Cadmium Induced Transcription and NFAT/cJun Proximal Promoter Binding in Breast Cancer Cells

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An Honors Thesis Submitted to the
University of Colorado
Department of Molecular, Cellular, and Developmental Biology
March, 2015

Defended April 2, 2015

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Abstract

In order to better comprehend onset and progression of diseases such as cancer, the means by which genes are regulated must be understood. RNA Polymerase II (Pol II) transcribes mRNAs that are translated into proteins, but this process is tightly controlled by transcription factors that recruit Pol II to promoter elements. These transcription factors determine if and when a gene is expressed at a basal level. This thesis focuses on the NFAT and AP-1 family of transcription activators. Each of these families has been characterized as important transcriptional activators in the onset and growth of cancer, but their mechanism of action is not well understood.

Genes up-regulated in mammary duct carcinoma in response to the environmental carcinogen cadmium chloride were identified. These genes where then used to determine the optimal cadmium concentration and time of treatment for maximal transcriptional induction. ChIP assays against NFATc2 and cJun were performed before and after treatment with cadmium. ChIP and qRT-PCR data collected suggests that genes MMP-1 and RAB11 are upregulated via cadmium chloride exposure and transcription factors NFATc2 and AP-1 are bound to promoter elements of each of these genes.

Acknowledgments

First, a big thank you to Jim Goodrich and Jen Kugel for allowing me to work in their lab for the past year. Their trust along with guidance created a learning experience unmatched by any other in my scientific academic career. Being allowed to work autonomously on this project allowed me to experience all the successes and failures that are part of the scientific research field. Having two positive and optimistic PIs to guide me through this process was a true blessing.

Next, I would like to thank David McSwiggen for taking me under his wing and teaching me many of the techniques I needed to know in order to work on this project. Without taking time out of his busy schedule to help me, I would not have been able to complete this manuscript. Also a big thank you to the rest of the Goodrich Lab: Rob Abrisch, Abby Horn, Joe Cardiello, Vijay Sekaran, Elina Ly and Tess Eidem who answered my questions and helped me along with troubleshooting and teaching me new techniques.

Thanks to Jim Goodrich, Marybeth Childers, and Al Childers for reviewing and helping me edit this manuscript.

Special thanks to my family who was very encouraging and helpful during this whole process.

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Introduction:

An Overview of Transcription

Transcription is the process by which cells use template DNA to make RNA, which codes for specific proteins. DNA is conserved throughout all cells and contains coding information needed to produce a vast library of over 20,000 proteins[1,2]. Every cell type does not need each of these proteins and tight regulation must occur from cell to cell in order to produce only necessary proteins. For example, cytotoxic T cells need to produce cytotoxins in order to kill infected cells, while neural cells must produce neurotransmitters important in cell-to-cell communication. Thus, each of these cell types must have mechanisms in-between DNA and RNA production to control expression of one gene over another. Understanding mechanisms vital to RNA transcriptional regulation of genomic information is fundamental for further improving treatments to diseases.

At the core of transcription is the enzyme RNA Polymerase II (Pol II) and associated general transcription factors. Transcription occurs when Pol II adds complementary nucleotide triphosphates (NTPs) together via phosphodiester bonding catalyzed in the active site of Poll II. This reaction is facilitated by the high-energy bonds between the three phosphate groups attached to each nitrogenous base in NTPs. As NTPs are incorporated into a strand, a nascent RNA product is formed[3]. Nascent RNA products are produced with high fidelity due to hydrogen bonding with template DNA and proofreading activity associated with Pol II[4,5].

When studying Pol II it is important to understand its tertiary structure and the effect this structure has on Pol II's enzymatic activity. Poll II is comprised of 12 protein

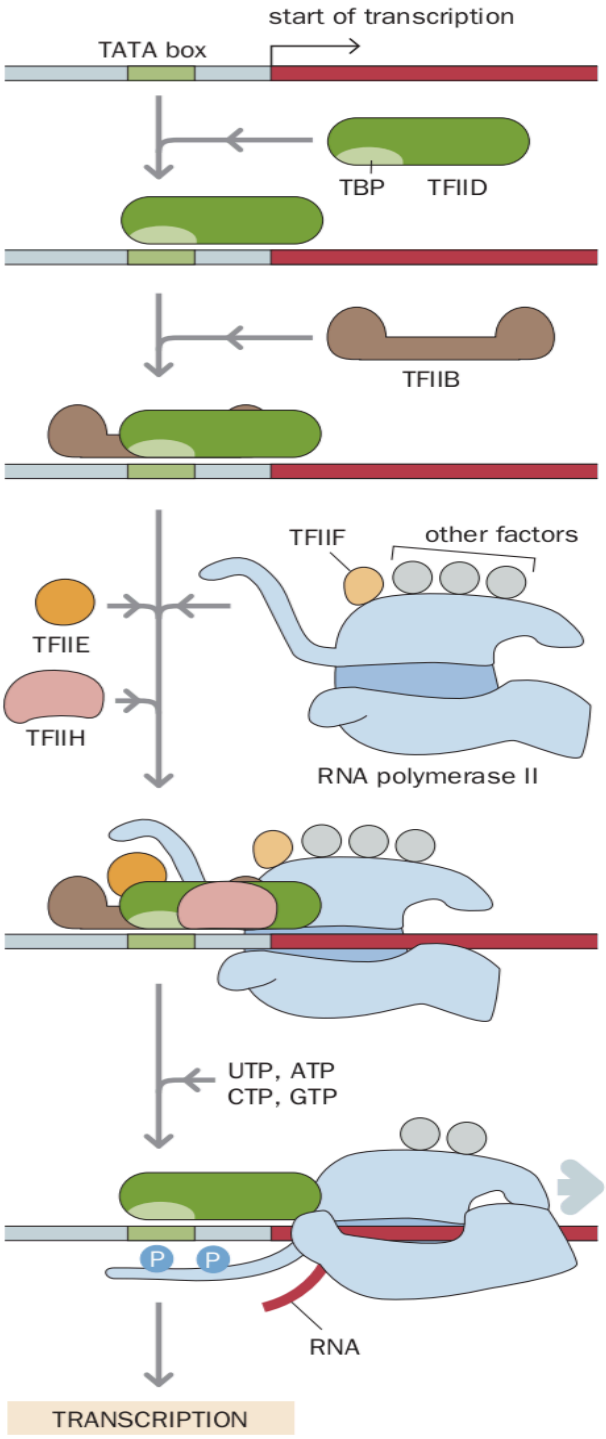

Figure 1: Schematic of PIC formation. PIC begins with TBP domain of TFIID binding to the -30 nucleotide TATA box upstream of the first exon (red rectangle) and transcription start site. TFIID recruits TFIIB, TFIIE, and TFIIH. TFIIH helicase activity opens up dsDNA for transcription to begin[18].

subunits (RPB1 - RPB12). Together these subunits make four domains within the Pol II enzyme: the active site that binds DNA for template mediated synthesis, a clamp to keep DNA in the active site, a funnel for NTP incorporation, and a "rudder" which removes the complementary RNA strand from template DNA[1,6]. Each of these domains is used in the three steps of transcription, which are initiation, elongation, and termination.

