

**Studying the effect of zinc on the interaction between C-terminal region  
of NFATc2 and cJun homodimers**

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## ABSTRACT

It is essential to study transcription factors that regulate synthesis of IL-2 mRNA because IL-2 is primarily regulated at the transcription level. Two transcription factors that have a unique ability to synergistically activate IL-2 transcription are NFATc2 and cJun. The putative zinc finger on the C-terminal domain of NFATc2 hinted us that zinc might affect the interaction between NFATc2 and cJun homodimers. In this research, we studied the effect of zinc on the interaction between NFATc2 and cJun homodimers. By using GST pull down assay, we found that zinc enhances the interaction between NFATc2 and cJun homodimers. Zinc was unique in enhancing this interaction because other divalent metal ions such as calcium, manganese and magnesium did not enhance the interaction. In addition, preliminary results from DNA pull down assay showed that NFATc2 might be recruiting cJun to the IL-2 promoter. Moreover, FRET assay results showed that there could be a change in the conformation of NFATc2 when zinc was present. This might be the reason behind the enhancement of interaction between NFATc2 and cJun homodimers in the presence of zinc because zinc changes the conformation of NFATc2, which might be the conformation that has higher interacting ability with cJun homodimers.

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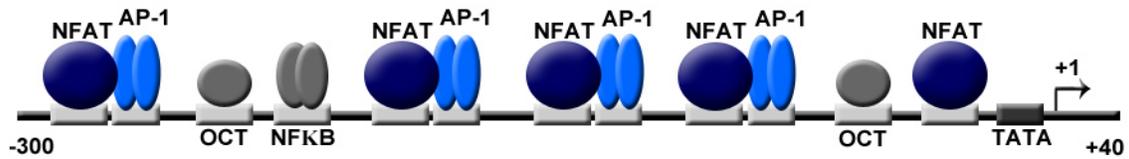
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## **Chapter I: General introduction to IL-2 transcription and the involvement of NFATc2 and cJun homodimers**

Transcription of protein-encoding genes is the process whereby RNA polymerase II synthesizes mRNA from a DNA template. This process is regulated by various transcription factors that can bind DNA in the promoter region of a gene and interact with other transcription factors and general transcription machinery to either repress or activate transcription. Transcription factors play a key role in the regulation of gene expression and can be activated by intracellular and extracellular signals.

Interleukin-2 (IL-2) is the first cytokine that is produced by naïve T cells when the T cells are stimulated by antigen presenting bodies (Avni and Rao, 2000). IL-2, after binding to the IL-2 receptor on lymphocytes, induces the growth and differentiation of cytotoxic T cells. Since IL-2 is primarily regulated at the transcription level (Jain *et al.*, 1995), it is essential to study the transcription factors that regulate synthesis of IL-2 mRNA. Two families of transcription factors that play an important role in IL-2 transcription are Nuclear Factor of Activated T-cells (NFAT) and Activator Protein 1 (AP-1). A schematic of the IL-2 promoter is shown in Figure 1.1, which shows that NFAT and AP-1 proteins bind at many sites to activate IL-2 transcription.

The NFAT family consists of five proteins: NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5. Members of this family of proteins contain an amino-terminal domain, a regulatory domain, a DNA-binding domain and a carboxy-terminal domain (Müller and Rao, 2010). In resting T cells, phosphorylated NFATc2 is localized in the cytoplasm, which is its inactive form (Figure 1.2). Upon T cell activation, calcium is released from the endoplasmic reticulum,



**Figure 1.1:** Schematic of human IL-2 promoter region. There are 4 NFAT/AP-1 composite sites, which have varying affinity for NFAT and AP-1 proteins. There is also a NFAT binding site at -45, which is not followed by AP-1 binding site. The arrow denotes the start site of transcription of the IL-2 gene.

activating calcineurin. Calcineurin then dephosphorylates NFATc2 leading to its translocation from the cytoplasm into the nucleus (Hogan *et al.*, 2003). In the nucleus, NFATc2 binds promoter DNA and interacts with other transcription factors, such as AP-1 family members, which then regulates several transcriptional pathways (Müller and Rao, 2010).

AP-1 is a family of transcription factors that consist of Fos (v-Fos, c-Fos, FosB, Fra1, Fra2), Jun (v-Jun, c-Jun, JunB, Jun D) and ATF (Activating Transcription Factor) proteins, which bind AP-1 elements on promoter DNA (Karin *et al.*, 1997). Members of the AP-1 family of transcription factors contain a highly conserved basic region and a leucine zipper region, which is termed the bZIP region, collectively. The basic region of AP-1 proteins is responsible for DNA binding, whereas the leucine zipper domain is responsible for the dimerization between two AP-1 proteins (Jain *et al.*, 1992). In addition, the leucine zipper domain also determines the stability and specificity of the dimers being formed (Hess *et al.*, 2004). A characteristic of the Jun family proteins is the ability to homodimerize, which is absent in Fos proteins. However, both Jun and Fos proteins are able to heterodimerize.

AP-1 proteins are regulated in the cell via physiological and pathological stimuli such as cytokines, growth factors, and stress. AP-1 proteins are present in the nucleus of the cell and are activated by the mitogen-activated protein kinase (MAPK) pathway (Figure 1.2). Dimerization of AP-1 proteins not only enhances their ability to bind DNA but also their ability to be recognized by MAPKs (Karin *et al.*, 1997).

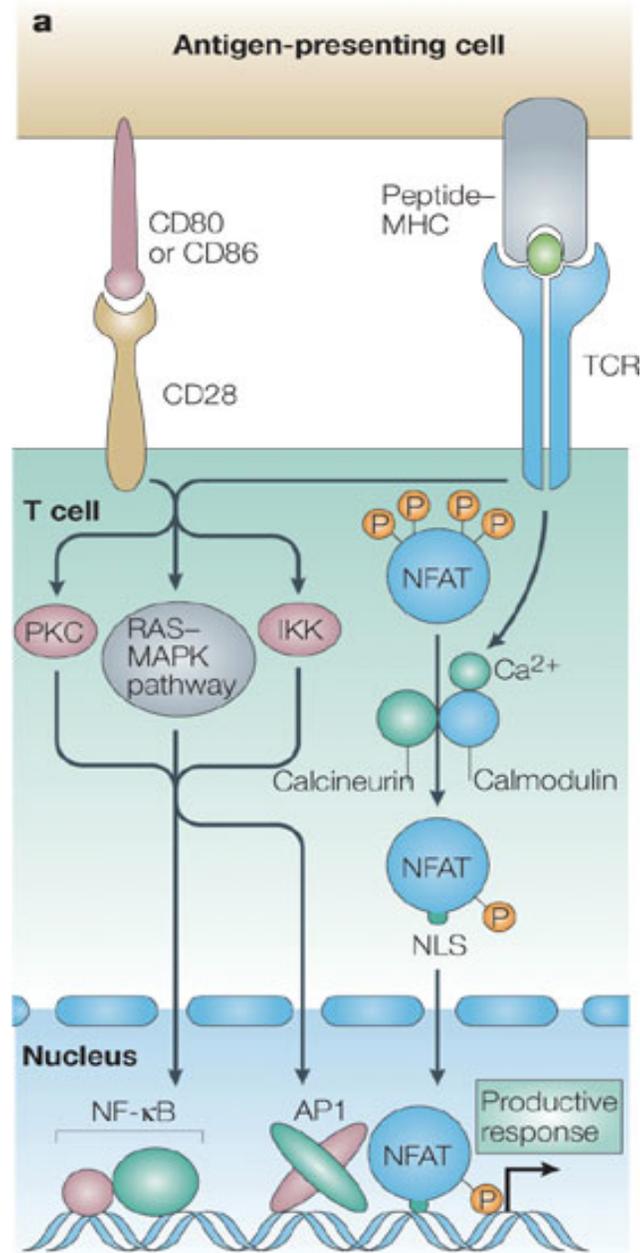


Figure adapted from (Macian, 2005)

**Figure 1.2:** Figure showing the overview of how IL-2 transcription is activated in activated T-cells. Antigen-presenting cells activate T-cells through a co-stimulation process. Once the T-cells are activated, dephosphorylated NFAT is translocated into the nucleus where it binds the IL-2 promoter. Simultaneously, AP-1 proteins are activated via a MAPK pathway and they, too, bind the IL-2 promoter with NFAT.

