IDENTIFICATION OF THE UPSTREAM REGULATORS OF INTERFERON REGULATORY FACTOR 4 (IRF4) EXPRESSION IN MELANOMA CELLS

by

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B.S., Molecular Biology and Genetics, Istanbul University, 2011

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University

2015

ACKNOWLEDGEMENTS

I would like to emphasize my strongest gratitude to my supervisor, Assist. Prof. Tolga Emre for his guidance and assistance along this thesis process. From the day he accepted me to his lab, his guidance during tough times, and scientific contributions not only made this thesis study possible but also improved me as a researcher.

I would like to thank Assoc. Prof. Arzu Çelik Fuss for sparing her valuable time to evaluate, criticize and improve this thesis.

I would also like to thank my third jury member Assoc. Prof. Ayça Sayı Yazgan for sparing her valuable time to evaluate, criticize and improve this thesis.

Last but not least, I would like to honor the manager of 4th North Dormitory, Metin Irmak. I am very happy to have his support during the disaster times of housing conditions in Bogazici University. I would like to thank to Tuncay Şeker, Ruçhan Karaman and Vahap Kapıkıran for their help in conducting luciferase reporter assays. I would like to thank to Professsor Nesrin Özöeren for sharing her pBVI plasmid with me. I would also like to thank my best friends Cansu Yerinde, Tuğberk Kaya, Lenny Nero, Ruçhan Karaman, Gürkan Mollaoğlu, Ali İşbilir, Mustafa Yalçınkaya, Emirhan Taşöz, Harun Niron, Ceren Saygı, Hasan Yıldırım, Seda Yaşa and Neslihan Zöhrap.

This study was supported by grants from Framework Programme 7 (FP7) Marie Curie – Career Integration Grant (CIG), EMBO – Integration Grant (IG) and TUBITAK-1001-113Z103.

ABSTRACT

IDENTIFICATION OF THE UPSTREAM REGULATORS OF INTERFERON REGULATORY FACTOR 4 (IRF4) EXPRESSION IN MELANOMA CELLS

Melanoma is derived from melanin pigment producing cells which are known as melanocytes. Late stage, metastatic melanoma is considered as one of the most aggressive malignancies with one of the worst prognosis. IRF4 belongs to interferon regulatory family member transcriptional regulators. IRF4 expression, which is regulated by mitogenic stimuli such as NF-κB in lymphocytes, is associated with certain cancers such as T-cell leukemia, multiple myeloma and B cell lymphoma. In addition to immune cell derived malignancies, IRF4 overexpression is found in melanoma cell lines and melanoma patient samples. Additionally, dependency of melanoma cell lines to IRF4 expression was recently found. Moreover, genome wide association studies (GWAS) identified a SNP in IRF4 locus as high risk factor for melanoma. The aim of this project is to find out how IRF4 expression is regulated, and which pathways are playing role in its overexpression in melanoma. Insights into IRF4 regulation holds promise for new therapeutic intervention strategies for melanoma. Most of our knowledge about upstream regulators of IRF4 comes from hematologic cells. Based on known regulators of IRF4 in these cells, we choose three pathways as candidate regulators of IRF4 expression in melanoma cells. Using small molecular compounds, we modulated the activities of these pathways. We used luciferase transactivation assays in order to prove the efficacy of these small molecules. Then we checked IRF4 expression at both mRNA and protein levels in treated cells. Our results suggest that one of these lymphocyte-related signaling pathways plays role in IRF4 expression in melanoma cells.

ÖZET

INTERFERON REGULATORY FACTOR 4 (IRF4) HEDEF GENLERİNİN MELANOMA HÜCRELERİNDE BELİRLENMESİ

Melanoma pigment üreten hücrelerden kökenlenir. Geç evrelerde ise metastatik melanoma en agresif hastalıklardan birisi olarak bilirnir ve kötü bir prognoza sahiptir. IRF4 interferon düzenleyici faktör transkripsiyon ailesine aittir. IRF4 ekspresyonu NF-κB gibi mitojenik uyarıcılar tarafından kontrol edilir ve bu ekspresyon T-hücre lösemisi, Multipl Miyelom (MM) ve B-hücre lenfoması ile ilişkilendirilir. İmmün hücrelerden kökenlenen kanserlere ek olarak, IRF4'un yüksek seviyelerde eksprese edilmesi melanoma hücre hatlarında ve hasta örneklerinde gözlemlenmiştir. Buna ilave olarak melanoma hücre hatlarının yaşamlarının IRF4'e bağımlı olduğu yakın zamanda gösterilmiştir. Genom düzeyindeki ilişkilendirme çalışmaları (GWAS) ise IRF4 lokusunda hastaların melanomaya yatkınlığını belirleyen bir SNP bulmuştur. Bu projedeki amaç ise hangi sinyal yolaklarının IRF4 ekspresyonunu melanomada düzenlediğinin araştırılmasıdır. Eğer IRF4'un anlatımının düzenlenmesine dair fikir sahibi olabilirsek, bu çalışma melanoma için yeni tedavi stratejilerinin gelişmesine yol açacaktır. IRF4'un bilinen düzenleyicilerine ait bilgilerimiz çoğunlukla immün hücrelerden gelmektedir. IRF4'un bilinen düzenleyicilerine bağlı olarak, üç aday yolağı, IRF4'un ekspresyonunu melanomada aday düzenleyici olarak seçtik. Küçük moleküler bileşenler kullanarak, bu yolakların aktivitesini modüle ettik. Daha sonra, IRF4 ekspresyonunu mRNA ve protein düzeyinde muamele edilmiş hücrelerde kontrol ettik. Sonuçlarımız bu lenfosit alakalı yolaklardan birisinin IRF4'un melanoma hücrelerindeki ekspresyonunda rol aldığını önermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS

bp	Base Pairs
gr	Gram
kb	Kilobase
kDa	Kilodalton
L	Liter
M	Molar
mA	Milliamper
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
s	Second
V	Volt
^{o}C	Centigrade degree
µg	Microgram
µl	Microliter
α	Alpha
β	Beta

LIST OF ACRONYMS / ABBREVIATIONS

ABC-DLBCL	Activated B-cell like diffuse large B-cell lymphoma
APS	Ammonium persulfate
BCL	B-cell lymphoma
BRAF	V-raf murine sarcoma viral oncogene homolog B
BRCA2	Breast cancer 2
CDK4	Cyclin dependent kinase 4
CDKN2A	Cyclin dependent kinase inhibitor 2A
cDNA	Complementary DNA
ddH_2O	Double-distilled water
DMEM	Dubecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2F1	E2F transcription factor 1
ECL	Enhanced chemiluminescent solution
EDTA	Ethylenediaminetetraacetate
ERK1/2	Mitogen-activated protein kinase 3/1
EtOH	Ethanol
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GWAS	Genome-wide association studies
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IRF4	Interferon regulatory factor 4
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2 homologue
MEK1/2	Dual specificity mitogen-activated protein kinase kinase 1/2
$MgCl_2$	Magnesium chloride

MITF	Microthalmia-associated transcription factor
MM	Multiple myeloma
mRNA	Messenger RNA
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
NF-κB	Nuclear factor of kappa B
NGS	Next-generation sequencing
NRAS	Neuroblastoma v-ras oncogene homolog
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	3-phosphoinositide dependent protein kinase-1
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-OH kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PS	Polystrol
PTEN	Phosphate and tensin homologue
PVDF	Polyvinylidene fluoride
RB	Retinoblastoma
RGP	Radial growth phase
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-qPCR	Reverse transcriptase - quantitative PCR
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TBS	Tris-buffered saline
TBS-T	Tris buffered saline with tween
TEMED	Tetramethylethylenediamine
Th	T helper cell
TLR	Toll-like receptor
TP53	Tumor protein p53
Tris	Tris (hydroxylmethyl) aminomethane
Tween	Polysorbate

TRYP1	Tyrosinase-related protein 1
TYR	Tyrosinase
UV	Ultraviolet
UVR	UV radiation
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase

1. INTRODUCTION

1.1. Cancer: Overview

Cancer is a result of abnormalities in the homeostasis of cells mostly due to genetic or epigenetic alterations in the genome. Through these genomic alterations, cancer cells acquire traits that enable them to evade from proliferation limiting cellular mechanisms such as replicative senescence and apoptosis. Once these cells arise from normal tissues, they start to gather and form the founding parts of the disease called primary tumors. However, all tumors are not cancerous, as benign tumors do not spread to other parts of the body. If cell growth and proliferation continue in an uncontrolled manner, the tendency of cancer cells to spread throughout the body accelerates the onset of the disease. This tendency mostly results in new colonies of cancer cells in both nearby and distant organs. This phenomenon is called as metastasis, and these cancers are termed malignant which can be fatal due to the eventual organ failure that can manifest itself as abnormal bleeding, a new lump, weight loss, changes in bowel habits (Preetha *et al*., 2008). Weinberg sum up the hallmarks of cancer under six categories as listed below (Weinberg *et al*., 2007):

(i) Constant growth signaling
(ii) Replicative immortality
(iii) Resistance against cell death
(iv) Unlimited replicative potential
(v) Activated tissue invasion & metastasis
(vi) Sustained angiogenesis

Sustained level of constant growth of cancer cells stems from either abnormalities in certain signal transduction pathways or stimulated normal cells within the microenvironment of tumor. Aberrant activation of signaling pathways occurs at different levels through the signaling such as abundance of ligand, overall activation of receptor of

certain growth factors and autocrine activation of downstream effectors of the pathway through mutations. For instance, BRAF (v-raf murine sarcoma viral oncogene homolog B) is a gene that makes one of the critical downstream effectors of MAPK (mitogen-activated protein kinase) pathway. One of the well-known driver mutations in BRAF results from a substitution of valine to glutamic acid which is designated as BRAF V600E (Wan *et al*., 2004). This mutation occurs in the active site of BRAF and results in a conformational change that causes elevated kinase activity regardless of its upstream effectors and constant targeting of its downstream effectors such as ERK. Such genes that have the potential to drive the normal cells to proliferative stage are called as proto-oncogenes and they are activated through gain of function mutations or excessive expression (Weinberg *et al*., 2007).

Growth-promoting genes comprise one side of the cancer while growth-balancing genes also exist throughout the genome. These anti-growth genes, which constrain cell proliferation, are called as tumor suppressor genes. Their involvement in the tumor formation relies on their loss or inactivation. One of the well-studied example of such genes is PTEN (phosphatase and tensin homolog) that encodes phosphatidylinositol-3,4,5-triphosphate 3-phosphatase (Molina A *et al*., 2013). PIP3, a second messenger lipid, is activated via activation of PI3K pathway by various extracellular signals and provides an anchoring site for the activation of AKT, which is associated with certain cancers (Phin *et al*., 2013). PTEN acts as a growth limiting gene by converting PIP3 to PIP2 and counterbalancing AKT activity (Xu *et al*., 2014). RB (retinoblastoma), TP53 (tumor protein p53) and NF1 (neurofibromatosis type 1) are the other well-characterized tumor suppressor genes, which counterbalance growth stimulatory signals in normal cells. Both copies of these gatekeeper and caretaker genes need to be functionally lost in cancers, therefore they show a recessive phenotype in familial cancers. This impairment includes loss of function mutations, global methylation and transcriptional silencing of tumor suppressor genes. After the loss of tumor suppressor genes, cancer cells progress to cell proliferation, DNA repair, pro-apoptosis and keep to accumulate new mutations within the cancer cell population.

Hyper-proliferation of cancer cells results in steady increase of tumor volume, which faces inner part of the tumor to starvation of oxygen, nutrients as well as the effective removal of metabolic waste. In order to overcome these limitations, tumors start to generate microvascular structures with the help of matrix metalloproteinases (MMPs), PDGF, interferon-γ fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) secretion. After the vascularization within the tumor area, epithelial cells may be forced to assume a mesencymal character, termed epithelial-mesenchymal transition (EMT) which enables them to acquire invasive characteristics, which allow them to establish vascular structures (Ombrato *et al*., 2014).

Cancer is multi-step continuum that includes activation of proto-oncogenes and deactivation of tumor suppressor genes in normal cells and proceeds to fatal stages through angiogenesis and metastasis. Cancer cells acquire new traits that enable them to survive, proliferate and migrate. In addition to the conventional cancer treatments including surgery, radiotherapy and chemotherapy, innovative treatment strategies are needed for a proper prognosis and treatment

1.2. Melanoma

1.2.1. Melanoma: Overview

Melanoma, which originates from pigment producing melanocytes, is considered as the most aggressive type of skin cancer with poor prognosis. Conventional treatment strategies such as surgical digestion of the primary tumor area are not adequate at the metastatic stage and mechanisms underlying the disease are poorly defined. Melanoma is more dangerous than other types of skin cancers since deaths from melanoma account for 80% of skin cancers while melanoma cases comprise only 4% all skin cancers (Gray-Schopfer *et al*., 2007). According to the current estimations of the American Cancer Society for new melanoma cases and related deaths, mortality rate of melanoma is fifth among all other cancer types (Gray-Schopfer *et al*., 2007).

Terminology of melanoma depends on the body site of the arising melanoma tumor in patients. If it originates from the skin, it is called cutaneous malignant melanoma but it can also originates from other sites of the body, such as vagina (vaginal melanoma), eye (uveal melanoma) and vulva (vulval melanoma). Melanocytes, which originate from neuronal-crest progenitors, are located at the basal layer of epidermis and hair follicles where they produce brown pigment called melanin. Melanocyte homeostasis is regulated by surrounding keratinocytes. After exposure to ultraviolet (UV) radiation, keratinocytes secrete factors that are critical for melanocyte survival, motility, proliferation and differentiation (Gray-Schopfer *et al*., 2007). This stimulation results in upregulation of melanin pigment production in the skin. Increased melanin pigment production protects body from deleterious effects of UV. However, exposure of melanocytes to UV impairs their genomic balance and leads accumulation of mutations in their genomes (Gray-Schopfer *et al*., 2007).

Melanocytes escape from tight regulation of keratinocytes after some critical mutations in growth stimulatory genes. Deregulation of melanocyte growth results in production of aoutocrine growth receptors and loss of adhesion receptors. Consequently, melanocytes start to dedifferentiate, proliferate and spread which results in formation of benign neoplasms called nevi (Gray-Schopfer *et al*., 2007). Nevi can proceed to radial-growth-phase (RGP) melanoma where they grow horizontally. RGP cells have the ability to progress to vertical-growth-phase (VGP) melanoma considered as primary malignant stage because of their ability to invade dermis. Figure 1.1. shows the transformation of melanocytes to malignant melanoma cells.

1.2.2. Melanoma: Deregulated Pathways

In-depth understanding the biology of initiation and progression of melanoma is required because of the complex nature of the disease. This will lead to identification of new biomarkers and better understanding of the disease staging and subtype classification, therefore better therapeutic strategies will be found.

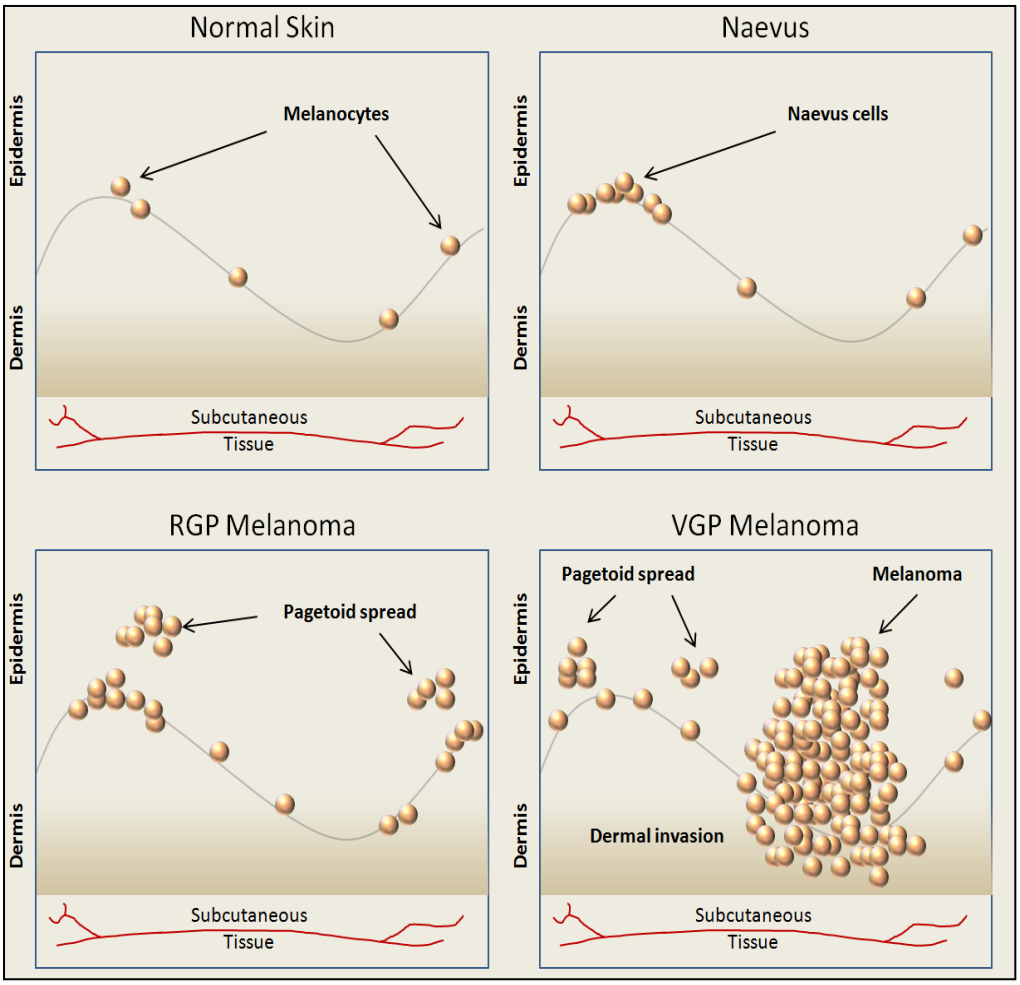

Figure 1.1. Transformation of melanocytes to malignant melanoma cells. Normal skin shows even distribution of dendritic melanocytes while nevus starts to accumulate them. Radial growth phase (RGP) melanoma is considered to be the primary malignant stage. Vertical growth phase melanoma (VGP) has increasing metastatic potential. (Adopted from Gray-Schopfer *et al*., 2007).

MAPK (Mitogen-activated protein kinase) pathway, which has regulatory role on cell fate decisions, is the most commonly deregulated pathway in melanoma as ERK (extracellular-signal regulated kinase), downstream effector of MAPK pathway, is hyper activated in up to 90 % of melanoma (Wellbrock *et al*., 2014). MAPK signaling depends on phosphorylation of the components in a signaling cascade. It can get stuck in "on" or

"off" stages when one of the components is mutated. In melanocytes, this pathway is activated by growth factors such as stem cell factor (SCF), hepatocyte growth factor, and fibroblast growth factor (FGF); however, these growth factors do not cause strong or sustained activation of MAPK pathway (Werner *et al*., 2007). Hyper-activation of ERK occurs via either autocrine signaling or mutational activation of growth factor receptor c-KIT or other components (Wellbrock *et al*., 2014). The most mutated component of this pathway BRAF that is a type of serine-theronin protein kinase (Wellbrock *et al*., 2014). 50-70% of melanomas have a point mutation of BRAF, which leads to valine to glutamic acid substitution in the active site and designated as BRAF V600E. Because of the conformational change in the active site of BRAF, it becomes constitutively active which exerts proliferation, survival and tumor maintenance functions through the hyper-activation of ERK signaling (Wellbrock *et al*., 2014). In addition to the stimulatory effect of BRAF V600E on ERK signaling, aberrant activation of MAPK pathway can occur independent of mutant BRAF. Under stress conditions such as targeted therapies of mutant BRAF, proliferative signals of MAPK pathway might switch to other RAF isoforms rather than acting through BRAF (Kugel *et al*.,2014). By stimulating VEGF secretion, BRAF also plays crucial role in neoangiogenesis (Kugel *et al*.,20146). Microphthalmia-associated transcription factor (MITF), tumor maintenance enzymes matrix metallaoproteinase-1, cell cycle regulators cyclin D1 and $p16^{INK4A}$ are the downstream effectors of BRAF V600E in melanoma. Another common mutation in MAPK pathway in melanomas is on NRAS (neuroblastoma RAS viral oncogene homolog). NRAS is mutated 15-30% of melanomas with the most common mutation resulting in leucine to glutamine change at position 61 (Q61L). NRAS and BRAF mutations are mutually exclusive and they rarely coincide (Mandala *et al*., 2014).

Another signaling pathway that fuels proliferation of melanoma cells is phosphoinositide-3-OH kinase (PI(3)K) pathway (Figure-1.2). Phosphoinositides are the major components of this pathway. These lipids are converted to second messengers by one of the PI(3)K a family members. Second messengers including PIP3 can activate various signaling pathways that have critical roles in cell survival, proliferation, tumor growth and motility. Cancerous activity of this pathway is blocked by PTEN in normal cells. Hyper-activation of PI(3)K pathway depends on loss of function in PTEN, activating

mutations in PI(3)K and high expression of protein kinase B (PKB) in late stage melanoma (Gray-Schopfer *et al*., 2007).

MITF, which is a basic helix-loop-leucine transcription factor, is master regulator of melanocyte development as it regulates expression of melanogenic proteins such as tyrosinase and melan-A (Hartman *et al*., 2015). Although MITF regulates melanoblast survival and melanocyte lineage commitment, it exerts crucial roles in melanoma since sustained MITF expression enables melanoma cell proliferation and survival. MITF amplification was shown in 10-20% of melanomas (Hartman *et al*., 2015). MITF expression in melanoma is significantly lower than melanocytes and both low and high level of MITF expression lead to cell cycle arrest by cell cycle regulators such as $p16^{INK4A}$ (Hartman *et al*., 2015).

