ABSTRACT

CD44 is a cell surface glycoprotein that serves as a multifunctional receptor aiding in trafficking and adhesion of immune cells. CD44 also serves as a recruitment platform for signaling molecules and has been shown to regulate proliferation. In several types of leukemia the presence or absence of CD44 expression is associated with different clinical outcomes, with patients who have increased expression of CD44 exhibiting a stronger response to conventional chemo- and radiotherapy. By using Jurkat T cells, which do not express CD44, to determine the effects of CD44 expression in a model Acute Lymphocytic Leukemia cell line, we have outlined two major areas of study. Firstly, upon expression of CD44, Jurkat T cells proliferate slowly compared to the control cells. This decrease in proliferation is coupled to an arrest in the cell cycle during the transition from the G1 phase into S phase. The dysregulation of the cell cycle induced by CD44 also leads to the induction of aneuploidy. CD44 expressing Jurkat T cells have reduced mRNA expression for several key regulators of chromosome separation and the mitotic spindle complex. This finding, coupled with decreased EGR-1 expression, which controls the cyclins responsible for transition from G1 into S phase, leads to an unstable cell phenotype which proliferates slowly and accumulates extra chromosomes in daughter cells. The second area of study focuses on the mechanism by which CD44 expression at the cell surface results in the observed decreases in proliferation, Akt activation, and EGR-1 expression. We observed that CD44 expressing Jurkat cells show four to five times higher calcium influx when at rest compared to the vector control cells. This influx in calcium is due to CD44 expression activating a cell surface inducible calcium release activated calcium channel. The excess calcium activates calcium-activated phosphatases and kinases, disrupting EGR-1 expression and inducing a hypophosphorylation of Akt. Together, these findings indicate that CD44 expression can regulate

cell proliferation and signal transduction pathways in addition to its role in adhesion. Thus, our data provide a further understanding of how CD44 expression modifies leukemic cells into cells that are favorable for therapeutic intervention.

EFFECT OF CD44 EXPRESSION ON T CELL ACUTE LYMPHOCYTIC LEUKEMIA

Dissertation

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CHAPTER I

INTRODUCTION

Acute Lymphocytic Leukemia

Acute Lymphocytic Leukemia (ALL) is a cancer of the B cells or T cells with numerous genetic mutation associations and disease profiles. The cancer can develop at any stage of lymphoid cell development which gives rise to precursor B cell, early T cell, mid T cell, mature T cell, and early thymic precursor cell leukemias[1-3]. Because of the multiple forms of ALL and the multiple underlying genetic mutations that give rise to it, treatment of ALL has one of the most complex treatment schedules and chemotherapy combinations used in oncology[3]. Long term survival is best in adolescents with an 80% survival rate at 5 years. In adults, however, the outcome is worse with a long term survival rate varying from 35-65% depending on the type of ALL and age of the individual[1,2]. Of each of the ALL subtypes, T cell lineage ALL has the worst outcome in adults and adolescents[1,2]. CD44 expression has been characterized in ALL patients, and most tumoral forms of the disease show high expression while circulating stages show varying levels of expression[4-6].

CD44 overview

CD44 is a surface glycoprotein that is the major receptor for hyaluronan, a glycosaminoglycan in the extracellular matrix. CD44 can serve as a recruitment platform for other signaling molecules such as kinases, providing closer association with related pathway proteins allowing for increased signaling and kinase activity[7]. CD44 has other functions such

as facilitating cell adhesion, cancer cell metastasis, cell signaling, and cell proliferation[8-10]. CD44 has also been shown to regulate the stem-like state of tumor cells which are highly malignant and resistant to chemotherapy[5,11]. It is also known that CD44 is a regulator of cell migration and metastasis in various cancers including breast[12], colon[13], Chronic Myeloid Leukemia[11], Acute Myeloid Leukemia[14,15], and ALL[5,16]. Targeting of CD44 with a depleting antibody has been shown to eradicate leukemic stem cells in Acute Myeloid Leukemia, which highlights this surface protein's importance and relevance to carcinogenesis. Gadhoum et al. has shown that activation of CD44 reduces the proliferation of acute myeloid leukemia cells[17]. Importantly, our lab has previously shown that CD44 induces a reduction in the proliferation of T cell lineage ALL cells in vitro, implicating CD44 as a negative regulator of proliferation in different types of acute leukemia[18].

CD44 in Leukemia

In patients with Acute Lymphocytic Leukemia there is increasing evidence of CD44's potential role as a bio-marker[19,20]. Patients with high levels of the standard isoform of CD44 expression are correlated with having a less aggressive disease and better response to therapy[21-23]. Lack of CD44 expression and expression of variant forms of CD44 has been correlated with a more aggressive disease[24,25].

Cell Cycle Checkpoints

The cell cycle is the progression of cells along a regular pattern of phases that denote quiescence (G_0 phase), DNA replication (S phase), growth (G_1 and G_2 phases), and mitosis (M phase). There are three main checkpoints which determine if the cell continues to the next phase, undergoes cell death, or diverts into a quiescent state. The first checkpoint exists between the G_1 and S phases, and it is often called the restriction point. The restriction point is controlled by

CKI-p16 which acts to inhibit the interaction of CDK4/6 with cyclin D1 or cyclin D2[26]. Inhibition of this interaction prevents the cell from passing the checkpoint, but this can be overcome through oncogenic-induced cyclin D expression[27]. This increased expression of cyclins D1 and D2 allows the cell to force past the checkpoint through competition with CKI-p16 for binding with CDK4/6[26]. The CDK4/6-Cyclin D complex phosphorylates Rb which stops the inhibition of E2F allowing for the expression of cyclin E which ultimately interacts with CDK2 to drive the progression into S phase[26]. Progression from G_2 into M phase requires the activation of Cdc25[28]. Activated Cdc25 removes the inhibition of Maturation Promoting Factor allowing the cell to begin mitosis. DNA damage will inactivate Cdc25 and prevent entry into M phase. Progression through M phase is restricted by the spindle assembly checkpoint[29]. This checkpoint relies on the Anaphase Promoting Complex (APC) as well as BUB, Cdc20, MAD1/2, and Aurora A/B/C[30]. This checkpoint ensures that each kinetochore is connected with a microtubule and that proper distribution of chromosomes can occur[29]. The APC also functions to maintain the cell in G_1 phase by degrading the cyclins necessary to progress into S phase again. In cancer the cell cycle is dysregulated to allow for the rapid, unrestricted growth of cancer cells. Commonly mutated cell cycle proteins in human cancer include Cdk4, Plk1, BUB1, and BUBR[30].

G1-S Checkpoint Cyclins

Cyclin D1, cyclin D2, and cyclin D3 are encoded by three different highly conserved genes, but serve the same purpose. They each can bind to CDK4 or CDK6 to form an active cyclin D-CDK4/6 complex. This active cyclin D-CDK4/6 complex partially phosphorylates

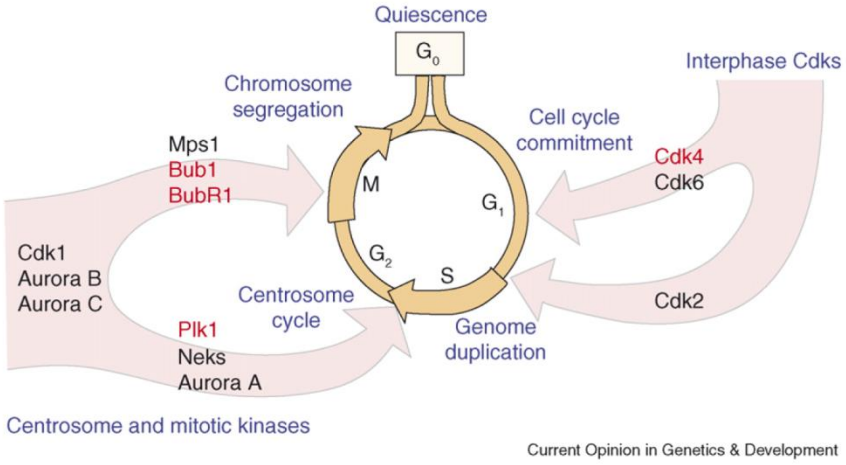

Figure 1. Main Cell Cycle Kinases and Their Involvement in Cell Cycle Progression.

The main cell cycle kinases are illustrated to correspond to the checkpoint that they modify. Kinases mutated in human cancer are shown in red. Used with permission[30].

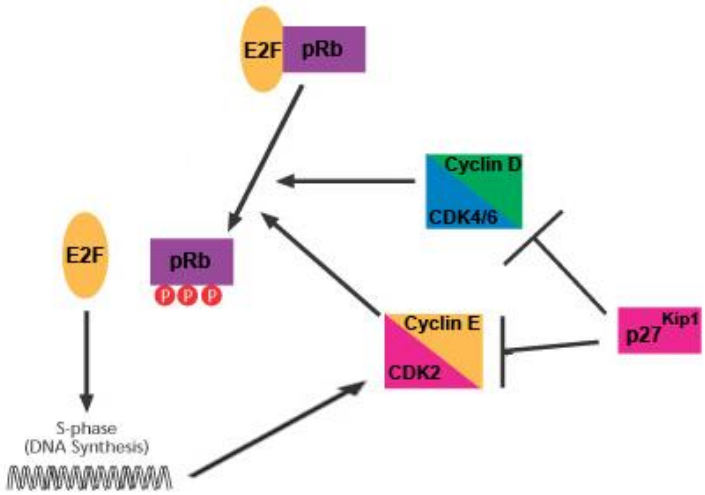

Figure 2. G_1-S Checkpoint Regulation.

This figure illustrates how the interactions between D and E cyclins and their target CDKs allows the cell to pass from G_1 phase into S phase once the E2F transcription factor is activated.

retinoblastoma tumor suppressor protein (Rb). Partially phosphorylated Rb then releases the E2F transcription factor that was bound to it, allowing E2F to function as a transcription factor. E2F induces the transcription of Cyclin E. Cyclin E forms a complex with CDK2 and drives the cell into S phase by fully phosphorylating Rb. p27(Kip1) can inhibit the activity of cyclin D and cyclin E by binding to them. The p27(Kip1)-Cyclin complex can still bind its target CDK, but p27(Kip1) blocks the active site of the CDK. p27 must be degraded via ubiquitin tagging before a cell can pass from G_1 into S phase. The phosphorylation and degradation via ubiquitin tagging of cell cycle proteins are the major mechanisms of cell cycle regulation.

Aneuploidy

Aneuploidy is when a cell does not possess the number of chromosomes appropriate for the species. Most cancer cells are aneuploid, largely due to the normal genomic stability functions of common oncogenes[31,32]. There are multiple mechanisms responsible for aneuploidy during mitosis. Nondisjunctions can occur due to alterations in mitotic checkpoint proteins such as MAD2A, which are responsible for assuring the chromosomes are aligned at the metaphase plate[33]. Mutations in p53, which are common in cancer, also result in aneuploidy due to increased genetic instability[34]. Calpain-1 is a calcium dependent peptidase that cleaves Rad21, a centromeric cohesion responsible for holding sister chromatids, causing chromosome separation[35].

Akt

Akt, also known as Protein Kinase B, is a serine/threonine protein kinase that regulates multiple cellular processes including proliferation[36], migration, transcription[36,37], and apoptosis[38-40]. Akt binds PIP3 at the cell surface before being phosphorylated at Thr308 by PDK1[41]. This initial phosphorylation allows the Akt protein to expose its second

phosphorylation site, Ser473[42]. Phosphorylation of Ser473 is performed by a large host of kinases, allowing Akt to be activated by numerous receptor and non-receptor mediated signaling pathways. The PIP3 required by Akt for membrane association is produced by PI3 Kinase by phosphorylating PIP2[43]. Akt activation can be inhibited by two distinct mechanisms. The tumor suppressor PTEN has the opposite function of PI3 Kinase; it converts PIP3 back into PIP2, meaning Akt cannot associate with the cell membrane and cannot be phosphorylated[44]. Several phosphatases can remove the activating phosphate groups from both Thr408 and Ser473. PP1, PP2A, PP2B (calcineurin), and PP2C can dephosphorylate both sites[45], while PHLPP phosphatases dephosphorylate Ser473 almost exclusively[46].

EGR-1

EGR-1 is a zinc finger protein that functions as a transcriptional regulator. EGR-1 is produced in the cytoplasm before it is transported into the nucleus by importin-7[47]. Once in the nucleus EGR-1 functions as a tumor suppressor that regulates p53, PTEN, and TGFβ1[48]. EGR-1 derives its early growth response name from its role in controlling the expression of cyclins D1 and D2. These two cyclins regulate the initiation of the transition from G1 into the S phase of the cell cycle. Induction of EGR-1 expression is caused by numerous signaling pathways, but importantly Akt activation and calcium influx have both been shown to regulate EGR-1 expression[18,49]. EGR-1 also regulates the expression of L-type calcium channels, SERCA, and the calcium pump regulating protein phospholamban[50]. EGR-1 expression is also increased by PLCγ induced signaling pathways, resulting in increased expression of STIM1 and calcium influx[51]. The regulation of EGR-1 and the genes that it regulates, like most signaling pathway mechanisms, are cell type dependent.

Calcium Signaling

Calcium is required for the proliferation of T cells. T cells possess the canonical components of store-operated calcium entry, including the inositol triphosphate receptor, stromal interaction molecule 1, and cytoplasmic calcium channels which are opened by stromal interaction molecule 1 due to endoplasmic reticulum calcium depletion[52,53]. Numerous drugs used for the treatment of myeloid and lymphoid leukemias cause alterations in calcium homeostasis, leading to tumor cell death[54,55]. For example, tipifarnib induces apoptosis of acute myeloid leukemia and multiple myeloma by modulating calcium influx[54]. Imatinib, the gold standard for treating chronic myeloid leukemia, causes the release of calcium from the endoplasmic reticulum, inducing stress and priming the tumor cells for drug-induced apoptosis[55].

CD44 Induced Calcium Signaling

CD44 expression in aortic endothelial cells shows that upon association with cholesterol lipid rafts, CD44 binds to IP3 receptor through ankyrin interactions. This association between CD44 and the IP3 receptor induces an increase in intracellular calcium concentrations that increases cell proliferation, adhesion, and nitric oxide production[56]. Upon binding of hyaluronan to CD44 on head and neck squamous cell carcinoma cells an influx of calcium into the cytoplasm is observed. It was determined that CD44 interacts with phospholipase C to create IP3 and activate the IP3 receptors on the endoplasmic reticulum surface[57]. Influx of extracellular calcium in T cells has been shown to be partially dependent on CD44 engagement of the ECM, resulting in the recruitment of Pyk2 and phospholipase C into lipid rafts before the calcium influx occurs[58]. CD44 also possesses a phosphorylation site that is essential for CD44 migration on hyaluronan. The kinase responsible for phosphorylation of CD44 at serine 325 is

Ca(2+)/calmodulin-dependent protein kinase II[59]. The involvement of extracellular calcium or endoplasmic reticulum calcium stores is cell type and stimulus dependent and has not been studied in Jurkat cells.

Significance

Acute leukemia is a cancer of the bone marrow and blood that can affect both myeloid and lymphocytic cells. Gadhoum et al. has shown that activation of the cell surface receptor, CD44 reduces the proliferation of acute myeloid leukemia cells[17]. Recently, our group reported for the first time that CD44 also reduces the proliferation rates of E6.1 Jurkat cells[18]. Thus, CD44 potentially has a broad role in negatively regulating cell proliferation in different types of acute leukemia. Dysregulation of the cell cycle is a key step of carcinogenesis[30], and CD44 expression has been shown by our group to decrease the expression of cyclins D1 and D2 in Jurkat (TALL) cells, two key regulators of cell cycle progression that drive the G_1-S phase transition[18]. CD44 standard form expression has been correlated with increased responsiveness to therapy and a less aggressive disease in ALL[21,24,25,60,61]. An understanding of the mechanisms by which CD44 reduces acute leukemia cell proliferation and influences tumorigenesis could lead to identification of biomarkers for diagnosis and prognosis or novel therapeutic targets and new treatment strategies for leukemia patients.

Specific Aims

Our laboratory has previously shown that CD44 expression in T Acute Lymphocytic Leukemia Jurkat cells induces a decrease in proliferation due to hypophosphorylation of Akt[18]. Akt is a kinase responsible for cell cycle regulation[36] and cell growth through a wide array of downstream targets[36,37]. Akt activity is responsible for control of cell proliferation past DNA damage checkpoints, and hypophosphorylation of Akt may yield abnormalities in chromosome

number and structure[38-40]. Previous work has also determined that EGR-1 expression is downregulated in CD44 expressing Jurkat cells, indicating potentially abnormal calcium influx and dysregulation of cell cycle checkpoints. **We hypothesize that CD44 expression induces aneuploidy and reduces proliferation through abnormal calcium signaling**. To test this hypothesis we propose the following specific aims. Firstly, to assess the cell cycle disruption induced by CD44 expression in Jurkat T cells. Secondly, to determine the molecular pathway regulated by CD44 in Jurkat T cells.

CHAPTER II

MATERIALS AND METHODS

Aim 1 – CD44 Induced Cell Cycle Disruption

Cell lines and Tissue Culture: E6.1 Jurkat cells were engineered to express CD44 or an open vector control as described previously[18]. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, and 800 µg/mL Hygromycin B. Calcium was removed from media via ethylene glycol tetraacetic acid (EGTA) chelation.

Cell Cycle Analysis: One million cells per mL were suspended in 70% ethanol and incubated at 20°C for 2 h. Cells were centrifuged and washed in PBS twice before being incubated in 60 µg/mL propidium iodide and 10 µg/mL RNase A for 1 h at 37°C. Cells were then analyzed via flow cytometry utilizing a Beckman Coulter Cytomics FC500.