Of the various combinations of NFAT and AP-1 proteins (Figure 1.3), NFATc2 and cJun homodimers have the unique ability to synergistically activate IL-2 transcription (Nguyen *et al.*, 2010). Transcriptional synergy is when the cooperative interaction between two or more proteins activates transcription to a greater extent than the sum of the effect of the individual proteins. For example, NFATc2 and cJun homodimers together activate IL-2 transcription approximately 3-fold more than the sum of the transcriptional activation by NFATc2 and cJun homodimers individually. *In vitro* protein-protein interaction assays revealed the region of NFATc2 required for the unique synergy with cJun in activating IL-2 transcription. The C-terminal activation domain (amino acids 688-921) of NFATc2 is required for synergistic IL-2 activation (Nguyen *et al.*, 2010). In fact, the involvement of the C-terminal activation domain of NFATc2 in synergy is consistent with the inability of NFATc1 to synergistically activate IL-2 transcription because NFATc1 lacks the activation domain on its C-terminal (Nguyen *et al.*, 2010).

Importantly, *in vitro* interaction assays showed that the region of NFATc2 (688-921) required for synergy interacted with cJun homodimers in the absence of DNA (Nguyen *et al.*, 2010). By contrast, NFATc2 (688-921) did not interact with Jun/Fos heterodimers in the absence of DNA (Nguyen *et al.*, 2010). This shows that synergy does not result from the cooperative DNA binding between AP-1 and NFATc2. If it did, then all AP-1 protein combinations should be able to synergistically activate IL-2 transcription with NFATc2 because every combination of AP-1 and NFATc2 can cooperatively bind DNA at composite elements of IL-2 promoter due to their conserved DNA binding

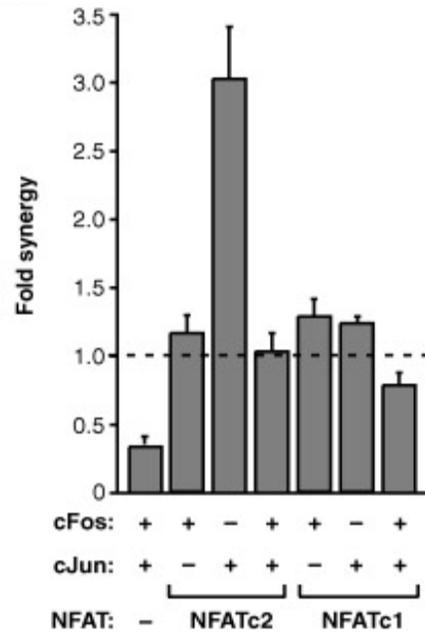


Figure adapted from (Nguyen *et al.*, 2010)

**Figure 1.3:** Fold synergy obtained for the various combinations of NFAT and AP-1 transcription factors. Fold synergy of 1 or above is considered to synergistically enhance luciferase activity. NFATc2 and cJun combination is enhancing luciferase activity the most.

domains (Erlanson *et al.*, 1996; Nguyen *et al.*, 2010). However, only the combination of NFATc2 and cJun homodimers was able to synergistically activate IL-2 transcription.

Experiments in cultured Jurkat T-cells showed that over expression of the NFATc2 C-terminal activation domain resulted in a loss of transcriptional synergy at the IL-2 promoter, presumably by blocking the interaction between the NFATc2 and cJun proteins (Nguyen *et al.*, 2010). This suggests that the interaction between the C-terminal activation domain of NFATc2 and cJun homodimers is critical for synergistic activation of IL-2 transcription in cells. By further characterizing this interaction, we aim to gain insight into how a specific protein-protein interaction at the promoter of a gene contributes to its overall transcriptional regulation.

## Chapter II: Role of zinc in the interaction between the C-terminal region (688-921) of NFATc2 and cJun homodimers

### Introduction:

Analysis of the primary sequence of the NFATc2 C-terminal activation domain revealed a putative zinc finger motif (Cys-x(2)-His-x(4)-His-x(2)-Cys) from amino acids 822-834. Zinc fingers are characterized by the presence of cysteine and histidine residues, which have ability to form a stabilized interaction with zinc ions. Different arrangements of cysteine and histidine residues within the primary amino acid sequence of a protein give rise to different structures of zinc fingers. For example, the Really Interesting New Gene (RING) family of proteins have a zinc finger which consists of Cys1-X<sub>2</sub>- Cys2-X<sub>(9-39)</sub>- Cys3-X<sub>(1-3)</sub>-His1-X<sub>(2-3)</sub>-Cys4-X<sub>2</sub>- Cys5-X<sub>(4-48)</sub>- Cys6-X<sub>2</sub>- Cys7 where X is any amino acid (Borden, 1998). These cysteine and histidine residues are used to coordinate zinc ions via a unique cross-brace system. Another type of zinc finger is present in the LIM domain. The LIM domain consists of (Cys-X<sub>2</sub>-Cys-X<sub>17-19</sub>-His-X<sub>2</sub>-Cys)-X<sub>2</sub> -(Cys-X<sub>2</sub>-Cys-X<sub>16-20</sub>-Cys-X<sub>2</sub>-His/Asp/Cys) residues where X is any amino acid (Kuroda *et al.*, 1996). Zinc fingers differ in their folded structures; however a common theme between zinc finger motifs is the conservation of cysteine and histidine residues.

Zinc fingers are found in a wide variety of protein structures and thus, exhibit diversity in their functions as well. Generally, zinc fingers are well known for their ability to mediate protein-protein interactions, DNA and RNA binding, and membrane association (Laity *et al.*, 2001). For example, a protein containing a LIM domain was found to interact with protein kinase C (PKC) via the LIM domain, which consists of a couple of zinc finger motifs (Kuroda *et al.*, 1996).

The suggestive zinc finger on the C-terminal region of NFATc2 consists of a Cys-His-His-Cys (CHHC) zinc finger motif (Figure 2.1). Recently, a CHHC zinc finger domain was discovered in spliceosomal proteins and tRNA methyl transferases. A solution structure of the CHHC zinc finger domain from the spliceosomal protein U11-48K showed that the domain is an independent folding unit and can bind zinc stoichiometrically in one-to-one ratio (Tidow *et al.*, 2009). It is possible that NFATc2 binds zinc in a functionally important manner.

Since zinc fingers are associated with mediating protein-protein interactions, we hypothesized that the putative zinc finger on NFATc2 would have a similar function in mediating the interaction between the C-terminal region of NFATc2 and cJun homodimers. As previously described, the synergistic activation of IL-2 transcription is mediated by the protein-protein interaction between the C-terminal activation domain of NFATc2 and cJun homodimers. Thus, we wanted to see if zinc would affect this interaction.