Initiation is the first part of RNA transcription and involves targeting Pol II to transcription start sites throughout the genome. Pol II can synthesis RNA, but alone is incapable of targeting protein-coding genes within DNA and needs help being recruited to promoter elements via general transcription factors. Pol II associates with general transcription factors in order to utilize their ability to bind to specific promoter elements upstream of protein coding genes. As well, one of the general transcription factors provides helicase activity that opens up double stranded DNA so that Pol II can begin transcription[2,7].

When Pol II associates with general transcription factors on promoter DNA, a preinitiation complex (PIC) forms (Figure 1). PIC formation starts with the TATA binding protein (TBP) subunit of TFIID binding the TATA box which is 30 basepairs upstream of the transcription start site. Although TBP is a common factor for recruitment of general transcription factors, other transcription factors can facilitate an analogous function. Upon TBP binding to DNA, two "kinks" are created in the DNA which assist in Poll II association[8]. TFIIB then targets DNA into the active site of Pol II with the aid of TFIIF. Finally, TFIIE and TFIIH are recruited the TFIIH helicase activity unwinds the double stranded DNA in order for Pol II to access the template strand[9]. Often, once Pol II has access to the template strand, it is held in a paused stage where small transcripts are produced. Once the PIC is able to escape pausing it enters the elongation stage in which complementary RNA is produced. Upon elongation most PIC proteins dissociate and initiation is ready to start elsewhere on the genome[10,11].

Regulatory Elements in Eukaryotes

Understanding the organization of promoters is essential to understanding regulation of RNA transcription. Promoters are elements within genomic DNA that are upstream of transcription start sites where transcription factors can bind. Transcription factor is a generic term for a set of proteins that bind DNA and regulate transcription[14]. Regulation can be achieved through recruitment of general transcription factors, interaction with PIC and looped bridging of mediator molecules to stabilize transcription complexes[15,16]. Transcription factors can be thought of as acting alone, but their ability to act cooperatively gives rise to a larger diversity of transcriptional regulation. A set of three factors could form many different homo and hetero dimers in order to regulate transcription of different genes or levels of a single gene[17].

There are three general categories of promoter elements; core promoter elements, proximal regulatory elements, and distal enhancer elements[12]. The core promoter elements consist of a TATA-box for TBP association and PIC recruitment via TFIID (Figure 1). Also core promoter elements consist of BRE elements that TFIIB binds to and aligns Pol II with the transcription start site[9]. Positioned within a few hundred bases of the core promoters are the proximal regulatory elements. Proximal regulatory elements allow for transcription factor binding to act in a repressive or activating manner (Figure 2). Distal enhancer elements are positioned up to thousands of bases away from transcription start site and also bind transcription factors in a repressive or activating

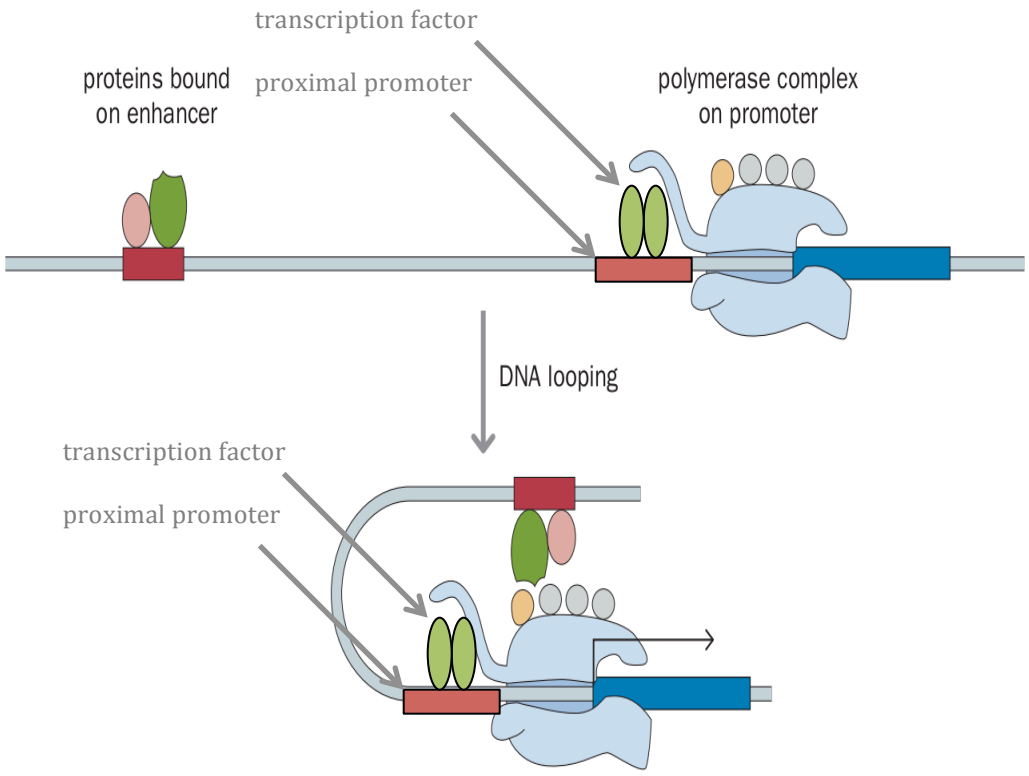

Figure 2: Schematic of regulatory elements. Transcription factors can bind to DNA at proximal and distal sites. Proximal promoters are within a couple hundred bases and bind transcription factors that interact with PIC formation. Distal enhancers are located thousands of bases away and bind transcription factors. These factors interact by looping DNA and interacting with mediators (grey circles)[18].

manner. The position of distal enhancers has been documented as irrelevant because relocation of these distal enhancers has shown not to affect transcription. Distal enhancers function by binding transcription factors and looping DNA to allow stabilization of transcription machinery (Figure 2).

AP-1 Family Transcription Factors

Notable among the transcription factors are members of the Activator Protein 1 (AP-1) family. The AP-1 family of transcription factors are composed of dimers of two alpha helical proteins. More specifically AP-1 dimers can be characterized as basic leucine zippers, each of which contains a stretch of five hydrophobic leucines on one side of an alpha helix (Figure 3). Past the five leucine residues, the alpha helixes diverge and the basic regions bind to DNA[18]. Upon binding to DNA the affinity of dimerization increases between each pair of AP-1 family members which helps facilitate DNA binding. AP-1 has been characterized to bind to TRE elements within proximal promoters of DNA and facilitate transcription specific to genes containing TRE elements[19].

