncovered an anti-neoplastic and suppressive role in regulating translation of HIF1α and TWIST1 mRNA [134]. However, it has become apparent that these findings are for the CPEB2A isoform. Indeed, our lab has published that dysregulated CPEB2 AS, marked by an increase in the CPEB2B isoform which promotes translation of HIF1α and TWIST1 mRNA, is prevalent in TNBC tumor samples [135]. Corresponding *in vivo* analysis indicated that a splicing ratio shift favoring expression of the CPEB2B isoform, which includes exon four in the mRNA transcript, contributed anoikis resistance (AnR), detachment-induced cell death, promoting tumor metastasis to the lung. Furthermore, we have shown that these splice variants regulate hypoxia and EMT in an opposing fashion through translational regulation of HIF1α and TWIST1 mRNA, transcription factors important in regulating these pathways [136]. However, the mechanism by which translational regulation is accomplished remains elusive.

Cytoplasmic polyadenylation element-binding protein 2 (CPEB2) is a member of the CPEB family of proteins that mediate polyadenylation of mRNA targets in the cytoplasm [137]. There are four family members in the CPEB protein family (1-4) divided into two categories based on protein sequence similarity. CPEB1 belongs to the first category and is the most distinct of the CPEB family members, whereas the second category consisting of CPEB2, CPEB3, and CPEB4 are closely related [138]. In terms of structure, all CPEB proteins are comprised of three regions: they have an N-terminal domain characterized as intrinsically disordered, highly structured c-terminal domains which contain two RNA recognition motifs (RRMs) and a cysteine-histidine region resembling a zinc finger [139]. The RRMs of CPEB proteins bind consensus

cytoplasmic polyadenylation element (CPE) sequences in 3' untranslated regions (UTR) mRNA, the most common of which is UUUUUAU [140]. A nuclear role for CPEB proteins, specifically CPEB1 and CPEB4, has been suggested in which these proteins bind to their targeted mRNAs in the nucleus, and the CPEB/mRNA complex is then exported to the cytoplasm [141]. In this manner, the CPEB protein can exhibit tight translational regulation of mRNAs crucial to stress responses.

As mentioned previously, the N-terminal end of the CPEB proteins are characterized as intrinsically disordered, and tier sequences vary significantly amongst the family members [142]. In CPEB1, the N-terminal region was shown to associate with proteins that belong to the polyadenylation complex, specifically GLD2 and PARN, and with the cap-binding eukaryotic initiation factor four E (eIF4E) [143]. This mechanism describes how CPEB1 regulates the translation of target mRNA. Richter and colleges demonstrated that CPEB1 binds the 3'UTR CPE sites in mRNA and interacts with the polyadenylation complex proteins GLD2 and PARN [144]. PARN, a deadenylating enzyme, outcompetes the poly(A) polymerase activity of GLD2, inhibiting the polyadenylation of the mRNA [145].

Additionally, CPEB1 interacts with a cap-binding protein, eIF4E, inhibiting the eIF4E/eIF4G interaction, synonymous with cap-dependent mRNA translation initiation, inhibiting the recruitment of the 43s preinitiation complex and thereby halting translation initiation [146]. Phosphorylation of CPEB1 via Aurora kinase results in a conformational change and disassociation with PARN, which then allows for GLD2 to polyadenylate mRNA [147]. Furthermore, CPEB1 phosphorylation also results in eIF4E release allowing interaction with eIF4G and recruitment of the 43s preinitiation complex and

initiating translation [148]. However, the mechanism for mRNA translation regulation by the other CPEB family members (2-4) differs from CPEB1, partly due to the absence of an Aurora kinase phosphorylation site in the N-terminal domain [149].

Progress towards understanding its mechanism of translational regulation of mRNA by CPEB2 has been reported. Chen and colleagues reported that CPEB2 (presumably the CPEB2A isoform) inhibits translation of hypoxia-inducible factor one-alpha (HIF1α) via association with its 3'CPE region and by binding to eukaryotic elongation factor 2 (eEF2) in Neuro 2A (N2A) murine cells. Upon oxidative stress, an interaction between CPEB2 (eEF2) is halted, allowing the ribosome to continue translation [150]. Another study by Nairisgami and colleagues proposed a mechanism of translational regulation by CPEB2 for Twist Family BHLH Transcription Factor 1 (TWIST1) in MCF-10A non-malignant breast epithelial cells [151]. In this mechanism, CPEB2 and CPEB1 regulate TWIST1 mRNA translation via association with CPE sites, of which there are two in the 3'UTR of TWIST1 mRNA, allowing polyadenylation of TWIST1 mRNA. Association with both CPE sites allows for the inclusion of microRNA (miRNA) regulatory binding sites, resulting in reduced TWIST1 protein translation.

Our lab has published findings indicating that the CPEB2 isoforms have opposing effects on the translation of HIF1α and TWIST1 mRNA. Furthermore, we have shown that ectopic expression of CPEB2B did not result in robust increases in HIF1α and TWIST1 mRNA nor proteolytic turnover. These results suggest a link between mRNA binding and the regulation of translational activity of the CPEB2A/B transcripts. Taken together, we hypothesized that the inclusion of exon four in CPEB2, the CPEB2B

isoform, changes the mRNA binding and that exon four is associating with novel protein complexes important in promoting the translation of mRNA.

In this study, our laboratory demonstrated that CPEB2A but not CPEB2B associates with 3'UTR CPE sites located in HIF1α and TWIST1 mRNA. Surprisingly, the binding of the CPEB2A isoform but not the CPEB2 isoform results in polyadenylation of TWIST1 mRNA; however, no differences in HIF1α mRNA polyadenylation was observed. We also found that CPEB2A and CPEB2B localize to both the nucleus and the cytoplasm, which indicate a nuclear role for CPEB2 similar to that which has been previously described for CPEB1 and CPEB4 [152].

We have also identified novel interactions with proteins identified as internal ribosome entry site (IRES) *trans*-acting factors (ITAFs), which are important in regulating IRES cap-independent translation of mRNAs in stress response pathways [153], with exon four of the CPEB2B isoform. Furthermore, we have identified that CPEB2A and CPEB2 proteins interact with each other. Taken together, these novel findings suggest that CPEB2B may bind CPEB2A inhibiting CPEB2As activity as a translational repressor and promoting CPEB2Bs function as a translational activator through an IRES-mediated cap-independent mechanism of transitional regulation. These findings provide evidence that shifts in the AS of CPEB2 towards increased CPEB2B isoform production promotes HIF1α and TWIST1 mRNA translation via CPEB2A/CPEB2B competitive interaction and activation of IRES cap-independent translation.

Material and Methods

Cell culture and reagents:

MDA-231 and MDA-468 cells were obtained from ATCC (Manassas, VA) and maintained in RPMI (Invitrogen). The cell lines were supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% Penicillin / Streptomycin (Invitrogen). Cell lines were maintained in a 95% air / 5% CO2 incubator at 37°C. Cells were passaged once every 3-5 days (~90% confluence), and all experiments were performed during the first 12 passages.

Western Blotting:

Total protein (10-30 µg) was electrophoretically separated on 10% SDS-polyacrylamide gels. Samples were transferred electrophoretically to PVDF membranes, then probed with the appropriate antibody (anti-3xFlag, eIF3H, eIF4/G, hnRNPR, hnRNPF/H, HA antibodies purchased from Cell Signaling.

Plasmid construction:

The 3xFlag-CPEB2A and 3xFlag-CPEB2A and the HA-CPEB2B plasmid constructs were previously reported. Briefly, genomic DNA fragments were amplified using standard PCR conditions and proofreading Taq polymerase (Phusion High Fidelity DNA Polymerase, New England Biolabs). The PCR conditions consisted of a denaturing step of 98 °C for 30 seconds followed by 30 cycles, 98 °C denaturing for 10 sec., 60 °C annealing for 30 seconds, 72 °C extension for 30 sec., and final extension step at 72 °C for 10 min. Site-directed mutagenesis was performed using the

QuickChange II XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions along with primers containing either the 3xflag-tag sequence (5'GACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAGGATG ACGATGACAAG3') or the HA-tag sequence (5'TACCCATACGATGTTCCAGATTAC GCT 3'). Fusion of amplified genomic DNA material with the pcDNA3.1$^{(+)}$ mammalian vector (Invitrogen) was accomplished using the In-Fusion HD cloning reaction (Clontech). With each step of PCR amplification and plasmid generation. All the primers used in cloning were synthesized by Integrated DNA Technologies (IDT).