Evading from cell cycle control mechanism is one of the hallmarks of cancer. Cell cycle is tightly regulated by cyclins and CDKs. Transitions through different stages of cell cycle is utilized by specific cyclin(s) matched with its CDK in normal cells. The Rb protein is an important gatekeeper of the cell cycle control. During transition of the cell cycle to S phase, CDK4 and CDK6 phosphorylate Rb leading to dissociation of its interaction partner E2F transcription family members, which activate S phase genes (Sheppard *et al*., 2013). $P16^{INK4A}$ checks cell cycle progression and inhibit tumor growth by inhibiting of CDK4 and CDK6 (Sheppard *et al*., 2013). About 75% of melanoma patients demonstrate decreased $p16^{INK4A}$ expression due to the point mutations, deletions, hyper-methylation of promoter and transcriptional silencing by over-expression of transcriptional suppressors such as inhibitor of differentiation 1 (ID1) (Sheppard *et al*., 2013).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is one of the hyperactivated pathways, which provides signals for survival, proliferation and resistance to apoptosis (Madonna *et al*., 2012). Some components of NF-κB pathway such as p50 and p65/RELA, are overexpressed in the nucleus of dysplastic nevi when compared to healthy melanocytes (Madonna *et al*., 2012). Increased nuclear localization of these transcription factors can be achieved via multiple ways. One of the

mechanisms of NF-κB hyper-activation relies on alterations in balance between IκBα degradation and re-synthesis. In Hs294T melanoma cells, there is shift, which favors the IκBα degradation rather than its re-synthesis thus leading to increase in nuclear localization (Madonna *et al*., 2012). According to the recent studies on gene expression profile of melanoma cells demonstrated increased expression profile of Osteopontin (OPN), which enhances IKK activity due to the phosphorylation, and degradation of IκBα, thereby induces NF-κB activity (Madonna *et al*., 2012).

Nuclear factor of activated T-cells (NFAT) pathway is a critical transcriptional regulator of T-cell development and function (Mancini *et al*., 2009). Activation of NFAT pathway is known to contribute to survival, growth, invasion and angiogenesis in certain malignancies (Macian *et al*., 2005). The activity of this pathway relies on several evidences. Melanoma cells have functional Ca^{2+}-dependent signaling mechanism (Mancini *et al*., 2009). Melanoma cells express certain members of NFAT family transcription factors some of which are transcriptionally active (Mancini *et al*., 2009). Apoptosis of melanoma cells can be promoted by the calcineurin inhibitor cyclosporin (CsA) that inhibits activity of NFAT pathway (Mancini *et al*., 2009). CsA was used in a phase II in advanced melanoma with a manageable toxicity (Mancian *et al*., 2005).

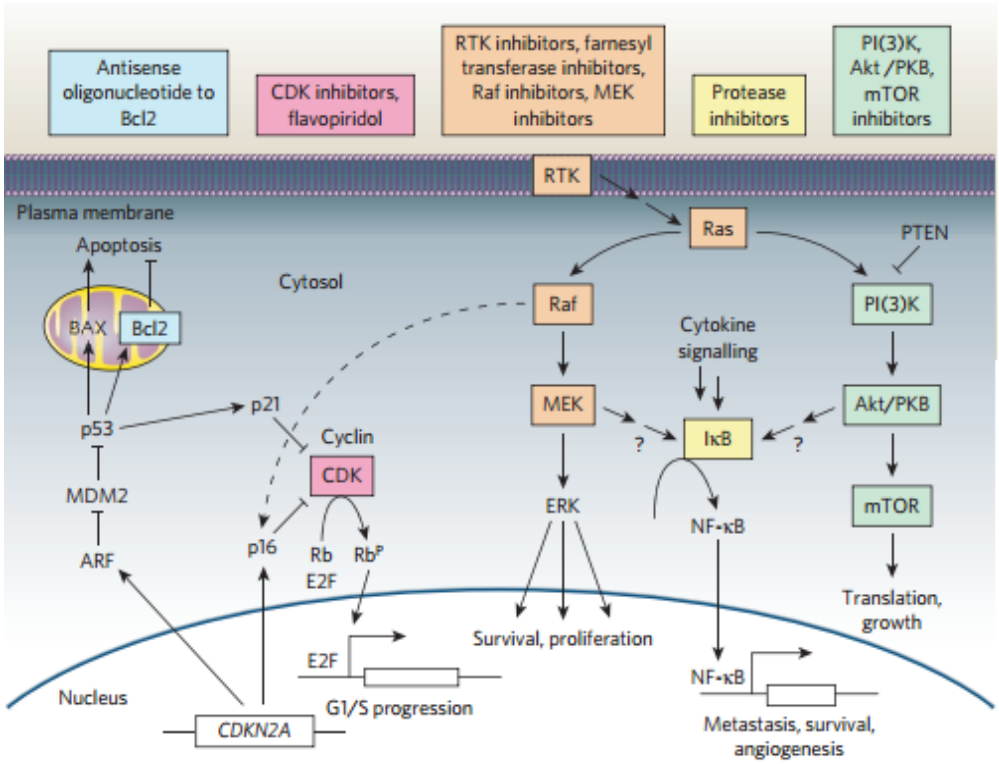

Figure 1.2. Common deregulated pathways of human melanoma. Mutations and deregulations are shown (Adopted from Gray-Schopfer *et al*., 2007).

1.3. Interferon Regulatory Factor 4 (IRF4)

1.3.1. IRF4: Overview

IRF4 is a member of Interferon Regulatory Factor family of transcription factors which has 9 members. Other designations of IRF4 are PIP (PU.1 interaction partner), MUM1 (Multiple Myeloma Oncogene 1), LSIRF (Lymphocyte specific interferon regulatory factor), or ICSAT (Interferon consensus sequence binding protein for activated T-cells). IRF family of proteins was initially identified because of their regulatory roles in interferon-inducible genes (De Silva *et al*., 2012).

IRF4 is composed of single polypeptide chain composed of three parts: N-terminal DNA binding domain DBD, functional domain composed of two transactivation domains (TAD) and ternary complex formation regions (De Silva *et al*., 2012). An intermediary linker domain allows conformational changes in IRF4 structure.

IRF4 has potential to act both as an activator and repressor depending on the presences of its interaction partners. These interaction partners confer IRF4 cell type and stage specific functional roles (Figure 1.3).

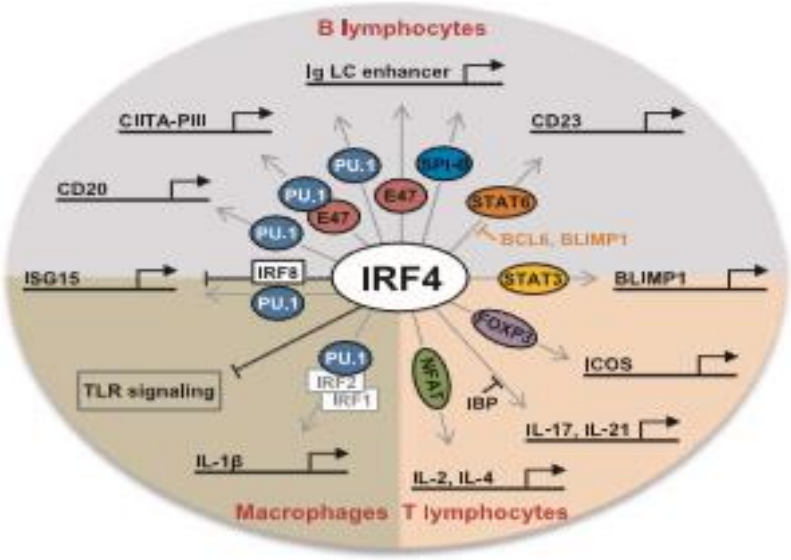

Figure 1.3. Roles of IRF4 in different cell types. IRF4 has cell type and stage dependent functions due to the interaction with different interaction partners (Adopted from De Silva *et al*., 2012).

1.3.2. Role of IRF4 in Immune Cells and Immune Cell Derived Cancers

IRF4 is an essential regulator in immune system depending on the cell type and stage. During early B-cell development in the bone marrow, rearrangement of immuno-

globin (Ig) genes occurs by a strictly regulated multistep process. In order to produce small pre-B cell, B cells proliferate and differentiate into large pre-B cell after a successful Ig heavy chain rearrangement. Together with IRF8, IRF4 regulates pre-B cell differentiation since IRF4 and IRF8 deficient B-cells are arrested at the large B-cell stage by overexpressing pre-B cell receptor (preBCR) on the cell surface (Lu *et al*., 2003). IRF4 has also roles in activation of immunoglobin light chain rearrangement by directly binding to the 3' kappa and gamma immunoglobin enhancers in small pre-B cells (Johnson, K. *et al* 2008). IRF4 also mediates receptor editing in B-cells independent of IRF8 (De Silva *et al*., 2012).

IRF4 is critical regulator T-cell driven immune responses. By controlling apoptosis and cytokine production, IRF4 regulates Th2 and Th17 development therefore it regulates T-helper-cell differentiation (Lohoff, M. *et al*., 2004)

In addition to its regulatory roles in B and T cells, IRF4 has also roles in myeloid cells in the context of dendritic cells and macrophages. Overexpression of IRF4 mRNA is detected during the transition of monocytes to macrophages or dendritic cells (Lehtonen, *et al* 2005). Role of IRF4 on the expression of major histocompability complex class II and subsequent antigen presentation to T cells was revealed (Suzuki *et al*., 2004). Through a competition with IRF5, an, IRF4 modulates Toll-like receptor signaling (TLR) in macrophages (Negishi *et al*., 2005).

Deregulation of IRF4 expression is associated with certain immune cell derived malignancies because of its tight regulatory role in both development and differentiation of certain immune cell types. Multiple myeloma (MM) is a malignancy of plasma cells. Genetic aberration of IRF4, which is a translocation t(6;14)(p25;q32) results in juxtaposition of immunoglobin heavy chain locus to IRF4 gene, is observed in MM (Iida *et al*., 1997). Overexpression of IRF4 in MM patients is associated with poor prognosis of the disease (Heintel, *et al*., 2008). Another study revealed that MM cell lines have dependency to IRF4 expression since 50% knock-down is sufficient to kill MM cell lines (Shaffer, *et al*., 2008). On the other side, overexpression of IRF4 is not adequate to cause the

disease onset in the lymphocytes of transgenic mice, suggesting that IRF4 doesn't have tumor promoting potential while it exerts non-oncogenic addiction (Saito, *et al*., 1999).

Activated B cell like diffuse large B cell lymphoma (ABC-DLBCL), which is an aggressive type of DLBCL, derives from late germinal center B cells (Figure-1.4). Constitutive activation of NF-κB signaling pathway induces IRF4 expression in ABC-DLBCL cells (Saito *et al*., 2007). Like MM, ABC-DLBCL has also dependency to IRF4 expression (Yang *et al*., 2012).

1.3.3. IRF4 in Non-immune Cells, Including Melanocytes and Osteoclasts

Until recently, IRF4 expression was thought to be restricted to immune cells; however, IRF4 expression in various other cell types was recently shown. Among them, adipocytes are one of the non-immune cells that have IRF4 expression. Adipocytes, which are a type of fat cells, store energy as triglycerides. In fasting conditions, triglycerides are broken down into free fatty acids and glycerol by lipolysis mechanism. IRF4 promotes this mechanism by regulating the expression of lipases (Eguchi *et al*., 2008).

IRF4 is also expressed in cardiac tissue and central nervous system (CNS) neurons. During adaptive response to pressure, IRF4 induces cardiac hypertrophy in heart muscle (Jiang *et al*., 2013). IRF4 expression in neurons protects them from apoptosis and death (Gua *et al*., 2014).

Early observations showed that IRF4 is expressed in a normal foreskin melanocyte (EST library) (Grossman *et al*., 1996). A SNP located on IRF4 (rs12203592) has been linked to hair color and skin pigmentation by several genome-wide association studies (GWAS) (Han *et al*., 2008). These studies implicate that IRF4 might have role in pigmentation. More recently, it was recently shown that MITF together with IRF4 activates one of the critical pigmentation genes tyrosinase (TYR) in melanocytes (Praetorius *et al*., 2013).

1.3.4. IRF4 in Melanoma

IRF4 expression was previously detected in G-361 melanoma cell line (Grossman *et al*., 1996). Another study revealed that IRF4 is expressed in protein level and mainly located into nucleus in melanoma cells (Natkunam *et al*., 2001). According to the further study, 92% of conventional primary and metastatic melanomas stained with IRF4 (Sundram, *et al*., 2003). Additionally, the germline SNP rs12203592 (described above) in IRF4 predisposes humans to melanoma and other skin cancers (Han *et al*., 2008).

1.4. Known Regulators of IRF4

IRF4 expression is regulated by certain signal transduction pathways in other contexts such as immune cells and immune cell derived cancers. In addition to these signaling pathways, some immunomodulatory drugs are known to down modulate IRF4 expression in certain cancer types.

1.4.1. NF-κB Pathway

The NF-κB family transcription factors consists of five members in mammals; RELA (also known as p65), RELB (also known as c-REL), REL (also known as c-REL), p50 and p62 that can form either homodimers or heterodimers (Pasparakis *et al*., 2009). In resting conditions, cells keep their NF-κB dimers in the cytoplasm via association with proteins of the inhibitor of NF-κB family, including IκBα, IκBβ, IκB-gamma, p100 and p105. Activation of NF-κB pathway is driven by IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKα (also known as IKK1) and IKKβ (also known as IKK2) and one regulatory subunit NEMO (Hinz *et al*., 2014). Upon cell stimulation, IKK complex phosphorylates IκB proteins from the serine residues and induces their subsequent polyubiquitinylation and proteasomal degradation which is followed by an unmasking of NF-κB dimers' nuclear localization signals. NF-κB dimers then translocate into nucleus where they can activate transcription of their target genes (Pasparakis *et al*., 2009). So far, there are two NF-κB activation pathways, the canonical (classical)

pathway is induced by several stimuli such as pro-inflammatory cytokines, bacterial and viral products, ultraviolet light and oxidative stress, while the non-canonical (alternative) pathway is induced by some lymphocyte tissue related receptors such as lymphotoxin-β receptor and receptor for B cell-activating factor (BAFF) (Hinz *et al*., 2014) (Figure 1.4).

NF-κB pathway is known to be deregulated in melanoma (Madonna *et al*., 2012). Madonna *et al* demonstrated that imbalance between production and degradation of IκBα cause increased nuclear translocation of p65 in the nucleus of melanoma cells when compared to healthy neavi. In addition to the importance of NF-κB pathway in melanoma, this pathway is also known to regulate IRF4 expression in B cells.

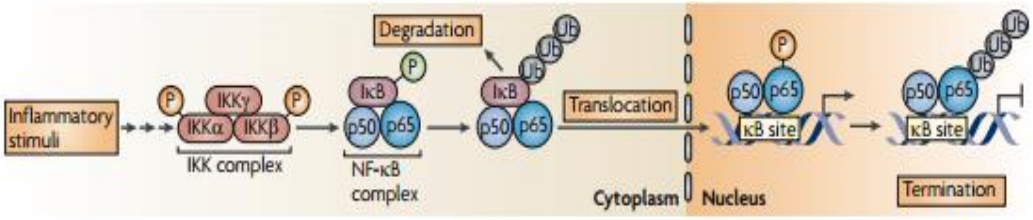

Figure 1.4. Activation of NF-κB pathway from classical pathway. Upon activation with inflammatory stimuli the IKK kinase complex is activated. IKK complex phosphorylates IκB, which masks nuclear localization signal of NF-κB complex. This phosphorylation leads to polyubiqutinylation and degradation of IκB and subsequent translocation of NF-κB complex to the nucleus where it activates its target genes (Adopted from Katia *et al*., 2015).

1.4.2. NFAT Pathway

Nuclear Factor of Activated T-cells (NFAT) pathway was identified in nuclear extracts of activated T cells via its inducible DNA-binding capacity to the interleukin-2 (Il2) promoter (Shaw *et al.,* 1988). The NFAT family consists of five proteins which are evolutionarily conserved and related to the c-Rel. The transcription factors in this family are: NFAT1 (also known as NFATc2 or NFATp), NFAT2 (also known as NFATc1 or

NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 or NFATx) and NFAT5 (also known as Ton EBP). NFAT proteins have an amino-terminal transactivation domain (TAD), a regulatory domain (also known as the NFAT homology region (NHR)), a carboxy-terminal domain and a highly conserved DNA binding domain (also known as the Rel-homology domain (RHD)) suggesting that all NFAT family members share the same DNA binding motif (Figure 1.5). The regulatory domain of NFAT transcription factors is moderately conserved (Müller *et al*., 2010). There are 14 NFAT export and maintenance kinase docking sites in amino-terminal region of NFAT transcription factors. These docking sites contain serine-rich regions (SRRs) which are phosphorylated by NFAT kinases such as casein kinase 1 (CK1), dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) and glycogen synthase kinase3 (GSK3) in resting cells (Müller *et al*., 2010) leading to their containment in the cytoplasm.

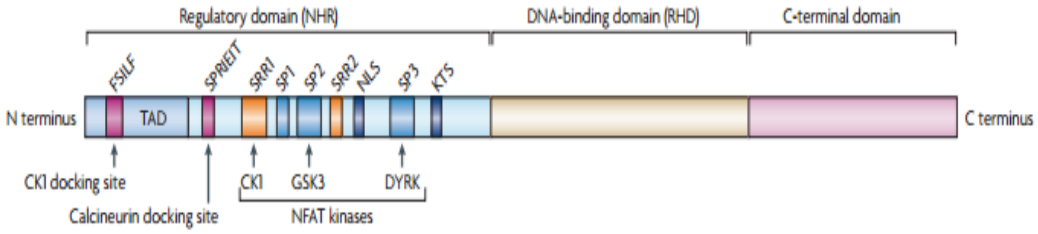

Figure 1.5. General scheme for NFAT family transcription factors. All five NFAT transcription factors share the DNA-binding domain similarity suggesting that they all bind to the same response element. They differ in their N and C terminal domain. N terminus contains a trans-activation domain (TAD) and several docking sites for NFAT kinases (Adopted from Mancian *et al*., 2005)

After the isolation and molecular characterization of all NFAT family transcription factors, it was realized that their expression was not restricted to only T cells and they are expressed almost in every cell types such as smooth and skeletal muscle cells, B cells, mast cells and neutrophins (Crabtree *et al*., 2002). Role of NFAT1, NFAT2 and NFAT4 in T-cell development and function is extensively studied. NFAT3 is not expressed by immune system cells (Lu *et al*., 2007). NFAT5 is the only NFAT family member that is not regulated by intracellular Ca^{2+} signaling. NFAT5 is expressed in all

cell types and is activated by osmotic stress. Activation of NFAT5 results in expression of several cytokines including tumor-necrosis factor (TNF) (Hogan *et al*., 2003).

Regulation of NFAT pathway is tightly controlled by intracellular calcium levels, calcineurin and NFAT kinases. Activation of NFAT pathway is induced by engagement of several receptors including Fc γ receptors. Engagement of these receptors leads to activation of phospholipase-C-y (PLC- γ) which is responsible for hydolization of phosphatidylinositol-4,5-biphosphate to produce inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ binds to the $InsP_3$ receptor ($InsP_3$ R) on the endoplasmic reticulum (ER) membrane and leads to steady increase in intracellular calcium levels through the efflux of Ca^{2+} (Müller *et al*.,2010). This efflux leads to triggering a process known as store-operated Ca^{2+} entry (SOCE) (Feske *et al*., 2007). Calcium binds to calcium sensing protein calmodulin which leads to activation of a phosphatase called as calcineurin (Hogan *et al*., 2005). Calcineurin, also known as phosphatase 2B, is a calmodulin-dependent serine/threonine phosphatase and consists of two subunits with following predicted molecular weights 59 kDa and 19 kDa (Furman *et al*., 1992). Calcineurin is expressed in eukaryotic cells including yeast (Cyert *et al*., 1991) and its expression is most abundant in brain (Stemmer *et al*., 1991). Upon calmodulin dependent activation of calcineurin, NFAT family transcription factors are dephosphorylated and translocated into nucleus to activate their target genes upon their interaction with their transcriptional partners such as activated protein 1 (AP1) (Mancini *et al*., 2009).

Roles of NFAT family transcription factors are determined by their redundancy. Gene knock-out mice screen in which individual NFAT family members are knocked-out, revealed that more than one knock-out of family members is needed for appearance of severe changes in many cells and functions of the immune cells (Hodge *et al*., 1996). However, only mild alterations are observed when just one NFAT transcription factor is depleted. According to following in vivo gene knock-out coupled functional screen studies, deficiencies due to absence of each NFAT transcription factors are discovered. Loss of NFAT1 results in splenomegaly with moderate hyper-proliferation, mildly enhanced B- and T-cell responses, sustained IL-4 expression, decreased IFN- gamma production

during response to T-cell receptor (TCR) ligation, slowed thymic braid and enhanced germinal-center formation (Yoshida *et al*., 1998).

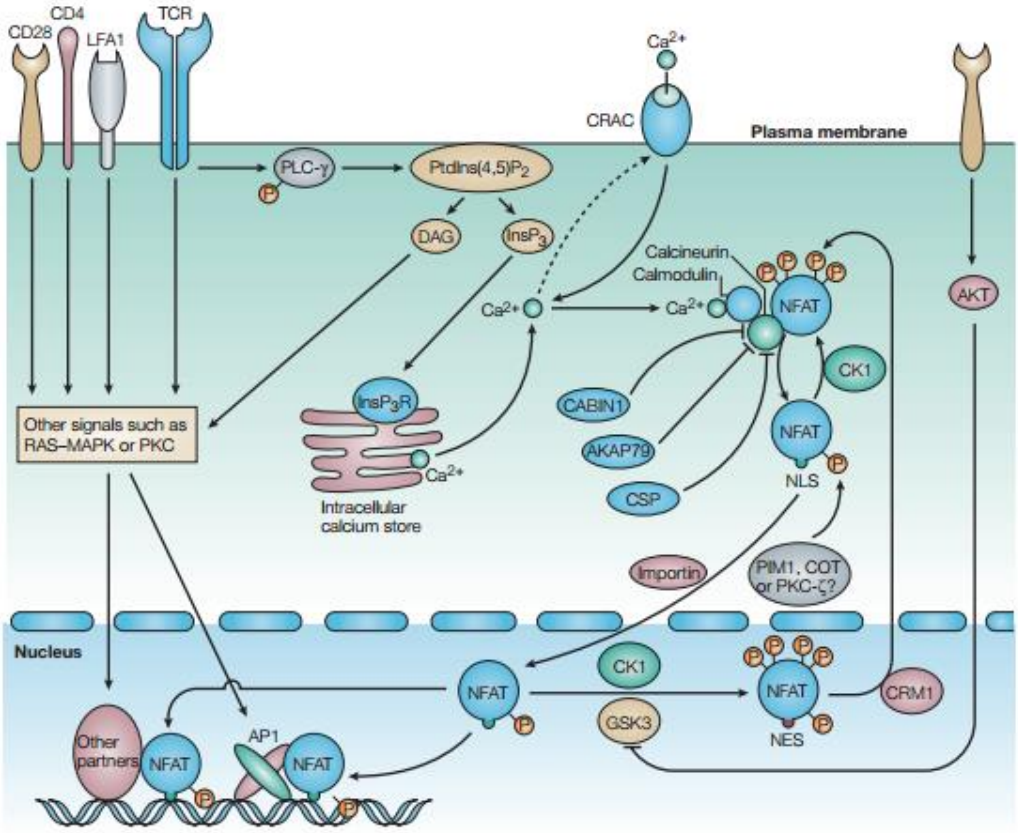

Figure 1.6. Regulation of NFAT pathway. Activation of PLC- γ upon receptor engagement triggers hydrolization of phosphatidylinositol-4,5-biphosphate to produce $InsP_3$ which binds to $InsP_3$ receptors in ER and leading to store-operated Ca^{2+} entry induced calcineurin activation. Calcineurin dephosphorylates NFAT transcription factors and favors their nuclear localization (Adopted from Macian *et al*., 2005).