Karyotyping: Vector control and CD44 expressing cells were cultured overnight in 40 ng/mL colcemid to arrest them in metaphase to preserve chromosome structure. Cells were washed in PBS and then suspended in a 1:1 solution of 75 mM KCl and 9% sodium citrate in PBS for 8 minutes at room temperature. The disrupted cells were then suspended in a 3:1 mixture of methanol and acetic acid for one hour at -20°C. The fixed cells were spun down and suspended in fresh methanol and acetic acid before being placed at -20°C overnight. Fixed cells were dropped from a 2 meter height onto glass slides which are chilled to -20°C in 100% ethanol. Two 10 uL drops were placed on each slide and then heavy breathing is used to moisten the slides and

encourage chromosome spreading. Slides were allowed to dry for 1 hour at room temperature before being coated in 600 nM DAPI solution for 30 minutes in the dark at room temperature. Slides were then washed with dH_2O before application of mounting media and drying overnight. Cells that are counted should have chromosomes that are spread out and not compact[62]. Cells were counted using a fluorescent microscope with a DAPI filter. A sample size greater than 30 cells per group is required to achieve statistical significance.

Myristoylated Akt: CD44 expressing cells were cultured in complete media with 60 MOI of Myr-Akt adenovirus for 24 to 72 hours[18,63]. Samples were taken every 24 hours and fixed in 75% ethanol at -20°C. Cells then proceed to cell cycle analysis protocol.

Akt Inhibition: For every instance of LY294002 use, the following protocol was used: cells were cultured for 20 hrs in media with 10μM LY294002, an inhibitor of Akt activation[18].

G1 to S phase Measurement: Cells were synchronized in the G_1 phase by serum starvation for 24 h prior to experiments. BrdU was added to media at 10 μM and cells were cultured under normal conditions for 1 hr. Samples were stained for BrdU according to kit manufacturer instructions (BD Biosciences) before analysis via flow cytometry. The percent BrdU positive cells at each time point will illustrate any delay entering S phase present in these cells.

S phase Duration: To calculate the duration of the S phase of the cell cycle for each group based on the measurement of the G1 to S phase of the cell cycle, the equation $S = N_0t/(Nt - N_0)$ was used, where t is the time between each time point, N_0 is the %BrdU+ at the first time point and N is the %BrdU+ at the final time point[64].

RT^2-qPCR Arrays and Analysis: RNA extracts were taken from Vector and CD44 cells using the Qiagen RNeasy Plus Mini kit. Briefly; cells were lysed by passage through a 22g needle in a lysis buffer containing a detergent and 2-mercaptoethanol followed by passage through a DNA

elimination column and an RNA isolation column. RNA purity was determined by the A260/A280 ratio and an equal amount of RNA per sample was converted into cDNA using SA Biosciences First Strand Kit. The two RT^2-qPCR arrays were the SA Biosciences Human Cell Cycle array and the SA Biosciences Human Signal Transduction Pathway Finder array. A 2-fold cutoff was used to determine down or up regulated genes of interest. GNCPro analysis was performed in order to determine the relationship between the genes of interest with respect to CD44 and Akt. A more detailed analysis was carried out using IPA software in order to predict which upstream regulators of the genes of interest are critical to the observed phenomenon based on IPA statistical modeling.

Western Blotting: Ten million cells were harvested and lysed with CelLytic M (Sigma-Aldrich) per the manufacturer's instructions. Protein concentration was assessed via the Bradford assay and equal amounts of protein were loaded into a 4-20% acrylamide gel for SDS-PAGE. Protein was transferred from SDS-PAGE gels onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in tris-buffered saline with 0.1% Tween 20 for 2 h before overnight incubation in primary antibody at 4°C. EGR-1 was detected at 1:1000 dilution with anti-hEGR1 antibody (R&D Systems). Phospho-Akt (Ser473) was detected at 1:500 dilution with anti-pAkt(Ser473) antibody (Cell Signaling). Beta-actin was used as a loading control and detected at 1:1000 dilution with anti-beta-actin antibody (Cell Signaling). Blotting with secondary goat anti-rabbit antibodies conjugated to alkaline phosphatase (Invitrogen) was performed before visualization with a UVP GelDoc-It imaging system.

PTEN Inhibition: The PTEN inhibitor SF1670 was added in increasing concentrations to media in a 96-well plate for 24 hrs. The wells were analyzed for proliferation according to the proliferation assay protocol described in Aim 2.

EGR-1 Nuclear Localization: An Active Motif Nuclear Extract Kit was used to obtain a cytosolic and nuclear protein fraction for CD44 expressing cells and vector control cells. Equal amounts of each fraction were loaded into the standard procedure for Western Blotting as described above.

Aim 2 – CD44 Induced Calcium Signaling and Pathway Regulation

Intracellular Calcium Measurements: One million cells per mL were suspended in PBS without calcium. Fura-2-AM was loaded into the cells at 6 µM and then incubated for 15 min at 37°C. The suspension was diluted a further one fourth with PBS and incubated for 15 min at 37°C. After washing, cells were suspended in a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mM NaH_2PO_4, and 5.5 mM glucose at pH 7.4. Cells were placed over coverslips coated in poly-L-lysine and incubated for 10 min at 37°C before imaging. Intracellular calcium was measured at 37ºC by the ratiometric technique using fura-2-AM (excitation at 340 nm and 380 nm, emission at 510 nm) (Invitrogen, Carlsbad, CA) as described by Dibas et al. utilizing a Nikon Eclipse TE2000-5 microscope and NIS-Elements AR3.2 software (Nikon Instruments, Melville, NY)[65]. The Grynkiewicz equation was used to convert the 340/380 ratio to internal levels of calcium in nanomolar concentrations[66].

Calcium Depletion Experiments: All experiments where calcium was removed from the cell culture used the following protocol. Complete media was combined with media containing 10mM EGTA to achieve a final concentration of 2.5mM EGTA.

Proliferation Assays: One hundred thousand cells per group were plated into 96 well plates in 200 µL of media. Cells were allowed to proliferate for 24 or 48 h. Proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega). To rule out the possibility that an observed decrease in proliferation was due to

a difference in viability, trypan blue staining was performed before and after incubation to assure that viability was maintained throughout incubation. Inducible calcium release activated channel inhibition was performed by adding 50 µM $NiSO_4$ to culture media.

Phosphatase Activity Assay: The calcineurin cellular activity assay kit from Enzo Life Sciences was used to measure phosphatase activity in the cells. Briefly, cell protein extracts were taken and chilled on ice in protease inhibitor lysis buffer. Extracts were then desalted using a resin column before a protein concentration was taken using the method of Bradford. Equal amounts of protein per sample were loaded into wells containing RII phosphopeptide substrate, target of PP1, PP2A, calcineurin, and PP2C. Samples run without the substrate were used as a background. Wells containing EGTA were used to eliminate the activity of calcineurin so that calcineurin activity could be measured by subtracting the EGTA wells from the total activity wells. Development with BIOMOL Green and measurement at A655nm using a standard curve of known phosphate concentrations produced values of nmole PO_4 per well.

Fluorescent Microscopy: One million cells were allowed to adhere to a slide coated in poly-lysine for 1 hour at room temperature. The slides were then exposed to 1% formaldehyde for 15 minutes. Slides were washed in PBS and then incubated with 0.5% Triton X-100 for 10 minutes. Slides were incubated with primary antibodies for Lck at 1:100 and CD44 at 1:100 for one hour at room temperature. Slides were washed in PBS for five minutes three times before incubation with fluorescent secondary antibodies at 1:100 for one hour at room temperature. Slides were washed for five minutes three times in PBS before being imaged on a fluorescent microscope.

Immunoprecipitation: Protein G coated magnetic beads from Active Motif were coated with 10µg of anti-phosphotyrosine antibody overnight at 4°C. Protein extracts from both CD44 expressing cells and vector cells were taken using CelLytic M from Sigma-Aldrich. Protein

extracts were combined with the anti-phosphotyrosine magnetic beads and rotated overnight at 4°C. The beads were spun down and the supernatant was discarded. The beads were washed twice in PBS containing 0.1% Tween-20. The beads were boiled for 10 minutes to release the immunoprecipitated phosphoytyrosine containing proteins, and that protein sample was loaded in equal amounts of total protein onto an SDS-PAGE gel. The protocol for western blotting was performed as described above.

CRAC Inhibition: CRAC channel inhibition was performed by the addition of 0.05mM Ni to complete media. The cells were cultured for 24 hours before being subjected to the proliferation assay as described above.

JC-1 Mitochondrial Staining: One million cells per sample were stained with 5μM JC-1 dye from Invitrogen by incubating at 37°C for 15 minutes. Cells were spun down and washed once in warm PBS. MFI were collected on a Beckman Coulter FC500 using the ratio of the FL-2 and FL-1 channels. An ISS PC1 spectrofluorometer was used to measure the fluorescence intensity of each cell sample at an ex/em of 485/530 for the monomeric form of JC-1 and an ex/em of 535/590 for the aggregate form of JC-1.

Invasion Assay: 2 million cells per group were placed in a transwell system with 0.8um pores atop 800uL matrigel. The media in the upper chamber was RPMI-1640 with 0% FBS. In the lower chamber the media was RPMI-1640 with 100U/mL of IL-2, 5ug/mL of fibronectin, and 10% FBS. Cells were allowed to invade through the gel for 48 hours. Gel inserts were removed and washed in dH_2O to remove non-invasive cells. Matrigel membranes were digested using 4mL of dispase at 10U/mL for 4 hours at 37°C. Invasive cells were counted from this dissolved gel solution using a hemocytometer.

Statistical Analysis: Experimental groups were compared by a Student's t test unless indicated otherwise. A p-value of less than 0.05 was considered significant. All results are shown as the mean ± SD. Each experiment was repeated thrice with similar results in the experiments.

CHAPTER III

CD44 INDUCED CELL CYCLE DISRUPTION

CD44 is a cell surface protein involved in hyaluronan metabolism and cellular adhesion. Studies have shown that CD44 can function as a recruitment platform, allowing various signaling pathway proteins to interact. To date, no known studies have examined the impact of CD44 expression on cell cycle progression in T cell Acute Lymphocytic Leukemia cells. Using E6.1 Jurkat cells as a model to investigate the cell cycle elements responsible for the observed decrease in proliferation upon CD44 expression, we can investigate the role of CD44 in a novel setting. Previous studies in our laboratory have determined that CD44 expression in Jurkat T cells induces a decrease in proliferation[18]. This defect in proliferation is due to decreased Akt activation which results in decreased EGR-1 expression[18], indicating that some elements of the cell cycle are disrupted.

CD44 Expression Induces Aneuploidy in Jurkat T Cells

To investigate the potential disruption in the cell cycle, we examined CD44 expressing cells and vector control cells for their DNA content. As seen in Fig. 3, 25% of the CD44 expressing cells possessed DNA content higher than 4N, indicating a severely aneuploid number of chromosomes. Over 40% of CD44 expressing cells showed DNA content of 4N, which was higher than the 10% of control cells at 4N. There were decreases in all other groups from less than 2N to the transition from 2N to 4N DNA content when comparing CD44 expressing cells to the vector control cells, but the differences are due to the buildup of aneuploid cells. To

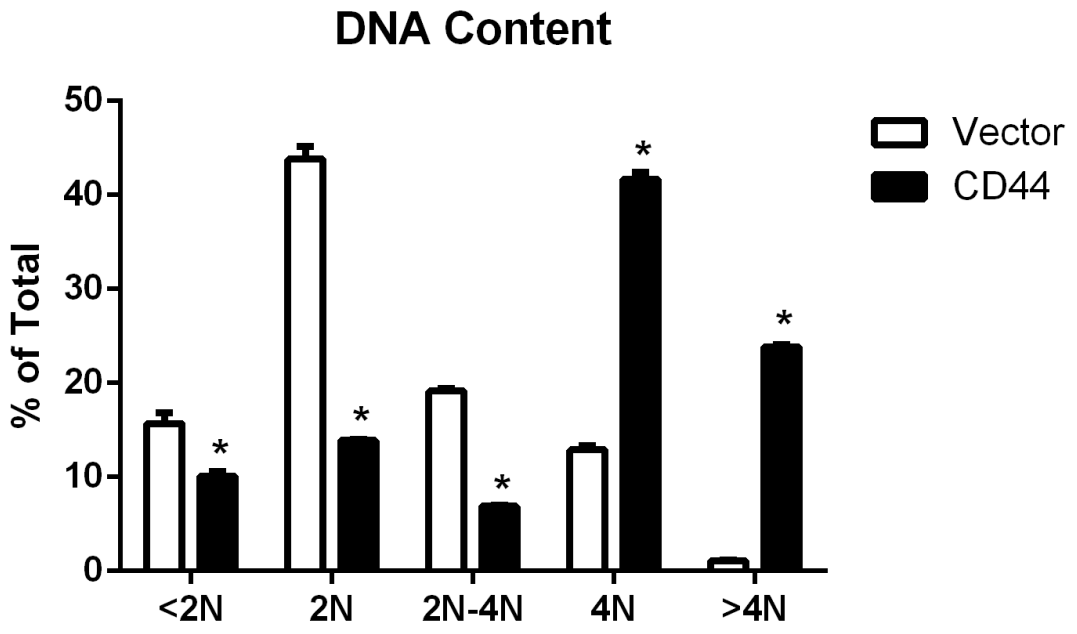

Figure 3. CD44 Expression Induces Aneuploidy in Jurkat T Cells.

DNA content analysis was performed via propidium iodide staining and flow cytometry. A Student's t test detected differences between CD44 cells and Vector cells. Results show representative data from four repeated experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

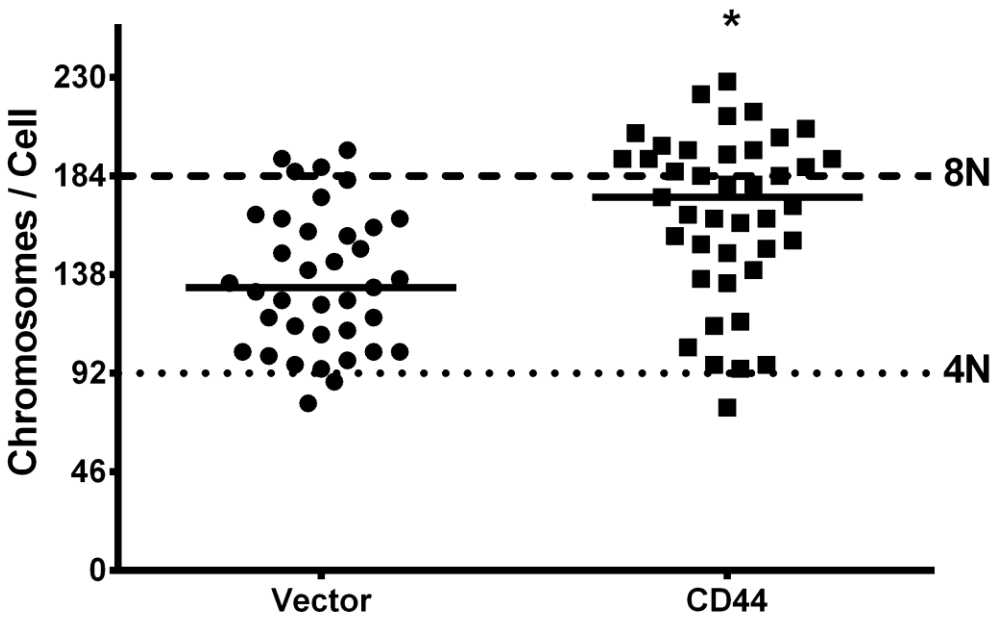

Figure 4. CD44 Expressing Jurkat T cells Have Significantly More Chromosomes.

Karyotyping was performed on Vector cells and CD44 cells by arresting the cells during mitosis with colcemid. Fixation and staining with DAPI followed by chromosome counting under a fluorescent microscope was performed to obtain counts. 4N is the DNA content of a normal cell undergoing mitosis. A Student's t test detected differences between CD44 cells and Vector cells. The chromosomes of 37 cells were counted per experiment. Results show representative data from two independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

determine the extent of the observed aneuploidy, CD44 and vector control cells were karyotyped. As seen in Fig. 4, CD44 expressing cells possessed mean chromosome number of 166 while the vector control had a mean chromosome number of 135. There were also cells observed between 4N and 8N in each group, but the number of cells coincides with the percent cells seen between these two intervals in Fig. 3. Therefore, these data indicate that CD44 expression induces aneuploidy, increasing the average number of chromosomes in CD44 expressing cells during mitosis by 20% compared to the vector control.

Akt Hypophosphorylation is Not Responsible for Aneuploidy

To determine the mechanism for the induction of aneuploidy, we examined the role of Akt in the establishment and maintenance of an aneuploid cell state in Jurkat T cells. Akt has been previously shown to be hypophosphorylated in CD44 expressing Jurkat T cells when compared to the vector control cells[18], and Akt is known to regulate chromosome stability[38-40]. We approached this problem from both sides of Akt activation. To determine if restoring Akt activity in CD44 expressing cells to match the vector control cell Akt activity we utilized myristoylated Akt transfection. When CD44 expressing Jurkat T cells were transfected with myristoylated Akt, a constitutively activated form of Akt, we observed no differences in aneuploid cell number when compared to the uninfected control as seen in Fig. 5. We next utilized LY294002 to inhibit Akt activity in the vector control cells to see if decreasing Akt phosphorylation, matching what occurs with CD44 expression, would induce aneuploidy. Inhibition of Akt activation did not induce aneuploidy in the vector control cells (Fig. 6), and the observed decreases in the phases of the cell cycle were due to the toxicity of the inhibitor over this time period, as shown by the increase in apoptotic cells. Therefore, Akt signaling is not involved in the induction of or rescue from aneuploidy in Jurkat T cells.

