EXAMINING THE REGULATION AND FUNCTION OF CANCER TESTIS ANTIGENS

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DEDICATION

First, I would like to thank my mentor Dr. Angelique Whitehurst for her many hours of training and guidance. I truly appreciate your dedication and support throughout my graduate career. I would like to thank Dr. Rolf Brekken, Dr. Melanie Cobb, and Dr. Jane Johnson for their continual support, I have learned a great deal from all of you. I would like to thank my parents for always encouraging me to reach for my dreams, and making so many sacrifices to help me achieve my goals. To my sister, thank you for your love and guidance. To my grandmother, thank you for always being my number one fan. Lastly, to my husband who has been there for me every day, supporting me and believing in me, I could never have done this without you.

EXAMINING THE REGULATION AND FUNCTION OF CANCER TESTIS ANTIGENS

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EXAMINING THE REGULATION AND FUNCTION OF CANCER TESTIS ANTIGENS

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Abstract: Cancer Testis Antigens (CTAs) are a class of genes whose expression is generally restricted to the testis, but are reactivated in cancer cells. The function and regulation of many CTAs are unknown, however several CTAs have been shown to impact tumor cell fitness and correlate with poor prognosis. Our lab became interested in CTAs after a pan-genomic-loss-of-function RNAi screen identified several CTAs as chemo-sensitizers. Acrosin Binding Protein (ACRBP) was identified in this screen and further analysis confirmed ACRBP's function as a microtubule stabilizer that protects cancer cells from the mitotic defects attributed to paclitaxel treatment. Because ACRBP is not expressed in normal somatic tissue, we are interested in how ACRBP is reactivated in cancer cells. In mouse spermatogonium, the ACRBP promoter is bound by Cyclic-amp Response Element Modulator (CREM) at a conserved Cyclic-amp response

element (CRE). Depletion of its close family member CREB results in a loss of ACRBP expression in cancer cells, while overexpression of CREB induces ACRBP expression. Interestingly, this regulation appears to be phospho-independent and seems to apply to other CTAs.

Another CTA, Chondrosarcoma Associated Genes 1, was identified by a loss-of-function RNAi proliferation screen as a supporter of melanoma cell proliferation. CSAG1 is highly expressed in Melanoma lines with little to no expression in normal tissues. Additional CSAG1 siRNA experiments have validated the screen data confirming CSAG1's involvement in melanoma cell proliferation. Additionally, CSAG1 loss-of-function reduces long-term melanoma cell viability and induces senescence in cancer cells. Consistent with this data, overexpression of CSAG1 enhances colony-forming ability in cancer cells. These data suggest that CSAG1 supports cancer cell viability. Further studies will help elucidate how CSAG1 supports tumor cell fitness. Understanding the regulation and function of CTAs may provide new insight into novel cancer therapeutics.

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Pavlovsky, A.A., et al., *Psychological stress, cocaine and natural reward each induce endoplasmic reticulum stress genes in rat brain.* Neuroscience, 2013. **246**: p. 160-9.

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CHAPTER ONE
Introduction

1.1 Discovery and characterization of cancer-testis antigens

Spermatogenesis is the process by which male primordial germ cells develop into functioning spermatozoa [1]. Mammalian male germ cells express several specialized genes that are not expressed in somatic tissues to complete this highly evolved process. For this reason, the mammalian testis has evolved as an immune privileged organ to prevent autoimmune attacks [1]. Many of these testis-selective genes are expressed in cancer cells and were first identified because they elicit an immunologic response from autologous cytolytic T-lymphocytes (CTLs) in cancer patients [2]. The Melanoma antigen (MAGE) family of CTAs was the first to be discovered this way, and expression analysis revealed regionally restricted expression of the MAGE A genes to testes and tumors only [3, 4]. B melanoma antigen (BAGE) and G melanoma antigen (GAGE) were also identified using CTLs mounting an immune response against melanoma cells [5, 6]. Several other testis-restricted antigens were identified using antibodies along with cDNA expression libraries [7, 8]. With the growing number of testis-restricted tumor antigens being discovered, the term cancer-testis antigen (CTA) was coined to describe these genes [8]. It is important to note that some CTAs can also be detected in fetal ovarian germ cells, trophoblasts, and adult stem cells [9].

1.2 Cancer-testis antigens in spermatogenesis and tumorigenesis

CTAs have been separated into two groups: CT-X antigens and non-CT-X antigens [10]. CT-X antigens are encoded on the X-chromosome and are more commonly expressed in proliferating germ cells, or spermatogonia; while non-CT-X antigens reside on other

chromosomes and are typically expressed in more differentiated germ cells, or spermatocytes/spermatids [10]. Additionally, CT-X antigens tend to be part of large gene families as a result of gene duplication in evolution, however it is uncommon for non-CT-X antigens to be a part of a large gene family [10]. Each stage of spermatogenesis requires the use of specialized genes which may be why many CTAs are restricted to specific stages of spermatogenesis. [11] (Figure 1).

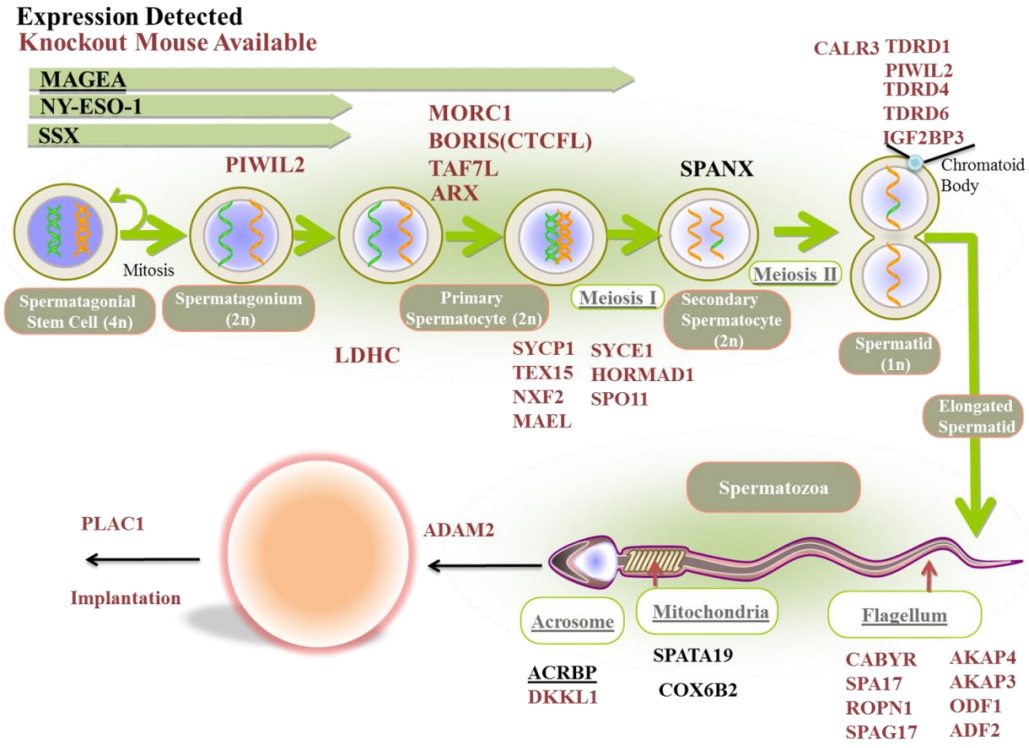

Figure 1. Cancer-testis antigen involvement in spermatogenesis [11] Cancer testis antigen expression depicted in the stages of spermatogenesis. Red genes have knockout mice available.

For many years, scientists have theorized that the process of spermatogenesis and tumorigenesis share common characteristics such as proliferation, migration, colonization, increased metabolic demand, and cell survival [1, 10-13]. It has been hypothesized that several CTAs are uniquely evolved for these processes, and tumor cells can utilize CTAs to enhance their own fitness [10, 13, 14].

The most characterized family of CTAs, the MAGE-I genes, function in many ways to block apoptosis in cancer cells. MAGE-I genes have been implicated in p53 ubiquitin-mediated degradation, as well as inhibiting p53 transactivation potential by recruiting Histone Deacetylate 3 (HDAC3) to MAGE-p53-chromatin complexes [15-17]. Additionally, MAGEA3 can block Caspase-12 activation to prevent apoptosis in cancer cells [18].

Several other CTAs have been implicated in tumor cell proliferation and survival. The SSX (Synovial Sarcoma, X Breakpoint) family of transcriptional repressors was shown to support cancer cell proliferation and survival by modulating WNT and MAPK signaling [19, 20]. CAGE (Cancer Antigen 1), another CTA, activates AP-1 and E2F1 transactivation of cell cycle genes such as cyclins D1 and E [21]. ATAD2 (ATPase family, AAA domain containing) acts as a co-activator of MYC and Estrogen Receptor 1, inducing the transcription of proliferation and cell survival genes such as B-MYB and EZH2 (Enhancer Of Zeste Homolog 2) [22, 23]. PRAME (Preferentially Expressed Antigen in Melanoma) binds to and inhibits Retinoic Acid Receptor's (RAR) transactivation of differentiation and apoptotic genes in melanoma cells [24]. PIWIL2 (Piwi-Like RNA-Mediated Gene Silencing 2), blocks apoptosis and increases cancer cell survival by activating the STAT3/BCL12 pathway [25]. Many of these CTAs can also protect cancer cells from growth inhibitory chemotherapy drugs such as paclitaxel, cisplatin, etc., making CTAs not only prognostic but predictive factors for cancer patients [26-28].

Another common phenotype of germ cells and cancer cells is their ability to migrate to and invade new tissues. Primordial germ cells must migrate from the yolk sac to the gonad where they proliferate and mature [29]. Spermatogonia and spermatocytes must also migrate from the basement membrane of the seminiferous tubule into the lumen as they mature into spermatozoa [1]. It is suggested that germ cell migration and invasion closely resembles tumor cell metastasis

and that some CTAs may support tumor cell migration and invasion by increasing cancer cell motility and driving epithelial-to-mesenchymal transition (EMT) [12]. For Example, MAGEC-2 is associated with breast cancer metastasis along with lower E-cadherin and cytokeratin expression and higher vimentin and fibronectin expression, suggesting a shift towards a mesenchymal phenotype [30]. CT45A1 was also found to enhance breast cancer cell migration and invasion through EMT [31]. CAGE was found to enhance melanoma cell motility by inducing EMT proteins Snail and c-FLIP via extracellular regulated kinase (ERK), Akt and Nuclear Factor kappaB (NF-kB) [32]. Additionally, GAGE12 was the most differentially overexpressed gene in metastatic gastric carcinoma compared to primary tumors in mice [33]. The authors found that GAGE12 overexpression enhanced gastric carcinoma cell migration and invasion in an *in vivo* model [33]. More research into the functions of CTAs in spermatogenesis and tumorigenesis will likely reveal several other CTAs that support tumor cell fitness.

1.3 Cancer-testis antigens as cancer biomarkers and therapeutic targets

CTAs' restricted tumor/testis expression and their functional roles in cancer make them useful biomarkers and therapeutic targets. For example, the above mentioned genes involved in metastasis also serve as progressed-disease biomarkers in cancer patients [14]. CTAs can also be biomarkers for early stage disease such as Sperm-Associated Antigen 9 (SPAG9) in early breast and cervical cancer detection [34, 35]. Several CTAs can also be used as biomarkers for response to treatment, such as PIWIL2, MAGE and GAGE genes which confer cisplatin resistance to cancer cells [27, 36]. Interestingly, some CTAs can predict cancer subtypes [37].

CTAs are also being used as targets for cancer immunotherapies such as CTA vaccines and adoptive transfer CTA-targeting T-cells [38]. CTA cancer vaccines work by fusing an

immunogenic CTA peptide with a highly immunogenic adjuvant to stimulate a T-cell response against a tumor that expresses that particular CTA [39]. MAGE-A3 cancer vaccines have been unsuccessful at extending disease-free survival (DFS) in non-small cell lung cancer (NSCLC) and melanoma patients (http://www.gsk.com). However, NY-ESO-1 vaccines have shown some success in inducing T-cell responses and extending DFS of ovarian and melanoma cancer patients [40, 41]. It is suggested that the success of NY-ESO-1vaccines over MAGE-A3 vaccines may be a due to NY-ESO-1's higher immunogenicity than MAGE-A3[12]. New studies are exploring combination CTA vaccines to improve efficacy by targeting more tumor cells than a single agent vaccine [10, 12].

Another type of cancer immunotherapy targeting CTAs is adoptive CTA-specific T-cell therapy. This strategy employs *in vitro* expanded T-cells genetically modified to target a CTA expressed in the patient's tumor [42]. Again, NY-ESO and MAGE-A3 were the first CTAs to be targeted using this method. NY-ESO-targeting T-cells have achieved significant clinical responses in melanoma and synovial sarcoma patients and did not result in cytotoxicity [43]. Unfortunately, MAGE-A3-targeting T-cells resulted in serious complications due to off-target cross-reactivity against proteins expressed in the brain and heart [44, 45]. These results suggest that CTAs can be successful targets for cancer immunotherapy, but more research needs to be done to understand the expression pattern and immunogenicity of potential targets.

Recent efforts combine CTA immunotherapy with another cancer therapy in hopes of preventing resistance [12]. Traditional chemotherapeutic agents work by inducing apoptosis in proliferating cells, however some cancer cells can avoid apoptosis by expressing anti-apoptotic CTAs such as MAGE, GAGE, and PIWI genes [14-16, 26, 34]. Combining chemotherapy with

immunotherapy targeting anti-apoptotic CTAs will likely kill more tumor cells than either treatment alone.

DNA-methyltransferase inhibitors (DNMTI) can induce the expression of epigenetically silenced genes in cancer cells to restore tumor-suppressor pathways that may activate apoptosis or senescence in cancer cells [46, 47]. An unforeseen side-effect of DNMT treatment is the induction of certain CTAs that trigger T-cell responses in cancer patients [48-50]. A new strategy is designed to treat a patient with DNMTs first to induce CTA expression, and then treat the patient with CTA immunotherapy. Again this strategy could target more of the tumor instead of just one specific population in the tumor.