## **Materials and Methods**

### *GST Pull Down Assay*

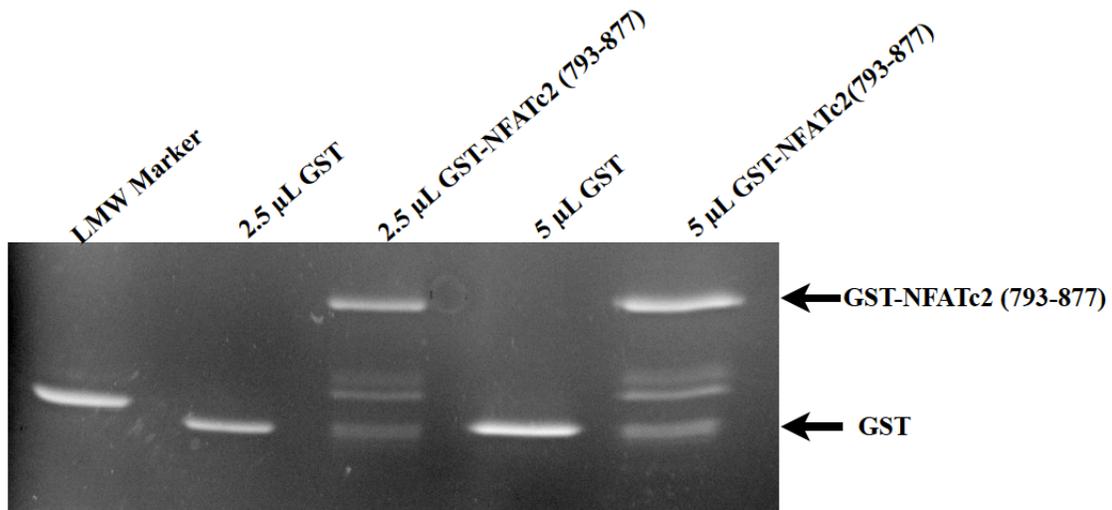
BL-21 cells (E-coli) were used for the protein expression. Two types of proteins were expressed for the experiment: 1) Glutathione S-transferase (GST) for the experimental control and 2) GST- NFATc2 (793-877) fusion proteins. After transformation of the bacterial cells with plasmids that encode the proteins, cultures were grown until the optical density at 600nm was 0.4. Expression of these proteins was induced with 0.6 mM Isopropylthio- $\beta$ -D-galactoside (IPTG) for 2 hrs at 37°C. Cells were



then harvested and resuspended in 15 ml of lysis buffer containing 20 mM Tris (pH 7.9), 20% Glycerol, 1mM EDTA, 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF, and sonicated four times for 20 seconds with a microtip on ice. The sample was centrifuged for 30 minutes at 4,000 rpm, and the supernatant was saved for protein purification.

Glutathione sepharose beads, which bind with high affinity to the GST protein, were used for protein purification. 200  $\mu$ L of GST and GST-NFATc2 fusion protein extracts were mixed with 100  $\mu$ L of equilibrated glutathione-sepharose beads in 0.1 M TGEM buffer containing 20 mM Tris (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM  $MgCl_2$ , 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF and 0.1 M NaCl. The protein-bead slurry was nutated for 30 minutes at 4°C to allow GST proteins to bind to the beads. After nutation, the immobilized GST proteins were washed 2 times with 10-bead volumes of 1.0 M TGEM (20 mM Tris (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM  $MgCl_2$ , 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 1 M NaCl) followed by 3 times with 10-bead volumes of 0.1 M TGEM. After the final wash, the beads were stored as 1:1 slurry in 0.1 M TGEM.

In order to have uniformity in the amount of our control (GST) and the protein of interest (GST-NFATc2), we determined the relative amount of protein on the beads using SDS-PAGE (Figure 2.2). For SDS-PAGE we used 2.5  $\mu$ L of the slurry, 2.5  $\mu$ L of 2X loading dye, and 2  $\mu$ L of 1M DTT per sample. 5  $\mu$ L of the Low Molecular Weight marker that we used was equivalent to 500 ng of protein. After calculating the relative amount of GST and GST-NFATc2 on beads, we performed various pull down assays with full-length cJun.



**Figure 2.2:** SDS-PAGE of control GST and GST-NFAT (793-877) purified from *E. Coli*. The marker band is equivalent to 500 ng of protein. Considering the marker as our reference we were able to calculate the amount of in our 1:1 immobilized bead slurry.

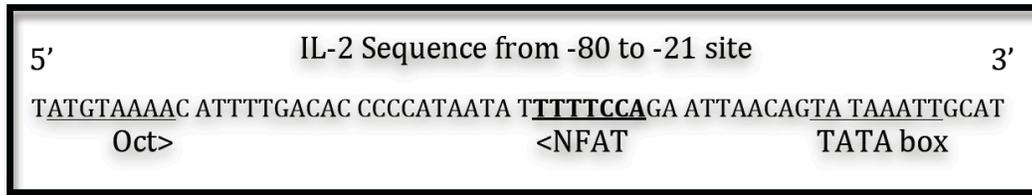
Each pull down assay was performed in 0.1 M TGEM, with each reaction sample containing 100  $\mu$ L of total volume. 20  $\mu$ M zinc chloride was used for samples with zinc. Different concentrations of GST proteins and cJun were used for reaction samples in order to find the optimal reaction conditions, which is discussed later in the results section. All the reaction samples were allowed to nutate at 4°C for 2.5 hours. These samples were then washed with 50  $\mu$ L of 0.1 TGEM buffer four times. After the final wash, the protein was boiled in 25  $\mu$ L of 2X loading buffer and resolved by SDS-PAGE. The pulled down cJun was detected using Western Blots with an antibody specific for cJun.

*DNA Pull down Assay:*

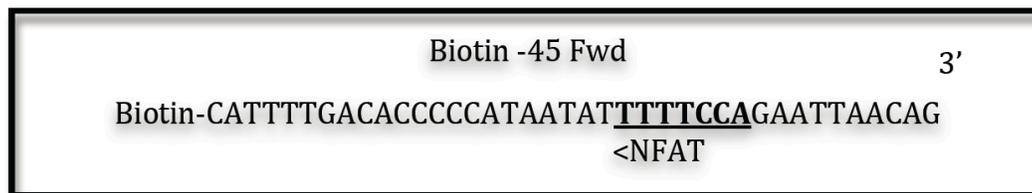
Biotin-DNA oligos were ordered, which contained the -45 site of the IL-2 promoter (Figure 2.3). The -45 site of IL-2 promoter contains the sequence, 5'TTTTCCA3'. Another biotin-DNA oligo had a mutated -45 site of IL-2 promoter, which contained the sequence, 5'TTTTAAA3'. The mutant biotin oligo was used as our experimental control because NFATc2 should not be able to bind this site. In a final volume of 50  $\mu$ L, a final concentration of 16  $\mu$ M Biotin oligo was allowed to anneal with 17.6  $\mu$ M of the reverse complement oligo in the presence of anneal buffer (20 mM Tris (pH 7.9), 2 mM MgCl<sub>2</sub>, 50 mM NaCl) and 36.6  $\mu$ L of ddH<sub>2</sub>O. The forward and reverse oligos were heated for 2 minutes at 95°C, 1 h at 65°C, and the samples were allowed to cool to 4°C in the PCR machine, to allow annealing.

