

ACTIVE RAS/MEK PATHWAY DOWNREGULATES EXPRESSION OF IFN-  
INDUCIBLE GENES BY TARGETING IRF1: IMPLICATIONS FOR  
UNDERSTANDING MOLECULAR MECHANISMS OF VIRAL ONCOLYSIS

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## ABSTRACT

Oncolytic viruses exploit common molecular changes in cancer cells, which are not present in normal cells, to target and kill cancer cells. Ras transformation and defects in type I interferon (IFN)-mediated antiviral responses are known to be the major mechanisms underlying viral oncolysis. The Hirasawa lab has previously demonstrated that oncogenic Ras/Mitogen-activated protein kinase kinase (Ras/MEK) activation suppresses the transcription of many IFN-inducible genes in human cancer cells, suggesting that Ras transformation underlies type I IFN defects in cancer cells. The objective of my PhD project was to elucidate the mechanisms underlying how Ras/MEK downregulates IFN-induced transcription.

By conducting promoter deletion analysis of IFN-inducible genes, the IFN regulatory factor 1 (IRF1) binding site was identified to be responsible for the regulation of transcription by MEK. MEK inhibition promoted transcription of the IFN-inducible genes in wild-type mouse embryonic fibroblasts (MEFs), but not in IRF1<sup>-/-</sup> MEFs. Furthermore, IRF1 expression was lower in RasV12 cells compared with vector control NIH3T3 cells, which was restored to equivalent levels by inhibition of MEK. Similarly, MEK inhibition restored IRF1 expression in human cancer cells. IRF1 re-expression in human cancer cells increased cellular resistance to infection by the oncolytic vesicular stomatitis virus strain. Together, these results indicate that Ras/MEK activation in cancer cells downregulates transcription of IFN-inducible genes by targeting IRF1 expression, resulting in increased susceptibility to viral oncolysis.

I further sought to determine how active Ras/MEK downregulates IRF1 expression. MEK inhibition restored IRF1 expression at the protein level prior to mRNA induction; however, it did not affect IRF1 protein stability. The expression of IRF1-targeting microRNA, activity of IRF1 5' and 3'-UTRs, and polysome loading of IRF1 mRNA in response to MEK inhibition were analyzed; however, the translational regulation of IRF1 mRNA by Ras/MEK remained inconclusive. To determine whether Ras/MEK modulates post-translational modifications (PTMs) of IRF1, phosphorylation, ubiquitination, sumoylation, and acetylation of IRF1 were examined. MEK inhibition promoted ubiquitination and inhibited sumoylation of IRF1, indicating that active Ras/MEK alters PTM of IRF1 protein.

Lastly, siRNA screens and overexpression experiments identified RSK3 and RSK4 to be the ERK downstream effectors involved in Ras/MEK-mediated IRF1 regulation.

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## LIST OF ABBREVIATIONS AND SYMBOLS

ARID3A: AT rich interactive domain-3A

CHIP: C-terminus of the Hsc (heat-shock cognate)-70-interacting protein

ChIP assay: Chromatin immunoprecipitation assays

CHX: Cycloheximide

cIAP2: Cellular inhibitor of apoptosis-2

dsRNA: Double stranded RNA

eIF4B: Eukaryotic translation initiation factor-4B

eIF4E: Eukaryotic translation initiation factor-4E

EMCV: Encephalomyocarditis virus

ERK: Extracellular signal regulated kinase

EtBr: Ethidium bromide

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Gbp2: Guanylate binding protein-2

Ifi47: Interferon gamma inducible protein-47

Ifit1: Interferon-induced protein with tetratricopeptide repeats-1

IFN- $\alpha$ : Interferon-alpha

Iigp2: Immunity-related GTPase family M member-2

KSHV: Kaposi's sarcoma associated herpesvirus

Il15: Interleukin-15

IRF: Interferon regulatory factor

IRFE: interferon regulatory factor (IRF)-binding element

ISRE: IFN-stimulated response element

Lys: Lysine

MEF: Mouse embryonic fibroblasts

MEK: Mitogen-activated protein kinase kinase

MDII genes: MEK-downregulated IFN-inducible genes

MDM2: Murine double minute-2

miRNA: microRNA

MNK: MAPK-interacting kinase

MOI: Multiplicity of infection

MSK: Mitogen- and stress-activated protein kinases

NEM: N-ethylmaleimide

PAX2: Paired box gene-2

PIAS: Protein inhibitor of activated STAT

PKR: Protein kinase R

PTM: Post-translational modification

Ptx3: Pentraxin related gene

Rig-I: Retinoic acid-inducible gene-1

RLU: Relative luciferase activities

rpS6: Ribosomal protein S6

RSK: Ribosomal s6 kinases

SCR siRNA: Scrambled siRNA

SIAH: Seven in absentia

SOX17: SRY-box containing gene-17

SOX10: SRY-box containing gene-10

STAT: Signal transducer and activator of transcription

SUMO: Small ubiquitin-like modifier

SV40: Simian virus 40

TRAF6: TNF receptor-associated factor-6

UTR: Untranslated region

VSV: Vesicular stomatitis virus

Xaf1: XIAP-associated factor-1

4E-BP: 4E-binding protein

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**CHAPTER 1**  
**INTRODUCTION**

## **1.1 Oncolytic viruses**

### **1.1.1 History of Oncolytic viruses**

Oncolytic viruses preferentially replicate within cancer cells, leading to destruction of cancer cells, while normal cells remain unharmed. The concept of using viruses as anticancer agents dates back to the mid-1800s when numerous reports described transient remission of hematological malignancies coincided with naturally acquired viral infections including influenza, chickenpox, measles, and hepatitis viruses (Kelly & Russell, 2007). Thereafter, viruses began to be examined experimentally as anti-cancer agents in clinical settings. In the 1940s, blood-borne virus was administered to 22 patients with Hodgkin's lymphoma, of which 7 patients showed improvements in clinical aspects of the disease. Unfortunately, 14 patients developed hepatitis from the treatment (Hoster et al., 1949). In the 1950s, infection of West Nile virus (Egypt 101 isolate) for treatment of multiple types of cancer allowed 4 of the 34 patients treated to show transient tumor regression. However, two patients developed encephalitis (Southam & Moore, 1952). In a different study, adenoidal-pharyngeal-conjunctival virus (adenovirus) was tested for treatment in 30 patients with cervical cancer. Although necrosis was confirmed at the tumor site in 26 patients, these patients suffered from hemorrhage, and the viruses were eventually eliminated by the host immune system (Georgiades et al., 1959). In the 1970s, non-attenuated mumps virus was tested for the treatment of patients with various types of terminal cancer. Strikingly, 37 out of 90 patients had complete regression or a more than 50 % decrease in tumor size with minimum side effects (Asada, 1974).

Around the same time as these clinical studies were being conducted, the efficacy of viral oncolysis was tested in animal cancer *in vivo* models (Kelly & Russell, 2007). Moore reported that the growth of the transplanted mouse sarcoma 180 tumor was inhibited when the mice were inoculated with tick-borne encephalitis virus (Moore, 1949). A number of studies subsequently demonstrated the oncolytic potential of many other viruses including adenovirus, Bunyamwera virus, dengue virus, Ilheus virus, mumps virus, Semliki Forest virus, vaccinia virus (VV), West Nile virus (WNV), and yellow fever virus (YFV) on *in vivo* rodent models of cancer (Huebner et al., 1956; Kelly & Russell, 2007; Moore, 1952; Newman & Southam, 1954; and Southam & Moore, 1951). Following the animal studies, many of these viruses were examined for their oncolytic abilities in clinical studies. In general, the efficacy of viral oncolysis was often higher in experimental animal models than in the patient. This was partly attributed to preexisting antiviral immunity, which rapidly eliminated the viruses (Kelly & Russell, 2007).

In order to bypass the preexisting immunity against human viruses, researchers began to test oncolytic activity of non-human viruses. Non-human viruses that were identified to possess oncolytic potential in early studies included herpesviruses (equine rhinopneumonitis and bovine rhinotracheitis), arenaviruses, avian influenza virus, Newcastle disease virus (NDV), and vesicular stomatitis virus (VSV) (Cassel & Garrett, 1965; Hammon et al., 1963; Lindenmann & Klein, 1967; Southam & Moore, 1951; Stojdl et al., 2003; and Yohn et al., 1968). Among these viruses, viral oncolysis of NDV and VSV continue to be extensively studied in animal models and in clinical trials (Kelly & Russell, 2007).

Toward the end of 20<sup>th</sup> century, as the manipulation of viral genomes became possible through the development of recombinant DNA technology, significant breakthroughs were made in the field due to the ability to engineer oncolytic viruses to increase cancer specificity. This was demonstrated by engineering of first generation of oncolytic herpes simplex virus (HSV) with a deletion of the viral thymidine kinase (*TK*) gene. TK is an enzyme required for DNA synthesis and highly expressed in actively proliferating cells such as cancer cells (Hallek et al., 1992). As the human TK can functionally replace viral TK for its replication (Chen et al., 1998), the deletion of the viral TK gene allows the virus to replicate only in cancer cells with high TK activity (Varghese & Rabkin, 2002). The injection of the TK mutant HSV inhibited tumor growth and prolonged the survival of mice bearing malignant glioma (Martuza et al., 1991). In addition to direct tumor lysis by oncolytic viruses, recent studies have demonstrated that viral infection can indirectly destroy uninfected cancer cells by disrupting the tumor vasculature as well as promoting antitumor immunity (Russell et al., 2012). As a result, cancer immune therapies using oncolytic viruses have been actively examined at both the basic and clinical research levels (Lichty et al., 2014).

### **1.1.2 Molecular mechanisms of viral oncolysis**

Oncolytic viruses exploit tumor-specific molecular changes in cancer cells for their replication such as p53 deficiency (Bischoff et al., 1996), oncogenic Ras activation (Strong et al., 1998) and defects in the type I interferon (IFN)-induced antiviral response (Stojdl et al., 2000 and Stojdl et al., 2003).

### **1.1.2.1 P53 deficiency**

The p53 tumor suppressor is functionally inactive in cancer cells due to frequent deletion or mutation of the gene. Oncolytic adenovirus ONYX-015 was engineered to exploit cancer-specific deficiency of p53 for its replication by deleting the viral E1B-55kDa gene (Bischoff et al., 1996). The viral protein E1B-55kDa can bind and inactivate cellular p53, which normally induces apoptosis as an antiviral response. The mutant virus cannot inactivate p53, thus selectively replicates only in cells lacking functional p53, which is a common defect in cancer cells (Patel & Kratzke, 2013).

### **1.1.2.2 Ras-dependency**

Ras-dependent oncolysis was first reported as the mechanism responsible for reovirus oncolysis (Strong et al., 1998). Although type III reovirus (Dearing) cannot infect NIH3T3 cells, transformation of NIH3T3 cells by epidermal growth factor receptor (EGFR) (Strong et al., 1993), v-erbB (Strong & Lee, 1996), or constitutively active son of sevenless (Sos) or Ras (Strong et al., 1998) makes NIH3T3 cells susceptible to reovirus infection. Following these studies, the ability of reovirus to destroy cancer cells has been extensively studied both in animal models and in clinical settings, making reovirus a promising anti-cancer agent (Norman & Lee, 2005).

Since the discovery of Ras-dependent oncolysis of reovirus, other viruses including adenovirus (VAI mutant), bovine herpesvirus 1, HSV, influenza virus (delNS1 strain), NDV, poliovirus, and VSV were found to similarly exploit the activated Ras signaling pathway for their oncolysis (Balachandran et al., 2001; Bergmann et al., 2001; Farassati et al., 2001; Cascallo et al., 2003; Goetz et al., 2010; Puhlmann et al., 2010; and

Cuddington & Mossman, 2014). Multiple cellular mechanisms have been identified that underlie the Ras-dependent viral oncolysis including inhibition of antiviral activity of dsRNA activated protein kinase R (PKR) (Strong et al., 1998 and Bergmann et al., 2001), promotion of uncoating and release of oncolytic reovirus (Marcato et al., 2007), increase in the efficiency of cap-independent translation of oncolytic poliovirus (Goetz et al., 2010), and disruption of type I IFN-induced antiviral response (Battcock et al., 2006; Christian et al., 2009).

### **1.1.2.3 IFN insensitivity**

Another concept of viral oncolysis is to exploit IFN defects in cancer cells by IFN-sensitive viruses (Stojdl et al., 2000). Insensitivity of cancer cells to IFN is one of the major obstacles of IFN therapy in cancer patients (B. X. Wang et al., 2011). By systematically testing a panel of human cancer cells, Stojdl et al. (2000) demonstrated that cancer cells generally have lower sensitivity to IFN than the normal cells.

Subsequently, the same group has found that IFN-sensitive mutant VSVdel51 efficiently replicates in cancer cells but shows limited replication in normal cells even in the absence of exogenous IFN (Stojdl et al., 2003). This was attributed to an inability of mutant VSV matrix protein to block the production of IFN in infected cells (Stojdl et al., 2003). Since then, disarming anti-IFN proteins of wild-type viruses became one of the common strategies in designing novel oncolytic viruses to increase tumor specificity. The examples of such viruses include the NS1 deletion mutant of influenza A virus (IAV) (Muster et al., 2004) and the V deletion mutant of NDV (Elankumaran et al., 2010).

## **1.2. Ras**

### **1.2.1 Ras-Raf-MEK-ERK pathway**

Ras belongs to the family of small GTPases that function as molecular switches to transduce external cellular signals to the nucleus by cycling between an inactive GDP-bound state and an active GTP-bound state. Three Ras genes, *H-Ras*, *N-Ras*, and *K-Ras* have been characterized in humans (Rocks et al., 2006). These isoforms have a high degree of sequence homology but can localize to different subcellular membrane compartments depending on post-translational lipid modifications of the C-terminus, which functions as a membrane anchor (Rocks et al., 2006). The activity of Ras is activated by guanine nucleotide exchange factors (GEFs) that facilitate exchange of GDP for GTP, and suppressed by GTPase-activating proteins (GAPs) that facilitate exchange of GTP for GDP (Rocks et al., 2006).

In an active GTP-bound state, Ras recruits and activates its downstream effector Raf kinase at the plasma membrane. Activated Raf further phosphorylates another serine/threonine kinase Mitogen-activated protein kinase/ERK Kinase (MEK) 1/2, which in turn, phosphorylates Extracellular-signal-Regulated Kinases (ERK) 1/2. Once activated, ERKs regulate transcriptional and translational activities that control multiple cellular processes including cell growth, differentiation, proliferation, adhesion, migration, and apoptosis (Santarpia et al., 2012).

### **1.2.2 Dysregulation of Ras-Raf-MEK-ERK pathway in cancer**

The Ras-Raf-MEK-ERK cascade is often dysregulated in human cancer cells (Santarpia et al., 2012). Nearly 30 % of all human cancers have activating point mutations in Ras

(Bos, 1989). The most common mutations are glycine to valine mutation at residue 12 (G12V) and glutamine to lysine mutation at residue 61 (Q61K) (Malumbres & Barbacid, 2003). Mutations at these residues interfere with the transition state of GTP hydrolysis, thereby resulting in a constitutively active Ras bound to GTP. As such, the rate of GTPase activity of oncogenic H-Ras has been shown to be approximately 300-fold lower than the activity of normal H-Ras (Malumbres & Barbacid, 2003). The frequency of Ras mutation varies depending on the cancer types. K-Ras is most commonly mutated in tumors originating from pancreas, large intestine, small intestine, lung, endometrium, or ovary, while N-Ras mutations are most frequently found in tumors originating from the skin, nervous system, and hematopoietic and lymphoid tissues. H-Ras mutations are most prevalent in tumors originating from the salivary gland and urinary tract (Santarpia et al., 2012).

The Ras-Raf-MEK-ERK pathway can be also activated by aberrant activation of its upstream signaling components of Ras, such as EGFR, erb-b2 receptor tyrosine kinase 2 (HER2/neu), or SRC proto-oncogene non-receptor tyrosine kinase (SRC). Furthermore, activating mutation of Raf is commonly found in malignant melanoma, thyroid, colorectal, and ovarian tumors (Santarpia et al., 2012). The B-Raf mutation, which has a substitution of valine for glutamic acid at residue 600 (V600E), is found in approximately 7 % of all cancers (Garnett & Marais, 2004). Overall, the majority of cancer cells have activated Ras-Raf-MEK-ERK pathway.

### **1.2.3 ERK downstream elements**

ERK1 and ERK2 are 44 and 42 kDa serine/threonine kinases that are expressed in most



mammalian tissues. MEK1/2 phosphorylate ERK1 at residue Thr202 and Tyr204 and ERK2 at residue Thr185 and Tyr187. Upon phosphorylation, ERK1/2 in turn phosphorylate and activate MAPK-interacting kinases (MNKs) as well as the Ribosomal S6 Kinases (RSKs) in the cytoplasm (Roux & Blenis, 2004). Furthermore, ERK1/2 can translocate into the nucleus where they activate Mitogen- and Stress-activated Protein Kinases (MSKs) (Yoon & Seger, 2006).

#### **1.2.3.1 Ribosomal S6 kinases**

Ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases that regulate multiple cellular processes including cell growth, motility, survival, and proliferation. Four members of RSK family (RSK1, RSK2, RSK3 and RSK4) have been identified in human (Anjum & Blenis, 2008). Although RSKs share a high degree of sequence homology in amino acid sequence (75-80%), increasing evidence suggests they have distinct roles in regulating cellular functions (Anjum & Blenis, 2008). RSKs are found in both the cytoplasm and the nucleus. RSK1-3 translocate into the nucleus upon their phosphorylation while RSK4 remains predominantly in the cytoplasm (Dummler et al., 2005). RSK4 is expressed at much lower level compared to the other members, and is constitutively active in the absence of upstream signal (Anjum & Blenis, 2008).

All RSKs consist of two functionally distinct kinase domains. The N-terminal kinase domain (NTKD) is homologous to the protein kinase A, G, and C families (AGC family kinases), and is responsible for substrate phosphorylation (Jones et al., 1988). In contrast, the C-terminal kinase domain (CTKD) belongs to the calcium/calmodulin-dependent protein kinase (CaMK) family, and autophosphorylates its NTKD (Fisher &

Blenis, 1996). These two kinase domains are connected by a linker region (Anjum & Blenis, 2008). The C-terminal tail contains a D domain, which serves as a docking site for ERKs (Anjum & Blenis, 2008). Activated ERKs bind to the D domain and phosphorylate the CTKD. Phosphorylated CTKD autophosphorylates its linker region, creating a docking site for 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1, in turn, phosphorylates the NTKD, resulting in a complete activation of RSKs (Anjum & Blenis, 2008).

RSKs regulate transcription through phosphorylation of various transcription factors involved in immediate-early gene expression, such as cyclic AMP response element-binding protein (CREB), c-FOS, c-JUN, and serum response factor (SRF) (Shahbazian et al., 2006). Immediate early genes, also known as primary response genes, can be expressed within 5 to 10 minutes of stimulation since they can be expressed without *de novo* protein synthesis. These genes are important regulators of the secondary response genes, which require *de novo* protein synthesis to be expressed (Fowler et al, 2011).

RSKs also promote mRNA translation by phosphorylating translational regulators including the translation initiation factor-4B (eIF4B) (Shahbazian et al., 2006) and the 40S ribosomal subunit protein S6 (rpS6) (Roux et al., 2007). They can also phosphorylate and inactivate glycogen synthase kinase 3- $\beta$  (GSK3 $\beta$ ) (Sutherland et al., 1993) and elongation factor-2 kinase (eEF2K) (X. Wang et al., 2001), which further promotes protein synthesis.

### **1.2.3.2 Mitogen- and stress-activated protein kinases**

Mitogen and stress activated protein kinase 1 (MSK1, also known as PLPK) and MSK2 (also known as RSKB) regulate transcription in response to various cellular stimuli (Vermeulen et al., 2009). Unlike RSKs, MSKs are activated by multiple upstream kinases. Mitogenic signals induced by epidermal growth factor (EGF) and 12-O-tetradecanoylphorbol-13-acetate (TPA) activate MSKs through ERKs, while stress signals induced by UV-radiation and hydrogen peroxide activate MSKs through p38 MAPKs (Vermeulen et al., 2009). Furthermore, proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- $\alpha$ ) activates MSKs through both ERK and p38 MAPK pathways (Tomas-Zuber et al., 2000). MSKs, structurally related to RSKs, have CTKD and NTKD separated by a linker region. MSK1 is activated by binding of ERKs or p38 MAPKs to the C-terminal docking domain, which phosphorylates MSK1 within the CTKD. The phosphorylated CTKD subsequently autophosphorylates and activates its NTKD region (Vermeulen et al., 2009). MSKs have a nuclear localization sequence in their C-terminus, and are predominantly found in the nucleus (Tomas-Zuber et al., 2001).

Functionally, MSKs regulate transcription of immediate early genes by phosphorylating various transcription factors and nucleosome associated proteins. MSKs regulate transcriptional activity of CREB, which is a transcription factor constitutively bound to the CRE promoter element (Montminy & Bilezikjian, 1987). Upon phosphorylation by MSKs, CREB recruits its transcriptional coactivators CREB-binding protein (CBP) and p300 to the promoter. The coactivators possess histone acetyltransferase activity and together activate transcription of the CREB-regulated genes

(Wiggin et al., 2002). MSKs can also regulate the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) in response to TNF- $\alpha$  (Vermeulen et al., 2003). NF- $\kappa$ B is sequestered in an inactive state in the cytoplasm by inhibitor of  $\kappa$ B (I $\kappa$ B), which masks the nuclear localization signal of NF- $\kappa$ B. I $\kappa$ B kinase (IKK) phosphorylates and degrades I $\kappa$ B, leading to nuclear translocation of NF- $\kappa$ B. In the nucleus, MSKs phosphorylate the p65 subunit of NF- $\kappa$ B to promote its interaction with CBP and p300. This cascade results in the activation of NF- $\kappa$ B-regulated genes (Vermeulen et al., 2003). Furthermore, MSK1 and 2 were both shown to phosphorylate histone 3 (H3) at Ser10, a chromatin modification linked to gene expression, indicating that they can modulate the chromatin environment as well (Soloaga et al., 2003).

### **1.2.3.3 MAPK-interacting kinases**

The mitogen-activated protein kinase (MAPK) interacting protein kinases 1 (*MNK1*) and *MNK2* are serine/threonine kinases that play an important role in mRNA translation. Similar to MSKs, MNKs can be activated by either ERKs or p38 MAPKs in response to growth factors, cellular stress, and proinflammatory cytokines.

MNKs consist of a C-terminal MAPK interacting domain and a catalytic domain that is similar to the CaMK family of kinases (Waskiewicz et al., 1997). ERKs or p38 MAPKs activate MNKs by binding to the C-terminal MAPK binding domain, and phosphorylating at least two threonine residues within its kinase domain (Waskiewicz et al., 1997).

The major downstream target of the MNKs is a cap binding eukaryotic initiation factor 4E (eIF4E), which acts as a component of the eukaryotic initiation factor eIF4F and a rate-limiting determinant of protein synthesis (Clemens & Bommer, 1999). MNKs do not directly bind to eIF4E, but interact with the scaffolding protein eIF4G to bring MNKs and eIF4E into close proximity. This enables MNKs to phosphorylate eIF4E (Joshi & Platanias, 2014). The consequence of eIF4E phosphorylation on mRNA translation has not been elucidated (Scheper & Proud, 2002). In addition, nuclear eIF4E can also bind to mRNAs containing an eIF4E-sensitive element in their 3'UTR to promote nuclear export of mRNA (Culjkovic et al., 2005). Phosphorylation of eIF4E by MNK promotes the nuclear export of eIF4E-bound mRNA (Phillips & Blaydes, 2008). Many of the mRNAs regulated by the nuclear eIF4E are known to promote cell growth (Strudwick & Borden, 2002).

Another downstream target of MNKs is the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1). HnRNPA1 is a RNA binding protein bound to the AU rich elements in the 3'UTR of mRNAs that blocks initiation of translation. Upon phosphorylation by MNKs, hnRNP1 dissociates from the 3'UTR, allowing translation to initiate (Buxade et al., 2005).

#### **1.2.4 MEK inhibitors**

Due to high prevalence of activation of the MAPK pathway in different types of tumors and its important roles in cancer growth and survival, a number of small molecule inhibitors of MEK have been developed and are currently being tested in clinical trials (Friday & Adjei, 2008). The first MEK inhibitor identified was PD98059. The compound

binds to an inactive form of MEK1/2 and prevents their activation by Raf. PD98059 has a higher affinity for MEK1 inhibition ( $IC_{50}=2-7\mu M$ ) compared to that of MEK2 inhibition ( $IC_{50}=50\mu M$ ) (Alessi et al., 1995 and Dudley et al., 1995). Another inhibitor commonly used in basic research is U0126 (Favata et al., 1998), which inhibits both MEK1 ( $IC_{50}=70nM$ ) and MEK2 ( $IC_{50}=60nM$ ) more potently than PD98059 (Duncia et al., 1998). U0126 and PD98059 are both allosteric inhibitors and are non-competitive with respect to MEK substrates and ATP (Favata et al., 1998). These inhibitors have been shown to exert anti-proliferative effects on various cancer cell lines (Alessi et al., 1995 and Favata et al., 1998). Since their discovery, these inhibitors have become powerful tools to study MAPK signal transduction in *in vitro* studies. Subsequently, a number of other small inhibitors of MEK have been developed, some of which progressed into clinical trials (Akinleye et al., 2013). In 2013, the FDA approved the first MEK inhibitor GSK1120212 (Mekinist) for treatment of advanced melanoma expressing B-Raf mutation. GSK1120212 is an orally bioavailable, potent, small allosteric inhibitor of MEK ( $IC_{50}=0.7-0.9nM$ ), which inhibits MEK in an ATP-non-competitive manner (Gilmartin et al., 2011).

## **1.3 Interferon (IFN)**

### **1.3.1 IFN Classification**

IFNs are a group of secreted cytokines that can function in both an autocrine and a paracrine manner to block virus replication (Borden et al., 2007). IFNs are classified into three groups (type I, II, and III) based on their sequence. Type I IFN is the largest group,

consisting of IFN- $\alpha$  (13 subtypes), IFN- $\beta$ , IFN- $\omega$ , IFN- $\epsilon$ , and IFN- $\kappa$  in humans (Gibbert et al., 2013). They belong to the helical cytokine family, which are mostly non-glycosylated proteins with size ranging from 165-200 amino acids (AA) (Borden et al., 2007). Members of type I IFN share approximately 30-85% amino acid sequence homology (Borden et al., 2007). Various cell types respond to viral infections by producing type I IFNs. While plasmacytoid dendritic cells (pDCs) are the most potent inducers of IFN- $\alpha$  (Asselin-Paturel & Trinchieri, 2005), IFN- $\beta$  is predominantly produced by fibroblasts and epithelial cells (Ivashkiv & Donlin, 2014). Type I IFNs signal through two transmembrane proteins, interferon (alpha, beta and omega) receptor 1 (IFNAR1) and IFNAR2, which are broadly expressed on most cell types (Borden et al., 2007).

The only member of the type II IFN group is IFN- $\gamma$ ; a single glycosylated protein of 140AA produced predominantly by natural killer (NK) or activated T cells (Shi & Van Kaer, 2006). Type II IFNs function through a receptor consisting of the heterodimer of two receptor chains, IFN gamma receptor 1 (IFNGR1) and IFNGR2. Unlike the other types of IFNs, the primary function of IFN- $\gamma$  is to activate cell-mediated immune responses against pathogen or tumor (Borden et al., 2007).

Type III IFNs have been recently identified as members in the IFN family. These consist of three subtypes, IFN- $\lambda$ 1, IFN- $\lambda$ 2 and IFN- $\lambda$ 3 (Kotenko et al., 2003 and Sheppard et al., 2003). While pDC are the most potent producers of IFN- $\lambda$ , most cell types can induce IFN- $\lambda$  in response to viral infection. These cytokines signal through a heterodimeric receptor complex composed of interleukin 10 receptor 2 (IL10R2) and IFN

lambda receptor 1 (IFNLR1) (Kotenko et al., 2003 and Sheppard et al., 2003). Although the type III IFNs activate distinct receptor complexes, they function through the same intracellular signaling pathway as type I IFNs and consequently activate similar antiviral responses. However, since the expression of IFN- $\lambda$  receptor is restricted to cells of the epithelial origin, they exert antiviral effects only in specific cell types (Donnelly & Kotenko, 2010).

### **1.3.2 IFN production**

IFNs are synthesized when pattern recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs) during virus infection. Viral dsRNA are recognized by transmembrane protein Toll-like receptor 3 (TLR3) localized at the endosomal membrane. RNA helicases including retinoic acid-inducible gene I (Rig-I) and melanoma differentiation associated protein 5 (MDA5), both localized in the cytoplasm, can also detect dsRNA or RNA with 5'-triphosphates. TLR7 and TLR9 at the endosomal membranes detect viral ssRNA or DNA, respectively, while TLR2 and TLR4 on the cell surface detect viral proteins (Kawai & Akira, 2010). Finally, DNA-dependent activator of IFN-regulatory factors (DAI) and cyclic GMP-AMP (cGAMP) synthase (cGAS) are recently identified members of PRRs that function as cytosolic DNA sensors (Paludan & Bowie, 2013 and L. Sun et al., 2013).