The AP-1 family is made of four smaller families of proteins: Jun, Fos, ATF, and MAF. Among these smaller families, members of the Jun family are able to form homodimers as well as heterodimers with the other family members. In contrast, Fos members can only form heterodimers with members of other families and lack the ability to form homodimers[20]. The AP-1 family of transcription factors become phosphorylated in response to external and internal stimuli and play a vital role in regulating the transcription of genes that encode proteins vital to differentiation, proliferation, and apoptosis[21]. The mechanism of AP-1 phosphorylation is through the mitogen-activated protein kinase (MAPK) which activates the Jun N-terminal kinase (JNK) and causes

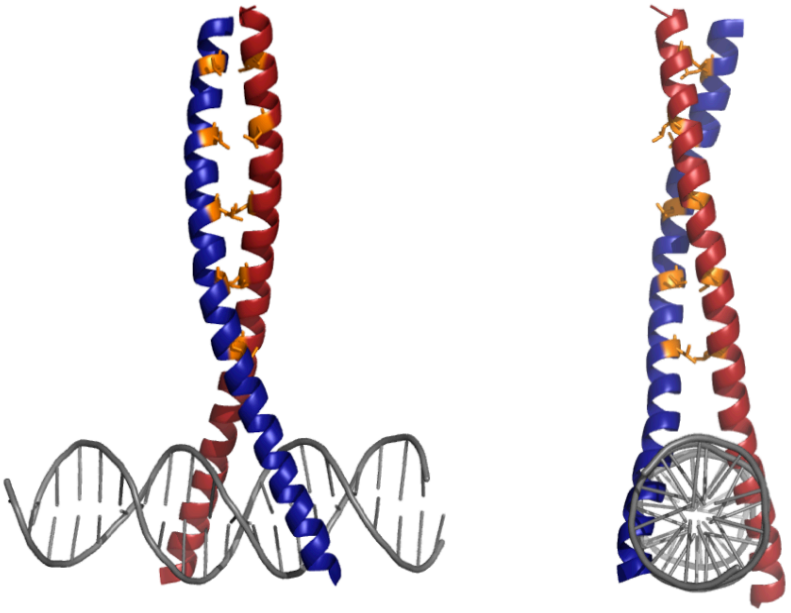

Figure 3: AP-1 Structure. AP-1 is a basic leucine zipper DNA binding protein. DNA is shown in grey. A heterodimer above is shown binding to DNA and contains cFos (red) and cJun (blue). Electrostatic interactions between the hydrophobic leucine residues are depicted in orange. Glover, J. N., and Harrison, S. C. Crystal structure of the heterodimeric bZIP transcription factor cFos-cJun bound to DNA, Nature 373, 257-261 (1995).

phosphorylation of AP-1 family proteins. Once phosphorylated, AP-1 protein characteristics are altered and they become more active transcription factors[22,23].

NFAT Family Transcription Factors

The nuclear factor of activated T cells (NFAT) is another family proteins that act as transcriptional activators. Typically NFAT family proteins are phosphorylated and localized to the cytosol in an inactive form that cannot bind DNA and activate transcription. Environmental conditions can cause an increase in cytosolic calcium concentration, which activates a calcium binding protein (calmodulin) and a calcium dependent phosphatase (calcineurin) (Figure 4A). Activation of both of these proteins leads to cleavage of phosphate groups from NFAT family members revealing their nuclear localization sequence, which induces relocalization to the nucleus[24]. This process is reversed by tyrosine phosphorylation regulated kinase 2, which re-phosphorylates NFAT family members targeting them for nuclear export[25]. Figure 4 shows DNA binding domains as well as phosphorylation domains within two of the most characterized NFAT family members: NFATc1 and NFATc2. This Thesis focuses specifically on NFATc2.

NFAT and AP-1 family cooperatively

Mechanisms of transcriptional regulation cannot be characterized in a single way. The same concept applies to transcriptions factors. By itself, no one-factor has the ability to regulate the vast information found within the genome. NFAT and cJun are two transcription factors that have been characterized to regulate genes in their own independent manner. NFAT and cJun, however, have been characterized to work in a cooperative manner, for example in the proximal promoter region of the IL-2 gene. The binding of NFAT and cJun to composite sites proximal to the IL-2 transcription start site

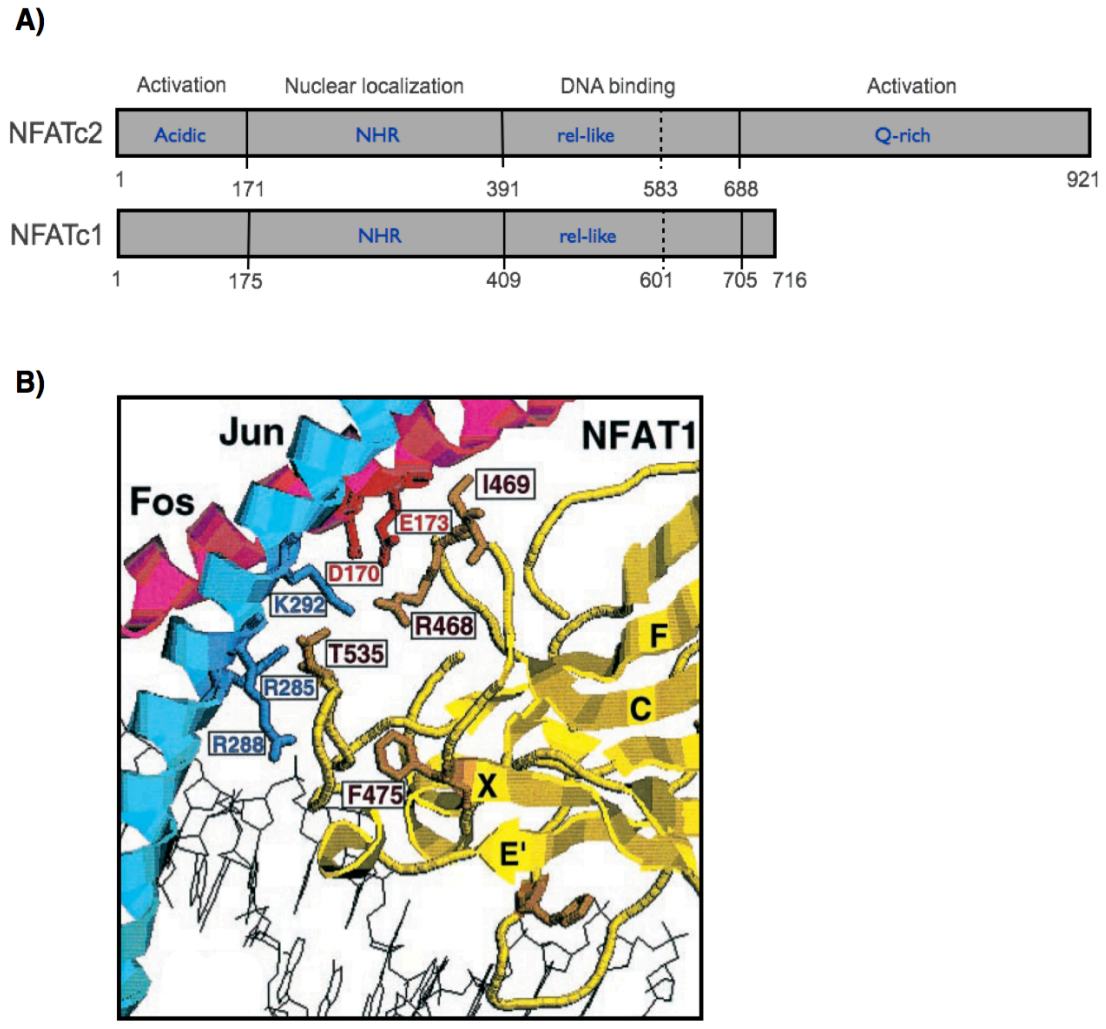

Figure 4: NFAT structure. **A)** NFATc1 and NFATc2 both contain a NHR domain for binding calcineurin as well as a rel-like DNA binding domain. **B)** Shows the interaction between NFAT and AP-1 in their tertiary structures. Macian, F., Lopez-Rodriguez, C. & Rao, a. Partners in transcription: NFAT and AP-1. Oncogene 20, 2476–89 (2001).

constitute a powerful method of transcriptional regulation[50] (Figure 4B). Our lab is interested in exploring other potential areas of biology that may use cooperative NFAT and cJun activation. Specifically we are interested in this cooperative effect in mammary duct carcinoma upon exposure to the carcinogen cadmium chloride. This thesis focuses on the effect of cadmium chloride on transcription as well as NFAT and cJun binding proximal to upregulated genes in mammary duct carcinoma cells.