Loss of NFAT2 results in decreased proliferative responses under RAG2-deficient complementation conditions, defective repopulation of the thymus and lymphoid organs and defective IL-4 production (Ranger *et al* 1998). Loss of NFAT4 results in moderate hyper-activation of peripheral T cells and apoptosis of double-positive thymocytes (Ranger *et al* 1998). Loss of NFAT5 results in impaired T-cell function during hyper-osmosis and attenuated cellularity of the thymus and spleen (Go *et al* 2004). All of these functional analyses explain why certain T-cell functions are specifically regulat-

ed by different NFAT family members because of having either different mechanisms of regulation or their different interaction partners through their less-conserved N- or C-terminal domains. Trisomy 21 results in triplication of the DSCR1 and Dyrk1a genes. Increased dosage of the former prevents nuclear translocation of NFATc by inhibiting calcineurin. While the latter export NFATc proteins from the nucleus. Investigation of calcineurin and NFATc deficient mice, Dscr-1 and Dyrk1a overexpressing mice, mouse models of Down's syndrome and human trisomy 21 indicate that destabilization of regulatory circuit of Dscr-1 and Dyrk1a might control the human disease (Crabtree *et al*., 2002). Deregulation of NFAT signaling through overexpression of its certain isoforms is associated with hematological malignancies and human solid tumors (Müller *et al*., 2010). Because of regulatory roles of NFAT pathway in cell survival, differentiation, angiogenesis, invasive migration and tumor microenvironment, safe and effective treatment strategies for NFAT driven cancers are needed. Clinically available NFAT pathway inhibitors with different mechanisms of actions in terms of different intracellular targets exist. Fk-506 and cyclosporine A (CsA) are the well-known small molecular compounds that interfere NFAT pathway activation through different mechanisms. CsA binds to cyclophilin while Fk 506 binds to Fk-506 binding protein-12 (FKBP-12) (Kincaid *et al*., 1987). Both cyclophilin and FKBP-12 affinity matrices bind to calcineurin in a Ca^{2+} dependent manner (Furman *et al*., 1992). This binding inhibits calcineurin's phosphatase activity which results in accumulation of NFAT transcription factors in cytosol (Jun *et al*., 1991).

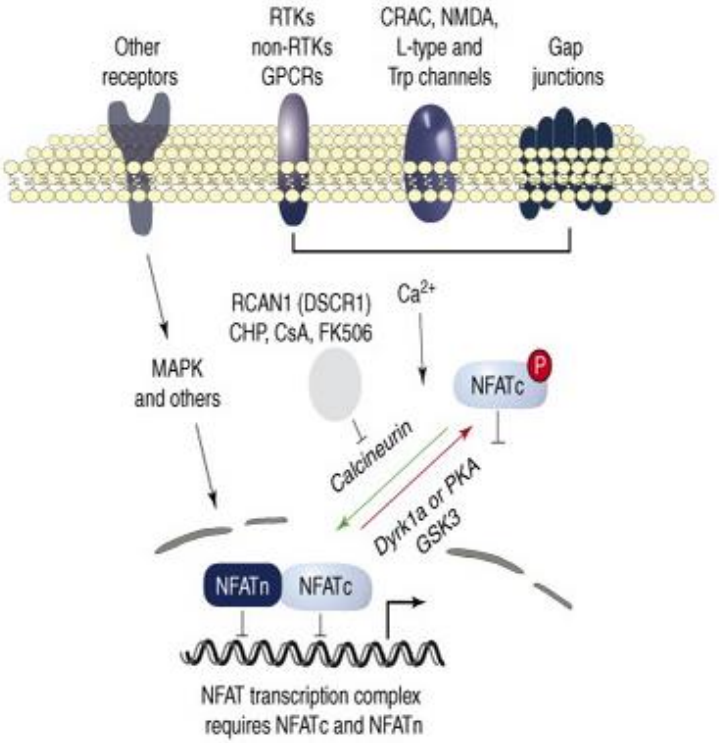

Figure 1.7. Inhibition of NFAT pathway. NFAT pathway can be targeted by multiple ways. Most common strategy to block this pathways activity is targeting calmodulin/calcineurin complex via CsA and Fk506. Both of the drugs by using different binding partners (Adopted from Crabtree *et al*., 2002).

Melanoma cells are known to have functional Ca^{2+} signaling system. They express certain members of NFAT family transcription factors. Moreover, treatment of melanoma cell lines with NFAT pathway inhibitors leads to apoptosis. In addition to NFAT pathways' role in melanoma, this pathway is also known to regulate IRF4 expression in ectopically M2 protein expressing primary B cells.

1.4.3. Vitamin D and its Nuclear Hormone Receptor

There are two major forms of vitamin D, vitamin D2 (ergocalciferol) and vitamin D3 (1,25(OH_2) D3) also known as calcitriol. Vitamin D2 is not produced by vertebrates while vitamin D3 is synthesized by human and vast majority of vertebrate animals through photosynthesis in the skin via conversion of 7-dehydroxycholestrol into previtamin D3 that is followed by immediate and temperature-dependent isomerization into

its active form 1,25(OH_2)D3 (Michelino *et al*., 2011). In order to produce metabolically active form of vitamin D3, vitamin D3 is first hyrdoxylated at the carbon 25-position by several cytochrome P450 (CYP) isoforms especially by CYP2R1 (Cheng *et al*., 2004). Hydroxylated form of vitamin D3 is transported to the proximal tubule of kidney via bloodstream (Michelino *et al*., 2011). 25-hydroxyvitamin D3 is hydrolysed at the 1α-position for the formation of calcitriol, the metabolically active form of vitamin D3, by the enzyme 25-hydroxyvitamin D-1 α-hydroxylase (CYP27B1) in the kidney (Jurutka *et al*., 2007).

Vitamin D3 exerts its biological function by binding to vitamin D receptor (VDR) and retinoid X receptor-α (RXR-α) in the nucleus. VDR is ligand activated transcription factor and nuclear hormone receptor (Bikle *et al*., 2009). VDR is expressed vast majority of immune cells including monocytes, stimulated macrophages, dendritic cells (DCs), natural killer cells and activated B and T cells (Provveddini *et al* 1983). Binding of calcitriol to VDR triggers conformational changes in VDR-RXR heterodimers in the nucleus (Carlberg *et al*., 1998). The conformational changes in VDR-RXR structure leads to dissociation of co-repressor protein such as nuclear receptor co-repressor (NCoR) and enables interaction of VDR-RXR heterodimer with co-activator family such as transcriptional intermediary factor2, steroid receptor co-activators-1 and receptor activated co-activators-3 (Torchia *et al*., 1998). Vitamin D receptor-interacting protein complex and other co-regulatory proteins, which control histone modifications, chromatin remodeling, RNA polymerase II binding and exchange of co-activators in the transcriptional complex of VDR targets, are recruited by ligand activated VDR-RXR complex and this recruitment results in ligand induced transactivation of VDR target genes and signaling pathways (Rachez *et al*., 1999) (Figure 1.8). VDR-RXR also interacts with the E-box type element of repressor vitamin D response elements (VDREs) through CANNTG-like motifs. This interaction leads to dissociation of p300 co-activator and association of the histone deacetylator co-repressor complex, which results in ligand induced trans-repression of VDR target genes and signaling pathways (Fujuki *et al*., 2005).

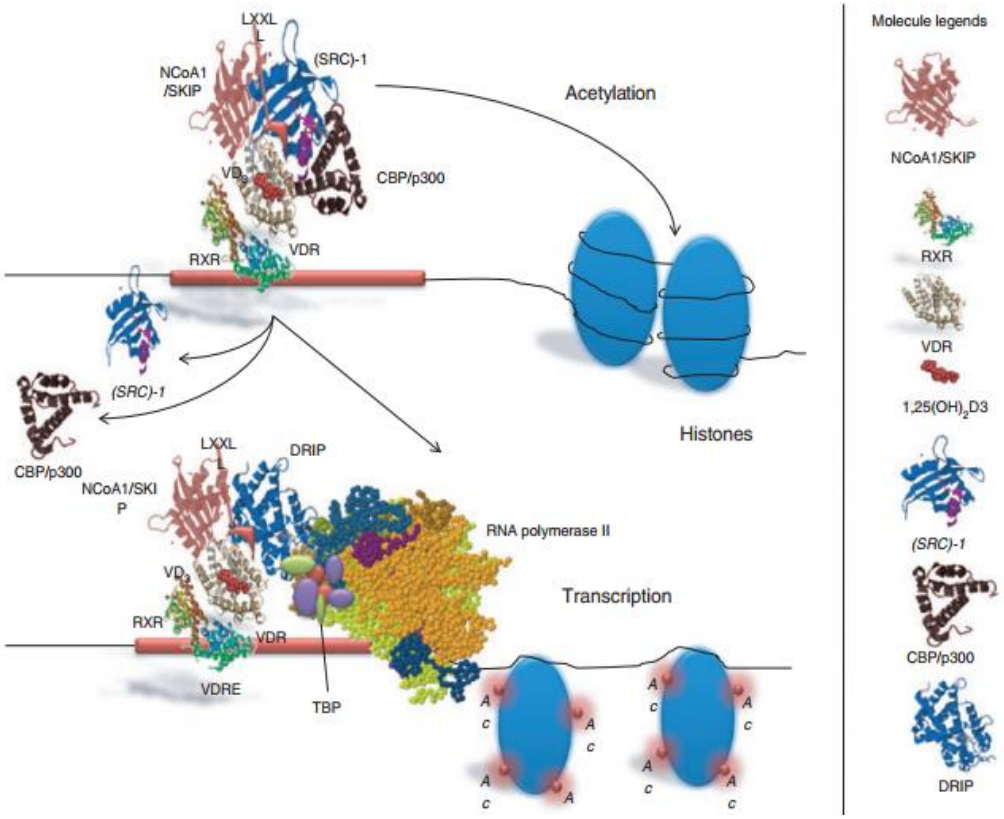

Figure-1.8. Biological activity of vitamin D. Upon binding of its ligand, calcitriol, VDR gets activated and exerts its biological function by forming heterodimer with RXR. VDR-RXR complex formation provides high-affinity DNA binding to vitamin D response elements (VDREs). (Adopted from Michelino, D. R. *et al*., 2011).

VDR activation has anti-proliferative, pro-differentiative and imunomodulatory effects on immune cells depending on the type of cell. Active VDR-RXR complex has suppressive effect on NF-κB signaling pathway in T-cells, monocytes and macrophages (Bao, B. Y. *et al*., 2010). However, ligand activated VDR has no effect on NF-κB signaling pathway as well as other transcriptional regulators such as Paired box-5 (PAX-5), B-cell lymphoma 6 (BCL-6), activation-induced cytidine deamiase (AID), B lymphocyte-induced maturation protein 1 (Blimp1) and IRF4 in B-cells (Calfon, M. *et al*., 2002). Thus vitamin D3 has different functions in different immune cell types. Ligand activated VDR-RXR stimulates the differentiation of monocytic precursors into mature cells in macrophages, suppresses antigen presentation to T cells and regulates negative DCs differentiation in dendritic cells, regulates T cell development and migration in T

cells, inhibits lineage commitment of Th17 cells and inhibits plasma cells differentiation in B lymphocytes (Michelino *et al*., 2011).

Recent clinical studies revealed that melanoma patients have reduced vitamin D3 serum levels. In addition to the clinical observations that highlight importance of vitamin D3 pathway, this pathway is known to suppress IRF4 expression in dendritic cells. And one of the ChIP on chip study revealed that vitamin D3 binds to IRF4 gene locus.

2. PURPOSE

The viability of melanoma cells has been shown to depend on IRF4 expression (Ayhan *et al*., 2013). However, the mechanism of IRF4 expression in melanoma cells has not been elucidated so far. To shed light on this issue, we aimed at taking a candidate pathway approach and identify signaling pathways that regulate IRF4 expression in melanoma cells. Understanding the regulatory mechanisms that lead to IRF4 expression in melanoma could in the future lead to new therapeutic approaches in treating melanoma.

3. MATERIALS

3.1. General Kits, Enyzmes and Reagents

Table 3.1. List of kits, enzymes and reagents.

BCA Protein Assay Kit	Thermo Scientific, USA
DMEM	Gibco, LifeTechnologies, USA
FBS	Gibco, LifeTechnologies, USA
Plasmid Midiprep Kit	Roche, Switzerland Qiagen, Germany
EndoFree Plasmid Maxi Kit	Qiagen, Germany Macherey Nagel, Germany
First Strand cDNA Synthesis Kit	New England Biolabs (AMV), USA Thermo Scientific (RevertAid), USA
XTT Kit	Roche, Switzerland
Non Essential Amino Acids	Gibco, LifeTechnologies, USA
Penicillin / Streptomycin	Gibco, LifeTechnologies, USA
RNA Isolation Kits	Roche (High Pure), Switzerland Macherey Nagel, Germany
Protein Molecular Weight Marker	New England Biolabs (P7709 V), USA
Protease Inhibitor Coctail Tablets	Roche, Switzerland
Taqman Mastermix	Roche, Switzerland Life Technologies, USA
Trypsin	Gibco, LifeTechnologies, USA
Chemiluminescent HRP Substrate	Thermo Scientific, USA
Dual Luciferase Assay Kit	Promega, USA
Luminaris Color High Green	Thermo Scientific, USA
Phosphatase Inhibitor	Roche (PhosStop), Switzerland
K2 Transfection Reagent	Biontex Labaroteries, Germany

3.2. Biological Materials

3.2.1. Bacterial Strains

Escherichia coli STBL3 strain was used for the transformation of all the vectors.

3.2.2. Cell Lines

SKMEL-28, G-361 (human melanoma cell lines; kindly provided by Dr. Marisol Soengas), MALME-3M (human melanoma cell line; kindly provided by Dr. Ali Osman Güre), A2058, SKMEL-5 (human melanoma cell lines; kindly provided by Yetiş Gültekin and Dr. David M. Sabatini), MEL-ST (human immortalized melanocyte cell line; kindly provided by Yetiş Gültekin and by Dr. David M. Sabatini), MCF-7 (human breast cancer cell line; kindly provided by Dr. Nesrin Özören), HEK-293FT (human embryonic kidney cell line; kindly provided by Dr. Nesrin Özören), cell lines were used in the experiments.

3.2.3. Plasmids

pBVI plasmid (kindly provided by Dr. Nesrin Özören), pGL2-NFAT luciferase (purchased from Addgene reference number 10959) and pGL3-NFAT luciferase (purchased from Addgene reference number 17870) were used for luciferase transactivation assays.

3.2.4. Primers

Table 3.2. Primers used in this study.

Primer ID	Sequence	Application
IRF4 – FAM	Probe: 5'-/56-FAM/AGCCCAGCA/ZEN/ GGTTCACAACTACAT/3IABkFQ/-3' Primer1: 5'-GGGACATTGGTACGGGATT-3' Primer2: 5'-CTACACCATGACAACGCCTTA-3'	qRT-PCR
IRF4 - HEX	Probe: 5'-/5HEX/AAGCAGGAC/ZEN/ TACAAC-CGCGAGG/3IABkFQ/-3' Primer1: 5'-GAGAACGAGGAGAAGAGCATC-3' Primer2: 5'-CTTTCCTTTAAACAGTGCCCAAG-3'	qRT-PCR
IRF4-FAM	Probe: 5'-/56-FAM/AAGCAGGAC/ZEN/ TACAAC-CGCGAGG/3IABkFQ/-3' Primer1: 5'-GAGAACGAGGAGAAGAGCATC-3' Primer2: 5'- CTTTCCTTTAAACAGTGCCCAAG-3'	qRT-PCR
GAPDH - FAM	Probe: 5'-/56-FAM/AAGGTCGGA/ZEN/ GTCAAC-GGATTTGGTC/3IABkFQ/-3' Primer1: 5'-TGTAGTTGAGGTCAATGAAGGG-3' Primer2: 5'-ACATCGCTCAGACACCATG-3'	qRT-PCR
GAPDH - FAM	Probe: 5'-/56-FAM/CCATCACCA/ZEN/ TCTTCCAG-GAGCGAG/3IABkFQ/-3' Primer1: 5'-TACTCAGCGCCAGCATC-3' Primer2: 5'-TGTTCCAATATGATTCCACCCA-3'	qRT-PCR
MITF - FAM	Probe: 5'-/56-FAM/CCAGTGCTC/ZEN/ TTGCTTCAGACTCTGT/3IABkFQ/-3' Primer1: 5'-GATTGTCCTTTTTCTGCCTCTC-3' Primer2: 5'-CTCACCATCAGCAACTCCTG-3'	qRT-PCR
MITF - FAM	Probe: 5'-/56-FAM/AGGAGTTGC/ZEN/ TGATGGTGAGGCC/3IABkFQ/-3' Primer1: 5'-CTTGATGGATCCTGCTTTGC-3' Primer2: 5'-TTATGTTGGGAAGGTTGGCT-3'	qRT-PCR
PGK1 - HEX	Probe: 5'-/5HEX/TCATCAAAA/ZEN/ ACCCAC-CAGCCTTCTGT/3IABkFQ/-3' Primer1: 5'-TTTGGCACTGCTCACAGAG-3' Primer2: 5'-TTGCAAAGTAGTTCAGCTCCT-3'	qRT-PCR

3.3. Chemicals

Table 3.3. Chemicals used in this study.

Ethanol	Merck, USA
Nonidet P40	Fluka, USA
Hexadimethrine bromide	Sigma Aldrich, USA
Chloroquine diphosphate	Applichem, Germany
Ammonium persulfate	Applichem, Germany
SDS	Applichem, Germany Sigma Aldrich, USA
Ampicilllin	Applichem, Germany
Acrylamide	Sigma Aldrich, USA
Sodium Deoxycholate	Sigma Aldrich, USA
N,N'-Methylenbisacrylamide	Sigma Aldrich, USA
DEPC-treated water	Fisher Scientific, USA
Agarose	Sigma Aldrich, USA
Bay 11-7085	Selleckchem, Germany
MLN 4924	Active Biochem, USA
Calcitriol	Selleckchem, Germany
Fk506	Selleckchem, Germany
Cyclosporine	Selleckchem, Germany
Cyclosporine A	Selleckchem, Germany
BSA	Applichem, Germany
Saponin	Sigma Aldrich, USA
Tris-Base	Sigma Aldrich, USA
Ponceau S powder	Applichem, Germany
Paraformaldehyde	Sigma-Aldrich, USA (P6148)
Isopropanol	Merck, USA
Milk Powder	Pinar, Turkey
HEPES	Gibco Invitrogen, USA

3.4. Buffers and Solutions

Table 3.4. Buffers and solutions used in this study.

10X SDS Running Buffer	1% SDS 1.92M Glycine 250 mM Tris-Base
10X Transfer Buffer	1.92M Glycine 250 mM Tris-Base
1X Transfer Buffer	10% 10X Transfer Buffer 20% Methanol
4X Protein Loading Dye	200 mM Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 4% β-mercaptoethanol 50 mM EDTA 0.8% Bromophenol Blue
Cell Lysis Buffer	150 mM NaCl 50 mM Tris (pH: 8.0) 0.1% SDS 0.5% Sodium deoxycholate 1.0% NP-40
10% SDS-PAGE Gel, Resolving Part (20ml)	8 ml ddH2O 6.66 ml 30% Acrylamide/Bisacrylamide mix 5ml 1.5M Tris, pH: 8.8 200 µl 10% SDS 120 µl 10% APS 20 µl TEMED
4% SDS-PAGE Gel, Stacking Part (8ml)	7788 µl ddH2O 1072 µl 30% Acrylamide/Bisacrylamide mix 1ml 1 M Tris, pH: 6.8 200 µl 10% SDS 50 µl 10% APS 10 µl TEMED
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X TBS-T	50 mM Tris-Base (pH: 7.4) 150 mM NaCl 0.1% Tween 20
Paraformaldehyde (4%)	1 g Paraformaldehyde 25 ml distilled water

Table 3.5. Buffers and solutions used in this study (cont.).

5% Blocking Solution	5% BSA (w/v) in 1X TBS-T 5% Milk Powder (w/v) in 1X TBS-T
10X PBS	1,37 M NaCl 270 mM KCl 80 mM NaH2PO4 20 mM KH2PO4
1X PBS+	1X PBS 0.5% BSA
1X PBS+S	1X PBS 0.5% BSA 0.3% Saponin
Freezing Medium	1X DMEM 20% FBS 1X Pen/Strep 100 µM MEM-NEAA 10% DMSO
400X Choloroquin	10 mM Choloroquin
1000X Polybrene	4 mg/ml Polybrene
2X HBS	50 mM HEPES (pH: 7.0) 280 mM NaCl 1.5 mM NaH2PO4
LB Medium	10 g/L Tryptone 5 g/L NaCl 5 g/L Yeast Extract
1000X Ampicillin	100 mg/ml in ddH2O
1000X Kanamycin	50 mg/ml in ddH2O

3.5. Antibodies

Table 3.6. Antibodies used in this study.

Name	**Species**	**Dilution**	**Source**
Anti-Actin	Rabbit	1:2500	4967S, Cell Signaling
Anti-IRF4 X (M-17)	Goat	1:5000	Sc-6059, Santa Cruz
Anti-IRF4 (M-17)	Goat	1:500	Sc-6059, Santa Cruz
Anti-MITF	Mouse	1:3000	Ab12039, Abcam
Anti-Rabbit-HRP	Mouse	1:2500	7074S, Cell Signaling

Table 3.7. Antibodies used in this study (cont.).