Once the viral products are recognized by the PRRs, a series of signaling events is induced that leads to the activation of transcriptional activators of IFN, including interferon regulatory factor (IRF) 3, IRF7 and NF- $\kappa$ B. TANK binding kinase 1 (TBK1) or inducible I $\kappa$ B kinase (IKK $\epsilon$ ) phosphorylate IRF3 and IRF7 in the cytoplasm to induce



their dimerization and translocation into the nucleus. The I $\kappa$ B kinase also activates NF- $\kappa$ B by phosphorylating the inhibitor of NF- $\kappa$ B (I $\kappa$ B) to causes its degradation and subsequently releases NF- $\kappa$ B to translocate into the nucleus. In the nucleus, these transcription factors are all assembled on the IFN promoter to activate its transcription (Borden et al., 2007).

### **1.3.3 Jak/Stat pathway**

IFN signaling components are sufficiently expressed under normal conditions and become activated by their phosphorylation upon IFN stimulation during viral infection. This rapid response is essential for IFNs to be an important first line of defense against viral infection. Briefly, the binding of IFN- $\alpha/\beta$  to their receptors leads to activation of the two receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which are both located at the cytoplasmic domain of each IFN receptor chain. Activated JAK1 and TYK2 phosphorylate the receptor chains to induce the recruitment and activation of signal transducer and activator of transcription 1 (STAT1) and STAT2. The complex of phosphorylated STAT1 and STAT2 associate with IRF9 to form a heterotrimeric complex called IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex then translocates into the nucleus and binds to IFN-stimulated response elements (ISRE; consensus sequence TTTCNNTTTC) present within the promoter of IFN-stimulated genes (ISGs). These signaling events lead to transcriptional activation of hundreds of ISGs with antiviral, antitumor, and immune-modulatory functions (Ivashkiv & Donlin, 2014).

### 1.3.4 IFN induced transcription

Although activated IFNAR signal primary through the Jak/STAT pathway and lead to the formation of ISGF3, different STAT homodimers or heterodimers can also be activated upon IFNAR stimulation. These alternate STATs promote expression of genes containing gamma-activated sequences (GAS) element (van Boxel-Dezaire et al., 2006). Type I IFN-induced transcription can be further regulated by other transcription factors, such as IRF1, IRF7, IRF8, and IRF9 that bind to IRF-binding element (IRFE) present in the promoter regions of many ISGs (Ivashkiv & Donlin, 2014). These IRFs are present at low basal levels, and IFN stimulation causes their *de novo* protein synthesis. IRFs in turn initiate the activation of secondary responsive ISGs (Ivashkiv & Donlin, 2014). In contrast to the IRFs, FOXO3 is a transcriptional repressor of several ISGs that reduces their expression to a basal level after their initial activation by IFN stimulation (Litvak et al., 2012).

In addition, post-translational modification of STATs can regulate the type I IFN signaling and therefore expression of ISGs. Addition of small ubiquitin related modifier (SUMO) proteins to STAT1 suppress ISG expression by interfering with the DNA binding activity of STAT1 (Shuai & Liu, 2005). ISG expression is regulated further by modulations at the level of chromatin. This is initiated by histone acetyltransferases, histone deacetylases, histone methyltransferases, and nucleosome-remodeling enzymes (Ivashkiv & Donlin, 2014).

The ISGs induced by IFNs protect host cells from virus infection in different ways. First, the ISGs include antiviral genes that play essential roles in IFN production

(Rig-I, MDA5, TLR) or IFN signaling (STAT1, STAT2, IRF7). Therefore, increased expression of these genes will further enhance cellular ability to inhibit viral replication. Second, IFNs induce chemokines and chemokine receptors that activate cell-to-cell communication of immune cells involved in antiviral immunity (CXCL9, CXCL10, CXCL11). Third, the ISGs encode proteins that directly inhibit viral entry, viral translation and replication or disrupt cellular machinery required for viral replication (PKR, OAS, RNASEL, Mx1/2, IFITM, TRIM). Lastly, IFN upregulates ISGs that promote apoptosis of virally infected cells (APO2L/TRAIL, Fas, XIAP) (Borden et al., 2007; Schneider et al., 2014, and Schoggins & Rice, 2011).

### **1.3.5 Clinical application of IFNs**

Type I IFNs have been clinically used for treatment of viral infection, cancer, and multiple sclerosis (MS) (Borden et al., 2007).

#### **1.3.5.1 Viral infection**

IFN- $\alpha$ 2a (Roferon-A) is used for treatment of hepatitis C virus (HCV) infection (Zeuzem et al., 2000). IFN efficacy was greatly improved by conjugating IFN- $\alpha$  with polyethylene glycol to form peg-interferon (PEG-IFN). The conjugation increases the IFN half-life by approximately 10-fold compared to non-pegylated IFN (Glue et al., 2000). Currently, HCV patients who do not have access to the newer direct-acting antiviral agents are treated with PEG-IFN in combination with a nucleoside inhibitor Ribavirin. Ribavirin interferes with the RNA metabolism of HCV (Hoofnagle & Seeff, 2006).

IFN- $\alpha$  is also used for treatment of infection with hepatitis B virus (HBV), (Borden et al., 2007). Current treatment for chronic HBV patients includes a combination of PEG-IFN- $\alpha$  and one of several reverse transcriptase inhibitors (lamivudine, adefovir, entecavir, or tenofovir) (R. M. Friedman, 2008).

### **1.3.5.2 Cancer**

Due to its antiproliferative and apoptotic effects, IFN- $\alpha$ 2b (Intron-A) is used for treatment of different types of cancers, including kaposi sarcoma (KS), melanoma, chronic myelogenous leukaemia (CML), and renal cell carcinoma (RCC) (B. X. Wang et al., 2011). Acquired immune deficiency syndrome (AIDS)-associated KS is a cancer caused by human herpes virus-8 (HHV-8) infection in AIDS patients. Approximately 35 % of these patients treated with IFN- $\alpha$ 2b have shown complete or partial remissions in their tumor (Qureshi et al., 2009). IFN- $\alpha$ 2b adjuvant therapy is currently used as a post-surgery treatment to improve both disease-free survival and overall survival in high-risk melanoma patients (Mocellin et al., 2010). Moreover, in CML patients, IFN- $\alpha$  treatment has been shown to induce long-term remission by activating cytotoxic T cells to target CML tumor antigens (Burchert & Neubauer, 2005). The most widely used systemic treatment for RCC patients is IFN- $\alpha$  and IL-2 therapy (Motzer & Russo, 2000). The combined treatment with IFN- $\alpha$  and IL-2 have lead to tumor regression in up to 80.6 % and disease-free survival at 200 days post-treatment in up to 63.6 % in RCC patients with lung metastasis (Akaza et al., 2010).

### **1.3.5.3 Multiple sclerosis**

MS is a progressive neurodegenerative disease characterized by the demyelination of nerves (Trapp et al., 1999). Approximately 85 % of the MS patients suffer from relapsing-remitting MS (RR-MS), which is characterized by a chronic immune response to the myelin (Borden et al., 2007). IFN- $\beta$ 1a (AVONEX; Rebif) and IFN- $\beta$ 1b (Betaseron) are currently used to treat these patients (Bermel & Rudick, 2007). IFN- $\beta$  treatment decreases both neurological symptoms and the numbers of lesions in the spinal cord (Rudick & Cutter, 2007). The therapeutic effect of IFN- $\beta$  on MS is attributed to immunoregulatory effects of ISGs, which inhibits inflammatory cells from crossing the blood-brain barrier (Bermel & Rudick, 2007).

Similar to any other medications, IFNs have side effects, especially when administered at high doses. Fever and chills are common symptoms, which can last for a few hours after the IFN treatment. Hematotoxic effects, including leucopenia and thrombocytopenia, may occur. Other symptoms include fatigue, anorexia, weight loss, and a reversible increase in the level of hepatic transaminases (Borden et al., 2007).

### **1.3.6 Cellular suppressors of the IFN pathway**

Although IFNs are used as a therapeutic for treatment of viral infections, cancer, and MS, they are not always effective (Borden et al., 2007). Cellular suppressors can interrupt the IFN signaling pathway and reduce the efficacy of IFNs. The p38 MAPK can phosphorylate type I IFN receptors, leading to additional phosphorylation by casein kinase-I. These series of phosphorylation events lead to the internalization, and

degradation of the IFNAR (Bhattacharya et al., 2010; Bhattacharya et al., 2011). The repression of IFNAR is considered to be one of the mechanisms used by cancer cells to evade the antitumor activities of IFNs (Fuchs, 2013).

In addition, many viruses have ability to evade the antiviral effects of IFN. These viruses are equipped with anti-IFN proteins that can counteract the IFN responses by inhibition of antiviral gene expression, inhibition of IFN production, interruption of the Jak-Stat signaling, or blocking the activity of IFN stimulated antiviral effectors (Randall & Goodbourn, 2008).

Protein kinase D2, which is often overexpressed or constitutively activated in cancer cells, can also phosphorylate to degrade IFNAR (Zheng et al., 2011). Furthermore, the SH2 domain-containing protein tyrosine phosphatase 1 (SHP1) and SHP2 dephosphorylate the signaling components of the Jak-Stat pathway to interrupt IFN response (Xu & Qu, 2008). The suppressor of cytokine signaling (SOCS) is another negative regulator of the Jak-Stat pathway, which competes with STATs for binding to IFNARs. Furthermore, SOCS contains a C-terminal SOCS box domain that recruits E2 ubiquitin ligase and promotes proteasome-mediated degradation of IFNARs (Yoshimura et al., 2007). Protein inhibitor of activated STAT-1 (PIAS1), which is known as an E3 SUMO protein ligase, inhibits STAT signaling by interfering with the DNA binding activity of STAT1 (B. Liu et al., 1998). Recent evidence indicates that certain microRNAs (miRNA)s can suppress the IFN signaling by decreasing the expression of the signaling components of the Jak-Stat pathway (Ivashkiv & Donlin, 2014).

Hirasawa lab and others have previously reported that activation of the Ras and its downstream element MEK, suppresses the host antiviral response induced by IFN,

clearly demonstrating that the two major mechanisms of viral oncolysis (Ras-dependency and IFN-deficiency) are indeed connected (Battcock et al., 2006 and Noser et al., 2007). A variety of mechanisms are involved in the suppression of IFN response by Ras, including downregulation of STAT1 (Klampfer et al., 2003) and/or STAT2 expression (Klampfer et al., 2003 and Christian et al., 2009).

#### **1.4 Connection between Ras-dependent and IFN-insensitivity-dependent viral oncolysis**

Our laboratory, under the direction of Dr. Hirasawa, has been investigating how cells can become susceptible to virus infection through activation of the Ras signaling pathway. Previously, our laboratory reported that an IFN-sensitive virus can replicate in cells with constitutively active Ras (RasV12 cells) despite the presence of IFN (Battcock et al., 2006). Noser et al. (2007) also reported that inhibition of Ras-Raf-MEK-ERK pathway in human cancer cell lines restored antiviral responses induced by IFN. These were the first reports to identify Ras/MEK as one of the cellular suppressors of IFN-induced antiviral response.

Follow up study by the Hirasawa lab further demonstrated that the transcription of STAT2, an essential component of IFN signalling, was suppressed by activated Ras/MEK (Christian et al, 2009). Both overexpression of STAT2 and MEK inhibition restored IFN sensitivity in RasV12 cells. However, while the MEK inhibition completely restored IFN-induced antiviral effects in RasV12 cells, the restoration by the STAT2 overexpression was only partial. This result suggested that the downregulation of STAT2

expression is not the sole mechanism involved in the Ras/MEK-mediated IFN suppression. Therefore, it was hypothesized that the Ras/MEK pathway also affects the transcription of other IFN-inducible genes in addition to STAT2 in order to suppress the antiviral response. This hypothesis was tested by examining the transcriptional changes induced by IFN treatment and MEK inhibition in human cancer cells through global gene expression profiling (Christian et al., 2012). Microarray analysis identified a novel group of IFN-inducible genes whose transcription is downregulated by the Ras/MEK pathway (MEK-Downregulated IFN-Inducible (MDII) genes). Together, these studies demonstrate that the two major mechanisms of viral oncolysis (Ras-dependency and IFN-deficiency) are indeed connected.

## **1.5 IRF1**

### **1.5.1 Biology of IRF1**

The IRF1 protein belongs to the interferon regulatory factor (IRF) family of transcription factors, which consists of nine members in mammals (IRF1-9). Many members of this family play critical roles in establishing host immune responses against pathogens or tumors. IRF1, which is involved in regulating many IFN inducible genes, is expressed by various cell types in response to viral infection or IFN stimulation (Miyamoto et al., 1988; Harada et al., 1989; Harada et al., 1990, and Yarilina et al., 2008). IRF1 has a N-terminal DNA binding domain, which consists of five-conserved tryptophan repeats forming a helix-turn-helix motif that binds to IRF-binding element (IRFE, G(A)AAA<sup>G/C</sup>T/CGAAA<sup>G/C</sup>T/C) or IFN-stimulated response element (ISRE,



<sup>A</sup>/GNGAAANNGAAACT) (Escalante et al., 1998).

### **1.5.2 Functions of IRF1**

IRF1 has antiviral and antitumor functions and is also known to regulate the development of immune cells. IRF1 exhibits its antiviral activity against a broad range of viruses (Schoggins & Rice, 2011). Viruses known to be sensitive to antiviral effects of IRF1 include HCV, human immunodeficiency virus (HIV), YFV, WNV, Venezuelan equine encephalitis virus, chikungunya virus, Sindbis virus (Schoggins et al., 2011), VSV (Stirnweiss et al., 2010 and Nair et al., 2014), and murine gammaherpesvirus 68 (Dutia et al., 1999). This is not surprising considering the ability of IRF1 to induce various IFN-stimulated antiviral effectors including OAS, ISG-15 (Au et al., 1992), Viperin (Stirnweiss et al., 2010). IRF1 also promotes expression of MHC-1, which is essential for displaying degraded antigenic peptides on the cell surface for recognition by cytotoxic T cells (Taniguchi et al., 2001). Expression of transporter associated with antigen presentation 1 (TAP1) and low molecular mass polypeptide 2 (LMP2) are regulated by IRF1, both of which positively regulate antigen processing and presentation by MHC class I (White et al., 1996). In addition, IRF1 promotes expression of MHC class II indirectly by activating the transcription of class II transactivator (CIITA), which functions as a transcriptional activator of MHC class II (Hobart et al., 1997 and Muhlethaler-Mottet et al., 1998).

The roles of IRF1 in immune cell development have been studied in IRF1<sup>-/-</sup> mice (Taniguchi et al., 2001). Mice deficient in IRF1 have defects in the development and activity of NK cells, most likely due to its inability to produce IL15, which is essential

for NK cell development (K. Ogasawara et al., 1998). CD8<sup>+</sup> T cell development is also impaired in IRF1<sup>-/-</sup> mice, thereby the cytotoxic T lymphocyte response to virally infected cells is greatly reduced in these mice. Furthermore, dendritic cell development is impaired in IRF1<sup>-/-</sup> mice, which is essential for T cell activation (Gabriele et al., 2006).

Another important function of IRF1 is the regulation of tumor suppressors and oncogenes. Tanaka et al. (1994) demonstrated that introduction of one oncogene (H-Ras) was sufficient to transform MEFs lacking IRF1 (Tanaka et al., 1994). This study clearly indicated a tumor suppressive role of IRF1, since wildtype MEFs are not transformed by a single oncogene, but requires the introduction of at least two independent oncogenes to be transformed (Weinberg, 1989). Subsequent to this study, IRF1 was reported to inhibit cell transformation induced by other oncogenes including c-myc, fos-B, IRF2, and EGFR (Harada et al., 1993; Tanaka, Ishihara, & Taniguchi, 1994; Kirchhoff & Hauser, 1999, and Kroger et al., 2003). The tumor suppressive effects of IRF1 are attributed to its ability to regulate genes involved in apoptosis and cell cycle arrest (Taniguchi et al., 2001). IRF1 mediates IFN-induced apoptosis in cancer cells by upregulating the expression and/or activity of caspase 1 (Kim et al., 2002), caspase 7 (Sanceau et al., 2000 and Tomita et al., 2003), caspase 8 (Ruiz-Ruiz et al., 2004), and TNF-related apoptosis-inducing ligand (Clarke et al., 2004). Furthermore, IRF1 induces cell cycle arrest upon DNA damage by upregulating the expression of cell cycle inhibitor p21<sup>WAF1/CIP1</sup> (Tanaka et al., 1996).

IRF1 expression is frequently dysregulated in cancer. The IRF1 gene is often lost in leukemia, preleukemic myelodysplastic syndrome (Boulwood et al., 1993; Willman et al., 1993), esophageal (Ogasawara et al., 1996) and gastric cancers (Tamura et al., 1996).

In addition, inactivating mutations of IRF1 have been identified in gastric cancer (Nozawa et al., 1998). Even without genetic abnormality to IRF1 sequence, its activity can be lost due to aberrant splicing (Harada et al., 1994 and Tzoanopoulos et al., 2002), overexpression of IRF1 binding proteins with inhibitory activity (Kondo et al., 1997 and Narayan et al., 2011), or increased level of IRF1 sumoylation which attenuates its transcriptional activity (Park et al., 2007).

### **1.5.3 Post-translational modifications of IRF1**

Several studies have demonstrated that post-translational modifications regulate transcriptional activity and protein stability of IRF1 (Nakagawa & Yokosawa, 2000; Nakagawa & Yokosawa, 2002; Qiu et al., 2014, and Watanabe et al., 1991) (Figure 1.1). In many cases, overexpression of IRF1 is not sufficient to restore its transcriptional activity, but further stimulation is required to maximize its function (Lallemant et al., 2007).

**Figure 1.1** Post-translational modification sites of IRF1. IRF1 consists of DNA binding domain (AA1-120), nuclear localization signal (NLS, AA115-139), transactivation domain (AA185-256), and enhancer domain (AA256-325) (Schaper et al., 1998). Previously reported PTM sites are indicated, with acetylation (Ac) in orange, phosphorylation (P) in yellow, sumoylation (S) in blue, and ubiquitination (Ub) in green.

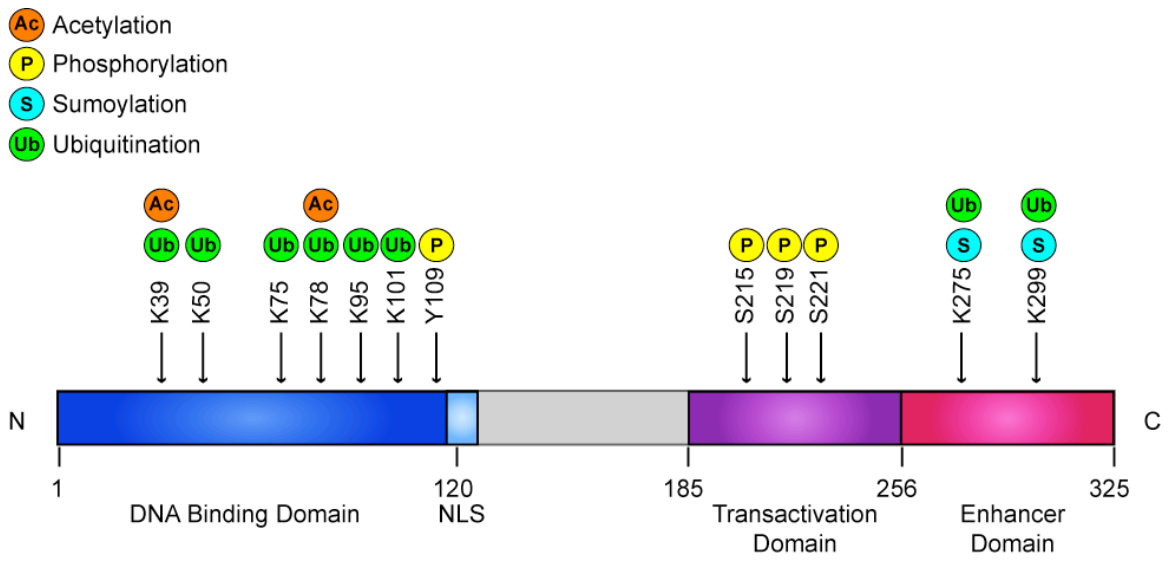


Figure 1.1

### 1.5.3.1 Phosphorylation

IRF1 phosphorylation was first reported in myeloid cell line U937 in response to IFN- $\gamma$  stimulation (Kautz et al., 2001 and Sharf et al., 1997). Several protein kinases have been identified to phosphorylate IRF1 in a cell type specific manner. IKK- $\alpha$  phosphorylated IRF1 during TLR9-induced IFN- $\beta$  production in dendritic cells (DCs) (Hoshino et al., 2010). IKK- $\beta$  phosphorylated IRF1 in response to IFN- $\gamma$  stimulation (Shultz et al., 2009). The serine/threonine kinase casein kinase II was demonstrated to phosphorylate IRF1 *in vitro* at two regions, AA138-150 and the C-terminal AA219-231. Mutation of C-terminal phosphorylation site AA219-231 disrupted ability of IRF1 to activate the IFN- $\beta$  promoter (Lin & Hiscott, 1999). In contrast, phosphorylation of IRF1 at Ser215, 219, and 221 by IKK- $\epsilon$  in primary CD4<sup>+</sup> T cells was shown to inhibit transcriptional activity of IRF1 (Sgarbanti et al., 2014).

### 1.5.3.2 Ubiquitination

Ubiquitin belongs to a family of modifier proteins that are covalently attached to target proteins. Addition of ubiquitin to target protein (ubiquitination) is regulated by three enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). A protein to be ubiquitinated carries a specific degradation signal (degron), which is recognized by the ubiquitin enzymes. The substrate specificity is mediated by the E3 ligase, which binds to the degron in a target protein, and assists in the transfer of ubiquitin molecule from E2 to lysine residues in the target protein (Ciechanover, 1994). Monoubiquitination, an addition of one ubiquitin molecule to a

target protein, alters protein functions involved in membrane trafficking and endocytosis (Komander, 2009). Polyubiquitination adds multiple ubiquitin molecules on a single lysine residue on the substrate protein in order to regulate proteosomal degradation and protein functions involved in endocytosis, DNA-damage response and cell signaling (Komander, 2009).

Several studies have demonstrated that IRF1 can be degraded by the ubiquitin-proteasome pathway and that the C-terminal enhancer domain of IRF1 is essential for directing its poly-ubiquitination (Nakagawa & Yokosawa, 2000 and Pion et al., 2009). Furthermore, a recent study reported that IRF1 function is promoted by Lys63-linked poly-ubiquitination in response to IL-1 stimulation in human embryonic kidney HEK293 cells, indicating that poly-ubiquitination of IRF1 is essential for regulation of its protein stability and transcriptional activity (Harikumar et al., 2014).

### **1.5.3.3 Sumoylation**

Small ubiquitin-related modifier (SUMO) proteins belong to a family of ubiquitin-like protein modifiers that are covalently attached to proteins to alter their function. The process of sumoylation is similar to that of ubiquitination in that it involves three enzymes, SUMO-activating enzyme (E1), SUMO conjugating enzyme (E2), and SUMO ligase (E3). A SUMO molecule is first activated by E1 enzyme, resulting in formation of a thioester bond between the C-terminal glycine of SUMO and catalytic cysteine of E1 enzyme. The E2 enzyme enables the formation of an isopeptide bond between the activated SUMO and a lysine residue on a target protein, and the E3 ligase stabilizes the interaction between the target protein and the E2 enzyme (Flotho & Melchior, 2013).

Sumoylation is a reversible process, since the SUMO tag, conjugated to the target protein, can be removed by SUMO proteases. Therefore, both SUMO specific enzymes and SUMO specific proteases regulate sumoylation.

IRF1 has been reported to undergo sumoylation mediated by the E2 enzyme Ubc9 and the E3 ligase PIAS3 (Nakagawa & Yokosawa, 2002). IRF1 sumoylation inhibits its ability to regulate transcription (Nakagawa & Yokosawa, 2002; Park et al., 2007, and Kim et al., 2008). However, the SUMO proteases responsible for desumoylating IRF1 have not been identified.

#### **1.5.3.4 Acetylation**

Acetylation is another type of post-translational modification that can regulate protein function in many ways (Choudhary et al., 2009). Lysine acetylation can alter transcription factor activity and interactions of proteins containing bromodomains. IRF1 is acetylated by the transcriptional co-activator p300 (Masumi & Ozato, 2001; Marsili et al., 2004; Qi et al., 2012, and Qiu et al., 2014). Acetylation promotes DNA binding and transcriptional activity of IRF1 (Qi et al., 2012 and Qiu et al., 2014).

### **1.6 mRNA translation**

#### **1.6.1 Regulation of mRNA translation**

The translation of mRNA is a critical regulatory point in gene expression, and is controlled primarily at the initiation stage, in which the elongation competent 80 S ribosomes assemble on mRNA. The canonical mechanism of eukaryotic translation initiation involves several steps starting with formation of the 43 S pre-initiation complex



consisting of a 40 S ribosomal subunit, eIF1, eIF1A, eIF3, eIF2-GTP-Met-tRNA. The mRNA is next activated by cap-binding complex eIF4F (consisting of eIF4E, eIF4G, and eIF4A). The 43 S complex is then attached to 5'-UTR, which then scans the mRNA until it reaches an initiation codon. Next 48 S initiation complex is formed by displacement of eIF1 and hydrolysis of eIF2-GTP and Pi release from 43 S complex. Lastly, initiation factors are displaced and 60 S ribosomal subunit is joined to 48 S complex, resulting in formation of an elongation-competent 80 S ribosomes on mRNA (Orom et al., 2008).

### **1.6.2 5'-and 3'-UTR of mRNA**

Initiation of translation is regulated by elements present within the untranslated regions (UTRs) of mRNA. The 5'-cap and the 3'-poly(A) tail are both essential for initiation of translation as it serves as a binding site for eIF4F and poly-(A) binding proteins (PABP)s, respectively.

The 5'-UTR may contain secondary structure or other elements that can serve as binding sites for regulatory proteins that interfere with the binding or scanning process during translational initiation (Wilkie et al., 2003). Furthermore, presence of upstream initiation codons or upstream open reading frames (ORFs) can reduce the rate of translation initiated from the main ORF, since ribosomes can detach from the mRNA after completing translation of the upstream ORF (Mignone et al., 2002).

Moreover, the length of poly-(A) tail and binding of PABPs to the 3'-UTR influence translation efficiency. They play essential roles in initiating and stabilizing circularization of the mRNA and enhancing recruitment of small ribosomal subunits to the 5'-UTR (Wilkie et al., 2003). In contrast, binding of 3'-UTR binding protein

cytoplasmic polyadenylation-element-binding protein (CPEB) represses translation by interfering with PABP binding to 3'-UTR (Wilkie et al., 2003). Moreover, non-coding RNAs can bind to either 3'- or 5'-UTR of mRNA to suppress (Fabian et al., 2010) or sometimes enhance efficiency of translation (Vasudevan et al., 2007 and Orom et al., 2008).

### **1.6.3 microRNA**

microRNAs (miRNAs) are non-coding small RNAs of approximately 22 nucleotides in length that play important roles in regulation of gene expression at the post-transcriptional level (Bartel, 2004). miRNAs suppress target gene expression by two major mechanisms. Binding of miRNA can lead to cleavage and degradation of the target mRNA when the base-pairing between the miRNA and the target mRNA matches fully complementary or nearly complementary. This mechanism is originally discovered in plants (Rhoades et al., 2002). In contrast, most animal miRNAs repress translation of the target mRNA without affecting its stability by base-pairing with partially complementary sequences (Olsen & Ambros, 1999). miRNAs are first transcribed into long primary transcripts called pre-miRNAs, which are then processed into mature miRNA duplex structure by two enzymes Droscha and Dicer. One strand of miRNAs is then loaded into an Argonaute family protein, forming RNA-induced silencing complex (miRISCs), which binds and represses gene expression of the target mRNA (Huntzinger & Izaurralde, 2011). Four types of translational repression mechanisms have been proposed in mammals, including inhibition of translation initiation, inhibition of translational elongation, co-translational protein degradation, and premature translation termination

(Huntzinger & Izaurralde, 2011). However the precise mechanisms underlying gene silencing by miRNAs still remain largely unknown. Hundreds of miRNAs have been identified in animals (Bartel, 2009), and computational approaches have predicted that as many as 30-50% of human transcriptome may be subject to miRNA regulation (Lewis et al., 2005 and Friedman et al., 2009).

Papers arising from this thesis:

**Oncogenic Ras inhibits IRF1 to promote viral oncolysis.** Yumiko Komatsu, Sherri. L Christian, Nhu Ho, Theerawat Pongnopparat, Maria Licursi, and Kensuke Hirasawa. *Oncogene* (2015). 23;34(30):3985-93.

The author primarily performed all work presented in this thesis except for the graciously acknowledged contribution of Dr. Sherri Christian for performing the microarray analysis in Figure 3.1A.

**CHAPTER 2**  
**MATERIALS AND METHODS**

## **2.1 Cell culture**

Murine fibroblast cells (NIH3T3 and L929) and human cancer cells (HT29, HT1080, DLD-1 and MDA-MB-468) were obtained from the American Type Culture Collection. These cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Life Technologies (Burlington, ON) with 10% fetal bovine serum (Cansera, Etobicoke, ON). Vector control NIH3T3 and RasV12 cells were generated as previously described (Battcock et al., 2006). IRF1 deficient and wildtype MEFs were established from C57BL/6-Irf1<sup>tm1Mak</sup> (Tanaka et al., 1994) and C57BL/6J mice purchased from the Jackson Laboratory (Bar Harbor, ME), respectively. MEF cell cultures were obtained from day 14 embryos that were washed three times in PBS, minced, and digested in 0.25 % trypsin/EDTA solution (Tanaka et al., 1994). After digestion, MEFs were maintained in DMEM supplemented with 10 % FCS, 2 mM L-Glutamine (Life Technologies), Antibiotic-Antimycotic (Life Technologies), and MEM Non-Essential Amino Acids (Life Technologies). Immortalized IRF1 deficient MEFs were established by serial passages of primary MEFs over the course of 7 passages over 21 days.