A schematic illustrating the DNA pull down assays is shown in Figure 2.4. 0.2 nMol of annealed biotinylated DNA (wild type and mutant in separate tubes) was added to 50 $\mu$ L of 0.1 M TGEM buffer. The mixture was then allowed to nutate with 50 $\mu$ L of

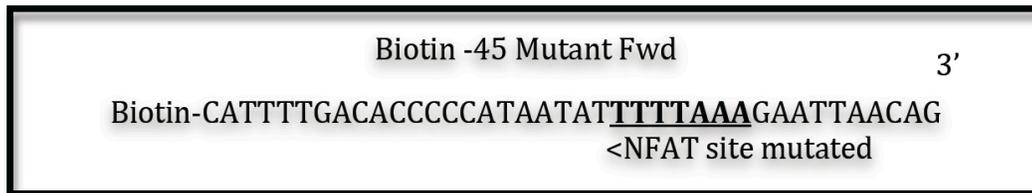
streptavidin agarose beads for 30 mins at 4°C. The beads were then washed 2 times with 0.1 M TGEM in order to remove any unbound annealed biotinylated DNA. 20 µL of the slurry was then mixed with 80 µL 0.1 M TGEM buffer, 30 ng of poly dG.dC to reduce nonspecific binding, and 50 nM of NFATc2. We made 2 samples for the wild type DNA and 2 for the mutant DNA. The samples were then allowed to incubate for an hour at 4°C. After incubation, one of each wild type and mutant sample received 500 µM zinc. Finally, 120 ng of full-length cJun was added to each of 4 tubes and was allowed to incubate for an hour at 4°C. Each sample was washed with 0.1 M TGEM buffer 3 times. The proteins were harvested with 20 µL of SDS-loading dye. Samples were run on SDS-PAGE and Western Blotting with antibodies against cJun and NFAT were used to detect proteins.



Panel A

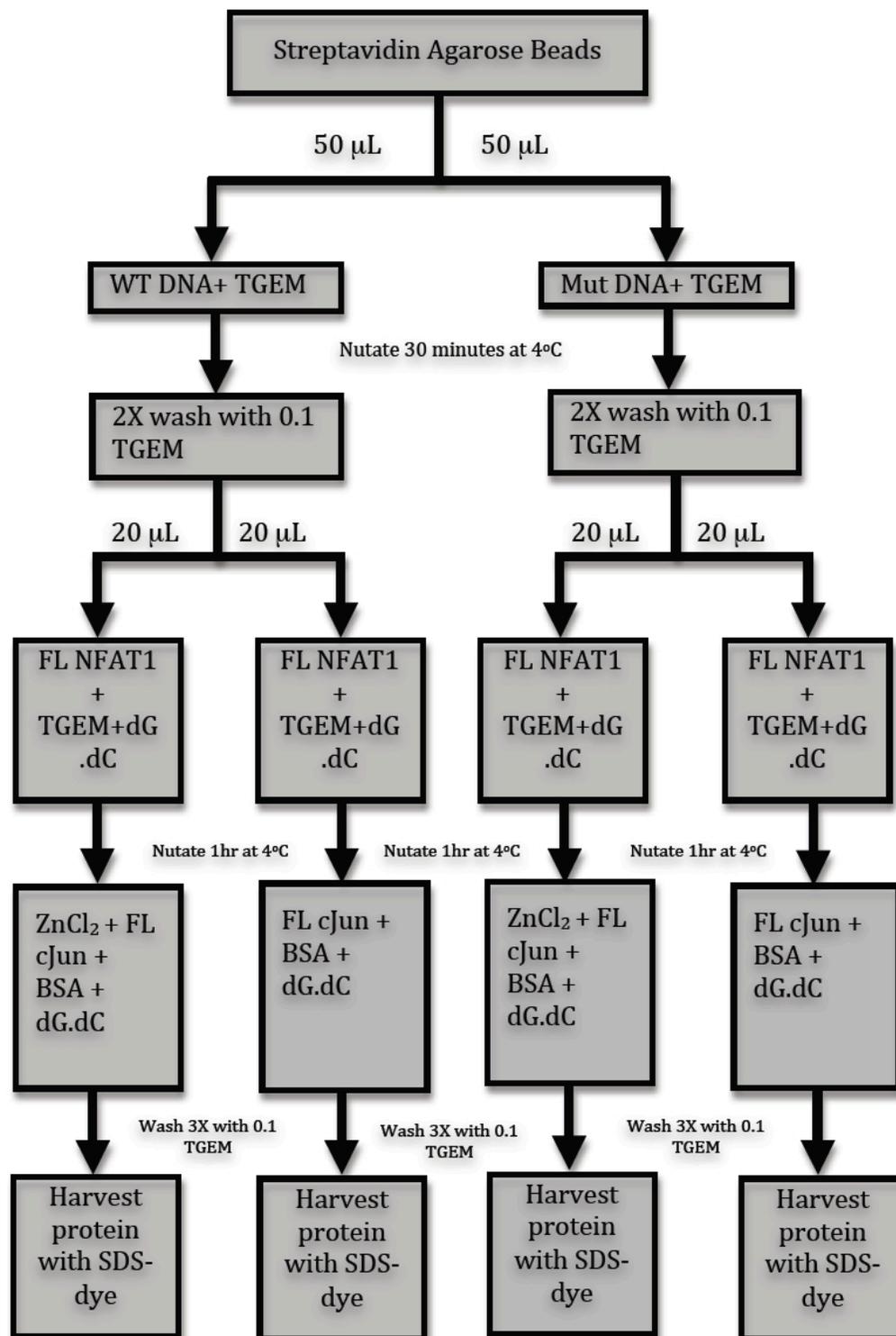


Panel B



Panel C

**Figure 2.3:** Panel A shows the non-template strand of IL-2 promoter site from -80 to -21. At -45 site IL-2 has the NFAT recognition site which is labeled in the figure. Panel B shows the wild type Biotin DNA with the nucleotide sequence like that of IL-2 NFAT binding site. Panel C shows the biotin DNA with mutated -45 site which makes it unable to bind NFAT.

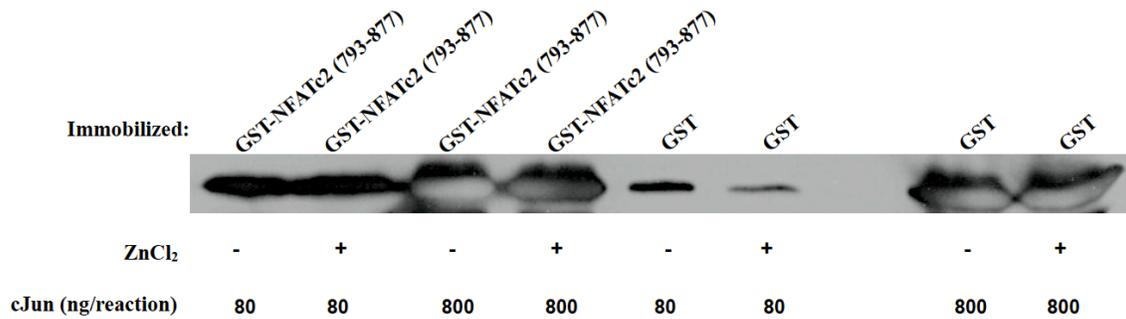


**Figure 2.4:** Flowchart showing the reaction sample set up for DNA pull down assay.

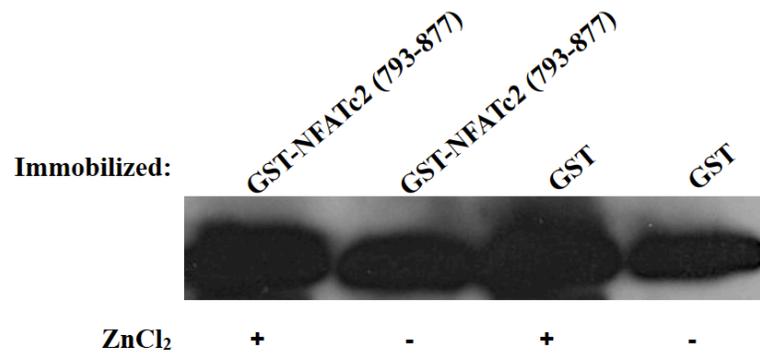
**Results:***Optimization of GST Pull Down Assay*

