CHARACTERIZATION OF IRF4 DEPENDENCY IN MELANOMA CELL LINES

by

Mustafa Can Ayhan

B.S., Molecular Biology and Genetics, Istanbul University, 2010

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

2013

ACKNOWLEDGEMENTS

First, I would like to express the greatest gratitude to my thesis supervisor Dr. N.C. Tolga Emre. His guidance was flawless, which always bring me appropriate scientific thinking and led me to research efficiently. He shaped every aspect of this project by unceasingly helping me with the though experiment designs and complex theoretical understandings. By all means, I'm proud to be a member of his laboratory, and the first M.Sc. student under his supervision.

Another special person I want to thank is Assos. Prof. Nesrin Özören. I had valuable work experience in her laboratory, before starting to work on my thesis with my supervisor. Her support during my work at her laboratory was truly inspirational for my scientific skills.

I would also like to thank to my third jury member Assoc. Prof. Ayten Kandilci for spending her valuable time to evaluate this thesis.

My laboratory colleges Yasemin Begüm Alankuş, Ekin Ece Erkan, Erdem Yılmaz, Ulduz Sobhiafshar and Ahmet Buğra Tufan was always very helpful and friendly. Their contribution to this work cannot be denied.

Last but not least, I would like to thank to my close friends Balkan Canher, Vahap Kapıkıran, Uğur Kaplan, Güner Kaçmaz, Güneş Tunçgenç, Burcu Nur Keçeli, Pelin Aksoy Kapıkıran, Ayşegül Taşcı, Tijen Bergin and Ece Kartal whose warm friendship and endless support always helped me a lot.

During this study, I was supported by Framework Programme 7 (FP7) Marie Curie – Career Integration Grant (CIG) and EMBO – Integration Grant (IG).

ABSTRACT

CHARACTERIZATION OF IRF4 IN MELANOMA CELL LINES

Interferon Regulatory Factor 4 (IRF4, also known as MUM1, LSIRF, NFEM5, and ICSAT) is a transcription factor with important regulatory roles on B-cell maturation and differentiation. Although the first identification of IRF4 is as lymphocyte specific IRF family member, its expression was later shown in melanocytes, with unknown functional roles, until its recently identified functions in pigmentation. Alongside its key functional roles in lymphocytes, IRF4 expression is shown to be associated with many lymphoid cancers. For example, multiple myeloma (MM) and activated B-cell like diffuse large B-cell lymphoma (ABC-DLBCL) cancer cells have high IRF4 expression levels and have non-oncogene addiction to IRF4, so that these cells die if IRF4 expression is knocked-down. Surprisingly, immunohistochemistry studies show remarkable IRF4 expression in melanoma samples compared to non-lymphoid cancer types. In addition, several genome wide association studies (GWAS) have also linked IRF4 polymorphisms to melanoma. In the light of these studies, we set out to identify the function of IRF4 in melanoma cell lines. First, by using publicly available gene expression databases we confirmed that IRF4 is highly expressed in melanomas, compared to the non-lymphoid cancers. We also validated that many melanoma cell lines we tested have remarkable endogenous IRF4 expression, while non-lymphoid cancer cell lines have no detectable expression. We then knocked-down IRF4 levels in melanoma cells by introducing short hairpin RNA (shRNA) using a lentiviral infection system and observed reduced competitive fitness upon IRF4 knock-down. Our results suggest that melanoma cells have non-oncogene addiction to IRF4 *in vitro*, similar to the observations in MM and ABC-DLBCL cells.

ÖZET

MELANOMA HÜCRE HATLARINDA IRF4 BAĞIMLILIĞININ KARAKTERİZASYONU

İnterferon düzenleyici faktör 4 (IRF4, aynı zamanda MUM1, LSIRF, NFEM5 ve ICSAT olarak da bilinir) B-hücrelerinin olgunlaşmasında ve farklılaşmasında önemli etkileri olan bir transkripsiyon faktörüdür. IRF4'un ilk olarak 'lenfositlere özgü IRF ailesi üyesi' ismiyle tanımlamasına karşın, melanositlerde de IRF4 anlatımı olduğu sonraki çalışmalarda gösterilmiştir. Yakın zamanda IRF4'un melanosit pigmentasyonunda rol aldığı tanımlanana kadar melanositlerdeki fonksiyonu bilinmemekteydi. Lenfositlerdeki önemli fonksiyonel işlevlerinin yanı sıra, IRF4 anlatımı birçok lenfoid kanseri ile ilişkili bulunmuştur. Örneğin, multipl mylelom (MM) ve aktifleşmiş B-hücre benzeri difuz büyük B-hücreli lenfoma (ABC-DLBCL) kanseri hücreleri yüksek miktarda IRF4 anlatımına sahiptir ve IRF4'a bağımlılıkları vardır, bu da eğer IRF4 susturulursa bu hücrelerin öleceği anlamına gelir. Şaşırtıcı şekilde, immünohistokimyasal çalışmalar ile melanoma örneklerinde lenfoid-olmayan kanser türlerine kıyasla dikkat çekici miktarda IRF4 seviyesine rastlanmıştır. Ek olarak, bazı genom ölçeğinde ilişkilendirme çalışmaları (GWAS) da IRF4 polimorfizmlerini melanoma ile ilişkilendirmiştir. Bu çalışmalar ışığında IRF4'un melanoma hücre hatlarındaki fonksiyonunu belirlemek için yola çıktık. İlk olarak halka açık gen anlatımı veritabanlarını kullanarak, lenfoid-olmayan kanserlere kıyasla, melanomalarda yüksek miktarda IRF4 anlatımı olduğunu doğruladık. Ayrıca *in vitro* deneyler ile; denediğimiz birçok melanoma hücre hattında dikkat çekici miktarda endojen IRF4 anlatımı olduğunu, diğer lenfoid-olmayan kanser hücre hatlarında da belirlenebilir seviyede bir anlatımı olmadığını teyit ettik. Daha sonra melanoma hücrelerinde, lentiviral enfeksiyon ve RNA interferaz sistemi kullanarak IRF4'u susturduk, ve hücrelerin rekabete dayanan sağlık durumlarında düşüş gözlemledik. Sonuçlarımız melanoma hücrelerinin, tıpkı MM ve ABC-DLBCL hücrelerinde gözlemlendiği gibi, IRF4'a karşı *in vitro* bağımlılığının olduğunu gösteriyor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

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

LIST OF ACRONYMS / ABBREVIATIONS

ABC-DLBCL	Activated B-cell like diffuse large B-cell lymphoma
APS	Ammonium persulphate
ATL	Adult T-cell leukemia
ATP	Adenosine 5’-triphosphate
BCL	B-cell lymphoma
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCLE	Cancer cell line encyclopedia
CDK4	Cyclin dependent kinase 4
CDKN2A	Cyclin dependent kinase inhibitor 2A
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CO_2	Carbon dioxide
CSR	Class switch recombination
C-terminal	Carboxyl terminal
ddH_2O	Double-distilled water
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
ECL	Enhanced Chemiluminescent Solution
EDTA	Ethylenediaminetetraacetate
Emp	Empty
EtOH	Ethanol
FBS	Fetal bovine serum
FSC	Forward scatter
gDNA	Genomic DNA
GTP	Guanosine 5’ -triphosphate
GFP	Green fluorescent protein

HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IRF4	Interferon regulatory factor 4
LB	Luria-Bertani broth
MAPK	Mitogen activated protein kinase
MCR1	Melanocortin-1 receptor
MCS	Multiple cloning site
MDM2	Mouse double minute 2 homologue
MEM-NEAA	Minimum Essential Medium Non-essential Amino Acid
$MgCl_2$	Magnesium chloride
MHCII	Major histocompatibility complex class II
MITF	Microthalmia-associated transcription factor
MM	Multiple myeloma
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
N-terminal	Amino terminal
ORF	Open reading frame
PI	Propidium Iodide
PI3K	Phosphoinositide-3-OH kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Steptomycin
PFA	Paraformaldehyde
PTEN	Phosphate and tensin homologue
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RB	Retinoblastoma
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SNP	Single nucleotide polymorphism
shRNA	Short hairpin RNA
SSC	Side scatter
TAE	Tris acetic acid
TBS	Tris-buffered saline
TBST	Tris Buffered Saline with Tween
TEMED	N,N’,N’,N’-Tetramethylethylenediamine
TFAP2A	Activating enchancer binding protein 2 alpha
Th	T helper cell
TLR	Toll-like receptor
Tris	Tris (hydroxylmethyl) aminomethane
Tween	Polysorbate
U	Units (enzyme units)
Unt	Untransduced
UV	Ultra Violet

1. INTRODUCTION

1.1. Molecular Biology of Cancer

Cancer is a neoplastic disease caused by genetic alterations in the genome. The disease is basically defined as uncontrolled growth and spread of abnormal cells and this uncontrolled cell spread is lethal due to vital organ failure if it can not be treated. Normal cells have strict control mechanisms preventing them from forming a tumor. These cells need to obtain several functional capabilities in order to proliferate, escape the cell death and activate metastasis. There are a number of traits that provide metastatic and growth capabilities to the tumor (Hanahan and Weinberg, 2000). These hallmarks of cancer are schematized in Figure 1.1.

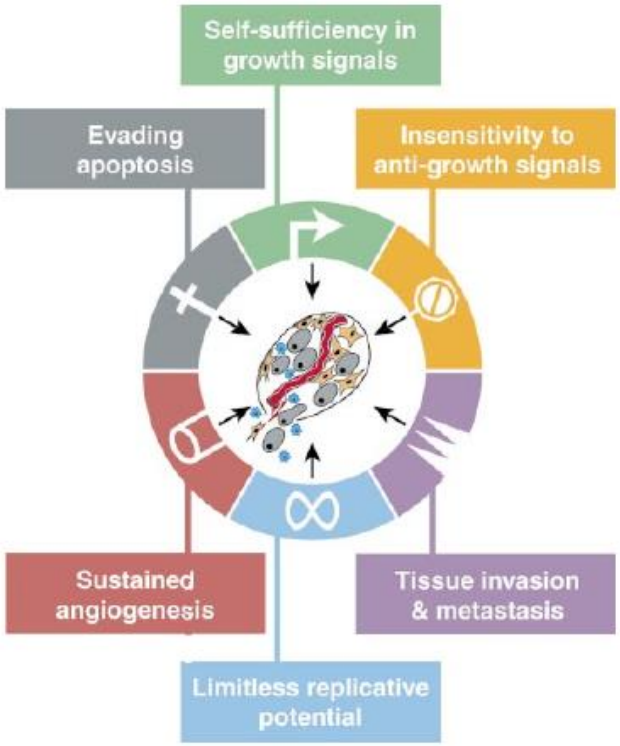


Figure 1.1. The hallmarks of cancer. Illustration of acquired functional capabilities of cancer during their development (Hanahan and Weinberg, 2000).

Proliferation capacity of the tumors plays the most fundamental role in oncogenesis. Normal cells control the growth signal production and release, thus controls the cell cycle and division. These signals are deregulated by cancer cells, making them able to re-write their destiny (Lemmon and Schlessinger, 2010). Cancer cells must activate promoting signals (Witsch *et al*., 2010) and bypass negative regulatory signals (Burkhart and Sage, 2008).

Programmed cell death is a natural barrier preventing the cancer development. During tumorigenesis, cancer cells encounter physiological stresses that activate the apoptotic pathway in these cells. Foretold stresses are caused by signaling imbalances in elevated oncogene levels, and DNA damage (Adams and Cory, 2007). Tumor cells develop escape mechanisms to get over apoptosis. The most commonly exploited strategy is the loss of TP53 tumor suppressor, which means the shutdown of an important damage sensor in the apoptosis induction. There are also other mechanisms used by cancers, involving the alteration of anti-apoptotic regulators (Bcl-2, Bcl-x_L) or pro-apoptotic factors (Bax, Bim, Puma) (Willis and Adams, 2005).

High proliferation rate in turn requires high nutrients and oxygen, and the disposal of metabolic waste. Normal cells are supplied with these needs by already existing blood vessels, which develops at the embryogenesis step of the organism. However, the vasculogenesis (formation of vascular tubes) and angiogenesis (sprouting of new tubes from established ones) are at a quiescent state after the morphogenesis and need to be turned on during carcinogenesis (Hanahan and Folkman, 1996). Vascular endothelial growth factor (VEGF) up-regulation by tumor cells accomplishes the purpose of angiogenesis activation (Ferrara, 2009) while fibroblast growth factor (FGF) up-regulation sustains the activated angiogenesis (Baeriswyl and Christofori, 2009).

Cancer cells undergo a series of biological changes before reaching the malignant status. First step is spreading over the local tissue, then the intravasation of cancer cells through the basal membrane reaching blood and lymphatic vessels. Cancer cells enter these vessel and transit until the extravasation step in which the cells exit the capillaries and enter distant tissues. This step is the metastasis of the cancer cells and pro-

ceeds into the growth of these cells in the distant tissues, termed colonization, which is the last step (Talmadge and Fidler, 2010).

Tumor cells need to obtain some capabilities in order to perform metastatic features. These capabilities are mostly alterations of their cell to cell, and cell to extracellular matrix (ECM) attachments. E-cadherin is the master cell to cell adhesion molecule, therefore its down-regulation helps cancer cells to increase their mobility (Berx and van Roy, 2009). N-cadherin is another adhesion molecule, normally expressed in migrating mesenchymal cells and neurons, which is observed to be up-regulated in many invasive tumor cells (Cavallaro and Christofori, 2004).

Oncogenes and tumor suppressors are genes which contribute to the conversion of a normal cell into a cancer cell by affecting the mechanisms mentioned above. Proteins encoded by oncogenes are involved in cell proliferation, apoptosis, or both. If an oncogene's activation is increased by a mutation, amplification or a translocation, it leads to the cancer formation (Bueno *et al*., 2008). Discovery of oncogenes resulted in an anti-oncogene theory that suggested the existence of a gene group with the opposite effects of oncogenes; blocking tumor development. Earliest studies identified these as tumor suppressor genes (Harris *et al*., 1969), with regulatory roles in cell cycle checkpoint, detection and repair of DNA damage and mitogenic signaling (Sherr, 2004).

Oncogene and tumor suppressor gene products are mainly transcription factors. These protein products, by regulating the expression of target genes, controls signaling pathways that are important in cancer. Aberrant transcription factor activity may be caused by an alteration in its expression, change in its stability or a modification at its protein interaction. This aberrant activity leads to deregulation of genes which are involved in tumor cell survival, proliferation, angiogenesis and metastasis (reviewed in Libermann *et al*., 2006).

1.2. Skin Cancer and Melanoma Biology

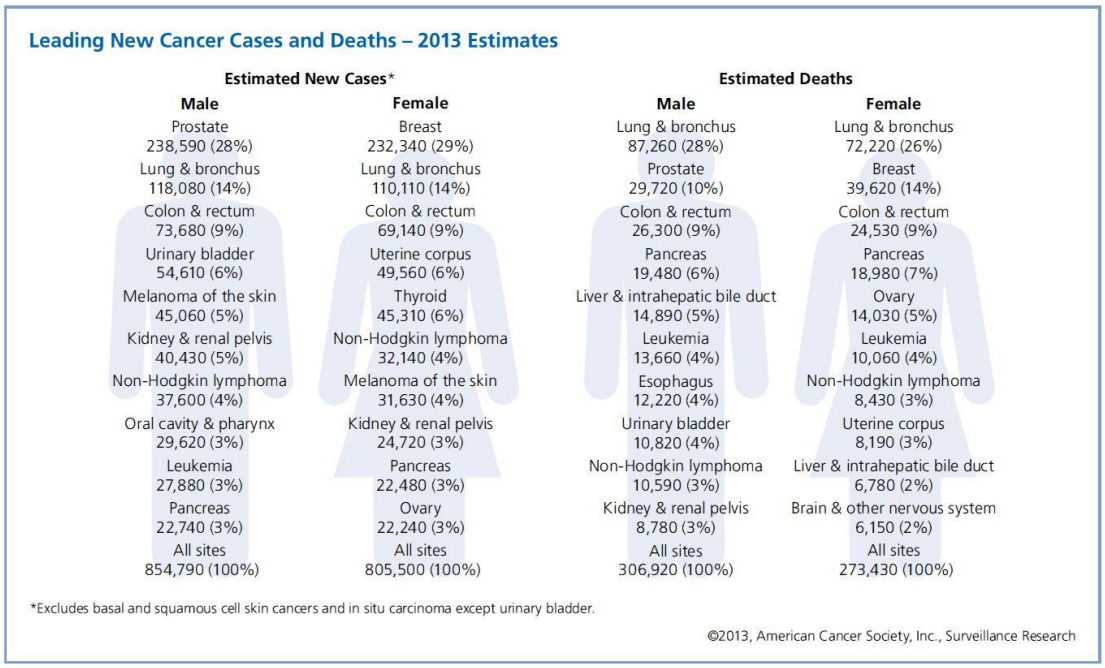
Two main types of skin cancer are present; non-melanoma and melanoma skin cancers. Non-melanoma skin cancer (NMSC) can arise from transformed keratinocytes, have a higher incidence rate compared to melanoma, but are less aggressive. Although NMSC is much more common than melanoma, melanoma is the major cause of death among all skin cancers, accounting up to 80 per cent of mortality from skin cancers.

There are some important warning signs of melanoma, which can be used to detect malignancy of a nevus (growth of melanocytes, forming a mole). These signs are described by a mnemonic rule: ABCDE of melanoma. A is for the asymmetry of the nevus; B is for borders irregularity; C is for the color (uniform and variegated pigmentation; D is for the diameter (greater than 6mm); E is for evolving over time (Abbasi *et al*., 2004).

Melanoma has a complex etiology. Environmental factors are effective in its genesis. Ultraviolet (UV) radiation exposure is determined as the primary environmental cause. Other risk factors are phenotypical characteristics such as green or blue eyes, red or blond hair, freckles score and melanocytic nevi count (100 or more). The last risk factor is a personal or family history of melanoma). Familial background constitutes up to 10% of melanoma cases. Relatives of patients who suffer from melanoma have an increased risk of melanoma development (Badenas *et al*., 2012). Also second primary melanoma risk of an individual increases eight-fold after the first melanoma diagnosis (Bradford *et al*., 2010).

Since most of the non-melanoma skin cancer (NMSC) cases are highly curable, dividing the skin cancer topic into two subgroups of melanoma and NMSC is statistically more accurate. Even though melanoma is also highly curable at the early stages if treated properly, it is more likely to metastasize compared to NMSC types.

In the United States, melanoma diagnosis is expected to be at a number about 76,690 in 2013 (Figure 1.2). Although this number is less than the 5% of all skin cancer cases, it is the majority of deaths caused by skin cancer. The incidence rates of melanoma have been increasing for more than 30 years. Skin cancer related 12,650 deaths are estimated for 2013. Out of this estimation, 3,170 of the deaths are from NMSC while 9,480 of the deaths are of melanoma origin.



Leading New Cancer Cases and Deaths – 2013 Estimates

Estimated New Cases*		Estimated Deaths	
Male	**Female**	**Male**	**Female**
Prostate 238,590 (28%)	Breast 232,340 (29%)	Lung & bronchus 87,260 (28%)	Lung & bronchus 72,220 (26%)
Lung & bronchus 118,080 (14%)	Lung & bronchus 110,110 (14%)	Prostate 29,720 (10%)	Breast 39,620 (14%)
Colon & rectum 73,680 (9%)	Colon & rectum 69,140 (9%)	Colon & rectum 26,300 (9%)	Colon & rectum 24,530 (9%)
Urinary bladder 54,610 (6%)	Uterine corpus 49,560 (6%)	Pancreas 19,480 (6%)	Pancreas 18,980 (7%)
Melanoma of the skin 45,060 (5%)	Thyroid 45,310 (6%)	Liver & intrahepatic bile duct 14,890 (5%)	Ovary 14,030 (5%)
Kidney & renal pelvis 40,430 (5%)	Non-Hodgkin lymphoma 32,140 (4%)	Leukemia 13,660 (4%)	Leukemia 10,060 (4%)
Non-Hodgkin lymphoma 37,600 (4%)	Melanoma of the skin 31,630 (4%)	Esophagus 12,220 (4%)	Non-Hodgkin lymphoma 8,430 (3%)
Oral cavity & pharynx 29,620 (3%)	Kidney & renal pelvis 24,720 (3%)	Urinary bladder 10,820 (4%)	Uterine corpus 8,190 (3%)
Leukemia 27,880 (3%)	Pancreas 22,480 (3%)	Non-Hodgkin lymphoma 10,590 (3%)	Liver & intrahepatic bile duct 6,780 (2%)
Pancreas 22,740 (3%)	Ovary 22,240 (3%)	Kidney & renal pelvis 8,780 (3%)	Brain & other nervous system 6,150 (2%)
All sites 854,790 (100%)	All sites 805,500 (100%)	All sites 306,920 (100%)	All sites 273,430 (100%)

*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Figure 1.2. Estimations of American Cancer Society for 2013. (American Cancer Society. Cancer Facts & Figures 2013. Atlanta: American Cancer Society; 2013)

1.3. Melanoma Molecular Biology

There are a number of penetrant genes included in melanoma susceptibility. Two of these genes, which are high penetrant, are cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase inhibitor 2A (CDKN2A). Both genes play a role in the cell cycle. CDKN2A is a tumor suppressor gene with two different transcripts, α and β, which encodes p16 and p14ARF protein, respectively. Protein p16 regulates G1-phase exit while p14ARF is involved in p53 pathway and induces apoptosis or cell cycle arrest

(Pomerantz *et al*., 1998, Serrano *et al*., 1993). CDK4, on the other hand, is an oncogene with a protein product which is one of the binding partners of p16 (Zuo *et al*., 1996).

There are also other genes linked with melanoma susceptibility. Two of them are medium penetrant genes, microthalmia-associated transcription factor (MITF) and melanocortin-1 receptor (MC1R). MITF have important regulatory roles on melanocyte development and differentiation. It has been implicated in melanoma susceptibility after recent studies, underlying its importance also in melanoma development and differentiation (Bertolotto *et al*., 2011, Levy *et al*., 2006). MC1R takes part in pigmentation process by encoding melanocyte-stimulating hormone. Some MC1R variants are associated with melanoma risk (Suzuki *et al*., 1996, Williams *et al*., 2011).

A number of key molecular pathways are implicated in melanoma tumorigenesis; mitogen activated protein kinase (MAPK), phosphoinositide-3-OH kinase (PI3K), retinoblastoma (RB) and p53. MAPK pathway is activated by the binding of growth factors to receptor tyrosine kinase (RTK). This binding activates Ras GTPase, which recruits Raf and leads to the phosphorylation of downstream elements. MAPK pathway is responsible for the control of cell growth, survival and invasion. Therefore its aberrant activation is an essential feature of many cancers, including melanoma (Panka *et al*., 2006). Activation of MAPK pathway in melanoma is generally through B-raf or N-ras activation. B-raf activating mutations are the most commonly found mutations in melanoma, covering 66% of all cases. A single base pair substitution at codon 600 constitutes up to 90% of all B-raf mutations that causes glutamate to valine change (V600E) and constitutive activation of B-raf (Davies *et al*., 2002). N-ras mutations are roughly seen at 15% of all melanomas. It is mutated mostly in codon 61 (Q61R and Q61K), making it constitutively active (Ball *et al*., 1994, Malumbres and Barbacid, 2003).

PI3K pathway is responsible for the regulation of cell survival, proliferation and motility. It resides in the downstream of Ras, and like B-raf, it is activated in the presence of oncogenic N-ras (Cully *et al*., 2006). Genetic alterations in this pathway are observed in low frequency in melanoma. However, phosphate and tensin homologue (PTEN) is a tumor suppressor gene that inhibits PI3K pathway and loss of its expression

is seen in 5-20% of primary melanomas. This expression loss can be caused by a deletion, mutation or promoter methylation (Wu *et al*., 2003).

p53 is a transcription factor that controls the stability of the genome (therefore named as the guardian of the genome) by blocking cell cycle, initiating DNA repair mechanisms or leading abnormal cells to apoptosis. The p53 pathway is disrupted in many malignancies by directly targeting p53 itself. However, regulators of p53 pathway are mainly found to be mutated in melanomas instead of the p53 itself (Albino *et al*., 1994). Two important regulators of this pathway are mouse double minute 2 homologue (MDM2) and p14ARF. MDM2 negatively regulates the p53 activity and promotes p53 degradation, while p14ARF negatively regulates MDM2 and helps the stabilization of p53 (Lowe and Sherr, 2003).

In order to proliferate and/or migrate, melanocytes need to obtain mutations to bypass the regulatory function of keratinocytes, which tightly controls them. By cadherins (a group of intercellular adhesion junctions), melanocytes are retained in epidermis. E-cadherin is expressed at all epidermal cells, while N-cadherin is expressed only in dermal fibroblasts and vascular endothelial cells. Loss of E-cadherin and gain of N-cadherin at a melanocyte provides increased motility to the cell, aiding its invasion potential.

After the invasion of epidermis, transformed melanocytes can form a benign nevus. Following the formation of a nevus, the tumor can become aggressive and go into a radial growth phase by invading nearby epidermal tissue. The next stage is the vertical growth phase where the melanoma becomes malignant and invades through the dermis, reaching to the lymphatic and the vascular system (Figure 1.3).