## **2.2 DNA Microarray Analysis**

DNA microarray analysis was conducted in collaboration with Dr. Sherri Christian at the department of Biochemistry, Memorial University of Newfoundland (St. John's, NL). RasV12 cells were treated with 20  $\mu$ M U0126 (Cell Signaling Technology, Danvers, MA) or 500 U/ml recombinant mouse IFN- $\alpha$  (PBL Interferon Source, Piscataway, NJ), or left untreated, for 6 hours. Total RNA was isolated using TRIzol® Reagent (Life

Technologies), treated with DNase using TURBO DNA-free™ kit (Ambion, ON), and then sent to the Centre for Applied Genomics (TCAG, ON) for analysis using Affymetrix 430 2.0 mouse DNA microarrays. RNA integrity number was determined to be greater than 8.9 for all samples (Agilent 2100 Bioanalyzer, Agilent, Santa Clara, CA). Data from three biological replicates were analyzed using GeneSpring (v7.3, Agilent). Genes with greater than 2.5 fold induction compared to the untreated control were determined. Data are deposited in the Gene expression omnibus (Barrett et al., 2013) (GEO accession number GSE49469).

### **2.3 Quantitative RT-PCR**

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed using the previously described validation strategies (Christian et al., 2012). Briefly, primers were validated using a duplicate 5-point, 5-fold dilution series starting from 100 ng of DNase-treated RNA isolated from RasV12 cells using SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR Kit with ROX (Life Technologies) and analyzed on the StepOnePlus qPCR system (Applied Biosystems, Foster City, CA). The cycling conditions were as per manufacturer's instructions: 50 °C for 3 minutes, 95 °C for 5 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds then 40 °C for 1 minute, and then followed by melt-curve analysis (Life Technologies). The absence of non-specific amplification was confirmed by observing a single peak in the melt-curve analysis, confirmation of the expected amplicon size as determined by agarose gel analysis as well as the absence of primer dimers, and by the absence of amplification in the no template control wells. The sequence, amplicon size, and

efficiency of each primer sets are shown in appendix table 1. The quantification of Gbp2, Ifi47, Il15, Rig-I, Stat2, Xaf1, Iigp2, Ifit1, Ptx3, and Irf1 expression in RNA isolated from three independent biological replicates were performed in duplicate using the above strategy using 50 ng of RNA as template. Gapdh was used as a reference gene.

In order to quantify miRNA, total RNA was isolated from Trizol (Life Technologies), reverse transcribed into cDNA using miScript II RT Kit (Qiagen, Toronto, ON). Expression of miR-23a was measured with Rnu6 as a reference gene using 1 ng of cDNA as template using miScript Primer Assay Kit (Qiagen) according to the manufacturer's instruction. The kit comes with miScript SYBR green, which contains miScript universal primer (reverse primer) and pre-designed, ready to use target specific forward primers [Qiagen, *miR-23a* (MS00032599), RNU6 (MS00033740)]. The cycling conditions were as per manufacturer's instructions: 95 °C for 15 minutes followed by 40 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds, 70 °C for 30 seconds, followed by melt-curve analysis (Qiagen).

## **2.4 Promoter and UTR reporter assays**

pISRE-Luc from was purchased from Stratagene (La Jolla, CA). Promoter regions of MDII genes were obtained from the NCBI database. Enough length of the promoter of each gene was included in the construct for it to be activated by IFN treatment, since these genes represent IFN-inducible genes. Promoter constructs of Gbp2, Ifi47, Rig-I, Irf1 variant 1&3, and Irf1 variant 2 were obtained by PCR amplification of mouse genomic DNA and ligation into the XhoI and HindIII sites of pGL3-Basic vector (Promega, Madison, WI). The promoter deletion constructs of Gbp2 and Ifi47 were made using the



Erase-a-Base™ System (Promega) according to the manufacturer's instructions followed by sequencing to determine the remaining promoter region. RasV12 cells ( $3 \times 10^4$  cells / well), wildtype ( $4 \times 10^4$  cells / well) or IRF1 deficient MEFs ( $4 \times 10^4$  cells / well) were cultured in 24-well plate and transiently transfected with 1  $\mu$ g of the reporter plasmids using SuperFect Transfection Reagent (Qiagen). Twenty-four hours after transfection, cells were treated with U0126 or IFN- $\alpha$  for additional 24 hours. To block the effect of endogenous IFN, cells were pretreated with anti-IFN- $\alpha/\beta$ R $\alpha$  (C-18) antibody for 6 hours (Santa Cruz Biotechnology, Santa Cruz, CA) prior to U0126 or IFN treatment and also for the remaining duration of the treatment time (24 hours). Luciferase activity was measured by the Luciferase Assay System (Promega) and luminescence measured using Fluoroskan Ascent FL (Thermo LabSystems, Waltham, MA). Transcription factor binding elements were identified using the JASPAR database (Mathelier et al., 2014).

For UTR reporter assay, 3' - or 5' -UTR sequence of IRF1 was PCR amplified using mouse pCMV-SPORT6-Irf1 (Thermo Fisher Scientific) as a template and sub-cloned into XbaI site or NcoI site in pGL3-Control vector, respectively. RasV12 cells were transfected with 0.5  $\mu$ g of reporter plasmids, treated with or without U0126 for 6 and 24 hours, and luciferase expression was measured as above. miRNA binding sites were identified using the miRbase (Kozomara & Griffiths-Jones, 2014) and the miRWalk databases (Dweep et al., 2011).

Detailed sequences of each MDII promoter construct and the IRF1 UTR constructs are presented in Appendix (Figure 1-4).

## **2.5 Cycloheximide experiment**

Mouse pCMV-SPORT6-Irf1 vector was purchased from Thermo Fisher Scientific, and human IRF1 expression vector was generated from incyte human IRF1 cDNA (Thermo Fisher Scientific) cloned into the pCMV-SPORT6 vector. RasV12 and HT1080 cells were transfected with mouse or human IRF1 pCMV-SPORT6, respectively. At 24 hours after transfection, cells were pretreated with U0126 (20  $\mu$ M) or left untreated for 1 hour, and then treated with cycloheximide (CHX, 30  $\mu$ g/ml) for 15, 30, 45, 60, 120 and 240 minutes. Protein expression of IRF1 and GAPDH were analyzed by western blot.

## **2.6 Viruses and infection**

Wild-type VSV (Indiana strain) and attenuated VSV (VSVM51R) provided by Dr. John C. Bell (Centre for Innovative Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, ON), were amplified and titered by plaque assay using L929 cells. For IRF1 knockdown experiments, RasV12 cells cultured in 24-well plate ( $3 \times 10^4$  cells / well) were transfected with 30 nM ON-TARGETplus SMARTpool Mouse Irf1 siRNA or ON-TARGETplus Non-Targeting pool siRNA (Thermo Fisher Scientific) using 1  $\mu$ l of DharmaFECT1 transfection reagent (Thermo Fisher Scientific). At 2 days after siRNA treatment, the cells were treated with IFN- $\alpha$  and/or U0126 for 16 hours, followed by infected with VSV (MOI=1). IRF1-overexpressing RasV12 cells were generated by transfecting 0.5  $\mu$ g of mouse pCMV-SPORT6-Irf1 or control pCMV-SPORT6 using SuperFect transfection reagent. At 24 hours after transfection, the cells were treated with IFN- $\alpha$  for 16 hours, followed by infection with VSV (MOI=1). IRF1-overexpressing

human cancer cells, DLD-1, HT1080, and HT29 cells were generated by transfecting with 0.5 µg of human pCMV-SPORT6-IRF1 or control pCMV-SPORT6 as above. At 24 hours after the transfection, the cells were infected with VSVM51R (MOI=5, 1.25, 0.3, 0.08, or 0.02). The level of IRF1 transfection in RasV12, HT1080, HT29, and DLD-1 were determined by Western blotting for IRF1 protein. Upon transfection with IRF1 expression vector, all of these cell lines were confirmed to express higher level of IRF1 compared to cells transfected with control vector.

## **2.7 Chromatin Immunoprecipitation (ChIP) Assay**

Approximately  $5 \times 10^6$  cells were cultured in 15-cm dish and treated with U0126 or IFN- $\alpha$ , or left untreated. After 4 hours post-treatment, chromatin was cross-linked with 37 % formaldehyde (Sigma-Aldrich, St. Louis, MO) for 10 minutes, followed by addition of 0.125 M glycine. Cells were washed twice with ice cold PBS, scraped into 20 ml tube, centrifuged at 2,000 rpm for 5 min at room temperature, and then cells were lysed in ChIP lysis buffer [1.0 % SDS, 10.0 mM EDTA, 50 mM Tris-HCl (pH 8.1)]. Chromatin was sonicated into 200-500 bp length fragments (9 cycles of 20 % amplitude, 10 sec pulse and 20 sec pause) using Sonic Dismembrator Model 500 (Fisher Scientific). The size ranges of sonicated fragments were confirmed by agarose gel analysis. Sonicated samples were cleared of debris by centrifugation at 8,000 rpm for 20 min, pre-cleared with 50 µl of protein A agarose bead (Thermo Fisher Scientific), which has been pre-blocked with Herring Sperm DNA (Life Technologies) and BSA (Promega), and protein concentration determined using BCA protein assay kit (Thermo Scientific). One

milligram of the cell lysate was diluted to a final volume of 1 mL with CHIP dilution buffer [0.01 % SDS, 1.1 % Triton X-100, 1.20 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl] supplemented with protease inhibitors and was incubated with 4 µg of anti-IRF1 (M-20) antibody or normal rabbit IgG (Santa Cruz) overnight at 4° C. The antibody-chromatin complex was incubated with 50 µl of preblocked protein A agarose beads for 2 hours at 4°C. After incubation, beads were washed twice each with 1 mL low salt wash buffer [0.1 % SDS, 1.0 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high salt wash buffer [0.1 % SDS, 1.0 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], LiCl wash buffer [250 mM LiCl, 1.0 % IGEPAL-CA630, 1 % Deoxycholic Acid, 1.0 mM EDTA, 10.0 mM Tris (pH 8.1)], and TE buffer [10.0 mM Tris (pH 8.1), 1.0 mM EDTA]. The chromatin complex was eluted from beads with elution buffer (1.0 % SDS, 100.0 mM NaHCO<sub>3</sub>), protein-DNA crosslinking reversed with 5 M NaCl, and RNA and proteins digested with RNase A (Qiagen) and Proteinase K (Qiagen), respectively. The recovered DNA was purified using QIAquick PCR purification kit (Qiagen) and eluted in 50 µl water. The CHIP and the total input DNA were analyzed by end-point PCR using primers listed in appendix table 2. CHIP qPCR was performed using *Power SYBR® Green Master Mix* (Life Technologies) and primers listed in appendix table 1. Background signal obtained from the control antibody was subtracted from CHIP IRF1, and the percentage of CHIP DNA relative to the input DNA was determined.

## **2.8 RNAi for ERK downstream elements**

The control siRNA and siRNA against mouse Erk1, Erk2, Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk2, Rsk3, Rsk4, human RSK1, RSK2, RSK3 and RSK4 were purchased from Santa Cruz Biotechnology. A day before transfection, RasV12 ( $2.5 \times 10^4$  cells / well), DLD-1 ( $3 \times 10^4$  cells / well), or MDA-MB-468 ( $5 \times 10^4$  cells / well) were plated in 24-well plates. The cells were transfected with transfection complex prepared by mixing 10 pmol of siRNA and 1  $\mu$ l of Lipofectamine RNAi MAX (Life Technologies) in serum-free medium. At 24 hours post-transfection, the transfection was repeated again for greater suppression of target genes. Protein and RNA samples were collected at 48 hours post-transfection. The protein expression of ERK1/2, human RSK1, and RSK2 were determined by Western blot analysis, while those of MNK1/2, MSK1/2, mouse RSK1, RSK3, and RSK4 silencing were determined by semi-quantitative RT-PCR using primers listed in appendix table 2.

## **2.9 RSK Overexpression**

RasV12 cells ( $8 \times 10^4$  cells / well) plated in 24-well plates were transfected with 1  $\mu$ g of mouse pCMV-SPORT6-Rsk3 (Thermo Fisher Scientific), mouse pCMV-SPORT6-Rsk4 (Thermo Fisher Scientific), or co-transfected with both vectors (1  $\mu$ g each) using 5  $\mu$ l of Superfect reagent (Qiagen). NIH3T3 cells ( $1 \times 10^5$  cells / well) plated in 24-well plate were transfected with the same amount of plasmids as above using 3  $\mu$ l of *TransIT*®-2020 transfection reagent (Mirus Bio LLC, Madison, WI), which gave us better transfection efficiency than the Superfect reagent. At 24 hours after transfection, RasV12

and NIH3T3 cells were treated with or without U0126 (20  $\mu$ M) for 24 hours and then protein samples were collected for Western blot analysis. The level of RSK overexpression were determined by western blotting for RSK3 or RSK4 protein. Both RasV12 and NIH3T3 cells were confirmed to overexpress RSKs compared to its control.

### **2.10 Western Blot Analysis**

Protein samples were separated on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, ON). The membrane was blocked with 5% skim milk or 1-5 % BSA in TBS (20 mM Tris and 137 mM NaCl [pH 7.3]) containing 0.05% Tween 20 (TBST) for 1 hour followed by primary antibody incubation overnight unless otherwise indicated in the appendix table 3. Next day, the membrane was washed three times with TBST, incubated with appropriate secondary antibody (peroxidase-conjugated goat anti-mouse IgG, anti-rabbit IgG, anti-goat IgG, or anti-rat IgG (Santa Cruz)) for 1 hour, washed three times again with TBST, and specific bands were detected by enhanced chemiluminescence (Amersham, Baie d'Urfe, QC) using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Baie d'Urfe, QC). ImageQuant Software (GE Healthcare Life Sciences) was used to quantify the intensity of bands.

Specific primary and secondary antibody conditions for each antibody are shown in appendix table 3. Antibody to VSV-G (VSVII-M) was purchased from Alpha Diagnostic (San Antonio, TX), phospho-ERK-1/2 (#9101) from Calbiochem, GAPDH (6C5) from Abcam (Toronto, ON), ERK (K-23), Sumo-1 (FL-101), RSK2 (E-1), RSK3 (A-16), RSK4 (JS-31) and mouse IRF1 (M-20) from Santa Cruz Biotechnology (Santa

Cruz, CA), human IRF1 (#612046) from BD Transduction Laboratories (Mississauga, ON), anti-phosphotyrosine (clone 4G10) from EMD Millipore (Billerica, MA), anti-phosphoserine (Clone PSR-45) from Life Technologies, anti-HA high affinity (clone 3F10) from Roche (Mississauga, ON), acetylated-lysine (#9441) and RSK1 (#9333) from Cell Signaling Technology. For densitometry analysis, band intensities were determined using ImageJ 1.48v (Schneider et al. 2012). Band intensities of target proteins were first normalized to endogenous control (GAPDH for all blots except for IP experiment, in which IRF1 was used instead) and then the shown as percentage compared to appropriate control as indicated under each figure legend.

## **2.11 Immunoprecipitation**

RasV12 cells ( $6 \times 10^5$  cells / dish) plated in 10-cm plates were transfected with 5  $\mu$ g of mouse pCMV-SPORT6-Irf1 (for acetylation and phosphorylation studies) or co-transfected with 5  $\mu$ g of pCMV-SPORT6-Irf1 and with either 5  $\mu$ g of pCMV-SPORT6-Sumo1 (Thermo Fisher Scientific) (for sumoylation study) or pRK5-HA-Ubiquitin (gift from Ted Dawson, Addgene plasmid # 17608) (for ubiquitination study) using 60  $\mu$ l of Superfect reagent. At 24 hours post-transfection, cells were treated with 20  $\mu$ M U0126 or DMSO for 6 hours. At 2 hours prior to cell lysis (4 hours after U0126 treatment), MG132 was added to a final concentration of 25  $\mu$ M to prevent protein degradation. Cells were washed twice with ice cold PBS and lysed with 1 mL 1 % Triton X-100 lysis buffer [20 mM Tris-HCl (pH8), 1 % Triton X-100, 10 % glycerol, 2 mM EDTA, 137 mM NaCl] supplemented with protease (PMSF and aprotinin, Sigma-Aldrich) and phosphatase inhibitors (halt phosphatase inhibitor cocktail, Thermo Fisher Scientific). For acetylation

and sumoylation studies, 10  $\mu$ M sodium butyrate (deacetylation inhibitor) (Sigma-Aldrich) or 20  $\mu$ M N-ethylmaleimide (desumoylation inhibitor) (Sigma-Aldrich) was further added to the above lysis buffers, respectively. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4 °C and the amount of protein was measured using the BCA protein assay kit (Thermo Fisher Scientific). One milligram of cell lysate was diluted up to 1 mL of 1 % Triton X-100 lysis buffer containing all appropriate inhibitors, pre-cleared for 1 hour with 30  $\mu$ l of pre-blocked protein A agarose beads, and immunoprecipitated with 2  $\mu$ g of IRF1 antibody (M-20) overnight at 4°C. The next day, the antibody-protein complex was captured with 30  $\mu$ l of pre-blocked protein A beads for 2 hours, washed extensively with 1 % Triton X-100 lysis buffer, and then eluted by boiling the beads in 1 x sample buffer for 10 minutes. Immunoprecipitated IRF1 and its post-translational modifications were determined by Western blot analysis using the primary antibodies listed in appendix table 3 and light chain specific secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

## **2.12 Polysome Analysis**

Polysome analysis was conducted in collaboration with Dr. Tommy Alain at Children's Hospital of Eastern Ontario (Ottawa, ON). RasV12 cells were cultured to approximately 80-90% confluency in 15-cm dish and treated with or without U0126 (20  $\mu$ M) for 2 hours. At the end of the U0126 treatment, cycloheximide was added to the culture media to a final concentration of 100  $\mu$ g/ml and incubated for 5 minutes to prevent ribosome runoff from mRNA. Cells were washed and scraped with ice-cold PBS supplemented



with cycloheximide (100 µg/ml), centrifuged, and lysed in hypotonic buffer [5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl<sub>2</sub>, 1.5 mM KCl, complete protease inhibitor cocktail (Roche, Mississauga, ON, Canada)]. The cell lysate was further supplemented with cycloheximide (100 µg/ml), DTT (2 mM), RNase inhibitor (100 U), 10 % triton X-100 (final concentration of 0.5 %), and 10 % sodium deoxycholate (final concentration of 0.5%), centrifuged and then the supernatant was separated on 10-50% sucrose gradient by centrifugation at 35,000 rpm for 2 hours. After separation, gradients were fractionated from the top of the gradient by pumping the chasing solution (60 % w/v sucrose, 0.02 % w/v bromophenol blue) from the bottom of the tube at 1.5 ml/min using an Isco density gradient fractionator (Teledyne Isco Inc., Lincoln, NE) and the RNA was monitored at 254 nm using the WinDaq data acquisition software (DATAQ Instruments, Akron, OH).

To isolate RNA for polysome analysis, 500 µl of each fraction was mixed with 750 µl Trizol. Chloroform (150 µl) was next added, vortexed for 15 seconds, incubated at room temperature for 3 minutes, centrifuged at 13,000 rpm for 15 minutes, and 800 µl of aqueous layer was transferred to a fresh tube. Ice-cold isopropanol (750 µl) was next added to the aqueous layer, inverted 10 times, and RNA was precipitated overnight at -20 °C. The next day, the samples were centrifuged at 13,000 rpm for 10 minutes, supernatant discarded, and RNA pellet air dried and resuspended in 20 µl of RNase-free water by incubating at 37 °C for 10 minutes. RNA was treated with DNase, and equal volume of RNA (5 µl) from individual fractions was used for cDNA synthesis using the RevertAid H minus first strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacture's instruction. The levels of polysome associated IRF1 and GAPDH

mRNA in each fraction was determined by semi-quantitative PCR using primers listed in appendix table 2. For quantitative analysis, RNA isolated from fractions representing subpolysomes (fraction #2-6), early polysomes (Fraction #7-10), or heavy polysomes (fraction #11-15) were pooled, and measured by RT-qPCR as described in section 2.3.

### **2.13 Statistical analysis**

One-way ANOVA with Tukey's post-hoc test was performed using GraphPad Prism 4.0c software (GraphPad Software, La Jolla, CA).

## CHAPTER 3

### **Oncogenic Ras inhibits IRF1 to promote viral oncolysis**

Most of the work in this chapter has been published in the journal *Oncogene*.

### **3.1 Rationale**

Oncolytic viruses exploit common molecular changes in cancer cells, absent from normal cells, to replicate in and kill cancer cells. Ras transformation and defects in type I interferon (IFN)-mediated antiviral responses have been known to be the major mechanisms underlying viral oncolysis. Our laboratory and others previously reported that activation of the Ras/Mitogen-Activated Protein Kinase Kinase (MEK) pathway suppresses the host antiviral response induced by IFN (Battcock et al., 2006; Christian et al., 2009, and Noser et al., 2007), clearly indicating that the two major mechanisms of viral oncolysis (Ras-dependency and IFN-insensitivity) are indeed connected. Furthermore, a group of IFN-inducible genes whose transcription is downregulated by Ras/MEK activation (MEK-Downregulated IFN-Inducible (MDII) genes) was identified by microarray analysis in human cancer cells (Christian et al., 2012). These results suggest that transcriptional dysregulation of the MDII genes in human cancer cells is one of the underlying mechanisms of Ras- and IFN insensitivity-dependent viral oncolysis. In this chapter, we sought to further clarify the precise mechanism of how Ras/MEK suppresses transcription of the MDII genes.

### **3.2 Results**

#### **3.2.1 Ras/MEK downregulates expression of MDII genes in RasV12 cells.**

The Hirasawa lab has previously reported the presence of MDII genes in human cancer cells (Christian et al., 2012). In order to clarify the molecular mechanisms of the regulation of MDII genes by Ras/MEK, we analyzed global gene expression in RasV12

transformed NIH3T3 (RasV12) cells that do not have the confounding mutations that occur in human cancer cells. RasV12 cells were treated with U0126, IFN, or left untreated for 6 hours. The gene expression profiles were determined using DNA microarrays (Figure 3.1 A). We identified 1264 genes and 1258 genes with  $\geq 2.5$ -fold increased expression by either U0126 or IFN treatment, respectively. Furthermore, we identified 619 genes that were upregulated by both MEK inhibition and IFN treatment as the responsive MDII genes in mouse fibroblast cells.

The expression changes of a subset of genes, guanylate binding protein 2 (Gbp2), interferon gamma inducible protein 47 (Ifi47), interferon-induced protein with tetratricopeptide repeats 1 (Ifit1), immunity-related GTPase family M member 2 (Iigp2), interleukin 15 (Il15), pentraxin related gene (Ptx3), retinoic acid-inducible gene 1 (Rig-I), signal transducer and activator of transcription 2 (Stat2) and XIAP associated factor 1 (Xaf1) were validated by RT-qPCR analysis (Figure 3.1 B). The expression of Gbp2, Ifi47, Il15, Rig-I, Stat2, and Xaf1 genes were significantly induced by U0126-only and IFN-only treatment, and identified as MDII genes. Interestingly, combined treatment of IFN and U0126 significantly increased expression of Gbp2, Il15 and Stat2 compared to those in cells treated with IFN only or U0126 only. Iigp2 and Ifit1, which were induced only by IFN treatment but not by U0126, represent non-MDII IFN-inducible genes. In contrast, Ptx3, which was induced by U0126, but not by IFN, represents a non-MDII MEK-downregulated gene.

**Figure 3.1** Identification of mouse MEK-downregulated IFN-inducible (MDII) genes.

(A) Venn diagrams from DNA microarray analysis showing gene upregulation ( $\geq 2.5$ -fold expression) in RasV12 cells treated with U0126 (20  $\mu$ M) or IFN- $\alpha$  (500 U/ml).

MDII genes represent genes upregulated by both MEK inhibition and IFN treatment. (B)

RT-qPCR analysis for Gbp2, Ifi47, Il15, Rig-I, Stat2, Xaf1, Iigp2, Ifit1 and Ptx3. RasV12

cells were treated with U0126 (20  $\mu$ M), IFN- $\alpha$  (500 U/ml) or U0126/IFN- $\alpha$  or left untreated for 6 hours. The relative expression levels were normalized to Gapdh and

reported as compared to the untreated controls. [n=3 (3 independent experiments),

significant upregulation compared to untreated controls denoted by \*P<0.05 and

\*\*P<0.01, significant upregulation by U0126/IFN- $\alpha$  combined treatment compared to that

by U0126-only or with IFN-only treatment denoted by #P<0.05 and ##P<0.01].

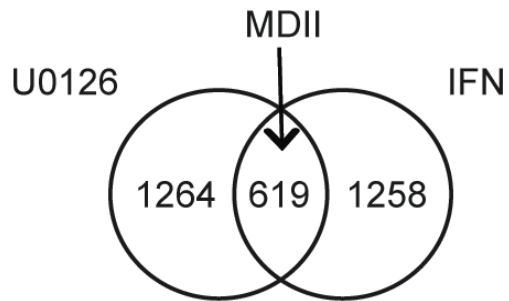
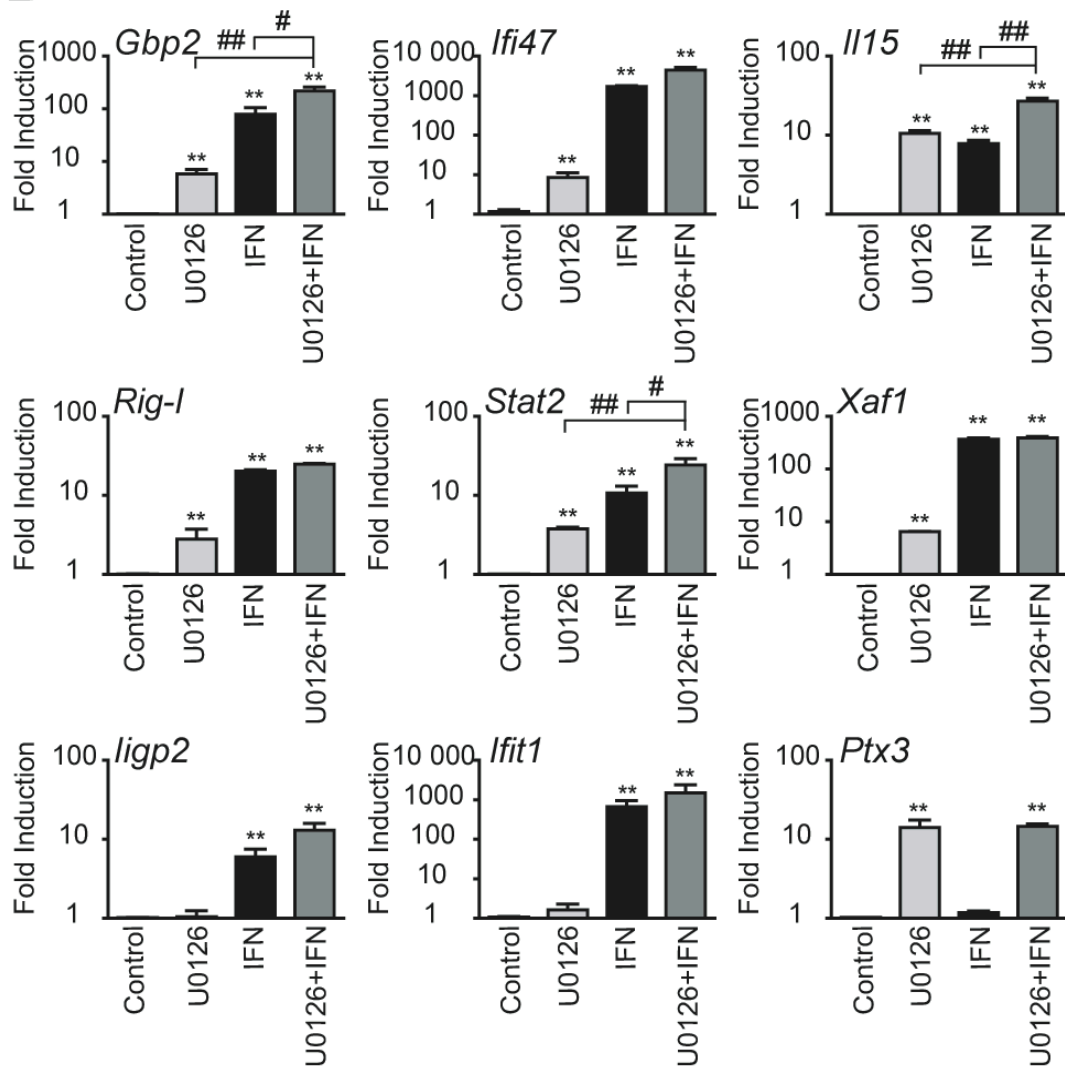
**A****B**

Figure 3.1

### **3.2.2 Ras/MEK suppresses the transcription of MDII genes.**

In order to determine whether Ras/MEK regulates the MDII gene transcription, we conducted promoter gene analysis for a subset of MDII genes. RasV12 cells were transfected with the pGL3-Basic vector containing the promoter region of either the Gbp2, Ifi47, or Rig-I genes, or the interferon-stimulated response element (ISRE) and then treated with U0126 or IFN for 24 hours (Figure 3.2 A). We found that IFN treatment significantly activated transcription of the ISRE reporter construct and all of the reporter constructs containing the MDII gene promoter region. MEK inhibition also significantly increased the promoter activity of Gbp2, Ifi47, and the ISRE in RasV12 cells. Although Rig-I promoter activity increased two-fold in RasV12 cells treated with U0126, the induction was not statistically significant. These results demonstrate that Ras/MEK suppresses the promoter activities of these MDII genes.