Combining different cancer immunotherapies has also been proposed to enhance efficacy. For example, treating a patient with a CTA vaccine will induce a T-cell response against the tumor which can then be enhanced by adoptive transfer of patient derived tumor-specific T-cells. One challenge to using CTA vaccines is immune checkpoint molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) which can block T-cell anti-tumor responses [51]. Treating the patient with an antibody targeting immune checkpoint molecules can enhance the CTA vaccine induced anti-tumor T-cell response [51]. Combination therapies could enhance anti-tumor responses while keeping the cytotoxicity relatively low.

1.4 Reactivation of CTAs in cancer

CTA expression is typically repressed in all normal tissues with the exception of the testis, but how CTAs are re-expressed in tumor cells is not well understood. It was mentioned above that DNMTI can induce CTA expression in cancer cells, suggesting that demethylation may be a means by which cancer cells reactivate CT-X antigen expression [46, 47, 52, 53].

DNA-methyltransferases methylate DNA at CpG islands which block transcriptional activators and recruit transcriptional repressors [54]. Interestingly, demethylation occurs naturally during the early stages of spermatogenesis and activates the expression of CT-X antigens in male germ cells [10, 55]. Large scale hypomethylation occurs in many cancers and is associated with CT-X antigen expression [56-58]. A recent study compared TCGA RNA-seq data with methylation array data from 8 tumor types to determine if a correlation exists between expression and methylation status of CTAs [37]. They found that about 30% of the CTAs they analyzed showed a negative correlation between mRNA expression and promoter/enhancer DNA methylation [37]. An additional study from our lab found that about 25% of CTAs can be induced by treatment with a demethylating agent, 5-azacytidine, in a highly methylated colon cancer line HCT116. Again, CT-X antigens were the majority of re-expressed genes. Together, this evidence suggests some CTAs, mainly CT-X antigens, are regulated by epigenetics and the transcription factors required for activating these genes are ubiquitously expressed. Hypomethylation may be one mechanism by which cancer cells re-activate certain CTAs. Little is known about the regulation of CTAs not regulated by epigenetics but the mechanisms are likely to be cell specific.

CHAPTER TWO
Examining the regulation of Acrosin Binding Protein in cancer

2.1 - Introduction

2.1.1 Discovery of ACRBP as a modulator of paclitaxel responsiveness

Our lab first became interested in CTAs after a pan-genomic-loss-of-function RNAi synthetic lethality screen in NSCLC line H1155 identified several CTAs that promote chemo-resistance (Figure 2) [59]. This screen was performed by treating cells with siRNA and a sub-lethal dose of paclitaxel to identify genes that modulate paclitaxel responsiveness [59]. Paclitaxel is a microtubule stabilizer that inhibits mitotic spindle dynamics [60, 61]. Microtubule dynamics is essential for bipolar spindle formation in mitotic cells [62]. Prolonged paclitaxel treatment can lead to mitotic arrest and eventually apoptosis [63]. Taxanes such as paclitaxel are used as first line therapies for NSCLC, however the 5 year survival rate is less than 4% due to chemo-resistance and toxicity of treatment [64]. Therapies that can be used in combination to sensitize cancer cells to paclitaxel would likely increase the efficacy of treatment for NSCLC. Depletion of genes identified in this screen resulted in synthetic lethality of H1155 cells treated with a low dose of paclitaxel [59]. Genes identified in this screen include components of the proteasome, genes involved in microtubule dynamics, and CTAs [59]. This was one of the first accounts of CTAs supporting tumor cell fitness.

Acrosin Binding Protein (ACRBP), a CTA, was identified in this screen and found to support tumor cell fitness in the presence of paclitaxel [59]. RNAi-mediated depletion of ACRBP did not affect tumor viability on its own, but did reduce tumor cell viability in the presence of a sub-lethal dose of paclitaxel (Figure 2) [59]. Depletion of ACRBP in addition to a sub-lethal dose of paclitaxel resulted in multipolar mitotic spindle formation and a loss of

viability [59]. These data suggest that ACRBP protects H1155 cancer cells from paclitaxel's anti-proliferative affects by supporting mitotic spindle formation.

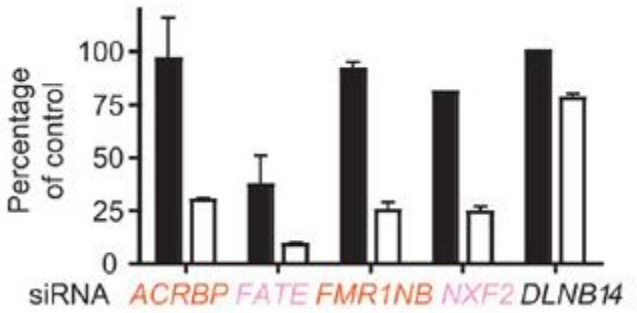

Figure 2. CTA sensitizes NCSLC cells to a sub-lethal dose of paclitaxel [59]
Results are viability of H1155 NSCLC cells after siRNA transfection of CT antigens in the presence of 0nM paclitaxel (Black bars) or 10nM paclitaxel (white bars) normalized to DLNB14 siRNA as a percentage of control. (Used with permission, License # 3743260792288)

2.1.2 ACRBP is a cancer-testis antigen

ACRBP, also known as OY-TES-1, was first identified as a CTA in 2001 by Ono, T., et al. [65]. The authors found that ACRBP mRNA was detected in the testis and in several cancers but not in other adult normal tissues [65]. Another study detected ACRBP protein in 60% of epithelial ovarian cancer (EOC) samples by immunohistochemistry, and found that ACRBP mRNA expression negatively correlates with DFS of EOC patients [66]. The authors also analyzed serum from EOC patients for ACRBP antibodies via ELISA. They found 1 response out of 10 ACRBP-positive patients while no ACRBP antibodies from 11 ACRBP-negative patients demonstrating that ACRBP can be immunogenic [66]. A more recent study found that ACRBP protein expression correlated with invasion and histological grade of colorectal cancer (CRC) as well as 9.6% of CRC patients had anti-ACRBP antibodies is their sera [67].

2.1.3 ACRBP in spermatogenesis

Little is known about human ACRBP in spermatogenesis, but an ACRBP pig homolog, sp32, was originally isolated from ejaculated pig sperm in 1994 [68]. The authors found that sp32 localized to the acrosome of pig sperm where it is bound to proacrosin [68]. The acrosome is a storage vesicle at the head of the sperm that makes contact with the oocyte and releases proteolytic enzymes that penetrate the zona pellucida to allow for fertilization [68, 69]. Proacrosin is a zymogen that is activated into one of these proteolytic enzymes [68]. The Proacrosin/sp32 complex localizes to high molecular weight complexes at the head of the acrosome during capacitation to allow for quick release at first contact with the zona pellucida [69].

2.1.4 ACRBP in cancer

ACRBP expression has been detected in several cancers, but is expressed in 60% of ovarian cancers which can be used as a background to study ACRBP's function in cancer [66]. Mentioned above, ACRBP was identified as a modulator of paclitaxel responsiveness in an RNAi synthetic lethality screen using a NSCLC line, H1155 [59]. ACRBP was then further characterized in a follow-up study. ACRBP expression was found to be higher in a chemotherapy-resistant serous adenocarcinoma ovarian cancer cell line, PEO4, than in a patient matched chemotherapy responsive line, PEO1, suggesting that ACRBP may play a role in chemoresistance in ovarian cancer [70]. Overexpression of ACRBP blocked paclitaxel induced mitotic defects in both chemo-sensitive lines H1155 and PEO1; again supporting the idea that ACRBP confers paclitaxel resistance to cancer cells (Figure 3) [70].

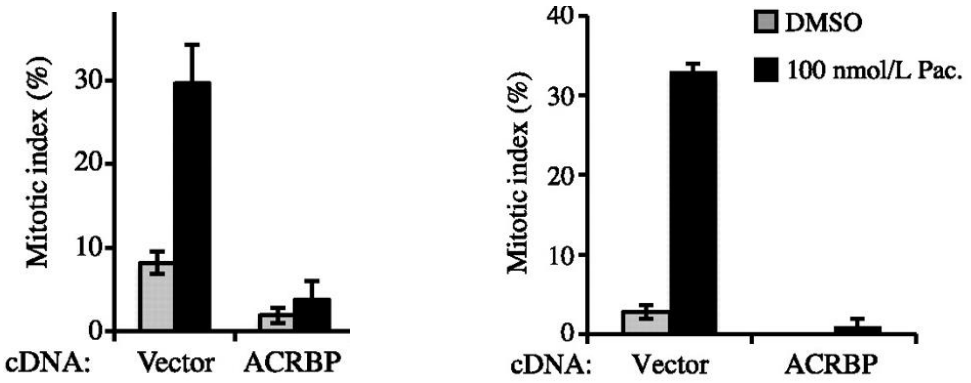

Figure 3. ACRBP gain of function protects cancer cells from paclitaxel induced mitotic defects- H155 (left) and PEO1 (right) cells were transfected for 24 hours and then treated with 100nmol/L paclitaxel for 12 hours. Cells were stained with DAPI and scored microscopically. Data is the mean of two independent experiments and expressed as the percentage of total cells. (adapted from [70]) (Used with permission, License #3743270063986)

To gain insight into how ACRBP gain of function contributes to paclitaxel resistance in cancer cells, ACRBP-interacting proteins were identified by immunoprecipitation followed by mass spectrometry analysis. One interesting interactor identified was Nuclear Mitotic Apparatus Protein (NUMA) [70]. NUMA is a high molecular weight protein with many functions and multiple binding partners [71]. Importantly, NUMA plays a major role in mitosis by tethering microtubule spindles at two poles to allow for equal division of two daughter cells [71]. However, NUMA overexpression in cancer can inhibit mitosis due to spindle multipolarity, aneuploidy, and genomic instability [71, 72]. Depletion of ACRBP in cancer cells results in an accumulation of NUMA protein as well as disordered mitotic spindle formation in the presence of paclitaxel [70]. This mitotic defect is rescued by co-depletion of ACRBP and NUMA, suggesting that the mitotic defect is NUMA dependent (Figure 4) [70]. Paclitaxel is a microtubule stabilizer that works by inhibiting mitotic spindle dynamics [73]. NUMA overexpression and paclitaxel both inhibit mitotic spindle assembly and organization which may be why ACRBP depletion sensitizes cancer cells to paclitaxel. Interestingly, both high ACRBP and high NUMA expression together correlate with poor prognosis in ovarian cancer patients,

further corroborating that ACRBP may be selectively expressed in order to counter high NUMA expression (figure 5) [70].

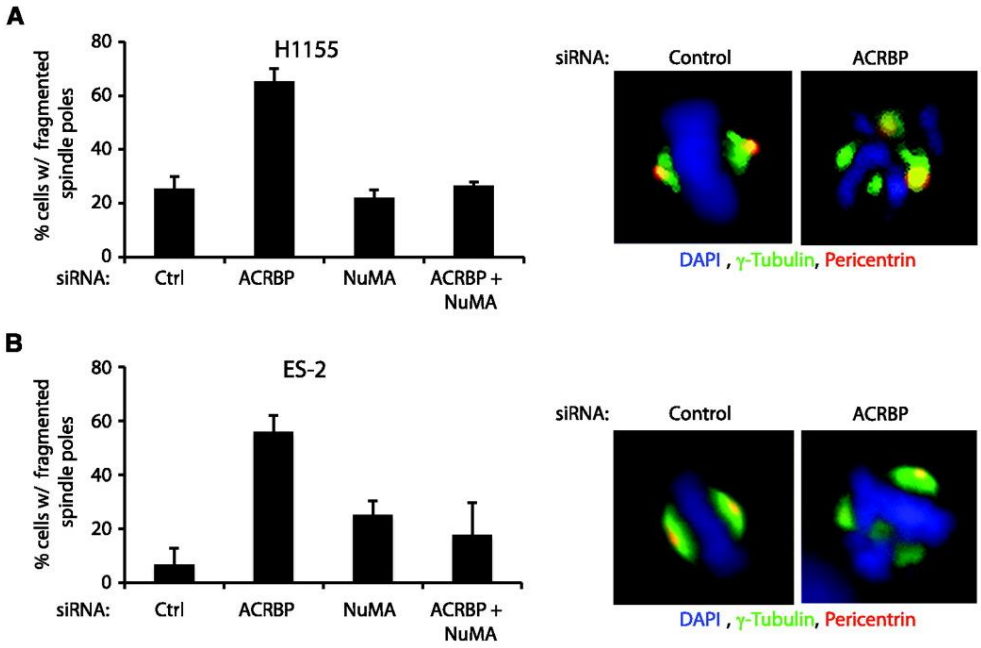

Figure 4. ACRBP depletion drives fragmented spindle poles – H1155 and ES-2 cells were transfected for 48 hours and then treated with 10 nmol/L paclitaxel for 24 h. Cells were immunostained with the indicated probes and scored microscopically. Data is expressed the average deviation from two independent experiments. [70] (Used with permission, license #3743270063986)

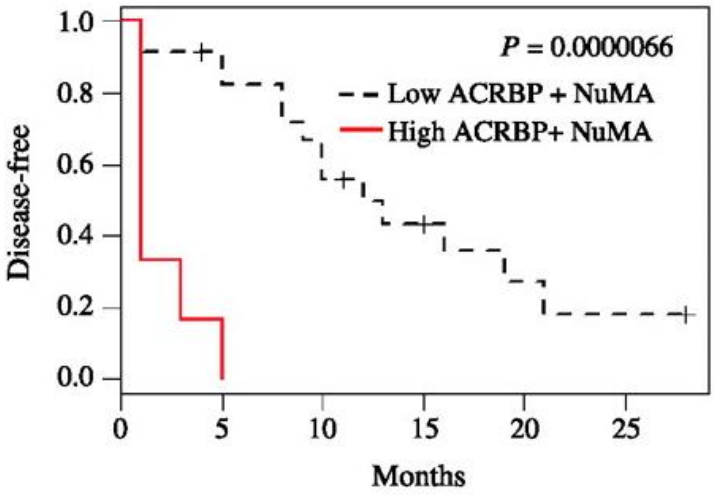

Figure 5. High NUMA and ACRBP expression correlate with poor prognosis in ovarian cancer patients- Kaplan-Meier plot of disease-free survival correlation with ACRBP and NUMA expression. (Used with permission, license #3743270063986)

Our lab has also explored ACRBP's paclitaxel protective effects *in vivo* utilizing an orthotropic xenograft model of ovarian cancer in mice. ShRNA-mediated depletion of ACRBP sensitized ovarian cancer cells to paclitaxel resulting in an extended survival of mice.