Plasmid Transfection:

All transfections were performed in duplicate with 10-cm tissue culture dishes. The plasmid transfections were accomplished using the Lipofectamine 3000 lipid-based transfection system (ThermoFisher) according to the manufacturer's instructions and using 5.0 μg total DNA per 10-cm tissue culture dish.

UV Cross-linking and Co-immunoprecipitation:

UV crossing-linking and co-immunoprecipitation of bound mRNA to CPEB2A and CPEB2B were performed according to previously described protocol []. Briefly, MDA-231 cells transfected with either 3xflag-CPEB2A or 3xflag-CPEB2B constructs were irradiated with 200 mJ UV and harvested. Lysates were incubated with anti-3xflag magnetic agarose beads (Millipore), followed by three stringent washes with a high-salt buffer wash and two washes with 1XPBS (Fisher). Bound mRNA was eluted with phenol-chloroform and purified with an mRNA purification kit (Zymogen).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR):

Primer/probe sets targeting the 5'UTR of HIF1α and TWIST1 mRNA were purchased from Integrated DNA Technologies. PCR was performed as described [154]. cDNA synthesis was accomplished using the Superscript III kit (Life Technologies) according to the manufacturer's instructions. Samples were amplified with the Bio-Rad CFX Connect qPCR machine and then calculated using the standard curve method.

RNA Binding Assays:

3'UTR Wild-type (WT) biotinylated RNA TWIST1 CPE sequences 1 and 2 (Bi:5'GUGAGAUCUAGUUUGCAGUUGCCAGCUUGGGGCUCAGAUUCACUCCAAGAUAGUUGGUGCACUGCAGCCGGAACAUCCAGAAUAGACCAG3') and HIF1α CPE sequence(bi:5'GUGAGAUCUAGUUUGCAGUUGCCAGCUUGGGGCUCAGAUUCACUCCAAGAUAGUUGGUGCACUGCAGCCGGAACAUCCAGAAUAGACCAG) were incubated with 3xFlagCPEB2A or 3xFlagCPEB2B transfected MDA-231 cell lysates, and RNA-bound proteins were precipitated as described [155]. Samples were subjected to immunoblotting with 10% polyacrylamide gels with an anti-flag antibody (ThermoFisher).

USB Poly(A) Tail-Length Assay:

USB Poly(A) Tail-Length Assay Kit (ThermoFisher) and manufactures instructions were used to determine poly(A) tail-length of HIF1α and TWIST1 mRNA. Briefly, MDA-231 cells were transfected with 3xFlagCPEB2A or 3xFlagCPEB2B, and mRNA was extracted. G/I tailing of mRNA was completed by adding Tail Stop Solution

and incubating at 37 for 60 minutes. Poly(G/I) tailed RNA was then subjected to RTPCR to produce cDNA transcripts. PCR of Poly(G/I) tailed cDNA was completed using primer sets designed to amplify poly(A) tail regions as described (Fig. 9-10).

Co-immunoprecipitation and Proteomic Analysis:

Co-immunoprecipitation was accomplished as previously described. Briefly, MDA-231 and MDA-468 cells lysates with transiently expressed 3xFlag-CPEB2A or 3xFlag-CPEB2B were incubated with anti-3xflag magnetic agarose beads (Millipore) followed by three stringent washes with a high-salt buffer wash and two washes with 1XPBS (Fisher). Bound proteins were eluted with (200ng/mL) 3xflag peptide (ThermoFisher) and subjected to proteomic analysis at the University of South Florida proteomics core (Tampa, FL). Statistical analysis of results was analyzed via Ingenuity Pathway Analysis (IPA).

CPEB2 Exon four Binding Assay:

Wild-type (WT) biotinylated exon four CPEB2B peptide sequence (5'VRSSLQLPAWGSDSLQDSWCTAAGTSRIDQ3') were incubated with MDA-231, and MDA-468 cellular lysates and bound proteins were precipitated as described [156]. Samples were subjected to immunoblotting with 10% polyacrylamide gels with antibodies targeting specific proteins.

Immunofluorescence:

Immunofluorescence was carried out with MDA-231 cells stably expressing either Flag-CPEBA or Flag-CPEB2B plasmids on coverslips for 24 hours and then fixed with 4% paraformaldehyde. The primary antibodies used for immunofluorescence were Alexa fluor 488 rabbit monoclonal anti-flag antibody (1:1000). The nuclei were visualized by DAPI (Sigma, St. Louis, MO). Images were taken using a Keyence BZ-X series microscope (Itasca, IL).

Subcellular Fractionation Assay:

Subcellular fractionation was performed using the subcellular protein fractionation kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, MDA-231 cell lysates transiently expressed 3xFlag-CPEB2A or 3xFlag-CPEB2B and treated with either arsenic (2ng/mL) or H2O (control) for 16 hours were separated according to the manufacturer's kit instructions. Western blot analysis of fractionated samples was accomplished with 10% polyacrylamide gels and immunoblotted with either anti-3xflag, anti-tubulin, anti-Lamin, or anti-Clv-Parp antibodies (Cell Signaling).

Results

CPEB2A binds to CPE 1 and 2 sites of TWIST1 and CPE 1 site of HIF1α 3' UTR and regulates polyadenylation.

Previously, our lab reported that CPEB2A and CPEB2B isoforms of CPEB2 regulate the translation of HIF1α and TWIST1 mRNA in an opposing manner. These findings led to the investigation of the mechanism driving CPEB2Aand CPEB2B

translational regulation of HIF1α and TWIST1 mRNA. To this end, a streptavidin-biotin affinity pull-down assay (SBAP) was utilized to determine binding affinities of the CPEB2A and CPEB2B protein isoforms for the 3'UTR CPE sites in HIF1α (CPE1) and TWIST1 (CPE1 and CPE2) (Fig. 8B & 9B).

Transfection of Flag-CPEB2A resulted in significant binding to HIF1α (CPE1) and both TWIST1 (CPE1 and CPE2) sites (Fig. 8B & 9B). Conversely, transfection of Flag-CPEB2B did not show an affinity for HIF1α (CPE1) and neither TWIST1 (CPE1 and CPE2) sites (Fig. 8B & 9B). This assay revealed that the CPEB2A protein isoform binds to the CPE regions of HIF1α and TWIST1 3'UTR, with competition for CPEB2A binding achieved with unlabeled HIF1α (CPE1) and TWIST1 (CPE1 and CPE2) RNA at access concentration (100x); while nonspecific competitor RNA showed no effect on the RNA: protein complex (Fig. 8B & 9B).

Additionally, while the CPEB2B protein isoform showed no binding affinity for HIF1α and TWIST1 CPE1 sites, it did indicate minimal binding to the CPE2 site of TWIST1 3' UTR. Competition for CPEB2B binding was achieved with unlabeled HIF1α (CPE1) and TWSIT1 (CPE1 and CPE2) RNA at access concentration (100x), while nonspecific competitor RNA showed no effect on the RNA: protein complex (Fig. 8B & 9B). These data demonstrate that the CPEB2A protein isoform binds to the HIF1α and TWIST1 3' CPE1 and TWIST1 CPE2 3'UTR mRNA while revealing a difference in binding affinity differences for the CPEB2 isoforms.