The role of environmental carcinogens in controlling NFAT and cJun activity

A recent study done by the American Cancer Society revealed that 23% of all deaths in America in 2014 were attributed some form of cancer[26]. This ranks cancer as the second leading cause of death among the American population. Understanding the fundamentals of this disease will help with the development of new treatments to lower this statistic and benefit those affected by cancer. Cancer is not easily treated. There is no foreign pathogen to be killed as seen in bacterial or viral infections. Rather, cells that the human immune system recognizes as self begin to demonstrate hallmark characteristics of cancer. Two of these hallmarks are uncharacteristic differentiation and loss of replication regulation. Cancerous cells lose the ability to communicate with other cells in proximity and differentiate independent of tissue type needs. Also, cancerous cells lose their ability to self regulate cellular replication leading to characteristic tumors[27]. Although predispositions such as BRCA-1 mutations can cause increased risk of cancer it is ultimately environmental factors known as carcinogens that lead to cancer onset[28,29]. In order to provide better preventative health information to the population at large, environmental carcinogens need to be studied in depth to understand their mechanisms of action.

Heavy metals are environmental carcinogens that are present near mining sites and in water runoffs potentially contaminating drinking water[30]. Not only is there potential for water contamination, but also heavy metals have been seen to collect in tissues of many animals that

we eat, namely fish[31]. Heavy metals are known carcinogens and have been shown to induce carcinomas in animal models and one hypothesis is that these small molecules mimic that of calcium and interrupt proper signaling within cells[32,33]. Of these heavy metals, cadmium has been shown in increase the levels of cytosolic calcium[35,36]. Based on the mechanism by which NFAT is regulated it could be assumed that these heavy metals would activate it. As well cadmium has been shown to activate the MAPK pathway in prostate cancer and increased activated cJun was observed[32,37].

Previously, this lab has shown that cytosolic calcium levels within MDA-MB-231 cells oscillate when stimulated with cadmium chloride. As well, artificial increases in cytosolic calcium using PMA and ionomycin lead to NFAT localization to the nucleus in MDA-MB-231 cells[38]. These data lead us to wonder what affects cadmium chloride has on transcription levels of genes known to play a role in progression and invasion of mammary duct carcinoma. More specifically we wanted to study the effects of cadmium on carcinoma related genes that are also regulated by NFAT or cJun.

Results:

Cadmium titration affects some nascent transcripts in MDA-MB-231 Cells.

To explore nascent transcription effects of cadmium chloride, we selected five genes whose upregulation is associated with mammary duct carcinoma: CD44[39], FasL[40] , MMP-1[41], IL-8[42], and ENPP-2 (autotaxin)[43]. To qualitatively determine the nascent expression patterns, we titrated in cadmium chloride from 1 to 25 micromolar. PMA+ionomycin and no treatment were used as positive and negative controls, respectively. Cells were exposed to each treatment for 6 hours based on immunomicroscopy data showing full NFAT localization to the nucleus 7 hours after cytosolic calcium efflux[38]. cDNA libraries of each of these samples where subject to RT-PCR using primers that spanned intron-exon junctions. The reason for use of intron-exon junction primers was to capture a snapshot of transcripts newly transcribed upon cadmium exposure. This method, in theory, would omit detection of any mature RNA products. Detection of these nascent RNAs would give us better insight into the effect cadmium has on transcription. The results (Figure 5) showed that MMP-1, a matrix metalloproteinase, appeared to be upregulated up to concentrations of 25 micromolar cadmium chloride. This finding would be consistent with research suggesting inhibition of MMP-1 leads to lower rate of metastasis and better prognosis in mammary duct carcinomas[41]. We also found that ENPP-2 was upregulated upon titration of cadmium chloride. Signals from IL-8 and FasL seemed to be less prominent than those from ENPP-2 and MMP-1, but their transcription did seem to respond to exposure to PMA+ionomycin. Finally, CD44 failed to show much of a signal and transcription appears to remain constant throughout the titration.

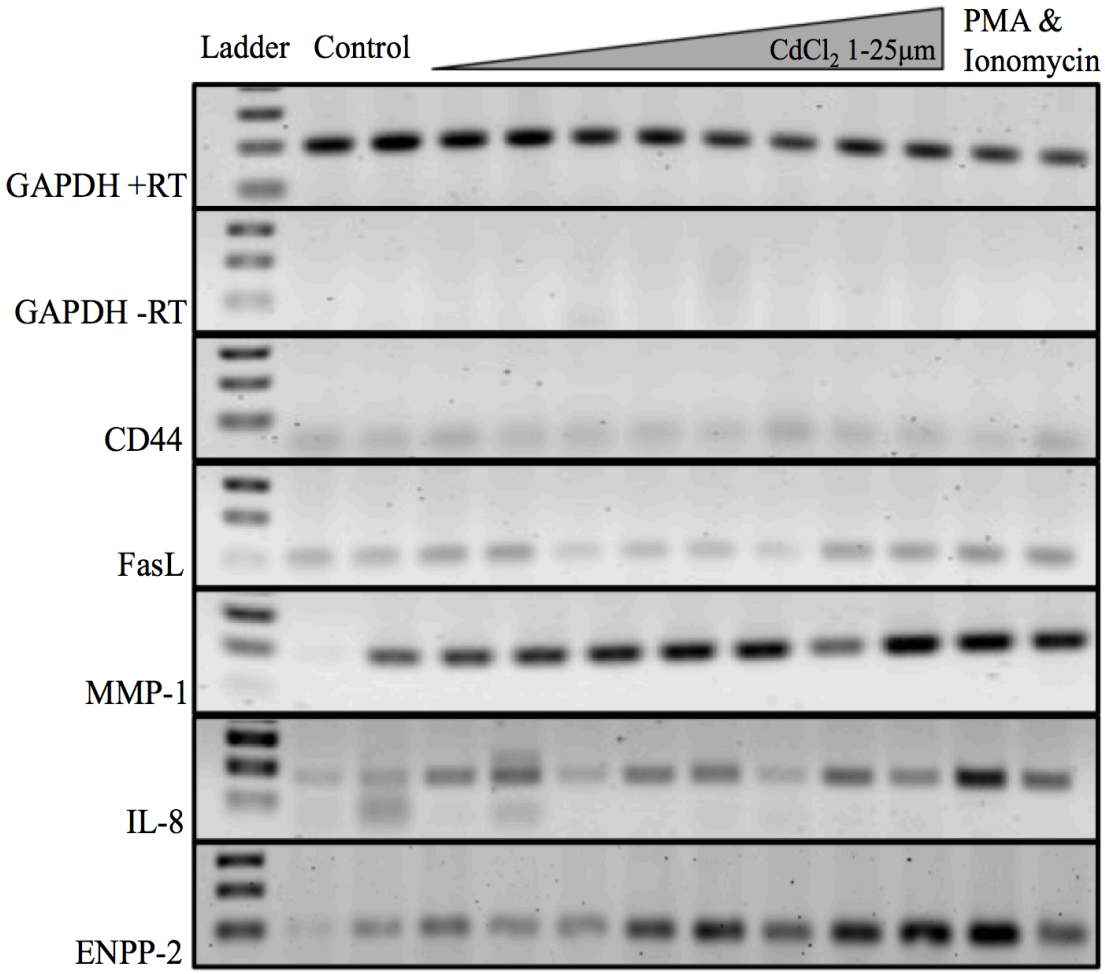

Figure 5: Cadmium gel titration. MDA-MB-231 cells were exposed to increasing concentrations of cadmium chloride form 1-25 micromolar for 6 hours. RNA was isolated and turned into cDNA through reverse transcription. Gene specific primers and PCR amplification was used for detection. Gel was 1.8% agrose. GAPDH –RT contained no reverse transcriptase.