ACRBP protects cancer cells from mitotic defects induced by paclitaxel *in vitro* and *in vivo.* ACRBP is an ideal therapeutic target due to its restricted expression profile and its importance to cancer cell viability in the presence of paclitaxel. Understanding how ACRBP is re-activated in cancer may lead to potential therapies that could be used to sensitize cancer cells to paclitaxel. Mentioned above, cancer cell hypomethylation has been linked to the reactivation of CTAs in cancer [74, 75]. However, a demethylation and expression array performed in our lab showed that ACRBP's expression is only modestly increased by demethylation. This data suggests that ACRBP's transcriptional regulation in cancer may be driven by genetic factors in addition to epigenetic factors. To determine which transcription factors may play a role in ACRBP's regulation, I aligned the human and mouse 5' promoter and searched for conserved transcription factor motifs using ECR Browser [76]. Three transcription factor motifs were identified: SP1, CRE, and an E2F1 motif (figure 6). Next, I mined the literature for ChIP-seq data on these transcription factors. One ChIP-seq performed in mouse spermatogonial cells identified an interaction between CREMt (Cyclic AMP Response Element Modulator tau) and the conserved CRE site in the ACRBP 5' promoter [77].

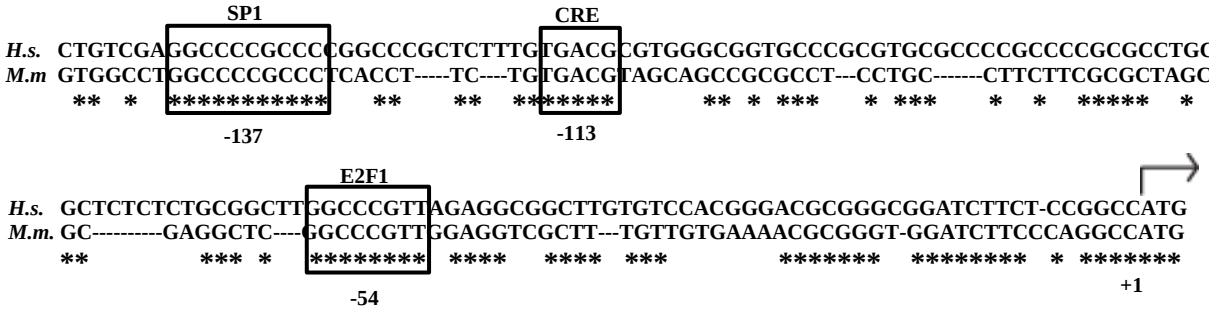

Figure 6. Conserved human and mouse ACRBP 5' promoter

2.1.5 CREB family of transcription factors

CREM is part of the Cyclic AMP Response Element Binding Protein (CREB) family of transcription factors that also includes Activating Transcription Factor 1 (ATF1) [78]. The CREB family of transcription factors consists of basic leucine zipper (bZip) transcription factors that bind to the same Cyclic AMP Response Element (CRE): full-CRE 5'-TGACGTCA-3' or half-CRE 5'-TGACG-3' and 5'-CGTCA-3' [78]. They can homodimerize and heterodimerize with other bZip transcription factors to create a diverse array of target genes [78]. The traditional mode of activation for the CREB family of transcription factors is through cAMP activation of Protein Kinase A (PKA), which then releases its catalytic subunits into the nucleus where it phosphorylates CREB, CREM, and ATF1[79]. Once phosphorylated, histone acetyltransferases, CREB Binding Protein (CBP) and P300, bind to CREB, CREM, and ATF1 and activate target gene transcription by catalyzing acetylation of promoter histones and recruiting Polymerase II [79]. In addition to PKA, several other kinases phosphorylate the CREB transcription factors in response growth factors and stress stimuli [79].

CREB/CREM can also be activated through a phosphorylation-independent mechanism by interacting with co-activators Transducer of CREB 1, 2 and 3 (TORC1, 2, and 3), also known as CRTC1, 2, and 3 (Figure 23) [80]. TORC1 and 2 bind to CREB's bZip domain and activate target gene transcription by recruiting $TAF_{II}130$, a subunit of TFIID (Figure 23) [80].

Another phosphorylation-independent mechanism of CREB/CREM activation is via interactions with co-activators four-and-a-half-LIM-domain (FHL) proteins [81]. A testis specific FHL protein, Activator of CREM in Testis (ACT), activates CREB/CREM target genes in spermatids [82]. This mechanism depends on KIF17b's transport of ACT into the nucleus and KIF17b's transport of newly transcribed mRNA to the cytoplasm [82]. CREMt, a testis specific

CREM isoform, is a very important regulator of spermatogenesis, activating several post meiotic genes involved in the final maturation of spermatozoon [77]. Mentioned above, a CREM ChIP-seq performed with mouse spermatogonia cells identified CREM bound to the ACRBP 5' promoter as well as other CTA promoters [77]. Because CREB can also bind to the same CRE sites and is highly upregulated in several cancers and correlates with poor prognosis, we hypothesize that CREB regulates ACRBP transcription in cancer [83-86]. Thus in Chapter 2.2, I explore ACRBP's transcriptional regulation via CREB in cancer cells.

2.2 Results

2.2.1 ACRBP Expression is dependent upon CREB in ovarian cancer cell lines

I used ovarian cancer cell lines to explore ACRBP's dependency on CREB because ACRBP is highly expressed and plays a functional role in ovarian cancer cells [70]. To determine if ACRBP mRNA expression is dependent upon CREB expression, I depleted CREB in six ovarian cancer cell lines using a pool of four siRNAs, and quantified ACRBP and CREB expression using quantitative real-time PCR (qPCR). In all six lines, CREB depletion resulted in a significant loss of ACRBP mRNA expression (Figure 7). Interestingly, the least responsive cell line, ES-2, expresses very low levels of ACRBP mRNA which may be why CREB knockdown did not dramatically change ACRBP expression. Additionally, all four independent CREB siRNAs reduce ACRBP mRNA expression, confirming that ACRBP's loss in expression is not due to an off-target effect (Figure 8). Next, I tested whether ACRBP protein expression is dependent on CREB expression in ovarian cancer cells. Remarkably, ACRBP protein was significantly reduced after CREB depletion, confirming the functional significance of CREB's

regulation of ACRBP (Figure 9). Taken together, these data support the hypothesis that CREB regulates ACRBP expression in ovarian cancer.

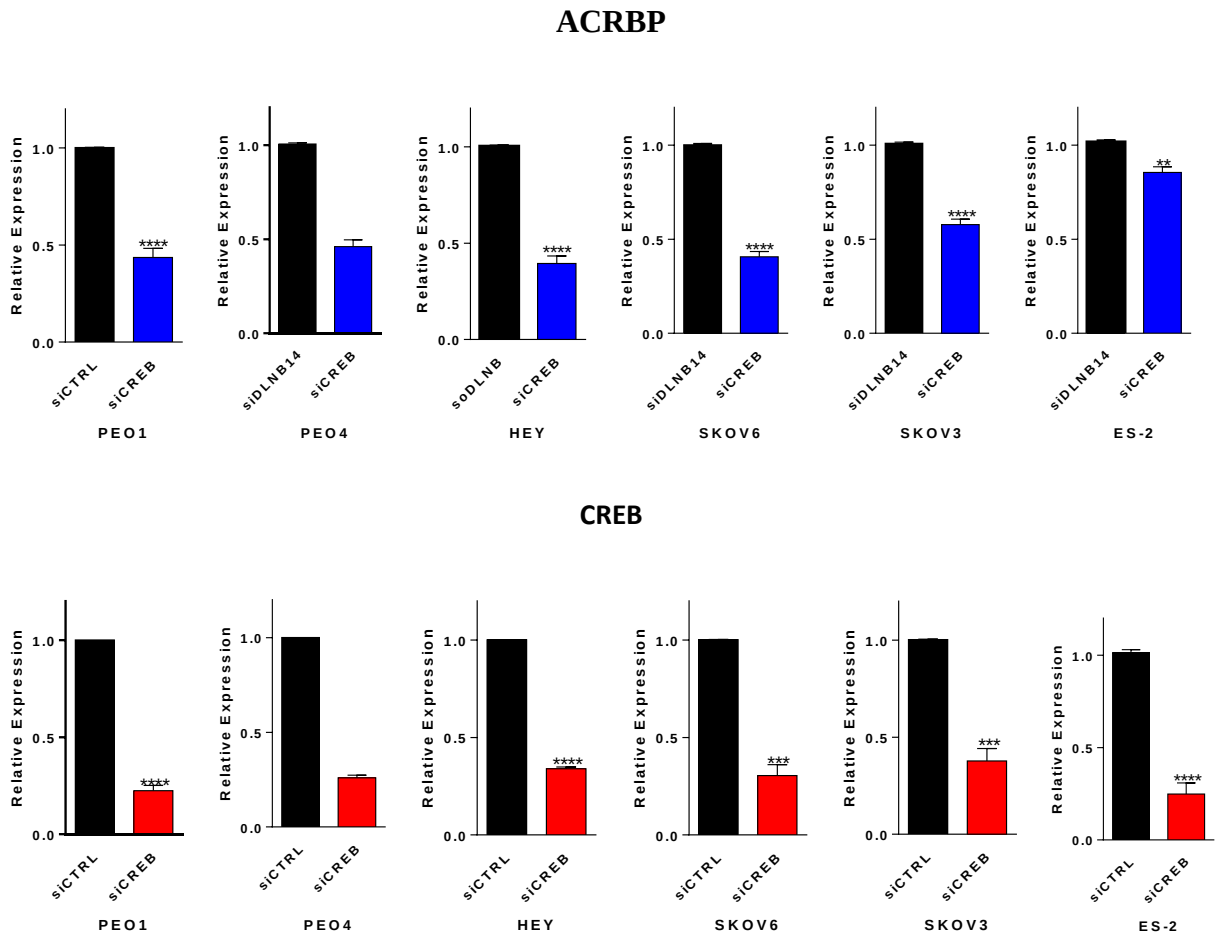

Figure 7. ACRBP expression is CREB dependent in ovarian cancer cell lines- Cells were transfected for 72 hours with indicated siRNAs (pools of 4). Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for ACRBP (top) and CREB (bottom) relative expression. Data represents the average of 6 PEO1 experiments, 2 PEO4 experiments, 3 HEY experiments, 3 SKOV6 experiments, 3 SKOV3 experiments, and 3 ES-2 experiments. Error bars represent range for PEO4 and SEM for all other cell lines. P-values were calculated by one-tailed, unpaired t-test. * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001

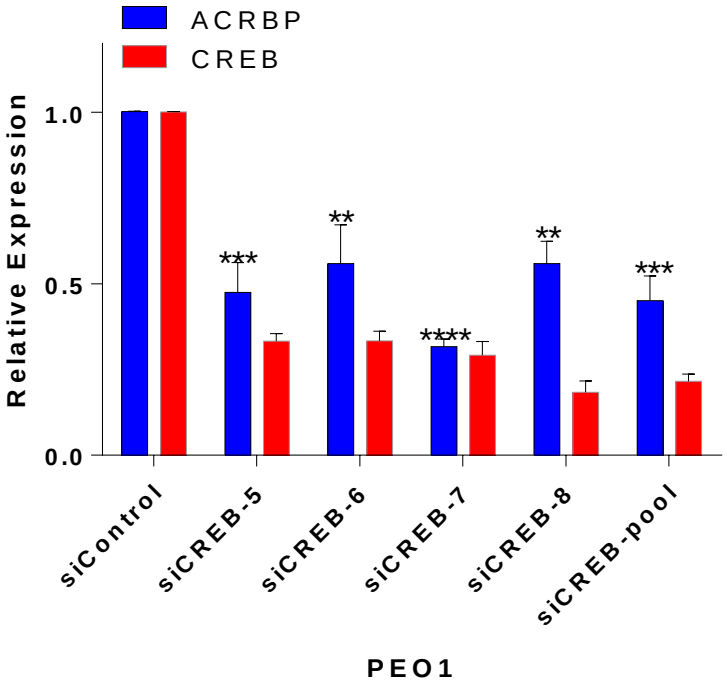

Figure 8. Four unique CREB siRNAs reduce ACRBP expression- PEO1 cells were transfected for 72 hours with indicated siRNAs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for ACRBP and CREB relative expression. Data represents the average of 4 experiments and error bars represent SEM. P-values were calculated by one-tailed, unpaired t-test. * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001.