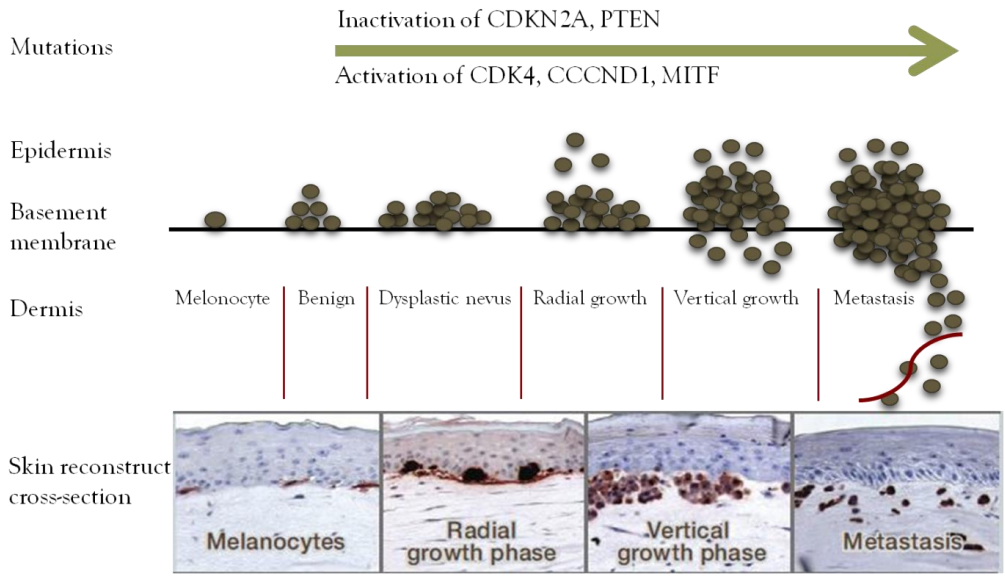


Figure 1.3. Melanoma Progression Chart. Adapted from (Vultur and Herlyn, 2013)

1.4. IRF4 and Its Connection to Cancer

1.4.1. Initial Characterization of IRF4

There are nine members of IRF family transcription factors in humans and mice. They are named with a number from one to nine: IRF1, IRF2, IRF3, IRF4 (MUM-1, LSIRF, ICSAT, and NFEM5), IRF5, IRF6, IRF7, IRF8 (ICSBP) and IRF9. Initial discovery of IRFs were describing them as downstream regulators at the interferon signaling pathway, emphasizing crucial roles of them in both adaptive and innate immune responses, and correlating them to tumorigenesis (Shaffer *et al*., 2009, Tamura *et al*., 2008). IRF4's first identification was as lymphocyte-specific nuclear factor (NFEM5), provided by the evidence of it binding to EM5 motif contiguous to PU.1 binding site in the κE3' enhancer sequence (Pongubala *et al*., 1992). The characterization of IRF4 proceed as lymphocyte-specific IRF (LSIRF) and interferon consensus sequence-binding protein (ICSAT) after IRF4 was cloned from mouse spleen (Matsuyama *et al*., 1995) and adult T-cell leukemia (Yamagata *et al*., 1996) respectively.

1.4.2. Structural Features of IRF4

IRF4 protein comprises three major parts that compose a single polypeptide chain. One of these three parts is N-terminal DNA binding domain (DBD) (Matsuyama *et al*., 1995), the other part includes intermediary linker domain which is important for the conformational changes of IRF4 protein, and the last part is responsible for regulating the function of protein and contains two transactivation domains (TAD) along with auto inhibition and ternary complex formation regions. These three domains have been highly studied, therefore their functional elements are identified (Figure 1.4). Compared by the structural features, IRF4 is most closely related to IRF8 among all other IRFs. Given in numbers, IRF4 and IRF8 have 83% similarity in their N-terminus, 51% in their C-terminus and considerable homology at their DBDs.

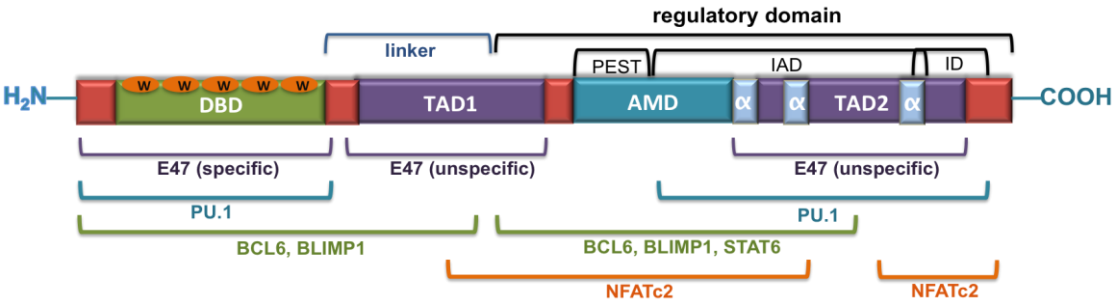


Figure 1.4. Schematic Representation of the IRF4 Protein. AMD, activation masking domain; DBD, DNA binding domain; IAD, IRF association domain; ID, inhibitory domain; TAD, transactivation domain; W, tryptophan; α, α-helix. Adapted from Silva *et al*., 2012.

With the assistance of its cofactors, IRF4 can play a role in the activation or repression of a gene transcription. These cofactors are cell type and stage specific, which results in a lineage and stage restricted activation of IRF4 and modulation of gene expression. Without the cofactors for IRF4 to interact, its IRF4 association domain (IAD) masks the DNA binding domain (DBD) thus makes the IRF4 protein incapable of both binding to DNA and forming a ternary complex (Yamagata *et al*., 1996).

1.4.3. Hematological Findings and Functions of IRF4

IRF4 is an important regulator in hematopoietic cells; it has critical roles in lymphocyte development (Shaffer *et al.*, 2009). Therefore it is deeply studied; the interaction partners of IRF4 and the major pathways that involve IRF4 are identified in immune cells to a considerable extent (Figure 1.5).

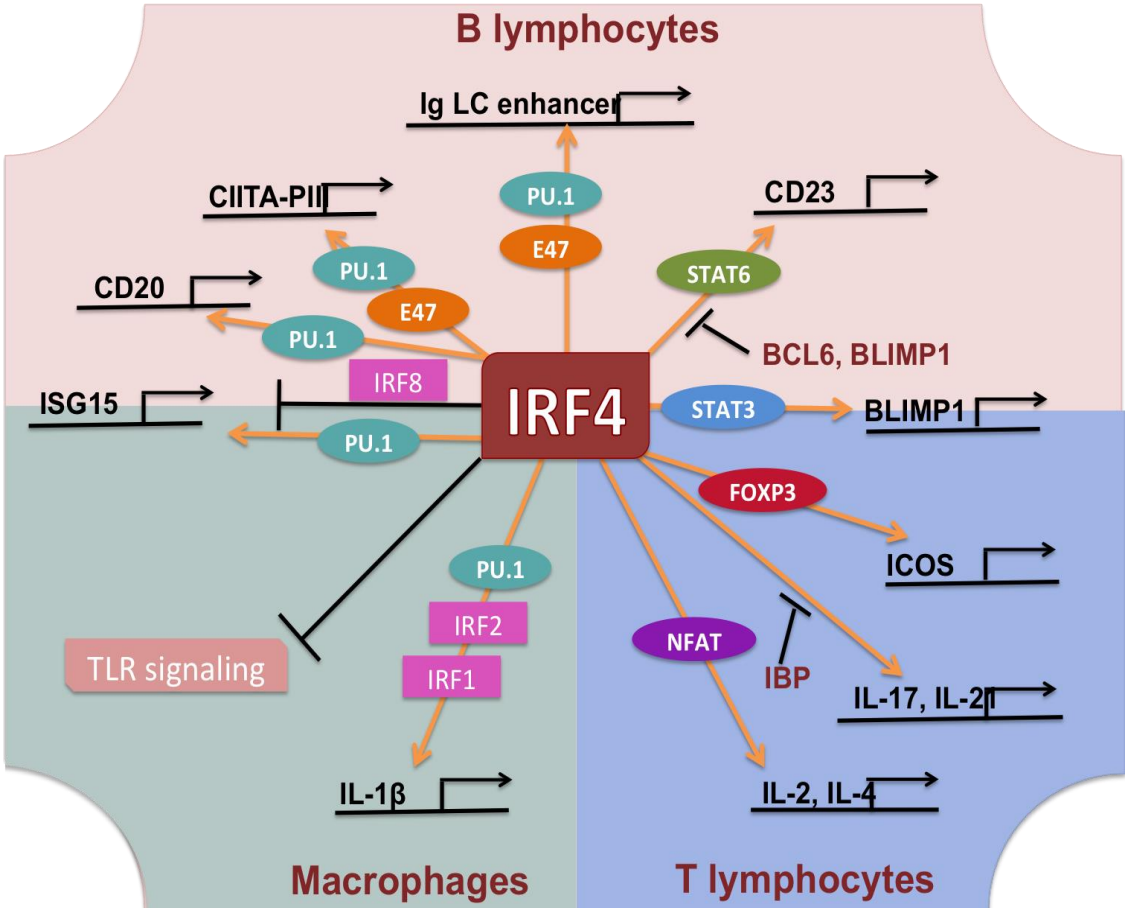


Figure 1.5. IRF4 Activity in Immune Cells. IRF4 can activate or repress various transcriptional targets in different immune cells. IBP, IRF4 binding protein; LC, light chain. Adapted from Silva *et al.*, 2012.

Studies with Irf4 $^{-/-}$ mice showed that IRF4 is dispensable in early B-cell differentiation but essential for late B-cell differentiation. In the absence of IRF4, Irf4 $^{-/-}$ mice lacked germinal centers (GCs) and plasma cells, along with decreased serum immuno-

globulin (Ig) levels that lead to failure in antibody responses (Mittrucker *et al*., 1997). IRF4 is found out to be required for plasma cell development with functional *in vivo* studies on Irf4 $^{-/-}$ B-cells. These studies also revealed that IRF4 has a critical role on immunoglobulin class switch recombination (CSR), which is a process that takes place before the plasma cell development (Sciammas *et al*., 2006). Follow-up studies shown that nuclear factor-κB (NF-κB) activation up-regulates IRF4, which represses the key regulator of GC B-cells, B-cell lymphoma 6 (BCL-6) (Klein and Dalla-Favera, 2008). In summary, IRF4 has important roles in, abolishing GC B-cell program, driving CSR process and programming centrocytes into plasma cell differentiation (De Silva *et al*., 2012).

In addition to the roles of IRF4 in B-cell lineage, it is an important regulator in T-cell and myeloid-cell differentiation. IRF4 controls cytokine expression and apoptosis, leading the differentiation progress in T-helper 2 (Th2) and Th17 cells (Zheng *et al*., 2009). In macrophages, IRF4 is required for toll-like receptor (TLR) signaling (Honma *et al*., 2005). In dentritic cells, it is required for the expression of surface major histocompatibility complex class II (MHCII) and antigen presentation of these cells (Suzuki *et al*., 2004). Overall, IRF4 is a critical regulator in immune cells, with developmental functions, determining the fate of the cells. (De Silva *et al*., 2012).

Apart from all the functions of IRF4 in hematopoietic cell lineages, recent studies emphasize its roles in non-hematopoietic cell types. In adipocytes, IRF4 determines the transcriptional response to nutrient availability and its reduction increases lipid synthesis (Eguchi *et al*., 2011).

1.4.4. IRF4 in Melanocytes

There was no identified functional role for IRF4 in melanocytes until recently. First implication of IRF4 in pigmentation is through some GWAS studies that found an IRF4 SNP (rs12203592-T) associated with increased freckles score, sensitivity of the

skin to sun exposure, blue eyes and brown hair color (Han *et al*., 2008, Sulem *et al*., 2007).

A recent study identified the mechanism of IRF4 in melanocytes along with the function of the SNP that was found to be associated with pigmentation through the GWAS studies. They investigated the SNP (rs12203592-T) which is in intron 4 of IRF4 and found out that it lies within an enhancer of IRF4 transcription. They showed that microphthalmia-associated transcription factor (MITF) and activating enhancer binding Protein 2 alpha (TFAP2A) cooperatively activates IRF4 expression, by interacting with the intron 4 that the SNP (rs12203592-T) is observed in. This SNP is shown to be decreasing the binding efficiency of TFAP2A to the enhancer, resulting in decreased IRF4 expression. They also showed that MITF and IRF4 cooperatively activate the transcription of tyrosinase (TYR), which encodes a protein that is involved in melanin synthesis. The functional change in pigmentation caused by IRF4 SNP (rs12203592-T) is therefore explained by the alteration of IRF4 expression, also emphasizing the role of IRF4 in increasing melanin synthesis (Praetorius *et al*., 2013).

1.4.5. Non-oncogene Addiction to IRF4

With all the important developmental functions of IRF4 in lymphocytes, it is not surprising that the deregulation of IRF4 can stimulate a tumorigenic effect. Recent studies uncovered many IRF4-dependent malignancies including multiple myeloma (MM), activated B-cell like diffuse large B-cell lymphoma (ABC-DLBCL), adult T-cell leukemia (ATL) and chronic lymphocytic leukemia (CLL) (Shaffer *et al*., 2009).

Human T-lymphotropic virus 1 (HTLV-I) and Epstein-Barr virus (EBV) are related to lymphoma progression due to their effect of NF-κB pathway activation and in turn IRF4 expression elevation (Sharma *et al*., 2000). Cytokine IL-15 is a growth factor in some adult T-cell leukemia (ATL) cases and IRF4 transactivates IL-15 receptor α chain, suggesting that IRF4 can possibly promote growth in ATL cases (Kukita *et al*., 2002, Mariner *et al*., 2002). EBV infection causes mature B-cells to transform into lym-

phoblastic cells, but when IRF4 is knocked-down in these lymphoblastic cells, cell division decreases and apoptosis increases (Xu *et al*., 2008).

Chronic lymphocytic leukemia (CLL) susceptibility is found to be strongly associated with a single nucleotide polymorphism (SNP) in the 3'UTR region of IRF4. The association is the result of a genome wide association study (GWAS) and is observed in several patient cohorts. Although the function of this SNP is unknown, it is associated more strongly with a CLL subtype that is of post-GC memory B-cell origin. Emphasizing the role of IRF4 in late B-cell differentiation, this SNP might be interrupting the plasma cell differentiation process of memory B-cells (Di Bernardo *et al*., 2008).

Multiple myeloma (MM) is a malignancy of plasma cells that possess extensive range of genetic anomalies such as c-myc, N-ras, K-ras and p53 (Bergsagel and Kuehl, 2005). The role of IRF4 in supporting carcinogenesis is most clearly shown in MM. In MM, direct targets of IRF4 are identified and it points out IRF4 as a master regulator; affecting metabolic control, membrane biogenesis, cell cycle progression, cell death, transcriptional regulation and plasmacytic differentiation. MYC is also identified as a direct target of IRF4 and it also transactivates IRF4 itself, creating an auto-regulatory loop. Knock-down of IRF4 by using short hairpin RNA (shRNA) is found to be toxic to myeloma cell lines, regardless of the combination of foretold genetic anomalies. In rare MM cases, a translocation juxtaposes IRF4 gene with immunoglobulin heavy chain locus, displaying the fact that IRF4 can act as an oncogene (Lidar *et al*., 1997). But in most of the cases, IRF4 is not genetically altered, suggesting a 'non-oncogene addiction' of myeloma cells to the aberrant function of normal IRF4 protein (Shaffer *et al*., 2008).

Diffuse large B-cell lymphoma (DLBCL) subtypes have different gene expression characterizations based on the stages of B-cell differentiation. In activated B-cell like (ABC) subtype of DLBCL, high expression of IRF4 is a distinctive characteristic (Staudt and Dave, 2005). This high IRF4 expression is largely mediated by constitutive NF-κB activity in ABC-DLBCL, and it is lowly expressed in GC B-cell like (GCB) subtype of DLBCL where NF-κB activity is also low (Davis *et al*., 2001, Lam *et al*., 2005). Similar to the 'non-oncogene addiction' observed in the MM samples, ABC-DLBCL

samples are addicted to IRF4, and cell death is induced when IRF4 is knocked-down by shRNA (Yang *et al*., 2012).

1.4.6. Therapeutic Features of IRF4

IRF4 directed therapy is a new approach in the field, with a benefit of not effecting most normal cells because of IRF4's tissue restricted expression. Lenalidomide, an immunomodulatory compound, is being used in MM and ABC-DLBCL malignancies recently, and the mechanism of action is identified in recent studies for both malignancies.

MM patients treated with lenalidomide have significantly longer overall survival. The prognostic significance is underlined when two patient groups with low and high IRF4 expression is compared. Normally, median survival of high IRF4 level patients is 19 months, while the median survival is 83 months for low IRF4 level patients. However, lenalidomide treatment abolished this significant difference between differently IRF4 expressing groups. As shown above, lenalidomide had an effect on MM treatment if the IRF4 level is high in the patient, this shows that lenalidomide targets IRF4 and functions through its regulation. An i*n vitro* study with MM cell lines showed that lenalidomide rapidly down-regulates IRF4 levels within 8 hours after the exposure. This down-regulation is found to be associated with a decrease in MYC levels, cell cycle arrest at G1 checkpoint, decreased cell proliferation and cell death (Lopez-Girona *et al*., 2011).

Early phase clinical trials of lenalidomide showed preferential activity against ABC-DLBCL, but its mechanism of actions was unknown (Hernandez-Ilizaliturri *et al*., 2011). A recent study identified the mechanism of lenalidomide in ABC-DLBCL cells by analyzing gene expression profile changes upon the lenalidomide treatment. This study showed that lenalidomide treated cells have increased interferon β (IFNβ) levels and blocked NF-κB. Transcription factors IRF4 and SPIB have functions of suppressing IFNβ and enhancing NF-κB. These transcription factors are found to be down-regulated by lenalidomide explaining the changes in IFNβ and NF-κB levels (Yang *et al*., 2012).

1.5. IRF4: A New Factor in Melanoma?

Melanoma is associated with various types of oncogenes, commonly found to be mutated in patients. Such oncogenes are B-Raf, N-Ras, TP53, PTEN, p16INK4a and p14ARF (Hodis *et al*., 2012). There is yet no scientific evidence showing the tumorigenesis effect of IRF4 over melanocytes, even though high level IRF4 expression in many melanoma cell lines has been shown in different studies as described below.

First discovery of IRF4 in melanoma is in G-361 cell line, along with its discovery in normal melanocytes of mice foreskin (Grossman *et al*., 1996). This finding is important for bringing out the argument that IRF4 should not be defined as a lymphocyte specific transcription factor. A comprehensive tissue micro array (TMA) study, including 1209 human malignancies and normal tissues showed that, IRF4 expression is remarkable in melanoma samples together with lymphomas. 5 out of all 22 melanoma samples tested stained positive for IRF4 protein in TMA, while rest of the non-lymphoid normal and tumor tissues were negative (Natkunam *et al*., 2001). Immunohistochemistry stainings of melanoma samples confirmed the TMA results, and indicate that IRF4 could be used as a specific and sensitive melanoma marker alongside the current panel of markers; S100, HMB45 and MelanA (Sundram *et al*., 2003).

Some genome wide association studies (GWAS) linked single nucleotide polymorphisms (SNP) in IRF4 to nevi counts, tanning tendency and color variations in skin, eye and hair (Nan *et al*., 2009) Sulem *et al*., 2008; Han *et al*., 2009). These skin phenotypes are known risk factor modulators of melanoma, pointing out the association between IRF4 and melanoma progression. As expected from these findings, later GWAS studies showed the link between an IRF4 SNP (rs12203592*T) and melanoma risk (Duffy *et al*., 2010; Han *et al*., 2011). This SNP was previously found to be increasing the IRF4 expression, supporting the role of IRF4 in melanoma (Do *et al*., 2010).

Bringing all together, IRF4 is an important transcription factor, not only in lymphocytes but also in melanocytes. With functions yet to be found out, it is evident that

IRF4 de-regulation increases melanoma predisposition. Unlike in any other non-lymphoid cancers, IRF4 expression is high in many melanoma samples, which also states it as a specific melanoma marker.

2. PURPOSE

The purpose of this thesis project is to identify the cellular mechanisms that IRF4 is involved in melanoma cell lines. For this purpose, we decided to investigate the possible phenotypic effects upon IRF4 manipulation in melanoma cell lines. This investigation is composed of three steps. First step is to verify the high expression levels of IRF4, which were shown in previous studies in many melanoma cell lines. These expression levels provide a background for the importance of IRF4 in melanoma cell lines, addressing a possible dependency for melanoma cell lines over IRF4. Second step is to down-regulate IRF4 with RNAi in the cell lines. The last step is to finally investigate the phenotype changes in cell lines upon IRF4 knock-down. There are a number of cancer hallmarks, such as escape from cell cycle checkpoints, resisting apoptosis, anchorage independency, migration and invasion potential, which are aimed to be investigated through different analysis methods in this study.

3. MATERIALS

3.1. General Kits, Enzymes and Reagents

Table 3.1. List of kits, enzymes and reagents.

BCA Protein Assay Kit	23227, Thermo Scientific, USA
Plasmid Midiprep Kit	12143, Qiagen, Germany
EndoFree Plasmid Maxi Kit	12362, Qiagen, Germany 740426.10, Macherey Nagel, Germany
First Strand cDNA Synthesis Kit	E6550S, New England Biolabs (AMV), USA K1622, Thermo Scientific (RevertAid), USA
RNA Isolation Kits	11828665001, Roche (High Pure), Switzerland 740955.50, Macherey Nagel, Germany
Protein Molecular Weight Marker	P7709V, New England Biolabs (P7709 V), USA
Protease Inhibitor Coctail Tablets	Roche, Switzerland
Taqman Mastermix	Roche, Switzerland Life Technologies, USA
Chemiluminescent HRP Substrate	34080, Thermo Scientific, USA
DNA Ladder	N3232S, Fermentas, USA
DNA Loading Dye	B7021S, Fermentas, USA

3.2. Biological Materials

3.2.1. Bacterial Strains

Transformations were done by using *Escherichia coli* STBL3 strain as competent cells. Recombination frequency of this strain is relatively lower for lentiviral vectors and it has been always used for this purpose. The genotype of *Escherichia Coli* STBL3 strain

is F– mcrB mrr hsdS20 (rB–, mB–) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 (StrR) xyl-5 λ– leu mtl-1.

3.2.2. Cell Lines

Table 3.2. List of cell lines.

Cell Line	Provider
SKMEL-5	Kindly provided by Prof. David M. Sabatini and Yetiş Gültekin
SKMEL-28	Kindly provided by Nesrin Özören
A2058	Kindly provided by David M. Sabatini and Yetiş Gültekin
G-361	Kindly provided by Prof. Maria Soengas
Malme-3M	Kindly provided by Maria Soengas
MeWo	Kindly provided by Nesrin Özören
MEL-9	Kindly provided by Nesrin Özören
MEL-10	Kindly provided by Nesrin Özören
MCF-7	Kindly provided by Nesrin Özören
MDA-MB-231	Kindly provided by Kuyaş Buğra
HEK293FT	Kindly provided by Nesrin Özören

3.2.3. Plasmids

Lentiviral RNAi vector KH1 (kindly provided by Dr. Maria Soengas) and packaging plasmids pRSV-rev, pHCMV-G, pMDLg-pRRE (kindly provided by Dr. Maria Soengas) were used.

3.2.4. Primers

Table 3.3. Primers used in this study.

Primer ID	Sequence	Application
KH1-seq	5'- TGTCGCTATGTGTTCTGGGA -3'	Sequencing
KH1-rev-seq	5'- TATCGTTTCAGACCCACCTC -3'	Sequencing
pLenti Forward	5'- GACTGTCGACATGAACCTGGAGGG -3'	PCR
pLenti Reverse	5'- GACTCTCGAGCAATTCTTGAA-TAGAGG -3'	PCR

3.3. Oligonucleotides

Table 3.4. List of oligonucleotides used in shRNA cloning

Oligonucleotide	Sequence
shIRF4b-sense	5'- CCGGGGTGCCATTTCTCAGGGAAGTATCCACAC-CATACTTCCCTGAGAAATGGCACTTTTTT -3'

Table 3.4. List of oligonucleotides used in shRNA cloning (cont.).

shIRF4b-antisense	5’- CTGAAAAAAGTGCCATTTCTCAGGGAAGTAGGT GTGGTACTTCCCTGAGAAATGGCACC -3’
shIRF4a1-sense	5’- CCGGGCCGCCATTCCTCTATTCAAGACCACACCT CTTGAATAGAGGAATGGCGGTTTTTT -3’
shIRF4a1-antisense	5’- CTAGAAAAAACCGCCATTCCTCTATTCAAGAGGT GTGGTCTTGAATAGAGGAATGGCGGC -3’
shLuc-sense	5’- CCGGGGTGGATTTCGAGTCGTCTTAATCCACACC ATTAAGACGACTCGAAATCCACTTTTTT -3’
shLuc-antisense	5’- CTAGAAAAAAGTGGATTTCGAG-TCGTCTTAATGGTGTGGATTAAGACGACTCGAAATCCACC-3’
shRPS13-sense	5’- CTAGAAAAAACCGCCATTCCTCTATTCAA-GAGGTGTGGTCTTGAATAGAGGAATGGCGGC -3’
shRPS13-antisense	5’- CTAGAAAAAAGGTGTAATCCTGAGAGAT-TCATGGTGTGGATGAATCTCTCAGGATTACACCC -3’

3.4. Chemicals, Plastic and Glassware

Chemicals were purchased from either Applichem (Germany), Merck (Germany) or Sigma-Aldrich (USA); cell culture plasticware from TPP (Switzerland); micropipette tips from BioPointe Scientific (Mexico); 2ml and smaller tubes from Axygen (USA), 5ml tubes from BD Biosciences (USA), 15ml and 50ml tubes from VWR (USA). Glassware was also purchased from VWR (USA). For sterilization, all tips and tubes were autoclaved at 121 ^{o}C for 20 minutes.

3.5. Buffers and Solutions

Table 3.5. Buffers and solutions used in this study.

10X SDS Running Buffer	250 mM Tris-Base (pH: 8.3) 1.90 M Glycine 1% SDS
10X Transfer Buffer	1.90 M Glycine 250 mM Tris-Base
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X TBST	1X TBS 0.1% Tween-20
12% SDS-PAGE Gel (20ml)	8.6 ml ddH2O 6 ml 40% Acrylamide mix 5ml Tris (1.5 M, pH: 8.8) 200 µl 10% SDS 200 µl 10% APS 20 µl TEMED
5% Blocking Buffer	5% BSA (w/v) in TBST
4X Protein Loading Dye (Laemmli)	250 mM Tris-Cl (pH: 6.8)
	8% SDS
	40% Glycerol
	20% β-mercaptoethanol
	0.008% Bromophenol Blue
Cell Lysis Buffer (RIPA)	150 mM NaCl
	50 mM Tris (pH: 8.0)
	0.1% SDS
	1.0% NP-40
	0.5% Sodium deoxycholate
Paraformaldehyde (4%)	1 g Paraformaldehyde
	25 ml ddH2O
Propidium Iodide Staining Solution	20 µg/ml Propidium Iodide 0.1% Triton X-100 200 µg/ml RNAse
10X PBS	1,37 M NaCl 270 mM KCl 80 mM NaH2PO4 20 mM KH2PO4
Dulbecco's Modified Eagle Medium (DMEM)	41966-029, Gibco Invitro-gen, USA

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

Freezing Medium	1X DMEM 20% FBS 1X Pen/Strep 100 µM MEM-NEAA 7.5% DMSO
0.05% Trypsin-EDTA	25300-054, Gibco Invitrogen, USA
Fetal Bovine Serum (FBS)	10270-106, Gibco Invitrogen, USA
Minimum Essential Medium Non-essentail Amino Acid (MEM-NEAA)	11140-035, Gibco Invitrogen, USA
100X Penicillin/Streptomycin (5000 u Penicillin + 5000 µg Streptomycin per ml)	15140-122, Gibco Invitrogen, USA
400X Choloroquin	10 mM Choloroquin
1000X Polybrene	4 mg/ml Polybrene
HEPES	Gibco Invitrogen, USA
2X HBS	50 mM HEPES (pH: 7.0) 280 mM NaCl 1.5 mM NaH2PO4
50X Tris acetic acid (TAE)	2 M Tris-acetate 50 mM EDTA (pH: 8.5)
LB Medium	10 g/L Tryptone 5 g/L NaCl 5 g/L Yeast Extract
LB Agar	10 g/L Tryptone 5 g/L NaCl 5 g/L Yeast Extract 15g/L Agar
1000X Ampicillin	100 mg/ml in ddH2O
1000X Kanamycin	50 mg/ml in ddH2O

3.6. Antibodies

Table 3.6. Antibodies used in this study.