The time course of the transcriptional response to cadmium in MDA-MB-231 Cells.

It is clear that cadmium caused increases in transcription for some nascent transcripts, based on a single time point of treatment. We were curious how varying exposure time would affect transcription from these genes. We wanted to identify the best time point at which to expose cells for chromatin immune-precipitation. In order to do this, we moved to a more quantitative method for determining transcript production through qualitative RT-PCR. Based off the information provided from the previous RT-PCR experiment, we exposed our cells to 25 micromolar cadmium chloride over various time intervals ranging from 2-12 hours. cDNA libraries were created for these samples and analyzed via qPCR. In order to create these data, Ct values for each biological and technical replicate were averaged together to minimize variations. Ct values were calculated into delta delta Ct by finding the difference between the target gene untreated minus the reference gene untreated and the target gene treated minus the reference gene treated. The reference gene used here was GAPDH. Fold change was finally calculated by raising 2 to the delta delta Ct value

MMP-1 shows a variety of expression levels at the different time points, all of which are much greater than that observed in the absence of cadmium. MMP-1 had an average increase of almost 37 fold and peaked at 60 at 6 hours. The peak around 6 hours would explain the large increase in band intensity that we saw in the previous RT-PCR experiments (Figure 6B). IL-8 and ENPP-2 had interestingly opposite correlations from each other. IL-8 showed a very large transcriptional induction that rapidly tapered off as the experiment progress out to 12 hours (Figure 6C&D). By contrast, the ENPP-2 nascent transcripts seemed to gradually increase through the experiment peaking around a 25-fold increase at

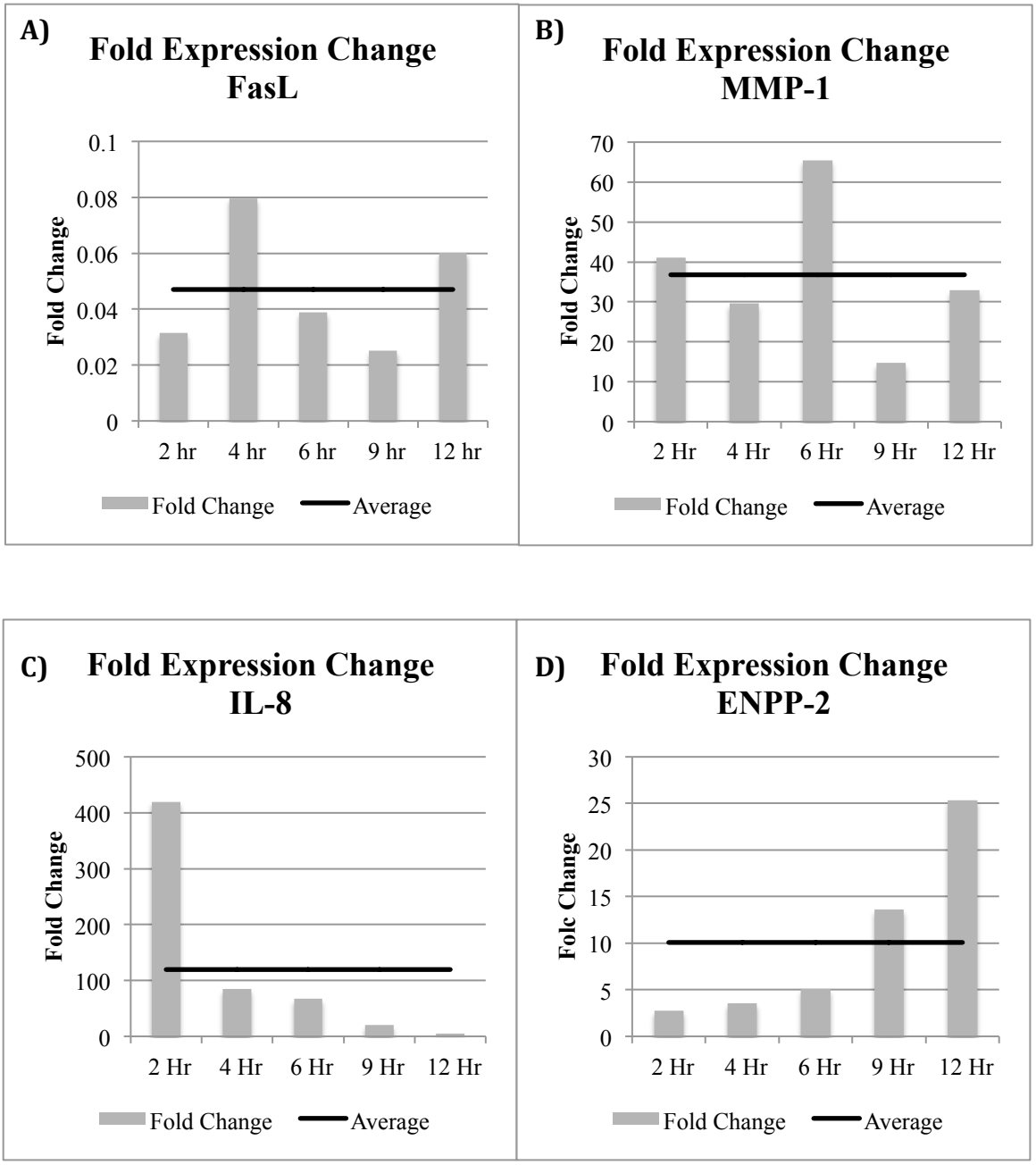

Figure 6: Time course qPCR. MDA-MB-231 cells were exposed to 25 micromolar $CdCl_2$ over a variety of time periods. RNA was isolated and reverse transcription used to make cDNA libraries. cDNA libraries were subject to qPCR and fold change was calculated from Ct values.

12 hours. Fold changes in FasL seemed to be decreased over the entire course of the experiment. We were optimistic from the data collected through RT-PCR that we would find more interesting data, but these data suggest that FasL transcription is not induced from exposure to cadmium chloride (Figure 6A). These data allowed us to monitor transcription over a large window of time and quantify the change in nascent transcription. These data also suggest that genes associated with mammary duct carcinoma seem to have notable fold increases in transcription when cells are exposed to cadmium chloride.

NFAT and cJun bind proximal promoter elements after cadmium exposure in MDA-MB-231 Cells and SKBR-3 Cells.