Anti-Mouse-HRP	Horse	1:2500	7076S, Cell Signaling
Anti-Goat-HRP	Rabbit	1:2500	Sc-2768, Santa Cruz
Anti-Goat-HRP	Mouse	1:2500	Sc-2354, Santa Cruz
Normal Rabbit IgG	Rabbit	-	Sc-2028, Santa Cruz
Normal Goat IgG	Goat	-	Sc-2028, Santa Cruz
Anti-Goat-IgG-PerCP	Goat	-	Sc-45091, Santa Cruz
Anti-Goat-IgG-PE	Goat	-	Sc-3743,Santa Cruz

3.6. Disposable Labware

Table 3.8. List of disposable labwares used in this study.

Western Blotting Paper	Whatman, UK
Parafilm	Brand, Germany
Scalpel	Swann-Morton, UK
Centrifuge Tubes, 15 ml	Vwr, USA
Centrifuge Tubes, 15 ml	Vwr, USA
Serological pipette, 5ml	Tpp, Switzerland
Serological pipette, 10ml	Tpp, Switzerland
Serological pipette, 25ml	Tpp, Switzerland
Pipette Tips, filtered	Biopointe, USA
Pipette Tips, bulk	Biopointe, USA
Microcentrifuge tubes	Axygen, USA
PCR Tubes, 0.2 ml	Axygen, USA
Medical Gloves	Vwr, USA Broche Medikal, Turkey
Syringe Filters	Sartorius, Germany
Cryovial	Tpp, Switzerland
Cell Culture Plates, 10 cm	Tpp, Switzerland
Cell Culture Plates, 6-well	Tpp, Switzerland
Cell Culture Plates, 96-well	Tpp, Switzerland
Cell Culture Flasks, $75cm^2$	Tpp, Switzerland
Insulin Syringes, 1 ml	Vwr, USA
Syringe, 10ml	Becton Dickinson, USA

Table 3.9. List of disposable labwares used in this study.

PS Test Tubes, 5 ml	Becton Dickinson, USA Isolab, Germany
Glass Pasteur Pipette, 230 mm	Witeg, Germany
Chemiluminescent Detection Film	Roche, Switzerland
96 well plates for qRT-PCR	Thermo Scientific, USA

3.7. Equipments

Table 3.10. List of equipment used in this study.

Beaker, 100 ml	Duran, Germany
Beaker, 600 ml	Duran, Germany
Erlenmeyer, 250 ml	Duran, Germany
Erlenmeyer, 500 ml	Duran, Germany
Bottle, 1000 ml	Vwr, USA
Bottle, 500 ml	Vwr, USA
Bottle, 100 ml	Vwr, USA
Forceps	RSG Solingen, Germany
Magnetic Stirring Bar	Vwr, USA
Cryobox	Tenak, Denmark
Autoclave Indicator Tape	Llg, Germany
Measuring Cylinder 100 ml	Kartell, Italy
Measuring Cylinder 250 ml	Kartell, Italy
Measuring Cylinder 1000 ml	Kartell, Italy
pH Meter	Hanna, USA
Microtube Racks	Vwr, USA
Electronic Balance	Sartorius AY123, Germany
Microcentrifuge	Vwr Galaxy Ministar, USA
Horizontal Electrophoresis	Cleaver Scientific Multi Sub Mini, UK
Micropipettes	Cleaver Scientific, UK Gilson Pipetman Neo PCR Kit, USA Axygen, USA

Table 3.11. List of equipment used in this study.

Heat Block	Cleaver Scientific EL-01, UK
Pipettor	Greiner Labopet 240, Germany
Vortex	Vwr, USA
Vertical Electrophoresis	Cleaver Scientific Omni-page Mini, UK
Centrifuges	J2-21, Beckman Coulter, USA Allegra X-22, Beckman Coulter, USA 5415R, Eppendorf, USA
Rotor	Beckman Coulter JA-14, USA
Cell Culture Incubator	Binder C-150, Germany
Documentation System	GelDoc XR System, Bio-Doc, Italy
Flow Cytometer	FACSCalibur , Becton Dickinson, USA
Freezers	-20°C, Ugur UFR 370 SD, Turkey -80°C, Thermo Scientific TS368, USA -150°C, Sanyo MDF1156, Japan
Microplate Reader	680, Biorad, USA
Microscopes	Inverted Microscope, Nikon Eclipse TS100, Japan Fluoroscence Microscope, Observer Z1, Zeiss, Germany
Thermal Cycler	Antarus MyCube ANT101, USA
Power Supply	Vwr, USA
Real Time PCR Machine	Thermo Scientific Piko Real 96, USA
Shaker	VIB Orbital Shaker, InterMed, Denmark Vwr Mini Orbital Shaker, USA
Spectrophotometer	NanoDrop 1000, USA
Stirrer - Heater	Dragonlab MS-H-S, China
Rotator	Grant Bio Multifunctional Rotator PTR-35, UK
Safe Light	Kodak Model B, USA
Safe Light Filter	Kodak GBX-2, USA
Hybridization Oven	Thermo Scientific Hybaid Shake'n'Stack, USA
Refrigerator	4°C, Ugur USS 374 DTKY, Turkey
Refrigerated Vapor Trap	Thermo Scientific SPD111V, USA
Oil-free Gel Pump	Thermo Scientific Savant VLP110, USA
Vacuum Pump Oil Filter	Thermo Scientific VPOF110, USA
SpeedVac	Thermo Scientific SPD111V, USA
Carbon dioxide Tank	Genc Karbon, Turkey
Ice Maker	Brema, Italy Scotsman Inc. AF20, Italy
Autoclave	Model ASB260T, Astell, UK

Table 3.12. List of equipment used in this study.

Dishwasher	Miele Mielabor G7783, Germany
Cold Room	Birikim Elektrik Soğutma, Turkey
Stella	Raytest, Germany
Freezing Container	Nalgene, USA
Oven	Nüve KD200, Turkey
Local Server with 5 x Quad-Core Intel Xeon Processor	HP, USA

4. METHODS

4.1. Cell Culture

4.1.1. Cell Line Maintenance

Cell lines used in this study were grown in DMEM - high glucose which contains the additives of 10% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids. They were incubated in an incubator with the following conditions, 5% CO_2 and 37°C. In order to passage cells, old medium was aspirated and cells were washed with 1X PBS. In order to detach the cells were incubated with trypsin (0.05%) in 37°C for 3-5 minutes. Equal amount of DMEM was added to the cells for the deactivation of the trypsin, and they were transferred into a 15ml falcon for the centrifugation at 2000 RPM for 2 min. Medium-trypsin mix aspirated and cells were suspended in a proper volume of fresh DMEM by pipetting. Then cells were seeded back into new flasks or plates.

In order to stock the cells for long periods; after cells were trypsinized and centrifuged, old medium was aspirated and cell pellet was resuspended in freezing medium (Table 3.4). If 10-cm cell culture plate was fully confluent, it was divided into 5 cryovials which contains 1ml of cell and freezing medium mix. Cryovials were placed into Nalgene Freezing Container filled with isopropanol. Container was kept at -80°C for a day and then cryovials were transferred into cryoboxes at -150°C.

In order to thaw the cells from cell line stocks which are kept at -150°C freezers; one vial for one 10-cm cell culture plate was thawed quickly in water bath. Immediately, after thawing was complete, they were transferred into 15ml falcon tubes previously filled with 5 ml of fresh and warm DMEM. Cells were centrifuged at 500g for 5 min. After aspirating the medium to remove DMSO, cells were resuspended and seeded into proper plates.

4.1.2. Drug Treatment of Cell Lines

All treated cell lines were seeded onto different type of plates or wells at 60-80% confluency 12 hours before the treatment. Working solutions of all drugs including Bay 11-7085, MLN 4924, Calcitriol, JQ1, Fk506 and CsA were prepared 1000 fold of the desired concentration. 1 to 1000 dilutions were done in the fresh and warmed medium. Thereafter, medium of the cells were aspirated and washed with PBS in order to completely remove the old medium. Drug containing medium was added to the cells along with the drug's control. .

4.1.3. Transient Transfection of Episomal Vectors with K2 Transfection System

Transfection by K2 system relies on a lipofection method in which cationic lipids were used for the formation of liposomes around DNA by "K2 Transfection Reagent". Another solution, K2 Multiplier, decreases the detectability of DNA by transfected cell as a foreign DNA therefore, it helps to avoid from immune reactions by the transfected cells.

Two hours before transfection, 40µl K2 Multiplier was mixed with 2ml of fresh medium (serum containing DMEM) and added onto cells in a 6-well plate. If there were multiple wells, master mix was prepared for K2 Multiplier - fresh medium mix. In a 96-well plate; solution A, containing 144µl of serum-free medium and 2µg plasmid, and solution B, containing 144µl of serum-free medium and 12µl of K2 Transfection Reagent were prepared in separate wells. At this step, they should not be kept in separate solutions for longer than 5 minutes. In the next step, solution A, containing the plasmid was added onto solution B, containing the K2 Transfection Reagent and mixed by pipetting. Mixed solution was incubated for 15 to 20 minutes and then, it was added onto cells in a drop-wise manner. 4,5 hours later, medium of the wells were refreshed with serum containing fresh DMEM. Transfection efficiency was determined by flow cytometry analysis, 1-2 days after transfection.

4.1.4. Dual Luciferase Assay

After transient transfection of firefly and renilla luciferase reporter plasmids, cells were incubated for 48 hours in order to express proper amount of firefly and renilla luciferin. At the end of the incubation, medium of the cells were collected to the 15 ml tube. Cells were washed with PBS and remaining PBS was also collected to the same tube. In order to detach the cells were incubated with trypsin (0.05%) in 37°C for 3-5 minutes. Equal amount of DMEM was added to the cells for the deactivation of the trypsin, and they were collected to the same 15 ml tube. The collection tube was centrifuged at 2000 RPM for 2 minutes. Medium-trypsin mix was aspirated and cells were resuspended in a proper volume of PBS and were centrifuged at 2000 RPM for 2 minutes in order to remove trypsin. Thereafter, PBS was aspirated and cells were resuspended in a proper volume of PBS. Cells were added to a black walled and bottom 96 well plates and were mixed with firefly luciferase substrate of Promega. 15 minutes of incubation was followed by shaking and five independent measurement of firefly luciferase activity by luminometer. Renilla luciferase substrate was freshly prepared in renilla solution according to the manufacturer's instructions. Fresh renilla substrate was added to the same wells in the black walled and bottom 96 well plates. Thereafter, 15 minutes of incubation was followed by shaking and five independent measurement of renilla luciferase activity by luminometer.

4.1.5. XTT Cell Viability Assay

7500 cells were seeded onto black walled and clear bottom 96 well plate 12 hours before the treatment. After drug exposure, cell viability was measured by using Roche XTT Cell Viability kit according to the manufacturer's instructions.

4.2. Molecular Biological Techniques

4.2.1. Plasmid Isolation

Plasmids isolated were always used either for transient transfection with K2 or pEI25b systems to melanoma cells to perform either luciferase reporter transactivation assays or overexpression of desired protein. Since such experiments require a pure plasmid batch, isolations were performed with endotoxin-free maxi plasmid isolation kits. For the plasmid isolation purposes either QIAGEN EndoFree Plasmid Maxi Kit or Macharey-Nagel NucleoBond Xtra MaxiPlus EF was used according to the manufacturer's instructions.

4.2.2. Isolation of Total RNA

Medium of cells was collected into 15 ml falcon tube. In the following step, cells were washed with PBS and remaining PBS was collected to the same 15 ml falcon tube. Cells were detached by trypsinization. Detached cells were transferred into the same 15ml falcon tube. Falcon tube was centrifuged at 2000RPM for 2 minutes and medium was aspirated. Afterwards, cells were resuspended in 1 ml of 1X PBS for washing. Centrifugation was repeated and cell pellet was lysed by lysis buffer, supplied with the kit. Roche High Pure RNA Isolation kit or Macherey-Nagel NucleoSpin RNA Isolation kit was used according to the manufacturer's instructions. Total RNA was stored at -20^{o}C for short periods (1-2 weeks), and for longer periods, samples were kept at -80^{o}C.

4.2.3. cDNA Synthesis

Reverse transcription and cDNA synthesis were performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instruc-

tions. Final volume of 20µl was diluted according to the quantity of total RNA. cDNA products were kept at -20^{o}C for further use.

4.2.4. Real Time Quantitative PCR (RT-qPCR)

After cDNA synthesis, samples were diluted 1 to 50. Master mix for each well prepared from 5µl of TaqMan Gene Expression Master Mix and 0,5µl of primer-probe mix. 4,5µl of sample was added to the wells after the master mix was added. Plate then centrifuged at 2000RPM for 2 minutes. This step enables that all the solution is at the bottom of the wells. Plate was placed into PikoReal 96 real time PCR machine and the protocol was prepared with initial denaturation at 50^{o} C for 2 minutes then at 95^{o} C for 10 minutes. In the next step, protocol includes 40 cycles of 95^{o} C for 15 seconds incubation and 60^{0} C for 1 minute for annealing-elongation. An automatic threshold for the amplification peaks was determined by the software at the end of the qPCR.

4.3. Western Blotting

4.3.1. Cell Lysis and Protein Extraction

Medium of the cells to be lysed was collected to 15 ml falcon tube and cells were washed with PBS once. Remaining PBS solution was collected to the same tube. Adherent cells were trypsinized and transferred to same 15ml falcon tube with the same amount of fresh DMEM. After centrifugation at 2000RPM for 2 minutes, medium was aspirated carefully, and cells were washed with PBS twice to completely remove trypsin. NP-40 cell lysis buffer was mixed with 50X protease inhibitor just before addition on to the cells. 300µl of protease inhibitor containing NP-40 buffer was used to lyse cells from either dishes or 6 well plates. Following the addition of the lysis buffer, the solution should be mixed harshly by pipetting to lyse the cell pellet efficiently. Protein lysate was transferred into a 1.5ml tube, then, was kept in ice for 30 minutes and syringed with a 22-gauge needle afterwards. Lysate was centrifuged at 14,000g for 15 minutes at 4^{o}C.

After leaving precipitates in the tube, total protein lysate was transferred into a new 1.5ml micro centrifuge tube.

4.3.2. BCA Assay for Protein Concentration Determination

Pierce BCA Protein Assay Kit was used for the determination of protein concentrations. BSA standards in the concentrations of 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml were prepared from the albumin stock given in the kit by serial dilution in 1X PBS. All the samples were prepared in at least 4-replicates. BCA solution was prepared by mixing 150µl of Reagent A and 3µl of Reagent B for each sample. After BCA solution was prepared as a master mix, 150µl per well was distributed into a 96-well plate after the addition of 5µl of standards and lysates. Plate was kept on ice while adding the protein lysates. Plate was incubated at 37°C for 30 minutes and absorbance values were determined at 570 nm on the plate reader with the addition of 3 shakes. Blanks were subtracted from median OD values of samples and standards. Standard blank is the PBS and sample blank is the NP-40 buffer. To calculate concentration of samples, firstly, a standard curve was prepared in Microsoft Excel for standards by adding the line to cross from (x,y) = 0,0 where x stands for concentration and y stands for the absorbance value. Formula of the standard curve was used to calculate the samples' concentrations.

4.3.3. Preparation of Protein Samples and SDS Gel Electrophoresis

Protein lysates were prepared by adding 4X laemni loading dye and boiling the mix at 95°C for 5 minutes. Those lysates were loaded into the wells of SDS-gel prepared at Cleaver Omni-Page Gel Casting System where resolving gel (10%) polymerized at the bottom and stacking gel (4%) at the upper part (Table 3.4). The amount of lysates loaded were calculated according to the BCA assay results and the lowest concentrated protein lysate was the limiting factor for how much protein would be loaded into each well. Lysates were loaded into the wells in the vertical gel electrophoresis chamber, filled with 1X running buffer and the system was run at 100V until the loading dye gets into resolving gel, and then at 120V until the loading dye reached to the end of the gel.

4.3.4. Blotting - Transfer from SDS Gel to PVDF Membrane

Blotting papers and PVDF membrane, cut in the size of the blotting pad, were prepared. PVDF membrane was activated in methanol for 10 seconds, then washed in dH_2O for 1 minute and kept in 1X transfer buffer until it was placed in the blotting system. Meanwhile, glasses of running apparatus were separated from each other, by leaving the SDS gel on one of the glasses. Under distilled water the stacking part was removed and the gel was placed on the blotting paper. Under the blotting paper, there was one of the blotting cassettes and one blotting pad. Membrane was placed on the gel by paying attention not to leave any bubbles between blotting paper and gel, and between gel and membrane for an efficient transfer. For that purpose, those steps were performed in a container partially filled with transfer buffer, so bubbles could be removed easily. Another blotting paper and blotting pad was placed onto the membrane. After other part of the cassette was placed on the pad, clips were closed to compress the system. Transfer cassette was placed into the transfer chamber in the right orientation. Membrane should be at positive, and gel should be at the side of negative pole because proteins are negatively charged due to SDS. Transfer was performed at 250 mA for 1hour and 45min. After transfer was complete, membranes were cut to a smaller size depending on the size of the protein of interest. Membranes were washed with 1X TBS-T (0.1% Tween 20) for several times to remove methanol.

4.3.5. Staining - Antibody Incubations

Prior to staining with antibodies, the membrane was blocked for 1 hour at room temperature with 5% BSA (w/v) prepared in TBS-T. Membrane was incubated overnight at 4^oC, in certain dilution of the desired antibody, also prepared in 5% BSA solution. Membrane was washed 3 times with TBS-T with 5 minute intervals before incubation with HRP linked secondary antibody, diluted in TBS-T. Secondary antibody incubation was performed at room temperature for 2 hours. Membranes were washed 3 times with TBS-T in 5 minute intervals. Blocking, washing and antibody incubations were performed on a bench top orbital shaker.

4.3.6. Chemiluminescence Detection

ECL (enhanced chemiluminescent solution) was prepared by mixing 1 volume of peroxide and 1 volume of substrate in a Falcon tube wrapped with aluminum foil to protect from light. Before placing the membrane on a glass plate for detection of luminescent signal at imaging system (Stella), membrane was incubated in the ECL solution in a small-size container for a minute, paying attention to keep it away from light. Then, membrane placed on a glass plate was put in Stella and exposure duration was determined by the help of Xstella software. Detection was done after focusing was adjusted.

4.4. Intracellular Flow Cytometry

In order to detect stained proteins in the cells with flow cytometry, intracellular flow cytometry assay was used. PBS-Saponin (PBS+S) and PBS-BSA (PBS+) were prepared to be used in the washing steps. Cells were collected into glass flow cytometry tubes and washed with PBS+ for twice. Then, cells were fixed by using 2% paraformaldehyde for 10 minutes at room temperature. Residual paraformaldehyde was removed by centrifugation. Cells were permeabilized by using PBS+S, which enables opening of the pores. After removal of the residual saponin by light centrifugation, primary antibody was added to the tube and the cells were incubated for 30 minutes at room temperature. After removal of unbound primary antibody with light centrifugation, cells were incubated with secondary antibody for 30 minutes at room temperature. After addition of the secondary antibody, all samples were kept in dark conditions due to the light sensitivity of the secondary antibody. After secondary antibody incubation, cells were centrifuged and unbound secondary antibody was aspirated and cells were washed with PBS+ twice. At the last step, cells were resuspended in fresh PBS. Samples were analyzed by using FACS Calibur.

5. RESULTS

5.1. Optimization of Intracellular Flow Cytometry for Protein Expression Analysis

In order to study IRF4 protein expression patterns of melanoma cell lines at different conditions, we implemented an intracellular flow cytometry protocol using IRF4-positive Sk-mel-28 and IRF4-negative Mewo cell lines. Unstained and isotype control normal goat serum stained cells were used as negative control as IRF4 primary antibody is goat origin. IRF4 positive melanoma cell line, Sk-mel-28, showed a distinguishable peak from both negative controls, when it was stained for target protein IRF4. However, the IRF4 negative melanoma cell line, Mewo, showed an overlapping peak with the isotype control antibody stained sample (Figure 5.1). As a proof of principle, this method is sufficient to detect IRF4 protein expression patterns of melanoma cells under various conditions.

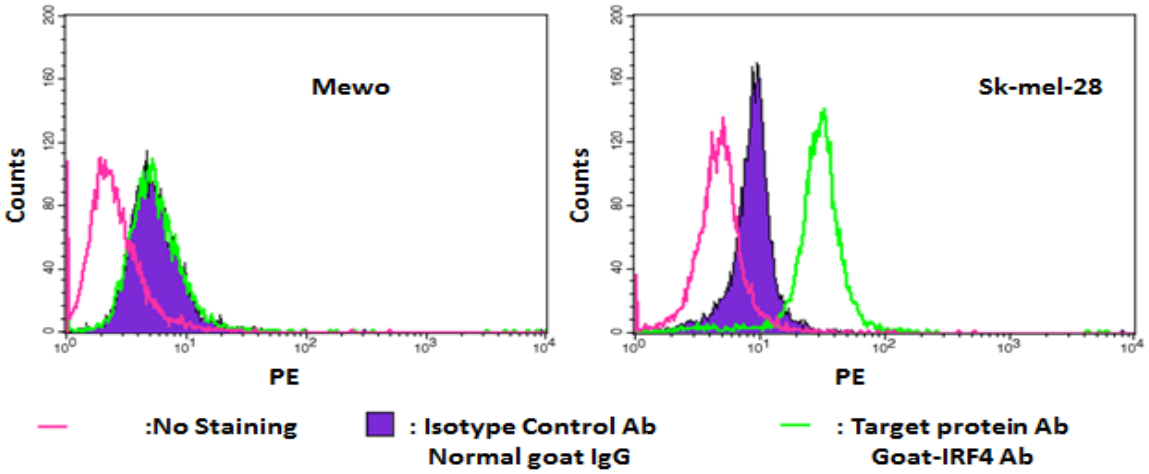

Figure 5.1. Optimization of intracellular flow cytometry. Intensities of fluorescence emitted from phycoerythrin (PE), which is conjugated to secondary antibodies are shown on the x-axis and cell counts are shown on the y-axis. Mewo cell line was used as negative control. Unstained and isotype control antibody stained samples were used as internal controls.

5.2. Testing the Regulatory Role of NF-κB Pathway on IRF4 Expression

5.2.1. Detection of the NF-κB Pathway Activity

IRF4 expression is known to be induced by binding of RELA, called as p65, one of the NF-κB transcription factor family members, to its binding motif, NGGRNTTYCC in promoter regions of B cells (Sharma *et al.*, 2003). In order to detect NF-κB pathway activity, we first analyzed the expression levels of p65. Herein, we assay p65 expression in the melanoma cell line Sk-mel-28 using intracellular flow cytometry. B-cell lymphoma cell line HBL was used as a positive control. Our analysis shows that p65 in melanoma cells is expressed as in the positive control line HBL-1.

