The Characterization and Role of RREB1 in Human Bladder Cancer

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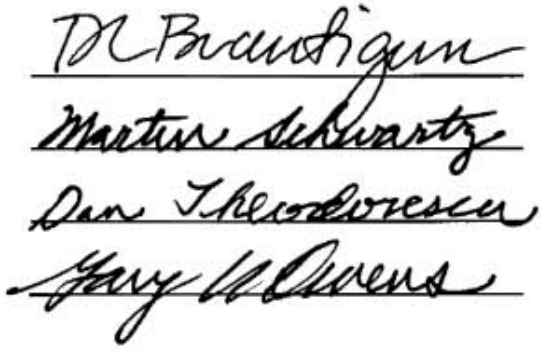

Abstract

RREB1 is a C_2H_2 zinc-finger protein that has previously been associated with malignancy but little is known about its expression, composition of splice variants, or role in cancer phenotypes. Total RREB1 expression decreased in human bladder cancer tumors compared to paired normal tissue. Rigorous comparison of commercial RREB1 antibodies allowed for the identification of RREB1 splice variants in the cytoplasm and nucleus on western blot. Five RREB1 splice variants were identified and designated: RREB1α, RREB1β, RREB1γ, RREB1δ, RREB1ε. Immunohistochemistry of RREB1 in a bladder cancer tissue microarray demonstrated that RREB1 expression positively correlates with the squamous cell histology. Transient depletion of RREB1 in bladder cancer cell lines (UMUC3 and KU7) decreased cell growth *in vitro* and similar results were seen in prostate cancer cell lines (LNCAP and PC3). siRNA duplexes that target RREB1 isoforms showed RREB1α and RREB1β, but not RREB1β alone, were necessary for *in vitro* UMUC3 cell growth and *in vivo* subcutaneous tumor growth. RREB1 depletion was found to decrease the expression of tumor associated protein CD24. Cloning of the CD24 promoter revealed a hypoxia response element, which led to the discovery of hypoxia induced CD24 RNA expression and promoter activity. RREB1 depletion decreased the expression of two additional hypoxia responsive genes (VEGFA and IGFBP3) without affecting the mRNA stability. Loss of RREB1 was found to reduce HIF1A protein levels and decrease its occupancy of the CD24 promoter.

Depletion of HIF1A caused a similar degree of cell growth inhibition as seen with RREB1 depletion. Thus, we propose the necessity of RREB1 in urologic malignancies at least partially acts by the stabilization of HIF1A protein and maintenance of downstream gene expression.

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Chapter 1: Introduction

1.1 The Clinical Manifestation of Bladder Cancer

1.1.1 Epidemiology of Bladder cancer

In 2009 it is estimated that nearly 71,000 American men and women will receive the diagnosis of bladder cancer and another 14,330 will die due to the disease [1]. Bladder cancer is a disease of the elderly with the median age at diagnosis being 73 years [2] and 90% of people with this disease are over the age of 55 [3]. Men have a 4:1 increased risk over women of developing this disease, mainly due to the American male having a higher rate of exposure to tobacco smoking and aromatic amines [4]. Other environmental exposures such as aniline dyes [5] or endemic infections of *Schistosoma haematobium* [6] have also been linked to increased risk of bladder cancer. Furthermore, environmental exposure to coffee, artificial sweetener, or hair dyes, which have been implicated as a cause of bladder cancer, lack sufficient evidence to make definitive conclusions at this time [7]. However, there is a definite genetic component to this disease as the risk of bladder cancer increases 50-100% in first-degree relatives of bladder cancer patients [8-10].

Tobacco is the largest risk factor in the western world; believe to contribute to the development of 50-80% of all bladder cancers [2]. The relative risk of developing bladder cancer in smokers is 2-4 fold greater than that of nonsmokers and increases with the number of cigarettes smoked and duration of

smoking [7]. Cessation of smoking decreases the risk 30-60% for current smokers, and continues to decline out to 25 years [11].

Currently, it is estimated that over 500,000 people are living with bladder cancer, making it the 4th most common cancer [12]. In addition, because the natural history of this disease is commonly characterized by multifocality and frequent recurrence, there is an enormous treatment cost, which is projected to be $2.9B for this year alone [12] and has also been ranked as the most costly cancer to treat due to long-term survival and the requirement of life-long monitoring [13]. Despite the growing need for better treatment, federal research funding for bladder cancer has decreased 31% over the past 5 years [14]. Decreases in research support have necessitated that current investigators focus on projects that have direct clinical relevance in addition to uncovering the underlying molecular pathology.

1.1.2 Normal Bladder Development

The bladder develops in two stages from the partitioning of the cloaca into the urogenital sinus and the dorsal rectum. The urogenital sinus eventually becomes the urachus, urinary bladder, and the proximal urethra. The bladder and urethral urothelium lining are derived from the endoderm while the underlying layers are of mesenchymal origin [15]. The embryonic stem cells that differentiate to form the urothelium exhibit a cellular organization where the basilar cells are p63-positive and the central-luminal urothelium is p63 negative.

Intriguingly, p63 is a marker of high grade urothelial carcinoma and is important for bladder cancer pathology [16-19].

1.1.3 Normal Bladder Histology

The urinary bladder is a muscular organ that resides within the pelvis and has a capacity of approximately 500 ml. The internal surface of the bladder is lined with transitional epithelium, approximately six cells thick, and rests on a thin basement membrane. Below this is the lamina propria that forms a thick layer of elastic connective tissue that allows the bladder to distend. The lamina propria also contains numerous blood vessels and the muscularis mucosa. Below the lamina propria is the smooth muscle of the bladder wall that forms interlacing bundles of loosely arranged fibers. This meshwork of muscle fibers is ideal for emptying the bladder [20].

1.1.4 Bladder Cancer diagnosis

Bladder cancer is most frequently suspected by the presence of hematuria (blood in the urine) or, less commonly, dysuria (painful urination) [15]. The tumors are most likely to occur on one of the bladder walls (40%) as opposed to other sites (ureteric orifice, trigone, dome, or neck) [7], thus the detection can be delayed due to an asymptomatic tumor development. Diagnosis is confirmed by urine cytology of cancer cells or, more commonly, cystoscopy. Cystoscopy is the procedure of using a thin, lighted scope (cystoscope) to get visual images within the bladder and possible tissue samples. Transurethral resection of the bladder

(TURB) remains the gold standard for bladder cancer diagnosis of low stage tumors [21].

There has been intense interest in developing a non-invasive urine test for bladder cancer that identifies specific bladder cancer biomarkers [22]. There are currently 7 commercial biomarker tests available but none have distinguished themselves to eliminate the need for cystoscopy; all suffering from poor sensitivity [23].

1.1.5 Types of Bladder Cancer

In North America, urothelial carcinoma (also called transitional-cell carcinoma) comprises 92-99% of all diagnosed bladder cancers [24]. There are some race variations with urothelial carcinoma accounting for 95% of bladder cancers in white and 85% in black American populations [7]. In Southeast Asia, the proportion of UC decreases to only 70-80% of the population and in Northern Africa it is less than 50% [24, 25]. Other types of bladder cancer include squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma is frequent in areas with high infections rates of the bladder parasite *Schistosoma haematobium* [6]. Other rare types of bladder cancer include: neuroendocrine bladder tumors (small-cell or carcinoid) and mesenchymal tumors (lymphomas or sarcomas) [24].

1.1.6 Bladder cancer staging

The staging most often used for bladder cancer follows the guidelines set by the American Joint Committee on Cancer (AJCC) which is most commonly known as the TNM system [26]. The T stands for "tumor" and is followed by a number 1 to 4 and describes the extent to which the tumor has grown into surrounding tissue (**Table 1**). The N stands for "node" and is given a number 1 to 3, which indicates the level of spread to lymph nodes surrounding the bladder. The M stands for "metastasis" and is given the number 0 or 1 depending on whether the tumor has spread to a distant site. A combination of each T, N, and M score determines the clinical stage, which is a strong predictor of survival. According to the SEER Data Base of the National Cancer Institute, Stage 0 bladder cancer has a 5-year survival rate of 98% while a Stage IV cancer is only 15% [2].

In addition to staging, bladder cancers can also be given pathologic grades according to the level of differentiation displayed by the tumor cells. Low grade lesions have variation in nuclear size, shape, and polarity, have minimal mitoses and have less aberrant cytogenetics. High grade lesions have prominent variation in nuclear size, shape and polarity, have common mitoses, and can have large genetic changes. Examples of low and high grade tumors are shown in **Figure 1**. Tumor grading is important as high grade lesions are independently prognostic for invasion, recurrence, and survival [15].

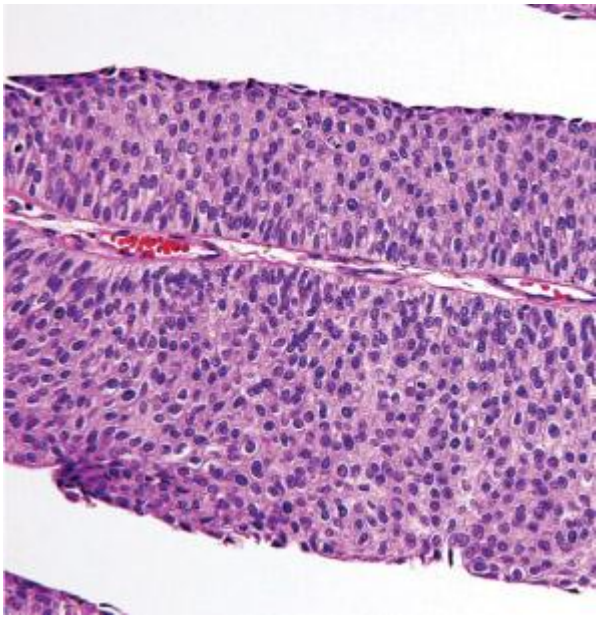
Table 1

Tumor Stage (T)	Description
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ (CIS)
T1	Invasion of the lamina propria
T2	Invasion of the muscularis propria
T3	Invasion of the perivascular fat
T4	Invasion of the prostate, uterus, vagina, pelvic or abdominal wall

Figure 1. Low and high grade papillary carcinoma. **A.** Low grade urothelial carcinoma is characterized by orderly urothelial cells with minimal nuclear enlargement and atypia. No mitoses are present. **B.** High grade urothelial carcinoma is characterized by gross mucosal cellular disorganization and pervasive anaplasia. Images were adapted from Peterson et al. [15].

Figure 1

A



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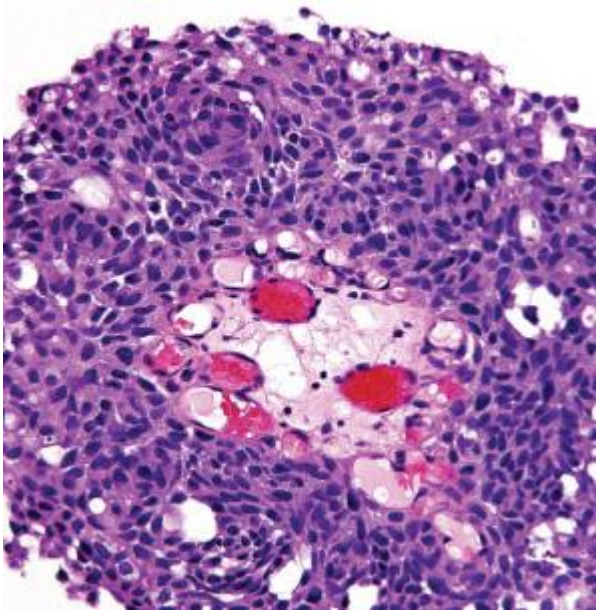


1.1.7 Natural History of Bladder Cancer

Of diagnosed bladder cancers in the US, 70-80% are nonmuscle invasive tumors (Ta, Tis, T1), 25% are muscle invasive (T2), and 5% are metastatic [27]. Of the nonmuscle invasive tumors, 60-70% will eventually recur [28], with 20-30% progressing to a higher stage or grade [29]. Transurethral resection of the bladder (TURB) remains the ideal choice for treatment of Ta and T1 tumors [21]. For carcinoma in situ (Tis), intravesical inoculation with bacillus Calmette-Guérin bacteria, which is thought to initiate an immune response against the tumor, is the main mode of treatment [30]. For muscle invasive tumors, radical cystectomy is the standard of care. Adjuvant chemotherapy is used post operatively though recent findings suggest that neoadjuvant chemotherapy may provide increased survival benefit [31]. **Figure 2** depicts current treatment of bladder cancer.

1.2 Molecular Insights into Bladder Cancer Progression

Despite sufficient need for understanding, the molecular pathogenesis of bladder cancer remains a field without clear consensus. No single mutation or pathway has emerged as the protagonist of bladder tumorigenesis. However, a compilation of mutations, deletions, and epigenetic changes have demonstrated their importance. Several of these changes are common to many cancers including the loss of tumor suppressors p53, RB, and TSC1 [32]. The Ras pathway has been implicated in driving initial bladder cancer tumorigenesis [33], while several proteins involved in the hypoxia response pathway, such as VEGF [34],

mTOR [35], and HIF1A [36, 37] serve to increase the aggressive nature of the disease.

1.2.1 Ras and Ral GTPases

The first identified human oncogene, HRAS, was discovered in the bladder cancer cell line EJ [38] and is mutated in approximately 30-40% of all bladder cancers [39, 40]. HRAS is typically mutated at the codons 12, 13, or 61, which locks the protein in an activated conformation [32]. GTPases cycle between guanine diphosphate (GDP) (inactive) and guanine triphosphate (GTP) (active) based on the interactions with guanine exchange factors (GEFs), which exchange GDP for GTP to activate the protein, or GTPase activating proteins (GAPs), which cause the Ras GTPase to hydrolyze GTP to GDP (**Figure 3**) [41]. Activation of the Ras signaling pathway in bladder cancer is not limited to direct HRAS gene mutations as overexpression of HRAS occurs in over half of human urothelial carcinomas; a higher frequency than that of HRAS mutations [42].

Apart from direct mutation or overexpression of Ras, receptor tyrosine kinases (RTKs) that activate the Ras GTPases play a large role in bladder cancer. Overexpression of EGFR [43-47] is associated with aggressive and metastatic tumor phenotypes and poor patient prognosis. ERBB2, also known as HER2, is overexpressed in bladder cancers and correlates with invasive tumors and poor prognosis [48, 49]. Interestingly, other mutated or overexpressed RTKs do not correlate with poor clinical parameters. FGFR3 is mutated in up to 70% of low grade, non-invasive papillary tumors [32]. ERBB3 and ERBB4 when overexpressed are linked to low grade, non-invasive papillary tumors [50].

Figure 2. Bladder cancer treatment diagram. Current treatment regimens for bladder cancer are shown, depending on the stage of the disease at presentation. Abbreviations: TUR: Transurethral resection; BCG: bacillus Calmette-Guérin; MVAC: methotrexate, vinblastine, doxorubicin, and cisplatin; CMV: cisplatin, methotrexate, and vinblastine.

Figure 2

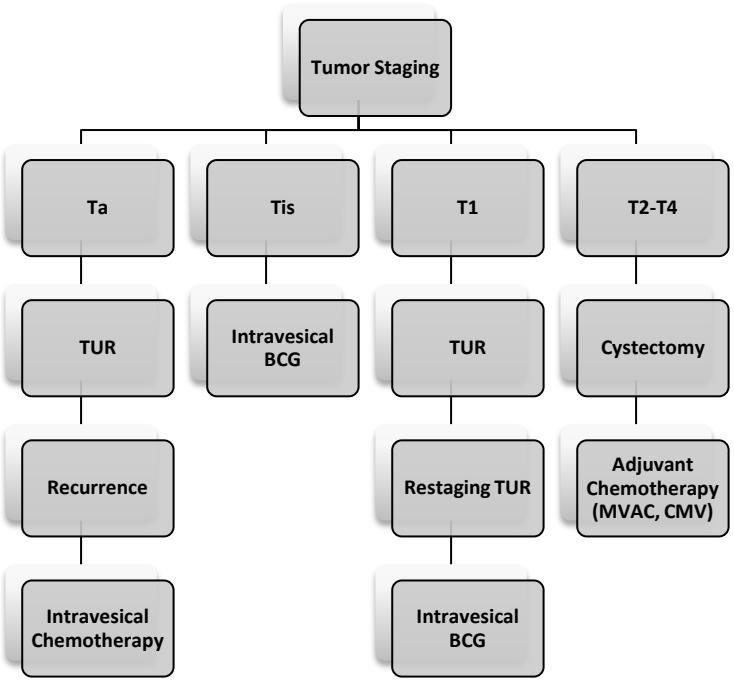
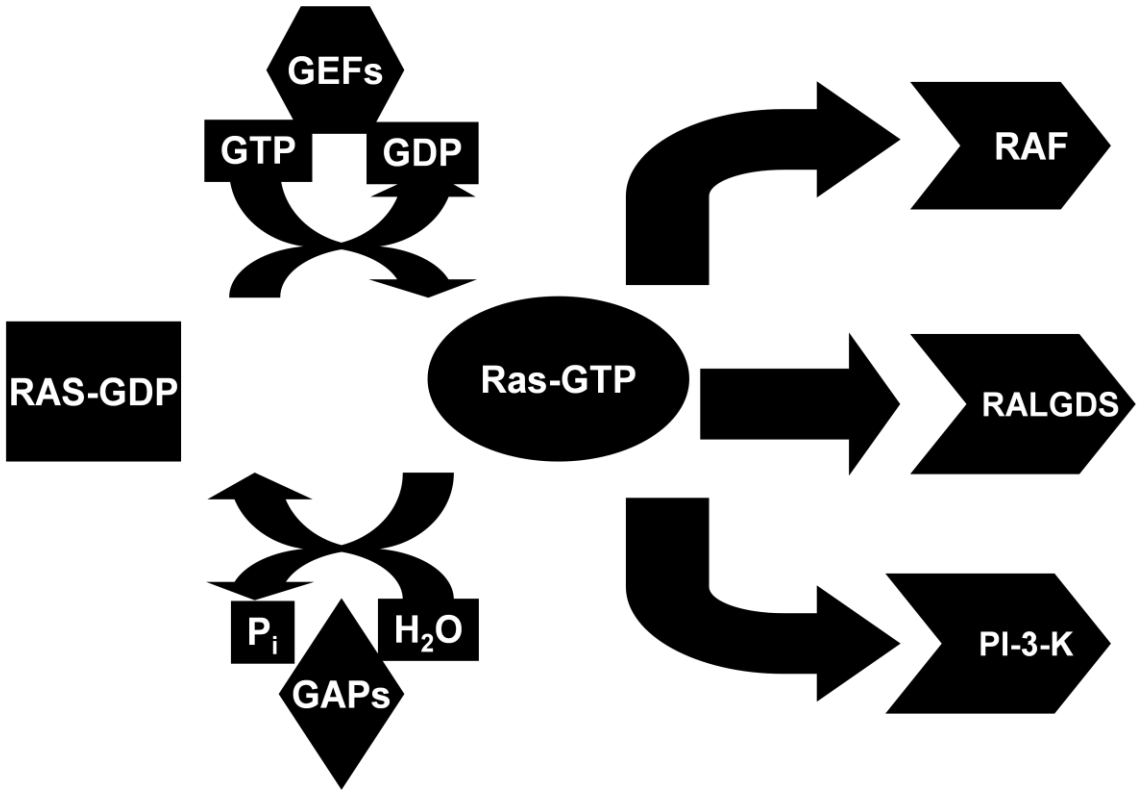

Tumor Staging
Ta
Tis
T1
T2-T4
TUR
Intravesical BCG
TUR
Cystectomy
Recurrence
Restaging TUR
Adjuvant Chemotherapy (MVAC, CMV)
Intravesical Chemotherapy
Intravesical BCG

Figure 3. Cycling of Ras GTPase leads to activation of downstream effectors. Ras is a GTPase that cycles between GDP (guanine diphosphate) and GTP (guanine triphosphate). Binding of a guanine exchange factor (GEF) exchanges GDP for GTP and activates the GTPase (Ras) to interact with downstream effectors. Ras binding to a GTPase activating protein (GAP) induces Ras to hydrolyze the terminal phosphate of GTP to form GDP, thus reducing Ras to a non-active state. There are three main effector pathways downstream of Ras: Raf/MAPK, RalGDS/Ral, and PI3K/Akt.

Figure 3



Research aimed at understanding the signaling pathways downstream of EGFR [51] have led us to focus on **Ra**s-**L**ike GTPases RalA and RalB, two closely related 23 kDa proteins, as important mediators of human bladder cancer aggressiveness and metastasis. RalA and RalB were first isolated based on sequence similarity to Ras GTPases [33, 41]. Human RalA and RalB are 82% identical and, when compared to the ~170 small GTPases, are structurally most related to the Ras subfamily.

RalA expression increases as a function of stage while RalB is overexpressed in both superficial and invasive bladder cancer [52]. More importantly, both RalA and RalB have higher GTP bound fractions (activated) in bladder tumors compared to normal bladder urothelium [52]. siRNA depletion of RalA and RalB decrease the growth and migration of the bladder cancer cell line UMUC3 [53] and loss of RalA abrogates EGF stimulated migration of the bladder cancer cell line EJ [54]. Taken together, the evidence suggests a correlation between the Ral pathway and invasive cancer and poor prognosis.

1.2.2 CD24 expression is dependent on Ral GTPases

Through investigating the gene expression changes in bladder cancer upon depletion of RalA and RalB, CD24 was found to highly downregulated [55]. The CD24 gene encodes an 80 amino acid pre-protein that is cleaved into a 27 amino acid protein that is heavily glycosylated and linked to cell membranes through a GPI anchor [56]. Originally described as a marker of developing B-cells, low levels of expression have been detected in other tissues [57]. Conversely,

CD24 is over expressed in multiple cancers arising from various tissues as depicted in **Table 2**.

CD24 expression also correlates with other histologic factors such as stage and grade, disease aggressiveness such as invasion and metastasis, and ultimately in prognosis (**Table 2**). While the correlation with cancer is not disputed, there is an ongoing investigation into the active participation of CD24 in the malignant phenotype. Our lab was the first to show the necessity of CD24 expression for survival of cancer cells from several tissue types [55]. Recently, these results were confirmed by a second study reporting siRNA depletion of CD24 in pancreatic and colorectal cancer cell lines abrogates growth *in vitro* and *in vivo* [58]. Overexpression of CD24 in breast cancer cells increased subcutaneous xenograft tumor growth [59]. We feel that these studies underscore the importance of CD24 in tumor biology and discredit the idea that CD24 is merely a passenger or marker of transformation.

However, less is known about the mechanism employed by CD24 to promote cell survival, invasion, and metastasis. Several studies have shown that CD24 binds P-selectin, an endothelial cell surface protein, which may contribute to an increased metastatic capacity by promoting efficient arrest of the circulating cells in the lungs [60-62]. CD24 may also associate with plasma membrane lipid rafts, a collection of glycosphingolipids and protein receptors that serve as organizing centers for cell signaling, leading to increased intracellular signaling [63, 64]. Recently, CD24 has become the subject of intense debate as its expression positively and negatively correlates with the “cancer stem cell” phenotype [65, 66].

Table 2

Tissue origin	Up in Cancer?	Grade?	Stage?	Invasion?	Metastasis?	Prognosis?
Bladder	Yes [67]			Yes [67, 68]		
Brain	Yes [69]			Yes [70]		
Breast	Yes [71-73]	Yes [71, 73]		Yes [74]	Yes [60, 75]	Yes [74, 76]
Colon	Yes [72]	Yes [77]	Yes [77]		Yes [77]	Yes [77]
Gastric	Yes [78]		Yes [72, 78]	Yes [78]		Yes [78]
Kidney	Yes [79, 80]	Yes [81]				Yes [81]
Liver	Yes [82]					Yes [83]
Lung	Yes [84]					Yes [74]
Lymphoma	Yes [85]					
Nasopharyngeal	Yes [86]					
Ovarian	Yes [87-89]			Yes [90]		Yes [87, 90-92]
Prostate	Yes [93]				Yes [94]	Yes [94]

1.2.3 RREB1 is downstream of Ral GTPases

In an effort to uncover the proteins responsible for Ral dependent CD24 transcription, we used an *in silico* tool (CARRIE) developed to uncover putative transcription factor-regulatory pathways associated with changes in transcriptional profiles. When RalA and RalB expression was depleted in the UMUC3 bladder cancer cell line, the differentially expressed genes were found to have a high representation of the RREB1 transcription factor consensus binding site in their promoters [95]. Thus, we inferred that RREB1 may form a critical link between Ral and CD24.

The **R**as-**R**esponsive **E**lement **B**inding protein 1 (RREB1) (also described in the literature as **F**inger protein **I**n **N**uclear **b**odies (Finb)) contains 13 – 15 classical C_2H_2 zinc-finger domains; depending on known alternative splicing of the pre-mRNA [96-98]. Four isoforms of RREB1 have been identified but no study has reported any differences in functionality or expression [96 98].

RREB1 has been reported to localize to the nucleus and function as a putative transcription factor [98]. Genes induced by RREB1 include Calcitonin [97], FSH[99], MT-IIA [98], p53[100], and Secretin [101], while genes repressed by RREB1 include Angiotensinogen [96], HLA-G [102], hZIP1[103], p16[104], and PSA [105]). Additionally, RREB1 has been found to bind other nuclear proteins such as the CtBP [106], NeuroD [101], and the androgen receptor (AR) [105]. As a component of the C-terminal binding protein (CtBP) complex, RREB1 is believed to be a component necessary in deacetylation and methylation of histones [106].

Though the role of RREB1 in cancer has not been explicitly studied, there have been several reports demonstrating its participation in the transformed phenotype. The initial report characterizing RREB1 found that the gene product bound the calcitonin promoter in medullary thyroid carcinomas in response to Ras transformation [97]. Of interest to bladder cancer where the tumor suppressor p16 is commonly lost as an early event in tumorigenesis [107], in the BALB/c mouse RREB1 was found to bind and repress transcription of the *Cdkn2a* locus, of which p16 is a product [104]. Depletion of RREB1 by siRNAs decreased migration in a wound closure assay and blocked cell spreading in the breast epithelial cell line MCF10A [108] In the osteosarcoma cell lines U2OS and SaOS-2, RREB1 was found to bind the p53 promoter and transactivate p53 expression in the presence of DNA damage [100].

The RREB1 locus is also a preferential site of integration in viral induced malignancies and gene amplification. In hepatocellular carcinoma, the Hepatitus B Virus integrates immediately upstream of the RREB1 transcription start site [109]. The Moloney Murine Leukemia Virus (MuLV), a retrovirus that randomly inserts throughout the DNA, was used to infect $p19^{ARF}$ and p53 null mice. When tumors arose in these mice the location of the retroviral insertion was mapped to the gene location. RREB1 was one of three potential oncogenes indentified by this method [110]. Furthermore, the location of the retroviral insertion was compared to the data from the Wellcome Trust Sanger Institute's Cancer Genome Project, where SNP arrays and copy number information have been performed on over 700 cell lines. The location on chromosome 6 that encodes RREB1 and

harbored the retroviral insertions was found to be amplified in cancer cell lines of breast, skin, lung, ovary, brain, and bone [110]. Finally, the human RREB1 locus has been found to be amplified in melanoma and is currently an area of intense investigation as a potential molecular diagnostic test [111-116].

1.2.4 The hypoxic response in tumors

Hypoxia is the result of an inadequate supply of oxygen to tissues within the body. A multitude of medical conditions can give rise to hypoxia, including atherosclerosis, vascular stenosis, vascular trauma, chronic obstructive pulmonary disease, shock and a variety of other pathological processes. Most notable for this discussion, the rapid growth of tumors and the aberrant and inadequate formation of a neovasculature to support them often lead to local areas of hypoxia within the tumor and surrounding tissue. If severe, hypoxia can result in cell death and organ failure. Hypoxia does play a role in normal biology in the case of tissue growth, where hypoxia stimulates formation of new vasculature. Certain organs, such as the liver, function chronically at a lower oxygen tension than other more oxygen intensive organs, such as the brain [117, 118]. The typical response by a cell when exposed to hypoxia is secretion of factors to increase flow of blood to area (angiogensis), decrease non-essential cellular processes, and a switch in energy metabolism from oxidative respiration involving the Krebs Cycle and oxidative phosphorylation to anaerobic glycolysis [119].

It has long been known that the cellular metabolism within solid tumors is different than the surrounding normal tissue. These major metabolic changes were first noticed more than 50 years ago by Otto Warburg [120]. Tumor cells rely on glycolysis, which requires high amounts of glucose, to generate ATP. This occurs when limiting oxygen levels prevent ATP production by oxidative phosphorylation, leading to preferential use of glycolysis by the tumors to generate cellular energy, even in non-limiting oxygen levels [121]. The reliance on glycolysis leads to low energy efficient tumor cells taking up high levels of glucose to maintain cellular function. This phenomenon is exploited by oncologists who use radioactive glucose to identify tumor growth. Tumor hypoxia is vitally important clinically as it has been correlated with metastasis, invasion, and poor prognosis [122, 123].

One of the main cellular responses to low oxygen levels is the stabilization of the HIF1A and HIF2A transcription factors. In normal oxygen levels (normoxia) HIF1A and HIF2A are hydroxylated by prolyl hydroxylases (PHDs), bound by the tumor suppressor VHL, and degraded by the proteosome [124]. In hypoxia, the PHDs no longer hydroxylate HIF1A, which allows it to form a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT), translocate to the nucleus, and bind gene promoters containing a hypoxia responsive element (HRE) [124] (**Figure 4**). The myriad genes induced by the HIF transcription factors participate in angiogenesis, glycolysis and glucose uptake, anti-apoptosis, and function as growth factors.

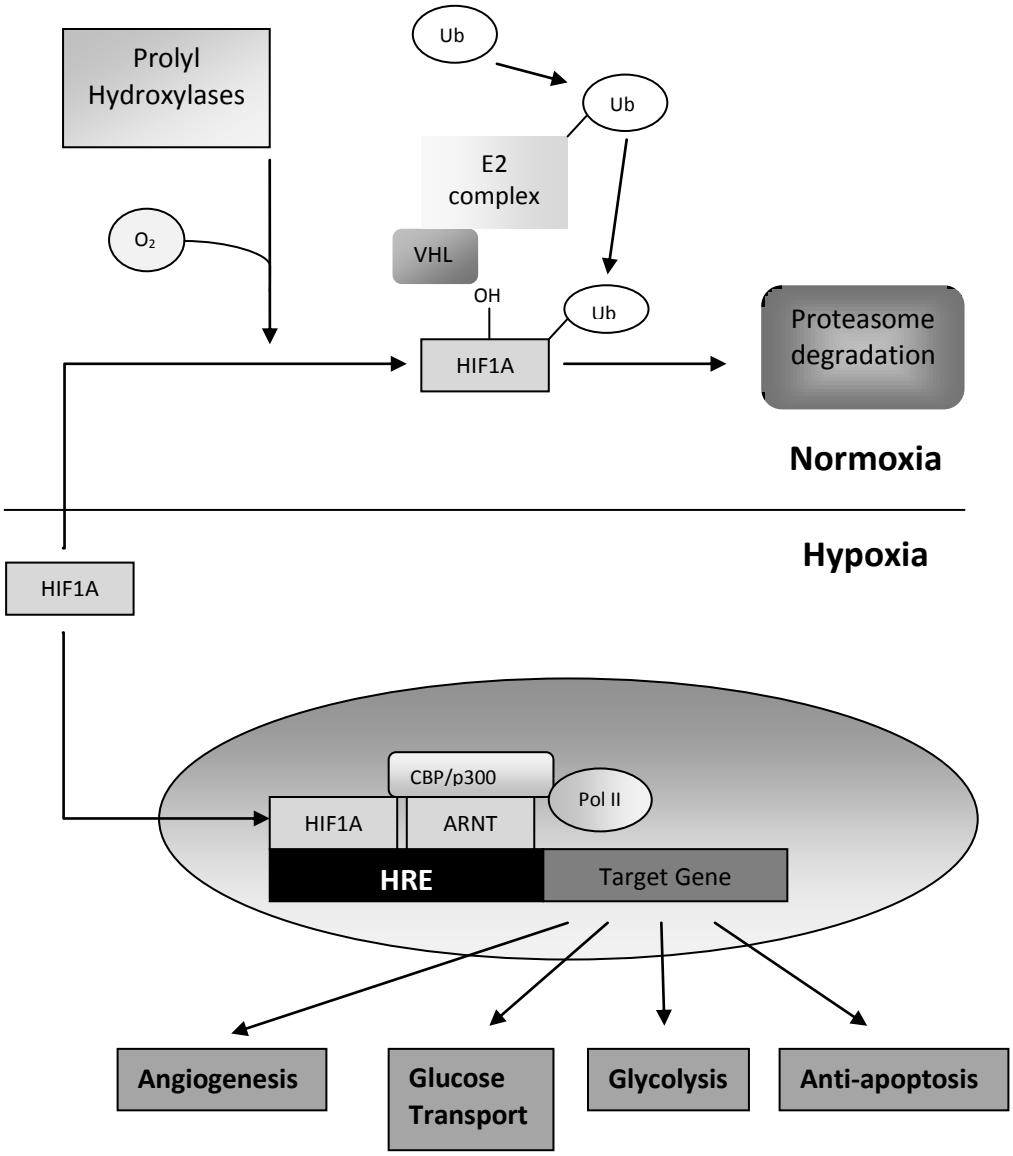
Protein stabilization of HIF1A an HIF2A can occur in non-hypoxic conditions due to aberrant oncogenic signaling. Constitutively activated Ras signaling [125] or overexpression of Ras activating tyrosine kinase receptors like ERBB2 [126] stabilize HIF1A and HIF2A in normoxia. The mechanism for this stabilization continues to be active area of research but it is thought to proceed through kinase signaling as O_2-independant stabilized HIF is hyperphosphorylated [121].

1.2.5 Hypoxia and Bladder Cancer

Hypoxia and the hypoxia responsive transcription factors HIF1A and HIF2A appear to have significant roles in bladder cancer progression. HIF1A expression positively correlates with grade [36, 127] and proliferation [36]. HIF1A and HIF2A are significantly associated with invasive disease [128, 129]. HIF1A and HIF2A expression are also important clinically as their expression in tumors predicts recurrence [128, 130, 131] and a poor prognosis [131-134]. In the xenograft tumor model system, bladder cancer cells VMRC that stably express HIF1A, showed significantly greater subcutaneous tumor volume than the control tumors [135]. Furthermore, the orthotopic growth of the bladder cancer cell line RT112 in mice can be blocked by systemic injection of anti-VEGF antibodies [136] underscoring the importance of hypoxic responsive genes in tumor growth.

Figure 4. The HIF1A Pathway. In normoxia (21% O_2) prolyl hydroxylases post translationally modify HIF1A by hydroxylating a proline, which allows the protein to interact with the VHL complex. Binding to VHL allows the ubiquitin conjugating enzyme (E2) to ubiquitilate (Ub) HIF1A. Unibiquitylation of HIF1A targets it for destruction in the proteasome. In low oxygen environments (hypoxia), the prolyl hydroxylases cannot hydroxylate HIF1A and it translocates to the nucleus to heterodimerize with ARNT and bind hypoxia response elements (HRE). Binding of the CBP/p300 complex is necessary for recruiting RNA polymerase II and inducing transcription of target genes.

Figure 4


Prolyl Hydroxylases
Ub
Ub
E2 complex
O2
VHL
OH
Ub
HIF1A
Proteasome degradation
Normoxia
Hypoxia
HIF1A
CBP/p300
Pol II
HIF1A
ARNT
HRE
Target Gene
Angiogenesis
Glucose Transport
Glycolysis
Anti-apoptosis

Drug developers have been keen to the importance of tumor hypoxia and have developed novel chemotherapeutic agents to target this phenomenon. The drug AQ4N (banoxantrone) is a prodrug that is enzymatically cleaved to a cytotoxic DNA-binding agent in hypoxia. Treatment with this drug increased the efficacy of cisplatin and radiation therapy to RT112 bladder cancer subcutaneous xenografts [137]. When AQ4N was administered to bladder cancer patients prior to surgery in a phase 1 clinical trial, the cleaved drug was found at significantly higher concentrations in the tumor than adjacent normal tissue [138]. Subsequent efficacy studies should provide additional evidence to the importance of hypoxia in bladder cancer.

Taking the necessity of hypoxia in bladder cancer progression into context with previous findings from our lab revealed interesting connections. Several of the genes most differentially expressed with Ral depletion are categorized as stress response genes. Upon closer investigation, many have been shown to be specifically regulated in hypoxia, such as MMP1 [139], Clusterin [140], and IGFBP3 [141]. CD24, whose expression is dependent on the Ral GTPases, was identified through mass spectrometry to be upregulated in endothelial cells after exposure to hypoxia [142]. While there are no direct reports linking RREB1 with hypoxia, it does regulate the expression of MT2A [98], a gene known to be upregulated in hypoxia [143]. Furthermore, RREB1 has been shown to bind the basic helix-loop-helix transcription factor NEUROD [101]. HIF1A also is a basic helix-loop-helix transcription factor [144]. In the end a connection can be drawn between the Ral GTPases, RREB1, CD24 and the hypoxic phenomenon.

The previously described importance of Ras and Ral GTPases led us to study CD24 and RREB1. Herein, we aim to characterize RREB1 and define its role in bladder cancer and CD24 expression. In this pursuit we identified hypoxia, specifically HIF1A, as the linchpin between RREB1 and CD24.

Chapter 2: The characterization of alternatively spliced RREB1

Matthew D. Nitz, Michael A. Harding, Steven C. Smith, Shibu Thomas, and Dan Theodorescu

Contributors

Matthew D. Nitz, UVA Department of Molecular Physiology and Biological Physics, designed and performed all experiments, analyzed the data, and wrote the manuscript.

Dr. Michael A. Harding, UVA Department of Urology, designed isoform specific RREB1 real time PCR primers, contributed substantially to the study design and preparation of the manuscript

Dr. Steven C. Smith, UVA Department of Urology, scored the IHC tissue microarray, provided statistical expertise, created Figure 2-10 and Table 2-1 and contributed substantially to the preparation of the manuscript.

Dr. Dan Theodorescu, UVA Department of Urology and Molecular Physiology and Biological Physics, provided resources, guidance, and advice on the design and interpretation of experiments.

2.1 Abstract

RREB1 is an alternatively spliced, C_2H_2 zinc-finger transcription factor implicated as a regulator of the androgen receptor in prostate cancer and mediator of Ras signaling in bladder cancer. However, little is known about the role of RREB1 isoforms in these diseases. Herein, we investigate the expression of alternatively spliced RREB1 in urologic malignancies. Characterization of RREB1 expression in prostate and bladder cancer cell lines in addition to corresponding human tissues identified four previously described isoforms designated: RREB1α, RREB1β, RREB1γ, and RREB1δ, and one novel isoform, RREB1ε. Comparison of currently available RREB1 antibodies for sensitivity and specificity in detecting RREB1 isoforms demonstrated isoform specific nuclear localization in cell fractionation studies. Immunohistochemical evaluation of bladder and prostate tumors confirmed the primary nuclear expression of RREB1 *in vivo*. To date, this is the most comprehensive examination of RREB1 isoforms in any cancer type.

2.2 Introduction

The Ras family of GTPases, their regulators and effectors have been implicated in progression of bladder and prostate cancer [33, 46, 145-149]. Recently we found that the Ral (Ras-like) GTPase pathway downstream of Ras plays an important role in bladder cancer cell migration [53, 54]. In addition, depletion of both Ral paralogs (RalA and RalB) leads to decreased growth of bladder cancer cells [53], coincident with significant decreases in expression of stress response genes [55, 95]. Interestingly, the RREB1 (Ras-Responsive Element Binding protein 1) transcription factor was identified as a putative Ral regulated gene through batch analysis of promoter sequences in Ral target genes [95]. Experiments confirmed that Ral manipulation affected RREB1 reporter activity in bladder cancer cells [95].