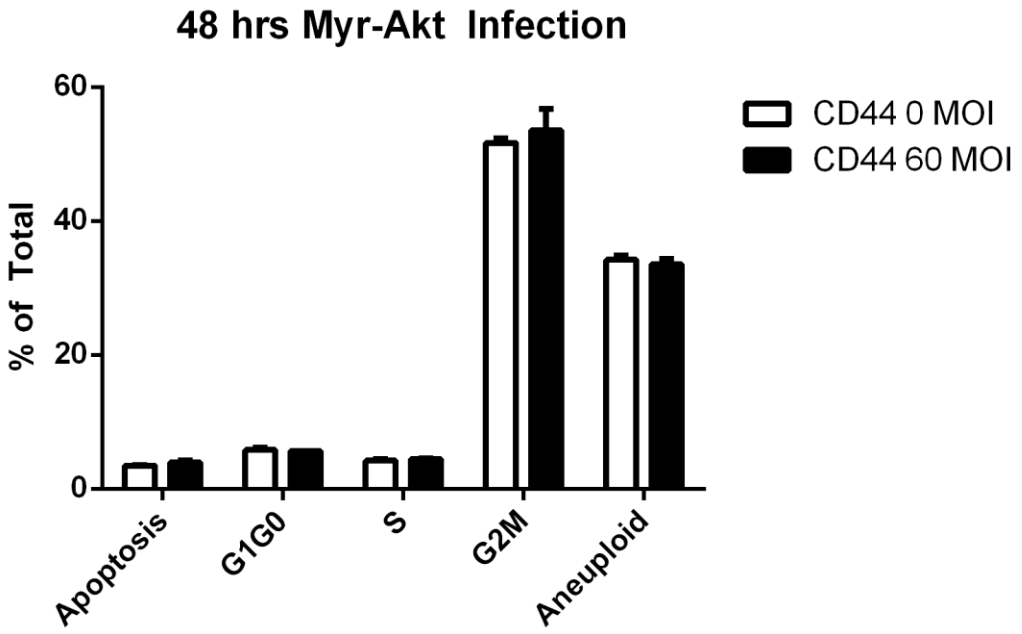

Figure 5. Myristoylated Akt Cannot Rescue CD44 Cells from Aneuploidy.

CD44 expressing cells were transfected with myristoylated-Akt using an adenoviral vector at 60 MOI. Cells were cultured for 48 hrs after transfection and the percent of cells that were aneuploid was determined by PI staining and flow cytometry. A Student's t test did not detect differences between transfected and untransfected cells. Fifty thousand events were counted per experiment. Results show representative data from three independent experiments. All data are expressed as the mean ± SD.

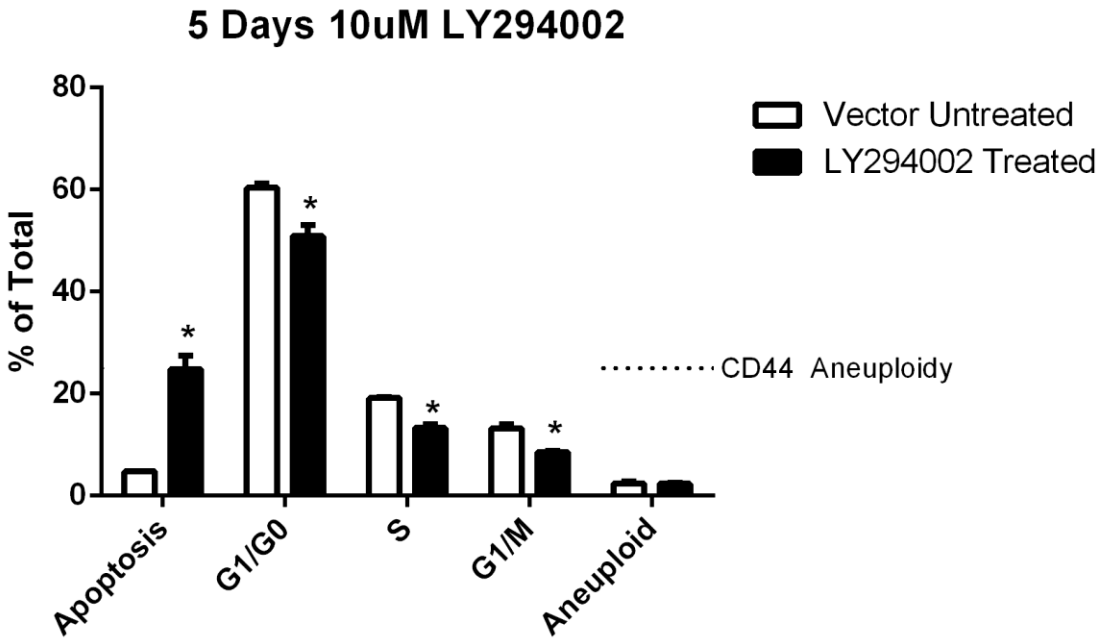

Figure 6. Akt Inhibition Does Not Induce Aneuploidy in Control Cells.

Vector control cells were treated with the Akt inhibitor LY294002 at 10μM for 5 days. The percent of cells that were aneuploid was determined by PI staining and flow cytometry. A Student's t test detected differences between treated and untreated cells. Fifty thousand events were counted per experiment. The dashed line indicates the average percent aneuploid cells in the CD44 expressing Jurkat cell cultures. Results show representative data from three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

The G1 to S Phase Transition is Slowed by CD44

EGR-1 expression and Akt activation are both related to the transition from the G1 phase of the cell cycle into the S phase. Since both EGR-1 expression and Akt activation were decreased in CD44 expressing cells compared to the control, we began our investigations into the cell cycle at the G1 to S restriction checkpoint. We synchronized cells in G1 phase and then introduced BrdU into the cell culture. This allowed us to determine how many cells in the culture at an observed time point had entered or completed the S phase of the cell cycle. As shown in Fig. 7A, 30 minutes after the cells were allowed to proliferate, fewer CD44 expressing cells had entered S phase compared to the control. This difference was short-lived however, as after one hour the difference between CD44 expressing cells and the control was not statistically significant. To determine if more than the transition from G1 into S phase is modified by CD44 expression, we next measured the length of the S phase. Although Fig. 7B shows a difference in the duration of the S phase between CD44 expressing cells and the vector control, the result was not statistically significant. The duration of S phase was highly variable on a per cell basis in both CD44 expressing and vector control cells. These results show that CD44 expressing Jurkat cells suffer from a delayed entry into S phase, but the duration of S phase is not impacted by CD44 expression.

Multiple Cell Cycle Components are Impacted by CD44 Expression

To expand our search for the mechanism of cell cycle disruption and aneuploidy due to CD44 expression we performed a RT^2-qPCR array. The array examines 84 genes involved in each cell cycle phase and checkpoint. As shown in Table 1, using a 2 fold cutoff we found that 2 genes were upregulated and 9 genes were downregulated when comparing mRNA expression in

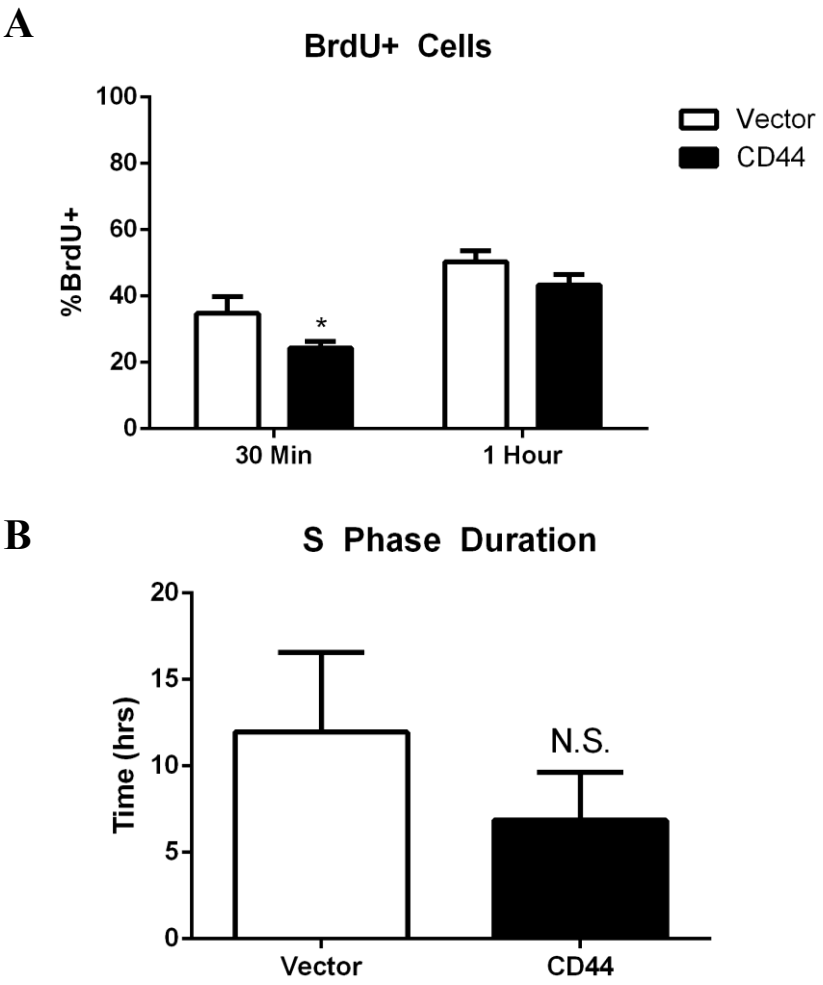

Figure 7. The G1 to S Phase Transition is Slowed by CD44 Expression.

Cells were synchronized into the G1 phase using serum starvation for 24 hours. Vector and CD44 cells were then cultured in the presence of 10mM BrdU for 1 hr. Cells were fixed at 30 minutes and 1 hr and counted by flow cytometry. (A) Shows the percent of cells that are BrdU+ at each time point. A two-way ANOVA with Tukey's HSD post-hoc test detected differences between CD44 and Vector cells at the 30 minute time point. (B) The equation $S = N_0t/(Nt - N_0)$ was used from the data collected in (A) to determine the duration of the S phase. A Student's t test did not detect differences between CD44 and Vector S phase duration. Fifty thousand events were counted per experiment. Results show representative data from three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

Gene Symbol	Name	Fold Regulation
Up-regulation:		
CCND1	Cyclin D1	4.73
TFDP2	E2F dimerization partner 2	2.77
Down-regulation:		
CDK5RAP1	CDK5 regulatory subunit 1	-2.03
RAD51	RAD51	-2.12
MKI67	Ki-67	-2.17
MAD2L1	Mitotic spindle assembly pro.	-2.22
BCL2	B-cell lymphoma 2	-2.29
MRE11A	Double-strand break repair protein	-2.36
CASP3	Caspase 3	-2.48
RB1	Retinoblastoma 1	-3.74
BRCA2	Breast cancer 2, early onset	-6.01

Table 1. Cell Cycle RT^2-qPCR Array Comparing Expression in CD44 cells to Vector.

The expression profiles of 84 different genes were evaluated using a commercial available RT2-qPCR array. A 2-fold cut-off was used to identify down- or up-regulated genes in CD44 expressing Jurkat T cells compared to the vector control.

CD44 expressing cells to the control cells. Retinoblastoma (Rb) was decreased 3.74 fold and Cyclin D1 was increased 4.73 fold in CD44 expressing cells compared to the control. These two proteins are involved in the transition from G1 into S phase[26,37]. MAD2A stops cells from entering anaphase until all chromosomes are properly aligned[67] and was downregulated 2.22 fold. RAD51 is involved in homologous recombination during double strand break repair[68] and was downregulated 2.12 fold. BRCA2 is a tumor suppressor that aids in the repair of DNA[33,69,70], interacting with RAD51, and was downregulated 6.01 fold. These results indicate that CD44 expressing cells have decreased mRNA expression of several key proteins involved in chromosome number integrity and proliferation. These results will be combined with another PCR array to generate pathway predictions.

CD44 Expression Could Modify Calcium Signaling and Invasion

We next utilized a RT^2-qPCR signal transduction array to broadly extend our search for signaling pathways modified by CD44 expression in Jurkat T cells. As shown by Table 2, we observed 4 upregulated genes and 21 downregulated genes in CD44 expressing cells compared to the control. SERPINE1 was upregulated 4.93 fold and is a serine protease inhibitor that blocks fibrinolysis and the activity of matrix metalloproteinases[71], decreasing invasiveness of tumors[72]. The EGF receptor was downregulated 2.03 fold and has been shown to interact with CD44 to induce calcium signaling[57]. These two genes led us to study invasion and calcium signaling, but the other results from this array need to be analyzed in order to establish other potential pathways.

Gene Symbol	Name	Fold Regulation
Up-regulation:		
SERPINE1	Serine peptidase inhibitor E1	4.93
CCND1	Cyclin D1	4.11
CEBPD	CCAAT/enhancer-binding protein	3.05
JAG1	Jagged 1	2.93
Down-regulation:		
EGFR	EGF Receptor	-2.03
NOTCH1	Notch 1	-2.1
BMP4	Bone morphogenetic protein 4	-2.17
GSR	Glutathione reductase	-2.32
WNT1	WNT1	-2.33
CSF1	Macrophage colony-stimulating f	-2.42
GATA3	GATA binding protein 3	-2.52
BCL2A1	Bcl-2-related protein A1	-2.59
TNF	TNF-alpha	-2.62
BCL2	B-Cell Lymphoma 2	-2.89
PTCH1	Patched 1	-2.89
ACSL5	CoA ligase 5	-2.97
AXIN2	Axin 2	-3
HEY2	Hairy/enhancer of split rel…	-3.04
RB1	Retinoblastoma 1	-3.09
VEGFA	VEGF A	-3.56
FCER2	CD23, FCεRII	-4.08
WISP1	WNT1 inducible signaling... pro 1	-4.95
EPO	Erythropoietin	-6.5
CA9	Carbonic anhydrase 9	-6.54
HES1	Hair and enhancer of split 1	-15.66

Table 2. Human Signal Transduction RT^2-qPCR Array Comparing Expression in CD44 cells to Vector.

The expression profiles of 84 different genes were evaluated using a commercial available RT2-qPCR array. A 2-fold cut-off was used to identify down- or up-regulated genes in CD44 expressing Jurkat T cells compared to the vector control.

CD44 Expression Regulates Genes Important for Aneuploidy and Proliferation

To examine signaling pathways potentially modified by CD44 expression, we took the data from the two RT^2-qPCR arrays and ran it through analysis software. The first analysis of data using GNCpro groups the genes found using a 2 fold cutoff by their function within the cell. We also added Akt and CD44 to the GNCpro software, as CD44 expression is being induced and we know Akt is hypophosphorylated in CD44 expressing Jurkat T cells. Any genes within the 2 fold cutoff that are not related to any other genes within the sample are not illustrated. As shown in Fig. 8, four distinct areas of related genes are regulated by CD44 expression. Grey lines between genes show that there is a proven relationship, such as direct interaction or transcriptional regulation, as evidenced by peer reviewed articles. Dashed lines indicate a predicted interaction between the genes that has been proposed in other literature. Four genes involved in the regulation and prevention of aneuploidy were downregulated by CD44 expression (Table 2), and are linked to a cluster of genes responsible for proliferation and anti-apoptosis signaling. Central to the proliferation and anti-apoptosis cluster is Akt, which is linked to every other member of this cluster, as well as providing a link to the Immune Function and Differentiation cluster. The cluster for Immune Function is not unexpected, as Jurkat cells are T cells. Isolated from the three main clusters is a NOTCH signaling cluster, which is tangentially linked to the Immune Function cluster and directly linked to the Proliferation cluster by way of the EGF receptor. This pathway analysis shows that the primary functional areas modified by CD44 expression within the two RT^2-qPCR arrays are aneuploidy and proliferation.

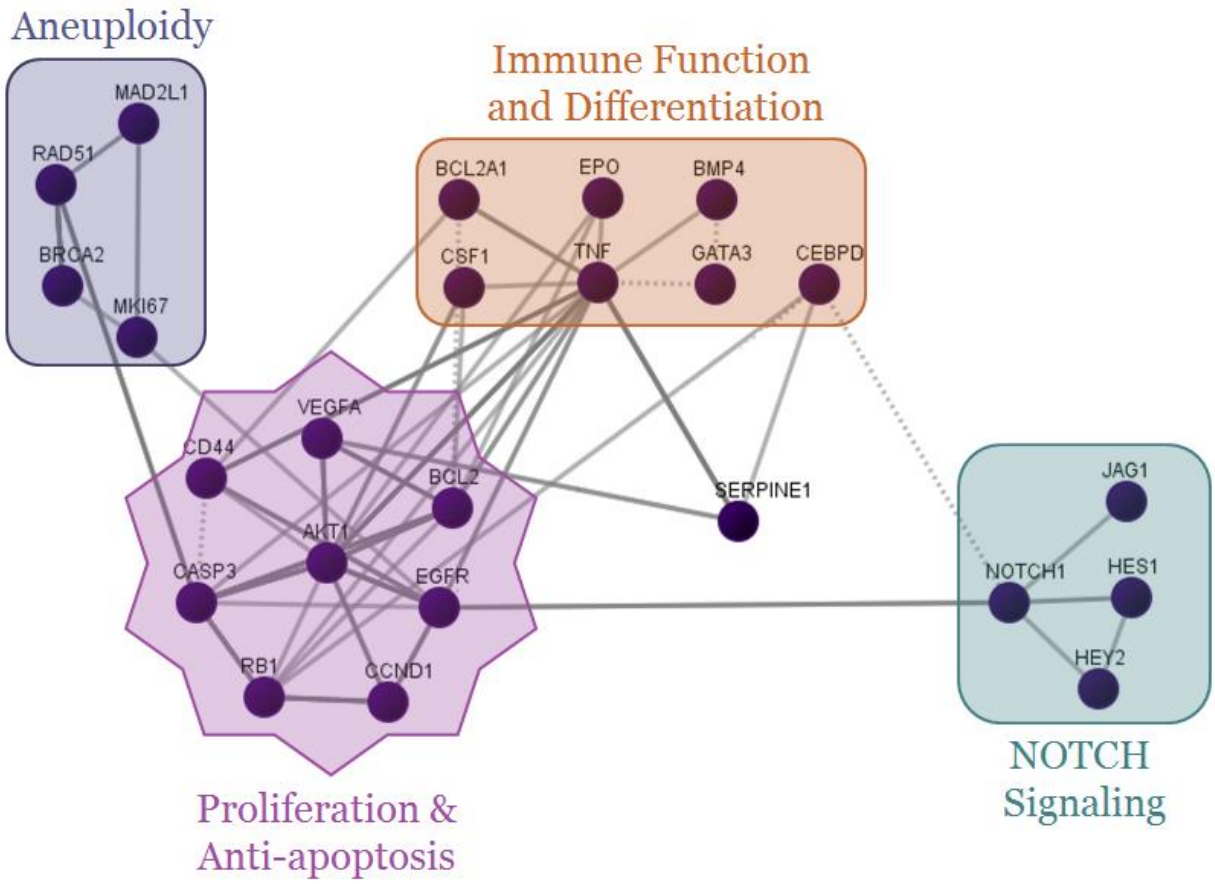

Figure 8. CD44 Expression Alters Gene Regulation in Four Distinct Areas.