To investigate if potential differences in CPEB2A and CPEB2B regulation of HIF1α and TWIST1 polyadenylation of mRNA, a poly(A) tail length assay was used. RNA of MDA-231 cells transfected with either Flag-CPEB2A or Flag-CPEB2B were

analyzed via RT-PCR with primers designed to target the HIF1α polyadenylation site (pA1) and the three polyadenylation sites of TWIST1 (pA1, pA2, and pA3) (Fig. 8A & 9A). Over-expression of CPEB2A resulted in no change in polyadenylation of HIF1α pA1 compared to the control and increased polyadenylation of TWIST1 mRNA at the pA2 and pA3 sites (Fig. 8C & 9C). Over-expression of the CPEB2B protein isoform resulted in no change in polyadenylation to either the HIF1α pA1 or TWIST1 (pA1, pA2, and pA3) sites compared to the control (Fig. 8C & 9C). These results demonstrate the differential regulation of polyadenylation of HIF1α and TWIST1 3'UTRs by the CPEB2A and CPEB2B protein isoforms.

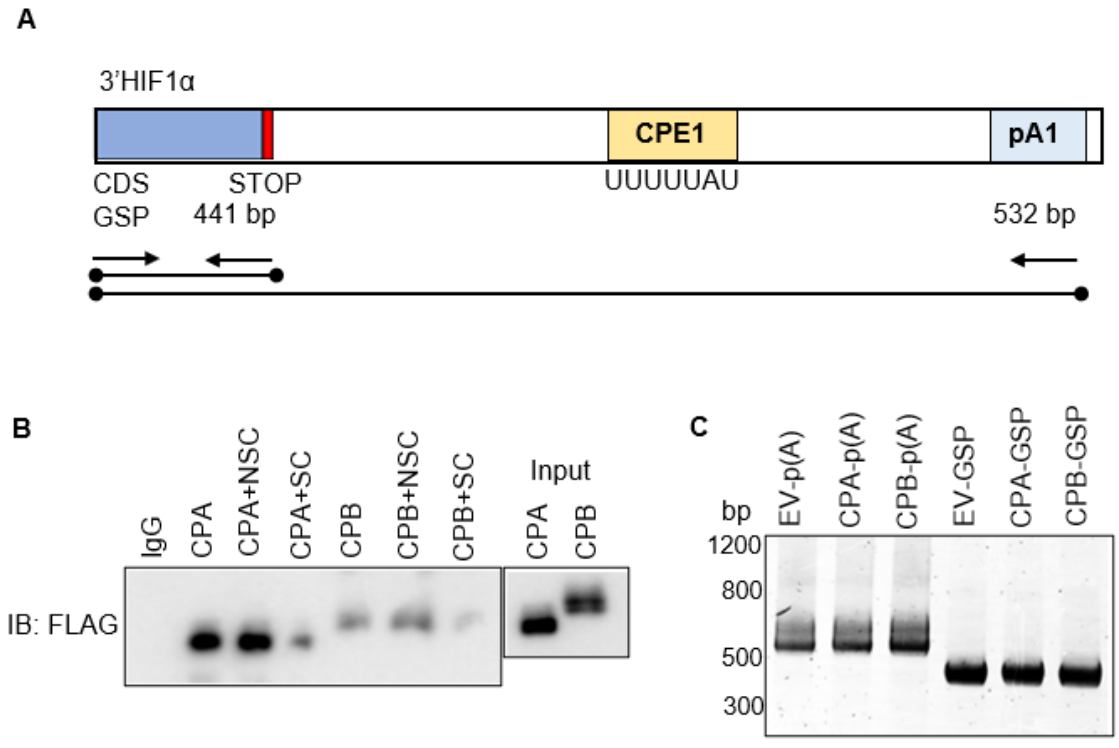

Figure 8: CPEB2A and CPEB2B HIF1α 3'CPE binding differs but poly(A) of HIF1α mRNA by both CPEB2 isoforms is consistent.

Figure 8 (continued): A) A graphical representation of HIF1α3'UTR mRNA indicating the CPE site (CPE1) and polyadenylation site (pA1) located in the 3' HIF1α UTR. Primer design scheme for poly(A) tail length assay is also represented. B) SBAP assay was used to detect Flag-CPEB2A (CPA) and Flag-CPEB2B (CPB) bound to CPE sequence 1 of HIF1α 3' UTR. MDA-231 cells were transfected with Flag-CPEB2A or Flag-CPEB2B plasmids and harvested after 48 hrs. Lysates were incubated with biotinylated HIF1α CPE 1 sequence RNA oligo. Samples were incubated with biotin-labeled HIF1α CPE 1 sequence + "cold" nonspecific competitor (NSC) or pre-incubated with 100X "cold" unlabeled HIF1α CPE1 3'UTR RNA as a specific competitor (+SC). C) USB Poly(A) Tail-Length Assay was used to determine poly(A) tail length of HIF1α mRNA. MDA-231 cells were transfected with Flag-CPEB2A (CPA) or Flag-CPEB2B (CPB), and RNA was extracted. G/I tailing of RNA was completed by adding Tail Stop Solution and incubating at 37C for 60 minutes. Poly(G/I) tailed RNA was then subjected to RT-PCR to produce cDNA transcripts. PCR of Poly(G/I) tailed cDNA was completed using primer sets designed to amplify poly(A) tail regions as described in (A).

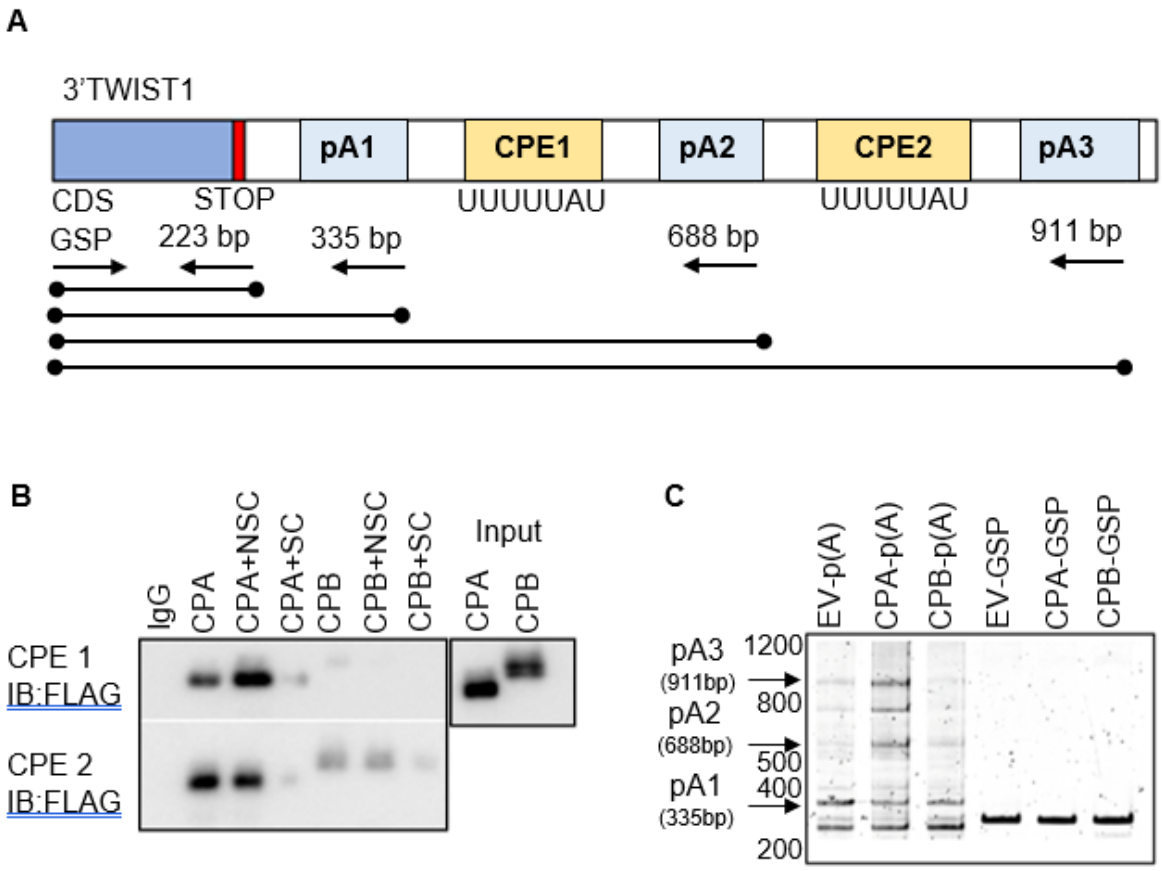

Figure 9: CPEB2A and CPEB2B TWIST1 3'CPE mRNA binding and poly(A) differ.