RREB1 is a transcription factor containing between 13-15 zinc finger domains depending on alternative splicing [96] and has been found through EMSA [97] and DNA footprinting [150] to bind promoter sequences and regulate transcription. In the prostate cancer cell line LNCaP, RREB1 was found to bind the PSA promoter only in association with AR to repress transcription of PSA [105]. Oncogenic Ras activation, a known modulator of RREB1 activity, was shown to abrogate the RREB1 repressive effects on PSA. RREB1 was also found to bind the promoter and decrease transcription [103] of hZIP1 a zinc transporter ubiquitously expressed prostate glands but downregulated in prostate adenocarcinoma [151].

However, as evidence accumulates concerning the importance of RREB1 in human malignancy, an urgent need exists for characterization of expressed RREB1 RNA and protein species. Though four unique RREB1 splice variants have been described, no systematic characterization of their expression patterns or functional roles in key cancer phenotypes has been undertaken. Herein, we address these limitations in our knowledge and understanding of RREB1, while discovering a new isoform and observing novel associations of this protein's expression with disease states and histological types in bladder and prostate cancer.

2.3 Materials and Methods

2.3.1 Cell Lines and Human tissues

Human cancer cell lines were grown as described [152]. TERT cells were a gift from Dr. MA Knowles[153]. LUL2 were isolated from lung tumors by successive passages of the UMUC3 cell line by tail vein injection into nude mice (*manuscript in preparation*). Strictly observing NIH and UVA guidelines and with approval of the Institutional Review Board, deidentified flash frozen and archival tissues of human bladder and prostate cancer and adjacent non-neoplastic epithelia, procured by the UVA Biotissue Repository Facility, were obtained for analysis of RREB1 expression. The tissue microarray described herein has been reported [154].

2.3.2 RNA isolation, Reverse transcription, and PCR

RNA isolation was carried out using an RNeasy® Mini Kit (Qiagen) and cDNA synthesis using iScript™ (Biorad). Q-RTPCR was performed on cDNA using the iQ™ SYBR® Green Supermix (Biorad). RNA quantification was performed as described [155] with RREB1 (total) used as the reference. Primers with annealing temperatures: RREB1 (total) F:5'-CTT-CCT-ATA-ACT-GCC-CCC-3'; R:5'-ATG-AGT-GGT-CGG-CTC-CTC-C-3'; RREB1α: F:5'-TGG-ATC-CCA-TGA-TAG-CAC-AGA-C-3'; R:5'-TGC-TCT-CTG-TCC-CGT-GAG-G-3'; RREB1β: F:5'-CAC-ATG-CTC-ACA-CAC-ACT-GAC-A-3'; R:5'-CCG-ACG-GCT-GCT-CTC-TGT-3'; RREB1δ: F:5'-ACC-AAC-TGC-CTG-CAG-AAG-ATC-A-3'; R:5'-GTA-TGG-CCT-TTC-CCC-AGT-GTG-T-3'; RREB1ε: F:5'-TAC-AGA-ACA-ACC-CTT-CAA-TTC-CT-3'; R:5'-TAT-GGC-CTT-TCC-CCT-GAG-3'. PCR for the 5' and 3' ends of RREB1 was carried out using the AccuPrime™ Supermix II (Invitrogen) for 35 cycles with the following primers: RREB1 5': F:5'- TCG-GAT-TGG-CAG-AAG-GAA-3'; R:5'-CAG-GCT-CAG-CAG-GTT-GGT-3'; RREB1 3': F:5'-CGG-AAC-TCG-TAC-ACC-AAC-TG-3'; R:5'-CGC-TGT-GGG-TGG-ACT-CAT-TC-3'; RREB1 full length: F:5'-GAT-CAA-GCT-TAC-GTC-AAG-TTC-GCC-CGC-T-3'; R:5'-GAT-CCT-CGA-GTC-ACT-CCA-TCC-CCA-CGA-G-3'.

2.3.3 Cloning of RREB1

RREB1δ and RREB1ε were cloned out of UMUC3. Sequences were submitted to Genbank: RREB1δ (HM369361) and RREB1ε (HM369360). Total

RNA was isolated as described above and cDNA synthesis using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) with 50μM random hexamers and 1μg of total RNA. The PCR reaction also included: 10X PfuUltra™ HF Reaction Buffer (Agilent), 100mM dNTP Mix (Agilent), 5% DMSO, 1M Betaine Solution (Sigma-Aldrich). The primers used were as follows: RREB1 Cloning: F:5'-GAT-CAT-CGA-TAT-GAC-GTC-AAG-TTC-GCC-C-3'; R:5'-GAT-CTC-TAG-ACT-CCA-TCC-CCA-CGA-GCT-G-3'. PCR products were isolated in a 1% agarose gel and purified using a Qiaquick® Gel Extraction Kit (Qiagen). Isolated products were digested at 37^{o}C by the enzymes ClaI and XbaI (New England Biolabs) and ligated into the p3XFLAG-CMV™-14 Expression Vector (Sigma-Aldrich). RREB1α and RREB1β were kindly provided by Dr. Akiyoshi Fukamizu (University of Tsukuba, Tsukuba, Japan) [96] and subcloned into the p3XFLAG-CMV™-14.

2.3.4 Antibodies, Immunoblotting, and IHC

The following antibodies were used in detection of RREB1: (Genway Biotech Inc., Cat no: 18-732-2922332), (Cosmo Bio Co. LTD, Cat. no: CBX-CBX00717), (Sigma-Aldrich, Cat. no: HPA001756), FLAG (Sigma-Aldrich, Cat. no: F1804), Tubulin (Santa Cruz Biotechnology, Cat. no: sc-58668), TBP (TFIID)(Santa Cruz Biotechnology, Cat. no: sc-204). Nuclear and cytoplasmic isolates were made with the NE-PER® kit (Thermo Scientific). Immunoblotting and detection were performed as described [53]. IHC was performed on a Dako Autostainer Instrument (Dako Inc.) with the following protocol: antigen retrieval (125^{o}C, Dako TRS9 buffer, 30sec.), Dako Duel Endogenous Enzyme Block

(10min.), RREB1 antibody (Sigma-Aldrich)(1:100 in Dako Antibody Diluent, 30min.), Detection (Dako Envision Duel Link – Anti Rabbit, 30min.), Chromagen (Dako Dab+ Substrate, 10min.), Counterstain (Hematoxalin, 5min.). Staining of RREB1 was scored semi quantitatively as: negative (absence of staining), low/focal (a blush or positivity of cells <10%), moderate (clearly detectable nuclear staining pattern in up to 50% of cells), high (a strong nuclear and/or cytoplasmic positivity in >50% of cells). Examples of these types of staining are shown in **Figure 4B**.

2.3.5 Transfection and siRNA

Transient vector transfection was performed using FuGENE®6 Tranfection Reagent (Roche) according to manufacturer instructions. Stable expression was achieved by cutting RREB1 expressing p3XFLAG-CMV™-14 vector with the ScaI restriction enzyme (New England Biolabs) and transfecting with FuGENE®6. Selection was performed for 14 days in 800µg/ml of G418 (Geneticin®, Invitrogen). Oligofectamine™ (Invitrogen) was used to transiently transfect siRNA according to manufacturer instructions. All siRNA was transfected at a final concentration of 25nM unless otherwise noted: RREB1 total 5'-GGA-GUU-UGU-UUG-CAA-GUA-U-3' and 5'-GUU-CAG-ACC-UAU-CUU-CCA-U-3' (used in combination at 12.5nM), GL2 5'-CGUACGCGGAAUACUUCGAdTdT-3', RREB1 Exon 7 5'-CCA-ACA-AGU-UCA-GUC-CGU-U-3', RREB1 Exon 8-1 5'-CCU-GAG-AAG-AAA-CGG-GCU-UUU-3', RREB1 Exon 8-2 5'-CGC-AAA-CAC-GGA-GUU-ACC-ACC-UGU-U-3', RREB1

Exon 9-1 5'-CAG-AGA-AGA-GCG-ACG-AUG-AdTdT-3', RREB1 9-2 5'-CCA-CCA-AGC-UCA-UGG-ACU-UUU-3'.

2.4 Results

2.4.1 Alternative splicing of C-terminal cassette exons create unique RREB1 splice variants

RREB1 was initially described as a 755 amino acid C_2H_2 zinc finger protein (RREB-1) [97] though subsequent analyses in chicken and human cells indicated RREB1 encoded a longer protein of 1656 (Finb) amino acids (AA) in humans [98, 156]. A second variant encoding 1397 amino acids (Finb (cl-32)) with a unique C-terminus was also identified [98]. Two additional RREB1 C-terminal isoforms exhibiting addition or removal of cassette exons were isolated and designated Finb188 (1742AA) and Finb159 (1476AA). They exhibit a translation start site 57 base pairs upstream of the prior described Finb and Finb (cl-32) [96]. The latter report also uncovered that Finb contained regions without homology to consensus protein sequence of Finb188. cDNA sequence alignment of Finb and Finb188 reveals that the former does not conform to sequences for the human genome; potentially resulting from cloning artifacts [96]. Thus, there is a critical need for consensus on the RREB1 proteins. For clarity, **Figure 5** diagrams the variants characterized thus far.

To uncover the RREB1 isoforms expressed in bladder and prostate cancer cell lines, primers were designed to interrogate for expression of variants

in the 3' region of RREB1, where alternative splicing has been described [96, 98] (**Figure 5**). Predicted PCR product sizes for the four RREB1 isoforms characterized thus far were 1237bp for Finb188, 1072bp for Finb182 (RREB-1, Finb), 439bp for Finb159, and 348bp for Finb (cl-32). In nine cell lines examined (6 bladder, 3 prostate), bands corresponding to all four splice variants were identified (**Figure 6A**).

Each band was sequenced to confirm the PCR products corresponded to predicted splice variants. To examine whether the 5' end of the RREB1 transcript underwent alternative splicing, primers spanning from the first to the seventh exon were tested on seven cell lines. Each cell line showed a single band leading us to conclude that splicing of RREB1 occurred predominantly at the 3' end of the RNA (**Figure 6B**).

To determine if other splice variants existed for RREB1, primers were designed to interrogate all 10 coding exons (**Figure 5**). PCR products for Finb188/182 and Finb (cl-32) were identified, as well as a novel variant running at a much smaller size (**Figure 7A**). Sequencing of this band (**Figure 7B**) revealed it to be a novel isoform of RREB1, lacking coding exons 7, 8, and 9 and causing a frame shift in the 10^{th} exon resulting in a loss of a C_2H_2 zing finger and a new translation stop site. This variant has been submitted to Genbank as HM369360. Given these findings we propose the following nomenclature to describe the five RREB1 splice variants: RREB1α, RREB1β, RREB1γ, RREB1δ, and RREB1ε.

Figure 5. Characterization of RREB1 alternative splicing. 10 coding exons of RREB and previously described RREB1 sequences were aligned. Finb and Finb(cl-32) contained several regions of frame shifts in the cDNA that resulted in non homologous protein sequence. These sequences also described an alternative translation start site that results in a protein without the N-terminal 19 amino acids. We propose using the following nomenclature to describe RREB1 alternative splicing: RREB1α, RREB1β, RREB1γ, RREB1δ, and RREB1ε.

Figure 5

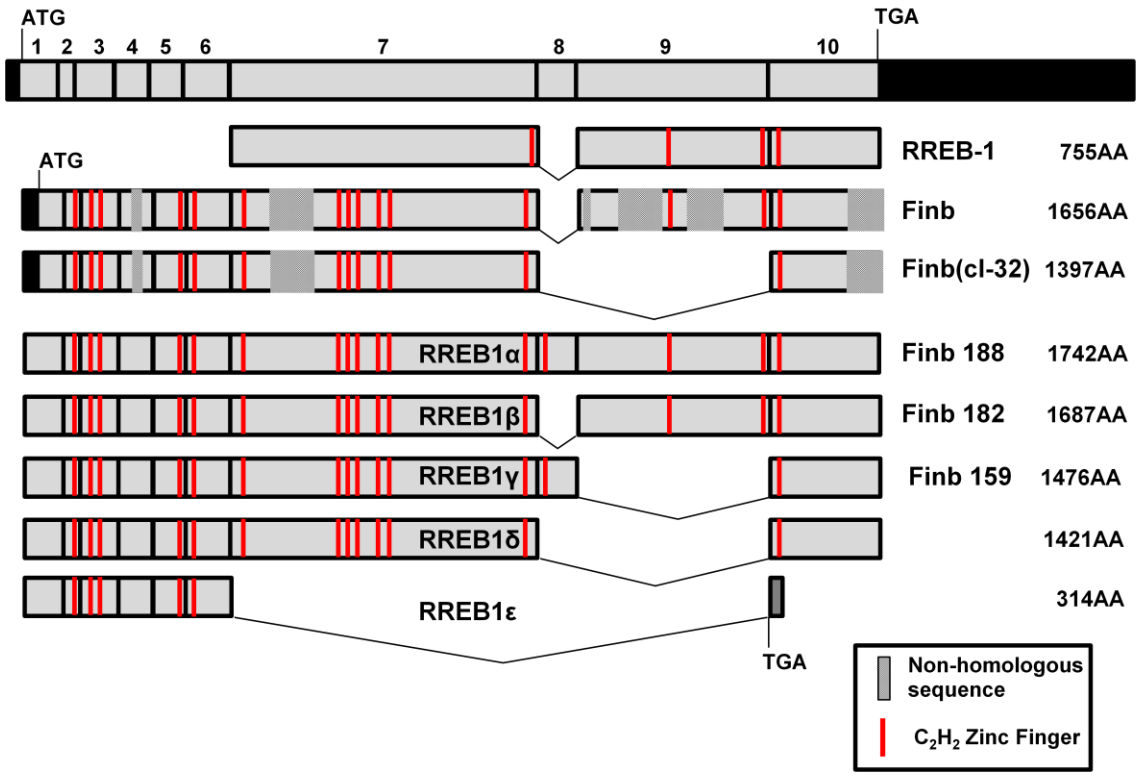
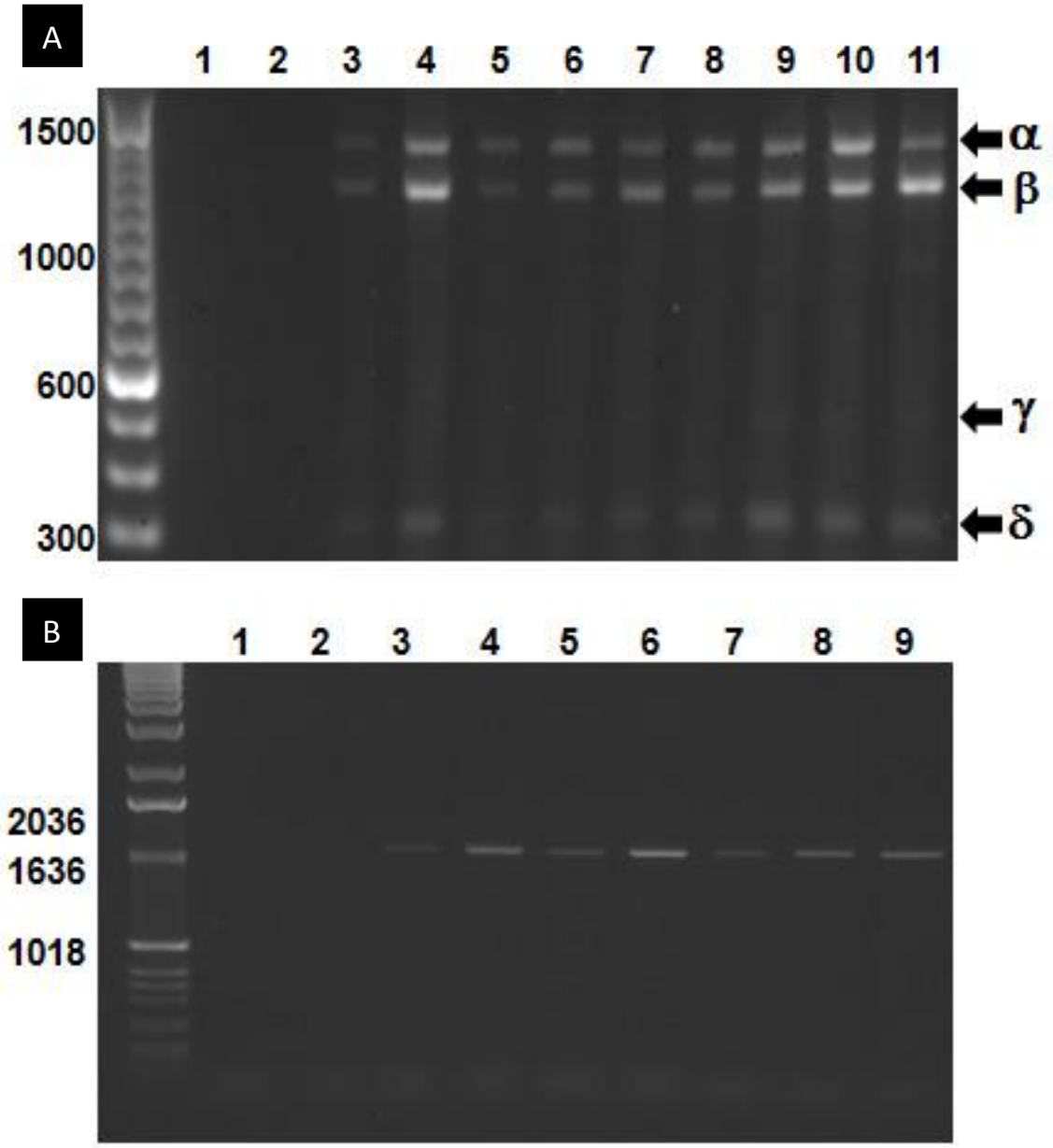

ATG
1 2 3 4 5 6 7 8 9 10
TGA
ATG
RREB-1 755AA
Finb 1656AA
Finb(cl-32) 1397AA
RREB1α
Finb 188 1742AA
RREB1β
Finb 182 1687AA
RREB1γ
Finb 159 1476AA
RREB1δ
1421AA
RREB1ε
314AA
TGA
Non-homologous sequence
C2H2 Zinc Finger

Figure 6. Detection of RREB1 isoforms in bladder and prostate cancer cell lines. A. Primers designed to span the region of RREB1 alternative splicing (exon 7 to exon10) were interrogated on the following bladder and prostate cancer cell lines: 1. Negative PCR control 2. Negative RT control 3. TERT 4.293T 5. UMUC3 6. LUL2 7. UMUC13D 8. J82 9. 1A6 10. PC3 11. LNCAP **B.** Primers were designed that spanned from exon 1 to exon 7 of RREB1 were used to amplify cDNA from the following samples: 1. Negative PCR control 2. Negative RT control 3. TERT 4. 293T 5. UMUC3 6. LUL2 7. UMUC13D 8. J82 9. 1A6

Figure 6


A
1 2 3 4 5 6 7 8 9 10 11
1500
1000
600
300
α
β
γ
δ
B
1 2 3 4 5 6 7 8 9
2036
1636
1018

2.4.2 RREB1 expression in bladder and prostate cell lines

No study has evaluated the expression of RREB1 in normal and neoplastic tissues, we examined a telomerase (TERT) immortalized urothelial cell line [153], 8 bladder cancer cell lines, and 3 prostate cancer cell lines. Primers spanning the second and third exons, the area we found not to be involved in differential splicing, were used to detect total expression of all known RREB1 splice variants via quantitative real-time polymerase chain reaction (Q-RTPCR). As seen in **Figure 8A**, total RREB1 expression was less in every bladder cancer cell line than in the TERT bladder line, which was unanticipated due to the association of RREB1 with various cancers.

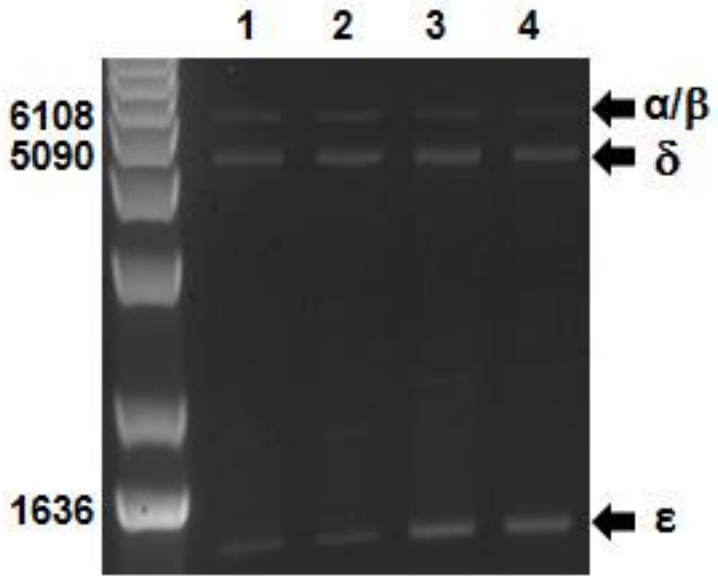
The expression of each isoform was evaluated by Q-RTPCR and each sample and RREB1ε was always <1% of total RREB1. This is the first description of an assay for specific, quantitative detection of RREB1 splice variants in human cells.

Next we examined frozen human tissues of paired normal and cancerous bladder (n=10) and prostate (n=4). The majority of cancers had lower RREB1 expression than their normal counterparts (Bladder 8/10, P=0.01, Prostate 3/4) (**Figure 9A**). RREB1 splice variant expression showed a similar pattern as seen in the cell lines where RREB1α and RREB1β comprised the majority of RREB1 expressed in these tissues (**Figure 9B**).

Figure 7. Detection of the novel splice variant RREB1ε **A.** Primers were designed that span Exon 1 to Exon 10 of RREB1 were used to amplify cDNA from the following samples: 1. UMUC3 2. UMUC3 3. LUL2 4. LUL2. **B.** Coding sequence of RREB1ε is shown. Exons are denoted by alternating font colors of red and black.

Figure 7

A



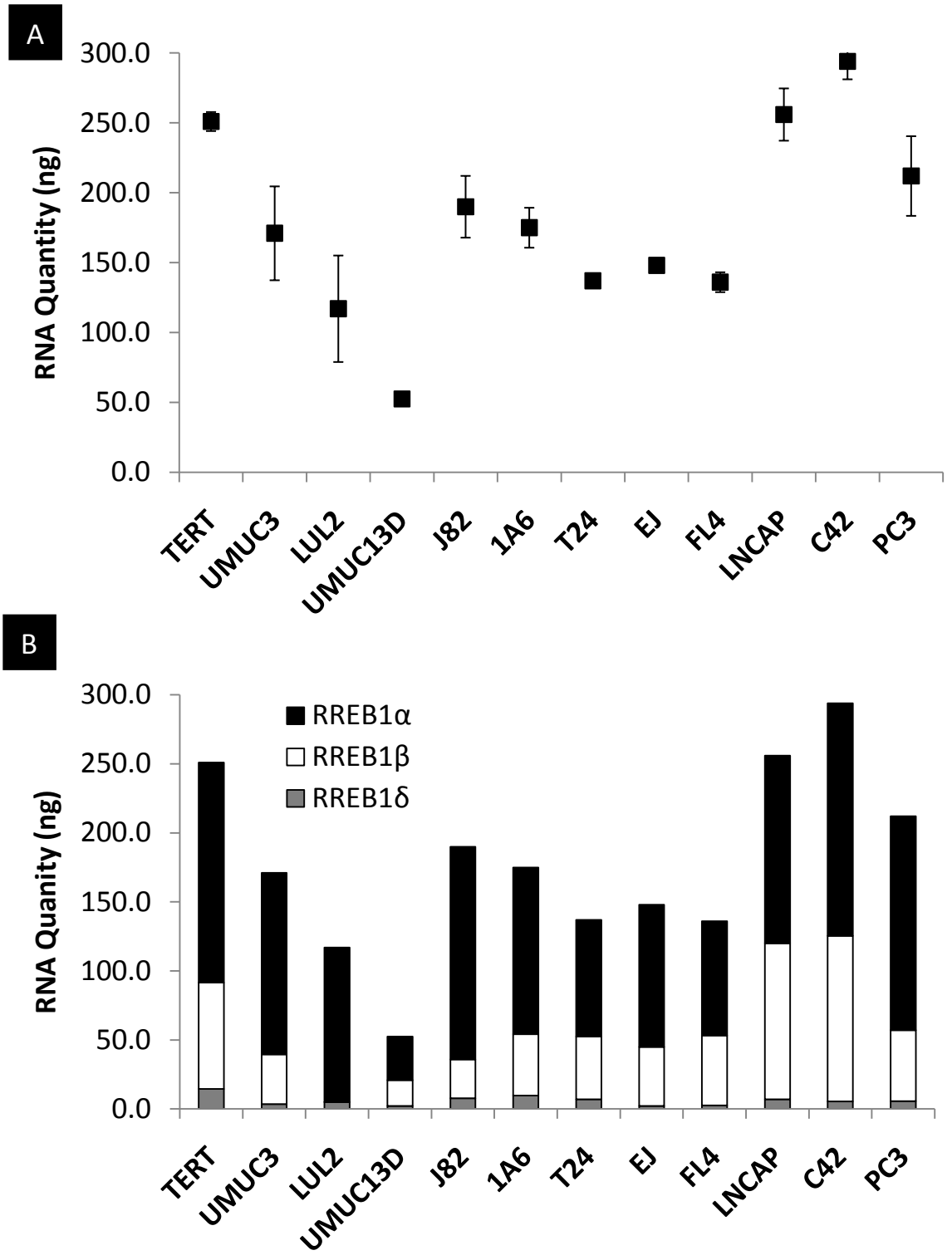
B

RREB1ε coding sequence

ATGACGTCAAGTTCGCCCGCTGGCTTGGAAGGTTCAGACCTATCTTC
CATCAACACCATGATGTCGGCGGTCATGAGTGTAGGGAAGGTCACAG
AGAATGGCGGGAGCCCCCAGGGGATCAAGTCCCCCTCGAAGCCTCC
AGGACCAAATCGGATTGGCAGAAGGAACCAGGAAACGAAAGAGGAG
AAGTCTTCCTATAACTGCCCCCTGTGTGAGAAGATTTGCACTACCCAG
CACCAGCTGACCATGCACATTCGCCAGCACAACACAGACACTGGAGG
AGCCGACCACTCATGCAGCATCTGCGGAAAGTCACTGAGCTCGGCC
AGCTCCCTCGATCGCCACATGCTGGTGCACTCTGGCGAGAGGCCTTA
CAAGTGCACTGTGTGTGGCCAGTCATTTACCACCAATGGGAACATGC
ACAGACATATGAAGATCCATGAGAAGGACCCTAACAGTGCCACAGCC
ACAGCCCCTCCATCTCCTCTGAAACGTAGGCGATTGTCCTCCAAGAG
GAAACTGAGTCACGATGCCGAGTCAGAGAGAGAAGACCCAGCACCA
GCTAAAAAGATGGTAGAAGACAGGCAGTCAGGTGACTTGGAGAAGAA
AGCTGATGAAGTCTTTCACTGCCCAGTATGTTTCAAGGAGTTTGTTTG
CAAGTATGGACTGGAGACCCACATGGAGACCCATTCAGATAACCCAC
TAAGATGTGACATTTGTTGTGTCACCTTTCGAACACATCGAGGACTGC
TGCGTCACAACGCGCTTGTCCACAAACAACTTCCCAGGGATGCAATG
GGCAGACCTTTCATACAGAACAACCCTTCAATTCCTGCTGGCTTCCAC
GACTTAGGATTCACGGACTTCTCCTGTAGGAAGTTTCCTCGCATTTCT
CAGGGGAAAGGCCATACAAATGTCAGACCTGCGAGCGAACCTTCACC
TTGA

Figure 8. RREB1 transcripts in cancer cell lines. A. Total RREB1 expression was measured using primers that span Exon 2 and 3 of RREB1, an area lacking alternative splicing. **B.** RNA expression of RREB1 splice variants were measured using primers designed to the unique splice junctions. RREB1γ was not included due to sub-quantifiable expression. Isoform expression levels were calculated using the Pfaffl method [155].

Figure 8


A
300.0
250.0
200.0
150.0
100.0
50.0
0.0
RNA Quantity (ng)
TERT
UMUC3
LUL2
UMUC13D
J82
1A6
T24
EJ
FL4
LNCAP
C42
PC3
B
RREB1α
RREB1β
RREB1δ
RNA Quanity (ng)

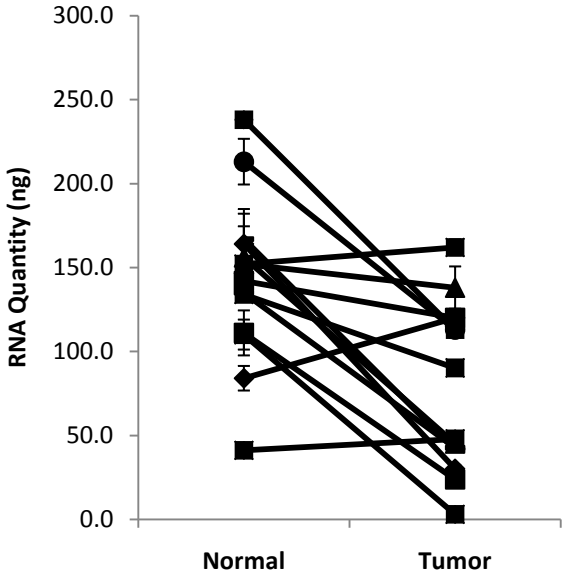
2.4.3 Characterization, detection and cellular localization of RREB1 protein

To date the expression of RREB1 splice variants at the protein level has not been examined. A major obstacle is the lack of a well characterized antibody. Here we define the sensitivity and specificity of three commercial RREB1 antibodies to detect the known RREB1 splice variants. We used cloned splice variants from UMUC3 (RREB1δ and RREB1ε) or previously published vectors [96] (RREB1α and RREB1β) that were cloned into a C-terminal 3X-FLAG tagged vector. RREB1γ was not examined since it is undetectable in the bladder cancer cell lines. Each isoform was expressed in 293T cells, and expression was confirmed by the anti-FLAG antibody on western (**Figure 10A**). The antibodies were tested against the RREB1 splice variants expressed in 293T. Two antibodies (Cosmo and GenWay) detected RREB1α and RREB1β, the latter antibody also detecting the RREB1δ transgene; but both antibodies suffered from low sensitivity in detecting endogenous RREB1 (**Figure 10A**). We observed that an antibody from Sigma-Aldrich exhibited the most robust detection of RREB1α, RREB1β, and RREB1δ tagged transgenes expressed in 293T cells.

Figure 9. RREB1 expression decreases in bladder and prostate tumors. A. Total RREB1 expression was measured in 10 bladder and 4 prostate samples with paired normal and cancer tissues. Total RREB1 expression was found to be lower in cancer than in normal tissue (p=0.01). **B.** RREB1 splice variant expression in paired normal and cancer bladder (BL) and prostate (PR) tissues.

Figure 9

A


300.0
250.0
200.0
150.0
100.0
50.0
0.0
RNA Quantity (ng)
Normal
Tumor

B

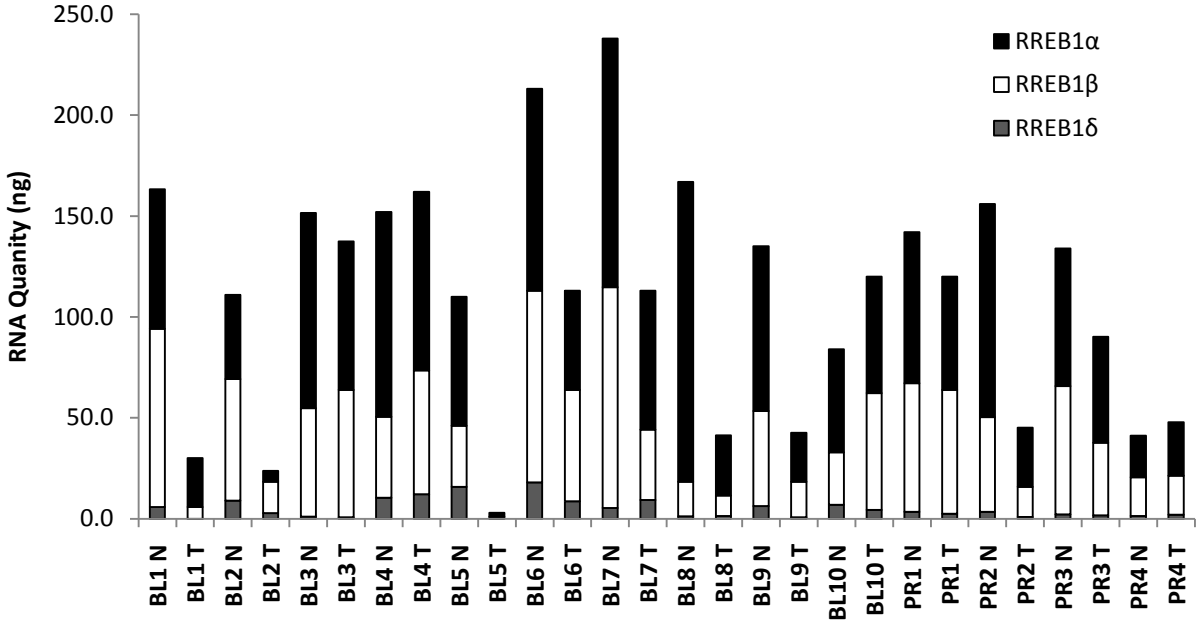
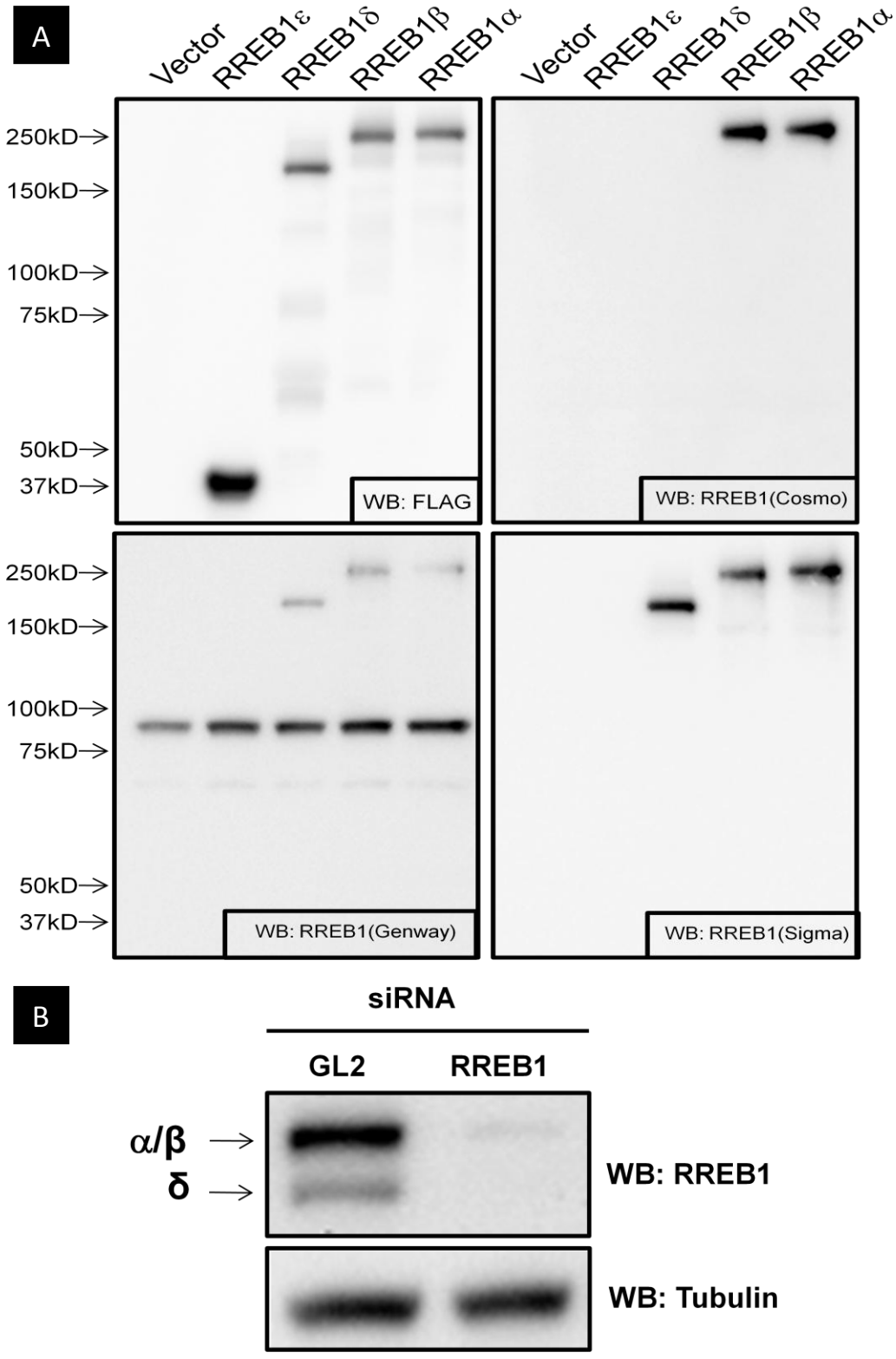

250.0
200.0
150.0
100.0
50.0
0.0
RNA Quanity (ng)
RREB1α
RREB1β
RREB1δ
BL1 N
BL1 T
BL2 N
BL2 T
BL3 N
BL3 T
BL4 N
BL4 T
BL5 N
BL5 T
BL6 N
BL6 T
BL7 N
BL7 T
BL8 N
BL8 T
BL9 N
BL9 T
BL10 N
BL10 T
PR1 N
PR1 T
PR2 N
PR2 T
PR3 N
PR3 T
PR4 N
PR4 T

Figure 10. Detection of RREB1 protein isoforms. A. RREB1 isoforms were either cloned out of the UMUC3 cell line (Isoforms δ and ε) or were a gift from Dr. Akiyoshi Fukimizu (Isoforms α and β). The splice variants were cloned into a C-terminal 3XFLAG tagged expression construct and were expressed in 293T cells. Detection with Anti-FLAG (1:1000), anti-RREB1 (Sigma) (1:1000), anti-RREB1 (Cosmo) (1:500), or anti-RREB1 (Genway) (1:1000) antibody is shown. **B.** RREB1 siRNA designed to knock down all splice variants was transfected into UMUC3 cells at 25nM. RREB1 antibody (Sigma) detection (1:1000) on western blot is shown.

Figure 10


A
Vector
RREB1ε
RREB1δ
RREB1β
RREB1α
Vector
RREB1ε
RREB1δ
RREB1β
RREB1α
250kD
150kD
100kD
75kD
50kD
37kD
WB: FLAG
WB: RREB1(Cosmo)
WB: RREB1(Genway)
WB: RREB1(Sigma)
B
siRNA
GL2
RREB1
α/β
δ
WB: RREB1
WB: Tubulin

Additional support for the antibody's specificity was provided by observing significant decreases in endogenous bands of appropriate sizes to the splice variants RREB1α, RREB1β, and RREB1δ upon treatment with siRNA to total RREB1 in UMUC3 cells (**Figure 10B**).