GNCPro software was used to analyze the data from Tables 1 & 2 with the addition of CD44 and Akt. Solid lines between genes indicate that there is a known interaction between the two genes. Dashed lines indicate a predicted interaction. Genes are grouped by function as determined by the SA Biosciences analysis software. Genes which did not interact with any others are not shown.

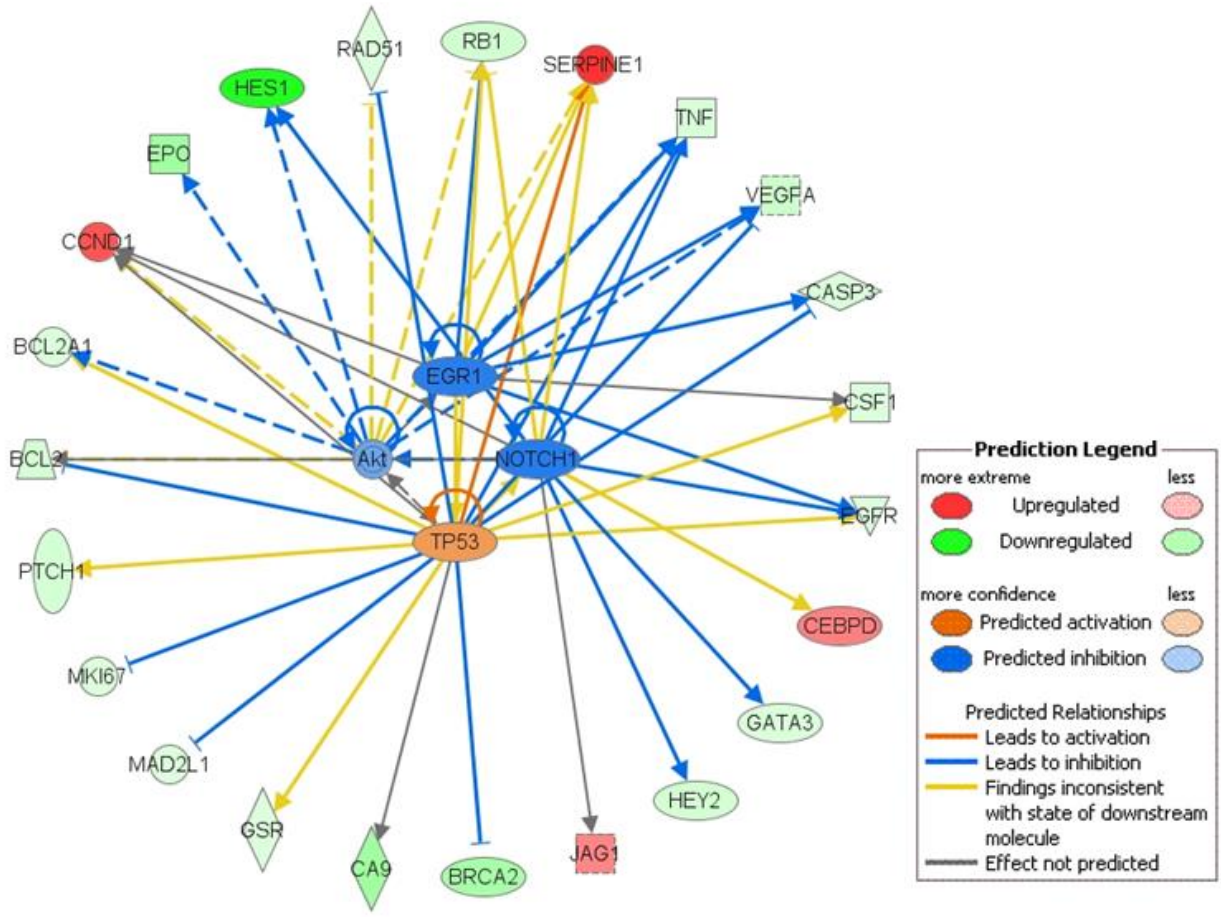

Figure 9. EGR-1, Akt, p53, and Notch are Predicted to be Regulated by CD44 Expression.

IPA software was used to analyze the data from Tables 1 & 2. Shown are the four proteins predicted to be inhibited or activated by CD44 expression based on the gene regulation data obtained from the RT^2-qPCR arrays. Not all findings are consistent, as indicated by yellows lines.

CD44 Expression is Predicted to Inhibit Akt and EGR-1 activity

Taking our RT^2-qPCR array data one step further, we utilized Ingenuity Pathway Analysis (IPA) software. This software allows us to predict which signaling pathways are regulated by CD44, based on what genes were up and down regulated according to our arrays. As shown in Fig. 9, IPA predicted that, based on the genes returned from our RT^2-qPCR arrays, Akt and EGR-1 activation would be inhibited compared to the control cells. Akt is hypophosphorylated in CD44 expressing Jurkat T cells compared to their control, and EGR-1 expression is decreased in CD44 expressing cells. The software also predicts NOTCH1 to be inhibited, echoing the fact that there was a Notch pathway cluster in Fig. 8. IPA also predicts that p53 is activated in CD44 expressing Jurkat cells compared to the control. This pathway analysis, coupled with GNCpro, expands upon what we know to be inhibited by CD44 expression as well as provides a future direction for this study via p53 and potentially NOTCH1.

The p53-Rb Signaling Pathway is Not Modified by CD44 Expression

Because the RT^2-qPCR array analysis indicated that p53 is potentially activated in CD44 expressing cells, we wanted to investigate the major players in the p53 pathway. We first began by determining if there was a difference in the expression of p53 between the cell groups. Western blotting for total p53 (Fig. 10A) revealed that neither CD44 expressing cells nor the vector control Jurkat cells express p53. This finding matches what has been reported previously, that E6.1 Jurkat cells are p53 null[73-75]. We next looked at p27Kip1, an inhibitor of cell cycle progression from G1 phase to S phase. Western blotting for total p27Kip1 (Fig. 10B) shows that neither CD44 expressing cells nor the vector control Jurkat cells express detectable amounts of p27Kip1. Rb was suggested by both RT^2-qPCR arrays to be downregulated in CD44 expressing cells. Western blotting for phosphor-Rb at S795 (Fig. 10C) shows that CD44 expressing cells

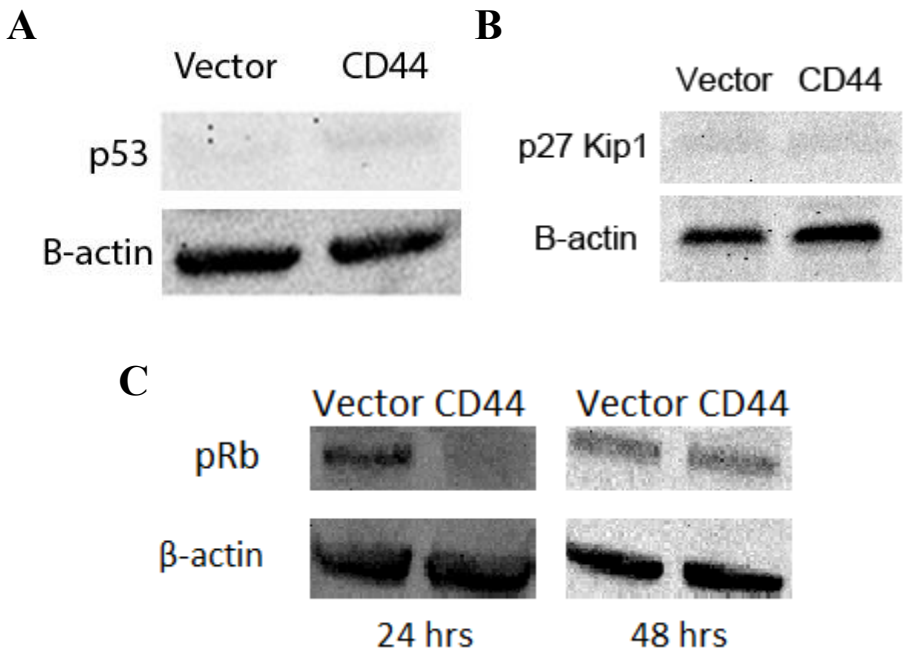

Figure 10. The p53 Pathway is Absent or Normally Cyclic in Jurkat Cells

Western blots for various components of the p53 regulatory pathway were performed on protein samples from CD44 and Vector cells. (A) Western blot for the presence of total p53 reveals that p53 expression is absent in Jurkat T cells. (B) Western blot for p27Kip1 reveals there is no detectable p27Kip1 expression in Jurkat T cells (C) Western blot for phospho-Rb (S795) reveals that the phosphorylation status of this serine is dependent on the time from last media change. Shown here are protein extracts taken 24 and 48 hrs after proving the cells with fresh media. Results show representative data from three independent experiments.

possess less pRb 24 hours after changing the media in the culture flask, but at 48 hours post media change there is no difference in pRb when compared to the control cells. Both RT^2-PCR arrays showed that Rb mRNA was downregulated 3 fold in CD44 cells compared to the control, but Western blotting revealed that total Rb is equal in both cell types (data not shown). Such a result helps illustrate the fact that mRNA data does not always equate to protein expression. Therefore, these results indicate that the p53 pathway is not a good target for continued study as Jurkat cells are null for several of the elements in the pathway. Elements of the pathway that exist, such as Rb, exhibit a cyclic nature of activation characteristic of cell cycle proteins.

PTEN is Not Activated by CD44 Expression

The array analysis reconfirmed that Akt was a major player in the effects of CD44 expression on other signaling pathways. Our next objective was to determine if CD44 expression was inducing hypophosphorylation of Akt by activating PTEN and preventing association of Akt with the plasma membrane. Western blotting for phosphorylated PTEN showed that CD44 expressing cells possess less activated PTEN compared to the control cells (Fig. 11A). To rule out the possibility that PTEN activation was transient, we performed longer term studies with the PTEN inhibitor SF1670. As shown in Fig. 11B, inhibition of PTEN with SF1670 did not induce proliferation in CD44 expressing cells. The proliferation of both CD44 expressing cells and the vector control was reduced as the concentration of the inhibitor increased, largely due to increasing toxicity of the inhibitor. Therefore, these data indicate that PTEN is not activated by CD44 expression and does not play a role in the decrease in proliferation observed in CD44 expressing Jurkat T cells.

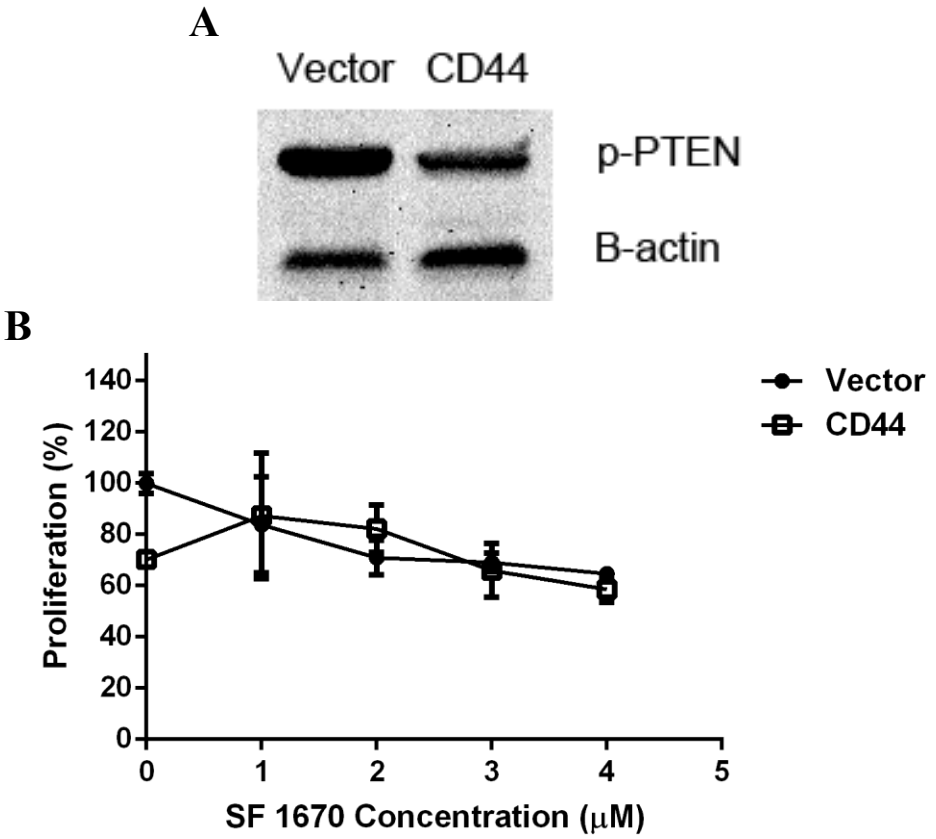

Figure 11. The Hypophosphorylation of Akt is Not Caused by PTEN Activation.

(A) Western blotting for phosphorylated PTEN reveals that Vector cells have more activated PTEN than CD44 cells. (B) Treatment of cells with the PTEN inhibitor SF1670 at varying concentrations for 24 hours has no effect on proliferation. The decrease in proliferation is due to increasing toxicity of the inhibitor. Results are normalized to Vector 0μM set to 100%. A Student's t test did not differences between CD44 and Vector S proliferation at any concentration of SF1670. Results show representative data from three independent experiments. All data are expressed as the mean ± SD.

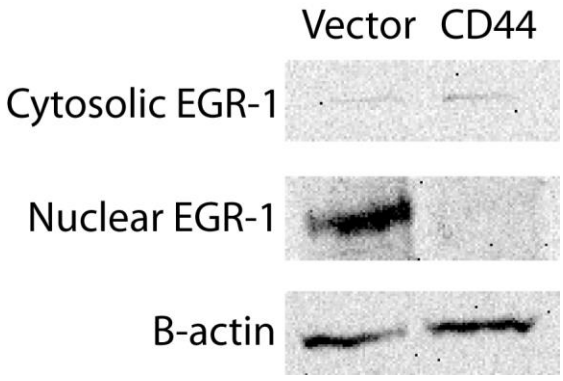

Figure 12. EGR-1 Nuclear Localization is Impaired by CD44.

Western blot showing the localization of EGR-1 in CD44 and Vector cells. CD44 expressing cells do not have detectable amounts of nuclear EGR-1. The β-actin band is from the nuclear fraction. Results show representative data from three independent experiments.

EGR-1 Localization is Disrupted by CD44 Expression

Previous data from our lab has demonstrated that EGR-1 expression is decreased in CD44 expressing cells[18], but EGR-1 must be imported into the nucleus in order to function as a transcription factor. To determine if the EGR-1 that is present in CD44 expressing cells is functional, we isolated nuclear and cytoplasmic extracts from CD44 expressing cells and the vector control. Western blotting for EGR-1 with both extracts (Fig. 12) revealed that all of the EGR-1 present in the vector control cells was located within the nucleus, while the little EGR-1 that was expressed in CD44 cells was located in the cytoplasm. This data indicates that not only is EGR-1 expression decreased in CD44 expressing Jurkat cells, but the EGR-1 that is present exists in a non-functional state outside of the nucleus.

Summary

The results presented in this chapter provide evidence that CD44 delays entry into the S phase of the cell cycle despite apparent upregulation of Cyclin D1 mRNA and the absence of p27Kip1 (Figs. 7A&10B, Table 1). CD44 expression induces aneuploidy by potentially downregulating several genes responsible for maintaining chromosome stability and separation during mitosis, but the hypophosphorylation of Akt is not responsible for the alteration in chromosome number (Figs. 3-6, 8, Tables 1&2). Our data demonstrates that Akt and EGR-1 are at the center of the observed gene regulation and phenotype of the CD44 expressing Jurkat cells (Figs. 8&9). We have shown that the hypophosphorylation of Akt due to CD44 expression is not caused by activation of PTEN by CD44, and CD44 also inhibits the nuclear translocation of EGR-1 (Figs. 11&12).

Other pathway targets suggested by array analysis were p53 and Notch signaling. Jurkat cells do not express p53 and other pathway components are either not expressed or exhibit cyclic

patterns of activation characteristic of cell cycle regulatory proteins (Figs. 8-10). The Notch pathway was not studied in this thesis, but future work could be performed to fully investigate this detailed pathway. For the first time, we outline the impact of CD44 on chromosome number and stability in Jurkat T cells while further implicating Akt and EGR-1 in a wide array of phenotypic changes.

CHAPTER IV

CD44 INDUCED CALCIUM SIGNALING AND PATHWAY REGULATION

The previous chapter demonstrated that CD44 expression in Jurkat T cells induces hypophosphorylation of Akt and decreases EGR-1, resulting in aneuploidy and an altered cell cycle. RT^2-qPCR array data also suggests that CD44 could be altering calcium influx in order to activate a broad spectrum of signaling pathways. Although previous studies have shown that CD44 can interact with elements of calcium signaling pathways, the role of CD44 expression in a leukemic T cell has not been defined. This chapter will focus on the role of CD44 in calcium signaling within Jurkat T cells.