A) A graphical representation of TWIST1 3'UTR mRNA indicating the CPE sites (CPE1, CPE2) and polyadenylation sites (pA1, pA2, pA3) located in the 3' TWIST1 UTR. Primer design scheme for poly(A) tail length assay is also represented. B) SBAP assay was used to detect Flag-CPEB2A (CPA) and Flag-CPEB2B (CPB) bound to CPE sequences 1 and 2 (CPE1, CPE2) of TWIST1 3' UTR. MDA-231 cells were transfected with Flag-CPEB2A or Flag-CPEB2B plasmids and harvested after 48 hrs. Lysates were incubated with either biotinylated TWIST1 CPE1 or CPE2 sequences RNA oligos. Samples were incubated with biotin-labeled TWIST1 CPE1 or CPE2 sequences + "cold" nonspecific competitor (NSC) or pre-incubated with 100X "cold" unlabeled TWIST1

Figure 9 (continued): CPE1 or CPE2 3'UTR RNA as a specific competitor (+SC). C) USB Poly(A) Tail-Length Assay was used to determine poly(A) tail length of TWIST1 mRNA. MDA-231 cells were transfected with Flag-CPEB2A (CPA) or Flag-CPEB2B (CPB), and RNA was extracted. G/I tailing of RNA was completed by adding Tail Stop Solution and incubating at 37C for 60 minutes. Poly(G/I) tailed RNA was then subjected to RT-PCR to produce cDNA transcripts. PCR of Poly(G/I) tailed cDNA was completed using primer sets designed to amplify poly(A) tail regions as described in (A).

CPEB2A and CPEB2B associate with polyadenylation complex proteins.

To investigate potential interaction with polyadenylation complex proteins which have been previously described as associating with CPEB proteins and regulating translation of mRNAs, a co-immunoprecipitation assay was used. MDA-231 cell lysates with transiently expressing Flag-CPEB2A or Flag-CPEB2B were subjected to pull-down with anti-3xflag magnetic agarose beads and immunoblotting (Fig. 10A). The results indicated that CPEB2A and CPEB2B associate with the polyadenylation complex proteins GLD2 and PARN with no difference in affinities between the CPEB2A and CPEB2B isoforms detected (Fig. 10A). Furthermore, neither the CPEB2A nor the CPEB2B protein isoforms associated with the eukaryotic initiation factors eIF4E and eIF4G (Fig. 10A), which are essential in the cap-dependent mechanism of mRNA translation. This was surprising as it has previously been reported that CPEB1 indirectly binds eIF4E, inhibiting eIF4Es recruitment of the preinitiation complex.

To determine differences in CPEB2A and CPEB2B novel protein interactions, co-immunoprecipitation followed by proteomic analysis was employed. MDA-231 cell lysates with transiently expressing Flag-CPEB2A or Flag-CPEB2B were subjected to

pull-down with anti-3xflag magnetic agarose beads, and bound proteins were analyzed by proteomic analysis. This resulted in the identification of the hnRNP proteins hnRNPR and hnRNPF/H, and the eukaryotic initiation factor 3H (eIF3H) as having a stronger association with the CPEB2B isoform (Fig. 10 B&C). Additionally, the association of hnRNPR with CPEB2 was significantly more robust (*p-value 0.003) than with CPEB2A (Fig. 10D). Interestingly, hnRNPR was previously reported as an ITAF associated with IRES-mediated cap-independent translation of HIF1α mRNA. Also, eIF3Hs association with the preinitiation complex suggests that CPEB2Bs interaction with these proteins may promote activation of the cap-independent translation mechanism.

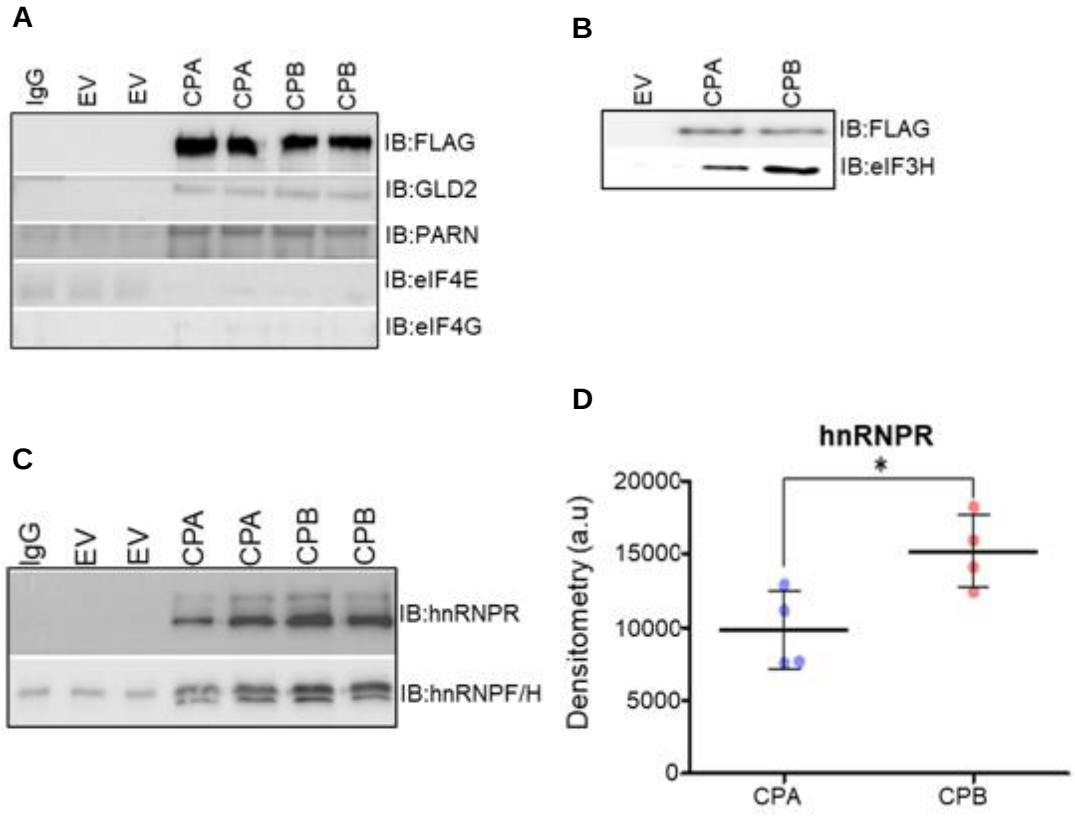

Figure 10: CPEB2B forms novel protein complexes.

Figure 10 (continued): A) Co-immunoprecipitation assays were performed using conical members of the polyadenylation complex. MDA-231 cell lysates transiently expressing Flag-CPEB2A or Flag-CPEB2B were exposed to anti-Flag-bound magnetic beads. The pull-down fraction was immunoblotted (IB) and compared to the (pcDNA3.1) (+) empty vector (EV) control. B-C) Co-immunoprecipitation immunoblotting of the novel proteins identified by proteomic analysis compared to the control (EV). D) The representative image for statistical significance analysis of hnRNPR bound to CPEB2B. Quantitation n = 4 ± standard deviation (sd) via densitometry. Statistical significance is reported as a p-value from oneway ANOVA pooled t-test (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).

CPEB2A and CPEB2B localize to the nucleus and cytoplasm.