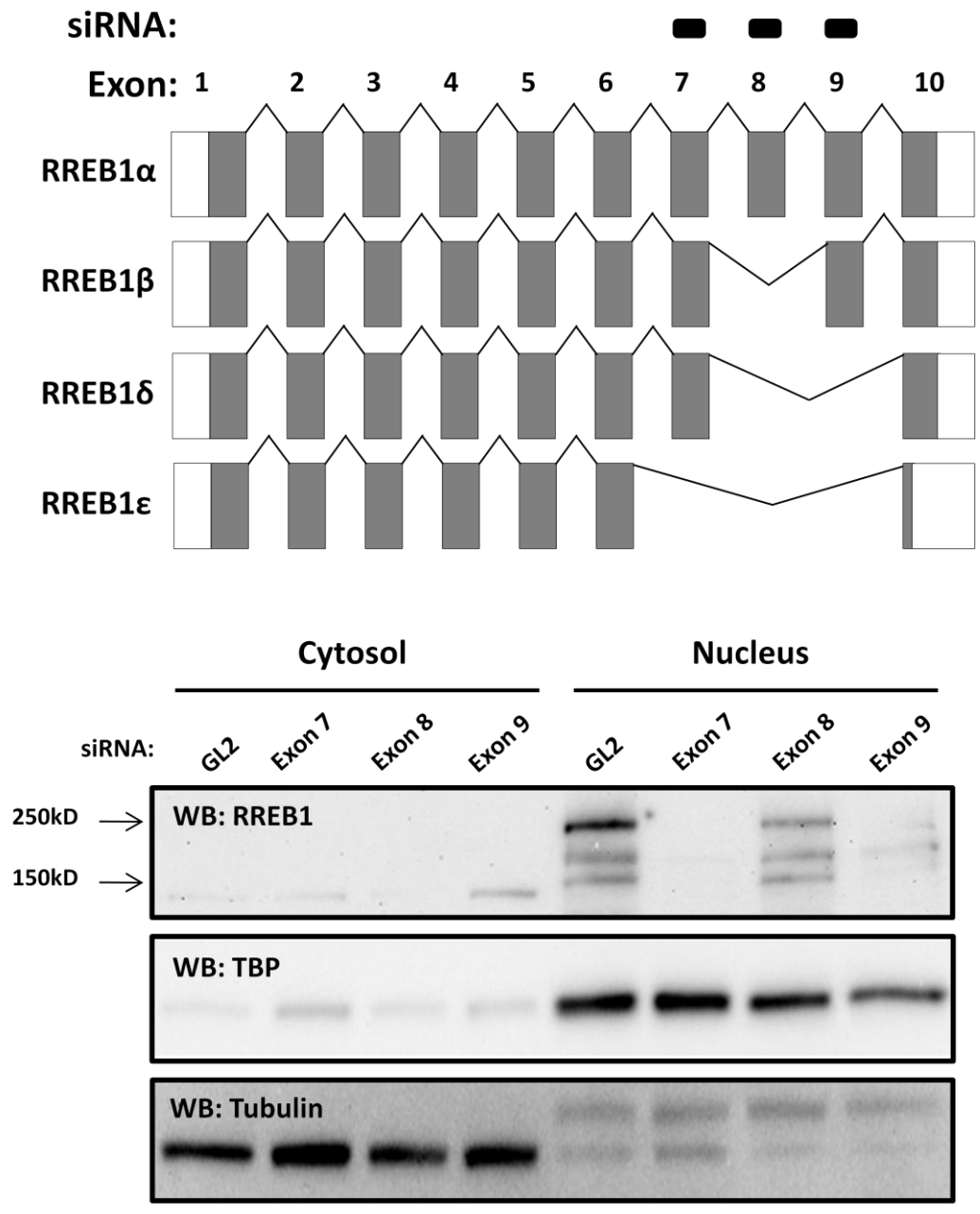
To connect protein bands on a western blot with RREB1 splice variants, we designed siRNAs targeting specific RREB1 exons. In parallel, the nuclei and cytosol were fractionated to examine splice variant localization. Despite observation of overexpressed RREB1 in nuclear bodies by immunofluorescence [98], no biochemical assay has been reported to confirm the localization of endogenous RREB1; much less individual splice variants. As seen in **Figure 11**, RREB1 primarily localizes to the nucleus. Tubulin and TATA box binding protein (TBP) were also blotted to estimate the purity of the cytosolic and nuclear fractions, respectively.

siRNA to Exon 8 knocks down the RREB1α and RREB1γ splice variants. Since RREB1γ mRNA was undetectable in UMUC3 cells we assumed all measurable protein depleted by siRNA to Exon 8 was RREB1α. As seen in **Figure 11**, Exon 8 siRNA decreased the intensity of the largest band, which, based on size (~250kD) is likely RREB1α. siRNA to Exon 9 depletes RREB1β in addition to RREB1α and resulted in an elimination of the largest RREB1 band, which due to a mere 3% difference in amino acid content between the splice variants, is likely both RREB1α and RREB1β. Surprisingly, a second band at ~150kD was also depleted with Exon 9 siRNA. This band could represent a

unique RREB1 isoform, either due to an unknown pre-mRNA splicing event or an alternative translation product from the RREB1β isoform. Finally, siRNA to Exon 7, which depletes RREB1α, RREB1β, and RREB1δ, eliminated the upper band of RREB1α and RREB1β and the second largest band (~200kD) which migrated at the same size as the RREB1δ transgene.

Figure 11. Detection of RREB1 protein isoforms using RREB1 exon specific siRNA. siRNA designed to exons 7, 8, and 9 was transfected into UMUC3 cells at 25nM. Cytoplasmic and nuclear fractions were isolated after 96 hours and RREB1 protein splice variants were detected on western blot (1:1000).

Figure 11


siRNA:
Exon: 1 2 3 4 5 6 7 8 9 10
RREB1α
RREB1β
RREB1δ
RREB1ε
Cytosol
Nucleus
siRNA:
GL2
Exon 7
Exon 8
Exon 9
GL2
Exon 7
Exon 8
Exon 9
250kD
150kD
WB: RREB1
WB: TBP
WB: Tubulin

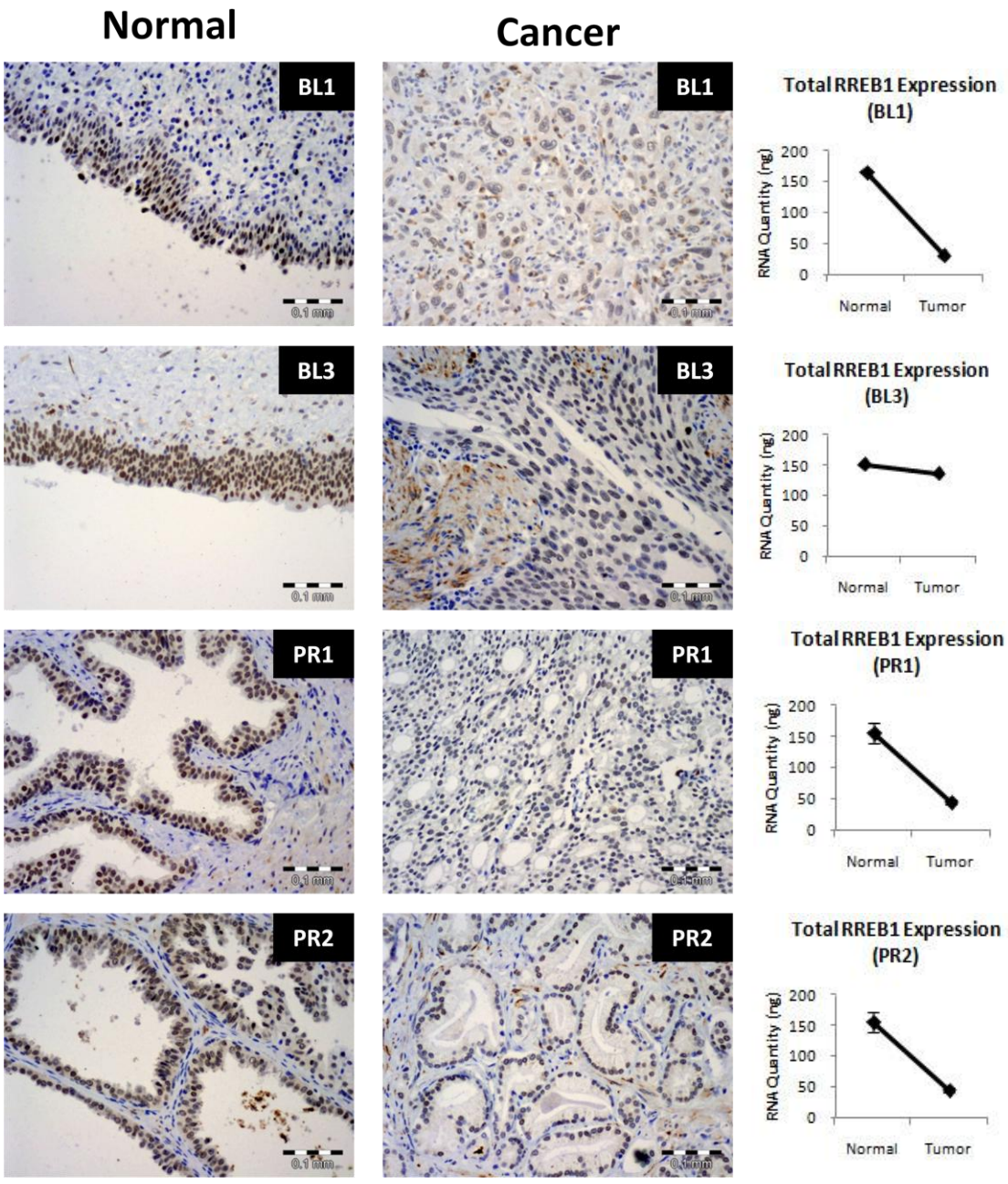
The presence of a cytosolic band around 140kD, that appears to disappear with siRNA to Exon 8, may also represent another unique RREB1 isoform that has yet to be identified. In conclusion, the Sigma-Aldrich RREB1 antibody showed the greatest sensitivity and specificity in detecting the exogenously expressed and endogenous isoforms RREB1α, RREB1β, and RREB1δ and was thus used in subsequent experiments.

2.4.4 Expression of RREB1 protein in normal and cancerous bladder and prostate cancer tissues

Having characterized the specificity of RREB1 antibodies, we were interested in performing immunohistochemical (IHC) staining to determine whether the expression patterns and subcellular localization we observed *in vitro* were relevant to RREB1 detected in intact, archival human tissue samples. Immunohistochemistry was carried out using the Sigma-Aldrich antibody on 2 samples of bladder cancer and 2 samples of prostate cancer, as well as adjacent non-neoplastic tissues from patient resection specimens. As we observed in our Q-RTPCR analysis of matched normal bladder mucosa and bladder tumors, we found a general decrease in RREB1 staining between each patient's matched normal bladder mucosa and invasive bladder tumors, as well as in patients' matched prostate normal prostate glands and prostate cancers (**Figure 12**). In general, nuclear expression of RREB1 was strongest. Though as we observed in cell line fractionation experiments, some cytoplasmic expression was detected, particularly in cases with stronger staining overall (**Figure 12**).

Figure 12. Immunohistochemistry of RREB1 shows a strong correlation to total RREB1 mRNA. RREB1 IHC (1:100) of two paired normal and cancer bladder (BL1 and BL3) and prostate (PR1 and PR2) tissues. Total RREB1 RNA levels from the same tissue are provided for reference.

Figure 12


Normal
Cancer
BL1
BL1
Total RREB1 Expression (BL1)
RNA Quantity (ng)
200
150
100
50
0
Normal
Tumor
BL3
BL3
Total RREB1 Expression (BL3)
PR1
PR1
Total RREB1 Expression (PR1)
PR2
PR2
Total RREB1 Expression (PR2)
0.1 mm

2.4.5 Significance of RREB1 expression in clinical and histopathologic disease states in bladder cancer.

To examine expression of RREB1 in a larger population, we used a tissue microarray reported before [154] to examine expression of RREB1 in 145 samples of human bladder cystectomy specimens. RREB1 expression was graded as negative, focal/low, moderate, and high; examples of each are shown (**Figure 13)**. RREB1 staining was not associated with pathologic tumor stage (pT) (P=0.43), node stage (pN) (P=0.69), or survival (P=0.86) (**Figure 14**). However, RREB1 was positively associated (P=0.001) with squamous cell carcinoma (SCC) of bladder; a subtype that comprises only 5% of bladder cancer incidence [157] (**Table 3** and **Figure 13**). There was a positive trend of RREB1 expression and the Adenocarcinoma variant of bladder cancer but the study lacked significant statistical power (n=7) to make a definitive assessment.

2.5 Discussion

Little consensus exists regarding the nature, expression, or function of RREB1 RNA and protein in cancer cells. We addressed this gap in the literature and characterized RREB1 mRNA and protein isoforms in bladder and prostate tumors. We were able to confirm expression of four previously described splicing events through DNA sequencing, which also served to clarify prior sequencing artifacts [96 98], and identify a novel RREB1 splice variant of significantly smaller

size. Characterization of currently available RREB1 antibodies failed to detect the RREB1ε isoform highlighting the need for further antibody development.

To avoid further confusion in the literature, we have proposed the following nomenclature to denote the RREB1 splice variants in the order of largest to smallest protein coding sequence: RREB1α, RREB1β, RREB1γ, RREB1δ, and RREB1ε.

Given its associations with cancer signaling, one might hypothesize that RREB1 is expressed at higher levels in tumors. Using Q-RTPCR and immunohistochemistry for RREB1 to examine its expression in matched normal and cancerous tissues of bladder and prostate cancer as well as by staining a large tissue microarray of bladder tumors, we found that overexpression of RREB1 was inversely associated with the transformed state. However, we observed that RREB1 was significantly associated with squamous cell carcinoma (SCC) of the bladder. This association was unexpected, and represents an exciting avenue for future research.

Even less is known concerning the expression of RREB1 proteins. We provide the first characterization of RREB1 isoform expression at the protein level, as well as provide an extensive workup of commercial antibodies to these proteins, using cloned RREB1 splice variants and RREB1 siRNA to determine their sensitivity and specificity. Additionally, we observed that more than one protein band could be attributed to RREB1β.

Figure 13. Immunohistochemistry of RREB1 on a bladder cancer tissue microarray reveals a high correlation with squamous cell carcinoma of the bladder. Archived bladder cancers (n=145) were assayed for RREB1 expression on a tissue microarray. RREB1 expression was graded as negative, low/focal, moderate, and high and examples of each level are provided. Squamous cell carcinoma (SCC) of the bladder was found to have a higher proportion of cases with increased RREB1 expression compared to urothelial carcinoma and an example of high RREB1 expression is provided.

Figure 13

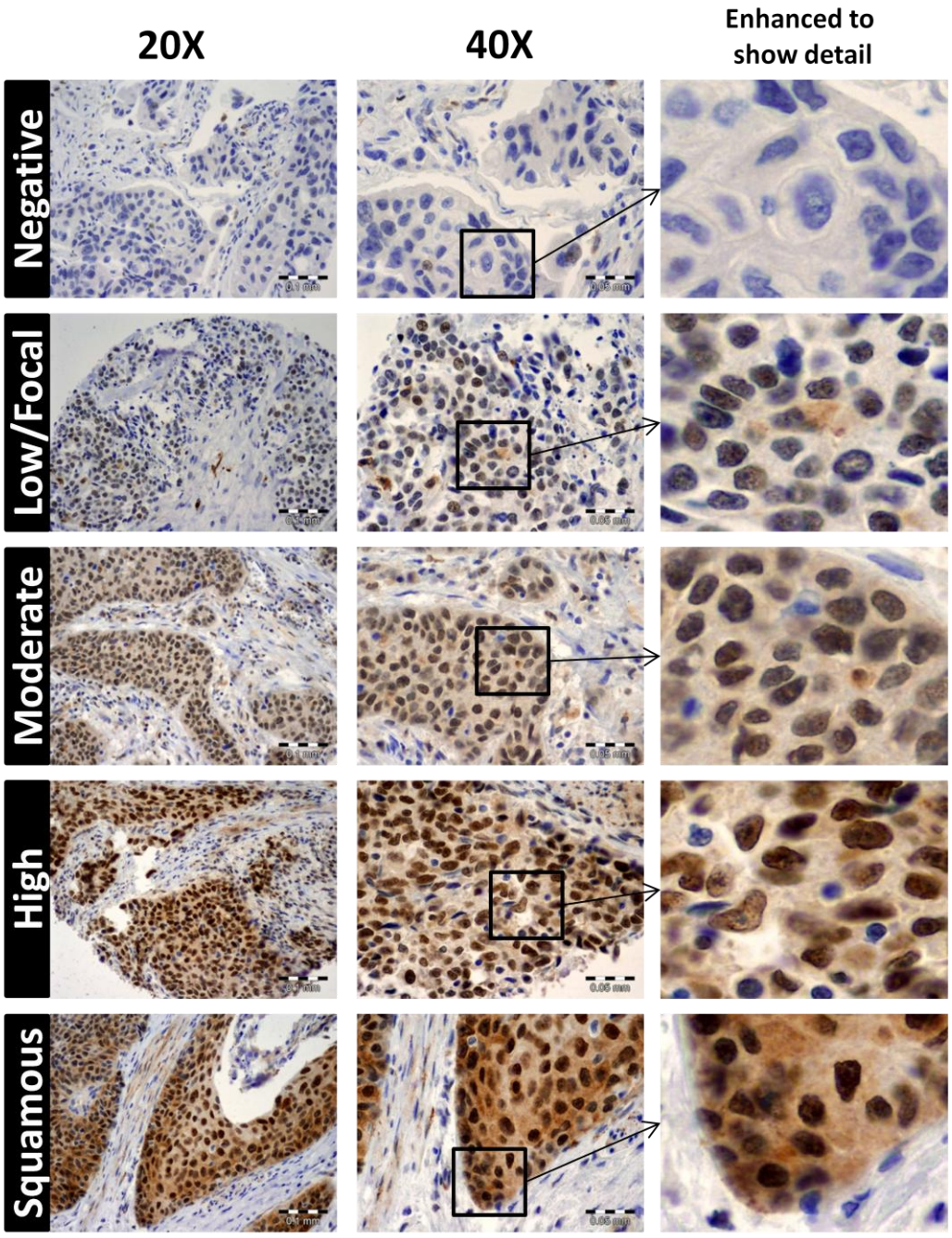

20X
40X
Enhanced to show detail
Negative
Low/Focal
Moderate
High
Squamous

Figure 14. RREB1 IHC staining does not correlate with clinical parameters of bladder cancer. A tissue microarray containing 145 unique bladder cancers was assayed for RREB1 IHC staining. Degrees of staining were classified as negative, focal/low, moderate, or high (**Figure 13**). RREB1 staining did not correlate with pathologic tumor stage (pT) (P=0.43), node stage (pN) (P=0.69), or survival (P=0.86).

Figure 14

RREB1 Staining *versus* Tumor Grade

Count, (%)

RREB1	Grade 2	Grade 3	Grade 4
Negative	2 (11.1)	10 (17.2)	10 (17.2)
Focal, Low	8 (44.4)	19 (32.8)	15 (32.8)
Moderate	6 (33.3)	25 (43.1)	16 (43.1)
High	2 (11.1)	4 (6.9)	8 (6.9)

X^2 P=0.62

RREB1 Staining *versus* Tumor Stage

RREB1	pTa, pT1	pT2	pT3	pT4
Negative	1 (11.1)	12 (25.5)	9 (14.1)	2 (8)
Focal, Low	4 (44.4)	15 (31.9)	19 (29.7)	11 (44)
Moderate	4 (44.4)	13 (27.7)	28 (43.8)	10 (40)
High	0 (0)	7 (14.9)	8 (12.5)	2 (8)

X^2 P=0.43

RREB1 Staining *versus* Nodal Metastasis

RREB1	pN0	pN1	pN2-3
Negative	17 (17)	4 (23.5)	0 (0)
Focal, Low	32 (32)	4 (23.5)	6 (42.9)
Moderate	39 (39)	7 (41.2)	6 (42.9)
High	12 (12)	2 (11.8)	2 (14.3)

X^2 P=0.69

RREB1 Staining *versus* Survival

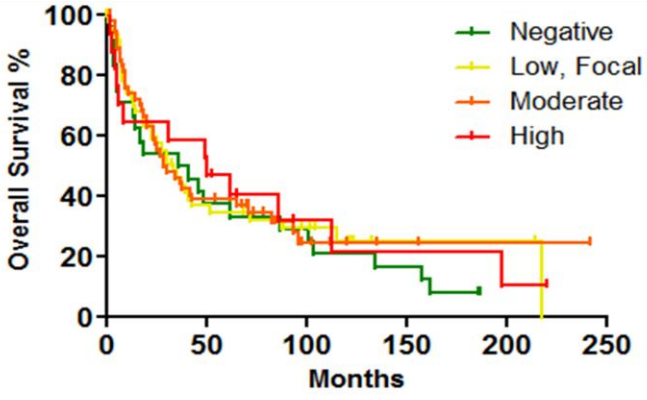


Table 3

Table 3. RREB1 Staining versus Variant of Bladder Carcinoma

RREB1IHC^	Adenocarcinoma	SCC	Urothelial
Negative	2 (28.6%)	0 (0%)	22 (19.5%)
Low, Focal	1 (14.3%)	5 (22.7%)	40 (35.4%)
Moderate	4 (57.1%)	10 (45.5%)	41 (36.3%)
High	0 (0%)	7 (31.8%)	10 (8.8%)
Total Cases	7	22	113

N=145$^{\pm}$, P=0.001*

*** P value for the Chi-Squared Test**

^ Semiquantitative scoring system per Materials and Methods

± Bladder carcinoma histology data were available for 142/145 cases where sufficient tissue was present for scoring.

Such a finding could be due to a still unknown RREB1 splice variant that results from an as yet uncharacterized alternative 5' promoter that excludes Exon 1, or it could result from alternative translation product of the RREB1β mRNA. With 25 in frame ATGs in the RREB1β mRNA, 21 of which have high predictive value as viable translation start sites (using NetStart 1.0 program, www.cbs.dtu.dk/services/NetStart/, data not shown), it is entirely possible that translation may proceed from more than one starting point within a single mRNA. We also observed that all RREB1 variants localized primarily to the nucleus, consistent with its function as a transcription factor, though sub-cellular localization of endogenous RREB1 had not been studied before.

More broadly, as advances in high throughput sequencing have made examination of the entire transcriptome possible, new data indicate that 95-100% of human pre-mRNAs from multi-exonic genes are alternatively spliced [158, 159]. Thus, alternative splicing can no longer be viewed as a biologic exception but rather must be assessed as an essential premise of study in evaluating the function of a gene product. The observations made in this study concerning the multiple gene products of RREB1 suggest that simply assaying the total expression of RREB1 could have led to confusing and limited snapshot of what appears to be far richer biology. Future studies of RREB1 thus must consider the functional consequences of manifold RREB1 transcripts and gene products with potentially divergent cellular functions, while the finding of RREB1 as a

mediator of core signaling pathways in bladder and prostate cancer justifies its careful elucidation.

2.6 Acknowledgements

The authors would like to thank Dr. Christopher Moskaluk of the Department of Pathology as well as the UVA Biotissue Repository Facility (BTRF) for their assistance in obtaining deidentified human clinical specimens for evaluation.

Chapter 3: The Role of RREB1 in Bladder Cancer

Matthew D. Nitz, Shibu Thomas, Michael A. Harding, Steven C. Smith, and Dan Theodorescu

Contributors

Matthew D. Nitz, UVA Department of Molecular Physiology and Biological Physics, designed and performed all experiments, analyzed the data, and wrote the manuscript.

Dr. Shibu Thomas, UVA Department of Urology, contributed substantially to the design of all mouse experiments.

Dr. Michael A. Harding, UVA Department of Urology, designed isoform specific RREB1 real time PCR primers, contributed substantially to the study design and preparation of the manuscript

Dr. Steven C. Smith, UVA Department of Urology, provided statistical expertise, contributed to the preparation of the manuscript.

Dr. Dan Theodorescu, UVA Department of Urology and Molecular Physiology and Biological Physics, provided resources, guidance, and advice on the design and interpretation of experiments.

3.1 Abstract

RREB1 is an alternatively spliced transcription factor that has been implicated in the pathology of urologic malignancies through interaction with androgen receptor, reduction of zinc transporters, and modulation by the oncogenic Ras pathway. However, no studies have examined the role of RREB1 in cancer phenotypes. Transient siRNA depletion of RREB1 led to decreased growth *in vitro* of bladder (UMUC3, KU7) and prostate (LNCaP, PC3) cancer cell lines while specific loss of RREB1α and RREB1β led to suppressed tumor formation *in vivo* of UMUC3 (P=0.001). However, attempts to rescue growth with individual or combinations of RREB1 isoforms were unsuccessful. Depletion of individual isoforms revealed an upregulation of the endogenous non-targeted isoforms, which were also incapable of rescuing cell growth. The RREB1 isoforms were stably overexpressed in UMUC3 cells but only RREB1δ was sufficient to significantly increase tumor volume *in vivo* (P=0.05), which, taken together, is the first report of *in vivo* loss or gain of function phenotypes for RREB1 in human cancer. In total this study provides evidence for the importance of RREB1 in the maintenance of proliferation in bladder and prostate cancer.

3.2 Introduction

Bladder and prostate cancer combine to cause over 41,000 deaths in the United States each year [2]. Current treatment strategy relies on early detection due to the nearly uniformly fatal prognosis of distant disease. Chemotherapeutic strategies aim to target the proliferation machinery of cancer cells so insights into these mechanisms could prove beneficial for future treatment regimens.

RREB1 is an alternatively spliced C_2H_2 zinc finger transcription factor that may play a role in bladder and prostate cancer. Originally reported to be a nuclear responsive element downstream of Ras in transformed thyroid cells [97], RREB1 activity has been shown to be responsive in bladder cancer cells to the Ras effector paralogs RalA and RalB [95]. The Ral (Ras-like) GTPase pathway downstream of Ras plays an important role in bladder cancer cell migration [53, 54]. In addition, depletion of both Ral paralogs (RalA and RalB) leads to decreased growth of bladder cancer cells [53].

In prostate cancer, RREB1 has been shown to bind the androgen receptor (AR) which leads to binding of the PSA promoter and decreased transcription [105]. Furthermore, RREB1 has been shown to bind the promoter of the zinc transporter hZIP1 and decrease its transcription [103]. These findings are important as AR cofactors have become an increasingly central topic of investigation in the field of androgen-independent prostate cancer [160] and the down regulation of hZIP1 has been demonstrated to be a critical event in the early development of prostate cancer [151].

Several studies have also shown that increased expression of RREB1 is associated and possibly causative in cancer development. $p19^{ARF}$ and p53 null mice were infected with the Moloney Murine Leukemia Virus (MuLV), a retrovirus that randomly inserts throughout the DNA and causes increased transcriptional activity in that region. Of the tumors developed in these mice, the RREB1 locus was one of a handful of genes found to be a common site of integration, implicating it as a possible oncogene [110]. Furthermore, the RREB1 gene locus has also been discovered to be a site of integration of the hepatitis B virus in hepatocellular carcinoma [109]. In melanoma, the human RREB1 locus has been found to be amplified and is currently an area of intense investigation as a potential molecular diagnostic test [111, 112 , 113-116].

Intriguingly, even with the aforementioned reports providing compelling grounds for further investigation of RREB1 in tumorigenesis; there is a dearth in the literature addressing this need. Therefore, we aim to determine the necessity of RREB1 in bladder and prostate cancer cell growth *in vitro* and the sufficiency of RREB1 to drive tumor growth *in vivo*. As we have previously shown, RREB1 is alternatively spliced with five known unique protein isoforms. Thus, our work on the role of RREB1 in cancer cell growth was performed with consideration to parsing out the function of these isoforms.

3.3 Materials and Methods

3.3.1 Cell Lines

Human cancer cell lines were grown in the following conditions: UMUC3, LUL2: Minimum essential media (MEM) (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco®, Invitrogen, Carlsbad, CA) and 1% sodium pyruvate (Gibco®, Invitrogen, Carlsbad, CA), KU7: MEM media supplemented with 10% FBS, 1% sodium pyruvate, and 1% non-essential amino acids (Gibco®, Invitrogen, Carlsbad, CA), LNCAP: RPMI medium 1640 (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% FBS, PC3: DMEM/F12 (1:1) (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% sodium pyruvate.

3.3.2 RNA isolation, Reverse transcription, and PCR

RNA isolation was carried out using an RNeasy® Mini Kit (Qiagen) and cDNA synthesis using iScript™ (Biorad). Q-RTPCR was performed on cDNA using the iQ™ SYBR® Green Supermix (Biorad). RNA quantification was performed as described [155] with RREB1 (total) used as the reference. Primers with annealing temperatures: RREB1 (total) F:5'-CTT-CCT-ATA-ACT-GCC-CCC-3'; R:5'-ATG-AGT-GGT-CGG-CTC-CTC-C-3'; RREB1α: F:5'-TGG-ATC-CCA-TGA-TAG-CAC-AGA-C-3'; R:5'-TGC-TCT-CTG-TCC-CGT-GAG-G-3'; RREB1β: F:5'-CAC-ATG-CTC-ACA-CAC-ACT-GAC-A-3'; R:5'-CCG-ACG-GCT-GCT-CTC-

TGT-3'; RREB1δ: F:5'-ACC-AAC-TGC-CTG-CAG-AAG-ATC-A-3'; R:5'-GTA-TGG-CCT-TTC-CCC-AGT-GTG-T-3'; RREB1ε: F:5'-TAC-AGA-ACA-ACC-CTT-CAA-TTC-CT-3'; R:5'-TAT-GGC-CTT-TCC-CCT-GAG-3'.

3.3.3 Cloning of RREB1

RREB1δ and RREB1ε were cloned out of UMUC3. Sequences were submitted to Genbank: RREB1δ (HM369361) and RREB1ε (HM369360). Total RNA was isolated as described above and cDNA synthesis using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) with 50μM random hexamers and 1μg of total RNA. The PCR reaction also included: 10X PfuUltra™ HF Reaction Buffer (Agilent), 100mM dNTP Mix (Agilent), 5% DMSO, 1M Betaine Solution (Sigma-Aldrich). The primers used were as follows: RREB1 Cloning: F:5'-GAT-CAT-CGA-TAT-GAC-GTC-AAG-TTC-GCC-C-3'; R:5'-GAT-CTC-TAG-ACT-CCA-TCC-CCA-CGA-GCT-G-3'. PCR products were isolated in a 1% agarose gel and purified using a Qiaquick® Gel Extraction Kit (Qiagen). Isolated products were digested at 37^{o}C by the enzymes ClaI and XbaI (New England Biolabs) and ligated into the p3XFLAG-CMV™-14 Expression Vector (Sigma-Aldrich). RREB1α and RREB1β were kindly provided by Dr. Akiyoshi Fukamizu (University of Tsukuba, Tsukuba, Japan) [96] and subcloned into the p3XFLAG-CMV™-14.

3.3.4 Antibodies, Immunoblotting, and IHC

The following antibodies were used: RREB1 (Sigma-Aldrich, Cat. no: HPA001756), FLAG (Sigma-Aldrich, Cat. no: F1804), Tubulin (Santa Cruz Biotechnology, Cat. no: sc-58668), TBP (TFIID) (Santa Cruz Biotechnology, Cat.

no: sc-204). Nuclear and cytoplasmic isolates were made with the NE-PER® kit (Thermo Scientific). Immunoblotting and detection were performed as described [53].

3.3.5 Transfection and siRNA

Transient vector transfection was performed using FuGENE®6 Tranfection Reagent (Roche) according to manufacturer instructions. Stable expression was achieved by cutting RREB1 expressing p3XFLAG-CMV™-14 vector with the ScaI restriction enzyme (New England Biolabs) and transfected with FuGENE®6. Selection was performed for 14 days in 800µg/ml of G418 (Geneticin®, Invitrogen). Oligofectamine™ (Invitrogen) was used to transiently transfect siRNA according to manufacturer instructions. All siRNA was transfected at a final concentration of 25nM unless otherwise noted: RREB1 total 5'-GGA-GUU-UGU-UUG-CAA-GUA-U-3' and 5'-GUU-CAG-ACC-UAU-CUU-CCA-U-3' (used in combination at 12.5nM), GL2 5'-CGUACGCGGAAUACUUCGAdTdT-3', RREB1 Exon 7-1 5'-CCA-ACA-AGU-UCA-GUC-CGU-U-3', RREB1 Exon 7-2 5'-GAC-UAC-AUC-GCC-GCC-GACA-dTdT-3', RREB1 Exon 8-1 5'-CCU-GAG-AAG-AAA-CGG-GCU-UUU-3', RREB1 Exon 8-2 5'-CGC-AAA-CAC-GGA-GUU-ACC-ACC-UGU-U-3', RREB1 Exon 8-3 5'-GAU-GUU-GGA-UCC-CAU-GAU-AUU-3', RREB1 Exon 9-1 5'-CAG-AGA-AGA-GCG-ACG-AUG-AdTdT-3', RREB1 Exon 9-2 5'-CCA-CCA-AGC-UCA-UGG-ACU-UUU-3', RREB1 Exon 9-3 5'-GGA-AGA-AGG-UCU-GCA-GCG-UdTdT-3'.

3.3.6 *In vitro* and *in vivo* cell growth assays, Cell Migration

In vitro cell growth assays were carried out by using Alamar Blue (Invitrogen) fluorescence emission as described previously [152]. The subcutaneous tumorigenicity of $5x10^5$ (RREB1 siRNA) or $1x10^6$ (RREB1 isoform stables) UMUC3 cells per site were evaluated as described previously {Wu, 2007 #52}. 72 hours after transfection after siRNA transfection, cells were harvested, counted in a hemacytometer, and resuspended in serum-free media. Cells (20,000) were added in triplicate to the upper chambers of transwell filters (8.0 Am pores, Becton Dickinson, Franklin Lakes, NJ) in a 24-well tissue culture plate. The lower chambers contained media with 2% FBS. Plating control assays were done in triplicate in adjacent wells containing the same media but with no transwell filters. After 6 hours (for UMUC-3) cells remaining on the upper surface of the filters were removed with cotton swabs and cells on the lower surface were fixed with 100% methanol, stained with crystal violet, and counted with the aid of a gridded coverslip. Cell numbers in plating control assay wells were estimated using CyQuant (Molecular Probes, Eugene, OR) according to manufacturer's instructions.

3.4 Results

3.4.1 Growth and migration of bladder and prostate cancer cell lines is dependent on RREB1

While RREB1 has been implicated in cancer by virtue of regulating key genes involved in urologic cancer such as the p16 tumor suppressor [104] and androgen receptor [105] a direct test of the functional relevance of this gene in bladder or prostate cancer cells had not been evaluated. Given a prior report that in MCF-10A breast epithelial cells depletion of RREB1 expression abrogated cellular motility and spreading [108], we hypothesized that RREB1 would also regulate migration in bladder cancer. UMUC3 and LUL2 cells were transiently depleted of RREB1 and assayed for serum-stimulated transwell migration. Consistent with the prior findings RREB1 depletion decreased transwell migration 70% in UMUC3 (P=0.038) and 79% in LUL2 (P=0.022) (**Figure 15A**).

To determine if RREB1 had an effect on cell growth in addition to migration, we treated bladder and prostate cancer cell lines *in vitro* with siRNAs designed to deplete all RREB1 variants. We observed significant decreases of *in vitro* proliferation of the bladder cancer cell lines UMUC3 ($P=6.0 \times 10^{-5}$) and KU7 ($P=4.3 \times 10^{-4}$), and the prostate cancer cell lines LNCAP ($P=2.7 \times 10^{-4}$) and PC3 (1.1×10^{-4}) (**Figure 15B**). These findings suggest RREB1, though not overexpressed in bladder or prostate tumors (as demonstrated in **Chapter 2**, **Figure 9A**), may still be necessary for key cancer phenotypes; begging the

question of which splice variants mediate the former, which we investigated below.

3.4.2 Rescue of UMUC3 growth due to RREB1 knockdown with RREB1 splice variants.

Having shown that RREB1 was necessary for growth *in vitro*, we hypothesized that cell line growth was dependent on a single splice variant. UMUC3 cells were stably transfected with RREB1α, RREB1β, RREB1δ, and RREB1ε and siRNA was designed to the 3' untranslated region (UTR) of the mRNA. In this model, the 3' UTR siRNA would deplete endogenous RREB1 while the stable RREB1 isoform transgenes, containing only the coding sequences, would continue to express. UMUC3 cells were used as they were demonstrated to be the most sensitive to RREB1 knockdown (**Figure 15B**). Cells were treated with 12.5nM of two independent siRNA duplexes designed to separate regions of the reported RREB1 3'UTR to decrease the possibility of non-specific hybridization of the siRNAs. Expression of individual RREB1 isoforms in UMUC3 was not sufficient to rescue loss of total endogenous RREB1 (**Figure 16A**).

Figure 15. Loss of RREB1 decreases the cell migration and growth of bladder and prostate cancer cell lines. **A.** 72 hours after treatment with control (GL2) or RREB1 siRNA, UMUC3 and LUL2 bladder cancer cells were harvested and equal numbers were plated in 0.5% serum media in the upper well of a Boyden chamber, with 2% serum media in the lower well. Six hours after plating, cells migrating to the lower chamber were counted. RREB1 knockdown significantly decreased serum induced migration in UMUC3 (P=0.038) and LUL2 (0.022) cells. **B.** Bladder (UMUC3 and KU7) and Prostate (LNCAP and PC3) cancer cell lines were transfected with control (GL2) and RREB1 siRNA at 25nM. Growth was measured every 24hrs up to 120 hours using the Alamar Blue assay. RREB1Western blots (1:1000) were used to confirm knock down of RREB1 protein. A student's ttest was used to determine significance of the growth differential with the following P values: UMUC3: $P=6.0 \times 10^{-5}$, KU7: $P=4.3 \times 10^{-4}$, LNCAP: $P=2.7 \times 10^{-4}$, PC3: $P=1.1 \times 10^{-4}$.