MEK inhibition may stimulate production of endogenous type I IFN, which leads to the activation of MDII gene promoter activities as a secondary effect. We tested this by analyzing the promoter activities of MDII genes in the presence or absence of type I IFN receptor antibody (anti-IFN- $\alpha/\beta$ -R) in order to block the effect of endogenous IFN in the culture supernatant. As expected, pre-treatment with anti-IFN receptor antibody abolished the IFN-induced activation of the ISRE and Gbp2 promoters, indicating that the anti-IFN antibody successfully neutralized the effect of exogenous IFN- $\alpha$  (Figure 3.2 B). In contrast, promoter activation of Gbp2 by U0126 treatment was not altered in the presence of the anti-IFN receptor antibody, which suggested that MEK inhibition directly induces MDII gene transcription by trans-acting factors other than the endogenous IFN- $\alpha/\beta$ .



In order to identify the promoter region responsive to Ras/MEK, we conducted promoter deletion analysis of the Gbp2 and Ifi47 genes as these showed the most robust up-regulation by U0126 treatment in Figure 3.2A (Figure 3.2 C). Deletion of a 21-bp region between -68 and -47 of the Gbp2 promoter significantly reduced the U0126-induced transcriptional response, indicating that this region contains elements essential for transcriptional regulation of Gbp2. Similarly, we observed a significant reduction in U0126-induced activation of the Ifi47 promoter when the region from -762 to -148 or from -148 to -80 was deleted. Importantly, the deletion between -148 and -80 of the Ifi47 promoter completely abolished activation of its promoter activity by U0126. The regulation of Ifi47 transcription by IFN was primarily regulated by the same region (-762 to -80) as the U0126 response region, although a region between -80 to -35 was found also to be responsive. In contrast, the elements responsible for regulation of Gbp2 by IFN are contained in regions from -512 to -83 and from -83 to -68 of the Gbp2 promoter, distinct from the U0126 responsive sites. These results suggest that independent promoter elements are required for MDII gene transcription by MEK inhibition compared to IFN stimulation. We next examined the Gbp2 and Ifi47 promoter regions essential for regulation by U0126 (Gbp2: -68 to -47 and Ifi47: -148 and -80) for transcription factor binding sites. Using the JASPAR database, we identified five putative transcription factor-binding sites [for paired box gene 2 (PAX2), interferon regulatory factor (IRF)-binding element (IRFE), AT rich interactive domain 3A (ARID3A), SRY-box containing gene 17 (SOX17) and SRY-box containing gene 10 (SOX10)] that were located in both of the U0126-responsive regions. Among the five candidate binding sites, we decided to focus on the IRFE binding element due to its relevance to antiviral responses (Taniguchi

et al., 2001). Among various IRF family members that can possibly bind to IRFE sites, IRF1 was chosen as our candidate since the JASPAR database specifically predicted the binding sites for IRF1 and IRF2, and that our preliminary experiment result suggested that IRF1 was under the regulation of the active Ras/MEK pathway, whereas IRF2 was not (data not shown).

### **3.2.3 IRF1 regulates the transcription of MDII genes.**

Chromatin immunoprecipitation (ChIP) assays were performed to determine whether IRF1 binds to the Gbp2 and Ifi47 gene promoters in response to MEK inhibition. We observed a relatively small amount of IRF1 present at the putative IRFE sites of Gbp2 and Ifi47 promoter in untreated RasV12 cells (Figure 3.3 A). Treatment with either U0126 or IFN substantially enhanced IRF1 recruitment at the Gbp2 and Ifi47 promoter. This interaction was specific as the IRFE sites of Gbp2 and Ifi47 promoter regions were not detected in the pull-down with control IgG, and the binding of IRF1 to the distal control sites in the two promoters was not observed. These results demonstrate specific binding of IRF1 to the IRFE sites of Gbp2 and Ifi47 upon Ras/MEK inhibition in RasV12 cells. As a means to validate and quantify these results, quantitative ChIP analysis was performed. U0126 treatment significantly increased IRF1 binding at the IRFE sites of both Gbp2 and Ifi47 (Figure 3.3 B).

We determined if IRF1 regulates MDII gene expression in response to inhibiting the Ras/MEK pathway by testing primary and immortalized mouse embryonic fibroblasts (MEFs), which represent different stages of transformation, from wild-type, or IRF1-

**Figure 3.2** Identification of MDII promoter regions responsible for transcriptional activation by U0126 or IFN. (A) Promoter activity of control pGL3-Basic plasmid, pISRE-Luc plasmid, or pGL3-Basic plasmid containing promoters of Gbp2, Ifi47 or Rlg-I in RasV12 cells treated with U0126 (20  $\mu$ M) or IFN- $\alpha$  (500 U/ml) for 24 hours. Relative luciferase activities (RLU) are reported as compared to the untreated controls. (B) Promoter activity of control pGL3-Basic plasmid, pISRE-Luc plasmid, or pGL3-Basic plasmid containing promoters of Gbp2 in RasV12 cells pre-incubated with or without anti-IFN- $\alpha/\beta$ -R antibody (Ab, 100 U/ml) for 0.5 hours and then treated as above. RLU of was reported as compared to the untreated controls, or compared in the presence or absence of anti-IFN- $\alpha/\beta$ -R antibody of the same treatment. (C) Promoter activity of control pGL3-Basic plasmid, or pGL3-Basic plasmid containing various deletion constructs of the Gbp2 and the Ifi47 promoters in RasV12 cells treated as above. RLU of each promoter construct was reported as compared to the next shorter construct of the same treatment [n=3 (3 replicates in 1 representative independent experiment), \*\*P<0.01].

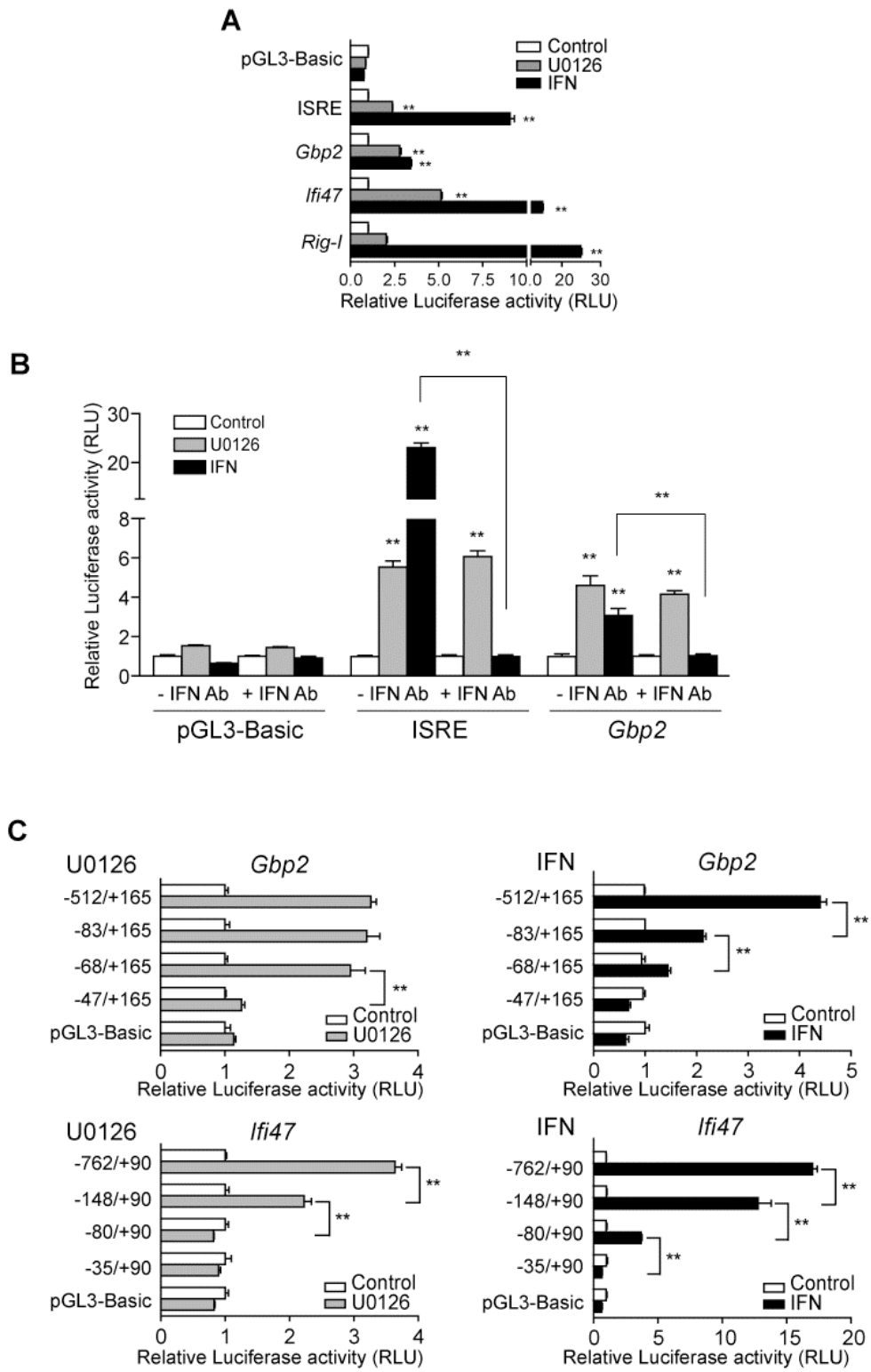


Figure 3.2

deficient, C57BL/6 mice. Expression of the MDII genes *Gbp2* and *Xaf1* was significantly induced by U0126 in primary wildtype MEFs (Figure 3.4 A). In support of the role of IRF1 in regulating MDII genes, U0126 did not increase transcription of *Gbp2* or *Xaf1* in primary IRF1-deficient MEFs. In contrast, there was no upregulation of *Ifi47*, *Ii15* and *Rig-I* in wild-type primary MEFs treated with U0126. Therefore, we examined the transcription of MDII genes in response to U0126 and IFN in immortalized MEFs, which are at a more advanced stage of transformation. Treatment with U0126 increased expression of all the MDII genes tested (*Gbp2*, *Ifi47*, *Ii15*, *Rig-I* and *Xaf1*) in immortalized wildtype MEFs treated with U0126 (Figure 3.4 B). This suggests that the degree of MDII gene induction by U0126 correlates with the level of cellular transformation and the level of IRF1 downregulation, which are in turn correlated with the degree of constitutive Ras activation. In contrast, induction of these genes was not observed in immortalized IRF1-deficient MEFs, confirming that IRF1 expression is necessary for this regulation. The absence of IRF1 also significantly reduced the induction of *Ifi47*, *Ii15* and *Rig-I* by IFN in both primary and immortalized MEFs and that of *Gbp2* in primary MEFs and that of *Xaf1* in immortalized MEFs, supporting the critical role of IRF1 in IFN-mediated transcription (Taniguchi et al., 2001). Taken together, these results demonstrated that IRF1 is the primary transcriptional regulator of these MDII genes.

**Figure 3.3** Effects of Ras/MEK inhibition on IRF1 binding to the Gbp2 and Ifi47 promoter. ChIP assay was performed on chromatin isolated from RasV12 cells treated with U0126 (20  $\mu$ M) or IFN- $\alpha$  (500 U/ml), or left untreated. The ChIP DNA and input DNA were analyzed by (A) end-point PCR and (B) qPCR using primers designed to amplify the U0126 responsive and distal promoter regions as shown. Vertical bars in the promoter diagram indicate the location of predicted IRFEs (Gbp2: -61 to -44. Ifi47: -156 to -145, -128 to -116, -74 to -56 and -46 to -35). Arrows, and the numbers below the arrows, indicate binding sites of the primer sets (thick arrows: amplifies IRFE regions, thin arrows: amplifies distal control regions). IRF1 binding to the promoter of Gbp2 or Ifi47 are presented as percentage of input (% Input) [n=3 (3 independent experiments) \*P <0.05; \*\*P <0.01].

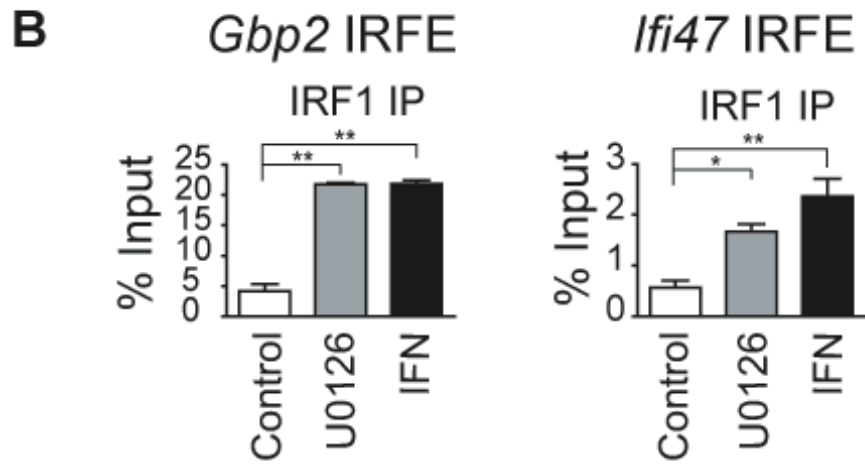
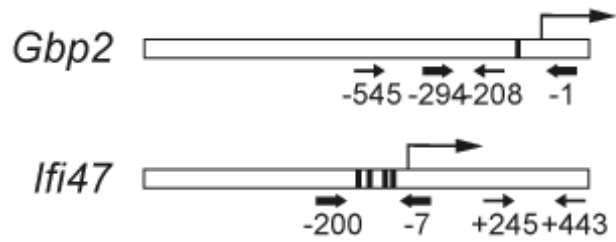
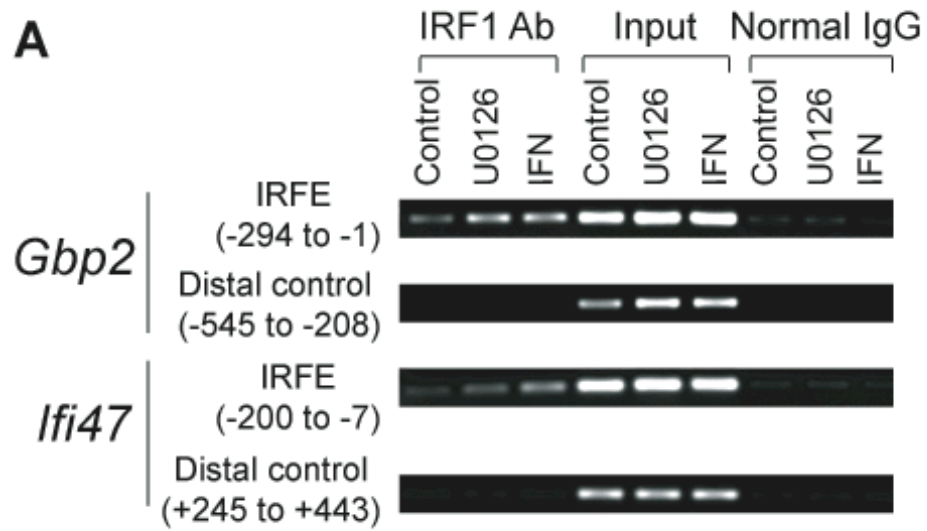


Figure 3.3

**Figure 3.4** IRF1 involvement in the modulation of MDII gene transcription by Ras/MEK. RT-qPCR analysis was conducted for the MDII genes (Gbp2, Xaf1, Ifi47, Il15 and Rig-I). Primary (A) and immortalized (B) MEFs derived from wild-type or IRF1-deficient mice were treated with U0126 (20  $\mu$ M) or IFN- $\alpha$  (500 U/ml), or left untreated for 6 and 12 hours, respectively. Relative expression levels of the MDII genes were normalized to Gapdh and the data from wildtype MEFs were compared to that of IRF1-deficient MEFs of the same treatment [n=3 (3 independent experiments), \*P<0.05; \*\*P<0.01].



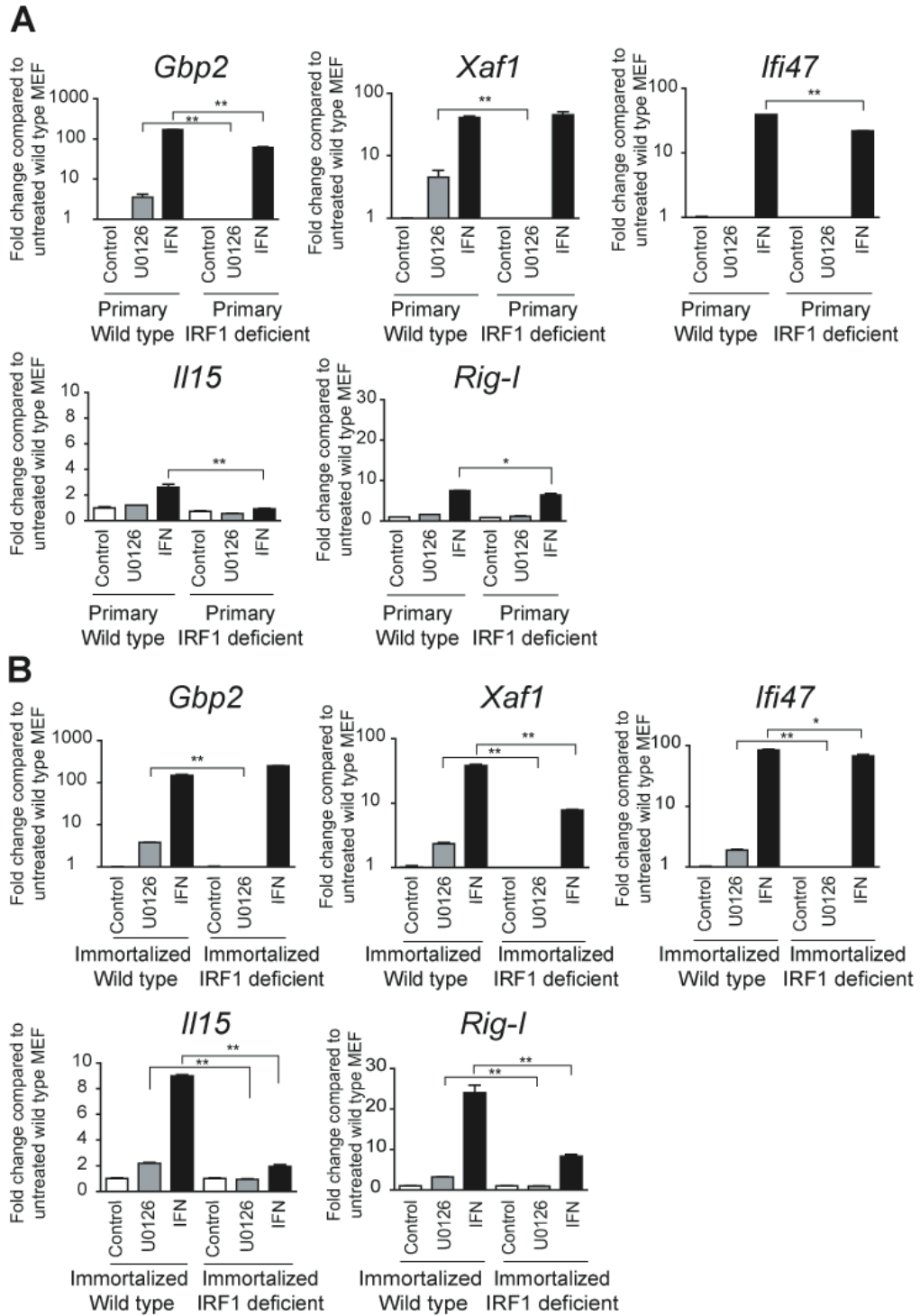


Figure 3.4

### **3.2.4 IRF1 expression is suppressed by the Ras/MEK pathway.**

To determine how the Ras/MEK pathway regulates IRF1 function, we examined IRF1 protein expression in vector control NIH3T3 cells and RasV12 cells treated with or without U0126 (Figure 3.5 A). The amount of IRF1 protein was lower in RasV12 cells than in vector control NIH3T3 cells and was restored by 6 hours of MEK inhibition. Double bands for IRF1 were observed in vector control NIH3T3 cells whereas only one band was apparent in RasV12 cells. We believe that the upper band is one of the IRF1 isoforms, however, it remains to be determined why it is present only in vector control cells. We also examined the mRNA and protein expression levels of IRF1 in RasV12 cells after treatment with U0126 (Figure 3.5 B). RT-qPCR analysis revealed that the IRF1 mRNA level was significantly increased by MEK inhibition after 4 hours of U0126 treatment, indicating that IRF1 mRNA levels are downregulated by the active Ras/MEK pathway. Similarly, we observed increased IRF1 protein levels as early as 2 hours after U0126 treatment. These results demonstrated that the Ras/MEK pathway downregulates IRF1 expression at both the mRNA and protein levels.

To determine whether the regulation of IRF1 by Ras/MEK also occurs in human cancer cells, we next analyzed the effect of MEK inhibition on IRF1 expression in the human breast carcinoma cells MDA-MB-468 and the human colon cancer cells DLD-1 (Figures 3.5 C and 3.5 D). We found that IRF1 protein is upregulated in both MDA-MB-468 and DLD-1 cells after 24 hours of treatment, even at sub-optimal concentrations of U0126, as determined by ERK phosphorylation levels. Furthermore, we found that IRF1 protein increased in response to three different MEK inhibitors (U0126, PD98059, or

SL327), demonstrating that the regulation of IRF1 was not due to non-specific effects of U0126 (Figure 3.5 D). Knockdown of ERK 1/2 in RasV12 cells also increased the IRF1 expression levels, further supporting the interaction between Ras/MEK and IRF1 (Figure 3.5 E).

### **3.2.5 Ras/MEK-mediated downregulation of IRF1 impairs the IFN anti-viral response.**

To determine whether IRF1 downregulation is the key factor in the impairment of anti-viral response in cells with activated Ras/MEK, we restored IRF1 expression in RasV12 cells and in human cancer cells. First, to determine if IRF1 overexpression is sufficient to restore IFN-induced anti-viral activity in RasV12 cells, the cells were transfected with either mouse IRF1 or control vector (pCMV-SPORT6), followed by treatment with different concentrations of IFN for 16 hours and then challenged with VSV. IRF1 introduction restored IFN's ability to protect RasV12 cells from VSV infection, particularly with 120 and 60 U/ml of IFN (Figure 3.6 A).

Next, we knocked down IRF1 in RasV12 cells using IRF1 siRNA to examine whether endogenous IRF1 upregulation is necessary for restoration of anti-viral IFN activity by U0126. Consistent with previous observations in our lab, (Battcock et al., 2006 and Christian et al., 2009), MEK inhibition restored the IFN-induced antiviral response in RasV12 cells (lane 9 compared to lane 7) (Figure 3.6 B). However, when we knocked-down IRF1, viral replication occurred, albeit at a lower level, even in the presence of both IFN and U0126 (lane 10 compared to lane 9). These data strongly support a critical role for IRF1 in the restoration of IFN sensitivity by MEK inhibition.

**Figure 3.5** Restoration of IRF1 expression by Ras/MEK inhibition. (A) Vector control NIH3T3 (control) or RasV12 cells were treated with the indicated concentration of U0126 for 6 hours, and IRF1 and ERK phosphorylation levels were detected by western blot. (B) RT-qPCR analysis for IRF1 mRNA (top panel) and western blot analysis for IRF1 protein (bottom panel) in RasV12 cells treated with U0126 (20  $\mu$ M) for the indicated period of time. IRF1 expression levels were normalized against the 0.5 hour controls [n=3 (3 independent experiments), \*P<0.05]. (C) MDA-MB-468 cells were treated with the indicated concentration of U0126 for 6 hours. (D) DLD-1 cells were treated with indicated concentration of the different MEK inhibitors U0126, PD98059, or SL327. IRF1 and ERK phosphorylation levels were analyzed by western blot. (E) RasV12 cells transfected with random nucleotide sequences (NG) or two independent ERK 1/2 oligonucleotides (#1 and #2) for indicated time points were analyzed by Western blot.

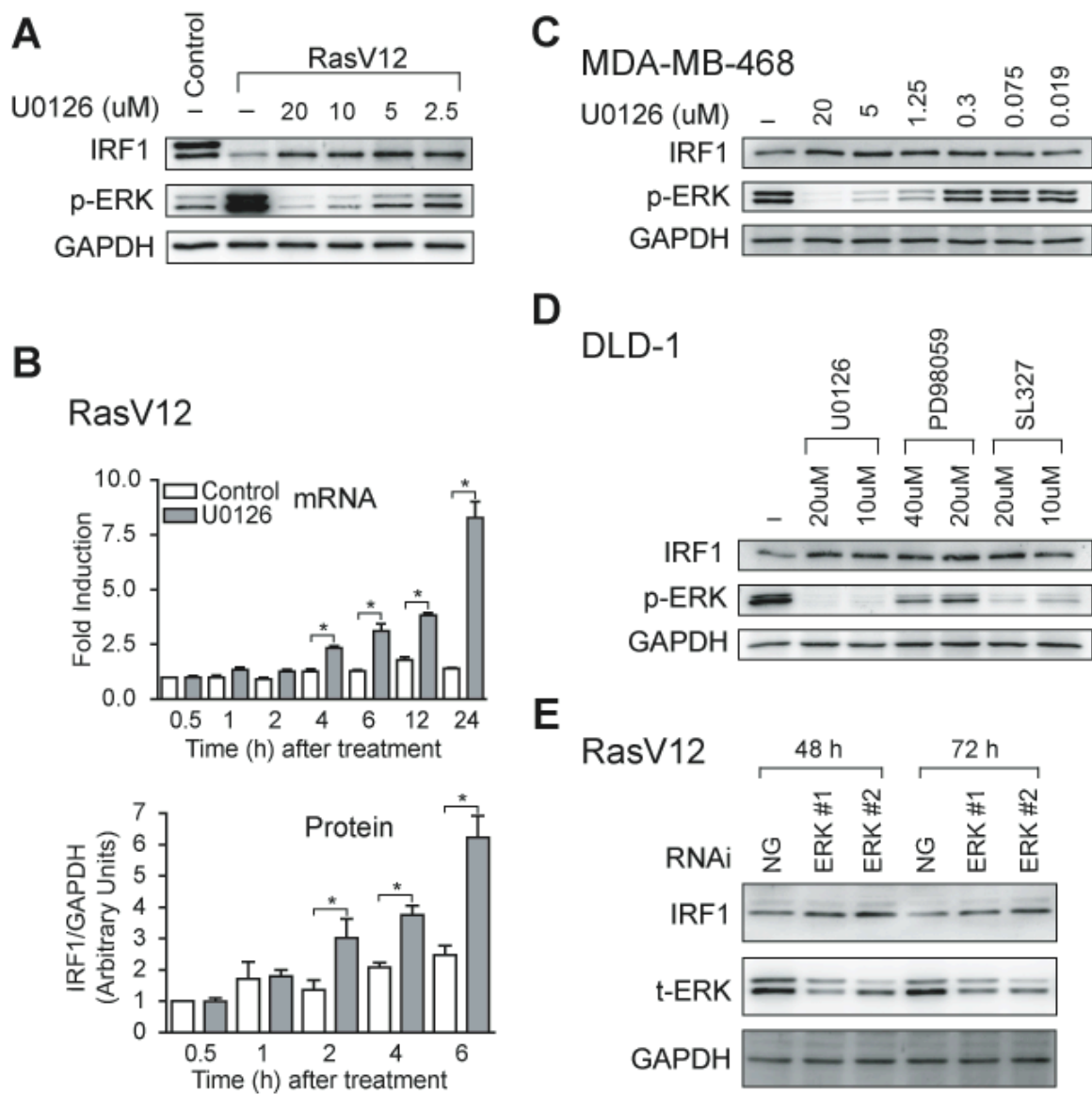


Figure 3.5

Next, we determined whether IFN-sensitive oncolytic viruses exploit Ras-mediated IRF1 downregulation in human cancer cells. We overexpressed IRF1 followed by infection with the oncolytic version of VSV (VSVM51R; AV1)(Stojdl et al., 2003). We found that the human cancer cell lines, HT1080 and HT29, were susceptible to the oncolytic virus when transfected with control vector plasmid (pCMV-SPORT6), but that viral oncolysis was severely restricted in a concentration dependent manner with overexpression of IRF1 (Figure 3.6 C). Next, human cancer DLD-1 cells were infected with different MOIs of VSVM51R after transfection with control or IRF1 vector (Figure 3.6 D). Similar to the results obtained above, the oncolytic virus replicated efficiently in cells transfected with control vector, whereas IRF1 overexpression substantially restricted viral oncolysis. Together these results demonstrated the critical role of IRF1 in defining the susceptibility of cancer cells to certain oncolytic viruses.

