NUCLEAR TRANSLOCATION OF NFAT FAMILY PROTEINS IN HUMAN REPORTER CELL LINES

by
ABDALSALAM OMER KMAIL

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
June 2009

NUCLEAR TRANSLOCATION OF NFAT FAMILY PROTEINS IN HUMAN REPORTER CELL LINES

APPROVED BY:

Assoc. Prof. Dr. Batu Erman .....
(Dissertation Supervisor)

Assist. Prof. Dr. Alpay Taralp .....

Assoc. Prof. Dr. Hikmet Budak .....

Assoc. Prof. Dr. Levent Ozturk .....

Assoc. Prof. Dr. Ugur Sezerman .....

DATE OF APPROVAL: 11.06.2009

ABSTRACT

Nuclear factor of activated T-cells (NFAT) proteins are transcription factors evolutionary related to the NFκB/Rel family. The NFAT family consists of five proteins and several isoforms that regulate the expression of various immune system genes. The mRNAs that encode NFAT proteins, which were first thought to be T-cell-specific, vary in distribution between different cells and tissues. In dormant cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon phosphorylation by a signal activated calcineurin phosphatase, NFATs are imported to the nucleus. NFAT proteins are exported back out of the nucleus by NFAT kinases such as CK1 and GSK3. This study aimed to investigate the domains necessary for the subcellular translocation of NFAT transcription factor proteins upon PMA/ionomycin treatment. The calcium phosphate transient transfection method was used to express different NFAT family members in mammalian cells. We found that ionomycin treatment results in the nuclear translocation of NFAT2 while NFAT3 does not follow this rule. We generated reporter human embryonic kidney 293 cell lines that express green fluorescent protein (GFP) upon NFAT nuclear translocation. Fluorescent microscopy and flow cytometry experiments were performed and showed that the nuclear localization of NFAT2 begins within 15 minutes after PMA/ionomycin stimulation, with most of the proteins entering the nucleus after two hours. We identified a domain in the NFAT2 protein called the regulatory domain which was important for translocation. Protein-protein interactions likely play a crucial role in NFAT nuclear import. Future experiments using biochemical techniques may help to bring these proteins to light.

ÖZET

Aktive edilmiş T hücrelerinin çekirdek faktörü (NFAT) proteinleri, evrimsel açıdan NFκB/Rel familyasıyla bağlantılı yazılma faktörleridir. NFAT protein ailesi, beş proteinden ve çeşitli bağışıklık sistemi genlerinin ifadesini düzenleyen bazı izoformlardan oluşur. NFAT proteinlerini kodlayan ve önceleri T hücresine özgü olduğu düşünülen mRNA'lar, çeşitli hücreler ve dokular arasında dağılım farkları gösterir. Atıl hücrelerde NFAT proteinleri yüksek oranda fosforilaze olmuştur ve sitoplazmada yer alır. Sinyalle aktive edilen kalsinürin fosfatazı sonucunda defosforalize olan NFAT'lar çekirdeğe taşınır. NFAT proteinleri, CK1 ve GSK3 gibi NFAT kinazları yoluyla tekrar hücreden çıkar. Bu çalışma, NFAT yazılma faktörü proteinlerinin PMA/iyonomisin uygulaması sonucunda hücre altı yer değişimi için gerekli bölgeleri araştırmayı hedeflemektedir. Memeli hücrelerinde farklı NFAT protein ailesi bireylerini ifade etmek için kalsiyum fosfat geçici transfeksiyon yöntemini kullandık. İyonomisin uygulaması NFAT2'nin çekirdeksel yer değişimini sağlarken, NFAT3'ün bu kurala uymadığını belirledik. NFAT'ın çekirdeksel yer değişimi sonucunda yeşil flüoresan protein (GFP) sentezleyen insan embriyosu böbrek hücre dizileri oluşturduk (HEK293). Yaptığımız flüoresan mikroskopi ve akım sitometre deneylerinde, NFAT2'nin çekirdeksel lokalizasyonunun PMA/iyonomisin uyarısından sonraki 15 dakika içinde başladığını ve proteinlerin çoğunluğunun çekirdeğe iki saat sonunda girdiğini gösterdik. NFAT2 proteininde, çekirdeğe taşınması için önemli olan ve düzenleyici bölge adı verilen bir bölge belirledik. Protein-protein etkileşiminin, NFAT çekirdeğe taşınmasında kritik bir rol oynaması muhtemeldir. İleride biyokimyasal yöntemler kullanılarak yapılacak deneyler, bu proteinlerin aydınlatılmasına yardımcı olacaktır.

DEDICATION

This thesis is dedicated to those I lost during my study; my father Omer Alowny; my brother Osamah, whom I lost in 2002. To whom still settling in my mind all the time I pray to their souls and dedicate them my work and success. This thesis is dedicated to my family who suffered from my absence, my kind mother, my loving wife, and my sweet son who I left when he was 55 days old. For my brothers and sisters, each by his name, who offered me unconditional love and support throughout the course of this thesis. For each of them I extend my deepest appreciation and dedication.

Also, this thesis is dedicated to the people in my home country who have special presence in my mind and gave me their support during my study; Jamal Abu Al-Rub, Ayman Alowny, Abdalkader Alowny, Jamal Sabany, Jamal Hanaysheh, Bassam Zakarny, Mohammad Hilmi, and Hasan Abu Al-Rub. Finally, this thesis is dedicated to all those who believe in the richness of learning.

ACKNOWLEDGMENTS

From the formative stages of this thesis, to the final draft, I owe an immense debt of gratitude to my supervisor Prof. Dr. Batu Erman for his guidance, teaching and insight throughout the research; I express him a sincere appreciation for his kind heart and patience. I would also like to thank the members of my thesis committee Prof. Dr. Alpay Taralp, Prof. Dr. Hikmet Budak, Prof. Dr. Levent Ozturk, and Prof. Dr. Ugur Sezerman, without your time and cooperation; this project would not have been possible.

Special thanks go to Prof. Dr. Bashar Saad for his following and help during the course of research and spending his valuable time. Full Appreciation goes to Prof. Dr. Zehra Sayers for her sound advice and encouragement. Dr. Ceren Tuncer, Serkan Belkaya, and Safak Isil Nalbant, I make them all my respect for their efforts and assistance in the experimental techniques. I express my thanks and appreciation to my family for their understanding, motivation and patience. Lastly, but in no sense the least, I am thankful to all colleagues; Gamze Gunal, Ilcim Ozlu, Dr. Belkis Atasever, and Nazli Keskin. Also to my friends; Iyad hashlamon, Moamin Abughazala, Mohammed Samara, Amer Faiz, Ahmed Alghareeb, Momen Yasin, and Aziz Tomi who made my stay at the university a memorable and valuable experience.

The foundation of my study was fully covered by Erasmus Mundus ECW (Lot 2) team in Brussels. It is not enough to thank them for giving me the opportunity to do a master. I am thankful to the European Union who covers the whole project.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
γ	Gamma
Bp	Base Pair
Ca^{+}_{2}	Calcium Ion
CIAP	Calf Intestinal Alkaline Phosphatase
CRAC	Ca^{+}_{2} Release-Activated Ca^{+}_{2} Channels
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ER	Endoplasmic Reticulum
EtOH	Ethyl Alcohol
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
HEK293	Human Embryonic Kidney 293 Fibroblast Cell Line
IFN	Interferon
IL	Interleukin
INF- γ	Interferon- Gamma
IP3	Inositol 1,4,5-triphosphate
ITAM	Immuno-Tyrosine Activation Motif
μg	Microgram
μg	Milligram
μl	Microliter
μl	Milliliter

NFAT	Nuclear Factor of Activated T-Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI-3	Phosphatidylinositol Triphosphate
PIP2	Phosphoinositol 4,5-biphosphate
PLC	Phospholipase C
rpm	Revolution Per Minute
TBE	Tris Borate EDTA
TCR	T Cell Receptor

1 INTRODUCTION

Cell growth, differentiation, regeneration, and apoptosis are regulated through the microenvironment. The microenvironment consists of various factors such as, cell-cell interactions, cell-substrate interactions, and soluble factors, such as hormones and cytokines. Cytokines are regulators of host responses to infections, immune responses, inflammation, and trauma. Inflammation is the first response of the immune system to infection or irritation. The two types of cytokines are regulated by pro-inflammatory and anti-inflammatory cytokines. Inhibitors of the pro-inflammatory cytokines have been considered as candidates for anti-inflammatory drugs [1]. Inflammatory responses are advantageous for eradicating bacteria, as long as the inflammatory response can stay under control under control when out of control. However, deregulated inflammation leads to the massive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) by macrophages which can cause tissue injury and multiple organ failure. The inflammatory process is controlled by immunosuppressant cytokines such as IL-10 and IL-4.

Many cytokines, such as IL-2, IL-4, granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α, CD40, and CD95 ligands are regulated by nuclear factor of activated T cells (NFAT) family proteins [2]. This protein family has five members which are listed in Table 1.1, namely, NFATc1/NF-ATC, also called NFATc or NFAT2, NFATc2/NF-ATP, also called NFATp, NFAT1, NFATc3/NFAT4, also called NFATX, NFATc4/NFAT3, also called NF-ATc4 or NFATC4, and NFATc5/TONEBP, also called KIAA0827, NFATL1, OREBP, NFATZ or NF-AT5 [1, 3, 4, 5, 6].

Each of NFAT family members can have several isoforms resulting from alternative RNA splicing. NFAT family proteins have the following characteristics:

1. They are regulated by Ca/calcineurin-dependent signaling such as TCR signal transduction pathway. NFAT family transcription factors were first characterized in T-lymphocytes and have an important role in coordinating the transcription of different genes during the immune response. Some of these transcription factor proteins play an important role in other cell types and tissues of the body [7].

2. They are highly phosphorylated transcription factors that reside in the cytoplasm of unsignaled dormant cells. The nuclear import of NFAT requires the calcium/calmodulin-dependent serine phosphatase called calcineurin. This enzyme is activated through increasing cytoplasmic calcium concentration [8].

3. Dephosphorylation of 13 serine residues found in the amino terminal domain of NFAT proteins leads to their nuclear translocation. Variation in the phosphorylated sites among different NFAT proteins suggests a possible mechanism for their differential control in different tissues. Although several kinases have been identified, there is no integrated picture of NFAT kinases [9].

4. NFAT proteins can form heterodimeric complexes with bZIP transcription factors called AP-1. NFAT proteins can form complexes with other factors such as, GATA, MEF2 transcription factors and histone deacetylases (HDACs). The association of NFAT proteins with other transcription factors determines the transcription activation function of this transcription factor [10].

1.1 Cell communication

Cells have membrane receptors that receive signals from the microenvironment, which mediate the intercellular cascade of protein activation. Extracellular signals, as illustrated in figure 1.1, must first be transmitted through the plasma membrane after binding to specific receptors and converting into biochemical forms in the interior part of the cell leading to the signal transduction cascades which control cellular processes such as, proliferation, differentiation, and immune responses. Cells have developed sophisticated and elegant methods for achieving sensitive yet controllable receptor-mediated responses. Signals transmitted through complicated pathways start from the cell membrane and cytoplasm and go to genome in the nucleus. Chemical substances, such as hormones, neurotransmitters and a variety of pharmaceuticals, physical and other environmental signals induce conformational changes in the cell membrane receptors leading to the activation of intracellular signaling pathways. These networks are not simply passive relay systems, but actively modulate the transmitted signals [11, 12, 13, 14].

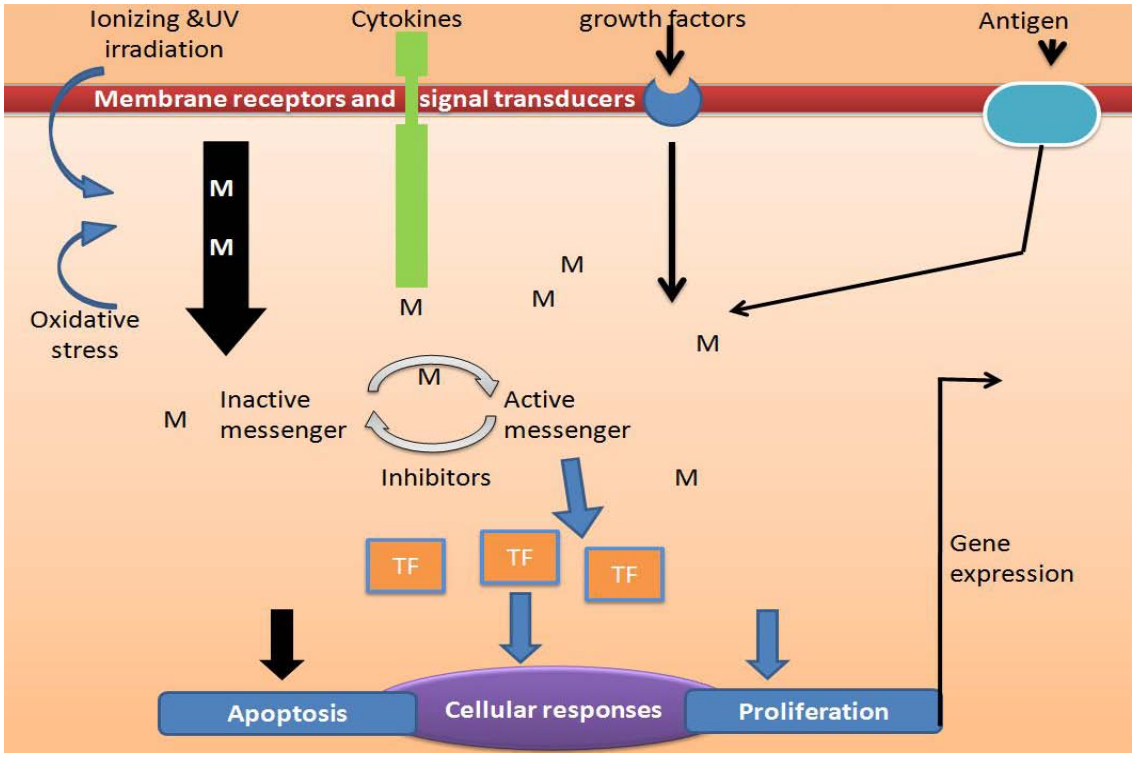


Figure 1.1: Cell communication

Signaling systems that are activated in cells respond either to external stimuli or to internal stimuli. External stimuli activate membrane receptors found on the cell membrane on the cell-surface that are coupled to membrane receptors or transducers, relay information into the cell using different second messengers (M) and activating transcription factors (TF). Various cellular responses such as proliferation or apoptosis are controlled by signals generated at the cell surface.

1.2 Signal transduction

1.2.1 Signal transduction pathways

T-lymphocyte development and function is regulated by signal transduction pathways mediated by cell-surface receptors, intracellular signaling molecules, and transcription factors. Lymphocyte signal transduction results in the production of cytokines and chemokines that are responsible for cell proliferation, development, and immune system activation [15, 16].

1.2.2 Calcium signaling

Intracellular calcium ions (Ca^{+}_{2}) which influxes from release activated Ca^{+}_{2} channels (CRAC), serve functions such as muscle contraction, synaptic transmission, and neuroendocrine secretion. Sustained $Ca+_{2}$ influxes induce the nuclear translocation and transcriptional activation of NFAT transcription factors [17, 18, 19].

Two storage sources for calcium Ca^{+}_{2} are used by cells in the pathway of signal transduction. The first Ca^{+}_{2} sources are from the extracellular fluid, while the second is internally generated from stores within the membrane systems of the endoplasmic and sarcoplasmic reticulum. The release of Ca^{+}_{2} from internal stores is controlled by various channels such as the inositol-1,4,5-triphosphate receptor (InsP3R) and ryanodine receptor (RYR) [20, 21]. This signaling pathway leads to the control of several cellular processes, such as cell growth, fertilization, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signaling [22, 23].

1.2.3 NFAT signaling

1.2.3.1 Importance of NFAT signaling

The NFAT signaling pathway is important in inducing the expression of transcription factors, signaling proteins, cell surface receptors, cytokines, and other effector proteins [24, 25, 26]. Cell surface receptor binding to an antigen presenting cell activates the signal transduction pathways required for T-lymphocyte development and function. TCR activation plays a role in immunologic stimulation for mature and peripheral T-cells [6]. NFAT is one of the most important transcription factors activated upon TCR engagement, along with NFAT and AP-1 [27].

NFAT has an important role in the transcription of cell membrane molecules such as FasL and CD40L [28]. In mast cells, both Ras and Rac-1 GTPases have important

regulation functions in NFAT transcription [29]. Ras have a role in NFAT3 translocation and transcriptional activity [30].

1.2.3.2 NFAT signaling pathway

Figure 1.2 illustrates the NFAT signal transduction pathway. The NFAT signaling in T-lymphocytes begins when the T-cell receptor (TCR) and the peptides presented by major histocompatibility complex molecules (MHC) which interact in an adhesive junction between the T-cell and antigen-presenting cell (APC), together with the ligation of additional surface molecules, called co-stimulatory receptors. Ligand binding results is in the activation of Immuno-Tyrosine Activation Motifs (ITAMs) which have tyrosine residues that are specific for receptor-associated protein tyrosine Src family kinases. The critical Src family kinase for TCR signal initiation is Lck. Lck recruitment to the TCR results in the phosphorylation of tyrosine residues in the ITAM regions of the cytoplasmic tails of the CD3 subunits of the TCR. Binding of Syk and ZAP-70 kinases to phosphorylated ITAMs is the next step in the signaling chain; these proteins phosphorylate adaptors such as linker for activation of T-cells (LAT) proteins resulting in the activation of phospholipase C-γ (PLC-γ). Phospho-inositol-biphosphate (PIP2) is then degraded into inositol triphosphate (IP3) and diacyl glycerol (DAG) by the help of PLC-γ activation. Generation of high concentrations of cytoplasmic IP3 results in the opening of endoplasmic reticulum resident IP3 regulated calcium channels, forcing $Ca+_2$ into the cytosol [31]. $Ca+_2$ are associates with calmudulin which form a complex that can bind to and activate the phosphatase calcineurin. When the calcineurin is activated, it begins to dephosphorylate NFAT serines reside in, at SP-repeats and the serine-rich region, causing accessibility of nuclear localization sequences and NFAT translocation to the nucleus. The translocation of NFAT allows it to reach its final destination by binding DNA in a sequence-specific fashion to energize the expression of a host of gene products involved in T-cell activation [32].

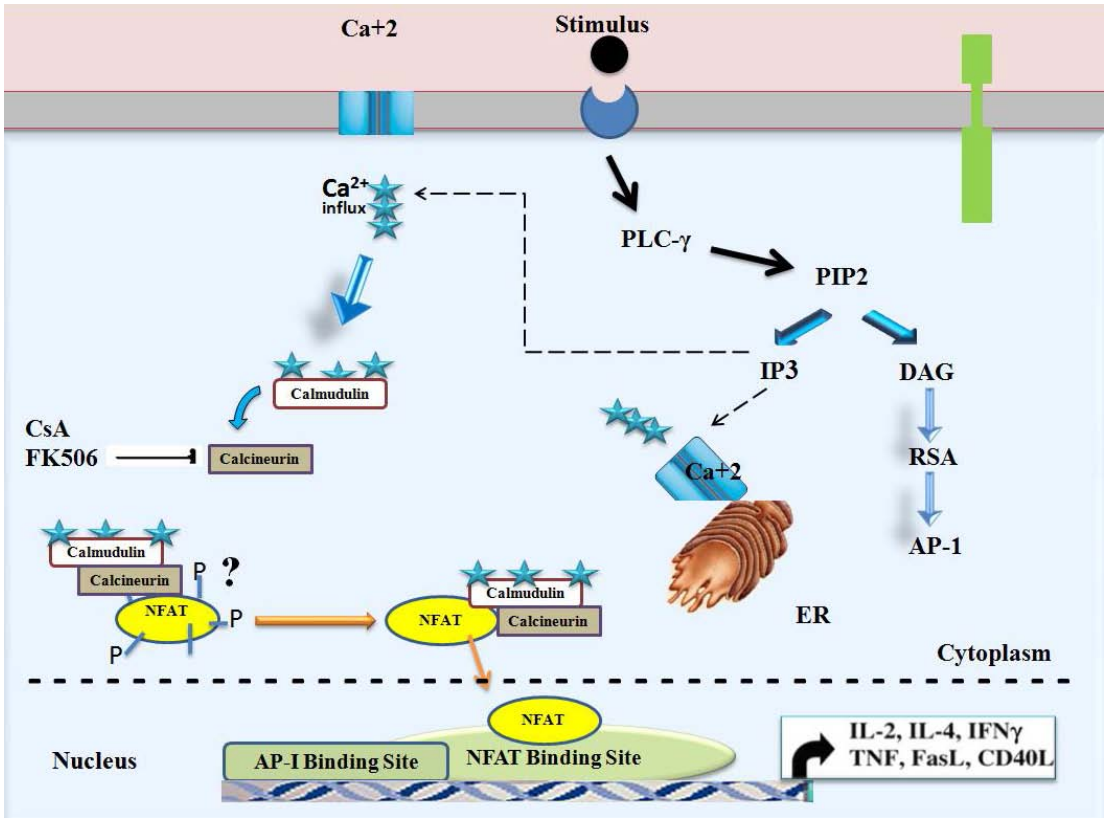


Figure 1.2: NFAT signal transduction pathway

Signals starting from the cell membrane activate various signaling proteins resulting in NFAT dependent gene transcription. Ca^{+}_{2}: Calcium ion. PLC-γ: Phospholipase C-gamma. PIP2: Phosphoinositol biphosphate. IP3: Inositol triphosphate. Ras: Small GTP-binding proteins encoded by ras genes. AP-1: Activating Protein-1. CsA: cyclosporine A. NFAT: Nuclear factor of activated T-cell.

1.3 NFAT transcription factor

NFAT was first discovered as a T-cell-specific factor that mediates the rapid induction of interleukin-2 (IL-2) gene expression upon T-cell activation. Evolutionarily, NFAT members (Table 1.1) are related to NFκB/Rel family of transcription factors [33]. NFAT genes of different family members are located on separate chromosomes and are expressed in various cell types [10]. While initial studies focused on the T lymphocyte activity of these transcription factors, NFAT target genes are also involved in the regulation of apoptosis and differentiation in non-immune cell types [34].

1.3.1 NFAT members

1.3.1.1 NFAT1

NFAT1 which was initially named NFATp and was first purified by McCaffrey et al. in 1993 from murine (Ar-5) and human (Jurkat) T-cell cDNA libraries. It binds to the NFAT site of the murine IL-2 promoter when translocated to the nucleus, resulting in the initiation of the transcription of IL-2 in cooperation with c-Fos and c-Jun, the two proteins that make up the AP-1 transcription factor, which results in the activation of the immune response [3]. In addition, it has been reported that NFAT1 has an important role in the growth of skeletal muscles [32]. It is also crucial in positive selection and survival of thymocytes in the developing immune system [35]. It has been found recently that NFAT1 knockout results in osteoarthritis through dysfunction of adult articular chondrocytes [36].

1.3.1.2 NFAT2

NFAT2 was cloned from a Jurkat T-cell cDNA library, isolated in 1994 by the group of Northrop and named NF-ATc [4]. It regulates immune responses by activating the IL-2 promoter in non T-lymphocytes; hence, it is crucial for IL-2 gene expression. In order to mediate transcription of IL-2, NFAT2 proteins physically bind to AP-1 proteins. NFAT2-AP1 complexes can also activate the calcium-dependent transcription of other cytokine genes in different immune system cell types [37]. It has been shown that NFAT2 knockout causes a partial block at the double-negative stage 3. NFAT2 deletion also causes a partial block in positive selection in T-lymphocytes [38].

1.3.1.3 NFAT3

NFAT3 was identified by Hoey et al. in the year (1995) [5]. NFAT3 is the only transcription factor from the NFAT family members that is not present in immune cells. It is produced mostly by cardiac muscles and neuronal tissues, whereas most other NFAT family members are produced in immune cells and skeletal muscle [34]. It has been found that angiotensin-II (ANG-II) plays an inhibiting role for NFAT3 in response to antioxidant enzyme catalase and the antioxidants N-acetyl-L-cysteine, phenyl-N-tert-butylnitrone, and lipoic acid [39]. Consistent with its expression profile, NFAT3 knockout mice have been shown to be viable and fertile with defects in axon outgrowth [40]. NFAT3 was recently shown to accumulate in the nucleus in granule neurons of the developing cerebellum mediating the survival of these neurons, in pulmonary vascular smooth muscle cells upon intracellular calcium concentration increase, in electrically paced cardiomyocytes, studied as an in vitro model for cardiac hypertrophy [41, 42, 43]. NFAT3 driven transcription has an important role in the survival of neurons. In cerebellar granule neurons of developing mice brain, inhibition of NFAT leads to cell apoptosis [44]. Consistent with its expression pattern and in addition to its role in heart muscle cells, NFAT3 was found to regulate smooth muscle physiological activity [45]. Ichida et al. identified Ras as an activator of NFAT3 functioning upstream of calcineurin [46].