Figure 15

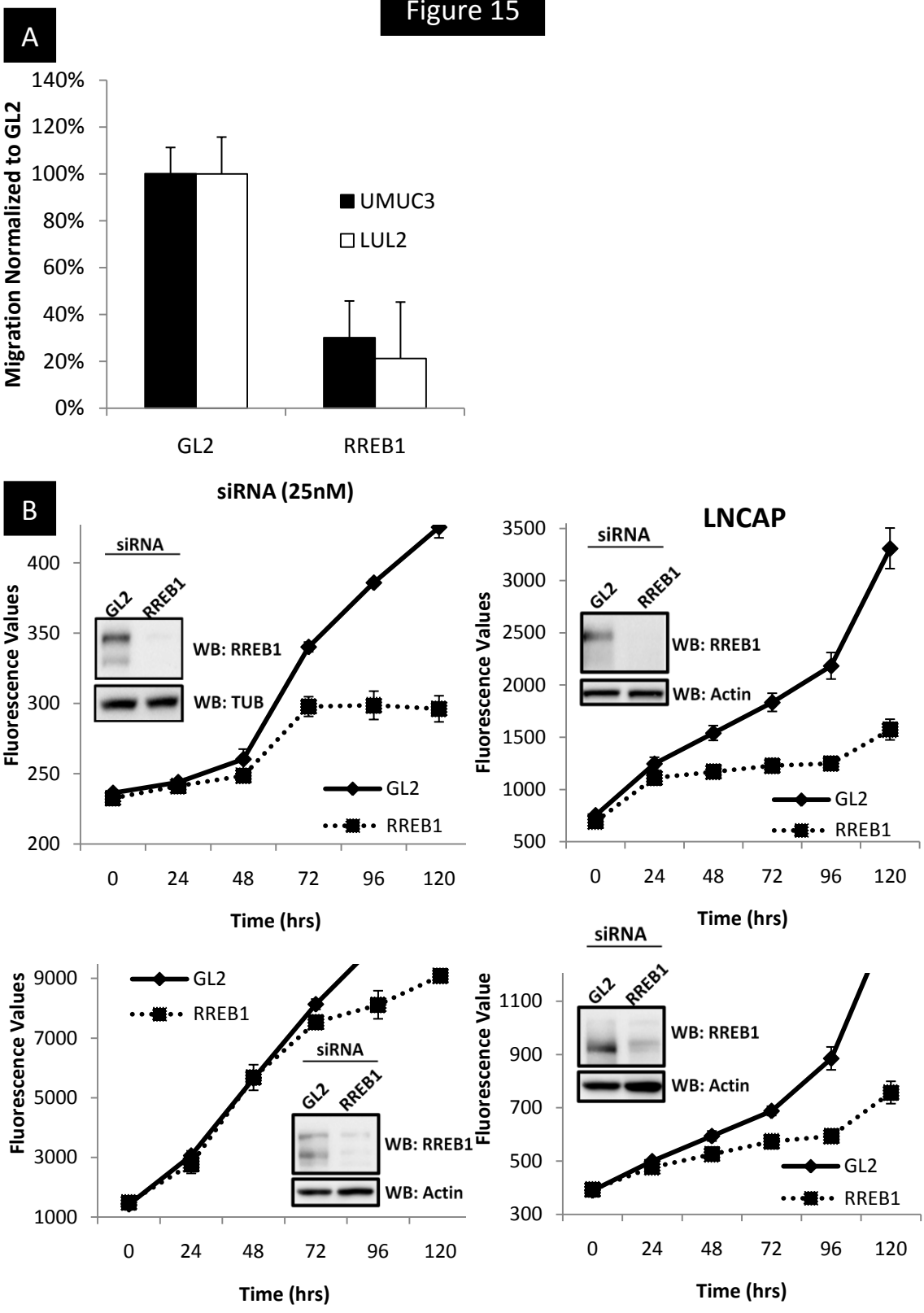
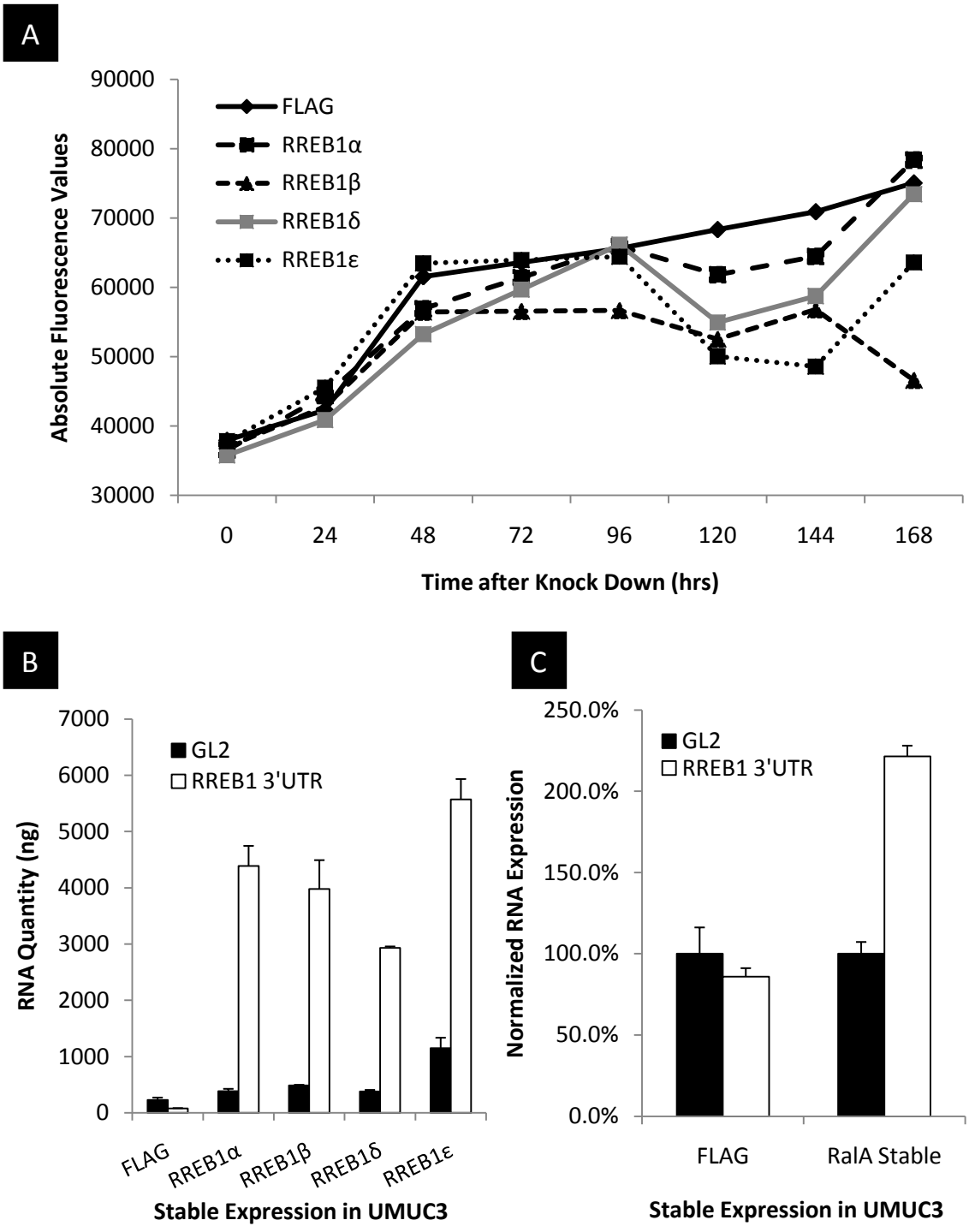
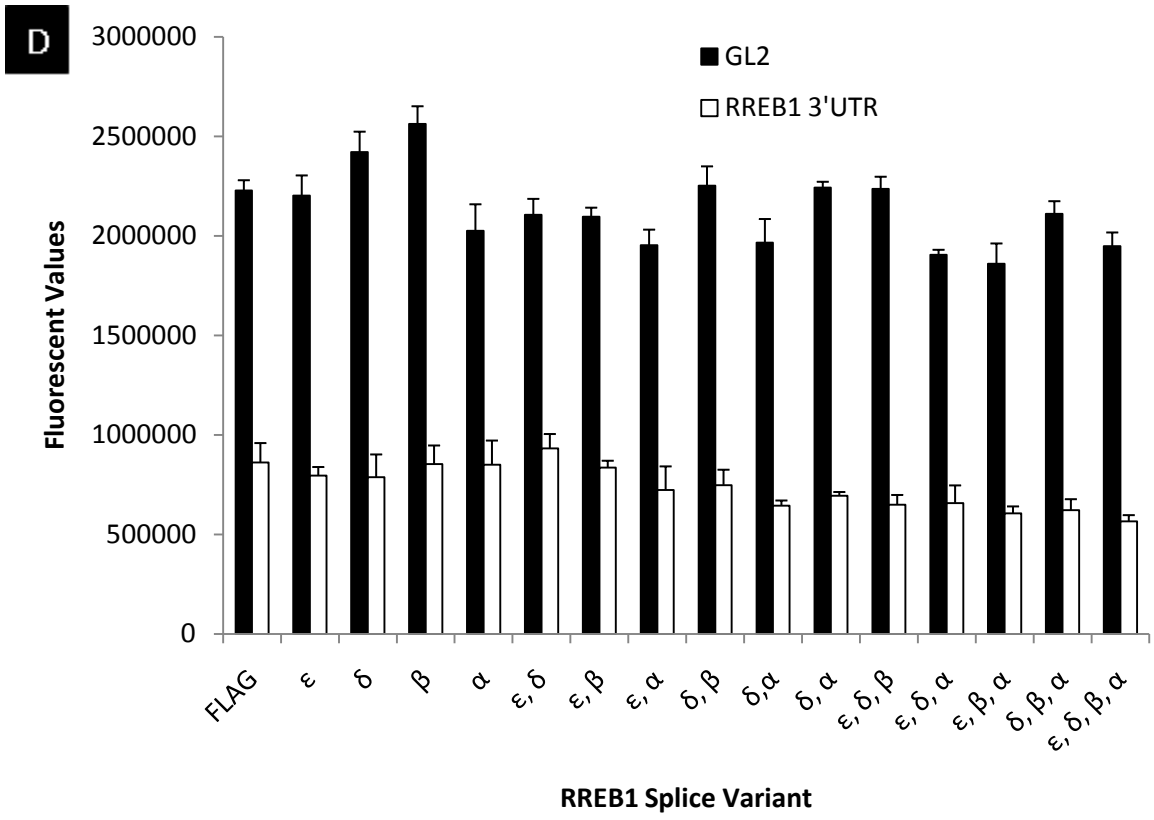

A
Migration Normalized to GL2
140%
120%
100%
80%
60%
40%
20%
0%
UMUC3
LUL2
GL2
RREB1
B
siRNA (25nM)
siRNA
GL2
RREB1
WB: RREB1
WB: TUB
Fluorescence Values
Time (hrs)
LNCAP
WB: Actin
GL2
RREB1

Figure 16. Over expression of RREB1 splice variants does not rescue RREB1 siRNa knockdown. A. RREB1 splice variants were stably transfected in UMUC3 cells and then treated with RREB1 3'UTR siRNA, which is designed to target the endogenous RREB1 expression but not the transgenes. Growth was measured every 24 hours after knockdown. **B.** Q-RTPCR of total RREB1 after control (GL2) or RREB1 3'UTR siRNA treatment (25nM) in UMUC3 cells stably expressing the RREB1 isoforms. **C.** Expression of RalA after treatment of UMUC3 cells stably expressing RalA (same vector as RREB1 isoform stables) with control (GL2) or RREB1 3'UTR siRNA (25nM). **D.** All permutations of RREB1 isoforms were transfected into UMUC3 cells 48 hours after treatment with RREB1 3'UTR siRNA. Growth was measured 96 hours after knock down.

Figure 16


A
90000
80000
70000
60000
50000
40000
30000
Absolute Fluorescence Values
FLAG
RREB1α
RREB1β
RREB1δ
RREB1ε
0
24
48
72
96
120
144
168
Time after Knock Down (hrs)
B
7000
6000
5000
4000
3000
2000
1000
0
RNA Quantity (ng)
GL2
RREB1 3'UTR
FLAG
RREB1α
RREB1β
RREB1δ
RREB1ε
Stable Expression in UMUC3
C
250.0%
200.0%
150.0%
100.0%
50.0%
0.0%
Normalized RNA Expression
GL2
RREB1 3'UTR
FLAG
RalA Stable
Stable Expression in UMUC3


D
3000000
2500000
2000000
1500000
1000000
500000
0
Fluorescent Values
GL2
RREB1 3'UTR
FLAG
ε
δ
β
α
ε, δ
ε, β
ε, α
δ, β
δ,α
δ, α
ε, δ, β
ε, δ, α
ε, β, α
δ, β, α
ε, δ, β, α
RREB1 Splice Variant

Real time PCR for total RREB1 expression after RREB1 3'UTR siRNA knockdown shows that in the stable isoform cells total RREB1 expression greatly increased (**Figure 16B**). This result confirmed the retention of RREB1 isoform transgene expression after siRNA treatment. However, these results also indicate that a single RREB1 isoform is most likely not responsible for UMUC3 growth. Intriguingly, we noticed that not only did RREB1 expression in the stable overexpressors not decrease, but it went up as much as 10 fold. To determine if endogenous RREB1 exhibited a negative feedback on RREB1 transgene expression, we knocked down endogenous RREB1 in UMUC3 cells with stable RalA expression of the same vector backbone as RREB1. As seen in **Figure 16C**, RalA expression increased after RREB1 knockdown whereas endogenous RalA expression does not change (**Figure 16C**). This result led us to conclude that endogenous RREB1 is negatively regulating the CMV promoter of the 3X-FLAG pCMV-14 vector of RREB1.

Despite the failure to rescue growth individually, the possibility remained that the RREB1 isoforms could work in combination to promote cell growth. Therefore, we transfected every permutation of RREB1 isoforms 48hrs after RREB1 3'UTR knock down. As seen in **Figure 16D**, no combination of RREB1 isoforms was sufficient to rescue growth after knockdown of endogenous protein.

3.4.3 Regulation of proliferation *in vitro* by RREB1

To determine the isoform specificity of the RREB1 growth phenotype, we tested isoform-specific siRNAs in depletion experiments in UMUC3. siRNA to

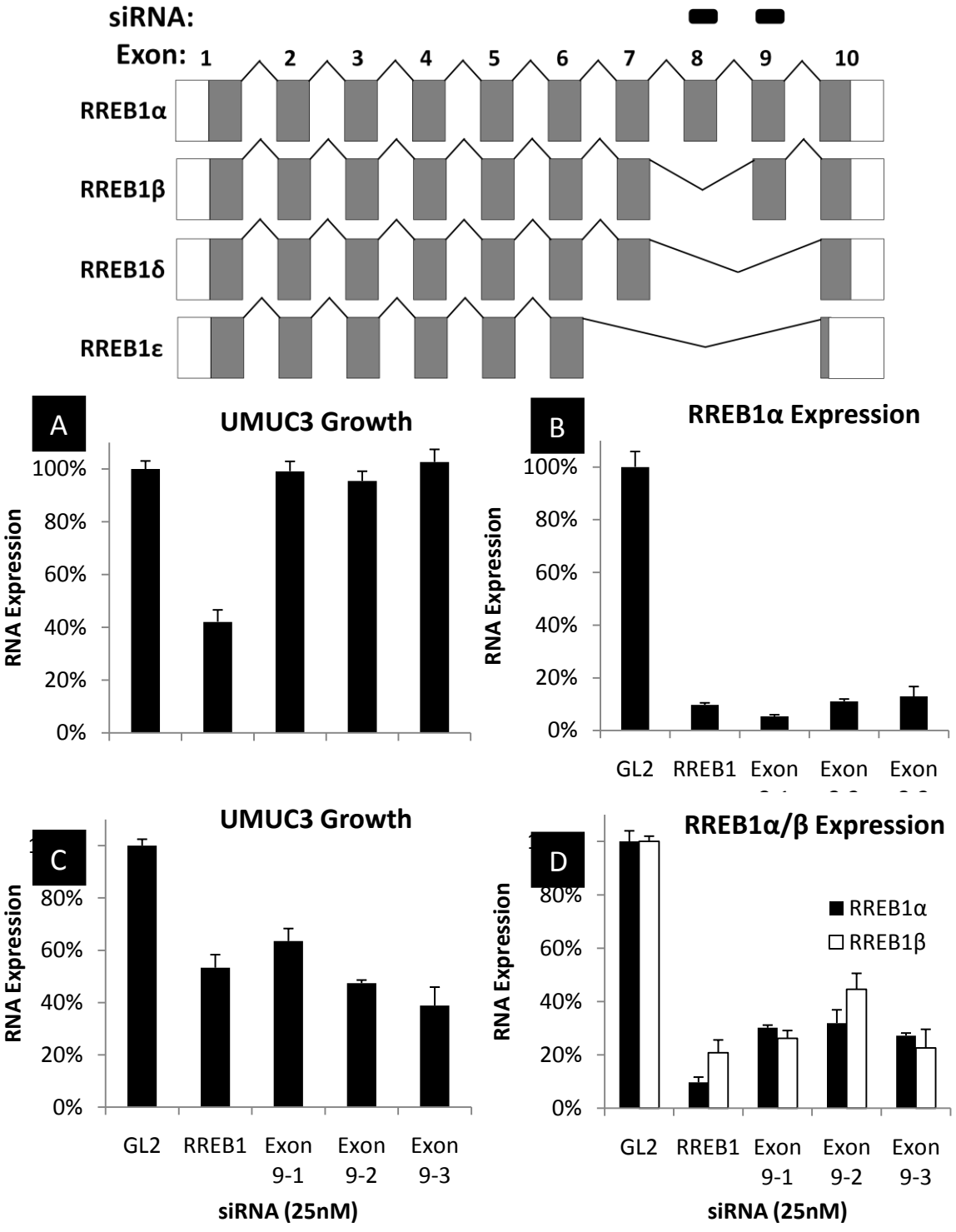
Exon 8, which targets RREB1α showed no decrease in UMUC3 growth *in vitro* (**Figure 17A**). Q-RTPCR for RREB1α was performed to judge the extent of knockdown and showed >80% loss of expression (**Figure 17B**).

To determine if this effect was specific, we designed two additional siRNAs to separate regions within Exon 8 (Exon 8-2 and Exon 8-3). **Figure 17A** shows that the additional siRNAs had nearly identical effects on UMUC3 growth while maintaining efficient knockdown of RREB1α (**Figure 17B**).

However, siRNA to Exon 9, which knocks down RREB1β in addition to RREB1α, caused a nearly identical decrease in growth compared to siRNAs targeting all RREB1 splice variants (**Figure 17C**). Isoform specific Q-RTPCR showed that RREB1α and RREB1β were robustly depleted in these cells (**Figure 17D**). Two additional siRNAs were designed to unique regions within Exon 9 to confirm the specificity of these phenotypic effects. The additional siRNAs targeted to Exon 9, labeled 9-2 and 9-3, showed identical results on UMUC3 growth (**Figure 17C**).

Figure 17. siRNA to exon 9 decreases UMUC3 growth *in vitro*. A. UMUC3 cells were treated with 25nM siRNA of control (GL2), RREB1 (all isoforms), and three unique siRNAs to exon 8 (8-1, 8-2 and 8-3). 96 hours after transfection growth was measured by Alamar Blue and real time PCR was performed to confirm knock down of RREB1α. **B.** UMUC3 cells were treated with 25nM siRNA of control (GL2), RREB1 (all isoforms), and three unique siRNAs to exon 9 (9-1, 9-2, and 9-3). RREB1α and RREB1β specific real time PCR was used to measure RREB1α and RREB1β knock down.

Figure 17

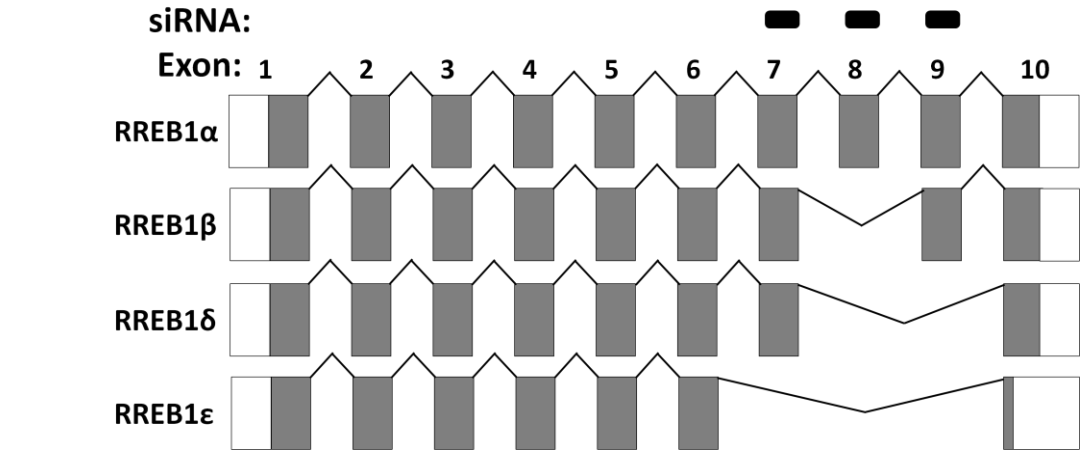

siRNA:
Exon: 1 2 3 4 5 6 7 8 9 10
RREB1α
RREB1β
RREB1δ
RREB1ε
A
UMUC3 Growth
RNA Expression
100%
80%
60%
40%
20%
0%
B
RREB1α Expression
GL2
RREB1
Exon
C
UMUC3 Growth
GL2
RREB1
Exon 9-1
Exon 9-2
Exon 9-3
siRNA (25nM)
D
RREB1α/β Expression
RREB1α
RREB1β

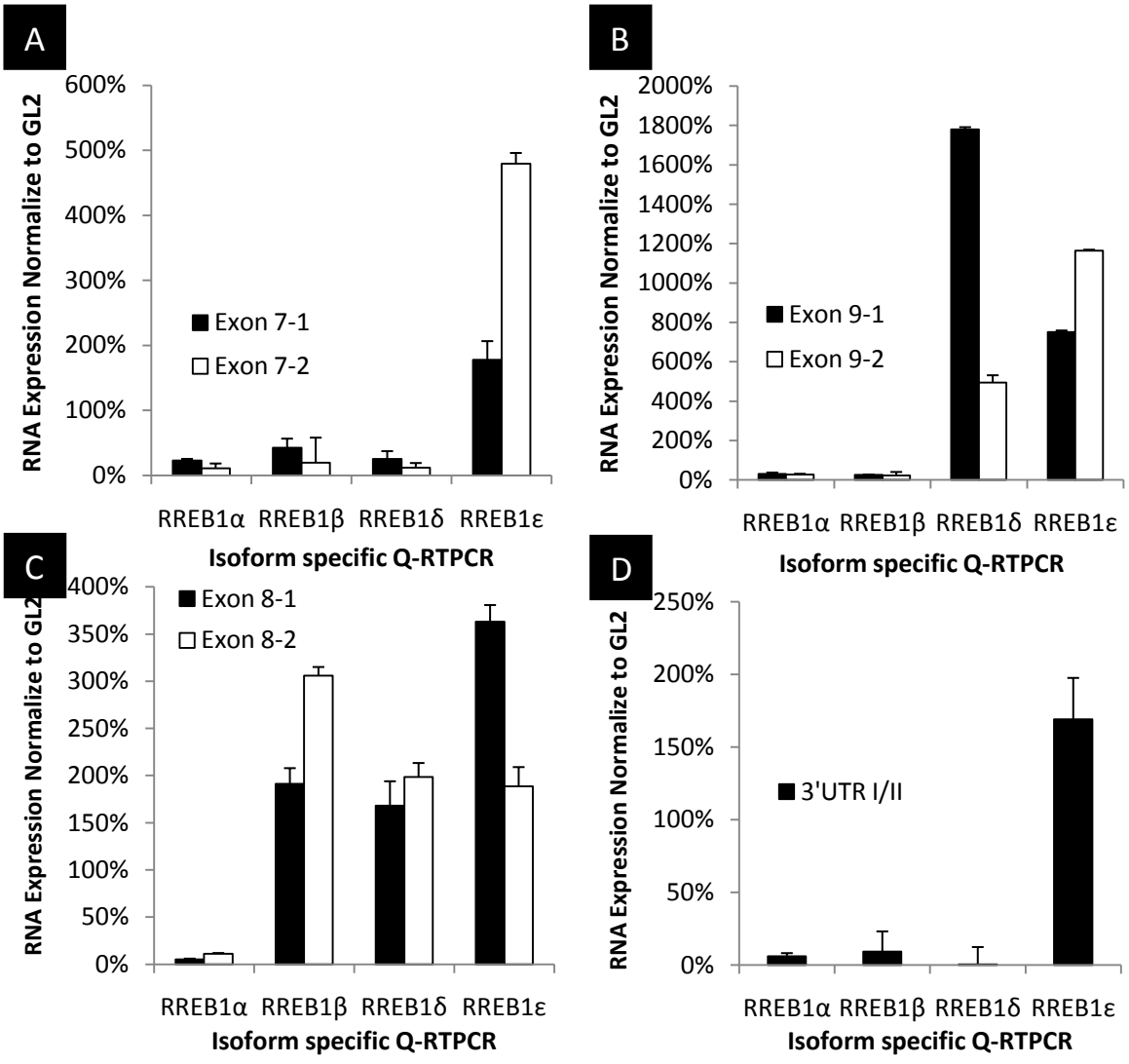
3.4.4 Upregulation non-targeted RREB1 isoforms after treatment with siRNA

As shown in **Figures 17B** and **17D**, Exon 8 and Exon 9 siRNA potently deplete UMUC3 cells of RREB1α and RREB1α/β, respectively. However, an unexpected finding seen in **Figure 18** shows the expression of isoforms not targeted by these siRNAs increase. Using siRNA designed to each of the exons found to be involved in alternative splicing, UMUC3 cells were treated for 96 hours prior to harvesting of the RNA. RREB1 isoform specific Q-RTPCR, described earlier, was used to uncover this effect. Each exon specific siRNA was repeated with a unique duplex to ensure reproducibility and specificity of the knockdown. These findings reveal the reduction in growth after depletion of RREB1α and RREB1β (**Figure 17C**) cannot be rescued by an endogenous upregulation of RREB1δ and RREB1ε. Furthermore, these findings appear to be a novel observation that could provide evidence concerning the mechanisms of alternative splicing.

Figure 18. Upregulation of targeted RREB1 isoforms after RREB1 exon specific knockdown. A. UMUC3 cells were transfected with two siRNA duplexes at 25nM that target unique sites within Exon 7 of RREB1. Exon 7 siRNA targets isoforms RREB1α, RREB1β, and RREB1δ. Q-RTPCR was used to measure isoform expression 96 hours after knockdown. **B.** UMUC3 cells were transfected with two siRNA duplexes at 25nM that target unique sites within Exon 9 of RREB1. Exon 9 siRNA targets isoforms RREB1α and RREB1β. Q-RTPCR was used to measure isoform expression 96 hours after knockdown. **C.** UMUC3 cells were transfected with two siRNA duplexes at 25nM that target unique sites within Exon 8 of RREB1. Exon 8 siRNA targets isoform RREB1α. Q-RTPCR was used to measure isoform expression 96 hours after knockdown. **D.** UMUC3 cells were co-transfected with two siRNA duplexes at 12.5nM each that target unique sites within the reported 3'UTR of RREB1 that were believed to target all RREB1 isoform. Q-RTPCR was used to measure isoform expression 96 hours after knockdown.

Figure 18


siRNA:
Exon: 1 2 3 4 5 6 7 8 9 10
RREB1α
RREB1β
RREB1δ
RREB1ε


A
RNA Expression Normalize to GL2
600%
500%
400%
300%
200%
100%
0%
Exon 7-1
Exon 7-2
RREB1α RREB1β RREB1δ RREB1ε
Isoform specific Q-RTPCR
B
RNA Expression Normalize to GL2
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
Exon 9-1
Exon 9-2
RREB1α RREB1β RREB1δ RREB1ε
Isoform specific Q-RTPCR
C
RNA Expression Normalize to GL2
400%
350%
300%
250%
200%
150%
100%
50%
0%
Exon 8-1
Exon 8-2
RREB1α RREB1β RREB1δ RREB1ε
Isoform specific Q-RTPCR
D
RNA Expression Normalize to GL2
250%
200%
150%
100%
50%
0%
3'UTR I/II
RREB1α RREB1β RREB1δ RREB1ε
Isoform specific Q-RTPCR

3.4.5 Regulation of proliferation *in vivo* by RREB1

Intrigued by the decrease of *in vitro* growth due to loss of RREB1α and RREB1β, we tested the effect of depleting RREB1α alone or both RREB1α and RREB1β on *in vivo,* subcutaneous, xenograft tumor growth. For these assays, we used transient depletion of RREB1 to assay kinetics of tumor formation, as has been reported for other genes in the past [161]. UMUC3 cells treated with non-targeting (luciferase) GL2 control, Exon 8 (RREB1β, or Exon 9 (RREB1α and RREB1β) siRNAs, and equal numbers of cells were injected subcutaneously 24 hours post-transfection. As expected, *in vitro* growth showed no difference with siRNA to Exon 8 (RREB1α) (P=0.46) but a negative growth effect with Exon 9 (RREB1α and RREB1β) ($P=1.4x10^{-9}$) (**Figure 19A**). We observed that depletion of RREB1α and RREB1β, but not RREB1α alone, had a significant negative effect on incidence (9/10 GL2 vs. 1/10 Exon 9, P=0.001) (**Fig. 19B**) and subcutaneous tumor size (P=0.005) (**Figure 19C**), which we interpret as supportive of an important role for RREB1 in tumorigenesis. Interestingly, though loss of RREB1α did not have a significant effect on *in vitro* growth or tumor incidence, it had a measureable, though not significant, negative effect on average tumor size (P=0.22) (**Figure 19C**). Tumors representative of each cohort are shown in **Figure 19D**.

Having observed that RREB1α and RREB1β were necessary for tumor growth *in vivo* we then asked whether RREB1 was sufficient to promote subcutaneous tumorigenesis. UMUC3 cells stably expressing the RREB1

isoforms were injected subcutaneously into nude mice. Only RREB1δ significantly increased UMUC3 average tumor volume compared to vector control (4,507mm^3 vs. 3,248mm^3, P=0.05) (**Figures 20A** and **20B**) while RREB1ε tumors showed a non-significant increase (3,802mm^3, P=0.16). RREB1α and RREB1β showed no change in tumor volume from the vector control (P=0.41 and P=0.38, respectively) (**Figures 20C and 20D**).

Figure 19. Loss of RREB1α and RREB1β decrease UMUC3 tumor formation *in vivo*. A. Populations of UMUC3 cells used for subcutaneous tumor growth were monitored for *in vitro* cell growth by the Alamar Blue Assay. Exon 8 (P=0.46) was not different 6 days post transfection but Exon 9 knockdown was highly significant (** $P=1.4x10^{-9}$). **B.** UMUC3 cells were transfected with 25nM of GL2 (control), RREB1 Exon 8, or RREB1 Exon 9 siRNA 24 hours prior to subcutaneous injection in nude mice. Palpable tumor formation was monitored twice a week and Exon 9 siRNA showed significantly less tumors than the GL2 control (P=0.001). **C.** The average tumor size for all injection sites were measured twice a week. Exon 9 knockdown showed a significant negative effect on average tumor size (** P=0.005) whereas Exon 8 did not (P=0.22). **D.** Representative tumors of each group are shown. The Exon 9 siRNA group only developed 1 tumor out of 10 injection sites.

Figure 19

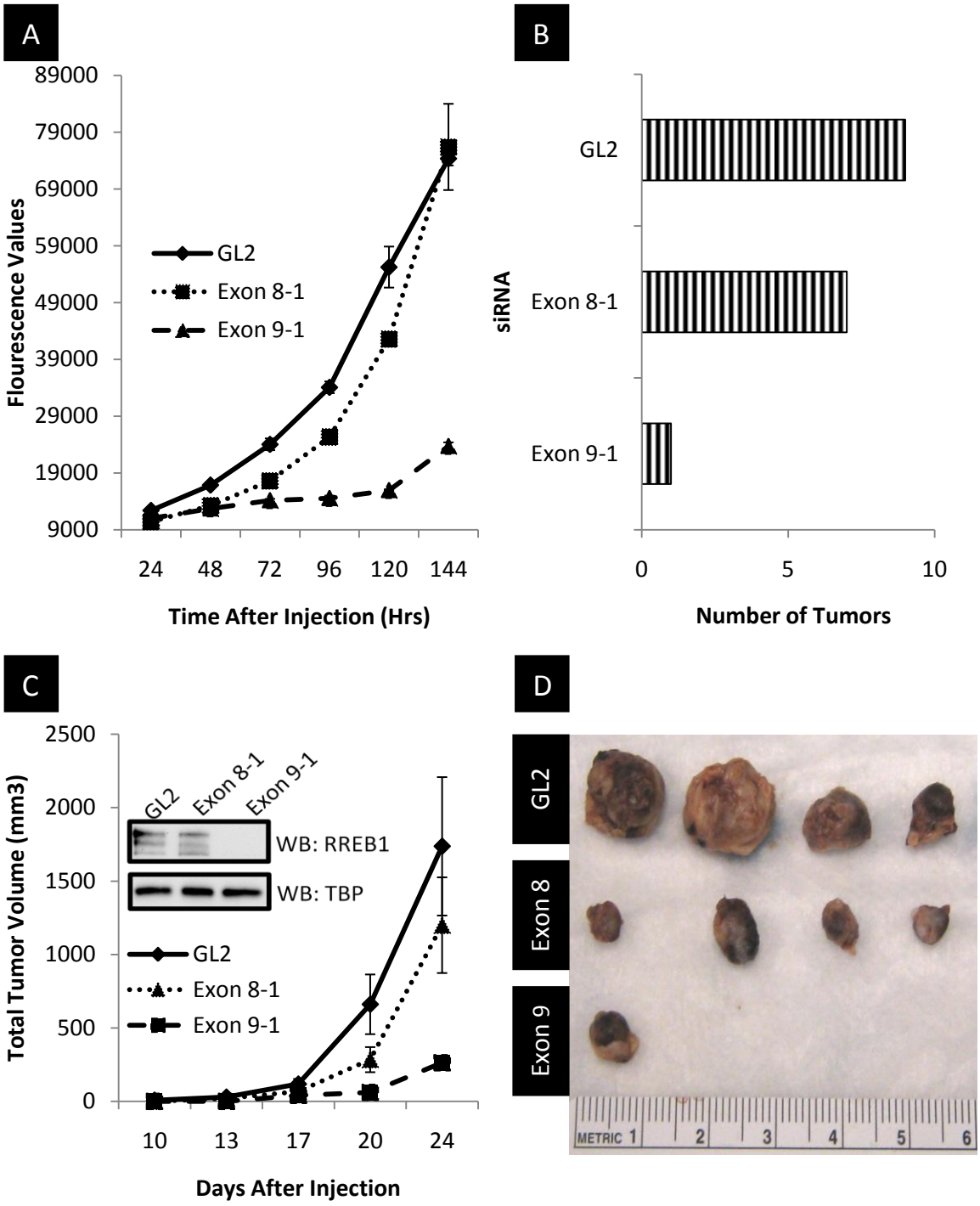
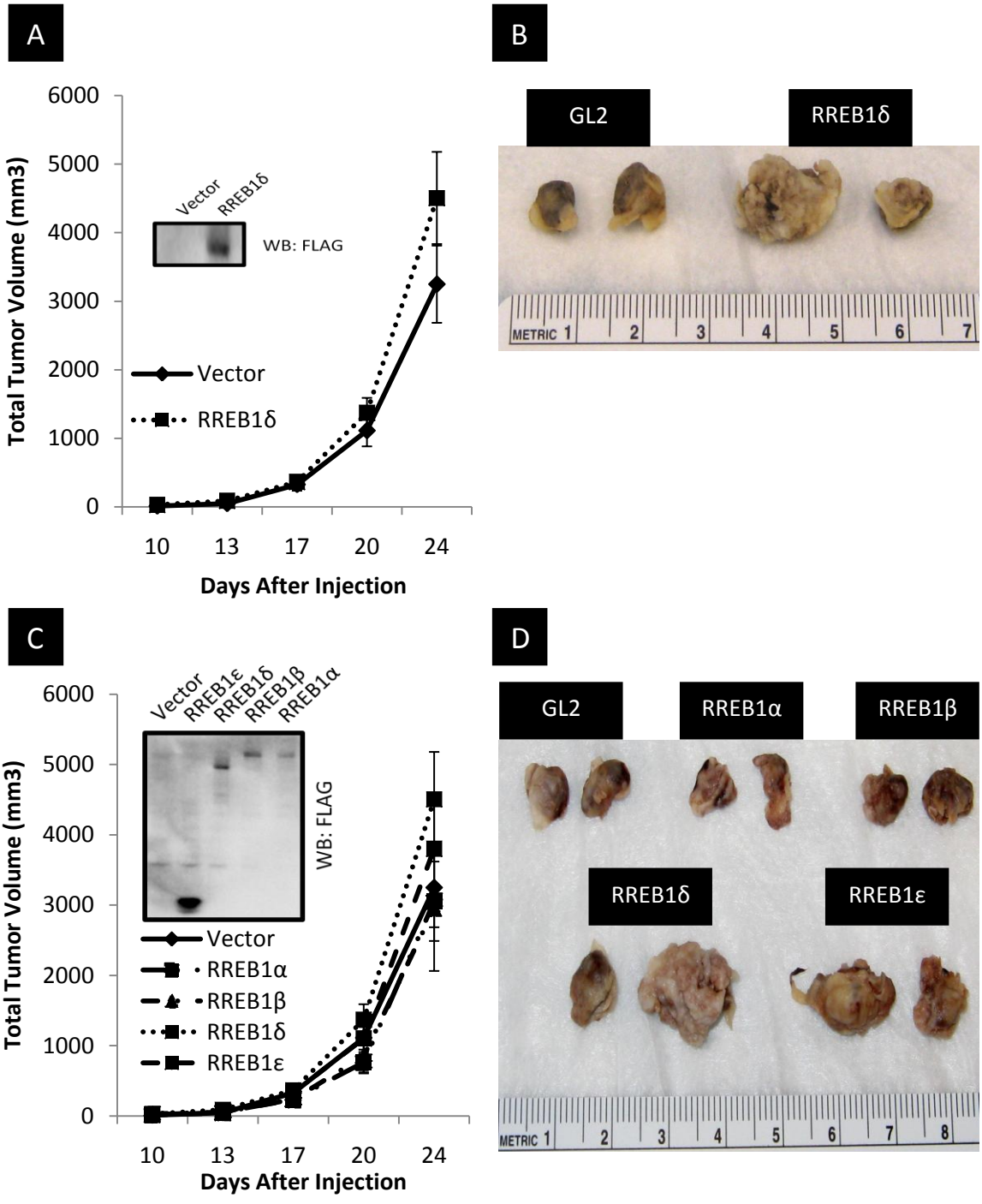

A
89000
79000
69000
59000
49000
39000
29000
19000
9000
Flourescence Values
GL2
Exon 8-1
Exon 9-1
24
48
72
96
120
144
Time After Injection (Hrs)
B
GL2
Exon 8-1
Exon 9-1
siRNA
0
5
10
Number of Tumors
C
2500
2000
1500
1000
500
0
Total Tumor Volume (mm3)
GL2
Exon 8-1
Exon 9-1
WB: RREB1
WB: TBP
GL2
Exon 8-1
Exon 9-1
10
13
17
20
24
Days After Injection
D
GL2
Exon 8
Exon 9
METRIC 1 2 3 4 5 6

Figure 20. RREB1δ expression increases UMUC3 tumor size *in vivo*. A. Vector control and RREB1δ stably overexpressing UMUC3 cells were injected subcutaneously into nude mice. Tumor growth was measured twice a week until 24 days post injection. (* P=0.05) **B.** Representative tumors are shown for each group. **C.** Vector control (FLAG) and RREB1 isoforms stably expressed in UMUC3 cells were injected subcutaneously into nude mice. Tumor growth was measured twice a week until 24 days post injection. **D.** Representative tumors are shown for each group.

Figure 20


A
B
C
D
Total Tumor Volume (mm3)
6000
5000
4000
3000
2000
1000
0
10
13
17
20
24
Days After Injection
Vector
RREB1δ
WB: FLAG
Vector
RREB1δ
GL2
RREB1δ
METRIC 1 2 3 4 5 6 7
Vector
RREB1ε
RREB1δ
RREB1β
RREB1α
WB: FLAG
Vector
RREB1α
RREB1β
RREB1δ
RREB1ε
GL2
RREB1α
RREB1β
RREB1δ
RREB1ε
METRIC 1 2 3 4 5 6 7 8

3.5 Discussion

Several transcription factors have been shown to be important in bladder cancer including GLI2 [162] and p63 [17]. Despite the reduced level of expression in cancer compared to normal we show that RREB1 is still necessary for the proliferation and tumorigenesis in bladder and prostate cancer models. Not only did we observe that depletion of both RREB1α and RREB1β was sufficient to abrogate growth *in vitro*, but we also observed that the loss of both isoforms or of just RREB1α was sufficient to impact tumorigenesis *in vivo*. To our knowledge, this is the first report of an *in vivo* loss of function phenotype for RREB1 in human cancer. While it is tempting to speculate, taking together our *in vitro* and *in vivo* phenotypes, that RREB1β may be essential for cellular proliferation, we cannot rule out the possibility that RREB1α and RREB1β have overlapping functions where growth effects are not seen until loss of both isoforms. Attempts to knock down solely RREB1β were unsuccessful due to the lack of a siRNA seed region in the small, unique sequence created by the Exon 7 and Exon 9 junction. RREB1δ, though not shown to be necessary for tumor growth through siRNA depletion, was the only splice variant observed to be sufficient to increase *in vivo* tumor growth of UMUC3 cells.