**Figure 3.6** IRF1 regulates viral oncolysis. (A) RasV12 cells transfected with control or mouse pCMV-SPORT6-Irf1 (0.5  $\mu$ g) were treated with indicated concentration of IFN- $\alpha$  for 16 hours and then challenged with VSV (MOI=1). (B) RasV12 transfected with scrambled (SCR) or IRF1 siRNA were treated with indicated concentration of IFN- $\alpha$  or U0126 for 16 hours and then challenged with VSV (MOI=1). Protein samples were obtained at 24 hours after infection. (C) HT1080 and HT29 cells transfected with control (1  $\mu$ g) or human pCMV-SPORT6-IRF1 (1, 0.5, 0.25, 0.12 and 0.06  $\mu$ g) were infected with VSVM51R (MOI=5). (D) DLD-1 cells transfected with control or human pCMV-SPORT6-IRF1 (1  $\mu$ g) were infected with VSVM51R (MOI=0, 5, 1.25, 0.3, 0.08, or 0.02) for 24 hours. Protein levels of VSV-G, IRF1 or GAPDH were determined by Western blot analysis.

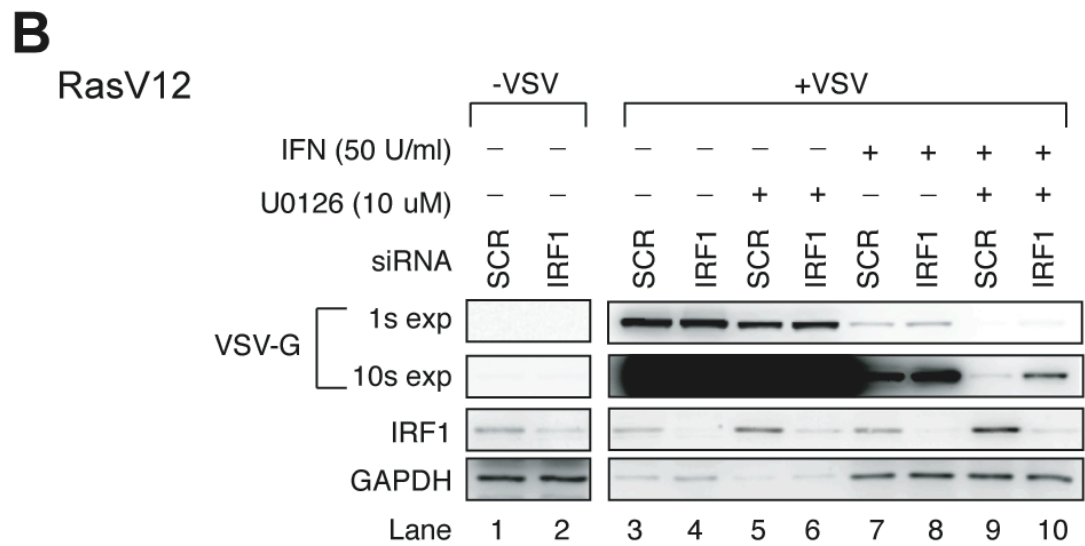
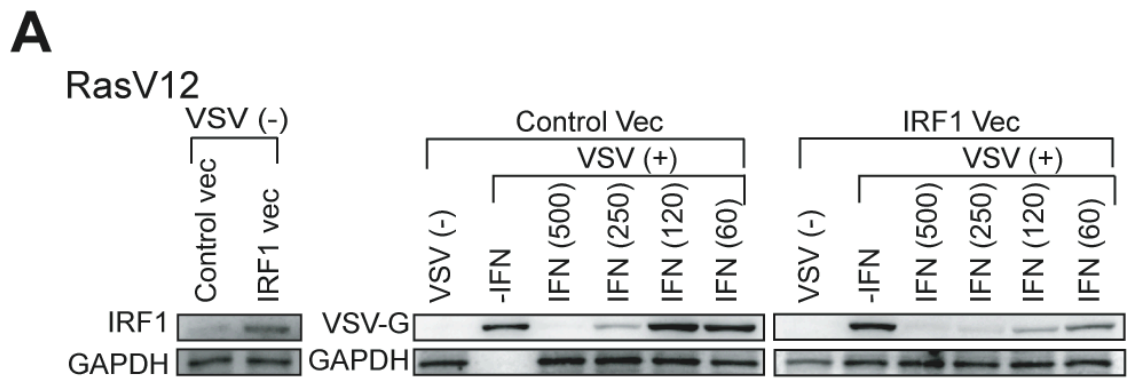


Figure 3.6



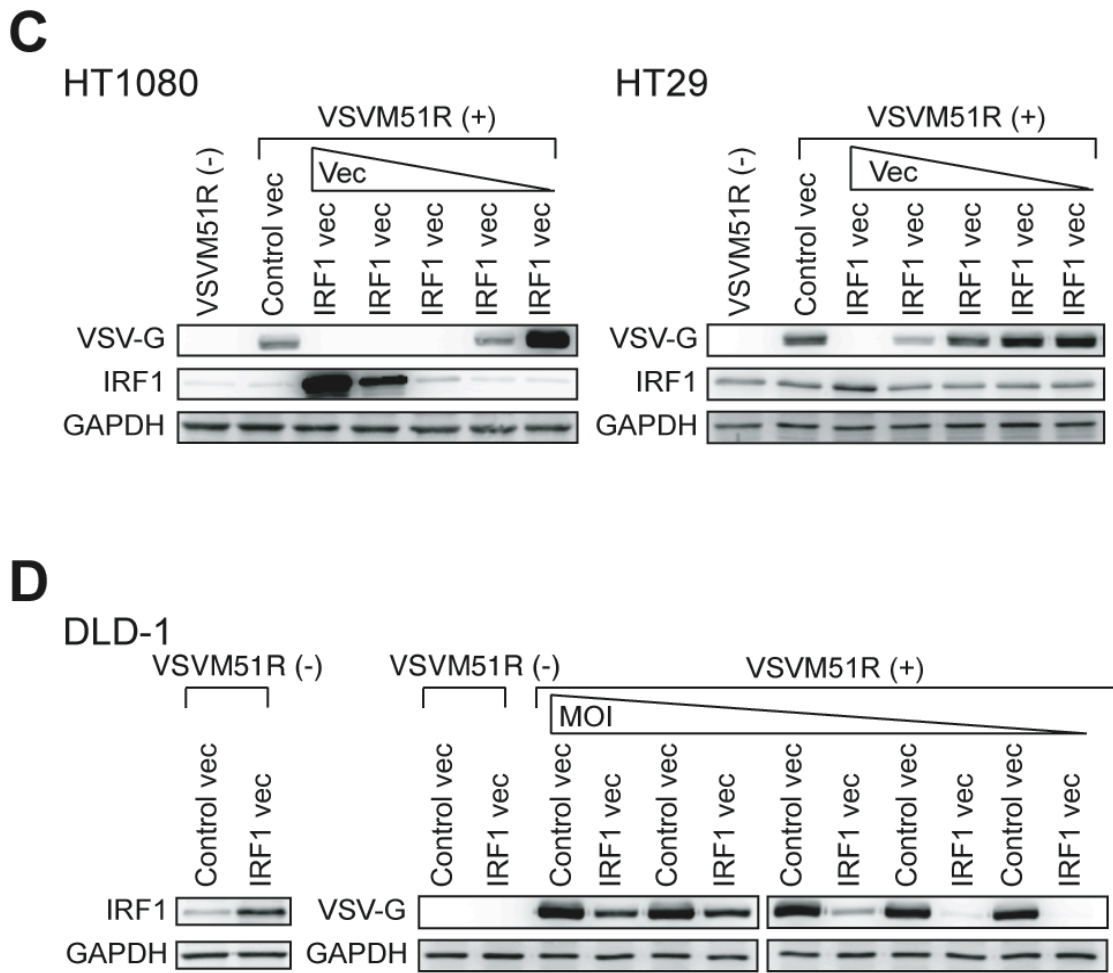


Figure 3.6

## **CHAPTER 4**

**Mechanisms underlying regulation of IRF1 expression and post-translational modifications by the Ras/MEK pathway.**

The work in chapter 4 has not been published.

## **4.1 Rationale**

In chapter 3, IRF1 downregulation by activated Ras/MEK was found to be the mechanism underlying transcriptional suppression of a group of IFN-inducible genes. Oncolytic VSV exploited the Ras/MEK-mediated IRF1 downregulation for its replication. The precise mechanism of IRF1 dysregulation remains to be elucidated. To this end, we next sought to clarify how Ras/MEK suppresses IRF1 expression. We focused on post-transcriptional regulation of IRF1 as U0126 treatment increased IRF1 protein level prior to IRF1 mRNA level (Figure 3.5 B). As IRF1 can function as its own transcriptional regulator (Reis et al., 1992), we hypothesized that the restoration of IRF1 protein leads to an increase in the level of IRF1 mRNA (Figure 4.1). In addition, we examined whether Ras/MEK regulates post-translational modifications (PTMs) of IRF1, as IRF1 PTMs have been reported to alter its biological activity (Ozato et al., 2007).

## **4.2 Results**

### **4.2.1 IRF1 protein is required to promote IRF1 mRNA expression in cells treated with the MEK inhibitor.**

We first sought to examine whether MEK inhibition increases IRF1 transcription in the absence of IRF1 protein. Three mouse IRF1 variants are reported in the NCBI database (NM\_008390.2, NM\_001159396.1, NM\_001159393.1), two of which (variant 1 and 3) have the same promoter. Variant 2 has an alternative promoter from variant 1 and 3. We constructed pGL3-Basic vector containing IRF1 variant 1 & 3 or variant 2 promoter and

**Figure 4.1** Working model: Ras/MEK activation interrupts the positive feedback loop of IRF1 expression by targeting IRF1 protein expression.

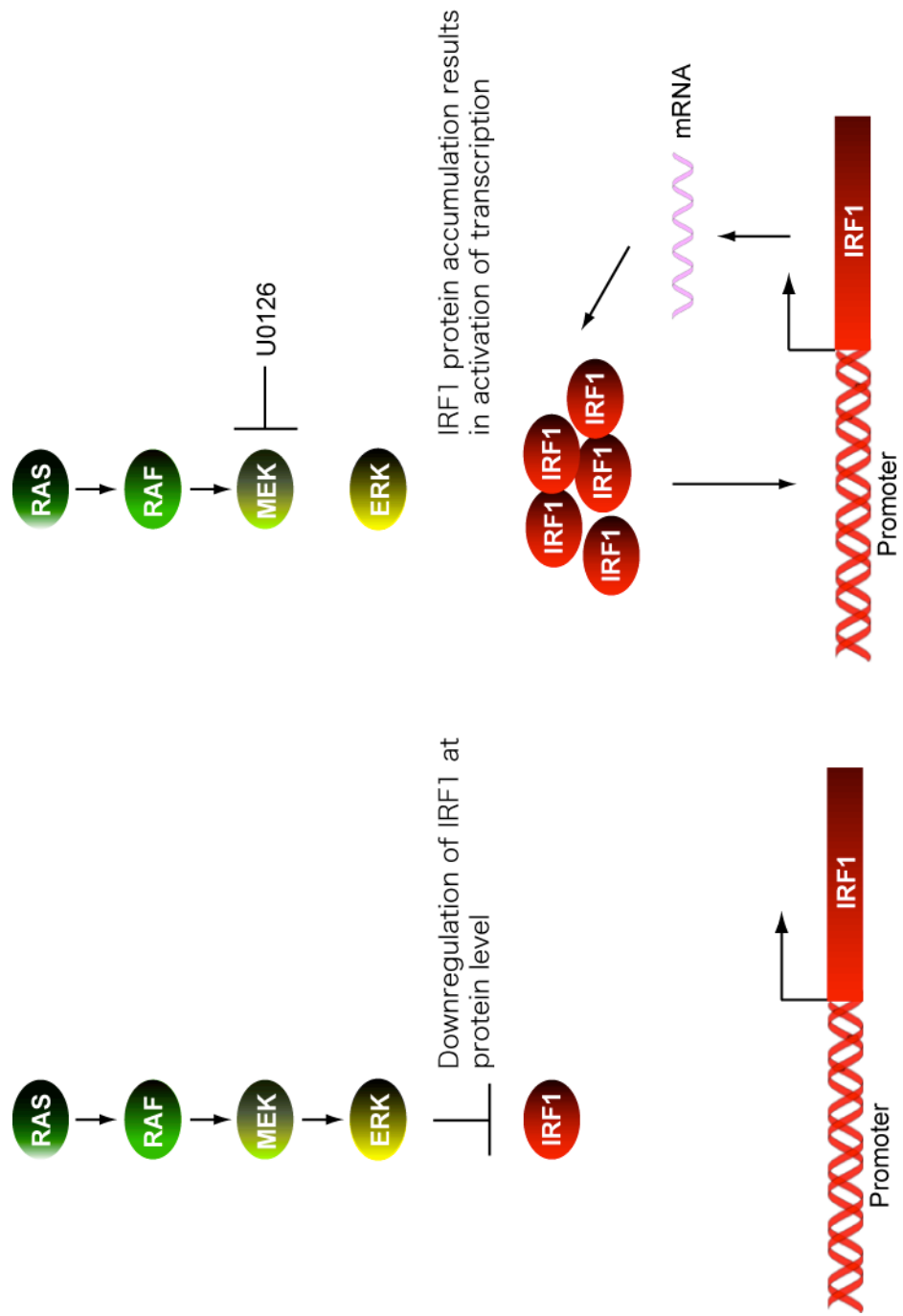


Figure 4.1

tested their promoter activities in RasV12 cells (Figure 4.2 A). Promoter activities of variant 1 & 3, and variant 2 to a lesser extent, significantly increased by 24 hours of MEK inhibition. Treatment with IFN- $\alpha$ , which is a well-known transcriptional activator of IRF1 (Taniguchi et al., 2001), significantly increased promoter activity of IRF1 variant 1 & 3, but not that of IRF1 variant 2. These data suggest that the IRF1 variant 2 promoter is not IFN-responsive. Therefore, we decided to use the promoter of IRF1 variant 1 & 3 for further study.

To determine whether IRF1 mRNA expression can be promoted by MEK inhibition in the absence of IRF1 protein, we next determined promoter activity of IRF1 variant 1 & 3 in RasV12, wild-type or IRF1<sup>-/-</sup> MEFs (Figure 4.2B). U0126 treatment significantly increased the level of IRF1 promoter activity in wild-type MEFs while the induction was lower in wild-type MEF compared to in RasV12 cells. This is likely due to less basal activation of Ras/MEK in wild-type MEF. In contrast, U0126-induced IRF1 promoter activity was not observed in IRF1<sup>-/-</sup> MEFs, suggesting that the induction of IRF1 mRNA by MEK inhibition is dependent on IRF1 protein. We also examined the promoter activity of Gbp2, which is one of the MDII genes regulated by IRF1. Similar to the IRF1 promoter, Gbp2 promoter activity was significantly higher in U0126-induced RasV12 than in wild-type MEFs treated with U0126, while U0126-induced Gbp2 promoter activity was completely abrogated in IRF1<sup>-/-</sup> MEFs. Together, these data indicate that MEK inhibition first increases the expression of IRF1 protein, which in turn exerts a positive feedback loop to activate its own transcription.

**Figure 4.2** MEK inhibition promotes IRF1 promoter activity. (A) Control pGL3-Basic plasmid, pGL3-Basic plasmid containing promoter of IRF1 variant 1 & 3 or IRF1 variant 2 were transfected into RasV12 cells. At 24 hours after transfection, the cells were treated with U0126 (20  $\mu$ M) or IFN- $\alpha$  (500 U/ml) for 24 hours. (B) pGL3-Basic plasmid containing promoter of IRF1 variant 1 & 3 or Gbp2 promoter was transfected into RasV12 cells, wild-type MEFs or IRF1<sup>-/-</sup> MEFs. At 24 hours after transfection, the cells were treated with U0126 (20  $\mu$ M) for 24 hours. Relative luciferase activities (RLU) were reported as compared with the untreated controls. [n =3 (3 replicates in 1 representative independent experiment), \*P<0.05, \*\*P<0.01].

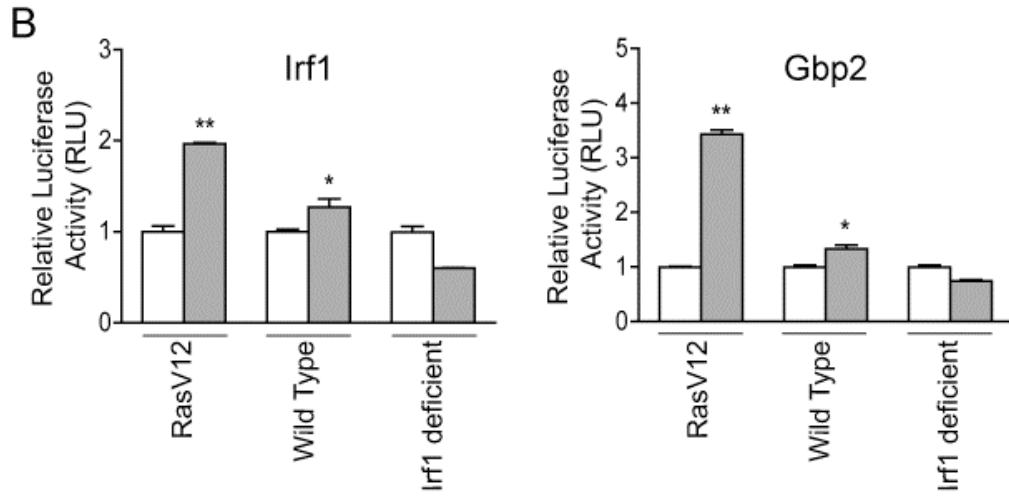
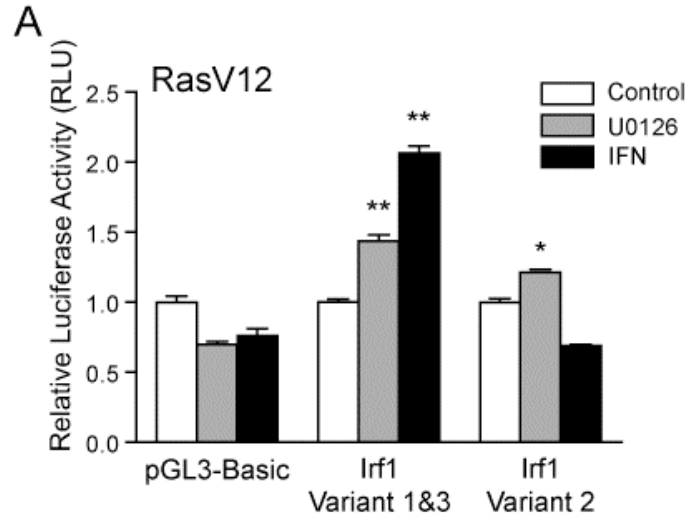


Figure 4.2



#### **4.2.2 Ras/MEK does not regulate IRF1 protein stability.**

Since MEK inhibition increased IRF1 at the protein level prior to mRNA level, we next examined whether Ras/MEK regulates IRF1 protein stability. RasV12 and human fibrosarcoma HT1080 cells were transfected with pCMV-SPORT6-IRF1 and then pretreated with or without U0126, followed by inhibition of *de novo* protein synthesis by cycloheximide treatment (Figure 4.3). IRF1 protein was rapidly degraded after the cycloheximide treatment, as demonstrated by decreasing level of IRF1 protein analyzed by IRF1 immunoblot. Inhibition of MEK did not change the stability of IRF1 protein in both RasV12 and in HT1080 cells. These data demonstrate that Ras/MEK regulates IRF1 protein in a manner independent from protein degradation.

#### **4.2.3 Role of Ras/MEK activity on post-translational modification of IRF1.**

The activity of IRF1 can be regulated by its post-translational modifications (PTMs) such as phosphorylation (Hoshino et al., 2010; Kautz et al., 2001; Lin & Hiscott, 1999; Sgarbanti et al., 2014, and Sharf et al., 1997), sumoylation (Kim et al., 2008; Nakagawa & Yokosawa, 2002, and J. Park et al., 2007), ubiquitination (Harikumar et al., 2014; Nakagawa & Yokosawa, 2000; Narayan, Pion et al., 2011, and Pion et al., 2009) and acetylation (Marsili et al., 2004; Masumi & Ozato, 2001; Qi et al., 2012, and Qiu et al., 2014) Here, we examined whether active Ras/MEK regulates PTMs of IRF1.

To determine whether active Ras/MEK modifies phosphorylation status of IRF1, RasV12 cells transfected with pCMV-SPORT6-IRF1 were treated with or without U0126

**Figure 4.3** IRF1 protein stability does not depend on Ras/MEK activity. RasV12 and HT1080 cells transfected with mouse or human pCMV-SPORT6-IRF1, respectively, were preincubated with or without U0126 (20  $\mu$ M) for 2 hours, and then treated with cycloheximide (CHX, 30  $\mu$ g/ml) for 15, 30, 45, 60, 120 and 240 minutes. Expression levels of IRF1 and GAPDH were determined by Western blot analysis.

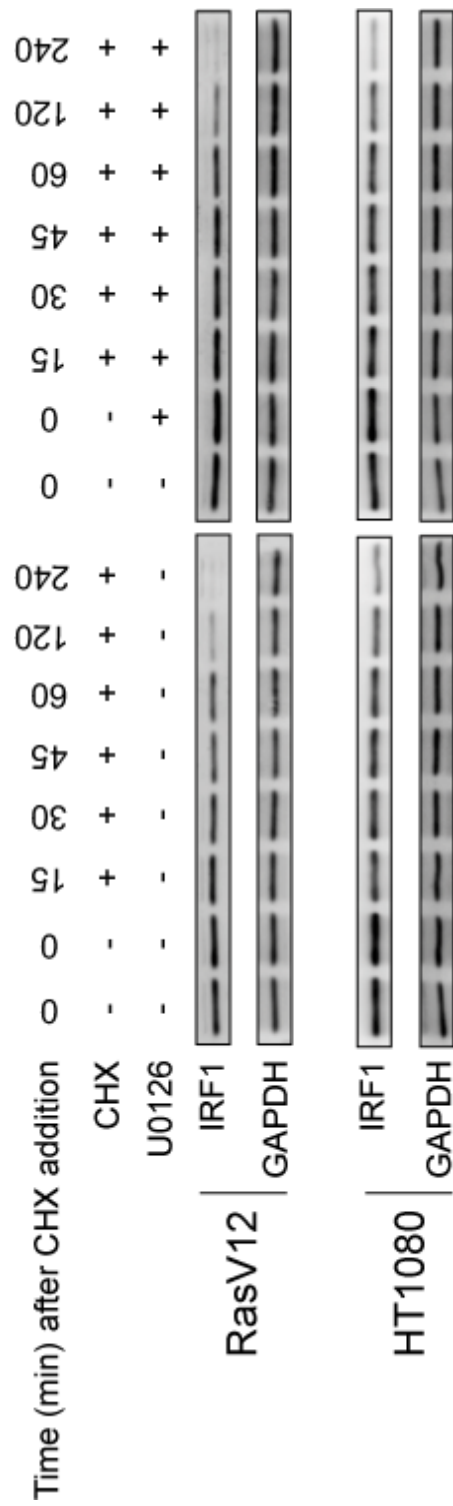


Figure 4.3

for 6 hours. Cell extracts were subjected to immunoprecipitation using anti-IRF1 antibody and phosphorylation status was determined by Western blot analysis using phospho-serine and phospho-tyrosine antibodies (Figure 4.4 A). IRF1 was efficiently immunoprecipitated from cell extracts as shown in IRF1 immunoblot (Figure 4.4 A, bottom panel). Phospho-serine antibody detected a band at approximately 48 kDa, which is the expected size for phosphorylated IRF1. U0126 treatment did not change the level of the serine phosphorylation of IRF1. Similarly, the level of IRF1 tyrosine phosphorylation was not changed by U0126 treatment in RasV12 cells, indicating that IRF1 phosphorylation is not regulated by Ras/MEK activity.

To investigate whether the Ras/MEK pathway regulates IRF1 ubiquitination, RasV12 cells were co-transfected with pCMV-SPORT6-IRF1 and pRK5-HA-Ubiquitin, and then treated with or without U0126 as described above. The cell lysates were immunoprecipitated using IRF1 antibody and the level of IRF1 ubiquitination determined by Western blot analysis with anti-HA antibody. As previously reported (Nakagawa & Yokosawa, 2000 and Pion et al., 2009), IRF1 ubiquitination was detected as smearing pattern of bands in the anti-HA western blots (Figure 4.4 B). The level of IRF1 ubiquitination was slightly increased upon treatment with U0126, suggesting the possibility that Ras/MEK activation may inhibit IRF1 ubiquitination.

Next we determined whether Ras/MEK regulates sumoylation of IRF1, a modification that changes its transcriptional activity and protein stability (Nakagawa & Yokosawa, 2002 and J. Park et al., 2007). RasV12 cells co-transfected with pCMV-SPORT6-IRF1 and pCMV-SPORT6-SUMO1 were treated with or without U0126. The cell lysates were subjected to immunoprecipitation using IRF1 antibody (Figure 4.4 C).

Since sumoylated proteins can be readily de-sumoylated by Sumo isopeptidases, N-ethylmaleimide (NEM), a de-sumoylation inhibitor, was supplemented in all the cell lysis and immunoprecipitation buffers. Park et al. (2007) previously demonstrated that sumoylated IRF1 migrates to approximately 75 kDa in the similar experimental setting. In the immunoblot using anti-SUMO1 antibody, we detected several bands ranging in size between 48-75 kDa, including the 75 kDa sumoylated IRF1 band which was observed in the membrane with longer exposure (Figure 4.4 C, middle panel). Importantly, U0126 treatment decreased the level of sumoylated IRF1 in RasV12 cells, providing a preliminary evidence that Ras/MEK activity may promote IRF1 sumoylation to suppress its function.

Lastly, we investigated whether Ras/MEK regulates the IRF1 acetylation, which increases its ability to act as a transcriptional activator (Qi et al., 2012 and Qiu et al., 2014). RasV12 cells were transfected with pCMV-SPORT6-IRF1 and treated as above. The cell lysates were immunoprecipitated using IRF1 antibody. Sodium-butyrate was added to all buffers in order to inhibit deacetylase activity during IRF1 immunoprecipitation. Acetyl-lysine antibody detected a band at the 48 kDa marker, similar to the size of IRF1 (Figure 4.4 D). The intensity of these bands, however, was not modulated by U0126 treatment, suggesting that IRF1 acetylation is not regulated by Ras/MEK activation. However, it should be noted that the size of acetylated IRF1 remains undefined in the field. Therefore, additional experiments are needed to confirm that the 48 kDa band represents acetylated IRF1. Taken together, these results provide preliminary evidence of Ras/MEK activity on promoting IRF1 sumoylation and reducing IRF1 ubiquitination.

**Figure 4.4** Effect of U0126 treatment on post-translational modifications of IRF1.

RasV12 cells were transfected with (A) pCMV-SPORT6-IRF1, (B) pCMV-SPORT6-IRF1 and pRK5-HA-Ubiquitin, (C) pCMV-SPORT6-IRF1 and pCMV-SPORT6-SUMO1 or (D) pCMV-SPORT6-IRF1 for 24 hours and next treated with DMSO control (-) or U0126 for 6 hours. At 2 hours prior to cell lysis, proteasome inhibitor MG132 (25  $\mu$ M) was added to the media. Expression levels of serine phosphorylation (P-Ser), tyrosine phosphorylation (P-Tyr), HA-tagged ubiquitination (HA), sumoylation (SUMO), acetylation (Ac-Lys) and IRF1 were analyzed by Western blot analysis. The density ratios of IRF1 phosphorylation (P-Ser/IRF1, P-Tyr/IRF1), ubiquitination (HA/IRF1), sumoylation (SUMO/IRF1), and acetylation (Ace-Lys/IRF1) to IRF1 in immunoprecipitated samples are shown as percentages normalized to values for cells treated with DMSO control (n=1, 1 independent experiment).