Recent studies have found that there is a close relationship and physical interaction between NFAT3 and the estrogen receptor (ER), both *in vitro* and *in vivo*. ER protein levels are elevated in some forms of breast cancer. NFAT3 is thought to cooperate with and enhance the binding of ER to ERE sequences and thus play a role in the development of breast cancer. In addition, NFAT3 plays a role in the expression of ER proteins itself by regulating the expression of this gene [47]. The activation of ER proteins by NFAT3 seems to be highly tissue specific because NFAT3 acts as a corepressor in kidney cells decreases the transcriptional activity of ER proteins [48].

1.3.1.4 NFAT4

NFAT4 was identified by Masuda et al. in the year (1995) [7]. It was later shown that NFAT4 protein can activate the IL-2 promoter in T-cells and is produced in high levels in the thymus [5, 6,]. In thymocyte development, NFAT4 is expressed in double positive thymocytes at much higher levels than the other family members [49]. NFAT4 is very important for positive selection of T lymphocytes, in the case of its absence, T lymphocyte development is blocked and immunodeficient animals that have very few mature, functional T lymphocytes are generated [35]. NFAT4 knockout mice develop massive lymphadenopathy and splenomegaly [2].

1.3.1.5 NFAT5

The fifth member of NFAT family transcription factors, NFAT5, was discovered much later by both Lopez-Rodriguez et al. and Jauliac et al., separately, in 1999 and 2002 respectively [28, 50]. NFAT5 was found to be more abundant in kidney than in the thymus and stomach. NFAT5 is unique in not responding to Ca/calcineurin signaling, but to osmotic stress, because it does not contain calcineurin binding sites in its regulatory domain and its dephosphorylation and transcription activity is initiated when cells respond to hypertonic stress [10]. This member of the NFAT family transcription factors is not activated by cytoplasmic Ca^{+}_{2} elevations, and so called tonicity-responsive transcription factor [9]. NFAT5 proteins are also unique because they bind to the DNA in the nucleus as homo-dimers, whereas all other family members are heterodimerize with AP-1 transcription factors [50]. NFAT5 transcription factor is important for cell proliferation and under hypertonic conditions; it induces an osmo-adaptive response that enhance cells to express fundamental regulators needed for cell cycle progression. Deletion of NFAT5 found to cause reduction of proliferation capacity and viability of lymphocytes and renal medullary cells under hypertonic stress [51].

Table 1.1: NFAT members in human

No.	**Approved Symbol**	**Aliases**	**Official Full Name**	**Gene Type**	**Chromosome**	**Source**	**Discovered by**	**Year**
1	**NFATC1**	NF-ATC, NFATc, NFAT2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	gene with protein product	18q23	Homo sapiens (human)	Northrop et al.	1994
2	**NFATC2**	NF-ATP, NFATp, NFAT1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	gene with protein product	20q13.2	Homo sapiens (human)	McCaffrey et al.	1993
3	**NFATC3**	NFAT4, NFATX	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	gene with protein product	16q22	Homo sapiens (human)	Hoey et al., Masuda et al. And Ho et al.	1995
4	**NFATC4**	NFAT3; NF-ATc4; NFATC4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	protein coding	14q11.2	Homo sapiens (human)	Hoey et al.	1995
5	**NFAT5**	TONEBP, KIAA0827, NFATL1, OREBP, NFATZ, NF-AT5	nuclear factor of activated T-cells 5, tonicity-responsive	gene with protein product	16q22.1	Homo sapiens (human)	Lopez-Rodriguez et al. And Jauliac et al.	1999 and 2002

1.3.2 NFAT expression

Although the NFAT name derived is from T-lymphocytes, NFAT proteins are expressed in different tissues and at different levels [4, 5, 6, 7, 52].

- NFAT1 is produced in peripheral T-cells, B-cells, mast cells, NK cells, monocytes, neuronal cell lines, endothelial cell lines, and peripheral lymphoid tissue.
- NFAT2 is found in peripheral T-cells, peripheral lymphoid tissue, activated T-cells and NK cells.
- NFAT3 is expressed in heart muscle, neurons, and in lymphoid tissue at low levels.
- NFAT4 is expressed at high levels in the thymus.

1.4 NFAT protein

Two conserved domains build up NFAT protein; the first domain is responsible for translocation to the nucleus upon Ca/calcineurin dephosphorylation [2]. The second domain, which is conserved between different NFAT family members, is the DNA binding domain (DBD) is responsible for DNA recognition in the promoters of different cytokines. NFAT proteins, which reside in the cytoplasm, are highly phosphorylated in resting cells with low affinity to bind DNA, and they became phosphorylated upon calcineurin activation. Ultimately, the NFAT protein complex translocates to the nucleus and binds to DNA activating the transcription of downstream genes. The activation and DBD domains of NFAT are required for the transcription activation function of this protein [1, 7, 8, 53, 54].

Due to alternative splicing, NFAT1, NFAT2, NFAT3 and NFAT4 proteins are found in several isoforms [1]. Human and mouse NFAT mRNAs were identified by

analyzing genomic, mRNA, and EST databases using bioinformatics tools. The analysis showed that the number of human isoforms of NFAT1, NFAT2, NFAT3, NFAT4 proteins were 3, 4, 8, and 8 isoforms respectively. While mouse screening showed that the number of isoforms of NFAT1, NFAT2, NFAT3, and NFAT4 proteins were 5, 4, 3 and 4 isoforms respectively. All mouse and human NFAT protein isoforms were more conserved in their regulatory and DNA binding domains but were variable in their N- and C-terminal domains [55].

1.4.1 Domains of NFAT proteins

NFAT proteins have four major domains: N-terminal domain (NTD), regulatory domain (RD), DNA-binding domain (DBD), and C-terminal domain (CTD) (Figure 1.3). The NTD of NFAT proteins have SP-repeats and a serine-rich region which contain serine residues that are serines dephosphorylated upon calcineurin activation [56]. The DBD is distantly related to the NF-κB DBD and is highly conserved among NFAT proteins. The DBD region also includes binding sites for other transcription factors such as AP-1 and CREB binding protein.

The RD is the critical domain for NFAT activation. This entire domain contains conserved sequences, such as calcineurin binding sites, phosphorylation sites, nuclear localization and export signals. Phosphorylated serine-rich regions are substrates for calcineurin SRR-1, the site adjacent to the calcineurin docking site it is preferentially dephosphorylated by calcineurin. The N-terminal regulatory region and the C-terminal DNA binding domain (Rel homology Region) of all members of the NFAT family except NFAT5 are highly conserved. The NTD and CTD domains are highly diverse among NFAT proteins, suggesting that the functional differences between different NFAT family members arises not by differential DNA binding but by post-translational modifications and protein-protein interactions [33, 47, 57, 58].

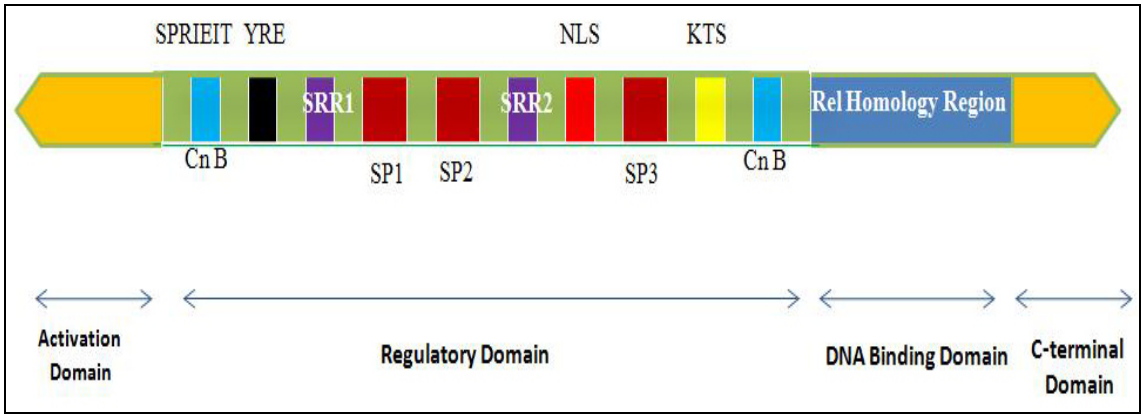


Figure 1.3: The structure of murine NFAT1 protein

The diagram shows the main domains of the NFAT protein. Orange region represents the N-terminal activation domain. Green region represents the regulatory domain which contains phosphorylation sites in conserved sequence motifs shown in brown. Violet colored motifs represent SRR-1, which is also a target site of phosphorylation, and SRR-2 region, which is not dephosphorylated by calcineurin. Light blue region represents the calcineurin contracts. The left region is the PxIxIT sequence found in all NFAT proteins, whereas the right region is found only in NFAT2 and in NFAT4. The red region in the C-terminus of the regulatory domain is the nuclear localization signal (NLS). The yellow KTS motif contains residues that are phosphorylated in the regulatory domain. The dark blue domain is the conserved Rel homology region which is responsible for DNA binding. The orange domain on the right is the C-terminal domain which has no known function [33, 57].

1.4.2 Specific regions in the NFAT regulatory domain are responsible for translocation

Four serine rich regions (SRRs); SRR-1, SPxx repeat, SRR-2, and KTS located in the NFAT regulatory domain are conserved in all NFAT members [33]. The dephosphorylation of these conserved serine residues is a prerequisite for NFAT nuclear translocation. The number and context of the serines in the SRRs differ among different NFAT members.

For nuclear translocation; Ser172 is critical for NFAT2, Ser168 is critical for NFAT1, Ser163 and Ser165 are critical for NFAT4, while Ser168 and Ser170 are critical in the case of NFAT3. The remaining conserved Ser residues in the SRR motifs are important but not sufficient to cause NFAT translocation to the nucleus. The rephosphorylations of Ser168 and Ser170 of NFAT3 in the cytoplasm are mediated by

the tonic protein kinase mTOR, while rephosphorylation in the nucleus is mediated by NFAT kinases such as Erk5, Ck1, and Gsk3 [59]. These kinases are activated upon calcineurin inhibition or when calcium is depleted. The kinases of NFAT kinase activation can be as fast as 15 minutes after calcium depletion [33]. GSK3-beta inhibits NFAT3-dependent cardiac-specific gene expression by phosphorylating and causing ubiquitinylation dependent degradation. The function of NFAT kinases is to inhibit NFAT-dependent transcription, for example, in cardiac muscle cells and Gsk3-β inhibits NFAT3-dependent transcription [60].

1.4.3 Selective activation of NFAT family members

In tissues where more than one NFAT family member is expressed, how does an individual family member get activated? The answer to this question lies in the differences in the regulatory domains of different NFAT family members. NFAT2–4 all contains the SRR-1 region, while NFAT3 lack both SP-2 and SP-3 motifs. Kinases, such as MAP kinases, p38 and JNK can selectively phosphorylate NFAT proteins at SP motifs resulting in nuclear export. JNK is responsible for NFAT2 and NFAT4 phosphorylation, whereas p38 can phosphorylate both NFAT1 and NFAT3. Thus in the same cell type, the selectivity of export kinases is thought to regulate the activity of different NFAT proteins [33].

1.5 NFAT DNA binding site

NFAT family proteins consist of two domains: The Rel Similarity Domain (RSD), which is homologous to a Rel homology region (RHR) commonly found in Rel family proteins such as NF-κB p50 and p65 and is responsible for DNA binding. The second conserved NFAT domain is the NFAT homology domain (NHD) which is responsible for binding to NFAT interacting proteins. Both the RSD and the RHR are responsible for recognizing specific proteins such as AP-1 complex which binds to RSD, IκB, and FOXP3 which bind to RHR [23]. The importance of these binding partners stems from the

requirement of these proteins for the transcription activation function of NFAT proteins [61]. For example, the IL-2 gene promoter has five sites for NFAT protein binding sites; four of which are associated with Fos and Jun (AP-1) binding. Thus it can be said that NFAT is a transcription factor that depends on combinational interactions with other proteins to activate transcription [62].

NFAT binding sites can be found in the promoters of several genes. It has the consensus sequence (A/T)GGAAA(A/N)(A/T/C)N [34], and has been found on both human and mouse antigen receptor response element-2 (ARRE-2) sites which NFAT binds to in response to TCR stimulation of T lymphocytes [63, 64]. NFAT and AP-1 bind to separate but adjacent sites that make up the ARRE2. The NFAT consensus is very similar in sequence to NFκB binding sites. This fact can be explained b y the similarity of the sequences of the DNA binding domains of these proteins [37].

Cytokine gene expression, which is regulated by NFAT members in T-cells, is controlled by cis-acting elements present in the promoters of cytokine genes [7]. For example, the ARRE-2 located in the IL-2 gene promoter where NFAT binds are well conserved in sequence and location among different species and therefore demonstrate the importance of NFAT in controlling this cytokine genes transcription and regulation [65, 66]. EBS1 and EBS2 are the two regions in the IL-2 promoter that are responsible for ets family transcription factors which bind to NFAT and contribute to IL-2 gene regulation [67]. IL-2 participates in the activation of B-cells, natural killer cells, cytolytic T-cells, and lymphokine-activated killer cells [68]. In proximal promoters of some Interleukin genes such as IL-4; multiple NFAT sites have been found that cooperatively bind NFAT and other transcription factors to mediate the activation of these interleukins [61].

1.6 NFAT expression

The mRNAs that encode NFAT proteins vary in distribution between different cells and tissues, and also vary within the same cell when in an activated versus cell resting state [2]. Different tissues differ in expression and activity for each member of the

NFAT family. NFAT2 transcriptional activity is abundant in peripheral blood lymphocytes, the thymus, spleen, placenta and muscle. NFAT3 is expressed mostly in the placenta, lung, kidney, testis and ovaries. Skeletal muscle tissues mostly express the NFAT1 gene, while NFAT4 expression is found in the thymus and muscles [69, 70].

1.7 NFAT functions

1.7.1 Diverse functions of NFAT transcription factors

NFAT activation induces the expression of different immune system genes such as IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and TNF-γ. In addition NFAT has an important role in the transcription of cell membrane immunoregulatory molecules of T-cells such as CD5, CD25, CD40L, FasL, and cytotoxic T-lymphocyte antigen-4 (CTLA-4). NFAT proteins have also been found to have crucial roles in B-cells and mast cells in the immune system [24, 32, 33, 34].

1.7.2 Dual function of NFAT

The NFAT transcription factor itself can act in a dual function as a T-cell activator or a transcriptional repressor depending on the partner it associates with. Transcriptional partners such as AP-1 mediated activation whereas Foxp3-associated NFAT may cause transcription inhibition. NFAT binding to AP-1, myocyte enhancer factor-2 (MEF2), or GATA transcription factors activates a set of genes that leads to lymphocyte activation. On the other hand, NFAT can bind to silencing complexes such as histone de-acelylases (HDAC) to repress the transcription of specific gene loci like the cdk4 gene promoter [34, 32].

1.8 Calcineurin

The protein phosphatase calcineurin is a serine threonine-specific protein that resides in the cytosol. It is conserved in all eukaryotes (including the brewer's yeast S. cerevisiae) and is unique among all phosphatases by responding to Ca^{+}_{2} through its activation by calmodulin [71]. Calcineurin activates NFAT proteins by dephosphorylation in participation with the Ca/calmodulin complex. Drug inhibitors such as cyclosporine, pimecrolimus and tacrolimus that inhibit calcineurin have been used effectively as immunosuppressors. Calcineurin can be activated by tyrosine kinase receptors, immune system cell receptors, and Ca^{+}_{2} dependent processes in the brain [72]. Cells must sustain elevated cytoplasmic Ca^{+}_{2} levels for continued activation of calcineurin and NFAT1-4 activation [10]. It has been found that Polycystin-1 (PC1) signaling can positively affect sustained activation of intracellular Ca^{+}_{2} levels, and this can activate NFATs [31]. Vascular endothelial growth factor (VEGF) can also activate heart muscle Ca^{+}_{2} levels to activate NFATs during heart development and heart valve formation [73].

The N-terminus of the regulatory domain of all NFAT members contains a PXIXIT motif, which is the most important docking site for calcineurin. This site is responsible for efficient NFAT dephosphorylation; it is thought that all upstream signals are delivered to NFAT when calcineurin binds this. Thus, this site is a good target for the development of immunosuppressive drugs [33, 47].

1.9 Transcriptional inhibition of NFAT

The fungal metabolites: cyclosporine A (CsA) and FK506, are immunosuppressive drugs that completely block signal transduction of the NFAT. These drugs competitively inhibit calcineurin's activity on NFAT pathway and thus, they interrupt the Ca^{+}_{2} dependent T-cell signal transduction pathways. CsA and FK506 inhibitors were first used as immunosuppressors in the field of organ transplantation starting in 1983 and 1989 respectively. CsA binds specifically to cyclophilin, while FK506 interacts with FKBP receptors inside the cell [74]. Both FK506 and CsA inhibitors prevent the binding of NFAT to DNA cause the relocalizaion of NFAT from nucleus to the cytoplasm [10].

1.9.1 Relationship between NFAT, AP-1, Foxp3, and Runx

NFAT2–AP-1 complexes formed at the promoter of the IL-2 gene can be inhibited by the forkhead/winged-helix transcriptional regulator (Foxp3), which is also known as forkhead box P3. Foxp3 has the same consensus binding site as the AP-1 site, which is located upstream and adjacent to the NFAT DNA binding site in this promoter leading to completive inhibition between Foxp3 and AP-1 [75, 76, 77].

The T-regulatory cells, which suppress immune responses, have Foxp3 teaming up with NFAT on the IL-2 gene promoter resulting in the inhibition of IL-2 gene transcription. On the other hand, in effector T-cells NFAT associates with AP-1 on the same promoter and results in Il-2 transcription activation. Thus the NFAT-Foxp3-DNA complex cooperatively inhibits the NFAT-Fos-Jun-DNA complex and represses NFAT-AP-1 transcriptional activity [78, 79].

The runt-related transcription factor (Runx) is another transcription factor which functions in gene silencing. Runx cooperates with both NFAT and Foxp3 in T-

regulatory cells. It binds DNA sequences other than where Foxp3 and AP-1 bind. The proteins, NFAT and Runx, associate in a sequence-specific manner with regulatory elements of the in target genes, repressing transcription [80].

1.10 NFAT and disease

Various viral infections affect NFAT signaling pathways. It has been found that NFAT nuclear translocation was inhibited in the early stages of a herpes simplex virus (HSV) infection. NFAT signaling is also inhibited during human immunodeficiency virus (HIV), hepatitis-C virus (HCV) and African swine fever infections, resulting in the repression of NFAT transcriptional activity. In the case of A238L African swine fever virus, the viral protein A238L binds to calcineurin blocking NFAT translocation and hence blocks transcription activation by NFAT [56]. On the other hand, the Kaposi sarcoma associated herpes virus and rhesus monkey rhadinovirus which infect B-cells, enhance NFAT transcriptional activity and thereby cause lympho-proliferative disorders [81].

1.11 Cells used in the study

In this study we used a human kidney cell line to examine the nuclear-cytoplasmic localization of NFAT proteins. The main cell types of the kidney are; parenchymal cells which have the essential function of the kidney, and the stroma which make up the connective supporting tissue. The human Embryonic Kidney 293 cell line (HEK293) was created by transforming normal healthy human fetus embryonic kidney cells with adenovirus-5 by Van der Eb et al (1977). HEK293 cells are characterized by fast growth and high transfection. HEK293 cells efficiency are commonly used for virology studies as packaging cell lines to produce retroviruses and are transfected to express recombinant proteins for studies in a number of research fields. Calcium transfection efficiency of HEK293 cells can reach up to 100% with the calcium phosphate

transfection method. HEK293 cells are sensitive to G418, making them amenable to stable transfection with plasmids containing neomycin resistance genes [82].

HEK293T cells are a sub-clone of the HEK293 cell line which expresses the SV40 large T-antigen protein. T-antigen protein mediates the episomal replication of plasmids which have the SV40 origin of replication. Plasmids containing the SV40 origin are replicated to a copy number of between 400-1000 plasmids per HEK293T cell, and therefore can be used to express proteins at high levels. HEK293T cells are resistant to G418 and therefore are not amenable to stable transfection unless antibiotic selection such as puromycin is used [82]. It is not known which combination of NFAT family members are expressed in HEK293 cells; however stimulation by para-methoxy-amphetamine (PMA) and ionomycin can mimic TCR signaling in these cells to activate NFAT driven reporter transcription [83].

2 PURPOSE OF THE STUDY

NFAT transcription factor activation induces the expression of signaling proteins, cell surface receptors, cytokines, and other effector proteins. NFAT proteins are transcription factors that belong to a family that contains 5 members (NFAT1-5), which are all expressed in immune cells except NFAT3. Although the NFAT (nuclear factor of activated T lymphocytes) name is derived from early studies with T-lymphocytes, these proteins are also expressed in different tissues at different levels. The chemical Ionomycin induces cytoplasmic calcium elevation activating the calcineurin phosphatase, which can directly dephosphorylate NFAT1-4. Dephosphorylated NFAT proteins can translocate into the cell nucleus in various cell types. We have found that ionomycin treatment causes different effects on the subcellular localization of two members of the NFAT family. Upon ionomycin treatment, NFAT2 proteins leave the cytoplasm and translocate into the nucleus while NFAT3 proteins do not. The main objective of this study was to gain an insight into the mechanisms controlling the subcellular localization of NFAT proteins (NFAT2 and NFAT3) using reporter cell lines. We identified the domain necessary for the movement of these NFAT proteins from the cytoplasm into the nucleus by examining the nuclear localization of different NFAT2/NFAT3 chimeric proteins in the human embryonic kidney HEK293T cell line. We also generated reporter cell lines, which originated from the HEK293 cell line, that express GFP reporter proteins upon nuclear translocation of NFAT proteins. We used this reporter cell line to determine the timing of NFAT nuclear translocation after induction with ionomycin.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The chemicals used in the study are listed below:

6X Loading Dye	Quality Biological Inc, Lithuania
Agarose	peQLab,UK
Ampicillin	Sigma, Germany
Boric Acid	Molekula,France
$CaCl_2$	Merck, Germany
DAPI	Invetrogen
DMEM	Sigma, Germany
DMSO	Sigma, Germany
EDTA	Riedel-de Haen,Germany
Ethanol	Riedel-de Haen,Germany
Ethidium Bromide	Merck, Germany
FBS	Biological Industries, Israel
G418 Sulfate	Celloro, USA
Glycerol	Riedel-de Haen,Germany
HEPES	AppliChem,Germany
HCl	Merck, Germany
Isopropanol	Riedel-de Haen,Germany
Kanamycin	Gibco, Germany
L-glutamine	Hyclone, Germany

Liquid Nitrogen	Karbogaz,Turkey
Luria agar	Difco, USA
Luria Broth	Difco, USA
$MgCl_2$	Sigma, Germany
Penicillin-Streptomycin	Biological Industries, Israel
PIPES	Sigma, Germany
KCl	Fluka,Switzerland
RPMI 1640	Biological Industries, Israel
NaCl	AppliChem,Germany
NaOH	Merck, Germany
Paraformaldehyde	Aldrich, USA
Tris Base	Amresco,USA
Trypan Blue	Sigma, Germany
Trypsin-EDTA	Biological Industries, Israel

3.1.2 Equipment

The equipments used in the study are listed below:

Autoclave	Hirayama,Hiclave HV-110,Japan
	Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP221S, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Cell Counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany
	Hitachi, Sorvall RC5C Plus, USA
CO_2 Incubator	Binder, Germany
Deepfreeze	-80 ^{0}C, Forma,Thermo ElectronCorp.,USA
	-20 ^{0}C,Bosch,Turkey
Distillator	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA

	Biorad Inc., USA
Filter Membranes	Millipore,USA
Flow Cytometer	BDFACSCanto,USA
Gel Documentation	UVITEC,UVIdocGel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Heater	Thermomixer Comfort,Eppendorf,Germany
Hematocytometer	Hausser Scientific, Blue Bell Pa.,USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Model 300, Germany
	Memmert, Model 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton,3000RS,USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microcentrifuge	Labnet International Inc., Korea
Microliter Pipettes	Gilson, Pipetman, France
	Eppendorf, Germany
Microscope	Olympus CK40,Japan
	Olympus CH20,Japan
	Olympus IX70,Japan
Microwave Oven	Bosch,Turkey
pH meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	Bosch,Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan
	Schimadzu, UV-3150, Japan
PCR Thermocycler	Eppendorf, Mastercycler Gradient, Germany
	Biorad, USA
Vortex	Velp Scientifica, Italy

3.1.3 Buffers and solutions

All Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in Sambrook et al., 2001 and listed below [84]:

- 10X Tris-Borate-EDTA (TBE) Buffer: 104 g tris base, 55 g boric acid and 40 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 L of distilled water.
- Agarose Gel: For 1% w/v agarose gel preparation, 1 g of agarose was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.
- Calcium Chloride ($CaCl_2$) Solution: used for competent cell preparation contained 60 μM $CaCl_2$ (diluted from 2M stock), 15% Glycerol,10mM PIPES (pH=7) and the solution prepared was autoclaved at 121 °C for 15 minutes and stored at 4 °C.
- Phosphate-Buffered Saline (PBS): 8 g NaCl, 0.2 g of KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 were dissolved in 800 ml distilled water. PH was adjusted to 7.4 by drop wise addition of concentrated HCl and the buffer was completed to 1 L with distilled water.
- 2X HEPES-Buffered Saline: 0.8 g NaCl, 0.027 g Na2HPO4.2H2O and 1.2 g HEPES were dissolved in 90 ml of distilled water. PH was adjusted to 7.05 with 0.5 M NaOH and the solution was completed to 100 ml with distilled water. The buffer was filter-sterilized.
- Trypan Blue Dye (0.4% w/v): 40 μg of trypan blue was dissolved in 10 ml PBS.