CD44 Expressing Jurkat T Cells Have Increased Calcium Influx

To investigate the potential role of CD44 expression in modifying the amount of calcium signaling in Jurkat T cells, we first loaded cells with fura-2-AM. In Fig. 13A cells were allowed to adhere to poly-lysine before fura-2-AM loading to allow for UV microscopy. CD44 expressing cells showed distinct pockets of lower fura-2-AM ratios, contrasting the high fluorescence ratio observed in the main body of each cell. Vector control cells display uniform ratios throughout the cell. Normalization with ionomycin and EDTA allows the application of the Grynkiewicz equation to determine the cytoplasmic concentration of calcium in each cell. CD44 expressing Jurkat T cells contain an average calcium concentration of 654nM, while vector control cells have an average calcium concentration of 119nM (Fig. 13B). Therefore, these data indicate that CD44 expression induces calcium influx in Jurkat T cells.

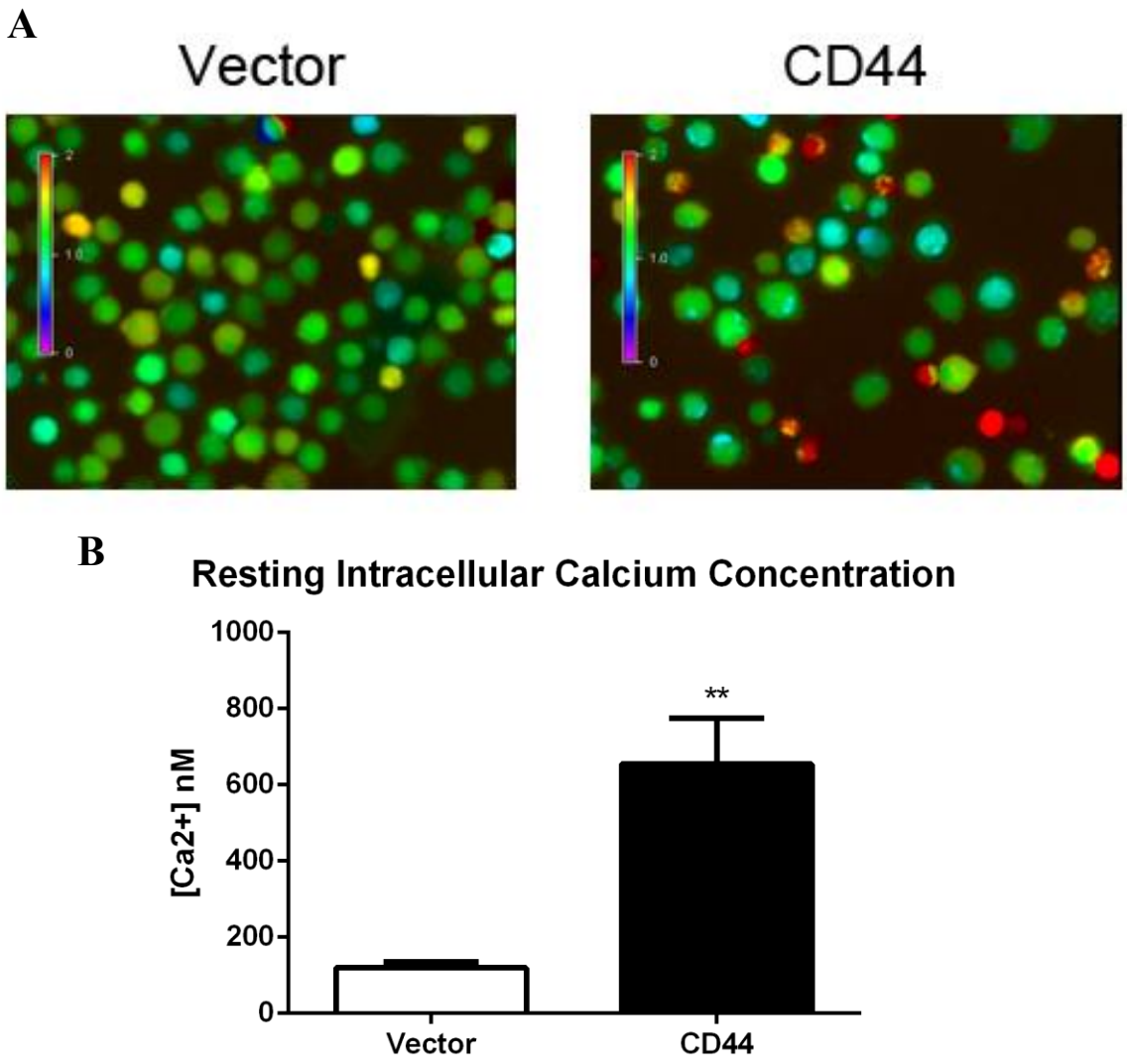

Figure 13. CD44 Expressing Cells Have a Higher Intracellular Calcium Concentration

CD44 and Vector cells were loaded with fura-2-AM under nonstimulating conditions in media containing calcium. (A) UV microscopy showing the ratio of fluorescence at 340nm to 380nm. Images were taken using two different exposures due to the calibrations required to record calcium concentrations. (B) Resting intracellular calcium concentration was assessed by ratiometric imaging of fura-2-am loaded cells with the Grynkiewicz equation. Multiple images were taken for ratiometric assessment and the data was pooled for statistical analysis using a Student's t-test (n = 40 cells). A Student's t test detected differences between CD44 and Vector cell calcium concentration. Results show representative data from three independent experiments. All data are expressed as the mean ± SD. (**$p<0.01$)

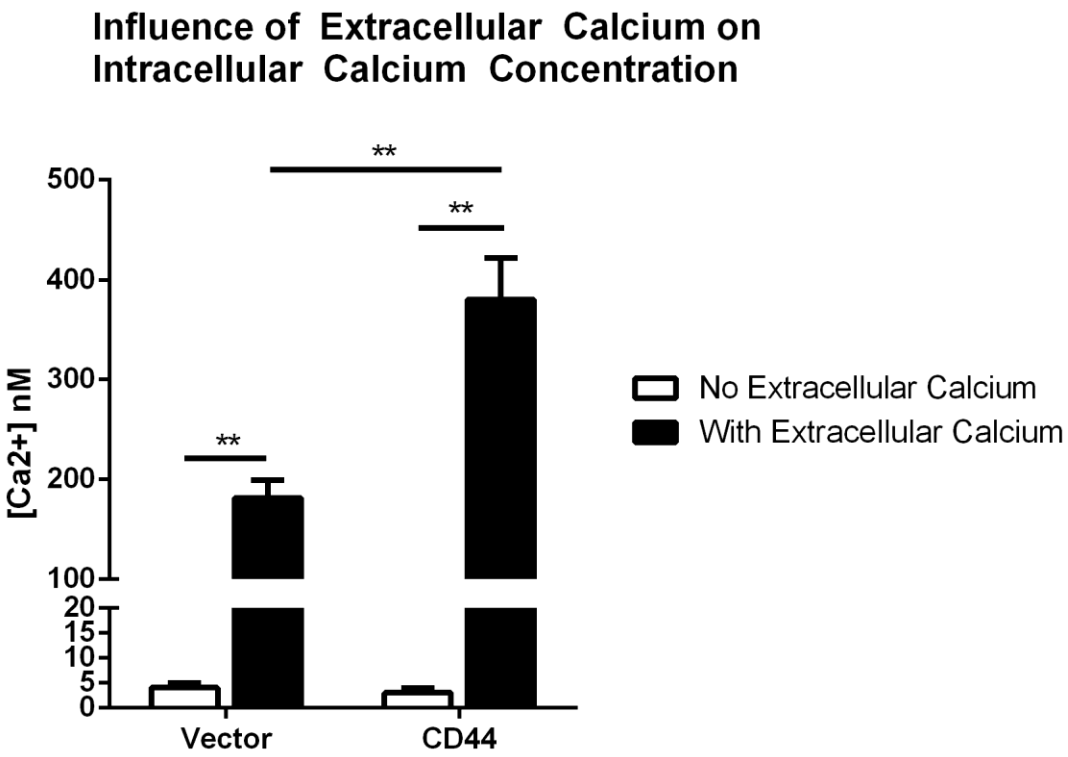

Figure 14. Extracellular Calcium Stores are Used for Influx.

Intracellular calcium concentration in the presence or absence of extracellular calcium. There was no difference between Vector and CD44 with no extracellular calcium ($p>0.05$, $n = 30$ cells). A two-way ANOVA with Tukey's HSD post-hoc test detected differences between CD44 and Vector with extracellular calcium between the cells with and without extracellular calcium. Results show representative data from three independent experiments. All data are expressed as the mean ± SD. (**$p<0.01$)

The Influx of Extracellular Calcium is the Reservoir Opened by CD44

We have shown that calcium concentrations are higher within CD44 expressing cells compared to the control, but the source of the calcium is unknown. To determine which store of calcium is increasing the intracellular calcium concentration of CD44 expressing cells, we first investigated the possibility that extracellular calcium was entering the cells. As shown in Fig. 14, when both CD44 expressing and vector control cells are cultured in media without calcium, the intracellular calcium concentration decreases. The intracellular calcium concentrations are 4nM and 3nM in the vector control and CD44 expressing cells, respectively. This data indicates that both CD44 expressing and vector control cells rely on extracellular calcium to sustain their calcium influx and that CD44 expression alters the influx of calcium from outside of the cell to the inside.

Calcium is Required for Proliferation

To investigate the role of the intracellular calcium concentration on proliferation, we began culturing cells without calcium in their media. When deprived of extracellular calcium CD44 and vector control cells have decreased proliferation compared to normal media (Fig. 15A). To determine if this impairment of proliferation altered the cells permanently or transiently, we returned cells that had been deprived of calcium back into media with calcium. As shown in Fig. 15B, when cells that have been deprived of calcium are given back normal concentrations of calcium they begin to proliferate. After four days of normal media calcium levels after deprivation, CD44 expressing cells still proliferate less than the vector control cells. The deprivation of calcium did not induce cytotoxicity, as the viability of cultures with and without calcium was not changed (data not shown). Therefore, this data shows that Jurkat cells

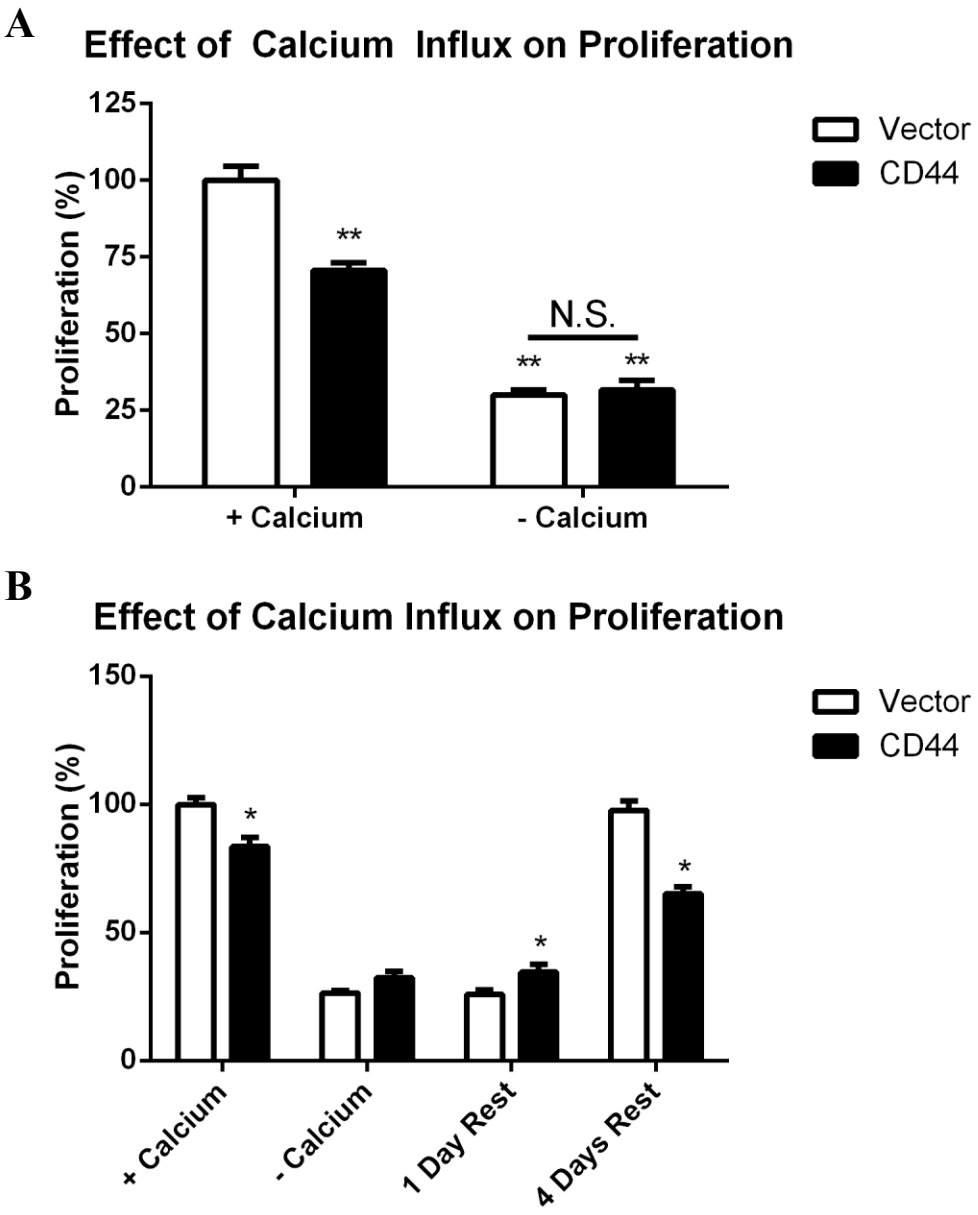

Figure 15. Calcium is Required for Proliferation

(A) Cell proliferation with and without extracellular calcium in the media. Results are normalized to percent of control cell line with calcium. (B) Cell proliferation before, during, and after a 24 h period without extracellular calcium. Results are normalized to percent of control cell line with calcium. There was no difference between Vector and CD44 cell lines without calcium ($p>0.05$). A two-way ANOVA with Tukey's HSD post-hoc test detected differences between CD44 and Vector as indicated by a *. Results show representative data from three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

require calcium to proliferate normally, and removing extracellular calcium does not permanently alter cell proliferation. The inherent difference in proliferation between CD44 expressing cells and the vector control cells is only present when extracellular calcium is able to enter the cells.

EGR-1 Expression is Regulated by Calcium

EGR-1 expression is decreased in CD44 expressing Jurkat cells compared to the vector control[18]. We wanted to determine whether calcium influx played a role in the regulation of EGR-1 expression. Western blotting of protein samples from cells with and without calcium in the media reveals that EGR-1 expression decreases in both CD44 expressing and vector control cells in the absence of calcium (Fig. 16A). To determine if removing calcium from the media had a permanent or transient effect on EGR-1 expression, we returned cells that had been deprived of calcium back into media with calcium and took protein samples. Similar to the proliferation data (Fig. 15B), the expression of EGR-1 is not permanently decreased by absence of calcium (Fig. 16B). These data show that EGR-1 expression requires calcium influx, and that the regulation of its expression is not permanently effected by removal of calcium stores.

EGR-1 Expression is Equally Regulated by Calcium and Akt

Our lab has previously shown that activated Akt was responsible for controlling EGR-1 expression in our Jurkat T cells[18]. We investigated the interplay between Akt activity and calcium influx in regulating EGR-1 expression. Cells were deprived of calcium in their media, cultured in the Akt inhibitor LY294002, or both. Western blotting shows that EGR-1 expression was equally decreased by all three experimental conditions in both CD44 expressing cells and vector control cells(Fig 17). These data suggest that Akt activation is potentially regulated by calcium signaling in Jurkat T cells.