A previously described nuclear role for CPEB proteins, specifically CPEB1 and CPEB4, suggested that these proteins bind to their targeted mRNAs in the nucleus, and the CPEB/mRNA complex is then exported to the cytoplasm. In this manner, the CPEB protein can exhibit tight translational regulation of mRNAs crucial to stress responses. To investigate the localization of the CPEB2A and CPEB2B protein isoforms, an immunofluorescent assay was used. MDA-231 cells transiently expressing either Flag-CPEB2A or Flag-CPEB2B were analyzed via microscopic analysis for nuclear and/or cytoplasmic localization (Fig. 11A). We found that both the CPEB2A and CPEB2B localized to the nucleus and cytoplasm (Fig. 11A). Examination of CPEB2A and CPEB2B under stress conditions was accomplished via a sub-cellular fractionation assay (Fig. 12). MDA-231 cells transiently expressing Flag-CPEB2A or Flag-CPEB2B

were subjected to either 2ng/mL arsenic or H_2O (control) for 16 hrs. Cell lysates were subjected to sub-cellular fraction and immunoblotted. The results indicated that CPEB2A and CPEB2B localized to both the cytoplasm (Fig. 12A) and the nucleus (Fig. 12B), and their localization is independent of cellular stress events (Fig. 12 A & B). These results suggest a nuclear and cytoplasmic role for CPEB2A and CPEB2B irrespective of cellar stress.

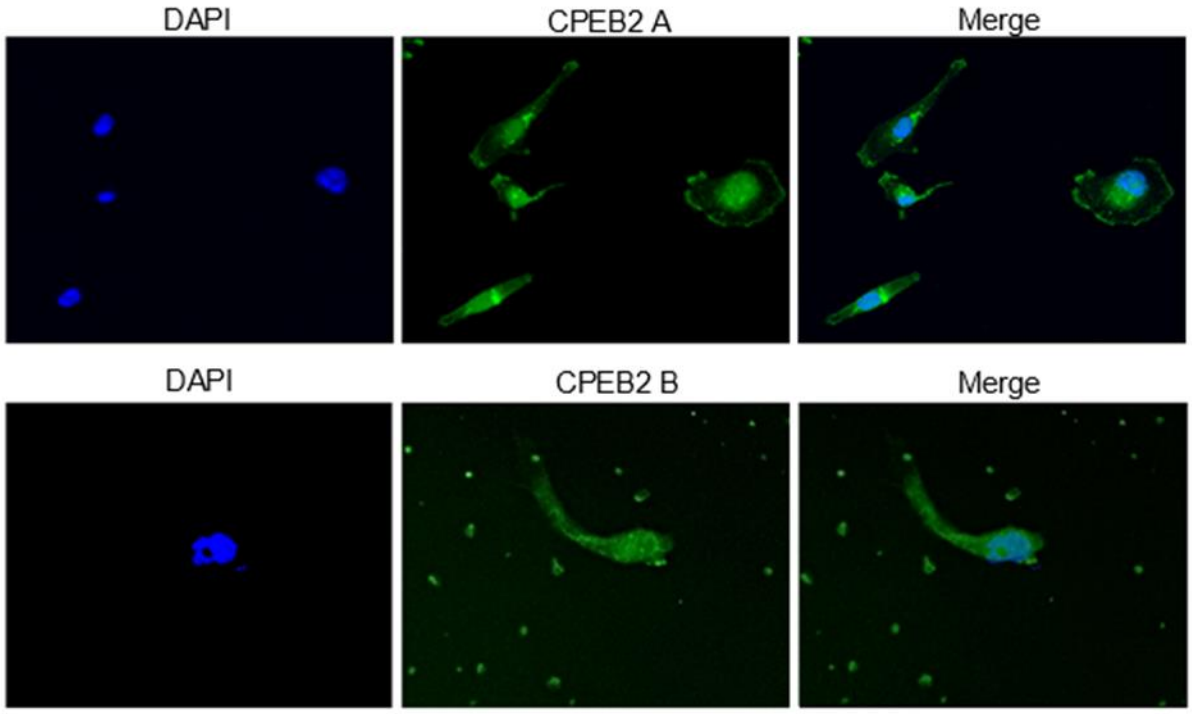

Figure 11: Nuclear and Cytoplasmic Localization of CPEB2A and CPEB2B.

MDA-231 cells stably expressing either Flag-CPEBA or Flag-CPEB2B were cultured on coverslips for 24 hours and then fixed with 4% paraformaldehyde. Cells were incubated with Alexa fluor 488 rabbit monoclonal antibody. Nuclei were with by DAPI. Images were taken using a Keyence BZ-X series microscope.

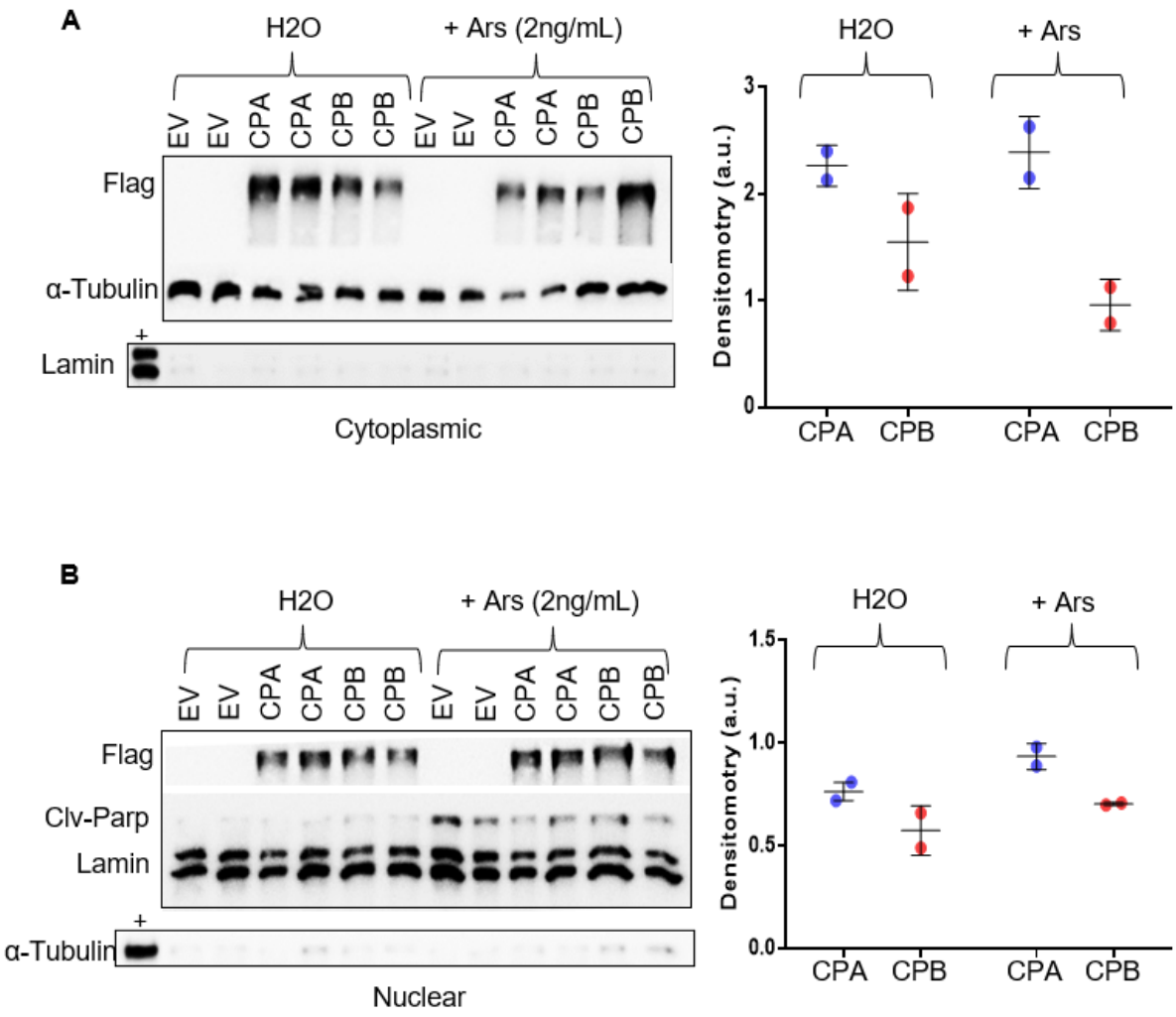

Figure 12: CPEB2A and CPEB2B protein isoforms localize to the cytoplasm and nucleus independent of cellular stress.