Taken together, we believe that the seemingly paradoxical finding of decreased expression in tumors can fit into a model that explains the function of RREB1 in bladder and prostate cancer. RREB1α and RREB1β, while comprising the majority of RREB1 expression, were, necessary, but not sufficient to drive

tumor growth. Therefore, there appears to be no advantage to upregulate RREB1α and RREB1β expression, provided their expression does not fall below a certain level. Thus, total RREB1 expression may decrease in tumors. RREB1δ and RREB1ε on the other hand do not appear to be necessary but possibly sufficient to drive tumor growth in the over expression scenario. Though we did not observe RREB1δ and RREEB1ε to be grossly overexpressed in the 14 tumors we interrogated, it does not preclude that possibility. Furthermore, the tissue microarray further reinforced the importance of RREB1 in bladder cancer is not based on expression level. Ergo, with the relevance of androgen receptor in prostate cancer or myriad tumors driven or modulated by Ras activity, RREB1, in the context of its differential isoforms, may play a more complex and important role in cancer progression than initially anticipated and our findings support a promising direction for future study.

Chapter 4: Ral, RREB1, and Hypoxia Regulation of the CD24 Promoter

Matthew D. Nitz, Shibu Thomas, Michael A. Harding, Steven C. Smith, and Dan Theodorescu

Contributors

Matthew D. Nitz, UVA Department of Molecular Physiology and Biological Physics, designed and performed all experiments described below, analyzed the data, and wrote the manuscript.

Dr. Shibu Thomas, UVA Department of Urology, performed the experiments for Figures 4-8B and 4-8C, helped with the design of all hypoxia experiments.

Dr. Michael A. Harding, UVA Department of Urology, designed the CD24 promoter cloning PCR primers, contributed substantially to the study design and preparation of the manuscript

Dr. Steven C. Smith, UVA Department of Urology, provided statistical expertise, contributed to the study design, and preparation of the manuscript.

Dr. Dan Theodorescu, UVA Department of Urology and Molecular Physiology and Biological Physics, provided resources, guidance, and advice on the design and interpretation of experiments.

4.1 Abstract

CD24 is a cell surface glycoprotein that is overexpressed in many cancers, including bladder cancer. Despite numerous studies linking high CD24 levels with poor histopathologic and clinical parameters such as invasion, metastasis, and survival, no study has examined the CD24 locus for clues as to its mechanism of overexpression. We report for the first time a consensus CD24 promoter sequence from seven cell lines (UMUC3, J82, LUL2, LNCaP, EJ, 293T, and TERT). Overexpression of activated RalA (G23V) positively regulated the CD24 promoter reporter, but did not increase endogenous RNA, whereas RalB negatively regulated the reporter but increased CD24 RNA levels. RREB1 isoforms beta and epsilon increased CD24 reporter activity but not RNA. However, siRNA to both RalB and RREB1 decreased CD24 promoter activity and RNA in concert. A hypoxia response element (HRE) was found 24 base pairs upstream of the CD24 transcription start site. We demonstrate that CD24 is a hypoxia responsive gene, driven by HIF1A. Taken together, these data contribute valuable tools and insights into the regulation of CD24 expression.

4.2 Introduction

CD24 is a 27 amino acid protein that is purported to reside primarily on the plasma membrane. The gene encodes an 80 amino acid pre-protein that is cleaved in the endoplasmic reticulum prior to the addition of a heavy glycosylation pattern and glycosylphosphatidylinisotol (GPI) anchor. Initially identified in developing lymphocytes, CD24 is overexpressed in several cancer types and its expression positively correlates with unfavorable clinical parameters (**Chapter 1, Table 1**). Recent work has confirmed that CD24 overexpression in cancer cells is not merely a nonfunctional passenger, but actively participates in maintaining the cancer phenotype [55 58]. However, insights into the mechanism of CD24 overexpression continue to remain unclear.

Work from our lab has uncovered the necessity of **Ra**s-**l**ike (Ral) GTPase expression in maintaining cancer cell CD24 expression [55]. Ral GTPases are the nexus for one of the three main signaling pathways downstream of Ras. Ras activation, either through mutation or endogenous activation of upstream receptors (EGFR, PDGFR, etc), promotes the binding to Ral guanine exchange factors (GEFs), which can then bind and activate the Ral paralogs, RalA and RalB [33]. Ral GTPases have previously been shown to be important for maintaining cancer cell proliferation and promoting cell migration [53]. Gene expression profiling revealed that transient depletion of RalA, RalB, or both with siRNA resulted in a synergistic decrease in CD24 expression [55]. Depletion of CD24 independently caused a significant reduction of cell proliferation *in vitro*

and in soft agar, which could only partially be attributed to an increase in apoptotic cells [55]. Thus the Ral to CD24 connection appears important for cancer biology.

In an effort to uncover what proteins were responsible for Ral dependent CD24 transcription, we used an *in silico* tool (**CARRIE**) developed to uncover putative transcription factor-regulatory pathways associated with changes in transcriptional profiles. When RalA and RalB expression was depleted using transient siRNA in the UMUC3 bladder cancer cell line, the promoters of differentially expressed genes were found to have a statistical overrepresentation of the RREB1 transcription factor consensus binding sites [95]. Using an RREB1 consensus binding site reporter, manipulation of the Ral GTPases had significant effects on reporter activity. It was found that depletion of RalB, but not RalA, decreased reporter activity while overexpression RalA or RalB was sufficient to increase reporter activity [95]; leading to the authors to conclude that the transcription factor RREB1 is a downstream effector of Ral signaling.

Intriguingly, several of the genes with the highest up-regulation with Ral depletion are categorized as stress response genes. Upon closer investigation, many have been shown to be specifically regulated in hypoxia: MMP1 [139], Clusterin [140], and IGFBP3 [141]. Furthermore, CD24 has been found by mass spectrometry to be up-regulated in endothelial cells after exposure to hypoxia [142]. The primary drivers of transcription in the context of hypoxia are the basic helix-loop-helix (bHLH) transcription factors HIF1A and HIF2A that form heterodimers with HIF1B (ARNT) to bind DNA [163]. The DNA sequence bound by these dimers

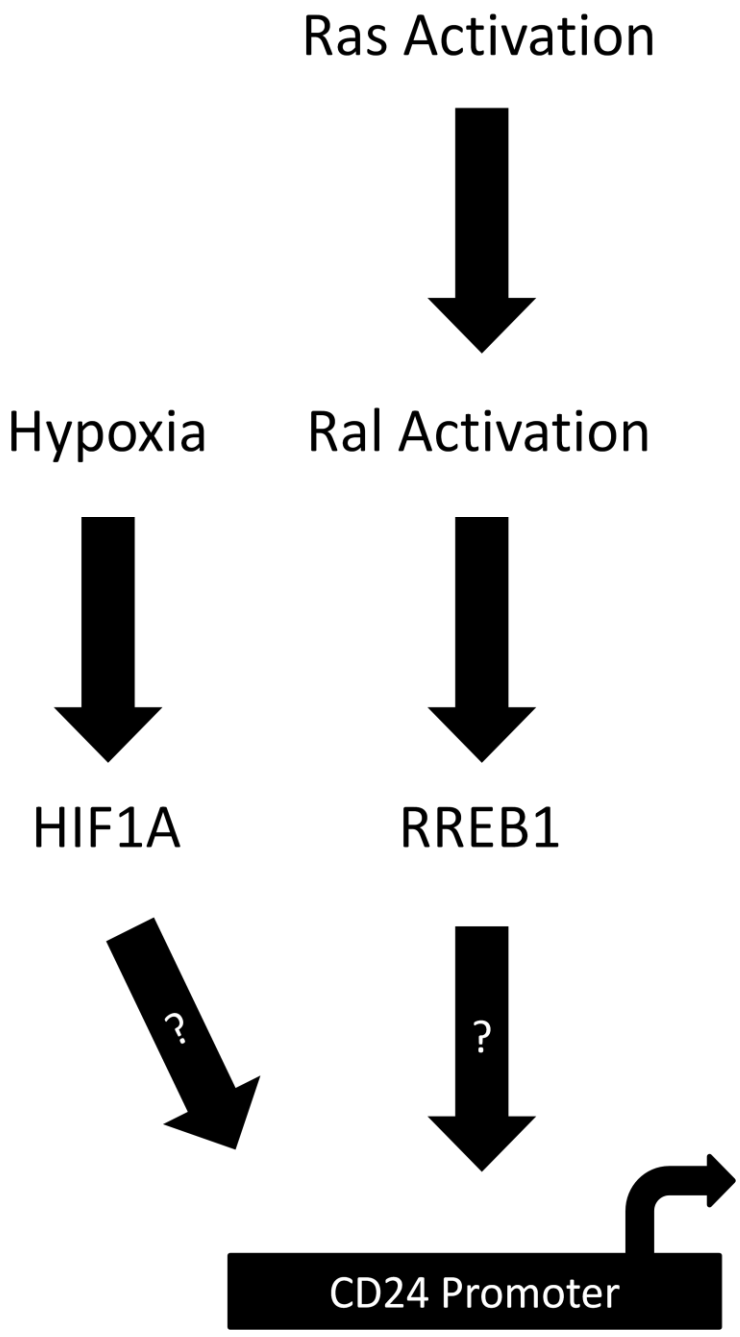
has previously been validated and has been dubbed the **h**ypoxia **r**esponsive **e**lement (**HRE**) [164].

As seen in **Figure 21**, the connection of Ral to RREB1 appears to infer a likely mechanism for CD24 overexpression. However, a significant hurdle remains in the study of CD24 expression. Despite the reported completion of the Human Genome Project [165] there remains a gap in our knowledge of the DNA sequence encompassing parts of the CD24 gene and promoter. The lack of an annotated DNA sequence in the repository for all human genetic information, the National Center for Biotechnology Information (NCBI) GenBank, has stalled further study of CD24 transcription.

Therefore, we aimed to clone and sequence the DNA immediately upstream of the CD24 gene locus and determine the role of RREB1 and HIF1A in CD24 transcription. We found that HIF1A and RREB1 dynamically regulate the CD24 promoter.

Figure 21. Proposed mechanism for regulation of the CD24 promoter. Based on previous findings we proposed two pathways that could regulate the activity of the CD24 promoter. Hypoxia may increase CD24 expression [142] thus we propose that the hypoxia responsive transcription factor HIF1A may regulate CD24. Ral has been shown to regulate RREB1 reporter activity [95] and CD24 expression [55], but it remains unknown if RREB1 regulates CD24 [166].

Figure 21


Ras Activation
Hypoxia
Ral Activation
HIF1A
RREB1
?
?
CD24 Promoter

4.3 Materials and Methods

4.3.1 Cell lines, growth conditions and clones

Human cancer cell lines were grown in the following conditions: UMUC3, LUL2: Minimum essential media (MEM) (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco®, Invitrogen, Carlsbad, CA) and 1% sodium pyruvate (Gibco®, Invitrogen, Carlsbad, CA), LNCAP: RPMI medium 1640 (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 293T: DMEM High Glucose (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% FBS, J82: MEM supplemented with 10% FBS and 1% amino acids (Gibco®, Invitrogen, Carlsbad, CA). TERT cells were a gift from Dr. MA Knowles and were grown as described [153]. Cells were grown at 37^{o}C supplemented with 5% CO_2. Hypoxic samples were placed in a hypoxia chamber (Billups-Rothenberg, Del Mar, CA) and incubated in a gas mixture of 1%O_2, 5% CO_2, and 94% N_2 for 24 hours. 5-Azacytadine (Sigma-Aldrich) was added to cells at a final concentration of 1 or 2μM. RREB1α and RREB1β were kindly provided by Dr. Akiyoshi Fukamizu (University of Tsukuba, Tsukuba, Japan) [96] and subcloned into the p3XFLAG-CMV™-14. RREB1δ and RREB1ε were cloned from UMUC3 into the p3XFLAG-CMV™-14 vector as described previously (**Chapter 2**). Wild type, constitutively active (G23V) mutants, and fast cycling (F39L) mutants of RALA and RALB were described previously [53]. pcDNA3.0 HIF1A was a gift from G. Semenza and was described previously [167].

4.3.2 RNA isolation, Reverse transcription, and Q-RTPCR

RNA isolation was carried out using an RNeasy® Mini Kit (Qiagen) and cDNA synthesis using iScript™ (Biorad). Q-RTPCR was performed on cDNA using the iQ™ SYBR® Green Supermix (Biorad). Primers with annealing temperatures: RREB1 (total) (62^{o}C) F:5'-CTT-CCT-ATA-ACT-GCC-CCC-3'; R:5'-ATG-AGT-GGT-CGG-CTC-CTC-C-3'; CD24 (62^{o}C) F:5'-CAA-TAT-TAA-ATC-TGC-TGG-AGT-TTC-ATG-3'; R5'-TCC-ATA-TTT-CTC-AAG-CCA-CAT-TCA-3'; GUSB (62^{o}C) F:5'-CCG-ACT-TCT-CTG-ACA-ACC-GAC-G-3', R:5'-AGC-CGA-CAA-AAT-GCC-GCA-GAC-G-3'.

4.3.3 Cloning and sequencing of the CD24 promoter

Genomic DNA was isolated using the Puregene™ DNA isolation kit (Gentra Systems) according to manufacturer's instructions. The PCR reaction also included: 10X PfuUltra™ HF Reaction Buffer (Agilent), 100mM dNTP Mix (Agilent), 5% DMSO, 1M Betaine Solution (Sigma-Aldrich) and 100ng of DNA. The primers used were as follows: CD24 promoter 2344 F:5'-GAA-CCC-GGC-ACT-CCT-GAG-TCA-3', R:5'-ACC-ATT-GCT-CTG-CCC-ATG-TCC-3'; CD24 PROMOTER 1896 F:5'-GAT-CGC-TAG-CCA-CGC-CCG-GCC-AAA-GTA-TTT-C-3', R:5'-GAT-CAA-GCT-TCA-GGA-TGC-TGG-GTG-CTT-GGA-G-3'. PCR products were isolated in a 1% agarose gel and purified using a Qiaquick® Gel Extraction Kit (Qiagen). Isolated products were digested at 37^{o}C by the enzymes NheI and HindIII (New England Biolabs) and ligated into the pGL4.20 Luciferase Reporter Vector (Promega). CD24 promoters were sequenced at the University

of Virginia DNA sequencing Core using the BigDye Terminator v3.1 (Applied Biosystems) chemistry on a 3730 DNA Analyzer (Applied Biosystems). Primers for sequencing were: RV primer 3: 5'- CTA-GCA-AAA-TAG-GCT-GTC-CCC-3', pGL4.20 R: 5'- CTT-AAT-GTT-TTT-GGC-ATC-TTC-C-3', CD24promSF1: 5'-CTC-CTC-TTT-GTG-CCG-GTT-CAT-T-3', CD24promSR1: 5'-CGG-TCC-TGG-AGC-AAG-TGC-A-3'. Sequences were submitted to GenBank.

4.3.4 *In silico* analyzing of the CD24 promoter

Detemination of the CD24 promoter G/C content and generation of the graph was performed by the EMBOSS Isochore program [168] (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). Discovery of a CpG island was found using the EMBOSS CpGPlot program [169] (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). To uncover transcription factor binding sites and their over or under representation in the CD24 promoter the Genomatix Region Miner [170] program was used (Genomatix, Ann Arbor, MI).

4.3.5 Antibodies and Western Blotting

The following antibodies were used: RREB1 (Sigma-Aldrich, Cat. no: HPA001756), Tubulin (Santa Cruz Biotechnology, Cat. no: sc-58668), CD24 (antibody SWA11, a gift of Dr. Peter Altevogt, Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany). Immunoblotting and detection were performed as described [53].

4.3.6 Transfection, siRNA and Luciferase assays

Transient vector transfection was performed using FuGENE®6 Tranfection Reagent (Roche) at a ratio of 1µg of DNA per 3µl of FuGENE. For luciferase assays, cells were lysed in 1X Report Lysis Buffer (Promega, Madison, WI) and assayed for light expression using the Luciferase Assay System (Promega) on a BioTek Synergy 2 plate reader (Winooski, VT). Oligofectamine™ (Invitrogen) was used to transiently transfect siRNA according to manufacturer instructions. All siRNA was transfected at a final concentration of 25nM unless otherwise noted: RREB1 total: 5'-GGA-GUU-UGU-UUG-CAA-GUA-U-3' and 5'-GUU-CAG-ACC-UAU-CUU-CCA-U-3' (used in combination at 12.5nM); GL2: 5'-CGU-ACG-CGG-AAU-ACU-UCG-AdTdT-3'; RALA: 5'- GAC-AGG-UUU-CUG-UAG-AAG-AdTdT-3'; RALB: 5'- AAG-CUG-ACA-GUU-AUA-GAA-AdTdT-3'; Scramble: 5'-UAG-CGA-CUA-AAC-ACA-UCA-AUU-3'.

4.4 Results

4.4.1 Transient depletion of RREB1 decreases CD24 expression

To test whether CD24 expression was dependent on RREB1 expression siRNA was designed to target all RREB1 splice variants. Depletion of RREB1 decreased CD24 RNA levels in UMUC3 and LUL2 cells compared to the GL2 control (luciferase) (**Figures 22A** and **22C**, respectively). GUSB expression was used to determine equal loading between GL2 and RREB1 knockdown samples

(**Figures 22B** and **22D**). In UMUC3 cells RREB1 depletion decreased CD24 protein expression (**Figure 22E**).

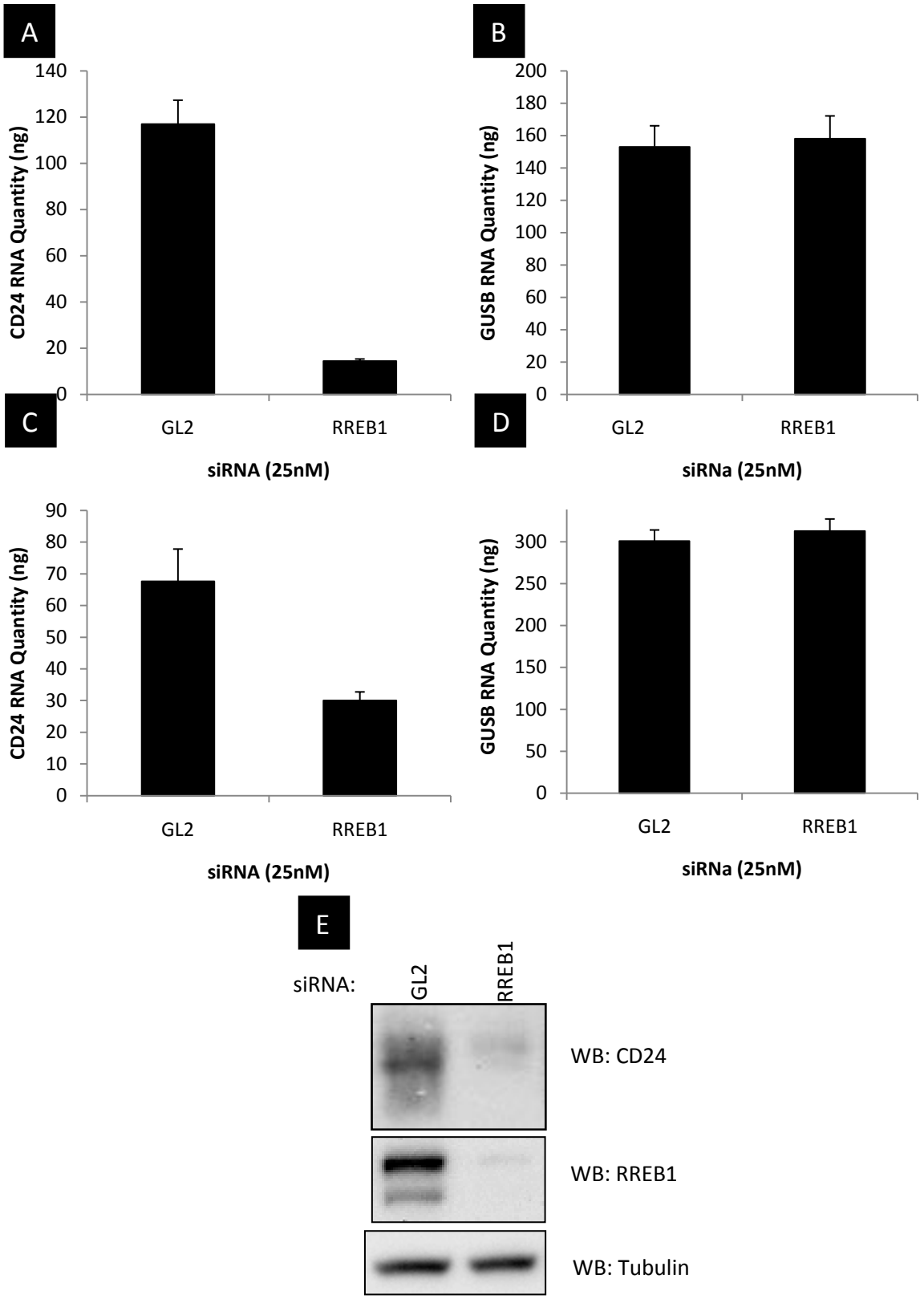
4.4.2 CD24 expression partially rescues UMUC3 cell growth after RREB1 knockdown

We have previously shown that RREB1 depletion in the bladder cancer cell line UMUC3 decreased cell growth (**Chapter 3**). Also, previous studies have demonstrated that transient CD24 depletion in UMUC3 also decreases cell growth [55]. Therefore, we asked whether stable CD24 expression could rescue UMUC3 growth after RREB1 depletion.

UMUC3 cells were stably transfected with a control or CD24 expression vector. RREB1 siRNA was transfected into both cell populations and Alamar Blue activity was measured 96 hrs post transfection. Stable CD24 expression, but not the vector control, reduced the decrease in cell growth due to loss of RREB1 to a nonsignificant value (**Figure 23A**) (Control P=0.03 vs. CD24 Stable P=0.40, respectively). **Figure 23B** shows CD24 protein expression of the vector control and CD24 stable cells with GL2 and RREB1 siRNA depletion. The decrease of CD24 expression in CD24 stably expressing cells was presumed to be the loss of endogenous CD24 expression, not the transgene.

Figure 22. CD24 expression is dependent on RREB1. A. UMUC3 cells were transfected with 25nM of GL2 control and RREB1 siRNA 96 hours prior to harvest. CD24 expression was measured using quantitative real time PCR (Q-RTPCR). **B.** Q-RTPCR for GUSB in GL2 and RREB1 transfected UMUC3 cells. **C.** LUL2 cells were transfected with GL2 and RREB1 siRNA as described above. **D.** GUSB expression for GL2 and RREB1 treated cells. **E.** UMUC3 cells were transfected with 25nM of GL2a and RREB1 siRNA 96 hours prior to protein harvest. Western blots for CD24 (1:3), RREB1 (1:1000) and Tubulin (1:1000) were performed.

Figure 22

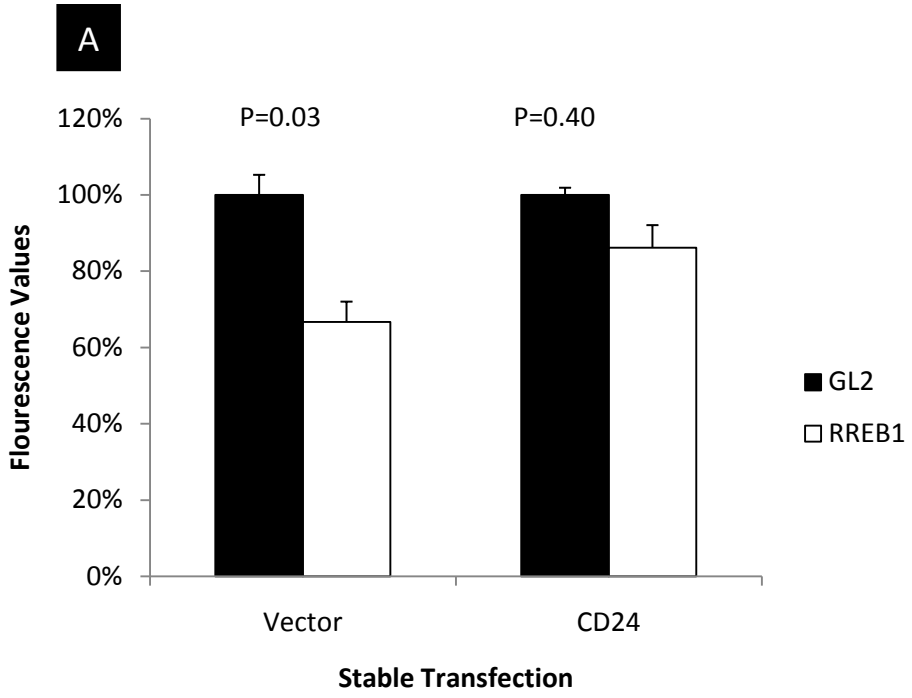

A
CD24 RNA Quantity (ng)
140
120
100
80
60
40
20
0
GL2
RREB1
siRNA (25nM)
B
GUSB RNA Quantity (ng)
200
180
160
140
120
100
80
60
40
20
0
GL2
RREB1
siRNa (25nM)
C
CD24 RNA Quantity (ng)
90
80
70
60
50
40
30
20
10
0
GL2
RREB1
siRNA (25nM)
D
GUSB RNA Quantity (ng)
300
250
200
150
100
50
0
GL2
RREB1
siRNa (25nM)
E
siRNA:
GL2
RREB1
WB: CD24
WB: RREB1
WB: Tubulin

4.4.3 Cloning of the CD24 promoter in multiple cell lines

To uncover the interaction of RREB1 with the CD24 promoter we aimed to test the promoter directly *in vitro*. However, a portion of the CD24 promoter in NCBI GenBank was incomplete with a 204 base pair sequence missing containing the 5' untranslated region (UTR) of CD24 and the initial 100 base pairs of the promoter (**Figure 24**). There have been two independently published CD24 promoter sequences. The first was cloned out of a pooled sample of human DNA [171] while the second was cloned out of a population of B lymphocytes [172]. These two promoters had considerable mismatched sequences between each other and the partial promoter from GenBank (**Figure 24**). Therefore, to determine the consensus CD24 promoter sequence in UMUC3 cells we cloned a 1896 base pair region that encompassed the first 79 base pairs of the CD24 5'UTR. Comparing the UMUC3 sequence to the previously reported sequences for the CD24 promoter several mismatches and deletions were observed (**Figure 24**). To gain a consensus CD24 promoter sequence we cloned the same region in four additional cancer cell lines (LUL2, EJ, J82, and LNCaP) and two non-cancerous cell lines: telomerase (TERT) immortalized urothelial cell line [153] and human embryonic kidney cells (293T).

Figure 23. Rescue of UMUC3 growth after RREB1 depletion with CD24 overexpression. **A.** pcDNA3.1 vector or pcDNA3.1-CD24 was stably transfected into UMUC3 cells. After selection, cells were transfected with 25nM siRNA to GL2 or RREB1. CD24 expressing cells did not show a significant decrease in growth (P=0.40) as opposed to the vector (P=0.03) 96 hours after transfection. **B.** Western blot showing CD24 expression of the vector and CD24 expressing cells with GL2 and RREB1 siRNA transfection.

Figure 23


A
120%
100%
80%
60%
40%
20%
0%
P=0.03
P=0.40
Flourescence Values
GL2
RREB1
Vector
CD24
Stable Transfection

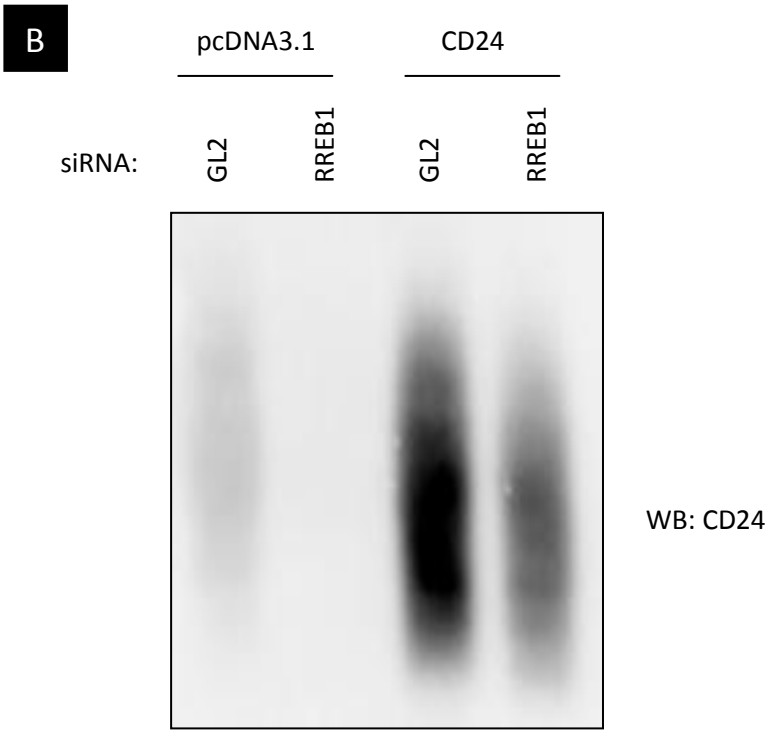
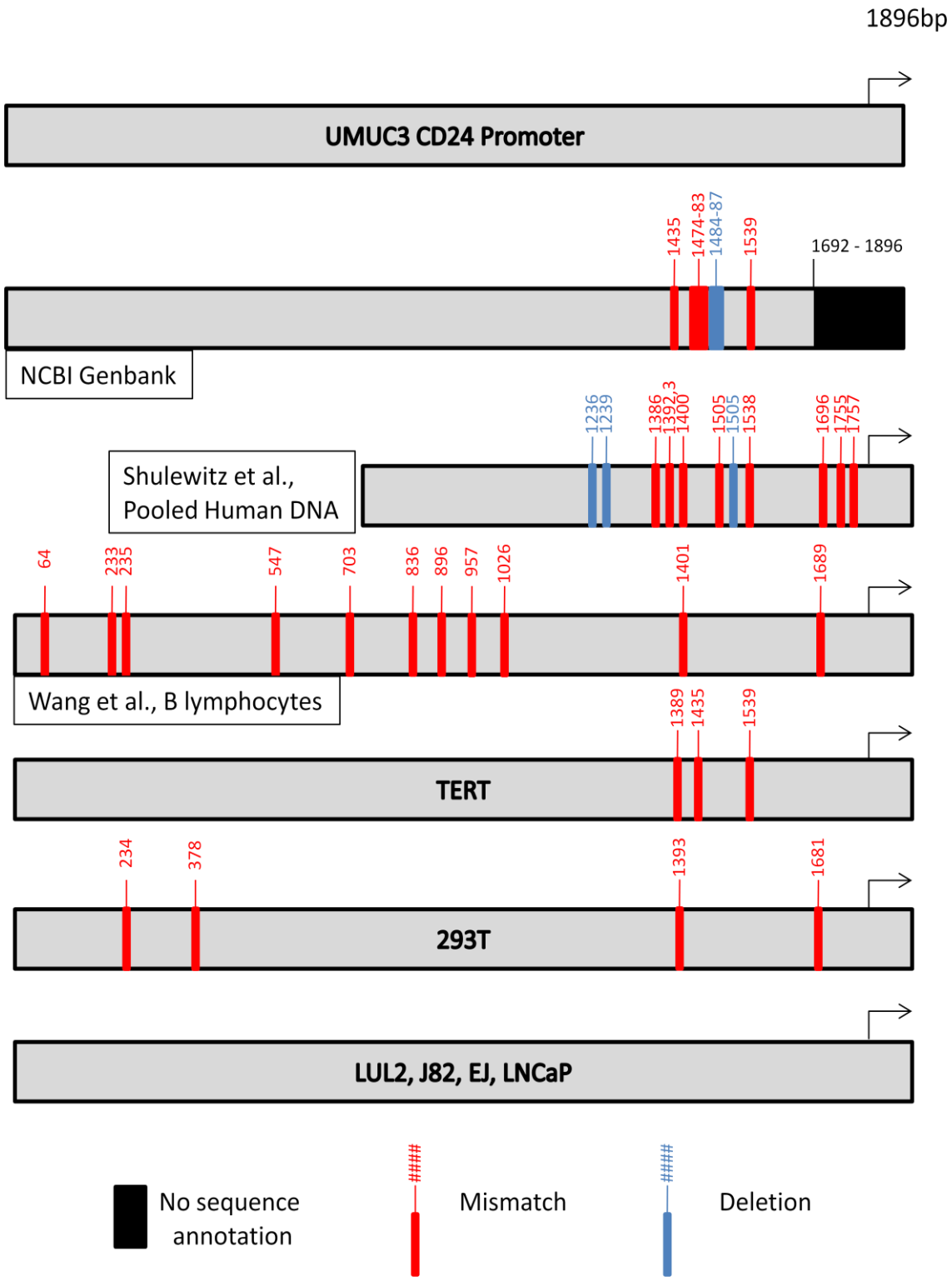

B
pcDNA3.1
CD24
siRNA:
GL2
RREB1
GL2
RREB1
WB: CD24

Figure 24. Cloning of the CD24 promoter. A 1,896 base pair region surrounding the transcription start site of CD24 (-1817 to +79) was cloned out of UMUC3 DNA. Comparison of the UMUC3 sequence with three published CD24 promoters: NCBI GenBank, pooled human DNA [171], and B lymphocytes [172]. Mismatches or insertions are red, sequence deletions are blue. The numbers on top indicate the position of the mismatches/deletions. LUL2, J82, EJ, and LNCaP CD24 promoters had identical sequences to UMUC3.

Figure 24


1896bp
UMUC3 CD24 Promoter
1435
1474-83
1484-87
1539
1692 - 1896
NCBI Genbank
1236
1239
1386
1392,3
1400
1505
1505
1538
1696
1755
1757
Shulewitz et al.,
Pooled Human DNA
64
233
235
547
703
836
896
957
1026
1401
1689
Wang et al., B lymphocytes
1389
1435
1539
TERT
234
378
1393
1681
293T
LUL2, J82, EJ, LNCaP
No sequence annotation
####
Mismatch
####
Deletion

The two non-cancerous lines showed far fewer mismatches when compared to UMUC (4 for 293T and 3 for TERT) while the four cancer cell lines were identical in sequence to UMUC3 (**Figure 24**). All CD24 promoter sequences have been submitted to GenBank.

4.4.4 The CD24 promoter contains a predicted CpG island

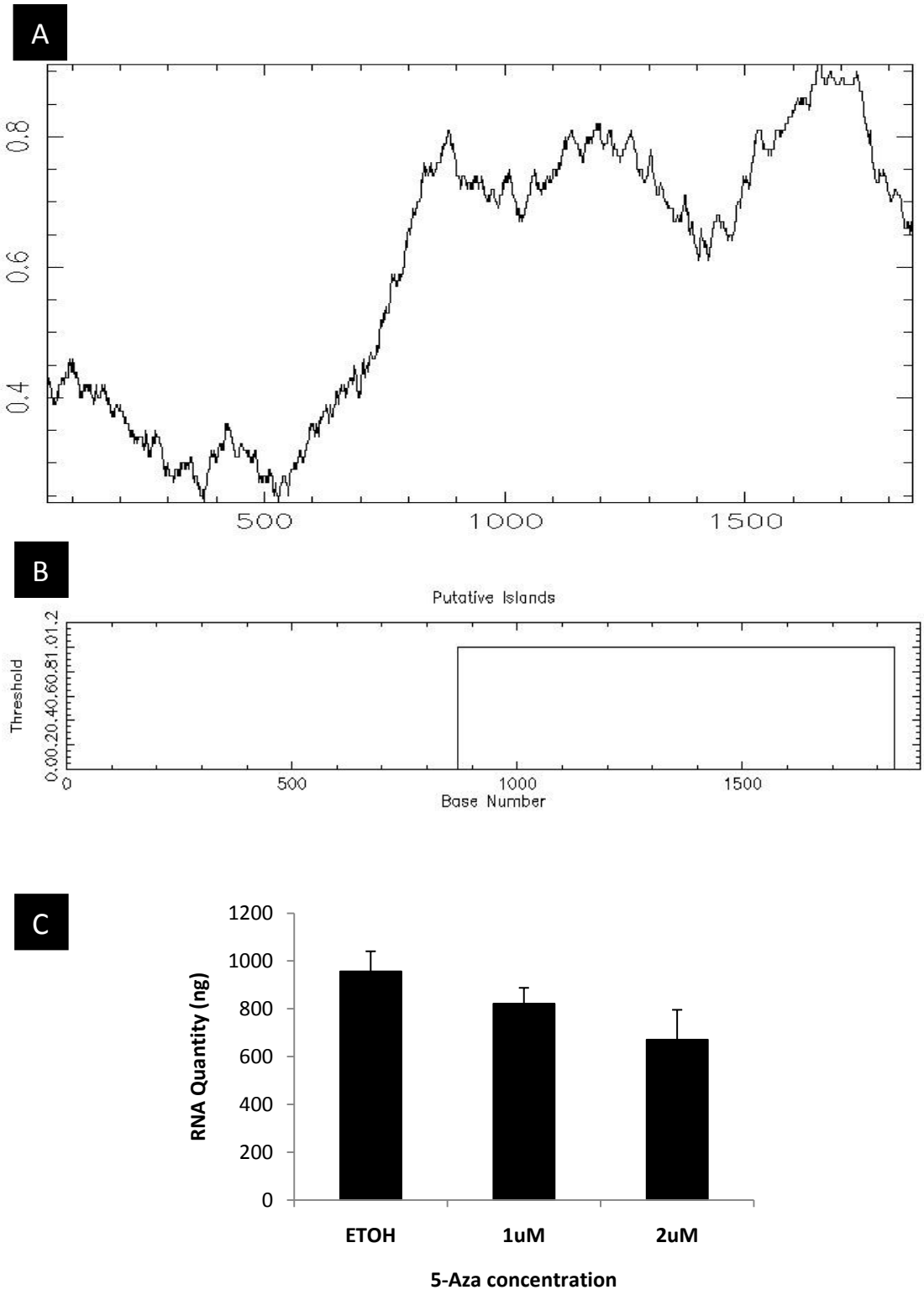
Considerable difficulty was met in cloning the CD24 promoter, the reason for which was uncovered after successful cloning and sequencing. A 1kb region of the promoter immediately upstream of the transcription start site contains a high G/C content that ranges between 65% and 95% (**Figure 25A**), which raises the temperature necessary to denature the DNA and makes the PCR very inefficient. G/C rich regions are fairly common among human promoters and have become an area of intense investigation due to their association with enhancing or silencing of gene expression. G/C rich regions have a higher frequency of CpG sites, which are a **c**ytosine nucleotide that occurs next to a **g**uanine nucleotide in a linear sequence (connected by a **p**hosphate bond). A high concentration of CpG sites within a defined region are referred to as CpG islands (CGIs). Human promoters show a bimodal distribution (either presence or lack) of CpG sites where 72% have high CpG concentration, making it the more common occurrence [173]. CGIs are typically found in the promoters of the most widely expressed housekeeping genes and are often hypomethylated [174].

The original definition of a CGI was an area of at least 200bp, with a G/C content >50%, and CpG frequency greater than 0.6 (observed/expected) [175].

Recent studies have shown that false positives can be reduced 90% by increasing the stringency to a >500bp length, >55% G/C content, and CpG frequency of 0.65% [176]. Therefore, to reduce the possibility of false positive even further, we used an even greater stringency of 500bp, >60%, and >0.70 to search for a CGI in the CD24 promoter.

Figure 25. The CD24 promoter contains a CpG island. A. G/C content across the CD24 promoter was calculated using the EMBOSS IsoChore program. The y-axis is proportion of G/C out of total bases for that region. **B.** EMBOSS CpGPlot prediction of a 970bp CpG island in the CD24 promoter extending from +21 to -949. **C.** UMUC3 cells were treated with 1 or 2 μM of 5-Azacytatdine for 24 hours, after which cells allowed to grow in fresh media for 48 hours. Q-RTPCR for CD24 expression was performed.