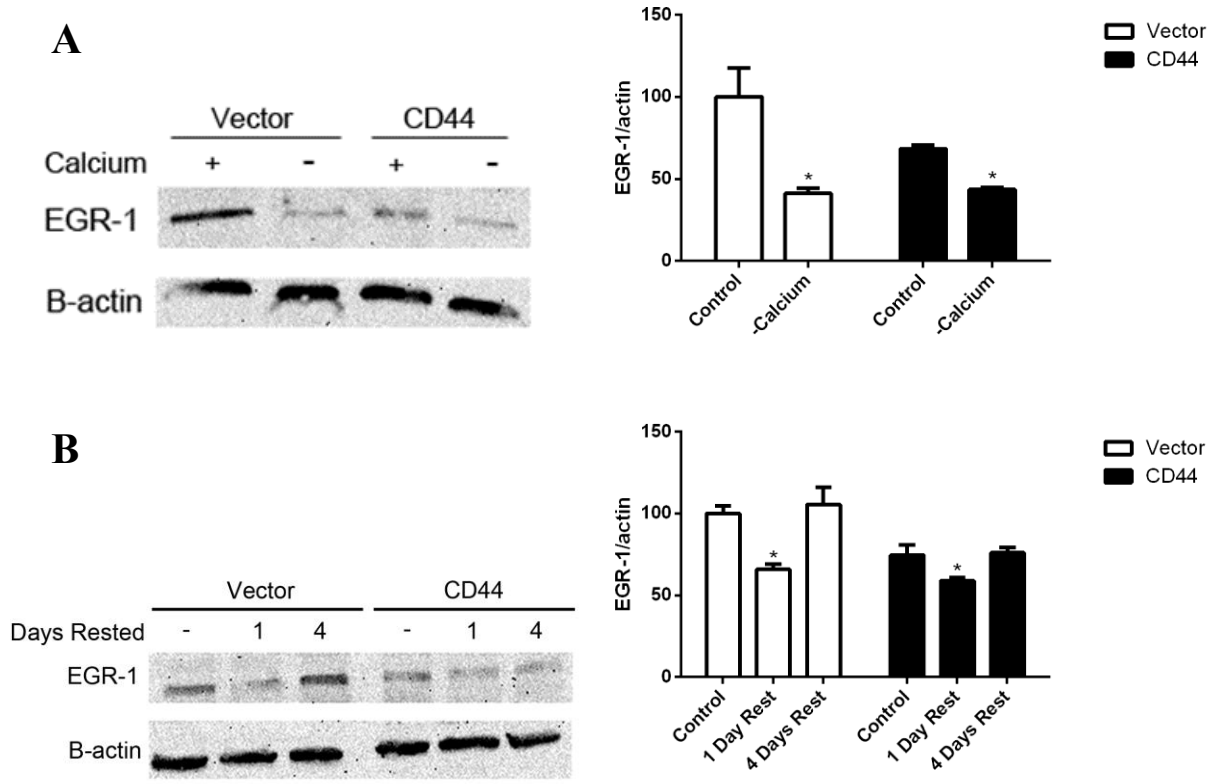

Figure 16. EGR-1 Expression is Regulated by Calcium in Jurkat T Cells

(A) Western blot for EGR-1 expression with and without extracellular calcium. Blot is representative of 3 independent experiments. (B) Western blot for EGR-1 expression before removal of extracellular calcium, after one day of rest following 24 h without extracellular calcium, and after four days of rest following 24 h without extracellular calcium. Three independent experiments were used to create the densitometry data where EGR-1 expression has been normalized to β-actin expression. The Vector cell control lane is set to 100 for comparison. A two-way ANOVA with Tukey's HSD post-hoc test detected statistical differences between bands within each cell line as indicated by a *. All data are expressed as the mean ± SD. (*$p<0.05$)

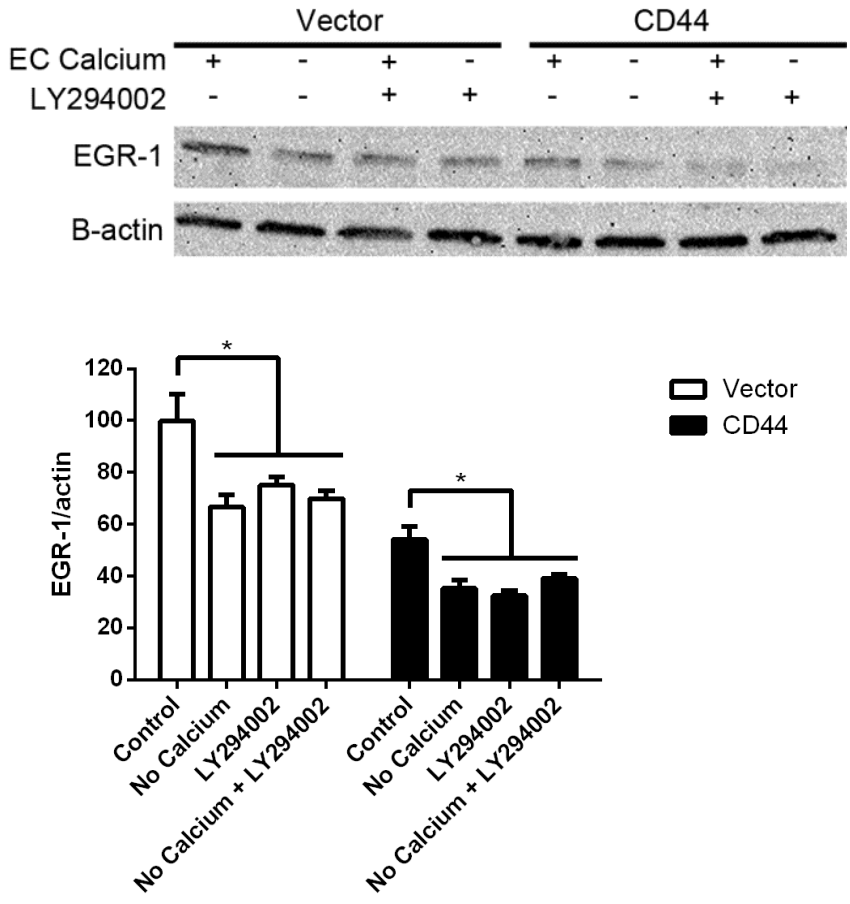

Figure 17. EGR-1 Expression is Equally Regulated by Calcium and Akt.

Western blot for EGR-1 expression with extracellular calcium (EC calcium), without EC calcium, with an inhibitor of Akt activation, and without EC calcium and an Akt inhibitor. Three independent experiments were used to create the densitometry data where EGR-1 expression has been normalized to β-actin expression. The Vector cell control lane is set to 100 for comparison. A two-way ANOVA with Tukey's HSD post-hoc test detected differences between the control lanes and the experimental lanes, but not between each experimental lane. All data are expressed as the mean ± SD. (*$p<0.05$)

Akt Activity is Regulated by Calcium Influx, but not by Calcineurin

In order to investigate the relationship between calcium influx and Akt activity we examined Akt phosphorylation in response to calcium deprivation. Western blotting of samples from cells cultured with and without calcium reveals that calcium is required for full phosphorylation of Akt at Ser473 (Fig. 18A). When calcium is not present in the culture media both CD44 expressing and vector control cells have half as much phosphorylated Akt. Despite the reduction in Akt activation due to the absence of calcium influx, the inherent difference in Akt activation between CD44 expressing cells and vector control cells remained. To determine if this inherent difference in Akt activation was due to calcineurin, a calcium dependent phosphatase, we measured phosphatase activity. As shown in Fig. 18B, total phosphatase activity is higher in CD44 expressing cells compared to the control cells. This assay specifically measures the activity of PP1, PP2A, calcineurin, and PP2C. When the activity of calcineurin, the only calcium dependent phosphatase measured by this assay, is inhibited, CD44 expressing cells still have higher phosphatase activity compared to the vector control. The activity of calcineurin is measured by subtracting the total phosphatase activity from the calcium independent activity, and there is no difference between CD44 expressing and vector control cells. Therefore, these results indicate that Akt phosphorylation is regulated by calcium, but the inherent hypophosphorylation of Akt in CD44 expressing cells is not caused by activated calcineurin.

Lck Associates with CD44 at the Cell Surface, but PLCγ1 is Not Activated

To determine how CD44 expression was leading to increased intracellular calcium concentrations, we first investigated the potential activation of Lck and PLCγ1. Cells were fixed to slides and stained for CD44 and Lck, an upstream component of calcium entry signaling that is known to associate with CD44. As shown in Fig. 19A, Lck can be seen colocalized with CD44

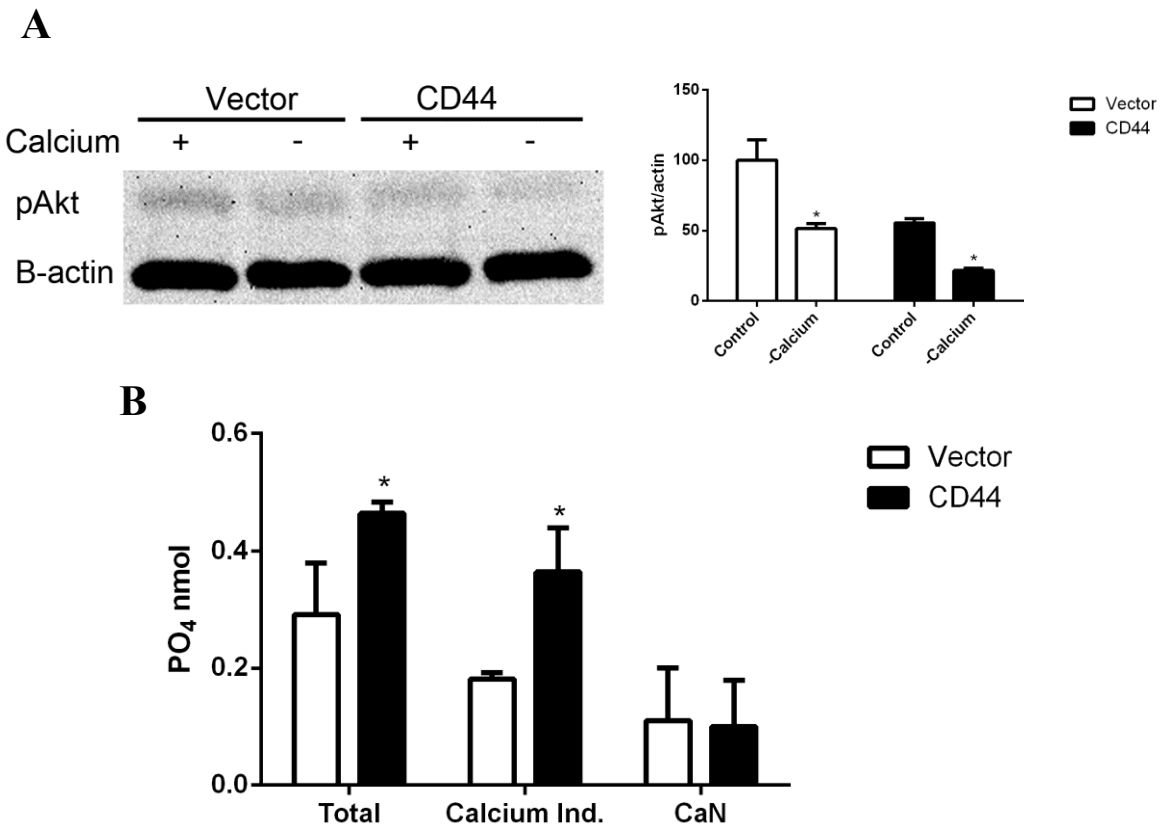

Figure 18. Akt Activity is Regulated by Calcium Influx, But Not by Calcineurin.

(A) Western blot for phosphorylated Akt (Ser473) with and without extracellular calcium. Three independent experiments were used to create the densitometry data where EGR-1 expression has been normalized to β-actin expression. The Vector cell control lane is set to 100 for comparison. A two-way ANOVA with Tukey's HSD post-hoc test detected differences between the control lane and the experimental lane for both cell groups. (B) A phosphatase activity assay specific for PP1, PP2A, calcineurin, and PP2C. Total represents all measured phosphatase activity. Calcium Independent represents the activity of PP1, PP2A, and PP2C. CaN represents the activity of just calcineurin. A Student's t test detected differences between the CD44 and Vector cells for Total and Calcium Independent but not calcineurin activity. All data are expressed as the mean ± SD. (*$p<0.05$)

at the cell surface when imaged using fluorescence microscopy. Technical difficulties arose when attempting to examine the slides with confocal microscopy due to abnormally high background and the extremely fragile nature of the cells fixed to the slides. Further technical difficulties occurred when attempting to immunoprecipitate CD44, as our lab has never been able to successfully western blot nor pull down CD44 from any protein sample. Due to the inability to further investigate potential interactions between CD44 and Lck, we next investigated whether PLCγ1, which is activated by Lck, was hyper-activated by CD44 expression. Western blotting revealed that total expression of PLCγ1 was not different in CD44 expressing cells and vector control cells (Fig. 19B). Activation of PLCγ1 is normally detected by measuring phosphorylation at Tyr783, and CD44 expressing cells had less phosphorylated PLCγ1 than vector control cells (Fig. 19D). There are multiple phosphorylation sites on PLCγ1 that can lead to its activation, and in order to determine if there was an overall difference in PLCγ1 activation we employed an immunoprecipitation reaction. Protein extracts from CD44 and vector control cells were immunoprecipitated for all phosphor-tyrosine containing proteins and then blotting for PLCγ1 was performed. As shown in Fig. 19D, vector control cells had slightly elevated amounts of activated PLCγ1 when compared to CD44 expressing cells. This result is the opposite of what one would expect since CD44 expressing cells show elevated levels of calcium influx and activation of PLCγ1 results in calcium influx. Therefore, these data show that Lck is potentially interacting with CD44 at the cell surface, but PLCγ1 is not hyper-activated in CD44 expressing cells. The increased calcium influx in CD44 expressing cells is due to another mechanism.

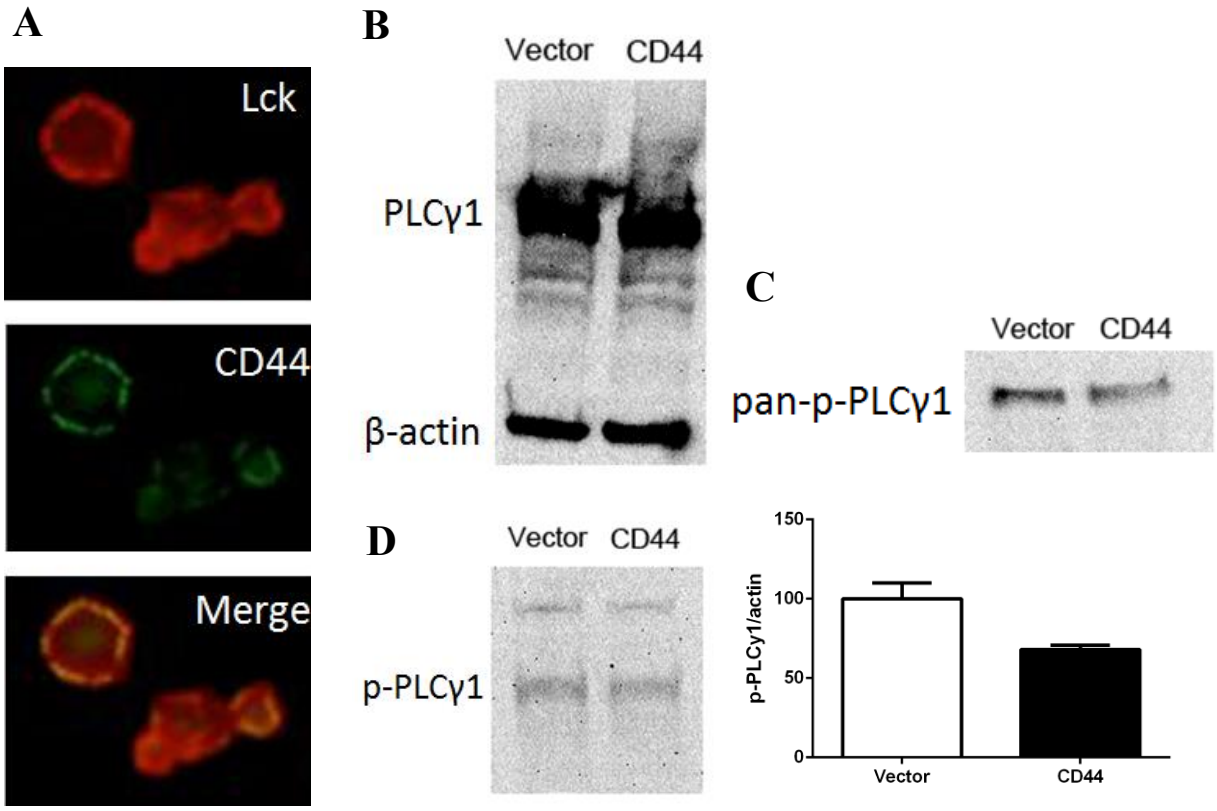

Figure 19. Lck Associates with CD44 at the Cell Surface, but PLCγ1 is Not Activated.

(A) Fluorescence microscopy for Lck (red) and CD44 (green). Merged images indicate localization of CD44 with Lck in yellow. Shown is one representative cell of 100 at 400x magnification. (B) Western blot showing total amounts of PLCγ1. (C) Western blot of immunoprecipitation product after precipitating all phosphor-tyrosine proteins and blotting for PLCγ1. (D) Western blot showing amounts of phospho-PLCγ1 (Tyr783). Two independent experiments were used to create the densitometry data where EGR-1 expression has been normalized to β-actin expression. The Vector cell control lane is set to 100 for comparison. Results show representative data from two or three independent experiments.

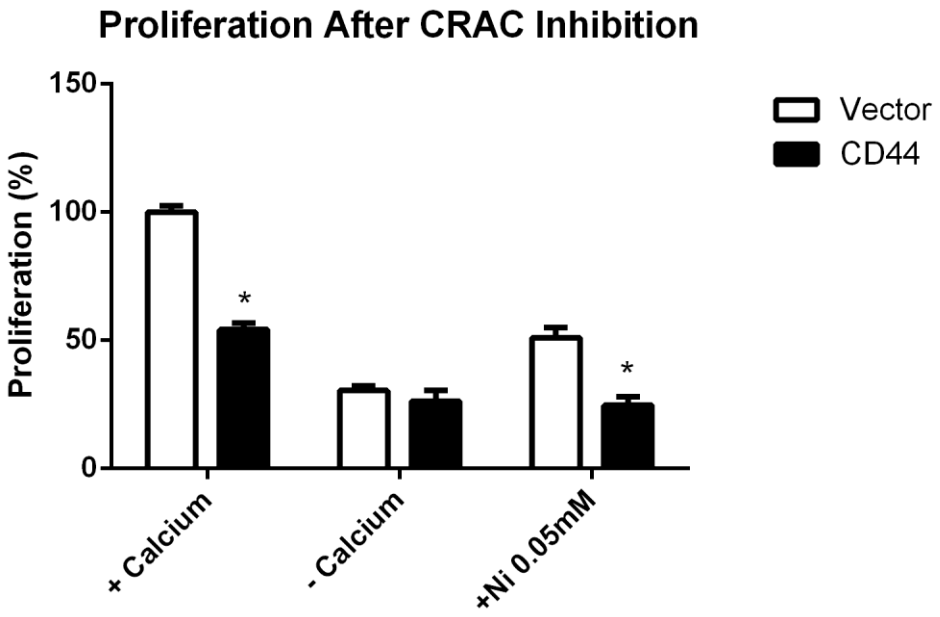

Figure 20. CD44 Expressing Cells have Activated CRAC Channels.

Cell proliferation of CD44 and Vector cells measured with extracellular calcium, without extracellular calcium, and with an inhibitor of CRAC channels. Results are normalized to percent of the vector control cells with calcium. A Student's t test detected differences between the CD44 and Vector cells for +Calcium and +Ni, but not without calcium. Results are representative of three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

CD44 Expressing Cells Have Activated CRAC Channels

To investigate the possibility that CD44 is directly interacting with a calcium channel at the cell surface resulting in increased calcium influx, we investigated the effect of channel inhibitors on proliferation. Nickel is a known inhibitor of inducible calcium release activated calcium channels (CRAC) that are commonly found on T cells[53,76,77]. In the presence of this CRAC inhibitor, CD44 expressing cells proliferate less than vector control cells (Fig. 20). The vector control cells treated with nickel proliferate more than those cells that are cultured without calcium, while the proliferation of the CD44 expressing cells with nickel and without calcium are equal. This data indicates that CD44 expressing cells required CRAC channels for proliferation, while the vector control cells only partially rely on these channels, as indicated by the partial proliferation impairment.