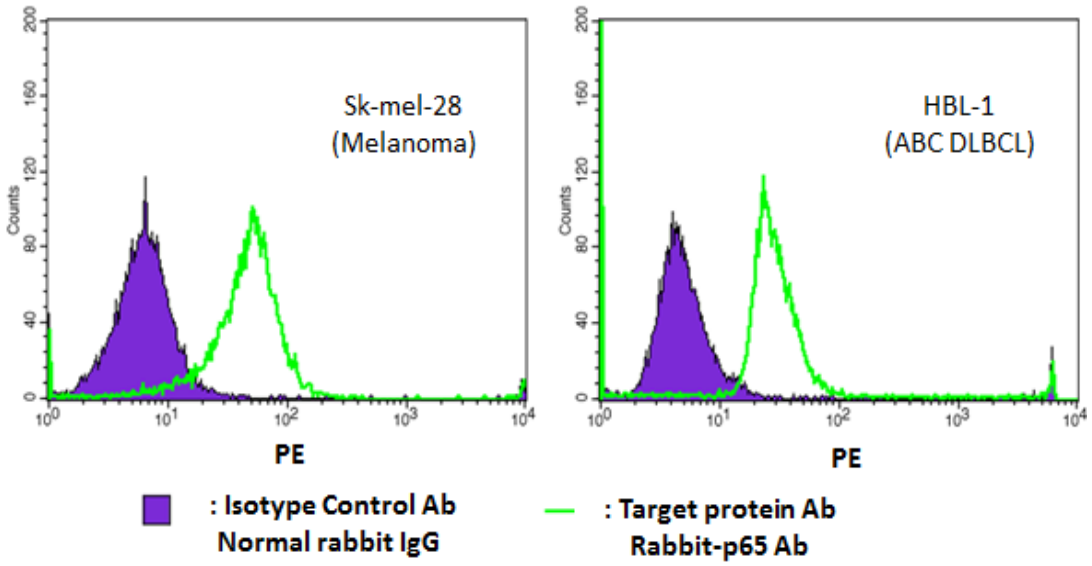

Figure 5.2. P65 expression in Sk-mel-28 melanoma cell line assayed by intracellular flow cytometry. Intensities of fluorescence emitted from phycoerythrin (PE), which is conjugated to secondary antibodies are shown on the x-axis and cell counts are shown on the y-axis. An isotype control antibody was used as the negative control.

After observing p65 expression in the melanoma cell line Sk-mel-28, we measured NF-κB pathway activity in melanoma. We transiently transfected A2058, G361, Mewo, Sk-mel-5 and Sk-mel-28 melanoma cell lines and an immortalized melanocyte cell line, Mel-st, with pBVI reporter plasmid (kindly gift of Professor Nesrin Özören) containing seven consecutive repeats of NF-κB binding motifs along with transfection control plasmids pSV40-renilla. Transfected cells are expected to express firefly luciferase enzyme and activate the transfected reporter if NF-κB transcription factor family members are present in the nucleus. Two days after transfection, activity of NF-κB pathway was measured using a dual luciferase reporter assay. In this assay, harvested and collected cells are incubated with firefly luciferase substrate. Due to the activation of the reporter, an enzymatic reaction occurs and a detectable signal is measured in luminometer. After recording the signals of firefly luciferase reporter, cells are treated with second substrate called as Stop&Go. This substrate blocks the previous enzymatic reaction and starts another enzymatic reaction which is catalyzed by renilla reporter. The results of latter reaction is used for transfection and seeding control.

As a result, our experiment shows that all tested melanoma cell lines have an active NF-κB pathway. After normalization of each cells' firefly to renilla luciferase activities, we normalized the results to highest value. Therefore, Sk-mel-5 showed the highest firefly luciferase activity while G361 showed the lowest firefly luciferase activity, indicating that nuclear localization of the NF-κB transcription factor family members are more abundant or active in Sk-mel-5 among the all other tested melanoma cell lines. Immortalized melanocyte cell line Mel-st showed very little NF-κB transcriptional activity when compared to melanoma cell lines.

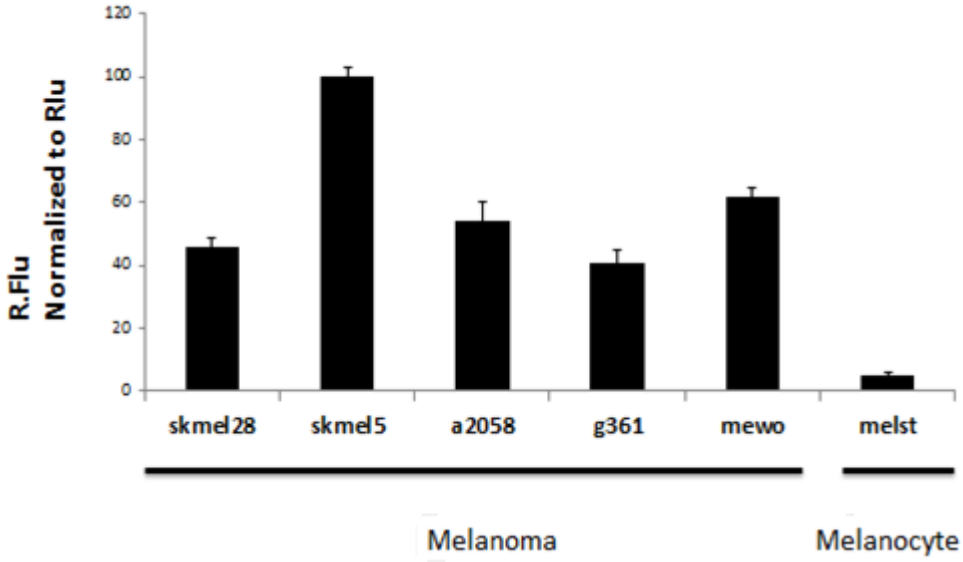

Figure 5.3. NF-κB pathway activity in melanoma. Melanoma cell lines; Sk-mel-28, Sk-mel-5, A2058, G361, Mewo and immortalized melanocyte cell line; Mel-st were transiently transfected with pBVI and SV40 Renilla plasmids. Relative firefly Luciferase activity was normalized to renilla luciferase and then to Sk-mel-5.

5.2.2. Validation of NF-κB Pathway Inhibition

After observing NF-κB pathway activity in melanoma cell lines, we looked at its possible positive regulatory role on IRF4 expression in melanoma cell lines as some of them express IRF4 (Ayhan, 2013; Yılmaz, 2014). In order to dissect a possible positive regulatory mechanism, we blocked NF-κB pathway activity. Before looking at effects of NF-κB pathway inhibition, we first validated inhibition of the NF-κB pathway by using two different small molecular compounds; Bay 11-7085 (Pierce *et al*., 1997) and MLN-4924 (Marblestone *et al*., 2009) using the dual luciferase system. For this, we combined our NF-κB pathway reporter dual luciferase system with drug treatment. In this system, we first transfected cells with a firefly luciferase reporter plasmid and a renilla luciferase plasmid using a K2 transfection method which is a transfection method of primary cell

lines. At the medium changing step of the transfection procedure, we applied drugs and their proper control; DMSO as all drugs were initially dissolved in it to the cells. 48 hours after the treatment, cells were collected and relative firefly and renilla luciferase activities were measured by using dual luciferase reporter assay (Figure 5.4).

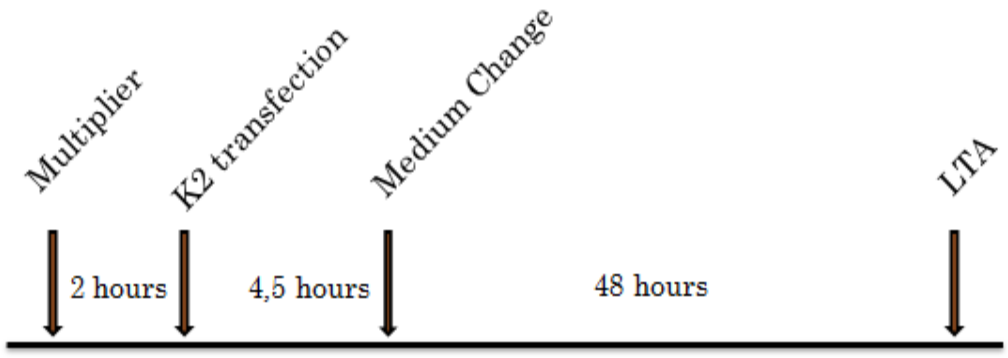

Figure 5.4. Schematic overview of the combination of drug screen assays with luciferase transactivation assays. Melanoma cell lines were transfected with reporter plasmids using the K2 transfection method. At the medium changing step, cells were treated with either drugs or their proper controls. 48 hours after the treatment, cells were collected and relative firefly luciferase activities were measured

In this experiment, the aim was to evaluate whether Bay 11-7085 and MLN 4924 are able to inhibit NF-κB pathway activity or not. For this purpose, different melanoma cell lines with a constitutively active NF-κB pathway transfected with pBVI and SV40 Renilla plasmids. In the medium changing step of the transfection, cells were treated with either drugs or vehicle DMSO as all drugs were initially dissolved in it. Relative firefly and renilla luciferase activities were detected 48 hours after treatment. NF-κB pathway activity in G361 melanoma cell lines was not much affected by Bay 11-7085 inhibition. But inhibition of the neddylation pathway by MLN 4924 impaired NF-κB pathway activity in G361 (Figure 5.5) indicating a presence of Cullin Ring Ligase (CRL) pathway dependent NF-κB activity in G361 because inhibitory effect of MLN 4924 on NF-κB pathway depends on CRL pathway (Soucy *et al*., 2009) and the inhibition of merely the canonical pathway by Bay 11-7085 didn't alter NF-κB pathway activity. The same experimental procedure was also applied to the Sk-mel-28 melanoma

cell line. NF-κB pathway activity in Sk-mel-28 was significantly affected by the same concentration of Bay 11-7085 and two different concentrations of MLN 4924 (Figure 5.5). We did XTT cell viability assay in order to see whether cells' viability is affected by Bay 11-7085 treatment or not (Figure B.1).

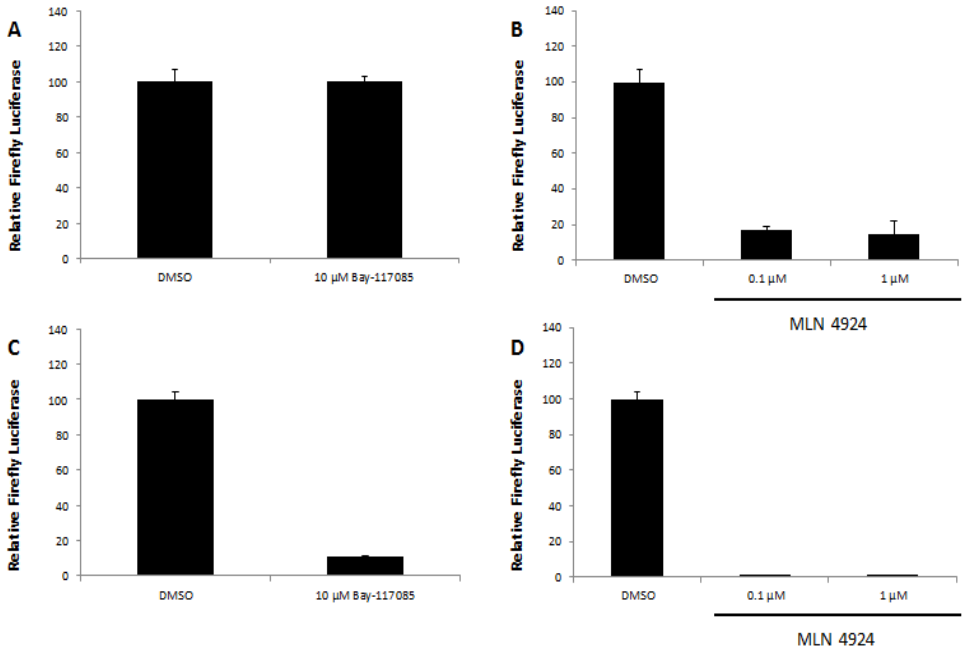

Figure 5.5. Validation of NF-κB pathway inhibitors Bay 11-7085 and MLN 4924. G361 and Sk-mel-28 melanoma cell lines were transfected with pBVI and SV40 Renilla plasmids. At the medium changing step of the transfection, G361 and Sk-mel-28 cells were treated with (A,C) Bay 11-7085 and (B,D) MLN 4924 for 48 hours. Relative luciferase activities were normalized to renilla and then to DMSO treated samples.

In summary, using dual luciferase system, we validated that both NF-κB pathway inhibitors, Bay 11-7085 and MLN 4924 effectively inhibits NF-κB pathway activity in melanoma cell lines except for Bay 11-7085 in G361.

5.2.3. Regulatory Role of NF-κB Pathway in IRF4 Expression

Our aim is to find whether NF-κB pathway positively regulates IRF4 expression in melanoma cell lines. For this purpose, we looked at the IRF4 expression levels after inhibition of NF-κB pathway activity in several melanoma cell lines using the inhibitors Bay 11-7085 and MLN 4924. IRF4 mRNA expression levels of the A2058 cell line was analyzed by Q-RT PCR after treatment with MLN 4924. Relative mRNA expression levels of IRF4 were normalized to reference gene UBC expression individually. We found that inhibition of the NF-κB pathway down-regulates IRF4 mRNA expression in the A2058 cell line within 24 hours after drug exposure (Figure 5.6)

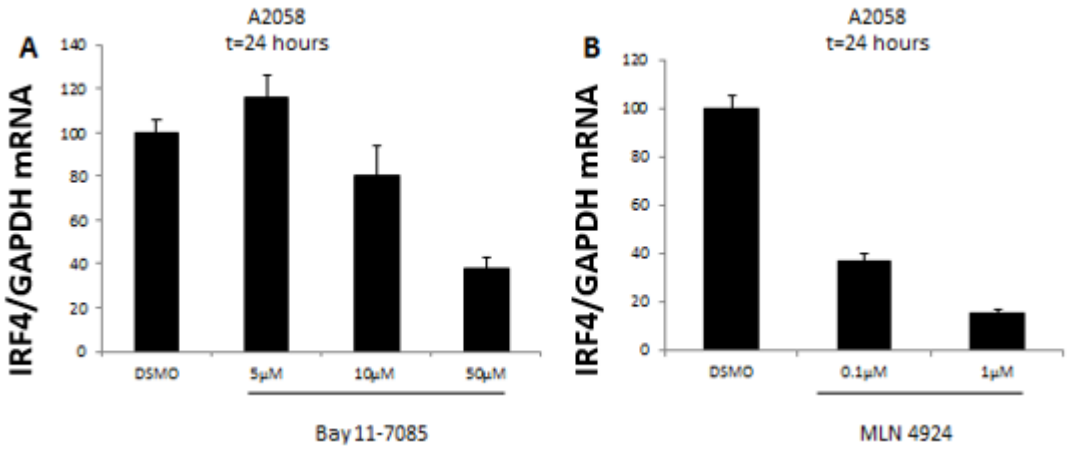

Figure 5.6. IRF4 mRNA expression levels in the A2058 melanoma cell line upon inhibition of the NF-κB pathway by MLN 4924 treatment. A2058 cells treated with MLN 4924 for 6 hours (A) and 24 hours (B). cDNAs were prepared from total RNA and relative mRNA expression level of IRF4 was normalized to GAPDH.

After observing a sharp decrease in IRF4 mRNA levels in the A2058 melanoma cell line due to the NF-κB pathway inhibition by MLN 4924, we analyzed the effect of the same inhibition at the IRF4 protein level. A2058 cells were treated with MLN 4924 and IRF4 protein expression levels were observed by using both western blotting (Figure 5.7) and intracellular flow cytometry (Appendix B).

Although the NF-κB pathway seems to positively regulate IRF4 expression in the A2058 melanoma cell line, inhibition of the NF-κB pathway did not affect IRF4 expression levels in other melanoma cell lines including G361, Sk-mel-5 and Sk-mel-28 because IRF4 protein expression levels did not change after inhibition of the NF-κB pathway with MLN 4924 in these cell lines (Figure 5.7). Therefore, NF-κB pathway doesn't have any consistent observable regulatory effect on IRF4 expression in melanoma cell lines in general.

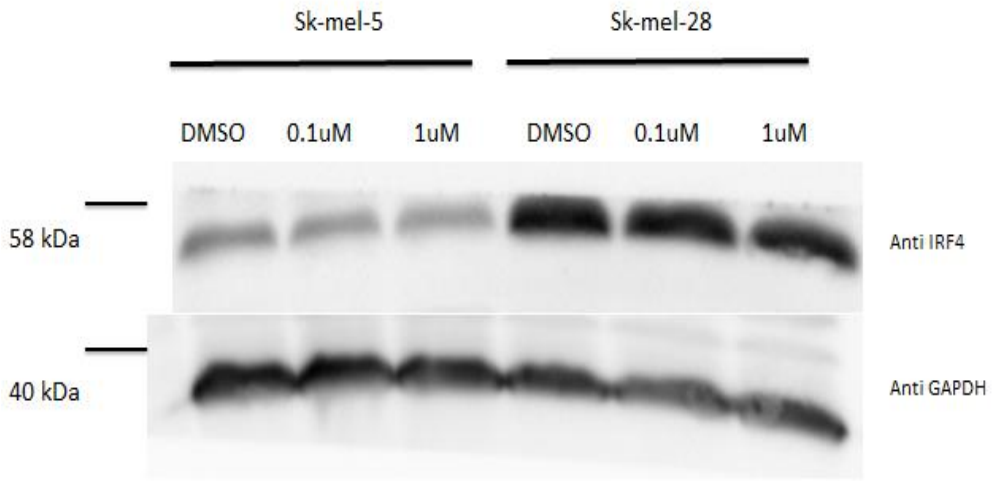

Figure 5.7. Western blotting result of MLN 4924 treated melanoma cell lines. Sk-mel-5 and Sk-mel-28 melanoma cell lines treated with either DMSO or two different concentrations of MLN 4924 for 24 hours. Total proteins were extracted and western blotting was done with anti IRF4 and anti GAPDH antibodies.

5.3. Testing the Regulatory Role of Vitamin D3 on IRF4 Expression

IRF4 expression is known to be significantly down-regulated by the metabolically active form of Vitamin D3 in dendritic cells (Gauzzi *et al*., 2005). In addition to the known regulatory role of Vitamin D3 on IRF4 expression, ChIP on chip data revealed binding of Vitamin D3 to the IRF4 gene locus (Meyer *et al*., 2010). Therefore, we tested the possible regulatory effect of Vitamin D3 on IRF4 expression in melanoma cell lines.

We first analyzed the expression of Vitamin D receptor in melanoma cell lines. Total cell lysates of G361, A2058, Sk-mel-28 and Mewo were blotted with Vitamin D receptor antibody. All tested cell lines express the Vitamin D receptor (Figure 5.8).

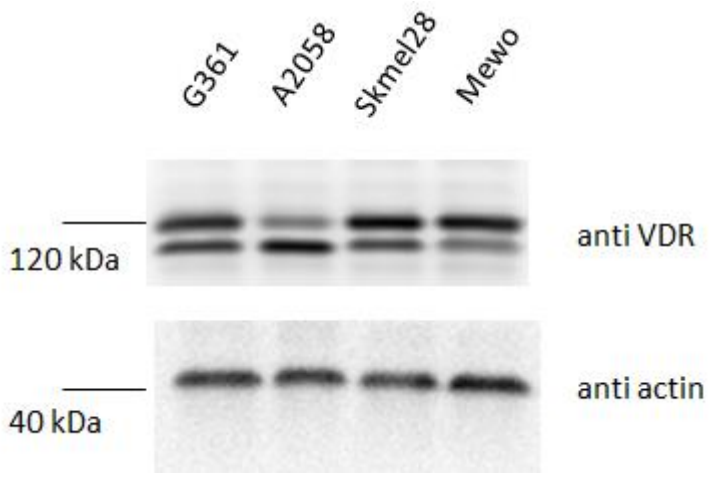

Figure 5.8. VDR expression in melanoma cell lines. Total cell lysates of G361, A2058, Skmel28 and Mewo were blotted with Vitamin D receptor antibody and actin antibody as a loading control.

We first tested the capability of the metabolically active form of vitamin D3 to act by analyzing the expression levels of one of the vitamin D3 targets in melanoma cell lines (Figure G.1).

Melanoma cell line the Sk-mel-28 was treated with the different concentrations of the metabolically active form of Vitamin D3, calcitriol, for 12 hours and assayed IRF4 mRNA expression by QRT-PCR. 12 hours of calcitriol treatment did not cause any down-regulation in the IRF4 mRNA level as expected instead a modest up-regulation was observed (Figure 5.9).

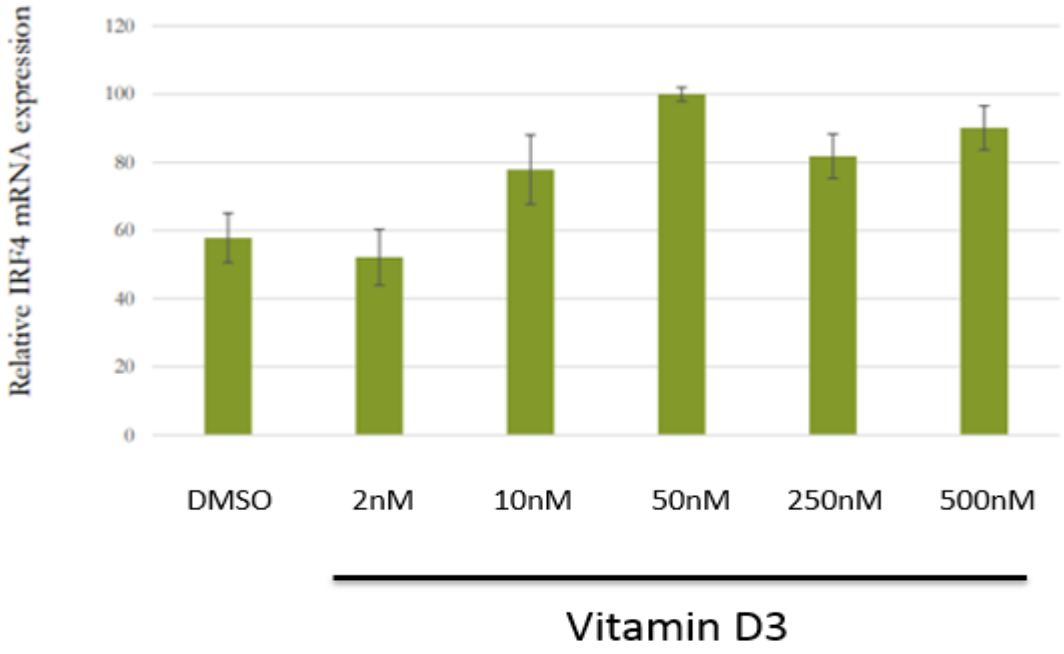

Figure 5.9. Calcitriol treatment of Sk-mel-28 melanoma cell line for mRNA expression level. Sk-mel-28 cells were treated with 2nM, 10nM, 50nM, 250nM and 500nM of calcitriol for 12 hours. Total RNAs were extracted and converted into cDNA. Relative IRF4 mRNA expression was normalized to relative PGK1 mRNA expression. The highest normalized expression was normalized to 100.