Subcellular fractionation was performed on MDA-231 cell lysates transiently expressed Flag-CPEB2A or Flag-CPEB2B and treated with either arsenic (2ng/mL) or H2O (control) for 16 hours. A) MDA-231 cytoplasmic fraction was subjected to immunoblot analysis with indicated antibodies. B) MDA-231 cytoplasmic fraction was subjected to immunoblot analysis with indicated antibodies. Quantitation n = 2 ± standard deviation

Figure 12 (continued): (sd) via densitometry. Statistical significance is reported as a p-value from oneway ANOVA pooled t-test (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).

CPEB2A and CPEB2B protein association.

Although direct interaction amongst CPEB proteins nor indirect interaction via association with the same complex has never been reported, we investigated this potential using co-immunoprecipitation. Mda-231 cells transiently expressing Flag-CPEB2A and HA-CPEB2B were subjected to pull-down with anti-3xflag magnetic agarose beads and immunoblotted (Fig. 13). Surprisingly, the results indicate that HA-CPEB2B either directly interacts or maybe interacting with a protein complex in which Flag-CPEB2A is bound (Fig. 13).

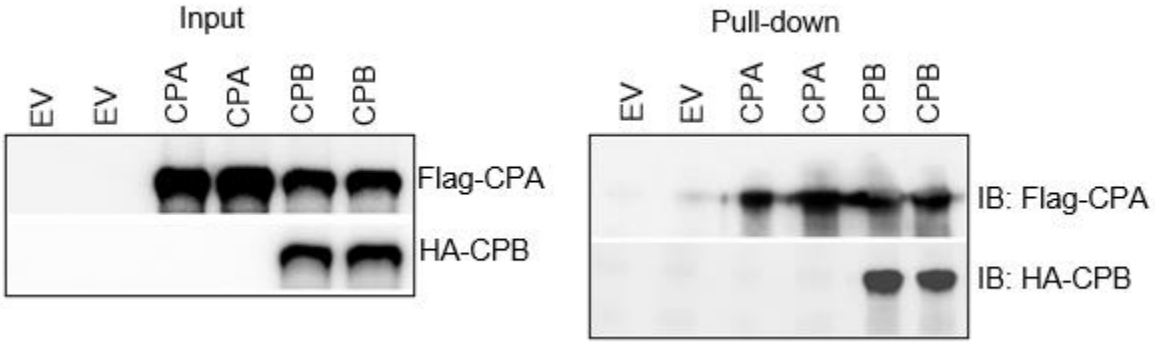

Figure 13: CPEB2A and CPEB2 protein interaction

A) Input: MDA-231 cell lysates transiently expressing Flag-CPEB2A (CPA) and HA-CPEB2B (CPB) were incubated with anti-Flag-bound magnetic beads and

Figure 13 (continued): immunoblotted for indicated proteins. B) Pull-down fraction was immunoblotted (IB) and compared to the (pcDNA3.1) (+) empty vector (EV) control.

Discussion

As presented herein, our laboratory has provided insight into the differential regulatory function of CPEB2A and CPEB2B in the context of HIF1α and TWIST1A mRNA binding and expression, including describing a mechanism for their translational regulation by the CPEB2 protein isoforms in TNBC. We have previously described a mechanism whereby altered mRNA splicing of CPEB2, via association with the *trans*-splicing factor serine-arginine splicing factor 3 (SRSF3), differentially regulates translation of HIF1α and TWIST1 mRNA, transcription factors crucial to the metastatic progression of TNBC. Specifically, we have previously reported that CPEB2A decreases TWIST1 and HIF1α protein levels. Conversely, we have reported that CPEB2B dramatically increases TWIST1 and HIF1α protein levels corresponding to an increase in the metastatic phenotype.

In this thesis, we describe the differential RNA binding displayed by the two CPEB2 isoforms. Specifically, the CPEB2A protein isoform binds 3'CPE sequences in both HIF1α and TWIST1; however, surprisingly, we observed limited to no binding affinity of CPEB2B to the CPE regions of HIF1α and TWIST1 as predicted by our original hypothesis. Furthermore, we show differences in 3' UTR poly(A) regulation of TWIST1 mRNA between the CPEB2A and CPEB2B isoforms. Specifically, overexpression of the CPEB2A isoform results in poly(A) extension of the TWIST1 3'UTR compared to the control. However, we did not see this result in the CPEB2B

overexpressing samples, which more closely resembled the TWIST1 poly(A) length in the control. These results were surprising as it is well characterized that CPEB proteins, upon binding to CPE sites of target mRNA species, inhibit their polyadenylation and subsequently their translation. Typically, under stress conditions, phosphorylation of CPEB proteins allows for mRNA polyadenylation and increased translation.

Interestingly, we observe that CPEB2A regulates the polyadenylation of TWIST1 in non-stress conditions by allowing the polyadenylation of TWIST1, which is not consistent with the original hypothesis. Taken together, these data suggest that CPEB2A regulates the translation of TWIST1 via binding to the TWIST1 3'UTR, promoting TWIST1 polyadenylation and decreasing translation. Indeed, Nairismägi and colleagues reported that translational suppression of TWIST1 was accomplished by CPEB1, CPEB2 (presumably CPEB2A isoform, as this study was carried out in MCF-10A cells), and mi-580 [157]. TWIST1 contains two CPE sequences within the 3'UTR and binding of both of these sequences by the CPEB (1,2) proteins promote the inclusion of regulatory miRNA sites within the 3'UTR of TWIST1 mRNA. In this manner, the association of CPEB1/CPEB2/miRNA results in reduced expression of TWIST1 protein. We have shown that CPEB2A associates strongly with both CPE elements in the TWIST1 3'UTR and that increased CPEB2A reduces translation of TWIST1 mRNA. We proposed that this result may be due to the miRNA site found between the two CPE sites in TWIST1 3'UTR (Fig.14).

As we seek to explain why overexpression of the CPEB2B protein isoform results in increased TWIST1 mRNA translation, we note that it has been well documented that

shortened poly(A) tails of mRNAs essential to stress response pathways promotes their rapid translation [158]. Indeed, it is plausible that whereas CPEB2A inhibits TWIST1 mRNA translation ploy(A) extension and subsequent inclusion of miRNA sites, CPEB2 may indirectly reduce poly(A) tail length of TWIST1 mRNA and increase its translation (Fig. 14). This finding is evidence for a novel function of the CPEB2 isoforms in regulating translation through differing polyadenylation of TWIST1 mRNA.

We have also demonstrated that CPEB2 (exon four included) associates with proteins that have been reported as IRES *trans*-acting factors (ITAFs) important in IRES-mediated cap-independent translation [159]. Indeed, our proteomic analysis of proteins bound to CPEB2B found that the CPEB2B protein isoform associates with the translation initiation factor eIF3H and the hnRNP proteins hnRNPR and hnRNPF/H. Interestingly, hnRNPR has been shown to function as an ITAF in promoting IRES cap-independent translation of mRNA [160]. Furthermore, we found that CPEB2B is associated with the translation initiation factors eIF4E or eIF4G, critical regulatory proteins in the cap-dependent translation mechanism [161]; however, we did find CPEB2B associated with eIF3H, which interacts with the preinitiation complex. Furthermore, an IRES site has been reported in the 5'UTR of HIF1α mRNA, activating the cap-independent translation of HIF1α mRNA [162]. Taken together, these findings suggest a mechanism by which CPEB2B interacts with known ITAFs, activating the IRES cap-independent mechanism of translation HIF1α.

Here we also report that the CPEB2A and CPEB2B protein isoforms interact with each other. This novel finding was very surprising as interactions amongst CPEB proteins have not been described. This interaction provides evidence of a

CPEB2A/CPEB2B axis of translational regulation of mRNA. In this mechanism, an alternative splicing switch promoting an increase in the CPEB2B isoform allows for increased binding to CPEB2A, thus blocking translational inhibition of HIF1α and TWIST1 mRNA while CPEB2B interacts with the ITAF (hnRNPR) and recruits the 43S preinitiation complex via association with eIF3H and promotes rapid translation in an IRES cap-independent mechanistic fashion (Fig. 15).