CD44 Expression Does Not Result in Destabilized Mitochondria

Our data show that CD44 expressing Jurkat cells have higher intracellular calcium concentrations than the vector control, indicating a disrupted calcium homeostasis. Such a disruption to the cell can destabilize the mitochondria[78], resulting in leaky mitochondria and a higher susceptibility to apoptosis[79,80]. To measure the integrity of the mitochondria we used the dye JC-1, which aggregates in the mitochondria fluorescing red and disperses through the cytoplasm fluorescing green. Measuring the ratio of red to green JC-1 staining provides a way to compare the stability of the mitochondria of two groups of cells. Measurement of JC-1 staining ratios using a spectrofluorometer and flow cytometry (Fig. 21A&B, respectively) shows that CD44 expressing cells do not have less mitochondrial stability than the vector control cells. This result indicates that although the intracellular calcium concentrations of CD44 expressing and vector control cells differ, this imparts no negative effects on mitochondrial integrity.

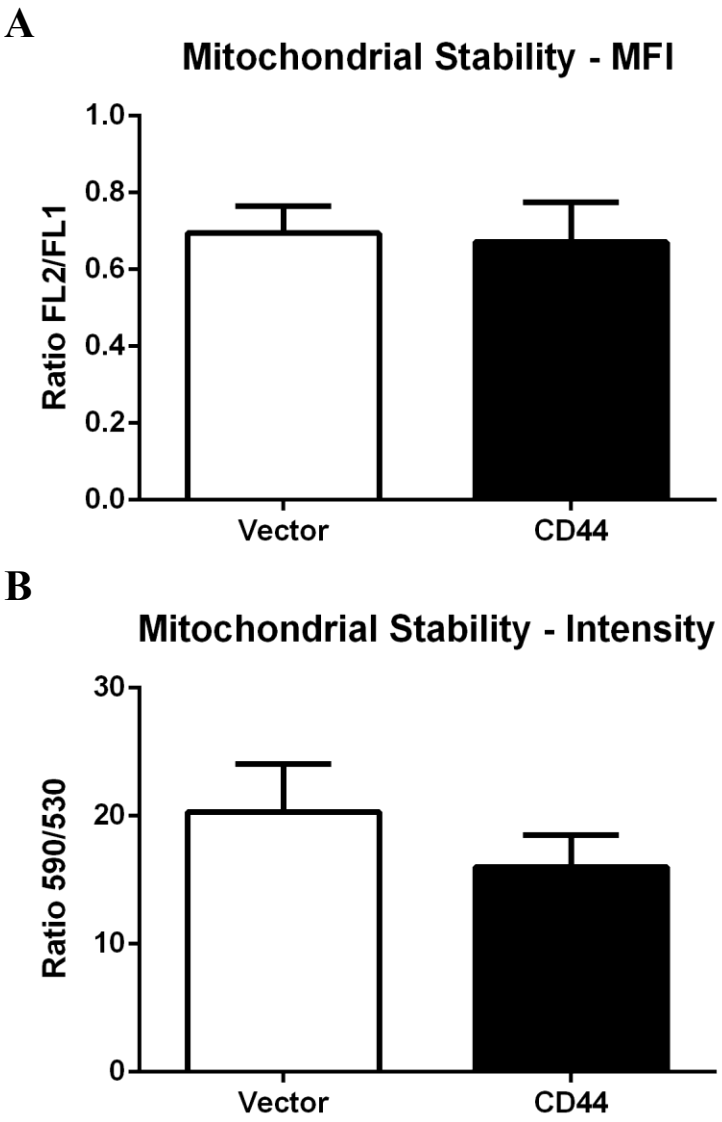

Figure 21. CD44 Expression Does Not Result in Destabilized Mitochondria.

(A) Ratio of MFI of JC-1 stained cells collected by flow cytometry. Fifty thousand cells were collected per experiment. (B) Ratio of fluorescence intensity of JC-1 stained cells collected by spectrofluorometer. A cell suspension of one million cells per mL was measured for a period of 20 seconds, recording intensity each second. A Student's t test did not detect differences between the CD44 and Vector cells in either experiment. Results are representative of three independent experiments. All data are expressed as the mean ± SD.

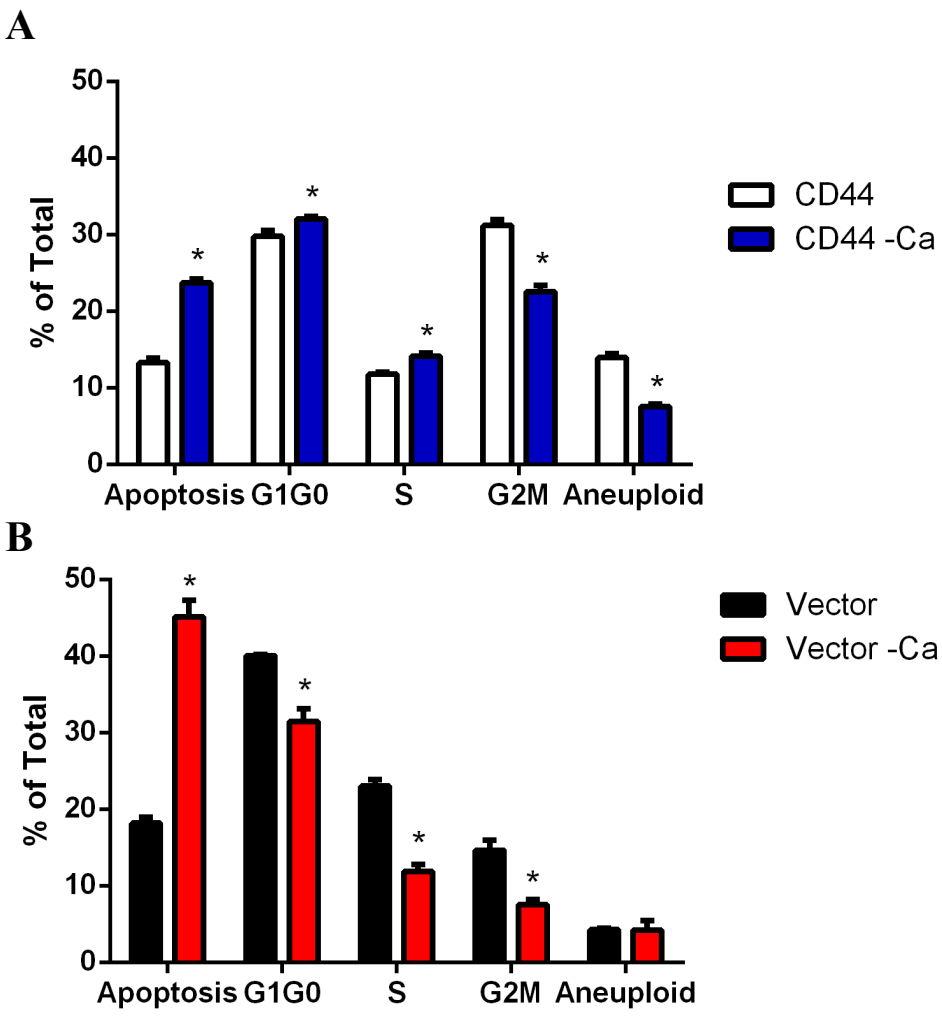

Figure 22. Absence of Calcium Decreases Aneuploidy.

Cell cycle staining and flow cytometry of (A) CD44 and (B) Vector control cells with and without extracellular calcium for 24 hrs. Fifty thousand cells per group were collected. A Student's t test detected differences between the cells with calcium and without as indicated by a *. Results are representative of three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

Absence of Calcium Decreases Aneuploidy

Our data shows that CD44 expression induces aneuploidy and an increased intracellular calcium concentration when compared to the vector control cells. In order to determine if there is a link between aneuploidy and the increased calcium within CD44 expressing cells, we deprived the cells of calcium and observed the occurrence of aneuploidy. Upon removal of calcium from culture media for one day, the CD44 expressing cells show decreases in the G2M and Aneuploid populations when compared to CD44 expressing cells with calcium in the media (Fig. 22A). The decreases in the populations containing the aneuploid cells coincide with a 10% increase in apoptosis and marginal increases in G1G0 and the S phase populations. Vector control cells exposed to the same treatment only show a large increase in apoptotic cells, corresponding to the decreases seen across all other populations (Fig. 22B). Therefore, these data suggest a role for the increased intracellular calcium concentration in the maintenance of the aneuploid cell state.

CD44 Expressing Jurkat Cells are Less Invasive

We have shown that CD44 expression induces decreases in proliferation, Akt phosphorylation, EGR-1 expression, and induces increases in the intracellular calcium concentration and aneuploidy. We next wanted to focus on the potential for these leukemic T cells to invade tissues. A matrigel invasion assay was performed, and CD44 expressing cells and vector control cells were allowed to invade across the matrigel membrane for two days. Out of two million cells 4% of the vector control cells invaded the matrigel insert, while only 1% of CD44 expressing cells were able to invade the gel (Fig. 23). This datum shows that CD44 expressing Jurkat T cells are less invasive *in vitro*, and that future *in vivo* tumorigenesis and metastasis studies should be performed.

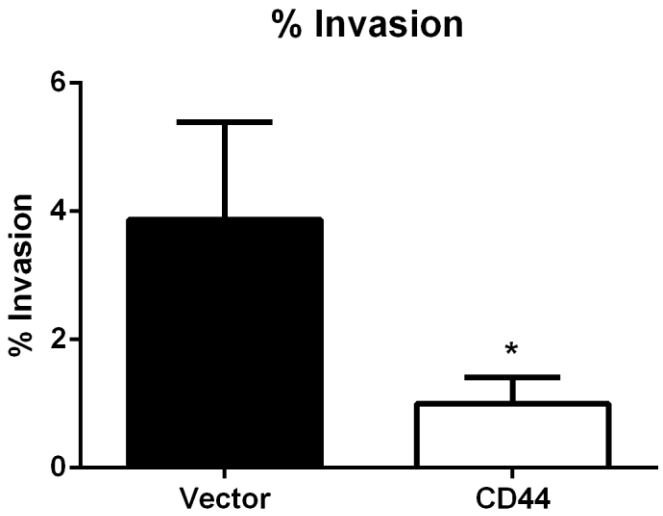

Figure 23. CD44 Expressing Jurkat Cells are Less Invasive.

Two million cells per group were placed into a transwell system and allowed to invade through matrigel for 48 hrs. The top chamber contained RPMI-1640 with 0% FBS. The bottom chamber contained RPMI-1640 with 100U/mL IL-2, 5μg/mL fibronectin, and 10% FBS. Inserts were washed to remove non-invading cells and gels were dissolved in dispase before counting. A Student's t test detected a difference in invasion between CD44 and Vector cells. Results are representative of three independent experiments. All data are expressed as the mean ± SD. (*$p<0.05$)

Summary

The results presented in this chapter provide evidence that CD44 causes a calcium influx that is potentially able to cause aneuploidy (Figs. 13&22). CD44 expressed on the cell surface opens a CRAC channel which causes extracellular calcium to flow into the cell without disrupting mitochondrial stability (Figs. 14,20,&21). Our data demonstrates that both CD44 expressing cells and vector control cells require calcium influx to proliferate, to express EGR-1, and to phosphorylate Akt (Figs. 15-18). For the first time, we outline the role of CD44 in sustaining a calcium influx without the activation of Lck and PLCγ1 (Fig. 19). CD44 expression also induces hypophosphorylation of Akt, potentially via PP1, PP2A, or PP2C (Fig. 18B), paving the way for future work that will fully describe this novel pathway. CD44 expression was also shown to reduce invasion in vitro (Fig. 23), which corresponds with data gathered from patients indicating that CD44 is a potential marker for less aggressive forms of leukemia.

CHAPTER V

DISCUSSION

CD44 has been shown to directly interact with cell surface inositol triphosphate receptor in aortic endothelial cells to induce an increase in intracellular calcium concentration, increasing proliferation and regulating nitric oxide production[56,81]. Interaction between CD44 and phospholipase C was shown in head and neck squamous cell carcinoma, resulting in store-operated calcium entry[57,82,83]. CD44 has been shown by numerous studies to directly interact with and activate the Src family kinase Lck[84-87]. Despite evidence for interaction between Lck and CD44 in Jurkat T cells (Fig. 19), the downstream target of Lck, PLCγ1, was hypophosphorylated in CD44 expressing cells. This finding contradicts the finding that CD44 expression causes calcium influx through a CRAC channel (Fig. 20). CRAC channels are traditionally opened when an initial event triggers the release of calcium from the endoplasmic reticulum, leading STIM1 to translocate to the plasma membrane and open a CRAC channel. Our findings suggest that CD44 alone, or through an unknown adaptor protein interacting with CD44 on the plasma membrane, is capable of inducing store operated calcium entry without the activation of a phospholipase C.

The exact mechanism linking CD44 to increased calcium influx is not known, and the effects of the excess calcium are equally unknown. EGR-1 is known to be regulated by calcium in other cell types[49]. We have observed that EGR-1 is also regulated by calcium in Jurkat T

cells that do and do not express CD44. The CD44 expressing Jurkat T cells possess four times more calcium than the vector control, but express less EGR-1. This may be the results of a signaling pathway similar to what happens in anergic T cells. NFAT is the transcription factor responsible for controlling EGR-1, EGR-2, and EGR-3[88,89]. Upon calcium influx, NFAT is activated and EGR-1 is expressed, but after prolonged or repeated calcium influx and NFAT activation, EGR-2 and EGR-3 are expressed. EGR-2 and EGR-3 inhibit the expression of EGR-1 and decrease cell proliferation. This may explain the link between EGR-1 expression, calcium, and proliferation observed in the Jurkat T cells with and without CD44 expression. Both CD44 expressing and vector control cells continuously require some amount of calcium to proliferate (Fig. 15) and express EGR-1 (Fig. 16), and the amount of EGR-1 expressed is directly proportional to the proliferation of the cells. If CD44 expressing and vector control Jurkat T cells are deprived of calcium and then the calcium is restored, it is likely that at some point between 1 and 4 days with restored calcium a threshold is crossed where CD44 expressing cells begin to have a higher intracellular calcium concentration than the vector control cells. Like T cells that are becoming anergic, these CD44 expressing Jurkat T cells could become insensitive to continued calcium signaling and switch from EGR-1 expression to EGR-2/EGR-3 expression, which would decrease their proliferation and EGR-1 expression. We have previously shown that EGR-1 expression is also induced by activated Akt in Jurkat T cells, and that the hypophosphorylation of Akt induced by CD44 expression causes the decrease in EGR-1 expression[18]. The regulation of EGR-1 in Jurkat T cells by Akt and calcium is equivalent, as shown in Fig. 17. Calcium influx is required for EGR-1 expression and Akt activation (Figs. 16-18), but CD44 may be regulating EGR-1 expression via Akt activation through an unknown mechanism.

The Akt pathway has been linked to calcium influx previously in other cell lines[90-95]. In non-small cell lung cancer, inhibition of the CRAC channel Orai3 causes a decrease in Akt phosphorylation and decreases in proliferation[90], in Th17 cells it has been shown that calcium activated kinases can activate the Akt signaling pathway[91], and upon exposure to herpes simplex virus Akt is activated in target cells which induces the calcium influx required to promote viral entry[92]. The inverse of these findings is found when studying renal cell carcinoma proliferation and leptin secretion from adipocytes[93,94]. Renal cell carcinoma cells were found to require extracellular calcium influx in order to activate Akt, inducing proliferation and migration[93]. Adipocytes relied on extracellular calcium influx to sustain robust Akt phosphorylation and leptin secretion[94]. While these findings illustrate that a link between Akt activation and calcium influx is present, they do not describe what is likely occurring in our Jurkat T cells. In differentiating keratinocytes it has been shown that extracellular calcium has an inhibitory effect on Akt phosphorylation and proliferation[95]. This may be similar to the pathway activated in our Jurkat T cells.

The phosphorylation of Akt at Ser473 is calcium dependent in Jurkat T cells (Fig. 18A), but the inherent hypophosphorylation of Akt in CD44 expressing cells still remains. As shown in Fig. 18B, calcineurin, a calcium dependent Akt phosphatase, is not activated due to CD44 expression. This finding suggests that Akt is being dephosphorylated by a calcium independent phosphatase that is activated in CD44 expressing cells. One or a combination of the calcium independent phosphatases PP1, PP2A, and PP2C are more active due to CD44 expression, and they are known to dephosphorylate Akt[45]. It is possible that one of these phosphatases, or an unknown phosphatase, is activated by CD44 in a calcium independent manner. CD44 has been shown to be immunoprecipitated with PP2A in EL4 T cell lymphoma cells[96], and cross-

linkage of CD44 has been shown to activate CD44-associated PP2A[96]. Activated PP2A is known to dephosphorylate Akt at Ser473, and this pathway could be responsible for the observed calcium independent dephosphorylation of Akt due to CD44 expression in Jurkat T cells. PP1 and PP2C activation has not been linked to CD44, but they remain strong potential targets for future experiments. Activated PP2A is also known to dephosphorylate Mad2[97], resulting in a decreased genomic stability and a failure of cell cycle checkpoints regulating aneuploidy. Future work needs to be performed assessing PP2A activity, localization with CD44 at the cell surface, and the potential interactions between PP2A and cell cycle machinery.