Name	**Species**	**Dilution**	**Source**
Anti-IRF4 (M-17)	Goat	1:5000	Sc-6059, Santa Cruz
Anti-Goat, HRP	Rabbit	1:2500	Sc-2768, Santa Cruz
Anti-Actin	Rabbit	1:2500	4967S, Cell Signaling
Anti-Mouse, HRP	Goat	1:2500	7074S, Cell Signaling

3.7. Equipments

Table 3.7. List of equipment used in this study.

Agarose Gel Electrophoresis	MSMINIDUO, Cleaver Scientific, UK
Agarose Imaging	Gel Doc XR System, Bio Rad, USA
Autoclaves	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	AY123, Satorius, Germany
Centrifuges	Allegra X-22, Beckman, USA J2-MC Centrifuge, Beckman, USA J2-21 Centrifuge, Beckman, USA
Chemiluminescence Imaging System	Stella 2000, Raytest, Germany
CO_2 Incubator	MCO-18AC, Sanyo, Japan
CO_2 Tank	Genç Karbon, TR
Deep Freezers (-20°C)	MZAA1, Hotpoint Ariston, Italy
Deep Freezers (-80°C)	Sanyo Ultra Low, UK,Thermo Scientific, USA
Dish Washer	Mielabor G7783, Miele, Germany
Flow Cytometer	FACSCalibur, Beckman Dickinson, USA
Ice Machine	Scotsman Inc. AF20, ITALY
Inverted Microscope	Z1 Axio Observer, Zeiss, USA
Laminar Flow Cabinet	Class II B, Tezsan, TR
Magnetic Stirrer	MS-H-S, Dragonlab, China

Table 3.7. List of equipment used in this study (cont.).

Micro-centrifuge	Himac CT15RE, Hitaci Koki, Japan
Microplate Reader	680, Bio Rad, USA
Micro-Spin	Galaxy Ministar, VWR, USA
Oven	Gallenkamp 300, UK
pH Meter	Hanna, USA
Pipettes	Pipetman, Gilson, USA
Pipettor	Labopet 204, Greiner, Germany
Power Supply	VWR, USA
Refrigerator (4°C)	MFAA1, Hotpoint Ariston, Italy
Rotors	Beckman JS-7.5, USA Beckman JA-14, USA Beckman JA-20, USA
SDS-PAGE Transfer System	Cleaver Scientific, UK
Spectrophometer	NanoDrop1000, Thermo Scientific, USA
Vertical Electrophoresis System	Omni-Page Mini, Cleaver Scientific, UK
Vortex	VWR, USA
Water Bath	Memmert, Germany

4. METHODS

4.1. Molecular Cloning

4.1.1. Plasmid Preparation

Plasmid DNA was isolated according to the manufacturer's instructions of the plasmid isolation kits. For mini scale isolation, 5 ml LB medium was seeded with plasmid containing bacteria, and in the last step 50 µl elution buffer was added on the column. This scale of isolation was used for preparing the samples for sequencing. For maxi scale isolation 200 ml LB medium was seeded with plasmid containing bacteria, and 500 µl elution buffer was used for the re-suspension of the plasmid DNA. This isolation step involves the use of endo-free kit, which is important for the isolation of plasmids in transfection grade purity.

4.1.2. Restriction Enzyme Digestion of Plasmids

To insert the gene of interest into the plasmid, 8 µg of lentiviral vector KH1 was double digested with 8 U of XmaI and 8 U of XbaI restriction enzymes for 2 hours at 37^{o}C. The digestion took place in 50 µl reaction volume. NEBuffer 4 was used for the reaction at 1X final concentration. After the digestion was completed, enzymes in the solution were heat inactivated by incubating it at 65^{o}C for 20 minutes.

Similar to the KH1 digestion, for the double digestion of pLenti CMV Blast vector, 8 µg of plasmid was digested for 2 hours at 37^{o}C with 8 U of XhoI and 8 U of XbaI restriction enzymes in 1X NEBuffer 4. After the completion of digestion, reaction was heat inactivated by 65^{o}C incubation for 20 minutes.

4.1.3. PCR amplification of IRF4 with cloning primers

IRF4 open reading frame (ORF) containing pDONR221 vector was ordered from PlasmID database, Harvard (Clone ID: HsCD00040446) and used as a template in cloning experiments. To clone IRF4 ORF into another vector, primers with desired restriction sites at the primer terminals were designed (Table 3.3). By the use of these cloning primers, IRF4 ORF was PCR amplified. Phusion DNA polymerase was used in the PCR reactions and the PCR master mix for one sample was as follows: 10 µl 5X phusion buffer; 2 µl $MgCl_2$ (2,5 mM final concentration); 1 µl dNTP (200 µM final concentration); 5 µl forward primer (1 µM final concentration); 5 µl reverse primer (1 µM final concentration); 1 µl Taq DNA polymerase and 26 µl dH_2O. The PCR reaction was as: initial denaturation at 98^oC for 1 minute; 35 cycles of denaturation, annealing and elongation at 98^oC for 10 seconds, 60^oC for 30 seconds and 72^oC for 30 seconds respectively; final elongation at 72^oC for 10 minutes.

4.1.4. Agarose Gel Electrophoresis

Double digested vectors and PCR amplifications were run on 1% agarose gel, in horizontal electrophoresis tank filled with 1X TAE (Table 3.5), along with undigested and single digested vectors to check if the enzymes worked. Undigested vector is in circular shape and has a number of super coil forms which is easy to identify in an agarose gel due to altered mobility, while digested vectors are linear and travel through the gel according to its molecular size.

4.1.5. Agarose Gel Extraction and PCR Purification

In order to isolate the double digested vector from the digestion solution, entire digested vector (8 µg) was run in agarose gel and was gel extracted. Gel was cut from the borders of digested vector with a scalpel under UV light, cutting it as small as possible. Then QIAquick gel extraction kit was used according to manufacturer's instructions.

4.1.6. Oligo-Annealing

Short hairpin RNA's (shRNA's) targeting IRF4, ribosomal subunit 13 (RPS13) and Luciferase are described in and sequences are shown in Table 3.4. They were ordered HPLC purified to avoid incomplete synthesis products. shRNA's were designed as two complementary single strands (sense and antisense) with a hairpin loop in the middle, target sequences flanking the loop, terminator at the 5' end and a restriction site at the 3' start. These complementary strands need to be annealed in order to form a double strand DNA helix that can be used in a ligation reaction. To achieve this, 1 µl of each sense and antisense oligonucleotide strands were mixed in 48 µl annealing buffer. They were heated in order to break their secondary structures and then were cooled down slowly to allow the double strand formation. Heating and cooling procedure was as in following order: incubation at 90^{o}C for 4 minutes, at 70^{o}C for 10 minutes, at 37^{o}C for 10 minutes, at 10^{o}C for 10 minutes and then they were cooled down to and were kept at 4^{o}C.

4.1.7. Ligation of Inserts and Plasmids

100 ng of double digested, gel extracted vector was ligated with calculated amount of annealed shRNA oligonucleotides for 2 hours at room temperature, using 1 U T4 polymerase. The insert calculation was done by applying 1:3 molar vector to insert ratio and reaction was performed in 20 µl with a final concentration of 1X T4 ligation buffer (Table 3.5). T4 ligation buffer aliquots were prepared for single use since the ATP in the buffer is sensitive to freeze thaw cycles.

4.1.8. Transformation of Ligation Products to Competent Bacteria

Escherichia coli Stbl3 competent strain was used for transformations. Competent cells were taken from -80^{o}C freezer and thawed on ice for 10 minutes. 5 µl of ligated plasmids were added on competent cells and were incubated for 10 minutes before heat shock at 42^{o}C for 1 minute. Samples were then cooled on ice for 2 minutes, 500 µl LB

was added on tubes and they were incubated in shaker at 37^{o}C for 1 hour to give them enough time to express the antibiotic resistance gene. 250 µl of the samples were spread at ampicillin containing LB agar plates and incubated overnight. Single colonies were taken into 5 ml ampicillin containing LB medium by micropipette tip and were incubated over-night for plasmid isolation.

4.1.9. Colony PCR

Colony PCR is a validation step that can be used to detect the insert that ligated into the vector. Transformed bacteria were used as a PCR template in this experiment. Bacterial DNA was PCR amplified by using the same primers which were used in the PCR amplification of the IRF4 ORF (Table 3.3). A number of single colonies were selected for colony PCR validation, and labeled by a number on the transformation plate. PCR ingredients were calculated based on the reaction information as follows: 3 µl 10X KCl buffer; 1,2 µl $MgCl_2$ (2,5 mM final concentration); 1,6 µl dNTP (200 nM final concentration); 1,5 µl forward primer (1 µM final concentration); 1,5 µl reverse primer (1 µM final concentration); 3 µl Taq DNA polymerase and 19,2 µl dH_2O. Distilled water was added on PCR tubes and then the colonies were taken into these PCR tubes by using the tip of a micropipette tip and labelled accordingly. Tips were mixed well with the water in tubes, making all the bacteria transfer into it. A backup plate was created by pipetting 1 µl from the PCR tubes to a fresh ampicillin containing LB agar plate. Backup plate was incubated overnight at 37^{o}C, then was put in 4^{o}C for later use. To continue with the colony PCR amplification, bacteria were lysed by an incubation step at 95^{o}C for 15 minutes. After the lysis step, PCR master mix was equally distributed to the all PCR tubes and the reaction was started. It involves 5 steps as: initial denaturation at 95^{o}C for 5 minutes; 35 cycles of denaturation, annealing and elongation at 95^{o}C for 30 seconds, 58^{o}C for 45 seconds and 72^{o}C for 6 seconds, respectively; final extension at 72^{o}C for 10 minutes. PCR products were loaded on 1% agarose gel stained with EtBr and analyzed at gel documentation system.

4.1.10. Sequencing of Constructed Plasmids

Sequencing of the plasmids was done by Macrogen – (S.Korea) sanger sequencing services.

4.2. Cell Culture

4.2.1. Maintenance of the Cell Lines

All the cell lines were incubated at 37^{o}C and 5% CO_2 environment, provided by the CO_2 incubator, and were cultured in complete high glucose DMEM: 10% FBS, 100U/ml penicillin & 100µg/ml streptomycin and 1X MEM non-essential amino acids (Table 3.5). Passaging was done by first detaching the adherent cells from the flask using 0.05% trypsin-EDTA solution and incubating the flask at 37^{o}C for 3 minutes. Then the trypsin activity was inhibited by adding complete DMEM at the equal amount of the trypsin added on the cells. In order to keep the cells healthy, they were sub-cultivated at a ratio of 1:6, and the process was repeated every 3 to 4 days.

4.2.2. Transfection of HEK293FT Cells

HEK293FT cell line is widely used for lentivirus production purposes. These cells stably express the SV40 large T antigen that allows them to express the proteins of SV40 origin containing vectors in high levels. Calcium phosphate transfection, which works highly efficient on HEK293FT cells, is a method that produces insoluble precipitates of DNA and calcium phosphate. These precipitates are then engulfed by HEK293FT cells. To transfect HEK293FT cells, they were seeded into 10 cm plates at 70% confluency 1 day before transfection. On the day of transfection, choloroquine was added to the medium at a final concentration of 25µM. Plasmid solution was prepared in a 2ml tube by adding 6ug of lentiviral plasmid KH1, along with 6ug of each packaging plasmids pRSV rev, pHCMV-G and pMDL/pRRE (Dull *et al*., 1998); $CaCl_2$ at a final concentration of 125 mM; then the solution was completed to 500µl with dH_2O. Plasmid

solution was then rigorously mixed with 500µl 2X HBS by pipetting the mixture up and down harshly. The mixture was incubated for 5 minutes and then was added to the medium of previously seeded HEK293FT cells. The medium was replaced with fresh medium 6 hours after the transfection. Successful transfections were determined by inverted fluorescence microscope (Figure J.1).

4.2.3. Harvesting Lentiviruses after the Transfection

Lentiviruses produced by transfected HEK293FT cells were harvested by passing these cells' medium, which contains the lentiviruses, through a 0.45µm filter 2 days after the transfection. This filtration step separates the lentiviruses from the HEK293FT cells completely, preventing cellular contamination. Lentivirus containing supernatants (10 ml) were then split into 10 equal aliquots and were frozen at -80^{o}C for later use.

4.2.4. Lentiviral Transduction

Cells to be transduced with lentiviruses were seeded into 6-well plates 1 day before the transduction. On the day of transduction, lentivirus aliquots were thawed from -80^{o}C, and polybrene was added to a final concentration of 4µg/ml. After the virus was mixed with polybrene, medium on the cells were replaced with 1 ml of virus containing medium, then filled up to 2ml with fresh medium. Mediums on transduced cells were replaced with fresh medium 10 hours after the transduction. Transduced cells were incubated in CO_2 incubator for at least 2 days before any measurement to give them enough time to synthesize the desired products.

4.3. GFP Competition Assay

Cells were detached by using trypsin, 2 days after transduction. ¼ of the detached cells were put back into the well to continue the experiment and wells were filled up to 2ml with fresh medium. The remaining ¾ of the cells were taken into 5 ml tubes, centrifuged and re-suspended in 1 ml PBS. Tubes with samples were then analyzed at

FACSCalibur flow cytometer device. To begin with the analysis, self-fluorescence intensity of the cells was determined by using untransduced cells in CellQuestPro software. At the next step, percentage of the cells with GFP intensity that crosses self-fluorescence threshold was calculated by dividing it to all population (Figure C.1 and Figure C.2). First detections were performed 2 or 3 days after the transduction process, and then the same procedure was repeated after 2 or 3 days following the previous detection, for about 3 weeks.

4.4. Cell Cycle Profiling with PI Staining

Cells to be stained ($1x10^6$) with PI were taken into 5ml tubes and centrifuged. Supernatant was discarded and the cell pellet was washed with 2 ml PBS then centrifuged again. Supernatant was discarded again and cells were fixed by adding 1ml of 70% ice cold EtOH slowly on the pellet while vortexing to avoid cell clumping. Cells were incubated on ice for 30 minutes then were centrifuged to get rid of the EtOH. Cell pellet was washed with PBS and centrifuged. Supernatant was discarded and cell pellet was re-suspended in 1ml of PI staining solution. Cells were incubated at 37^oC for 40 minutes in PI staining solution, then was centrifuged again to remove the staining solution. Cell pellet was washed with PBS and centrifuged. Supernatant was discarded and the cell pellet was re-suspended in 500µl PBS then analyzed at FACSCalibur flow cytometer. All the centrifugations were carried out at 200 x g for 5 minutes. Analysis was done with the CellQuestPro software as explained in Figure H.1.

4.5. Western Blotting

4.5.1. Sample Preparation

Cell lysates were collected first by removing the medium over the cell monolayer in 6-well plates and the cells were detached using a cell scraper after adding 1ml PBS. Cell suspension was taken into 1.5ml tube and centrifuged at 3000 rpm for 5 minutes. Supernatant was discarded and cell pellet re-suspended in 1ml of cold RIPA buffer

(Table 3.5) with freshly added protease inhibitors. The tube was incubated on ice, vortexed every 10 minutes, and centrifuged at 14,000 x g for 15 minutes at 4^{o}C after 30 minutes of incubation. The total protein lysate, which was in the supernatant, was transferred into a new 1.5 ml tube and stored at -80^{o}C freezer.

4.5.2. SDS-PAGE Gel Preparation

10ml SDS-PAGE gel (Table 3.5) was prepared in a beaker and poured between the glasses which were placed in the gel casting system. Comb was inserted on the gel and shaken if necessary to get rid of any bubbles that could have been stuck between the gel and the comb. After the polymerization of the gel (approximately 30 minutes), comb was removed and the gel was moisten with dH2O and stored in 4°C for later use or was put in the vertical electrophoresis system which was filled with 1X running buffer (Table 3.5).

4.5.3. Protein Gel Electrophoresis

Proteins were taken from freezer and then necessary volume of thawed protein equivalent to 15 µg of protein was mixed with 4X laemmli buffer (Table 3.5). Protein samples were then incubated at 95°C for 5 minutes. After the incubation samples were taken into an ice tray until they were loaded in the gel. Prepared gel was placed in the vertical electrophoresis system which was filled with 1X running buffer, then the protein ladder and the samples were loaded using a 10 µl micropipette. Gel was run at 150 V until the loading dyes reach the end of the gel (approximately 2 hours).

4.5.4. Transfer of Proteins to PVDF Membrane

Proteins that run in the gel were transferred into a PVDF membrane by wet transfer method as follows: The run gel was put into 1X transfer buffer (Table 3.5) for 5 minutes. In the meantime, PVDF membrane was cut at the size of the gel then it was first incubated in 100% methanol for 10 seconds, then in dH_2O for 1 minute, then in

transfer buffer for 5 minutes. Preparation of the gel and the membrane to the transfer was as follows; one pressure pad, one filter paper, membrane, gel, one filter paper and one pressure pad was put on each other respectively and the system cassette was closed. Any bubbles left between the sandwich layers were removed by rolling a 15 ml tube over the layers. Then with correct orientation (proteins run towards the positive pole), the sandwich was put in the transfer tank which was filled with 1X transfer buffer and was run at 250 mA for 1 hour and 45 minutes.

4.5.5. Antibody Incubation

After the transfer, membrane was taken into a case filled with 1X TBST, then was incubated in fresh 5% blocking solution (Table 3.5) for 1 hour at room temperature. The primary antibody was diluted in 5% blocking solution as recommended in its datasheet and the membrane was incubated with it over-night at 4^{o}C. The membrane was washed three times with TBST and was then incubated in horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBST as recommended in it datasheet) for 1 hour at room temperature.

4.5.6. Chemiluminescence Imaging of the Membrane

By the end of the secondary antibody incubation, the membrane was washed three times with TBST. In the meantime, enhanced chemiluminescent solution (ECL) was prepared by mixing 1 volume of peroxide and 1 volume of substrate solutions at a final volume depending on the size of the membrane (approximately 1ml for a standard membrane). Membrane was placed under the imaging system and then ECL was poured on the membrane in a drop wise manner, while paying attention to cover it completely. By using the Xstella software, the membrane was then exposed and observed with Stella chemiluminescence imaging system.

4.6. RT-qPCR

4.6.1. RNA Isolation from Cell Lines

$1x10^6$ cells were collected from 6-well plates by detaching them using trypsin. Cells were centrifuged, supernatant was discarded and the pellet was re-suspended in 200 µl 1X PBS (Table 3.5).To lyse the cells and isolate total RNA from them, Nucleo-Spin RNA kit was used and the manufacturer's instructions were followed. Freshly isolated RNA's were stored at -80°C freezer.

4.6.2. cDNA Synthesis (reverse transcription) from RNA Samples

Total RNA samples, isolated from the cells, were used as a template for cDNA synthesis. RevertAid First Strand cDNA Synthesis kit was used and the manufacturer's instructions were followed. 1 µg of RNA and oligo d(T) primer was used at the beginning of the reaction. Oligo d(T) binds to the poly(A) tails of the mRNA's and starts their reverse transcription in the presence of reverse transcriptase, resulting in the production of the total cDNA. After the cDNA synthesis was complete, 20 µl reaction volume was increased to 200 µl by adding nuclease free dH_2O, it was then further diluted 1:50 in dH_2O.

4.6.3. Real Time PCR (qPCR) Procedure

In order to compare the gene expression level differences between the cell lines, mRNA levels of IRF4 was measured by real time PCR. TaqMan Gene Expression Master Mix was used according to the manufacturer's instructions. One typical reaction includes 5 µl 2X master mix, 0.5 µl primer/probe mix, 2 µl cDNA (1:50 diluted) and 2.5 µl dH_2O. Reaction was run using PikoReal 96 real time PCR system. Steps of PCR reaction was as follows: initial denaturation step first at 50°C for 2 minutes then at 95°C for 10 minutes, then 40 identical cycles of denaturation at 95°C for 15 seconds followed by annealing and elongation step at 60°C for 1 minute. After every cycle, fluorescence data

was acquired by real time PCR system. Analysis of the raw data was done by ΔΔct method, by basicly calculating the median of the replicates (for both IRF4 mRNA and GAPDH mRNA), then exponentiation was done by taking number 2 as base and using the calculated median as exponent. This calculation for IRF4 mRNA was then divided to the GAPDH mRNA calculation.

4.7. Analysis of Gene Expression Databases

4.7.1. Oncomine Database

http://www.oncomine.com website (Rhodes *et al*., 2004) offers free online access to the database to the ones that have non-profit interest. We signed up using the Bogazici University provided e-mail domain and logged in to the system. From the search bar at the top left of the screen, we set the search parameter as IRF4. This parameter brought us disease summary for IRF4 (Figure 5.1). From the filters we added cancer vs. cancer analysis and selected individual studies for the analysis from the datasets that includes melanoma data in them (Figure B.1).

4.7.2. Cancer Cell Line Encyclopedia

http://www.broadinstitute.org/ccle/home website (Barretina *et al*., 2012) provides open access to everyone with internet connection. From the search bar at the top right of the screen, we set IRF4 as search parameter. In the next page we selected IRF4 which is shown under gene symbol title. That link provided us the gene summary view of IRF4 with nearly one thousand cell lines used in (Figure 5.2).

5. RESULTS

5.1. Determination of IRF4 Levels for Melanoma Cell Lines through Publicly Available Gene Expression Databases

There are several online databases where mRNA expression profiles for cancer cell lines are available to public. We have signed up to these databases to investigate the IRF4 expression level in cancer types.

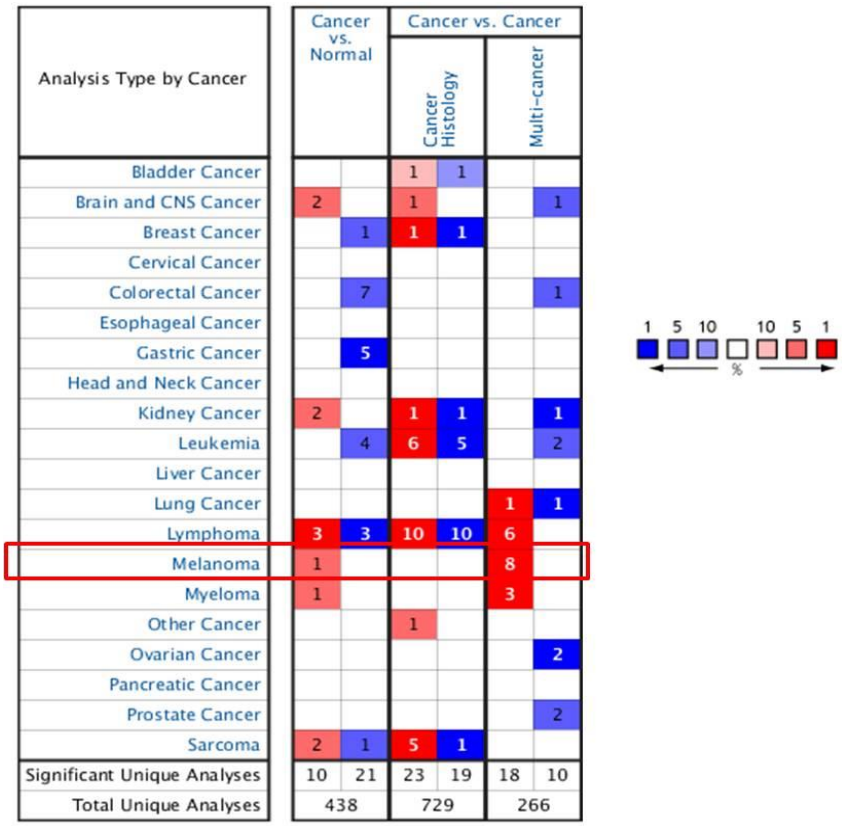


Figure 5.1. IRF4 mRNA levels in oncomine database. Disease summary for IRF4 is shown in the figure. Numbers indicate significant analysis counts and colors indicate gene rank percentile. Multiple-cancer title refer to the comparative analysis of gene expression data of multiple cancer types. Red box highlight melanoma.

We evaluated the available data in two of these databases: oncomine (Rhodes *et al*., 2004) and Broad institute cancer cell line encyclopedia (CCLE) (Barretina *et al*., 2012). In Figure 5.1, IRF4 gene expression rank was in top 5% for 9 melanoma samples, with high significance. By analyzing CCLE database and individual studies in oncomine, we found that IRF4 expression is slightly lower than B-cell origin cancer cell lines but remarkably higher than any other cancer cell lines, which is consistent with previous studies (Figure 5.2, Figure B.1).