We have shown that CPEB2A and CPEB2B interact and that CPEB2B is associated with the hnRNPR, hnRNPF/H, and eIF3H proteins. Since hnRNP proteins are multifunctional in both the nucleus and cytoplasm and a nuclear role for CPEB proteins has been described, we wanted to determine if CPEB2A and/or CPEB2B also can localize to the nucleus. Interestingly, we found that both CPEB isoforms are indeed localized to the nucleus and cytoplasm. This finding was intriguing and suggests CPEB2A may bind nuclear HIF1α and TWIST1 mRNA before being shuttled out to the cytoplasm. In this manner, CPEB2 can exhibit tight translational control of the mRNAs. Furthermore, we previously found that siRNA targeting hnRNPF/H bound to and promoted the inclusion of exon four in the CPEB2 pre-mRNA transcript (CPEB2B) (Table 1). Here we also reported that the CPEB2B protein isoform was found to associate with hnRNPF/H. This suggests an additional level of CPEB2 regulation in which CPEB2B may bind nuclear hnRNPF/H and inhibit its association with exon four and allowing association with SRSF3 with exon four of CPEB2 pre-mRNA, thus promoting inclusion of exon four in the CPEB2 transcript (CPEB2B).

In conclusion, we have identified a novel mechanism for HIF1 and TWIST1 mRNA translational regulation by the CPEB2A and CPEB2B isoforms. The mechanism

described here demonstrates that the CPEB2A/CPEB2B interaction directly relates to translational activation or inhibition of HIF1 and TWIST1. A splicing switch favoring the production of CPEB2B leads to increased HIF1 and TWIST1 translation, promoting the acquisition of AnR and metastasis in TNBC. Additionally, we provide evidence that the regulation of translation begins in the nucleus by the binding of CPEB2A to the mRNA of HIF1 and TWIST1. We have also provided evidence that CPEB2B make act in a regulatory feedback loop through binding hnRNPF/H in the nucleus and promoting increased CPEB2B production. Future studies are necessary to investigate potential binding motifs within the CPEB2 proteins, allowing their dimerization. This study provides insight into the regulatory process governing the translational control of HIF1a and TWIST1 mRNA and TNBC progression and its potential therein to translate to novel therapeutic targets for the treatment of breast cancer carcinoma.

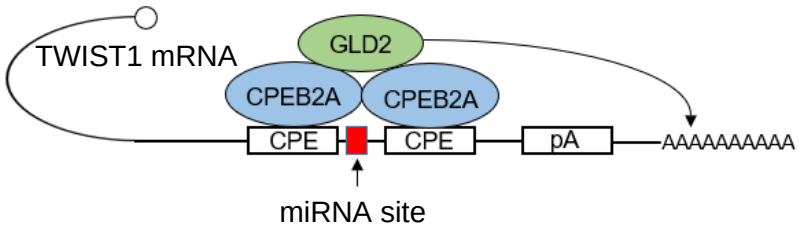

Figure 14: CPEB2A protein isoform inhibits TWIST1 mRNA translation.
CPEB2A inhibits the translation of TWIST1 mRNA by binding both CPE elements located in TWIST1 3'UTR, promoting poly(A) and inclusion of miRNA regulatory sites between the CPE sites.

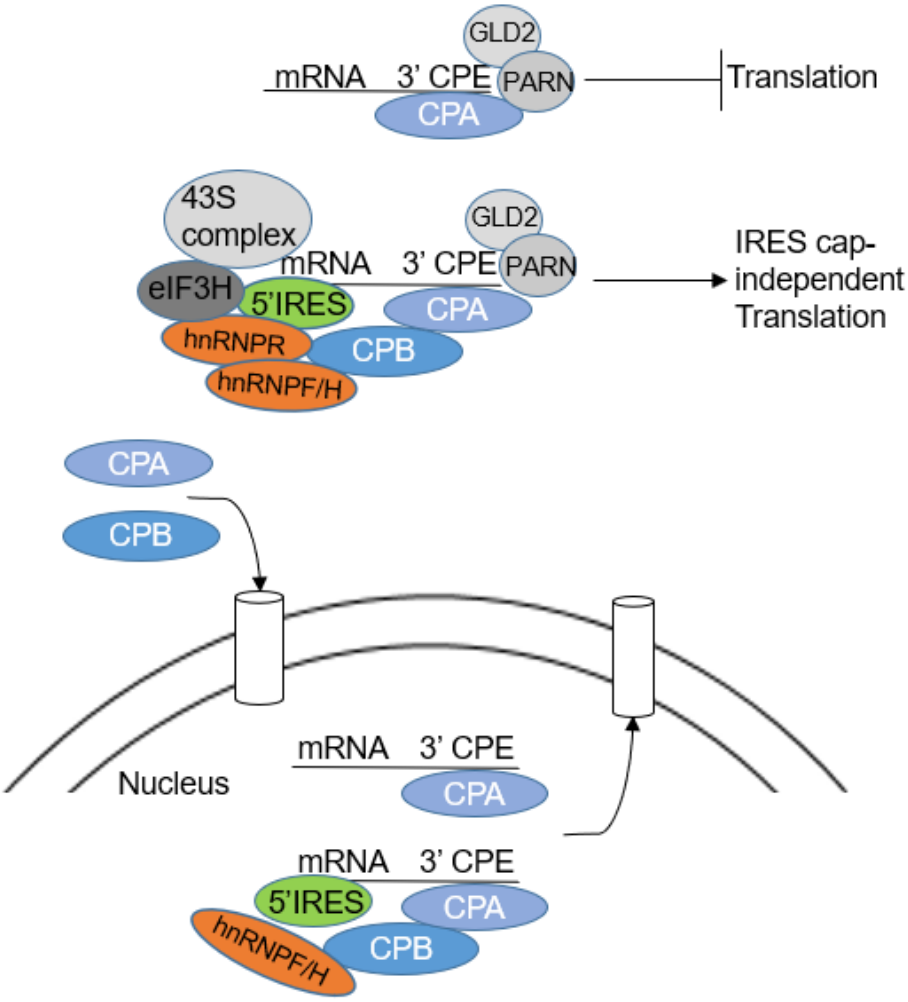

Figure 15: Mechanism of Translational regulation by CPEB2A/CPEB2B interaction and IRES cap-independent translational activation.

CPEB2A and CPEB2B protein isoforms locate the nucleus. CPEB2A binds target mRNA and is exported to the cytoplasm, where it interacts with the poly(A) protein complex (GLD2 and PARN) and inhibits translation. Increases levels of the CPEB2B isoform binds the A isoform and interacts with hnRNPF/H, which is then exported. In the cytoplasm, CPEB2B interaction the ITAF hnRNPR, recruits the 43s preinitiation complex through eIF3H interaction, and initiates cap-independent translation.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

TNBC is a heterogeneous cancer with high morbidity and mortality. This heterogeneity increases the complexity of its clinicopathological features and influences the survival and outcomes of this disease. Further attempts to classify TNBC into the basal-like subtype (1-2) (BL1 and BL2), mesenchymal-like and mesenchymal stem-like (M and MSL), and luminal androgen receptor (LAR) subclasses have been made. These classification attempts aim to generate “precision” treatment approaches based on specific genetic and biochemical targets to increase favorable outcomes for TNBC patients. As an example, the BL1/BL2 subtypes display increased expression of genes that promote cell proliferation and DNA damage repair, which allows for focused clinical trials using antimitotic and DNA-damaging agents. Furthermore, the IM subtype of TNBC has been treated using monoclonal antibodies, activating an immune response towards cells that display high immunogenicity and neoantigen expression. It's estimated that over ninety percent of TNBC tumors overexpress the TROP2 glycoprotein, which promotes proliferation, invasion, and survival. The use of an antibody-drug conjugate with active irinotecan metabolite to treat TNBC resulted in a thirty percent overall response rate and an overall survival rate of 16.6 months in patients displaying metastatic TNBC. These examples provide evidence that targeted (or “personalized”) treatment of TNBC based on the patient's molecular profile is a viable option in pursuing increased survival and outcomes of the disease. The studies presented in this dissertation attempt to improve our understanding of crucial regulatory

pathways active in TNBC progression, specifically during the early earliest phases of metastasis when cells depend on the acquisition of anoikis resistance and EMT to invade surrounding tissue and enter circulation. Two genes critical to these processes, HIF1α and TWIST1, are translationally activated to promote these phenotypes. These genes both contain CPE consensus sequences within their 3'UTR mRNA, in which binding of the CPEB1 protein represses their translation in maturing oocytes. It was also demonstrated that the CPEB2 member of the CPEB family binds to the HIF1α and TWIST1 3'PE sites. In chapter two of this dissertation, we demonstrated that the splice variants of CPEB2, CPEB2A, and CPEB2B interact with the 3'CPE sites in HIF1α and TWIST1; however, CPEB2Bs interaction is significantly weaker. These interactions directly influence the transitional fate of HIF1α and TWIST1 mRNAs.