Additionally, IRF4 protein expression levels in the Sk-mel-28 melanoma cell line were evaluated upon treatment with different concentrations of the calcitriol for 48 hours. 48 hours calcitriol treatmetn didn't cause any down-regulation of IRF4 protein level (Figure 5.10). Together with the mRNA expression data, these results indicate that Vitamin D3 does not have any regulatory effect on IRF4 expression in the tested melanoma cell lines.

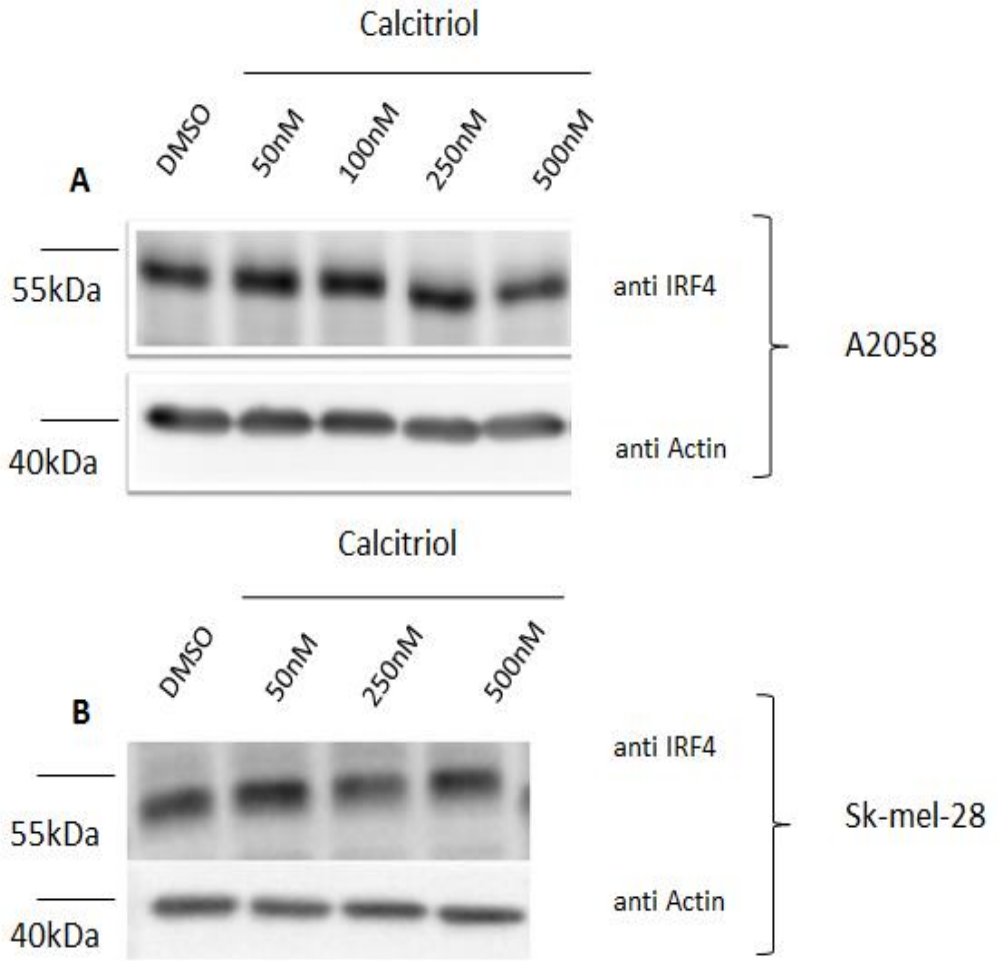

Figure 5.10. IRF4 protein expression of A) A2058 and B) Sk-mel-28 melanoma cell lines upon Calcitriol treatment. A2058 and Sk-mel-28 cells were treated with calcitriol for 48 hours and total protein lysates were blotted with anti IRF4 antibody and anti actin antibody as a loading control.

5.4. Testing the Regulatory Role of NFAT Pathway in IRF4 Expression

5.4.1. Detection of the NFAT Pathway Activity in Melanoma

The Nuclear Factor of Activated T cells (NFAT) pathway is thought to promote certain aspects of tumor biology, including growth, survival, invasion and angiogenesis (Mancini *et al.*, 2009). Melanoma cells express members of the Ca^{2+} /calcineurin-regulated NFAT family of transcription factors some of which are transcriptionally active (FLockart *et al.*, 2009). In addition to the significance of NFAT pathway in melanoma, IRF4 expression is known to be regulated by NFAT pathway in ectopically M2 protein, a component of influenza B, expressing primary murine B cells (Rangaswamy *et al.*, 2014). Therefore, we hypothesized that the NFAT pathway might regulate IRF4 expression in melanoma cells.

We first confirmed whether NFAT family transcription factors are expressed in melanoma cell lines. Total RNAs of A2058, G361 and Sk-mel-28 melanoma cell lines were extracted and were converted into cDNA. We looked at mRNA expression levels of 5 different NFAT family transcription factors by using q-RT PCR. Among the 5 NFAT family transcription factors, only NFATc3 (NFAT4) is expressed in the tested melanoma cell lines (Figure 5.11). This data was technically repeated (Data not shown).

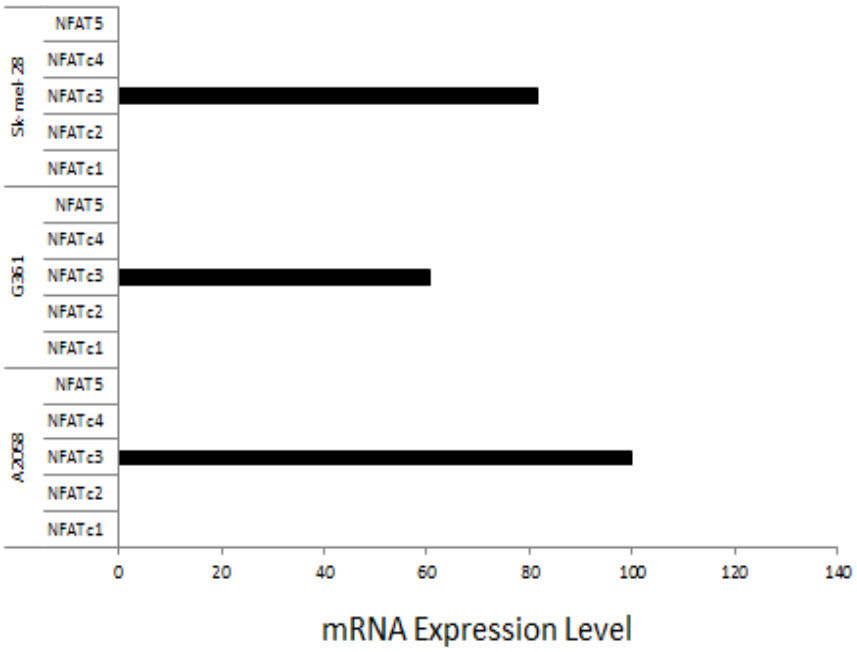

Figure 5.11. Expression levels of 5 different NFAT family transcription factors in A2058, G361 and Sk-mel-28 melanoma cell lines. Total RNAs were extracted from the melanoma cell lines and were converted into cDNA. QRT-PCR was performed using sybr green dye and mRNA expression levels of NFAT transcription factors were normalized to GAPDH expression.

In order to measure transcriptional activity of the NFAT pathway, we used a dual luciferase reporter system. We started with two different NFAT reporter plasmids with different backbones containing firefly luciferase gene whose expression is driven by a promoter with three tandem repeats of NFAT binding motifs. As a proof of principle, we compared strength of these two plasmids in detecting NFAT pathway transcriptional activity. One of the reporter constructs has pGL2 backbone (Ichida *et al*., 2001) and the other one has pGL3 backbone (Clipstone *et al*., 1992). In this study, relative firefly luciferase activities of these two constructs were compared upon their transient co-transfection with constitutively active renilla luciferin expressing construct to the melanoma cell line A2058. The NFAT reporter construct with the pGL3 backbone showed a stronger signal when compared to the other NFAT reporter construct with

pGL2 backbone (Figure 5.12). Therefore we decided to continue to perform luciferase transactivation assays using the plasmid with the pGL3 backbone.

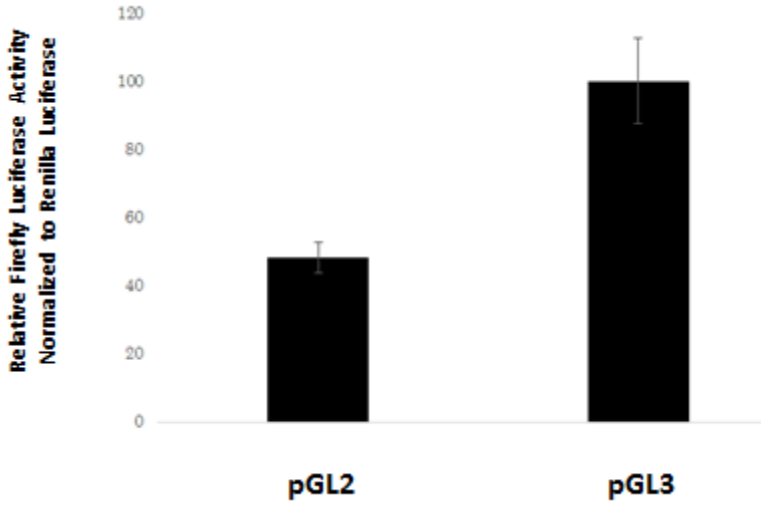

Figure 5.12. Comparison of the NFAT reporter plasmids. NFAT reporter plasmids with pGL2 and pGL3 backbones were transiently co-transfected to the A2058 melanoma cell line. Relative firefly luciferase activities were measured 48 hours after the transfection and were normalized to the renilla luciferase activities. Error bars indicate five independent measurement replicates.

5.4.2. Validation of NFAT Pathway Inhibition

The inhibitory potential of one of the NFAT pathway inhibitors, Fk506 (Ochiai *et al.*, 1987), was evaluated using dual luciferase transactivation assay with a NFAT reporter plasmid in order to test whether Fk506 is able to inhibit NFAT pathway transcriptional activity. For this purpose, the A2058 melanoma cell line was transiently co-transfected with NFAT and renilla reporter plasmid under constitutively active promoter (Clipstone *et al.*, 1992). At the last step of K2 transfection, A2058 cells were either treated with 25µM of Fk506 or DMSO. This drug treatment assay was coupled with the luciferase transactivation assay in order to detect the inhibitory effect of Fk506 in NFAT pathway activity (Figure 5.13).

48 hours after the treatment, cells were collected and their relative firefly luciferase activities were measured and normalized to their relative renilla luciferase activity (Figure 5.12). Thus, the potential of the Fk506 to down-regulate the NFAT pathway was verified.

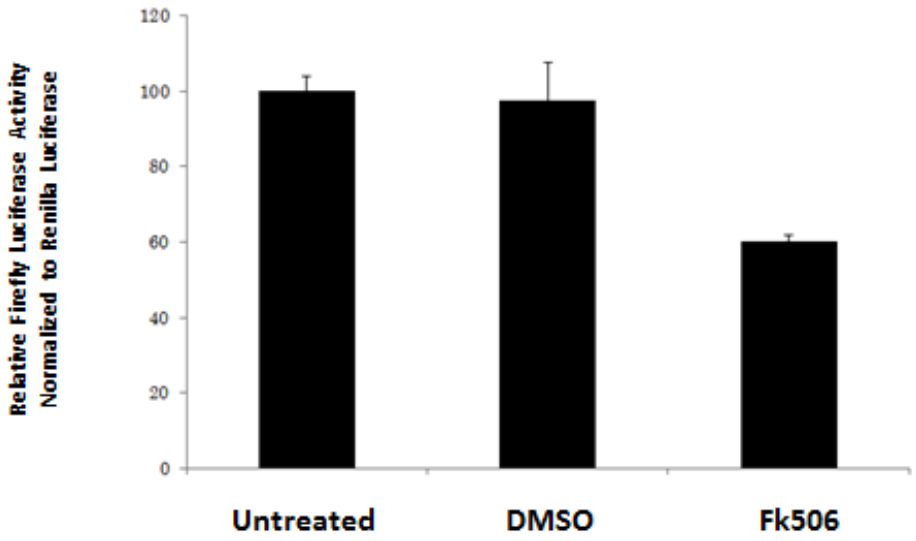

Figure 5.13. Verification of the inhibitory effect of Fk506 on NFAT pathway transcriptional activity. A2058 melanoma cell line transiently co-transfected with NFAT reporter and renilla plasmids. At the last step of the transfection, cells were either treated with the drug Fk506 or DMSO or left untreated. 48 hours after the treatment, relative firefly luciferase activities were measured and normalized to renilla luciferase activities. Error bars indicate five independent measurements.

5.4.3. Regulatory Role of NFAT Pathway on IRF4 Expression

In order to explore out whether the NFAT pathway positively regulates IRF4 expression in melanoma cells, IRF4 mRNA and protein expression was analyzed after NFAT pathway inhibition Fk506.

Melanoma cell lines A2058 and Sk-mel-28 were treated with Fk506 and CsA for 3 and 9 hours. Total RNAs were extracted at the end of each time point and converted into cDNA for the detection of the relative IRF4 mRNA expression by QRT-PCR. The analysis showed that IRF4 mRNA expression was diminished upon treatment with two different NFAT inhibitors, CsA and Fk506, in the A2058 and Sk-mel-28 melanoma cell lines in early (Figure 5.13) and late time points suggesting that NFAT pathway might positively regulate IRF4 mRNA expression in tested melanoma cell lines.

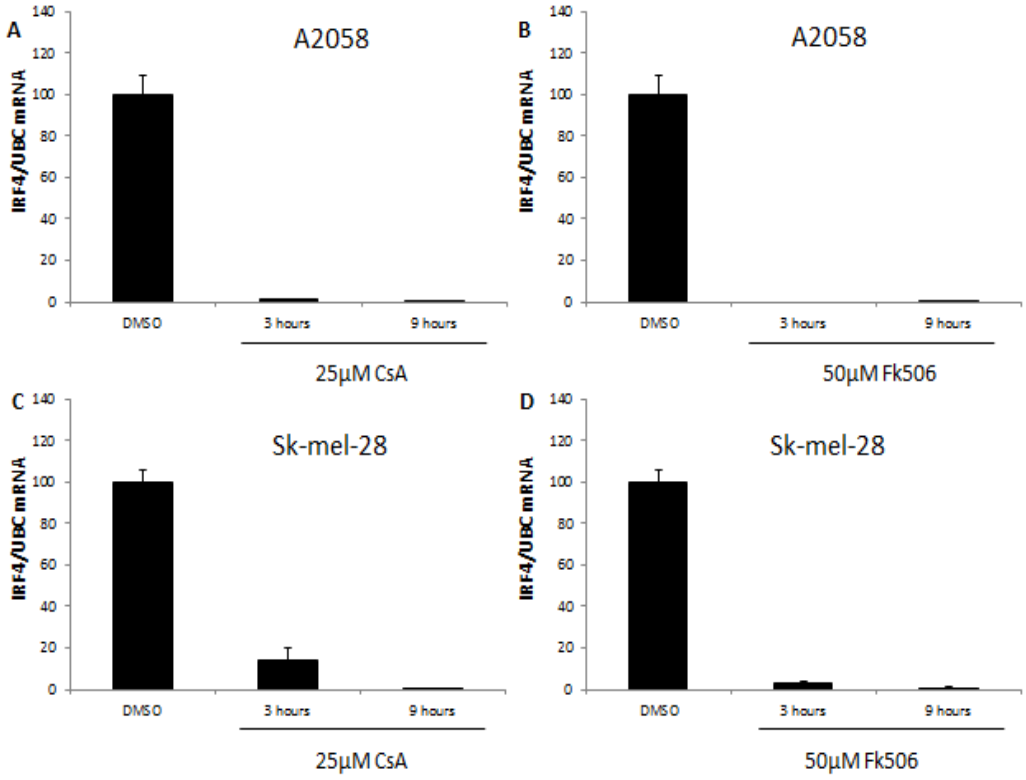

Figure 5.14. Down-regulation of IRF4 mRNA expression upon treatment with two different NFAT pathway inhibitors, CsA and Fk506. A2058 and Sk-mel-28 cells were treated with NFAT inhibitors for 3 and 9 hours. Relative IRF4 mRNA expression was normalized to relative UBC mRNA expression for each sample. Relative IRF4 mRNA expression in drug treated samples at each time point was normalized to DMSO.

The inhibitory effect of NFAT inhibition on IRF4 mRNA expression was further analyzed at the protein level after treatment of the melanoma cell lines, A2058, G361,

Sk-mel-5 and Sk-mel-28 with NFAT pathway inhibitors was determined. Protein lysates were taken at both early and late time points and the protein expression level of IRF4 was visualized on western blotting experiments (Figure 5.14). IRF4 protein expression levels decreased upon treatment of melanoma cell lines A2058, G361, Sk-mel-5 and Sk-mel-28 with two structurally different NFAT inhibitors, CsA and Fk506. These results indicate that the NFAT pathway positively regulates IRF4 expression in the tested melanoma cell lines. Also similar results were shown in Appendix K.

5.4.4. Regulatory Role of NFAT Pathway in MITF Expression

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development and pigment production (Cheli *et al*., 2010). In melanoma, MITF is considered as an oncogene and plays roles in proliferation, cell survival, cell cycle, metastasis and invasion (Levy *et al*., 2006). Synergy between NFAT and MITF has important roles during bone formation but it is unknown whether the NFAT pathway has a regulatory role on MITF expression or not.

According to our research team's unpublished data, MITF binds to the IRF4 gene locus. Thus, we asked whether inhibition of the NFAT pathway has any effect on MITF expression in melanoma cell lines or not. The A2058 melanoma cell line was treated with two different NFAT pathway inhibitors for 24 hours. Total RNA was extracted and converted to cDNA and relative MITF mRNA expression levels by QRT-PCR. 24 hours of NFAT inhibition at different concentrations of two different NFAT inhibitors led to down-regulation of MITF mRNA expression in A2058 and Sk-mel-28 melanoma cell lines (Figure 5.15).

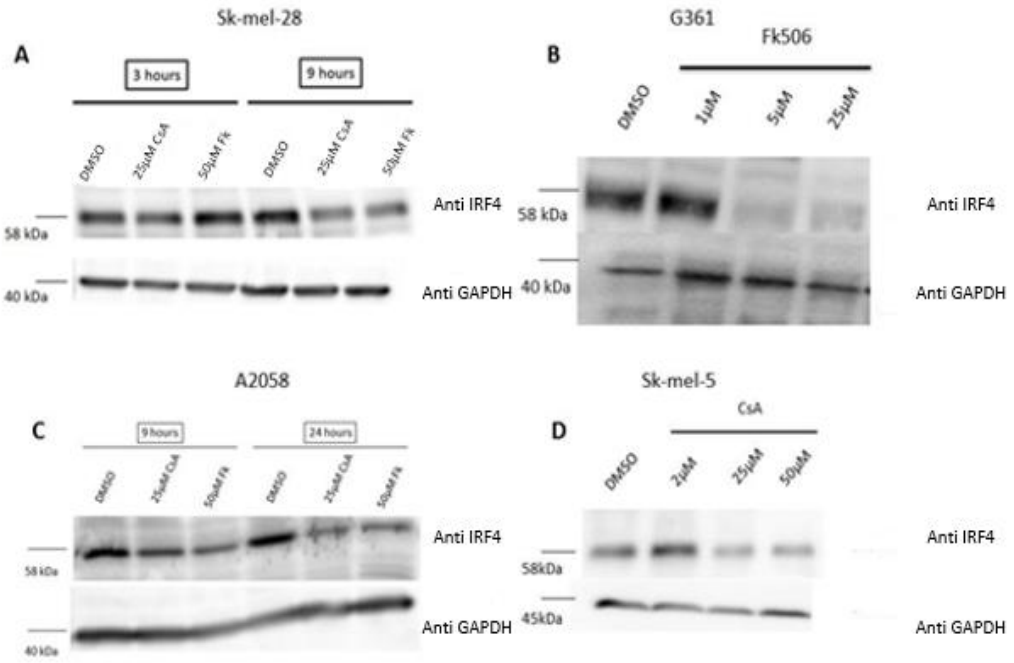

Figure 5.15. Down-regulation of IRF4 protein express in melanoma cell lines upon treatment with two structurally different NFAT pathway inhibitors; CsA and Fk506. A) Sk-mel-28 and C) A2058 melanoma cell lines were treated with NFAT inhibitors; CsA and Fk506, for 3, 9 and 24 hours. B) G361 cells were treated with different concentrations of Fk506 for 24 hours. D) Sk-mel-5 cells were treated with CsA.