Although cadmium exposure appears to induce transcription of genes documented to be upregulated in mammary duct carcinoma the mechanism of their control is unknown. Documented increases in cJun and NFAT activity upon cadmium exposure lead us to question if cadmium could cause binding of these two factors to proximal promoter elements in the genes identified above. To do this we performed chromatin immuno-precipitation (ChIP) on cells that had been exposed to cadmium for 6 hours along with positive and negative controls, PMA+ionomycin and no treatment, respectively. ChIP assays were performed with a NFAT antibody, cJun antibody, or no antibody. DNA recovered was analyzed with qPCR using primers designed to flank known and potential NFAT and cJun binding sites in the genes of interest. Data are represented as percent of input pulled down during precipitation (%IP).

At this point, we decided to broaden our scope of cells lines and include another mammary duct carcinoma line SKBR-3. In both MDA-MB-231 as well as SKBR-3 cell

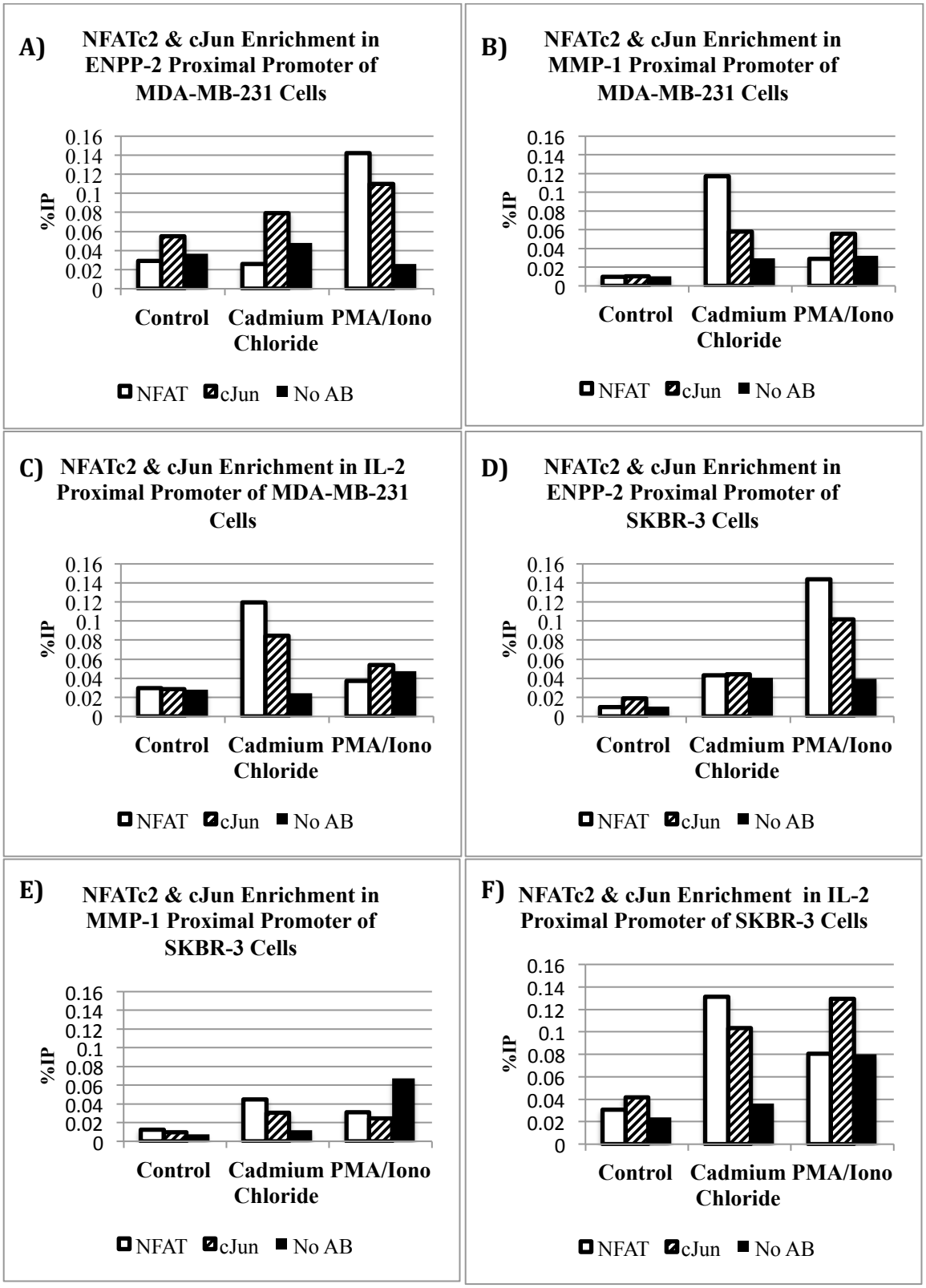

Figure 7: IL-2, MMP-1, ENPP-2 ChIP data. MDA-MB-231 and SKBR-3 cells were exposed to 25 uM $CdCl_2$ for 6 hours and immune precipitated with NFAT and cJun antibodies. Primers flanking potential proximal promoter NFAT and cJun binding sites were used to detect %IP.

lines we used IL-2 as an indicator that the ChIP experiment was working correctly. NFAT was originally characterized in IL-2 as a transcription factor needed for T cell differentiation. Recently this lab has also done studies showing that NFAT and homodimers of cJun work as cooperative transcription factors in the regulation of IL-2[50]. The signal received from ChIP-qPCR shows enrichment of both cJun and NFAT at a sight previously characterized as one that cooperatively binds NFAT and cJun (Figure 7C&F).

Analysis of ENPP-2 and MMP-1 promoter regions showed binding motifs for both NFAT and cJun within close proximity of each other and within a few hundred bases of the transcription start site[51,52] ChIP signal from these sites shows an enrichment of NFAT and cJun in ENPP-2 when treated with PMA+ionomycin, but not with exposure to cadmium (Figure 7A&D). Contrary to this, MMP-1 ChIP signal favors NFAT and cJun enrichment in cadmium exposed cells over PMA+ionomycin treated cells(Figure 7B&E). .

Identification of other genes upregulated by cadmium that bind NFAT and cJun in MDA-MB-231 cells.