Figure 25


A
0.4
0.6
0.8
500
1000
1500
B
Putative Islands
Threshold
0.0 0.2 0.4 0.6 0.8 1.0 1.2
0
500
1000
1500
Base Number
C
RNA Quantity (ng)
1200
1000
800
600
400
200
0
ETOH
1uM
2uM
5-Aza concentration

Using the EMBOSS CpGPlot program a CGI of 970 base pairs was found that extended from +21 to -949 within the CD24 promoter (**Figure 25B**). Because CD24 shows variable tissue expression and extensive CD24 overexpression in tumors, it has been hypothesized that methylation of its promoter is a mechanisms used to modulate CD24 RNA expression. We treated UMUC3 cells with 5-Azacytadine (5-AZA), which inhibits DNA methyltransferases and has been shown to cause reexpression of genes silenced by promoter methylation [177]. UMUC3 cells were used because our lab has found that this cell line expresses 10-20 fold lower CD24 expression than its metastatic daughter cell line LUL2 (unpublished data). We found that 5-AZA did not increase CD24 expression which, taken together, led us to conclude that CD24 expression is not regulated by promoter methylation in UMUC3 cells (**Figure 25C**).

4.4.5 Transcription factor binding site overrepresentation in the CD24 promoter

To determine if specific transcription factor binding sites were over or under represented in the CD24 promoter we used Genomatix Region Miner [170]. This program searches for published transcription factor binding sites within the submitted sequence and then compares the frequency of those sites to the frequency that would be expected over the entire genome or within vertebrae promoters. As shown in **Table 4**, several over represented transcription factor

binding sites were identified while only a single site, Oct1, was found to be significantly under represented. Statistical significance was determined by calculating a Z-score for the abundance of each site, which can be approximated to a P-value (Z-score of 2 or -2 is approximately P=0.05). The most significantly over represented site, NRF1, was found in greater than 90 fold abundance in the CD24 promoter but does not have a strong association with malignancy. However, other transcription factors such as SP1 [178] and NFκB [179] have been implicated in several types of cancers.

Interestingly, only a single RREB1 site (-110) and HRE (-24) were identified; neither of which were significantly under or over represented (**Table 4**). However, it was noted their proximity to the transcription start site and to each other. The over represented transcription factor binding sites, while intriguing and possibly important to CD24 transcription, were not further examined in this study.

Table 4

TF Family	Description	Over Represented (Genome) ‖	Z-Score†	Over Represented (Promoter) ‡	Z-Score †
V$NRF1	Nuclear respiratory factor 1	91.29	37.81	5.72	7.85
O$TF2B	RNA polymerase II transcription factor II B	43.45	11.23	5.55	3.27
O$MTEN	Motif Ten Element - Binding site of RNA Pol II	32.92	17.59	5.43	5.96
V$ZF5F	ZF5 POZ domain zinc finger (ZFP161)	31.48	14.38	3.95	3.85
V$CTCF	CTCF and BORIS gene family, transcriptional regulators with 11 highly conserved zinc finger domains (CTCF/CTCFL)	15.37	15.09	3.49	5.44
V$EGRF	EGR/nerve growth factor induced protein C & related factors (ZBTB7B)	11.32	14.37	2.98	5.33
V$CHRE	Carbohydrate response elements, consist of two E box motifs separated by 5 bp (MLXIPL)	10.82	4.22	4.26	2.14
V$ZBPF	Zinc finger with KRAB and SCAN domains 3	7.47	8.5	1.94	2.34
V$NRSF	Neuron-restrictive silencer factor (REST/PRICKLE1)	7.05	5.98	3.22	3.19
V$SP1F	GC-Box factors SP1/GC (SP2)	6.84	8.31	2.15	2.85
V$NFKB	Nuclear factor kappa B/c-rel	5.64	5.81	3.53	3.97
V$PAX3	PAX-3 binding sites	5.58	3.29	3.92	2.45
V$E2FF	E2F-myc activator/cell cycle regulator	3.98	4.42	2.03	2.06
V$RREB	Ras-responsive element binding protein (RREB1)	1.14	-0.4	0.6	-0.91
V$HIFF	Hypoxia inducible factor, bHLH/PAS protein family	1.14	-0.4	0.6	-0.9
V$OCT1	Octamer binding protein (Oct1/Oct2)	0.12	-3.8	0.2	-2.71

‖ Overrepresentation against genomic background: Fold factor of match numbers in regions compared to an equally sized sample of the background (i.e. found versus expected). Genomic background comprises all chromosomes of the selected organism.

‡ Overrepresentation against promoter background: Fold factor of match numbers in regions compared to an equally sized sample of the background (i.e. found versus expected). The promoter background comprises all Genomatix defined promoters of optimized length (about 500/100bp up/downstream of the transcription start site).

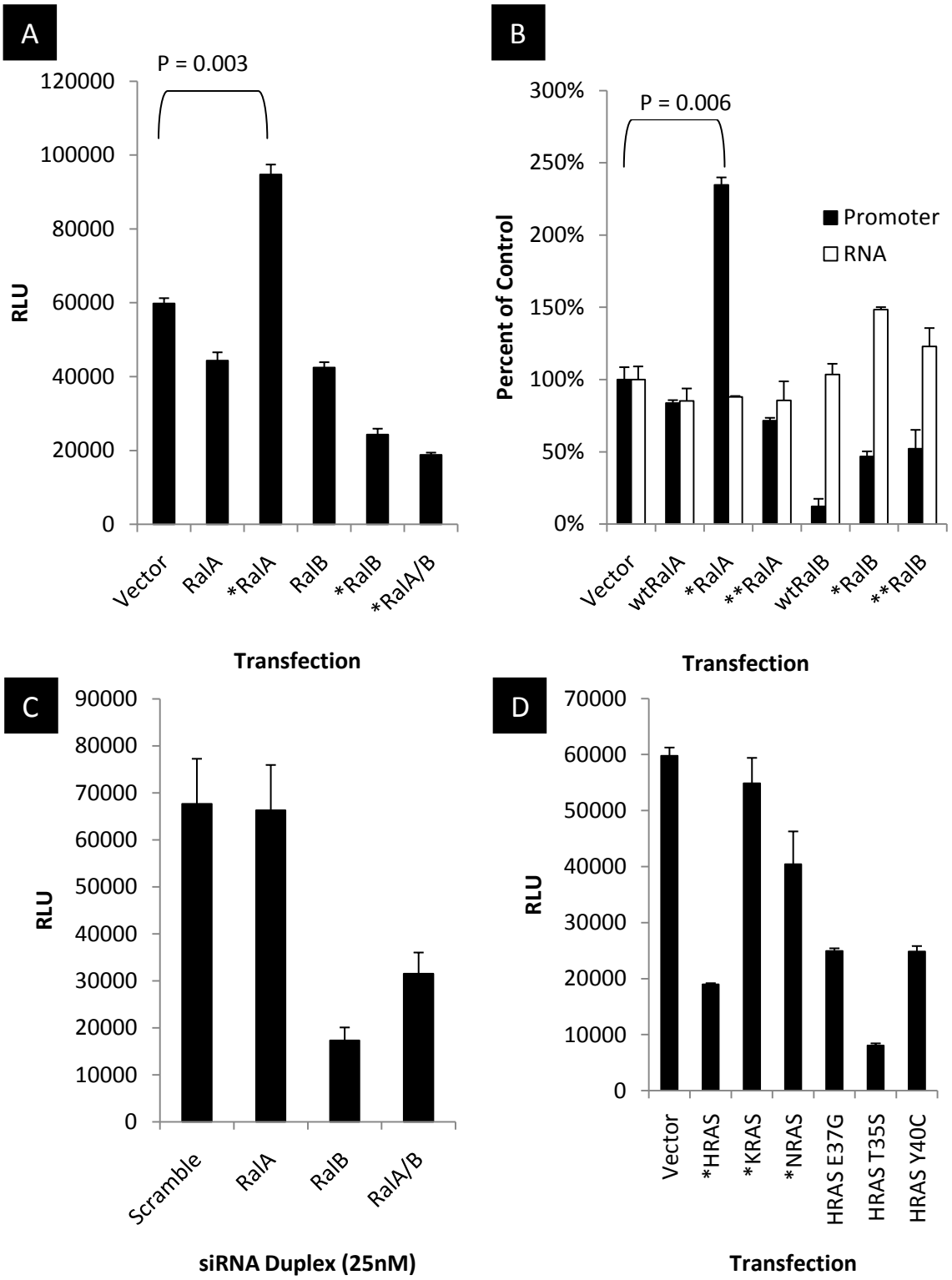
† Z-score of overrepresentation against genomic or promoter background: The distance from the population mean in units of the population standard deviation. Here, the Z-scores are calculated with a continuity correction using the formula z = (x-E-0.5)/S, where x is the number of found matches in the input data, E is the expected value and S is the standard deviation. A Z-score below -2 or above 2 can be considered statistically significant, it corresponds to a P-value of about 0.05.

4.4.5 Ras and Ral affect CD24 promoter activity

Initial observations found CD24 expression dependent on Ral GTPase expression, thus, we tested the effect of Ral manipulation on the activity of the CD24 promoter. The CD24 promoter cloned from the UMUC3 bladder cancer cell line was inserted into the pGL4 luciferase reporter vector. Cotransfection of wild type and constitutively activated mutants of Ral paralogs revealed that only active RalA was capable of increasing CD24 reporter activity (**Figure 26A**). In fact, constitutively active RalB or active RalA and RalB decreased reporter activity nearly 70%. However, RalA (wild type, constitutively active mutant, and fast cycling mutant) transfection decreased the total CD24 RNA levels whereas the opposite was seen for RalB. Wild type, constitutively active mutant, and fast cycling mutant of RalB all increased endogenous CD24 expression but caused a decrease in the CD24 promoter luciferase activity (**Figure 26B**). RalA and RalB were transiently depleted and only the RalB knockdown decreased CD24 promoter activity (**Figure 26C**) confirming the decrease that was observed in endogenous CD24 expression [55]. Because we had conflicting observations between overexpression of the Ral paralogs with the endogenous CD24 RNA, we tested the effect of overexpressing activated Ras on the CD24 promoter. We had previously made the observation that activated HRAS overexpression in UMUC3 cells decreases CD24 expression (unpublished observation) and RREB1 reporter activity [95].

Figure 26. Ral and Ras GTPases regulate the CD24 promoter. A. The 1,896bp CD24 promoter was cloned into the pGL4.2 luciferase reporter vector. The reporter and Ral GTPases were transfection of into UMUC3 cells and incubated for 48 hours prior to determination of luciferase activity. * indicates constitutively active mutants (G23V). Student t-test demonstrates a significant difference (P=0.003) between vector and activated RalA effect on the CD24 promoter reporter. **B.** UMUC3 cells were transfected with the promoter or the RalGTPases. Duplicate transfections were done to assay for either luciferase activity or CD24 RNA expression. * indicates constitutively active mutants (G23V), ** indicates rapid cycling mutants (F39L). Student t-test demonstrates a significant difference (P=0.006) between vector and activated RalA effect on the CD24 promoter reporter. **C.** UMUC3 cells were transfected with scramble (control) or Ral paralog specific siRNA (25nM) [53] 48 hours prior to transfection with the CD24 promoter luciferase vector. 96 hours after siRNA depletion cells were harvested to detect luciferase activity. **D.** Ras GTPases were transfected into UMUC3 cells with the CD24 luciferase reporter 48 hours prior to harvest. The effector mutants are: E37G (Ral pathway), T35S (MAPK pathway), Y40C (PI3K pathway). (Error bars for all experiments were derived from the standard deviation from 2 biological replicates.)

Figure 26


A
P = 0.003
120000
100000
80000
60000
40000
20000
0
RLU
Vector
RalA
*RalA
RalB
*RalB
*RalA/B
Transfection
B
P = 0.006
300%
250%
200%
150%
100%
50%
0%
Percent of Control
Promoter
RNA
Vector
wtRalA
*RalA
**RalA
wtRalB
*RalB
**RalB
Transfection
C
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
RLU
Scramble
RalA
RalB
RalA/B
siRNA Duplex (25nM)
D
70000
60000
50000
40000
30000
20000
10000
0
RLU
Vector
*HRAS
*KRAS
*NRAS
HRAS E37G
HRAS T35S
HRAS Y40C
Transfection

Confirming what had been observed with endogenous CD24 expression, the CD24 promoter activity significantly decreased (**Figure 26D**). The lack of an effect on the CD24 promoter with transfection of activated KRAS (G12V) is not wholly unexpected due to the UMUC3 cells harboring an endogenously mutated KRAS [180]. Further investigation revealed that the Ras effector mutant T35S, which preferentially activates the MEK pathway, had greater repression of CD24 promoter activity than the E37G (Ral pathway) or Y40C (PI3K pathway) mutants.

4.4.6 RREB1 isoform specific regulation of the CD24 promoter

RREB1 was predicted, and later demonstrated, to be regulated by the Ral GTPases [95] CD24 was also found to be dependent their expression [166]. We confirmed through transient siRNA that CD24 expression was also dependent on RREB1 (**Figure 22**). However, overexpression of the RREB1 isoforms has failed to show an increase in endogenous CD24 expression, which does not preclude the possibility that RREB1 may still interact with the CD24 promoter. Therefore, we overexpressed the four RREB1 isoforms found in UMUC3 cells (**Chapter 2**) and measured the CD24 promoter luciferase activity. RREB1β and RREB1ε showed the greatest increases in promoter activity (**Figure 27A**); however, all RREB1 isoforms decreased endogenous CD24 RNA expression. **Figure 27B** compares the increases in promoter activity seen with RREB1 isoform over expression to the changes in endogenous CD24 RNA. Measurement of total RREB1 mRNA expression levels after transfection confirmed successful transfection and approximately equal expression between isoforms (**Figure 27C**).

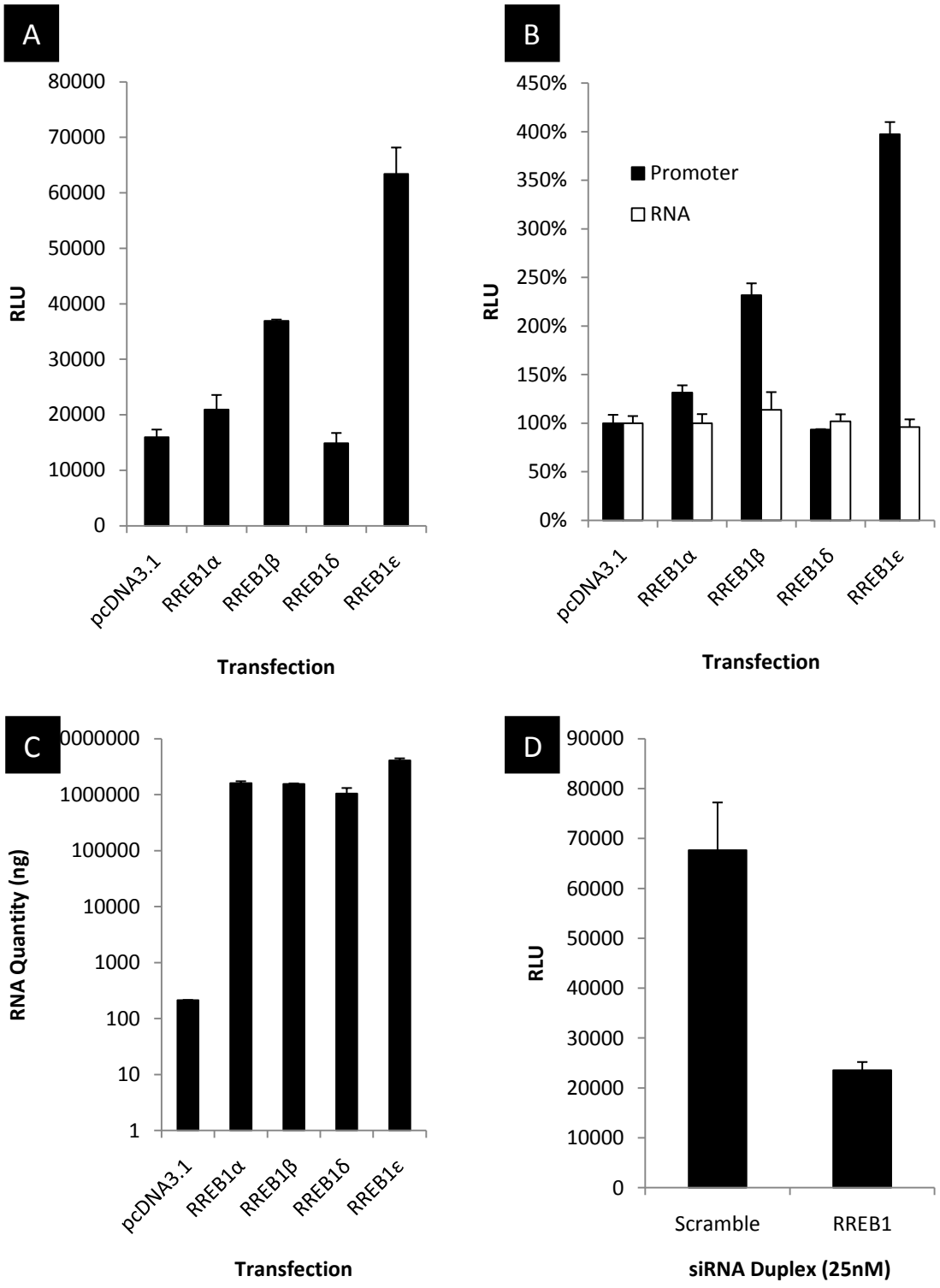
Despite superphysiologic levels of RREB1 RNA the end effect on the CD24 promoter produced a mere 2-4 fold increase in activity and no change in endogenous CD24 transcription. However, similar to what was seen with the Ral GTPases, RREB1 siRNA decreased CD24 promoter activity and reduced endogenous RNA (**Figures 27D** and **22A**).

4.4.7 The CD24 promoter contains a functional hypoxia response element

As shown in **Table 4**, the CD24 promoter has a hypoxia response element (HRE) 24 base pairs prior to the published transcription start site. An HRE is a consensus sequence that is bound by HIF1A or HIF2A after they heterodimerize with ARNT [163]. The HIF (**H**ypoxia **I**nducible **F**actor) quantity in a cell is typically controlled through protein stability not RNA transcription. In normal oxygen tension, HIF proteins are hydroxylated by prolyl hydroxylases and sequestered for proteosomal degradation by VHL. However, in low oxygen levels (hypoxia) the non-hydroxylated HIF proteins bind the chaperone protein HSP90 and are shuttled to the nucleus where they dimerize to the constitutively expressed ARNT and bind HREs in DNA [164].

Figure 27. RREB1 isoforms beta and epsilon regulate the CD24 promoter. **A.** RREB1 isoforms were transfected with the CD24 promoter luciferase reporter 48 hours prior to harvest. **B.** RREB1 isoforms were transfected with the CD24 promoter into duplicate UMUC3 cells to measure reporter activity and CD24 RNA levels. **C.** Total RREB1 RNA levels were measured by Q-RTPCR 48 hours after transfection with the RREB1 isoforms. **D.** CD24 promoter luciferase reporter activity measured 96 hours after siRNA depletion of scramble (control) or RREB1 and 48 hours after transfection with the luciferase vector.

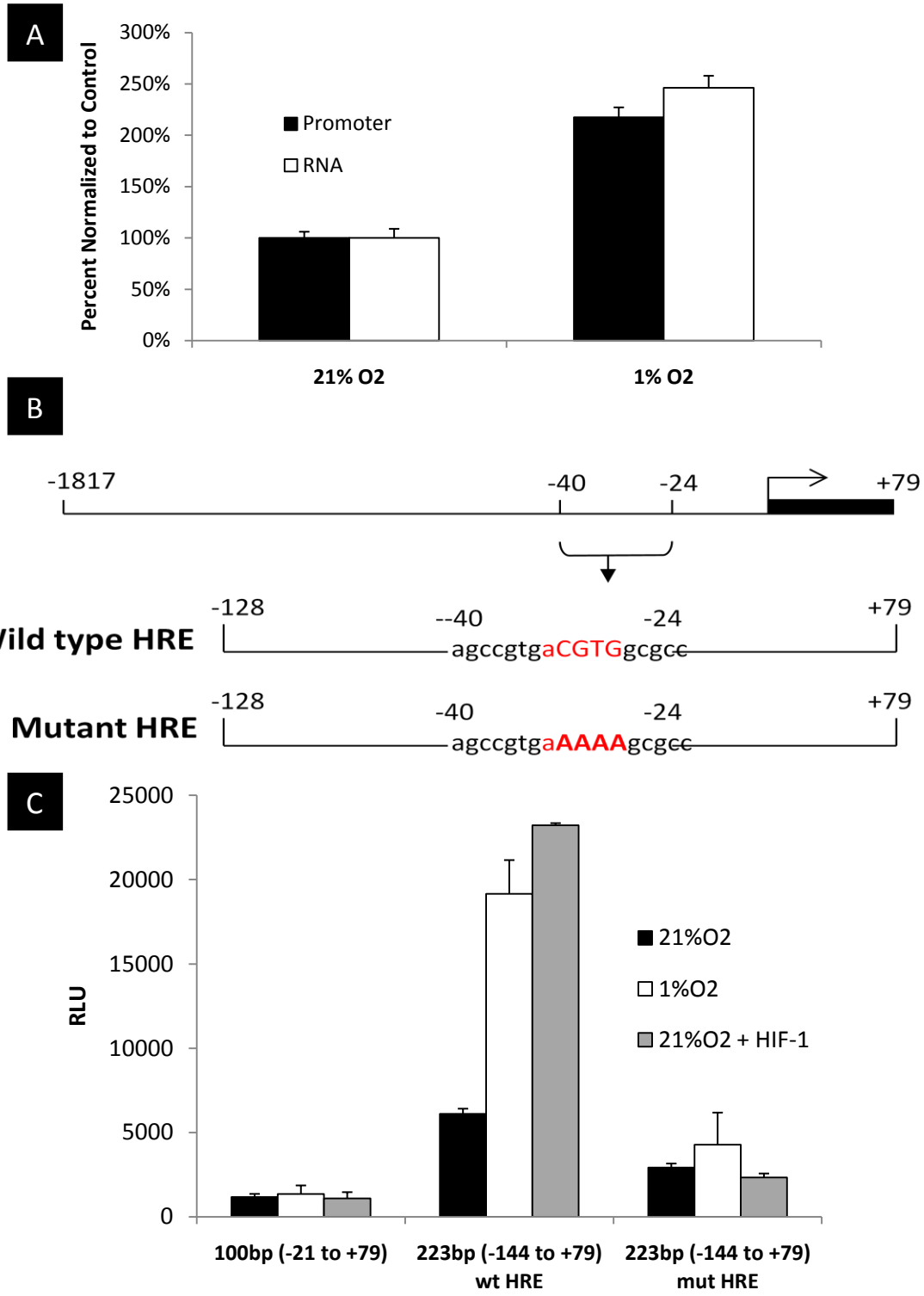
Figure 27


A
80000
70000
60000
50000
40000
30000
20000
10000
0
RLU
pcDNA3.1
RREB1α
RREB1β
RREB1δ
RREB1ε
Transfection
B
450%
400%
350%
300%
250%
200%
150%
100%
50%
0%
Promoter
RNA
RLU
pcDNA3.1
RREB1α
RREB1β
RREB1δ
RREB1ε
Transfection
C
1000000
100000
10000
1000
100
10
1
RNA Quantity (ng)
pcDNA3.1
RREB1α
RREB1β
RREB1δ
RREB1ε
Transfection
D
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
RLU
Scramble
RREB1
siRNA Duplex (25nM)

To test the consensus HRE in the CD24 promoter we incubated UMUC3 cells in 1% oxygen for 24 hours, 24 hours after transfection with the CD24 promoter. **Figure 28A** shows that hypoxia doubled CD24 promoter activity and endogenous CD24 RNA. Knowing the CD24 promoter luciferase vector was responding similar to the endogenous in hypoxia, we next asked whether the HRE identified was responsible for the hypoxia induced increase in promoter activity. The core HRE sequence: CGTG, was mutated to: AAAA to remove the HIF DNA binding recognition site. This was done in a 231bp section of the CD24 promoter that contains the HRE (**Figure 28B**). The mutated CD24 promoter activity was severely diminished in hypoxia compared to the non-mutated CD24 promoter activity (**Figure 28C**). To determine if HIF1A was responsible for the increased promoter activity seen in hypoxia, HIF1A was transfected into normoxic UMUC3 cells with the 231bp CD24 promoter. **Figure 28C** shows that overexpressed HIF1A was able to recapitulate the increase in promoter activity seen in hypoxia. These data suggest that CD24 is a hypoxic responsive gene.

Figure 28. Hypoxia and HIF1A increase CD24 promoter activity. A. UMUC3 cells were transfected with the CD24 promoter luciferase reporter 24 hours prior to treatment with 1% O_2. After 24 hours of hypoxia (1% O_2) the luciferase activity and CD24 RNA expression was measured. **B.** The hypoxia responsive element in the CD24 promoter was mutated from CGTG to AAAA. **C.** Transfection of UMUC3 cells with the wild type or mutant HRE CD24 promoter reporter 24 hours prior to exposure to hypoxia. UMUC3 cells were also transfect with the CD24 reporter and HIF1A without exposure to hypoxia and luciferase activity was measured 48 hours after transfection.

Figure 28


A
300%
250%
200%
150%
100%
50%
0%
Percent Normalized to Control
Promoter
RNA
21% O2
1% O2
B
-1817
-40
-24
+79
-128
--40
-24
+79
Wild type HRE
agccgtgaCGTGgcgcc
-128
-40
-24
+79
Mutant HRE
agccgtgaAAAAgcgcc
C
25000
20000
15000
10000
5000
0
RLU
21%O2
1%O2
21%O2 + HIF-1
100bp (-21 to +79)
223bp (-144 to +79) wt HRE
223bp (-144 to +79) mut HRE

4.5 Discussion

Cancer remains the second leading cause of deaths in the United States but is projected to be the top killer in the near future due to improvement in the management of chronic heart disease. The majority of deaths occur due to systemic metastasis. Recently, hypoxia has emerged as a driver and mediator of metastatic cancer with HIF1A and HIF2A shown to play important roles in this process [181]. The identification of a hypoxia responsive element in the promoter of CD24 suggests that its expression may increase due to hypoxic stress.

There exists considerable ambiguity surrounding the DNA sequence of the CD24 promoter. The official NCBI GenBank DNA sequence for CD24 lacks a 204 base pair region encompassing the 5' UTR and 100 base pairs of the promoter. Published sequences, while more complete, further complicate the picture due to a high frequency of dissimilarity between each other and the GenBank sequence. Thus, there was significant need for proper cloning and sequencing of the CD24 promoter to bring clarity and tools to this field. We cloned an 1896 base pair DNA fragment from UMUC3 cells that extended from 1817bp upstream to 79bp downsteam of the transcription start site. We also cloned the same region from six additional cell lines to determine sequence variability. The promoters cloned by our lab had at least three fold less variability compared to the previously published sequences, with 4 out of 6 having identical CD24 promoter sequences. The majority of sequence variability was found in the GC rich region, which could be due to sequencing error, deamination of

methylated cytosine, replication error in bacteria, or cloning artifact. CpG islands, similar to the one that encompasses 970 base pairs of the CD24 promoter, are areas reported to be methylated in silenced genes [182]. However, treatment of UMUC3 cells with the methyltransferase inhibitor 5-Azacytadine did not alter the expression of CD24. We interpreted this result to suggest that the CD24 promoter, in the UMUC3 cell line at least, was not transcriptionally regulated through promoter methylation. To further address this question we could bisulfite sequence the CD24 promoter, which allows for identification of methylated cytosines.

Hundreds of transcription factor binding sites were found to lie within the CD24 promoter. We chose to focus on two, RREB1 and HIF1A, due to prior evidence linking these two transcription factors with CD24 expression [95, 142]. However, several transcription factors listed in **Table 4** have prior associations with gene transcription in malignant cells. Further investigation into importance of these transcription factors in CD24 expression is warranted.

Because our lab had initially arrived at CD24 due to its dependency on Ral expression, we examined the activity of the CD24 promoter after Ral manipulation. We found that CD24 promoter activity did not correspond to RNA expression when RalA or B was over expressed. However, CD24 promoter activity and RNA showed excellent concordance when Ral was depleted. Similar results were found with RREB1. Specific isoforms of RREB1, when over expressed, caused significant increases in CD24 promoter activity without an increase in endogenous CD24 RNA. Mutation of the RREB1 site was also not

capable of abrogating the increased activity after RREB1 overexpression [166]. But, transient depletion of RREB1 showed concordant decrease in CD24 promoter activity and endogenous RNA. The discordance seen between CD24 promoter/RNA with Ral and RREB1 over expression likely reflects the complex biological mechanisms regulating transcription at this gene. It is possible that the transgene promoter used in these studies does not accurately reflect the endogenous promoter due to epigenetic modifications present on the endogenous promoter, other upstream or downstream promoter elements not present on the isolated sequence, or enhancer sequences that are only present in the native DNA.

Unlike Ral and RREB1, hypoxia and specifically HIF1A, was capable of increasing CD24 promoter activity and RNA levels. Mutation of the HIF1A site successfully diminished the hypoxia induced effect confirming its necessity in the CD24 hypoxic response. The increase of CD24 in response to hypoxia, where most genes are down regulated, raises the teleological question of CD24's role in hypoxia. Further investigation into answering this question may lead to novel ideas and avenues of treatment modalities. Furthermore, it is not lost upon the authors that the connection of CD24 and hypoxia and HIF1A could also link the Ral GTPases and RREB1 to the hypoxic response.

We attempted to address the connection between RREB1, CD24, and the UMUC3 growth phenotype in this study. Independently, both RREB1 and CD24 depletion cause a decrease in cell number (approximated by Alamar Blue metabolism) over time. Furthermore, we know that CD24 expression decreases

with RREB1 depletion. Thus, could the decreased CD24 expression in RREB1 depleted cells be responsible for decreased UMUC3 growth? We attempted to address this question by stably expressing CD24 in UMUC3 cells prior to RREB1 siRNA exposure. The logic presumed RREB1 regulated CD24 at the transcriptional level and that RREB1 depletion would not affect the transcription or translation of a CMV driven transgene. To our surprise, RREB1 depletion decreased CD24 expression in the stable cell line more than anticipated. We attributed this decrease to the loss of endogenous CD24 but we cannot rule out that loss of RREB1 also affect the expression of the transgene. Anecdotal observations with other transgenes in RREB1 knockdown cells have shown less expression than the control when normalized for total protein. These observations may hint that RREB1 may participate in more cellular functions than currently known.

As interest in CD24 continues to increase and our knowledge evolves to better understand its function, there will be more incentive to understanding the mechanism of its overexpression. Here we provide the first large scale characterization of the CD24 promoter from several different cell lines. These tools allowed us to examine and discover a novel regulator of CD24 expression, HIF1A. Further examination into these findings could lead to novel and exciting associations in the regulation of CD24.

Chapter 5: RREB1 binds and stabilizes the HIF1A transcription factor

Matthew D. Nitz, Michael A. Harding, Shibu Thomas, Steven C. Smith, and Dan Theodorescu

Contributors

Matthew D. Nitz, UVA Department of Molecular Physiology and Biological Physics, designed and performed all experiments described below, analyzed the data, and wrote the manuscript.

Dr. Michael A. Harding, UVA Department of Urology, contributed substantially to the study design and preparation of the manuscript

Dr. Shibu Thomas, UVA Department of Urology, helped with the design of all hypoxia experiments.

Dr. Steven C. Smith, UVA Department of Urology, provided statistical expertise, contributed to the study design, and preparation of the manuscript.

Dr. Dan Theodorescu, UVA Department of Urology and Molecular Physiology and Biological Physics, provided resources, guidance, and advice on the design and interpretation of experiments.

5.1 Abstract

Hypoxia induced factor 1 alpha (HIF1A) plays an important role in the ability of cancer to survive in low oxygen environments and has been found to promote invasion, metastasis, and chemoresistance resulting in decreased patient survival. CD24 is a heavily glycosylated, cell surface protein that is overexpressed in cancer whose expression has recently been identified to be induced in hypoxic conditions. RREB1, the C_2H_2 zinc-finger domain protein, is necessary for CD24 expression but is not sufficient to drive its transcription. The mechanism of RREB1 mediated CD24 expression remains unknown. To that end, we hypothesized that RREB1 mediates the hypoxia induced transcriptional response of the cell. CD24 and two additional hypoxia responsive genes (VEGF and IGFBP3) failed to increase mRNA levels in hypoxia after depletion of RREB1. HIF1A mRNA expression was not affected by depletion of RREB1 but RREB1 is necessary for HIF1A protein stability, and reciprocally, RREB1 protein is dependent on HIF1A. Depletion of RREB1 decreases the occupancy of HIF1A on the CD24 promoter, which may explain how RREB1 affects CD24 expression levels. Finally, depletion of HIF1A abrogates UMUC3 cell growth, mimicking the phenotype seen with RREB1 depletion and confirming the necessity of HIF1A for maintenance of bladder cancer cell growth.

5.2 Introduction

Hypoxia is the result of inadequate supply of oxygen within the body. A multitude of medical conditions can give rise to hypoxia, including atherosclerosis, vascular stenosis, vascular trauma, chronic obstructive pulmonary disease, shock and a variety of other pathological processes. Most notable for this discussion, the rapid growth of tumors and the aberrant and inadequate formation of a neovasculature to support them often lead to local areas of hypoxia within the tumor and surrounding tissue. If severe, hypoxia can result in cell death and organ failure. Hypoxia does play a role in normal biology in the case of tissue growth, where it stimulates formation of new blood vessels. Certain organs, such as the liver, function chronically at a lower oxygen tension than other more oxygen intensive organs, such as the brain [117, 118]. The typical response by a cell when exposed to hypoxia is secretion of factors to increase flow of blood to area (angiogenesis), decrease non-essential cellular processes, and switch energy metabolism from oxygen demanding oxidative respiration to anaerobic glycolysis [119].

Tumors have long been recognized for having lower oxygen tension than normal tissue [124]. Decreased oxygen tension results in a number of different cellular changes, the best studied is the stabilization of the HIF proteins, HIF1A and HIF2A. HIF1A RNA is constitutively expressed but the cellular quantity is regulated at protein level. In normal oxygen tension (normoxia) HIF1A protein is hydroxylated by prolyl hydroxylases (PHDs), bound by the tumor suppressor

VHL, and degraded by the proteasome [124]. In hypoxia, the PHDs no longer hydroxylate HIF1A, which allows it to form a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT), translocate to the nucleus, and bind gene promoters containing a hypoxia response element (HRE) [124]. Genes, whose expression increase during hypoxia, include angiogenesis, glycolysis and glucose uptake, anti-apoptosis, and growth factors. Hypoxia has also been correlated with metastasis, invasion, and poor prognosis [122, 123].

In bladder cancer, hypoxia and HIF1A have shown positive tumor promoting and negative clinical effects. Vascular endothelial growth factor (VEGF) is a well studied hypoxia responsive gene that can be measured in the urine of bladder cancer patients [183] and has been shown to mediate angiogenesis [184]. VEGF expression is also highly correlated with poor outcome in invasive bladder cancer [185]. Furthermore, HIF1A expression has been shown to be negatively prognostic in bladder cancer [37, 186]. Thus, hypoxia and its downstream sequelae appear to be an important mediator of this disease.

CD24 is a 27 amino acid, cell surface glycoprotein that is widely overexpressed in cancers, including bladder cancer [68]. Elevated CD24 in bladder cancer significantly correlates with increased invasiveness [67] and poor survival [55]. CD24 has been found by mass spectrometry to be upregulated in endothelial cells after exposure to hypoxia [142]. We recently identified an HRE in the CD24 promoter and demonstrated that reporter activity and endogenous RNA expression increase in the bladder cancer cell line UMUC3 after exposure to hypoxia (**Chapter 4**, **Figure 28**). RREB1 (**R**as-**R**esponsive **E**lement **B**inding

protein **1**) is a putative C_2H_2 zinc-finger transcription factor that, when depleted in bladder cancer cell lines, abrogates CD24 expression but is insufficient to drive CD24 RNA transcription (**Chapter 4, Figure 22**).

There are no direct reports indicating that RREB1 may play a role in hypoxia, however, RREB1 does regulate the expression of MT2A, a gene known to be upregulated in hypoxia [143]. Furthermore, RREB1 has been shown to bind the basic helix-loop-helix transcription factor NEUROD [101]. HIF1A also is a basic helix-loop-helix transcription factor [144]. Therefore, we hypothesized that RREB1 is necessary for CD24 expression through modulating the hypoxia transcriptional response. In this study we demonstrate that RREB1 is necessary for expression of hypoxia responsive genes and HIF1A protein. Depletion of RREB1 leads to a loss of HIF1A protein and decreased occupancy of the CD24 promoter.

5.3 Materials and Methods

5.3.1 Cell lines, growth conditions and clones

Human cancer cell lines were grown in the following conditions: UMUC3: Minimum essential media (MEM) (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco®, Invitrogen, Carlsbad, CA) and 1% sodium pyruvate (Gibco®, Invitrogen, Carlsbad, CA). Actinomycin D (Sigma-Aldrich, St. Louis, MO) was added to UMUC3 cells for a final concentration of 5µg/ml. For hypoxia experiments, cells were placed in a

hypoxia chamber (Billups-Rothenberg, Del Mar, CA) and flushed with the gas mixture: 94% N_2, 5% CO_2, and 1% O_2 for five minutes prior to the chamber being sealed. Cells were incubated in the chamber for 24 hours at 37^{o}C. pcDNA3.0 HIF1A was a gift from G. Semenza and was described previously [167]. A 1,896 base pair genomic DNA sequence encompassing the CD24 promoter (-1817 to +79) was cloned out of UMUC3 DNA into the pGL4.2 luciferase vector (Promega) as described previously (**Chapter 4**).

5.3.2 RNA isolation, Reverse transcription, and Q-RTPCR

RNA isolation was carried out using an RNeasy® Mini Kit (Qiagen) and cDNA synthesis using iScript™ (Biorad). Q-RTPCR was performed on cDNA using the iQ™ SYBR® Green Supermix (Biorad). Primers with annealing temperatures: RREB1 (total) (62^{o}C) F:5'-CTT-CCT-ATA-ACT-GCC-CCC-3'; R:5'-ATG-AGT-GGT-CGG-CTC-CTC-C-3'; CD24 (62^{o}C) F:5'-CAA-TAT-TAA-ATC-TGC-TGG-AGT-TTC-ATG-3'; R:5'-TCC-ATA-TTT-CTC-AAG-CCA-CAT-TCA-3'; VEGF (62^{o}C) F:5'-AGA-AGG-AGG-AGG-GCA-GAA-TCA-T-3', R5'-ACA-GGA-TGG-CTT-GAA-GAT-GTA-CTC-G-3'; IGFBP3 (64^{o}C) F:5'-GCG-CTA-CAA-AGT-TGA-CTA-CGA-GTC-T-3', R:5'-CCA-TTT-CTC-TAC-GGC-AGG-GAC-3'; BNIP3L (58^{o}C): F:5'-CAA-CAA-CAA-CAA-CAA-CTG-CGA-GGA-A-3', R:5'-TTA-TCA-TTG-CCA-TTG-CTG-CTG-TTC-A-3'; CD24 promoter (58^{o}C)(supplemented with 1M Betaine Solution (Sigma-Aldrich) and 5% DMSO) F:5'-ACG-GCT-ATT-GTG-GCT-TTC-CTG-GTA-T-3', R:5'-GCT-TGG-AGA-ACC-GCT-GGC-TC-3'; HIF1A

(58^{o}C) F:5'- TGA-GGA-AAT-GAG-AGA-AAT-GCT-T-3', R:5'- TCA-TAG-TTC-TTC-CTC-GGC-TAG-T-3'.