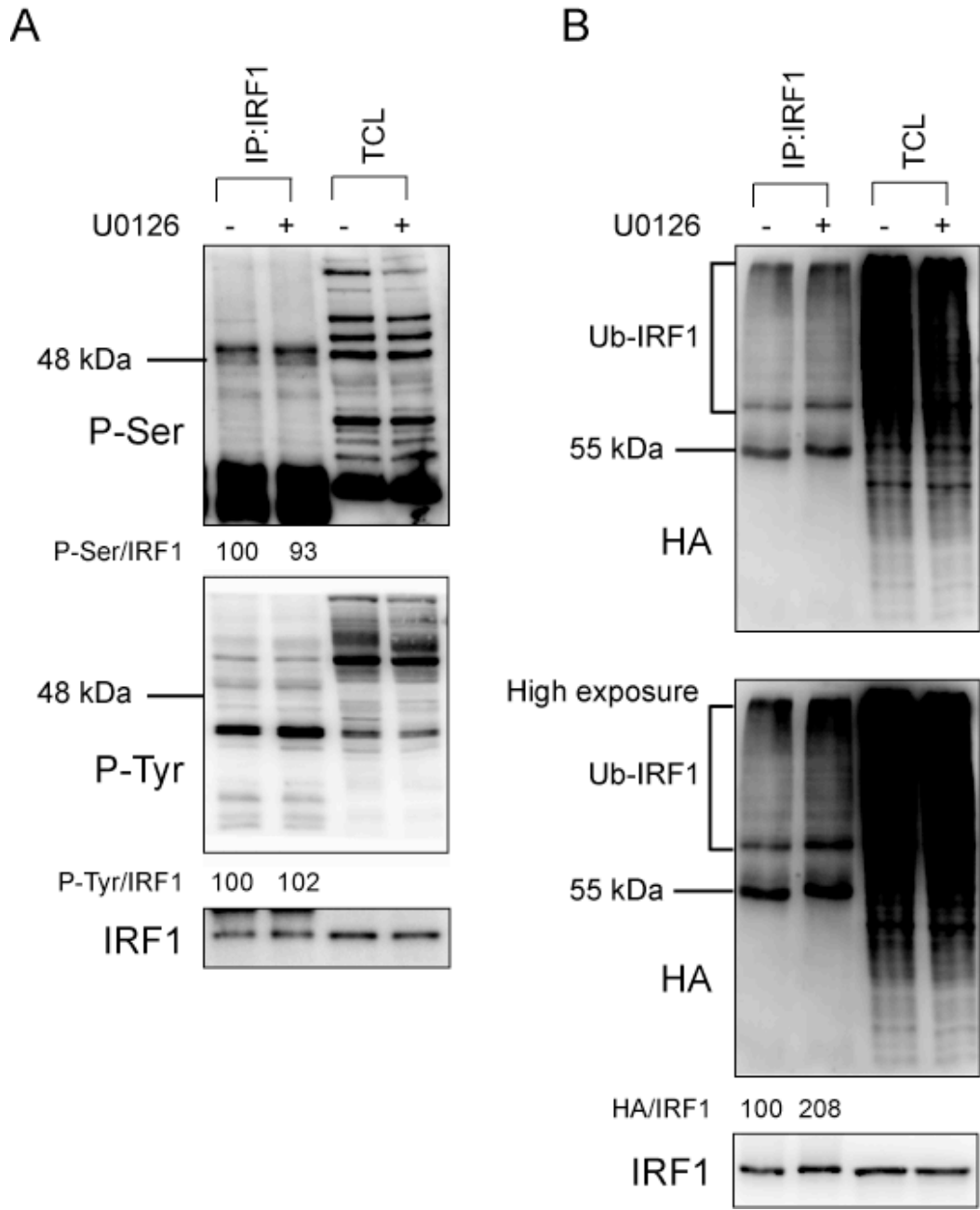


Figure 4.4

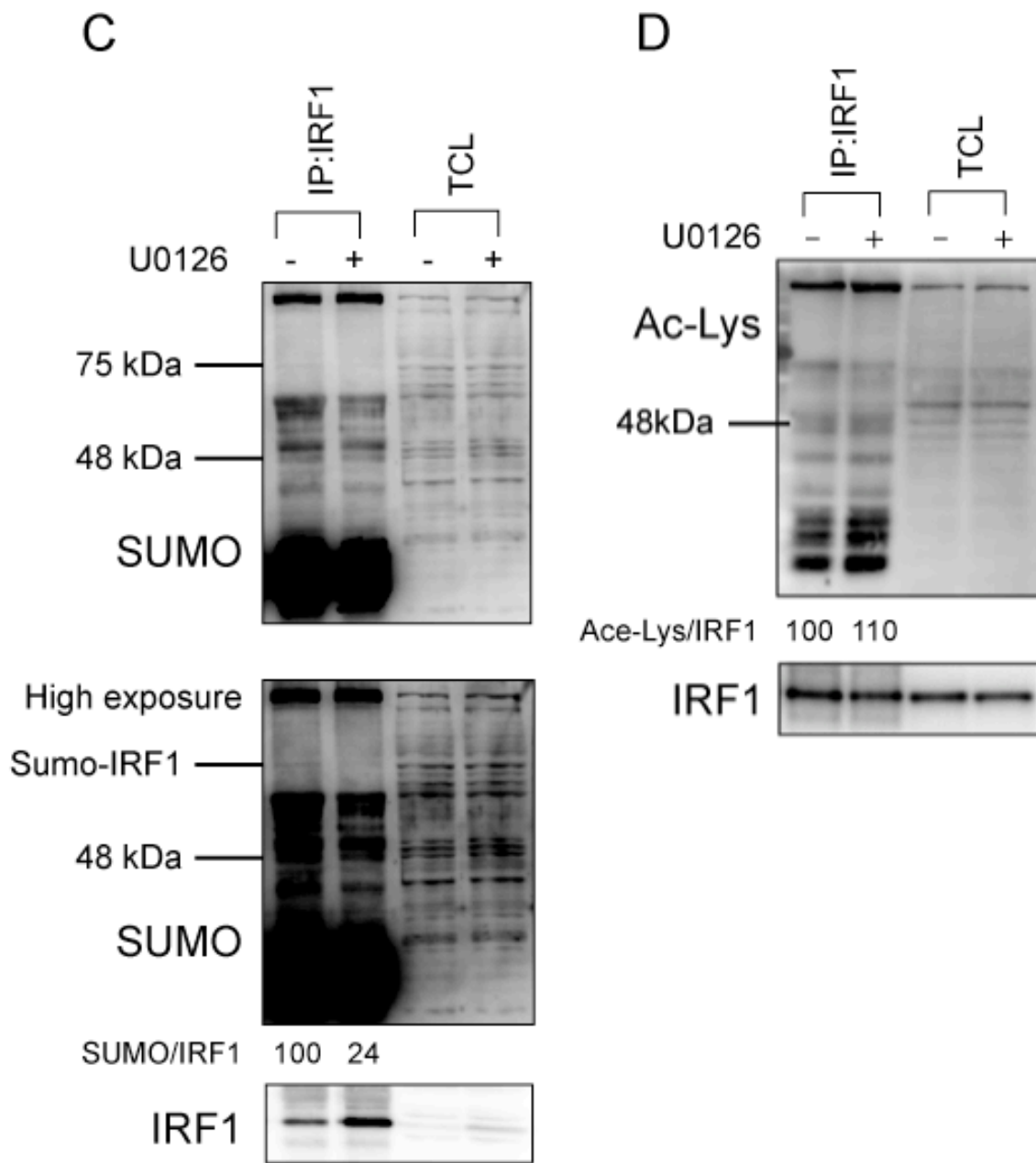


Figure 4.4



#### **4.2.4 Ras/MEK does not regulate expression of the IRF1-targeting miR-23a.**

In chapter 3, we found evidence that Ras/MEK downregulates IRF1 expression at the protein level (Figure 3.5B). However, the stability of IRF1 was not modulated by Ras/MEK activity (Figure 4.3). Based on these observations, we next sought to investigate whether the Ras/MEK pathway regulates IRF1 translation. Growing evidence suggests that miRNA can regulate protein synthesis by repressing translation or, in some cases, by promoting translation (Fabian et al., 2010 and Vasudevan et al., 2007). Therefore, Ras/MEK may modulate the expression of an IRF1-binding miRNA(s) to regulate IRF1 mRNA translation. To examine this possibility, we conducted *in silico* analysis for miRNAs that may bind to IRF1 mRNA at 3'- and 5'-untranslated region (UTR) using miRbase and miRWalk (Dweep et al., 2011; Kozomara & Griffiths-Jones, 2014).

We identified a group of 26, and another of 14, putative miRNAs that could target both mouse and human 3'- or 5'-UTR of IRF1, respectively (Table 4.1). Among this list, miR-23a was chosen for further analysis since it has been previously validated to bind to the 3'-UTR of IRF1 in gastric cancer cells (Liu et al., 2013), and its expression reported to be upregulated by oncogenic Ras in colorectal cancer cells (Ota et al., 2012 and Tsunoda et al., 2011). First, we tested whether Ras/MEK regulates miR-23a expression in our experimental system. RasV12 or DLD-1 cells were left untreated or treated with U0126 for 6 hours, and expression of miR-23a was examined by RT-qPCR. Expression level of miR-23a was not altered upon U0126 treatment in both RasV12 and DLD-1 cells (Figure 4.5 A), indicating that Ras/MEK does not downregulate IRF1 expression by modulating miR-23a expression in these cells.

**Table 4.1** *In silico* analysis of miRNAs predicted to bind to 3'- or 5'-UTR of mouse and human IRF1.

<b>IRF1 3'-UTR</b>	<b>IRF1 5'-UTR</b>
miR-301a	miR-671-5p
miR-383	miR-744
miR-130b	miR-326
miR-301b	miR-124
miR-130a	miR-675-5p
miR-20a	miR-423-5p
miR-345-5p	miR-296-3p
miR-17	miR-151-3p
miR-125b-5p	miR-298
miR-23b	let-7i
miR-23a	let-7b
miR-93	let-7d
miR-340-5p	let-7e
miR-423-3p	let-7g
miR-335-5p	
miR-204	
miR-20b	
miR-214	
miR-384-3p	
miR-31	
miR-412	
miR-124	
miR-370	
miR-9	
miR-129-5p	
miR-504	

**Figure 4.5** Effect of MEK inhibition on miR-23a expression. RT-qPCR analysis of miR-23a expression in RasV12 or DLD-1 cells treated with or without U0126 (20  $\mu$ M) for 6 hours. The relative expression level was normalized to Rnu6 and reported as compared with untreated controls. (n=3, 3 independent experiments).

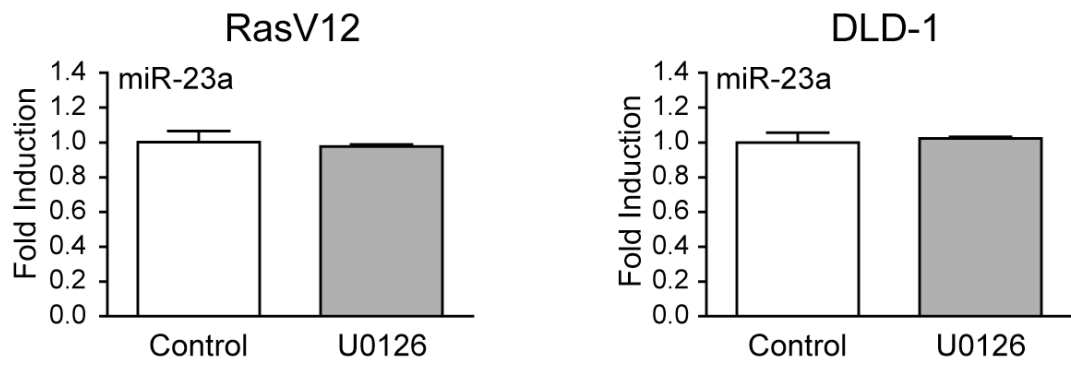


Figure 4.5

#### **4.2.5 Ras/MEK does not regulate translation of IRF1 mRNA.**

As described in the introduction, there are cellular mechanisms other than miRNA that can regulate translation of mRNA. The key *cis-acting* elements for the translational control are found at 5' - and 3' -UTR of mRNAs. To examine whether Ras/MEK regulates translation of IRF1 mRNA by factors that target its UTRs, luciferase reporter constructs containing 5' - or 3' -UTR of IRF1 were generated (Figure 4.6 A). RasV12 cells were transfected with control pGL3-Control, or pGL3-Control-IRF1-3' - or 5' -UTR reporter constructs, and then left untreated as a control or treated with U0126 (Figure 4.6 B, C). Although Ras/MEK inhibition significantly upregulated translation efficiency of IRF1 3' -UTR at both 6 and 24 hours, the expression of pGL3-Control construct was also significantly increased by U0126 treatment at both of these time points. Similarly, U0126 treatment induced luciferase activity in cells transfected with either pGL3-Control or IRF1 5' -UTR construct at 6 and 24 hours. While the increase of luciferase activity in RasV12 cells transfected with pGL3-control construct at 8 hours was not statistically significant, its activity was significantly elevated after 24 hours of U0126 treatment. We believe that the promotion of luciferase activities in all the reporter constructs was due to non-specific activation of SV40 promoter by U0126 treatment. Therefore the results of these experiments were inconclusive.

Polysome analysis was conducted next, in collaboration with Dr. Tommy Alain at Children's Hospital of Eastern Ontario (Ottawa, ON), to address the question of whether the activated Ras/MEK pathway controls IRF1 mRNA translation. RasV12 cells were treated with or without U0126 for 2 hours, as this was the time point when IRF1 protein level was significantly up but not its mRNA level (Figure 3.5 B). Cells were then treated

with cycloheximide to inhibit translation elongation and to prevent ribosome run-off, and then the cell lysates were separated on 10-50 % sucrose gradient in order to fractionate mRNAs based on the number of ribosomes loaded. Analysis of polysome profiles revealed that the levels of 40S, 60S, 80S, and polysome complexes did not change upon Ras/MEK inhibition, indicating that the global mRNA translation was not affected by 2 hours of MEK inhibition (Figure 4.7 A).

To determine whether Ras/MEK regulates polysome-loading of IRF1 mRNA, RNA isolated from each individual fractions that was converted into cDNA was analyzed by semi-quantitative RT-PCR for IRF1 and GAPDH (Figure 4.7 B). Amounts of 40S, 60S and 80S rRNA were examined on ethidium bromide gel to determine fractions containing polysomes (top panel, fractions #6-15 contain polysomes). Comparison of semi-quantitative RT-PCR analysis of ribosome-associated IRF1 mRNA between non-treatment control and U0126 treatment revealed that IRF1 mRNA were more equally broadly distributed among the fractions # 5-13 in the control group. In contrast, although IRF1 mRNA were observed in same number of fractions, U0126 treatment shifted a peak of ribosome-associated IRF1 mRNA to the fractions # 9-13, suggesting the possibility that MEK inhibition may promote translation of IRF1 mRNA (Figure 4.7 B, middle panel). Polysome loadings of GAPDH mRNA were examined as a negative control, which did not change by inhibition of MEK (Figure 4.7 B, bottom panel). To further confirm these results, the fractions representing sub-polysomes (fraction # 1-5), light polysomes containing 2-5 ribosomes (fraction # 6-9), and heavy polysomes containing 6 or more ribosomes (fraction # 10-15) were pooled, and analyzed by RT-qPCR for IRF1 and GAPDH (Figure 4.7 C). Although the level of IRF1 mRNA in the sub-polysome

fractions slightly decreased and that of the highly translating heavy polysome slightly increased by U0126 treatment, these differences were not statistically significant. Based on these observations, it is unlikely that Ras/MEK controls IRF1 expression at the translational level, though the RT-qPCR analysis needs to be conducted on each individual fraction to further confirm these results to be negative.

**Figure 4.6** Activity of 3'- and 5'- UTR of IRF1 in response to Ras/MEK inhibition (A) Illustration of IRF1 3'- and 5'- UTR luciferase reporter constructs. Luciferase activity was measured in RasV12 cells transfected with pGL3-Control, (B) pGL3-Control-IRF1-3'-UTR or (C) pGL3-Control-IRF1-5'- UTR treated with or without U0126 treatment (20  $\mu$ M) for indicated periods of time. RLU were reported as compared with the untreated controls [n=3 (3 replicates in 1 representative independent experiment), \*P<0.05, \*\*P<0.01].



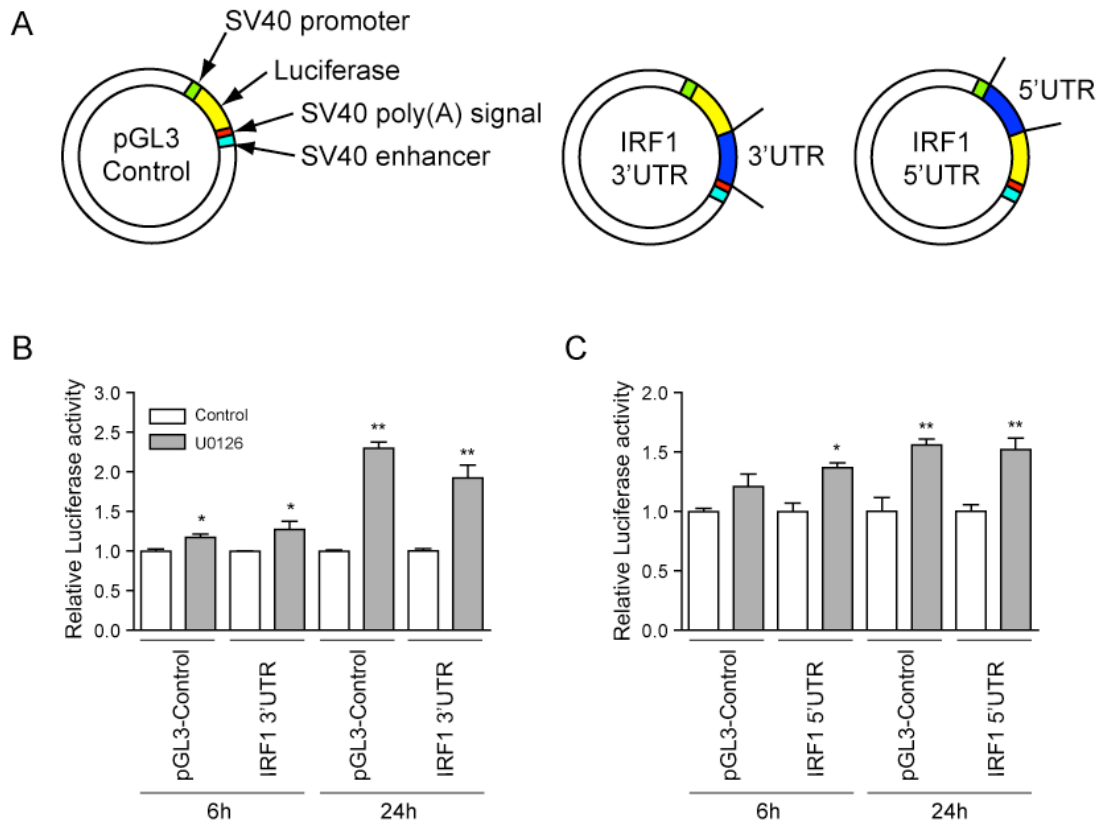


Figure 4.6

**Figure 4.7** Polysome analysis of IRF 1 in RasV12 cells. (A) Polysome profiles of RasV12 cells left untreated or treated with U0126 (20  $\mu$ M) for 2 hours were determined by recording the optical density (OD) of fractionated gradients at 254 nm. Peaks corresponding to 40S, 60S, 80S, and polysomes are indicated. (B) Ethidium bromide (EtBr) staining of RNA isolated from fraction # 1 to # 15 (top panel). Semiquantitative RT-PCR analysis of IRF1 (middle panel) and GAPDH (bottom panel) in each fraction. (C) RT-qPCR analysis of IRF1 and GAPDH in pooled fractions representing the sub-polysomes (fraction # 1-5), the light polysomes (fraction # 6-9), and the heavy polysomes (fraction # 10-15). Data was represented as percentage of polysome-associated mRNA/total mRNA (n=3, 3 independent experiments).

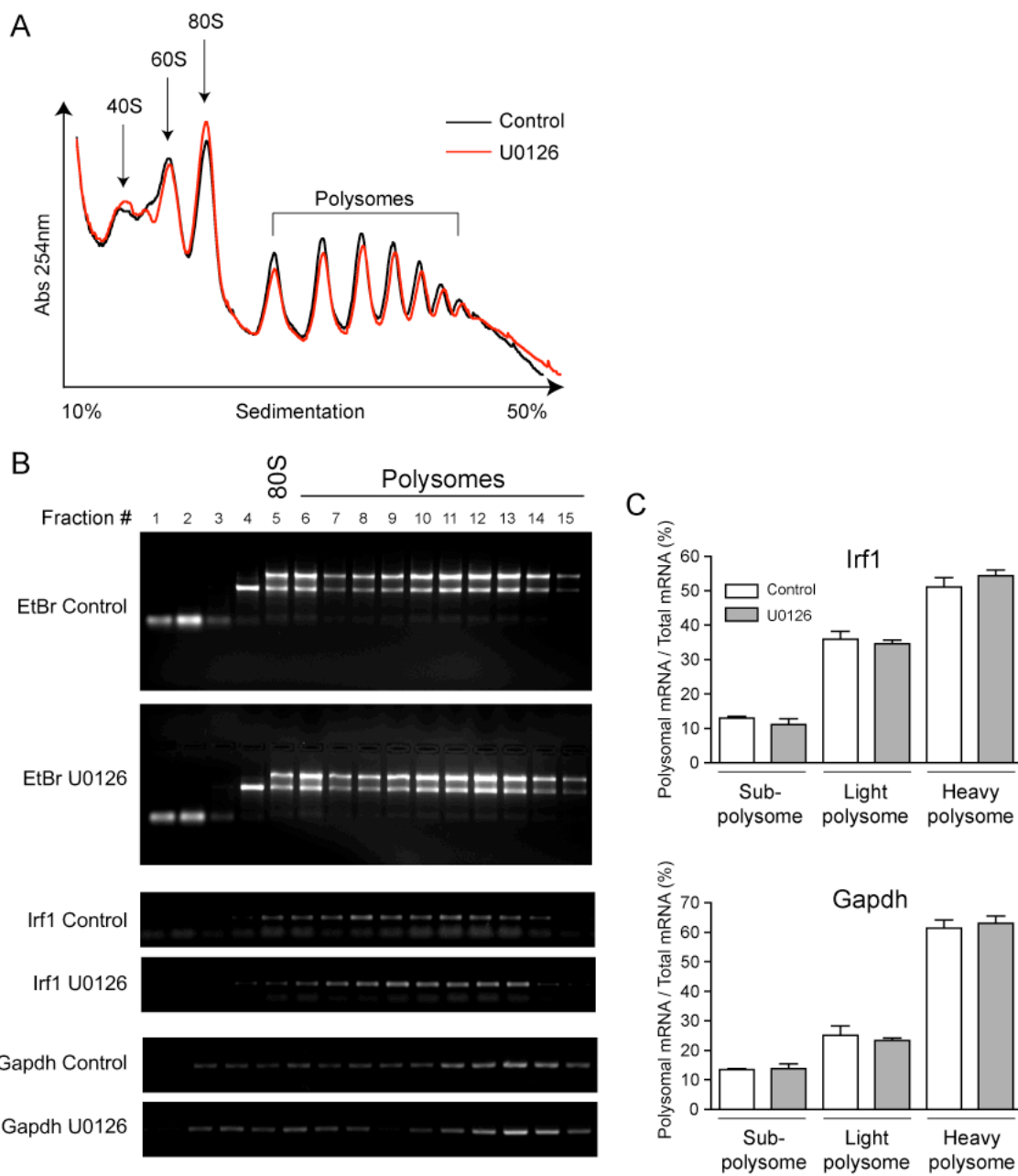


Figure 4.7

## **CHAPTER 5**

### **Identification of ERK downstream elements mediating IRF1 downregulation by the**

### **Ras/MEK pathway**

The work in chapter 5 has not been published.

## **5.1 Rationale**

ERK RNAi experiments in chapter 3 (Figure 3.5) demonstrated that IRF1 downregulation is mediated by the Ras/MEK/ERK1/2 pathway. ERKs activate a broad range of downstream elements, including Mnk1-2, Msk1-2 and Rsk 1-4 (Figure 5.1). Here, RNAi screenings against the downstream elements of ERKs were conducted to further elucidate which of these downstream ERK element(s) are involvement in the IRF1 regulation.

## **5.2 Results**

### **5.2.1 RSK3 and RSK4 downregulates IRF1 expression in RasV12 cells but not in DLD-1 and MDA-MB-468 cells.**

RasV12 cells were transfected with siRNA targeting Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk2, Rsk3 and Rsk4 for 48 hours, and the levels of IRF1 and GAPDH were analyzed by Western blot (Figure 5.2 A). Erk1/2 were also knocked down as a positive control for IRF1 promotion. Knockdowns of Erk1/2 and Rsk2 were confirmed by Western blot analysis. Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk3, and Rsk4 required RT-PCR confirmation due to lack of antibodies for their detection (Figure 5.2 B). Although the siRNA oligos showed different levels of knockdown of their targets, the expression levels were sufficiently decreased by 48 hours after siRNA transfection. IRF1 protein expression was promoted upon transfection with Erk1, Erk2, and Erk1/2 siRNAs (Figure 5.2 B), confirming our previous results (Figure 3.5 E). Single knockdown of Rsk3 and Rsk4 or co-knockdowns of Rsk3/4 also upregulated IRF1 expression in all 5 independent experiments conducted. In contrast, IRF1 expression was upregulated upon transfection

of Msk1, Msk2, Msk1/2, and Rsk2 siRNAs in a few sets of experiments, which was not always consistent (compare Figure 5.2 A top and bottom panel). Mnk1 and Mnk2 were not involved in the IRF1 regulation since IRF1 level was not altered by their knockdown in any of the experiments (Figure 5.2 A, top panel).

Rsk3 and Rsk4 silencing most consistently upregulated IRF1 protein expression in RasV12 cells, and so we next determined whether overexpression of Rsk3 and Rsk4 would modulate IRF1 expression (Figure 5.3). RasV12 and NIH3T3 cells were left non-transfected as a control, transfected with pCMV-SPORT6-Rsk3 or pCMV-SPORT6-Rsk4, or co-transfected with both vectors for 24 hours and then treated with or without U0126 for 6 hours. Overexpression of Rsk3, Rsk4 or co-overexpression of Rsk3/4 all decreased the level of IRF1 in both RasV12 and NIH3T3 cells. Furthermore, U0126 treatment increased IRF1 expression in non-transfected control RasV12 and NIH3T3 cells, but not in those co-transfected with Rsk3/4. Taken together, these results suggest that Rsk3 and Rsk4 are responsible for downregulating IRF1 expression downstream of ERK1 and ERK2.

Since Rsk3 and Rsk4 downregulated IRF1 expression in mouse fibroblasts, we next investigated whether RSKs similarly function in human cancer cells. DLD-1 and MDA-MB-468 cells were selected for this experiment since we previously observed clear restoration of IRF1 expression by MEK inhibition (Figure 3.5 C, D). Transfection of DLD-1 cells with RSK1, RSK2, or RSK4 siRNAs did not alter the level of IRF1 expression at both 48 and 72 hours post-transfection. In contrast, transfection of RSK3 siRNA decreased the level of IRF1 expression at both time points, which was opposite from our observation in RasV12 cells (Figure 5.4 A, left panel). Interestingly,

knockdowns of RSK1, RSK2, RSK3, or RSK4 all downregulated IRF1 expression in MDA-MB-468 cells at both 48 and 72 hour after siRNA transfection, suggesting that RSKs positively regulate IRF1 expression in MDA-MB-468 cells (Figure 5.4 A, right panel). Western blot analysis confirmed RNAi knockdown of RSK 1 and RSK2 and semi-quantitative RT-PCR confirmed RSK3 and RSK4 (Figure 5.4 B). Together, these results provide evidence that Ras/MEK/ERK/RSK3/4 pathway underlie IRF1 downregulation in mouse fibroblasts, but not in DLD-1 and MDA-MB-468 cells.

**Figure 5.1** ERK downstream elements. ERK downstream elements possibly involved in IRF1 downregulation include four members of ribosomal protein S6 kinases (Rsk1/2/3/4), two members of mitogen-and stress-activated kinases (Msk1/2), and two members of mitogen-activated protein kinase (MAPK)-interacting kinases (Mnk1/2).



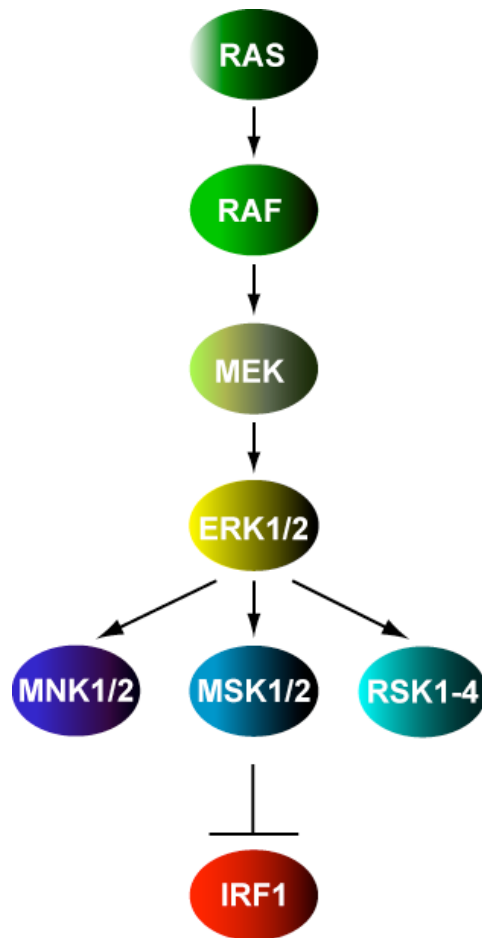


Figure 5.1

**Figure 5.2** Involvement of ERK downstream elements in IRF1 downregulation in RasV12 cells. (A) IRF1 expression levels in RasV12 cells transfected with random control (NG), Erk1, Erk2, Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk2, Rsk3, or Rsk4 oligonucleotides for 48 hours were analyzed by western blot analysis. (B) Confirmation of RNAi-mediated knockdown of Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk3, and Rsk4 by semi-quantitative RT-PCR, and ERK1/2 and RSK2 by Western blot analysis. The density ratios of IRF1 (IRF1/GAPDH), t-ERK (t-ERK/GAPDH), and RSK2 (RSK2/GAPDH) to GAPDH in Western blots are shown as percentages normalized to values for cells transfected with negative scrambled siRNA (NG). Knockdowns of Mnk1, Mnk2, Msk1, Msk2, Rsk1, Rsk2, Rsk3, Rsk4, Erk1, and Erk2 were compared to NG (1 $\mu$ l); co-knockdowns of Mnk1/2, Msk1/2, Rsk1/2, Rsk3/4, and Erk1/2 were compared to NG (2 $\mu$ l); and co-knockdowns of Rsk1/2/3/4 were compared to NG (4 $\mu$ l) (n=2, 2 independent experiments).