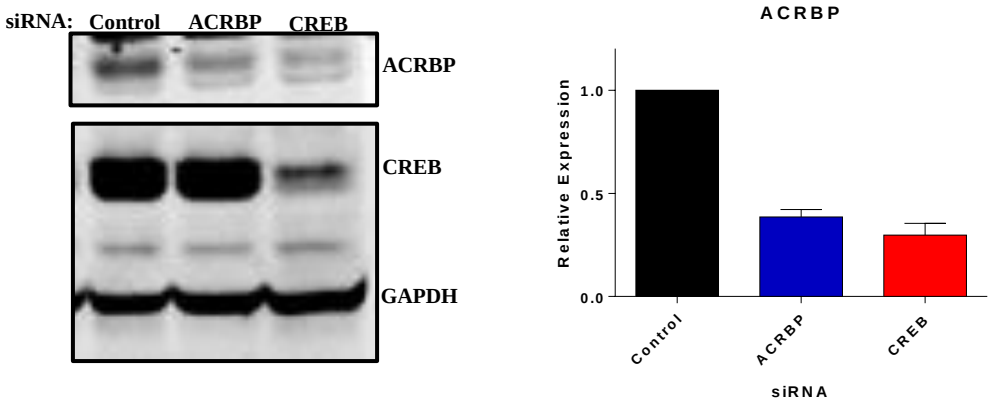

Figure 9. ACRBP protein expression is CREB dependent in ovarian cancer cells- SKOV6 cells were transfected for 96 hours with indicated siRNAs. Whole cell lysates were harvested and then immunoblotted with indicated antibodies. Data represents the average of 2 experiments and error bars represent range.

2.2.2 CREB is sufficient to drive ACRBP expression in cancer

I overexpressed CREB in HELA cells to determine if CREB is sufficient to drive ACRBP expression in cancer cells. In fact, CREB is sufficient to induce ACRBP expression in HELA cells (Figure 10). As mentioned above, CREB can be phosphorylated by several kinases at serine 133 which allows CREB to interact with CBP and p300 to activate transcription of target genes such as FOS and NURR1 [87]. Interestingly, the CREB construct I used was not a constitutively active CREB mutant but an unstimulated wild-type CREB which does not induce the expression of phospho-dependent CREB target genes FOS and NURR1 (Figure 10). Additionally, a S133A CREB mutant was also able to stimulate ACRBP expression (Figure 11). These data suggest that CREB can activate ACRBP expression in a phospho-independent manner. I also tested whether phosphorylated endogenous CREB can activate ACRBP by stimulating HELA cells with forskolin and IBMX. Forskolin and IBMX increases cellular cAMP which activates PKA which, in turn, phosphorylates CREB and stimulates transcription of phospho-dependent target genes [87]. Forskolin stimulation did not induce ACRBP transcription, although it did stimulate FOS transcription (Figure 12). All together these data suggest that CREB regulates ACRBP in a phospho-independent manner.

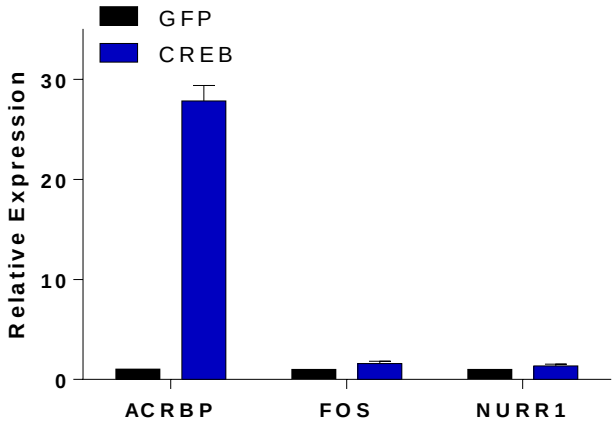

Figure 10. Wild-type CREB induces ACRBP expression but not FOS and NURR1- HELA cells were transfected for 40 hours with indicated constructs. Total RNA was analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. Data represents the average of 2 experiments and error bars represent range.

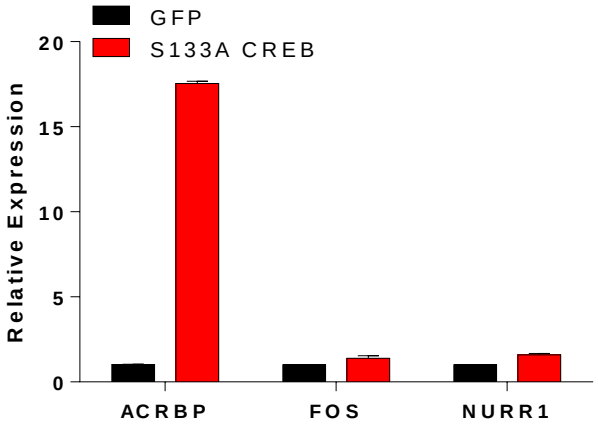

Figure 11. S133A CREB induces ACRBP expression but not FOS and NURR1- HELA cells were transfected for 40 hours with indicated constructs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. Data represents the average of 2 experiments and error bars represent range.

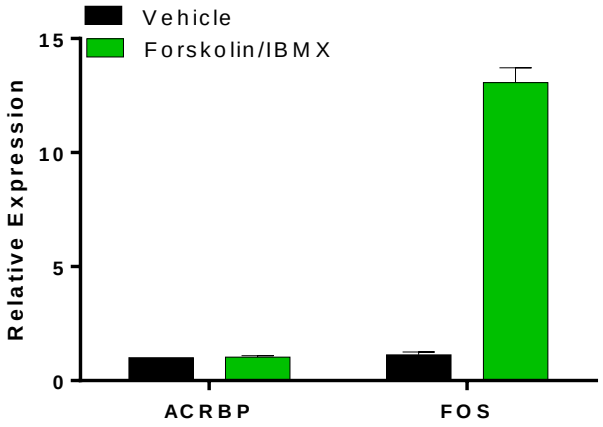

Figure 12. Forskolin/IBMX treatment induces FOS but not ACRBP- HELA cells were treated with vehicle or 10uM Forskolin and 200uM IBMX for 1 hour and total RNA was harvested and total mRNA was analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. Data represents the average of 2 experiments and error bars represent range.

2.2.3 ACRBP expression is dependent upon TORC/CRTC genes

Mentioned above, CREB can activate transcription of target genes through various phospho-independent mechanisms involving different co-activators [88]. One group of co-activators is the TORC/CRTC genes which can bind to CREB and activate transcription of CREB target genes [88]. Overexpression of TORC genes induces CREB target genes without stimulating CREB phosphorylation [88]. To test whether ACRBP expression is dependent on CRTC genes in ovarian cancer, I knocked down CRTC genes in SKOV6 cells. Remarkably, depletion of 2 CRTC genes significantly reduced ACRBP expression (Figure 13). ACRBP expression is dependent on both CREB and CRTC genes, suggesting that ACRBP could be regulated by the CREB/CRTC pathway in cancer. More experiments are required to confirm this hypothesis.

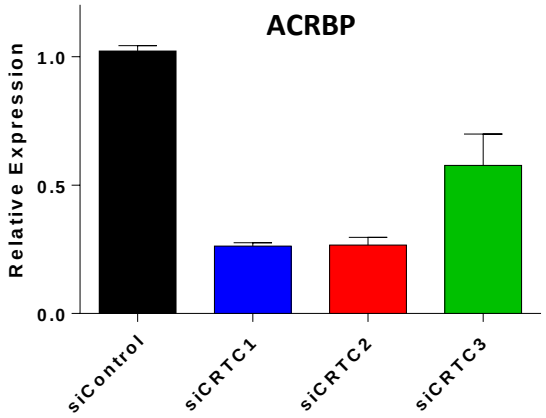

Figure 13. ACRBP expression is dependent upon CRTC genes- SKOV6 cells were transfected for 72 hours with indicated siRNAs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for ACRBP relative expression. Data represents the average of 2 experiments and error bars represent range.

2.2.4 Does CREB regulate ACRBP by a direct or in direct mechanism?

CREM was identified bound to the promoter/enhancer regions of several CTAs in mouse spermatids [77]. One of these CTAs is ACRBP, which has a CRE site 113 base pairs upstream of the transcription start site. CREB binds to the same CRE sites as CREM, suggesting that CREB could activate ACRBP transcription in cancer via this CRE site. However, I have performed a series of ChIP-qPCR experiments that did not detect enrichment of the ACRBP promoter in a CREB immunoprecipitation assay (Figure14). Enrichment of the FOS promoter was identified suggesting that the assay was functionally working, however I cannot rule out completely other technical issues or specificities that may be particular to the type of CREB interaction with the ACRBP promoter. Mutant promoter reporter assays may help elucidate the importance of the CRE site in the ACRBP promoter. However, even if the CRE site is important for CREB's regulation of the ACRBP promoter, it does not mean that CREB is directly binding to that site. It is possible that CREB induces the expression of another CRE-binding transcription factors like ATF1 or somatic versions of CREM. To test this, I would analyze the gene expression of other

CRE-binding transcription factors to determine if any are induced by CREB overexpression. Additional experiments would then need to be done to confirm that ACRBP expression is dependent on other CREB family transcription factors. Of course, it is also possible that all CREB family transcription factors can bind to the CRE site and activate ACRBP transcription. Additional ChIP-qPCR experiments could help elucidate if another transcription factor binds to the CRE site.

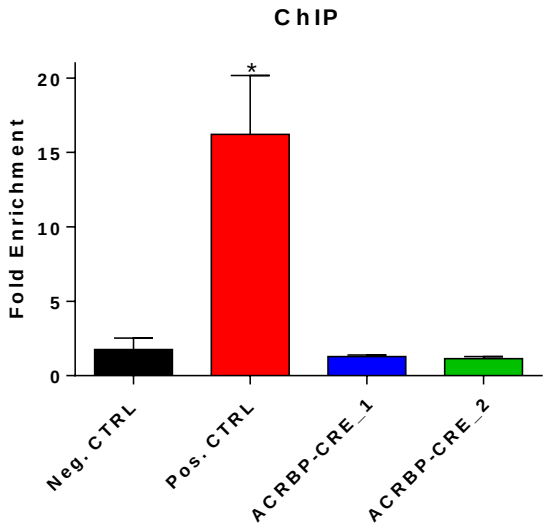

Figure 14. ChIP Assay- SKOV3 nuclei were isolated and sonicated. Then an immunoprecipitation was performed with a total CREB antibody. DNA was cleaned and analyzed via qPCR. Fold enrichment is compared to IgG. Negative control primers detect an open reading frame in ACRBP, positive control primers detect a CRE site in the FOS promoter, and both ACRBP-CRE_1 and 2 detect the conserved CRE site in the ACRBP promoter. Data is the average of 3 experiments. Error bars represent SEM. P-values were calculated by one-tailed, unpaired t-test. * indicates p-value < 0.05.

2.2.5 SP1 and E2F1 in the regulation of ACRBP

Mentioned above, two other transcription factor motifs are present in the conserved ACRPB 5' promoter, Specificity protein 1 (SP1) and E2F1 (Figure 6). SP1 is in a family of SP zinc finger transcription factors that bind to GC-rich motifs and are implicated in development and tumorigenesis [89]. SP1 can act as both an activator and repressor of transcription,

depending on its specific interactors [90] [91]. To determine if SP1 regulates ACRBP expression, I knocked down SP1 in ovarian cancer cells. Interestingly, SP1 depletion resulted in an induction of ACRBP mRNA, suggesting that SP1 represses or activates a repressor of ACRBP expression (Figure 15). More experiments are required to explore how SP1 could be inhibiting ACRBP expression.

E2F1 is part of the E2F family of transcription factors that control cell fate. E2F1, 2, and 3 are transcriptional activators that drive G1/S cell cycle progression and have been implicated in tumorigenesis [92]. E2F1 is sequestered and suppressed by tumor suppressor Retinoblastoma Protein (RB) in a cell cycle dependent manner [93]. In early G1, Cyclin Dependent Kinases (CDK) 4 and 6 phosphorylate RB, facilitating the release of E2F1 where it can activate cell cycle gene transcription [93]. I depleted E2F1 in ovarian cancer cells to determine if E2F1 activates ACRBP. E2F1 depletion resulted in a loss of ACRBP expression suggesting that E2F1 supports ACRBP expression in ovarian cancer. More information is needed to further characterize this regulation.

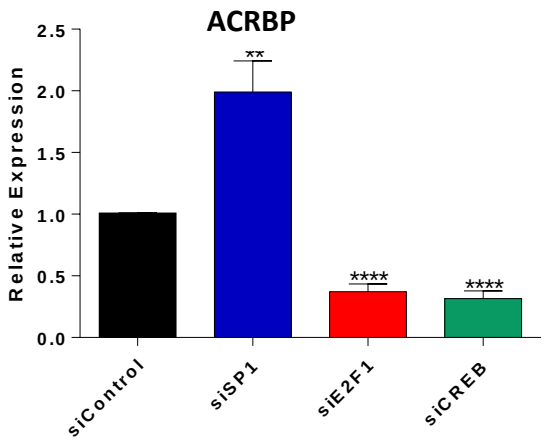

Figure 15. SP1 inhibits ACRBP expression while E2F1 supports ACRBP expression- SKOV6 cells were transfected for 72 hours with indicated siRNAs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for ACRBP relative expression. Data represents the average of 3 experiments for siCREB and 4 experiments for siSP1 and siE2F1 and error bars represent SEM. P-values were calculated by one-tailed, unpaired t-test. * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001.

2.2.6 CREB regulates other CTAs expression

Mentioned above, CREM and CREB play important roles in spermatogenesis. CREM knockout mice are completely void of mature spermatozoon due to the lack of post-meiotic, sperm specific gene transcription [94]. Genuinely, CREB is expressed in the earlier spermatogonia stages while CREM is involved in later stage haploid stage of spermatogenesis [77]. The CREM ChIP-seq mentioned earlier that identified CREM bound to the ACRBP promoter in mouse haploid spermatids also identified CREM bound to several other CTA promoters (Table 1) [77]. Additionally, they performed a trimethylated lysine 4 of histone H3 (H3K4me3) ChIP-seq to identify actively transcribed promoters [77]. All CTA promoters identified in the CREM ChIP-seq were also associated with H3K4me3 (Table 1).