In order to determine whether the amount of cJun pulled down by the C-terminal domain of NFATc2 changed in the presence of zinc, it was essential that we used amounts of cJun and NFATc2 (793-877) proteins that resulted in a signal that was in the linear range of the Western Blots. The result from an initial pull down assay is shown in Figure 2.5, where the bands represent the amount of pulled down cJun. Out of 8 reaction samples, 4 of these reaction samples contained 80 ng of cJun and the other 4 contained 800 ng of cJun. All the samples had 2  $\mu$ g of the immobilized protein (GST or GST-NFATc2 (793-877)). The amount of cJun pulled down in the reactions that received 800 ng of cJun appear to be out of the linear range of the Western Blot gel because differences between the GST samples and the GST-NFATc2 (793-877) were not observed. By contrast, with 80 ng of cJun we were able to observe more cJun pulled down in the GST-NFATc2 (793-877) sample compared to the GST control. Also, samples with and without zinc seemed to be pulling down the same quantity of cJun. Thus, we proceeded with 80 ng of cJun per sample and scaled down the amount of immobilized proteins to 100 ng per reaction to hopefully detect differences in the presence of zinc.

We next set up reactions that contained 100 ng of the immobilized proteins and 80 ng of cJun. The results are shown in Figure 2.6. There seemed to be some non-specific interaction between cJun and the immobilized beads because both of the immobilized proteins (GST and GST-NFATc2 (793-877)) were able to pull down approximately the



**Figure 2.5:** Pull down assay with two amounts of cJun was performed. Signals for the samples with 800 ng of cJun were not on the linear range of the Western Blot. The background seems to be better for 80 ng of cJun samples.



**Figure 2.6:** The figure shows that more cJun is pulled down when the sample is treated with zinc. However, both the GST protein and the GST-NFATc2 (793-877) protein seem to interact with the cJun at the same level, indicating a high level of non-specific background binding in this experiment.

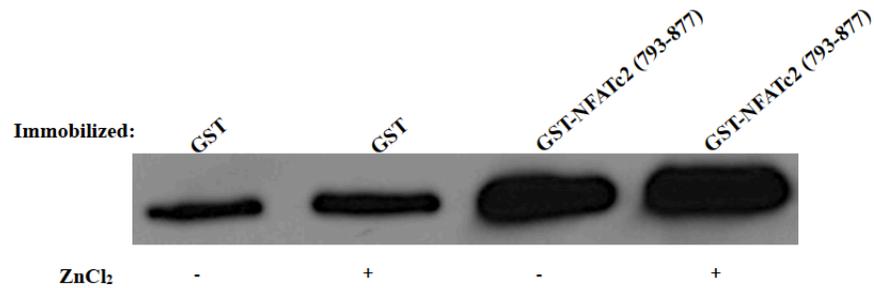


Figure (a)

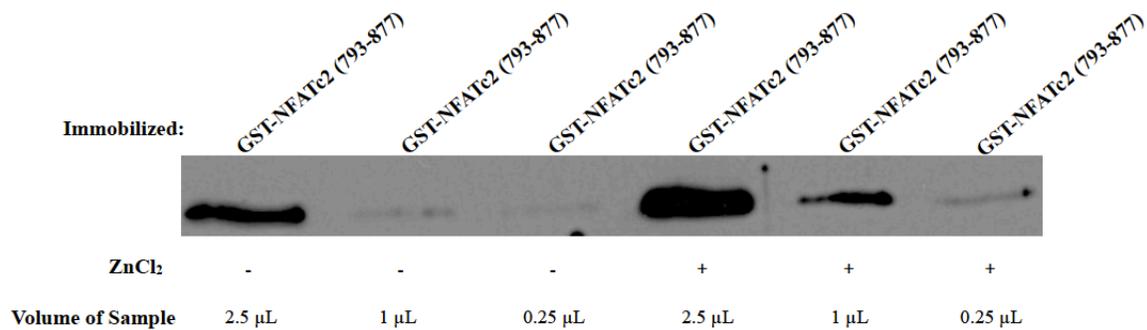


Figure (b)

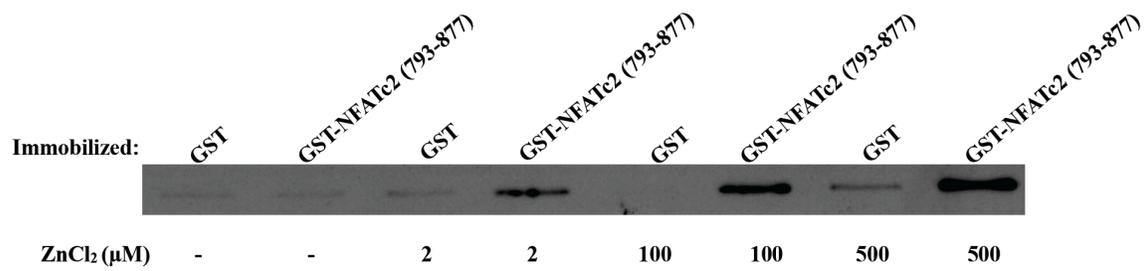
**Figure 2.7:** Figure (a) shows that immobilized GST-NFATc2 (793-877) protein is binding more cJun than immobilized GST protein. Also, immobilized GST-NFATc2 (793-877) protein in the presence of zinc is binding slightly more cJun than GST-NFATc2 (793-877) protein without zinc. Figure (b) shows different volumes of pull down samples loaded on the gel. The figure clearly shows the enhancement of cJun binding to GST-NFATc2 (793-877) with zinc.

same amount of cJun in the presence and absence of zinc. In order to eliminate non-specific interactions between the bead proteins and cJun, we decided to increase the strength of our TGEM washing buffer by increasing the salt concentration. We also decided to use 4 ng of bovine serum albumin (BSA) per 100  $\mu$ L of the sample and increase the amount of detergent. BSA and detergent help to reduce non-specific interactions.