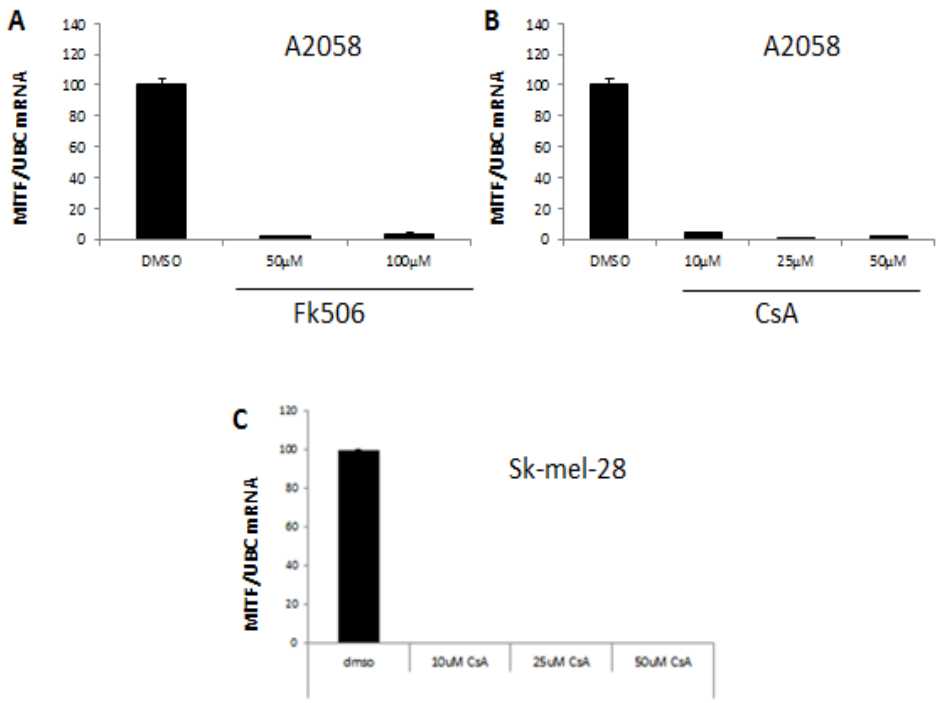

Figure 5.16. Down-regulation of MITF mRNA expression level upon treatment with two different NFAT pathway inhibitors, CsA and Fk506. A2058 cells were treated with different concentrations of A) Fk506 and B) CsA for 24 hours. C) Sk-mel-28 cells were treated with different concentrations of CsA for 24 hours.

Inhibition of the NFAT pathway led to a decrease in MITF mRNA expression in the tested melanoma cell lines. To evaluate if this decrease is also reflected at the protein level, we looked at the effect of NFAT inhibition on MITF protein expression in melanoma cell lines. The melanoma cell line A2058 was treated with two different NFAT inhibitors for 48 hours and protein lysates were extracted in order to perform western blotting. 48 hours of NFAT pathway inhibition led to full or partial reduction of MITF protein levels in A2058 [other lines] melanoma cell line (Figure 5.16). Loss of MITF protein expression upon NFAT pathway inhibitor treatment indicates that the NFAT pathway directly or indirectly regulates MITF expression in all tested melanoma cell lines.

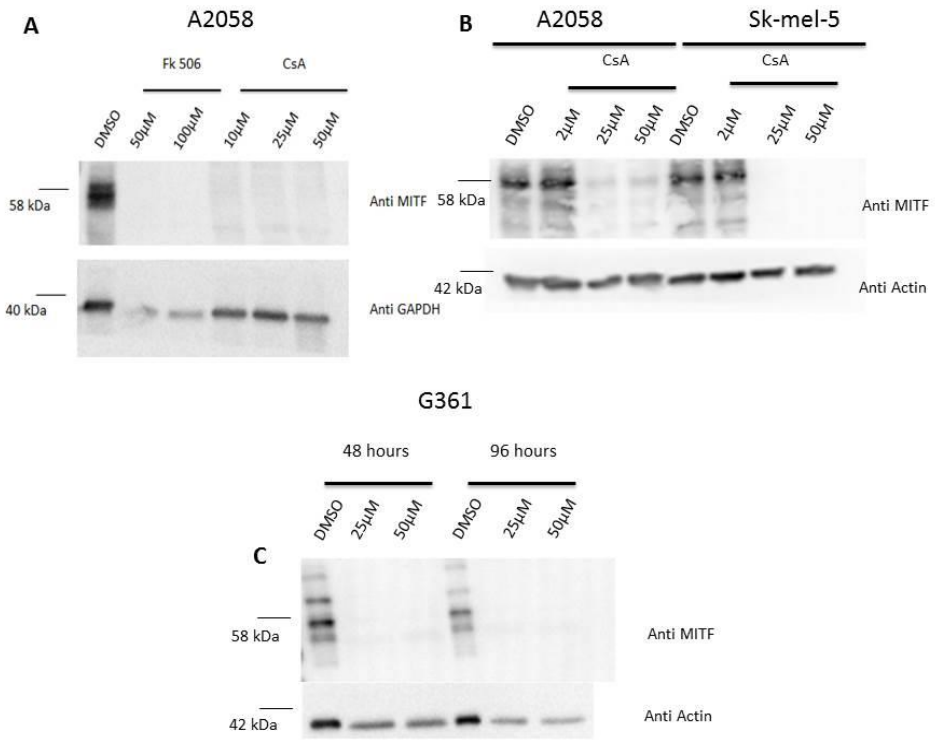

Figure 5.17. Loss or reduction of MITF protein expression level upon treatment with different concentrations of Fk506 and CsA. A) A2058 cells were treated with different concentrations of Fk506 and CsA for 24 hours. B) A2058 and Sk-mel-5 cells were treated with different concentrations of CsA for 48 hours. C) G361 cells were treated with different concentrations of CsA for 48 and 96 hours.

6. DISCUSSION

IRF4 has been studied in immune cells, more importantly in lymphocytes, since IRF4 has important roles in the development and function of lymphocytes. Additionally, high IRF4 expression was previously associated with immune cell derived malignancies like activated B-cell-like diffuse large B-cell lymphoma (ABC DLBCL) and multiple myeloma (MM). Shaffer *et al*. demonstrated that both ABC-DLBCL and MM cells have a non-oncogenic addiction to IRF4 expression as these cells' viability was impaired by IRF4 knockdown (Yang *et al*., 2012). We noticed that IRF4 is also expressed in melanoma cells and melanoma cell viability depends on IRF4 expression (Ayhan, 2014).

As its expression is critical for melanoma cell viability, identification of the regulatory pathways that induce its expression in melanoma is important for the development of better strategies for melanoma therapy. Most of our knowledge about upstream regulators of IRF4 expression comes from immune cells and immune cell derived cancers. Based on a candidate approach, regulatory roles of those candidate pathways on IRF4 expression in melanoma cell lines were tested in this study.

There are several reasons that make the NF-κB pathway a candidate regulator of IRF4 expression in melanoma. The NF-κB pathway is known to be altered in melanoma. Some components of the NF-κB pathway, such as p50 and p65 proteins, are overexpressed in the nucleus of dysplastic nevi compared to normal cells (McNulty *et al*., 2004). Moreover, high IKK enzyme activity in melanoma leads to more rapid degradation of IκB, thus an enhanced trans-activating capacity of the NF-κB pathway (Shattuck *et al*., 1997). The NF-κB pathway elevates IRF4 expression by binding of p65 to its promoter region during lymphocyte activation and differentiation (Sharma *et al*., 2000). Therefore, we hypothesized that the NF-κB pathway might positively regulate IRF4 expression in melanoma cell lines.

We first looked at the expression of p65 in one of the melanoma cell lines, Sk-mel-28 (Figure 5.2). Thereafter, activity of the NF-κB pathway was measured in all tested melanoma cell lines by using a dual luciferase reporter assay. In Figure 5.3, we verified that the NF-κB pathway is transcriptionally active in all tested melanoma cell lines. Therefore, we aimed to block the activity of this pathway and determine whether it induces IRF4 expression or not. For this purpose, we used two different NF-κB pathway inhibitors, Bay 11-7085 and MLN 4924. In Figure 5.5, we demonstrated that we could block transcriptional activity of the NF-κB pathway by using MLN 4924 in all melanoma cell lines tested. Although MLN 4924 was able to block the transcriptional activity of the NF-κB pathway in all tested melanoma cell lines, the other small molecular compound, Bay 11-7085, blocked the pathway in only the Sk-mel-28 melanoma cell line. These different results can be attributed to the presence of cullin ring ligase (CRL)-dependent NF-κB pathway activation in other melanoma cell lines except the Sk-mel-28 melanoma cell line. After the verified blockage of NF-κB pathway in melanoma cell lines, IRF4 mRNA and protein expression was analyzed. Although we detected down-regulation of IRF4 mRNA expression in only A2058 melanoma cell line, we could not detect any down-regulation of IRF4 protein expression in any of the melanoma cell lines. Thus, we concluded that the NF-κB pathway does not have any positive regulatory role on IRF4 expression in the tested melanoma cell lines. Therefore, there are different regulatory mechanism of IRF4 expression in melanoma when compared to lymphoma as NF-κB pathway regulates IRF4 in B-cell cancers (Yang *et al*., 2013). This different effect might be a result of inaccessibility of the NF-κB response elements in the IRF4 promoter in melanoma cell lines.

The Vitamin D pathway is also considered as a possible regulator of IRF4 expression in melanoma cell lines. Gauzzi *et al*., 2005 showed that treatment of dendritic cells with the metabolically active form of vitamin D3, calcitriol, diminished IRF4 protein expression (Gauzzi *et al*., 2005). ChIP on chip data of Meyer *et al*., 2010 demonstrated that vitamin D3 binds to the distal promoter region of IRF4 (Meyer *et al*., 2010). Vitamin D3 deficiency has been observed in melanoma patients (Oliveira *et al*., 2014) and melanoma cells are responsive to vitamin D3 treatment as some of them can be killed by vitamin D3 treatment (Reichrath *et al*., 2014), suggesting that absence of vitamin D3 might result in elevated IRF4 expression levels in melanoma. Therefore, we

treated melanoma cell lines with a metabolically active form of Vitamin D3. However, we could not observe any down-regulation of IRF4 protein and mRNA expression (Figure 5.9, Figure 5.10). Thus, we could not obtain any evidence that vitamin D3 pathway has a regulatory role on IRF4 expression in any of the tested melanoma cell lines.

NFAT pathway has certain roles in tumor biology, including growth, survival, invasion and angiogenesis (Mancini *et al*., 2009). Indeed, melanoma cells express certain members of the NFAT family of transcription factors, some of which are transcriptionally active (FLockart *et al.,* 2009). Besides the essential roles of the NFAT pathway in melanoma, IRF4 expression is known to be regulated by the NFAT pathway in ectopically M2 protein expressing primary murine B cells (Rangaswamy *et al*., 2014). Therefore, we asked whether NFAT pathway positively regulates IRF4 expression in melanoma or not. For this purpose, we first analyzed the mRNA expression levels of different NFAT family transcription factors, NFATc1/NFAT2, NFATc2/NFAT1, NFATc3/NFAT4, NFATc4/NFAT3 and NFAT5 in different melanoma cell lines. As shown in Figure 5.11., we were able to detect only NFATc3/NFAT4 expression in all tested melanoma cell lines (Figure 5.11). Thereafter, NFAT transcriptional activity was measured using dual luciferase assay (Figure 5.12) the inhibitory effect of one of the NFAT inhibitors was also verified by using the same dual luciferase reporter system. After treatment of melanoma cell lines with two structurally different NFAT inhibitors with distinct molecular targets in the pathway (Mancini *et al*., 2009), we observed a striking down-regulation in IRF4 mRNA and protein expression levels. Thus, we concluded that the NFAT pathway directly or indirectly regulates IRF4 expression in melanoma cells.

During the verification experiments of the NFAT inhibitor Fk506, we barely detected blockage of the NFAT pathway. This could be due to low concentration of Fk506. Therefore, we need to either replicate this assay or look at the protein expressions of some of the downstream effectors of the NFAT pathway in melanoma, such as p53 and Apollon, after treatment with NFAT inhibitors. In order to figure out whether the down-regulation of IRF4 expression in the tested melanoma cell lines upon treatment with NFAT inhibitors is a direct effect of NFAT or not, a chromatin immunoprecipitation

(ChIP) assay should be performed with a NFATc3/NFAT4 antibody as there are two binding NFAT binding motifs in the IRF4 promoter (Figure 6.1).

Figure 6.1. NFAT binding motifs in IRF4 promoter. Colored sequences indicate presence of three different NFAT binding motifs in IRF4 promoter.

The NFAT pathway also has roles in bone formation in which it synergies with MITF (Torein *et al*., 2006), which is a master regulator of melanocyte development and is considered as an oncogene for melanoma. Given its critical role in melanoma, we also looked at its protein and mRNA expression level upon NFAT pathway inhibition. We observed total down-regulation of both MITF protein and mRNA level in NFAT inhibitor treated melanoma cell lines. Thus, the NFAT pathway appears to directly or indirectly regulate MITF expression in melanoma. In order to figure out whether this regulation is direct or indirect, a luciferase assay could be performed because there are not apparent binding motifs of NFAT in the MITF locus. In this luciferase reporter assay, the MITF promoter can be cloned into a luciferase plasmid. This reporter system could then be transfected to HEK cells with and without NFAT family transcription factor cDNAs. The luciferase signals in this context will give an idea whether NFAT directly or indirectly regulates MITF expression in melanoma.

Taken together, we treated melanoma cell lines with different pathway inhibitors and analyzed IRF4 expression under these conditions. Based on a candidate approach, we found that the NF-κB pathway doesn't have any regulatory role on IRF4 expression in melanoma, rather the NFAT pathway appears to regulate IRF4 expression in melanoma. In order to determine the molecular mechanisms of NFAT-dependent IRF4 expres-

sion in melanoma, ChIP experiment should be done in the future. In addition to proposing a NFAT-dependent IRF4 expression mechanism in melanoma, we also claimed that the NFAT pathway might have a regulatory role on MITF expression in melanoma. Therefore, our results implicate three possible regulatory mechanisms of IRF4 expression in melanoma cells. First, NFAT might positively regulate MITF expression. MITF then might positively regulate IRF4 expression in melanoma cell lines. Second, NFAT might positively regulate both MITF and IRF4 expression in melanoma cell lines. Third, NFAT might positively regulate MITF expression. MITF then might positively regulate IRF4 expression but NFAT might also contribute to IRF4 expression in melanoma cell lines

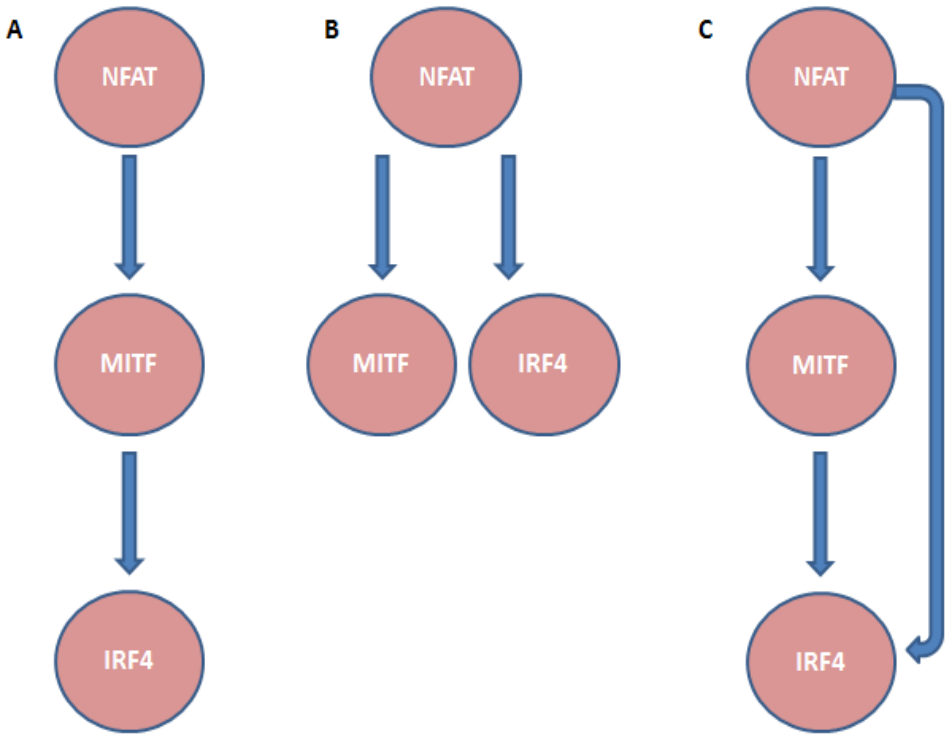

Figure 6.2. Possible regulatory mechanisms of IRF4 expression in melanoma. A) NFAT driven MITF expression triggers IRF4 expression, B) NFAT regulates both IRF4 and MITF expression and C) NFAT driven MITF expression triggers IRF4 expression but NFAT also contributes to IRF4 expression.

APPENDIX A: MICROSCOPE IMAGES OF BAY 11-7085 TREATED SK-MEL-28 MELANOMA CELL LINES

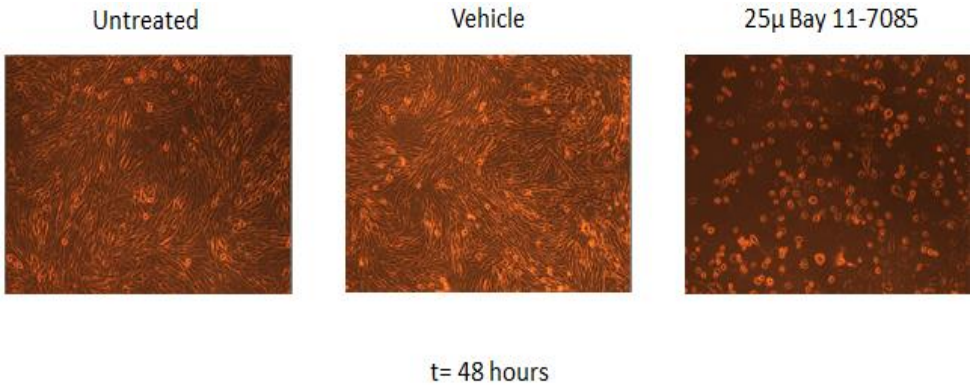

Figure A.1. Brightfield images of Sk-mel-28 melanoma cell line after 48 hours of 25µM Bay 11-7085 treatment. 10X light microscopy images were taken after 48 hours of incubation with or without DMSO and Bay 11-7085.

APPENDIX B: DETECTION OF THE EFFECT OF BAY 11-7085 TREATMENT ON SK-MEL-28 MELANOMA CELL LINE VIABILITY BY XTT

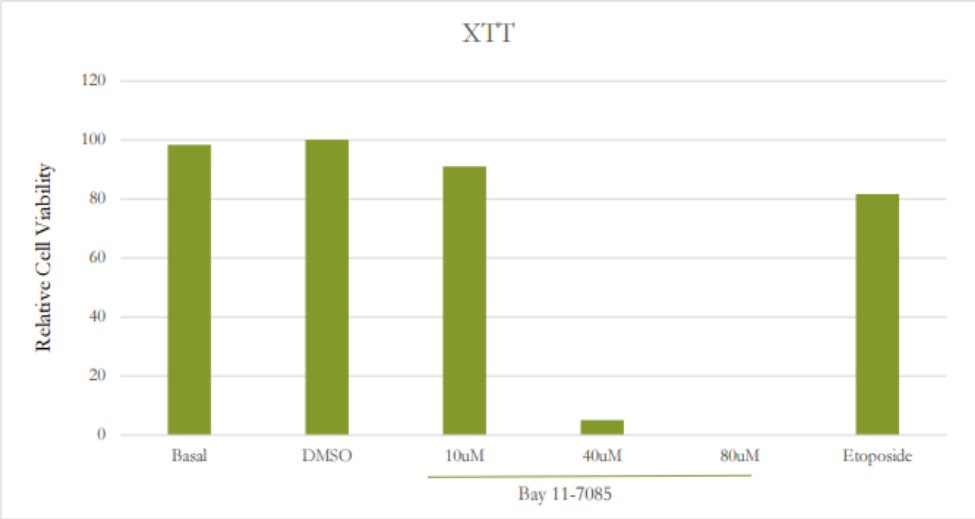

Figure B.1. XTT result of Bay 11-7085 treated Sk-mel-28 melanoma cell line. Sk-mel-28 cells were treated with different concentrations of Bay 11-7085 for 24 hours. Relative cell viability was measure by using XTT cell viability kit.

APPENDIX C: REPLICATION OF MLN 4924 VALIDATION IN A2058 MELANOMA CELL LINE

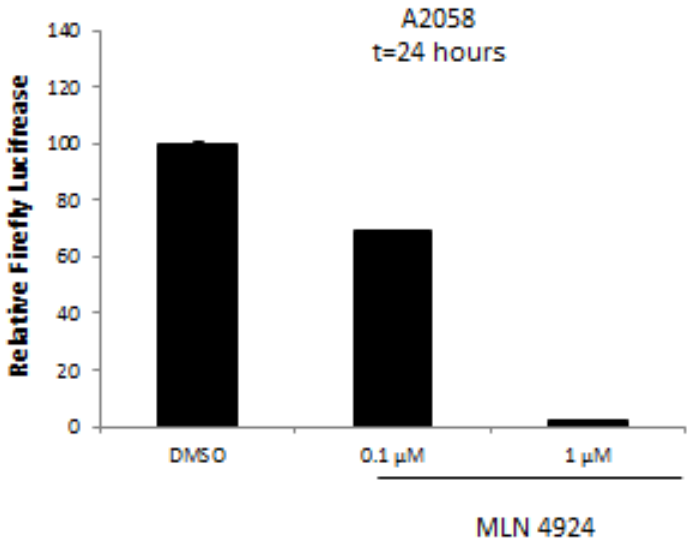

Figure C.1. Validation of inhibitory effect of MLN 4924 on the NF-κB pathway. A2058 cells were transfected with pBVI reporter plasmid using the K2 transfection method. At the last step of the transfection, cells were treated with different concentrations of MLN 4924 and DMSO for 24 hours. At the end of the treatment, relative firefly luciferase activities were measured and normalized to relative renilla luciferase.

APPENDIX D: DETECTION OF THE EFFECT OF BAY 11-7085 TREATMENT ON IRF4 PROTEIN EXPRESSION IN SK-MEL-28 MELANOMA CELL LINE BY WESTERN BLOTTING

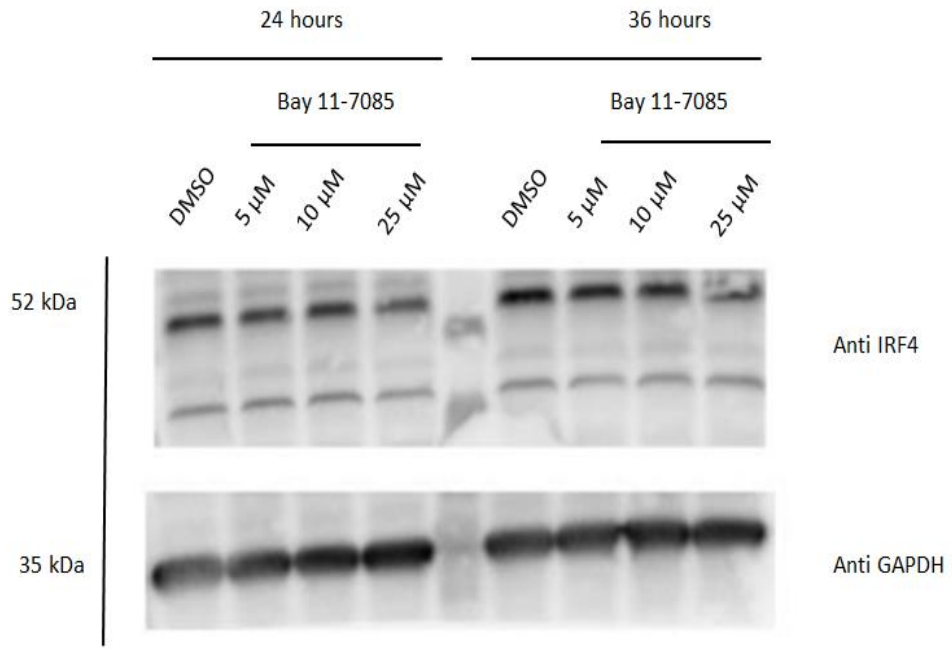

Figure D.1. Effect of Bay 11-7085 treatment on IRF4 expression in the Sk-mel-28 melanoma cell line. Sk-mel-28 cells were treated with different concentrations of Bay 11-7085 for 24 and 36 hours. Total lysates were obtained and western blotting was done with anti-IRF4 and anti-GAPDH antibodies.