My data suggests that ACRBP is regulated by CREB in cancer cells, so I tested whether other CREM-target CTAs may be driven by CREB in cancer cells. First, I depleted CREB in

SKOV6 and SKOV3 cells and looked at the change in gene expression of CREM-target CTAs expressed in those cell lines. A handful of the genes analyzed showed dependency on CREB expression (Figure 16). Next, I overexpressed wild-type CREB and S133A CREB in HELA cells and analyzed the expression of CREM target genes and found that many were induced by CREB overexpression (Figure 17). Importantly, the expression of a CTA that is not a CREM-target gene (CSAG1) was not activated by CREB (Figure 17. I also checked the expression of four other non-CTA genes and none were induced by CREB, suggesting that this mechanism is selective to CTAs (Figure 18). Together this data suggests that other CREM-target CTAs are regulated by CREB in cancer cells and in a similar mechanism to ACRBP's regulation, since no stimulation of CREB is required for gene induction.

CTA Gene name	**Fold Enrichment**	**Full CRE**	**CRE with 0 or 1 mismatch**	**Half CRE**	**H3K4Me3**
KIAA0100	8.19	No	No	Half CRE	yes
TAF7L	9.61	No	No	Half CRE	yes
Acrbp	19.71	No	No	Half CRE	yes
Akap3	16.38	No	CRE	No	yes
Atad2	12.64	No	No	Half CRE	yes
Brdt	10.56	No	No	Half CRE	yes
Cabyr	20.54	No	No	Half CRE	yes
Calr3	11.96	No	CRE	Half CRE	yes
Casc5	9.38	No	CRE	Half CRE	yes
Cbll1	6.88	No	No	No	yes
Ccdc36	10.81	No	No	Half CRE	yes
Ccdc62	13.09	No	No	Half CRE	yes
Cep55	13.95	No	CRE	Half CRE	yes
Ctage5	9.53	No	CRE	Half CRE	yes
Dcaf12	8.89	No	No	Half CRE	yes
Dkkl1	8.42	No	No	No	yes
Fam46c	7.92	No	No	Half CRE	yes
Fth1	12.99	No	No	Half CRE	yes
Hormad1	14.53	No	CRE	No	yes
Igf2bp3	7.85	No	No	Half CRE	yes
Imp3	9.16	No	No	Half CRE	yes
Kdm5b	8.55	No	No	Half CRE	yes
Kif2c	27.44	No	No	Half CRE	yes

Ldhc	23.52	No	No	Half CRE	yes
Mael	7.22	No	No	No	yes
Maged1	6.58	No	No	No	yes
Mll3	9.57	No	CRE	Half CRE	yes
Mov10l1	7.72	No	No	No	yes
Mtag2	9.75	No	CRE	Half CRE	yes
Nol4	10.18	TGACGTCA	CRE	Half CRE	yes
Nr6a1	16.89	TGACGTCA	CRE	Half CRE	yes
Nxf2	9.85	No	CRE	Half CRE	yes
Odf2	8.36	No	No	Half CRE	yes
Prm1	8.9	No	CRE	No	yes
Rbm46	8.73	No	No	No	yes
Slco6b1	19.2	No	CRE	Half CRE	yes
Spag17	23.36	No	No	No	yes
Spag4	16.44	TGACGTCA	CRE	Half CRE	yes
Spag6	20.16	TGACGTCA	CRE	Half CRE	yes
Spag9	21.75	TGACGTCA	CRE	Half CRE	yes
Spag9	12.67	No	CRE	Half CRE	yes
Ssx2ip	12.91	No	CRE	Half CRE	yes
Sycp1	12.01	No	No	No	yes
Tdrd1	15.23	No	CRE	Half CRE	yes
Tekt5	11.89	No	No	Half CRE	yes
Tex101	8.25	No	CRE	No	yes
Tfdp1	12.93	No	No	Half CRE	yes
Tmeff1	18.75	No	No	Half CRE	yes
Tsga10	11.11	No	No	No	yes
Tssk6	7.43	No	No	Half CRE	yes
Ttk	13.18	No	No	Half CRE	yes
Tulp2	14.41	No	CRE	No	yes

Table 1. CREM bound CTA loci- CTAs identified in a CREM and H3K4me3 ChIP-seq [77]

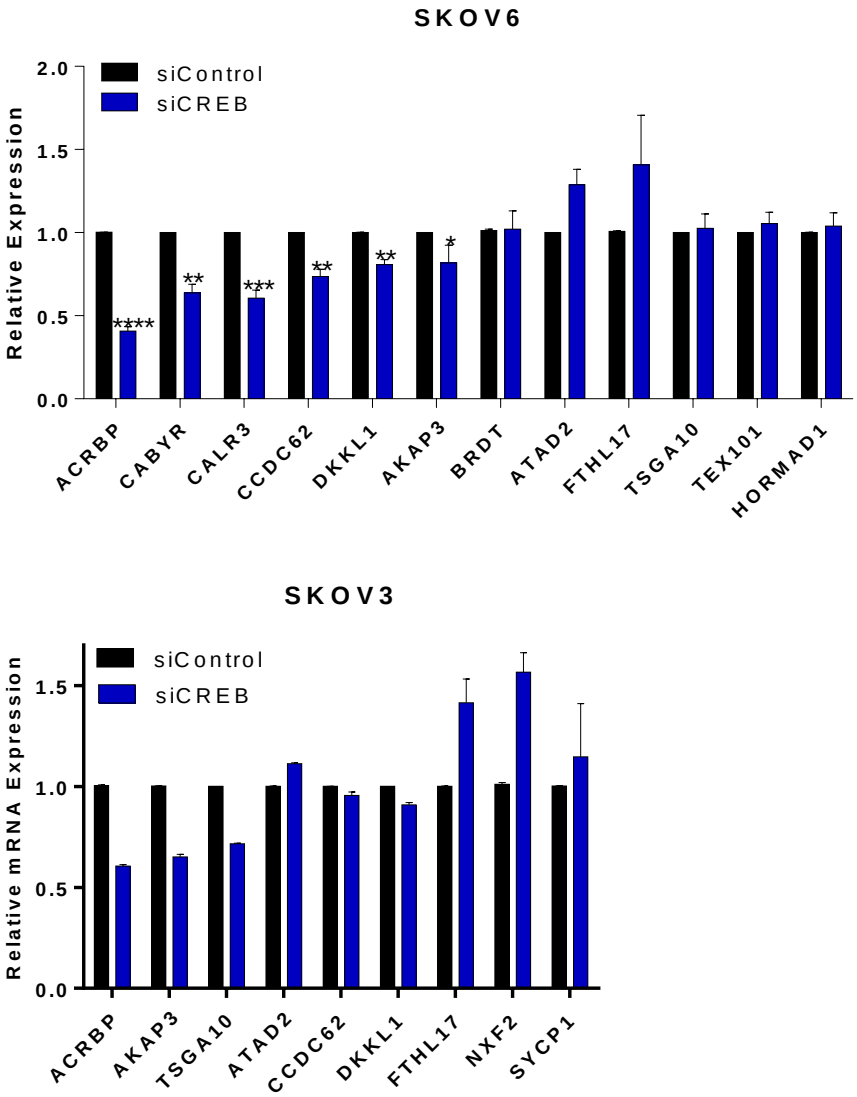

Figure 16. Other CREM-target CTAs are dependent on CREB in cancer cells- SKOV6 and SKOV3 cells were transfected for 72 hours with indicated siRNAs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. SKOV6 Data represents the average of 3 experiments for ACRBP, CABYR, CALR3, DKKL1, ATAD2 and 4 experiments for AKAP3, BRDT, FTHL17, TSGA10, TEX101, HORMAD1.SKOV6 error bars represent SEM. P-values were calculated by one-tailed, unpaired t-test. * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001. SKOV3 data are the average of two experiments and error bars represent range.

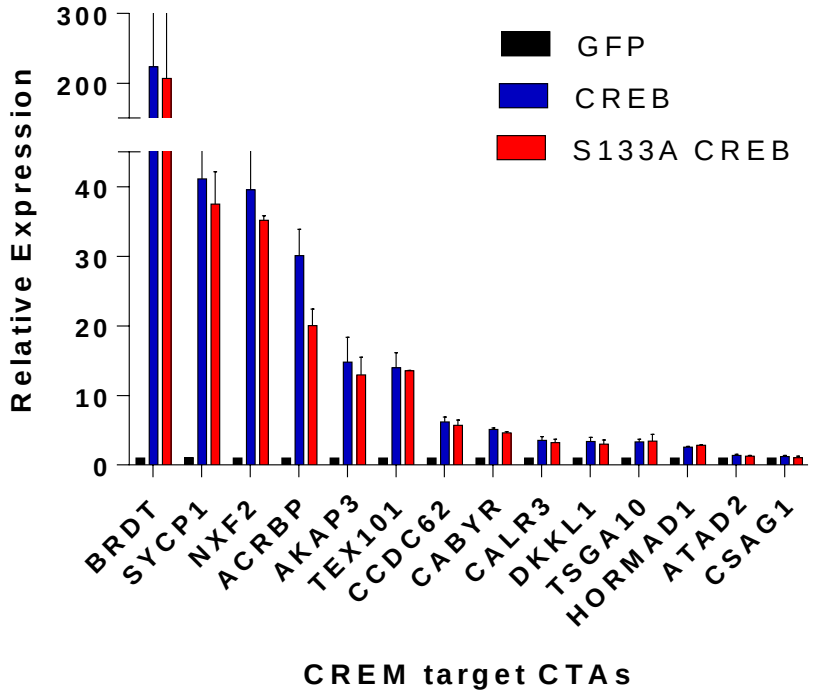

Figure 17. CREM target CTAs are induced by CREB overexpression- HELA cells were transfected for 40 hours with indicated constructs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. Data represents the average of 2 experiments and error bars represent range.

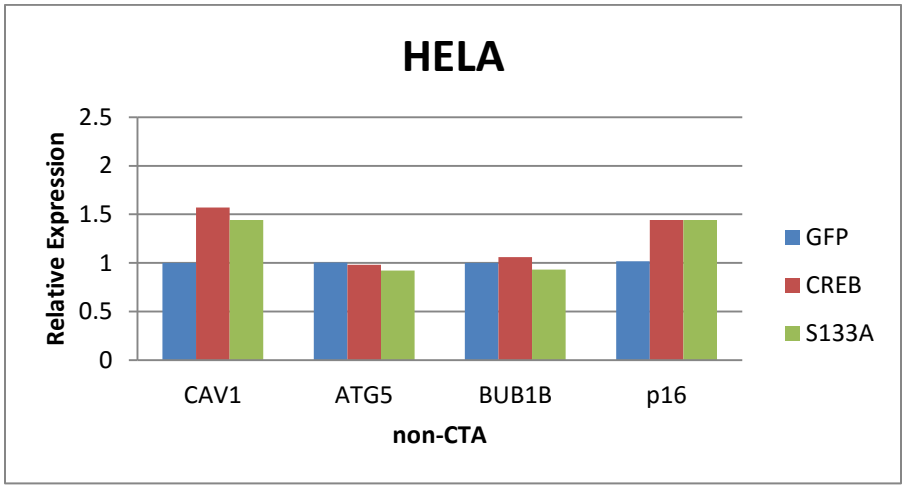

Figure 18. Negative control genes not induced by CREB overexpression- HELA cells were transfected for 40 hours with indicated constructs. Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression. Data represents the 1 experiment.

2.3 Materials and Methods

Cells and Reagents: HELA cells were purchased from ATCC and maintained in DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS). PEO1, PEO4, ES-2, HEY, SKOV3, and SKOV6 cells were maintained in RPMI medium (Gibco) supplemented with 10 % FBS. Forskolin and IBMX were purchased from Caymen Chemicals and were dissolved in dimethyl sulfoxide (DMSO).

Quantitative PCR (qPCR): Total RNA was collected from cells using the GenElute Mammalian Total RNA Miniprep kit (Sigma). 1-2 μg of total RNA was then reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR (qRT-PCR) was performed with TaqMan gene expression assays.Taqman probes are from Life Technology (Hs) or GE healthcare (AX): (sequences are proprietary) ACRBP- Hs0103587_m1, AKAP3- Hs00179042_m1, ATAD2- Hs00204205_m1, BRDT- Hs00266121_m1, CABYR- AX-012436-00-0100, CALR3- Hs00376767_m1, CCDC62- Hs00261486_m1, CREB- Hs00231713_m1, CSAG1- Hs00395635_m1, DDKL1- AX-020522-00-0100, FOS- Hs99999140_m1, HORMAD1- Hs00611993_m1, NXF2- Hs00903814_m1, RPL27- Hs03044961_g1, SYCP1- Hs00172654_m1, TEX101- Hs00758335_m1, TSGA10- Hs00228873_m1. Data is normalized to Ribosomal Protein L27 (RPL27) as an internal control using the ddCT method. The probes span exon boundaries to avoid genomic contamination.

siRNA Transfection: Reverse transfections conditions were performed in 6-well dishes with 50nM siRNA pool or 100nM siRNA individual with 7.5ul of RNAimax (Thermo). Cells were transfected for 72 or 96 hours before collected. Control siRNA transfections were performed with DLNB14 siRNA pool.

cDNA Transfection: Forward Transfections were performed on HELA cells using 1ug of cDNA and 3ul of Lipofectamine 2000 (Thermo). Cells were transfected for 40 hours with pCMV-CREB, pCMV-CREB S133A, and compared to pC2-GFP as a control.

Immunoblotting: Cells were lysed in boiling 2X Laemmli sample buffer. ACRBP was immunoblotted with Abcam Rabbit anti-ACRBP antibody (ab6480), 1:500. CREB was immunoblotted with Cell Signaling mouse anti-CREB antibody (86B10), 1:500. GAPDH was immunoblotted with Sigma mouse anti-GAPH (G8795), 1:2500. All results were normalized to GAPDH as a loading control.