Attempting to identify target genes for testing through individual papers documenting a genes relation to mammary duct carcinoma as well as those documenting NFAT or cJun control was tedious. At this point we came up with the idea to use current ChIP-seq data for binding sites of cJun in HeLa cells[53]. This data was extracted to an excel document and paired down to only genes with cJun binding sites within -300 and +100 of the transcription start site. The set of genes produced was then cross referenced with a paper depicting gene expression profiles in breast cancer patients with poor prognosis signatures. Those that had a cJun-binding site in the -300 and +100 range and were upregulated in patients with poor prognosis were selected. Finally, the selected genes were put into Snap Gene Viewer to identify genes that had a NFAT binding

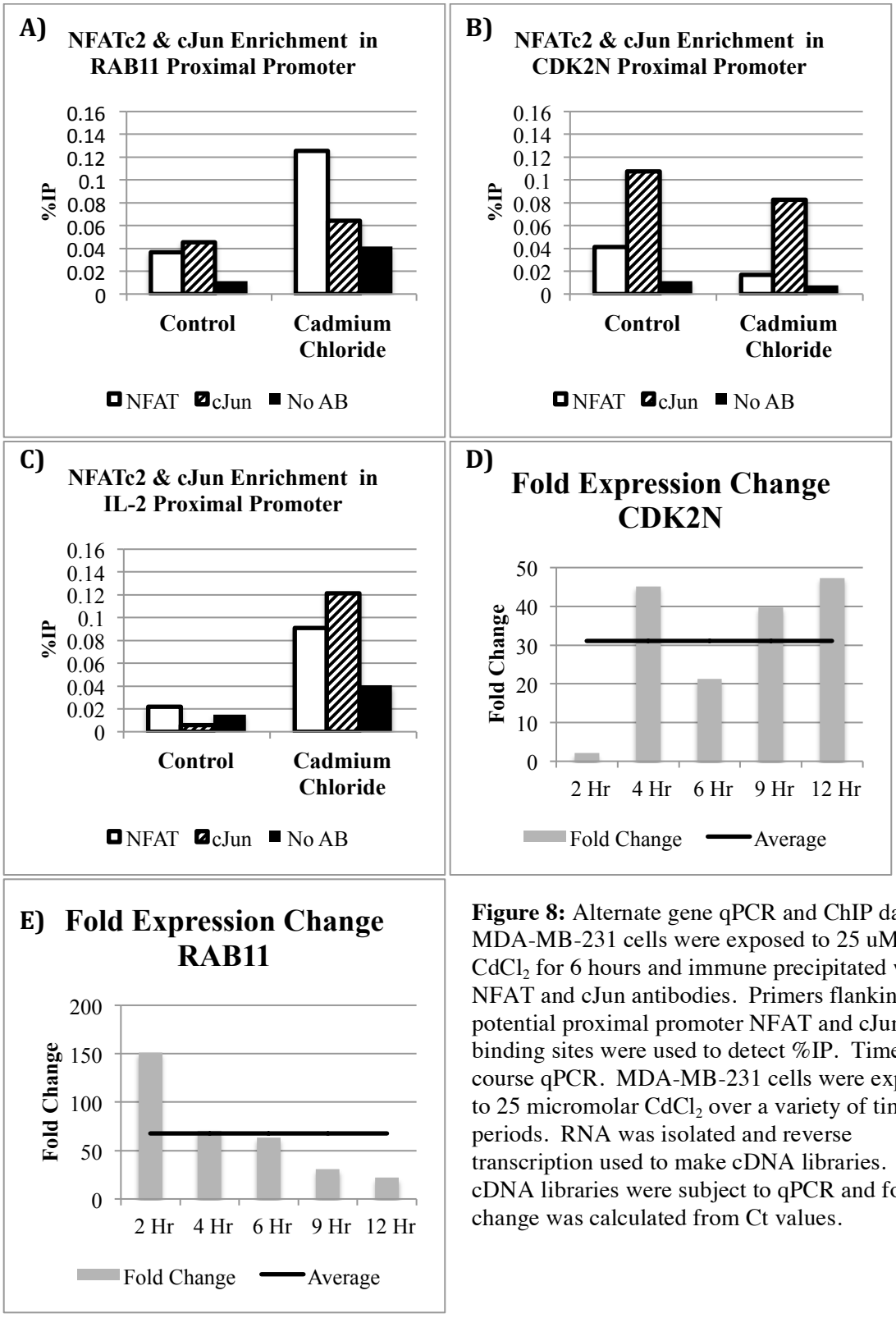

Figure 8: Alternate gene qPCR and ChIP data. MDA-MB-231 cells were exposed to 25 uM $CdCl_2$ for 6 hours and immune precipitated with NFAT and cJun antibodies. Primers flanking potential proximal promoter NFAT and cJun binding sites were used to detect %IP. Time course qPCR. MDA-MB-231 cells were exposed to 25 micromolar $CdCl_2$ over a variety of time periods. RNA was isolated and reverse transcription used to make cDNA libraries. cDNA libraries were subject to qPCR and fold change was calculated from Ct values.

Motif proximal to a cJun binding site. These genes were analyzed for expression changes upon cadmium exposure as well as binding of both NFAT and cJun in their proximal promoters.

The RAB11 and CDK2N genes were identified as potential targets. Again cells were exposed to a time course of 25 micromolar cadmium chloride from 2 to 12 hours and total RNA isolated. CDK2N showed strong induction over the untreated control after the 4-hour mark. Induction was variable over the 4-12 hour period, but exceeded 20 fold at all time points (Figure 8D). RAB11 showed similar transcription characteristics to IL-8. A signal is seen at 2 hours, but dampens out over time (Figure 8E). Nonetheless, RAB11 appears to be upregulated as well upon cadmium exposure.

The RAB11 and CDK2N proximal promoter binding of NFAT and cJun was studied next. ChIP was used again with qPCR and primers flanking the previously analyzed NFAT and cJun binding sites. The data revealed that the proximal promoter for RAB11 showed much better enrichment of NFAT and cJun after cadmium exposure (Figure 8A). On the contrary, CDK2N had higher enrichment of NFAT and cJun in the control sample indicating that NFAT and cJun binding to this region was not induced by cadmium exposure (Figure 8B).

Discussion

Cadmium is known as a carcinogen that has induced cancer in animal models. However, the mechanisms through which cadmium induces cancer need to be studied. Specifically, we looked at a few of the multitude of genes that are involved in cancer in order to see how cadmium affects their transcription within a mammary duct carcinoma cell line. To do this, we used RT-PCR coupled with titration of cadmium chloride to show that cadmium is in fact causing induction. Secondly, qRT-PCR was used to determine quantitative expression changes with in our cell line.

The set of genes we investigated was selected based off previous research that has shown these genes are also under control of transcription factors NFAT or cJun[44-48]. A hypothesized model is that oscillation in calcium signals, previously demonstrated in this lab, could activate NFAT and cJun and yield stronger transcriptional induction over steady state levels of calcium[38]. Increased oscillatory induction of NFAT responsive genes has been observed in T cells[24,25]. We believe that these findings along with others that support activation of cJun through the MAPK pathway could be a possible mechanism for up regulation of the genes studied above.

Our data show increased transcription of genes after treatment with cadmium, which is the framework for future experiments. Strong transcriptional induction is the end result of upregulation via activating transcription factors such as NFAT and cJun. Previous information on the cooperative binding of NFAT and cJun to the IL-2 promoter suggests that these two transcription factors could synergize to regulate transcription of specific genes. These experiments do not prove cooperatively, however increased nascent mRNA products as well as enrichment of both NFAT and cJun at proximal promoters encourages further conclusive experiments for cooperative binding of NFAT and cJun. To better determine if these genes are

under cooperative control some sort of knock out must be done for each and the ChIP as well as qRT-PCR data compared to control cells before and after cadmium treatment. On a genome wide scale, ChIP-seq as well as RNA-seq data could be collected in order to determine the effect of cadmium chloride genome wide. Regardless, we are enthusiastic about the observed cadmium induced increase in these genes coupled with NFAT and cJun binding to proximal promoters.

Materials and Methods

Chemicals and reagents

Cadmium chloride was dissolved in ultra-pure water to a stock concentration of 1 M and stored at -20°C for up to one month.