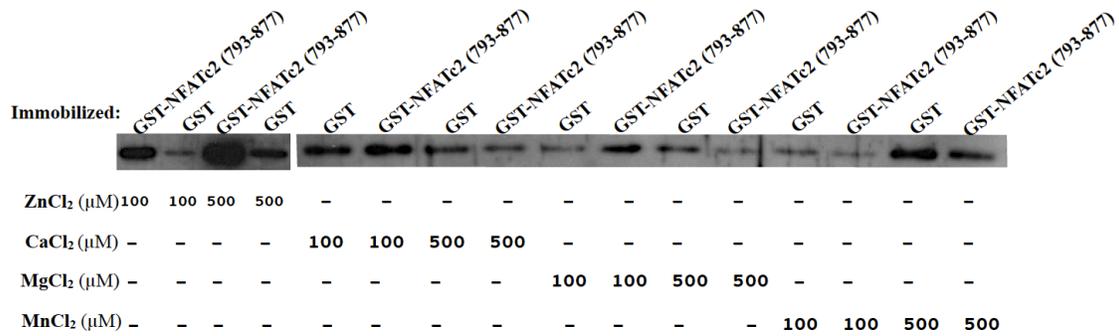
We next set up reactions that contained the same amount of proteins as in Figure 2.6, except in this experiment BSA was added to the samples and the stringency of the washing buffer was increased. The samples were washed with 0.15 M TGEM buffer with 0.2% NP-40, as opposed to the previous 0.1 M TGEM buffer with 0.1 % NP-40. The result from this experiment is shown in Figure 2.7a. GST-NFATc2 (793-877) pulled down significantly higher amounts of cJun than the GST proteins. Moreover, the amount of pulled down cJun increased with zinc for the GST-NFATc2 protein. In order to observe this result more precisely, we ran another Western Blot with diluted volumes of the pull down samples (Figure 2.7b). If we compare the 2.5  $\mu$ L samples of GST-NFATc2 with and without zinc, it is reasonable to conclude that approximately 3-fold more cJun was pulled down when zinc was present.

*Zinc facilitates the interaction between NFATc2 and cJun.*

With the assay optimized we wanted to further test if zinc enhanced the interaction between NFATc2 and cJun. We performed an assay with a titration of zinc, and the result is shown in Figure 2.8. We found that zinc enhanced the interaction between immobilized GST-NFATc2 and full-length cJun. The amount of cJun pulled



**Figure 2.8:** Various concentrations of zinc applied to samples in order to see whether the amount of cJun pulled down changed with increasing zinc concentration. The first two lanes are our controls where we did not add zinc chloride. The concentration of zinc chloride was increased for the rest of the lanes, which is indicated on the figure.



**Figure 2.9:** Immobilized GST and GST-NFATc2 proteins were allowed to incubate with cJun at various concentrations of zinc, calcium, magnesium and manganese. There is an enhancement in the interaction of cJun with GST-NFATc2 (793-877) when zinc is present. However, no such significant enhancement is seen with other divalent metal ions.

down by NFATc2(793-877) increased as the concentration of zinc was increased. The sample without zinc chloride added did not have significant interaction between GST-NFATc2 and cJun.

*Other divalent metal ions do not show enhancement of the interaction between NFATc2 and cJun.*

Other divalent metal ions such as calcium, magnesium and manganese were used to see their effect on the binding of GST-NFATc2 (793-877) with cJun because we wanted to determine if the effects were specific to zinc. We let immobilized GST and GST-NFATc2 (793-877) incubate with cJun in the presence of zinc, calcium, magnesium and manganese to see how these divalent metal ions influenced the interaction between the proteins. The result from this experiment is shown in Figure 2.9. We observed an enhancement in the interaction between NFATc2 (793-877) and full-length cJun when zinc was present. However, the enhancement was lost when calcium, manganese, or magnesium was in the reaction sample.

*The -45 site of IL-2 promoter binds NFATc2, which can recruit cJun in a higher quantity in the presence of zinc.*

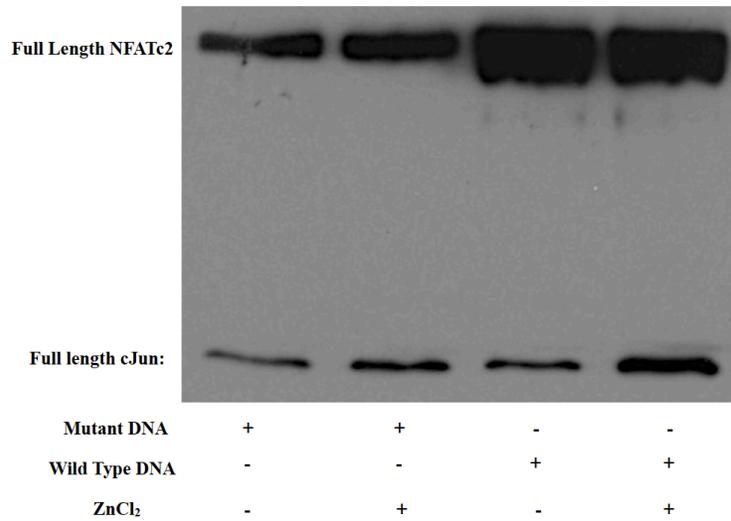
We next performed DNA pull down assays to obtain insight into the mechanism of NFATc2 and cJun recruitment to the IL-2 promoter. Because the interaction between NFATc2 (793-877) and cJun does not require DNA (Nguyen *et al.*, 2010), we asked if DNA-bound NFATc2 could recruit cJun in the absence of DNA sequences that bind to cJun, and do so in zinc facilitated manner. Our lab has shown that the -45 NFAT binding site in the IL-2 promoter is important for the transcriptional synergy observed between NFATc2 and cJun (R. Walters, unpublished data). We therefore used the sequence of the -45 NFAT site in DNA pull down assays.

Different samples (with and without zinc) of immobilized wild type and mutant DNA were incubated with NFATc2, and then cJun was added to the mixture as described in Figure 2.4. Western Blot results from the DNA pull down assay are shown in Figure 2.10. Wild type DNA sequence of the -45 site of the IL-2 promoter was able to bind significantly more NFATc2 than the mutated the -45 site sequence, as expected. The binding of NFATc2 at the -45 site is a zinc-independent process. Preliminary results suggest that NFATc2 can potentially recruit cJun when zinc is present; however, more experiments need to be performed because the level of cJun that is being recruited with zinc is only slightly higher than the background cJun with mutated -45 site DNA.

**Discussion:**

We studied the involvement of a putative zinc finger, which is present in the C-terminal domain (793-877) of the NFATc2 protein, in facilitating an interaction with cJun homodimers. The zinc finger in NFATc2 runs from amino acid residues 822-834, which has the following Cys and His pattern: Cys-X<sub>2</sub>-His-X<sub>4</sub>-His-X<sub>3</sub>-Cys. It has been previously shown that zinc fingers help in mediating interactions between proteins and DNA as well as protein-protein interactions (Laity *et al.*, 2001). It has also been shown that NFATc2 can bind cJun homodimers even in the absence of DNA (Nguyen *et al.*, 2010). So we investigated whether zinc had the ability to enhance the interaction of NFATc2 with cJun in the absence of DNA. We found out that in the presence of zinc, the interaction between NFATc2 (793-877) and cJun was increased.

When we used other divalent metal ions, there was no enhancement in the interaction between NFATc2 (793-877) and cJun. This suggests that zinc mediates the



**Figure 2.10:** DNA pull down result detecting NFATc2 and cJun using Western Blot. We see two thick bands of NFATc2 for the wild type DNA sequence both in the presence and absence of zinc. Moreover, we were able to pull down the most amount of cJun with wild type DNA when zinc was present.

interaction between NFATc2 (793-877) and cJun through the putative zinc finger in the C-terminal activation domain of NFATc2. If the charges on the zinc were solely responsible for the enhancement, then we should have seen similar results when we used other divalent metal ions (Figure 2.9). But, there was no enhancement in the interaction between NFATc2 and cJun when calcium, magnesium and manganese ions were present.

In order to conclude that the putative zinc finger on NFATc2 was responsible for binding zinc and thus enhancing the interaction with cJun, we need to mutate the cysteine residues and see if the interaction is still enhanced by zinc. This mutation should disrupt the ability of the Cys residues to coordinate zinc. The interaction should no longer show an enhancement in the presence of zinc if the cysteine residues are crucial for the coordination of zinc. Furthermore, we can also mutate the histidine residues on the zinc finger and study their involvement in coordinating zinc and the interaction with cJun.