ChIP: SKOV3 cells were grown to 75 % confluency and cross-linked with 1 % formaldehyde for 10 minute sat room temperature. Cross-linking was quenched with 0.125M glycine for 5 minutes at room temperature. Nuclei were isolated by dounce homogenization in hypotonic buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10 % glycerol, 1μg/mL pepstatin, 2 μg/mL leupeptin, 2 μg/mL aprotinin and 50 μM bestatin) followed by centrifugation at 600g for 5 minutes and then lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 1 % Deoxycholate, 0.1 % SDS, 1 mM EDTA, 1 μg/mL pepstatin, 2 μg/mL leupeptin, 2 μg/mL aprotinin and 50 μM bestatin). DNA was sheared using Diagenode Bioruptor® to a range of 300-500 bp fragments. Chromatin was immunoprecipitated using 2 μg of ChIP-Grade anti-CREB (Cell Signaling 48H2 Rabbit mAb #9197) over night at 4 °C followed by a 2-hour incubation with Protein A/G beads. DNA was recovered by reverse cross linking with an overnight incubation at 65 °C. Excess RNA and protein were removed with 100μg RNAse and 10 μg Proteinase K, respectively and the remaining DNA was purified using the Zymogen Zymo-Spin™ ChIP-Grade DNA Clean-Up Kit. DNA was analyzed via qPCR with Sybr Green assay using custom designed primers. Primer Sequences are as follows:

FOS promoter positive control forward CCCCTTACACAGGATGTCCATATT and reverse GGAAAGGCCGTGGAAACCT, ACRBP open reading frame negative control forward AGCCGACACAAGAACACAAG and reverse TGTCCTTCTTCCTGCTTTCC, ACRBP-CRE_1 forward AGCTGTTTGCCATTCCTACC reverse CCCACGCGTCACAAAGAG, ACRBP-CRE_2 forward TGGGCTCTGGCCACTTTAGG reverse GCCCACGCGTCACAAAG.

Statistics: Error bars represent range for an average of 2 experiments and SEM for average of 3 or more experiments. P-values were calculated by one-tailed, unpaired t-test: * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001.

2.4 Conclusions and Future Directions

2.4.1 Summary

The mechanisms by which CTAs are re-activated in cancer cells are not well known. Here, I have shown that ACRBP expression is dependent on CREB in ovarian cancer cells. In addition, ACRBP is induced by CREB in a phospho-independent manner. ACRBP expression is also dependent on phospho-independent CREB co-activators, CRTCs, in ovarian cancer cells, suggesting that CREB may support ACRBP expression through a CRTC-mediated mechanism. Understanding the regulation of ACRBP may provide insight into possible therapeutic avenues that could be used to sensitize cancer cells to paclitaxel. Interestingly, other CTAs were also found to be regulated by CREB in a phospho-independent manner. Given this unique mechanism of CTA activation, I propose the following future directions to further investigate this mechanism.

2.4.2 Future Directions

Does CREB regulate other CTAs?

Several other CTAs were identified in the CREM spermatid ChIP-seq. Interestingly, almost all of the CTAs identified are non-CT-X antigens that are involved in post-meiotic sperm development [37, 77]. Mentioned above, many CT-X antigens are induced in cancer cells by hypomethylation, but this has little effect on most non-CT-X antigens, supporting the idea that other mechanisms may drive the expression of this class of CTAs [37]. CREM regulation of post-meiotic spermatid genes has evolved to control the spatial and temporal specific regulation of this class of genes. The re-activation of these genes in cancer may be a result of the re-activation of this haploid spermatid specific mechanism of gene regulation. CREM/CREB in the testis activates genes through a phospho-independent mechanism by binding to FHL co-activator ACT [82]. The FHL family consists of 5 members; FHL1-4 and FHL5, also known as ACT [81]. With the exception of FHL1, the expression of FHL proteins is tissue specific. FHL4 and 5 are testis specific, while FHL2 is heart specific and FHL3 is muscle specific [81]. CREB can interact with FHL2, 3 and 5 to induce transcription of target genes [81]. Interestingly, FHL2 is expressed in many types of cancer and is implicated in tumor progression [95, 96]. I detected both FHL 2 and 3 mRNA in HELA and SKOV6 cells. It is possible that the CREB family of transcription factors could bind to FHL2 or 3 and activate target genes via this mechanism. To test this hypothesis, I would first knockdown FHL2 and 3 (together and separately) in cancer cells that express them, to determine if CTA expression is lost. Next I would deplete FHL2 and 3 while overexpressing CREB to determine if CREB's activation of CTAs is FHL dependent. It would also be interesting to overexpress FHL proteins along with CREB overexpression to determine if together they can induce a greater activation of non-X-CTAs. FHL proteins may provide the

specificity needed to induce only CTAs and no other CREB target genes. More research into FHL functions may uncover potential ways to target FHL/CREB protein interactions that could inhibit the expression of non-X-CTAs in cancer.

Does CREB regulate ACRBP through CRTC genes?

Another phospho-independent mechanism of CREB target gene activation is through interactions with the CRTC genes. CRTCs can be phosphorylated by Salt Inducible Kinases (SIK), which keeps CRTCs in the cytoplasm where they cannot activate CREB [88]. A tumor suppressor protein Liver Kinase B1 (LKB1) activates SIK via phosphorylation [97]. As a result, LKB1 deficient cells have constitutively active (nuclear) CRTCs [98]. Interestingly, HELA cells are LKB1 deficient which would allow overexpressed CREB to induce target genes such as ACRBP [99]. Additionally, ACRBP expression is dependent upon CREB and CRTCs in ovarian cancer cells suggesting that they are both important in the regulation of ACRBP. Again additional knockdown and overexpression experiments could help elucidate the role CRTCs play in CTA activation. There are other co-factors of CREB not mentioned here, but the emerging idea in the CREB field is that the co-activators of CREB are how cells selectively express CREB target genes. It is likely that CREB overexpression selectively activates CTAs in HELA cells due to specific co-activators that are expressed in HELA cells. Additionally, co-immunoprecipitation assays and ChIP-qPCR for various CREB co-activators may determine which is functionally important in a specific cellular environment.

Are SP1 and E2F1 involved in ACRBP regulation?

An SP1 and E2F1 binding motifs were also identified in the conserved mouse and human ACRBP 5' promoter. Depletion of SP1 resulted in an induction of ACRBP expression, suggesting it may inhibit ACRBP expression. E2F1 knock down dramatically reduced ACRBP expression suggesting that E2F1 is driving ACRBP expression as well as CREB. Initially, more cell lines should be tested to confirm these results. Next, overexpression experiments can determine if SP1 suppresses ACRBP expression and if E2F1 can induce ACRBP expression. Mutant reporter assays and ChIP-qPCR would be performed next, to determine if there is a physical interaction between these transcription factors and the ACRBP promoter. Additionally there may be other transcription factors involved that do not involve the conserved 5' promoter and may not even involve the promoter at all. Deletion promoter/enhancer reporter assays could determine both activator and repressor sites within the whole ACRBP promoter and enhancer regions.

2.4.3 Conclusion

In conclusion I examined the functional role that CREB plays in regulating ACRBP's expression in cancer. I have shown that ACRBP's expression is CREB dependent in ovarian cancer cells, and that CREB can induce ACRBP expression in cancer cells in a phospho-independent mechanism. I have also shown that other non-X-CTAs are also regulated in this way. ACRBP as well as other CTAs support tumor cell fitness, and are ideal cancer therapy targets due to their selective cancer/testis expression. Understanding how CTAs are re-activated in cancer may help identify ways to target CTAs for cancer therapy.

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CHAPTER THREE

Understanding the Function of CSAG1, a Cancer Testis Antigen, in Cancer

3.1 Introduction

Many CTAs have no known functions, so our lab has performed a series of biological screens to help elucidate the roles CTAs may play in tumorigenesis. In these screens each CTA was depleted via RNAi, one by one in several cancer cell lines, and the effects on cell survival, proliferation, and signaling pathways were analyzed. CSAG1 (Chondrosarcoma associated gene 1) was one of the top hits in an EDU/proliferation screen performed in two melanoma cell lines, suggesting that CSAG1 supports melanoma cell proliferation (Figure 19).

The CSAG family consists of CSAG1, 2, and 3, all of which are uncharacterized CT-X-Antigens. CSAG2 and 3 are identical 127 amino acid proteins while CSAG1 is a smaller 78 amino acid protein. I found that CSAG1 mRNA expression was limited to the testis in adult tissues (Figure 20). Additionally, I found that CSAG1 expression in cancer cell lines is expressed even higher than the testis (Figure 20). The CSAG gene evolved after primate speciation occurred and has since duplicated twice [100]. Consequently, there is no mouse homolog for CSAG1 to study. No known function is published for CSAG genes but mRNA expression has been detected in pre-meiotic spermatocytes as well as chondrosarcoma and melanoma [101, 102]. Thus in 3.2 we explore the function of CSAG1 in melanoma cells.

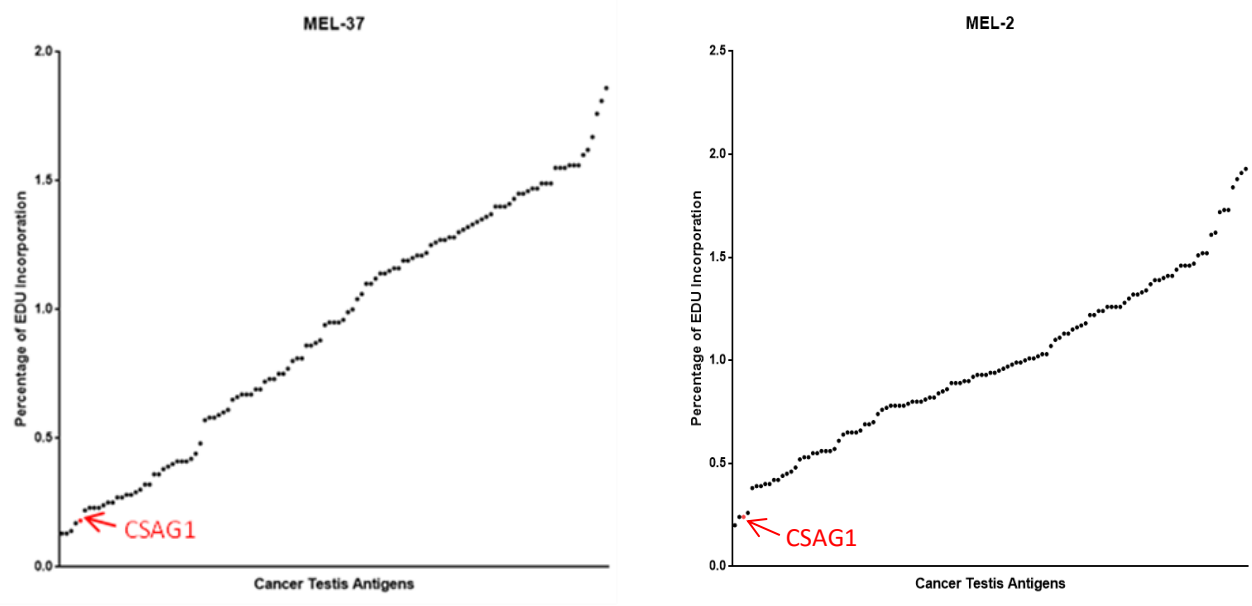

Figure 19. CSAG1 depletion reduces melanoma cell proliferation in EDU screen- cells were transfected for 72 hours. MEL-37 cells were treated with EDU for 24 hours and MEL-2 cells were treated for 3 hours with EDU to allow for incorporation into newly synthesized DNA. Each dot represents a CTA that was depleted with a pool of 4 siRNAs. EDU positive cells were counted and expressed at a percentage of total cells counted and compared to a control non-targeting siRNA, MM5. (Kathleen Corocoran and Josh Wooten)

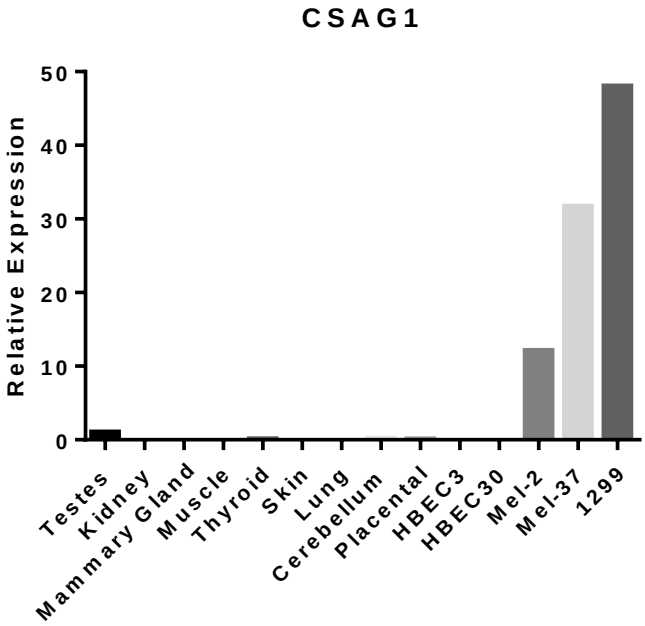

Figure 20. CSAG1 mRNA expression- Total RNA was harvested and then analyzed via quantitative real time PCR. RPL27 was used as an internal control for relative expression, and all data was normalized to testes. Data represents N=1.

3.2 Results

3.2.1 Explore CSAG1 loss of function phenotypes

CSAG1 depletion resulted in a significant reduction of melanoma cell proliferation in a previous screen. To validate this phenotype, I transfected two high CSAG1 expressing melanoma lines (RPMI8322 and MEL-37, CT values around 21) and one low CSAG1 expressing melanoma line (MEL-505 CT values around 34) with CSAG1 siRNA for 96 hours and then measured EDU incorporation. CSAG1 depletion greatly decreased cancer cell proliferation in the two melanoma lines that express high levels of CSAG1 (MEL-37 and RPMI8322) while CSAG1 siRNA had no effect on a melanoma line that does not express CSAG1 (MEL-505), suggesting the proliferation defect is not a result of an off-target effect (figure 21). These data confirm that CSAG1 supports melanoma cell proliferation.