Cell culture

Human mammary adenocarcinoma cells (MDA-MB-231) were cultured in Libovitz's L-15 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 °C and 0% CO_2 in a humid incubator; they were trypsinized with 0.25% trypsin and sub-cultured roughly every four days. Cells were treated with $CdCl_2$ by exchanging medium with fresh medium containing various concentrations of $CdCl_2$. In all cases, the amount of DMSO in the medium never exceeded 0.3% (v/v).

Human mammary adenocarcinoma cells (SKBR-3) were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 °C and 5% CO_2 in a humid incubator; they were trypsinized with 0.25% trypsin and sub-cultured roughly every four days. Cells were treated with $CdCl_2$ by exchanging medium with fresh medium containing various concentrations of $CdCl_2$. In all cases, the amount of DMSO in the medium never exceeded 0.3% (v/v).

mRNA extraction

MDA-MB-231, and SKBR-3 cells at 70% confluence were treated for 6 to 12 hours with $CdCl_2$. Post treatment, cells were scraped from the plate and spun at 1100x g for 5 minutes. The supernatant was discarded and the cells were suspended in 200 μL TRIzol (Life Technologies) followed by incubation for 5 minutes. 40 μL of chloroform were added and, after 15 seconds of

gentle agitation, the solution was centrifuged at 16,000x g for 15 minutes. The aqueous phase was removed and the RNA was precipitated with 100 μL isopropanol. The collected RNA was pelleted, and washed with 75% ethanol before suspension in 1x RQ-1 DNase buffer. Samples were treated with 1 unit of RQ-1 DNase (Promega) for 20 minutes at 37 °C, followed by the addition of 1 μL DNase stop solution and a 10 minute incubation at 65 °C. cDNA was synthesized from the extracted RNA using 1 unit of Multiscribe RT, 10 μM random hexamer primers, and 2 mM dNTPs in 1x RT buffer (Promega). Samples were incubated at 25 °C for 5 minutes, then at 37 °C for 1 hour before ramping up to 65 °C to inactivate the Multiscribe. Individual mRNA products were detected using custom primers in 30-34 cycles of PCR. PCR products were resolved on a 1.8% agarose gel and detected using fluorescence scanning (Typhoon).

As well, qPCR was conducted on RNA extractions. Reactions were set up using Life Technologies 96 well plates. Master mixes for each primer set were made with the following for one reaction; 11 μL SYBR green (Life Technologies), 0.66 μL forward primer, 0.66 μL reverse primer, 5.68 μL water, 4 μL cDNA from RNA extraction. 18 μL of master mix with out cDNA was pipetted into 0.65 μL tubes and 4 μL of cDNA added for a total volume of 22 μL. From these 22 μL tubes 10 μL was pipetted into adjacent wells in the plate for technical duplicates. Samples were analyzed in Life Technologies StepOnePlus™ Real-Time PCR System.

Chromatin Immuno-Precipitation

Human mammary adenocarcinoma cells (MDA-MB-231 and SKBR-3) were grown to confluence in T-175 adherent cell flasks. The control group, cadmium chloride, and the PMA+ionomycin group were each assigned one T-175. The PMA+ionomycin group and the cadmium chloride were exposed for 9 hours. Post exposure cross-linking of protein to DNA was achieved by adding 810 uL of 37% formaldehyde for a final concentration of 1% to each flask

and placed on a shaker for 8 minutes. 1.5 mL of 2.5M glycine was added to give a final concentration of 0.125M and incubated for 5 minutes in order to stop the cross linking reaction. Media was aspirated and the cells were washed twice with PBS. 10 mL of cold PBS was added to each flask and cells were scraped and transferred to 15 mL TPX conical. 50 uL of each TPX conical was saved in order to determine cell count.

In order to isolate the nuclei of the cells each TPX conical was spun down at 1-2K RPM at 4 °C for 5 minutes. Cells where then washed with cold PBS. NRO buffer was added in a volume of 80 uL per million cells counted previously and incubated for 5 minutes. Samples where then spun at 3K at 4 °C for 5 minutes and the supernatant aspirated. Pellets were washed with the same volume of NRO buffer, and pellets were then frozen in liquid nitrogen.

To shear the chromatin for immunoprecipitation, pellets were resuspended in lysis buffer at a volume of 30 uL per million cells and incubated on a rocker for 10 minutes a 4 °C. Dilution buffer was then added in a 2:1 ratio. Chromatin were sheared via sonication in a Diagenode bioruptor with 30 seconds on and 30 second off for 25 minutes for the MDA-MB-231 cell line and 35 minutes for the SKBR3 cell line. After shearing samples where spun at 14K at 4 °C for 10 minutes and supernatant removed into new tubes which were stored at -80 °C. Immunoprecipitation (IP) started with pre-clearing the sonicated chromatin with beads in order to minimize non-specific pull down. This was done by pipetting 100 ul of each of control, cadmium chloride and ionomycin/PMA chromatin into eppendorf tubes corresponding to: Input, NFATc2, c-Jun, and minus antibody for a total of 12 tubes. Beads were equilibrated by washing 3 times in 10 column volumes of IP buffer. 15 uL equilibrated packed bead volume was pipetted into each tube and incubated for 2 hours at 4 °C. Beads were spun down, and the supernatant was pipetted into new tubes. Inputs were set aside for later precipitation. IP against the protein of interest was done by adding 4ug of NFAT or cJun antibody (NFAT: SC7296 cJun: SC1694)

to 100 ul precleared chromatin samples diluted to 300 ul with IP buffer and then placed on a rocker overnight at 4 °C. Beads were blocked by equilibration in IP buffer and then left overnight at 4 °C with 0.4 mg/ml yeast RNA and 0.5 mg/ml BSA. Preblocked beads were then washed twice with IP buffer and 20 ul packed bead volume was added to the antibody chromatin samples. Samples were rocked at 4 °C for 2 hours and then washed with low salt, high salt, lithium chloride, and TE buffers (spin, aspirate, add new buffer). Cross linked protein to DNA was then reverses by adding 250 ul of elution buffer and incubated for 1 hour at 37 °C. Samples and input sodium chloride concentration was raised to 200 mM and incubated at 65 °C for 12 hours. Inputs and samples where then proteinase K treated at 55 °C for 1 hour. Beads were spun and supernatants removed and phenol chloroform extracted. Extractions where then ethanol precipitated with 1/10 volume 3 M sodium acetate, 5 ug glycogen blue, and 2.5 volumes of 100% ethanol. Pellets were washed with 70% ethanol and dried in speed vac. Sample pellets were resuspended in 40 uL of water and inputs in 100 uL of water.

qPCR of elutes from precipitation was done to detect %IP. Reactions were set up using Life Technologies 96 well plates. Master mixes for each primer set were made with the following for one reaction; 11 μL SYBR green (Life Technologies), 0.66 μL forward primer, 0.66 μL reverse primer, 5.68 μL water, 4 μL sample from elutes. 18 μL of master mix with out cDNA was pipetted into 0.65 μL tubes and 4 μL of cDNA added for a total volume of 22 μL. From these 22 μL tubes 10 μL was pipetted into adjacent wells in the plate for technical duplicates. Samples were analyzed in Life Technologies StepOnePlus™ Real-Time PCR System. Relative standard curves were determined by diluting input samples from one to one thousand and quantities of elute samples determine form the standard curve in Life Technologies software.

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