APPENDIX E: DETECTION OF THE EFFECT OF BAY 11-7085 TREATMENT ON IRF4 PROTEIN EXPRESSION IN SK-MEL-28 MELANOMA CELL LINE BY INTRACELLULAR FLOW CYTOMETRY

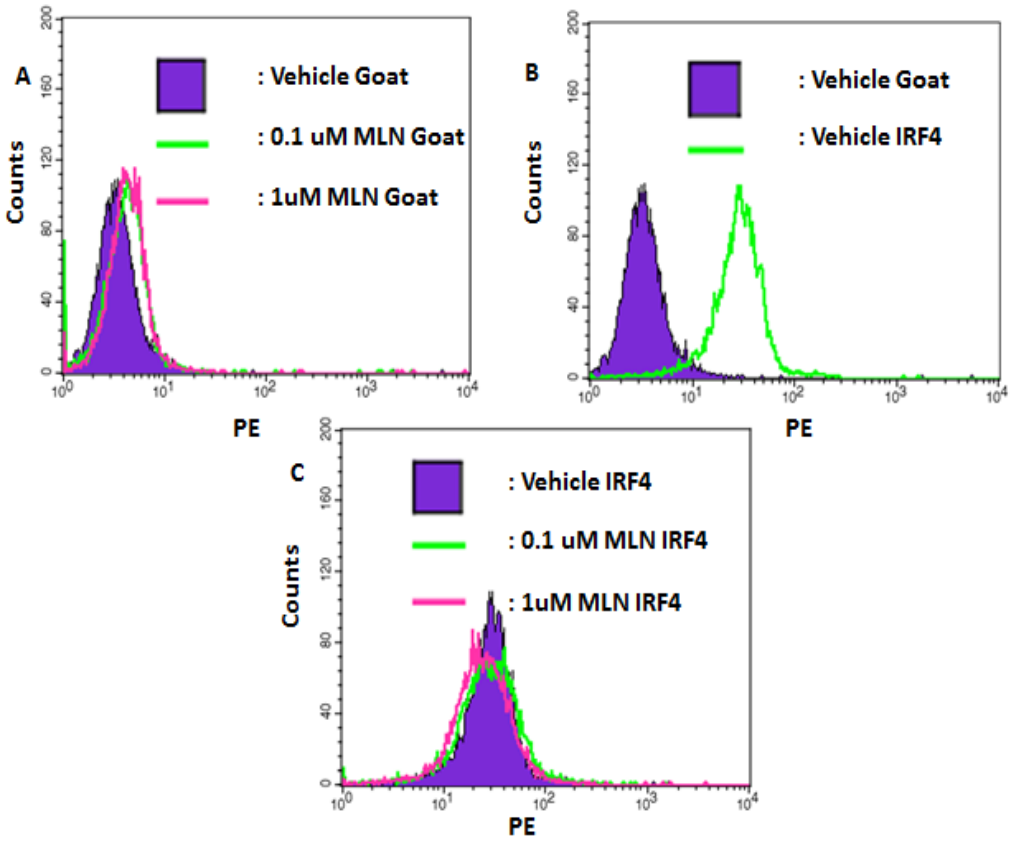

Figure E.1. Effect of MLN 4924 treatment on IRF4 expression in Sk-mel-28 melanoma cell line. Sk-mel-28 cells were treated with different concentrations of MLN 4924 for 24 hours. At the end of the treatment, IRF4 expression was detected using an intracellular flow cytometry method.

APPENDIX F: DETECTION OF THE EFFECT OF BAY 11-7085 TREATMENT ON IRF4 PROTEIN EXPRESSION IN A2058 MELANOMA CELL LINE BY INTRACELLULAR FLOW CYTOMETRY

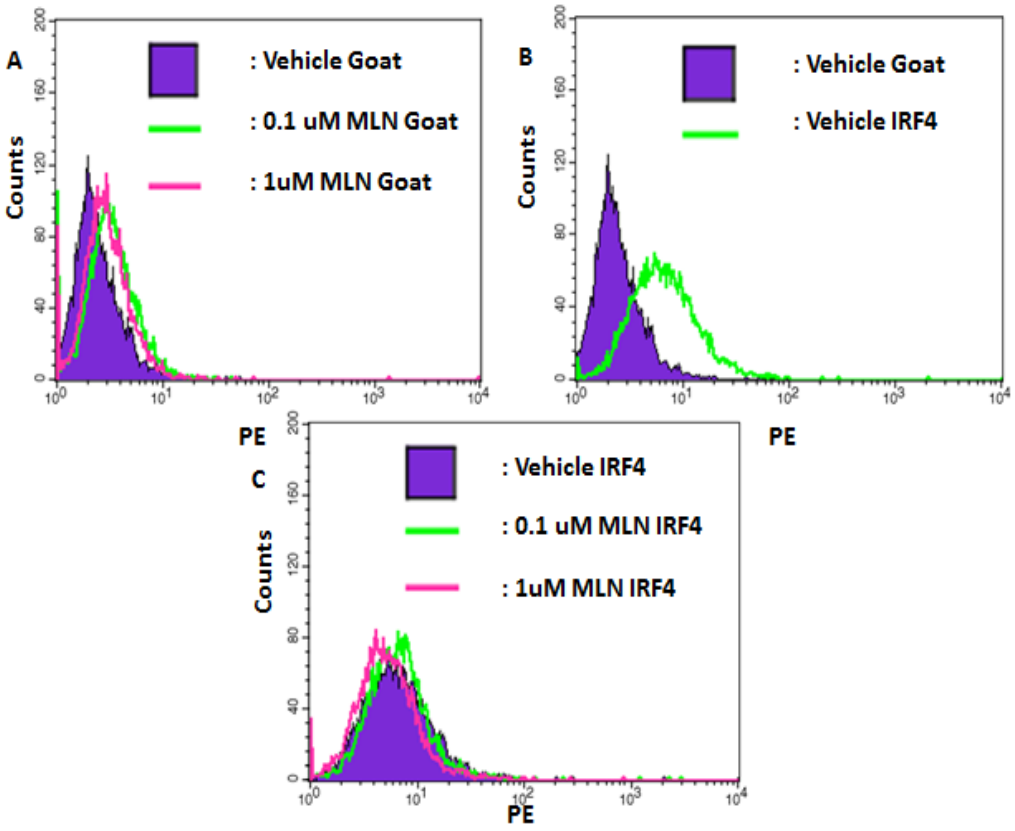

Figure F.1. Effect of MLN 4924 treatment on IRF4 expression in A2058 melanoma cell line. Sk-mel-28 cells were treated with different concentrations of MLN 4924 for 24 hours. At the end of the treatment, IRF4 expression was detected using an intracellular flow cytometry method.

APPENDIX G: VALIDATION OF CALCITRIOL EFFECT ON VITAMIN D3 PATHWAY IN MELANOMA CELL LINES

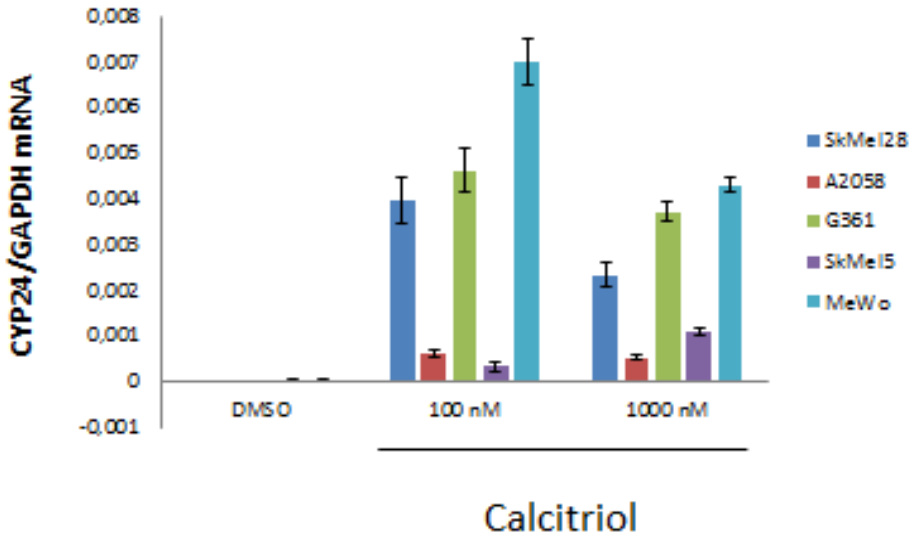

Figure G.1. Validation of calcitriol's capability to induce the Vitamin D3 pathway. Different melanoma cell lines were treated with different concentrations of calcitriol. Total RNAs were extracted and were converted into cDNA. Relative CYP24 levels, one of the downstream effectors of Vitamin D pathway, expression was normalized to GAPDH expression (Ogmen *et al*., unpublished data).

APPENDIX H: IRF4 MRNA EXPRESSION LEVELS OF MELANOMA CELL LINES UPON CYCLOSPORINE TREATMENT

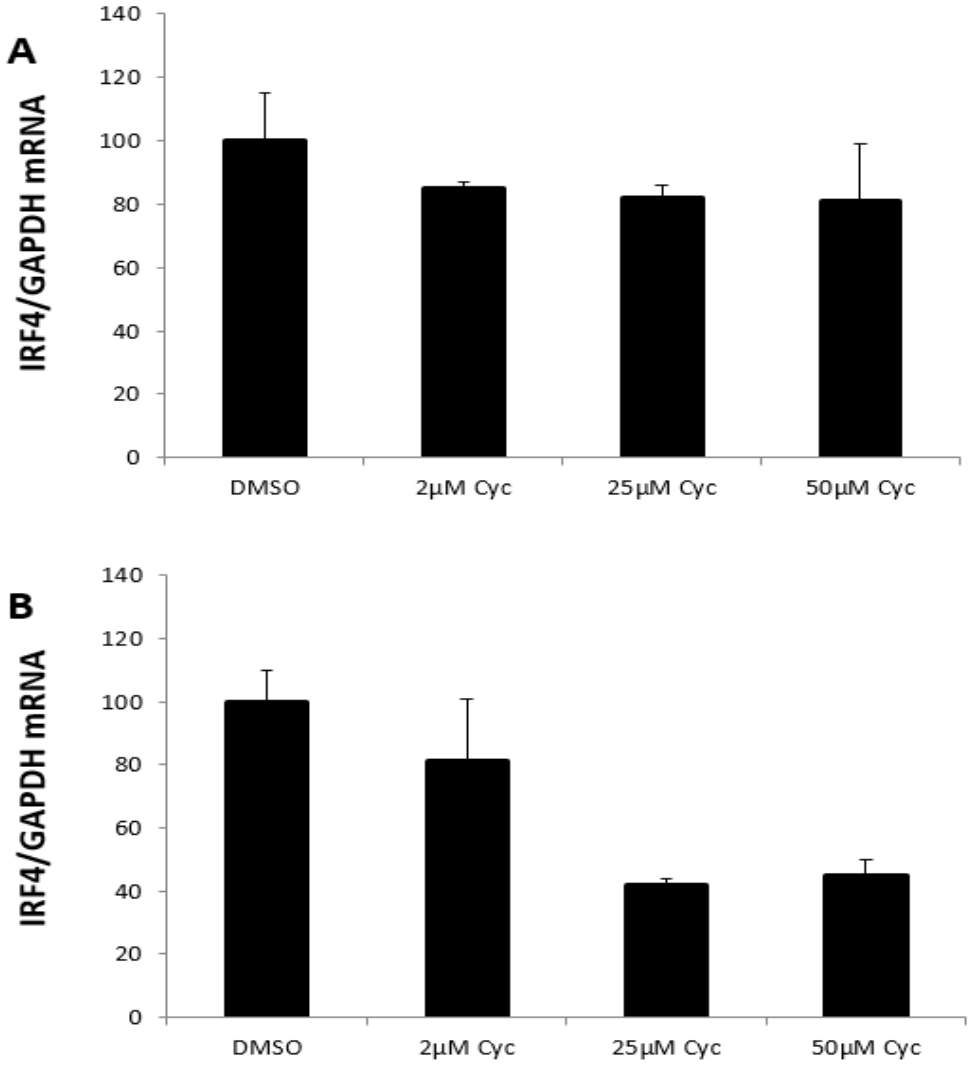

Figure H.1. IRF4 mRNA expression level upon cyclosporine treatment. A) A2058 and B) Sk-mel-5 melanoma cell lines were treated with cyclosporine for 24 hours. Total RNAs were extracted and were converted into cDNA. Relative IRF4 mRNA levels were normalized to GAPDH and vehicle treated sample.

APPENDIX I: MAP OF NFAT REPORTER PLASMID PGL2

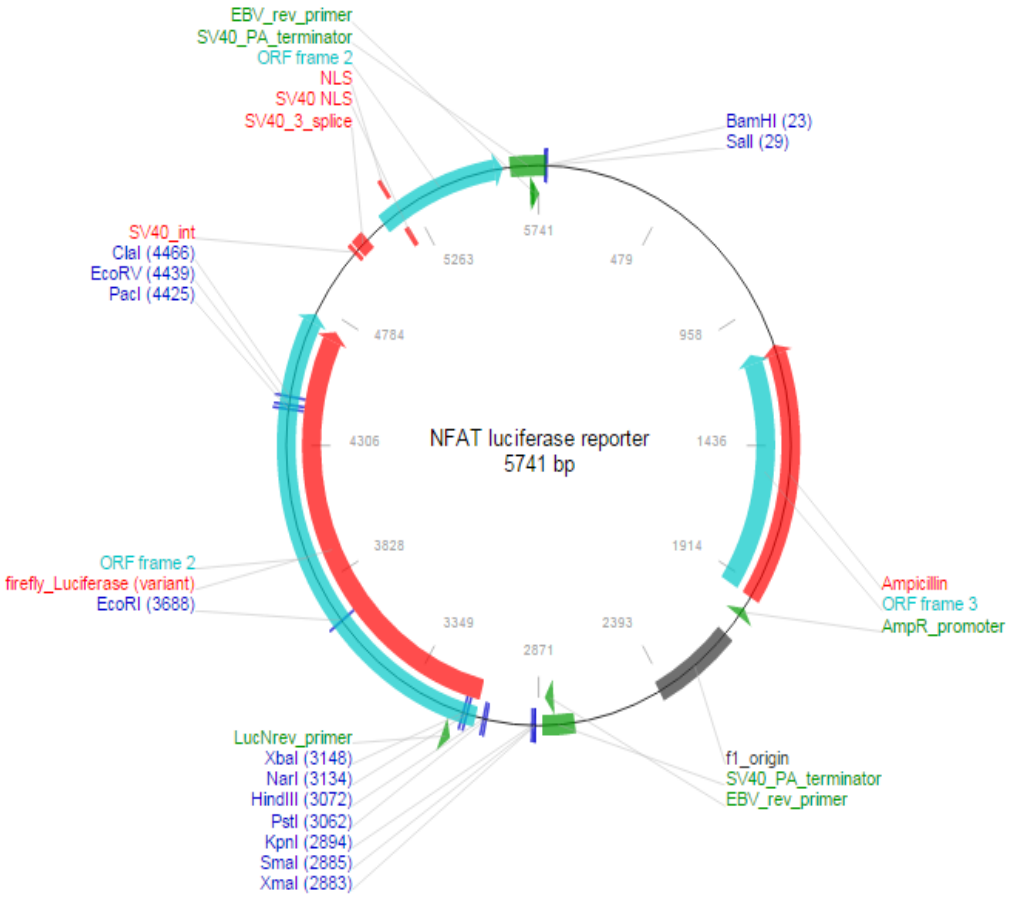

Figure I.1. Map of PGL2 NFAT reporter plasmid.

APPENDIX J: MAP OF NFAT REPORTER PLASMID PGL3

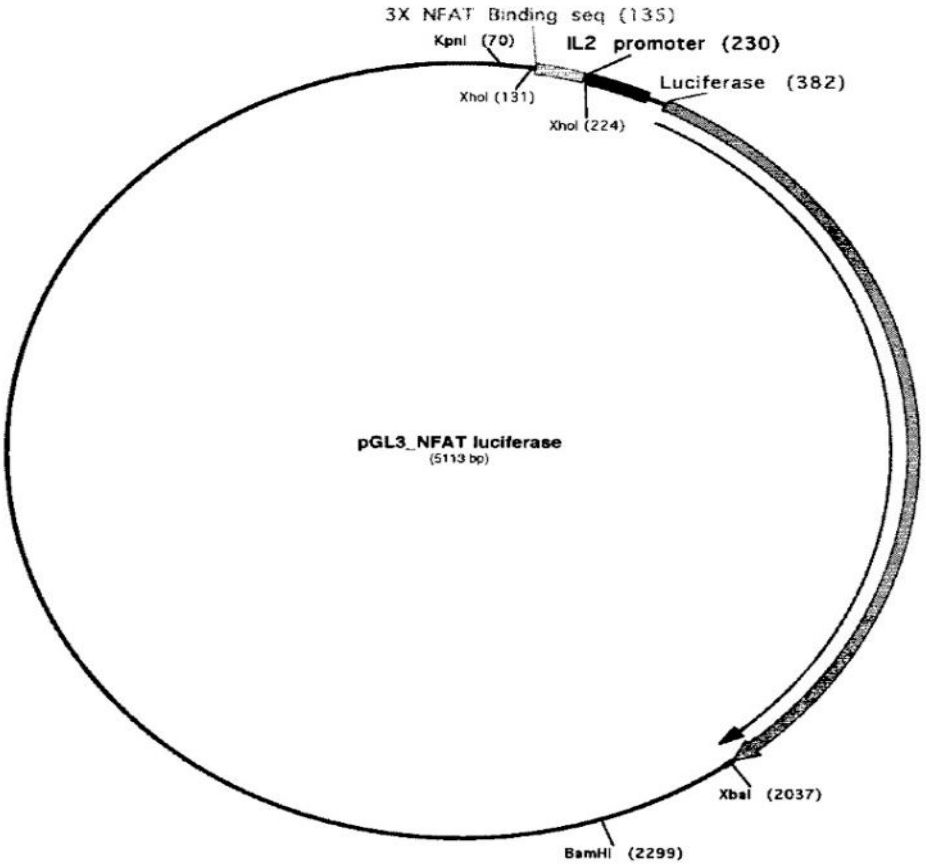

Figure J.1. Map of PGL3 NFAT reporter plasmid.

APPENDIX K: CYCLOSPORINE TREATMENT DEPENDENT DOWN REGULATION OF IRF4 PROTEIN EXPRESSION IN MELANOMA CELLS

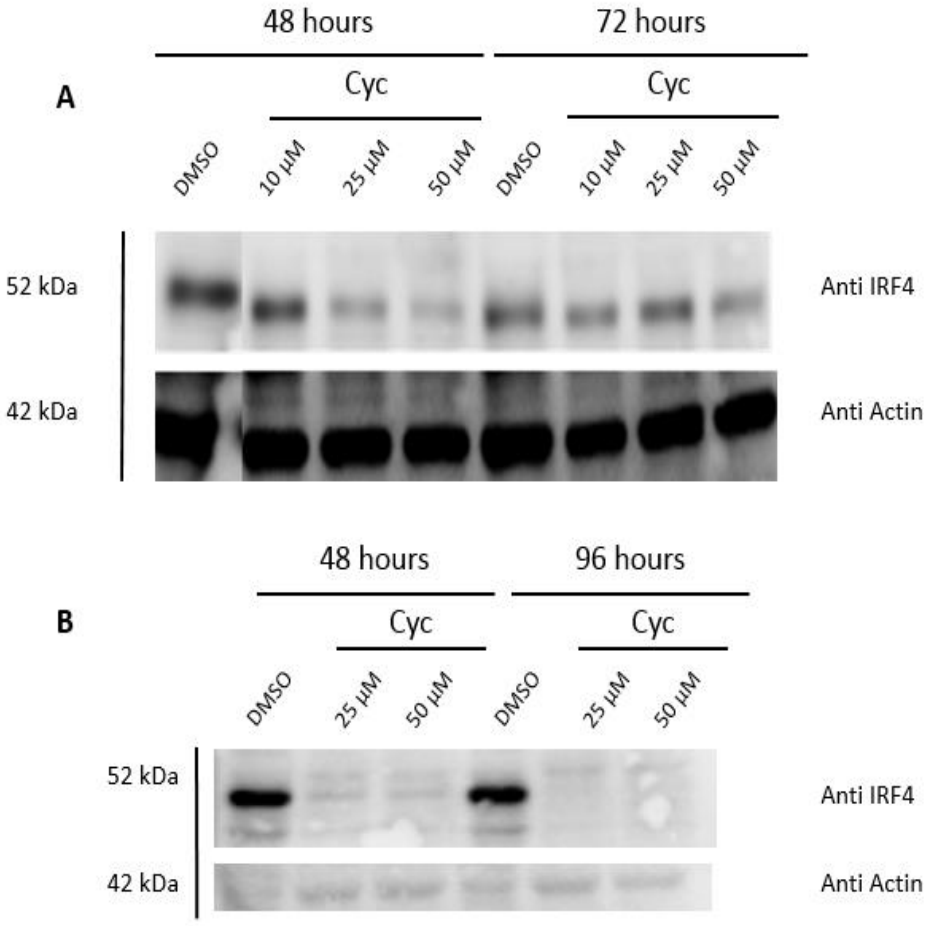

Figure K.1. Cyclosporine treatment dependent down-regulation of IRF4 protein expression in A) Sk-me-28 and B) G361 melanoma cell lines.

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