3.1.4 Growth media

Bacterial growth media: Luria Broth from Difco. was used to prepare the bacterial liquid culture. 20 g of LB broth were dissolved in 1 L of distilled water and autoclaved at 121 °C for 20 minutes. Kanamaycin 50 μg of a final concentration and

ampicillin 100 μg of a final concentration were added to the medium for selection purposes. LB Agar from Difco. was used to prepare solid medium plates for the growth of bacteria. 40 g of LB Agar was dissolved in 1 L distilled water and autoclaved at 121 °C for 20 minutes. Kanamaycin 50 μg of a final concentration and ampicillin 100 μg of a final concentration added to the medium at 55 °C for selection purposes. The medium then was poured into sterile Petri plates (~20 ml/plate) and left to cool to room temperature under sterile conditions.

Mammalian growth media: Human Embryonic Kidney (HEK293) cells and stable transfected cell lines originated from the HEK293 cell line. HEK293 cells are attached cells cultured in Dulbecco's Modified Eagle's (DMEM) medium containing 10% newborn calf serum, 2 mM L-Glutamine, 100 unit/ml of penicillin and 100 unit/ml of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The Human Embryonic Kidney (HEK293T) originated cell line, which are slightly attached cells, were cultured in RPMI 1640 medium containing 10% newborn calf serum, 2 mM L-Glutamine , 100 unit/ml penicillin and 100 unit/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

3.1.5 Freezing medium and glycerol stocks

The freezing medium was prepared by dissolving fetal bovine serum in DMSO (9:1 v/v) respectively and stored at 4 °C for further use. Bacterial cells were grown overnight (12-16 hours) at 37 °C with shaking at 270 rpm in Luria Broth. Glycerol was added to the cell suspension to a final concentration of 15%. Cells were frozen overnight in liquid nitrogen and then stored at -80 °C.

3.1.6 Molecular biology kits

- QIAGEN Plasmid Maxi Kit, QIAGEN,Germany
- QIAGEN Plasmid Midi Kit, QIAGEN,Germany
- Miniprep Kit, ROCHE,Switzerland
- Qiaquick Gel Extraction Kit, QIAGEN,Germany

3.1.7 Enzymes

Enzymes and corresponding reaction buffers of digestions used for cloning and other purposes are listed below:

Enzyme	Buffer	Fermentas
Bgl1	Orange (O)	Fermentas
CIAP	CIAP	Fermentas
EcoRI	Tango	Fermentas
Hind III	Red (R)	Fermentas
NcoI	Tango	Fermentas
T4 DNA Ligase	T4	Fermentas
Xho I	Red (R)	Fermentas
Xho I + EcoRI	Tango	Fermentas
Xho I + Hind III	Red (R)	Fermentas

3.1.8 Cells and plasmids

DH5α cells were used to create competent cells. These competent cells were used for plasmid transformation purposes. HEK293 kidney originated cell line was used to create stable transfected cell lines. HEK293T cell line and different stably transfected HEK293 originated cell lines were used for plasmid transient transfection.

The plasmids used in the study are listed below:

Plasmid	Selective marker	Purpose of use	Provided by
pCS2+MT-N1	Ampicillin	Transfection	Lab. constructed
pCS2+MT-N2	Ampicillin	Transfection	Lab. constructed
pCS2+MT-N3	Ampicillin	Transfection	Lab. constructed
pCS2+MT-N4	Ampicillin	Transfection	Lab. constructed
pEGFP-C3	Kanamycin	Transfection	Novagen
pEGFP-CP	Kanamycin	Transfection	Lab. constructed
pEGFP-N2	Kanamycin	Transfection	Lab. constructed
pEGFP-N3	Kanamycin	Transfection	Lab. constructed
pEGFP-NP	Kanamycin	Transfection	Lab. constructed
pEGFP-RP	Kanamycin	Transfection	Lab. constructed
pMEX-HA	Kanamycin	Cloning	Dualsystems
pPO4AT 2-5	Kanamycin	Transfection	Lab. constructed
PUC-19	Ampicillin	Transfection	GIBCO

3.1.9 Computer based programs

The software and online programs used are: AVID-VISTA, Flowjo, VectorNTI, BD FACSDiva Software.

3.2 Methods

3.2.1 Culture growth

Bacterial culture growth: The bacterial cells were grown at 37 °C overnight (12-16 hours) with shaking at 270 rpm in Luria Broth prior to any application. Depending on the application, selective antibiotics were added to the media. LB Agar was used to grow bacterial cultures on Petri plates. The bacterial cells were either spread or streaked and were incubated overnight (12-16 hours) at 37 °C.

Mammalian cell culture: The HEK293T cell line was grown in RPMI 1640 medium with high glucose content (4.5 g/l), supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 unit/ml penicillin, and 100 unit/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cell cultures were split twice per week at 70-80% confluence by 1/20 dilutions. Both HEK293 and HEK293 stable transfected cell lines were grown in a DMEM medium with a high glucose content (4.5 g/l), supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cell cultures were split twice per week at 70-80% confluence by 1/20 dilutions.

3.2.2 Culture maintenance

The pH of the media for growing cells was maintained at 7.4 and under 5% CO_2 at 37 °C. The medium of cells from cell lines was changed twice weekly, when the growth reaches almost 70-80% confluence.

3.2.3 Mammalian cell thawing and splitting

Cell thawing: Cryovials of frozen cells were rapidly thawed by immersing the vial in a 37 °C water bath. Immediately after the vials were wiped from the outside with 70% EtOH, the contents were then transferred to a 15 ml plastic falcon tube containing 10 ml of the DMEM medium. The cell suspension was spanned, the supernatant was discarded and the cells were washed from DMSO again by PBS and spinning. The cells were resuspended into 15 ml of fresh medium before pouring in 10 cm-plate. To distribute cells evenly over the growth surface, the medium was gently rocked, and culture was then placed in a 37 °C of 5% CO_2 humidified incubator. Healthy cells displaying a flat morphology and adhering well to the plate under the microscope were medium aspirated and replaced with fresh and pre-warmed growth medium in the next day for thawing. The culture was expanded as needed and cells were split every 2–4 days when they reached not more than 70–80% confluence.

Cell splitting: When cells in the plate reach 70–80% confluence, medium was discarded, and then cells washed with pre-warmed sterile PBS. 1-2 ml of trypsin-EDTA solution was added to the cells for 1–2 minutes to detach cells. As soon as possible 5–10 ml of growth medium was added to stop trypsinization. Cells then resuspended gently and centrifuged in a 15 ml falcon tube at 1000 rpm, then washed by 5-10 ml pre-warmed PBS. Finally cells were again resuspended with DMEM medium and grown in a new 10 cm plate in a dilution of 1:20 with 20 cm of pre-warmed DMEM medium and incubated in a 37 °C, 5% CO_2, and a humidified incubator.

3.2.4 Preparing frozen stocks

Very early passages of cell cultures were used to prepare frozen stocks of cells to ensure a renewable source of cells. The media was changed 1-2 hours before freezing the cells. Cells were first trypsinized from the plate and trypsin was neutralized by 10 ml DMEM medium which have 10% serum, then harvested in a 15 ml tube. Cells were counted before spinning down at 1000 rpm for 5 minutes at room temperature,

supernatant was removed and the cells were dispersed. Ice cold freezing medium was added to reach a concentration of 1-2X10^6 of cells/ml on ice. 1 ml of cell suspension was added to cryovials and placed as soon as possible in a cryobox to allow them to cool down gradually and cells were preserved at -80 °C for 24-48 hours. Finally, cryovials were placed in a liquid nitrogen container for long term storage.

3.2.5 Cell treatment

Transiently transfected cells were treated after 20 hours by addition of 1 mM ionomycin (Calbiochem Corp., La Jolla, Calif.) and 10 ng of phorbol myristate acetate (PMA) per ml (Sigma) for 4 hours before examination under fluorescent microscope or flow cytometry. The stable cell lines which have the chimeric NFAT permanently were treated with Ionomycin alone, PMA alone, and both of them for 15 minutes for 2 hours and 4 hours.

3.2.6 Competent cell preparation

DH5-α competent cells prepared by inoculating a single colony of a previously streaked DH5-α plate in 50 ml of LB medium with no antibiotics in a 100 ml flask and incubated overnight at 37 °C at 270 rpm. 4 ml of the culture were added to 400 ml of LB medium in a 2 L flask and incubated once more at 37 °C, 270 rpm. OD590 of the culture followed until it reached 0.345. The culture was split into 8 x 50 ml falcons on ice for 5-10 minutes, and then centrifuged at 1600 g for 20 minutes at 4 °C to pellet the cells. Gently, pellets were resuspended in 80 ml ice cold 2 M $CaCl_2$ solution and centrifuge at 4 °C and 1100 g for 10 minutes. Cells resuspended again in 80 ml 2 M $CaCl_2$ and left on ice for 30 minutes before a centrifuge at 4 °C and 1100 g for 10 minutes. Pellets were resuspended in 16 ml ice cold $CaCl_2$ solution. Aliquots of 200 µl were filled into pre-chilled eppendorfs on ice. Tubes were immediately put into liquid nitrogen and stored at -80 °C. The competency of the cells was tested by transforming 1 ng, 10 ng, and 100 ng of pUC19 plasmid.

3.2.7 Vector construction

DH5-α transformation: Frozen competent cells were removed from -80 °C and placed on ice until thawing. 50 ng of the plasmids were mixed by flicking the tube gently. The tubes were then stored on ice for 30 minute. Cells were heat shocked for 90 seconds at 42 °C with no shaking. Immediately the tubes placed on ice for 2 minutes. 800 µl of room temperature LB medium was added to tubes and incubated for 45 minutes at 37 °C. The transformation mix precipitated at 1000 r/min for 5 minutes and then the cells were resuspended in a 100 µl LB medium plated onto Luria Agar plates containing the appropriate antibiotic which was placed in the 37 °C incubator and grew overnight.

Isolation of plasmid DNA: Plasmid DNA was isolated either by the alkaline lysis protocol in Sambrook et al., 2001 [84], or the Qiaprep Spin Miniprep, or the QIAGEN Plasmid Midi Kits. A single colony of E coli from an overnight grown Luria Agar plate or glycerol stock was grown overnight at 37 °C at 270 rpm in liquid culture containing the selective antibiotic, prior to the plasmid isolation. The concentration of the isolated plasmid DNA was determined by UV spectrophotometry and quantitatively on an agarose gel.

Restriction enzyme digestion: Digestion reactions mixtures were set by mixing the DNA, enzyme and the compatible buffer and then incubated at the optimum temperature of the enzyme 2.5 hours. 6X DNA Loading Dye was added to the reaction products and loaded on a gel in 2 wells.

Digestion of cloned plasmids: To diagnose cloned plasmids; 5 µl miniprep DNA, 0.5 µl appropriate restriction enzyme/s, 1 µl buffer, and 14.5 µl dH_2O were mixed in an eppindorf tube and incubated at 37 ^oC water bath for 2.5 hours. 4 µl 6X DNA Loading Dye added to mixture and loaded on gel for 45 minutes.

Agarose gel preparation: Agarose gel electrophoresis is a method used to separates molecules based on charge, size, and shape. It is particularly useful in

separating charged biomolecules such as DNA, RNA and proteins. Agarose gel is made by dissolving agarose powder in different concentrations ranging from 0.7% to 2% depending on the size of the fragments to be separated in boiling 0.5 X TBE (Tris Borate EDTA) buffer solutions. The solution is then cooled to approximately 55 °C and poured into a mold containing a comb.

Sample preparation and loading: Samples were prepared for electrophoresis by mixing them with 6X loading dye making them visible and denser than the electrophoresis buffer. These samples were loaded using a micropipette into wells that were created in the gel by a template during casting. The dense samples sink through the buffer and remain in the wells. A direct current power supply is connected to the electrophoresis apparatus and a current is applied. Gels were run at 100 volts for 40-60 minutes and the bands were observed under UV light. Charge molecules in the sample enter the gel through the walls of the wells. Within a range, the higher the applied voltage, the faster the samples migrate.

Ligation: The ligation reactions were carried out using T4 Ligase (Fermentas), in a ratio of 3:1 insert to vector respectively using 100 ng of vector. The Appropriate buffer was added to the mix and a total volume of 20 µl was completed using sterile distilled water. The reaction then was incubated at 16 °C for 16 hours before transforming 10 µl of the mix into competent cells.

3.2.8 Mammalian cell transfection

For transfection, cells were seeded in 6-well plates or 10 cm plate -transient or stable transfection respectively, and supplemented with 5% bovine serum DMEM for HEK293 (5.5 stable transfected) cells and RPMI for HEK293T cells. The cells were transfected using plasmids with a GFP reporter, 1 µg of C3-EGFP expression vector, and 2.5 µg of the expression vectors for NFAT2, NFAT3, NP-EGFP, RP-EGFP, and CP-EGF. 12 hours later, the medium was refreshed. The transfected cells were treated after 20 hours of transfection with 10 nM PMA and 1 µg of Ionomycin for 4 hours. GFP

expression was determined both under fluorescent microscope and by flow cytometry. All experiments were repeated three times.

3.2.8.1 Stable cell transfection ($CaCl_2$ transfection method)

Cell preparation: 24 hours before transfection, 1 x 10^6 cells were seeded per 10 cm Petri dish in 15 ml DMEM medium of 10% FBS and incubated overnight at 37 °C, humidity, and 5% CO_2 incubator.

Transfection solutions preparation: Solution A composed of 10 µg of plasmid DNA, 60 µl of 2 M $CaCl_2$, and distilled water to reach a volume up to 500 µl. The contents were vortexed and left, to stand, in the Laminar flow hood for 5 minutes. Solution B composed of 500 µl 2XHEPES buffered saline in a 15 ml plastic centrifuge tube. While vortexing the 2X HEPES solution, the plasmid solution was added drop by drop and fast in less than 30 seconds. The mix was left stand for 15 minutes in the Laminar flow hood.

Transfection: The prepared mixture of 1 ml final volume added drop by drop to a 10 cm Petri dish containing 15 ml DMEM of 5% FBS. After 24 hours of incubation in the CO_2 the incubator the media was refreshed. 24 hours later, 48 hours after the time of transfection, the medium was replaced by a medium containing 1 mg G-418/ml medium.

Selection: The medium was replaced every 2 to 4 days by G-418 containing medium for selection purpose. When colonies became ready, 12 to 17 days after the time of transfection, large, healthy and well separated colonies were marked and detached by trypsin and transferred into 24 well-plate with 1 ml DMEM containing 1 mg G-418/ml. Cells were detached and transferred to 6 a well-plate when cells became 70% confluent, then again to 10 cm plates. Finally cells were frozen and stored in liquid nitrogen at a density of 1 to 2 x 106 cells / in freezing medium.

3.2.8.2 Transient cell transfection ($CaCl_2$ transfection method)

Cell preparation: 24 hours before transfection, 3 x 10^5 cells were seeded per well in a 6 well-plate with 3 ml DMEM medium of 10% FBS and incubated overnight at 37 °C, humidity, and 5% CO_2 incubator.

Solutions preparation: Solution A composed of 2.5 µg of plasmid DNA, 15 µl of 2 M $CaCl_2$, and distilled water to reach a volume up to 125 µl. The contents were vortexed and left, to stand, in the Laminar flow hood for 5 minutes. Solution B composed of 125 µl 2XHEPES buffered saline in eppindorf plastic tube. While vortexing the 2X HEPES solution, the plasmid solution was added drop by drop in less than 30 seconds. The mix was left to stand for 15 minutes in the Laminar flow hood.

Transfection: The prepared mixture of 250 µl final volume was added drop by drop to a 6 well-plate containing 3 ml DMEM of 5% FBS. After 20 hours of incubation in the CO_2 incubator. The time was determined experimentally; cells were treated or taken for examination according to the experiment.

3.2.9 UV spectrophotometry

To determine the yield after plasmid DNA isolation, a UV Spectrophotometer was used. Several dilutions of plasmid DNA miniprep, midiprep, or maxiprep was prepared. Quartz cuvettes UV absorbance was used for measurement at 260 nm.

3.2.10 Flow cytometric analysis

Cells to be examined were first detached from plate by trypsin and counted under a microscope by the aid of Hematocytometer using trypan blue for identification of dead cells. 10^6 cells from each sample were centrifuged at 1000 rpm for 5 minutes at 25 °C.

Precipitated cells were washed with PBS and centrifuged again. Cells were then resuspended in 0.5 ml PBS. Cell suspension was directly used for flow cytometric analysis. The analyses were made using BD FACSCanto. GFP was excited by the argon laser and fluorescence was detected using FITC 530/30 nm band pass filter.

3.2.11 NFAT nuclear translocation assay (DAPI counterstaining)

The transfected cells grown on cover slips in 6 well-plates were washed by PBS. 300 µl of 4% paraformaldehyde was added to the cells and was then incubated for 20 minutes in the dark at room temperature. Cells were washed twice by PBS, air dried, mounted with antifade (0.1% SDS) for 30 minutes in the dark at room temperature.

The cells were rinsed three times by PBS, then counterstained with 300 µl of 500 µM DAPI solution and incubated for 5 minutes in the dark at room temperature, cover slips were taken out of wells and rinsed several times by PBS, and the excess buffer was drained from the cover slip. Cover slips were inverted on slides; the edges were flushed by nail polish and kept dry. Finally samples were viewed using a fluorescence microscope with appropriate filters. Green indicated (GFP expression) and blue (DAPI), which stains the double stranded DNA in the nucleus .Images were merged to distinguish cytosolic and nuclear NFAT.

4 RESULTS

In order to test the effects of PMA and Ionomycin on the subcellular localization of NFAT proteins, we decided to generate NFAT2 and NFAT3 chimeric constructs which were fused to a GFP reporter protein. These constructs were used to express NFAT2/3 chimeras in mammalian tissue culture cells.

4.1 Construction of NFAT2, NFAT3, NP, RP, CP chimeras

Five different plasmids NFAT2, NFAT3 and chimeras containing different regions of these proteins (Table 4.1) fused to GFP reporter proteins were previously generated by Pinar Ayata in Prof. Batu Erman's laboratory (Sabanci University) to study the domains controlling the subcellular localization of NFAT2 and NFAT3.

Table 4.1: NFAT2/NFAT3 chimeras

No.	Plasmid chimera	Abbreviation	Contain
1	pEGFP-T2	T2	NFAT2 cDNA
2	pEGFP-T3	T3	NFAT3 cDNA
3	pEGFP-NP	NP	N-Terminal domain of NFAT3 cDNA and other three domains from NFAT2 cDNA
4	pEGFP-RP	RP	Regulatory domain of NFAT3 cDNA and other three domains from NFAT2 cDNA
5	pEGFP-CP	CP	C-Terminal domain of NFAT3 cDNA and other three domains from NFAT2 cDNA

These chimeras were designed to be transfected into mammalian cells and to investigate their response to PMA and ionomycin treatment. The cDNAs of chimeras listed in Table 4.1 were cloned into the pGFP-C3 mammalian expression plasmid. The overlap extension PCR was used for creating and cloning these chimeras.

4.1.1 Overlap extension PCR

Different regions of the NFAT2 and NFAT3 cDNAs were amplified by polymerase chain reaction (PCR) using forwards and reverse primers with flanking spacers carrying Xho I and Hind III restriction sites, respectively. As depicted in Figure 4.1, the dark green line represents the coding (top) strand of NFAT3 and the light green line represents its template (bottom) strand. Similarly the dark blue line represents the coding (top) strand of NFAT2 and the light blue line represents its (bottom) template strand. The pink lines represent the Hind III and Xho I restriction sites.

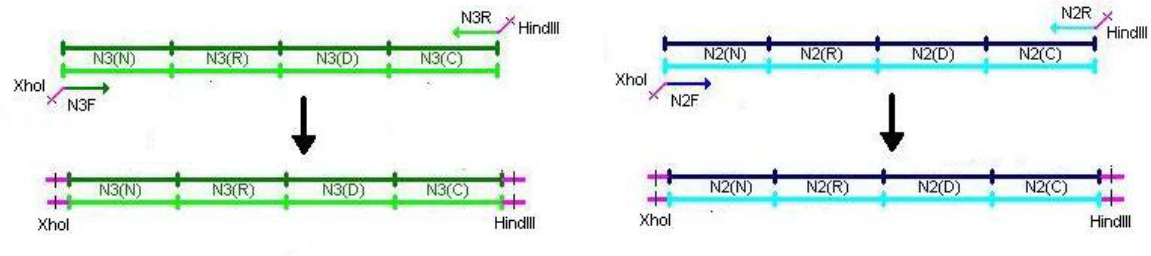


Figure 4.1: Explanatory scheme for NFAT2 and NFAT3 proteins

The dark green line indicates the coding strand of NFAT3 (N3) and the light green line indicates its template strand, similarly the dark blue line is the coding strand of NFAT2 (N3) and the light blue line is its template strand. The pink lines represent restriction sites. N, R, and C represent the N-terminal, Regulatory DNA binding, and C-terminal domains respectively.

The three PCR reactions were performed to generate the NP, RP, and CP chimeras illustrated in Figures 4.2, 4.3, and 4.4. For the NP chimera (Figure 4.2); N-Terminal domain (NTD) was from NFAT3 while Regulatory domain (RD), DNA binding domain (DBD), and C-Terminal domain (CTD) were from NFAT2. Briefly, a cDNA encoding NFAT3 was amplified with N3F and NT2 oligos, and a cDNA

encoding NFAT2 was amplified with NT3 and N2R oligos, these two PCR products were gel purified and mixed in a second reaction that contained oligos N3F and N2R.

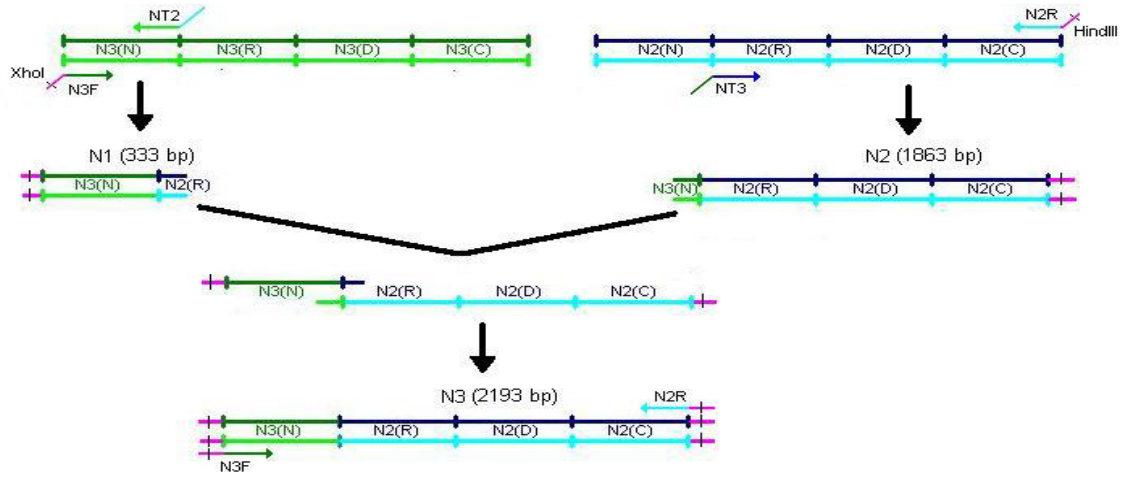


Figure 4.2: Overlap extension of NP

The diagram shows the overlapping extension of NP, which is built up from N-terminal domain of NFAT3 and the rest of domains from NFAT2.

For the RP chimera illustrated in Figure 4.3; Regulatory domain (RD) was from NFAT3, while N-Terminal domain (NTD), DNA binding domain (DBD), and C-Terminal domain (CTD) were from NFAT2. Briefly, a cDNA encoding NFAT3 was amplified with RD2 and RD1 oligos, and a cDNA encoding NFAT2 was separately amplified with N2F and N2R, or RD4 and RD5 oligos, these three PCR products were gel purified and mixed in a second reaction that contained oligos N2F and N2R.