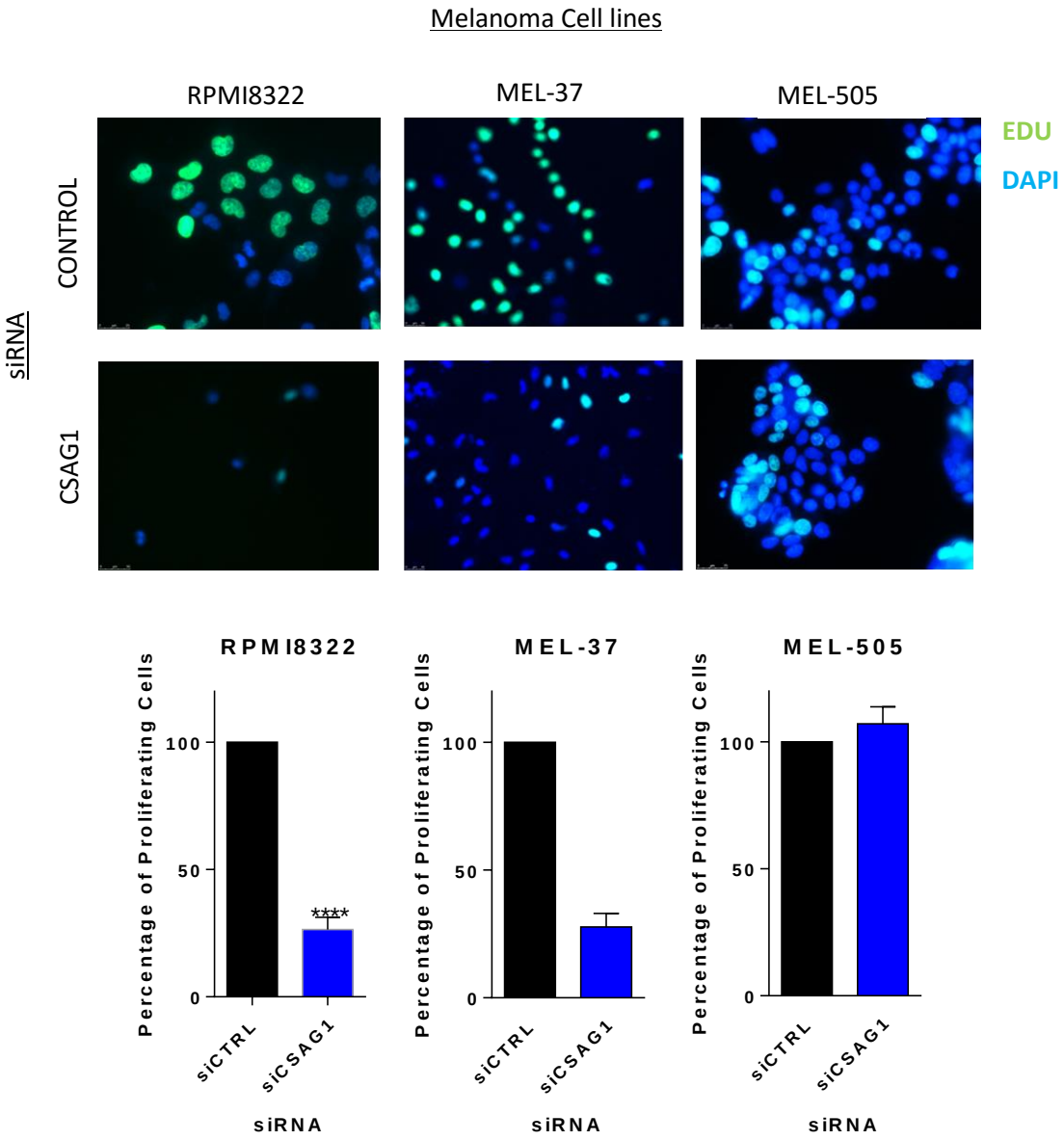

Figure 21. CSAG1 supports melanoma cell proliferation: Cells were transfected for 96 hours, then treated with 20uM EDU for 24 hours (MEL-37) and 3 hours (RPMI8322 and MEL-505). EDU incorporation was assessed by counting cells and EDU positive cells are expressed as a percentage of total cells counted. Data represents the average of 3 experiments for RPMI8322 and MEL-505 and 2 experiments for MEL-37. RPMI8322 and MEL-505 error bars represent SEM while MEL-37 error bars represent range. P-values were calculated by one-tailed, unpaired t-test. **** indicates p-value < 0.001

To determine if CSAG1 depletion results in a long term viability defect, I performed a colony formation re-plating assay. A colony formation assay tests the ability of a cell to continue

to divide indefinitely and form a colony. CSAG1 depletion in Mel-37 melanoma cells resulted in a loss of colony forming ability, signifying a long term viability defect (Figure 22). A long term defect in cell division may suggest that the cells are under a permanent cell cycle arrest. Cellular senescence is a form of permanent cell cycle arrest that mitotic cells undergo after stress that may by oncogenic [103]. Cancer cells must evade cellular senescence cues to continue dividing indefinitely [103]. One way to identify senescent cells is to check for B-galactosidase activity, which accumulates in the lysosomes of senescent cells [103]. To determine if CSAG1 depletion leads to a senescence phenotype, I performed a senescence assay, staining for an accumulation of B-galactosidase in the lysosomes of melanoma cells. In fact, 96 hours after CSAG1 depletion, Mel-37 and Mel-2 melanoma cells had an accumulation of B-galactosidase in the lysosomes as well as an enlarged cytoplasm, indicative of senescent cells (Figure 23). All together, these data suggest that CSAG1 blocks cell cycle arrest in melanoma cells, allowing for the cells to continually divide.

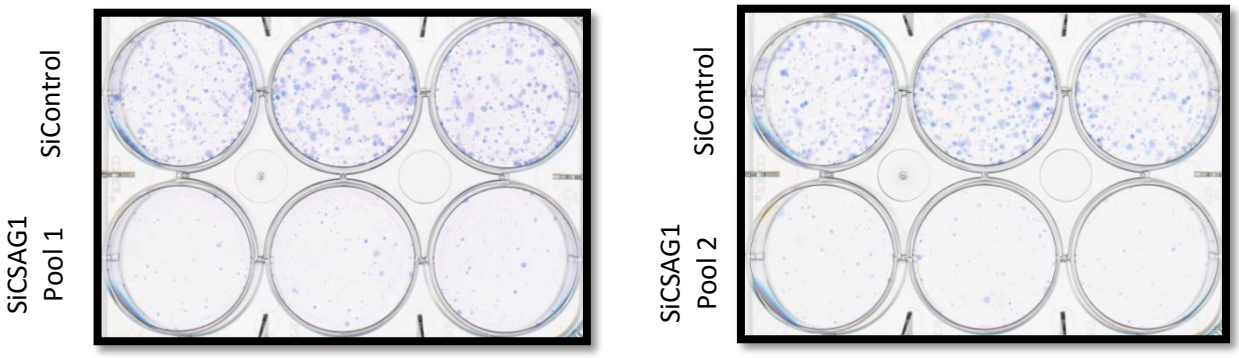

Figure 22. CSAG1 supports long term viability of melanoma cancer cells- Mel-37 Cells were transfected with siRNA for 96 hours then removed from the plate, counted and re-plated 1000 cells/well. Cells were allowed to grow colonies for 10 days and then fixed and stained. Control siRNA was a non-targeting control, MM5. Experiment was performed twice with two different siCSAG1 pools.

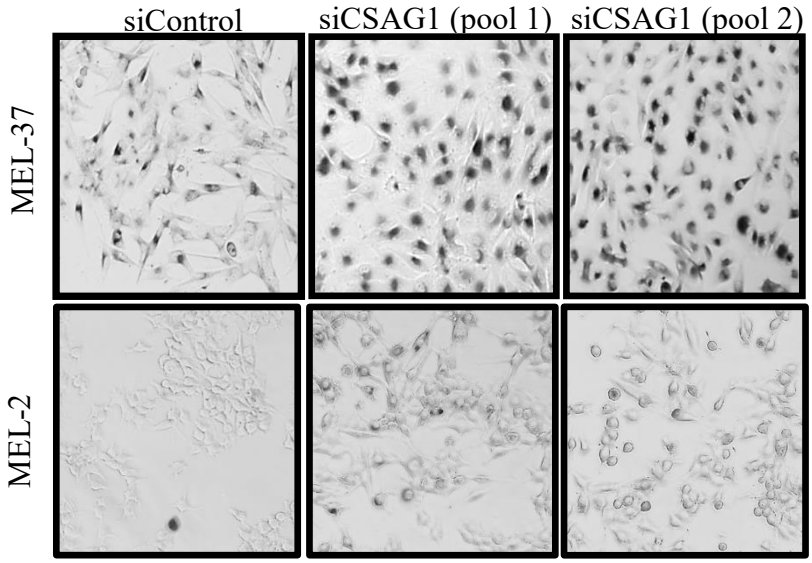

Figure 23. CSAG1 blocks senescence cues in melanoma cancer cells- Cells were transfected with indicated siRNA for 96 hours then stained with X-gal to determine B-galactosidase activity. Assays were repeated for an N=2.

3.2.2 Explore CSAG1 gain of function phenotypes

CSAG1 depletion results in a loss of long term viability in cancer cells, so I asked whether CSAG1 overexpression could enhance long term viability in cancer cells. To test this, I attempted to establish a stable CSAG1-V5 expressing line in immortalized fibroblasts and HELA cells that do not express CSAG1 endogenously. However, I could not detect CSAG1-V5 protein after infection until I treated the cells with a proteasome inhibitor, MG132, suggesting that CSAG1 protein is degraded in these cells (Figure 24). Next, I established CSAG1-V5 stable h1299 and PMWK cells, both express high levels of CSAG1 mRNA (CT values of around 21 and 25 respectively). CSAG1-V5 protein was detectable without treatment of MG132 (Figure 25). For this reason, I used h1299 and PMWK cells for CSAG1 gain of function experiments. I compared the colony forming ability of CSAG1-V5 cells to control, HCRED, cells. Remarkably, CSAG1-V5 cells have improved colony forming ability over HCRED control cells, suggesting

that CSAG1 does support tumor cell viability (Figure 26). Additionally, CSAG1 localization has not been reported, but CSAG1-V5 expression in 1299 cells localizes to the nucleus (Figure 27). Knowing that CSAG1-V5 is localized to the nucleus may provide a clue as to how CSAG1 functions.

I also tested whether CSAG1-V5 can contribute to tumorigenesis *in vivo*. I performed a subcutaneous xenograft study comparing 1299 CSAG1-V5 and HCRED tumor growth. Tumor size and growth rate was not significantly different between cell types (Figure 28). H1299 cells grow very well in xenograft models so it may be difficult to enhance the growth of these cells *in vivo*. More xenograft studies using different cell types and different number of cells injected will help determine if CSAG1 can contribute to tumorigenesis *in vivo*.

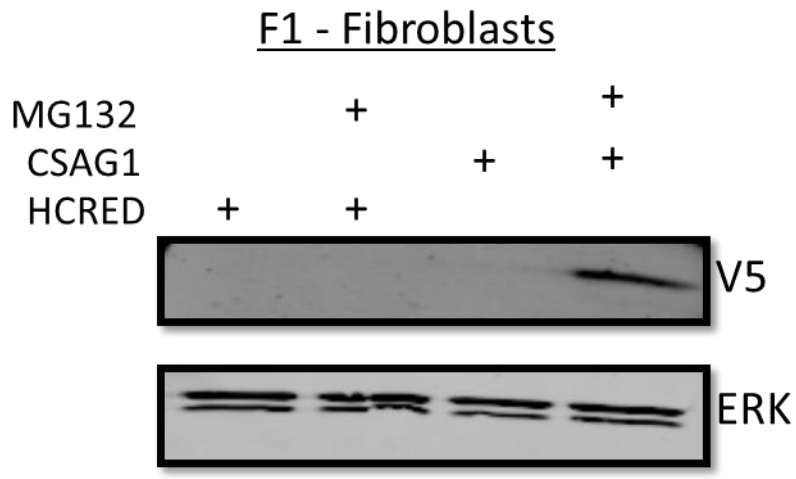

Figure 24. Detection of CSAG1-V5 in F1-Fibroblasts- F1 fibroblasts were infected with plx-302-CSAG1 or plx-302-HCRED and then treated with 10uM MG132 for 8 hours. Whole cell lysates were harvested and then immunoblotted with indicated antibodies.

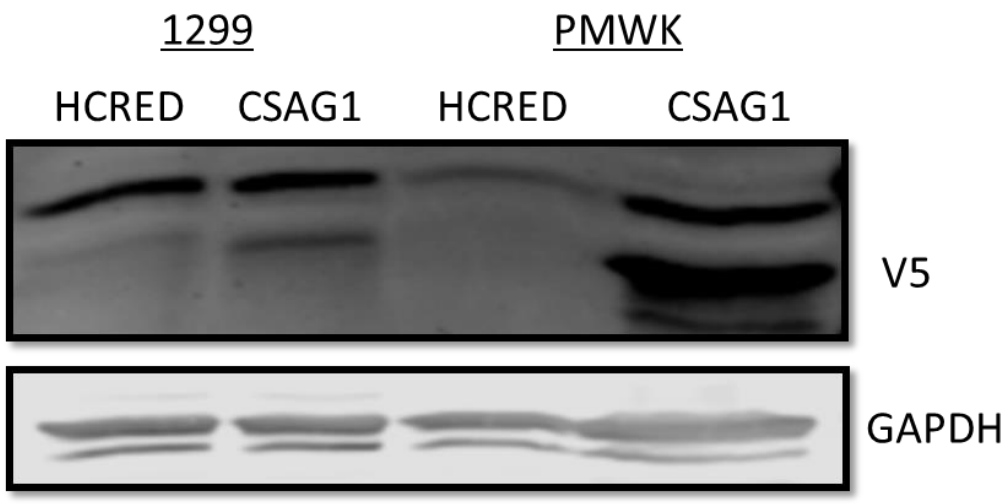

Figure 25. Detection of CSAG1-V5 in 1299 and PMWK cells- Cells were infected with plx-302-CSAG1 or plx-302-HCRED. Whole cell lysates were harvested and then immunoblotted with indicated antibodies.