Even though the results from DNA pull down assay were preliminary, and more experiments need to be done to validate this conclusion, it is possible that NFATc2 might be recruiting cJun to the IL-2 promoter. We hypothesized two models for the recruitment of cJun homodimers by NFATc2 to the IL-2 promoter, which are depicted in Figure 2.11. Since there is no AP-1 site adjacent to the -45 site of IL-2 promoter, cJun cannot bind with the DNA with high affinity. However, zinc might facilitate and stabilize the interaction between cJun and DNA. Another potential model is that cJun is recruited to -45 site as a coactivator, and cJun may not interact with DNA at all.



**Figure 2.11:** Figure A illustrates a model of zinc stabilizing the interaction between cJun and the DNA. Figure B shows a model with cJun being recruited to NFATc2 as a coactivator, not having any direct contacts with DNA.

### **Chapter III: Application of a Zinc Sensor in studying the interaction between the C-terminal region of NFATc2 and zinc.**

#### **Introduction**

Zinc sensors are proteins that are typically expressed in cells and used to study the cellular concentration of zinc in an organelle. A zinc sensor consists of a zinc detector domain that is flanked by two fluorescent proteins on each end (Dittmer *et al.*, 2009). When the zinc-detecting domain binds zinc, there is a change in the conformation of the domain. The terminal fluorescent proteins detect the change in conformation because the fluorescent proteins are capable of undergoing fluorescence resonance energy transfer (FRET) as the change in conformation occurs. The amount of FRET observed is dependent on the distance between and the orientation of the two fluorescence proteins (Palmer *et al.*, 2011).

We created a zinc sensor using the C-terminal region of NFATc2, between two fluorescent proteins: cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Figure 3.1). If this region of NFATc2 can bind zinc, then it should lead to a conformational change in NFATc2. The conformational change results in the change in FRET because the distance between the two fluorophores will change. When the change in FRET occurs, the intensity of donor fluorescence protein (CFP) emission decreases, and the intensity of the acceptor fluorescence protein (YFP) emission increases. This technique can provide insight into the nature of the interaction between NFATc2 and zinc, and it could potentially lead us in understanding the interaction between NFATc2 and cJun.

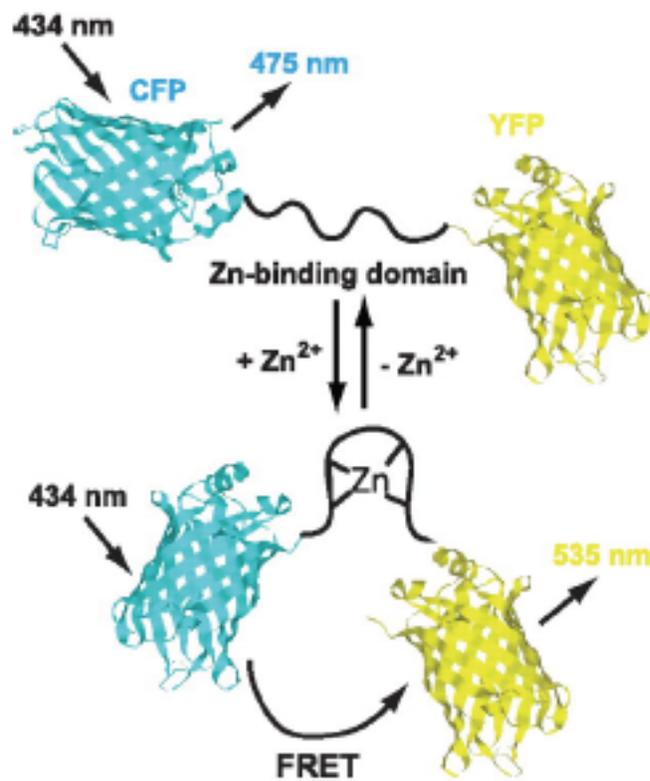


Figure adapted from (Dittmer et al., 2009)

**Figure 3.1:** Schematic of a zinc sensor. In our case, the Zn-binding domain is the C-terminal region of NFATc2. There is a conformational change in this domain when zinc is bound, bringing two fluorescence proteins close together that results in the change in FRET.

**Materials and Methods:***Making the construct*

A plasmid containing CFP and YFP with a calcium-binding region in between them was obtained from Palmer lab. Since we were interested in studying the zinc-binding region of NFATc2, we removed the calcium-binding region using the restriction enzymes SacI and SpH1, simultaneously. Oligonucleotides coding for the C-terminal region of NFATc2 were ligated into the vector between SacI and SpH1 restriction sites. Ligated plasmid was used to transform E. coli XL 1-Blue cells and the cells were plated on LB-Amp plates. A colony was grown up in 5 mL LB/Amp culture and the plasmid DNA was extracted.

*Expressing and purifying the sensor*

The NFATc2 zinc sensor proteins were expressed in TOP10 E. coli cells. TOP10 E. coli cells were mixed with 50ng of the zinc sensor plasmid and incubated on ice for 45 min. After incubation, the mixture was heat shocked for 45 s at 42°C. Heat shocked cells were plated on a LB-Amp plate and incubated overnight at 37°C. A colony was picked to grow up a 5ml starter culture, which was incubated overnight at 37°C. The starter culture was then used to inoculate a 150ml culture. L-(+) arabinose (0.2%) was used to induce protein expression, once the optical density of the cells reached 0.4 at 600 nm wavelength. The protein was expressed overnight at room temperature and the cells were harvested by centrifugation at 12,000xg for 20 minutes.

For zinc sensor protein purification, we lysed the bacteria pellet in 5 mL of BPER reagent (Thermo Scientific) in the presence of EDTA-free protease inhibitors (Roche).

The resuspended solution was shaken at 200 rpm for 60 minutes (room temperature), with intermittent light vortexing. The zinc sensor contained a 6xHis tag allowing the protein to be purified over a nickel column. 5 ml of Ni-NTA agarose beads (qiagen) were used for purification. The beads was pre-conditioned with 2 mL of TNS (50 mM Tris Buffer, 300 mM NaCl, pH 7.4)/ 20 mM imidazole Buffer. The resuspended cell pellet in BPER buffer was centrifuged at 1400 rpm for 15 minutes at 5°C. The supernatant from the centrifugation was mixed with imidazole (final concentration of 20 mM), and the mixture was added to pre-conditioned resin, and it was allowed to rotate in the cold room for an hour.

The resin and supernatant mixture containing 20 mM imidazole was poured in the column. The first wash was performed with 25 ml of TNS/20 mM imidazole buffer. The second wash contained 10 ml of TNS/40 mM imidazole buffer. Finally, the protein was eluted from the column with 10 ml TNS/100 mM imidazole buffer. Eluted fluorescent protein was dialysed with 10 mM MOPS, 100 mM KCl (pH 6.8).

#### *Performing the FRET assay*

Purified zinc sensor was incubated with different concentrations of zinc: 0  $\mu\text{M}$ , 50  $\mu\text{M}$ , 125  $\mu\text{M}$ , and 250  $\mu\text{M}$ . The samples were prepared in a buffer containing 100 mM KCl, 5 mM beta mercaptoethanol and 20 mM Tris (pH 7.5). Each sample contained 40  $\mu\text{L}$  of the purified protein and 125  $\mu\text{M}$  EDTA. 100  $\mu\text{L}$  of each sample was added to a 96-well plate, and the samples were analyzed by a fluorimeter courtesy of the Palmer lab. The excitation wavelength was set to 430 nm and the emission was measured from 450-600 nm.