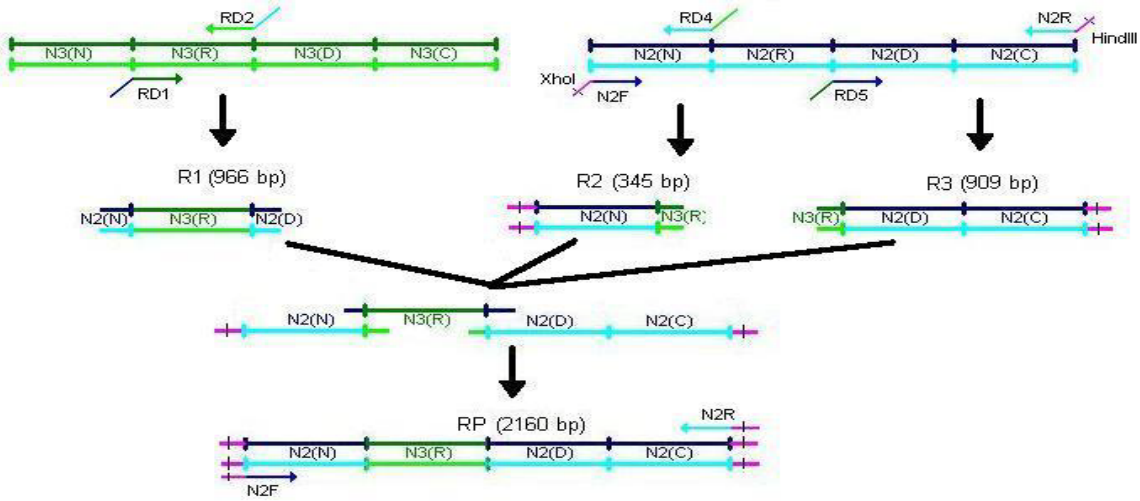


Figure 4.3: Overlap extension of RP

The diagram show the overlap extension of RP, which is built up from regulatory domain of NFAT3, and the rest of domains from NFAT2.

For the CP chimera depicted in Figure 4.4; the C-Terminal domain was from NFAT3, while, N-Terminal domain (NTD), Regulatory domain (RD), DNA binding domain (DBD), and C-Terminal domain (CTD) were from NFAT2. Briefly, a cDNA encoding NFAT3 was amplified with the N3R and CT1 oligos, and a cDNA encoding NFAT2 was amplified with N2F and CT4 oligos, these two PCR products were gel purified and mixed in a second reaction that contained oligos N2F and N3R.

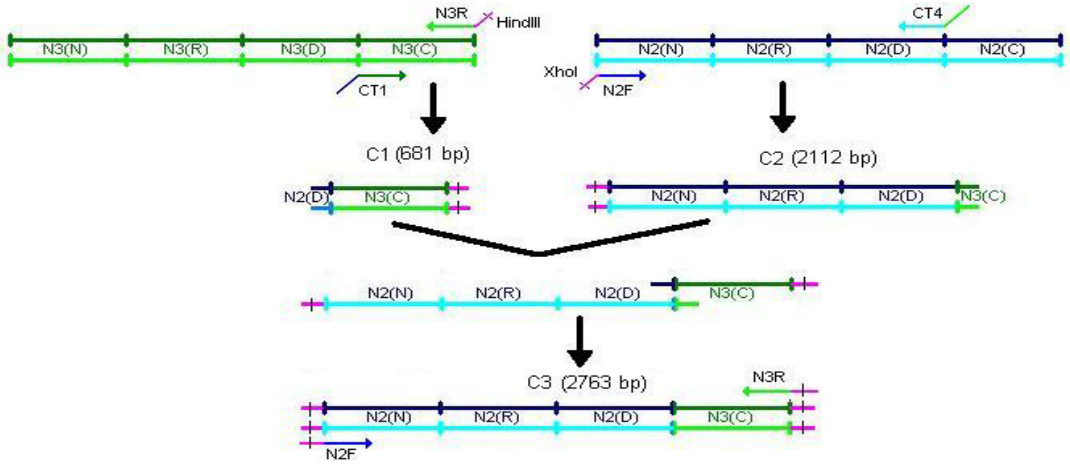


Figure 4.4: Overlap extension of CP

The diagram shows the overlapping extension of RP, which is built up from C-terminal domain of NFAT3, and the rest of domains from NFAT2.

4.1.2 Cloning of N2, N3, NP, RP, and CP

The pGFP-C3 mammalian expression plasmid used to make GFP fusions was provided by Prof. Batu Erman (Sabanci University). All PCR products, except NFAT3, and plasmid were digested with 10 units of both, Xho I and Hind III (Fermentas) simultaneously for 4 hours at 37˚C in 1X Buffer R (Fermentas) in a 30 µl reaction. Because NFAT3 contained a Hind III site in the DNA-binding domain, it was only digested with Xho I. The vector for NFAT3 was first cut with 10 units of Hind III for 2 hours and blunted directly by addition of 0.1 mM dNTP and 2.5 units of T4 Polymerase for 15 minutes at 37 ˚C. After the polymerase inactivation for 20 minutes at 70 ˚C, 10

units of Xho I was added to the reaction and the digestion continued for another two hours.

All digestion products were purified into 10 µl ddH2O using DNA Extraction Kit (Fermentas) directly from reaction. Since the concentrations were similar, all ligation reactions contained 5 µg insert (except NFAT3) and 500 ng vector with 1X T4 Ligase Buffer (Fermentas) and 1 unit T4 Ligase (Fermentas). In NFAT3 reaction 4.5 µg insert and 5% (w/v) PEG 4000 (Fermentas) were used. The ligation reaction was carried out at 16 ˚C overnight. Two control reactions were carried out in the absence and presence PEG 4000, where water instead of insert was used. The ligation reactions were transformed into the DH5α E.coli strains, which were grown on LB plates supplemented with a 50 µg/ml Kanamycin.

Colonies were selected, DNA purified, and verification of the constructs was performed. Figure 4.5 shows maps of the eukaryotic expression plasmids containing NFAT2/3 chimeras.

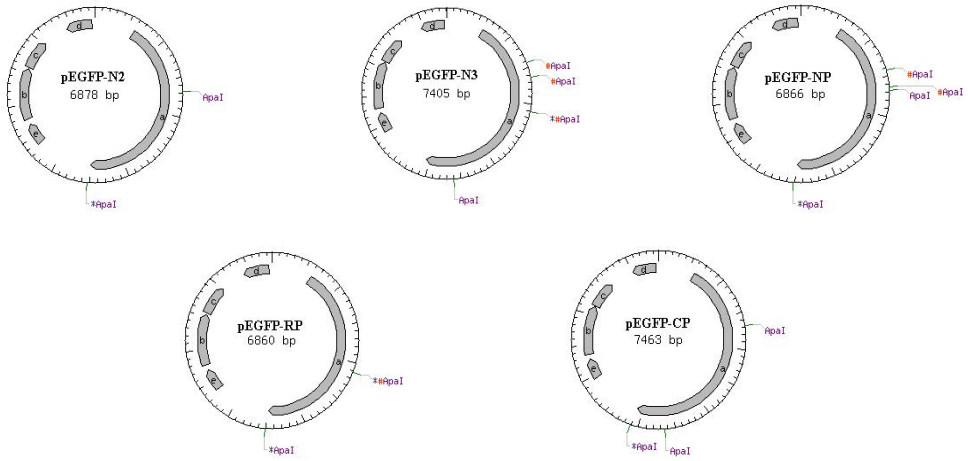


Figure 4.5: Chimeric cloning products

The design of the GFP plasmid reporter having different NFAT chimeric constructs.

4.2 Stable transfection of GFP reporter constructs activated by NFAT binding sites

In order to generate a reporter cell line system that is activated when NFAT is translocated into the nucleus, we decided to create a cell line that permanently carries binding sites of the NFAT transcription factor driving the expression of the GFP reporter gene. In the proximal promoters of some interleukins; multiple NFAT sites have been found to cooperate in the production and activation of these interleukins. For example, the IL-2 gene promoter has five repetitive sites specific for endogenous NFAT protein binding. Therefore, the plasmid to be transfected (pPO4AT 2-5), was chosen to have four NFAT DNA binding sites. The pPO4AT 2-5 was previously generated by Pinar Onal in Prof. Batu Erman's laboratory (Sabanci University) [85]. The designed primers are listed in appendix A.

The HEK293 cell line was transfected with pPO4AT 2-5 plasmid, which has four NFAT binding sites, a promoter from the human Fos gene and a GFP c DNA, along with a neomycin resistance gene. We tried to optimize the transfection efficiency by modifying the number of cells transfected and the linear/circular state of plasmid. For this purpose eight different transfections were performed, as detailed in Table 4.2. Cells were transfected in 10 cm Petri-plates using the calcium precipitation method for 48 hours before the medium was replaced with Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 2 mg/ml G418 antibiotic for selection. The colonies that appeared in the transfection plates were first transferred into 24 well-plates, then into 6 well-plates, and finally into 10 cm plates. Stocks of each colony were frozen in the laboratory liquid nitrogen tank at a concentration 2 X 10^6 cells/ml (appendix). As shown in the table 4.2, there was no significant difference between the numbers of colonies generated when cells were transfected with linear or circular DNA. On the other hand, cell number was crucial; we observed that when cell number exceeds 2.25 X 10^6 cell/10 cm plate (Plates 7 and 8) no colonies appeared in the plate.

Table 4.2: Colony selection from stable transfection optimization

Colonies from different transfections transferred from 10 cm plate into 24 well-plates then to 6 well-plates then to 10 cm plates before freezing. Total frozen stocks from all transfections were (51) vials. L: Linear, C: Circular.

No.	**DNA state**	**Plate & Dilution**	**Colonies (24 well-plate)**	**Transfer into (6 well-plate)**	**Transfer into (Petri-plate)**	**Frozen**
1	L	0.75×10^6	24	16	15	15
2	C	0.75×10^6	30	5	5	5
3	L	1.5×10^6	12	11	11	10
4	C	1.5×10^6	36	4	4	4
5	L	2.25×10^6	24	6	6	5
6	C	2.25×10^6	24	13	13	12
7	L	3×10^6	-	-	-	-
8	C	3×10^6	-	-	-	-

We observed that a significant number of colonies which appeared in the 24 well-plate were not viable when transferred into 6 well-plates. This death resulted not due to the fact that cells were not transfected by plasmid DNA, but rather because they had overgrown before they were transferred from the 10 cm plate to the 24 well-plate. Frozen cells from different transfections were named by taking two numbers for each; the first number represents the number of the original transfection, while the second number represents the colony number. For example, colony number 5.5 is the fifth colony isolated from plate number five which was seeded with 2.25×10^6 cells prior to transfection and was transfected with linear pPO4AT 2-5 plasmid (appendix).

4.3 Identification of stable HEK293-NFAT-GFP cell lines activated by NFAT

Cells stably transfected cells with the pPO4AT 2-5 reporter plasmid were treated with both ionomycin (1 μM) and PMA (10 ng/ml). Fluorescent microscope and flow cytometry examination showed no fluorescence compared with the control untransfected HEK293 cells (data not shown). This could result because either the cells were not properly transfected by the pPO4AT 2-5 plasmid or because, the cells did not have enough endogenous NFAT protein to translocate to the nucleus and activate our reporter construct. To test the the latter hypothesis, we decided to transiently transfect cells derived from numerous colonies shown in table 4.2 with an NFAT2 protein expression plasmid (named pCS2+MT-N2).

Six different stably transfected cell cultures (1.8, 2.3, 3.2, 4.6, 5.5, 6.11) were randomly chosen from the stable transfected cells shown in Table 4.2. These cells were transiently transfected with NFAT2 expression plasmids for 16 hours, followed by treatment (or no treatment) by PMA (10 ng/ml) and ionomycin (1 μM) for four hours. Cultures were then examined under the fluorescent microscope (Figure 4.6). Surprisingly we observed GFP protein expression in all NFAT2 transfected stable reporter cells regardless of the treatment by PMA/Ionomycin.

Stably transfected cells from colony number five, from transfection plate five hereafter named named the HEK293-5.5 stable cell line was selected as a reporter cell line that can express GFP proteins after NFAT nuclear translocation. Using the HEK293-5.5 stable reporter cell line, NFAT2 expression plasmid DNA concentration and incubation time after transfection were optimized in an additional two experiments, to identify the optimal conditions for NFAT nuclear translocation and GFP reporter expression.

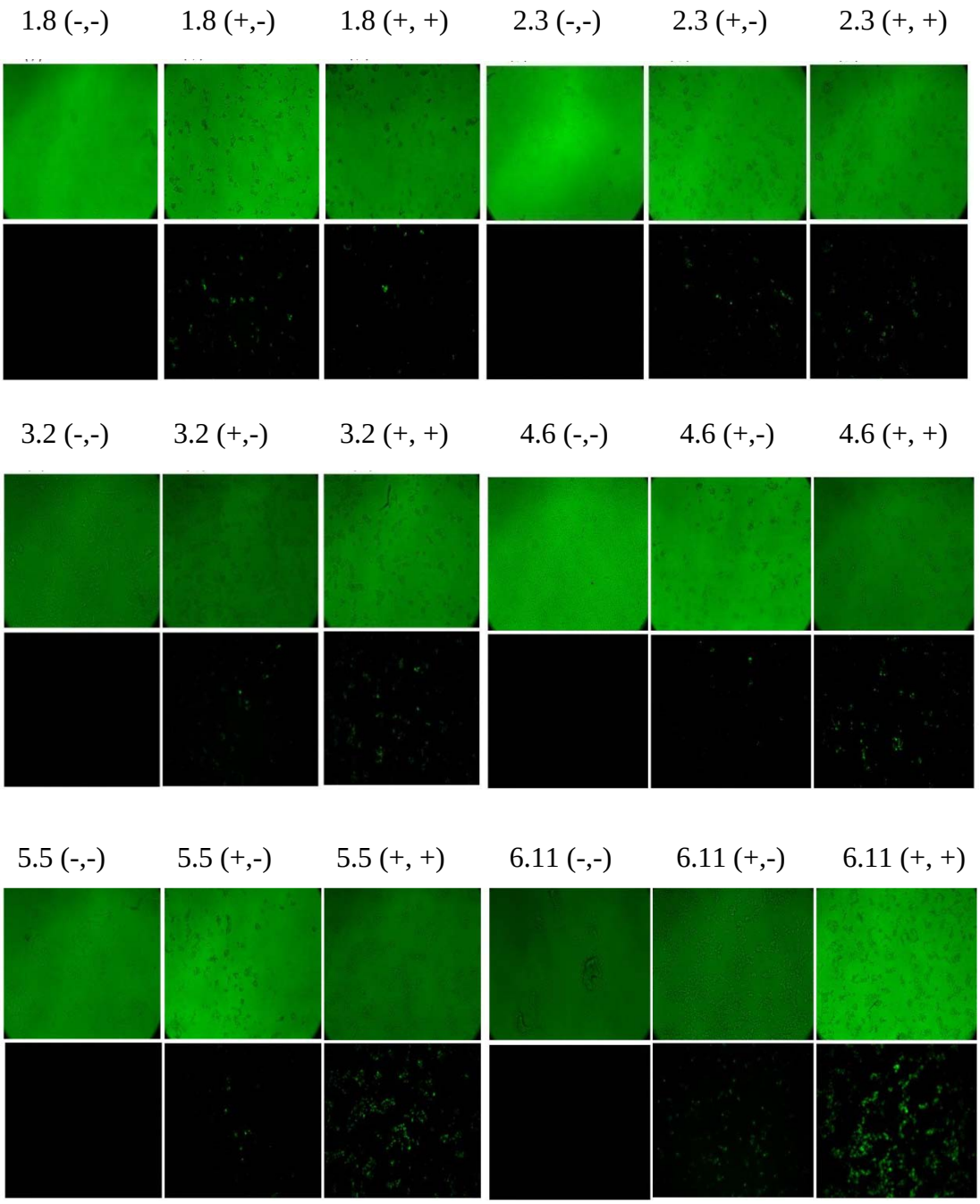


Figure 4.6: Generation stable transfected cells

Six different cell cultures representing the six transfections: Left, untransfected and PMA/Ionomycin untreated cells (-,-). Middle, transfected and PMA/Ionomycin untreated cells (+,-). Right, transfected and PMA/Ionomycin treated cells (+, +). Cultures were transiently transfected with 2 μg NFAT2 expression plasmid in 6 well-plates.

4.4 Transfection efficiency

4.4.1 Determination of DNA concentration in transfection efficiency

To determine the optimum concentration of plasmid DNA for transfection efficiency, three DNA concentrations of NFAT2, (1, 2.5, and 5 µg), were used to transfect the HEK293 derived HEK293-5.5 stable reporter cell line. The experiment was done in 6 well-plates; cells were incubated for 16 hours after transfection. Flow cytometry results (Figure 4.7) showed a significant increase in the transfection efficiency, determined by the percentage of FITC positive cells, when DNA concentration increased. 13%, 35%, and 73% of the transfected cell populations were FITC positive, when 1, 2.5, and 5 µg of DNA were transfected respectively. In future experiments we decided to transfect 2.5 µg of DNA, because higher DNA concentrations caused extensive cell death.

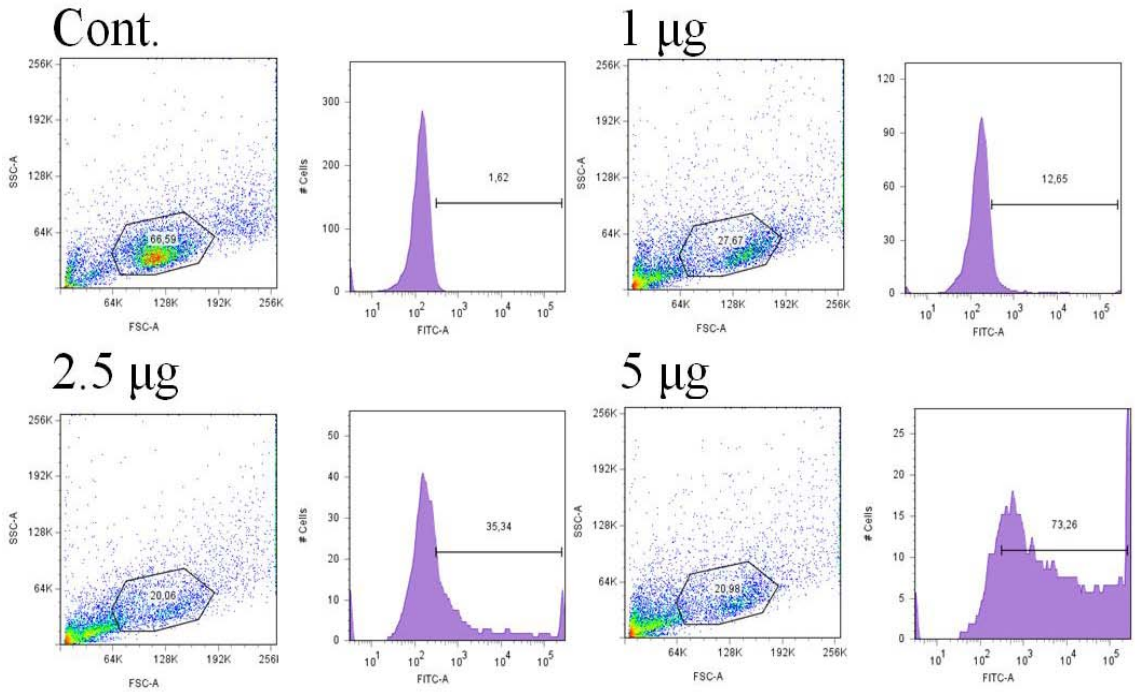


Figure 4.7: Transfection efficiency (DNA concentration).

Flow cytometry readings of HEK293 cells transfection with; 1, 2.5, and 5 µg pEGFP-N2 plasmid/6 well-plate compared to the untransfected cells (Cont.). FSC-A versus the plots allow the gating of live cells. The GFP fluorescence measured in the

FITC-A channel is shown only for live gated cells. Numbers in the histograms indicate the percentage of the population expresses GFP.

4.4.2 Determination of the effect of post transfection incubation time in transfection efficiency

To determine the appropriate duration of post transfection period needed to achieve the best reporter gene expression, HEK293 cultures were transfected with 2.5 μg of NFAT2 in 6 well-plates. Cells incubated for 8, 16, 20, 24, and 32 hours after transfection before were analyzed for GFP expression. Flow cytometry results showed a significant increase in the transfection efficiency, determined by the percentage of GFP positive cells, when incubation time increased. GFP positivity of cells incubated for 8, 16, 20, and 24, hours were 4%, 29%, 46%, and 56% respectively. After this time point GFP positivity decreased to 46% at 32 hours of incubation. Comparing the cell viability showed that the viability began to decrease after 20 hours after transfection, we noticed a significant decrease of cell viability noticed when post transfection incubation exceeded 24 hours. Cell viability of 8, 16, 20, 24, and 32 hours were 62%, 45%, 51%,46%, and 22% respectively. Accordingly, we decided to perform further experiments by incubating cells for 20 hours post transfection. These results are also shown as an overlay plot demonstrating that 20 hours after transfection is the optimal condition with regard to cell death and reporter protein expression (Figure 4.8).

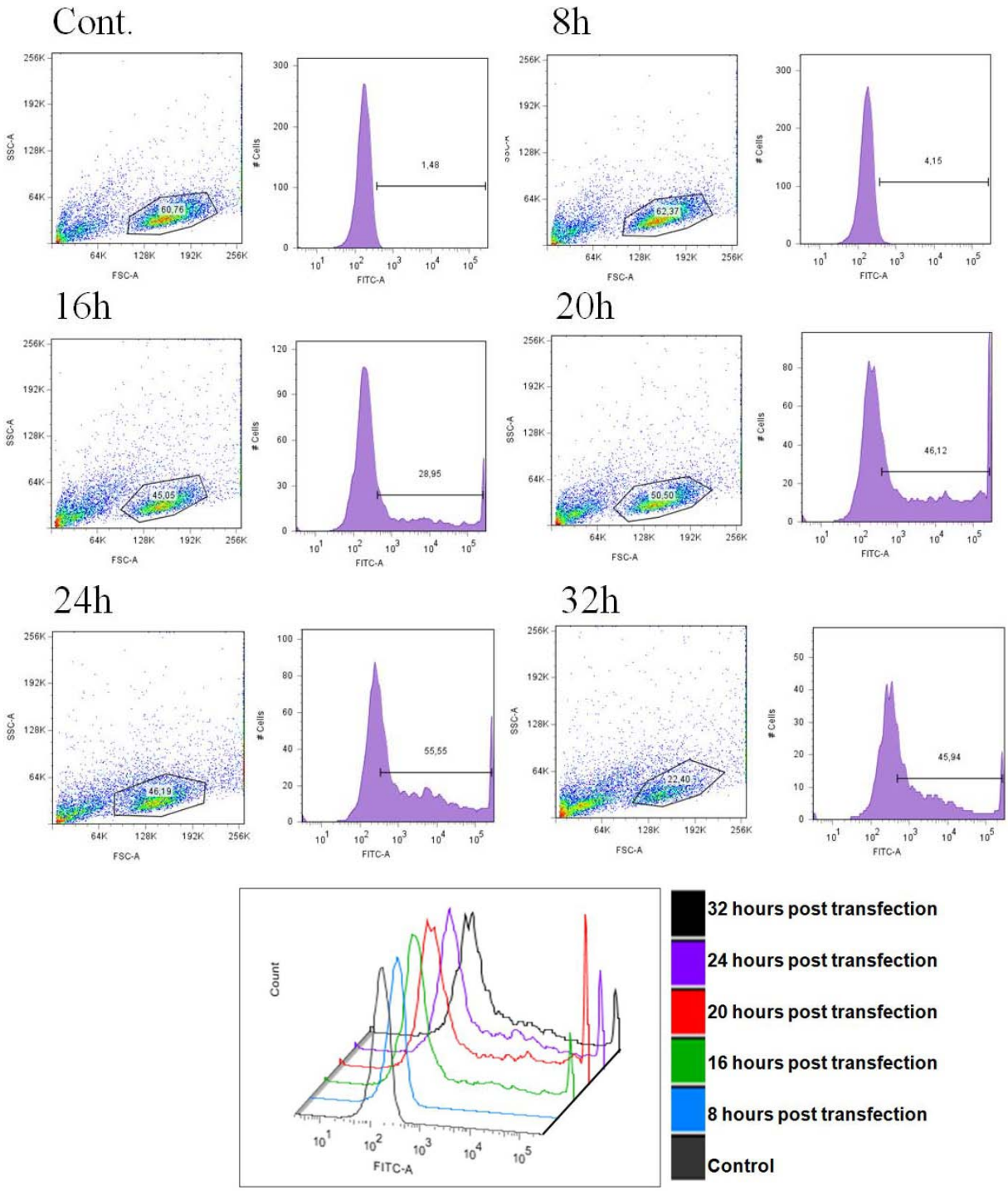


Figure 4.8: Transfection efficiency as a function of Post transfection incubation.

The flow cytometry results of HEK293 cells. The cells were transfected with 2.5 µg pEGFP-N2 reporter plasmid in 6 well-plates. Cells were incubated for different periods (8, 16, 20, 24, and 32 hours) after transfection and compared to the untransfected cells (Cont.) as in figure 4.7 live cells were gated arbitrarily using forward and side scatter measurements. GFP expression was measured by the fluorescence measured by the FITC detected for on live gated cells. Lower diagram shows the laying over of the six histograms in the figure above.