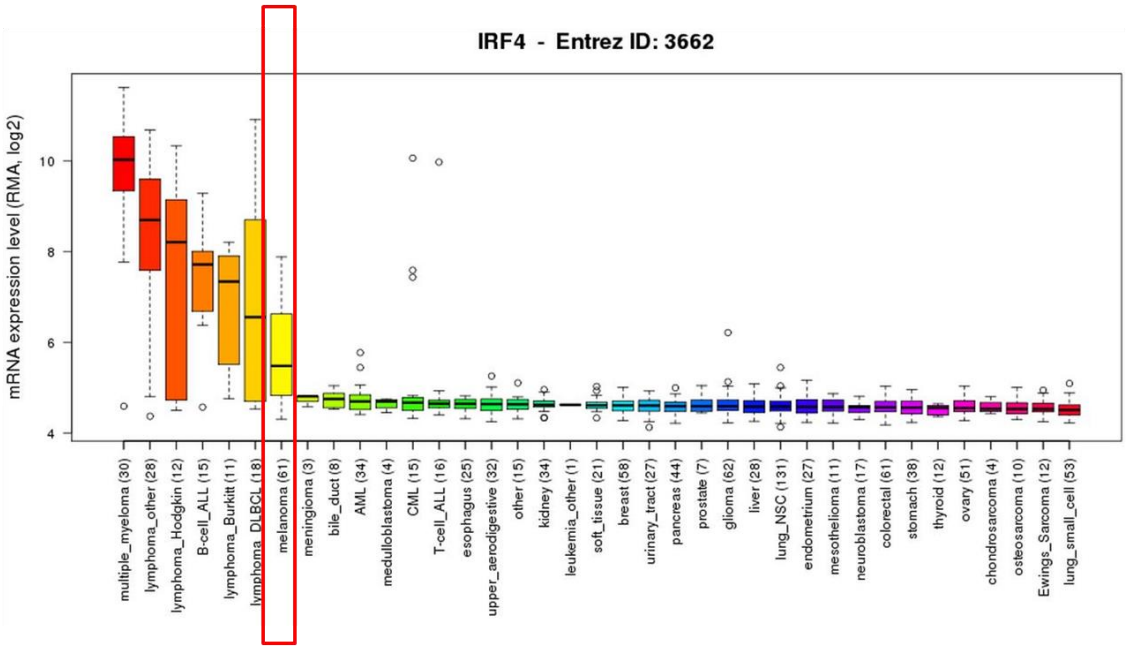


Figure 5.2. IRF4 mRNA levels in cancer cell line encyclopedia. Box plot of nearly one thousand cancer types were arranged from the highest IRF4 expression to the lowest. Numbers in brackets indicate sample numbers. Red box highlight melanoma. RMA mean robust multi-array average.

5.2. Determination of IRF4 Expression in Melanoma Cells *in vitro*

Database analysis and previous studies show that most melanoma cell lines express high levels of IRF4 mRNA. To replicate the results *in vitro*, we obtained various melanoma cell lines for experiments and breast cancer cell lines for use as negative con-

trols. We collected whole cell protein lysates and total RNA extracts from the cell lines we obtained. IRF4 levels both in mRNA and protein level were analyzed using these samples.

5.2.1. qPCR Analysis of Melanoma and Breast Cancer Cell Lines

To determine the IRF4 mRNA levels of the cell lines, we first collected total RNA extracts from them, then synthesized cDNA's and analyzed them using qPCR with an IRF4 primer/probe mix. Melanoma cell lines A-2058, SKMEL-5, SKMEL-28 and G-361 and Malme-3M had amplification while melanoma cell lines MeWo, MEL-9 and MEL-10 and, breast cancer cell line MCF-7 had no detectable amplification. (Figure 5.3).

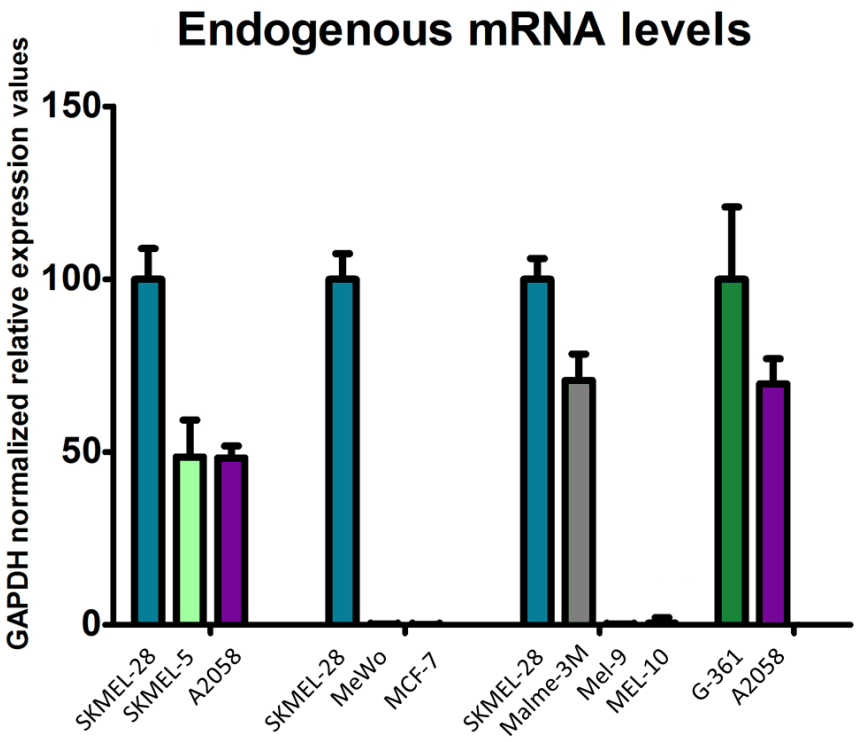


Figure 5.3. IRF4 mRNA levels of indicated cell lines. Each group of column represent an individual qPCR result. GAPDH normalized expression values were normalized to the first cell line in the group. Error bars represent the SEM of replicates.

5.2.2. Western Blot Analysis of Melanoma and Breast Cancer Cell Lines

For the determination of IRF4 protein levels in the cell lines, we collected whole cell lysates from them. Western blot analysis of this collected lysates showed that endogenous IRF4 protein levels were high in melanoma cell lines G-361, A-2058, SKMEL-5, SKMEL-28 and Malme-3M, while melanoma cell lines MeWo, MEL-9 and MEL-10, and breast cancer cell lines MCF-7 and MDA-MB-231 had no detectable IRF4 protein (Figure 5.4). These results are similar with the qPCR (Figure 5.3) and database results (Figure 5.1 and Figure 5.2), and previous studies.

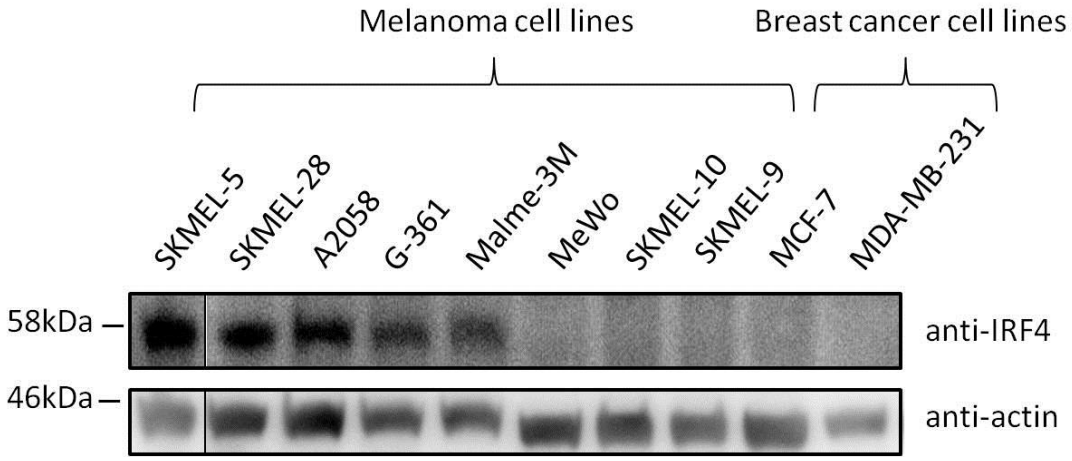


Figure 5.4. Western blot analysis of indicated cell lines.

5.3. Knock-down of IRF4 in Melanoma Cells Using Short Hairpin RNA (shRNA)

It is evident that IRF4 levels are remarkably high in many melanoma cell lines. To investigate the importance of its expression, we decided to knock-down IRF4 in melanoma cell lines by using a short hairpin RNA (shRNA). For this purpose, we constructed lentiviral vectors containing shRNA which targets IRF4 mRNA (shIRF4). Each cell line subject to the test were infected with lentiviruses and had shIRF4 integrated into their genomes in the result of this process. We then checked IRF4 mRNA and protein levels of these cell lines to confirm the decrease in IRF4 levels.

5.3.1. Construction of shRNA Expressing Lentiviral Vector KH1

KH1 is a 3rd generation lentiviral vector that contains GFP as a marker and H1 promoter for the recruitment of RNA polymerase II, which is responsible for the synthesis of short RNA's. From previous studies in our department (Sumer, 2009) we know that KH1 originated lentiviruses have high transduction efficiency on melanoma cell lines. Cloning strategy and insert sequences are described in Section 4.1. Restriction enzyme digested vector photographs, before and after the gel extraction, are shown in Figure 5.5.

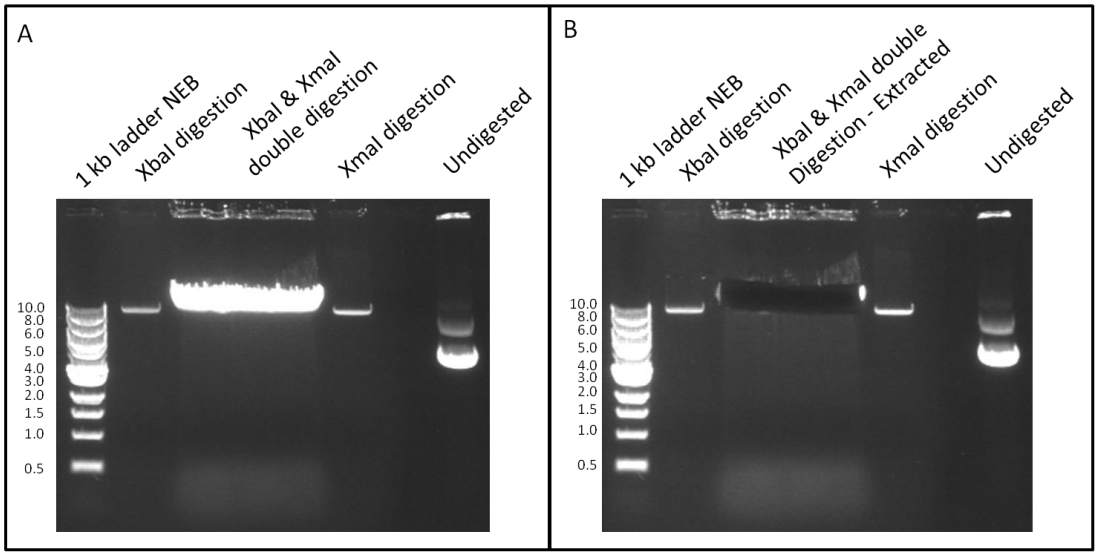


Figure 5.5. Agarose gel photographs of digested KH1 vector. A) Before the gel extraction. B) After the gel extraction of the digested vector.

We confirmed the successful cloning results by sequencing (Figure 5.6). All the sequencing reactions were performed by Macrogen – S.Korea sanger sequencing services.

Figure 5.6. Sequencing result of shIRF4b cloning. This result is used as a representative, all the cloning results were clear and correct. Red box indicates the cloned sequence, shIRF4b.

5.3.2. qPCR Analysis of shIRF4 Transduced Cells

shRNA's find their target mRNA by making nucleotide match in the cells and perfect match leads to mRNA degradation. Thus, introducing shIRF4 in IRF4 positive cell lines should result in a decrease in IRF4 mRNA levels. To determine this decrease, we first introduced stably shRNA-expressing constructs to the cell lines by using lentiviral transduction method. One of these constructs target IRF4 mRNA and the other one targets firefly luciferase gene (shLuc), which was used as a negative control. We then collected total RNA extracts from transduced cells and synthesized cDNA from them. Finally, we analyzed these cDNA's using qPCR. We had IRF4 knockdown efficiency ranging from 40% to 80% (Figure 5.7).

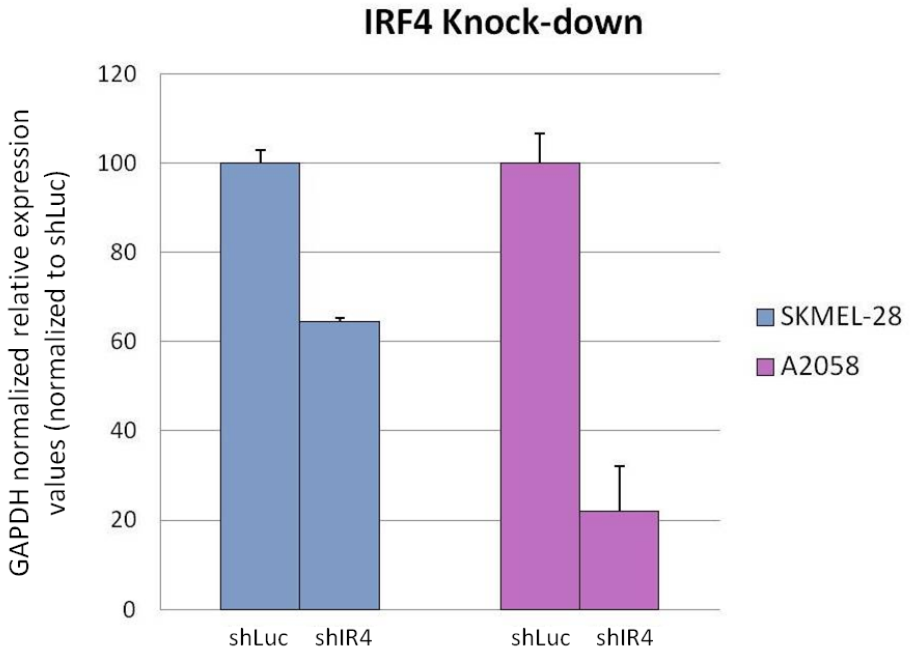


Figure 5.7. Detection of the decrease in IRF4 mRNA levels by qPCR. Cell lysates were collected 5 days after the transduction. Error bars represent SEM of replicates.

5.3.3. Western Blot Analysis of shIRF4 Transduced Cells

To investigate the viability effects of IRF4 on melanoma cell lines, we need to knock-down IRF4 protein. So far we have showed that the knock-down system which we are using decreases IRF4 mRNA levels in *vitro* (Figure 5.7). This mRNA decrease should also lead to the decrease of IRF4 protein levels in these cells. To determine this decrease we collected whole protein lysates from separate empty vector, shIRF4, shLuc introduced and untransduced cell lines. By western blot analysis of these lysates, we showed that IRF4 protein level decreases with the introduction of shIRF4 to the cells.

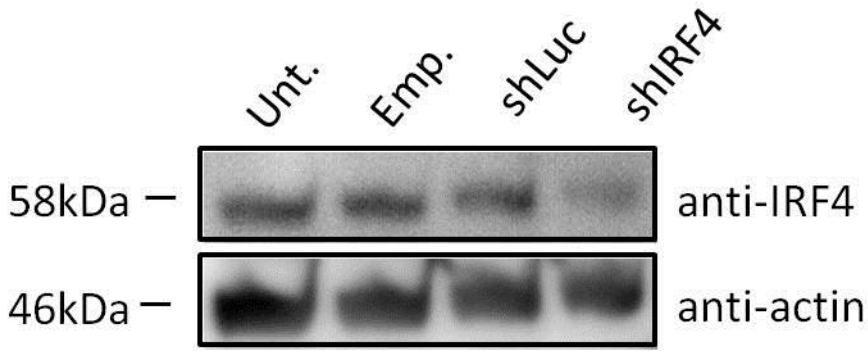


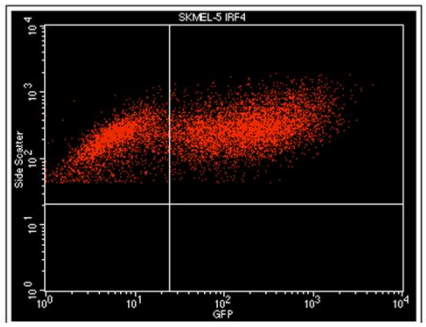
Figure 5.8. Determination of IRF4 knock-down on protein level by western blot analysis. SKMEL-28 cell lines was used. Unt. represent untransduced, emp. represent empty vector transduction.

5.4. Detection of the Viability Effects of IRF4 Knock-down in Melanoma Cell Lines

After the knock-down of IRF4 in melanoma cell lines, we wanted to investigate the changes in their viability. From previous studies (Shaffer *et al*., 2008; Yang *et al*., 2012) we know that even a 50% decrease in IRF4 mRNA level is sufficient to kill ABC-DLBCL and MM cell lines. Although it is effective, this partial knock-down of IRF4 causes the viability change in cells to be observed in 2 to 3 weeks time. In this regard, we decided to use an assay termed 'GFP competition assay', which was also used in the previous studies, where the transduced and un-transduced cells share the same tissue culture plate.

To perform the GFP competition assay, we infected the cell lines with shRNA containing lentiviruses with an estimated efficiency of 50% which means the infection of the half of the population in a tissue culture plate. With half of the population infected, only half of the cells will be able to produce shRNA along with the GFP marker. We observed the change in GFP percentage in the population over time and used it as a marker for shRNA's effect on cells' competitive fitness (Figure C.2).

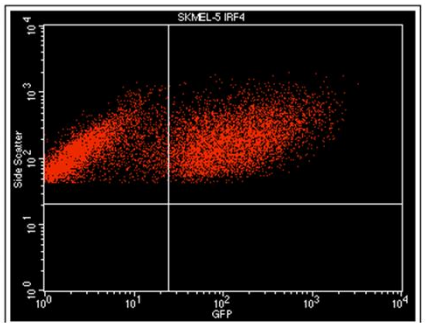
We first separately infected the cell lines with a positive control shRNA and a negative control empty vector. shRPS13, which targets an essential ribosomal subunit, was used as a positive control for the experiments and achieved its goal by diminishing the GFP positive population, which means shRPS13 expressing cells rather died or had cell cycle arrest. As expected, negative control empty vector transductions were un-effective in the cells' competitive fitness (Figure E.01). These results suggest that GFP competition assay is a reliable tool in terms of determining viability changes.



Quadrant Statistics

File: SKMEL-5 IRF4 Acquisition Date: 14-Nov-12
Gate: G1 X Parameter: GFP (Log)
Y Parameter: Side Scatter (Log)

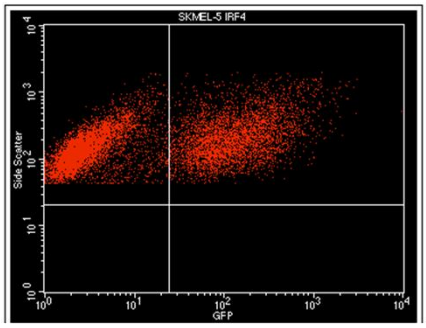
Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	3536	35.35	8.83	7.60	6.24	217.05	186.53
UR	6467	64.65	16.15	296.60	184.72	336.47	285.08
LL	0	0.00	0.00	***	***	***	***
LR	0	0.00	0.00	***	***	***	***



Quadrant Statistics

File: SKMEL-5 IRF4 Acquisition Date: 20-Nov-12
Gate: G1 X Parameter: GFP (Log)
Y Parameter: Side Scatter (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	5278	48.10	9.94	4.33	2.96	153.45	122.77
UR	5694	51.90	10.72	215.56	134.04	243.25	187.95
LL	0	0.00	0.00	***	***	***	***
LR	0	0.00	0.00	***	***	***	***



Quadrant Statistics

File: SKMEL-5 IRF4 Acquisition Date: 29-Nov-12
Gate: G1 X Parameter: GFP (Log)
Y Parameter: Side Scatter (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	5828	57.55	16.30	3.99	3.08	166.64	135.42
UR	4298	42.45	12.02	201.67	131.16	280.27	210.31
LL	0	0.00	0.00	***	***	***	***
LR	0	0.00	0.00	***	***	***	***

Figure 5.9. GFP percentage change in time of shIRF4 introduced cells analyzed by flow cytometer. SKMEL-5 cell line is used as a representative, similar observations were made with other melanoma cell lines. Results of day 3, 10 and 18 are shown In quadrant statistics; red boxes highlight GFP percentage of the population. UL represent upper left, UR upper right.

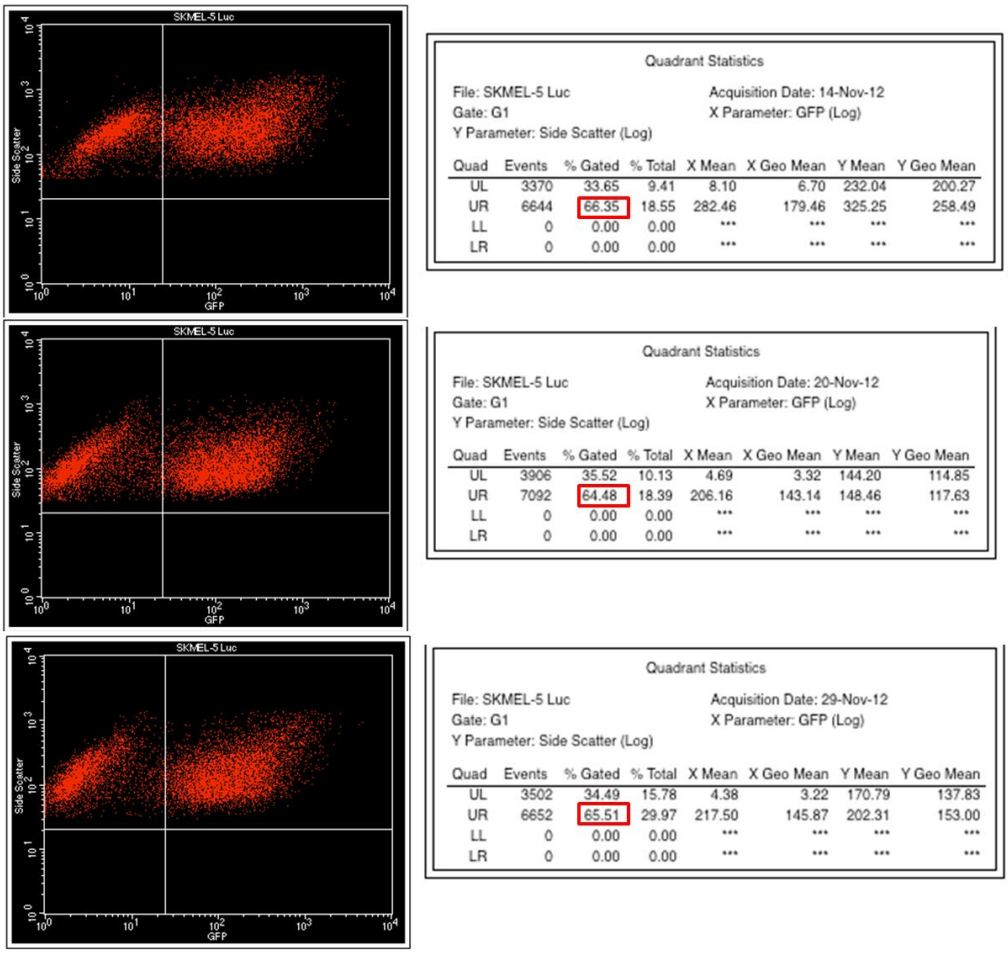


Figure 5.10. GFP percentage change in time for shLuc introduced cells analyzed by flow cytometer. SKMEL-5 cell line is used as a representative, similar observations were made with other melanoma cell lines. Results of day 3, 10 and 18 are shown. In quadrant statistics; red boxes highlights GFP percentage of the population. UL represent upper left, UR upper right.

In order to investigate the effect of IRF4 knock-down on cells' viability, we separately introduced shIRF4 and shLuc to cell lines and analyzed them with GFP competition assay (Figure D.1). Here we showed that IRF4 positive cell lines had a decrease in the GFP percentage when we introduced shIRF4 to them (Figure 5.9), but not when we introduced shLuc (Figure 5.10). Furthermore, IRF4 negative control cell lines had no change in the GFP percentage with either of the shRNA's. Flow cytometer results of the all cell lines and time points are shown in Figure 5.11 in a line graph, and the significance of the final results are shown in Figure F.1.

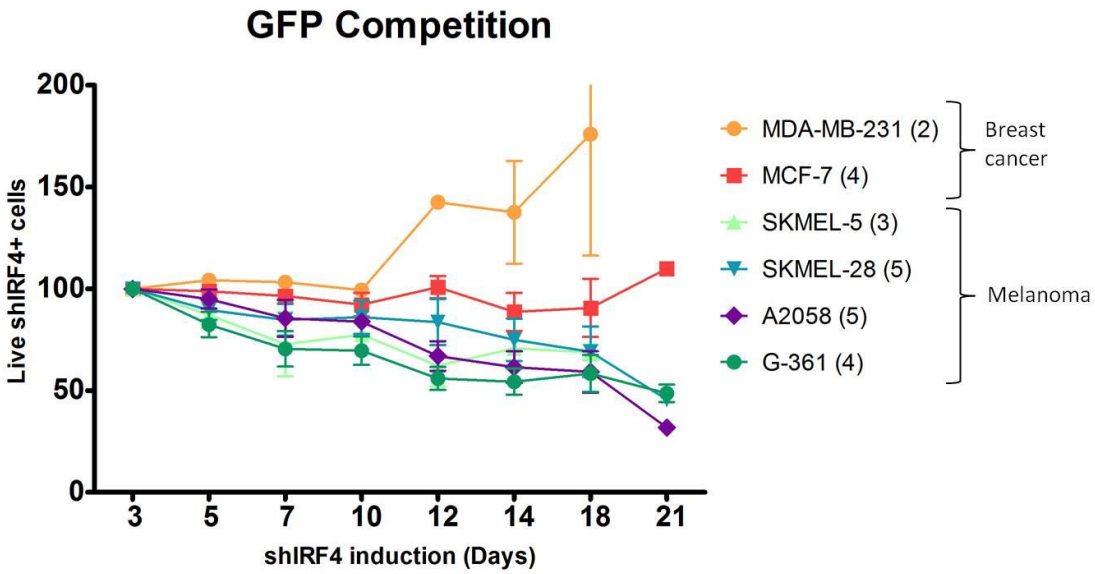


Figure 5.11. Determination of reduced competitive fitness in shIRF4 introduced cell lines. All time points were normalized to day 3 and shLuc transduction results. Numbers in brackets indicate the number of independent infection replicates. Error bars represent SEM of replicates.