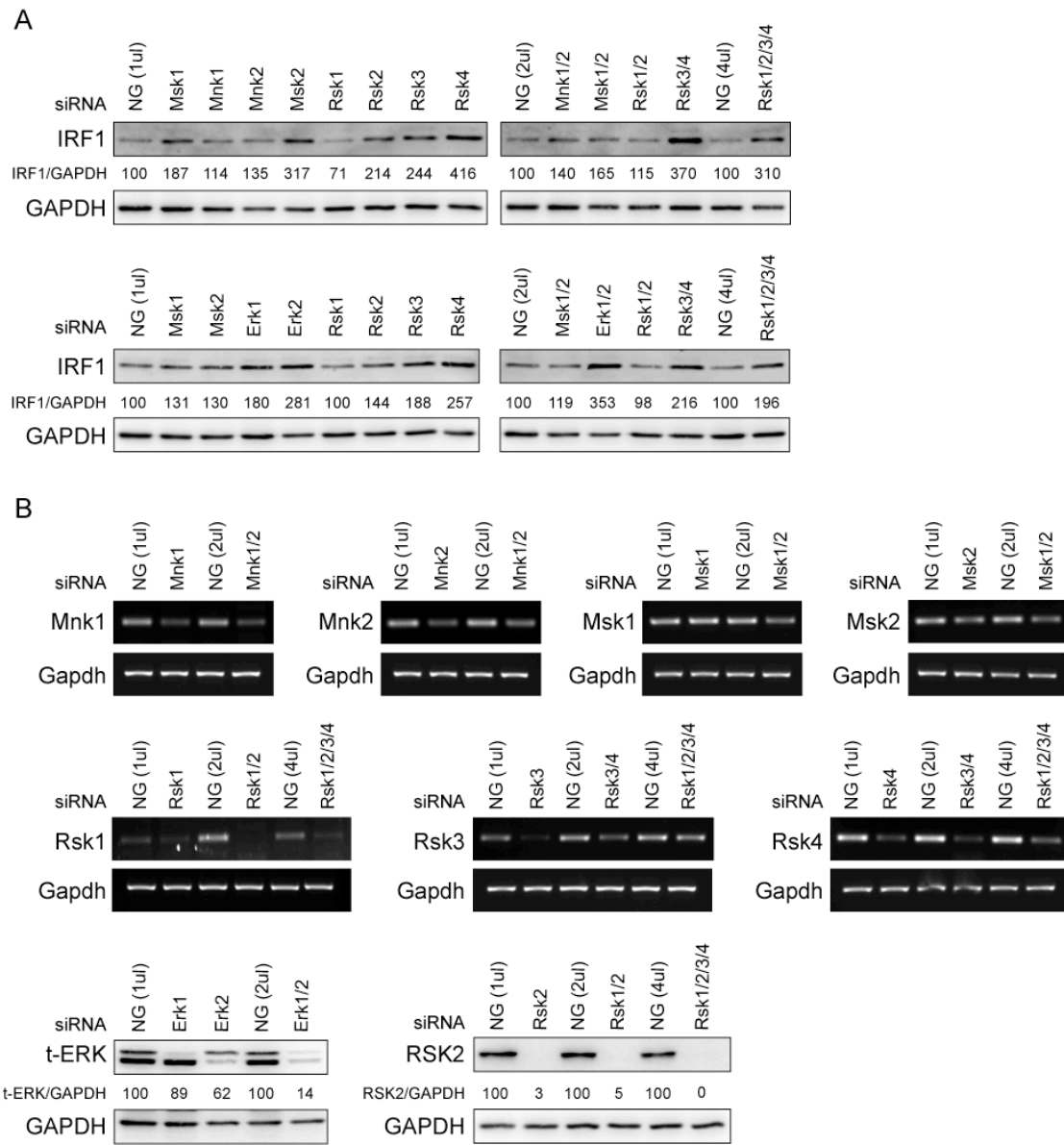


Figure 5.2

**Figure 5.3** Reduction of IRF1 expression by Rsk3 and Rsk4 overexpression in RasV12 and NIH3T3 cells. RasV12 and NIH3T3 cells were left untransfected, or transfected with pCMV-SPORT6-Rsk3 or pCMV-SPORT6-Rsk4 or co-transfected with both vectors. At 24 hours post-transfection, cells were treated with or without U0126 (20  $\mu$ M) for 6 hours. The expression levels of IRF1, RSK3, RSK4, and GAPDH were determined by Western blot analysis. The density ratios of IRF1 (IRF1/GAPDH), RSK3 (RSK3/GAPDH), and RSK4 (RSK4/GAPDH) to GAPDH are shown as percentages normalized to values for non-transfected control cells (-). (n=1, 1 independent experiment).

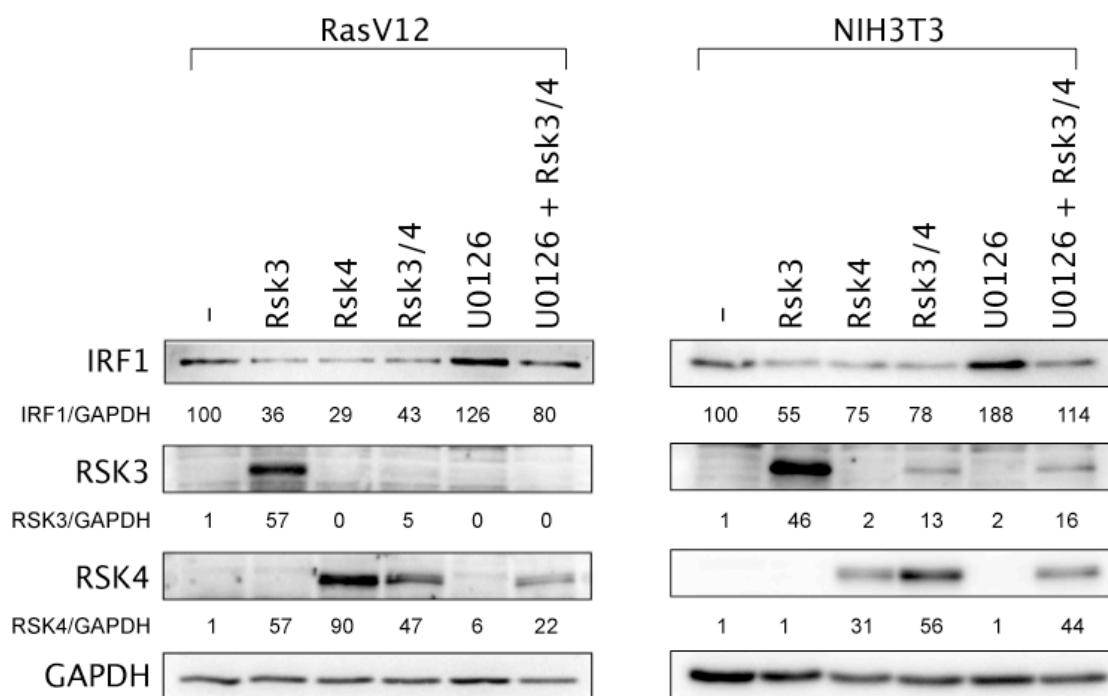


Figure 5.3

**Figure 5.4** Modulation of IRF1 expression by RSK knockdowns in human cancer cell lines. (A) DLD-1 (left) and MDA-MB-468 cells (right) transfected with negative scrambled siRNA (NG), human RSK1, RSK2, RSK3, or RSK4 oligonucleotides for indicated time points. IRF1 and GAPDH expression levels were determined by Western blot analysis. (B) Confirmation of siRNA-mediated knockdown of RSKs by Western blot or by semi-quantitative RT-PCR. The density ratios of IRF1 (IRF1/GAPDH), RSK1 (RSK1/GAPDH), and RSK2 (RSK2/GAPDH) to GAPDH in Western blots are shown as percentages normalized to values for cells transfected with negative scrambled siRNA (NG). Knockdowns of Rsk1, Rsk2, Rsk3, and Rsk4 were compared to NG (1 $\mu$ l); and co-knockdowns of Rsk1/2/3/4 were compared to NG (4 $\mu$ l) (n=1, 1 independent experiment).

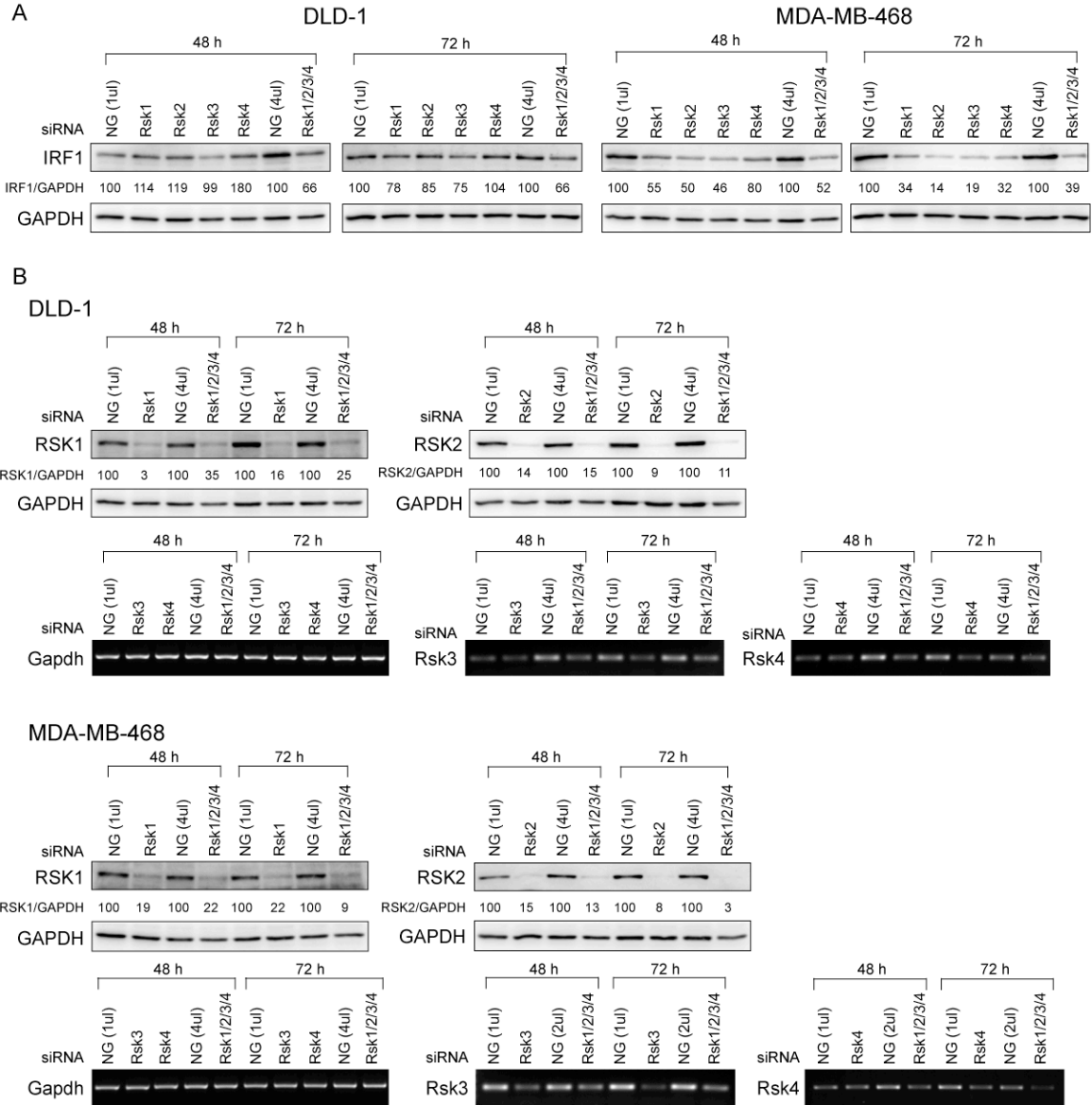


Figure 5.4

## **CHAPTER 6**

### **DISCUSSION**



### **6.1 Chapter 3 discussion: Oncogenic Ras inhibits IRF1 to promote viral oncolysis**

The Hirasawa lab previously reported that MEK downregulated IFN-inducible (MDII) genes were suppressed by Ras/MEK in human cancer cells (Christian et al., 2012). In this study, I clarified the molecular mechanisms underlying Ras/MEK-mediated downregulation of MDII gene transcription. This study demonstrates that 1) IRF1 is the transcriptional regulator of MDII genes, 2) activated Ras/MEK reduces IRF1 expression in mouse fibroblast cells and human cancer cells, and 3) the expression level of IRF1 modulates cellular susceptibility to viral oncolysis.

Initially identified as a transcriptional activator of the IFN- $\beta$  gene, IRF1 is broadly involved in regulating gene transcription important for innate immune responses (Kimura et al., 1994; Miyamoto et al., 1988, and Taniguchi et al., 2001). IRF1 is a potent antiviral effector, and its deficiency dramatically increases cellular susceptibility to different types of viruses (Brien et al., 2011; Kanazawa et al., 2004, and Pine, 1992).

IRF1 also functions as a tumor suppressor that simultaneously upregulates the transcription of tumor suppressor genes while downregulating that of oncogenes, in order to induce apoptosis and growth inhibition (Tamura et al., 1995). Therefore, it is likely beneficial for tumor cells to acquire oncogenic cellular characteristics that dysregulate the antitumor functions of IRF1 during their development. In fact, the IRF1 gene is lost, mutated, or downregulated in several types of cancer (Willman et al., 1993) and is negatively correlated with the tumor grade and risk of recurrence in breast cancer and hepatocellular carcinoma (Doherty et al., 2001; Moriyama et al., 2001, and Cavalli et al., 2010). Activating mutations of the *RAS* have been found in approximately 30% of all

human cancers (Adjei, 2001). Moreover, in cancers where direct activating mutations of *RAS* are absent, the upstream or downstream signaling components of the Ras pathway are often found to be over-active instead (Downward, 2003), suggesting that Ras/MEK activation may underlie IRF1 impairment in a broad range of human cancer cells. Such observations suggest that the IRF1 downregulation may be a common aberrant characteristic in human cancer cells that can be exploited by Ras-dependent or IFN-sensitive oncolytic viruses.

As I show here, IRF1 is the key transcriptional regulator of the MDII genes that are responsible for antiviral response, IFN production or Jak/STAT activation (Figure 6.1). Therefore, the low expression IRF1/MDII genes in cancer cells results in a delay in establishing antiviral responses, and allows oncolytic viruses to replicate. However, in normal cells expressing sufficient levels of IRF1, as the expression of MDII genes are maintained, and the antiviral responses can be induced rapidly and efficiently upon oncolytic virus infection. The IRF1/MDII genes-downregulation by MEK is particularly important at the initial stage of viral oncolysis. In contrast, other tumor-specific molecular changes may play critical roles in maintaining cancer cell susceptibility to oncolytic viruses at the later phase of infection when IFNs and cytokines are actively produced. Recent studies have reported that the steady state levels of antiviral genes correlate with sensitivity of cancer cells to viral oncolysis (Iankov et al., 2014 and Kurokawa et al., 2014).

Some of the MDII genes identified from this study including Gbp2, Rig-I, Il-15, Xaf1, and Stat2 were previously reported ISGs (Schneider et al., 2014). In addition to these previously known ISGs, we also identified a novel ISG, Ifi47, which was potently induced

by IFN- $\alpha$  treatment in RasV12 cells. The function of Ifi47 remains to be elucidated. Among the six MDII genes validated by qPCR analysis, three MDII genes (*Gbp2*, *Rig-I*, and *Stat2*) were common in our previously conducted human HT1080 microarray analysis (Christian et al., 2012). To determine exactly how many of 619 MDII genes overlap between the mouse (RasV12) and the human system (HT1080), independent bioinformatic analysis needs to be conducted in the future. A number of IRF1 regulated MDII genes identified from this study have antiviral functions. IFN-induced GTPase, *GBP2* inhibits VSV and EMCV replication (Carter et al., 2005). *XAF-1* is an important mediator of IFN-induced apoptosis and potential tumor suppressor that binds and antagonizes the anti-apoptotic function of XIAP (Liston et al., 2001). *XAF-1* plays a critical role in antiviral defense by inducing apoptosis of virus-infected cells as its expression is highly induced upon infection by influenza virus (Sutejo et al., 2012) and dengue virus (Long et al., 2013). A cytokine IL-15 stimulates proliferation and activity of lymphocytes that kill virus-infected cells or tumors (Steel et al., 2012). dsRNA helicase *RIG-I* functions as an intracellular pattern recognition receptor for dsRNA or ssRNA containing 5'-phosphate that are produced during viral replication. Activation of *RIG-I* stimulates antiviral innate immune response (Pichlmair et al., 2006 and Yoneyama et al., 2004). Considering the broad range of antiviral functions of IRF1 regulated MDII genes, their suppression by Ras/MEK-mediated IRF1 downregulation is one of the major mechanisms of how oncolytic viruses replicate in cancer cells, but not in normal cells. It still remains to be studied how the downregulation of each MDII genes contributes to viral oncolysis, which may lead to identification of therapeutic target genes to enhance the efficacy of oncolytic therapy.

Both type I and type II IFNs are known as potent inducers of IRF1 (Taniguchi et al., 2001). IRF1 promotion by Ras/MEK inhibition may be a secondary effect of IFN induction and activation of the IFN pathway. However, this is unlikely the case because MEK inhibition did not activate the Jak/STAT pathways (Christian et al., 2009) and because transcriptional activators of IFNs, such as IRF3 and IRF7, were not induced by U0126 treatment in the microarray analysis. In support of these observations, treatment of cells with anti-IFN- $\alpha/\beta$ -R antibody did not abrogate induction of IRF1 and the MDII genes by U0126 treatment (Figure 3.2 B), indicating that IRF1 promotion by Ras/MEK inhibition does not involve type I IFN production.

Within cancer cells, Ras activation and IFN insensitivity are the two major cellular characteristics targeted by oncolytic viruses. Ras activation promotes replication of oncolytic viruses in multiple ways, such as inhibition of antiviral function of PKR, or enhancement of virus entry and uncoating, viral particle release and translation of viral mRNA (Goetz et al., 2010; Marcato et al., 2007; Strong et al., 1998). Deficiencies in IFN signaling components (Sun et al., 1998) and epigenetic modifications of chromatin (Nguyen et al., 2008) have also been reported to contribute to the generation of a defective innate immune response. Here, we demonstrate that IRF1 downregulation is responsible for transcriptional suppression of IFN-inducible genes in cells with activated Ras/MEK. By conducting knockdown and overexpression of IRF1, we demonstrate that replication of both wild-type and mutant strain of VSV was dependent on the expression levels of IRF1. In RasV12 cells, silencing of IRF1 did not alter its sensitivity to VSV infection, but promoted infection when cells were treated with IFN or with combination of IFN and U0126. This

could be that IRF1 knockdown did not make a significant difference in the level of IRF1 expression in RasV12 cells because basal IRF1 expression is already low.

We observed more infection in HT1080 and HT29 cells transfected with the lowest amount of IRF1 construct compared to in those transfected with empty vectors (Control vector vs IRF1 vector with the lowest amount of IRF1 transfection) (Figure 3.6 C). This is probably because protein samples were taken after the peak of infection in cells transfected with control vector. In fact, we observed higher cytopathic effects in cells transfected with control vector than those transfected with the lowest amount of IRF1 construct.

As shown in figure 3.5 A, Western blot analysis revealed that there is an additional IRF1 band in cell lysate of NIH3T3 cells but not in that of RasV12 cells. The additional band could be 1) post-translational modified (PTM) IRF1, 2) an IRF1 isoform, or 3) non-specific band. As examined in chapter 4, IRF1 can be modulated by sumoylation or ubiquitination, processes regulated by the Ras/MEK activity. However, since the size of the additional band is different from that of SUMO-IRF1 or ubiquitinated-IRF1, it is unlikely that the band represents a PTMs form of IRF1. A new transcript variant of mouse IRF1, named variant X1 (XM\_006532308.1) recently has been reported in NCBI database. This variant includes an additional 137AA or 162AA compared to mouse IRF1 isoform A (NP\_032416.1) and isoform B (NP\_001152865.1), respectively. As the expected size of variant XI is similar to the additional band observed in NIH3T3 cells, it is possible that the additional band could represent one of the different isoforms of IRF1. Lastly, the observed band X could be non-specific signal for two reasons. First, it was only detected by IRF1 M-20 antibody (Santa Cruz) but not by IRF1 NBP1-78761 antibody (NEB) (data not shown).

Secondly, the protein X was more stable than IRF1 when its stability was examined by cycloheximide experiment (data not shown).

In chapter 3, promoter activity of Gbp2 was unchanged in response to U0126 treatment either in the presence or absence of IFN- $\alpha/\beta$  receptor antibody which blocked the receptor from binding to endogenous IFN; therefore it was hypothesized that MEK inhibition induced MDII gene transcription by transacting factor other than IFN (Figure 3.2B). Of note, there are other types of IFN, including type II or type III IFNs, which signal through different receptor complexes as described in the chapter 1.3.1. Therefore, we cannot exclude the possibility of MEK inhibition having an effect on expression of other types of IFNs, and this needs to be examined in future study.

**Figure 6.1** A schematic diagram illustrating the suppression of MDII gene expression by Ras/MEK via inhibition of IRF1. The expression of the MDII genes is regulated by the level of IRF1 expression, which is in turn regulated by Ras/MEK activity.

The MDII genes are involved in promoting host defense against oncolytic viruses at the different stages, such as antiviral response (Gbp2, Xaf1), IFN production (Rig-I, Il15) and IFN sensitivity (Stat2). The decreased expressions of the MDII genes by active Ras/MEK support replication of oncolytic virus in cancer cells, but not in normal cells. The relative expression levels of the proteins are symbolized by the size of the protein.

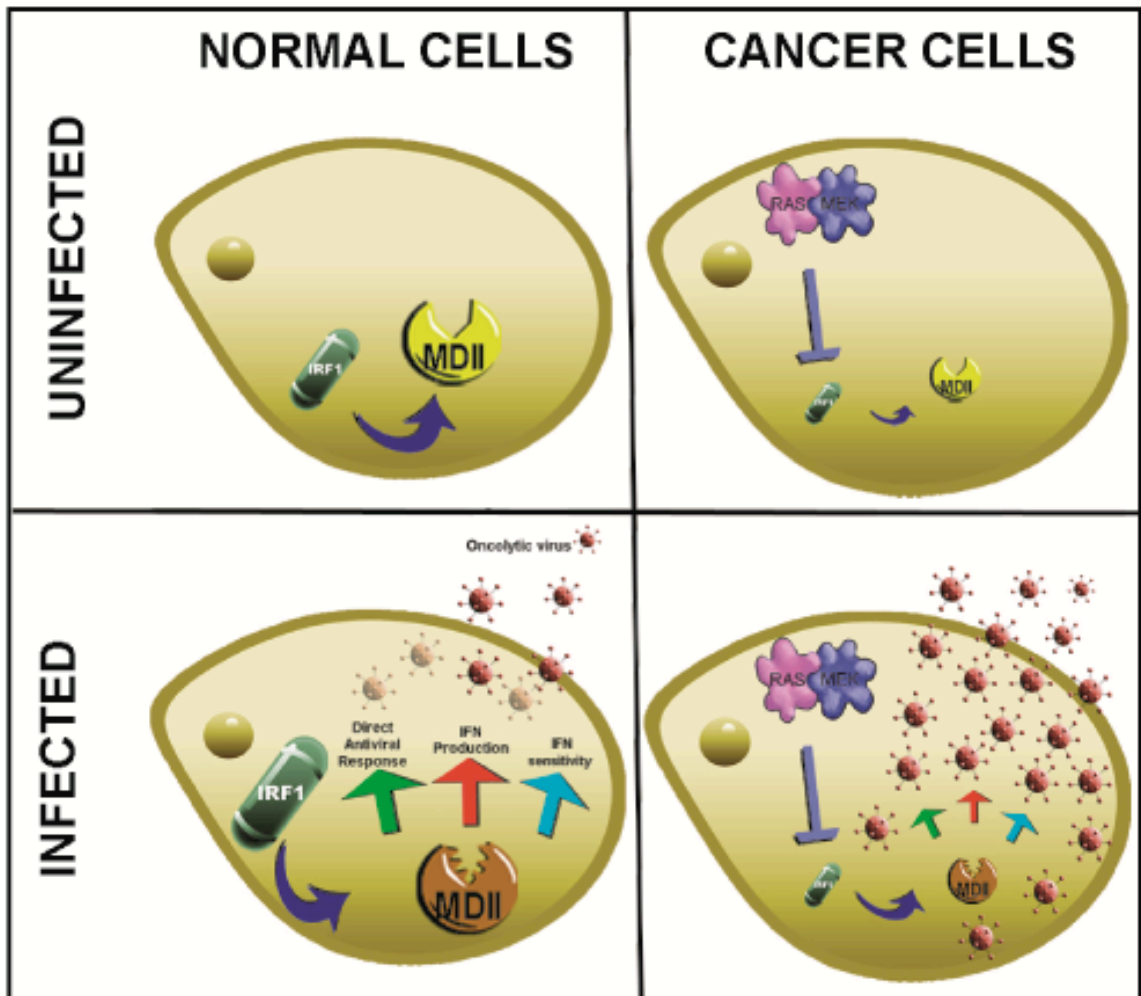


Figure 6.1



## **6.2 Chapter 4 discussion: Mechanisms underlying regulation of IRF1 expression and post-translational modifications by the Ras/MEK pathway.**

By conducting IRF1 immunoprecipitation (IP) experiment, we found preliminary evidence that suggests that Ras/MEK activation may suppress IRF1 ubiquitination, and promote IRF1 sumoylation. It should be noted however, that additional controls for IP and alternative experiments must be conducted to confirm these results. For Figure 4.4 B and C, IRF1 IP was conducted on RasV12 co-transfected with pCMV-SPORT6-IRF1 and pCMV-SPORT6-SUMO1 or pRK5-HA-Ubiquitin expression vectors. To ensure that the bands we saw were Ubiquitin-IRF1 or Sumo-IRF1 and not non-specific bands, future IP experiment should include cells transfected with pCMV-SPORT6-IRF1 only or pCMV-SPORT6-SUMO1 or pRK5-HA-Ubiquitin expression vector only. In addition, IgG control group can be included for additional control. If the bands we saw were specific for Ubiquitin-IRF1 or Sumo-IRF1, then these bands should only come up in co-transfection group immunoprecipitated with the IRF1 antibody.

It remains to be further elucidated whether these PTMs modulate the stability and/or function of IRF1. In addition to its roles in protein degradation, ubiquitination plays a critical role in regulating the activities of transcriptional regulators (Komander & Rape, 2012). Importantly, nonproteolytic ubiquitylation can modulate DNA binding and activity of transcriptional regulators (Geng et al., 2012). Therefore, IRF1 ubiquitination induced by Ras/MEK inhibition may increase IRF1 expression by promoting IRF1 transcriptional activity that can in turn promote its own mRNA expression. While IRF1 poly-ubiquitination via Lys48-linkage is essential for its degradation by proteasomes,

poly-ubiquitination via Lys63-linkage functions in nonproteolytic processes (Geng et al., 2012). This is supported by a recent study demonstrating that transcriptional activity of IRF1 is activated by Lys63-linked polyubiquitylation following IL-1 stimulation in human embryonic kidney HEK293 cells (Harikumar et al., 2014). Considering that Ras/MEK activation does not modulate IRF1 stability (Figure 4.3), Ras/MEK may regulate polyubiquitination of IRF1 via Lys63-linkage to activate its transcriptional activity. Interestingly, the activities of other members of the IRF transcription factor family are also regulated by Lys-63 linked polyubiquitination. Lys-63 linked polyubiquitination of IRF5 and IRF7 by E3 ubiquitin ligase TRAF6 leads to nuclear translocation and binding to its target gene promoters (Balkhi et al., 2008; Ning et al., 2008). In addition, IRF3 is activated by Lys63-linked polyubiquitination in response to viral infection (Zeng et al., 2009). As a next step, it will be critical to determine if Ras/MEK regulates ubiquitination via Lys48, Lys63 or both of them.

Sumoylation can alter activity, stability, or protein-protein interactions in response to various types of cellular stimuli. Preliminary evidence from this study suggested that Ras/MEK inhibition decreases IRF1 sumoylation in RasV12 cells. Previous studies have shown that sumoylation of IRF1 increases its stability and inhibits its transcriptional activity (Nakagawa & Yokosawa, 2002; Park et al., 2007, and Kim et al., 2008). IRF1 sumoylation was elevated in ovarian tumor compared to in normal tissue (Park et al., 2007). Moreover, introduction of sumoylated IRF1 interferes with anticancer activities of endogenous IRF1 and induces transformation of NIH3T3 cells. (Park et al., 2010). In contrast, effects of IRF1 sumoylation on antiviral responses remain to be studied. Recent studies indicate that viral infection can promote sumoylation of other IRFs, such as IRF3

and 7, which can be used by viruses to evade the host's antiviral response (Kubota et al., 2008; Chang et al., 2009, and Bentz et al., 2012). Interestingly, Kaposi's sarcoma associated herpesvirus (KSHV) encodes viral Sumo E3 ligase K-bZIP that can sumoylate IRF1, suggesting that IRF1 sumoylation is one of targets for immune evasion during KSHV infection (Chang et al., 2013). Based on these findings, promotion of IRF1 sumoylation by Ras/MEK activation may increase cellular susceptibility to oncolytic virus in cancer cells. This needs to be further confirmed in the future study.