The induction of aneuploidy in CD44 expressing Jurkat T cells over time may also be due to the increased intracellular calcium concentration. CD44 expressing cells have decreased expression of MAD2A, RAD51, and BRCA mRNA compared to the vector control (Table 2). While these genes are important for proper chromosome separation, there is a crucial role for calcium in the maintenance of proper chromosome number. Calpains are a family of calcium dependent intracellular cysteine proteases that are ubiquitously expressed[35,98]. Increases in calcium concentration activate calpains, causing them to cleave the cohesin, Rad21, which keeps the sister chromatids bound together during metaphase[35,98]. The prolonged calcium elevation in CD44 expressing Jurkat T cells could be hyper-activating the calpains, resulting in early dissociation of chromatids that over time builds up into severe aneuploidy. This hypothesis is backed up by the finding that removing calcium from the media of CD44 expressing cells causes a reduction in the aneuploid cells (Fig. 22). The removal of calcium may allow the cells to restore the mitotic checkpoints responsible for stopping proliferation if aneuploidy occurs, which could also explain why the vector control cells simply become apoptotic upon removal of calcium. As mentioned previously, the removal of calcium from the media does not cause an

increase in cell death, despite the increases to the apoptotic population shown in Figure 20. Future work should focus on the expression levels and activity of these calpains. Initial work needs to be performed using Western blotting to determine the relative expression of DNA damage checkpoint proteins like MAD2A and RAD51. Knockdowns of potential target mechanisms should be performed in the control cells to check for induction of aneuploidy. Work should then focus on the mechanism behind CD44 expression induced modification of these targets, such as the recruitment of phosphatases to deactivate checkpoint kinases or the reduction of protein levels of checkpoint proteins.

The Notch signaling pathway was predicted to be inhibited in CD44 expressing cells based on the analysis of the RT^2-PCR arrays (Figures 8&9). Notch signaling requires direct contact between cells, and both the CD44 expressing and vector control cells adhere in clumps in culture, illustrating the potential for Notch pathway involvement in the observed phenotypes. Activation of the Notch pathway is essential for commitment of lymphoid progenitor cells into the T cell lineage[99], and the E6.1 Jurkat cells used in this study are cancerous recent T cell lineage committed cells that have not yet matured. Indeed, faulty activated notch signaling is seen in most cancers; contributing to more than half the cases of TALL[100]. Interestingly, inhibition of this pathway in mouse tal1 cells, a TALL cell line, reduces proliferation[101,102]. The same result was also reported in various human TALL patient samples in vitro as well as an inducible mouse TALL model in vivo[103]. While direct inhibition of Notch signaling using pharmacological reagents may reduce proliferation of TALL cells, we do not know how CD44 could interact with Notch pathway proteins to produce a similar inhibition. Notch signaling requires direct interaction between the isoform of NOTCH and a ligand, and CD44 may be functioning as a recruitment platform, either sequestering ligands or NOTCH proteins in a way

that prevents interaction with other cells. Akt is activated by Notch pathway activation, and Notch also regulates cyclin expression in order to induce proliferation[104-106]. Inhibition of Akt has been shown to cause impairment of Notch signaling in Jurkat cells in vivo[107], suggesting that there is also a potential mechanism by which Akt regulates Notch. The Notch protein contains a signaling domain that must be cleaved by γ-secretase so that it can translocate to the nucleus where it functions[108]. This secretase also cleaves the CD44 intracellular domain, allowing it to also translocate to the nucleus[109,110]. It is possible that forced CD44 expression in E6.1 Jurkat cells is somehow forcing interactions between it and γ-secretase that deprive Notch proteins of their necessary cleavage. Future work on the Notch pathway should include an analysis of the protein levels of all four isoforms of Notch as well as an activity assay for γ-secretase. Treatment of the control Jurkat T cells with Notch and Akt inhibitors while looking at both signaling pathways will aid in determining the upstream event responsible for the decrease in proliferation.

Our data show that CD44 expressing Jurkat T cells have a higher intracellular calcium concentration when compared to the control cells (Fig. 13), but this does not tell the full story. The method used to determine the calcium concentration does not discriminate between calcium in the cytoplasm and calcium stored in organelles as fura-2-AM penetrates the membrane of the endoplasmic reticulum and the mitochondria[111]. It is possible that the excess calcium that is taken up by CD44 cells is stored instead of being available for use. This could explain why an increase in calcium concentrations is observed at the same time as a decrease in calcium regulated events such as EGR-1 expression and Akt activation (Figs. 16-18). One piece of data that adds to this conclusion is that PLCγ1 is less activated in CD44 expressing Jurkat cells than the control (Fig. 19). Activated PLCγ1 is an integral part of store operated calcium entry

(SOCE). PLC generates IP_3 which activates the IP_3 receptor, releasing calcium from endoplasmic reticular stores. This makes sense when compared to the actual data. Normally, PLCγ1 activation would occur and SOCE would induce STIM1 to open CRAC channels, but we found that CD44 expression opens a CRAC channel without requiring the activation of PLCγ1. In this way it is possible for CD44 to cause a calcium influx through direct interaction with a channel or some other influx mechanism while the inactivation of PLCγ1 causes calcium stores to quickly remove this excess calcium from the cytoplasm. It has been reported that Akt can activate PLCγ1 through direct interaction, resulting in the production of IP_3 and classical activation of SOCE[112,113]. In our CD44 expressing Jurkat cells Akt is hypophosphorylated, potentially causing a decrease in the activation of PLCγ1 resulting in a lack of the ability to properly release calcium from stores. This would cause a decrease in the cytoplasmic calcium concentration compared to normal cells, but when coupled with an increase in extracellular influx will show up as an increased calcium concentration when using fura-2-AM.

Based on our data we propose the following models of CD44 induced signaling pathways in Jurkat T cells. As shown in Fig. 24, CD44 expressed on the cell surface is interacting with either a CRAC channel directly or an intermediary protein (Fig. 20). This interaction causes a sustained calcium influx that raises intracellular calcium levels (Figs. 13&14). Calcium influx is normally required for Akt activation, proliferation and EGR-1 expression (Figs. 15-18), but the excess calcium is also causing the prolonged activation of calpains. CD44 is also interacting with PP2A resulting in its activation (Fig 18B). Activated PP2A is dephosphorylating Akt, which in turn decreases the expression of EGR-1. The hypophosphorylation of Akt and the decrease in EGR-1 expression both result in a decrease in proliferation. Activated PP2A also deactivates

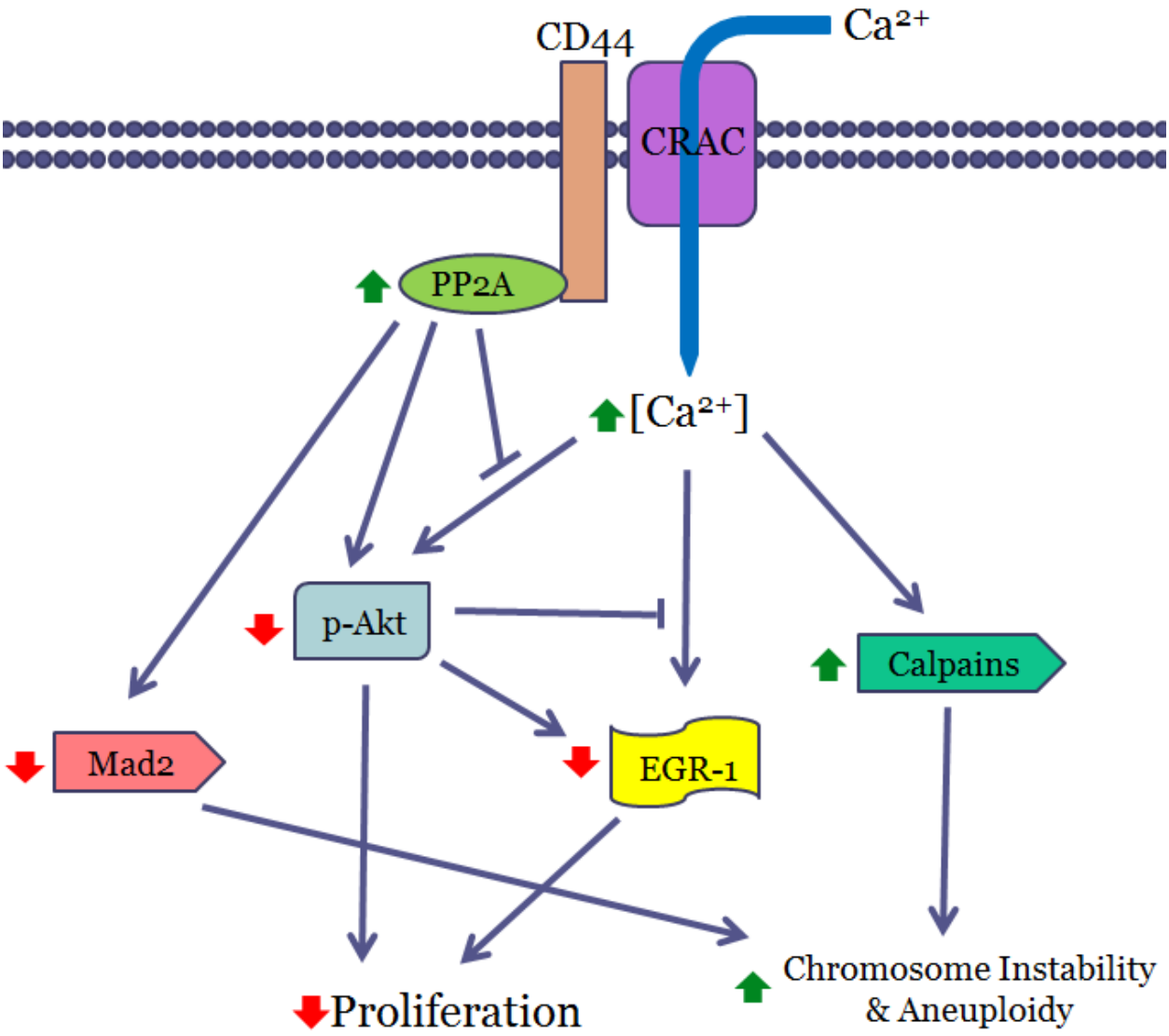

Figure 22. Proposed CD44 Signaling Pathway.

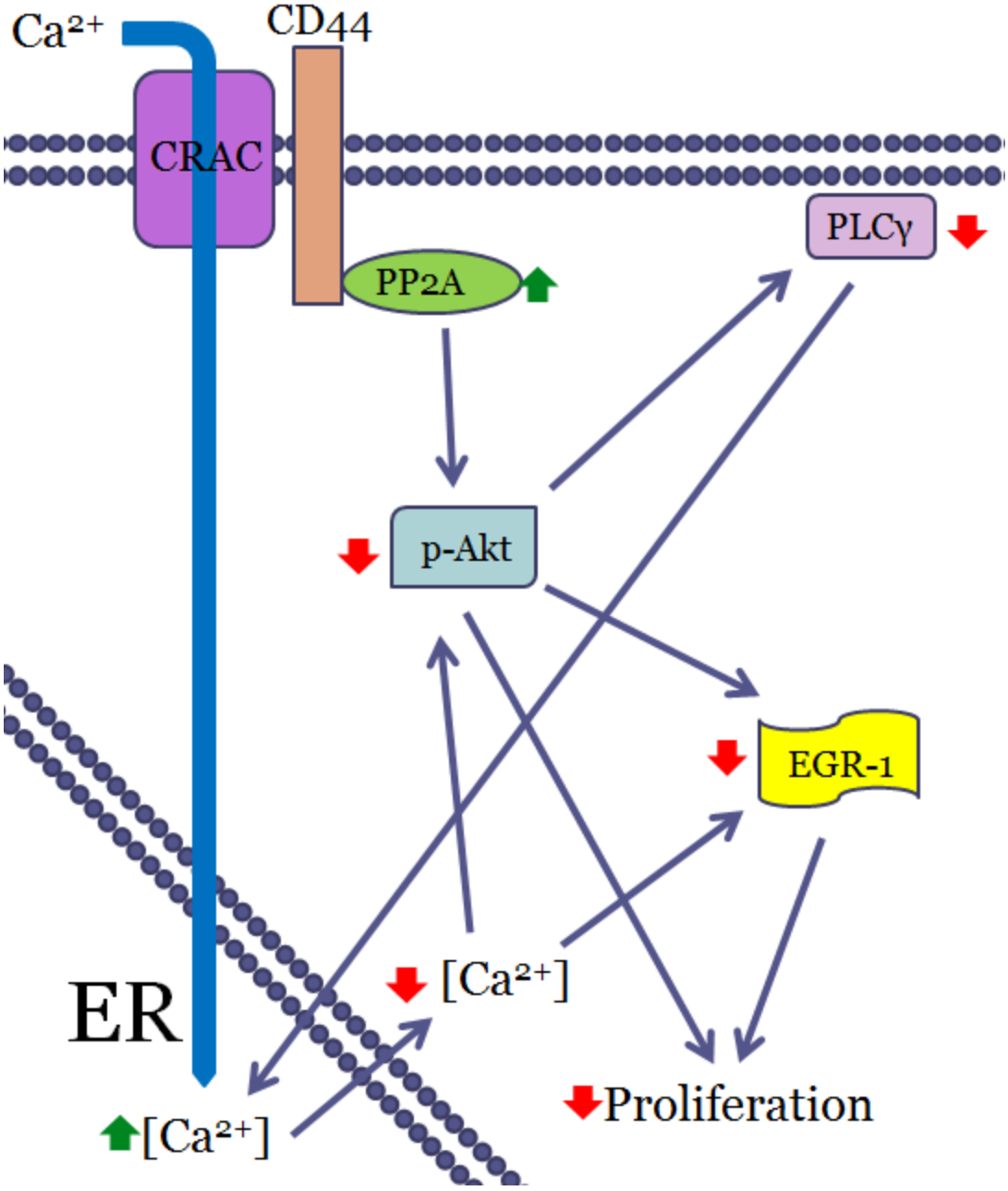

Figure 23. Proposed Alternative CD44 Signaling Pathway

Mad2, causing genomic instability by disabling the checkpoints responsible for ensuring aneuploidy does not occur (Table 2). The calpains, which are constantly activated by the calcium influx, cause chromosomes to prematurely separate during mitosis, resulting in daughter cells that have extra chromosomes.

An alternative CD44 signaling pathway is proposed in Fig. 25. CD44 interacting with the CRAC channel induces a calcium influx, but that calcium is quickly stored in the endoplasmic reticulum. PLCγ normally activates SOCE to release this stored calcium, but PLCγ is hypophosphorylated(Fig. 19). This hypophosphorylation of PLCγ is caused by an initial hypophosphorylation of Akt that is induced by an activated phosphatase such as PP2A. The inactivation of Akt leads to the lack of a signal to activate EGR-1 expression. The lack of calcium due to its sequestration also helps maintain the hypophosphorylation of Akt and fails to provide signaling to induce EGR-1 expression. The absence of free calcium in the cytoplasm, the decreased EGR-1 expression, and the hypophosphorylation of Akt result in a decrease in proliferation.

The results in this study provide a strong basis for future clinically relevant work. Our finding that CD44 expression induces a decrease in proliferation, which corresponds to what has been reported clinically in TALL patients, is promising. The pathways described in the three proposed models offer many points for intervention with current therapeutics in order to exploit the weaknesses induced by CD44 expression. Future work on the Notch pathway will strengthen the potential for combination therapy to target the Notch pathway in addition to the Akt pathway. Patients whose leukemic cells express CD44 could be treated with a spectrum of drugs that act on the notch pathway alone, as the Akt pathway is already inhibited. Other labs have shown that, when treating TALL, if Notch is inhibited without inhibiting Akt, the risk of encountering a

resistance to the Notch inhibition increases[100,114]. The resistance to treatment is presumably due to a compensation mechanism where Notch's normal function is to induce PTEN activation and inhibit the Akt pathway and proliferation[114]. By treating the cancer that contains dysregulated active Notch with an inhibitor you remove the controls on the Akt pathway and the cells survive treatment through Akt induced survival pathways. The fact that CD44 expressing Jurkat T cells have less activated PTEN (Fig. 11) coincides with a potentially inactivated Notch pathway (Figs 8&9). We could then assume that TALL patients whose cells express CD44, which causes a hypophosphorylation of Akt, may be more responsive to Notch inhibitor therapy. Future work on the Notch pathway will help understand if the Notch pathway is still partially active in these cells, providing the basis for use of Notch inhibitors as a potential therapy.

The invasion assay performed in this study was performed with the intention of continuing into an in vivo model. Experiments should be performed first in mice using a xenograft ALL model in nude mice. The CD44 expressing Jurkat T cells would be predicted to be less invasive as measured by spinal cord infiltration when compared to the vector control cells. The mechanism behind the observed decrease in invasion (Fig. 23) is unknown, but it is possible that CD44 expression could make the Jurkat T cells more prone to adhesion to the extracellular matrix, preventing invasion and migration. The results of the in vivo model will help describe the role of CD44 in a translational setting. More work should be done to study the impact of CD44 expression on matrix metalloproteinases, collagenases, and other enzymes needed to pass through endothelial barriers during metastasis. Activated T cells are CD44 positive, yet can still extravasate from the blood into tissues. This contradicts the finding that CD44 expressing Jurkat cells cannot invade matrigel as easily as the control cells. CD44 could potentially be altering expression or localization of proteins and enzymes needed to pass through

extracellular matrix, as the addition of a hyaluronan adhering protein does not stop normal T cells from extravasation due to adherence to hyaluronan when they express CD44.

Future directions should focus on several distinct areas of the CD44 signaling pathway in Jurkat T cells. The CD44-PP2A interaction is a novel signaling pathway that has only been reported in one other instance[96], but never before in Jurkat T cells. The potential for CD44 to influence genomic stability by maintaining a constitutively active phosphatase is a novel concept. This work could explain why CD44 expression in leukemia patients is correlated with a better response to chemotherapy[60]; potentially by inactivating Notch and Akt signaling pathways. We could also target PP2A with other drugs in patients whose tumor cells are CD44 positive to greater enhance the efficacy of conventional treatments. Overall, these data contribute to our knowledge regarding the function of CD44, which is necessary in order to understand and exploit its presence in many types of cancer.

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