4.5 Comparison of reporter gene activation by NFAT2 and NFAT3

We next induced both NFAT2 and NFAT3 expression in HEK293-5.5 reporter cells to answer the question: Can overexpressed NFAT2 or NFAT3 translocate to the nucleus to activate reporter gene transcription? To answer this question, HEK293-5.5 cells were transfected by 2.5 μg of NFAT2 or NFAT3 with or without PMA/Ionomycin treatment. Cultures were incubated for 20 hours after transfection, according to the previously described optimization, and reporter gene activation was examined either by fluorescent microscopy (Fig 4.9) or by flow cytometry (Fig 4.10).

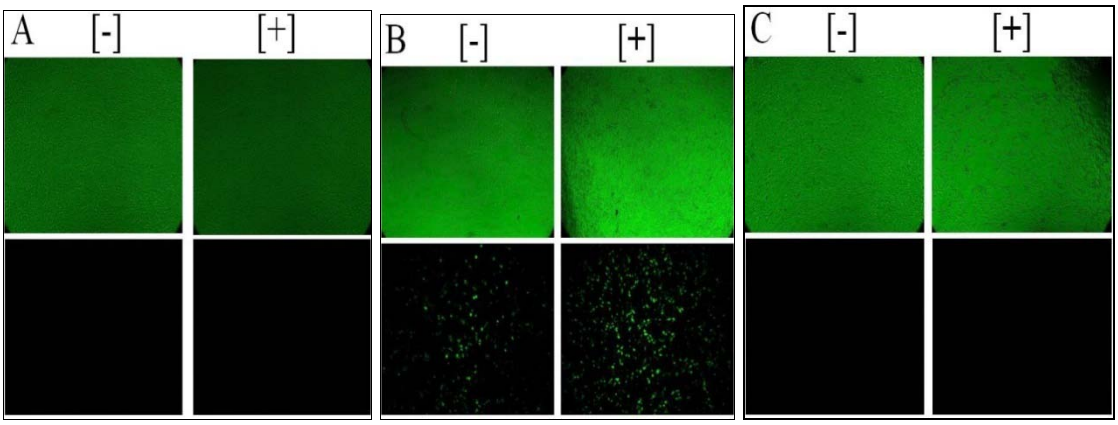


Figure 4.9: Fluorescent microscopy showing GFP reporter expression after for NFAT2 and NFAT3 transfection of HEK293-5.5 reporter cell lines

HEK293-5.5 cells were seeded in 6 well plates; (A) Untransfected and untreated cells (-), and treated with 10 ng PMA and 1 μM ionomycin (+). (B) Cells transfected with 2.5 μg NFAT2 but not treated with PMA/Ionomycin (-), and treated with 10 ng PMA and 1 μM ionomycin (+). (C) Cells transfected with 2.5 μg NFAT3 but not treated with PMA/Ionomycin (-), and treated with 10 ng PMA and 1 μM ionomycin (+). In all microscope pictures: Top line: DIC image, bottom line fluorescent image.

Results obtained under the fluorescent microscope (Figure 4.9) showed that, when HEK293-5.5 reporter cells were transfected with an NFAT2 cDNA expression plasmid (pCS2+MT-N2) GFP reporter expression was observed. In contrast, when cells were transfected with NFAT3 cDNA expression plasmids (pCS2+MT-N3) no significant GFP expression was observed. We noticed that in NFAT2 transfected reporter cells that

were treated with 10 ng PMA and 1 μM ionomycin (B +), more cells seem to be expressing the GFP reporter compared to the untreated cultures in (B -), however; the slight increase in fluorescence results from the fact that the field where the picture was taken contained more the cells compared to (B -) as can be seen in the DIC image showing the total number of cells in the field.

Quantitative results obtained by flow cytometry also showed no significant increase in the fluorescence upon PMA+Ionomycin treatment of NFAT2 transfected cells as measured by GFP fluorescence in the FITC detector, 17.43% FITC positive in PMA and ionomycin untreated cells in (B-), and 19.55% FITC positive in PMA and ionomycin treated cells (B+). Notably, we could not detect any GFP reporter expression in NFAT3 cDNA expression plasmid transfected HEK293-5.5 reporter cells, in the presence or absence of PMA+Ionomycin treatment.

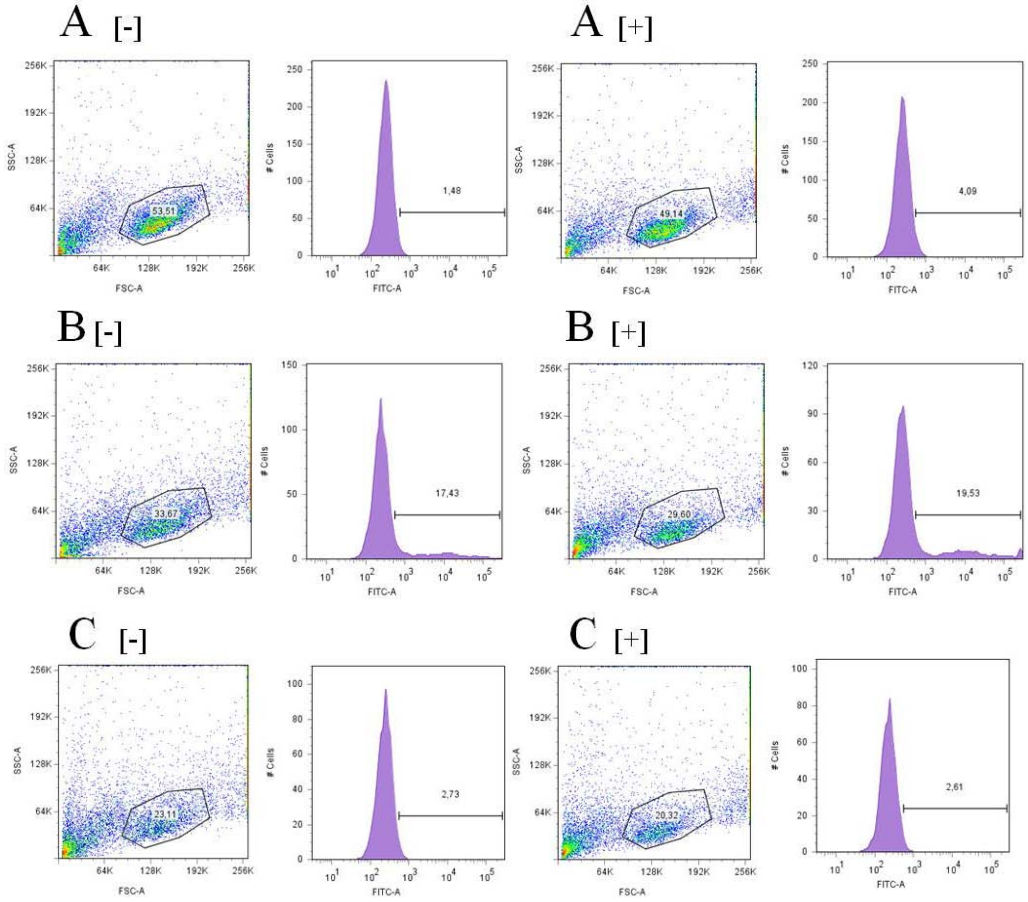


Figure 4.10: Flow cytometric investigation of GFP reporter activation upon NFAT2 and NFAT3 expression in the HEK293-5.5 reporter cell line

Flow cytometry results for HEK293-5.5 reporter cells transfected with pCS2+MT-N2 and pCS2+MT-N2 plasmids as explained in Figure 4.9.

4.6 Transfection of NFAT1-4 into the HEK293-5.5 reporter cell line

To inspect if NFAT1 and NFAT4 expression follow NFAT2 or NFAT3 when they are transfected into HEK293-5.5 reporter cell lines, cells were transfected with 10 μg of NFAT1, and NFAT4 in 10 cm plates in parallel with NFAT2 and NFAT3 under the same conditions. Cells were incubated for 20 hours after transfection, and then examined by flow cytometry. The transfected plates were split after transfection, $1X10^6$ of the cells were used for flow cytometry examination. To exclude dead cells from the analysis, 1 ng/ml of 7-Amino-actinomycin D (7AAD) dye, which intercalates into double-stranded nucleic acids in the cells, but can be excluded by live cells were added to the cell suspension and incubated for 10 minutes at room temperature. Flow cytometric results are gated on 7AAD negative live cells.

The HEK293-5.5 reporter cell line was transfected by NFAT1-4 and examined first under fluorescent microscope (data no shown), and then measured by BD FACS Canto flow cytometry machine (Figure 4.11). We observed that only NFAT2 transfected cells could activate the expression of the GFP reporter gene (FITC channel positive cells are about 59%). The positive control, pEGFP-C3 expression vector, expresses a GFP protein to demonstrate that transient transfection conditions are appropriate, has about 53% FITC while the rest of the NFAT expression plasmids do not activate the GFP reporter (FITC channel positive cells were less than 2%).

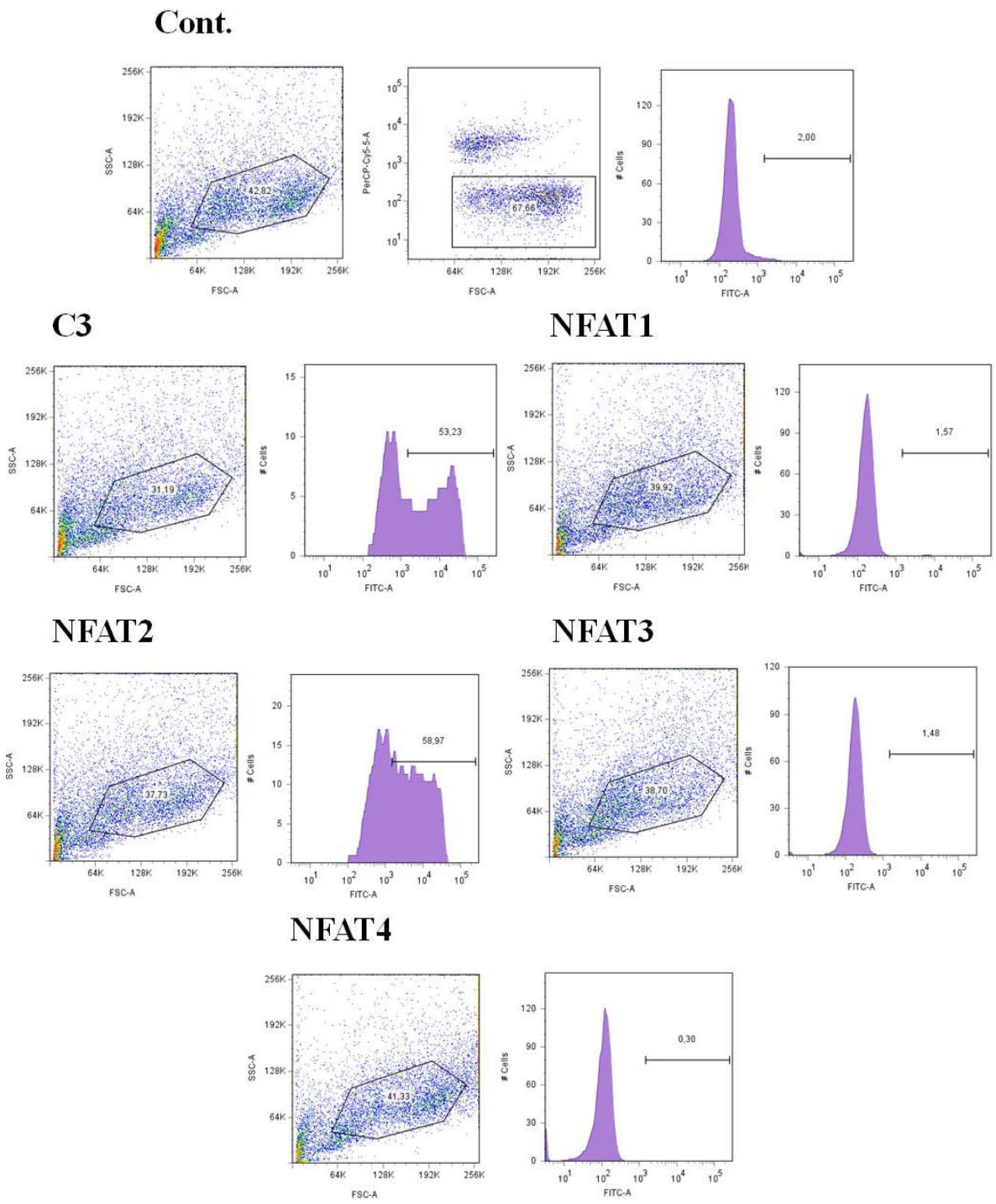


Figure 4.11: NFAT1-4 transfection into the HEK293-5.5 cell line (flow cytometry).

The HEK293-5.5 reporter cell line transfected with 10 µg of NFAT1-4 and the pEGFP-C3 (C3) expression vector, or left without transfection (Cont.). Cells were seeded in 10 cm plates and incubated for 20 hours after transfection, $1X10^6$ cells of the culture were examined by flow cytometry. 1 ng/ml of 7-Amino-actinomycin D (7AAD) dye were added to the cell suspension for 10 minutes and examined by flow cytometry.

4.7 Identification of NFAT2 domains that are necessary for nuclear translocation and reporter gene activation

In order to determine the domain of the NFAT2 protein responsible for nuclear translocation and reporter gene activation, the five plasmids in Figure 4.5 which encodes the both NFAT2 and NFAT3, and the three chimeras between NFAT2 and NFAT3 were transfected into HEK293T cell lines. These chimeras have the following characteristics; NFAT2, NFAT3, NP (N-terminal domain from NFAT3 and the rest domains from NFAT2), RP (Regulatory domain from NFAT3 and the rest domains from NFAT2), CP (C-terminal domain from NFAT3 and the rest domains from NFAT2). In addition, these chimeras have a GFP protein fused to their C-terminal tails that allows the identification of their nuclear or cytoplasmic localization. These constructs were transfected into HEK293T cell lines. Transfection was performed in cells seeded on glass cover slips placed in 6 well-plates, using 2.5 μg of plasmid DNA/well. Transfected cells were incubated for 20 hours before fluorescence microscopic examination (Fig 4.12).

Fluorescence microscopy results obtained under 40X and 100X magnifications for each transfection showed that; NFAT2, NP, and CP chimeric proteins translocated from the cytoplasm into the nucleus. On the contrary, both NFAT3 and RP chimeric proteins were found exclusively in the cytoplasm. Because the RP chimera contains the regulatory domain from NFAT3 and the remaining domains from NFAT2, we conclude that the regulatory domain of NFAT2 is responsible for nuclear translocation. It is possible that there are cytoplasmic factors associating with the NFAT2 regulatory domain to mediate nuclear translocation, or alternatively there may be factors associating with the NFAT3 regulatory domain to prevent nuclear translocation.

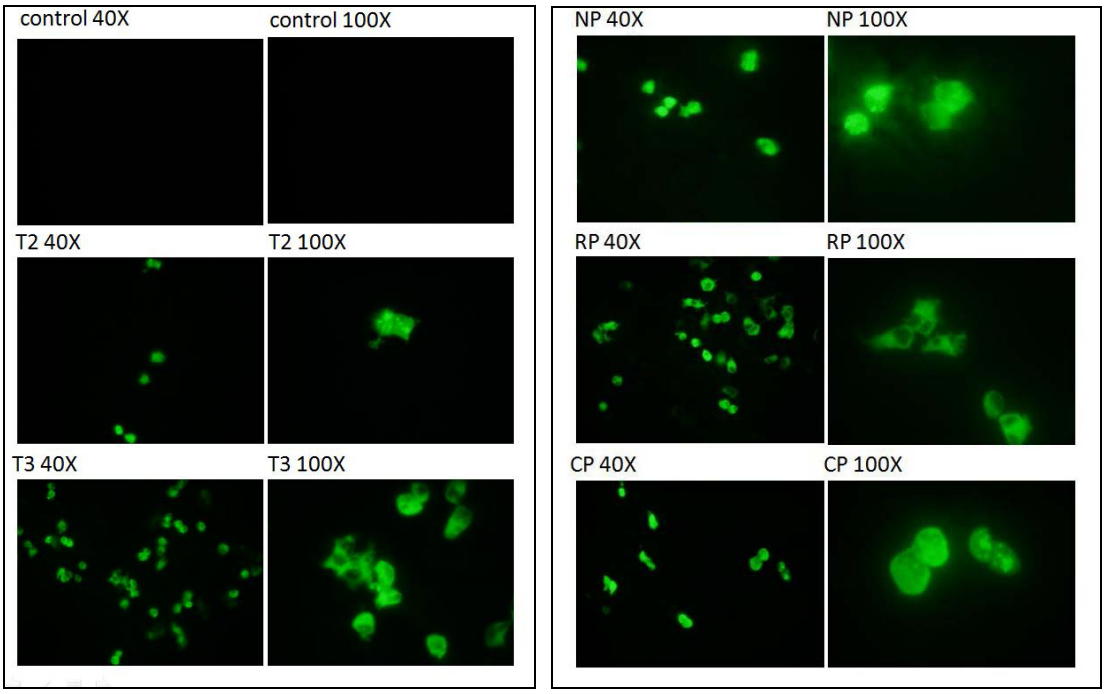


Figure 4.12: Chimeras transient transfection into HEK293T cells

Five chimeras (Table 4.1 and Figure 4.5), transfected into HEK293T cells in 6 well-plates on glass cover slips using 2.5 µg plasmid DNA in 6 well-plates and incubated for 20 hours, then examined under both 40X and 100X magnifications of fluorescent microscope. Untransfected cells were taken as control.

To confirm the translocation results, the same experiment was repeated (Figure 4.13) with a DAPI nuclear stain, to definitively identify the nucleus of transfected cells. 500 nM DAPI was added to the cells after fixation, the stain binds the double stranded DNA in the nucleus and stained it in blue colour under the fluorescent microscope. This experiment was performed under the same conditions as the previous experiment. Fluorescent microscope photographs were recorded under 100X magnification for each field of cells (Figure 4.13), using visible light (A), or fluorescent light and a GFP filter (B), or using fluorescent light and a DAPI blue filter. Overlaying of B and C of each slide indicated that NFAT3 and RP chimeric proteins were localized to the cytoplasm. The blue colour of the nucleus as a result of DAPI stain did not overlay with GFP fluorescence which appeared only in the cytoplasm. On the other hand, the rest of the proteins (NFAT2, NP, and CP) were translocated to the nucleus, the blue nuclear stain overlaid with the GFP green fluorescence in the nucleus.

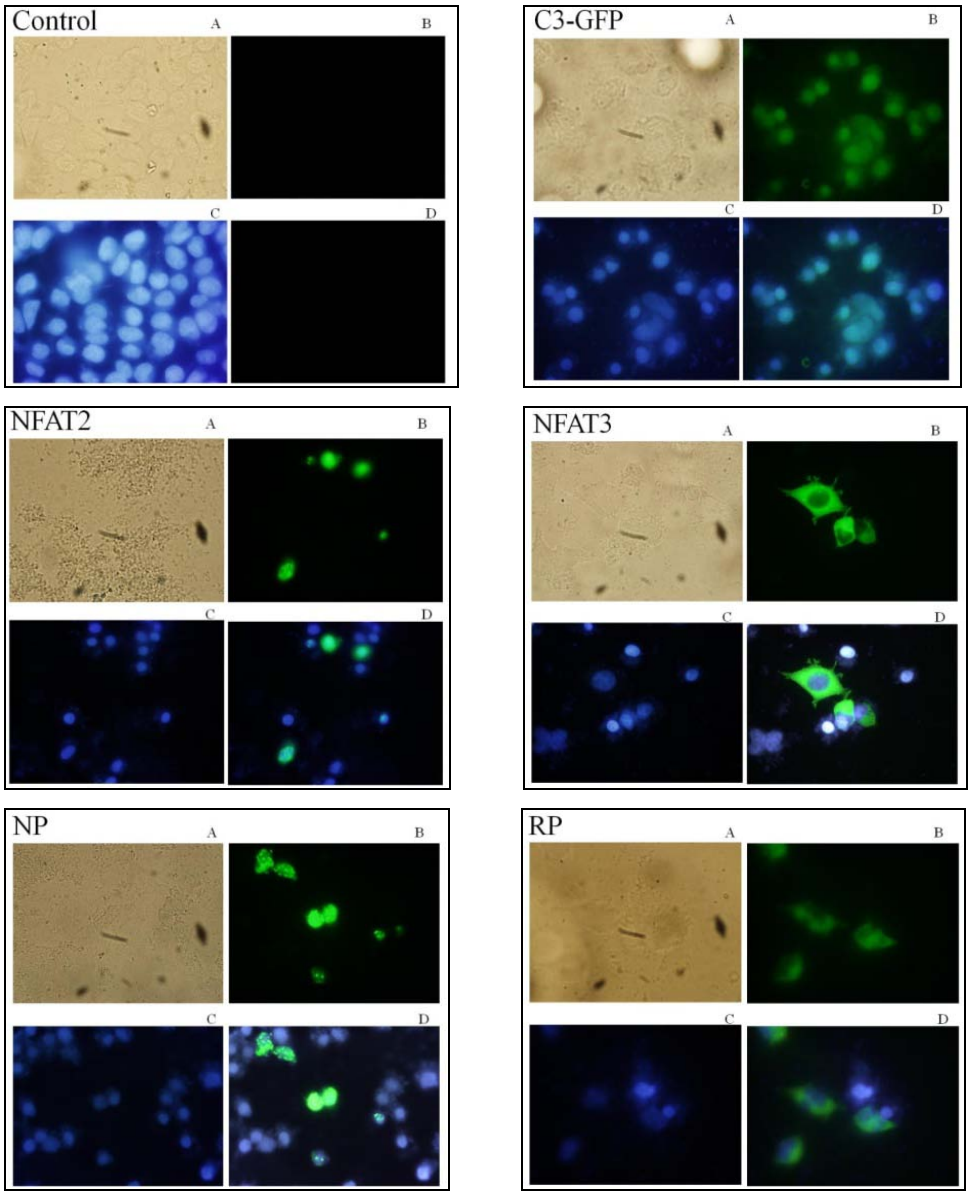


Figure 4.13: Chimeras transient transfection into HEK293T cells (DAPI staining)

Five chimeras (Table 4.1 and Figure 4.5), were transfected into HEK293T cells in 6 well-plate on glass cover slips using 2.5 µg plasmid DNA, incubated for 20 hours. Cells were stained with 500 nM DAPI stain after fixation for five minutes, and then examined under 100X magnification of fluorescent microscope. Control: Untransfected cells. C3-GFP: pEGFP-C3 expression vector. (A): Normal light. (B): GFP fluorescent filter. (C): DAPI blue fluorescent filter. (D): Overlaying of B, and C.

4.8 Chimeric sable transfections

It is well- known that, Ca^{+}_{2} results in the movement of NFAT proteins from the cytoplasm to the nucleus by activating calcineurin-NFAT binding. In the previous experiments, ionomycin, induced the release of Ca^{+}_{2} ions in the cell, but did not affect the translocation of all NFAT family proteins. These results oriented us to test the translocation and expression of NFAT proteins in different ways. In order to study the effects of PMA/ionomycin treatment on the nuclear translocation of NFAT proteins, we decided to create stable cell lines expressing both NFAT2 and NFAT3 in addition to protein chimeras NP, RP, and CP (Table 4.1).

The HEK293 cell line was used to create new stable cell lines. Cells at 30-50% confluence were seeded in 10 cm plates. Five plates were used for the 5 transfections, after 24 hours, each plate was transfected with 10 μg plasmid DNA illustrated in Figure 4.5. Cultures were incubated for 24 hours in a 37 °C tissue culture incubator, 5% CO_2 before changing the medium. Selection of transfected cells started 48 hours after transfection as mentioned in section (4.2). Briefly, transfected cells were incubated under G418 selection for approximately three weeks, until colonies appeared. Twelve colonies from each transfection isolated and transferred into 24 well-plates, then into 6 well-plates. At this point, cells were examined under the fluorescent microscope, all colonies of all transfections showed cytoplasmic GFP fluorescence indicating that cells were transfected with the NFAT GFP fusion proteins. Finally, 5 colonies from each transfection were selected, grown in 10 cm plates, and frozen in serum containing DMSO as stocks. The colonies that have been chosen for further work were labelled as:

HEK293-T2: Colony 5
HEK293-T3: Colony 1
HEK293-NP: Colony 4
HEK293-RP: Colony 5
HEK293-CP: Colony 1

The new stable cell lines were named depending on the chimeras that were used in transfection and the original cell line that was used for transfection (Table 4.3).