While IRF1 variant 1 & 3 promoter was activated either by MEK inhibition or IFN- $\alpha$  treatment (Figure 4.2 A), variant 2 promoter did not respond at all to IFN- $\alpha$  treatment and responded to a much lesser extent to U0126 treatment. This suggests that IRF1 variant 2 may play a different role from type I IFN-mediated antiviral defense. It also is possible that cytokines other than type I IFN activate its transcription, which needs to be further studied. By conducting IRF1 promoter reporter analysis in IRF1<sup>-/-</sup> MEFs (Figure 4.2 B), we found that IRF1 protein is necessary for promotion of IRF1 mRNA by U0126. These data suggest that Ras/MEK inhibition first restores IRF1 protein expression or function that can then activate its own transcription. As Ras/MEK does not regulate IRF1 stability (Figure 4.3), it regulates either the IRF1 transcriptional activity by modulating IRF1 PTMs or the translation efficiency of IRF1 mRNA.

To this end, we next examined whether MEK inhibition leads to promotion of IRF1 mRNA translation by conducting polysome analysis of IRF1 mRNA. The Ras/MEK pathway is known to play a significant role in translational regulation by phosphorylating the components of the translational machinery. U0126 treatment has been shown to

change the profile of polysome-associated mRNA in glial progenitor cell with activated Ras (Rajasekhar et al., 2003). In addition, the Ras/MEK pathway promotes phosphorylation of translational repressor 4E-BP1 at multiple sites, which upon phosphorylation, release eIF4E for initiation of translation (Herbert et al., 2002). Unlike these studies, we did not observe any significant difference in the level of global translation in RasV12 cells in response to MEK inhibition (Figure 4.8 A).

In order to determine whether Ras/MEK modulates miRNA to control translation of IRF1 mRNA, we first conducted *in silico* analysis and identified 40 miRNAs that have putative binding sites in IRF1 5'- or 3'- UTR. Among the candidate mRNAs, I analyzed expression of miR-23a, which was previously found to be upregulated by oncogenic K-Ras activation in colorectal cancer cells HCT116 and DLD-1 (Tsunoda et al., 2011 and Ota et al., 2012). The U0126 treatment, however, did not change miR-23a expression level in both RasV12 and DLD-1, suggesting that other Ras downstream elements are likely involved in regulating its expression.

Since the 5'- and 3'-UTRs of mRNA are essential for translational regulation or miRNA binding, we also conducted IRF1 UTR reporter assay. However, this result was inconclusive as U0126 treatment activated the promoter activity of control pGL3 vector, likely due to activation of its SV40 promoter. Therefore, alternative approach such as using reporter constructs with a different promoter that does not respond to U0126 treatment, or *in vitro*-transcribed RNA reporter constructs should be utilized in the future study. Overall, we did not find clear evidences to demonstrate that Ras/MEK modulates translation of IRF1 mRNA via IRF1 5'- or 3'-UTR or miRNA.

### **6.3 Chapter 5 discussion: Identification of ERK downstream elements mediating IRF1 downregulation by the Ras/MEK pathway.**

During siRNA screening of ERK downstream elements, we identified RSK3 and RSK4 to be responsible for Ras/MEK-mediated downregulation of IRF1 expression in RasV12 cells. In contrast, these kinases were not involved in the IRF1 downregulation in DLD-1 and MDA-MB-468 cells. In fact, knockdown of RSKs further downregulated IRF1 expression in these cells. There are many other signaling pathways deregulated in human cancer cells while only Ras and its downstream elements are activated in RasV12 cells. These differences in signaling environment may attribute to the discrepancy between mouse and human systems. Moreover, the functions of human RSK3 and RSK4 may be different from those in mouse. The functions of human RSK3 and RSK4 have been well characterized while there are very few reports on the function of mouse RSK3 and RSK4. If this is the case, we will need to conduct the RNAi screening using human cancer cells, as other Ras downstream elements may be involved in the IRF1 downregulation. IRF1 expression was upregulated by MSK1 or MSK2 silencing in two out of five experiments conducted with RasV12 cells. This also should be further determined by using different approaches such as introduction of constitutively active mutants or a 2<sup>nd</sup> set of siRNA oligos.

In a follow-up study, we examined the RSK-IRF1 connection using BI-D1870, an inhibitor of the N-terminal kinase activity of RSKs (Komatsu, data not shown). BI-D1870 treatment however, did not alter the level of IRF1 expression in RasV12 cells. This suggests that the IRF1 inhibition by RSKs is independent from their N-terminal kinase activity, but dependent on other regulatory domains such as C-terminal kinase domain. It

is also possible that non-specific effects of the inhibitor may mask IRF1 restoration. BI-D1870 is the only compound that has been confirmed to inhibit RSK3 and RSK4 activities *in vivo* (Sapkota et al., 2007). However, it has been reported to modulate signaling molecules other than RSK (Bain et al., 2007; Neise et al., 2013, and Sapkota et al., 2007).

RSK3 and RSK4 knockdowns promoted the expression of IRF1 in RasV12 cells (Figure 5.2 A). Moreover, overexpression of RSK3 or/and RSK4 suppressed IRF1 expression in NIH3T3 and RasV12 cells (Figure 5.3). Therefore, regardless of the inconsistencies observed between RasV12 vs DLD-1 and MDA-MB-468 and between the RSK RNAi and the RSK inhibitor experiments, we believe that RSK3 and RSK4 are the ERK downstream elements responsible for IRF1 downregulation.

Based on the previous publications, RSK3 and RSK4 have either tumor-suppressive or tumor-promoting roles depending on the cell type. RSK3 inhibits colony formation and increases apoptosis in ovarian cancer cells, and its expression level is often lower or absent in ovarian cancer cells compared to normal ovarian cells (Bignone et al., 2007). Similarly, RSK4 expression is downregulated in colon and renal tumor tissues (Lopez-Vicente et al., 2009). RSK4 suppresses colony formation and the *in vivo* tumor growth of breast cancer cell line MDA-MB-231 (Thakur et al., 2008). In contrast to these observations, RSK3 and RSK4 are both overexpressed in breast cancer cell line MCF7, and inhibit induction of apoptosis and promote protein synthesis by increasing phosphorylation of ribosomal protein S6 (rpS6) and eIF4B (Serra et al., 2013). Furthermore, RSK4 promotes cell motility in lung cancer cell line A549, but not in another lung cancer cell line H23 (Lara et al., 2011).

Interestingly, RSK4 has been reported as a negative regulator of the Ras/MEK pathway (Myers et al., 2004). Inhibition of Ras/MEK signaling by RSK4 depends on the 96AA at its N-terminus, which is present only in RSK4, but not in RSK1-3 (Myers et al., 2004). Furthermore, regulation of RSK4 seems to be distinct from other RSK members. Unlike RSK1-3, which are activated by growth factor-dependent signaling, RSK4 is constitutively active (Anjum & Blenis, 2008). Despite of the importance of RSK3 and RSK4 in cancer development, there has not been data published to indicate the involvement of RSK3 and RSK4 in antiviral immunity or PTMs. Therefore, it still remains to be further studied how RSK3 and RSK4 regulates IRF1 expression. We have conducted the tandem mass spec analysis of IRF1 binding proteins in RasV12 treated with or without U0126; however, RSK3 and RSK4 were not in the list of the IRF1 binding proteins (Komatsu, data not shown). Therefore, it is likely that RSK3 and RSK4 regulate IRF1 indirectly by interacting with other IRF1-regulatory protein(s).

**CHAPTER 7**  
**FUTURE DIRECTION**



## **7.1 IRF1 and oncolytic virotherapy**

In chapter 3, we demonstrated that IRF1 plays critical roles in controlling cancer cell susceptibility to Ras-dependent and IFN-sensitive oncolytic virus. By targeting IRF1, we can establish novel therapeutic strategies to enhance the efficacy and safety of oncolytic virotherapy. For example, treatment of IFN- $\gamma$ , a strong inducer of IRF1, may be effective to completely eliminate oncolytic viruses from patients after completion of oncolytic virotherapy or when any signs of side effects are observed. In addition, if any oncolytic viruses possess anti-IRF1 protein, such gene should be deleted or inactivated in order to increase specificity for cancer cells over normal cells. This is also critical for enhancing the safety of the virus. Moreover, it is likely that cancer populations that remain resistant against oncolytic virotherapy have relatively high expression levels of IRF1. As an alternative approach, anticancer IFN therapy could be effective to eliminate cancer cell populations with high IRF1 expression levels.

## **7.2 IRF1 post-translation modifications by Ras/MEK**

In chapter 4, we found preliminary evidence that active Ras/MEK suppress IRF1 ubiquitination and promotes sumoylation. These findings can be validated using alternative technique such as His-Sumo pull down assay which does not involve use of antibody (Tatham et al., 2009). In this approach, Sumo is tagged with 6 x His tag. The 6His-Sumo vector is co-transfected with pCMV-SPORT6-IRF1, and the cell lysate is purified on nickel affinity chromatography, which binds to 6 x His tags, pulling down all the sumoylated proteins. The purified pull down product can be analyzed by Western blot for IRF1 to determine whether IRF1 is sumoylated under various conditions. Unlike

IP, which was conducted under non-denaturing conditions, this experiment is carried out under denaturing conditions; therefore, it should reduce the risk of false positive result.

Among the three enzymes (E1, E2, E3) responsible for catalyzing ubiquitination, E3 ubiquitin ligase mediates substrate specificity and therefore is an important regulator of ubiquitination. Several IRF1 E3 ubiquitin ligases have been identified, including CHIP [C-terminus of the Hsc (heat-shock cognate) 70-interacting protein], MDM2 (murine double minute 2) (Landre et al., 2013 and Narayan et al., 2011) and cIAP2 (cellular inhibitor of apoptosis 2) (Harikumar et al., 2014). Therefore, a future study should examine whether Ras/MEK modulates IRF1 sumoylation via MDM2, cIAP2, or SIAH. Considering the important roles of ubiquitination on transcription factor activity, it will also be important to further study whether Ras/MEK-mediated suppression of IRF1 ubiquitination alters transcriptional activity of IRF1 at MDII gene promoters.

Sumoylation is tightly regulated by actions of SUMO-specific enzymes that catalyze attachment of SUMO in ATP-dependent manner as well as SUMO isopeptidases that cleaves SUMO from target proteins. Previous studies have shown that IRF1 undergoes sumoylation in the presence of Sumo E2 enzyme Ubc9 and Sumo E3 ligase PIAS3 in human embryonic kidney 293T cells (Nakagawa & Yokosawa, 2002). Interestingly, IRF1 is sumoylated at the same lysine residues as its ubiquitination sites (Park et al., 2007), indicating that IRF1 sumoylation and ubiquitylation may be competing for the same residues. This may explain why U0126 treatment increases IRF1 ubiquitination and decreases IRF1 sumoylation. To this end, a future study should determine which sumoylation or ubiquitination of IRF1 is the primary target of Ras/MEK by examining activities of the SUMO specific enzymes in the presence and absence of

MEK inhibitor.

### **7.3 IRF1 post-translational modifications and viral evasion**

Interestingly, many viruses have evolved to exploit sumoylation system as a strategy to evade host antiviral responses (Wimmer et al., 2012). It also is known that virus infection often activates the Ras/MEK pathway (Huber et al., 1999; Planz et al., 2001; Barber et al., 2002; Luo et al., 2002, and Kong et al., 2004). Since IRF1 is a potent antiviral protein, it will be important to address whether IRF1 sumoylation induced by Ras/MEK activation is a target of viral evasion strategies. Future study should first determine whether IRF1 sumoylation can be induced during infection of different viruses. If this is the case, additional experiments can be conducted to determine whether the viral sumoylation of IRF1 is initiated by Ras/MEK activation during infection.

### **7.4 IRF1 modulation by RSK 3 and 4**

In chapter 5, we demonstrated that ERK downstream elements RSK3 and RSK4 are responsible for downregulating IRF1 expression in RasV12 cells. However, the precise mechanisms of IRF1 regulation by RSK3 and RSK4 remain to be clarified. As a first step, it will be important to determine whether RSK 3 and 4 modulate PTM of IRF1. In addition, activation levels of RSK3 and RSK4 during infection should be examined in order to determine if different viruses regulate IRF1 PTMs to evade host antiviral responses.

**CHAPTER 8**  
**CONCLUSION**

Understanding the differences in cellular machinery between normal and cancer cells is especially important during the design of anticancer therapeutics that are highly specific to cancer cells without damaging normal cells. Defects in type I IFN-induced antiviral responses, oncogenic Ras activation, and p53 defects are the major cancer-specific changes exploited by oncolytic viruses. Prior to my studies, the Hirasawa lab identified that the Ras/MEK pathway suppresses the IFN-induced antiviral responses, showing that the two mechanisms of viral oncolysis are linked. In my thesis, I demonstrated that activation of the Ras/MEK pathway downregulates a group of IFN-inducible genes [MEK downregulated IFN-inducible (MDII) genes] by downregulating IRF1 expression. Ras/MEK-mediated downregulation of IRF1 was exploited by oncolytic VSV in various cancer cell lines. Among the several downstream effectors of ERKs, Ras/MEK downregulated IRF1 through RSK3 and RSK4 in RasV12 cells. In addition, I found preliminary evidence that Ras/MEK activation modulates post-translational modification (PTM) of IRF1, typically ubiquitination and sumoylation. Given that IRF1 is an important determinant of viral oncolysis, further characterization of IRF1 PTM by RSK3 and 4 could lead to the development of novel anticancer therapeutics that improve the efficacy and safety when using oncolytic viruses.

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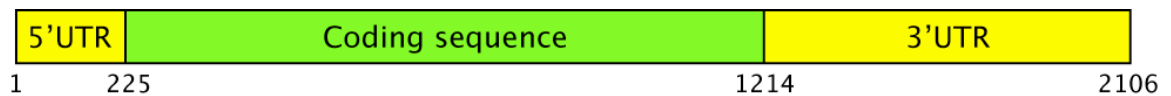
## **APPENDICES**



**Figure 1.** IRF1 mRNA sequence and the position of untranslated regions (UTR). The 5'- and the 3'-UTR sequence of IRF1 were determined from the NIBI database and are highlighted in yellow. Coding sequences are highlighted in green.

5'---1 ccgctcggg gcctcttgg agcccagagt ggccgtcgc cgcaccccg agcagcgccg  
61 ggggactcgg atgtccgct gcgcgcactt ttggcggtcg cagcttggt cccagctctt  
121 gctttcggac gcgcagtgtt cggtcagag gttggctcgc tgccttgact ggctggaccg  
181 ggtcgtgaac tactgggctt tgggaggag gtgccacagc caccatgcca atcaactcga  
241 tgcggatgag acctggcta gagatgcaga ttaattcaa ccaaatcca gggctgatct  
301 ggatcaataa agaagagatg atctccaga ttccatggaa gcacgtgct aageacggct  
361 gggacatcaa caaggatgcc tgtctgtcc ggagctgggc cattcacaca ggccgataca  
421 aagcaggaga aaaagagcca gatcccaaga catggaaggc aaacttccgt tgtgcatga  
481 actcctgcc agacatgag gaagtgaagg atcagagtag gaacaagggc agctctgctg  
541 tgcgggtgta ccggatgctg ccaccctca ccaggaacca gaggaaagag agaaagtcca  
601 agtccagccg agacactaag agcaaaaacca agaggaagct gtgtggagat gtagcccg  
661 acatttctc tgatggactc agcagctcta cctacctga tgaccacagc agttacacca  
721 ctcagggcta cctgggtcag gacttgata tggaaagga cataactca gcaactgca  
781 cgtgtgctg cagcagcagt ctctctgagt ggcatatgca gatggacatt ataccagata  
841 gcaccactga tctgtataac ctacaggtgt cacccatgcc ttccacctcc gaagccgcaa  
901 cagacgagga tgaggaagg aagatagccg aagacctat gaagctctt gaacagtctg  
961 agtggcagcc gacacacatc gatgcaagg gatactgct caatgagcca gggaccagc  
1021 tcttctctg ctatggagac tcagctgca aagaggaacc agagattgac agccctcgag  
1081 gggacattgg gataggcata caacatgct tcacggagat gaagaatag gactccatca  
1141 tgtggatgga cagcctgctg ggcaactctg tgaggctgcc gcctctatt caggccatc

1201 cttgtgcacc atagtttggg tctctgacce gtcttgccc tctgagtga gttaggcctt  
 1261 ggcacatgg tggctgtgat acaaaaaag ctagactcct gtgggccct tgacacatgg  
 1321 caaagcatag tcccactgca aacaggggac catcctcctt gggtcagtgg gctctcaggg  
 1381 cttaggaggc agagtctgag tttcttgtg aggtgaagct ggccctgact cctaggaaga  
 1441 tggattgggg ggtctgaggt gtaaggcaga ggccatggac aggagtcatc ttctagcttt  
 1501 ttaaaagcct tgtgcatag agagggtctt atcgtgggc tggccctgag gggaatagac  
 1561 cagcggccac agaagageat agcactggcc ctagagctgg ctctgtacta ggagacaatt  
 1621 gcactaaatg agtcctatc ccaagaact gctgccctc ccaaccgagc cctgggatgg  
 1681 ttccaagcc agtgaaatgt gaagggaaaa aaaatggggg cctgtgaagg ttgctcct  
 1741 tagcctcaga gggaaatgc ctactacct gctccagctg tggggctcag gaaaaaaaaa  
 1801 tggcacttc tctgtggact ttgccacatt tetgatcaga ggtgtacct aacatttctc  
 1861 cccagtctag gccttgcatt ttattatat agtccttgc ctggtgctg ctgtctctc  
 1921 aggccttggc agtctcagc aggccaggg aaaagggggg ttgtgagcgc cttggcgtga  
 1981 ctctgacta tctattagaa acgccaccta actgctaaat ggtgtttggt catgtggtgg  
 2041 acctgtgtaa atatgtatat ttgtctttt ataaaaattt aagtgttta caaaaaaaaaa  
 2101 aaaaaa----3'





CACACACACTGGCATATCAAGTCTCTGTTAGGCTAGGCGCATCCTCTCCCACTGAGGTCA  
GACAAGACTGCCAGCTAGAAGAACATATCCCACGGACAGGCAACAGCTTTTGGGACAGC  
CACGCTCCAGTTGTTTGGGACTCATAAAAGACTAAACTCACACCTGCTACAAAAGTGCAG  
GGAGGCCTAGGTCCAGCCTGTGTGTGCTCTTTGCTTAGTGGTGTCTCTGAGAGCCCTAAG  
GATCAAAGTTTGTGACTCTGTTGGTCTTCCTGTGGAGTTCCTATCCCCTTCGTCCCCCT  
CCCCACCCTGCAATCTTCCCTCAACTCTTCTTTAGGCCTGGTGGTGGTGGTGTGGTGGC  
ATGGAGGAGGTGGTGGAGGGGGTGGGGTCTTTAATCCCGGTTCTTGTGAGACCGAAGCA  
GGGACGATTTCCGAGCTCTGTGAGTTTGAGGCCAGCCTGGTCTATAGATCTAGTTCCAGG  
ACAGTCAGAGCTA **CATAGAGAAACC** CTGCCCCGAGGGGGGGGGGGCGCGGGGAATGGTTA  
AAGATTATTGCAGGACCCAGCTGATCTGTGGAAGAGGTAACGGGTGTTTATGTTTTTCGA  
AACTCATTGAACAATGCACTTCAATTGTGCGCACTTTAGAAATATAAAGCCACCACGCGA  
AAAGCTGCGCCCCAACTTAAAGGCAATTTCCAAGGTACTTCTGGGTCCCTTGC GGTT CAGT  
GGCTGTCTAGGTTC **AGAAACGAAACT** GGATCCCCGCCCCGCCCCCCCCGCCCCCCCCCTCC  
CCAGCGCCCTGAGGC **AGT** TT

**Figure 3.** Sequence of the Ifi47 promoter construct. IRFE sites were determined using the JASPAR database and highlighted in yellow. Transcription start site was determined from the NCBI database and highlighted in pink.

ACACAGAGCTGGAAAAAAAAAGACACCATTTTAATTAACAGGAGCATGTAATC  
TGTTCTTCATATCTGATTACATGTGTTAAAATTACGCAGAACTCCGGGCGTG  
GTGGTACATGCCTTTAATCCCAGCACTCGGGAAGCAGAGGCAGGAAGATTTC  
TGAGTTCGAGGCCAGCCTGGTCTACAAAGTAAGTTCAGGACAGCCAGGGCT  
ATACAGAGAAACCCTGTCTCAAAAATACAAAACAACAACAACAACAACAA**CAAA**  
**AACCAAACCAAAACA**AAACAAAACAAAAAATTATGCAGAAACACTGTCAAT  
ATAACCCAAACCATCCATGTTTCAGAGACAGACTTCACCAAGGACAGAACATG  
TCAG**GCAACAGCTTCTTTTCCCAAC**ATGGCAGCAAATGTCTTTCCTCCATGGTG  
ATAAACGTTAAAACCAACAAAACCTGTGAGGGATTATCTCATTGCATCAAAT  
GATGTTTTTGAAAACCTGTCTTCACTTTGTGCCAGACTACGCTCTCATGAGG  
GAATCCTGAGGTAGATAA**AATGAGAAAGGAAACTAGGAA**GACTCCATTACTC  
TCTTTCCTGAGTTGTCTTGGTGGCTCTGGATCCTGTGAGACCCCAGGTTTCATG  
GGTGCCTTGGGGCTGAAATGAGGTTAGTCAGCCACAGTGTTCTTGAGAAGGA  
CAAGGAGGCAGACGCATGGCACAGTCCTTCCCGATGGGCAGTGTGTTTCAGT  
CATTCACTGGCGTCTTTGGAAAACCCCTCATCTCTTTCATCCTTGTCCCCAGGA  
ATCCTTTGGCTTCAGAGAACTCATTCCCCTGATGTCTTGGCCGAGTGACCCTT  
CACAGGCCAAGTGGAATGTCTTCTGGCCTTGAATCTTCCAGGCTTCTTTCA  
TTCTCTGACCAAGTTA**AAGTCCTTTTCTTTTTCCTGG**AAATGTTTATGCTGCAG  
GGGAAACAAAGAAAGAG**GCTTTAGTTTCACTTTTGTTT**CCTGCAA**ATATATCTT**  
**TCATTTTTTCT**CTCTGCTAGGGCTCATTGCTTCAGACTTTCCTGAAGGAGGGC

AGACCCGTGCCGTTGGTGGTGGCTGTGCTGTGGGCTGCAGTGAGAAACAGAC

CCGGTATTTCTACTTCTTGGATCTGG

**Figure 4.** Sequence of the Gbp2 promoter construct. IRFE sites were determined using the JASPAR database and highlighted in yellow. Transcription start site was determined from the NCBI database and highlighted in pink.

TCAGATTCACTTTCACTTTTCAGTAATTTTTCTTCCTCCCTCCTTCCCTCTCTTTG  
TCTCCCCATCTTTCTCCTTCCTTTCTCTCTCCTTGTCCCTCCAAATTCTCACCT  
TTTTCGTTCTATTTTTATTCTTTCACAGTACAGAGAAATGATTTTTAAACGATT  
CATGTCCACTTCTGAATTGCACAATTGCTTGCTAACTTTGTGTAACACAGTGG  
GGCTGGCAACTTCACAAAACAAACACTAATGGACAAAAAAGGACTGAAACA  
TAAGAGAATGATTTCAAACCACACCCTTTTCACCCTACATTTTGAAACACCC  
ACAAGAAATGGCACCATTTATCAATCTCTACCTGAGAAGTCCTGAGACCCTCC  
TCCCATGAGCAAACCCCTCATTCTGCAGTGCTGGTTGAGTCATCCCTCCAAC  
CCCACCCAGTTAGGAACTCTTTT **AGTTTCACTTTCACTGTC** TTAAGCATAAAT  
AAAGAGCGAACTCCTCACAAGTTCTCTGGCA **AAA** ATCTGGA ACTTCCCGGGT  
TACTACAGGGTCTATGTACAGTGCCTGTGAGAGAGGACAGAGCACTCTGCA  
GCCAGCCTCAGAGGCAA

**Table 1.** qPCR primers

<b>Gene name</b>	<b>5' primer</b>	<b>3' primer</b>	<b>Amplicon size</b>	<b>Efficiency (%)</b>
Gapdh	ATGTGTCCGTCGTG GATCTGA	TGCCTGCTTCAC CACCTTCTT	79	109
Gbp2	AGGTAAACGGAAA ACCCGTCA	CACAGTCGCGGC TCATTAAG	100	128
Ifi47	TGAGCTTCATCCCC TTCATGA	CCAAGCCAAAAT AGCTTCGGT	93	126
Ifit1	CCTGGATTCCTGAC TGTTGTCG	TGGCACATGCAC AGCAAGAT	121	101
Iigp2	TGTGGGTAAGGAT AGGATGGA	CAAGCAGGTGGA AGGTGAGT	92	139
Il15	CAGCACAGCTCCA TGGATTT	TCATGTGATCCA AGTGGCTCA	83	100
Ptx3	ACGGAGGAGCCCA GTATGTT	ACGCACCGAAGT TTTCAGAC	108	145
Rig-1	GGCAGACAAAGAG GAGGAGA	CGGACATCGTGG AAGAAGG	150	134
Stat2	TTCGGCTTCTTGAC TCTGGT	CTCAACCACGAA GCTGATGA	150	130
Xaf1	GCCTGCGCTTCATA GTCCTTT	GGTGCACAACCTT CCATGTGCT	83	108
Irf1	TCTTGCCCTCCTGA GTGAGT	GGGACTATGCTT TGCCATGT	102	113
ChIP Gbp2	CCCACCCAGTTAG GAACTC	TAACCCGGGAAG TTCCAGAT	109	107
ChIP Ifi47	TGCAGGGGAAACA AAGAAAG	GAAGCAATGAGC CCTAGCAG	108	100



**Table 2.** Semiquantitative PCR primers.

<b>Gene name</b>	<b>5' primer</b>	<b>3' primer</b>	<b>Annealing Temperature</b>	<b>Cycles</b>	<b>Amplicon size</b>
Gbp2 IRFE	GCTGGCAACTT CACAAAACA	TGCCAGAGAA CTTGTGAGGA	66	34	295
Gbp2 distal control	TGATTTCCCAG CATTTGACA	AGGGTGAAAA GGGTGTGGTT	66	34	337
Ifi47 IRFE	CAGGCCAAGTG GAAATGTCT	CTGAAGCAAT GAGCCCTAGC	66	34	193
Ifi47 distal control	GGTATTCACAA AGCCCTCCA	TCTCCACAGTC CCAACATCA	61	34	198
Irf1	TCTTGCCCTCCT GAGTGAGT	TCTAGGGCCA GTGCTATGCT	66	35	363
Gapdh	GGGTGGAGCCA AACGGGTCA	GGAGTTGCTG TTGAAGTCGC A	58-66	28	531
Mnk1	AGGTCTTTAGG GACGAGGCT	TTTCTGGAGCT TGCCCTTGT	66	33	336
Mnk2	CTGACAAGGAC TGGTCCCAC	AGCTGTTCCCTC TGCAGAACC	66	35	174
Msk1	GAGCGACTGTT TCAGGGCTA	ATGGACCAGC CATGTCCAAG	58	34	486
Msk2	CTTTCATGGCGT TCAACCGAG	GGAGGGGAGG GCAATTCCTA	66	33	323
Rsk1	GAGAGACATCC TCGCTGACG	TGCCTAGCTTC GCCTTCAA	60	34	473
Rsk3	CTCCCAAGGGG TTGTCCATC	CACGGGTGCT TCAACACTTG	66	34	441
Rsk4	GTTGGCTGGCT ACACTCCAT	ATATGGTGCT GCCACTGCTT	66	34	280
huGAP DH	CCATCACCATC TTCCAGGAG	CCTGCTTCACC ACCTTCTTG	55.5	33	600
huRSK 3	GCTGGCAGGAT TTACCCCTT	CACGGGTGTT TGAGCACTTG	66	35	201
huRSK 4	ATGTTTCTCGG AACGGGAGG	GGAGTGTAGC CAGCCAACAT	66	35	324

**Table 3.** Primary and secondary antibody conditions for Western blot

<b>Antibody</b>	<b>Primary antibody condition</b>	<b>Secondary antibody condition</b>
Acetylated Lysine (#9441)	1:1000 in 5 % BSA	1:5000 Anti-rabbit IgG in TBST
p-ERK (#9101)	1:1000 in TBST	1:5000 Anti-rabbit IgG in TBST
t-ERK (K-23)	1:30,000 in TBST	1:5000 Anti-rabbit IgG in TBST
GAPDH (6C5)	1:60,000 in TBST (1 hour)	1:5000 Anti-mouse IgG in TBST
HA (3F10)	1:1000 in 1 % BSA (1 hour)	1:5000 Anti-rat IgG in 1 % BSA
Human IRF1 (BD)	1:2000 in TBST	1:5000 Anti-mouse IgG in 5 % milk
Mouse IRF1 (M-20)	1:2000 in 5 % milk	1:5000 Anti-rabbit IgG in 5 % milk
Phosphoserine (PSR-45)	1:1000 in 3 % BSA	1:5000 Anti-mouse IgG in TBST
Phosphotyrosine (4G10)	1:1000 in 3 % BSA	1:5000 Anti-mouse IgG in TBST
RSK1 (#9333)	1:1000 in 5 % BSA	1:5000 Anti-rabbit IgG in 5 % BSA
RSK2 (E-1)	1:1000 in 5 % milk	1:5000 Anti-mouse IgG in 5 % milk
RSK3 (A-16)	1:1000 in TBST	1:5000 Anti-goat IgG in 5 % milk
RSK4 (JS-31)	1:1000 in TBST	1:5000 Anti-mouse IgG in 5 % milk
SUMO (FL-101)	1:500 in TBST	1:5000 Anti-rabbit IgG in TBST
VSV-G (VSV11-M)	1:10,000 in 5 % milk	1:5000 Anti-mouse IgG in 5 % milk