5.5. Over-expression of IRF4 in Cell Lines

Knock-down of IRF4 in melanoma cell lines showed that these cell lines are addicted to its expression. However, shIRF4b that we introduce into the cells might have effects on mRNA's other than IRF4 mRNA, which is defined as off-target effect. We used the same shIRF4 sequence which was used in previous B-cell studies with known no off-target effects (Shaffer *et al*., 2008), but we still need to show it in melanoma cell lines.

In order to eliminate the possibility of off-target effect related results in melanomas, we designed rescue experiment. This experiment is based on the integration of IRF4 open reading frame (ORF) into the cell lines, creating a stably IRF4 ORF expressing cells, and transducing them again with KH1-shIRF4b originated lentiviruses. shIRF4b targets 3'UTR of the IRF4 mRNA, thus it can not target the mRNA transcribed

from the IRF4 ORF. If the cells with reduced competitive fitness upon IRF4 down-regulation become unaffected with IRF4 ORF integration in them, this means IRF4 is the only factor responsible for the changes in cells' fitness.

5.5.1. Cloning of IRF4 ORF into Lentiviral Vector pLenti CMV Blast

pLenti CMV Blast is a 3rd generation lentiviral vector with a CMV promoter before the multiple cloning site, and a blasticidin (mammalian selection) antibiotic resistance gene. We cloned IRF4 open reading frame (ORF) into the multiple cloning site of this vector. Cloning strategy is described in 4.1. We run the PCR amplification of IRF4 insert with molecular cloning primers (Table 3.3) and double digested pLenti CMV Blast vector in 1% agarose gel and extracted them separately (Figure 5.12).

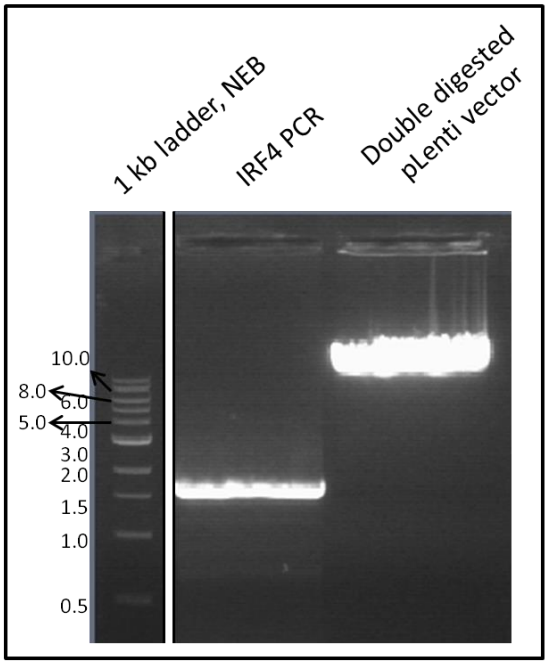


Figure 5.12. Gel extraction of PCR product and digested vector.

Before sequence verification, we did colony PCR analysis on bacterial colonies which were transformed with cloned vector. Colony PCR results showed that we created IRF4 ORF containing vector (Figure 5.13). For further validation, we sent colonies from

1 to 4 for sequencing to Macrogen – S.Korea sanger sequencing services and confirmed the successful cloning of IRF4 ORF into pLenti CMV Blast vector.

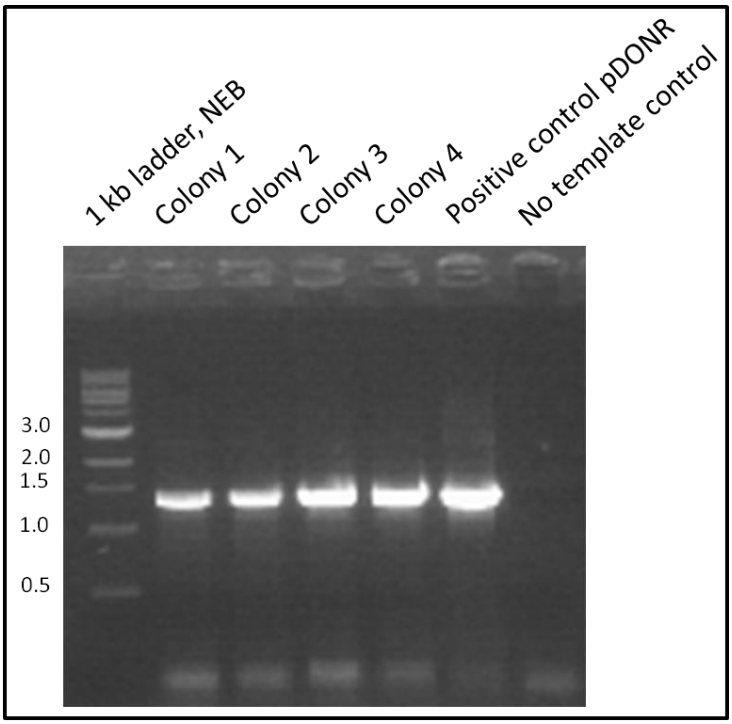


Figure 5.13. Colony PCR results of 4 different colonies along with positive and negative controls.

5.5.2. Antibiotic Selection of pLenti CMV Blast Transduced Cells

Blasticidin is an antibiotic with translational inhibition function in eukaryotic cells. It causes rapid cell death even at low antibiotic concentrations. pLenti CMV Blast lentiviral vector include *bsd* gene which grants blasticidin resistance to the integrated cells, and we used it for selection. We treated the transduced and untransduced cells with blasticidin antibiotic at a final concentration of 10 µg/ml for 2 weeks. By this treatment process with blasticidin antibiotic, we successfully selected resistant colonies in the tissue culture plates. All the untransduced cells died with the treatment (Figure 5.14A), while pLenti transduced cells had resistant colonies (Figure 5.14B). We had the same

result for SKMEL-28, G-361, A-2058, SKMEL-5, MeWo and MDA-MB-231 cell lines for two different pLenti transductions: one with the empty vector and one with the IRF4 ORF cloned vector.

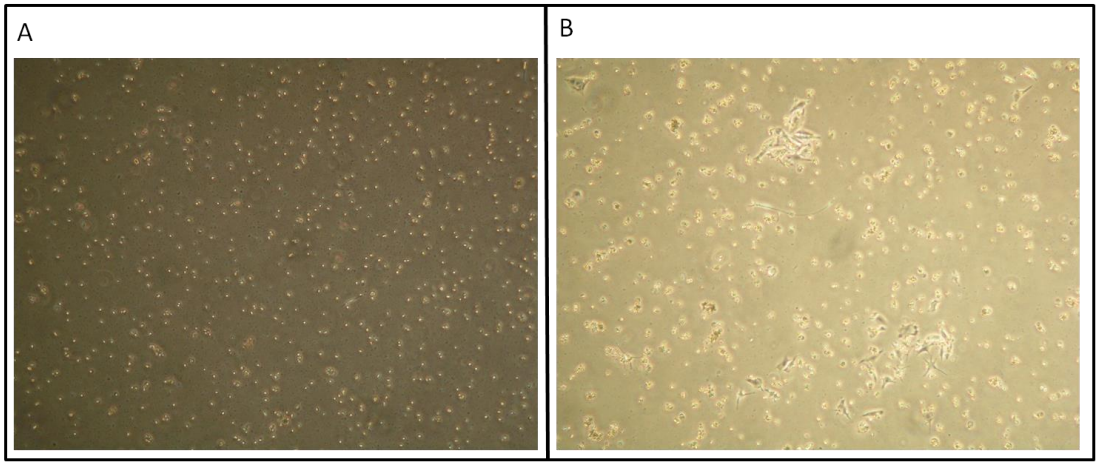


Figure 5.14. Antibiotic selection of pLenti transduced cells. SKMEL-28 cell line photographs are used as a representative, similar results are observed in all cell lines. A) Untransduced cells. B) pLenti transduced cells.

5.5.3. Verification of IRF4 Over-expression

After the selection of transduced cells with blasticidin antibiotic, we grew them in tissue culture plates until their number increased to a level enough to both maintain a healthy culture and extract lysates from them. We extracted total RNA lysates from cell lines transduced with (IRF4 ORF cloned pLenti CMV Blast vector originated) lentiviruses and analyzed them by RT-qPCR. We observed a remarkable increase in IRF4 mRNA levels of all transduced cell lines except G-361 cell line, which has a slight increase (Figure 5.15).

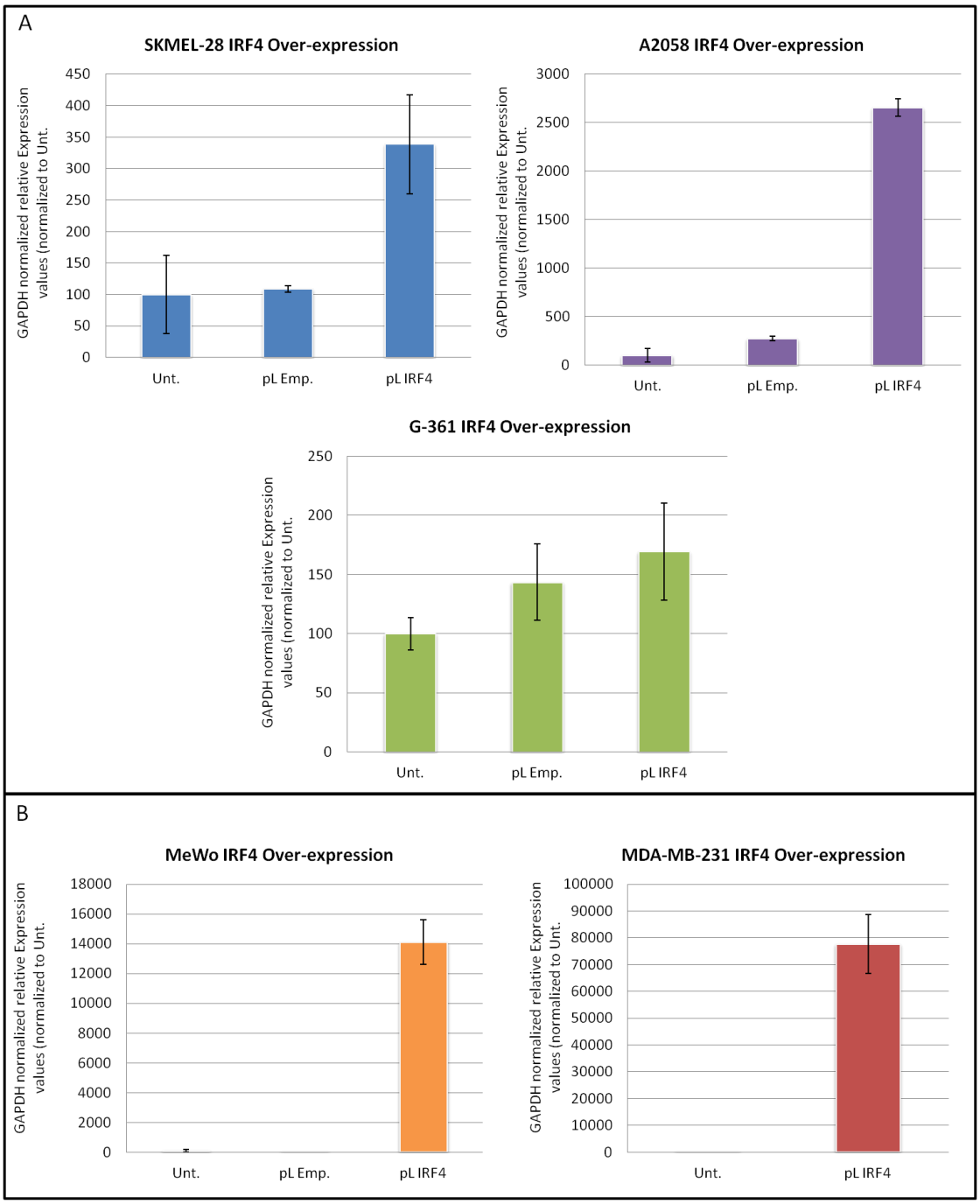


Figure 5.15. Determination of IRF4 mRNA in IRF4 ORF integrated cell lines. Over-expression results in cell lines: A) with endogenous IRF4 expression, B) with no detectable endogenous IRF4 expression. Unt. refer to untransduced, pL Emp. refer to pLenti empty vector transductions; pL IRF4 refer to IRF4 ORF cloned pLenti vector transductions. Error bars represent SEM of replicates.

6. DISCUSSION

IRF4 is highly expressed in lymphocytes and plays a key role in lymphocyte development. Alongside its normal functional roles, it is associated with specific hematopoietic cancer cases and has potential oncogene-like functions (Shaffer *et al*., 2008; Yang *et al*., 2012). Regarding the remarkable IRF4 expression in melanomas (Grossman *et al*., 1996; Sundram *et al*., 2003), IRF4 might be contributing to the melanoma progression. Supporting this suggestion, some SNP's in IRF4 locus were found to be associated with melanoma predisposition through GWAS studies (Duffy *et al*., 2010; Han *et al*., 2011). Although association of IRF4 with melanoma is somewhat identified, its functions are yet to be elucidated.

As a preliminary study, we first evaluated the IRF4 expression of cancer samples on online databases (oncomine.org: Rhodes *et al*., 2010 and Broad-Novartis cancer cell line encyclopedia: Barretina *et al*., 2012) and successfully came up with the data which correlates with the previous studies, showing high level IR4 expression in melanoma and lymphoid cancers compared to the other cancer types (Figure 5.1, Figure 5.2).

We obtained melanoma cell lines to conduct *in vitro* studies. Before going on with the experiments, we wanted to validate the IRF4 expression profiles of the melanoma cell lines in hand. Determination of IRF4 expression both in mRNA and protein level for the melanoma cell lines were performed by reverse transcriptase quantitative PCR (RT-qPCR) and western blotting, respectively. All the expression results were positively correlated with the current databases and previous studies; we showed that many melanoma cell lines that we tested have high IRF4 expression, while breast cancer cell lines, which were used as negative controls, had no detectable expression (Figure 5.3, Figure 5.4).

To investigate the role of IRF4 in melanoma cell lines, we wanted to decrease the intracellular level of IRF4 in melanoma cell lines and observe the changes in these cells

phenotype. To achieve this decrease, we decided to use a genetic tool that efficiently works on melanoma cell lines, which is lentiviral transduction, since the transfection method is not very efficient on melanoma cell lines along with its limitations in the use of long term experiments. For this purpose, we constructed lentiviral vectors, capable of integrating an shRNA into the cells genome and knocking-down the targeted gene. Transductions were tracked with a GFP marker which is present in the vector and integrated into the genome along with the shRNA (Table 3.4).

We successfully manipulated the melanoma cell lines and the negative control breast cancer cell lines with the shIRF4-introducing lentiviral system. As a control, we also included a non-targeting shRNA against the firefly luciferase gene, and a no shRNA containing transduction groups for all the tests. These controls are to assess the possibility that the observed phenotype is whether caused by the artificial shRNA production and/or transduction procedure or not. IRF4 knock-down efficiency was determined by both RT-qPCR and western blotting. We observed 40% to 80% decrease in IRF4 mRNA levels (Figure 5.7) and a similar effect in protein levels (Figure 5.8) after the knock-down process, while the control transductions did not have an effect on IRF4 levels which is similar to the previous studies (Shaffer *et al*., 2008; Yang *et al*., 2012).

Finally, we wanted to test the effects of IRF4 knock-down on the viability of the melanoma cell lines. From previous B-cell origin cancer studies, we know that MM and ABC-DLBCL cell lines are addicted to IRF4 expression and even a 50% decrease in the IRF4 mRNA level leads to the death of the cells (Shaffer *et al*., 2008; Yang *et al*., 2012). However, this cell death is not a rapid cell death. It is a prolonged effect that takes weeks to observe in cell culture. In this respect, an assay termed “GFP competition assay” is well suited for the monitoring of the competitive fitness.

GFP competition assay makes use of the transduction heterogeneity of the cell population in a single tissue culture plate. This heterogeneity was accomplished by aiming 50% transduction efficiency. Flow cytometer analysis of the population gave us the exact percentage of GFP (shRNA) expressing cells in them. After two weeks of analysis of the shIRF4 introduced cell lines, we observed a gradual and significant decrease in

the GFP percentage of the IRF4 positive melanoma cell line populations (Figure 5.11, Figure F.1). When control results are also taken into account, which did not have a significant change in the GFP percentage, it is safe to say that the reduction in the competitive fitness is triggered by IRF4 knock-down. In the light of previous association and expression studies combined with our results, we suggest that melanoma cell lines have non-oncogene addiction to IRF4, and reduced competitive fitness effect is similar to the observations in MM and ABC-DLBCL cell lines.

Although we are using the same shIRF4 sequence which was used in previous B-cell studies with known no off-target effects (Shaffer *et al*., 2008), we have to demonstrate that it also does not have off target effects in melanoma. To eliminate this possibility that can be the cause of the reduced competitive fitness, we are working on rescue experiments. In this direction, we constructed another lentiviral vector that integrates IRF4 open reading frame (ORF) into the target cell genome. IRF4 ORF is not targetable by the shIRF4 that we are using, which means melanoma cells do not get IRF4 knock-down after the lentiviral shRNA transduction. We successfully created IRF4 over-expressing melanoma cell lines using this vector. Although the increase in protein levels are yet to be determined, we showed elevated IRF4 mRNA levels (Figure 5.15). In the future experiments, we will repeat the GFP competition assay including these cells in the experiment groups.

There is still much unknown about IRF4's contribution to melanoma. First question to be answered is the mechanism of reduced fitness, whether the cells are dying or subject to an arrest at a cell cycle checkpoint. Type of cell death is also another question to be answered if it is the issue. If the cells are dying, defining the cell death mechanism will give some insight about the pathways that IRF4 may be important in melanoma cells. Even if reduced competitive fitness is a finding that leads us to study viability related effects, there are many other hallmarks of cancer that IRF4 may be playing role in. Changes in anchorage independency and metastasis and invasion potential are in the scope of this project and some preliminary studies regarding the investigation of some of these hallmarks have already been done, as described below.

An assay named ‘wound healing assay’ measures the migration potential of cell lines cultured *in vitro*. By scratching a confluent tissue culture plate with a micropipette tip we created an artificial wound in cell monolayer. If the cells have migration capacity, they migrate through the wound and resemble a healing effect. We observed their migration potential by measuring the percentage of the healed wound by taking the pictures of the wound after 4 and 8 hours, posterior to wounding. We did not detect a difference between the shIRF4 introduced and control samples, but the experiment needs to be replicated to ensure the results are significant (Figure G.3).

Another assay we performed as a preliminary study involves the detection of the cell cycle profile of the cell lines, using propidium iodide (PI) staining. We treated shIRF4 introduced and control transduction cells with PI staining and checked their cell cycle profiles. A2058 and SKMEL-28 cell lines had an increase in the G2/M phase when shIRF4 was introduced, while neither the control transductions nor the negative control cell line MeWo had a cell cycle profile change (Figure H.2, Figure H.3). Although this analysis looks promising, it needs to be replicated to provide its significance.

Finally, we also worked on the improvement of the knock-down system, trying to increase the knock-down efficiency. In this respect, we designed and ordered 4 different shRNA’s that target different sequences on IRF4 mRNA. As we did before with the present shIRF4, we also cloned these 4 new shRNA’s into the KH1 plasmid and used it to produce lentiviruses. By using these lentiviruses we successfully introduced the shRNA’s into the target cells’ genome and knocked IRF4 down. RT-qPCR analysis of these cell lines showed that we could not achieve higher knock-down efficiency with this method (Figure I.1). Right now, we are working on the cloning of the current shIRF4 to a miR30 backbone, which is described as a more efficient way of delivering shRNA’s (Stegmeier *et al*., 2005).

To sum up, we suggest that IRF4 positive melanoma cells have non-oncogene addiction to IRF4 and their competitive fitness decreases if IRF4 is knock-down *in vitro* in melanoma cell lines (Figure 5.11). The reason behind this reduced competitive fitness might be due to a cell cycle arrest at G2/M checkpoint (Figure H.3), but this suggestion

requires verification through more experiments. Along with all the possible viability controlling mechanisms of IRF4 over melanomas, there are also other hallmarks of cancer that may be affected by IRF4 regulation, such as anchorage independency, migration and invasion potential. We checked the migration potential of IRF4 knocked-down cell lines but did not find a difference between the samples (Figure G.3), suggesting that IRF4 is not involved in the migration pathway in melanomas, but this suggestion also requires some more experiments to be conclusively shown. The very next step of this study is to complete the rescue experiments and replicate the preliminary results.

APPENDIX A: VECTORS

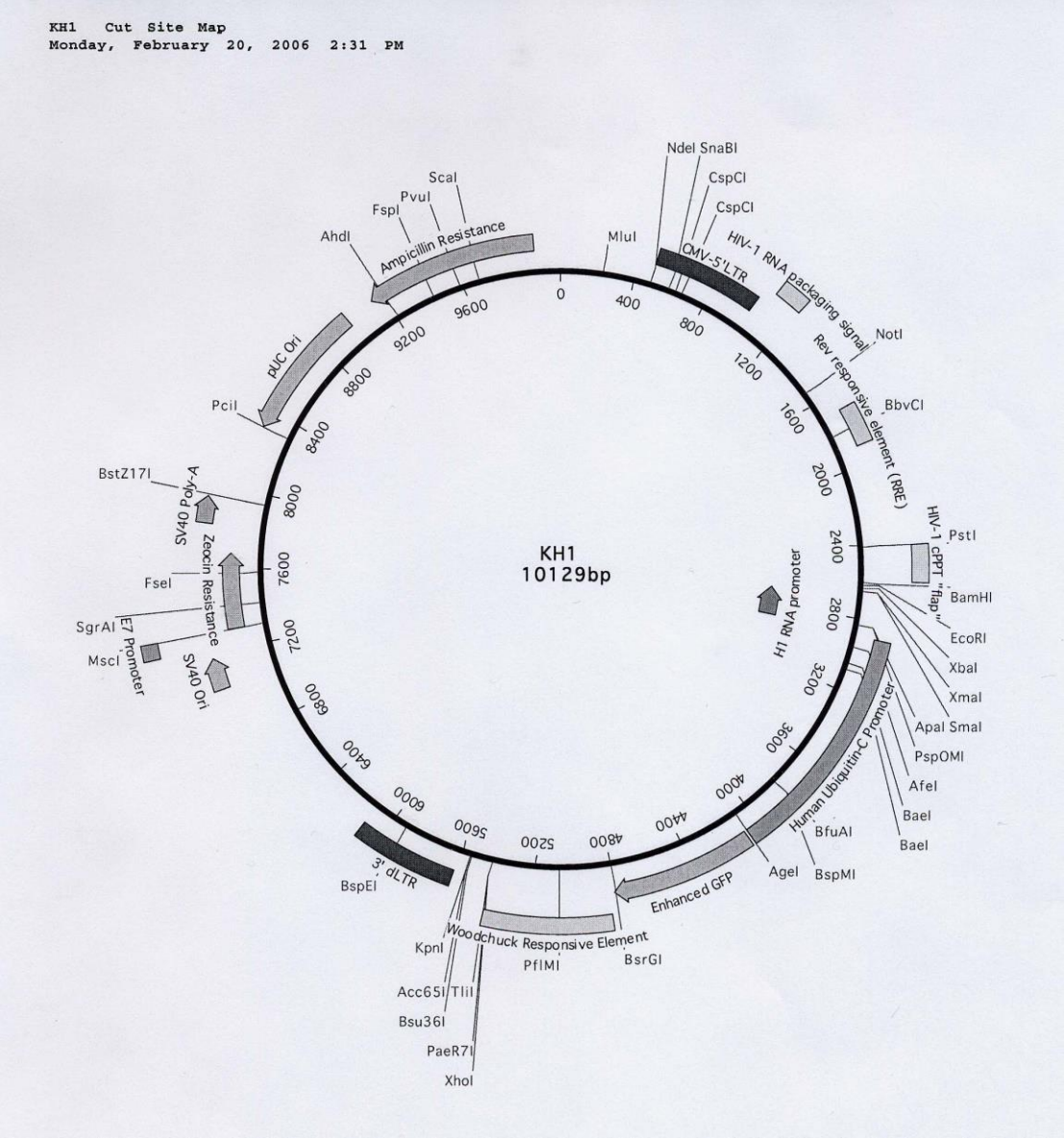


Figure A.1. Vector map of KH1.