5.3.3 Antibodies, Western Blotting, and ChIP

The following antibodies were used: RREB1 (Sigma-Aldrich, Cat. no: HPA001756), Tubulin (Santa Cruz Biotechnology, Cat. no: sc-58668), CD24 (antibody SWA11, a gift of Dr. Peter Altevogt, Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany), TBP (TFIID)(Santa Cruz Biotechnology, Santa Cruz, CA, Cat. no: sc-204), HIF1A (BD Biosciences, San Jose, CA). Immunoblotting and detection were performed as described [53]. Chromatin Immunoprecipitation (ChIP) was performed using the *ChIP*-IT™ Express kit (Active Motif, Carlsbad, CA) according to manufacturer instructions. Antibodies used for ChIP: Rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), RNA Pol II (Active Motif), and HIF1A (Novus Biologicals, Littleton, CO, Cat. no: NB100-134)

5.3.4 Transfection, siRNA, and Luciferase assays

Transient vector transfection was performed using FuGENE®6 Transfection Reagent (Roche) at a ratio of 1µg of DNA per 3µl of FuGENE. Oligofectamine™ (Invitrogen) was used to transiently transfect siRNA according to manufacturer instructions. All siRNA was transfected at a final concentration of 25nM unless otherwise noted: RREB1 total: 5'-GGA-GUU-UGU-UUG-CAA-GUA-U-3' and 5'-GUU-CAG-ACC-UAU-CUU-CCA-U-3' (used in combination at

12.5nM); GL2: 5'-CGU-ACG-CGG-AAU-ACU-UCG-AdTdT-3'; RREB1 Exon 7 5'-CCA-ACA-AGU-UCA-GUC-CGU-U-3'; RREB1 Exon 8 5'-CCU-GAG-AAG-AAA-CGG-GCU-UUU-3'; RREB1 Exon 9 5'-CAG-AGA-AGA-GCG-ACG-AUG-AdTdT-3', HIF1A: 5'-CAA-AGU-UCA-CCU-GAG-CCU-AdTdT-3'; HIF2A: 5'-GCA-AAU-GUA-CCC-AAU-GAU-AdTdT-3'. For luciferase assays, cells were lysed in 1X Report Lysis Buffer (Promega, Madison, WI) and assayed for light expression using the Luciferase Assay System (Promega) on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT).

5.3.5 *In vitro* growth assays

In vitro cell growth assays were carried out by using Cyquant® Cell Proliferation Assay (Invitrogen) according to manufacturer instructions. Briefly, replicate plates were made for each 24 hour time point. Cells were harvested every 24 hours after siRNA knockdown with GL2 or HIF1A and stored at -80^{o}C. After the last time point, all cells were thawed at room temperature and lysed using Cyquant® Cell Lysis Buffer. Cell lysates were diluted 1:200 and added to the Cyquant® GR Dye and measured at 480/520 (excitation/emission) on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT).

5.4 Results

5.4.1 Depletion of RREB1 decreases RNA expression of hypoxia responsive genes.

To determine if RREB1 modulates the broader hypoxia response, we transfected UMUC3 cells with RREB1 siRNA prior to hypoxia exposure (1% O_2) to examine the change in RNA expression of two additional hypoxia responsive genes, namely VEGF-A, and IGFBP3. **V**ascular **E**ndothelial **G**rowth **F**actor A (VEGF) is the classic angiogenic growth factor, whose expression in response to hypoxia has been extensively studied a chemotherapeutic target in cancer treatments [187]. **I**nsulin-like **G**rowth **F**actor **B**inding **P**rotein **3** (IGFBP3), another secreted protein, whose function in cancer is still being elucidated [188], has been be identified to be upregulated bladder cancer cells exposed to hypoxia [186] [141]. **Figure 29A** shows an increase in mRNA expression for all three genes after exposure to hypoxia. However, RREB1 depletion decreased expression off all genes in hypoxia.

The canonical regulation of hypoxic gene expression is mediated by the transcription factor Hypoxia Inducible Factor (HIF). HIF is a heterodimer composed of an oxygen-sensitive alpha subunit, either HIF1alpha (HIF1a) or HIF2alpha (HIF2a), and a constitutively expressed beta subunit, ARNT (Aryl-hydrocarbon Receptor Nuclear Translocator). To confirm that hypoxic gene expression was mediated through these two transcription factors we depleted both in UMUC3 cells prior to exposure to hypoxia. HIF1A depletion blocked the response of CD24 and IGFBP3, while VEGF was unaffected (**Figure 29B**).

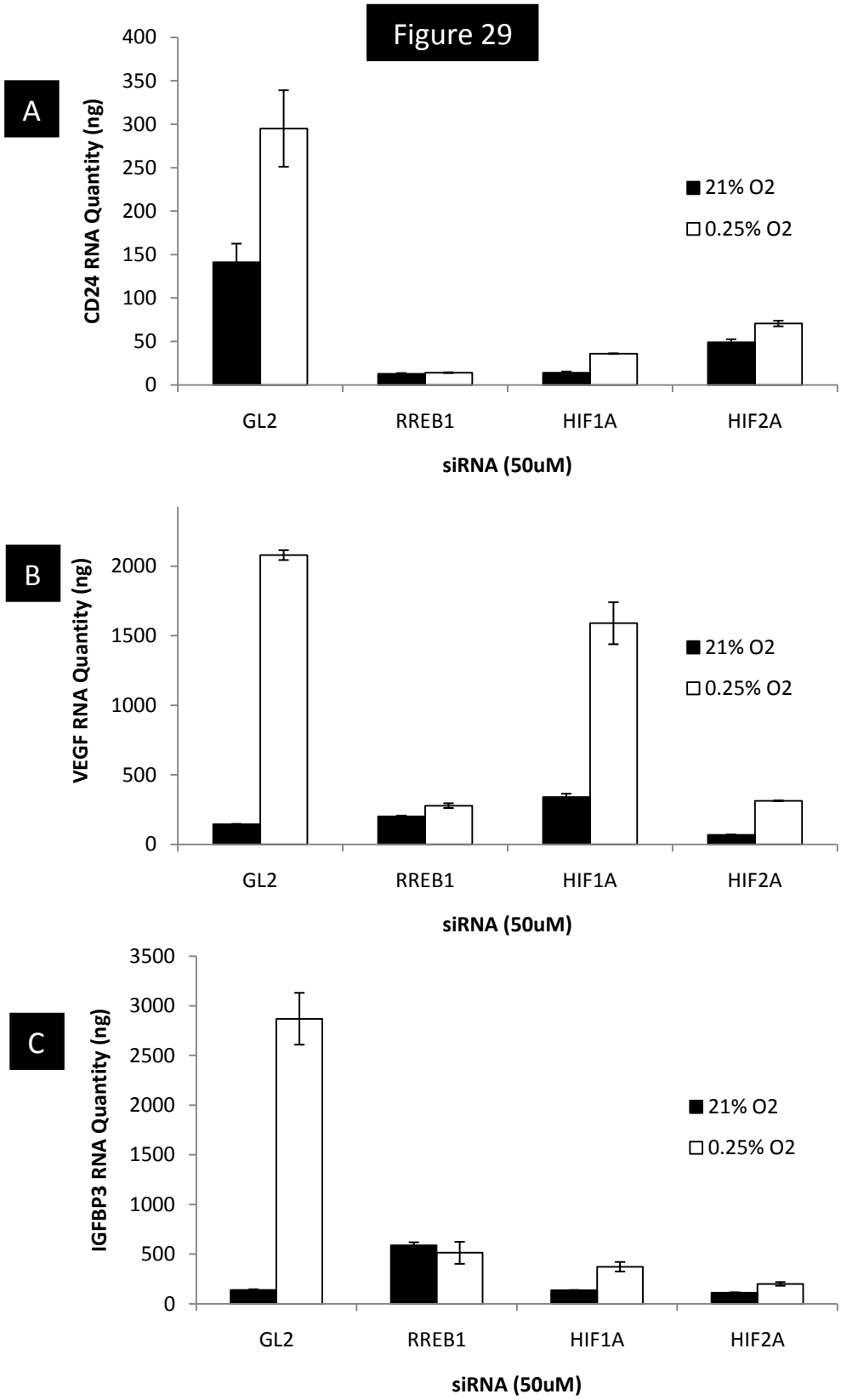
Depletion of HIF2A also decreased expression of CD24 and IGBP3, but also decreased VEGF levels (**Figure 29C**). Hypoxia induced gene expression in UMUC3 cells appears to be mediated by both HIF1A and HIF2A, and dependent on RREB1.

5.4.2 RREB1 affects hypoxia-responsive mRNA transcription, not mRNA stability

Next, we asked whether RREB1 affected the mRNA transcription or stability. RREB1 is a nuclear protein with up to 15 canonical C_2H_2 zinc finger domains, depending on the splice variant (Chapter 2). These domains are classically reported to bind DNA; however, emerging research has demonstrated their ability to interact with RNA and other proteins. In fact, C_2H_2 zinc-finger domain proteins can bind RNA to stabilize the message by preventing RNase degradation [189]. Therefore, it is possible that RREB1 may bind the RNA of these hypoxia induced genes to increase their longevity; which results in decreased RNA levels when RREB1 is depleted.

Figure 29. RREB1 decreases expression of hypoxia responsive genes. RNA expression levels of hypoxia responsive genes were measured using Q-RTPCR in UMUC3 cells grown in normoxic or hypoxic conditions (21% O_2 vs. 1% O_2, respectively). The following siRNA duplexes were transfected into the UMUC3 cells at 25nM: GL2, **A.** RREB1, **B.** HIF1A, and **C.** HIF2A.

Figure 29


A
CD24 RNA Quantity (ng)
400
350
300
250
200
150
100
50
0
21% O2
0.25% O2
GL2
RREB1
HIF1A
HIF2A
siRNA (50uM)
B
VEGF RNA Quantity (ng)
2000
1500
1000
500
0
21% O2
0.25% O2
GL2
RREB1
HIF1A
HIF2A
siRNA (50uM)
C
IGFBP3 RNA Quantity (ng)
3500
3000
2500
2000
1500
1000
500
0
21% O2
0.25% O2
GL2
RREB1
HIF1A
HIF2A
siRNA (50uM)

UMUC3 cells were transfected with GL2 or RREB1 siRNA (25nM) 72 hours prior to hypoxia exposure. Immediately after removal from hypoxia, actinomycin D was added to the media (10μM) to inhibit transcription. RNA was harvested over the course of 10 hours to measure the rate of decay. Q-RTPCR revealed that RREB1 did not affect the rate of RNA decay of VEGF or CD24 (**Figures 30A and 30B**, respectively) leading us to conclude that the mechanism of RREB1 is not through RNA stability.

The most likely mechanism for RREB1 dependant hypoxia gene expression entails RREB1 participation in gene transcription. If RREB1 affects transcription, the expectation would be that RREB1 regulates the promoter activity of the hypoxic responsive gene. The CD24 promoter was cloned from UMUC3 cells into the pGL4.20 luciferase reporter vector (Chapter 4).

Depletion of RREB1 in UMUC3 cells containing the CD24 promoter showed lower reporter activity than the control (GL2) cells (**Figure 30C**); making it likely that RREB1 is necessary for hypoxia induced transcription. However, overexpression of RREB1 isoforms did not increase CD24 expression (Chapter 4, **Figure 27**). Therefore, the data suggest RREB1 is necessary for CD24 transcription but not sufficient.

5.4.3 HIF1A and RREB1 protein levels are reciprocally dependant

If RREB1 is not sufficient to drive CD24 expression, then perhaps it interacts with a protein that is capable. We have previously observed HIF1A overexpression increases CD24 promoter activity and RNA levels in normoxia

(Chapter 4, **Figure 28C**, and data not shown). We hypothesized that RREB1 may function in hypoxic gene expression by interacting with the HIF1A transcription factor. This idea is bolstered by the previous finding that RREB1 binds the basic helix-loop-helix transcription factor NEUROD *in vivo* [101]. HIF1A is a basic helix-loop-helix transcription factors [144].

The previous finding of RREB1 mediated hypoxic transcription (**Figures 29A and 30C**) made it possible that HIF1A RNA transcription was dependent on RREB1. HIF1A is constitutively expressed at the RNA level while expression is typically controlled through protein stability. But, a loss of HIF1A RNA expression would preclude any hypoxic HIF1A protein induction. RREB1 was depleted in UMUC3 cells and Q-RTPCR was used to determine HIF1A expression levels. RREB1 transient depletion (**Figure 31A**) did not decrease HIF1A RNA (**Figure 31B**). Furthermore, HIF1A transient depletion (**Figure 31C**) did not have an effect on RREB1 RNA (**Figure 31D**).

Because HIF1A is regulated at the protein level, we asked whether RREB1 had an effect on HIF1A protein stability. RREB1 and HIF1A were depleted in UMUC3 cells prior to hypoxia exposure.

Figure 30. Depletion of RREB1 affects RNA transcription, not RNA stability. **A.** UMUC3 cells were transfected with GL2 or RREB1 siRNA 96 hours prior to addition of 5μM of Actinomycin D. RNA was harvested at specific time points and Q-RTPCR was used to determine the amount of VEGF RNA. **B.** Q-RTPCR for CD24 after addition of Actinomycin D. **C.** CD24 promoter luciferase reporter activity after siRNA depletion of RREB1.

Figure 30

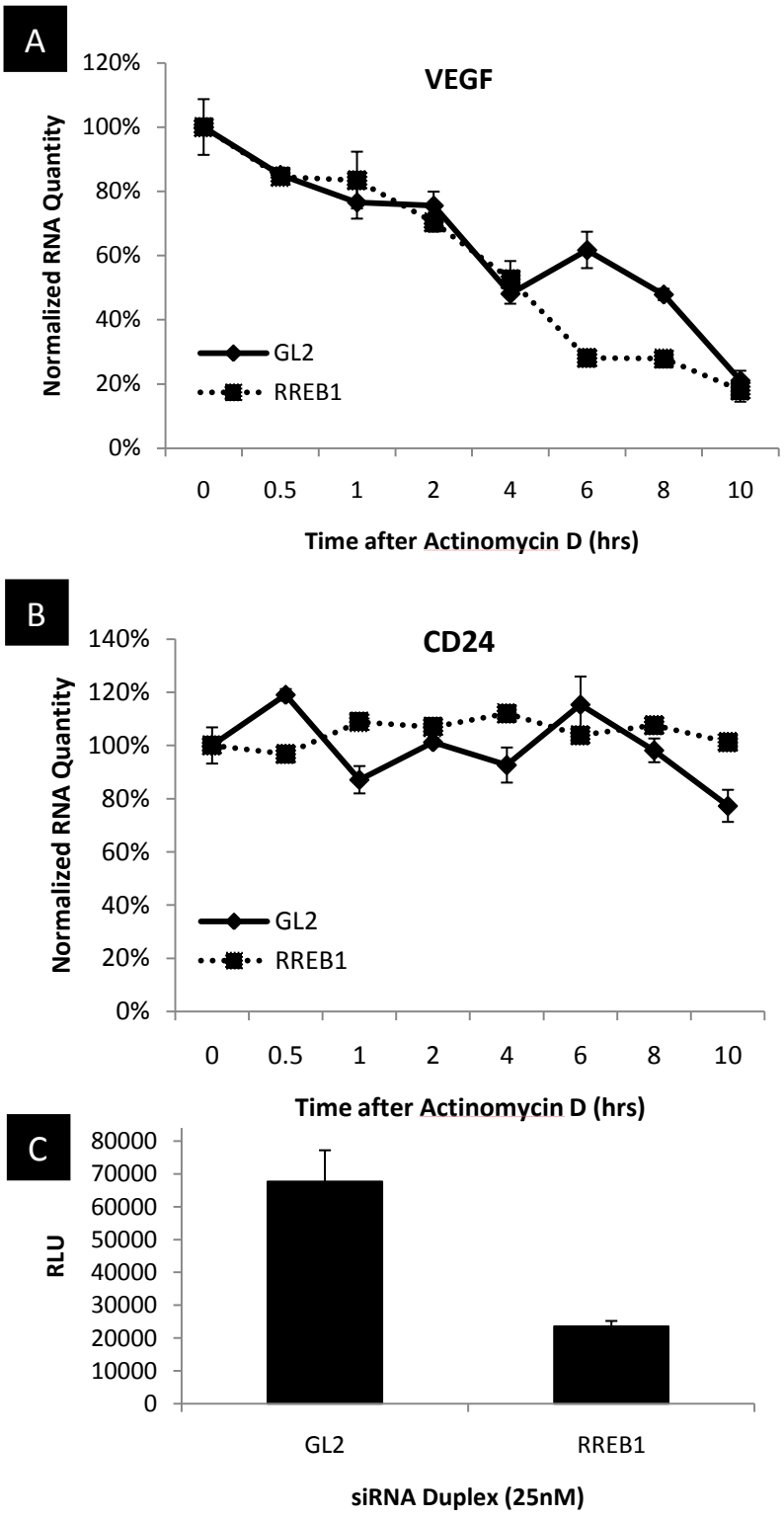
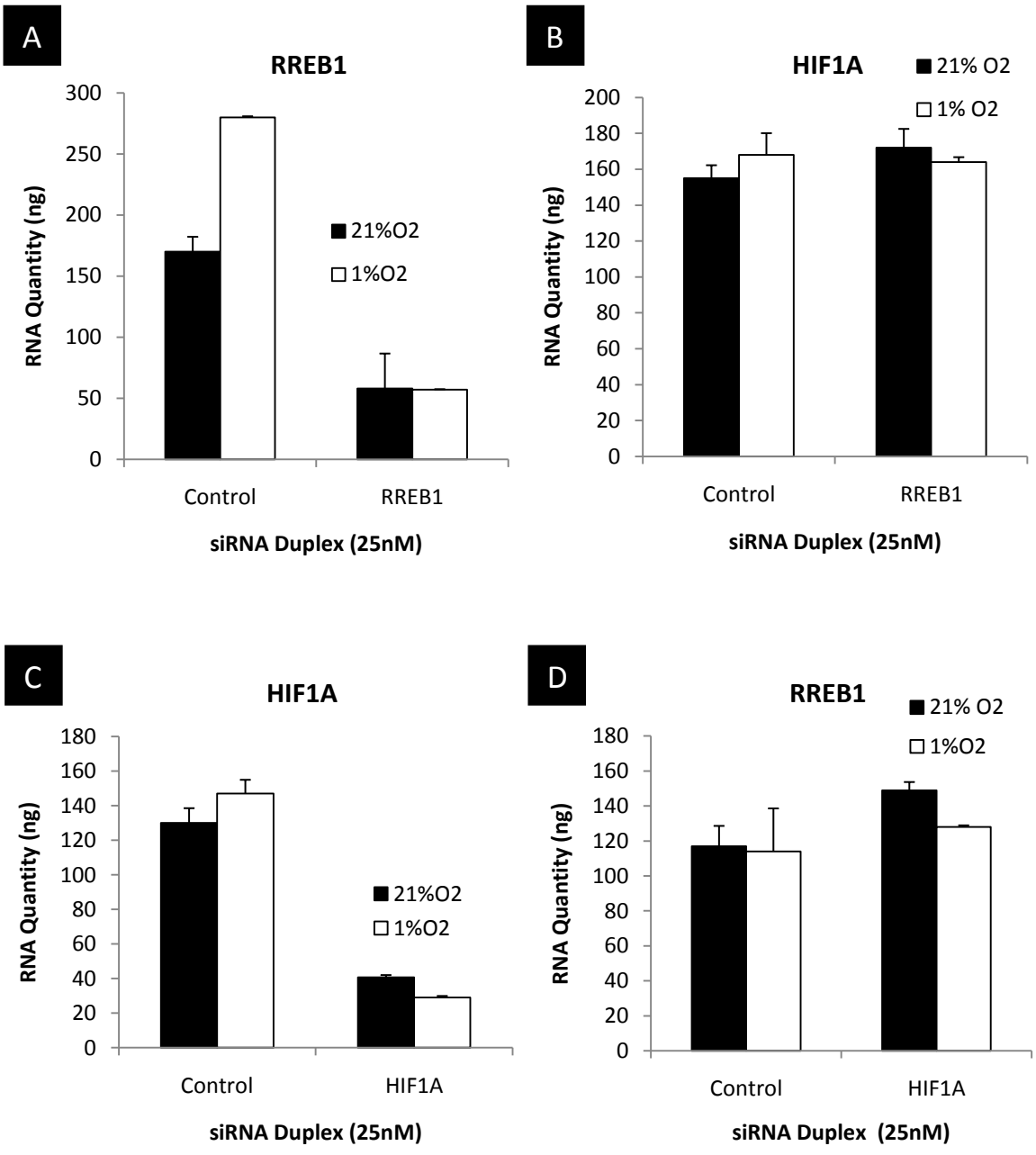

A
VEGF
120%
100%
80%
60%
40%
20%
0%
Normalized RNA Quantity
GL2
RREB1
0
0.5
1
2
4
6
8
10
Time after Actinomycin D (hrs)
B
CD24
140%
120%
100%
80%
60%
40%
20%
0%
Normalized RNA Quantity
GL2
RREB1
0
0.5
1
2
4
6
8
10
Time after Actinomycin D (hrs)
C
80000
70000
60000
50000
40000
30000
20000
10000
0
RLU
GL2
RREB1
siRNA Duplex (25nM)

Figure 31. RREB1 and HIF1A depletion do not decrease the RNA levels of HIF1a and CD24, respectively. UMUC3 cells were transfected with 25nM siRNA to GL2 or RREB1. 96 hours after transfection RNA was harvested and Q-RTPCR was performed for RNA expression levels of **A.** RREB1 or **B.** HIF1A. UMUC3 cells were transfected with 25nM siRNA to GL2 or HIF1A. 96 hours after transfection RNA was harvested and Q-RTPCR was performed for RNA expression levels of **C.** HIF1A or **D.** RREB1.

Figure 31


A
RREB1
RNA Quantity (ng)
300
250
200
150
100
50
0
21%O2
1%O2
Control
RREB1
siRNA Duplex (25nM)
B
HIF1A
21% O2
1% O2
RNA Quantity (ng)
200
180
160
140
120
100
80
60
40
20
0
Control
RREB1
siRNA Duplex (25nM)
C
HIF1A
RNA Quantity (ng)
180
160
140
120
100
80
60
40
20
0
21%O2
1%O2
Control
HIF1A
siRNA Duplex (25nM)
D
RREB1
21% O2
1%O2
RNA Quantity (ng)
180
160
140
120
100
80
60
40
20
0
Control
HIF1A
siRNA Duplex (25nM)

Western blotting for RREB1 and HIF1A protein expression revealed that RREB1 and HIF1A protein levels are reciprocally dependent (**Figure 32A**). Depletion of RREB1 decreased HIF1A expression and HIF1A depletion decreased RREB1 expression. The reciprocal dependency hinted that the two proteins may interact, so we asked whether RREB1 binds HIF1A. The nuclei of UMUC3 were isolated and endogenous HIF1A was immunoprecipitated. Western blot for endogenous RREB1 showed a distinct band in the HIF1A pull down but not in the IgG control (**Figure 32B**). Therefore, we concluded that RREB1 and HIF1A bind in the nucleus of UMUC3 cells which may act to stabilize both proteins.

5.4.4 HIF1A protein levels are dependent on RREB1α and RREB1β

We have previously shown RREB1 isoform specific effects on cancer cell growth *in vitro* and *in vivo* (**Chapter 3, Figure 17**). To determine if HIF1A was dependent on specific RREB1 isoforms, exon specific siRNA was used. Depletion of RREB1α and RREB1β, but not RREB1α alone was found to decrease HIF1A protein levels (**Figure 33**). siRNA to Exon 7, which depletes RREB1α, RREB1β, and RREB1δ, confirmed that the alpha and beta isoforms were primarily responsible for HIF1A protein stability as additional depletion of the delta isoform did not have an added decrease of HIF1A protein expression.

5.4.5 RREB1 depletion reduces HIF1A occupancy of the CD24 promoter.

HIF1A was previously shown to regulate the CD24 promoter and RNA expression (**Chapter 4, Figure 27B and Figure 29B**). HIF1A protein levels are

also dependent on RREB1 (**Figure 32**). To determine if RREB1 depletion decreased HIF1A binding to the CD24 promoter, a chromatin Immunoprecipitation (ChIP) assay was performed. UMUC3 cells were depleted of RREB1 and nuclear chromatin was isolated.

ChIP for rabbit IgG (control) showed no amplification using Q-RTPCR with primers designed to the location of the hypoxia response element in the CD24 promoter. However, HIF1A ChIP enriched the CD24 promoter DNA five fold over the RNA Pol II (Control) ChIP (**Figure 34A**). RREB1 depletion decreased CD24 promoter enrichment four fold. Western blotting of RREB1 knockdown cells confirmed that HIF1A and CD24 protein levels decreased in these cells (**Figure 34B**). Taken together, it appears that RREB1 depletion causes a decrease in HIF1A protein levels that accounts for less occupancy by HIF1A on the CD24 promoter, which may explain the decrease seen in CD24 expression after RREB1 depletion.

5.4.6 HIF1A depletion decreases UMUC3 growth

We have previously shown that transient RREB1 depletion in UMUC3 cells decreased cell growth (**Chapter 3, Figure 15**) and HIF1A protein levels (**Figure 32**). To determine if HIF1A depletion decreased cell growth, we transfected UMUC3 cells with 25nM GL2 or HIF1A siRNA. DNA content was measured every 24 hours for 5 days. **Figure 35** shows that HIF1A potently decreased UMUC3 cell growth *in vitro*. Thus it remains a possibility that the decrease in growth seen with RREB1 knockdown is due to HIF1A depletion.

Figure 32. Reciprocal dependency of RREB1 and HIF1A protein levels. RREB1 and HIF1A were transiently depleted from UMUC3 cells in the context of normoxia (21% O_2) or hypoxia (1% O_2) over the course of 96 hours. Western blots for RREB1 (1:1000), CD24 (1:3), HIF1A (1:1000), and TUB (1:1000) are shown.

Figure 32

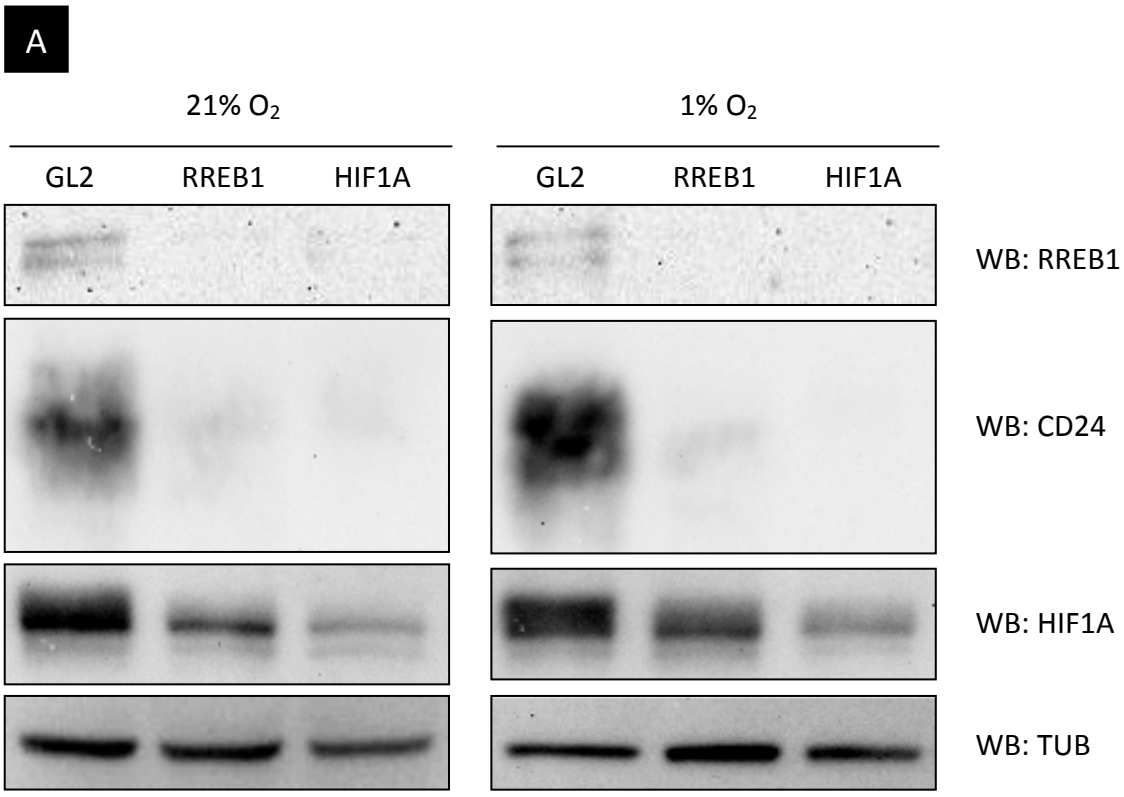

A
21% O2
1% O2
GL2
RREB1
HIF1A
GL2
RREB1
HIF1A
WB: RREB1
WB: CD24
WB: HIF1A
WB: TUB

Figure 33. RREB1α and RREB1β are necessary for HIF1A protein stability. UMUC3 cells were transfected with 25nM siRNA to GL2 or the RREB1 exons that target specific splice variants: Exon 7 (RREB1α, RREB1β, and RREB1δ), Exon 8 (RREB1α), and Exon 9 (RREB1α and RREB1β). 96 hours after siRNA transfection, cells were harvested and separated into their cytoplasmic and nuclear fractions. Western blotting for RREB1 (1:1000), HIF1A (1:1000), TBP (1:1000) and TUB (1:1000) is shown.

Figure 33

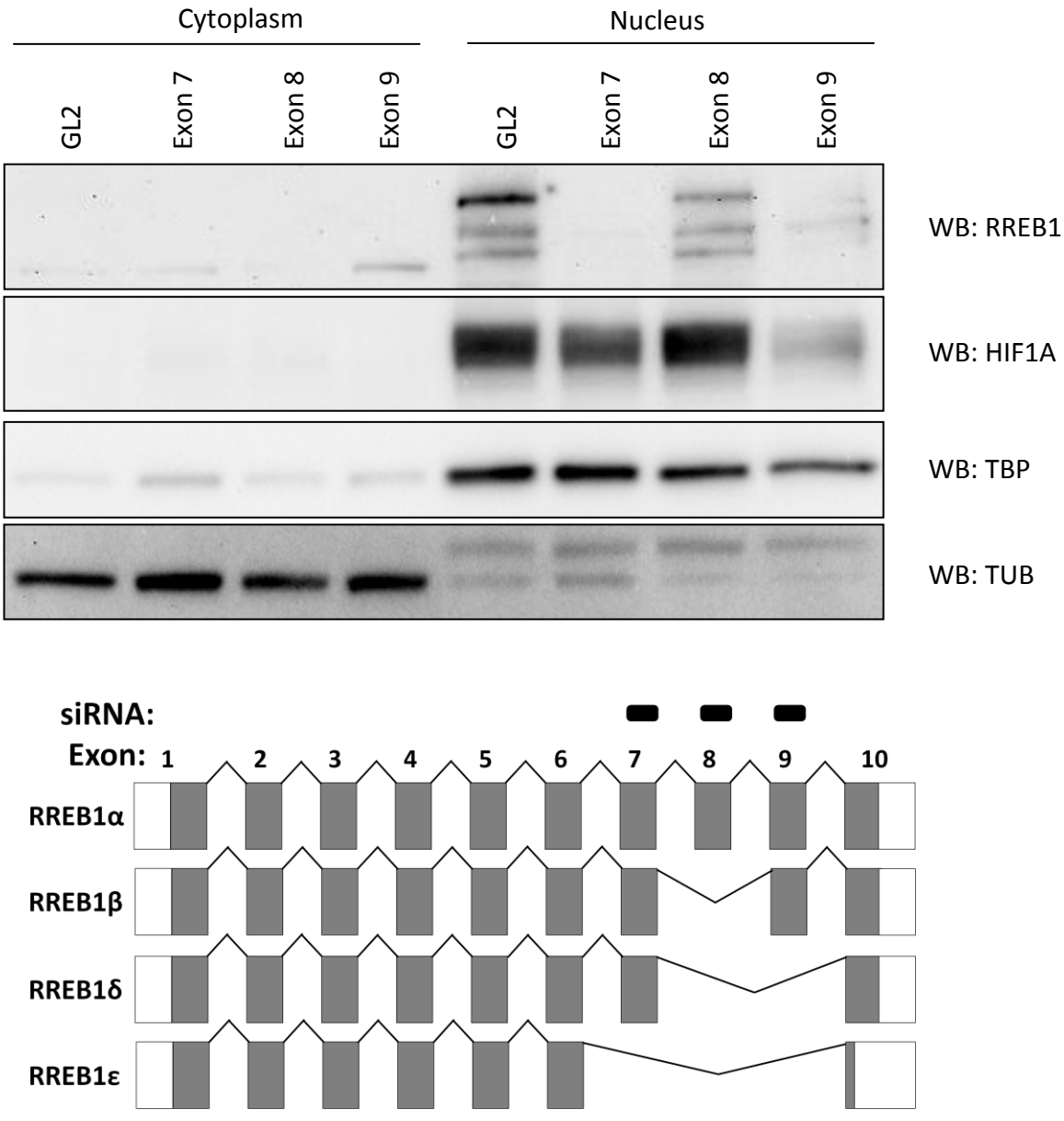
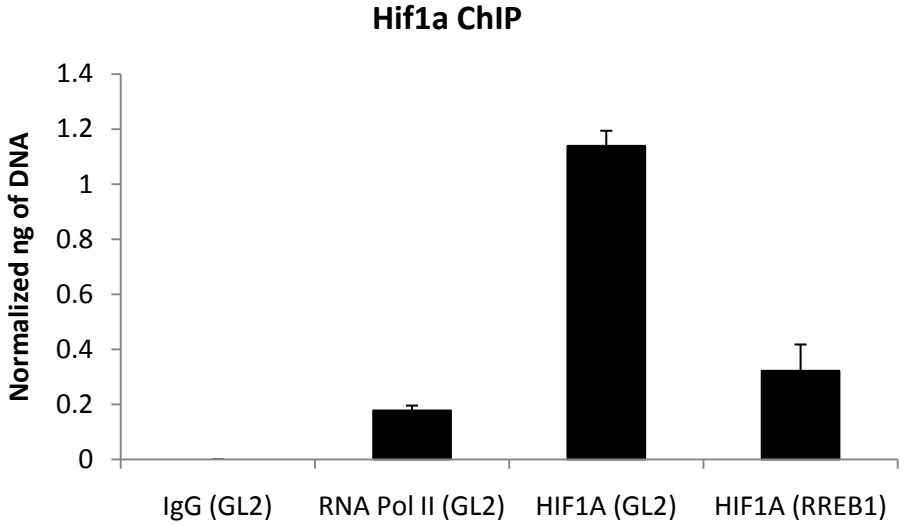

Cytoplasm
Nucleus
GL2
Exon 7
Exon 8
Exon 9
GL2
Exon 7
Exon 8
Exon 9
WB: RREB1
WB: HIF1A
WB: TBP
WB: TUB
siRNA:
Exon: 1 2 3 4 5 6 7 8 9 10
RREB1α
RREB1β
RREB1δ
RREB1ε

Figure 34. RREB1 depletion decreases HIF1A occupancy of the CD24 promoter in a ChIP assay. A. UMUC3 cells were transfected with 25nM of siRNA to GL2 or RREB1. 96 hours after transfection cells were harvested and chromatin was isolated. Immunoprecipitation with 25µg of antibody to rabbit IgG, RNA polymerase II, or HIF1A was used to isolate DNA. Q-RTPCR was used to quantitate the level of CD24 promoter isolated in the ChIP. **B.** Western blots of the previous experiment for CD24 (1:3), HIF1A (1:1000), RREB1 (1:1000), and TUB (1:1000).

Figure 34

A


Hif1a ChIP
Normalized ng of DNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IgG (GL2)
RNA Pol II (GL2)
HIF1A (GL2)
HIF1A (RREB1)

B

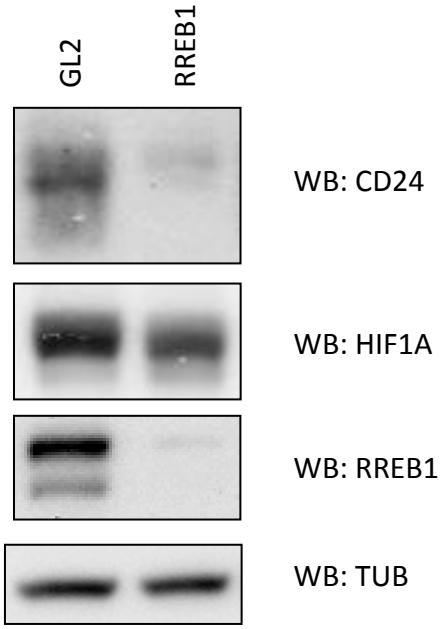
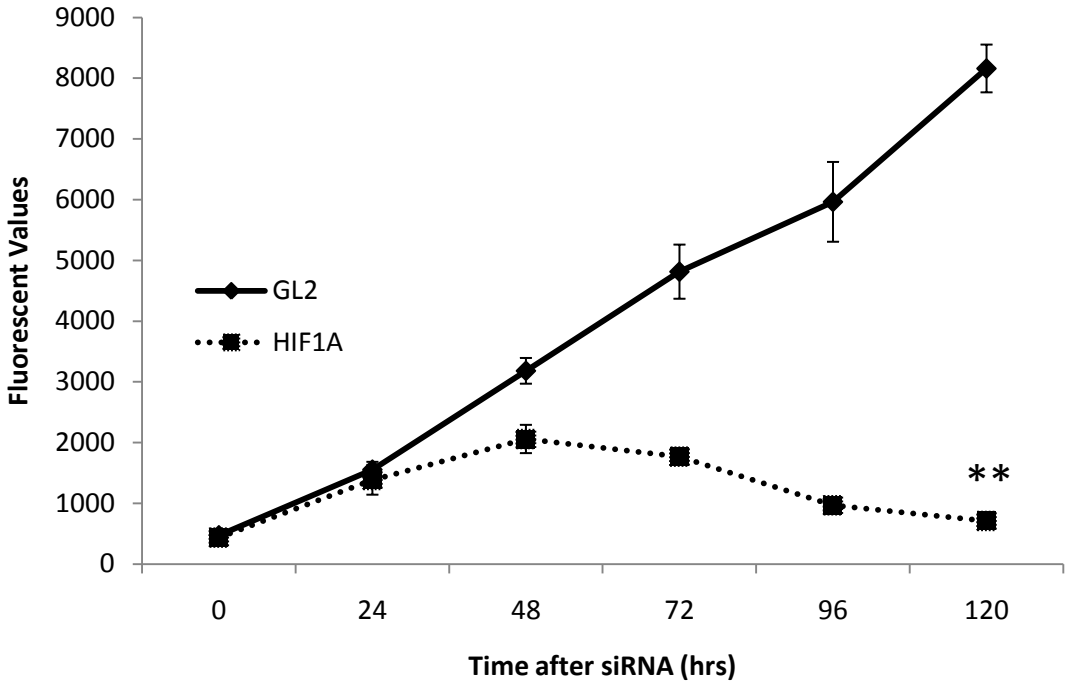

GL2
RREB1
WB: CD24
WB: HIF1A
WB: RREB1
WB: TUB

Figure 35. HIF1A depletion decreases UMUC3 cell growth. UMUC3 cells were transiently depleted of HIF1A and DNA content was measured every 24 hours using the Cyquant assay. At 120 hours HIF1A depleted UMUC3 cell DNA was significantly less than the control (GL2). ** ($P=5.6x10^{-6}$)

Figure 35


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Fluorescent Values
GL2
HIF1A
**
0
24
48
72
96
120
Time after siRNA (hrs)

5.5 Discussion

Adaptation to growth and survival in low oxygen environments is a hallmark of cancer. There is increasing evidence that the cellular changes undergone in hypoxia are not merely survival tactics by the cell but create a more aggressive malignancy with increased invasion of surrounding tissues and distant metastasis [190] [191] [192]. The hypoxia response pathway, which includes HIF1A and other proteins such as mTOR, is currently under intense evaluation as a new avenue of chemotherapeutic design [193] [194].