Table 4.3: Chimeric stable cell lines

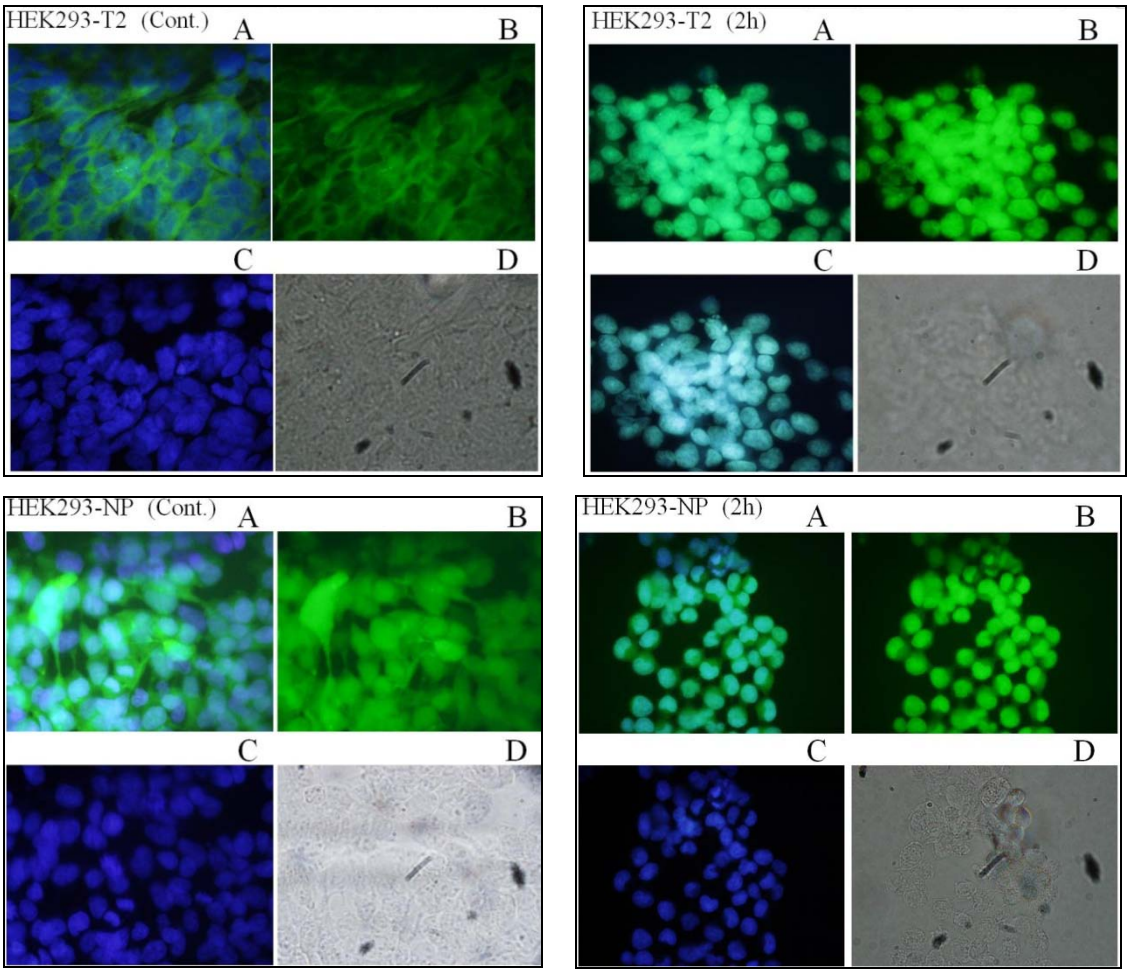
No.	Original cell line	Transfected Plasmid	Main features	New cell line name
1	HEK293	pEGFP-T2	NFAT2 cDNA	HEK293-T2
2	HEK293	pEGFP-T3	NFAT3 cDNA	HEK293-T3
3	HEK293	pEGFP-NP	N-Terminal domain of NFAT3 cDNA and other three domains from NFAT2 cDNA	HEK293-NP
4	HEK293	pEGFP-RP	Regulatory domain of NFAT3 cDNA and other three domains from NFAT2 cDNA	HEK293-RP
5	HEK293	pEGFP-CP	C-Terminal domain of NFAT3 cDNA and other three domains from NFAT2 cDNA	HEK293-CP

4.9 Treatment of stable cells lines expressing chimeric NFAT proteins with PMA and ionomycin

Newly created stable cell lines (Table 4. 3) were treated with 10 ng/ml PMA and 1 μM ionomycin for different periods of time (15 minutes, 2 hours, and 4 hours) to follow the nuclear translocation of different chimeric NFAT proteins. Cells were grown on cover slips placed in 6 well-plates and stained with 500 nM nuclear DAPI stain for 5 minutes after fixation with 4% paraformaldehyde for 20 minutes. Photomicrographs were recorded by the fluorescent microscope under 100X magnification and GFP/DAPI filters.

We observed that PMA and ionomycin can induce the nuclear translocation of NFAT2, NP, and CP chimeric proteins in the cell lines HEK293-T2, HEK293-NP, and HEK293-CP respectively. In these cell lines, GFP fluorescence was observed in the cytoplasm in untreated cells. While the proteins found to reside the nucleus upon PMA + ionomycin treatment for two hours. We found that the translocation of NFAT2, NP,

and CP protein chimeras began within 15 minutes of PMA + ionomycin treatment and most of the protein translocated to the nucleus within two hours (Picture of 15 minutes treatment not shown). On the other hand, the NFAT3 GFP chimeric protein could not translocate from the cytoplasm into the nucleus even when the HEK293-T3 cells were treated for up to four hours. Surprisingly, when HEK293-RP cells were induced by PMA/ionomycin, GFP could be observed in both the nucleus and in the cytoplasm before (Cont.) and after (2h) of PMA/ionomycin treatment (Figure 4.14).



Continue next page …

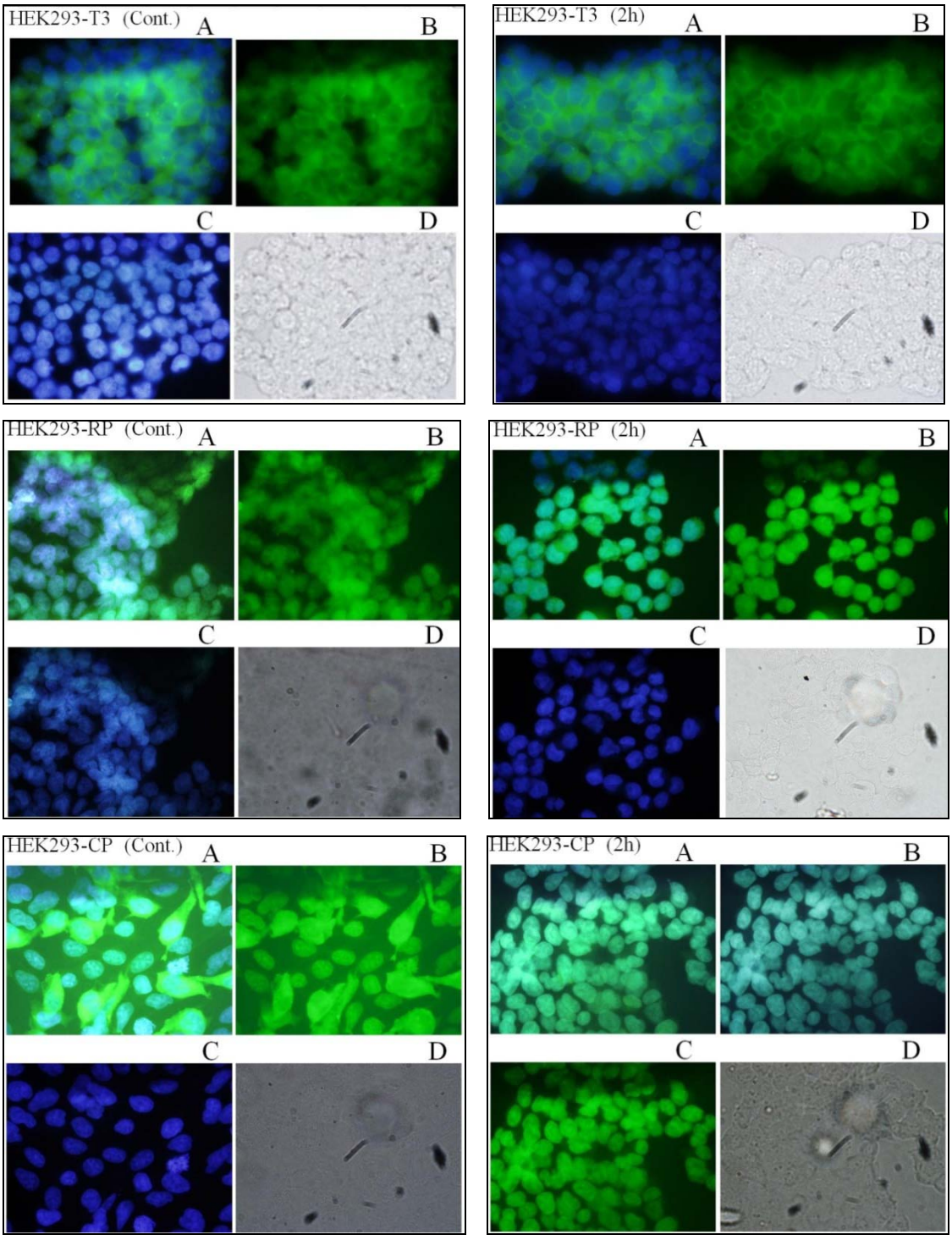


Figure 4.14: PMA/Ionomycin Treatment for Cell Lines Stably Expressing Chimeric NFATs (100X magnification)

Stable cell lines (Table 4.3) treated with 10 ng PMA and 1 μM ionomycin for 2 hours on cover slips placed in 6 well-plates. Left: Cells left untreated as a control (Cont.). Right: Cells were treated with both PMA and ionomycin for 2 hours. (A): Overlaying of B and C. (B): GFP fluorescent filter. (C): DAPI blue fluorescent filter. (D): Normal light with no fluorescence.

4.10 Construction of NFAT2/NFAT3 chimeras that are not fused to GFP proteins

After examining the NFAT2/NFAT3 chimeras to identify the domain necessary for nuclear translocation, we decided to examine the ability of these chimeric proteins to activate NFAT binding site dependent transcription. To do this, we decided to use our HEK293-5.5 reporter cell line. The three expression plasmids encoding pEGFP-NP, pEGFP-RP, and pEGFP-CP, which we used to generate stable cell lines (Table 4.3), expresses different NFAT2/3 chimeras (NP, RP, and CP respectively) which are fused to the GFP protein. Because our stable-transfected HEK293-5.5 cells also express the GFP reporter, we needed to re-clone the three NFAT2/NFAT3 chimeras in a eukaryotic expression vector not expressing GFP.

The pMEX-HA plasmid (Dualsystems) has no GFP reporter and was used as a eukaryotic expression vector. It has both Xho I and Hind III restriction sites in its multiple cloning site which were used to sub-clone NP, RP, and CP cDNAs. The (pEGFP-NP, pEGFP-RP, and pEGFP-CP plasmids) were digested with Xho I and Hind III, purified into the Xho I and Hind III sites of the pMEX-HA plasmid (Figure 4.15).

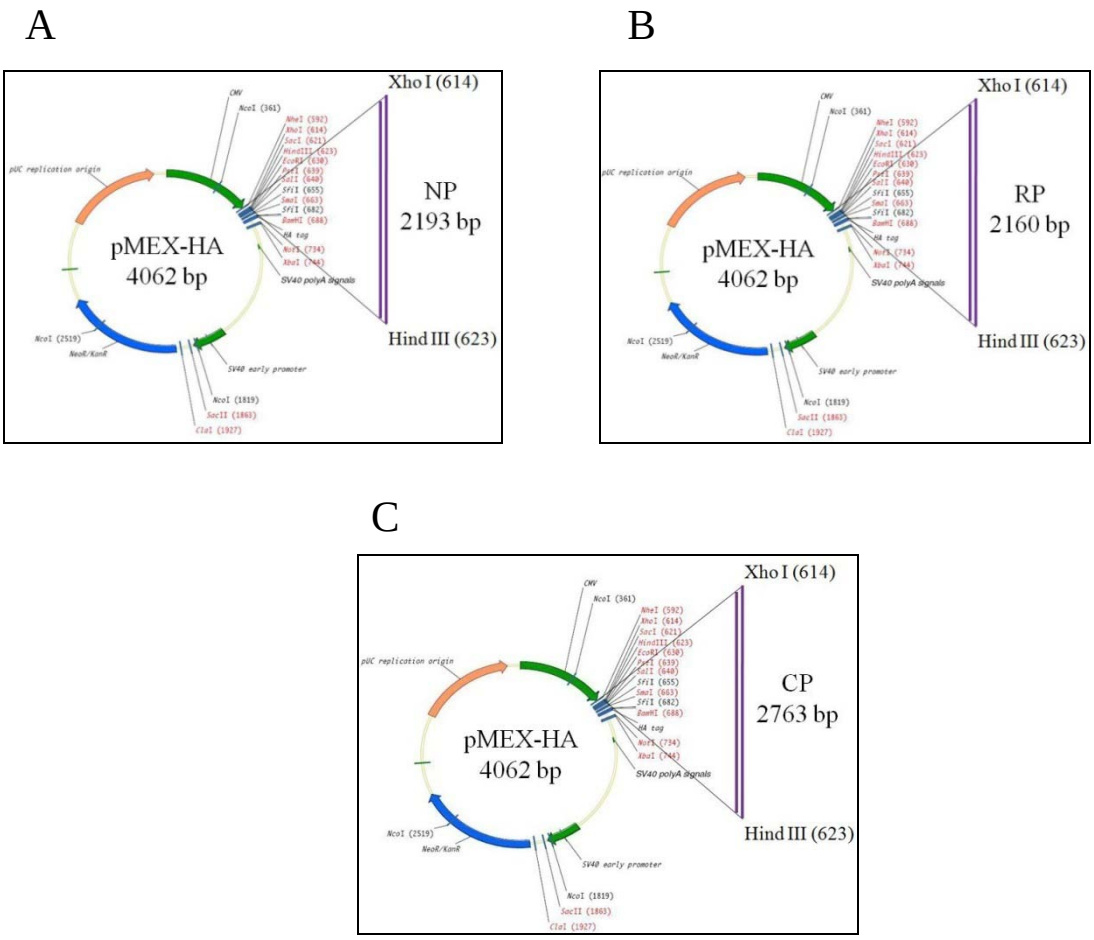


Figure 4.15: Restriction map of the pMEX-HA and inserted regions

(A): pMEX-HA-NP constructs. (B): pMEX-HA-RP constructs. (C): pMEX-HA-CP constructs.

All three chimera plasmids were digested at the same time in parallel with the vector using Xho I and Hind III restriction enzymes (Fermentas). The digest yielded in fragments of the following lengths as shown in Figure 4.16: pEGFP-NP (2193 bp), pEGFP-RP (2160 bp), and pEGFP-CP (2763 bp).

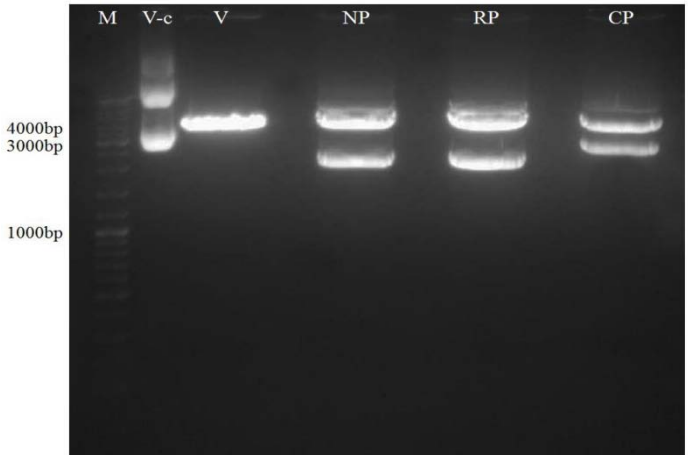


Figure 4.16: Digestion of the pMEX-HA, pEGFP-NP, pEGFP-RP, and pEGFP-CP plasmids.

V-c: Undigested vector (control). V: Xho I single digestion for the vector. NP, RP, and CP: pEGFP-NP, pEGFP-RP, and pEGFP-CP respectively double digested by both Xho I and Hind III restriction enzymes.

The pMEX-HA vector was double digested with Xho I and Hind III (Fermentas) in two reaction tubes, one with the calf intestinal alkaline phosphatase (CIAP) enzyme (Fermentas) and the other without to test the effect of removing the terminal phosphate residues on the linear plasmid ends on the efficiency of insert ligation (Figure 4.17).

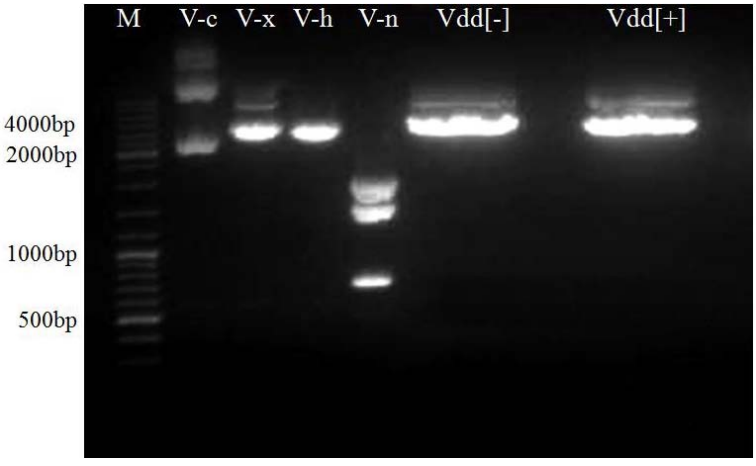


Figure 4.17: pMEX-HA vector double digestion

Digestion for the pMEX-HA vector. V-c: Uncut vector. V-x: Single digestion of vector by Xho I. V-h: Single digestion of vector by Hind III. V-c: Single digestion of vector by NcoI which gives three bands of (700, 1458, and 1904 bp).

The inserts of NP, RP, and CP in (Figure 4.16) and vector (Figure 4.17) were gel extracted using a DNA Extraction Kit (Qiagen). Extracted DNA was loaded on a 1%

agarose gel (Figure 4.18) to compare the DNA concentrations of inserts and vector to determine the amount to be used in the ligation reaction.

Three separate ligation reactions were performed at the same time, one with vector DNA that was not subjected to CIAP enzyme treatment, and the other two using vector DNA that was dephosphorylated using CIAP enzyme (Fermentas). The latter two ligation reactions were performed with two different concentrations of insert (1:10 and 1:2 molar ratio of vector: insert). The reaction was incubated at 16 ˚C overnight. The ligation reactions were transformed into the DH5α bacterial strain grown on LB plates, supplemented by 50 µg/ml Kanamycin antibiotics.

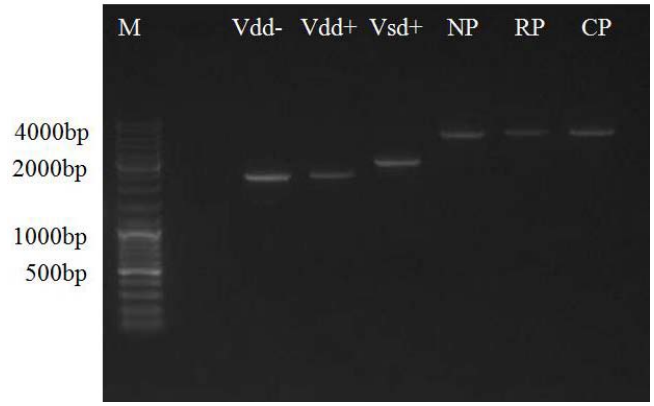


Figure 4.18: Concentration comparison between vector and insert DNA

1 µl of gel extracted vector DNA and 2 µl of insert loaded to compare concentrations for ligation reactions. Vdd-: Double Digested vector without treatment with CIAP. Vdd+: Double digested vector with treatment with CIAP. Vsd+: Sequential digested vector with treatment with CIAP. NP, RP, and CP: The three different insert DNAs containing dimeric NFAT DNAs.

The number of colonies obtained from ligation reactions with CIAP treated vectors was significantly more than the other two, detailed in table 4.4. 8-9 colonies from the plates that contained treated vector and all colonies from plates that contained CIAP treated vectors were collected and cultured. Plasmids were extracted by miniprep extraction kits (Qiagen), and digested with both Xho I and Hind III restriction enzymes (Fermentas). Most colonies contained inserts properly ligated into vectors shown in figures 4.19, 4.20, and 4.21.

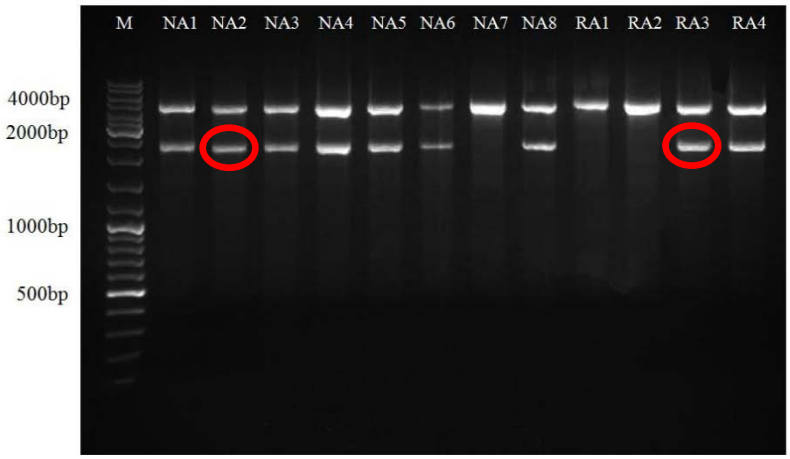


Figure 4.19: NP and RP colony digestion

M: Marker. NA: NP inserts + vector, Ligation 10/1, No CIAP treatment. RA: RP insert + vector, Ligation 10/1, No CIAP treatment. 1, 2, 3, etc.: Colony number.

Eight colonies from NP (NA1-8) and four colonies from RP (RA1-4), (Figure 4.19) originating from the ligation of inserts in the CIAP untreated vector. Colonies extracted by miniprep extraction kit (Qiagen), and double digested with both Xho I and Hind III restriction enzyme. The gel picture (Figure 4.19) shows that 7 of 8 colonies of NP contained the appropriate insert (2193 bp) and 2 of 4 colonies of RP contained the appropriate insert (2160 bp). Circles in (Figure 4.19 and 4.20) indicate the colonies that were selected for further verification.

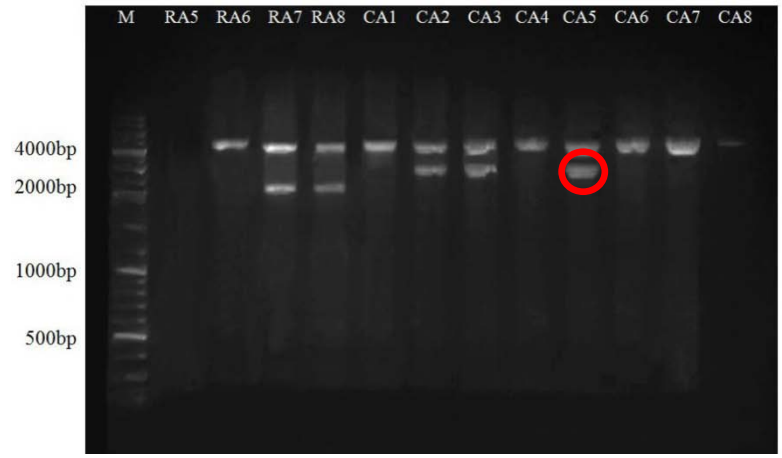


Figure 4.20: RP and CP colony digestion

M: Marker. RA: RP insert + vector, Ligation 10/1, No CIAP treatment. CA: CP insert+ vector, Ligation 10/1, No CIAP treatment. 1, 2, 3, etc.: Colony number.

Four colonies from RP (RA4-8) and nine colonies from CP (CA1-9) (Figure 4.20) originated from the ligation reactions with vector not treated with CIAP. Colonies extracted by miniprep extraction kits, and double digested with both Xho I and Hind III restriction enzymes. The gel picture (Figure 4.20) shows that 2 of 4 colonies of RP contained the appropriate insert (2160 bp) and 3 of 8 colonies of CP contained the appropriate insert (2763 bp). Circles on bands (Figure 4.19 and 4.20) identify the three colonies from NP, RP, and CP ligations and expected to have the insert.