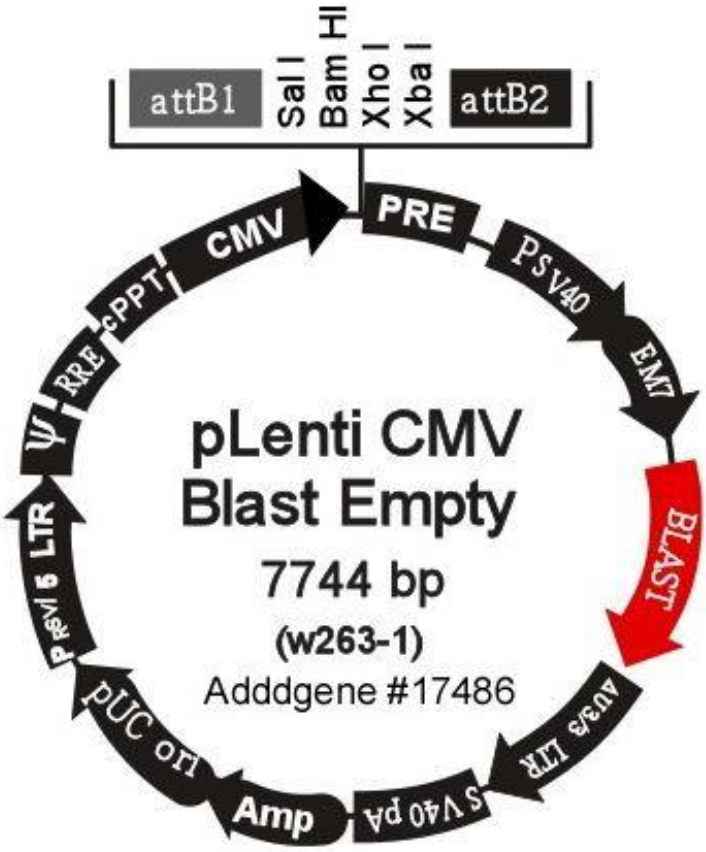


Figure A.2. Vector map of pLenti CMV Blast

APPENDIX B: STUDY BASED RESULTS OF ONCOMINE DATABASE

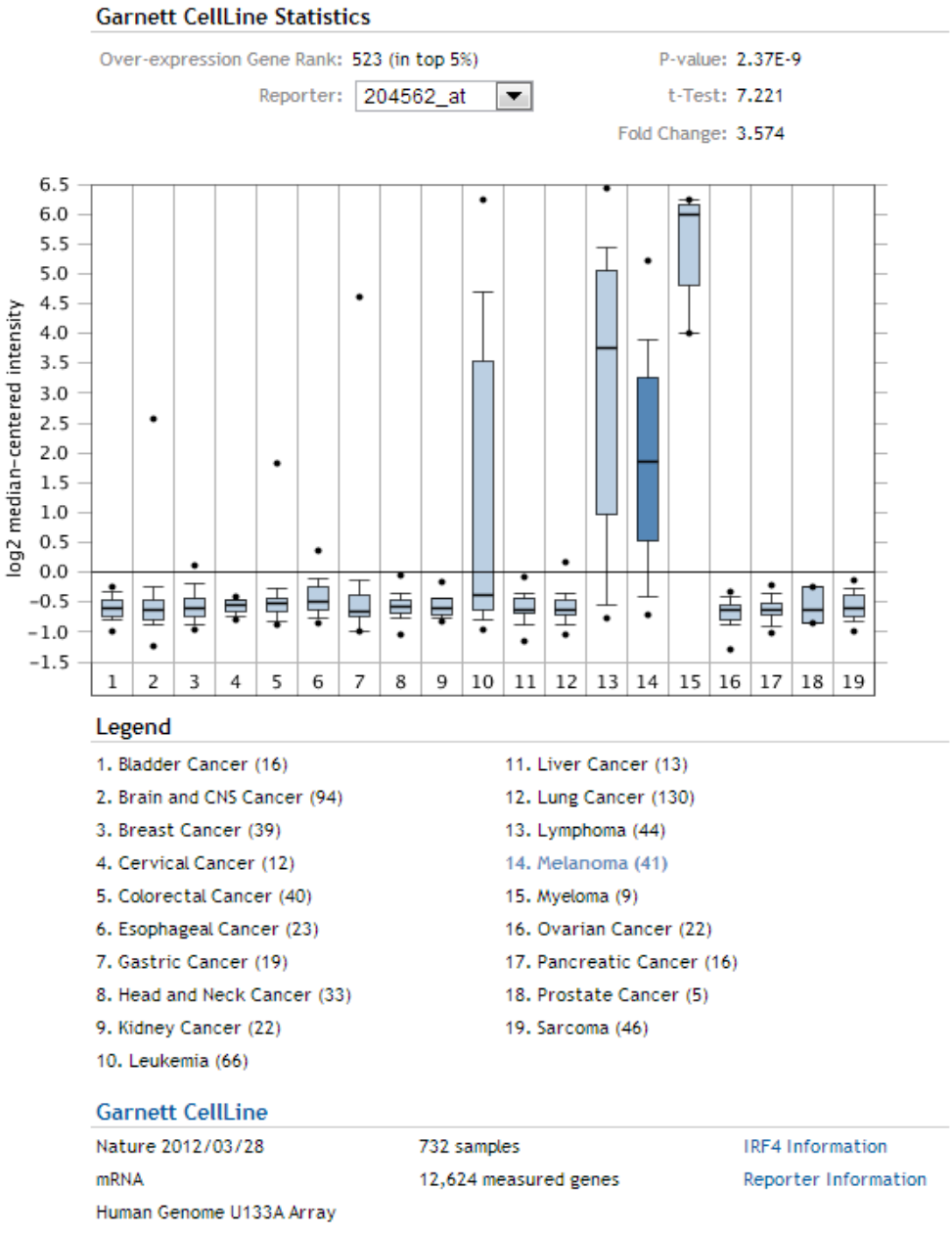


Figure B.1. An example study in oncomine database. Dark blue box highlights melanoma cells. Brackets indicate sample numbers. A fold change of nearly 3.5 with P-value of 2.37×10^{9} and a gene rank number of 523 in 14500 genes (makes up to top 5 percentile) demonstrates the significance of IRF4 in melanoma cells.

APPENDIX C: GFP COMPETITION PROCEDURE

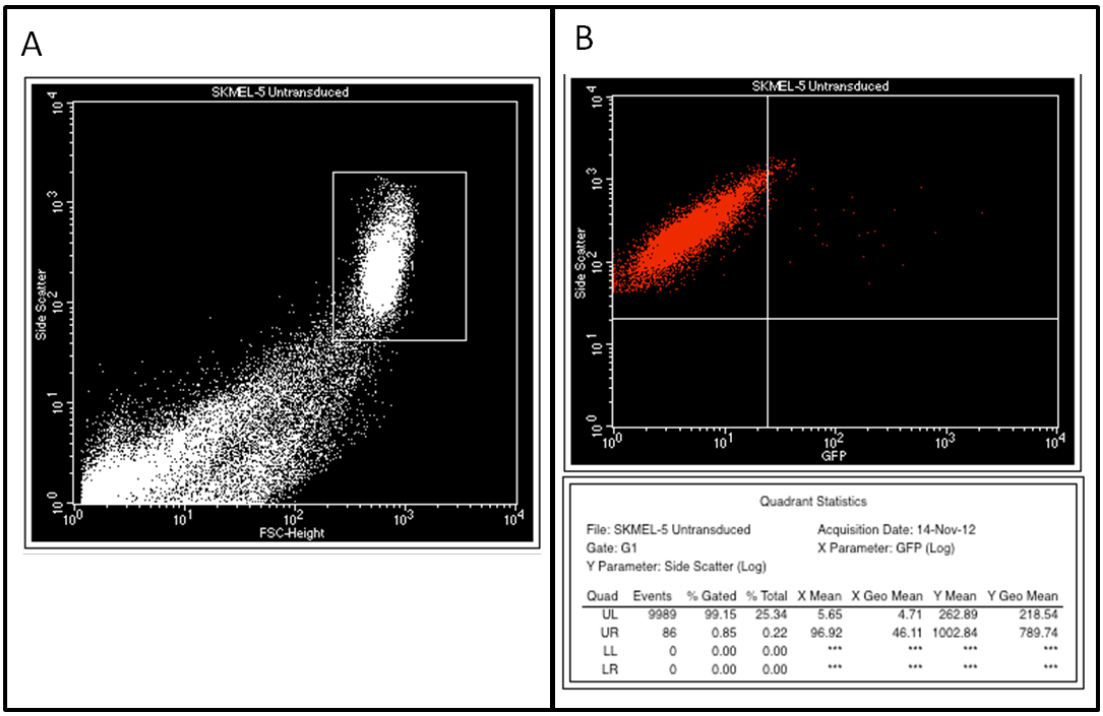


Figure C.1. Gating and self-fluorescence determination of untransduced cells. SKMEL-5 cell line is used as a representative, all applications are similar in other cell lines. A) Gating of the cells in a scatter plot. B) Threshold determination by a dot plot. In quadrant statistics; UL represent upper-left, UR upper right.

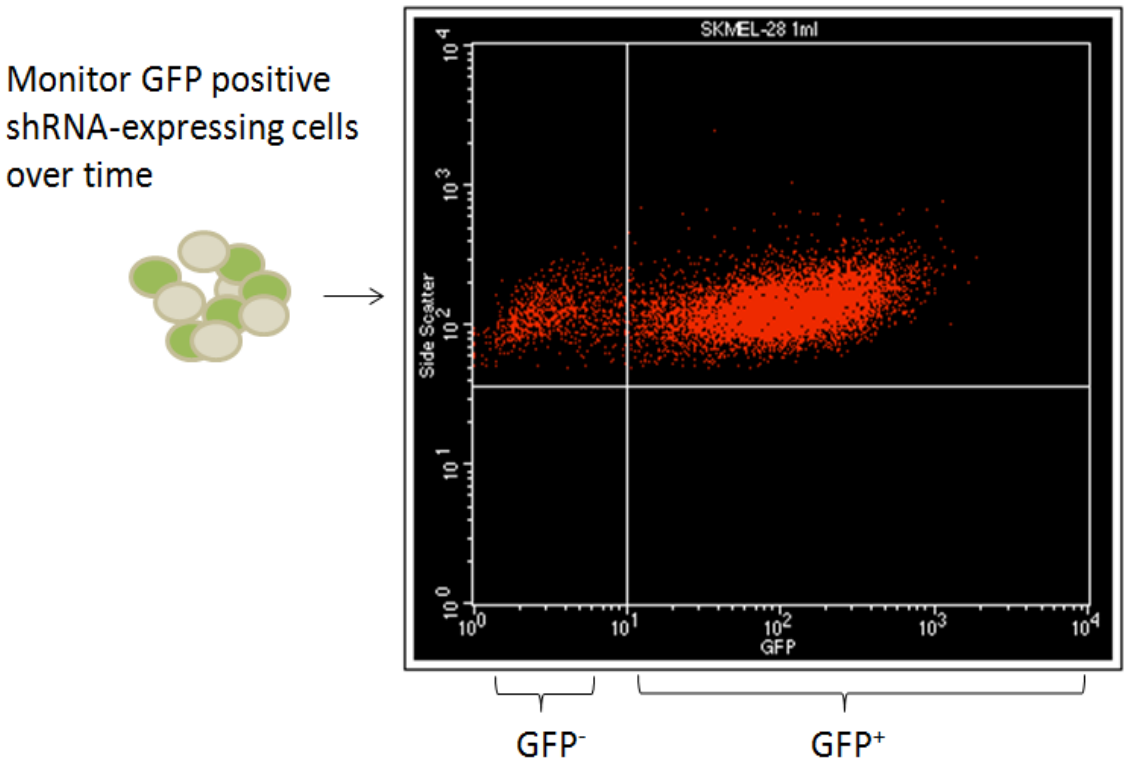


Figure C.2. GFP competition procedure. Detached cells were analyzed by flow cytometer. FSC and SSC values were adjusted specifically for each cell line and cell population was gated. No GFP containing cells were used for the determination of the self fluorescence threshold, resembling the image above.

APPENDIX D: GFP COMPETITION WITHOUT shLUC NORMALIZATION

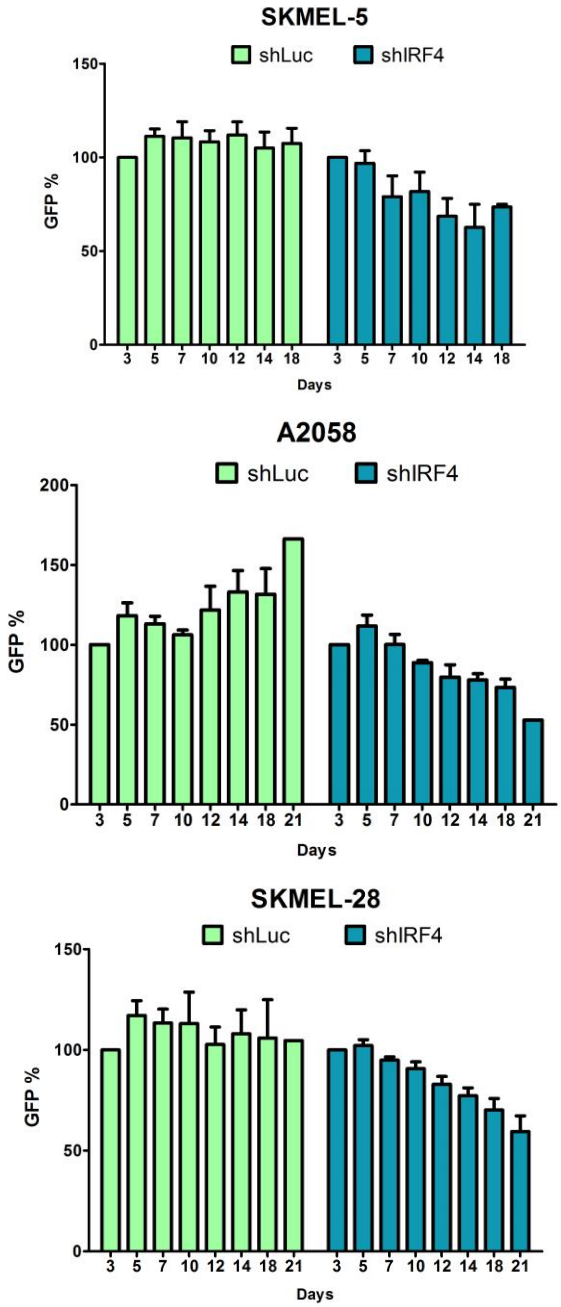


Figure D.1. GFP competition time points without shLuc normalization. All the time points were normalized to only the untransduced cells' data. Error bars represent SEM of replicates.

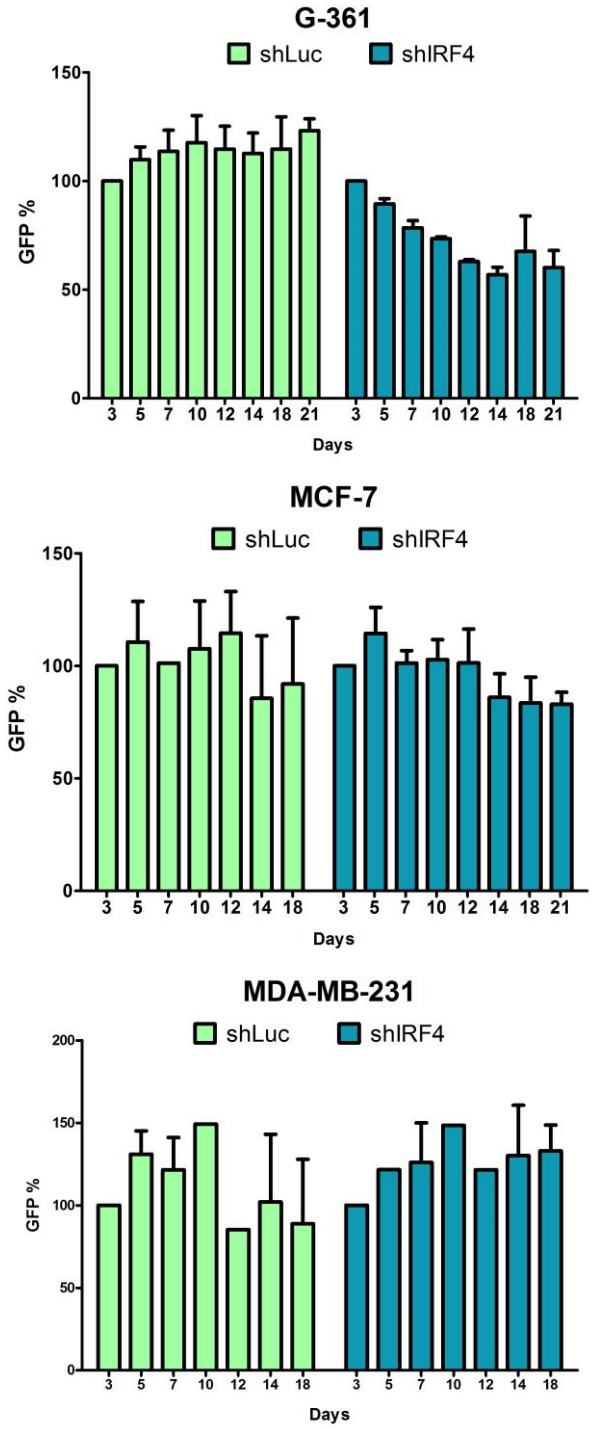


Figure D.1. GFP competition time points without shLuc normalization (cont.). All the time points were normalized to only the untransduced cells' data. Error bars represent SEM of replicates.

APPENDIX E: GFP COMPETITION CONTROLS

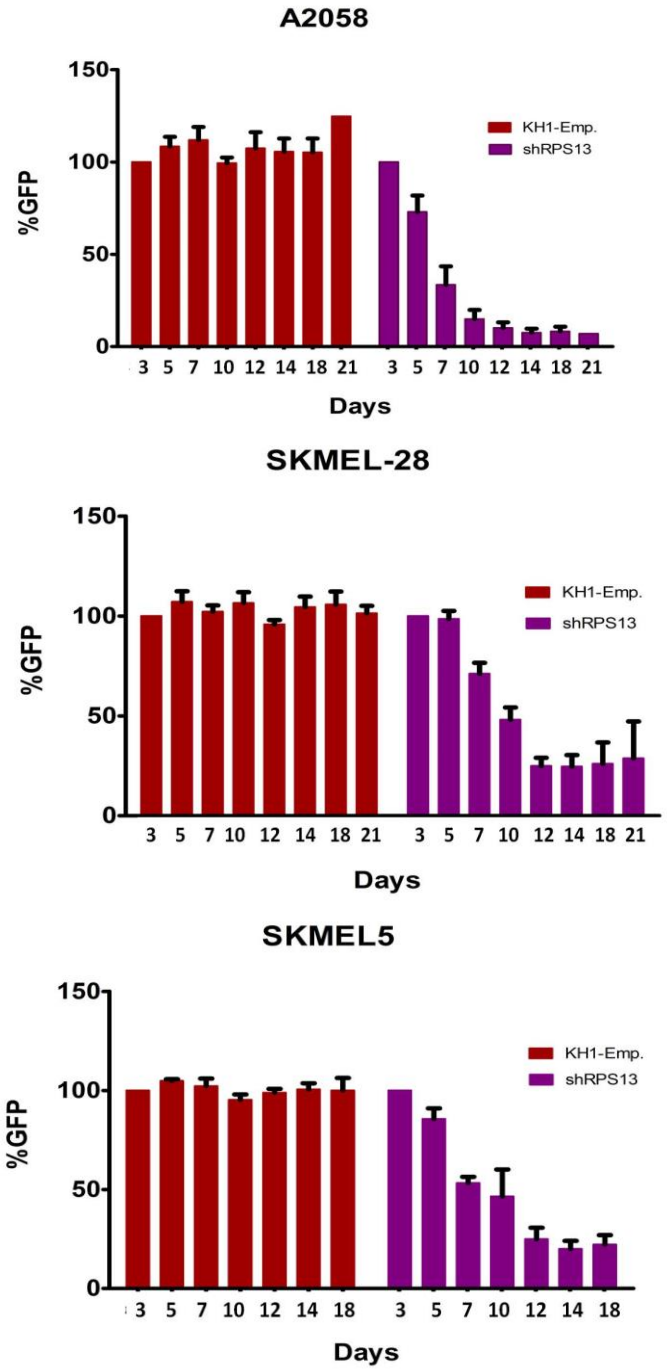


Figure E.1. Control experiments for the GFP competition assay. KH1-Emp. represents empty KH1 vector transductions. All the time points were normalized to untransduced cells' data. Error bars represent SEM of replicates.

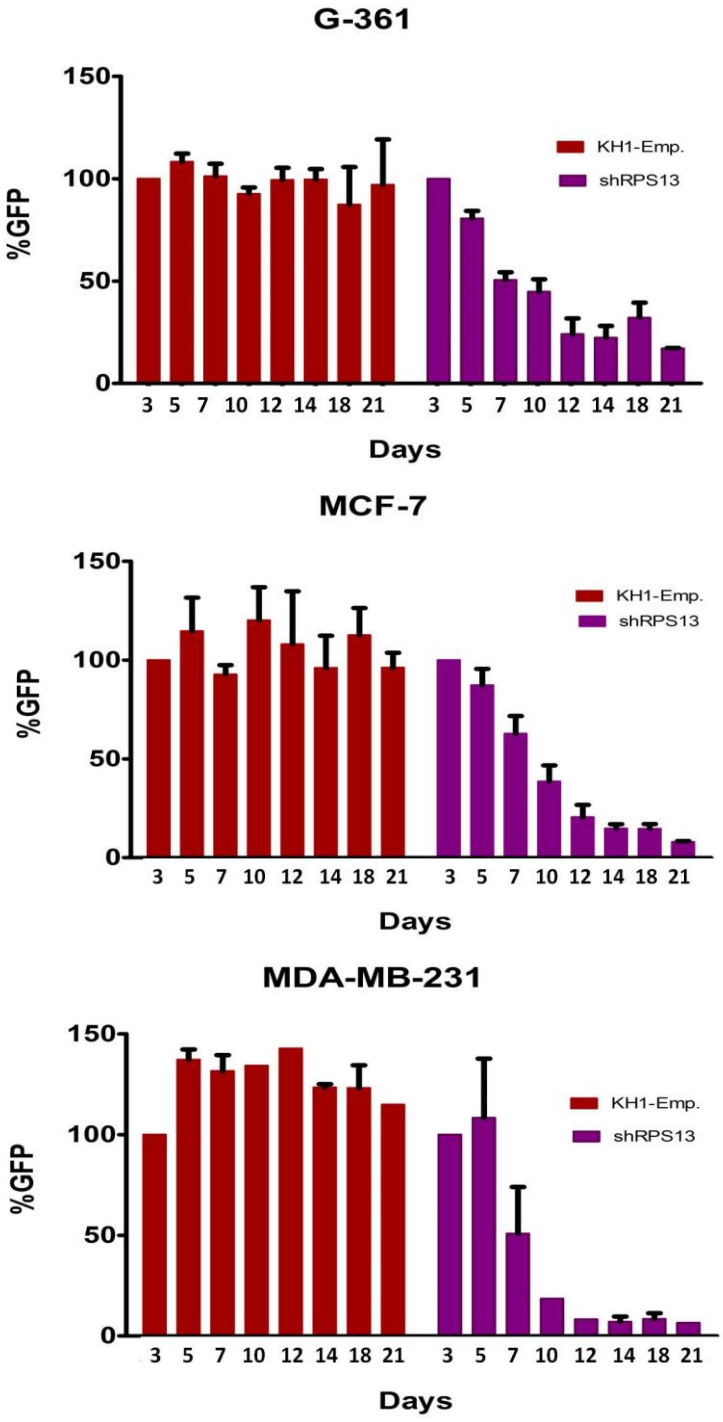


Figure E.01. Control experiments for the GFP competition assay (cont.). KH1-Emp. represents empty KH1 vector transductions. All the time points were normalized to un-transduced cells' data. Error bars represent SEM of replicates.

APPENDIX F: GFP COMPETITION ASSAYS' SIGNIFICANCE

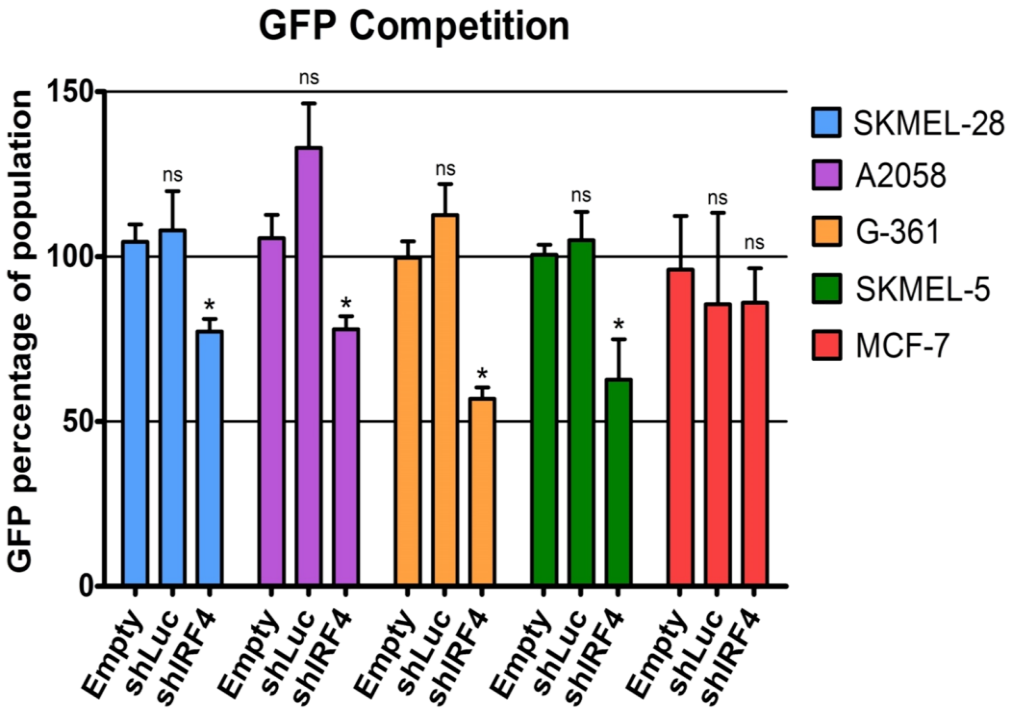


Figure F.1. Significance of GFP competition assays. Day 18 data were normalized to Day 3. (*) indicate p-value lower than 0.05, ns refers to not significant. Error bars represent SEM values of replicates.