From our findings, RREB1 appears vitally necessary for expression of hypoxia responsive genes. Interestingly, many of the genes examined were also expressed at relatively high levels in normoxia, which also decreased with RREB1 depletion. HIF1A expression was also detectable in normoxic UMUC3 cells and protein levels decreased with RREB1 knockdown (**Figure 32**). Prior reports have shown that HIF1A protein is stabilized in normoxia due to Ras pathway signaling [195-197]. UMUC3 cells have an activated KRAS mutation [180] which may explain how HIF1A protein is stabilized in normoxic UMUC3 cells. The evidence showing that RREB1 is a downstream effector of Ras [97] provides the framework of a possible mechanism of HIF1A protein stability through Ras activation of RREB1.

While Co-immunoprecipitation experiments have yet to identify a direct interaction between RREB1 and HIF1 the reciprocal dependency shown in the above figures suggests a functional interaction. HIF1A has been shown to

interact with other proteins in an oxygen independent manner for regulation of protein stability. HSP90, the chaperone protein that protects its binding partners from misfolding and degradation through its ATPase activity, binds HIF1A in its PAS-A domain, the area used for dimerization with ARNT [198, 199] [124]. RACK1, the multifunctional scaffolding protein that plays a role in intracellular signaling [200] and assembly of the 80S ribosome [201], competes with HSP90 for binding to the HIF1A PAS-A domain [202]. As opposed to the HSP90 stabilization, RACK1 binding to HIF1A recruits the E3 ubiquitin ligase complex and targets the HIF1A for 26S proteasomal degradation [203]. The RREB1 protein stability effect may occur by a similar mechanism as HSP90 in the nucleus. In this hypothesis, binding to RREB1 prevents HIF1A transport out of the nucleus and proteasomal degradation. Experiments are currently on going aiming to answer this question.

We initially demonstrated that RREB1 was able to block hypoxic induction of genes regulated by both HIF1A and HIF2A; suggesting that RREB1 interacted with both. However, all subsequent experiments on protein stability were performed solely on HIF1A. The authors recognize that RREB1 may have a similar effect on HIF2A protein stability. To this point, we have been unable to provide evidence for a dependency between RREB1 and HIF2A due to a lack of a reliable HIF2A antibody. Experiments are currently ongoing to identify a HIF2A antibody and determine its interaction with RREB1.

Chapter 6: Discussion

6.1 RREB1 in bladder and prostate cancer

6.1.1 Context of RREB1

Study of RREB1 was hindered by lack of clarity surrounding the nature of RREB1, specifically, what protein(s) did the RREB1 loci encode? The original cloning of RREB1 identified mRNA from Ras transformed thyroid cells that translated to a 756 amino acid protein [97]. Further studies on RREB1 mRNA described proteins with alternative translation starting points and differential splicing at the C-terminus. However, nearly every published study simply describes using the "RREB1" protein, with little mention of the precise species used in experiments. The same was true for siRNA directed at RREB1. Every study published to date describes a depletion of RREB1 without a description of duplex hybridization location. The considerable ambiguity surrounding RREB1 motivated us to systematically identify the RREB1 isoforms expressed in bladder and prostate cancer cells.

We first came to study RREB1 when the computer program CARRIE identified RREB1 transcription factor binding sites to be overrepresented in the promoters of genes differentially expressed by Ral [95]. In initial experiments we used the most commonly cited RREB1 construct originally identified from an expression library of the breast cancer cell line MDA-MB453 [98]. Sequencing of this transgene revealed multiple regions of the translated protein to be non-

homologous with the reported sequence of RREB1, which led us to conclude the transgene significantly differed from the endogenous mRNA. It became apparent that our results with the overexpressed RREB1 transgene may not be recapitulating the endogenous RREB1 activity.

It is intriguing to speculate whether confusion surrounding splice variants led other researchers to abandon a careful cataloguing of the isoforms. Date et al. describes identifying three unique RREB1 splice variants in cDNA of normal human keratinocytes and the liver carcinoma cell line HepG2; and goes on to describe how RREB1 represses the angiotensin gene [96]. It is perplexing that after making an effort to describe three unique RREB1 isoforms no experiments addressed any isoform differences. Furthermore, no attempt was made to provide a standard nomenclature to describe the RREB1 isoforms.

The current knowledge of RREB1 protein is less clear. In review of the literature no careful characterization of RREB1 proteins has been found. This may be due to the absence of a reliable, commercial RREB1 antibody. Western blots of RREB1 were performed with antibodies developed “in-house”, without any accompanying data showing their specificity to RREB1. Our lab managed to obtain one of these antibodies and found that it had been made to only a 12 amino acid region of RREB1β. Western blot with this protein showed a ladder of detectable bands [166], but the authors claim that this antibody could specifically isolate RREB1 bound DNA in a ChIP assay. At the time of our study, three unique commercial antibodies for RREB1 were available. Therefore, for proper

study of this protein it was imperative to have a reliable, specific, and sensitive antibody for detection of RREB1 protein. Furthermore, it was necessary to also know which isoform corresponded to the bands seen on Western blot.

The dearth of basic tools for studying RREB1 has led to a general lack of RREB1 understanding. Though there are significant associations between RREB1 and malignancy, there have been no studies attempting to understand whether its expression correlates with cancer. It is a paradigm that oncogenes are either mutated or overexpressed in cancer. However, it is now recognized that some genes are not mutated or overexpressed to be oncogenic; their splice variant expression pattern can change to favoring one isoform over another [204]. Therefore, we asked whether specific RREB1 splice variants participated in bladder cancer.

Likewise, the question of the necessity of RREB1 in the transformed phenotype has not been directly addressed. We have found only a single study where an *in vitro* cancer phenotype was tested by manipulating RREB1. MCF10A cells were depleted of RREB1 and their migration decreased [108]. However, a recent study depleted RREB1 in chicken mesenchymal cells during chondrogenesis which resulted in decreased cellular proliferation [205]. Abnormal cellular proliferation is a hallmark of cancer, but, it remained unknown whether RREB1 was necessary for tumor growth.

6.1.2 RREB1 Isoform Characterization

One major complication in characterizing the RREB1 splice variants was obtaining reliable expression of each individual isoform. Primers had to be developed that hybridized to unique exon junctions for each isoform. Technically this proved to be quite difficult as there was very little unique sequence between the isoforms. Splice variants made from addition and removal of cassette exons only create one, when removed, or two, when added, unique sequence locations. The primers would have to specific for the unique sequence and not bind the other splice variants nonspecifically. Initially we failed in this attempt because the primers were designed to be centered over the unique junctional sequence; there was very high nonspecific binding of the other splice variants. We determined that the 3' end of the primer, where cDNA extension would begin, needed to be matched to the junctional sequence. If the 3' end does not hybridize with the RNA the polymerase complex machinery essentially falls off after initiation. This designed allowed us to specifically amplify only a single splice variant using real time PCR.

Matching what we had observed with end point PCR, RREB1γ was expressed at undetectable or extremely low levels in all cell lines and tissues examined. RREB1γ may have a negative effect on tumor malignancy as its only identification outside of this report was in normal kerotinocytes [96], explaining why its expression is suppressed. We hoped to obtain these cells to determine if the lack of expression was due to a technical artifact or the isoform was truly not expressed well in the tissues examined. Another approach would be to look for

the RREB1γ isoform across an mRNA library of human tissues. It could be that this isoform is very tissue specific.

Though we made a systematic and thorough approach to identifying alternative splicing within the first and tenth coding exons, we failed to examine the non coding regions. To fully uncover all alternative splicing events we would need to identify the 5' and 3' ends the RREB1 mRNA in each splice variant. 5' and 3' **R**apid **A**mplification of **c**DNA **E**nds (RACE) would allow for the cloning and sequencing of the UTRs. The primers used for RACE initiation would have to sit within an exon that is shared by all splice variants. The most logical exon to use would be one that is shared by all known splice variants and is determined to be necessary for the observed RREB1 phenotypes. This approach would not be an academic exercise to prove that RREB1 does not have further splicing events that do not have biological consequences. There are several examples of genes whose expression is regulated post transcriptionally by sequence of the UTR such as addition and removal miRNA sites [206] [207]. In fact, some diseases have even been traced to SNPs in the UTR affect mRNA stability and protein expression [208] [209].

We observed that depleting of one or more specific RREB1 splice variants increased the expression of the non-targeted isoform. There are a couple of explanations for this phenomenon. First, the depleted isoforms may have negative a feedback effect on the non-targeted isoforms. Or, siRNA to specific exons may shift the splicing machinery to making non-targeted isoforms. We believe that the latter hypothesis is the likely mechanism. Personal

communication with the technical representatives of Gene Tools, LLC., a leading company in the development of morpholinos, revealed that they had observed similar findings. Morpholinos are 25-mer nucleic acids linked through morpholine rings that specifically hybridize to their RNA target and sterically block translation without RNase-H or RISC complex activity; components necessary for siRNA inhibition of translation [210]. They observed that when a mophonlino is targeted to an exon, it will bind that sequence in the pre-mRNA. The pre-mRNA is the immediate product of DNA transcription that still contains both introns and exons. When the splicing machinery binds this product, it becomes processed to mature mRNA by removal of introns and possibly exons to create alternatively spliced RNA. An exon containing a bound morpholino will always be excised by the splicing machinery, creating mature mRNA missing that exon. Thus, they observe an increase in the absolute expression of mRNA isoforms that are not targeted by the morpholino. Thus, it appears that siRNA to a specific exon does not necessary deplete the pool of RNA, but merely shifts the splicing machinery to excising that exon and creating a higher quantity of non-targeted isoforms.

Despite exhaustive literature investigation, we have not found a similar description by other labs. This could be due to the technical hurdles of developing specific quantitative real time PCR primers and siRNA for each isoform. Furthermore, due to the complex and difficult nature of studying multiple alternative protein products of a single gene, there has been resistance to conducting a rigorous study as we have described. However, as sequencing technology has vastly improved efficiency while decreasing cost, more studies

are beginning to study the transcriptome. Somewhat unexpectedly, these studies have found that the majority of human genes are alternatively spliced and only a small percentage of these isoforms have been described [158, 159]. Therefore, we believe alternative splicing, as opposed to being viewed as an exception, will eventually be understood as a chief mechanism of genetic diversity and possibly a major contributor to human disease.

6.1.3 RREB1 protein expression in bladder cancer

We found the RREB1 antibody produced by Sigma-Aldrich had the highest specificity and sensitivity of the three commercial antibodies. This antibody was produced by immunizing rabbits to a 115 amino acid (AA) peptide fragment that encompassed part of exon 7 (85 AA) and exon 8 (30AA). Theoretically, the Sigma-Aldrich antibody should detect only RREB1α if the epitope spans the junction between the two exons. But, we found that it also detects RREB1β and RREB1δ using overexpression of these isoforms and siRNA that eliminates specific splice variants. We interpreted this data to mean that the Sigma-Aldrich RREB1 antibody epitope is in exon 7. It is also interesting that the sensitivity and specificity of the RREB1 antibodies was directly related to the amount of human protein used for immunization. The Genway RREB1 antibody used full length mouse RREB1α, which is 79% sequence similar to human at the protein level, was more sensitive but less specific than the Cosmo RREB1 antibody, which only used 50 amino acids from human RREB1α.

Using the Sigma-Aldrich RREB1 antibody we demonstrated that RREB1 localizes primarily to the nucleus of UMUC3 cells. RREB1 had previously been described as a nuclear protein using a non-commercial antibody in an immunofluorescence assay [98]. However, the band detected in the cytoplasmic fraction proved more intriguing. Several reports have shown that RREB1 functions as a transcription factor so it makes teleological sense that the protein would have a nuclear localization signal (NLS). Using the PredictNLS Server (http://cubic.bioc.columbia. edu/services/predictNLS/) a NLS was found in all known RREB1 splice variants. It is located in exon 4, an exon that has not been shown to be removed in alternative splicing. But, this cytoplasmic RREB1, which does not correspond to any RREB1 nuclear bands, can be hypothesized to have lost the NLS sequence or have it masked through protein binding. To lose the NLS sequence, RREB1 RNA could be made without the first four exons through transcription from an alternative promoter. Also, RRBE1 protein may be made without the NLS through an alternative ATG start site downstream of the NLS.

One limitation of the study of RREB1 proteins was the lack of antibodies to differentiate between the splice variants. We discussed creating these reagents by using the peptide sequence at each unique splice junction; hoping that there would be enough diversity among the isoforms to obtain reliable specificity. We chose to not create theses antibodies because of the incredible cost, time, and effort needed to complete such a project. However, this work would become valuable should a specific splice variant be demonstrated to be upregulated in malignancy.

Using the Sigma antibody that detects RREB1α, RREB1β and RREB1δ we analyzed a bladder cancer tissue micro array to determine if RREB1 expression differed based on patient survival or histologic subtype. Despite examining mRNA expression between normal and malignant bladder cells, not large scale analysis was performed at the protein level. This was not performed due to the lack of normal bladder tissue available for TMA. Comparing normal versus tumor RREB1 protein expression will help to answer the question whether RREB1 is downregulated in tumors. Our limited mRNA analysis suggests that RREB1 decreases in tumors. The Oncomine database is a repository of gene expression microarrays where the expression of a single gene can be explored over multiple data sets [211]. RREB1 is overexpressed in cancers compared to normal spanning multiple cancer types in 9 studies while under expressed in 7. Thus, a thorough examination of protein expression may add evidence to the direction of RREB1 expression change in cancer.

6.1.4 Results on the necessity of RREB1 in cancer cell growth

We observed that depletion of RREB1 decreased bladder cancer cell growth. To further characterize this finding we aimed to determine which splice variant was necessary for growth. This question was approached from both ends: siRNA was to be designed that specifically depleted RREB1 isoforms and endogenous RREB1 would be depleted and rescued with transgenes of each isoform. However, we encountered problems with each approach.

To deplete each isoform individually, siRNA would have to be designed to the unique exon junctions of each isoform. Unfortunately, we could not find viable siRNA seed sequences in these junctions to be predicted by any commercial siRNA design software. We could have taken a trial and error approach by walking siRNA sequences along the splice junction and screening for specific RREB1 splice variant depletion. We decided against this method due to the intense cost and effort necessary to perform this approach correctly. Though, we did observe that depletion of RREB1α and RREB1β, but not RREB1α alone, was able to reduce UMUC3 growth *in vitro* and *in vivo*. We originally thought RREB1β may be solely responsible for UMUC3 growth. But, because RREB1α and RREB1β make up the majority of the expressed RREB1 in bladder cancer cells, the loss of RREB1α and RREB1β may reflect a dosage effect. This conclusion is supported by the observation of decreased subcutaneous tumor size, albeit non-significantly, in RREB1α depleted UMUC3 cells.

Next, we asked which of the four expressed RREB1 splice variants in UMUC3 were necessary for growth. Depletion of endogenous RREB1 with 3'UTR siRNA showed in cells stably expressing the isoform transgenes was not sufficient to rescue growth. We concluded from this experiment that a single isoform was not sufficient to rescue growth from depletion of all endogenous RREB1 isoforms. It is possible there may be a coordinate dependency between RREB1 isoforms; creating a model where binding between differing isoforms is necessary for their activity. However, despite expressing all permutations of

RREB1 isoforms transiently after depletion of RREB1, UMUC3 growth could not be rescued after RREB1 knockdown. Our inability to salvage UMUC3 growth with the RREB1 splice variants may reflect a technical artifact such as insufficient transgene expression, interference by the 3X FLAG C-terminal epitope tag with protein binding, or off target effects of the RREB1 siRNA. Also, there could be a biological reason such as additional unknown splice variants that are necessary for growth.

There are numerous possibilities that could explain the inability to rescue UMUC3 growth with the RREB1 transgene expression, some of which are mentioned above. However, the failure of the RREB1 rescue does not negate the observation of RREB1 necessity made with siRNA. After the Horizon meeting on RNA interference (RNAi) in 2002 the editors of the journal *Nature Cell Biology* published a list of RNAi controls that still serves as a basis for judging the quality of RNAi data [212]. The five requirements include: 1. Scrambled siRNAs, 2. Show reduction at the RNA and protein level, 3. siRNA titration, 4. Rescue by expression of target gene, and 5. siRNA multiplicity. We employed all 5 controls including 11 unique siRNAs to RREB1 that demonstrated decreased UMUC3 growth and titrating the siRNAs down to 12.5 nanomolar concentration. Only the rescue failed. However, the authors go on to state that a rescue is not entirely necessary: “The rescue control has to be regarded as the control of choice, given the multiple as yet ill-defined modes of action of this powerful mechanism. However, this may not always be possible and we will not insist on it if convincing alternative controls are enclosed, especially of category 5.” We feel

that 11 unique siRNAs to RREB1 fulfills the requirement of demonstrating siRNA multiplicity and that our data of RREB1 necessity in bladder cancer cell growth remains convincing.

6.1.5 Future directions in the study of characterizing RREB1

Though rigorous investigation into RREB1 alternative splicing was undertaken, additional splice variants may have escaped our detection. Our focus centered on detecting all RREB1 splice variants that contained the first and the last coding exons because siRNA to either of these exons produced similar growth phenotypes in UMUC3 cells *in vitro*. However, alternative splicing or transcription start sites could create RREB1 isoforms that have not been detected. To further search for RREB1 RNA isoforms 5' and 3' RACE could be employed. Though RACE could answer whether additional RREB1 RNA species exist, it does not answer the more important question of the composition of RREB1 proteins. As we demonstrated, there were additional bands on the RREB1 western that could not be accounted for by the known RREB1 RNA isoforms. Furthermore, we also described how the RREB1 RNA has multiple in frame ATG codons that have high predictive value as start sites based on their Kozak sequences. So, it appears likely that additional RREB1 proteins exist. Using the Sigma-Aldrich antibody, RREB1 could be isolated from UMUC3 lysates and individual proteins sequence after separation on a gel. Sequencing individual bands on a gel is preferred to using mass-spec to sequence all proteins from a RREB1 pull down as it would be far more difficult to align each

RREB1 sequence correctly with a compilation of short amino acid sequences from several different isoforms.

The decrease in RREB1 RNA expression in tumors was one of the more interesting observations. Examining the promoter of RREB1 may uncover some clues as to a mechanism of decreased transcription. Using the RREB1 promoter cloned into a reporter vector, different pathways could be activated and assayed for their effect on RREB1 promoter activity. Most intriguing would be to observe the effect of overexpressed RREB1 isoforms or depleted endogenous RREB1 on the RREB1 promoter to identify any feedback control of RREB1 upon itself. Understanding how RREB1 is expressed may also provide insight into its function.

Along these same lines, the mechanism of RREB1 isoform expression remains unknown. We observed that siRNA to a specific RREB1 splice variant increased the expression of the non-targeted splice variants. While the study of this mechanism alone is quite interesting, we believe there could be broader implications of molecular biology that could be tested. MicroRNAs (miRNAs) are short RNAs that bind mRNA, typically in the 3' end, and target it for degradation. However, we conjecture that it may be possible for miRNAs to bind specific exons in the pre-mRNA, which could shift the composition of splice variants. Analysis of RREB1 cassette exons revealed several intriguing miRNA binding sites that will serve as future areas of research.

Finally, our observation of increased RREB1 expression in squamous cell carcinoma (SCC) of the bladder represents a novel and exciting avenue of

research. Squamous cell carcinoma of the bladder is predominant in areas of endemic trematode bladder infection [6]. Perhaps in SCC of the bladder, RREB1 expression may be a driver of malignancy. To better evaluate this, *in vitro* experimentation of the bladder SCC cell line SCABER [213] could be used to assess the composition and role of RREB1. Also, RREB1 expression should be further evaluated on a larger tissue microarray that is comprised of SCC of the bladder. Finally, investigation of the role of RREB1 in SCCs of other locations, such as lungs, skin, and esophagus, may reveal its importance in the broader category of squamous histopathology.

6.2 Defining CD24 transcriptional control

6.2.1 Context

CD24 is grossly overexpressed in multiple cancer types and its expression often negatively correlates with clinical outcome (**Chapter 1**, **Table 2**). Despite this connection, there have been no studies to date that have examined the CD24 promoter in cancer cells to determine the mechanism of overexpression. This may be partly due to the incomplete sequencing of the CD24 locus in GenBank. There are two reported sequences of the CD24 promoter but a comparison between the two shows considerable disagreement. Therefore, there is a clear need in the field for a careful characterization of the CD24 promoter.

Prior work by our lab has shown that CD24 expression is dependent on Ral expression [55] and possibly RREB1 activity [95, 166]. Therefore, we were originally interested in examining the CD24 promoter for RREB1 binding sites. To do this we first needed to sequence the promoter as a reliable sequence had not been published.

6.2.2 Results on cloning the CD24 promoter

Considerable difficulty was encountered in cloning the CD24 promoter as initial attempts failed to produce a PCR product of the appropriate size. We made an assumption that the CD24 promoter contained a G/C rich region that was preventing efficient PCR. To combat this, DMSO and Betaine were added to the PCR mix for their ability to stabilize the polymerase, eliminate base pair composition dependence, and destabilize the DNA double helix [214-217]. Use of these additives resulted in a PCR that created single band at the correct size. This band was cloned into the pGL4.20 luciferase reporter vector and sequenced. The CD24 promoter sequence was cloned from several cell lines (TERT, 293T, LUL2, EJ, J82, and LNCaP) to gain a consensus sequence. We observed very little sequence variation in the CD24 promoter between these cell lines.

Our prediction proved correct in that the CD24 promoter contained very high G/C rich region. In fact, over several hundred base pairs it extended to over 90% G/C. G/C rich regions tend to have a higher frequency of CpG sites, which are a **c**ytosine nucleotide that occurs next to a **g**uanine nucleotide in a linear

sequence (connected by a **p**hosphate bond). A high concentration of CpG sites within a defined region is referred to as a CpG island (CGI). Using the most stringent criteria to define a CGI, we found a 970 base pair predicted CGI in the CD24 promoter. CGIs are typically found in the promoters of the most widely expressed housekeeping genes and tend to be hypomethylated [174]. Hypermethylation is a natural mechanism employed by the cell to decreased gene expression but is also a common mechanism of silencing tumor suppressors in cancer [218]. If gene expression is being controlled through hypermethylation of the promoter, a common method for determining this is treatment with 5-azacytadine, a DNA methylation inhibitor. Treatment of UMUC3 cells with 5- azacytadine did not increase expression of CD24. We interpreted these results to indicate CD24 expression was controlled less through epigenetic signals and more through transcription factor activity on the promoter.

Examination of the CD24 promoter sequence identified dozens of transcription factor binding sites. Some of these sites occurred multiple times, such as NRF, SP1, and NFKB, and were found to occur at a statistically significant higher rate than what would be expected in a similar length sequence in the genome or in other promoters. Though we found the identification of transcription factors intriguing and useful for future studies, we focused on the RREB1 and HIF1A binding sites. Each was found to have a single binding site within the CD24 promoter, which made the study much more amenable to testing the interaction between transcription factor and promoter.

Despite our work with depletion of HIF1A and RREB1 we failed to examine the multiple other transcription factor binding sites identified after cloning and sequencing of the CD24 promoter. Both feasibility and direction were reasons cited for not pursuing intriguing transcription factors. Cost, time, and manpower limitations prevented screening of every transcription factor with a binding site in the CD24 promoter, forcing us to focus on a limited few that had ties to other ongoing projects and could be placed in a model to explain CD24 expression. But, this is not the most accurate method for determining the relevant transcription factors. First, we could employ a common technique used to determine the promoter area necessary for transcription. The cloned promoter would be placed in a luciferase reporter vector. Multiple variants of this promoter would be made in decreasing size; typically 100 to 200 base pairs would be removed from the 5' end of the DNA. In this "promoter walking" the important area would be identified with a decrease in the luciferase activity. Once the important region is identified, mutagenesis could help isolate the exact promoter binding sequence. Or, EMSA could be used to identify the transcription factor. Secondly, we could also directly query the transcription factors with a custom siRNA library to all the ones identified to have binding sites. Though this method would most likely be more costly, it has the potential of finding the important players faster.

6.2.3 Results on CD24 promoter activity

CD24 expression was shown in prior experiments to be dependent on Ral expression [55]. Depletion of RALA and RALB decreased CD24 expression but their overexpression failed to increase CD24 mRNA or protein. It this study the authors concluded that the Ral proteins were necessary but not sufficient for CD24 expression. Likewise, overexpression of Ral paralogs did not show consensus between CD24 promoter activity and endogenous RNA transcription. However, depletion of Ral with siRNA, CD24 promoter activity and RNA decreased. We concluded Ral was necessary but not sufficient to drive CD24 transcription. A similar result was seen for RREB1. Overexpression of RREB1 splice variants had either no effect (RREB1α and RREB1δ) or increased CD24 promoter activity (RREB1β and RREB1ε). But, CD24 RNA expression did not change with RREB1 splice variant overexpression. Mutation of the RREB1 binding site did not change the reporter responsiveness, leading us to conclude that the effects observed on the CD24 promoter with RREB1 overexpression were not mediated through the RREB1 binding site. However, as had been observed with depletion of Ral, RREB1 depletion similarly decreased CD24 promoter activity and RNA expression.

It is difficult to envision a scenario where the promoter activity is of greater biological importance than what occurs with endogenous RNA. Apart from demonstrating direct interaction of the protein with the promoter DNA, any effects that are observed on the promoter that does not match what is occurring at the endogenous RNA is often dismissed as artifact. However, useful information can

still be gleaned from the promoter studies. The CD24 promoter reporter is a far different context than endogenous CD24 promoter within chromosome 6. There are epigenetic modifiers, more complex DNA strand and chromosome structure, and additional flanking sequence that can exert cis or trans effects on RNA transcription. When viewed in this context, it becomes clearer how these proteins may function. The observations we made with the CD24 promoter by Ral GTPases and RREB1 manipulation suggest that both of these proteins are necessary but not sufficient to drive CD24 transcription. They are not the limiting factor in CD24 transcription, but serve some vitally important role in maintaining this expression. For RREB1, we believe to have uncovered that role: stabilizing HIF1A.

Similar to a prior study where CD24 was identified through mass spectrometry of hypoxic endothelial cells [142], we found that CD24 expression increased when UMUC3 cells were incubated in hypoxic conditions. A nearly identical increase in CD24 promoter activity was also observed in hypoxia. Cloning and sequencing of the CD24 promoter allowed for identification of a hypoxia response element (HRE), binding site of HIF1A, merely 24 base pairs downstream of the CD24 transcription start site. Mutation of the HRE eliminated the hypoxia induced increase in promoter activity. Overexpression of HIF1A in normoxia led to an increase in promoter activity suggesting that the hypoxia induced increase in CD24 expression was mediated by HIF1A acting on the CD24 promoter.

As opposed to Ral and RREB1, HIF1A was observed to be sufficient to drive CD24 promoter and RNA transcription. But, the hypoxic induction seen with UMUC3 was underwhelming when compared to other cell lines. Comparison of HIF1A expression between UMUC3 and LNCAP at normoxia revealed that UMUC3 has very high levels of HIF1A (data not shown). We believe this is due to an activated KRAS mutation in UMUC3 [180] that promotes HIF1A stabilization in normoxia [125]. We asked the question, if HIF1A is stabilized at normoxia, does it drive CD24 expression? Depletion of HIF1A dramatically decreased CD24 expression in normoxia. After making this observation it became obvious, we had been studying a hypoxia response in the context of normoxia.

6.2.4 Future directions on CD24 transcription

One of the more intriguing findings of this study was the overrepresentation of transcription factor binding sites in the CD24 promoter. Some of these transcription factors have been implicated to upregulate gene expression in cancer. SP1 sites are canonical binding sites for the Sp/KLF family of transcription factors. These family members are implicated in growth control and angiogenesis of cancer, two effects that can be related to CD24 [178 55]. NFKB binding sites are bound by hetero and homodimers of the proteins with the Rel homology domain. Some have activation domains it their C-terminus (RelA, RelB, c-Rel) while others are inhibitory (p105 (NFKB1) and p100 (NFKB2)) until cleaved into a mature DNA binding protein (p50 and p52 respectively) [179]. These

genes are found to be amplified, overexpressed, and mutated which leads to many solid tumors have a constitutively activated NFKB pathway [179] including bladder cancer [219]. Therefore, the constitutive activation of these pathways in cancer may partially explain how CD24 expression is overexpressed in so many malignancies. To answer this question, Initial screens should block these pathways with siRNA or drugs to determine the effect on CD24 expression. The CD24 promoter could then be used to map out the transcription factor binding sites responsible for maintaining high CD24 expression due to the pathway activation.

The connection between CD24 expression and hypoxia was demonstrated to be mediated through the HIF1A transcription factor. This led us to ask the question whether RREB1 was connected to the hypoxia pathway as RREB1 depletion decreased CD24 expression. We found that HIF1A protein stability was dependent on the RREB1 protein (**Chapter 5**). Seeing that we came to study CD24 and RREB1 through our studies of Ral, these results raise additional questions concerning the role of Ral in hypoxia. To address this question, depletion of Ral could be observed in hypoxia. Does it also abrogate the CD24 induction? Does it alter HIF1A protein stability? Our lab is currently working to answer these questions.

The role of HIF1A in the transcriptional activation of CD24 raises some interesting questions. CD24 has been identified as a marker of the cancer stem cell phenotype [65]. Recently, the HIF transcription factors have been found to promote a stem cell phenotype in malignant cells [192]. Thus, could CD24 be a

marker, or even a driver, of HIF induced cancer stem cells? To answer this question, the HIF transcription factors could be overexpressed in cells with and without CD24 depletion through stable shRNA. These groups would then be assayed for the properties defining of cancer stem cells such as increased proliferation, self renewal, tumorgenicity, and metastatic seeding [220]. These studies would help give greater significance to the previous findings.

6.3 Defining the interaction between RREB1 and HIF1A

6.3.1 Context

Having made the connection between hypoxia, HIF1A and CD24 expression, we were curious if RREB1 participated in hypoxia. A high level of UMUC3 HIF1A protein was observed in normoxia when compared to other cell lines (data not shown) that was attributed to an active KRAS mutation stabilizing HIF1A protein [125]. This HIF1A protein was functional and was found to drive CD24 expression. Thus HIF1A and RREB1 both decreased CD24 expression in normoxia. Furthermore, HIF1A protein stability may be maintained by activated KRAS while RREB1 activity is driven by Ras. Upon initial examination of the literature, there were no direct connections between RREB1 and hypoxia. However, closer examination revealed a study demonstrating RREB1 protein regulating the MTIIA gene promoter, a gene known to be upregulated in hypoxia [143]. Hence, if RREB1 has been found to regulate two hypoxic responsive genes (CD24 and MTIIA), could it be important for the global hypoxic gene response? If

that is the case, then what is the mechanism employed by RREB1 to be necessary for the hypoxic gene response? It was these questions that led us to into studying RREB1 in hypoxia.

6.3.2 Results on RREB1 in Hypoxia

VEGFA was used as a marker of HIF activity because it is universally accepted as being hypoxia responsive with known HREs in its promoter [187]. IGFBP3 was also studied because it was found to be the most differentially expressed gene in hypoxic bladder cancer cells [186]. 3 hypoxia responsive genes were studied to determine if the RREB1 effect on hypoxia was blocking just HIF1A responsive genes or if HIF2A was also inhibited. Because we did not have evidence that any of these genes were responsive to HIF2A, we were fortunate to have VEGF primarily dependent on HIF2A for hypoxia induced expression.

RREB1 depletion blocked the hypoxic induction of the genes CD24, VEGFA, and IGFBP3. Surprisingly, HIF1A depletion only blocked CD24 an IGFBP3, while HIF2A depletion blocked induction of CD24, and VEGFA. CD24 expression appears to require both HIF1A and HIF2A. RREB1 inhibited the hypoxic induction of all tested genes, suggesting that the RREB1 effect is on both HIF1A and HIF2A. One question that may be raised is whether RREB1 depletion blocks transcription of all genes. We have in fact observed that is not the case as RREB1 does not change the RNA levels of RALA, RALB, HIF1A, HIF2A, ARNT, and ERBB2 (unpublished data). C_2H_2 zinc-finger proteins can

bind RNA and protect it from degradation [189]; therefore, we asked whether RREB1 changes the stability or transcription of hypoxia responsive genes. We found that VEGFA and CD24 mRNA stability did not change with RREB1 depletion, leaving us to conclude that RREB1 decreases the transcription of these genes.

The only protein observed to this point to directly drive CD24 transcription is HIF1A. Therefore, we asked whether RREB1 affected HIF1A. Because RREB1 is a putative transcription factor we first looked at HIF1A RNA expression after depletion of RREB1; which it did not change. Next, we asked whether RREB1 affected HIF1A protein stability. Depletion of RREB1 eliminated the detection of HIF1A protein on western blot. To our surprise, HIF1A depletion also decreased RREB1 protein on western blot but had no effect on RREB1 RNA expression. Therefore, we concluded that RREB1 and HIF1A have a reciprocal protein dependency.

To uncover of RREB1 stabilization of HIF1A protein, we looked for a physical interaction between these two proteins. We hypothesize that RREB1 could protect HIF1A from degradation by binding it in the nucleus and preventing its transport to the cytoplasm and proteasome. We believed this hypothesis had some merit as there is some precedence suggesting that RREB1 is capable of binding HIF1A. RREB1 has been shown to bind the basic helix-loop-helix (BHLH) transcription factor NEUROD [101]; and HIF1A is a BHLH transcription factor. Multiple pull downs of native proteins and overexpressed transgenes failed to find a binding interaction between RREB1 and HIF1A (data not shown).

Next we wanted to know if the destabilized HIF1A after RREB1 depletion had the functional consequence of reducing HIF1A protein on the CD24 promoter. This would give us direct physical evidence that RREB1 depletion decreased CD24 expression, at least in part, by reducing HIF1A protein on the CD24 promoter. RREB1 was depleted in hypoxic UMUC3 cells and nuclear extracts were prepared. Chromatin immuoprecipitation (ChIP) for HIF1A, RNA polymerase II (control), or IgG (control) was performed. We found that RREB1 decreased HIF1A occupancy of the CD24 promoter to control levels.

Lastly, we previously observed RREB1 depletion to have a severely negative effect on growth of UMUC3 cells. This growth effect was partially rescued by overexpression of CD24. HIF1A protein decreases in RREB1 depletion and know that CD24 expression is also dependent on HIF1A. Therefore, we hypothesized that the RREB1 growth effect was due to loss of HIF1A. To test this, we depleted HIF1A in normoxic UMUC3 cells and measured the growth over 5 days. We found a striking decrease in UMUC3 growth, even greater than what was observed in RREB1 depletion. We tentatively concluded that the loss of HIF1A protein stability after RREB1 depletion accounted for the decrease in UMUC3 growth.

6.3.3 Future directions in the study of RREB1 binding partners

One of the most exciting implications of this work is the connection between Ras, RREB1, and HIF1A. As has been previously mentioned, activated Ras is known to stabilize HIF1A and HIF2A in normal oxygen tension [125].

However, the mechanism of this connection remains unknown. RREB1 was originally discovered as a Ras responsive transcription factor and several studies have shown that it does not bind DNA or shown transcription activity unless activated by the Ras pathway [95, 97-99, 101, 105, 150]. Therefore, could RREB1 be the connection between Ras and HIF1A? In this model RREB1 and HIF1A would bind after an activating mutation from Ras. To test this a cell line not harboring an alteration leading to over activity of the Ras pathway would be used so that activated Ras could be added to the system to see if there was a change in the binding affinity between RREB1 and HIF1A. In bladder cancer, the cell line 5637 lacks a Ras mutation or tyrosine kinase receptor amplification [221].

At this point, it is also unknown if RREB1 and HIF1A directly interact. Though our experiments suggest this is not the case, it is possible that the interaction is weak or indirect, as in a complex, making it more difficult to detect. To test for a weak interaction cellular protein can be cross-linked prior to lysis. The crosslinking will covalently bind proteins in close proximity. The sensitivity of the pull down greatly increases at the sacrifice of specificity. This method increases the likelihood of detecting a nonspecific protein.

Another explanation is that RREB1 and HIF1A interact through a complex but are not directly bound. To enrich a HIF1A and RREB1 complex, the tandem affinity purification (TAP, also referred to as TAP tagging) method would be used transiently overexpressed vectors of RREB1 and HIF1A [222]. This would also have the advantage of being able to use the individual RREB1 isoforms so

identify differences in composition of the complex. Mass spectrometry would be employed to identify binding partners in this novel interaction.

6.4 Future Directions of RREB1

6.4.1 Remaining Questions

One of the key findings of this work was the necessity of RREB1 for cancer cell growth. However, we did not completely investigate into how RREB1 decreased growth. Was an apoptotic pathway activated or did the cells go through karyorrhexis? What cellular machinery was affected? A TUNEL assay, which detects DNA strand nicks that are typically present in late stage apoptosis, will help identify the fraction of cells undergoing apoptosis [223]. Gene expression profiling, either through microarrays or transcriptome sequencing, would identify genes up or down regulated after depletion of RREB1. Through grouping these genes by their known function we would get an idea of the cellular processes that have been affected.

To date we have only investigated the necessity of RREB1 in urologic malignancies. It remains an interesting question whether similar results would be seen in other cancer types. By expanding the scope of tumors that necessitate RREB1 for growth it would strengthen the evidence to pursuing RREB1 based treatment strategies. Furthermore, a pattern may emerge (epithelial versus glandular vs sarcomatoid, etc.) that hints toward the type differentiation or environment where RREB1 is most important.

Initially this project was to focus on the connection between Ral and RREB1. After several discouraging experiments and the realization that further characterization of RREB1 was needed, we put this aspect of the project on hold. The question of how these two proteins interact remains a valid and interesting question. A database of interacting *drosophila melanogaster* proteins (http://dip.doe-mbi.ucla.edu) identified a homolog of RREB1 in *d. melanogaster*, pebbled, bound the exocyst complex protein sec10L (EXOC5 in humans). Interestingly, while RalA is well known to bind the exocyst components sec5 (EXOC2) and exo84 (EXOC8), RalA was also found to associate with sec10 (EXOC5) in a RalA pull down of brain lysates [224]. We isolated EXOC5 and coexpressed it with RREB1 in 293T cells but were unable to identify a direct interaction in pull down experiments. As discussed above, a method for uncovering how this connection is mediated is mass spectrometry. Isolating endogenous RREB1 would be ideal to identify binding partners that could be linked to the Ral pathway. Another area of focus would be the post translational modifications of RREB1, such as phosphorylation. RREB1 contains multiple predicted phosphorylation sites, including ones specific for MAPK, that have not been described [225]. Phosphorylation of RREB1 could be detected after Ral manipulation (depletion or overexpression) mass spectrometry.

Perhaps the most exciting result for future research concerns the dependence of HIF1A protein on RREB1 expression. While many caveats still need to be worked out, such as the effect on HIF2A, this connection may reveal a novel level of hypoxic control and a new avenue for cancer treatments. We

looked at whether RREB1 and HIF1A bind in a pull down assay but did not detect an interaction. A direct interaction would be the easiest way to explain the connection between these two proteins. RREB1 could then be postulated to be involved in HIF1A transport, cellular location, and availability for binding partner interaction. HIF1A protein stability is regulated through hydroxylation of its proline residues in an oxygen rich environment that targets it for proteosomal degradation. Perhaps RREB1 plays a role along this pathway that has yet to be described.

6.4.2 Conclusions

Our work aimed to create an environment that would encourage and enable future research into RREB1. We identified novel splice variants and clarified their nomenclature. We created and validated multiple siRNAs that specifically deplete RREB1 and PCR primers that accurately measure individual splice variants. We characterized all commercial RREB1 antibodies in the specific splice variants they detect. Using these tools we then conducted the first interrogation of RREB1 mRNA and proteins in cell lines and human tissue. We are the first to describe the necessity of RREB1 in the growth of cancer cell lines *in vitro* and in a xenograft mouse model. Finally, we are the first to describe the necessity of RREB1 in the hypoxia gene response and stability of HIF1A protein. Our research of RREB1 has also lead to other discoveries, such as the cloning of the CD24 promoter and identifying the HIF1A binding site. These contributions

have advanced the knowledge of the field and have given the possibility of another target for cancer chemotherapeutic development.

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