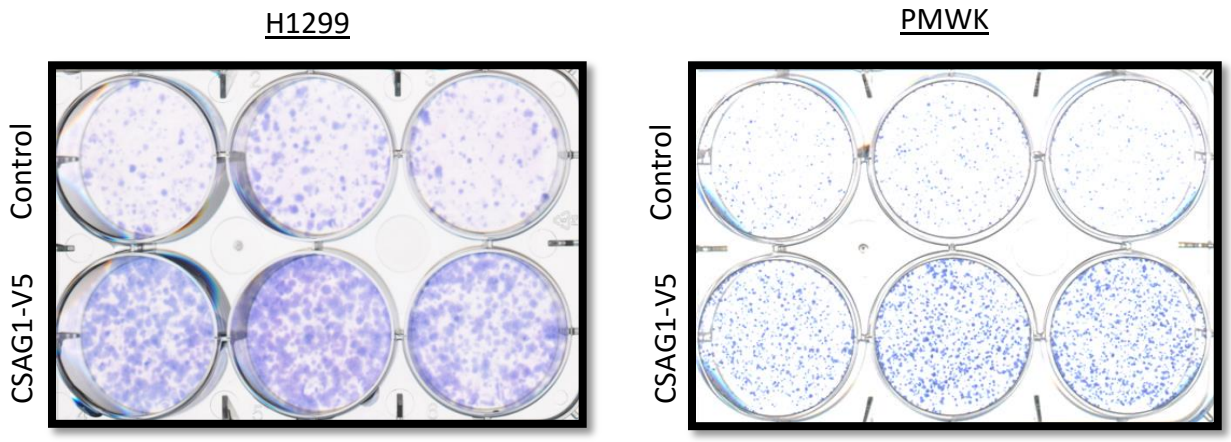

Figure 26. CSAG1-V5 supports cancer cell viability- Cells were infected with plx-302-CSAG1 or plx-302-HCRED. Cells were counted and then re-plated at 1000 cells per-well. Cells were allowed to grow for 10 days and then fixed and stained.

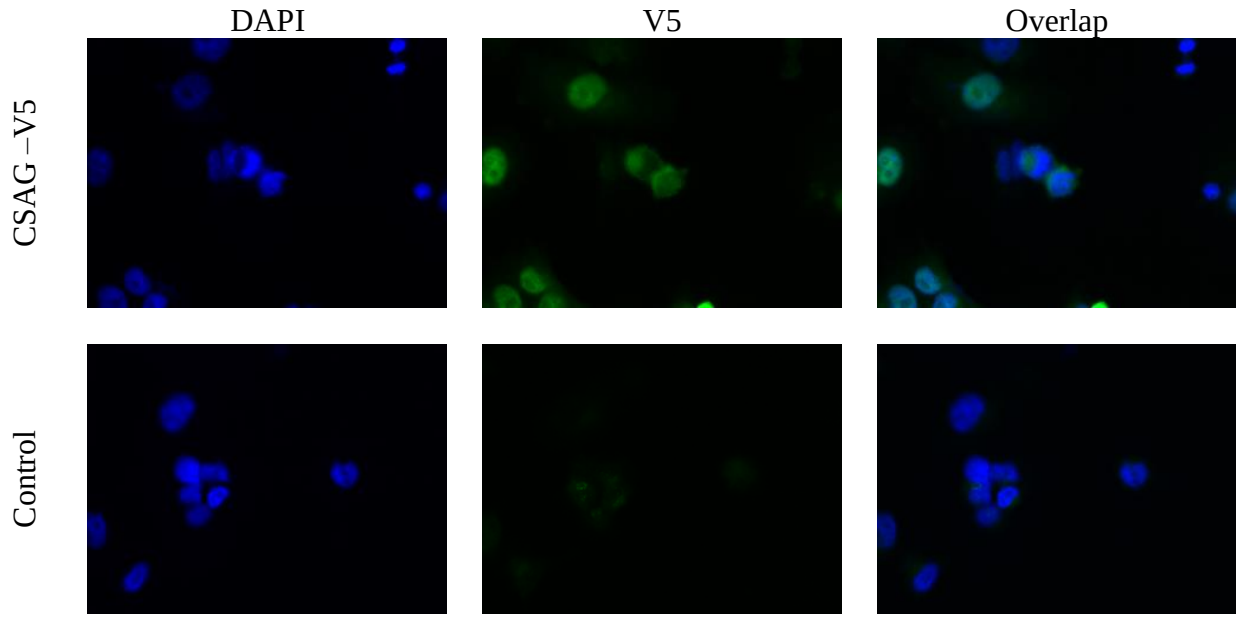

Figure 27. CSAG1-v5 localizes to the nucleus- 1299 stable CSAG1-V5 and HCRED cells were plated and then fixed 24 hours later and immunostained for V5 and stained with DAPI.

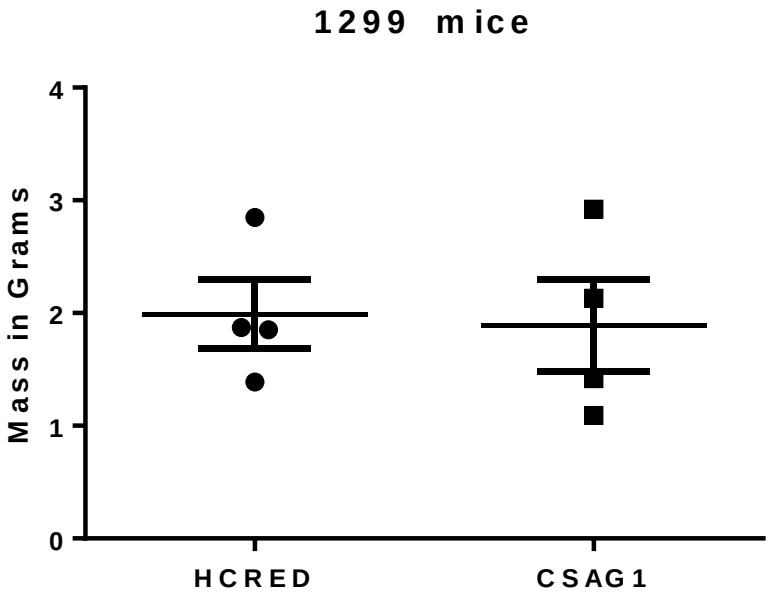

Figure 28. 1299 CSAG1 xenograft study- 500,000 HCRED and 500,000 CSAG1-V5 cells were injected subcutaneously on the mouse flank and allowed to grow until tumors reached 2cm in length. Tumors were then removed and weighed. Each dot represents a tumor and experiment was performed with 4 mice for each group.

3.3 Materials and Methods

Cells and reagents: MEL-37 cells were maintained in DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS). H1299 cells were maintained in RPMI media (Gibco) supplemented with 5 % FBS. MEL-2 cells were maintained in MEM alpha media (Life Technologies) supplemented with 10% FBS.

siRNA transfection: Reverse transfection conditions were performed in 6-well dishes with 50nM siRNA (pool of 3 or 4) and 7.5ul of RNAimax (Thermo). Cells were transfected for 96 hours before collected. Control siRNA transfections were performed with MM5 non-targeting siRNA pool.

Stable cell lines: 293t cells were transfected with 3ug of VSVG, 3ug of Delta 8.9, and 3ug of either PLX-302-HCRED or PLX-302-CSAG1 plasmid, and 18ul of Fugene 6. The media was changed the next morning and then given 24 hours to make virus. The virus media was then removed from the 293t cells and added to the desired cells for infection for 3 hours. After 3 days, the cells were selected with puromycin for one week and then lysates were immunoblotted to confirm expression.

EDU proliferation assays: Cells were treated with 20uM EDU for the last 3 hours of a 96 hour transfection, then cells were fixed and a Click-it EDU assay was performed by manufactures protocol (Thermo Scientific). Immunofluorescence was used to detect EDU positive cells, which were then counted and normalized back as a percentage of proliferating cells.

Mice xenograft study: 500,000 CSAG1-V5 and HCRED stably expressing 1299 cells were injected in the right flank of NOD SCID mice. Tumors were measured twice weekly using a digital caliper. According to IACUC guidelines, mice bearing tumors longer than 2cm were

sacrificed. Tumors were surgically removed and weighed. All studies were conducted in accordance with a UTSW IACUC approved protocol

Colony formation assays: Cells were counted and then plated 1000 per-well in 6 well plates. Cells were allowed to grow for 10 days and were then fixed and stained with Giemsa.

B-galactosidase assay: Cells were transfected for 96 hours in 6-well plates and then fixed. B-galactosidase activity was detected using a senescence β-Galactosidase staining kit (Cell Signaling).

Immunofluorescence: Immunofluorescence detection of CSAG1-V5 was done with a primary anti V5 antibody (Life Technologies R960-25) and a secondary Alexa Fluor 488 goat anti mouse secondary antibody (Invitrogen A-21121). Cells were also stained with dapi to detect nuclei.

Statistics: Error bars represent range for an average of 2 experiments and SEM for average of 3 or more experiments. P-values were calculated by one-tailed, unpaired t-test: * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.005, **** indicates p-value < 0.001.

Quantitative PCR (qPCR): Total RNA was collected from cells using the GenElute Mammalian Total RNA Miniprep kit (Sigma). 1μg of total RNA was then reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR (qRT-PCR) was performed with Sybr Green Master Mix. Sequences are as follows: RPL27 forward- GTTCATGAAACCTGGGAAGG and reverse- CTTCACGATGACAGCTTTGC, CSAG1 forward- ATGTCGGCGACTACAGCCTG and reverse- CTTGGGAACCTCTTTCCTCT. Data is normalized to Ribosomal Protein L27 (RPL27) as an internal control using the ddCT method. The probes span exon boundaries to avoid genomic contamination.

3.4 Conclusions and Future Directions

3.4.1 Summary

Up to this point, it was not known whether CSAG1 expression impacted tumor cell fitness. Here, I have shown that melanoma cell proliferation is dependent on CSAG1 expression. In addition, CSAG1 appears to support long term viability by blocking cellular senescence cues. Induction of CSAG1 expression in h1299 cells greatly enhanced their ability to form colonies. The mechanism by which CSAG1 supports tumor cell fitness is not known, so I propose the following future directions to further investigate CSAG1 function in cancer.

3.4.2 Future Directions

Is endogenous CSAG1 protein reduced after siRNA transfection, and is it localized to the nucleus?

Detecting knockdown of CSAG1 protein has been difficult due to the lack of efficient commercially available antibodies and the small size of CSAG1. I have tested three commercially available antibodies and none detect endogenous protein. It may be necessary to have an antibody made, since very few commercial antibodies available. If an antibody can be made to detect endogenous CSAG1 expression, we can then validate CSAG1 knockdown and also confirm its localization in the nucleus. Knowing CSAG1's localization could provide us insight into how it functions.

Is CSAG1 involved in blocking the Retinoic Acid signaling pathway?

In another biological CTA screen, knockdown of CSAG1 resulted in a significant induction of all-trans-retinoic-acid (ATRA) signaling in h1299 cells. This suggests that CSAG1 can block ATRA signaling. ATRA signaling involves ATRA binding to its nuclear receptors,

which then activate the transcription of their target genes. ATRA can halt cellular proliferation and even induce cellular senescence in cancer cells [104]. If CSAG1 does in fact block ATRA signaling, it could be a mechanism by which CSAG1 enhances melanoma cell proliferation and blocks senescence cues. To test this hypothesis, first the screen data should be validated with independent CSAG1 siRNAs. Additionally, I would test whether CSAG1 overexpression can sensitize cells to ATRA. If these results suggest that CSAG1 does block ATRA signaling, it would be interesting to determine if CSAG1 is a binding partner of RAR receptor utilizing a co-immunoprecipitation assay.

Does CSAG1 block oncogene induced senescence?

Another mechanism by which cancer cells stop proliferating is through oncogene-induced senescence. 63% of melanomas were found to have an activating BRAF or NRAS mutation [105]. These oncogenes can activate oncogene-induced senescence, which must be overcome in melanoma cells to continue proliferation [106]. I have established a CSAG1-V5 stable melanoma cell line, PMWK, with an inducible V600E BRAF mutation. When active, the V600E BRAF mutation initiates oncogene-induced-senescence. To determine if CSAG1 can block these senescence cues, I would activate the V600E BRAF mutation in CSAG1-V5 and HCRED PMWK cells and perform a senescence β-Galactosidase assay.

Does CSAG1 impact tumorigenesis *in vivo*?

The previous data suggests that CSAG1 enhances tumor cell viability *in vitro,* but we do not know if CSAG1 is necessary for tumorigenesis *in vivo*. I have performed a pilot xenograft study in mice to explore h1299 CSAG1-V5 phenotype *in vivo,* however tumor size and progression was comparable to HCRED control cells. Additional xenograft experiments should

be done to determine CSAG1's impact on tumor size, vasculature, and metastases. Also, shRNA xenograft experiments should be done as well.

What are CSAG1's binding partners?

CSAG1 has no known binding partners, or a common domain that could point to its function. To determine CSAG1 interactors, I would perform an immunoprecipitation of CSAG1-V5 in h1299 cells using a V5 antibody, and then run the samples on an acrylamide gel to compare bands to HCRED control. Unique bands should be cut out and sent to the mass spectrometry core for identification. Potential interactors should be validated and further characterized. Identifying CSAG1's physical interactors should provide information about CSAG1's function in cancer.

3.4.3 Conclusion

In conclusion, these data support the hypothesis that CSAG1 is essential to melanoma cell viability. Little is known about the mechanism by which CSAG1 supports tumor cell fitness, but more studies will help elucidate its role in cancer. Understanding the function of CSAG1 may lead to novel therapeutics of an ideal cancer target.

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