APPENDIX G: WOUND HEALING ASSAY

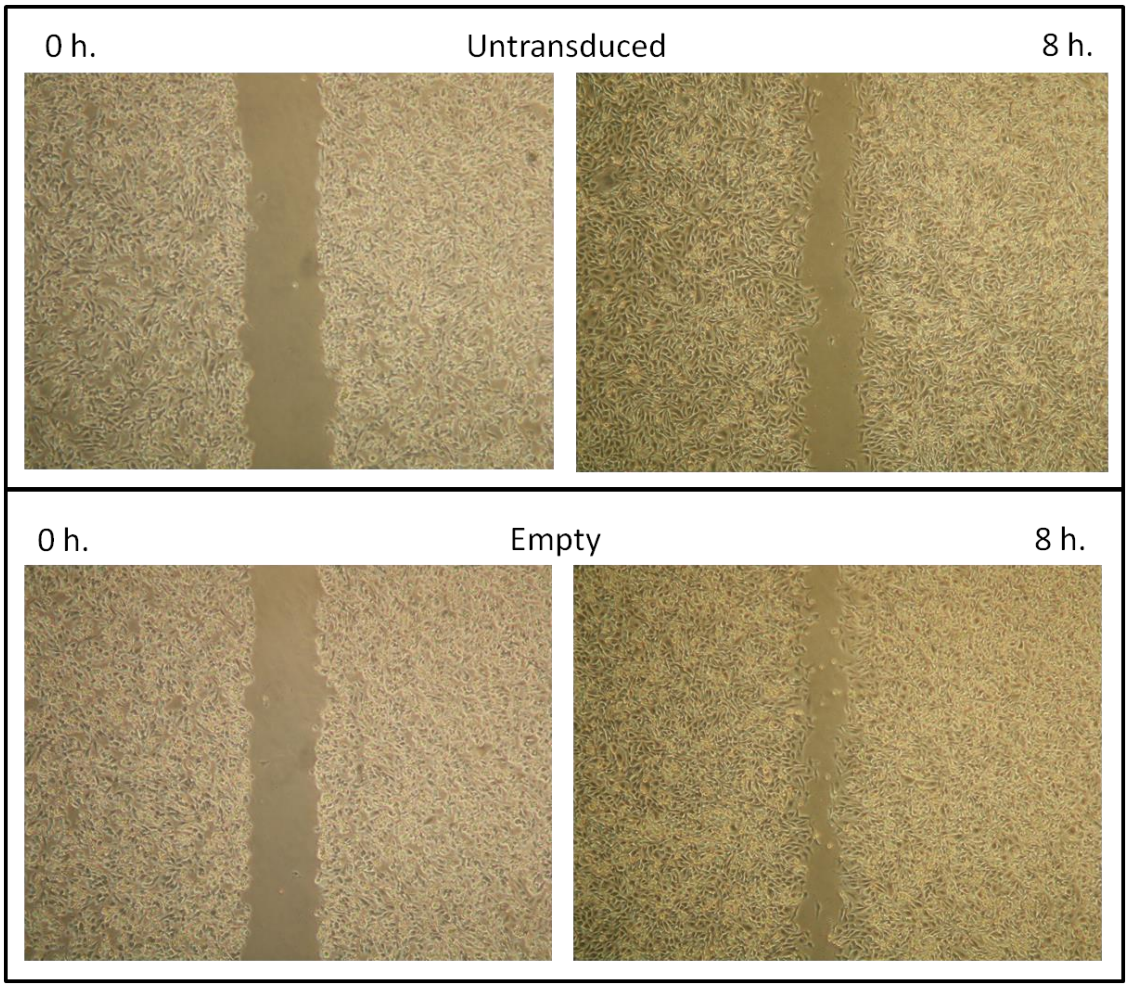


Figure G.1. Photographs of wounded SKMEL-28 cells, untransduced or empty vector introduced. Two photos were taken; one right after the wounding and one after 8 hours. Wound width was measured with plastic ruler for 10 different spots which are in the same place of the culture plate for both time points. 2 different parts of the plate, similar to the photos, was also calculated and the values were averaged.

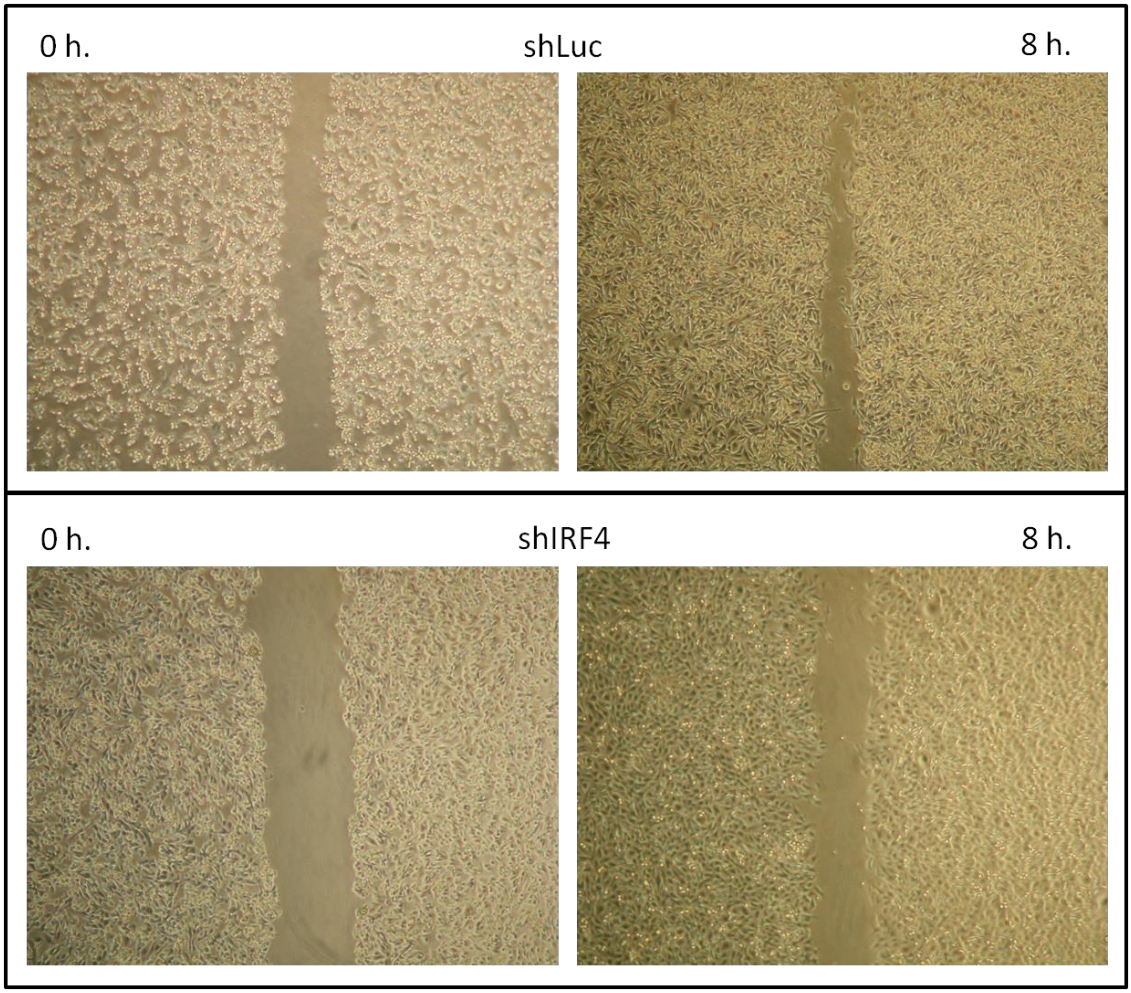


Figure G.2. Photographs of wounded SKMEL-28 cells, transduced by shLuc or shIRF4. Two photos were taken; one right after the wounding and one after 8 hours. Wound width was measured with plastic ruler for 10 different spots which are in the same place of the culture plate for both time points. 2 different parts of the plate, similar to the photos, was also calculated and the values were averaged.

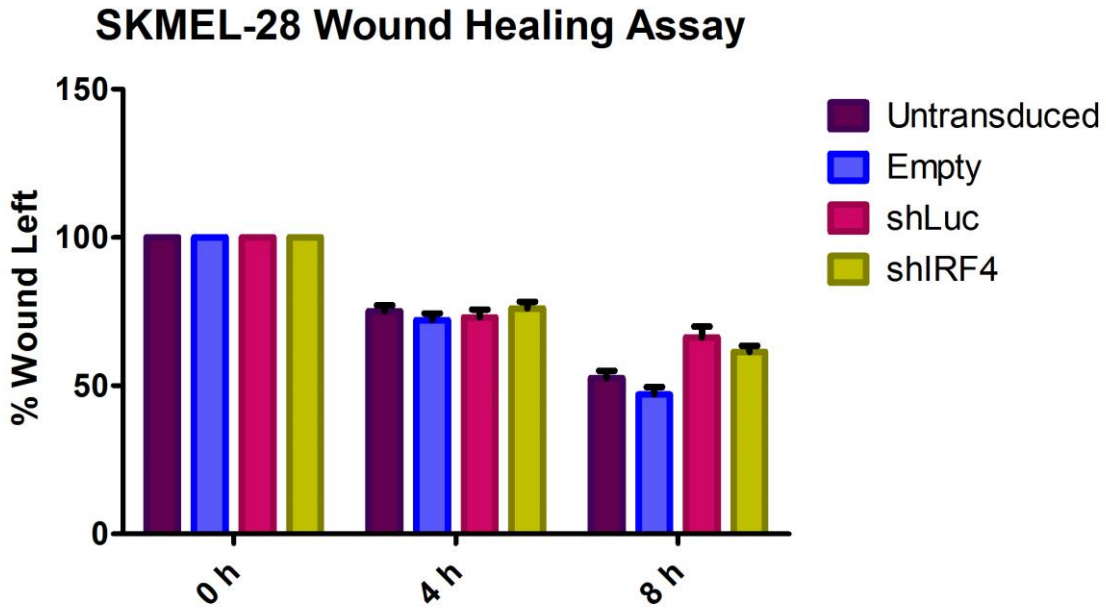


Figure G.3. Wound Healing Assay of SKMEL-28 Cell Line with different transduction conditions. 4 hours and 8 hours' data were normalized to 0 hour. Error bars represent SEM of replicates.

APPENDIX H: CELL CYCLE PROFILING

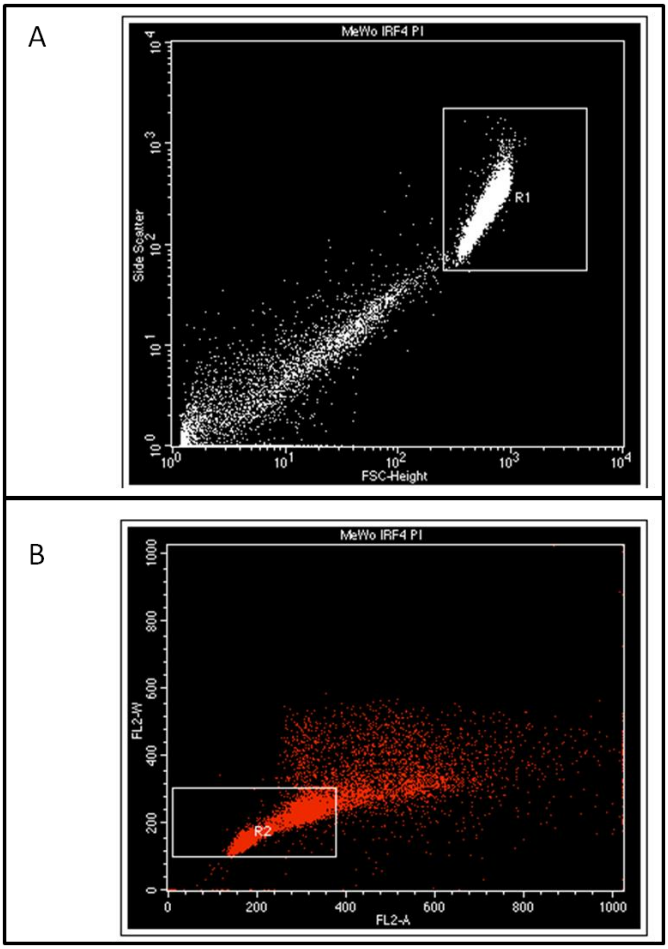


Figure H.1. Gating of PI staining samples in flow cytometer. MeWo cell lines is used as a representative, all applications are similar in other cell lines. A) Basic gating procedure, described in GFP competition assay. B) Gating of single cells using FL2-Width and FL2-Area parameters.

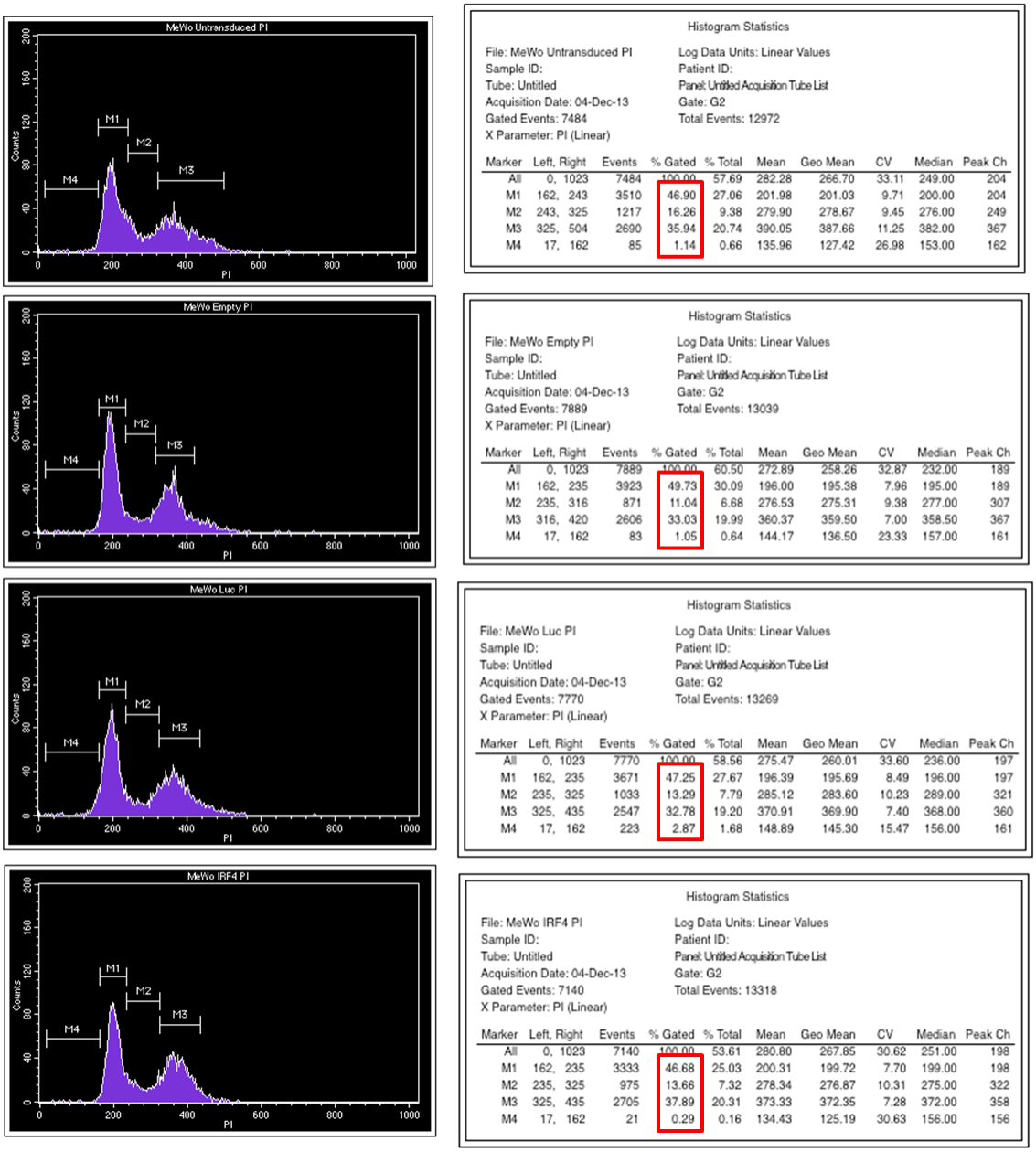


Figure H.2. Determination of cell cycle profile by PI staining. MeWo cell lines is used as a representative, similar results were observed in other cell lines. Untransduced, Empty vector transduced, shLuc introduced and shIRF4b introduced cells' cycle profiles are shown respectively. M1 represents G1 phase, M2 S phase, M3 G2/M phase and M4 sub-G1. Red box indicates the percentages of the phases in the population.

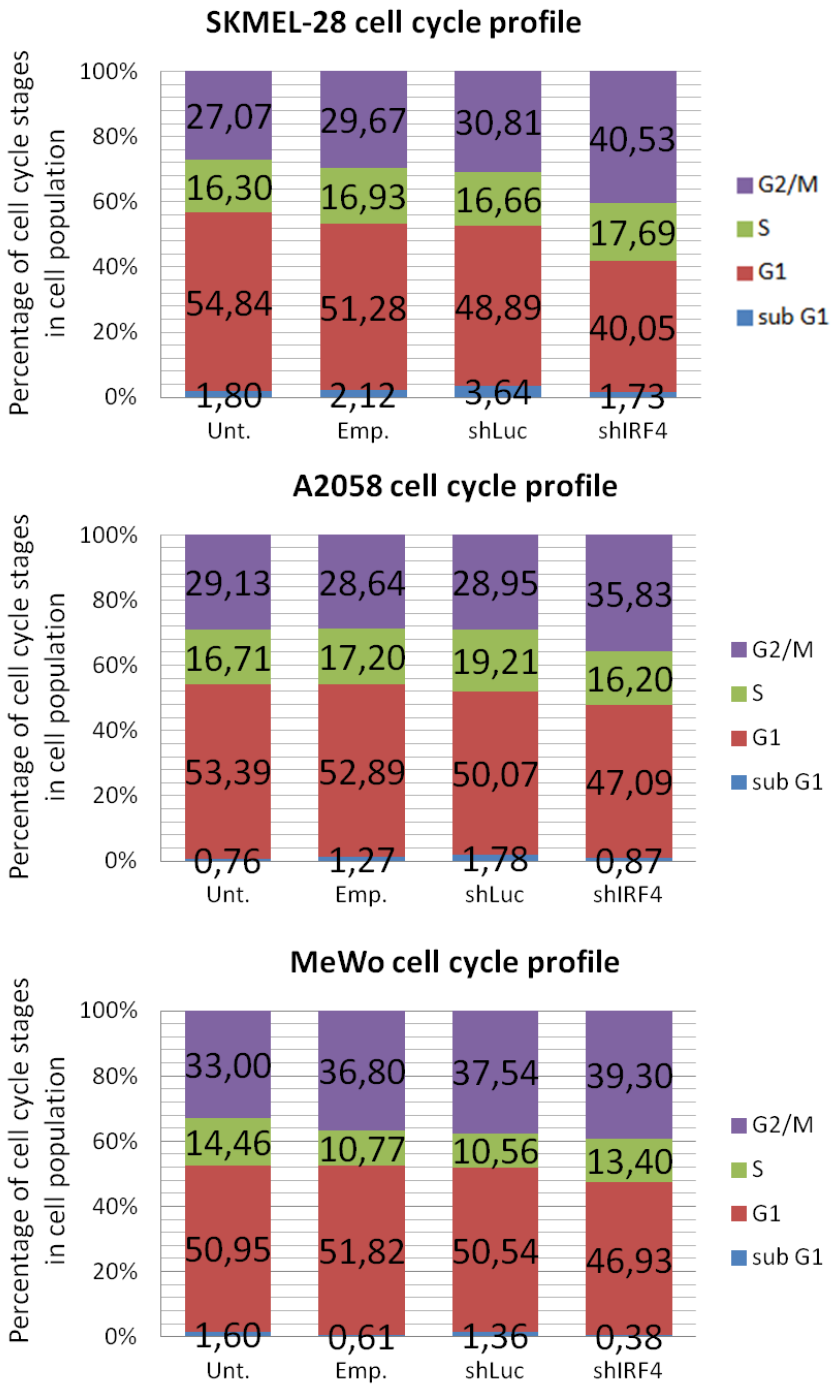


Figure H.3. Cell cycle changes in cell lines, after shIRF4 induction and control transductions using PI staining.

APPENDIX I: KNOCK-DOWN EFFICIENCY OF DIFFERENT shRNA'S

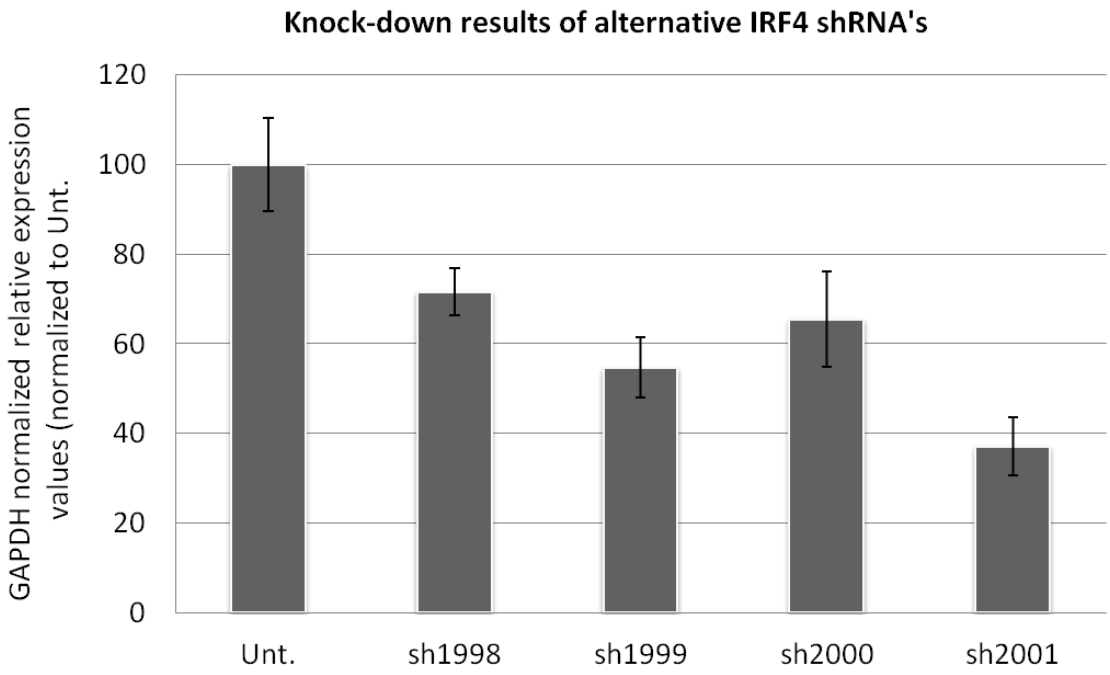


Figure I.1. Knock-down efficiency of different shRNA's on A2058 cell line. mRNA levels of IRF4 were determined by RT-qPCR. Error bars represent SEM of replicates.

APPENDIX J: GFP MARKER IN TRANSFECTED HEK293FT CELLS

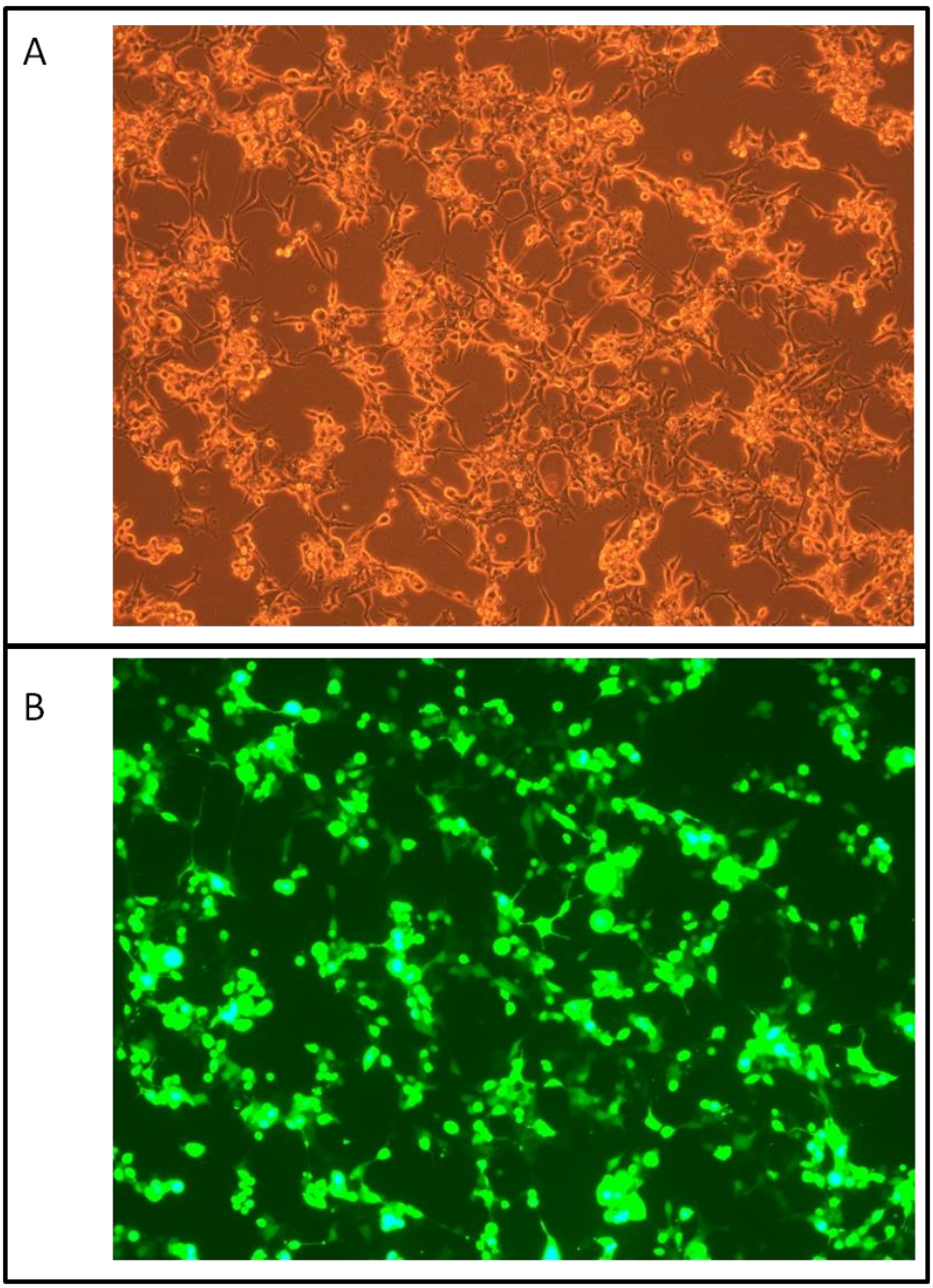


Figure J.1. Photographs of HEK293FT cells transfected with shIRF4b cloned KH1 vector along with the packaging plasmids. Photos were taken 2 days after the transfection. A) Bright field photo of HEK293FT cells. B) Fluorescence photo of A.

REFERENCES

Abbasi, N.R., H.M. Shaw, D.S. Rigel, R.J. Friedman, W.H. McCarthy, I. Osman, A.W. Kopf and D. Polsky, 2004, "Early Diagnosis of Cutaneous Melanoma: Revisiting the Abcd Criteria", *JAMA : the journal of the American Medical Association*, Vol. 292, No. 22, pp. 2771-2776.

Adams, J.M. and S. Cory, 2007, "Bcl-2-Regulated Apoptosis: Mechanism and Therapeutic Potential", *Current Opinion in Immunology*, Vol. 19, No. 5, pp. 488-496.

Albino, A.P., M.J. Vidal, N.S. McNutt, C.R. Shea, V.G. Prieto, D.M. Nanus, J.M. Palmer and N.K. Hayward, 1994, "Mutation and Expression of the P53 Gene in Human Malignant Melanoma", *Melanoma Research*, Vol. 4, No. 1, pp. 35-45.

Badenas, C., P. Aguilera, J.A. Puig-Butille, C. Carrera, J. Malvehy and S. Puig, 2012, "Genetic Counseling in Melanoma", *Dermatologic Therapy*, Vol. 25, No. 5, pp. 397-402.