A characteristic of TNBC and other cancer types is their ability to hijack alternative splicing pathways and promote transcriptome changes beneficial to their growth and survival. As a crucial regulator of pre-mRNA splicing, the global *trans*-splicing factor SRSF3 is estimated to have over two hundred splicing targets. We have demonstrated that SRSF3 mRNA levels are significantly increased in TNBC compared to other breast cancer subtypes in the BRCA cohort of TCGA. Examination of this cohort also revealed that alteration of SRSF3 expression correlates to reduced survival. Furthermore, SRSF3 protein expression is enhanced after acquisition of anoikis resistance in multiple TNBC cell lines. While these data provide evidence that SRSF3 affects the metastatic phenotype of TNBC *in vitro,* in humans, the effects may be more subtle due in part to the effects of post-translational modification of SRSF3 and its

function. Therefore, it would be useful to examine protein kinases regulating SRSF3 phosphorylation at the RS domain in future studies.

Interestingly, patients that exhibited the lowest SRSF3 expression levels were either stage II or stage III, stages in which lymph node involvement is common, and micrometastases are generally present. While these patients may exhibit low SRSF3 expression in the primary tumor, a different expression profile may be found in metastatic sites. Future investigations combining PTM status, profiling of metastatic RNA, and pathological staging taken in concert may better predict patient outcomes.

In chapter two, we endeavored to elucidate a mechanism to determine the location of SRSF3 binding within exon four of the CPEB2 pre-mRNA, thereby promoting its inclusion in the CPEB2B mature mRNA transcript. In TNBC anoikis-resistant cells, increased SRSF3 protein levels directly correlate with a decrease in the CPEB2A/CPEB2B isoform ratio implicating SRSF3 as the RNA *trans*-factor modulating the AS of CPEB2. In addition, reduced SRSF3 expression via siRNA increased the CPEB2A/CPEB2B ratio (reduced exon four inclusion) in both MDA-231 parental and anoikis resistant cells. We also identified an RNA cis-element in exon four in which SRSF3 binds and mutation of this cis-element ablated interaction with SRSF3. We further investigated this result by designing a minigene reporter construct which allowed for modulation of SRSF3 in a manner that mimics endogenous CPEB2A and CPEB2B isoform expression, and that mutation of the CPEB2 RNA *cis*-element increased the CPEB2A/CPEB2B isoform ratio *in vitro*. We were able to "rescue" the anoikis-resistant phenotype in TNBC cells that were treated with siSRSF3 by overexpression of the CPEB2B isoform. Taken together, we see evidence that SRSF3 acts as the trans-

splicing factor regulating the AS of CPEB2 via binding an RNA *cis*-element in exon four of CPEB2 in TNBC.

In chapter three, we investigated the mechanism of translational regulation by the CPEB2A and CPEB2B isoforms. First, we explored the potential of the CPEB2 isoforms to bind to conserved CPE sequences in the 3'UTR of HIF1α and TWIST1 mRNA since both CPEB2 isoforms contain identical conserved RBD's in their C-terminal ends. We reported that the CPEB2A protein isoform binds 3'CPE sequences in both HIF1α and TWIST1, but the CPEB2B isoform displayed a weak interaction. We also reported an increase in poly(A) of TWIST1 3'UTR corresponding to CPEB2A binding both CPE sequences. We propose that this result is due to a miRNA site found between the two CPE sites in TWIST1 3'UTR results in a decrease in TWIST1 mRNA translation and an increase in anoikis sensitivity in TNBC. With regards to the CPE sequence binding potential of the CPEB2B isoform, further investigation is needed. We have previously reported that increased CPEB2B protein levels promote the translation of both HIF1α and TWIST1 mRNA; however, we saw that the CPEB2B isoform interaction with the CPE sequence in TWIST1 and HIF1α was weak how then is CPEB2B regulating their translation.

A potential answer was found when we investigated possible protein interaction differences between the two isoforms. Using co-immunoprecipitation and proteomic analysis, we identified numerous proteins which showed a strong binding affinity for CPEB2B but not CPEB2A. We identified these proteins as important trans-factors in IRES cap-independent translation. Also, we found via co-immunoprecipitation that the CPEB2A and CPEB2B isoforms were interacting with each other. This finding was

certainly unique and revealed the potential of a novel mechanism for translational regulation of mRNA by CPEB2. We propose that CPEB2A binds 3' CPE sequences in targeted mRNA and inhibits their translation; however, upon interaction with the CPEB2B isoform and its binding of ITAF factors, IRES cap-independent translation is initiated. This would also explain CPEB2Bs weak interaction with 3'CPE sequences of mRNA as it is bound to CPEB2A, which has a strong affinity to the CPE sequences of HIF1α and TWIST1.

Furthermore, we reported that CPEB2A and CPEB2B localize to both the nucleus and the cytoplasm. This finding was not novel for the CPEB2A protein isoform as a nuclear function for the CPEB family member CPEB1 has been reported in which CPEB1 binds nuclear mRNA before export, thereby eliciting tight translational control of targeted mRNA. However, it is surprising in the CPEB2B protein isoform context since we demonstrated a weak interaction of CPEB2B and 3' CPE sequences within HIF1α and TWIST1 mRNA. The answer to this question may lie in the details of our chapter two findings. When we analyzed potential trans-splicing factors bound to exon four of CPEB2 pre-mRNA using a siRNA screen, we found that knock-down of hnRNPF/H resulted in a reduced ratio in CPEB2A/CPEB2B mRNA transcripts. Later in chapter three, we detailed a strong affinity between the CPEB2B and hnRNPF/H proteins. We now think it is plausible that CPEB2B may bind nuclear hnRNPF/H in the nucleus initiating a regulator feedback loop. In this mechanism, the CPEB2B isoform binds to nuclear hnRNPF/H, thereby reducing the levels of available hnRNPF/H to bind the exon four sequence of CPEB2 pre-mRNA. This mechanism, combined with increases in SRSF3, promotes rapid and robust CPEB2B mRNA isoform expression. The increased

presence of the CPEB2B allows for increased CPEB2A/CPEB2B interaction, resulting in increased translational activation of HIF1α and TWIST1 mRNA.

Although these conclusions still need a considerable amount of investigation for confirmation, the mechanism would provide significant insight into the role of CPEB proteins and their mechanism of translational regulation.

With the seemingly limitless potential of bioinformatics, advances in the treatment of TNBC based on precision medicine have been limited and still rely, in large part, on systemic delivery of chemotherapeutics. Biomedical research's continued and future goal is to focus on tailoring patient therapy by utilizing the genetic profiles of individual cancer subsets. As bioinformatic technologies including transcriptomics, immune-omics, radiomics as well as other “omics” become less expensive, their use will allow for individual tumor analysis and will help provide treatment guidance for patients. For example, this approach could be used to investigate the dysregulated cellular pathways which define the tumor's genetic signature and help guide treatment options towards therapeutics which target those pathways. Indeed, a recent clinical trial using this approach found that inhibition of the PD-L1 immune checkpoint via treatment with atezolizumab (monoclonal anti-PD-L1 antibody) yielded positive results in TNBC treatment. The research contained herein provides new insight into the dysregulated cellular pathways which promote the aggressive nature of TNBC and contribute to potential treatment strategies in the clinic. These insights allow for the possibility of developing novel therapeutics targeting unique aspects of both the transcriptome and proteome with consideration of specific sequence properties and molecular interactions of TNBC metastatic progression.

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