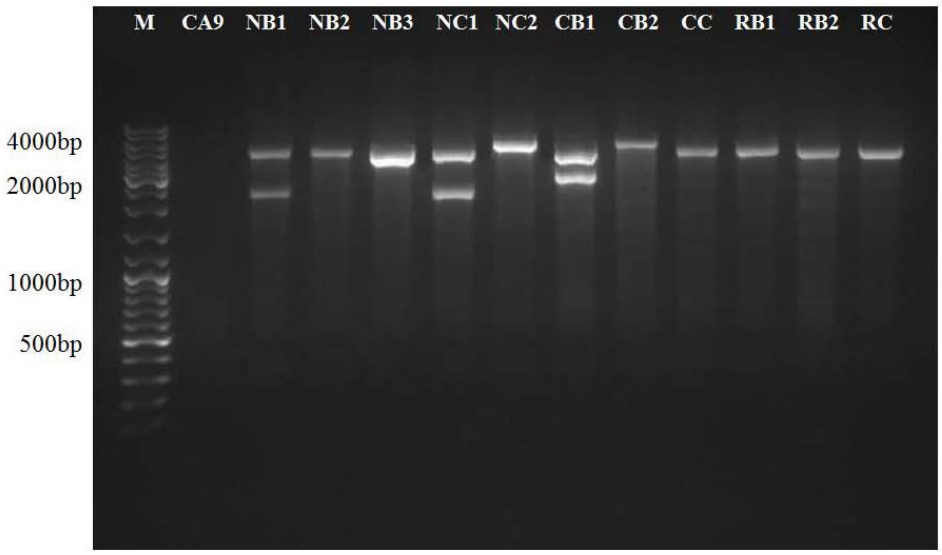


Figure 4.21: NP, RP and CP colony digestion

M: Marker. N, R, C: NP, RP, CP inserts respectively. A: Vector (dd & not treated with CIAP), 1/10. B: Vector (dd & treated with CIAP), 1/10. C: Vector (dd & treated with CIAP), 1/2. 1, 2, 3, etc.: Colony number.

In the ligation reactions containing vectors not treated with CIAP: insert; B (1:10), and C (1:2). Only 11 colonies representing these two ligation reactions grew on kanamycin plates. All colonies were collected and extracted by miniprep extraction kit, then double digested with both Xho I and Hind III restriction enzymes. The gel picture in Figure 4.21 and Table 4.4 show that only 3 of the 11 colonies from RP, RP, and CP were contained the appropriate insert.

Table 4.4: Colonies generated from ligation reactions

Plate	No. of colonies	Picked for digestion	Got the insert
A	200-250		
NA	8	8	7
RA	100-150	8	4
CA	150-200	9	3
B	100-150		
NB	3	3	1
RB	2	2	1
CB	2	2	-
C	100-150		
NC	2	2	1
RC	1	1	-
CC	1	1	-

Three colonies that contained NP, RP, and CP cDNAs cloned into the pMEX-HA plasmid; (circles in figures 4.19 and 4.20) were digested with different restriction enzymes to confirm ligation. All enzyme digestions of the three constructs confirmed that the vector contained the appropriate the right insert (Figures 4.22, 4.23, 4.24 and 4.25).

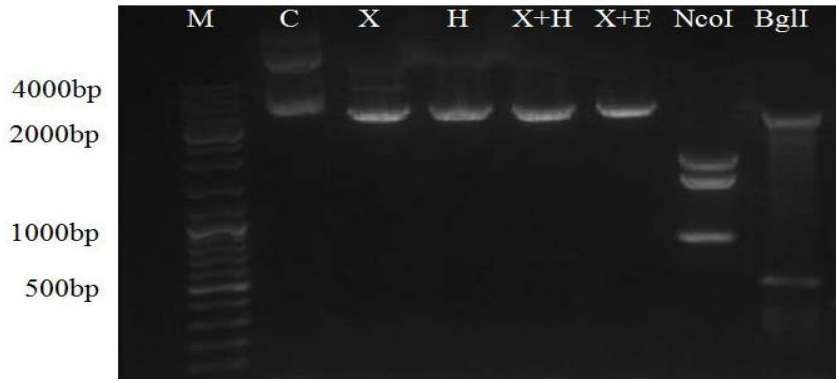


Figure 4.22: pMEX-HA plasmid diagnostic restriction digestions

The original pMEX-HA vector digestion by X: Xho I, H: Hind III, H+H: Xho I Hind III, X+E: Xho I+ EcoRI, NcoI, Bgl I, and C: control.

Diagnostic restriction digest reactions were performed on the PMEX-HA empty vector (Figure 4.22), the pMEX-HA-NP which is expected to have the NP chimera (Figure 4.23), the pMEX-HA-RP which is expected to have the RP chimera (Figure 4.24) and the pMEX-HA-CP which is expected to have the CP chimera (Figure 4.25).

To confirm the proper construction of these plasmids, different enzymes were used in the reaction with appropriate buffers and incubated at 37 ^{0}C for 2.5 hours.

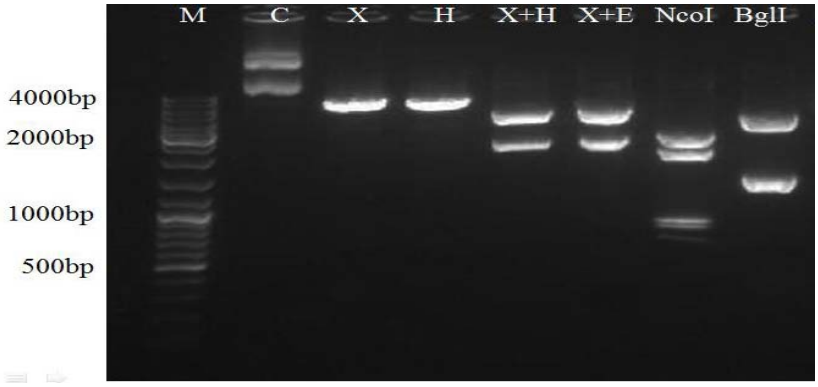


Figure 4.23: pMEX-HA-NP ligation diagnoses

pMEX-HA-NP vector digestion by X: Xho I, H: Hind III, H+H: Xho I+Hind III, X+E: Xho I+ EcoRI, NcoI, Bgl I, and C: control.

Double digestion of constructs by Xho I and Hind III or Xho I and EcoRI confirm that all the constructs have got the right insert. Hence, the new plasmids can be used for transfecting mammalian cell lines to express NFAT chimeric proteins that are not fused to GFP proteins.

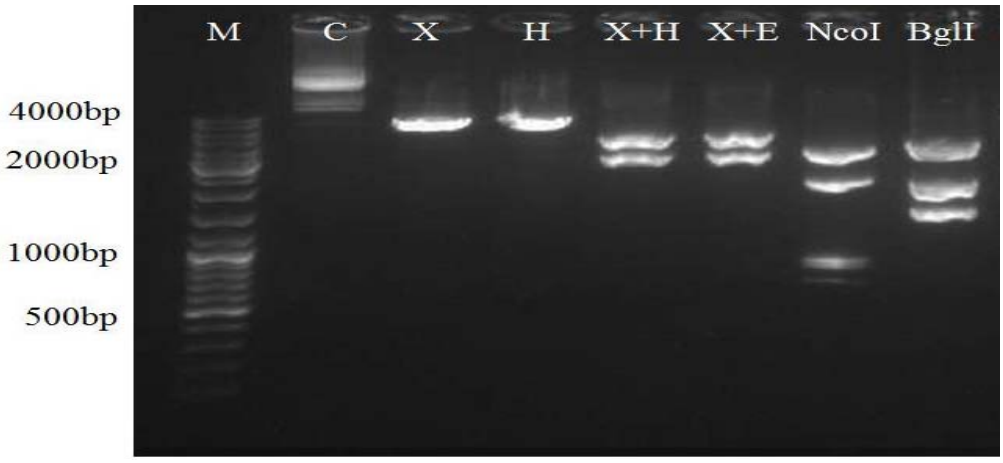


Figure 4.24: pMEX-HA-RP ligation diagnoses

pMEX-HA-RP vector digestion by X: Xho I, H: Hind III, H+H: Xho I+Hind III, X+E: Xho I+ EcoRI, NcoI, Bgl I, and C: control.

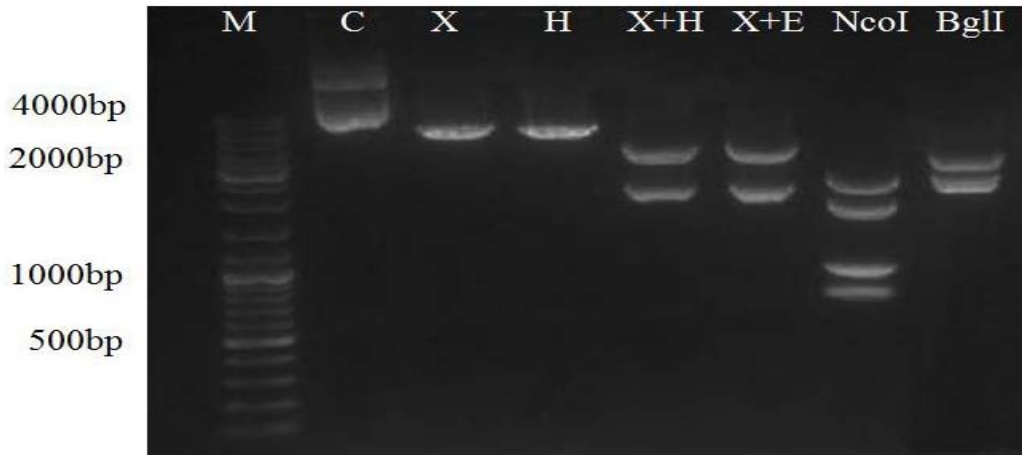


Figure 4.25: pMEX-HA-CP control diagnoses

pMEX-HA-CP vector digestion by X: Xho I, H: Hind III, H+H: Xho I+Hind III, X+E: Xho I+ EcoRI, NcoI, Bgl I, and C: control.

These plasmids (pMEX-HA-NP, pMEX-HA-RP, and pMEX-HA-CP) which do not have GFP proteins fused to NFAT proteins will be used in future transfection experiments in the HEK293-5.5 reporter cell line to identify the domains necessary for transcription activation.

5 DISCUSSION

NFAT binding sites can be found at the promoters of several genes. NFAT binds to the sequence (A/T)GGAAA(A/N)(A/T/C)N16 and is found on both human and mouse ARRE-2 sites in locations having NF-κB-like sites; indicating that, NFAT is related to the NF-κB family [63, 64]. The promoters of several interleukin genes have been found to have multiple NFAT sites, which cooperate to activate the expression of these interleukin genes [68]. For this reason, our plan was to create stable cell lines having multiple NFAT DNA binding sites. The pPO4AT 2-5 plasmid had four DNA binding sites (AGGAAAAAC) for NFAT protein binding. Mammalian HEK293 cells were used for stable transfection, because they are easy transfected cells by the pPO4AT 2-5 plasmid. The stable cell line created using this technique was named HEK293-5.5.

To create the HEK-293-5.5 cell line, we optimized the stable transfection conditions by changing both the plasmid state (linear or circular) and the number of transfected cells. Results in Table 4.3 showed that the number of cells was crucial for transfection, especially when cell number exceeds 2.25 X 10^6 cell/10 cm plates. We observed that plates seeded with $3X10^6$ cells yielded no stably transfected colonies in the plate. We think that cell number is important because cells plated at high density overgrow and die before the G418 antibiotic selection begins at 48 hours after transfection. This long period resulted in cell over growth in the plates, and hence, caused the death of the minority of truly infected cells. These results also showed that there was no significant difference between cells transfected with linear or circular DNA.

When HEK293-5.5 cells were transiently transfected with NFAT protein, they expressed the GFP protein, which confirms that, HEK293-5.5 cells encoding plasmids contained NFAT DNA binding sites. Surprisingly, these cells controlling GFP

transfection showed GFP expression regardless to the treatment by PMA and Ionomycin. The most likely reason for this unexpected result is that the intracellular Ca^{+}_{2} ions were already induced in the cells by the transfection technique itself, because the cells were transfected by $CaCl_2$ transfection. Alternatively translocation may have been caused by the over expression of the NFAT proteins.

Chimeras used in this study were designed to be transfected into mammalian cells in order to investigate their response to PMA and ionomycin treatment, and therefore to examine the domains responsible for the nuclear translocation of NFAT2 and NFAT3. The decision to use NFAT2 and NFAT3 was based on the experiments which were previously performed on cervical cancer cells and brain tumor cells by Pinar Ayata, a member of the Erman Lab. These experiments showed that NFAT2 translocates to the nucleus upon both PMA and ionomycin treatment while NFAT3 does not follow this rule. Two of the five chimeras (Figure 4.5), represent NFAT2 and NFAT3 cDNAs fused to the GFP reporter, while the remaining three were built by including various domains coming from N2 and N3. These chimeras contain the N-terminal domain (NP), regulatory domain (RP), and C-terminal domain (CP) of NFAT3 expressed in the context of the rest of the NFAT2 protein. The DNA binding domain of NFAT3 was not subjected to examination because it is highly conserved among different NFAT family members.

Although, the the N-terminal and C-terminal domains are highly diverse among NFAT proteins, indicating a possible criterion for functional differences [33, 57], they were not responsible for NFAT sub-cellular localization as shown in figures 4.12, 4.13, and 4.14. The results of our experiments confirm that, the NFAT regulatory domain which has conserved regions such as calcineurin binding sites, phosphorylation sites, nuclear localization and export sequences is the critical domain for NFAT nuclear translocation.

Two experiments were done to optimize transient transfection efficiency; one to determine the DNA concentration, and the other to adjust the incubation time after transfection. Flow cytometry experiments (Figure 4.7) showed significant increases in the transfection efficiency DNA concentration was increased. Transfecting the cell

cultures with 5 μg/6 well-plate of pEGFP-NP plasmid resulted in the highest fluorescence (73% FITC positive).

For the optimization of the duration of incubation time after transfection efficiency, flow cytometry experiments (Figure 4.8) showed a significant increase in the efficiency when post transfection period increased from 8-16 hours and from 16-20 hours, but no significant increase in the transfection efficiency was observed when the incubation increased over 20 hours, while there was a slight decrease in the cell viability. We decided to perform further experiments by incubating cells for 20 hours after transfection.

Here, it is interesting to mention that transfected but untreated reporter cells showed GFP fluorescence, indicating the treatment of HEK293-5.5 cells with PMA and ionomycin did not affect the nuclear translocation of NFAT protein in this case. In the previous experiments we could not detect the impact of PMA and ionomycin in the translocation of NFAT proteins. Therefore, there was a need to re-examine their effect again but with a different strategy. We decided to create cell lines permanently expressing these proteins, for this purpose, five stably transfected cell lines were created, each express one of the five chimeras (Table 4.4). The new stable cell lines were generated in the HEK293 cell line, and permanently express the chimeras listed in (Table 4.1).

Cells treated with 10 nM PMA and 1 μg ionomycin for different periods of time to follow the nuclear translocation of the different NFAT chimeras. The results (Figure 4.14) confirm the effect of both PMA and ionomycin in making the NFAT2 transcription factor move to the nucleus. The translocation of NFAT2, NP, and CP protein chimeras all begin within 15 minutes and most of the protein translocates within two hours. On the other hand, the NFAT3 protein could not translocate from cytoplasm into the nucleus even when the cells were treated with PMA and ionomycin for up to four hours. Surprisingly, when HEK293-RP cells expressing the RP chimera were induced by PMA/ionomycin, the nuclear translocation also occurred. This result contradicts the hypothesis that the regulatory domain is responsible for the difference in the nuclear translocation phenotype between NFAT2 and NFAT3. The most possible

reason for this result is that the RP chimeric cDNA may have mutations affecting its nuclear translocation, because the cells showed GFP in both the nucleus and cytoplasm before and after PMA/ionomycin treatment (Figure 4.14). There is a need to confirm the RP construct by sequencing and to generate new stable cell lines expressing this NFAT chimera of RP.

When HEK293-5.5 cells transfected with NFAT1-4 CDNAs, flow cytometry results (Figure 4.11) showed that the only protein that could activate the reporter was NFAT2. It is possible that the inability of the other proteins to activate the reporter stems from their mobility to be expressed. There is a need to perform western blotting on lysates from these transfected cells in order to determine the expression of transfected proteins. Unexpected results also were found when the constructs; pMEX-HA-NP, pMEX-HA-RP, and pMEX-HA-CP were transfected into HEK293-5.5 cells. We could not detect any GFP expression under the fluorescent microscope. In these experiments, transfection efficiency needs to be optimized. It is important to sequence the new constructs to ensure the proper expression potential of these plasmids.

Our observations in this study show that NFAT2 protein can move from the cytoplasm into the nucleus as a result of PMA and ionomycin induction, while NFAT3 could not. There are two possible reasons for this phenotype; the first possibility is the presence of putative activator proteins (Figure 5.1-A) that bind to NFAT2 and induce the protein to move from the cytoplasm into the nucleus. It is possible that this protein which resides in the cytoplasm cannot bind to NFAT3 resulting in cytoplasmic localization of NFAT3 even after treatment. The second possibility is the presence of a putative inhibitor protein (Figure 5.1-B), that can bind to NFAT3 protein preventing it from nuclear translocation, while the same protein may not able to bind NFAT2, and so does not stop its nuclear import.

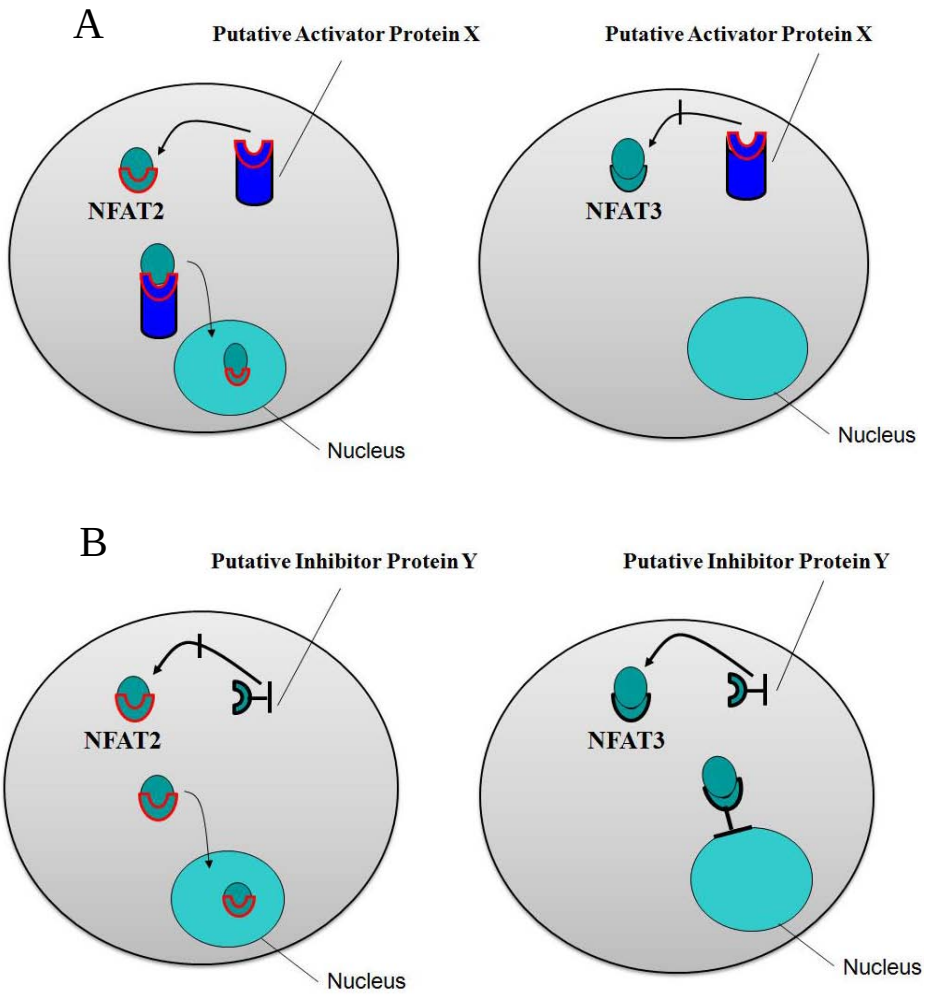


Figure 5.1: Proposed model for NFAT2 and NFAT3 nuclear translocation and activation of transcription

(A). A putative activator protein that binds to NFAT2 but not NFAT3 resulting in the translocation of NFAT2 but not NFAT3. (B). A putative inhibitor protein that binds to NFAT3 but not NFAT2 resulting in the prevention of nuclear translocation of NFAT3 but not NFAT2.

From the results we can say that, putative protein/proteins should bind the regulatory domain of the NFAT transcription factors, either to activate or to inhibit the protein translocation. Although we have not experimentally demonstrated a direct interaction between NFAT family members and the calcineurin phosphatase, the N-terminal region of all NFAT members contain the PXIXIT motif, which is responsible for recognition by calcineurin. For this reason we think that both NFAT2 and NFAT3 proteins can be bound by calcineurin. However, the putative protein/proteins that bind to (or act through) the regulatory domains of NFAT2 or NFAT3 may be preventing or accelerating the dephosphorylation of NFAT by calcineurin.

6 CONCLUSION

NFAT is a family of Ca^{+}_{2}/calcineurin-dependent transcription factor that were initially characterized in T-lymphocytes with an important role in coordinating the transcription of different genes during the immune response. NFAT proteins are highly phosphorylated transcription factors that reside in the cytoplasm of dormant cells. The nuclear import of NFAT proteins requires calcium/calmodulin-dependent serine phosphatase calcineurin.

In this study we followed the subcellular localization of NFAT2 and NFAT3 upon PMA/ionomycin treatment by two complementary approaches. In the first, by transient transfection of HEK293T cells by different GFP-tagged NFAT chimeras. The second, we created reporter cell lines permanently expressing NFAT2 and NFAT3 chimeras. We found that NFAT2 begins to leave the cytoplasm to the nucleus within 15 minutes of stimulation and most of the proteins were translocated to the nucleus within two hours. We found that the regulatory domain of NFAT proteins is the critical domain for nuclear import. We created another set of stable cell lines having four NFAT DNA binding sites driving the expression of the GFP reporter using the calcium phosphate method. We used this cell line to test the ability of NFAT family members to translocate to the nucleus and activate transcription. We found that NFAT2 was unique in activating transcription in this cell line system. Taken together with the domain mapping studies, these experiments indicate that the regulatory domain of NFAT proteins is important in their nuclear translocation and transcription activation. These experiments are the first step forwards the identification of novel putative proteins participating in the NFAT transcription/translocation pathway. Further studies should be conducted to discover these proteins.

7 FURTHER STUDIES

In the NFAT transcription pathway, Ca^{+}_{2} ions are associated with calmodulin, forming a complex that binds with high affinity to the calcineurin protein phosphatase. Although several kinases have been identified to play a role in NFAT translocation, there is no integrated picture of NFAT kinases and no proteins have been identified to be responsible for NFAT2 translocation but not NFAT3 upon PMA/ionomycin treatment.

We think that novel proteins may be responsible for binding the NFAT regulatory domain and controlling nuclear translocation. There is a need to search for the putative protein/proteins using methods such as the yeast two-hybrid assay and to analyze the protein complexes using biochemical techniques such as crosslinking and co-immunoprecipitation.

A search for point differences between the regulatory domain sequences in both NFAT2 and NFAT3 using bioinformatics programs will likely identify putative protein-protein interaction regions within this regulatory domain.

8 REFERENCES

1. Luo, C., Burgeon, E., & Rao, A. Mechanisms of transactivation by nuclear factor of activated T cells-1. *J Exp Med* **184**, 141-7 (1996).

2. Stroud, J., & Chen, L. Structure of NFAT bound to DNA as a monomer. *J Mol Biol* **334**, 1009-22 (2003).

3. McCaffrey, PG., Luo, C., Kerppola, TK., Jain, J., Badalian, TM, Ho, AM., Burgeon, E., Lane, WS., Lambert, JN., Curran, T., Verdine, G., Rao, A., & Hogan, P. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750–4 (1993).

4. Northrop, JP., Ho, SN., Chen, L., Thomas, DJ., Timmerman, LA., Nolan, GP., Admon, A., & Crabtree, GR. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* **369**, 497–502 (1994).

5. Hoey, T., Sun, YL., Williamson, K., & Xu, X. Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* **2**, 461–72 (1995).

6. Ho, SN., Thomas, DJ., Timmerman, LA., Li, X., Francke, U., & Crabtree, GR. NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. *J Biol Chem* **270**, 19898–907 (1995).

7. Masuda, ES., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K., & Arai, N. NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. *Mol Cell Biol* **5**, 2697–706 (1995).

8. Timmerman, LA., Healy, JI., Ho, SN., Chen, L., Goodnow, CC. & Crabtree, GR. Redundant expression but selective utilization of nuclear factor of activated T cells family members. *J Immunol* **159**, 2735–40 (1997).

9. Hogan, PG., Chen, L., Nardone, J., & Rao, A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* **17**, 2205-32 (2003).

10. Sin-Hyeong, I., & Rao, A. Activation and deactivation of gene expression by Ca^{+}_{2}/calcineurin-NFAT-mediated signaling. *Molecules and Cells* **18**, 1-9 (2004).

11. McClean, MN., Mody, A., Broach, JR., & Ramanathan, S. Cross-talk and decision making in MAP kinase pathways. *Nat Genet* **39**, 409–14 (2007).

12. Kiessling, LL., Gestwicki, JE., & Strong, LE. Synthetic Multivalent Ligands as Probes of Signal Transduction. *Angew Chem Int Ed Engl* **45**, 2348 –68 (2006).

13. Komarova, NL., Zou, X., Nie, Q., & Bardwell, L. A theoretical framework for specificity in cell signaling. *Mol Syst Biol* **1**, 2005-23 (2005).