Baeriswyl, V. and G. Christofori, 2009, "The Angiogenic Switch in Carcinogenesis", *Seminars in Cancer Biology*, Vol. 19, No. 5, pp. 329-337.

Ball, N.J., J.J. Yohn, J.G. Morelli, D.A. Norris, L.E. Golitz and J.P. Hoeffler, 1994, "Ras Mutations in Human Melanoma: A Marker of Malignant Progression", *Journal of Investigative Dermatology*, Vol. 102, No. 3, pp. 285-290.

Barretina, J., G. Caponigro, N. Stransky, K. Venkatesan, A.A. Margolin, S. Kim, C.J. Wilson, J. Lehar, G.V. Kryukov, D. Sonkin, A. Reddy, M. Liu, L. Murray, M.F. Berger, J.E. Monahan, P. Morais, J. Meltzer, A. Korejwa, J. Jane-Valbuena, F.A. Mapa, J. Thibault, E. Bric-Furlong, P. Raman, A. Shipway, I.H. Engels, J. Cheng, G.K. Yu, J. Yu, P. Aspesi, Jr., M. de Silva, K. Jagtap, M.D. Jones, L. Wang, C. Hatton, E. Palescandolo, S. Gupta, S. Mahan, C. Sougnez, R.C. Onofrio, T. Liefeld, L. MacConaill, W. Winckler, M. Reich, N. Li, J.P. Mesirov, S.B. Gabriel, G. Getz, K. Ardlie, V. Chan, V.E. Myer, B.L. Weber, J. Porter, M. Warmuth, P. Finan, J.L. Harris, M. Meyerson, T.R. Golub,

M.P. Morrissey, W.R. Sellers, R. Schlegel and L.A. Garraway, 2012, "The Cancer Cell Line Encyclopedia Enables Predictive Modelling of Anticancer Drug Sensitivity", *Nature*, Vol. 483, No. 7391, pp. 603-607.

Bergsagel, P.L. and W.M. Kuehl, 2005, "Molecular Pathogenesis and a Consequent Classification of Multiple Myeloma", *Journal of Clinical Oncology*, Vol. 23, No. 26, pp. 6333-6338.

Bertolotto, C., F. Lesueur, S. Giuliano, T. Strub, M. de Lichy, K. Bille, P. Dessen, B. d'Hayer, H. Mohamdi, A. Remenieras, E. Maubec, A. de la Fouchardiere, V. Molinie, P. Vabres, S. Dalle, N. Poulalhon, T. Martin-Denavit, L. Thomas, P. Andry-Benzaquen, N. Dupin, F. Boitier, A. Rossi, J.L. Perrot, B. Labeille, C. Robert, B. Escudier, O. Caron, L. Brugieres, S. Saule, B. Gardie, S. Gad, S. Richard, J. Couturier, B.T. Teh, P. Ghiorzo, L. Pastorino, S. Puig, C. Badenas, H. Olsson, C. Ingvar, E. Rouleau, R. Lidereau, P. Bahadoran, P. Vielh, E. Corda, H. Blanche, D. Zelenika, P. Galan, G. French Familial Melanoma Study, F. Aubin, B. Bachollet, C. Becuwe, P. Berthet, Y.J. Bignon, V. Bonadona, J.L. Bonafe, M.N. Bonnet-Dupeyron, F. Cambazard, J. Chevrant-Breton, I. Coupier, S. Dalac, L. Demange, M. d'Incan, C. Dugast, L. Faivre, L. Vincent-Fetita, M. Gauthier-Villars, B. Gilbert, F. Grange, J.J. Grob, P. Humbert, N. Janin, P. Joly, D. Kerob, C. Lasset, D. Leroux, J. Levang, J.M. Limacher, C. Livideanu, M. Longy, A. Lortholary, D. Stoppa-Lyonnet, S. Mansard, L. Mansuy, K. Marrou, C. Mateus, C. Maugard, N. Meyer, C. Nogues, P. Souteyrand, L. Venat-Bouvet, H. Zattara, V. Chaudru, G.M. Lenoir, M. Lathrop, I. Davidson, M.F. Avril, F. Demenais, R. Ballotti and B. Bressac-de Paillerets, 2011, "A Sumoylation-Defective Mitf Germline Mutation Predisposes to Melanoma and Renal Carcinoma", *Nature*, Vol. 480, No. 7375, pp. 94-98.

Berx, G. and F. van Roy, 2009, "Involvement of Members of the Cadherin Superfamily in Cancer", *Cold Spring Harbor Perspectives in Biology*, Vol. 1, No. 6, pp. a003129.

Bradford, P.T., D.M. Freedman, A.M. Goldstein and M.A. Tucker, 2010, "Increased Risk of Second Primary Cancers after a Diagnosis of Melanoma", *Archives of Dermatology*, Vol. 146, No. 3, pp. 265-272.

Bueno, M.J., I. Perez de Castro, M. Gomez de Cedron, J. Santos, G.A. Calin, J.C. Cigudosa, C.M. Croce, J. Fernandez-Piqueras and M. Malumbres, 2008, "Genetic and Epigenetic Silencing of Microrna-203 Enhances Abl1 and Bcr-Abl1 Oncogene Expression", *Cancer Cell*, Vol. 13, No. 6, pp. 496-506.

Burkhart, D.L. and J. Sage, 2008, "Cellular Mechanisms of Tumour Suppression by the Retinoblastoma Gene", *Nature Reviews: Cancer*, Vol. 8, No. 9, pp. 671-682.

Cavallaro, U. and G. Christofori, 2004, "Cell Adhesion and Signalling by Cadherins and Ig-Cams in Cancer", *Nature Reviews: Cancer*, Vol. 4, No. 2, pp. 118-132.

Cully, M., H. You, A.J. Levine and T.W. Mak, 2006, "Beyond Pten Mutations: The Pi3k Pathway as an Integrator of Multiple Inputs During Tumorigenesis", *Nature Reviews: Cancer*, Vol. 6, No. 3, pp. 184-192.

Davies, H., G.R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M.J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B.A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G.J. Riggins, D.D. Bigner, G. Palmieri, A. Cossu, A. Flanagan, A. Nicholson, J.W. Ho, S.Y. Leung, S.T. Yuen, B.L. Weber, H.F. Seigler, T.L. Darrow, H. Paterson, R. Marais, C.J. Marshall, R. Wooster, M.R. Stratton and P.A. Futreal, 2002, "Mutations of the Braf Gene in Human Cancer", *Nature*, Vol. 417, No. 6892, pp. 949-954.

Davis, R.E., K.D. Brown, U. Siebenlist and L.M. Staudt, 2001, "Constitutive Nuclear Factor Kappab Activity Is Required for Survival of Activated B Cell-Like Diffuse Large B Cell Lymphoma Cells", *Journal of Experimental Medicine*, Vol. 194, No. 12, pp. 1861-1874.

De Silva, N.S., G. Simonetti, N. Heise and U. Klein, 2012, "The Diverse Roles of Irf4 in Late Germinal Center B-Cell Differentiation", *Immunological Reviews*, Vol. 247, No. 1, pp. 73-92.

Di Bernardo, M.C., D. Crowther-Swanepoel, P. Broderick, E. Webb, G. Sellick, R. Wild, K. Sullivan, J. Vijayakrishnan, Y. Wang, A.M. Pittman, N.J. Sunter, A.G. Hall, M.J. Dyer, E. Matutes, C. Dearden, T. Mainou-Fowler, G.H. Jackson, G. Summerfield, R.J. Harris, A.R. Pettitt, P. Hillmen, D.J. Allsup, J.R. Bailey, G. Pratt, C. Pepper, C. Fegan, J.M. Allan, D. Catovsky and R.S. Houlston, 2008, "A Genome-Wide Association Study Identifies Six Susceptibility Loci for Chronic Lymphocytic Leukemia", *Nature Genetics*, Vol. 40, No. 10, pp. 1204-1210.

Do, T.N., E. Ucisik-Akkaya, C.F. Davis, B.A. Morrison and M.T. Dorak, 2010, "An Intronic Polymorphism of Irf4 Gene Influences Gene Transcription in Vitro and Shows a Risk Association with Childhood Acute Lymphoblastic Leukemia in Males", *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, Vol. 1802, No. 2, pp. 292-300.

Dull, T., R. Zufferey, M. Kelly, R.J. Mandel, M. Nguyen, D. Trono and L. Naldini, 1998, "A Third-Generation Lentivirus Vector with a Conditional Packaging System", *Journal of Virology*, Vol. 72, No. 11, pp. 8463-8471.

Eguchi, J., X. Wang, S. Yu, E.E. Kershaw, P.C. Chiu, J. Dushay, J.L. Estall, U. Klein, E. Maratos-Flier and E.D. Rosen, 2011, "Transcriptional Control of Adipose Lipid Handling by Irf4", *Cell Metab*, Vol. 13, No. 3, pp. 249-259.

Ferrara, N., 2009, "Vascular Endothelial Growth Factor", *Arteriosclerosis, Thrombosis, and Vascular Biology*, Vol. 29, No. 6, pp. 789-791.

Grossman, A., H.W. Mittrucker, J. Nicholl, A. Suzuki, S. Chung, L. Antonio, S. Suggs, G.R. Sutherland, D.P. Siderovski and T.W. Mak, 1996, "Cloning of Human Lymphocyte-Specific Interferon Regulatory Factor (Hlsirf/Hirf4) and Mapping of the Gene to 6p23-P25", *Genomics*, Vol. 37, No. 2, pp. 229-233.

Han, J., P. Kraft, H. Nan, Q. Guo, C. Chen, A. Qureshi, S.E. Hankinson, F.B. Hu, D.L. Duffy, Z.Z. Zhao, N.G. Martin, G.W. Montgomery, N.K. Hayward, G. Thomas, R.N. Hoover, S. Chanock and D.J. Hunter, 2008, "A Genome-Wide Association Study Identifies Novel Alleles Associated with Hair Color and Skin Pigmentation", *PLoS Genetics*, Vol. 4, No. 5, pp. e1000074.

Hanahan, D. and J. Folkman, 1996, "Patterns and Emerging Mechanisms of the Angiogenic Switch During Tumorigenesis", *Cell*, Vol. 86, No. 3, pp. 353-364.

Hanahan, D. and R.A. Weinberg, 2000, "The Hallmarks of Cancer", *Cell*, Vol. 100, No. 1, pp. 57-70.

Harris, H., O.J. Miller, G. Klein, P. Worst and T. Tachibana, 1969, "Suppression of Malignancy by Cell Fusion", *Nature*, Vol. 223, No. 5204, pp. 363-368.

Hernandez-Ilizaliturri, F.J., G. Deeb, P.L. Zinzani, S.A. Pileri, F. Malik, W.R. Macon, A. Goy, T.E. Witzig and M.S. Czuczman, 2011, "Higher Response to Lenalidomide in Relapsed/Refractory Diffuse Large B-Cell Lymphoma in Nongerminal Center B-Cell-Like Than in Germinal Center B-Cell-Like Phenotype", *Cancer*, Vol. 117, No. 22, pp. 5058-5066.

Hodis, E., I.R. Watson, G.V. Kryukov, S.T. Arold, M. Imielinski, J.P. Theurillat, E. Nickerson, D. Auclair, L. Li, C. Place, D. Dicara, A.H. Ramos, M.S. Lawrence, K. Cibulskis, A. Sivachenko, D. Voet, G. Saksena, N. Stransky, R.C. Onofrio, W. Winckler, K. Ardlie, N. Wagle, J. Wargo, K. Chong, D.L. Morton, K. Stemke-Hale, G. Chen, M. Noble, M. Meyerson, J.E. Ladbury, M.A. Davies, J.E. Gershenwald, S.N. Wagner, D.S. Hoon, D. Schadendorf, E.S. Lander, S.B. Gabriel, G. Getz, L.A. Garraway and L. Chin, 2012, "A Landscape of Driver Mutations in Melanoma", *Cell*, Vol. 150, No. 2, pp. 251-263.

Honma, K., H. Udono, T. Kohno, K. Yamamoto, A. Ogawa, T. Takemori, A. Kumatori, S. Suzuki, T. Matsuyama and K. Yui, 2005, "Interferon Regulatory Factor 4 Negatively Regulates the Production of Proinflammatory Cytokines by Macrophages in Response to Lps", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 102, No. 44, pp. 16001-16006.

Klein, U. and R. Dalla-Favera, 2008, "Germinal Centres: Role in B-Cell Physiology and Malignancy", *Nature Reviews: Immunology*, Vol. 8, No. 1, pp. 22-33.

Kukita, T., N. Arima, K. Matsushita, K. Arimura, H. Ohtsubo, Y. Sakaki, H. Fujiwara, A. Ozaki, T. Matsumoto and C. Tei, 2002, "Autocrine and/or Paracrine Growth of Adult T-Cell Leukaemia Tumour Cells by Interleukin 15", *British Journal of Haematology*, Vol. 119, No. 2, pp. 467-474.

Lam, L.T., R.E. Davis, J. Pierce, M. Hepperle, Y. Xu, M. Hottelet, Y. Nong, D. Wen, J. Adams, L. Dang and L.M. Staudt, 2005, "Small Molecule Inhibitors of Ikappab Kinase Are Selectively Toxic for Subgroups of Diffuse Large B-Cell Lymphoma Defined by Gene Expression Profiling", *Clinical Cancer Research*, Vol. 11, No. 1, pp. 28-40.

Lemmon, M.A. and J. Schlessinger, 2010, "Cell Signaling by Receptor Tyrosine Kinases", *Cell*, Vol. 141, No. 7, pp. 1117-1134.

Levy, C., M. Khaled and D.E. Fisher, 2006, "Mitf: Master Regulator of Melanocyte Development and Melanoma Oncogene", *Trends in Molecular Medicine*, Vol. 12, No. 9, pp. 406-414.

Lidar, T., A. Christian, S. Yakar, P. Langevitz, G. Zeilig, A. Ohry, R. Bakimer, H. Sorek and A. Livneh, 1997, "Clinically Insignificant (Natural) Autoantibodies against Acetyl Cholinesterase in the Sera of Patients with a Variety of Neurologic, Muscular and Autoimmune Diseases", *Immunology Letters*, Vol. 55, No. 2, pp. 79-84.

Lopez-Girona, A., D. Heintel, L.H. Zhang, D. Mendy, S. Gaidarova, H. Brady, J.B. Bartlett, P.H. Schafer, M. Schreder, A. Bolomsky, B. Hilgarth, N. Zojer, H. Gisslinger, H. Ludwig, T. Daniel, U. Jager and R. Chopra, 2011, "Lenalidomide Downregulates the Cell Survival Factor, Interferon Regulatory Factor-4, Providing a Potential Mechanistic Link for Predicting Response", *British Journal of Haematology*, Vol. 154, No. 3, pp. 325-336.

Lowe, S.W. and C.J. Sherr, 2003, "Tumor Suppression by Ink4a-Arf: Progress and Puzzles", *Current Opinion in Genetics & Development*, Vol. 13, No. 1, pp. 77-83.

Malumbres, M. and M. Barbacid, 2003, "Ras Oncogenes: The First 30 Years", *Nature Reviews: Cancer*, Vol. 3, No. 6, pp. 459-465.

Mariner, J.M., Y. Mamane, J. Hiscott, T.A. Waldmann and N. Azimi, 2002, "Ifn Regulatory Factor 4 Participates in the Human T Cell Lymphotropic Virus Type I-Mediated Activation of the Il-15 Receptor Alpha Promoter", *Journal of Immunology*, Vol. 168, No. 11, pp. 5667-5674.

Matsuyama, T., A. Grossman, H.W. Mittrucker, D.P. Siderovski, F. Kiefer, T. Kawakami, C.D. Richardson, T. Taniguchi, S.K. Yoshinaga and T.W. Mak, 1995, "Molecular Cloning of Lsirf, a Lymphoid-Specific Member of the Interferon Regulatory Factor Family That Binds the Interferon-Stimulated Response Element (Isre)", *Nucleic Acids Research*, Vol. 23, No. 12, pp. 2127-2136.

Mittrucker, H.W., T. Matsuyama, A. Grossman, T.M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P.S. Ohashi and T.W. Mak, 1997, "Requirement for the Transcription Factor Lsirf/Irf4 for Mature B and T Lymphocyte Function", *Science*, Vol. 275, No. 5299, pp. 540-543.

Nan, H., P. Kraft, D.J. Hunter and J. Han, 2009, "Genetic Variants in Pigmentation Genes, Pigmentary Phenotypes, and Risk of Skin Cancer in Caucasians", *International Journal of Cancer*, Vol. 125, No. 4, pp. 909-917.

Natkunam, Y., R.A. Warnke, K. Montgomery, B. Falini and M. van De Rijn, 2001, "Analysis of Mum1/Irf4 Protein Expression Using Tissue Microarrays and Immunohistochemistry", *Modern Pathology*, Vol. 14, No. 7, pp. 686-694.

Panka, D.J., M.B. Atkins and J.W. Mier, 2006, "Targeting the Mitogen-Activated Protein Kinase Pathway in the Treatment of Malignant Melanoma", *Clinical Cancer Research*, Vol. 12, No. 7 Pt 2, pp. 2371s-2375s.

Pomerantz, J., N. Schreiber-Agus, N.J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H.W. Lee, C. Cordon-Cardo and R.A. DePinho, 1998, "The Ink4a Tumor Suppressor Gene Product, P19arf, Interacts with Mdm2 and Neutralizes Mdm2's Inhibition of P53", *Cell*, Vol. 92, No. 6, pp. 713-723.

Pongubala, J.M., S. Nagulapalli, M.J. Klemsz, S.R. McKercher, R.A. Maki and M.L. Atchison, 1992, "Pu.1 Recruits a Second Nuclear Factor to a Site Important for Immunoglobulin Kappa 3' Enhancer Activity", *Molecular and Cellular Biology*, Vol. 12, No. 1, pp. 368-378.

Praetorius, C., C. Grill, S.N. Stacey, A.M. Metcalf, D.U. Gorkin, K.C. Robinson, E. Van Otterloo, R.S. Kim, K. Bergsteinsdottir, M.H. Ogmundsdottir, E. Magnusdottir, P.J. Mishra, S.R. Davis, T. Guo, M.R. Zaidi, A.S. Helgason, M.I. Sigurdsson, P.S. Meltzer, G. Merlino, V. Petit, L. Larue, S.K. Loftus, D.R. Adams, U. Sobhiafshar, N.C. Emre, W.J. Pavan, R. Cornell, A.G. Smith, A.S. McCallion, D.E. Fisher, K. Stefansson, R.A. Sturm and E. Steingrimsson, 2013, "A Polymorphism in Irf4 Affects Human Pigmentation through a Tyrosinase-Dependent Mitf/Tfap2a Pathway", *Cell*, Vol. 155, No. 5, pp. 1022-1033.

Rhodes, D.R., J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, T. Barrette, A. Pandey and A.M. Chinnaiyan, 2004, "Oncomine: A Cancer Microarray Database and Integrated Data-Mining Platform", *Neoplasia*, Vol. 6, No. 1, pp. 1-6.

Sciammas, R., A.L. Shaffer, J.H. Schatz, H. Zhao, L.M. Staudt and H. Singh, 2006, "Graded Expression of Interferon Regulatory Factor-4 Coordinates Isotype Switching with Plasma Cell Differentiation", *Immunity*, Vol. 25, No. 2, pp. 225-236.

Serrano, M., G.J. Hannon and D. Beach, 1993, "A New Regulatory Motif in Cell-Cycle Control Causing Specific Inhibition of Cyclin D/Cdk4", *Nature*, Vol. 366, No. 6456, pp. 704-707.

Shaffer, A.L., N.C. Emre, L. Lamy, V.N. Ngo, G. Wright, W. Xiao, J. Powell, S. Dave, X. Yu, H. Zhao, Y. Zeng, B. Chen, J. Epstein and L.M. Staudt, 2008, "Irf4 Addiction in Multiple Myeloma", *Nature*, Vol. 454, No. 7201, pp. 226-231.

Shaffer, A.L., N.C. Emre, P.B. Romesser and L.M. Staudt, 2009, "Irf4: Immunity. Malignancy! Therapy?", *Clinical Cancer Research*, Vol. 15, No. 9, pp. 2954-2961.

Sharma, S., Y. Mamane, N. Grandvaux, J. Bartlett, L. Petropoulos, R. Lin and J. Hiscott, 2000, "Activation and Regulation of Interferon Regulatory Factor 4 in Htlv Type 1-Infected T Lymphocytes", *AIDS Research and Human Retroviruses*, Vol. 16, No. 16, pp. 1613-1622.

Sherr, C.J., 2004, "Principles of Tumor Suppression", *Cell*, Vol. 116, No. 2, pp. 235-246.

Staudt, L.M. and S. Dave, 2005, "The Biology of Human Lymphoid Malignancies Revealed by Gene Expression Profiling", *Advances in Immunology*, Vol. 87, No. pp. 163-208.

Stegmeier, F., G. Hu, R.J. Rickles, G.J. Hannon and S.J. Elledge, 2005, "A Lentiviral Microrna-Based System for Single-Copy Polymerase Ii-Regulated Rna Interference in Mammalian Cells", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 102, No. 37, pp. 13212-13217.

Sulem, P., D.F. Gudbjartsson, S.N. Stacey, A. Helgason, T. Rafnar, K.P. Magnusson, A. Manolescu, A. Karason, A. Palsson, G. Thorleifsson, M. Jakobsdottir, S. Steinberg, S. Palsson, F. Jonasson, B. Sigurgeirsson, K. Thorisdottir, R. Ragnarsson, K.R. Benediktsdottir, K.K. Aben, L.A. Kiemeney, J.H. Olafsson, J. Gulcher, A. Kong, U. Thorsteinsdottir and K. Stefansson, 2007, "Genetic Determinants of Hair, Eye and Skin Pigmentation in Europeans", *Nature Genetics*, Vol. 39, No. 12, pp. 1443-1452.

Sundram, U., J.D. Harvell, R.V. Rouse and Y. Natkunam, 2003, "Expression of the B-Cell Proliferation Marker Mum1 by Melanocytic Lesions and Comparison with S100, Gp100 (Hmb45), and Melana", *Modern Pathology*, Vol. 16, No. 8, pp. 802-810.

Suzuki, I., R.D. Cone, S. Im, J. Nordlund and Z.A. Abdel-Malek, 1996, "Binding of Melanotropic Hormones to the Melanocortin Receptor Mc1r on Human Melanocytes Stimulates Proliferation and Melanogenesis", *Endocrinology*, Vol. 137, No. 5, pp. 1627-1633.

Suzuki, S., K. Honma, T. Matsuyama, K. Suzuki, K. Toriyama, I. Akitoyo, K. Yamamoto, T. Suematsu, M. Nakamura, K. Yui and A. Kumatori, 2004, "Critical Roles of Interferon Regulatory Factor 4 in Cd11bhighcd8alpha- Dendritic Cell Development", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 101, No. 24, pp. 8981-8986.

Talmadge, J.E. and I.J. Fidler, 2010, "Aacr Centennial Series: The Biology of Cancer Metastasis: Historical Perspective", *Cancer Research*, Vol. 70, No. 14, pp. 5649-5669.

Tamura, T., H. Yanai, D. Savitsky and T. Taniguchi, 2008, "The Irf Family Transcription Factors in Immunity and Oncogenesis", *Annual Review of Immunology*, Vol. 26, No. pp. 535-584.

Vultur, A. and M. Herlyn, 2013, "Snapshot: Melanoma", *Cancer Cell*, Vol. 23, No. 5, pp. 706-706 e701.

Williams, P.F., C.M. Olsen, N.K. Hayward and D.C. Whiteman, 2011, "Melanocortin 1 Receptor and Risk of Cutaneous Melanoma: A Meta-Analysis and Estimates of Population Burden", *International Journal of Cancer*, Vol. 129, No. 7, pp. 1730-1740.

Willis, S.N. and J.M. Adams, 2005, "Life in the Balance: How Bh3-Only Proteins Induce Apoptosis", *Current Opinion in Cell Biology*, Vol. 17, No. 6, pp. 617-625.

Witsch, E., M. Sela and Y. Yarden, 2010, "Roles for Growth Factors in Cancer Progression", *Physiology (Bethesda)*, Vol. 25, No. 2, pp. 85-101.

Wu, H., V. Goel and F.G. Haluska, 2003, "Pten Signaling Pathways in Melanoma", *Oncogene*, Vol. 22, No. 20, pp. 3113-3122.

Xu, D., L. Zhao, L. Del Valle, J. Miklossy and L. Zhang, 2008, "Interferon Regulatory Factor 4 Is Involved in Epstein-Barr Virus-Mediated Transformation of Human B Lymphocytes", *Journal of Virology*, Vol. 82, No. 13, pp. 6251-6258.

Yamagata, T., J. Nishida, S. Tanaka, R. Sakai, K. Mitani, M. Yoshida, T. Taniguchi, Y. Yazaki and H. Hirai, 1996, "A Novel Interferon Regulatory Factor Family Transcription Factor, Icsat/Pip/Lsirf, That Negatively Regulates the Activity of Interferon-Regulated Genes", *Molecular and Cellular Biology*, Vol. 16, No. 4, pp. 1283-1294.

Yang, Y., A.L. Shaffer, 3rd, N.C. Emre, M. Ceribelli, M. Zhang, G. Wright, W. Xiao, J. Powell, J. Platig, H. Kohlhammer, R.M. Young, H. Zhao, Y. Yang, W. Xu, J.J. Buggy, S. Balasubramanian, L.A. Mathews, P. Shinn, R. Guha, M. Ferrer, C. Thomas, T.A. Waldmann and L.M. Staudt, 2012, "Exploiting Synthetic Lethality for the Therapy of Abc Diffuse Large B Cell Lymphoma", *Cancer Cell*, Vol. 21, No. 6, pp. 723-737.

Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J.M. Kim, T.T. Chu, L. Corcoran, P. Treuting, U. Klein and A.Y. Rudensky, 2009, "Regulatory T-Cell Suppressor Program Co-Opts Transcription Factor Irf4 to Control T(H)2 Responses", *Nature*, Vol. 458, No. 7236, pp. 351-356.

Zuo, L., J. Weger, Q. Yang, A.M. Goldstein, M.A. Tucker, G.J. Walker, N. Hayward and N.C. Dracopoli, 1996, "Germline Mutations in the P16ink4a Binding Domain of Cdk4 in Familial Melanoma", *Nature Genetics*, Vol. 12, No. 1, pp. 97-99.