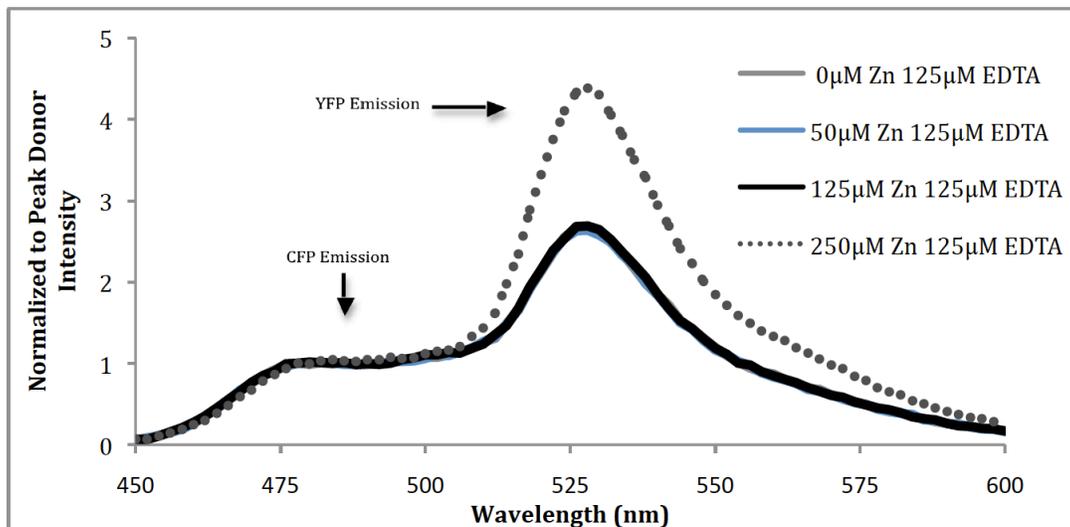
**Results:**

*There is an apparent change in FRET of the zinc sensor when zinc is present*

Our zinc sensor contained CFP and YFP separated by a region of NFATc2. We wanted to know if the fluorescence emission would change with different concentrations of zinc present in the sample. Thus, we tested samples with different zinc concentrations. Our excitation wavelength was 430 nm and the emission was measured at 450-600 nm. CFP typically has an emission peak at 480 nm and YFP has its emission peak at 545 nm. Sample containing no zinc was compared against samples containing different concentrations of zinc. As shown in Figure 3.2, significant change in FRET occurred with the sample containing 250  $\mu\text{M}$  of zinc. YFP emission was higher for this sample compared to YFP emission when there was no zinc present. We also found out that lower levels of zinc concentration such as 50  $\mu\text{M}$  and 125  $\mu\text{M}$  were not sufficient to observe significant change in FRET. In figure 3.2, the spectra for each sample was normalized to donor peak emission.

**Discussion:**

We saw a significant change in FRET with 250  $\mu\text{M}$  zinc. The change in FRET with higher zinc concentration could be due to the change in the conformation of NFATc2 peptide. When the distance and the orientation between two fluorescent proteins changes, there is a change in FRET. Our result suggested that 250  $\mu\text{M}$  of zinc concentration could be enough to significantly change the conformation of the NFATc2 peptide, resulting in the change in FRET. Our result was consistent with published values of zinc concentration that resulted in a significant change in FRET. For a canonical



**Figure 3.2:** Normalized emission intensity of CFP and YFP from 450 nm to 600 nm. The Fluorescent proteins were excited at 430 nm. Sample with zinc concentrations of 250 μM had the highest change in FRET

Cys<sub>2</sub>His<sub>2</sub> zinc finger, 180  $\mu$ M of zinc was able to produce a significant change in FRET (Dittmer *et al.*, 2009).

Our result with the NFATc2 zinc sensor might give us insight into the interaction that occurs with cJun and NFATc2 in the presence of zinc. We saw an enhanced interaction between NFATc2 and cJun when zinc was present. This might have to do with a change in the conformation of the C-terminal region of NFATc2 when it binds zinc. This new conformation might have higher affinity for cJun, resulting in positive interaction. In order to further test this model, we need to perform other experiments: performing the FRET assay with other divalent metal ions, and FRET assays with mutated amino acid residues in the putative zinc finger that is believed to be responsible for coordinating zinc.

## References

- Avni, O. & Rao, A. (2000).** T cell differentiation: a mechanistic view. *Curr Opin Immunol* **12**, 654–659.
- Borden, K. L. B. (1998).** RING fingers and B-boxes: zinc-binding protein-protein interaction domains. *Biochem Cell Biol* **76**, 351–358.
- Dittmer, P. J., Miranda, J. G., Gorski, J. A. & Palmer, A. E. (2009).** Genetically encoded sensors to elucidate spatial distribution of cellular zinc. *J Biol Chem* **284**, 16289.
- Erlanson, D. A., Chytil, M. & Verdine, G. L. (1996).** The leucine zipper domain controls the orientation of AP-1 in the NFATĚAP-1ĚDNA complex. *Chem Biol* **3**, 981–991.
- Hess, J., Angel, P. & Schorpp-Kistner, M. (2004).** AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* **117**, 5965.
- Hogan, P. G., Chen, L., Nardone, J. & Rao, A. (2003).** Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* **17**, 2205.
- Jain, J., Loh, C. & Rao, A. (1995).** Transcriptional regulation of the IL-2 gene. *Curr Opin Immunol* **7**, 333–342.
- Jain, J., Valge-Archer, V. E. & Rao, A. (1992).** Analysis of the AP-1 sites in the IL-2 promoter. *The Journal of Immunology* **148**, 1240.
- Karin, M., Liu, Z. & Zandi, E. (1997).** AP-1 function and regulation. *Curr Opin Cell Biol* **9**, 240–246.
- Kuroda, S., Tokunaga, C., Kiyohara, Y., Higuchi, O., Konishi, H., Mizuno, K., Gill, G. N. & Kikkawa, U. (1996).** Protein-protein interaction of zinc finger LIM domains with protein kinase C. *J Biol Chem* **271**, 31029.

**Laity, J. H., Lee, B. M. & Wright, P. E. (2001).** Zinc finger proteins: new insights into structural and functional diversity. *Trends in Biotechnology* **11**, 39–46.

**Macian, F. (2005).** NFAT proteins: key regulators of T-cell development and function. *Nature Reviews Immunology* **5**, 472–484.

**Müller, M. R. & Rao, A. (2010).** NFAT, immunity and cancer: a transcription factor comes of age. *Nature Reviews Immunology* **10**, 645–656.

**Nguyen, T. N., Kim, L. J., Walters, R. D., Drullinger, L. F., Lively, T. N., Kugel, J. F. & Goodrich, J. A. (2010).** The C-terminal region of human NFATc2 binds cJun to synergistically activate interleukin-2 transcription. *Molecular Immunology* **47**, 2314–2322.

**Palmer, A. E., Qin, Y., Park, J. G. & McCombs, J. E. (2011).** Design and application of genetically encoded biosensors. *Trends in Biotechnology*

**Tidow, H., Andreeva, A., Rutherford, T. J. & Fersht, A. R. (2009).** Solution structure of the U11-48K CHHC zinc-finger domain that specifically binds the 5'splice site of U12-type introns. *Structure* **17**, 294–302.