14. Kin, NW., & Sanders, VM. It takes nerve to tell T and B cells what to do. *J Leukoc Biol* **79**, 1093-104 (2006).

15. Lyakh, L., Ghosh, P., & Rice, NR. Expression of NFAT-family proteins in normal human T cells. *Mol Cell Biol* **17**, 2475-84 (1997).

16. Torgersen, KM., Aandahl, EM., & Taskén, K. Molecular architecture of signal complexes regulating immune cell function. *Handb Exp Pharmacol* **186**, 327-63 (2008).

17. Katzav, S. Vav1: an oncogene that regulates specific transcriptional activation of T cells. *Blood* **103**, 2443-51 (2004).

18. Quintana, A., Griesemer, D., Schwarz, E., & Hoth, M. Calcium-dependent activation of T-lymphocytes. *Pfluquers Arch* **450**, 1-12 (2005).

19. Puri, S., Magenheimer, BS., Maser, RL., Ryan, EM., Zien, CA., Walker, DD., Wallace, DP., Hempson, SJ., & Calvet, JP. Polycystin-1 Activates the Calcineurin/NFAT (Nuclear Factor of Activated T-cells) Signaling Pathway. *J Biol Chem* **279**, 55455-64 (2004).

20. Clapham, DE. Calcium signaling. *Cell* **80**, 259–68 (1995).

21. Berridge, MJ. Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–25 (1993).

22. Elena, A., & Valery, K. The influence of diffusion on stability in continuous/discrete media. *Nonlinear Analysis* **30**, 4799-804 (1997).

23. Ferrari, D., Stroh, C., & Schulze-Osthoff, K. P2X7/P2Z purinoreceptor-mediated activation of transcription factor NFAT in microglial cells. *J Biol Chem* **274**, 13205-10 (1999).

24. Lazarevic, V., Zullo, AJ., Schweitzer, MN., Staton, TL., Gallo, EM., Crabtree, GR., & Glimcher, LH. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol* **10**, 306-13 (2009).

25. Cockerill, GW., Bert, AG., Ryan, GR., Gamble, JR., Vadas, MA., & Cockerill, PN. Regulation of granulocyte-macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT. *Blood* **86**, 2689-98 (1995).

26. Kelly, K., & Siebenlist, U. Immediate-early genes induced by antigen receptor stimulation. *Curr Opin Immunol* **7**, 327-32 (1995).

27. Quintana, A., Griesemer, D., Schwarz, EC., and Hoth, M. Calcium-dependent activation of T-lymphocytes. *Pflugers Arch* **450**, 1-12 (2005).

28. Pan S, Tsuruta, R., Masuda, ES., Imamura, R., Bazan, F., Arai, K., Arai, N., & Miyatake, S. NFATz: a novel rel similarity domain containing protein. *Biochem Biophys Res Commun* **272**, 765-76 (2000).

29. Turner, H., Gomez, M., McKenzie, E., Kirchem, A., Lennard, A., & Cantrell, DA. Rac-1 regulates nuclear factor of activated T cells (NFAT) C1 nuclear translocation in response to Fcepsilon receptor type 1 stimulation of mast cells. *J Exp Med* **188**, 527-37 (1998).

30. Ichida, M., & Finkel, T. Ras regulates NFAT3 activity in cardiac myocytes. *J Biol Chem* **276**, 3524-30 (2001).

31. Puri, S., Magenheimer, BS., Maser, RL., Ryan, EM., Zien, CA., Walker, DD., Wallace, DP. Polycystin-1 Activates the Calcineurin/NFAT Signaling Pathway. *J Biol Chem* **279**, 55455-64 (2004).

32. Horsley, V., Friday, BB., Matteson, S., Kegley, KM, G., & Gephart, J. Regulation of the Growth of Multinucleated Muscle Cells by an NFATC2-dependent Pathway . *J Cell Biol* **153**, 329-38 (2001).

33. Hogan, PG., Chen, L., Nardone, J. & Rao, A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Development* **17**, 2205-32 (2003).

34. Rao, A., Luo, C., & Hogan, PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15**, 707–47 (1997).

35. Cante-Barrett, K., Winslow, MM., Crabtree, GR. Selective Role of NFATc3 in Positive Selection of Thymocytes. *The Journal of Immunology* **179**, 103–10 (2007).

36. Wang, J., Gardner, BM., Lu, Q., Rodova, M., Woodbury, BG., Yost, JG., Roby, KF., Pinson, DM., Tawfik, O., and Anderson, HC. Transcription factor Nfat1 deficiency causes osteoarthritis through dysfunction of adult articular chondrocytes. *J Pathol* **21,** Epub ahead of print (2009).

37. Rao, A. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. *Immunol Today* **15**, 274-81 (1994).

38. Canté-Barrett, K., Winslow, MM., and Crabtree, GR. Selective role of NFATc3 in positive selection of thymocytes. *J Immunol* **179**, 103-10 (2007).

39. Tu, VC., Sun, H., Bowden, GT., & Chen, QM. Involvement of oxidants and AP-1 in angiotensin II-activated NFAT3 transcription factor. *Am J Physiol Cell Physiol* **292**, C1248-55 (2007).

40. Graef, IA., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M., & Crabtree, GR. Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**, 657-70 (2003).

41. Benedito, AB., Lehtinen, M., Massol, R., Lopes, UG., Kirchhausen, T., Rao, A., & Bonni, A. The transcription factor NFAT3 mediates neuronal survival. *The Journal of Biological Chemistry* **280**, 2818-25 (2004).

42. Yaghi, A., & Sims, SM. Constrictor-induced translocation of NFAT3 in human and rat pulmonary artery smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **289**, L1061-74 (2005).

43. Xia, X., McMillin, JB., Lewis, A., Moore, M., Zhu, WG., Williams, RS., & Kellems, RE. Electrical stimulation of neonatal cardiac myocytes activates the NFAT3 and GATA4 pathways and up-regulates the Adenylosuccinate Synthase 1 gene. *J Biol Chem* **275**, 1855-63 (2000).

44. Benedito, AB., Lehtinen, M., Massol, R., Lopes, UG., Kirchhausen, T., Rao A, & Bonni, A. The transcription factor NFAT3 mediates neuronal survival. *J Biol Chem* **280**, 2818-25 (2005).

45. Yaghi, A., & Sims, SM. Constrictor-induced translocation of NFAT3 in human and rat pulmonary artery smooth muscle. *Am J Physiol Lung Cell Mol* Physiol **289**, L1061-74 (2005).

46. Ichida, M., & Finkel, T. Ras regulates NFAT3 activity in cardiac myocytes. *J Biol Chem* **276**, 3524-30 (2001).

47. Zhang, H., Xie, X., Zhu, X., Zhu, J., Hao, C., Lu, Q., Ding, L., Liu, Y., Zhou, L., Liu, Y., Huang, C., Wen, C, & Ye, Q. Stimulatory cross-talk between NFAT3 and estrogen receptor in breast cancer cells. *J Biol Chem* **280**, 43188-97 (2005).

48. Zhang, H., Wang, X., Li, J., Zhu, J., Xie, X., Yuan, B., Yang, Z., Zeng, M., Jiang, Z., Li, J., Huang, C., & Ye, Q. Tissue type-specific modulation of ER transcriptional activity by NFAT3. *Biochem Biophys Res Commun* **353**, 576-81 (2006).

49. Oukka, M., Ho, IC., de la Brousse, FC., Hoey, T., Grusby, MJ., & L. Glimcher, LH. The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* **9**, 295–304 (1998)**.**

50. Lee, SD., Colla, E., Sheen, MR., Na, KY., & Kwon, HM. Multiple domains of TonEBP cooperate to stimulate transcription in response to hypertonicity. *J Biol Chem* **278**, 47571−7 (2003).

51. Drews-Elger, K., Ortells, MC., Rao, A., López-Rodriguez, C., and Aramburu, J. The transcription factor NFAT5 is required for cyclin expression and cell cycle progression in cells exposed to hypertonic stress. *PLoS One* **4**, e5245 (2009).

52. Ranger, AM., Oukka, M., Rengarajan, J., & Glimcher, LH. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* **9**, 627–35 (1998).

53. Jain, PK., Fukushima, K., Deshmukh, D., Ramesh, A., Thomas, E., Lalwani, AK., Kumar, S., Plopis, B., Skarka, H., & Srisailapathy, CR. A human recessive neurosensory nonsyndromic hearing impairment locus is potential homologue of murine deafness (dn) locus. *Hum Mol Genet* **4**, 2391-4 (1995).

54. Shaw KT, Ho. AM., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A., & Hogan, PG. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc Natl Acad Sci* **92**, 11205-9 (1995).

55. Vihma, H., Pruunsild, P., and Timmusk, T. Alternative splicing and expression of human and mouse NFAT genes. *Genomics* **92**, 279-91 (2008).

56. Crabtree, GR., & Olson, EN. NFAT Signaling: Choreographing the Social Lives of Cells. *Cell* **109**, **S67-**79 (2002).

57. Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, JP., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, PG., & Rao, A. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* **6**, 539-50 (2000).

58. Yang, TT., Xiong, Q., Enslen, H., Davis, RJ., & Chow, CW. Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. *Mol Cell Biol* **11**, 3892-904 (2002).

59. Yang, TT., Yu, RY., Agadir, A., Gao, GJ., Campos-Gonzalez, R., Tournier, C., & Chow, CW. Integration of protein kinases mTOR and extracellular signal-regulated kinase 5 in regulating nucleocytoplasmic localization of NFATc4. *Mol Cell Biol* **10**, 3489-501 (2008)**.**

60. Fan, Y., Xie, P., Zhang, T., Zhang, H., Gu, D., She, M., & Li, H. Regulation of the stability and transcriptional activity of NFATc4 by ubiquitination. *FEBS lett* **582**, 4008-14 (2008).

61. Burke, F., Casolaro, V., & Georas, S. Characterization of P5, a novel NFAT/AP-1 site in the human IL-4 promoter. *Biochem Biophys Res Commun* **270**, 1016-23 (2000).

62. Rooney, JW., Sun, YL., Glimcher, LH., & Hoey, T. Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. *Mol Cell Biol* **15**, 6299-310 (1995).

63. McCaffrey, PG., Goldfeld, AE. & Rao, A. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-α gene transcription. *J Biol Chem* **269**, 30445–50 (1994).

64. McCaffrey, PG., Luo, C., Kerppola, TK., Jain, J., Badalian, TM., Ho, AM., Burgeon, E., Lane, WS., Lambert, JN., & Curran T. Isolation of the

cyclosporinsensitive T cell transcription factor NFATp. *Science* **262**, 750–4 (1993).

65. Shaw, JP., Utz, PJ., Durand, DB., Toole, JJ., Emmel, EA., & Crabtree, GR. Identification of a putative regulator of early T cell activation genes. *Science* **241**, 202–5 (1988).

66. Novak, T., White, P., & Rothenberg E. Regulatory anatomy of the murine interleukin-2 gene. *Nucleic Acids Res* **18**, 4523-33 (1990).

67. Thompson, C., Wang, C., H., Bohjanen, P., Petryniak, B., June, C., Miesfeldt, S., Zhang, L., Nabel G., & Karpinski, B. Cis-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. *Mol Cell Biol* **12**, 1043-53 (1992).

68. Waldmann, TA. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem* **58**, 875-911 (1989).

69. Meyer, KB., & Ireland, J. PMA/ionomycin induces Ig kappa 3' enhancer activity which is in part mediated by a unique NFAT transcription complex. *Eur J Immunol* **5**, 1467-80 (1998)**.**

70. Shibasaki, F., Price, E.R., Milan, D., & McKeon, F. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370–3 (1996)**.**

71. Aramburu, J., Heitman, J., & Crabtree, GR. Calcineurin: a central controller of signalling in eukaryotes. *EMBO Rep* **5**, 343-8 (2004).

72. Klee, C., Crouch, T., & Krinks, M. Calcineurin: a calcium and calmodulin-binding protein of the nervous system. *Proc Natl Acad Sci* **76**, 6270–3 (1979).

73. Lambrechts, D., & Carmeliet, P. Sculpting Heart Valves with NFATc and VEGF. *Cell* **118**, 532-4 (2004).

74. Clipstone, N., & Crabtree, GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **6380**, 695-7 (1992).

75. Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, AD., Stroud, JC., Bates, DL., Guo, L., Han, A., Ziegler, SF., Mathis, D., Benoist, C., Chen, L., & Rao A. FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell* **126**, 375–87 (2006).

76. Freedman, B. Mechanisms of calcium signaling and function in lymphocytes. *Crit Rev Immunol* **26**, 97-111 (2006).

77. Hori, S., Nomura, T., & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–61 (2003).

78. Fontenot, J., Gavin, M., & Rudensky, A. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330–6 (2003).

79. Ho, L., &Crabtree, GR. A Foxy tango with NFAT. *Nat Immunol* **7**, 906-8 (2006).

80. Hu, H., Djuretic, I., Sundrud, MS., & Rao, A. Transcriptional partners in regulatory T cells: Foxp3, Runx and NFAT. *Trends Immunol* **8**, 329-32 (2007).

81. Scott, ES., Malcomber S., & O'Hare, P. Nuclear Translocation and Activation of the Transcription factor NFAT Is Blocked by Herpres Simplex Virus Infection. *J Virol* **75**, 9955-65 (2001).

82. Graham, FL., Smiley, J., Russell, WC., & Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type **5**. *J Gen Virol* **36**, 59–74 (1977).

83. Nau, GJ., Kim, DK., and Fitch, FW. Agents that mimic antigen receptor signaling inhibit proliferation of cloned murine T lymphocytes induced by IL-2. *J Immunol* **141**, 3557-63 (1988).

84. Sambrook, Joseph & Russell, David W. *Molecular cloning : a laboratory manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).

85. Pinar, O. Screening of plant factors that modulate the activation of the NF-kB transcription factor in mammalian cells. Sabanci University, *Master thesis* (2007).

APPENDIX A

The list below shows the primers designed using to the PCR. Tm indicates the melting temperature of the primer, without the flanking regions. Calculations are made by the *Primer Melting Temperature Calculation* program by ITT Biotech.

Primer	Sequence (5' to 3')	Tm / ºC
N2F	attagcctcgagatgccaaataccagctttcca	57.5
N2R	ccgttcaagctttcagtaaaaacctcctctca	54.2
N3F	attagcctcgagatgggggccgcaagctgcgag	71.7
N3R	ccgttcaagctttcaggcaggaggctcttctc	63.7
NT2	gggtctggtgttgcctgcattacctcctgg	51.4
NT3	ccaggaggtaatgcaggcaacaccagaccc	58.4
RD1	tacttcctgtcctctgggggtgctgggggt	69.4
RD2	aagctcgtatggaccatactggctgggtaa	48.5
RD4	acccccagcacccccagaggacaggaagta	48.1
RD5	ttacccagccagtatggtccatacgagctt	50.9
CT1	cgtttcacgtaccttcctgtggtcttcaag	50.1
CT4	cttgaagaccacaggaaggtacgtgaaacg	48.6

APPENDIX B

The stable transfected cell colonies which have been frozen as stocks in the liquid nitrogen tank are listed below:

Box 3.2

Colonies from the HEK293 cells line which was stably transfected with pEGFP-N2 plasmid in A+B rows, pEGFP-Np in C+D rows, pEGFP-RP in E+F rows, and pEGFP-N3 in G+H rows. x and y axis: (x); 2: NFAT2 chimera, 3: NFAT3 chimera, NP: NP chimera, RP: RP chimera. (y); colony number.

	1	2	3	4	5	6	7	8	9
A	2.1	2.1	2.1	2.2	2.2	2.2	2.3	2.3	2.3
B	2.4	2.4	2.4	2.5	2.5	2.5	-	-	-
C	NP1	NP1	NP1	NP2	NP2	NP2	NP3	NP3	NP3
D	NP4	NP4	NP4	NP5	NP5	NP5	-	-	-
E	RP1	RP1	RP1	RP2	RP2	RP2	RP3	RP3	RP3
F	RP4	RP4	RP4	RP5	RP5	RP5	RP6	RP6	RP6
G	3.1	3.1	3.1	3.2	3.2	3.2	3.3	3.3	3.3
H	3.4	3.4	3.4	3.5	3.5	3.5	3.6	3.6	3.6

Box 3.3

Colonies from the HEK293 cell line that was stably transfected with pPO4AT 2-5 plasmid which have 4 NFAT binding sites in A-E raws. Colonies from the HEK293 cell line that was stably transfected with pEGFP-CP plasmid in H+I rows. x and y axis: (x); CP: CP chimera. (y); colony number.

	1	2	3	4	5	6	7	8	9
A	1.22	1.22	1.22	2.3	2.3	2.3	2.4	2.4	-
B	2.2	2.2	2.2	4.1	4.1	-	-	4.2	4.2
C	4.3	4.3	4.3	4.5	4.5	4.5	4.7	4.7	4.7
D	2.3	2.3	2.3	4.10	4.10	4.10	-	4.8	4.8
E	4.9	4.9	4.9	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-
G	CP1	CP1	CP1	CP2	CP2	CP2	CP3	CP3	CP3
H	CP4	CP4	CP4	CP5	CP5	CP5	CP6	CP6	CP6

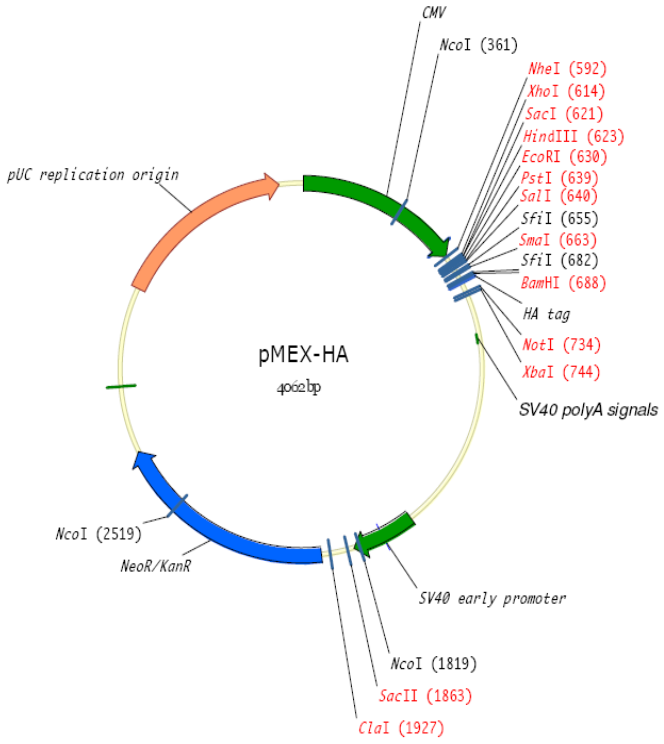
Box 3.4

Colonies from the HEK293 cell line was stably transfected with pPO4AT 2-5 plasmid which have 4 NFAT binding sites. x and y axis: (x); plate number. (y); colony number.

	1	2	3	4	5	6	7	8	9
A	5.6	5.6	5.6	5.7	5.7	5.7	5.20	5.20	5.20
B	5.8	5.8	5.8	5.4	5.4	5.4	5.15	5.15	5.15
C	5.18	5.18	5.18	5.2	5.2	5.2	5.1	5.1	5.1
D	5.3	5.3	5.3	5.3	5.3	4.10	-	5.5	5.5
E	3.2	3.2	-	4.12	4.12	4.12	4.4	4.4	4.4
F	-	-	-	-	-	-	-	-	-
G	1.18	1.18	1.18	1.20	1.20	1.20	1.21	1.21	1.21
H	1.17	1.17	1.17	1.16	1.16	1.16	1.15	1.15	1.15
I	1.6	1.6	1.6	1.4	1.4	1.4	1.3	1.3	1.3

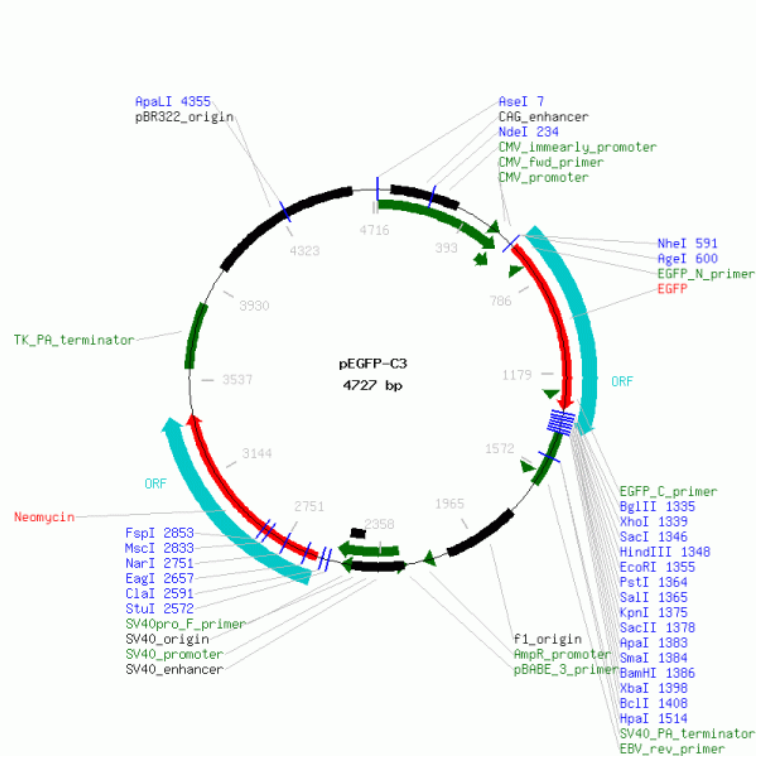
APPENDIX C

pMEX-HA



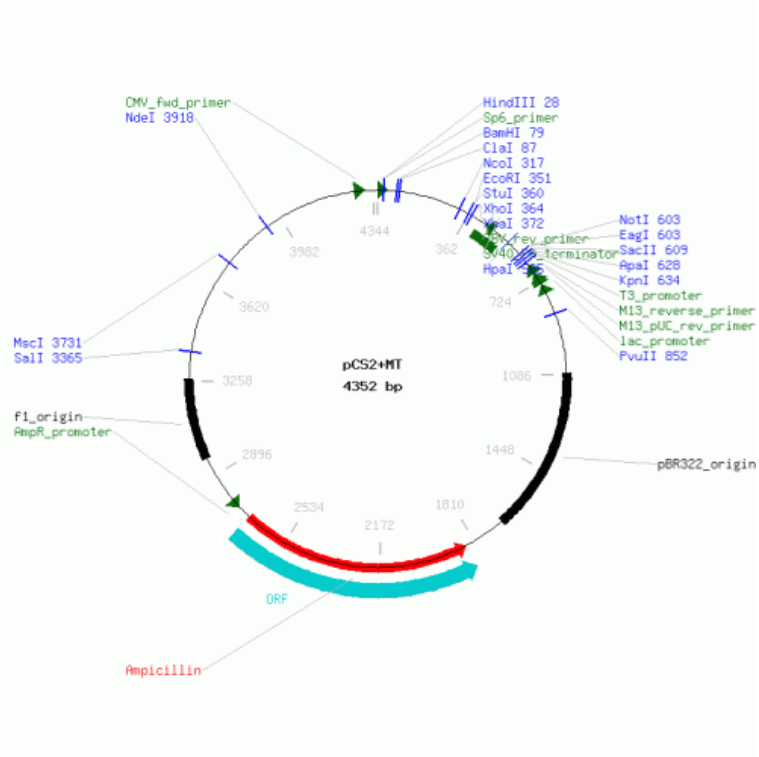
Feature	Position
CMV promoter	1 – 589
HA epitope tag	696 – 725
SV40 polyadenylation signal	884 – 917
SV40 early promoter	1610 – 1839
NeoR/KanR coding sequence	1958 – 2752
pUC origin of replication	3337 - 3980

pEGFP-C3



Feature	Position
CAG enhancer	65 – 352
CMV immearly promoter	10 – 562
CMV fwd primer	519 – 539
CMV promoter	520 – 589
EGFP N primer	679 – 658
EGFP	613 – 1329
ORF frame 1	613 – 1410
EGFP C primer	1266 – 1287
SV40 PA terminator	1408 – 1636
EBV rev primer	1606 – 1625
f1 origin	2081 – 1775
AmpR promoter	2160 – 2188
pBABE 3 primer	2274 – 2254
SV40 enhancer	2475 – 2260
SV40 promoter	2272 – 2540
SV40 origin	2439 – 2516
SV40pro F primer	2501 – 2520
ORF frame 1	2623 – 3417
Neomycin	2626 – 3414
TK PA terminator	3592 – 3861
pBR322 origin	4009 – 4628

pCS2+MT



Feature	Position
Sp6 primer	35 – 52
EBV rev primer	35 – 52
SV40 PA terminator	530 – 411
T3 promoter	666 – 647
M13 reverse primer	701 – 683
M13 pUC rev primer	722 – 700
Lac promoter	765 – 736
pBR322 origin	1693 – 1074
ORF frame 3	2708 – 1848
Ampicillin	2708 – 1848
AmpR promoter	2778 – 2750
f1 origin	3277 – 2971
CMV fwd primer	3277 – 2971